Pertanika Journal of

TROPICAL AGRICULTURAL SCIENCE

JTAS

VOL. 44 (1) FEB. 2021

PERTANIKA JOURNALS

A scientific journal published by Universiti Putra Malaysia Press

Pertanika Journal of Tropical Agricultural Science
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About the Journal

Overview

Pertanika Journal of Tropical Agricultural Science is an official journal of Universiti Putra Malaysia. It is an open-access online scientific journal. It publishes the scientific outputs. It neither accepts nor commissions third party content.

Recognised internationally as the leading peer-reviewed interdisciplinary journal devoted to the publication of original papers, it serves as a forum for practical approaches to improving quality in issues pertaining to tropical agriculture and its related fields.

Pertanika Journal of Tropical Agricultural Science is a **quarterly** (*February*, *May*, *August*, and *November*) periodical that considers for publication original articles as per its scope. The journal publishes in **English** and it is open for submission by authors from all over the world.

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Pertanika Journal of Tropical Agricultural Science aims to provide a forum for high quality research related to tropical agricultural research. Areas relevant to the scope of the journal include agricultural biotechnology, biochemistry, biology, ecology, fisheries, forestry, food sciences, genetics, microbiology, pathology and management, physiology, plant and animal sciences, production of plants and animals of economic importance, and veterinary medicine.

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Currently, as an interdisciplinary journal of agriculture, the revamped journal, a leading agricultural journal in Malaysia now focuses on tropical agricultural research and its related fields.

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Pertanika is now over 42 years old; this accumulated knowledge has resulted in Pertanika Journal of Tropical Agricultural Science being abstracted and indexed in SCOPUS (Elsevier), Clarivate Web of Science (ESCI), EBSCO, DOAJ, Agricola, ASEAN CITATION INDEX, ISC, Microsoft Academic, Google Scholar, National Agricultural Science (NAL), and MyCite.

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The abbreviation for Pertanika Journal of Tropical Agricultural Science is *Pertanika J. Trop. Agric. Sci*.

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Pertanika Journal of Tropical Agricultural Science: e-ISSN 2231-8542 (Online).

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Most scientific papers are prepared according to a format called IMRAD. The term represents the first letters of the words ***I****ntroduction*, ***M****aterials and Methods*, ***R****esults*, ***A****nd* ***D****iscussion*. IMRAD is simply a more 'defined' version of the "IBC" (*Introduction*, *Body*, *Conclusion*) format used for all academic writing. IMRAD indicates a pattern or format rather than a complete list of headings or components of research papers; the missing parts of a paper are: *Title*, *Authors*, *Keywords*, *Abstract*, *Conclusions*, *References*, and *Acknowledgement*. Additionally, some papers include *Appendices*.

The *Introduction* explains the scope and objective of the study in the light of current knowledge on the subject; the *Materials and Methods* describes how the study was conducted; the *Results* section reports what was found in the study; and the *Discussion* section explains meaning and significance of the results and provides suggestions for future directions of research. The manuscript must be prepared according to the journal's **Instruction to Authors** (http://www.pertanika.upm.edu.my/Resources/regular_issues/Regular_Issues_Instructions_to_ Authors.pdf).

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Notification of the editorial decision is usually provided within 90 days from the receipt of manuscript. Publication of solicited manuscripts is not guaranteed. In most cases, manuscripts are accepted conditionally, pending an author's revision of the material.

As articles are double-blind reviewed, material that may identify authorship of the paper should be placed only on page 2 as described in the first-4-page format in *Pertanika*'s **Instruction to Authors** (http://www.pertanika.upm.edu.my/Resources/regular_issues/Regular_Issues_Instructions_to_Authors.pdf).

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In the peer review process, 2 or 3 referees independently evaluate the scientific quality of the submitted manuscripts. At least 2 referee reports are required to help make a decision.

Peer reviewers are experts chosen by journal editors to provide written assessment of the **strengths** and **weaknesses** of written research, with the aim of improving the reporting of research and identifying the most appropriate and highest quality material for the journal.

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What happens to a manuscript once it is submitted to *Pertanika*? Typically, there are 7 steps to the editorial review process:

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2. The Chief Executive Editor sends the article-identifying information having been removed, to 2 or 3 reviewers. They are specialists in the subject matter of the article. The Chief Executive Editor requests that they complete the review within 3 weeks.

   Comments to authors are about the appropriateness and adequacy of the theoretical or conceptual framework, literature review, method, results and discussion, and conclusions. Reviewers often include suggestions for strengthening of the manuscript. Comments to the editor are in the nature of the significance of the work and its potential contribution to the research field.

3. The Editor-in-Chief examines the review reports and decides whether to accept or reject the manuscript, invite the authors to revise and resubmit the manuscript, or seek additional review reports. In rare instances, the manuscript is accepted with almost no revision. Almost without exception, reviewers' comments (to the authors) are forwarded to the authors. If a revision is indicated, the editor provides guidelines to the authors for attending to the reviewers' suggestions and perhaps additional advice about revising the manuscript.

4. The authors decide whether and how to address the reviewers' comments and criticisms and the editor's concerns. The authors return a revised version of the paper to the Chief Executive Editor along with specific information describing how they have answered' the concerns of the reviewers and the editor, usually in a tabular form. The authors may also submit a rebuttal if there is a need especially when the authors disagree with certain comments provided by reviewers.

5. The Chief Executive Editor sends the revised manuscript out for re-review. Typically, at least 1 of the original reviewers will be asked to examine the article.

6. When the reviewers have completed their work, the Editor-in-Chief examines their comments and decides whether the manuscript is ready to be published, needs another round of revisions, or should be rejected. If the decision is to accept, the Chief Executive Editor is notified.

7. The Chief Executive Editor reserves the final right to accept or reject any material for publication, if the processing of a particular manuscript is deemed not to be in compliance with the S.O.P. of *Pertanika.* An acceptance notification is sent to all the authors.

   The editorial office ensures that the manuscript adheres to the correct style (in-text citations, the reference list, and tables are typical areas of concern, clarity, and grammar). The authors are asked to respond to any minor queries by the editorial office. Following these corrections, page proofs are mailed to the corresponding authors for their final approval. At this point, **only essential changes are accepted**. Finally, the manuscript appears in the pages of the journal and is posted on-line.

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A scientific journal published by Universiti Putra Malaysia Press

PJTAS

Pertanika Journal of Tropical Agricultural Science

AN INTERNATIONAL PEER-REVIEWED JOURNAL

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URL: www.journals-jd.upm.edu.my

PUBLISHER

UPM Press
Universiti Putra Malaysia
43400 UPM, Serdang, Selangor, Malaysia.
Tel: +603 9769 8855
E-mail: penerbit@putra.upm.edu.my
URL: http://penerbit.upm.edu.my

ABSTRACTING AND INDEXING OF PERTANIKA JOURNALS

Pertanika is over 40 years old; this accumulated knowledge has resulted in the journals being abstracted and indexed in SCOPUS (Elsevier), Clarivate-Emerging Sources Citation Index [ESCI (Web of Science)], BIOSIS, National Agricultural Science (NAL), Google Scholar, MyCite and ISC.

Pertanika Journal of Tropical Agricultural Science
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Foreword

Welcome to the First Issue of 2021 for the Pertanika Journal of Tropical Agricultural Science (PJTAS)!

PJTAS is an open-access journal for studies in Tropical Agricultural Science published by Universiti Putra Malaysia Press. It is independently owned and managed by the university for the benefit of the world-wide science community.

This issue contains 14 articles; 10 are regular articles and the rest are review articles. Articles submitted in this issue cover the scope of animal production; aquaculture; biotechnology; crop and pasture production; food and nutrition development; microbiology; soil and water sciences; forestry sciences; and zoology. The authors of these articles come from different countries namely Columbia, Malaysia, Pakistan, Singapore, and Thailand.

Douglas Law and his teammates from Universiti Kebangsaan Malaysia and Universiti Malaysia Kelantan focused on the finding of a sex marker for the *Oxyeleotris marmorata* using the amplified fragment length polymorphism (AFLP) method. The found SCAR marker has the potential to be used for the sexual identification of the *O. marmorata* at the juvenile stage; thereby it is useful in the breeding programs. Further details of this study are found on page 107.

A regular article entitled "Effectiveness of Bioinoculants *Bacillus cereus* and *Trichoderma asperellum* as Oil Palm Seedlings Growth Promoters" tested on the plant growth promotion potentials of bacterium, *Bacillus cereus* (UPM15) and fungus *Trichoderma asperellum* (UPM16). The production of indole acetic acid (IAA) was considered as the indicator of the plant growth-promoting potentials. Both *B. cereus* and *T. asperellum* were capable of producing IAA. In addition, the mixture of these isolates yielded good vegetative growth. Full information of this study is presented on page 157.

A special thanks to the Editor-in-Chief, Prof. Dr. Mohd. Zamri Saad, and all the Editorial Board Members of PJTAS (2018-2020) for serving Pertanika Journal of Tropical Agricultural Science for the past two years, in ensuring Pertanika plays a vital role in shaping the minds of researchers, enriching their lives, and encouraging them to continue their quest for new knowledge. We welcome the new Editor-in-Chief, Prof. Dr. Amin Ismail, and new

Editorial Board Members on board. We hope that their involvement and contributions towards Pertanika would not only improve its quality but also support the development efforts in making it an international journal of good standing. We anticipate that you will find the evidence presented in this issue to be intriguing, thought-provoking and useful in reaching new milestones in your own research. Please recommend the journal to your colleagues and students to make this endeavour meaningful.

All the papers published in this edition underwent Pertanika's stringent peer-review process involving a minimum of two reviewers comprising internal as well as external referees. This was to ensure that the quality of the papers justified the high ranking of the journal, which is renowned as a heavily-cited journal not only by authors and researchers in Malaysia but by those in other countries around the world as well.

We would also like to express our gratitude to all the contributors, namely the authors, reviewers, Editor-in-Chief and Editorial Board Members of PJTAS, who have made this issue possible.

PJTAS is currently accepting manuscripts for upcoming issues based on original qualitative or quantitative research that opens new areas of inquiry and investigation.

Chief Executive Editor
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Pertanika J. Trop. Agric. Sci. 44 (1): 1 - 23 (2021)

PERTANIKA JOURNALS

TROPICAL AGRICULTURAL SCIENCE

Journal homepage: http://www.pertanika.upm.edu.my/

Review Article

Holistic Approaches to Reducing *Salmonella* Contamination in Poultry Industry

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ABSTRACT

Salmonella are widely found in the poultry industry, which subsequently may pose a risk to animal and human health. The aim of this review is to highlight strategies for the prevention and control of *Salmonella* at each stage in the poultry production chain by monitoring risks from the farm to the retailer. Among the primary approaches for control of *Salmonella* at the farm level includes the administration of synthetic and natural compounds to live chickens (vaccination and antibiotic), litter management as well as fortification of feed and acidification of drinking water. In the poultry processing plant, multiple hurdle technology and different chilling conditions to reduce *Salmonella* were discussed. In the retail level, an effective monitoring program to control *Salmonella* contamination by good manufacturing practices and hazard analysis and critical control points has been reviewed. Overall, we conclude that these approaches play a role in reducing the dissemination of *Salmonella* in the poultry industry. However, there is no published data related to logistic scheduling of poultry processing.-

Keywords: Contamination, control, poultry industry, prevention, *Salmonella*

ARTICLE INFO

Article history:
Received: 28 May 2020
Accepted: 24 August 2020
Published: 24 February 2021

DOI: https://doi.org/10.47836/pjtas.44.1.01

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ISSN: 0128-7680
e-ISSN: 2231-8526

INTRODUCTION

Foodborne diseases can be caused by biological, physical, or chemical hazards. Biological hazards such as bacteria, especially from the genus *Salmonella*, have been reported in many cases of foodborne-related illness (Cummings et al., 2010). Poultry product consumption may be a leading cause of salmonellosis (Bryan, 1980; Painter et al., 2013; Scallan et al., 2011). Rouger et al. (2017) stated that the prevalence of *Salmonella* in live chickens and in many types of raw chicken products could indicate that the microbe had already established its route in the chicken industry.

Salmonella dissemination may result from not using best practices (cross-contamination from unhygienic personnel, polluted water, and dirty processing facility) during transporting, processing or packaging of poultry meat and poultry products. Numerous studies have found that these bacteria are now becoming resistant towards antibiotics, causing these microbes to increase in number even if the chickens have been administered orally with antibiotics (Bansal et al., 2006; Gebreyes et al., 2004; Li et al., 1993; Salomonsson et al., 2005; Seo & Lee, 2004). The high number of poultry-associated *Salmonella* outbreaks in humans (Gaffga et al., 2012) highlights the need for well-developed approaches for combatting *Salmonella* contamination throughout the poultry production chain. In the year 2015, a national outbreak investigation was carried out in Canada as *Salmonella* Enteritidis was identified in frozen raw breaded chicken products. There were a total of 51 cases reported over a 6-month period by which 45% of these cases were hospitalised (Public Health Notice, 2015). Therefore, regulators recommended that industry strengthen messaging to all the poultry handlers and consumers to handle raw poultry and poultry products at appropriate condition including storage temperature as well as cooking temperature to reduce contamination along the poultry supply chain.

In addition to public health concerns, *Salmonella*-contaminated poultry have negative impacts on the economic status of a country, as it will be rejected for international trading; consequently, the competitiveness of the industry is affected (Rodríguez & Suárez, 2014). The growing world population has triggered a rise in the consumer demand for a wide variety of foods, resulting in an increasingly complex global food chain (Wirsenius et al., 2010). These challenges have placed greater responsibility on food producers and handlers to ensure food safety. Therefore, the level of microbial contamination in poultry at each stage of the poultry production line must be monitored thoroughly, from the poultry-farming level to the retailer. A reduction in microbial contamination would not only reduce the prevalence of food-borne illnesses, but also ensure a high standard of quality and economic stability in the poultry industry.

Characteristics of *Salmonella* and its Pathogenicity

Salmonella (family: Enterobacteriaciae) is generally grouped into two major species:

Salmonella enterica and *Salmonella bongori*. According to Grimont and Weill (2007), *S. enterica* is further divided into six subspecies designated by Roman numerals as follows: *enterica* (I), *salamae* (II), *arizonae* (IIIa), *diarizonae* (IIIb), *houtenae* (IV), and *indica* (VI). When *Salmonella* is observed under the microscope, it appears as a flagellated, straight rod gram-negative bacterium. *Salmonella* could be motile or non-motile (variants like *Salmonella* Gallinarum and *Salmonella* Pullorum) (van Asten et al., 2004).

According to Botteldoorn et al. (2003), *Salmonella* can colonise a wide range of hosts such as poultry, cattle, and pigs. *Salmonella* can also persist in a wide variety of environmental conditions, where it can grow in foods stored at temperatures as low as 2 – 4°C and also at temperatures as high as 54°C (Juneja et al., 2007). They are sensitive to heat and usually killed at temperatures ≥ 70°C. *Salmonella* grow in the pH range of 4 – 9 with the optimum pH range of 6.5 – 7. 5. They require high water activity (a_w) between 0.99 and 0.94 (pure water $a_w = 1.00$), but they can survive at $a_w < 0.2$. Complete inhibition of growth occurs at temperatures < 7°C, pH < 3.8, or water activity < 0.94 (D'Aoust & Maurer, 2007).

The highly ubiquitous species of *Salmonella* serovars can be divided into typhoidal and non-typhoidal *Salmonella* (NTS) serovars (Gal-Mor et al., 2014). Despite their genetic similarity, these two groups elicit very different diseases and distinct immune responses in humans. An example of typhoidal *Salmonella* is *Salmonella* Typhimurium which causes typhoid fever through ingestion into intestinal tract. It will then multiply in the liver and spleen and may persist in gall bladder for years as it triggers ulceration of intestine and also causes delirium (Cooper et al., 1994). In contrast, non-typhoidal *Salmonella* causes gastroenteritis, bacteraemia, and focal infection. Foodborne illnesses in humans are commonly caused by non-typhoidal *Salmonella* such as *Salmonella* Enteritidis, which commonly found in chicken egg and meat. Graham (2002) reported that approximately 1% of immunocompromised individuals and children below five years of age risked death due to the consumption of poultry contaminated with non-typhoidal *Salmonella*.

The most common reason for the spread of *Salmonella* from the environment to a flock is unhygienic farming activities, overcrowding, and a lack of biosecurity measures in poultry houses (Frederick & Huda, 2011). The application of preventive and control measures in the poultry supply chain, from farm to retailer, are put into place to significantly minimize *Salmonella* contamination and its subsequent transmission to humans. *Salmonella* can reside in the gut of a chicken and be excreted in its faeces (Shivaprasad et al., 1990). However, when *Salmonella*-contaminated chicken is consumed by humans, it can result in serious systemic disease (Chappell et al., 2009). *Salmonella* has several attributes that enable it to multiply much faster and infect new cells. Among the features are long

tails, which are called flagella because of their movement; they can inject protein by piercing cells using a complex needle which is present on their membrane (Schraidt & Marlovits, 2011). The action mechanism by which *Salmonella* infects humans is through the gastrointestinal tract, from where it gains access to the epithelium (Schikora et al., 2011). *Salmonella* activates its virulence mechanism once it reaches the submucosa of the stomach, which allows it to survive and replicate in the host (Reed et al., 2002).

Poultry Industry: From Farm to Retailer

Salmonella can enter the poultry production chain at many points. In general, there are many interlinked segments in the poultry industry which allows maximum control of produced products. This industry implements the ladder concept (as illustrated in Figure 1), in which chickens are produced based on demand. By this means, the poultry industry is able to produce a high-quality product efficiently. The structure of the poultry industry starts from the primary breeder, feed mill, breeders, hatchery, grow-out farms, processing plants, and further processing before the poultry is transported and marketed (Van der Vorst et al., 2007).

At the primary breeder level, poultry companies aim to produce chickens that have desirable characteristics, such as an abundance of white meat and efficient feed conversion (Raji, 2018). To achieve this, chickens are raised from a natural process

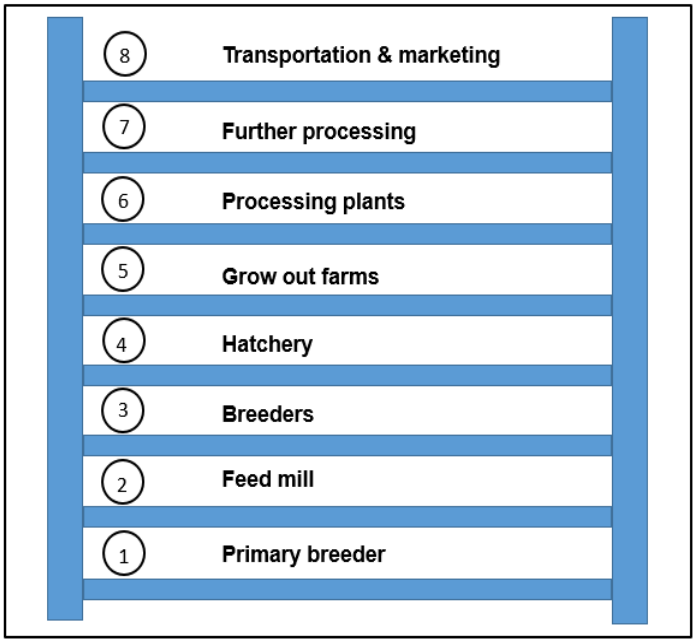

Figure 1. Ladder concept in the structure of poultry industry (modified from https://www.uspoultry.org)

of selecting and crossbreeding birds with the most desirable qualities. In nearly all cross-breeding programmes, the cross-breed birds exhibit considerably better egg production and growth rate. At the feed-mill level, companies produce different formulas to accommodate the different nutrition stages of chickens. Since 1996, farmers in poultry's industry have fed genetically modified corn and soybeans which are commercially available to the chickens. Next, the offspring of breeder parents will be raised to become larger and healthier broilers for markets. Modern advances in farming including advanced housing, climate controls, biosecurity, and good animal husbandry as well as cooperation between farmers and veterinarians helped in raising desirable broilers to meet the increasing demands of poultry meats. At the hatchery level, fertile eggs are placed in incubators under optimum incubation conditions until nearly the end of hatchery, when the eggs are transferred to trays; chicks hatch by pecking through the large end of the eggs. The newly hatched chicks are then transported to grow-out farms by independent farmers.

After approximately six to seven weeks, chickens at the grow-out farm are taken to the processing plant (Bailey et al., 2002). In a modern poultry processing plant, every step is taken to ensure that the chickens are processed quickly and painlessly (G. C. Mead, 2012). Ten stages are involved in poultry processing; the first stage, *stunning*, is performed to render chickens unconscious prior to slaughter. After slaughter, the chickens will be placed in a hot water bath to loosen their feathers, a procedure called *scalding* (Slavik et al., 1995). The next step in processing is *evisceration*, during which the internal organs of the poultry are removed. The carcasses are then washed and

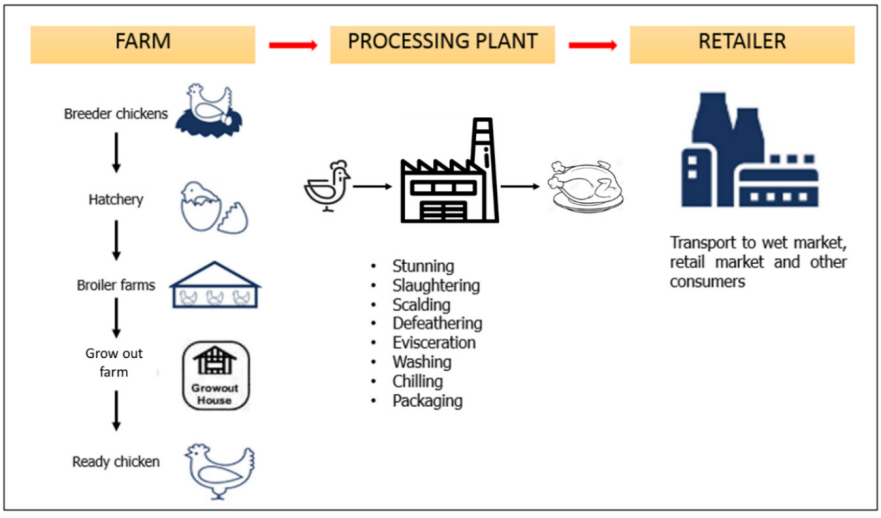

Figure 2. Poultry production chain from farm to retailer

chilled to limit the growth of bacteria. After chilling they are packaged for distribution or cut into parts (Thomas, 1977). After processing, the poultry is transported from the processing plant to grocery stores, wet markets, and other retail outlets via refrigerated trucks. Figure 2 shows the poultry production chain, beginning with the farm and ending with the retailer.

Route of *Salmonella* Contamination in the Poultry Industry

The first entry point for *Salmonella* in chickens begins as early at the hatchery stage in the farm (Foley et al., 2011). Hence, hens that appear to be healthy may be undiscernibly infected with *Salmonella* and could contaminate their eggs by sitting on them. These bacteria can be present on faeces and passed to other egg shells (Cox et al., 2000). Insects can also serve as a vehicle for *Salmonella* contamination in poultry farm. Chen et al. (2011) identified houseflies as a source of *Salmonella* contamination at a poultry farm in Selangor, Malaysia due to the close association of houseflies with faeces and garbage. A high prevalence of *Salmonella* at farms where chickens are raised eventually leads to high levels of bacterial contamination in slaughterhouses. During transportation from farm to processing plant, chickens experience stress, and excrete faeces that may contain *Salmonella*, thus bacterial infection easily occurred (Marin & Lainez, 2009). Earlier studies have shown that the longer the transportation time, the higher the incidence of bacterial infection in poultry (Mulder, 1995).

Salmonella can gain access and contaminate poultry at any processing step, for instance, when multiple chickens are placed in the same water bath during scalding, allowing cross-contamination between *Salmonella*-infected chickens and *Salmonella*-free chickens (Mulder et al., 1978; Rasschaert et al., 2008). Furthermore, *Salmonella*-infected chickens and its *Samonella*-contaminated faeces can easily disseminate through multiple movements during feather removal process (Nde et al., 2007). Evisceration (process of organ removal) may contribute to the contamination of chicken carcasses. For example, *Salmonella* can be easily transferred from the intestines of a contaminated chicken to other chickens which were initially free from *Salmonella* bacteria (Gast & Porter, 2020). A study by Carramiñana et al. (1997) revealed that poultry livers were heavily contaminated with *Salmonella* whereby, 55% of the samples were *Salmonella*-positive indicate cross contamination had possibly occurred. *Salmonella* contamination may also happen if processed chickens are being washed, chilled, and pre-packaged using insanitary machines (Jones et al., 1991). Moreover, contamination can occur via improper handling of poultry meat by poultry workers who do not follow proper sanitation procedures, such as using sanitized utensils and gloves (Mazengia et al., 2015).

During transportation from processing plants to retail markets, *Salmonella* contamination or populations may increase if the transport period is too long or if

preservation conditions are neglected (Corry et al., 2002). Improper handling practices by wholesalers at retail markets are also considered to be a risk factor for *Salmonella* cross-contamination in poultry meat, for example when the same chopping board is used for raw and ready-to-eat food (e.g. vegetables) (El-Aziz, 2013). Table 1 presents a summary of the routes of *Salmonella* contamination in poultry.

Table 1

Summary of route for Salmonella *contamination in poultry at farm, processing plant and at the retailer*

Route	References
Farm	
Hatchery	Foley et al. (2011)
Transportation	Marin and Lainez (2009)
Processing plant	
Scalding	Mulder et al. (1978)
Defeathering	Nde et al. (2007)
Evisceration	Carramiñana et al. (1997)
Retailer	
Long transport period	Corry et al. (2002)
Handling	El-Aziz (2013)

Control Measures to Reduce *Salmonella* Contamination in Poultry Farm

The aim to reduce the prevalence of *Salmonella* in the poultry industry has always been a challenging issue, indicating that a proactive approach to control contamination levels is needed. As discussed earlier, *Salmonella* can begin its entry in the poultry production in the farm during hatchery (White et al., 1997). Thus, it is crucial for farms to implement promising preventive measures at this primary stage. Among the current strategies used to reduce contamination at the farm level is vaccination with both live and dead strains of *Salmonella*. Barrow (2007) reported that a live, attenuated *Salmonella* strain provided better protection than a dead strain in terms of antibody production by chickens. This is because the dead strain cannot induce cytotoxic T cells or secrete IgA antibodies, which are important for the protection of mucosal surfaces. At this point, vaccination with an attenuated *Salmonella* Typhimurium strain has proven to prevent *Salmonella* Typhimurium infection in layer chickens. Additionally, this vaccination also provides additional cross-protective immunity against infections with *Salmonella* Enteritidis, and *Salmonella* Gallinarum serovars (Lee, 2015).

Another immune strategy incorporated in recent years to reduce the pathogen is passive immunity, in which antibodies

are fed to hatchlings. It is expected that this technique will enable the maternal antibodies to be transferred from the yolk to the chick and thus prevent *Salmonella* colonization (Chalghoumi et al., 2009). This hypothesis was confirmed by a study conducted by Rahimi et al. (2007), in which the authors observed that three-day old chicks with *S.* Enteritidis that received purified yolk IgY in drinking water showed lower levels of *S.* Enteritidis in caecum when twenty-eight days old. Antimicrobial activity may involve agglutination of the pathogen to the egg yolk, generating a competition for adhesion sites or stimulation of the immune system by the egg-yolk components (Vandeplas et al., 2010).

The use of antimicrobial compounds is also an option for combatting *Salmonella* in poultry. Gallinacins, cationic antimicrobial peptides that are naturally present in chickens, are effective against *Salmonella*, as they can facilitate interactions with negatively charged membrane pathogens (Milona et al., 2007). Colony-counting assay experiments conducted by van Dijk et al. (2007) showed a strong bactericidal and fungicidal activity against food-borne pathogens such as *Campylobacter jejuni*, *Salmonella* Typhimurium, *Clostridium perfringens*, and *Escherichia coli*. Studies have explored the incorporation of antimicrobial peptides into chicken feed to act as an antibiotic with which to limit *Salmonella* contamination (Bennoune et al., 2009; Joerger, 2003). However, issues regarding economy and ecology must be resolved before this idea can be applied into practice.

Salmonella normally does not survive at pH 3.0; thus, an approach using acidification of feed and drinking water with short chain fatty acids (SCFA) and medium chain fatty acids (MCFA) could result in bactericidal or bacteriostatic (Corrier et al., 1990). MCFA is much more effective than SCFA against *Salmonella*, as only 25mM could cause *Salmonella* to become bacteriostatic compared to SCFA, which would require 100mM (Van Immerseel et al., 2006). This is because MCFA has more carbon molecules (6 – 10), making it more effective. In short, acidification of the chicken environment can be an effective control measure at the farm level, as it can be applied for use with young chickens.

Farm litter modification with various acidification compounds, such as organic acids, formalin, sodium bisulfate, aluminium sulphate, and sulfuric acid has been reported to be a more practical and cost-effective strategy for reducing *Salmonella*-shedding in the litter. According to Vicente et al. (2007), when the pH of the litter is modified, ammonia emissions are reduced, resulting in a significantly lower *Salmonella* count. However, a meaningful control of *Salmonella* requires a careful removal of all litter and subsequent cleaning and disinfection between batches of chicken. Apart from litter modification, acidification of the poultry diet has also proved to be an efficient way to reduce the microbial load. In this approach, the poultry are fed with fermented

liquid feed (FLF). In order to acidify the FLF, lactic acid bacteria are introduced to the liquid feed. Hence, when the poultry consume the FLF, the Enterobacteriaceae along the gastrointestinal tract are reduced (Heres et al., 2003). Usually, fermented feed will contain high lactic and acetic acid contents, which help to reduce *Salmonella* in the anterior part of the gastrointestinal tract (GIT).

Colonization of *Salmonella* in the avian GIT can also be controlled through dietary modification. Klasing (1998) claimed that the dietary characteristics of poultry directly impact the fowls' susceptibility to various pathogens. Hence, the dietary characteristics of poultry should be monitored thoroughly. There have been several studies of dietary modification for poultry, such as introducing high-fibre feed. It is assumed that poultry feeding on dietary fibre will likely prefer the utilization of normal microbial populations, such as *Lactobacillus* and *Bifidobacterium* species (Bedford, 1996). These bacteria can produce lactic acid and maintain a low pH in the GIT. For example, alfalfa is a high-fibre dietary source that helps to promote lactic acid production in the GIT, thus inhibiting *Salmonella* (Donalson et al., 2008).

Nutritional strategy is another approach to combatting the growth of *Salmonella* along the GIT. This is because a damaged GIT may increase the chance for an invasion of pathogens (McDevitt et al., 2006). Hence, to ensure gut health in poultry, the chickens' nutrition can be formulated with several amino acids, such as arginine, glutamine, serine, and threonine. These amino acids can limit the growth of *Salmonella* (Hume et al., 1997). Effective nutrient absorption is an important aspect of poultry diet intervention. It has been hypothesized that the remaining nutrients (i.e. those unabsorbed by the poultry) could possibly be utilized by pathogenic microbes to aid in their growth. Thus, to ensure an efficient absorption process of nutrients, exogenous enzymes, such as alpha-amylase, could be incorporated into corn-based products in the poultry diet (Santos, 2005). It has been shown that poultry growth performance increases significantly as *Salmonella* decreases (Mamber & Katz, 1985).

In addition, to prevent pathogen colonization, antibiotics have been incorporated into poultry feed. However, for the past few years, the emergence of antibiotic-resistant bacteria has become an issue of concern, which may be due to the practice of adding antibiotics to poultry feed (Singh et al., 2006) resulting in the use of antimicrobials in feed being prohibited by law in most parts of the world. Therefore, alternative options, such as the use of probiotics and prebiotics, have been used to promote the growth of microflora communities. The probiotic microbe that is commonly used in poultry feed is of the *Lactobacillus* genus. Jin et al. (1996) found that *Lactobacillus fermentum* and *Lactobacillus acidophilus* blocked the attachment of *Salmonella* Typhimurium and *Salmonella* Pullorum to the epithelial cells due to competition. As for prebiotics,

the mechanism of action for eliminating pathogens is through the direct binding of fermentable carbohydrates and sugar alcohols (e.g. lactose, lactosucrose, and lactulose) to pathogens in intestinal lumen, which prevents bacteria from adhering to epithelial cells (Chambers et al., 1997; Tellez et al., 1993).

Byrd et al. (2008) found that when chlorate was added to poultry feed or drinking water, the incidence of *Salmonella* Typhimurium was low. The reason for this is that in the process of *Salmonella* respiration, nitrate reductase helps to convert nitrate into nitrite. However, nitrate reductase does not have the ability to distinguish between nitrate and chlorate (Rusmana & Nedwell, 2004); hence, in use, the chlorate is reduced to chlorite, which in turn creates a toxic environment and eventually kills the *Salmonella*.

In most developed countries, such as the United Kingdom, Sweden, and Denmark, the control programme for *Salmonella* has been effective, as these countries have adopted a few strategies for the frequent monitoring of poultry farms. For instance, Sweden has implemented an uncompromising strategy in the bacteriological examination of pooled faecal samples. If *Salmonella* is identified in the samples, the flock is destroyed (Wierup et al., 1995). In Japan, *Salmonella* control continues to be an important topic, as the country ranks as one of the highest consumers of eggs in the world. Japanese consumers eat a large portion of their eggs raw, making *Salmonella* control even more important whereby the use of vaccines at poultry farm is more common (Esaki et al., 2013).

Control Measures to Reduce *Salmonella* Contamination in Poultry Processing Plant

Poultry processing is a highly automated industry; therefore, if *Salmonella*-positive chickens from a farm enter the processing plant, a significant amount of cross-contamination is possible. Thus, the best practice for controlling the spread of *Salmonella* is to use multiple approaches at multiple points at which chickens may be contaminated (Stopforth et al., 2007). To address this, several chemicals are currently being applied as antimicrobial controls during reprocessing, poultry chilling and post-chilling (Yang et al., 1998). Antimicrobial chemicals are generally sprayed, but in this technique, the effectiveness is limited due to short contact times and inadequate coverage.

Previously, chlorine has been used in poultry chillers at a concentration of 20 – 50 ppm (G. G. Mead & Thomas, 1973). However, several other antimicrobial chemicals have been discovered that offer a more significant reduction of *Salmonella* in comparison with chlorine. Acidified sodium chlorite, bromine, chlorine dioxide, cetylpyridinium chloride, organic acids, peracetic acid, trisodium phosphate, sodium metasilicate, monochloramine, electrolyzed water, and hypochlorous acid (chlorine) are examples of approved antimicrobials for poultry (Nagel et al., 2013). Among these

antimicrobials, organic acid and peracetic acid are considered environmentally friendly, but they could change the colour of the meat product (Mani-Lopez et al., 2012). Hence, a possible ideal application for poultry would be to combine a few antimicrobials. For example, a combination of peracetic acid and hydrogen peroxide achieves a synergistic effect in reducing *Salmonella* contamination (Bauermeister et al., 2008).

At the slaughterhouse, another approach to the reduction of *Salmonella* contamination that significantly impacts the safety of poultry is chilling. The main aim of chilling is to limit the growth of food-spoilage microbes and *Salmonella*. According to James et al. (2006), immersion chilling using an ice-water mixture causes a larger reduction in the number of microbes compared to the spray chilling method. When chlorine is added to ice water during chilling, the process kills the microbes more effectively. Another effective chilling method is air chilling by which the air-chilled chicken is cooled by passing the birds through several chambers where cold, purified air is circulated to cool the meat, resulting in no added moisture, stronger flavour and decrease chance for contamination (Kim et al., 2017). In the air chilling system, birds travel across the belt for almost three hours passing three different cooling chambers by which each chamber has controlled temperature and humidity. Air chilling is a significant contributor to the tenderness and flavour of the meat as compared to conventional water chilling system. The water chilling system operates differently whereby thousands of chickens are submerged into a cold-water bath mixed with chlorine (to control bacterial contamination), which consequently dilutes the natural juices of the bird (Micciche et al., 2018).

In order to improve the safety of poultry meat and poultry products, the poultry industries take on a process control system called "hazard analysis critical control point" (HACCP). In general, this system helps to ensure product safety by identifying possible health hazards before they occur. The concept of HACCP was developed over 60 years ago by the Pillsbury Company as they were trying to develop microbiologically safe food products for space (NASA) (Gehring & Kirkpatrick, 2020). Thereafter, the HACCP system was implemented and the United States Department of Agriculture Food Safety and Inspection Service (USDA-FSIS) had enforced rule whereby they required meat and poultry plants to incorporate HACCP in their safety operations.

HACCP is considered as an evaluation and control system of the whole food production chain solely for reducing potential health risks to consumers. Safety program in HACCP in poultry processing aims to maintain the safety of the poultry meat as hazards may occur during processing. Thus, possible hazards are defined, evaluated, controlled so that they can be prevented. 'Hazard' means any biological, physical or

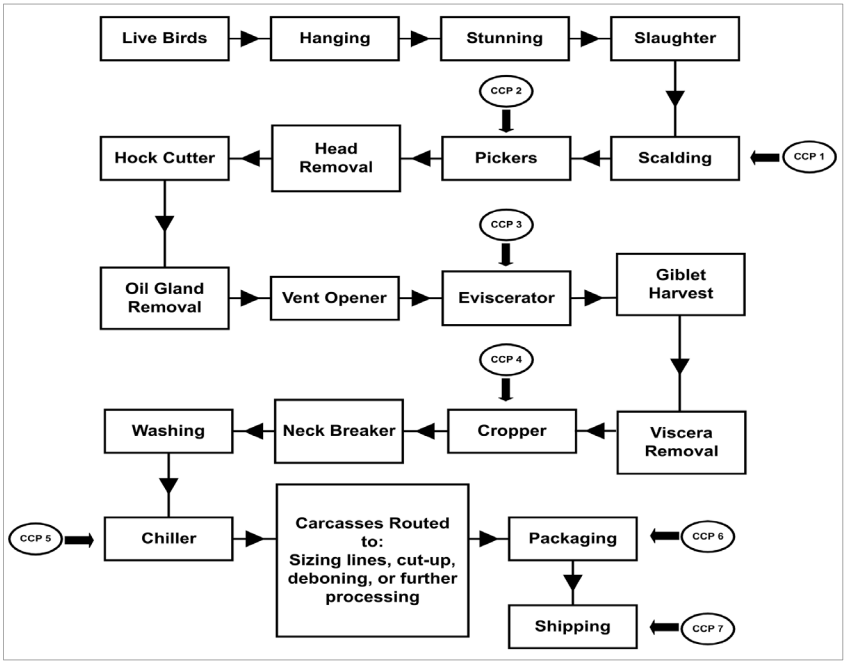

Figure 3. Example of poultry processing HACCP flow diagram (Northcutt and Russell, 2010)

chemical property that can cause adverse health effects for consumers (Park et al., 2014). Poultry processing plants are required to have a HACCP plan. Figure 3 shows the general poultry processing HACCP flow diagram in a poultry processing plant.

Control Measures to Reduce *Salmonella* Contamination in Poultry Retail Market

The final supply chain of poultry meat ends at retail levels. Here, as mentioned above, poultry meat is easily contaminated with *Salmonella* or other microbial pathogens via many routes, which include unhygienic conditions or practices by food handlers or improper storage temperatures of raw poultry meat (Donado-Godoy et al., 2012). In the farm-to-table continuum, post-processing food handling is very important in the reduction of foodborne illnesses caused by *Salmonella*. Therefore, adequate training for the personnel is of a prime factor in the handling of meat that is safe and suitable for human consumption (Zanin et al., 2017). Poultry handlers who are engaged in meat hygiene activities should be well-trained to have an excellent level of knowledge and practice to carry out specified meat hygiene tasks, e.g., post-mortem inspection and HACCP. It is expected that from an appropriate training program, poultry handlers at retailing stage who come into direct contact with meat would maintain appropriate personal cleanliness while working. Besides, these workers

would also have a positive attitude towards the importance of personal cleanliness like hand washing as well as adequate protective clothing appropriate to the circumstances. Good hygiene practices especially washing of hands properly before sales of meat or usage of proper clothing such as hand gloves, masks and head covers can reduce the spread of this microbe to the raw poultry meat.

Foodborne diseases usually arise when the causative organism, initially present in low numbers can multiply on the chicken carcass surface during transport and distribution of poultry to the retail stores. The potential risks may arise through poultry-handling mistakes, such as leaving meat at inappropriate temperature during transportation. It is important to note that *Salmonella* multiplies very slowly at 10 °C, and not at all at 6 °C to 7 °C (Ha et al., 2020). In light with this information, applicable precautionary measures could be done. FDA has prescribed the shippers, carriers, receivers and others who engage in food transport to monitor safe temperatures during poultry meat transport between distribution centres and retail stores as the growth of *Salmonella* on carcasses can be entirely prevented with attentive temperature control (Alfama et al., 2019). On that account, equipment for constant monitoring of temperatures should accompany transport vehicles and bulk containers wherever required. Additionally, the conditions of transport should cater for sufficient protection from any biological, physical or chemical contamination, and should reduce the growth of pathogenic bacteria. If meat is mistakenly exposed to unfavourable temperature conditions or sources of contamination that may affect the quality and safety of poultry meat, an inspection should be conducted by a qualified person before further transport or distribution is granted (Biswas et al., 2019).

When considering the storage of poultry meat, it is supposed that the chickens are already cleaned to be chilled or frozen, and ready for selling. Studies have shown that storage conditions of processed broilers at the retail outlet can affect the bacterial load on the meat (Masoumbeigi et al., 2017). If broiler chickens are not packaged separately, cross-contamination can happen, increasing the occurrence of *Salmonella* within a batch. Shafini et al. (2017) conducted a study to compare the prevalence of *Salmonella* in packed and unpacked chicken sampled from retail outlets. The results showed contamination of *Salmonella* spp. in unpacked chicken were higher (84.8%) compared to the packaged chicken (54.5%). *Salmonella* can also multiply if other storage conditions (including storage temperature, relative humidity and broiler moisture, pH, and storage density) are neglected (Trinetta et al., 2019). Fresh meat, poultry and seafood are considered as the most difficult items to store as these food items are rich in carbohydrate and protein suitable for the utilization of most microbes. Therefore, when keeping this item in the refrigerator, various food safety aspects need to be considered. First of all, these food items have to be kept in a safe temperature which

is 4 °C or lower (Silva et al., 2018). Besides, all carcass meats should be unwrapped and hung so that air can circulate around them. It is important to put absorbent paper beneath the meats for quick clean-up of drips. Fresh meat must not be kept too long whereby maximum period to keep boned meat should be no longer than three days while chicken parts should be discarded within two days. Additionally, fresh meat should be covered with plastic or stainless-steel trays and should be packed in ice when they are stored in the lower shelves of the refrigerator (Masson et al., 2017).

Apart from favourable transport and storage condition, the weather is a key influence on salmonellosis. In Malaysia, the tropical weather temperature allows faster replication of *Salmonella*, increasing the contamination risk throughout the entire poultry supply chain. *Salmonella* is known to proliferate at a warmer climate with higher relative humidity (Ishihara et al., 2020). However, the influence of weather on salmonellosis is not always immediate but most often delayed for around 2 – 4 weeks which possibly because *Salmonella* colonisation takes place earlier during a hot day, however, the consequent human salmonellosis cases only occur when those infected chickens are consumed weeks later (Akil et al., 2014). To address climatic factor-driven *Salmonella*-contamination risk, quality control (QC) officer assure that temperature control schemes are adequate in tropical climate weather. Additionally, public health agencies should review food temperature control guidance for meats handled in retail establishments. Such preventive measures at retailing stage should aim to reduce bacterial transmission and improve food producer and consumer awareness.

Another strategy with which to control *Salmonella* contamination in poultry is through the implementation of hazard analysis and critical control points programs in the entire poultry marketing chain (González-Miret et al., 2006). It is important that the use of the HACCP (hazard analysis critical control point) approach, based on the use of multi-functional strategies (combining the innovative use of sanitizers and modern disinfection techniques) and supervised by professional food handlers and food regulators with a visionary commitment by management from the production, through the processing, preservation, handling, and final preparatory stages, be imposed to help eliminate or reduce significantly the prevalence of *Salmonella*, *Escherichia coli*, and other food-borne pathogens or contaminants and the consequent food poisoning in the society. HACCP is now helping to decrease health risks connected to the distribution of fresh food in wet markets across China. The improvements that HACCP promoted in China's wet markets can be achieved in other countries where local markets are prominent. Continued efforts to enhance food safety and hygiene in wet markets across the globe will also strengthen food security by generating greater access to local food (Zhu et al., 2017).

CONCLUSIONS

The continuation of the poultry supply chain, which begins at the farm and ends at the retailer, requires the monitoring of multiple risk factors in order to control the level of *Salmonella* contamination. Hence, it is important to determine those risk factors that contribute to the *Salmonella* contamination of raw poultry meat so that appropriate control measures can be taken. This review highlights the most recently developed strategies for use in the significant reduction of *Salmonella* in the poultry supply chain. To summarize, the strategies discussed in this review include preventive measures that start at the farm such as the modification of feed and drinking water, immune strategies and feed additives such as antibiotics, probiotics and prebiotics. These approaches have significantly impacted the reduction of *Salmonella* at the farm level. Prevention of bird flock contamination based on the good manufacturing practices and good agricultural practices are most useful ways to prevent contamination of bird flocks before slaughter. Approaches that have been practised at the slaughterhouse include a multiple hurdle strategy (combination of few technologies and approaches), the use of antimicrobial compounds during reprocessing and various poultry-chilling techniques. It has been shown that these strategies play a role in reducing the dissemination of *Salmonella* in poultry meat. However, there is no published data related to logistic scheduling of poultry processing. Therefore, further research on the optimization of logistic scheduling of poultry processing conditions is indicated to fill in the data gap on the effects of applying different logistic scheduling conditions to eliminate *Salmonella* in poultry meat.

ACKNOWLEDGEMENTS

The authors would like to acknowledge the Ministry of Higher Education, Malaysia for the financial support through the Higher Institution Centres of Excellence (HICoE) research grant.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

REFERENCES

Akil, L., Ahmad, H. A., & Reddy, R. S. (2014). Effects of climate change on *Salmonella* infections. *Foodborne Pathogens and Disease*, *11*(12), 974-980. https://doi.org/10.1089/fpd.2014.1802

Alfama, E. R. G., Hessel, C. T., de Oliveira Elias, S., Magalhães, C. R. P., Santiago, M. F. T., Anschau, M., & Tondo, E. C. (2019). Assessment of temperature distribution of cold and hot meals in food services and the prediction growth of *Salmonella* spp. and *Listeria monocytogenes*. *Food Control*, *106*, 106725. https://doi.org/10.1016/j.foodcont.2019.106725

Bailey, J. S., Cox, N. A., Craven, S. E., & Cosby, D. E. (2002). Serotype tracking of *Salmonella* through integrated broiler chicken operations. *Journal of Food Protection*, *65*(5), 742-745. https://doi.org/10.4315/0362-028X-65.5.742

Bansal, N. S., Gray, V., & McDonell, F. (2006). Validated PCR assay for the routine detection of *Salmonella* in food. *Journal of Food Protection*,

69(2), 282-287. https://doi.org/10.4315/0362-028X-69.2.282

Barrow, P. A. (2007). *Salmonella* infections: Immune and non-immune protection with vaccines. *Avian Pathology*, *36*(1), 1–13. https://doi.org/10.1080/03079450601113167

Bauermeister, L. J., Bowers, J. W., Townsend, J. C., & McKee, S. R. (2008). Validating the efficacy of peracetic acid mixture as an antimicrobial in poultry chillers. *Journal of Food Protection*, *71*(6), 1119-1122. https://doi.org/10.4315/0362-028X-71.6.1119

Bedford, M. R. (1996). Interaction between ingested feed and the digestive system in poultry. *Journal of Applied Poultry Research*, *5*(1), 86-95. https://doi.org/10.1093/japr/5.1.86

Bennoune, O., Melizi, M., Khazal, K., & Bourouba, R. (2009). Avian cationic antimicrobial peptides in health and disease: A mini review. *Journal of Animal and Veterinary Advances*, *8*(4), 755-759.

Biswas, C., Leboveic, A., Burke, K., & Biswas, D. (2019). Post-harvest approaches to improve poultry meat safety. In *Food safety in poultry meat production* (pp. 123-138). Springer. https://doi.org/10.1007/978-3-030-05011-5_6

Botteldoorn, N., Heyndrickx, M., Rijpens, N., Grijspeerdt, K., & Herman, L. (2003). *Salmonella* on pig carcasses: Positive pigs and cross contamination in the slaughterhouse. *Journal of Applied Microbiology*, *95*(5), 891-903. https://doi.org/10.1046/j.1365-2672.2003.02042.x

Bryan, F. L. (1980). Foodborne diseases in the United States associated with meat and poultry. *Journal of Food Protection*, *43*(2), 140-150. https://doi.org/10.4315/0362-028X-43.2.140

Byrd, J. A., Burnham, M. R., McReynolds, J. L., Anderson, R. C., Genovese, K. J., Callaway, T. R., Kubena, L. F., & Nisbet, D. J. (2008). Evaluation of an experimental chlorate product as a preslaughter feed supplement to reduce *Salmonella* in meat-producing birds. *Poultry Science*, *87*(9), 1883-1888. https://doi.org/10.3382/ps.2007-00502

Carramiñana, J. J., Yangüela, J., Blanco, D., Rota, C., Agustin, A. I., Ariño, A., & Herrera, A. (1997). *Salmonella* incidence and distribution of serotypes throughout processing in a Spanish poultry slaughterhouse. *Journal of Food Protection*, *60*(11), 1312-1317. https://doi.org/10.4315/0362-028X-60.11.1312

Chalghoumi, R., Beckers, Y., Portetelle, D., & Théwis, A. (2009). Hen egg yolk antibodies (IgY), production and use for passive immunization against bacterial enteric infections in chicken: A review. *Biotechnology, Agronomy, and Society and Environment*, *13*(2), 295-308.

Chambers, J. R., Spencer, J. L., & Modler, H. W. (1997). The influence of complex carbohydrates on *Salmonella* Typhimurium colonization, pH, and density of broiler ceca. *Poultry Science*, *76*(3), 445-451. https://doi.org/10.1093/ps/76.3.445

Chappell, L., Kaiser, P., Barrow, P., Jones, M. A., Johnston, C., & Wigley, P. (2009). The immunobiology of avian systemic salmonellosis. *Veterinary Immunology and Immunopathology*, *128*(1-3), 53-59. https://doi.org/10.1016/j.vetimm.2008.10.295

Chen, C. L., Aziz, S. A., Soe, W. S., & Nordin, F. (2011). Isolation of *Campylobacter* and *Salmonella* from houseflies (*Musca domestica*) in a university campus and a poultry farm in Selangor, Malaysia. *Tropical Biomedicine*, *28*(1), 16-20.

Cooper, G. L., Venables, L. M., Woodward, M. J., & Hormaeche, C. E. (1994). Invasiveness and

persistence of *Salmonella* Enteritidis, *Salmonella* Typhimurium, and a genetically defined *S.* Enteritidis *aroA* strain in young chickens. *Infection and Immunity*, *62*(11), 4739-4746.

Corrier, D. E., Hinton Jr., A., Ziprin, R. L., Beier, R. C., & DeLoach, J. R. (1990). Effect of dietary lactose on cecal pH, bacteriostatic volatile fatty acids, and *Salmonella* Typhimurium colonization of broiler chicks. *Avian Diseases*, *34*(3), 617-625. https://doi.org/10.2307/1591254

Corry, J. E. L., Allen, V. M., Hudson, W. R., Breslin, M. F., & Davies, R. H. (2002). Sources of *Salmonella* on broiler carcasses during transportation and processing: Modes of contamination and methods of control. *Journal of Applied Microbiology*, *92*(3), 424-432. https://doi.org/10.1046/j.1365-2672.2002.01543.x

Cox, N. A., Berrang, M. E., & Cason, J. A. (2000). *Salmonella* penetration of egg shells and proliferation in broiler hatching eggs - A review. *Poultry Science*, *79*(11), 1571-1574. https://doi.org/10.1093/ps/79.11.1571

Cummings, P. L., Sorvillo, F., & Kuo, T. (2010). Salmonellosis-related mortality in the United States, 1990-2006. *Foodborne Pathogens and Disease*, *7*(11), 1393-1399. https://doi.org/10.1089/fpd.2010.0588

D'Aoust, J. Y., & Maurer, J. (2007). *Salmonella* species. In *Food microbiology: Fundamentals and frontiers* (3rd ed.) (pp. 187-236). American Society for Microbiology.

Donado-Godoy, P., Clavijo, V., León, M., Tafur, M. A., Gonzales, S., Hume, M., & Doyle, M. P. (2012). Prevalence of *Salmonella* on retail broiler chicken meat carcasses in Colombia. *Journal of Food Protection*, *75*(6), 1134-1138. https://doi.org/10.4315/0362-028X.JFP-11-513

Donalson, L. M., McReynolds, J. L., Kim, W. K., Chalova, V. I., Woodward, C. L., Kubena, L. F., Nisbet, D. J., & Ricke, S. C. (2008). The influence of a fructooligosaccharide prebiotic combined with alfalfa molt diets on the gastrointestinal tract fermentation, *Salmonella* Enteritidis infection, and intestinal shedding in laying hens. *Poultry Science*, *87*(7), 1253-1262. https://doi.org/10.3382/ps.2007-00166

El-Aziz, D. M. A. (2013). Detection of *Salmonella* Typhimurium in retail chicken meat and chicken giblets. *Asian Pacific Journal of Tropical Biomedicine*, *3*(9), 678-681. https://doi.org/10.1016/S2221-1691(13)60138-0

Esaki, H., Shimura, K., Yamazaki, Y., Eguchi, M., & Nakamura, M. (2013). National surveillance of *Salmonella* Enteritidis in commercial eggs in Japan. *Epidemiology and Infection*, *141*(5), 941-943. https://doi.org/10.1017/S0950268812001355

Foley, S. L., Nayak, R., Hanning, I. B., Johnson, T. J., Han, J., & Ricke, S. C. (2011). Population dynamics of *Salmonella enterica* serotypes in commercial egg and poultry production. *Applied and Environmental Microbiology*, *77*(13), 4273-4279. https://doi.org/10.1128/AEM.00598-11

Frederick, A., & Huda, N. (2011). *Salmonella*, poultry house environments and feeds: A review. *Journal of Animal and Veterinary Advances*, *10*(5), 679-685. https://doi.org/10.3923/javaa.2011.679.685

Gaffga, N. H., Behravesh, C. B., Ettestad, P. J., Smelser, C. B., Rhorer, A. R., Cronquist, A. B., Comstock, N. A., Bidol, S. A., Patel, N. J., Gerner-Smidt, P., Keene, W. E., Gomez, T. M., Hopkins, B. A., Sotir, M. J., & Angulo, F. J. (2012). Outbreak of salmonellosis linked to live poultry from a mail-order hatchery. *New England Journal of Medicine*, *366*(22), 2065-2073. https://doi.org/10.1056/nejmoa1111818

Gal-Mor, O., Boyle, E. C., & Grassl, G. A. (2014). Same species, different diseases: How and why typhoidal and non-typhoidal *Salmonella enterica* serovars differ. *Frontiers in Microbiology*, *5*, 391. https://doi.org/10.3389/fmicb.2014.00391

Gast, R. K., & Porter Jr., R. E. (2020). *Salmonella infections*. https://onlinelibrary.wiley.com/doi/abs/10.1002/9781119371199.ch16

Gebreyes, W. A., Thakur, S., Davies, P. R., Funk, J. A., & Altier, C. (2004). Trends in antimicrobial resistance, phage types and integrons among *Salmonella* serotypes from pigs, 1997–2000. *Journal of Antimicrobial Chemotherapy*, *53*(6), 997-1003. https://doi.org/10.1093/jac/dkh247

Gehring, K. B., & Kirkpatrick, R. (2020). Hazard analysis and critical control points (HACCP). In *Food safety engineering* (pp. 191-204). Springer.

González-Miret, M. L., Escudero-Gilete, M. L., & Heredia, F. J. (2006). The establishment of critical control points at the washing and air chilling stages in poultry meat production using multivariate statistics. *Food Control*, *17*(12), 935-941. https://doi.org/10.1016/j.foodcont.2005.06.012

Graham, S. M. (2002). Salmonellosis in children in developing and developed countries and populations. *Current Opinion in Infectious Diseases*, *15*(5), 507-512. https://doi.org/10.1097/00001432-200210000-00009

Grimont, P. A., & Weill, F. X. (2007). *Antigenic formulae of the* Salmonella *serovars*. https://www.pasteur.fr/sites/default/files/veng_0.pdf

Ha, J., Park, E., Kim, J. S., Lee, S., Kim, S., Lee, J., Choi, Y., Yoon, Y., Oh, H., Kim, Y., Lee, Y., Seo, Y., & Kang, J. (2020). Prevalence of *Salmonella* in cucumbers, antibiotic and acid resistances and description of the kinetic behavior with dynamic model during storage. *Journal of Food Safety*, *40*(2), 12760. https://doi.org/10.1111/jfs.12760

Heres, L., Engel, B., Van Knapen, F., De Jong, M. C., Wagenaar, J. A., & Urlings, H. A. (2003). Fermented liquid feed reduces susceptibility of broilers for *Salmonella* Enteritidis. *Poultry Science*, *82*(4), 603-611. https://doi.org/10.1093/ps/82.4.603

Hume, M. E., Nisbet, D. J., & DeLoach, J. R. (1997). *In vitro* ^{14}C-amino acid fermentation by CF3™, a characterized continuous-flow competitive exclusion culture of caecal bacteria. *Journal of Applied Microbiology*, *83*(2), 236-242. https://doi.org/10.1046/j.1365-2672.1997.00224.x

Ishihara, K., Nakazawa, C., Nomura, S., Elahi, S., Yamashita, M., & Fujikawa, H. (2020). Effects of climatic elements on *Salmonella* contamination in broiler chicken meat in Japan. *Journal of Veterinary Medical Science*, *82*(5), 646-652. https://doi.org/10.1292/jvms.19-0677

James, C., Vincent, C., de Andrade Lima, T. I., & James, S. J. (2006). The primary chilling of poultry carcasses — A review. *International Journal of Refrigeration*, *29*(6), 847-862. https://doi.org/10.1016/j.ijrefrig.2005.08.003

Jin, L. Z., Ho, Y. W., Ali, M. A., Abdullah, N., & Jalaludin, S. (1996). Effect of adherent *Lactobacillus* spp. on *in vitro* adherence of salmonellae to the intestinal epithelial cells of chicken. *Journal of Applied Bacteriology*, *81*(2), 201-206. https://doi.org/10.1111/j.1365-2672.1996.tb04501.x

Joerger, R. D. (2003). Alternatives to antibiotics: Bacteriocins, antimicrobial peptides and bacteriophages. *Poultry Science*, *82*(4), 640–647. https://doi.org/10.1093/ps/82.4.640

Jones, F. T., Axtell, R. C., Rives, D. V., Scheideler, S. E., Tarver Jr., F. R., Walker, R. L., &

Wineland, M. J. (1991). A survey of *Salmonella* contamination in modern broiler production. *Journal of Food Protection*, *54*(7), 502-507. https://doi.org/10.4315/0362-028X-54.7.502

Juneja, V. K., Melendres, M. V., Huang, L., Gumudavelli, V., Subbiah, J., & Thippareddi, H. (2007). Modeling the effect of temperature on growth of *Salmonella* in chicken. *Food Microbiology*, *24*(4), 328-335. https://doi.org/10.1016/j.fm.2006.08.004

Kim, K. T., Kim, J. H., Park, Y. M., Myung, K. S., & Park, T. W. (2017). Comparison of preservation in poultry carcasses processed by different chilling systems. *Korean Journal of Veterinary Service*, *40*(4), 245-251. https://doi.org/10.7853/kjvs.2017.40.4.245

Klasing, K. C. (1998). Nutritional modulation of resistance to infectious diseases. *Poultry Sci*ence, *77*(8), 1119–1125. https://doi.org/10.1093/ps/77.8.1119

Lee, J. H. (2015). Protection against *Salmonella* Typhimurium, *Salmonella* Gallinarum, and *Salmonella* Enteritidis infection in layer chickens conferred by a live attenuated *Salmonella* Typhimurium strain. *Immune Network*, *15*(1), 27-36. https://doi.org/10.4110/in.2015.15.1.27

Li, E. K., Cohen, M. G., Ho, A. K., & Cheng, A. F. (1993). *Salmonella* bacteraemia occurring concurrently with the first presentation of systemic lupus erythematosus. *Rheumatology*, *32*(1), 66-67. https://doi.org/10.1093/rheumatology/32.1.66

Mamber, S. W., & Katz, S. E. (1985). Effects of antimicrobial agents fed to chickens on some Gram-negative enteric bacilli. *Applied and Environmental Microbiology*, *50*(3), 638-648.

Mani-Lopez, E., García, H. S., & López-Malo, A. (2012). Organic acids as antimicrobials to control *Salmonella* in meat and poultry products. *Food Research International*, *45*(2), 713-721. https://doi.org/10.1016/j.foodres.2011.04.043

Marin, C., & Lainez, M. (2009). *Salmonella* detection in faeces during broiler rearing and after live transport to the slaughterhouse. *Poultry Science*, *88*(9), 1999-2005. https://doi.org/10.1016/j.foodres.2011.04.043

Masoumbeigi, H., Tavakoli, H. R., Koohdar, V., Mashak, Z., & Qanizadeh, G. (2017). The environmental influences on the bacteriological quality of red and chicken meat stored in fridges. *Asian Pacific Journal of Tropical Biomedicine*, *7*(4), 367-372. https://doi.org/10.1016/j.apjtb.2017.01.006

Masson, M., Delarue, J., & Blumenthal, D. (2017). An observational study of refrigerator food storage by consumers in controlled conditions. *Food Quality and Preference*, *56*(Part B), 294-300. https://doi.org/10.1016/j.foodqual.2016.06.010

Mazengia, E., Fisk, C., Liao, G., Huang, H., & Meschke, J. (2015). Direct observational study of the risk of cross-contamination during raw poultry handling: Practices in private homes. *Food Protection Trends*, *35*(1), 8-23.

McDevitt, R. M., Brooker, J. D., Acamovic, T., & Sparks, N. H. C. (2006). Necrotic enteritis; A continuing challenge for the poultry industry. *World's Poultry Science Journal*, *62*(2), 221-247. https://doi.org/10.1079/WPS200593

Mead, G. C. (Ed.). (2012). *Processing of poultry*. https://www.springer.com/gp/book/9781461358541

Mead, G. G., & Thomas, N. L. (1973). Factors affecting the use of chlorine in the spin-chilling of eviscerated poultry. *British Poultry Science*, *14*(1), 99-117. https://doi.org/10.1080/00071667308416000

Micciche, A. C., Feye, K. M., Rubinelli, P. M., Wages, J. A., Knueven, C. J., & Ricke, S. C. (2018). The implementation and food safety issues associated with poultry processing reuse water for conventional poultry production systems in the United States. *Frontiers in Sustainable Food Systems*, *2*, 70. https://doi.org/10.3389/fsufs.2018.00070

Milona, P., Townes, C. L., Bevan, R. M., & Hall, J. (2007). The chicken host peptides, gallinacins 4, 7, and 9 have antimicrobial activity against *Salmonella* serovars. *Biochemical and Biophysical Research Communications*, *356*(1), 169-174. https://doi.org/10.1016/j.bbrc.2007.02.098

Mulder, R. W. A. W. (1995). Impact of transport and related stresses on the incidence and extent of human pathogens in pig meat and poultry. *Journal of Food* Safety, *15*(3), 239-246. https://doi.org/10.1111/j.1745-4565.1995.tb00136.x

Mulder, R. W. A. W., Dorresteijn, L. W. J., & Van Der Broek, J. (1978). Cross-contamination during the scalding and plucking of broilers. *British Poultry Science*, *19*(1), 61-70. https://doi.org/10.1080/00071667808416443

Nagel, G. M., Bauermeister, L. J., Bratcher, C. L., Singh, M., & McKee, S. R. (2013). *Salmonella* and *Campylobacter* reduction and quality characteristics of poultry carcasses treated with various antimicrobials in a post-chill immersion tank. *International Journal of Food Microbiology*, *165*(3), 281-286. https://doi.org/10.1016/j.ijfoodmicro.2013.05.016

Nde, C. W., McEvoy, J. M., Sherwood, J. S., & Logue, C. M. (2007). Cross contamination of turkey carcasses by *Salmonella* species during defeathering. *Poultry Science*, *86*(1), 162-167. https://doi.org/10.1093/ps/86.1.162

Northcutt, J. K., & Russell, S. M. (2010). *General guidelines for implementation of HACCP in a poultry processing plant*. https://athenaeum.libs.uga.edu/handle/10724/12487

Painter, J. A., Hoekstra, R. M., Ayers, T., Tauxe, R. V., Braden C. R., Angulo, F. J., & Griffin, P. M. (2013). Attribution of foodborne illnesses, hospitalizations and deaths to food commodities by using outbreak data, United States, 1998-2008. *Emerging Infectious Diseases*, *19*(3), 407. https://doi.org/10.3201%2Feid1903.111866

Park, S. H., Aydin, M., Khatiwara, A., Dolan, M. C., Gilmore, D. F., Bouldin, J. L., Ahn, S., & Ricke, S. C. (2014). Current and emerging technologies for rapid detection and characterization of *Salmonella* in poultry and poultry products. *Food Microbiology*, *38*, 250-262. https://doi.org/10.1016/j.fm.2013.10.002

Public Health Notice. (2015). *Public Health Notice - Outbreak of* Salmonella *infections linked to frozen raw breaded chicken products*. https://www.canada.ca/en/public-health/services/public-health-notices/2018/outbreaks-salmonella-infections-linked-raw-chicken-including-frozen-raw-breaded-chicken-products.html

Rahimi, S., Zahra Moghadam, S., Taghi Zahraei, S., Torshizi, M. A. K., & Grimes, J. L. (2007). Prevention of *Salmonella* infection in poultry by specific egg-derived antibody. *International Journal of Poultry Science*, *6*(4), 230-235. https://doi.org/10.3923/ijps.2007.230.235

Raji, A. O. (2018). Comparative evaluation of some properties of chicken and Japanese quail eggs subjected to different storage methods. *Poultry Science Journal*, *6*(2), 155-164. https://doi.org/10.22069/psj.2018.14403.1308

Rasschaert, G., Houf, K., Godard, C., Wildemauwe, C., Pastuszczak-Frak, M., & De Zutter, L. (2008). Contamination of carcasses with *Salmonella* during poultry slaughter. *Journal of Food Protection*, *71*(1), 146-152. https://doi.org/10.4315/0362-028X-71.1.146

Reed, K. A., Hobert, M. E., Kolenda, C. E., Sands, K. A., Rathman, M., O'Connor, M., & Madara, J. L. (2002). The *Salmonella* Typhimurium flagellar basal body protein FliE is required for flagellin production and to induce a proinflammatory response in epithelial cells. *Journal of Biological Chemistry*, *277*(15), 13346-13353. https://doi.org/10.1074/jbc.M200149200

Rodríguez, D. M., & Suárez, M. C. (2014). *Salmonella* spp. in the pork supply chain: A risk approach. *Revista Colombiana de Ciencias Pecuarias*, *27*(2), 65-75.

Rouger, A., Tresse, O., & Zagorec, M. (2017). Bacterial contaminants of poultry meat: Sources, species, and dynamics. *Microorganisms*, *5*(3), 50. https://doi.org/10.3390/microorganisms5030050

Rusmana, I., & Nedwell, D. B. (2004). Use of chlorate as a selective inhibitor to distinguish membrane-bound nitrate reductase (Nar) and periplasmic nitrate reductase (Nap) of dissimilative nitrate reducing bacteria in sediment. *FEMS Microbiology Ecology*, *48*(3), 379-386. https://doi.org/10.1016/j.femsec.2004.02.010

Salomonsson, A. C., Aspán, A., Johansson, S., Heino, A., & Häggblom, P. (2005). *Salmonella* detection by polymerase chain reaction after pre-enrichment of feed samples. *Journal of Rapid Methods and Automation in Microbiology*, *13*(2), 96-110. https://doi.org/10.1111/j.1745-4581.2005.00012.x

Santos, A. A. (2005). *Poultry intestinal health through diet formulation and exogenous enzyme supplementation* [Doctoral thesis, North Carolina State University]. NC State University Libraries. https://repository.lib.ncsu.edu/handle/1840.16/4359

Scallan, E., Hoekstra, R. M., Angulo, F. J., Tauxe, R. V., Widdowson, M. A., Roy, S. L., & Griffin, P. M. (2011). Foodborne illness acquired in the United States — Major pathogens. *Emerging Infectious Diseases*, *17*(1), 7. https://doi.org/10.3201%2Feid1701.P11101

Schikora, A., Virlogeux-Payant, I., Bueso, E., Garcia, A. V., Nilau, T., Charrier, A., Pelletier, S., Menanteau, P., Baccarini, M., Velge, P., & Hirt, H. (2011). Conservation of *Salmonella* infection mechanisms in plants and animals. *PLOS One*, *6*(9), e24112. https://doi.org/10.1371%2Fjournal.pone.0024112

Schraidt, O., & Marlovits, T. C. (2011). Three-dimensional model of *Salmonella*'s needle complex at subnanometer resolution. *Science*, *331*(6021), 1192-1195. https://doi.org/10.1126/science.1199358

Seo, S., & Lee, M. (2004). The serogroup and antimicrobial resistance of *Salmonella* spp. isolated from the clinical specimens during 6 years in a tertiary university hospital. *Korean Journal of Clinical Microbiology*, *7*(1), 72-76.

Shafini, A. B., R. Son, N. A., Mahyudin, N. A., Rukayadi, Y., & Tuan Zainazor, Y. C. (2017). Prevalence of *Salmonella* spp. in chicken and beef from retail outlets in Malaysia. *International Food Research Journal*, *24*(1), 437-449.

Shivaprasad, H. L., Timoney, J. F., Morales, S., Lcio, B., & Baker, R. C. (1990). Pathogenesis of *Salmonella* Enteritidis infection in laying chickens. I. Studies on egg transmission, clinical signs, fecal shedding and serological responses. *Avian Diseases*, *34*(3), 548-557. https://doi.org/10.2307/1591243

Silva, F., Domingues, F. C., & Nerín, C. (2018). Trends in microbial control techniques for poultry products. *Critical Reviews in Food Science and Nutrition*, *58*(4), 591-609. https://doi.org/10.1080/10408398.2016.1206845

Singh, B. R., Singh, P., Verma, A., Agrawal, S., Babu, N., Chandra, M., & Agarwal, R. K. (2006). A study on prevalence of multi-drug-resistant (MDR) *Salmonella* in water sprinkled on fresh vegetables in Bareilly, Moradabad, and Kanpur (northern Indian cities). *Journal of Public Health*, *14*(3), 125. https://doi.org/10.1007/s10389-005-0015-3

Slavik, M. F., Kim, J. W., & Walker, J. T. (1995). Reduction of *Salmonella* and *Campylobacter* on chicken carcasses by changing scalding temperature. *Journal of Food Protection*, *58*(6), 689-691. https://doi.org/10.4315/0362-028X-58.6.689

Stopforth, J. D., O'connor, R., Lopes, M., Kottapalli, B., Hill, W. E., & Samadpour, M. (2007). Validation of individual and multiple-sequential interventions for reduction of microbial populations during processing of poultry carcasses and parts. *Journal of Food Protection*, *70*(6), 1393-1401. https://doi.org/10.4315/0362-028X-70.6.1393

Tellez, G., Dean, C. E., Corrier, D. E., DeLoach, J. R., Jaeger, L., & Hargis, B. M. (1993). Effect of dietary lactose on cecal morphology, pH, organic acids and *Salmonella* Enteritidis organ invasion in Leghorn chicks. *Poultry Science*, *72*(4), 636–642. https://doi.org/10.3382/ps.0720636

Thomas, N. L. (1977). The continuous chilling of poultry in relation to EEC requirements. *International Journal of Food Science and Technology*, *12*(2), 99-114. https://doi.org/10.1111/j.1365-2621.1977.tb00092.x

Trinetta, V., McDaniel, A., Magossi, G., Yucel, U., & Jones, C. (2019). Effects of different moisture and temperature levels on *Salmonella* survival in poultry fat. *Translational Animal Science*, *3*(4), 1369-1374. https://doi.org/10.1093/tas/txz090

van Asten, F. J., Hendriks, H. G., Koninkx, J. F., & van Dijk, J. E. (2004). Flagella-mediated bacterial motility accelerates but is not required for *Salmonella* serotype Enteritidis invasion of differentiated Caco-2 cells. *International Journal of Medical Microbiology*, *294*(6), 395-399. https://doi.org/10.1016/j.ijmm.2004.07.012

Van der Vorst, J. G., Da Silva, C. A., & Trienekens, J. H. (2007). *Agro-industrial supply chain management: Concepts and applications*. http://www.fao.org/3/a-a1369e.pdf17.pdf

van Dijk, A., Veldhuizen, E. J., Kalkhove, S. I., Tjeerdsma-van Bokhoven, J. L., Romijn, R. A., & Haagsman, H. P. (2007). The β-defensin gallinacin-6 is expressed in the chicken digestive tract and has antimicrobial activity against food-borne pathogens. *Antimicrobial Agents and Chemotherapy*, *51*(3), 912-922. https://doi.org/10.1128/AAC.00568-06

Van Immerseel, F., Russell, J. B., Flythe, M. D., Gantois, I., Timbermont, L., Pasmans, F., Haesebrouck, F., & Ducatelle, R. (2006). The use of organic acids to combat *Salmonella* in poultry: A mechanistic explanation of the efficacy. *Avian Pathology*, *35*(3), 182–188. https://doi.org/10.1080/03079450600711045

Vandeplas, S., Dauphin, R. D., Beckers, Y., Thonart, P., & Thewis, A. (2010). *Salmonella* in chicken: Current and developing strategies to reduce contamination at farm level. *Journal of Food Protection*, *73*(4), 774-785. https://doi.org/10.4315/0362-028X-73.4.774

Vicente, J. L., Higgins, S. E., Hargis, B. M., & Tellez, G. (2007). Effect of poultry guard litter amendment on horizontal transmission of *Salmonella* Enteritidis in broiler chicks. *International Journal of Poultry Science*, *6*(5), 314–317. https://doi.org/10.3923/ijps.2007.314.317

White, P. L., Baker, A. R., & James, W. O. (1997). Strategies to control *Salmonella* and *Campylobacter* in raw poultry products. *Revue Scientifique et Technique-Office International des Epizooties*, *16*(2), 525-541. https://doi.org/10.1016/0168-1605(94)00090-S

Wierup, M., Engström, B., Engvall, A., & Wahlström, H. (1995). Control of *Salmonella* Enteritidis in Sweden. *International Journal of Food Microbiology*, *25*(3), 219-226. https://doi.org/10.1016/0168-1605(94)00090-S

Wirsenius, S., Azar, C., & Berndes, G. (2010). How much land is needed for global food production under scenarios of dietary changes and livestock productivity increase in 2030?. *Agricultural Systems*, *103*(9), 621-638. https://doi.org/10.1016/j.agsy.2010.07.005

Yang, Z., Li, Y., & Slavik, M. (1998). Use of antimicrobial spray applied with an inside–outside birdwasher to reduce bacterial contamination on prechilled chicken carcasses. *Journal of Food Protection*, *61*(7), 829-832. https://doi.org/10.4315/0362-028X-61.7.829

Zanin, L. M., da Cunha, D. T., de Rosso, V. V., Capriles, V. D., & Stedefeldt, E. (2017). Knowledge, attitudes and practices of food handlers in food safety: An integrative review. *Food Research International*, *100*, 53-62. https://doi.org/10.1016/j.foodres.2017.07.042

Zhu Y., Lai H., Zou L., Yin S., Wang C., Han X., Xia X., Hu K., He L., Zhou K., Chen S., Ao, X., & Liu, S. (2017). Antimicrobial resistance and resistance genes in *Salmonella* strains isolated from broiler chickens along the slaughtering process in China. *International Journal of Food Microbiology*, *259*, 43-51. https://doi.org/10.1016/j.ijfoodmicro.2017.07.023

Pertanika J. Trop. Agric. Sci. 44 (1): 25 - 48 (2021)

PERTANIKA JOURNALS

TROPICAL AGRICULTURAL SCIENCE

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Review Article

Growth and Reproductive Performance of the Indigenous Kedah-Kelantan (KK) Cattle: A Review

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ABSTRACT

Kedah-Kelantan (KK) cattle plays a significant role for beef industry in Malaysia. KK cattle is a well-adapted local breed reared by traditional farming system with low quality feeds. KK cows normally produce a calf per year, which attracts farmers for commercial production. Currently, KK cattle is playing an important role for profitable beef production in Malaysia since the imported exotic breeds, crossbreds, and synthetic breeds of cattle could not perform to their full potential for sustainability of the livestock industry in the country. Consequently, nowadays, importance of the unique Malaysian beef breed (KK) has been increasing gradually in consideration to the changing climatic situation and adaptibility. Meanwhile, it is required to know the productive and reproductive performance of KK cattle for a further long term sustainable breeding program. As such reviewing growth performance, age at puberty and maturity, semen quality, scrotal biometry, libido efficiency, conception rate, service per conception, and calving interval are fundamental. There exist limited systemic studies and in-depth reviews based on these key reproductive, growth characteristics, and indexes for KK cattle. Therefore, reproductive key parameters of KK cattle were reviewed with the aim of understanding the challenges on the production of KK cattle and to suggest possible strategies to alleviate those challenges.

Keywords: Growth, KK cattle, puberty, reproductive performance, semen quality

ARTICLE INFO

Article history:
Received: 20 June 2020
Accepted: 23 September 2020
Published: 24 February 2021

DOI: https://doi.org/10.47836/pjtas.44.1.02

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ISSN: 1511-3701
e-ISSN: 2231-8542

INTRODUCTION

Kedah-Kelantan (KK) cattle is an indigenous unique cattle breed in Malaysia. It is a well-adapted but slower growth performing local breed comprising 85% (593, 299 heads) of the total beef cattle population (Department of Veterinary Services [DVS], 2014). It is highly tolerant to extreme temperature and humidity. It is reared by traditional farming system with low quality feeds. Large genetic variations are found within KK cattle than the synthetic breeds. It is a great scope to improve the KK cattle through long term appropriate breeding effort. KK cattle are suitable for commercial beef production (Warzukni & Haron, 2010) which is playing a fundamental role for profitable beef production in Malaysia (Ariff et al., 2015; Thundathil et al., 2016).

Growth performance is one of the most important economic traits of beef cattle production. KK cows have been crossed with Brahman, Hereford, Angus, Shorthorn, Charolais, Limousin, and Semental Sire and so on for increasing their growth performance. Consequences, all animals crossed with KK cows have shown higher growth rate and body weight compared to KK straight bred cattle. On the contrary, KK cows crossing with different breeds affected the purity of Kedah-Kelantan cattle. Furthermore, genetic resource of Malaysian unique local beef cattle is declining as more emphasis has been given on crossing KK cows with different exotic beef breeds in order to increase beef production. Moreover, reduced survivability and fertility caused a decline in the profitability and sustainability of KK crossbred cattle in the tropical environment of Malaysia (Jamaludin et al., 2014). As a result, KK purebred cattle have been considered the natural heritage and unique natural animal genetic resouce for sustainable beef production in the country.

Better reproductive performance of breeding animals is the pre-requisite for efficient beef production. It can be said that bulls with good quality semen are the half of a cattle herd (Thundathil et al., 2016). Semen from one superior fertile bull can be used to thousands cows by exploiting that male genetic potentiality to a specific cattle population (Devkota et al., 2008). Approximately 40% lower fertility was observed in breeding bull that were not selected on the basis of their semen quality (Sofienaz et al., 2014). It has been reported that more than 90% KK cows reared by small-scale farmers are not bred by superior KK bulls. Furthermore, few practices of selective breeding program to improve the KK cattle population do exist based on the evaluation of bulls for their semen quality. Consequently, farmers are usually breeding their cows with poor quality KK bulls without proper breeding records which is a great challenge for KK cattle production (Ariff, et al., 2015; Johari & Jasmi, 2009).

Fertility is of primary economic consideration in the beef cattle industry. The importance of fertility, growth rate, and carcass quality has been correlated at the ratio of 10:2:1. KK cattle has higher fertility rate for producing a calf per year which is one of the most important traits of KK breed (A. W. M. Hafiz, 2019;

Clayton, 1983; Johari & Jasmi, 2009; M. A. R. Hafiz et al., 2009; Tan et al., 1985). Fertility was found as 46.3% to 52.5% in KK cattle reared by small-holder farmers at rural areas of Malaysia (Ariff et al., 2015). It is seen that, in terms of reproductive characteristics, especially fertility trait, KK cattle shows better performance than that of KK crossbred cattle in the country.

Selection of beef bulls for high quality semen is always much more important rather than a cow (Okere et al., 2014). Beef bulls are usually selected based on their growth performance, age, and weight at puberty, scrotal biometry, testosterone level and semen quality (Brito, 2014; Perumal, 2014; Sarsaifi et al., 2013). Body weight and scrotal circumference are commonly used to predict the semen producing efficiency of a breeding bull (Yimer et al., 2011). Recent studies revealed that beef bull selection on basis of scrotal circumference at growing stage was more reliable than adult stages (Gopinathan et al., 2018; Penitente-Filho et al., 2018). Therefore, reproductive key parameters of KK cattle with more emphasis on KK bulls have been reviewed with the aim of understanding the challenges on the production of KK cattle and to suggest possible strategies to alleviate those challenges.

BEEF CATTLE PRODUCTION IN MALAYSIA

The livestock industry plays a significant role in producing valuable animal protein for the human population of Malaysia (Kusriatmi et al., 2014). The livestock sector comprises beef cattle, buffalo, goat and sheep sub-sectors. Beef supply alone contributed 0.011% to the GDP and earned around RM 62 million in the agricultural sector in 2010 (Ariff et al., 2015). The KK cattle are the most important indigenous cattle in Malaysia. KK cattle are unique potential genetic resource for commercial beef production. The KK cattle are small-sized breed ranging in matured weight of 300 kg to 312 kg for male, and from 219 to 240 for female. In comparison, the mature weight of Brahman cattle from India is between 800 and 1100 kg but they were not profitable due to high mortality as well as poor reproductive performance. Malaysia needs to increase livestock production to meet up at least 50% of the local market needs. Presently, the local production of beef is only able to supply about 30% of the local consumption. In 2017, Malaysia produced 52,000 tonnes of beef worth RM 169 million and imported beef worthy of RM1.14 billion annually to meet the local demand of about 191, 000 tonnes (Loh, 2004). Therefore, KK beef cattle have been playing a significant role to meet up the growing demand of animal protein in Malaysia. However, KK cattle are normally reared in the subsistence farming system by smallholder farmers in Malaysia that makes KK cattle more sustainable in relation to climate change in the country. Role of three types of beef cattle breeds in Malaysia are as follows:

Exotic Beef Cattle Breeds

Malaysia has taken some initiatives to improve the beef production for fulfilling

the existing gap and growing demand of the beef of the country. Exotic beef breed importation is one of the most important efforts for accelerating the beef industry in the country. For that reason, Malaysia has imported more than 15 exotic breeds like Brahman, Branggus, Hereford, Aberdeen, Nelore, Angus, Droughmaster, Santa Gertudis, Bali, Shorthorn, Charolais, Limousin, Semental, and Chineese Yellow cattle from different regions of the world. However, most of the exotic breeds did not sustain in the Malaysian environment except a few breeds (Jamaludin et al., 2014; J. J. Abdullah, 1993; Johari & Jasmi, 2009). Breeds did not significantly contribute within 55 years of effort for the development of beef industry in Malaysia because of their poor reproductive performance and high mortality rate due to hot environment. Exotic breeds from various environmental conditions did not sustain in the tropical environment of Malaysia. Heat stress of this environment might have a direct effect on their production performance, fertility and survivability.

Crossbred Beef Cattle

Malaysia started the cross-breeding program in 1970 for increasing the production performance of KK cattle reported by Flint (1971). Initial crossbreeding program for beef production was started at UPM by crossing KK cows with Brahman and Hereford bulls (Flint, 1971). KK cows were crossed with more than fifteen exotic beef breeds for accelerating the beef industry in Malaysia (Ariff et al., 1993; Dahlan, 1985; A. W. M. Hafiz et al., 2014; Raymond, 2012). The Brakmas and Charoke synthetic cattle breeds were produced by 50% KK cows with 50% Brahman and 50% Charolais, respectively. All animals crossed with KK have shown higher growth rate and body weight in comparison to KK straight bred cattle. Crossbred cattle have low reproductive performance despite more growth efficiency (Abdulla et al., 2016). Furthermore, KK cows crossing with exotic breeds affected the purity of Kk cattle population. Moreover, survivability and fertility problem of KK crossbred cattle are acute in the hot environment of Malaysia (Jamaludin et al., 2014).

Indigenous Kedah-Kelantan (KK) Cattle (*Bos indicus*)

KK cattle are small sized Zebu type cattle with fatty hump and poorly developed dewlap. KK's are local beef breed like local Thai cattle and Yellow cattle of Southern China. KK's are mostly brown coat with solid body conformation along with reddish, black and grey spot. They have a broad and short head. Their neck is narrow and deep while their horns are short with variable size. The tail is long with switch about reaching the ground (around 70-78cm). They are highly fertile with strong mothering ability (Johari et al., 1994). They are well adapted to Malaysian tropical environment and mostly resistant to common diseases. KK cattle are considered as the natural animal genetic resource and it is called natural heritage and unique genetic resource of beef. They are reared with low quality feed

by rural small farmers through grazing and tethering system. Growth rate of KK is slow despite the type of the beef cattle. However, KK cattle are better for lean meat production in comparison to their crossbreds (Raymond & Hasan, 2012). Table 1 summarizes the key features, productive, and reproductive traits of KK cattle.

Advantages of Kedah-Kelantan Cattle over Exotic Breeds and Crossbreds in Malaysia

KK cattle are fully adapted with Malaysian environment of higher temperature, humidity, and rainfall. Despite this scope, Malaysia has to import about 80% of the total requirement of beef due to low production performance (Warzukni & Haron, 2010). Profitability and sustaianbility of livestock farming depends on the availability, cost and quality of raw ingredients for animal feeds. Feeds incur most part of production cost that involves about 70% to 80% cost of production in any livestock industry. Whereas, KK cattle mostly depends on locally available feedstuff on grazing lands with only some concentrate supplementation like palm kernel cake (PKC) and salt lick (Loh, 2004). The cost of animal feeds that contribute to the total cost of production is between 5.4% and 19.3% for both dairy and beef cattle production in Malaysia which is very low in comparison to the proportion of total production cost in other countries of the world. However, crossbred cattle need to be balanced ration with improved housing

Table 1
Key features of Malaysian Kedah-Kelantan cattle production

Characteristics	Results	References
Breed	Tropical breed (*Bos indicus*)	Devendra and Choo (1975a); Devendra et al. (1975)
Origin	Malaysia	Devendra and Choo (1975a)
Coat colour	Mostly brown	Raymond (2012)
Rearing purpose	Subsistence farming	Johari and Jasmi (2009)
Feeding habit	Low quality feeds	Loh (2004)
Rearing system	Traditional	Jamaludin et al. (2014)
Adaptability	Fully adapted to Malaysia	Ariff et al. (2015)
Diseases resistance	High resistance against many diseases	Jamaludin et al. (2014)
Calf mortality (%)	5.50	Sivarajasingam (1984)
Mortality rate (%)	5-10	Mohamed et al. (2013)
Dressing percentage	45-55	Devendra and Choo (1975a)
	55.90	Dahlan et al. (1992)
Meat: Bone ratio	5.4:1	Devendra and Choo (1975a)
	3-7:1	Dahlan et al. (1992)
Calf production (no.)/year	One calf	Johari and Jasmi (2009)
Feed conversion ratio (FCR)	6.55	Ariff et al. (1993)
Average dry matter intake (kg)	3.03	Sukri and Idris (1982)

system whereas KK cattle needs traditional housing system with low quality feeds. KK cattle has higher fertility rate for producing a calf per year which is one of the most important trait of KK breed (Johari & Jasmi, 2009). Fertility was found to be 46.3% to 52.5% in KK cattle reared by small-holder farmers at rural areas of Malaysia (Ariff et al., 2015). So, it can be certainly said that in terms of reproductive characteristics, especially fertility trait of KK cattle shows the better performance than that of KK crossbreds. Fertility is a primary economic consideration in the beef cattle industry. The importance of fertility, growth rate, and carcass quality has been correlated at the ratio of 10:2:1. Higher genetic variations were found within Kedah-Kelantan cattle than the synthetic breeds. So, there is a great scope to improve the KK cattle through long term selective breeding program. KK cattle are suitable for commercial beef production in Malaysia (Johari et al., 1994), which is playing a fundamental role for profitable beef production in Malaysia (Jamaludin et al., 2014). On the other hand, crossbred cattle have lower fertility in comparison to KK cattle. KK cattle are strongly resistant against the most viral and bacterial diseases. They are also highly resistant against the most external and internal parasities. For that reason, only FMD and HS vaccines are given to KK cattle as disease prevention. Parasitic diseases are fully controlled by deworming of animals twice or sometimes once a year. No or minimum cost is required in bio-security measures to control and prevent any diseases in subsistence farming system. Furthermore, KK cattle can be reared by minimum cost for disease prevention and control measures because of their high resistance to diseases. Thus, KK cattle can contribute more and more for beef cattle production in Malaysia due to their well adaptability with progressive climate changes.

GROWTH PERFORMANCE OF KK CATTLE

Growth performance is one of the most important traits for beef production system. Growth performance is normally measured by body weight and average daily gain of an animal in a certain period of time. Body weight is the most essential tool to assess the reproductive efficiency and growth performance of breeding animals. It also helps to determine the correct amount of feed to be provided to animal thereby avoiding overfeeding or underfeeding. Body weight measurement is the most vital, easier, faster, and cheaper technique to take decision for breeding, feeding, and veterinary services of animals. Moreover, body weight helps to determine the age at puberty of male and female animals. Methods for body weight measurements are electronic scale, weight band and height, width, and heart girth. Electronic scale is a generally used device to determine the body weight of animal perfectly. Growth is frequently measured in body weight gain per unit of time. Growth is also defined as a progressive increase in size or weight of an animal in a specific time period (Bures & Barton, 2012). Growth can also be associated with accumulation

of nutrients in the body over the lifetime of an animal along with other factors like breed type, management, and environment. Growth performance in the peri-pubertal age is a good indicator of reproductive efficiency of bulls for artificial insemination program or natural services. Growth performance is directly related to breed, age, scrotal circumference, and testicular hormones (Lee et al., 2009; Silva et al., 2017). On the other hand, growth affects greatly the semen quality especially sperm motility, sperm concentration, and sperm morphology of beef bulls. For better understanding, meta-analysis was performed on body weight and average daily gain of KK bulls and cows by one sample *t*-test using SPSS computer program.

KK BULLS

Body weight and average daily gain (ADG) of KK bulls in different ages were summarized in Table 2. Table 3 showed the meta-data analysis on body weight and average daily gain of KK bulls in different ages. Mean birth weight of KK bull calves were found to be 15.28 kg with significant difference ($p<0.001$) among different researchers. Mean body weight of KK bulls were found to be 73.89kg and 102.25kg at 6 months and 12 months, respectively and differed significantly ($p<0.05$). Average daily gain was 0.291kg/day/bull of KK with a highly significance difference ($p<0.001$) which clearly showed the large variation on average daily gain by the finding of different researchers in different ages of KK bulls. This variation might be due to different location, period, and management. It indicates the further systematic research for better understanding of growth performance of KK bulls. However, body weight and growth performance are comparatively lower in KK bulls rather than other breed types like crossbreds, synthetic breeds, and exotic breeds.

Table 2
Body weight and average daily gain of KK bulls in different ages

Parameters	Results	References
Birth weight (kg)	15.80	Devendra et al. (1975)
	15.00	Sivarajasingam (1984)
	15.60	Dahlan (1985)
	14.00	Mak et al. (1986)
	17.50	Idris and Moin (2002)
	17.00	Johari and Jasmi (2009)
	14.31	M. A. R. Hafiz et al. (2009)
	13.97	Warzukni and Haron (2010)
	14.27	Sofienaz et al. (2014)
3 month weight (kg)	44.95	Sofienaz et al. (2014)
6 month weight (kg)	59.60	Devendra et al. (1975)
	79.50	Idris and Moin (2002)
	77.10	Dahlan (1985)

Table 2 *(continue)*

Parameters	Results	References
6 month weight (kg)	80.00	Johari and Jasmi (2009)
	70.02	M. A. R. Hafiz et al. (2009)
	67.55	Sofienaz et al. (2014)
12 month weight (kg)	95.50	Sivarajasingam (1984)
	100.80	Dahlan (1985)
	130.00	Johari and Jasmi (2009)
18 month weight (kg)	87.45	M. A. R. Hafiz et al. (2009)
	144.00	Dalhan (1985)
	120.41	M. A. R. Hafiz et al. (2009)
24 month weight (kg)	188.70	Dahlan (1985)
	156.19	M. A. R. Hafiz et al. (2009)
30 month weight (kg)	190.00	Johari and Jasmi (2009)
	217.40	Dahlan (1985)
Mature weight (kg)	214.50	Devendra et al. (1975)
	306.00	Sivarajasingam (1984)
ADG from 0 -3 month	0.324	Sofienaz et al. (2014)
ADG from 0-6month	0.296	Sivarajasingam (1984)
	0.341	Dahlan (1985)
ADG from 0-12 month	0.350	M. A. R. Hafiz et al. (2009)
	0.243	M. A. R. Hafiz et al. (2009)
ADG from 0-18 month	0.223	M. A. R. Hafiz et al. (2009)
	0.225	Mak et al. (1986)
ADG from 0-24 month	0.216	M. A. R. Hafiz et al. (2009)
ADG from 3 -6 month	0.252	Sofienaz et al. (2014)
ADG from 6-12 month	0.132	Dahlan (1985)
ADG from 6-24 month	0.339	Sivarajasingam (1984)
ADG from 12-18 month	0.239	Dahlan (1985)
	0.448	Budiono (1985)
ADG from 18-24 month	0.583	Mak et al. (1986)

Note. ADG = Average daily gain

Table 3
Meta-analysis of body weight and average daily gain of KK bulls in different ages

Parameters	Minimum	Maximum	Range	Mean ±SD	*p*-value
Birth weight (kg)	13.97	17.50	3.53	15.28±1.23	0.000
6 month weight (kg)	59.60	80.40	20.80	73.89±7.44	0.031
12 month weight (kg)	87.45	130.00	42.55	102.25±16.27	0.019
24 month weight (kg)	156.19	190.00	33.81	178.30±17.11	0.374
ADG(kg/day/animal)	0.12	0.58	0.46	0.291±0.11	0.000

Note. ADG = Average daily gain

KK COWS

Weight at birth, 3-month, 6-month, 12-month, 24-month, 26-month, 48-month, at maturity and their average daily gain from birth to 3 month, 6 month, 12 month, 18 month, 24 month, 3-6month, 6-12 month, 12-18 month, and 6-24 month of ages are illustrated in Table 4. The results of different researchers from 1975 to 2014 in various locations with traditional and improved management system are highlighted in this review. Table 5 summarizes the meta analysis of body weight and average daily gain of KK cows in different ages. Body weight at birth, 6-months and 12-months of ages were 14.30 kg, 65.43kg, and 92.06kg, respectively with significant difference (p<0.05). The average daily gain was 0.267kg/day/animal which was found highly significant difference (p<0.001) among the results of various previous researchers in different ages of KK cows.

Significant differences were obtained among the findings of various researchers for body weight and average daily gain in both the sex of KK cattle which clearly indicated the need of more attention for in depth research for further better understanding of the growth performance of KK cattle. More detailed research is needed on the body weight and growth performance of Kedah-Kelantan bulls before undertaking long term plan of selective breeding program to augment the KK cattle production in Malaysia.

Table 4
Body weight and average daily gain of KK cows in different ages

Parameters	Results	References
Birth weight (kg)	14.70	Devendra et al. (1975)
	15.12	Dahlan (1985)
	15.60	Liang et al. (1991)
	12.93	Warzukni and Haron (2010)
	13.95	M. A. R. Hafiz et al. (2009)
	13.50	Sofienaz et al. (2014)
3 month weight (kg)	45.40	Sofienaz et al. (2014)
6 month weight	74.90	Dahlan (1985)
	64.35	M. A. R. Hafiz et al. (2009)
	66.90	Sofienaz et al. (2014)
	55.60	Devendra et al. (1975)
	94.80	Dahlan (1985)
12 month weight (kg)	101.00	Ariff et al. (1993)
	80.37	M. A. R. Hafiz et al. (2009)
18 month weight (kg)	131.90	Dahlan (1985)
	111.90	M. A. R. Hafiz et al. (2009)
24month weight (kg)	195.60	Dahlan (1985)
	138.61	M. A. R. Hafiz et al. (2009)

Table 4 *(continue)*

Parameters	Results	References
30 month weight (kg)	212.80	Dahlan (1985)
36 month weight (kg)	175.80	Ariff et al. (1993)
48 month weight (kg)	197.40	Ariff et al. (1993)
Mature weight (kg)	227.80	Ariff et al. (1993)
	173.70	Devendra et al. (1975)
	234.50	Sivarajasingam (1984)
Rate of maturing	0.0523	Ariff et al. (1993)
ADG from 0-3month	0.310	J. J. Abdullah (1993)
	0.365	Sofienaz et al. (2014)
ADG from 0-6month	0.270	J. J. Abdullah (1993)
	0.332	Dahlan (1985)
	0.321	M. A. R. Hafiz et al. (2009)
ADG from 0-12 month	0.223	M. A. R. Hafiz et al. (2009)
ADG from 0-18 month	0.207	M. A. R. Hafiz et al. (2009)
ADG from 0-24 month	0.192	M. A. R. Hafiz et al. (2009)
ADG from 3 - 6 month	0.238	Sofienaz et al. (2014)
ADG from 6-12 month	0.410	Sukri and Idris (1982)
	0.109	Dahlan (1985)
ADG from 12-18 month	0.209	Dahlan (1985)
ADG from 6-24 month	0.294	Devendra and Choo (1975b)

Note. ADG = Average daily gain

Table 5
Meta-analysis of body weight and average daily gain of KK cows in different ages

Parameters	Minimum	Maximum	Range	Mean ± SD	*p*-value
Birth weight (kg)	12.93	15.60	2.67	14.30±1.01	0.016
6 month weight (kg)	55.60	74.90	19.30	65.43±7.95	0.007
12 month weight (kg)	80.37	101.00	20.63	92.06±10.58	0.047
Mature weight (kg)	173.70	234.50	60.80	212.00±33.33	0.148
ADG (kg/day/animal)	0.11	0.41	0.30	0.267±0.07	0.001

Note. ADG = Average daily gain

REPRODUCTIVE PERFORMANCE OF KK CATTLE

KK Bulls

Age at Puberty and Sexual Maturity of KK Bulls. Puberty is the age of first breeding potential, while sexual maturity is the age of maximum breeding potential of a breeding bull. It is basically defined as the period whenever the sexual organs of a bull are functionally developed for reproduction. Onset of puberty is typically demarcated when a bull can produce at least 50×10^6 sperm /ml with at least 10% progressive motility in the first ejaculation (Ismaya,

1987). Puberty is usually characterised by different male reproductive traits like age, body weight, scrotal circumference, hormone concentration, libido and semen quality (Barth, 2004; Menegassi et al., 2011). Growth performance, testosterone level, and age at puberty are positively correlated with good quality semen (Thundathil et al., 2016). Semen traits are commonly evaluated in every 30 days of interval from 8 month to about 16 months. Studies reported that age at puberty were 11, 10, 11, 9, and 8 months for Hereford, Angus, Red Poll and Brown Swiss respectively with a minimum of 50 x 10 spermatozoa/ml with at least 10% progressive motility (Argiris et al., 2018). It is clearly seen that different breeds of beef bulls are generally used for breeding ranging from 8-14 months. In addition, beef bulls routinely produce good quality semen within 3-4 months of age after attaining the age at puberty. About 33%, 60% bulls, and almost all bulls can produce good quality semen within the age of 12, 14, and 16 month of age, respectively (Argiris et al., 2018; Persson & Söderquist, 2005; Watson, 2000). Semen quality are gradually improved after attainment of age at puberty (about 10 months) and age at maturity (about 12 months) reported by Brito et al. (2012). Likewise, beef bulls within 12-15 months of age are normally considered for satisfactory semen quality approximately 50 days after puberty. Besides, age at puberty of KK bulls were more than 16 months with sperm concentration of $320x10^6$/ml and body weight 151.5 kg (Ismaya, 1987). Body weight during peri-pubertal age of bulls is a good indicator of early pubertal age. However, data are not available on peri-pubertal age of KK bulls to determine actual age of puberty and sexual maturity for selecting appropriate breeding bulls for commercial purpose to enhance the productivity of KK cattle in the country.

Scrotal Circumference. Scrotal circumference is the major key component of breeding soundness evaluation of breeding bulls. Scrotal circumference is a vital indicator of high quality semen production in young bulls (McGowan, 2018). Scrotal circumference is highly repeatable and heritable reproductive trait of male animal (Corbet et al., 2013). It is phenotypically and genetically correlated with reproductive traits of male especially sperm motility, morphology and concentration that greatly influence the bull fertility (Melis et al., 2010; Paterno et al., 2017). Moreover, scrotal circumference is also a good sign of puberty producing at least 50 million sperm with 10% or higher motility in an ejaculation. Thus, scrotal circumference (SC) has been considered a very useful reproductive tool for determining the age at puberty and is performed to improve the reproductive performance of beef cattle production (Silvio et al., 2019). Scrotal circumference is highly correlated with body weight, age with testis' weight linked fertility (Devkota et al., 2008; Waldner et al., 2010). Brito et al. (2003) reported that testicular diameter, testicular length, testicular volume along with scrotal circumference were an important part of breeding soundness evaluation of a breeding bull. The bulls with

larger testes ejaculate more than bulls with smaller testes. Testicular size are positively related to body weight and age (Engelken, 2008). However, measurement of these parameters especially scrotal circumference has a great value on onset of puberty, total semen production, semen quality, pathological conditions of reproductive system, and the fertility or infertility status of breeding bulls (Menegassi et al., 2011). Moreover, testicular measurements have been utilized as the indicators for reproductive performance in the pre and post pubertal age of bulls (I. Ahmad et al., 2013). Scrotal circumference were 26.9cm, 33.5cm, 30.6 cm, 34.1 cm, and 32.2 cm for Nellore, Angus, Brangus, Hereford, and Brafard beef breeds with the age ranging 15-17 month (Silvio et al., 2019). Scrotal circumference performance of Kedah-Kelantan bulls with different age period were reported by different researchers that were summarized in Table 6. Mean scrotal circumference of Kedah-Kelantan purebred bulls in various age ranging from 24 month to more than 36 months were 22.5 cm to 36 cm which is lower than the standard beef breeds which may be due to smaller body size of KK cattle (Abdulla et al., 2016). Furthermore, mean scrotal circumference of Kedah-Kelantan straight bred bulls was found to be 22.5 cm whereas Senepol purebred beef bulls resemble to *Bos taurus* possessed 30.1 cm (Abdulla et al., 2016; M. I. Abdullah et al., 2010). However, scrotal circumference of KK bulls needs to measure for prediction of optimum age at puberty for further commercial use to have clear understanding of bull reproduction.

Table 6
Scrotal circumference of Kedah-Kelantan purebred bulls

Age (months)	Scrotal circumference (cm)	References
12	15.80	Ismaya (1987)
18	17.90	Ismaya (1987)
24	22.50	M. I. Abdullah et al. (2010)
	22.70	Ismaya (1987)
36	24.90	Ismaya (1987)
Above 36	36.00	Yimer et al. (2011)
	27.80	Ismaya (1987)

Testosterone Hormone (T) Production

Testosterone hormone (T) plays a vital role in the reproductive efficiency of bulls. Testosterone is essential for normal spermatogenesis and expression of secondary sexual traits (Okere et al., 2014). Testosterone is produced by Leydig cell of testicular parenchyma that tremendously affects the fertility and semen quality of bulls. Different values of T level in blood serum were found in different beef breeds with various ages. The minimum and maximum T level were 0.05 ng/ml and 2.96 ng/ml in blood serum respectively, in Simental beef bull (Melis et al., 2010). Hormonal changes at puberty are commonly caused by several mechanisms (Brito et al., 2012). It has been reported that pituitary gland becomes more responsive to GnRH at pubertal age (Harstine et al., 2018). As T concentration is always more at pubertal stage than pre-pubertal bulls (Stradaioli et al., 2017). Testosterone levels are normally lower in young bulls then gradually increases with the increasing

age of bulls (Byrne et al., 2017). The mean T concentrations of KK bulls were also reported with an increasing trend from peri-pubertal to pubertal age (Ismaya, 1987). The mean values of T were 0.42, 0.65, 1.38, and 3.01ng/ml in blood serum within the age of 12 months, 18 months, 24 months, and 36 months of KK bulls, respectively. However, T concentration in the KK bull from pre-pubertal age to old age needs to be thoroughly investigated to relate with libido, semen output, and its quality.

Semen Production and Quality

Semen volume varies between and within species, and breeds and even the same male during various time of collection (Kastelic & Thundathil, 2008; Penny, 2018; Thundathil et al., 2016). It may also vary with environmental condition, age of breed, body condition score, frequency of collection, exercise, teasing, nutritional status, season, and method of collection. In general, young bulls and those of smaller size within a species, produce smaller amount of semen. Table 7 represents the semen production and quality traits of KK bulls found by different researchers. Volume of semen means the total amount of semen produced in each ejaculation that is expressed in millilitres. The volume of semen is found to be 2 to 5.4ml per ejaculation in different ages of KK bulls. KK bull's semen is normally milky to whitish creamy colour with good wave pattern. Sperm concentration is measured as the concentration of spermatozoa in an ejaculation expressed in millions per millilitre (Suchocki & Szyda, 2015). Sperm motility means the ability of sperm to move progressively forward (Brito et al., 2003). Wave pattern or mass activity is a subjective score measured by a standard scale of 1-5 or 1-3 (Suchocki & Syzda, 2015). But Gredler et al. (2007) did not apply any scoring system for wave motion. However, more motility score is always desirable for quality semen. Forward swimming of sperm in a straight lines or large scales are called progressive motility (Utt, 2016). Motility and progressive motility both are scored in percentage in each ejaculation. At least 100 spermatozoa are counted for motile and non-motile sperm under microscope. Sperm

Table 7
Semen production and quality of Kedah-Kelantan bulls

Parameters	Results	References
Volume of semen (ml)	2.60 to 5.40	Ismaya (1987)
Sperm concentration ($\times 10^6$ sperm/ml)	1265.00	Yimer et. al. (2011)
	1167.00	M. I. Abdullah et. al. (2010)
	320 .00 to1018.00	Ismaya (1987)
Sperm motility (%)	67.00	Ashrafzadeh et al. (2013)
	67.00	Sauerweina et al. (2000)
	47.50 to 81.00	Ismaya (1987)
Live sperm (%)	78.30 to 82.50	Ismaya (1987)
	20.60 to 30.00	Ismaya (1987)

concentration, sperm motility and sperm morphology are essential parameters of evaluating the semen quality (Ashrafzadeh et al., 2013). Different researchers reported sperm concentration (320 to 2000 million/ml), sperm motility (47.5 to 82.50%) and abnormal sperm morphology (20 to 30%) in KK breeding bulls of different age period (Ismaya 1987; M. I. Abdullah et al., 2010; Yimer et al., 2011).

Libido

Sexual aggressiveness or **s**exual behaviour is an important factor that highly influences the reproductive performance of bulls (Rehman et al., 2014). It depends mostly on breed, age of bull, genetic makeup and their environmental conditions (Beran et al., 2011; Galina et al., 2007; Kondracki et al., 2013; Petherick, 2005). The level of sexual aggressiveness or libido can directly affect the ejaculatory efficiency and the quality of semen in ejaculations (Levis & Reicks, 2005; Pound et al., 2002). Libido is typically defined as the lapsed time between exposure to stimuli and first service (Beran et al., 2011). It is a good parameter to predict the reproductive competence of bulls (M. Ahmad et al., 2005). Libido is also helpful to assess the soundness of bulls for selecting a suitable bull for artificial insemination program. Thus, bulls with higher libido can ensure optimum level of production with good quality semen during breeding seasons as well multiple ejaculations. There are many environmental factors affecting expression of libido of bull. It is difficult to draw specific conclusion about the libido and reproductive performance of bulls without studies involving females. However, as not enough work was done in this regard, it is difficult to draw any valid inference.

KK Cows

Results of reproductive traits of Kedah-Kelantan cow by different researchers are presented in Table 8. Reproductive performance is measured by the number of pregnant cows out of the total number of eligible cows to be pregnant of a cattle herd within one year. Calving interval, convention rate, conception rate are the most important reproductive characteristics of female animals for the efficient reproductive performance of bovine production. Estrous cycle is considered as the time between periods of estrous. The average of estrous cycles is almost similar in farm animals.

Puberty. Puberty can be defined when a heifer shows heat or estrous with ovulation first time. Heifers of exotic breeds normally reach puberty by 13-14 month of ages. Age at puberty of heifers is directly influenced by age, breed, body weight and environmental factors. Heifers can attain their puberty when they reach about 65-67% of their mature weight. The mean age at first estrous was 11 months of age for KK cows reported by Sivarajasingam (1984).

Conception Rate. High rate of conception is important for effective breeding program. The conception rate is defined as the percentage of services that result in conception. Age and weight at first conception were 18 months

to 28.60 month and 166 kg respectively, in KK cows revealed by various researchers. Age at first calving was 35.50 month to 38.20 month while weight at first calving was 250.70kg in KK cows.

Calving Interval. Calving interval (CI) is considered as the duration of time between two successive parturitions consisting the post -partum interval, conception length and the length of gestation. The range of calving interval was 12.10 to 13.20 month of age which is most prominent reproductive trait of KK cattle. Reproduction is the major factor influencing the efficiency beef cattle production. Optimum reproductive efficiency of beef cow mostly relies on the calving interval. On the other hand, calving interval is greatly depended on the postpartum interval from parturition to first estrous. Better reproductive efficiency were found in KK cows rather than Brangus, Bradford and Sahiwal against cystic ovarian diseases (COD) and abnormal ovarian cyclicity (AOC), which are the key factors

Table 8
Reproductive performances of Kedah-Kelantan cows

Parameters	Results	References
Mean age at first estrous (month)	11.00	Sivarajasingam (1984)
Age at first conception (month)	18.00	Sivarajasingam (1984)
Weight at first conception (kg)	166.00	Sivarajasingam (1984)
Mean age at conception (month)	28.60	Warzukni and Haron (2010)
Age at first calving (month)	35.50	Sivarajasingam (1984)
Age at first calving (month)	37.40	Johari et al. (1994)
Age at first calving (month)	38.20	Warzukni and Haron (2010)
Weight at first calving (kg)	250.70	J. J. Abdullah (1993)
First calving to conception (days)	81.00	Sivarajasingam (1984)
Calving to conception (days)	83.40	J. J. Abdullah (1993)
Calving to first breeding (days)	73.90	J. J. Abdullah (1993)
Calving to first ovulation (days)	66.30	J. J. Abdullah (1993)
Calving interval (month)	13.20	Sivarajasingam (1984)
Calving interval (month)	12.60	J. J. Abdullah (1993)
Calving interval (month)	12.10	Johari and Jasmi (2009)
Calving interval (month)	13.03	Warzukni and Haron (2010)
Number of services/conception	1.80	J. J. Abdullah (1993)
First service conception rate (%)	50.00	J. J. Abdullah (1993)
First service conception rate (%)	50.00	Johari et al. (1994)
Conception rate (%)	80-95	Sivarajasingam (1984)
Calving rate (%)	92.40	Johari et al. (1994)
	70-75	Mohamed et al. (2013)
Pregnancy rate (%)	84.20	J. J. Abdullah (1993)
Gestation length (days)	279.90	Liang et al. (1991)

of reproductive failures directly influencing the calving interval in cows (Yimer et al. 2010 & 2018).

Breeding. KK cows are normally bred by bulls through natural services. The number of services per conception was 1.80 in KK cows (J. J. Abdullah, 1993). First service conception rate was 50% reported (Johari et al., 1994) while calving rate 92.40% was found by Sivarajasingam (1984).

Calving interval of KK cows is about one year while conception rate is 85%-95% which are the prominent reproductive capability of KK cows. Due to lack of improved management and appropriate breeding techniques, the productivity of KK cows is comparatively low despite its high fertility and calf production ability of one calf in each year. However, published information on reproductive performance of KK cows based on systematic procedure of investigation is very limited.

CONCLUSION

KK cattle are small size and well-adapted local breed reared with traditional farming system with minimum feed costs. Large genetic variations are found within KK cattle than the synthetic breeds indicating a great scope to improve the KK cattle in future. Malaysia imported more than 15 foreign breeds from differents regions of the world. Unfortunately, most of the exotic breeds did not play any significant contribution within 55 years of effort for the development of beef industry in Malaysia due to their poor reproductive performance and high mortality rate. Simultaneously, crossing KK cows with the exotic beef breeds like Brahman, Hereford, Angus, Shorthorn, Friesian, Charolais, Limousin, and Semental, which caused genetic erosion and dilution of KK purity are the major challenges to maintain the original genomic characteristics of KK cattle.

Growth performance of an animal is the outmost important factor for economic beef production system. Monitoring growth performance is the most vital, easier, faster, and cheaper technique to take decision for breeding, feeding and veterinary services of animals. Moreover, it can be used as a means to determine the age at puberty and maturity of both cows and bulls. Based on the literature reviewed, it is apparent that the KK cattle have been found to be superior in terms of reproductive performances (Calving interval (CI) of about one year and a conception rate of 85%-95%) and semen quality but relatively lower growth performance compared to other cattle breeds such as crossbreds, synthetic breeds and exotic breeds. However, evidences have shown that growth performance of KK cattle can be enhanced. Based on the meta data analysis of KK cattle performance, a significantly wide variation among the findings of previous researchers for body weight and average daily gain in both sexes have been found; which implies presence of opportunities improvement in growth performance and the need of further research in sorting out the important factors that lead to superior production performance of the KK cattle. There is

lack of information on KK bulls based on scrotal biometry, semen quality, testosterone and libido at pre, puberty and post puberty ages for selecting appropriate breeding bulls. Scrotal circumference of KK bull was found to range from 22.5 to 36 cm for ages of 24 months to more than 36 months. Scrotal circumference is highly repeatable and heritable reproductive trait that needs to be measured for prediction of optimum age at puberty and maturity of KK bulls. Generally, though there may be several studies conducted on KK cattle, published information on reproductive performance of KK cows based on systematic researches is scarce. Therefore, any information pertinent to the production and reproduction performance and economics of the Malaysian indigenous KK cattle should be properly documented for further research and we believe that the current review will benefit in advancing further research and development work on KK cattle breeds and subsequently augmenting the staggering beef industry of Malaysia.

RECOMMENDATIONS FOR FUTURE RESEARCH

1. Special emphasis needs to be employed to well adapted indigenous KK cattle rather than exotic breeds or crossbreds with a view to protect the genetic erosion and dilution of KK cattle purity.
2. KK cattle are measured as the natural animal genetic resource and it can be considered "The Natural Heritage and Own Animal Genetic Resource of Malaysia" for sustainable and profitable beef production. So, long term plan for conservation and multiplication should be focused on priority basis to improve the KK cattle by the respective government institutes.
3. Genomic characterization of KK cattle based on common phenotypic traits must be considered as a top most priority work to determine the genetic potentiality through whole genome sequencing (WGS) for future research works.
4. More detailed research is needed on the body weight and growth performance of KK cattle under different rearing systems before undertaking the long term plan of selective breeding program to enhance the KK cattle production in Malaysia.
5. It can be strongly recommended that growth and reproductive performance KK cattle can be improved through appropriate and long term selective breeding program which may be the significant way for commercialization of KK cattle in this tropical region with progressive climate change situation.
6. Superior breeding bulls of Kedah-Kelantan can significantly contribute to develop the KK local beef cattle population. Bulls should examine from growing to post pubertal age considering all productive and reproductive parameters especially growth, reproductive traits, hormones, libido, age at puberty and maturity, and semen quality. Hence, very limited

in depth works so far have been done on Kedah-Kelantan breeding bull evaluation before. So, in-depth study based on the key reproductive traits of KK bulls would be given more emphasis for selecting the KK best breeding bulls to enhance the reproductive performance of KK cows.

7. Need to establish standard selection criteria for phenotypic characterization, breeding bull evaluation, age at puberty and maturity of KK cattle for taking the suitable steps for breeding, feeding and management.

Eventually, it can be recommended that KK cattle would be used for sustainable and economical beef production in Malaysia due to their adaptability behaviour with progressive climate changes by implementing selective breeding program.

CONFLICT OF INTEREST

None of the authors of this paper has any financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of paper.

ACKNOWLEDGMENTS

The authors would like to thank the Government of Bangladesh and National Agricultural Technology Project Phase 2(NATP-2), Bangladesh Agricultural Research Council, Bangladesh for awarding the full funded PhD scholarship to Mr. Mohammed Sirajul Islam.

AUTHOR'S CONTRIBUTION

All authors have contributed by giving their ideas, searching literature and repeated revision of the draft for preparing the manuscript.

REFERENCES

Abdulla, I., Mohamed, A. F., Bala, B. K., Bach, N. L., & Mohammadi, S. (2016). Management of beef cattle production in Malaysia: A step forward to sustainability. *American Journal of Applied Sciences*, *13*(9), 976–983. https://doi.org/10.3844/ajassp.2016.976.983

Abdullah, J. J. (1993). *Postpartum reproductive performance of Kedah-Kelantan cattle its crossbreds* [Master's thesis, Universiti Putra Malaysia]. Universiti Putra Malaysia Institutional Repository. http://psasir.upm.edu.my/id/eprint/12304/

Abdullah, M. I., Saharee, A. A., & Haron, A. W. (2010). *Breeding soundness examination in Kedah-Kelantan bulls* [Doctoral's dissertation, Universiti Putra Malaysia]. Universiti Putra Malaysia Institutional Repository. http://psasir.upm.edu.my/id/eprint/30947/

Ahmad, I., Fiaz, M., Manzoor, M. N., Ahmad, T., Yaqoob, M., & Jo, I. H. (2013). Comparative growth performance of calves of different cattle breeds under a feedlot fattening system. *Journal of Animal Science and Technology*, *55*(6), 539–543. https://doi.org/10.5187/jast.2013.55.6.539

Ahmad, M., Asmat, M. T., & Rehman, N. (2005). Relationship of testicular size and libido to age and season in Sahiwal bulls. *Pakistan Veterinary Journal*, *25*(2), 67-70.

Argiris, A., Ondho, Y. S., Santoso, S. I., & Kurnianto, E. (2018). Effect of age and bulls on fresh semen quality and frozen semen production of Holstein bulls in Indonesia. In *Intstitute of Physics Conference Series: Earth and Environmental*

Science (pp. 1-10). Intstitute of Physics Publishing. https://doi.org/10.1088/1755-1315/119/1/012033

Ariff, O. M., Sharifah, N. Y., & Hafidz, A. W. (2015). Status of beef industry of Malaysia. *Journal of Animal Science*, *18*(2), 1-21.

Ariff, O. M., Johari, J. A., & Dahlan, I. (1993). Growth pattern for body weight of straightbred and crossbred Kedah-Kelantan cattle. *Malaysian Agricultural Research and Development Institute Research Journal*, *21*(2), 129–134.

Ashrafzadeh, A., Nathan, S., & Karsani, S. A. (2013). Comparative analysis of mafriwal (*Bos taurus* × *Bos indicus*) and Kedah Kelantan (*Bos indicus*) sperm proteome identifies sperm proteins potentially responsible for higher fertility in a tropical climate. *International Journal of Molecular Sciences*, *14*(8), 15860-15877. https://doi.org/10.3390/ijms140815860

Barth, A. D. (2004). Pubertal development of *Bos taurus* beef bulls. In *Proceedings of the 23rd World Buiatrics Congres* (pp. 1-5). IVIS Publisher.

Beran, J., Stádník, L., Ducháček, J., Toušová, R., Louda, F., & Štolc, L. (2011). Effect of bulls' breed, age and body condition score on quantitative and qualitative traits of their semen. *Acta Universitatis Agriculturae et Silviculturae Mendelianae Brunensis*, *5*(6), 37-44. https://doi.org/10.11118/actaun201159060037

Brito, L. F. C. (2014). *Bull development: Sexual development and puberty in bulls*. The Wiley Online Libray. https://doi.org/10.1002/9781118833971.ch5

Brito, L. F. C., Barth, A. D., Wilde, R. E., & Kastelic, J. P. (2012). Effect of growth rate from 6 to 16 months of age on sexual development and reproductive function in beef bulls. *Theriogenology*, *77*(7), 1398-405. https://doi.org/10.1016/j.theriogenology.2011.11.003

Brito, L. F. C., Silva, A. E. D. F., Barbosa, R. T., Unanian, M. M., & Kastelic, J. P. (2003). Effects of scrotal insulation on sperm production, semen quality, and testicular echotexture in *Bos indicus* and *Bos indicus* x *Bos taurus* bulls. *Animal Reproduction Science*, *79*(1-2),1-15. https://doi.org/10.1016/S0378-4320(03)00082-4

Budiono, R. S. (1985). *Utilization of* Setaria sphacelata *variety* splendida *by Kedah-Kelantan cattle* [Master's thesis, Universiti Putra Malaysia]. Universiti Putra Malaysia Institutional Repository. http://psasir.upm.edu.my/id/eprint/12269/

Bures, D., & Barton, L. (2012). Growth performance, carcass traits and meat quality of bulls and heifers slaughtered at different ages. *Czech Journal of Animal Science*, *57*(1), 34-43. https://doi.org/10.17221/5482-cjas

Byrne, C. J., Fair, S., English, A. M., Urh, C., Sauerwein, H., Crowe, M. A., Lonergan, P., Kenny, D. A. (2017). Effect of breed, plane of nutrition and age on growth, scrotal development, metabolite concentrations and on systemic gonadotropin and testosterone concentrations following a GnRH challenge in young dairy bulls. *Theriogenology*, *96*(1), 58-68. https://doi.org/10.1016/j.theriogenology.2017.04.002

Clayton, A. M. (1983). Large scale commercial cattle operation in Darabif. In *Proceedings of the 7th Annual Malaysian Society of Animal Production Conference* (pp. 7-13). Malaysian Society of Animal Production.

Corbet, N. J., Burns, B. M., Johnston, D. J., Wolcott, M. L., Corbet, D. H., Venus, B. K., Li, Y., McGowan, M. R., & Holroyd, R. G. (2013). Male traits and herd reproductive capability in tropical beef cattle. 2. Genetic parameters of bull traits. *Animal Production Science*, *53*(2), 101-113. https://doi.org/10.1071/AN12163

Dahlan, I. (1985). *Growth performance, body conformation, carcass characteristics and*

palatability of KK cattle and its crossbred [Master's thesis, Universiti Putra Malaysia]. Universiti Putra Malaysia Institutional Repository. http://psasir.upm.edu.my/id/eprint/12266/

Dahlan, I., Haron, A. R., & Sukri, M. H. I. (1992). Effect of agricultural by-product diets on carcass characterisrtics of four types of cattle in the feedlot. *Asian-Australian Journal of Animal Sciences*, *5*(3), 455-459. https://doi.org/10.5713/ajas.1992.455

Department of Veterinary Services (2014). *Livestock statistics*. DVS. http://www.dvs.gov.my/statistik;jsessionid

Devendra, C., & Choo, T. L. K. (1975a). Studies on Kedah-Kelantan cattle: Body composition and carcass characteristics. *Malaysian Agricultural Research and Development Institute Resarch Bulletin*, *4*(2), 81-89.

Devendra, C., & Choo, T. L. K. (1975b). Studies on Kedah-Kelantan cattle II . The effect of feeding increasing levels of tapioca (*Manihot utilissima* Pohl.) on growth. *Malaysian Agricultural Research and Development Institute Research Journal*, *4*(1), 80-89.

Devendra, C., Hasan, M. N., Hodge, R., Lee, K. C., & Pathmasingam, M. (1975). Kedah-Kelantan cattle in Malaysia. *Malaysian Agricultural Journal*, *49*, 25-47.

Devkota, B., Koseki, T., Matsui, M., Sasaki, M., Kaneko, E., Miyamoto, A., Amaya Montoya, C., & Miyake, Y.-I. (2008). Relationships among age, body weight, scrotal circumference, semen quality and peripheral testosterone and estradiol concentrations in pubertal and postpubertal Holstein bulls. *Journal of Veterinary Medical Science*, *70*(1), 119-121. https://doi.org/10.1292/jvms.70.119

Engelken, T. J. (2008). The development of beef breeding bulls. *Theriogenology*, *70*(3), 573-575. https://doi.org/10.1016/j.theriogenology.2008.05.038

Flint, B. (1971). *Crossbreeding Kedah-Kelantan cows with American Hereford and Brahman bulls by artificial insemination*. College of Agriculture.

Galina, C. S., Horn, M. M., & Molina, R. (2007). Reproductive behaviour in bulls raised under tropical and subtropical conditions. *Hormones and Behavior*, *15*(1), 26-31. https://doi.org/10.1016/j.yhbeh.2007.03.026

Gopinathan, A., Sivaselvam, S. N., & Kulasekar, K. (2018). Effect of body weight and scrotal circumference on semen production traits in crossbred Holstein Friesian bulls. *Indian Journal of Animal Reproduction*, *39*(1), 24-27.

Gredler, B., Fuerst, C., Fuerst-Waltl, B., Schwarzenbacher, H., & Sölkner, J. (2007). Genetic parameters for semen production traits in Austrian dual-purpose simmental bulls. *Reproduction in Domestic Animals*, *42*(3), 326-328. https://doi.org/10.1111/j.1439-0531.2006.00778

Hafiz, A. W. M., Idris, I., & Yaakub, H. (2014). Growth pattern for body weight, hip height and body length of Brakmas cattle. *Pakistan Journal of Biological Sciences*, *17*(7), 952-955. https://doi.org/10.3923/pjbs.2014.952.955

Hafiz, A. W. M., Marini, A. M., Rosly. S. M., Hifzan, M. R., Bahtiar, I, A. J., & Shanmugavelu, S. (2019). Describing growth pattern of selected Kedah-Kelantan cows using non-linear regression models. *Malaysian Journal of Animal Science*, *22*(1), 47-54.

Hafiz, M. A. R., Salleh, S. I., & Raymond, A. K. (2009). Growth performance of Kedah-Kelantan cattle in Malaysia. In *Proceedings of the 21st Veterinary Association of Malaysia Congress* (pp. 204-208). Veterinary Association of Malaysia.

Harstine, B. R., Cruppe, L. H., Abreu, F. M., Rodrigues, A. D., Premanandan, C., DeJarnette, J. M., & Day, M. L. (2018). Impact of a timed-release FSH treatment from 2 to 6 months of age in bulls I: Endocrine and testicular development of beef bulls. *Theriogenology*, *105*(1), 141-149. https://doi.org/10.1016/j.theriogenology.2017.09.018

Idris, I., & Moin, S. (2002). Productive performance levels from local cows through crossbreeding. In *Proceedings of the 3rd International Seminar on Tropical Animal Production* (pp. 17-22). Gadjah Mada University Press.

Ismaya, D. Y. (1987). *Studies in Kedah-Kelantan, Brahman and crossbred bulls at puberty and sexual maturity* [Master's thesis, Universiti Putra Malaysia]. Universiti Putra Malaysia Institutional Repository. http://psasir.upm.edu.my/id/eprint/12272/

Jamaludin, M. H., Hassan, M. H., Amin, M. R., & Zulhisyam, A. K. (2014). The future of the Malaysian beef industry. *Journal of Tropical Resources Sustainable Sciences*, *2*(1), 23-29.

Johari, J. A, Ariff, M. O., & Adnan, S. (1994). Reproductive performance of Kedah-Kelantan crossbred cattle. *MARDI Research Journal*, *22*(1), 91–95.

Johari, J. A., & Jasmi, Y. (2009). Breeds and breeding program for beef production in Malaysia. In *Proceedings of the 8th Malaysia Congress on Genetics* (pp. 22-28). Malaysian Society of Animal Science.

Kastelic, J. P., & Thundathil, J. C. (2008). Breeding soundness evaluation and semen analysis for predicting bull fertility. *Reproduction in Domestic Animals*, *43*(2), 368-373. https://doi.org/10.1111/j.1439-0531.2008.01186.x

Kondracki, S., Iwanina, M., Wysokińska, A., & Górski, K. (2013). The use of sexual activity measurements to assess ejaculatory performance of boars. *Archives Animal Breeding*, *56*(106), 1052-1059. https://doi.org/10.7482/0003-9438-56-106

Kusriatmi, Oktaviani, R., Syaukat, Y., & Said, A. (2014). Analysis of the effects of beef import restrictions policy on beef self-sufficiency in Indonesia. *Journal of the International Society for Southeast Asian Agricultural Sciences*, *20*(1),115-130.

Lee, W. S., Khan, M. A., Kim, H. S., Kim, J. H., Yang, S. H., Ki, K. S., Lee, H. J., Kim, S. B., Baek, K. S., & Ha, J. K. (2009). Feed consumption, growth performance and carcass evaluation of Korean Holstein bulls fed either conventional or organic diets. *Animal Production Science*, *49*(4), 306-310. https://doi.org/10.1071/EA08229

Levis, D. G., & Reicks, D. L. (2005). Assessment of sexual behavior and effect of semen collection pen design and sexual stimulation of boars on behavior and sperm output: A review. *Theriogenology*, *63*(2), 630-642. https://doi.org/10.1016/j.theriogenology.2004.09.037

Liang, J. B., Samiyah, M. N., & Shokri, O. A. (1991). Energy requirement for pregnancy and some reproductive characteristics of Kedah-Kelantan, Brahman×Kedah-Kelantan crossbred and buffalo heifers. *Malaysian Agricultural Research and Development Institute Research Journal*, *19*(1), 101-109.

Loh, T. C. (2004). Livestock production and the feed industry in Malaysia. *Animal Science*, *18*, 329–339.

Mak, T., Hutagalung, R., & Togimin, T. (1986). Performance of Kedah-Kelantan cattle fed palm kernel cake. *Pertanika*, *9*(1), 75–80.

McGowan, M. (2018). Evaluation of the fertility of breeding males. In *Veterinary reproduction and obstetrics* (pp. 619-634). Elsvier. https://doi.org/10.1016/b978-0-7020-7233-8.00035-5

Melis, M. H., Eler, J. P., Rosa, G. J. M., Ferraz, J. B. S., Figueiredo, L. G. G., Mattos, E. C., & Oliveira,

H. N. (2010). Additive genetic relationships between scrotal circumference, heifer pregnancy, and stayability in Nellore cattle. *Journal of Animal Science*, *88*(12), 3809-3813. https://doi.org/10.2527/jas.2009-2127

Menegassi, S. R. O, Barcellos, J. O., Peripolli, V., Pereira, P. R. R., Borges, J. B., & Lampert, V. (2011). Measurement of scrotal circumference in beef bulls in Rio Grande do Sul. *Arquivo Brasileiro de Medicina Veterinária e Zootecnia*, *63*(1), 87-93. https://doi.org/10.1590/s0102-09352011000100014

Mohamed, Z., Hosseini, A., & Kamarulzaman, N. H. (2013). Analysis of Malaysian beef industry in peninsular Malaysia under different importation policies scenarios and rate management systems. *Pertanika Journal of Social Science and Humanities*, *21*(August), 1–16.

Okere, C., Keith, L., & Bolden-Tiller, O. (2014). The relationship between body conformation, testicular traits and serum testosterone levels in pre-pubertal male boer goat crosses. *American Journal of Experimental Agriculture*, *4*(12), 1812–1819. https://doi.org/10.9734/ajea/2014/4929

Paterno, F. M., Buzanskas, M. E., Koury Filho, W., Lôbo, R. B., & Queiroz, S. A. (2017). Genetic analysis of visual assessment and body weight traits and their relationships with reproductive traits in Nellore cattle. *Journal of Agricultural Science*, *155*(4), 679-687. https://doi.org/10.1017/S0021859617000028

Penitente-Filho, J. M., Castaño Villadiego, F. A., E Silva, F. F., Camilo, B. S., León, V. G., Peixoto, T., & Guimarães, J. D. (2018). Can scrotal circumference-based selection discard bulls with good productive and reproductive potential?. *PLOS One*, *13*(3), e0193103. https://doi.org/10.1371/journal.pone.0193103

Penny, C. D. (2018). *Examinations of bulls for breeding soundness - An illustrated guide*. Tegasse Agriculture and Food Development Authority.

Persson, Y., & Söderquist, L. (2005). The proportion of beef bulls in Sweden with mature spermiograms at 11-13 months of age. *Reproduction in Domestic Animals*, *40*(2), 131-135. https://doi.org/10.1111/j.1439-0531.2005.00567.x

Perumal, P. (2014). Scrotal circumference and its relationship with testicular growth, age, and body weight in Tho Tho (*Bos indicus*) bulls . *International Scholarly Research Notices*, *2014*, 249537. https://doi.org/10.1155/2014/249537

Petherick, J. C. (2005). A review of some factors affecting the expression of libido in beef cattle, and individual bull and herd fertility. *Applied Animal Behaviour Science*, *90*(3-4), 185-205. https://doi.org/10.1016/j.applanim.2004.08.021

Pound, N., Javed, M. H., Ruberto, C., Shaikh, M. A., & Del Valle, A. P. (2002). Duration of sexual arousal predicts semen parameters for masturbatory ejaculates. *Physiology and Behavior*, *76*(4-5), 685-689. https://doi.org/10.1016/S0031-9384(02)00803-X

Raymond, A. K. (2012). A genetic improvement program for Kedah-Kelantan cattle. *Malaysian Journal of Veterinary Research*, *3*(2), 19–28.

Raymond, A. K., & Abu Hassan, M. A. (2012). A genetic improvement programme for Kedah-Kelantan cattle. *Malaysian Journal of Veterinary Research*, *3*(2), 19-28.

Rehman, F. U., Qureshi, M. S., & Khan, R. U. (2014). Effect of soybean based extenders on sperm parameters of holstein-friesian bull during liquid storage at 4°C. *Pakistan Journal of Zoology*, *46*(1), 185-189.

Sarsaifi, K., Rosnina, Y., Ariff, M. O., Wahid, H., Hani, H., Yimer, N., Vejayan, J., Naing, S. W., & Abas, M. O. (2013). Effect of semen collection methods on the quality of pre- and post-thawed Bali cattle (*Bos javanicus*) spermatozoa. *Reproduction in*

Domestic Animals, *48*(6), 1006-1012. https://doi.org/10.1111/rda.12206

Sauerweina, H., Breier, B. H., Gallaher, B. W., Götz, C., Küfner, G., Montag, T., Schallenberger, E. (2000). Growth hormone treatment of breeding bulls used for artificial insemination improves fertilization rates. *Domestic Animal Endocrinology*, *18*(1), 145-158. https://doi.org/10.1016/S0739-7240(99)00070-3

Silva, L. H. P., Paulino, P. V. R., Assis, G. J. F., Assis, D. E. F., Estrada, M. M., Silva, M. C., Chizzotti, M. L. (2017). Effect of post-weaning growth rate on carcass traits and meat quality of Nellore cattle. *Meat Science*, *123*, 192-197. https://doi.org/10.1016/j.meatsci.2016.10.005

Silvio, M. R. O., Pereira, G. R., McManus, C., Roso, V. M., Bremm, C., Koetz Jr., C., Lopes, J. F., & Barcellos, J. O. J. (2019). Evaluation and prediction of scrotal circumference in beef bulls. *Theriogenology*, *140*, 25-32. https://doi.org/10.1016/j.theriogenology.2019.08.008

Sivarajasingam, S. (1984). Cattle evaluation studies and future priorities in Malaysia. In *Proceedings of the Australian centre for International Agricultural Research (ACIAR): Evaluation of large and ruminants for the Tropics* (pp. 44-51). Australian Centre for International Agricultural Research.

Sofienaz, I., Fuad, A., Amin, R., & Rusli, N. D. (2014). Effect of some non-genetic factors on birth weight and pre-weaning growth pattern in Kedah-Kelantan calves. *Journal of Tropical Resources and Sustainable Science*, *2*, 10–15.

Stradaioli, G., Peric, T., Montillo, M., Comin, A., Corazzin, M., Veronesi, M. C., & Prandi, A. (2017). Hair cortisol and testosterone concentrations and semen production of *Bos taurus* bulls. *Italian Journal of Animal Science*, *16*(4), 631-639. https://doi.org/10.1080/1828051X.2017.1303339

Suchocki, T., & Szyda, J. (2015). Genome-wide association study for semen production traits in Holstein-Friesian bulls. *Journal of Dairy Science*, *98*(8), 5774-5780. https://doi.org/10.3168/jds.2014-8951

Sukri, M., & Idris, H. J. (1982). The performance of Kedah-kelantan cattle fed with pelleted pineapple bran ration. *Malaysian Agricultural Research and Development Institute Research Journal*, 9(2), 69-73.

Tan, H. S., Kassim, H., & Mak, T. K. (1985). Postpartum fertility in suckled indigenous cattle of Malaysia. In *Proceedings of the 3rd Australasian Association of Animal Production Societies (AAAP) Animal Science Congress* (pp. 441-443). Australasian Association of Animal Production Societies.

Thundathil, J. C., Dance, A. L., & Kastelic, J. P. (2016). Fertility management of bulls to improve beef cattle productivity. *Theriogenology*, *86*(1), 397–405. https://doi.org/10.1016/j.theriogenology.2016.04.054

Utt, M. D. (2016). Prediction of bull fertility. *Animal Reproduction Science*, *169*, 37-44. https://doi.org/10.1016/j.anireprosci.2015.12.011

Waldner, C. L., Kennedy, R. I., & Palmer, C. W. (2010). A description of the findings from bull breeding soundness evaluations and their association with pregnancy outcomes in a study of western Canadian beef herds. *Theriogenology*, *74*(5), 871-883. https://doi.org/10.1016/j.theriogenology.2010.04.012

Warzukni, N. A., & Haron, A. W. (2010). Reproductive performance of Kedah-Kelantan cattle at Pusat Ternakan Haiwan Pantai Timur, Malaysia [Doctoral's dissertation, Universiti Putra Malaysia]. Universiti Putra Malaysia Institutional Repository. http://psasir.upm.edu.my/id/eprint/27814/

Watson, P. (2000). The causes of reduced fertility with cryopreserved semen. *Animal Reproduction Science*, *60–61*, 481–492. https://doi.org/10.1016/S0378-4320(00)00099-3

Yimer, N., Haron, A. W., & Rosnina, Y. (2018). Determination of ovarian cysts in cattle with poor reproductive performance using ultrasound and plasma progesterone profile. *Veterinary Medicine – Open Journal*, *3*(1), 1-9. http://dx.doi.org/10.17140/VMOJ-3-126

Yimer, N., Rosnina, Y., Haron, A. W., Saharee, A. A., Yap, K. C., & Ganesmurthi, P. (2010). Ovarian activity in beef and dairy cows with prolonged postpartum period and heifer that fail to conceive. *Tropical Animal Health and Production*, *42*, 607-615. https://doi.org/10.1007/s11250-009-9464-y

Yimer, N., Rosnina, Y., Wahid, H., Saharee, A. A., Yap, K. C., Ganesamurthi, P., & Fahmi, M. M. (2011). Trans-scrotal ultrasonography and breeding soundness evaluation of bulls in a herd of dairy and beef cattle with poor reproductive performance. *Pertanika Journal of Tropical Agricultural Science*, *34*(2), 217–228.

Pertanika J. Trop. Agric. Sci. 44 (1): 49 - 67 (2021)

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A Longitudinal Study of Lameness Incidence and Association with Animal-Based Welfare Measures in Dairy Cows

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ABSTRACT

The objective of this study was to investigate time to lameness event, prevalence of claw lesions, and their associations with animal-based welfare measures (ABWMs) in dairy cows. A total of 120 non-lame lactating cows (n = 30 each from four intensive dairy farms) having no claw lesion were enrolled in the study. The cow-level enrollment criteria included good body condition score (BCS), normal hock condition score, absence of body injuries, and normal claw length. Information on cows' parity, milk yield, and previous lameness event (PLE) were recorded. The cows were observed twice monthly (every 2 weeks) for locomotion scores (LS), and ABWMs, whereas claw lesions were recorded upon onset of lameness and at the end of the observation period. Cows were considered lame when 2 consecutive LS = 3, or any assessment with score 4. Cox regression models were used to investigate lameness incidence and the association with ABWMs, while association between the latter and claw horn lesions (CHL) prevalence were analyzed using logistic regression models. Twenty-four percent (29/120) of the cows were lame during the study period. Lameness risk was associated with PLE (Hazard ratio; HR = 7.4; 95% CI 2.4-23.0), presence of overgrown claw (HR = 3.7; 95% CI 1.1-12.6) and low BCS pre-lame (HR = 4.5; 95% CI 1.3-16.6). Amongst the cows affected with claw

ARTICLE INFO

Article history:
Received: 17 April 2020
Accepted: 01 September 2020
Published: 24 February 2021

DOI: https://doi.org/10.47836/pjtas.44.1.03

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ISSN: 1511-3701
e-ISSN: 2231-8542

lesions, 68.4% (37/44) were lame and CHL were predominant (75.4%) compared to infectious claw lesions (24.7%). Cows with lower BCS (< 3.0) (OR = 5.7; 95% CI 1.6-20.4) and those with PLE (OR = 7.2; 95% CI = 2.1-24.7) were more likely to have CHL. Management practices such as maintaining cows' body condition, improved care for those with history of lameness, and proper claw trimming could assist farmers to reduce lameness incidence in the studied herds.

Keywords: Claw lesions, claw trimming, dairy cows, lameness, welfare

INTRODUCTION

Lameness is an important health problem in dairy cows (Cook et al., 2016). Lameness is also a major animal welfare issue due to the manifestation of pain and behavior changes in affected cows (Ramanoon et al., 2018b; Thomas et al., 2016). The condition continues to be the cause of economic losses to farmers resulting from reduced milk yield (Green et al., 2014; Sadiq et al., 2019), maintenance and treatment of affected cows and higher culling risk (Charfeddine & Cabral-Perez, 2017).

Claw lesions are responsible for most locomotion disorders in dairy herds (Bryan et al., 2012; Sadiq et al., 2017a). The prevalence of lesions causing lameness varies under different management systems, with intensively managed dairy cows experiencing higher lameness prevalence compared to pasture-based systems (Hund et al., 2019). However, the risk factors for lameness and specific claw lesions on intensively managed dairies are multifaceted at cow and herd levels (Olechnowicz et al., 2010; Sadiq et al., 2017b; Solano et al., 2015). For instance, the development of claw horn lesions (CHL) has been linked to the overloading on softer regions of the lateral hind claws, due to disproportionate heel height between the medial and lateral claws, biomechanical reaction at the floor-claw interface (Nuss & Paulus, 2006), and peri-calving metabolic and hormonal changes leading to negative energy balance (Lim et al., 2015; Newsome et al., 2017a). For infectious claw lesions (ICL), factors such as presence of an infected cattle, poor leg hygiene, and biosecurity issues have been reported to increase the prevalence of digital dermatitis (DD) and other related lesions in dairy herds (Oliveira et al., 2017; Relun et al., 2013).

Animal-based welfare measures (ABWMs) are vital parameters in assessing the well-being and performance of dairy cows (Robichaud et al, 2019). They include, but not limited to factors such as hock and body condition, leg hygiene, lying behavior, social grooming, and body injuries (Ramanoon et al., 2018b; Sadiq et al., 2017b). These factors have been associated with lameness and specific claw lesions prevalence on dairies (Bran et al., 2018; Solano et al., 2015). Cows with low body condition score (BCS), poor leg hygiene, and hock injuries had greater odds of being lame (Sadiq et al. 2017a; Solano et al., 2015). Likewise, the risk for CHL increased in thin cows and they were more susceptible to future lameness event. (Randall et al., 2015). However, majority of the research

were cross-sectional; hence, the direction of the relationship between lameness and welfare parameters could not be elucidated.

Lameness and claw lesions are major health and welfare issues in Malaysian dairy herds (Sadiq et al., 2017a, 2020a). Recent studies conducted in dairy farms in Peninsular Malaysia reported lameness and claw lesions prevalence of 33% and 46%, respectively (Sadiq et al., 2020a, 2021). The associations between cow-level risk factors and lameness prevalence depicted poor animal welfare (Sadiq et al., 2017a, 2020a). Moreover, only a few farms practiced routine claw trimming (CT) as a lameness management strategy. Lack of claw trimming and hoof care contributed to the high prevalence of overgrown claws and CHL in dairy cows in Peninsular Malaysia (Ramanoon et al., 2018a, Sadiq et al., 2021).

To gain more knowledge on the welfare indicators of lameness risk at cow level and the direction of the events, there is a need for a longitudinal assessment of dairy cows from their non-lame to lameness status. The objective of this study was to determine the time-related changes of animal-based welfare indicators and risk of new lameness event in dairy cows.

MATERIALS AND METHODS

Study Design and Herd Selection

Farms were recruited from the list and contacts of dairy farms registered with the Department of Veterinary Services (DVS), Selangor, Malaysia. The inclusion criteria included location of farm within Selangor State in Malaysia, free-stall housing, adequate farm records on animal health, milk yield and fertility, herd size of 50 or more milking cows, periodic trimming of the cows either at dry-off or during lactation, and absence of pasture grazing. A total of 18 farms were contacted and 6 of them agreed to participate in the study. Upon further examination, 2 farms were excluded based on the floor design, management routine during calving, and uncertainty of the cows remaining in the study for the planned period. The factors considered for the management and herd characteristics of the enrolled farms are presented in Table 1. The farms were located in Batang Kali (n = 1), Semenyih (n = 2), and Kajang (n = 1) while the herd size ranged from 145-200 cows (mean of 172 cows). All the enrolled cows were Australian Friesian Sahiwal (crossbreed) and the average 305-day milk yield ranged from 2, 700 to 3, 200 kg.

Lameness Control

All the farms practiced routine claw trimming either during dry off or mid-lactation and footbathing as herd health programs targeted to improve claw health. The farmers were informed about the methodology of the study, and that no interruption of routine farm and management practices would be attempted except the suspension of CT in the sampled cows during the study period.

Cow Enrollment

Information on animal health status, DIM, parity and previous lameness event (PLE) were obtained from each farms' health and production records. Next, all the

Table 1
Herd characteristics of the studied farms

Features	Farm 1	Farm 2	Farm 3	Farm 4
Location	Semenyih	Batang Kali	Semenyih	Kajang
Herd size	145	170	175	200
No. of milking cows	63	68	85	74
305-day milk yield(kg/cow)	3100	2700	2900	3200
Management system				
Housing	Free-stall	Free-stall	Free-stall	Free-stall
Access to pasture	No	No	No	No
Stocking rate(cow/ stall)	One	One	One	One
Floor and stall design				
Floor type	Concrete	Concrete	Concrete	Concrete
Stall base	Rubber mats	Rubber mats	-	-
Location	Milking, walking alley and resting barn	Milking parlour and resting barn	Resting barn	Resting barn
Frequency of cleaning (per day)	2	3	3	>3
Use of footbath	Yes	Yes	Yes	Yes
Claw trimming (per year)	Once	Once	Twice	Once

lactating cows in each herd were assessed for locomotion score (LS) using the four-point LS system developed by DairyCo (n. d.). Briefly, the LS system entailed the observation of postural (head bob and back presentation) and gait (stride length, steps, weight transfer or bearing) features during cows' locomotion, which were then aggregated to classify lameness severity. Based on the scale, LS 1 = sound cows; absence of head bob, straight back presentation, symmetrical gait, and normal stride, LS 2 = non-lame; absence of head bob and/or ached back and shortened stride length, LS 3 = lame; Presence of head bob and/or ached back and shortened stride length, LS 4 = severely lame; as presented in LS 3 and non-weight bearing or recumbent. Only the non-lame cows that were within 40 days in milk (DIM) and apparently free from other disease conditions were selected for further evaluation. Priority was given to the animals expected to remain in the herd for the study period.

The hind limbs were examined for hock condition score (HCS) on a 3-point scale where, 1 = normal area with no alopecia and inflammation, 2 = hair loss but absence or slight swelling (< 1- 2cm), 3 = hair loss and substantial swelling (> 2cm) (Cook, 2006). Cows with the two latter scores were not enrolled. Upon hoof examination, only the cows without lesions were enrolled; however, cows with history of PLE or claw

lesions (one month before study period) were only considered for enrollment after complete recovery from lameness and resolution of lesions. The latter was defined by the presence of LS 1 and non-withdrawal of the previously affected limb/claw upon applying pressure using a hoof tester. The dorsal wall angle was used to measure the presence of claw overgrowth on the hind feet as described by Solano et al. (2015). Values < 45 degree were classified as overgrown and such cows were not considered for enrollment.

Other ABWMs such as body condition score (BCS), body injuries (BDI), and leg hygiene were recorded during cow enrollment. The BCS was measured using a 4-point scale recorded as thin, poor, moderate and fat respectively (Elanco Animal Health, 1997). Cows with BCS of 3 were enrolled for the study. Presence of injuries on body regions (neck, brisket, carpal joint area, rib-cage area, area over the tuber coxae, ischial area, hock joint area, teats, and udder) was observed as described by Cook et al. (2016). The signs considered as indicators of BDI included presence of external wounds, ulcerations, swellings, localized hair loss, scars, and skin hyperkeratosis. The lateral lower hind limbs were assessed for cleanliness from the coronary band to the tarsal joint region based on degree of manure contamination using a 0-3 scale: 0 = fresh manure for < 50% of the area; 1 = fresh manure for >50% of the area; 2 = dried caked and fresh manure for < 50% of the area; and 3 = entire area with dried caked manure (Gibbons et al., 2012). Finally, a purposive sample of 30 Australian Friesian Sahiwal cows were selected from each farm (total = 120) and their tag numbers were recorded. The cows were enrolled at early-lactation and followed for 9 months.

Locomotion Scoring and Cow Characteristics

One investigator visited the farms twice every month for locomotion scoring of the cows and collection of animal-based data. The locomotion scoring took place immediately after the cows were enrolled until the dry-off period. Two successive LS 3 or a single LS 4 was necessary for a cow to be considered lame as described by Thomas et al. (2016). Immediately a cow fulfilled the lameness definition (censored), lactation stage and DIM were recorded, and all the front and rear feet were examined for claw lesions or other definite cause of lameness.

Assessment of Claw Health and Lesions Recording

Claw lesions were recorded based on the description of previous authors (Green et al., 2014; Solano et al., 2016) and in conjunction with the International Committee of Animal Recording (ICAR) Claw Health Atlas (Egger-Danner et al., 2015). Infectious lesions consisted of digital dermatitis (DD), heel horn erosion, and swelling of the coronet area (SC), whereas non-infectious group included sole lesions (haemorrhage, ulcer, bruises, and double sole), toe ulcer, white line disease (WLD), heel lesions, (HL). Lesions such as wall fissures, interdigital

hyperplasia and corkscrew were recorded as "others". The presence of different lesions on a single foot or different feet was considered as multiple lesions, whereas the presence of more than one of the same lesion on one foot or two feet was recorded as a single lesion.

Management of Dropout and Missing Data

The cows that became lame during the study were managed according to the farms' protocol. Locomotion scores and animal-based data were collected from all the enrolled animals at 18 different time points (every two weeks for 9 months). All the enrolled cows (n = 120) remained in the study throughout the study period.

Statistical Analysis

Descriptive statistics was used to summarize the proportion of lame cows and distribution of claw lesions. To determine the association between ABWMs and lameness occurrence, a multivariable cox proportional regression model was built following a two-stage procedure. Parity and PLE were recorded as present during enrollment. Since similar BCS, HCS, leg hygiene, and claw length were considered before cow enrollment from each farm, the last record of each parameter before the occurrence of two consistent lame scores (LS3 or LS4) was recorded for the affected cows. For the non-lame cows, median scores of each parameter were computed. Next, a univariable cox proportional regression model was constructed to evaluate the association between lameness and the covariates: leg hygiene (clean, dirty, and very dirty), parity, HCS (normal, hair loss, and swelling/ulcer), BCS (thin, good, and fat), BDI (present or absent), claw overgrowth (present or absent), PLE (present or absent), and claw lesion (present or absent). In the next stage, factors were introduced into the multivariable cox proportional regression model if the *p*-value < 0.10. Farm was introduced into the model as a random effect. A forward method was applied and changes in the remaining coefficients were checked as factors were excluded in the model. *P*-value < 0.05 was used for the final model.

For lesions prevalence, the outcome was the odds for claw lesion at the end of the follow-up period. Binary logistic regression model was only conducted for ICL as the prevalence of other lesions was relatively small. As such, analysis was done for cows with CHL compared with those without lesions. The final records of the welfare parameters (BDI, HCS, BCS, leg hygiene, and claw length) were used for the analysis, whereas parity and PLE were recorded as present during enrollment. A binary logistic regression model similar two step model building process described earlier was used to build the final multivariable logistic regression models. Comparisons between the predicted proportions in the model and the actual proportions in the data set were used in determining the model fit. The results were presented in odds ratios and at 95% confidence interval (CI).

RESULTS AND DISCUSSION

Descriptive Statistics and Characteristic of the Study Population

This work investigated the association between ABWM, lameness occurrence, and claws lesions prevalence in dairy cows. One reason for this piece of work was to elucidate the direction of event between welfare measures such as BCS, body injuries, hock condition, claw length, and the lameness incidence during lactation in the dairy cow. The descriptive statistics for the enrolled cows is presented in Table 2. Fifty-two percent (63/120) of the cows were within first 30 DIM, and majority (85%; 102/120) of them had moderate BCS during enrollment. The mean (± SD) milk yield of the sampled cows was 14.3±1.7 kg/day and all of them were having normal HCS, claw length, and absence of BDI when they were enrolled.

Table 2
Descriptive statistics of the 120 cows enrolled from the 4 studied farms

Factors	Frequency	%
DIM		
1-30	63	52.5
31-60	57	47.5
Parity		
Primiparous	41	34.2
Second	47	39.2
Third and above	32	26.6
BCS		
3.0	102	85
>3.0	18	15
Body injuries		
Normal (absent)	120	100
Previous lameness event		
Yes	27	77.5
No	93	22.5
Leg hygiene		
Clean	75	62.5
Dirty	35	29.2
Very dirty	10	8.3
HCS		
Normal	120	100
Claw length (all claws)		
Normal (≥ 45º)	120	100
Milk yield (kg)/cow/day[2]	14.3± 1.7	

Note. [1]DIM = days in milk, [2]Milk yield is presented in mean ± standard deviation. Other variables are presented in categories and proportions

Lameness Incidence and Association with ABWMs

Twenty-four percent (29/120) of the cows were lame during the study period. At every monthly observation period, the proportion of non-lame cows never went below 60%. The incidence of lameness (24.2%) reported in the present study was higher than that of Bryan et al. (2012), similar to that of Olechnowicz et al. (2010), but lower compared to that of Green et al. (2014). The difference might be related to the definition of lameness, potential risk factors, and variation in milk yield during lactation. Since the absence of overgrown claw was one of the enrollment criteria, the higher proportion of sound cows might be related to benefits of proper claw length and maintenance of sole thickness. These events improve weight distribution between the medial and lateral hind claws, and frictional properties at the floor-claw interface (Nuss & Paulus, 2006; Sadiq et al., 2020b; van der Tol et al., 2004).

The factors in the univariable cox regression model included BCS pre-lame, claw length, and PLE. Cows with overgrown claw (Hazard ratio; HR = 3.4; 95% CI 1.2-11.4) had higher risk of being lame compared to those with normal claw length. Higher risk for lameness was observed in cows with PLE (HR = 6.9; 95% CI 2.3-23.2) than those without lameness history in previous lactations (Table 3). BCS pre-lame was associated ($p = 0.03$) with the lameness incidence in the study population. Other factors such as parity, HCS, leg hygiene, and DIM (during enrollment) were not associated with lameness incidence. Similar proportions (44%) of the cows became lame around early (within 120 DIM) and mid-lactation (181-200 DIM), while less than 15% of the lameness cases occurred during late lactation (above 200 DIM) (Figure 1). The predictors of lameness in the final cox regression model were claw length, BCS pre-lame, and previous lameness event. Cow with PLE were 7 times more likely (95% CI 2.4-23.0) to be lame during the study period compared to those without PLE. Likewise, those with overgrown claw (HR = 3.7; 95% CI 1.1-12.6) and thin BCS pre-lame (HR = 4.5; 95% CI 1.3-16.6) were at higher risk of being lame than those with normal claw

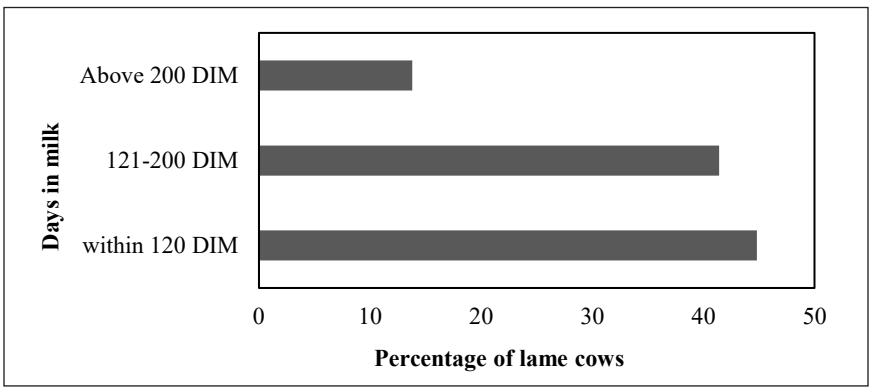

Figure 1. Percentage of lame cows during various stages of lactation

Table 3
Cox proportional hazard model estimates of the association between ABWM and time to lameness event based on the univariable and multivariable cox proportional hazard model

	Univariable model						Multivariable model					
	B	S. E.	Wald	*p*-value	HR	95% CI	B	S. E.	Wald	*p*-value	HR	95% CI
Claw length												
Overgrown	-1.2	0.6	3.7	0.04	3.4	1.2-11.4	1.3	0.6	4.6	0.03	3.7	1.1-12.6
Normal					Ref						Ref	
BDI												
Present	0.5	0.8	0.4	0.4	1.7	0.3-8.9						
Absent					Ref							
BCS pre-lame			6.6	0.03					6.0	0.05		
< 3.0	1.5	0.9	2.8	0.1	4.8	0.7-30.0	-1.4	0.6	5.8	0.01	4.5	1.3-16.6
3.0	-0.1	0.8	0.1	0.8	0.8	0.1-4.3	-1.3	0.8	2.3	0.1	3.8	0.6-20.8
> 3.0					Ref						Ref	
HCS pre-lame			0.9	0.6								
Normal	1.3	1.4	0.8	0.3	3.6	0.2-61.2						
Hair loss	0.9	1.5	0.4	0.5	2.6	0.1-51.9						
Swollen					Ref							
Leg hygiene pre-lame			1.9	0.3								
Clean	-0.3	1.0	0.0	0.7	0.7	0.09-5.8						
Dirty	-1.2	1.1	1.0	0.2	0.2	0.03-2.9						
Very dirty					Ref							
Previous lameness												
Present	-1.9	0.6	10.2	0.001	6.9	2.3-23.2	-0.01	0.5	12.2	0.01	7.4	2.4-23.0
Absent					Ref						Ref	
Parity			1.7	0.4								
First	-0.9	0.7	1.5	0.2	0.3	0.09-1.7						
Second	-0.6	0.6	1.0	0.3	0.5	0.1-1.9						
Third					Ref							
DIM												
1-30	-1.8	0.3	0.2	0.6	0.8	0.3-1.8						
31-60					Ref							

Note. B = Beta, S. E. = Standard error, Wald = Chi-square statistic, HR = Hazard ratio, CI = Confidence interval, DIM = Days in milk, Ref = Reference group, *p*-value < 0.05 was considered for significant associations in the multivariable model

length and good body condition (BCS = 3), respectively.

Previous studies have demonstrated positive association between overgrown claw and lameness occurrence in dairy cows (Sadiq et al., 2017a; Solano et al., 2015). Accordingly, overgrown claw affects cows' weight distribution especially around the rear claw; thus, enhancing conformational changes those results to altered locomotion (Sadiq et al., 2020b; van der Tol et al., 2004). The lateral claw of the rear foot bears a larger part of the weight directed unto the limb and the function is affected when the claw length is overgrown (Alsaaod et al., 2017). Claw overgrowth affects the placement of the foot and displaces the stride during locomotion (Alsaaod et al., 2017).

Lame cows experience behavioral changes such as reduced frequency time, frequency of visits to feed bunk, and lower energy balance to compete for feed, which may result to body condition loss (Green et al., 2014; Ramanoon et al., 2018b). These behavioral changes are defensive mechanisms against the on-going pain in lame cows (Whay & Shearer, 2017). In this study, majority of the lame cows had low BCS prior to the onset of clinical lameness. Randall et al. (2015) reported that dairy cows with low BCS were more likely to become lame in the future. The finding suggests that BCS loss occurred for certain period before the onset of gait changes. The changes in BCS as shown in this study could be used to identify cows requiring prompt management; thus, reducing the chances of future lameness event.

Cows with previous history of lameness event had higher risk of being lame in the present lactation. Although claw health records were not sufficient to identify the causes of previous lameness episodes in the enrolled cows, other related studies have shown that cows with previous lameness history are more likely to be lame in subsequent lactation (Huxley et al., 2013; Randall et al., 2015). Such relapses in lameness event are common in CHL due to pathological changes within the capsule that destabilizes the pedal bone (Newsome et al., 2017b; Räber et al., 2006). Moreover, most of the studied cows underwent routine CT once before enrollment and no further trimming was conducted during the study period. Repeated CT might be required improve the stability of pedal bone and reduce the tendency of relapse of previous horn lesions (Sadiq et al., 2020b). Preventive claw trimming at dry off and again at DIM 40–60 could reduce the lameness prevalence during early lactation (Green et al., 2002).

Factors such as HCS, leg hygiene, and parity were not associated with lameness incidence in the present study. In cross-sectional studies, higher odds of lameness were observed in cows with poor HCS (swollen hock or hair loss) (Solano et al., 2015), higher parity (Lim et al., 2015; Machado et al., 2010) and poor leg hygiene (Relun et al., 2013; Sadiq et al., 2017a). However, the direction of the events could not be determined in these studies. All the sampled cows in the present study had normal HCS during enrollment and majority of the lame cows had no changes in their

hock condition during their first lameness events. This finding reinstates that claw-related changes were more important in influencing lameness occurrence in the studied cows than hock injuries. The lack of association between lameness, parity, leg hygiene, and BDI could be related to the various causes of lameness and their severity in the sampled cows. Moreover, studies have shown that parity influences the occurrence of infectious claw lesions and CHLs in different ways. Cows at higher parity had higher incidence of CHL at drying off (Machado et al., 2010) and during lactation (Oberbauer et al., 2013). In contrast, the risk of DD was lower in greater parity cows compared with those at first and second parity (Holzhauer et al., 2006), whereas incidence of DD was twice in heifers that calved at younger age compared to those that calved at older age (Mellado et al., 2018).

Herd cleanliness is another factor associated with lameness risk in dairy herds. The overall herd cleanliness can be estimated using the leg hygiene of individual cows (Cook, 2006). Herein, we found no association between lameness risk and leg hygiene at cow level. This finding contradicts those of previous studies where poor leg hygiene increased the chances of lameness and infectious claw lesions in dairy cows (Solano et al., 2015, 2016). However, one of the study designs was cross-sectional and thus; limits further information on the events' direction. Since the present study entailed a longitudinal approach, the result suggests that leg hygiene may not be as important as other welfare parameters influencing lameness incidence during lactation. However, the subjectivity of the leg hygiene scoring method and lesions prevalence could also contribute to the disparity in results. Majority of the claw lesions observed in this study were CHL, and leg hygiene may play more significant role in the development of claw lesions of infectious origin (Relun et al. 2013; Wilson-Welder et al., 2015).

Claw Lesions Analysis

A total of 57 claw lesions were recorded in 37% (44/120) of the enrolled cows during the study. Thirty-nine cows (68.4%) were lame amongst the cows affected with claw lesions. CHL were predominant (75.4%; 43/57) compared to ICL (24.7%; 14/57) (Table 4).

The proportion of lame cows amongst each lesion category was highest for sole lesions (SU/SH) (16/20; 80%) and ICL (12/14; 86%) (Figure 2).

The lesion prevalence is comparable to the reports in previous studies (Becker et al., 2014; Cramer et al., 2008). Regarding the higher prevalence of CHL compared to infectious types, our previous study in selected dairy farms in Selangor showed a similar result, with sole lesions and WLD being the most prevalent at cow level (Sadiq et al. 2017a). CHL are multifactorial and intensive dairy facilities are highly susceptible to claw disorders (Cook et al., 2016; Charfeddine & Perez-Cabal, 2017). Preventive measures entail appropriate stall designs to improve cow comfort, as

Table 4
Prevalence of claw lesions at during lactation of 120 cows enrolled in the study by categories of claw lesions and proportion of lame cows

	Claw lesions	Lame cows
Claw horn lesions		
SL	20	16
WLD	10	5
TU	5	4
HU	1	1
Others[3]	7	1
Total[1] (%)	43 (75.4)	27 (62.7)
ICL[2]	14 (24.6)	12 (85.7)
Total[4] (Overall)	57 (100)	39 (68.4)

Note.
[1]Total number and percentage of claw horn lesions
[2]Infectious claw lesions comprise of digital and interdigital dermatitis, heel horn erosion, and swollen coronet
[3]Others comprise of corkscrew claw, interdigital hyperplasia
[4]Total number of claw lesions recorded in both groups
Cows were counted more than one if they had more than one claw lesion
ICL = infectious claw lesion, SL = sole lesions, WLD = white line disease, TU = toe ulcers, HU = heel ulcer

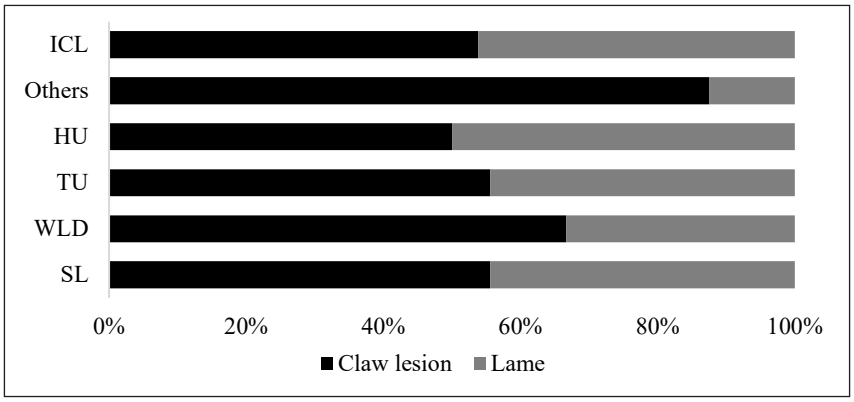

Figure 2. Proportion of lame cows among those affected with various claw lesions

well as specific management during high risk period. For instance, the cows were housed under completely confined systems without provision for pasture grazing. Recent reports suggest that pasture access is important for the reduction of CHL occurrence (Armbrecht et al., 2018) and these lesions elicit greater pain than other foot lesions based on the evaluation of nociceptive threshold (Passos et al., 2017). An indication of such event in this study was the higher record of lameness cases amongst those affected with CHL.

Covariates in the univariable model were median BCS and PLE. Cows with poor BCS (< 3.0) (OR = 5.7; 95% CI 1.6-20.4)

and PLE (OR = 7.27; 95% CI = 2.1-24.7) had higher lameness prevalence in relation to those good BCS and no lameness history. At the multivariable level, the chances of having CHL tended to be associated with lower BCS (p = 0.08), whereas higher odds of CHL (OR = 5.4; 95% CI 2.4-23.3) was observed in cows with PLE (Table 5).

The finding regarding association between BCS and CHL is consistent with recent studies reporting significant BCS loss before (Sepúlveda-Varas et al., 2018; Stambuk et al., 2019) and after (Sepúlveda-Varas et al., 2018) CHL incidence. Various changes occurring around calving that induces body condition loss has been linked to the increased risk of CHL (Bicalho et al., 2009; Machado et al., 2010; Newsome et al., 2017a). Thickness of DC; a protective apparatus for the sensitive structures during limb loading, is reduced during body condition loss (Räber et al., 2006; Bicalho et al., 2009). Hence, thinner cows in the present study might be developed thinner DC; thus, reducing the protective function and increasing the chance of corium injury. Newsome et al. (2017b) showed that thinness of the DC, corium (soft tissues), and back fat thickness increased the likelihood of CHL lameness. However, CHL occurred independent of both parameters, indicating that thinness of the DC could result from other physiological changes (aside body fats) affecting the suspensory apparatus (Newsome et al., 2017b).

Cows in the present study had positive association between PLE and CHL prevalence. The result is in agreement with that of Randall et al. (2015), where severe WLD and sole lesions were associated with increased risk of future lameness events. Likewise, Foditsch et al. (2016) demonstrated that cows affected with CHL in the previous lactation and those lame at drying off had greater risk of developing CHDL in the subsequent lactation. These events could be due to pathological changes in the claw capsule resulting from similar lesion type during previous lactations. The relapse of such lesions in subsequent lactation was mostly reported during the high risk period for lameness (Foditsch et al., 2016). For instance, the claw affected with CHDL had significant bone development that majorly influences the locomotion score in the future (Newsome et al., 2016). Our finding supports the suggestion linking lifetime lameness history and future risk of CHL.

Both overgrown claw and BDI were not associated with CHL. The different floor designs and herd cleanliness might contribute to such finding, since these factors mediate the growth and wear of hoof horn tissues (Alsaaod et al., 2017). Moreover, normal claw length was considered as a criterion for cow enrollment. BDI frequently results from poor stall designs and lying surfaces that are unfavorable to cow welfare (Cook et al., 2016). The lack of association between BDI and CHL prevalence could be the low severity of horn lesions and similarity in stall designs in the studied farms. Severity of lesions might not be sufficient to induce prolonged lying down time and onset of BDI. Likewise, factors

Table 5
Associations between ABWMs and presence of claw horn lesion during lactation, displayed as odds ratio based on the univariable and multivariable model

Variables	Univariable model						Multivariable model					
	B	S. E.	Wald	*p*-value	HR	95% CI	B	S. E.	Wald	*p*-value	HR	95% CI
Claw length												
Overgrown	0.07	0.7	0.3	0.3	2.0	0.1-9.9						
Normal					Ref							
BDI												
Present	0.05	0.6	0.01	0.9	1.0	0.2-3.9						
Absent					Ref							
BCS			7.4	0.02					4.9	0.08		
< 3.0	-1.7	0.6	7.4	0.01	5.7	1.6-20.4	-1.2	0.5	4.8	0.03	0.2	0.1-0.8
3.0	-0.9	0.8	1.2	0.2	0.3	0.1-2.0	-0.6	0.7	0.5	0.4	0.5	0.1-2.5
> 3.0					Ref						Ref	
HCS			0.2	0.9								
Normal	-0.1	0.6	0.09	0.7	0.8	0.2-2.7						
Hair loss	-0.4	1.1	0.1	0.7	0.6	0.1-6.0						
Swollen					Ref							
Leg hygiene			2.8	0.2								
Clean	-0.9	0.6	2.2	0.1	0.3	0.1-1.3						
Dirty	0.5	0.9	0.3	0.5	1.7	0.2-10.7						
Very dirty					Ref							
Previous lameness												
Present	1.9	0.6	10.1	0.01	7.2	2.1-24.7	2.0	0.5	12.1	0.01	5.4	2.4-23.3
Absent					Ref						Ref	
Parity			1.9	0.3								
First	-0.1	0.6	0.07	0.7	0.8	0.2-2.8						
Second	0.6	0.6	1.0	0.3	1.9	0.5-7.0						
Third					Ref							
DIM												
1-30	-0.6	0.5	1.4	0.2	0.5	0.1-.1.5						
31-60					Ref							

Note. B = Beta, S. E. = Standard error, Wald = Chi-square statistic, OR = Odds ratio, CI = Confidence interval, DIM = Days in milk, Ref = Reference group, *p*-value < 0.05 was considered for significant associations in the multivariable model

relating to stall designs differ in their impact on CHL and BDI in dairy herds (Adams et al., 2017; Cook et al., 2016).

Limitations inherent in this study are well identified. The findings in this study are specific to cows kept in free-stalls with no access to outdoor grazing. The management system was selected based on reports of increased risk of lameness and claw pathologies in confined cows. Though the analysis was carried out at cow level, a larger sample size might have provided higher chances of obtaining significant associations between lameness and other explanatory variables. Other factors such as herd hygiene and handling of the animals by farm staff might influence some of the findings, despite selecting the farms based on similar management routines and practices. Longer observational period and inclusion of other vital ABWMs for further elucidation of factors associated with lameness occurrence could be considered in future studies.

ACKNOWLEDGEMENT

This study was supported by the Universiti Putra Malaysia (UPM/800-3/3/1/GP-IPS/2016/9507600). Our sincere appreciation goes to the Department of Veterinary Services (DVS), Selangor and all the technical staff of the Department of Farm and Exotic Animals Medicine and Surgery, Faculty of Veterinary Medicine UPM Serdang Selangor, Malaysia.

COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest

The authors declare that they have no conflict of interest.

Ethical Statement

This study was approved by the Institution of Animal Care and Use Committee (UPM/IACUC/AUP-R028/2017). Examination of the animals was conducted by trained professionals in lameness assessment and diagnosis. All the participating farmers were informed about the purpose and methods in the study and participation was voluntary.

REFERENCES

Adams, A. E., Lombard, J. E., Fossler, C. P., Román-Muñiz, I. N., & Kopral, C. A. (2017). Associations between housing and management practices and the prevalence of lameness, hock lesions, and thin cows on US dairy operations. *Journal of Dairy Science*, *100*(3), 2119–2136. https://doi.org/10.3168/jds.2016-11517

Alsaaod, M., Huber, S., Beer, G., Kohler, P., Schupbach-Regula, G., & Steiner, A. (2017). Locomotion characteristics of dairy cows walking on pasture and the effect of artificial flooring systems on locomotion comfort. *Journal of Dairy Science*, *100*(10), 8330-8337. https://doi.org/10.3168/jds.2017-12760

Armbrecht, L., Lambertz, C., Albers, D., & Gauly, M. (2018). Does access to pasture affect claw condition and health in dairy cows?. *The Veterinary Record*, *182*(3), 79. https://doi.org/10.1136/vr.104554

Becker, J., Steiner, A., Kohler, S., Koller-Bahler, A., Wuthrich, M., & Reist M. (2014). Lameness and foot lesions in Swiss dairy cows: II. Risk factors.

Schweiz Arch Tierheilkd, *156*(2), 79-89. https://doi.org/10.1024/0036-7281/a000554

Bicalho, R. C., Machado, V. S., & Caixeta, L. S. (2009). Lameness in dairy cattle: A debilitating disease or a disease of debilitated cattle? A cross-sectional study of lameness prevalence and thickness of the digital cushion. *Journal of Dairy Science*, *92*(7), 3175-84. https://doi.org/10.3168/jds.2008-1827

Bran, J. A., Daros, R. R., von Keyserlingk, M. A. G., LeBlanc, S. J., & Hötzel, M. J. (2018). Cow and herd-level factors associated with lameness in small-scale grazing dairy herds in Brazil. *Preventive Veterinary Medicine*, *151*(1), 79–86. https://doi.org/10.1016/j.prevetmed.2018.01.006

Bryan, M., Tacoma, H., & Hoekstra, F. (2012). The effect of hindclaw height differential and subsequent trimming on lameness in large dairy cattle herds in Canterbury, New Zealand. *New Zealand Veterinary Journal*, *60*(6), 349–55. https://doi.org/10.1080/00480169.2012.698443

Charfeddine, N., & Perez-Cabal, M. A. (2017). Effect of claw disorders on milk production, fertility, and longevity, and their economic impact in Spanish Holstein cows. *Journal of Dairy Science*, *100*(1), 653-665. https://doi.org/10.3168/jds.2016-11434

Cook, N. B. (2006). *Footbath alternatives*. http://www.milkproduction.com/Library/Scientific-articles/Animal-health/Footbath-alternatives/

Cook, N. B., Hess, J. P., Foy, M. R., Bennett, T. B., & Brotzman, R. L. (2016). Management characteristics, lameness, and body injuries of dairy cattle housed in high-performance dairy herds in Wisconsin. *Journal of Dairy Science*, *99*(7), 5879-5891. https://doi.org/10.3168/jds.2016-10956

Cramer, G., Lissemore, K. D., Guard, C. L., Leslie, K. E., & Kelton, D. F. (2008). Herd- and cow-level prevalence of foot lesions in Ontario dairy cattle. *Journal of Dairy Science*, *91*(10), 3888-3895. https://doi.org/10.3168/jds.2008-1135

DairyCo. (n. d.). *DairyCo mobility score*. https://www.farmhealthonline.com/wp-content/uploads/2013/11/dairy_co_mobility_score.pdf

Egger-Danner, C., Nielsen, P., Fiedler, A., Müller, K., Fjeldaas, T., Döpfer, D., Daniel, V., Bergsten, C., Cramer, G., Christen, A.M., Stock, K. F., Thomas, G., Holzhauer, M., Steiner, A., Clarke, J., Capion, N., Charfeddine, N., Pryce, J. E., Oakes, E., Burgstaller, J., Heringstad, B., Ødegård, C., & Kofler, J. (2015). *ICAR claw health atlas*. http://www.icar.org/Documents/ICAR_Claw_Health_Atlas.pdf

Elanco Animal Health. (1997). *Body condition scoring in dairy cattle*. https://www.vet.cornell.edu/sites/default/files/1e_Elanco%20Cow%20Body_condition_scoring_V3.pdf

Foditsch, C., Oikonomou, G., Machado, V. S., Bicalho, M. L., Ganda, E. K., Lima, S. F., & Bicalho, R. C. (2016). Lameness prevalence and risk factors in large dairy farms in upstate New York. Model development for the prediction of claw horn disruption lesions. *PLOS One*, *11*(1), e0146718. https://doi.org/10.1371/journal.pone.0146718

Gibbons, J., Vassuer, E., Rushen, J., & de Passillé, A. M. (2012). A training programme to ensure high repeatability of injury scoring of dairy cows. *Animal Welfare*, *21*(3), 379–388. https://doi.org/10.7120/09627286.21.3.379

Green, L. E., Hedges, V. J., Schukken, Y. H., Blowey, R. W., & Packington, A. J. (2002). The impact of clinical lameness on the milk yield of dairy cows. *Journal of Dairy Science*, *85*(9), 2250–2256. https://doi.org/10.3168/jds.S0022-0302(02)74304-X

Green, L. E., Huxley, J. N., Banks, C., & Green, M. J. (2014). Temporal associations between low body condition, lameness and milk yield in a

UK dairy herd. *Preventive Veterinary Medicine*, *113*(1), 63-71. https://doi.org/10.1016/j.prevetmed.2013.10.009

Holzhauer, M., Hardenberg, C., Bartels, C. J., Frankena, K. (2006). Herd- and cow-level prevalence of digital dermatitis in the Netherlands and associated risk factors. *Journal of Dairy Science*, *89*(2), 580-588. https://doi.org/10.3168/jds.S0022-0302(06)72121-X

Hund, A., Logrono, J. C., Ollhort, R. D., & Kofler, J. (2019). Aspects of lameness in pasture based dairy farms. *Veterinary Journal*, *244*, 83-90. https://doi.org/10.1016/j.tvjl.2018.12.011

Huxley, J. N. (2013). Impact of lameness and claw lesions on health and production. *Livestock Science*, *156*(1-3), 64–70. https://doi.org/10.1016/j.livsci.2013.06.012

Lim, P. Y., Huxley, J. N., Willshire, J. A., Green, M. J., Othman, A. R., & Kaler, J. (2015). Unravelling the temporal association between lameness and body condition score in dairy cattle using a multistate modelling approach. *Preventive Veterinary Medicine*, *118*(4), 370–377. https://doi.org/10.1016/j.prevetmed.2014.12.015

Machado V. S., Caixeta L. S., McArt J. A. A., & Bicalho R. C. (2010). The effect of claw horn disruption lesions and body condition score at dry-off on survivability, reproductive performance, and milk production in the subsequent lactation. *Journal of Dairy Science*, *93*(9), 4071–4078. https://doi: 10.3168/jds.2010-3177

Mellado, M., Saavedra, E., Gaytán, L., Véliz, F., Macías-Cruz, U., Avendaño-Reyes, L., García, J. (2018). The effect of lameness-causing lesions on milk yield and fertility of primiparous Holstein cows in a hot environment. *Livestock Science*, *217*, 8-14. https://doi.org/10.1016/j.livsci.2018.09.008

Newsome, R. F., Green, M. J., Bell, N. J., Bollard, N. J., Mason, C. S., Whay, H. R., & Huxley, J. N. (2017a). A prospective cohort study of digital cushion and corium thickness. Part 1: Associations with body condition, lesion incidence, and proximity to calving. *Journal of Dairy Science*, *100*(6), 4745–4758. https://doi.org/10.3168/jds.2016-12012

Newsome, R. F., Green, M. J., Bell, N. J., Bollard, N. J., Mason, C. S., Whay, H. R. & Huxley, J. N. (2017b). A prospective cohort study of digital cushion and corium thickness. Part 2: Does thinning of the digital cushion and corium lead to lameness and claw horn disruption lesions?. *Journal of Dairy Science*, *100*(6), 4759-4771. https://doi.org/10.3168/jds.2016-12013

Newsome, R., Green, M. J., Bell, N. J., Chagunda, M. G. G., Mason, C. S., Rutland, C. S., Sturrock, C. J., Whay, H. R., & Huxley, J. N. (2016). Linking bone development on the caudal aspect of the distal phalanx with lameness during life. *Journal of Dairy Science*, *99*(6), 4512–4525. https://doi.org/10.3168/jds.2015-10202

Nuss, K., & Paulus, N. (2006). Measurements of claw dimensions in cows before and after functional trimming: A post-mortem study. *Veterinary Journal*, *172*(2), 284–292. https://doi.org/10.1016/j.tvjl.2005.04.031

Oberbauer, A. M., Berry, S. L., Belanger, J. M., McGoldrick, R. M., Pinos-Rodriquez, J. M., & Famula, T. R. (2013). Determining the heritable component of dairy cattle foot lesions. *Journal of Dairy Science*, *96*(1), 605–613. https://doi.org/10.3168/jds.2012-5485

Olechnowicz, J., Jedrzej, M., & Jaskowski, J. M. (2010). Impact of clinical lameness, calving season, parity, and month of lactation on milk, fat, protein, and lactose yields during early lactation of dairy cows. *Bulletin of the Veterinary Institute in Pulawy*, *54*(4), 605–610.

Oliveira, V. H. S., Sørensen, J. T., & Thomsen, P. T. (2017). Associations between biosecurity practices and bovine digital dermatitis in Danish

dairy herds. *Journal of Dairy Science*, *100*(10), 8398–8408. https://doi.org/10.3168/jds.2017-12815

Passos, L. T., da Cruz, E. A., Fischer, V., da Porciuncula, G. C., Werncke, D., Dalto, A. G. C., Stumpf, M. T., Vizzotto, E. F., & da Silveira, I. D. B. (2017). Dairy cows change locomotion score and sensitivity to pain with trimming and infectious or non-infectious lesions. *Tropical Animal Health and Production*, *49*(4), 851-856. https://doi.org/10.1007/s11250-017-1273-0

Räber, M., Scheeder, M. R., Ossent, P., Lischer, C. J., & Geyer, H. (2006). The content and composition of lipids in the digital cushion of the bovine claw with respect to age and location – A preliminary report. *The Veterinary Journal*, *172*(1), 173-177. https://doi.org/10.1016/j.tvjl.2005.03.009

Ramanoon, S. Z., Sadiq, M. B., Abdul Razak, N. M., Shaik Mossadeq, W. M., Mansor, R., & Syed-Hussain, S. S. (2018a). Retrospective analysis of lameness cases in cattle reported to the University Veterinary Hospital, University Putra Malaysia, from 2013-2017. *Jurnal Veterinar Malaysia*, *30*(1), 1-6.

Ramanoon, S. Z., Sadiq, M. B., Shaik Mossadeq, W. M., Mansor, R., & Syed-Hussain, S. S. (2018b). *The impact of lameness on dairy cattle welfare: Growing need for objective methods of detecting lame cows and assessment of associated pain*. https://www.intechopen.com/books/animal-welfare/the-impact-of-lameness-on-dairy-cattle-welfare-growing-need-for-objective-methods-of-detecting-lame-

Randall, L. V., Green, M. J., Chagunda, M. G., Mason, C., Archer, S. C., Green, L. E., & Huxley, J. N. (2015). Low body condition predisposes cattle to lameness: An 8-year study of one dairy herd. *Journal of Dairy Science*, *98*(6), 3766-3777. https://doi.org/10.3168/jds.2014-8863

Relun, A., Lehebel, A., Chesnin, A., Guatteo, R., & Bareille, N. (2013). Association between digital dermatitis lesions and test-day milk yield of Holstein cows from 41 French dairy farms. *Journal of Dairy Science*, *96*(4), 2190-2200. https://doi.org/10.3168/jds.2012-5934

Robichaud, M. V., Rushen, J., de Passillé, A. M., Vasseur, E., Orsel, K., & Pellerin, D. (2019). Associations between on-farm animal welfare indicators and productivity and profitability on Canadian dairies: I. On freestall farms. *Journal of Dairy Science*, *102*(5), 4341-4351. https://doi.org/10.3168/jds.2018-14817

Sadiq M. B., Ramanoon, S. Z., Shaik Mossadeq, W. M., Mansor, R., & Syed-Hussain, S. S. (2020a). Cow- and herd-level factors associated with lameness in dairy farms in Peninsular Malaysia. *Preventive Veterinary Medicine*, *184*, 105163. https://doi.org/10.1016/j.prevetmed.2020.105163

Sadiq M. B., Ramanoon, S. Z., Shaik Mossadeq, W. M., Mansor, R., Syed-Hussain, S. S. (2021). Prevalence and risk factors for hoof lesions in dairy cows in Peninsular Malaysia. *Livestock Science*, *245*, 104404. https://doi.org/10.1016/j.livsci.2021.104404

Sadiq, M. B., Ramanoon, S. Z., Mansor, R., Syed-Hussain, S. S., & Shaik Mossadeq, W. M. (2017a). Prevalence of lameness, claw lesions, and associated risk factors in dairy farms in Selangor, Malaysia. *Tropical Animal Health and Production*, *49*(8), 1741-1748. https://doi: 10.1007/s11250-017-1387-4

Sadiq, M. B., Ramanoon, S. Z., Mansor, R., Syed-Hussain, S. S., & Shaik Mossadeq, W. M. (2017b). Association between lameness and indicators of dairy cow welfare based on locomotion scoring, body and hock condition, leg hygiene and lying behavior. *Animals (Basel)*, *7*(11), 79. https://doi: 10.3390/ani7110079

Sadiq, M. B., Ramanoon, S. Z., Shaik Mossadeq, W. M., Mansor, R., & Syed-Hussain, S. S. (2020b). Claw trimming as a lameness management practice and the association with welfare and

production in dairy cows. *Animals*, *10*(9), 1515. https://doi.org/10.3390/ani10091515

Sadiq, M. B., Ramanoon, S. Z., Shaik Mossadeq, W. M., Mansor, R., & Syed-Hussain, S. S. (2019). Dairy farmers' perceptions of and actions in relation to lameness management. *Animals*, *9*(5), 270. https://doi: 10.3390/ani9050270

Sepúlveda-Varas, P., Lomb, J., von Keyserlingk, M. A. G., Held, R., Bustamante, H., & Tadich, N. (2018). Claw horn lesions in mid-lactation primiparous dairy cows under pasture-based systems: Association with behavioral and metabolic changes around calving. *Journal of Dairy Science*, *101*(10), 1–12. https://doi.org/10.3168/jds.2018-14674

Solano, L., Barkema, H. W., Mason, S., Pajor, E. A., LeBlanc, S. J., & Orsel, K. (2016). Prevalence and distribution of foot lesions in dairy cattle in Alberta Canada. *Journal of Dairy Science*, *99*(8), 6828-6841. https://doi.org/10.3168/jds.2016-10941

Solano, L., Barkema, H. W., Pajor, E. A., Mason, S., LeBlanc, S. J., Zaffino Heyerhoff, J. C., Nash, C. G., Haley, D. B., Vasseur, E., Pellerin, D., Rushen, J., de Passille, A. M., & Orsel, K. (2015). Prevalence of lameness and associated risk factors in Canadian Holstein-Friesian cows housed in freestall barns. *Journal of Dairy Science*, *98*(10), 6978-6991. https://doi.org/10.3168/jds.2015-9652

Stambuk, C. R., McArt, J. A. A., Bicalho, R. C., Miles, A. M., & Huson, H. J. (2019). A longitudinal study of digital cushion thickness and its function as a predictor for compromised locomotion and hoof lesions in Holstein cows. *Translational Animal Science*, *3*(1), 74-83. https://doi.org/10.1093/tas/txy107

Thomas, H. J., Remnant, J. G., Bollard, N. J., Burrows, A., Whay, H. R., Bell, N. J., Mason, C., & Huxley, J. N. (2016). Recovery of chronically lame dairy cows following treatment for claw horn lesions: A randomised controlled trial. *Veterinary Record*, *178*(5), 116. http://dx.doi.org/10.1136/vr.103394

van der Tol, P. P., van der Beek, S. S., Metz, J. H., Noordhuizen-Stassen, E. N., Back, W., Braam, C. R., & Weijs, W. A. (2004). The effect of preventive trimming on weight bearing and force balance on the claws of dairy cattle. *Journal of Dairy Science*, *87*(6), 1732–1738. https://doi.org/10.3168/jds.S0022-0302(04)73327-5

Whay, H. R., & Shearer, J. K. (2017). The impact of lameness on welfare of the dairy cow. *Veterinary Clinics of North America: Food Animal Practice*, *33*(2), 153-64. https://doi.org/10.1016/j.cvfa.2017.02.008

Wilson-Welder, J. H., Alt, D. P., & Nally, J. E. (2015). Digital dermatitis in cattle: Current bacterial and immunological findings. *Animals (Basel)*, *5*(4), 1114-1135. https://doi.org/10.3390/ani5040400

Pertanika J. Trop. Agric. Sci. 44 (1): 69 - 82 (2021)

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Review Article

Synbiotic Efficacy as Therapeutic Approach in Human Disease: A Review

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ABSTRACT

In combatting the increase in healthcare costs, at present, one of the preventive approaches to medicine has been developed with the upliftment of new synbiotic products. Synbiotic is the synergistic effect of probiotics and prebiotics which exert multiple beneficial effects and have been increasingly used in preventing or treating human diseases since the last ten years. Several trials have reported that synbiotic therapy could help in the treatment of human disease prevention. PubMed, Science Direct, and Google Scholar were searched by keywords 'prebiotic', 'probiotic', and 'synbiotic' for relevant literature from 2000 to 2020. A total of 58 articles were selected and revised. This paper evaluates the effect of synbiotic supplementation on different diseases, for instance, obesity, insulin resistance syndrome, diabetes, and non-alcoholic fatty liver disease. The progressive knowledge on the outcome of synbiotic supplementation on health, recent trends and developments in this field are summarised. However, further research is required to understand the mechanism of how synbiotics affect in different diseases.

Keywords: Diabetes, obesity, prebiotic, probiotic, synbiotic

ARTICLE INFO

Article history:
Received: 19 June 2020
Accepted: 18 December 2020
Published: 24 February 2021

DOI: https://doi.org/10.47836/pjtas.44.1.04

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INTRODUCTION

Synbiotic supplementation has significant effects in gut environment and human immunity system compared with the consumption of probiotic or prebiotic alone (Frece et al., 2009). Considerable evidence indicated the important function of synbiotics in human metabolic regulation

ISSN: 1511-3701
e-ISSN: 2231-8542

(Tajabadi-Ebrahimi et al., 2017). The modulation can be performed through the oral administration of good bacteria, such as *Lactobacillus* and *Bifidobacterium*, which are commonly known as probiotics. The appropriate intake of prebiotics, which are mainly indigestible oligosaccharides, results in a positive improvement in the formation of gut microbiota including unique functional properties (Tajabadi-Ebrahimi et al., 2017). Synbiotics mainly consist of probiotics and prebiotics with synergistic effects only. Synbiotics have diverse forms and are consumed as ingredients of yogurt or fermented milk, cheese, synbiotic juice and bread (Singh et al., 2011). By altering the gut microbiota probiotics and prebiotics, type 2 diabetes mellitus (T2DM) and cardiovascular diseases may be affected through the regulation of insulin signalling pathway and decrease the cholesterol level as well (Yoo & Kim, 2016). According to Ipar et al. (2015), the serum total cholesterol and total oxidative stress levels are significantly reduced after a synbiotic intervention in a study group.

An altered gut microbiota is linked with hypertension, which leads to the development of chronic kidney disease (CKD) (Yang et al., 2018). The intestinal microbes can be deliberated as best suitable candidate for the treatment and prevention of several diseases, such as weight loss regimens and weight management (Heiss, 2018). Moreover, human gut microbes are linked with various health conditions including respiratory tract infections (RTIs) via the gut–lung axis. Studies have reported that synbiotic therapy could help prevent RTIs (Chan et al., 2020). The findings of random clinical intervention trials using synbiotic supplementation in obesity, insulin resistance syndrome (IRS), T2DM, and non-alcoholic fatty liver disease (NAFLD) have been summarized in Table 1. Diet containing high protein and less carbohydrate is often efficiently followed during weight loss program but has been linked with the decrement of beneficial bacteria inside the body (Russell et al., 2011). These diets induce protein fermentation with metabolic by-product formation by the gut microbiota and can trigger inflammation in the colon (Yao et al., 2016). The synbiotic dietary intervention aims to correct the breakdown of upset gut microbiota found in obese people and improve health conditions by facilitating the weight reduction process (Anhê et al., 2015; Martinez et al., 2017). The structure of gut microbiota can influence the environmental factors and cause intrahepatic fat accumulation (Çakır et al., 2017). Consequently, the modulated interaction between liver and microbes has become the principle objective in the maintenance of NAFLD (Quigley, 2015). The incident of bacterial overgrowth in small intestinal can alter intestinal integrity and play a significant role in the pathogenesis of NAFLD. This situation can be regulated by modulating synbiotic content on the human diet (dos Santos et al., 2019; Mehal, 2013). A graphical representation of synbiotic effect on obesity, NAFLD, IRS and T2DM is reflected in Figure 1 (Sáez-Lara et al., 2016).

Table 1
Summarized information of random clinical trial intervention of synbiotics in obesity, insulin resistance syndrome, type 2 diabetes mellitus, and non-alcoholic fatty liver disease

Subjects	Dose/Strain/Prebiotics	Time	Outcomes	References
		Obesity		
153 men and women with obesity	*Lactobacillus rhamnosus* CGMCC1.3724, 6×10^8 CFU, with inulin	36 weeks	Reduces leptin and increase in Lachnospiraceae	Sanchez et al. (2014)
High BMI containing 73 children and adolescents	*Lactobacillus casei*, *L. rhamnosus*, *Streptococcus thermophilus*, *Bifidobacterium breve*, *L. acidophilus*, *B. longum*, *Lactobacillus bulgaricus*, containing FOS	8 weeks	Reduction in BMI z-score and waist circumference	Sáez-Lara et al. (2016)
77 overweight children	*Lactobacillus acidophilus*, *L. rhamnosus*, *Bifidobacterium bifidum*, *B. longum*, *Enterococcus faecium*, including FOS	4 weeks	Alteration of anthropometric measurements, reduction in TC, LDL-C, and serum level of oxidative stress	Ipar et al. (2015)
		Insulin resistance syndrome		
IRS (38 subjects)	*Lactobacillus casei*, *L. rhamnosus*, *S. thermophilus*, *B. breve*, *L. acidophilus*, *B. longum, L. bulgaricus*, with FOS	28 weeks	Fasting blood sugar level and insulin resistance level improved remarkably	Sáez-Lara et al. (2016)
		Type 2 diabetes		
54 T2DM patients Age- 35 to 70 years	*Lactobacillus acidophilus*, *L. casei*, *L. rhamnosus*, *L. bulgaricus*, *B. longum*, *B. breve*, *S. thermophilus*, 10^9 CFU, including 100 mg FOS	8 weeks	Improved TGL plasma level, HOMA-IR, and decreased CRP in serum level	Sáez-Lara et al. (2016)
81 patients (T2DM)	*Lactobacillus sporogenes*, 1×10^8 CFU with 0.07 g inulin in per gram	8 weeks	Remarkable reduction of serum insulin level, HOMA-IR, and assessment of homeostatic model β-cell function	Tajadadi-Ebrahimi et al. (2014)
78 T2DM patients	*Lactobacillus sporogenes*, 1×10^8 CFU including 0.07 g inulin in per gram	8 weeks	Serum lipid profile reduction especially TAG, TC/HDL-C with a significant increase in HDL-C serum level	Tajadadi-Ebrahimi et al. (2014)
20 T2DM patients	*Lactobacillus acidophilus* 10^8 CFU/mL, *B. bifidum* 10^8 CFU/mL, and oligofructose (2g)	2 weeks	Raised HDL-C level with decreasing fasting glycemia	Moroti et al. (2012)

Table 1 *(continue)*

Subjects	Dose/Strain/Prebiotics	Time	Outcomes	References
	Non-alcoholic fatty liver disease			
20 study patients with NASH	*Lactobacillus plantarum*, *Lactobacillus delbrueckii* spp., *L. bulgaricus*, *L. acidophilus*, *L. rhamnosus*, *B. bifidum*, and inulin	26 weeks	Reduction in intrahepatic triacylglycerol content (IHTG)	Sáez-Lara et al. (2016)
NAFLD individuals (52 adults)	*Lactobacillus casei*, *S. thermophilus*, *B. breve*, *L. rhamnosus*, *L. acidophilus*, *B. longum*, *L. bulgaricus*, with FOS	30 weeks	Prohibition of NF-$_{k}$B and decreased TNF-α	Eslamparast et al. (2014a)

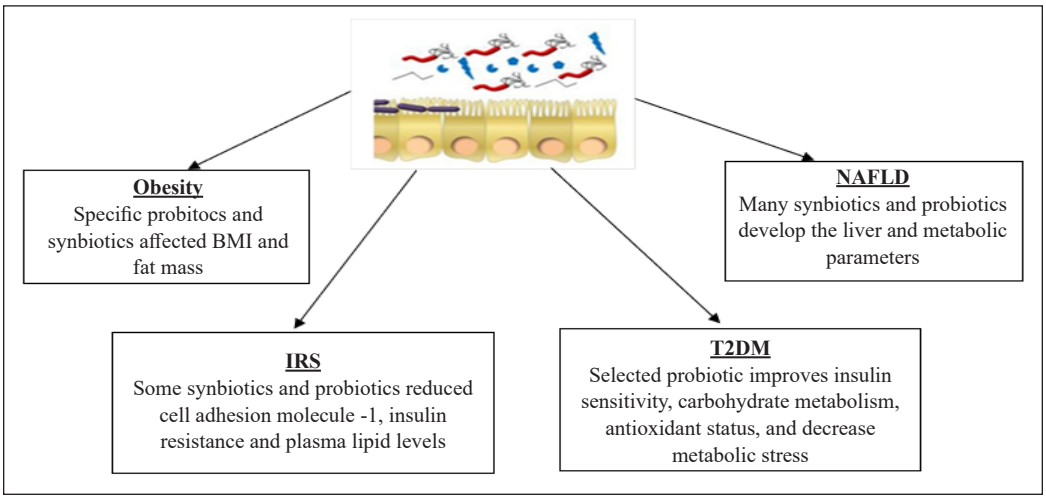

Figure 1. Graphical representation of synbiotic effect in obesity, insulin resistance syndrome, type 2 diabetes mellitus, and non-alcoholic fatty liver disease (Sáez-Lara et al., 2016)

EFFECTS OF SYNBIOTICS ON OBESITY

The intestinal microbiota affects energy balance in the human body and *Eubacteria rectale*, *Lactobacillus*, *Blautia coccoides*, and *Bifidobacterium* bacteria are associated with obesity (Sáez-Lara et al., 2016). The developed gut manipulating field can be valuable to the promotion of therapeutic strategies for managing obese problem and related metabolic diseases. Synbiotic supplementation can result in the proper balance in the gut microbiota and thus have advantageous effects, such as fat storage reduction, peptide secretion, glucose and insulin metabolism development, inflammatory biomarker, and body weight reduction (Rabiei, 2019). Enteroendocrine cells (EEC) in the gastrointestinal tract regulate gut motility and peptide hormone secretion to control food consumption and insulin production (Covasa et al., 2019).

Gut microbiota produces active signaling molecules like short-chain fatty acids (SCFAs) by the fermentation process of dietary fibers. The interaction between gut microbes and G protein-coupled receptors (GPCRs) affects insulin sensitivity and regulate energy metabolism. Transient changes in the gut ecosystem disrupt the host physiology and increase the risk of developing metabolic disorders including low-grade inflammation (Boulangé et al., 2016). The effect of supplementation with *Lactobacillus rhamnosus* (CGMCC1.3724), oligofructose, and inulin was investigated in obese men and women subjected to a 24-week weight loss program (Sanchez et al., 2014). The results showed that *L. rhamnosus* could induce weight loss in women with a remarkable decrease in fat mass and the abundance of bacteria (Lachnospiraceae) in the faeces from Firmicutes phylum family, a taxonomic group that is related to obesity. In brief, supplementation with selected synbiotics can reduce body mass index (BMI), body fat percentage, and serum leptin levels in women with a growing level of Lachnospiraceae family in their faeces. In children, treatment with synbiotics shows the reduction of BMI z-score, waist circumference (WC), total cholesterol (TC), low density lipoprotein cholesterol (LDL-C), and triacylglycerol (TAG) serum levels (Sáez-Lara et al., 2016). Results observed from another study in obese children showed a dramatic reduction in BMI score, WC, and the risk factors of the cardiometabolic conditions such as TC, LDL-C, and TAG, after the intake of synbiotics (Zarrati et al., 2014). Furthermore, a protein-rich, less carbohydrate and restricted-energy diet can be efficiently utilized to reduce the excess weight of an obese person (Noakes et al., 2005). In the large intestine, microbial breakdown of proteins is considered as the principle reason for generating genotoxic and cancer related metabolites, such as N-nitroso compounds and ammonia (Hinai et al., 2019). Evidence from the previous study revealed that the synbiotic food could alter the microbial composition and exerts beneficial effects on weight loss and maintenance (Ferrarese et al., 2018). A 3-month intervention using synbiotic supplementation with four strains of *Bifidobacterium* and *Lactobacillus acidophilus* and galactooligosaccharides significantly increased the abundance of the probiotic group in intestinal *Bifidobacterium* (Sergeev et al., 2020). In this study, significant modulation of the intestinal microbiota with a decrease in *Prevotella* and *Gardnerella* genera were observed after having this intervention.

EFFECTS OF SYNBIOTICS ON INSULIN RESISTANCE SYNDROME, TYPE 2 DIABETES MELLITUS AND CORONARY HEART DISEASES

Insulin Resistance Syndrome

There are an increasing number of people with IRS, in which individuals are suffering from hypertension, obesity, glucose intolerance, and dyslipidemia. Synbiotic supplementation can decrease plasma fasting insulin and triglyceride concentrations (Asemi et al., 2014). However, IRS can

increase the overall morbidity and mortality rate of cardiovascular diseases (Hong et al., 2014). Visceral adipose tissues increase free fatty acid flux due to low insulin sensitivity, inhibit the activity of insulin-sensitive tissues, and can be allied with the development of diabetes mellitus (Sáez-Lara et al., 2016). Synbiotic capsules containing fructooligosaccharide (FOS) with preselected strains significantly alleviate the insulin resistance and improve the fasting blood sugar level of patients with IRS (Eslamparast et al., 2014b). In another study, IRS patients were treated with synbiotic capsules consisting of seven strains and fructooligosaccharide with placebo capsules for the investigation of lipid profile and insulin resistance (Sáez-Lara et al., 2016). The results exhibited a significant improvement of the fasting blood sugar and insulin level in synbiotic group compared to control group (Eslamparast et al., 2014b). Probiotics decrease cell adhesion molecule-1 levels; for instance, *Lactobacillus plantarum* reduces TC, glucose and homocysteine levels in postmenopausal women (Plaza-Diaz et al., 2019). Prebiotic and probiotic mixtures alleviate insulin resistance and high-density lipoprotein (HDL) and decrease the TAG and TC levels in individuals with IRS (Sáez-Lara et al., 2016).

Type 2 Diabetes Mellitus

The salutary effects of synbiotic on metabolic activity were reported beforehand in patients with NAFLD, gestational diabetes mellitus, T2DM, and non-obese T2DM (Farrokhian et al., 2019). Evidence from a new study suggested that the structure of gut microbes is associated with T2DM development (Han & Lin, 2014). A translucent relationship was found between T2DM and compositional alteration in patients with T2DM, whose intestinal microbiotas had a decreased amount of Firmicutes and high amounts of Bacteroidetes and Proteobacteria (Sáez-Lara et al., 2016). The outcome of synbiotic bread consumption contributes to decrease insulin level, serum lipid profile (TAG, TC/HDL), homeostatic model assessment of insulin resistance (HOMA-IR), homeostatic model assessment cell function, and to rise high-density lipoprotein cholesterol (HDL-C) level comparing to control bread consumption (Sáez-Lara et al., 2016; Tajadadi-Ebrahimi et al., 2014). The administration of probiotic mixture shows significant improvement in their lipid profile, decreasing total cholesterol (TC), LDL-C level, and increasing insulin sensitivity (Sáez-Lara et al., 2016). Lastly, a diet (for 24 weeks) containing *Bifidobacterium longum* with fructooligosaccharides results in a significant reduction in HOMA-IR in people with non-alcoholic steatohepatitis (Malaguarnera et al, 2012).

Coronary Heart Diseases

The outbreak of T2DM has significantly increased world-wide; in addition, the risk for coronary heart diseases (CHDs) has increased twofold (Farrag et al., 2013). Differences in intestinal microflora of diabetic and non-diabetic patients and between lean and obese subjects are

significant with respect to composition and function (Larsen et al., 2010; Turnbaugh et al., 2009). In patients with T2DM, impaired insulin metabolism can influence individuals with CHD and dyslipidemia (Gaede et al., 2003). Consequently, parameters for glucose homeostasis and lipid profile control can successfully reduce the morbidity and mortality rate of T2DM and CHD patients (Chillarón et al., 2014). The supplementation of synbiotic capsule for 84 days showed significant upshot on diabetic patients with CHD, particularly on their serum insulin and HDL-cholesterol levels (Tajabadi-Ebrahimi et al., 2017). Synbiotic consumption can promote insulin function by providing beneficial effects on hepatic insulin signalling and decreasing the phosphorylation of insulin receptor (substrate-1) and inflammatory cytokine production (Raso et al., 2014). Through the assembly of good bacteria in the intestinal microbiota, synbiotics can suppress the growth of Gram-negative bacteria in the inner lining of the intestinal tract and decrease the disposal of pathogens in the bloodstream by maintaining the unity of the mucosal barrier (Kellow et al., 2014). A study was conducted on patients with T2DM but without previous histories of CHD. After consuming the food full of *Lactobacillus sporogenes* and inulin for six weeks, the patients showed not much difference in lipid profile but showed difference in serum triglyceride level (Asemi et al., 2014). Following the food consumption consisting *L. sporogenes* (1×10 colony-forming-unit (CFU) with inulin 0.04g in per gram for nine weeks, a pregnant woman in good health expressed a drastic reduction in serum triglyceride and low-density lipoprotein cholesterol level (Taghizadeh et al., 2014). In another study synbiotic food containing *L. sporogenes* (27×10^7 CFU) with inulin (1.1g) for 42 days intervention in diabetic patients showed a promising decrease in the serum level (Asemi et al., 2014). However, new therapeutical strategies by modulating the gut microbiota can be a useful invention to reduce the vulnerability of CHD (DiRienz, 2014).

EFFECTS OF SYNBIOTICS IN NON-ALCOHOLIC FATTY LIVER DISEASE

NAFLD is a state when an excess amount of fat accumulated in the liver and correlated to obesity (Vajro et al., 2012). Results from synbiotic cases showed lower fat production in the liver and tumour necrosis factor (TNF) to restrain NAFLD development (Sáez-Lara et al., 2016). A clinical trial on study patients expressed that those consumed synbiotic yogurts for twenty-four weeks have improved the lipid profile, glycaemic variables, liver enzyme, oxidative stress, gut peptide concentrations, steatosis, and adipokine concentrations. However, no significant changes observed in insulin and HDL cholesterol levels (Bakhshimoghaddam et al., 2018). This study was conducted on synbiotic supplementation that included seven probiotic strains and fructooligosaccharide for 28 weeks. This intervention was performed on adult patients with NAFLD, who subsequently showed

lifestyle modification that is superior to that of the non-synbiotic group (Eslamparast et al., 2014a). Synbiotic supplementation can control inflammatory markers and reduce BMI and waist circumference. This effect was observed after 14 weeks of treatment and was maintained till the treatment was completed. In another study, steatosis in rats that received synbiotic supplementation was alleviated (dos Santos et al., 2019). An experimental study model of hypercholesterolemia involving prebiotic and probiotic supplementations showed significant changes in the gene expression of toll-like receptor 4 (TLR-4), nuclear factor kappa B (NF-$_k$B) and tumour necrosis factor α (TNF-α) (Gurry, 2017). Prebiotic and synbiotic supplementations can increase TLR-4 and NF-$_k$B levels. TNF-α raised only in those rats treated with prebiotics; moreover, the potential immunomodulatory function of prebiotics also found on monocytes and T cells (Capitán-Cañadas et al., 2014). A 4-month synbiotic intervention involving a change in lifestyle patterns decreased nearly two-third of sonographic grade in children with fatty liver disease (Çakır et al., 2017). This study also observed that fatty liver condition was alleviated after an increase in LDL level in synbiotic-supplemented children (Hernandez-Rodas et al., 2015). High LDL level is the risk factor of cardiovascular disease in NAFLD patients and reduction in LDL level after synbiotic supplementation mitigates the complications for fatty liver and cardiovascular diseases (Katsiki et al., 2016).

EFFECTS OF SYNBIOTICS IN RESPIRATORY TRACT INFECTIONS

According to World Health Organization (WHO) (2018), RTIs have various clinical symptoms, including the normal flu, rhinitis, nasopharyngitis, bronchitis, inflammation of the epiglottis, laryngitis, inflammation of the trachea and bronchi, pneumonia, upper, and lower RTIs. Apart from providing the synergistic effects of probiotics and prebiotics in immunity system, synbiotics may be essential to nutritional strategies for managing global problems associated with respiratory infections and abuse of antibiotics on RTIs (Markowiak, 2017). Synbiotic intervention decreases the outbreak and ratio of RTI cases by 16% (Chan et al., 2020). Moreover, their potential beneficial effects against this disease may be imposed to their anti-inflammatory function that was discovered in gastrointestinal diseases (Gurry, 2017).

EFFECTS OF SYNBIOTICS IN CHRONIC KIDNEY DISEASE

CKD is advanced and irretrievable damage of the kidney function, which requires dialysis or kidney transplant after developing to the last stage (Kocełak et al., 2012). The study shows that a synbiotic supplement of 500 mg two times a day for 42 days could lessen the level of urea nitrogen in blood of stage 3 or 4 CKD patients (Dehghani et al., 2016). CKD changes the formation and function of intestinal microbiota and illustrates a dysbiotic state with hostile consequences (Yang et al., 2018). Toxic solutes, such as

trimethylamine-N-oxide, *ρ*-cresol sulphate (PCS), and indoxyl sulphate (IS) reduce the micronutrients that help to mitigate systemic inflammation, CKD development, and complications of cardiovascular disease (Niwa, 2011; Vaziri, 2016). The findings of the study expose that synbiotic therapy considerably decreases serum PCS and IS levels in study patients who have not received antibiotics (Nakabayashi et al., 2011).

CONCLUSION

The present review focuses on the synbiotic efficacy as a therapeutic approach in the treatment of diseases such as obesity, IRS, T2DM, and NAFLD. Recent findings have noted that synbiotic consumption along with changing lifestyle may help in patients with NAFLD and promote serum lipid levels in T2DM patients. Scientific evidence from other studies showed a dramatic reduction in abdominal adiposity, IRS, and BMI in patients with obesity who undergone synbiotic interventions. Moreover, the availability of beneficial bacteria for modulating gut microbes demonstrates a tremendous contribution in mitigating the risk factors of chronic disease. This study provides an opportunity to design advanced dietary intervention with synbiotic supplementation as a new therapeutic approach to treat diseases such as metabolic syndrome, NAFLD, diabetes, CKD, obesity, and many more. Without a doubt, further research is required to analyse the superlative dose-response effect, follow up experiment, and long-term intervention effects of synbiotics for the betterment of human life.

ACKNOWLEDGEMENT

Authors would like to thank the School of Industrial Technology at Universiti Sains Malaysia (USM) and USM RUI grant - Ref. No.: 1001/PTEKIND/8011116 for supporting this research project financially. Financial assistance of the Graduate Assistance scheme from USM for author Beauty Akter was gratefully acknowledged.

REFERENCES

Anhê, F. F., Varin, T. V., Le Barz, M., Desjardins, Y., Levy, E., Roy, D., & Marette, A. (2015). Gut microbiota dysbiosis in obesity-linked metabolic diseases and prebiotic potential of polyphenol-rich extracts. *Current Obesity Reports*, *4*(4), 389–400. https://doi.org/10.1007/s13679-015-0172-9

Asemi, Z., Khorrami-Rad, A., Alizadeh, S. A., Shakeri, H., & Esmaillzadeh, A. (2014). Effects of synbiotic food consumption on metabolic status of diabetic patients: A double-blind randomized cross-over controlled clinical trial. *Clinical Nutrition*, *33*(2), 198-203. https://doi.org/10.1016/j.clnu.2013.05.015

Bakhshimoghaddam, F., Shateri, K., Sina, M., Hashemian, M. S., & Alizadeh, M. (2018). Daily consumption of synbiotic yogurt decreases liver steatosis in patients with non-alcoholic fatty liver disease: A randomized controlled clinical trial. *The Journal of Nutrition*, *148* (8), 1276-1284. https://doi.org/10.1093/jn/nxy088

Boulangé, C. L., Neves, A. L., Chilloux, J., Nicholson, J. K., & Dumas, M. (2016). Impact of the gut microbiota on inflammation, obesity, and metabolic disease. *Genome Medicine*, *8*(1), 42. https://doi.org/10.1186/s13073-016-0303-2

Çakır, M., Aksel İşbilen, A., Eyüpoğlu, İ., Sağ, E., Örem, A., Mazlum Şen, T., Kaklıkkaya, N., & Kaya, G. (2017). Effects of long-term synbiotic supplementation in addition to lifestyle changes in children with obesity-related non-alcoholic fatty liver disease. *Turkish Journal of Gastroenterology*, *28*(5), 377–383. https://doi.org/10.5152/tjg.2017.17084

Capitán-Cañadas, F., Ortega-González, M., Guadix, E., Zarzuelo, A., Suárez, M. D., de Medina, F. S., & Martínez-Augustin, O. (2014). Prebiotic oligosaccharides directly modulate proinflammatory cytokine production in monocytes via activation of TLR4. *Molecular Nutrition Food Research*, *58*(5), 1098-1110. https://doi.org/10.1002/mnfr.201300497

Chan, C. K. Y., Tao, J., Chan, O. S., Li, H. B., & Pang, H. (2020). Preventing respiratory tract infections by synbiotic interventions: A systematic review and meta-analysis of randomized controlled trials. *Advances in Nutrition*, *11*(4), 979-988. https://doi.org/10.1093/advances/nmaa003

Chillarón, J. J., Roux, J. A. F., Benaiges, D., & Pedro-Botet, J. (2014). Subclinical cardiovascular disease in type 2 diabetes mellitus: To screen or not to screen. *World Journal of Clinical Cases*, *2*(9), 415–421. https://doi.org/10.12998/wjcc.v2.i9.415

Covasa, M., Stephens, R. W., Toderean, R., & Cobuz, C. (2019). Intestinal Sensing by gut microbiota: Targeting gut peptides. *Frontiers in Endocrinology*, *10*, 82. https://doi.org/10.3389/fendo.2019.00082

Dehghani, H., Heidari, F., Mozaffari-Khosravi, H., Nouri-Majelan, N., & Dehghani, A. (2016). Synbiotic supplementations for azotemia in patients with chronic kidney disease: a randomized controlled trial. *Iranian Journal of Kidney Diseases*, *10*(6), 351-357.

DiRienz, D. B. (2014). Effect of probiotics on biomarkers of cardiovascular disease: Implications for heart-healthy diets. *Nutrition Reviews*, *72*(1), 18–29. https://doi.org/10.1111/nure.12084

dos Santos, A. C., Ribeiro, D. A., da Cruz Ferreira, J. A., Aguiar, O., Waitzberg, D. L., & Alves, C. C. (2019). Inflammatory gene expression analysis after prebiotic, probiotic and synbiotic supplementation in experimental non-alcoholic fatty liver disease. *Nutrition and Food Science*, *49*(1), 75-84. https://doi.org/10.1108/NFS-04-2018-0119

Eslamparast, T., Poustchi, H., Zamani, F., Sharafkhah, M., Malekzadeh, R., & Hekmatdoost, A. (2014a). Synbiotic supplementation in non-alcoholic fatty liver disease: A randomized, double-blind, placebo-controlled pilot study. *The American Journal of Clinical Nutrition*, *99*(3), 535-42. https://doi.org/10.3945/ajcn.113.068890

Eslamparast, T., Zamani, F., Hekmatdoost, A., Sharafkhah, M., Eghtesad, S., Malekzadeh, R., & Poustchi, H. (2014b). Effects of synbiotic supplementation on insulin resistance in subjects with the metabolic syndrome: A randomised, double-blind, placebo-controlled pilot study. *British Journal of Nutrition*, *112*(3), 438–445. https://doi.org/10.1017/S0007114514000919

Farrag, A., Bakhoum, S., Salem, M. A., El-Faramawy, A., & Gergis, E. (2013). The association between extracoronary calcification and coronary artery disease in patients with type 2 diabetes mellitus. *Heart Vessels*, *28*(1), 12–18. https://doi.org/10.1007/s00380-011-0205-6

Farrokhian, A., Raygan, F., Soltani, A., Tajabadi-Ebrahimi, M., Esfahani, M. S., Karami, A. A., & Asemi, Z. (2019). The effects of synbiotic supplementation on carotid intima-media thickness, biomarkers of inflammation and oxidative stress in people with overweight, diabetes and coronary heart disease: A randomized, double-blind, placebo-controlled trial. *Probiotics and Antimicrobial Proteins*,

11(1), 133-142. https://doi.org/10.1007/s12602-017-9343-1

Ferrarese, R., Ceresola, E., Preti, A., & Canducci, F. (2018). Probiotics, prebiotics and synbiotics for weight loss and metabolic syndrome in the microbiome era. *European Review for Medical and Pharmacological Sciences*, *22*(21), 7588–7605. https://doi.org/10.26355/eurrev_201811_16301

Frece, J., Kos, B., Svetec, I. K., Zgaga, Z., Beganović, J., Lebos, A., & Susković, J. (2009). Synbiotic effect of *Lactobacillus helveticus* M92 and prebiotics on the intestinal microflora and immune system of mice. *Journal of Dairy Research*, *76*(1), 98-104. https://doi.org/10.1017/S0022029908003737

Gaede, P., Vedel, P., Larsen, N., Jensen, G. V. H., Parving, H., & Pedersen, O. (2003). Multifactorial intervention and cardiovascular disease in patients with type 2 diabetes. *The New England Journal of Medicine*, *348*(5), 383-393. https://doi.org/10.1056/NEJMoa021778

Gurry, T. (2017). Synbiotic approaches to human health and well-being. *Microbial Biotechnology*, *10*(5), 1070–1073. https://doi.org/10.1111/1751-7915.12789

Han, J.-L., & Lin, H.-L. (2014). Intestinal microbiota and type 2 diabetes: From mechanism insights to therapeutic perspective. *World Journal of Gastroenterology*, *20*(47), 17737–17745. https://doi.org/10.3748/wjg.v20.i47.17737

Heiss, C. N., & Olofsson, L. E. (2018). Gut microbiota-dependent modulation of energy metabolism. *Journal of Innate Immunity*, *10*(3), 163–171. https://doi.org/10.1159/000481519

Hernandez-Rodas, M. C., Valenzuela, R., & Videla, L. A. (2015). Relevant aspects of nutritional and dietary interventions in non-alcoholic fatty liver disease. *International Journal of Molecular Science*, *16*(10), 25168-25198. https://doi.org/10.3390/ijms161025168

Hinai, E. A. A., Kullamethee, P., Rowland, I. R., Swann, J., Walton, G. E., & Commane, D. M. (2019). Modelling the role of microbial *ρ*-cresol in colorectal genotoxicity. *Gut Microbes*, *10*(3), 398–411. https://doi.org/10.1080/19490976.2018.1534514

Hong, H. R., Ha, C. D., Kong, J. Y., Lee, S. H., Song, M. G., & Kang, H. S. (2014). Roles of physical activity and cardiorespiratory fitness on sex difference in insulin resistance in late elementary years. *Journal of Exercise Nutrition and Biochemistry*, *18*(4), 361–369. https://doi.org/10.5717/jenb.2014.18.4.361

Ipar, N., Aydogdu, S. D., Yildirim, G. K., Inal, M., Gies, I., Vandenplas, Y., & Dinleyici, E.C. (2015). Effects of synbiotic on anthropometry, lipid profile and oxidative stress in obese children. *Beneficial Microbes, 6*(6), 775–781. https://doi.org/10.3920/BM2015.0011

Katsiki, N., Mikhailidis, D. P., & Mantzoros, C. S. (2016). Non-alcoholic fatty liver disease and dyslipidemia: An update. *Metabolism*, *65*(8), 1109-23. https://doi.org/10.1016/j.metabol.2016.05.003

Kellow, N. J., Coughlan, M. T., Savige, G. S., & Reid, C. M. (2014). Effect of dietary prebiotic supplementation on advanced glycation, insulin resistance and inflammatory biomarkers in adults with pre-diabetes: A study protocol for a double-blind placebo-controlled randomised crossover clinical trial. *BMC Endocrine Disorders*, *14*(1), 55. https://doi.org/10.1186/1472-6823-14-55

Kocełak, P., Olszanecka-Glinianowicz, M., & Chudek, J. (2012). Fibroblast growth factor 23 – Structure, function and role in kidney diseases. *Advances in Clinical and Experimental Medicine*, *21*(3), 391–401.

Larsen, N., Vogensen, F. K., van den Berg, F. W., Nielsen, D. S., Andreasen, A. S., Pedersen, B. K., Al-Soud, W. A., Sørensen, S. J., Hansen, L. H., & Jakobsen, M. (2010). Gut microbiota in

human adults with type 2 diabetes differs from non-diabetic adults. *PLOS One*, *5*(2), e9085. https://doi.org/10.1371/journal.pone.0009085

Malaguarnera, M., Vacante, M., Antic, T., Giordano, M., Chisari, G., Acquaviva, R., Mastrojeni, S., Malaguarnera, G., Mistretta, A., Volti, G. L. & Galvan, F. (2012). *Bifidobacterium longum* with fructo-oligosaccharides in patients with non-alcoholic steatohepatitis. *Digestive Diseases and Sciences*, *57*(2), 545–553. https://doi.org/10.1007/s10620-011-1887-4

Markowiak, P. & Śliżewska, K. (2017). Effects of probiotics, prebiotics, and synbiotics on human health. *Nutrients*, *9*(9), 1021. https://doi.org/10.3390/nu9091021

Martinez, K. B., Leone, V., & Chang, E. B. (2017). Western diets, gut dysbiosis, and metabolic diseases: Are they linked?. *Gut Microbes*, *8*(2), 130–142. https://doi.org/10.1080/19490976.2016.1270811

Mehal, W. Z. (2013). The Gordian knot of dysbiosis, obesity and NAFLD. *Nature Reviews Gastroenterology and Hepatology*, *10*(11), 637-644. https://doi.org/10.1038/nrgastro.2013.146

Moroti, C., Magri, L. F. S., Costa, M. d. R., Cavallini, D. C. U., & Sivieri, K. (2012). Effect of the consumption of a new symbiotic shake on glycemia and cholesterol levels in elderly people with type 2 diabetes mellitus. *Lipids in Health and Disease*, *11*(1), 29. https://doi.org/10.1186/1476-511X-11-29

Nakabayashi, I., Nakamura, M., Kawakami, K., Ohta, T., Kato, I., Uchida, K., & Yoshida, M. (2011). Effects of synbiotic treatment on serum level of *p*-cresol in haemodialysis patients: A preliminary study. *Nephrology Dialysis Transplantation*, *26*(3), 1094–1098. https://doi.org/10.1093/ndt/gfq624

Niwa, T. (2011). Role of indoxyl sulfate in the progression of chronic kidney disease and cardiovascular disease: Experimental and clinical effects of oral sorbent AST-120. *Therapeutic Apheresis and Dialysis*, *15*(2), 120–124. https://doi.org/10.1111/j.1744-9987.2010.00882.x

Noakes, M., Keogh, J. B., Foster, P. R., & Clifton, P. M. (2005). Effect of an energy-restricted, high-protein, low-fat diet relative to a conventional high-carbohydrate, low-fat diet on weight loss, body composition, nutritional status, and markers of cardiovascular health in obese women. *American Journal of Clinical Nutrition*, *81*(6), 1298-306. https://doi.org/10.1093/ajcn/81.6.1298

Plaza-Diaz, J., Ruiz-Ojeda, F. J., Gil-Campos, M. & Gil, A. (2019). Mechanisms of action of probiotics. *Advances in Nutrition*, *10*(1), S49–S66. https://doi.org/10.1093/advances/nmy063

Quigley, E. M., & Monsour, H. P. (2015). The gut microbiota and non-alcoholic fatty liver disease. *Seminars in Liver Disease*, *35*(3), 262-269. https://doi.org/10.1055/s-0035-1562946

Rabiei, S., & Hekmatdoost, A. (2019). New therapeutic agents to treat obesity and its related disorders: Prebiotic, probiotic and synbiotic supplementation. *International Journal of Food and Nutritional Science*, *6*(1), 43-53.

Raso, G. M., Simeoli, R., Iacono, A., Santoro, A., Amero, P., Paciello, O., Russo, R., D'Agostino, G., Di Costanzo, M., Canani, R. B., Calignano, A., & Meli, R. (2014). Effects of a *Lactobacillus paracasei* B21060 based synbiotic on steatosis, insulin signaling and toll-like receptor expression in rats fed a high-fat diet. *The Journal of Nutritional Biochemistry*, *25*(1), 81–90. https://doi.org/10.1016/j.jnutbio.2013.09.006

Russell, W. R., Gratz, S. W., Duncan, S. H., Holtrop, G., Ince, J., Scobbie, L., Duncan, G., Johnstone, A. M., Lobley, G. E., Wallace, R. J., Duthie, G. G., & Flint, H. J. (2011). High-protein, reduced-carbohydrate weight-loss diets promote metabolite profiles likely to be detrimental to colonic health. *The American Journal of*

Clinical Nutrition, *93*(5), 1062–1072. https://doi.org/10.3945/ajcn.110.002188

Sáez-Lara, M. J., Robles-Sanchez, C., Ruiz-Ojeda, F. J., Plaza-Diaz, J., & Gil, A. (2016). Effects of probiotics and synbiotics on obesity, insulin resistance syndrome, type 2 diabetes and non-alcoholic fatty liver disease: A review of human clinical trials. *International Journal of Molecular Science*, *17*(6), 928. https://doi.org/10.3390/ijms17060928

Sanchez, M., Darimont, C., Drapeau, V., Emady-Azar, S., Lepage, M., Rezzonico, E., Ngom-Bru, C., Berger, B., Philippe, L., Ammon-Zuffrey, C., Leone, P., Chevrier, G., St-Amand, E., Marette, A., Doré, J., & Tremblay, A. (2014). Effect of *Lactobacillus rhamnosus* CGMCC1.3724 supplementation on weight loss and maintenance in obese men and women. *The British Journal of Nutrition*, *111*(8), 1507–1519. https://doi.org/10.1017/S0007114513003875

Sergeev, I. N., Aljutaily, T., Walton, G., & Huarte, E. (2020). Effects of synbiotic supplement on human gut microbiota, body composition and weight loss in obesity. *Nutrients*, *12*(1), 222. https://doi.org/10.3390/nu12010222

Singh, K., Kallali, B., Kumar, A., & Thaker, V. (2011). Probiotics: A review. *Asian Pacific Journal of Tropical Biomedicine*, *1*(2), S287-S290. https://doi.org/10.1016/S2221-1691(11)60174-3

Taghizadeh, M., Hashemi, T., Shakeri, H., Abedi, F., Sabihi, S., Alizadeh, S., & Asemi, Z. (2014). Synbiotic food consumption reduces levels of triacylglycerols and VLDL, but not cholesterol, LDL, or HDL in plasma from pregnant women. *Lipids*, *49*(2), 155–161. https://doi.org/10.1007/s11745-013-3867-2

Tajabadi-Ebrahimi, M., Sharifi, N., Farrokhian, A., Raygan, F., Karamali, F., Razzaghi, R., Taheri, S., & Asemi, Z. (2017). A randomized controlled clinical trial investigating the effect of synbiotic administration on markers of insulin metabolism and lipid profiles in overweight type 2 diabetic patients with coronary heart disease. *Experimental and Clinical Endocrinology and Diabetes*, *125*(1), 21–27. https://doi.org/10.1055/s-0042-105441

Tajadadi-Ebrahimi, M., Bahmani, F., Shakeri, H., Hadaegh, H., Hijijafari, M., Abedi, F., & Asemi, Z. (2014). Effects of daily consumption of synbiotic bread on insulin metabolism and serum high-sensitivity C-reactive protein among diabetic patients: A double-blind, randomized, controlled clinical trial. *Annals of Nutrition and Metabolism*, *65*(1), 34–41. https://doi.org/10.1159/000365153

Turnbaugh, P. J., Hamady, M., Yatsunenko, T., Cantarel, B. L., Duncan, A., Ley, R. E., Sogin, M. L., Jones, W. J., Roe, B. A., Affourtit, J. P., Egholm, M., Henrissat, B., Heath, A. C., Knight, R., & Gordon, J. I. (2009). A core gut microbiome in obese and lean twins. *Nature*, *457*(7228), 480–484. https://doi.org/10.1038/nature07540

Vajro, P., Lenta, S., Socha, P., Dhawan, A., McKiernan, P., Baumann, U., Durmaz, O., Lacaille, F., McLin, V., & Nobili, V. (2012). Diagnosis of non-alcoholic fatty liver disease in children and adolescents: Position paper of the ESPGHAN hepatology committee. *Journal of Pediatric Gastroenterology and Nutrition*, *54*(5), 700–713. https://doi.org/10.1097/MPG.0b013e318252a13f

Vaziri, N. D. (2016). Effect of synbiotic therapy on gut–derived uremic toxins and the intestinal microbiome in patients with CKD. *Clinical Journal of the American Society of Nephrology*, *11*(2), 199–201. https://doi.org/10.2215/CJN.13631215

World Health Organization. (2018). *Diseases of the respiratory system*. https://www.who.int/classifications/icd/revision/en/

Yang, T., Richards, E. M., Pepine, C. J. & Raizada, M. K. (2018). The gut microbiota and the brain-

gut-kidney axis in hypertension and chronic kidney disease. *Nature Reviews Nephrology*, *14*(7), 442–456. https://doi.org/10.1038/s41581-018-0018-2

Yao, C. K., Muir, J. G., & Gibson, P. R. (2016). Review article: Insights into colonic protein fermentation, its modulation and potential health implications. *Alimentary Pharmacology and Therapeutics*, *43*(2), 181–196. https://doi.org/10.1111/apt.13456

Yoo, J. Y., & Kim, S. S. (2016). Probiotics and prebiotics: Present status and future perspectives on metabolic disorders. *Nutrients*, *8*(3), 173. https://doi.org/10.3390/nu8030173

Zarrati M., Salehi E., Nourijelyani K., Mofid V., Zadeh M. J. H., Najafi F., Ghaflati Z., Bidad K., Chamari M., Karimi M., & Shidfar F. (2014). Effects of probiotic yogurt on fat distribution and gene expression of proinflammatory factors in peripheral blood mononuclear cells in overweight and obese people with or without weight-loss diet. *Journal of the American College of Nutrition*, *33*(6), 417–425. https://doi.org/10.1080/07315724.2013.874937

Pertanika J. Trop. Agric. Sci. 44 (1): 83 - 105 (2021)

PERTANIKA JOURNALS

TROPICAL AGRICULTURAL SCIENCE

Journal homepage: http://www.pertanika.upm.edu.my/

Review Article

Review of Antioxidant-rich Natural Dietary Products as Protective and Therapeutic Factors against Cadmium Toxicity in Living Organisms

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ABSTRACT

Advances in civilization processes and industrialization have doubled the release of toxic heavy metals into the environment, consequently elevating their presence in the food chains. Cadmium (Cd) is one of the severe toxic metals widely present in the atmosphere. The major route of animal or human exposure to Cd is through water or food ingestion and inhalation of particles or inhalation of fumes during various industrial processes. Continuous exposure to low levels of Cd results in a gradual deposition in different tissues of the body, causing toxic effects on the liver, kidneys, testes, and other vital organs. The beneficial effect of natural antioxidants against chemical induced toxicity is receiving more attention. Antioxidant-rich dietary products and their function in tempering free radicals produced in the body under different pathological conditions is an active research field. In the current review, we attempted to highlight the current research progress in the field of using antioxidant-rich natural dietary products and their function in mitigating or

ARTICLE INFO

Article history:
Received: 08 July 2020
Accepted: 27 August 2020
Published: 24 February 2021

DOI: https://doi.org/10.47836/pjtas.44.1.05

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ISSN: 1511-3701
e-ISSN: 2231-8542

preventing health issues and tissue damage associated with Cd induced toxicity along with its mechanism.

Keywords: Cadmium toxicity, dietary products, mechanisms, natural antioxidants

INTRODUCTION

The incidence of several adverse health effects in humans and animals associated with exposure to toxic heavy metals in the environment is a general issue and a matter of grave concern (Vardhan et al., 2019). In recent years the concentration of heavy metals in the environment has increased abundantly; industries have played a major role in this regard (H. Zhang & Reynolds, 2019). Cd is one of the severe toxic metals widely present in the atmosphere (Ataei et al., 2019; Lane et al., 2015; Patra et al., 2011; Satarug et al., 2010). It is extensively present in the environment. Advances in civilization processes and industrialization have doubled the increase in Cd level in the environment consequently elevating its presence in the food chains and risking human and animal health (World Health Organization [WHO], 2010). Cd is present in PVC pipes, phosphatic fertilizers, and color pigments, with few of Cd containing products recycled (Järup & Åkesson, 2009). Cigarette smoke and food are the most important sources of Cd exposure in the human population (Piasek et al., 2001), while animals are exposed through several other sources (H. Zhang & Reynolds, 2019). It can enter the body via daily eating, which contains a considerable amount of Cd in normal condition (X. Wang et al., 2021). Cd causes damage to various tissues and vital organs such as the kidney, liver, lungs, bones, and brain (Geng & Wang, 2019). Endocrine modulative properties of Cd have been suggested by several studies, and therefore, it has been included in the category of endocrine disruptors (WHO, 2017). In the case of acute Cd poisoning, the liver is the main target organ of toxicity resulting in hepatotoxicity that can lead to Cd caused lethality (Rikans & Yamano, 2000). Several studies also showed an association between chronic Cd-toxicity and nephrotoxicity (El-Demerdash et al., 2004). Various studies on female rats revealed that Cd, a heavy metal with a considerably long half-life, accumulates in the female reproductive organ (S. Wang et al., 2019). Oxidative stress plays an important role in Cd-induced toxicity in the brain, kidneys, liver, bone, testes, and ovarian tissues. Oxidative stress is recognized as one of the many mechanisms of Cd toxicity which involves stimulating the synthesis of reactive oxygen species (ROS), thus causes oxidative disruption in RBCs and several other tissues, resulting in loss of membrane function (Nasiadek et al., 2014). Thus, Cd can cause oxidative damage in various tissues by increasing the peroxidation of membrane lipids and interfering with the antioxidant mechanistic of the cells. Cellular components may lead to disruption due to the damage caused by the peroxidation of cellular membrane and interference of metal ions with organelles (Sarkar et al., 2013).

The beneficial effects of natural antioxidants against chemically induced toxicity are receiving more attention (Mężyńska & Brzóska, 2019; Spencer, 2003). Research on naturally occurring antioxidants and their function in tempering free radicals produced in the body under different pathological conditions has remained to be a focus point (Flora et al., 2012). Dietary and supplementary antioxidants may inhibit carcinogenesis (Calabrese, 2002). Various naturally occurring compounds are reported to show a protective role against oxidative stress such as ROS lipid peroxidation (Dailiah Roopha & Padmalatha, 2012). The understanding of oxidative stress as an active mechanism by which Cd causes its toxic effect indicates that naturally occurring antioxidants can play a role in the treatment of Cd poisoning (Abdelaziz et al., 2013). The main purpose of this review is, therefore, to highlight the ongoing development in the area of research related to the use of naturally occurring antioxidant-rich sources against heavy metal toxic agents especially Cd and to point out possible knowledge gaps and future directions.

In preparation of this review, we searched for the data in bibliographic databases like Elsevier, Scopus, Medline, and Google Scholar using keywords such as natural antioxidants, bio elements, honey, green tea, cadmium/Cd, exposure, adverse effects, kidney damage, ginger, hepatotoxicity, and mechanism of Cd toxicity, green tea, reproductive toxicity, curcumin, and preventive measures.

MECHANISMS OF Cd TOXICITY

Various mechanisms are responsible for Cd toxicity. The main mechanisms reported include oxidative stress, DNA damage and apoptosis. Synergism of several mechanisms is responsible for the entire effect of Cd on any tissue or cell (Figure 1) (Khan et al., 2019; Rani et al., 2014; Thompson & Bannigan, 2008).

Oxidative Stress

Oxidative stress is the outcome of the imbalance between the generation of oxidants and their elimination systems (Puppel et al., 2015). Oxidative stress plays a vital role in Cd-induced toxicity in the brain, kidneys, liver, bone, testes, and ovarian tissues. It is recognized as one of the many mechanisms of Cd toxicity mediated by stimulating the synthesis of reactive oxygen species (ROS), thus causing oxidative disruption in RBCs and in several other tissues, resulting in membrane function loss (Giaginis et al., 2006; Nasiadek et al., 2014). Cd causes oxidative stress and induces lipid peroxidation by either inhibiting the antioxidant enzymatic system or through depletion of glutathione (GSH) (Rikans & Yamano, 2000). ROS are produced during the metabolism of mitochondria and in cell responses to xenobiotics (S. Wang et al., 2019). ROS are often responsible for the Cd-induced detrimental health effects. There is direct evidence of the free radicals generation in animals following acute Cd exposure and indirect evidence of ROS responsible for chronic Cd toxicity and carcinogenesis (Patra et al., 2011). Cd

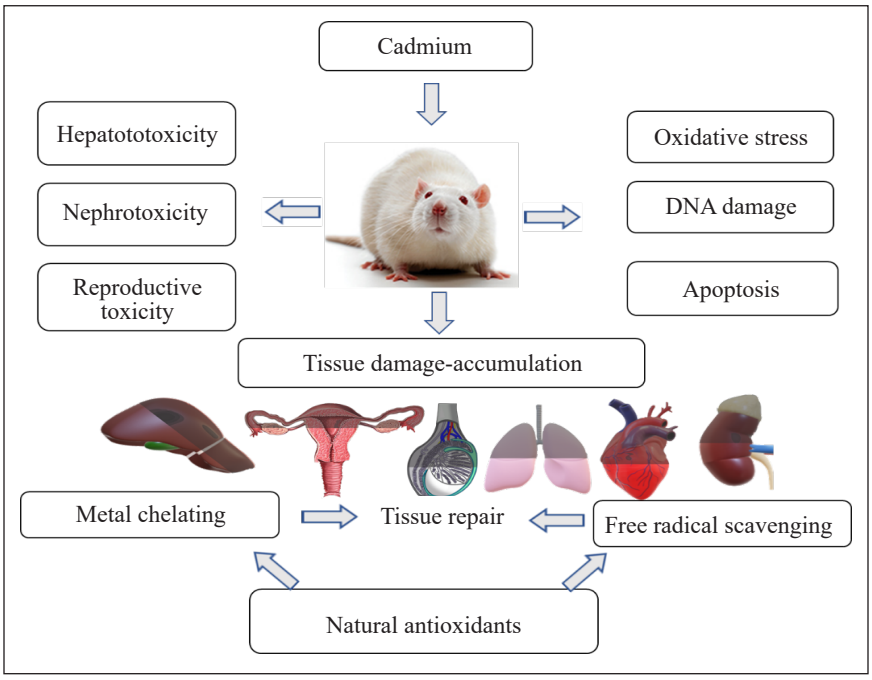

Figure 1. The possible biological pathways of Cd-induced oxidative injury and tissue damage

can cause oxidative damage in various tissues by increasing the peroxidation of membrane lipids and interfering in the antioxidant mechanistic of the cells. Cellular components may lead to disruption due to the damage caused by the peroxidation of cellular membrane and interference of metal ions with organelles (Sarkar et al., 2013).

DNA Damage

Cd is responsible for the disruption of DNA synthesis, cell cycle progression, cell proliferation and differentiation, and other various cellular processes (Aimola et al., 2012). The inhibition of the DNA repair mechanism may be another impact of Cd toxicity (Giaginis et al., 2006). Furthermore, several other possible indirect mechanisms have also been proposed to elucidate Cd carcinogenesis, including oxidative stress, alteration of DNA methylation, protooncogene activation, and dysregulated gene expression (Beyersmann & Hechtenberg, 1997). *In vitro* experiments demonstrate clearly that Cd induces oxidative stress, damage to the DNA and programmed cell death in cells containing human liver carcinoma (Skipper et al., 2016). In a study conducted by J. M. Yang et al., (2003), cadmium was found to be directly toxic to primary Leydig cells, and the decreased percentage of normal cells and increased levels of DNA damage in cadmium exposed Leydig cells was evident. Lysosome is one of the main targets for cadmium toxicity and can be responsible for other cellular events, including DNA damage (Fotakis et al., 2005).

Apoptosis

Among the various harmful responses, apoptosis is also responsible for the Cd toxicity effect. As a primary defense mechanism against the free pre-filtering transformed or mutated cells, apoptosis that is usually found in Cd-exposed cells supposed to have an anti-cancer function (Ospondpant et al., 2019). Some studies have shown that only a proportion of Cd-exposed cells in a population die by apoptosis, while the remaining can become apoptosis-resistant (S. Wang et al., 2019; Waisberg et al., 2003). Moreover, it has been observed that resistance to apoptosis is enhanced in Cd-adapted or transformed cells (Hart et al., 2001). Disturbance in the apoptosis mechanism is considered crucial in tumor development, acquired resistance to apoptosis, and malignant progression is a common sign of cancer (van der Wall, 2010).

Cd-INDUCED ORGAN TOXICITY

Nephrotoxicity

The kidney is the main target organ in chronic Cd exposure. It has been long observed that Cd-Metallothionein (MT) complex mediates the Cd-induced nephrotoxicity (Klaassen et al., 2009). Accumulation of Cd in the kidney found in human population studies indicates that a considerable number of people may have toxic levels of Cd in their kidneys (Satarug et al., 2000). Several studies showed evidence of an association between chronic Cd exposure and nephrotoxicity. Initial stages of Cd nephrotoxicity include specific disruptions in proximal tubule cell adhesion, cellular signaling, cascade, and autophagic response that occur before the occurrence of apoptosis or necrosis of proximal tubule cells (Prozialeck & Edwards, 2012). A high degree of Cd exposure can result in interference of calcium metabolism and formation of kidney stones, osteoporosis, and softening of the bones, which have been observed in human individuals living or working in Cd-abundant areas or exposed groups (WHO, 2010). Cd induces renal damage, characterized by proximal re-absorptive tubular dysfunction (Järup & Åkesson, 2009).

Hepatotoxicity

In the case of acute Cd-poisoning, the liver is the main primary organ of toxicity, and hepatotoxicity can also lead to lethality (Klaassen et al., 2009). Cd is delivered to target organs via pulmonary or gastrointestinal absorption into the systemic circulation. The liver receives much of the Cd absorbed in the intestines through the portal circulation, bound mainly to albumin, where it reaches to hepatocytes from sinusoidal capillaries (Figure 2) (DelRaso et al., 2003). Cd-induced damage occurs to all liver cells; the Cd-induced inflammatory response generates an infiltration and activation of phagocytic cells that generates more inflammatory mediators including cytokines or ROS (Arroyo et al., 2013). Cd hepatotoxicity is closely associated with inflammation. Following acute exposure, the damaged liver is often intruded by polymorphonuclear neutrophils (PMN), which along with damage to Kupfer cells;

contribute to the hepatotoxicity by elevating inflammatory mediators and promoting necrosis. Two pathways induce the hepatotoxicity; the early injury resulted from the direct effects of the metal deposit and the other injury that follows the inflammatory process (Rikans & Yamano, 2000).

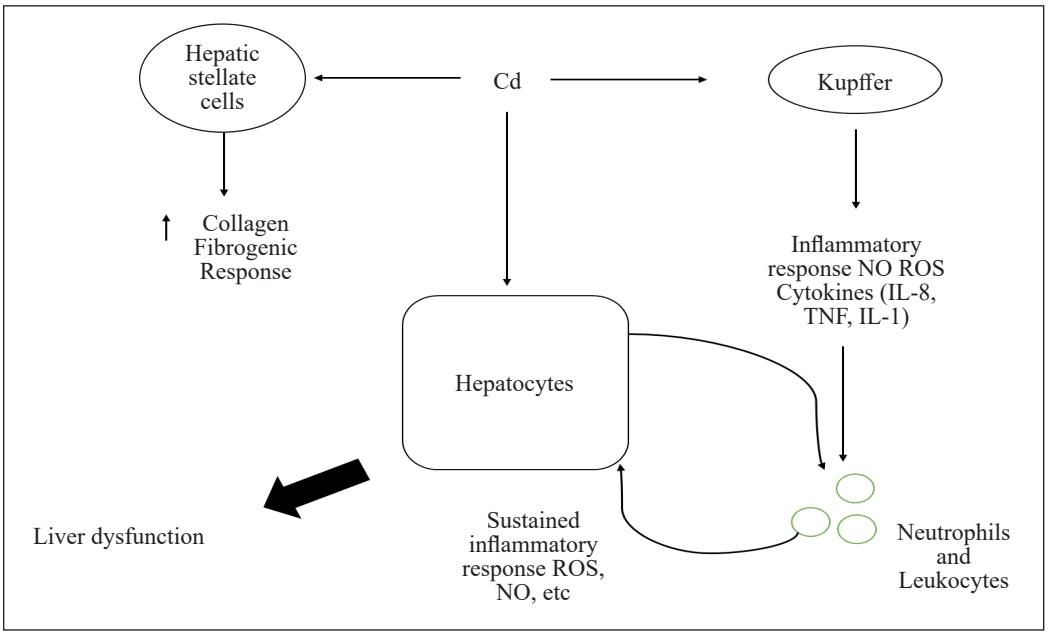

Figure 2. General pathway of Cd-induced liver dysfunction. Cd toxic reaction involves different types of hepatic cells, resulting in liver damage (Arroyo et al., 2013)

Reproductive Toxicity

Cd exposure is linked with several toxic effects on the mammalian reproductive system, thereby affecting the normal reproductive functions. It has been placed under the list of recognized endocrine-disrupting chemicals (EDCs) due to its defined ability to disturb the rates of placental and ovarian steroidogenesis process (Chedrese et al., 2008; Nna et al., 2017). For example, progesterone biosynthesis in human luteal cells is either enhanced or inhibited due to the accumulation of Cd, therefore, affecting reproductive morphology. Additionally, premature birth and decreased birth weights were reported to have been associated with Cd exposure in women (Miceli et al., 2005).

Moreover, Cd is known to cause blockage of the pathway that delivers cholesterol precursors for steroidogenesis from the maternal peripheral circulation due to an increase in placental low-density lipoprotein receptor. Another possible potential mode of actions by which Cd may disrupt steroidogenesis includes alteration of the DNA bind zinc through the replacement of Cd for Zn (Henson & Chedrese, 2004). Cd persuades expression of estrogen target genes in mammalian cell culture, thus

initiating activation of cytoplasmic kinases (W. Zhang & Jia, 2007). Cd accumulation in embryos increases from the four-cell stage ahead, and blastocyst stage development is inhibited by high dose exposure, which can cause decompaction and degeneration in blastocysts. Besides, Cd has also been incorporated in the chromatin structure of developing gametes. Ovarian tissues accumulate high amount of Cd over a span of time and with increasing age, consequently leading to deterred progression of oocyte growth from the primary to the secondary stage and failure to ovulate. Another mechanism by which ovulation is considered ineffective is the failure of tubal cilia to pick up the oocytes (Thompson & Bannigan, 2008). Incidences of reproductive anomalies linked with Cd exposure are higher in women as shown in different studies (Akesson et al., 2002; Berglund et al., 1994; Järup, 2003). Nevertheless altered testicular function associated with Cd exposure has also been reported in men (Amit et al., 2019; de Angelis et al., 2017; T. Zhang et al., 2019). Mechanisms of deleterious effects due to Cd exposure in the testis include impairment to Sertoli and Leydig cells, intercellular connections, vascular endothelium, oxidative stress induction, interference in antioxidant defense system, and interference in the inflammatory responses, which leads to functional and morphological changes like impairment of spermatogenesis and inhibition of testosterone synthesis (Figure 3) (Taha et al., 2013). Cd also disrupts prostate function by altering its secretion, hormonal activity, and consequently leads to infertility problems in men (Sarkar et al., 2013).

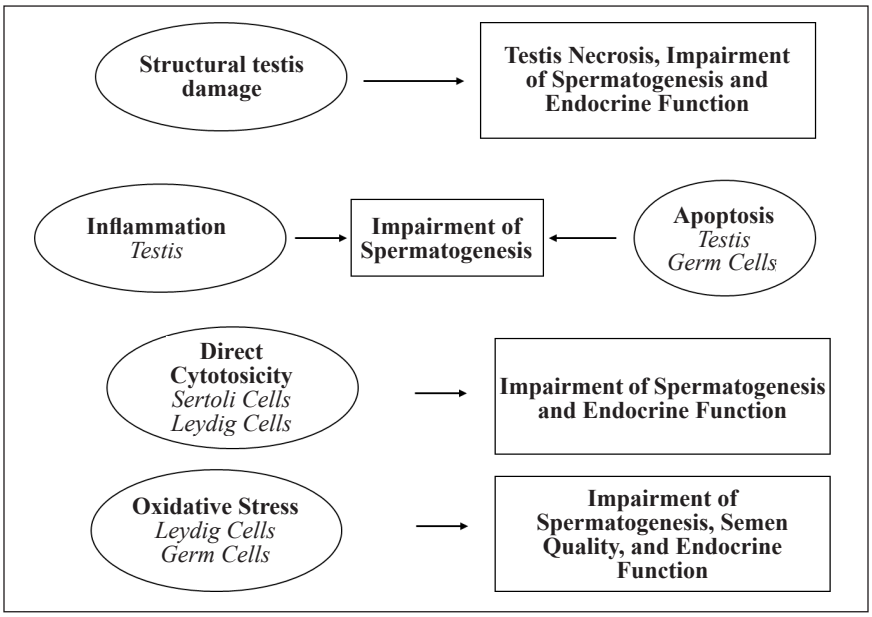

Figure 3. Overview of main proposed pathways of Cd-induced reprotoxicity

NATURALLY OCCURRING ANTIOXIDANT SUBSTANCES AND THEIR AMELIORATING EFFECTS ON Cd TOXICITY

Nowadays, natural antioxidants and their protective effects against chemically induced toxicity are receiving more and more attention (Mężyńska & Brzóska, 2019). Naturally occurring antioxidants and their function in tempering free radicals produced in the body under different pathological conditions are also active research fields (Flora et al., 2012). Dietary and supplementary antioxidants have been believed to inhibit carcinogenesis (Calabrese, 2002). Consequently, a great deal of attention has been given towards the biochemical functions of natural substances in biological systems (Matés et al., 2010). Various naturally occurring substances are reported to show a protecting role against ROS and lipid peroxidation (Table 1) (Alkhedaide et al., 2016; Amamou et al., 2015; J. J. Kim et al., 2019; Nna et al., 2017) and below are some of them considered in this review.

Curcumin

Curcumin, a turmeric plant's active component, is an efficient antioxidant against oxidative tissue damage because it significantly hampers the formation of both *in vitro* and *in vivo* ROS (K. S. Kim et al., 2018). It was revealed by Eybl et al. (2006) that pre-treatment of curcumin effectively protected against Cd-induced lipid peroxidation and ameliorated Cd adverse effects in mice. Attia et al. (2014a) carried out a study on rats, and their research finding suggested that curcumin increased the antioxidant glutathione peroxidase (GPx) activity of Cd-exposed rats and decreased lipid peroxidation and hemolysis of erythrocytes (Attia et al., 2014a). $CdCl_2$ decreases sperm motility, reduces sperm density, and the amount of serum testosterone, it also decreased glutathione peroxidase (GSH-Px), testicular total superoxide dismutase (T-SOD), and glutathione (GSH), and increased malondialdehyde (MDA) levels, while all of these parameters were ameliorated by the supplementation of curcumin (S. H. Yang et al., 2019). A study conducted by Abu-Taweel (2016) in mice showed that curcumin improved both behavioral and biochemical parameters of blood analyzed and decreased the Cd toxicity effect also in a dose-dependent manner (Abu-Taweel, 2016). Blood indices such as red and white blood cells, platelets, hemoglobin, and packed cell volume were reduced in Cd exposed mice but remained at normal levels in mice treated with nanoparticles of curcumin (Ahmad et al., 2018). Curcumin treatment significantly reduced the urinary excretion of kidney damaging molecules-1 (Kim-1), osteopontin (OPN), metalloproteinases tissue inhibitor 1 (TIMP-1), neutrophil gelatinase-associated lipocalin (GRL), and netrin-1 in comparison with $CdCl_2$ group treatments (K. S. Kim et al., 2018).

Honey

In addition to its sugar component, honey also includes numerous bioactive

Table 1
Different natural antioxidants and their effect on tissues

Natural substance	Dosage/Route of exposure of Cd	Studied animal	Exposure length	Target tissue	Mechanism of action	References
Curcumin	25 mg/kg b.w./P.O.	Sprague Dawley male rats	7 days	Kidney	Curcumin treatment significantly improved the biomarkers associated with nephrotoxicity compared with that in the $CdCl_2$-treated group	K. S. Kim et al. (2018)
	7 mg/kg b.w./SQ	Male CD mice	3 days	Liver, Kidney, Testes, Brain	Curcumin pretreatment effectively protected against Cd-induced lipid peroxidation and improved the negative impact of cadmium on antioxidant status without reducing tissue cadmium rates	Eybl et al. (2006)
	10 mg/kg b.w./P.O.	Male albino rats	24 days	Blood	Curcumin due to its antioxidant mechanisms had a protective role against cadmium-induced hematotoxicity in rats, and may have therapeutic relevance	Attia et al. (2014a)
	2 mg/kg b.w./IP	Mice	5 days	Male reproductive system	By activating the Nrf2/ARE (antioxidant responsive element) signaling pathway, curcumin may protect against Cd-induced testicular injury	S. H. Yang et al. (2019)
Honey	0.5 mg/kg b.w./IP	Male albino Wistar rats	30 days	Liver and Kidney	Honey administration with Cd induced improvement in all parameters examined.	Abdel-Moneim and Ghafeer (2007)
	0.67 mg/kg b.w./IP	Male albino mice	8 days	Male reproductive system	Honey administration improved the level of Cd-induced chromosomal aberrations and sperm abnormalities	Asmaa et al. (2016)
	200 mg/kg b.w./ P.O.	Albino rats	21 days	Liver	Honey found protective against cadmium-induced liver cell injury as evidenced by the ability of each test substance to minimize increased liver enzyme plasma activity and total liver function marker concentration of bilirubin	Anyia (2016)

Table 1 *(continue)*

Natural substance	Dosage/Route of exposure of Cd	Studied animal	Exposure length	Target tissue	Mechanism of action	References
Green tea extract (GTE)	3 µmoles/kg b.w./P.O.	Wistar rats	6 months	Liver	Green tea extract significantly increased the activity of enzymatic antioxidants in rat's liver compared to those treated with cadmium alone	Hamden et al. (2009)
	1.25 mg/kg b.w./IP	Male albino rats	Not defined	Liver	Hepatoprotective effect of green tea in cadmium chloride toxicated rats is reported	
	6 mg/L P.O.	Albino rats	10 days	Liver and kidney	GTE intake can effectively reduce Cd-induced cell injury in liver and kidney tissues	Hussain and Al-taee (2014)
Olive	5 mg/kg b.w./P.O.	Albino rats	4 weeks	Kidney and brain	Olive oil administration with $CdCl_2$ has been effective in improving the altered biochemical and oxidative-antioxidant parameters as well as the percentage of DNA fragmentation to almost those of the control group	E. Mohammed et al. (2014)
	50 mg/L P.O.	Wistar rats	8 weeks	Liver	Olive oil co-treatment significantly improved Cd oxidative damage. The potential for antioxidants in plasma and liver was significantly restored and MDA levels also significantly decreased	Amamou et al. (2015)
	1.8 mg/kg b.w./ P.O.	Swiss Albino mice	28 days	Kidney	The treatment with olive oil significantly protected the cadmium-induced oxidative stress	Wani et al., (2018)
Grape seed extract (GSE)	5 mg/kg b.w./P.O.	Male Wistar rats	4 weeks	Liver, kidney, brain, and testes	GSE enhanced antioxidant potential in all tissues, and reduced blood plasma and liver MDA levels	Evcimen et al. (2018)
	5 mg/kg b.w./P.O.	Kunming mice	30 days	Kidney	GSE administration attenuated Cd-induced lipid peroxidation, and antagonized renal apoptosis, possibly associated with Bax and Bcl-2 expression	Chen et al. (2013)
	60 mg/L P.O.		20 weeks	Prostate	The prostatic oxidative stress and fibrosis caused by $CdCl_2$ were improved by the GSE	Lei et al. (2017)

Table 1 *(continue)*

Natural substance	Dosage/Route of exposure of Cd	Studied animal	Exposure length	Target tissue	Mechanism of action	References
Rosemary	30 mg/kg b.w./P.O.	Albino rat	8 weeks	Liver	The toxic effects of $CdCl_2$ on the liver were improved by rosemary aqueous extract demonstrated by histological observation, decreased MDA and increased CAT, SOD, and GSH in the liver	Sakr et al. (2015)
	5 mg/kg b.w./P.O.	Guinea pigs	28 days	Liver	The serum alanine aminotransferase, aspartate aminotransferase, and alkaline phosphatase activity, as well as complete and direct serum bilirubin, were increased due to Cd-induced toxicity, all these parameters were declined by co-administration of rosemary extract	Albasha (2014)
	15 mg/kg b.w./P.O.	Wistar rats	4 weeks	Kidney and liver	Cd-induced cellular disorganization of the kidney and liver has been restored through the co-treatment of rosemary extract.	Virk et al. (2013)
Ginger	5 mg/kg b.w./P.O.	Wistar albino rats	30 days	Kidney	Enhancement of the kidney tissue in histological examinations reflects improvements in functional kidney markers and significantly supports the possible protective role of ginger against renal toxicity due to cadmium	Gabr et al. (2019)
	200 mg/kg b.w./ P.O.	Rabbits	12 weeks	Kidney and liver	Caspase3 and MKI67's immunohistochemical expression was high in hepatocytes and tubular epithelium in (Cd) group, while hepatocytes and tubular epithelium were slightly stained in cadmium and ginger group, suggesting ginger's protective effect against cadmium toxicity	Baiomy and Mansour (2016)
	10 mg/kg b.w./P.O.	Female albino rats	26 days	Blood	Cd-exposed animals with ginger decreased the concentration of MDA and hemolysis by 20% and 17%, respectively	Attia et al. (2014b)

Note. b.w. = Body weight; P.O. = *per os* (Orally); SQ = Subcutaneous; IP = Intraperitoneal

compounds such as phenolic compounds, flavonoids, and carotenoids, which act as antioxidants and scavenging free radicals (Elmenoufy, 2012). A study conducted by Abdel-Moneim and Ghafeer (2007) in male albino rats showed that honey in combination with Cd reduced its hazards. It was also revealed in the same research that honey could protect against Cd-induced oxidative stress by reducing free radicals and increasing antioxidant levels (Abdel-Moneim & Ghafeer, 2007). According to Abdelaziz et al. (2013), honey treatment is reported to be the most effective as compared to vitamin C and B complex in recovering the affected blood parameters in Cd-exposed rabbits. Asmaa et al. (2016) revealed that the administration of honey improved the chromosomal aberration rate and sperm abnormality caused by Cd. Among the beneficial effects of honey reported also include the capability to prevent oxidative damage, protect the liver and kidney tissues and restore the natural metabolic processes (Elmenoufy, 2012). Furthermore, honey with Kola seed was shown to have an anti-hepatotoxic function and decreased plasma total bilirubin concentration and enzyme activity in rats exposed to Cd acetate (Anyia, 2016).

Green Tea Extract

Green tea intake is correlated with various health-promoting properties (Venables et al., 2008). It helps detoxify heavy metals by inhibiting absorption and promoting excretion (El-Shahat et al., 2009). According to Hamden et al (2009), oral administration of green tea with Cd improves liver dysfunction and oxidative stress in rats. A study conducted by Kumar et al. (2010) showed that green tea extract (GTE) had hepatoprotective effects on the liver of Cd-exposed rats. As discussed before, Cd has cytotoxic effects on rat liver and kidney cells, and according to Hussain et al. (2014), GTE intake can minimize cell damage in these tissues effectively. GTE supplement also showed protective effects on testes against Cd through inhibition of oxidative damage and apoptosis (Abdelrazek et al., 2016). The ameliorating effect of GTE against Pb and Cd-induced testicular toxicity in rats was also proven by Hussein et al. (2014). Supplementing rats with GTE along with Cd boosts the antioxidant or detoxification process, thus reducing oxidative stress in rat testes (Hussein et al., 2014). The study conducted by Mahmood et al. (2015) in female Wistar rats showed ameliorating effects of GTE on gonadotropin hormones (FSH and LH) against Cd-induced toxicity. It was found by Singh et al. (2013) that GTE, due to its antioxidant properties, reversed changes in hematological parameters of rats, thus reducing the toxic effects of Cd. According to Z. I. Mohammed (2014), GTE's phenolic compound protects the kidney tissue against Cd toxicity.

Onion and Garlic

Onion and garlic are essential bioactive natural ingredient sources, including polyphenols and organosulfur compounds (Mężyńska & Brzóska, 2019). Garlic and garlic extracts have been reported to

defend body against free radical damage (Chung, 2006). An investigation carried out by Massadeh et al. (2007) demonstrated that Cd-induced immunosuppression in mice was substantially reversed by garlic extract. According to Ola-Mudathir et al. (2008), garlic extract offers protection against Cd-induced oxidative damage and spermiotoxicity in rats possibly through reduction of the peroxidation of lipids and enhance the antioxidant protection mechanism (Ola-Mudathir et al., 2008). It was revealed by Lawal et al.(2011) that garlic extract had positive actions in 1321N1 human astrocytoma cells and HEK 293 human embryonic kidney cells to combat the Cd-induced toxicity, and these include membrane damage prevention and lipid peroxidation reduction. Garlic and onion extracts are suggested to have their protective effects by reducing lipid peroxidation and enhancing antioxidant resistance (Suru, 2008). Upon Cd exposure in rats, histological abnormalities including myofibril degradation, cytoplasm vacuolization, and myofibril irregularity were observed in cardiac tissue. These histological changes were successfully mitigated by onion extract (Alpsoy et al., 2014).

Olive Oil

There has been considerable interest in the antioxidant ability of phenolic compounds in olive oil (Owen et al., 2000). Administration of olive oil with $CdCl_2$ has successfully enhanced and improved altered biochemical and oxidative- antioxidant parameters and fragmentation of DNA (E. Mohammed et al., 2014). Consumption of olive oil or colocynth oil protected rat liver against Cd-induced injury by increasing enzyme activity and reducing oxidative stress (Amamou et al., 2015). It was found that serum creatinine, blood urea, and the antioxidant markers (glutathione peroxidase, superoxide dismutase, and catalase) levels were decreased in Cd-exposed rats while these parameters were improved in olive oil-treated groups (Wani et al., 2018). In addition to a reduction in the number of chromosomes, several chromosomal anomalies in albino rats were caused by $CdCl_2$, while olive oil supplementation reversed these chromosomal shifts (Aly et al., 2018).

Rosemary

Results from several studies showed that the rosemary essential oil had antimicrobial, antioxidant, anti-carcinogenic, and cognitive effects, it was mostly researched in light of its anti-cancer, antioxidant, and anti-infectious properties, covering 55% of the studies (Andrade et al., 2018; Sakr et al., 2015). According to a research conducted by Sakr et al. (2015), administration of rosemary extract in rats exposed to $CdCl_2$ showed that the concentration of GSH and the activity of catalase (CAT) and superoxide dismutase (SOD) were elevated while a decrease in malonaldehyde MDA were noticed as compared with the rats treated with the metal alone (Sakr et al., 2015). In Cd-exposed guinea pigs, aspartate aminotransferase, serum alanine aminotransferase, glutamyl

transferase, and alkaline phosphatase, serum total, and specific bilirubin were elevated. The fenugreek, rosemary, and cinnamon co-administration considerably improved structural changes in the liver and significantly reduced all of the biochemical parameters described above (Albasha, 2014). It was found that with the co-treatment of the rosemary, Cd-induced cell disorganizations of the kidney and liver were restored, SOD and CAT were elevated while MDA levels were lowered (Virk et al., 2013).

Ginger

Ginger has been reported to have antioxidant activity driven by the removal of free radicals (Maisuthisakul et al., 2007). Ginger's pharmacological activities are primarily due to its active phytocompounds; 6-gingerol is the abundant bioactive compound with numerous pharmacological effects, including analgesic, anti-inflammatory, antipyretic, and antioxidant properties (Ali et al., 2018). According to Gabr et al. (2019), Cd intoxicated rats display renal dysfunction, damage to the kidney tissue and an oxidative stress effect along with a decline in the total antioxidant status (TAC) and DNA content. Meanwhile, treatment with ginger leads to significant recovery of biomarkers of renal function, TAC, molecular DNA, and histological improvements, which may occur via free radical scavenging and regenerative mechanisms. Administration of Cd increased the mRNA expression of the observed apoptotic cells, the proliferation of MKI67, antioxidant (GST) expressions whereas decreased anti-apoptotic expression (Bcl2). The effect of Cd was counteracted by the administration of the ginger extract, resulting in the down-regulation of the previously mentioned upgraded genes (Baiomy et al., 2016). Cd exposure in rats resulted in an elevation of parameters of liver function. However, ginger-containing treatment reduced ALT and normalized the other liver function parameters. The altered renal total serum cholesterol parameters were returned to near typical values after treatment (Ugwuja et al., 2016). It was found that ginger treatment of Cd-exposed rats increased hemoglobin content compared to Cd alone group. Ginger treatment also increased Glutathione Peroxidase (GPx) activity of Cd-exposed rats compared to Cd alone group (Attia et al., 2014b).

Grape Seed Extract

The grape seed extract (GSE) has shown to have excellent antioxidant potential and a greater scavenging capability of free radicals (Evcimen et al., 2018). According to Chen et al. (2013), Cd causes a decrease in GSH and SOD activities, and elevation in MDA level, induces renal apoptosis in mice. Co-administration of GSE, however, mitigates Cd-induced lipid peroxidation and antagonized renal apoptosis, possibly associated with Bax and Bcl-2 expression. GSE is also reported to improve prostatic oxidation stress and fibrosis induced by $CdCl_2$. It inhibits the over-generation of prostatic expressions of transforming

growth factors. According to Bashir et al. (2016), increasing levels of proinflammatory cytokines, lowers levels of cell defense proteins, and glucose transporters, as well as enhancing rates of signals of apoptosis molecules, observed in pancreas of Cd-intoxicated rats. Blood and liver MDA levels in Cd-treated rats were increased relative to controls. GSE improved the antioxidant potential in all tissues and decreased blood plasma and liver MDA levels (Evcimen et al., 2018). It was found that Cd treatment increased MDA and decreased Monoamine oxidase-A(MAO-A), acetylcholinesterase, and glutathione reductase (GR), while the GSE treatment caused improvements in those parameters (El-Tarras et al., 2016).

CONCLUSION AND FUTURE PERSPECTIVES

Since environmental and occupational exposure to Cd remains a serious health issue (Gaur & Agnihotri, 2019; Järup et al.,1998; J. J. Kim et al., 2019; Patra et al., 2011; Sarkar et al., 2013), especially in developing and industrialized countries, it is very important to find an effective solution to protect against adverse effects of Cd-exposure. Antioxidant-rich dietary products seem to be among the potential protective and therapeutic agents. They are particularly more promising in prevention than reversing treating the adverse health effects of the metal poisons in humans. Several experimental studies have shown that antioxidants exert a beneficial action against Cd toxicity in different organs of the body by averting oxidative stress as well as other adverse effects of this xenobiotic. The natural products rich in antioxidants can reduce the Cd-accumulation in tissues and hence humans may benefit from the consumption of these products. The existing literature shows that the researchers have recently focused their attention on ways to mitigate the absorption and accumulation of Cd and improve the organism's resistance to the toxic element. Interest has been given to agents with antioxidant ability, primarily naturally occurring substances. The search for more potent antioxidant-rich natural products is expected to continue with additional, relatively new products coming into the investigation. One of the examples is Edible Bird's Nest (EBN) which is currently under investigation in our laboratory to see its protective role against Cd toxicity. In our recent studies, EBN has been confirmed to play a significant protective role against lead (Pb), another heavy metal toxic agent (Albishtue et al., 2019). More research on the prophylactic and therapeutic use of antioxidant containing natural substances is warranted, as therapeutically active chelation treatment of Cd is currently lacking.

ACKNOWLEDGEMENT

This work was supported by the Ministry of Education Malaysia under the FRGS research grant scheme (No. 5540114/FRGS/1/2018/SKK08/UPM/02/6).

REFERENCES

Abdel-Moneim, W., & Ghafeer, H. (2007). The potential protective effect of natural honey against cadmium-induced hepatotoxicity and nephrotoxicity. *Mansoura Journal of Forensic Medicine and Clinical Toxicology*, *15*(2), 75–98. https://doi.org/10.21608/mjfmct.2007.48851

Abdelaziz, I., Elhabiby, M. I., & Ashour, A. A. (2013). Toxicity of cadmium and protective effect of bee honey, vitamins C and B complex. *Human and Experimental Toxicology*, *32*(4), 362–370. https://doi.org/10.1177/0960327111429136

Abdelrazek, H. M. A., Helmy, S. A., Elsayed, D. H., Ebaid, H. M., & Mohamed, R. M. (2016). Ameliorating effects of green tea extract on cadmium induced reproductive injury in male Wistar rats with respect to androgen receptors and caspase-3. *Reproductive Biology*, *16*(4), 300–308. https://doi.org/10.1016/j.repbio.2016.11.001

Abu-Taweel, G. M. (2016). Effects of curcumin on the social behavior, blood composition, reproductive hormones in plasma and brain acetylcholinesterase in cadmium intoxicated mice. *Saudi Journal of Biological Sciences*, *23*(2), 219–228. https://doi.org/10.1016/j.sjbs.2015.05.010

Ahmad, M., Taweel, G. M. A., & Hidayathulla, S. (2018). Nano-composites chitosan-curcumin synergistically inhibits the oxidative stress induced by toxic metal cadmium. *International Journal of Biological Macromolecules*, *108*, 591–597. https://doi.org/10.1016/j.ijbiomac.2017.12.054

Aimola, P., Carmignani, M., Volpe, A. R., Di Benedetto, A., Claudio, L., Waalkes, M. P., van Bokhoven, A., Tokar, E. J., & Claudio, P. P. (2012). Cadmium induces p53-dependent apoptosis in human prostate epithelial cells. *PLOS One*, *7*(3), e33647. https://doi.org/10.1371/journal.pone.0033647

Akesson, A., Berglund, M., Schütz, A., Bjellerup, P., Bremme, K., & Vahter, M. (2002). Cadmium exposure in pregnancy and lactation in relation to iron status. *American Journal of Public Health*, *92*(2), 284–287. https://doi.org/10.2105/AJPH.92.2.284

Albasha, M. O. (2014). Effect of cadmium on the liver and amelioration by aqueous extracts of fenugreek seeds, rosemary, and cinnamon in guinea pigs: Histological and biochemical study. *Cell Biology*, *2*(2), 7–17. https://doi.org/10.11648/j.cb.20140202.11

Albishtue, A. A., Yimer, N., Zakaria, M. Z. A., Haron, A. W., Babji, A. S., Abubakar, A. A., & Almhanawi, B. H. (2019). Effects of EBN on embryo implantation, plasma concentrations of reproductive hormones, and uterine expressions of genes of PCNA, steroids, growth factors and their receptors in rats. *Theriogenology*, *126*, 310–319. https://doi.org/10.1016/j.theriogenology.2018.12.026

Ali, A. M. A., El-Nour, M. E. A. M., & Yagi, S. M. (2018). Total phenolic and flavonoid contents and antioxidant activity of ginger (*Zingiber officinale* Rosc.) rhizome, callus and callus treated with some elicitors. *Journal of Genetic Engineering and Biotechnology*, *16*(2), 677–682. https://doi.org/10.1016/j.jgeb.2018.03.003

Alkhedaide, A., Alshehri, Z. S., Sabry, A., Abdel-Ghaffar, T., Soliman, M. M., & Attia, H. (2016). Protective effect of grape seed extract against cadmium-induced testicular dysfunction. *Molecular Medicine Reports*, *13*(4), 3101–3109. https://doi.org/10.3892/mmr.2016.4928

Alpsoy, S., Kanter, M., Aktas, C., Erboga, M., Akyuz, A., Akkoyun, D. C., & Oran, M. (2014). Protective effects of onion extract on cadmium-induced oxidative stress, histological damage, and apoptosis in rat heart. *Biological Trace Element Research*, *159*(1–3), 297–303. https://doi.org/10.1007/s12011-014-9968-9

Aly, F. M., Kotb, A. M., Haridy, M. A. M., & Hammad, S. (2018). Impacts of fullerene C_{60} and virgin olive oil on cadmium-induced genotoxicity in rats. *Science of the Total Environment*, *630*, 750–756. https://doi.org/10.1016/j.scitotenv.2018.02.205

Amamou, F., Nemmiche, S., kaouthar Meziane, R., Didi, A., Yazit, S. M., & Chabane-Sari, D. (2015). Protective effect of olive oil and colocynth oil against cadmium-induced oxidative stress in the liver of Wistar rats. *Food and Chemical Toxicology*, *78*, 177–184. https://doi.org/10.1016/j.fct.2015.01.001

Amit, P., Ranjan, D. S., Sutapa, B., & Sukumar, K. (2019). Study on the effects of cadmium chloride on liver and testis in albino rats. *Research Journal of Chemistry and Environment*, *23*(8), 85–93.

Andrade, J. M., Faustino, C., Garcia, C., Ladeiras, D., Reis, C. P., & Rijo, P. (2018). *Rosmarinus officinalis* L.: An update review of its phytochemistry and biological activity. *Future Science OA*, *4*(4), FSO283. https://doi.org/10.4155/fsoa-2017-0124

Anyia, A. F. (2016). Protective effect of *Garcinia kola* seed and honey mixture against cadmium-induced hepatotoxicity in Wistar albino rats. *International Journal of Science and Research*, *5*(5), 356–363.

Arroyo, V. S., Flores, K. M., Ortiz, L. B., Gómez-quiroz, L. E., & Gutiérrez-ruiz, M. C. (2013). Liver and cadmium toxicity. *Journal of Drug Metabolism and Toxicology*, *S5*(001). https://doi.org/10.4172/2157-7609.s5-001

Asmaa, S., Mohamed, Z., & Khaled, S. (2016). The protective role of honey against cytotoxicity of cadmium chloride in mice. *African Journal of Biotechnology*, *15*(46), 2620–2626. https://doi.org/10.5897/ajb2016.15426

Ataei, N., Aghaei, M., & Panjehpour, M. (2019). Evidences for involvement of estrogen receptor induced ERK1/2 activation in ovarian cancer cell proliferation by cadmium chloride. *Toxicology in Vitro*, *56*, 184–193. https://doi.org/10.1016/j.tiv.2019.01.015

Attia, A. M. M., Ibrahim, F. A. A., Abd El-Latif, N. A., & Aziz, S. W. (2014a). Antioxidant effects of curcumin against cadmium chloride-induced oxidative stress in the blood of rats. *Journal of Pharmacognosy and Phytotherapy*, *6*(3), 33–40. https://doi.org/10.5897/JPP2014.0316

Attia, A. M. M., Ibrahim, F. A. A., Abd El-Latif, N. A., Aziz, S. W., & Moussa, S. A. A. (2014b). Protective effects of ginger (*Zingiber officinale* Roscoe) against cadmium chloride-induced oxidative stress in the blood of rats. *Journal of Medicinal Plants Research*, *8*(39), 1164–1172. https://doi.org/10.5897/jmpr2014.5531

Baiomy, A. A., & Mansour, A. A. (2016). Genetic and histopathological responses to cadmium toxicity in rabbit's kidney and liver: Protection by ginger (*Zingiber officinale*). *Biological Trace Element Research*, *170*(2), 320–329. https://doi.org/10.1007/s12011-015-0491-4

Bashir, N., Manoharan, V., & Miltonprabu, S. (2016). Grape seed proanthocyanidins protects against cadmium induced oxidative pancreatitis in rats by attenuating oxidative stress, inflammation and apoptosis via Nrf-2/HO-1 signaling. *The Journal of Nutritional Biochemistry*, *32*(3), 128–141. https://doi.org/10.1016/j.jnutbio.2016.03.001

Berglund, M., Akesson, A., Nermell, B., & Vahter, M. (1994). Intestinal absorption of dietary cadmium in women depends on body iron stores and fiber intake. *Environmental Health Perspectives*, *102*(12), 1058–1066. https://doi.org/10.2307/3431993

Beyersmann, D., & Hechtenberg, S. (1997). Cadmium, gene regulation, and cellular signalling in mammalian cells. *Toxicology and Applied Pharmacology*, *144*(2), 247–261. https://doi.org/10.1006/taap.1997.8125

Calabrese, E. J. (2002). Part 1. The role of ROS in health disease: Part 2. Proposing a definition of hormesis. *Human and Experimental Toxicology*, *21*(2), 59. https://doi.org/10.1191/0960327102ht209oa

Chedrese, P., Piasek, M., & Henson, M. (2008). Cadmium as an endocrine disruptor in the reproductive system. *Immunology, Endocrine and Metabolic Agents in Medicinal Chemistry*, *6*(1), 27–35. https://doi.org/10.2174/187152206775528941

Chen, Q., Zhang, R., Li, W. M., Niu, Y. J., Guo, H. C., Liu, X. H., Hou, Y. C., & Zhao, L. J. (2013). The protective effect of grape seed procyanidin extract against cadmium-induced renal oxidative damage in mice. *Environmental Toxicology and Pharmacology*, *36*(3), 759–768. https://doi.org/10.1016/j.etap.2013.07.006

Chung, L. Y. (2006). The antioxidant properties of garlic compounds: Alyl cysteine, alliin, allicin, and allyl disulfide. *Journal of Medicinal Food*, *9*(2), 205–213. https://doi.org/10.1089/jmf.2006.9.205

Dailiah Roopha, P., & Padmalatha, C. (2012). Effect of herbal preparation on heavy metal (cadmium) induced antioxidant system in female Wistar rats. *Journal of Medical Toxicology*, *8*(2), 101–107. https://doi.org/10.1007/s13181-011-0194-y

de Angelis, C., Galdiero, M., Pivonello, C., Salzano, C., Gianfrilli, D., Piscitelli, P., Lenzi, A., Colao, A., & Pivonello, R. (2017). The environment and male reproduction: The effect of cadmium exposure on reproductive functions and its implication in fertility. *Reproductive Toxicology*, *73*, 105–127. https://doi.org/10.1016/j.reprotox.2017.07.021

DelRaso, N. J., Foy, B. D., Gearhart, J. M., & Frazier, J. M. (2003). Cadmium uptake kinetics in rat hepatocytes: Correction for albumin binding. *Toxicological Sciences*, *72*(1), 19–30. https://doi.org/10.1093/toxsci/kfg009

El-Demerdash, F. M., Yousef, M. I., Kedwany, F. S., & Baghdadi, H. H. (2004). Cadmium-induced changes in lipid peroxidation, blood hematology, biochemical parameters and semen quality of male rats: Protective role of vitamin E and β-carotene. *Food and Chemical Toxicology*, *42*(10), 1563–1571. https://doi.org/10.1016/j.fct.2004.05.001

El-Shahat, A. E.-R., Gabr, A., Meki, A.-R., & Mehana, E.-S. (2009). Altered testicular morphology and oxidative stress induced by cadmium in experimental rats and protective effect of simultaneous green tea extract. *International Journal of Morphology*, *27*(3), 757–764. https://doi.org/10.4067/s0717-95022009000300020

El-Tarras, A. E. S., Attia, H. F., Soliman, M. M., El Awady, M. A., & Amin, A. A. (2016). Neuroprotective effect of grape seed extract against cadmium toxicity in male albino rats. *International Journal of Immunopathology and Pharmacology*, *29*(3), 398–407. https://doi.org/10.1177/0394632016651447

Elmenoufy, G. A. M. (2012). Bee honey dose-dependently ameliorates lead acetate- mediated hepatorenal toxicity in rats. *Life Science Journal*, *9*(4), 780–788.

Evcimen, M., Aslan, R., & Gulay, M. S. (2018). Protective effects of polydatin and grape seed extract in rats exposed to cadmium. *Drug and Chemical Toxicology*, *43*(3), 225–233. https://doi.org/10.1080/01480545.2018.1480629

Eybl, V., Kotyzova, D., & Koutensky, J. (2006). Comparative study of natural antioxidants - Curcumin, resveratrol and melatonin - In cadmium-induced oxidative damage in mice. *Toxicology*, *225*(2–3), 150–156. https://doi.org/10.1016/j.tox.2006.05.011

Flora, G., Gupta, D., & Tiwari, A. (2012). Toxicity of lead: A review with recent updates. *Interdisciplinary Toxicology*, *5*(2), 47–58. https://doi.org/10.2478/v10102-012-0009-2

Fotakis, G., Cemeli, E., Anderson, D., & Timbrell, J. A. (2005). Cadmium chloride-induced DNA and lysosomal damage in a hepatoma cell line. *Toxicology in Vitro*, *19*(4), 481–489. https://doi.org/10.1016/j.tiv.2005.02.001

Gabr, S. A., Alghadir, A. H., & Ghoniem, G. A. (2019). Biological activities of ginger against cadmium-induced renal toxicity. *Saudi Journal of Biological Sciences*, *26*(2), 382–389. https://doi.org/10.1016/j.sjbs.2017.08.008

Gaur, S., & Agnihotri, R. (2019). Health effects of trace metals in electronic cigarette aerosols — A systematic review. *Biological Trace Element Research*, *188*, 295–315. https://doi.org/10.1007/s12011-018-1423-x

Geng, H. X., & Wang, L. (2019). Cadmium: Toxic effects on placental and embryonic development. *Environmental Toxicology and Pharmacology*, *67*(2), 102–107. https://doi.org/10.1016/j.etap.2019.02.006

Giaginis, C., Gatzidou, E., & Theocharis, S. (2006). DNA repair systems as targets of cadmium toxicity. *Toxicology and Applied Pharmacology*, *213*(3), 282–290. https://doi.org/10.1016/j.taap.2006.03.008

Hamden, K., Carreau, S., Ellouz, F., Masmoudi, H., & El Feki, A. (2009). Improvement effect of green tea on hepatic dysfunction, lipid peroxidation and antioxidant defence depletion induced by cadmium. *African Journal of Biotechnology*, *8*(17), 4233–4238. https://doi.org/10.5897/AJB2009.000-9409

Hart, B. A., Potts, R. J., & Watkin, R. D. (2001). Cadmium adaptation in the lung - A double-edged sword?. *Toxicology*, *160*(1–3), 65–70. https://doi.org/10.1016/S0300-483X(00)00436-4

Henson, M. C., & Chedrese, P. J. (2004). Endocrine disruption by cadmium, a common environmental toxicant with paradoxical effects on reproduction. *Experimental Biology and Medicine*, *229*(5), 383–392. https://doi.org/10.1177/153537020422900506

Hussain, B. I., & Al-taee, N. H. (2014). Ameliorated effect of green tea extract on cadmium toxicity in liver and kidney of rats. *Journal of Pure and Applied Sciences*, *22*(6), 1746–1753.

Hussein, S. A., Ragab, O. A., & El-Eshmawy, M. A. (2014). Protective effect of green tea extract on cyclosporine A: Induced nephrotoxicity in rats. *Journal of Biological Sciences*, *14*(4), 248–257. https://doi.org/10.3923/jbs.2014.248.257

Järup, L. (2003). Hazards of heavy metal contamination. *British Medical Bulletin*, *68*(1), 167–182. https://doi.org/10.1093/bmb/ldg032

Järup, L., & Åkesson, A. (2009). Current status of cadmium as an environmental health problem. *Toxicology and Applied Pharmacology*, *238*(3), 201–208. https://doi.org/10.1016/j.taap.2009.04.020

Järup, L., Berglund, M., Elinder, C. G., Nordberg, G., & Vahter, M. (1998). Health effects of cadmium exposure - A review of the literature and a risk estimate. *Scandinavian Journal of Work, Environment and Health*, *24*(Suppl. 1), 1–51.

Khan, A., Ikram, M., Muhammad, T., Park, J., & Kim, M. O. (2019). Caffeine modulates cadmium-induced oxidative stress, neuroinflammation, and cognitive impairments by regulating Nrf-2/HO-1 *in vivo* and *in vitro*. *Journal of Clinical Medicine*, *8*(5), 680. https://doi.org/10.3390/jcm8050680

Kim, J. J., Kim, Y. S., & Kumar, V. (2019). Heavy metal toxicity: An update of chelating therapeutic strategies. *Journal of Trace Elements in Medicine and Biology*, *54*, 226–231. https://doi.org/10.1016/j.jtemb.2019.05.003

Kim, K. S., Lim, J. S., Son, J. Y., Lee, J., Lee, B. M., Chang, S. C., & Kim, H. S. (2018). Curcumin ameliorates cadmium-induced nephrotoxicity in Sprague-Dawley rats. *Food and Chemical*

Toxicology, *114*, 34–40. https://doi.org/10.1016/j.fct.2018.02.007

Klaassen, C. D., Liu, J., & Diwan, B. A. (2009). Metallothionein protection of cadmium toxicity. *Toxicology and Applied Pharmacology*, *238*(3), 215–220. https://doi.org/10.1016/j.taap.2009.03.026

Kumar, P. V., Pricy, A. A., Kumar, C. S., & Goud, K. K. (2010). Hepatoprotective effect of green tea (*Camellia sinensis*) on cadmium chloride induced toxicity in rats. *Journal of Chemical and Pharmaceutical Research*, *2*(6), 125–128. https://doi.org/10.5483/BMBRep.2005.38.5.563

Lane, E. A., Canty, M. J., & More, S. J. (2015). Cadmium exposure and consequence for the health and productivity of farmed ruminants. *Research in Veterinary Science*, *101*, 132–139. https://doi.org/10.1016/j.rvsc.2015.06.004

Lawal, A. O., & Ellis, E. M. (2011). The chemopreventive effects of aged garlic extract against cadmium-induced toxicity. *Environmental Toxicology and Pharmacology*, *32*(2), 266–274. https://doi.org/10.1016/j.etap.2011.05.012

Lei, Y., Chen, Q., Chen, J., & Liu, D. (2017). Potential ameliorative effects of grape seed-derived polyphenols against cadmium induced prostatic deficits. *Biomedicine and Pharmacotherapy*, *91*, 707–713. https://doi.org/10.1016/j.biopha.2017.05.006

Mahmood, B., Mokhtar, M., & Esfandiar, S. (2015). The impact of green tea (*Camellia sinensis*) on the amount of gonadotropin hormones (LH, FSH) in immature female rats poisoned with cadmium chloride. *Biomedical and Pharmacology Journal*, *8*(1), 261–268. https://doi.org/10.13005/bpj/607

Maisuthisakul, P., Suttajit, M., & Pongsawatmanit, R. (2007). Assessment of phenolic content and free radical-scavenging capacity of some Thai indigenous plants. *Food Chemistry*, *100*(4), 1409–1418. https://doi.org/10.1016/j.foodchem.2005.11.032

Massadeh, A. M., Al-Safi, S. A., Momani, I. F., Alomary, A. A., Jaradat, Q. M., & Alkofahi, A. S. (2007). Garlic (*Allium sativum* L.) as a potential antidote for cadmium and lead intoxication: Cadmium and lead distribution and analysis in different mice organs. *Biological Trace Element Research*, *120*(1–3), 227–234. https://doi.org/10.1007/s12011-007-8017-3

Matés, J. M., Segura, J. A., Alonso, F. J., & Márquez, J. (2010). Roles of dioxins and heavy metals in cancer and neurological diseases using ROS-mediated mechanisms. *Free Radical Biology and Medicine*, *49*(9), 1328–1341. https://doi.org/10.1016/j.freeradbiomed.2010.07.028

Mężyńska, M., & Brzóska, M. M. (2019). Review of polyphenol-rich products as potential protective and therapeutic factors against cadmium hepatotoxicity. *Journal of Applied Toxicology*, *39*(1), 117–145. https://doi.org/10.1002/jat.3709

Miceli, F., Minici, F., Tropea, A., Catino, S., Orlando, M., Lamanna, G., Sagnella, F., Tiberi, F., Bompiani, A., Mancuso, S., Lanzone, A., & Apa, R. (2005). Effects of nicotine on human luteal cells *in vitro*: A possible role on reproductive outcome for smoking women. *Biology of Reproduction*, *72*(3), 628–632. https://doi.org/10.1095/biolreprod.104.032318

Mohammed, E., Hashem, K., & Rheim, M. (2014). Biochemical study on the impact of *Nigella sativa* and virgin olive oils on cadmium-induced nephrotoxicity and neurotoxicity in rats. *Journal of Investigational Biochemistry*, *3*(2), 71. https://doi.org/10.5455/jib.20140716041908

Mohammed, Z. I. (2014). Effect of phenolic compound extract of green tea to ameliorate the cadmium sulphate toxicity on the female rat kidneys. *Journal of Pharmacy and Biological Sciences*, *9*(2), 44–50.

Nasiadek, M., Skrzypińska-Gawrysiak, M., Daragó, A., Zwierzyńska, E., & Kilanowicz, A. (2014). Involvement of oxidative stress in the mechanism of cadmium-induced toxicity on rat uterus. *Environmental Toxicology and Pharmacology*, *38*(2), 364–373. https://doi.org/10.1016/j.etap.2014.07.007

Nna, V. U., Usman, U. Z., Ofutet, E. O., & Owu, D. U. (2017). Quercetin exerts preventive, ameliorative and prophylactic effects on cadmium chloride-induced oxidative stress in the uterus and ovaries of female Wistar rats. *Food and Chemical Toxicology*, *102*, 143–155. https://doi.org/10.1016/j.fct.2017.02.010

Ola-Mudathir, K. F., Suru, S. M., Fafunso, M. A., Obioha, U. E., & Faremi, T. Y. (2008). Protective roles of onion and garlic extracts on cadmium-induced changes in sperm characteristics and testicular oxidative damage in rats. *Food and Chemical Toxicology*, *46*(12), 3604–3611. https://doi.org/10.1016/j.fct.2008.09.004

Ospondpant, D., Phuagkhaopong, S., Suknuntha, K., Sangpairoj, K., Kasemsuk, T., Srimaroeng, C., & Vivithanaporn, P. (2019). Cadmium induces apoptotic program imbalance and cell cycle inhibitor expression in cultured human astrocytes. *Environmental Toxicology and Pharmacology*, *65*, 53–59. https://doi.org/10.1016/j.etap.2018.12.001

Owen, R. W., Giacosa, A., Hull, W. E., Haubner, R., Würtele, G., Spiegelhalder, B., & Bartsch, H. (2000). Olive-oil consumption and health: The possible role of antioxidants. *The Lancet Oncology*, *1*(2), 107-112. https://doi.org/10.1016/s1470-2045(00)00015-2

Patra, R. C., Rautray, A. K., & Swarup, D. (2011). Oxidative stress in lead and cadmium toxicity and its amelioration. *Veterinary Medicine International*, *2011*, 457327. https://doi.org/10.4061/2011/457327

Piasek, M., Blanuša, M., Kostial, K., & Laskey, J. W. (2001). Placental cadmium and progesterone concentrations in cigarette smokers. *Reproductive Toxicology*, *15*(6), 673–681. https://doi.org/10.1016/S0890-6238(01)00174-5

Prozialeck, W. C., & Edwards, J. R. (2012). Mechanisms of cadmium-induced proximal tubule injury: new insights with implications for biomonitoring and therapeutic interventions. *Journal of Pharmacology and Experimental Therapeutics*, *343*(1), 2–12. https://doi.org/10.1124/jpet.110.166769

Puppel, K., Kapusta, A., & Kuczyńska, B. (2015). The etiology of oxidative stress in the various species of animals, a review. *Journal of the Science of Food and Agriculture*, *95*(11), 2179–2184. https://doi.org/10.1002/jsfa.7015

Rani, A., Kumar, A., Lal, A., & Pant, M. (2014). Cellular mechanisms of cadmium-induced toxicity: A review. *International Journal of Environmental Health Research*, *24*(4), 378-399. https://doi.org/10.1080/09603123.2013.835032

Rikans, L. E., & Yamano, T. (2000). Mechanisms of cadmium-mediated acute hepatotoxicity. *Journal of Biochemical and Molecular Toxicology*, *14*(2), 110–117. https://doi.org/10.1002/(SICI)1099-0461(2000)14:2<110::AID-JBT7>3.0.CO;2-J

Sakr, S. A., Bayomy, M. F., & El-Morsy, A. M. (2015). Rosemary extract ameliorates cadmium-induced histological changes and oxidative damage in the liver of albino rat. *The Journal of Basic and Applied Zoology*, *71*, 1–9. https://doi.org/10.1016/j.jobaz.2015.01.002

Sarkar, A., Ravindran, G., & Krishnamurthy, V. (2013). A brief review on the effect of cadmium toxicity: From cellular to organ level. *International Journal of Biotechnology Research*, *3*(1), 17–36.

Satarug, S., Garrett, S. H., Sens, M. A., & Sens, D. A. (2010). Cadmium, environmental exposure, and health outcomes. *Environmental Health*

Perspectives, *118*(2), 182–190. https://doi.org/10.1289/ehp.0901234

Satarug, S., Haswell-Elkins, M. R., & Moore, M. R. (2000). Safe levels of cadmium intake to prevent renal toxicity in human subjects. *British Journal of Nutrition*, *84*(6), 791–802. https://doi.org/10.1017/s0007114500002403

Singh, N., Rani, P., Gupta, M., & Tandan, N. (2013). Role of green tea on cadmium toxicity on haematological profile of albino rats. *American Journal of Phytomedicine and Clinical Therapeutics*, *1*(5), 537–542.

Skipper, A., Sims, J. N., Yedjou, C. G., & Tchounwou, P. B. (2016). Cadmium chloride induces DNA damage and apoptosis of human liver carcinoma cells via oxidative stress. *International Journal of Environmental Research and Public Health*, *13*(1), 1–10. https://doi.org/10.3390/ijerph13010088

Spencer, J. P. E. (2003). Metabolism of tea flavonoids in the gastrointestinal tract. *The Journal of Nutrition*, *133*(10), 3255S–3261S. https://doi.org/10.1093/jn/133.10.3255S

Suru, S. M. (2008). Onion and garlic extracts lessen cadmium-induced nephrotoxicity in rats. *BioMetals*, *21*(6), 623–633. https://doi.org/10.1007/s10534-008-9148-5

Taha, E. A., Sayed, S. K., Ghandour, N. M., Mahran, A. M., Saleh, M. A., Amin, M. M., & Shamloul, R. (2013). Correlation between seminal lead and cadmium and seminal parameters in idiopathic oligoasthenozoospermic males. *Central European Journal of Urology*, *66*(1), 84–92. https://doi.org/10.5173/ceju.2013.01.art28

Thompson, J., & Bannigan, J. (2008). Cadmium : Toxic effects on the reproductive system and the embryo. *Reproductive Toxicology Journal*, *25*(3), 304–315. https://doi.org/10.1016/j.reprotox.2008.02.001

Ugwuja, E. I., Erejuwa, O. O., & Ugwu, N. C. (2016). Spices mixture containing garlic, ginger and nutmeg has protective effects on the kidneys and liver of cadmium exposed rats. *Advanced Pharmaceutical Bulletin*, *6*(2), 271–274. https://doi.org/10.15171/apb.2016.038

van der Wall, E. E. (2010). Increasing recognition of NHJ: A first-time impact factor of 1.4! *Netherlands Heart Journal*, *18*(9), 399. https://doi.org/10.1007/BF03091804

Vardhan, K. H., Kumar, P. S., & Panda, R. C. (2019). A review on heavy metal pollution, toxicity and remedial measures: Current trends and future perspectives. *Journal of Molecular Liquids*, *290*, 111197. https://doi.org/10.1016/j.molliq.2019.111197

Venables, M. C., Hulston, C. J., Cox, H. R., & Jeukendrup, A. E. (2008). Green tea extract ingestion, fat oxidation, and glucose tolerance in healthy humans. *American Journal of Clinical Nutrition*, *87*(3), 778–784. https://doi.org/10.1093/ajcn/87.3.778

Virk, P., Elobeid, M., Hamad, S., Korany, Z., Al-Amin, M., Omer, M. D. S., AIOlayan, E., Siddiqui, M. I., & Mirghani, N. M. (2013). Ameliorative effects of *Embilica officinalis* and *Rosmarinus officinalis* on cadmium-induced oxidative stress in Wistar rats. *Journal of Medicinal Plants Research*, *7*(14), 805-818. https://doi.org/10.5897/JMPR13.2593

Waisberg, M., Joseph, P., Hale, B., & Beyersmann, D. (2003). Molecular and cellular mechanisms of cadmium carcinogenesis. *Toxicology*, *192*(2–3), 95–117. https://doi.org/10.1016/S0300-483X(03)00305-6

Wang, X., Cui, W., Wang, M., Liang, Y., Zhu, G., Jin, T., & Chen, X. (2021). The association between life-time dietary cadmium intake from rice and chronic kidney disease. *Ecotoxicology and Environmental Safety*, *211*, 111933. https://doi.org/10.1016/j.ecoenv.2021.111933

Wang, S., Ren, X., Hu, X., Zhou, L., Zhang, C., & Zhang, M. (2019). Cadmium-induced apoptosis through reactive oxygen species-mediated mitochondrial oxidative stress and the JNK signaling pathway in TM3 cells, a model of mouse Leydig cells. *Toxicology and Applied Pharmacology*, *368*, 37–48. https://doi.org/10.1016/j.taap.2019.02.012

Wani, F. A., Ibrahim, M. A., Moneim, M. M. A., & Almaeen, A. R. H. A. (2018). Cytoprotectant and anti-oxidant effects of olive oil on cadmium induced nephrotoxicity in mice. *Open Journal of Pathology*, *8*(1), 31–46. https://doi.org/10.4236/ojpathology.2018.81004

World Health Organization. (2010). *Exposure to cadmium: A major public health concern*. WHO. http://www.who.int/ipcs/features/cadmium.pdf

Yang, J. M., Arnush, M., Chen, Q. Y., Wu, X. D., Pang, B., & Jiang, X. Z. (2003). Cadmium-induced damage to primary cultures of rat Leydig cells. *Reproductive Toxicology*, *17*(5), 553–560. https://doi.org/10.1016/S0890-6238(03)00100-X

Yang, S. H., He, J. Bin, Yu, L. H., Li, L., Long, M., Liu, M. Da, & Li, P. (2019). Protective role of curcumin in cadmium-induced testicular injury in mice by attenuating oxidative stress via Nrf2/ARE pathway. *Environmental Science and Pollution Research*, 34575–34583. https://doi.org/10.1007/s11356-019-06587-9

Zhang, H., & Reynolds, M. (2019). Cadmium exposure in living organisms: A short review. *Science of the Total Environment*, *678*, 761–767. https://doi.org/10.1016/j.scitotenv.2019.04.395

Zhang, T., Gao, X., Luo, X., Li, L., Ma, M., Zhu, Y., Zhao, L., & Li, R. (2019). The effects of long-term exposure to low doses of cadmium on the health of the next generation of mice. *Chemico-Biological Interactions*, *312*, 108792. https://doi.org/10.1016/j.cbi.2019.108792

Zhang, W., & Jia, H. (2007). Effect and mechanism of cadmium on the progesterone synthesis of ovaries. *Toxicology*, *239*(3), 204–212. https://doi.org/10.1016/j.tox.2007.07.007

Pertanika J. Trop. Agric. Sci. 44 (1): 107 - 115 (2021)

PERTANIKA JOURNALS

TROPICAL AGRICULTURAL SCIENCE

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Use of Amplified Fragment Length Polymorphism and Sequence Characterized Amplified Region Marker for Identifying the Sex of the *Oxyeleotris marmorata*

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ABSTRACT

There is a huge demand for the *Oxyeleotris marmorata*, especially in Asian markets. However, farmers are unable to provide a constant supply of this fish to meet the demand, which is estimated to be around 100 metric tonnes per annum. One of the reasons that are hindering the supply is the low success rate of *O. marmorata* breeding programs. These breeding programs rely on many factors for their success, one of which is the use of genuine male and female adults, although determining these could be a daunting task. This research was carried out in an attempt to determine a sex marker for the *O. marmorata* using the amplified fragment length polymorphism (AFLP) method. Of the 30×30 AFLP primer mixtures screened, the E-TAA and M-CTT primer pair had an amplified ~600 bp marker that was specific to the female. This ~600 bp AFLP marker was later used to design a 464 bp sequence characterized amplified region (SCAR) marker. Thus, it has been suggested that the SCAR marker obtained has the potential to be used for the sexual identification of the *O. marmorata* at the juvenile stage, thereby enabling them to be used in breeding programs.

Keywords: AFLP, *Oxyeleotris marmorata*, sex determination, sex marker

ARTICLE INFO

Article history:
Received: 03 August 2020
Accepted: 23 November 2020
Published: 24 February 2021

DOI: https://doi.org/10.47836/pjtas.44.1.06

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ISSN: 1511-3701
e-ISSN: 2231-8542

INTRODUCTION

In several Asian countries such as China, Thailand, Vietnam, and Malaysia, the *Oxyeleotris marmorata* (marble goby) is conventionally raised in cages located in freshwater waterways and ponds. It fetches between USD 20/kg to USD 50/kg in Asian markets (Herawati et al., 2016; Yong et al., 2013). Due to its pricey market value, the *O. marmorata* has the potential to be raised as an alternative profitable aquaculture species. Multiple efforts have been undertaken by various parties to perfect the culturing technique for the *O. marmorata* (Lam et al., 2014a, 2014b; Seetapan et al., 2012). However, the sexing of the *O. marmorata* is one of the biggest challenges faced by farmers in culturing this fish. False sexing of this fish will lead to failure in producing proper brood stocks for the breeding program. What is more challenging is that a system for determining the sex of this fish remains elusive.

The sexual dimorphism of the adult *O. marmorata* can be determined by observing the morphometric differences in its urogenital papilla, caudal fin and caudal peduncle. The female has a longer urogenital papilla in comparison to the male. On the other hand, both the caudal fin and caudal peduncle of the male are longer than those of the female (Idris et. al., 2012). The longer urogenital papilla observed in the female *O. marmorata* is attributed to the need to fulfil the spawning ritual, during which; the female uses its urogenital papilla to deposit its eggs on the surface of a substance. The longer caudal fin and caudal peduncle observed in the male *O. marmorata* are needed for adequate agitation and aeration for the mixing and milting of the eggs during the fertilization process (Idris et. al., 2012). This sexual dimorphism, however, is sometimes hard to determine by the untrained eye and can sometimes lead to false sexing in the field or farm.

In this study, the amplified fragment length polymorphism (AFLP) technique (Vos et. al., 1995) was proposed for the sex-specific marker identification of the *O. marmorata* using genomic DNA obtained from phenotypically sex-based morphometric features (Idris et al., 2012). The advantages of the AFLP are that it can be conducted without any prior sequence information; it has pre-designed generic primers, and a high multiplexing ratio. The AFLP had previously been successfully utilised for the sex identification of various organisms, including fishes such as the *Acipenser schrenckii*, *Clarias gariepinus*, *Scophthalmus maximus*, *Dicentrarchus labrax*, *Hypophthalmichthys nobilis*, *Hypophthalmichthys molitrix*, *Pseudobagrus ussuriensis*, *Pelteobagrus fulvidraco*, and *Oncorhynchus mykiss* (Cheng et al., 2013; Felip et al., 2000, 2005; Kovács et al., 2000; Liu et al., 2018; Pan et al., 2015; Vale et al., 2014; Xiao et al., 2014). To enable the sex marker to be used in breeding programs on a large scale in a more cost-efficient and labour-intensive manner, the AFLP marker was converted into a polymerase chain reaction-based marker such as the SCAR marker for marker-associated selections (Wang et al., 2011).

MATERIALS AND METHODS

Oxyeleotris marmorata Source and Genomic DNA Isolation

A total of 14 adult *O. marmorata* were collected from Empangan Kelau, Bentong Pahang, Malaysia (Coordinates 3° 34' 40.9224"N 101° 59'17.7504" E) using both fish traps and gill nets in the months of May 2018 and March 2019. The fish samples were kept alive at the Makmal Genomik 1, Universiti Kebangsaan Malaysia. The fish were kept in fish tank equipped with proper aeration and were fed once a day ad libitum with live feed. The determination of the sex of the *O. marmorata* was based on their morphometric features, as described by Idris et al. (2012). The lengths of the adult fish used in this study were between 20 to 30 cm, while their average weight was 300 g (± 30 g). The fish were separated into two groups. The first group, comprising 3 females and 3 males, was used for the construction of the gene pool, while the second group, comprising 4 males and 4 females, was used to verify the isolated sex markers. The care of the fish and all the experiments were conducted in compliance with the UKM Animal Ethics Guidelines, as approved by the UKM Ethics Committee (Animal Ethics approval number: FST/2018/MOHDSHAZRUL/28-MAR./905-MAR-2018-AUG-2019) on 11 April 2018. All the fish were euthanized at the end of the experiments using methods previously described by Blessing et al. (2010). The sexual organs were also observed to confirm the gender of the fish.

The genomic DNA was isolated from the caudal fin of the fish, according to the phenol-chloroform method with modifications (Sambrook et al., 1989). About 0.5 g of the caudal fin was taken from the *O. marmorata* and preserved in 95 % ethanol immediately after collection for storage prior to the genomic extraction process. The preserved sample was frozen using liquid nitrogen prior to homogenisation using a pre-chilled mortar and pestle. A total volume of 500 µL of extraction buffer (10 mM EDTA, 100 mM Tris-HCl, 200 mM NaCl, and 0.7% SDS) were added to the homogenised sample. The mixture was vortexed for 30 seconds. Proteinase K (50 µg) was added, and the mixture was incubated for 60 minutes at 50°C. Next, 650 µL of phenol, chloroform and isoamyl alcohol (25:24:1) were added to the sample, after which, the mixture was vortexed for 30 seconds, followed by centrifugation at 4°C for 5 minutes at a speed of 12, 000 x*g*. Later, the aqueous phase was transferred into a fresh tube, and the same volume of chloroform and isoamyl alcohol (24:1) was added to the sample. The mixture was vortexed for 30 seconds, followed by centrifugation at 4°C for 5 minutes at a speed of 12, 000 x*g*. The aqueous phase was transferred into a fresh tube, and two volumes of ethanol were added to the sample. The mixture was inverted several times and was incubated at -20°C for 16 to 20 hours. The mixture was then centrifuged at 4°C for 10 minutes at a speed of 12, 000 x*g*. The pellet was washed in 70% cold ethanol. The sample was then dried, and resuspended in sterile dH_2O containing

RNase A (10 µg/mL). The quality of all the genomic DNA obtained was checked using a NanoDrop™ Spectrophotometer ND-2000. Only the genomic DNA at a 260/280 ratio of 1.8 and above was used for all the assays.

AFLP Reaction Assay

Approximately 0.5 mg of genomic DNA from the first fish group was digested and ligated simultaneously at room temperature overnight using 0.5 M of NaCl, 5 U *Eco*RI (Promega Corporation), 10 U *Mse*I (New England Biolabs), 0.55 µL(1.0 mg/mL) of 100× BSA, 1 µL of *Mse*I adapter (20 pmol/µL), 1 µL of *Eco*RI adapter (20 pmol/µL), 200 U T4 DNA ligase (Promega), 1.1 µL of T4 DNA ligase buffer, and 5.5 µL of distilled water. The digested and ligated mixture was then diluted to 200 µL with Tris-EDTA buffer (20mM Tris-HCl, 0.1 mM EDTA) and kept at -20°C for use in the pre-amplification process.

For the pre-amplification process, a mixture was prepared with the following content: 7.5 µL of ligated DNA, 2.0 µL of 10× *Taq* polymerase buffer, 0.6 µL of 50 mM $MgCl_2$, 0.5 µL of 10 mM dNTPs (Invitrogen), 0.25 µL of *Eco*RI pre-amplification primer (20 pmol/µL), 0.25 µL of *Mse*I pre-amplification primer (20 pmol/µL), 2.5 U *Taq* polymerase (Invitrogen) and distilled water, to make a final volume of 20 µL. The cycling parameters used for the pre-amplification process were 94°C for 5 minutes, followed by 20 cycles at 94°C for 45 seconds, 56°C for 30 seconds, and 72°C for 2 minutes, followed by a final extension at 72°C for 30 minutes. The pre-amplification products were then diluted to 100 µL with Tris-EDTA buffer (20 mM Tris-HCl, 0.1 mM EDTA) and kept at -20°C for use in the selective amplification process.

Each 10 µL of the mixture for the selective amplification reaction contained 0.5 µL of the pre-amplified products, 1.0 µL of 10× *Taq* polymerase buffer, 0.6 µL of 50 mM $MgCl_2$, 0.5 µL of 10 mM dNTPs (Invitrogen), 1 µL of *Eco*RI + 3 primer (10 pmol/µL), 0.25 µL of *Mse*I + 3 primers (10 pmol/µL), 2.5 U of *Taq* polymerase (Invitrogen) and distilled water to make a final volume of 10 µL. The AFLP products were amplified using touchdown PCR. Stage 1: 94°C for 5 minutes (1 cycle); Stage 2: 94°C for 45 seconds, 65°C for 30 seconds, and 72°C for 2 minutes. The annealing temperature was then lowered by 1.0°C per cycle (10 cycles); Stage 3: 94°C for 30 seconds, 56°C for 30 seconds, and 72°C for 2 minutes (20 cycles); and this was followed by a final extension stage at 72°C for 15 minutes. The selective amplification products were separated in 1.5% agarose.

The sex-specific fragments were excised and purified from the agarose gel using NucleoSpin® Gel and PCR Clean-up Kit (MACHEREY-NAGEL), and were sent to Apical Sdn. Bhd. for sequencing.

Adaptation of AFLP Marker as SCAR Marker

Primer blast (NCBI) software was used in designing the SCAR primers for the AFLP marker sequences. These primers were used to amplify the genomic DNA of the second group of fishes, consisting of four males and

4 females. The SCAR reaction was carried out in 2.0 µL of 10× PCR reaction buffer, 0.5 µL of 25 mM $MgCl_2$, 0.6 µL of 2 mM dNTPs, 20 pmol each of forward and reverse primers, 0.5 µL of genomic DNA, 2.0 U *Taq* polymerase, 0.5 µL of genomic DNA and distilled water to bring it to a volume of 20 µL. The cycling parameters used were 94°C for 5 minutes, followed by 30 cycles at 94°C for 45 seconds, optimised annealing temperature for 45 seconds, 72°C for 75 seconds, and final extension cycle at 72°C for 15 minutes. All the PCR products were examined in 1.5% agarose to determine the success of the SCAR primer conversion.

RESULTS AND DISCUSSIONS

Combinations of 30×30 AFLP primer pairs (Appendix 1) were used to screen potential sex markers from groups of males and females of the *O. marmorata* genomic DNA. To verify that these markers were associated to the genomic sex DNA sequences, the genomic DNA from 6 male and female *O. marmorata* were used as the DNA templates for these primer pairs (Figure 1). The study showed that only the E-TAA and M-CTT primer pair specifically amplified the DNA amplicon size of ~600 bp from the female genomic DNA, making it a specific marker for the female *O. marmorata*. The ~600 bp amplicon was sequenced, giving 574 bp. The sequence was deposited at the GenBank with accession number: MW148239. However, from the nucleotide BLAST analysis that was done, the sequence revealed that the amplicon did not contain any homology to any other species sequence in the GenBank database (data not shown). The non-homology sequence was expected as the AFLP is able to detect random DNA variations and highly likely non-coding DNA (Figure 2).

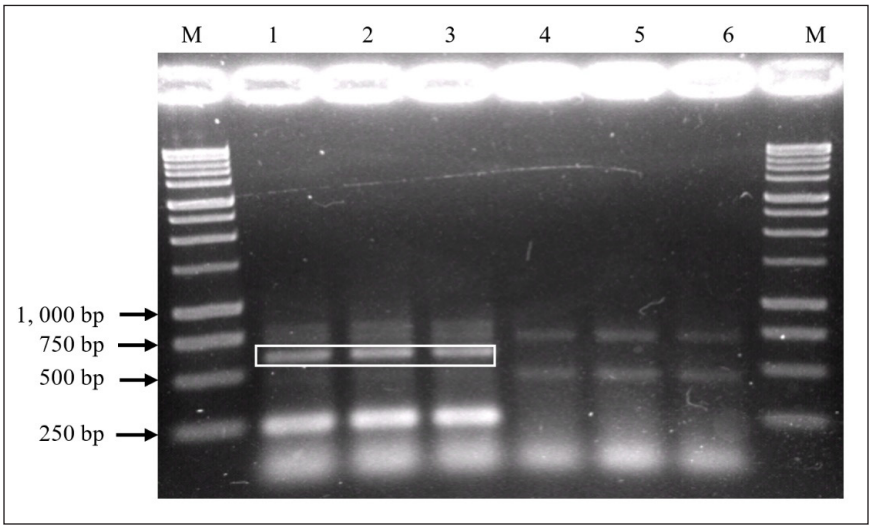

Figure 1. A ~600 bp band of the female-specific AFLP marker discovered with the E-TAA/M-CTT primer pair (marked in white box). Lanes 1 to 3 are the female samples; Lanes 4 to 6 are the male samples; Lane M is 1 kb DNA ladder marker

A primer pair (SM1: 5' GTCGGAATGTACCAAAGACAT 3' and SM2: 5'TATGAGTCCGTGAGTAACGC 3') was designed based on the sequence obtained from the female specific AFLP fragment from the E-TAA/M-CTT primer pair screening. The designed primer pair optimal annealing temperature was at 54°C, producing the expected ~480 bp amplicon, which was observed only in the female *O. marmorata*, but not in any male *O. marmorata* sample (Figure 3).

As far as is known, this is the first ever reported sex marker for the *Oxyeleotris marmorata* determined through the AFLP. The identified specific sex marker has multiple possible uses in basic research and commercial brood stock raising (Purcell et al., 2018). In basic research, the characterization of a sex marker for mature *O. marmorata* can provide invaluable data for understanding the sex determination mechanism. In commercial brood stock raising of the *O. marmorata*, the fish require

```
  1 GACTGCGTAC CAATTCTAAG AACCATAATA CATAAAGAAC AGGGCGAGCG GACAGCTCCC  60
 61 GGGCTCCATC CCTCCGGGGA GGCCCAACGT GTCGGAATGT ACCAAAGACA TCTATTATAC 120
                                    SM1
121 GGCCGAATCC TTAGATTTGA GCATCACTCC TCTCGCTGTG CGACACCCGG CGTATCTGAA 180
181 AGACACTTCG CACTTCATAG AGTCCATAAT GGATCTGCAC GTCCCGGGAG ACGCCTTCTT 240
241 CTTCTCCCTG GACATTGACT CTTTAAATAC CAACATTCCC ATCCAGGCCG GCATTCAGGC 300
301 CGTCAGAGAA ATTTTCCAAC TGTACCCTGA CCCCGCCAGG CCGGACGCGG AGCTCCTCCG 360
361 GTTACTGGAG ATCAATTTGA CTAAGAACGA CTTTGTCTTT GACGGCAAGT TCTACCTCCA 420
421 GGTCAAAGGG ACCGCCATGG GCAAACGCTT TGCCCCCGCT TACGCTAATA TCTTCATGGC 480
481 CAAATGGGAA AGACAAGTAC TGGCCACCTG CCCTAAAAAA CCGCTGCACT ACCTGCGTTA 540
541 CTCACGGACT CATACAAGTT ACTCAGGACT CATC
        SM2
```

Figure 2. The female-specific AFLP marker sequence and bold section indicate the primer designed for the SCAR marker

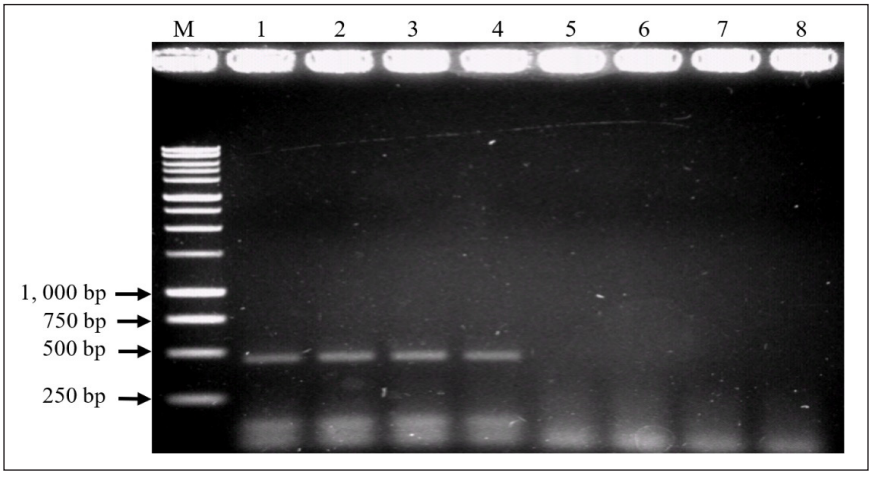

Figure 3. The SCAR marker for *Oxyeleotris marmorata* sexes determination. A ~ 480 bp band is present in the females (F) but not in the males (M). Lanes 1 to 4 are female sample. Lanes 5 to 8 are male sample. Lane M, 1 kb DNA ladder marker

almost 2 years before sexual identification can be done based on their reproductive organs, and only a highly-trained and experienced person will be able to do this as there are different distinct numbers of brood stocks to be maintained between the two sexes (Idris et al., 2012). Therefore, it will be more efficient and economical, in the long run if early sex identification can be done through molecular methods without having to wait for the fish to enter the maturity phase (Al-Ameri et al., 2016).

CONCLUSION

This study has succeeded in determining a female-specific AFLP and SCAR marker for *Oxyeleotris marmorata* samples from Tasik Klau, Bentong, Pahang. Both the female-specific AFLP and SCAR marker can be potentially used as a quick method for determining the sex of the *O. marmorata* for their potential use at the juvenile stage, thereby reducing the time and economic resources required to breed the species for commercialisation.

ACKNOWLEDGEMENTS

The authors would like to thank the Malaysia Ministry of Higher Education (FRGS:A07.00/01543A/001/201700436) for funding this research activity. The authors are grateful to the Makmal Genomik 1, Faculty of Science and Technology, Universiti Kebangsaan Malaysia for providing facilities throughout the research period.

REFERENCES

Al-Ameri, A. A., Al-Qurainy, F., Gaafar, A. R. Z., Khan, S., & Nadeem, M. (2016). Molecular identification of sex in *Phoenix dactylifera* using inter simple sequence repeat markers. *BioMed Research International*, *2016*, 4530846. https://doi.org/10.1155/2016/4530846

Blessing, J. J., Marshall, J. C., & Balcome, S. R. (2010). Humane killing of fishes for scientific research: A comparison of two methods. *Journal of Fish Biology*, *76*(10), 2571-2577. https://doi.org/10.1111/j.1095-8649.2010.0263 3.x

Cheng, D., Mei, J., Wang, D., & Gui, J. F. (2013). Genetic differentiation and efficient sex-specific marker development of a pair of Y- and X-linked markers in yellow catfish. *International Journal of Biological Science*, *9*(10), 1043–1049. https://doi.org/10.7150/ijbs.7203

Felip, A., Martinez-Rodriguez, G., Piferrer, F., Carrillo, M., & Zanuy, S. (2000). AFLP analysis confirms exclusive maternal genomic contribution of meiogynogenetic sea bass (*Dicentrarchus labrax* L.). *Marine Biotechnology*, *2*(3), 301–3066. https://doi.org/10.1007/s101260000000

Felip, A., Young, W. P., Wheeler, P. A., & Thorgaard, G. H. (2005). An AFLP-based approach for the identification of sex-linked markers in rainbow trout (*Oncorhynchus mykiss*). *Aquaculture*, *247*(1-4), 35–43. https://doi.org/10.1016/j.aquaculture.2005.02.014

Herawati, T., Yustiati, A., Nurhayati, A., & Natadia, S. S. (2016). Domestication of marble goby [*Oxyeleotris marmorata* (Bleeker, 1852)] indogenous fish of Citarum River, Indonesia. *Aquatic Procedia*, *7*, 247-253. https://doi.org/10.1016/j.aqpro.2016.07.035

Idris, H. B., Ambak, M. A., & Ikhwanuddin, M. (2012). Sex determination in *Oxyeleotris marmorata* (Bleeker, 1852) based on morphometric features. *Advances in Natural Applied Sciences*, *6*(6), 763-771.

Kovács, B., Egedi, S., Bártfai, R., & Orbán, L. (2000). Male-specific DNA markers from African catfish (*Clarias gariepinus*). *Genetica*, *110*(3), 267-276. https://doi.org/10.1023/A:1012739318941

Lam, S. S., Ambak, M. A., Jusoh, A., & Law, A. T. (2014a). Growth performance and waste excretion of marble goby (*Oxyeleotris Marmorata* Bleeker) in relation to different culture system and diet. *Aquaculture*, *29*(1), 41-45. https://doi.org/10.1016/ j.aquaculture. 2007.11.023

Lam, S. S., Ma, N. L., Jusoh, A., & Ambak, M. A. (2014b). A study on the optimal tank design and feed type to the growth of marble goby (*Oxyeleotris marmorata* Bleeker) and reduction of waste in a recirculating aquaponic system. *Desalination and Water Treatment*, *52*(4), 1044-1053. https://doi.org/10.1080/19443994. 2013.826854

Liu, H., Pang, M., Yu, X., Zhou, Y., Tong, J., & Fu, B. (2018). Sex-specific markers developed by next-generation sequencing confirmed an XX/ XY sex determination system in bighead carp (*Hypophthalmichehys nobilis*) and silver carp (*Hypophthalmichthys molitrix*). *DNA Research*, *25*(3), 257–264. https://doi.org/10.1093/dnares/ dsy009

Pan, Z. J., Li, X. Y., Zhou, F. J., Qiang, X. G., & Gui, J. F. (2015). Identification of sex-specific markers reveals male heterogametic sex determination in *Pseudobagrus ussuriensis*. *Marine Biotechnology*, *17*(4), 441–451. https:// doi.org/10.1007/s10126-015-9631-2

Purcell, C. M., Seetharam, A. S., Snodgrass, O., Ortega-García, S., Hyde, J. R., & Severin, A. J. (2018). Insights into teleost sex determination from the *Seriola dorsalis* genome assembly. *BMC Genomics*, *19*(1), 31. https://doi.org/10.1186/ s12864-017-4403-1

Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989). *Molecular cloning: A laboratory manual* (2nd ed.). Cold Spring Harbour Press.

Seetapan, K., Puanglarp, N., & Meunpol, O. (2012, November 22-24). *Study of optimal culture conditions for juvenile marble goby (*Oxyeleotris marmorata *Bleeker, 1852)* [Paper presentation]. Proceedings of 2nd the Annual International Conference Syiah Kuala University-Life Sciences and Engineering, Banda Aceh, Indonesia. http://jurnal.unsyiah.ac.id/AICS-SciEng/article/view/1713/1611

Vale, L., Dieguez, R., Sánchez, L., Martínez, P., & Viñas, A. (2014). A sex-associated sequence identified by RAPD screening in gynogenetic individuals of turbot (*Scophthalmus maximus*). *Molecular Biology Report*, *41*(3), 1501–1509. https://doi.org/10.1007/s11033-013-2995-3

Vos, P., Hoger, R., & Blecker, M. (1995). AFLP: A new technique for DNA fingerprinting. *Nucleic Acids Research*, *23*(21), 4407–4414. https://doi. org/10.1093/nar/23.21.4407

Wang, D. W., Li, Y., & Li, Z. Q. (2011). Identification of a male-specific amplified fragment length polymorphism (AFLP) and a sequence characterized amplified region (SCAR) marker in *Eucommia ulmoides* Oliv. *International Journal of Molecular Sciences*, *12*(1), 857-864. https:// doi.org/10.3390/ijms12010857

Xiao, T. Q., Lu, C. Y., Li, C., Cheng, L., Cao, D. C., & Sun, X. W. (2014). An AFLP-based approach for the identification of sex-linked markers in Amur sturgeon *Acipenser schrenckii* Brandt, 1869. *Journal of Applied Ichthyology*, *30*(6), 1282-1285. https://doi.org/10.1111/jai.12553

Yong, S., Ooi, S., & Shapawi, R. (2013). The utilization of soybean meal in formulated diet for marble goby, *Oxyeleotris marmoratus*. *Journal of Agricultural Science*, *5*(11), 139-149. https:// doi.org/10.5539/jas.v5n11p139

APPENDIX 1

Supplementary Table
List of AFLP primer pairs used in the screening

No.	*Eco*RI AFLP primers (E-XXX)	No.	*Mse*I AFLP primers (M-XXX)
1	*5' GACTGCGTACCAATTCTAA 3'	1	5' GATGAGTCCTGAGTAAACA 3'
2	5' GACTGCGTACCAATTCTAT 3'	2	5' GATGAGTCCTGAGTAAACT 3'
3	5' GACTGCGTACCAATTCTAC 3'	3	5' GATGAGTCCTGAGTAAACC 3'
4	5' GACTGCGTACCAATTCTAG 3'	4	5' GATGAGTCCTGAGTAAACG 3'
5	5' GACTGCGTACCAATTCTTA 3'	5	5' GATGAGTCCTGAGTAAAGA 3'
6	5' GACTGCGTACCAATTCTTT 3'	6	5' GATGAGTCCTGAGTAAAGT 3'
7	5' GACTGCGTACCAATTCTTC 3'	7	5' GATGAGTCCTGAGTAAAGC 3'
8	5' GACTGCGTACCAATTCTTG 3'	8	5' GATGAGTCCTGAGTAAAGG 3'
9	5' GACTGCGTACCAATTCTCA 3'	9	5' GATGAGTCCTGAGTAACAA 3'
10	5' GACTGCGTACCAATTCTCT 3'	10	5' GATGAGTCCTGAGTAACAT 3'
11	5' GACTGCGTACCAATTCTCC 3'	11	5' GATGAGTCCTGAGTAACAC 3'
12	5' GACTGCGTACCAATTCTCG 3'	12	5' GATGAGTCCTGAGTAACAG 3'
13	5' GACTGCGTACCAATTCTGA 3'	13	5' GATGAGTCCTGAGTAACTA 3'
14	5' GACTGCGTACCAATTCTGT 3'	14	*5' GATGAGTCCTGAGTAACTT 3'
15	5' GACTGCGTACCAATTCTGC 3'	15	5' GATGAGTCCTGAGTAACTC 3'
16	5' GACTGCGTACCAATTCTGG 3'	16	5' GATGAGTCCTGAGTAACTG 3'
17	5' GACTGCGTACCAATTCAAA 3'	17	5' GATGAGTCCTGAGTAACCA 3'
18	5' GACTGCGTACCAATTCAAT 3'	18	5' GATGAGTCCTGAGTAACCT 3'
19	5' GACTGCGTACC AATTCAAC 3'	19	5' GATGAGTCCTGAGTAACCC 3'
20	5' GACTGCGTACCAATTCAAG 3'	20	5' GATGAGTCCTGAGTAACCG 3'
21	5' GACTGCGTACCAATTCATA 3'	21	5' GATGAGTCCTGAGTAACGA 3'
22	5' GACTGCGTACCAATTCATT 3'	22	5' GATGAGTCCTGAGTAACGT 3'
23	5' GACTGCGTACCAATTCATC 3'	23	5' GATGAGTCCTGAGTAACGC 3'
24	5' GACTGCGTACCAATTCATG 3'	24	5' GATGAGTCCTGAGTAACGG 3'
25	5' GACTGCGTACCAATTCACA 3'	25	5' GATGAGTCCTGAGTAAGAA 3'
26	5' GACTGCGTACCAATTCACT 3'	26	5' GATGAGTCCTGAGTAAGAT 3'
27	5' GACTGCGTACCAATTCACC 3'	27	5' GATGAGTCCTGAGTAAGAC 3'
28	5' GACTGCGTACCAATTCACG 3'	28	5' GATGAGTCCTGAGTAAGAG 3'
29	5' GACTGCGTACCAATTCAGA 3'	29	5' GATGAGTCCTGAGTAAGTA 3'
30	5' GACTGCGTACCAATTCAGT 3'	30	5' GATGAGTCCTGAGTAAGTT 3'

Note.
1. XXX represent the last 3 nucleotides of the primer
2. * The AFLP selective-primer pairs that were used for development of SCAR marker

Pertanika J. Trop. Agric. Sci. 44 (1): 117 - 137 (2021)

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Journal homepage: http://www.pertanika.upm.edu.my/

Evaluation of Antagonism Activity and Control of *Vibrio alginolyticus* in *Artemia* Culture Using Mixed Probiotic

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ABSTRACT

Supplementation with mixed probiotic in aquaculture has been proven to benefit the hosts as disease resistance tool. In this study, a mixed probiotic which consisted of three isolated strains (*Lysinibacillus fusiformis* strain SPS11, A2, and *Bacillus megaterium* strain I24) was formulated for the *in vitro* assays against *Vibrio alginolyticus* and *in vivo* preliminary study towards *Artemia* nauplii. These strains showed antagonism activities against *V. alginolyticus* in *in vitro* assay. An increase in biofilm formation of this mixed probiotic was observed which indicated that the strains could work synergistically with each other to confer benefits to the hosts. Enrichment of *Artemia* nauplii with the formulated mixed probiotic was done to investigate its role in enhancing resistance against the *V. alginolyticus*. *Artemia* nauplii were cultured in two different concentrations of mixed probiotic (10^6 and 10^8 CFU mL^{-1}) and challenged via immersion method. The mixed probiotic at both concentrations resulted in significantly higher survival of *Artemia* compared to the challenged group with no probiont added (10^6 CFU mL^{-1}, 65.00 ± 0.00 % and 10^8 CFU mL^{-1}, 77.50 ± 3.53 %). Significant reduction of *Vibrio* loads was observed in *Artemia* and its culture water supplemented with mixed probiotic at 10^8 CFU mL^{-1} whereas there was

ARTICLE INFO

Article history:
Received: 14 September 2020
Accepted: 04 January 2021
Published: 24 February 2021

DOI: https://doi.org/10.47836/pjtas.44.1.07

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ISSN: 1511-3701
e-ISSN: 2231-8542

no reduction of *Vibrio* at 10^6 CFU mL^{-1}. This study suggests that the usage of formulated mixed probiotic at high concentration (10^8 CFU mL^{-1}) as opposed to single-strain probiotic can confer protection against *V. alginolyticus* infection towards *Artemia*.

Keywords: Antagonism, *Artemia*, biofilm formation, mixed probiotic, *Vibrio alginolyticus*

INTRODUCTION

Aquaculture is the cultivation of aquatic species in both coastal and inland areas involving interventions in the rearing process to enhance production. Accounting for 50% of the world's food-fish supply, it is one of the fastest-growing food production sectors. In 2015, fish contributed to 17% of animal protein consumed by the global population (Food and Agriculture Organization [FAO], 2018). As marine species are most commonly cultured with semi-intensive or intensive techniques in the sea or coastal waters, disease outbreak is often a risk in farms as it results in mass mortalities, translating to severe economic losses for farmers. Infection by water-borne pathogens such as *Vibrio* spp., and coliforms are a common consequence of intensive aquaculture (Rengpipat et al., 2008) due to the combination of high stocking densities and deterioration of water quality.

In order to combat disease outbreaks in farms, the most universal treatment is the application of antibiotics. However, the usage of antibiotics generates drug residues and proliferation of antibiotic-resistance among bacteria populations. It has been approximated that 90% of bacteria populations stemming from the marine environment are resistant to one or more antibiotics, and up to 20% of that is resistant to at least five (Fingerman et al., 2003). The development of antibiotic-resistant bacteria would increase the risk of spread to consumers as bacterial strains in commercial seafood products carrying resistance includes human pathogenic bacteria (Chiu et al., 2013; Kumar et al., 2016). Therefore, with this knowledge, it is important that alternative environmentally friendly solutions are developed to counteract bacterial infections.

Probiotics are microorganisms that confer health benefits to the host when administered at the appropriate dose. They are supplemented in fish rearing to increase the growth performance, appetite, digestibility, and control diseases by improving immune response (Shefat, 2018). However, most studies involved the use of single probiotic strains and there is little research on the use of mixed probiotics as a treatment method. Combination of different species and genera or different strains from same genus can be considered as a multi-strain probiotics (MSP). Multi-species probiotics are characterised as the incorporation of strains of different probiotic species belonging to one or, preferably, more genera (Timmerman et al., 2004).

In order to study the effects of the developed potential probiotics, the brine shrimp *Artemia* was selected as a model system and preliminary test organism. It is an exemplary model organism to study the

modes of action of probiotic and pathogenic bacteria, as it can be easily cultivated under controlled environments (Marques et al., 2005). Furthermore, being a continuous, non-selective and particulate filter feeder, *Artemia* is considered a multipurpose vector in aquaculture (Seenivasan et al., 2012). *Artemia* has been used as a vector to administer nutrients, vaccines, and most importantly, probiotics. Patra and Mohamed (2003) proved that the enrichment of *Artemia* nauplii with probiont *Saccharomyces boulardii* increased resistance to pathogenic *Vibrio*. In addition, a study by Haq et al. (2012) supported the finding and observed that the use of probiotics in *Artemia* was effective against marine pathogenic bacteria.

Bio-enrichment of *Artemia* spp. with probiotics and subsequent feeding to live aquatic animals also showed positive resistance against diseases. An investigation by Touraki et al. (2012) indicated that fish treated with *Bacillus subtilis*-enriched nauplii showed significantly elevated survival rates as compared to untreated group of fish when challenged with *Vibrio anguillarum*. Thus, this study aims to develop a mixed probiotic and to determine its effectiveness against pathogenic marine bacteria via *in vitro* and *in vivo* studies.

MATERIALS AND METHODS

Bacterial Culture of Probionts and Pathogens

The probionts used in this study were previously isolated and identified from previous research at the Laboratory of Fish Diseases, Department of Aquaculture, Faculty of Agriculture, Universiti Putra Malaysia (Table 1). Meanwhile the marine pathogen, *Vibrio alginolyticus* NBRC 15630 is a ATCC 17749 strain. Prior to the commencement of *in vitro* and *in vivo* assays, probionts and pathogen were sub-cultured in Tryptic Soy Broth (TSB, Difco Company, USA) supplemented with 1.5% NaCl, in individual, sterile 50 mL conical centrifuge tubes. All tubes were incubated at 30°C for 24 hours with continuous shaking.

In vitro Screening Assays

In order to utilize the probionts to produce mixed probiotic, the probiont strain must be able to exhibit inhibitory properties against *V. alginolyticus*. Agar-well diffusion and spot assays were used before formulating the mixed probiotic.

Agar-Well Diffusion Assay

The agar-well diffusion assay was conducted according to Tagg and McGiven (1971),

Table 1
List of probionts used in this study and their GenBank accession numbers

Code	Species/Strain	GenBank accession number	Origin of isolation	References
I24	*Bacillus megaterium*	KR150755	*Penaeus monodon* (Tiger shrimp)	Jasmin et al. (2016)
A2	*Lysinibacillus fusiformis*	MK764895	*Amphora* sp. (Microalgae)	Rosland (2018)
SPS11	*Lysinibacillus fusiformis*	MK757974	*Spirulina* sp. (Microalgae)	Zabidi (2018)

with some modifications where the indicator strain (pathogen) was swabbed on the agar first before inoculating the tester strain (probiont) in the wells as opposed to the method whereby, the tester strain is first inoculated in the well before flooding the agar with indicator strain. The optical density at 550 nm (OD_{550}) of pathogen *V. alginolyticus* was first measured with a UV spectrophotometer and the concentration of pathogen was adjusted to 10^7 CFU mL^{-1}. A sterile cotton bud immersed with pathogenic bacteria was swabbed evenly onto the Tryptic Soy Agar (TSA, Difco Company, USA) supplemented with 1.5% NaCl. Wells with a diameter of approximately 5mm was punched into the agar at equal distance apart (± 20mm). A fixed volume of 10 µL of each probiont (10^9 CFU mL^{-1}) was loaded into the respective wells. The plates were then incubated at 30°C for 24 hours. Following incubation, diameter of inhibition zone was measured and recorded. This assay was conducted in triplicate.

Spot Assay

Spot assay was conducted as secondary screening step to ascertain the inhibition of pathogen by the probionts as seen in results from the agar-well diffusion assay. The assay was conducted according to Wang et al. (2017). A sterile cotton bud was dipped into pathogen (10^7 CFU mL^{-1}) broth suspension and swabbed evenly onto the surface of TSA + 1.5% NaCl. Next, 2.5 µL of probiont (10^9 CFU mL^{-1}) suspension was spotted onto the agar plate. The plates were then incubated at 30°C for 24 hours. Following incubation, diameter of inhibition zone was measured and recorded. This assay was conducted in triplicate.

Formulation of Mixed Probiotic

The *V. alginolyticus*-inhibiting probionts which were preliminarily selected (*in vitro*) for formulation of the mixed probiotic were checked for their compatibility between strain. Compatibility was determined using the agar-well diffusion assay. A probiont strain (indicator strain) was swabbed onto the TSA + 1.5% NaCl and remaining strains of selected probionts were aliquoted into the well punched on the agar and allowed to dry completely. Zones of inhibition were observed after plates were incubated at 30°C, overnight. Mixed probiotic was then formulated via the addition of equal volumes of each individual probiont strain and mixed thoroughly by vortex. The mixed probiotic was incubated at 30°C for 15-30 minutes prior to usage.

Biofilm Formation Assay

The quantification of biofilm production was measured using crystal violet assay described by Bruhn et al. (2007). The mixed probiotic, individual probiont strain belonging to the mix and pathogen were cultured overnight in TSB + 1.5% NaCl at 30°C. Next, 200 µL of bacterial culture was transferred into a glass bottle containing 2 mL of TSB + 1.5% NaCl broth. The formation of biofilm was observed at 6 hours interval for the first 12 hours and 12 hours intervals subsequently, from 0 to 72

hour(s). At every sampling interval, contents in the glass bottle were discarded and the bottle was gently washed with sterile saline to rinse off poorly attached cells. Then, 200 µL of 0.2% crystal violet solution was added into the glass bottles before washing with sterile saline and dried at room temperature. The addition of 95% ethanol eluted the stain and concentration of biofilm formation was measured using UV spectrophotometer at OD_{550}.

In vivo Challenge of *Artemia* Nauplii

Experimental Design. The possibility of the mixed probiotic being beneficial probiotics against vibriosis was assessed preliminarily in *Artemia* culture. Freshly hatched *Artemia* nauplii were divided into 50 mL Falcon tubes, 20 *Artemia* in each tube, containing 30 mL of filtered, sterile seawater. Prior to challenge with pathogen, *Artemia* was incubated with the mixed probiotic at two different concentrations (10^6 CFU mL^{-1} and 10^8 CFU mL^{-1}), and the constituent single strain probiont for 24 hours. A control set-up containing 20 *Artemia* nauplii was incubated with filtered, sterile seawater. After 24 hours, *Artemia* were challenged with *V. alginolyticus* by immersing the pathogen (10^6 CFU mL^{-1}) in the culture water. All treatment tubes were incubated with shaking (120 rpm) on an orbital shaker for aeration purposes. *Artemia* was fed with dry yeast once daily. Daily observations were made, and the challenge test ceased when 50% mortality occurred in group of *Artemia* challenged with *V. alginolyticus* only. Susceptibility of *Artemia* to *V. alginolyticus* infection was determined by survival rates and *Vibrio* counts on Thiosulphate Citrate-Bile Salt (TCBS, Difco Company, USA) agar plates.

***Vibrio* Counts.** At the end of the challenge, *Artemia* from each treatment was passed through a sterile mesh to separate from culture water. Harvested *Artemia* was rinsed with filtered sterile seawater thrice and homogenised in 1 mL sterile saline water (1.5% NaCl). Serial dilution of up to 10^{-6} was performed, and 100 µL of each sample was spread onto TCBS agar plate in triplicates. Likewise, 1 mL of culture water from each treatment was collected and serially diluted to 10^{-6}. Next, 100 µL of sample was spread onto TCBS agar plates in triplicates. All agar plates were incubated at 30°C, overnight. Colonies of vibrios were counted using Rocker Galaxy 230 Colony Counter following incubation and calculated as CFU mL^{-1} using the formula:

$$\text{CFU mL}^{-1} = (\text{No. of colonies} \times \text{dilution factor}) / \text{Volume of culture plate (mL)}$$

Statistical Analysis

Statistical analysis was performed with IBM SPSS Statistics 20 software. All data collected from the biofilm formation assay and preliminary *in vivo* assessment were analysed using one-way analysis of variance (ANOVA). Tukey's test was applied to determine significant differences among treatments. Results were expressed as mean ± standard deviation at significance level $p<0.05$.

RESULTS

Single Strain Probiotic Antagonistic Assay

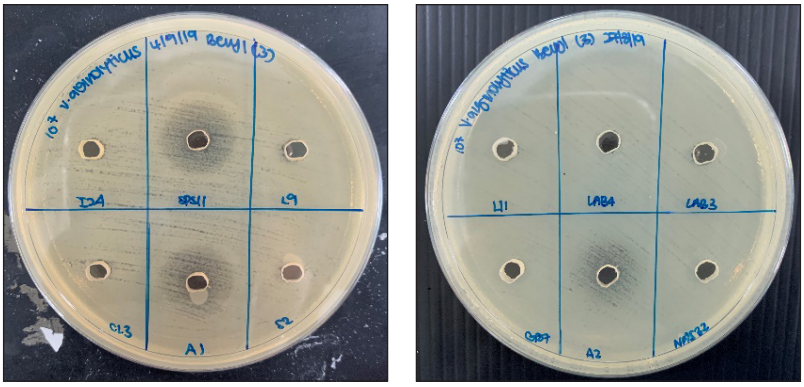
The probiont strains *L. fusiformis* SPS11, *B. megaterium* I24, and *L. fusiformis* A2 showed positive inhibition against *V. alginolyticus* (Figure 1). The inhibition zone produced by *B. megaterium* I24 was denoted as immeasurable because there was slight inhibition observed but it was insufficient to be measured. Furthermore, the strain I24 did not produce inhibitory zone in spot assay when tested against *V. alginolyticus*. The results from the antagonistic assays were summarised in Table 2.

Compatibility of Probiotic Strains

The three strains were also tested for their compatibility with each other using agar-well diffusion assay, to evaluate the suitability for application in a mixed probiotic (Figure 2). The 3 strains were compatible with each other. No inhibition zones were observed in the well diffusion assay. Thus, these 3 strains (*Lysinibacillus fusiformis* SPS11, *L. fusiformis* A2, *Bacillus megaterium* I24) can be used to produce mixed probiotic.

Biofilm Formation Assay

The biofilm formation ability of the mixed probiotic as compared to single strains and pathogen, *V. alginolyticus* was

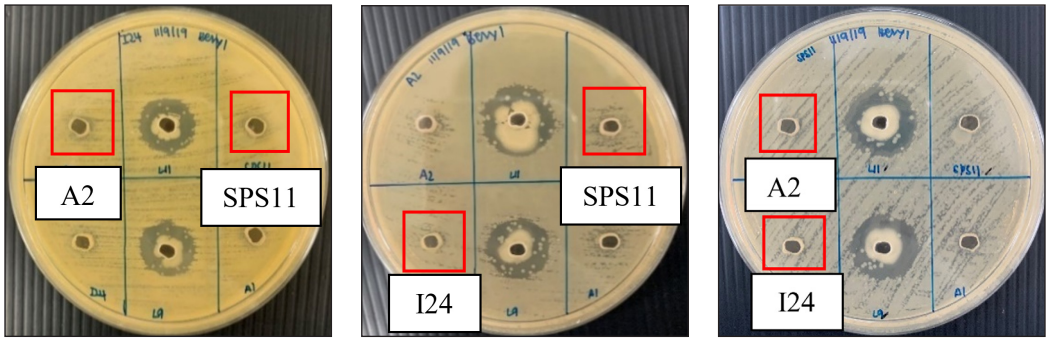


(a) (b)

Figure 1. Inhibition of *Vibrio alginolyticus* by single-strain probionts: *Lysinibacillus fusiformis* on TSA + 1.5% NaCl plates using agar-well diffusion assay: (a) SPS11; and (b) A2

Table 2
Diameter of inhibition zone (± size of well/colony growth) in mm by single strain probiotics (10^9 CFU mL^{-1}) against Vibrio alginolyticus *(10^7 CFU mL^{-1})*

	Zone of inhibition (mm)	
Probiont	Agar-well diffusion assay	Spot assay
Lysinibacillus fusiformis SPS11	19 ± 5	8 ± 6
Bacillus megaterium I24	immeasurable	8 ± 5
Lysinibacillus fusiformis A2	15 ± 5	10 ± 6



(a) (b) (c)

Figure 2. Compatibility assay done using agar-well diffusion method whereby the indicator strains: (a) *Bacillus megaterium* I24; (b) *Lysinibacillus fusiformis* A2; and (c) *Lysinibacillus fusiformis* SPS11, and the tester strains were labelled, respectively (*Note*. The rectangle box shows that the 3 strains showed no inhibiton zone when tested against each other)

Table 3
Absorbance (OD_{550}) of biofilm formed by various bacteria at each sampling time interval (hour)

Bacteria	Time interval (hour)				
	6	12	24	48	72
Control (TSB + 1.5% NaCl only)	0.132 ± 0.038^{b}	0.275 ± 0.029^{b}	0.213 ± 0.051^{d}	0.371 ± 0.113^{c}	0.151 ± 0.025^{c}
Lysinibacillus fusiformis SPS11	0.792 ± 0.163^{a}	1.240 ± 0.331^{a}	1.045 ± 0.012^{bc}	1.307 ± 0.591^{b}	0.632 ± 0.062^{ab}
Bacillus megaterium I24	0.533 ± 0.089^{a}	0.822 ± 0.257^{ab}	0.797 ± 0.261^{cd}	6.793 ± 1.990^{ad}	0.572 ± 0.030^{b}
Lysinibacillus fusiformis A2	0.574 ± 0.110^{a}	1.245 ± 0.381^{a}	0.523 ± 0.134^{cd}	0.805 ± 0.121^{bc}	0.581 ± 0.112^{b}
Mixed probiotic (*Lysinibacillus fusiformis* SPS11 + *Bacillus megaterium* I24 + *Lysinibacillus fusiformis* A2)	0.649 ± 0.082^{a}	1.312 ± 0.174^{a}	1.981 ± 0.492^{a}	8.693 ± 2.050^{a}	1.020 ± 0.304^{a}
Vibrio alginolyticus	0.651 ± 0.100^{a}	0.516 ± 0.119^{b}	1.651 ± 0.393^{ab}	7.577 ± 4.792^{a}	0.677 ± 0.162^{ab}

Note. All values are expressed as mean ± standard error. Within columns, different alphabets in superscript denotes significant difference (p<0.05)

analysed (Table 3). At 12 hours interval, the absorbance readings of the biofilm formed by the mixed probiotic (*Lysinibacillus fusiformis* SPS11 + *Bacillus megaterium* I24 + *Lysinibacillus fusiformis* A2) were significantly higher than *V. alginolyticus*. Biofilm formation in all probiont groups was increased at 12 hours interval except for *V. alginolyticus* which increased at 24 hours post incubation. The biofilm formation by mixed probiotic peaked at 48 hours, along with strain *B. megaterium* I24 and pathogen, *V. alginolyticus*. The absorbance reading for mixed probiotic (8.693 ± 2.050) was higher than the absorbance reading of *V. alginolyticus* (7.577 ± 4.792) at 48 hours.

Moreover, the absorbance reading of the mixed probiotic was significantly higher than single strains at 48h. Reduction of biofilm was observed in all treatments at 72 hours.

Preliminary *in vivo* Challenge in *Artemia* Culture

Survival Rate. Two different concentrations (10^6 and 10^8 CFU mL^{-1}) of mixed probiotic were given to *Artemia* and the survival of the *Artemia* after challenged with *V. alginolyticus* in the corresponding treatments were recorded and presented in Tables 4 and 5 as well as Figures 3 and 4, respectively.

Among the four treatment groups challenged with *V. alginolyticus*, *Artemia* treated with probiont *L. fusiformis* A2 (T9) as a single strain showed the highest survival (75.00 ± 5.00%), followed by *Artemia* treated with mixed probiotic (T10) and single strain *B. megaterium* I24 (T8) at 65.00 ± 0.00% and 62.67 ± 2.52%, respectively (Table 4, Figure 3). *Artemia* culture treated with single strain *L. fusiformis* SPS11 and challenged with *V. alginolyticus* (T7) showed the lowest survival at 50.00 ± 5.00%. The results showed significant differences ($p<0.05$) between the survival of *Artemia* treated with mixed probiotic (T10) and *Artemia* challenged with *V. alginolyticus* only (T6).

On the other hand, in *Artemia* cultures treated with 10^8 CFU mL^{-1} of probionts and challenged with *V. alginolyticus*, the highest survival (82.50 ± 3.53%) was observed in *Artemia* treated with *L. fusiformis* SPS11 (T17) (Table 5, Figure 4). This was closely followed by treatment with mixed probiotic (T20, 77.50 ± 3.53%) and thereafter, single strain *L. fusiformis* A2 treatment (T19, 65.00 ± 7.07%). Among the four challenge treatments, *Artemia* treated with single strain *B. megaterium* I24 recorded the lowest survival at 62.50±3.53%.

Table 4
Survival of Artemia *pre-treated with 10^6 CFU mL^{-1} single and mixed probionts and challenged with 10^6 CFU mL^{-1} Vibrio alginolyticus*

Treatments	Description	Survival (%)
T1	*Artemia* only (Control)	57.67 ± 2.52^{ed}
T2	*Lysinibacillus fusiformis* SPS11	57.57 ± 2.52^{ed}
T3	*Bacillus megaterium* I24	52.67 ± 2.52^{df}
T4	*Lysinibacillus fusiformis* A2	80.00 ± 0.00^{b}
T5	Mixed probiotic	90.00 ± 0.00^{a}
T6	*Vibrio alginolyticus*	47.67 ± 2.52^{f}
T7	*Lysinibacillus fusiformis* SPS11 + *Vibrio alginolyticus*	50.00 ± 5.00^{df}
T8	*Bacillus megaterium* I24 + *Vibrio alginolyticus*	62.67 ± 2.52^{ce}
T9	*Lysinibacillus fusiformis* A2 + *Vibrio alginolyticus*	75.00 ± 5.00^{b}
T10	Mixed probiotic + *Vibrio alginolyticus*	65.00 ± 0.00^{c}

Note. All values are expressed as mean ± standard error. Different alphabets in superscript represent significant differences between treatments ($p<0.05$)

The survival of *Artemia* cultured at the two different concentrations of mixed probiotic administered was compared. Results demonstrated that *Artemia* treated with 10^8 CFU mL^{-1} mixed probiotic (T20, 77.50 ± 3.53%) had higher survivability as compared to *Artemia* treated with 10^6 CFU mL^{-1} mixed probiotic (T10, 65.00 ± 0.00%) after challenged. Moreover, non-challenged *Artemia* supplemented with 10^6 and 10^8

Table 5
Survival of Artemia *pre-treated with 10^8 CFU mL^{-1} single and mixed probionts and challenged with 10^6 CFU mL^{-1} Vibrio alginolyticus*

Treatments	Description	Survival (%)
T11	*Artemia* only (Control)	42.50 ± 3.53^{d}
T12	*Lysinibacillus fusiformis* SPS11	47.50 ± 3.53^{d}
T13	*Bacillus megaterium* I24	52.50 ± 3.53^{d}
T14	*Lysinibacillus fusiformis* A2	47.50 ± 3.53^{d}
T15	Mixed probiotic	97.50 ± 3.53^{a}
T16	*Vibrio alginolyticus*	47.50 ± 3.53^{d}
T17	*Lysinibacillus fusiformis* SPS11 + *Vibrio alginolyticus*	82.50 ± 3.53^{b}
T18	*Bacillus megaterium* I24 + *Vibrio alginolyticus*	62.50 ± 3.53^{c}
T19	*Lysinibacillus fusiformis* A2 + *Vibrio alginolyticus*	65.00 ± 7.07^{c}
T20	Mixed probiotic + *Vibrio alginolyticus*	77.50 ± 3.5^{3}b

Note. All values are expressed as mean ± standard error. Different alphabets in superscript represent significant differences between treatments (p<0.05)

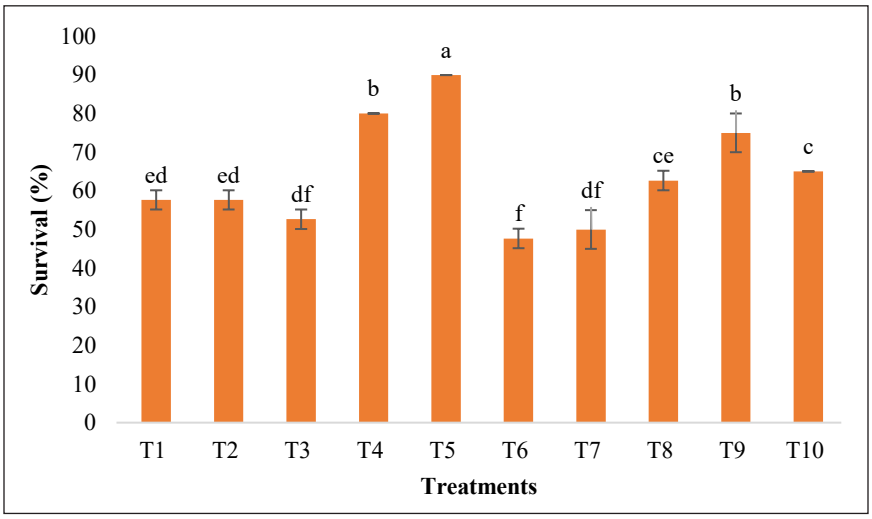


Figure 3. Survival of *Artemia* pre-treated with single and mixed probionts at 10^6 CFU mL^{-1} and challenged with 10^6 CFU mL^{-1} *Vibrio alginolyticus*. Error bars indicate standard error (SE). Different alphabets indicate significant differences among treatments (p<0.05). T1 (*Artemia* only), T2 (*Lysinibacillus fusiformis* SPS11), T3 (*Bacillus megaterium* I24), T4 (*Lysinibacillus fusiformis* A2), T5 (Mixed probiotic), T6 (*Vibrio alginolyticus*), T7 (*Lysinibacillus fusiformis* SPS11 + *Vibrio alginolyticus*), T8 (*Bacillus megaterium* I24 + *Vibrio alginolyticus*), T9 (*Lysinibacillus fusiformis* A2 + *Vibrio alginolyticus*), and T10 (Mixed probiotic + *Vibrio alginolyticus*)

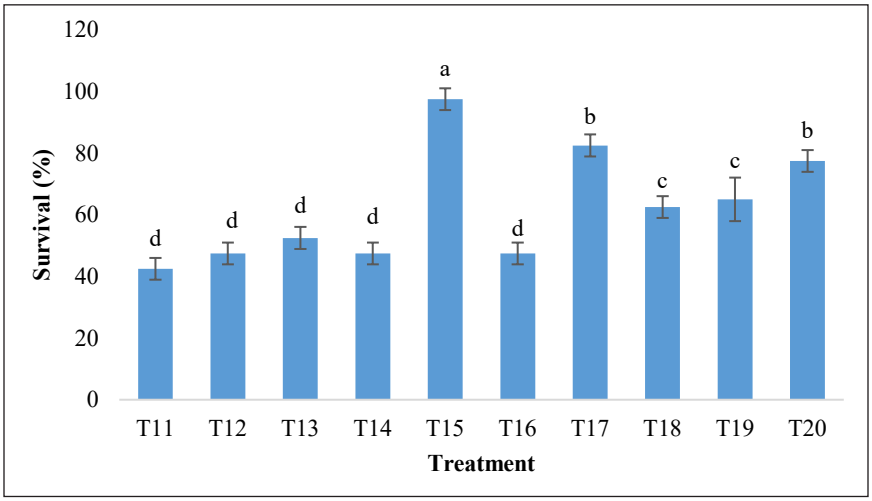


Figure 4. Survival of *Artemia* pre-treated with single and mixed probionts at 10^8 CFU mL^{-1} and challenged with 10^6 CFU mL^{-1} *Vibrio alginolyticus*. Error bars indicate standard error (SE). Different alphabets indicate significant differences among treatments ($p<0.05$). T11 (*Artemia* only), T12 (*Lysinibacillus fusiformis* SPS11), T13 (*Bacillus megaterium* I24), T14 (*Lysinibacillus fusiformis* A2), T15 (Mixed probiotic), T16 (*Vibrio alginolyticus*), T17 (*Lysinibacillus fusiformis* SPS11 + *Vibrio alginolyticus*), T18 (*Bacillus megaterium* I24 + *Vibrio alginolyticus*), T19 (*Lysinibacillus fusiformis* A2 + *Vibrio alginolyticus*), and T20 (Mixed probiotic + *Vibrio alginolyticus*)

Table 6
Vibrio *counts in* Artemia *pre-treated with 10^6 CFU mL^{-1} single and mixed. probiont and challenged with 10^6 CFU mL^{-1}* Vibrio alginolyticus

Treatments	Description	Log10 CFU mL^{-1}
T6	*Vibrio alginolyticus*	3.37 ± 0.64^{b}
T7	*Lysinibacillus fusiformis* SPS11 + *Vibrio alginolyticus*	4.57 ± 0.53^{a}
T8	*Bacillus megaterium* I24 + *Vibrio alginolyticus*	4.14 ± 0.18^{ab}
T9	*Lysinibacillus fusiformis* A2 + *Vibrio alginolyticus*	3.46 ± 0.45^{ab}
T10	Mixed probiotic + *Vibrio alginolyticus*	3.75 ± 0.18^{ab}

Note. All values are expressed as mean ±standard error. Different alphabets in superscript represent significant differences between treatments ($p<0.05$)

CFU mL^{-1} of mixed probiotic only (T5 and T15) showed the highest survival among all the other treatments.

Vibrio* Counts in *Artemia. There was no reduction of *Vibrio* loads in the *Artemia* cultures across the treatments with probionts (10^6 CFU mL^{-1}) excluding group T7. Across the four probiotic treatments, *Vibrio* loads peaked in *Artemia* culture immersed with single strain *L. fusiformis* SPS11 (T7) at Log10 4.57 ± 0.53. The increase of *Vibrio* in T7 was also significantly different ($p<0.05$) to T6. There were no significant differences ($p<0.05$) in the *Vibrio* loads in *Artemia* treated with 10^6 CFU mL^{-1} of mixed probiotic (T10) and single strain probiotics (T8 and T9) (Table 6).

On the other hand, there was significant reduction (p<0.05) in *Vibrio* loads in *Artemia* cultures immersed in mixed probiotic (T20) at concentration of 10^8 CFU mL^{-1}as compared to *Artemia* cultures with *V. alginolyticus* only (T16) (Table 7). Mixed probiotic (T20) treatment resulted in a lower *Vibrio* loads at Log10 1.60 ± 0.52 compared to Log10 2.43 ± 0.12 in *Artemia* cultures challenged with *V. alginolyticus* (T16) only. Among all treatments treated with probionts, only group T18 showed no significant reduction of *Vibrio* loads compared with T16. There was no colony growth in *Artemia* treated with *L. fusiformis* SPS11 (T17).

***Vibrio* Counts in Culture Water.** In the culture water collected from *Artemia* cultures at 10^6 CFU mL^{-1} of probionts (T7-T10), there were no significant reduction of *Vibrio* loads compared to culture water with *V. alginolyticus* only (T6) (Table 8).

In contrast, there was a significant reduction (p<0.05) of *Vibrio* loads in culture water collected from *Artemia* cultures treated with 10^8 CFU mL^{-1} mixed probiotic (T20) as compared to culture

Table 7
Vibrio *count in* Artemia *pre-treated with 10^8 CFU mL^{-1} probiotics and challenged with 10^6 CFU mL^{-1}* Vibrio alginolyticus

Treatments	Description	Log10 CFU mL^{-1}
T16	*Vibrio alginolyticus* (10^6 CFU mL^{-1})	2.43 ± 0.12[a]
T17	*Lysinibacillus fusiformis* SPS11 (10^8 CFU mL^{-1}) + *Vibrio alginolyticus* (10^6 CFU mL^{-1})	-
T18	*Bacillus megaterium* I24 (10^8 CFU mL^{-1}) + *Vibrio alginolyticus* (10^6 CFU mL^{-1})	3.09 ± 0.10[a]
T19	*Lysinibacillus fusiformis* A2 (10^8 CFU mL^{-1}) + *Vibrio alginolyticus* (10^6 CFU mL^{-1})	1.62 ± 0.15[b]
T20	Mixed probiotic (10^8 CFU mL^{-1}) + *Vibrio alginolyticus* (10^6 CFU mL^{-1})	1.60 ± 0.52[b]

Note. All values are expressed as mean ± standard error. Different alphabets in superscript represent significant differences between treatments (p<0.05)

Table 8
Vibrio *count in culture water pre-treated with 10^6 CFU mL^{-1} single and mixed probiont and challenged with 10^6 CFU mL^{-1}* Vibrio alginolyticus

Treatments	Description	Log10 CFU mL^{-1}
T6	*Vibrio alginolyticus*	4.56 ± 0.30[a]
T7	*Lysinibacillus fusiformis* SPS11 + *Vibrio alginolyticus*	4.39 ± 0.33[a]
T8	*Bacillus megaterium* I24 + *Vibrio alginolyticus*	4.47 ± 0.86[a]
T9	*Lysinibacillus fusiformis* A2 + *Vibrio alginolyticus*	3.85 ± 0.52[a]
T10	Mixed probiotic + *Vibrio alginolyticus*	4.38 ± 0.60[a]

Note. All values are expressed as mean ± standard error. Different alphabets in superscript represent significant differences between treatments (p<0.05)

Table 9
Vibrio *count in culture water pre-treated with 10^8 CFU mL^{-1} single and mixed probiont and challenged with 10^6 CFU mL^{-1}* Vibrio alginolyticus

Treatments	Description	Log10 CFU mL^{-1}
T16	*Vibrio alginolyticus*	5.57 ± 0.06^{a}
T17	*Lysinibacillus fusiformis* SPS11 + *Vibrio alginolyticus*	3.48 ± 0.31^{c}
T18	*Bacillus megaterium* I24 + *Vibrio alginolyticus*	3.89 ± 0.26^{c}
T19	*Lysinibacillus fusiformis* A2 + *Vibrio alginolyticus*	5.00 ± 0.21^{ab}
T20	Mixed probiotic + *Vibrio alginolyticus*	4.90 ± 0.10^{b}

Note. All values are expressed as mean ± standard error. Different alphabets in superscript represent significant differences between treatments ($p<0.05$)

water with pathogen only (T16) (Table 9). The culture water from the mixed probiotic treatment T20 resulted in a lower *Vibrio* loads (Log10 4.90 ± 0.10) compared with *Artemia* challenged with pathogen only, T16 (Log10 5.57 ± 0.06). Significant reduction ($p<0.05$) of *Vibrio* was also demonstrated in treatments T17 and T18.

DISCUSSION

In the present study, isolated probiont strains *L. fusiformis* SPS11, A2, and *B. megaterium* I24 able to inhibit pathogenic *V. alginolyticus* when tested via *in-vitro* antimicrobial assay using agar well diffusion and spot assay. Furthermore, a reduction in *Vibrio* counts was recorded in the culture water collected from treatments with 10^8 CFU mL^{-1} probionts when tested *in vivo*. These suggest that the probiont strains may have the ability to produce or secrete antibacterial compounds or inhibitory substances that are antagonistic towards *V. alginolyticus*. As aforementioned, the production of inhibitory compounds is one of the modes of actions of probiotics. Extracellular substances such as bacteriocins, hydrogen peroxide, siderophores, lysozymes, and proteases released by probionts may have antagonistic consequences on another microflora. Additionally, the production of acids, like lactic acid, by probionts may decrease gut pH of aquatic species, thwarting the proliferation of pathogens (Zorriehzahra et al., 2016).

Lysinibacillus fusiformis is a gram-positive, rod-shaped, lysine producing bacteria belonging to the genus *Lysinibacillus*, in the family of *Bacillaceae* (Abideen & Babuselvam, 2014). They are generally encountered in plant soil but have been identified in plant tissues (Melnick et al., 2011), fermented plant seed products (Parkouda et al., 2010) and puffer fish liver samples (Wang et al., 2010). A study by Ahmad et al. (2014) reported that bacteriocin produced by *L. fusiformis* can counteract a wide variety of foodborne bacteria and fungi and had the potential to be used as a substitutive disease control tool against pathogenic microbes. This is supported in a separate study by Adebo et al. (2016) which documented that extracellular proteins in a series of bacterial cells including *L. fusiformis*, had the ability to breakdown and detoxify toxic metabolites

in contaminated food and feed materials. *In vivo* study conducted also endorses the result that *L. fusiformis* may releases extracellular substances that are effective against *V. alginolyticus*. *Vibrio* count in culture water treated with 10^8 CFU mL^{-1} *L. fusiformis* SPS11 revealed a significant decrease in colonies.

Bacillus megaterium belong to the genus *Bacillus*, in the family of Bacillaceae. *Bacillus megaterium* is a large, gram-positive and rod-shaped, predominantly aerobic spore-forming bacteria found in various environments (Vary et al., 2007). Al-Thubiani et al. (2018) identified a compound originating from *B. megaterium* with an extensive range of antimicrobial action towards both gram-positive and negative bacteria. In addition, a study by Jasmin et al. (2016) established that *B. megaterium* can inhibit the growth of *Vibrio* spp. in solid and liquid *in vitro* conditions. This is also supported in this study, both in *in vitro* and in *in vivo*. Significant reduction in the number of *Vibrio* was recorded in culture water treated with 10^8 CFU mL^{-1} of *B. megaterium*.

Bacillus species are known to secrete a variety of extracellular compounds targeting a wide spectrum of pathogens (Yilmaz et al., 2006). A study by Amin et al. (2015) endorsed the theory, demonstrating that several *Bacillus* species had the inherent ability to generate antimicrobial substances effective in containing diseases. Luis-Villaseñor et al. (2011) isolated *Bacillus* sp. from the intestine of shrimp with antagonistic activity against *Vibrio* spp. In a similar study, *Bacillus* spp. obtained from the gastrointestinal tract of white shrimp (*Litopennaeus vannamei*) exhibited antimicrobial activity against *Vibrio parahaemolyticus* (Liu et al., 2014). It is evident that both bacterial species in this study (*Bacillus megaterium* and *Lysinibacillus fusiformis*) showed functionality as probiotic. Previous study that applied rice bran fermented with both *Bacillus* and *Lysinibacillus* improved the growth performance and survival of Pacific white shrimp (*Penaeus monodon*) (Liñan-Vidriales et al., 2020). Hence the combination of both *Bacillus* and *Lysinibacillus* in a mixed probiotic could be explored further for its effectiveness in different aspect of fish or shrimp culture.

The quantification of biofilm formation by the respective probionts as a mixed probiotic in this study showed the ability of the strains to effectively form biofilm. Biofilm is the aggregation of microbial cells on a surface that cannot dislodge with delicate washing (Donlan, 2002). The formation of biofilm by potential probionts served as an indication of their capability to possibly adhere themselves to the intestinal mucosa of aquatic species. Since pathogens require attachment to the gut mucosa to bring about negative impacts, adhesion by probionts to gut epithelial cells and intestinal mucus may serve as a form of competition and henceforth ultimately preventing the colonisation of pathogenic bacteria in the host (Lee & Salminen, 2009). Furthermore, adhesion ability to intestinal walls is also considered criteria for probiotics to regulate immunity of host.

The biofilm formation assay conducted in this study revealed that all potential probionts were able to form biofilms. Absorbance readings exceeding the value of one indicated high adherence (Zhao, 2014) of the probionts, and potential for biofilm production and efficient competition with pathogen *V. alginolyticus* for adhesion sites in the gut. This study had also revealed that attachment abilities of the probionts are improved when formulated as a mixed probiotic. As mentioned in the previous section, the quantification of biofilm is correlated to the attachment ability of probiotics. In this study, the highest absorbance reading for the mixed probiotic was recorded at 48 hours (8.693 ± 2.050) post-incubation. This reading was also the highest as compared to single strain probiotics and pathogen, *V. algnolyticus*. This is an indication that the mixed probiotic is profoundly adherent (Zhao, 2014) and could potentially outcompete *V. alginolyticus* for adhesion sites in the gastrointestinal tract.

Furthermore, the absorbance reading of mixed probiotic was maintained at a value above one (1.020 ± 0.304) even after 72 hours, whereas the absorbance of single-strain probiotics decreased below value one after 72 hours. The effectiveness of *B. subtilis* supplemented to *Artemia franciscana* which showed an increased in survival rate after challenged with *Vibrio angullarium* is further supported by its high biofilm forming capability (Zoumpourtikoudi et al., 2018). This is similar to the effects shown by the mixed probiotic in this study.

A study on the efficacy of mixed *Bacillus* probiotics on early development of white shrimp by Nimrat et al. (2012) reported that the vast improvement of developmental and survival rates of postlarvae shrimp were associated to the establishment of mixed *Bacillus* probiotics in the gut. The results were in line with studies carried out by Boonthai et al. (2011) which observed an increase in *Bacillus* spp. in the hepatopancreas and intestine of black tiger prawns (*Penaeus monodon*) after feeding with mixed *Bacillus* probiotics (*Bacillus subtilis*, *B. megaterium*, and *B. thuringiensis*), proving the proficiency of mixed probiotics to propagate in digestive tracts.

The ability of mixed probiotic to form better biofilms may be attributed to the synergistic effects generated by each individual strain. The formation of biofilm relies on the interactions between bacterial species by intraspecies signalling, interspecies communications or chemical cues (Gallegos-Monterrosa et al., 2017). For example, aggregation of *Lactobacillus paracasei* strains and *Saccharomyces cerevisiae* was intensified when cultured together as a result of the interactions between the proteins on cell surface of *L. paracasei* and the polysaccharides in *S. cerevisiae* (Xie et al., 2011). Therefore, the adhesion of probiotics to intestinal wall of aquatic species could improve with the supplementation of multi-species probiotic supplement. However, it is important to note that the actual mechanism of biofilms and the interactions of probionts in this study

is still relatively unexplored and would require further studies to draw conclusions. Since the mixed probiotic in our study was able to produce positive results in the biofilm assay, it is possible that the mixed probiotic can serve as a strong competitor for attachment sites in the intestinal mucosa of aquatic species as compared to pathogen, *V. alginolyticus*.

The performance of the probionts in *in vitro* conditions may not coincide with *in vivo* conditions (Kesarcodi-Watson et al., 2008); hence, *Artemia* was used in preliminary *in vivo* challenge test against *V. alginolyticus* to assess the effectiveness of the mixed probiotic as compared to single strain probiotics. The treatment of *Artemia* cultures with 10^6 CFU mL^{-1} probionts showed that the highest survival rate is observed in single strain treatment of *L. fusiformis* A2 at 75.00 ± 5.00%. The higher effectiveness of a single strain (*L. fusiformis* A2) than mixed strain could only be observed when a comparative evaluation is done, such as the one conducted in this study. Hence, to evaluate the effectiveness of mixed probiotic, one of the main criteria that should be focused on is the comparative evaluation with its constituent single-strain. Comparative evaluation is important to highlight the functionality of mixed probiotics in comparison with single-probiotic and also to determine whether mixed probiotics are indeed better than single-strain probiotics. *Artemia* in mixed probiotic treatment showed the second highest survival rate at 65.00 ± 0.00%. The competency of *L. fusiformis* A2 in producing the culture with the highest *Artemia* survival rates is in line with study conducted on *Bacillus* spp. as potential probiotics in pacific white shrimp. Guo et al. (2009) reported that supplementing shrimps with *Bacillus fusiformis* at a dose as low as 10^5 CFU mL^{-1} could increase survival.

The mixed probiotic applied at both concentrations of 10^6 and 10^8 CFU mL^{-1} did not produce the highest survival rate when challenged with *V. alginolyticus*, among the treatment groups. This may be due to the low concentration of mixed probiotic, resulting in increased residue in the culture water rather than the transfer of potential benefits to the *Artemia*. Nonetheless, the survival rate of *Artemia* in mixed probiotic treatment was still significantly ($p<0.05$) higher than the survival rate of *Artemia* without probiotic treatment. On the contrary, the unchallenged *Artemia* fed with the mixed probiotic at both concentrations (T5 and T15) showed the highest survival rate, 90.00 ± 0.00% and 97.50 ± 3.53% in comparison to *Artemia* supplemented with single strain probiotic only. This is contradicting to research findings by Touraki et al. (2012) who observed a decrease in survival of *Artemia* nauplii fed with *Bacillus subtilis* and *Lactobacillus plantarum*. Supplementing the mixed probiotic to *Artemia* might not necessarily confer benefits in terms of disease control. Based on the high survival of *Artemia* fed with mixed probiotic recorded in this study, it suggests that this particular mix of probiotics could be bioencapsulated in *Artemia* and fed to the host for improvement of growth, feeding parameters and immune response (Jafaryan et al., 2010).

Survival rates of *Artemia* across all treatments challenged with 10^6 CFU mL^{-1} *V. alginolyticus* was higher as compared to *Artemia* without probiotic treatments. The results from our study is in line with studies by Nimrat et al. (2012), which recommended that a combination of *Bacillus* probiotics given at 10^9 CFU mL^{-1} would notably enhance growth performance and survival rates of white shrimps. Furthermore, improved immunity and resistance against *Aeromonas hydrophila* was observed in rohu (*Labeo rohita*) provided with 10^8 CFU g^{-1} $diet^{-1}$ probiotic (Giri et al., 2013).

Although the survival rates of *Artemia* fed with single probiont only in treatment T12 (*Lysinibacillus fusiformis* SPS11), T13 (*Bacillus megaterium* I24), and T14 (*Lysinibacillus fusiformis* A2) were lower than *Artemia* challenged with pathogen only in T16 (*Vibrio alginolyticus*), the difference was not significant. Furthermore, the survival of *Artemia* fed with single-strain probiont were found to be significantly lower than *Artemia* fed with probiotics and challenged with *V. alginolyticus* in treatment T17 (*Lysinibacillus fusiformis* SPS11 + *Vibrio algnilyticus*), T18 (*Bacillus megaterium* I24 + *Vibrio alginolyticus*) and T19 (*Lysinibacillus fusiformis* A2 + *Vibrio alginolyticus*). This could be due to the mode of action of the supplemented probiotics. One possible explanation on the high survival of *Artemia* fed with probiotics and challenged with pathogen could be due to the competitive inhibition which causes aggressive hindrance for attachment site on intestinal epithelial layer (Chauhan & Singh, 2019). Antagonism mechanism is offered by probiont for the purpose of colonization and competition with pathogen (Verschuere et al., 2000). Hence, in this aspect, the high survival of the treatment groups could be caused by the probiotics action to defend the gut flora from pathogen (Skjermo & Vadstein, 1999). Probiotics could have utilized all the available nutrients which restrict the presence of pathogen due to unavailability of nutrients to survive (Chauhan & Singh, 2019).

Comparison of two different concentration of mixed probiotic showed that higher dosage of mixed probiotic (10^8 CFU mL^{-1}) administered had significantly ($p<0.05$) higher *Artemia* survival rates in comparison to the group with pathogen only. The direct correlation in survival rates of *Artemia* and concentration of probionts in this study was also documented by Jasmin et al. (2016), which stated that the survival of *Artemia* rose with the increase in concentration of probiotic administered.

In view of attachment and colonisation of the gut as a mode of action of probiotics, the quantification of *V. alginolyticus* in *Artemia* was studied. Successful attachment of probiotics in the organism would be indicated by the reduction in *Vibrio* count on TCBS agar. In *Artemia* cultures treated with 10^6 CFU mL^{-1}, there was no reduction in the *V. alginolytius* load in *Artemia* from all treatments. Instead, elevated *Vibrio* counts were recorded. A study conducted by Interaminense et al. (2018) on the probiotic effects of *B. subtilis* and *Shewanella algae* also noted that *Vibrio*

counts in the intestine and faeces of pacific white shrimp (*L. vannamei*) increased during probiotic treatment. The low concentration of single strain probiotics and mixed probiotic administered to the *Artemia* cultures may be the reason for the failure of the probionts to act as selective pressure in the gastrointestinal tract of the *Artemia*, hence decreasing the ability to adhere to intestinal mucosa. However, it is good to note that despite the increase in *Vibrio* counts in treated *Artemia* in contrast to non-treated *Artemia*, the survival rate remains higher in *Artemia* cultures supplied with probionts. This may indicate an underlying factor conferring increased immunity and resistance against *V. alginolyticus* which would require further research.

On the contrary, it was observed that there was a decreased in *Vibrio* loads in *Artemia* treated with 10^8 CFU mL^{-1} of both single and mixed probiotic respectively. Significant ($p<0.05$) reduction of *Vibrio* was recorded in *Artemia* cultured with the mixed probiotic. This may signify that higher concentration of probionts in the mixed probiotic was able to outcompete pathogenic *V. alginolyticus* for adhesion sites in the gut, as well as successfully establishing themselves in *Artemia*. The use of commercial probiotics to control a series of pathogenic bacteria in *Artemia* cultures have proven that pathogenic bacterial load in *Artemia* can be reduced (Haq et al., 2012), thereby, supporting the results in the present study. In culture water from 10^8 CFU mL^{-1} mixed probiotic treatment, significant reduction in *Vibrio* counts was recorded. The reduction of *Vibrio* in culture waters treated with mixed probiotic was frequently reported in studies (Boonthai et al., 2011; Ferreira et al., 2017). Choosing an optimal concentration of a suitable probiotic is important to offer protection to *Artemia* (Touraki et al., 2012).

The reduction in pathogenic *Vibrio* loads in culture waters attributing to the mixed probiotic treatment may be beneficial to the survival of *Artemia.* Since there is a reduction in pathogenic bacteria in culture water, it can be assumed that the probability of infection would be reduced as well. This may also explain the reduction of *Vibrio* counts in *Artemia* culture at 10^8 CFU mL^{-1}.

CONCLUSION

In conclusion, the results suggested that mixed bacterial strains in this study have substantial potential as probiotics against *Vibrio alginolyticus* infection. The mixed probiotics demonstrated antagonism and biofilm activity in *in vitro* study. Moreover, in *in vivo* study, the mixed probiotic was able to confer protections towards *Artemia* and reduced the number of *Vibrio* loads in *Artemia* and culture water. However, it is crucial to note that the mixed probiotic is only more effective when used at a higher dose as compared to a lower dose.

ACKNOWLEDGEMENT

This research was supported by Universiti Putra Malaysia under High Performance Individual Research Grants UPM/700-2/1/GPB/2017/9553100, Ministry of Higher Education Malaysia (MOHE) through

SATREPS JICA-JST COSMOS 2016-2021 and Long Research Grant Scheme (LRGS) by Ministry of Education Malaysia, LRGS/1/2019/UPM//1.

REFERENCES

Abideen, S., & Babuselvam, M. (2014). Antagonistic activity of *Lysinibacillus fusiformis* n 139 strain isolated from marine fish *Triacanthus strigilifer* and genome sequence. *International Journal of Current Microbiology and Applied Sciences*, *3*(4), 1066-1072.

Adebo, O., Njobeh, P., & Mavumengwana, V. (2016). Degradation and detoxification of AFB_1 by *Staphylococococcus warneri*, *Sporosarcina* sp. and *Lysinibacillus fusiformis*. *Food Control*, *68*, 92-96. https://doi.org/10.1016/j.foodcont.2016.03.021

Ahmad, V., Muhammad Zafar Iqbal, A., Haseeb, M., & Khan, M. (2014). Antimicrobial potential of bacteriocin producing *Lysinibacillus jx416856* against foodborne bacterial and fungal pathogens, isolated from fruits and vegetable waste. *Anaerobe*, *27*, 87-95. http://doi.org/10.1016/j.anaerobe.2014.04.001

Al-Thubiani, A., Maher, Y., Fathi, A., Abourehab, M., Alarjah, M., Khan, M., & Al- Ghamdi, S. (2018). Identification and characterization of a novel antimicrobial peptide compound produced by *Bacillus megaterium* strain isolated from oral microflora. *Saudi Pharmaceutical Journal*, *26*(8), 1089-1097. https://doi.org/10.1016/j.jsps.2018.05.019

Amin, M., Rakhisi, Z., & Zarei Ahmady, A. (2015). Isolation and identification of *Bacillus* species from soil and evaluation of their antibacterial properties. *Avicenna Journal of Clinical Microbiology and Infection*, *2*(1), 23233. https://doi.org/10.17795/ajcmi-23233

Boonthai, T., Vuthiphandchai, V., & Nimrat, S. (2011). Probiotic bacteria effects on growth and bacterial composition of black tiger shrimp (*Penaeus monodon*). *Aquaculture Nutrition*, *17*(6), 634–644. https://doi.org/10.1111/j.1365-2095.2011.00865.x

Bruhn, J. B., Gram, L., & Belas, R. (2007). Production of antibacterial compounds and biofilm formation by *Roseobacter* species are influenced by culture conditions. *Applied and Environmental Microbiology*, *73*(2), 442-450. https://doi.org/10.1128/AEM.02238-06

Chauhan, A., & Rahul, S. (2019). Probiotics in aquaculture: A promising emerging alternative approach. *Symbiosis*, *77*(2), 99-113. https://doi.org/10.1007/s13199-018-0580-1

Chiu, T., Kao, L., & Chen, M. (2013). Antibiotic resistance and molecular typing of *Photobacterium damselae* subsp. *damselae*, isolated from seafood. *Journal of Applied Microbiology*, *114*(4), 1184-1192. https://doi.org/10.1111/jam.12104

Donlan, R. M. (2002). Biofilms: Microbial life on surfaces. *Emerging Infectious Diseases*, *8*(9), 881–890. https://doi.org/10.3201/eid0809.020063

Ferreira, M. G. P., Melo, F. P., Lima, J. P. V., Andrade, H. A., Severi, W., & Correia, E. S. (2017). Bioremediation and biocontrol of commercial probiotic in marine shrimp culture with biofloc. *Latin American Journal of Aquatic Research*, *45*(1), 167–176.

Fingerman, M. (Ed.). (2003). *Recent advances in marine biotechnology: Molecular genetics of marine organisms* (Vol. 10). CRC Press.

Food and Agriculture Organization. (2018). *The state of world fisheries and aquaculture 2018 - Meeting the sustainable development goals*. FAO. https://tethys.pnnl.gov/publications/

state-world-fisheries-aquaculture-2018-meeting-sustainable-development-goals

Gallegos-Monterrosa, R., Kankel, S., Götze, S., Barnett, R., Stallforth, P., & Kovács, Á. T. (2017). *Lysinibacillus fusiformis* M5 induces increased complexity in *Bacillus subtilis* 168 colony biofilms via hypoxanthine. *Journal of Bacteriology*, *199*, e00204-17. https://doi.org/10.1128/JB.00204-17

Giri, S. S., Sukumaran, V., & Oviya, M. (2013). Potential probiotic *Lactobacillus plantarum* VSG3 improves the growth, immunity, and disease resistance of tropical freshwater fish, *Labeo rohita*. *Fish and Shellfish Immunology*, *34*(2), 660–666. http://doi.org/10.1016/j.fsi.2012.12.008

Guo, J.-J., Liu, K.-F., Cheng, S.-H., Chang, C.-I., Lay, J.-J., Hsu, Y.-O., Yang, J.-Y., & Chen, T.-I. (2009). Selection of probiotic bacteria for use in shrimp larviculture. *Aquaculture Research*, *40*(5), 609–618. https://doi.org/10.1111/j.1365-2109.2008.02140.x

Haq, M. B., Vijayasanthi, P., Vignesh, R., Shalini, R., Chakraborty, S., & Rajaram, R. (2012). Effect of probiotics against marine pathogenic bacteria on *Artemia franciscana*. *Journal of Applied Pharmaceutical Science*, *2*(4), 38-43.

Interaminense, J. A., Vogeley, J. L., Gouveia, C. K., Portela, R. W., Oliveira, J. P., Andrade, H. A., Silvio, M. P., Roberta, B. S., Diego, S., & Bezerra, R. S. (2018). *In vitro* and *in vivo* potential probiotic activity of *Bacillus subtilis* and *Shewanella algae* for use in *Litopenaeus vannamei* rearing. *Aquaculture*, *488*, 114–122. https://doi.org/10.1016/j.aquaculture.2018.01.027

Jafaryan, H., Mehdi, T. M., & Mohammad, M. N. (2010). The effects of probiotic *Bacillus* for promotion of growth and feeding parameters in beluga (*Huso huso*) larvae via feeding by bioencapsulated *Artemia*. *Aquaculture, Aquarium, Conservation and Legislation*, *3*(4), 273-280.

Jasmin, M. Y., Wagaman, H., Yin, T. A., Inasalwany, M. Y., Daud, H. M., & Karim, M. (2016). Screening and evaluation of local bacteria isolated from shellfish as potential probiotics against pathogenic Vibrios. *Journal of Environmental Biology*, *37*(4), 801–809.

Kesarcodi-Watson, A., Kaspar, H., Lategan, M., & Gibson, L. (2008). Probiotics in aquaculture: The need, principles and mechanisms of action and screening processes. *Aquaculture*, *274*(1), 1-14. https://doi.org/10.1016/j.aquaculture.2007.11.019

Kumar, S., Lekshmi, M., Parvathi, A., Nayak, B., & Varela, M. (2016). Antibiotic resistance in seafood-borne pathogens. In O. V. Singh (Ed.), *Foodborne pathogens and antibiotic resistance* (pp. 397-415). Wiley. https://doi.org/10.1002/9781119139188.ch17

Lee, Y. K., & Salminen, S. (Eds.) (2009). *Handbook of probiotics and prebiotics* (2nd ed.). Wiley. https://doi.org/10.1002/9780470432624

Liñan-Vidriales, M. A., Peña-Rodríguez, A., Tovar-Ramírez, D., Elizondo-González, R., Barajas-Sandoval, D. R., Ponce-Gracía, E. I., Rodríguez-Jaramillo, C., Rodríguez-Jaramillo, J. L., & Quiroz-Guzmán, E. (2020). Effect of rice bran fermented with *Bacillus* and *Lysinibacillus* species on dynamic microbial activity of Pacific white shrimp (*Penaeus vannamei*). *Aquaculture*, *531*, 735958. https://doi.org/10.1016/j.aquaculture.2020.735958

Liu, H., Li, Z., Tan, B., Lao, Y., Duan, Z., Sun, W., & Dong, X. (2014). Isolation of a putative probiotic strain S12 and its effect on growth performance, nonspecific immunity and disease-resistance of white shrimp, *Litopenaeus vannamei*. *Fish and Shellfish Immunology*, *41*(2), 300-307. http://doi.org/10.1016/j.fsi.2014.08.028

Luis-Villaseñor, I. E., Macías-Rodríguez, M. E., Gómez-Gil, B., Ascencio-Valle, F., & Campa-Córdova, Á. I. (2011). Beneficial effects of four *Bacillus* strains on the larval cultivation of *Litopenaeus vannamei*. *Aquaculture*, *321*(1-2), 136-144. https://doi.org/10.1016/j.aquaculture.2011.08.036

Marques, A., Dinh, T., Ioakeimidis, C., Huys, G., Swings, J., Verstraete, W., Dhont, J., Sorgeloos, P., & Bossier, P (2005). Effects of bacteria on *Artemia franciscana* cultured in different gnotobiotic environments. *Applied and Environmental Microbiology*, *71*(8), 4307-4317. https://doi.org/10.1128/AEM.71.8.4307-4317.2005

Melnick, R., Suárez, C., Bailey, B., & Backman, P. (2011). Isolation of endophytic endospore-forming bacteria from *Theobroma cacao* as potential biological control agents of cacao diseases. *Biological Control*, *57*(3), 236-245. http://doi.org/10.1016/j.biocontrol.2011.03.005

Nimrat, S., Suksawat, S., Boonthai, T., & Vuthiphandchai, V. (2012). Potential *Bacillus* probiotics enhance bacterial numbers, water quality and growth during early development of white shrimp (*Litopenaeus vannamei*). *Veterinary Microbiology*, *159*(3-4), 443–450. http://doi.org/10.1016/j.vetmic.2012.04.029

Parkouda, C., Thorsen, L., Compaoré, C., Nielsen, D., Tano-Debrah, K., & Jensen, J., Diawara, B., & Jakobsen, M. (2010). Microorganisms associated with Maari, a Baobab seed fermented product. *International Journal of Food Microbiology*, *142*(3), 292-301. http://doi.org/10.1016/j.ijfoodmicro.2010.07.004

Patra, S., & Mohamed, K. (2003). Enrichment of *Artemia* nauplii with the probiotic yeast *Saccharomyces boulardii* and its resistance against a pathogenic *Vibrio*. *Aquaculture International*, *11*(5), 505-514. https://doi.org/10.1023/b:aqui.0000004193.40039.54

Rengpipat, S., Rueangruklikhit, T., & Piyatiratitivorakul, S. (2008). Evaluations of lactic acid bacteria as probiotics for juvenile seabass *Lates calcarif*er. *Aquaculture Research*, *39*(2), 134-143. https://doi.org/10.1111/j.1365-2109.2007.01864.x

Rosland, N. A. (2018). *Evaluation of potential probiotic bacteria for microalgae propagation and* Artemia franciscana *(Kellog, 1906) bioencapsulation*. [Unpublished Master's thesis]. Universiti Putra Malaysia.

Seenivasan, C., Saravana-Bhavan, P., Radhakrishnan, S., & Shanthi, R. (2012). Enrichment of *Artemia* nauplii with *Lactobacillus sporogenes* for enhancing the survival, growth and levels of biochemical constituents in the post larvae of the freshwater prawn *Macrobrachium rosenbergii*. *Turkish Journal of Fisheries and Aquatic Sciences*, *12*(1).

Shefat, S. H. T. (2018). Probiotic strains used in aquaculture. *International Research Journal of Microbiology*, *7*(2), 43-55. http:/doi.org/10.14303/irjm.2018.023

Skjermo, J., & Vadstein, O. (1999). Techniques for microbial control in the intensive rearing of marine larvae. *Aquaculture*, *177*(1-4), 333-343. https://doi.org/10.1016/S0044-8486(99)00096-4

Tagg, J., & McGiven, A. (1971). Assay system for bacteriocins. *Applied Microbiology*, *21*(5), 943.

Timmerman, H. M., Koning, C. J. M., Mulder, L., Rombouts, F. M., & Beynen, A. C. (2004). Monostrain, multistrain and multispecies probiotics — A comparison of functionality and efficacy. *International Journal of Food Microbiology*, *96*(3), 219-233. https://doi.org/10.1016/j.ijfoodmicro.2004.05.012

Touraki, M., Karamanlidou, G., Karavida, P., & Chrysi, K. (2012). Evaluation of the probiotics *Bacillus subtilis* and *Lactobacillus plantarum* bioencapsulated in *Artemia* nauplii against vibriosis in European sea bass larvae

(*Dicentrarchus labrax*, L.). *World Journal of Microbiology and Biotechnology*, *28*(6), 2425-2433. https://doi.org/10.1007/s11274-012-1052-z

Vary, P. S., Biedendieck, R., Fuerch, T., Meinhardt, F., Rohde, M., Deckwer, W.-D., & Jahn, D. (2007). *Bacillus megaterium* — From simple soil bacterium to industrial protein production host. *Applied Microbiology and Biotechnology*, *76*(5), 957-967. https://doi.org/10.1007/s00253-007-1089-3

Verschuere, L., Rombaut, G., Sorgeloos, P., & Verstraete, W. (2000). Probiotic bacteria as biological control agents in aquaculture. *Microbiology and Molecular Biology Reviews*, *64*(4), 655-671. https://doi.org/10.1128/mmbr.64.4.655-671.2000

Wang, J., Fan, Y., & Yao, Z. (2010). Isolation of a *Lysinibacillus fusiformis* strain with tetrodotoxin-producing ability from puffer fish *Fugu obscurus* and the characterization of this strain. *Toxicon*, *56*(4), 640-643. http://doi.org/10.1016/j.toxicon.2010.05.011

Wang, J., Woo, M., & Yan, C. (2017). Spot plating assay for the determination of survival and plating efficiency of *Escherichia coli* in sub-MIC levels of antibiotics. *Journal of Experimental Microbiology and Immunology*, *1*, 26-29.

Xie, N., Zhou, T., & Li, B. (2011). Kefir yeasts enhance probiotic potentials of *Lactobacillus paracasei* H9: The positive effects of coaggregation between the two strains. *Food Research International*, *45*(1), 394–401. http://doi.org/10.1016/j.foodres.2011.10.045

Yilmaz, M., Soran, H., & Beyatli, Y. (2006). Antimicrobial activities of some *Bacillus* spp. strains isolated from the soil. *Microbiological Research*, *161*(2), 127-131. https://doi.org/10.1016/j.micres.2005.07.001

Zabidi, N. A. (2018). *Isolation and screening of bacteria from microalgae as potential probiont* [Unpublished Bachelor's thesis]. Universiti Putra Malaysia.

Zhao, W. (2014). *Characterization of the probiotic mechanism of* Phaeobacter gallaeciensis *S4 against bacterial pathogens* [Doctoral's dissertation, University of Rhode Island]. DigitalCommons@*URI*. https://doi.org/10.23860/diss-zhao-wenjing-2014

Zorriehzahra, M. J., Delshad, S. T., Adel, M., Tiwari, R., Karthik, K., Dhama, K., & Lazado, C. C. (2016) Probiotics as beneficial microbes in aquaculture: an update on their multiple modes of action: A review. *Veterinary Quarterly*, *36*(4), 228–241. https://doi.org/10.1080/01652176.2016.1172132

Zoumpourtikoudi, V., Pyrgelis, N., Chatzigrigoriou, M., Tasakis, R. N., and Touraki, M. (2018). Interactions among yeast and probiotic bacteria enhance probiotic properties and metabolism offering augmented protection to *Artemia franciscana* against *Vibrio anguillarum*. *Microbial Pathogenesis*, *125*, 497-506. https://doi.org/10.1016/j.micpath.2018.10.022

Pertanika J. Trop. Agric. Sci. 44 (1): 139 - 155 (2021)

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Journal homepage: http://www.pertanika.upm.edu.my/

Effects of Harvesting *Mucuna bracteata* on the Legume Biomass and Soil Properties under Mature Oil Palm

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ABSTRACT

The under-utilized legume *Mucuna bracteata* is a potential biomass resource in Malaysia. A 24-month study was conducted under 10-year-old mature oil palm trees to determine the effects of several harvesting frequencies of *M. bracteata* on the legume biomass and soil properties. The experimental design was a randomized complete block design (RCBD) for the biomass and a two-factorial RCBD for the soil properties. The treatments were the harvesting frequencies, which were once every two, four, six, and twelve months. The control treatment was without harvest. There were significant effects on the legume's cumulative biomass, standing biomass, leaf area, nutrient contents, and total nutrient harvested for N, Ca, Mg, and cellulose content. Generally, the more frequent the harvest, the more biomass was obtained, but the more legume standing biomass and leaf area were reduced. Despite the reduction in legume growth and leaf area in the field, harvesting the legume did not affect any of the soil physicochemical properties. The biomass N, Ca, and Mg contents and nutrient harvested were also affected by harvesting. This was due to the production of relatively more young shoots after harvesting, which would remove most of the aboveground plant parts. The cellulose content in the legume also increased for the same reasons. Results showed that harvesting *M. bracteata* once every six months was an acceptable compromise between collecting large

ARTICLE INFO

Article history:
Received: 30 April 2020
Accepted: 07 September 2020
Published: 24 February 2021

DOI: https://doi.org/10.47836/pjtas.44.1.08

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ISSN: 1511-3701
e-ISSN: 2231-8542

amounts of legume biomass and having a reduced legume growth recovery and leaf area in the field, but yet not detrimentally affecting the soil properties.

Keywords: Biomass quality, legume, *Mucuna bracteata*, soil conservation, soil nutrient

INTRODUCTION

Malaysia produced about 168 million tons of agricultural wastes in 2007, and the production of the biomass increases every year. These biomass resources are from oil palm, rubber, rice, horticulture, coconut, and orchard (C. S. Goh et al., 2010). However, most of the biomass (more than 90%) is from oil palm. Being the fourth largest contributor to the Malaysian's Gross National Income (GNI) and generating billions of revenue (Agensi Inovasi Malaysia [AIM], 2013), oil palm industries have been reported to produce 50 to 70 million tons of solid and liquid biomass in 2007 (Shuit et al., 2009), which further increased to 60 to 83 million tons of biomass in 2012. The biomass production of oil palm industries is expected to further increase to between 85 – 110 million tons by this year 2020 (AIM, 2013). Agriculture biomass has multiple uses, such as for renewable energy (Lior, 2008), bio-based materials in furniture, building materials, electronics, packaging, automobile industries, and value-added products (such as animal food, fertilizers, absorbent, as well as pulp and paper) (Abdul Khalil et al., 2009, 2010; Chaikitkaew et al., 2015).

For these reasons, Malaysia is interested in finding sources for biomass. The Malaysian Biomass Initiatives (MBI) was established on 9th May 2012 to support the national vision to increase biomass usage and encourage the growth and adaptation of green technologies in the country. This effort and support from the government are part of the many national policies, such as National Green Technology Policy 2009, Economic Transformation Programme 2010, Renewable Energy Act 2011, National Biomass Strategy 2020, and Malaysia Biomass Industry Action Plan 2020.

Among the potential biomass supplementation to oil palm is *Mucuna bracteata* DC. ex Kurz, and the potential of its use is substantial. *Mucuna bracteata* is under-utilized because this leguminous plant is currently grown only as a cover crop in oil palm plantations since its introduction in Malaysia in 1991 (Mathews, 1998). This legume grows fast (new shoots regrow approximately after 30 days), is tolerant to drought (can survive up to four months), and shade tolerant, as well as quick to produce thick and uniform cover compared to other conventional leguminous cover crops namely, *Pueraria phaseoloides* (*Pueraria javanica*), *Centrosema pubescens*, *Calopogonium mucunoides*, and *Calopogonium caeruleum* (Chiu et al., 2001). In addition, *M. bracteata* experiences less pest infestation and disease attacks, and the legume is non-palatable to cattle due to high content of phenolic compounds (Kothandaraman et al., 1989).

Considering *M. bracteata*'s rapid growth and high biomass yield, it is desirable if this legume, in addition to its role as a cover crop to protect and conserve soil, can act as a source of biomass that could be harvested regularly. Unfortunately, much less is known about the quality and quantity of *M. bracteata* coupled with or without harvesting effects after several frequencies because the use of this legume as a biomass resource in Malaysia and the effects of harvesting have not been explored. The only study was by Chiu and Basad (2006) that showed that fully established *M. bracteata* was tolerant to repeated cutting at bimonthly intervals. Most studies emphasized on the soil properties (for e.g., chemical, physical, and biological), soil erosions and runoff, soil water, suppressing noxious weeds, pest control (*Rhinocerous orycite*) as well as agronomic and the economic planting of *M. bracteata*. Therefore, this study was carried out with the general objective to determine the effect of frequent harvesting of *M. bracteata* on the legume and soil properties. The specific objectives were: (i) to determine the suitability of *M. bracteata* above ground parts as a biomass resource regarding on its quantity and quality properties, (ii) to determine the cumulative and standing biomass weights of *M. bracteata* after being harvested at several harvesting frequencies, and (iii) to determine whether *M. bracteata* can remain as an effective cover crop under oil palm to maintain soil fertility even after being harvested.

MATERIALS AND METHODS

Study Site and Soil Profile Background

The study was carried out at Kombok Estate, Rembau, Negeri Sembilan (2.6216° N 101.9836° E) on Durian series soil. According to the Food and Agriculture Organization (FAO) classification, the Durian series is classified as Plinthaquic, clayey, mixed, isohyperthermic, Ferric Acrisol. The soil was developed from sedimentary and low-grade metamorphic rocks. According to Department of Agriculture (DoA) (2008), this soil series is considered suitable for oil palm (*Elaeis guineensis*), cocoa (*Theobromae cocoa*), rubber (*Hevea brasiliensis*), coconut (*Cocos nucifera*), and fruits, as well as short term crops, and this soil has moderate nutrient contents. This soil is normally located on undulating terrain with a CEC range of 5 – 10 $cmol_+ kg^{-1}$, and the colour of this soil is yellowish brown (10YR 5/4, 5/6, 5/8). The soil at this experimental site was measured as having a clay texture, with a mean (± std. error) of 43.4 ± 0.30% clay and 25.3 ± 0.17% sand.

Fertilizer Management and Rainfall at the Study Site

The fertilization routine carried out in the mature oil palm area is under Applied Agricultural Resources (AAR) Sdn. Bhd. The fertilizers used were OPCOM 65 and Fertibor (Table 1). OPCOM 65 is a compound fertilizer that contains 13.65% ammonium sulphate, 0.99% rock phosphate, and 19.2% potassium chloride that provides nitrogen (N), phosphorus (P), and potassium

(K), respectively. Fertibor is a straight fertilizer that provides approximately 15% of B. The study was conducted from March 2015 to March 2017. Before the study was conducted, the application of fertilizer had been conducted in February 2015 with the dosage between 2.00 and 2.25 kg palm^{-1} (OPCOM 65). The routine continued into May, August, and September for the years 2015 and 2016, and the oil palm was supplemented with 0.10 kg palm^{-1} of Fertibor in July.

Table 1
Type, month, and rate of application of fertilizers in year 2015 and 2016

Fertilizer type	Month of application	Rate (kg palm^{-1} yr^{-1})	
		2015	2016
OPCOM 65	February	2.00	2.25
OPCOM 65	May	2.00	2.25
Fertibor	July	0.10	0.10
OPCOM 65	August	2.00	2.25
OPCOM 65	September	2.25	2.00

Table 2
Monthly (mm month^{-1}) and annual (mm yr^{-1}) rainfall in year 2015 and 2016

Month	2015	2016
January	144	159
February	38	141
March	136	55
April	306	169
May	94	209
June	128	214
July	42	192
August	87	95
September	116	115
October	164	181
November	260	74
December	328	153
Total	1843	1757

The rainfall distribution in the area was very variable (Table 2). In particular, due to the El Nino phenomenon between mid-December 2015 and mid-April 2016, there was a large decline in rainfall during those months, with monthly rainfall of between 55 and 328 mm. This directly affected the average rainfall distribution in a year, and subsequently, detrimentally affected the quantity of legume biomass.

Experimental Units and Designs

Mucuna bracteata was already planted and established under the oil palm (8 to 10 years) before the field experiment. The experimental designs in the study were: (i) randomized completely block design (RCBD) for *M. bracteata* biomass, where the factor was the harvesting frequencies, and (ii) factorial RCBD for the soil parameters, where the factors were the harvesting frequencies and soil depths. There were five harvesting frequencies, where the first four harvesting frequencies were harvesting the legume once every: i) two (HF 2), ii) four (HF 4), iii) six (HF 6), and iv) twelve (HF 12) months. The fifth harvesting frequency was control (HF0), which did not involve any harvesting. Each harvesting treatment was replicated four times, and means comparisons were done for months 12 and 24. Soil properties were measured from two soil depths (0-15 and 15-30 cm).

Sampling and Preparation of *Mucuna bracteata* Biomass

In each sampling time, the leaves and stolon were harvested over a 1-m^2 area at 2 cm

height above the soil surface. The sample was placed in a plastic bag, labelled, and brought to the laboratory. At the laboratory, the leaves and stolon were separated, cleaned thoroughly to remove the dirt and soil, and air-dried for 24 hours. The leaf blades were measured for the leaf area determination. The samples were then kept in carbon free envelops and oven-dried at 65°C for 5 days, then they were reweighed. The initial and final weights were used to determine the cumulative and standing biomass (Table 3).

The biomass were ground into 1-mm size using MK 10 basic IKA WERKE mill (IKA-Works Inc., USA) with 3,750 rpm, and then kept in dry screw-capped bottles to avoid ambient air moisture. Some ground samples were delivered to the Agriculture and Food Analytical Laboratory, Malaysian Agricultural Research and Development Institute (MARDI) for the determination of cellulose, hemicellulose, and lignin contents. All other analyses were done at the Material Characterization Lab, Department of Chemical and Environmental Engineering and Analytical Laboratory 2, Faculty of Engineering or the Department of Land Management, Faculty of Agriculture, Universiti Putra Malaysia.

The total harvested biomass nutrient (kg ha^{-1} yr^{-1}) was calculated via the following formula:

$$Total\ harvested\ biomass\ nutrient = c/100 \times w \quad [1]$$

Table 3
Methods used to determine the quantitative and quality properties of Mucuna bracteata

Property	Method
Cumulative dry weight (t ha^{-1} yr^{-1})	Accumulation of *M. bracteata* biomass dry weight from all previous harvests
Standing dry weight (t ha^{-1})	*Mucuna bracteata* current biomass dry weight in the field
Leaf area (m^2 m^{-2})	i. PAR Ceptometer (AccuPAR LP – 80, Decagon Devices Inc., USA) before month 8 (Zarate-Valdez et al., 2012) ii. LI–COR Leaf Area Meter (Model LI – 3100C, LI-COR Inc., USA) (Campostrini & Yamanishi, 2001) at month 8 onwards
C and N contents (%)	Analyzed using LECO TruMac® CNS Auto Analyzer Version 1.1x. (LECO Corporation, USA)
P, K, Ca, and Mg contents (%)	Standard established wet ashing method (Van Lierop, 1976) and analyzed by XYZ Auto Sampler ASX – 520 Series (SD Acquisition, Inc., USA) and PerkinElmer Atomic Absorption Spectrometer PinAAcle™ 900T (PerkinElmer Inc., USA)
Cellulose, hemicellulose, and lignin contents (%)	Standard acid detergent fiber (ADF) and Standard acid detergent lignin (ADL) assays (Saura-Calixto et al., 1983)
Ash content and volatile matter (%)	Thermogravimetric analysis (TGA) (Idris et al., 2010) and analyzed by METTLER TOLEDO TGA/SDTA 851e (METTLER TOLEDO, Canada) (Ludwig et al., 2007)
Calorific value (MJ kg^{-1})	Analyzed using IKA C2000 Bom Calorimeter (IKA® Works Inc., USA) (Gardner et al., 2015)

where *c* is the biomass nutrient content (%), and *w* is the cumulative harvested biomass weight (kg ha^{-1} yr^{-1}).

Sampling and Preparation of Soil

The soils were sampled for the chemical and physical parameters, as shown in Table 4. Two hundred and fifty grams of soil were sampled at each of two soil depths: 0 – 15 and 15 – 30 cm in a 1-m^2 area at sampling times once after every 2, 4, 6, and 12 months, meanwhile for control (no-harvest) was sampled at months 2 and 24. The soil samples were air-dried for 7 days, after which the air-dried soil was crushed by using pestle and mortar, and sieved into 2.0 mm size using Endecotts 2.0-mm siever (Endecotts Limited, London, UK).

Statistical Analysis

All data were analyzed with analysis of variance (ANOVA) using SAS statistical software package, Version 9.4 (SAS Institute, North Carolina State University, USA). For significant treatment effects (p<0.05), Tukey's test was used to separate the treatment means.

RESULTS AND DISCUSSION

ANOVA showed there was no significant effect of harvesting *M. bracteata* on any of the soil physicochemical properties (Table

Table 4
Methods used to determine the soil physicochemical properties

Property	Method
pH (H_2O)	Soil water ratio 1:2.5 (Mc Lean, 1982) and analyzed using MeterLab®PHM standard pH meter (Radiometer Analytical, Copenhagen, Denmark)
CEC ($cmol_+$ kg^{-1})	Leaching method (Thomas, 1982), and the extractant is analyzed using PerkinElmer Atomic Absorption Spectrophotometer PinAAcle™ 900T (PerkinElmer Inc., USA)
Total C and N (%)	Analyzed using LECO TruMac® CNS Auto Analyzer Version 1.1x. (LECO Corporation, USA)
Available P ($cmol_+$ kg^{-1})	Bray and Kurtz no. 2 extracting solution (Olsen & Sommers, 1982), and the extractant is analyzed using XYZ Auto Sampler ASX – 520 Series (SD Acquisition, Inc., USA)
Exchangeable K ($cmol_+$ kg^{-1})	Leaching method (Thomas, 1982), and the extractant is analyzed using XYZ Auto Sampler ASX – 520 Series (SD Acquisition, Inc., USA)
Exchangeable Ca and Mg ($cmol_+$ kg^{-1})	Leaching method (Thomas, 1982), and the extractant is analyzed using PerkinElmer Atomic Absorption Spectrophotometer PinAAcle™ 900T (PerkinElmer Inc., USA)
Texture	Pipette method (Teh & Jamal, 2006)
Bulk density (Mg m^{-3}), Volumetric water content (%) Porosity (%)	Core method (Teh & Jamal, 2006)
Aggregate stability (%)	Wet sieving (Teh & Jamal, 2006)
Mean weight diameter (aggregation) (mm)	Dry sieving (Teh & Jamal, 2006)

5) or on any of the legume's biomass quality properties. Only the legume's biomass weight, leaf area, and N, Ca, and Mg plant contents were significantly affected by the harvesting treatments.

Cumulative biomass increased with increasing harvesting frequencies (Figure 1a). However, the standing biomass and leaf area decreased substantially (Figure 1b and c). *Mucuna bracteata* harvested once every two (HF 2), four (HF 4), six (HF 6), and twelve (HF 12) months obtained biomass quantities ranging between 7.7 to 24.5 t ha^{-1} yr^{-1}. Recall that HF 2 denotes harvesting

Table 5
Mean (± std. error) of soil physicochemical properties at 0-15 and 15-30 cm soil depths at the end of the field experiment (month 24)

	(cm)	Treatments				
Property	Depth	HF 0	HF 2	HF 4	HF 6	HF 12
C	0-15	2.45 ± 0.02	2.43 ± 0.02	2.40 ± 0.03	2.39 ± 0.03	2.41 ± 0.03
(%)	15-30	2.42 ± 0.01	2.42 ± 0.01	2.42 ± 0.01	2.43 ± 0.01	2.41 ± 0.01
N	0-15	0.15 ± 0.01	0.15 ± 0.01	0.14 ± 0.02	0.16 ± 0.02	0.15 ± 0.02
(%)	15-30	0.15 ± 0.02	0.15 ± 0.03	0.15 ± 0.03	0.13 ± 0.03	0.16 ± 0.03
P	0-15	3.96 ± 0.35	3.70 ± 0.34	3.36 ± 0.30	3.18 ± 0.26	3.42 ± 0.28
(mg kg^{-1})	15-30	3.21 ± 0.17	3.12 ± 0.17	3.09 ± 0.17	2.89 ± 0.10	2.83 ± 0.09
K	0-15	0.13 ± 0.01	0.12 ± 0.01	0.08 ± 0.02	0.13 ± 0.01	0.10 ± 0.02
($cmol_+$ kg^{-1})	15-30	0.11 ± 0.03	0.13 ± 0.02	0.10 ± 0.01	0.15 ± 0.03	0.16 ± 0.03
Ca	0-15	0.30 ± 0.06	0.33 ± 0.08	0.34 ± 0.08	0.31 ± 0.08	0.29 ± 0.09
($cmol_+$ kg^{-1})	15-30	0.40 ± 0.03	0.34 ± 0.03	0.31 ± 0.03	0.33 ± 0.04	0.29 ± 0.04
Mg	0-15	0.25 ± 0.03	0.21 ± 0.01	0.22 ± 0.03	0.24 ± 0.04	0.23 ± 0.04
($cmol_+$ kg^{-1})	15-30	0.19 ± 0.03	0.11 ± 0.04	0.12 ± 0.04	0.14 ± 0.04	0.12 ± 0.06
CEC	0-15	9.72 ± 1.19	9.98 ± 1.18	9.95 ± 1.19	9.91 ± 1.12	10.53 ± 1.10
($cmol_+$ kg^{-1})	15-30	4.48 ± 0.27	4.88 ± 0.32	4.88 ± 0.35	4.75 ± 0.34	4.85 ± 0.33
pH (H_2O)	0-15	4.86 ± 0.03	4.80 ± 0.03	4.78 ± 0.05	4.72 ± 0.05	4.76 ± 0.05
	15-30	4.66 ± 0.03	4.60 ± 0.03	4.55 ± 0.04	4.49 ± 0.04	4.51 ± 0.04
Bulk density	0-15	1.49 ± 0.07	1.39 ± 0.06	1.47 ± 0.09	1.46 ± 0.06	1.40 ± 0.08
(Mg m^{-3})	15-30	1.54 ± 0.06	1.52 ± 0.04	1.52 ± 0.03	1.52 ± 0.06	1.53 ± 0.11
Aggregate	0-15	25.4 ± 0.10	30.0 ± 0.32	23.2 ± 0.29	22.8 ± 0.22	25.5 ± 0.26
stability (%)	15-30	25.1 ± 0.09	28.4 ± 0.31	22.5 ± 0.30	22.7 ± 0.17	26.8 ± 0.27
Porosity	0-15	45.8 ± 0.15	47.1 ± 0.19	44.3 ± 0.33	44.6 ± 0.23	47.2 ± 0.27
(%)	15-30	46.8 ± 0.11	46.8 ± 0.13	47.3 ± 0.16	46.4 ± 0.20	46.8 ± 0.35
Aggregation	0-15	3.51 ± 0.02	3.40 ± 0.02	3.51 ± 0.03	3.54 ± 0.05	3.63 ± 0.05
(mm)	15-30	3.50 ± 0.02	3.60 ± 0.04	3.60 ± 0.04	3.51 ± 0.03	3.48 ± 0.05
Soil water	0-15	36.0 ± 0.2	34.6 ± 0.6	36.2 ± 0.6	36.5 ± 0.4	35.8 ± 0.5
(m^3 m^{-3})	15-30	35.7 ± 0.4	35.3 ± 0.9	35.2 ± 0.6	34.0 ± 0.5	37.2 ± 0.7

For a given soil property and soil depth, ANOVA revealed no significant effect ($p>0.05$*) by all the harvesting frequency treatments (HF n treatment indicates harvest once every n months, and HF 0 is the control, without harvest)*

done once every 2 months (therefore, most frequent), and on the other extreme, treatment HF 12 denotes harvesting done once every 12 months (therefore, least frequent). Without harvest (control; HF 0), the biomass was comparable to that harvested once after every six and twelve months (HF 6 and HF 12). Harvesting once after every two months (HF 2) yielded the highest biomass followed by HF 6, HF 4, and HF 12.

Similar findings were also observed from other studies, such as on the giant reed (*Arundo donax* L.) (Dragoni et al.,

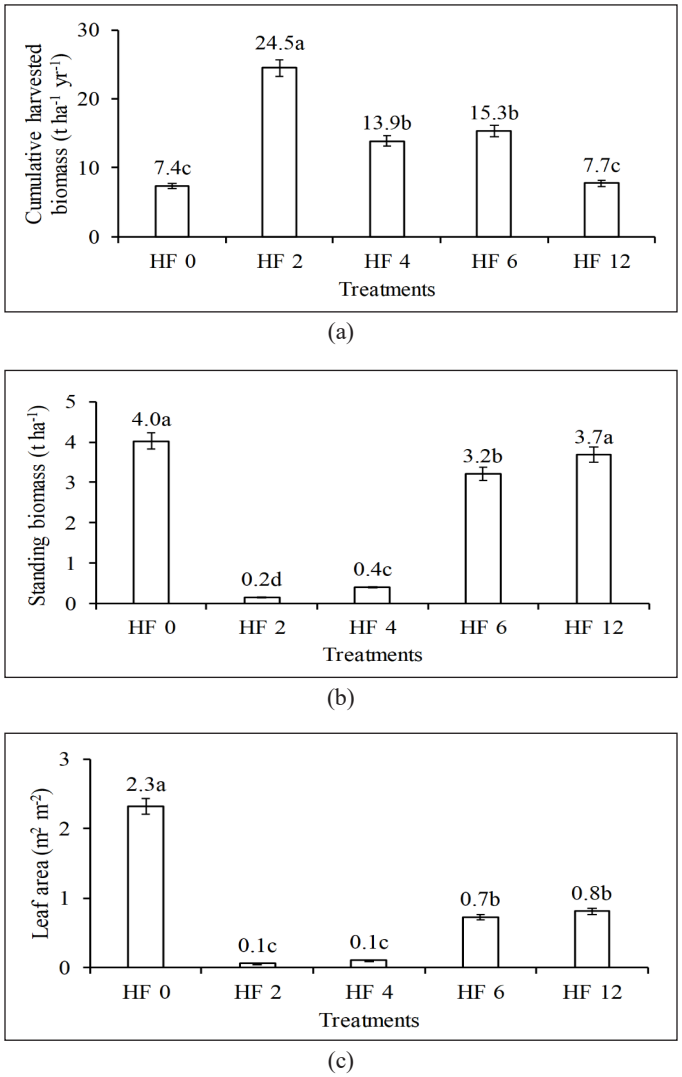


Figure 1. Mean (± S.E) at month 24 of *Mucuna bracteata*: (a) cumulative harvested biomass, (b) standing biomass, and (c) leaf area. For the same property, means with the same letter are not significantly different from one another (p>0.05) (*Note*. HF *n* treatment indicates harvest once every *n* months; HF 0 is the control, without harvest)

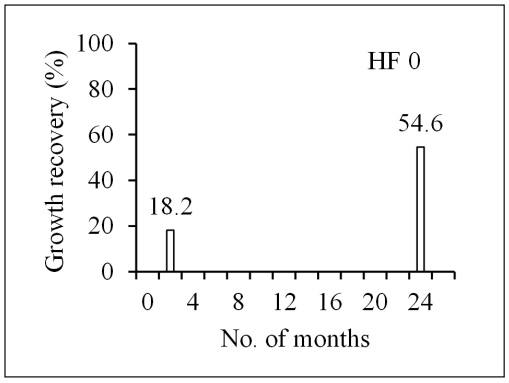
2015), King Napier grass (Lounglawan et al., 2014), cassava (Hue et al., 2012), soybean (Aqeel, 2011), ryegrass/white clover swards (Vinther, 2006), and *Pueraria lobata* (Willd.). Terrill et al. (2003) affirmed that the more frequent the harvesting, the more cumulative crop biomass weights were obtained but the lower the standing biomass weights and leaf area. Figure 1b shows that at month 24 the above ground standing biomass for HF 2 and HF 4 were greatly decreased by 94 and 89%, respectively, compared with HF 0, HF 6, and HF 12. Both HF 6 and HF 12 were slightly smaller than HF 0 by about 9 and 20%, respectively. Similarly to the standing biomass trend, the leaf area also showed a similar trend (Figure 1c).

The leaf area greatly decreased for HF 2 and HF 4 by 86 and 96%, respectively, compared with HF 0, HF 6, and HF 12. There was not much difference in leaf area between HF 6 and HF 12; however, they still decreased by 69 and 65%, respectively, compared with HF 0. Without harvest (HF 0), the standing biomass and leaf area of *M. bracteata* were higher than the other harvesting frequencies.

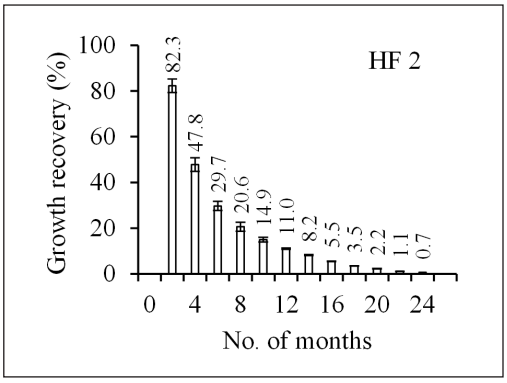
The biomass growth recovery was expressed, in percentage, as the ratio between the current and previous weights of standing biomass harvested. This measure indicates relatively how much, in percentage, new aboveground biomass had developed since the previous harvest. Recovery rates smaller and larger than 100% indicate that the standing biomass weights at current harvest are, respectively, smaller and larger than the weights at previous harvest. Figure 2 shows the biomass recovery decreased quickly in the order of HF 2 > HF 4 > HF 6 > HF 12. The more frequent the harvest, the lower the biomass recovery. For instance, HF 2's the final biomass recovery was 0.7%, but for HF 4, HF 6, and HF 12, their final biomass recovery rates were at 2.8, 20.6, and 47.6%, respectively. HF 0 instead had an increased biomass recovery from 18.2% initially to 54.6% in the final month.

The trends for total biomass N, Ca, and Mg harvested were generally the opposite of the respective biomass nutrient contents (Figure 3). The more frequent the harvest, the higher the total biomass nutrient harvested, but the lower the biomass nutrient content. Harvesting *M. bracteata* affected its biomass N, Ca, and Mg contents, which subsequently caused a difference in their total amount of nutrients harvested, as indicated in Figure 3. Without harvest (HF 0), the nutrient contents and nutrient harvested (N, Ca, and Mg) were always the lowest compared with the other harvesting frequencies.

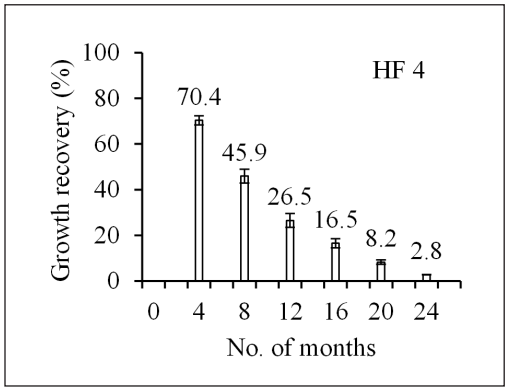
The biomass N content generally increased with decreasing harvesting frequency, in the order of HF 0 < HF 2 = HF 12 < HF 4 < HF 6. But total biomass N harvested decreased in the opposite direction, in the order of HF 2 > HF 4 > HF 6 > HF 0 > HF 12. N concentration in *M. bracteata* ranged between 2.3 to 3.2%, which was lower compared with other legumes (> 3% of N), as reported by Mills and Jones (1996) as well as Vose (1963). The concentration of N in leaves does not often correlate with the growth and yield of a



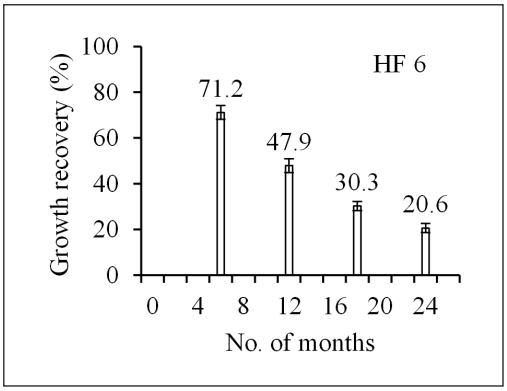
(a)



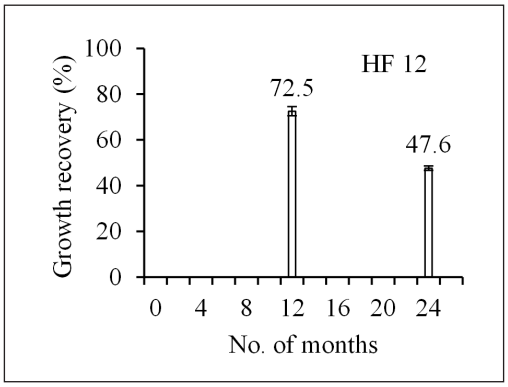
(b)



(c)



(d)



(e)

Figure 2. Growth recovery of *Mucuna bracteata* for all harvesting frequencies (*Note*. HF *n* treatment indicates harvest once every *n* months; HF 0 is the control, without harvest)

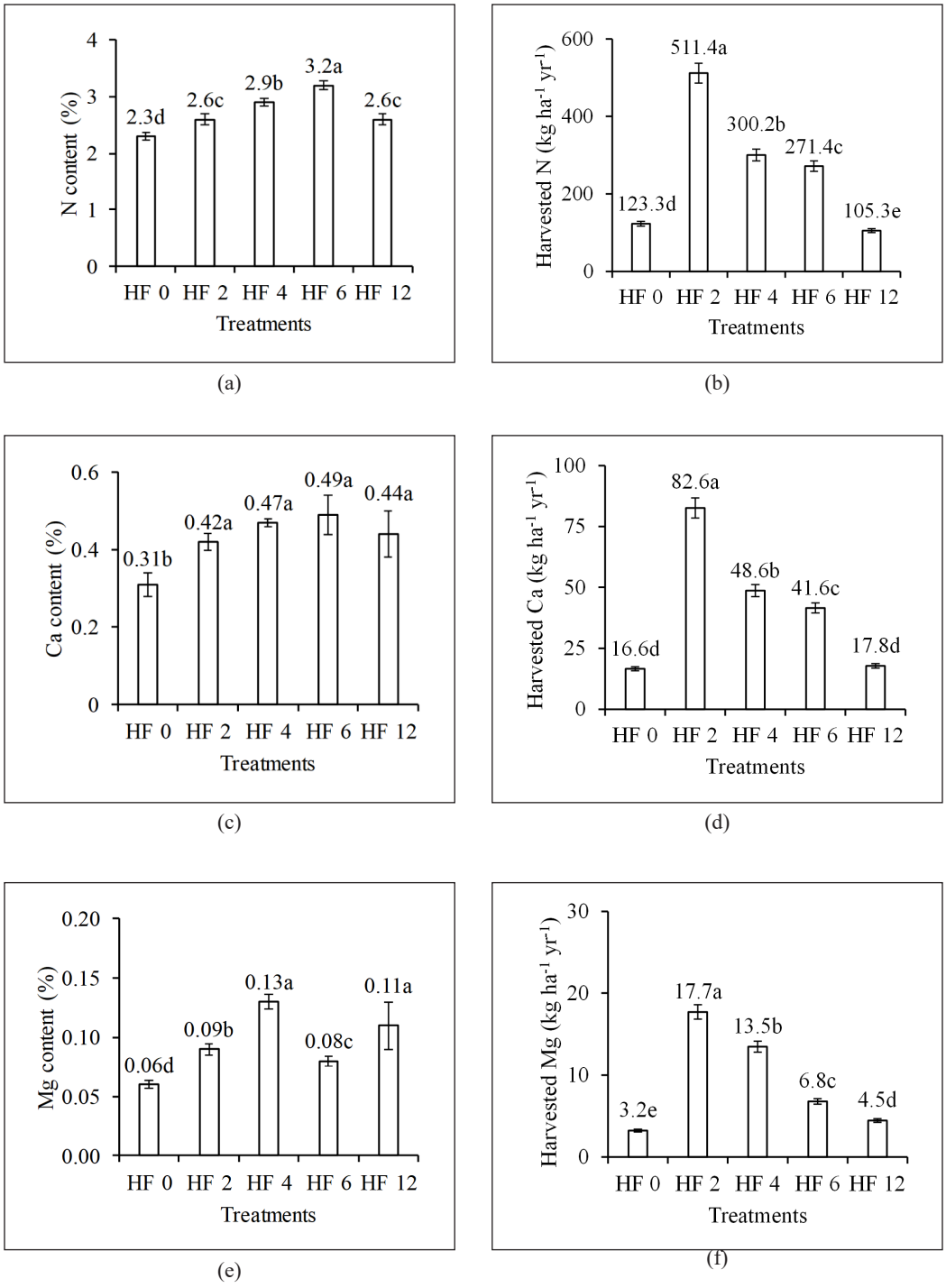


Figure 3. Mean (± S.E) *Mucuna bracteata*'s N, Ca, and Mg contents and nutrients harvested (*Note*. HF *n* treatment indicates harvest once every *n* months; HF 0 is the control, without harvest)

plant. It is instead more correlated with plant maturity stages in the leaf, stem (stolon), and roots. This also depends on the plant type, whether the plant is annual, biennial, or perennial (Marschner, 2012).

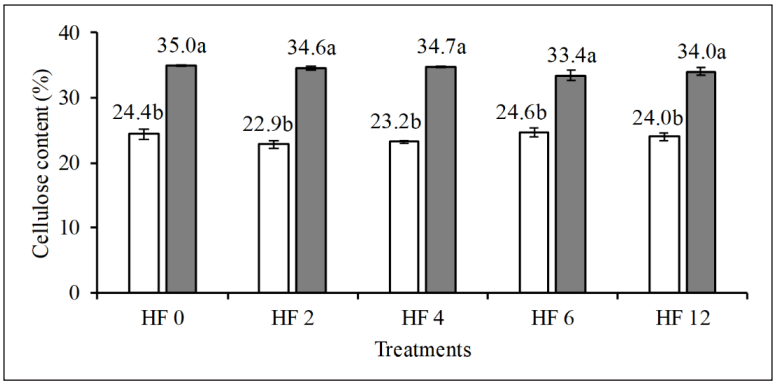
Harvesting would remove most of the aboveground parts of *M. bracteata*, and this would indirectly influence the legume's maturity, assimilate allocation (photosynthesis, translocation, deposition, and accumulation), growth (vegetative), and the nutrient contents. At the early plant growth stage, N concentration in the plant would be high. Results previously showed that the more frequent *M. bracteata* was harvested, the lower the standing biomass and leaf area (Figure 1). More frequent removal of biomass meant there was relatively younger to old plant parts, and thus, more N was allocated to these young plant parts (Mills & Jones, 1996; Vose, 1963; Yoneyama et al., 2003). In other words, frequent harvesting affected the maturity of *M. bracteata*. More frequent harvesting induced the legume to sprout relatively more new and young shoots and stolon.

Unlike the N content, there was no difference in the biomass Ca content between the various harvesting frequencies. HF 2 to HF 12, however, had higher Ca content than control (HF 0). This meant that harvesting *M. bracteata*, even by once every 12 months, would increase the biomass Ca content. And similar to the trend for N, the total biomass Ca harvested decreased in the order of HF 2 > HF 4 > HF 6 > HF 12 > HF 0.

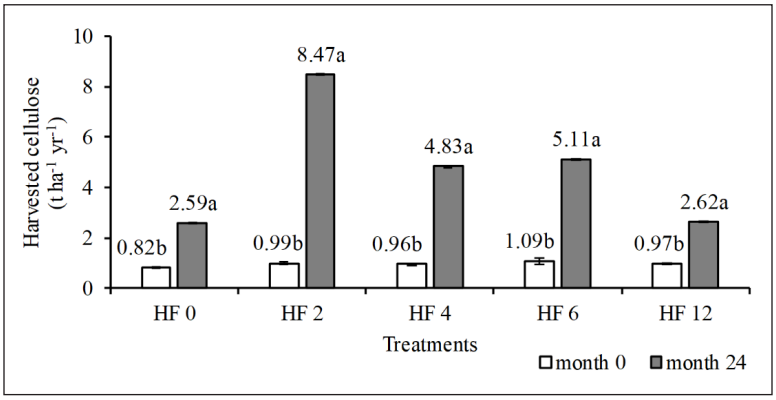
Mg content decreased in the order of HF 4 = HF 12 > HF 2 > HF 6 and Mg harvested HF 2 > HF 4 > HF 6 > HF 12. This showed harvesting *M. bracteata* would affect the Mg content and its amount harvested, following the general trend of N.

Several studies have been conducted on the shoots of 16 cover crop species across various soil conditions. In particular, Fageria et al. (2014) as well as Reuter and Robinson (1986) reported that, on average, the plant N content ranged between 2.5 and 5.0%, the Ca content between 0.7 and 3.0%, and the Mg content between 0.3 and 0.5%. The N content of *M. bracteata* observed in this study was within the range, but for the Ca and Mg contents, they were lower than the average of the 16 cover crops, as previously mentioned. However, in this study, the biomass nutrient contents were additionally affected by biomass removal from harvesting.

Figure 4a shows that harvesting did not affect the biomass cellulose content. Cellulose content, even for the legume in the control plots, increased from between 22.9 - 24.6% at month 0 to between 33.4 - 35.0% at month 24, but there was no significant difference between treatments for a given month. Figure 4b, however, shows that cellulose yield was affected by the harvesting frequencies. More frequent harvest would return more biomass amounts (Figure 1) and thus, more cellulose yields. HF 2 showed the highest quantity yielded of cellulose, followed by HF 6, HF 4, and HF 12.



(a)



(b)

Figure 4. Mean (± S.E) *Mucuna bracteata*'s cellulose: a) content and b) yield (harvested quantity). For the same treatment, means the same letter are not significantly different from one another (p>0.05) (*Note*. HF *n* treatment indicates harvest once every *n* months; HF 0 is the control, without harvest)

Similarly to other common legumes, such as *P. javanica*, *C. pubescens*, *C. caeruleum*, and *C. mucunoides*, harvesting *M. bracteata* would cause the legume to produce relatively more new and young shoots and stolon (K. J. Goh et al, 2007). During plant regrowth, cell walls are rebuilt, which would cause an increase in the plant cellulose content, more than for hemicellulose and lignin (McDonald et al., 2002). One of the major components of plant cell walls is cellulose microfibrils, comprising between 15 and 30% of the dry mass of primary walls (McQueen-Mason et al., 2003). But even without harvesting, the legume in HF 0 still showed an increase in cellulose content from month 0 to 24. This is probably due to the increase in canopy shade (i.e., oil palm trees maturing and their canopies becoming larger and more complete in ground cover) that would inhibit more light penetration and thus increase the dieback of *M. bracteata*'s older leaves and stolon so that young shoots would be produced (K. J. Goh et al., 2007), and this would synthesize more plant cellulose.

More frequent harvesting of *M. bracteata* resulted in increasing amounts of legume biomass collected from the field. But harvesting the legume detrimentally affected the growth of the legume. Legume growth recovery decreased and never recovered even after 12 months after harvest. The leaf area of the legume was also much reduced compared with plots without harvest (HF 0). But although the legume growth and leaf area were reduced by harvesting, their reduction did not impact the soil properties. Soils in plots with harvest were no different than those without harvest. This could be because the soils were regularly well-fertilized under conventional management practices (Table 1) and that, despite biomass removal during harvest, the legume could still re-establish, with varying degrees of recovery, in the field (Figure 1 and 2). Results from this study suggest that *M. bracteata* could be harvested but not too frequently, such as once every two (HF 2) to four (HF 4) months, as this would greatly reduce the legume standing biomass and leaf area in the field. Instead, harvesting the legume once every six months (HF 6) is an acceptable compromise between collecting large amounts of legume biomass and having a reduced legume growth recovery and leaf area in the field, but yet not detrimentally affecting the soil properties.

CONCLUSIONS

Harvesting *Mucuna bracteata* affected the legume biomass but not the soil properties. The more frequent the harvest, the more biomass was obtained, but the legume standing biomass and leaf area were reduced. Despite the reduction in legume growth and leaf area in the field, harvesting the legume did not affect any of the soil physicochemical properties. The biomass N, Ca, and Mg contents were also affected by harvesting. This was due to the production of relatively more young shoots after harvesting, which would remove most of the aboveground plant parts. The cellulose content in the legume also increased for the same reasons. Results showed that too frequent harvesting (such as once every two or four months) is not recommended, as it would cause too much reduction in legume standing biomass and leaf area (despite allowing for large amounts of biomass to be collected). Instead, harvesting *M. bracteata* once every six months is recommended as this harvesting frequency provided for large amounts of legume biomass without having excessive reduction in legume growth, as compared with other harvesting frequencies, and without detrimentally affecting soil physicochemical properties.

ACKNOWLEDGEMENTS

We would like to thank Mr. Goh Kah Joo and Applied Agriculture Research Sdn. Bhd. for the research funds and field logistics; the management of Kombok Estate; Department of Land Management, UPM; Department of Crop Science, UPM; Institute of Tropical Agriculture and Food Security (ITAFoS), UPM; Department of Environment and Chemical Engineering, UPM; and Malaysian Agriculture Research and Development Institute (MARDI) for their help.

REFERENCES

Abdul Khalil, H. P. S., Kang, C. W., Khairul, A., Ridzuan, R., & Adawi, T. O. (2009). The effect of different laminations on mechanical and physical properties of hybrid composites. *Journal of Reinforced Plastics and Composites*, *28*(9), 1123–1137. https://doi.org/10.1177/0731684407087755

Abdul Khalil, H. P. S., Poh, B. T., Issam, A. M., Jawaid, M., & Ridzuan, R. (2010). Recycled polypropylene-oil palm biomass: The effect on mechanical and physical properties. *Journal of Reinforced Plastics and Composites*, *29*(8), 1117–1130. https://doi.org/10.1177/0731684409103058

Agensi Inovasi Malaysia. (2013). *National Biomass Strategy 2020: New wealth creation for Malaysia's biomass industry* (Version 2.0, 2013). Agensi Inovasi Malaysia.

Aqeel, A. M. (2011). *Using manual defoliation to simulate soybean rust: Effect on growth and yield formation* [Unpublished Doctoral dissertation]. University of Kentucky.

Campostrini, E., & Yamanishi, O. K. (2001). Estimation of papaya leaf area using the central vein length. *Scientia Agricola*, *58*(1), 39-42. https://doi.org/10.1590/S0103-90162001000100007

Chaikitkaew, S., Kongjan, P., & Thong, S. O. (2015). Biogas production from biomass residues of palm oil mill by solid state anaerobic digestion. *Energy Procedia*, *79*, 838–844. https://doi.org/10.1016/j.egypro.2015.11.575

Chiu, S. B., & Basad, M. (2006). *Mucuna bracteata* – Biomass, litter and nutrient production. *The Planter*, *82*(961), 247– 254.

Chiu, S. B., Farida, E., & Siow, A. (2001). *Mucuna bracteata* - A super legume cover crop revisited. *The Planter*, *77*(907), 575-583.

Department of Agriculture. (2008). *Panduan siri-siri tanah utama di Semenanjung Malaysia* [Guide to main soil series in Peninsular Malaysia] (2nd ed.). Ministry of Agriculture and Food Industries.

Dragoni, F., o Di Nasso, N. N., Tozzini, C., Bonari, E., & Ragaglini, G. (2015). Aboveground yield and biomass quality of giant reed (*Arundo donax* L.) as affected by harvest time and frequency. *BioEnergy Research*, *8*(3), 1321-1331. https://doi.org/10.1007/s12155-015-9598-x

Fageria, N. K., Baligar, V. C., & Li, Y. C. (2014). Nutrient uptake and use efficiency by tropical legume cover crops at varying pH of an Oxisol. *Journal of Plant Nutrition*, *37*(2), 294-311. https://doi.org/10.1080/01904167.2013.851695

Gardner, J. L., He, W., Li, C., Wong, J., Sale, K. L., Simmons, B. A., & Tanjore, D. (2015). Calorimetric evaluation indicates that lignin conversion to advanced biofuels is vital to improving energy yields. *RSC Advances*, *5*(63), 1092-1101. https://doi.org/10.1039/C5RA01503K

Goh, C. S., Tan, K. T., Lee, K. T., & Bhatia, S. (2010). Bio-ethanol from lignocellulose: Status, perspectives and challenges in Malaysia. *Bioresource Technology*, *101*(13), 4834–4841. https://doi.org/10.1016/j.biortech.2009.08.080

Goh, K. J., Gan, H. H., & Ng, H. C. P. (2007). Agronomy of *Mucuna bracteata* under oil palm. In K. J. Goh (Ed.), *Mucuna bracteata: A cover crop and living green manure* (pp. 45-84). Agricultural Crop Trust (ACT).

Hue, K. T., Van, D. T. T, Ledin, I., Wredle, E., & Sporndly, E. (2012). Effect of harvesting frequency, variety and leaf maturity on nutrient composition, hydrogen cyanide content and cassava foliage yield. *Asian-Australasian Journal of Animal Science*, *25*(12), 1691-1700. https://doi.org/10.5713/ajas.2012.12052

Idris, S. S., Rahman, N. A., Ismail, K., Alias, A. B., Rashid, Z. A., & Aris, M. J. (2010). Investigation on thermochemical behaviour of low rank

Malaysian coal, oil palm biomass and their blends during pyrolysis via thermogravimetric analysis (TGA). *Bioresource Technology*, *101*(12), 4584-4592. https://doi.org/10.1016/j.biortech.2010.01.059

Kothandaraman, R., Mathew, J., Krishnakumar, A. K., Joseph, K., Jayarathnam, K., & Sethuraj, M. R. M. (1989). Comparative efficiency of *Mucuna bracteata* DC and *Pueraria phaseoloides* Benth. on soil nutrient enrichment, microbial population and growth of *Hevea*. *Indian Journal of Natural Rubber Research*, *2*, 147-150.

Lior, N. (2008). Energy resources and use: The present situation and possible paths to the future. *Energy*, *33*(6), 842-857. https://doi.org/10.1016/j.energy.2007.09.009

Lounglawan, P., Lounglawan, W., & Suksombat, W. (2014). Effect of cutting interval and cutting height on yield and chemical composition of King Napier grass (*Pennisetum purpureum* x *Pennisetum americanum*). *APCBEE Procedia*, *8*, 27-31. https://doi.org/10.1016/j.apcbee.2014.01.075

Ludwig, C., Wochele, J., & Jörimann, U. (2007). Measuring evaporation rates of metal compounds from solid samples. *Analytical Chemistry*, *79*(7), 2992-2996. https://doi.org/10.1021/ac0622173

Marschner, H. (2012). *Marschner's mineral nutrition of higher plants* (3rd ed.). Academic Press.

Mathews, C. (1998). The introduction and establishment of a new leguminous cover crop, *Mucuna bracteata* under oil palm in Malaysia. *The Planter*, *74*(868), 359-368.

McDonald, P., Edward, R. A., Greenhalgh, J. F. D., & Morgan, C. A. (2002). *Animal nutrition* (6th ed.). Wiley.

McLean, E. O. (1982). Soil pH and lime requirement. In A. L. Page (Ed.), *Methods of soil analysis. Part 2. Chemical and microbiological properties* (2nd ed., pp. 199-224). American Society for Agronomy.

McQueen-Mason, S., Darley, C., Roberts, P., & Jones, L. (2003). Cell growth. In B. Thomas, D. J. Murphy, & B. G. Murray (Eds.), *Encyclopedia of applied plant sciences* (pp. 523-532). Academic Press.

Mills, H. A., & Jones Jr, J. B. (1996). *Plant analysis handbook II. Plant Nutrition*. MicroMacro Publishing.

Olsen, S. R., & Sommers, L. E. (1982). Phosphorus. In A. L. Page (Ed.), *Methods of soil analysis. Part 2. Chemical and microbiological properties* (2nd ed., pp. 403-430). American Society of Agronomy, Soil Science Society of America.

Reuter, D. J., & Robinson, J. B. (1986). *Plant analysis: An interpretation manual*. CSIRO Publishing.

Saura-Calixto, F., Cañellas, J., & Soler, L. (1983). Dietary fibre and components of the nitrogen-free extract of almond kernels. *Journal of the Science of Food and Agriculture*, *34*(12), 1419-1422. https://doi.org/10.1002/jsfa.2740341216

Shuit, S. H., Tan, K. T., Lee, K. T., & Kamaruddin, A. H. (2009). Oil palm biomass as a sustainable energy source: A Malaysian case study. *Energy*, *34*(9), 1225-1235. https://doi.org/10.1016/j.energy.2009.05.008

Teh, C. B. S., & Jamal, T. (2006). *Soil physics analyses* (Vol. 1). UPM Press.

Terrill, T. H., Gelaye, S., Mahotiere, S., Amoah, E. A., Miller, S., & Windham, W. R. (2003). Effect of cutting date and frequency on yield and quality of kudzu in the Southern United States. *Grass and Forage Science*, *58*(2), 178-183. https://doi.org/10.1046/j.1365-2494.2003.00369.x

Thomas, G. W. (1982). Exchangeable cations. In A. L. Page (Ed.), *Methods of soil analysis. Part 2. Chemical and microbiological properties* (2nd ed., pp. 159-165). American Society for Agronomy.

Van Lierop, W. M. (1976). Digestion procedures for simultaneous automated determination of NH_4,

P, K, Ca and Mg in plant material. *Canadian Journal of Soil Science*, *56*(4), 425-432. https://doi.org/10.4141/cjss76-051

Vinther, F. P. (2006). Effects of cutting frequency on plant production, N-uptake and N_2 fixation in above- and below-ground plant biomass of perennial ryegrass–white clover swards. *Grass and Forage Science*, *61*(2), 154-163. https://doi.org/10.1111/j.1365-2494.2006.00519.x

Vose, P. B. (1963). Varietal differences in plant nutrition. *Herbage Abstracts*, *33*(1), 1-13.

Yoneyama, T., Ito, O., & Engelaar, W. M. H. G. (2003). Uptake, metabolism and distribution of nitrogen in crop plants traced by enriched and natural ^{15}N: Progress over the last 30 years. *Phytochemistry Reviews*, 2, 121-132. https://doi.org/10.1023/B:PHYT.0000004198.95836.ad

Zarate-Valdez, J. L., Whiting, M. L., Lampinen, B. D., Metcalf, S., Ustin, S. L., & Brown, P. H. (2012). Prediction of leaf area index in almonds by vegetation indexes. *Computers and Electronics in Agriculture*, *85*, 24-32. https://doi.org/10.1016/j.compag.2012.03.009

Pertanika J. Trop. Agric. Sci. 44 (1): 157 - 170 (2021)

PERTANIKA JOURNALS

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Effectiveness of Bioinoculants *Bacillus cereus* and *Trichoderma asperellum* as Oil Palm Seedlings Growth Promoters

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ABSTRACT

In the establishment of oil palm seedlings, apart from the application of adequate amount of fertilizers, other sustainable plant nutrient sources are known to have the potential in enhancing vegetative growth and improve plants' resistance against pests and diseases. The application of plant growth promoters is known to contribute towards sustaining healthy plant growth leading to strong plant defense mechanisms. The present study was conducted to determine plant growth promotion potentials of bacterium, *Bacillus cereus* (UPM15) and fungus *Trichoderma asperellum* (UPM16). Isolates *B. cereus* and *T. asperellum* were assessed on their effectiveness as plant growth promoters for oil palm seedlings. Plant growth-promoting potentials were evaluated in terms of their ability to produce indole acetic acid (IAA), a naturally occurring plant hormone of the auxin class, iron-chelating compounds or siderophores, and phosphate solubilisation, considered to be one of the most important traits associated with plant phosphate nutrition. A series of treatments was applied to establish the potential of *B. cereus* and *T. asperellum* as microbial inoculants in singles and mixed applications in an *in vivo* nursery study. The ability to solubilize precipitated phosphate and to produce siderophores was positively demonstrated by *T. asperellum*. Both *B. cereus* and *T. asperellum* were capable of producing IAA. The results showed that the former significantly contributed towards growth enhancement of roots and the later in growth promotion of aerial parts of oil palm seedlings. Mixture of these isolates

ARTICLE INFO

Article history:
Received: 14 August 2020
Accepted: 04 January 2021
Published: 24 February 2021

DOI: https://doi.org/10.47836/pjtas.44.1.09

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ISSN: 1511-3701
e-ISSN: 2231-8542

yielded good vegetative growth. The study revealed the benefits of microbial inoculants that extended beyond their capacity as biofertilizers.

Keywords: *Bacillus cereus*, IAA production, phosphate solubilisation, plant growth promoter, siderophore, *Trichoderma asperellum*

INTRODUCTION

Synthetic or chemical fertilizers have been continuously used in agriculture for decades. Although effective in most cases, continuous and excessive use of synthetic fertilizers could cause negative impacts on the hydrological systems and soil environment (Salman et al., 2011; Wuana et al., 2011). The nutrients present in fertilizers can be mobilized by rainfall and eventually cause eutrophication (the nutrient enrichment of surface water bodies). Eutrophication enables aquatic plants and algal to grow uncontrollably and causes the reduction of dissolved oxygen which leads to adverse effects on aquatic life (M. N. Khan et al., 2018). Nitrogen-based fertilizers can cause nitrate contamination in the hydrological system, and in the worst case scenario, the consumption of nitrate-contaminated water can cause blue baby syndrome in infants and stomach cancer in adults (Nolan et al., 2002; Wolfe & Patz, 2002). According to S. Khan et al. (2008), land application of fertilizers may cause soil contamination due to the accumulation of heavy metals and metalloids in the soil. Heavy metals can remain in the soil for a longer period after their introduction and their presence can severely inhibit the biodegradation of organic contaminants (Adriano, 2003; Maslin & Maier, 2000). The situation remains a concern and thus, it has become crucial to explore alternative methods of crop fertilization with the objective of minimizing or even preventing chemical fertilizer application. Therefore, studies beneficial microbes or plant growth promoters, as alternative fertilizers, being regarded as a sustainable approach, are on the increase. Bacteria of the genus *Bacillus* spp. and *Trichoderma* spp. in the fungi family have been reported to have significantly enhanced plant growth and development of a number of species, boosting their defense mechanism towards biotic and abiotic stresses (Harman et al., 2004; Musa et al., 2018; Shoresh et al., 2005; Vinale et al., 2008; Yedidia et al., 2003). L. Zhao et al. (2011) and Naher et al. (2012) proposed that *Bacillus* spp. and *Trichoderma* spp. species could be potential plant growth promoters in agriculture. Against this background, the present study was conducted to examine the effectiveness of bioinoculants *Bacillus cereus* (UPM 15) and *Trichoderma asperellum* (UPM 16) as plant growth promoters on oil palm seedlings' growth and development.

MATERIALS AND METHODS

Plant Materials and Soil Preparation

A total of 96 oil palm seedlings of variant GH500 (*Dura*×*Pisifera*) were purchased from a commercial nursery. The 3-month old seedlings were certified as basal stem rot disease-free. The seedlings were maintained following standard nursery

practices in a plant nursery facility at Faculty of Agriculture, Universiti Putra Malaysia, Serdang, Selangor. Standand NPK (15:15:15) fertilizer was applied to all treatments once a fortnight throughout the trial duration. The seedlings were irrigated twice daily at 11.00 a. m. and after 4.00 p. m. Plant trays containing the 3-month old seedlings were kept on hold at the nursery facility for two weeks before transferring them to polybags to stabilize and adapt to the new nursery environment. Polythene bags of 30 cm × 38 cm with a thickness of 500 gauges (0.125 mm) were each used to hold 3 kg of soil mixture. Prior to transplanting, a soil mixture of 3:2:1 v/v/v topsoil: peat: sand was prepared and sterilized in an autoclave at 121ºC with 100 kPa pressure for 30 minutes at Microbe Control Laboratory, Faculty of Agriculture, UPM.

Treatments on Oil Palm Seedlings

In the present study, bacterium *B. cereus* and fungi *T. asperellum* previously isolated from oil palm roots in two different studies focusing on biological control of oil palm basal stem rot disease by Musa et al. (2018) and Nusaibah et al. (2017) respectively, were used. Isolates of *B. cereus*, cultured on nutrient agar (NA) for 48 hours, were used to prepare inoculum suspension. A CFU mL^{-1} of 10^8 was used to fix the inoculum suspension (Zaiton et al., 2008). An amount of 150 mL of *B. cereus* inoculum suspension was used to drench the seedling soil as shown in Table 1. A booster application of *B. cereus* was applied with similar protocol after 21 days from the initial application. Booster application was chosen to be applied at day 21 after testing a few time frames in a preliminary study based on the recovery data of each microbe used in the current study.

Preparation of conidial suspension of *T. asperellum* was carried out based on Izzati and Abdullah (2008) with some modifications. Muslin cloth was used to replace the filter paper Grade 1 (11 µm). Seven-day old conidia from *T. asperellum* culture grown on potato dextrose agar (PDA) were harvested. The conidia were dislodged using an L-shaped glass rod with 10 mL of sterile distilled water. The conidia suspension was then filtered through Whatman® Grade 1 filter paper. Subsequently, the filtered suspension was made up to 1 L with sterile distilled water. Conidia counts were fixed in the range of 10^8 conidia/mL. Precisely, 250 mL of the filtered suspension was used to drench the soil around the stem of each seedling as

Table 1
Treatments for in vivo *nursery trial to assess oil palm seedling growth promotion*

Treatment	Description
BT	Plant + *Trichoderma asperellum* + *Bacillus cereus*
T	Plant + *Trichoderma asperellum*
B	Plant + *Bacillus cereus*
UC	+ Plant (Untreated negative control)

set in Table 1. A booster application with similar concentration was applied 21 days after initial application.

Vegetative Growth Assessment of Oil Palm Seedlings

In determining the effects of treatments on oil palm seedling growth and development, parameters such as plant height, root dry weight, top dry weight (stem to leaf), bole girth size, bole weight, and chlorophyll content were recorded throughout the nursery trial on a monthly basis followed by destructive sampling at the end of the treatments after a duration of 6 months. A measuring tape was used to measure plant height from soil level to the most elevated seedling leaf. Harvested seedling parts, such as dry top and root weights, were measured and recorded using analytical balance (A&D Company, GF-300) after a drying process in an oven at 70°C for 72 hours. Chlorophyll content or "greenness" was measured by Spectrum SPAD 502 Plus meter.

Experimental Design and Statistical Analysis

A randomised complete block design (RCBD) of 4 treatments with 12 oil palm seedlings for each treatment was used as the experimental design for the *in vivo* nursery trial. The 48 oil palm seedlings in the polythene bags were arranged on 6 benches in a randomized manner. Therefore, each bench would be a block, and all treatments were randomly assigned to every block. Block factors, namely different light, temperature, and moisture conditions that could affect the response variable were also taken into consideration.The selection of RCBD design in this study was able to eliminate the bias factors particularly the light factor. Light is a vital factor of plant growth via photosynthesis. Therefore, the current study managed to be assessed solely on the effects by plant growth promoters applied. All data were subjected to analysis of variance (ANOVA) with means comparison by least significant difference (LSD) at $p \leq 0.05$ using SAS® software version 9.4 (SAS Institute Inc., 1995).

Production of Indole Acetic Acid (IAA)

Indole acetic acid (IAA), synthesized by *B. cereus* and *T. asperellum* isolates, was measured calorimetrically using Salkowski's reagent following procedures of Glickmann and Dessaux (1995) as well as Gordon and Weber (1951) respectively. The method quantified the amount of IAA produced in aqueous solution containing precursor L-tryptophan. A standard curve using a series of IAA dilutions were generated at 0, 50, 100, 150, 200, 250, 300, 350, and 400 µg/mL to quantify the amount of IAA produced by both *B. cereus* and *T. asperellum*.

Bacillus cereus isolate was cultured in a nutrient broth (NB) (Difco™) at 28 ± 2°C on a shaker at 150 rpm. After 24 hours, 1 mL of bacterial culture from previous NB was pipetted into 100 mL of modified NB (Difco™) (added with 5 mL of 100 mg L^{-1} of L-tryptophan solution). The bacterium was allowed to grow in the modified NB

(Difco™) for 48 hours. An amount 1.5 mL of bacterial culture was centrifuged for 5 minutes at 1, 7709 x*g*. Upon completion of centrifugation, 1 mL of supernatant was pipetted out and added to 2 mL of Salkowski's reagent. A spectrophotometer (Thermo Fisher Scientific, Finland) was used to record colour densities of the mixture at 530 nm after incubating for 25 minutes at room temperature.

For culture of *T. asperellum,* 200 mL of potato dextrose broth (PDB) (Difco™), modified by adding 5 mL of 100 mg L^{-1} L-tryptophan solution was used. Conidial suspension at 10^8 of *T. asperellum* was used to inoculate the modified (added with L-tryptophan solution) PDB (Difco™) and incubated for 8 days at room temperature. Subsequently, conidial suspension was centrifuged for ten minutes at 1, 107 x*g* and filtered through a 0.22 µm syringe filter. An amount 1 mL of supernatant was pipetted out and mixed with 2 drops of orthophosphoric acid (Sigma-Aldrich, USA) and 2 ml. of Salkowski's reagent (2% of 0.5 M $FeCl_3$ in 35% $HClO_4$ solution). Thermo Scientific Multiskan GO spectrophotometer (Thermo Fisher Scientific, Finland) was used to record colour densities of the mixture after incubating for 20 minutes in the dark at room temperature. The spectrometer was set at 530 nm absorbance.

Siderophore Production Assay

Siderophore production assay was carried out following procedure used by Alexander and Zuberer (1991). Four solutions were prepared, sterilized separately and mixed to produce chrome azurol sulphonate (CAS) agar based on their formula. Chrome azurol sulphonate (CAS) agar was poured into Petri plates and allowed to solidify. A cork-borer (5 mm in size) was used to punch four holes on each of the 14 CAS agar plate. Bacterial inocula and conidia suspension each at 100 µL were dispensed into the holes of the agar in separate plates. The Petri plates were incubated for 7 days at 28 ± 2ºC. The capability to yield siderophore was determined by measuring the diameter of orange halos exhibited after the duration of incubation.

Phosphate Solubilization Test

Phosphate solubilization test was done following the procedure of Mehta and Nautiyal (2001). Both UPM 15 and UPM 16 isolates were inoculated into NB (Difco™) and PDB (Difco™) respectively. A cork-borer (5mm in size) was used to punch four holes on each of the National Botanical Research Institute (NBRIP) agar plate (14 replicates were prepared for each treatment). After 48 hours of inoculation, 100 µL of bacterial inoculum and conidial suspension were dispensed into the holes of the agar medium. The Petri plates were incubated for 7 days at 28 ± 2ºC. The presence of clear zones around the bacterial and fungal colonies was used as indicators for positive phosphate solubilization. The effectiveness of the microbes to solubilize phosphate was determined by measuring the diameter of the clear zones.

RESULTS

Influence of Plant Growth Promoters on Vegetative Growth Oil Palm Seedlings

Single applications of *T. asperellum* treatments recorded the highest plant height (86.9 cm), followed by the single applications of *B. cereus* with 85.3 cm (Table 2). Nevertheless, the plant height for all treatments significantly increased between the start of treatments and 6 months after. Figure 1 shows visible differences on oil palm seedling height for all treatments.

Table 2
Impact of application of plant growth promoters on oil palm seedling vegetative parameters

Treatments	Vegetative parameters (average readings)				
	Height (cm)	Bole size (cm)	Bole dry weight (g)	Top dry weight (g)	Root dry weight (g)
BT	81.0 ± 2.3^{b}	3.80 ± 0.08^{a}	36.0 ± 2.5^{a}	215.0 ± 36.7^{a}	80.0 ± 10.9^{a}
T	86.9 ± 3.1^{a}	3.40 ± 0.05^{b}	31.4 ± 2.1^{a}	219.8 ± 24.0^{a}	63.8 ± 6.8^{ab}
B	85.3 ± 2.7^{a}	3.40 ± 0.08^{b}	32.3 ± 1.2^{a}	161.3 ± 10.8^{ab}	71.3 ± 13.8^{ab}
UC	77.1 ± 2.2^{c}	3.10 ± 0.05^{c}	31.1 ± 0.9^{a}	117.5 ± 5.6^{b}	45.0 ± 9.1^{c}

Note. Values are the means ± S.E. (n = 12). Means followed by same letters in the same columns and rows are not significantly different at $p = 0.05$ using Duncan's multiple range test. Treatments: BT = *Bacillus cereus* + *Trichoderma asperellum*, T = *Trichoderma asperellum*, B = *Bacillus cereus*, UC = Untreated control

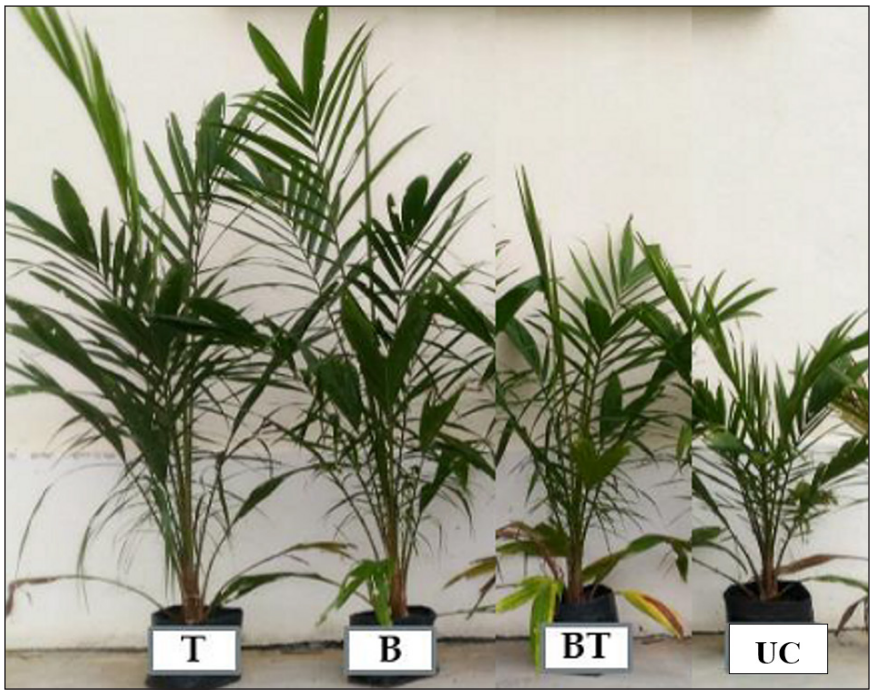

Figure 1. Visible view of oil palm seedling height for all the treatments at 6 months after treatment. Scale bar represents 10 cm (*Note*. Treatments: BT = *Bacillus cereus* + *Trichoderma asperellum*, T = *Trichoderma asperellum*, B = *Bacillus cereus*, UC = Untreated control)

The present study recorded that bole size in almost all treatments showed no significant difference except for *B. cereus* and untreated control treatments (UC). Mixed application of *B. cereus* contributed the highest bole girth size of 3.80 cm. Mixed application also recorded the weightiest dry bole weight of 36.0 g (Table 2).

Data on top weight implied that *T. asperellum* enhanced the growth of top parts of the seedlings. Single applications of *T. asperellum* and mixed applications of *B. cereus* and *T. asperellum* were significantly superior to other treatments, recording 219.8 g and 215.0 g of dry top weight respectively (Table 2). Mixed applications of *B. cereus* and *T. asperellum* were able to produce the heaviest root dry weight (80.0 g), and single applications of *B. cereus* yielded 71.3 g of dry weight (Table 2). Figure 2 shows the visible abundance of oil palm roots in the treatments.

Data on the chlorophyll content demonstrated that chlorophyll content started to decrease at 2 months. A slight increase was noted at 6 months. At 6 months, treatment with *T. asperellum* gave the highest chlorophyll content with 41.9^{a} SPAD unit, followed by untreated control (40.9^{a} SPAD unit), treatment *B. cereus* (41.1^{a} SPAD unit), and mixture treatment (39.8^{a} SPAD unit). However, there were no significant differences in chlorophyll content between all treatments (Table 3).

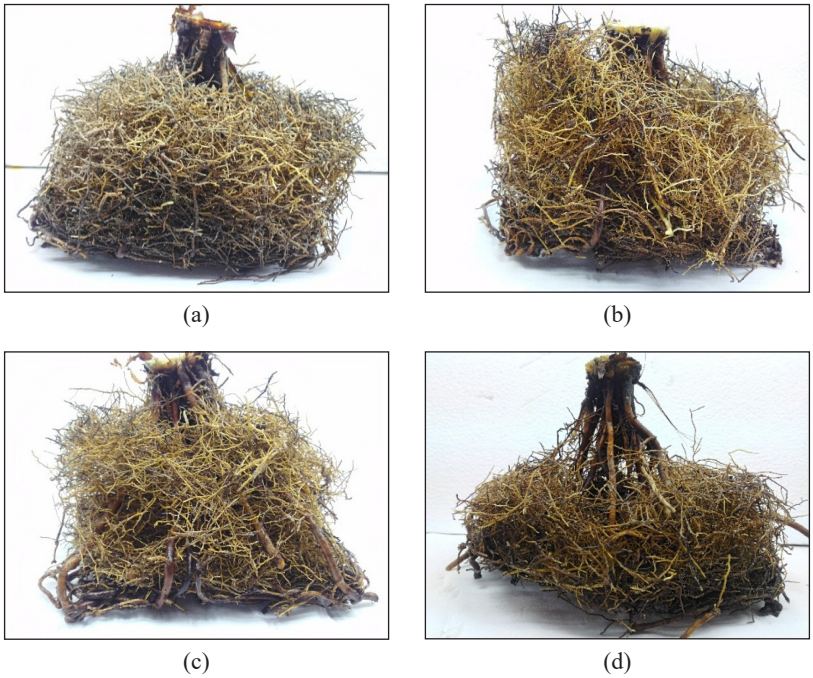

Figure 2. Visibility of oil palm seedling roots for all the treatments at 6 months after treatment: (a) BT = *Bacillus cereus* + *Trichoderma asperellum*; (b) T = *Trichoderma asperellum*; (c) B = *Bacillus cereus*; and (d) UC = Untreated control

Table 3
Effect of Trichoderma asperellum *and* Bacillus cereus *on chlorophyll content of oil palm*

Treatments	Month after inoculation (MAI) (SPAD unit)						
	MAI 0	MAI 1	MAI 2	MAI 3	MAI 4	MAI 5	MAI 6
BT	50.0 ± 1.7^{ab}	52.4 ± 1.5^{a}	47.5 ± 1.4^{a}	40.4 ± 1.4^{a}	37.5 ± 1.7^{a}	35.3 ± 1.3^{a}	39.8 ± 2.3^{a}
T	50.7 ± 1.7^{ab}	51.6 ± 1.9^{a}	38.7 ± 1.6^{b}	38.2 ± 1.7^{a}	37.4 ± 1.5^{a}	35.6 ± 2.3^{a}	41.9 ± 2.6^{a}
B	53.3 ± 1.8^{a}	52.3 ± 1.6^{a}	45.4 ± 1.8^{a}	43.1 ± 1.8^{a}	41.2 ± 1.7^{a}	38.8 ± 2.6^{a}	41.1 ± 1.8^{a}
UC	47.3 ± 1.8^{b}	50.7 ± 2.6^{a}	45.1 ± 1.4^{a}	42.4 ± 1.7^{a}	36.3 ± 2.2^{a}	40.1 ± 1.8^{a}	40.9 ± 1.5^{a}

Note. Values are the means ± S.E. (n=12). Means followed by same letters in the same columns and rows are not significantly different at $p = 0.05$ using Duncan's multiple range test. Treatments: BT = *Bacillus cereus* + *Trichoderma asperellum*, T = *Trichoderma asperellum*, B = *Bacillus cereus*, UC = Untreated control

Table 4
Plant growth promotion traits exhibited by bioinoculants on oil palm seedlings

Bioinoculant	IAA production	Siderophore production assay	Phosphate solubilization test
	[Mean IAA production (ug/ml)]	[Mean orange halos (cm)]	[Mean clear zone (cm)]
Bacillus cereus	9.99 ± 0.03^{a}	0.40 ± 0.01^{b}	0.60 ± 0.01^{b}
Trichoderma asperellum	5.83 ± 0.02^{b}	2.90 ± 0.04^{a}	1.00 ± 0.03^{a}

Note. Values are the means ± S.E. (n=12). Means followed by same letters in the same columns and rows are not significantly different at $p = 0.05$ using Duncan's multiple range test

Indole Acetic Acid (IAA) Production

In the present study, the treatment with *B. cereus* (9.99 ± 0.03^{a} µg/mL) produced more IAA than with *T. asperellum* (5.83 ± 0.02^{b} µg/mL) as shown in Table 4. However, both isolates were able to produce IAA which was one of the most vital aspects in selecting plant growth promoters in terms of plant growth promotion traits.

Siderophore Production Ability

The presence of orange halos indicated the production of siderophore by the isolates. After 7 days of incubation in the culture chamber, *T. asperellum* showed significantly larger measurement of orange halos at 2.9 ± 0.04 cm compared to *B. cereus* which exhibited only 0.4 ± 0.01 cm (Table 4). Both microbes confirmed the ability to produce siderophore.

Phosphate Solubilization Test

Both isolates in the present study were found to be able to solubilize phosphate when tested using NBRIP medium. After 7 days of incubation at room temperature, *T. asperellum* gave significantly higher average clear zones which were 1.0 cm compared to *B. cereus* that gave only an average of 0.6 cm clear zone (Table 4). In the test, the bigger the average measurement of clear zones, the greater the ability of the microbes to solubilize phosphate.

DISCUSSION

Awareness on the importance of sustainable practices in the application of fertilizers in the plantation sector occurred quite recently. In the past, agricultural producers had been relying on chemical fertilizers to be applied in the plantations. Planters had little or no awareness of the long-term harmful effects of chemical fertilizer application. They routinely used chemical fertilizers due to being readily available, easy to use and typically result is instant (Cawoy et al., 2011; Ntow et al., 2006). However, awareness on the possible harmful effects to the planters themselves and the environment at large has encouraged use of bioinoculants as an alternative to chemical fertilizers, approaching towards minimal use of chemicals (Cawoy et al., 2011). According to Chen et al. (2012), microbes that live in the soil have the potential to be used as plant growth promoters as the soil rhizospheres act as the first line of defense for plant's roots against pathogens. *Bacillus cereus* and *T. asperellum* isolates used in the present study were previously isolated from oil palm roots in two different studies by Musa et al. (2018) and Nusaibah et al. (2017), respectively. The two isolates were proven to be excellent *Ganoderma boninense* growth suppressors via *in vitro* and *in vivo* tests and trials (Musa et al., 2018; Nusaibah et al., 2017).

In the present study, enhanced vegetative growth was demonstrated in treated seedlings when compared to untreated seedlings. Mixed treatments of *T. asperellum* and *B. cereus* demonstrated a significant increase in bole, top and root weights. These discoveries were in agreement with Nusaibah et al. (2017). Another significant finding was on the application of *T. asperellum* which yielded superior plant height compared to other treatments. The findings were in consistence with Harman et al. (2004) who reported that *Trichoderma* sp. improved nutrient uptake in maize and subsequently enhanced plant growth.

Inoculant of *B. cereus* performed better in increasing the bole and root weights of the seedlings. A previous study by J. L. Zhao et al. (2010) demonstrated that bacterial polysaccharide extracted from *B. cereus* significantly improved the biomass of *Salvia miltiorrhiza* hairy roots. Dawwam et al. (2013) reported that the application of *B. cereus* as a biofertilizer constituent showed positive plant growth promoting trait.

The present study revealed that the application of both microbes did not have any significant impact on chlorophyll content. These findings were consistent with Pereira et al. (2015), who reported that *Azospirillum brasilense* did not significantly influence chlorophyll content of maize. The present findings did not complement a research conducted by Anuar et al. (2015), where fungus *Phlebia* sp. isolate increased all vegetative growth parameters of oil palm including total chlorophyll content.

Production of IAA, phosphate solubilisation ability, and siderophore production were parameters assessed for plant growth promotion activities of the microbes under study. Work by Simon et al. (2013) showed that both *B. cereus* and

T. asperellum demonstrated the ability to produce IAA, the most active auxin. The present findings were also consistent with other studies which demonstrated the ability of *Bacillus* spp. and *T. asperellum* to produce IAA that, in turn, promoted plant growth and played roles in plants defense responses (Hermosa et al., 2012; Husen, 2003).

Phosphorus is one of the most inadequate elements present in soil compared to other macronutrients (Bünemann et al., 2010). However, there are some microbes that have the ability to solubilize precipitated phosphate into a suitable form for plant uptake (Kang et al., 2002; Pradhan & Sukla, 2006). Thus, in selecting excellent plant growth promoters, the ability of microbes to solubilize phosphate should be considered. The present study conducted using NBRIP gar medium, demonstrated that *B. cereus* and *T. asperellum* had the ability to solubilize phosphate. These findings were in agreement with L. Zhao and Zhang (2015) which disclosed that *T. asperellum* could solubilize inorganic and organic phosphates. Maheswar and Sathiyavani (2012) reported that *Bacillus cereus* and *Bacillus subtilis* were active in solubilization of tricalcium phosphate under *in vitro* conditions. Jones and Oburger (2011) also stated that several species of soil bacteria such as *Pseudomonas*, *Azotobacter*, *Burkholderia*, *Bacillus*, and *Rhizobium* were able to solubilize precipitated phosphates.

Various bacteria, fungi, plants, and yeast have been reported to produce and release siderophore to up take ferric ion from the environment (Chu et al., 2010). Iron is required in vital processes such as chlorophyll production (Encarnação et al., 2012) and enzyme functions (Ghasemian & Ghalavand, 2010; Grotz & Guerinot, 2006) in maintaining plant health. Therefore, one the most essential traits of potential plant growth promoters is the ability to produce siderophore. The results of the present study indicated that both *B. cereus* and *T. asperellum* exhibited positive siderophore production. The findings were consistent with Qi and Zhao (2013) that showed *T. asperellum* with capabilities of producing siderophores of up to 96.6% siderophore units after 2 days on CAS agar medium. Triveni et al. (2013) also demonstrated that *Trichoderma–Bacillus* and *Trichoderma–Pseudomonas* biofilms exhibited higher siderophore production.

The *in vitro* assessment conducted on isolates *B. cereus* and *T. asperellum* demonstrated their ability in contributing towards plant growth promotion traits in a controlled environment. However, the *in vitro* studies supported the physiological parameters assesed via *in vivo* study. For instance, highest concentration of IAA was produced by *B. cereus in vitro* which correlates with the highest root dry weight in *B. cereus* treated oil palm seedlings. IAA is the most abundant and basic auxin hormone produced in the roots, stem and bud. Nonetheless, the highest top dry weight could be interrelated with the efficientcy of *T. asperellum* in producing siderophore and solubilizing phosphate, which is involved in the synthesis of chorophyll and adenosine 5'-triphosphate

(ATP), respectively. Phosphorus is a dynamic component structure of ATP used for performing photosynthesis. Results provided further support that these 2 isolates could be considered as potential plant growth promoters and could be tested via nursery trial (*in vivo*) to evaluate their effectiveness against basal stem rot disease of oil palm.

CONCLUSION

The present study concluded that *Trichoderma asperellum* contributed significantly towards growth of aerial parts while *Bacillus cereus* towards root growth of oil palm seedlings. Mixed treatments of *T. asperellum* and *B. cereus* complemented each other in contributing towards a wholesome plant growth promotion effects on the oil palm seedlings. As bioinoculants, the study proved that the *in vitro* assessment conducted on *B. cereus* and *T. asperellum* isolates demonstrated their abilities in producing compounds that might have contributed towards significant plant growth promotion activities of oil palm seedlings.

ACKNOWLEDGMENT

Putra Grant-IPS via Research and Management Centre (RMC), Universiti Putra Malaysia (UPM) (Grant No: GP-IPS/2016/9503700).

REFERENCES

Adriano, D. C. (2003). *Trace elements in terrestrial environments: Biogeochemistry bioavailability and risks of metals* (2nd ed.). Springer. https://doi.org/10.1007/978-0-387-21510-5

Alexander, D. B., & Zuberer, D. A. (1991). Use of chrome azurol S reagents to evaluate siderophore production by rhizosphere bacteria. *Biology and Fertility of Soils*, *12*(1), 39-45. https://doi.org/10.1007/BF00369386

Anuar, E. N., Nulit, R., & Idris, A. S. (2015). Growth promoting effects of endophytic fungus *Phlebia* GanoEF3 on oil palm (*Elaeis guineensis*) seedlings. *International Journal of Agriculture and Biology*, *17*(1), 135-141.

Bünemann, E. K., Oberson, A., & Frossard, E. (Eds.). (2010). *Phosphorus in action: Biological processes in soil phosphorus cycling*. Springer. https://doi.org/10.1007/978-3-642-15271-9

Cawoy, H., Bettiol, W., Fickers, P., & Ongena, M. (2011). Bacillus-*based biological control of plant diseases*. https://www.intechopen.com/books/pesticides-in-the-modern-world-pesticides-use-and-management/bacillus-based-biological-control-of-plant-diseases

Chen, Z. H., Chen, L. J., & Wu, Z. J. (2012). Relationships among persistence of *Bacillus thuringiensis* and Cowpea trypsin inhibitor proteins, microbial properties and enzymatic activities in rhizosphere soil after repeated cultivation with transgenic cotton. *Applied Soil Ecology*, *53*, 23-30. https://doi.org/10.1016/j.apsoil.2011.10.019

Chu, B. C., Garcia-Herrero, A., Johanson, T. H., Krewulak, K. D., Lau, C. K., Peacock, R. S., Slavinskaya, Z., & Vogel, H. J. (2010). Siderophore uptake in bacteria and the battle for iron with the host; a bird's eye view. *Biometals*, *23*(4), 601-611. https://doi.org/10.1007/s10534-010-9361-x

Dawwam, G. E., Elbeltagy, A., Emara, H. M., Abbas, I. H., & Hassan, M. M. (2013). Beneficial effect of plant growth promoting bacteria isolated from the roots of potato plant. *Annals of Agricultural Sciences*, *58*(2), 195-201. https://doi.org/10.1016/j.aoas.2013.07.007

Encarnação, T., Burrows, H. D., Pais, A. C., Campos, M. G., & Kremer, A. (2012). Effect of N and P on the uptake of magnesium and iron and on the production of carotenoids and chlorophyll by the microalgae *Nannochloropsis* sp.. *Journal of Agricultural Science and Technology*, *2*, 824-832.

Ghasemian, V., & Ghalavand, A. (2010). The effect of iron, zinc and manganese on quality and quantity of soybean seed. *Journal of Phytology*, *2*(11), 73-79.

Glickmann, E., & Dessaux, Y. (1995). A critical examination of the specificity of the Salkowski reagent for indolic compounds produced by phytopathogenic bacteria. *Applied Environmental Microbiology*, *61*(2), 793-796. https://doi.org/10.1128/AEM.61.2.793-796.1995

Gordon, S. A., & Weber, R. P. (1951). Colorimetric estimation of indoleacetic acid. *Plant Physiology*, *26*(1), 192-195. https://doi.org/10.1104/pp.26.1.192

Grotz, N., & Guerinot, M. L. (2006). Molecular aspects of Cu, Fe and Zn homeostasis in plants. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research*, *1763*(7), 595-608. https://doi.org/10.1016/j.bbamcr.2006.05.014

Harman, G. E., Howell, C. R., Viterbo, A., Chet, I., & Lorito, M. (2004). *Trichoderma* species-opportunistic, avirulent plant symbionts. *Nature Reviews Microbiology*, *2*(1), 43-56. https://doi.org/10.1038/nrmicro797

Hermosa, R., Viterbo, A., Chet, I., & Monte, E. (2012). Plant-beneficial effects of *Trichoderma* and of its genes. *Microbiology*, *158*(1), 17-25. https://doi.org/10.1099/mic.0.052274-0

Husen, E. (2003). Screening of soil bacteria for plant growth activities *in vitro*. *Indonesia Journal of Agriculture Science*, *4*(1), 27-31. https://doi.org/10.21082/ijas.v4n1.2003.p27-31

Izzati, M. Z. N. A., & Abdullah, F. (2008). Disease suppression in *Ganoderma*-infected oil palm seedlings treated with *Trichoderma harzianum*. *Plant Protection Science*, *44*(3), 101-107. https://doi.org/10.17221/23/2008-PPS

Jones, D. L., & Oburger, E. (2011). Solubilization of phosphorus by soil microorganisms. In E. K. Bünemann, A. Oberson, & E. Frossard (Eds.), *Phosphorus in action* (pp. 169-198). Springer. https://doi.org/10.1007/978-3-642-15271-9_7

Kang, S. C., Ha, C. G., Lee, T. G., & Maheshwari, D. K. (2002). Solubilization of insoluble inorganic phosphates by a soil-inhabiting fungus *Fomitopsis* sp. PS102. *Current Science*, *82*(4), 439-442.

Khan, M. N., Mobin, M., Abbas, Z. K., & Alamri, S. A. (2018). Fertilizers and their contaminants in soils, surface and groundwater. *Encyclopedia of the Anthropocene*, *5*, 225-240. https://doi.org/10.1016/B978-0-12-809665-9.09888-8

Khan, S., Cao, Q., Zheng, Y. M., Huang, Y. Z., & Zhu, Y. G. (2008). Health risks of heavy metals in contaminated soils and food crops irrigated with wastewater in Beijing, China. *Environmental Pollution*, *152*(3), 686-692. https://doi.org/10.1016/j.envpol.2007.06.056

Maheswar, N. U., & Sathiyavani, G. (2012). Solubilization of phosphate by *Bacillus* sp., from groundnut rhizosphere (*Arachishypogaea* L.). *Journal of Chemical and Pharmaceutical Research*, *4*(8), 4007-4011.

Maslin, P., & Maier, R. M. (2000). Rhamnolipid-enhanced mineralization of phenanthrene in organic-metal co-contaminated soils. *Bioremediation Journal*, *4*(4), 295-308. https://doi.org/10.1080/10889860091114266

Mehta, S., & Nautiyal, C. S. (2001). An efficient method for qualitative screening of phosphate-solubilizing bacteria. *Current Microbiology*, *43*(1), 51-56. https://doi.org/10.1007/s002840010259

Musa, H., Nusaibah, S. A., & Khairulmazmi, A. (2018). Assessment on *Trichoderma* spp. mixture as a potential biocontrol agent of *Ganoderma boninense* infected oil palm seedlings. *Journal of Oil Palm Research*, *30*(3), 403-415. https://doi.org/10.21894/jopr.2018.0035

Naher, L., Yusuf, U. K., Siddiquee, S., Ferdous, J., & Rahman, M. A. (2012). Effect of media on growth and antagonistic activity of selected *Trichoderma* strains against *Ganoderma*. *African Journal of Microbiology Research*, *6*(48), 7449–7453. https://doi.org/10.5897/AJMR12.1216

Nolan, B. T., Hitt, K. J., & Ruddy, B. C. (2002). Probability of nitrate contamination of recently recharged groundwaters in the conterminous United States. *Environmental Science and Technology*, *36*(10), 2138-2145. https://doi.org/10.1021/es0113854

Ntow, W. J., Gijzen, H. J., Kelderman, P., & Drechsel, P. (2006). Farmer perceptions and pesticide use practices in vegetable production in Ghana. *Pest Management Science*, *62*(4), 356-365. https://doi.org/10.1002/ps.1178

Nusaibah, S. A., Saad, G., & Tan, G. H. (2017). Antagonistic efficacy of *Trichoderma harzianum* and *Bacillus cereus* against *Ganoderma* disease of oil palm via dip, place and drench (DPD) artificial inoculation technique. *International Journal of Agriculture and Biology*, *19*(2), 299-306. https://doi.org/10.17957/IJAB/15.0280

Pereira, L. D. M., Pereira, E. D. M., Revolti, L. T. M., Zingaretti, S. M., & Môro, G. V. (2015). Seed quality, chlorophyll content index and leaf nitrogen levels in maize inoculated with *Azospirillum brasilense*. *Revista Ciência Agronômica*, *46*(3), 630-637. https://doi.org/10.5935/1806-6690.20150047

Pradhan, N., & Sukla, L. B. (2006). Solubilization of inorganic phosphates by fungi isolated from agriculture soil. *African Journal of Biotechnology*, *5*(10), 850-854.

Qi, W., & Zhao, L. (2013). Study of the siderophore-producing *Trichoderma asperellum* Q1 on cucumber growth promotion under salt stress. *Journal of Basic Microbiology*, *53*(4), 355-364. https://doi.org/10.1002/jobm.201200031

Salman, J. M., Njoku, V. O., & Hameed, B. H. (2011). Batch and fixed-bed adsorption of 2,4-dichlorophenoxyacetic acid onto oil palm frond activated carbon. *Chemical Engineering Journal*, *174*(1), 33-40. https://doi.org/10.1016/j.cej.2011.08.024

Shoresh, M., Yedidia, I., & Chet, I. (2005). Involvement of jasmonic acid/ethylene signaling pathway in the systemic resistance induced in cucumber by *Trichoderma asperellum* T203. *Phytopathology*, *95*(1), 76-84. https://doi.org/10.1094/PHYTO-95-0076

Simon, S., Kubeš, M., Baster, P., Robert, S., Dobrev, P. I., Friml, J., Petrášek, J., & Zažímalová, E. (2013). Defining the selectivity of processes along the auxin response chain: a study using auxin analogues. *New Phytologist*, *200*(4), 1034-1048. https://doi.org/10.1111/nph.12437

Triveni, S., Prasanna, R., Shukla, L., & Saxena, A. K. (2013). Evaluating the biochemical traits of novel *Trichoderma*-based biofilms for use as plant growth-promoting inoculants. *Annals of Microbiology*, *63*(3), 1147-1156. https://doi.org/10.1007/s13213-012-0573-x

Vinale, F., Sivasithamparam, K., Ghisalberti, E. L., Marra, R., Barbetti, M. J., Li, H., Woo, S. L., & Lorito, M. (2008). A novel role for *Trichoderma* secondary metabolites in the interactions with plants. *Physiological and Molecular Plant Pathology*, *72*(1-3), 80-86. https://doi.org/10.1016/j.pmpp.2008.05.005

Wolfe, A. H., & Patz, J. A. (2002). Reactive nitrogen and human health: Acute and long-term implications. *AMBIO: A Journal of the Human Environment*, *31*(2), 120-125. https://doi.org/10.1579/0044-7447-31.2.120

Wuana, R. A. & Okieimen, F. E. (2011). Heavy metals in contaminated soils: A review of sources, chemistry, risks and best available strategies for remediation. *International Scholarly Research Notices*, *2011*, 402647. https://doi.org/10.5402/2011/402647

Yedidia, I., Shoresh, M., Kerem, Z., Benhamou, N., Kapulnik, Y., & Chet, I. (2003). Concomitant induction of systemic resistance to *Pseudomonas syringae* pv. lachrymans in cucumber by *Trichoderma asperellum* (T-203) and accumulation of phytoalexins. *Applied Journal of Environmental Microbiology*, *69*(12), 7343-7353. https://doi.org/10.1128/aem.69.12.7343-7353.2003

Zaiton, S., Sariah, M., & Ahmad, Z. A. M. (2008). Effect of endophytic bacteria on growth and suppression of *Ganoderma* infection in oil palm. *International Journal of Agriculture Biology*, *10*(2), 127-132.

Zhao, J. L., Zhou, L. G., & Wu, J. Y. (2010). Promotion of *Salvia miltiorrhiza* hairy root growth and tanshinone production by polysaccharide–protein fractions of plant growth-promoting rhizobacterium *Bacillus cereus*. *Process Biochemistry*, *45*(9), 1517-1522. https://doi.org/10.1016/j.procbio.2010.05.034

Zhao, L., & Zhang, Y. Q. (2015). Effects of phosphate solubilization and phytohormone production of *Trichoderma asperellum* Q1 on promoting cucumber growth under salt stress. *Journal of Integrated Agriculture*, *14*(8), 1588-1597. https://doi.org/10.1016/S2095-3119(14)60966-7

Zhao, L., Xu, Y., Sun, R., Deng, Z., Yang, W. & Wei, G. (2011). Identification and characterization of the endophytic plant growth promoter *Bacillus cereus* strain MQ23 isolated from *Sophora alopecuroides* root nodules. *Brazilian Journal of Microbiology*, *42*(2), 567–575. https://doi.org/10.1590%2FS1517-838220110002000022

Pertanika J. Trop. Agric. Sci. 44 (1): 171 - 192 (2021)

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Journal homepage: http://www.pertanika.upm.edu.my/

Molecular Characterisation of the GdhA$^-$ Derivative of *Pasteurella multocida* B:2

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ABSTRACT

Pasteurella multocida B:2 is an important veterinary pathogen causing fatal and acute haemorrhagic septicaemia (HS) in bovine. A live vaccine candidate, *P. multocida* B:2 GDH7 was reported to enable protection in cattle and buffaloes via intranasal (i. n.) administration. This potential vaccine was also reported to be self-transmitted from the vaccinated animal to the free-ranging animals allowing wider vaccination coverage. Prior to commercialisation, this potential vaccine requires further characterisation in accordance with the authoritative guidelines from the World Organisation for Animal Health (OIE). Hence, in this study, the potential vaccine strain, *P. multocida* B:2 GDH7 and the virulent parent strain were characterised through genomic and proteomic profiling. A crucial first step was to develop a sensitive yet simple and robust identification test to differentiate both strains which has been achieved by the development of a precise yet straightforward PCR method. In genomic profiling, Repetitive Extragenic Palindromic sequence-PCR (REP-PCR)

ARTICLE INFO

Article history:
Received: 10 September 2020
Accepted: 15 November 2020
Published: 24 February 2021

DOI: https://doi.org/10.47836/pjtas.44.1.10

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ISSN: 0128-7680
e-ISSN: 2231-8526

was manipulated and both strains have a different display of genomic DNA band patterns. Some of the major OMPs were observed and prominent immunogens of *P. multocida*, OmpA and OmpH were observed to be expressed differently between these strains through SDS-PAGE analysis. In conclusion, a reproducible PCR detection method has enabled differentiation of both strains. Further characterisation of these strains shows a significantly different profile through genomic and proteomic profiling.

Keywords: Haemorrhagic septicaemia, live-attenuated vaccine, OMPs, *Pasteurella multocida*, REP-PCR

INTRODUCTION

Hemorrhagic septicaemia (HS) is a major disease in cattle and buffaloes which is caused by the infection of *Pasteurella multocida* B:2 or E:2. In Asia, the serotype of the bacteria responsible for the disease is *P. multocida* B:2 (Zamri-Saad, 2013). This endemic disease causes great economic losses particularly towards farmers due to high mortality rate and rapid transmission of an outbreak field to the adjacent field (Zamri-Saad & Annas, 2016). Transmission occurred from diseased animals or carriers through intranasal and oral routes (Abubakar & Zamri-Saad, 2011). Invasion of the bacteria through endothelial cells resulted in rapid infiltration of the animals' bloodstream (Galdiero et al., 2001). High occurrence of outbreak happened during monsoon or raining season due to the weakening of immune response of the animals making them susceptible to the infection (Zamri-Saad, 2013). Vaccination is an effective routine for controlling outbreak of HS especially in Asian countries due to semi-wild rearing methods of the animals (Zamri-Saad, 2013).

Vaccines for HS usually are registered prior to rainy season using oil-adjuvant vaccine or alum-precipitated vaccine. Despite both vaccines containing bacterin, only short-termed protection was detected (Zamri-Saad, 2013) and also tedious administration process, resulting in ineffective disease outbreak control. Therefore, live-attenuated vaccine was recommended to control HS outbreak efficiently such that this type of vaccine enabled mimicry of natural route of infection like the wild-type (De Alwis, 1999). Live-attenuated vaccine consisted of live organisms such as the attenuated bacteria with reduced virulence when compared to the wild-type (Zamri-Saad, 2013). A local strain of *P. multocida* B:2 from previous outbreak was attenuated into a derivative known as *P. multocida* B:2 GDH7. The mutant was generated through the disruption of the *gdhA* gene by the insertion of kanamycin cassette (Othman et al., 2017). This resulted in the arrested metabolism and thus weakened the pathogenicity of the mutant. Since currently available vaccines such as alum-precipitated vaccine and oil-adjuvant vaccine were discovered to be less effective, a new alternative is paramount. The aforementioned *gdhA* gene disruption has been found to be a promising manipulation for non-pathogenic *P. multocida* B:2 vaccine

development (Othman et al., 2012). Field studies were performed previously to show its effectiveness and apathogenicity when challenged (Rafidah et al., 2012; Rafidah & Zamri-Saad, 2013). However, the characteristics of the mutant were yet to be thoroughly scrutinized. Therefore, further information of the developed live vaccine can enable comprehension on its efficacy for HS control and prevention.

For commercialisation purposes, upscaling the potential vaccine strain *P. multocida* B:2 GDH7 with an economical media is recommended when compared to the commercial available media (Sarwar et al., 2013). This establishment will provide a more competitive and sustainable product in the market. Hence, a minimal media known as YDB media was formulated with the purpose to reduce production cost of *P. multocida* B:2 GDH7 as vaccine strain for HS (Hazwani-Oslan et al., 2017; Oslan et al., 2018). The formulated media has enabled significant reduction by about 10-fold per litre of the cell production with no significant effect on cells concentration when compared to the commercially available media (Oslan et al., 2018).

The organisation specified in the animals' health concerns, World Organisation for Animal Health or also initially known as Office International des Epizooties (OIE) has regulated the production of vaccines in order to assure safe vaccines for animal diseases control in each country (World Organisation for Animal Health [OIE], 2008, 2016). However, the profile characterisation of the attenuated strain, *P. multocida* B:2 GDH7 has yet to be fully apprehended. In this study, a conventional PCR assay was established as a detection method to distinguish *P. multocida* B:2 GDH7 from its parent strain. Hence, unique primers to the attenuated *P. multocida* B:2 GDH7 were generated. Genotypic and phenotypic profiling of a modified bacterial strain is vital in ensuring vaccine quality according to the OIE's vaccine production guidelines. Therefore, the information on the genomic and proteomic profiles of the bacterium used for vaccination will enable better understanding towards the vaccine and its efficacy prior to commercialisation.

METHODS

Bacterial Strains

Pasteurella multocida strains used in this study were listed in Table 1. All *P. multocida* strains were cultured on Brain-Heart Infusion (BHI) (Oxoid, UK) or YDB modified media agar plates or in broth. YDB is a minimal media developed by Oslan et al. (2018) to replace BHI media for the purpose of high-scale production of *P. multocida* B:2 GDH7 as a live-attenuated vaccine for HS. The bacteria were cultured in 37°C and shaken at 180 rpm if in broth.

Genomic DNA Extraction

Genomic DNA extraction of all *P. multocida* strains were performed using overnight cultures. Overnight cultures of each *P. multocida* were pelleted down by centrifugation at 6, 000 x*g* at 4°C. The pellets were used for the genome extraction

Table 1

List of bacteria used in this study

Bacteria name	Description	References
Pasteurella multocida B:2 wild-type	Isolated from an outbreak case in Malaysia, 2006	Othman (2007)
Pasteurella multocida B:2 GDH7	Δ*gdhA* mutant derived from the wild-type strain above	Othman (2007)
Pasteurella multocida B:2 JRMT12	Δ*aroA* mutant derived from the wild-type strain from Sri Lanka	Tabatabaei et al. (2002)

using MYgen™ Genomic DNA Prep Kit from Gene Express (Gene Xpress PLT, Malaysia) according to the manufacturer's protocol. The concentration of the extracted genomic DNA was measured using Implen NanoPhotometer™. The extracted genomic DNA was maintained and stored at -20°C for long-term storage.

PCR Analysis

PCR amplification was carried out with 20 µL of PCR mixture consisting of 50 ng genomic DNA as template, 10 µM of both forward and reverse primers pairs (Table 2), 1X PCR buffer, 20 µM dNTPs mix, 40 µM $MgCl_2$ and 6 U *Taq* polymerase (Thermo Fisher Scientific, US). Eppendorf Mastercycler® Nexus Thermal Cycler was used to amplify with initial denaturation at 95°C for 3 min following with 30-35 cycles of denaturation at 95°C for 30 sec, annealing of the primers at selected temperature (Table 2) for 30 sec and extension at 72°C for 1-5 min. A final extension step was added at 72°C for 10 min. The amplified PCR products were electrophoresed in 1-2% agarose gel (Vivantis, Malaysia) stained with HealthView™ nucleic acid stain (Genomics BioSci &Tech, Taiwan). The nucleic acid stain is a safe option compared to ethidium bromide.

Table 2

List of primers used for detection between Pasteurella multocida *B:2 strains*

Primer name	Primer sequence	Targeted region	Expected fragment size (bp)	Annealing temperature (°C)
Kan Forward	TCGAGCATCAAATGAAACTG	Kanamycin resistance gene (KR)	794[a]	51.3
Kan Reverse	TATTCAACGGGAAACGTCTT			

Table 2 *(Continued)*

Primer name	Primer sequence	Targeted region	Expected fragment size (bp)	Annealing temperature (◦C)
CD1 Forward	CCGCCTTCACTTCTGTTGAATACTC	Probable region containing kanamycin cassette (*gdhA* upstream)	475[ab]	60.7
CD1 Reverse	CCCCATTGGTAATGTGGTTAACGCG			
CD2 Forward	ATGGCGTACAATTAGTGGCTGAAGG	Probable region containing kanamycin cassette (*gdhA* downstream)	389[ab]	59.8
CD2 Reverse	CTTTCTTGACGAACAAAGTGCGGTG			

Note. [a]The expected size was derived from preliminary *in silico* analysis of primers and the targeted sequence respectively based on the parent strain's genome. [b]Addition of a kanamycin cassette with 1, 200 bp is expected to be present in *Pasteurella multocida* B:2 GDH7

REP-PCR Analysis

Repetitive element palindromic-PCR (REP-PCR) analysis in this study was based from Townsend et al. (1997) with minor modifications. PCR mixture with 25 µL containing 50 ng of genomic DNA template, 20 pmol of primer pairs REP1R-Dt (NNNNCGNCGNCATCNGGC) and REP2-IDt (NCGNCTTATCNGGCCTAC), 1.5 U *Taq* polymerase, 125 µM of dNTPs mix, 80 µM $MgCl_2$ and 1X *Taq* Buffer (Thermo Fisher Scientific, US). REP amplification was performed using Eppendorf Mastercycler® nexus thermal cycler with initial denaturation at 95◦C for 3 min following with 30 cycles of denaturation at 95◦C for 30 s, annealing at 45◦C for 1 min and annealing at 72◦C for 3 min. Final extension step was added at 72◦C for 10 min. The amplified REP-PCR products were electrophoresed using agarose gel of 2%.

Cell-Free Proteins Extraction of *Pasteurella multocida* B:2 Strains

Cell-free proteins extraction method was slightly modified from Ataei et al. (2009). Cell-free extracts (CFEs) are proteins obtained by mechanical disruption of bacterial cells to release the cytosolic proteins and the disrupted membrane proteins. A few colonies of each strain of *P. multocida* B:2 was inoculated into respective broth (5 mL) to culture overnight at 37◦C, shaken at 180 rpm. Each 16-18 h cultures was further inoculated into fresh 100 mL (1:500) of broth to grow overnight until it reached OD_{600nm} >1.2. The cultures were

harvested by centrifugation at 6, 000 x*g*, 30 min, 4°C. The cell pellets were washed with PBS and centrifuged at 6, 000 x*g*, 30 min, 4°C. The pellets were resuspended in 5 mL of cold PBS and sonicated for 5 min with 40% amplitude. The sonicated suspension was centrifuged at 6, 000 x*g* for 30 min at 4°C to pellet down the remaining unbroken cells. The CFEs of each *P. multocida* B:2 strains were quantified using Bradford's assay (Bradford, 1976) and stored in -20°C.

Outer Membrane Proteins (OMPs) Extraction of *Pasteurella multocida* B:2 Strains

Extraction of *P. multocida* B:2 strains' OMPs in this study was referred from Prasannavadhana et al. (2014) and Wheeler et al. (2009) with modifications. A few colonies of each strain of *P. multocida* B:2 was inoculated into respective broth (5 mL) to culture overnight at 37°C, shaken at 180 rpm. Each of the overnight cultures (800 µL) was further inoculated into fresh 400 mL (1:500) of broth to grow overnight until it reached OD_{600nm} >1.2. The cultures were harvested by centrifugation at 6, 000 x*g* for 30 min at 4°C. 45 mL of cold 20 mM Tris-HCl (pH 7.2) was added to wash pellet and centrifuged at 6, 000 x*g* for 30 min at 4°C. The pellets were resuspended in 5 mL of cold 20 mM Tris-HCl (pH 7.2) and sonicated for 10 min with 40% amplitude. The sonicated suspension was centrifuged at 6, 000 x*g* for 30 min at 4°C to collect the remaining unbroken cells. The supernatant containing cytoplasmic proteins, cell membrane elements and OMPs was collected and cold 20 mM Tris-HCl (pH 7.2) was added to the suspension until it reached 10 mL. The supernatant was centrifuged using Beckman Coulter Optima™ ultracentrifuge at 100, 000 x*g* for 1 h at 4°C. The resulting pellet was resuspended with 5 mL of 0.5% (w/v) sodium N-lauroylsarcosine (Sarkosyl) (Thermo Fisher Scientific, US) using sterile long-form Pasteur pipette. The sarkosyl solubilisation was done at room temperature for 20 min with intermittent resuspension of the protein pellet. The 0.5% sarkosyl was added to the suspension until it reached 10 mL and then centrifuged at 100, 000 x*g* for 1 h at 4°C to separate the soluble cytoplasmic proteins with the insoluble OMPs. The OMPs pellet was washed with 10 mL of cold 20 mM Tris-HCl (pH 7.2). The suspension was again centrifuged at 100, 000 x*g* for 1 h at 4°C. The OMPs pellet was resuspended in ≤500 µL of 20 mM Tris-HCl (pH 7.2). The OMPs of each strain of *P. multocida* B:2 were quantified with Bradford's assay (Bradford, 1976) and then stored in -20°C.

SDS-PAGE Analysis of Proteins

The proteins were analysed through SDS-PAGE method by Laemmli (1970) with slight modifications. The gel was run with 1X SDS-PAGE running buffer at 70 V for 1 h 45 min. Coomassie-blue staining method was used to visualize and to approximate the proteins bands from the respective proteomic profiles.

RESULTS AND DISCUSSION

Identification and Differentiation Between *Pasteurella multocida* B:2 Wild-type and *P. multocida* B:2 GDH7

The amplification of *gdhA* gene from *P. multocida* B:2 wild-type only was successful whereas identification and differentiation of *P. multocida* B:2 GDH7 was not as expected from previous study (Othman, 2007). Amplification of the *gdhA* gene-based primers was only observed from the wild-type strain whereas no amplifications observed from our mutant strain (Figure 1a). Similar observation was determined through the amplification with the multiplex primers as shown in Figure 1b. In addition, the specifically designed KanR primers has enabled in the amplification of the KR cassette (Table 2) of the mutant when compared to the wild-type. As shown in Figure 1c, only the mutant was observed to possess the ~800 bp DNA band when compared to the wild-type strain. This showed the presence of KR cassette only in the mutant and the positive control which was a vector harbouring the same KR cassette as the mutant.

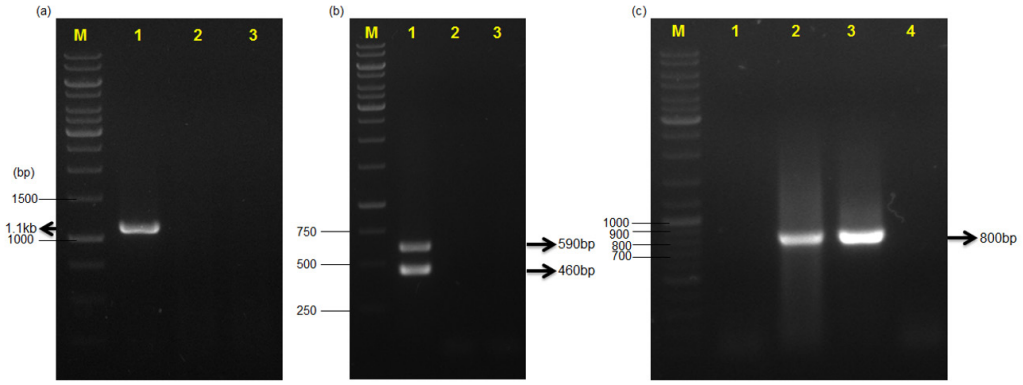


Figure 1. Identification and differentiation between *Pasteurella multocida* B:2 wild-type and *P. multocida* B:2 GDH7. (a) PCR products by using *gdhA* gene-based primers (Othman, 2007): Lane M, DNA ladder; Lane 1, *Pasteurella multocida* B:2 wild-type; Lane 2, *Pasteurella multocida* B:2 GDH7, Lane 3, Negative control. (b) PCR products by using multiplex primers (Townsend et al., 1998): Lane M, DNA ladder; Lane 1, *Pasteurella multocida* B:2 wild-type; Lane 2, *Pasteurella multocida* B:2 GDH7, Lane 3, Negative control. (c) PCR products by using KanR primers: Lane M, DNA ladder; Lane 1, *Pasteurella multocida* B:2 wild-type; Lane 2, *Pasteurella multocida* B:2 GDH7; Lane 3, Positive control; Lane 4, Negative control

Two primer pairs were designed in order to confirm the location of the kanamycin resistance cassette within the genome of *P. multocida* B:2 GDH7 (Figure 2). The primers were designed based on the genome sequence of the wild-type. The strategy in Figure 2 was derived to amplify the targeted part of the mutant's genome to locate the kanamycin gene. Amplification was successful for *P. multocida* B:2 wild-type's

native gene which acted as a negative control in this experiment (Figure 3). Therefore, the primers based on the kanamycin sequence (KanR primers) were sufficient to be used to distinguish both the wild-type and the attenuated *gdhA* derivative. The presence of the kanamycin sequence in the mutant *P. multocida* B:2 GDH7 was evidently distinct but was absent in the *P. multocida* B:2 wild-type as initially expected (Figure 1c). Furthermore, the kanamycin resistance gene (KR) sequence amplified from the mutant was confirmed to be highly similar to the kanamycin resistance gene initially inserted in this mutant strain (Othman et al., 2017) and also to the positive control of pET28 harbouring the same gene (Supplementary material 1).

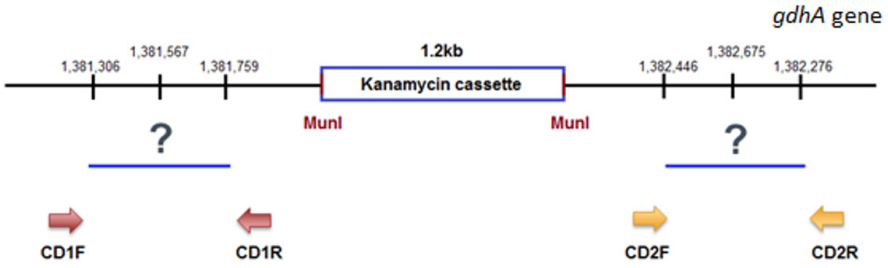


Figure 2. Primers and target size map of kanamycin sequence predicted by *in silico* analysis. The kanamycin sequence was suggested to be located in the region marked '?' after the analysis of the amplification by the respective two primer pairs above

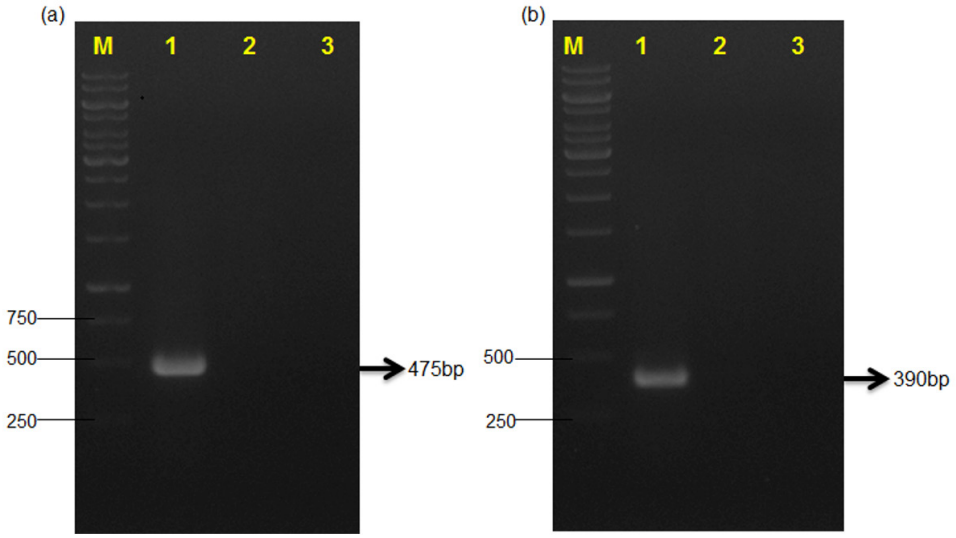


Figure 3. PCR amplification using CD1 and CD2 primers. (a) PCR products amplified by using CD1 primers: (b) PCR products amplified by using CD2 primers: Lane M, DNA ladder; Lane 1, *Pasteurella multocida* B:2 wild-type; Lane 2, *Pasteurella multocida* B:2 GDH7; Lane 3, Negative control

From the PCR amplification based on the strategy in Figure 2, only the wild-type possessed the expected amplified DNA sizes (Figure 3). The amplification by using CD1 primers has produced a DNA band with 475 bp whereas CD2 produced DNA band of 390 bp in wild-type. In contrast, no amplification was observed from the genome of *P. multocida* B:2 GDH7. This could indicate a total disruption of the *gdhA* gene in the *P. multocida* B:2 GDH7 as compared to the wild-type. None of the investigated areas of the gene were amplified when using the CD1 and CD2 primers (Table 2). Further sequencing of the mutant strain can be implemented to investigate the location of the KR cassette in the mutant genome. It was also reported that *GDH1* gene encoding the glutamate dehydrogenase was found to be a hotspot for genetic mutation in *S. cerevisiae*. Manipulation of *GDH1* gene has contributed to genome shuffling and also phenotype change of the organism (Biot-Pelletier et al., 2018). Hence, it can be proposed that KR cassette could have been reshuffled to other region of the bacterial genome. This could have contributed to the inability of kanamycin cassette localisation due to unknown vicinity sequences post-reshuffling. El-Brolosy and Stainier (2017) also commented that the genome reshuffling could have compensated the gene annotations. Hence, in this study this resulted in inability of the targeted product to be amplified from the mutant strain by using the multiplex primers (Figure 1b).

Genomic Profiling of *Pasteurella multocida* B:2 Strains

All the DNA bands profile of *P. multocida* B:2 strains in this study were seen with different patterns and allocated with different molecular weight (MW) sizes. The genomic patterns observed from REP-PCR were reproducible throughout this study. The genomic profile of wild-type strain possessed eight DNA bands comprising of sizes 2.9 kb, 2.3 kb, 1.7 kb, 1.4 kb, 1.1 kb, 850 bp, 700 bp, and 425 bp. Meanwhile, the profile of *P. multocida* B:2 GDH7 consisted of DNA bands with sizes 2.2 kb, 1.6 kb, 1.3 kb, 800 bp, and 425 bp. Only three bands were observable in the profile of *P. multocida* B:2 JRMT12 which were 1.5 kb, 1.0 kb, and 600 bp. Nonetheless, both *P. multocida* B:2 wild-type and *P. multocida* B:2 GHD7 shared a similar DNA band of 425 bp that was not observed in the profile of *P. multocida* B:2 JRMT12. The major heterogeneity between REP profiles of the bacterial strains could be represented from the pathogenicity of respective strains (Biswas et al., 2004). Saxena et al. (2006) reported two DNA bands at 1000 bp and 800 bp could be observed in every strains and isolates of *P. multocida*. However, in this study, both bands could not be observed from the *P. multocida* B:2 GDH7 mutant's genome (Figure 4). In contrast, the genome pattern of *P. multocida* B:2 wild-type possessed both of the respective DNA bands with slight difference in sizes. A plausible band of 425 bp was seen to be present in both profiles of *P. multocida* B:2 wild-type

and *P. multocida* B:2 GDH7 (Figure 4). Previously it was reported that repetitive elements are localised in particular regions of the bacterial isolates (Turni et al., 2018). Hence, this could indicate the 425 bp band was conserved only between the two strains. Nevertheless, whole genome sequencing is crucial in order to confirm this occurrence.

Amplification of REP profiles generated multiple DNA bands with different sizes due to multiple locations of REP sequences distinctly located within the bacterial genome. Thus, the multiple DNA bands observed post-gel electrophoresis depicted the genomic distance between the binding sites of the REP-based primers and the adjacent repetitive elements within the genomic content (Ishii et al., 2009). As shown in Figure 4, each strain's genome profile comprised multiple DNA bands with various molecular weights showing the REP sequences location variety within each genome. Despite the similar ancestral background, the genome fingerprints between the strains in this study were seen to be completely diverse to each other. This confirmed that attenuation of the mutant not only altered the bacterial metabolism (Kamal et al., 2017) but could also have altered the genome organization. Moreover, the attenuated mutant was observed capable of providing mass protection towards buffaloes when challenged with the wild-type strain in field tests (Rafidah et al., 2012;

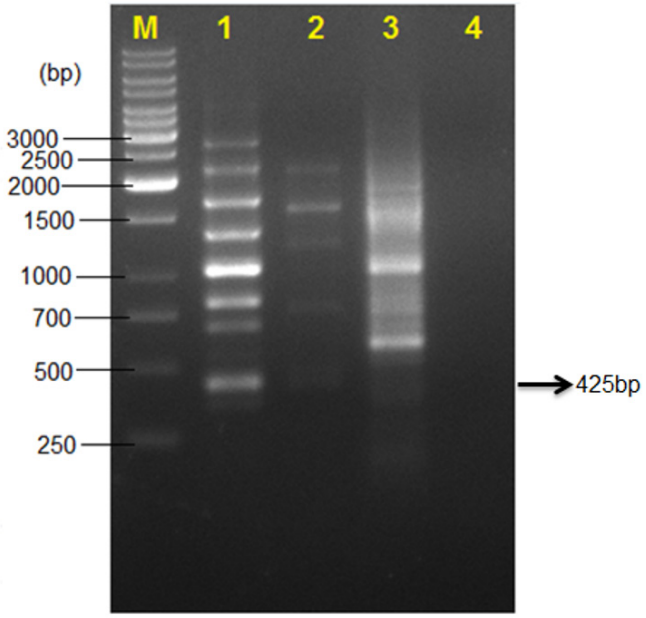


Figure 4. REP-PCR assay for genotyping analysis of *Pasteurella multocida* B:2 strains. Comparison between *P. multocida* B:2 wild-type, *P. multocida* B:2 GDH7, and *P. multocida* B:2 JRMT12: Lane M, DNA ladder; Lane 1, *Pasteurella multocida* B:2 wild-type; Lane 2, *Pasteurella multocida* B:2 GDH7; Lane 3, *Pasteurella multocida* B:2 JRMT12; Lane 4, Negative control

Rafidah & Zamri-Saad, 2013). Opportunistic pathogens evolved rapidly with the highest rate of homologous recombination events in order to adapt to the host environment (González-Torres et al., 2019). Homologous recombination and antibiotic selection was found to induce mutagenesis and this lead to genome instability and genome rearrangement (Darmon et al., 2014; Oliveira et al., 2015). However, contribution of the mutant strain's genome instability towards its apathogenicity remains unknown.

Fingerprint typing analysis towards another mutant strain, *P. multocida* B:2 JRMT12, was also conducted to compare the genetic organisation of the mutant with the rest of the bacterial strains in this study. From the REP-PCR profile, all of the strains possessed significant differences of their genomic content (Figure 4). In addition, the genomic profile of *P. multocida* B:2 JRMT12 was observed to contain more DNA bands when compared to *P. multocida* B:2 GDH7 and the wild-type strains. The excessive presence of multiple DNA bands in the profile of *P. multocida* B:2 JRMT12 observed causes the profile to smear when compared to other strains' profiles. Similar to *P. multocida* B:2 GDH7, this occurrence also could be contributed from the attenuation process of *P. multocida* B:2 JRTM12. Hence, the attenuation of the *aroA* mutant could also contribute to its genome reorganisation (Tabatabaei et al., 2002).

Proteomic Profiling of *Pasteurella multocida* B:2 Strains' Cell-Free Extracts

The CFE profiles were observed with proteins ranging from 12 kDa to 115 kDa (Figure 5). Conspicuous protein bands were observed with differences in the respective CFE profiles of each bacterial strain. The CFE of wild-type strain possessed conspicuous band of 28 kDa whereas *P. multocida* B:2 GDH7 strain possessed a 30 kDa band. These bands are high likely to be identified as OmpH (Ataei et al., 2009). As expected, there were no significant different observed between the CFE profiles of *P. multocida* B:2 GDH7 cultured in BHI or YDB broth media. However, the protein bands above 15 kDa of the latter sample were observed as faint bands. The minimum nutrients provided by YDB media could possibly limit the expressions of these proteins as compared to proteins expressed with the commercial media. Moreover, the CFE profile of the reference mutant strain, *P. multocida* B:2 JRMT12 was shown to possess more proteins as compared to the other strains. It was also interesting to note that the were no visible protein bands of sizes >15 kDa for CFE from *P. multocida* B:2 wild-type when compared to the others. Overall, the prominent protein bands of each CFE profile were distinctively visible and each CFE profiles were distinctive towards the specific strains.

From these findings, the colony morphology (unpublished data) and

genomic pattern changes are high likely contributed by genomic alterations of the mutants as compared to the virulent strain. This was observed in a study of attenuated *Mycobacterium tuberculosis* strain H37Ra, when compared against its virulent strain in order to observe the respective genomic organization (Zheng et al., 2008). The spontaneous attenuation introduced towards *M. tuberculosis* H37Ra strain had caused multiple mutations within its genome. The multiple mutations of the attenuated strain had caused genetic variations that affected the metabolism and growth related genes. The attenuation also contributed to colony morphology differences of the mutant with the virulent strains (Zheng et al., 2008). The latter observation was also recorded in this study with *P. multocida* B:2 GDH7 and its wild-type strain. Thus, the attenuation against *P. multocida* B:2 GHD7 has possibly contributed to its colony morphology changes (unpublished data) and consequently towards the genomic alteration. Nevertheless, a subsequent thorough comparative genomic analysis is required for further information between the genomic organisations of both strains.

In *P. multocida*, cell-free extracts (CFEs) are less commonly profiled as compared to outer membrane proteins (OMPs). In a report by Ataei et al. (2009), the CFEs profile of a Sri Lankan strain *P. multocida* B:2 (*P. multocida* B:2 strain 85020) showed numerous protein bands with sizes that ranged from 16 to 90 kDa with a conspicuous protein band at 30 kDa. The 30 kDa was also observed in the OMP profile and identified as OmpH from mass spectrometry (MS) analysis. However, this protein was proven to be non-immunogenic due to no response in immunoblotting with the vaccinated animal sera (Ataei et al., 2009). In contrast, this study obtained different sizes of conspicuous protein bands in the respective strains' CFEs profile (Figure 5) as compared to Ataei et al. (2009).

The use of different bacterial strains in the respective experiments could contribute to the differences observed in each CFEs profile. Although targeting similar bacterial serotypes, but the strain of *P. multocida* B:2 used by Ataei et al. (2009) was of Sri Lankan origin. Differences of the CFEs profiles were also observed from other serotypes of *P. multocida* B:2 isolated from Indian strain (Chaudhuri et al., 2001). Thus, it can be assumed that different strains had represented different proteomic profiles. This is likely due to differential environment adaptation in each geographical region of respective bacterial strains. Even so, the CFE profiles cannot be further scrutinised other than the presence of the conspicuous bands. Therefore, OMP profiling was performed to further examine the proteomic profiles of each bacterial strain in this study.

Proteomic Profiling of *Pasteurella multocida* B:2 Strains' OMPs

Similar to the CFE profiles, the OMP profiles of each strain was also seen to be distinct with each other (Figure 6). The OMP profiles consisted of protein bands with sizes

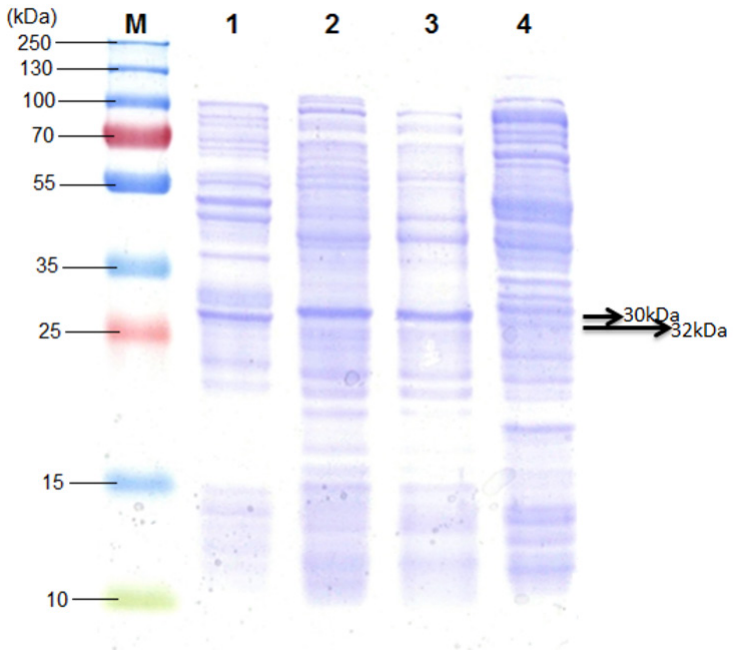


Figure 5. CFE profiles of each *Pasteurella multocida* B:2 strains represented on a Coomassie blue-stained SDS-PAGE gel. Lane 1, *Pasteurella multocida* B:2 wild-type; Lane 2, *Pasteurella multocida* B:2 GDH7 grown in BHI media; Lane 3, *Pasteurella multocida* B:2 GDH7 grown in YDB media; Lane 4, *Pasteurella multocida* B:2 JRMT12; Lane M, protein ladder

ranging from 14 kDa to 90 kDa. The high molecular weights (HMW) protein bands were faintly observed in the OMP profile of mutant strain *P. multocida* B:2 GDH7 as compared to the other two strains. However, the pattern of the HMW proteins of the two latter strains was also positioned at a different location when compared to one another. The HMW proteins of *P. multocida* B:2 GDH7 were observed between 55 kDa to 70 kDa size. Meanwhile, the HMW proteins of *P. multocida* B:2 wild-type were seen to be higher than 70 kDa in which the wild-type possessed two protein bands when compared to *P. multocida* B:2 GHD7's HMW proteins.

In each of the bacterial OMP profiles, they comprised two distinct and highly expressed protein bands within the range of about 22 kDa to 32 kDa. The sizes of these two protein bands were different to each other such that *P. multocida* B:2 wild-type possessed a visibly dense protein band at about 28 kDa and 23 kDa. Both *P. multocida* B:2 GDH7 grown in BHI and YDB have similar OMP profile with distinct bands of about 30 kDa and 22 kDa, proving that using a minimal media did not have significant effect on OMP expression. Simultaneously, two bands of about 34 kDa and 32 kDa were observed from the OMP profile of *P. multocida* B:2 JRMT12. Proteins with

lower molecular weight (LMW) were also observed from the OMP profiles. Both *P. multocida* B: wild-type and *P. multocida* B:2 JRMT12 shared the same protein band size of about 14 kDa whereas *P. multocida* B:2 GDH7 OMP profiles possessed protein band of about 15 kDa. Furthermore, all OMP profiles shared one similar protein size at around 18 kDa.

OMP typing or profiling was used in this study to characterise between *P. multocida* B:2 strains (Figure 6). It is understood that most of the virulent factors that include OMPs, resides on the surface of *P. multocida* due to their association with hosts' cells colonisation and invasion. (Boyce et al., 2006). The OMPs were expressed in iron-rich environment in order to understand on the proteins that contributed to the pathogenicity of the wild-type *P. multocida* B:2 and the apathogenicity of both mutants, *P. multocida* B:2 GDH7 and *P. multocida* B:2 JRMT12 strains. Hence, from the SDS-PAGE analysis, respective strains' OMP profiles showed notable differences and also similarities with each other. In this study, it was identified that two conspicuous bands were present with different sizes in each OMP profile. These OMPs could be determined as OmpA or OmpH which are known as major OMPs in *P. multocida* (Prasannavadhana et al., 2014; Wheeler, 2009).

Various strains of *P. multocida* B:2 were targeted for OMP profiling to determine

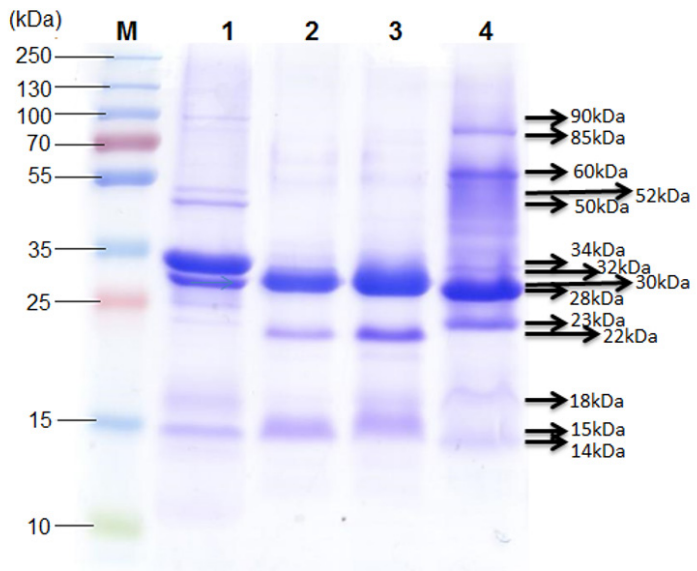


Figure 6. OMP profiles of each *Pasteurella multocida* B:2 strains represented on a Coomassie blue-stained SDS-PAGE gel. Lane 1, *Pasteurella multocida* B:2 wild-type; Lane 2, *Pasteurella multocida* B:2 GDH7 grown in BHI media; Lane 3, *Pasteurella multocida* B:2 GDH7 grown in YDB media; Lane 4, *Pasteurella multocida* B:2 JRMT12; Lane M, protein ladder

the strain specific markers as a preliminary typing analysis for isolates differentiation (Munir et al., 2007). In 2007, an OMP profile of *P. multocida* serotype B:2 isolated from Pakistan showed presence of six polypeptide bands ranging from 15 kDa to 91 kDa (Munir et al., 2007). Furthermore, two predominant bands were observed with 32 kDa and 39 kDa and with less conspicuous bands, 15 kDa, 44 kDa, 72 kDa, and 91 kDa. Four Malaysian outbreak isolates of *P. multocida* B:2 were targeted for OMP profiling that showed six protein bands ranging from 26 kDa to 100 kDa (Tan et al., 2010). Each outbreak strains possessed slight differences in respective OMP profiles. Moreover, major OMP, OmpH was identified from protein size of 37 kDa after immunoblotting analysis (Tan et al., 2010).

A report on *P. multocida* serotype B's OMP profiling by Somshekhar et al. (2014) has stated minor differences between the Indian field isolates and the vaccine strain (*P. multocida* B:2 P52) with polypeptide bands ranging from 16 kDa to 123 kDa. In addition, a prominent band of 32 kDa was identified as any of the two major OMPs for all bacterial strains of the study. Meanwhile, protein bands of 46 kDa and 123 kDa were only observed in the vaccine strain's OMPs but not found in the rest of field isolates' OMPs. Nevertheless, *P. multocida* B:2 P52 is a local strain isolated from previous outbreak in India and is used as formalin-killed bacterin vaccine production for HS outbreak control in India (Chaudhuri et al., 2001). Hence, the OMP profiles of the respective bacteria should not have major differences to each other. In contrast, this study used a genetically manipulated *P. multocida* B:2 (*P. multocida* B:2 GDH7) to profile its OMPs and to be compared to the profile of its parent strain, isolated from previous outbreak in Malaysia (Othman, 2007). Another notable report of *P. multocida* B:2 OMP profiling in 2014 by Prasannavadhana et al. (2014) had shown two major OMPs with bands sizes of 33 kDa and 38 kDa. These bands then were confirmed by mass spectrometry analysis as OmpH at 33 kDa and OmpA at 38 kDa. Minor polypeptide bands were also observed in the OMP profile with 14-15 kDa bands on the gel. Therefore, the conspicuous bands identified in this study can be predicted as either major OmpH or OmpA.

A low molecular protein known as Omp16 was also found to be expressed in capsular types A, D and F of *P. multocida* but with protein size heterogeneity across avian, bovine, porcine, and ovine isolates (Wheeler, 2009). Moreover, this protein was also expressed in all of the previous studies of *P. multocida* B:2 with protein sizes ranging from 14 kDa to 19 kDa. Thus, this protein is a highly conserved protein in the OMP profile of *P. multocida*. However, unlike the previous reports, in this study, two low molecular weight proteins were observed at 14 kDa/15 kDa, and 18 kDa. This low molecular weight proteins pattern was also observed in *P. haemolytica* isolated from bovine but of unknown identity and function (Davies et al., 1992). Moreover, these proteins were only reported

in selected strains of *P. multocida* B:2 such as *P. multocida* P52 and four isolates of *P. multocida* serotype B isolated from England and Wales (Aiswarya et al., 2017; Davies et al., 2003). Furthermore, several isolates of *P. multocida* of capsular type A were also found with this protein pattern (Davies et al., 2003). The reason behind the occurrence of this protein pattern was not known due to limited information of these proteins aside from the reported immunogenic Omp16. Although, the band was observed in multiple serotypes and isolates of *P. multocida*, it could have an importance in each pathogenesis.

Proteins of HMW were frequently reported in iron-limited conditions due to the proteins association in the iron uptake and regulation in iron deprived environment (Wheeler, 2009). However, a protein that is present in either iron rich or iron-limited conditions which is 89 kDa TbpA was reported to be present only in bovine and ovine isolates (Wheeler, 2009). TbpA was also detected in *P. multocida* B:2 with the size of 87 kDa and 89 kDa (Prasannavadhana et al., 2014). Aside from that, another HMW found in OMP profile *P. multocida* to be constitutively expressed in both conditions is Omp87 of 87 kDa.

The reason behind the obvious differences between the OMP profiles of each *P. multocida* B:2 strains is still unclear. However, further proteomic analysis such as mass spectrometry is highly able to provide clarification on this phenomenon. Still, one can assume that the differences observed between the conspicuous bands in all strains of *P. multocida* B:2 used in this study was most likely due to the differences of genetic makeup between the strains. The attenuation performed in both mutant strains might have altered the genomic content. And the alteration was shown through the different patterns of each OMP profile. However, genetic make-up differences cannot be represented solely by the bacterial OMP (Davies, 2004).

It was understood that the differences between the OMP profiles between each *P. multocida* strains is represented by the rapid evolution of the respective OMPs when compared to housekeeping genes due to introduction of diversified selection pressures (Davies, 2004). Therefore, media selection of *P. multocida* B:2 GDH7 might also influenced the unique pattern of its OMP profile when compared to the parent strain. It could also be postulated that the conspicuous bands exhibited in respective profiles were different due to the up-regulation and down-regulation of the selected conspicuous bands and its similar sizes of protein bands.

The differences of protein expression might also be reflected from the attenuation process of the mutants than the wild-type. An initial OMPs determination analysis was performed on the genome of *P. multocida* B:2 PMTB2.1, which was known as the parent strain used in this study. The selected housekeeping gene *gdhA*, disrupted in *P. multocida* B:2 GDH7, was found to be located in the outer membrane of the bacteria. Hence, alteration of this outer membrane protein gene could have shifted

the biogenesis of the membrane in order to survive the post-attenuation (Bernardini et al., 1993; Bochner, 2009; Godlewska et al., 2009; Martorana et al., 2014). This phenomenon could also address the weakening of both mutants' pathogenicity and their ability in giving protection when vaccinated to the animals (Rafidah et al., 2012; Tabatabaei et al., 2002). Aside from GdhA, the proteins TbpA, OmpA and also OmpH were identified in the initial OMPs determination analysis of *P. multocida* B:2 PMTB2.1 genome. Hence, the presence of these proteins might explain their contributions towards the virulence of the wild-type and the apathogenicity of the mutant strains.

CONCLUSION

This study has enabled characterisation between the *Pasteurella multocida* B:2 wild-type and its attenuated derivative, *P. multocida* B:2 GDH7. It was revealed that kanamycin-based primers enabled discrimination between the mutant strain *P. multocida* B:2 GDH7 and wild-type strain *P. multocida* B:2 by using conventional PCR analysis. The conventional detection method may enable simple but accurate identification and maintenance of the potential live attenuated vaccine that can be applied to ensure the consistency and the quality of the vaccines during commercialisation process. The genotypic and proteomic profiles of each strain were distinguished in this study. The differences in both genomic and OMP profiles were uniformed and consistent that could be represented as differences contributing to the apathogenicity of each strain in this study.

ACKNOWLEDGEMENT

FMA was supported by the academic grant awarded from SEARCA for UPM-KU Dual Degree program. FMA was also supported by the Graduate Research Fellowship awarded from School of Graduate Studies of UPM. This work was a part of the UPM Putra Research Grant (GP/2017/9560000) and Malaysian Ministry of Education Research Grant (PRGS/1/2015/WAB01/UPM/01/1).

REFERENCES

Abubakar, M. S., & Zamri-Saad, M. (2011). Clinico-pathological changes in buffalo calves following oral exposure to *Pasteurella multocida* B:2. *Basic and Applied Pathology*, *4*(4), 130–135. https://doi.org/10.1111/j.1755-9294.2011.01113.x

Aiswarya, V., Mathakiya, R. A., Bhanderi, B. B., & Roy, A. (2017). Characterization of *Pasteurella multocida* isolates of buffalo origin from Gujarat state of India by outer membrane protein profile analysis. *Buffalo Bulletin*, *36*(2), 313-322.

Ataei, S., Burchmore, R., Christopher Hodgson, J., Finucane, A., Parton, R., & Coote, J. G. (2009). Identification of immunogenic proteins associated with protection against haemorrhagic septicaemia after vaccination of calves with a live-attenuated *aroA* derivative of *Pasteurella multocida* B:2. *Research in Veterinary Science*, *87*(2), 207–210. https://doi.org/10.1016/j.rvsc.2009.01.007

Bernardini, M. L., Sanna, M. G., Fontaine, A., & Sansonetti, P. J. (1993). OmpC is involved in invasion of epithelial cells by *Shigella*

flexneri. *Infection and Immunity*, *61*(9), 3625–3635. https://doi.org/10.1128/IAI.61.9.3625-3635.1993

Biot-Pelletier, D., Pinel, D., Larue, K., & Martin, V. J. J. (2018). Determinants of selection in yeast evolved by genome shuffling. *Biotechnology for Biofuels*, *11*(1), 282. https://doi.org/10.1186/s13068-018-1283-9

Biswas, A., Shivachandra, S. B., Saxena, M. K., Kumar, A. A., Singh, V. P., & Srivastava, S. K. (2004). Molecular variability among strains of *Pasteurella multocida* isolated from an outbreak of haemorrhagic septicaemia in India. *Veterinary Research Communications*, *28*(4), 287–298. https://doi.org/10.1023/B:VERC.0000026656.77847.5b

Bochner, B. R. (2009). Global phenotypic characterization of bacteria. *FEMS Microbiology Reviews*, *33*(1), 191–205. https://doi.org/10.1111/j.1574-6976.2008.00149.x

Boyce, J. D., Cullen, P. A., Nguyen, V., Wilkie, I., & Adler, B. (2006). Analysis of the *Pasteurella multocida* outer membrane sub-proteome and its response to the *in vivo* environment of the natural host. *Proteomics*, *6*(3), 870–880. https://doi.org/10.1002/pmic.200401342

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, *72*(1–2), 248–254. https://doi.org/10.1016/0003-2697(76)90527-3

Chaudhuri, P., & Goswami, P. P. (2001). Cloning of 87 kDa outer membrane protein gene of *Pasteurella multocida* P52. *Research in Veterinary Science*, *70*(3), 255–256. https://doi.org/10.1053/rvsc.2001.0470

Darmon, E., & Leach, D. R. F. (2014). Bacterial genome instability. *Microbiology and Molecular Biology Reviews*, *78*(1), 1–39. https://doi.org/10.1128/MMBR.00035-13

Davies, R. L. (2004). Genetic diversity among *Pasteurella multocida* strains of avian, bovine, ovine and porcine origin from England and Wales by comparative sequence analysis of the 16S rRNA gene. *Microbiology*, *150*(12), 4199–4210. https://doi.org/10.1099/mic.0.27409-0

Davies, R. L., MacCorquodale, R., & Caffrey, B. (2003). Diversity of avian *Pasteurella multocida* strains based on capsular PCR typing and variation of the OmpA and OmpH outer membrane proteins. *Veterinary Microbiology*, *91*(2–3), 169–182. https://doi.org/10.1016/S0378-1135(02)00300-0

Davies, R. L., Parton, R., Coote, J. G., Gibbs, H. A., & Freer, J. H. (1992). Outer-membrane protein and lipopolysaccharide variation in *Pasteurella haemolytica* serotype-A1 under different growth conditions. *Journal of General Microbiology*, *138*(5), 909–922.

De Alwis, M. C. L. (1999). *Haemorrhagic septicaemia*. Australian Centre for International Agricultural Research.

El-Brolosy, & M. A, Stainier D. Y. R. (2017). Genetic compensation: A phenomenon in search of mechanisms. *PLoS Genetics*, *13*(7), e1006780. https://doi.org/10.1371/journal.pgen.1006780

Galdiero, M., De Martino, L., Pagnini, U., Pisciotta, M. G., & Galdiero, E. (2001). Interactions between bovine endothelial cells and *Pasteurella multocida*: Association and invasion. *Research in Microbiology*, *152*(1), 57–65. https://doi.org/10.1016/S0923-2508(00)01168-2

Godlewska, R., Wiśniewska, K., Pietras, Z., & Jagusztyn-Krynicka, E. K. (2009). Peptidoglycan-associated lipoprotein (*Pal*) of Gram-negative bacteria: Function, structure, role in pathogenesis and potential application in immunoprophylaxis:

Minireview. *FEMS Microbiology Letters*, *298*(1), 1–11. https://doi.org/10.1111/j.1574-6968.2009.01659.x

González-Torres, P., Rodríguez-Mateos, F., Antón, J., & Gabaldón, T. (2019). Impact of homologous recombination on the evolution of prokaryotic core genomes. *MBio*, *10*(1), e02494-18. https://doi.org/10.1128/mBio.02494-18

Hazwani-Oslan, S. N., Tan, J. S., Saad, M. Z., Halim, M., & Ariff, A. B. (2017). Improved cultivation of *gdhA* derivative *Pasteurella multocida* B:2 for high density of viable cells through *in situ* ammonium removal using cation-exchange resin for use as animal vaccine. *Process Biochemistry*, *56*, 1–7. https://doi.org/10.1016/j.procbio.2017.02.016

Ishii, S., & Sadowsky, M. J. (2009). Applications of the rep-PCR DNA fingerprinting technique to study microbial diversity, ecology and evolution: Minireview. *Environmental Microbiology*, *11*(4), 733–740. https://doi.org/10.1111/j.1462-2920.2008.01856.x

Kamal, N. M., Zamri-Saad, M., Masarudin, M. J., & Othman, S. (2017). Interaction between *Pasteurella multocida* B:2 and its derivatives with bovine aortic endothelial cell (BAEC). *BMC Veterinary Research*, *13*(1), 1-8. https://doi.org/10.1186/s12917-017-1109-1

Laemmli, U. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, *227*(5259), 680–685. https://doi.org/10.1038/227680a0

Martorana, A. M., Motta, S., Di Silvestre, D., Falchi, F., Dehò, G., Mauri, P., Sperandeo, P., & Polissi, A. (2014). Dissecting *Escherichia coli* outer membrane biogenesis using differential proteomics. *PLOS One*, *9*(6), 100941. https://doi.org/10.1371/journal.pone.0100941

Munir, R., Shahwar, D., Farooq, U., Nawaz, I., Shahzad, I., & Khanum, A. A. (2007). Outer membrane protein profiling of *Pasteurella multocida*. *Pakistan Veterinary Journal*, *27*(1), 1–4.

Oliveira, P. H., Prazeres, D. M. F., Monteiro, G. A., & Pedro H., Oliveira, G. A. M. (2015). DNA instability in bacterial genomes: Causes and consequences. In M. Poptsova (Ed.), *Genome analysis: Current procedures and applications* (pp. 261-284). Caister Academic Press.

Oslan, S. N. H., Tan, J. S., Saad, M. Z., Halim, M., Mohamed, M. S., & Ariff, A. B. (2018). Influence of amino acids and vitamins on the growth of *gdhA* derivative *Pasteurella multocida* B:2 for use as an animal vaccine. *Bioprocess and Biosystems Engineering*, *42*(3), 355–365. https://doi.org/10.1007/s00449-018-2040-y

Othman, S., Parton, R., & Coote, J. (2012). Interaction between mammalian cells and *Pasteurella multocida* B:2. Adherence, invasion and intracellular survival. *Microbial Pathogenesis*, *52*(6), 353–358. https://doi.org/10.1016/j.micpath.2012.03.005

Othman, S. S., Zamri-Saad, M., Abdul Rahim, R., & Zunita, Z. (2017). Pasteurella multocida *B:2 GDH7* (Malaysian Patent No. MY-168662-A). IP UPM Malaysia. http://www.upmip.upm.edu.my/action.do?view=ipdetail&cluster=1&ipid=887&ipdetailid=870&projectleaderid=47&page=search&search=pasteurella&searchkey=1

Othman, S. S. (2007). *Contstruction of an attenuated Pasteurella multocida B:2 by mutation in the gdhA gene* [Master's thesis, University Putra Malaysia]. Universiti Putra Malaysia *Institutional Repository.* http://psasir.upm.edu.my/id/eprint/11652/1/FPV_2007_11_A.pdf

Prasannavadhana, A., Kumar, S., Thomas, P., Sarangi, L. N., Gupta, S. K., Priyadarshini, A., Nagaleekar, V. K., & Singh, V. P. (2014). Outer membrane proteome analysis of Indian strain of *Pasteurella multocida* serotype B:2 by MALDI-TOF / MS analysis. *Scientific World Journal*, *2014*, 617034. https://doi.org/10.1155/2014/617034

Rafidah, O., & Zamri-Saad, M. (2013). Effect of dexamethasone on protective efficacy of live *gdhA* derivative *Pasteurella multocida* B:2 vaccine. *Asian Journal of Animal and Veterinary Advances*, *8*(3), 548–554. https://doi.org/10.3923/ajava.2013.548.554

Rafidah, O., Zamri-Saad, M., Shahirudin, S., & Nasip, E. (2012). Efficacy of intranasal vaccination of field buffaloes against haemorrhagic septicaemia with a live *gdhA* derivative *Pasteurella multocida* B:2. *Veterinary Record*, *171*(7), 175. https://doi.org/10.1136/vr.100403

Sarwar, N., Muhammad, K., Rabbani, M., Younus, M., Sarwar, M., Ali, M. A., Hanif, K., & Kamran, M. (2013). Optimization of physico-chemical factors augmenting *in vitro* biomass production of *Pasteurella multocida*. *Journal of Animal and Plant Sciences*, *23*(4), 1085–1088.

Saxena, M. K., Singh, V. P., Kumar, A. A., Chaudhuri, P., Singh, V. P., Shivachandra, S. B., Biswas, A., & Sharma, B. (2006). REP-PCR analysis of *Pasteurella multocida* isolates from wild and domestic animals in India. *Veterinary Research Communnications*, *30*(8), 851–861. https://doi.org/10.1007/s11259-006-3321-5

Somshekhar, S. H., Veeregowda, B. M., Suryanarayana, V. V. S., Leena, G., Dhama, K., & Chakraborty, S. (2014). Outer membrane protein (OMP) profiles of *Pasteurella multocida* isolates associated with haemorrhagic septicaemia by SDS-PAGE and Western blot analysis. *Asian Journal of Animal and Veterinary Advances*, *9*(8), 513-518. https://doi.org/10.3923/ajava.2014.513.518

Tabatabaei, M., Liu, Z., Finucane, A., Parton, R., & Coote, J. (2002). Protective immunity conferred by attenuated *aroA* derivatives of *Pasteurella multocida* B:2 strains in a mouse model. *Infection and Immunity*, *70*(7), 3355–3362. https://doi.org/10.1128/IAI.70.7.3355

Tan, H. Y., Nagoor, N. H., & Sekaran, S. D. (2010). Cloning, expression and protective capacity of 37 kDa outer membrane protein gene (*ompH*) of *Pasteurella multocida* serotype B:2. *Tropical Biomedicine*, *27*(3), 430–441.

Townsend, K. M., Dawkins, H. J., & Papadimitriou, J. M. (1997). REP-PCR analysis of *Pasteurella multocida* isolates that cause haemorrhagic septicaemia. *Research Veterinary Science*, *63*(2),151-155. https://doi.org/10.1016/s0034-5288(97)90009-6

Townsend, K. M., Frost, A. J., Lee, C. W., Papadimitriou, J. M., & Dawkins, H. J. S. (1998). Development of PCR assays for species- and type-specific identification of *Pasteurella multocida* isolates. *Journal of Clinical Microbiology*, *36*(4), 1096–1100.

Turni, C., Singh, R., & Blackall, P. J. (2018). Genotypic diversity of *Pasteurella multocida i*solates from pigs and poultry in Australia. *Australian Veterinary Journal*, *96*(10), 390–394. https://doi.org/10.1111/avj.12748

Wheeler, R. (2009). Outer membrane proteomics of *Pasteurella multocida* isolates to identify putative host-specificity determinants. *Bioscience Horizons*, *2*(1), 1–12. https://doi.org/10.1093/biohorizons/hzp002

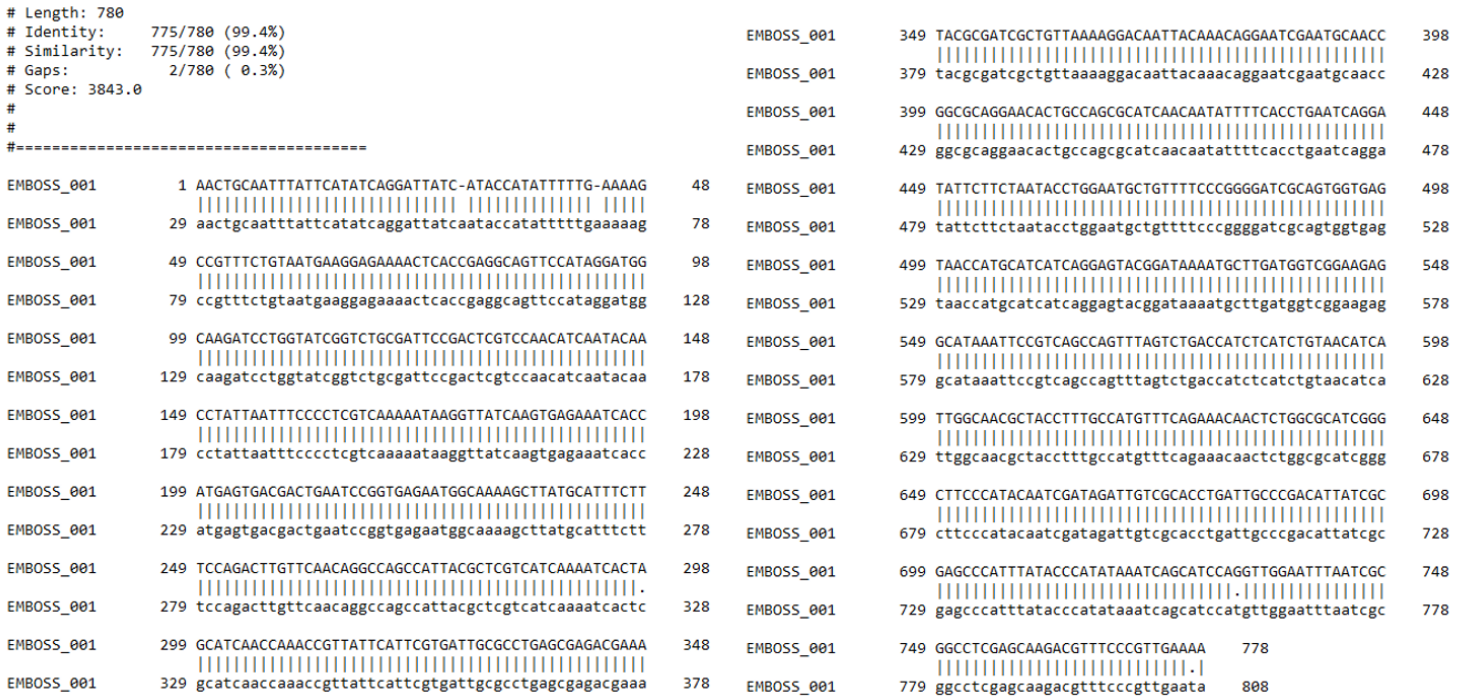
World Organisation for Animal Health. (2008). *Principle of veterinary vaccine production*: *OIE terrestrial manual*. OIE. http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2008/pdf/1.01.06_VACCINE_PRODUCTION.pdf

World Organisation for Animal Health. (2016). *1.1.8. Principles of veterinary vaccine production: Manual of diagnostic tests and vaccines for terrestrial animals 2016*. OIE. http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/1.01.08_VACCINE_PRODUCTION.pdf

Zamri-Saad, M. (2013). *Haemorrhagic septicaemia of cattle and buffaloes in Asia*. UPM Press.

Zamri-Saad, M., & Annas, S. (2016). Vaccination against haemorrhagic septicaemia of bovines: A review. *Pakistan Veterinary Journal*, *36*(1), 1–5. https://doi.org/10.1097/QCO.0b013e3283638104

Zheng, H., Lu, L., Wang, B., Pu, S., Zhang, X., Zhu, G., Shi, W., Lu, Z., Wang, H., Wang, S., Zhao, G., & Zhang, Y. (2008). Genetic basis of virulence attenuation revealed by comparative genomic analysis of mycobacterium tuberculosis strain H37Ra versus H37Rv. *PLOS One*, *3*(6), 2375. https://doi.org/10.1371/journal.pone.0002375



Supplementary material 1. Sequence homology of the amplified and sequenced kanamycin sequence from *Pasteurella multocida* B:2 GDH7. The sequence of the kanamycin gene region was assembled into a contig sequence prior to homology analysis. The figure above showed the sequence alignment result against the kanamycin gene of pUC4k vector. The top strand was the sequenced sample whereas the bottom strand of the alignment was the kanamycin of pUC4k

Pertanika J. Trop. Agric. Sci. 44 (1): 193 - 203 (2021)

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The Diversity of *Hoya* (Apocynaceae: Asclepiadoideae) in Some Parts of Kedah and Perak, Peninsular Malaysia

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ABSTRACT

This study provided data on the diversity of *Hoya* species reported to occur in some parts of the northern region of Peninsular Malaysia. Seven locations were chosen between two states, Kedah (Lata Celak, Sedim, and Bukit Hijau) and Perak (Mahang, Belukar Semang, Sungai Rui, and Pondok Tanjung). This extensive fieldwork was carried out from 2018 until the end of 2019 to provide a baseline checklist for this genus in this northern region. All living specimens found were recorded, and some species were cultivated for further identification and *ex-situ* conservation purposes. A total of 27 species and one variety were recorded, out of which 15 species were newly reported for the northern region of Peninsular Malaysia. The newly described species, *Hoya peninsularis* Rodda & Zakaria, was also reported in this study. One unknown species was found and labelled as *Hoya* sp. cf. *scortechinii*. Further identification process together with molecular analysis of this plant is still ongoing. The most diverse forests are Sungai Rui and Lata Celak, having the Shannon index of 2.741 and 2.622, respectively. Both forests possess the richest in *Hoya* species, but with low dominance index. The Sorenson similarity index is 6.74%, indicating a very low similarity of *Hoya* species between the studied sites. Due to the high diversity recorded, the number of *Hoya* species in the country is expected to increase if the genus is explored continuously. However, most of the study areas were exposed to extreme logging activities and had a high degree of human disturbances, indicating the need for intensive conservation efforts in this genus.

Keywords: Checklist, conservation, diversity, *Hoya*, northern region, Peninsular Malaysia

ARTICLE INFO

Article history:
Received: 31 August 2020
Accepted: 10 November 2020
Published: 24 February 2021

DOI: https://doi.org/10.47836/pjtas.44.1.11

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ISSN: 1511-3701
e-ISSN: 2231-8542

INTRODUCTION

The epiphytic genus *Hoya* R. Br. is one of the most species-rich genera of the tribe Marsdenieae of Asclepiadoideae (Apocynaceae). This genus includes 350–450 currently accepted species found throughout the tropics and sub-tropics regions of Asia and Australia (Lamb & Rodda, 2016). Some of the species of this genus are still being newly discovered and described, particularly from the Malaysian part of Borneo Island (Rodda, 2015). In Peninsular Malaysia, *Hoya* has been revised three times (Rodda & Simonsson, 2013). This includes 23 species by King and Gamble (1908), 25 species by Ridley (1923), and 25 species by Rintz (1978). Other revisions that were published later reported 27 species of *Hoya* (Kiew, 1989; Rodda & Simonsson, 2012). However, a recent study proved that numerous species could be discovered by searching through the available herbarium specimens (Salim & Nikong, 2020).

Hoya species are lactiferous epiphytic lianas mostly characterised as having milky saps. They are commonly known as waxy plants due to the waxy appearance of their leaves and flowers. Hoyas are evergreen plants whose leaves are positioned oppositely on each node, and the leaf shapes vary from orbicular to linear (Kleijn & van Donkelaar, 2001; Wanntorp, 2009). They have unusual floral morphology, i.e. extra-axillary umbelliform inflorescence and star-shaped corona. This makes *Hoya* plants popular ornamentals (Wanntorp & Kunze, 2009). Generally, hoyas can be found in places with high humidity and good sunlight exposures (Hansen et al., 2007; Rahayu et al., 2018; Rintz, 1980). These climbers can be seen along the rivers and streams, and usually occupy the lower canopy of forests (Rahayu et al., 2010).

Usually, to maximise exposure to sunlight, *Hoya* species use the big trees as host plants. The host plants have rough and notched bark textures that accumulate humus and litter, thereby facilitating the growth of *Hoya* species (Sulaeman et al., 2019). Many of the epiphytes are associated with arboreal ants (Davidson & Epstein, 1989). Therefore, *Hoya* plants are known as myrmecophytes or called as ant-plants (Kleijn & van Donkelaar, 2001; Orivel & Leroy, 2011). They usually provide shelter and food for these types of ants. In return, these ants build carton nests that contain organic materials for the plants as nutrients (Corbara et al., 1999). The presence of these ants can also promote seed dispersal (Wagner, 1997).

Hoyas are not only recognised for the beauty of their flowers, but are also plants with several uses such as pollutant-absorbers (Yang et al., 2009). They are also known as sources of certain medicinal and pharmaceutical compounds (Barukial & Sarmah, 2011; Gautam et al., 2013; Mollik et al., 2010). Based on Wanntorp et al. (2006), new species of *Hoya* are added each year. However, the most common threat faced by hoyas is habitat degradation. Clearing of the host plants has threatened *Hoya* populations and may lead to the extinction of species. The hoyas are rarely found blooming in the

wild. Therefore, continuous visits to similar sites are often required to ensure recording and collecting plants in bloom.

This study aimed to develop an inventory of *Hoya* species found in certain parts of the northern region of Peninsular Malaysia. Previous studies (Kiew, 1989; Rintz, 1978; Rodda & Simonsson, 2012) were mostly focused on central and eastern parts of the peninsular. Thus, it is essential to have an adequate understanding of the presence, conservation status, and ecological role of *Hoya* species in the forest by conducting the quantitative monitoring of its diversity.

MATERIALS AND METHODS

The sampling of *Hoya* species was conducted from January 2018 until December 2019. The sampling was focused on some parts of the northern region of Peninsular Malaysia (Kedah and Perak). Seven study sites were chosen between the two states, involving rivers or streams that were easily accessible and containing good habitats for hoyas (Table 1). The fieldwork was focused on areas near streams and lower canopy, as hoyas prefer to occupy areas with high humidity and good exposure to sunlight. Each of the sampling sites was accessed multiple times to maximise the finding. Modified line transect method was used for the sampling. A line transect of 1,000 meters was set at each site along the river bank as the primary reference route. The specimens of hoyas were collected and identified to the lowest taxa using the taxonomic keys (Lamb & Rodda, 2016; Rintz, 1978). The identification process was made easier by one of the authors, who was a taxonomist and *Hoya* specialist.

Digital images of the whole plants or parts of it that were available at the collection time were also obtained to facilitate species identification. The plants without flowers and difficult to identify were grown *ex-situ* at Universiti Sains Malaysia (USM) plant nursery. Voucher specimens were deposited at the Herbarium Unit, USM.

The PAST 4.0 software (Hammer et al., 2001) was used to quantify the diversity indices, including Shannon index, Simpson index, and species evenness. A non-parametric rarefaction and extrapolation analysis with 500 bootstrap replicates was used to estimate the species richness of *Hoya* at the sampled sites (Chao et al., 2016). This

Table 1
List of study sites

Study sites	Type	Gps coordinate	Altitude (m)
Lata Celak, Kedah	Lowland dipterocarp	5°34'N 100°51'E	200-300
Sedim, Kedah	Lowland dipterocarp	5°24'N 100°46'E	200-300
Bukit Hijau, Kedah	Lowland dipterocarp	5°30'N 100°46'E	200-300
Mahang, Perak	Hill dipterocarp	5°19'N 100°45'E	450-800
Belukar Semang, Perak	Lowland dipterocarp	5°34'N 100°59'E	200-300
Sungai Rui, Perak	Lowland dipterocarp	5°26'N 101°06'E	200-300
Pondok Tanjung, Perak	Peat swamp forest	4°59'N 100°43'E	100-200

analysis was based on avoiding the bias that involved species with only single individuals at any of the sites. The confidence intervals of the species curves were used to determine the significant differences in the species richness between the sites. This analysis was done using iNEXT online software (Chao et al., 2016). The Sorenson similarity index was determined to assess the similarities in the species between the sampled sites, using the equation below:

$$Sc = (2W/a) \times 100\% \text{ (Sorenson, 1948)}$$

where *Sc* is the similarity coefficient, *W* represents the number of species common to all sites, and *a* means the sum of all the number of species observed at each of the study sites.

RESULTS AND DISCUSSION

A total of 784 *Hoya* plants were recorded from the seven study sites, which consist of 27 species and one variety (Table 2). Earlier researchers recorded 27 species of *Hoya* in Peninsular Malaysia (Kiew, 1989; Rintz, 1978; Rodda & Simonsson, 2012), and this study recorded 89% of them in the sampling areas. From this study, 15 taxa were newly reported for the northern region of Peninsular Malaysia. They include *Hoya beccarii*, *Hoya coronaria*, *Hoya diversifolia*, *Hoya elliptica*, *Hoya erythrina*, *Hoya erysthrostemma*, *Hoya flagellata*, *Hoya forbesii*, *Hoya ignorata*, *Hoya javanica*, *Hoya lasiantha*, *Hoya mitrata*, *Hoya parviflora*, *Hoya verticillata*, and *Hoya verticillata* var. *hendersonii*. The newly described species, *Hoya peninsularis* Rodda & Zakaria (Rodda & Zakaria, 2020) was also found in this study (Figure 1). This species has been long confused with *Hoya finlaysonii* due to their similar leaf morphology. One unknown species was found in this study and was labelled as *Hoya* sp. cf. *scortechinii* (Figure 2). It has similar inflorescences with *Hoya scortechinii* but differs in colour and the leaves. Further identification process with the aid of molecular analysis is still ongoing to clarify the status of this particular species. *H. ignorata* (Figure 3), which was recently rediscovered in Terengganu (Salim & Nikong, 2020), as this species was previously reported by Corner in 1934 at Kemaman, Terengganu (Trân et al., 2011) was also found in three different sites. The most common and abundantly distributed species from all the study sites is *Hoya revoluta* (101; Figure 4), followed by *H. finlaysonii* (81; Figure 5). Both species were found to adapt to a vast range of environmental parameters and are distributed at different levels of altitude.

A few uncommon species with fewer individuals such as *Hoya curtisii* (1), *H. flagellata* (2), *Hoya mappigera* (2), *H. verticillata* var. *hendersonii* (2), and *Hoya imperialis* (3) were observed. This result suggests that a broader collection effort should be conducted in the future to update the record of *Hoya* by focusing on the selected forests around Kedah and Perlis. It is observed that a few species, namely *H. ignorata*, *H. imperialis*, and *H. mappigera*, which are also found in some

Table 2
List of Hoya *species found at each of the study sites*

S/N	Name of species	Study sites						
		Lata Celak	Sedim	Bukit Hijau	Mahang	Belukar Semang	Sungai Rui	Pondok Tanjung
1	*Hoya beccarii* Rodda & Simonsson (voucher: RZ/19/021)	√	x	x	x	√	√	x
2	*Hoya caudata* Hook. f. (voucher: RZ/19/036)	√	√	√	√	√	√	√
3	*Hoya coriacea* Blume (voucher: RZ/18/016)	√	x	x	√	x	√	√
4	*Hoya coronaria* Blume (voucher: RZ/19/033)	√	x	x	x	√	√	√
5	*Hoya curtisii* King & Gamble (voucher: RZ/19/032)	√	x	x	x	x	x	x
6	*Hoya diversifolia* Blume (voucher: RZ/18/008)	√	x	x	x	x	x	√
7	*Hoya elliptica* Hook. f. (voucher: RZ/18/005)	√	√	√	x	√	√	√
8	*Hoya erythrina* Rintz (voucher: RZ/19/031)	√	x	x	x	√	√	√
9	*Hoya erysthrostemma* Kerr (voucher: RZ/18/011)	x	x	x	x	x	√	x
10	*Hoya finlaysonii* Wight (voucher: RZ/18/019)	√	x	√	√	√	√	√
11	*Hoya flagellata* Kerr (voucher: RZ/19/035)	√	√	x	x	x	x	x
12	*Hoya forbesii* King & Gamble (voucher: RZ/18/012)	√	√	√	√	√	√	√
13	*Hoya ignorata* T. B. Tran, Rodda, Simonsson & Jongku Lee (voucher: RZ/19/036)	√	x	x	√	x	√	x
14	*Hoya imperialis* Lindl. (voucher: RZ/19/026)	x	x	x	x	x	√	x
15	*Hoya javanica* Boerl. (voucher: RZ/18/006)	√	x	x	√	x	√	x
16	*Hoya lacunosa* Blume (voucher: RZ/18/007)	√	x	x	x	√	√	√
17	*Hoya lasiantha* Korth. ex Miq. (voucher: RZ/18/016)	√	√	√	x	x	√	√
18	*Hoya latifolia* G. Don (voucher: RZ/18/27)	x	x	x	x	x	√	x

Table 2 (*continue*)

S/N	Name of species	Study sites						
		Lata Celak	Sedim	Bukit Hijau	Mahang	Belukar Semang	Sungai Rui	Pondok Tanjung
19	*Hoya mappigera* Rodda & Simonsson (voucher: RZ/19/024)	x	x	x	x	x	√	x
20	*Hoya mitrata* Kerr (voucher: RZ/19/020)	x	x	x	√	x	√	x
21	*Hoya obtusifolia* Wight (voucher: RZ/18/013)	√	x	x	x	x	√	x
22	*Hoya parviflora* Wight (voucher: RZ/18/009)	√	x	√	x	√	√	x
23	*Hoya revoluta* Wight ex Hook. f. (voucher: RZ/18/006)	√	√	√	√	√	√	√
24	*Hoya scortechinii* King & Gamble (voucher: RZ/18/003)	x	√	x	√	x	x	x
25	*Hoya verticillata* (Vahl) G. Don (voucher: RZ/18/017)	x	√	√	√	x	√	x
26	*Hoya verticillata* (Vahl) G. Don var. *hendersonii* (Kiew) Veldkamp (voucher: RZ/18/015)	√	x	x	x	x	x	x
27	*Hoya peninsularis* Rodda & Zakaria (voucher: RZ/19/29)	x	x	x	x	x	x	√
28	*Hoya* sp. cf. *scortechinii* (voucher: RZ/19/038)	x	x	x	x	x	x	√

Note. √ means present, x means absent

Figure 1. The inflorescence of *Hoya* sp. cf. *scortechinii* at the study sites

Figure 2. The inflorescence of *Hoya peninsularis* at the study sites

Figure 3. The inflorescence of *Hoya ignorata* at the study sites

Figure 4. The inflorescence of *Hoya revoluta* at the study sites

Figure 5. The inflorescence of *Hoya finlaysonii* at the study sites

parts of Thailand, are well distributed in the northern part of the peninsular, as this region is closer to Thailand (Rodda & Simonsson, 2012; Trân et al., 2011; Wai et al., 2008).

The major factors that limit the distribution of epiphytes are light, water, and mineral nutrition (Benzing, 2008; Luttge, 2008). The majority of hoyas were found hanging on phorophytes near water stream or river, except a few of them, such as *H. erythrina* and *H. verticillata*, which grow on damp boulders and rocks. Although *Hoya* species mostly occur in areas with high humidity, it could be also be found in very dry habitats for extended periods (Rahayu, 2012). Habitat heterogeneity probably affects epiphyte distribution, whether it is seedling germination or recruitment success (Winkler et al., 2005). Through observation, no specific host plant selection for hoyas was found in this study. This finding is supported by previous studies stating that there is no particular association between hoyas and the host plants (Damayanti et al., 2017; Zakaria et al., 2019). Hoyas are observed to choose their host plants mainly for sunlight exposure.

It was observed that Sungai Rui had the highest number of *Hoya* species (21), the highest number of individuals (281), and the highest Shannon index ($H' = 2.741$), followed by Lata Celak with 19 species of *Hoya* and Shannon index of 2.622 (Table 3). The higher *Hoya* diversity could be due to several factors that may be investigated in future studies. The lowest number of species (8) was recorded in Sedim and Bukit Hijau. These two studied sites are known as recreational forests with some degree of

Table 3
The Hoya *diversity indices of the study sites*

Parameters	Lata Celak	Sedim	Bukit Hijau	Mahang	Belukar Semang	Sungai Rui	Pondok Tanjung
No. of taxa (*S*)	19	8	8	10	10	21	13
Individuals	75	26	52	105	115	281	130
Dominance (*D*)	0.09227	0.2012	0.2345	0.2472	0.1341	0.07575	0.1336
Shannon (*H'*)	2.622	1.778	1.669	1.688	2.097	2.741	2.243
Evenness *e*H/S*	0.7241	0.7399	0.6633	0.5409	0.8146	0.7385	0.7245

human disturbances marked with logging and other activities (Rahmad & Akomolafe, 2019). Hence, this might be responsible for the lower number of species and the lowest number of individuals of the species. Bukit Hijau and Mahang forests were observed to have the lowest diversity of Hoya species with Shannon index of 1.669 and 1.688, respectively. The same forests possessed the highest dominance index of 0.2345 and 0.2472, respectively. This is very understandable because some species are expected to dominate the site with a lower diversity of plant species; hence, a higher dominance index is expected (Sasaki & Lauenroth, 2011). The highly diverse forests could be regarded as more functional and stable than the less diverse ones (Allan et al., 2011).

It is worthy to note that the Bukit Hijau and Mahang forests were the forests with the lowest species evenness index 0.6633 and 0.5409, respectively. This could only suggest a direct relationship between Shannon index and evenness index because the sites with the lowest Shannon index also had the lowest evenness index; hence, rendered less productive (Allan et al., 2011). The rarefaction and extrapolation estimation of the species richness revealed

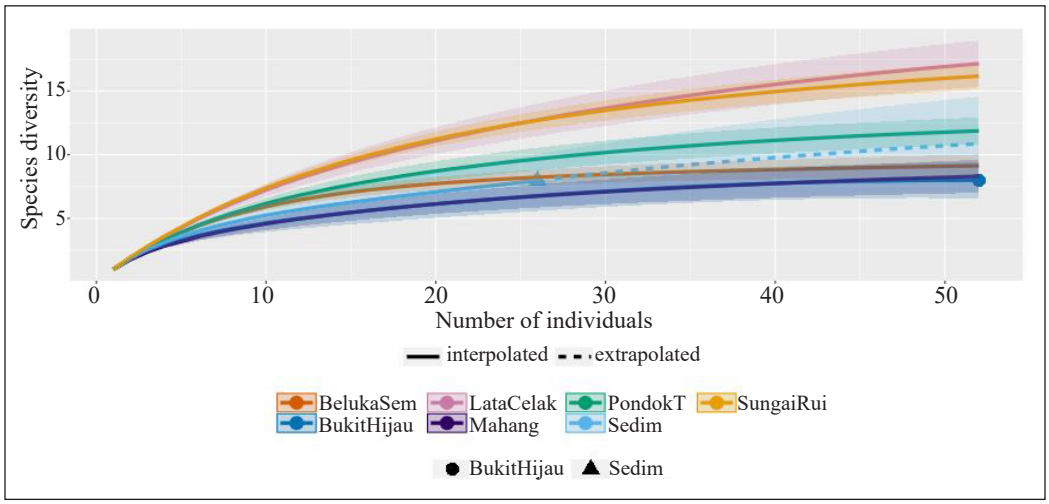

Figure 6. Sample-based rarefaction and extrapolation curve for the species richness of *Hoya* at the study sites

that Sungai Rui and Lata Celak forests had the highest species richness. However, their differences are not significant based on the overlapped confidence intervals (Figure 6). However, their species richness is significantly different from the other forests. The low number of Sorenson similarity index (6.74%) indicated the low similarities of Hoya species between the sampled sites.

CONCLUSION

This current checklist could be considered as a new and updated *Hoya* collection for northern Peninsular Malaysia. This study provided a view of the richness and diverse nature of the studied forests in terms of *Hoya* species, mainly in the northern area. More undiscovered or new species could be identified if a more comprehensive study was undertaken. The quantitative monitoring of *Hoya* diversity can provide fundamental understanding in detecting the changes in the species population. The results of this study can be useful in providing practical strategies for the conservation of *Hoya* species. Moreover, it is also important that the strategies are strictly implemented to protect forests that are essential for the survival and spread of this species.

ACKNOWLEDGEMENT

The authors would like to thank Universiti Sains Malaysia for providing study opportunity and staff assistance. The financial support of the project was provided under the Short Term Grant from Universiti Sains Malaysia (304/PBIOLOGI/6315202).

REFERENCES

Allan, E., Weisser, W., Weigelt, A., Roscher, C., Fischer, M., & Hillebrand, H. (2011). More diverse plant communities have higher functioning over time due to turnover in complementary dominant species. *Proceedings of the National Academy of Sciences*, *108*(41), 17034-17039. https://doi.org/10.1073/pnas.1104015108

Barukial, J., & Sarmah, J. N. (2011). Ethnomedicinal plants used by the people of Golaghat District, Assam, India. *International Journal of Medicinal and Aromatic Plants*, *1*(3), 203-211.

Benzing, D. H. (2008). *Vascular epiphytes*. Cambridge University Press.

Chao, A., Ma, K. H., & Hsieh, T. C. (2016). *iNEXT (iNterpolation and EXTrapolation) online: Software for interpolation and extrapolation of species diversity*. https://chao.stat.nthu.edu.tw/wordpress/sofware_download

Corbara, B., Dejean, A., & Orivel, J. (1999). Ant gardens, a unique epiphyte-ant association. *Année Biologique*, *38*, 73-89. https://doi.org/10.1016/S0003-5017(99)80027-0

Damayanti, I., Siregar, I. Z., & Rahayu, R. (2017). Asosiasi pohon dengan epifit *Hoya* spp. di Taman Nasional Bukit Duabelas [Tree association with epiphytes *Hoya* spp. in Bukit Duabelas National Park]. *Journal of Tropical Silviculture*, *8*(3), 191-196.

Davidson, D. W., & Epstein, W. W. (1989). Ephiphytic associations with ants. In U. Lüttge (Ed.), *Vascular plants as epiphytes* (pp. 200-233). Springer. https://doi.org/10.1007/978-3-642-74465-5_8

Gautam, R. K., Singh, D., & Nainwani, R. (2013). Medicinal plants having anti-arthritic potential: A review. *International Journal of Pharmaceutical Sciences Review and Research*, *19*(1), 96-102.

Hammer, O., Harper, D. A., & Ryan, P. D. (2001). PAST: Paleontological Statistics software

package for education and data analysis. *Paleontologia Electronica*, *4*(1), 9.

Hansen, D. M., Olesen, J. M., Mione, T., Johnson, S. D., & Muller, C. B. (2007). Coloured nectar: distribution, ecology, and evolution of an enigmatic floral trait. *Biological Reviews*, *82*(1), 83-111. https://doi.org/10.1111/j.1469-185X.2006.00005.x

Kiew, R. (1989). *Hoya endauensis* (Asclepiadaceae) and *Licuala dransfieldii* (Palmae), two new species from Ulu Endau, Peninsular Malaysia. *Malayan Nature Journal*, *42*(4), 262-265.

King, G., & Gamble, J. S. (1908). *Hoya* - Materials for a flora of the Malayan Peninsula. *Journal of the Asiatic Society of Bengal*, *74*, 559-580.

Kleijn, D., & van Donkelaar, R. (2001). Notes on the taxonomy and ecology of the genus *Hoya* (Asclepiadaceae) in Central Sulawesi. *Blumea*, *46*(3), 457–483.

Lamb, A., & Rodda, M. (2016). *A guide to hoyas of Borneo*. Natural History Publications (Borneo).

Luttge, U. (2008). *Physiological ecology of tropical plants* (2nd ed.). Springer. Where

Mollik, M. A. H., Hossan, M. S., Paul, A. K., Taufiq-Ur-Rahman, M., Jahan, R., & Rahmatullah, M. (2010). A comparative analysis of medicinal plants used by folk medicinal healers in three districts of Bangladesh and inquiry as to mode of selection of medicinal plants. *Ethnobotany Research and Applications*, *8*, 195-218. https://*doi.*org/*10.17348/era.8.0.195-218*

Orivel, J., & Leroy, C. (2011). The diversity and ecology of ant gardens (Hymenoptera: Formicidae; Spermatophyta: Angiospermae). *Myrmecological News*, *14*, 73-85.

Rahayu, S. (2012). *Hoya* (Apocynaceae: Asclepiadoideae) diversity in Gunung Gede Pangrango National Park, West Java, Indonesia. *Reinwardtia*, *13*(4), 331-339. https://doi.org/10.14203/reinwardtia.v13i4.436

Rahayu, S., Fakhrurrozi, Y., & Putra, H. F. (2018). *Hoya* species of Belitung Island, Indonesia, utilization and conservation. *Biodiversitas Journal of Biological Diversity*, *19*(2), 369-376. https://doi.org/10.13057/biodiv/d190203.

Rahayu, S., Jusuf, M., Suharsono, S., Kusmana, C., & Abdulhadi, R. (2010). Morphological variation of *Hoya multiflora* Blume at different habitat type of Bodogol Research Station of Gunung Gede Pangrango National Park, Indonesia. *Biodiversitas Journal of Biological Diversity*, *11*(4), 187-193. https://doi.org/10.13057/biodiv/d110404

Rahmad, Z., & Akomolafe, G. F. (2019). Taxonomic diversity of ferns of two recreational forests in Kedah, Malaysia. *Malaysian Journal of Science*, *38*(3), 1-11. https://doi.org/10.22452/mjs.vol38no3.1

Ridley, H. N. (1923). A botanical excursion to northern Sumatra. *Journal of the Malayan Branch of the Royal Asiatic Society*, *1*(87), 46-113.

Rintz, R. E. (1978). The Peninsular Malaysian species of *Hoya* (Asclepiadaceae). *Malaysian Nature Journal*, *30*(3&4), 467-522.

Rintz, R. E. (1980). The Peninsular Malayan species of *Dischidia*. *Blumea*, *26*(1), 81-126.

Rodda, M. (2015). Two new species of *Hoya* R.Br. (Apocynaceae, Asclepiadoideae) from Borneo. *PhytoKeys*, *53*, 83-93. https://doi.org/10.3897/phytokeys.53.5079

Rodda, M., & Simonsson, J. N. (2012). (Apocynaceae, Asclepiadoideae), a new campanulate flowered species from Peninsular Malaysia and southern Thailand. *Feddes Repertorium*, *122*(5-6), 337-343. https://doi.org/10.1002/fedr.201100019

Rodda, M., & Simonsson, J. N. (2013). The taxonomy of *Hoya micrantha* and *Hoya revoluta*

(Apocynaceae, Asclepiadoideae). *Webbia*, *68*(1), 7-16. https://doi.org/10.1080/00837792.2013.802937

Rodda, M., & Zakaria, R. (2020). *Hoya peninsularis* (Apocynaceae, Asclepiadoideae), a new species from Peninsular Malaysia, and notes on *Hoya maingayi* and *Gongronema wrayi*. *Nordic Journal of Botany*, *38*(10). https://doi.org/10.1111/njb.02854

Salim, J. M., & Nikong, D. (2020). Notes on *Hoya* of Terengganu, Peninsular Malaysia. *Malayan Nature Journal*, *72*(1), 43-51.

Sasaki, T., & Lauenroth, W. K. (2011). Dominant species, rather than diversity, regulates temporal stability of plant communities. *Oecologia*, *166*(3), 761-768. https://doi.org/10.1007/s00442-011-1916-1

Sorenson, T. (1948). A method of establishing groups of equal amplitude in plant sociology based on similarity of species content and its application to analyses of the vegetation on Danish commons. *Biologiske Skrifter*, *5*(4), 1-34.

Sulaeman, M., Sulistijorini, S., & Rahayu, S. (2019). Habitat suitability for *Hoya* spp. (Apocynaceae) in the Bodogol Conservation Area, West Java. *Biosaintifika: Journal of Biology and Biology Education*, *11*(1), 91-99. https://doi.org/10.15294/biosaintifika.v11i1.13021

Trân, T. B., Kim, J. H., Kim, D. K., Lee, J., Bui, T. H., Simonsson, J. N., & Rodda, M. (2011). *Hoya ignorata* (Apocynaceae, Asclepiadoideae): An overlooked species widely distributed across South East Asia. *Novon*, *21*(4), 508-514. https://doi.org/10.3417/2010068

Wagner, D. (1997). The influence of ant nests on *Acacia* seed production, herbivory and soil nutrients. *Journal of Ecology*, *85*(1), 83-93. https://doi.org/10.2307/2960629

Wai, J. S., Sridith, K., & Thaithong, O. (2008). *Hoya imperialis* Lindl. (Apocynaceae: Asclepiadaceae), a new record for Thailand. *Thai Forest Bulletin (Botany)*, (36), 81-85.https://li01.tci-thaijo.org/index.php/ThaiForestBulletin/article/view/24184

Wanntorp, L. (2009). Phylogenetic systematics of *Hoya* (Apocynaceae). *Blumea-Biodiversity, Evolution and Biogeography of Plants*, *54*(1-3), 228-232. https://doi.org/10.3767/000651909X476201

Wanntorp, L., & Kunze, H. (2009). Identifying synapomorphies in the flowers of *Hoya* and *Dischidia* — Toward phylogenetic understanding. *International Journal of Plant Sciences*, *170*(3), 331-342. https://doi.org/10.1086/596329

Wanntorp, L., Kocyan, A., van Donkelaar, R., & Renner, S. S. (2006). Towards a monophyletic *Hoya* (Marsdenieae, Apocynaceae): Inferences from the chloroplast *trnL* region and the *rbcL-atpB* spacer. *Systematic Botany*, *31*(3), 586-596. https://doi.org/10.1600/036364406778388593

Winkler, M., Hulber, K., & Hietz, P. (2005). Effect of canopy position on germination and seedling survival of epiphytic bromeliads in a humid montane forest. *Annals of Botany*, *95*(6), 1039-1047. https://doi.org/10.1093/aob/mci115

Yang, D. S., Pennisi, S. V., Son, K. C., & Kaysl, S. J. (2009). Screening indoor plants for volatile organic pollutant removal efficiency. *Hortscience*, *44*(5), 1377-1381. https://doi.org/10.21273/HORTSCI.44.5.1377

Zakaria, R., Mansor, A., Syafiq, M., Azmi, A., Rusdi, N. M., Harun, A. H., Harun, M. S., & Azmi, M. F. (2019). Notes on the *Hoya* (Apocynaceae, Asclepiadoideae) of Pulau Pangkor, Perak, Peninsular Malaysia. *The Malaysian Forester*, *82*(1), 197-203.

Pertanika J. Trop. Agric. Sci. 44 (1): 205 - 220 (2021)

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TROPICAL AGRICULTURAL SCIENCE

Journal homepage: http://www.pertanika.upm.edu.my/

In vitro Assessment of Bacterial Strains Associated with Microalgae as Potential Probiotics

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ABSTRACT

Bacteria and microalgae are essential elements in the aquatic ecosystem, co-existing and having constant interactions with each other which help microalgae to exert its beneficial effect as probiotics in aquaculture. This research aims to isolate and identify potential probiotics from different species of microalgae and to evaluate their antimicrobial activity against pathogenic *Vibrio* spp. via series of *in vitro* assays; disc diffusion, well diffusion, and co-culture assays. A total of 18 bacterial strains were isolated from five species of microalgae; *Chlorella* sp., *Nannochloropsis* sp., *Amphora* sp., *Chaetoceros* sp., and *Spirulina* sp.. The isolated strains were tested in *in vitro* antagonistic assay against four *Vibrio* spp. (*Vibrio harveyi*, *Vibrio alginolyticus*, *Vibrio vulnificus*, and *Vibrio parahaemolyticus*). Seventeen strains demonstrated antimicrobial activity with the highest inhibition was observed by strain SPS11 against *V. parahaemolyticus* (12.6 ± 0.36 mm) in disc diffusion assay and strain NAS32 showed 13.2 ± 0.45 mm clear zone against *V. vulnificus* in well diffusion assay. In co-culture assay, both the SPS11 and NAS32 were able to reduce the growth of *V. parahaemolyticus* and *V. harveyi* at concentration of 10^6 and 10^8 CFU mL^{-1}, respectively. Strains SPS11 and NAS32 were characterized as gram positive bacteria with rod shape and further identified as *Lysinibacillus fusiformis* (SPS11) and *Lysinibacillus sphaericus* (NAS32) using 16s rRNA. These two strains should be further studied in *in vivo* challenged experiments in fish and shellfish to explore their probiotic effects.

Keywords: *Lysinibacillus fusiformis*, *L. sphaericus*, microalgae, probiotics, *Vibrio* spp.

ARTICLE INFO

Article history:
Received: 27 August 2020
Accepted: 22 December 2020
Published: 24 February 2021

DOI: https://doi.org/10.47836/pjtas.44.1.12

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ISSN: 1511-3701
e-ISSN: 2231-8542

INTRODUCTION

Microalgae are microscopic algae, usually found in fresh and marine water that varies in size depending on species. In aquaculture, microalgae are often used as supplements for additional nutritional value, serve as immunostimulants, improve defense mechanisms as well as enhance disease resistance towards pathogenic bacteria (Shah et al., 2018). Microalgae have great potential as antiviral agents, antifungal, antibacterial, enzyme inhibiting, immunostimulant, and antiplasmodial due to their ability to synthesize active substances (Ghasemi et al., 2004). The most common species of microalgae used in aquaculture are *Chlorella*, *Tetraselmis*, *Isochrysis*, *Nannochloropsis*, *Pavlova*, *Chaetoceros*, *Skeletonema*, and *Thalassiosira* (Charoonnart et al., 2018). There are positive interactions between microalgae and bacteria in which bacteria can enhance the growth of microalgae by producing growth-promoting factors such as vitamins and regeneration of inorganic nutrients (Fuentes et al., 2016). In return, microalgae synthesize exudates that can be a source of fixed carbon to be absorbed by the bacteria (Yao et al., 2019). Microalgae are able to produce antibacterial compounds that inhibit bacterial growth and *vice versa* (Amin et al., 2012), which is species-specific and can be influenced by culture conditions (Grossart et al., 2006).

Disease outbreaks caused by infectious diseases have become the major limiting factor causing significant economic losses in aquaculture farms. This infectious disease can be caused by pathogenic bacteria, viruses and fungi that lead to severe damage in hatcheries and grow-out ponds. Pathogenic bacteria are the foremost significant pathogens in aquaculture which can cause heavy mortalities. The most common disease in aquaculture appears to be caused by *Vibrio* spp. and their virulence may be resistant to antibiotic treatments (Abraham, 2016). *Vibrio* spp. already exist as normal microbiota in marine and estuarine environments that associated with fish and other aquatic animals and act as primary or secondary opportunistic pathogens which can increase its populations in cultured pond water systems (Priyadarsani & Abraham, 2013). For instance, *V. parahemolyticus* has been identified as the causative agent for acute hepatopancreatic necrosis disease (AHPND) in shrimp including *Penaeus vannamei*, *Penaeus monodon*, and *Penaeus chinensis* (Food and Agriculture Organization [FAO], 2013).

Antibiotics remain as one of the preferred choices in dealing with the outbreaks. However, uncontrollable usage of antibiotics in aquaculture may result in disease resistance of bacteria, and negative impacts on the environment. It is estimated that 75 % of the antibiotic used for feeding are excreted back into the water (Burridge et al., 2010), as fish do not effectively metabolize the antibiotic. On top of that, the residue of antibiotics may accumulate in the flesh of cultured animals which can be transferred to humans through food handling and consumption that poses a high risk to human health. In the long term, antibiotic usage will result in the emergence of antibiotic resistant pathogens. It is

reported that many pathogenic *Vibrio* have been determined to be resistant to almost all available antibiotics (Baker-Austin et al., 2008; Sarter et al., 2007). Abraham (2016) also reported on resistance of *Vibrio* spp. that affected finfish, crustaceans and mollusc production, reduced larval growth as well as inhibited the defence mechanisms of fish larvae. Due to the negative impacts of antibiotic usage towards the environment and human, antibiotic used in aquaculture has been banned in some countries.

Uses of probiotics are known to be harmless as microorganisms obtained from the probiotic are endemic to their purpose, which avoids the introduction of other bacteria into the system (Abraham, 2016). Thus, probiotics have been widely used as an alternative method as a replacement of antibiotics as disease prevention measures in aquaculture. The range of probiotics in aquaculture varies from both Gram-negative and Gram-positive bacteria, bacteriophages, yeasts, as well as microalgae (Irianto & Austin, 2002). Microalgae play a major role as primary live food for fish and shellfish hatcheries and at the same time involves in the overall health management and disease prevention. Each microalgae species has its potential probiotic organism which allows them to strive in their natural environment. Probiotics are not only recognized to prevent common diseases (Sharifah & Eguchi, 2011), but also able to improve the overall growth of microalgae culture.

Thus, the purpose of this study was to identify potential probiotics isolated from different species of microalgae consists of *Chlorella* sp., *Chaetoceros* sp., *Spirulina* sp., *Amphora* sp., and *Nannochloropsis* sp. and its ability to inhibit pathogenic *Vibrio* spp. through series of *in vitro* assays.

MATERIALS AND METHODS

Phytoplankton Collection

Live microalgae culture of *Chlorella* sp., *Nannochloropsis* sp., *Spirulina* sp., *Amphora* sp., and *Chaetoceros* sp. used in this experiment were obtained from Aquatic Bioproduct Laboratory, Department of Aquaculture, Faculty of Agriculture, Universiti Putra Malaysia. Live microalgae were harvested during the late log phase (cell density reached 10^8 cells/mL).

Isolation of Potential Probiotic Candidates

The selected microalgae species were centrifuged at 4,000 ×*g* for 15 minutes. Both supernatant and pellet were placed in tubes following ten-fold serial dilution up to 10^3. A 50 µL from each diluted microalgae sample was pipetted on top of Trypticase Soy Agar (TSA, Difco™, USA) supplemented with 1.5% sodium chloride (NaCl) in triplicates and was spread evenly using a sterile glass stick. All plates were incubated overnight at 30ºC. Pure cultures of individual probiont candidates were preserved at -80°C in 20% sterile glycerol solution for future used.

Elimination of Pathogenic *Vibrio* Strains

Pure cultures of each isolate were streaked onto Thiosulfate Citrate Bile Salt Sucrose (TCBS, Difco™, USA) agar and incubated at

30°C for 24 hours to eliminate any possible pathogen species among the isolates. Isolated bacterial strains with negative growth on TCBS agar were selected for screening in *in vitro* assay.

Pathogenic Bacterial Strains

Four strains of marine pathogenic *Vibrio* sp., *V. harveyi* (NBRC 15634), *V. alginolyticus* (NBRC 15630), *V. vulnificus* (CMCP6), and *V. parahaemolyticus* were obtained from Fish Health Laboratory, Department of Aquaculture, Faculty of Agriculture, Universiti Putra Malaysia and cultured in TCBS agar. The cultures were incubated at 30°C for 24 hours prior used.

Screening of Probiotic Candidates

The potential probionts underwent three assays in *in vitro* screening; disc diffusion, well diffusion, and co-culture.

Disc Diffusion Assay. A pure culture of each potential probiont was inoculated aseptically into Tryptic Soy Broth (TSB, Difco™, USA) with 1.5% NaCl and incubated at the same time and temperature as the pathogen culture. Inoculum densities of pathogens were adjusted to 10^5 CFU mL^{-1} (Jasmin et al., 2016) using saline seawater. Pathogens were then swabbed onto TSA + 1.5% NaCl agar plates using sterile polyester-tipped swabs. Sterile paper discs were dipped into each of these overnight cultures of potential probiont suspensions (10^9 CFU mL^{-1}) while negative control was done by dipping a sterile disk into a 0.22 mm filtered sterile saline seawater and placed onto the agar surface that already swabbed evenly with the pathogen. All agar plates were then incubated at 30°C for overnight. Plates were examined and the diameter of inhibition zone was measured and recorded.

Well Diffusion Assay. The potential probionts were further confirmed for their antagonistic activity in a well-diffusion agar against the target strains. The *Vibrio* strains were grown in TSB + 1.5% NaCl for overnight at 30ºC. On the next day, the cell density was adjusted to 10^5 CFU mL^{-1}. The *Vibrio* strains were swab onto TSA + 1.5% NaCl agar plate. Then, a hole with a diameter of 3 mm was punched aseptically using a sterile cork borer and filled with 2 µL of potential probiont overnight culture with cell density of 10^8 CFU mL^{-1}. The plates were then incubated at 30ºC for 24 hours and inhibition zones were recorded.

Co-culture Assay. Selected potential probionts with the highest inhibition zone towards the virulent *Vibrio* strains from previous screening were further tested in co-culture assay. Overnight culture of *V. harveyi* and *V. parahaemolyticus* was inoculated into TSB + 1.5% NaCl at an initial cell density of 10^5 CFU mL^{-1}, whereas the initial cell density of probiont candidates were 10^4, 10^6, and 10^8 CFU mL^{-1}. Each pathogen and potential probionts were co-cultured in 10 mL of TSB + 1.5% NaCl and incubated at 30 ºC with shaking. Samples were taken at time interval of 0, 6, 12, 24, 48, and 96 hour(s) of incubation for the determination of *Vibrio* densities by spreading the treatment samples

onto TCBS plate and incubated at 30 ºC for 24 hours. The number of colonies for each inoculum was counted and recorded as CFU mL^{-1} using the formula:

$$\text{CFU mL}^{-1} = \frac{(\text{No.of colonies} \times \text{dilution factor})}{\text{Volume of culture plate}}$$

Identification of Potential Probionts

Potential probionts obtained through the screening assays were preliminary identified through Gram staining (Bartholomew & Mittwer, 1952) for morphological characteristics observation and further identified using molecular identification method 16S rRNA sequence analysis (Labreuche et al., 2012; Walling et al., 2010).

Gram Staining. Gram staining was performed by using a loopful of a single bacterial colony and smeared onto a glass slide and heat fixed. The smear was stained with crystal violet for one minute and then washed with gentle water. Iodine reagent was added for one minute and decolorized using acetone for 3 to 5 seconds. The smear was then counterstained with safranin for 45 seconds, washed with water and air-dried. The slide was then observed for its shape and color under an oil immersion lens using a microscope.

Molecular Identification through 16s rRNA Sequence Analysis. A total genomic DNA of the potential probiotics was extracted using Genaeid™ Genomic DNA Mini Kit (Genaied Biotech, Taiwan). The universal primer used to amplify the 16s rRNA gene sequence from each DNA template extracted were; forward primer (5′ AGAGTTTGATCCTGGCTCAG 3′) and reverse primer (5′ ACGGCTACCTTGTTACGACTT 3′) (Amin et al., 2012). The PCR protocol involved the initial denaturation at 95ºC for 4-5 minutes, and then 40 amplification cycles of denaturation at 95ºC for 1 minute, annealing at 55ºC for 1 minute and extension at 72ºC for 2 minutes and a final extension at 72ºC for 5 minutes. Amplified PCR products were detected on agarose gel (1%) electrophoresis in 1x TAE buffer and visualized under UV light. The PCR products were sent for sequencing (First Base Laboratories Sdn. Bhd, Malaysia). The sequence identity of the concatenated 16S rRNA sequence was Blast using NCBI blast online tool.

Statistical Analysis

All the data collected were analyzed using one-way analysis of variance (ANOVA). Multiple comparison tests (Tukey's test) was used (IBM SPSS Statistic 2.0 software). Results were expressed as the mean ± standard error and the differences were considered significant at $p < 0.05$.

RESULTS

Isolation of Potential Probiotic Candidates

A total of 18 bacterial strains were successfully isolated from different species of microalgae as potential probionts (Table

1). Results showed 34% strains were isolated *Chlorella* sp., 22% from *Nannochloropsis* sp., 22% from *Amphora* sp., 11% from *Chaetoceros* sp., and 11 % from *Spirulina* sp..

Screening of Probiont Candidates

Elimination of Pathogenic Vibrio Strains. All 18 potential probiotics showed no growth on TCBS agar, hence it is confirmed that these potential probiotics did not belong to *Vibrio* spp..

Disc and Well Diffusion Assays. Positive inhibitory activities against four *Vibrio* strains were observed on each potential probionts in both assays except for strain CAS11 (Table 2). The highest inhibition was

Table 1
Potential probionts isolated from five different species of microalgae

Microalgae	Samples isolated	Label
Chlorella sp.	6	CP11, CP12, CP31, CS21, CS22, CS31
Nannochloropsis sp.	4	NAP11, NAS21, NAS31, NAS32
Chaetoceros sp.	2	CAP11, CAP31
Amphora sp.	4	AMP11, AMP31, AMS21, AMS31
Spirulina sp.	2	SPS11, SPS31

Table 2
Diameter of inhibition zone (mm, mean ± SE) by potential probionts (10^9 CFU mL^{-1}) against Vibrio *spp. (10^5 CFU mL^{-1}) in disc and well diffusion assays*

Isolate	*Vibrio vulnificus*		*Vibrio parahaemolyticus*		*Vibrio harveyi*		*Vibrio alginolyticus*	
Assay	Disc	Well	Disc	Well	Disc	Well	Disc	Well
AMP11	8.5 ± 0.58	7.6 ± 0.58	8.3 ± 0.15	9.0 ± 0.15	-	7.0 ± 0.06	-	10.6 ± 0.06
AMP31	8.4 ± 0.53	8.4 ± 0.53	8.6 ± 0.12	8.0 ± 0.06	-	8.4 ± 0.06	-	11.2 ± 0.12
AMS21	6.0 ± 0.58	7.8 ± 0.42	7.0 ± 0.15	10 ± 0.25	-	7.0 ± 0.06	-	8.6 ± 0.06
AMS31	7.2 ± 0.32	9.0 ± 0.58	2.0 ± 0.26	9.0 ± 0.06	-	8.8 ± 0.10	-	10.2 ± 0.10
SPS11	8.0 ± 0.32	8.8 ± 0.35	12.6 ± 0.36	8.0 ± 0.17	-	3.2 ± 0.06	-	10.0 ± 0.15
SPS31	5.7 ± 0.26	8.4 ± 0.52	8.8 ± 0.06	5.8 ± 0.15	-	5.8 ± 0.06	-	11.2 ± 0.06
CAP31	9.6 ± 0.25	9.0 ± 0.26	9.0 ± 0.06	9.0 ± 0.06	-	4.2 ± 0.12	-	4.2 ± 0.12
CS21	7.0 ± 0.12	7.0 ± 0.58	7.0 ± 0.06	7.0 ± 0.06	-	7.2 ± 0.12	-	11.2 ± 0.10
CS31	7.6 ± 0.31	9.0 ± 0.58	9.0 ± 0.06	9.0 ± 0.21	-	7.4 ± 0.06	-	9.6 ± 0.06
CS22	8.4 ± 0.32	9.0 ± 0.15	9.0 ± 0.06	9.0 ± 0.06	-	6.0 ± 0.15	-	11.0 ± 0.17
CP11	-	-	-	-	-	6.0 ± 0.12	-	-
CP31	4.6 ± 0.32	8.0 ± 0.38	10.8 ± 0.10	8.0 ± 0.15	-	7.2 ± 0.10	-	8.4 ± 0.10
CP12	5.4 ± 0.17	8.6 ± 0.20	9.5 ± 0.10	7.6 ± 0.06	-	6.4 ± 0.06	-	14.2 ± 0.17
NAS31	5.8 ± 0.06	8.2 ± 0.29	8.3 ± 0.21	7.8 ± 0.10	-	7.0 ± 0.06	-	8.0 ± 0.06
NAS21	7.6 ± 0.20	7.6 ± 0.11	8.0 ± 0.12	8.6 ± 0.15	-	4.0 ± 0.12	-	9.2 ± 0.15
NAP11	6.0 ± 0.10	8.0 ± 0.17	8.5 ± 0.15	10.0 ± 0.06	-	9.6 ± 0.10	-	8.4 ± 0.06
NAS32	13.2 ± 0.45	9.0 ± 0.10	9.6 ± 0.06	10.6 ± 0.10	-	7.5 ± 0.06	-	12.0 ± 0.21

observed on NAS32 against *V. vulnificus* with a diameter of 13.2 ± 0.45 mm in disc diffusion and *V. alginolyticus* with a diameter of 12.0 ± 0.21 mm in well diffusion assay. Meanwhile, SPS11 demonstrated highest inhibition zone against *V. parahaemolyticus* with a diameter of 12.6 ± 0.36 mm in disc diffusion assay. NAS32 and SPS11 showed high inhibition zones against all *Vibrio* strains tested with a range of inhibition between 7.5 to 13.0 mm. Strain CP12 had the highest inhibition against *V. alginolyticus* at 14.2 ± 0.17 mm, however, it was later identified to be as the same species as NAS32. Thus, only NAS32 and SPS11 were selected for co-culture assay.

Co-Culture Assay. Potential probionts SPS11 and NAS32 which had the highest inhibition towards *Vibrio* strains in previous screening assays were tested in co-culture assay to identify the optimum concentration of potential probionts that could inhibit the growth of *V. harveyi* and *V. parahaemolyticus*.

In this assay, the growth of pathogenic *V. parahaemolyticus* was inhibited by isolate SPS11 at three different initial concentrations of 10^4, 10^6, and 10^8 CFU mL^{-1} (Figure 1). A higher concentration of SPS11 (10^8 CFU mL^{-1}) reduced the growth of *V. parahaemolyticus* after 6 hours incubation period. Whereas, lower concentrations of SPS11 (10^4 and 10^6 CFU mL^{-1}) managed to reduce *V. parahaemolyticus* effectively at 12 hours co-incubation until 96 hours.

Potential probiont NAS32 able to inhibit *V. parahaemolyticus* at all concentrations (10^4, 10^6, and 10^8 CFU mL^{-1}) from 6 to 24 hours (Figure 2). NAS32 with the highest concentration (10^8 CFU mL^{-1}) inhibited *V. parahaemolyticus* better compared to the other concentrations from 48 hours onwards. Higher concentrations of NAS32 (10^6 and

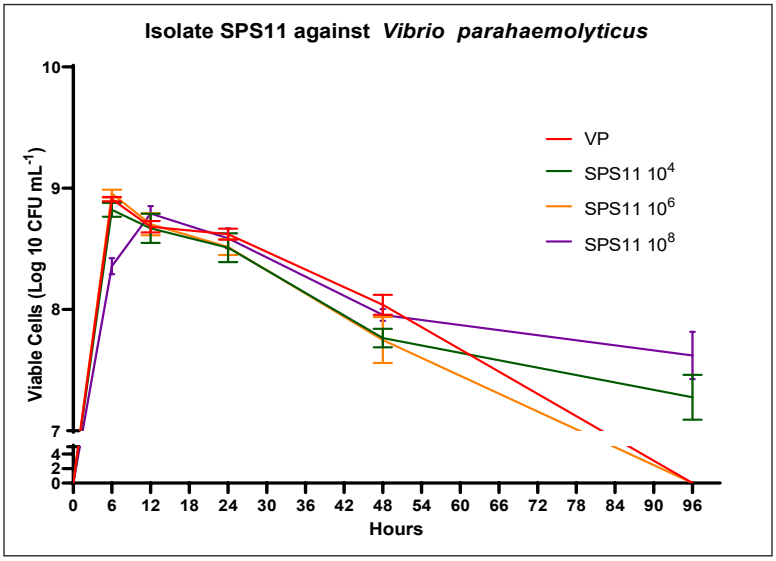


Figure 1. Growth pattern of *Vibrio parahaemolyticus* (VP) CFU mL^{-1} incubated with different concentrations of potential probiont SPS11 (10^4, 10^6, and 10^8 CFU mL^{-1}) against time. Error bars indicate standard error (SE)

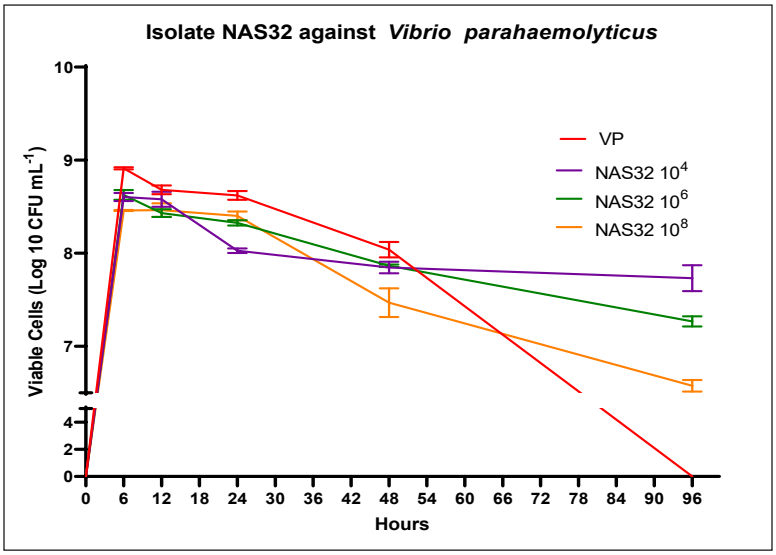


Figure 2. Growth pattern of *Vibrio parahaemolyticus* (VP) CFU mL^{-1} incubated with different concentrations of potential probiont NAS32 (10^4, 10^6, and 10^8 CFU mL^{-1}) against time. Error bars indicate standard error (SE)

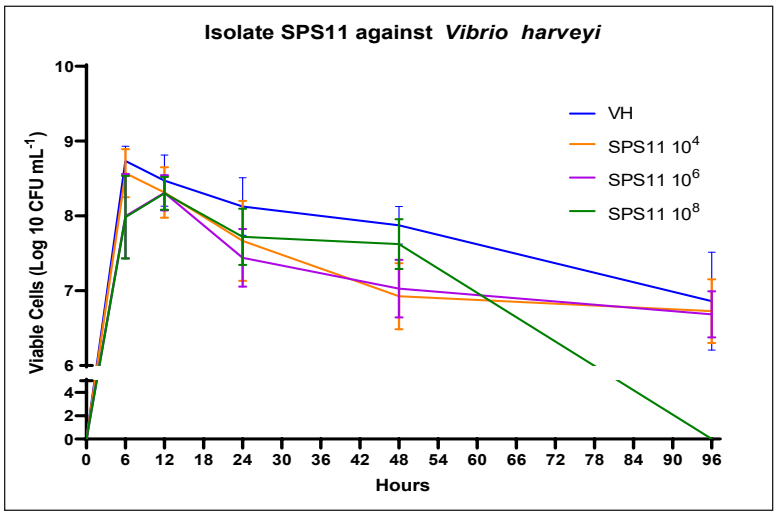


Figure 3. Growth pattern of *Vibrio harveyi* (VH) CFU mL-1 incubated with different concentrations of potential probiont SPS11 (10^4, 10^6, and 10^8 CFU mL-1) against time. Error bars indicate standard error (SE)

10^8 CFU mL^{-1}) able to reduce the total viable counts compared to the lowest concentration of NAS32 (10^4 mL^{-1}).

SPS11 at concentrations of 10^4 and 10^6 CFU mL^{-1} had significant inhibition against *V. harveyi* compared with the growth of *V. harveyi* with no probiont added (Figure 3). However, only slight inhibition of *V. harveyi* occurred at 10^8 CFU mL^{-1} of SPS11 from 24 to 96 hours. SPS11 strain at 10^6 CFU mL^{-1} was the most effective concentration in reducing the growth of *V. harveyi*.

Strain NAS32 at 10^8 CFU mL^{-1} showed significant inhibition towards *V. harveyi* from 6 to 48 hours compared to the lower concentrations. However, the other two NAS32 concentrations; 10^4 and 10^6 CFU mL^{-1}, did not show any viable growth (probionts and vibrios) until the end of the experimental period at 96 hours, hence the results for these two concentrations were omitted (Figure 4).

Identification of Potential Probiotics

Gram Staining. Preliminary identification of potential probionts, SPS11 and NAS32 using Gram staining revealed that these two strains were characterized as Gram positive with rod in shape (Figure 5).

Molecular Identification. Both strains were identified using 16S rRNA sequence analysis (Labreuche et al., 2012; Walling et al., 2010).

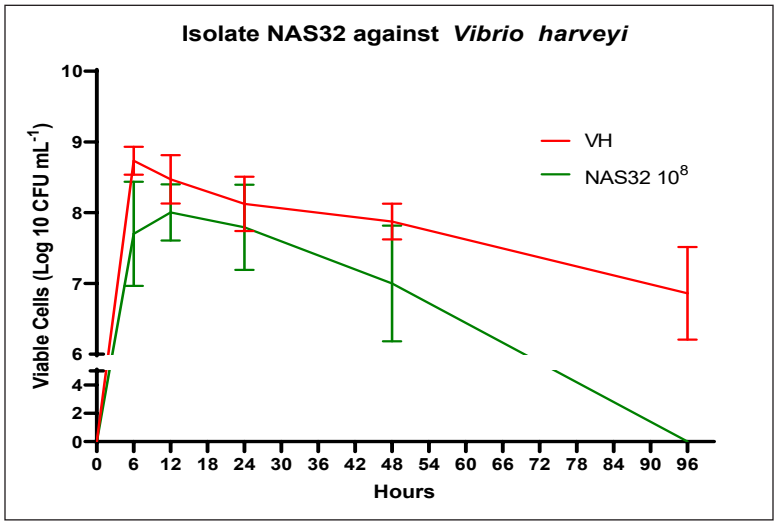
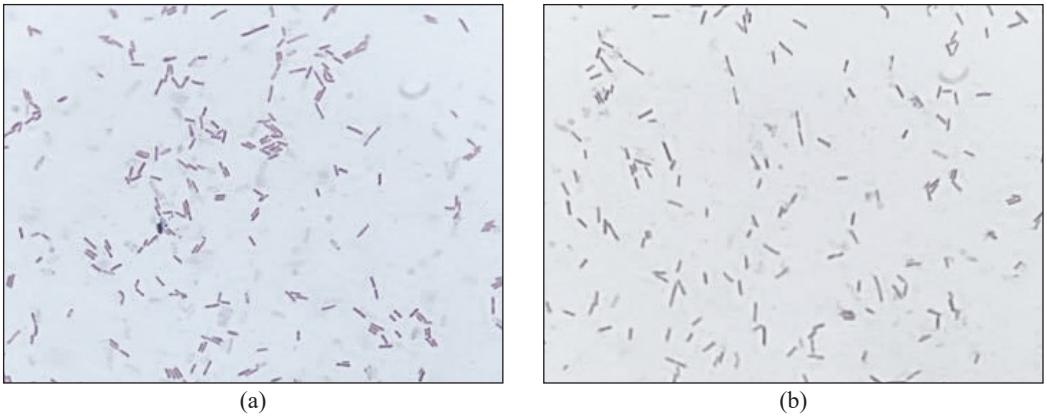


Figure 4. Growth pattern of *Vibrio harveyi* (VH) CFU mL^{-1} incubated with different concentrations of potential probiont NAS32 (10^8 CFU mL^{-1}) against time. Lower concentration of NAS32 (10^4 and 10^6 did not show a valid data). Error bars indicate standard error (SE)



(a) (b)

Figure 5. Gram staining of potential probionts (a) SPS11 and (b) NAS32 shows blue staining and bacillus in shape

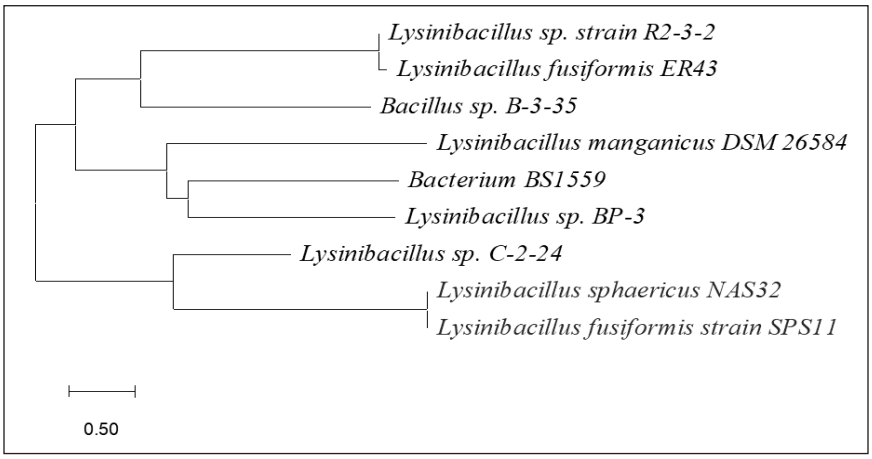


Figure 6. Phylogenetic tree of sequence analysis of SPS11 identified as *Lysinibacillus fusiformis* and NAS32 as *Lysinibacillus sphaericus*. Samples were determined by comparing the homology with the existing GenBank database

The PCR products were sequenced and the results were blast using National Centre for Biotechnology Information (NCBI). The phylogenetic tree was determined by comparing the homology with the existing GenBank database as shown in Figure 6. The comparative analysis revealed their closest neighbors. Results showed that SPS11 was identified as *Lysinibacillus fusiformis* with 98% similarity while NAS32 was identified as *Lysinibacillus sphaericus* with 99% similarity.

DISCUSSION

Probiotic strains isolated from various hosts and sources have been proven to be beneficial in enhancing diseases resistance as well as growth promoters in aquaculture. In this study, 90% of bacterial strains isolated from five species of microalgae showed potential as probiotics. The two most effective strains were SPS11 which was isolated from *Spirulina* sp. and NAS32 isolated from *Nannochloropsis* sp.. These potential probionts demonstrated the highest inhibition activities against *V. parahaemolyticus* and *V. vulnificus* among other isolated strains and were identified as *Lysinibacillus fusiformis* for SPS11 and *Lysinibacillus sphaericus* for NAS32.

The present study successfully isolated a total of 18 potential probiotics from different species of microalgae; *Amphora* sp., *Chaetoceros* sp., *Chlorella* sp., *Spirulina* sp., and *Nannochloropsis* sp.. Most of the isolates were obtained from *Chlorella* sp., which had been found to have a selection of symbionts, which included bacteria that may have potential as probiotics (Ferro et al., 2019; Myers, 2016; Watanabe et al., 2005).

Microalgae have been used in aquaculture especially for shrimp and fish larvae cultures as a growth promoter as well as to increase the antimicrobial activity of the cultured species. *Chlorella*, *Tetraselmis*, *Isochrysis*, *Pavlova*, *Phaeodactylum*, *Chaetoceros*,

Nannochloropsis, *Skeletonema*, and *Thalassiosira* are among the most frequently used microalgae species in aquaculture. The synergistic relationship between microalgae and bacteria may increase the density of both in which will improve the growth performance of microalgae (Han et al., 2016). Bacteria are able to successfully uptake the dissolved oxygen and organic materials produced by microalgae for their benefits (Ethier et al., 2011).

A previous study by Kokou et al. (2012) on microalgae strains of *Chlorella minutissima*, *Tetraselmis chuii*, *Nannochloropsi*s sp., and *Isochrysis* sp. showed antibacterial activity against *Vibrio alginolyticus*, *Vibrio lentus*, *Vibrio splendidus*, *Vibrio scophthalmi*, *Vibrio parahaemolyticus*, and *Vibrio anguillarum* when co-cultivated with the microalgae. The antibacterial activity showed by these microalgae against the pathogenic bacteria might be due to the active compounds with antibacterial properties synthesized by the microalgae which were able to inhibit bacterial growth. These active compounds include fatty acids (Desbois et al., 2009), terpenoids, carbohydrates (Duff & Bruce, 1966) peptides, polysaccharides, and alkaloids (Borowitzka, 1995).

In this study, 17 potential probionts exhibited antibacterial activity on both disc and well diffusion assay against four strains of pathogenic *Vibrio* spp.. The highest inhibition was observed by isolates SPS11 and NAS32 against *V. harveyi* and *V. parahaemolyticus*. This may be due to the production of antibacterial compounds that was diffused through the media inhibiting the growth of vibrios as observed in previous studies (Karim & Hasan, 2019; Ravi et al., 2007; Vaseeharan & Ramasamy, 2003). SPS11 and NAS32 were further tested in co-culture assay to test their ability in inhibiting the growth of *V. harveyi* and *V. parahaemolyticus* in motile conditions.

In the co-culture assay, each potential probiotic was able to inhibit the growth of *V. harveyi* and *V. parahaemolyticus* with different patterns and time intervals. SPS11 at lower concentrations (10^4 and 10^6 CFU mL^{-1}) were able to inhibit *V. parahaemolyticus* and *V. harveyi* while NAS32 performed better with a higher concentration (10^8 CFU mL^{-1}) against both pathogens. These findings are correlated with a recent study by Jasmin et al. (2016) which stated that increasing the amount of probiotic at a specific time might improve the effectiveness in inhibiting pathogens. The inhibition of pathogens by the potential probionts might be due to the production of bacteriocin-like compounds, competition for attachment sites, competition for nutrients (particularly iron in marine microbes), alteration of enzymatic activity of pathogens and immunostimulatory functions (Kesarcodi-Watson et al., 2008). This could explain the decrease of *V. harveyi* and *V. parahaemolyticus* population when co-cultured with potential probionts in the study. Based on the period of incubation interval, early incubation periods from 0 to 12 hour(s) exhibit the rapid increase in the growth of *V. harveyi*, *V. parahaemolyticus*, and potential probiotics (Chen et al., 2019; Stalin & Srinivasan, 2017; Wang et al., 2017).

Several probiotic strains have been reported to be able to reduce and inhibit the growth of pathogens in aquaculture and at the same time improve resistance against disease infection. The most common probiotic strains; *Bacillus* sp., are effective in inhibiting vibriosis (Doroteo et al., 2018; Tepaamorndech et al., 2019). Giri et al. (2013) reported Lactobacillus *plantarum* VSG3 was beneficial towards *Labeo rohita* by improving the growth performance, immunity, and disease resistance. *Leuconostoc mesenteroides* was found to inhibit pathogenic bacteria in Nile tilapia (Zapata & Lara-Flores, 2013). Meanwhile, *Lactobacillus rhamnosus* and *Lactobacillus sporogenes* were able to improve the disease resistance of common carp (Harikrishnan et al., 2010). The ability of probiotics in inhibiting pathogenic bacteria may be due to the competitive action of the probiotics against the pathogenic bacteria for adhesion sites. In order for the pathogenic bacteria to initiate the development of a disease, it requires abundance of attachment at the mucosal layer of the host gastrointestinal tract (Adams, 2010). Besides, probiotic itself needs to have bactericidal effects on other microbial populations by synthesizing active compounds such as bacteriocins, hydrogen peroxide, siderophores, lysozymes, proteases (Panigrahi & Azad, 2007), organic acid, and volatile fatty acids that can reduce pH in gastrointestinal tract, which can prevent opportunistic pathogenic bacteria to grow (Tinh et al., 2007).

Both isolates SPS11 and NAS32 were observed to be Gram positive bacteria with rod shape. Through 16s rRNA molecular identification, SPS11 was identified as *Lysinibacillus fusiformis* while NAS32 was identified as *Lysinibacillus sphaericus*. *Lysinibacillus fusiformis* and *L. sphaericus* were previously known as *Bacillus fusiformis* and *Bacillus sphaericus*, respectively. Reclassification from genus *Bacillu*s to *Lysinibacillus* is based on the fact that the *Lysinibacillus* genus contains peptidoglycan with lysine, aspartic acid, alanine, and glutamic acid, which bacteria form genus *Bacillus* do not have. Several reports on *Lysinibacillus* sp. as probiotics have been reported. *Lysinibacillus fusiformis* isolated from Nile tilapia reportedly exhibits antagonistic characteristics towards *Aeromonas* sp. (Reda et al., 2018). Similar findings were reported on *L. fusiformis* isolated from rainbow trout against pathogen *Flavobacterium psychrophilum* (Burbank et al., 2011, 2012). Compared to *L. fusiformis*, there are limited researches on the probiotic activity of *L. sphaericus* in aquaculture. Recently, *L. sphaericus* had been found isolated from Catla, *Catla catla* (Seelam et al., 2017). In India, *L. sphaericus* was isolated from the gut of Asian catfish, *Clarias batrachus*, and showed positive antagonistic activity towards common *Vibrio* pathogens; *V. harveyi*, *V. vulnificus*, and *V. parahaemolyticus* (Ganguly et al., 2019).

These current findings are the key in developing potential probiotics that can benefit both microalgae and marine aquaculture since not many studies have been done on the application of *L. fusiformis* and *L. sphaericus* as probiotics in the marine aquaculture systems.

CONCLUSION

In conclusion, two potential probiotics; *Lysinibacillus fusiformis* which was isolated from *Spirulina* sp. and *Lysinibacillus sphaericus* from *Nannochloropsis* sp. displayed inhibitory effects against *V. harveyi* and *V. parahaemolyticus*, in *in vitro* assay. These bacteria may have positive inhibition towards pathogenic *Vibrio* spp. in laboratory condition yet the efficiency of these potential probiotics in *in vivo* studies is still unclear. Thus, further study in *in vivo* is very much needed to determine the efficiency and to understand better the mechanism of the two potential probiotics. Although there are still more researches that need to be done before it can be considered to be used in the culture systems, the potential of the two potential probiotics, *L. fusiformis* SPS11 and *L. sphaericus* NAS32 are promising and deserve to be further evaluated.

ACKNOWLEDGEMENTS

This research was supported by Ministry of Higher Education Malaysia (MOHE) through Fundamental Research Grant Scheme FRGS/1/2020/WAB04/UPM/02/9. Also special thanks to SATREPS JICA-JST COSMOS 2016-2021 and Higher Institution Centre of Excellence (HiCoE) grant of Innovative Vaccine and Therapeutics against Fish Diseases for providing facilities at Laboratory and Hatchery Unit in Institute of Bioscience, UPM. The first author is under the scheme of Graduate Research Fellowship Scheme with Universiti Putra Malaysia.

REFERENCES

Abraham, T. J. (2016). Transferable chloramphenicol resistance determinant in luminous *Vibrio harveyi* from penaeid shrimp *Penaeus monodon* larvae. *Journal of Fisheries*, *4*(3), 428-430. https://doi.org/10.17017/jfish.v4i3.2016.158

Adams, C. A. (2010). The probiotic paradox: Live and dead cells are biological response modifiers. *Nutrition Research Reviews*, *23*(1), 37-46. https://doi.org/10.1017/s0954422410000090

Amin, S. A., Parker, M. S., & Ambrust, E. V. (2012). Interactions between diatoms and bacteria. *Microbiology and Molecular Biology Reviews*, *76*(3), 667–684. https://doi.org/10.1128/mmbr.00007-12

Baker-Austin, C., McArthur, J. V., Tuckfield, R. C., Najarro, M., Lindell, A. H., Gooch, J., & Stepanauskas, R. (2008). Antibiotic resistance in the shellfish pathogen *Vibrio parahaemolyticus* isolated from the coastal water and sediment of Georgia and South Carolina, USA. *Journal of Food Protection*, *71*(12), 2552-2558. https://doi.org/10.4315/0362-028x-71.12.2552

Bartholomew, J. W., & Mittwer, T. (1952). The Gram stain. *Bacteriological Reviews*, *16*(1), 1. https://doi.org/10.1128/br.16.1.1-29.1952

Borowitzka, M. A. (1995). Microalgae as sources of pharmaceuticals and other biologically active compounds. *Journal of Applied Phycology*, *7*(1), 3–15. https://doi.org/10.1007/bf00003544

Burbank, D. R., LaPatra, S. E., Fornshell, G. & Cain, K. D. (2012). Isolation of bacterial probiotic candidates from the gastrointestinal tract of rainbow trout, *Oncorhynchus mykiss* (Walbaum), and screening for inhibitory activity against *Flavobacterium psychrophilum*. *Journal of Fish Diseases*, *35*(11), 809-816. https://doi.org/10.1111/j.1365-2761.2012.01432.x

Burbank, D. R., Shah, D. H., LaPatra, S. E., Fornshell, G., & Cain, K. D. (2011). Enhanced resistance to coldwater disease following feeding of probiotic

bacterial strains to rainbow trout (*Oncorhynchus mykiss*). *Aquaculture*, *321*(3-4), 185-190. https://doi.org/10.1016/j.aquaculture.2011.09.004

Burridge, L., Weis, J. S., Cabello, F., Pizarro, J., & Bostick, K. (2010). Chemical use in salmon aquaculture: A review of current practices and possible environmental effects. *Aquaculture*, *306*(1-4), 7-23. https://doi.org/10.1016/j.aquaculture.2010.05.020

Charoonnart, P., Purton, S., & Saksmerprome, V. (2018). Applications of microalgal biotechnology for disease control in aquaculture. *Biology*, *7*(2), 24. https://doi.org/10.3390/biology7020024

Chen, Y. R., Hwang, C. A., Huang, L., Wu, V. C., & Hsiao, H. I. (2019). Kinetic analysis and dynamic prediction of growth of *Vibrio parahaemolyticus* in raw white shrimp at refrigerated and abuse temperatures. *Food Control*, *100*, 204-211. https://doi.org/10.1016/j.foodcont.2019.01.013

Desbois, A. P., Mearns-Spragg, A., & Smith, V. J. (2009). A fatty acid from the diatom *Phaeodactylum tricornutum* is antibacterial against diverse bacteria including multi-resistant *Staphylococcus aureus* (MRSA). *Marine Biotechnology*, *11*(1), 45–52. https://doi.org/10.1007/s10126-008-9118-5

Doroteo, A. M., Pedroso, F. L., Lopez, J. D. M., & Apines-Amar, M. J. S. (2018). Evaluation of potential probiotics isolated from saline tilapia in shrimp aquaculture. *Aquaculture International*, *26*(4), 1095-1107. https://doi.org/10.1007/s10499-018-0270-2

Duff, D. A., Bruce, D. L., & Antia, N. J. (1966). The antibacterial activity of marine planktonic algae. *Canadian Journal of Microbiology*, *12*(5), 877-884. https://doi.org/10.1139/m66-120

Ethier, S., Woisard, K., Vaughan, D., & Wen, Z. (2011). Continuous culture of the microalgae *Schizochytrium limacinum* on biodiesel-derived crude glycerol for producing docosahexaenoic acid. *Bioresource Technology*, *102*(1), 88–93. https://doi.org/10.1016/j.biortech.2010.05.021

Ferro, L., Colombo, M., Posadas, E., Funk, C., & Muñoz, R. (2019). Elucidating the symbiotic interactions between a locally isolated microalga *Chlorella vulgaris* and its co-occurring bacterium *Rhizobium* sp. in synthetic municipal wastewater. *Journal of Applied Phycology*, *31*(4), 2299-2310. https://doi.org/10.1007/s10811-019-1741-1

Food and Agriculture Organization. (2013). *Report of the FAO/MARD technical workshop on early mortality syndrome (EMS) or acute hepatopancreatic necrosis syndrome (AHPNS) of cultured shrimp* (under TCP/VIE/3304). FAO. http://www.fao.org/3/i3422e/i3422e00.htm

Fuentes, J. L., Garbayo, I., Cuaresma, M., Montero, Z., González-del-Valle, M., & Vilchez, C. (2016). Impact of microalgae-bacteria interactions on the production of algal biomass and associated compounds. *Marine Drugs*, *14*(5), 100. https://doi.org/10.3390/md14050100

Ganguly, A., Banerjee, A., Mandal, A., Khan, M. A., & Mohapatra, P. K. D. (2019). Isolation and characterization of bacteria from the intestine of *Clarias batrachus* for probiotic organism. *Proceedings of the Zoological Society*, *72*(4), 411-419. https://doi.org/10.1007/s12595-018-0283-x

Ghasemi, Y., Yazdi, M. T., Shafiee, A., Amini, M., Shokravi, S., & Zarrini, G. (2004). Parsiguine, a novel antimicrobial substance from *Fischerella ambigua*. *Pharmaceutical Biology*, *42*(4-5), 318-322. https://doi.org/10.1080/13880200490511918

Giri, S. S., Sukumaran, V., & Oviya, M. (2013). Potential probiotic *Lactobacillus plantarum* VSG3 improves the growth, immunity, and disease resistance of tropical freshwater fish. *Fish and Shellfish Immunology*, *34*(2), 660–666. https://doi.org/10.1016/j.fsi.2012.12.008

Grossart, H. P., Czub, G., & Simon, M. (2006). Algae-bacteria interactions and their effects on aggregation and organic matter flux in the sea. *Environmental Microbiology*, *8*(6), 1074–1084. https://doi.org/10.1111/j.1462-2920.2006.00999.x

Han, J., Zhang, L., Wang, S., Yang, G., Zhao, L., & Pan, K. (2016). Co-culturing bacteria and microalgae in organic carbon containing medium. *Journal of Biological Research-Thessaloniki*, *23*(1), 8. https://doi.org/10.1186/s40709-016-0047-6

Harikrishnan, R., Balasundaram, C., & Heo, M. S. (2010). Potential use of probiotic and triherbal extract-enriched diets to control *Aeromonas hydrophila* infection in carp. *Disease of Aquatic Organisms*, *92*(1), 41–49. https://doi.org/10.3354/dao02240

Irianto, A., & Austin, B. (2002). Probiotics in aquaculture. *Journal of Fish Diseases*, *25*(11), 633–642. https://doi.org/10.1046/j.1365-2761.2002.00422.x

Jasmin, M. Y., Wagaman, H., Yin, T. A., Inasalwany, M. Y., Daud, H. M., & Karim, M. (2016). Screening and evaluation of local bacteria isolated from shellfish as potential probiotics against pathogenic Vibrios. *Journal of Environmental Biology*, *37*, 801-809.

Karim, M., & Hasan, F. (2019). A comparative study of antibiotics and probiotics against pathogens isolated from coastal shrimp aquaculture system. *Jordan Journal of Biological Sciences*, *12*(3), 323-328.

Kesarcodi-Watson, A., Kaspar, H., Lategan, M. J., & Gibson, L. (2008). Probiotics in aquaculture, the need, principles and mechanisms of action and screening processes. *Aquaculture*, *274*(1), 1-14. https://doi.org/10.1016/j.aquaculture.2007.11.019

Kokou, F., Makridis, P., Kentouri, M., & Divanach, P. (2012). Antibacterial activity in microalgae cultures. *Aquaculture Research*, *43*(10), 1520-1527. https://doi.org/10.1111/j.1365-2109.2011.02955.x

Labreuche, Y., Pallandre, L., Ansquer, D., Herlin, J., Wapotro, B., & Le Roux, F., (2012). Pathotyping of *Vibrio* isolates by multiplex PCR reveals a risk of virulent strain spreading in New Caledonian shrimp farms. *Microbial Ecology*, *63*(1), 127–138. https://doi.org/10.1007/s00248-011-9951-3

Myers, J. E. (2016). *Untangling algae-bacteria consortia: The ecological, phylogenetic, and bioremediation properties of freshwater microbiota*. University of Tulsa.

Panigrahi, A., & Azad, I. S. (2007). Microbial intervention for better fish health in aquaculture: The Indian scenario. *Fish Physiology and Biochemistry*, *33*(4), 429–440. https://doi.org/10.1007/s10695-007-9160-7

Priyadarsani, L., & Abraham, T. J. (2013). Ecology of antibiotic resistant vibrios in traditional shrimp farming system (*bhery*) of West Bengal, India. *Journal of Coastal Life Medicine*, *1*(4), 265-272.

Ravi, A. V., Musthafa, K. S., Jegathammbal, G., Kathiresan, K., & Pandian, S. K. (2007). Screening and evaluation of probiotics as a biocontrol agent against pathogenic Vibrios in marine aquaculture. *Letters in Applied Microbiology*, *45*(2), 219-223. https://doi.org/10.1111/j.1472-765x.2007.02180.x

Reda, R. M., Selim, K. M., El-Sayed, H. M., & El-Hady, M. A. (2018). *In vitro* selection and identification of potential probiotics isolated from the gastrointestinal tract of Nile tilapia, *Oreochromis niloticus*. *Probiotics and Antimicrobial Proteins*, *10*(4), 692-703. https://doi.org/10.1007/s12602-017-9314-6

Sarter, S., Nguyen, H. N. K., Hung, L. T., Lazard, J., & Montet, D. (2007). Antibiotic resistance in Gram-negative bacteria isolated from farmed catfish. *Food Control*, *18*(11), 1391–1396. https://doi.org/10.1016/j.foodcont.2006.10.003

Seelam, N. S., Akula, H., Katike, U., & Obulam, V. S. R. (2017). Production, characterization and optimization of fermented tomato and carrot juices by using *Lysinibacillus sphaericus* isolate. *Journal of Applied Biology and Biotechnology*, *5*(4), 66-75. https://doi.org/10.7324/jabb.2017.50410

Shah, M. R., Lutzu, G. A., Alam, A., Sarker, P., Chowdhury, M. A. K., Parsaeimehr, A., Liang, Y., & Daroch, M. (2018). Microalgae in aquafeeds for a sustainable aquaculture industry. *Journal of Applied Phycology*, *30*(1), 197–213. https://doi.org/10.1007/s10811-017-1234-z

Sharifah, E. N., & Eguchi, M. (2011). The phytoplankton *Nannochloropsis oculata* enhances the ability of *Roseobacter* clade bacteria to inhibit the growth of fish pathogen *Vibrio anguillarum*. *PLOS One*, *6*(10), e26756. https://doi.org/10.1371/journal.pone.0026756

Stalin, N., & Srinivasan, P. (2017). Efficacy of potential phage cocktails against *Vibrio harveyi* and closely related *Vibrio* species isolated from shrimp aquaculture environment in the south east coast of India. *Veterinary Microbiology*, *207*, 83-96. https://doi.org/10.1016/j.vetmic.2017.06.006

Tepaamorndech, S., Chantarasakha, K., Kingcha, Y., Chaiyapechara, S., Phromson, M., Sriariyanun, M., & Visessanguan, W. (2019). Effects of *Bacillus aryabhattai* TBRC8450 on vibriosis resistance and immune enhancement in Pacific white shrimp, *Litopenaeus vannamei*. *Fish and Shellfish Immunology*, *86*, 4-13. https://doi.org/10.1016/j.fsi.2018.11.010

Tinh, N. T. N., Dierckens, K., Sorgeloos, P., & Bossier, P. (2007). A review of the functionality of probiotics in the larviculture food chain. *Marine Biotechnology*, *10*(1), 1-12. https://doi.org/10.1007/s10126-007-9054-9

Vaseeharan, B., & Ramasamy, P. (2003). Control of pathogenic *Vibrio* spp. by *Bacillus subtilis* BT23, a possible probiotic treatment for black tiger shrimp *Penaeus monodon*. *Letters in Applied Microbiology*, *36*(2), 83-87. https://doi.org/10.1046/j.1472-765x.2003.01255.x

Walling, E., Vourey, E., Ansquer, D., Beliaeff, B., & Goarant, C. (2010). *Vibrio nigripulchritudo* monitoring and strain dynamics in shrimp pond sediments. *Journal of Applied Microbiology*, *108*(6), 2003–2011. https://doi.org/10.1111/j.1365-2672.2009.04601.x

Wang, Y., Barton, M., Elliott, L., Li, X., Abraham, S., O'Dea, M., & Munro, J. (2017). Bacteriophage therapy for the control of *Vibrio harveyi* in greenlip abalone (*Haliotis laevigata*). *Aquaculture*, *473*, 251-258. https://doi.org/10.1016/j.aquaculture.2017.01.003

Watanabe, K., Takihana, N., Aoyagi, H., Hanada, S., Watanabe, Y., Ohmura, N., & Tanaka, H. (2005). Symbiotic association in *Chlorella* culture. *FEMS Microbiology Ecology*, *51*(2), 187-196. https://doi.org/10.1016/j.femsec.2004.08.004

Yao, S., Lyu, S., An, Y., Lu, J., Gjermansen, C., & Schramm, A. (2019). Microalgae–bacteria symbiosis in microalgal growth and biofuel production: A review. *Journal of Applied Microbiology*, *126*(2), 359-368. https://doi.org/10.1111/jam.14095

Zapata, A. A., & Lara-Flores, M. (2013). Antimicrobial activities of lactic acid bacteria strains isolated from Nile tilapia (*Oreochromis niloticus*) intestine. *Journal of Biology and Life Science*, *4*(1), 123-129. https://doi.org/10.5296/jbls.v4i1.2408

Pertanika J. Trop. Agric. Sci. 44 (1): 221 - 236 (2020)

PERTANIKA JOURNALS

TROPICAL AGRICULTURAL SCIENCE

Journal homepage: http://www.pertanika.upm.edu.my/

Incorporation of Rice Husk Ash with Palm Oil Mill Wastes in Enhancing Physicochemical Properties of the Compost

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ABSTRACT

Rice husk ash (RHA), palm oil mill effluent (POME) sludge and decanter cake can be utilized together in compost production to minimize the environmental pollution. This study aims to evaluate the role of different composition of RHA in enhancing the physicochemical properties of palm oil-based compost. The composts were prepared by mixing different composition of RHA, in the range of 0% to 30%, with 1:1 (wt/wt) weight ratio of POME sludge and decanter cake. The moisture content, water holding capacity, pH, nitrogen (N), phosphorus (P), potassium (K), silica (Si) contents, and C:N ratio of raw materials were analyzed by using CHNS and WDXRF analyzers. The composting process was conducted in compost containers for 60 days, in which the temperature and pH of the composts were monitored daily. The finished composts were analyzed for physicochemical properties as same as raw materials. For physical properties of finished composts, RHA_{30} had the highest moisture content and water holding capacity which was 1.9 to 23.8% (wt/wt) and 4.2 to 26.8% higher compared to other finished composts, respectively. For chemical properties, the highest N and P contents were recorded by control compost. However, for K and Si content, the elements were found to be higher in RHA_{10} and RHA_{30}, respectively, compared to other finished composts. Overall, RHA, POME sludge and decanter cake combination in compost production can create a well-balanced condition for the compost to perform effectively as an organic fertilizer. The addition of 5% to 10% RHA in compost formulation made from palm oil mill wastes is suggested to achieve the desirable condition.

Keywords: Compost, decanter cake, POME sludge, rice husk ash

ARTICLE INFO

Article history:
Received: 10 September 2020
Accepted: 17 November 2020
Published: 24 February 2021

DOI: https://doi.org/10.47836/pjtas.44.1.13

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ISSN: 1511-3701
e-ISSN: 2231-8542

INTRODUCTION

The demand of rice, which is one of the most crucial crop in the world, has triggered the expansion of rice processing industries in many countries including Malaysia, India, and China (Babaso & Sharanagouda, 2017). One of the valuable wastes produced by this industry is rice husk ash (RHA). RHA is the ashes produced through the burning process of rice husk. Although it is considered as a waste product, it can be exploited in agricultural industry as it has the ability to influence the hydro-physical properties of the soil and amend the acidity level as buttressed by Islabão et al. (2014). Other than that, RHA contains an enormous amount of silica components which can be as high as 89% (Badar & Qureshi, 2014). Silica plays a crucial role in retaining soil moisture content due to the presence of highly porous structure of this element (Phonphuak & Chindaprasirt, 2015).

Apart from rice processing industries, Malaysia is also leading towards palm oil industry, which can trigger the production of enormous amounts of wastes. Palm oil mill effluent (POME) is one of the wastes generated from palm oil mill. The effluent is generated from various processing units including sludge clarification, drain-off of hydrocyclone, and condensates sterilization (Akhbari et al., 2020). Approximately 0.50 to 0.75 tons of POME will be generated for every ton of FFB from the milling process (Wun et al., 2017). If the effluent is not treated accordingly, POME can cause a significant environmental pollution, especially water pollution, due to its characteristics that contain a very high level of biochemical oxygen demand (BOD) and chemical oxygen demand (COD). Thus, POME undergoes several treatment process in the treatment ponds before being discharged into the river, whereby the process produces a sediment known as POME sludge (Khairuddin et al., 2017). POME sludge has been widely utilized as a fertilizer through compost making process to reduce its disposal to the environment. Khairuddin et al. (2016) had suggested that, POME sludge could be experimented on crops to prove its effectiveness as an organic fertilizer as this material contained several essential nutrients that were crucial for plant growth.

Other from POME sludge, decanter cake (DC) is another type of solid waste produced in palm oil mill industry. For every ton of palm fresh fruit bunch (FFB), approximately 0.035 tons of DC is produced (Sahad et al., 2014). The direct dumping of the waste has caused numerous hazards to the environment such as leaching, odor, water pollution, pests, and rodents as claimed by Embrandiri et al. (2012). However, as mentioned by Dewayanto et al. (2009), DC can be beneficially utilized as fertilizer as it contains a significant amount of nitrogen (N), potassium (K), and phosphorus (P).

As the abundance of those types of waste available in Malaysia, study related to utilization of those types of wastes into valuable and environmental friendly material is worth to be explored. Hence, as suggested by Anwar et al. (2015), the wastes from rice mill and palm oil mill industries can be converted into an environmentally

friendly waste by utilizing them as an organic fertilizer through composting process. The compost produced through the process may promote the production of healthier plants. Other than that, the process is also practical and convenient. Moreover, composting process can be conducted with low operating and capital costs, and it is a great alternative to reduce landfill space.

Although there have been many studies that were conducted to prove the effectiveness of palm oil wastes in enhancing the physicochemical properties of compost, the incorporation of RHA with the palm oil mill wastes in compost production is worth to be explored. Previous research made by Theeba et al. (2012) had attempted to investigate the effect of RHA in combination with other organic substrates, such as chicken manure and rice bran. The authors had studied the addition of 4% and 6% of RHA to the compost and found that, 4% RHA could improve the moisture and nutrient content of the compost produced. Similar as Theeba et al. (2012), Frimpong-Manso et al. (2011) also discovered that, RHA could serve as an addition to composted sawdust, in which the composition was varied between 2% to 20% in their study. However, based on the authors, 2% RHA addition could enhance the growth nutrient content and growth of oyster mushroom. The findings obviously show that the best composition of RHA would vary when different compost raw materials were used.

Even so, based on Theeba et al. (2012) and Frimpong-Manso et al. (2011)'s study, the percentage of RHA utilized in the research is not varied broadly. There may be a tendency of the chosen percentage of RHA not being the best composition of RHA to be added as an additive to the compost in composting process. Therefore, this study attempted to vary the RHA composition in a broader range, which was between 0 to 30% of RHA composition. This is because; RHA could benefit the agricultural activity by enhancing the nutrients uptake by the plant due to the presence of high content of silica that can improve the crop production.

Moreover, the source of raw materials used in Theeba et al. (2012) and Frimpong-Manso et al. (2011) were different compared to the materials utilized in this work. This research had utilized the palm oil mill wastes as they contained high amount of N, P, and K, which were essential for the plant growth. Since the palm oil mill wastes were available in abundance in Malaysia, therefore it was worthwhile to investigate the potential of these wastes to be converted into compost materials. Thus, this research was aimed to evaluate the effectiveness of RHA addition at different composition in enhancing the physicochemical properties of palm oil-based compost. The physicochemical parameters of the raw materials and compost were measured in terms of pH, moisture content, water holding capacity, N, P, K, and Si contents.

MATERIALS AND METHODS

Sample Preparation

Rice husk ash (RHA) was purchased from a nursery in Kuantan, Pahang. Meanwhile, palm oil mill effluent (POME) sludge and

decanter cake were collected from LKPP Lepar, Pekan, Pahang. All raw materials were stored at ambient temperature before ready to be used in experimental work.

Physicochemical Properties Analysis of Raw Material

RHA, POME sludge, and decanter cake were analyzed for physical and chemical properties, which includes the analysis of moisture content, water holding capacity, pH, and elemental contents. Three replicates of samples were prepared in the analysis for each physical and chemical property in order to obtain the mean and standard deviation of the data.

For pH analysis, 5 g of sample was taken and diluted in 50 mL of water and stirred by using magnetic stirrer. Then, the supernatant liquid was obtained from the mixture through filtration. The pH meter probe (METTLER TOLEDO S20 SevenEasy™) was inserted into the supernatant liquid and the reading was taken (Ramli et al., 2016).

For moisture content analysis, the sample was weighed to obtain the fresh weight. Then, the sample was dried at 105 ± 2°C for 5 h in an oven. The sample was then reweighed again to obtain the dry weight and moisture content was calculated based on standard test ASTM D4442-16.

Meanwhile, for the analysis of water holding capacity, 10 g of sample was mixed-well with 50 mL of distilled water and allowed to stand for 30 min. Then, the sample was transferred on filter paper in a funnel. The drop-off water was recorded every 30 min until the sample began to dry. The weight of wet sample in the filter was taken when the weight remain unchanged. The sample was dried in an oven for 48 h at 105°C ± 2°C and reweighed again. Standard test ASTM D2980-02 was referred to calculate the water holding capacity of the sample.

For chemical analysis, the sample was oven-dried for 24 h at 105°C ± 2°C and pulverized by using a grinder until a powder form was obtained. Standard test ASTM E1621 - 13 was used to determine the content of potassium (K), phosphorus (P), and silica (Si). Wavelength Dispersive X-ray Fluorescence (WDXRF) spectrometer instrument (model Axios^{mAX} made in Netherlands by PANalytical) was employed to determine the elements. Meanwhile, CHNS analyser (model vario MICRO cube made in Germany by Elementar) was utilized to detect the carbon (C) and nitrogen (N) contents.

Preparation of Rice Husk Ash

In this work, seven samples with different composition of RHA were prepared. The RHA composition was varied in the range of 0 to 30%. However the weight ratio of POME sludge to decanter cake was kept constant at ratio 1:1 (wt/wt) as referred to the previous work by Ramli et al. (2016). Other than that, the total weight of composting material was fixed at 5 kg. The variation of weight composition for material in the sample is presented in Table 1.

Table 1
Weight of material used in composting process

Treatments	Composition of RHA (%) added	Weight of RHA (kg)	Weight of POME sludge (kg)	Weight of decanter cake (kg)	Total weight (kg)
Control	0	0	2.50	2.50	5.00
RHA_5	5	0.25	2.375	2.375	5.00
RHA_{10}	10	0.50	2.25	2.25	5.00
RHA_{15}	15	0.75	2.125	2.125	5.00
RHA_{20}	20	1.00	2.00	2.00	5.00
RHA_{25}	25	1.25	1.875	1.875	5.00
RHA_{30}	30	1.50	1.75	1.75	5.00

Composting Process

The composting process was conducted at Universiti Malaysia Pahang based on the procedure stated in Ramli et al. (2019). The process was conducted in seven containers based on the composition as shown in Table 1. Each composition was developed with a total weight of 5 kg and placed inside containers with a size of 16 cm (H) x 30 cm (L) x 18 cm (W). For control treatment, the formulation was carried out without RHA component. The sample only contained main component of the compost, which were POME sludge and decanter cake, with weight ratio of 1:1 (wt/wt). Meanwhile, for RHA_5, RHA_{10}, RHA_{15}, RHA_{20}, RHA_{25}, and RHA_{30} treatments, the amount of RHA added to the palm oil mill wastes was based on the weight of materials as tabulated in Table 1. All materials were mixed together in the containers and the treatments were allowed to be decomposing for a period of 60 days. The pH and temperature of the composts were monitored daily for 60 days in the afternoon by using the 4 in 1 Soil Survey Instrument. In order to control the odor and aid the decomposition process, the composts were turned by using a pitch-fork once a week. Other than that, 200 mL of tap water was added to each container once a week to maintain moisture level at desired condition (40-60 %) (Zakarya et al., 2018). All samples were analyzed once the composting process was completed. For physical properties analysis, moisture content and water holding capacity were measured. Meanwhile, chemical properties analysis was performed to determine the N, P, K, and Si contents of finished compost. Both analyses were conducted based on the procedure as described in part 'Physicochemical Properties Analysis of Raw Material'. All experiments were conducted by using a completely randomized design (CRD) with three replications of sample for each experiment.

Statistical Analysis

The data obtained was subjected to Analysis of Variance (ANOVA) using MINITAB®18 Statistical Software (Version 18.1, Minitab, Inc., State College, PA). Least significant difference (LSD) at significant level of $p \leq 0.05$ was performed to determine the significant difference among means.

RESULTS AND DISCUSSION

Physical Properties of Raw Materials

The data obtained for the physical properties analysis is tabulated in Table 2. Based on Table 2, the highest pH was recorded by RHA while the lowest was obtained by decanter cake. For moisture content, the highest percentage was achieved by decanter cake, with 70.59%, followed by POME sludge and RHA. Meanwhile, the analysis for water holding capacity had clearly shown that, RHA had the highest percentage, which was 83.61%, compared to POME sludge and decanter cake.

Based on Table 2, the pH of RHA was slightly alkaline, which is similar with the findings obtained by Islabão et al. (2014), Saranya et al. (2018), and Persaud et al. (2018). The alkaline condition of RHA is crucial to reduce the acidity of soil as buttressed by Okon et al. (2005). For POME sludge, the pH obtained for this material was close to neutral level. However, it still lies within an acceptable range between 6 to 9 as this material was directly retrieved from aerobic pond of palm oil mill (Akhbari et al., 2020). Meanwhile, the pH for decanter cake was acidic, which might be due to the release of humic acid caused by the material degradation as reported by Osman et al. (2019). The presence of humic acid could increase the concentration of hydrogen (H^+) ions which can be detected by pH analysis.

According to the results tabulated in Table 2, the percentage of moisture content obtained for POME sludge and decanter cake in this study was close to the results obtained by Khairuddin et al. (2016), Razak et al. (2012), and Sahad et al. (2014). Meanwhile, RHA contained as high as 59.17% of moisture content, which might be related to high specific surface area and highly porous structure of silica in its components that could absorb water efficiently (Phonphuak & Chindaprasirt, 2015). Other than that, the presence of high content of silica also influences the water holding capacity of this material. The presence of silica allows the material to retain more water due to the highly porous structure of the element that can increase the moisture content as well. Other than that, the water holding capacity for POME sludge and decanter cake were impressively high with 69.53% and 76.28%, respectively, which proved the significantly high moisture content obtained by these materials.

Chemical Properties of Raw Materials

The data obtained for carbon (C), nitrogen (N), potassium (K), phosphorus (P), and silica (Si) contents existed in the raw

Table 2
Physical properties of raw materials

Parameters	Materials (Mean ± SD)		
	Rice husk ash	POME sludge	Decanter cake
pH	8.70 ± 0.15	7.20 ± 0.10	4.43 ± 0.15
Moisture content (%)	59.17 ± 0.94	64.47 ± 0.85	70.59 ± 0.58
Water holding capacity (%)	83.61 ± 0.54	69.53 ± 0.64	76.28 ± 0.96

materials are presented in Table 3. Based on Table 3, the highest N, P, K contents were recorded by POME sludge while RHA obtained the highest Si content and C:N ratio compared to other materials.

Based on Table 3, POME sludge contained an appreciable amount of N, which was in agreement with Bala et al. (2014). The authors had claimed that POME sludge contained high amount of essential nutrients in its components such as N, P, and K. Since POME sludge is formed from the treatment of palm oil mill effluent (POME), it contains a considerably high amount of organic matter (Kamyab et al., 2018) which can act as a reservoir of nutrients for plants including N, P, and K. Meanwhile, for RHA, it only consists of a small amount of N as this material was widely utilized to enhance the availability of nutrient as buttressed by Islabão et al. (2014), and not as the main source of N.

For phosphorus (P), the high content of this element attained by POME sludge lies within the range of P content mentioned by Khairuddin et al. (2016) and Zaini et al. (2014) in their studies. For RHA, it achieved the lowest amount of P which proved the statement made by Dizaji et al. (2019). The authors stated in their work that, RHA only consisted a small amount of nutrient elements, which prevented them from being consumed for land usage and it was usually directly disposed.

Meanwhile, for potassium (K), this element was found to be higher in POME sludge compared to other materials, which was supported by the previous research by Nizar et al. (2018). However, for RHA, K content obtained for this material was the least compared to other raw materials. This is because the content of elements existing in RHA will vary based on temperature and time when the husk is burnt as claimed by Priyadharshini and Seran (2010).

For silica (Si) content, the highest content was recorded by RHA, which proved the role of RHA as the main source of Si (Rambo et al., 2011). The Si content in RHA may reach up to 95%. For POME sludge, although the Si content was the lowest compared to RHA and decanter cake, the value obtained in this study was almost similar to the Si content reported by Zaini et al. (2014).

Apart from that, RHA had the highest carbon to nitrogen (C:N) ratio, followed by decanter cake and POME sludge. According

Table 3
Chemical properties of raw materials

Parameters	Materials (Mean ± SD)		
	Rice husk ash	POME sludge	Decanter cake
N (%)	0.85 ± 0.07	4.17 ± 0.10	2.74 ± 0.06
P (%)	0.70 ± 0.93	3.71 ± 0.11	2.29 ± 0.89
K (%)	1.65 ± 0.11	5.00 ± 0.34	1.98 ± 0.20
Si (%)	69.28 ± 0.82	8.48 ± 0.35	20.26 ± 0.71
C/N	43.8 ± 0.64	6.74 ± 0.16	14.63 ± 0.30

to Misra et al. (2003), C:N ratio greater than 35 will trigger the microbial immobilization, prolong the composting period and limit the microorganisms' growth. Meanwhile, for POME sludge and decanter cake, the C:N ratio obtained for both materials were between 6 to 15, which were quite low. C:N ratio value in the range of 1 to 15 will result in rapid release of N into the soil for immediate crop use and enhance the mineralization process (Brust, 2019). In composting process, the available C:N ratio must be kept at a proper level to ensure the microorganisms remain active.

Temperature Profile of Compost

Temperature is considered as an important parameter that needs to be closely monitored as to ensure the completion of composting process. The temperature profile for all composts is illustrated in Figure 1.

As shown in Figure 1, the temperature of the composts began to increase from day 9 until day 18, which indicates the mesophilic stage of composting process. Mesophilic stage had a range of temperature between 20 to 45°C as stated by Misra et al. (2003). In this stage, RHA_{15} recorded the highest temperature which was more than 47°C, recorded on day 16. Meanwhile, control compost recorded the lowest temperature at this stage. Compost that contained an appropriate moisture content level may improve the consumption of oxygen by aerobic microorganisms that will enhance the microbial activity (Kim et al., 2016). As a result, more heat would be generated as the biodegradation process by

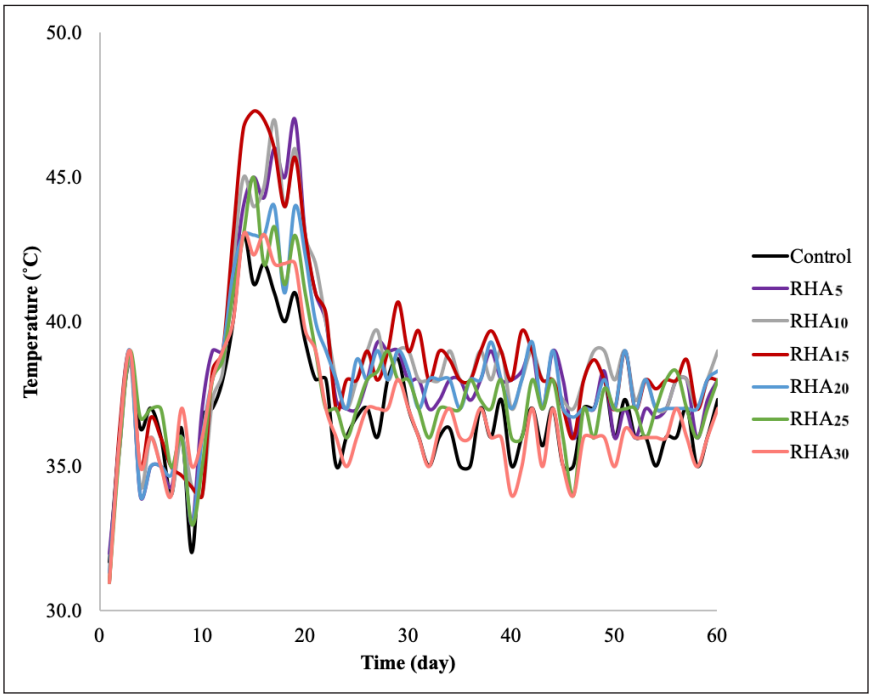

Figure 1. Temperature profile for a period of 60 days

the microorganisms was enhanced (Trisakti et al., 2018). However, excessive moisture content may reduce the microbial activity as the oxygen transport to the composting zone was greatly reduced (Makan et al., 2013). This scenario was proven by the composts with higher RHA composition that had a lower temperature level compared to RHA_{15}, although the moisture content of these composts were higher than RHA_{15}.

However, based on Figure 1, the temperature of all samples only fell within the mesophilic stage temperature, which was similar to the findings obtained by Hayawin et al. (2016) and Trisakti et al. (2018). The temperature obtained in this study was insufficient to enter the thermophilic stage. This occurrence was due to the minimal microbial activity that prevented the temperature to increase until the required level (Hayawin et al., 2016). Apart from that, the lower C/N ratio of POME sludge and decanter cake might also prevent the temperature to reach the temperature required to enter thermophilic stage since lower C/N ratio will result in lower compost temperature as mentioned by Neugebauer et al. (2017).

Starting from day 19 onwards, the temperature of the composts were gradually declined and fluctuated due to the deceleration of microbial activity. The curing stage was noted when the temperature of all samples remained close to the surrounding temperature that indicates the full consumption of residual substances by the microorganisms and the composts were already stabilized as stated by Lee (2016).

pH Profile of Compost

The data obtained for pH profile throughout 60 days of composting process is presented in Figure 2.

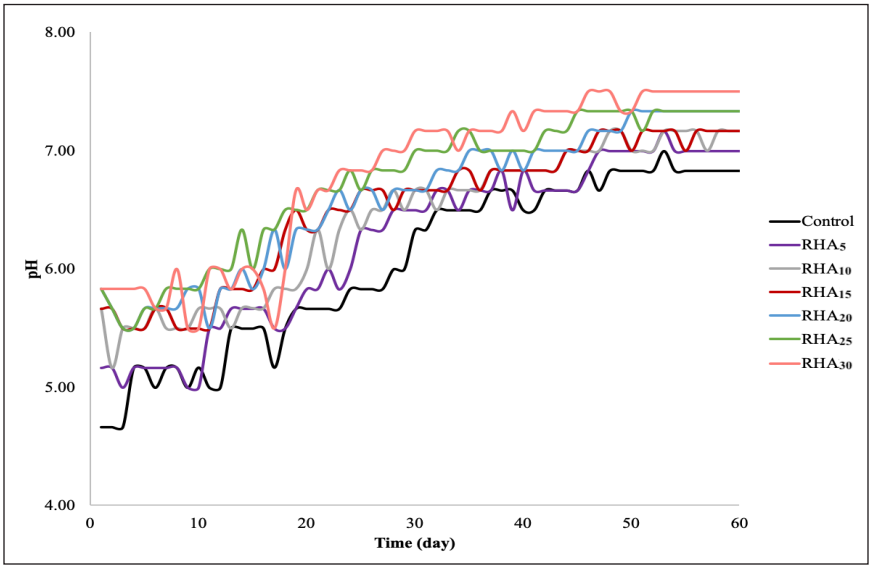

Figure 2. pH profile for a period of 60 days

According to Figure 2, the composts had recorded pH value in the range of 4.6 to 5.8, which was observed at day 1 until 10. The pH level of the samples was then increased from pH 5 to pH 7 between day 10 until 40, which was caused by the organic acids degradation (Hock et al., 2009) and the nitrogen (N) transformation into ammonium (NH_4^+) or ammonia (NH_3) via ammonification process (Irvan et al., 2019). In ammonification process, the NH_3 is be protonated and formed the NH_4^+ and hydroxide (OH^-), that eventually increase the alkalinity of the compost (Krause et al., 2018). This process is performed by the ammonifying bacteria.

At the beginning of composting process, control compost recorded the lowest level of pH, which was due to the properties of raw materials, POME sludge and decanter cake, which were slightly alkaline and acidic, respectively. Meanwhile, RHA_{30} recorded the highest pH level, which was due to the alkaline condition of RHA. Okon et al. (2005) had stated that, the presence of RHA was able to minimize the acidity level of composts, which was proven in this study. Other than that, alkaline condition is important in composting process to enhance the activity of microbes that can increase the rate of degradation as mentioned by Ameen et al. (2016).

Starting from day 26 onwards, the pH level of all composts gradually reached neutral level of pH 7. This level was reached when the organic acids existed in the compost was neutralized by the humic substances' buffering effects (Hock et al., 2009). Next, the pH level of all samples that contained RHA had exceeded the neutral level and became slightly alkaline starting from day 30 onwards. However, according to Jain et al. (2019), pH level of 7 to 8 can improve the organic matter decomposition by microorganisms. The completion of composting process could be noted from day 45 onwards when the pH of all composts was relatively stable. The final pH level achieved by all composts lies within an acceptable range of 6 to 8 for finished compost as mentioned by Sharma et al. (2017).

Physical Properties of Finished Compost

In this part, the results obtained for moisture content and water holding capacity were presented in Table 4. According to Table 4, RHA_{30} has recorded the highest percentage of moisture content, which was 1.9 to 23.8% higher compared to other samples. The

Table 4
Physical properties of finished composts

Parameters	Treatments (Mean ± SD)						
	Control	RHA_5	RHA_{10}	RHA_{15}	RHA_{20}	RHA_{25}	RHA_{30}
Moisture content (%)	47.37 ± 0.38^{e}	48.19 ± 0.36^{e}	53.21 ± 0.32^{d}	56.35 ± 0.40^{c}	57.50 ± 0.36^{b}	57.56 ± 0.42^{ab}	58.64 ± 0.56^{a}
Water holding capacity (%)	55.39 ± 0.85^{e}	57.21 ± 0.78^{e}	59.52 ± 0.82^{d}	64.02 ± 0.66^{c}	65.45 ± 0.70^{bc}	67.45 ± 0.68^{b}	70.26 ± 0.57^{a}

Note. Means in column with the same letters are not significantly different at $p \leq 0.05$

value obtained by RHA_{30} was significantly different from other treatments at $p \leq 0.05$, except for RHA_{25}. Besides, water holding capacity of RHA_{30} was also the highest with 4.2 to 26.8% higher than other finished composts and was significantly different compared to other treatments.

Based on the results presented in Table 4, RHA_{30} was found to depict the highest percentage of moisture content and water holding capacity. This is caused by the presence of high amount of silica present in RHA_{30} as linked to the data presented in Table 5. Previous research by Badar and Qureshi (2014) had claimed that, RHA could contain as high as 89% of silica in its components. The silica component contains a large specific surface area and highly porous structure (Phonphuak & Chindaprasirt, 2015). These special characteristics of silica allow it to retain high amount of liquid which can enhance the moisture content and water holding capacity of the material. Schaller et al. (2020) had also proven that, the amorphous structure of Si with high surface area had improved the water holding capacity of soil that would enhance the availability of water in soil. High availability of water can increase the nutrient uptake by plant via roots.

Apart from that, Kim et al. (2016) had suggested that, an ideal range of moisture content for a compost is between 40% to 60 %. This range was achieved by all finished composts in this study, although the percentage was lower compared to the percentage obtained by RHA_{30}. The microbial activity will be restricted if the moisture content is below 40% and decomposition of organic matter will be reduced if the percentage exceeds 60% (Kim et al., 2016).

Chemical Properties of Finished Compost

In this part, the data obtained for the chemical properties analysis of finished composts is presented in Table 5. Based on Table 5, the highest N content was recorded by control treatment, in which the difference was significant compared to other treatments at $p \leq 0.05$, except for RHA_5. Other than that, control and RHA_{10} treatments recorded the highest P and K contents, respectively,

Table 5
Chemical properties of finished composts

Parameters	Treatments (Mean ± SD)						
	Control	RHA_5	RHA_{10}	RHA_{15}	RHA_{20}	RHA_{25}	RHA_{30}
N (%)	3.31 ± 0.02^{a}	3.20 ± 0.04^{a}	2.97 ± 0.08^{b}	2.53 ± 0.07^{c}	2.29 ± 0.01^{d}	1.92 ± 0.05^{e}	1.73 ± 0.03^{f}
P (%)	0.87 ± 0.03^{a}	0.72 ± 0.02^{b}	0.67 ± 0.02^{bc}	0.59 ± 0.01^{d}	0.59 ± 0.01^{d}	0.61 ± 0.04^{cd}	0.67 ± 0.02^{bc}
K (%)	3.46 ± 0.04^{b}	3.41 ± 0.06^{b}	3.74 ± 0.07^{a}	2.81 ± 0.01^{c}	2.75 ± 0.06^{c}	2.28 ± 0.02^{e}	2.54 ± 0.03^{d}
Si (%)	18.32 ± 0.87^{c}	25.94 ± 0.91^{b}	28.12 ± 0.66^{b}	35.31 ± 0.98^{a}	35.40 ± 0.62^{a}	35.84 ± 0.98^{a}	36.00 ± 0.96^{a}

Note. Means in column with the same letters are not significantly different at $p \leq 0.05$

which was significantly higher than other treatments. Meanwhile, the highest Si content was obtained by RHA_{30}. However, the Si content in RHA_{30} was not significantly different from the treatment of RHA_{15}, RHA_{20}, and RHA_{25}.

As presented in Table 5, control compost possessed the highest N and P contents as it contained the largest weight composition of POME sludge and decanter cake. These palm oil mill wastes were the main source of N and P, which contributed to the high percentage of these nutrients in control compost. The addition of RHA into the compost had diminished the N content as the weight composition of palm oil mill wastes was reduced. Moreover, the presence of RHA did not contribute in enhancing the N and P content of the composts as much as the palm oil mill wastes could do since this material only contained a small amount of N and P as linked to the findings tabulated in Table 3.

Apart from that, it was also expected that control compost would have the highest amount of K. However, the actual result was contradicted with the predicted result, in which RHA_{10} had the highest composition of this element compared to control. As K is highly soluble in water, this element can be easily leached which could lead to the reduction of this element inside the compost as buttressed by Mendes et al. (2016). Alfaro et al. (2017) had also claimed that, K could be lost through leaching since K was a mobile ion and the loss could be expected when the inputs of K exceeded the retention capacity of the soil. Therefore, the addition of RHA, which act as a nutrient binder, can minimize nutrient leaching. However, RHA_{15} until RHA_{30} shows slightly lower amount of K. This is due to the availability of K in the raw materials of RHA_{15} to RHA_{30} that were lower compared to control, RHA_5 and RHA_{10} since the weight proportion of palm oil mill wastes was decreased as shown in Table 1.

Meanwhile, for Si, this element was found in abundance in RHA_{30} as this formulated compost had the highest RHA composition compared to other composts. This finding was similar to the prior findings obtained by Hisham and Ramli (2019). The authors also found that, the addition of higher percentage of RHA could enhance the Si content in the compost.

Hence, based on the results obtained in this study, the utilization of RHA as a compost material could enhance the moisture content, water holding capacity and Si contents in the finished compost. The addition of RHA could potentially enhance the nutrient contents, which is N, P and K, in finished compost. This is well-correlated with the trend obtained by Hisham and Ramli (2019). However, the authors suggested that $RHA_{7.5}$ was the best option, which is slightly different to the current work since the source of raw materials obtained in both studies were different. The results tabulated in Table 3 clearly show that, the POME sludge and decanter cake were the main source of N, P, and K. The composition of the important nutrients would be higher when lower percentage of RHA was added to the compost material.

Thus, the addition of RHA in the range of 5% to 10% is recommended for compost production as they gave a balance condition of physicochemical properties.

CONCLUSIONS

The addition of RHA in composting process involving palm oil mill wastes has proven that this combination can improve the physical properties of finished compost. An appropriate pH, moisture content and water holding capacity are important to boost the plant's nutrient uptake. Based on the findings, the presence of RHA did not contribute significantly towards increasing the macronutrient contents of the finished compost. Even so, this material can slightly enhance the nutrient contents of the compost. The addition of 5% to 10% RHA to palm oil mill wastes in compost formulation is suggested to improve the moisture content, water holding capacity, and NPK contents of the finished compost. Hence, the reported results in this study can give more options to the farmers and manufacturers in formulating and producing the compost according to their needs. The composition of RHA should be reduced if high NPK content is aimed for the compost. Meanwhile, high RHA composition is recommended if the compost is aimed to maintain a high level of moisture content and water holding capacity. Thus, it is important to have an appropriate composition of NPK, water holding capacity and moisture content in the compost since these properties can beneficially help farmers to enhance their crops' growth.

ACKNOWLEDGEMENTS

The authors are grateful for the financial support from Ministry of Education Malaysia and Universiti Malaysia Pahang through Fundamental Research Grant Scheme (RDU190136) with reference code FRGS/1/2018/TK10/UMP/02/7.

REFERENCES

Akhbari, A., Kutty, P. K., Chuen, O. C., & Ibrahim, S. (2020). A study of palm oil mill processing and environmental assessment of palm oil mill effluent treatment. *Environmental Engineering Research*, *25*(2), 212–221. https://doi.org/10.4491/eer.2018.452

Alfaro, M. A., Rosolem, C. A., Goulding, K. W. T., & Wang, H. (2017). Potassium losses and transfers in agricultural and livestock systems. In T. S. Murrell & R. L. Mikkelsen (Eds.), *Frontiers of Potassium Science Conference* (Issue January, pp. 25–27). International Plant Nutrition Institute.

Ameen, A., Ahmad, J., & Raza, S. (2016). Effect of pH and moisture content on composting of Municipal solid waste. *International Journal of Scientific and Research Publications*, *6*(5), 35–37.

Anwar, Z., Irshad, M., Fareed, I., & Saleem, A. (2015). Characterization and recycling of organic waste after co-composting - A review. *Journal of Agricultural Science*, *7*(4), 70–81. https://doi.org/10.5539/jas.v7n4p68

Babaso, P. N., & Sharanagouda, H. (2017). Rice husk and its applications: Review. *International Journal of Current Microbiology and Applied Sciences*, *6*(10), 1144–1156. https://doi.org/10.20546/ijcmas.2017.610.138

Badar, R., & Qureshi, S. A. (2014). Composted rice husk improves the growth and biochemical parameters of sunflower plants. *Journal of Botany*, *2014*, 427648. https://doi.org/10.1155/2014/427648

Bala, J. D., Lalung, J., & Ismail, N. (2014). Palm oil mill effluent (POME) treatment "Microbial communities in an anaerobic digester": A review. *International Journal of Scientific and Research Publications*, *4*(6), 1–24.

Brust, G. E. (2019). Management strategies for organic vegetable fertility. In D. Biswas & S. A. Micallef (Eds.), *Safety and practice for organic food* (1st ed., pp. 193–212). Elsevier. https://doi.org/10.1016/B978-0-12-812060-6.00009-X

Dewayanto, N., Sahad, M. Z., & Nordin, M. R. (2009). *Waste to valuable by-product: Palm oil mill decanter cake and its ability to remove Cd, Cu and Pb ions*. http://umpir.ump.edu.my/id/eprint/8861/

Dizaji, H. B., Zeng, T., Hartmann, I., Enke, D., Schliermann, T., Lenz, V., & Bidabadi, M. (2019). Generation of high quality biogenic silica by combustion of rice husk and rice straw combined with pre- and post-treatment strategies - A review. *Applied Sciences (Switzerland)*, *9*(6), 1–27. https://doi.org/10.3390/app9061083

Embrandiri, A., Rupani, P., ShIrene, Q., Ibrahim, H., & Singh, R. (2012). *Palm oil mill wastes (decanter cake) as soil amendments; heavy metal analysis and SEM studies of* Abelmoschus esculentus *(lady's finger) plants*. https://www.researchgate.net/publication/237072254_Palm_oil_mill_wastes_Decanter_cake_as_soil_amendments_Heavy_metal_Analysis_and_SEM_studies_of_Abelmoschus_ESCULENTUS_Lady%27s_finger_PLANTS

Frimpong-Manso, J., Obodai, M., Dzomeku, M., & Apertorgbor, M. M. (2011). Influence of rice husk on biological efficiency and nutrient content of *Pleurotus ostreatus* (Jacq. ex. Fr.) Kummer. *International Food Research Journal*, *18*(1), 249–254.

Hayawin, Z. N., Astimar, A. A., Ropandi, M., Faizah, J. N., & Hamid, F. A. (2016). Rapid composting of oil palm biomass by using aerobic thermophilic composter. *Palm Oil Engineering Bulletin*, *119*, 19–26.

Hisham, N. E. B., & Ramli, N. H. (2019). Effect of rice husk ash on the physicochemical properties of compost. *Indonesian Journal of Chemistry*, *19*(4), 967–974. https://doi.org/10.22146/ijc.39704

Hock, L. S., Baharuddin, A. S., Ahmad, M. N., Hassan, M. A., Shah, U. K. M., Rahman, N. A. A., Abd-Aziz, S., & Shirai, Y. (2009). Physicochemical changes in windrow co-composting process of oil palm mesocarp fiber and palm oil mill effluent anaerobic sludge. *Australian Journal of Basic and Applied Sciences*, *3*(3), 2809–2816.

Irvan, Rahman, M., Anwar, D., Trisakti, B., & Daimon, H. (2019). Production of compost from non-shredded empty fruit bunches mixed with activated liquid organic fertilizer in tower composter. *Malaysian Journal of Analytical Science*, *23*(1), 138–146. https://doi.org/10.17576/mjas-2019-2301-17

Islabão, G. O., Vahl, L. C., Timm, L. C., Paul, D. L., & Kath, A. H. (2014). Rice husk ash as corrective of soil acidity. *Revista Brasileira de Ciência Do Solo*, *38*(3), 934–941. https://doi.org/10.1590/S0100-06832014000300025

Jain, M. S., Daga, M., & Kalamdhad, A. S. (2019). Variation in the key indicators during composting of municipal solid organic wastes. *Sustainable Environment Research*, *29*(9), 1–8. https://doi.org/10.1186/s42834-019-0012-9

Kamyab, H., Chelliapan, S., Din, M. F. M., Rezania, S., Khademi, T., & Kumar, A. (2018). Palm oil mill effluent as an environmental pollutant. In V. Waisundara (Ed.), *Palm oil* (pp. 13–24). InTech. https://doi.org/10.5772/intechopen.75811

Khairuddin, M. N., Md Isa, I., Zakaria, A. J., & Syahlan, S. (2017). Ameliorating plant available water by addition of treated palm oil mill

effluent (POME) sludge on entisols. *Journal of Agricultural Science*, *9*(7), 218–228. https://doi.org/10.5539/jas.v9n7p218

Khairuddin, M. N., Zakaria, A. J., Md Isa, I., Jol, H., Wan Abdul Rahman, W. M. N., & Salleh, M. K. S. (2016). The potential of treated palm oil mill effluent (POME) sludge as an organic fertilizer. *AGRIVITA Journal of Agricultural Science*, *38*(2), 142–154. https://doi.org/10.17503/agrivita.v38i2.753

Kim, E., Lee, D.-H., Won, S., & Ahn, H. (2016). Evaluation of optimum moisture content for composting of beef manure and bedding material mixtures using oxygen uptake measurement. *Asian-Australasian Journal of Animal Sciences*, *29*(5), 753–758. https://doi.org/10.5713/ajas.15.0875

Krause, S., Liebetrau, V., Löscher, C. R., Böhm, F., Gorb, S., Eisenhauer, A., & Treude, T. (2018). Marine ammonification and carbonic anhydrase activity induce rapid calcium carbonate precipitation. *Geochimica et Cosmochimica Acta*, *243*(October), 116–132. https://doi.org/10.1016/j.gca.2018.09.018

Lee, Y. (2016). Various microorganisms' roles in composting : A review. *APEC Youth Scientist Journal*, *8*(1), 11–15.

Makan, A., Assobhei, O., & Mountadar, M. (2013). Effect of initial moisture content on the in-vessel composting under air pressure of organic fraction of municipal solid waste in Morocco. *Iranian Journal of Environmental Health Science and Engineering*, *10*(3), 1–9. https://doi.org/10.1186/1735-2746-10-3

Mendes, W. D. C., Alves Júnior, J., da Cunha, P. C. R., da Silva, A. R., Evangelista, A. W. P., & Casaroli, D. (2016). Potassium leaching in different soils as a function of irrigation depths. *Revista Brasileira de Engenharia Agricola e Ambiental*, *20*(11), 972–977. https://doi.org/10.1590/1807-1929/agriambi.v20n11p972-977

Misra, R. V., Roy, R. N., & Hiraoka, H. (2003). *On-farm composting methods*. Food and Agriculture Organization of the United Nations.

Neugebauer, M., Sołowiej, P., Piechocki, J., Czekała, W., & Janczak, D. (2017). The influence of the C:N ratio on the composting rate. *International Journal of Smart Grid and Clean Energy*, *6*(1), 54–60. https://doi.org/10.12720/sgce.6.1.54-60

Nizar, M. K., Isharudin, M. I., Jamil, A. Z., & Hazandy, A. H. (2018). Influence of treated palm oil mill effluent sludge on maize (*Zea mays*) growth performance and gas exchange. *Sains Malaysiana*, *47*(5), 961–969. https://doi.org/10.17576/jsm-2018-4705-11

Okon, P. B., Ogeh, J. S., & Amalu, U. C. (2005). Effect of rice husk ash and phosphorus on some properties of acid sands and yield of okra. *Communications in Soil Science and Plant Analysis*, *36*(7–8), 833–845. https://doi.org/10.1081/CSS-200049460

Osman, S. F., Hashim, M. M., Sulaiman, A., & Abdullah, M. Y. (2019). Hot water treatment of oil palm decanter cake (OPDC) for bio-organic media of oil palm seedlings. *Konvensyen Kebangsaan Kejuruteraan Pertanian Dan Makanan*, *2019*, 22–26.

Persaud, T., Homenauth, O., Fredericks, D., & Hamer, S. (2018). Effect of rice husk biochar as an amendment on a marginal soil in Guyana. *World Environment*, *8*(1), 20–25. https://doi.org/10.5923/j.env.20180801.03

Phonphuak, N., & Chindaprasirt, P. (2015). Types of waste, properties, and durability of pore-forming waste-based fired masonry bricks. In F. Pacheco-Torgal, P. B. Lourenco, J. A. Labrincha, S. Kumar, & P. Chindaprasirt (Eds.), *Eco-efficient masonry bricks and blocks* (pp. 103–127). Elsevier Ltd. https://doi.org/10.1016/B978-1-78242-305-8.00006-1

Priyadharshini, J., & Seran, T. H. (2010). Paddy husk ash as a source of potassium for growth and yield

of cowpea *Vigna unguiculata* L.). *The Journal of Agricultural Sciences - Sri Lanka*, *4*(2), 67–76. https://doi.org/10.4038/jas.v4i2.1646

Rambo, M. K. D., Cardoso, A. L., Bevilaqua, D. B., Rizzetti, T. M., Ramos, L. A., Komdorfer, G. H., & Martins, A. F. (2011). Silica from rice husk ash as an additive for rice plant. *Journal of Agronomy*, *10*(3), 99–104. https://doi.org/10.3923/ja.2011.99.104

Ramli, N. H, Badrul Hisham, N., Mohd Said, F., & Mariyappan, T. (2016). The effect of weight ratio on the physiochemical properties of compost from palm oil mill effluent (POME) sludge and decanter cake. *Australian Journal of Basic and Applied Sciences*, *10*(17), 34–39.

Ramli, N. H., Hisham, N. E. B., Said, F. M., Hisham, N. E. B., & Olalere, O. A. (2019). The role of wood vinegar in enhancing the microbial activity and physicochemical properties of palm oil-based compost. *Pertanika Journal of Tropical Agricultural Science*, *42*(4), 1391–1403.

Razak, M. N. A., Ibrahim, M. F., Yee, P. L., Hassan, M. A., & Abd-Aziz, S. (2012). Utilization of oil palm decanter cake for cellulase and polyoses production. *Biotechnology and Bioprocess Engineering*, *17*(3), 547–555. https://doi.org/10.1007/s12257-011-0590-9

Sahad, N., Md. Som, A., Baharuddin, A. S., Mokhtar, N., Busu, Z., & Sulaiman, A. (2014). Physicochemical characterization of oil palm decanter cake (OPDC) for residual oil recovery. *BioResources*, *9*(4), 6361–6372. https://doi.org/10.15376/biores.9.4.6361-6372

Saranya, P., Sri Gayathiri, C. M., & Sellamuthu, K. M. (2018). Potential use of rice husk ash for enhancing growth of maize (*Zea mays*). *International Journal of Current Microbiology and Applied Sciences*, *7*(3), 899–906. https://doi.org/10.20546/ijcmas.2018.703.105

Schaller, J., Cramer, A., Carminati, A., & Zarebanadkouki, M. (2020). Biogenic amorphous silica as main driver for plant available water in soils. *Scientific Reports*, *10*(1), 1–7. https://doi.org/10.1038/s41598-020-59437-x

Sharma, D., Varma, V. S., Yadav, K. D., & Kalamdhad, A. S. (2017). Evolution of chemical and biological characterization during agitated pile composting of flower waste. *International Journal of Recycling of Organic Waste in Agriculture*, *6*(1), 89–98. https://doi.org/10.1007/s40093-017-0155-9

Theeba, M., Bachmann, R. T., Illani, Z. I., Zulkefli, M., Husni, M. H. A., & Samsuri, A. W. (2012). Characterization of local mill rice husk charcoal and its effect on compost properties. *Malaysian Journal of Soil Science*, *16*(1), 89–102.

Trisakti, B., Mhardela, P., Husaini, T., Irvan, & Daimon, H. (2018). Production of oil palm empty fruit bunch compost for ornamental plant cultivation. *IOP Conference Series: Materials Science and Engineering*, *309*(1), 1–8. https://doi.org/10.1088/1757-899X/309/1/012094

Wun, W. L., Chua, G. K., & Chin, S. Y. (2017). Effect of palm oil mill effluent (POME) treatment by activated sludge. *Journal CleanWAS*, *1*(2), 6–9. https//doi.org/10.26480/jcleanwas.02.2017.06.09

Zaini, M. A. A., Cher, T. Y., Zakaria, M., Kamaruddin, M. J., Mohd. Setapar, S. H., & Che Yunus, M. A. (2014). Palm oil mill effluent sludge ash as adsorbent for methylene blue dye removal. *Desalination and Water Treatment*, *52*(19-21), 3654–3662. https://doi.org/10.1080/19443994.2013.854041

Zakarya, I. A., Khalib, S. N. B., & Mohd Ramzi, N. (2018). Effect of pH, temperature and moisture content during composting of rice straw burning at different temperature with food waste and effective microorganisms. In *E3S Web of Conferences* (Vol. 34, pp. 1–8). EDP Sciences. https://doi.org/10.1051/e3sconf/20183402019

Pertanika J. Trop. Agric. Sci. 44 (1): 237 - 254 (2021)

PERTANIKA JOURNALS

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Efficiency of Bioconversion of Coffee Pulp using *Hermetia illucens* (Diptera: Stratiomyidae) Larvae

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ABSTRACT

In this study, we evaluate the capacity of *Hermetia illucens* (L.) to degrade coffee pulp, which is a highly contaminating residue and a by-product of processing coffee beans. The larvae were fed with dietary dosages of 60, 100, 160, and 200 mg/larva/day of *Coffea arabica* (L.) Castillo variety (0.5% caffeine), until they reached pupae stage. With a dietary supply of 100 mg/larva/day, the highest reduction percentage (%R) on a wet basis (62.88%) and efficiency of conversion of ingested food (ECI) (7.89%) were achieved, while with a dietary supply of 160 mg/larva/day the highest weight reduction index (WRI) in a wet basis was achieved (0.85%). Larvae fed 200 mg/larva/day had the highest weight (115.9 mg) and shortest average development time (38.65 days). In this study we were able to define which dietary supplies of 160 and 200 mg/larva/day, allowing for efficient bioconversion of coffee pulp.

Keywords: Bioconversion, coffee pulp, *Hermetia illucens*

INTRODUCTION

In coffee-growing countries such as Colombia, the most abundant residue during the processing of coffee beans is the pulp, which is one of the main contaminants when it is dumped into the environment (Blandón et al., 1999; Pandey et al., 2011). This situation has impelled the coffee-producing countries to seek for alternatives for the management and use of the coffee pulp (CP), aiming with this to mitigate its impact. Among the alternatives that are handled are the use of coffee pulp as a raw material for the production of animal feed, beverages, vinegar, biogas, caffeine, peptic enzymes, protein, and fertilizer, among others (Noriega et al., 2008; Rodríguez &

ARTICLE INFO

Article history:
Received: 17 August 2020
Accepted: 23 November 2020
Published: 24 February 2021

DOI: https://doi.org/10.47836/pjtas.44.1.14

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ISSN: 1511-3701
e-ISSN: 2231-8542

Zambrano, 2010). The use of coffee pulp as part of the diet of cattle, sheep, pigs, poultry, fish, rabbits, among others, has been limited by the presence of partially negative compounds, such as tannins and caffeine (Noriega et al., 2008; Rubio, 1973), the latter being considered the most nutritionally negative compound (Molina et al., 1990) given than its average concentrations, in coffee varieties such as Arabica, varies between 0.5 to 1.3 % (Elías, 1978).

A potential alternative, which can efficiently minimize the impact caused by CP dumping, is bioconversion with *Hermetia illucens* (L.) larvae, a Diptera from the Stratiomyidae family, commonly known as "black soldier fly", whose larvae are characterized by being voracious consumers of organic matter of plant or animal origin (Diener, 2009), manure (Banks et al., 2014; Gamboa & Jasbón, 2008; Newton et al., 2005b) and corpses (Lord et al., 1994; Pujol-Luz et al. , 2008). Due to the presence of strong mouthparts and digestive enzymes, the larvae can consume large amounts of waste much faster and more efficiently than any other fly larvae (Kim et al., 2011). These characteristics make the black soldier fly a potential organism in the management of organic solid waste such as CP.

An implementation of the use of *H. illucens* larvae in CP management will have a great impact in developing countries such as Colombia, since in comparison with other biodegradation processes, such as composting and worm farming, it will not only reduce up to 56% of the initial volume of fresh waste (Newton et al., 2005a), but will as well generate by-products such as larval biomass, which can be used as biodiesel when its fat is extracted (Zheng et al., 2011, 2012), as larvae flour for feeding animals such as birds, pigs, fish, and amphibians (Arango et al., 2004; Bondari & Sheppard, 1987; Hale, 1973; Newton et al., 2005b) and for the extraction of chitin, for use in the medical industry. All these by-products would support local economies, representing an additional source of revenue (Warburton & Hallman, 2002).

Worldwide, there are many studies on the efficiency of bioconversion of *H. illucens* larvae on cattle, pig, and poultry manure. In Colombia, bioconversion studies with *H. illucens* larvae have focused on the production of larvae flour for animal feed (Canary, 2009), reduction of urban solid waste (Jaramillo & Zapata, 2008) and reduction of vegetables (Parra et al., 2015). Here we seek to evaluate the efficiency of *H. illucens* in bioconversion of CP, based on parameters such as percentage reduction (%R), reduction index (WRI) (Diener et al., 2009), and efficiency of conversion of ingested food (ECI) (Oonincx et al., 2015a). Additionally, the effect of feeding with CP on the development of these immatures is evaluated.

MATERIAL AND METHODS

The trials were carried out under greenhouse and laboratory conditions at the Universidad del Valle Meléndez, Colombia location (3°22'37.18 "N., 76°31'48.60 "W). The specimens used in the tests were obtained from the *H. illucens* colony at the Universidad del Valle.

Fresh pulp of *Coffea arabica* (L.) Castillo variety (0.5% of caffeine) was used, sourced from coffee farms located in the village of El Faro, Corregimiento Los Andes, in Valle del Cauca Colombia (3°25'26.3 "N 76°35'34.6 "W). In order to avoid biological contamination and aerobic fermentation, the pulp was transported in dark plastic containers.

For comparison purposes, organic fruit-based residues (papaya, melon, banana, and pineapple) were used. To ensure the homogeneity of all waste, they were reduced to sizes similar to CP (1.5-2 cm).

In order to determine the base physical composition (fixed), taking into account that all residues were used fresh, their moisture was determined and moisture percentages were defined on a wet basis. The moisture percentage was determined by taking fresh 5g samples and drying them in an oven at 105°C for 24 h (Diener et al., 2009), the remaining moisture was measured on an Ohaus analytical balance [Equation (1)].

$$\%H = \left(\frac{(W1 - W2)}{W1}\right) * 100 \qquad [1]$$

%H =Moisture percentage (%)
W_1 = Initial wet weight in grams
W_2 = Final dry weight in grams

Bioconversion Speed

Based on the works of Diener et al. (2009, 2011) and Myers et al. (2008), four treatments were performed with four repetitions each. Each treatment contained 200 larvae, selected following the suggestions by Sheppard et al. (2002). Each treatment contained 60, 100, 160, and 200 mg/larva/day (fresh weight with 80% humidity), corresponding to 12, 20, 32, and 40 mg/larva/day dry weight, identified as T1, T2, T3, and T4, respectively.

Six-day-old larvae were deposited in "larval incubators" or acrylic containers built to scale, following the specifications proposed by Parra et al. (2015), and covered with a plastic film to prevent moisture loss and contamination by other arthropods (Banks et al., 2014). Each "larval incubator" had a capacity of 1.2 larvae/cm^2.

Every three days, the food from the different treatments was replaced. For this purpose, all the surviving larvae were moved to another container with the initial standardized conditions, and the residues or remaining "larvacomposite" (excrement and undigested material contents), were weighed with the help of an analytical balance, both fresh and dry, the latter being obtained after drying the residues at 105°C for 24 hours, thus determining the dry weight.

All trials were carried out until 50% of the larvae reached the prepupa stage, a prepupa being identified when its coloration changes from light to dark (Diener et al., 2009).

Bioconversion Time, Survival Rate and Larval Weight Gain

Every six days, from each treatment, 20 larvae were selected at random and their weight was recorded. Once weighed, the specimens were returned to the corresponding "larval incubator".

Likewise, as they developed, each prepupa was weighed until the end of the trials.

To determine the larval survival rate, the number of live larvae at the end of the trials was divided with respect to the initial number of larvae per treatment (Oonincx et al., 2015a).

Bioconversion Efficiency

The bioconversion efficiency was determined by the reduction percentage (%R) on a dry basis [Equation (2)] (Diener et al., 2009).

$$\%R = \left(\frac{T - R}{T}\right) * 100 \quad [2]$$

T = material added to the system
R = Non-ingested feed + Excrement

The waste reduction rate (WRI) [Equation (3)] is obtained from the reduction percentage [Equation (3)] and the number of days (t) that the reduction process takes. According to Diener et al. (2009), a high reduction rate relates to high reduction efficiency.

$$WRI = \left(\%\frac{R}{t}\right) \quad [3]$$

R = reduction percentage
t = bioconversion rate, defines as the time
taken by 50% of larvae to become prepupa

The efficiency of conversion of ingested food (ECI) measures the larval capacity to utilize the ingested feed in growth (Waldbauer, 1968). It is calculated by taking into account the ingested feed in relation to the weight gained [Equation (4)].

$$ECI = \left(\frac{Lwf - Lwi}{I}\right) * 100 \quad (4)$$

I = Ingested feed (Supplied Feed - Non-ingested Feed + Excrement)
L_{wi} = Initial larval weight
L_{wf} = Final larval weight

The relative growth rate (RGR) (Scriber & Slansky, 1981), considers the relationship between the efficiency of conversion of ingested food and the relative feed consumption. It takes into account the food consumed, the excreted, as well as the assimilated [Equation (5)] (Manurung et al., 2016).

$$RGR = \frac{Lwf - Lwi}{T * Lwi} \quad (5)$$

L_{wf} = Final larval weight
L_{wi} = Initial larval weight
T = Time

Statistical Analysis

With a randomized experimental design, analysis of variance (ANOVA) was used for comparisons between treatments, while an LSD test allowed defining the best treatment. All analyses were performed in the R program version 3.0.0.

Effect of Caffeine on the Development of *Hermetia illucens*

To evaluate the effect of caffeine on the duration of immature stages, four trials were performed, all using 667 cm^2 "larval

incubators", designed to scale according to the specifications of Parra et al. (2015), covered with a plastic film to prevent moisture loss and contamination by other arthropods (Banks et al., 2014).

Five hundred (500) newly emerged *H. illucens* larvae were deposited in "larval incubators" and fed 40 mg/larva/day (dry weight), two diets were used: 1) solid vegetal waste (SVW) and 2) coffee pulp (CP), each diet with four repetitions. All larvae were fed until 50% had molted into prepupa, however, observations were continued until all larvae either molted into prepupa or died (Tomberlin et al., 2002). Daily, grown prepupae were transferred to containers containing moist soil and covered with organza as a medium for pupa formation. The duration of the pupa was recorded (Figure 1).

RESULTS

Bioconversion Efficiency

A dietary supply of 100, 160, and 200 mg/larva/day (80% humidity), produced an average reduction (dry weight) of 32, 30.22, and 30.96% respectively (Table 1). No significant differences were found among all four treatments. However, a dietary supply of 100 mg/larva/day, presented the highest average percentage of reduction (32%). Regarding the average percentage of reduction on a wet basis, significant differences were found between these and the treatments, T_2 (100 mg/larva/day) reaching an average percentage of reduction of 62.88%. The average percentage of reduction on a dry basis ranged from 30.22 to 32%.

In Table 1, it can be seen that, for the average percentage reduction in dry and wet weight, it has an inverse linear relationship (Figure 2).

The index of reduction (WRI) in the analyses with wet basis (WW) and dry basis (DW), can be appreciated in the Table 1, where significant differences were found in both trials. For the wet weight the higher index of reduction (0.85) was observed in the T_3 treatment (160 mg/larva/day), while,

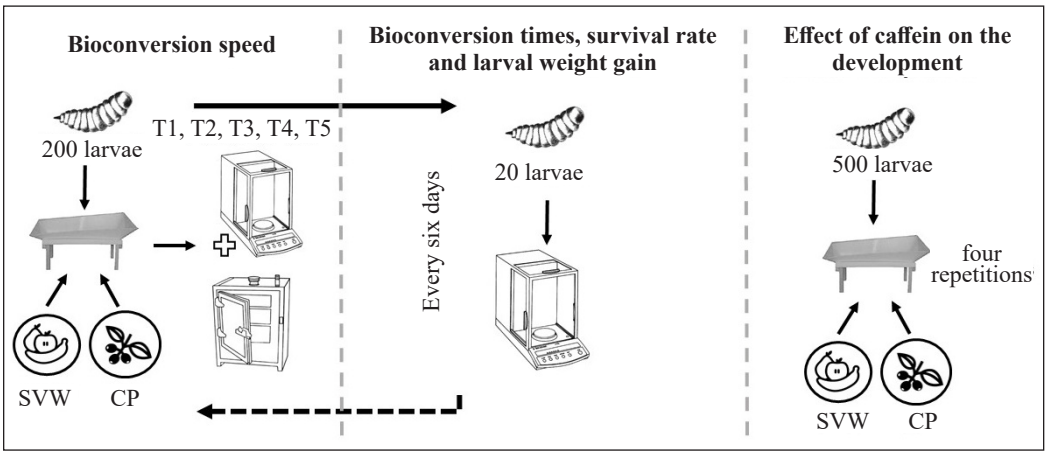

Figure 1. Methodology flow diagram

Table 1
Parameters of bioconversion efficiency of Hermetia illucens *larvae fed with CP*

Parameters	T_2 100mg/larva/day		T_3 160mg/larva/day		T_4 200mg/larva/day	
	Average	SE	Average	SE	Average	SE
Bioconversion time (days)	77.95a	1.08	46.45b	0.47	**38.65c**	0.32
Survival rate (%)	76.00a	4.17	80.90a	4.11	89.11a	4.11
%R (reduction percentage) WW*	**61.88a**	0.66	51.25b	3.15	29.79c	2.03
%R (reduction percentage) DW*	32.00a	0.47	30.22a	0.68	30.96a	0.5
WRI (reduction index) WW	0.61b	0.01	**0.85a**	0.03	0.63b	0.04
WRI (reduction index) DW	0.31a	0.005	0.50b	0.01	**0.66c**	0.02
ECI (Efficiency of Conversion of Ingested Food) DW expressed in %	3.9b	0.11	**7.89a**	0.29	**6.18a**	0.24

Note. DW*: dry weight; WRI: weight reduction index; WW*: wet weight. Average values followed by an identical letter means that they do not vary significantly (p<0.05)

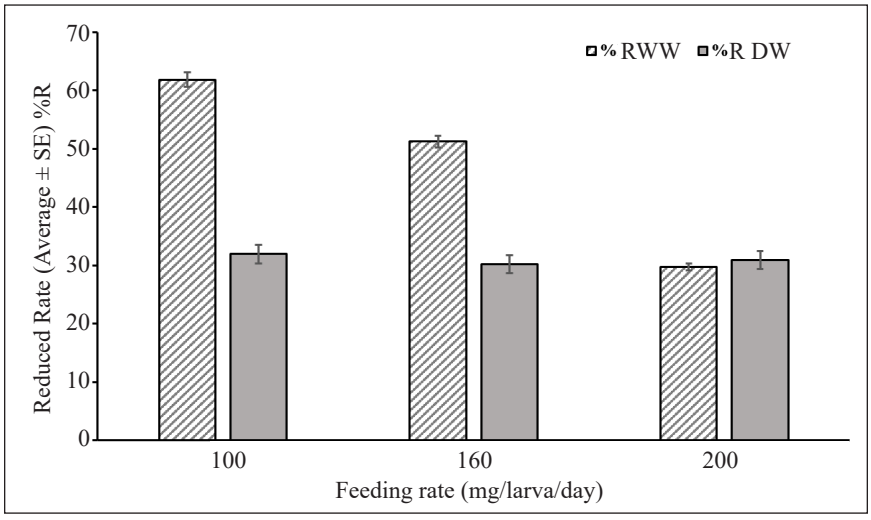

Figure 2. Reduction percentage (%R) of *Hermetia illucens* larvae on three dietary supplies

for the dry weight analysis, the higher index of reduction (0.66) was observed in the T_4 treatment (200 mg/wet/day) (Figure 3).

High WRI values indicate good reduction efficiency (Diener et al., 2009) and these were reported, for dry basis analysis, in the T_4 treatment (200 mg/larva/day).

As for efficiency of conversion of ingested food (ECI), maximum feed metabolization (7.89%) was reached when fed 100 mg/larva/day, however, ECI of 6.18% with a diet of 200 mg/larva/day did not have significant differences (p>0.0018). From the values reported by Lardé (1990), the ECI was calculated for *H. illucens* larvae fed in CP, obtaining an ECI value (6.03%) very similar to the one found in this study (Table 2).

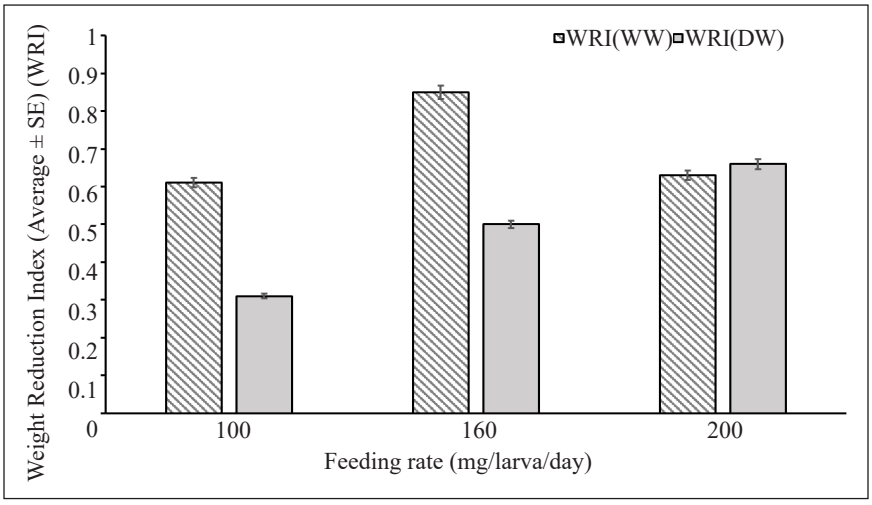

Figure 3. Weight Reduction Index (WRI) of *Hermetia illucens* larvae on three dietary supplies

Table 2
Larval and prepupal weight (mg) of Hermetia illucens *fed different dietary supplies of CP (60, 100, 160, and 200 mg/larva/day)*

Developmental stage	T_1 60 mg		T_2 100 mg		T_3 160 mg		T_4 200 mg	
	Average	N	Average	N	Average	N	Average	N
Larva* WW (mg)	72.17 ±13.2a	20	96.94±16.7b	20	95.80±19.1b	20	115.92±20.8c	20
Prepupa WW (mg)	57.84±12.5a	51	64.14±14.3b	320	65.99±10.8b	320	73.40±12.6c	320

Note. * The measurement of the day in which 50% of the prepupae had migrated, except for T_1

From the formula (4), the ECI value for larvae fed with chicken, pig and cow manure was calculated with the data presented by Oonincx et al. (2015a), these were respectively 8.92, 10.89, and 8.08%. Additionally, from the data provided by Banks et al. (2014) we calculated the ECI values for larvae fed with pig manure (Newton et al., 2005b) and with municipal solid waste (Sheppard et al., 1994) obtaining values of 10.4 and 17.3% respectively.

The high values of ECI (7.89%) obtained in this study, reflect the capacity that larvae of *H. illucens* have to use in growth (weight gained) the ingested CP.

Weight Gain and Development Time

Larvae fed 100, 160, and 200 mg/larva/day (wet weight 60% moisture, 40 mg/larva/day DW), took respectively 77.95, 46.45, and 38.65 days to complete the bioconversion process. In terms of weight gained during the process for dietary supplies of 60, 100, 160, and 200 mg/larva/day, the larval weight was 72.17, 96.9, 95.8, and 115.9 mg respectively and for pre-pupas it was 57.7, 64.7, 65.9, and 73.4 mg, respectively (Table 2).

In terms of larval and prepupal weight, significant differences were found between

treatments T_1 (60 mg/larva/day) and T_4 (200 mg/larva/day), between treatments T_2 (100 mg/larva/day) and T_3 (200 mg/larva/day) no significant differences were found (p>0.8431, p>0.0636 larva and prepupa respectively) (Figure 3).

Larvae fed with 200 mg/larva/day of CP, presented the highest weight (average 115.9 mg). These larvae required a shorter average time of development (38.65 days), the larvae fed with the lowest dietary supply (60 mg/larva/day), presented the lowest weight at the end of the process (57. 7 mg); as for the prepupae a dietary supply of 200 mg/larva/day presented the highest weight (73.4 mg), where dietary supplies of 100 mg/larva/day, resulted in a prepupal weight of 64.7 mg, corresponding to a larval duration of 77.95 days, the longest of the whole process (Table 2, Figure 4).

Observations in T_1 (60 mg/larva/day), were suspended at 103 days, when only 6.4% of prepupae had migrated. From a biological point of view, low dietary supplies increase larval development time and accumulated weight (Figure 4), the fact that larval development time is prolonged, with poor dietary supplies, representing a high adaptability in bioconversion operations with variable food supplies (Diener et al., 2009).

The maximum percentage of survival (89.9%) was reached in those larvae fed with 200 mg/larva/day of CP, less than 24% of the larvae could not reach the prepupa stage. No significant differences were found between the percentage of survival and the different treatments, however, a direct linear relation is appreciated, between the percentage of survival and the dietary availability (Table 1).

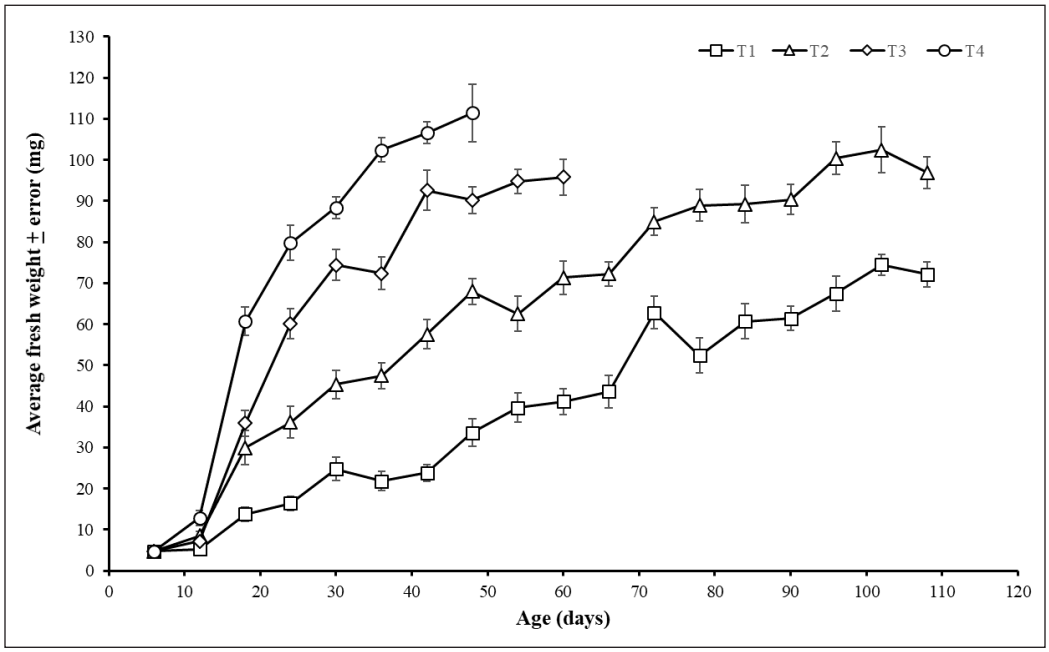

Figure 4. Increase in weight on a wet basis (Average ± Error) of *Hermetia illucens* larvae with respect to development time, in four different treatments T1 = 60, T2 = 100, T3 = 160, and T4 = 200 mg/larva/day

The Relative Growth Rate (RGR) presented the best values with a diet of 200 mg/larva/day of CP, this value is related to the shorter time of larval development (Figure 5).

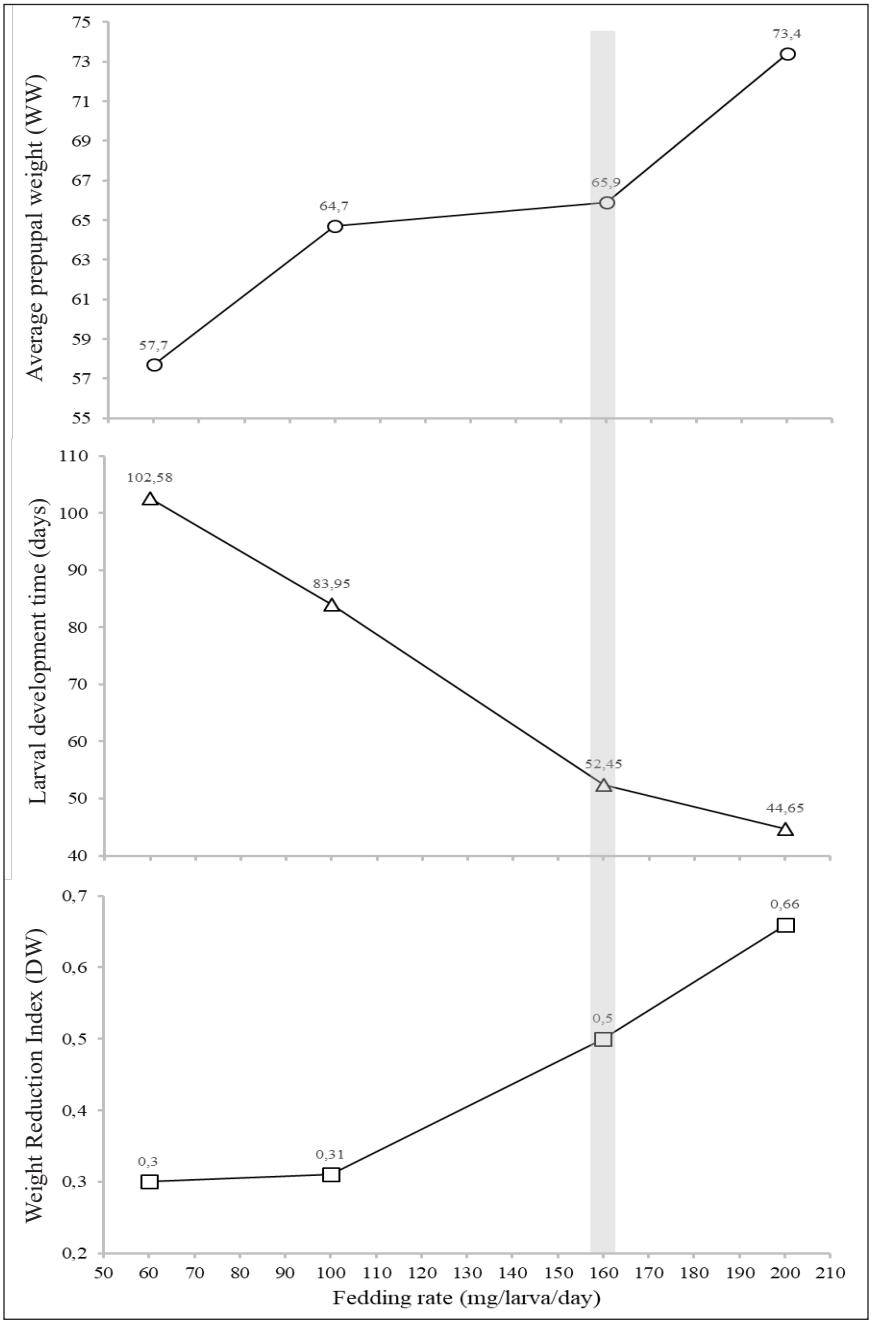

Figure 5. Prepupa weight (mg), larval development time (days), and reduction index (WRI), related to dietary supplies (60, 100, 160, and 200 mg/larva/day). Shaded area indicated the optimal feeding rate

Effect of Caffeine on the Development of *Hermetia illucens*

Both the pH (Mann Whitney, df = 40, F =319, p = 0.0142) and temperature (Mann Whitney, df = 40, F = 176, p = 0.272) values were not found to affect the larval development of *H. illucens* (Figure 6A, 6B). The temperature of development varied between 26.49 and 36.63°C.

Initially, the Solid Vegetable Waste (SVW) presented more acidic values (3.33), than the CP (6.76). This was due to the fact that the SVW was composed, among others, by pineapple residues, which has a pH of 3.6 (Ulloa, 2007). However, soon after (less than three days), the pH of both the CP and the SVW became basic. In addition, both CP and SVW were observed to decrease

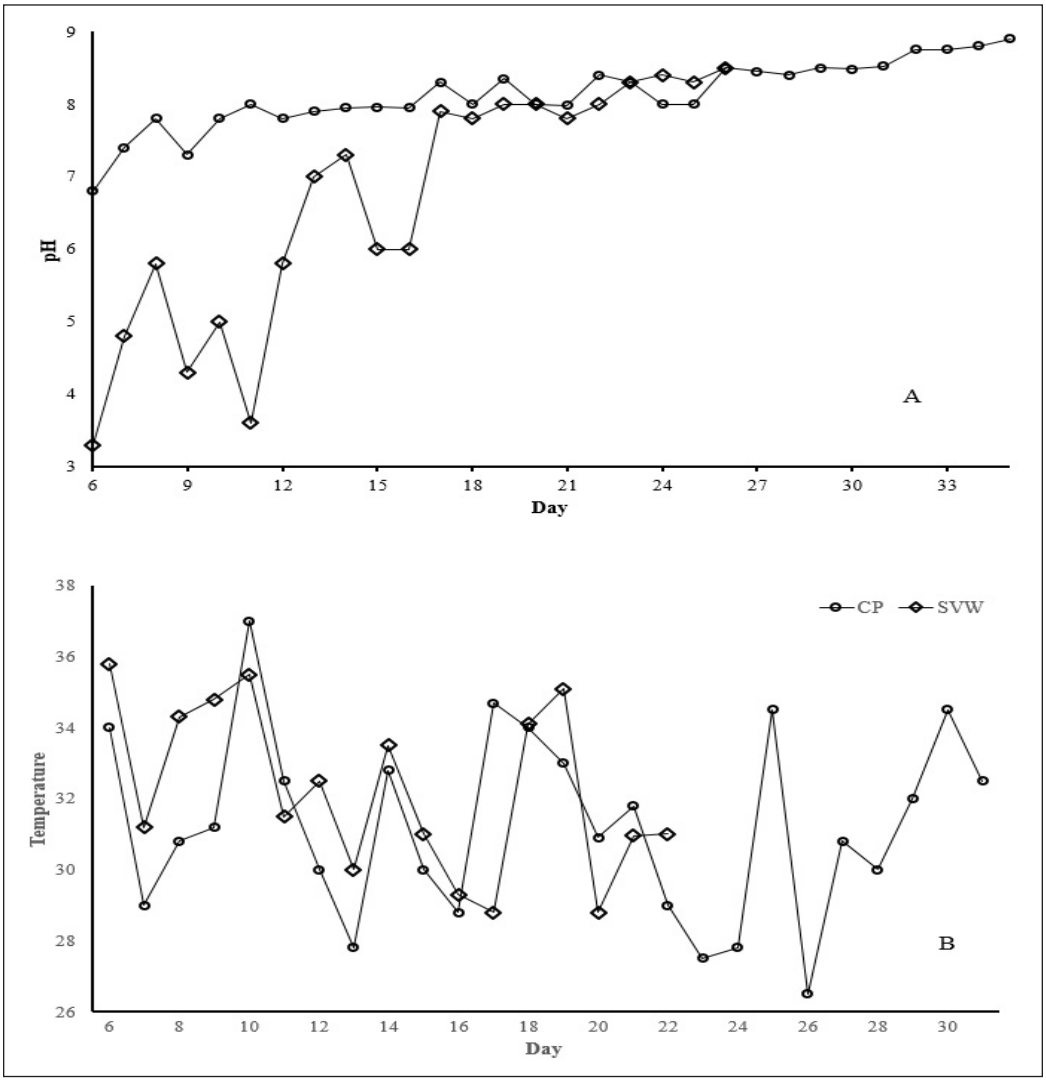

Figure 6. Variations in pH (A) and temperature (B), between treatments during the larval development of *Hermetia illucens*

in pH each time new food was supplied (Figure 6A).

Under average environmental conditions of 31.74 ± 2°C, RH of 51.85 ± 5%, and CP with 0.5% caffeine, it was found that the duration of larval development was longer in CP than in SVW (F = 7023.73, p = 0.000) (Table 3).

Although no significant differences were found in pupal duration in CP and SVW (F = 3.39, p = 0.066), the percentage of survival was higher in SVW (83.7%) than in CP (71.17%) (Table 3) and the migration of prepupae was first in SVW (19 days) than in CP (26 days) (Figure 7).

Table 3
Survival rate and duration in days, in the different stages of development of Hermetia illucens*, fed with two different diets*

Diet	Egg (n = 6570)		Development				Adult development	
			larva to prepupa		prepupa to adult		Male	Female
	S (%)	D (days)	S (%)	D (days)	S (%)	D (days)	D (days)	D (days)
CP (n = 2000)	88.73	3.57±0.53	74,8	34.8±4.4a	71.17	18.99±1.74a	3.9±2.9a	3.7±2.44a
SVW (n = 2000)	-	-	78.4	24.3±2.1b	83.7	19.3±1.56a	3.6±2.72a	3.5±3.23a

Note. SVW = solid vegetal waste, CP = coffee pulp, S = survival, D = duration
Average values followed by an identical letter means that they do not vary significantly (p<0.05)

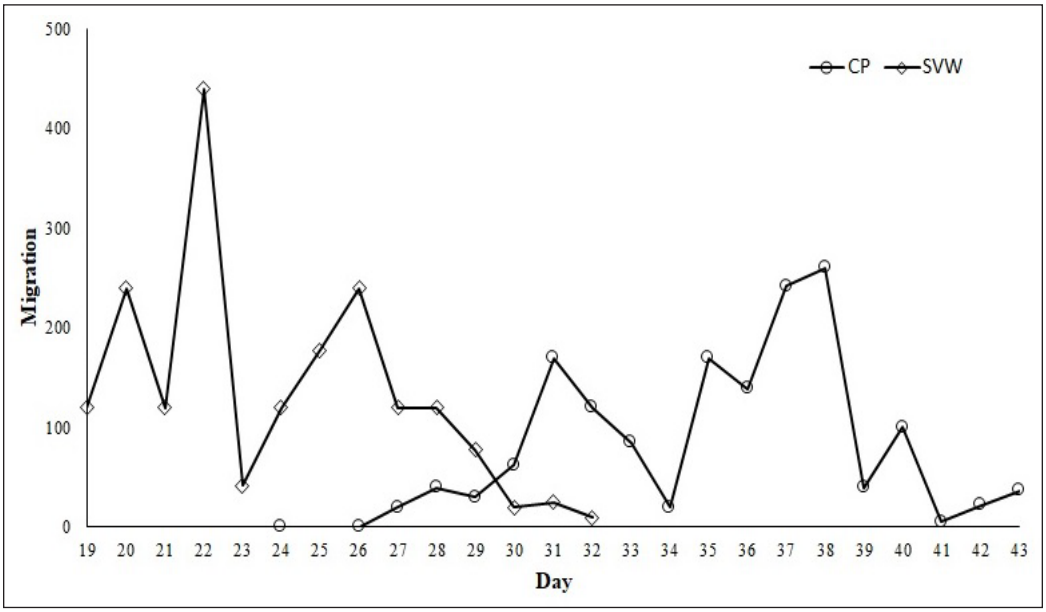

Figure 7. Start and end of prepupal migration of *Hermetia illucens*, fed with CP and SVW

DISCUSSION

The values of the average percentage reduction in dry base (30.22 and 32%) were similar to those obtained by Oonincx et al. (2015a) in chicken, pig and cow excrement (36.7, 37.7, and 36.8%, respectively), while Lardé (1990) reported a reduction of 29 in CP. 8%. The same author, mentioned in the analysis of his results that Booram et al. (1977) obtained a percentage reduction between 20.3 and 21.7% in pig excrement, percentages lower than those obtained in this study.

The trend in the linear relationship found between the average percentage of reduction and feeding rate, coincides with that expressed by Myers et al. (2008), who by feeding *H. illucens* larvae with 90 and 233 mg/larva/day of cow manure, found a reduction of 58 and 33% respectively. Similar results were obtained by Manurung et al. (2016), when feeding larvae with 12.5 and 200 mg/larva/day of rice "husk", the percentage of reduction being 31.5 and 9.58% respectively, maintaining an inverse linear relationship.

Authors such as Myers et al. (2008) and Manurung et al. (2016), agreed that an inverse relationship between the amount of food and the percentage of reduction was explained by the fact that, with less food available, the larvae consumed the totality of it, which of course compromised the development duration and the biology of the adult (Tomberlin et al., 2002).

The WRI values found, are similar to those obtained by Manurung et al. (2016), which are 0.58-0.24 for dietary supplies of 12.5-200 mg/larva/day respectively. However, the WRI/diet ratio in unit of "mg" maintains for Manurung et al. (2016), an inverse relationship, these results may find justification in the loss of moisture.

The high ECI values (7.89%) obtained in this study reflect the capacity that *H. illucens* larvae have to use the ingested CP for growth (weight gained). These values are low compared with the data obtained from Oonincx et al. (2015a) and Banks et al. (2014), indicating the low digestibility of CP, which the larvae have, does not significantly affect either their bioconversion efficiency or their biology.

In terms of weight gain and developmental time, the results obtained in this study were similar to those presented by authors such as Supriyatna et al. (2016) when *H. illucens* larvae were fed cassava, chicken were fed supplement (Diener et al. 2009), and Furman et al. (1959) found that under poor dietary conditions, the larval period was extended by more than four months.

Diener et al. (2009) stated, supported by Nijhout and Williams (1974) that under poor nutritional conditions, larvae would feed until they reached the minimum energy reserves required to reach the pupal stage.

The relationship between the duration of larval development and dietary supply is consistent with those reported by Diener et al. (2009) when feeding larvae with chicken feed, with the larval development of 10.6 days, while larval development was 41.1 days when the larvae were fed with rice husks (Manurung et al. 2016). The

differences found can be justified in the nutritional components of the different diets, that is, the shortest duration (10.6 days) was found in larvae fed with chicken feed. This type of feed has a balanced component, with a protein content (20%) higher than that found in CP (approximately 11%). On the other hand, the low digestibility of structural carbohydrates such as lignin, cellulose, and hemicellulose present in diets based on rice husks and CP, have an effect, increasing the larval development time. Nguyen et al. (2013), stated that proteins were the most important dietary component in the larval development of *H. illucens*, so that the higher the proteins content, the shorter the time of larval development. The diet used in this study, unlike all those mentioned, contained 0.5% caffeine. According to the data obtained, caffeine should be considered as a dietary element that can affect (although not significantly) the time of larval development.

Regarding the linear relationship found between the percentage of survival and dietary availability, the results observed are related to those reported by Manurung et al. (2016), who with dietary supplies (rice husk) of 12.5, 25, 50, 100, and 200 mg/larva/day, presented survival rates of 51.21, 74.24, 80.90, 91.79, and 98.27% respectively, despite finding significant differences, the direct linear relationship is maintained. Similar results were obtained by Myers and collaborators (2008), who when feeding larvae with 133.3 mg/larva/day of cow manure, reported a survival rate of 84.50%.

The relationship found between the shortest development time and the best RGR values is justified by Manurung et al. (2016), who stated that RGR values would depend on factors such as feed availability, that is, higher RGR values occurred in those cases where feed availability was the highest. Under optimal nutritional conditions, larvae adjust their energy consumption, and prioritize energy utilization for body growth and metabolism. Under poor dietary conditions, larvae slow down their growth and prioritize their energy on sustaining their survival and once the nutritional conditions change, the strategy may shift towards increasing size, this characteristic behavior of holometabolic insects, denominated by Manurung et al. (2016) as phenotypic plasticity.

In Figure 8, the results obtained in this study are compared with those reported by Manurung et al. (2016), who fed rice hull to *H. illucens* larvae.

Development occurred well at temperatures between 26.49 and 36.63°C, ranges that coincide with the results reported by Alattar (2012), in studies with *H. illucens* larvae, fed with leachate of plant origin.

Concerning the pH, our results are consistent with those obtained by Alattar (2012), who stated that *H. illucens* larvae modified the pH of the medium, developing in ranges between 0.7 and 13.7. These results match those presented by Meneguz et al. (2018), where they stated that larval activity modified the pH from the fourth day, and that they tolerated wide ranges of pH (4.0-9.5).

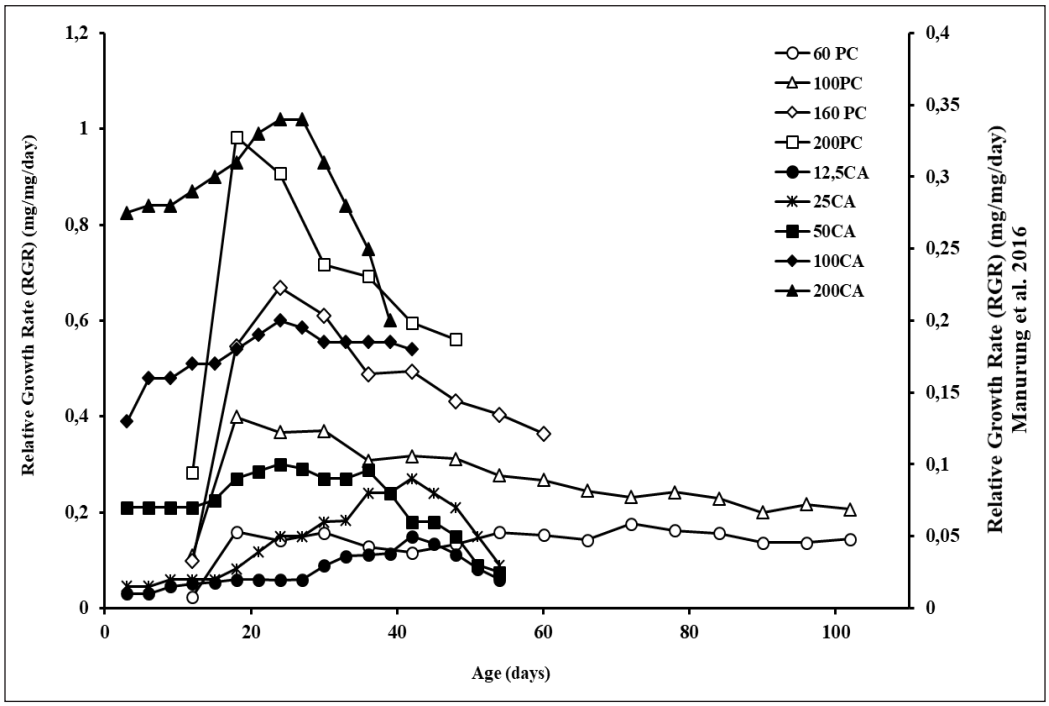

Figure 8. Comparative Relative Growth Rate (RGR) *Hermetia illucens* larvae fed with CP

CONCLUSIONS

Hermetia illucens has the capacity to efficiently digest CP. The survival rate of immature stages, the bioconversion time, the percentage and reduction index, are positive in regard to its bioconversion efficiency. Dietary supplies of 160-200 mg/larva/day allow for efficient bioconversion of CP. Caffeine present in fresh CP does not affect the development speed of immature *H. illucens*. The use of the "black soldier fly" in the degradation of the CP in coffee farms, is envisioned as a valid alternative to mitigate the enormous environmental impact caused by the spill of these to the ecosystem, and additionally offers a good opportunity in the harnessing of the generated by-products.

ACKNOWLEDGEMENTS

The authors would like to thank the Administrative Department of Science, Technology and Innovation (COLCIENCIA), for funding the project Valorisation of agri-food residuals with insect technologies (EntoWaste) in the framework of the Second Transnational Call EraNet-LAC of (ELAC2015-T03-0580); special thanks to José Noé Castro, José Guerrero, Fernando Capote, and Raúl Canal for facilitating the coffee pulp used during this research; to the Universidad del Valle (Grupo de Investigaciones Entomológicas, Professors Gloria Delfina Lasso de Fernández of the Chemical Engineering Department, and Enrique Peña and Wilmar Bolivar of the Biology Department) for facilitating the different equipment used.

REFERENCES

Alattar, M. A. (2012). *Biological treatment of leachates of microaerobic fermentation* [Master's thesis, Portland State University]. Pdxscholar. https://pdxscholar.library.-pdx.edu/open_access_etds/905/

Arango, G., Vergara, R., & Mejía, H. (2004). Análisis composicional, microbiológico y digestibilidad de la proteína de la harina de larvas de *Hermetia illucens* L. (Diptera: Stratiomyidae) en Angelópolis-Antioquia, Colombia [Compositional, microbiological and digestibility analysis of the protein of the larvae meal of *Hermetia illucens* L. (Diptera: Stratiomyidae) in Angelopolis-Antioquia, Colombia]. *Revista Facultad Nacional de Agronomía*, *57*(2), 2491-2499.

Banks, I., Gibson, W., & Cameron, M. (2014). Growth rates of black soldier fly larvae fed on fresh human faeces and their implication for improving sanitation. *Tropical Medicine International Health*, *19*(1), 14-22. https://doi.org/10.1111/tmi.12228

Blandón, C., Rodríguez, V., & Dávila, M. (1999). Caracterización microbiológica y físico-química de los subproductos del beneficio del café en proceso de compostaje [Microbiological and physical-chemical characterization of coffee processing by-products in the composting process]. *Cenicafé*, *49*, 169-185.

Bondari, K., & Sheppard, C. (1987). Soldier fly, *Hermetia illucens* L., larvae as feed for channel catfish, *Ictalurus punctatus* (Rafinesque), and blue tilapia, *Oreochromis aureus* (Steindachner). *Aquaculture Research*, *18*(3), 209-220. https://doi.org/10.1111/j.1365-2109.1987.tb00141.x

Booram, C. V. Jr., Newton, G. L., Hale, O. M., & Barker, R. W. (1977). *Manure residue as a substrate for protein production via* Hermetia illucens *larvae*. https://agris.fao.org/agris-search/search.do?recordID=US19780337139

Canary, G. (2009). *Diseño y gestión de un proceso para reciclar desechos orgánicos con la larva* Hermetia illucens *para producir harina de larva* [Design and management of a process to recycle organic waste with the larva *Hermetia illucens* to produce larva flour]. https://www.semanti-cscholar.org/paper/Dise%C3%B1o-y-gesti%C3%B3n-de-un-proceso-para-reciclar-con-la-Canary-Gonzalez/2750a9d99f3b265bd067ac186279aca1-79a7f403

Diener, S., Studt, N. M., Roa, F., Zurbügg, C., & Tockner, K. (2011). Biological treatment of municipal organic waste using black soldier fly larvae. *Waste Biomass Valorization*, *2*(4), 357-363. https://doi.org/10.1007/s12649-011-9079-1

Diener, S., Zurbrügg, C., & Tockner, K. (2009). Conversion of organic material by black soldier fly larvae: Establishing optimal feeding rates. *Waste Management and Research*, *27*(6), 603-610. https://doi.org/10.1177/0734242X09103838

Elías, L. (1978). Composición química de la pulpa de café y otros subproductos [Chemical composition of coffee pulp and other by-products]. In J. E. Braham (Ed.), *Pulpa de café: Composición, tecnología y utilización* [Coffee pulp: Composition, technology and use] (pp. 19-29). Institute of Nutrition of Central America and Panama.

Furman, D., Young, R., & Catts, P. (1959). *Hermetia illucens* as a factor in the natural control of *Musca domestica* Linnaeus. *Journal of Economic Entomology*, *52*(5), 917-921. https://doi: 10.1177/0734242X09103838

Gamboa, E., & Jasbón, N. (2008). *Degradación de residuos de almidón y cárnicos, mediante la producción de la larva soldado negra (*Hermetia illucens *L.)* [Degradation of starch and meat residues by production of the black soldier larva (*Hermetia illucens* L.)]. https://ciencia.lasalle.edu.co/-ing_ambiental_sanitaria/669

Hale, O. M. (1973). Dried *Hermetia illucens* larvae (Diptera: Stratiomyidae) as a feed additive for poultry. *Journal of the Georgia Entomological Society*, *8*, 16-20.

Jaramillo, G., & Zapata, L. (2008). *Aprovechamiento de los residuos sólidos orgánicos en Colombia* [Use of organic solid waste in Colombia]. http://www.enjambre.gov.co/enjambre/file/download/125298

Kim, W., Bae, S., Park, K., Lee, S., Choi, Y., Han, S., & Koh, Y. (2011). Biochemical characterization of digestive enzymes in the black soldier fly, *Hermetia illucens* (Diptera: Stratiomyidae). *Journal of Asia-Pacific Entomology*, *14*(1), 11-14. https://doi.org/10.1016/j.aspen.2010.11.003

Lardé, G. (1990). Recycling of coffee pulp by *Hermetia illucens* (Diptera: Stratiomyidae) larvae. *Biological Wastes*, *33*(4), 307-310. https://doi: 10.1016/0269-7483(90)90134-E

Lord, W. D., Goff, M. L., & Adkins, T. R. (1994). The black soldier fly *Hermetia illucens* (Diptera: Stratiomyidae) as a potential measure of human post-mortem interval: Observations and case histories. *Journal of Forensic Sciences*, *39*(1), 215-22. https://doi: 10.1111/j.1556-4029.2008.00659.x

Manurung, R., Supriatna, A., Esyanthi, R., & Putra, R. E. (2016). Bioconversion of rice straw waste by black soldier fly larvae (*Hermetia illucens* L.): Optimal feed rate for biomass production. *Journal of Entomology and Zoology Studies*, *4*(4), 1036-1041. https://www.entomoljournal.com/archives/2016/vol4issue4/PartK/4-3-163-796

Meneguz, M., Gasco, L., & Tomberlin, J. K. (2018). Impact of pH and feeding system on black soldier fly (*Hermetia illucen*s, L; Diptera: Stratiomyidae) larval development. *PLoS One*, *13*(8), e0202591. https://doi.org/10.1371/journal.pone.0202591

Molina, M., Lechuga, R., & Bressan, R. (1990). Valor nutritivo de la pulpa de café sometida a fermentación sólida usando *Aspergillus niger* en pollos y cerdos [Nutritional value of coffee pulp subjected to solid fermentation using *Aspergillus niger* in chickens and pigs]. *Agronomía Mesoamericana*, *1*, 79-82. https://doi.org/10.15517/am.v1i0.25350

Myers, H., Tomberlin, J., Lambert, B., & Kattes, D. (2008). Development of black soldier fly (Diptera: Stratiomyidae) larvae fed dairy manure. *Environmental Entomology*, *37*(1), 11-15. https://doi.org/10.1093/ee/37.1.11

Newton, G. L., Sheppard, D. C., Watson, D. W., Burtle, G. J., Dove, C. R., Tomberlin, J. K., & Thelen, E. (2005a). *The black soldier fly,* Hermetia illucens, *as a manure management/resource recovery tool*. https://www.researchgate.net/publication/237345975_The_black_soldier_fly_Hermetia_illucens_as_a_manure_managementresource_recovery_tool

Newton, G. L., Sheppard, D. C., Watson, D. W., Burtle, G., & Dove, R. (2005b). *Using the black soldier fly,* Hermetia illucens*, as a value-added tool for the management of swine manure*. https://www.researchgate.net/publication/267377822_Using_the_Black_Soldier_Fly_Hermetia_Illucens_as_a_Value-Added_Tool_for_the_Management_of_Swine_Manure

Nguyen, T., Tomberlin, J., & Vanlaerhoven, S. (2013). Influence of resources on *Hermetia illucens* (Diptera: Stratiomyidae) larval development. *Journal of Medical Entomology*, *50*(4), 898-906. https://doi.org/10.1603/ME12260

Nijhout, H. F., & Williams, C. M. (1974). Control of molting and metamorphosis in tobacco hornworm, *Manduca sexta* (L): Growth of last-instar larva and decision to pupate. *Journal of Experimental Biology*, *61*(2), 481-491.

Noriega, A., Silva, R., & García de Salcedo, M. (2008). Revisión: Utilización de la pulpa de café en alimentación animal [Review: Use of coffee pulp in animal feed]. *Zootecnia Tropical*, *26*(4), 411-419.

Oonincx, D., Van Huis, A., & Van Loon, J. (2015a). Nutrient utilization by black soldier flies fed with chicken, pig, or cow manure. *Journal of Insects as Food and Feed*, *1*(2), 131-139. https://doi.org/10.3920/JIFF2014.0023

Pandey, A. K., Mishra, B. K., Arora, A., Singh, S., & Lata-Ray, R. C. (2011). Bioaugmentation and biovalourization of agro-food and beverage industry effluents. In A. Singh, N. Parmar, & R. Kuhad (Eds.) *Bioaugmentation, biostimulation and biocontrol* (pp. 85-106). Springer. https://doi.org/10.1007/978-3-642-19769-7_5

Parra, S., Carrejo, N., & Gómez, C. (2015). Effects of larval density and feeding rates on the bioconversion of vegetable waste using black soldier fly larvae *Hermetia illucens* (L.), (Diptera: Stratiomyidae). *Waste Biomass Valorization*, *6*(6), 1059-1065. https://doi.org/10.1016/j.jclepro.2018.10.017

Pujol-Luz, J. R., Francez, P. A. C., Ururahy-Rodrigues, U., & Constantino, R. (2008). The black soldier by, *Hermetia illucens* (Diptera, Stratiomyidae), used to estimate the postmortem interval in a case in Amapá State, Brazil. *Journal of Forensic Sciences*, *53*(2), 476-478. https://doi.org/10.1111/j.1556-4029.2008.00659.x

Rodríguez. V., & Zambran, D. (2010). *Los subproductos del café: Fuente de energía renovable* [Coffee by-products: A source of renewable energy]. Cenicafé Press.

Rubio, J. (1973). *Composición química y digestibilidad* in vitro *de la pulpa de café* [Chemical composition and *in vitro* digestibility of coffee pulp] [Master's thesis, Universidad Nacional]. Universidad Nacional Research Repository. http://hdl.handle.net/20.500.12324/23189

Scriber, J., & Slansky, F. (1981). The nutricional ecology of immature insects. *Annual Review of Entomology*, *26*(1), 183-211. https://doi.org/10.1146/annurev.en.26.010181.001151

Sheppard, D. C., Newton, G. L., Thompson, S. A., & Savage, S. (1994). A value-added manure management-system using the black soldier fly. *Bioresource Technology*, *50*(3), 275-279. https://doi.org/10.1016/0960-8524(94)90102-3

Sheppard, D. C., Tomberlin, J. K., Joyce, J. A., Kiser, B. C., & Sumner, S. M. (2002). Rearing methods for the black soldier fly (Diptera: Stratiomyidae). *Journal of Medical Entomology*, *39*(4), 695-698. https://doi.org/10.1603/0022-2585-39.4.695

Supriyatna, A., Manurung, R., Rachmi-Esyanti, R., & Putra, R. E. (2016). Growth of black soldier larvae fed on cassava peel wastes, an agriculture waste. *Journal of Entomology and Zoology Studies*, *4*(6), 161-165.

Tomberlin, J., Shepard, D., & Joyce, J. (2002). Selected life-history traits of black soldier flies (Diptera: Stratiomyidae) reared on three artificial diets. *Annals of the Entomological Society of America*, *95*(3), 379-386. https://doi.org/10.1603/00138746(2002)095-[0379:SLHTOB]2.0.CO;2

Ulloa, J. A. (2007). *Frutas auto estabilizadas en el envase por la tecnología de obstáculos* [Fruits self-stabilized in the package by the obstacle technology]. Universidad Autónoma de *Nayarit Press.*

Waldbauer, G. P. (1968). The consumption and utilization of food by insects. In *Advances in Insect Physiology*, *5*, 229-288. https://doi.org/10.1016/S0065-2806(08)60230-1

Warburton, K., & Hallman, V. (2002). *Processing of organic materials by the soldier fly*, Hermetia illucens. https://www.agrifutures.com.au/wp-content/uploads/publications/01-174.pdf

Zheng, L., Cai, H., Garza, E., Yu, Z., & Zhou, S. (2011). From organic waste to biodiesel: Black soldier fly, *Hermetia illucens*, makes if feasible. *Fuel*, *90*(4), 1545-1548. https://doi.org/10.1016/j.fuel.2010.11.016

Zheng, L., Zhang, J., & Yu, Z. (2012). Double the biodiesel yield: Rearing black soldier fly larvae, *Hermetia illucens*, on solid residual fraction of restaurant waste after grease extraction for biodiesel production. *Renewable Energy*, *41*, 75-79. https://doi.org/10.1016/j.renene.2011.10.004

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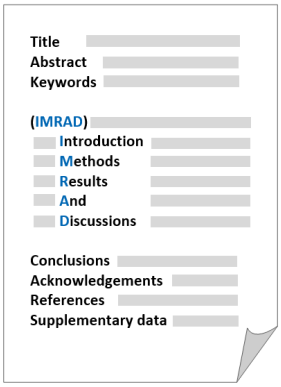

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Pertanika Journal of Tropical Agricultural Science
Vol. 44 (1) Feb. 2021

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