Resistant starch from underutilised legumes as prebiotic and its effect on the growth of *Danio rerio* and *Lates calcarifer*

BSc Ivan Kar Mun Chiew

Thesis submitted to the University of Nottingham for the degree of

Doctor of Philosophy, 2017

School of Biosciences

University of Nottingham Malaysia Campus

Abstract

Aquaculture is an important protein source for Malaysia. However, the susceptibility of fish to disease leads to heavy losses and hinders production. A potential solution is the supplementation of diets with prebiotics: non-digestible carbohydrates which improve growth and health by modulating gut microbiota to favour beneficial bacteria. Resistant starch (RS) is a prebiotic commonly fed to terrestrial animals, but little work has been performed on aquatic animals. Therefore, this study investigated the use of RS isolated from underutilized crops as a prebiotic in fish. Six species of underutilised legumes (adzuki beans, mung beans, black-eyed peas, pigeon peas, Bambara groundnuts and red lentils) were used for starch isolation via alkaline steeping, followed by processing involving enzyme or acid hydrolysis, and lastly gelatinisation and retrogradation to increase RS yield. Starch was isolated with yields of 25 – 40%, while enzyme hydrolysis pre-treatment was more effective and improved RS content up to 18.06% in most legumes. The starch and RS samples were then supplemented at 5% (w/v) in nutrient broth to investigate their prebiotic effect on fish gastrointestinal lactic acid bacteria. Enzyme pre-treatment improved the growth of *W. cibaria*, *L. garvieae* and *E. gilvus* by up to 43.9% for most legumes tested. Red lentil and adzuki bean enzyme-RS showed highest prebiotic potential and was applied in the zebrafish growth trial, while only red lentil was used in the Asian sea bass growth trial. Supplementation of diets with RS from legumes provided no significant difference in the growth and performance parameters measured in both zebrafish and Asian sea bass when supplemented at 2.5% and fed for five and six weeks respectively. Further studies involving analysis of immune parameters is necessary to identify prebiotic potential on targeted fish. Nevertheless, this study contributed to promote future work in using underutilised legumes as prebiotic source to improve the fish health under commercial conditions of rearing.

Acknowledgements

Throughout the journey of completing my PhD thesis, I am very grateful to the colleagues and friends whom I have m*et al*ong the way.

Firstly, I would like to thank the supervisors in my supervisor committee Dr. Lim Yin Sze and Prof. Andrew Salter for their continuous support of my PhD study, for their patience, encouragement and brilliance throughout the study. Their guidance throughout research and writing have been invaluable.

For financial support, I'd like to thank Crops for the Future (CFF) for the funding of this project and the studentship.

For technical support, I'd like to thank Prof. Tim Parr and the technicians at the North Laboratory and Food Sciences at the Sutton Bonnington Campus for their assistance throughout my research at the UK campus. I would also like to thank Prof Ian Young, Kieran Magee and the technicians at the University of Liverpool for facilitating the zebrafish feeding trial. I would also like to thank Dr. Kumar Katya, Giva and Zufar from FishPLUS of CFF for their support and facilitating the Asian sea bass feeding trial. Not forgetting, I would also like to thank the technicians in Block C in the Malaysia Campus for their technical help. I am grateful for all the support provided, which has been instrumental throughout my journey.

Lastly, I would like to thank colleagues, friends and family whom have been around to support me throughout the PhD. I am grateful for all the pillars of support provided in forms of laughter, encouragement and stimulating discussions whether in times of ups or downs, which have certainly made the entire journey a more comfortable one.

Supervisory Committee

The thesis submitted to the University of Nottingham has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee are as follows:

Dr Yin Sze Lim

- School of Biosciences, Faculty of Science, University of Nottingham Malaysia Campus

Professor Andrew Salter

- Division of Nutritional Sciences, School of Biosciences, Faculty of Science, University of Nottingham

Dr Sean Mayes

- Crops For the Future

List of conferences

Conference proceedings

Chiew, I.K., Lim, Y.S., Salter, A.M. and Wood, J., 2015. The effects of resistant starch from underutilised legumes on the growth of probiotic *Weisella cibaria* isolated from barramundi (*Lates calcarifer*). In Korean Society of Fisheries Science, pp.66.

Chiew, I.K.M, Salter, A.M. Parr, T., Lim, Y.S., 2016. The effects of resistant starch from underutilised legumes on the growth of potential probiotic *Lactococcus lactis* isolated from barramundi (*Lates calcarifer*). In The Proceedings of the British Society of Animal Science 2016, Chester, pp. 126.

Conferences attended

Oral presenter at DTP 2014 CFFRC UNMC Doctoral Training Programme (DTP), Kuala Lumpur, Malaysia, 17-21 November 2014

Oral presenter at World Aquaculture 2015, Jeju, Korea, 26-30 May 2015

Oral presenter at DTP 2015 Doctoral Training Partnership Week 2015, Selangor, Malaysia, 21-25 September 2015

Oral presenter at British Society of Animal Science Annual Conference 2016, Chester, UK, 6-7 April 2016

Oral presenter at DTP 2016 Future Agriculture: crops and crop researchers for a changing climate, Selangor, Malaysia, 13-16 December

Table of Contents

List of Figures

List of Tables

Abbreviations

Common abbreviations

acid-RS	Acid-hydrolysed resistant starch
Adz EH	Basal diet supplemented with adzuki bean enzyme-RS
Adz ST	Basal diet supplemented with adzuki bean untreated-S
AFLP-PCR	Amplified fragment length polymorphism with polymerase chain reaction
AMG	Amyloglucosidase
AOAC	Association of Analytical Communities
CFU	Colony forming units
DNA	Deoxyribonucleic acid
EDTA	Ethylenediamine tetra-acetic acid
EMB	Eosin methylene blue
enzyme-RS	Enzyme-hydrolysed resistant starch
ETB	Enterobacteriaceae
FCR	Feed conversion ratio
FOS	Fructo-oligosaccharides
GIT	Gastrointestinal tract
GOS	Galacto-oligosaccharides
LAB	Lactic acid bacteria
Lens EH	Basal diet supplemented with red lentil enzyme-RS
Lens ST	Basal diet supplemented with red lentil untreated-S
MOS	Mannan-oligosaccharide
MRS	De Man, Rogosa and Sharpe
PCR	Polymerase chain reaction

PCR-DGGE	Polymerase chain reaction with denaturing gradient gel electrophoresis
PER	Protein efficiency ratio
REP-PCR	Repetitive element palindromic polymerase chain reaction
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
RS	Resistant starch
SCFA	Short-chain fatty acid
SGR	Specific growth rate
untreated-RS	Untreated isolated starch
WG	Weight gain
WG%	Weight gain percentage

Units

%	Percent
⌀	Diameter
° C	Degrees Celsius
µL	Microlitre
µM	Micrometer
cm	Centimetre
g	Gravity
g	Gram
hr	Hour
kg	Kilogram
kJ	Kilojoule
M	Molar

mbar	Millibar
mg	Milligram
min	Minute
MJ	Megajoule
mL	Millilitre
mM	Millimolar
mm	Millimetre
N	Normality
ng	Nanogram
nm	Nanometre
pg	Page
psi	Pound per square inch
s	Seconds
U	Units
v/v	Volume/volume
w/v	Mass/volume
w/w	Mass/mass

1 Chapter 1: Literature review and experimental objectives

1.1 Introduction

Currently, seafood is one of the key protein sources, as well as the main source of omega-3-fatty acids essential for brain development (FAO & WHO 2011; Hibbeln *et al.* 2007). However, the reliance of capture fisheries for seafood production has many drawbacks such as overfishing and depletion of stocks which can affect the quality of the output and ultimately alter the ecosystem food chain (Smith *et al.* 2010; Choi *et al.* 2004; Frank & Brickman 2000). Hence, aquaculture is highlighted as an alternative source for seafood production.

Aquaculture is an underwater farming technique and can surpass the yield limit of capture fisheries (Garcia & Grainger 2005). However, various problems are also associated with the current practices of aquaculture such as the unsustainable dependency on capture fisheries for fish meal production and disease occurrences and outbreaks present in various farms; in addition, both problems aggravate as the aquaculture industry continues to intensify (Tacon & Metian 2008; Bondad-Reantaso *et al.* 2005; Naylor *et al.* 2000). The reliance on trash fish for the production of fish meal ultimately creates greater gaps for food security especially in poorer countries while antibiotic resistance was found to be developing in not only disease-causing bacteria but also possibly transferred to terrestrial bacteria as well (Alder *et al.* 2008; Sapkota *et al.* 2008; Defoirdt *et al.* 2007). Hence, different alternatives for the improvement of health and growth of fish can be considered, such as modification of gastrointestinal tract (GIT) through the inclusion of prebiotic and probiotic components in diet based on its effect observed in humans (Ringø *et al.* 2010b).

1.2 Role of microbiota in the gastrointestinal tract

Microorganisms are present throughout the human GIT with varying distribution of species and populations at particular regions. However, the colon is a subject of particular interest due to the slower transit time of digesta as compared to the stomach and small intestine, which is 12-24 hr versus two to five hr respectively in human adults (Guerra *et al.* 2012). This encourages the colonisation and growth of microbial populations in the colon, supported by the near neutral pH and relatively low absorptive state of the colon (Guerra *et al.* 2012). Among these microbial populations, intestinal bacteria can play a role in the health of the host. The intestinal bacteria can be divided into various categories, which are based on the effect exerted on the host; they either provide health benefits or are harmful towards the host (Salminen *et al.* 1999). As the colon harbours a wide range of microbiota, the attachment and overgrowth of pathogenic bacteria usually result in acute diarrhoeal infections and inflammatory bowel diseases (Tlaskalová-Hogenová *et al.* 2011; Spiller & Garsed 2009; Spiller 2007). On the other hand, beneficial microbiota, such as the *Bifidobacteria* spp., are also present and contributes towards promoting health benefits (Licht *et al.* 2012). In brief, various experiments have presented that *Bifidobacteria* spp. regulates the intestinal environment not only via modulation of immune responses, but also by interfering with the pathogenicity of harmful bacteria (Hord 2008; Gibson *et al.* 2004). Other groups of bacteria which play a role also include *Lactobacillus* spp. and some *Clostridium* spp., which the latter are key producers of butyrate for the maintenance of a healthy epithelium (Louis & Flint 2009; Pryde *et al.* 2002). These studies promote the concept of probiotics as a supplement that is beneficial to host health (Oelschlaeger 2010).

1.3 Probiotics and prebiotics

Probiotics and prebiotics serve as a form of functional food which aims to affect functions in the body in a targeted way to illicit a positive effect on health (Roberfroid 2000). Generally, both probiotics and prebiotics function similarly to improve host health via manipulation of GIT microbiota populations. However, probiotics revolve around the supplementation of microbial cells to elicit health benefits in the host (Salminen *et al.* 1999). Meanwhile, prebiotics are non-digestible which survive host digestion and arrives in the colon for microbial fermentation, resulting in specific changes in the GIT microflora which confers benefits to host health (Roberfroid 2007).

1.3.1 Probiotics: benefits and their shortcomings

The concept of probiotics revolves around the supplementation of the GIT with health-promoting bacteria to increase their population and thus, promoting beneficial effects for the host. Probiotics compose mainly of lactic acid bacteria (LAB) and provide several benefits to the GIT, such as the production of inhibitory compounds that act antagonistically towards pathogenic bacteria, competition of adhesion sites that prevent pathogenic bacteria from colonising, modulation of host immune system as well as competition for nutrients, chemicals and available energy in the GIT, as well as contribute to colon health via production of health related bacterial metabolites (Nayak 2010; Gatesoupe 2008; Panigrahi & Azad 2007; Ringø *et al.* 2010b; Roberfroid 2007; Verschuere *et al.* 2000). These benefits ultimately prevent the multiplication and entry of pathogenic bacteria via GIT and hence, reducing the likeliness of disease.

Nevertheless, probiotic treatments are limited by several shortcomings. For example, resistance to antibiotics in probiotic bacterial strains is a critical factor. The main concern in this aspect is the possibility of gene transfer, in which probiotic bacteria often possess antibiotic genes which could be transferred to pathogenic bacteria (Gueimonde *et al.* 2013; van Reenen & Dicks 2011). In addition, the colonisation of bacteria on the GIT is also a critical factor to the success of the probiotic treatment (Ohashi & Ushida 2009; Bezkorovainy 2001). Studies conducted found that bacterial cells were no longer detected in faeces a few days following the cessation of treatment, indicating that the probiotic was not successful in colonising or adhering onto the GIT (Satokari *et al.* 2001; Tannock *et al.* 2000). Lastly, another key factor for the success of probiotic treatments is the delivery methods of the bacteria. Various stress factors are involved prior to the arrival at the desired site of action, such as highly acidic stomach environment as well as bile secretions in the small intestine (Gibson & Rastall, 2006). In addition, feed preparation techniques such as extrusion exposes feed to harsh conditions such as high temperatures – this also adversely affects the effectiveness of probiotics especially when used as supplements in diets (Ganguly *et al.* 2013). Therefore, prebiotics can be considered as an alternative in resolving some of the issues faced by probiotics.

1.3.2 Prebiotics: concepts and benefits

Prebiotics were introduced in 1995 as an approach to the modulation of the GIT microbiota (Gibson & Roberfroid 1995). By definition, prebiotics are selectively fermented ingredients that allow specific changes, both in the composition and/or activity of the GIT microbiota that confers benefits upon host well-being and health (Roberfroid 2007). Therefore, prebiotics are associated with several characteristics, such as the resistance towards host GIT activity, fermented by intestinal bacteria, as

well as selective stimulation of growth and/or activity of intestinal bacteria associated with health benefits (Licht *et al,* 2012).

Prior to the concept of prebiotics, fermentative substrates of dietary origin have already been involved in supporting the growth of microflora in the GIT. These fermentative substrates are composed either of non-digestible carbohydrates or proteins which have escaped digestion (Gibson & Rastall 2006). A large portion of these non-digestible carbohydrates are composed of resistant starch (RS), which are starches recalcitrant to the activities of human digestive enzymes. The remainder of the carbohydrates are composed of non-starch polysaccharides, unabsorbed sugars and oligosaccharides, which become targets for fermentation (Macfarlane *et al.* 2006). It should also be noted that dietary fibre are not identical to prebiotics. By definition, dietary fibre refers to the overall components of non-digestible carbohydrates; apart from prebiotics such as oligosaccharides, dietary fibre includes lignin, pectin and other associated plant substances (Bellei & Haslberger 2012). However, prebiotics exclusively refers to the potential of particular carbohydrates to promote growth or metabolic effects of selected bacteria species in the GIT that contribute to health benefits, and therefore can be considered as unique dietary fibres (Roberfroid 2007).

Prebiotics are usually associated with carbohydrates, as they are the preferred and principal substrate during fermentation. In the absence of carbohydrates, certain groups of microbes, such as *Clostridium* spp. turn to protein fermentation which can produce harmful nitrogenous metabolites into the lumen (Rajilić-Stojanović *et al.* 2013; Davila *et al.* 2013). Excessive protein fermentation has been linked with diseases such as colon cancer, thereby highlighting the importance of carbohydrates

in the GIT (Rajilić-Stojanović 2013; Russell *et al.* 2011). Nevertheless, one of the key products of carbohydrate fermentation in the GIT are short chain fatty acids (SCFA), such as acetate, propionate and butyrate (Hijova & Chmelarova 2007). The other products produced include electron sinks, such as lactate, pyruvate, ethanol, succinate, as well as gasses such as hydrogen, carbon dioxide, methane and hydrogen sulphide (Rajilić-Stojanović 2013; Davis & Milner 2009; Hijova & Chmelarova 2007). The SCFA produced are rapidly absorbed and contribute to the health of the host. For example, acetate is metabolised in the muscle, brain and heart, while butyrate is metabolised at the colon epithelium where it serves as a regulator of cell growth and differentiation (Gibson & Rastall, 2006).

1.3.3 Established prebiotics and their benefits

The understanding of the relationship between the microbiota of the GIT and the health of the host eventually led to the development of more prebiotics. Key factors in the design of prebiotics are that the compounds are not only abundant but also inexpensive (Gibson & Rastall, 2006). Newer compounds such as dextran and bacterial polysaccharides are being studied for the possibility of prebiotic effects (Korakli *et al.* 2002; Olano-martin *et al.* 2000). On the other hand, established prebiotics include fructans (includes inulin and FOS), galacto-oligosaccharides (GOS) and lactulose (Gibson *et al.* 2004). In addition, RS has also gained recognition as a functional ingredient (Fuentes-Zaragoza *et al.* 2010). While various types of prebiotics exist and are currently in development, this review will focus on RS as a growing prebiotic.

1.3.3.1 Resistant starch (RS)

RS is the most quantitatively important prebiotic and is included under dietary fibre (Englyst *et al.* 1996; Cummings & Macfarlane 1991). By definition, RS is the fraction of starch which is not hydrolyzed to D-glucose in the small intestine within 120 min of being consumed, but which is fermented in the colon (Fuentes-Zaragoza *et al.* 2011). These allows RS to exert prebiotic properties when fermented by beneficial microbiota (Yao *et al.* 2009).

Like other forms of starch, RS are polysaccharides composed of a-D-glucopyranosyl units linked together with α-D- (1–4) and/or α-D-(1–6) linkages, and are comprised of two molecular types: amylose, the straight chain polyglucan comprised of approximately 1000, α-D-(1–4) linked glucoses; and amylopectin, the branched glucan, comprised of approximately 4000 glucose units with branches occurring as α-D-(1–6) linkages (Haralampu 2000). In general, RS can be found in starch containing food, such as the starch from chloroplast storage granules in leaves, as well as in the amyloplast of seeds and tubers (Sajilata *et al.* 2006). RS from native sources can also be prepared, which results vary depending on a large range of factors (Perera *et al.* 2010). Briefly, preparation methods influence the types of RS produced, which can be divided into 4 categories: RS1, RS2, RS3, and RS4, as summarised in Table 1.1.

Generally, RS grades are based on their increasing resistance towards digestion, as well as methods which can reduce this resistance (Fuentes-Zaragoza *et al,* 2010). Properties of RS is summarised from the review of Sajilata *et al,* (2006) and pictured in Figure 1.1:

- RS1 is associated with the physical inaccessibility of enzymes towards the starch, such as due to the presence of grain walls

Table 1.1: Types of resistant starch, resistance to digestion in small intestine and food sources (Adapted from Fuentes-Zaragoza *et al.* 2010)

Type	**Description**	**Digestion in small intestine**	**Resistance reduced by**
RS1	Physically inaccessible to digestion by entrapment in a non-digestible matrix	Slow rate; partial degree. Totally digested If properly milled	Milling, chewing
RS2	Ungelatinized resistant granules with type B crystallinity, slowly hydrolysed by α-amylase	Very slow rate; little degree. Totally digested when freshly cooked	Food processing and cooking
RS3	Retrograded starch formed when starch- containing foods are cooked and cooled	Slow rate; partial degree. Reversible digestion: digestibility improved by reheating	Processing conditions
RS4	Selected chemically-modified RS and industrially processed food ingredients	As a result of chemical modification, can resist hydrolysis	Less susceptible to digestion *in vitro*

- RS2 is associated with raw and uncooked starch, where crystallinity limits hydrolysis due to conformation and structure of the granule
- RS3 is a completely hydrated starch granule produced via retrogradation. Amylose leaches from the starch granules into the solution as a random coil polymer and re-associates as double helices stabilized by hydrogen bonds upon cooling
- RS4 is associated with starch obtained by chemical treatment (e.g.: di-starch phosphate ester)

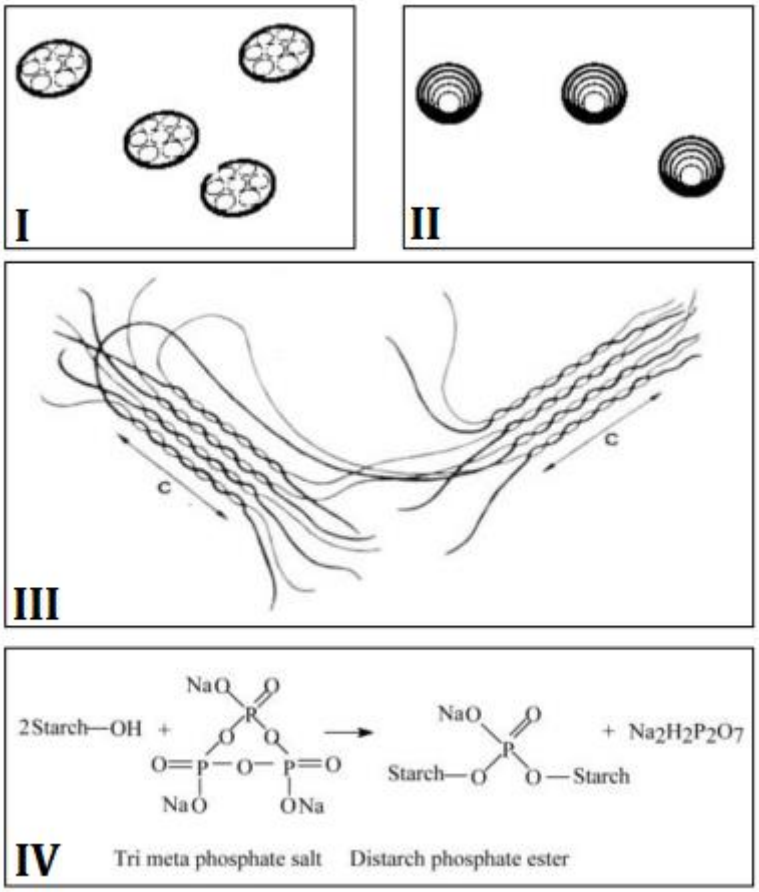


Figure 1.1: Structure of RS, where I = RS1, II = RS2, III = RS3 and IV = RS4; C refers to the ordered crystalline structure composed of double helices over a region of the chain and is interspersed with amorphous, enzyme degradable regions (Adapted from Sajilata *et al.* 2006)

A key factor of RS as a potential prebiotic is due to its capability to pass through the small intestine, allowing it to behave as a growth substrate instead for microorganisms, especially probiotic types in the colon (Sajilata *et al.* 2006). This is associated with studies which have shown that the consumption of RS produces increased SCFA production, such as butyrate, which is commonly produced following bacterial fermentation of prebiotics in the colon (Topping *et al.* 2003; Topping & Clifton 2001; Cummings & Macfarlane 1991). This contributes to health benefits in the colon; for example, butyric acid is considered as an important energy source for

colonocytes while also having an inhibitory effect on the growth of and proliferation of tumour cells *in vitro* via cell cycle arrest at G_1 (Sharma *et al.* 2008; Elmstahl 2002). This benefit can be linked to the availability of RS as a prebiotic, resulting in the production of butyric acid in the colon as a result of fermentation (Brouns *et al.* 2002). Apart from that, analysis of populations and RS consumption concludes that by doubling the uptake of dietary fibre, which includes RS, risk of colorectal cancer can be reduced by up to 40% (Bingham *et al.* 2003). This was further supported by studies which found that RS suppresses the growth and development of neoplastic lesions in the colon, highlighting its potential as a preventive agent for colon cancer development (Liu & Xu 2008).

Several studies have also investigated the effect of RS as a prebiotic in livestock, such as pigs (Regassa & Nyachoti 2018). These studies aimed improve to survival of piglets during post-weaning periods, protecting them against enteric infections and subsequently various diseases (Zimmermann *et al.* 2001; Pluske *et al.* 1998). The supplementation of RS in pigs via raw potato starch (RPS) was found to increase total caecal SCFA concentration and improve faecal consistency in weaning piglets (Heo *et al.* 2014). In addition, feeding pigs with RPS also improved microbial composition in the GIT, which subsequently improved intestinal morphology and also reduced pathogenic microorganisms (Haenen *et al.* 2013; Hedemann & Knudsen 2007). Lastly, RS was also shown to reduce skatole in the distal colon, potentially reducing the piggy odour from pork (Lösel & Claus 2005). Hence, RS can be said to possess potential for the applications such as in aquaculture as well.

1.4 Prebiotics from underutilised crops

Prebiotics may originate from various sources. For example, oligosaccharides can originate from beet, cow's milk, and also soybean (Gibson & Rastall, 2006). Other prebiotics include inulin and RS, which are major components in chicory roots and legumes respectively (Sajilata *et al.* 2006; Gupta *et al.* 2003a). Nevertheless, studies are focused on alternative sources for functional food apart from the major crops which are currently cultivated, which are defined as underutilised crops. Underutilised crops also include a large variety of legumes, which also contains high contents of RS (Hoover & Zhou 2003). Hence, underutilised crops are selected as targets for prebiotic extractions due to additional benefits attained apart from their functional food composition (Mayes *et al.* 2012; Bowe 2007).

1.4.1 The concept of underutilised crops

In general, the term underutilised crops is commonly applied to crops which potential has not fully been realised; however, this can also be applied to major crops and commodities which were abandoned or in decline, which does not highlight the type of crops which are in focus (Padulosi & Hoeschle-Zeledon 2004). Therefore, the term 'underutilised crops' is more suitably labelled to crops which have not received proper research, excluding neglected cultivars of major crops (Padulosi *et al.* 2011). Padulosi & Hoeschle-Zeledon (2004) defined underutilized species as "those non-commodity crops, which are part of a larger biodiversity portfolio, once more popular and today neglected by users' groups for a variety of agronomic, genetic, economic, social and cultural factors." A common factor leading to the neglect of specific crops is due to the advent of major crops, which led to domestic species becoming no longer competitive with the demand of major crops such as maize (Padulosi & Hoeschle-Zeledon, 2004). However, in terms of food

security, major crops would require additional improvements – this is where genetic modification may play a role but also leads to reduced genetic diversity in agriculture (Janick 1999). On the other hand, underutilised crops provide the other alternative with several advantages which had led them to be a focus of research (Mayes *et al.* 2012).

A key strength of native species and underutilised crops are their resilience in a wide range of conditions. This capability to resist climate change drew interest, especially in terms of possible traits which can be applied to major crops. The transfers of yield and quality traits to major crops have been beneficial previously, with contributions up to US$ 350 million annually (Prescott-Allen & Prescott-Allen 1996). Following the ongoing occurrence of climate change, a desired trait following the exploitation of underutilised crops is their resilience in adverse conditions. One example is the Bambara groundnut (*Vigna subterranea*), which was shown to be capable of drought tolerance and is cultivated on harsh climates and marginal soil throughout the sub-Saharan Africa (Andika *et al.* 2008). However, Bambara groundnuts are neglected due to unpredictable yields and long cooking time (Mayes *et al.* 2009). Other drought tolerant crops include coarse cereals in South Asia (Padulosi *et al.* 2011). On the other hand, perennial species such as the sea buckthorn (*Hippophae rhamnoides*) and tamarind (*Tamarindus indica*) were found not only to be more resilient to abiotic stress such as drought resistance and survival on marginal lands (Van den Bilcke *et al.* 2013; Hegde & Mishra 2009; Xu *et al.* 2009a). There are also cases where underutilized crops can be used as drought indicators in many cultures, such as the tree *Dobera glabra* in Ethiopia (Tsegaye *et al.* 2007). However, not all underutilised plants may survive climate changes, such as from rising sea level or reduced temperature in mountain regions, where the latter houses various medicinal herbs

and aromatic plants (Heywood 2011; Tanton & Haq 2008). Various studies have been published regarding the effect of climate change on several plant species, in which some face extinction (Lira *et al.* 2009; Lane & Jarvis 2007). However, it should be noted that these findings may not be entirely conclusive due to the inclusion of a wide range of plants and animal species and is not specifically to underutilized species (Padulosi *et al.* 2011). Nevertheless, the reported possibility of extinction for certain species can serve as an indication on research priorities for species of underutilized crops. Therefore, underutilized crops can be a subject of study in determining which species are most suited for environmental resilience requirements for future volatile environments, such as in Africa (Padulosi *et al.* 2011).

1.4.2 Resistant starch from underutilised legumes

Like other underutilised crops, legumes also possess several advantages which support their use as a suitable crop in agriculture. Legumes are recognised as the 2nd most valuable plant source for human and animal nutrition and have the 3rd largest family among flowering plants (Bhat & Karim 2009). Several types of legumes have been a subject of study for dietary fibre values and prebiotic contents, some of which are grown and consumed on a local scale only (reviewed in Bhat & Karim 2009). Taking into consideration the size of the legume family, other legumes can be a subject of further studies for potential prebiotic content. In addition, the cultivation of legumes can also assist in improving soil conditions – a problem faced by various farmers especially not only in Africa but also soil in developing nations (Graham & Vance 2003; Dakora & Keya 1997).

Legumes contain a high amount of carbohydrate which is mainly abundant in the seed, which is between 22-45% of total weight (Hoover & Sosulski 1985). However, native legume starch, which is the key component of the total leguminous carbohydrate, is less digestible than native cereal starch (Yadav *et al.* 2010). This reduced bioavailability is associated with several characteristics of leguminous starch, such as high levels of amylose, high content of viscous soluble dietary fibre components, as well as strong interactions between amylose chains (Hoover & Sosulski 1985). This highlights the use of legumes for prebiotic preparation due to high native dietary fibre content. For example, lima beans was found to have high total dietary fibre content and amylose levels, which were found to be at 22.0% and 35.2% respectively (Betancur-Ancona *et al.* 2003). As RS is a component of dietary content, legumes can be associated with high RS levels. Evidently, RS contents analysed in raw legumes, such as lentils and pulses were found to be higher than in cereals and potatoes due to their variations in their starch crystalline structures (Fuentes-Zaragoza *et al.* 2010; Yadav *et al.* 2010; Hoover & Zhou 2003; Tharanathan & Mahadevamma 2003).

Additional benefits of legumes include the unique due to the ability to develop root nodules and fix nitrogen following symbiosis with compatible rhizobacteria. Generally, legumes release flavonoids from their roots to trigger nodulation genes in compatible rhizobacteria, leading to the formation of lipochitooligosaccharide molecules which then trigger the development of root nodules (Long 1996). The presence of nitrogen-fixing bacteria in the roots of legumes was found to have contributed significantly, fixing up to 60 million metric tons of nitrogen from agriculturally important plants, while legumes in natural ecosystems fix up to 5 million metric tons (Smil 1999). As nitrogen nutrition is one of the key determinants

of crop productivity in Africa, management of biological nitrogen fixation can greatly benefit as a cheap and effective tool in maintaining sustainable yields in African agriculture (Dakora & Keya 1997).

1.5 Probiotics and prebiotics in aquaculture

The benefits of certain strains of bacteria in the GIT of fish indicate an opportunity for the improvement of health of the fish via manipulation of the microbiota composition. As success was observed in terrestrial animals, fish have also become targets for prebiotic and probiotic treatments. Due to the importance of maintaining the health of the fish GIT, prebiotics and probiotics have been studied as possible alternatives, especially following the European Union moratorium on the banning of antibiotic growth promoters in animal feeds, including fish (Dimitroglou *et al.* 2011).

1.5.1 Gastrointestinal tract microflora of fish

The occurrence of a dynamic and complex microbial ecosystem in the GIT of organisms are an important factor for the health of the host, which plays a role in the pathological, physiological as well as nutritional point of view (Nayak 2010). In mammalians, the GIT is widely composed of both aerobic and anaerobic microorganisms which interact in an ecosystem (Rajilić-Stojanović 2013). However, in terms of aquatic organisms, such as fish, the microbiota composition in the GIT is believed to be much simpler; initially, studies were conducted on fish GIT microbiota with emphasis in terms of food spoilage, nutritional aspects, as well as changes in farms and antibiotic resistance (Spanggaard *et al.* 2001). However, as studies highlight several significance of the GIT in mammalians, such as in terms of health and nutrition and the prevention of colonisation of infectious agents, emphasis has

been placed in studying the microbiota of fish to further relate the GIT microbes to their possible functional roles in fish (Nayak 2010).

1.5.2 Importance of healthy gastrointestinal tract microflora

There are a few benefits associated with the GIT in terms of contribution to the host, which were studied using organisms with a known microbiota. For fish, the absence of microbiota was shown to lead to the arrest of differentiation in the GIT epithelium – this was caused by the lack of intestinal alkaline phosphatase activity and mature patterns of glycan expression, which are contributed by microbiota (Bates *et al.* 2006). Apart from playing a role in the formation of the intestinal structure, microbiota in the GIT also contribute in health management of the host in terms of nutrition and disease outbreak (Nayak 2010).

The role of bacteria in the GIT towards the well-being of their hosts has been evidenced in many terrestrial animals. The microbiota in the GIT of fish contributes towards the nutrition of fish, which include the synthesis of vitamins, amino acids, digestive enzymes and metabolites. Several metabolites such as vitamin B_{12} and SCFA have been found in the GIT of various fish as a result of bacterial fermentation (Kihara & Sakata 2002; Sugita *et al.* 1991). In addition, the production of enzymes for the digestion of compounds not absorbed by the intestine could also contribute towards additional nutrition for the host. For example, while most fish are unable to utilise carbohydrate efficiently, they harbour microbial populations which aid in digestion of the plant materials through the production of enzymes (Saha *et al.* 2006). One of these compounds is cellulose, which requires the enzyme cellulase to cleave β-1, 4-glycosidic bonds in the polymer to release glucose units (Barr *et al.* 1996). GIT bacteria in fish have been shown to be capable of degrading plant-based

substances, such as chitin and cellulose (Saha *et al.* 2006). This increases nutrient availability to fish, such as carnivorous fish which are not capable of producing such enzymes (Smith 1989). Similarly, a large amount of starch degrading enzymes were also found to be contributed by the intestinal microflora of omnivorous fish (Sugita *et al.* 1997). This highlights the contribution of GIT microbiota which confers nutritional benefits towards overall health improvement. However, the exact nutritional contribution from the GIT can be difficult to conclude due to variation in the ecology of the microbiota in the GIT.

The GIT of fish also plays a role in the occurrence of disease in the host. Generally, the health of the host is maintained by a balance between the endogenous microbiota of the host and the host's control mechanism. However, transient pathogens can cause diseases if conditions become favourable for their multiplication (Gomez *et al.* 2007). Entry of pathogens via the GIT has been shown in several studies. For example, the oral ingestion of *Streptococcus iniae* at 1×10^3 colony-forming-units (CFU) lead to acute forms of the disease in Asian sea bass (*Lates calcarifer*) (Bromage & Owens 2002). Apart from that, GFP-labelled *Vibrio anguillarum* was found to localise in the GIT of zebrafish, indicating that the GIT represents a site of infection for the pathogen (Toole *et al.* 2004). In addition to the penetration of the GIT, studies have also shown that pathogenic bacteria can damage the intestinal lining by releasing toxins and extracellular enzymes, leading to the establishment of lethal disease (Ruwandeepika *et al.* 2012). However, under normal circumstances, autochthonous bacteria in the GIT confer protection towards the host by acting as a protective barrier. This can either be in terms of nutrient depletion or the secretion of antimicrobial compounds, which can be observed through studies *in vitro* (Vine *et al.* 2004; Sugita *et al.* 1996). *In vitro* studies showed

that the the co-culture of LAB was found to be capable of inhibiting the growth of *Aeromonas hydrophillia* under culture conditions of pH 4.5 (Rengpipat *et al.* 2008). In addition, increased populations of LAB in Asian sea bass also produced greater survival rates of fish in waters infected with *A. hydrophila*, as well as higher survival rates as compared to fish which are not fed with the fortified diet (Rengpipat *et al.* 2008). Apart from that, the presence of *Lactobacillus rhamnosus* in the GIT when fed as a probiotic was capable of improving the immunity of fish against pathogenic bacteria strains (Panigrahi *et al.* 2011). In addition, up to 40% of the GIT microbiota of sole (*Solea senegalensis*) larvae was antagonistic towards pathogens such as *V. anguillarum* after 6 weeks of feeding (Makridis *et al.* 2005). It was suggested that the inhibition is caused by the production of lactic acid and bacteriocin compounds by the LAB (De Vuyst & Vandamme 1994). The production of bacteriocin is also observed in other bacteria, such as *Carnobacterium piscicola* which is a common member of the endogenous microbiota in the GIT in many fish (Denev *et al.* 2009; Stoffels *et al.* 1992). Therefore, microbiota in the GIT are key players and are related to disease, especially against pathogens which uses the GIT as a site of infection.

1.5.3 The application of probiotics in aquaculture

Essentially, probiotics refer to the stimulation of health via supplementation of health-promoting bacteria. However, due to variation in the GIT microbiota of fish, commonly used probiotics in terrestrial animals were not applicable in fish. Therefore, various studies were conducted in order determine suitable probiotics, which has been reviewed extensively (Mohapatra *et al.* 2013; Merrifield *et al.* 2010; Irianto & Austin 2002a). Briefly, key groups of probiotic bacteria are found to comprise of LAB, mainly from the *Lactobacillus* and *Carnobacterium* strains. Another common strain found to be probiotic in some studies are *Aeromonas*, although most

strains are pathogenic (Irianto & Austin 2002b; Gibson *et al.* 1998). These bacteria are usually administered as probiotics directly via feed, though environmental supplementation of prebiotics was also performed (Balcázar *et al.* 2006). This highlights a key difference between probiotics of terrestrial and aquatic animals, as the microbiota of fish GIT often contain high levels of transient bacteria as a result of constant ingestion of the surrounding water (Ringø & Birkbeck 1999). Several general benefits have been associated with the supplementation of probiotics, particularly from LAB due to survival in stomach acid and bile salts (Marteau *et al.* 1997). These benefits include competitive exclusion of pathogenic bacteria, source of nutrients and enzymatic contribution to digestion, direct uptake of dissolved organic material mediated by the bacteria, and also enhancement of immune response towards pathogenic bacteria (Ringø 2008; Balcázar *et al.* 2007b; Vine *et al.* 2004; Gomez-gil *et al.* 2000; Moriarty 1997). Therefore, LAB are said to be an important class of probiotics, which can be categorised into allochthonous and autochthonous strains.

Autochthonous strains of probiotics involve the colonisation in the GIT of fish. These often include LAB as candidates, as they are generally harmless while some strains beneficial for the health of fish. For example, several studies show that many *Carnobacterium* strains were found to be effective antagonistic towards pathogens when tested *in vivo*. Mortality of salmon and rainbow trout fed with *Carnobacterium inhibens* reduced when challenged with pathogens *A. salmonicida*, *V. ordalii* and *Yersinia ruckeri* (Robertson *et al.* 2000). However, the colonisation of *C. inhibens* was found to be ineffective in reducing mortality caused by *V. anguillarum* (Robertson *et al.* 2000). Other strains, such as *C. divergens* were found to be effective against *V. anguillarum* when administered on Atlantic cod. In the study of Gildberg & Mikkelsen (1998), it was found that reduced cumulative mortality was recorded in the fish (80-

84%) and that the bacteria could colonize the internal mucus layer of the cod fry pyloric caeca. Apart from *Carnobacterium*, other LAB such as *Lactococcus* sp. also shows positive results. For example, the application of dietary probiotic, *Lactococcus lactis* subsp. *lactis* I2 on olive flounder (*Paralichthys olivaceus*) led to 100% survival when challenged with *S. iniae* (Heo *et al.* 2013). This was in contrast to the control group, which experienced 90% mortality rates. Despite being able to colonise the GIT of fish, generally, probiotic bacteria are usually not detected after 3-4 weeks of reverting to non-probiotic feed (Balcázar *et al.* 2007a). This was believed to be associated to the dominance indigenous microbiota, which usually constitute a larger component of the resident microbiota (González *et al.* 1999). Therefore, continuous supply of probiotics might be necessary and can be a target of future research.

Allochthonous strains of probiotics refer to the probiotics which are transient in nature and are usually not found in the GIT after 1-2 weeks of application (Gatesoupe 2008). However, allochthonous probiotics were shown to have beneficial effects not only in fish, but also terrestrial animals and humans (Gatesoupe 2008). While the benefits revolved around general increase in health, it was observed that the increase in immunity levels are one of the key benefits following probiotic application of allochthonous bacteria. For example, stimulation of immune system has been observed in many studies, such as through the application of *Carnobacterium* sp. in rainbow trout, where live bacteria were found to be more efficient than heat inactivated ones (Panigrahi *et al.* 2005). In another study, *L. rhamnosus* was used in rainbow trout to significantly reduce mortality from furunculosis (Nikoskelainen *et al.* 2003). This treatment was also verified to be transient, as immunity levels returned to normal after two weeks of treatment (Nikoskelainen *et al.* 2003; Panigrahi *et al.* 2005). Similar trends were observed when

heat-killed cells of bacteria were used for immunostimulation despite low adhesion on intestinal epithelium (Salinas *et al.* 2005; Villamil *et al.* 2003). Apart from immunostimulation, general health improvement was also noted following the introduction of probiotics; the use of *Lactobacillus acidophilus* and other probiotics were found to improve improved water quality, lysozyme activity, and resistance against heat shock and *V. anguillarum* infection (Taoka *et al.* 2006). However, specific contributions of the probiotics were not taken into consideration to allow concluding that these benefits originated directly from the LAB. Nevertheless, these studies highlight the benefits of allochthonous probiotics which mainly revolves around immune stimulation.

1.5.4 The application of prebiotics in aquaculture

Apart from probiotics, prebiotics are also another form of microbiota manipulation. Prebiotics have several advantages over the use of probiotics, where low viability of bacteria after feed production is observed (Merrifield *et al.* 2010). Other problems involving probiotics include the leaching of particles in rearing water as well as low viability during storage (Merrifield *et al.* 2010; Wang *et al.* 2008). Similar to prebiotics in terrestrial animals, prebiotics for fish must also qualify for the key requirement – to stimulate selectively the growth and/or activity of intestinal bacteria associated with health and well-being. However, compared to probiotics, studies on prebiotics on fish are limited and insufficient to conclude specific effects of their application (Ringø *et al.* 2010b). Therefore, greater research effort is required to expand on studies on the effect of prebiotics on the benefits in fish. So far, some of the benefits observed in fish revolve around host growth parameters, immunity levels as well as effect on the microbiota of the GIT from commonly applied prebiotics in terrestrial animals, such as inulin and oligosaccharides (Yousefian & Amiri 2009).

1.5.4.1 Inulin

Inulin is a commonly used as a prebiotic in terrestrial animals with various benefits. Hence, despite not being as part of the natural diet of fish, inulin was also selected as one of the candidates of prebiotics for fish (Ringø *et al.* 2006). Generally, inulin is capable of stimulating the growth of bacteria, such as LAB, which has also been suggested as potential probiotics of aquaculture. In addition, inulin has been found to be fermented by LAB extracted from the GIT of fish, such as the genus *Carnobacterium* sp. (Ringø 2004). Therefore, inulin has been used in the study of many types of fish.

When Artic charr were fed with inulin, variation in microbiota was observed. While the inclusion of inulin in diet led to an increase of probiotic strains such as *Bacillus* spp. and *C. maltaromaticum*, a reduction in autochtonous bacteria was observed (Ringø *et al.* 2006). This was believed to be associated with the selectivity of specific strains and effect on enterocytes due to high inulin content of 15%. In another study, when Artic charr (*Salvelinus alpinus* L.) were fed with diets containin 15% inulin, intestinal cell damage was observed (Olsen *et al.* 2001). The destructive effect of inulin was mainly detected on the microvillus organisation, which were found lacking in some areas and less straight compared to control animals. This shows deleterious effects of inulin inclusion in the diet. A study conducted on red drum (*Sciaenops ocellatus*) also did not reveal any health benefits, in terms of weight gain and food efficiency ratios when inulin was supplemented (Burr & Gatlin III 2009). On the other hand, Cerezuela *et al.* (2008) also concluded that inulin is unsuitable as a immunostimulant for sea bream (*Sparus aurata* L.). However, in some studies, merit has been obtained from inulin as a prebiotic. The supplementation of 0.5% inulin in nile tilapia (*Oreochromis niloticus*) shows slight improvement in growth rates and

resistance to *A. hydrophila* (Ibrahem *et al.* 2010). Apart from that, inulin also improved weight gain, intestinal absorption of calcium and reduced intestinal populations in farmed rainbow trout (Ortiz *et al.* 2013). As the effect of inulin varies in species, the effect of inulin cannot be concluded without greater coverage of other species which can be candidates for prebiotic-inclusion diets.

1.5.4.2 Oligosaccharides

Oligosaccharides are another form of prebiotic which are widely used in terrestrial animals, and even in various commercial products for human consumption. As oligosaccharides are substrates fermentable by microbiota in the GIT, they are also applied in aquaculture as prebiotics (Ringø *et al.* 2010b). In aquaculture, various studies have been conducted regarding oligosaccharides as a prebiotics, where established types include MOS and FOS, which have been applied in a wide variety of fish (Ringø *et al.* 2010b).

MOS are glucomannoprotein-complexes derived from the cell wall of yeast (*Saccharomyces cerevisiae*) (Sohn *et al.* 2000) and have been used in terrestrial animals such as sheep (Klebaniuk *et al.* 2008). Generally, the effect of MOS was believed to improve immunity levels through the production of cytokines, which could have a role in innate immune function (Gazi & Martinez-Pomares 2009). Hence, the effect of MOS was evaluated in a wide range of fish. When Atlantic salmon was fed with MOS, benefits such as 11% lower oxygen consumption, 5% lower protein and 3% higher energy concentration in the whole body and 7% greater energy retention was observed (Grisdale-Helland *et al.* 2008). However, immune response towards pathogen was not recorded. In other studies, such as by Torrecillas *et al.* (2007), MOS improved the growth, feed utilization and immune status of

European sea bass (*Dicentrarchus labrax*), which could be associated with enhanced amino acids absorption (Iji *et al.* 2001). More positive results were observed in rainbow trout, such as enhanced growth rates and intestinal folding (Staykov *et al.* 2007; Genc *et al.* 2007). However, many of these studies did not address immunity and disease susceptibility, except for the case where MOS inclusion diets reduced mortality of rainbow trout when challenged with *V. anguillarum* (Rodrigues-Estrada *et al.* 2008). However, the inclusion of MOS may also have no effect, as observed when fed to sturgeons (*Acipenser oxyrinchus)* (Pryor *et al.* 2003), which could be associated to differences in microbiota, which was not analysed in this study.

FOS are another class of oligosaccharides which have been studied as a prebiotic in aquaculture. Generally, FOS are short and medium chains of β-d-fructans in which fructosyl units are bound by β-(2-1) glycosidic linkages and attached to a terminal glucose unit and includes all non-digestible oligosaccharides composed of fructose and glucose units (Swanson *et al.* 2002). As FOS is fermented by LAB, it might contribute to health benefits in some fish and therefore has been a subject of study (Ringø *et al.* 2010b). In Atlantic salmons, positive effect was observed following the supplementation of FOS – approximately 5% higher feed efficiency and 6% energy retention was observed when compared to control diets. However, there was no improvement in the growth and nutrient digestibility. In the study of Ye *et al.* (2011), the supplementation of FOS alone in Japanese flounder (*Paralichthys olivaceus*) did not produce any benefits. However, the supplementation of FOS with *Bacillus clausii* as a synbiotic led to higher weight gain rates in the fish. This could be associated to the lack of microbiota capable of utilizing FOS, which was not analysed in the study. In another study involving hybrid tilapia, FOS did not improve growth rates (He *et al.*

2003). However, FOS-inclusion in diets led to increased innate defence mechanisms measured by lysozyme and alternative complement activity (ACH50).

1.5.4.3 Resistant starch

At present, RS as a prebiotic have not been tested in aquaculture. One of the benefits of the use of RS as a prebiotic is the the production of SCFA following the fermentation. This is more common in herbivorous fish; however, it is also observed in carnivorous red seabream (Kihara & Sakata 1997) and European sea bass (Gatesoupe *et al.* 2014). While no beneficial effect was found apart from strengthening the GIT of red seabream, the increased production of SCFA, such as acetate, following bacteria modulation shown in the European sea bass may also benefit Asian sea bass (Gatesoupe *et al.* 2014). In addition, while carnivorous fish are incapable of digesting complex carbohydrates, several benefits such as higher weight gain, improved nutrient digestibility and survival was observed when supplemented with amylolytic probiotic feed which results in a more favourable microbiota in the fish GIT (De *et al.* 2015).

1.6 Aquaculture of Asian sea bass (*Lates calcarifer*)

Asian sea bass or better known as barramundi in Australia and Siakap in Malay was suggested as a perfect candidate as food security for aquaculture when first discovered in the 1980s (Greenberg 2010). With emphasis on the Asian region and climate, a few types of fish are commonly used in aquaculture, such as the tilapia. However, despite being considered sustainable, the vegetarian diets of tilapia cause them to have reduced amounts of omega-3 oils, which are the highlight of nutrition in fish (Powell 2003). In contrast, the long chain omega-3 content of farmed Australian barramundi was found to equal farmed salmon, at 2,960 and 2,985 mg per

150 g wet weight respectively (Nichols *et al.* 2010). This could be associated with the diets of these fish, which are carnivorous in nature and fed with fish products in feed (Nichols *et al.* 2010). However, while salmon contains high amount of omega-3 fatty acids, their carnivorous nature might require up to 5 kg of wild fish to produce 1 kg of salmon (Covington 2004; Powell 2003). Asian sea bass are said to require less – approximately 20% is sufficient (Greenberg 2011), while other studies contradict this, stating that higher protein content is more beneficial for growth (Glencross 2006). Nevertheless, the use of Asian sea bass for aquaculture is also supported by high survivability in wide variety of conditions (Bermudes *et al.* 2010; Katersky & Carter 2007; Katersky & Carter 2005).

Asian sea bass aquaculture initiated approximately in the 1960s, which experienced slow growth until the 1980s where aquaculture production experienced increased growth (FAO 2017b). Currently, aquaculture of Asian sea bass contributes to approximately 70,000 metric tonnes (FAO 2015). Although far from the contribution of salmons and carps farmed, Asian sea bass can still be considered an important aquaculture in several regions, such as Australia, Thailand, Indonesia and Malaysia. For Australian aquaculture, silver perch, Asian sea bass, eels, aquarium fish and other native fish make up to 14.22% of the total finfish aquaculture in 2007-2008 (Australian Bureau of Statistics 2012). In the consecutive years of 2008-2009 and 2009-2010, the total amount represented for the category above increased to 15.80% and 17.64% respectively; this indicates the increasing contribution of other finfish, which includes Asian sea bass, towards the total aquaculture production of finfish (Australian Bureau of Statistics 2012). On the other hand, in Malaysia, the aquaculture of Asian sea bass is also significant for brackish water based aquaculture. In 2015, Asian sea bass is the highest cultured fish in brackish water conditions,

comprising of 22.66% of the total weight of total cultured species (DOFM 2016). Other species which are highly cultured are the white shrimps and cockles which represent 37.56% and 13.12% of total brackish water aquaculture in terms of weight respectively (DOFM 2016). Effectively, wholesale value of Asian sea bass in Malaysia also contributes up to 18.71% of the total revenue gained from brackish water aquaculture (DOFM 2016). This highlights Asian sea bass as one of the key species for aquaculture in Malaysia.

1.6.1 Functional food in Asian sea bass aquaculture

Disease prevalence in Asian sea bass resulted in studies on the improvement of survival. Prebiotic studies have not been conducted in Asian sea bass, although similar studies have been conducted with a certain level of efficacy. These methods include probiotic-supplemented diets as well as additional ingredient in diets.

Several studies been conducted revolving around the efficiency of probiotic-supplemented diets. For example, Asian sea bass was provided feed supplemented with amylolytic bacteria isolated from the GIT. Supplementation of probiotic in the diet throughout a 30 day feeding trial resulted in a higher weight gain (141.42%), protein efficiency ratio (1.18), survival rate (91.5%) and a lower food conversion ratio (2.15). In addition, nutrient digestibility was also improved (De *et al.* 2013). In other studies, when LAB isolated from the GIT of mature Asian sea bass was supplemented as a probiotic, significantly higher survivability (80%) was conferred when exposed to the common aquatic pathogen *Aeromonas* sp. (Rengpipat *et al.* 2008). This highlights the efficiency of probiotics when applied in Asian sea bass.

A few studies investigated the application of common prebiotics, namely inulin, MOS and FOS on Asian sea bass. These studies investigated the effect of the prebiotics supplemented at different levels in the diets of fingerlings, weighing between 7 g and 12 g. For inulin, diets supplemented above 1% improved specific growth rates and feed conversion ratios while having no effect on weight gain. These were believed to be caused by changes in the GIT microbial community of the fish based on polymerase chain reaction and denaturing gradient gel electrophoresis (PCR-DGGE) (Ali *et al.* 2016). On the other hand, the inclusion of MOS at 1% increased weight gain, specific growth rate and increased blood glucose, triglyceride and cholesterol content while having no effect on whole body composition (Ali *et al.* 2017b). The authors suggested that these benefits was due to the improved absorptive surface area of the intestine from the 1% MOS supplemented. Meanwhile, the inclusion of 1% FOS in diets improved fish survival up to 97.7% and immune parameters, such as lysozyme and superoxide dismutase levels at 77.4 U ml^{-1} and 75.2 U ml^{-1} respectively (Ali *et al.* 2017a). The improvements observed following the supplementation of these prebiotics were believed to be able to contribute to Asian sea bass in aquaculture.

The concept of other added ingredients into diets was another approach which was applied in the improvement of feed for Asian sea bass. The inclusion of *Rhodovulum sp*. biomass as nutritional ingredient in feed was also studied throughout a 12-week period. It was found that at 0.3% biomass concentration, highest weight gain (419 g), feed conversion ratio (1.95) and survival rate (86.7%) was obtained (Shapawi *et al.* 2012). This was comparable to previous study involving the supplementation of probiotics (De *et al.* 2013). In other studies, the addition of neem leaf (*Azadirachta indica*) as a supplement in diets also achieved high survival rates (80%) when

exposed to the common pathogen *V. harveyi* (Talpur & Ikhwanuddin 2013). This could be related to the antibacterial and antifungal content of the leaves which has been a subject of previous studies (Girish & Shankara Bhat 2008). Hence, these studies indicate the responsiveness of Asian sea bass towards additional ingredients in their diets.

1.7 Experimental objectives

Currently, the beneficial effects of prebiotics have been documented in both animal models and terrestrial animals. Significant work has also been carried out to investigate the effect of established prebiotics (e.g. inulin, FOS, MOS) in aquatic animals. However, the effects of these prebiotics vary when fed to different species of fish. In addition, little work has been done on resistant starch (RS) as a prebiotic in particular. Hence, this study seeks to explore effective alternatives by using a natural prebiotic source of RS from underutilised legumes to improve the growth and health of Asian sea bass, which is an important commodity in Malaysia.

Therefore, the objectives of this study were;

1. To assess the potential of six underutilised legumes as a source for RS
2. To compare the prebiotic effect of RS extracted from underutilised legumes on the growth of probiotic bacterium isolated from fish intestinal tract in an *in vitro* setting
3. To compare the effects of selected RS on the growth and health of zebrafish (*Danio rerio*) and Asian sea bass (*Lates calcarifer*) in *in vivo* setting

The project was separated into three studies with the first study comparing the amount of RS present in six underutilised legume species and the effect of acid- and enzyme-hydrolysis as pre-treatments in improving the RS content from the legume starches. The second study compared the effects of the RS produced on the *in vitro* growth and acid production of probiotic bacteria isolated from the gut of zebrafish and Asian sea bass. The third study compared the effect of supplementation of selected RS on the growth and intestinal microflora content of zebrafish and Asian sea bass.

2 Chapter 2: Determination and quantification of resistant starch present in underutilised legumes

2.1 Introduction

Resistant starch (RS) refers to the fraction of starch which remains undigested until arriving at the colon, which is fermented by microbial populations and subsequently resulting in health benefits to the host (Zaman & Sarbini 2015). Currently, several studies have developed or modified methods used to analyse RS contents in diets, which is based on the quantity of indigestible starch remaining after digestion with amylases (McCleary *et al.* 2002; Englyst *et al.* 1996; Goñi *et al.* 1996). However, as RS extraction processes and detection methods differ, the quantity of RS measured varied; this creates an issue of defining RS content in food while highlighting the issue of having no standard method to date (Perera *et al.* 2010). Thus, another challenge was to develop a reliable method to quantify the RS presents in legumes.

The RS content in starch increased when subject to heating and cooling in the presence of excess water, via the processes of gelatinisation and retrogradation (Morales-Medina *et al.* 2014; Polesi & Sarmento 2011). This increment is due to the formation of B-type crystalline structure and annealing of starch during retrogradation (Fuentes-Zaragoza *et al.* 2010). When consumed, this B-type crystalline structure resists intestinal enzyme digestion which allows RS to arrive at the colon as substrates for the growth of beneficial microorganisms (probiotics) and subsequently benefiting host health (Zaman & Sarbini 2015).

Hence, the current study investigates the RS availability in a range of underutilised legumes for use as sustainable prebiotic ingredients in aquafeeds.

2.1.1 Hypothesis

Underutilised legumes are high in RS content and the production of RS could be improved following optimised hydrolysis, gelatinisation and retrogradation processes. Based on published literature, the Megazyme Resistant Starch assay method should be a precise quantification method for RS (Perera *et al.* 2010).

2.1.2 Aims

The current study determines the RS availability in six underutilised legumes, namely adzuki beans (*Vigna angularis*), mung beans (*Vigna radiata*), black-eyed peas (*Vigna unguiculata* subsp. *Unguiculata*), pigeon peas (*Cajanus cajan*), Bambara groundnuts (*Vigna subterranea*) and red lentils (*Lens culinaris*).

Specific aims:

- To determine the amount of starch present in these underutilised legumes
- To screen for the most reliable RS quantification and production methods for legumes
- To determine the amount of RS present in underutilised legumes

2.2 Materials and methods

2.2.1 Experimental overview

Six underutilised legumes were investigated in this study. The legume starches were isolated via alkaline steeping as reported by Wani *et al.* (2010) with slight modification. Then, two RS quantification methods were compared which were the modified method of McCleary *et al.* (2002) (McCleary method) and the Megazyme Resistant Starch Assay Kit (Megazyme, Ireland). Lastly, the legume RS was produced via hydrolysis, gelatinisation and retrogradation steps based on the study of Polesi & Sarmento (2011). Two methods of hydrolysis, acid and enzyme, (followed by identical gelatinisation and retrogradation steps) were compared.

2.2.2 Materials

2.2.2.1 Legumes

The legumes selected in this study were based on the list of underutilised crops published by INFOODS (2012). The choices of legumes were: adzuki beans (*Vigna angularis*), mung beans (*Vigna radiata*), black-eyed peas (*Vigna unguiculata* subsp. *Unguiculata*), pigeon peas (*Cajanus cajan*), Bambara groundnuts (*Vigna subterranea*) and red lentils (*Lens culinaris*), which were purchased from a supermarket (The Store, Semenyih, Malaysia). Meanwhile, Bambara groundnuts were provided by Crops for the Future (CFF) research centre, Malaysia.

2.2.2.2 Starch samples

Starch samples used in this study were isolated from the legumes as based on the method described in Section 2.2.3.1 and are labelled as isolated starch.

2.2.3 Methods

2.2.3.1 Isolation of starch

The method applied for the isolation of starch from legumes were adapted from the method of Wani *et al.* (2010). In this method, the milled legume flour was used as the starting material instead of soaked legumes due to higher yields obtained and the steps for removal of pre-soaking and seed coat removal were omitted. In addition, sedimentation was also included to replace the limitations of the centrifugation step.

Thus, in this modified method, the legumes were milled into fine powder by using a miller operated on cycles of 15 s for approximately 5 min. The milled flour was sieved through a 1 mm sieve to ensure homogeneity in particle size. Flour that passes through the sieve was collected, while particles which were retained by the sieve were milled again for 3 min. Finally, the total flour was collected and stored at room temperature prior to use and is labelled as legume flour.

A legume flour suspension was produced by mixing 100 g of legume flour in 1 L of distilled water. The pH of the suspension was adjusted to 10.0 using a 30% (w/v) sodium hydroxide solution (R&M Chemicals) and was stirred using a magnetic stirrer at room temperature for 1 hr. Then, the suspension was filtered through a 125 µm mesh sieve to separate the fibre. The filtrate was then stored overnight in a 4 °C chiller for the sedimentation of the starch. After sedimentation, the sediment was centrifuged at 3000 *g* at 10 °C for 30 min. After centrifugation, the supernatant was discarded. The washing step proceeded with the sediment mixed with 30 mL of 50% (v/v) ethanol solution, followed by centrifugation at 3000 *g* at 10 °C for 30 min. The supernatant was discarded and this washing step was repeated once. After that, the

sediment was dried at 45 °C in a convection oven. The upper portion consisting of insoluble fibre was carefully discarded and the lower white portion was retrieved as starch.

2.2.3.2 Quantification of RS content

RS is measured as the portion of starch surviving digestion to be partially or wholly fermented in the large intestine (Fuentes-Zaragoza *et al.* 2010). Quantification of RS was performed by simulating conditions in the human digestive tract and has been modified in a range of studies to replicate results obtained by healthy ileostomy subjects (Perera *et al.* 2010). While modifications in the method affects the final RS content quantified, fewer studies investigate sources of the enzymes used, which is explored in this study.

In this study, two methods were compared, which is: a) "Modified method of McCleary *et al.* (2002)" and b) "Method of Megazyme RS Kit", where the key difference is the use of fungal amylase instead of porcine pancreatic amylase in the methods of McCleary *et al.* (2002) in the first stage of the digestion of starch using the α-amylase solution, as described in detail in Section 2.2.3.2.a.

a. Modified method of McCleary *et al.* (2002)

The method applied for the quantification of resistant starch from legumes was adapted from the method of McCleary *et al.* (2002) and will be labelled as the McCleary method. In this study, the pancreatic α-amylase was replaced with α-amylase from *Aspergillus oryzae* (Sigma 10065) while the amyloglucosidase (AMG) solution was replaced with AMG from *Aspergillus niger* (Sigma 10113). Lastly, the glucose content was measured using the Glucose Assay Kit (GO; Sigma GAGO20).

Instructions for the preparation of reagents were provided in the study of McCleary *et al.* (2002). In this study, the reagents were prepared with similar methods to reduce the possibility of variation and are as listed in Appendix A.

The resistant starch assay can be separated into two components: i) conversion of resistant starch into glucose and ii) measurement of glucose content.

i. Conversion of RS into glucose

A total of 100 mg of sample was mixed with 4 mL α-amylase solution via vortex and was incubated in a shaking incubator set at 37 °C with 200 strokes/min for 16 hr. After that, an initial washing step was performed, where 4 mL of 95% (v/v) ethanol was added and mixed via vortex. The mixture was centrifuged at 1500 *g* for 15 min and supernatant was discarded carefully. Then, a second washing step was performed, where 8 mL of 50% (v/v) ethanol was added and mixed vigorously via vortex, followed by centrifugation at 1500 *g* for 15 min. The supernatant was discarded carefully. The second washing step was repeated once and the sediment was dried by placing the tubes in an inverted position on absorbent paper for 1 hr.

After drying, the sediment was re-suspended with the addition of 2 mL of 4 M potassium hydroxide solution and the mixture was mixed by tapping the tubes gently. The samples were then shaken in ice at 200 rpm for 20 min. Then, 8 mL of 1.2 M sodium acetate solution (1.2 M, pH 3.8) was added into the samples and mixed by swirling, followed by the addition of 100 µL of concentrated AMG solution. The samples were mixed vigorously and incubated at in a water bath set at 50 °C for 30 min with intermittent mixing via vortex within approximately 5 min intervals. The

mixture was then subjected to centrifugation at 1500 *g* for 15 min and the supernatant was collected for the measurement of glucose content via Glucose (GO) Assay Kit (Sigma GAGO20).

ii. Measurement of glucose content via GO

Glucose measurement was performed using a Glucose (GO) Assay Kit (Sigma GAGO20). Using this kit, glucose was oxidised to gluconic acid and hydrogen peroxide by glucose oxidase. Hydrogen peroxide reacted with o-dianisidine in the presence of peroxidase and formed a coloured product, which reacted with sulfuric acid to form a more stable coloured product. The intensity of the pink colour was then measured at 540 nm is proportional to the original glucose concentration

An aliquot of 100 µL of samples were pipetted into sterile 1.5 mL microcentrifuge tubes and 200 µL of Assay Reagent was added to each tube after 30 s and mixed well via inversion of the tubes. Samples were then incubated for 30 min at 37 °C. At the end of the incubation, the reaction was immediately terminated by the addition of 200 µL of 12 N sulfuric acid and was mixed well via inversion of the tubes. An aliquot of 200 µL was transferred into 96-well microtiter plates for measurement of glucose content against the reagent blank at a wavelength of 540 nm. For sample that exceed absorbance reading of 2.0, a 10-fold dilution will be carried out and the measurement of glucose content was repeated accordingly.

A standard curve was developed by using 0-80 µg/mL of glucose as shown in Appendix B. The following equation generated from the glucose standard curve was used in the determination of glucose concentration (µg/mL):

Glucose concentration (µg/mL) = (Absorbance at OD-540nm)/(0.00266)

The glucose concentration measured was then multiplied by a factor of 0.9 to determine its resistant starch content (Cho *et al.* 1999). For samples which exceed 10% RS content, a 10-fold dilution is performed and the measurement of glucose content was repeated and calculated accordingly.

b. Method of Megazyme RS Kit

The Megazyme Resistant Starch Assay Kit was developed by Megazyme (K-RSTAR, Ireland) based on the McCleary *et al.* (2002). This assay kit is commonly used as a colorimetric method for determination of resistant starch in cereal products and feeds. This Megazyme RS kit standardizes the RS measurements across studies and is accepted as the official method for RS measurement in starch by the Association of Official Analytical Chemists (AOAC; Method 2002.02).

Instructions for the preparation of reagents were included in the manual provided by the kit and reagents were prepared as listed in Appendix C.

The resistant starch assay can be separated into two components: i) conversion of resistant starch into glucose and ii) measurement of glucose content via GOPOD.

i. Conversion of RS into glucose

The method of a) conversion of resistant starch into glucose is identical to the method described in Section 2.2.3.2 (a, i).

ii. Measurement of glucose content via GOPOD

Glucose measurement was performed based on the protocol provided by the Megazyme RS kit with slight modification to suit the application in 96-well microtiter plates. The glucose oxidase peroxidase (GOPOD) reagents convert glucose into a quinonemine dye. The intensity of the pink colour was then measured at 510 nm is proportional to the original glucose concentration

For sample measurement, a total 50 µL of the supernatant was transferred each into 2.0 mL microcentrifuge tubes containing 1.5 mL of GOPOD reagent and is performed in duplicate. For glucose standard measurements, a total 50 µL aliquot of the glucose standard (1 mg/mL, provided by the kit) was transferred each into 2.0 mL microcentrifuge tubes containing 1.5 mL of GOPOD reagent and is performed in quadruplicate. For blank measurements, a total of 50 µL aliquot of the blank sample was transferred into 2.0 mL microcentrifuge tubes containing 1.5 mL of GOPOD reagent and was performed in triplicates. All tubes were incubated at 50 °C in a water bath for 20 min, followed by 200 µL aliquot from each sample and transferred into a 96-well microtiter plate for measurement at a wavelength of 510 nm.

Calculations for resistant starch content (% w/w, on a dry weight basis) of the test samples were performed based on the instructions provided by the kit, which are as shown below. In this study, the percentage of RS content measured was based on the isolated starch fraction. After measurement, samples which exceed 10% RS content was diluted via a 10-fold dilution is performed and the measurement of glucose content and the calculation of RS content was repeated.

For non-diluted sample:

Resistant Starch content (g/100 g sample; % w/w)

= ΔE x F x (10.3/0.1) x (1/1000) x (100/W) x (162/180)

= ΔE x (F/W) x 9.27

For 10-fold diluted sample:

Resistant Starch content (g/100 g sample; % w/w)

= ΔE x F x (100/0.1) x (1/1000) x (100/W) x (162/180)

= ΔE x (F/W) x 90

Where:

- ΔE = absorbance (reaction) read against the reagent blank;
- F = conversion from absorbance to micrograms (the absorbance obtained for 100 µg of D-glucose in the GOPOD reaction is determined and F = 100 (µg of D-glucose) divided by the GOPOD absorbance for this 100 µg of D-glucose;
- 100/0.1 = volume correction (0.1 mL taken from 100 mL 10-fold diluted samples);
- 1/1000 = conversion from micrograms to milligrams;
- W = dry weight of sample analysed = "as is" weight x [(100-moisture content)/100];
- 100/W = factor to present RS as a percentage of sample weight;
- 162/180 = factor to convert from free D-glucose, as determined, to anhydro-D-glucose as occurs in starch;
- 10.3/0.1 = volume correction (0.1 mL taken from 10.3 mL non-diluted samples) for samples containing 0-10% RS where the incubation solution is not diluted and the final volume is approximately 10.3 mL.

2.2.3.3 Production of RS from legumes

The application of heat to starch in the presence of moisture was observed to have a positive impact on RS levels. This treatments cause gelatinisation of amylose and amylopectin fractions to assume a rubbery state, allowing them to interact to form double helices to increase the overall stability of the granule to disruption, increasing RS (Chung *et al.* 2009). In this study, the effect of two pre-treatments were compared (Section a and b), which aimed to increasing the apparent levels of amylose via debranching amylopectin molecules or via acid treatment. This was

followed with gelatinisation and retrogradation and lyophilisation (Section c and d). The preparation of reagents of the methods used in this study were also as listed in Appendix D.

a. Enzyme-hydrolysis method

The enzyme-hydrolysis procedure applied in this study was adapted from the study of Polesi & Sarmento (2011) with no modification. Pullulanase was used for the hydrolysis of the 1-6 glycosidic bonds produced more free linear chains which can participate in crystal formation by chain elongation and folding (Vasanthan & Bhatty, 1998). This increases crystal formation during retrogradation, increasing RS content.

A 10% (w/v) starch suspension was produced by adding 100 g of isolated starch into 1 L of sodium acetate buffer (0.1 M, pH 5.3). The starch in the suspension was gelatinised via incubation in a water bath set at 100 °C for 15 min with constant stirring with a spatula. The starch suspension was then removed and allowed to cool to approximately 60 °C. Then, an aliquot of 380 µL of pullulanase microbial (Sigma E2412) was added into the suspension to produce a concentration of 40 units per g of starch and was mixed with a spatula. The beaker was covered with aluminium foil and was subjected to incubation for 10 hr in a water bath set at 60 °C. The starch suspension was then subjected to gelatinisation and retrogradation. Isolated starch which were treated using this method are labelled as enzyme-RS.

b. Acid-hydrolysis method

The acid-hydrolysis procedure applied in this study was adapted from the study of Polesi & Sarmento (2011) with slight modification. Reduction of molecular weight via acid treatment generates a larger number of linear chains to facilitate pairing,

consequently increasing RS content (Polesi & Sarmento 2011). In this study, an additional washing step with 80% (v/v) ethanol was performed based on the methods of Vasanthan & Bhatty (1998).

A 20% (w/v) starch suspension was produced by adding 100 g of isolated starch in 500 mL of 2.0 M hydrochloric acid. The beaker was covered with aluminium foil and was incubated in a water bath set at 45 °C for 150 min. The pH was then neutralised to 6.0 using a 30% (w/v) sodium hydroxide solution. Then, the suspension was transferred into 50 mL centrifuge tubes and was subjected to centrifugation of at 3000 *g* for 15 min. The supernatant was discarded and the sediment was washed by adding approximately 25 mL of 80% ethanol solution (v/v) and vortexed, followed by centrifugation of at 3000 *g* for 15 min. The washing step was repeated once and the sediment was then transferred into a beaker. Distilled water was added to produce a 10% (w/v) starch suspension and the starch suspension was then subjected to gelatinisation and retrogradation Isolated starch which were treated using this method are labelled as acid-RS.

c. Gelatinisation and retrogradation

Gelatinisation was performed by subjecting samples to similar autoclave conditions of 121 °C at 15 psi for 15 min to gelatinise the starch. After the autoclave cycle, the samples were stirred with a spatula and the contents were transferred into shallow rectangular plastic containers up to a height of approximately 1 cm. The plastic containers were covered and were transferred into a chiller set at 4 °C for 24 hr for retrogradation. After the incubation retrogradation has been completed, the samples are subject to lyophilisation.

d. Lyophilisation

Prior to lyophilisation, samples were transferred into -80 °C freezer and stored for overnight. Lyophilisation was performed using freeze dryer (Alpha 1-2 LD_{plus}, Martin Christ, Germany). The freeze dryer was set at 0.12 mbar and vacuum at -40 °C. The lyophilised samples were stored at room temperature until used.

2.2.3.4 Proximate analysis of legume samples

Proximate analysis of legume samples and feed samples were conducted by ALS Technichem Sdn. Bhd (Shah Alam, Malaysia). The principles of the methods used for the respective test parameters are as described in Table 2.1, meanwhile the method reference is listed in Appendix E as provided by the company.

Samples were prepared and stored in plastic containers, containing 50 g of sample in each. Samples were delivered and analysed for the following parameters:

Table 2.1: Proximate analysis test parameters and methods used by ALS Technichem Sdn Bhd

	Test Parameter	Method and Principle
1	Energy (by calculation)	Measurement by calculation*
2	Total fat	Measurement of free lipid via direct solvent extraction using organic solvents
3	Protein/ crude protein	Measurement of organic nitrogen content via Kjeldahl procedure (N × 6.25)
4	Moisture	Measurement of weight loss of water due to evaporation when heated with infrared via moisture analyser
5	Ash	Measurement of inorganic residue after ignition of organic matter at 500-550 °C
6	Total carbohydrate	Measurement by calculation**
7	Dietary fibre**	Measurement of fibre remaining after enzyme digestion of defatted samples (α-amylase, protease, amyloglucosidase)

Notes:

* Total energy is determined as total sum of protein, fat and carbohydrate measured multiplied with the following factors: protein: 16.7 kJ/g; fat: 37.4 kJ/g; carbohydrate: 16.7 kJ/g.

** Total carbohydrate (g/100g; % w/w) is determined by the formula below:

= 100% - (Total fat + crude protein + moisture + ash)

** Dietary fibre is not included within the total % (g/100g; % w/w) of parameters 1-6.

2.2.4 Experimental design

2.2.4.1 Isolation of starch from underutilised legumes

Starch was isolated from the legumes based on the modified method of Wani *et al.* (2010) based on alkaline steeping as described in detail in Section 2.2.3.1. A total of 200 g of legume flour was used for starch isolation. The weight of the isolated starch obtained was measured and compared.

2.2.4.2 Determination of quantification method for RS analysis

The RS content of isolated starch from each legume was measured by two methods, namely the McCleary method and the Megazyme RS Kit. A positive control with 44% RS content was used in both methods and each assay was performed in triplicates for each legume sample. The selected method was then used for quantification of RS content for the rest of the study.

2.2.4.3 Evaluation of production methods of RS from legumes

Isolated starch samples from each legume were subjected to three treatments to improve RS content as highlighted in Section 2.2.3.3: a) acid-RS; b) enzyme-RS, and c) untreated-S (isolated starch with no further processing steps). Samples produced by acid-hydrolysis and enzyme-hydrolysis were further subjected to gelatinisation and retrogradation steps and samples were then lyophilised prior to assay. Treatments applied to each legume were conducted in single batches and were performed as described in Section 2.3. RS content of all samples was measured in triplicate as using the Megazyme RS kit as described in Section 2.2.3.2.

2.2.4.4 Proximate analysis of mung bean samples

Proximate analysis was performed on mung bean due to preliminary prebiotic properties observed when supplemented in bacteria broth cultures. The mung bean samples analysed comprised of 1) milled flour, 2) isolated starch (untreated-S), 3) mung bean enzyme-hydrolysis treated starch (enzyme-RS) and 4) mung bean acid-hydrolysis treated starch (acid-RS), which were all produced as highlighted in Section 2.2.3.3. These samples were obtained from the same batch of mung beans used in the assays listed above. The milled flour was produced by direct milling of mung beans without additional processing steps. These four samples were then submitted to ALS Technichem Sdn. Bhd. for the analysis of proximate parameters as stated in Section 2.2.3.4.

2.2.5 Statistical analysis

All values reported are means ± standard deviation, unless otherwise stated. As all samples came from a single batch of each legume, replicates represent technical rather than biological variability. Statistical analysis was performed using Genstat 18th Edition. Normality of data was determined using the Shapiro-Wilk test and statistical comparisons of non-normal data were performed using the Kruskal-Wallis H test. Statistical comparisons of normal data were performed using ANOVA between samples and statistical significance is indicated via labels of different letters.

2.3 Results

2.3.1 Isolation and quantification of starch from underutilised legumes

The quantity of starch isolated from the six underutilised legumes is shown in Table 2.2. The results suggest that Bambara groundnuts, red lentils and pigeon peas have significantly higher amounts of starch ($p < 0.05$) than the other legumes. Pigeon pea had 10.53 – 14.53% more starch than the adzuki bean, black-eyed pea and mung bean.

Table 2.2: The total amount of starch isolated from six underutilised legumes using the modified method of Wani *et al.* (2010).

Legume	Total amount of starch (% w/w)
Adzuki bean	25.80 ± 4.23 [b]
Bambara groundnut	35.30 ± 1.42 [a]
Black-eyed pea	27.83 ± 1.91 [b]
Mung bean	29.80 ± 2.46 [b]
Red lentil	35.67 ± 1.55 [a]
Pigeon pea	40.33 ± 4.67 [a]

Notes:

- Values reported are means ± standard deviation; n=3.
- [a,b] Different superscripts in a column are significantly different at $p < 0.05$.

2.3.2 Comparison of RS quantification methods

The RS content of the isolated starch from six underutilised legumes, determined using the McCleary method and a commercial test kit – Megazyme RS Assay kit (Megazyme, Ireland) are presented in Table 2.3. A control sample, with known 44% (w/w) RS was used to compare the accuracy of both methods. The RS content determined by the Megazyme RS kit was found to be closer to the control than the

McCleary method which overestimated the RS content of the control by approximately 26%.

Table 2.3: Comparison of RS quantification methods for isolated legume starches

Legume	RS content (% w/w)	
	Megazyme RS Kit	McCleary method
Adzuki bean	11.86 ± 0.74 [a]	45.56 ± 8.58 [b]
Bambara groundnut	6.10 ± 0.07 [b]	53.49 ± 0.55 [ab]
Black-eyed pea	3.93 ± 0.55 [c]	62.03 ± 2.78 [a]
Mung bean	6.82 ± 1.74 [b]	59.77 ± 1.60 [a]
Red lentil	11.61 ± 0.95 [a]	62.93 ± 2.41 [a]
Pigeon pea	7.3 ± 1.15 [b]	55.71 ± 6.60 [ab]
Control	37.25	70.18

Notes:

- Values reported are means ± standard deviation; n=3.
- [a, b, c] Different superscripts in a column are significantly different at $p < 0.05$
- "Control" is provided by the Megazyme RS kit that contained 44% w/w RS; n=1.

Overall, the McCleary method produced significantly higher RS content as compared to the Megazyme RS kit ($p < 0.05$). The highest difference was observed in black-eyed peas, where the RS content measured via the McCleary method is approximately 15-fold higher than the Megazyme RS kit. Meanwhile, the smallest difference was observed in adzuki beans, where the RS content measured via the McCleary method is approximately 4-fold higher than the Megazyme RS kit.

The two different methods also appear to perform differently depending on the nature of the legume. The McCleary method indicated that adzuki bean had the lowest RS content among the tested legumes, but this is the highest when quantified using the Megazyme RS kit. Similar observations were found for black-eyed pea,

which had the highest RS content via the McCleary method but was the lowest of all the legumes when quantified using the Megazyme RS kit.

Based on the McCleary method the ranking of RS content found in the tested legumes is as follows: red lentil > black-eyed pea > mung bean > pigeon pea > Bambara groundnut > adzuki bean. Whereas based on the Megazyme RS kit, the ranking of RS content found in tested legumes is as follows: adzuki bean > red lentil > pigeon pea > mung bean > Bambara groundnut > black eyed pea.

2.3.3 Comparison of RS production methods of RS from legumes

Isolated starch from all legumes were treated via hydrolysis, gelatinisation and retrogradation and lyophillisation based on the study of Polesi & Sarmento (2011). Acid- and enzyme-hydrolysis methods were compared in this study with the untreated starch being used as a control. Based on the results obtained in Section 2.3.2, the Megazyme RS kit was selected to be used to quantify the RS content in samples, as shown in Table 2.4.

The method of hydrolysis had significant effect on the production of RS from tested legumes. The RS produced by acid-hydrolysis treatment (acid-RS) of Bambara groundnuts and pigeon pea were significantly higher than the untreated starches (untreated-S), while adzuki bean and red lentil acid-RS were lower than the untreated-S ($p < 0.05$). The highest RS content of the acid-RS was found in Bambara groundnut and the lowest in adzuki bean. The ranking of acid-RS is as follows: Bambara groundnut > pigeon pea > mung bean > red lentil > black-eyed pea > adzuki bean.

Table 2.4: RS content of legumes starches and their respective treatments

Legume	RS content and treatments (% w/w)		
	Acid-RS	Enzyme-RS	Untreated-S
Adzuki bean	2.83 ± 0.82 $^{a; A}$	17.73 ± 0.84 $^{c; C}$	11.86 ± 0.74 $^{c; B}$
Bambara groundnut	20.42 ± 0.35 $^{d; C}$	18.06 ± 0.40 $^{c; B}$	6.10 ± 0.07 $^{ab; A}$
Black-eyed pea	2.88 ± 0.41 $^{a; A}$	16.99 ± 0.82 $^{c; B}$	3.93 ± 0.55 $^{a; A}$
Mung bean	7.62 ± 0.36 $^{b; A}$	15.15 ± 0.27 $^{b; B}$	6.82 ± 1.74 $^{b; A}$
Red lentil	4.83 ± 1.93 $^{a; A}$	12.83 ± 0.44 $^{a; B}$	11.61 ± 0.95 $^{c; B}$
Pigeon pea	13.20 ± 1.75 $^{c; B}$	12.40 ± 0.43 $^{a; B}$	7.34 ± 1.15 $^{b; A}$

Notes:

- Values reported are means ± standard deviation; n=3.
- $^{a-d}$ Within a column, values with different superscripts are significantly different at $P < 0.05$.
- $^{A-C}$ Within a row, values with different superscripts are significantly different at $P < 0.05$.

The RS produced by enzyme-hydrolysis treatment (enzyme-RS) were significantly higher than the untreated starches (untreated-S), for all legumes except red lentil. The highest RS content was observed in Bambara groundnut which was 45.65% higher than pigeon pea (which had the lowest enzyme-RS content). The enzyme-RS can be ranked as follows: Bambara groundnut > adzuki bean > black-eyed pea > mung bean > red lentil > pigeon pea.

For both acid-RS and enzyme-RS, Bambara groundnut was highly ranked, in contrast to untreated Bambara groundnut which was ranked 5th. On the other hand, adzuki bean enzyme-RS was ranked 2nd versus adzuki bean acid-RS, which was ranked lowest.

2.3.4 Nutritional composition of legume after processing

The changes in the nutritional composition of the legume after different RS processing steps (milled flour, untreated-S, acid-RS and enzyme-RS) were investigated using mung bean as the targeted legume (Table 2.5).

Table 2.5: Nutritional composition of mung bean flour, isolated starch and processed starches

Nutritional composition	Mung bean samples			
	Legume flour	Untreated-S	Acid-RS	Enzyme-RS
Energy content (kJ/100g)	1336	1433	1483	1323
Total fat (%)	1.9	0.4	0.4	0.5
Total crude protein (%)	23.7	0.3	0.2	0.4
Moisture (%)	9.7	8.4	11.8	10.8
Ash (%)	3.3	1.5	0.2	4.2
Total carbohydrate (%)	61.4	89.4	87.4	84.1
Dietary fibre (%)	19.7	10.6	0.4	13.8

Notes:

- Values reported in % (g/100g; % w/w) except for Energy content (kJ/100g); n=1.

In terms of energy content, enzyme-RS was the lowest and acid-RS the highest though the difference was only 12%. The total carbohydrate content of all samples was higher than raw bean flour. The dietary fibre content varies between the RS processes and was considerably lower than in the milled flour. The most dramatic difference was in acid-RS where the fibre content was reduced to negligible levels.

In comparison, total crude protein and lipid content of the processed samples were lower than raw bean flour, suggesting that these RS extraction processes had successfully removed most of the nutrients.

2.4 Discussion

This study depicts a sequential processing method in which the RS from six underutilised legumes could be produced for applications including as a sustainable prebiotic ingredient in aquafeeds.

The first part of the study involved the isolation of starch from legumes via the alkaline steeping method. The alkali steeping method revolves around the exposure of starch to an alkali solution to promote the dispersal of the matrix protein, solubilizing and thereby removing the proteins.

Based on the finding in Table 2.5, 98.7% of protein in mung bean flour was successfully removed after alkali steeping step, confirming that at high pH most legume proteins can be solubilized (Boye *et al.* 2010; Lee *et al.* 2007; Baldwin 2001). The alkali steeping method has also been found to be highly effective in isolating starch granules from the protein matrices from various types of grains and legumes (Villarreal *et al.* 2013; Lee *et al.* 2007; Han & Hamaker 2002).

The alkali steeping method of Wani *et al.* (2010) was used in this study as it was developed by use on a legume, kidney bean. In this method, the alkaline steeping was performed prior to filtration, which varies slightly from the published method by Hoover & Sosulski (1985). However, the changes in the filtration sequence do not appear to affect the amount of starch isolated. The method of Hoover & Sosulski (1985) yielded approximately 35.75% (w/w) of starch from red lentil (Hoover & Ratnayake 2002), which is close to the 35.67% starch yield from the same legume in this study (Table 2.2).

When starch yields from the current study (Table 2.2) were compared with other published studies, the yield from adzuki bean starch was higher than that reported by Yoshimoto *et al.* (2001). However, the additional defatting of starch, via dimethyl sulfoxide and precipitation with ethanol may have reduced the yield obtained in the study by Yoshimoto *et al.* (2001). The yield of legume starch in this study were comparable to the published literatures by Hoover *et al.* (2010 and 1997), except for black-eyed peas. However, the starch yield for red lentil was slightly lower when compared to the findings of Lee *et al.* (2007), which were ranging from 42-50% (w/w) but close to the yield obtained in the study of Wang *et al.* (2009) which was 35% (w/w).

The starch content of the most of the other legumes, including Bambara groundnut, pigeon pea, red lentil and mung bean, all fall within the expected range of 20-40% as reported by published studies (Oyeyinka *et al.* 2015; Liu & Shen 2007; Hoover *et al.* 1993). On the other hand, the cowpea starch yield in this study was lower than those reported by Huang *et al.* (2007) who noted that cowpea starches are difficult to isolate due to the presence of fine fibre, which co-settles with starch to give a light and loose deposit. This results in larger amounts of starch being excluded in order to maintain its purity.

Current findings suggested that a certain percentage of loss in starch content could have been attributed to the washing steps applied, or the effectiveness of the alkaline steeping method applied in removing starch granule-based proteins (Boye *et al.* 2010; AACC International. 1999; Mistry *et al.* 1992). Starch granule-based proteins may also be the cause of yield loss due to starch adhering to insoluble-fibre which may require repeated resuspension in water and filtration to separate fully (Hsieh *et*

al. 1999). The yield of the starch isolated from a wide range of legumes has been reviewed by Hoover *et al.* (2010) and suggested that the discrepancy in starch yields may vary due to botanical origin, which also affect the structure and size of granules (Themeier *et al.* 2005).

In this study, the modified method of McCleary *et al.* (2002) and the Megazyme RS Kit were used to quantify the RS found in legumes. The key advantage of these two methods are the consistency in results when tested across various laboratories on samples such as native potato starch and regular maize starch (Moongngarm 2013; Themeier *et al.* 2005; Champ *et al.* 2003; McCleary *et al.* 2002).

Based on the control used (44% RS) for optimisation of RS quantification method, the RS content measured using Megazyme RS kit was closer to control. Whereas the RS content measured with the McCleary method was at least 1.5-fold higher than the control (Table 2.3). Similar trends were observed in the RS contents of the isolated legume starches tested, where analysis using the McCleary method were several folds higher than those presented by other studies (Hoover *et al.* 2010). The difference in the RS content measured in both methods could potentially due to the α-amylase solution used to remove all digestible starches within the 16 hr incubation period. In the case of the McCleary method, the removal of digestible starch may not be complete and the residues remained may have caused an over-estimation of RS content. In contrast, the α-amylase provided by Megazyme RS kit may remove the digestible starch effectively, resulting in a more accurate result.

Furthermore, in the McCleary method, the α-amylase from *Aspergillus oryzae* was used, instead of porcine pancreatic amylase. Fungal amylases are widely used in

industry and have not been suggested to be inferior to porcine pancreatic amylase (Souza & Magalhães 2010). However, in this study, the enzyme showed lower efficiency when compared to porcine α-amylase which is similar to the observation reported by Sandstedt & Gates (1954).

Although no recent study has been published on the efficiency of fungal and porcine amylases, several studies on the efficiency of α-amylase from different sources was reviewed by Gupta *et al.* (2003b). A key observation between α-amylases from different sources was differences in their optimum temperatures and pH, which are usually associated to the optimum growth conditions of the host (Saranraj & Stella 2013; Gupta *et al.* 2003b).

According to the manufacturer's product specification, the incubation temperature for α-amylase of *Aspergillus oryzae* was at 25 °C, and amyloglucosidase was at 60 °C as opposed to the incubation of temperature of 37 °C used in the McCleary method. This variation in incubation temperature may have contributed to reduce enzyme activity. However, a study by Su *et al.* (2005) suggested that α-amylase activity from *Aspergillus oryzae* increases with temperature. Hence current incubation at 37 °C might have been expected to enhance the α-amylase activity. Nevertheless, such an increment was not observed in this study.

In contrast, the incubation temperature of both α-amylase and amyloglucosidase provided by the Megazyme RS kit was 40°C and hence very closed to the 37 °C recommended by the kit. The current findings suggest that further optimization is required to enhance the efficiency of enzymes used in the McCleary method.

Based on the findings described above, the Megazyme RS kit was used for quantification of RS content in this study. The RS content measured in the six legume starches were found to vary between 3% in black-eyed peas to 11% in red lentils (Table 2.4). Variations in the RS content measured across different legumes could be related to the granule sizes (Abia *et al.* 1993; Svihus *et al.* 2005). Legumes with smaller starch granules were found to be more resistant to enzymatic degradation; this occurs possibly due to the presence of lipid:starch complexes, which reduces contact between enzyme and substrate and may be more severe when the starch granules exhibits greater surface to volume ratio (Svihus *et al.* 2005). Similar findings were observed in this study, as adzuki beans have a larger granule size as compared to black-eyed peas, lentils and mung beans, resulting in higher enzymatic degradation and hence, lower RS content (Hoover *et al.* 2010).

Generally, starch from legumes contain high amounts of RS due to the types of crystalline formations, which tend to favour C-type starch (Hoover *et al.* 2010). These crystalline structures restrict enzyme activity, especially when paired with smooth surfaces present on the granule of native starch (Božić *et al.* 2017; Hoover *et al.* 2010). The combination of these factors result in an overall higher amount of RS measured as compared to a granule with pores or with a crystallinity more accessible by enzyme attack (Božić *et al.* 2017; Abia *et al.* 1993).

In this study, the efficiency of acid- and enzyme-hydrolysis methods in the production of RS were compared. The hydrolysis step reduces the molecular weight of starch chains to facilitate retrogradation (Thompson 2000). This can be done through the application of debranching enzymes, such as pullulanase, or through acid treatment.

The enzyme-hydrolysis treatment applied in this study produced significantly higher amount of RS than the acid-hydrolysis method (Table 3.3). Enzyme-hydrolysis produces polymer molecules with approximately 100-300 units of glucose, facilitating the production of double helix formation and contributes to higher levels of RS produced (Polesi & Sarmento 2011). Similar to current study, the application of enzyme-hydrolysis treatment has been found to increase RS content of legumes such as chickpeas, lentils and field peas in several published studies (Morales-Medina *et al.* 2014; Polesi & Sarmento 2011; Vasanthan & Bhatty 1998).

In this study, enzyme-hydrolysis treatment was found to be more effective than acid-hydrolysis for most legumes. The highest RS increment in black-eyed-pea, of approximately 4-fold, could be attributed to the crystalline structure of its starch, ensuring stability throughout freezing and maintaining high RS content (Huang *et al.* 2007). Meanwhile, no significant changes in RS were observed in red lentils. It is possible that both red lentil untreated-S and enzyme-RS share relatively similar crystalline structure following gelatinisation and retrogradation, producing a similar highly ordered molecular structure which produces similar amylase resistance (Morales-Medina *et al.* 2014). In another study, the crystallinity of lentils was reduced following treatment, which was attributed to the possible formation of granular porosity (Chung *et al.* 2010). This was caused by the interruption of double helixes forming starch crystallites, ultimately increasing the susceptibility of the starch towards enzymes (Chung *et al.* 2010). These factors may have contributed towards the lack of any significant increment of RS content of red lentils following treatment, as a wide range of potential changes that occur following gelatinisation and retrogradation (Zavareze & Dias 2011).

Acid-hydrolysis reduces the molecular weight of starch molecules by the hydrolysis of α-1,4 glucosidic bonds to promote efficient crystal formation during retrogradation. From this study, RS contents reduced in most legume starches following the acid-hydrolysis treatment (Table 2.4). Several factors could be contributing to this effect including reduction in molecular weight of amylose and the plasticisation of starch.

Firstly, the extreme processing of starch in the presence of acid could reduce amylose molecular weight to an extent which inhibited reformation during retrogradation to produce RS (Htoon *et al.* 2010; Vasanthan & Bhatty 1998). However, this was not found to be the case for pigeon pea and Bambara groundnut, which could be attributed to their higher amylose content which may be sufficient overcome this effect (Sandhu & Lim 2008; Hoover *et al.* 2010).

Secondly, the neutralisation performed during the acid-hydrolysis process in this study could have affected the starch. It is possible that during the process of neutralisation of the acid-starch suspension, plasticisation occurs due to high temperatures and salts produced. This could lead to gelatinisation and plasticisation, reducing the crystallinity of the structure as has been shown in several studies (Zhang & Han 2010; Liu *et al.* 2009; Srichuwong *et al.* 2005; Chiotelli *et al.* 2002).

Overall, the results obtained suggest the acid-hydrolysis pre-treatment applied in this study could be further optimised to prevent the negative effects listed above. Alternatively, other pre-treatment methods could be selected to enhance the resistant starch content of the starches.

Key factors contributing to the RS content are amylose and amylopectin content, where higher levels of amylose corresponds with higher RS produced (Morales-Medina *et al.* 2014; Hallstrom *et al.* 2011; Thompson 2000; Eerlingen *et al.* 1993). Based on the review of Hoover *et al.* (2010) and the study of Oyeyinka *et al.* (2015), the ranking of the legumes tested based on the amylose content of their starch (w/w) was as follows: mung beans (39.0%) > pigeon pea (36.7%) > black-eyed pea (29.4%) > red lentils (27.9%) > Bambara groundnut (27.5%) > adzuki beans (26.3%). However, in current study, the amylose content of the legume starch was not determined and hence a direct comparison is not feasible.

2.5 Summary

In summary, this study investigated the RS availability in six underutilised legumes, namely adzuki beans, mung beans, black-eyed peas, pigeon peas, Bambara groundnuts and red lentils for application as sustainable prebiotic ingredients in aquafeeds. The alkaline steeping method applied was effective in the isolation of starch, obtaining a yield of 25-40% depending on the species. RS quantification using the Megazyme RS Assay kit was also found to be the more effective. Enzyme-hydrolysis was shown to be more effective in maximizing RS content producing significantly higher levels than untreated-S or acid-RS. RS content alone is insufficient to determine its efficiency as a prebiotic. Hence, Chapter 3 describes how the samples produced were used for *in vitro* analysis with bacteria to determine their prebiotic efficiencies.

3 Chapter 3: *In vitro* study on the prebiotic potential of legume resistant starch

3.1 Introduction

As the gastrointestinal tract (GIT) is a key site for pathogenesis, modification of the intestinal microflora has been suggested as a method to improve fish health. Nutritional benefits may include synthesis of functional compounds by the microbiota, while disease mitigation revolves around the maintenance of the balance between endogenous microbiota of the host and the host's control mechanism (Merrifield *et al.* 2010). Nevertheless, the studies of microbiota improvement in fish are limited due to difficulty in conclusively elucidating benefits to the host, compared to terrestrial animals and humans, where specific genera of bacteria are acknowledged to confer benefits to the host.

Recently, several researchers have taken the approach of investigating the relationship between prebiotics and the growth of fish. The prebiotics applied in aquatic animals were based on those commonly applied in terrestrial animals such as inulin, fructo-oligosaccharides (FOS) and galacto-oligosaccharides (Ringø *et al.* 2010b; Barclay *et al.* 2010; Torres *et al.* 2010). Ringø *et al.* (2010b) reported that supplementation of inulin in diets improved intestinal growth in Atlantic salmon (*Salmo salar*) and increased growth rates in turbot larvae (*Scophthalmus maximus*). Meanwhile, FOS was also effective in hybrid tilapia (*Oreochromis* sp.), improving survival and growth rates.

On the other hand, while resistant starch (RS) is commonly fed to terrestrial animals, such studies are less common in aquatic animals. When included as a prebiotic, RS improved short-chain fatty acid (SCFA) production in the intestinal tract in European sea bass (Gatesoupe *et al.* 2014). These metabolites produced by intestinal

microflora were utilised by enterocytes as an energy source (Ohashi & Ushida 2009). While maize represents one of the most commonly studied sources of RS in the form of high amylose maize, underutilised legumes have also been shown to be a potentially important source of RS, which can be improved further via processing as demonstrated in the previous chapter. To our knowledge no studies have yet compared the effect of this novel RS source on indigenous potential probiotic bacteria from fish intestines. Such studies could provide preliminary evidence of the benefits of using RS towards potential probiotic bacteria, which may then confer health benefits to the host (Kihara & Sakata 2002).

Hence, using the novel approach of producing prebiotic RS from sustainable legume sources, the effects of RS on indigenous probiotics from fish intestines were investigated. Using an *in vitro* study, the preliminary effects of legume RS on the growth of these indigenous probiotics were investigated, which may potentially contribute towards fish health. In this study, the RS of interest was selected for further studies as supplement in fish feed.

3.1.1 Hypothesis

RS extracted from six underutilised legumes could improve the growth and acid production of probiotics isolated from Asian sea bass (*Lates calcarifer*) and zebrafish (*Danio rerio*).

3.1.2 Aims

The current study aims to investigate the prebiotic effect of RS extracted from six underutilised legumes on the growth of probiotic bacterium isolated from fish intestinal tract in an *in vitro* setting. The outcomes of this study will provide

important insights on the suitability of these sources of RS to be used as prebiotic ingredients in aquafeed.

Specific aims:

- To isolate and identify potential probiotic lactic acid bacteria (LAB) from Asian sea bass fish and zebrafish
- To determine the effects of legume RS on the growth and acid production of probiotic LAB

3.2 Materials and methods

3.2.1 Experimental overview

A total of two potential probiotic bacteria were isolated from each fish of interest, namely zebrafish and Asian sea bass and identified using biochemical and molecular methods. The prebiotic potential of six legume RS (as reported in Chapter 2) in promoting the growth of these probiotics in *in vitro* setting were investigated. Experiments conducted for the zebrafish and Asian sea bass were conducted at the U.K. and Malaysia respectively.

3.2.2 Materials

3.2.2.1 Preparation of medium

Bacteria medium were prepared based on the manufacturer's instructions provided for each medium and was sterilised on the day of preparation via autoclave conditions of 121 °C at 15 psi for 15 min. Bacteria agar plates were prepared by allowing the medium to cool to approximately 60 °C before being poured into 75-mm petri dishes using aseptic techniques. Bacteria broth and agar media were stored either at room temperature or at 4 °C, depending on the requirements of the manufacturer.

3.2.2.2 Prebiotic samples and cultures

The 18 prebiotic samples used in this study are: the untreated-starch (untreated-S), acid-hydrolysed resistant starch (acid-RS) and enzyme-hydrolysed resistant starch (enzyme-RS) of the six underutilised legumes listed in the previous chapter. The preparation procedures of these samples are described in detail in Section 2.2.3.1 and 2.2.3.3.

To prepare the RS/starch broth for bacteria culture, 1 g of sample was transferred into a universal bottle, followed by 20 mL of freshly prepared nutrient broth, to produce a 5% (w/v) resistant starch broth. The mixture was sterilized via autoclave (121 °C at 15 psi for 15 min). After autoclave, the mixture was cooled down to room temperature before use.

3.2.2.3 Fish samples

A healthy adult Asian sea bass, approximately 500 g in weight and 30 cm in length, was procured from Asealot Aquaculture in Selangor, Malaysia.

Adult zebrafish, approximately 200 mg in weight and 20 mm in length, were obtained from the Institute of Integrative Biology aquarium facility at the University of Liverpool.

3.2.3 Methods

3.2.3.1 Bacteria culture and maintenance

Two types of bacteria are used mainly, namely lactic acid bacteria (LAB) and Enterobacteriaceae. LAB are cultured using De Man, Rogosa and Sharpe (MRS) medium for up to 48 hr at 37 °C. Enterobacteriaceae were cultured using Eosin methylene blue (EMB) agar and MacConkey broth and incubated for up to 48 hr at 37 °C. Glycerol stocks were prepared from 24-hr old broth cultures by adding 700 µL of broth cultures into 300 µL of glycerol. Glycerol stocks were stored in -20 °C freezers to be used within 6 months and in -80 °C freezers for long term storage.

3.2.3.2 Isolation of bacteria from fish intestine

Fish used for bacteria isolation from fish intestine were procured as detailed in Section 3.2.2.3. For intestinal samples from smaller fish (approximately below 15 cm in length), the intestine collected in 1.5 mL centrifuge tubes were mixed with buffered peptone water equivalent to 9 mL per 100 mg of intestine sample. The intestine was disrupted using a pellet pestle followed by vigorous vortex for 1 min. For intestinal samples from larger fish (above 15 cm in length), intestines collected in 50 mL centrifuge tubes were removed and cut into small segments of 0.5 cm each with a sterile scissor. The segments were weighed and mixed with buffered peptone water equivalent to 9 mL per 100 mg of intestine sample. The mixture was then vortexed vigorously for 1 min.

From the mixture, a series of 10-fold dilutions of the sample was performed by transferring 100 µL of the sample into 900 µL of peptone buffer water (Oxoid CM1049). A ten-fold serial dilution of 10^1 till 10^5 was carried out. Then, 100 µL of diluted samples were plated on agar plates and incubated at 37 °C for 72 hr under anaerobic conditions in a 2.5 L Anaerobic Jar with a sachet of 2.5 L Anaerogen for anaerobic atmosphere generation (Thermo Scientific).

Colonies with different appearance were sub-cultured and subsequently identified using API 50 CHL and 16S ribosomal RNA (rRNA) gene sequencing as biochemical and molecular methods of identification respectively, as described in Section 3.2.3.3 and Section 3.2.3.4. The bacteria isolated in this study were then used for subsequent experiments.

3.2.3.3 Phenotypic identification of lactic acid bacteria isolates

a. Carbohydrate fermentation patterns

The selected LAB isolated from fish intestine were identified using the API 50 CHL identification kit (bioMérieux). The API 50 CHL identification kit is based on the carbohydrate fermentation pattern of the LAB. Prior to performing the API test, the cell morphology of LAB isolates, such as Gram reaction, cell shape and catalase test were determined.

The API test was prepared and conducted based on the manufacturer's protocol and is as listed in Appendix F. The data was then analysed with the Apiweb™ database.

3.2.3.4 Molecular identification of lactic acid bacteria

The selected LAB isolates were further identified using 16S ribosomal RNA (rRNA) gene sequencing by using polymerase chain reaction (PCR) and primers which specifically target the 16S rRNA genes of the bacteria genome. Prior to PCR and DNA sequencing, DNA extraction from samples was performed and is described below:

a. DNA extraction from samples

Prior to DNA extraction, the lytic enzyme solution was prepared by adding 50 mg of lysozyme (Sigma L6876) into 5 ml of 50 mM EDTA (pH 8.0) and mixed thoroughly via inverting. The lytic enzyme solution was used on the day of preparation.
DNA extraction from bacteria and intestinal samples was performed using the Wizard® Genomic DNA Purification kit (Promega).

i) For bacterium culture, a 1 mL aliquot of broth culture was transferred into a sterile 1.5 mL microcentrifuge tubes and were subjected to centrifugation at

13000 *g* for 2 min to pellet the cells. The supernatant was discarded and the pellet was resuspended with 480 µL of 50 mM EDTA (pH 8.0). A total of 120 µL of lytic enzyme solution was added to the sample and was incubated at 37 °C in a water bath for 60 min. Then, the sample was centrifuged at 13000 *g* for 2 min and the pellets were collected.

ii) For intestinal samples, the whole intestine of approximately 100 mg was transferred into a sterile 1.5 mL microcentrifuge tube and was mixed with 480 µL of 50 mM EDTA (pH 8.0). The sample is disrupted using pellet pestles gently (Sigma Z359947). Then, 120 µL of lytic enzyme solution was added to the sample and the mixture as vortexed vigorously. The mixture was then incubated at 37 °C in a water bath for 60 min, followed by vigorous vortexing before removing the intestines with a tweezer. The mixture was then centrifuged at 13000 *g* for 2 min and the pellets were collected.

The pellets collected were added with 600 µL of Nuclei Lysis Solution (Promega) and mixed well via gentle pipetting. The solution was incubated at 80 °C in a water bath for 5 min for cell lysis, followed by cooling down to room temperature. Then, 3 µL of RNase Solution (Promega) was added to the cell lysate and the tube was inverted gently for mixing. The mixture was incubated at 37 °C in a water bath for 30 min. After cooling the mixture to room temperature, 200 µL of Protein Precipitation Solution (Promega) was added to the cell lysate and was subjected to vigorous vortex for 20 s. The mixture was then incubated on ice for 5 min, followed by centrifugation at 13000 *g* for 3 min.

A total of 600 µL of the supernatant was added into 600 µL of isopropanol and was gently mixed via inversion until thread-like DNA strands form a visible mass. The tube was then centrifuged at 13000 *g* for 2 min. The supernatant was removed carefully via pipetting and excess isopropanol was drained using clean absorbent paper. Then, 600 µL of 70% (v/v) ethanol was added and the tube was gently inverted to wash the DNA pellet, followed by centrifugation at 13000 *g* for 2 min. The supernatant was removed carefully via pipette and the tube was drained on clean absorbent paper. The pellet was air-dried for 15 min, followed by the addition of 50 µL of DNA Rehydration Solution (provided by the kit) and was incubated at 65 °C in a water bath for 1 hr. The DNA was stored at 4 °C for use within the week and stored at -20 °C for long term storage. DNA quantity and purity was measured using a spectrophotometer (Epoch, BioTek), followed by dilution of the DNA samples to the concentration of 100 ng/µL using the DNA Rehydration Solution.

b. Polymerase chain reaction amplification and sequencing of bacteria 16S rRNA gene

PCR amplification and sequencing of bacteria 16S rRNA gene can be separated into two components: a) amplification of the 16S rRNA gene using PCR and b) sequencing of the PCR product via services from 1st BASE DNA Sequencing services (Selangor, Malaysia) and Source BioScience (Nottingham, United Kingdom).

The universal primers sets applied in this study are according to James (2010) and Balcázar *et al.* (2007c):

I. 8F forward primer: 5'-AGA GTT TGA TCC TGG CTC AG-3' and U1492 reverse primer: 5'-GGT TAC CTT GTT ACG ACT T-3’ (James 2010)

II. plb16 forward primer: 5'-AGA GTT TGA TCC TGG CTC AG-3' and mlb16 reverse primer: 5’- GGC TGC TGG CAC GTA GTT AG-3’ (Balcázar *et al.* 2007c)

The PCR master mix preparation was performed using the DreamTaq DNA Polymerase kit (Thermo Scientific). The PCR master mix for 10 reactions was prepared based on Table 3.1, by adding the reagents into the sterile distilled water in a descending order in a sterile 1.5 mL microcentrifuge tube while kept on ice. A total of 19 µL aliquots of the master mix is distributed into 200 µL PCR tubes and 1 µL of sample with a concentration of 100 ng/µL is added into each except for one tube, where 1 µL of distilled water is used instead as the PCR no-template control.

The 200 µL PCR tubes were then transferred into a thermal cycler with settings as follows: a) 1 cycle of initial denaturation at 95 °C for 5 min; b) 35 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 30 s and extension at 72 °C for 30 s; c) 1 cycle of final extension at 72 °C for 5 min and, d) storage at 10 °C till used.

Table 3.1: Preparation of master mix for 10 reactions for PCR for bacteria identification

Reagent	Stock concentration	Volume (µL)	Final concentration
DreamTaq Polymerase Buffer	10x	20	1x
DNTP Mix	2 mM	16	0.16 mM
Forward primer	10 mM	6	0.3 mM
Reverse primer	10 mM	6	0.3 mM
DreamTaq Polymerase	5 U/µL	2	1 U/µL
Sterile distilled water	n/a	140	n/a

The PCR products obtained from was subjected to DNA sequencing by 1st BASE DNA Sequencing services (Selangor, Malaysia) and Source BioScience (Nottingham, United Kingdom) which includes PCR clean-up. Hence, PCR samples were submitted without further purification.

3.2.3.5 Viable cell count of lactic acid bacteria

The viable cell count of LAB determined using conventional plate count method as reported by Miles and Misra (1938) and was expressed in terms of colony forming units (CFU). The sample (either pure culture or intestinal sample) was mixed well with vortex to ensure homogeneity. Then, 10-fold serial dilution of the sample from 10^1 till 10^8 was performed by transferring 100 µL of the sample into 900 µL of peptone buffer water. Then, 5 aliquots of 10 µL of diluted sample were plated on MRS agar plate and incubated at 37 °C for 24 hr under anaerobic condition. All assays were carried out in triplicate.

After incubation, the viable cell count (CFU/mL) of the bacteria in the sample was enumerated based on the following formula:

$$\text{Viable cell count (CFU/mL)} = \frac{C}{D \times V}$$

- Where:
- C = number of colonies counted
- D = dilution factor
- V = volume of sample plated (mL)

3.2.3.6 pH measurement of bacteria broth culture

The pH of the broth culture was measured using a pH meter (Sartorius PB-11-P10.1). Calibration was performed using buffer solutions of pH 4, 7 and 10 (Eutech Instruments; ECPHBUFKITC).

3.2.4 Experimental design

An aliquot of 4% (v/v) of bacteria cultures of *W. cibaria* NM1 and *L. garvieae* NM2 from glycerol stock was added to the prebiotic cultures, including a negative control of nutrient broth without RS/starch supplement. All cultures were incubated at 37°C. At 0 hr and 24 hr intervals, 1 mL of sample was withdrawn for measurement of viable cell count (see Section 3.2.3.5) and pH (see Section 3.2.3.6). All assays were carried out in triplicate.

Following the results obtained using the bacteria isolated from Asian sea bass, the prebiotic RS samples were narrowed down to using each legume sample from enzyme-RS. The procedure was repeated as described above using the bacteria culture of *E. gilvus* V1 isolated from zebrafish.

3.2.5 Statistical analysis

All values reported are means ± standard deviation. Statistical analysis was performed using Genstat 18th Edition. Normality of data was determined using the Shapiro-Wilk test, where statistical comparisons of non-normal data were performed using the Kruskal-Wallis H test. Statistical comparisons of normal data were performed using ANOVA between samples and statistical significance is indicated via labels of different letters. All assays were carried out in triplicate.

3.3 Results

3.3.1 Isolation and identification of potential probiotics from gastrointestinal tract of fish

A total of two LAB isolates, LC-A and LC-B were isolated from the gastrointestinal tract of Asian sea bass fish by using De Man, Rogosa and Sharpe (MRS) selective medium. These LC-A and LC-B isolates were Gram-positive, coccus- (LC-A) and rod-shaped (LC-B), respectively.

A total of two LAB isolates, DR-A and DR-B were isolated from the gastrointestinal tract of zebrafish by using MRS selective medium. These DR-A and DR-B isolates were Gram-positive and coccus shaped.

All isolates were then identified using a carbohydrate fermentation pattern API 50 CHL test kit and 16S rRNA gene sequencing and the results are shown in Section 3.3.1.1 and 3.3.1.2.

3.3.1.1 Carbohydrate fermentation pattern of LAB from Asian sea bass

API 50 CHL carbohydrate fermentation patterns of isolates LC-A and LC-B are shown in Figure 3.1 and further detailed in the Appendix G. The isolate LC-A showed 99.2% similar identity to *Weissella confusa.* On the other hand, the isolate LC-B showed 98.6% similar identity to *Lactococcus lactis* ssp *lactis 1.*

(A)

DOUBTFUL PROFILE						
Strip	API 50 CHL V5.1					
Profile	- - - - + - + - - - - + + + - - - - - - - - + + + + + + + - - + - - - - - - - + - - - - - - - + - -					
Significant taxa	% ID	T	Tests against			
Weissella confusa	99.2	0.74	LARA 19%	GAL 100%		
Next taxon	% ID	T	Tests against			
Lactobacillus brevis 1	0.6	0.7	GAL 99%			

(B)

GOOD IDENTIFICATION						
Strip	API 50 CHL V5.1					
Profile	- - - - - + - - - - + + + + - - - - + - - - + + + + + + + + - - - + - - - + - - + - - - - - - - + - -					
Significant taxa	% ID	T	Tests against			
Lactococcus lactis ssp lactis 1	98.6	0.87	LAC 90%			
Next taxon	% ID	T	Tests against			
Lactococcus lactis ssp lactis 2	0.7	0.54	MAN 20%	LAC 99%	GNT 1%	

Figure 3.1: Identification of bacteria strains isolated from Asian sea bass using API 50 CHL test kit: A) LC-A; B) LC-B

3.3.1.2 Carbohydrate fermentation pattern of LAB from zebrafish

API 50 CHL carbohydrate fermentation patterns of isolates DR-A and DR-B are as shown in Figure 3.2 and further detailed in the Appendix H. The isolate DR-A showed 84.9% similar identity to *Bronchothrix thermosphacta.* On the other hand, the isolate DR-B showed 92.8% similar identity to *Lactobaccillus paracasei* ssp *paracasei 1.*

A)

DOUBTFUL PROFILE	
Strip	API 50 CHL V5.2
Profile	- + - - - + - - - - + + + + - - - - + - - - + + + + + + - - - - + - - - - - - + - - - - - - - - - -
Note	ID. NOT VALID BEFORE 48 HOURS

Significant taxa	% ID	T	Tests against			
Brochothrix thermosphacta	84.9	0.62	GLY 21%	GAL 25%	MAL 100%	
Lactobacillus paracasei ssp paracasei 3	10.2	0.55	GLY 0%	MAL 80%	LAC 80%	
Lactococcus lactis ssp lactis 1	3.5	0.59	GLY 1%	MAL 95%	LAC 90%	
Lactococcus lactis ssp lactis 2	1.2	0.42	GLY 1%	MAN 20%	MAL 91%	LAC 99%

Next taxon	% ID	T	Tests against			
Carnobacterium maltaromaticum	0.1	0.28	GLY 14%	MDG 99%	MAL 99%	SAC 100%
			INU 86%			

B)

GOOD IDENTIFICATION	
Strip	API 50 CHL V5.2
Profile	- + - - - + - - - - + + + + - - - - + + - - + + + + + + + - - + + - + - - - - + - - + - - - - + - -
Note	POSSIBILITY OF Lactobacillus casei

Significant taxa	% ID	T	Tests against			
Lactobacillus paracasei ssp paracasei 1	92.8	0.71	GLY 20%	LAC 99%	TUR 80%	

Next taxon	% ID	T	Tests against			
Lactobacillus paracasei ssp paracasei 2	6.5	0.63	GLY 16%	MDG 83%	TUR 100%	

Figure 3.2: Identification of bacteria strains isolated from zebrafish using API 50 CHL test kit: A) DR-A; B) DR-B

3.3.1.3 Identification of Asian sea bass LAB via 16S rRNA gene sequencing

The isolates LC-A and LC-B were further identified via 16S rRNA gene sequencing (Appendix I). The top five hits for LC-A and LC-B using both primer sets (plb16 and ml16; U8F and U1492R) showed E-values of approximately 0 (Table 3.2 and 3.3).

The results showed that LC-A had 98% and 99% similarity to *Weissella cibaria* strain CH2 when analysed using plb16 & ml16 and U8F and U1492R primers, respectively. Plb16 and ml16 primers resulted 96% similarity to *Weissella oryzae* and *Weissella paramesenteroides*, whereas only 94% and 93% similarity was found to *Weissella ceti* and *Weissella* minor strain, respectively, when analysed using U8F and U1492R primers. With this, LC-A was identified as *W. cibaria* and is designated as *W. cibaria* NM1 in this study.

LC-B shared 98% similarity with *Lactococcus garvieae* LG2 when amplified by primer sets of plb16 & ml16 and U8F & U1492R. About 88% similarity was recorded with *Lactococcus piscium* and *Bacillus azotoformans* when analysed using plb16 & ml16 primers, whereas 95% and 93% similarity was recorded with *Lactococcus lactis* and *Lactococcus piscium*, respectively, when analysed using U8F and U1492R primers. Taken together, LC-B was identified as *L. garvieae* and is designated as *L. garvieae* NM2 in this study

Table 3.2: Top five identities of isolate LC-A via 16S rRNA gene sequence

Primer Sets and Sequence Alignments	Query cover	E-value	Identity	Accession
plb16 & mlb16				
Lactococcus garvieae Lg2 DNA, complete genome	0.97	0	98%	NC_017490.1
Lactococcus piscium MKFS47 genome assembly *L_piscium*, chromosome : I	0.97	2E-166	88%	NZ_LN774769.1
Bacillus azotoformans LMG 9581 contig81, whole genome shotgun sequence	0.97	2E-160	88%	NZ_AJLR01000081.1
Streptococcus henryi DSM 19005 F601DRAFT_scaffold00033.33_C, whole genome shotgun sequence	0.97	2E-155	87%	NZ_AQYA01000005.1
Streptococcus sanguinis SK36 chromosome, complete genome	0.97	2E-151	87%	NC_009009.1
U8F & 1492R				
Lactococcus garvieae Lg2 DNA, complete genome	0.98	0	98%	NC_017490.1
Lactococcus lactis subsp. lactis Il1403 chromosome, complete genome	0.98	0	95%	NC_002662.1
Lactococcus piscium MKFS47 genome assembly *L_piscium*, chromosome : I	0.94	0	93%	NZ_LN774769.1
Streptococcus criceti HS-6 Contig1217313612, whole genome shotgun sequence	0.95	0	92%	NZ_AEUV02000002.1
Streptococcus equi subsp. zooepidemicus H70, complete genome	0.95	0	92%	NC_012470.1

Table 3.3: Top five identities of isolate LC-B via 16S rRNA gene sequence

Primer Sets and Sequence Alignments	Query cover	E-value	Identity	Accession
plb16 & mlb16				
Weissella cibaria strain CH2, complete genome	0.99	0	98%	NZ_CP012873.1
Weissella oryzae SG25 DNA, scaffold: sequence37, whole genome shotgun sequence	0.99	0	96%	NZ_DF820520.1
Weissella paramesenteroides ATCC 33313 genomic scaffold SCAFFOLD4, whole genome shotgun sequence	0.99	0	96%	NZ_GG697131.1
Weissella kandleri strain DSM 20593 Scaffold13, whole genome shotgun sequence	0.99	0	95%	NZ_JQBP01000013.1
Weissella viridescens strain DSM 20410 Scaffold6, whole genome shotgun sequence	0.99	0	95%	NZ_JQBM01000006.1
U8F & 1492R				
Weissella cibaria strain CH2, complete genome	0.96	0	99%	NZ_CP012873.1
Weissella ceti strain WS105, complete genome	0.97	0	94%	NZ_CP009224.1
Weissella minor strain DSM 20014 NODE_15, whole genome shotgun sequence	0.96	0	93%	NZ_JQCD01000006.1
Weissella koreensis KACC 15510, complete genome	0.96	0	93%	NC_015759.1
Weissella kandleri strain DSM 20593 Scaffold13, whole genome shotgun sequence	0.96	0	93%	NZ_JQBP01000013.1

3.3.1.4 Identification of zebrafish LAB via 16S rRNA gene sequencing

The isolates DR-A and DR-B were further identified via 16S rRNA gene sequencing (Appendix J). The top five hits for DR-A and DR-B using both primer sets (plb16 and ml16; U8F and U1492R) showed E-values of approximately 0 (Table 3.4 and 3.5).

The results show that DR-A had 98% and 99% similarity to *Enteroccocus gilvus* when analysed using plb16 & ml16 and U8F and U1492R primers, respectively. The plb16 and mlb16 primers resulted in 97% similarity to *Enterococcus avium*, whereas resulting in 98% similarity to both *Enterococcus avium* and *Enterococcus casseliflavus* when analysed using U8F and U1492R primers. Identification of DR-A using both primer sets suggest DR-A to be *E. gilvus* and is designated as *E. gilvus* V1.

Using the plb16 & mlb16 primers, DR-B was identified as *Enterococcus faecalis* with an identity similarity of 99%. This was followed by *Enterococcus haemoperoxidus* and *Enterococcus faecium*, with an identity similarity of 94% and 92% respectively. Using the U8F and U1492R primers, the identity of DR-B was identified as *Enterococcus faecalis* with an identity similarity of 99%. This was followed *by Enterococcus haemoperoxidus*, *Enterococcus durans* and *Enterococcus faecium*, with the former having an identity similarity of 96% while the latter two sharing an identity similarity of 95%. Identification of DR-B using both primer sets suggest DR-B to be *E. faecalis* and is designated as *E. faecalis* V1.

Table 3.4: Top five identities of isolate DR-A via 16S rRNA gene sequence

Primer Sets and Sequence Alignments	Query cover	E-value	Identity	Accension
plb16 & mlb16				
Enterococcus gilvus ATCC BAA-350 acOtF-supercont2.1, whole genome shotgun sequence	0.95	0	98%	NZ_ASWH01000001.1
Enterococcus avium ATCC 14025 genomic scaffold acyDI-supercont2.5, whole genome shotgun sequence	0.95	0	97%	NZ_KE136507.1
Enterococcus avium ATCC 14025 genomic scaffold acyDI-supercont2.1, whole genome shotgun sequence	0.95	0	97%	NZ_KE136500.1
Enterococcus casseliflavus EC20, complete genome	0.95	0	96%	NC_020995.1
Enterococcus phoeniculicola ATCC BAA-412 acOtz-supercont2.2, whole genome shotgun sequence	0.95	0	96%	NZ_ASWE01000002.1
U8F & 1492R				
Enterococcus gilvus ATCC BAA-350 acOtF-supercont2.1, whole genome shotgun sequence	0.96	0	99%	NZ_ASWH01000001.1
Enterococcus avium ATCC 14025 genomic scaffold acyDI-supercont2.5, whole genome shotgun sequence	0.96	0	98%	NZ_KE136507.1
Enterococcus casseliflavus EC20, complete genome	0.96	0	98%	NC_020995.1
Enterococcus phoeniculicola ATCC BAA-412 acOtz-supercont2.2, whole genome shotgun sequence	0.96	0	97%	NZ_ASWE01000002.1
Enterococcus faecium DO chromosome, complete genome	0.96	0	97%	NC_017960.1

Table 3.5: Top five identities of isolate DR-B via 16S rRNA gene sequence

Primer Sets and Sequence Alignments	Query cover	E-value	Identity	Accension
plb16 & mlb16				
Enterococcus faecalis V583 chromosome, complete genome	0.93	0	99%	NC_004668.1
Enterococcus haemoperoxidus ATCC BAA-382 genomic scaffold acOSg-supercont2.2, whole genome shotgun sequence	0.93	0	94%	NZ_KE136480.1
Enterococcus haemoperoxidus ATCC BAA-382 genomic scaffold acOSg-supercont2.1, whole genome shotgun sequence	0.93	0	94%	NZ_KE136479.1
Enterococcus faecium DO chromosome, complete genome	0.93	0	92%	NC_017960.1
Enterococcus phoeniculicola ATCC BAA-412 acOtz-supercont2.6, whole genome shotgun sequence	0.93	0	92%	NZ_ASWE01000006.1
U8F & 1492R				
Enterococcus faecalis V583 chromosome, complete genome	0.73	0	99%	NC_004668.1
Enterococcus haemoperoxidus ATCC BAA-382 genomic scaffold acOSg-supercont2.2, whole genome shotgun sequence	0.73	0	96%	NZ_KE136480.1
Enterococcus haemoperoxidus ATCC BAA-382 genomic scaffold acOSg-supercont2.1, whole genome shotgun sequence	0.73	0	96%	NZ_KE136479.1
Enterococcus faecium DO chromosome, complete genome	0.73	0	95%	NC_017960.1
Enterococcus durans strain KLDS 6.0930, complete genome	0.73	0	95%	NZ_CP012384.1

3.3.2 *In vitro* prebiotic potential of legume RS

In this section, the LAB isolated from Asian sea bass intestines, *W. cibaria* NM1 and *L. garvieae* NM2 were then used as the indicator bacteria to evaluate the prebiotic effect of acid-RS, enzyme-RS and untreated-S extracted from six underutilised legumes (as reported in Chapter 2). The reduction of pH indicates production of organic acid following the fermentation of carbohydrates by these bacteria for growth (Fusco *et al.* 2015; Vendrell *et al.* 2006). Overall, the supplementation of 5% (w/v) acid-RS, enzyme-RS and untreated-S in nutrient broth enhanced the growth of both indicator bacteria (Table 3.6 and 3.8). While both RS and starch enhanced the acid production of *L. garvieae* NM2 (Table 3.9), only enzyme-RS has enhanced the acid production of *W. cibaria* NM1 (Table 3.7).

Following the results obtained using LAB isolates from Asian sea bass intestines, the LAB isolated from zebrafish intestines, *E. gilvus* V1 was then used as an indicator bacteria to evaluate the prebiotic effect of enzyme-RS. The prebiotic effects of enzyme-RS of six underutilised legumes on the growth and acid production of the potential probiotic LAB *E. gilvus* V1 isolated from zebrafish are shown in Table 3.10 and Table 3.11 respectively.

In summary, the current findings further suggest enzyme-RS is a good prebiotic source in enhancing the growth and acid production of *L. garvieae* NM2, *W. cibaria* NM1 and *E. gilvus* V1. Among the bacteria tested, *L. garvieae* NM2 was also a better acid producer than both *W. cibaria* NM1 and *E. gilvus* V1.

3.3.2.1 Effect of legume RS on the growth and acid production of Weissella cibaria NM1

Table 3.6 and 3.7 show the effect of RS and starch supplementation on the growth and acid production of *W. cibaria* NM1. Enzyme-RS produced higher growth than untreated-S Bambara groundnut, pigeon pea and red lentil and acid-RS Bambara groundnut and pigeon pea. Only the adzuki bean untreated-S showed significantly higher growth increment than acid-RS and enzyme-RS. No difference in growth increment was observed for black eyed pea and mung bean. Based on the increased growth of *W. cibaria* NM1, enzyme-RS appears to be more suitable than the other two treatments as a prebiotic supplement. Overall the prebiotic effect of enzyme-RS to enhance the growth of *W. cibaria* NM1 could be ranked as following: red lentil > pigeon pea > mung beans > black eyed pea > negative control > adzuki bean > Bambara groundnut.

Similar to the observation in growth of *W. cibaria* NM1, the pH of the nutrient broth supplemented with enzyme-RS was found to be significantly lower than acid-RS, untreated-S, and the negative control. Similarly, reduction of pH was also observed in untreated-S, except for Bambara groundnut and pigeon pea, in which increment of pH were observed. In contrast, increment of pH was found in nutrient broth supplemented with acid-RS, except Bambara groundnut. This result shows that the growth enhancement of *W. cibaria* NM1 by enzyme-RS was also evidenced by significant reduction in pH of the growth medium, and thus indicating a good production of end product – organic acid.

Table 3.6: The growth of *W. cibaria* NM1 in medium supplemented with 5% (w/v) of legume RS/starch samples at 0 and 24 hr of incubation

Legume	Viable cell count of *W. cibaria* NM1 in medium supplemented with 5% (w/v) legume RS/starch samples (Log_{10} CFU/ml)								
	Untreated-S			Acid-RS			Enzyme-RS		
	0 hr	24 hr	Growth increment	0 hr	24 hr	Growth increment	0 hr	24 hr	Growth increment
Adzuki Bean	5.89 ± 0.03	7.99 ± 0.05	2.10 ± 0.09 $^{ab; C}$	5.91 ± 0.02	7.61 ± 0.07	1.70 ± 0.07 $^{c; A}$	5.87 ± 0.02	7.77 ± 0.09	1.90 ± 0.10 $^{c; B}$
Bambara Groundnut	5.90 ± 0.03	7.63 ± 0.03	1.73 ± 0.03 $^{cd; A}$	5.96 ± 0.03	7.68 ± 0.03	1.72 ± 0.04 $^{c; A}$	5.92 ± 0.05	7.80 ± 0.04	1.88 ± 0.11 $^{c; B}$
Black Eyed Pea	5.96 ± 0.09	8.00 ± 0.01	2.04 ± 0.12 $^{ab; A}$	5.96± 0.06	7.91 ± 0.06	1.95 ± 0.07 $^{b; A}$	5.83 ± 0.11	7.90 ± 0.01	2.06 ± 0.14 $^{abc; A}$
Mung Bean	5.67± 0.01	7.95± 0.01	2.25 ±0.01 $^{a; A}$	5.78± 0.04	7.85± 0.07	2.08 ± 0.09 $^{b; A}$	5.73± 0.09	7.91± 0.04	2.18 ± 0.16 $^{ab; A}$
Pigeon Pea	5.84 ± 0.03	7.95 ± 0.05	2.12 ± 0.03 $^{ab; A}$	5.85 ± 0.03	7.94 ± 0.03	2.09 ± 0.06 $^{b; A}$	5.77 ± 0.05	7.98 ± 0.02	2.21 ± 0.03 $^{ab; B}$
Red Lentil	5.49 ± 0.06	7.06 ± 0.19	1.57 ± 0.27 $^{d; A}$	5.53 ± 0.02	7.83 ± 0.04	2.31 ± 0.03 $^{a; B}$	5.48 ± 0.08	7.74 ± 0.04	2.26 ± 0.14 $^{a; B}$
Negative Control*	5.66 ± 0.04	7.62 ± 0.21	1.97 ± 0.19 ^{bc}	5.66 ± 0.04	7.62 ± 0.21	1.97 ± 0.19 ^{b}	5.66 ± 0.04	7.62 ± 0.21	1.97 ± 0.19 ^{bc}

Notes:

Values reported are means ± standard deviation; n=3.

*Negative control refers to broth culture without supplementation of RS/starch.

$^{a-d}$ Within a column, values with different superscripts are significantly different at $P < 0.05$.

$^{A-C}$ Within a row, values with different superscripts are significantly different at $P < 0.05$.

Table 3.7: The pH changes in medium supplemented with 5% (w/v) legume RS/starch samples during growth of *W. cibaria* NM1

Legume	pH of medium supplemented with 5% (w/v) of RS/starch samples during growth of *W. cibaria* NM1								
	Untreated-S			Acid-RS			Enzyme-RS		
	0 hr	24 hr	pH change	0 hr	24 hr	pH change	0 hr	24 hr	pH change
Adzuki Bean	5.78 ± 0.05	5.56 ± 0.00	-0.22 ± 0.06 $^{a; B}$	5.18 ± 0.04	5.24 ± 0.03	0.06 ± 0.02 $^{c; C}$	5.85 ± 0.00	5.35 ± 0.02	-0.50 ± 0.03 $^{a; A}$
Bambara Groundnut	5.45 ± 0.01	5.85 ± 0.00	0.40 ± 0.02 $^{e; C}$	4.28 ± 0.02	4.12 ± 0.01	-0.16 ± 0.03 $^{a; B}$	5.54 ± 0.01	5.30 ± 0.01	-0.24 ± 0.03 $^{d; A}$
Black Eyed Pea	5.80 ± 0.02	5.59 ± 0.04	-0.21 ± 0.04 $^{a; B}$	5.36 ± 0.00	5.38 ± 0.01	0.00 ± 0.01 $^{b; C}$	5.85 ± 0.02	5.41 ± 0.01	-0.44 ± 0.03 $^{b; A}$
Mung Bean	5.78 ± 0.05	5.56 ± 0.00	-0.22 ± 0.06 $^{a; B}$	5.81 ± 0.04	5.24 ± 0.03	0.06 ± 0.02 $^{c; C}$	5.85 ± 0.00	5.35 ± 0.02	-0.50 ± 0.03 $^{a; A}$
Pigeon Pea	5.59 ± 0.02	5.64 ± 0.05	0.05 ± 0.04 $^{c; B}$	5.28 ± 0.07	5.46 ± 0.08	0.18 ± 0.03 $^{e; C}$	5.76 ± 0.01	5.40 ± 0.03	-0.36 ± 0.03 $^{c; A}$
Red Lentil	5.55 ± 0.00	5.50 ± 0.02	-0.05 ± 0.03 $^{b; B}$	5.31 ± 0.02	5.41 ± 0.04	0.10 ± 0.04 $^{cd; C}$	5.73 ± 0.02	5.36 ± 0.00	-0.37 ± 0.03 $^{c; A}$
Negative Control*	5.53 ± 0.02	5.67 ± 0.00	0.14 ± 0.02 ^{d}	5.53 ± 0.02	5.67 ± 0.00	0.14 ± 0.02 ^{de}	5.53 ± 0.02	5.67 ± 0.00	0.14 ± 0.02 ^{e}

Notes:

Values reported are means ± standard deviation; n=3.

*Negative control refers to broth culture without supplementation of RS/starch.

$^{a-d}$ Within a column, values with different superscripts are significantly different at $P < 0.05$.

$^{A-C}$ Within a row, values with different superscripts are significantly different at $P < 0.05$.

3.3.2.2 Effect of legume RS on the growth and acid production of Lactococcus garvieae NM2

Table 3.8 and 3.9 show the effect of RS and starch supplementation on the growth and acid production of *L. garvieae* NM2. When comparing between the treatments, the growth increment caused by supplementation of enzyme-RS was higher than acid-RS, except for Bambara groundnut where no significant difference was observed. Enzyme-RS also produced a higher growth increment than adzuki bean, Bambara groundnut and red lentil of untreated-RS, but no difference when compared to black eyed pea, mung bean and pigeon pea. Overall the prebiotic effect of enzyme-RS to enhance the growth of *L. garvieae* NM2 could be ranked as following: red lentil > adzuki bean > mung bean > Bambara groundnut > pigeon pea > black eyed pea > negative control.

As with the growth of *L. garvieae* NM2, the pH of the nutrient broth supplemented with enzyme-RS was found to be significantly lower than acid-RS, untreated-S and negative control, except for black eyed pea. The pH of black eyed pea of untreated-S was lower than enzyme-RS. While not all legumes and treatments follow similar trends of high growth accompanied by high acid production, highest growth and acid production were found in enzyme-RS, further suggesting that enzyme-RS as the best prebiotic source to enhance the growth and acid production of *L. garvieae* NM2.

Table 3.8: The growth of *L. garvieae* NM2 in medium supplemented with 5% (w/v) of legume RS/starch samples at 0 and 24 hr of incubation

Legume	Viable cell count of *L. garvieae* NM2 in medium supplemented with 5% (w/v) legume RS/starch samples (Log_{10} CFU/ml)								
	Untreated-S			Acid-RS			Enzyme-RS		
	0 hr	24 hr	Growth	0 hr	24 hr	Growth	0 hr	24 hr	Growth
Adzuki Bean	6.40 ± 0.03	8.18 ± 0.00	1.79 ± 0.03 $^{bc; B}$	6.36 ± 0.01	7.58 ± 0.04	1.22 ± 0.03 $^{c; A}$	6.23 ± 0.15	8.35 ± 0.03	2.13 ± 0.17 $^{ab; C}$
Bambara Groundnut	6.29 ± 0.03	7.64 ± 0.07	1.35 ± 0.10 $^{d; A}$	6.33 ± 0.03	8.08 ± 0.06	1.76 ± 0.07 $^{a; B}$	6.32 ± 0.05	8.25 ± 0.04	1.93 ± 0.02 $^{b; B}$
Black Eyed Pea	6.27 ± 0.01	8.14 ± 0.07	1.87 ± 0.08 $^{b; B}$	6.21 ± 0.04	7.60 ± 0.10	1.39 ± 0.13 $^{bc; A}$	6.36 ± 0.03	8.13 ± 0.07	1.77 ± 0.04 $^{b; B}$
Mung Bean	6.30 ± 0.02	8.33 ± 0.02	2.02 ± 0.04 $^{a; B}$	6.30 ± 0.02	7.86 ± 0.10	1.56 ± 0.09 $^{ab; A}$	6.40 ± 0.04	8.34 ± 0.02	1.95 ± 0.06 $^{ab; B}$
Pigeon Pea	6.28 ± 0.04	8.09 ± 0.01	1.81 ± 0.04 $^{bc; B}$	6.32 ± 0.02	7.72 ± 0.09	1.41 ± 0.07 $^{bc; A}$	6.23 ± 0.08	8.06 ± 0.07	1.83 ± 0.14 $^{b; B}$
Red Lentil	6.30 ± 0.02	7.94 ± 0.14	1.64 ± 0.16 $^{c; A}$	6.30 ± 0.04	7.83 ± 0.04	1.53 ± 0.06 $^{b; A}$	6.09 ± 0.33	8.39 ± 0.02	2.30 ± 0.34 $^{a; B}$
Negative Control*	6.38 ± 0.02	7.23 ± 0.21	0.84 ± 0.21 ^{e}	6.38 ± 0.02	7.23 ± 0.21	0.84 ± 0.21 ^{d}	6.38 ± 0.02	7.23 ± 0.21	0.84 ± 0.21 ^{c}

Notes:

Values reported are means ± standard deviation; n=3.

*Negative control refers to broth culture without supplementation of RS/starch.

$^{a-d}$ Within a column, values with different superscripts are significantly different at $P < 0.05$.

$^{A-C}$ Within a row, values with different superscripts are significantly different at $P < 0.05$.

Table 3.9: The pH changes in medium supplemented with 5% (w/v) of RS/starch samples during growth of *L. garvieae* NM2

Legume	pH of medium supplemented with 5% (w/v) legume RS/starch samples during growth of *L. garvieae* NM2								
	Untreated-S			Acid-RS			Enzyme-RS		
	0 hr	24 hr	pH change	0 hr	24 hr	pH change	0 hr	24 hr	pH change
Adzuki Bean	6.40 ± 0.07	4.77 ± 0.05	-1.63 ± 0.11 $^{a; B}$	6.53 ± 0.00	5.17 ± 0.05	- 1.36 ± 0.05 $^{a; C}$	6.50 ± 0.01	4.56 ± 0.01	-1.94 ± 0.02 $^{a; A}$
Bambara Groundnut	6.53 ± 0.00	6.21 ± 0.02	-0.32 ± 0.02 $^{d; C}$	5.67 ± 0.10	4.54 ± 0.04	- 1.14 ± 0.08 $^{b; B}$	5.85 ± 0.04	4.41 ± 0.01	-1.44 ± 0.03 $^{d; A}$
Black Eyed Pea	5.77 ± 0.06	4.74 ± 0.03	-1.03 ± 0.03 $^{c; A}$	5.95 ± 0.04	5.37 ± 0.08	- 0.58 ± 0.04 $^{d; C}$	5.47 ± 0.02	4.63 ± 0.01	-0.84 ± 0.02 $^{e; B}$
Mung Bean	5.96 ± 0.02	4.69 ± 0.01	-1.27 ± 0.01 $^{bc; A}$	6.13 ± 0.06	5.33 ± 0.11	- 0.80 ± 0.15 $^{c; B}$	5.80 ± 0.01	4.43 ± 0.04	-1.37 ± 0.05 $^{d; A}$
Pigeon Pea	6.40 ± 0.02	4.87 ± 0.03	-1.53 ± 0.04 $^{ab; AB}$	6.57 ± 0.00	5.15 ± 0.06	- 1.42 ± 0.06 $^{a; B}$	6.28 ± 0.04	4.67 ± 0.01	-1.61 ± 0.04 $^{c; A}$
Red Lentil	6.44 ± 0.07	5.23 ± 0.30	-1.21 ± 0.28 $^{c; B}$	6.46 ± 0.01	5.15 ± 0.02	- 1.31 ± 0.02 $^{a; B}$	6.39 ± 0.05	4.61 ± 0.00	-1.78 ± 0.05 $^{b; A}$
Negative Control*	6.22 ± 0.01	4.87 ± 0.01	-1.34 ± 0.00 ^{bc}	6.22 ± 0.01	4.87 ± 0.01	-1.34 ± 0.00 ^{a}	6.22 ± 0.01	4.87 ± 0.01	-1.34 ± 0.00 ^{e}

Notes:

Values reported are means ± standard deviation; n=3.

*Negative control refers to broth culture without supplementation of RS/starch.

$^{a-d}$ Within a column, values with different superscripts are significantly different at $P < 0.05$.

$^{A-C}$ Within a row, values with different superscripts are significantly different at $P < 0.05$.

3.3.2.3 Effect of legume RS on the growth and acid production of Enterococcus gilvus V1

Table 3.10 and 3.11 show the effect of enzyme-RS supplementation on the growth and acid production of *E. gilvus* V1. Supplementation with legume enzyme-RS increased bacteria growth for all legumes except for black-eyed pea and mung beans. Among bacteria cultures with legume enzyme-RS, cultures supplemented with red lentil enzyme-RS showed the highest growth, which was significantly higher than cultures of black-eye pea and mung bean enzyme-RS while being statistically similar to the other three legumes. Overall the prebiotic effect of enzyme-RS to enhance the growth of *E. gilvus* V1 could be ranked as following: red lentil > Bambara groundnut > pigeon pea > adzuki bean > black eyed pea > mung bean.

Unlike with the observation in growth of *E. gilvus* V1, the pH change of the control nutrient broth was found to be significantly lower than enzyme-RS cultures. For enzyme-RS, the highest acid production was observed in black-eyed pea, which was significantly higher than all other legume enzyme-RS supplements. This was followed by cultures supplemented with red lentil enzyme-RS.

Table 3.10: The growth of *E. gilvus* V1 in medium supplemented with 5% (w/v) of legume enzyme-RS samples at 0 and 24 hr of incubation

Legume	Viable cell count of *E. gilvus* V1 in medium supplemented with 5% (w/v) legume enzyme-RS samples (Log_{10} CFU/ml)		
	0 hr	24 hr	Growth
Adzuki Bean	6.91 ± 0.06	7.96 ± 0.25	1.05 ± 0.22 [ab]
Bambara Groundnut	6.77 ± 0.06	8.00 ± 0.15	1.23 ± 0.16 [ab]
Black Eyed Pea	6.76 ± 0.09	7.63 ± 0.07	0.87 ± 0.06 [bc]
Mung Bean	6.56 ± 0.30	7.42± 0.13	0.85 ± 0.35 [bc]
Pigeon Pea	6.86 ± 0.15	8.04 ± 0.05	1.18 ± 0.20 [ab]
Red Lentil	7.00 ± 0.00	8.29 ± 0.05	1.29 ± 0.05 [a]
Negative control	6.62 ± 0.16	7.14 ± 0.07	0.52 ± 0.21 [c]

Notes:

Values reported are means ± standard deviation; n=3.

*Negative control refers to broth culture without supplementation of enzyme-RS

[a-c] Within a row, values with different superscripts are significantly different at $P < 0.05$.

Table 3.11: The pH changes in medium supplemented with 5% (w/v) legume enzyme-RS samples during growth of *E. gilvus* V1

Legume	pH of medium supplemented with 5% (w/v) legume enzyme-RS samples during growth of *E. gilvus* V1		
	0 hr	24 hr	pH change
Adzuki Bean	5.80 ± 0.04	5.07 ± 0.00	-0.72 ± 0.04 [d]
Bambara Groundnut	5.82 ± 0.03	5.10 ± 0.01	-0.72 ± 0.01 [d]
Black Eyed Pea	6.19 ± 0.01	5.14 ± 0.00	-1.05 ± 0.13 [b]
Mung Bean	5.75 ± 0.04	5.03 ± 0.10	-0.72 ± 0.04 [d]
Pigeon Pea	5.72 ± 0.05	5.06 ± 0.01	-0.66 ± 0.04 [e]
Red Lentil	5.92 ± 0.01	5.14 ± 0.01	-0.78 ± 0.01 [c]
Negative control*	6.39 ± 0.02	4.44 ± 0.01	-1.94 ± 0.01 [a]

Notes:

Values reported are means ± standard deviation; n=3.

*Negative control refers to broth culture without supplementation of enzyme-RS.

[a-d] Within a row, values with different superscripts are significantly different at $P < 0.05$.

3.4 Discussion

The current study was aimed to investigate the prebiotic effect of RS and starch extracted via acid- and enzyme-hydrolysis methods from six underutilised legumes on the growth of probiotic bacterium isolated from fish intestinal tract. In this study, two Gram positive isolates, LC-A and LC-B, were isolated from the intestinal tract of Asian sea bass to be used as probiotic indicators. These bacteria were identified using API 50 CHL test kit as a biochemical identification and 16S rRNA gene sequencing as a molecular identification.

The API 50 CHL carbohydrate fermentation patterns of LC-A and LC-B suggested that these isolates were highly similar to *Weissella confusa* and *Lactococcus lactis*. However, the results from 16S rRNA gene sequencing suggested that LC-A and LC-B were highly similar to *Weissella cibaria* and *Lactococcus garvieae*, which contradicts the findings obtained via API 50 CHL. Similarly, the API 50 CHL carbohydrate fermentation patterns of DR-A and DR-B were also contradictory.

The API 50 CHL kit tests the ability of bacterium to utilise 49 types of carbohydrates and hence produce a pattern that is unique to the bacterium. However the APIWEB database used has limitations, that mean a large variety of bacteria found exclusively in aquatic conditions may be excluded and misidentified (Janda & Abbott 2002). Hence, sequencing on the highly conserved 16S rRNA gene region of bacteria is a more accurate alternative (Wilson 1995). The use of 16S rRNA gene sequencing alleviates the limitations of commercial phenotypic methods; the number of 16S rRNA gene sequences published in the GenBank has increased vastly over the years (Rappé & Giovannoni 2003).

W. cibaria share extremely similar characteristics to *W. confusa* (Björckroth *et al.* 2002; Fusco *et al.* 2015). Fortunately, phenotypic analysis from the API kit used in this study revealed LC-A to be capable of L-arabinose fermentation and acid production. Based on the study of Björckroth *et al.* (2002), this was a key phenotypic difference between *W. cibaria* and *W. confusa*. Hence, the identity of LC-A is suggested to be *W. cibaria* instead of *W. confusa.* A few studies have identified *W. cibaria* as a potential probiotic for aquaculture (Maji *et al*. 2016; Muñoz-Atienza *et al.* 2014; Muñoz-Atienza *et al.* 2013; Mouriño *et al*. 2012), through *in vitro* investigation of the antimicrobial properties, as well as in application as a probiotic or symbiotic in fish feeding trials.

The identification of LC-B is contradictory based on the molecular and biochemical methods applied. Upon comparison to the published literatures (Itoi *et al*. 2008; Evans *et al.* 2006; Chang *et al*. 2002), it was suggested that the identity of LC-B is identical to *L. garvieae.* This is because the lactose utilisation ability is absent in *L. garveiae* but present in *L. lactis* (Itoi *et al*. 2008; Evans *et al.* 2006; Chang *et al*. 2002). It has been reported that *L. garvieae* is a common LAB in sturgeons and has also been selected as a potential probiotic (Hoseinifar *et al.* 2014). On the other hand, only one study found beneficial effects of *L. garvieae* as a probiotic (Zhang *et al.* 2016), while most other studies suggested that *L. garvieae* is a common aquatic pathogen which causes disease in a wide range of aquatic species (Vendrell *et al*. 2006; Cheng *et al.* 2002). However, it has not been reported to be found in *Lates calcarifer* and may be a probiotic of interest due to potential antimicrobial capabilities (Dodamani & Kaliwal 2014).

Two bacteria species from the *Enterococcus* genus were found to be the most common type of bacteria isolated from the intestinal tract of zebrafish housed at the University of Liverpool. Based on the carbohydrate fermentation profiles alone, it is suggested that both identifications are inaccurate, possibly due to limitations of the database; the kit failed to accurately identify the genus of both bacteria to be *Enterococcus*, which was suggested by the presence of acid production from glycerol fermentation (Bergey & Holt 1994). However, as *Enterococcus* spp. have been reported to have highly similar 16S rRNA gene sequences, up to 99%, additional biochemical tests become a necessity for accurate identification (Lebreton *et al.* 2014).

In the case of DR-A, identification, using 16S rRNA gene sequencing suggested the identity of the bacteria to be *Enterococcus gilvus*, which is a relatively new species of bacteria, having been discovered within the past 20 years (Tyrrell *et al.* 2002). Compared to the study of Tyrrell *et al.* (2002), acid production profiles were similar for all compounds compared, except for melibiose, raffinose, sorbitol and sorbose, for which a negative reaction was obtained in this study.

Many studies comparing *Enterococcus* have not included *E. gilvus* due to the species being relatively new, resulting in difficulty in comparing carbohydrate fermentation profiles. However, *E. gilvus* has been reported to display atypical phenotypic threads, suggesting that various strains may exist (Scheidegger *et al.* 2009). While *E. gilvus* has been isolated as a pathogenic *Enterococcus* in humans, and from dairy products, there have been no reports of *E. gilvus* being isolated from marine environments. This may be potentially due to *E. gilvus* being relatively new and only recently included in databases, or that the bacteria originated from human waste (Oprea &

Zervos 2007). Due to its novelty, *E. gilvus* V1 was selected for *in vitro* testing for the prebiotics in this study.

In the case of DR-B, identification using 16S rRNA gene sequencing suggested the identity of the bacteria to be *Enterococcus faecalis*. The carbohydrate fermentation profile obtained from DR-B was also identical to *E. faecalis*, apart from the negative reaction of lactose fermentation obtained in this study (Bergey & Holt 1994). Studies comparing identification methods revealed high levels of accuracy in the identification of *E. faecalis* by comparing 16S rRNA gene sequences with GeneBank, which supports the method and result obtained in this study (Moore *et al.* 2006). Nevertheless, other methods such as repetitive element sequence PCR (REP-PCR) and amplified fragment length polymorphism PCR (AFLP-PCR) can be used to improve the accuracy of the identification (Pangallo *et al.* 2008; Domig *et al.* 2003).

E. faecalis is frequently found in food and was also detected in the aquatic environment, thereby resulting in its presence in aquatic animals as well (Lebreton *et al.* 2014). *E. faecalis* has also been reported to produce enterocins, potential factors associated with its use as a probiotic, and has been used as a prebiotic in humans, poultry and pigs (Franz *et al.* 2011; Foulquié-Moreno *et al.* 2006). While not commonly used in studies involving aquatic animals as compared to *E. faecium*, *E. faecalis* was found to provide increments to the growth and immune responses of rainbow trout (Rodriguez-Estrada *et al.* 2009).

In this study, it was found that the supplementation of both RS and starch from legumes improved growth of potential probiotic LAB isolated of aquatic origin, *in vitro*. Similar studies involving prebiotics have been performed in the past, with

emphasis on microbial communities from faecal samples of healthy individuals and probiotic bacteria used in terrestrial animals (Patel & Goyal 2012; Pylkas *et al.* 2005). However, a literature search suggested that studies involving prebiotics from legumes are relatively less common than other prebiotics, such as inulin. A few studies have investigated the effects of two types of prebiotics from legumes, namely raffinose and RS (Zhou *et al.* 2013; Fernando *et al.* 2010). Overall, these studies found that the supplementation of both raffinose and RS *in vitro* led to prebiotic benefits in LAB, such as improving the growth of LAB, but not to other pathogenic bacteria. However, the bacteria applied in this study vary in origin and differ from the studies reported, leading to interest in the prebiotic effect of the RS derived from legumes which were treated to enhance RS contents.

The supplementation of both RS and starch significantly improved the growth of bacteria in this study. These results compliment the effect found in studies of *in vitro* cultures supplemented with raffinose, where the viable cell counts of *Lactobacillus acidophillus* and *Bifidobacteria bifidum* increased significantly when compared to the controls (Bednarczyk *et al.* 2011). On the other hand, the results of this study also compliment studies investigating the *in vitro* prebiotic potential of RS, where studies reported improved the growth of butyrate-producing bacteria (Scott *et al.* 2014) and *Bifidobacteria* spp. (Beards *et al.* 2010). This suggests that the samples applied in this study possess comparable prebiotic properties to improve the growth of bacteria. In addition, variation in the type of RS were also found to affect microbiota compositions (Martinez *et al.* 2010). Hence, for the application of the RS as prebiotics in aquatic animals, it may be useful to investigate its properties *in vitro* using bacteria isolated from such conditions (Jonathan *et al.* 2012).

The various treatments on the legume starch resulted in varying effects on the growth and acid production of all three bacteria tested. A key difference between the untreated starch and treated starches could be their granular structures, where smooth granular surfaces are usually less accessible to bacteria mainly due to reduced sites for adhesion (Topping *et al.* 2003; Abia *et al.* 1993). On the other hand, treatment may have resulted in structural changes, such as shown in studies by Wronkowska *et al.* (2006), where smooth irregular shaped granules native starch granules are transformed into fibrous bunches which form a three-dimensional matrix containing occasional globular units (Wronkowska *et al.* 2006). The author also suggested that the adhesion of bacteria to the modified starch, after disruption of the granule, may have contributed to increased growth and acid production, such as observed in this study (Wang *et al.* 1999). In this study, these structural changes may have improved bacteria accessibility to starch granules following treatment. Overall, all starches were suitable as prebiotics whether untreated or not. Slight variations between samples could be attributed to variation in starch granules properties, potentially influenced by their genetic or botanical differences (Ma *et al.* 2017; Hoover *et al.* 2010).

In this study, the *in vitro* experiments suggest enzyme-RS to be the most effective supplement, with overall higher growth and acid production following supplementation. Several factors may play a role in affecting the activity of enzyme-RS. While previous chapters highlight higher RS content as a feature of enzyme-RS, this property may not contribute to improved bacterial utilisation. Studies suggest that higher RS is associated with decreased enzyme accessibility and therefore reduced bacteria utilisation (Abia *et al.* 1993). In contrast, a study also suggested that highly organised structures of RS may promote SCFA production (Zhou *et al.* 2013).

However, both trends were not observed in this study. Instead, the RS contents of supplements did not correlate with the growth of the bacteria in this study. This was also observed in other studies which compared growth and the amount of SCFA produced following the fermentation of different RS (Giuberti *et al.* 2013; Siew-Wai *et al.* 2010). Further studies may be required to further elucidate the relationship between RS and its fermentation by bacteria. Nevertheless, high RS content is still a desirable trait as they can be associated with increased amounts of starches surviving host digestion to be used as nutrients for microbiota in the intestines (Regmi *et al.* 2011).

A possible factor for the improved growth following the supplementation of enzyme-RS could be the increased ash content, observed from the nutritional analysis of mung bean starch following all treatments. The ash content in nutritional analysis is comprised of minerals, which could compliment the growth of LAB which have complex nutrient requirements. Studies show that LAB require metal ions such as iron and manganese for growth and metabolic activities (Hayek & Ibrahim 2013; Hébert *et al.* 2004; Elli *et al.* 2000). While complex media, such as MRS, include additional minerals for supporting the growth of LAB, the basal medium used in this study does not contain these additional minerals (de Man *et al.* 1960). Hence, the increased ash content of enzyme-RS supplements may have contributed to metal ion requirements (Korkeala *et al.* 1992), improving growth and acid production of the bacteria.

The results from this study suggest that the enzyme-RS may possess prebiotic properties, which could have health benefits to the host. While *in vivo* studies may be more appropriate for investigating the prebiotic effect of RS, preliminary studies

in vitro reduce the costs and ethical issues involved in, *in vivo*, high throughput screening (Papadimitriou *et al.* 2015). Based on the growth and acid production of the LAB tested in this study, red lentil enzyme-RS appear to be most effective in the improvement of growth. Various factors may have played a role, such as red lentil's relatively smaller granule sizes, granule structure after treatment, and granule surface architecture which could have contributed to improving bacteria utilisation (Morales-Medina *et al.* 2014; Chung *et al.* 2010; Hoover *et al.* 2010). Following red lentils, adzuki beans also displayed high growth and acid production. Hence, red lentils and adzuki beans were selected from this study to be applied for further *in vivo* studies.

3.5 Summary

In summary, this study investigated the prebiotic effect of RS/starch supplements to improve the growth of LAB isolated from fish intestine via *in vitro* setting. The legume RS prepared via enzyme-hydrolysis method were the most effective in terms of growth and acid production, especially red lentil and adzuki beans. Hence, in subsequent studies, the red lentil and adzuki bean enzyme-RS were used in fish feeding trials to assess *in vivo* prebiotic potential.

4 Chapter 4: Effect of legume resistant starch on the growth performance of zebrafish (*Danio rerio*)

4.1 Introduction

The importance of the gastrointestinal tract (GIT) microbiota of fish was first demonstrated when 212 genes were significantly affected in a conventionally raised fish as opposed to a microbe-free fish (Rawls *et al.* 2004). The relationship between GIT microbiota and the fish differs from mammals; GIT microbiota in fish are transitory according to its environment and diet and are not associated for the entire lifespan (Sullam *et al.* 2012). This has prompted various studies to investigate the relationship between GIT microbiota and fish.

While many studies on prebiotics have been directed towards improving human health, relatively few studies have specifically investigated the use of prebiotics in fish (Ringø *et al.* 2010b). Several prebiotics such as inulin, oligosaccharides and mannan-oligosaccharides have been applied in aquaculture studies and have resulted in improvement of growth rate, innate immune responses (e.g. lysozyme, complement system, etc.) and survival of the fish (Merrifield *et al.* 2010; Yousefian & Amiri 2009). On the other hand, despite health benefits observed in terrestrial animals and humans, little has been performed relating to the application of legume resistant starch (RS) as prebiotic in fish. Hence, this study aimed to investigate the effect of RS in fish.

Zebrafish (*Danio rerio*) were selected for current *in vivo* study as an established fish model, with lots of benefits such as ease in handling for breeding and experimentation, as well as the ability to consume of a wide variety of food (Ulloa *et al.* 2014). To date, only a handful of studies related to probiotics have been reported

in zebrafish, including supplementation of the combination of probiotics and prebiotics which resulted in increased growth and survival rates. The application of probiotics also improved innate immunity and hepatic stress tolerance (Gioacchini *et al.* 2014; Nekoubin *et al.* 2012). In addition, the supplementation of the dietary probiotic, *Bacillus coagulans,* also improved survival rates of zebrafish against *Vibrio vulnificus* infection by enhancing the expression of immune-related genes (Pan *et al.* 2013). However, studies on the supplementation of prebiotics such as RS is scarce.

Hence this chapter aims to investigate the effect of legume RS, namely from adzuki bean (*Vigna angularis*) and red lentil (*Lens culinaris*), on the growth performance of zebrafish. The outcome of this study will provide useful insights and knowledge for future studies on Asian sea bass.

4.1.1 Hypothesis

Legume RS and starch samples could enhance the growth performance of zebrafish through beneficial effects of the microbiome.

4.1.2 Aims

This study aims to investigate the effect of RS and starch from adzuki bean and red lentil on the growth performance of zebrafish.

- To investigate the effect of RS on the growth performance of zebrafish as measured by weight gain

4.2 Material and methods

4.2.1 Experimental overview

Zebrafish housed in the University of Liverpool were used in this study. Diets consisting of four samples of legume RS samples, namely adzuki bean enzyme-RS, adzuki bean untreated-S, red lentil enzyme-RS and red lentil untreated-S were prepared by the University of Liverpool and analysed in the University of Nottingham. Fish were fed for six weeks and fish growth was assessed at the end of the feeding trial.

4.2.2 Materials

4.2.2.1 Preparation of RS samples

Adzuki bean and red lentil enzyme-RS and untreated-S were prepared from legumes according to the methods described in Section 2.2.3.1 and 2.2.3.3 and are labelled as RS samples.

4.2.2.2 Preparation of zebrafish experimental diets

A total of five zebrafish diets, comprising of the enzyme-RS diet and untreated-S diet for adzuki bean and red lentils, and basal diet were formulated by Mr Kieran Magee at the University of Liverpool using an in-house formulation method. The diet formulations are shown in Table 4.1. Diets supplemented with red lentil untreated-S and enzyme-RS are labelled Lens ST and Lens EH respectively, while diets supplemented with adzuki bean untreated-S and enzyme-RS are labelled as Adz ST and Adz EH respectively. Initial diets aimed at inclusion levels ranging between 1.0% and 5.0%. However, due to limitations where several inclusion levels could not be tested, 2.5% was selected as midpoint in range.

After formulation, the ingredients required were mixed thoroughly using a Hobart food mixer. Water was added until the mixture achieved dough like consistency. The dough mixture was then spread out on trays and dried for 24 hr at 50 °C using a nine shelf Parallexx Excalibur food dehydrator. Once dried, the diet was crushed and processed through a series of sieves with apertures of 425 μm and 850 μm. The desired pellet size should within this range. All diets were stored at 4 °C till used.

Table 4.1: Ingredients of diet formulated by the University of Liverpool

Ingredient	RS/starch diet (% w/w)	Basal diet (% w/w)
Fish Meal	36.54	38.74
Rapeseed Oil	4.39	4.37
Vitamins	0.31	0.31
Minerals	0.41	0.42
Wheat Gluten	26.46	22.91
Corn Starch	27.82	31.82
Binder	0.51	0.52
RS samples	2.51	0.00
Lysine	1.01	0.90
Arginine	0.05	0.00
Total	100.00	100.00

4.2.3 Methods

4.2.3.1 Nutritional analysis of diets

Nutritional analysis of diets were conducted in the University of Nottingham. These samples included the Lens EH, Lens ST, Adz EH, Adz ST and the basal diets. The analysis conducted included energy, crude protein, lipid, crude fibre, moisture content and ash content and carbohydrates. Principles of analysis conducted are as listed below while details on the procedures are as listed in Appendix K.

a. Energy content analysis

Energy content analysis was performed using a bomb calorimeter (6400 Automatic Isoperibol Calorimeter, Parr Instrument Company), where the gross energy is measured in terms of heat produced when a sample is completely combusted completely into carbon dioxide and water.

b. Crude protein analysis

Protein analysis was performed via the Dumas method, which revolves around the conversion of all nitrogen forms into nitrogen oxides through combustion at 800-1000 °C, reduction of these forms to nitrogen gas and subsequent measurement by use of a thermal conductivity device (Jung *et al.* 2003)

.

c. Lipid analysis

Lipid analysis was performed by lipid extraction using a Sohxlet extractor based on the AOAC method 991.36, followed by gravimetric measurement of extracted sample.

d. Crude fibre analysis

Crude fibre content analysis was performed using the fibrebag (Gerhardt) method (Method no. AN-04-203), where starch and glucose were removed via digestion in acid and proteins were removed via digestion in alkali, with crude fibre left as the remaining residue.

.

e. Moisture content analysis

Moisture content of a samples were measured based on the AOAC method 935.29 based on the weight lost due to evaporation of water..

f. Ash content analysis

Ash analysis was performed based on the AOAC method 942.05 based on the inorganic residue remaining after the organic matter has been burnt away.

4.2.3.2 Maintenance of zebrafish housing conditions

Fish were housed in groups of 15 individuals in 15 identical 3 L zebrafish tanks. Five replicate tanks were given the same experimental diet with a total of five diets (as mentioned in Section 5.2.2)

The fish tanks were made by Aquatic Habitats, each measuring 25 cm x 10 cm x 15 cm (L x W x H). The tanks were connected to a central system which was maintained by a sump filtration system with 50% weekly water changes. Due to the small size of the fish, tanks were fitted with a 400 µm fry mesh baffle; cleaning was conducted weekly during the weighing of the fish to prevent further disturbances. Water quality was subsequently kept stable with the following parameters, Ammonia (NH_4); 0 mg/L, Nitrite (NO_2); 0 mg/L, Nitrate (NO_3); <50 mg/L and pH; 7.0. Fish were maintained at 28±1 °C and exposed to a 12:12 hr light cycle.

4.2.3.3 Measurement of zebrafish weight

Zebrafish were weighed once a week using a laboratory balance (Kern 770). In brief, all zebrafish from the tank was retrieved via net and transferred into a tarred container on the laboratory balance. Absorbent paper was used to remove excess water from the zebrafish while in net before being weighed.

4.2.4 Experimental design

All fish were bred in house and maintained at The Institute of Integrative Biology aquarium facility at the University of Liverpool. A total of 225 juvenile zebrafish (AB wild type strain), of approximately 70 mg in weight, were used in this trial and were approximately 2 months old at trial start. These zebrafish were allocated into five treatment groups with three tanks for each treatment (n=15) and an average fish weight of 70 mg. The tanks were arranged in a completely randomised design.

Treatment groups were fed diets containing 2.5% supplements of untreated-S, or enzyme-RS, or the basal diet. Diets were fed for the 6-week period, excluding the acclimatisation period prior to the feeding trial.

The zebrafish were fed once daily. At the start of each week, each tank of fish was weighed and one week's worth of daily feed was pre-measured up to four decimal places at 4% body weight per day using a laboratory balance (Kern 770). This was repeated weekly to maintain a 4% body weight feed regime throughout the 6-week trial for maximum growth and health. On the days when fish were weighed, feed was given after they were weighed, while on all other days feed was given in the morning.

4.2.4.1 Calculations

During the trial, the following feed efficiency and growth performance indicators were assessed: Feed conversion ratio (FCR), protein efficiency ratio (PER), weight gain (WG), increment (WG %), and specific growth rate (SGR). Calculations are based on Halver & Hardy (2002) and are as shown below:

i. Weight gain (WG):

$$WG = W_F - W_0$$

Where:

- W_F = weight of fish at the end of the feeding trial
- W_0 = weight of fish at the start of the feeding trial

ii. Increment (WG %):

$$WG\ \% = \frac{W_F - W_0}{W_0}$$

Where:

- W_F = weight of fish at the end of the feeding trial
- W_0 = weight of fish at the start of the feeding trial

iii. Specific growth rate (SGR):

$$SGR = \frac{\ln W_F - \ln W_0}{T}$$

Where:

- W_F = weight of fish at the end of the feeding trial
- W_0 = weight of fish at the start of the feeding trial
- T = feeding trial duration in days

iv. Feed conversion ratio (FCR):

$$FCR = \frac{\text{total weight of feed (g; dry weight basis)}}{\text{weight gain of fish (g; wet weight basis)}}$$

v. Protein efficiency ratio (PER):

$$PER = \frac{\text{weight gain (g)}}{\text{protein fed (g; dry weight basis)}}$$

4.2.5 Ethical issues

It was anticipated that the welfare standards of the subject fish would not be affected; they were being housed using standard methods and fed on balanced diets formulated to meet nutritional requirements. The fish were also bred at the University of Liverpool zebrafish facility and so endured minimal transport or housing stress prior to this experiment starting. However, welfare was monitored independently, if any individual fish exhibiting adverse reactions was removed from the trial.

4.2.6 Statistical analysis

All values reported are means ± standard deviation, unless otherwise stated. Statistical analysis was performed using Genstat 18th Edition. Normality of data was determined using the Shapiro-Wilk test, where statistical comparisons of non-normal data were performed using the Kruskal-Wallis H test. Statistical comparisons of normal data were performed using ANOVA between samples and statistical significance is indicated via labels of different letters.

4.3 Results

The zebrafish feeding trial was conducted to investigate the effect of RS on the growth performance of zebrafish. Diets were prepared with a 2.5% replacement of test diets with the prebiotics of interest, which were red lentil untreated-S, red lentil enzyme-RS, adzuki bean untreated-S and adzuki bean enzyme-RS. Proximate analysis was performed to determine the nutritional qualities of the diet, followed by a 6-week feeding trial using the diets mentioned above.

4.3.1 Nutritional analysis of the experimental diets of zebrafish

Replacement of basal diets with 2.5% legumes resulted in some changes in the nutritional qualities as shown in Table 4.2. Compared to the basal diets, Lens EH had significantly higher energy content (<2% higher). In terms of fat content, Adz EH and Lens ST had significantly lower fat as compared to the basal diet. In terms of protein content, all test diets contained significantly higher crude protein, with Lens ST being the highest. Lastly, as expected, crude fibre in test diets was significantly higher than the basal diet. However, Adz EH contained unexpectedly low crude fibre content

Table 4.2: Nutritional composition of diets used in zebrafish feeding trial for basal diets and basal diets supplemented with 2.5% of samples

	Analysis [1]						
Diet	Calories (kJ/100g)	Fat, total (%)	Protein/ crude protein (%)	Crude fibre (%)	Moisture (%)	Ash (%)	NFE (%) [2]
Basal diet	2081 ± 40 [b]	7.57 ± 0.02 [a]	50.66 ± 0.24 [c]	0.00 ± 0.00 [d]	3.23 ± 0.14 [c]	7.06 ± 0.01 [a]	31.49
Lens EH	2118 ± 13 [a]	7.56 ± 0.18 [a]	58.69 ± 1.82 [b]	0.45 ± 0.13 [b]	2.97 ± 0.13 [c]	6.50 ± 0.04 [c]	23.83
Adz EH	2093 ± 5 [ab]	6.43 ± 0.01 [c]	55.62 ± 1.49 [b]	0.05 ± 0.06 [c]	3.91 ± 0.23 [b]	6.64 ± 0.08 [bc]	27.35
Lens ST	2101 ± 7 [ab]	6.99 ± 0.08 [b]	62.78 ± 2.56 [a]	0.50 ± 0.04 [b]	3.75 ± 0.08 [b]	6.50 ± 0.09 [c]	19.49
Adz ST	2076 ± 15 [b]	7.48 ± 0.23 [a]	55.60 ± 0.53 [b]	0.53 ± 0.03 [a]	4.37 ± 0.02 [a]	6.69 ± 0.05 [b]	25.33

Notes:

[1] Values reported are means ± standard deviation; n=3.

[2] Nitrogen-free-extract (NFE) was calculated by subtracting all values from 100%

[a-d] Within a column, values with different superscripts are significantly different at $P < 0.05$.

4.3.2 Effect of diets supplemented with RS and untreated starch from adzuki beans and red lentils on the growth of zebrafish

The results of the feeding trial are described in 2 categories: 1) growth and health parameters, and 2) feed conversion and efficiency ratios and are as shown in Table 4.3.

The growth and health measurements of the fish includes its total weight gain (WG), the growth increment in terms of percentage (WG %) and specific growth rate (SGR). No significant effect of diet was seen on any of these parameters.

In comparing FCR across all diets, significant differences were observed in fish fed with different diets ($p < 0.05$). The lowest FCR was observed in fish fed with Lens ST at 1.76, followed by fish fed with Lens EH and Adz ST with both at 1.83, all of which was significantly lower than the fish fed with basal diet of 2.01.

In terms of PER, there was no significant differences in fish fed with the different diets ($p > 0.05$).

Overall, no differences in growth were observed in zebrafish when fed with diets supplemented with RS regardless of legume and treatment applied. However, higher FCR was observed when fed with untreated-S from red lentils which suggest that these supplements could be beneficial to zebrafish health. Meanwhile, the supplementation of RS did not appear to have a negative effect in the zebrafish in terms of growth.

Table 4.3: Growth and feed conversion and efficiency ratios of zebrafish after 6 weeks of being fed with a basal diet and diets with 2.5% supplement with various legumes and respective treatments

	Growth and health measurement [1,2]				Feed conversion and efficiency ratios [1,2]	
Diet	Weight gain (mg)	Increment (%)	Specific growth rate (%)	Survival (%)	Feed conversion ratio	Protein efficiency ratio
Basal diet	85.11 ± 12.55	221.20 ± 10.26	1.93 ± 0.11	100	2.01 ± 0.12 [b]	0.99 ± 0.05
Lens EH	90.67 ± 6.77	237.27 ± 10.70	2.11 ± 0.11	100	1.83 ± 0.09 [ab]	0.93 ± 0.04
Adz EH	98.89 ± 25.87	224.78 ± 15.28	1.97 ± 0.17	100	1.98 ± 0.13 [b]	0.91 ± 0.06
Lens ST	97.33 ± 6.43	242.13 ± 7.12	2.16 ± 0.07	100	1.76 ± 0.07 [a]	0.90 ± 0.04
Adz ST	108.22 ± 33.15	240.75 ± 7.78	2.14 ± 0.08	100	1.83 ± 0.09 [ab]	0.99 ± 0.05

Notes:

Values reported are means ± standard deviation; n=3.

[a-d] Within a column, values with different superscripts are significantly different at $P < 0.05$.

4.4 Discussion

Although the use of prebiotics in fish is much more poorly studied than in mammals , several studies have shown beneficial prebiotic effects in aquatic animals (Ganguly *et al.* 2013). These benefits include improved live body weight gain, improved immune responses, increased feed efficiency and improved GIT microbial populations. (Lv *et al.* 2007; Mahious *et al.* 2006; Li & Gatlin III 2004). These reports suggest that the inclusion of prebiotics could play a role to improve the overall effectiveness of fish feed.

In this study, the 2.5% inclusion of adzuki bean and red lentil RS and starch did not significantly affect the growth of zebrafish. Meanwhile, inclusion of other prebiotics, such as MOS was found to increase growth of zebrafish when fed for six weeks (Forsatkar *et al.* 2017). A possible cause for the lack of growth observed in this study could be reduced growth rates observed as the fish approaches sexual maturity, or approximately 60 days post fertilization (Gómez-Requeni *et al.* 2010). Due to lower growth rates, the effect of different diets on the growth may be less obvious, resulting in no significant difference following statistical analysis. For the same reason, most studies investigate the effect on diets on zebrafish in the larvae stage and this can be put into consideration for future feeding trials (Nekoubin *et al.* 2012; Kaushik *et al.* 2011). As this study aimed to investigate the microbiota in the GIT of zebrafish, fish were required to be of sufficient size for ease of dissection and may have indirectly resulted in less obvious growth observations, in addition to the time constraints faced when the trial was carried out.

Another factor to consider is a possible lack of beneficial bacteria overall in the rearing waters or intestines of the zebrafish in this study. In such cases, several

studies find that the re-establishment of the balance between beneficial and pathogenic bacteria via probiotics may be effective (Wang *et al.* 2008). To overcome the limitations of probiotics, several studies have combined both probiotics with prebiotics as synbiotics to be supplemented in feed (Nayak 2010). In a study comparing the effect of the supplementation of the synbiotic Biomar on zebrafish larvae, significant improvements were observed in growth, SGR and FCR when diets were supplemented with up to 1.5 g/kg of the synbiotic (Nekoubin *et al.* 2012). It was suggested that the combination of both probiotics and prebiotics in the diet, which was not specified by the author, may have contributed to extracellular enzyme production which assisted in increased nutrient utilisation, resulting in the improved growth (Nekoubin *et al.* 2012). Hence, the application of synbiotics may be another strategy to be considered for future studies.

In this study, improved FCR were observed in zebrafish following supplementation of RS and starch from red lentils and adzuki beans in diets. However, the FCR measured in this study may not be conclusive due to the nutritional content variation found in the diets fed. As a key nutrient contributing to growth, the variation of protein content in diets may have influenced fish growth: in this study, fish fed diets with approximately 4% protein was found to have higher weight increment as well as specific growth rates, which may have affect FCR values. Similar results can also be seen in other studies where fish grow better and possess reduced FCR when fed with more protein (Sissener *et al.* 2010; Jauncey 1982). Meanwhile, while feeding to satiation was the preferred method for FCR analysis due to accuracy in measured feed intake (Robaina & Izquierdo 2000), the 4% body weight feeding was based on optimisation and was found to be most effective for zebrafish growth while having little variation when compared to feeding to satiation (K Magee 2016, pers. comm.

12 Feb). Nevertheless, future studies should incorporate measurement of feed intake to reduce potential inaccuracies in FCR measurements.

While prebiotics applied in other studies have ranged from 1 – 75 g/kg supplementation of feed (Ringø *et al.* 2010b), it is possible that further benefits may be observed with higher levels of RS and starch supplementations. However, higher growth was observed in studies with reduced levels of starch (7.7% vs 12.80%) (Siccardi III *et al.* 2009). This suggests that zebrafish may have a specific tolerance level of starch content in diets, which may be useful for consideration in future studies. While Siccardi III *et al.* (2009) did not elaborate on the effect of different level of starches, the author suggested that trace minerals and ingredient sources may play a role in physiological, cellular or molecular processes which contribute to differences in growth rates. In addition, the author also highlighted the importance of Fulton's condition factor as an indicator of fish health in selecting a diet (Siccardi III *et al.* 2009). In the current study, none of the diets appeared to have any detrimental effect on the fish health.

Based on the parameters of this study, it is suggested that RS supplementation did not affect growth. Meanwhile, it is inconclusive whether RS and starch improved FCR. In addition, the enzyme-hydrolysis treatment did not appear to improve the benefits provided by the legume starch. It could be that the higher crude protein in Lens ST eventually resulted in higher growth for the zebrafish as a primary source of growth (Ulloa *et al.* 2014). In addition, as untreated-S is less resistant towards digestion versus enzyme-RS, it may have been utilised by the zebrafish as a nutrient as zebrafish are capable of carbohydrate utilisation (Robison *et al.* 2008). If that is the case, enzyme-RS may be a better candidate as prebiotic in terms of surviving

host digestion and improving bacteria growth, as shown in the previous chapter (Zaman & Sarbini 2015). Lastly, it was undetermined whether the RS supplementation provided health benefits to the zebrafish, such as through improved complement activity or respiratory bursts and should be included in future studies based on effects shown from other prebiotics (Guerreiro *et al.* 2017b).

.

While zebrafish may serve as a candidate animal model, further studies are required on other species as well. A key factor is that the effect of prebiotic supplementation often varies between species (Ganguly *et al.* 2013; Ringø *et al.* 2010b). In addition, not many studies on RS in aquaculture have been reported. As the feeding trial on zebrafish showed no detrimental effects on health, the RS and starch applied in this study could be further applied in other species. Asian sea bass are one of the main aquaculture species reared in Malaysia and any improvements in growth efficiency could have significant economic and environmental benefits. In addition, the role of microbes in such carnivorous fish is less studied (Gatesoupe 2005). Hence, the studied described in the next chapter investigates the effect of red lentil enzyme-RS on the health and growth of this commercial aquaculture species.

4.5 Summary

In summary, the inclusion of adzuki beans and red lentil untreated-S and enzyme-RS did not affect the growth but may have an effect on the feed conversion ratios of zebrafish during a 6-week feeding trial. Inclusion of RS and starch had no apparent adverse effects on the growth and health of the fish. It was therefore decided to extend the investigation the effect of red lentil enzyme-RS on the health and growth benefits in a commercial aquaculture species, Asian sea bass.

5 Chapter 5: Effect of legume resistant starch on the growth performance of Asian sea bass (*Lates calcarifer*)

5.1 Introduction

The introduction of aquaculture as an alternative source of protein has reduced the reliance of ocean capture for seafood. However, the intensification of the industry has resulted in several issues, such as increased demand of ocean by-catch as food for aquaculture species, as well as pollution and the development of antibiotic-resistant strains of bacteria due to intensification. Studies have begun to investigate the alternative diets for aquaculture species involving lesser known crops to replace the reliance on fishmeal. Furthermore, the use of prebiotic and probiotics as alternatives to the application of antibiotic in farms has gained increasing interest (Glencross *et al.* 2016; Ibrahem *et al.* 2010; Burr *et al.* 2008; Gatesoupe 2005; Panigrahi *et al.* 2005). As aquaculture represents an important and growing industry in Malaysia, such studies are important for the sustainability and growth of the sector.

Asian sea bass (*Lates calcarifer*) is commonly farmed in the Southeast Asia and is mainly produced in Indonesia, Malaysia and China. In 2010, up to 66,694 tonnes were produced with approximately 20,000 tonnes from Malaysia (FAO 2017; DOFM 2013). Farmed Asian sea bass were marketed at approximately 500 g to 900 g and is commonly consumed locally (FAO 2017c). While these fish were commonly fed fishmeal-based diets, some studies investigate potential replacement of fishmeal as alternative protein sources (Glencross *et al.* 2016; Boonyaratpalin *et al.* 1998). In addition, the use of several novel types of feed additives to improve growth and health were also investigated. The application of edible mushroom (*Schizophyllum commune*) at 1% inclusion rates improved serum antibody levels and reduced

mortality rates when challenged with *Vibrio harveyi* via intraperitoneal injection (Chong *et al.* 2016). On the other hand, the supplementation of neem leaves (*Azadirachta indica*) at 0.5% improved growth as well as immune parameters, such as serum lysozyme and bactericidal activity (Talpur & Ikhwanuddin 2013). These studies suggest that these feed supplements can potentially be used for the benefit of Asian sea bass in aquaculture farms.

As mentioned previously, studies on the use of resistant starch (RS) as a prebiotic on aquaculture species is limited and, to our knowledge, no such studies in Asian sea bass have been published. RS has been shown to be an effective prebiotic in terrestrial animals, via modulations of gastrointestinal tract (GIT) microbiota in rats and humans (Rodríguez-cabezas *et al.* 2010; Lesmes *et al.* 2008; Queiroz-monici *et al.* 2005). It has also been shown to improve acetate production in seabass (*Dicentrarchus labrax*) (Gatesoupe *et al.* 2014). The studies described in Chapters 3 and 4 showed that enzyme-RS produced from red lentils (*Lens culinaris*), as a prebiotic, was more effective than other legumes in improving bacteria growth while also potentially improving feed conversion ratios in zebrafish (*Danio rerio*). Hence, the current study aimed to investigate the effect of red lentil enzyme-RS as a sustainable prebiotic ingredient for aquafeed for Asian sea bass. Furthermore, bacteria from the GIT in the fish was also measured using conventional methods.

5.1.1 Hypothesis

Legume resistant starch samples improve the growth of GIT probiotic lactic acid bacteria (LAB), reduce the growth of bacteria from Enterobacteriaceae (ETB) family and thus enhance the growth performance of Asian sea bass.

5.1.2 Aims

This study aims to investigate the effect of RS from red lentil on the growth performance and intestinal health of Asian sea bass.

Specific aims:

- To investigate the effect of RS on the growth performance of Asian sea bass as measured by weight gain
- To investigate the effect of RS on the GIT microbial population

5.2 Materials and methods

5.2.1 Experimental overview

Asian sea bass housed in the CFF field site were used in this study. Both basal and EH-Lens diets were also prepared in the CFF field site. Fish were fed for five weeks and fish and growth and GIT bacterial populations were assessed at the end of the feeding trial.

5.2.2 Materials

5.2.2.1 Preparation of RS samples

Red lentil enzyme-RS and untreated-S were prepared from legumes according to the methods described in Section 2.2.3.1 and 2.2.3.3 and are labelled as RS samples.

5.2.2.2 Preparation of Asian sea bass experimental diets

A total of two Asian sea bass diets, namely red lentil enzyme-RS diet and basal diet were formulated in the Crops for the Future (CFF) field site with the assistance of Dr. Kumar Katya which were described to be adequate for sea bass growth in experimental feeds (Glencross 2006). Diet formulations are shown in Table 5.1. Diets supplemented with red lentil enzyme-RS were labelled as EH-Lens.

After formulation, the ingredients were combined in an industrial mixer to form a paste and mixed until homogenous. The homogenous feed paste was then passed through a meat mincer to produce noodle-like strands (3 mm ⌀) and were collected in large plastic trays. These strands were then broken down to short pellet lengths of approximately 5 - 10 mm carefully and was subsequently dried in an oven set at 45 °C for approximately 3 days. Twice a day, the trays were removed from the oven for

the pellets to be turned over to allow drying on both sides. Dried feeds were collected in re-sealable plastic bags and stored at 4 °C until use.

Table 5.1: Ingredients of diets for Asian sea bass formulated with assistance from CFF

Ingredient	EH-Lens (% w/w)	Basal diet (% w/w)
Fish meal	54.795	56.200
Starch samples	2.500	0.000
Dextrin	14.430	14.800
Soybean meal	11.310	11.600
Corn meal	6.240	6.400
Fish oil	5.850	6.000
Vitamin premix	2.925	3.000
Mineral premix	1.950	2.000
Total	100.000	100.000

5.2.3 Methods

5.2.3.1 Nutritional analysis of diets

Nutritional analysis of diets were performed by ALS Technichem Sdn. Bhd, which included energy, fat, protein, moisture, ash, carbohydrate and dietary fibre. The analysis conducted and their respective principles are as listed in Section 2.2.3.4. Resistant starch content was measured using the Megazyme method as detailed in Section 2.2.3.2. b.

5.2.3.2 Maintenance of Asian sea bass housing condition

Glass aquariums of 120 L were used in this study and were separated into two halves of 60 L each. Two diets were prepared and were fed in triplicate to groups of four fish, for a total of six groups. The six groups of fish were then randomly allocated to half a tank each. The experimental design is further elaborated in Section 5.2.4.

A 10-hr light and 14-hr dark cycle was used, while water temperature was unregulated and kept at ambient room temperature of approximately 27 °C. Oxygen was provided by gentle aeration via fountain using a submersible aquarium air ball. Water quality was maintained by constant water exchange via recirculation system, which included sponge filtration of water. The siphoning of faecal debris was also performed daily, while uneaten feed was siphoned after feeding. Several conditions were not regulated, including the temperature and ammonia and nitrogen content of the water in the holding tank.

5.2.3.3 Measurement of Asian sea bass weight

Asian sea bass were weighed once a week using a laboratory balance (Sartorius ENTRIS 2202-1S Digital Scale). In brief, each fish was retrieved via net and transferred into a tarred container on the laboratory balance. Absorbent paper was used to remove excess water from the fish before it was weighed.

5.2.3.4 Animal euthanasia

Asian sea bass were euthanized humanely via anaesthesia with benzocaine, followed by destruction of the brain, as per Schedule 1 of the UK Animals (Scientific Procedures) Act 1986. The procedure is as described in Section 5.2.2.1 with dark blue pails (approximately 30 cm Ø) containing 5 L of benzocaine solution (0.35% v/v) being used to accommodate the difference in fish size.

5.2.3.5 Measurement of bacteria composition of fish

Intestines were extracted from Asian sea bass from each treatment. Samples were used in the isolation of bacteria, which were spread onto M17 and Eosin methylene

blue (EMB) agar as detailed in Section 2.4.3 (n=6). All intestine samples from each treatment were subject to identical procedures.

Measurement of bacteria composition of fish was performed using conventional spread plate. Bacteria spread onto M17 agar and EMB agar were quantified as LAB and ETB respectively. M17 agar was used instead of MRS due to higher efficiency of isolating LAB from Asian sea bass.

5.2.3.6 Nutritional analysis of fish carcass

Nutritional analysis of diets were performed in the University of Nottingham Malaysia Campus. The analysis conducted and their respective principles are as listed below while details on the procedures are as listed in Appendix K.

a. Crude protein analysis

Protein analysis was performed via the Kjeldahl method (Buchi K-360 Distillation Unit), supplemented with a digester (Buchi KjelDigester K-446) based on the AOAC method 976.06-1977 for the analysis of crude protein in animal feed.

b. Crude lipid analysis

Lipid analysis was performed by lipid extraction using a Sohxlet extractor based on the AOAC method 991.36, followed by gravimetric measurement of extracted sample.

c. Moisture content analysis

Moisture content of a samples were measured based on the AOAC method 935.29 based on the weight lost due to evaporation of water content.

5.2.4 Experimental design

Asian sea bass were purchased from BD Aquaculture Sdn. Bhd, Johor, Malaysia. A total of 24 fingerlings which were approximately 2.5 inches in length and 10 g in weight at the start of the trial were used. The Asian sea bass were allocated to two treatment groups (basal diet and diets supplemented with red lentil enzyme-RS) with three tanks for each treatment (n=4) and an average fish weight of 10 g. The tanks were arranged in a completely randomised design. Diets were fed for a 5-week period, excluding the acclimatisation period prior to the feeding trial.

The Asian sea bass were fed twice daily. After each tank of fish had been weighed, one week's worth of daily feeds was premeasured using a laboratory balance (Sartorius ENTRIS 2202-1S Digital Scale) up to two decimal places at 4% body weight per day. This was repeated weekly to maintain a 4% body weight feed regime throughout the 5-week trial for maximum growth and health. On the days when fish were weighed, feed was given after fish had been weighed and in the evening, while on all other days feed was given in the morning and evening.

At the end of the trial, the fish were sacrificed according to the procedure in Section 5.2.3.4. Upon dissection, viscera were removed from the fish. The entire GIT was separated from the viscera and the intestines were snipped from the end stomach to the anus of the fish and was transferred into weighed sterile 1.5 mL centrifuge tubes and stored on ice. Then, the entire fish was measured (Appendix L) transferred into a sterile 50 mL centrifuge tube and stored on ice. All samples were stored on ice no longer than 2 hr before being processed for measurement of bacterial composition of fish as described in Section 5.2.3.5.

Fish carcass from each treatment were pooled together and were selected at random for nutritional analysis of fish carcass, which was performed as described in Section 5.2.3.6. A total of three fish were used for the measurement of moisture content while the remaining fish were lyophilized and milled for analysis of crude protein and crude lipid content of fish carcass.

5.2.4.1 Calculations

During the trial, the following feed efficiency and growth performance indicators were assessed: feed conversion ratio (FCR), protein efficiency ratio (PER), weight gain (WG), increment (WG %), and specific growth rate (SGR) (Halver & Hardy 2002). Details of the calculations are as shown in Section 4.2.4.1.

5.2.5 Ethical issues

The Asian sea bass feeding trial was conducted under the approval (Ref. No. UNMC8) of the Animal Welfare and Ethical Review Body of the University of Nottingham

5.2.6 Statistical analysis

All values reported are means ± standard deviation, unless stated otherwise. Statistical analysis was performed using Genstat 18th Edition. Normality of data was determined using the Shapiro-Wilk test, where statistical comparisons of non-normal data were performed using the Kruskal-Wallis H test. Statistical comparisons of normal data were performed using ANOVA between samples and statistical significance is indicated via labels of different letters.

5.3 Results

5.3.1 Nutritional analysis of the experimental diets of Asian sea bass

Diets formulated for the Asian sea bass feeding trial were not designed to be identical in nutritional qualities due to the replacement supplementation of the test diet. However, the diets did not vary in nutritional quality as shown in Table 5.2.

As the analysis of nutritional composition of both diets was done without replicates, significant differences were not measured. Notable differences were observed in carbohydrate and dietary fibre content, with EH-Lens diets containing 6.87% and 8.54% higher content respectively. Also, RS content in the EH-Lens diet was significantly higher, at 0.23% versus the basal diet.

Table 5.2: Nutritional composition of diets used in Asian sea bass feeding trial for basal diets and basal diets supplemented with 2.5% of samples

	Diet	
Analysis [1]	Basal diet	EH-Lens
Energy (by calculation, kcal/100g)	398	396
Energy (by calculation, kJ/100g)	1672	1665
Fat, total (%)	13.6	13.3
Protein/ crude protein (%)	47.2	45.9
Carbohydrate, total (by calculation, %)	21.7	23.3
Dietary fibre (%)	7.5	8.2
Moisture (%)	8.7	8.5
Ash (%)	8.8	9.0
RS content (%)	0.10 ± 0.02 [a]	0.33 ± 0.05 [b]

Notes:

[1] Analysis of samples except resistant starch content (%) was performed by ALS Technichem Sdn Bhd without replicates (n=1). Values of RS content are means ± standard deviation; n=3.

[a-b] Within a column, values with different superscripts are significantly different at $P < 0.05$

5.3.2 Effect of diets supplemented with RS from red lentils on the growth of Asian sea bass

The data collected from the Asian sea bass feeding trial can be categorised into the following categories: 1) fish growth measurements and feed conversion ratios, 2) bacteria composition of fish and, 3) nutritional analysis of fish carcass.

5.3.2.1 Fish growth measurements and feed conversion ratio

A feeding trial on Asian sea bass was conducted using 2 types of diet, as shown in Table 5.3. The results are categorised into 2 categories: 1) growth and health parameters, and 2) feed conversion and efficiency ratios and are as shown in Table 6.3.

The growth and health measurements of the fish include total weight gain (WG), the growth increment in terms of percentage (WG %) and specific growth rate (SGR). No significant differences were observed in any of the growth parameters measured. Slight contradiction exist between weight gain and specific growth rates, which could be attributed to variation in starting weight of the fish. In addition, fish health appeared to be similar with Fulton condition factors of 2.14 and 2.01 for Asian sea bass fed with EH-Lens and the basal diet respectively.

The measurements of feed conversion and efficiency ratios includes feed conversion ratio (FCR) and protein efficiency ratio (PER). No significant differences were also observed in all conversion ratios measured.

Overall, the growth and health of Asian sea bass fed with diets supplemented by red lentil enzyme-RS were similar with basal diet.

Table 5.3: Growth, health and feed conversion and efficiency ratios of Asian sea bass after 6 weeks of being fed with a basal diet and diets with 2.5% supplement of EH-Lens

	Growth and health measurement [1,2]				Feed conversion and efficiency ratios [1,2]	
Diet	Weight gain (g)	Increment (%)	Specific growth rate (%)	Survival (%)	Feed conversion ratio	Protein efficiency ratio
Basal diet	8.45 ± 1.70	192.10 ± 20.82	2.24 ± 0.36	100	2.15 ± 0.37	1.01 ± 0.18
Lens EH	8.82 ± 2.40	186.55 ± 17.17	2.14 ± 0.31	100	2.33 ± 0.42	0.96 ± 0.18

Notes:

Values reported are means ± standard deviation; n=3.

[a-d] Within a column, values with different superscripts are significantly different at $P < 0.05$.

5.3.2.2 Measurement of bacterial composition of fish

Bacteria from the intestines of Asian sea bass fed with different diets were isolated and spread on M17 agar for LAB and EMB for bacteria in the family of ETB (such as *E. coli*, *Salmonella* etc.) and are as shown in Table 5.4.

Based on the measurements obtained via conventional spread plate, fish fed with EH-Lens showed no significant effects from diet based on the bacteria count.

Table 5.4: GIT LAB and ETB count of Asian sea bass fed basal diet and EH-Lens diet

	Analysis [1]	
Diet	LAB (log_{10} CFU/ml)	ETB (log_{10} CFU/ml)
Basal diet	5.15 ± 1.43	5.30 ± 0.61
EH-Lens	5.30 ± 1.10	5.02 ± 1.33

Notes:

[1] Values reported are means ± standard deviation; n=3.

5.3.2.3 Nutritional analysis of fish carcass

Nutritional analysis of the fish carcass was performed and the results are presented based on the wet weight of the sample (Table 5.5)

Table 5.5: Crude protein, total fat and moisture content of Asian sea bass carcass (wet weight basis)

	Analysis [1]		
Diet [3]	Protein/ crude protein (%)	Fat, total (%)	Moisture (%)
Basal diet	15.91 ± 0.25	2.73 ± 0.10 [a]	75.46 ± 1.65
EH-Lens	16.16 ± 0.46	4.75 ± 0.20 [b]	74.92 ± 0.18

Notes:

[1] Values reported are means ± standard deviation; n=3.

[a-b] Within a column, values with different superscripts are significantly different at $P < 0.05$.

Overall, no significant differences were observed in protein content or moisture content of Asian sea bass when fed different diets. However, the total fat content (% w/w) of the carcass was found to be significantly higher for Asian sea bass fed EH-Lens, which was 2.03% higher than Asian sea bass fed the basal diet.

5.4 Discussion

In this study, the effect of RS supplementation in fish feed was investigated in a fish feeding trial involving the aquaculture species Asian sea bass. Reported benefits of supplementation with prebiotics improved growth, immune parameters and overall survival rates (Ringø *et al.* 2010b).

One of the desired traits for fish in aquaculture is increment in growth. Prebiotic supplementation has been suggested to improve growth, via the improvement of nutrient digestibility via increments in populations of beneficial microbes. This results in microbial metabolism of substrates and allowing these products to be accessible as nutrients for the host, and thus improving nutrient digestibility (Burr *et al.* 2005). Such effects have been exhibited in studies involving supplementation with galacto-oligosaccharides (GOS) and mannan-oligosaccharides (MOS) into the diets of red drum (*Sciaenops ocellatus*). This was shown to improve nutrient digestibility either via the mitigation of anti-nutritional factor or through a microbial-mediated pathway (Burr *et al.* 2008). On the other hand, other studies which involved the inclusion of stachyose and raffinose as a prebiotic in Atlantic salmon (*Salmo salar*), showed no effect on nutrient digestibility (Sørensen *et al.* 2011). In addition, contrasting information has been presented on whether prebiotics induce increased enzyme production in the intestines (Anguiano *et al.* 2013; Xu *et al.* 2009b). Hence, it can be said that further understanding on the relationship between a prebiotic and the host may be necessary to understand its potential for improving nutrient digestibility.

In this study, it was found that the supplementation of RS in diets as a prebiotic did not significantly affect the growth of Asian sea bass. Previous studies on the

supplementation of inulin showed significant improvement of growth, which was suggested to be due to the improvement of nutrient digestion and absorption (Ali *et al.* 2016). On the other hand, studies on other prebiotics including fructo-oligosaccharides (FOS) failed to show any improved growth of the fish (Ali *et al.* 2017a). Meanwhile, other studies report FCR content between 1.47 to 1.80, which was significantly lower than the 2.15 reported in this study (Ali *et al.* 2017a; Ali *et al.* 2016). This may be caused by improper feeding practices which led to feed wastage, which was incorrectly measured as feed consumed. Hence, it is highlighted that the estimation of nutritional requirements, including FCR requires fish to be fed to satiation instead of providing fixed feeding regimes (Robaina & Izquierdo 2000). Nevertheless, the overall lack of effect were attributed to other possible factors, such as variation dependent on fish species, feeding duration the type of prebiotics (Ta'ati *et al.* 2011).

The lack of improvement in growth of Asian sea bass could be due to insufficient change of the microbiota population, as no significant changes were detected in the populations of LAB in this study. A contributing factor to the increment of growth following the supplementation of prebiotics has been attributed microbial metabolism of indigestible substrates to be accessible as nutrients for the hosts. A few studies have demonstrated the effect of probiotic-inclusion in feeds, in which the modification of microbiota populations led to significant increment in weight gain and other growth parameters measured (Mohapatra *et al.* 2013; Son *et al.* 2009; Yanbo & Zirong 2006). These studies suggest that the improvement of microbiota in the GIT can improve growth of fish. However, the alternative strategy introduced in this study of using prebiotics to stimulate beneficial bacteria growth did not produce

similar results, suggesting that there are other factors which may have to be addressed.

Another possible factor influencing the lack of effect following RS supplementation in diets may be the lack of LAB present in the GIT of the Asian sea bass used in this study. LAB are often associated with beneficial effects to the host, such as such as antagonising pathogenic bacteria as well as general improvement in health which has been exhibited in several studies (Ringø *et al.* 2010a; Gatesoupe 2008; Balcázar *et al.* 2007b). While LAB are usually present in the GITs of aquatic animals at low population levels, the actual relationship between the bacteria, its host and the environment are poorly understood (Gatesoupe 2008). In addition, studies have shown that only specific species of LAB stimulate beneficial effects in Asian sea bass (Rengpipat *et al.* 2008). Furthermore, due to variations in microbiota populations in rearing waters, it may also be possible that beneficial species of LAB may be lacking in the GIT of the Asian sea bass used in this study. Hence, while it is unclear which species of LAB may be beneficial to Asian sea bass, further studies should be performed, using next-generation-sequencing, to verify the presence and identity of LAB associated with improved health and growth of the fish (Ghanbari *et al.* 2015). Once the beneficial bacteria have been positively identified, further studies investigating the role of probiotics in enhancing their growth could be performed.

In this study, the populations of LAB and ETB were enumerated as indicators of potential probiotic bacteria and potential pathogenic bacteria. The use of culture-dependent methods in earlier parts of the study allowed isolation of potential probiotic bacteria and hence, the method was applied again in this study to estimate the populations of potential probiotic bacteria.

The supplementation of diets with EH-Lens resulted in no significant shift in bacterial populations. The current results differ from a previous study which demonstrated significant effects of slowly-digestible-starch on microbial composition of European sea bass significantly when compared to the basal diet (Gatesoupe *et al.* 2014). Different levels of inclusion may have resulted in the shift in intestinal microbial composition, which could be considered in future studies. In addition, several other studies have shown that the microflora in Asian sea bass are susceptible to changes based on the diets consumed. In the study of Ali *et al.* (2016), the supplementation of inulin led to an increment in bacteria not previously detected in Asian sea bass. In another study, the replacement of fishmeal with plant proteins also led to an increment of LAB in Asian sea bass (Apper *et al.* 2016). These studies suggest that the microflora of Asian sea bass can be modulated, contradicting results from this study.

The 2.5% inclusion rate of RS used in this study may have been insufficient to initiate an effect. Previous work has shown that, supplementation with FOS does not elicit a prebiotic effect in Asian sea bass until increased to 1% (Ali *et al.* 2017a). While FOS required only 1% to elicit an effect, this level of RS required may differ due to differing nature of prebiotics, as well as other factors as well (Ta'ati *et al.* 2011). Hence, future studies at increased inclusion levels of RS may demonstrate specific effects in Asian sea bass. In the case of Asian sea bass, studies by Glencross found that diets could have a starch content of up to 30% without significantly affecting protein digestibility of the fish (Glencross *et al.* 2012). However, it has been suggested that the levels of RS supplemented in feed may require regulation to prevent unwanted stimulation of opportunistic bacteria (Gatesoupe *et al.* 2014).

Fish represent an important protein source for many consumers. Whole body composition of the fish is often used as an indicator of quality of the fish as a food product and as a function of nutritional properties (Mumba & Jose 2005). A key nutritional quality attributed to fish is the protein content. In this study, analysis of Asian sea bass carcass showed no changes in crude protein content following the supplementation with RS. Similar results were obtained for Asian sea bass fed with diets supplemented with up to 1% MOS (Ali *et al.* 2017b). On the other hand, other studies which supplemented with inulin and FOS in diets resulted in significant increment in crude protein content for both prebiotics (Ali *et al.* 2017; Ali *et al.* 2016). It is generally accepted that fish whole body protein content is controlled within narrow limits based on their weight and life cycle stages (Shearer 1994). Nevertheless, as no changes were observed in protein efficiency ratios, the Asian sea bass fed with RS in this study did not appear to convert feed into protein more effectively, hence resulting in no changes in carcass protein content.

In this study, total fat content of fish carcass fed with the RS-supplemented diet was significantly higher compared to fish fed the basal diet. While similar results were observed when sea bass were fed 0.5% FOS, the results appear to deviate from general proximate composition of fish carcass, where protein and ash contents can be predicted accurately according to regression models while water and lipid contents vary (Ali *et al.* 2017a; Shearer 1994). Meanwhile, sea bass fed diets with inulin did not result in changes in lipid and ash content (Ali *et al.* 2016). In most studies, differences in fat content of fish muscle are often attributed to variations in dietary lipid and/or energy content (Grigorakis 2007; Alasalvar *et al.* 2002; Shearer 1994). However, in the present study both diets had similar fat and energy contents. It appears unlikely that the effect is due to dietary fibre which has been reported not

to influence on the fat content of fish carcass (Shearer 1994). Hence, due to the unusual trend of lipid content increment, further work is encouraged to verify the effect of RS on the composition changes of fish carcass or possible analytical errors.

Studies on the application on other prebiotics in other fish species resulted in both increase and decreases on fat content in carcass. Many studies performed on prebiotics resulted in no significant changes for fat content in fish carcass. It was suggested that the supplementation of polysaccharides entrapped bile salts, reducing lipid solubilisation and hence preventing lipid accumulation (Sinha *et al.* 2014). However, the supplementation of 0.2% MOS in giant sturgeon (*Huso huso*) increased crude lipid carcass, which has been attributed to improved lipid utilisation (Mansour *et al.* 2011). This may have been the case resulting in the increased crude lipid content of the carcass. Nevertheless, as lipid content is a significant contributor to meat composition and quality, and therefore taste and texture, further studies should be conducted to investigate the effect of increased crude lipid content in Asian sea bass carcass (Grigorakis 2007). Investigation into the composition of these lipids may also reveal potential accumulation of omega-3 long-chain polyunsaturated fatty acids, a desired trait from marine fish consumption in general.

5.5 Summary

In summary, the supplementation of the diets of Asian sea bass with EH-Lens did not result in significant improvements of growth after a 5-week feeding trial. Microbial composition of the GIT was not significantly altered as well. Lastly, it is inconclusive if the lipid content of the Asian sea bass carcass was affected, while protein and moisture content remain unchanged.

6 Chapter 6: General discussion and conclusion

6.1 Introduction

In the Southeast Asia region, Asian sea bass (*Lates calcarifer*) is an important aquaculture species produced in Malaysia, Thailand, and Indonesia (FAO 2017a). As a growing industry, fish aquaculture faces the issue of disease susceptibility because of intensification, resulting in reduced growth rates and high mortality (Naylor *et al.* 2000). A potential solution to optimise production, via eco-friendly methods without the use of antibiotics, is potentially through the use of functional food ingredients, including probiotics and prebiotics (Merrifield *et al.* 2010).

Prebiotics are non-nutritional food components which confer health benefits on the host via modulation of the gastrointestinal tract (GIT) microbiota. Motivation for the use of prebiotics in fish feed originated from the beneficial effects observed when terrestrial animals are fed with prebiotics. Thus, prebiotics such as inulin and fructo-oligosaccharides have been studied extensively across a wide range of farmed fish, including salmonids and carp (Merrifield *et al.* 2010; Ringø *et al.* 2010b; Guerreiro *et al.* 2017b). However, while resistant starch (RS) is commonly fed to terrestrial animals, less emphasis has been placed on this prebiotic for fish. Hence, RS was selected as the prebiotic of interest in this study.

The properties of RS as a prebiotic have been investigated through several studies, ranging from *in vitro* fermentation studies to *in vivo* studies conducted on both terrestrial animals and aquatic animals (Ringø *et al.* 2010b; Barclay *et al.* 2010; Torres *et al.* 2010). Overall, *in vitro* and *in vivo* studies show that RS leads to increments in the beneficial populations of bacteria and increments in short-chain fatty acid (SCFA) production both in terrestrial animals and their faecal bacteria (Giuberti *et al.* 2013;

Haenen *et al.* 2013; Zhou *et al.* 2013). Furthermore RS was found to improve SCFA production in fish (Gatesoupe *et al.* 2014) to a level comparable to other commonly used prebiotics, such as inulin, suggesting that RS may be potentially effective for use in aquaculture.

Several studies have also shown that different properties of RS may influence its prebiotic properties (Zhou *et al.* 2013; Wronkowska *et al.* 2006). Apart from high-amylose maize, which represents one of the most commonly studied sources of RS, legumes were suggested as an important source of RS due to the type of starch accumulated (Hoover & Zhou 2003). While several studies have investigated RS content across various types of legumes, little information is available on the *in vitro* fermentation of legume RS as a prebiotic. It was therefore decided to focus on legumes as an underutilised crop and an effective source of RS for both *in vitro* and *in vivo* studies.

Hence, this study aimed to investigate the use of leguminous RS as prebiotic to improve the growth and health of the Asian sea bass as a Malaysian aquaculture farmed fish. It involved the screening of legume starches and further processing to improve the RS contents, followed by an *in vitro* investigation of their prebiotic properties based on potential probiotic lactic acid bacteria (LAB) isolated from the intestines of the fish of interest. Finally, the effect of RS from legumes were investigated *in vivo* in fish feeding trials.

6.2 General discussion

6.2.1 Extraction of RS from underutilised legumes

In this section, underutilised legumes were screened as potential sources of resistant starch. Starch was isolated from several types of legumes via alkaline steeping, followed by the analysis of their RS content via Megazyme RS kit. Then, the legume starches were processed via hydrolysis, gelatinisation and retrogradation to increase their RS content.

Legumes were shown to be an effective source of starch, with content ranging from 35.30% to 40.33% (w/w) from red lentils, Bambara groundnuts and pigeon peas via alkaline steeping. These starch amounts were within the expected range of studies reported in a review (Hoover & Zhou 2003). Other starch isolation methods exist, which differ depending on the types of plant sources and the end use of the starch, potentially resulting in changes in properties, such as granule structure (Singh *et al.* 1997). For example, while the application of bisulphite for protein removal resulted in higher starch yields from potato (56.1% vs 38.9%), the kinetic parameters of the starch isolated via alkaline steeping showed greater resistance towards enzyme attack, suggesting alkaline steeping to be favourable for RS production (Djabali *et al.* 2009).

Apart from alkaline steeping, enzymatic removal of proteins via protease were also shown to be effective with no difference in starch yields, which ranged between 79.9% to 88.5% for chestnut and acorn flours respectively (Correia *et al.* 2012). However, the use of enzymes is costly compared to the other methods, especially for starch which is generally cheap (Correia & Beirao-Da-Costa 2010). Many studies also show that alkaline steeping was effective in reducing protein levels, reducing protein

contents of approximately 20% to the range of 1% to 0.3% in Bambara groundnuts and barley (Adebowale *et al.* 2002; Andersson *et al.* 2001). Similar results were shown in this study, with mung bean protein being reduced from 23.7% to 0.3% in mung bean flour. Hence, legume starch isolated via alkaline steeping was shown, not only to be superior in purity, but also cost and had higher RS content (Correia *et al.* 2012; Djabali *et al.* 2009), reducing the need for further optimisation.

Following starch isolation the processing of starches via hydrolysis, gelatinisation and retrogradation was also found to improve RS content. Between the two methods applied in this study, enzyme-hydrolysis was shown to be more effective than acid-hydrolysis as a pre-treatment prior to gelatinisation and retrogradation. The RS content of the legumes screened in this study ranged from 3.93% to 11.86% (Table 2.4). The RS content reported was significantly higher than those from cereals (0.2% to 0.7% w/w), except for high-amylose maize (65.8% to 66.5% w/w), due to the enzyme-susceptibility of the A-type starch crystalline structure found in cereals (Alsaffar 2011). However, while high-amylose maize possesses high RS content, further processing did not improve RS content further (Htoon *et al.* 2009). In addition, as maize is one of the major crops and was already commonly studied, it was not included in this study. Comparing between these two crop categories, legumes appear to be more suitable due to their relatively higher RS contents.

6.2.2 *In vitro* prebiotic potential of leguminous RS

In the previous section, the RS from six underutilised legumes were successfully produced via the enzyme-hydrolysis method. These legumes contained substantial amounts of RS, ranging from 12.40 to 18.06 % (w/w) (Table 2.4). In this section, the

prebiotic potential of these leguminous RS was investigated on the viability of three LAB isolated from the intestines of zebrafish (*Danio rerio*) and Asian sea bass.

The bacteria showed improved growth and acid production when cultured in nutrient broth supplemented with 5% (w/v) of leguminous starch and RS prepared in the previous chapters. Bacterial growth and acid production of *Weissella cibaria* NM1 and *Lactococcus garvieae* NM2 improved significantly for three of the leguminous enzyme-RS following treatment, with growth increments ranging from 4.24% to 43.9%. This contrasts with acid-RS, where the growth and acid production of the two bacteria increased with only two legumes after treatment and were generally reduced by approximately 21.81%. In addition, screening of RS from different legumes *in vitro* via *Enterococcus gilvus* V1 indicated differences in prebiotic potential, where red lentil was shown to be more effective than the other legumes.

In this study, the supplementation of 5% (w/v) red lentil enzyme-RS led to bacteria viable count increment of 2.3 $\log_{10}$ CFU/ml and an end point viable count of 8.39 $\log_{10}$ CFU/ml after 24 hr of culture (Table 4.7). Meanwhile, the pH change and end point pH after 24 hr of culture was observed to be -1.78 and 4.61 respectively. The growth and acid production of the bacteria in this study were similar to studies using other prebiotics, such as RS and fructo-oligosaccharide (FOS) (Siew-Wai *et al.* 2010; Wronkowska *et al.* 2006). Processed starches showed improved growth of approximately 1.0 $\log_{10}$ CFU/ml when *Bifidobacteria pseudolongum* were cultured with 1% processed wheat, potato and pea starches when compared to their native starch counterparts. However, only processed pea starches showed lower end point pH of 5.5 as compared to the native pea starch end point pH of 6.1 (Wronkowska *et al.* 2006). On the other hand, cultivation of *Lactobacillus casei* on 0.75% sago RS was

shown to be more effective than 0.5% FOS, with end point viable counts of 8.8 $\log_{10}$ CFU/ml and pH of 5.40 versus 7.69 $\log_{10}$ CFU/ml and pH of 5.91 respectively (Siew-Wai *et al.* 2010).

While it is uncertain if the concentrations of prebiotics applied in this *in vitro* study may have affected the growth and acid production, the carbohydrate is usually not consumed completely in 24 hr of culture and could be analysed in future studies due to its potential correlation with growth (Siew-Wai *et al.* 2010). The pH reductions observed in this study also suggested that SCFA were produced (Zhou *et al.* 2013). While studies suggest that the fermentation of RS mainly results in acetate and butyrate production (Zaman & Sarbini 2015; Zhou *et al.* 2013; Brouns *et al.* 2002), it may still be useful to investigate the types of SCFA produced via gas-chromatography, as fermentation of modified starches have been reported to produce different SCFA profiles (Wronkowska *et al.* 2006). Hence, the data suggest that leguminous enzyme-RS exhibits prebiotic properties *in vitro* similar to other more recognised prebiotics.

While RS content differs between each of the samples supplemented in culture, its role in bacterial growth and acid production is still not fully understood. While the application of the treatments in Chapter 2 resulted in higher RS content as a desirable trait as a prebiotic (Regmi *et al.* 2011), the increased RS content measured in some leguminous RS after treatment this study was not reflective of the bacteria growth and acid production measured. For example, although mung bean enzyme-RS showed a 178.52% RS increase following treatment, their growth *in vitro* did not differ (Table 3.3, Table 4.7). The results in this study do not agree with studies which suggest that higher RS content reduces bacteria utilisation and therefore growth

(Abia *et al.* 1993). This creates a need for future studies to investigate other factors to elucidate the mechanism of starch utilisation of bacteria.

Several studies have suggested potential factors that may influence bacterial utilisation of starch. Some studies highlight properties of the starch granule as a key factor, where a smooth granule surface prevents adhesion and bacteria accessibility, while pores present on the surface allows endogenous fermentation (Wronkowska *et al.* 2006; Sotomayor *et al.* 1999; Abia *et al.* 1993). However, this was not the case shown in another study, where amorphous and completely disrupted legume starch granules maintained resistance towards degradation, suggesting this to be an inherent property (Wyatt & Horn 1988). In addition, utilisation of starch as a substrate may also revolve around the capability of bacteria to utilise cell surface proteins for adhesion, which improves substrate hydrolysis and provides an advantage due to close physical proximity (Crittenden *et al.* 2001). Lastly, as fructan chain lengths were shown to influence its fermentability by bacteria (Scott *et al.* 2014), varying amylopectin chain lengths and its interactions in the starch chains may also play a role in bacteria utilisation due to its effect on enzyme degradation (Sandhu & Lim 2008; Zhou *et al.* 2004). Nevertheless, while the specific mechanisms whereby treatment improved bacteria utilisation are uncertain, enzyme-RS produced from red lentil starch was selected to be used in fish feeding trials based on the prebiotic properties displayed in the *in vitro* study performed.

6.2.3 Resistant starch as prebiotic supplemented *in vivo*/fish feeding trials

In the previous section, three types of RS and starch from six different underutilised legumes were screened for the prebiotic properties of increasing LAB growth and acid production. As these prebiotic properties were higher for red lentils and adzuki

bean enzyme-RS supplements, these two RS were selected to be included as supplements in fish feed to investigate the prebiotic effects *in vivo*.

The fish used in this study were zebrafish (a freshwater species commonly used in academic research) and a Malaysian aquaculture fish, the Asian sea bass. While zebrafish have the potential to be used in nutritional studies, it is important to recognise that the conditions under which they were routinely bred and housed were strictly controlled and, as such caution should be shown in extrapolating the results to other fish species farmed under commercial conditions (Ulloa *et al.* 2014). However, there relatively small size, rapid growth rate and extensive knowledge of their genome, together with the ability to carefully control environmental conditions, make them a useful model for testing specific effects of nutrients.

For fish feeds, growth of the fish and feed efficiencies are key traits desired by farmers. In this study, feeding both zebrafish and Asian sea bass with diets supplemented with leguminous starch at 2.5% did not lead to any adverse effects on its health. Meanwhile, while growth did not differ when fed different diets, zebrafish fed with RS had decreased food conversion ratios from 2.01 to 1.76. Similarly, fishmeal conversion ratio was also lower at 0.64 versus the basal diet at 0.78. On the other hand, no difference was observed in growth and feed conversion ratios in Asian sea bass fed with leguminous RS.

While studies on RS as a prebiotic in fish feed are scant, many studies have been conducted on other prebiotics. Several studies highlight similar results as shown in this study, where the supplementation with prebiotics such as mannan-oligosaccharides (MOS) (Torrecillas *et al.* 2011; Dimitroglou *et al.* 2010), short chain

FOS (Guerreiro *et al.* 2015), xylo-oligosaccharides (XOS) and galacto-oligosaccharides (Guerreiro *et al.* 2017a) did not result in increased growth. The dosage applied in the studies above ranged from 0.2% to 1.0% and may have been a factor resulting in no differences in growth. Nevertheless, despite the higher dosage of 2.5% applied in this study, no differences were also observed.

Various factors may influence growth and feed conversion efficiencies following prebiotic supplementation, such as changes in gut morphology or changes in digestive enzyme activity (Guerreiro *et al.* 2017b). Digestive enzyme activity may improve following the production of bacterial digestive enzymes, increasing the amounts of nutrients absorbed by the host (Wu *et al.* 2013; Soleimani *et al.* 2012). Meanwhile, the absorption of SCFA production by microbiota improves GIT structure in terms of microvilli density, height villi structure complexity and absorptive area, overall improving nutrient absorption for the host (Anguiano *et al.* 2013). As these observations were not performed on zebrafish in this study due to time constraints and logistic limitations, the potential of RS in promoting these factors is not conclusive despite reduced feed conversion ratios. Hence, future studies including the analysis of GIT morphology could be useful in elucidating whether a prebiotic has an effect or not *in vivo*.

When Asian sea bass were fed with RS from red lentils, no changes were observed in population counts of bacteria of Enterobacteriacieae family or LAB. (Table 6.4). Several other studies also highlight the lack of microbiota changes following supplementation of prebiotics, such as the use of inulin (Cerezuela *et al.* 2013), MOS (Dimitroglou *et al.* 2010) or XOS (Guerreiro *et al.* 2017a) in other fish species .On the other hand, the GIT microbial community of the Asian sea bass has been shown to

change following the supplementation with 2% (w/w) inulin, which resulted in increments in microbial species which may have benefited the host, via assistance in digestion of the complex protein-rich feed through their ability to degrade complex organic compounds and release of other extracellular enzymes, antibiotics and exo-polysaccharides (Ali *et al.* 2016).

Changes in fish GIT microbial communities are also influenced by variations in aqueous environments, such as temperature, salinity and surrounding bacterial populations (Merrifield & Rodiles 2015). Various studies compare microbial communities using PCR-DGGE, which may not be sensitive enough to detect changes in microbial populations (Zhou *et al.* 2014). Similarly, the conventional culture-dependent method applied in this study also faces this limitation. Future studies should implement more sensitive techniques, such as next-generation sequencing to improve coverage of microbiota changes which is one of the key aspects of a prebiotic (Zhou *et al.* 2014).

The nutritional content of the fish carcass may serve as an indicator of the nutritional content of the edible fish flesh (Mumba & Jose 2005). In this study, one of the potential changes in the nutritional content of the Asian sea bass carcass following feeding with RS from red lentils was the increment of fat content from 11% to 18%. Although the result from this study is inconclusive, the inclusion of prebiotics in other studies have observed increments in carcass fat content. Asian sea bass were fed FOS led to carcass fat content increasing from 2.75% to 4.17% (% wet weight), although the mechanism was not elucidated (Ali *et al.* 2017b). Meanwhile, increase of carcass fat content from 3.26% to 3.53% (% wet weight) in giant sturgeon (*Huso huso*) juveniles fed with MOS were associated with increased fatty acid utilisation

(Mansour *et al.* 2011). These results contradict other studies where fish fed prebiotics such as inulin (Ali *et al.* 2016), and MOS (Ali *et al.* 2017a) showed no change in carcass fat content. Apart from increased fatty acid utilisation, lipid accumulation has been attributed to excess energy storage in fish, as well as high protein content in feed causing proteins to be used as an energy source (Grigorakis 2007; Shearer 1994). As the factors influencing lipid accumulation in this study are undetermined, further investigation is warranted to confirm mechanisms associated with increased fat accumulation in Asian sea bass fed RS-supplemented diets. Investigation of the impact of RS-supplemented diets on the fatty acid composition, especially in terms of omega-3 long-chain polyunsaturated fatty acids of Asian sea bass, would also be of interest in terms of fish quality. However, as Asian sea bass have been found to be unable to convert dietary alpha-linolenic acid into omega-3 long chain polyunsaturated fatty acids, the accumulation of lipids in the carcass may represent a negative effect of RS supplementation which could be investigated and potentially applied in other aquaculture species as well (Tu *et al.* 2013).

6.3 Conclusion

From the results presented in this thesis, it can be concluded that RS derived from legumes may be processed to be an effective prebiotic *in vitro*, while further studies are required to investigate its application as a prebiotic *in vivo*. Starch was effectively isolated from underutilised legume and was effectively processed to prepare RS. These RS were shown to possess prebiotic properties on potential probiotic LAB (isolated from fish) when cultured *in vitro*, with enzyme-hydrolysed red lentils promoting better growth and acid production. Results obtained *in vivo* were not conclusive with little evidence of benefits in both zebrafish and Asian sea bass. Overall, while several questions remained unanswered, this study demonstrates the

potential use of RS as a prebiotic in commercial fish species, but highlights the need for further *in vivo* trials, preferably within an environment that reflects commercial production as well as variation in prebiotic dosage. Furthermore, studies should also address the impact of such RS in fish challenged with pathogenic bacteria or if innate immune responses are enhanced.

References

AACC International., 1999. Method 76-13.01. Total Starch Assay Procedure (Megazyme Amyloglucosidase/alpha-Amylase Method) Approved November 3, 1999. In *Approved Methods of Analysis, 11th Ed.* St. Paul, MN, U.S.A.: AACC International.

Abia, R., Buchanan, C.J. & Eastwood, M.A., 1993. Structural changes during the retrogradation of legume starches modify the *in vitro* fermentation. *Journal of Agricultural and Food Chemistry*, 41, pp.1856–1863.

Adebowale, K.O., Adeniyi Afolabi, T. & Lawal, O.S., 2002. Isolation, chemical modification and physicochemical characterisation of Bambarra groundnut (*Voandzeia subterranean*) starch and flour. *Food Chemistry*, 78(3), pp.305–311.

Alasalvar, C. *et al*., 2002. Differentiation of cultured and wild sea bass (*Dicentrarchus labrax*): total lipid content, fatty acid and trace mineral composition. *Food Chemistry*, 79, pp.145–150.

Alder, J. *et al*., 2008. Forage fish: From ecosystems to markets. *Annual Review of Environment and Resources*, 33, pp.153–166.

Ali, S.S.R. *et al*., 2017a. Effect of dietary fructooligosaccharide supplementation on growth, body composition, hematological and immunological parameters of Asian seabass (*Lates calcarifer*). *Aquaculture International*, 25, pp.837–848.

Ali, S.S.R. *et al*., 2017b. Effect of dietary mannan oligosaccharide on growth, body composition, haematology and biochemical parameters of Asian seabass (*Lates calcarifer*). *Aquaculture Research*, 48, pp.899–908.

Ali, S.S.R. *et al*., 2016. Effect of dietary prebiotic inulin on growth, body composition and gut microbiota of Asian seabass (*Lates calcarifer*). *Animal Feed Science and Technology*, 217, pp.87–94.

Alsaffar, A.A., 2011. Effect of food processing on the resistant starch content of cereals and cereal products - a review. *International Journal of Food Science and Technology*, 46, pp.455–462.

Andersson, A.A.M., Andersson, R. & Aman, P., 2001. Starch and by-products from a laboratory-scale barley starch isolation procedure. *Cereal Chemistry*, 78, pp.507–513.

Andika, D.O., Onyango, M.O.A. & Onyago, J.C., 2008. Role of Bambara groundnuts (*Vigna subterranea*) in cropping systems in Western Kenya. In J. Smartt & N. Haq, eds. *International Symposium on New Crops and Uses: Their role in a rapidly changing world*. Southampton: Centre of Underutilised Crops, University of Southampton, pp. 204–212.

Anguiano, M. *et al*., 2013. The effects of prebiotics on the digestive enzymes and gut histomorphology of red drum (*Sciaenops ocellatus*) and hybrid striped bass (*Morone chrysops x M . saxatilis*). *British Journal of Nutrition*, 109, pp.623–629.

Apper, E. *et al*., 2016. Hydrolysed wheat gluten as part of a diet based on animal and plant proteins supports good growth performance of Asian seabass (*Lates calcarifer*), without impairing intestinal morphology or microbiota. *Aquaculture*, 453, pp.40–48.

Australian Bureau of Statistics, 2012. *Fishing production and trade*, Australian Bureau of Statistics. Available at: http://www.abs.gov.au/ausstats/abs@.nsf/Lookup/by Subject/1301.0~2012~Main Features~Fishing~182.

Balcázar, J.L. *et al*., 2007a. Changes in intestinal microbiota and humoral immune response following probiotic administration in brown trout (*Salmo trutta*). *The British Journal of Nutrition*, 97, pp.522–527.

Balcázar, J.L. *et al*., 2007b. Enhancement of the immune response and protection induced by probiotic lactic acid bacteria against furunculosis in rainbow trout (*Oncorhynchus mykiss*). *FEMS Immunology and Medical Microbiology*, 51, pp.185–193.

Balcázar, J.L. *et al*., 2007c. Sequencing of variable regions of the 16S rRNA gene for identification of lactic acid bacteria isolated from the intestinal microbiota of healthy salmonids. *Comparative Immunology, Microbiology and Infectious Diseases*, 30, pp.111–118.

Balcázar, J.L. *et al*., 2006. The role of probiotics in aquaculture. *Veterinary Microbiology*, 114, pp.173–186.

Baldwin, P.M., 2001. Starch granule-associated proteins and polypeptides: A review. *Starch - Stärke*, 53, p.475.

Barclay, T. *et al*., 2010. Inulin - a versatile polysaccharide with multiple pharmaceutical and food chemical uses. *Journal of Excipients and Food Chemicals*, 1, pp.27–50.

Barr, B.K. *et al*., 1996. Identification of two functionally different classes of exocellulases. *Biochemistry*, 35, pp.586–592.

Bates, J.M. *et al*., 2006. Distinct signals from the microbiota promote different aspects of zebrafish gut differentiation. *Developmental Biology*, 297, pp.374–386.

Beards, E., Tuohy, K. & Gibson, G., 2010. Bacterial, SCFA and gas profiles of a range of food ingredients following *in vitro* fermentation by human colonic microbiota. *Anaerobe*, 16, pp.420–425.

Bednarczyk, M. *et al*., 2011. Field and *in vitro* study on prebiotic effect of raffinose family oligosaccharides in chickens. *Bull Vet Inst Pulawy*, 55, pp.465–469.

Bellei, G. & Haslberger, A., 2012. Dietary fibre and prebiotics. *Journal für Ernährungsmedizin*, 14, pp.12–15.

Bergey, D.H. (David H. & Holt, J.G., 1994. *Bergey's manual of determinative bacteriology*, Williams & Wilkins.

Bermudes, M. *et al*., 2010. The effects of temperature and size on the growth, energy budget and waste outputs of barramundi (*Lates calcarifer*). *Aquaculture*, 306, pp.160–166.

Betancur-Ancona, D., López-Luna, J. & Chel-Guerrero, L., 2003. Comparison of the chemical composition and functional properties of *Phaseolus lunatus* prime and tailing starches. *Food Chemistry*, 82, pp.217–225.

Bezkorovainy, A., 2001. Probiotics: determinants of survival and growth in the gut. *The American Journal of Clinical Nutrition*, 73, p.399S–405S.

Bhat, R. & Karim, A.A., 2009. Exploring the nutritional potential of wild and

underutilized legumes. *Comprehensive Reviews in Food Science and Food Safety*, 8, pp.305–331.

Van den Bilcke, N., Simbo, D.J. & Samson, R., 2013. Water relations and drought tolerance of young African tamarind (*Tamarindus indica L.*) trees. *South African Journal of Botany*, 88, pp.352–360.

Bingham, S.A. *et al*., 2003. Dietary fibre in food and protection against colorectal cancer in the European Prospective Investigation into Cancer and Nutrition (EPIC): an observational study. *The Lancet*, 361, pp.1496–1501.

Björkroth, K.J. *et al*., 2002. Taxonomic study of *Weissella confusa* and description of *Weissella cibaria* sp. nov., detected in food and clinical samples. *International Journal of Systematic and Evolutionary Microbiology*, 52, pp.141–148.

Bondad-Reantaso, M.G. *et al*., 2005. Disease and health management in Asian aquaculture. *Veterinary Parasitology*, 132, pp.249–272.

Boonyaratpalin, M., Suraneiranat, P. & Tunpibal, T., 1998. Replacement of fish meal with various types of soybean products in diets for the Asian seabass, *Lates calcarifer*. *Aquaculture*, 161, pp.67–78.

Bowe, C., 2007. Potential answers to the adaptation to and mitigation of climage change through the adoption of underutilised crops. *Tropical Agricultural Association Newsletter*, pp.9–12.

Boye, J., Zare, F. & Pletch, A., 2010. Pulse proteins: Processing, characterization, functional properties and applications in food and feed. *Food Research International*, 43, pp.414–431.

Božić, N. *et al*., 2017. Raw starch degrading α-amylases: an unsolved riddle. *Amylase*, 1, pp.12–25.

Bromage, E.S. & Owens, L., 2002. Infection of barramundi *Lates calcarifer* with *Streptococcus iniae*: Effects of different routes of exposure. *Diseases of Aquatic Organisms*, 52, pp.199–205.

Brouns, F., Kettlitz, B. & Arrigoni, E., 2002. Resistant starch and "the butyrate revolution''. *Trends in Food Science & Technology*, 13, pp.251–261.

Burr, G. *et al*., 2008. Effects of prebiotics on nutrient digestibility of a soybean-meal-based diet by red drum *Sciaenops ocellatus* (Linnaeus). *Aquaculture Research*, 39, pp.1680–1686.

Burr, G., Gatlin, D. & Ricke, S., 2005. Microbial ecology of the gastrointestinal tract of fish and the potential application of prebiotics and probiotics in Finnish aquaculture. *Journal of the World Aquaculture Society*, 36, pp.425–435.

Burr, G. & Gatlin III, D.M., 2009. Effects of the prebiotics GroBiotic®-A and inulin on the intestinal microbiota of red drum, *Sciaenops ocellatus*. *Journal of the World Aquaculture Society*, 40, pp.440–449.

Cerezuela, R. *et al*., 2013. Changes in intestinal morphology and microbiota caused by dietary administration of inulin and *Bacillus subtilis* in gilthead sea bream (*Sparus aurata* L.) specimens. *Fish and Shellfish Immunology*, 34, pp.1063–1070.

Cerezuela, R. *et al*., 2008. Effects of inulin on gilthead seabream (*Sparus aurata* L.) innate immune parameters. *Fish & Shellfish Immunology*, 24, pp.663–668.

Champ, M. *et al*., 2003. Advances in dietary fibre characterisation. Definition of dietary fibre, physiological relevance, health benefits and analytical aspects. *Nutrition Research Reviews*, 16, pp.71–82.

Chang, P.H., Lin, C.W. & Lee, Y.C., 2002. *Lactococcus garvieae* infection of cultured rainbow trout, *Oncorhynchus mykiss*, in Taiwan and associated biophysical characteristics and histopathology. *Bulletin of the European Association of Fish Pathologists*, 22, pp.319–327.

Cheng, W., Liu, C.H. & Chen, J.C., 2002. Effect of nitrite on interaction between the giant freshwater prawn *Macrobrachium rosenbergii* and its pathogen Lactococcus garvieae. *Diseases of Aquatic Organisms*, 50, pp.189–197.

Chiotelli, E., Pilosio, G. & Le Meste, M., 2002. Effect of sodium chloride on the gelatinization of starch: A multimeasurement study. *Biopolymers*, 63, pp.41–58.

Cho, S.S., Prosky, L. & Dreher, M., 1999. *Complex Carbohydrates in Foods*,

Choi, J.S. *et al*., 2004. Transition to an alternate state in a continental shelf ecosystem. *Canadian Journal of Fisheries and Aquatic Sciences*, 61, pp.505–510.

Chong, V., Al-Azad, S. & Shapawi, R., 2016. Comparison of two edible mushroom extract as aquaculture feed additive to enhance immune response of Asian Seabass. *Transactions on Science and Technology*, 3, pp.427–432.

Chung, H.-J., Liu, Q. & Hoover, R., 2009. Impact of annealing and heat-moisture treatment on rapidly digestible, slowly digestible and resistant starch levels in native and gelatinized corn, pea and lentil starches. *Carbohydrate Polymers*, 75, pp.436–447.

Chung, H.J., Liu, Q. & Hoover, R., 2010. Effect of single and dual hydrothermal treatments on the crystalline structure, thermal properties, and nutritional fractions of pea, lentil, and navy bean starches. *Food Research International*, 43, pp.501–508.

Correia, P.R. *et al*., 2012. Starch isolation from chestnut and acorn flours through alkaline and enzymatic methods. *Food and Bioproducts Processing*, 90, pp.309–316.

Correia, P.R. & Beirao-Da-Costa, M.L., 2010. Chestnut and acorn starch properties affected by isolation methods. *Starch/Staerke*, 62, pp.421–428.

Covington, M.B., 2004. Omega-3 fatty acids. *American Family Physician*, 70, pp.133–140.

Crittenden, R. *et al*., 2001. Adhesion of Bifidobacteria to granular starch and its implications in probiotic technologies. *Applied and Environmental Microbiology*, 67, pp.3469–3475.

Cummings, J.H. & Macfarlane, G.T., 1991. The control and consequences of bacterial fermentation in the human colon. *Journal of Applied Bacteriology*, 70, pp.443–459.

Dakora, F.D. & Keya, S.O., 1997. Contribution of legume nitrogen fixation to sustainable agriculture in Sub-Saharan Africa. *Soil Biology and Biochemistry*, 29, pp.809–817.

Davila, A.M. *et al*., 2013. Re-print of “intestinal luminal nitrogen metabolism: Role of

the gut microbiota and consequences for the host." *Pharmacological Research*, 69, pp.114–126.

Davis, C.D. & Milner, J.A., 2009. Gastrointestinal microflora, food components and colon cancer prevention. *The Journal of Nutritional Biochemistry*, 20, pp.743–752.

De, D. *et al*., 2015. Growth performance, nutrient digestibility and digestive enzyme activity in Asian seabass, *Lates calcarifer* juveniles fed diets supplemented with cellulolytic and amylolytic gut bacteria isolated from brackishwater fish. *Aquaculture Research*, 46, pp.1688–1698.

Defoirdt, T. *et al*., 2007. Alternatives to antibiotics to control bacterial infections: Luminescent vibriosis in aquaculture as an example. *Trends in Biotechnology*, 25, pp.472–479.

Denev, S. *et al*., 2009. Microbial ecology of the gastrointestinal tract of fish and the potential application of probiotics and prebiotics in finfish aquaculture. *International Aquatic Research*, 1, pp.1–29.

Dimitroglou, A. *et al*., 2010. Effects of mannan oligosaccharide (MOS) supplementation on growth performance, feed utilisation, intestinal histology and gut microbiota of gilthead sea bream (*Sparus aurata*). *Aquaculture*, 300, pp.182–188.

Dimitroglou, A. *et al*., 2011. Microbial manipulations to improve fish health and production – A Mediterranean perspective. *Fish & Shellfish Immunology*, 30, pp.1–16.

Djabali, D. *et al*., 2009. Relationship between potato starch isolation methods and kinetic parameters of hydrolysis by free and immobilised a-amylase on alginate (from *Laminaria digitata* algae). *Journal of Food Composition and Analysis*, 22, pp.563–570.

Dodamani, S. & Kaliwal, B., 2014. Production of bacteriocin by *Lactococcus garvieae* and influence of various supplements on its antimicrobial activity. *Indo American Journal of Pharmaceutical Research*, 4, p.2.

DOFM, 2016. *Annual Fisheries Statistics 2015*, Putrajaya. Available at: http://www.dof.gov.my/dof2/resources/Perangkaan/Perangkaan 2015/13.1_.2015_(11_files_merged)_.pdf.

Domig, K.J., Mayer, H.K. & Kneifel, W., 2003. Methods used for the isolation, enumeration, characterisation and identification of *Enterococcus* spp. 2. Pheno- and genotypic criteria. *International Journal of Food Microbiology*, 88, pp.165–188.

Eerlingen, R.C., Deceuninck, M. & Delcour, J., 1993. Enzyme-resistant starch. II. Influence of amylose chain length on resistant starch formation. *Cereal Chem*, 70, pp.345–350.

Elli, M. *et al*., 2000. Iron requirement of *Lactobacillus* spp. in completely chemically defined growth media. *Journal of Applied Microbiology*, 88, pp.695–703.

Elmstahl, H.L., 2002. Resistant starch content in a selection of starchy foods on the Swedish market. *European Journal of Clinical Nutrition*, 56, pp.500–505.

Englyst, H.N. *et al*., 1996. Measurement of resistant starch *in vitro* and *in vivo*. *British Journal of Nutrition*, 75, pp.749–755.

Evans, J.J. *et al*., 2006. First report of *Streptococcus agalactiae* and *Lactococcus garvieae* From a wild bottlenose dolphin (*Tursiops truncatus*). *Journal of Wildlife Diseases*, 42, pp.561–569.

FAO, 2017a. FAO Fisheries & Aquaculture - Cultured Aquatic Species Information Programme - *Lates calcarifer* (Block, 1790). Available at: http://www.fao.org/fishery/culturedspecies/Lates_calcarifer/en

FAO, 2017b. Global Aquaculture Production 1950-2015. *Food and Agriculture Organization of the United Nations Fisheries Statistics - Aquaculture*. Available at: http://www.fao.org/fishery/statistics/global-aquaculture-production/query/en.

FAO, 2017c. *Lates calcarifer* (Bloch, 1790). Available at: http://www.fao.org/fishery/species/3068/en

FAO, 2015. Species Fact Sheets - *Lates calcarifer* (Bloch, 1790). *FAO Fisheries & Aquaculture*. Available at: http://www.fao.org/fishery/species/3068/en

FAO & WHO, 2011. *Report of the Joint FAO/WHO Expert Consultation on the Risks and Benefits of Fish Consumption*, Rome; Geneva. Available at: http://www.fao.org/docrep/014/ba0136e/ba0136e00.pdf.

Fernando, W.M.U. *et al*., 2010. Diets supplemented with chickpea or its main oligosaccharide component raffinose modify faecal microbial composition in healthy adults. *Beneficial Microbes*, 1, pp.197–207.

Forsatkar, M.N. *et al*., 2017. Effects of prebiotic mannan oligosaccharide on the growth, survival, and anxiety-like behaviors of zebrafish (*Danio rerio*). *Journal of Applied Aquaculture*, 29, pp.183–196.

Foulquié-Moreno, M.R. *et al*., 2006. The role and application of enterococci in food and health. *International Journal of Food Microbiology*, 106, pp.1–24.

Frank, K.T. & Brickman, D., 2000. Allee effects and compensatory population dynamics within a stock complex. *Canadian Journal of Fisheries and Aquatic Sciences*, 57, pp.513–517.

Franz, C.M.A.P. *et al*., 2011. Enterococci as probiotics and their implications in food safety. *International Journal of Food Microbiology*, 151, pp.125–140.

Fuentes-Zaragoza, E. *et al*., 2010. Resistant starch as functional ingredient: A review. *Food Research International*, 43, pp.931–942.

Fuentes-Zaragoza, E. *et al*., 2011. Resistant starch as prebiotic: A review. *Starch - Stärke*, 63(7), pp.406–415.].

Fusco, V. *et al*., 2015. The genus *Weissella*: Taxonomy, ecology and biotechnological potential. *Frontiers in Microbiology*, 6, p.155.

Ganguly, S. *et al*., 2013. Supplementation of prebiotics in fish feed: A review. *Reviews in Fish Biology and Fisheries*, 23, pp.195–199.

Garcia, S.M. & Grainger, R.J.R., 2005. Gloom and doom? The future of marine capture fisheries. *Philosophical Transactions of the Royal Society of London B: Biological Sciences*, 360, pp.21–46.

Gatesoupe, F., 2005. Probiotics and prebiotics for fish culture , at the parting of the ways. *Aqua Feeds: Formulation & Beyond*, 2, pp.3–5.

Gatesoupe, F., 2008. Updating the importance of lactic acid bacteria in fish farming: Natural occurrence and probiotic treatments. *Journal of Molecular Microbiology and Biotechnology*, 14, pp.107–114.

Gatesoupe, F.J. *et al*., 2014. The effects of dietary carbohydrate sources and forms on metabolic response and intestinal microbiota in sea bass juveniles (*Dicentrarchus labrax*). *Aquaculture*, 422–423, pp.47–53.

Gazi, U. & Martinez-Pomares, L., 2009. Influence of the mannose receptor in host immune responses. *Immunobiology*, 214, pp.554–561.

Genc, M.A. *et al*., 2007. Effects of dietary mannan oligosaccharides (MOS) on growth ,body composition, and intestine and liver histology of the hybrid tilapia (*Oreochromis niloticus x O . aureus*). *The Israeli Journal of Aquaculture - Bamidgeh*, 59, pp.10–16.

Ghanbari, M., Kneifel, W. & Domig, K.J., 2015. A new view of the fish gut microbiome: Advances from next-generation sequencing. *Aquaculture*, 448, pp.464–475.

Gibson, G. & Roberfroid, M.B., 1995. Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. *The Journal of Nutrition*, 125, pp.1401–1412.

Gibson, G.R. *et al*., 2004. Dietary modulation of the human colonic microbiota: updating the concept of prebiotics. *Nutrition Research Reviews*, 17, pp.259–275.

Gibson, L.., Woodworth, J. & George, A.., 1998. Probiotic activity of *Aeromonas media* on the Pacific oyster, *Crassostrea gigas*, when challenged with *Vibrio tubiashii*. *Aquaculture*, 169, pp.111–120.

Gildberg, A. & Mikkelsen, H., 1998. Effects of supplementing the feed to Atlantic cod (Gadus morhua) fry with lactic acid bacteria and immuno-stimulating peptides during a challenge trial with *Vibrio anguillarum*. *Aquaculture*, 167, pp.103–113.

Gioacchini, G. *et al*., 2014. The Influence of probiotics on zebrafish *Danio* rerio innate immunity and hepatic stress. *Zebrafish*, 11, pp.98–106.

Giuberti, G. *et al*., 2013. *In vitro* production of short-chain fatty acids from resistant starch by pig faecal inoculum. *Animal*, 7, pp.1446–1453.

Glencross, B. *et al*., 2012. An assessment of cereal grains and other starch sources in diets for barramundi (*Lates calcarifer*) – implications for nutritional and functional qualities of extruded feeds. *Aquaculture Nutrition*, 18, pp.388–399.

Glencross, B. *et al*., 2016. An evaluation of the complete replacement of both fishmeal and fish oil in diets for juvenile Asian seabass, *Lates calcarifer*. *Aquaculture*, 451, pp.298–309.

Glencross, B., 2006. The nutritional management of barramundi, *Lates calcarifer* – a review. *Aquaculture Nutrition*, 12, pp.291–309.

Gomez-gil, B., Roque, A. & Turnbull, J.F., 2000. The use and selection of probiotic bacteria for use in the culture of larval aquatic organisms. *Aquaculture*, 191, pp.259–270.

Gómez-Requeni, P. *et al*., 2010. A reference growth curve for nutritional experiments in zebrafish (*Danio rerio*) and changes in whole body proteome during development. *Fish Physiology and Biochemistry*, 36, pp.1199–1215.

Gomez, R.G.D., Balcazar, J.L. & Shen, M.A., 2007. Probiotics as control agents in aquaculture. *Journal of Ocean University of China*, 6, pp.76–79.

Goñi, I. *et al*., 1996. Analysis of resistant starch: a method for foods and food products. *Food Chemistry*, 56(4), pp.445–449.

González, C.-J. *et al*., 1999. Bacterial microflora of wild brown trout (*Salmo trutta*), wild pike (*Esox lucius*), and aquacultured rainbow trout (*Oncorhynchus mykiss*). *Journal of Food Protection*, 62, pp.1270–1277.

Graham, P.H. & Vance, C.P., 2003. Legumes: Importance and constraints to greater use. *Plant Physiology*, 131, pp.872–877.

Greenberg, P., 2010. *Four Fish : the future of the last wild food*, New York: Penguin Books.

Grigorakis, K., 2007. Compositional and organoleptic quality of farmed and wild gilthead sea bream (*Sparus aurata*) and sea bass (*Dicentrarchus labrax*) and factors affecting it : A review. *Aquaculture*, 272, pp.55–75.

Grisdale-Helland, B., Helland, S.J. & Gatlin, D.M., 2008. The effects of dietary supplementation with mannanoligosaccharide, fructooligosaccharide or galactooligosaccharide on the growth and feed utilization of Atlantic salmon (*Salmo salar*). *Aquaculture*, 283, pp.163–167.

Gueimonde, M. *et al*., 2013. Antibiotic resistance in probiotic bacteria. *Frontiers in Microbiology*, 4, pp.1–6.

Guerra, A. *et al*., 2012. Relevance and challenges in modeling human gastric and small intestinal digestion. *Trends in Biotechnology*, 30, pp.591–600.

Guerreiro, I. *et al*., 2017a. Prebiotics effect on growth performance, hepatic intermediary metabolism, gut microbiota and digestive enzymes of white sea bream (*Diplodus sargus*). *Aquaculture Nutrition*, pp.1–11.

Guerreiro, I., Enes, P. & Oliva-Teles, A., 2015. Effects of short-chain fructooligosaccharides (scFOS) and rearing temperature on growth performance and hepatic intermediary metabolism in gilthead sea bream (*Sparus aurata*) juveniles. *Fish Physiology and Biochemistry*, 41, pp.1333–1344.

Guerreiro, I., Oliva-Teles, A. & Enes, P., 2017b. Prebiotics as functional ingredients: Focus on Mediterranean fish aquaculture. *Reviews in Aquaculture*, 0, pp.1–33.

Gupta, A.K., Kaur, N. & Kaur, N., 2003. Preparation of inulin from chicory roots. *Journal of Scientific and Industrial Research*, 62, pp.916–920.

Gupta, R. *et al*., 2003. Microbial a-amylases: A biotechnological perspective. *Process Biochemistry*, 38, pp.1599–1616.

Haenen, D. *et al*., 2013. A diet high in resistant starch modulates microbiota composition, SCFA concentrations, and gene expression in pig intestine. *The Journal of Nutrition*, 143, pp.274–283.

Hallstrom, E. *et al*., 2011. A novel wheat variety with elevated content of amylose

increases resistant starch formation and may beneficially influence glycaemia in healthy subjects. *Food and Nutrition Research*, 55, pp.1–8.

Halver, J.E. (John E. & Hardy, R.W., 2002. *Fish nutrition* 3rd ed. J. E. (John E. Halver & R. W. Hardy, eds., Amsterdam: Elsevier.

Han, X.Z. & Hamaker, B.R., 2002. Partial leaching of granule-associated proteins from rice starch during alkaline extraction and subsequent gelatinization. *Starch/Starke*, 54, pp.454–460.

Haralampu, S.G., 2000. Resistant starch — a review of the physical properties and biological impact of RS3. *Carbohydrate Polymers*, 41, pp.285–292.

Hayek, S.A. & Ibrahim, S.A., 2013. Current limitations and challenges with lactic acid bacteria: A review. *Food and Nutrition Science*, 4, pp.73–87.

He, S. *et al*., 2003. Effects of IMO and FOS on the growth performance and non-specific immunity in hybrid tilapia. *Chinese Feed*, 23, pp.14–15.

Hébert, E.M., Raya, R.R. & De Giori, G.S., 2004. Nutritional requirements of *Lactobacillus delbrueckii* subsp. *lactis* in a chemically defined medium. *Current Microbiology*, 49, pp.341–345.

Hedemann, M.S. & Knudsen, K.E.B., 2007. Resistant starch for weaning pigs — Effect on concentration of short chain fatty acids in digesta and intestinal morphology. *Livestock Science*, 108, pp.175–177.

Hegde, V.S. & Mishra, S.K., 2009. Landraces of cowpea, *Vigna unguiculata* (L.) *Walp*., as potential sources of genes for unique characters in breeding. *Genetic Resources and Crop Evolution*, 56, pp.615–627.

Heo, J.M. *et al*., 2014. Feeding a diet containing resistant potato starch influences gastrointestinal tract traits and growth performance of weaned pigs 1. *Journal of Animal Science*, 92, pp.3906–3913.

Heo, W. *et al*., 2013. Effects of dietary probiotic, *Lactococcus lactis* subsp. lactis I2, supplementation on the growth and immune response of olive flounder (*Paralichthys olivaceus*). *Aquaculture*, 376–379, pp.20–24.

Heywood, V.H., 2011. Ethnopharmacology, food production, nutrition and biodiversity conservation: Towards a sustainable future for indigenous peoples. *Journal of Ethnopharmacology*, 137, pp.1–15.

Hibbeln, J.R. *et al*., 2007. Maternal seafood consumption in pregnancy and neurodevelopmental outcomes in childhood (ALSPAC study): an observational cohort study. *The Lancet*, 369, pp.578–585.

Hijova, E. & Chmelarova, A., 2007. Short chain fatty acids and colonic health. *Bratislavské lekárske listy*, 108, pp.354–8.

Hoover, B.R. & Sosulski, F., 1985. Studies on the functional characteristics and digestibility of starches from *Phaseolus vulgaris* biotypes. *Starch/Stärke*, 6, pp.181–191.

Hoover, R. *et al*., 2010. Composition, molecular structure, properties, and modification of pulse starches: A review. *Food Research International*, 43, pp.399–413.

Hoover, R. *et al*., 1997. Physicochemical characterization of mung bean starch. *Food*

Hydrocolloids, 11, pp.401–408.

Hoover, R. & Ratnayake, W.S., 2002. Starch characteristics of black bean, chick pea, lentil, navy bean and pinto bean cultivars grown in Canada. *Food Chemistry*, 78, pp.489–498.

Hoover, R., Swamidas, G. & Vasanthan, T., 1993. Studies on the physicochemical properties of native, defatted, and heat-moisture treated pigeon pea (*Cajanus cajan* L) starch. *Carbohydrate Research*, 246, pp.185–203.

Hoover, R. & Zhou, Y., 2003. *In vitro* and *in vivo* hydrolysis of legume starches by α-amylase and resistant starch formation in legumes — A review. *Carbohydrate Polymers*, 54, pp.401–417.

Hord, N.G., 2008. Eukaryotic-microbiota crosstalk: Potential mechanisms for health benefits of prebiotics and probiotics. *Annual Review of Nutrition*, 28, pp.215–231.

Hoseinifar, S.H. *et al*., 2014. Probiotic, prebiotic and synbiotic supplements in sturgeon aquaculture: A review. *Reviews in Aquaculture*, 8, pp.89–102.

Hsieh, H.M., Swanson, B.G. & Lumpkin, T.A., 1999. Starch gelatinization and microstructure of Azuki An granules prepared from whole, abraded, or ground beans. *LWT - Food Science and Technology*, 32, pp.469–480.

Htoon, A. *et al*., 2009. Effects of processing high amylose maize starches under controlled conditions on structural organisation and amylase digestibility. *Carbohydrate Polymers*, 75, pp.236–245.

Htoon, A.K. *et al*., 2010. The effect of acid dextrinisation on enzyme-resistant starch content in extruded maize starch. *Food Chemistry*, 120, pp.140–149.

Huang, J. *et al*., 2007. Characterization of differently sized granule fractions of yellow pea, cowpea and chickpea starches after modification with acetic anhydride and vinyl acetate. *Carbohydrate Polymers*, 67, pp.11–20.

Ibrahem, M.D. *et al*., 2010. Effect of dietary supplementation of inulin and vitamin C on the growth, hematology, innate immunity, and resistance of Nile tilapia (*Oreochromis niloticus*). *Fish and Shellfish Immunology*, 29, pp.241–246.

Iji, P.A., Saki, A.A. & Tivey, D.R., 2001. Intestinal structure and function of broiler chickens on diets supplemented with a mannan oligosaccharide. *Journal of the Science of Food and Agriculture*, 81, pp.1186–1192.

Irianto, A. & Austin, B., 2002a. Probiotics in aquaculture. *Journal of Fish Diseases*, 25, pp.1–10.

Irianto, A. & Austin, B., 2002b. Use of probiotics to control furunculosis in rainbow trout, *Oncorhynchus mykiss* (Walbaum). *Journal of Fish Diseases*, 25, pp.333–342.

Itoi, S. *et al*., 2008. Isolation of halotolerant *Lactococcus lactis* subsp. lactis from intestinal tract of coastal fish. *International Journal of Food Microbiology*, 121, pp.116–121.

James, G., 2010. Universal Bacterial Identification by PCR and DNA Sequencing of 16S rRNA Gene. In *PCR for Clinical Microbiology*. Dordrecht: Springer Netherlands, pp. 209–214.

Janda, J.M. & Abbott, S.L., 2002. Bacterial identification for publication: When is enough enough? *Journal of Clinical Microbiology*, 40, pp.1887–1891.

Janick, J., 1999. New Crops and the Search for New Food Resources. In *Perspectives on New Crops and New Uses*. Alexandria: ASHS Press, pp. 104–110.

Jauncey, K., 1982. The effects of varying dietary protein level on the growth, food conversion, protein utilization and body composition of juvenile tilapias (*Sarotherodon mossambicus*). *Aquaculture*, 27(1), pp.43–54.

Jonathan, M.C. *et al*., 2012. *In vitro* fermentation of 12 dietary fibres by faecal inoculum from pigs and humans. *Food Chemistry*, 133, pp.889–897.

Jung, S. *et al*., 2003. Comparison of Kjeldahl and Dumas methods for determining protein contents of soybean products. *Journal of the American Oil Chemists' Society*, 80, p.1169.

Katersky, R.S. & Carter, C.G., 2005. Growth efficiency of juvenile barramundi, *Lates calcarifer*, at high temperatures. *Aquaculture*, 250, pp.775–780.

Katersky, R.S. & Carter, C.G., 2007. High growth efficiency occurs over a wide temperature range for juvenile barramundi *Lates calcarifer* fed a balanced diet. *Aquaculture*, 272, pp.444–450.

Kaushik, S., Georga, I. & Koumoundouros, G., 2011. Growth and body composition of zebrafish (*Danio rerio*) larvae fed a aompound feed from first feeding onward: Toward implications on nutrient requirements. *Zebrafish*, 8, pp.87–95.

Kihara, M. & Sakata, T., 1997. Fermentation of dietary carbohydrates to short-chain fatty acids by gut microbes and its influence on intestinal morphology of a detritivorous teleost tilapia (*Oreochromis niloticus*). *Comparative Biochemistry and Physiology - A Physiology*, 118, pp.1201–1207.

Kihara, M. & Sakata, T., 2002. Production of short-chain fatty acids and gas from various oligosaccharides by gut microbes of carp (*Cyprinus carpio* L.) in micro-scale batch culture. *Comparative Biochemistry and Physiology Part A*, 132, pp.333–340.

Klebaniuk, R. *et al*., 2008. Effectiveness of BIO-MOS in sheep nutrition. *Annals of Animal Science*, 8, pp.369–380.

Korakli, M., Ganzle, M.G. & Vogel, R.F., 2002. Metabolism by bifidobacteria and lactic acid bacteria of polysaccharides from wheat and rye, and exopolysaccharides produced by *Lactobacillus sanfranciscensis*. *Journal of Applied Microbiology*, 92, pp.958–965.

Korkeala, H., Alanko, T. & Tiusanen, T., 1992. Effect of sodium nitrite and sodium chloride on growth of lactic acid bacteria. *Acta veterinaria Scandinavica*, 33, pp.27–32.

Lane, A. & Jarvis, A., 2007. Changes in climate will modify the geography of crop suitability: Agricultural biodiversity can help with adaptation. *Journal of Semi-Arid Tropical Agricultural Research*, 4, pp.1–12.

Lebreton, F., Willems, R.J.L. & Gilmore, M.S., 2014. *Enterococcus Diversity, Origins in Nature, and Gut Colonization*, Massachusetts Eye and Ear Infirmary. Available at: http://www.ncbi.nlm.nih.gov/pubmed/24649513.

Lee, H.C., Htoon, A.K. & Paterson, J.L., 2007. Alkaline extraction of starch from Australian lentil cultivars Matilda and Digger optimised for starch yield and starch and protein quality. *Food Chemistry*, 102, pp.551–559.

Lesmes, U. *et al*., 2008. Effects of resistant starch type III polymorphs on human colon microbiota and short chain fatty acids in human gut models. *Journal of Agricultural and Food Chemistry*, 56, pp.5415–5421.

Li, P. & Gatlin III, D.M., 2004. Dietary brewers yeast and the prebiotic Grobiotic AE influence growth performance, immune responses and resistance of hybrid striped bass (*Morone chrysops x M . saxatili*s) to *Streptococcus iniae* infection. *Aquaculture*, 231, pp.445–456.

Licht, T.R., Ebersbach, T. & Frøkiær, H., 2012. Prebiotics for prevention of gut infections. *Trends in Food Science and Technology*, 23, pp.70–82.

Lira, R., Téllez, O. & Dávila, P., 2009. The effects of climate change on the geographic distribution of Mexican wild relatives of domesticated *Cucurbitaceae*. *Genetic Resources and Crop Evolution*, 56, pp.691–703.

Liu, H. *et al*., 2009. Thermal processing of starch-based polymers. *Progress in Polymer Science*, 34, pp.1348–1368.

Liu, R. & Xu, G., 2008. Effects of resistant starch on colonic preneoplastic aberrant crypt foci in rats. *Food and Chemical Toxicology*, 46, pp.2672–2679.

Liu, W. & Shen, Q., 2007. Structure analysis of mung bean starch from sour liquid processing and centrifugation. *Journal of Food Engineering*, 79, pp.1310–1314.

Long, S.R., 1996. Rhizobium symbiosis: Nod factors in perspective. *The Plant Cell*, 8, pp.1885–1898.

Lösel, D. & Claus, R., 2005. Dose-dependent effects of resistant potato starch in the diet on intestinal skatole formation and adipose tissue accumulation in the pig. *Journal of Veterinary Medicine Series A: Physiology Pathology Clinical Medicine*, 52, pp.209–212.

Louis, P. & Flint, H.J., 2009. Diversity, metabolism and microbial ecology of butyrate-producing bacteria from the human large intestine. *FEMS Microbiology Letters*, 294, pp.1–8.

Lv, H. *et al*., 2007. Effects of dietary short chain fructo-oligosaccharides on intestinal microflora , mortality and growth performance of *Oreochromis aureus* ♂ × *O . niloticus* ♀. *Chinese Journal of Animal Nutrition*, 19, pp.691–697.

Ma, M. *et al*., 2017. Physicochemical properties and *in vitro* digestibility of legume starches. *Food Hydrocolloids*, 63, pp.249–255.

Macfarlane, S., Macfarlane, G.T. & Cummings, J.H., 2006. Review article: Prebiotics in the gastrointestinal tract. *Alimentary Pharmacology and Therapeutics*, 24, pp.701–714.

Mahious, A.S. *et al*., 2006. Effect of dietary inulin and oligosaccharides as prebiotics for weaning turbot, *Psetta maxima* (Linnaeus, C. 1758). *Aquaculture International*, 14, pp.219–229.

Maji, U.J. *et al*., 2016. Diversity and probiotic potentials of putative lactic acid bacteria

for application in freshwater aquaculture. *Turkish Journal of Fisheries and Aquatic Sciences*, 16, pp.805–818.

Makridis, P. *et al*., 2005. Evaluation of candidate probiotic strains for gilthead sea bream larvae (*Sparus aurata*) using an *in vivo* approach. *Letters in Applied Microbiology*, 40, pp.274–277.

de Man, J.D., Rogosa, M. & Sharpe, M.E., 1960. A medium for the cultivation of Lactobacilli. *Journal of Applied Bacteriology*, 23, pp.130–135.

Mansour, M.R. *et al*., 2011. Effect of dietary mannan oligosaccharide (MOS) on growth performance, survival, body composition, and some hematological parameters in giant sturgeon juvenile (*Huso huso Linnaeus*, 1754). *Fish physiology and biochemistry*, 38, pp.829–835.

Marteau, P. *et al*., 1997. Survival of lactic acid bacteria in a dynamic model of the stomach and small intestine: Validation and the effects of bile. *Journal of Dairy Science*, 80, pp.1031–1037.

Martinez, I. *et al*., 2010. Resistant starches types 2 and 4 have differential effects on the composition of the fecal microbiota in human subjects. *PLoS ONE*, 5(11), p.e15046.

Mayes, S. *et al*., 2009. BAMLINK - a cross disciplinary programme to enhance the role of Bambara groundnut (*Vigna subterranea* l. Verdc.) for food security in Africa and India. *Acta Horticulturae*, 806, pp.137–150.

Mayes, S. *et al*., 2012. The potential for underutilized crops to improve security of food production. *Journal of Experimental Botany*, 63, pp.1075–1079.

McCleary, B. V. *et al*., 2002. Measurement of resistant starch by enzymatic digestion in starch and selected plant materials: Collaborative study. *Journal of AOAC International*, 85, pp.1103–1111.

Merrifield, D.L. *et al*., 2010. The current status and future focus of probiotic and prebiotic applications for salmonids. *Aquaculture*, 302, pp.1–18.

Merrifield, D.L. & Rodiles, A., 2015. The fish microbiome and its interactions with mucosal tissues. In B. H. Beck & E. Peatman, eds. *Mucosal Health in Aquaculture*. Cambridge, MA: Academic Press, pp. 273–295.

Mistry, A.H. *et al*., 1992. Alkali extraction of starch from corn flour. *Starch/Stärke*, 44, pp.284–288.

Mohapatra, S. *et al*., 2013. Aquaculture and stress management: A review of probiotic intervention. *Journal of Animal Physiology and Animal Nutrition*, 97, pp.405–430.

Moongngarm, A., 2013. Chemical compositions and resistant starch content in starchy foods. *American Journal of Agricultural and Biological Sciences*, 8, pp.107–113.

Moore, D.F. *et al*., 2006. Comparison of 16S rRNA sequencing with conventional and commercial phenotypic techniques for identification of enterococci from the marine environment. *Journal of Applied Microbiology*, 100, pp.1272–1281.

Morales-Medina, R. *et al*., 2014. Production of resistant starch by enzymatic debranching in legume flours. *Carbohydrate Polymers*, 101, pp.1176–1183.

Moriarty, D.J.W., 1997. The role of microorganisms in aquaculture ponds. *Aquaculture*,

151, pp.333–349.

Mouriño, J.L.P. *et al*., 2012. Effect of dietary supplementation of inulin and *W. cibaria* on haemato-immunological parameters of hybrid surubim (*Pseudoplatystoma* sp). *Aquaculture Nutrition*, 18, pp.73–80.

Mumba, P.P. & Jose, M., 2005. Nutrient composition of selected fresh and processed fish species from lake Malawi: A nutritional possibility for people living with HIV/AIDS. *International Journal of Consumer Studies*, 29, pp.72–77.

Muñoz-Atienza, E. *et al*., 2013. Antimicrobial activity, antibiotic susceptibility and virulence factors of lactic acid bacteria of aquatic origin intended for use as probiotics in aquaculture. *BMC microbiology*, 13, p.15.

Muñoz-Atienza, E. *et al*., 2014. *In vitro* and *in vivo* evaluation of lactic acid bacteria of aquatic origin as probiotics for turbot (*Scophthalmus maximus L.*) farming. *Fish and Shellfish Immunology*, 41, pp.570–580.

Nayak, S.K., 2010. Role of gastrointestinal microbiota in fish. *Aquaculture Research*, 41, pp.1553–1573.

Naylor, R.L. *et al*., 2000. Effect of aquaculture on world fish supplies. *Nature*, 405, pp.1017–1024.

Nekoubin, H. *et al*., 2012. The influence of synbiotic (Biomin imbo) on growth factors and survival rate of Zebrafish (*Danio rerio*) larvae via supplementation with biomar. *Global Veterinaria*, 8, pp.503–506.

Nichols, P.D., Petrie, J. & Singh, S., 2010. Long-chain omega-3 oils – An update on sustainable sources. *Nutrients*, 2, pp.572–585.

Nikoskelainen, S. *et al*., 2003. Immune enhancement in rainbow trout (*Oncorhynchus mykiss*) by potential probiotic bacteria (*Lactobacillus rhamnosus*). *Fish & Shellfish Immunology*, 15, pp.443–452.

Oelschlaeger, T.A., 2010. Mechanisms of probiotic actions – A review. *International Journal of Medical Microbiology*, 300, pp.57–62.

Ohashi, Y. & Ushida, K., 2009. Health-beneficial effects of probiotics: Its mode of action. *Animal Science Journal*, 80, pp.361–371.

Olano-martin, E. *et al*., 2000. *In vitro* fermentability of dextran, oligodextran and maltodextrin by human gut bacteria. *British Journal of Nutrition*, 83, pp.247–255.

Olsen, R.E. *et al*., 2001. Damaging effect of dietary inulin on intestinal enterocytes in Arctic charr (*Salvelinus alpinus* L.). *Aquaculture Research*, 32, pp.931–934.

Oprea, S.F. & Zervos, M.J., 2007. Enterococcus and its Association with Foodborne Illness. In *Infectious Disease: Foodborne Diseases*. Totowa, New Jersey: Humana Press, pp. 157–174.

Ortiz, L.T. *et al*., 2013. Effects of inulin and fructooligosaccharides on growth performance, body chemical composition and intestinal microbiota of farmed rainbow trout (*Oncorhynchus mykiss*). *Aquaculture Nutrition*, 19, pp.475–482.

Oyeyinka, S., Adebola, P. & Gerrano, A.S., 2015. Physicochemical properties of starches with variable amylose contents extracted from bambara groundnut genotypes. *Carbohydrate Polymers*, 133, pp.171–178.

Padulosi, S. *et al*., 2011. Underutilized species and climate change: Current status and outlook. In *Crop Adaptation to Climate Change*. New Jersey: John Wiley & Sons, pp. 507–521.

Padulosi, S. & Hoeschle-Zeledon, I., 2004. Underutilized plant species: What are they ? *Leisa Magazine*, pp.5–6.

Pan, C.Y., Wang, Y.D. & Chen, J.Y., 2013. Immunomodulatory effects of dietary Bacillus coagulans in grouper (*Epinephelus coioides*) and zebrafish (Danio rerio) infected with Vibrio vulnificus. *Aquaculture International*, 21, pp.1155–1168.

Pangallo, D. *et al*., 2008. Evaluation of different PCR-based approaches for the identification and typing of environmental enterococci. *Antonie van Leeuwenhoek*, 93, pp.193–203.

Panigrahi, A. *et al*., 2005. The viability of probiotic bacteria as a factor influencing the immune response in rainbow trout *Oncorhynchus mykiss*. *Aquaculture*, 243, pp.241–254.

Panigrahi, A. & Azad, I.S., 2007. Microbial intervention for better fish health in aquaculture: the Indian scenario. *Fish Physiology and Biochemistry*, 33, pp.429–440.

Panigrahi, A., Viswanath, K. & Satoh, S., 2011. Real-time quantification of the immune gene expression in rainbow trout fed different forms of probiotic bacteria *Lactobacillus rhamnosus*. *Aquaculture Researc*, 42, pp.906–917.

Papadimitriou, K. *et al*., 2015. Discovering probiotic microorganisms: *In vitro*, *in vivo*, genetic and omics approaches. *Frontiers in Microbiology*, 6, pp.1–28.

Patel, S. & Goyal, A., 2012. The current trends and future perspectives of prebiotics research: A review. *3 Biotech*, 2, pp.115–125.

Perera, A., Meda, V. & Tyler, R.T., 2010. Resistant starch: A review of analytical protocols for determining resistant starch and of factors affecting the resistant starch content of foods. *Food Research International*, 43, pp.1959–1974.

Pluske, J.R. *et al*., 1998. Confirmation of the role of rapidly fermentable carbohydrates in the expression of swine dysentery in pigs after experimental infection. *The Journal of Nutrition*, 128(10), pp.1737–1744.

Polesi, L.F. & Sarmento, S.B.S., 2011. Structural and physicochemical characterization of RS prepared using hydrolysis and heat treatments of chickpea starch. *Starch/Staerke*, 63, pp.226–235.

Powell, K., 2003. Eat your veg. *Nature*, 426, pp.378–379.

Prescott-Allen, R. & Prescott-Allen, C., 1996. *Assessing the Sustainability of Uses of Wild Species: Case Studies and Initial Assessment Procedure*, Gland, Switzerland and Cambridge, UK.

Pryde, S.E. *et al*., 2002. The microbiology of butyrate formation in the human colon. *FEMS Microbiology Letters*, 217, pp.133–139.

Pryor, G.S. *et al*., 2003. Mannanoligosaccharides in fish nutrition: Effects of dietary supplementation on growth and gastrointestinal villi structure in Gulf of Mexico sturgeon. *North American Journal of Aquaculture*, 65, pp.106–111.

Pylkas, A.M., Juneja, L.R. & Slavin, J.L., 2005. Comparison of different fibers for *in vitro* production of short chain fatty acids by intestinal microflora. *Journal of Medicinal Food*, 8, pp.113–116.

Queiroz-monici, K.S. *et al*., 2005. Bifidogenic effect of dietary fiber and resistant starch from leguminous on the intestinal microbiota of rats. *Nutrition*, 21, pp.602–608.

Rajilić-Stojanović, M., 2013. Function of the microbiota. *Best Practice and Research: Clinical Gastroenterology*, 27, pp.5–16.

Rajilić-Stojanović, M. *et al*., 2013. Long-term monitoring of the human intestinal microbiota composition. *Environmental Microbiology*, 15, pp.1146–1159.

Rappé, M.S. & Giovannoni, S.J., 2003. The uncultured microbial majority. *Annual Review of Microbiology*, 57, pp.369–394.

Rawls, J.F., Samuel, B.S. & Gordon, J.I., 2004. Gnotobiotic zebrafish reveal evolutionarily conserved responses to the gut microbiota. *Proceedings of the National Academy of Sciences of the United States of America*, 101, pp.4596–4601.

van Reenen, C.A. & Dicks, L.M.T., 2011. Horizontal gene transfer amongst probiotic lactic acid bacteria and other intestinal microbiota: What are the possibilities? A review. *Archives of Microbiology*, 193, pp.157–168.

Regassa, A. & Nyachoti, C.M., 2018. Application of resistant starch in swine and poultry diets with particular reference to gut health and function. *Animal Nutrition*.

Regmi, P.R. *et al*., 2011. Starch with high amylose content and low *in vitro* digestibility increases intestinal nutrient flow and microbial fermentation and selectively promotes bifidobacteria in pigs. *The Journal of Nutrition*, 141, pp.1273–1280.

Rengpipat, S., Rueangruklikhit, T. & Piyatiratitivorakul, S., 2008. Evaluations of lactic acid bacteria as probiotics for juvenile seabass *Lates calcarifer*. *Aquaculture Research*, 39, pp.134–143.

Ringø, E., 2004. Lactic Acid Bacteria in Fish and Fish Farming. In *Lactic Acid Bacteria: Microbiological and Functional Aspects*. Madison Avenue, New York: Mcarcel Dekker, pp. 581–610.

Ringø, E., Lövmo, L., *et al*., 2010a. Lactic acid bacteria vs. pathogens in the gastrointestinal tract of fish: A review. *Aquaculture Research*, 41, pp.451–467.

Ringø, E., Olsen, R.E., *et al*., 2010b. Prebiotics in aquaculture: A review. *Aquaculture Nutrition*, 16, pp.117–136.

Ringø, E., 2008. The ability of carnobacteria isolated from fish intestine to inhibit growth of fish pathogenic bacteria: a screening study. *Aquaculture Research*, 39, pp.171–180.

Ringø, E. *et al*., 2006. The effect of dietary inulin on aerobic bacteria associated with hindgut of Arctic charr (*Salvelinus alpinus* L.). *Aquaculture Research*, 37, pp.891–897.

Ringø, E. & Birkbeck, T.H., 1999. Intestinal microflora of fish larvae and fry. *Aquaculture Research*, 30, pp.73–93.

Robaina, L. & Izquierdo, M., 2000. Methodological strategies for the determination of

nutrient requirements in finfish. In *Cahiers Options Méditerranéennes* . Zaragoza: Centre International des Hautes Etudes Agronomiques Mediterraneennes, pp. 25–41.

Roberfroid, M., 2007. Prebiotics: The concept revisited. *The Journal of Nutrition*, 137, p.830S–837S.

Roberfroid, M., 2000. Prebiotics and probiotics: Are they functional foods? *American Journal of Clinical Nutrition*, 71, pp.1682–1687.

Robertson, P.A.W. *et al*., 2000. Use of Carnobacterium sp. as a probiotic for Atlantic salmon (*Salmo salar L.*) and rainbow trout (*Oncorhynchus mykiss*, *Walbaum*). *Aquaculture*, 185, pp.235–243.

Robison, B.D. *et al*., 2008. Sexual dimorphism in hepatic gene expression and the response to dietary carbohydrate manipulation in the zebrafish (*Danio rerio*). *Comparative Biochemistry & Physiology*, 3, pp.141–154.

Rodrigues-Estrada, U. *et al*., 2008. Studies of the effects of mannan-oligosaccharides, *Enterococcus faecalis*, and poly hydrobutyric acid as immune stimulant and growth promoting. In *5th World Fisheries Congress*. Yokohama, Japan, p. 158.

Rodríguez-cabezas, M.E. *et al*., 2010. The combination of fructooligosaccharides and resistant starch shows prebiotic additive effects in rats. *Clinical Nutrition*, 29, pp.832–839.

Rodriguez-Estrada, U. *et al*., 2009. Effects of single and combined supplementation of *Enterococcus faecalis*, mannan oligosaccharide and polyhydroxybutyrate acid on growth performance and immune response of rainbow trout *Oncorhynchus mykiss*. *Aquaculture Science*, 57, pp.609–617.

Russell, W.R. *et al*., 2011. High-protein, reduced-carbohydrate weight-loss diets promote metabolite profiles likely to be detrimental to colonic health. *American Journal of Clinical Nutrition*, 93, pp.1062–1072.

Ruwandeepika, A.H.D. *et al*., 2012. Pathogenesis, virulence factors and virulence regulation of vibrios belonging to the Harveyi clade. *Reviews in Aquaculture*, 4, pp.59–74.

Saha, S. *et al*., 2006. Characterization of cellulase-producing bacteria from the digestive tract of tilapia, *Oreochromis mossambica* (Peters) and grass carp, *Ctenopharyngodon idella*. *Aquaculture Research*, 37, pp.380–388.

Sajilata, M.G., Singhal, R.S. & Kulkarni, P.R., 2006. Resistant starch — a review. *Comprehensive Reviews in Food Science and Food Safety*, 5, pp.1–17.

Salinas, I. *et al*., 2005. Dietary administration of *Lactobacillus delbrueckii* and *Bacillus subtilis*, single or combined, on gilthead seabream cellular innate immune responses. *Fish and Shellfish Immunology*, 19, pp.67–77.

Salminen, S. *et al*., 1999. Probiotics: How should they be defined? *Trends in Food Science and Technology*, 10, pp.107–110.

Sandhu, K.S. & Lim, S.T., 2008. Digestibility of legume starches as influenced by their physical and structural properties. *Carbohydrate Polymers*, 71, pp.245–252.

Sandstedt, R.M. & Gates, R.L., 1954. Raw starch digestion: a comparison of the raw starch digesting capabilities of the amylase systems from four alpha-amylase

sources. *Journal of Food Scienceod*, 19, pp.190–199.

Sapkota, A. *et al*., 2008. Aquaculture practices and potential human health risks: Current knowledge and future priorities. *Environment International*, 34, pp.1215–1226.

Saranraj, P. & Stella, D., 2013. Fungal amylase - A review. *International Journal of Microbiological Research*, 4, pp.203–211.

Satokari, R.M. *et al*., 2001. Polymerase chain reaction and denaturing gradient gel electrophoresis monitoring of fecal bifidobacterium populations in a prebiotic and probiotic feeding trial. *Systemic and Applied Microbiology*, 24, pp.227–231.

Scheidegger, E.M.D. *et al*., 2009. RFLP analysis of a PCR-amplified fragment of the 16S rRNA gene as a tool to identify *Enterococcus* strains. *Memorias do Instituto Oswaldo Cruz*, 104, pp.1003–1008.

Scott, K.P. *et al*., 2014. Prebiotic stimulation of human colonic butyrate-producing bacteria and bifidobacteria, *in vitro*. *FEMS Microbiology Ecology*, 87, pp.30–40.

Sharma, A., Yadav, B.S. & Ritika, 2008. Resistant starch: Physiological roles and food applications. *Food Reviews International*, 24, pp.193–234.

Shearer, K.D., 1994. Factors affecting the proximate composition of cultured fishes with emphasis on salmonids. *Aquaculture*, 119, pp.63–88.

Siccardi III, A.J. *et al*., 2009. Growth and survival of zebrafish (*Danio rerio*) fed different commercial and laboratory diets. *Zebrafish*, 6, pp.275–280.

Siew-Wai, L. *et al*., 2010. Fermentation of *Metroxylon sagu* resistant starch type III by Lactobacillus sp. and *Bifidobacterium bifidum*. *Journal of Agricultural and Food Chemistry*, 58, pp.2274–2278.

Singh, S.K. *et al*., 1997. Comparison of laboratory and pilot-plant corn wet-milling procedures. *Cereal Chemistry Journal*, 74, pp.40–48.

Sinha, A.K. *et al*., 2014. Non-starch polysaccharides and their role in fish nutrition — A review. *Food Chemistry*, 127, pp.1409–1426.

Sissener, N.H. *et al*., 2010. Zebrafish (*Danio rerio*) as a model for investigating the safety of GM feed ingredients (soya and maize); performance, stress response and uptake of dietary DNA sequences. *British Journal of Nutrition*, 103, pp.3–15.

Smil, V., 1999. Nitrogen in crop production: An account of global flows adds recycled in organic up by harvested and quantification of N losses from crop to 26-60. *Global Biogeochemical Cycles*, 13, pp.647–662.

Smith, L.S., 1989. Digestive functions in teleost fishes. In J. Halver, ed. *Fish Nutrition*. San Diego, pp. 331–421.

Smith, M.D. *et al*., 2010. Sustainability and Global Seafood. *Science*, 327, pp.784–786.

Sohn, K.S. *et al*., 2000. The role of immunostimulants in monogastric animal and fish - review. *Asian-Australasian Journal of Animal Sciences*, 13, pp.1178–1187.

Soleimani, N. *et al*., 2012. Dietary supplementation of fructooligosaccharide (FOS) improves the innate immune response, stress resistance, digestive enzyme activities and growth performance of Caspian roach (*Rutilus rutilus*) fry. *Fish &*

Shellfish Immunology, 32, pp.316–321.

Son, V.M. *et al*., 2009. Dietary administration of the probiotic, *Lactobacillus plantarum*, enhanced the growth, innate immune responses, and disease resistance of the grouper *Epinephelus coioides*. *Fish and Shellfish Immunology*, 26, pp.691–698.

Sørensen, M. *et al*., 2011. Effect of stachyose, raffinose and soya-saponins supplementation on nutrient digestibility, digestive enzymes, gut morphology and growth performance in Atlantic salmon (*Salmo salar*, L). *Aquaculture*, 314, pp.145–152.

Sotomayor, C. *et al*., 1999. Lentil starch content and its microscopical structure as influenced by natural fermentation. *Starch*, 51, pp.152–156.

Souza, P.M. de & Magalhães, P. de O., 2010. Application of microbial α-amylase in industry - a review. *Brazilian Journal of Microbiology*, 41, pp.850–861.

Spanggaard, B. *et al*., 2001. The probiotic potential against vibriosis of the indigenous microflora of rainbow trout. *Environmental Microbiology*, 3, pp.755–756.

Spiller, R. & Garsed, K., 2009. Infection, inflammation, and the irritable bowel syndrome. *Digestive and Liver Disease*, 41, pp.844–849.

Spiller, R.C., 2007. Role of infection in irritable bowel syndrome. *Journal of Gastroenterology*, 42, pp.41–47.

Srichuwong, S. *et al*., 2005. Structure of lintnerized starch is related to X-ray diffraction pattern and susceptibility to acid and enzyme hydrolysis of starch granules. *International Journal of Biological Macromolecules*, 37, pp.115–121.

Staykov, Y. *et al*., 2007. Effect of mannan oligosaccharide on the growth performance and immune status of rainbow trout (*Oncorhynchus mykiss*). *Aquaculture International*, 15, pp.153–161.

Stoffels, G., Nes, I.F. & Gubmundsdottlr, A., 1992. Isolation and properties of a bacteriocin-producing *Carnobacterium piscicola* isolated from fish. *Journal of Applied Bacteriology*, 73, pp.309–316.

Su, N.W. *et al*., 2005. Effects of temperature and sodium chloride concentration on the activities of proteases and amylases in soy sauce koji. *Journal of Agricultural and Food Chemistry*, 53, pp.1521–1525.

Sugita, H. *et al*., 1996. Antibacterial abilities of intestinal bacteria in freshwater cultured fish. *Aquaculture*, 145, pp.195–203.

Sugita, H., Kawasaki, J. & Deguchi, Y., 1997. Production of amylase by the intestinal microflora in cultured freshwater fish. *Letters in Applied Microbiology*, 24, pp.105–108.

Sugita, H., Miyajima, C. & Deguchi, Y., 1991. The vitamin B12-producing ability of the intestinal microflora of freshwater fish. *Aquaculture*, 92, pp.267–276.

Sullam, K.E. *et al*., 2012. Environmental and ecological factors that shape the gut 2 bacterial communities of fish: A meta-analysis - Supplementary. *Molecular Ecology*, 21, pp.1–16.

Svihus, B., Uhlen, A.K. & Harstad, O.M., 2005. Effect of starch granule structure, associated components and processing on nutritive value of cereal starch: A

review. *Animal Feed Science and Technology*, 122, pp.303–320.

Swanson, K.S. *et al*., 2002. Fructooligosaccharides and *Lactobacillus acidophilus* modify gut microbial populations, total tract nutrient digestibilities and fecal protein catabolite concentrations in healthy adult dogs. *The Journal of Nutrition*, 132, pp.3721–3731.

Ta'ati, R. *et al*., 2011. Growth performance, carcass composition, and immunophysiological indices in juvenile great sturgeon (*Huso huso*) fed on commercial prebiotic, Immunoster. *Iranian Journal of Fisheries Sciences*, 10, pp.324–335.

Tacon, A.G.J. & Metian, M., 2008. Aquaculture feed and food safety: The role of the Food and Agriculture Organization and the Codex Alimentarius. *Annals of the New York Academy of Sciences*, 59, pp.50–59.

Talpur, A.D. & Ikhwanuddin, M., 2013. *Azadirachta indica* (neem) leaf dietary effects on the immunity response and disease resistance of Asian seabass, *Lates calcarifer* challenged with *Vibrio harveyi*. *Fish & Shellfish Immunology*, 34, pp.254–264.

Tannock, G.W. *et al*., 2000. Analysis of the fecal microflora of human subjects consuming a probiotic product containing *Lactobacillus rhamnosus* DR20. *Applied and Environmental Microbiology*, 66, pp.2578–2588.

Tanton, T. & Haq, N., 2008. Climate change: an exciting challenge for new and underutilised crops. In J. Smartt & N. Haq, eds. *International Symposium on New Crops and Uses: Their role in a rapidly changing world* . Southampton: Centre of Underutilised Crops, University of Southampton, pp. 15–22.

Taoka, Y. *et al*., 2006. Growth, stress tolerance and non-specific immune response of Japanese flounder *Paralichthys olivaceus* to probiotics in a closed recirculating system. *Fisheries Science*, 72, pp.310–321.

Tharanathan, R.N. & Mahadevamma, S., 2003. Grain legumes — a boon to human nutrition. *Trends in Food Science and Technology*, 14, pp.507–518.

Themeier, H. *et al*., 2005. Structural and morphological factors influencing the quantification of resistant starch II in starches of different botanical origin. *Carbohydrate Polymers*, 61, pp.72–79.

Thompson, D.B., 2000. Strategies for the manufacture of resistant starch. *Trends in Food Science and Technology*, 11, pp.245–253.

Tlaskalová-Hogenová, H. *et al*., 2011. The role of gut microbiota (commensal bacteria) and the mucosal barrier in the pathogenesis of inflammatory and autoimmune diseases and cancer: contribution of germ-free and gnotobiotic animal models of human diseases. *Cellular & molecular immunology*, 8, pp.110–20.

Toole, R.O. *et al*., 2004. Visualisation of Zebrafish infection by GFP-labelled Vibrio anguillarum. *Microbial Pathogenesis*, 37, pp.41–46.

Topping, D.L. & Clifton, P.M., 2001. Short-chain fatty acids and human colonic function: roles of resistant starch and nonstarch polysaccharides. *Physiological Reviews*, 81, pp.1031–1064.

Topping, D.L., Fukushima, M. & Bird, A.R., 2003. Resistant starch as a prebiotic and

synbiotic: state of the art. In *The Proceedings of the Nutrition Society 62*. Edinburgh, pp. 171–176.

Torrecillas, S. *et al*., 2007. Immune stimulation and improved infection resistance in European sea bass (*Dicentrarchus labrax*) fed mannan oligosaccharides. *Fish and Shellfish Immunology*, 23, pp.969–981.

Torrecillas, S. *et al*., 2011. Improved feed utilization, intestinal mucus production and immune parameters in sea bass (*Dicentrarchus labrax*) fed mannan oligosaccharides (MOS). *Aquaculture Nutrition*, 17, pp.223–233.

Torres, P.M.D. *et al*., 2010. Galacto-oligosaccharides: production, properties, applications, and significance as prebiotics. *Comprehensive Reviews in Food Science and Food Safety*, 9, pp.438–454.

Tsegaye, D. *et al*., 2007. *The role of Garsa (Dobera glabra) for household food security at times of food shortage in Aba`ala Wereda, North Afar: Ecological adaptation and socio-economic value*. Available at: https://www.zef.de/uploads/tx_zefportal/Publications/8cfe_Report%2049.pdf

Tu, W. *et al*., 2013. Dietary alpha-linolenic acid does not enhance accumulation of omega-3 long-chain polyunsaturated fatty acids in barramundi (*Lates calcarifer*). *Comparative Biochemistry and Physiology B- Biochemistry and Molecular Biology*, 164, pp.29–37.

Tyrrell, G.J. *et al*., 2002. *Enterococcus gilvus* sp. nov. and *Enterococcus pallens* sp. nov. isolate from human clinical specimens. *Journal of Clinical Microbiology*, 40(4), pp.1140–1145.

Ulloa, P.E., Medrano, J.F. & Feijo, C.G., 2014. Zebrafish as animal model for aquaculture nutrition research. *Frontiers in Genetics*, 5, pp.1–6.

Vasanthan, T. & Bhatty, R.S., 1998. Enhancement of resistant starch (RS3) in amylomaize, barley, field pea and lentil starches. *Starch - Stärke*, 50, pp.286–291.

Vendrell, D. *et al*., 2006. *Lactococcus garvieae* in fish: A review. *Comparative Immunology, Microbiology and Infectious Diseases*, 29, pp.177–198.

Verschuere, L. *et al*., 2000. Probiotic bacteria as biological control agents in aquaculture. *Microbiology and Molecular Biology Reviews*, 64, pp.655–671.

Villamil, L. *et al*., 2003. Control of Vibrio alginolyticus in Artemia culture by treatment with bacterial probiotics. *Aquaculture*, 219, pp.43–56.

Villarreal, M.E., Ribotta, P.D. & Iturriaga, L.B., 2013. Comparing methods for extracting amaranthus starch and the properties of the isolated starches. *LWT - Food Science and Technology*, 51, pp.441–447.

Vine, N.G. *et al*., 2004. Competition for attachment of aquaculture candidate probiotic and pathogenic bacteria on fish intestinal mucus. *Journal of Fish Diseases*, 27, pp.319–326.

De Vuyst, L. & Vandamme, E.J., 1994. Antimicrobial potential of lactic acid bacteria. In *Bacteriocins of Lactic Acid Bacteria*. Boston, MA: Springer US, pp. 91–142.

Wang, N. *et al*., 2009. Influence of cooking and dehulling on nutritional composition of several varieties of lentils (*Lens culinaris*). *LWT - Food Science and Technology*, 42, pp.842–848.

Wang, X.I.N. *et al*., 1999. *In vitro* utilization of amylopectin and high-amylose maize (amylomaize) starch granules by human colonic bacteria. *Applied and Environmental Microbiology*, 65, pp.4848–4854.

Wang, Y., Li, J. & Lin, J., 2008. Probiotics in aquaculture: Challenges and outlook. *Aquaculture*, 281, pp.1–4.

Wani, I.A. *et al*., 2010. Physico-chemical properties of starches from Indian kidney bean (*Phaseolus vulgaris*) cultivars. *International Journal of Food Science and Technology*, 45, pp.2176–2185.

Wilson, K.H., 1995. Molecular biology as a tool for Taxonomy. *Clinical Infectious Diseases*, 20, pp.S117–S121.

Wronkowska, M. *et al*., 2006. *In vitro* fermentation of new modified starch preparations-changes of microstructure and bacterial end-products. *Enzyme and Microbial Technology*, 40, pp.93–99.

Wu, Y. *et al*., 2013. Effects of dietary supplementation of fructooligosaccharide on growth performance, body composition, intestinal enzymes activities and histology of blunt snout bream (*Megalobrama amblycephala*) fingerlings. *Aquaculture Nutrition*, 19, pp.886–894.

Wyatt, G.M. & Horn, N., 1988. Fermentation of resistant food starches by human and rat intestinal bacteria. *Journal of the Science of Food and Agriculture*, 44, pp.281–288.

Xu, B. *et al*., 2009. Effect of prebiotic xylooligosaccharides on growth performances and digestive enzyme activities of allogynogenetic crucian carp (*Carassius auratus gibelio*). *Fish Physiology and Biochemistry*, 35, pp.351–357.

Xu, G., Li, C. & Yao, Y., 2009. Proteomics analysis of drought stress-responsive proteins in *Hippophae rhamnoides* L. *Plant Molecular Biology Reporter*, 27, pp.153–161.

Yadav, B.S., Sharma, A. & Yadav, R.B., 2010. Resistant starch content of conventionally boiled and pressure-cooked cereals, legumes and tubers. *Journal of Food Science and Technology*, 47, pp.84–88.

Yanbo, W. & Zirong, X., 2006. Effect of probiotics for common carp (*Cyprinus carpio*) based on growth performance and digestive enzyme activities. *Animal Feed Science and Technology*, 127, pp.283–292.

Yao, N., Paez, A. V. & White, P.J., 2009. Structure and function of starch and resistant starch from corn with different doses of mutant amylose-extender and floury-1 alleles. *Journal of Agricultural and Food Chemistry*, 57, pp.2040–2048.

Ye, J.D. *et al*., 2011. Single or combined effects of fructo- and mannan oligosaccharide supplements and *Bacillus clausii* on the growth, feed utilization, body composition, digestive enzyme activity, innate immune response and lipid metabolism of the Japanese flounder *Paralichth*. *Aquaculture Nutrition*, 17, pp.e902–e911.

Yoshimoto, Y. *et al*., 2001. Molecular structure and pasting properties of legume starches. *Journal of Applied Glycoscience*, 48, pp.317–324.

Yousefian, M. & Amiri, M.S., 2009. A review of the use of prebiotic in aquaculture for fish and shrimp. *African Journal of Biotechnology*, 8, pp.7313–7318.

Zaman, S.A. & Sarbini, S.R., 2015. The potential of resistant starch as a prebiotic. *Critical Reviews in Biotechnology*, 0, pp.1–7.

Zavareze, E.D.R. & Dias, A.R.G., 2011. Impact of heat-moisture treatment and annealing in starches: A review. *Carbohydrate Polymers*, 83, pp.317–328.

Zhang, T. *et al*., 2016. Effect of a potential probiotics *Lactococcus garvieae* B301 on the growth performance, immune parameters and caecum microflora of broiler chickens. *Journal of Animal Physiology and Animal Nutrition*, 100, pp.413–421.

Zhang, Y. & Han, J.H., 2010. Crystallization of high-amylose starch by the addition of plasticizers at low and intermediate concentrations. *Journal of Food Science*, 75, pp.N8–N16.

Zhou, Y., Hoover, R. & Liu, Q., 2004. Relationship between α-amylase degradation and the structure and physicochemical properties of legume starches. *Carbohydrate Polymers*, 57, pp.299–317.

Zhou, Z. *et al*., 2014. Methodological approaches used to assess fish gastrointestinal communities. In D. L. Merrifield & E. Ringö, eds. *Aquaculture Nutrition*. Chichester, UK: John Wiley & Sons, pp. 101–127.

Zhou, Z., Cao, X. & Zhou, J.Y.H., 2013. Effect of resistant starch structure on short-chain fatty acids production by human gut microbiota fermentation *in vitro*. *Starch/Starke*, 65, pp.509–516.

Zimmermann, B., Bauer, E. & Mosenthin, R., 2001. Pro- and prebiotics in pig nutrition - potential modulators of gut health? *Journal of Animal and Feed Sciences*, 10, pp.47–56.

Appendix A

Preparation of reagents for Modified method of McCleary *et al.* (2002)

Sodium maleate buffer (0.1 M, pH 6.0) was prepared by dissolving 23.2 g of maleic acid (Sigma M0375) in 1600 mL of distilled water and the pH was adjusted to 6.0 using 4 M sodium hydroxide. A total of 1.47 g of calcium chloride dihydrate ($CaCl_2.2H_2O$, Merck Millipore 102382) and 0.4 g of sodium azide (R&M Chemicals) was added and dissolved in the solution. The volume was then adjusted to 2 L and stored at 4 °C for up to 12 months.

Sodium acetate buffer (1.2 M, pH 3.8) was prepared by adding a total of 69.6 mL of glacial acetic acid (1.05 g/mL, Sigma A6283) into 800 mL of distilled water. Then, the pH was adjusted to 3.8 using 4 M sodium hydroxide and the volume of the solution was adjusted to 2 L using distilled water. The solution was stored at room temperature.

Potassium hydroxide solution (2 M) was prepared by dissolving a total of 112.2 g of potassium hydroxide granules (R&M Chemicals) in 900 mL of distilled water. The volume was adjusted to 1 L and was stored at room temperature.

Aqueous ethanol (50%) was prepared by adding 500 mL of 95% (v/v) ethanol (R&M Chemicals) into 500 mL of distilled water and stored in a well-sealed bottle at room temperature.

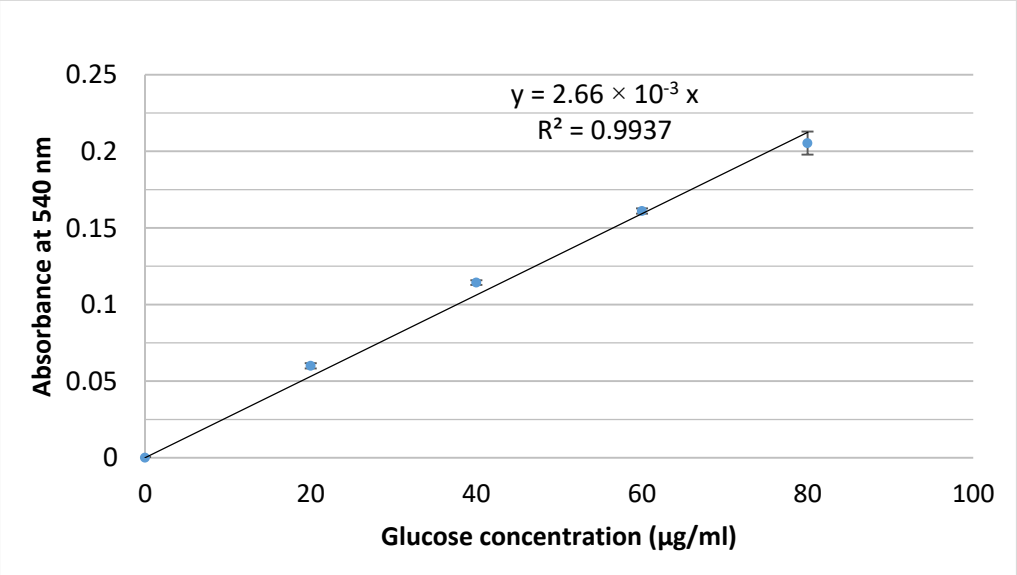
The concentrated AMG solution (3300 U/mL) and dilute AMG (300 U/mL) solution were prepared separately. For the concentrated AMG solution, 110 mg of AMG powder from *Aspergillus niger* (Sigma 10113, Lot # BCBF3497V) was weighed and

added into 4 mL of distilled water. The contents were mixed via inversion and used as the concentrated AMG solution. The dilute AMG solution was prepared by transferring an aliquot of 1 mL of the concentrated AMG solution into 11 mL of sodium maleate buffer (0.1 M, pH 6.0) and mixed via inversion. The solution was used as the dilute AMG solution. Both AMG solutions were used on the day of preparation and stored at 4 °C until use.

The α-amylase solution was prepared fresh accordingly using α-amylase from *Aspergillus oryzae* (Sigma 10065, Lot # BCBL0370V), instead of pancreatic α-amylase. In brief, a total of 1 g of α-amylase from *Aspergillus oryzae* was dissolved in 100 mL of sodium maleate buffer (0.1 M, pH 6.0) and stirred for 5 min. Then, 1 mL of the dilute AMG solution was added and mixed well and followed by centrifugation at 1500 *g* for 10 min. The supernatant was carefully decanted and used as the α-amylase solution on the day of preparation.

Appendix B

Standard curve of glucose concentrations and wavelengths of 540 nm



Appendix C

Preparation of reagent for method of Megazyme RS Kit

Sodium maleate buffer (0.1 M, pH 6.0), sodium acetate buffer (1.2 M, pH 3.8), potassium hydroxide solution (2 M) and aqueous ethanol (50%) was prepared as described in Appendix A.

Glucose determination (GOPOD) reagent was prepared based on the protocol provided by the kit. In brief, the contents of GOPOD reagent buffer from bottle 3 (Megazyme RS kit) added and diluted with distilled water into a total of 1 L. A total of 20 mL of GOPOD reagent buffer was aliquot and added into bottle 4 (Megazyme RS kit) and mixed. Then, the contents of bottle 4 were transferred into the GOPOD reagent buffer enclosed in aluminium foil to protect from light. The solution was mixed and separated into aliquots of 30 mL in foil-wrapped 50 mL centrifuge tubes and was stored at -20 °C.

Dilute AMG solution was prepared based on the protocol provided by the kit. In brief, a total of 2 mL of concentrated AMG solution was aliquot from bottle 1 (Megazyme RS kit) and added to 22 mL of 0.1 M sodium maleate buffer (0.1 M, pH 6.0). The solution was mixed via vortex and separated into 5 mL aliquots in 15 mL centrifuge tubes and stored at -20 °C.

The α-amylase solution was prepared fresh based on the protocol provided by the kit. In brief, a total of 1 g of pancreatic α-amylase from bottle 2 (Megazyme RS kit) was dissolved in 100 mL of 0.1 M sodium maleate buffer (0.1 M, pH 6.0) and stirred for 5 min. Then, 1 mL of dilute AMG solution was added and mixed well and followed

by centrifugation at 1500 *g* for 10 min. The supernatant was collected and used as the α-amylase solution on the day of preparation.

Appendix D

Preparation of reagent for Production of RS from legumes

Prior to the production of resistant starch, two reagents – sodium maleate buffer and 2 M hydrochloric acid were prepared as detailed below: (i) Sodium maleate buffer (0.1 M, pH 5.3) was prepared by dissolving 23.2 g of maleic acid in 1600 mL of distilled water and the pH was adjusted to 5.3 using 4 M sodium hydroxide. The volume was then adjusted to 2 L and stored at 4 °C for up to 12 months. (ii) 2.0 M hydrochloric acid was prepared by adding a total of 328.47 mL of concentrated hydrochloric acid (37%, Sigma 320331) into 500 mL of deionized water. Then, the total volume was adjusted to 2 L and stored at room temperature for up to 1 month.

Appendix E

Method reference for proximate analysis of legume samples used by ALS Technichem Sdn. Bhd.

Test Parameter	Method Reference
Calories (by calculation)	Method of Analysis for Nutrition Labeling (1993)* pg. 5 & 106
Total fat	In-House Method QWI-OF/17-10 based on Method of Analysis for Nutrition Labeling (1993) Chapter 18 and Pearson's (1991)** pg. 24
Protein/ crude protein	In-House Method QWI-OF/17-6 based on Method of Analysis for Nutrition Labeling (1993) Chapter 28 and Pearson's (1991) pg. 106
Moisture	In House Method QWI-OF/17-38 Moisture Analyser
Ash	Method of Analysis for Nutrition Labeling (1993) Chapter 10
Total carbohydrate, (by calculation)	Method of Analysis for Nutrition Labeling (1993) pg. 106***
Dietary fibre	In-House Method QWI-OF/17-14 based on Methods AOAC 985.29****

Notes:

* Sullivan, D.M. and Carpenter, D.E., 1993. Methods of Analysis for Nutrition Labeling. Association of Official Analytical Chemists Inc. (AOAC).

** Kirk, S. and Sawyer, R., 1991. Pearson's composition and analysis of foods (No. Ed. 9). Longman Group Ltd.

*** Total carbohydrate (g/100g; % w/w) is determined by the formula below:

= 100% - (Total fat + crude protein + moisture + ash)

**** Dietary fibre is not included within the total % (g/100g; % w/w) of parameters 1-6.

Appendix F

Preparation of API test kit for phenotypic identification of LAB isolates

The API test was prepared based on the manufacturer's protocol. In brief, the wells on the plastic tray were filled with sterile distilled water. The API 50 CHL test strips were placed in the tray according to their number sequence. Then, 200 µL of the 24 hr LAB culture was transferred into a 15-mL centrifuge tube and added with 5 mL of API CHL medium. The medium was mixed well and approximately 150 µL were transferred onto each capsule on the API CH50 trays. The capsules were then sealed with two drops of sterile mineral oil (BioMérieux) and covered with the plastic lid provided with the kit. The test kit was incubated at 37 °C for 48 hr and colour change from purple to yellow was recorded at 24 hr and 48 hr of incubation. The data was then analysed with the Apiweb^{TM} database.

Appendix G

Carbohydrate fermentation pattern of LC-A & LC-B via API CHL 50

Carbohydrate test	Bacteria	
	LC-A	LC-B
Glycerol	-	-
Erythritol	-	-
D-arabinose	-	-
L-arabinose	+	-
D-ribose	-	+
D-xylose	+	-
L-xylose	-	-
D-adonitol	-	-
Methyl-bd-xylopyranoside	-	-
D-galactose	-	+
d-glucose	+	+
Fructose	+	+
D-mannose	+	+
L-sorbose	-	-
L-rhamnose	-	-
Dulcitol	-	-
Inositol	-	-
D-mannitol	-	+
D-sorbitol	-	-
metyhl-ad-mannopyranoside	-	-
Methyl-ad-glucopyranoside	-	-
n-acetyl-glucosamine	+	+
amygdalin	+	+
arbutin	+	+
esculin	+	+
salicin	+	+
d-cellobiose	+	+
d-maltose	+	+
d-lactose	-	-
d-melibiose	-	-
d-sucrose	+	-
d-trehalose	-	+
inulin	-	-
d-lmelezitose	-	-
d-rafinose	-	-
starch	-	+
glycogen	-	-
xylitol	-	-
gentibiose	+	+
d-turanose	-	-

d-lyxose	-	-
d-tagatose	-	-
d-fucose	-	-
l-fucose	-	-
d-arabitol	-	-
l-arabitol	-	-
gluconate	+	+
2-ketogluconate	-	-
5-ketogluconate	-	-

Notes:
Analysis of bacteria biochemical profile performed without replicates; n=1
+ refers to a positive result; - refers to a negative result

Appendix H

Carbohydrate fermentation pattern of DR-A & DR-B via API CHL 50

Carbohydrate test	Bacteria	
	LC-A	LC-B
Glycerol	+	+
Erythritol	-	-
D-arabinose	-	-
L-arabinose	-	-
D-ribose	+	+
D-xylose	-	-
L-xylose	-	-
D-adonitol	-	-
Methyl-bd-xylopyranoside	-	-
D-galactose	+	+
d-glucose	+	+
Fructose	+	+
D-mannose	+	+
L-sorbose	-	-
L-rhamnose	-	-
Dulcitol	-	-
Inositol	-	-
D-mannitol	+	+
D-sorbitol	-	+
metyhl-ad-mannopyranoside	-	-
Methyl-ad-glucopyranoside	-	-
n-acetyl-glucosamine	+	+
amygdalin	+	+
arbutin	+	+
esculin	+	+
salicin	+	+
d-cellobiose	+	+
d-maltose	-	+
d-lactose	-	-
d-melibiose	-	-
d-sucrose	-	+
d-trehalose	+	+
inulin	-	-
d-lmelezitose	-	+
d-rafinose	-	-
starch	-	-
glycogen	-	-
xylitol	-	-
gentibiose	+	+
d-turanose	-	-

d-lyxose	-	-
d-tagatose	-	+
d-fucose	-	-
l-fucose	-	-
d-arabitol	-	-
l-arabitol	-	-
gluconate	-	+
2-ketogluconate	-	-
5-ketogluconate	-	-

Notes:
Analysis of bacteria biochemical profile performed without replicates; n=1
+ refers to a positive result; - refers to a negative result

Appendix I

Identification of LC-A & LC-B via 16S rRNA gene sequencing

a) 16S rRNA gene sequence of LC-A using primer sets plb16 & mlb16

NNNAAGGGGGGGCTATAATGCAGTCGAACGCTTTGTGGTTCAACTGATTTGAAGAGCTTGC
TCAGATATGACGATGGACATTGCAAAGAGTGGCGAACGGGTGAGTAACACGTGGGAAACC
TACCTCTTAGCAGGGGATAACATTTGGAAACAGATGCTAATACCGTATAACAATAGCAACCG
CATGGTTGCTACTTAAAAGATGGTTCTGCTATCACTAAGAGATGGTCCCGCGGTGCATTAGT
TAGTTGGTGAGGTAATGGCTCACCAAGACGATGATGCATAGCCGAGTTGAGAGACTGATCG
GCCACAATGGGACTGAGACACGGCCCATACTCCTACGGGAGGCAGCAGTAGGGAATCTTCC
ACAATGGGCGAAAGCCTGATGGAGCAACGCCGCGTGTGTGATGAAGGGTTTCGGCTCGTA
AAACACTGTTGTAAGAGAAGAATGACATTGAGAGTAACTGTTCAATGTGTGACGGTATCTT
ACCAGAAAGGAACGGCTAACTACTGCCCAGCCAGCCAAAA

b) 16S rRNA gene sequence of LC-A using primer sets U8F & U1492R

NNCANTCTGTTCACCGTTAGACGGCTGGCTCCCGAAGGTTACCCCACCGGCTTTGGGTGTTA
CAAACTCTCATGGTGTGACGGGCGGTGTGTACAAGACCCGGGAACGTATTCACCGCGGCGT
GCTGATCCGCGATTACTAGCGATTCCGACTTCATGTAGGCGAGTTGCAGCCTACAATCCGAA
CTGAGACGTACTTTAAGAGATTAGCTCACCCTCGCGGGTTGGCAACTCGTTGTATACGCCAT
TGTAGCACGTGTGTAGCCCAGGTCATAAGGGGCATGATGATTTGACGTCATCCCCACCTTCC
TCCGGTTTGTCACCGGCAGTCTCACTAGAGTGCCCAACTAAATGCTGGCAACTAGTAATAAG
GGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAACCATGC
ACCACCTGTCACCTTGTCCCCGAAGGGAACGCTCCATCTCTGGAGTTGTCAAGGGATGTCAA
GACCTGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACATGCTCCACCGCTTGTGCGGGT
CCCCGTCAATTCCTTTGAGTTTCAACCTTGCGGTCGTACTCCCCAGGCGGAGTGCTTAATGCG
TTAGCTGCGGCACTTAAGGGCGGAAACCCTCAAACACCTAGCACTCATCGTTTACGGTGTGG
ACTACCAGGGTATCTAATCCTGTTTGCTACCCACACTTTCGAGCCTCAACGTCAGTTACAGTC
CAGAAAGCCGCCTTCGCCACTGGTGTTCTTCCATATATCTACGCATTTCACCGCTACACATGG
AGTTCCACTTTCCTCTACTGCACTCAAGTCATCCAGTTTCCAAAGCAATTCCTCAGTTGAGCT
GAGGGCTTTCACTTCAGACTTAAATAACCGTCTGCGCTCGCTTTACGCCCAATAAATCCGGA
TAACGCTTGGAACATACGTATTACCGCGGCTGCTGGCACGTATTTAGCCGTTCCTTTCTGGTA
AGATACCGTCACACATTGAACAGTTACTCTCAATGTCATTCTTCTCTTACAACAGTGTTTTAC
GAGCCGAAACCCTTCATCACACACGCGGCGTTGCTCCATCAGGCTTTCGCCCATTGTGGAAG
ATTCCCTACTGGTGCCTCCCGTAAGAATATGGGCNCGGGTCTCAGTTCCCATTGTGGGCCGA
ACAAGTCTCTCAACTCCGGCTATGGAACCATCGGCCTTGGTGGAGCCCTTTCNCTCACCAAC
TTACCTAATGGC

c) 16S rRNA gene sequence of LC-B using primer sets plb16 & mlb16

NNNAGNGGCGGCGTGCTATAATGCAGTCGAGCGATGATTGAAGATAGCTTGCTATTTTCAT
GAAGAGCGGCGAACGGGTGAGTAACGCGTGGGAAATCTGCCGAGTAGCGGGGGACAACG
TTTGGAAACGAACGCTAATACCGCATAACAATGAGAATCGCATGATTCTTATTTGAAAGAAG
CAATTGCTTCACTACTTGATGATCCCGCGTTGTATTAGCTAGTTGGTAGTGTAAAGGACTACC
AAGGCGATGATACATAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGG
CCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGGGGCAACCCTGACCG
AGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAACTCTGTTGTTAGAGAAGAAC
GTTAAGTAGAGTGGAAAATTACTTAAGTGACGGTATCTAACCAGAAAGGGACGGCTAACTA
CGTGCCAAGCAGCCAAAANN

d) 16S rRNA gene sequence of LC-B using primer sets U8F & U1492R

NNNCCANCGGGTGTACTTAGGAGCGCCTCCTTGCGGTTAGGCAACCTACTTTGGGTACTCC
CAACTTCCGTGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGCGGCGTG
CTGATCCGCGATTACTAGCGATTCCGACTTCATGCAGGCGAGTTGCAGCCTGCAATCCGAAC
TGAGAATGGTTTTAAGAGATTAGCGCACCCTCGCGGGTTGGCGACTCGTTGTACCATCCATT
GTAGCACGTGTGTAGCCCAGGTCATAAGGGGCATGATGATTTGACGTCATCCCCACCTTCCT
CCGGTTTATCACCGGCAGTCTCACTAGAGTGCCCAACTTAATGATGGCAACTAGTAATAAGG
GTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAACCATGCAC
CACCTGTATCCCGTGTCCCGAAGGAACTCCTTATCTCTAAGGATAGCACGAGTATGTCAAGA
CCTGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCC
CCGTCAATTCCTTTGAGTTTCAACCTTGCGGTCGTACTCCCCAGGCGGAGTGCTTAATGCGTT
AGCTGCGCTACAGAGAACTTATAGCTCCCTACAGCTAGCACTCATCGTTTACGGCGTGGACT
ACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGAGCCTCAGTGTCAGTTACAGGCCA
GAGAGCCGCTTTCGCCTCCGGTGTTCCTCCATATATCTACGCATTTCACCGCTACACATGGAA
TTCCACTCTCCTCTCCTGCACTCAAGTCTCCCAGTTTCCAATGCACACAATGGTTGAGCCACT
GCCTTTTACATCAGACTTAAGAAACCACCTGCGCTCGCTTTACGCCCAATAAATCCGGACAA
CGCTTGGGACCTACGTATTACCGCGGCTGCTGGCACGTAGTTAGCCGTCCCTTTCTGGTTAG
ATACCGTCACTTAAGTAATTTTCCACTCTACTTAACGTTCTTCTCTAACAACAGAGTTTTACGA
TCCGAAAACCTTCTTCATTCACGCGGCGTTGCTCGGTCAGGGTTGCCCCCATTGCCGAAGAT
TCCCTACTGCTGCCTCCCGTAGGAATCTGGGGCCGGGTCTCAGTCCCAAGTGGGGCCGATTC
ACCCTCCCAGGGCGGCTATGGATCAACGGCCTTGGGAGGCCCTTTANCCTACCCAATTNG

Appendix J

Identification of DR-A & DR-B via 16S rDNA sequencing

a) 16S rDNA sequence of DR-A using primer sets plb16 & mlb16

NNNNNNNNNNNNNNCNANACNTGCANGTCGANGCTTTTTCTTTCACCGGAGCTTGCTCCA
CCGAAAGAAAAAGAGTGGCGAACGGGTGAGTAACACGTGGGTAACCTGCCCATCAGAAGG
GGATAACACTTGGAAACAGGTGCTAATACCGTATAACAAGTAAAACCGCATGGTTTTACTTT
GAAAGGCGCTTTTGCGTCACTGATGGATGGACCCGCGGTGCATTAGCTAGTTGGTGAGGTA
ACGGCTCACCAAGGCAACGATGCATAGCCGACCTGAGAGGGTGATCGGCCACATTGGGAC
TGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGACGAA
AGTCTGACCGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAACTCTGTTGTT
AGAGAAGAACAAGGATGAGAGTAAAATGTTCATCCCTTGACGGTATCTAACCAGAAAGCCA
CGGCTAACTACGTGNNCAGCAGNNNN

b) 16S rDNA sequence of DR-A using primer sets U8F & U1492R

NNNNNNNNNNNNNNNCNNNANNTGCAGTCGAACGCTTTTTCTTTCNCCGGAGCTTGCTC
CACCGAAAGAAAAAGAGTGGCGAACGGGTGAGTAACACGTGGGTAACCTGCCCATCAGAA
GGGGATAACACTTGGAAACAGGTGCTAATACCGTATAACAAGTAAAACCGCATGGTTTTAC
TTTGAAAGGCGCTTTTGCGTCACTGATGGATGGACCCGCGGTGCATTAGCTAGTTGGTGAG
GTAACGGCTCACCAAGGCAACGATGCATAGCCGACCTGAGAGGGTGATCGGCCACATTGG
GACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGAC
GAAAGTCTGACCGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAACTCTGTT
GTTAGAGAAGAACAAGGATGAGAGTAAAATGTTCATCCCTTGACGGTATCTAACCAGAAAG
CCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATT
TATTGGGCGTAAAGCGAGCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAA
CCGGGGAGGGTCATTGGAAACTGGGAAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCAT
GTGTAGCGGTGAAATGCGTAGATATATGGAGGAACACCAGTGGCGAANGCGGCTCTCTGG
TCTGTAACTGACGCTGANGCTCGAAAGCGTGGGGGAGCAAACAGGATTAGATACCCTGGT
AGTCCACGCCGTAAACGATGAGTGCTAAGTGTTGGANGGTTTCCGCCCTTCAGTGCTGCAG
CTAACGCATTAAGCACTCCGCCTGGGGAGTACGANCGCAAGGTTGAAACTCAAAGGAATTG
ACGGGGGCCCGCACAAGCGGTGGAGCATGTGGGTTTAATTCGAAGCAACGCGAAGAACCT
TACCAGGTCTTGACATCCTTTGACCACTCTAGAGATAGAGCTTCCCNTTCGGGGGCAAGTGA
CNNNNNNNNCATNNNNNCGTCAGCTCNNGNCNNGANATNNNGNNNAGTCCCCGNANN
ANCGCNNCNNNNNNNNNNCNTCNTTNNNNNNNACNNNNNCNNNACNNNNNNNNAAN
CGNANNNNNNNGNNNNNNGTNNNTCNNCNNNNNNNNGANNNNNNNNNNNNNNNNC
NACNANNGGNNNNTNNNNCNNNNNNNNNNN

c) 16S rDNA sequence of DR-B using primer sets plb16 & mlb16

NNNNNNNNNNNNNNNNNACNTGCAGTCGAACGCTTCTTTCCTCCCGAGTGCTTGCACTCA
ATTGGAAAGAGGAGTGGCGGACGGGTGAGTAACACGTGGGTAACCTACCCATCAGAGGG
GGATAACACTTGGAAACAGGTGCTAATACCGCATAACAGTTTATGCCGCATGGCATAAGAG
TGAAAGGCGCTTTCGGGTGTCGCTGATGGATGGACCCGCGGTGCATTAGCTAGTTGGTGAG
GTAACGGCTCACCAAGGCCACGATGCATAGCCGACCTGAGAGGGTGATCGGCCACACTGG
GACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGAC
GAAAGTCTGACCGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAACTCTGTT
GTTAGAGAAGAACAAGGACGTTAGTAACTGAACGTCCCCTGACGGTATCTAACCAGAAAGC
CACGGCTAACTANNNNNNNNANCAGNN

d) 16S rDNA sequence of DR-B using primer sets U8F & U1492R

NNNNNNNNNNNNNNNCNNNNNTGCAGTCGACGCTTCTTTCCTCCCGAGTGCTTGCACTC
AATTGGAAAGAGGAGTGGCGGACGGGTGAGTAACACGTGGGTAACCTACCCATCAGAGGG
GGATAACACTTGGAAACAGGTGCTAATACCGCATAACAGTTTATGCCGCATGGCATAAGAG
TGAAAGGCGCTTTCGGGTGTCGCTGATGGATGGACCCGCGGTGCATTAGCTAGTTGGTGAG
GTAACGGCTCACCAAGGCCACGATGCATAGCCGACCTGAGAGGGTGATCGGCCACACTGG
GACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGAC
GAAAGTCTGACCGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAACTCTGTT
GTTAGAGAAGAACAAGGACGTTAGTAACTGAACGTCCCCTGACGGTATCTAACCAGAAAGC
CACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTT
ATTGGGCGTAAAGCGAGCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAAC
CGGGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGAGAGTGGAATTCCAT
GTGTAGCGGTGAAATGCGTANATATATGGAGGAACACCAGTGGCGAANGCGGCTCTCTGG
TCTGTAACTGACGCTGAGGCTCGAAAGCGTGGGGAGCAAACAGGATTANATACCCTGGTA
GTCCACGCCGTANACGATGAGTGCTAAGTGTTGGAGGGTTTCCGCCCTTCAGTGCTGCAGC
ANCGCATTAAGCACTCCGCCTGGGGGAGTACNACCGCAAGGTTGAAACTCAAANGAATTG
ACGGGGGCCCGCACAAGCNGTGGAGCATGTGGTTTANNTCNNANCAACGCNNNAANCTT
ACNNNGTCNGACNTCCTTGACCACTCNNNNNNNANANCTTTCCNTTCNNNNCNAANTGAC
NNGNGNNGCANGCTTGTCGTCAGCNCNNNNNNNNNNANGNNNGGGATTNNNNCNNNN
ANCNANCNNCNNNCCNTNANTNNNNANTTNNNCATNCNNTNNNNNNNNNNNCTNCN
ANNNNGNNCTGNCNNNNNNNNNCNNNNNNNNNNNNNNNNNNANNNNNNNNNNN
ANNNCNNNNNNNNNNGNNTANNNNNNNNNNNNNNCNNNNNNNNNNNTNNNNNN

Appendix K

Nutritional analysis of diets

I. Energy content analysis

Energy content analysis was performed using a bomb calorimeter (6400 Automatic Isoperibol Calorimeter, Parr Instrument Company), where the gross energy is measured in terms of heat produced when a sample is completely combusted completely into carbon dioxide and water.

Prior to measurement of energy content of samples, the water re-circulating system and oxygen valves were switched on and set at 400 psi respectively. The calorimeter's pre-test was performed to ensure that the machine was functioning properly, followed by the measurement of benzoic acid as a standard, which should give 26.454 MJ/kg gross energy.

Approximately 1 g of sample was weighed into crucibles up to 4 decimal places and was compressed with the compressing tool. The crucible was placed into the holder and ignition thread was tied to connect the heating wire to the sample. The bomb head was inserted into the calorimeter and locked. The identity and weight of the sample was input into the calorimeter and the machine was set to start. After approximately 20 s, the run was completed and the bomb head was removed. The energy measurement obtained was recorded while the underside of the bomb head and the heating wire was dried thoroughly before proceeding with the next sample.

Energy measurement provided by the bomb calorimeter is expressed as kJ/100 g in this study.

II. Protein content analysis

Protein analysis was performed via the Kjeldahl method (Buchi K-360 Distillation Unit), supplemented with a digester (Buchi KjelDigester K-446) based on the AOAC method 976.06-1977 for the analysis of crude protein in animal feed. The method can be separated into 3 components: a) preparation and digestion of samples, b) distillation and, c) titration and calculations

a. Preparation and digestion of samples

Briefly, a total of 100 mg of sample was weighed and transferred into each sample tube. The weight of each sample was recorded up to 3 decimal places. Then, two Titanium micro tablets (Buchi) was added into each sample tube, followed by the addition of 15 mL of concentrated sulfuric acid. The tubes were transferred to the digester and heated to a final temperature of 380 °C for digestion for 1 hr and 30 min. Then the tubes were removed and allowed to cool down for approximately 15 min before proceeding with distillation.

b. Distillation

Distillation was performed on the distillation unit with the following settings:

Parameter	Settings
32% NaOH	63 mL
4% boric acid (pH 4.65)	60 mL
dH2O	60 mL
Reaction time	5 s
Steam power	100%
Distillation time	4 min
Aspirate samples	Yes

The sample tubes were transferred to the distillation unit and the process was initiated on the machine. A 250-mL glass conical flask was used to collect the solution, which was then used for titration when distillation was completed.

c. Titration and calculation

A burette containing 0.1 N sulfuric acid and a pH meter was prepared prior to the titration process. Prior to titration, the pH of the sample was measured continuously while the sample was being stirred. The pH of the sample solution was then adjusted to pH 4.65 via titration of 0.1 N sulfuric acid. The total volume of 0.1 N sulfuric acid used was recorded.

The protein content % (w/w) of the sample was measured based on the formula below:

$$\text{Protein content (\%)} = \frac{(V_S - V_0) \times N \times 1.4}{W} \times F_P \times 100$$

Where:

V_s = volume of sulfuric acid used in titration of sample;

V_0 = volume of sulfuric acid used in titration of blank;

N = normality of sulfuric acid used;

W = weight of the sample;

F_P = protein factor, applied in this study as 6.25

III. Lipid analysis

Lipid analysis was performed using a Sohxlet extractor based on the AOAC method 991.36 for the analysis of crude fat in meat and meat products. The method can be separated into three components: a) sample preparation and setup, b) sample extraction, and c) calculation of crude lipid content

a. Sample preparation and setup

Approximately 1 g of sample was weighed up to three decimal places and transferred onto an 11-cm qualitative grade filter paper (Whatman), which was loosely folded to fit into the bottom of a thimble. The sample was labelled and was transferred into a labelled thimble, which was covered with a plug of cotton wool. The thimble was then transferred into a Sohxlet extractor. Meanwhile, a 500-mL round-bottom flask was also labelled and weighed. Then, an aliquot of 250 mL of petroleum ether was transferred into the round bottom flask and the setup was set up in a fume hood on heating blocks with running water for the reflux condenser.

b. Sample extraction

The flask was heated continuously under the highest heat setting until the petroleum ether was observed to be boiling. Sample extraction was run for 6 hr and was monitored constantly to ensure that sufficient petroleum ether was present for lipid extraction from the samples. After 6 hr, the setup was dismantled and the round bottom flasks were transferred onto the heating blocks to evaporate the remaining petroleum ether from samples. When the petroleum ether was completely removed, the flasks were then weighed.

c. Calculation of lipid content

The crude lipid content of samples was measured based on the formula below:

$$\text{Crude lipid content } (\%) = \frac{W_F - W_0}{W_S} \times 100$$

Where:

W_F = weight of flask after extraction was performed

W_0 = weight of empty flask

W_S = weight of sample inserted into filter paper

IV. Crude fibre content analysis

Crude fibre content analysis was performed using the fibrebag (Gerhardt) method (Method no. AN-04-203), where starch and glucose were removed via digestion in acid and proteins were removed via digestion in alkali, with crude fibre left as the remaining residue. The method can be separated into three components: a) sample preparation, b) sample digestion, and c) calculation of crude fibre content

a. Sample digestion

Prior to use, fibrebags were dried in an oven set at 105 °C for 4 hr and then transferred into a desiccator to cool for 5 min. Fibrebags were weighed and labelled with a waterproof marker. Then, approximately 1 g of de-fatted samples were weighed up to three decimal places and inserted into the fibrebag. The fibrebags were then ready for sample digestion and stored in a desiccator until use.

b. Sample digestion

A spacer was added into each fibrebag and was transferred into the carousel. Meanwhile, a total of 360 mL of sulfuric acid (0.128 M) was added into the extraction beaker. Using the handling tool, the carousel was lowered into the extraction beaker and rotated gently to mix for approximately 1 min. The handling tool was removed and the extraction beaker was transferred to a cold hotplate. The condenser was attached to the apparatus and the extraction beaker was set to boil via the hotplate. The heating was reduced when boiling was achieved to produce a simmer in the extraction beaker solution. After 30 min of boiling, the extraction beaker was removed from the hotplate and the hotplate was turned off. Then, the carousel was removed from the extraction beaker and the contents of the extraction beaker were discarded.

Approximately 300 mL of boiling distilled water was added into the extraction beaker and the carousel was gently lowered into the extraction beaker. The carousel and the contents were washed by vigorous rotation of the carousel, followed by the removal of the carousel. The extraction beaker was emptied and then filled with approximately 300 mL of boiling distilled water. The washing was repeated and the pH of the water measured using a pH paper. If the pH was not neutral, the washing was repeated until a neutral pH was obtained. Then, the carousel was removed and the contents of the extraction beaker was discarded.

Then, a total of 360 mL of hot sodium hydroxide (0.313 M) was added into the extraction beaker and the carousel was lowered slowly into the extraction beaker. The extraction beaker was then transferred onto the hotplate and the condenser was reattached to the apparatus. The extraction beaker was then set to boil via the hotplate. The heating was reduced when boiling was achieved to produce a simmer in the extraction beaker solution. After 30 min of boiling, the extraction beaker was removed from the hotplate and the hotplate was turned off. The washing process was performed using hot distilled water once, followed by the same volume of 0.1 M of hydrochloric acid, and lastly with twice with hot distilled water.

Fibrebags were then removed from the carousel and the spacers were removed from fibrebags under a stream of water for rinsing. Fibrebags were then placed on absorbent paper for a few minutes, followed by drying in an oven set at 105 °C for 4 hr. The fibrebags were then desiccated for 15 min, cooled and weighed as fibrebag with residue. Then, ash content of the fibrebags and samples were measured using

the method described in Section 2.5.6. However, weight of the crucible without samples was measured after ash content was brushed off.

c. Calculation of crude fibre content

Crude fibre content was calculated based on the formula below:

$$\text{Crude fibre content (\%)} = \frac{(W_{B1} - W_{B0}) - (W_{A1} - W_{A0})}{W_S} \times 100$$

Where:

W_{B1} = weight of fibrebag with residue

W_{B0} = weight of empty fibrebag

W_{A1} = weight of crucible after heating overnight with samples

W_0 = weight of crucibles without samples

W_S = weight of samples

V. Moisture content analysis

Moisture content of a samples were measured based on the AOAC method 935.29 and is separated into two components: a) sample preparation and drying, and b) calculation of moisture content.

a. Sample preparation and drying

Prior to use, glass pots were dried in an oven set at 105 °C for 3 hr to remove moisture and subsequently stored in a desiccator. Then, the glass pots were labelled and weighed with their lids to three decimal places. A total of 3 g of sample were also weighed to three decimal places and was transferred into a glass pot. The glass pots were then transferred into an oven set at 105 °C and was dried for 3 hr. After

drying, the glass pots were removed and transferred into a desiccator before being weighed again.

b. Calculation of moisture content

Ash content was calculated based on the following formula, where weight after = weight of crucible after heating overnight with samples; weight before = weight of crucibles without samples:

$$\text{Moisture content (\%)} = \frac{W_0 - W_F}{W_S} \times 100$$

Where:

W_0 = weight of glass pot before drying

W_F = weight of glass pot after drying

W_S = weight of sample

VI. Ash content analysis

Ash analysis was performed based on the AOAC method 942.05. The method can be separated into two components: a) sample preparation and heating, and b) calculation of ash content

a. Sample preparation and heating

Prior to use, crucibles were heated in a furnace set at 550 °C overnight to remove impurities and subsequently stored in a desiccator. Then, crucibles were labelled and weighed to three decimal places. A total of 2.5 g of sample were also weighed to three decimal places and was transferred into a crucible. The crucibles were then transferred into a furnace set at 550 °C and was heated overnight. Then, crucibles were removed and allowed to cool down in a desiccator before being weighed again.

b. Calculation of ash content

Ash content was calculated based on the following formula, where weight after = weight of crucible after heating overnight with samples; weight before = weight of crucibles without samples:

$$\text{Ash content } (\%) = \frac{W_F - W_0}{W_S} \times 100$$

Where:

W_F = weight of crucible after heating overnight with samples

W_0 = weight of crucibles without samples

W_S = weight of sample

Appendix L

Measurement of length of Asian sea bass (*Lates calcarifer*)