

Bacillus mycoides improves health of gastrointestinal tract in marron (*Cherax cainii*, Austin 2002)

Abstract

The present study examined the health status of the gastrointestinal tract (GIT) in marron after a two-month feeding trial with a diet supplemented with host origin, *Bacillus mycoides*. Two groups of marron were fed with either a basal diet or probiotic added at 108cfu/g of feed. Microbial density, microvilli length and number, intestinal epidermis layer morphology and hepatopancreas indices of weight and moisture content were evaluated. Supplementation with *B. mycoides* in marron feed significantly improved intestinal bacterial density (4,007±121 million cfu/g of GIT) compared to basal diet fed marron (723.7±45.2 million cfu/g of GIT). Microvilli density (per 100 µm²) was also significantly higher (10.50±0.25 µm) compared to 5.71±0.24 in basal diet fed marron. Significantly higher villous length (4.93±0.11 µm) was observed in probiotic fed compared to basal diet fed marron (3.91±0.18 µm).

The intestinal epidermis layer of probiotic fed marron showed increased folding and thickness compared to basal diet fed marron. Higher hepatosomatic indices (Hiv) and low moisture content (HM%) in probiotic fed marron indicated efficient functioning of the healthy gut. The present study suggests that supplementing host origin customised probiotic in feed improves gut health as measured by microbial density, microvilli length and number, intestinal epidermis layer and hepatosomatic indices.

Keywords: Probiotic, Microbial density, Microvilli, Hepatosomatic indices, Marron

Volume 2 Issue 2 - 2015

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Received: December 01, 2014 | **Published:** April 25, 2015

Abbreviations: BA, Blood Agar; CfU/g, Colony Forming Unit Per Gram; Cm, Centimetre; GIT, Gastrointestinal Tract; GLL, Glycerol Lab Lemco broth; H & E, Hematoxylin and Eosin; Hiv and H_{id}, hepatosomatic wet and dry; µm, micrometre; HMDS, Hexamethyldisilazane; (HM%), Moisture Content; L, Litre; L/min, Litre/minute; M, Molar; MGA, Marron Growers Association; SEM, Scanning Electron Microscope; W_{wh}, Weight of wet hepatopancreas; W_{dh}, Weight of dry hepatopancreas; W_t, Total Weight of Marron

Introduction

It is widely established that in addition to skin and gills, the gastrointestinal tract (GIT) is considered one of the major routes for pathogenic invasion in aquatic animals.¹⁻⁴ Therefore, study of the GIT of aquatic animals as a physical and immunological barrier is increasingly important, and it is accepted that digestion and immunity are complicated physiological processes that have co-evolved.^{4,5} The GIT of aquatic animals plays an important role in non-specific immune defences, as it provides an initial barrier to pathogen entry.^{5,6-10} The first step in bacterial invasion of the intestine is mediated by adhesion of pathogenic bacteria to mucosal surfaces and disruption of the microbial balance.^{7,11} In most fish hatcheries, intestinal microbial disorders caused by bacterial disease are considered to be a major cause of mortality, thus stability of the intestinal microbes and gut health are essential for the health of an organism.¹⁰ As a result, much attention has been focused on the development of probiotics in order to maintain a stable, beneficial gut microbial population.¹² Study of morphology and intestinal health of aquatic animals using prebiotics has been evaluated in red drum, *Sciaenopsocellatus*,^{13,14} rainbow trout, *Oncorhynchus mykiss*,¹⁵ gilthead sea bream, *Sparusaurata*,¹⁶ tilapia, *Oreochromis niloticus*,¹⁷ channel catfish *Ictalurus punctatus*,¹⁸ Pacific white shrimp, *Litopenaeusvannamei*,¹⁹ marron *Cherax tenuimanus*²⁰ and its combination with probiotic.^{4,21-24}

Although the use of probiotics in aquatic animals has been reviewed by many authors,^{7,25-30} information for probiotics effect on intestinal health and morphology is extremely limited.^{18,31} To date, probiotic effects on intestinal health and morphology have been studied only in Nile tilapia, *Oreochromis niloticus*,³² seabream, *Sparusaurata* L.³¹ and beluga, *Husohuso*.³³ *Bacillus mycoides* is a bacterium found in marron and the environment that has favourable probiotic properties including growth inhibition of *V. mimicus* and *V. cholerae* non-01, is susceptible to a majority of antibiotics, non-pathogenic to marron, produces a wide range of enzymes³⁴ and improved the immunity and health of marron.³⁵ The aim of the present study was to examine the effects of *Bacillus mycoides* on intestinal health and morphology in marron³⁶ as determined by bacterial density, hepatopancreas indices including moisture content, microvilli density and length, and histological examination of intestinal cells.

Materials and Methods

Culture system, experimental animal and feed preparation

The experimental units were cylindrical plastic tanks (80 cm diameter, 50 cm high and 250 L in capacity). The tanks were filled with freshwater and supplied with constant aeration, and sufficient number of marron shelters of PVC pipes with appropriate diameters. Each tank was also equipped with a submersible thermostat set to 24°C and a recirculating biological filtration system. The water in the tank was recirculated continuously at a rate of approximately 3 L/min. To maintain good water quality in the tanks, water exchange at a rate of 10-15% of the total water volume was performed twice a week, after siphoning out the faeces and uneaten feed. Marron (weight 33-65g) were obtained from the Marron Growers Association (MGA) in Northcliffe and Manjimup, Western Australia. The 250 L tanks were stocked with marron at a density of 12 marron/tank. Before

commencement of the experiment, marron were kept for two weeks in the experimental tanks for acclimation. During the two month experimental period, a commercial pelleted diet (26% protein, 47-50% carbohydrate, 9% fats and 8.9% ash) from Enviroplus Pty Ltd., Perth Australia was fed to marron at a rate of 1.5 % body weight per day. *Bacillus mycoides* was isolated from a number of healthy marron.

The isolate was identified by the Bacteriology Laboratory, Animal Health Laboratories, Department of Agriculture and Food, Western Australia, using a matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometer (Bruker Bioscience Corporation), Vitek Compact II (Biomérieux) and conventional biochemical methods according to standard procedures and identification methods.³⁷ Subsequently, the strain was suspended into 1 mL aliquots of GLL (Glycerol Lab Lemco broth) and stored at -80°C. Supplementation using the probiotic strain was performed as per Hai and Fotedar.²¹ In brief, prior to probiotic supplementation of the experimental diet, a pure culture of *B. mycoides* was grown on 5% horse blood agar (BA) plates for 24h at 25°C. Colonies in logarithmic phase of growth were emulsified in sterilised distilled water and serially diluted. The optical reading of each serial dilution was recorded, and a viable count performed to obtain a standard curve for inoculum density. From the standard curve, the amount of the diluted probiotic was calculated to achieve the desirable supplementation density of 10⁸ colony forming unit (cfu) per gram of feed. The pellets were air dried, packed and stored at 4°C until used.

Data collection

At the termination of the experiment, the GIT health status of marron was determined through analysis of bacterial density, microvilli length and density, histologic assessment of GIT epithelium, and moisture content and weight of the hepatopancreas. All animals used for analysis from both treatment groups were of equal in weight or length size in order to minimise misinterpretations due to size variations.

Bacterial density: The bacterial density of marron GIT after feeding with probiotic supplemented feed was measured at the beginning and end of the experiment. Ten marron from each treatment group were sacrificed by placing them at -20°C for 5 minutes before aseptic removal of the GIT. The marron dorsal shell was cut-off horizontally from tail to head until the hepatopancreas and intestine were exposed. The hepatopancreas was removed, placed in a sterilised pestle, weighed and then homogenised. Similarly, the intestine from individual animals was collected aseptically and homogenised with a micropestle in a 1.5 ml microfuge tube. The homogenised hepatopancreas, and the homogenised intestine were serially (10⁻¹, 10⁻², 10⁻³, 10⁻⁴, 10⁻⁵ and 10⁻⁶) diluted. Fifty microliter of each serial dilution was inoculated onto a BA plate and incubated overnight in a CO₂ incubator at 25°C. A colony count was performed for each dilution to determine the total number of aerobic bacteria.

GIT microvilli assessment by micrograph: The distal part of the marron intestine was observed using a scanning electron microscope (SEM) following an established method.^{38,39} The intestinal tract of five marron from each treatment group was dissected and immersed in 3% glutaraldehyde in 0.1M cacodylate buffer overnight. Following overnight immersion, the GIT was washed in 3 change of the cacodylate buffer and 3 changes in distilled water for 5min per change. The intestine was immersed in 2% OsO₄ for 2h followed by 3 washes in distilled water for 5 min per wash. Dehydration of the sample was performed through solutions of 50%, 75%, 95% ethanol for 5 min per solution and finally 3 times in 100% ethanol for 5min per change

followed by chemical drying by washing in a series of 50%, 75% and 100% (twice) hexamethyl disilazane (HMDS) in ethanol solutions for 5 min per change. The final stage involved drying the samples at room temperature, mounting on stub using carbontape and then coating with gold before viewing the samples under a pressure scanning electron microscope (LX30). The images obtained from SEM were used to describe villous height and density (number per group surface area) in the GIT. The height of microvilli (µm) was measured following established methods.^{31,32} At least 10 villous per section were randomly selected and measured using a computerised morphometric technique. The height of each villous was measured from the villous bottom to the tip, and the average height of these 10 villi was expressed as the mean villous height. Villous density (villous/100 µm surface area) and villi per group were counted according to Sang and Fotedar.³⁹

Histological assessment of the intestine: Histological preparation and assessment of marron GIT post-feeding with probiotic and basal diets were prepared by Animal Health Laboratories, Department of Agriculture and Food Western Australia. Five marron GIT from each treatment group were dissected and fixed in 10% buffered formalin for 24h. Dehydration of the tissue was performed by passing through a series of 70%, 85% and 98% alcohol solutions. The samples were vacuum embedded in paraffin. The histological sections of 4-5µm was stained with Hematoxylin and eosin (H&E). The sections were examined and photographed using an Olympus BX50 microscope.

Hepatosomatic indices (HiW): The hepatosomatic indices (Hiw) of marron fed with probiotic supplemented diet and basal diet were calculated as per established equations.^{40,41} In brief, the hepatopancreas of ten marron from each treatment group were removed placed in foil and weighed. For hepatopancreas moisture content, the hepatopancreas was dried at 110°C for 24 h. The results, expressed as wet hepatosomatic indices (Hiw), dry hepatosomatic indices (H_{id}) and hepatopancreas moisture content (HM) were calculated as follows;

$$Hiw = W_{wh} \times 100 W_t^{-1}$$

$$Hid = W_{dh} \times 100 W_t^{-1}$$

$$HM = (W_{wh} - W_{dh}) \times 100 W_{wh}^{-1}$$

Where;

Hiw: Wet hepatosomatic indices (%)

Hid: Dry hepatosomatic indices (%)

W_{wh}: Weight of wet hepatopancreas (g)

W_{dh}: Weight of dry hepatopancreas

W_t: Total weight of marron (g)

HM: Hepatopancreas moisture content (%)

Data analysis

Data were analysed using SPSS statistical program version 22. Comparison of the mean values using T-test was performed to determine significance and the results were presented in tables 1-3 and graphs.

Results and Discussion

Results

Bacterial density: The mean bacterial density in marron intestine fed probiotic supplemented diet was significantly increased (4007±121million cfu/g of GIT) compared to the bacterial density in

the gut of basal diet fed marron (723.7 ± 45.2 million cfu/g of GIT). The diversity of the bacterial population was greater in the GIT of probiotic supplemented diet fed marron compared to basal diet fed, as observed by colonial morphology on BA plates after 24h incubation at 25°C (Figure 1).

Table 1 Ingredients of the basal diet

Ingredients	Percentage (%)
Wheat Flour	49.35
Fish Meal ^a	33.78
Soybean Meal	10.15
Fish Oil ^b	3.2
Wheat Starch	1.85
Betaine ^c	1.20
Cholesterol	0.25
Premix ^d	0.15
Ascorbic Acid	0.05
Calcium Carbonate	0.02
Total	100

All ingredients were supplied by Specialty Feeds Pty Ltd WA, Australia.

^aPeruvian fishmeal, 56 % CP

^bCod liver oil

^cBetaine anhydrous 97%

^dCommercial vitamin and mineral premix for trout

Table 2 Mean \pm SE (n=5) of villous height (μ m), villous number per group and villous density (per 100 μ m²) of marron hindgut fed probiotic supplemented diet and basal diet

Parameters	Probiotic Diet	Basal Diet
Villous Height	4.93 \pm 0.11 ^b	3.91 \pm 0.18 ^a
Villous Per Group	10.50 \pm 0.25 ^b	5.71 \pm 0.24 ^a
Villous Density	20.28 \pm 0.70 ^b	13.93 \pm 0.4 ^a

*Mean value in the same row having different superscript indicates significantly different at P<0.05

Table 3 Mean \pm SE (n=10) of hepatosomatic indices (%) and moisture content (%) of marron fed basal and probiotic supplemented diet

Treatment	Hiw (%)	Hid (%)	HM (%)
Basal diet	6.20 \pm 0.02 ^a	2.71 \pm 0.06 ^a	62.16 \pm 1.09 ^a
Probiotic diet	7.11 \pm 0.34 ^b	3.32 \pm 0.25 ^b	53.43 \pm 1.68 ^b

*Mean values in the same column with different superscript indicate significantly different (P< 0.05)

Hiw: hepatosomatic indices (wet)

Hid: hepatosomatic indices (dry)

HM: Hepatopancreas moisture content

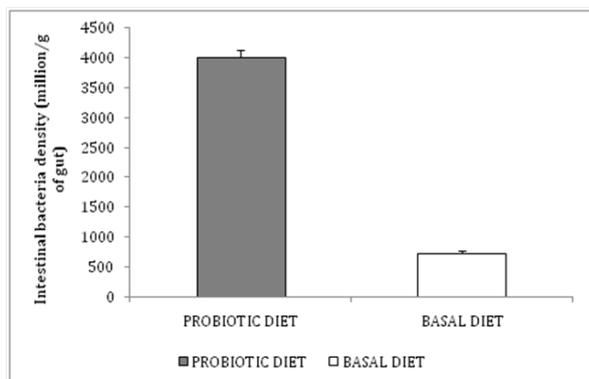


Figure 1 Mean \pm SE (n=10) of intestinal bacteria population (million/g of gut) of marron fed basal and probiotic supplemented diet.

GIT microvilli assessment by micrograph

The morphology of marron intestines after feeding with probiotic supplemented diet compared to basal diet is shown in Figure 2. The density and length of the microvilli per GIT surface area was significantly higher in marron fed *B. mycoides* supplemented diet (A) than microvilli of basal diet fed marron (B). The average density of microvilli per group (number of villous in a row) of marron fed the probiotic diet was 10.50 ± 0.94 compared to 5.71 ± 0.91 in basal diet fed marron (Figure 3).

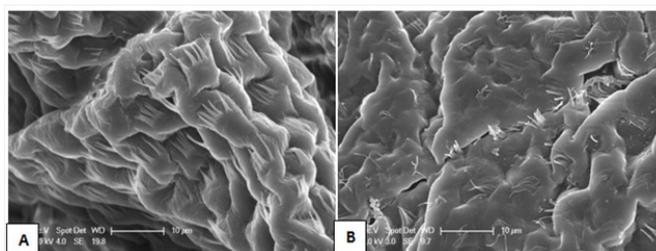


Figure 2 Scanning electron microscopy micrograph of marron hindgut fed probiotic supplemented diet (A) and basal diet (B). ($\times=2500$. Bar=10 μ m). In the probiotics fed marron there is an increase in folds and the villi are longer and more numerous.

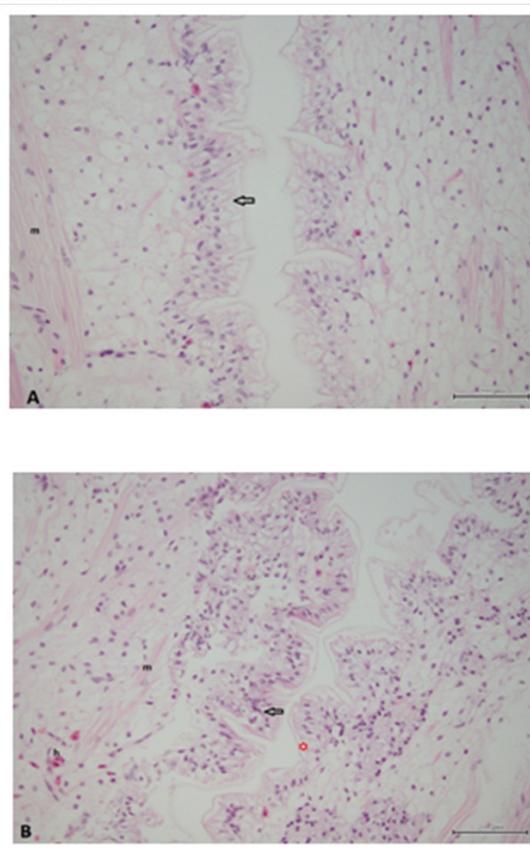


Figure 3 Histological sections of the hindgut from marron fed the control diet (A) and the probiotic supplemented diet (B). The epidermal cells (open arrow) in the probiotic supplemented group are larger and have a more foamy appearance. In both treatments there is some shrinkage artifact of the cuticle from the epithelial cells (red star). Muscle cells (m) and mixed populations of haemocytes (h) can be seen in the lamina propria in both the control and probiotic supplemented animals. Bars indicate 200 μ m.

Histological assessment of intestine: Histologically there were no major differences seen in the hepatopancreas of marron from the

different treatment groups. However, the foregut and hindgut of the probiotic fed marron had more folds and fewer haemocytes compared to the marron fed the control diet without probiotics.

Hepatopancreas weight: Hepatosomatic indices (Hiw) of probiotic fed marron were significantly higher (7.11 ± 0.34) compared to basal diet fed marron (6.20 ± 0.02). In addition, hepatopancreas moisture content (HM%) was lower (53.43 ± 1.68 %) in probiotic fed marron whereas in basal diet fed marron it was 62.16 ± 1.09 %.

Discussion

Balcazar et al.⁴² Zhou and Wang⁴³ suggest that probiotics act in several ways: Firstly by maintaining and restoring normal intestinal microbiota and gut homeostasis; secondly by contributing to the competitive exclusion of bacteria⁴⁴⁻⁴⁷ and thirdly by acting as a source of nutrients and enzymes.^{7,48-52} Most authors suggest that probiotics of host origin are more favourable compared to other sources as is believed that autochthonous bacteria are able to colonise, multiply and remain predominant in the same host.^{7,10,25,53,54} The health status of the GIT is most likely determined by the microbial balance of indigenous microbiota^{49,55} with the density and diversity of bacteria in the intestine having the most impact on intestinal health.⁵⁶ The main parameters commonly used to assess GIT health in aquatic animals are intestinal bacterial density and diversity, microvilli height and number, gut epithelium, hepatopancreas size and digestive enzyme activity.^{4,15,31,36,39,56-58}

In the present study marron fed a diet supplemented with *Bacillus mycoides* had improved intestinal morphology, increased density of bacteria in the intestine and a heavier hepatopancreas. All of these features suggest that the marron benefited from the probiotic supplementation. A number of studies show intestinal bacteria density increases after probiotic supplementation which is thought to be due to a probiotic of host origin providing a favourable environment for the indigenous intestinal bacteria. In shrimp *Penaeus monodon*, the number of intestinal bacteria increased by up to 803% after supplementation with three *Bacillus* species (*B. pumilus*, *B. sphaericus*, and *B. subtilis*) isolated from the host.⁵⁹ In grouper *Epinephelus coioides* potentially beneficial bacteria were stimulated, whereas some potentially harmful strains such as *Staphylococcus saprophyticus*, were suppressed after supplementation with probiotic *Psychrobacter* sp.⁶⁰ Reduction of either diversity or quantity of the indigenous microbiota is likely to reduce the effective barrier mechanism normally provided by the commensal microbiota.^{11,55}

Another feature of a healthy digestive system is the density and length of microvilli. In the present study, supplementation with *B. mycoides* significantly improved the height and number of villi. Other studies on probiotics had similar findings including one on marron using mannan-oligosaccharide (MOS) diet,²⁰ and others using *Lactobacillus* sp. in Nile tilapia *Oreochromis niloticus*,³² *Bacillus* sp. in European lobster *Homarus gammarus* L.²⁴ and *Pedococcus acidilactici* in rainbow trout.⁶¹ Contrary to these findings, observed no improvements in microvilli in rainbow trout fed with *Bacillus* sp. or *Enterococcus faecium* supplemented feeds, whereas Cerezuola et al.³⁴ found shorter villi in gilthead seabream (*Sparus aurata*) fed diets containing *B. Subtilise*, suggesting that the effect of probiotics on microvilli may not be consistent in all species.

In aquatic animals, longer intestinal villi provide greater absorption ability due to their increased surface area.^{26,34,35,62} They also provide a larger surface area for bacterial colonisation as was reported for Arctic charr, *Salvelinus alpinus*. Ringo et al.⁶³ observed large populations of

bacteria associated with the villous brush borders, while Hellberg and Bjerkas⁶⁴ detected the bacteria between the microvilli in common wolfish, *Anarhichas lupus* (L). In addition, Merrifield et al.⁶⁵ observed greater bacterial colonization between the folds of the mucosal surface because bacteria could become established and sustained more easily at the base of the villi and between the mucosal folds. Merrifield²³ suggested that more dense and regular villi may also play a role in disease prevention by reducing exposure to enterocyte tight junctions in rainbow trout, *Oncorhynchus mykiss* (Walbaum). Various hepatosomatic indices of marron have been reported in several studies. Jussila⁶⁶ compared hepatosomatic indices of marron at molt and post-molt at different feeding status and found that the lowest (3.8 ± 0.2 %) hepatosomatic indices (Hiw) of marron was observed in non-fed post-molt marron, followed by fed post-molt marron (5.4 ± 0.3 %) and the highest (5.6 ± 0.3 %) in fed-intermolt stage marron.

Sang and Fotedar⁶⁷ observed Hiw of marron fed β -1,3 glucan supplemented diet ranged between 6.35-7.17 %. In the present study Hiw of probiotic fed marron was 7.11 ± 0.34 % compared to 6.20 ± 0.02 % for basal diet fed marron. The improved intestinal health in the probiotic fed marron most likely resulted in the higher hepatosomatic index of these marron. The hepatopancreas, as the main energy reserve in crustaceans and source of various enzymes, has been used as an indicator of crayfish condition [40,41]. A heavier hepatopancreas could be an indication of higher digestive enzyme activities.⁶⁸ Our previous study also indicated that marron fed *B. mycoides* had a larger hepatopancreas especially at day 35.³⁵ *B. mycoides* produces a wide range of enzymes,³⁴ including many exo-enzymes important for digestion⁶⁹ and is a reason why *Bacillus* sp. have been used widely as probiotics.⁷⁰

Conclusion

Overall, supplementation of host origin *B. mycoides* in marron feed improved the health of the marron gastrointestinal tract as indicated by an increase in bacterial density, increased and longer microvilli, thicker intestinal epithelium and higher hepatosomatic indices. Consequently the use of host origin (particularly mucosal inhabitants) strains of bacteria with probiotic properties is recommended as these bacteria are able to maintain microbial homeostasis, are well adapted to the host GIT environment and mucosal attachment, and can protect the epithelium layer from potential pathogens, which in turn preserve optimal function of the gastrointestinal tract.

Acknowledgements

The authors wish to thank Directorate General of Higher Education (DIKTI) of Indonesia which financially supported this study. Sincere gratitude to Dr. Fran Stephens and Mr. Tai Le from Animal Health Laboratories, Department of Agriculture and Food Western Australia for histological samples preparation and analysis. Mr. Le TrungKy for SEM samples preparation, Mr. and Mrs. Harris for the supply of marron and Mr Albert Cooper for proof-reading of the manuscript.

References

1. Ringø E, Birkbeck T. Intestinal microflora of fish larvae and fry: a review. *Aquac Res.* 1999;30(2):73–93.
2. Ringø E, Myklebust R, Mayhew Tm, et al. Bacterial translocation and pathogenesis in the digestive tract of larvae and fry. *Aquacult.* 2007;268(1–4):251–264.
3. Dimitroglou A, Merrifield DL, Carnevali O, et al. Microbial manipulations to improve fish health and production – a Mediterranean perspective. *Fish Shellfish Immunol.* 2011;30(1):1–16.

4. Cerezuela R, Fumanal M, Tapia-Paniagua ST, et al. Changes in intestinal morphology and microbiota caused by dietary administration of inulin and *Bacillus subtilis* in gilthead sea bream (*Sparus aurata* L.) specimens. *Fish Shellfish Immunol.* 2013;34(5):1063–1070.
5. Liu W, Ren P, He S, et al. Comparison of adhesive gut bacteria composition, immunity, and disease resistance in juvenile hybrid tilapia fed two different *Lactobacillus* strains. *Fish Shellfish Immunol.* 2013;35(1):54–62.
6. Sugita H, Tsunohara M, Ohkashi T, et al. The establishment of an intestinal microflora in developing goldfish *Carassius auratus* ponds. *Microbiol Ecol.* 1988;15(3):333–344.
7. Verschuere L, Rombaut G, Sorgeloos P, et al. Probiotic Bacteria as Biological Control Agents in Aquaculture. *Microbiol Mol Biol Rev.* 2000;64(4):655–671.
8. Ramirez RF, Dixon BA. Enzyme production by obligate intestinal anaerobic bacteria isolated from oscars (*Astronotus ocellatus*), angelfish (*Pterophyllum scalare*) and southern flounder (*Paralichthys lethostigma*). *Aquaculture.* 2003;227(1–4):417–426.
9. Ringø E, Olsen RE, Mayhew TM, et al. Electron microscopy of the intestinal microflora of fish. *Aquacult.* 2003;227(1–4):395–415.
10. Rollo A, Sulpizio R, Nardy M, et al. Live microbial feed supplement in aquaculture for improvement of stress tolerance. *Fish Physiol Biochem.* 2006;32(2):167–177.
11. Ige BA. Probiotics use in intensive fish farming. *International Journal of Agricultural Research and Natural Resources.* 2013;1(1):1–11.
12. Merrifield DL, Dimitroglou A, Bradley G, et al. Probiotic applications for rainbow trout (*Oncorhynchus mykiss* Walbaum) I. Effects on growth performance, feed utilization, intestinal microbiota and related health criteria. *Aquacult Nutrition.* 2010;16(5):504–510.
13. Zhou QC, Buentello JA, Iii DMG. Effects of dietary prebiotics on growth performance, immune response and intestinal morphology of red drum (*Sciaenops ocellatus*). *Aquacult.* 2010;309(1–4):253–257.
14. Cheng Z, Buentello A, Iii DMG. Dietary nucleotides influence immune responses and intestinal morphology of red drum *Sciaenops ocellatus*. *Fish Shellfish Immunol.* 2011;30(1):143–147.
15. Dimitroglou A, Merrifield DL, Moate R, et al. Dietary mannan oligosaccharide supplementation modulates intestinal microbial ecology and improves gut morphology of rainbow trout, *Oncorhynchus mykiss* (Walbaum). *J Anim Sci.* 2009;87(10):3226–3234.
16. Dimitroglou A, Merrifield DL, Spring P, et al. Effects of mannan oligosaccharide (MOS) supplementation on growth performance, feed utilisation, intestinal histology and gut microbiota of gilthead sea bream (*Sparus aurata*). *Aquacult.* 2010;300(1–4):182–188.
17. Merrifield DL, Harper GM, Mustafa S, et al. Effect of dietary alginic acid on juvenile tilapia (*Oreochromis niloticus*) intestinal microbial balance, intestinal histology and growth performance. *Cell Tissue Res.* 2011;344(1):135–146.
18. Zhu H, Liu H, Yan J, et al. Effect of yeast polysaccharide on some hematologic parameter and gut morphology in channel catfish (*Ictalurus punctatus*). *Fish Physiol Biochem.* 2012;38(5):1441–1447.
19. Zhang J, Liu Y, Tian L, et al. Effects of dietary mannan oligosaccharide on growth performance, gut morphology and stress tolerance of juvenile Pacific white shrimp, *Litopenaeus vannamei*. *Fish Shellfish Immunol.* 2012;33(4):1027–1032.
20. Sang HM, Fotedar R. Prebiotic Mannan Oligosaccharide Diet Improves Health Status of the Digestive System of Marron, *Cherax tenuimanus* (Smith 1912). *Journal of Applied Aquaculture.* 2010;22(3):240–250.
21. Hai NV, Fotedar R. Comparison of the effects of the prebiotics (Bio-Mos® and β -1,3-D-glucan) and the customised probiotics (*Pseudomonas synxantha* and *P. aeruginosa*) on the culture of juvenile western king prawns (*Penaeus latisulcatus* Kishinouye, 1896). *Aquaculture.* 2009;289(3–4):310–316.
22. Ganguly S, Paul L, Sk M. Immunostimulant, probiotic and prebiotic—their applications and effectiveness in aquaculture. *Israeli J Aquacult–Bamidgeh.* 2010;62(3):130–138.
23. Merrifield DL, Dimitroglou A, Bradley G, et al. Soybean meal alters autochthonous microbial populations, microvilli morphology and compromises intestinal enterocyte integrity of rainbow trout, *Oncorhynchus mykiss* (Walbaum). *J Fish Dis.* 2009;32(9):755–766.
24. Daniels CL, Merrifield DL, Boothroyd DP, et al. Effect of dietary *Bacillus* spp. and mannan oligosaccharides (MOS) on European lobster (*Homarus gammarus* L.) larvae growth performance, gut morphology and gut microbiota. *Aquaculture.* 2010;304(1–4):49–57.
25. Gatesoupe FJ. The use of probiotics in aquaculture. *Aquaculture.* 1999;180(1–2):147–165.
26. Irianto A, Austin B. Probiotics in aquaculture. *Journal of fish diseases.* 2002;25(11):633–642.
27. Balcázar JL, Blas ID, Ruiz-Zarzuela I, et al. The role of probiotics in aquaculture. *Veterinary microbiology.* 2006;114(3–4):173–186.
28. Sahu MK, Swarnakumar NS, Sivakumar K, et al. Probiotics in aquaculture: importance and future perspectives. *Indian J Microbiol.* 2008;48(3):299–308.
29. Nayak SK. Probiotics and immunity: A fish perspective. *Fish Shellfish Immunol.* 2010;29(1):2–14.
30. Newaj-Fyzul A, Al-Harbi AH, Austin B. Review: Developments in the use of probiotics for disease control in aquaculture. *Aquacult.* 2014;431:1–11.
31. Cerezuela R, Fumanal M, Tapia-Paniagua ST, et al. Histological alterations and microbial ecology of the intestine in gilthead seabream (*Sparus aurata* L.) fed dietary probiotics and microalgae. *Cell Tissue Res.* 2012;350(3):477–489.
32. Pirarat N, Pinpimai K, Endo M, et al. Modulation of intestinal morphology and immunity in Nile tilapia (*Oreochromis niloticus*) by *Lactobacillus rhamnosus* GG. *Res Vet Sci.* 2011;91(3):e92–e97.
33. Salma W, Zhou Z, Wang W, et al. Histological and bacteriological changes in intestine of beluga (*Huso huso*) following *ex vivo* exposure to bacterial strains. *Aquaculture.* 2011;314(1–4):24–33.
34. Ambas I, Buller N, Fotedar R. Isolation and screening of probiotic candidates from marron, *Cherax cainii* (Austin, 2002) gastrointestinal tract (GIT) and commercial probiotic products for the use in marron culture. *Journal of Fish Diseases.* 2014;38(5):467–476.
35. Ambas I, Suriawan A, Fotedar R. Immunological responses of customised probiotics–fed marron, *Cherax tenuimanus*, (Smith 1912) when challenged with *Vibrio mimicus*. *Fish Shellfish Immunol.* 2013;35(2):262–270.
36. Nugroho RA, Fotedar R. Effects of dietary organic selenium on immune responses, total selenium accumulation and digestive system health of marron, *Cherax cainii* (Austin, 2002). *Aquaculture Research.* 2013;1–11.
37. Buller NB. Bacteria from Fish and Other Aquatic Animals: A Practical Identification Manual. CABI Publishing, Oxfordshire, UK, 2004.
38. Dunlap M, Adaskaveg JE. Introduction to the Scanning Electron Microscope: Theory, Practice, & Procedures. Facility for advanced instrumentation, UC Davis. 1997;p. 1–52.
39. Sang HM, Fotedar R. Prebiotic Mannan Oligosaccharide Diet Improves Health Status of the Digestive System of Marron *Cherax tenuimanus* (Smith 1912). *Journal of Applied Aquaculture.* 2010;22(3):240–250.
40. Jussila J. Carapace mineralisation and hepatopancreatic indices in natural and cultured population of marron (*Cherax tenuimanus*) in Western Australia. *Marine and Freshwater Research.* 1997;48(1):67–72.
41. Fotedar R. Nutrition of marron, *Cherax tenuimanus* (Smith) under different culture environments – a comparative study. Perth, Western Australia: Curtin University of Technology, 1998;pp. 174.

42. Balcazar JL, Rojas-Luna T, Cunningham Dp. Effect of the addition of four potential probiotic strains on the survival of pacific white shrimp (*Litopenaeus vannamei*) following immersion challenge with *Vibrio parahaemolyticus*. *J Invertebr Pathol*. 2007;96(2):147–50.
43. Zhou X, Wang Y. Probiotics in Aquaculture – Benefits to the Health, Technological Applications and Safety. In: Edmir Carvalho (Ed.), *Health and Environment in Aquaculture*. 2012;pp. 215–226.
44. Vine NG, Leukes WD, Kaiser H, et al. Competition for attachment of aquaculture candidate probiotic and pathogenic bacteria on fish intestinal mucus. *J Fish Dis*. 2004;27(6):319–326.
45. Seehanat S, Budtacup L, Kangklang S, et al. Growth inhibition of shrimp pathogens by isolated gastrointestinal microflora of Macrobrachium rosenbergii de Man. *Songklanakar J Sci Technol*. 2005;27(Suppl 1):265–274.
46. Hai NV, Fotedar R, Buller N. Selection of probiotics by various inhibition test methods for use in the culture of western king prawns, *Penaeus latisulcatus* (Kishinouye). *Aquaculture*. 2007;272(1–4):231–239.
47. Ariole CN, Oha EC. Antimicrobial Activity of Estuarine Isolates against Shrimp Pathogenic *Aeromonas Species*. *Nature and Science*. 2013;11(2):123–128.
48. Bairagi A, Ghosh KS, Sen SK, et al. Enzyme producing bacterial flora isolated from fish digestive tracts. *Aquaculture International*. 2002;10(2):109–121.
49. Ramirez RF, Dixon BA. Enzyme production by obligate intestinal anaerobic bacteria isolated from oscars (*Astronotus ocellatus*), angelfish (*Pterophyllum scalare*) and southern flounder (*Paralichthys lethostigma*). *Aquaculture*. 2003;227(1–4):417–426.
50. Ray AK, Roy T, Mondal S, et al. Identification of gut-associated amylase, cellulase and protease-producing bacteria in three species of Indian major carps. *Aquaculture Research* 2010;41(10):1462–1469.
51. Ray AK, Ghosh K, Ringo E. Enzyme-producing bacteria isolated from fish gut: a review. *Aquacult Nutrition*. 2012;18(5):465–492.
52. Lazado CC, Caipang CMA, Kiron V. Enzymes from the bacteria of Atlantic cod, *Gadus morhua* and their influence on intestinal enzyme activity. *Aquaculture Nutrition*. 2012;18(4):423–431.
53. Merrifield DL, Dimitroglou A, Foey A, et al. The current status and future focus of probiotic and prebiotic applications for salmonids. *Aquaculture*. 2010;302(1–2):1–18.
54. Hai NV, Buller N, Fotedar R. The use of customised probiotics in the cultivation of western king prawns (*Penaeus latisulcatus* Kishinouye, 1896). *Fish & Shellfish Immunol*. 2009;27(2):100–104.
55. Denev S, Staykov Y, Moutafchieva R, et al. Microbial ecology of the gastrointestinal tract of fish and the potential application of probiotics and prebiotics in finfish aquaculture. *International Aquatic Research*. 2009;1: 1–29.
56. Ringø E, Salinas I, Olsen RE, et al. Histological changes in intestine of Atlantic salmon (*Salmo salar* L.) following *in vitro* exposure to pathogenic and probiotic bacterial strains. *Cell Tissue Res*. 2007;328(1):109–116.
57. Cheng Z, Buentello A, Iii DMG. Effects of dietary arginine and glutamine on growth performance, immune responses and intestinal structure of red drum, *Sciaenops ocellatus*. *Aquaculture*. 2011;319(1–2):247–252.
58. Geda F, Rekeckie A, Decostee A, et al. Changes in intestinal morphology and amino acid catabolism in common carp at mildly elevated temperature as affected by dietary mannanoligosaccharides. *Animal Feed Science and Technology*. 2012;178(1–2):95–102.
59. Purivirojkul W, Maketon M, Areechon N. Probiotic Properties of *Bacillus pumilus*, *Bacillus sphaericus* and *Bacillus subtilis* in Black Tiger Shrimp (*Penaeus monodon* Fabricius) Culture. *Kasetsart J (Nat Sci)*. 2005;39: 262–273.
60. Yang HL, Sun YZ, Ma RL, et al. Probiotic *Psychrobacter* sp. improved the autochthonous microbial diversity along the gastrointestinal tract of grouper *Epinephelus coioides*. *Journal of Aquaculture Research & Development*, 2011.
61. Merrifield DL, Harper G, Baker RTM, et al. Possible influence of probiotic adhesion to intestinal mucosa on the activity and morphology of rainbow trout (*Oncorhynchus mykiss*) enterocytes. *Aquaculture Research*. 2010;41(8):1268–1272.
62. Caspary WF. Physiology and pathophysiology of intestinal absorption. *Am J Clin Nutr*. 1992;55(1 Suppl):299–308.
63. Ringø E, Lodemel JB, Myklebust R, et al. Epithelium-associated bacteria in the gastrointestinal tract of Arctic charr (*Salvelinus alpinus* L.). An electron microscopical study. *J Appl Microbiol*. 2001;90(2):294–300.
64. Hellberg H, Bjerkas I. The anatomy of the oesophagus, stomach and intestine in common wolffish (*Anarhichas lupus* L.): a basis for diagnostic work and research. *Acta Vet Scand*. 2000;41(3):283–297.
65. Merrifield DL, Burnard D, Bradley G, et al. Microbial community diversity associated with the intestinal mucosa of farmed rainbow trout (*Oncorhynchus mykiss* Walbaum). *Aquaculture Research*. 2009;40(9):1064–1072.
66. Jussila J. Comparison of Selected Condition Indices Between Intermolt and Post-Molt Marron, *Cherax tenuimanus*, of Different Feeding Status Raised Under Intensive Culture Conditions. *Journal of Applied Aquaculture*. 1999;9(3):57–66.
67. Sang H M, Fotedar R. Effects of dietary beta-1,3-glucan on the growth, survival, physiological and immune response of marron, *Cherax tenuimanus* (Smith, 1912). *Fish Shellfish Immunol*. 2010;28(5–6):957–960.
68. Hammer HS, Bishop CD, Watts SA. Activities of Three Digestive Enzymes During Development in the Crayfish *Procambarus Clarkii* (Decapoda). *Journal of the Crustacean Biology*. 2000;20(4):614–620.
69. Moriarty DJW. Control of luminous *Vibrio* species in penaeid aquaculture ponds. *Aquaculture*. 1998;164(1–4):351–358.
70. Ziaei-Nejad S, Rezaei MH, Takami GA, et al. The effect of *Bacillus* spp. bacteria used as probiotics on digestive enzyme activity, survival and growth in the Indian white shrimp *Fenneropenaeus indicus*. *Aquaculture*. 2006;252(2–4):516–524.