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(54) Title **Fish feed comprising probiotics**

(57) Abstract

The present application relates to fish feed. More particularly, the invention provides a fish feed comprising probiotics, particularly lactic acid bacteria. The invention further provides a method of producing a granular fish feed comprising lactic acid bacteria in a coating layer. The invention also relates to a method of treating fish and provides feed for use in improving intestinal health or innate immune response.

Fish feed comprising probiotics

Field of the invention

The present application relates to fish feed. The invention provides a fish feed comprising probiotics, and more particularly comprising lactic acid bacteria. The invention further provides a method of producing a fish feed comprising lactic acid bacteria. The invention also relates to a method of treating fish and provides feed for use in improving intestinal health or innate immune response.

Background of the invention

Fish feed is the highest cost factor in fish farming, such as in farming of Atlantic salmon, and efficient utilization is a key to keep the production cost low and economic turnover high in farming of Atlantic salmon. Disease prevention and control with reduced mortality during on-growth phase of the fish is also vital for a sustainable development of aquaculture as well as to keep control with the production costs. The fish is exposed to pathogens in the water and Atlantic salmon is equipped with a rather complex immune system including an innate immune system serving as the first line defence and is one adaptive branch to maintain its health. The first line defence includes the barriers in skin and gills. The epithelial cells in these organs are covered by mucus layer, which is mainly secreted by mucous cells. The mucus is considered to be one of the most important innate immune responses consisting of several innate immune molecules such as protease, lysozyme, esterase, complement protein, antibodies and antimicrobial peptides (AMP) which could chemically inactivate the pathogen. Another function of the mucus is to dispel the pathogens from forming colonies. Moreover, mucosal surfaces accommodate commensal or symbiotic microbiota which prevent colonization of pathogenic microorganisms through chemical inhibition or competitive exclusion and thereby improve the health of the fish. In addition, gut mucus aids in lubricating the digesta thus ensuring integrity of intestinal mucosa during digestion. Therefore, intact mucosal surface with adequate number of mucous cells is essential to maintain the barrier functions of the first line defence organs. Mucin and AMP related genes can be used as the bio markers in order to assess the mucosal health of fish. In Atlantic salmon, at least seven mucin secreting exist; two muc2 genes have been found

mainly in the intestine while five muc5 have been observed in other tissues such as pyloric ceca, gill or skin. The AMP are a diverse group, and some are transcribed by genes called cathelicidins and defensin.

Mucins and antimicrobial peptides are important for keeping the fish healthy, but there is limited information about how these genes are affected by feed composition. During ontogenetic development, microorganism colonize the gut of the fish. Microbiota can also be modulated by different feed ingredients and use of probiotics as shown by Gupta et al., 2019a *Lacotbacillus* dominate in the intestine of Atlantic salmon fed dietary probiotics. Frontiers in Microbiology, doi:10.3389/fmicb.2018.03247, as well as by pre-biotics as disclosed by Gupta et al., 2019b (Macroalga-derived alginate oligosaccharide alters intestinal bacteria of Atlantic salmon. Frontiers in Microbiology 10, 2037. The commensal microbiota is important to prevent ill-health and is preventing colonization of pathogenic microorganisms through chemical inhibition or competitive exclusion and is considered an important component of the mucosal immune system. The composition of the microbiota is however shifting with feed ingredients. Since 1990, the salmon feed has gradually shifted from marine to plant-derived ingredients. Plant protein concentrates, such as soy protein concentrate, which does not cause enteropathy in salmon, is a key ingredient, while other protein concentrates such as wheat gluten, corn gluten, faba beans, sunflower meal, pea protein concentrate and other plant proteins are incorporated at lower levels. Fish oil has to a large extent been replaced with rapeseed oil in European salmon feeds and in 2016, fish oil and rapeseed oil (mixed with small proportions of camelina oil) constituted 10.6 and 19.8%, respectively, of the ingredients used by the Norwegian aqua feed industry (Aas et al., 2019. Utilization of feed resources in the production of Atlantic salmon (*Salmo salar*) in Norway: An update for 2016. Aquaculture Reports 15. However, use of plant oils with unfavourable n-6/n-3 fatty acid ratio or feeds without eicosapentaenoic acid (EPA) may alter histo-morphology of the intestine of fish. Moreover, less refined ingredients, in particular soybean meal, containing saponins, has been found to cause enteritis in fish. The intestinal inflammation is characterized by widening of intestinal villi width, shortening of villi length, thickening of lamina propria, disappearance of supra nuclear vacuoles in enterocytes and infiltration of inflammatory cells into lamina propria.

There is currently about a 20% mortality in the growth phase of farmed salmon at sea. High mortality can largely be attributed to challenges with bacteria that cause winter sores in the fish, viral diseases and parasites (salmon lice). Strengthening health that contributes to a decrease in mortality has great value both in relation to fish welfare, reputation and not least higher profits. A reduction in mortality of 1% will in the current market (2020) have a value of more than 5 million Euros. In addition, a significant reputation value for the salmon industry lies in reducing mortality and improving fish health.

There is hence a need for improving the intestinal health of farmed fish, such as by the provision of new feed compositions.

Brief summary of the invention

The present invention relates to fish feed compositions comprising probiotics. The invention provides new fish feed compositions, methods to produce such fish feed compositions, feed compositions for use in treatment of fish, and methods to treat fish.

The invention provides a fish feed composition comprising lactic acid bacteria. More particularly, in a first aspect the invention provides a fish feed composition comprising at least one of the lactic acid bacteria *Lactobacillus fermentum* (LF) and *Lactobacillus plantarum* (LP).

In another aspect, the invention provides a method of producing a fish feed comprising at least one lactic acid bacteria comprising a step of vacuum coating the lactic acid bacteria on fish feed granulates.

In yet another aspect, the invention provides a fish feed for use in treatment of fish, particularly for improving at least one of intestinal health and innate immune response, administering a fish feed composition comprising at least one of the lactic acid bacteria *Lactobacillus fermentum* and *Lactobacillus plantarum* to the fish.

Brief description of the drawing

Figure1 shows the ratio between number of skin mucous cells and total area of skin epithelium (SNE) of Atlantic salmon (*Salmo salar*) fed different diets; BG1 – a marine,

BG5 – a plant and BG2 – a soybean meal based feed. For each feed, white colour bar (left side) represents control diet (CT) without probiotics and shaded pattern bar (right side) represents diet with probiotics (LP+LF). The effects of main factors (feed and probiotics) and their interaction were determined by two-way ANOVA and p values are indicated in the upper right corner. Different lower-case letters denote significant difference ($p < 0.05$) among all diet combinations after conducting a post-hoc (Tukey HSD) test. Values are presented as mean ± SEM.

Figure 2 shows the ratio between total area of gill mucous cells and total area of gill epithelium (GME) of Atlantic salmon (*Salmo salar*) fed different diets; BG1 – a marine, BG5 – a plant and BG2 – a soybean meal based feed. For each feed, white colour bar (left side) represents control diet (CT) without probiotics and shaded pattern (right side) represents diet with probiotics (LP+LF). The effects of main factors (feed and probiotics) and their interaction were determined by two-way ANOVA and p values are indicated in the upper right corner. Different lower-case letters denote significant difference ($p < 0.05$) among all diet combinations after conducting a post-hoc (Tukey HSD) test. Values are presented as mean ± SEM.

Figure 3 provides the ratio between number of gill mucous cells and total area of gill epithelium (SNE) of Atlantic salmon (Salmo salar) fed different diets; BG1 – a marine, BG5 – a plant and BG2 – a soybean meal based feed. For each feed, white colour bar (left side) represents control diet (CT) without probiotics and shaded pattern (right side) represents diet with probiotics (LP+LF). The effects of main factors (feed and probiotics) and their interaction were determined by two-way ANOVA and p values are indicated in the upper right corner. Different lower-case letters denote significant difference ($p < 0.05$) among all diet combinations after conducting a post-hoc (Tukey HSD) test. Values are presented as mean ± SEM.

Figure 4 shows the average width of lamina propria (LPW) of Atlantic salmon *(Salmo salar*) fed different diets; BG1 – a marine, BG5 – a plant and BG2 – a soybean meal based feed. For each feed, white colour bar (left side) represents control diet (CT) without probiotics and shaded pattern (right side) represents diet with probiotics (LP+LF). The effects of main factors (feed and probiotics) and their interaction were determined by two-way ANOVA and p values are indicated in the upper right corner. Different lower-case letters denote significant difference ($p < 0.05$) among all diet

combinations after conducting a post-hoc (Tukey HSD) test. Values are presented as mean ± SEM.

Figure 5 shows the average score for number of mucous cells (NM) per villus of Atlantic salmon (Salmo salar) fed different diets; BG1 – a marine, BG5 – a plant and BG2 – a soybean meal based feed, without (CT) or with (LP+LF) lactic acid bacteria. A semi-quantitative assessment of NM was used and the different patterns are accompanied with numbers from 1-5, as further explained in Table 3, indicating size and number of mucous cells throughout the complex villi in distal intestine.

Figure 6 shows the average score for supranuclear vacuoles (SNV) of Atlantic salmon (Salmo salar) fed different diets; BG1 – a marine, BG5 – a plant and BG2 – a soybean meal based feed without (CT) or with (LP+LF) lactic acid bacteria. A semi-quantitative assessment of SNV was used and the different patterns are accompanied with numbers from 1-5 indicating presence of SNV per villus, from absent (1) to highly vacuolated (5).

Figure 7 shows the relative mRNA levels of mucin gene, muc5ac1 in the skin of Atlantic salmon fed different diets; BG1 – a marine, BG5 – a plant and BG2 – a soybean meal based feed. For each feed, white colour bar (left side) represents control diet (CT) without probiotics and shaded pattern (right side) represents diet with probiotics (LP+LF). The effects of main factors (feed and probiotics) and their interaction were determined by two-way ANOVA and p values are indicated in the upper right corner. Different lower-case letters denote significant difference ($p < 0.05$) among all diet combinations after conducting a post-hoc (Tukey HSD) test. Values are presented as mean ± SEM.

Figure 8 provides the relative mRNA levels of mucin gene, muc5b in the skin of Atlantic salmon fed different diets; BG1 – a marine, BG5 – a plant and BG2 – a soybean meal based feed. For each feed, white colour bar (left side) represents control diet (CT) without probiotics and shaded pattern (right side) represents diet with probiotics (LP+LF). The effects of main factors (feed and probiotics) and their interaction were determined by two-way ANOVA and p values are indicated in the upper right corner. Different lower-case letters denote significant difference ($p < 0.05$)

among all diet combinations after conducting a post-hoc (Tukey HSD) test. Values are presented as mean ± SEM.

Figure 9 provides the relative mRNA levels of antimicrobial peptide gene, defensin3 in the intestine of Atlantic salmon fed different diets; BG1 – a marine, BG5 – a plant and BG2 – a soybean meal based feed. For each feed, white colour bar (left side) represents control diet (CT) without probiotics and shaded pattern (right side) represents diet with probiotics (LP+LF). The effects of main factors (feed and probiotics) and their interaction were determined by two-way ANOVA and p values are indicated in the upper right corner. Different lower-case letters denote significant difference ($p < 0.05$) among all diet combinations after conducting a post-hoc (Tukey HSD) test. Values are presented as mean ± SEM.

Figure 10 shows the relative mRNA levels of antimicrobial peptide gene, cathelcidin1 in the intestine of Atlantic salmon fed different diets; BG1 – a marine, BG5 – a plant and BG2 – a soybean meal based feed. For each feed, white colour bar (left side) represents control diet (CT) without probiotics and shaded pattern (right side) represents diet with probiotics (LP+LF). The effects of main factors (feed and probiotics) and their interaction were determined by two-way ANOVA and p values are indicated in the upper right corner. Different lower-case letters denote significant difference ($p < 0.05$) among all diet combinations after conducting a post-hoc (Tukey HSD) test. Values are presented as mean ± SEM. Figures 11-16 provide the survival rate of probiotic bacterial cells for different feed groups during a storage period of 8 months, wherein:

Figure 11 provides the viability of *Lactobacillus plantarum* bacterial cells in feed A after coating with / without saline and fish / plant oil during storage at 4°C.
Pellets A1 - 1st layer: *L. plantarum* probiotic cells resuspended in saline, 2nd layer: fish oil.
Pellets A2 - 1st layer: *L. plantarum* probiotic cells resuspended in saline, 2nd layer: plant oil.

Pellets A3 - coating layer: *L. plantarum* probiotic cells resuspended in fish oil.
Pellets A4 - coating layer: *L. plantarum* probiotic cells resuspended in plant oil

Figure 12 provides the viability of *Lactobacillus plantarum* bacterial cells in feed A after coating with / without saline and fish / plant oil during storage at 22°C.
Pellets A1 - 1st layer: *L. plantarum* probiotic cells resuspended in saline, 2nd layer: fish oil.
Pellets A2 - 1st layer: *L. plantarum* probiotic cells resuspended in saline, 2nd layer: plant oil.
Pellets A3 - coating layer: *L. plantarum* probiotic cells resuspended in fish oil.
Pellets A4 - coating layer: *L. plantarum* probiotic cells resuspended in plant oil.
a - significant differences between A1 and A2, b - significant differences between A1 and A3, c - significant differences between A1 and A4, d - significant differences between A2 and A3, e - significant differences between A2 and A4.

Figure 13 provides the viability of Lactobacillus plantarum bacterial cells in feed A after coating with lecithin 1.5% / 3% in fish / plant oil during storage at 4°C Pellets A5 - coating layer: *L. plantarum* probiotic cells resuspended in fish oil + 3% lecithin.
Pellets A6 - coating layer: *L. plantarum* probiotic cells resuspended in fish oil + 1.5% lecithin.
Pellets A7 - coating layer: *L. plantarum* probiotic cells resuspended in plant oil + 3% lecithin.
Pellets A8 - coating layer: *L. plantarum* probiotic cells resuspended in plant oil + 1.5% lecithin.
b - significant differences between A5 and A7.

Figure 14 provides the viability of *Lactobacillus plantarum* bacterial cells in feed A after coating with lecithin 1.5% / 3% in fish / plant oil during storage at 22°C.
Pellets A5 - coating layer: *L. plantarum* probiotic cells resuspended in fish oil + 3% lecithin.
Pellets A6 - coating layer: *L. plantarum* probiotic cells resuspended in fish oil + 1.5% lecithin.
Pellets A7 - coating layer: *L. plantarum* probiotic cells resuspended in plant oil + 3% lecithin.
Pellets A8 - coating layer: *L. plantarum* probiotic cells resuspended in plant oil + 1.5% lecithin.
b - significant differences between A5 and A7, c - significant differences between A5

and A8, d - significant differences between A6 and A7, e - significant differences between A6 and A8, f – significant differences between A7 and A8.

Figure 15 provides the viability of *Lactobacillus plantarum* and *Lactobacillus fermentum* bacterial cells in feed B after coating with saline during storage at 4°C.
Pellets B1 - coating layer: *L. plantarum* probiotic cells resuspended in saline.
Pellets B2 - coating layer: *L. plantarum* + *L. fermentum* probiotic cells resuspended in saline.
Pellets B3 - coating layer: *L. fermentum* probiotic cells resuspended in saline.
a - significant differences between B1and B2, b - significant differences between B1 and B3, c - significant differences between B2 and B3.

Figure 16 provides the viability of *Lactobacillus plantarum* and *Lactobacillus fermentum* bacterial cells in feed B after coating with saline during storage at 22°C.
Pellets B1 - coating layer: *L. plantarum* probiotic cells resuspended in saline.
Pellets B2 - coating layer: *L. plantarum* + *L. fermentum* probiotic cells resuspended in saline.
Pellets B3 - coating layer: *L. fermentum* probiotic cells resuspended in saline.
a - significant differences between B1and B2, b - significant differences between B1 and B3, c - significant differences between B2 and B3.

For more details about the Figures, please see the Examples.

Detailed description of the invention

Unless otherwise defined, all terms of art, notations and other scientific terms or terminology used herein are intended to have the meanings commonly understood by those of skill in the art to which this invention pertains. In some cases, terms with commonly understood meanings are defined herein for clarity and/or for ready reference, and the inclusion of such definitions herein should not necessarily be construed to represent a substantial difference over what is generally understood in the art.

A list of abbreviations is provided at the start of the Example section.

The parameter Colony Forming unit (CFU) is used herein for the number of bacterial cells. This may be provided as logarithmic numbers. Converting back and forth

between arithmetic and log numbers in Excel or LibreOffice can be done by typing the following formula into a cell in the spreadsheet:

Convert to log_{10}: “=log_{10}(150)”

Convert from log_{10}: “=10^2.18”

For example, converting the value 150 CFU/g to the log_{10} scale results in 2.176 log_{10}CFU/g.

The invention provides fish feed compositions found useful in improving the intestinal health of farmed fish. The invention provides a feed, preparation of this, and use of this, wherein one or more types of lactic acid bacteria is included in the fish feed for improving the fish's general health through better intestinal health. Microflora in the intestine in both humans and animals is important for a good general condition and can protect against pathogenic bacteria.

Numerous studies have reported that use of plant ingredients in fish feed interferes with intestinal health of fish. However, fewer studies have focused on the effect of incorporating probiotics with feed on the mucosal health in fish. Therefore, the applicant has now studied and revealed the effects of probiotics with different combinations of plant and marine ingredients on the skin, gills and intestinal tissues, the multiple mucosal sites, of Atlantic salmon. The mucosal surface on the skin, gills and intestine is vital for the health of the fish, and abrasions to the mucosal surfaces make the fish susceptible to disease.

Preliminary studies have been carried out to investigate two different lactic acid bacteria that are isolated from salmonids - and whether these a) colonize the intestines of salmon, b) can prevent intestinal inflammation and c) can repair intestinal damage. Preliminary studies show that these bacteria certainly have the potential to increase the barrier status of the fish - which in turn improves the fish's health status and prevents disease.

Studies are also performed to develop a method to apply the bacteria to the feed, and ways to increase storage stability of feed has been investigated with regard to bacterial survival.

The inventors have discovered that a fish feed comprising lactic acid bacteria can positively impact intestinal health and innate immune response. The findings are at least partly based on a study conducted on Atlantic salmon, investigating the intestinal health and innate immune response of feed ingredients along with, or without, administration of the probiotics *Lactobacillus fermentum* and *Lactobacillus plantarum,* as detailed in Example 1. The aim of the study was to investigate if there were any positive effects on the mucosal barriers in Atlantic salmon post smolts if they were fed a diet with or without probiotics, and whether the feed was plant based or marine based.

The invention hence involves adding one or more specific lactic acid bacteria to fish feed, which will strengthen the fish's intestinal health, and the barrier status on the body's surfaces (gills, mucus layer on body and intestine). Surprisingly, the inventors have found that a new feed can improve barrier status in that the fish can produce more mucus for capturing and inactivating pathogens.

The two lactic acid bacteria *Lactobacillus fermentum* and *Lactobacillus plantarum* have been isolated, documented and registered as probiotics.

In one aspect, the invention provides a feed for aquaculture animals, particularly for farmed fish, comprising at least one of the lactic acid bacteria *Lactobacillus fermentum* and *Lactobacillus plantarum.*

The *Lactobacillus plantarum* (LP) R2 Biocenol™ (CCM 8674) and *Lactobacillus fermentum* (LF) R3 Biocenol™ (CCM 8675) have been used in the disclosed studies. Both bacteria were isolated from the intestinal content of rainbow trout from a fish farm, Rybárstvo – Požehy s.r.o. Dubové in the Slovak Republic (Fečkaninová et al., 2019). The strains are deposited in Czech Collection of Microorganisms (CCM) of Masaryk University in Brno under the Budapest Treaty.

Pure cultures were grown on de Man, Rogosa and Sharpe (MRS) agar plates (HiMedia Laboratories, Mumbai, India) before they were inoculated into MRS broth and incubated. The culture was centrifuged, and the resulting cell pellets were washed and resuspended in sterile saline.

In some embodiments, other probiotics than LP and LF may be included in the feed. Hence, further lactic acid bacteria may be included in the feed, such that the feed may comprise one or more, such as a mixture of several, lactic acid bacteria (cocktail). Such additional probiotics are selected from, but not limited to, the group of lactic acid bacteria which are considered as GRAS (generally regarded as safe), they are common commensals of the digestive tract of fish and some strains have a positive effect on the fish organism. In one embodiment, such other probiotics may comprise any of the following: autochthonous lactic acid bacteria of species such as Lactobacillus brevis, Lactic acid bacteria such as *Lactobacillus spp., Pediococcus spp., Lactococcus spp., Carnobacterium spp.,* and *genus Leuconostoc, Bacillus sp., Lactobacillus sp., Enterococcus sp., Carnobacterium sp*., and the yeast *Saccharomyces cerevisiae*.

In a preferred embodiment, the feed comprises both *Lactobacillus fermentum* and *Lactobacillus plantarum.* The two bacteria strains are registered as probiotics and have been selected in the studies partly because of their ability to withstand the conditions in the gastrointestinal tract of the fish. Both strains have been found effectful, individually. In some embodiments, the feed comprises at least the LP bacteria, particularly as this has stronger tendency to colonise in the gastrointestinal tract after administration of the feed, and as this has a better viability during storage of the feed.

The feed comprises the one or more bacteria cultures themselves, as alive and active. In one embodiment the feed may further comprise metabolites produced from the LP or LF strains, such as bacteriocins, vitamin B and organic acids, such as formic acid, acetic acid and lactic acid. However, the bacteria cultures are not included in the feed for the purpose of fermentation, or in other ways which may affect or change the properties of the other ingredients of the feed. Rather, the bacteria cultures are included in the feed, in a stabilised form, that enables the bacteria culture to survive to they have been administered, i.e. till they have been fed the fish and entered the intestinal track of this. The feed hence comprises the lactic acid bacteria as living and active culture of bacteria.

Hence, the invention provides a method and a formulation wherein the one or more lactic acid bacteria is added to the feed in a way that allows them to survive until they

enter the fish. Hence, the bacteria are incorporated in the feed such that they provide a benefit to the consuming fish. The bacteria of the feed are viable and can colonize the gut to provide the benefits further discussed below. This has been achieved by including the bacteria in a coating. In on embodiment, the lactic acid bacteria are coated on and/or into the feed. Hence, the invention provides a fish feed comprising lactic acid bacteria, wherein the lactic acid bacteria are coated on a feed formulation, wherein the feed formulation comprises the basal feed ingredients. Hence, in one embodiment the lactic acid bacteria is included in a coating, e.g. coated layer for the basal feed. The basal feed may be provided in different stable and concentrated forms, such as usually provided in the form of a granular feed, of granulates or pellets, such as in the form of dry pellets. The form of the basal feed is selected e.g. based on the need for digestibility and refining the balance of nutrients to match the needs of the different species of fish at different periods of development, in addition to the need for sustainability of the ingredients used. In one embodiment, the feed of the invention is a granular feed of pellets or granulates coated with a coating comprising the lactic acid bacteria. In one embodiment, the feed is in the form of pellets, particularly dry pellets. The basal feed pellets are typically prepared by extrusion, providing a porous structure of the pellets. When coating such porous structures with lactic acid bacteria these will at least partly be absorbed into the pores of the pellets. Hence, in one embodiment, the feed comprises pellets wherein the lactic acid bacteria are coated onto, adsorbed onto, and/or absorbed into pores of the pellets. Hence, in one embodiment, the one or more lactic acid bacteria is/are adsorbed onto and/or adsorbed and/or absorbed into the granular feed, such as a feed pellet. Accordingly, the granular feed may be seen as a delivery system for the release of the one or more lactic acid bacteria, wherein the delivery system comprises a granular feed, such as a feed pellet, and one or more lactic acid bacteria is loaded on the granular feed, and wherein the carrier material consists of a granular feed, such as a porous dry basal feed pellet.

As further explained below, vacuum-coating may be used in the preparation of the feed pellets. Vacuum-coating systems offer a method by which to more effectively use higher levels of oil coating. Available since the mid-1980s, vacuum coaters for feeds have evolved into sophisticated machines that allow the addition of oil up to 40 percent. The best units combine a double-paddle mixer with the vacuum system.

Double-paddle units ensure proper mixing of the pellets with the oil, which is applied from spray nozzles located at strategic locations to ensure proper dispersion onto the feed. The vacuum-coating process is more successful with extruded products that have porous cell structures. Closing the feed pores with oil can improve water stability. The oil is hydrophobic and having it inside the porous structure reduces the opportunity for water to penetrate the feed.

The coating layer comprising the one or more lactic acid bacteria may further include additional ingredients. The lactic acid bacteria are applied to the basal feed granulates typically using a bacterial suspension, e.g. comprising the lactic acid bacterial suspended in a suspension media. Such suspension media is e.g. saline or an oil and is preferably an oil. Such oil may be selected from a marine oil or a plant oil and is preferably a fish oil or rapeseed oil. The feed of the invention, comprising the lactic acid bacteria, may hence include remains of the oil used in the application of the bacteria suspension. Further, the oil used in the bacterial suspension may also have the effect of stabilizing the final feed. As shown in Example 2, it is the bacteria of the feed pellets wherein the lactic acid bacteria are applied to the pellets as suspended in an oil, that show the best viability after storage, i.e. more bacteria survived storage. Alternatively, or additionally, the applied lactic acid bacteria suspension, and hence the final feed, may further comprise ingredients improving the stability of the feed composition. Such further ingredients are preferably emulsifiers, such as an emulsifier selected from the group of commercially available emulsifiers, such as lecithin, different oils, glycerides, and is preferably a lecithin. The lecithin may originate from legumes, such as more specifically soybeans or rapeseed. The applicant has shown that for feed pellets comprising LAB suspended in saline, the survival of the bacterial cells decreased more significantly than in samples prepared without saline. Preferably, when preparing the feed pellets of the invention, the lactic acid bacteria is beneficially suspended in a suspension media comprising an oil, as this increases the survival and shelf life of the bacteria. Further, it was seen that when lecithin was used as an emulsifier to homogenize the feed coating mixture of oil and probiotic bacterial cell pellets after centrifugation this also had an antioxidant effect creating suitable conditions for the survival of probiotic bacterial cells in feed.

Another factor that has positively affected the survival of probiotics is the presence of polyunsaturated fatty acids (PUFA) in fish oil, which increases the number of lactic acid bacteria in aquafeeds. The reason for the increase in LAB in fish fed comprising PUFAs may be that dietary fatty acids influence intestinal membrane composition, function and fluidity which may affect the attachment sites of the gut mucosa.

The fish feed of the invention, such as in the form of dry feed pellets comprising lactic acid bacteria in a coating, are stable for relatively long periods, for convenient storage and distribution. In one embodiment, the feed of the invention is a dry feed, preferably in the form of dry feed pellets. To ensure hygienic quality during storage and avoid problems with e.g. fungus, such as bacterial and fungal multiplication, the moisture content of the final feed should be kept low, such as at about 5-10% moisture, e.g. 7-8% moisture. A storage study of a feed of the invention, wherein bacterial cultures are included in a coating of coated dry feed pellets, has been carried out, please see Example 2. In this study the viability of probiotic bacterial cells in different groups of coated pellets was monitored during storage at refrigerator temperature (4 °C) or room temperature (22 °C). It was found that the storage conditions are crucial for how long the bacteria survive in the feed. The two different bacteria, LP and LF, have shown different survivability in applicant's studies, and one of the bacteria survived as long as 8 months at 4 °C storage temperature. Storing temperature is considered as a critical factor that influences probiotic viability and survival during the storage period. Generally, the best viability of probiotic cells was observed when storing the feed at 4 °C. In one embodiment, the feed of the invention should be stored at temperatures of 2 to 24 °C, such as at 4 to 22 °C, such as at 4 to 15 °C. However, also the composition of the coating plays an important role for the viability of the bacteria, and the inclusion of an oil, particularly a fish oil, as a suspension media for the bacteria, improves and prolongs the viability of the bacteria during storage. Further, when a stabilizer, such as lecithin, is comprised in the coating material the survival and viability of the bacteria is increased, Accordingly, when the feed is a granular feed with a coating comprising the lactic acid bacteria, an oil, and preferably also lecitin, the feed withstands higher storage temperatures. Further tests are conducted in larger scale to test reproducibility and to provide stability data.

In one embodiment the bacterial feed of the invention can be characterized as a special feed or functional feed, as it can be used in critical phases of the salmon's developmental stages.

The fish feed comprises an effective amount of the lactic acid bacteria. In one embodiment, the feed comprises at least a bacteria number of $1x10^7$ cells/gram feed, such as between $1x10^7$ and $1x10^{11}$ cells/gram feed. As shown in Example 1, a number of $\sim 10^8$ cells/g final feed, as determined by spread plating on MRS agar plates, was found suitable. In one embodiment, the fish feed comprises less than 25 grams, preferably less than 18 grams, such as less than 12 grams, preferably less than 1 grams of bacteria per kg fish feed. In one embodiment, the bacteria comprise from 0.0.5 to 0.1 weight% of feed.

It is important to consider the concentration of probiotic bacterial cells in fish feed. For the preparation of probiotic feed, the applicant used probiotic cells in the number of 10^8 CFU/g (Colony Forming unit/gram). Previous experiments have concluded that the number of bacterial cells 10^7 CFU/g of feed is sufficient to control fish pathogens and the use of higher concentrations of probiotics does not lead to better protection against the disease or to reduce mortality. Continuous application of probiotic cells with a dry diet containing $10^5 – 10^9$ CFU/g has earlier been shown to colonize the intestinal epithelium, intestinal mucosa and pyloric pendants.

In one embodiment the feed comprises both the LP and LF bacteria, and the ratio is from 2:1 to 1:2 and is preferably in a ratio of 1:1. LP is homofermentative and LF is heterofermentative. The ratio of 1:1 is most beneficial as the two will then not compete. Excessive amount of one strain can lead to inhibition of the another.

The feed of the invention comprises a mixture of ingredients providing the balanced nutrition needed by farmed fish. Hence, the feed of the invention comprises fats (lipids), proteins, carbohydrates, and preferably also vitamins, amino acids and minerals. The feed, e.g. providing the basal ingredients, preferably in the form of a granular feed, e.g. granulates or pellets, provide the nutrition in a stable and concentrated form, enabling the fish to feed efficiently and grow to their full potential.

The applicant has tested the effect of lactic acid bacteria included in different types of feed, i.e. identifying and comparing the effect of the lactic acids with a marine based feed versus a plant based feed, versus a soy bean meal based feed, as shown in Table A below. As an overall conclusion the applicant has found that the inclusion of at least one of LP and LF in fish feed is beneficial, independent of the basal feed ingredients.

Table A:

Feed type	**Feed name**	**Main components**
Marine based feed	BG1	Fish meal and fish oil
Marine based feed + lactic acids	BG1 LP+LF	Fish meal and fish oil + LP and LF
Soybean meal based feed	BG2	Soybean meal and fish oil
Soybean meal based + lactic acids	BG2 LP+LF	Soybean meal and fish oil + LP and LF
Plant based feed	BG5	Plant ingredients:marine ingredients 70:30
Plant based feed + lactic acids	BG5 LP+LF	Plant ingredients:marine ingredients 70:30 + LP and LF

In the reported studies, please see Example 1, the six experimental diets, listed above in Table A, were prepared by coating LP+LF to the three basal feeds, respectively. Table 1 of Example 1 shows the more detailed ingredient compositions of the basal feeds, prior to addition of the bacteria cultures. Table 2 provides the nutrient and amino acid composition of the tested diets.

In all the tested feeds, the amount of fat and protein is balanced to provide sufficient protein and energy for good growth of the fish. In the feed of the invention, the weight% of protein in the final feed is 30-65%, such as 35-55%, such as about 40-45%. The amount of lipid in the feed of the invention is 10-40%, such as 20-30%, such as about 25-30% by weight. In a preferred embodiment, the ratio of protein to lipid is 35-55:2-40%, depending on the size of the fish.

The protein source for the fish feed of the invention may be fish meal, or commercially used plant protein ingredients as traditionally used, but may also be selected from, but is not limited to, the group of fish meal, wheat meal, insect meal, microalgae, animal by-products, soybean meal, or other plant proteins, such as cereal grains, peas or beans. In one embodiment, the protein source is not soybean meal. The reason for excluding soybean meal, is that the study has shown that precautions should be taken when probiotics are incorporated with feed ingredients like soybean meal, because under inflammation conditions the fish did not produce more mucous cells (NM) and intraepithelial lymphocytes (IEL). Preferably, plant protein concentrates, such as soy protein concentrate, which does not cause enteropathy, may be included as a main ingredient, while other protein concentrates such as wheat gluten, corn gluten, faba beans, sunflower meal, pea protein concentrate and other plant proteins can be included in low amounts.

The fat component, or lipid component, of the feed may originate from, or may be, a marine oil such as a fish oil, or may be a plant oil, or oils from animal by-products such as poultry fat, or mixtures thereof. In one embodiment, the feed comprises a marine oil, such as fish oil, either as raw oil or as a concentrated or refined product. Preferably, the feed comprises fatty acids, such as polyunsaturated fatty acids (PUFAs), and particularly long chain polyunsaturated fatty acids, and more preferably omega-3 PUFAs. In one embodiment the feed comprises eicosapentaenoic acid (EPA) and/or docosahexaenoic acid (DHA), such as at least 1.6% EPA and/or /DHA. Further, in one embodiment, the diet comprises a balanced ratio between omega-3 fatty acids and omega-6 fatty acids.

From the studies it was concluded that the probiotics tested showed similar influence or capability regardless of feed ingredients, suggesting that the lactic acid bacteria can be utilized as immune regulators on skin, gills and intestine, independent of the basal feed ingredients. Hence, an effect of the LAB is seen independently of the basal feed ingredients. However, as cultured fish fed on a marine based feed generally has a better overall health, and particularly a better intestinal health, than fish fed on a plant based feed, the level of the effect of the LAB may be higher for fish fed a plant based feed of the invention than a marine based feed of the invention. The LAB reduced the number of inflammatory cells in the distal intestine of fish fed

the BG2 diet containing soybean meal. Supplementing LAB to the fish feeds resulted in a reduction in short chain fatty acids (SCFs) in chyme collected from distal intestine, and a shift in composition of the SCFs. In total 7 SCFs were detected in the digesta and the sum of these SCFAs varied from 31 - 60 mmol/L, based on values from fish fed the different diets. The highest concentration of SCF's were observed in fish fed BG1 (marine based) and this feed also had the highest concentration of adherent lactic acid (LP+LF) bacteria to the intestinal mucus 6.11 ± 0.04 log_{10}CFU.g-1. Feeding with probiotics resulted in a significant reduction in acetoacetic acids and a tendency towards reduction in succinic acids (p = 0.051) in digesta compared to those fish fed diets without probiotics. Table B below provides the short chain fatty acids in fish fed three different diets without or with the *Lactobacillus plantarum* and *Lactobacillus fermentum.*

Table B:

Parameters:	**Probiotics (LP+LF)**	
	Without	With
Formic acids	3.58 ± 0.30	3.35 ± 0.22
Acetoacetic acids	12.45 ± 0.79^{Y}	11.59 ± 0.43^{X}
Lactic acids	9.09 ± 0.43	9.75 ± 0.55
Succinic acids	7.39 ± 0.38	6.71 ± 0.35
Acetic acids	10.52 ± 0.86	9.48 ± 0.65
Propionic acids	3.06 ± 0.36	2.75 ± 0.27
Butyric acids	1.94 ± 0.40	1.08 ± 0.15
Total acids	47.21 ± 1.56^{Y}	43.73 ± 1.53^{X}

X, Y indicate significate differences between groups fed without or with lactic acid bacteria

In one embodiment, the feed is a marine based feed, i.e. comprising both a marine oil, e.g. fish oil as the main lipid component, and fish meal as the main protein component. As there may be a greater need for the invention, i.e. the inclusion of lactic acid bacteria, when the fish feed is plant-based rather than marine-based, another embodiment of the invention is a feed which is mainly plant based comprising vegetable oil as a main ingredient.

The feed of the invention is a fish feed for farmed fish. The disclosed feed is useful for farmed fish wherein this is from the group of bony fish (teleost), particularly for

teleost fish species that are farmed commercially, and particularly for salmonid species. The fish is e.g. selected from the group comprising Atlantic salmon (*Salmo salar*), brown trout (*Salmo trutta*), Steelhead/Rainbow trout, Chinook salmon, Coho salmon and other Pacific salmonid fishes (*Oncorhynchus s.p.p.*). In a preferred embodiment, the feed is for Atlantic salmon. For salmonids, the feed of the invention may be used in different phases of the life cycle of the fish including the pre-smolt phase (fry, parr), the smoltification phase, and the post smolt phase, i.e. the grow-out sea phase. The presence and content of different feed ingredients like salts, amino-acids and peptides etc. influencing the phase of the fish is relevant to assess and adjust dependent on which life cycle the feed is for. In one embodiment the feed is a post-smolt feed, i.e. for fish in the sea phase. In another embodiment, the feed is a pre-smolt feed, i.e. for fish that is still in the fresh-water phase.

In a further aspect, the invention provides a method for producing fish feed comprising lactic acid bacteria. In one embodiment, the invention provides a method of coating fish feed granulates, such as pellets with a bacterial suspension at an evacuated atmosphere, such as by vacuum coating. When using the term “coating” this is intended to cover also the “loading” of the one or more LAB onto, adsorbed onto, and/or absorbed into pores of the feed. The vacuum coating method uses vacuum technology to create a negative atmospheric pressure environment and an atomic or molecular condensable vapor source (i.e. bacteria suspension) to deposit thin films and coatings on the feed. The invention hence provides a method of producing a granular fish feed comprising at least one lactic acid bacteria, wherein the method comprising a step of coating feed granulates with the at least one lactic acid bacteria, by applying the bacteria from a bacterial suspension at an evacuated atmosphere, wherein the bacterial suspension comprises the at least one lactic acid bacteria, such as in a concentration of 9 – 10 $\log_{10}$CFU/ml. In one embodiment, feed granulates are coated with a bacterial suspension, e.g. using a vacuum coater, at a negative pressure, such as at a pressure of 200-700 In the method, the basal feed formulation, preferably in the form of dry pellets (granulates), to be coated is placed inside the vacuum chamber. A bacteria suspension is sprayed on to the pellets under vacuum. Bacteria from the vaporized suspension embed themselves onto the surface of the feed granulates, and preferably into the pores of the pellets, in the vacuum chamber. In this vacuum process, the applied vacuum removes air from the feed

pellet pores, leaving them as open cells. Once the vacuum pressure achieved, the bacterial suspension, of LAB in saline, oil and/or stabiliser (e.g. lecithin), sprayed on from nozzles located at strategic locations to ensure proper dispersion onto the feed, while the paddles mix the feed with the coating mixture to coat all particles. Once the application is completed under vacuum, the vacuum pressure is released slowly back to atmospheric pressure. The created pressure differential “pulls” the coating liquid (bacterial suspension) deep into the pellet pores. Hence, to ensure the feed granulates are evenly coated, the feed is carefully positioned and rotated during the coating process. When vacuum is released to ambient pressure, the bacteria suspension will be absorbed onto and into the granulate or pellet. Hence, each granulate or pellet is covered by a layer of the at least one lactic acid at approximately 10^8 bacteria per gram.

The temperature of the bacterial suspension while being applied onto the surface of the feed should be below 50 °C, such as about 40 °C. In some embodiments it may be relevant to use lower temperatures, such as below 25 °C, such as below 20 °C, preferably below 15 °C. The bacteria are applied to the granulates e.g. by vaporisation, spraying or sprinkling. Preferably, the basal feed and the bacterial suspension should have about the same temperature.

The bacterial suspension used for the coating comprises lactic acid bacteria. In one embodiment, the bacterial suspension used for the coating comprises cultures of at least one of *Lactobacillus fermentum* and *Lactobacillus plantarum,* as disclosed in the first aspect, and preferably both. As discussed for the feed, other probiotics may also be included.

When preparing the bacterial suspension, firstly the one or more autochtonous lactic acid bacteria cultures were isolated from a source. Such source may be selected from the group of either plant or isolated from animal origin. The lactic acid bacteria including the strains used herein are universal and can have different origin. In the example study, the LP and LF, specific bacteria strains were isolated from the intestinal content of rainbow trout. The isolated pure cultures are then grown, such as on agar plates for a sufficient time, e.g. for 48 h, before they are inoculated into a broth and incubated. The incubation takes place e.g. for 18 h at a temperature of 25-40 °C, such as at about 37 °C. The culture may so be centrifuged to provide a cell

pellet of bacteria. In one embodiment the centrifugation may take place at low temperature, such as at 4,500 rpm for 20 minutes, at 4 °C in a centrifuge. In another embodiment, cooling centrifugation has been seen not to be necessary. The resulting bacteria cell pellets is resuspended in a liquid, e.g. saline or an oil, and preferably in an oil. Hence, the suspension liquid for the bacteria suspension is preferably an oil. The bacteria suspension may be provided by mixing the bacteria cell pellets with oil or water. In one embodiment, the bacteria are mixed into oil, preferably a marine oil such as fish oil, or rape seed, but also other oils can be used such as soya, palm, olive, sunflower oil. Particularly, the oil further comprises a stabilizer, such as an emulsifier, to stabilise the one or more bacteria cultures. The emulsifier, preferably lecithin, is mixed into the suspension oil, e.g. at a weight% of 1.5-3.0, and e.g. homogenised by vortex.

In the method, feed granulates are e.g. pellets which are prepared by extrusion, or alternatively by expansion or a pelletizing process. Prior to coating with lactic acid bacteria, the basal feed granulates are dried, such as to a moisture content of about 5-10% moisture, e.g. 7-8% moisture, and preferably coated with an oil coating, such as under evacuated pressure, e.g. by vacuum coating.

More particularly, the basal feed granulates, prior to coating with lactic acid bacteria, are prepared by homogenizing the feed ingredients, followed by a preconditioning step wherein water and steam are added, such as in an atmospheric preconditioning step followed by extrusion of the feed mash. The temperature of the feed mash that was fed into the extruder was 86-100 °C. The temperature of the extruded feeds is usually between 100-130 °C and is usually optimised to give the right pellet quality. Appropriate feed ingredients are discussed in the first aspect. In one option, the wet extrudates are cut with a rotating knife of the extruder. The extruded pellets are further dried, e.g. in a hot air dual layer carousel dryer, e.g. at 60-80 °C, to obtain pellets with approximately 7-8% moisture. Next, the granulates, preferably the pellets, are coated with oil, such as fish oil or a plant oil such as rapeseed oil, using a vacuum coater. Immediately after the oil coating, the feed is packed, such as in sealed plastic containers or buckets. In the method of the invention, this basal feed, preferably in the form of oil coated pellets, is coated (loaded) with the bacteria suspension as disclosed above.

In a third aspect, the invention provides a fish feed for use in treatment of fish, particularly for improving at least one of intestinal health and innate immune response, administering a fish feed composition comprising at least one of the lactic acid bacteria *Lactobacillus fermentum* and *Lactobacillus plantarum* to the fish.

Likewise, the invention provides a method of treatment of fish, particularly for improving at least one of intestinal health and innate immune response, comprising administering a fish feed composition comprising at least one of the lactic acid bacteria *Lactobacillus fermentum* and *Lactobacillus plantarum* to the fish.

And likewise, the invention provides the use of a feed composition comprising an effective amount of lactic acid bacteria as disclosed herein, for improving at least one of intestinal health and innate immune response.

It has been found that the feed for use in treatment can strengthen the mucosal surfaces covering the gills, skin and fish intestine. The mucosal surfaces are covered by a mucus layer which is mainly secreted by mucus cells. The mucus also contains innate immune molecules such as protease, lysozyme, esterase, complement protein, antibodies and antimicrobial peptides which chemically inactivate pathogens. The mucosal surface is therefore an important barrier – and the barrier status is a term that says something about how well the fish's innate immune system copes with pathogens in the environment. Skin and mucous membranes are important parts of the fish's physical barrier to prevent pathogens from entering through the gills, skin and intestines. Surprisingly, it has been found that the new feed can improve barrier status in that the fish can produce more mucus which in turn contains components that are important for capturing and inactivating pathogens. Hence, there is a barrier strengthening effect of the lactic acid bacteria.

In the studies of the applicant, it has been investigated how the probiotics, as provided to the fish in the form of a feed comprising one or more lactic acid bacteria, affect fish health with regard to barrier status in the gills, intestines and skin, as well as whether the bacteria prevent feed-induced inflammation in the intestine. Several experiments have been performed under controlled conditions in tanks. Experiments with salmon have shown that both the LP and LF bacteria "survive" in salmon. The LP colonizes the intestine, i.e. "attaches" to the mucosa, while the LF lives in the

chyme). Studies have also been carried out to investigate whether these bacteria can have a preventive effect on intestinal health and also whether the probiotics can repair intestinal damage that has occurred. Positive effects of the probiotic have been found as detailed below. In summary it has been shown that these bacteria certainly have the potential to improve the barrier status of the fish - which in turn improves the fish's health status and prevents disease.

Results from the studies have shown that the fish feed as disclosed in the first aspect, for use in treatment provides either of an improvement of the fish's barrier status, e.g. so that it is better equipped to handle pathogens before they enter the body and can cause disease, or a reduction of inflammation in the intestine.

Initial studies including an infection test with rainbow trout indicates that the probiotics also can have a positive effect and increase survival.

A comprehensive trial has been conducted to study the effects of lactic acid bacteria on feed consisting of different raw materials, as shown in Table 1. One feed was designed to cause inflammation in the gut of salmon, named BG2, comprising a high content of soybean meal. The lactic acid bacteria showed positive effects when applied to any of the diets, but more significant effects were observed when they were supplemented to feeds based on more plant ingredients.

Reference is made to the feeding trial, see Example 1, wherein particularly two main factors have been assessed, namely the feed ingredients (marine based, vegetable, or soybean based), and the probiotics (LP+LF present or absent). At the end of the trial, fish were sampled for histology and the expression of selected mucosal immune related genes in the skin, gills and distal intestine.

Based on the study it was concluded that the number of mucous cells in skin, gills and intestine were affected by the presence of lactic acid bacteria. Probiotics increased the number of mucus cells in the skin. The increased number of mucus cells in the skin was accompanied by an up-regulation of the mucine genes muc5ac1 and muc5b. A positive correlation between the number of mucus cells and the gene expression of antimicrobial peptide cathl1 supports the barrier strengthening effects of lactic acid bacteria, in particular when it is supplemented feeds containing soybean

meal or based on high proportion of plant ingredients, which is known to compromise fish health. The gill mucosa also showed increased number of mucus cells for all diet groups when the probiotics were applied to the feed. The higher number of mucous cells can be interpreted as an overall immune response to the probiotics. The distal intestinal histomorphology of fish differed due to different feed ingredient composition. The morphology of fish fed plant-based feed was almost similar to fish fed marine based feed. However, fish fed soybean meal-based feed developed enteritis. Addition of probiotics to soybean meal-based feed groups did not completely prevent formation of the enteritis. However, positive effects were observed on the intestinal health when probiotics were added to the soybean meal group, such as increased villi height, improved score for number of mucus cells per villus, reduced width of lamina propria accompanied with reduced number of intraepithelial lymphocytes and reappearance of supra nuclear vacuoles were observed in post-smolt Atlantic salmon. Fish fed the marine based or the plant-based diets also showed increased number of goblet cells when fed probiotics, associated with increased mucus secretion. The increase in goblet cells was accompanied by an increase in the antimicrobial peptide genes defensin1, defensin4 and cathelcidin1 in the intestine by supplementation of lactic acid bacteria, indicating an immune response and improved barrier function of the intestinal mucosa. In order to boost innate immune response and enhance intestinal health, these probiotics can be incorporated with marine or plant-based feed without compromising growth.

Effect on mucous cells in dorsal skin: It was further found that the number of skin mucous cells per skin epithelium (SNE) was significantly influenced by both feed ingredients and probiotics, and it was found that the probiotics groups (LP+LF) had significantly more SNE. Hence, feeding fish the probiotics boosted the number of mucous cells per unit area of epithelium, but this response was only significant in fish fed the marine based ingredients (BG1). The number of mucous cells signifies the health status of the mucosal tissues and enhance skin barrier functions. Supplementation of probiotics, in particular to the plant or soybean meal based feeds, increased number of mucous cells in the skin of Atlantic salmon. Hence, in one embodiment, the feed may be used in treatment comprising increasing the number of mucous cells in epithelium, and hence enhance the skin barrier functions.

In another embodiment, the treatment is for increasing the number of mucous cells in the fish skin. The mucosal surface is the outermost tissue layer that separates the fish from the environment while communicating with it. When a fish encounters pathogens on its skin, it physically sheds the pathogens from the surface by continuous secretion of mucus secreted by mucous cells that are located in the epidermis part of skin.

The present study has tested the relative expression levels of mucin genes; muc2, muc5ac1, muc5ac2, and muc5b in the skin. Only the muc5ac1, muc5ac2, and muc5b were expressed in skin. The plant and soybean meal based feeds caused down-regulation of muc5ac2. Supplementation of probiotics did not influence the expression of muc5ac2 in any of the diets, but increased the expression of muc5ac1 and muc5b. The combination of plant-based feed and probiotics as well as feeding the soybean meal feed in combination with probiotics resulted in an up-regulation of the mRNA of those mucin genes. Hence, a feed comprising lactic acids as disclosed, can be used in a treatment including up-regulation of mucin genes.

Effect on gills mucous cells: Significant effects of both feed ingredients and probiotics were found on the gills mucous cells. The ratio between the total area of gill mucous cells (GM) and total area of gill epithelium (GE), i.e. the parameter GME, was used to assess the total area of mucous cells that cover the unit area of epithelium. Incorporation of probiotics to all the diets; marine, plant and soybean meal based diets significantly increased the GME. Fish fed the BG1 LP+LF, BG5 LP+LF and BG2 LP+LF increased the number of gills mucous cells by 2.3, 1.4 and 1.5 times, respectively, compared to their control groups. Hence, a feed comprising lactic acids as disclosed, can be used in a treatment of fish by increasing the number of gill mucous cells, e.g. with at least 1.4 times.

Effect on distal intestinal morphology: The parameters villi height (VH) and villi with, (VW), height of enterocyte (EH), width of lamina propria (LP) and reappearance of supra nuclear vacuoles (SNV) were assessed. The studies showed that fish fed the feed with soybean meal had lowest values for most morphological parameters and showed all the signs of enteritis. The present study showed that administration of probiotics reduced the width of the lamina propria in fish fed BG1 LP+LF and BG2 LP+LF. Probiotics also reduced the number of intraepithelial lymphocytes in groups

fed BG2 LP+LF suggesting that probiotics alleviate the progression of inflammation caused by soybean meal. The feed, i.e. marine based or plant based, comprising the lactic acid bacteria tended to increase the VM, EH and tended to reduce NM, IEL. Significant reduction in LPW and SNV were noted, thus it is evident that supplementation of LAB had a beneficial effect for the gut health and barrier status of the distal intestine.

Effect on gene expression: Relative expression of mucin genes (muc5ac1, muc5ac2, muc5b and muc2) in skin, gills and distal intestine of Atlantic salmon were tested and the expression were found to be tissue specific. Results showed that expression of the genes were affected by LAB. In dorsal skin, the probiotics significantly up-regulated expression of the muc5ac1 and muc5 genes. Expression of mucine genes in gills and distal intestine were not affected by probiotics supplementation to the feeds. Supplementation of probiotics to the feed significantly increased expression for AMP genes, especially cathl1.

The invention shall not be limited to the shown embodiments and examples. While various embodiments of the present disclosure are described herein, it will be obvious to those skilled in the art that such embodiments are provided by way of example only. Numerous modifications and changes to, and variations and substitutions of, the embodiments described herein will be apparent to those skilled in the art without departing from the disclosure. It is to be understood that various alternatives to the embodiments described herein can be employed in practicing the disclosure.

It is to be understood that every embodiment of the disclosure can optionally be combined with any one or more of the other embodiments described herein. Elements disclosed for one aspect also apply for other aspects, hence the details provided for the feed also apply for the method or use when relevant.

It is to be understood that each component, compound, or parameter disclosed herein is to be interpreted as being disclosed for use alone or in combination with one or more of each and every other component, compound, or parameter disclosed herein. It is further to be understood that each amount/value or range of amounts/values for each component, compound, or parameter disclosed herein is to

be interpreted as also being disclosed in combination with each amount/value or range of amounts/values disclosed for any other component(s), compound(s), or parameter(s) disclosed herein, and that any combination of amounts/values or ranges of amounts/values for two or more component(s), compound(s), or parameter(s) disclosed herein are thus also disclosed in combination with each other for the purposes of this description. Any and all features described herein, and combinations of such features, are included within the scope of the present invention provided that the features are not mutually inconsistent.

It is to be understood that each lower limit of each range disclosed herein is to be interpreted as disclosed in combination with each upper limit of each range disclosed herein for the same component, compound, or parameter. Thus, a disclosure of two ranges is to be interpreted as a disclosure of four ranges derived by combining each lower limit of each range with each upper limit of each range. A disclosure of three ranges is to be interpreted as a disclosure of nine ranges derived by combining each lower limit of each range with each upper limit of each range, etc. Furthermore, specific amounts/values of a component, compound, or parameter disclosed in the description or an example is to be interpreted as a disclosure of either a lower or an upper limit of a range and thus can be combined with any other lower or upper limit or a range or specific amount/value for the same component, compound, or parameter disclosed elsewhere in the application to form a range for that component, compound, or parameter.

Examples

Abbreviations:

CF: Condition factor (g/cm^3)

CFU: Colony Forming unit

EH: Height of enterocyte

FL: Final fork length (cm)

FW: Final body weight of fish (g)

GE: total area of gill epithelium

GM: total area of gill mucous cells

GME: Ratio between total area of gill mucous cells (GM) and total area of gill epithelium (GE)

GN number of gill mucous cells

GNE: Number of gill mucous cells (GN) per total area of gill epithelium (GE)

IEL: Intraepithelial lymphocytes

IF: Initial fork length (cm)

IL: Initial fork length

IW: Initial body weight of fish (g)

LF: *Lactobacillus fermentum*

LP: *Lactobacillus plantarum*

NM: Number of mucous cells

SE: Total area of skin epithelium

SM: Total area of skin mucus

SME: ratio between SM and SE

SN: number of skin mucous cells

SNE: number of skin mucous cells per skin epithelium (SN per SE)

SGR: Specific growth rate

TGC: Thermal growth coefficient

WG: Weight gain

VH: Villi height

VW: Villi width

Example 1: Atlantic salmon fed marine or plant-based diets supplemented with probiotics

The example describes as study wherein the aim was to document the effect of lactic acid bacteria (probiotics), when applied to a variety of feed ingredient compositions (different combinations of plant and marine ingredients), on the performance and health of Atlantic salmon.

1.1 Experimental diet preparation

Experimental diets: Six experimental feeds were prepared for this study. Three extruded feeds based on respectively fish meal and fish oil (BG1 CT), plant ingredients: marine ingredients in a ratio 70:30 (BG5 CT), and incorporation of soybean meal with marine ingredients (BG2 CT), were produced, see Table 1 providing the ingredient composition (%) of the three basal feeds. Six experimental

diets were prepared by coating LP+LF to the three basal feeds. Feeds without probiotics are denoted (÷), and feeds with probiotics are denoted (+).

Table 1:

	BG1 «fish meal and fish oil»	BG2 «Soy bean meal»	BG5 «Plant:marine 70:30»
Fishmeal	50	30	10
Wheat meal	13.85	6.55	6.05
Wheat gluten	5	10	10
Soy protein concentrate	0	0	20
Soybean meal	0	20	0
Corn Gluten	0	0	9
Pea protein concentrate	0	0	9
Fish oil	25	26.4	7.7
Rapeseed oil	0	0	19.8
Mineral premix	0.59	0.59	0.59
Vitamin premix	2	2	2
Monosodium Phosphate	2.5	2.5	2.5
Carop. Pink (10% Astax)	0.05	0.05	0.05
Yttrium oxide	0.01	0.01	0.01
Choline	0.5	0.5	0.5
Methionine	0.3	0.6	0.9
Lysine	0	0.5	1.2
Threonine	0	0.1	0.4
Histidine	0.2	0.2	0.3

The nutrient and amino acid composition of the diets is given in Table 2, which provides the analyzed proximate composition (% as is) and amino acid composition (% as is) of the experimental feeds.

Table 2:

	BG 1	BG 2	BG 5
Moisture	5.3	4.9	6.3
Protein	42.5	42.2	42.8
Lipid	29.0	28.6	26.0
Ash	11.2	9.45	7.02
Energy (KJ/100 g)	2000	2029	1994
Amino acid			
Alanine	2.44	2.03	2.04

Arginine	2.35	2.33	2.35
Aspartic acid	3.50	3.43	3.51
Glutamic acid	6.92	8.03	9.04
Glycine	2.61	2.18	1.75
Histidine	1.01	1.02	1.17
Hydroxyproline	0.31	0.22	0.16
Isoleucine	1.66	1.64	1.66
Leucine	3.01	2.93	3.54
Lysine	2.89	2.85	3.05
Phenylalanine	1.67	1.79	2.10
Proline	2.19	2.47	2.88
Serine	1.81	1.91	2.04
Threonine	1.64	1.64	1.9
Tyrosine	1.25	1.35	1.50
Valine	1.96	1.86	1.88
Tryptophan	0.43	0.44	0.41
Cysteine	0.41	0.50	0.53
Methionine	1.37	1.67	1.68

Three more experimental diets were produced by coating on two strains of lactic acid bacteria named as LP+LF, comprising *Lactobacillus plantarum* R2 Biocenol™ (CCM 8674) and *Lactobacillus fermentum* R3 Biocenol™ (CCM 8675) to the three extruded feeds. The three feeds with lactic acid bacteria (LAB) were called BG1 LP+LF, or BG1(+) (marine based feed with probiotics), BG5 LP+LF, or BG5(+) (plant-based feed with probiotics) and BG2 LP+LF, or BG2(+) (soybean meal based feed with probiotics).

Feed preparation: The ingredients of the experimental feeds were first homogenized (30 min) using a horizontal ribbon mixer and then they were subjected to a preconditioning step. During this step, water and steam were added into an atmospheric double differential preconditioner (DDC). The preconditioning step was followed by extrusion on a TX-52 co-rotating, fully intermeshing twin-screw extruder (Wenger Manufacturing Inc., Sabetha, KS, USA). While the temperature of the feed mash that was fed into the extruder was 86-88 °C, temperature of the extruded feeds were different; 120, 128, and 137 °C for BG1, BG2, and BG5, respectively. Two of the feeds, BG2 and BG5 had lower wheat content; consequently, more moisture in the form of steam was added into the DDC to ensure good expansion of the feed pellets. The wet extrudates, expelled out of the 24 circular 2.5 mm dies at the

extruder outlet, were cut with a rotating knife of the extruder The extruded pellets were dried in a hot air dual layer carousel dryer (Paul Klockner, Nistertal, Germany) at constant air temperature (77 °C) to obtain pellets with approximately 7-8% moisture. Next, the feeds were coated with oil using a vacuum coater (Pegasus PG-10VC LAB, Dinnissen B.V., the Netherlands). Immediately after the oil coating, feeds were packed in sealed plastic buckets.

Probiotics coating on feed pellets: LP and LF were isolated from the intestinal content of rainbow trout from a fish farm. Pure cultures of LP and LF were grown on de Man, Rogosa and Sharpe (MRS) agar plates (HiMedia Laboratories, Mumbai, India) anaerobically at 37 °C for 48 h before they were inoculated into 1,000 ml of MRS broth and incubated for 18 h at 37 °C on a shaker. The culture was centrifuged at 4,500 rpm for 20 min at 4 °C in a cooling centrifuge (Universal 320 R, Hettich, Germany). The resulting cell pellets were washed twice and resuspended in 30 ml of 0.9% (w/v) sterile saline. The feeds (batches of 1,600 g) were thoroughly coated with the bacterial suspensions using a vacuum coater (Rotating Vacuum Coater F-6-RVC, Forberg International AS, Norway) at 70 kPa at the feed laboratory of Nord University, Bodø, Norway. The viscosity of fish and rapeseed oil is almost the same at 40 ºC which allowed the same penetration into the pores of the pellet content. We observed this even after cutting the pellet with knife after coating. And, also the temperature up to 40 ºC is suitable for viability of lactic acid bacteria. The feed pellets and oil were heated to 40 ºC prior to coating. The lactic acid bacterial number on diets were $\sim 10^8$ cells g^{-1} as determined by spread plating on MRS agar plates and incubating anaerobically at 37 °C for 48 h. The control diets (BG1 CT, BG2 CT and BG5 CT) were coated with 0.9% of sterile saline. The coated diets were stored at 4 °C until they were fed to the experimental fish.

1.2 Fish, experimental design and feeding

Atlantic salmon post-smolts were obtained from Cermaq, Hopen, Bodø, Norway (Aquagen strain, Aquagen AS, Trondheim, Norway) There were two replicate tanks for each treatment, and each tank contained 40-43 fish. The average initial weight of the fish was 122.6 ± 2.1 g (mean ± standard error of mean, SEM).

The feeding experiment was carried out in 12 circular fiberglass tanks (1100 L) that were connected to a flow-through system. Each tank was supplied with water

pumped from Saltenfjorden, from a depth of 250 m. During the experiment, water flow rate was maintained at 1000 L per h, and the average temperature and salinity of the rearing water were 7.6 °C and 35 ‰, respectively. Oxygen saturation was always above 85%, measured at the water outlet. A 24 h photoperiod was maintained throughout the 38-day feeding trial. The fish were fed ad libitum using automatic feeders (Arvo Tech, Finland) for 12 h per day between 08:00 and 20:00 (7 feedings: 08:00-10:00, 10:00-12:00, 12:00-14:00, 14:00-16:00, 16:00-18:00, 18:00-19:00 and 19:00-20:00).

1.3 Sampling and data collection

At the beginning and end of the feeding experiment, all fish were individually weighed, and their fork lengths recorded. Fish were anesthetized using tricainemethanesulfonate (MS 222, 140 mg/L) before handling. Twelve fish per tank were sacrificed for obtaining the dorsal skin (left), gills (second arch) and intestine (approximately 2 cm of the anterior part of the distal intestine). Tissues from 6 fish were immediately placed in 10% neutral buffered formalin (NBF) for 24 h at room temperature for the histological evaluation, and tissues from remaining 6 fish were transferred to tubes filled with RNA later® (Ambion Inc., Austin, Texas, United States), and stored at -20 °C for gene expression analysis.

1.4 Histological evaluations

The standard histological procedure was conducted at the histology laboratory of the Research station, Nord University, Bodø, Norway. Fixed tissues were dehydrated with increasing concentrations of ethanol, followed by immersion in xylene and paraffin (Sørensen et al., 2011). Next, tissue sections of 4 µm were prepared using microtome and mounted onto a glass slide, after which they were stained with haematoxylin and Alcian blue - periodic acid–Schiff (pH 2.5). Stained slides (one per fish) were covered with a coverslip after adding a drop of glue, Pertex® (Histolab Products AB, Askim, Sweden). Microphotographs were captured at 40× magnification by a camera (Leica MC170HD, Heersbrugg, Switzerland) fitted on a light microscope (Leica DM1000, Wetzlar, Germany), and using a software, Leica Application Suite (LAS V4.12.INK, Heerbrugg, Switzerland). All the images were examined with ImageJ 1.52a (Schneider et al., 2012).

Dorsal skin: Tissues (approximately 2 cm) were sliced transversely into 3 equal parts after removing most of the muscles that were attached to the skin and decalcified with 10% formic acid (25 blocks per L) for 5 h. The tissues were rinsed with phosphate-buffered saline (PBS) prior to standard histological procedure. Approximately 600-900 µm (length) skin microphotographs (108 per diet) were generated to investigate the skin mucous cells.

Gills: To count the number of mucous cells in gills, 10 secondary lamellae from 5 different filaments per fish were chosen. Thus, in this study 600 secondary lamellae per diet group were examined to understand the effect of the diets.

Image analysis of dorsal skin and gills: To evaluate the mucous cells, 'Freehand selections' tool of ImageJ was selected to demarcate the total area of skin epithelium (SE) and then 'Brightness and Hue' under 'Colour threshold' of the 'Image' menu was adjusted, while keeping 'Thresholding method' as 'Default', 'Threshold colour' set to red and 'Colour space' to HSB (hue, saturation and brightness). Next step was to select the 'Analyze' menu to measure the SE (Gong et al., 2020). The 'Wand tool' was used to select individual mucous cells. The background was cleared using 'Edit' and then the image was converted to 8 bit to retain only the mucous cells. The total area of skin mucous cells (SM) and number of skin mucous cells (SN) were determined by selecting 'Threshold' under 'Image' menu, and by setting 'Analyze particles' to '30 to infinity' under the 'Analyze' menu in ImageJ. The SE, SM and SN were used to calculate 2 parameters: SME (ratio between SM and SE) and SNE (SN per SE). The same image analysis procedure that is described for skin, was employed for gills to examine the total area of gill epithelium (GE), the total area of gill mucous cells (GM) and number of gill mucous cells (GN). The obtained values were used to calculate 2 parameters: GME (ratio between GM and GE) and GNE (GN per GE).

Distal intestine: The gut contents were first rinsed off with 10% NBF prior to fixation. The excess tissues were trimmed before processing and embedded longitudinally. For the morphometric analysis, 10 simple, long, well-oriented and intact villi per fish were selected from 3 - 5 different locations. Approximately, 120 microphotographs per diet were generated.

Morphometric analysis of distal intestine: Height (VH) and width (VW) of villi, height of enterocytes (EH), and width of the associated lamina propria (LP) were measured to understand the diet-induced aberrations in intestinal structure. Width of the villus varies along its height, therefore, to measure VW, each villus was partitioned into 6 equal parts from the base to tip (Supplementary figure 3). From these 5 points, VW, EH and LP were gauged employing the analysing tools ('straight' and 'segmented lines') of the ImageJ, and the average of the 5 values was registered.

Semi-quantitative assessment was adopted to study the morphological changes of parameters; number of mucous cells (NM), number of intraepithelial lymphocytes (IEL), and supra nuclear vacuoles (SNV) of intestinal villi. Scoring strategy was developed (Table 3) based on Baeverfjord and Krogdahl, (1996), Bakke-McKellep et al., (2007), Knudsen et al., (2008), Uran et al., 2008) and Silva et al., (2015), wherein the table provides the Semi-quantitative scoring system adapted to study the number of mucous cells (NM), number of intraepithelial lymphocytes (IEL) and presence of supranuclear vacuoles in enterocytes (SNV) per villus.

Table 3:

Parameter / Score		Description
NM	1	Above 31 NM per simple villi, densely distributed small NM throughout the complex villi
	2	26 to 31 NM per simple villi, more of small and large NM throughout the complex villi
	3	21 to 26 NM per simple villi, more small and few large NM throughout the complex villi
	4	16 to 21 NM per simple villi, more large and few small NM throughout the complex villi
	5	Below 16 NM per simple villi, large NM evenly distributed throughout the complex villi
IEL	1	Above 15 IEL per simple villi, densely distributed in the complex villi
	2	13 to 15 IEL per simple villi
	3	11 to 13 IEL per simple villi
	4	9 to 11 IEL per simple villi
	5	Below 9 IEL per simple villi, evenly distributed in the complex villi
SNV	1	Completely absent or no vacuoles seem to be present
	2	Scattered, tiny few vacuoles appear at least in some part of enterocytes
	3	Obviously reduced, few small vacuoles still present in many

		enterocytes
	4	Mildly reduced, more medium sized vacuoles present almost half of the enterocytes
	5	Highly vacuolated, large vacuoles appear almost on entire apical part of enterocytes

1.5 Gene expression analysis

Genes: For this experiment, relative mRNA levels of mucin genes (muc2, muc5ac1, muc5ac2, and muc5b) in the skin, gills and distal intestine, and AMP genes (defensin 1 - def1, defensin 2 - def2, defensin 3 - def3, defensin 4 - def4, and cathelicidin 1 - cathl1) in the skin and distal intestine were studied. Details about the gene expression analysis can be found in Sørensen et al., 2021 (article "Nutrient Digestibility, Growth, Mucosal Barrier Status, and Activity of Leucocytes from Head Kidney of Atlantic Salmon Fed Marine- or Plant-Derived Protein and Lipid Sources", DOI: 10.3389/fimmu.2020.623726)

1. 6 Results:

Growth performance: The growth performance parameters for the Atlantic salmon fed the different combination of feed ingredients with and without probiotics were analysed. The fish weight increased from an average of 122.6 g to 194.4 g during the course of the experiment. There were no significant differences in growth among diet groups.

Histological evaluation of mucous cells in dorsal skin: The results showed that fish fed the probiotics (LP+LF) had significantly higher number of skin mucous cells per skin epithelium (SNE), meaning that the feed comprising LAB can be used in improving the barrier status by increasing the number of mucous cells, as shown in **Figure 1**. The number of mucous cells signifies the health status of the mucosal tissues and enhance skin barrier functions.

Histological evaluation of mucous cells in gills showed that the ratio between total area of gill mucous cells and total area of gill epithelium (GME) as well as the ratio between number of gill mucous cells and total area of gill epithelium (SNE) differed significantly between diets without and with LP+LF, GME presented in **Figure 2** and GNE in **Figure 3**. The increase in number of mucus cells increase the protection of the mucosal surfaces and correlation between number of mucus cells and mucus

secretion has been reported in scientific literature. Mucus secretion is an innate immune response to pathogens. The parameter GME was used to assess the total area of gills mucous cells that cover the unit area of gills epithelium. For fish fed marine based feed without probiotics (BG1÷), it was 0.024, indicating that 100 μm^2 of gills epithelium is covered by 2.4 μm^2 of mucous cells. For the plant based (BG5÷) and soybean meal (BG2÷) based groups, GME were 0.039 and 0.049, respectively. Incorporation of probiotics to all the three feeds; marine, plant and soybean meal based diets significantly increased the GME by 1.7, 1.4 and 1.5 times than their control groups without LAB, respectively. Histo-morphometric analysis of fish gills showed a similar trend for GNE and GME. There was positive correlation between GME and GNE, indicating that the GME might have increased due to increased GNE. Fish fed feeds without probiotics groups (÷) had lower value for SNE compared to feeds groups with probiotics (+). Fish fed the marine based feed (BG1÷) had on average 300 mucous cells per 1 mm^2. For fish, fed the plant (BG5÷) and soybean meal (BG2÷) based feed, the GNE were 2 and 3 times higher than marine based feed (BG1÷) fed groups, respectively. Fish fed the BG1+, BG5+ and BG2+ had increased number of gills mucous cells per unit area of gills epithelium (SNE) by 2.3, 1.4 and 1.5 times, respectively, compared to their feed groups without probiotics. Consequently, fish fed probiotics had improved barrier function in the gills.

Histological evaluation of distal intestine: Gut health and barrier statues of the distal intestine was analysed by the morphological parameters of distal intestine, the hight of villi (VH), Width of villi (VW), hight of enterocyte (EH), lamina propria (LP), Number of distal mucous cells (NM), number of intraepithelial lymphocytes (IEL) and supra nuclear vacuoles (SNV). The marine based feed group had an overall better status of all these parameters, except for LP, indicating a better gut health compared to the fish fed diets with plant protein ingredients. These observations are supported by findings reported by Sørensen et al (2021). Supplementation of LAB to all the diets reduced the thickness of the lamina propria, as shown in **Figure 4**, providing the average width of lamina propria (LPW) of Atlantic salmon fed different diets without or with LAB. A widening of lamina propria is associated with infiltration of lymphocytes as a response to an immune activation. A thinner lamina propria is therefore indicating no or less inflammation. The width of lamina propria was significantly affected by supplementation of probiotic. The LPW was significantly

reduced in fish fed BG1+ and BG2+ while no changes were observed for fish fed the BG5+. A thin lamina propria indicate less inflammatory cells.

Figure 5 shows the average score for number of mucous cells (NM) per villus, reference is made to Table 3. The NM per villi in distal intestine of fish fed BG1+ and BG5+ were significantly increased compared the fish fed these diets without probiotics. More mucus cells per villie indicate immune stimulation. Hence, an increased number of mucus cells per villus was observed with supplementation of LAB to the feed indicating a stimulatory effect and improved barrier status.

The average score for supranuclear vacuoles (SNV) is provided in **Figure 6**, in this the scores means:

1 Completely absent or no vacuoles.

2 Scattered, tiny few vacuoles present at least in some part of enterocytes.

3 Obviously reduced, few small vacuoles present in many enterocytes.

4 Mildly reduced, more medium-sized vacuoles present almost half of the enterocytes.

5 Highly vacuolated, large vacuoles present almost along the entire apical part of enterocytes.

Fish fed the soybean meal without probiotic (BG2÷) had almost no SNV, but they reappeared slightly when the fish was fed probiotics. The supranuclear vacuoles were large and well distributed in fish fed marine diets but was absent in fish fed soybean meal. The BG2÷ fed fish had the lowest score (almost no SNV on the enterocytes) and showed a significant increase in SNV when fish were fed probiotics. Absence of SNV's is a pathological condition.

Gene expression: Relative expression of mucin genes were tested in skin, gills and distal intestine of Atlantic salmon and the expression were found to be tissue specific. The skin expressed muc5ac1, muc5ac2 and muc5b. The gills expressed muc5ac2 and muc5b. The distal intestine expressed only muc2. Relative expression of AMP genes (def1, def2, def3, def4 and cathl1) in skin and distal intestine of Atlantic salmon were also tissue specific. The skin expressed def1 and cathl1. The distal intestine expressed def3, def4 and cathl1.

Gene expression - Dorsal skin: The expression of mucin and AMP genes in the dorsal skin were significantly affected by LAB. Expression of muc5ac1 and muc5b both showed the same pattern; a tendency towards decreased expression in fish fed marine based feed and increased expression in fish fed feeds with plant ingredients. **Figures 7** and **8** show the relative mRNA levels of mucin gene, muc5ac1 and muc5b, respectively, in the skin of Atlantic salmon fed different diets. The gene expression of these two genes were increased in fish fed BG2+ and BG5+ and is consistent with the increased number of mucus cells observed in the skin.

Gene expression - Distal intestine: Reference is made to **Figures 9** and **10** wherein **Figure 9** provides the relative mRNA levels of antimicrobial peptide gene, defensin3 in the intestine of Atlantic salmon fed different diets. Expression of the defensine 3 gene was significantly increased in fish fed BG1+ and tended to increase in fish fed BG5+ while no differences were noted in the fish fed BG2 without or with probiotics. Increased expression of AMP is associated with the improved host defense capacity. The AMP bind and destabilize the bacterial cell resulting in cell death. **Figure 10** shows the relative mRNA levels of antimicrobial peptide gene, cathelcidin1 in the intestine of Atlantic salmon fed different diets. Fish fed diets supplemented with probiotics significantly increased the expression of Cathelcidin1 in fish fed BG2 and BG5 and tended to increase the expression of this gene in fish fed BG1. Increased expression of AMP is associated with the improved host defense capacity, killing bacterial pathogens. Hence, expression of mucin gene, muc2 was affected only by the feed ingredient composition. Fish fed BG2 had significantly less mucin mRNA levels compared to other two feed groups. Probiotics did not influence the mucin expression in any of the feed group. Feed ingredient composition affected the expression of all AMP genes. Compared to other feed groups, fish fed BG2 had lower levels of mRNA for def3 and def4. However, fish fed BG1 had lower levels of mRNA for cathl1. Supplementation of probiotics to the diet groups revealed significant influences for AMP genes, especially cathl1. All probiotics incorporated diet groups had significantly increased expression of cathl1 compared to the control groups. A significant interaction between feed and probiotics was observed for def3. The relative mRNA level of def3 was up-regulated in fish fed BG1 LP+LF and BG5 LP+LF compared to their controls, while no such changes were observed for BG2. The mRNA level of def4 was down-regulated in fish fed feed BG1, while fish fed BG5

and BG2 were up-regulated. The interaction between feed and probiotics were not statistically significant.

Example 2: Survival and shelf life of probiotic strains in fish feed

The aim of the present study was to develop protocols for supplementation of probiotics to fish feeds, and to examine their viability in an 8-month shelf life study at different feed storage conditions. Two strains of Lactobacilli, *Lactobacillus plantarum* R2 Biocenol™ (CCM 8674) and *Lactobacillus fermentum* R3 Biocenol™ (CCM 8675), with a potential to be used as probiotics in aquaculture, were incorporated in experimental feeds. Overall, 11 groups of probiotic pellets were prepared.

Overall findings were that *L. plantarum* had better viability in both storage conditions compared to *L. fermentum* that could not withstand room temperature for more than 3 months. The number of bacterial cells of *L. plantarum* dropped from 9.04 ± 0.14 $\log_{10}$CFU.g^{-1} to 8.47 ± 0.12 $\log_{10}$CFU.g^{-1} during the 8-month storage period at 4 °C. Storage in refrigerator helped probiotic bacterial cells to survive in the fish feed. Thus, temperature is considered as a critical factor that influences probiotic viability and survival during the storage period. Further, the feeds comprising the probiotics in oil, rather than in saline, had better survival.

2.1 Material and methods

Lecithin (Denofa AS, Norway); Saline 9 g/l NaCl; Fish oil (Polarfeed Sales AS, Norway); Plant oil (Sunflower oil, Eldorado, Norway);

Probiotic strains: *Lactobacillus plantarum* R2 Biocenol™ (CCM 8674) and *Lactobacillus fermentum* R3 Biocenol™ (CCM 8675)

Pellets (A) basal feed; uncoated fish feed pellets (12 mm)

Pellets (B) basal feed; coated fish feed (3 mm), commercial feed; Spirit Supreme, Skretting AS.

Coated pellets:

A1. 1st layer: *L. plantarum* probiotic cells resuspended in saline, 2nd layer: fish oil

A2. 1st layer: *L. plantarum* probiotic cells resuspended in saline, 2nd layer: plant oil

A3. Coating layer: *L. plantarum* probiotic cells resuspended in fish oil

A4. Coating layer: *L. plantarum* probiotic cells resuspended in plant oil
A5. Coating layer: *L. plantarum* probiotic cells resuspended in fish oil + 3% lecithin
A6. Coating layer: *L. plantarum* probiotic cells resuspended in fish oil + 1.5% lecithin
A7. Coating layer: *L. plantarum* probiotic cells resuspended in plant oil + 3% lecithin
A8. Coating layer: *L. plantarum* probiotic cells resuspended in plant oil + 1.5% lecithin
B1. Coating layer: *L. plantarum* probiotic cells resuspended in saline
B2. Coating layer: *L. plantarum* + L. fermentum probiotic cells resuspended in saline
B3. Coating layer: *L. fermentum* probiotic cells resuspended in saline

2.2 Cultivation of probiotic bacteria for feed preparation

Liquid nutrient medium MRS broth (HiMedia, India) was used to cultivate probiotic strains, *L. plantarum* or *L. fermentum*. For each group of pellets, an 18-hour culture of probiotic baceria was prepared in 1 L of MRS broth at 37 °C on a shaker, which was then centrifuged (4500 rpm for 20 min) at 22 °C (ROTINA 420R, Germany). The resulting cell pellets were washed twice and resuspended in 30 mL of 0.9 % (w/v) sterile saline or in 400 mL fish / plant oil.

2.3 Preparation of probiotic feed by vacuum coating

The incorporation of probiotic bacteria, *L. plantarum* and *L. fermentum* into the fish feed was performed using a Forberg Rotating Vacuum Coater F-6-RVC, No 3041. In total, 11 groups of probiotic feed were prepared, named A1 – A8, B1 – B3, by vacuum coating. In the vacuum coating process, vacuum was applied at 0.7 bar of absolute pressure to remove air from the feed pellet pores, leaving them as open cells. Once the vacuum pressure was achieved, a mixture of probiotics and saline (in the case of pellets A1 – A2 and B1 – B3) or a mixture of oil with lecithin and probiotic bacterial cells (groups of pellets A5 – A8) was sprayed on from nozzles located at strategic locations to ensure proper dispersion onto the feed, while the paddles mixed the feed with the coating mixture to coat all pellets. Once the oil addition sequence was completed under vacuum, the vacuum pressure was released slowly back to atmospheric pressure of 1.015 mbar. This created a pressure differential which “pulled” the coating liquid deep into the pellet pores. For the pellets A1 and A2 multiple layers of coating were applied. The second layer of fish oil (FO) or plant oil (PO) was applied on the 1st layer of probiotic bacteria cells resuspended in saline.

Tables 4-6 below provide an overview of the components used for the different feeds A1-A4, A5-A8 and B1-B3, respectively.

Table 4 Amount of components in pellets A1 – A4

Probiotic strain/additive	Amount (g / log_{10}CFU.ml^{-1})			
	Pellets A1	Pellets A2	Pellets A3	Pellets A4
	1600	1600	1600	1600
L. plantarum + saline	30 / 10.96	30 / 10.87	-	-
Fish oil (FO)	400	-	-	-
Plant oil (PO)	-	400	-	-
L. plantarum + FO	-	-	400 / 10.15	-
L. plantarum + PO	-	-	-	400 / 9.90

Table 5 Amount of components in pellets A5 – A8

Probiotic strain/additive	Amount (g / CFU.ml-1)			
	Pellets A5	Pellets A6	Pellets A7	Pellets A8
	1600	1600	1600	1600
L. plantarum + FO	400 / 10	400 / 9.3	-	-
L. plantarum + PO		-	400 / 9	400 / 9.67
Lecithin	12	6	12	6

Table 6 Amount of components in pellets B1 – B3

Probiotic strain in saline	Amount (g / log_{10}CFU.ml-1)		
	Pellets B1	Pellets B2	Pellets B3
	1600	1600	1600
L. plantarum R2 + saline	30 / 10.32	-	-
L. fermentum R3 + saline	-	30 / 10.08	-
R2 + R3 + saline	-	-	30 / 10.28

2.4 Determination of probiotic viability in the feed during storage period

For all 11 groups of coated pellets the viability of probiotic bacterial cells was monitored during storage at refrigerator temperature (4 °C) or room temperature (22 °C). Sampling for the number of bacterial cells in the pellets was performed at 1-month intervals during 8 months of storage period. The number of viable cells was determined by culture method onto MRS agar plates based on the number of colonies i) before the incorporation of probiotics into the feed, ii) after coating and iii) during the storage period. From each group of pellets, 1 gram of sample was taken at specified intervals, mashed and suspended in 9 mL sterile saline and homogenized for 1 minute by vortex. Afterwards a volume of 0.1 mL of serial dilutions (10^{-1} – 10^{-8}) were spread on plates of de Man Rogosa and Sharp (MRS) agar (HiMedia, India) and incubated anaerobically (Oxoid Gas Pack Anaerobic system) at 37 °C for 48 hours to colony count.

2.5 Results

Figures 11 – 16 represent the survival rate of probiotic bacterial cells during the storage period of 1-month intervals during storage period of 8 months. The highest survival rates of the probiotic organisms were observed in L. plantarum, while the lowest values were recorded in L. fermentum throughout the period of storage. The best storage temperature was observed to be 4 °C (refrigerator temperature), at which the highest viability of the organisms was recorded in all feeds during the storage period. The best viability of probiotic cells was observed in the diet called A5 (coating layer: probiotic cells in fish oil with 3 % lecithin) that was stored at 4 °C during 8 months (Figure 13). For this, the initial number of alive cells was 9.044±0,137 $\log_{10}$CFU/g and the number of live probiotic cells after 8 months storage at 4°C decreased to 8.470±0.121 $\log_{10}$CFU/g. On the other hand, the viability of probiotics cells was significantly decreased in groups of pellets, which were prepared with saline (A1, A2) (Figure 11, 12).

Figure 11 shows the viability of LP bacterial cells in feeds A1-A4 after coating with either saline, fish oil or plant oil. During storage at 4 °C, the A1 pellet group (coating layers: 1st mixture of probiotics in saline, 2nd fish oil) was significantly different in the number of live probiotic bacterial cells than other groups of pellets A2 – A4 from

4th month of storage. The number of viable bacterial cells significantly dropped from 8.65 ± 0.49 to 7.52 ± 0.19 log_{10}CFU/g. This phenomenon of significant decreasing of live probiotic bacterial cells in the pellet group A1 was observed until the last day of sampling. In the A2 pellet group a significant decrease was also observed of number of live bacterial cells, which dropped to 7.73 ± 0.01 log_{10}CFU/g in the 8th month of storage at 4 °C. The best survival of *L. plantarum* cells was demonstrated in feed samples A3 and A4 (in which no saline was used for coating), which viability throughout storage was over 8 log_{10}CFU/g (Figure 11).

Figure 12 shows the viability of LP bacterial cells in feeds A1-A4 after coating with either saline, fish oil or plant oil. During storage of probiotic feed at room temperature, the best survival rate of probiotic bacterial cells was recorded in group of pellets A3 and A4, which were coated without saline, and even after 8 months of storage, more than 6 log_{10}CFU/g of viable bacterial cells were presented in the pellets. On the other hand, the A1 feed did not show any presence of lactic acid bacteria in sample after 5th month of storage period (Figure 12).

Figure 13 shows the viability of LP bacterial cells in feeds A5-A8 after coating with lecithin 1.5% or 3% in fish oil or plant oil, during storage at 4 °C. The best probiotic bacterial cell viability during storage at 4 °C was observed in the A5 feed samples. These pellets were coated with bacterial cells resuspended in fish oil with addition of 3 % lecithin. Even after 8 months of storage in all groups of pellets (A5-A8), the viability of bacterial cells was above 8 log_{10}CFU/g in these feed samples (Figure 13).

Figure 14 shows the viability of LP bacterial cells in feeds A5-A8 after coating with lecithin 1.5% or 3% in fish oil or plant oil, during storage at 22 °C. The highest viability of L. plantarum bacterial cells was observed in the group of pellets A7, which were coated mixture of plant oil with 3% lecithin and probiotic bacteria (Figure 14). In contrast, in A5 and A6 feed stored at 22 °C, probiotic cell survival was not highest because the presence of PUFA in fish oil caused oxidation in the feed, which affects the nutritional quality of individual feed components (Turchini, Ng, Tochler, 2010). We assume that in our case it negatively affected the survival of probiotic bacteria. The lowest number of viable probiotic bacterial cells, 6,79 ± 0.08 log_{10}CFU/g, was

observed in group of pellets A6 after 8 month of storage period. On the other hand, the highest viability of L. plantarum bacterial cells was observed in the group of pellets A7, which were coated mixture of plant oil with 3% lecithin and probiotic bacteria (Fig 14). The number of viable probiotic bacterial cells after 8 months of storage was 7,53 ± 0.08 log_{10}CFU/g.

The **Figures 15** and **16** show the viability of *Lactobacillus plantarum* and *Lactobacillus fermentum* bacterial cells in pellets B, i.e. wherein the probiotic cells were resuspended in saline. The feed group B1 showed high survival of *L. plantarum* bacterial cells even after coating with mixture of saline and bacteria. During 8 months of storage at 4 °C, the value of live bacterial cells decreased from 8.964 ± 0.043 log_{10}CFU/g to 7.901 ± 0.045 log_{10}CFU/g (Figure 15), but during storage at 22 °C the number of live probiotic bacterial cells decreased to 3.522 ± 0.065 log_{10}CFU/g. The bacterial cells of *L. fermentum* (B3) had much lower viability at both storage temperatures. No probiotic bacterial cells survived in the feed even after 4 months of storage at 22 °C. In the case of pellets coated with the mixture of strains (B2), the number of live bacterial cells decreased more significantly than in the feed group prepared by coating with probiotic bacterial cells of *L. plantarum* at both temperatures during storage (Figure 16).

From the study and the results it has been found that for the saline-supplemented pellet samples, the survival of probiotic bacterial cells decreased more significantly than in samples prepared without saline. Preferably, when preparing the feed pellets of the invention, the lactic acid bacteria is beneficially suspended in a suspension media comprising an oil, as this increases the survival and shelf life of the bacteria. For several of the feeds, lecithin was used as an emulsifier to homogenize the feed coating mixture of oil and probiotic bacterial cell pellets after centrifugation.The antioxidant effect of lecithin created suitable conditions for the survival of probiotic bacterial cells in feed stored at 4 °C, which explains the highest viability of lactic acid bacteria in A5 feed samples, which were coated with 3% lecithin in fish oil with probiotic microorganisms. The samples of A5 pellets showed the best survival of probiotics compared to other samples. During 8 months of storage at 4 °C, the number of bacteria decreased minimally, from 9.04 ± 0.14 log_{10}CFU/g to 8.47 ± 0.12

log_{10}CFU/g. Another factor that has positively affected the survival of probiotics is the presence of polyunsaturated fatty acids (PUFA) in fish oil, which increases the number of lactic acid bacteria survived in the feeds. In contrast, for the A5 feed stored at 22 °C, probiotic cell survival was not highest because the presence of PUFA in fish oil likely caused oxidation in the feed.

At a storage temperature of 4 °C, probiotic cells survived in higher numbers in all feed groups than during storage at 22 °C. In our study, probiotic bacterial cells in A5 – A 8 feed survived the longest storage time at 22 °C, their numbers did not fall below 8 log_{10}CFU/g during the four months of storage.

Claims:

1. Fish feed composition comprising at least one of the lactic acid bacteria *Lactobacillus fermentum* and *Lactobacillus plantarum.*

2. Fish feed composition as claimed in claim 1 comprising both *Lactobacillus fermentum* and *Lactobacillus plantarum as* living and active cultures of bacteria.

3. Fish feed composition as claimed in claim 1 or 2, wherein the feed is shaped into pellets or granulates comprising a coating with the lactic acid bacteria.

4. Fish feed composition as claimed in any of the claims 1 to 3, wherein the feed comprises pellets wherein the lactic acid bacteria are coated onto, adsorbed onto, and/or absorbed into pores of the pellets.

5. Fish feed composition as claimed in any of the claims 1 to 4, wherein further lactic acid bacteria are included in the feed.

6. Fish feed composition as claimed in any of the claims 1 to 5, wherein the feed is a granular feed comprising a coating comprising the lactic acid bacteria and wherein the coating further comprises an oil selected from the group of a plant oil and fish oil.

7. Fish feed composition as claimed in any of the claims 1 to 6, wherein the feed is a granular feed comprising a coating comprising the lactic acid bacteria and wherein the coating further comprises at least one stabilizer, such as lecithin.

8. Fish feed composition as claimed in any of the claims 1 to 7, wherein the feed comprises fats (lipids), proteins, and carbohydrates, and optionally also either of vitamins, amino acids and minerals.

9. Fish feed composition as claimed in any of the claims 1 to 8 comprising polyunsaturated fatty acids.

10. Method of producing a granular fish feed comprising at least one lactic acid bacteria, the method comprising a step of coating feed granulates with the at least one lactic acid bacteria, applying the bacteria from a bacterial suspension at an evacuated atmosphere, wherein the bacterial suspension comprises the at least one lactic acid bacteria and an oil and/or a stabilizer.

11. Method as claimed in claim 8 wherein the bacterial suspension used for the coating comprises cultures of at least one of *Lactobacillus fermentum* and *Lactobacillus plantarum.*

12. Method as claimed in claim 10 or 11 wherein the stabilizer is an emulsifier selected from the group of lecithins.

13. Fish feed for use in treatment of fish, for improving at least one of intestinal health and innate immune response, administering a fish feed composition comprising at least one of the lactic acid bacteria *Lactobacillus fermentum* and *Lactobacillus plantarum* to the fish.

14. Fish feed for use according to claim 11, wherein the feed is a granular feed comprising a coating which comprises the lactic acid bacteria.

15. Fish feed for use according to claim 13 or 14, wherein the use strengthens the fish's intestinal health by improving the barrier status on the body's surfaces on either of the gills and mucus layer on body and intestines.

16. Fish feed for use according to claims 13 to 14, wherein the use either of increases the number of mucous cells in epithelium, enhancing the skin barrier functions; prevents or reduces inflammation in the intestines; or includes an up-regulation of mucin genes.

Figures

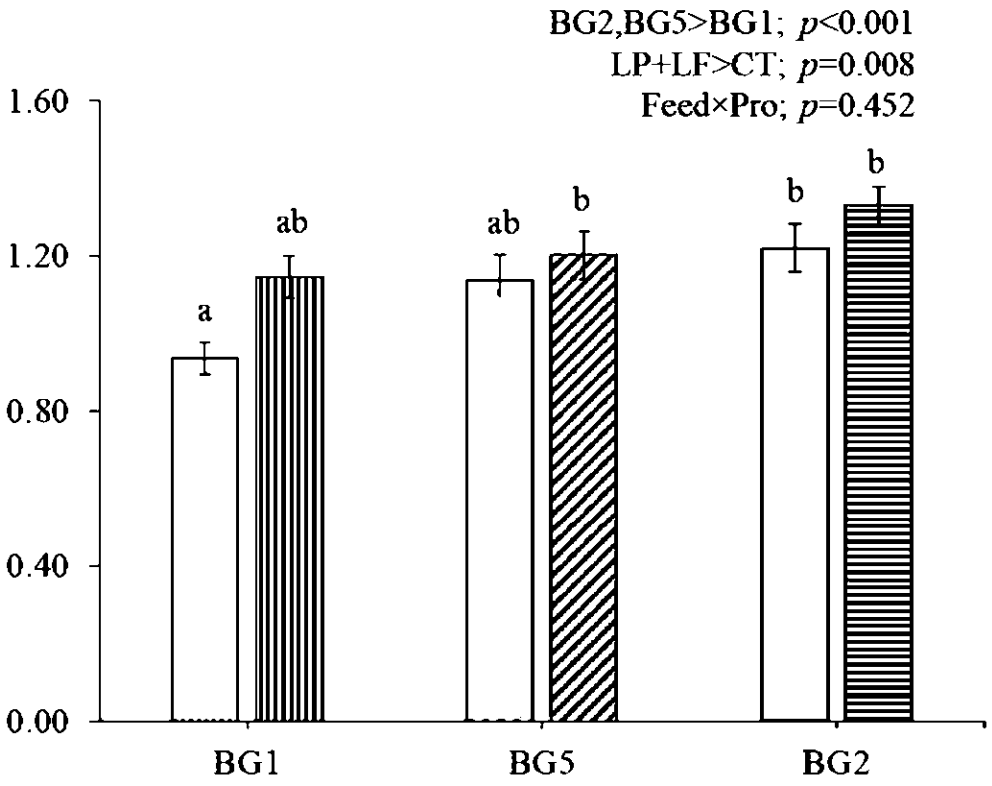
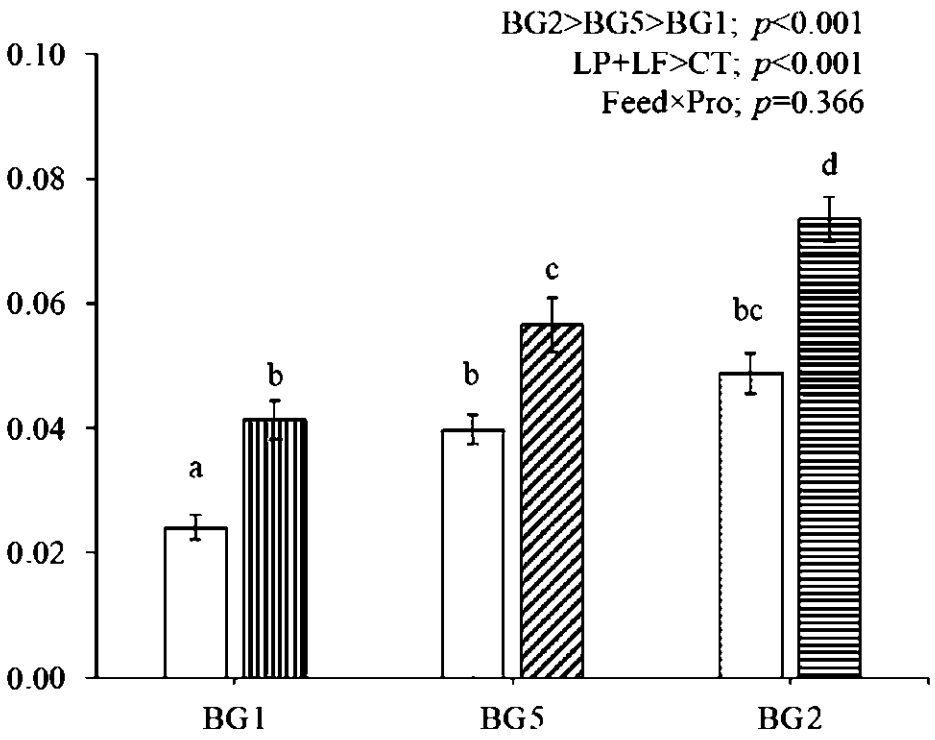


Figure 1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0.02
0.00
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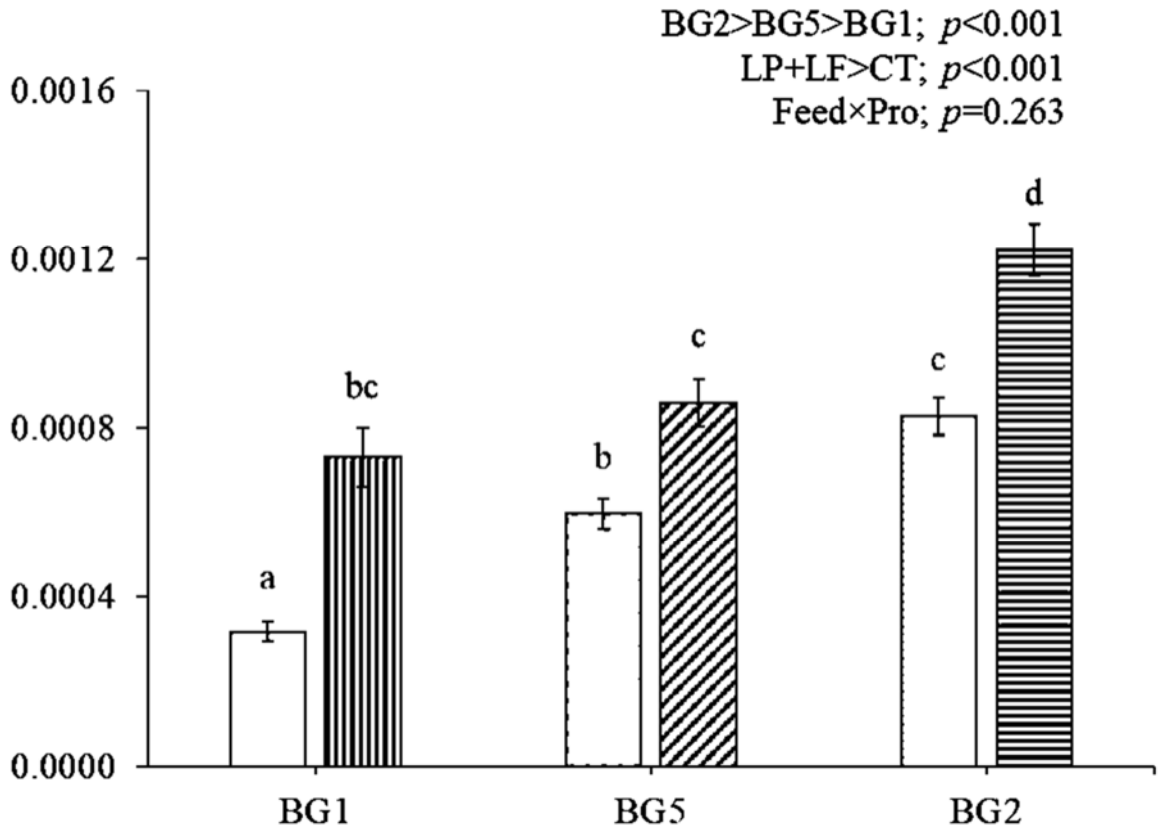
b
b
c
bc
d
BG1
BG5
BG2

Figure 2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0.0004
0.0000
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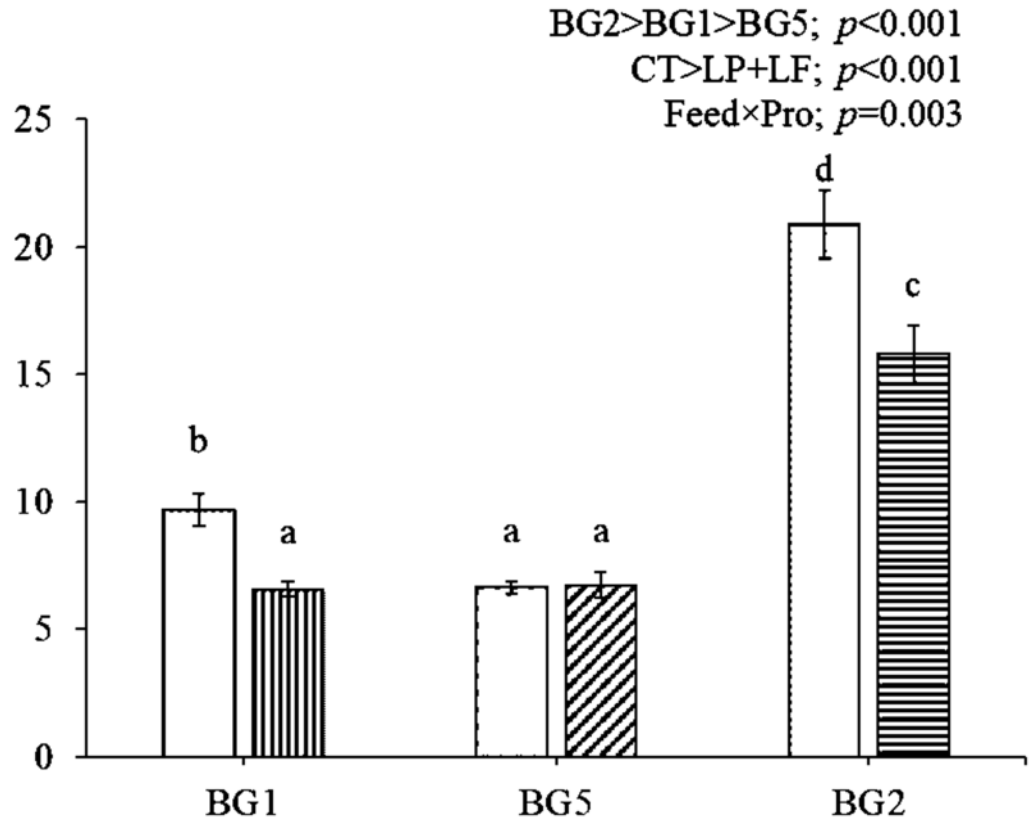
bc
b
c
c
d
BG1
BG5
BG2

Figure 3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15
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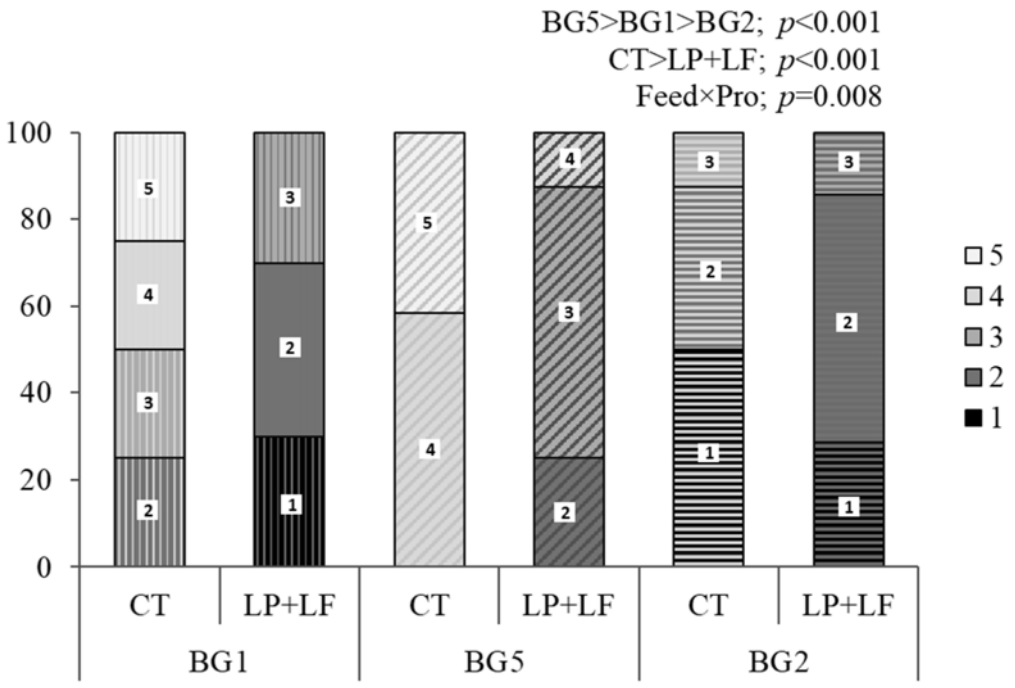
a
a
d
c
BG1
BG5
BG2

Figure 4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40
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CT
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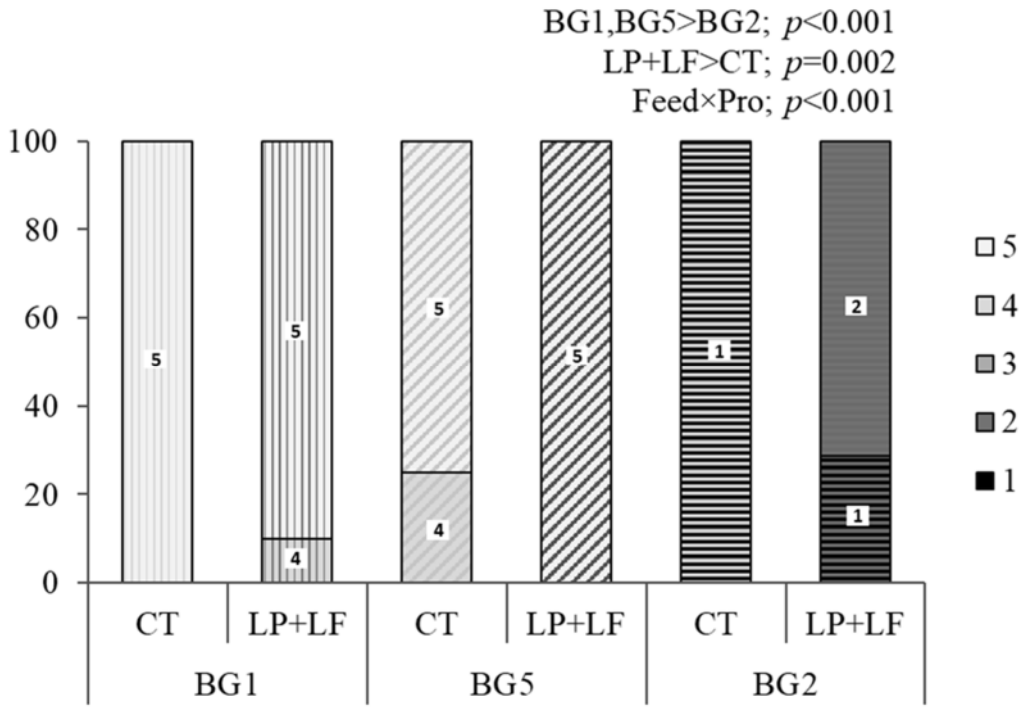
CT
LP+LF
CT
LP+LF
BG1
BG5
BG2
5
4
3
2
1

Figure 5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60
40
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4
3
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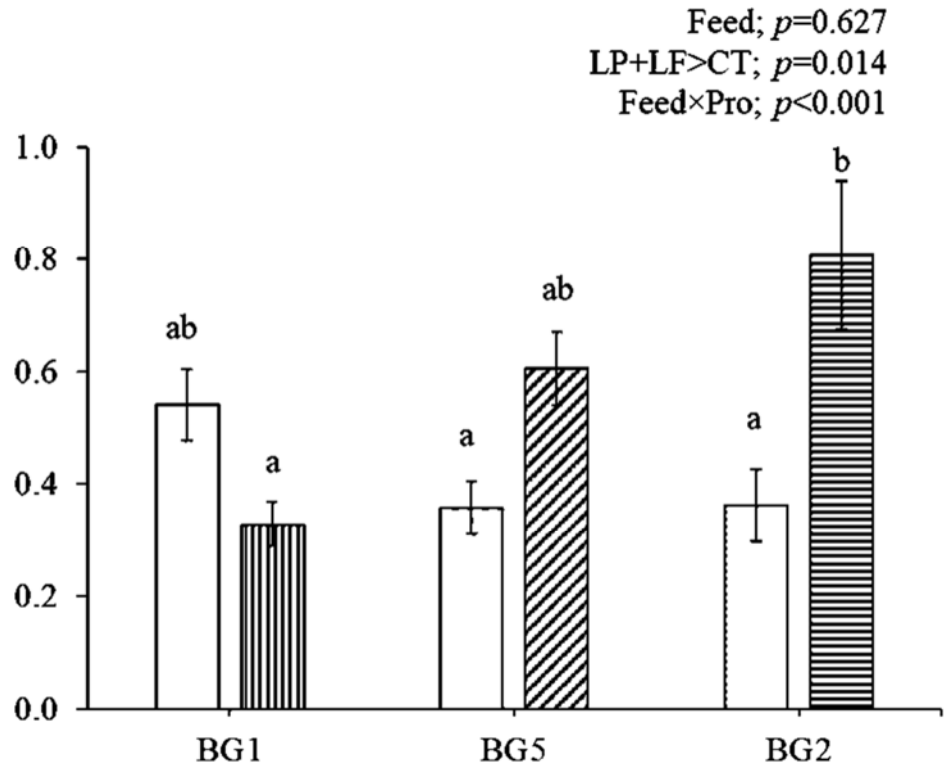
CT
LP+LF
CT
LP+LF
CT
LP+LF
BG1
BG5
BG2

Figure 6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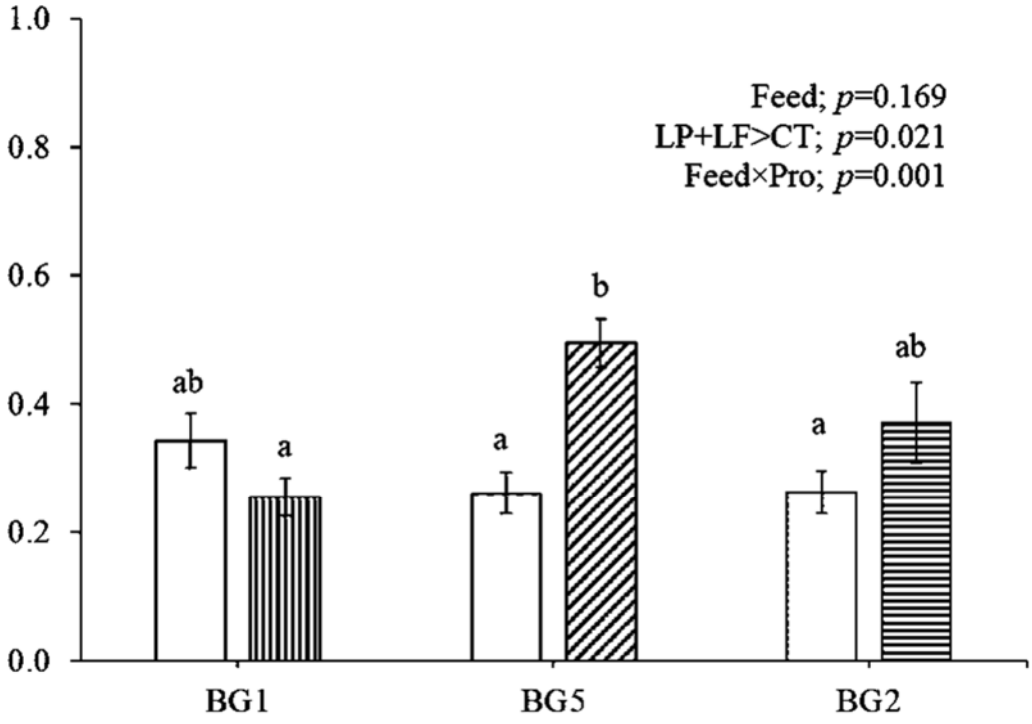
a
ab
a
b
BG1
BG5
BG2

Figure 7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0.4
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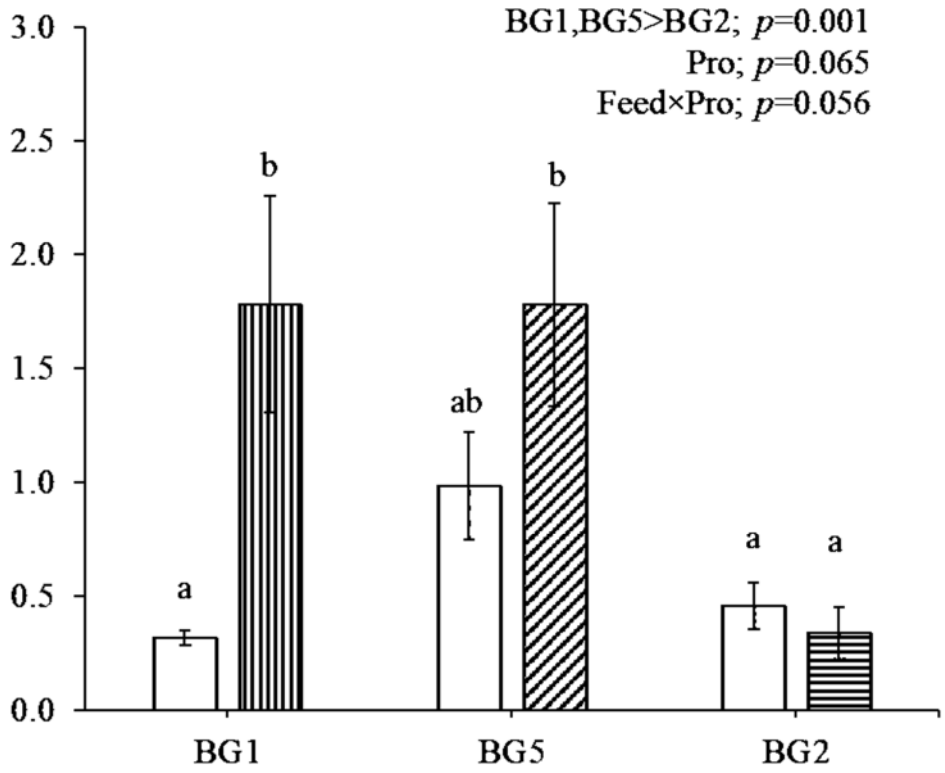
a
a
b
a
ab
BG1
BG5
BG2

Figure 8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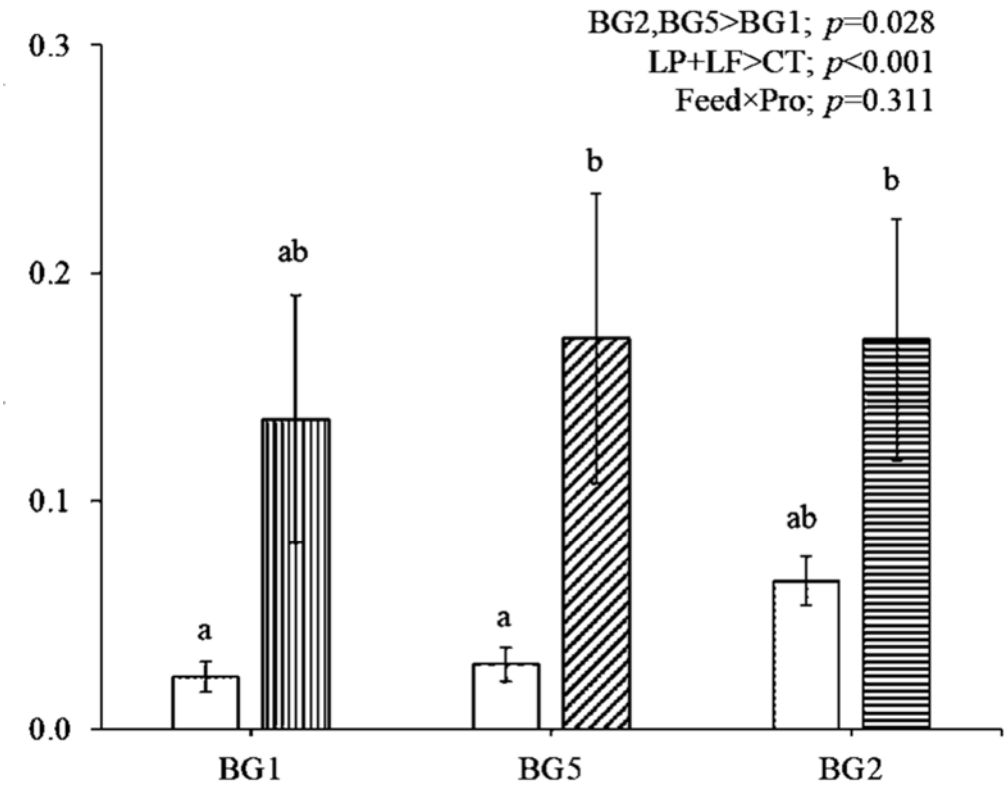
ab
b
a
a
BG1
BG5
BG2

Figure 9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ab
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b
ab
b
BG1
BG5
BG2

Figure 10

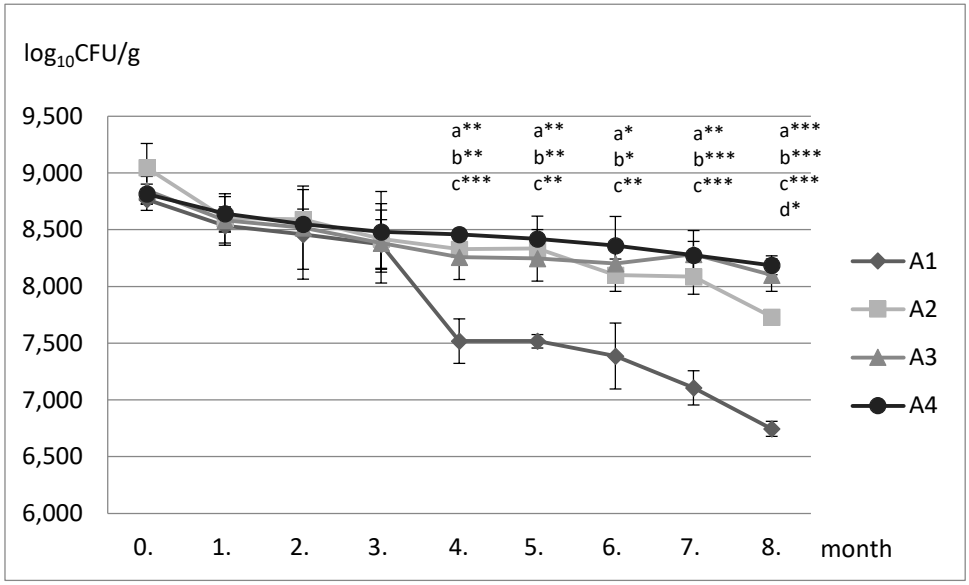

Figure 11

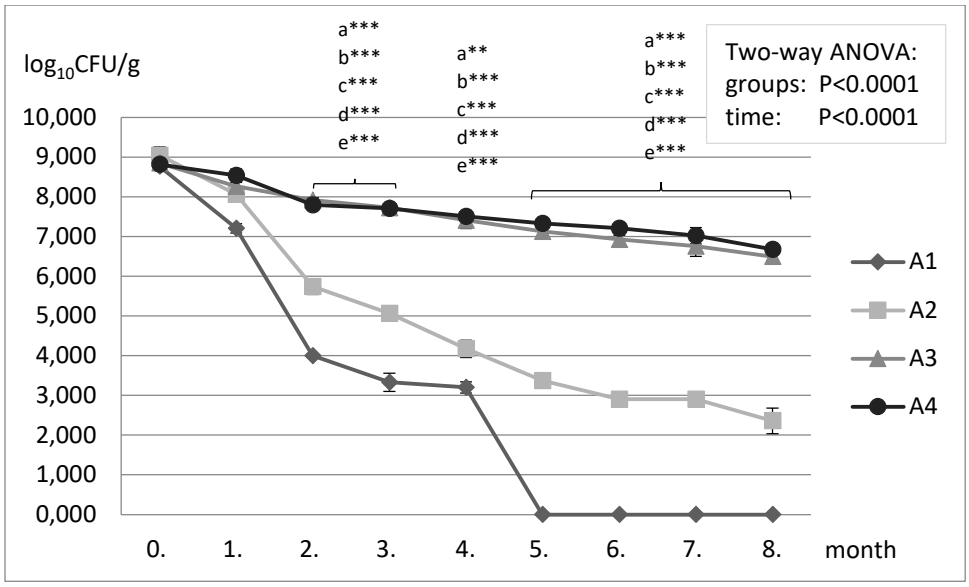

Figure 12

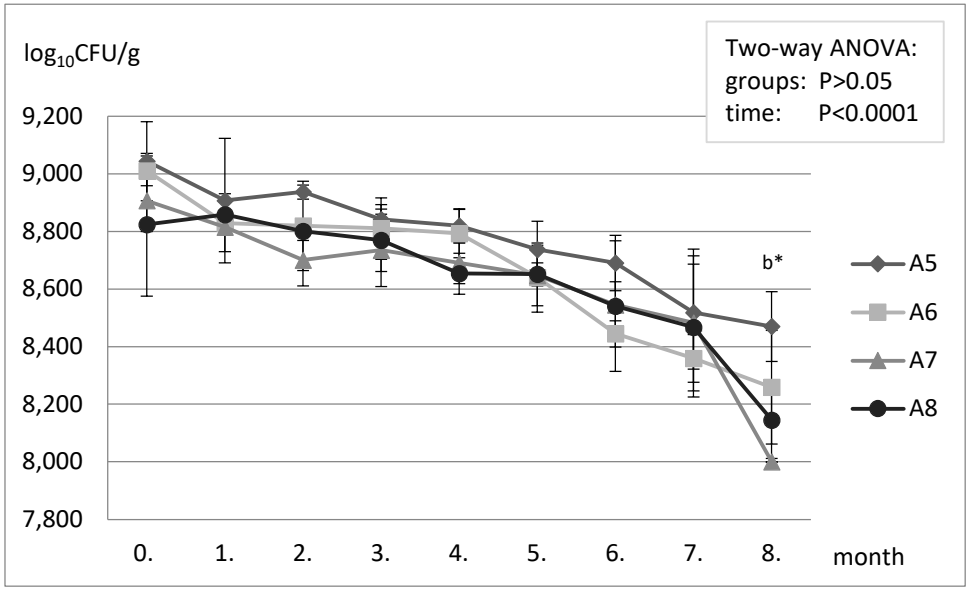

Figure 13

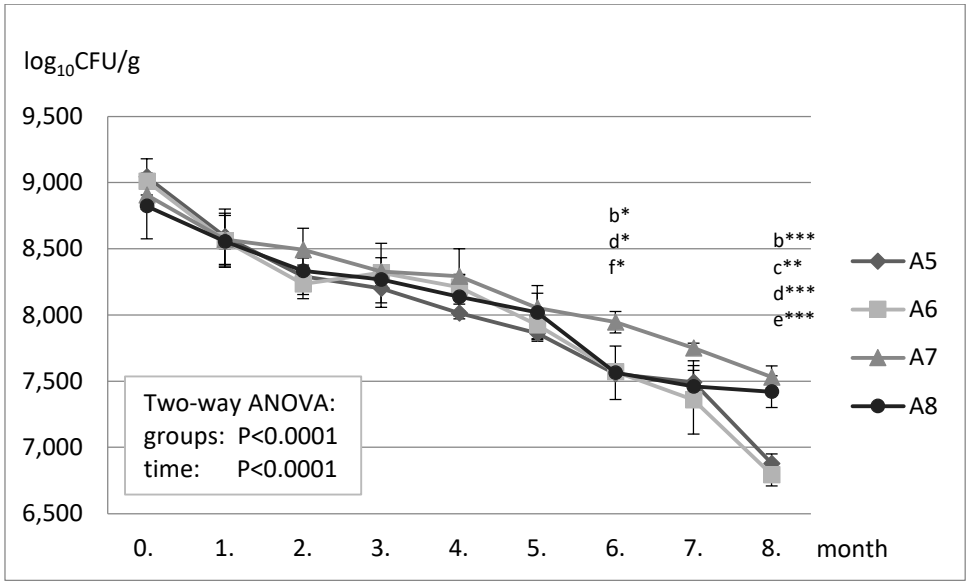

Figure 14

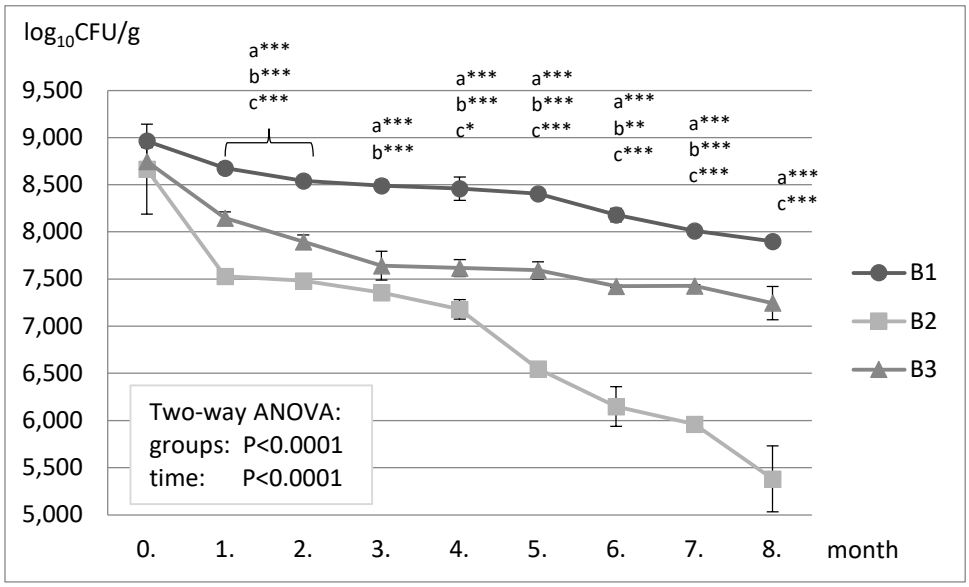

Figure 15

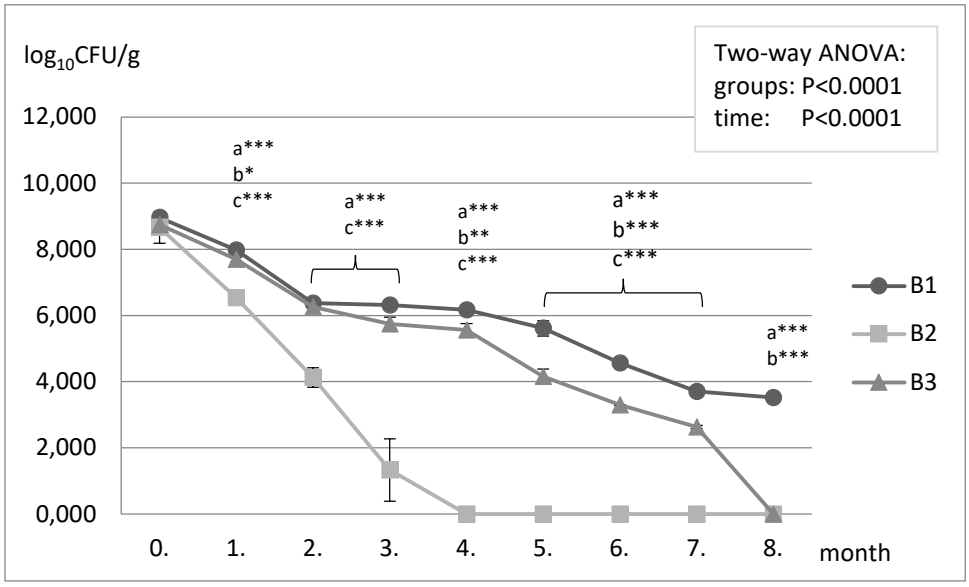

Figure 16