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Patent application no. 20210585
Nord Universitet

Reference is made to the official action dated 4 November 2023.

It is acknowledged that the amendments made in response to the first office action dated 3 June 2022 have overcome objections relating to the novelty of the invention. Herewith, the applicant provides detailed arguments in support of the patentability of the claims as filed 3 June 2022.

Inventive step:
The closest prior art, D1 (EP2659786), relates to probiotic feed for salmonids containing at least one lactic acid strain. It discloses different probiotic bacteria isolated from salmonids, amongst others, *Lactobacillus plantarum* isolated from Atlantic salmon. The feed production technology of D1 is very different from the method claimed and is not likely to be implemented in modern commercial scale feed production. The adherence properties to different mucus were performed with in vitro studies and disease preventive properties were tested in infection trials with *Aeromonas hydrophilia* and *Yersinia ruckeri*. D1 also describes production of probiotic feed with probiotic bacteria. However, the feed processing technology disclosed in D1 is different from the one the claimed invention. It is not likely that the method described in D1 will be commercially implemented.

The subject-matter of claim 1 differs from D1 in that it comprises a feed comprising *L. fermentum* (LF) and *L. plantarum* (LP).

The effect of this distinguishing feature is at least that the claimed composition, when fed to the fish, has positive effects on the mucosal barriers in the fish, such as on fish gut health.

An objective technical problem to be solved might thus be seen as providing an alternative fish feed starting from the probiotic feed of D1, which has positive effects on the mucosal barriers in the fish, such as on fish gut health.

There is no teaching, or even a hint, in D1, that a fish feed comprising both LP and LF should be provided.

The examiner considers the selection of lactic acid bacteria in the present invention merely one of several possibilities from which the skilled person would select based on the knowledge from D2-D6 and/or D8 in combination with D1. Further, the examiner states that there can only be an inventive step in the lactic acid bacteria selection if the selection presents unexpected effects or properties in relation to the lactic acid bacteria in D1 in combination with D2-D6 and/or D8.

As presented in the applicant's response to the first office action of 3 June 2022, the applicant does not share the view of the examiner that D2-D6 and/or D8 teach that LP and LF should be provided in a fish feed to solve the objective technical problem.

D2 relates to a novel strain *Lactobacillus fermentum* isolated from a human body. A skilled person seeking to solve the present objective technical problem is unlikely to seek literature of bacterial strains isolated from a human body and directly transfer such information for implementation into a fish feed.

D3 describes use of *Lactobacillus plantarum* isolated from colostrum of sows and feces from sows and piglets. The documented health benefit was to prevent diarrhoea in piglets during weaning. The effects were studied in vitro as well as in vivo. The pathogens creating illness in pigs are different from those in fish. This strain of plantarum has not been documented in fish and may not colonize the intestine of salmon – important to give the health benefit. It is not likely that the strains colonizing an omnivore homothermic gastrointestinal tract will colonize the intestine of a fish, such as a cold-water adapted carnivore fish.

D4 describes the use of a feed supplemented with the metabolites of a probiotic bacteria, *Lactobacillus fermentum*, UL4. D4 is limited to describing the metabolites of probiotic LAB and the supplementation of a product – an emulsion of probiotic bacteria in combination with furanone (that inhibits the colonization of microorganism on surfaces). The metabolites produced by *Lactobacillus plantarum* include bacteriocins, vitamin B and organic acids such as formic acid, acetic acid, and lactic acid. Hence, the technology is very different from the claimed invention.

D5 reports disease preventing effects when feeds were supplemented with the two LAB strains *Lactobacillus casei* and *Lactobacillus plantarum*. The two LAB strains were fed individually to different groups of rainbow trout.

D6 refers to a study of three lactic acid bacteria isolated from intestinal microbiota of rainbow trout. The LAB were *Lactococcus lactis*, *Lactobacillus plantarum*, *Lactobacillus fermentum*. An in vitro study was performed to study the adhesion of the fish bacteria Aeromonas hydrophilia, *Aeromonas salmonicida, Yersinia ruckeri* and *Vibrio anguillarum* to host intestinal mucus. D6 is only reporting data from in vitro studies, and there is no teaching that LP and LF should be provided in a fish feed.

D8 is studying the feeding of *Lactobacillus fermentum* and ferulic acid to common carp. The authors reported positive effects on haematological parameters as well as serum antioxidant enzymes.

In the present invention, the fish feed composition comprising LF and LP is contributing to a positive gut health and further a systemic positive effect on gill health and skin health. Thus, the overall effect of the feed of the invention relates to general welfare to the fish fed the feed. This is in contrast to D1, providing a fish feed providing a protective effect against bacterial infections. The different technical effects of the present invention and D1 is also reflected in the choice of parameters used to assess the effect of the fish feed.

In D1, the positive effect of the probiotic fish feed comprising LPS47 LP and LPS148 LL is assessed by immunological parameters such as haematocrit i.e. the proportion of red blood cells in the blood, and immune parameters. D1 demonstrates the protective effect against bacteria by using tests in which the lactic acid strains are challenged with a virulent strain of *Aeromonas salmonicida*.

For the present invention and examples of technical effect, reference is made to the feeding trial, see Example 1. This study aimed to document the effect of the probiotics (LP + LF), when applied to a variety of feed ingredient compositions (marine based, vegetable, or soybean based), on the performance and health of Atlantic salmon. 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.

Results from histological evaluation of mucous cells in dorsal skin 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 lactic acid bacteria (LAB) can be used in improving the barrier status by increasing the number of mucous cells, shown in Figure 1. The number of mucous cells signifies the health status of the mucosal tissues and enhance skin barrier functions.

The increased number of mucous 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.

The study of Example 1 also demonstrated that supplementation of LAB to all the diets reduced the average width of the lamina propria (LPW), as shown in Figure 4. 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. A thin lamina propria indicates less inflammatory cells.

The examiner states that the skilled person seeking to solve the objective technical problem knows that the fish epithelial surfaces are covered by mucus. The epidermal mucus contains innate immune components that provide the primary defence against different pathogenic microbes and act as a barrier between the fish and environment.

The applicant agrees that the skilled person knows that the mucosal surfaces of the fish including the epithelia and the mucosa-associated lymphoid tissues (MALTs) mainly including gut-, skin-, gill-, and nasopharynx-associated lymphoid tissue make up the mucosal immune system in teleost fish. The MALTs share common features as mentioned by the examiner, such as their coating of mucus on the epithelial surfaces and the presence of innate immune components. Another common feature of the MALTs is the presence of a complex and diverse microbiota, contributing to maintaining the physiology homeostasis of the fish host.

However, despite these mucosal surfaces sharing similar features, it is important to consider differences between fish species which will impact the effect of probiotic fish feed supplementation.

As D1 describes in [0035], one of the most important stages in the final selection of a probiotic is its capacity to colonise the epithelium of relevant organisms. The capacity to colonise the epithelium of relevant organisms is dependent on many factors. For example, variation in the digestive systems of the relevant organisms may influence how the probiotics colonise in the host, and impact nutrient absorption. Another important factor is the host's microbiota, whose complex composition varies between different fish species. Generally, this composition will influence the effect of the probiotic feed in a species-dependent manner. For example, the microbiota in the host's gastrointestinal tract can compete with the probiotic strains introduced by the fish feed for nutrients and attachment sites, thus affecting the colonisation and ability to elicit their effects. Similarly, interactions between the host microbiota and the bacteria supplied via feed may result in modulation of immune responses. Importantly, how this modulation occurs will depend on the inherent microbiota of the host.

Moreover, in the example study of the present invention, the LP and LF bacteria strains were isolated from the intestinal content of rainbow trout, a freshwater species. It is not trivial for the skilled person that both these lactic acid bacteria will be capable to colonise the gut of a saltwater fish species and have a positive contribution on the fish welfare.

In light of these species-dependent differences and their potential implications, the applicant argues that the skilled person reading either of the D1, D2-D6 and/or D8 would not be motivated to provide a combination of LP and LF in the same feed. It is unlikely to successfully predict the technical effect of the selected LAB combination based on information about the individual bacterial strains in other animals

or species, and therefore the solution of the invention is not obvious. Thus, the subject-matter of claim 1 involves an inventive step, and dependent claims 2-11 involves an inventive step by way of their dependencies.

Claim 10 relates to how to produce a fish feed comprising both LP and LF, wherein the method uses a step of coating feed granulates with the lactic acid bacteria. In the method disclosed in the present invention, the pellets or feed granulates are made through high temperature short term extrusion. The LABs LP and LF are in a suspension together with an oil and/or stabiliser, and the LAB suspension is coated onto the pellets or feed granulates under vacuum. As indicated in the description (page 13, line 26-28) it is beneficial to have the LABs suspended in a suspension media comprising an oil, as this increases the survival and shelf life of the bacteria. This method for producing a fish feed is industrially scalable. This is in contrast to D1, which applies a different method based on cold extrusion followed by drying of the pellet, with no scalability for industry manufacturing. No prior art has been identified disclosing the claimed method, accordingly the subject-matter of claim 10 involves an inventive step, and dependent claim 11 involves an inventive step by way of its dependency.

Claim 12 relates to a fish feed for use in a method of treatment of fish, for improving at least one of intestinal health and innate immune response. Reference is made to the argumentation presented above for the inventive step of independent claim 1 and dependent claims 2-9, as the applicant view this reasoning relevant also for claim 12 and dependent claims 13-14. Thus, the subject-matter of claim 12 involves an inventive step, and it follows that dependent claims 13-14 also involve an inventive step.

Certain defects
Claim 1 provides a composition comprising two LABs as living and active cultures of bacteria. As disclosed in the description (page 19, line 31-34 and page 20, line 1-12) the active cultures are incorporated e.g coated or loaded onto the feed granulate or pellet under vacuum. When the vacuum is released to ambient pressure, the bacteria suspension will be absorbed onto and into the granulate or pellet. More details on the preparation of the bacterial suspension is provided in the description (page 20, line 24-32 and page 21, line 1-12).
Claim 2 is novel and inventive as explained above.
Claims 3 and 4 relate to how to add the LAB to the feed pellet. Reference is made to Example 2, section "Coated pellets" on page 39-40, providing alternatives for how LF and LP can be comprised in the feed.

The applicant believes that the objections raised in the official action dated 4 November 2023 have been addressed, and that patentability of the invention has been demonstrated, and thus requests favourable consideration.

Yours sincerely,
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