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Probiotic potential of *Lactobacillus* sp. strains capable of phytate breakdown isolated from dairy products for using in rainbow trout (*Oncorhynchus mykiss* Walbaum, 1792) diet

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Abstract

This research investigated the feasibility of using three strains *Lactobacillus* sp. viz. AM11, AM13, and AM14 previously isolated from dairy products to be used as probiotics in fish diet for phytase production. The bacteria grown in oxygenated media, bile salt, acid and supernatant of digestive tract were examined for their antibiotic resistance and non-pathogenicity for using in fish feed. Results indicated that the isolates might be attractive candidates as probiotic strains in rainbow trout. All strains were capable of growth and produced phytase in a pH range from 3.0 to 8.0. The isolated strains were also resistant in bile salts and supernatant of digestive tract. No mortality has been observed in the fish injected by bacterial strains. No bacterial growth has observed in the liver, kidney and spleen media after 24 h. Therefore, the examined *Lactobacillus* strains were non-pathogenic. Due to the low resistance of these strains to tetracycline (antibiotic), it is suggested to avoid using tetracycline in rainbow trout farms when the bacterial strains are used as probiotic. According to the results, the three isolated resistant strains can be employed as probiotics in fish feed.

Keywords: Probiotic, *Lactobacillus* sp., Phytase, Fish feed

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Introduction

A probiotic is outlined as a live microbial adjunct that exerts a helpful impact on the host (Ibrahem, 2015). Probiotic products increase the appetite, improve digestibility (Cruz *et al*., 2012), stimulate immune system (Bricknell and Dalmo, 2005), improve growth (Didinen *et al.,* 2016; Najmi *et al.,* 2018), and can be beneficial for fish health either indirectly by regulating gut microbiota, or by direct signals mainly targeting the digestive and immune functions of the host (Gomez-Gil *et al*., 2000). Probiotics also stimulate the immune system (Bricknell and Dalmo, 2005) through production of antibiotics, bacteriocin, siderophore, lysozyme, protease, and hydrogen peroxide. Other known benefits of probiotics are changing pH and organic acid sequences (formic acid, acetic acid, lactic acid) (Verschuere *et al*., 2000), increased digestive enzymes, and improved growth (Didinen *et al.*, 2016). Additionally, probiotic bacteria have been introduced into animal feed due to their contribution to the health of farmed animals and as biological control agents against diseases and stress conditions in aquaculture (Verschuere *et al*., 2000; Gatesoupe, 2008; Kesarcodi-Watson *et al*., 2008). A wide range of probiotic groups have been investigated for using in aquaculture, which can be categorized into living bacteria of both Gram-positive and Gram-negative, unicellular algae, bacteriophages and yeasts (Ibrahem, 2015).

Dairy products are sources of probiotic bacteria, including Lactobacilli (Gharaei Fathabad and Eslamifar, 2011). Milk and other dairy products are an acceptable and suitable medium for the growth of probiotic bacteria because of the availability of many essential nutrients (Narimani *et al*., 2015). These bacteria are the main components of starters that are used in the fermentation process, especially for dairy products (Pelinescu *et al.,* 2009). Lactic acid bacteria (LAB) are also used as probiotics in aquaculture because of their properties such as low or no virulence, producing antimicrobial substances against harmful bacteria, adherence capacity and colonizing in the digestive tract, and competing with pathogens for adhesion sites (Mota *et al*., 2006).

Probiotic bacteria can be an enzyme sources (Khodaii *et al.,* 2013; Kanpiengjai *et al*., 2013; Savita *et al*., 2017). Production of enzymes such as β-galactosidase, phytase and bile hydrolase vary among probiotic LAB (Saraniya and Jeevaratnam, 2015).

Phosphorus is one of the most important minerals for fish and acts as a major component of nucleic acid structure, cell membrane, and bone. Phosphorus in grains and plant proteins is the form of phytic acid or phytate that cannot be used in the digestive tract of monogastric animals due to the lack or inadequate amount of the enzymes degrading phytate (Liebert and Portz, 2005). Phytase is one of the several widely used enzymes in feed industry and the dietary supplementation of phytase can enhance the bioavailability of minerals and proteins for animals (Singh and Satyanarayana, 2007).

Digestibility and accessibility of phosphorus from dietary plant protein sources are important for fish nutrition. Research on probiotics in aquaculture, particularly in rainbow trout farming, has focused on LAB, most notably *Lactobacillus* spp., *Carnobacterium* spp., *Enterococcus faecium* and *Pediococcus* spp. for improving fish immune system (Nikoskelainen *et al*., 2001; Balcazar *et al.,* 2006; Balcazar *et al.,* 2007; Gatesoupe, 2008; Merrifield *et al*., 2010; Grześkowiak *et al*., 2011; Soltani *et al.,* 2019). Rainbow trout (*Oncorhynchus mykiss*) as a cold fresh water fish is a highly commercial sport and market fish.

Probiotic bacteria as fish feed supplement must have the ability to tolerate gastric acid, bile salts, endure the supernatant of digestive tract, resist antibiotics and to be non-pathogenic for fish (Schillinger *et al*., 2005). Accordingly, the present study has sought to find some bacteria with phytase activity from dairy products that can be used as probiotics to enhance the availability of phosphorus in fish feed. Initial screening was performed with respect to phytase activity and bacterial growth at different pH and temperature levels. Then, three isolates were selected based on farming conditions of rainbow trout (*O. mykiss*) followed by characterization of probiotic properties of *Lactobacillus* sp. strains AM11, AM13 and AM14.

Matrials and methods

Isolation and culture of Lactobacillus sp.

Thirty one *Lactobacillus* species have been isolated from sheep and cow's milk and yogurt samples using serial dilutions and cultured in specific media for Lactobacilli followed by testing for gram positivity and catalase activity. Each isolate was streaked on Man Rogosa and Sharp (MRS) agar media and incubated in an anaerobic system in the presence of a gas-generating kit (Oxoid) at 15 °C for 48 h. All isolates were stored at -20 °C in MRS broth supplemented with 20% (v/v) glycerol (Hartemink *et al*., 1997).

Phytase assay

Phytase activity was assayed by measuring the amount of liberated phosphate from sodium phytate. Phytase activity was determined by the modified method of Raghavendra and Halami (2009). A reaction mixture containing 400 µl of extracellular enzyme and 200 µl of 100 mM sodium acetate buffer and 200 µl sodium acetate buffer containing 2 mM sodium phytate as substrate was incubated at 15°C for 15 min (Selected strain with maximum phytase activity will be used as probiotic for cold-fresh water fishes such as rainbow trout, this is the reason why temperatures in 15 °C was studied). The reaction was stopped by adding 800 µl of 10% (w/v) trichloroacetic acid solution (Songré-Ouattara *et al*., 2008). The released inorganic phosphate was measured by adding 800 µl of color reagent, prepared daily by mixing 4 volumes of solution

A and 1 volume of solution B. The fresh Solution A was prepared daily by dissolving 2 g ammonium molybdate in 80 ml distilled water slowly add 5.5 ml sulfuric acid (98%) and diluted to 20ml with water. Solution B contains 2% ferrous sulfate (2 g in 100 ml distilled water). The mixture was transferred to an eppendorf tube and centrifuged at 10,000 rpm for 5 min at 4°C. After 15 min, the tubes containing the reactions were measurement of absorbance in the 660 nm range, and finally carried out using a spectrophotometer (BEL PHOTONICS- UV-M51 UV/VIS spectrophotometer- Italy) (Raghavendra and Halami, 2009). One unit of phytase activity was defined as the amount of enzyme that produces 1 µmol of inorganic phosphorous per 15 min. The results were compared to a standard curve prepared with inorganic phosphate (K2HPO4).

The isolates showing higher levels of phytase activity and growth were selected from the cultured bacteria. One unit of phytase activity was defined as the amount of enzyme producing 1 µmol of inorganic phosphorous per 15 min. The unit of enzyme was estimated in one ml of bacteria culture.

Oxygen tolerance of bacteria

In order to investigate the bacterial resistance to ambient oxygen, the isolates were cultured in aerobic condition as described previously (Kandler and Weiss, 1984).

Bacterial resistance to acidity

The isolates were cultured in MRS medium in a range of pH values. The pH levels of buffers were adjusted from 3 to 8 by using 1 M NaOH and HCl (Seme *et al.*, 2015).

Isolation of supernatant from fish gastrointestinal tract

In order to prepare supernatant from different gastrointestinal sections, rainbow trout samples were have been on starving for 48 h prior to the sampling to allow full discharge of their digestive system (Elkhalil *et al*., 2007). The fish were then anaesthetized with clove powder (300 mg L^{-1}) and immediately placed in the vicinity of the ice to die off (Merrifield *et al.*, 2010). The stomach, pyloric caeca and intestines were carefully separated and the contents well drained off, washed up with distilled water (Chang and Liu, 2002) to remove the remaining feed in the stomach and intestines. Subsequently, they were immediately stored at -20 °C. For supernatant preparation, samples thawed at ambient conditions, weighed, mixed with 1 to 5 mM sodium chloride (W/V), and then homogenized in the presence of ice. The supernatant was obtained by centrifuging at 5000 rpm for 30 min at 4 °C (model Hettich, Germany). Afterward, the bacterial suspension was mixed with 0.3% of supernatant and placed on a solid MRS medium in three replicates at 37 ° C for 48 h (Chang and Liu, 2002). The absorbance of growth measured at 600nm by spectrophotometer (BEL PHOTONICS-UV-M51 UV/VIS spectrophotometer - Italy).

Bacterial resistance to bile

The resistance of *Lactobacilli* isolates against bile was measured by Arihara's method (Arihara *et al.,* 1998). First, the LAB were cultured at 37 °C for 24 h. Fish's bile was collected by aseptically puncturing the gall bladders of rainbow trout, then bacterial suspension was mixed with 0.3% of bile and cultured in three replicates on a solid MRS medium at 37 °C for 48h. The bacterial colonies were observed after incubation.

Bacterial resistance to the antibiotic

Antibiotic susceptibility of bacteria was investigated using disk diffusion method in which tetracycline has chosen as one of the commonly used antibiotics. The antibiotic disc (bioDisc) was purchased from Padtan Teb Company (Iran). Tetracycline with a concentration of 30 µg was placed on plates containing lactobacilli and cultured on MRS agar. Plates were incubated at 37 °C for 24 h. Inhibition zones (mm) around the disc were measured after the incubation (Chang *et al.,* 2011).

Non-pathogenicity test for fish

The potential harmful effects of isolated bacteria were measured according to Brunt and Austin (2005). For this purposes, the bacteria have cultured, washed up and the bacterial suspension (100 µl) containing 3×10^8 CFU ml^{-1} was injected intraperitoneally to rainbow trout (each treatment with two replicates; three fish per replicate) and the fish were cultured in fiberglass basins (20 L). Control group received only physiological serum. Bacteriological examinations have performed after one month. In completely sterile conditions, samples from the liver, kidney and spleen were removed and transferred to a solid MRS medium and incubated at 37 °C for 24 h (Klesius *et al.,* 2006).

Statistical analysis

This experiment was conducted with a completely randomized design. Data were first normalized using Kolmogorov-Smirnov method, then phytase activity and growth rate of three bacterial strains in different pH levels were compared by two-way analysis of variance (ANOVA). The resistance of three isolates to tetracycline was compared by one-way ANOVA. Duncan's test was applied to compare the mean values of treatments. All the statistical analyses were carried out with SPSS 17.

Results

Selection of bacteria (based on phytase activity and growth)

Of 31 LAB isolated from sheep and cow's milk, three isolates, viz. *Lactobacillus* sp. strains of AM11, AM13 and AM14 have been selected that showed higher levels of phytase activity and growth rates. The phytase activities of the three strains were 0.901±0.10, 0.912±0.14 and 0.771±0.05 U ml^{-1}, with growth rates of 0.415±0.012, 0.404±0.037 and 0.9±0.10, respectively.

Oxygen tolerance of bacterial strains

All selected bacteria survived more than 72 h of exposure to atmospheric

oxygen (Table 1). The results showed that the studied lactobacilli grew both in aerobic and anaerobic conditions indicating that the examined strains were facultative anaerobes.

Table 1: Oxygen tolerance of lactobacilli strains.

Lactobacillus strain	Oxygen tolerance (h)
AM11	> 72
AM13	> 72
AM14	> 72

Bacterial resistance to acidity

The strains AM11, AM13 and AM14 showed tolerance to pH ranges of 3.0-8.0 during 24 h of incubation. No significant differences were found between strains at pH of 5.0 (p>0.05). The strain AM14 had the highest biomass among the three isolated strains in pH values of 6.0-8.0 (p<0.05). The pH levels of 3.0 and 4.0 resulted in the highest biomass of the strains AM11 and AM13 (p<0.05; Table 2). Two-way ANOVA revealed significant interactions between the pH and the bacterial strains (p=0.00) with significant biomass differences among groups exposed to different pH and strains (p=0.00 and 0.00, respectively).

Table 2: Lactobacilli strains' growth at different pH levels after 24 h.

pH	*Lactobacillus* sp. strain			*p*-value		
	AM11	**AM13**	**AM14**	**pH**	**Strains**	**pH ×strains**
3.0	0.439 ± 0.01^{Aab}	0.382 ± 0.01^{Bb}	0.378 ± 0.03^{Bc}	0.00	0.00	0.00
4.0	0.101 ± 0.005^{Ce}	0.152 ± 0.01^{Ae}	0.132 ± 0.001^{Bc}			
5.0	0.193 ± 0.001^{Ad}	0.218 ± 0.02^{Ad}	0.196 ± 0.01^{Ac}			
6.0	0.281 ± 0.001^{Bc}	0.302 ± 0.001^{Bc}	0.705 ± 0.07^{Ab}			
7.0	0.415 ± 0.012^{Bb}	0.404 ± 0.037^{Bab}	0.900 ± 0.10^{Aa}			
8.0	0.470 ± 0.06^{Ba}	0.447 ± 0.04^{Ba}	0.75 ± 0.06^{Ab}			

Values are mean±SD. Different lowercase and uppercase letters show significant differences in each column and raw, respectively (p<0.05).

The results of pH effects on phytase activity of lactobacilli strains are shown in Table 3. The activities of strains AM11, AM13 and AM14 varied from 0.340 to 0.923, 0.317 to 0.960, and 0.320 to 0.790 U ml^{-1}, respectively.

The lowest phytase activities of the strains AM11, AM13 and AM14 have been observed in pH values of 3.0-5.0, 3.0-6.0, and 4.0, respectively (p<0.05). The phytase activities of all strains were uppermost in neutral and weak alkaline pH (7.0 and 8.0). Except for a pH of 8.0, the phytase activity was not significantly different among all bacterial strains (p>0.05). Two-way ANOVA revealed no significant interactions between the pH and bacterial strains (p=0.11). The phytase activity was significantly different among groups exposed to different pH and strains (p=0.00 and 0.006, respectively).

Table 3: Lactobacilli strains' phytase activity (U ml^{-1}) at different pH values after 24 h.

pH	*Lactobacillus* sp. strain			*p* value		
	AM11	AM13	AM14	pH	Strain	pH × strains
3.0	0.450 ± 0.03^{Abc}	0.406 ± 0.07^{Ab}	0.406 ± 0.04^{Abc}	0.00	0.00	0.11
4.0	0.340 ± 0.02^{Ac}	0.317 ± 0.02^{Ab}	0.320 ± 0.05^{Ad}			
5.0	0.400 ± 0.04^{Abc}	0.387 ± 0.06^{Ab}	0.381 ± 0.01^{Ac}			
6.0	0.446 ± 0.05^{Ab}	0.463 ± 0.04^{Ab}	0.452 ± 0.03^{Ab}			
7.0	0.901 ± 0.10^{Aa}	0.912 ± 0.14^{Aa}	0.771 ± 0.05^{Aa}			
8.0	0.923 ± 0.05^{Aa}	0.960 ± 0.05^{Aa}	0.790 ± 0.05^{Ba}			

Values are mean±SD. Different lowercase and uppercase letters show significant differences in each column and raw, respectively (p<0.05).

Bacterial resistance to supernatant of digestive tract

Based on the results, the isolates could survive well in digestive tract supernatant condition after 48 hours of incubation (Fig. 1). The control indicates bacterial growth in a medium without digestive supernatant.

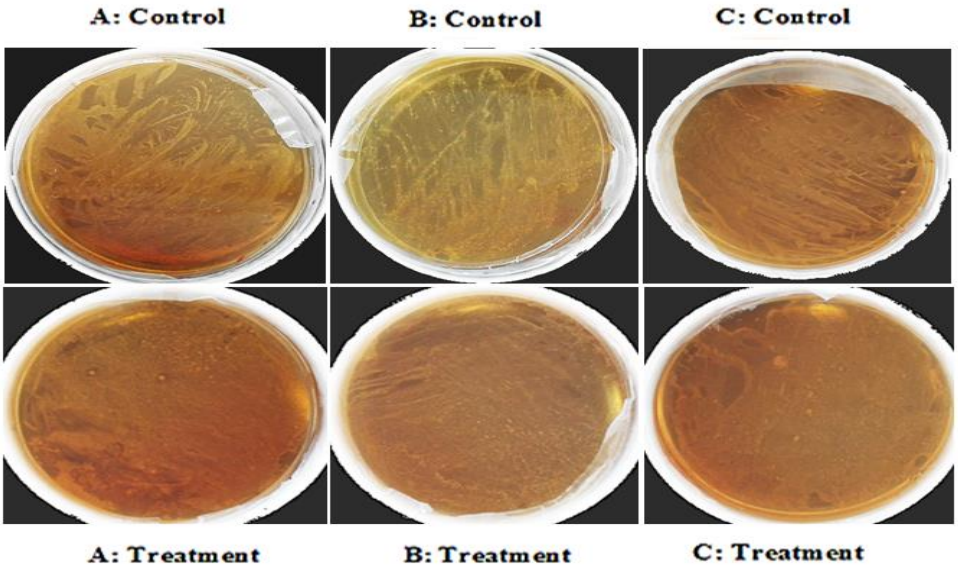

Figure 1: The growth rate of *Lactobacillus* sp. strains AM11(A), AM13 (B), and AM14 (C) in the medium containing digestive supernatant; The control indicates bacterial growth in a medium without digestive supernatant.

Bacterial resistance to bile

In this study, the bile exerted had not any effects on the growth of *Lactobacillus* sp. strains AM11, AM13, and AM14 indicating that these strains are able to tolerate bile in the fish intestines (Fig. 2).

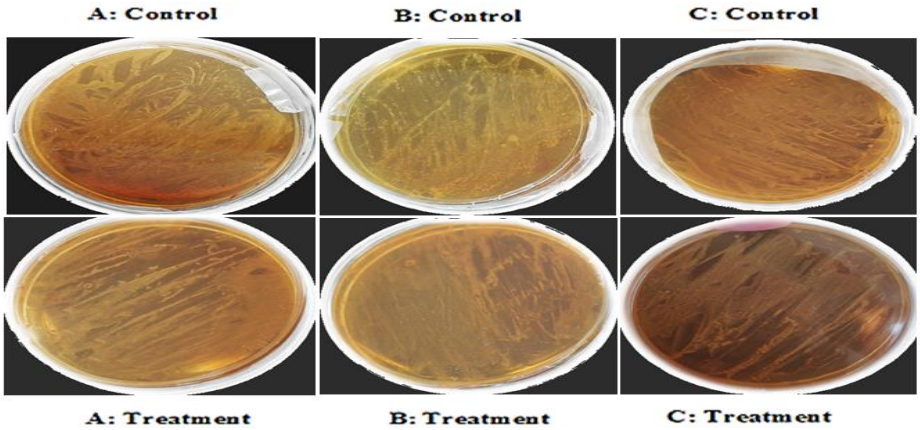

Figure 2: The growth rate of *Lactobacillus* sp. strains AM11 (A), AM13 (B), and AM14 (C) in the presence of bile. the control indicates bacterial growth in a bile free medium.

Antibiotic resistance

The result of tetracycline tolerance of the LAB strains are shown in Table 4. Accordingly, AM14 is more resistant to tetracycline compared to both AM11 and AM13 strains (p<0.05).

Table 4: Tetracycline resistance levels of LAB strains.

Antibiotic	**Inhibition zone (mm) of *Lactobacillus* sp. strains**		
	AM11	AM13	AM14
Tetracycline 30 µg	27±0.9[a]	25 0.5[b]	20±0.57[c]

Values are means±SD. Different superscript letters show significant differences (p<0.05).

Non-pathogenicity to fish

During one month, no mortality has been observed in the fish injected by bacterial strains and showed a good health status as the control group. Even symptoms of disease were not observed in the treatments. After one month of bacteriological examinations, samples from the liver, kidney and spleen did not show any bacterial growth after 24 h. Therefore, these *Lactobacillus* strains were not pathogenic to our fish samples (Fig. 3).

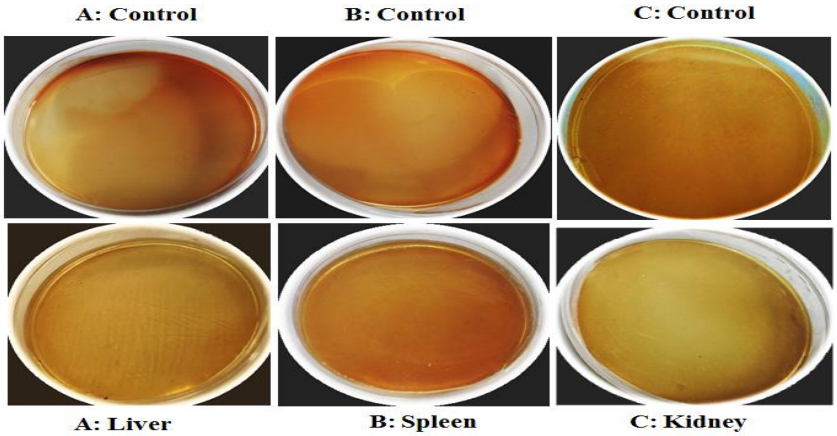

Figure 3: The bacterial culture after injection (3×10^8 CFU ml^{-1}) to fish. The control indicates bacteria culture not injected to fish. A) Liver; B) Spleen; C) Kidney.

Discussion

The present study was conducted to find probiotic bacteria capable of phytate breakdown from dairy products to be used in cold – fresh water fish systems, in particular rainbow trout (*O. mykiss*). In recent years, researchers have sought to introduce better probiotics in order to enhance the efficiency of aquaculture production. It has been shown that lactobacilli are good probiotics and that LAB from dairy products can be a suitable probiotic source for fish nutrition (Narimani *et al.,* 2015).

Supplemental phytase effectively releases phosphate groups of phytate in rainbow trout (Cain and Garling, 1995). There are still limited sources of phytase applicable in all food and animal feed. Therefore, a phytase producing probiotic can be a good alternative to supplement phytase. Phytate-bound P is not available to monogastric animals including fishes (National Research Council, 2011). Furthermore, phytate may interfere with the availability of other minerals and can bind trypsin, thereby, decrease protein availability in fish (Singh and Krikorian, 1982).

According to our results, the three lactobacilli strains with high growth rates and marked potential of phytase activity were isolated from dairy products (milk and yogurt) and studied for their probiotic characterization. Several studies evaluated the probiotic effects of LAB on crayfish (*Astacus leptodactylus*) (Didinen *et al.,* 2016) and *Artemia nauplii* (Lamari *et al*., 2013). Also, several LAB strains viz. *L. acidophilus* BS (Commercial fermented milk; 0.02 U ml^{-1}), *L. fermentum* DSM 20052 (Fermented beets; 0.19 U ml^{-1}), *L. plantarum* JBPRS (Plant origin; 0.36 U ml^{-1}), and *L. casei* DSM 20011 (cheese; 0.53 U ml^{-1}) (Haros *et al*., 2013) isolated from different ecosystems have been studied in terms of their phytase activities.

L. reuteri has been reported to show the highest phytate degrading activity (0.90 U ml^{-1}) (Hayek *et al*., 2014), similar to that of phytase detected in our isolated bacterial strains.

One of the primary conditions for probiotic microorganisms is oxygen-tolerance (Rolf *et al*., 1977). LABs are recognized as oxygen-tolerant anaerobes (Ianniello *et al.,* 2016; Karami *et al.,* 2017). Anaerobic bacteria differ in their ability to survive in the presence of oxygen (Loeschew, 1969; Tally *et al*., 1977). In this study, all strains could tolerate oxygen for more than 72 h. Rolf *et al*. (1977) studied the effect of atmospheric oxygen on the viability of 13 strains and reported that *L. plantarum* could tolerate oxygen for over 72 h.

Another probiotic characterization is resistance to acidity. In some studies, acidity resistance was assessed by investigating the ability of strains to survive in a simulated gastric juice (SGJ) (Gibson *et al*., 2005). Lactobacilli used as probiotic adjuncts are commonly delivered in a food and/or feed systems. To establish in the intestine, the bacteria should pass through the stomach so they must be resistant to the acidic conditions. In most fishes, the digestive system is

acidic, hence, tolerance of acidic conditions is one of the characteristics of probiotic microorganisms (Zago *et al.,* 2011). The stomach and intestines pH level in rainbow trout were estimated to be 2.5 - 3.5 and 7.0-8.0, respectively (Lavelle and Harris, 1997). Considering that the pH of fish intestines is 7.0-8.0, and that the bacteria colonize in the intestines, improving the intestinal microbial balance can help inhibit the colonization of fish pathogens in the digestive tract (Conway *et al*., 1987; Wong and Rawls, 2012), phytase activity, and bacterial biomass in such pH ranges. Our strains grew up at different pH levels, showing phytase activities in a range of 3.0 to 8.0 after 24 h. Similar results were reported by Turchi *et al*. (2013), where LAB strains showed to be tolerant of acidic pH (pH=3).

Kanpiengjai *et al*. (2013) demonstrated optimum pH values ranging from 6.0 to 9.0 for the production of phytase from a new source of phytase producing bacterial strain, *Anoxybacillus* sp. MHW14. Belduz *et al*. (2003) first reported a novel species, *A. gonenesis*, which had a broad range of optimum pH (5.5 - 9.5) while *A. flavithermus* grew well at pH levels of 5.5 - 9.0. *A. kamchatkensis* also displayed a broad optimum pH of 6.8 - 9.5 (Kevbrin *et al*., 2005).The effect of pH on the biosynthesis of phytase was probably corresponded to the optimum pH for the bacterial growth. A thermophilic bacterial strain, *A. gonensis* MHW14, produced phytase (0.20 U ml^{-1}) at a pH of 7.0 (Kanpiengjai *et al.,* 2013). In the present experiment, the amounts of phytase produced by *Lactobacillus* sp. AM11 (0.340-0.923 U ml^{-1}), AM13 (0.317-0.960 U ml^{-1}) and AM14 (0.320-0.790 U ml^{-1}) were obtained at pH values of 3.0 to 8.0 while phytase activities extracted from *Aspergillus niger* and *Saccharomyces cerevisae* were recorded at pH ranging between 4.5 and 5.0 (Naves *et al*., 2012). Overall, maximum phytase activity and bacterial biomass were demonstrated in pH values of 7.0 - 8.0.

The isolates of *Lactobacillus* sp. strains (*AM11, AM13,* and *AM14*) survived well under the action of gastrointestinal tract supernatant. Conway *et al*. (1987) showed the ability to survive in gastric juice varied significantly for the strains tested; *L. acidophilus* ADH survived better than both *L. bulgaricus*, and *Streptococcus thermophiles*. *Leuconostoc lactis* was evaluated *in vitro* for the tolerance of the simulated gastric and intestinal juices. The isolated strain was able to survive well in the trypsinase and pepsin solutions (Zhang *et al*., 2013).

Moreover, the survivability of LABs under stimulated gastric juice have already been documented supporting the present study (Faye *et al*., 2012; Turchi *et al.,* 2013; Garcia-Ruiz *et al.,* 2014). Our findings clarify that the LAB isolates could resist in the stimulated gastric conditions.

Probiotic bacteria subsequently enter the upper intestinal tract exposed to bile that secreted by pancreas. Previous studies reported that bile tolerance could also be considered an important

factor affecting LAB's viability (Pacheco *et al*., 2010). The concentrations of bile salts in the gastrointestinal tract averaged between ≈0.3 to 1.5 percent. In the present study, all isolates were resistant to bile concentrations. Chateau *et al*. (1994) showed the effects of bile salts on the growth of 38 LAB isolates, a half of which were slowly influenced by 0.3% bile salts with reported growth retardation for less than one hour in MRS medium compared to control. Rainbow trout bile tolerance accounts for an essential parameter for the survival of probiotic bacteria in the gut (Merrifield *et al.,* 2010). The bile concentrations in this study , showed no inhibitory effects on bacterial growth and all the three strains were able to survive when exposed to 0.3% of rainbow trout`s bile. The results suggest that these isolates have the potential to survive in fish digestive tract, hence, likely survivable while passing through the gasterointestinal tract, as observed in previous studies as well (Strahinic *et al.,* 2013; Trivedi *et al*., 2013).

With the widespread application of antibiotics in some fish farms, knowledge of probiotic resistance against antibiotic treatment seems to be critical. Some LABs are reportedly antibiotic-resistant. It is noteworthy that when using a probiotic bacteria, its antibiotic resistance should be measured at the time of use. Tetracycline is a broad spectrum antibiotic exhibiting inhibitory activity against a wide range of gram-positive and gram-negative bacteria (Chang *et al.,* 2011). Kattla *et al*. (2001) and Choi *et al*. (2003) also confirmed a generally lower resistance of LAB species towards tetracycline and chloramphenicol. Zago *et al*. (2011) represented that among 25 *L. plantarum* strains isolated from Italian and Argentinean cheeses tested for susceptibility to four antibiotics belonging to the clinically most relevant antibiotic classes, only two were highly resistant to tetracycline and carried the tetM gene. Vizoso-Pinto *et al*. (2006), however, did not detect any antibiotic resistant strains within *L. plantarum* isolated from traditional African fermented products. Conversely, a high frequency of tetracycline-resistant strains was observed in *L. plantarum* from Italian fermented dry sausages (Zonenschain *et al*., 2009). In our study, *Lactobacillus* sp. strains of AM11, AM13 and AM14 were sensitive to tetracycline as a commonly used antibiotic, in aquaculture. Since the resistance of these strains to tetracycline is low, it is suggested that tetracycline is not used in fish farms when these strains are used as probiotic.

One of the most important features of a probiotic is, it must not be pathogenic or toxic to its host (Kesarcodi-Watson *et al.,* 2008). The isolated bacteria in this study caused no harmful effects on rainbow trout and resulted in no infectious in the experimental fish. Furthermore, no visible disease symptoms or mortalities were observed following injection of every three isolated bacteria tested as probiotics. Burbank *et al*. (2012) identified two *Enterobacter* sp. strains (C6-6 and C6-

8) to be non-pathogenic to rainbow trout, inhibiting *Flavobacterium psychrophilum in vitro*. Didinen *et al*. (2016) also showed that *Hafnia alvei* did not cause any harmful effects on the crayfish Stage II juveniles upon challenge even at a dose of 10^{-7} cells mL^{-1}. Overall, *Lactobacillus* sp. is generally recognized as safe (GRAS) by the FDA (Food and Drug Administration, USA), therefore, rendering this genus a good probiotic (Zhang *et al*., 2013). Further studies are in progress in the authors' laboratories to evaluate the impacts of *Lactobacillus* sp. strain AM14 on growth performance, nutrient digestibility, and body composition of *O. mykiss*.

According to the results of the present study, isolated *Lactobacillus* strains from dairy products have probiotic properties that make them potential candidates for probiotic applications in cold-fresh water fish farms. Also, as these strains have phytase activity in a broad pH medium, it is possible to increase plant ingredients as protein resources replaced with dietary fish meal by using these strains, which reduces the cost of diet preparation and can help the future of the aquaculture industry by reducing fish meal consumption.

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