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Supplementation of Microencapsulated Fish-Derived Probiotic Lactic Acid Bacteria to Enhance Antioxidant Activity in Animal Feed

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ABSTRACT

The present global trend is finding potent probiotics in addition to animal feed. This study aimed to investigate the ability of lactic acid bacteria (LAB) isolated from Thai silver BARB fish (*Barbonymus gonionotus*) as potential probiotics for use in animal feed. Isolated strains were screened based on their resistance to lysozyme, and 10 selected strains showed higher activity than others. Those strains were identified using biochemical characteristics and the sequencing of 16S rDNA. Identification revealed that those isolates are belong to: *Lactocaseibacillus rhamnosus*, *Lactoplantibacillus plantarum*, *Pediococcus acidilactici*, *Pediococcus pentosaceus*, and *Enterococcus faecalis*. The 10 isolates were subjected to the probiotic tests, including (antimicrobial activity, antibiotic susceptibility, acid and bile salt tolerance, and hydrophobicity) to determine which isolate had the most potent probiotic effect. As a result, *L. rhamnosus* KKU-D89 isolate exhibited strong antimicrobial activity against *Aeromonas hydrophila* and *Escherichia coli*, high tolerance against acid and bile salt conditions, and an interesting hydrophobicity percentage. *L. rhamnosus* KKU-D89, as a probiotic potent was selected for encapsulation by glutinous rice flour mixed with inulin (GRF-inulin) using the freeze-drying technique. Microcapsules used to prepare probiotic feed pellets are named (microencapsulated-pro KKU-D89 pellets). Another feed pellets prepared from an uncoated isolate named are (uncoated-pro KKU-D89 pellets). Especially, microencapsulated-pro KKU-D89 pellets showed excellent encapsulation efficiency (100%), high stability efficiency (96.9%), and cell viability ranging from log 11.7-12.8 CFU/g compared with uncoated pellets. Additionally, microencapsulated-pro KKU-D89 pellets revealed releasing high viable cells (log 10.7 CFU/mL) in gastric juice pH 2.0 and (log 12.3 CFU/mL) in intestinal juice pH 7.2. This microcapsule pellets showed interesting free radicals scavenging activity against both DPPH and ABTS inhibition assays with 54.3 and 43.2%, respectively. In conclusion, the microencapsulated-pro *L. rhamnosus* KKU-D89 pellets using GRF-inulin have the potential to develop as a novel feed formulation by enabling viable probiotic bacteria to reach the large intestine through feeding.

Keywords: GRF-inulin capsules, Probiotic additive, Animal feeding, *In vitro* release, *In vitro* antioxidant

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INTRODUCTION

The addition of antibiotics and chemical drugs is continuing to be used in aquaculture and animal production to prevent contamination by microbes. Consumers are increasingly concerned about the possibility of multidrug resistance and cross-resistance of antibiotics in humans and livestock due to the use of such drugs (Rai et al., 2013). Consequently, in order to effectively handle the biosafety hazards associated with animal production, in particular aquaculture, a paradigm shift in this area was required. In August 2019, during their 10th session in Trondheim, Norway, the FAO Fisheries Committee and the COFI Aquaculture Subcommittee adopted a global progressive management program to enhance aquaculture biosafety. Additionally, innovative technological advancements in the fields of feed, genetic selection, bio-protection, and disease control, advanced technologies, and new approaches, etc. The first part of this program is "encouraging responsible fish farming, including lowering antimicrobial resistance in aquaculture, and the second part is using appropriate alternatives to antimicrobials, as well as other scientifically sound and technology-proven measures to better prevent diseases in aquaculture," in accordance with FAO (2020). Utilizing growth enhancers and safe microorganisms/generally recognized as safe (GRAS), such as lactic acid bacteria (LAB), as a probiotic microbiome, which improves human and animal health and their performance, constitutes a way to lessen these issues (Chizhayeva et al., 2022).

Importantly, members of fish microflora species, mostly LAB consisting of (Gram (+ve) bacteria, homo/hetero fermentative, facultative anaerobe, and non-sporeforming bacteria), exhibited broad-spectrum antagonistic action against opportunistic pathogenic bacterial (e.g., *Aeromonas hydrophila, Vibrio harveyi,* and *Escherichia coli)*, fungal, and viruses that contaminate waterways, microbiologically harm feed, and infect aquatic animals (Phupaboon et al., 2022a). The main genera of fish microflora species are *Lactobacillus*, *Pediococcus*, *Enterococcus*, *Streptococcus*, *Lactococcus*, *Vagococcus*, *Leuconostoc*, *Oenococcus*, *Weissella*, *Carnobacterium*, and *Tetragenococcus* (Ringø et al., 2018). LAB's metabolites can be used as biological food preservatives containing organic acids (e.g., lactic and acetic acid), biocides carbon dioxide, hydrogen peroxide, lysozyme, phenyl lactic acid, fatty acids, and antibiotics or bacteriocins (Phupaboon et al., 2022a). Fish-LAB species can be an extremely promising microbe due to their advantage in food production (e.g., fermented fish or *plaa-som* (Phupaboon et al., 2022b) and fermented pork or *nham* (Phupaboon et al., 2022c) as starter cultures) and/or feed supplementations (Kanwal et al., 2021), because they possess several important advantages that are needed for probiotic candidates. Numerous research endeavors have examined the LABs as a probiotic potential derived from fish microbiota, including: *Lacticaseibacillus casei* KKU-KK1, *Lactiplantibacillus pentosus* KKU-KK2, *Lactobacillus acidophilus* KKU-KK3 (Phupaboon et al., 2022c), followed by feed mixture formulations: *Lactococcus lactis* subsp. *lactis*, *Enterococcus* spp., *Lactiplantibacillus plantarum*, and some genus of bacilli (Alonso et al., 2019; Ringø et al., 2020). However, there are limitations to adding these probiotic cultures to the food and/or feed of humans and animals. These limitations are represented by their instability during food transport and their susceptibility to enzyme levels in the oral cavity. Specifically, they are unable to reach the gastrointestinal tract under the lowest acidity conditions and highest levels of bile acids produced by the pancreas (Phupaboon et al., 2022c).

In order to develop a solution to the issue of minimizing cell death during the gastrointestinal tract (GIT) transit and a chance to regulate the release of these cells throughout the digestive system, is by preparing these probiotics into microcapsules using spray and/or freeze-drying methodologies (Cook et al., 2011; Samedi & Charles, 2019). Freeze-drying technology is currently widespread interest relies on immobilizing or encapsulating bacteria into an encapsulant matrix (such as polymers, plant/animal-based protein extracts, and carbohydrate ingredients) which, in contrast to most controlled release devices for tiny chemicals, holds its structure in the stomach before breaking down and dissolving in the intestine by intestinal juice at different pH condition (Ibrahim et al., 2021). Therefore, the hypothesis of this research was to develop highly viable and stable probiotic animal feed through prebiotic supplements (e.g., inulin extracted from garlic) using encapsulation technology by freeze-drying technique. The current research aimed to evaluate the probiotic characteristics of LABs obtained from fish microflora species, especially those isolated from Thai silver BARB (*Barmonymus gonionotus*), and to investigate selected LAB strains with probiotic potential to encapsulate with glutinous rice flour and inulin (GRF-inulin) in feed formulation. Additionally, the feed probiotic formula was characterized by encapsulation efficiency, stability, *in vitro* release profile at different pH conditions, and *in vitro* antioxidant capacity.

MATERIALS & METHODS

Animal Ethics Approval

The ethics committee of Khon Kaen University approved all procedures involving animals used to obtain fish samples. This approval was granted in accordance with Khon Kaen University's guidelines for the Institutional Animals Care and Use Committee and was authorized by the Institute of Animals for Scientific Purpose Department (IAD), Thailand. The record numbers for this approval are IACUC-KKU 86/66 and U1-10937-2566.

Isolation of Fish Microflora Species

All 218 strains of lactic acid bacteria (LAB) were isolated from different parts of five healthy silver BARB (*Barbonymus gonionotus*) fish of approximately 400-500g cultured in farm at the Department of Fisheries, Faculty of Agriculture, Khon Kaen University, Thailand. Briefly, isolation of fish microflora (LAB) using a membrane-trapping technique was prepared by mixing homogeneous different organs of fish parts (digestive tract, flesh fish, gills, and water pool) with sterile normal saline containing 0.1% peptone media and filtering through a 0.45µm size of

cellulose acetate membrane. Each membrane is placed on MRS agar (Himedia, India) supplemented with 1% calcium carbonate and incubated at 37°C, and then the colony's growth is transferred into fresh media for isolation and purification. LABs microflora were isolated from the digestive tract (32 isolates), flesh fish (68 isolates), gills (40 isolates), and water in a pool (78 isolates) according to the previous study by Phupaboon et al. (2022a). The obtained strains were classified based on various parameters, including physiological characteristics of colonization, Gram stain, cell morphology, and biochemical characteristics including catalase production, and gas production from glucose in addition to verifying temperature conditions of the growth culture according to the method of Schillinger and Lücke (1987).

Resistance to Lysozyme

Isolated LAB (218 isolates) of fish microflora species from four parts were preliminary screened for the first fortification of saliva lysozyme. The effect of lysozyme on the growth of fish microflora strains was tested using the microplates method described by Jacobsen et al. (1999). Each isolate grown overnight in MRS broth (Himedia, India) at 37°C was pelleted by centrifugation, washed twice with phosphate buffer solution pH 7.0, and resuspended in the same buffer. The bacterial suspensions (10^7 CFU/mL; OD_{600} = 1) were inoculated with MRS broth (control test) with or without lysozyme (Sigma-Aldrich, USA) at different concentrations of 10, 20, and 30mg/mL (sample test). After incubation overnight, the absorbance (Abs) at 600nm of each strain was measured using iMark™ Microplate Reader (Bio-Rad, USA). The results were expressed in the percentage of growth index (GI) in accordance with Bevilacqua et al. (2010) following the equation (1):

$$\text{GI}\ (\%) = \frac{\text{Abs of sample}}{\text{Abs of control}} \times 100 \quad (1)$$

Antimicrobial Activity

Selected LABs (10 strains) from the previous experiment were continuing to be tested for antimicrobial activity as described by Lewus et al. (1991). One milliliter of modified MRS broth supplemented with 0.2% (w/v) dextrose (pH 6.0±0.2) was inoculated with 1% (v/v) of overnight culture and incubated in anaerobic conditions to rule out any inhibition due to hydrogen peroxide production at 37°C for 18 hours. The supernatants were collected by centrifugation, sterilized by passing them through a 0.2µm filter, and tested for antimicrobial activity. The test was achieved against two strains (*Aeromonas hydrophila* TISTR 1321 and *Escherichia coli* TISTR 780) using the paper disc diffusion method. Pathogenic suspensions (10^7 CFU/mL) after their growth at 37°C for 24 hours in nutrient broth (Himedia, India) were swapped onto the surface of nutrient agar (NA). Fifteen µL of each LABs culture was petted into the filter paper disc (6mm diameter, Whatman) and placed on the inoculated agar surfaces. Streptomycin antibiotic discs (15µg) were used as a positive control. All plates were incubated at 37°C for 18 to 24 hours depending on the species of bacteria used in the test. After the incubation, the plates were examined for inhibition zones. The inhibition zones were measured using calipers and compared with the diameter of the paper disc. The test was conducted in duplicate to ensure reliability (Rattanachaikunsopon & Phumkhachorn, 2006).

Antibiotic Susceptibility

Antibiotic susceptibility test of selected isolates was examined using the agar overlay diffusion method (Syal et al., 2017). The antibiotic strips were purchased from Oxoid, UK consisting of 10µg penicillin G (P), 15µg erythromycin (E), and 30µg tetracycline (TE) as well as chloramphenicol (C) were used in this study. All isolates grown overnight in the culture broth (10^6 CFU/mL) were spread onto an MRS agar plate (thin layer ≥20mm) and disc-specific antibiotic strips were placed on surface of the medium plates. Further, the plate was incubated overnight at 37 °C, and the diameters of zone inhibition were measured. The breakpoints (susceptible or resistant) were determined according to the Clinical and Laboratory Standards Institute (NCCLS) guideline for gram-positive microorganisms.

Acid and Bile Salt Tolerance

Acid and bile salt tolerance was carried out using isolates selected from the results of antimicrobial activity. The experiment was performed according to the method described by Kimoto-Nira et al. (2010). Overnight cultures of each isolate (approximately 10^6 CFU/mL) were harvested by centrifugation for use as an initial stage (t_0). The cell pellet was sub-cultured in the modified MRS broth with various pH at 2, 2.5, and 3 mixing with 0.3% (w/v) of bile salt (Himedia, India). Cultures were incubated at 37°C for 4 hours (t_4) and the results was expressed as the percentage of survival rate calculated using the following equation (2):

$$\text{Survival rate}\ (\%) = \frac{\text{t4}_{\ (\text{Log CFU/ml})}}{\text{t0}_{\ (\text{Log CFU/ml})}} \times 100 \quad (2)$$

Hydrophobicity

Bacterial cell surface hydrophobicity was assessed by measuring microbial adhesion to xylene and *n*-hexane as described by Collado et al. (2007). Bacterial cells grown in a culture medium were collected, washed, re-suspended in PBS and the initial absorbance at 655 nm was measured (Abs_0). An equal proportion of xylene and *n*-hexane were added to cell suspensions and vortexed well for about 2 minutes to form a two-phase system. Successively, the water and both hydrocarbon reagents phases were left to separate by incubation for 1 hours at room temperature. After that, the aqueous phase was carefully removed while the absorbance of the organic phase was measured at 655nm (Abs_1). The test was carried out in triplicate and hydrophobicity was expressed in percentage which was calculated using the following equation (3):

$$\text{Hydrophobicity}\ (\%) = \left(1 - \frac{\text{Abs1}}{\text{Abs}_0}\right) \times 100 \quad (3)$$

Genetic Identification of Potent Probiotic Strains

Based on data obtained from screening the probiotic properties of LAB isolates, 10 strains were selected and subjected to identification throughout 16S rDNA sequencing analysis according to the procedure of

Phupaboon et al. (2022a) and aligned the sequence into nucleotide BLAST in the NCBI database.

Microencapsulation of Probiotic *L. rhamnosus* KKU-D89 Strain

The microencapsulation process of the probiotic *L. rhamnosus* KKU-D89, a selected strains (pro- KKU-D89) was prepared according to the procedure of Phupaboon et al. (2022c; 2022d). Briefly, the pure culture was used for preparing the cell suspension which was grown in enrichment medium using modified lactose-MRS medium at 37ºC for overnight. Cells were sub-cultured into larger scale for 12 hours. Then, the harvested cells were centrifuged and washed twice with a sterile normal saline solution. After that cells were re-suspended (initial ≥10^{12} CFU/g) with 10% (w/v) of sterile glutinous rice flour (GRF) solution (Erawan Elephant Glutinous Rice Flour, Chonburi, Thailand). Consequently, the microcapsules were processed by mixing cell suspension with a previously prepared coating matrix. The coating matrix was prepared by adding 10% (w/v) sterile GRF solution to the inulin from garlic extract (Sigma-Aldrich, USA) at a ratio of 1:0.5 (v/v). The microencapsulation process was achieved by lyophilization using the freeze-drying technique (frozen at -80ºC for 60min, dried at -58 to -60ºC, and pressure <0.016mbar for overnight). Finally, the microencapsulated-pro KKU-D89 in form of lyophilized product was harvested and kept in a vacuum bag at -20ºC.

Feed Design of Probiotic *L. rhamnosus* KKU-D89 Strain and Preparation of Feed-pellets

The microencapsulated-pro KKU-D89 was voted for feed formulation. The feed-pellet formula used in the current study was prepared by following the procedure of Phesatcha et al. (2023) with slight modifications. The pellet ingredients were consisted of cricket meal (*Gryllus bimaculatus*) powder (90%), cassava starch (8%), and molasses (1%) of dry matter basis, in addition to (1%; 10^{12} CFU/g) consisting of microencapsulated-pro KKU-D89 and cell suspension or uncoated-pro KKU-D89. In case of using microencapsulated probiotic isolate, the obtained feed pellets are named (microencapsulated-pro KKU-D89 pellets). If adding uncoated probiotic isolate, the resulted pellets are named (uncoated-pro KKU-D89 pellets). All ingredients were well mixed into a chamber and the produced pellets were processed through an animal feed pellet machine (Gemco Energy, Anyang Gemco Energy Machinery Co., Ltd. P.R.C.). After that, the dried pellets were stored at room temperature in a closed drum for further use.

Physical Characterization of Microencapsulated-Probiotics *L. rhamnosus* KKU-D89 and their Uncoated Cells in Feed Products

The microstructure size and surface morphology characteristics of lyophilized cells, and both forms of pellets in addition to the cross-section of feed pellets were observed using a field emission microscope-SEM (Tescan mira, Cranberry Township, PA, USA) according to the procedure reported by Phupaboon et al. (2022c).

Cell Enumeration and Encapsulation Efficiency

All groups were subjected to a cell viability test and calculated for encapsulation efficiency using the drop-plate technique on modified MRS-$CaCO_3$ medium agar, as previously reported in an article published by Phupaboon et al. (2022c). Cell number expressed in log CFU/g was calculated from the mean of triplicate readings, encapsulation efficiency (EE) and stability efficiency (SE) at room temperature (30.2±0.2) were determined following equation (4):

$$\text{EE or SE (\%)} = \frac{\log\left(\frac{\text{CFU}}{\text{g}}\right)\text{of encapsulated/pelleted powders}}{\log\left(\frac{\text{CFU}}{\text{g}}\right)\text{of initial starter in suspension}} \times 100 \quad (4)$$

***In vitro* Release of Probiotic *L. rhamnosus* KKU-D89 from Microcapsules**

The feed pellets were investigated for *in vitro* bacterial release properties by adapting the procedure of Cook et al. (2011). The kinetic release of cells from the microcapsules and uncoated pellets was measured by incubating them separately in a water bath (37ºC) under anaerobic conditions in a screw bottle (1%; w/v) in different buffers. They were weighed and placed in each bottle condition containing simulated gastric juice (citrate buffer pH 2.0) and simulated intestinal juice (phosphate buffer pH 7.2) at 4 hours for incubation. After the incubation process, an aliquot sample was taken from the liquid medium to count the cell viability and expressed in log CFU/mL by the plate count method.

***In vitro* Antioxidant Activity of Probiotic *L. rhamnosus* KKU-D89 Strain and their Microcapsules**

The antioxidant activity of selected LAB strains (lyophilized, uncoated and microencapsulated pellets was evaluated in term of DPPH and ABTS radical scavenging activity following the method of Phupaboon et al. (2023) with slight modifications. *L. rhamnosus* KKU-D89 was grown in MRS media for 18 hours. After that, the cultured medium was collected from the cell pellets by centrifuging at 5,000rpm at 4ºC for 5 minutes and washed twice with PBS buffer containing 1% peptone. Then, it was adjusted to the optimum absorbance of 1.0 (approximately 10^8–10^9 CFU/mL) at an 600nm, then filtered through a 0.20µm sterile syringe filter (Whatman, US) for further use as a living cell supernatant (LCS).

Briefly, the DPPH and ABST radical inhibition of the selected strains were tested using a 96-well microplate assay through a PerkinElmer microplate reader (PerkinElmer, USA) with two reagents namely 0.2mM DPPH in 70% ethanol and 7mM ABTS solution containing 2.45mM potassium persulphate (Sigma-Aldrich, USA). Consequently, the reaction was started by mixing the LCS and each reagent solution at a ratio of 1:2 (v/v) in 250µL of the total volume per well and incubated in a dark condition at 37ºC for 30 minutes (Kim et al., 2022). Absorbance (Abs) was measured using the DPPH method at 517nm and the ABTS method at 734nm. The negative control and positive control groups were PBS and L-ascorbic acid, respectively. The radical scavenging activity was calculated using the equation (5):

$$\text{Radical scavenging inhibition (\%)} = \frac{\text{Ab0} - \text{Ab1}}{\text{Ab1}} \times 100 \quad (5)$$

Where Ab_0 is the absorbance of the control (no tested sample) and Ab_1 is the absorbance of the test sample.

Statistical Analysis

For statistical analysis, all data were presented as the mean±SD of measurements taken three times. Using IBM SPSS-KKU Statistics Version 27.0 software, an analysis of variance (ANOVA) was conducted with Duncan's new multiple range test ($P<0.05$) to determine the statistical significance of the observed variations in means.

RESULTS & DISCUSSION

Quantification of Isolated LAB-Fish Microflora

The relative frequency of LAB (total 218 isolates) isolated from silver BARB fish was recorded as seven genera namely *Pediococcus* (17.4%), *Enterococcus* (62.4%), *Streptococcus* (11.0%), *Aerococcus* (3.2%), *Tetragenococcus* (0.5%), *Lactococcus* (5.0%), and *Lactobacillus* (0.5%) as showed in Fig. 1. In addition, all strains were confirmed the morphological and biochemical characteristics following as Gram (+ve) bacilli and/or cocci shapes using microscopy technique, (+ve) test of glucose fermentation, and (-ve) test of catalase, oxidase as well as hemolytic, data referred to our earlier publication (Phupaboon et al., 2022a). The most striking result to emerge from the data is that fish microbiota are important components of fish mucosa, skin, gills, and gut make up a major portion of the first stage of defense against infections also, the microbiota of these organs gives rise to host immunity which is consistent with outcome reported by Benhamed et al. (2014).

Probiotic Characteristics of LAB Isolated from fish Microflora Species

LAB strains were evaluated for the following probiotic characteristics: lysozyme resistance, acid and bile salt tolerance, hydrophobicity, antimicrobial activity, and antibiotic susceptibility. Ten isolates represented potential probiotic properties as shown in Table 1. Selected strains showed Lysozyme resistance property ($P<0.05$), and data of lysozyme resistance were reported in percentage of growth index (GI). *L. rhamnosus* KKU-D89 isolate was achieved for the highest resistance activity at different lysozyme concentrations (10, 20, and 30mg/mL) with (GI%) at 99.5, 80.6, and 89.9%, respectively. Those findings are consistent with outcomes reported by Phupaboon et al. (2022d) and Zhang et al. (2020) who found that some LAB stains of *Lactobacillus* sp. and *S. thermophilus* isolated from fermented products of fish and pork in addition to the digestive tract in pickles demonstrated the highest lysozyme resistance in the survival rate ranging from 30.2 to 100.2%.

Table 1: Different characteristics of LAB species as potential probiotics obtained from fish (silver BARB) microflora

Strains	Resistance to lysozyme (%GI)			Survival rate of acids and 0.3% BS condition (%)			Hydrophobicity (%)		Antimicrobial activity (mm)		Antibiotic susceptibility			
	10 mg/mL	20 mg/mL	30 mg/mL	pH 2.0	pH 2.5	pH 3.0	Xylene	*n*-hexane	*E. coli*	*A. hydrophila*	C	P	TE	E
KKU-D3	71.8±0.3^{e}	71.5±0.8^{b,c}	48.0±0.4^{b}	72.6±1.6^{a}	76.5±6.7^{b}	89.4±1.3^{a}	65.7±0.2^{b}	65.0±2.5^{b,c}	37.5±1.1^{a,b}	25.9±1.2^{c}	S	S	S	S
KKU-D86	77.3±0.8^{c}	69.4±1.4^{d,e}	39.7±4.7^{c}	64.2±0.1^{c}	77.8±0.6^{b}	89.2±0.9^{a}	68.1±0.2^{a}	69.6±0.1^{b}	27.5±1.1^{c}	27.5±1.1^{c}	S	S	S	S
KKU-D89	99.5±0.7^{a}	80.6±0.6^{a}	89.9±0.6^{a}	62.7±0.7^{c}	83.1±0.9^{a}	88.5±5.7^{a}	68.7±0.2^{a}	66.7±0.2^{b}	39.2±1.2^{a}	45.9±1.2^{a}	S	S	S	S
KKU-G11	58.7±0.3^{f}	44.7±1.8^{f}	18.4±2.7^{d}	66.6±2.4^{c}	82.7±1.3^{b}	87.1±1.6^{a}	66.4±0.1^{a}	73.4±2.0^{b,c}	20.9±1.2^{d}	16.7±0.0^{d}	S	S	S	S
KKU-G48	88.9±3.2^{b}	81.1±2.8^{a}	47.4±0.9^{b}	67.4±3.9^{b}	76.4±7.0^{b}	89.7±0.5^{a}	68.9±0.3^{a}	63.9±2.7^{c}	27.5±1.1^{c}	16.7±0.0^{d}	S	S	S	S
KKU-G67	74.8±1.1^{d}	68.9±1.3^{d,e}	56.6±0.9^{b}	67.1±2.6^{b}	68.8±3.0^{b}	73.9±1.6^{a}	72.1±0.1^{a}	72.3±0.1^{a}	35.0±2.4^{b}	33.3±0.0^{b}	S	S	S	S
KKU-G70	39.9±1.4^{h}	12.2±0.2^{h}	10.9±0.7^{e}	70.6±6.7^{b}	77.7±0.9^{c}	90.5±1.7^{a}	65.4±2.5^{a}	69.5±2.3^{a}	33.3±0.0^{b}	33.3±0.0^{b}	S	S	S	S
KKU-F28	49.9±0.3^{g}	28.5±0.2^{g}	12.3±0.3^{f}	76.1±0.7^{b}	78.1±0.4^{b}	90.6±0.0^{a}	70.2±0.2^{a}	69.3±0.1^{b}	20.0±0.0^{d}	16.7±0.0^{d}	S	S	S	S
KKU-F36	94.3±2.2^{a}	73.2±1.5^{b}	81.1±2.8^{a}	77.3±0.5^{a}	88.6±1.4^{b}	90.0±0.6^{a}	86.6±0.3^{a}	69.6±0.3^{b}	35.0±2.4^{b}	34.2±1.2^{b}	S	S	S	S
KKU-F97	90.1±0.6^{b}	67.3±0.4^{e}	39.3±0.4^{c}	70.6±3.2^{b}	70.6±0.9^{c}	90.5±0.4^{a}	67.6±2.4^{a}	67.5±2.4^{b}	20.0±0.0^{d}	20.9±1.2^{c,d}	S	S	S	S

Note: %GI as growth index; S as susceptibility led from susceptible inhibition zone ≥19mm; P as penicillin G; E as erythromycin; TE as tetracycline; C as chloramphenicol. Values are expressed as the mean±SD (n=3); the different superscript letters indicate the significant differences in each column ($P<0.05$).

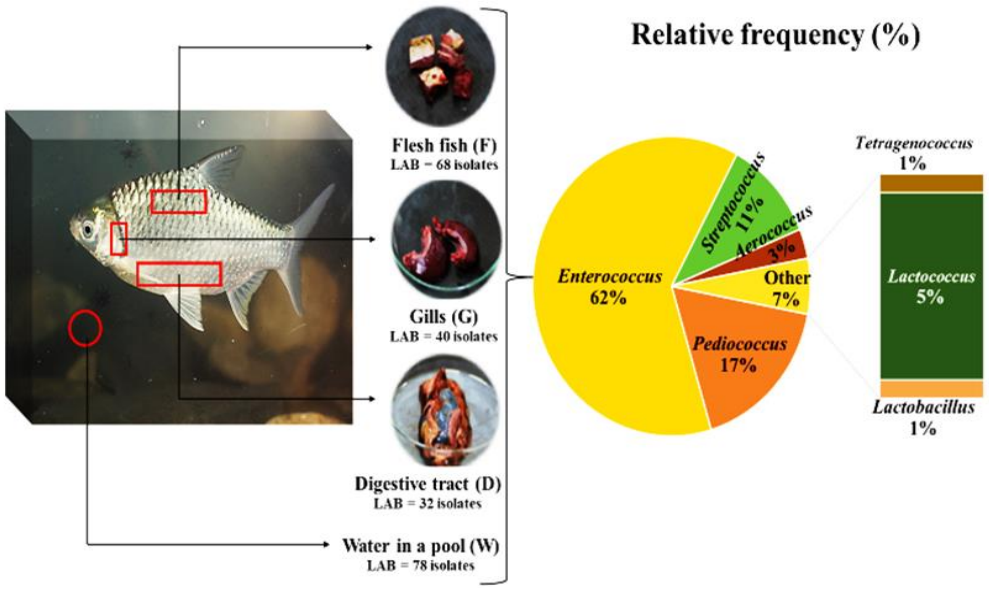

Fig. 1: Fish parts and relative frequencies of fish microbiota species mainly, genera of lactic acid bacteria (LAB)

The highest survival rate in acid combined with 0.3% of bile salt was recorded by *P. pentosaceus* KKU-F36 (77.3, 88.6, and 90.0%) at pH (2.0, 2.5, and 3.0) respectively. Similarly, 12 LAB strains belonging to *P. acidilactici*, *P. pentosaceus*, and *L. plantarum* isolated from Tilapia and Salmonids survived between 34.18% and 49.9% at low pH (1.5) when combined with pepsin for 3 hours, while growth rate ranged from 0.92 to 21.46% when exposed to 0.3% bile salt (Coulibaly et al., 2023; Vargas-González et al., 2024).

The ability to adhere on mucus and epithelial cells was importantly studied in the current research by *in vitro* model system and results were reporting in (%) hydrophobicity. The current data represents interesting hydrophobicity of selected stains throughout adhering to the non-polar hydrophobic surface substances such as xylene and *n*-hexane. Selected LAB isolates have hydrophobicity effect ranging from 86.6 to 65.4% and 73.4 to 65.0% with xylene and *n*-hexane, respectively. Results of Coulibaly et al. (2023) reported degree of cell surface hydrophobicity of LAB strains in presence of hexane ranging from 1.53 to 16.30% for LB195 and LB156 isolates, in xylene (51.1%) for LB137 isolate, while in chloroform (1.17%) for LB82 isolate.

Antibacterial activity is considered as crucial factor in the selection of probiotics isolates based on "Guidelines for the Evaluation of Probiotics in Food" (de Melo Pereira et al., 2018). This assay was achieved by the agar well diffusion method to evaluate the antibacterial activity of the selected LAB stains. Activity of *L. rhamnosus* KKU-D89 isolate against to *E. coli* and *A. hydrophila* was stated in 39.2 and 45.9mm, respectively, which accords with the data of Coulibaly et al. (2023) and Vargas-González et al. (2024).

The antibiotic disc diffusion method on MRS agar plates was used to test the antibiotic susceptibility of the LAB strains. Tetracycline, erythromycin, penicillin G, and chloramphenicol are used as inhibitors of protein synthesis and cell wall synthesis. Selected strains showed susceptibility to the four antibiotics used in the assay. In an additional study of Phupaboon et al. (2022c) and Cheon et al. (2020) involving various strains, the probiotic capacity of *Lactobacillus* strains was assessed, with particular attention paid to *L. casei*, *L. plantarum*, *L. acidophilus*, *L. rhamnosus*, *L. delbrueckii*, and *L. buchneri*. Hence, based on the mentioned results, LAB-fish microflora species particularly *L. rhamnosus* KKU-D89 has a potent probiotic effect, such as enzymatic digestion in addition to tolerance to both gastric acid and bile salt conditions in order to be used as feed supplements.

Genetic Identification of Selected Potent Probiotic Strains

From previous assays, 10 potential LABs were selected as potent probiotic strains consisting of *P. acidilactici* KKU-D3, *P. acidilactici* KKU-D86, *L. rhamnosus* KKU-D89, *P. pentosaceus* KKU-G11, *P. acidilactici* KKU-G48, *P. acidilactici* KKU-G67, *L. plantarum* KKU-G70, *P. acidilactici* KKU-F28, *P. pentosaceus* KKU-F36, and *E. faecalis* KKU-F97. The genetic identification of selected strains showed a high similarity index of 98-99% with different accession numbers according to MW227426, MW227424, OQ784244, KF193908, KX611572, AY852248, AP018405, MN82465, ON564549, and JQ388685 referring to our previous research (Phupaboon et al., 2022a).

Physical Properties

In addition, the probiotic strain of *L. rhamnosus* KKU-D89 microcapsules was subjected to morphological investigation using FE-SEM microphotographs. The result indicated that the particle size of microcapsule was round or square in shape and varied in size, ranging from 1.22 to 17.66µm (Fig. 2a). The cell suspensions were shaped like a single rod (in size 1.2µm) (Fig. 2b). Certain microcapsules possessed smooth surfaces, but other microcapsules had surfaces with concavities. In the recorded photos, there were no visible pores or cracks. This study produces results that corroborate the findings of a great deal of the previous work in this field (Chen et al., 2005; Li et al., 2023; do Carmo Alves et al., 2023). The appearance of the new pellets of each probiotic formula in size ranged from 10.2-10.5mm was found to be a light brown or sugar brown color from the components of cricket protein powder, which has been used as an alternative protein source in fish or animal feed formulations for this research (Fig. 2c-d). Under cross-section of microencapsulated-pro KKU-D89 pellets and uncoated-pro KKU-D89 pellets, the fragments displayed many different characteristics, such as fiber-like, round shape, rod-like and/or reticulated. These fragments were of different sizes and were difficult to detect by FE-SEM microphotographs as shown in Fig. 2e and f.

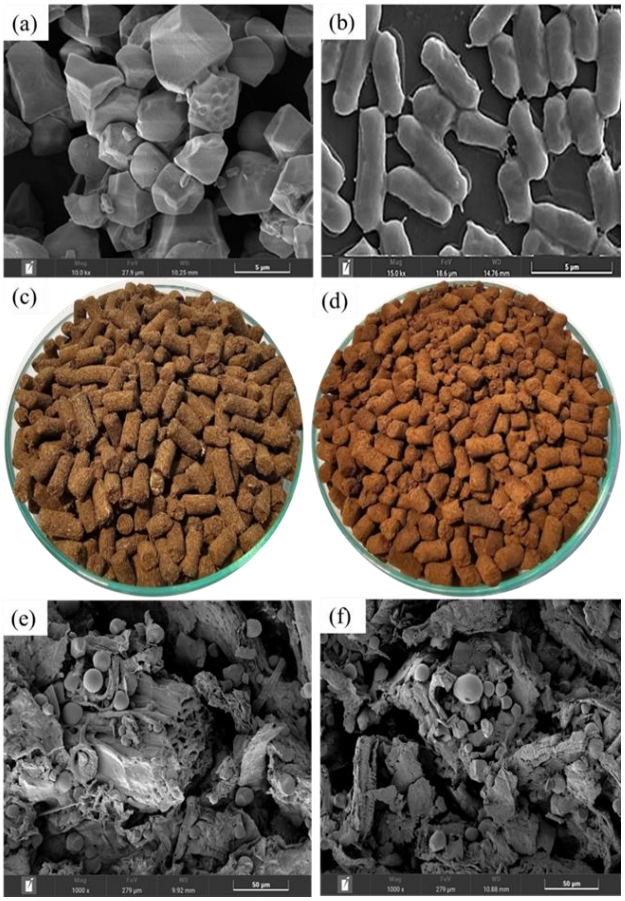

Fig. 2: FE-SEM micrographs of (a), microencapsulated-pro KKU-D89; (b), uncoated-pro KKU-D89; (c), microencapsulated-pro KKU-D89 pellets; (d), uncoated-pro KKU-D89 pellets; (e), cross-section of microencapsulated-pro KKU-D89 pellets; (f), cross-section of uncoated-pro KKU-D89 pellets.

This result corresponds in accordance with the findings of Giri et al. (2021), who hypothesized that microencapsulated-probiotic bacteria formulated in dietary may be extremely resilient to changes in environmental conditions, osmotic pressure, pH levels, and bile salt concentrations. They may also be able to inhibit opportunistic microorganisms.

Cell Enumeration and Encapsulation Efficiency

Viable cells (probiotic *L. rhamnosus* KKU-D89) were counted in both forms: microencapsulated-pro KKU-D89 pellets and uncoated-pro KKU-D89 pellets when compared with lyophilized cells (Table 2). The number of initial viable cells was approximately log 13.0 CFU/g of powders, and the number of live cells in encapsulated and/or uncoated pellets ranged from 11.7 to 12.8 CFU/g pellets. Additionally, encapsulation efficiency was (100%) and stability efficiency ranged from 90.7 to 100% in GRF-inulin based microcapsules. These results are consistent with other published articles which found that four probiotics (*L. acidophilus*, *L. casei*, *Bifidobacterium bifidum*, and *B. longum*) in addition to encapsulated *L. paraplantarum*, *E. faecalis*, *L. plantarum* and *W. paramsenteroides* which were encapsulated with prebiotics (fructooligosaccharides or isomaltooligosaccharides, and arrowroot strach), a growth stimulant (peptide), maltodextrin, and sodium alginate using freeze-drying showed enhancing the viabily of probiotic cells at 10^6 to 10^7 CFU/mL and stability ranged from 68.2 to 79.2% after stored at 30 days (Samedi & Charles, 2019; Chen et al., 2005). In similar with the outcomes of Li et al. (2023) reported that the microencapsulated *L. rhamnosus* LB1 (10^9 CFU/g) as feed additive for pigs was previously shown to reduce enterotoxigenic *E. coli* and *Salmonella* sp. infections and showed the highest stability efficiency in feed pellets at log 1.06 and 1.54 CFU/g when completed at 30 days.

***In vitro* Release Profiles of Bacterial Cell**

Several studies have demonstrated the importance of feeding probiotics at the ideal concentration and quantity. The main advantage is bacterial releasing under various circumstances in simulated intestinal juice (pH 7.2) and simulated gastric juice (pH 2.0) to increase their survival rate during passage through the simulated GIT condition (Cook et al., 2011). Table 3 presents a summary of *in vitro* release rates based on the cell viability of microencapsulated-pro KKU-D89 lyophilized and two different feed pellet formulas (microencapsulated- pro KKU-D89 pellets and uncoated-pro KKU-D89 pellets) in simulated gastric juice as followed by intestinal juice conditions at pH 2.0 and 7.2, respectively. Both buffers (pH 2.0 and 7.2) were log 7.3 to 10.7 CFU/mL and log 11.1 to 12.3 CFU/mL released at 4 hours after incubation at 37ºC. This finding is in agreement with Samedi & Charles (2019) finding which found that the possibility that probiotic bacteria microencapsulated in arrowroot starch and maltodextrin consists of encapsulated *L. paraplantarum*, *E. faecalis*, *L. plantarum* and *W paramsenteroides* survived in a simulated GIT, and their viability was 68.2, 73.6, 77.3, and 73.7% versus 69.5, 79.2, 77.7, and 72.8%, respectively. According to Babot et al. (2023) found distinct differences result the release at pH 8.0 for 3 hours, *L. salivarius* CRL2217 from soy protein isolated-alginate microcapsules was examined. The amount of cell viable released at log 6.22 microcapsules/mL in simulated intestinal juice, as measured with a Neubauer chamber. At the onset of the therapy, nearly all of the imprisoned lactobacilli cells were released, resulting in cell counts of log 8.32 CFU/mL. In addition, *B. adolescentis* was encapsulated in chickpea protein-alginate particles by Wang et al. (2014). The majority of the encapsulated bacteria were released at the start of the simulated intestinal juice treatment, and it was hypothesized that the protective effects of free biopolymers in solution (rather than in encapsulating form) could explain the higher viable cell count for cells released from the capsules compared to free cells.

Antioxidant Activity

In vitro antioxidant capacities in terms of DPPH and/or ABTS radical-scavenging inhibition obtained from probiotic *L. rhamnosus* KKU-D89 of live cell suspensions, microencapsulated-lyophilized, microencapsulated-pro KKU-D89 pellets and uncoated-pro KKU-89 pellets were recorded at 15.1, 34.9, 54.3, and 35.6%, followed by 12.2, 35.1, 43.2, and 22.4%, individually (Table 3). Additionally, the positive result obtained from ascorbic acid showed the

Table 2: Comparison of GRF-inulin microcapsules of encapsulated probiotic pellets, uncoated pellets and lyophilized KKU-D89 based on encapsulation and stability efficiencies.

Samples	Enumeration of cell viability (log CFU/g)		Encapsulation efficiency	Stability efficiency at 30 days
	Initial cells	encapsulated/pelleted	(%)	(%)
Microencapsulated-pro KKU-D89 lyophilized	12.9±0.0^a	12.8±0.2^a	100	100
Microencapsulated-pro KKU-D89 pellets	12.9±0.0^a	12.5±0.0^a	ND	96.9
Uncoated-pro KKU-D89 pellets	12.2±0.0^a	11.7±0.0^b	ND	90.7

Note: pro as probiotic; ND as not detected. Values are expressed as the mean±SD (n=3); the different superscript letters indicated the significant differences in each column (P<0.05).

Table 3: Effects of GRF-inulin microcapsules with both *in vitro* release profiles and *in vitro* antioxidant activities obtained from probiotic free cells, microcapsules, and different feed formula

Samples	*In vitro* release rate-based cell viability (log CFU/mL)		*In vitro* antioxidant activity (%)	
	gastric juice (pH 2.0)	intestinal juice (pH 7.2)	DPPH	ABTS
Live cell suspensions	ND	ND	15.1±0.3^c	12.2±0.3^d
Microencapsulated-pro KKU-D89 lyophilized	9.8±0.3^b	12.0±0.2^a	34.9±0.0^b	35.1±0.1^b
Microencapsulated-pro KKU-D89 pellets	10.7±0.3^a	12.3±0.0^a	54.3±0.3^a	43.2±0.0^a
Uncoated-pro KKU-D89 pellets	7.3±0.3^c	11.1±0.1^b	35.6±0.1^b	22.4±0.2^c

Note: pro as probiotic; ND as not detected. Values are expressed as the mean±SD (n=3); the different superscript letters indicated the significant differences in each column (P<0.05).

scavenging activity of DPPH and ABTS at 85.2 and 75.1%, respectively. Interestingly, from these data, microencapsulated pro KKU-D89 pellets is highly affected in an *in vitro* release property at pH 2.0 (increased 31.0%) and pH 7.2 (increased 90.2%) also *in vitro* antioxidant capacity in terms of DPPH inhibition (increased 65.6%) as well as ABTS inhibition (increased 51.9%) when compared with the formulation of uncoated-pro KKU-D89 pellets. Numerous researchers have examined probiotics based on their capacity to scavenge free radicals obtained from intact cells, extracellular liquid, and intracellular liquid (Mishra et al., 2015; Feng and Wang, 2020). The current results are similar to that of Coulibaly et al. (2023), who exhibited cell suspension and intact cells of following nine LAB strains, LB45 (90.23%), LB82 (51.92%), LB98 (74.29%), LB100 (84.06%), LB143 (75.57%), LB156 (64.52%), LB166 (63.85%), LB187 (57.84%), and LB194 (76.60%), which remarkably showed the highest DPPH scavenging activities from cell suspension than in intact cells. Additionally, *in vitro* studies of Yasmin et al. (2020), the *Bifidobacterium* showed the significant antioxidant activity in the cell supernatant, with values ranging from 80.72-87.72%.

Conclusion

Protecting the viability of probiotic microbes during their passage through the gastrointestinal system is a high priority at the moment. In animal production, many researchers are seeking many microbes from different sources for addition to animal feed. LABs of Thai silver BARB fish were selected in the current study. Ten strains out of 218 isolated microbes including: *L. rhamnosus*, *L. plantarum*, *P. acidilactici*, *P. pentosaceus*, and *E. faecalis* have effective resistance to lysozymes, low acid, high bile salt and board-spectrum antimicrobial activity. Especially, *L. rhamnosus* KKU-D89 showed high probiotic potential throughout different assays including (antimicrobial activity, antibiotic susceptibility, acid and bile salt tolerance, and hydrophobicity). Feed pellets of microencapsulated-pro *L. rhamnosus* KKU-D89 encapsulated with a GRF-inulin coating matrix exhibited high cell's viability and high stability in various conditions including gastric juice (pH 2.0) and intestinal juice (pH 7.2) as well as has scavenging activity against free radicals. Overall, microcapsules are a promising and effective tool in animal feeding and should be applied for further investigations.

Author's Contributions

SP, FJH, and SP conceived and designed the experiment. SP and FJH performed the study, and NK and PK conducted lab analyses. SP, PP, and PR supervised and coordinated the experiments, and BP provided feed formula. SP and SP performed statistical analyses of experimental data and prepared the manuscript format. SP, FJH, PP, and PR prepared the manuscript draft. All authors critically revised the manuscript and approved the final version.

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