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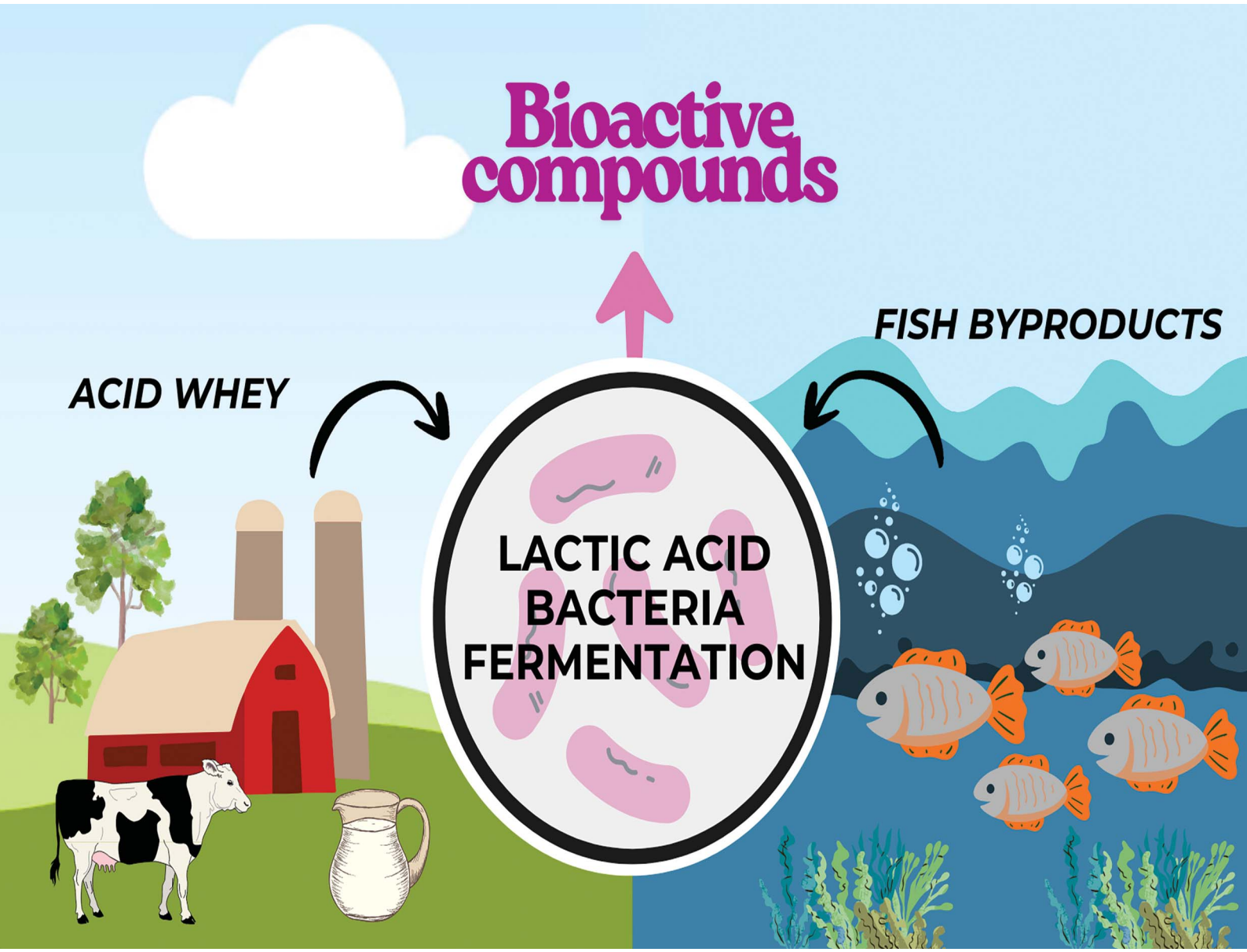

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Sustainable valorization of fish byproducts and acid whey through lactic acid bacteria fermentation into bioactive hydrolysates

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The growing accumulation of food byproducts and waste imposes environmental and economic challenges, highlighting the need for sustainable valorization strategies. This study investigated the usage of fish byproducts and acid whey through lactic acid bacteria (LAB) fermentation to produce bioactive fish protein hydrolysates (FPHs) with potential antioxidant and antibacterial properties. The effects of three formulations: acid whey (FAw), a starter culture (FLr), and their combination (FAwLr) on the fermentation and its products were systematically evaluated. LAB fermentation triggered protein hydrolysis, as demonstrated by the increased degrees of hydrolysis (DH). The fermentation process was also marked by significant microbial growth using FAwLr, with LAB populations above 10^8 CFU mL^{-1} by Day 3, accompanied by a rapid pH decline (<4.5). Viscosity of the fermented samples decreased in all formulations, showing smaller consistency indexes and larger flow behavior indexes, indicating enhanced protein hydrolysis and a reduced structural complexity of the system over time. Antioxidant activity, measured by DPPH (2,2-diphenyl-1-picrylhydrazyl), ABTS 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid), and FRAP (Ferric Reducing Antioxidant Power) assays, was significantly improved in all formulations containing acid whey. The antibacterial activity against *Listeria innocua* and *Escherichia coli* showed formulation-dependent effects. The combination of acid whey and LAB (FAwLr) exhibited the strongest antibacterial properties, lowering both the minimum inhibitory and bactericidal concentrations. These results highlight the synergistic effects of acid whey and LAB in producing multifunctional bioactive hydrolysates with both antioxidant and antibacterial activities, showcasing a sustainable approach to converting waste streams into value-added ingredients.

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Sustainability spotlight

Massive quantities of nutrient-rich byproducts such as acid whey and fish byproducts are generated from the global food industry, yet are often discarded due to processing challenges, contributing to environmental pollution and resource inefficiency. This study presents an innovative bioprocessing approach using lactic acid bacteria fermentation to valorize these underutilized streams into bioactive protein hydrolysates with antioxidant and antibacterial functions. By enabling upcycling of food-processing waste into functional ingredients, this work supports a circular economy and offers an alternative to disposal-intensive practices. It directly advances UN Sustainable Development Goals SDG 12 (Responsible Consumption and Production) and SDG 3 (Good Health and Well-being) by reducing waste and promoting natural antimicrobial alternatives that enhance food safety and preservation.

1. Introduction

Waste streams have become a serious global challenge, particularly within the food industry, where approximately one-third of food products are wasted throughout the global food supply chain.[1,2] Such waste not only depletes resources but also exacerbates greenhouse gas emissions, highlighting the need for innovative waste management solutions.

Fish byproducts, comprising up to 75% of the total catch, pose environmental and economic challenges. Despite being rich in proteins, bioactive peptides, omega-3 fatty acids, and minerals, improper disposal leads to marine pollution.[3,4] Similarly, acid whey, a liquid co-product from dairy industries, is challenging to valorize due to its high biochemical oxygen demand (BOD), low pH, and high lactic acid and mineral content, which complicate processing methods such as membrane concentration and spray drying.[5–7] Despite these challenges, acid whey contains valuable nutrients, including

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proteins, calcium, phosphorus, and lactose, and is a natural source of lactic acid bacteria (LAB), which facilitate fermentation by inhibiting spoilage microorganisms and promoting bioactive compound production.[8–11]

Traditionally, fish byproducts are processed into fish meal, a high-protein product, used as a major component in aquaculture feed.[12,13] However, fish meal production is often limited to large-scale industrial operations, rendering it impractical for smaller producers.[14] Thus, from a value-upgrading perspective, these byproducts have the potential to be transformed into higher-value ingredients rather than being directly processed into fish meal.[15] Alternative methods, such as fish silage and fish protein hydrolysate (FPH) production, have gained attention as feasible valorization strategies. Fish silage involves enzymatic breakdown of proteins using acids or microbial fermentation, enhancing amino acid availability for animal feed.[12] Meanwhile, enzymatic hydrolysis produces FPH rich in peptides with antioxidant, antihypertensive, and antimicrobial properties, but the process is not cost-effective and easily scalable.[16]

LAB fermentation is a promising solution to valorize food byproducts. LAB lower pH by producing lactic acid, thereby inhibiting spoilage microorganisms and improving the safety and shelf life of fermented products.[11] LAB fermentation is a natural, safe, and environmentally friendly bioprocess that enhances the nutritional and functional properties of protein-rich waste streams. Combined with acid whey, LAB fermentation not only mitigates environmental issues but also produces antimicrobial and antifungal compounds.[17,18] This sustainable approach holds potential for valorizing fish byproducts and acid whey into functional ingredients, addressing challenges in both the dairy and fishing industries.

Despite advancements in valorization techniques, key gaps remain in integrating fish and acid whey byproducts into a single fermentation process. Individually, fish byproducts undergo rapid proteolysis and microbial spoilage, while acid whey alone often leads to undesirable mold growth due to its low pH and nutrient content. However, their combination in fermentation resulted in a unique microbial environment that facilitated controlled hydrolysis and bioactive compound formation. This study investigated the synergistic effects of LAB fermentation on these combined waste streams, focusing on bioactive compound production with antioxidant and antibacterial properties. We hypothesized that LAB fermentation, particularly in the presence of acid whey and starter culture, could generate different compounds to enhance the bioactivity of FPH, offering an innovative solution for valorizing these underutilized resources. Therefore, this study aims to evaluate the potential of LAB fermentation to valorize fish byproducts and acid whey into bioactive hydrolysates. Specifically, the effects of acid whey addition on fermentation performance, microbial dynamics and physicochemical changes (including pH and degree of hydrolysis), as well as an assessment of the antioxidant and antibacterial properties of the resulting fish protein hydrolysates, were examined. To further emphasize the innovation of this work, this study explores a novel valorization approach by combining two underutilized byproducts, fish byproducts and acid whey, as fermentation substrates, offering a unique strategy to reduce food waste while unlocking new functional ingredient potentials. By addressing these gaps, this research has the potential to advance sustainable waste valorization and highlights the potential for producing high-value functional ingredients with applications in the food, feed, and nutraceutical industries.

2. Materials and methods

2.1 Materials

Frozen fish byproducts from surimi production using Pacific whiting were provided by Pacific Seafood (Warrenton, OR) in January 2023. These fish byproducts consisted of suspended solids rich in proteins and oils and were characterized by a crude protein content of 15.7%, moisture content of 69.7%, total solids of 30.3%, and fat content of 20.2%. Acid whey, a byproduct of cottage cheese production, was donated by Superior Dairy (Canton, OH). Both materials were stored at −20 °C prior to use. Blackstrap molasses (Grandma's Original, US) was used as an additional carbon source to enhance LAB growth and fermentation kinetics. The inclusion of molasses was based on our prior work,[19,20] where it was shown to be essential for achieving optimal lactic acid production and protein hydrolysis in similar fish–whey fermentation systems.

Lactobacillus rhamnosus OSU-PECh-69 (*L. rh*), a strain belonging to a generally recognized as safe (GRAS) species, was used to ensure the process remains suitable for food-related applications. This strain was selected due to its high proteolytic activity, as previously demonstrated in fish and acid whey fermentations.[19] Fermentation was conducted using raw, non-sterilized materials to preserve the natural microbiota of the byproducts and evaluate the impact of starter culture inoculation. Three formulations were prepared: (1) FAw: fish and acid whey; (2) FLr: fish and *L. rh*; (3) FAwLr: fish, acid whey, and *L. rh*.

2.2 Preparation of fermentation

The fermentation process was adapted from previously established methods with modifications.[19,20] Water was used to replace acid whey in the FLr formulation to maintain comparable dilution across treatments. Given the low protein content of acid whey, typically 0.55–0.75%, the total protein content among the three formulations was expected to remain similar.[21] Fish byproducts were mixed with acid whey according to the formulations listed in **Table 1**. Fermentations were carried out in 1 L water-jacketed spinner flasks (Chemglass Life Science, Wineland, NJ) with two side arms: one fitted with a GL-45 two-way port assembly and 0.2 μm vent filter (Whatman, Piscataway, NJ) to minimize contamination, and the other port connected to a vinyl tube with a 1/4″ pinch clamp for sampling.

The starter culture, *L. rh*, was activated from −80 °C glycerol stock in 10 mL sterilized MRS broth medium (BD Difco™, Franklin Lakes, NJ) and incubated aerobically at 37 °C for 16 h. Subculturing involved transferring 100 μL to 10 mL of fresh MRS broth and incubating under the same conditions. Bacterial cells were harvested by centrifugation (3500 × *g*, 20 min),

Table 1 Composition of the formulations of fermentation

Abbreviation	Fish byproducts (w/w%)	Acid whey (w/w%)	DI water (w/w%)	Molasses (w/w%)	Starter culture *L. rh* (CFU g^{-1})
FAw	50	47.5	0	2.5	0
FLr	50	0	47.5	2.5	10^8
FAwLr	50	47.5	0	2.5	10^8

washed twice with 0.85% (w/v) sodium chloride, and inoculated into the fish–whey mixture at 10^8 CFU g^{-1}.

A spinner flask was attached to an overhead stirrer (Fisher Scientific, Hampton, NH) and operated at 45 rpm with an impeller blade. Temperature was maintained at 37 °C using an external aquarium heater connected to the flask water jacket. Fermentation was conducted for five days with samples collected on Days 0, 1, 3, and 5, heat-treated at 85 °C for 10 min to inactive microbial reactions and stored at −20 °C until subsequent analyses.

The fermentation conditions (37 °C, 5 days) were adapted from prior work,[19] with modifications based on preliminary tests. The temperature was set to match the optimal growth temperature of *L. rhamnosus*, while the fermentation period was shortened from 14 to 5 days based on observed pH stabilization and to improve process efficiency.

2.3 Monitoring of the fermentation process

Fermentation was monitored by measuring pH with a calibrated meter and microbial viability *via* the pour plate method on selective agars. LAB viability was assessed on DeMan, Rogosa, and Sharpe (MRS) agar (37 °C, 48 h), coliforms on Eosin Methylene Blue (EMB) agar (37 °C, 24 h), and yeast and molds on Dichloran Rose-Bengal Chloramphenicol (DRBC) agar (25 °C, 5–7 days). Yeast and mold on DRBC agar are differentiated based on their colony morphology. Viability was expressed as colony-forming units per milliliter (CFU mL^{-1}), using plates containing 15–150 colonies; for DRBC agar, counts outside this range were included when necessary. Microbial counts at Day 0 were recorded immediately after blending the respective substrates for each formulation. These measurements represent the initial microbial load of the full mixture used in each treatment and served as the baseline for monitoring microbial dynamics during fermentation.

2.4 Viscosity

Viscosities of the fish–whey mixtures were measured fresh at different fermentation times using a Discovery HR-3 rheometer (TA Instruments, New Castle, DE) equipped with a Peltier-controlled concentric cylinder with a Stainless Steel Vaned Rotor (radius: 14 mm; length: 42 mm) and a standard cup (radius: 15 mm). Measurement was conducted at 25 °C through a flow sweep test with shear rates ranging from 0.1 to 100 s^{-1}. The flow curve (stress *versus* shear rate) was fitted using the power-law equation (eqn (1)), where σ is the shear stress (Pa), k is the flow consistency index (Pa s^n), n is a dimensionless power-law index, and $\dot{\gamma}$ is the shear rate (s^{-1}). n characterizes the fluid's rheological properties, with $n = 1$ indicating Newtonian flow, $n < 1$ indicating shear-thinning behavior, and $n > 1$ indicating shear-thickening behavior. Data acquisition and analysis were performed using TRIOS software (TA Instruments).

$$\sigma = K\dot{\gamma}n \quad (1)$$

2.5 Moisture content

Moisture content was determined using the oven-drying method.[22] Approximately 3 g of the fish–whey mixture were placed on aluminum trays, weighed, and dried at 105 °C for 5 h. Moisture content was calculated as the percentage of weight loss, representing the difference between the initial and dried masses.

2.6 Degree of hydrolysis

A modified *O*-phthalaldehyde (OPA) method based on Kuo *et al.*[20] and Nielsen *et al.*[23] was used to determine the degree of hydrolysis (DH). Total amino acid content was accessed by complete hydrolysis of the Day 0 sample treated with 6 N HCl and heated at 120 °C for 24 h. A 200 μL sample or complete hydrolysis sample was then mixed with 1.5 mL OPA reagent at room temperature for 2 min. Absorbance was measured at 340 nm. DH was calculated using eqn (2).

$$\mathrm{DH}\ (\%) = \frac{\mathrm{OD}_{\mathrm{sample}} - \mathrm{OD}_{\mathrm{blank}}}{\mathrm{OD}_{\mathrm{complete\ \ hydrolysis}} - \mathrm{OD}_{\mathrm{blank}}} \times 100\% \quad (2)$$

2.7 Preparation of FPH for bioactivity assays

Fish–whey mixtures were centrifuged at 16 000 × *g* for 15 min to separate into three layers: an upper lipid/emulsion layer, a middle aqueous layer, and a bottom layer of insoluble fish pellets. The aqueous phase was further centrifuged at 3200 × *g* for 10 min. The clarified aqueous phase was then freeze-dried using a 4.5 L FreeZone benchtop freeze-dryer (Labconco, Kansas City, MO, USA). The resulting FPH powder was reconstituted in deionized water (200 mg mL^{-1}) for bioactivity assays.

2.8 Antioxidant activity assays

2.8.1 DPPH. DPPH (2,2-diphenyl-1-picrylhydrazyl) assay was performed following Martí-Quijal *et al.*[24] and Shimamura *et al.*[25] with minor modifications. Briefly, 200 μL of FPH (20 mg mL^{-1}) was mixed with 800 μL 0.1 M Tris–HCl buffer (pH 7.4) to eliminate pH effects, followed by adding 1 mL of DPPH solution (0.1 mM in methanol). After mixing for 30 s, the mixture was incubated in the dark for 30 min prior to measuring the

absorbance at 517 nm using a UV-Vis spectrophotometer (Thermo Scientific, GENESYS™, Waltham, MA). A Trolox standard curve (0–250 μM) was used to calculate antioxidant activity. The results are expressed as Trolox Equivalent Antioxidant Capacity (TEAC) and reported as μmol TE g^{-1} FPH.

2.8.2 ABTS. ABTS (2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)) assay was adapted from Memarpoor-Yazdi *et al.*[26] and Chalamaiah *et al.*[27] Fresh $ABTS^+$ stock solution was prepared by reacting 7 mM $ABTS^+$ with 2.45 mM potassium persulphate (1 : 1 v/v) for 16 h in the dark and diluted with methanol to an absorbance of 1.1 ± 0.02 at 734 nm. 75 μL of FPH (8 mg mL^{-1}) was mixed with 1425 μL of the $ABTS^+$ solution, incubated in the dark for 30 min, and absorbance was measured at 734 nm. A Trolox standard curve (0–600 μM) was prepared as described by Ortizo *et al.*,[28] and the results were expressed as TEAC, reported as μmol TE g^{-1} FPH.

2.8.3 Ferric reducing antioxidant power (FRAP). The FRAP assay was conducted following Torres-Fuentes *et al.*[29] 0.5 mL FPH (16 mg mL^{-1}) was combined with 0.5 mL each of 1% potassium ferricyanide and 0.2 mM phosphate buffer (pH 6.6). After incubation at 50 °C for 20 min, 0.5 mL of 10% (w/v) trichloroacetic acid was added. Samples were centrifuged at 3000 × *g* for 10 min. The supernatant (1 mL) was mixed with 0.2 mL of 0.1% ferric chloride, incubated for 10 min, and absorbance was measured at 700 nm.

2.8.4 Ferrous (Fe^{2+}) ion chelating activity (%). Ferrous (Fe^{2+}) ion chelation was evaluated following Nalinanon *et al.*[30] 200 μL of FPH (16 mg mL^{-1}) was mixed with 10 μL of 2 mM ferrous chloride and 20 μL of 5 mM ferrozine, incubated at room temperature for 20 min, and absorbance was measured at 562 nm. The chelating activity (%) was calculated using eqn (3):

$$\text{Chelating activity } (\%) = \frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{conntrol}}} \times 100\% \quad (3)$$

2.9 Antibacterial activity assays

2.9.1 Bacterial strains and sample preparation. *Listeria innocua* (ATCC 51742) and *Escherichia coli* (BL 21) were used to evaluate the antibacterial activity of FPH. Glycerol stocks stored at −80 °C were streaked onto Brain Heart Infusion agar for *L. innocua* and Luria-Bertani agar for *E. coli* (Sigma-Aldrich, Cleveland, OH), and incubated overnight at 37 °C. A single colony was selected and subcultured in the respective broth at 37 °C overnight to prepare bacterial suspensions. Nisin was selected as the positive control for *Listeria innocua*, as it is primarily effective against Gram-positive bacteria,[31] whereas ampicillin was used for *Escherichia coli* due to its broad-spectrum activity against Gram-negative bacteria.[32] 20 mg mL^{-1} nisin (*L. innocua*) and 1 mg mL^{-1} ampicillin (*E. coli*) were used as positive controls for all tests.

2.9.2 Inhibition growth curve. The inhibitory effects of FPH on bacterial growth were assessed in a 96-well plate following Adnan *et al.*[33] Each well contained 200 μL sterile nutrition broth, 20 μL FPH (200 mg mL^{-1}), and 10 μL bacterial suspension. Plates were incubated at 37 °C for 12 h, and bacterial growth was monitored hourly by measuring absorbance at 600 nm.

2.9.3 Agar well diffusion method. The agar well diffusion method adapted from Geis *et al.*[34] was used. Briefly, 100 μL of overnight bacterial culture of *L. innocua* and *E. coli* were added into 10 mL of fresh broth and incubated at 37 °C for 4 h and 6 h, respectively. The bacterial suspension was mixed with 25 g of Mueller Hinton agar (Oxoid; Thermo Fisher Scientific, Basingstoke, UK) to achieve a final concentration of 10^6–10^7 CFU g^{-1}, which was then poured into Petri dishes. Wells (9 mm diameter) were cut into the agar, and 100 μL of FPH (200 mg mL^{-1}) was added. Plates were incubated at 37 °C for 24 h, and the results were presented as the diameter of inhibition zones (DIZ) in millimeters.

2.9.4 Minimum inhibition concentration (MIC). MIC was defined as the lowest FPH concentration that inhibits bacterial growth. MIC of FPH was determined in 96-well microtiter plates following the method of Elshikh *et al.*[35] For *L. innocua* and *E. coli*, 100 μL of Tryptic Soy broth or Mueller–Hinton (MH) broth, respectively, was added to each well. Serial two-fold dilutions of FPH samples were prepared, followed by the addition of 30 μL of bacterial suspension (10^6–10^7 CFU mL^{-1}). Plates were incubated at 37 °C for 24 h, then 30 μL of a freshly prepared 15 mg mL^{-1} resazurin (filtered through a 0.2 μm membrane) was added. After 2 h of incubation, blue/purple indicated no growth, while pink/colorless indicated bacterial growth.

2.9.5 Minimum bactericidal concentration (MBC). MBC was defined as the lowest FPH concentration that killed bacteria completely. After the MIC assay, 10 μL from each well was plated onto Tryptic Soy agar (*L. innocua*) and MH agar (*E. coli*) and incubated at 37 °C for 24 h. MBC was determined as the lowest concentration where no colonies were observed.

2.10 Statistical analysis

Fermentations for each formulation (FAw, FLr, and FAwLr) were conducted in three individual biological replicates. Measurements for each day (Day 0, 1, 3, and 5) and formulation were also performed in triplicate. Two-way ANOVA was used to analyze the main and interaction effects of day and formulation, with Tukey's *post hoc* test identifying significant differences ($p < 0.05$). The results were reported as mean values with standard deviation as error bars in both tables and graphical data. Statistical analyses were performed using JMP Pro 16 (SAS Institute, Cary, NC), and figures were generated with OriginLab software (OriginLab Corporation, Northampton, MA, USA).

3. Results and discussion

3.1 Microbial changes and pH

Effects of fermentation time and formulation on pH and microbial viability are presented in **Fig. 1.** Significant effects of time and formulation, and their interaction effect ($p < 0.0001$), were observed, indicating the dynamic interplay between the fermentation process and formulations.

3.1.1 pH. All formulations showed a significant pH drop after Day 2 ($p < 0.05$). Initial pH values for FAw (5.09) and FAwLr (5.13) were lower than FLr (5.5) due to the inclusion of acid whey (pH 4.2). In FAw, which lacked starter culture (*L. rh*), pH

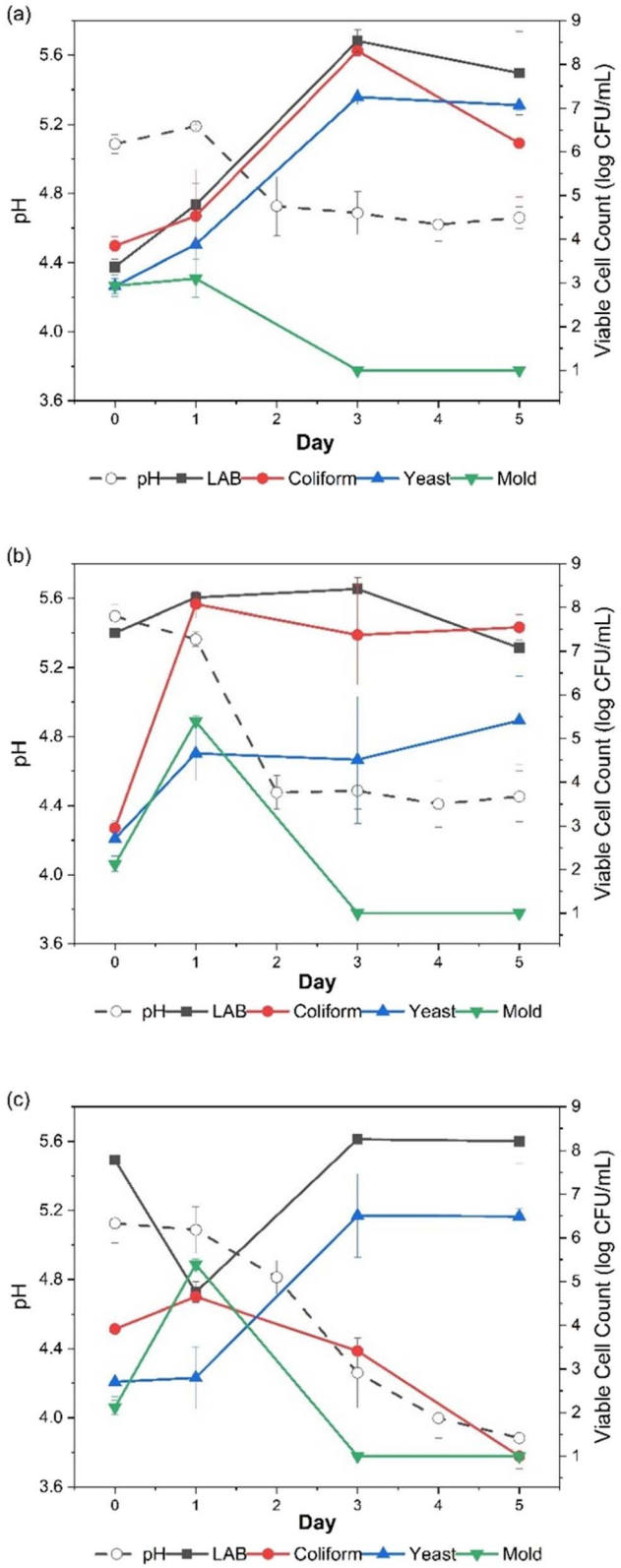

Fig. 1 Microbial and pH changes during fermentation by (a) FAw, (b) FLr, and (c) FAwLr. Note: data points are presented as mean ± standard deviation.

gradually decreased to 4.7 by Day 5, driven by lactic acid content in acid whey. In addition, the natural lactic acid bacteria present in acid whey likely contributed to further lactic acid production during fermentation, enhancing the pH reduction in this treatment. However, in FAwLr, the pH reduction was more pronounced, reaching 4.3 by Day 3 and stabilizing below 4.0 through Day 5, indicating a synergistic effect between *L. rh* and acid whey. In FLr, where acid whey was absent, pH decreased more slowly, stabilizing at 4.5 by Day 5. The absence of acid whey in FLr diminished the rate and extent of acidification compared to FAwLr, demonstrating the critical role of acid whey in enhancing the efficacy of LAB fermentation. FAwLr showed a significantly greater pH reduction than uncontrolled putrefaction, where microbial spoilage causes unregulated proteolysis. FAw supported LAB growth but with slower microbial succession, reinforcing the role of acid whey in fermentation control. LAB-generated organic acids, such as lactic acid, were responsible for reducing pH levels, while acid whey moderated this process by providing buffering capacity and ensuring a gradual decline. These findings emphasize the combined roles of LAB inoculation and acid whey in achieving controlled acidification during fermentation.

3.1.2 LAB. LAB count increased significantly over the fermentation period across all formulations, with notable differences in growth rates and final concentrations. Without starter culture, FAw exhibited significantly lower LAB counts compared to FLr and FAwLr ($p < 0.05$). However, LAB counts in FAw increased rapidly after Day 1, reaching 7.8 log CFU mL^{-1} by Day 5, likely due to the natural LAB present in acid whey. FLr and FAwLr showed similar initial LAB counts (FLr: 7.42; FAwLr: 7.78 log CFU mL^{-1}). LAB counts in FLr peaked at 8.4 log CFU mL^{-1} on Day 3 but declined slightly to 7.0 log CFU mL^{-1} by Day 5, indicating moderate growth. Conversely, FAwLr exhibited the highest LAB counts, with counts peaking at 8.26 log CFU mL^{-1} by Day 3 and maintaining this level through Day 5, despite a significant drop on Day 1. This highlights the synergistic role of acid whey and *L. rh* in enhancing LAB proliferation.

3.1.3 Coliforms. Coliform counts varied significantly across formulations and fermentation days. In FAw, coliforms increased steadily from an initial mean of 3.9 log CFU mL^{-1} on Day 0 to 4.5 log CFU mL^{-1} on Day 1, spiking to 8.3 log CFU mL^{-1} on Day 3 before declining to 6.2 log CFU mL^{-1} by Day 5. These fluctuations suggest that the natural LAB in acid whey was insufficient to consistently suppress coliform growth, particularly under favorable conditions for coliform proliferation on Day 3. In contrast, FAwLr demonstrated a steady decline in coliform counts, with complete inhibition (<1 log CFU mL^{-1}) achieved by Day 5, highlighting the combined inhibitory effects of *L. rh* and acid whey. In FLr, coliform counts rose sharply from 2.95 log CFU mL^{-1} on Day 0 to 8.07 log CFU mL^{-1} on Day 1, remaining above 7.0 log CFU mL^{-1} through Day 5. The absence of acid whey limited coliform suppression in FLr compared to FAwLr, underscoring the importance of acid whey in enhancing antibacterial efficacy.

3.1.4 Yeasts and molds. Monitoring yeast and mold is critical to maintaining fermentation quality, as molds can compromise safety by producing mycotoxins, while yeasts can

synergize with LAB to enhance fermentation.[36] In FAw, yeast counts increased significantly from 2.93 log CFU mL^{-1} on Day 0 to 7.07 log CFU mL^{-1} by Day 5, paralleling LAB growth. This trend suggests that natural LAB and yeast in acid whey synergistically promote yeast proliferation. Mold counts, initially detectable, fell below the detection limit (<1 log CFU mL^{-1}) after Day 3, likely due to inhibitory effects of natural LAB in acid whey. In FAwLr, yeast counts significantly rose from 2.70 log CFU mL^{-1} on Day 0 to 6.49 log CFU mL^{-1} by Day 5. Enhanced LAB growth in this formulation indirectly supported yeast growth by inhibiting molds and coliforms and stabilizing the pH. Mold counts in FAwLr dropped below the detection limit after Day 3 and remained undetectable, highlighting the combined effects of LAB and acid whey. In contrast, yeast counts in FLr increased modestly from 2.71 log CFU mL^{-1} on Day 0 to 5.41 log CFU mL^{-1} by Day 5, reflecting less pronounced growth due to the absence of acid whey.

The absence of acid whey in FLr limited the availability of nutrients, resulting in less pronounced yeast growth compared to FAw and FAwLr. Mold counts followed a similar trend to FAw and FAwLr, being detectable at the start of the fermentation but dropping below the detection limit after Day 3, likely due to the inhibitory activity of *L. rh*. The combination of acid whey and *L. rh* created more favorable conditions for yeast growth while effectively suppressing mold and coliform populations. In contrast, FLr demonstrated limited capacity to support yeast growth without the additional nutrients and growth factors from acid whey. These results suggest that LAB and yeast co-ferment, contributing complementary metabolic activities to the fermentation process.

Fish byproducts alone undergo proteolysis and spoilage, while acid whey promotes mold growth. However, their combination under LAB fermentation with a starter culture established a controlled fermentation process, evident in FAwLr's rapid pH drop, LAB growth, coliform suppression, and mold inhibition. In FAw, the absence of *L. rh* slowed LAB proliferation, though acid whey supported natural LAB growth and acidification, consistent with findings by Lasrado & Rai.[9] Acid whey's contribution to acidification aligns with studies on fish silage preservation, where LAB-produced organic acids such as lactic, acetic, succinic, and propionic acids produced by LAB were key drivers of pH reduction.[37] The rapid pH declines in FAwLr to below 4.5 are consistent with similar studies on LAB fermentation of fish byproducts,[38] demonstrating its efficacy in creating an inhospitable environment for spoilage microorganisms.

The suppression of coliforms and molds further corroborates the antibacterial effects of LAB fermentation. By Day 5, coliform counts in FAwLr dropped below detection limits, consistent with studies where LAB fermentation eliminated coliforms and *E. coli* within days.[37,39] These results underscore the efficacy of combining acid whey and LAB to enhance microbial safety in non-sterile fermentation systems. Additionally, the acidifying and nutrient-enriching properties of acid whey align with findings from studies on fish byproducts and molasses, where synergistic substrate–LAB interactions improved microbial suppression and product preservation.[40] The dual role of acid whey as a nutrient source and acidifying agent in FAwLr was instrumental in controlling spoilage microorganisms.[41]

The growth of yeast, particularly in FAw and FAwLr, suggests a potential co-fermentation process. In natural fermentation systems, various interactions between LAB and yeast have been reported, including both synergistic and antagonistic effects.[42,43] A previous study demonstrated that interactions between LAB and yeasts such as *Debaryomyces hansenii* and *Candida* spp. can enhance fermentation and inhibit spoilage organisms.[36] LAB can promote yeast growth through the production of metabolites such as carbon dioxide and organic acids, while yeast can enhance LAB growth by supplying essential vitamins and amino acids.[43] Conversely, certain LAB-derived compounds (*e.g.*, 4-hydroxy-phenyl lactic acid) and yeast-produced metabolites (*e.g.*, ethanol and fatty acids) may exert inhibitory effects on one another.[44,45] While specific metabolites were not analyzed in this study, the observed yeast-LAB co-growth in FAw and FAwLr suggests that such interactions may have influenced fermentation dynamics in this system.

3.2 Degree of hydrolysis (DH)

DH increased in all formulations (FAw, FLr, and FAwLr) during fermentation, demonstrating active protein hydrolysis by enzymatic and microbial processes (**Table 2**). On Day 0, no significant differences ($p > 0.05$) in DH were observed between FLr (9.86 ± 0.98%), FAw (7.25 ± 1.07%), and FAwLr (6.43 ± 0.29%), suggesting minimal initial hydrolysis and comparable baseline protein structures. By Day 1, significant increases in DH were evident, with FLr achieving the highest value (36.57 ± 2.64%), significantly greater than FAw (22.13 ± 1.67%) and FAwLr (25.19 ± 4.30%) ($p < 0.05$). The absence of acid whey in FLr likely resulted in a less buffered environment, accelerating protein degradation by LAB. In contrast, the buffering capacity of acid whey in FAwLr appeared to modulate hydrolysis, leading to a more gradual protein breakdown.

At the end of fermentations, DH values stabilized, with FLr achieving the highest DH on Day 5 (69.89 ± 5.77%), which is not significantly different from Day 3 (64.17 ± 2.12%). FAw increased significantly to 50.96 ± 5.81%, while FAwLr slightly declined to 39.66 ± 6.57% ($p > 0.05$), suggesting substrate

Table 2 Degree of hydrolysis (%) across different formulations (FAw, FLr, and FAwLr) over a five-day fermentation period[a]

Day	FAw	FLr	FAwLr
0	7.25 ± 1.07^{dA}	9.86 ± 0.98^{cA}	6.43 ± 0.29^{cA}
1	22.13 ± 1.67^{cB}	36.57 ± 2.64^{bA}	25.19 ± 4.30^{bB}
3	37.90 ± 4.75^{bB}	64.17 ± 2.12^{aA}	41.25 ± 7.52^{aB}
5	50.96 ± 5.8^{aB}	69.89 ± 5.77^{aA}	39.66 ± 6.57^{aC}

[a] Values are presented as mean ± standard deviation. Different lowercase superscripts within a column indicate significant differences ($p < 0.05$) among days in specific formulations; different uppercase superscripts within a row indicate significant differences ($p < 0.05$) among formulations on specific days.

depletion or equilibrium between hydrolysis and microbial activity in FAwLr. In FAw, ongoing enzymatic activity in the absence of LAB inoculum likely drove the continued increase. These results demonstrate that *L. rh* inoculation (FLr and FAwLr) significantly enhances protein hydrolysis compared to natural fermentation (FAw). Overall, these findings highlight the importance of substrate composition and microbial interactions in achieving desired hydrolysis outcomes during fermentation. Similar results were reported by Ruthu *et al.*, where the fermentation of fish heads with lactic acid bacteria resulted in a DH range of 29–38.4% by the end of the fermentation period, depending on the LAB isolates used.[46] Likewise, Khiari & Mason demonstrated that fish bycatch fermented with lactic acid bacteria exhibited a rapid increase in DH within the first three days, stabilizing thereafter and reaching over 40%.[47] Moreover, the DH curve observed in the present study follows a typical pattern of enzymatic hydrolysis in fish-based substrates, characterized by an initial rapid hydrolysis phase followed by a plateau as substrate availability and enzymatic activity reach equilibrium.

3.3 Moisture content

Moisture contents (**Table 3**) across all formulations (FAw, FLr, and FAwLr) remained relatively stable throughout the fermentation, with minor fluctuations. On Day 0, FLr (70.5 ± 2.65%) exhibited significantly higher moisture content compared to FAwLr (66.9 ± 2.66%), while FAw (68.7 ± 1.29%) was intermediate. The observed differences in initial moisture content are due to the distinct composition of each formulation at the start of formulation. The slightly lower moisture levels in formulations containing acid whey (FAw and FAwLr) can be attributed to the total solids content of acid whey, which typically ranges from 3.3% to 6.5%.[21] By Day 1, the difference in moisture content across all formulations diminished, stabilizing at approximately 70% with no significant variation ($p > 0.05$). This stabilization underscores the system's capacity to maintain consistent moisture levels, which is essential for ensuring product uniformity and minimizing spoilage risks. A balanced moisture content during fermentation is critical for preventing undesirable microbial activity and maintaining the quality and safety of the final product, consistent with observations from silage fermentation studies.[48]

Table 3 Moisture content of the fish–whey mixture in different formulations across the fermentation period[a]

Day	FAw	FLr	FAwLr
0	68.69 ± 1.29^{AB}	70.46 ± 2.65^{A}	66.88 ± 2.66^{B}
1	68.96 ± 2.93^{A}	70.66 ± 2.00^{A}	68.04 ± 3.37^{A}
3	69.10 ± 1.69^{A}	70.91 ± 2.99^{A}	68.95 ± 2.10^{A}
5	68.87 ± 2.99^{A}	72.05 ± 2.46^{A}	69.93 ± 3.43^{A}

[a] Values are presented as mean ± standard deviations. Different uppercase superscripts on the same row indicate significant differences ($p < 0.05$) among formulations on specific days. No significant difference ($p > 0.05$) was found in the same formulations across days.

3.4 Viscosity

The viscosity profiles of all formulations (FAw, FLr, and FAwLr) exhibited shear-thinning behavior, *i.e.*, a decrease in viscosity with increasing shear rate (**Fig. 2**). Since moisture content remained relatively stable across formulations during fermentation (Section 3.3), the observed viscosity changes can be primarily attributed to protein hydrolysis and structural breakdown, rather than to differences in moisture. On Day 0, FAw (Fig. 2a) displayed the lowest viscosity compared to FAwLr (Fig. 2c) and FLr (Fig. 2b), which may be attributed to the absence of *L. rh*. The presence of *L. rhamnosus* in FLr and FAwLr may have initiated early hydrolysis shortly after inoculation, increasing the breakdown of protein structures and contributing to the observed higher initial viscosity in those treatments. A limitation of the experimental setup for viscosity analysis was the time lag between sample collection and measurement. After samples were collected, microbial plating was carried out first, delaying viscosity testing by approximately two hours. During this interval, the high viable cell count of the inoculated cultures (10^8 CFU g^{-1}) might have initiated microbial activity, including hydrolysis, potentially altering the viscosity before measurement. This delay could have slightly underestimated the initial viscosity of the inoculated formulations, particularly FAwLr and FLr, due to early-stage microbial-driven hydrolysis.

During fermentation, viscosity decreased across all formulations by Day 1, with the largest reductions observed in FAwLr and FLr, which contained the inoculum *L. rh*, potentially due to protein hydrolysis and structural breakdown induced by the enhanced LAB fermentation. By Day 5, all formulations reached stabilized viscosities, indicating the completion of enzymatic hydrolysis and structural breakdown of high-molecular-weight compounds. These trends are consistent with the natural progression of fermentations, where LAB enzymes degrade proteins and polysaccharides, leading to liquefaction.[49]

The power-law consistency index k and the flow behavior index n further validated the samples' shear-thinning behavior (**Table 4**). On Day 0, formulations containing *L. rh* (FAwLr and FLr) had significantly higher k values, suggesting formulations with greater structural integrity compared to FAw. As fermentation progressed, k values decreased across all formulations, with the most substantial reductions in FLr (from 0.33 Pa s^{-n} on Day 0 to 0.013 Pa s^{-n} on Day 5), followed by FAwLr (from 0.26 Pa s^{-n} to 0.021 Pa s^{-n}). The decline in k reflects the extensive hydrolysis of macromolecules during fermentation, mirroring the viscosity reduction trends.

Conversely, n values increased significantly after Day 3 for all formulations, indicating a shift toward less shear-thinning behavior. This shift reflects the progressive breakdown of complex macromolecules into smaller, more homogeneous components. By the end of fermentation (Days 3 and 5), no significant differences in n values were observed among all formulations, suggesting that the hydrolysis process had reached a stable state. The increase in n values across all formulations reflects a reduction in structural complexity, with the system transitioning to a solution state less dependent on

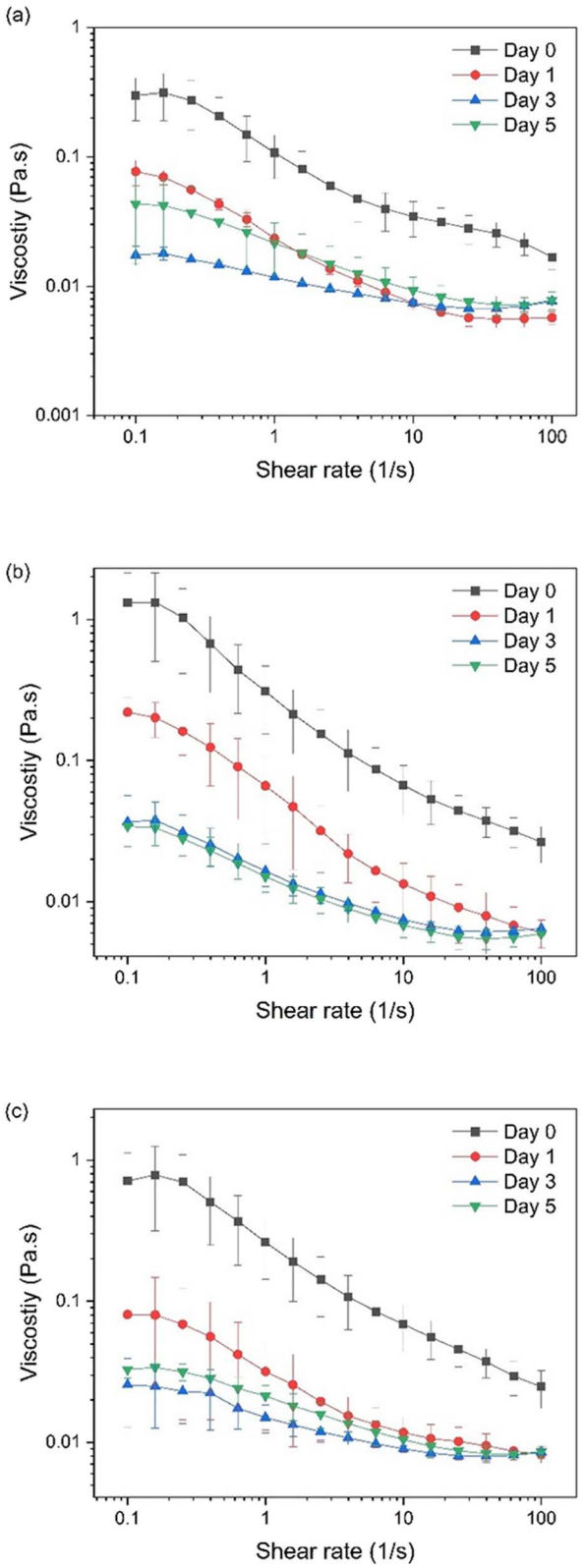

Fig. 2 Apparent viscosity of fermented fish-whey in different formulations: (a) FAw, (b) FLr, and (c) FAwLr on different days. Data points are presented as mean ± standard deviation.

the alignment of the molecules (Newtonian behavior). Differences in rheological behavior among formulations underscore the critical role of acid whey in modulating the texture and flow properties of fermented products, as its presence not only enhances microbial activity but also provides structural stability, as evidenced by the slower decline in k values for FAwLr. These results are consistent with previous reports of protein and polysaccharide hydrolysis during fermentation, a process often referred to as liquefaction in fish silage.[48] The relatively higher k value (Day 5) in FAwLr highlights the role of acid whey and *L. rh* in maintaining structural integrity by contributing proteins and buffering capacity, which delayed extensive degradation. In contrast, the rapid decrease in k values in FLr underscores that the absence of acid whey makes the system more susceptible to breakdown by microbial activity.

3.5 Antioxidant activity

To evaluate the antioxidant potential of FPH samples from all formulations during fermentation, four complementary assays were performed.

3.5.1 DPPH. The DPPH assay is based on the reduction of the stable, purple DPPH radical to a yellow compound (diphenylpicrylhydrazine) upon reaction with antioxidants.[50] The Trolox equivalent antioxidant capacity (TEAC, μmol TE g^{-1} FPH) of all formulations measured with the DPPH assay over the fermentation period is illustrated in **Fig. 3a**. At Day 0, FLr exhibited the highest TEAC (7.95 μmol TE g^{-1} FPH), significantly greater than FAw (5.26 μmol TE g^{-1} FPH) and FAwLr (5.45 μmol TE g^{-1} FPH). The absence of acid whey in FLr likely facilitated direct enzymatic activity, enhancing antioxidant release. During fermentation, TEAC significantly increased ($p < 0.05$) in FAw and FAwLr, peaking by Day 3 (FAw: 7.34 μmol TE g^{-1} FPH and FAwLr: 7.46 μmol TE g^{-1} FPH) and stabilizing by Day 5 (FAw: 7.62 μmol TE g^{-1} FPH and FAwLr: 7.24 μmol TE g^{-1} FPH). FLr maintained consistently high TEAC throughout, with minimal variation between Day 0 and Day 5. These results indicate that, by the end of the fermentation process, the natural LAB activity in FAw achieved an antioxidant activity level comparable to that of FAwLr. This suggests that the fermentation process driven by acid whey in FAw was sufficient to compensate for the absence of starter culture, eventually generating similar bioactive antioxidant compounds.

Peng *et al.* reported that whey protein isolate hydrolysates prepared with Alcalase demonstrated increased antioxidant activity in the DPPH assay as hydrolysis time progressed.[51] The increased TEAC in FAw and FAwLr may be due to the presence of whey proteins in acid whey, which undergo structural modifications during fermentation. The fermentation process may disrupt the native whey protein structure, unfolding the molecules and exposing amino acid residues with electron-donating properties. This increased availability of reactive sites could enhance the proteins' ability to quench free radicals, stabilizing them and terminating the free radical chain reaction.[51] Our results also highlight the balancing effect of acid whey on hydrolysis dynamics and its capacity to support natural

Table 4 *k* value and *n* value of the fish–whey fermented mixture in different formulations through the fermentation period[a]

	k value (Pa s^n)			*n* value		
Day	FAw	FLr	FAwLr	FAw	FLr	FAwLr
0	0.114 ± 0.041^{aB}	0.331 ± 0.165^{aA}	0.260 ± 0.123^{aA}	0.558 ± 0.037^{bA}	0.408 ± 0.058^{bB}	0.478 ± 0.062^{cAB}
1	0.025 ± 0.003^{aA}	0.048 ± 0.029^{bA}	0.033 ± 0.020^{bA}	0.571 ± 0.013^{bAB}	0.492 ± 0.212^{bB}	0.686 ± 0.157^{bA}
3	0.016 ± 0.008^{aA}	0.017 ± 0.004^{bA}	0.016 ± 0.004^{bA}	0.818 ± 0.057^{aA}	0.714 ± 0.079^{aA}	0.823 ± 0.088^{aA}
5	0.024 ± 0.009^{aA}	0.013 ± 0.007^{bA}	0.021 ± 0.003^{bA}	0.715 ± 0.062^{aA}	0.704 ± 0.030^{aA}	0.759 ± 0.024^{abA}

[a] Values are presented as mean ± standard deviation. In *k*-value columns/rows, different lowercase superscripts within a column indicate significant differences ($p < 0.05$) among days in specific formulations; different uppercase superscripts within a row indicate significant differences ($p < 0.05$) among formulations on specific days. In *n*-value columns/rows, different lowercase superscripts within a column indicate significant differences ($p < 0.05$) among days in specific formulations; different uppercase superscripts within a row indicate significant differences ($p < 0.05$) among formulations on specific days.

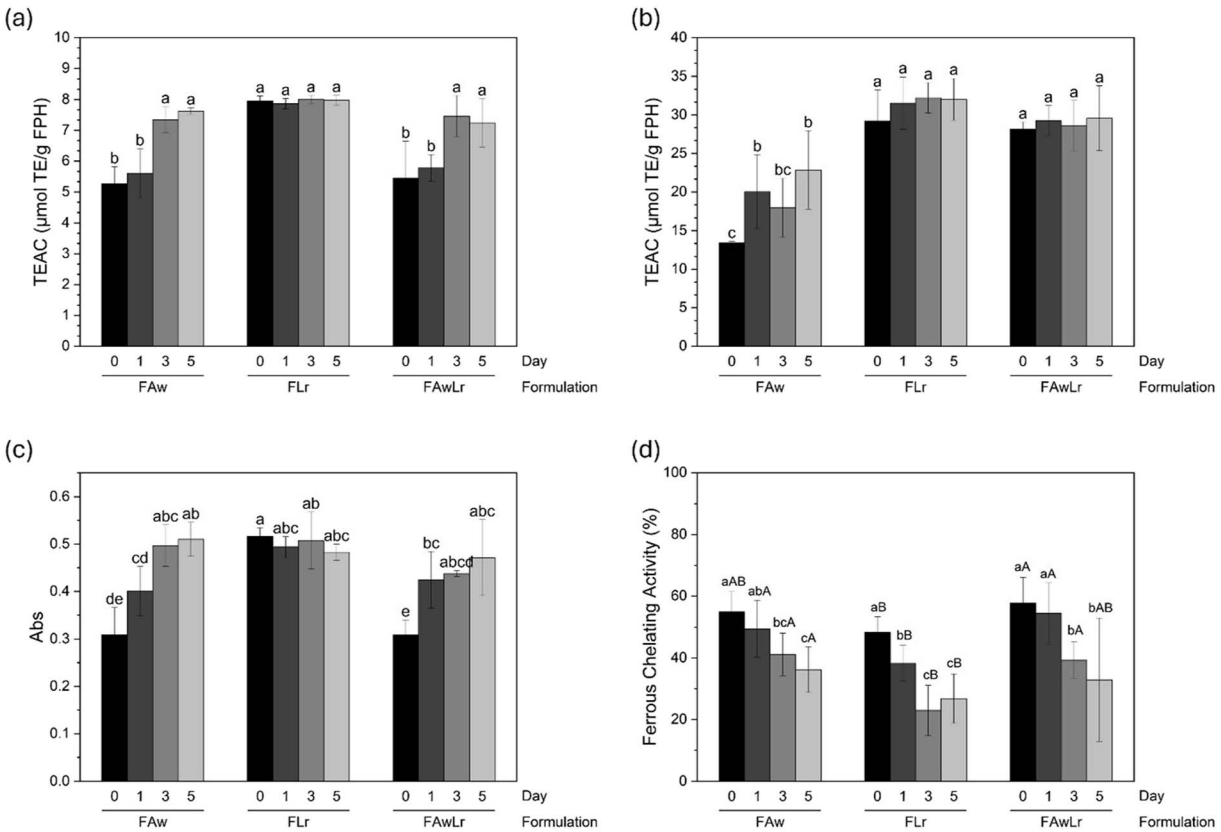

Fig. 3 Antioxidant activity of fish protein hydrolysates (FPH) with different assays: (a) DPPH, (b) ABTS, (c) FRAP, and (d) ferrous ion chelating activity on different days. Note: results for (a) and (b) are expressed as Trolox Equivalent Antioxidant Capacity (TEAC, μmol TE g^{-1} FPH) and presented as mean ± standard deviation. Results for (c) are presented as absorbance at 593 nm (mean ± standard deviation). Results for (d) are presented as % chelation activity (mean ± standard deviation). For (a)–(c), different lowercase superscripts indicate significant differences ($p < 0.05$). For (d), different lowercase superscripts indicate significant differences ($p < 0.05$) among days in the same formulation; different uppercase superscripts indicate significant differences ($p < 0.05$) among formulations on the same day.

LAB growth and bioactive compound generation. Overall, the results confirm that fermentation significantly enhances the antioxidant activity of FPH, with both acid whey and *L. rh* contributing to this improvement. The lack of a significant increase in TEAC between Day 3 and Day 5 across all formulations suggests that the production of bioactive compounds had reached an equilibrium by this stage of fermentation.

3.5.2 ABTS. The Trolox equivalent antioxidant capacity (TEAC, μmol TE g^{-1} FPH) of the formulations with the ABTS assay was monitored over the fermentation period (**Fig. 3b**). At Day 0, FLr and FAwLr exhibited significantly higher TEAC values (FLr: 29.2 μmol TE g^{-1} FPH and FAwLr: 28.17 μmol TE g^{-1} FPH) than FAw (13.43 μmol TE g^{-1} FPH), indicating enhanced antioxidant capacity provided by *L. rh*. In addition, the higher baseline TEAC in the *L. rh*-inoculated formulations (FLr and FAwLr) may also be influenced by antioxidant-active components introduced with the LAB starter culture. These may include intracellular components released from lysed cells,

such as antioxidant enzymes and other bioactive substances,[52] as well as LAB-derived exopolysaccharides,[53] both of which are known to contribute to antioxidant activity. During fermentation, all formulations showed significant TEAC increases over time. By Day 1, FLr and FAwLr reached peak TEAC levels (29.20 μmol TE g^{-1} FPH and 29.27 μmol TE g^{-1} FPH, respectively) with no significant differences observed, suggesting starter culture-driven protein hydrolysis and the release of components with antioxidant capacities. At the end of the fermentation period (Day 5), FLr achieved the highest (32.02 μmol TE g^{-1} FPH) antioxidant capacity, followed by FAwLr (29.57 μmol TE g^{-1} FPH) and FAw (22.85 μmol TE g^{-1} FPH), suggesting the absence of acid whey in FLr allows for more extensive and direct enzymatic activity without interference from whey proteins or buffering effects. In contrast, FAwLr showed no significant change in TEAC between Days 1, 3, and 5, suggesting that acid whey contributes to a more gradual release of antioxidant peptides or stabilizes antioxidant compounds earlier in the fermentation process. The significant increase in TEAC for FAw after Day 1 indicates that natural fermentation eventually releases antioxidant compounds, albeit at a slower rate compared to the LAB-inoculated formulations. These results highlight the key role of *L. rh* in accelerating enzymatic hydrolysis and peptide release, as well as the modulating effects of acid whey in controlling the rate and extent of antioxidant activity.

3.5.3 Ferric reducing antioxidant power (FRAP). The FRAP assay measures the ability of antioxidants to reduce Fe^{3+} (ferric) to Fe^{2+} (ferrous), with higher absorbance indicating greater reducing power. **Fig. 3c** illustrates that at Day 0, FLr exhibited the highest value (0.52), significantly greater than FAw (0.31) and FAwLr (0.31). This suggests a strong initial reducing power in FLr without acid whey, confirming the buffering effect of acid whey and its role in slowing down the release of reducing compounds at this stage. However, upon fermentation, FAw and FAwLr showed notable increases in FRAP values. In FAw, the increase was steady over time, reflecting the slower and natural fermentation process where endogenous enzymes gradually hydrolyze proteins to release antioxidant compounds. In contrast, FAwLr demonstrated a significant increase in FRAP values between Day 0 and Day 1, followed by a steady maintenance of similar values until the end of fermentation. This could be due to the structural alternation of whey protein in acid whey induced by pH shift, binding affinity, and the generation of phenolic compounds during the fermentation process.[54] FLr maintained consistently high FRAP values, with no significant changes over time. These results suggest that acid whey moderates the release of reducing compounds, supporting a controlled hydrolysis process. At the end of fermentation, FAw (0.51) exhibited the highest FRAP value, closely followed by FLr (0.48) and FAwLr (0.47). The findings indicate that while *L. rh* accelerates the hydrolysis process, acid whey modulates and stabilizes the release of antioxidant compounds.

3.5.4 Ferrous ion chelating activity. Ferrous ion (Fe^{2+}) chelating activity was measured to assess the ability of FPH to chelate Fe^{2+} ions. Overall, a decrease in the chelating activity across fermentation was observed (**Fig. 3d**). At Day 0, FAw (54.9%) and FAwLr (57.7%) exhibited the highest chelating activity than FLr (48.3%), reflecting acid whey's inherent metal-binding properties. Whey proteins, such as lactoferrin, are known for their ability to bind metal ions, which can help maintain chelating activity during fermentation.[55] By Day 5, all formulations showed reduced activity (FAw: 36.2%; FAwLr: 32.9%; FLr: 26.8%). The significant decrease in chelating activity over time, particularly in FLr, could be attributed to the degradation of chelating compounds during fermentation or the transformation of bioactive compounds into other functional forms that do not contribute to metal-binding properties.[56] The presence of acid whey in FAw and FAwLr appeared to moderate this decline, suggesting a buffering effect in maintaining metal ion-binding compounds during fermentation. Metal–protein interactions could also be affected by pH due to the changes in the protonation state of amino acid residues within the protein.[55] Especially in an acidic environment, the attractive forces involved in metal binding may be weakened due to the protonation of these metal–protein complexes.[57]

3.6 Antibacterial activity

3.6.1 Inhibition growth curve. Growth inhibition against *Listeria innocua* and *Escherichia coli* by FAw, FLr, and FAwLr across fermentation days (Day 0, 1, 3, and 5) was evaluated using their growth curves (Fig. S1). These inhibition growth curves are presented as visual representations of bacterial growth trends over time. No statistical comparisons were performed for these time-series curves, as quantitative antibacterial effects were evaluated separately using MIC, MBC, and inhibition zone measurements. Negative controls (sterile water alone) followed a typical exponential growth pattern, reaching stationary phases at 5 h for *L. innocua* and 8 h for *E. coli*. Positive controls (nisin for *L. innocua* and ampicillin for *E. coli*) showed consistent growth inhibition.

Strong inhibition of both bacteria was observed in FAw across the fermentation days, with slightly reduced inhibition at Day 0, suggesting inherent antibacterial properties of acid whey, even without fermentation. In FAwLr, the inhibition was similar to FAw, indicating that the addition of *L. rh* did not enhance early antibacterial effects, likely due to acid whey's dominant role at the initial stage. In FLr, no inhibition was observed on Day 0 and Day 1, as bacterial growth followed an exponential trend. However, by Day 3, strong inhibition was evident, attributed to pH reduction and the production of antibacterial hydrolysates during fermentation.

3.6.2 Agar well diffusion method. The antibacterial activity of FPH was evaluated using the agar well diffusion method alongside MIC and MBC assays against *L. innocua* and *E. coli*. The results (**Table 5**) revealed notable differences in antibacterial activity across formulations (FAw, FLr, and FAwLr) and days (Day 0 to Day 5). Representative images of inhibition zones of fish protein hydrolysates are shown in SI, **Fig. S2**.

DIZ values, which indicate bacterial growth inhibition zones, varied significantly between formulations and over time. For *L. innocua*, FAw demonstrated inhibition across all fermentation days, with significantly larger inhibition zones on Day 3 (11.3 ± 0.06 mm) and Day 5 (11.7 ± 0.07 mm) compared to Day 0 (10.2 ±

Table 5 Diameter of inhibition zone (mm), minimum inhibitory concentration (mg mL^{-1}), and minimum bacterial concentration (mg mL^{-1}) of freeze-dried samples against indicator bacteria[a]

		Diameter of inhibition zone (mm)			MIC (mg mL^{-1})			MBC (mg mL^{-1})		
	Day	FAw	FLr	FAwLr	FAw	FLr	FAwLr	FAw	FLr	FAwLr
Listeria innocua	0	10.20 ± 0.21^{ef}	ND	9.58 ± 0.09 ^{fg}	100	200	100	>200	>200	>200
	1	10.90 ± 0.07^{de}	9.75 ± 0.47^{g}	10.13 ± 0.07^{f}	25	100	25	>200	>200	>200
	3	11.32 ± 0.06 ^{cd}	13.36 ± 0.13 ^{ab}	12.70 ± 0.12^{b}	12.5	12.5	12.5	>200	100	50
	5	11.73 ± 0.07^{c}	13.42 ± 0.13 ^{ab}	13.48 ± 0.09^{a}	6.25	12.5	6.25	200	100	50
E. coli BL21	0	ND	ND	ND	>200	>200	> 200	>200	>200	>200
	1	ND	ND	ND	50	50	25	>200	>200	>200
	3	ND	9.27 ± 0.18^{b}	9.75 ± 0.38^{b}	12.5	6.25	6.25	200	50	50
	5	ND	9.52 ± 0.19^{b}	11.53 ± 0.63^{a}	12.5	6.25	6.25	100	50	25

[a] Values are presented as mean ± standard deviation. The diameter of the agar well is 9 mm. Different lowercase superscripts indicate significant differences ($p < 0.05$) across treatments.

0.21 mm). This suggests inherent antibacterial properties of acid whey, attributed to its low pH and bioactive components such as organic acids and peptides. FAwLr exhibited a steady and more pronounced increase in DIZ, starting at 9.6 ± 0.09 mm (Day 0) and reaching 13.5 ± 0.09 mm (Day 5). This highlights the synergistic effect of acid whey and *L. rh*, accelerating fermentation and enhancing the generation of bioactive antibacterial compounds. In contrast, FLr showed no inhibition on Day 0 and minimal inhibition on Day 1 (9.8 ± 0.47 mm). However, by Day 3 (13.4 ± 0.13 mm) and Day 5 (13.4 ± 0.13 mm), a significant inhibition zone emerged, indicating that the absence of acid whey delayed the production of bioactive antibacterial compounds. Notably, after Day 3, DIZ values in FLr and FAwLr surpassed those in FAw, underscoring the critical role of *L. rh* in promoting hydrolysis and efficiently releasing bioactive peptides. By Day 5, no significant difference was observed between FLr and FAwLr, suggesting that both formulations reached their maximum antibacterial potential as fermentation progressed. The DIZ results demonstrate the inhibitory effects of acid whey and fermentation dynamics, with a sensitivity not fully captured by the growth curve assay. For example, while FAw's growth curve suggested similar inhibition effects from Day 0 to Day 5, DIZ values showed significant enhancement by Day 5.

For *E. coli*, FAw exhibited no inhibition zones throughout fermentation, indicating that acid whey's inherent antibacterial effects are selective for Gram-positive bacteria such as *L. innocua*. This selectivity can be attributed to the structural resistance of Gram-negative bacteria, particularly their outer membrane, which acts as a barrier to bioactive compounds.[58] However, when *L. rh* was introduced, inhibition against *E. coli* became evident, especially on Day 3 and Day 5. FAwLr exhibited the largest inhibition zone (11.5 ± 0.63 mm) on Day 5, significantly surpassing both Day 3 and the corresponding FLr values. This enhanced activity may result from the combined effects of acid whey and *L. rh*, producing bioactive compounds capable of chelating ions or disrupting bacterial membranes to overcome Gram-negative resistance.

Overall, the results highlight acid whey's pivotal role in early antibacterial activity against *L. innocua*, with *L. rh* inoculation amplifying and accelerating these effects during fermentation. The delayed response against *E. coli* underscores the importance of overcoming Gram-negative structural resistance through extended fermentation and optimized bioactive compound production. Furthermore, the DIZ assay's ability to differentiate antibacterial activity across fermentation days complements the growth curve results, providing a more nuanced understanding of FPH's antibacterial potential.

3.6.3 Minimum inhibition concentration (MIC). MIC and MBC results (**Table 5**) provide additional insights into bacterial inhibition and killing by exposing bacteria to serially diluted FPH dispersions, leading to a more precise determination of the minimal effective concentration. MIC reflects the lowest FPH concentration required to inhibit visible bacterial growth. In FAw, MIC values of 100 mg mL^{-1} on Day 0 against *L. innocua* suggest acid whey's inherent antibacterial activity, likely due to its acidity and residual compounds. MIC values decreased gradually during fermentation, reaching 6.25 mg mL^{-1} by Day 5, indicating bioactive compound generation. In contrast, in FLr, MIC was >200 mg mL^{-1} on Day 0 but dropped to 12.5 mg mL^{-1} by Day 3 and remained stable, showing a time-dependent effect of *L. rh* inoculation in producing inhibitory compounds in the absence of acid whey. For FAwLr, MIC was 100 mg mL^{-1} on Day 0, similar to FAw, indicating the immediate effect of acid whey, but decreased to 6.25 mg mL^{-1} by Day 5, highlighting the synergistic effects of acid whey and *L. rh*.

MIC values against *E. coli*, in contrast, were consistently higher across all formulations, exceeding 200 mg mL^{-1} on Day 0, aligning with the absence of inhibition zones in the agar well diffusion method. This result reinforces the limited antibacterial efficacy of FPH against Gram-negative bacteria. However, by Day 3, MIC values decreased to 12.5 mg mL^{-1} in FAw and 6.25 mg mL^{-1} in FLr and FAwLr, indicating that bioactive compounds generated during fermentation had limited but measurable inhibitory effects at later fermentation stages.

3.6.4 Minimum bactericidal concentration (MBC). MBC, the lowest concentration required to kill 99.9% of bacterial cells, aligned with the MIC trend. For *L. innocua*, MBC values in FAw reduced from >200 mg mL^{-1} (Day 0) to 200 mg mL^{-1} (Day 5). FLr showed no bactericidal effect on Day 0 and Day 1 (>200

mg mL^{-1}) but dropped significantly to 100 mg mL^{-1} by Day 3 and Day 5. FAwLr exhibited the largest effective inhibition of bactericidal activity, with MBC decreasing from >200 mg mL^{-1} on Day 0 to 50 mg mL^{-1} by Day 3 and Day 5. For *E. coli*, MBC values remained above 200 mg mL^{-1} across all formulations on Days 0 and 1, indicating that while some inhibitory effects were observed, complete bacterial killing was not achieved. However, MBC values further dropped to 100 mg mL^{-1} in FAw, 50 mg mL^{-1} in FLr, and 25 mg mL^{-1} in FAwLr on Day 5.

MIC and MBC results confirm that fermentation enhances antibacterial activity, particularly in FAwLr, where acid whey and *L. rh* synergistically accelerate bioactive compound production. Acid whey contributes to early antibacterial effects in FAw and FAwLr, while FLr shows delayed activity, indicating that *L. rh* generates antimicrobial compounds over time. *L. innocua* (Gram-positive) is more susceptible to FPH than *E. coli* (Gram-negative), likely due to the absence of an outer membrane. Combining acid whey and *L. rh* is an effective strategy to enhance antibacterial properties, particularly against Gram-positive bacteria.

LAB fermentation also plays a crucial role in food safety by combating contamination from pathogenic microorganisms and fungi.[17] It achieves this through enzymatic hydrolysis of toxic fungal compounds and the production of antibacterial substances that inhibit the growth of foodborne pathogens.[59,60] Among these antibacterial compounds, bacteriocins, ribosomally synthesized antimicrobial peptides, are particularly effective against pathogenic bacteria.[61] These bioactive peptides target bacterial membranes, leading to cell lysis and death, while LAB-produced organic acids, such as lactic acid, further enhance antimicrobial effects by lowering pH and destabilizing bacterial structures.

Peptides derived from whey protein through LAB fermentation exhibit notable antibacterial activity by disrupting bacterial membranes and inhibiting microbial growth.[62] Papademas & Kotsaki highlighted that the bioactive peptides generated from whey not only possess antibacterial properties but also demonstrate anti-inflammatory and antioxidant activities, making them valuable functional ingredients for food and nutraceutical applications.[63] Similarly, research by Hati *et al.* identified specific peptides from whey protein hydrolysates with potent antimicrobial activity against foodborne pathogens, further supporting the role of LAB fermentation in enhancing the functional properties of whey-derived bioactive compounds.[62]

The production of antibacterial compounds by LAB during the fermentation of fish byproducts has been well-documented. Sahnouni *et al.* reported that bacterial strains isolated from the gastrointestinal tracts of Atlantic horse mackerel, European pilchard, and Atlantic bonito exhibited antibacterial activity against *E. coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *L. innocua*, *Salmonella* spp., and *Vibrio* spp.[64] Likewise, Rai *et al.* found that LAB strains isolated from fermented fish products produced bacteriocins and organic acids that effectively inhibited the growth of *E. coli*, *Micrococcus luteus*, *Salmonella enteritidis*, *Salmonella typhi*, and *Listeria murreyi*.[65] Their study further showed that antimicrobial activity increased over time, suggesting that the accumulation of bioactive peptides and organic acids plays a key role in pathogen inhibition.

These findings confirm that LAB fermentation of fish byproducts generates antibacterial compounds effective against foodborne pathogens. FAwLr, combining acid whey and *L. rh*, showed enhanced inhibition of *L. innocua*, likely due to synergistic bacteriocins and organic acids. This highlights the potential of LAB-fermented fish byproducts as natural antibacterial agents for food preservation.

3.7 General discussion, limitations, and future perspectives

Some degree of variability is inherent in biological byproducts such as fish byproducts and acid whey, depending on factors such as species, harvesting season, and processing methods. To minimize compositional differences, both fish byproducts and acid whey used in this study were sourced from single batches, and all fermentations were carried out in biological replicates under controlled conditions. Although batch-to-batch variation across different raw material sources was not assessed, the consistent trends observed across replicates, such as pH reduction, LAB proliferation, and mold suppression, suggest minimal variability within this experimental setup. Future studies should evaluate variability across different raw material sources and production seasons to support scalability and broader industrial applicability.

While this study demonstrated promising results at the laboratory scale, further investigation is needed to evaluate the long-term stability and scalability of the fermented hydrolysates. Storage stability, including microbial safety, antioxidant activity retention, and potential for phase separation, should be examined under different processing and packaging conditions. Additionally, scaling up the fermentation process would require optimization of operational parameters such as mixing efficiency, oxygen control, and temperature uniformity. Addressing these factors in future research will be essential for advancing this valorization strategy toward industrial implementation.

In addition, this study did not evaluate the microbiological status of individual raw materials (fish byproducts and acid whey) prior to blending. Although Day 0 microbial counts captured the total load of the combined mixture for each formulation, they do not reveal the specific microbial contributions from each component. This limits our ability to fully interpret the dynamics of spontaneous fermentation, particularly in the uninoculated FAw group.

Furthermore, the fermented samples were not neutralized prior to antimicrobial activity testing. As a result, the observed inhibition may be partially or predominantly attributed to organic acids rather than antimicrobial peptides or bacteriocins. While our goal was to capture the overall antibacterial potential of the hydrolysates, future studies should incorporate neutralization or isolate specific compounds to clarify their individual contributions to bioactivity.

4. Conclusions

This study demonstrates an innovative, sustainable approach for valorizing fish and dairy byproducts through lactic acid bacteria (LAB) fermentation. The synergistic use of acid whey

and LAB starter culture (FAwLr) enhanced fermentation efficiency, promoted controlled protein hydrolysis, and generated multifunctional bioactive hydrolysates exhibiting both antioxidant and antibacterial activities, underscoring their potential for food preservation, nutraceutical, or feed applications. The use of LAB fermentation further positions this approach as a clean-label, sustainable bioprocess compatible with food industry standards. Moreover, the use of a GRAS-status LAB strain enhances the practical applicability of this fermentation strategy for food-grade product development. Importantly, the process was conducted under non-sterile conditions, leveraging the natural microbiota of fish byproducts and acid whey, supplemented with LAB starter cultures, which highlights its practicality and cost-effectiveness for industrial applications. By converting low-value food byproducts into functional hydrolysates through microbial fermentation, this study contributes directly to circular bioeconomy initiatives focused on waste minimization and resource valorization. The integration of fish and dairy waste streams offers potential for developing functional food and feed ingredients, reinforcing the sustainability and industrial relevance of this approach. Future work should focus on scaling up the process and further characterizing the bioactivities of the resulting hydrolysates to maximize their industrial value.

Conflicts of interest

The authors declare no known conflict of interest.

Author contributions

Chih-Chun Kuo: writing – original draft, methodology, validation, investigation, formal analysis, data curation. Da Chen: methodology, writing – review and editing. Rafael Jiménez-Flores: conceptualization, resources, writing – review and editing. Macdonald Wick: conceptualization, writing – review and editing. Osvaldo Campanella: conceptualization, resources, project administration, supervision, writing – review and editing.

Data availability

Data can be obtained by request to the authors.

Supplementary information is available. See DOI: **https://doi.org/10.1039/d5fb00444f**.

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