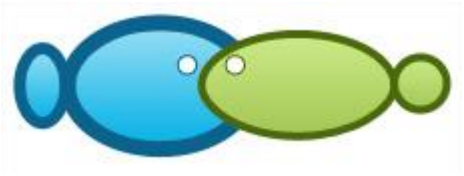

Improvement of duckweed (*Azolla microphylla*) nutrition by *Leuconostoc* sp. isolated from Nile tilapia (*Oreochromis niloticus*)

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Abstract. Probiotics are microbiological agents that can potentially increase fish growth performance and fight microbial disease in aquatic animals. The type of inoculum and fermentation time while improving the duckweed's nutrient content are the determining factors for the fermentation process's success. The research objective was to improve the nutrient content of duckweed (*Azolla microphyla*) by fermenting with lactic acid bacteria isolated from the intestine of Nile tilapia (*Oreochromis niloticus*). Lactic acid bacteria were isolated using a deMann Rogosa Sharpe agar supplemented with 0.5% CaCO3. Bacterial colonies that showed a clear zone around were isolated and purified. The pure isolates were tested for the ability to produce protease and cellulase enzymes, by using semi-qualitative methods. The study was conducted experimentally using a completely randomized design with a factorial pattern with two replications. The result of protease and cellulase tests showed that two isolates that can produce the best enzymes were identified molecularly and could be applied for fermenting duckweed. Identification results using molecular techniques showed that isolate A2 was *Leuconostoc pseudomesenteroides*, and isolate A3 was *Leucosnostoc* sp. *A. microphyla* was fermented by *Leucosnostoc* sp. The proximate and amino acids content was evaluated. The proximate composition of fermented *A. microphylla* was crude protein 14.19%, fat 1.47%, water 22.65%, and ash 55.47%. Amino acids content in fermented ingredients has increased, especially for essential amino acids. Hence, it was concluded that the nutritional properties of *A. microphylla* could be improved by *Leuconostoc* sp. isolated from *O. niloticus*.
Key Words: lactic acid bacteria, fermentation, isolates, enzymes.

Introduction. Protein substitution in fish feed is necessary because it allows alternative protein sources that are more sustainable and cost-effective than traditional fishmeal and fish oil. Fishmeal and fish oil are typically made from wild-caught fish and are becoming increasingly scarce and expensive. By using alternative protein sources, such as plant-based proteins, fish farmers can reduce their reliance on wild-caught fish and lower the overall cost of production. Additionally, alternative protein sources can reduce the environmental impact of fish farming by reducing the population of wild fish captured for food. However, it is essential to remember that not all substitute proteins are nutritionally similar to fishmeal and fish oil, so it is important to take precautions to ensure the fish get the right balance of nutrient (Aragao et al 2022).

Duckweed, *Azolla microphyla*, a small aquatic flowering plant, has the potential as a protein source for fish feed because it is a highly efficient protein producer. It can be grown in low-cost, low-impact systems and on non-arable land and wastewater. *A. microphyla* has a high protein content, up to 45% of its dry weight, higher than most other aquatic plants (Xu et al 2021). In addition, it can be grown in open ponds and requires minimal inputs, making it a low-cost and sustainable alternative protein source. Also, duckweed can be grown on wastewater. Thus, it can help treat and clean the water simultaneously (Liu et al 2020). However, one of the limitations of using *A. microphyla* as fish feed protein is that it may contain lower levels of some essential amino acids that fish need for growth and development. Thus, *A. microphyla* is usually

used as a partial replacement for fishmeal and fish oil rather than a complete replacement (Fiordelmondo et al 2022). Furthermore, *A. microphyla* is palatable to a limited range of fish species, and some research suggests that it may have adverse effects on the growth of some species, so it is important to conduct experiments to see which fish species are suitable for *A. microphyla* feed before it can be applied on a large scale (Vinogradskaya & Kasumyan 2019).

Fermentation improves the nutritional value of plant-based proteins by breaking down the complex carbohydrates and indigestible fibers found in many plant-based protein sources, making them more easily digestible for fish. Fermentation can also increase the bioavailability of certain nutrients, such as amino acids and minerals (Christensen et al 2022). During fermentation, microorganisms, such as bacteria and yeast, break down the plant material and release nutrients that are otherwise unavailable to fish. This includes increasing certain amino acids, such as lysine, which is important for fish growth and development. Fermentation also increases the digestibility of plant proteins by breaking down the indigestible fibers and complex carbohydrates that are present in plant-based proteins (Alrosan et al 2022). As a result, it makes the protein more readily available for fish to use. Fermentation also can reduce anti-nutritional factors such as phytates and tannins, which are compounds that can inhibit the absorption of minerals and other nutrients in the gut. Fermentation can also produce beneficial compounds like vitamins and antioxidants, enhancing the feed's nutritional value (Jan et al 2022).

Nile tilapia (*Oreochromis niloticus*) is fresh fish and one of the leading fishery commodities with increasing market demand. Therefore, productivity must be boosted continuously with various intensive system aquaculture technologies (Maryam 2010). Increasing fish productivity can be done with an approach to improving feed and overcoming microbial and parasitical diseases. One way is to provide probiotics that produce the enzyme proteinase and the cellulase (Albances et al 2022). This study was carried out to investigate the effect of fermentation on the nutrition value of *A. machrophila* by using lactic acid bacteria from the intestines of *O. niloticus*, which has the ability to produce proteinase and cellulase enzymes.

Material and Method

Materials. *O. niloticus* were obtained from the Freshwater Fish Laboratory, Faculty of Fisheries and Marine Sciences, Brawijaya University, Malang. The length of *O. niloticus* was 6-7 cm. *A. microphylla* was purchased from Purwodadi, Indonesia. *Leuconostoc* sp. was isolated from the gut of *O. niloticus*. The probiotics were cultivated on deMan Rogosa (MRS) Broth and Potato Dextrose Broth (PDB). All chemical materials were reagent grade.

Isolation of probiotic candidates. Isolation of lactic acid bacteria was carried out based on Prihanto et al (2021). *O. niloticus* intestines were taken aseptically and transferred to sterile petri dishes. First, the intestines were crushed and 1 g was put into a test tube containing Natrium thiosulfate at 0.95% (1:10). Prior to be cultured, the samples were serially diluted into 10^{-5}. Then, 25 µL of samples were grown on medium de Man Rogosa and Sharpe (MRS) +0.5% $CaCO_3$, then incubated at 37°C for 2-4 days. Colonies with clear zone and different appearances were purified by subculturing into MRS Agar for further identification.

Species identification. The DNA of the isolates was extracted using Promega system following the company manual. Specific identification was performed by molecular identification using 16S rRNA. The 16S ribosomal RNA (16S rRNA) gene has a conserved region so it is appropriately used in Polymerase Chain Reaction (PCR). The universal primers 27 F (5'-AGA GTT TGA TCC TGC CTC AG-3') and 907R (5'−CCG TCA ATT CCT TTG AGT TT-3') were used in the PCR reaction and resulted in a 900 bp band size (Lane 1991). The following amplification conditions were used: a 7 min denaturing step at 94°C, followed by 30 denaturation cycles (94°C for 1 min), annealing (55°C for

1 min), extension (72°C for 1 min), and a final extension (72°C for 15 min). PCR products were analyzed on 1% horizontal agarose gels. The sequencing analysis determined taxonomy, phylogeny and diversity between species. The sequenced isolates were compared to the published sequences using the BLAST search algorithm (GenBank National Centre for Biotechnology Information, NCBI) at https://blast.ncbi.nlm.

Screening for protease, cellulase and antimicrobial activity. Isolate of probiotic candidates was screened for protease activity using Skim Milk Agar medium. Bacterial pure isolates were grown on selective media and subsequently incubated for 24–48 hours at 30°C (MEMMERT INB200). Bacterial cultures that grow and form a clear zone around the colony are further separated and purified.

Cellulolytic enzyme production was tested using Nutrient Agar media (2.8 g) and Carboxymethylcellulose (CMC) 1% (w/v). The bacterial isolates to be tested were inoculated on the selective medium as much as one culture and incubated for 24-48 hours at 30°C (using a precision incubator Memmert INB200). Cellulolytic activity test was carried out by the Congo Red method. First, Congo Red solution (0.1% w/v) is poured into the culture and left for 15 minutes. The solution was then discarded and rinsed with NaCl 0.2 M for 15 minutes, thrice. The clear zone that appears around the bacterial colony indicates the occurrence of the cellulose hydrolysis process. On the other hand, if the hydrolysis process does not occur, there will be no clear visible zone around the growing colony (Augustine & Joseph 2018).

The probiotic isolate was tested for antimicrobial activity against pathogenic bacteria using a disk diffusion assay with the following steps (Amin et al 2020): First, each lab isolate was cultured in 10 mL MRS broth and incubated at room temperature for 24 hours. The bacterial cells were harvested by centrifugation at 13,000×g for 10 minutes, then the supernatant collected in sterile tubes. *Aeromonas hydrophila* was obtained from culture collections at Brawijaya University's Microbiology Laboratory, Faculty of Fisheries and Marine Sciences. *A. hydrophila* was grown in 10 mL Muller Hinton (MH) broth for 24 hours at 28°C. The bacteria cells were harvested by centrifugation at 13,000×g for 10 minutes, resulting in a cell concentration of 10^6 CFU mL^{-1}. Next, a 25 µL aliquot of bacterial pathogen was cultured on MH agar plates using the spreading technique. The base tip of a 200 µL sterilized pipette was then used to aseptically puncture disc paper (6 mm diameter). Then, 10 µL of each isolate was added to disc paper and incubated at 37°C. After a 24 hour incubation period, a clear antimicrobial zone was determined.

Duckweed fermentation. The leaves of duckweed were dried and ground sun-dried for two days. 20 g of grinded leaves were weighed and put into a 100 mL jar. Starter, 1 mL inoculum and 1 mL molasse were added and mixed. The sample was moved to zip-lock pouches. The fermentation occurred for three days at 30ºC. Prior to the experiment, all equipment was sterilized. Duckweed with molasses was used as a control.

Proximate analysis. Proximate analysis of duckweed samples was carried out following AOAC (2005) procedures. Protein was calculated as crude protein using the Kjedahl method. Humidity was determined by the gravimetric method by drying the sample in an oven at 105°C until the weight became constant. Fat content was determined by the Soxhlet method by extracting the residue with petroleum ether at 40–60°C for 7–8 hours in a Soxhlet apparatus. The ash content was determined by burning samples at 550°C in a Muffle furnace, to a constant weight.

Amino acid analysis. Amino acid was tested using Ultra Performance Liquid Chromatography (UPLC). The sample was hydrolyzed with acid and then analyzed with an AccQ Tag Ultra 18 column 1.7 µm (2.1 x 100 mm) into separated compounds. The column temperature was set at 49°C and a 0.5 mL min^{-1} flow rate. The standard solution used α-Amino Butyric Acid (AABA) and was detected at a wavelength of 260 nm.

Data analysis. All data were analyzed using variance analysis (ANOVA) followed by Duncan's multiple range test, except for amino acid data. The presented data was the average from two replications plus the standard deviation.

Results

Lab associated with intestine of Nile tilapia. Eight isolates suspected to be lactic acid bacteria were isolated and purified. The bacteria were tested for their ability to produce protease and cellulase enzymes. The test results showed that some bacteria could produce protease and cellulase enzymes. Isolates A2, A3, A4, A5, A6, A7, and A8 were identified as being able to produce enzymes (protease and cellulolytic). However, only isolates A2 and A3 were identified as capable of producing antibacterial activity against *A. hydrophila*, as indicated by the diameter of the clearance zone. The test results of lab isolates capable of producing protease and cellulase enzymes, and also having an antibacterial activity can be seen in Table 1 and Figure 1.

Table 1

Enzyme-producing characteristics and antibacterial activity

Isolate	*Protease index*	*Cellulolytic index*	*Antibacterial activity*
A1	1.11	-	+
A2	0.28	0.9	+
A3	0.22	0.83	+
A4	0.5	0.57	-
A5	0.14	2.27	-
A6	0.25	0.94	-
A7	0.5	1.15	-
A8	0.14	2.27	-

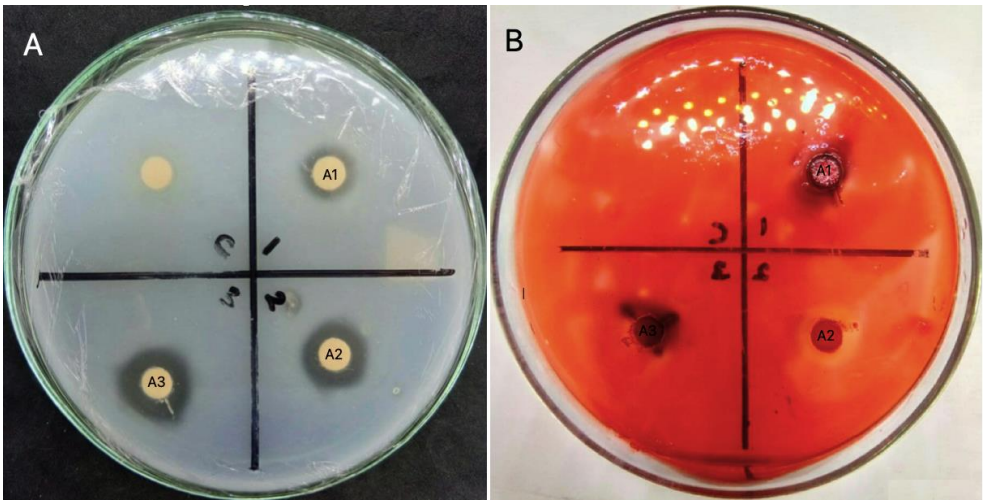

Figure 1. (A) Identification of protease enzyme-producing bacteria, (B) Identification of cellulose enzyme-producing bacteria.

Eight isolates suspected to be lactic acid bacteria were isolated and purified. Phylogenetic trees were constructed from sequences of 16S rRNA genes from isolates and the closest species was obtained by the BLAST function from the gene bank. Identification results using molecular techniques show that isolate A2 was *Leuconostoc pseudomesenteroides*, and isolate A3 was *Leucosnostoc sp*. (Figure 2). This result showed *Leuconostoc* could be isolated from the fish intestine. The results of the phylogenetic analysis of isolates A2 and A3 can be seen in Figure 2.

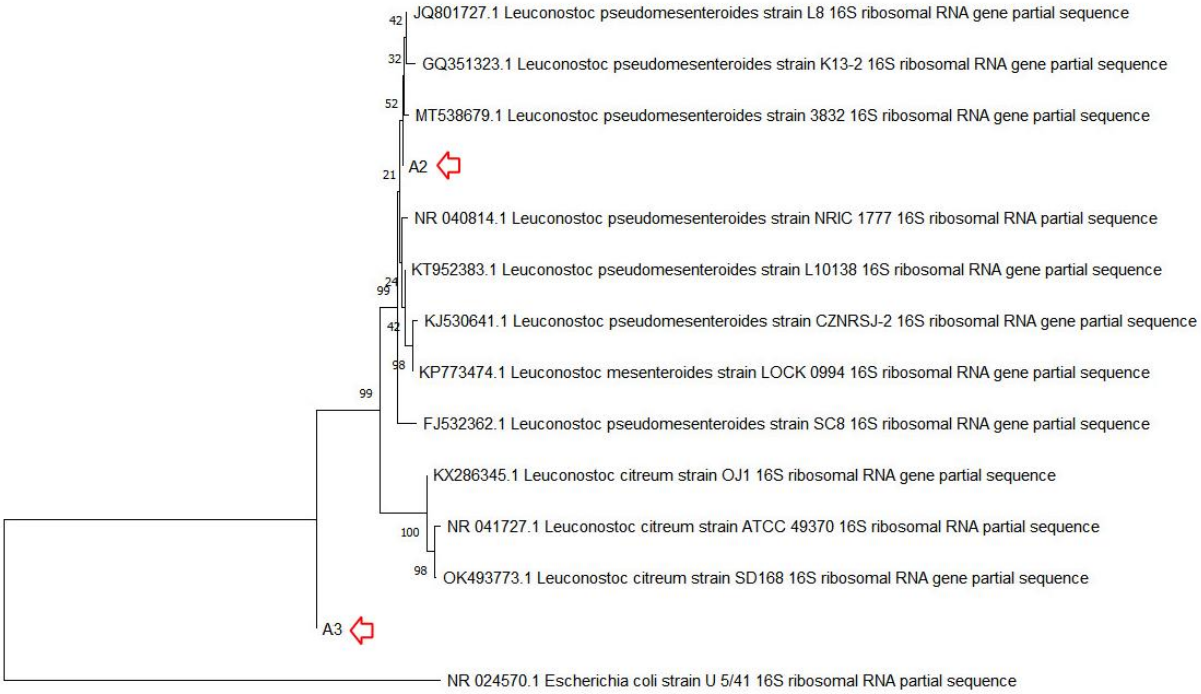

Figure 2. Phylogenetic tree analysis of isolate A2 and A3.

Proximate profile of Azolla microphylla. The proximate profile of fermented *A. microphylla* indicated an increased protein and fat content and a reduced water and ash content. The proximate composition fermentation of *A. microphylla* showed a higher nutrient content than the control. The proximate profile of *A. microphylla* can be seen in Table 2.

Table 2

Proximate profile of *Azolla microphylla*

Proximate composition (%)	*Control*	*Fermentation*
Crude Protein	11.91	14.19
Fat	1.05	1.47
Water	25.01	22.65
Ash	57.19	55.47

Amino acid profile of Azolla microphylla. Based on the analysis results, the amino acid profile of fermented *A. microphylla* showed the highest amino acid profile was leucine 0.908 g (100 g)$^{-1}$ in essential amino acids, and glutamatic acid was 1.107 g (100 g)$^{-1}$ in non-essential amino acids. The amino acid profile of *A. microphylla* was presented in Table 3.

Table 3

The amino acid profile of *Azolla microphylla*

Amino acids	*Content in Azolla microphylla* (g (100 g)$^{-1}$)	
	Raw material	*Fermented*
L-Phenylalanine	0.503	0.558
L-Isoleucine	0.357	0.475
L-Valine	0.478	0.602
L-Lycine	0.359	0.468
L-Leucine	0.658	0.908
L-Threonine	0.499	0.508

Amino acids	*Content in Azolla microphylla* (g (100 g)$^{-1}$)	
	Raw material	*Fermented*
L-Histidine	0.205	0.179
L-Serine	0.553	0.566
L-Glutamic acid	0.849	1.107
L-Alanine	0.541	0.645
L-Arginine	0.576	0.613
Glycine	0.558	0.599
L-Aspartic acid	0.715	0.880
L-Tyrosine	0.323	0.335
L-Proline	0.372	0.462

Discussion. The digestive system of fish is a source for isolating protease-producing bacteria. The characteristics of bacteria that produce certain enzymes associated with the fish gut are known to aid in host digestion and nutrition. Isolation, identification and characterization of protease-producing bacteria from the gut of *O. niloticus* have been carried out in this study. One of the proteolytic bacteria is *Bacillus* sp. *Bacillus thuringiensis* has a proteolytic ability leading to a significant increase in enzymatic hydrolysis of proteins (Chovatiya et al 2014).

In this study, eight isolates had the ability to produce protease enzymes. The highest protease index was found in isolate A1. Isolates that produced the lowest protease were isolates A5 and A8 which had a protease index value of 0.14. All isolates produced protease enzymes and the index value ranged from 0.14 to 1.11. Previous studies of the enzymes found in catfish digestion waste recorded a higher protease index, ranging from 0.03-2.25 (Prihanto et al 2021). In addition, 19 bacterial isolates were derived from mud grouper (*Epinephelus tauvina*) digestive tract samples, with proteolytic indices ranging from 0.5 to 6.0 (Ariana et al 2003). Different proteolytic abilities due to the different optimal conditions, such as pH, temperature and nutrients, required by each microbial species to produce maximum amounts of enzymes.

Seven isolates showed cellulolytic enzyme activity. The highest activity was shown in isolates A5 and A8, with a value of 2.27. Isolate A1 was unable to produce cellulolytic enzymes. The index value of the cellulolytic enzyme produced in the study is relatively high (2.27). According to Istiqomah et al (2019), isolates from the octopus's digestive tract (*Octopus* sp.) have an index value of 1.1-1.8. Meanwhile, Syukri et al (2021) reported that cellulolytic bacteria from the digestive tract of Cantang grouper had an index value of 0.14-3.3. Tanaka et al (2021) said isolates of cellulose-producing bacteria from the intestines of blackfish marine teleosts (*Girella melanichthys*) could be well grown in media with artificial seawater, with optimal growth at 20-35°C, but no growth at 40°C. In addition, Augustine & Joseph (2018) found four new strains of cellulolytic symbiotic bacteria isolated from the GI tract of carnivorous marine fish. Adding cellulases to fish rations may positively affect growth, feed utilization, and nutrient digestibility, potentially reducing feed costs.

This study found that the lab isolates have no inhibitory effects on pathogenic bacteria. Isolates A2 and A3 showed the ability to inhibit *A. hydrophila*. *A. hydrophyla* is a disease that affects many tilapia fish. Albances et al (2022) reported that probiotic bacteria isolated from saltwater green tilapia could prevent *A. hydrophila* infection in juvenile tilapia. *Staphylococcus aureus* showed the highest inhibition zone against *A. hydrophila*. In general, the ability to inhibit bacteria could be due to several factors, including the production of antibiotics, bacteriocins, siderophores, lysosomes, proteases and hydrogen peroxide or to affect the pH of the media by producing certain organic acids. A clear zone was formed due to the inhibition of antimicrobial compounds against microbial cells. The mechanism of an antimicrobial compound consists of disrupting or damaging cell wall constituents, reacting with cell membranes that cause increased cellular permeability, inactivation of essential enzymes and destruction or inactivation of functions and genetic material (Jubair et al 2021). Research by Allameh et al (2012) has successfully isolated lactic acid bacteria *Leuconostoc mesenteroides* sp. from the intestine of cork fish (*Channa striatus*). These bacteria also showed inhibitory activity

against three tested fish pathogens: *A. hydrophila*, *Pseudomonas aeruginosa* and *Shewanella putrefaciens*.

This study showed that fermenting might enhance the duckweed's protein quality. Sembada & Faisal (2022) explained that duckweed protein levels ranged from 10-37%, and lipids ranged from 4-9%. The protein and lipid content in duckweed can easily change according to the environmental conditions in which it lives. Yu et al (2011) showed that the protein content reached 0.34 g dry weight. *A. microphylla* contains 28-43% crude protein, 5% fiber (dry weight), and high concentrations of minerals, such as phosphorus, potassium, xanthophylls and carotenes. A research on *A. microphylla* as a substitute for soybean meal, performed by Solomon & Okomoda (2012), showed that *Lemna minor*, due to a high protein and amino acid content, corresponds to fish needs and could be used in fish farming feed formulations. The high protein content is a unique property of duckweed, which may make it suitable for supplementation or for substituting other plant or animal proteins in fish diets. *A. microphylla* can be processed through fermentation to lower its crude fiber level. Therefore, the high concentration of crude fiber could be decreased during fermentation. Moreover, fermentation can improve some nutritional value (Virnanto et al 2016).

Amino acids are essential biomolecules for building proteins and are intermediates in various metabolic pathways (Mohanty et al 2014). The findings of this study were consistent with Srirangam (2016), who reported that the fermentation process might be used to increase the quantities of protein, particularly amino acids. The activity of protease enzymes, which breaks down proteins to make them more easily soluble in water, causes a rise in nitrogen throughout fermentation.

Conclusions. According to the study's findings, *Leuconostoc* sp. isolated from *O. niloticus* is, potentially, a lactic acid-producing microorganism with proteolytic and cellulolytic abilities. These bacteria can be used to improve the nutritional properties of *A. microphylla*.

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Conflict of interest. The authors declare no conflict of interest

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