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Aquafeed Production from Fermented Fish Waste and Lemon Peel

Alessia Tropea [1,*], Angela Giorgia Potortì [1], Vincenzo Lo Turco [1], Elisabetta Russo [2], Rossella Vadalà [1], Rossana Rando [1] and Giuseppa Di Bella [1]

[1] BioMorf Department, University of Messina, 98168 Messina, Italy; agpotorti@unime.it (A.G.P.); vloturco@unime.it (V.L.T.); rosvadala@libero.it (R.V.); rrando@unime.it (R.R.); gdibella@unime.it (G.D.B.)
[2] Department of Environmental Sciences, Informatic and Statistics, Cà Foscari University of Venice, 30123 Venice, Italy; elisabetta.russo@unive.it
* Correspondence: atropea@unime.it

Abstract: In order to obtain a high-protein-content supplement for aquaculture feeds, rich in healthy microorganisms, in this study, *Saccharomyces cerevisiae* American Type Culture Collection (ATCC) 4126 and *Lactobacillus reuteri* ATCC 53608 strains were used as starters for fermenting fish waste supplemented with lemon peel as a prebiotic source and filler. Fermentation tests were carried out for 120 h until no further growth of the selected microorganisms was observed and the pH value became stable. All the samples were tested for proteins, crude lipids, and ash determination, and submitted for fatty acid analysis. Moreover, microbiological analyses for coliform bacteria identification were carried out. At the end of the fermentation period, the substrate reached a concentration in protein and in crude lipids of 48.55 ± 1.15% and 15.25 ± 0.80%, respectively, representing adequate levels for the resulting aquafeed, whereas the ash percentage was 0.66 ± 0.03. The main fatty acids detected were palmitic, oleic, and linoleic acids. Saturated fatty acids concentration was not affected by the fermentation process, whereas monounsaturated and polyunsaturated ones showed an opposite trend, increasing and decreasing, respectively, during the process. Coliform bacteria were not detected in the media at the end of the fermentation, whereas the amount of *S. cerevisiae* and *L. reuteri* were around 10^{11} and 10^{12} cells per g, respectively.

Keywords: fish waste; citrus peel; fermentation; aquafeed; *Saccharomyces cerevisiae*; *Lactobacillus reuteri*

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1. Introduction

World capture fishery production reached a peak of approximately 96 million tons in 2018; the most recent estimates suggest that 52% of marine stocks are fully exploited, 17% are overexploited, and 7% are totally depleted, while human population and the demand for marine and other aquatic resources continue to increase [1]. Global aquaculture would make a considerable contribution toward bridging the gap between supply and demand. Unfortunately, its development is hampered by an inadequate supply of feed, particularly fishmeal, which is scarce and expensive [2]. This has stimulated the evaluation of a variety of alternative dietary protein sources with the objective of partially, or totally, replacing fishmeal protein in aquafeeds [3]. The use of food industry waste as animal feed is an alternative of high interest because it stands to bring both environmental and public benefits, besides reducing the costs of animal production [4–6]. In particular, with reference to the fishing industry, the cost of waste management for aquaculture is typically in the range of USD 0.05 to USD 0.065 per pound of fish produced, representing concerns from both an economic and an environmental point of view. Waste management, in fact, contributes to the overall costs of production and reduces farmers' net income. Moreover, the improper management of fish wastes could have a negative environmental impact, such as eutrophication effects, on natural aquatic ecosystems [7].

By-products coming from the fishing industry, such as viscera, skin, scales and bones, representing up to 30–80% of the fish body weight, are discarded as solid wastes by industrial fish-processing operations [8] but, due to their composition, they have great potential to be used as protein supplements in aquaculture feeds [9,10]. Their conversion to aquafeed is also encouraged by the significant advantage that they do not require any thermal–chemical and/or enzymatic hydrolysis pretreatment steps. Since the pretreatment step is neither economically favorable nor environment-friendly, its elimination from the process makes the utilization of fish waste economic and more environmentally friendly [7].

Biotechnological methods like fermentation with microbe cultures are gaining more popularity for the treatment of waste [11,12].

Among the different microbes used, especially for the fermentation of animal/fish processing wastes, lactic bacteria have advantages over other microbes, as they are generally recognized as safe (GRAS) [13]. In addition, the products obtained upon fermentation with *Lactobacillus* are also reported to have additional beneficial effects on various aquatic animal intestines (anti-microbial properties, antioxidative properties), making them suitable for food/feed applications. In fact, they easily adapt to the intestinal environment of both aquatic and domestic animals, making them favorable for use in probiotic aquaculture feeds [14–18].

Among the microorganisms applied, yeasts have also been used as inoculum, along with lactic bacteria, to ferment fish waste [19] for converting it to a useful product that can be used as an ingredient to balance the food rations of animals. Yeast has many different immunostimulatory compounds, e.g., nucleic acid, b-glucans, and mannan oligosaccharides [20,21]. These compounds may enhance the growth of different fish species and therefore can be considered as the best health promoters for fish culture [22].

Feed nutritional composition is important; the major growth-promoting factors are proteins and lipids, since they are known to influence the growth and the body composition of fish [23,24].

Fermented fish waste is a liquid product, obtained by the liquefaction of tissues carried out by the enzymes already present in the fish and accelerated by an acid pH [10]. Natural fillers, such as agricultural by-products, can also be added to the substrate [25].

Citrus peel can be used as filler [26] during fermentation, playing at the same time an important role as a prebiotic source [27–29]. Among its beneficial effects, it has been reported that prebiotics can elevate fish resistance to pathogens and improve growth performance, feed utilization and lipid metabolism, as well as stimulating the immune response through the modulation of intestinal microbiota [18,30–32].

The aim of this research was to process non-sterilized fish wastes, supplemented with lemon peel as a filler and prebiotic source, by biological fermentation using combined starter cultures of *Saccharomyces cerevisiae* American Type Culture Collection (ATCC)4126 and *Lactobacillus reuteri* ATCC 53608 for bio-transforming these by-products into a high protein content supplement, rich in healthy microorganisms, for aquaculture feeds.

For this purpose, and to verify the optimum nutritional composition of aquafeed, proteins, crude lipids, ash and lipid content percentages were monitored throughout the process. The influence of the fermentation process on fatty acid concentrations was also evaluated. Finally, microbiological analyses of the starters and total and fecal coliform bacteria quantification were carried out, to evaluate the healthiness of the final product.

2. Materials and Methods

2.1. Substrate

Fish by-products (non-edible parts) of *Dicentrarchus labrax*, represented by the head, viscera, skin and bones, were provided by Acqua Azzurra S.p.a. (Pachino, Italy). Samples were collected directly at the farm, forwarded to the laboratory under refrigerated conditions, and stored at −20 °C until tests were performed. Lemon peel was provided by Simone Gatto S.r.l. (San Pier Niceto, Italy) and stored at −20 °C until use.

2.2. Microorganisms

Saccharomyces cerevisiae ATCC 4126 was maintained on yeast medium (YM) agar (yeast extract 3 g/L, malt extract 3 g/L, peptone 5 g/L, glucose 10 g/L, agar 20 g/L; Oxoid, Basingstoke, UK) and *Lactobacillus reuteri* ATCC 53608, then maintained on MRS (de Man, Rogosa, Sharpe) agar (peptone 10 g/L, "Lab-Lemco" powder 8 g/L, yeast extract 4 g/L, sorbitan mono-oleate 1 mL, di-potassium hydrogen phosphate 2 g/L, sodium acetate tri-hydrate 5 g/L, tri-ammonium citrate 2 g/L, magnesium sulfate heptahydrate 0.2 g/L, manganese sulfate tetrahydrate 0.05 g/L, agar 10 g/L; Oxoid, Basingstoke, UK) at 4 °C. To carry out the tests, *S. cerevisiae* and *L. reuteri* were cultured overnight at 35 °C and 37 °C, respectively, on a rotary shaker (INNOVA 44, Incubator Shaker Series, New Brunswick Scientific, Edison, NJ, USA) at 200 rpm, in tubes containing 20 mL YM medium for the yeast and MRS broth for the bacteria.

2.3. Experimental Set-Up

Fermentation tests were carried out in a 5 L batch fermenter (Biostat Biotech B, Sartorius Stedim Biotech, Goettingen, Germany. The fermenter was equipped with one four-bladed Rushton turbine and the usual control systems: temperature, pH, pO_2 and a foam detector. Fish waste and lemon peel (2:1 *w/w*) were homogenized in a blender for 5 min.

The resulting homogenate, with a dry matter content of 40% (*w/w*) was supplemented with 20 mL of *S. cerevisiae* (10^8 cells per mL) and 20 mL of *L. reuteri* culture (10^8 cells per mL), simultaneously. No sterilization procedures were adopted.

Fermentation parameters were 35 °C, with pH 5.0 and constant stirring at 200 rpm, and a final working volume of 3.5 L.

All fermentations were carried out for 120 h until no further growth of the selected microorganisms was observed, and the pH value became stable. The pH was not controlled by alkali addition during cultivation.

Medium samples were withdrawn daily from the reaction vessel using a sterile 20 mL syringe and immediately frozen at −20 °C until analysis.

2.4. Yeast Cell, Lactic Acid Bacteria and Coliform Bacteria Numbers

Suspended yeast cells and lactic acid bacteria were counted via the dilution plating method, whereas coliform bacteria were counted via the MPN (most probable number) method.

The CFU (colony-forming unit) of suspended yeast was counted by culturing at 35 °C in yeast media agar (pH 5.8) containing 100 mg/mL chloramphenicol (Oxoid, Basingstoke, UK). Lactic acid bacterial colonies were counted by assessing acid formation at 37 °C in the MRS agar (pH 6.8) containing 50 mg/mL bromocresol purple (Oxoid, Basingstoke, UK). Coliform bacteria grown at 37 °C in lauryl tryptose broth (Oxoid, Basingstoke, UK) (pH 6.8) and the gas-forming bacteria were confirmed on green bile medium (Oxoid, Basingstoke, UK) (pH 7.4).

2.5. Protein, Moisture, and Ash Determination

Representative samples were drained off daily for protein content testing, using the method suggested by the AOAC (Association of Official Agricultural Chemists, Rockville, MD, USA) [33]. The protein percentage was calculated considering a conversion factor of 6.25. The dry weights, both of the fresh waste and fermentation samples, were calculated as steady weights after 2 h at 110 °C, using a Mettler PM 200 equipped with a Mettler LP16 IR balance (Mettler-Toledo GmbH, Laboratory & Weighing Technologies, Greifensee, Switzerland). Ash determination was carried out according to the AOAC method [33]. All samples were analyzed in triplicate.

2.6. Crude Fat and Fatty Acid Determination

Samples were extracted with a mixture of chloroform and methanol (2:1). The mixture was allowed to stand overnight and the lower lipid layer, transferred into a pretreated and weighed flask, was dried off. The difference in the two weights established the weight of the fat [24].

The fatty acid analysis was performed by gas chromatography after transmethylation with 2% H_2SO_4 in methanol at 80 °C. The separation and quantification of fatty acid methyl esters were conducted with a Dani Master GC 1000, equipped with an FID detector (Dani Instruments, Milan, Italy) and a capillary column Supelco SLB-IL100 60 m × 0.25 mm, film 0.20 µm(Merck KGaA, Darmstadt, Germany), using the following experimental conditions: injector temperature 220 °C; oven temperature from 120 °C to 200 °C (10 min hold) at a rate of 1 °C/min; detector temperature 240 °C; carrier gas He at a constant velocity rate of 34 cm/sec; and a split ratio of 1:50.

Fatty acids were identified by comparing the samples with reference standards, using Supelco 37 component Fatty Acid Methyl Esters (FAME) mix in methylene chloride. All samples were analyzed in triplicate.

All chemicals were provided by Merk Life Science (Merck KGaA, Darmstadt, Germany).

2.7. Statistical Analysis

The studies of significant differences were carried out via Kruskal–Wallis tests, using the SPSS 13.0 software package for Windows (SPSS Inc., Chicago, IL, USA). The H statistic and asymptotic significance are the Kruskal–Wallis test output values that allow the significance evaluation of differences when more than two groups are considered.

3. Results and Discussion

3.1. Substrate Fermentation

The time course of fermentation by yeast and lactobacilli is shown in Figure 1, as well as the pH trend.

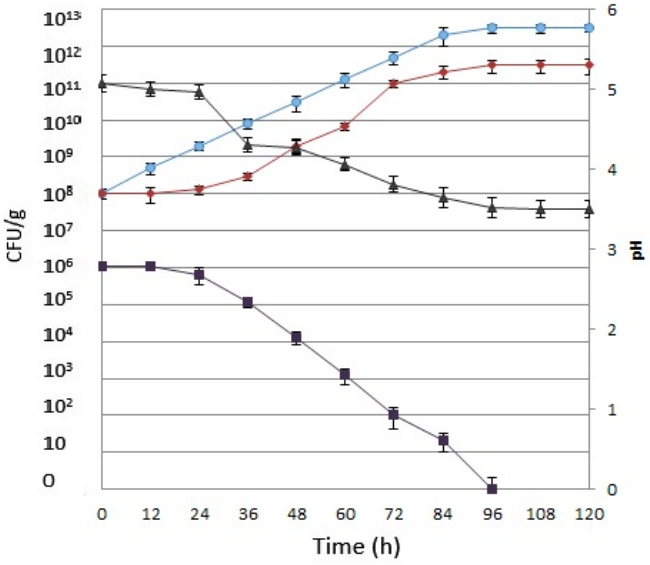

Figure 1. *Lactobacillus reuteri* (circle), *Saccharomyces cerevisiae* (diamond), and coliform (square) concentrations, reported as colony-forming unit (CFU) per g, and pH (triangle) values recorded during the fermentation.

The growth of *S. cerevisiae* was slow during the first 24 h of fermentation, maintaining a concentration of 10^8 CFU/g. The amount of *S. cerevisiae* reached a concentration of 10^{11} CFU/g after 72 h, remaining stable until the end of the process.

L. reuteri increased constantly from the beginning of the fermentation until after 96 h, rising from 10^8 CFU/g up to 10^{12} CFU/g, reaching a steady state until the end of the process. According to Giraffa et al. [17] and Hoseinifar et al. [18], this represents an added value for the resulting aquafeed.

The reduction in pH was slow during the first 24 hours of fermentation because of microorganism adaptations at the beginning of the process [19,34]. In the presence of acid lactic bacteria and yeast, after 24 h the pH of the mixture became stable at 3.5 after 96 h. The decrease in pH in the substrate offers evidence of good acidification through lactic fermentation by the starter cultures and represents the most important factor to control in biotransformation. Acidification must be achieved as quickly as possible, in order to inhibit the growth of pathogenic and spoilage microorganisms in the substrate, increasing the shelf life of the resulting fermented substrate [10,35,36]. Moreover, considering that no sterilization procedures were carried out, the quick drop in pH was found to be necessary for maintaining microbial hygiene, along with retaining the quality of the product as an aquaculture feed [37].

In fact, the amount of initial substrate total coliforms was 10^6 CFU/g, whereas no fecal coliforms were detected. The microbiological analysis for total coliform determination showed a net decrease during the fermentation, to reach a complete absence after 96 h (Figure 1).

The reduction in coliform numbers could be due to some inhibitory compounds (bacteriocins) formed by the microorganisms employed during lactic acid fermentation and/or to the acidification of the medium [19]. Moreover, the decrease in coliforms may ensure good biopreservation against undesirable and/or hazardous microorganisms.

The final fermented products were low in spoilage microorganisms and rich in healthy microorganisms, representing a healthy final substrate enriched by added value.

The starter cultures' capability of growing at low pH can be ascribed to the lemon peel supplementation since polysaccharides, such as pectins, show a protective effect on lactic acid bacteria LAB against low pH [38,39]. Their ability to achieve this on fermenting fish waste supplemented by lemon peel was confirmed by the protein level's increasing during the process, up to 48.55%, making these wastes an excellent raw material for aquafeed production with *Lactobacillus reuteri* and *Saccharomyces cerevisiae*.

3.2. Substrate Protein, Ash and Crude Lipids Concentration

In Table 1, the protein, crude lipid, and ash percentages at different fermentation times are reported. All statistical evaluations were performed at $\alpha = 0.05$.

Table 1. Protein, crude lipid, and ash percentages, after different fermentation times.

		Protein %	**Crude Lipid %**	**Ash %**
H statistic		16.290	7.058	13.386
Asymp. Sign.		**0.006**	0.0216	**0.020**
0 h	Mean ± S.D.	11.68 ± 0.48 (A)	13.74 ± 0.72 (A)	0.83 ± 0.04 (A)
24 h	Mean ± S.D.	15.46 ± 0.40 (A)	14.04 ± 0.74 (A)	0.82 ± 0.04 (A)
48 h	Mean ± S.D.	20.01 ± 0.26 (A)	14.65 ± 0.77 (A)	0.78 ± 0.04 (AB)
72 h	Mean ± S.D.	32.09 ± 0.77 (B)	14.85 ± 0.78 (A)	0.71 ± 0.04 (AB)
96 h	Mean ± S.D.	48.55 ± 1.15 (B)	15.05 ± 0.79 (A)	0.66 ± 0.03 (B)
120 h	Mean ± S.D.	48.55 ± 1.15 (B)	15.25 ± 0.80 (A)	0.66 ± 0.03 (B)

Bold values are significant at $p < 0.05$. A and B indicate homogeneous groups at $\alpha = 0.05$: fermentation times that do not differ from each other are designated by the same letter.

The substrate's initial protein content was 11.68 ± 0.48%. It increased slowly by 72 h, reaching up to 32.09 ± 0.77%. The highest protein percentage in the substrate, 48.55 ± 1.15%, was reached after 96 h. This value remained stable until the end of the fermentative process, allowing to obtain a substrate rich in protein, achieving a suitable percentage for aquafeed formulation according to Nasseri et al. [34]. The protein content against the CFU of the yeast and LAB is reported in Figure 2.

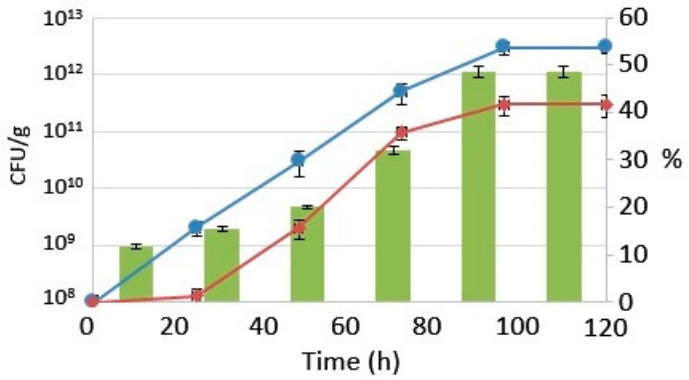

Figure 2. *Lactobacillus reuteri* (circle), *Saccharomyces cerevisiae* (diamond), reported as colony-forming unit (CFU) per g, and protein (bars) levels increasing during the fermentation process, reported as percentages.

During all fermentations, ash concentrations decreased significantly, from 0.83 ± 0.04% to 0.66 ± 0.03%. This could be due to partial ash utilization by the yeast as a source of minerals [40].

The crude lipid content calculated on the initial substrate was 13.74 ± 0.72%. Throughout the process, this value did not increase significantly; at the end of the fermentation period, it reached just 15.25 ± 0.80%.

According to the literature [8,35,41], final protein and lipid contents had reached adequate levels in the resulting aquafeed, offering a way to ameliorate the problem of a lack of protein sources in aquaculture by encouraging the conversion of fish waste into feed, using a low-cost process such as lactic fermentation.

3.3. Fatty Acid Composition

Fatty acid contents at different fermentation times are shown in Table 2. At the end of the fermentation period, as confirmed by statistical tests with significances higher than 95%, MUFAs (monounsaturated fatty acids) increased significantly (+23.5%) and PUFAs (polyunsaturated fatty acids) decreased (−22.7%), whereas the SFA (saturated fatty acids) content was not affected by the fermentation process. Changes in fatty acid composition during the fermentation process are shown in Table 3. The main fatty acids detected throughout the process were C18:1 n-9 cis (33.02%), C18:2 n-6 cis (20.49%) and C16:0 (17.53%). C14:1, C17:0, C17:1, C20:0, and C21:0 were detected, starting after 72 h of the process. A significant increase in concentration for C20:1 n-9, C 20:2 n-6 and C 24:1 n-9 was observed, whereas C18:2 n-6 cis, C20:3 n-6, C20:4 n-6, C20:5 n-3, C22:2, C23:0 and C24:0 showed a significant opposite trend. According to Nadège et al. [23] and Babalola and Apata [42], this fatty-acid composition is suitable for aquafeed formulations in which the shelf life could be extended because of the decrease in polyunsaturated fatty acids.

Table 2. Fatty acid contents at different fermentation times.

		SFA (%)	MUFA (%)	PUFA (%)
H statistic		5.398	13.070	13.819
Asymp. Sign.		0.369	**0.023**	**0.017**
0 h	Mean ± S.D.	28.92 ± 1.51	39.02 ± 2.04 (A)	31.73 ± 1.66 (A)
24 h	Mean ± S.D.	28.45 ± 1.49	39.68 ± 2.08 (A)	29.44 ± 1.54 (A)
48 h	Mean ± S.D.	27.68 ± 1.45	42.20 ± 2.21 (A)	28.08 ± 1.47 (A)
72 h	Mean ± S.D.	27.53 ± 1.44	44.90 ± 2.35 (A)	26.43 ± 1.38 (B)
96 h	Mean ± S.D.	26.96 ± 1.41	47.48 ± 2.49 (B)	24.63 ± 1.29 (B)
120 h	Mean ± S.D.	26.49 ± 1.39	48.19 ± 2.52 (B)	24.53 ± 1.29 (B)

Values in bold are significant at $p < 0.05$. A and B indicate homogeneous groups at $\alpha = 0.05$; fermentation times that do not differ from each other are designated by the same letter.

Table 3. Fatty acid contents (%) at different fermentation times.

		C14:0	C15:0	C16:0	C17:0	C18:0	C20:0	C21:0	C22:0	C23:0	C24:0	
H statistic Asymp. Sign.		13.757 0.017	11.294 0.031	4.273 0.511	14.537 0.013	10.584 0.060	14.868 0.011	14.434 0.013	7.602 0.207	15.690 **0.008**	15.409 **0.009**	
0 h	Mean S.D.	2.38 0.12	0.24 0.01	17.53 0.92	0.005 * 0.00	3.72 0.19	0.005 * 0.00	0.005 * 0.00	0.55 0.03	3.85 (B) 0.20	0.65 (B) 0.03	
24 h	Mean S.D.	2.57 0.13	0.22 0.01	17.69 0.93	0.005 * 0.00	3.92 0.21	0.005 * 0.00	0.005 * 0.00	0.60 0.03	3.20 (AB) 0.17	0.26 (AB) 0.01	
48 h	Mean S.D.	3.09 0.16	0.26 0.01	17.21 0.90	0.005 * 0.00	3.63 0.19	0.005 * 0.00	0.005 * 0.00	0.58 0.03	2.75 (AB) 0.14	0.17 (A) 0.01	
72 h	Mean S.D.	2.31 0.12	0.22 0.01	17.62 0.92	0.20 0.01	3.21 0.17	0.23 0.01	0.06 0.00	0.52 0.03	3.00 (AB) 0.16	0.15 (A) 0.01	
96 h	Mean S.D.	2.62 0.14	0.25 0.01	17.16 0.90	0.15 0.01	3.65 0.19	0.10 0.01	0.05 0.00	0.57 0.03	2.25 (A) 0.12	0.16 (A) 0.01	
120 h	Mean S.D.	2.62 0.14	0.25 0.01	17.16 0.90	0.15 0.01	3.65 0.19	0.10 0.01	0.05 0.00	0.57 0.03	2.25 (A) 0.12	0.16 (A) 0.01	
		C14:1	C16:1	C17:1	C18:1n9t	C18:1n9c	C20:1n9	C22:1n9	C24:1n9			
H statistic Asymp. Sign.		14.434 0.013	12.251 0.031	14.110 0.015	15.058 0.011	10.708 0.057	15.199 **0.010**	10.081 0.073	16.579 **0.005**			
0 h	Mean S.D.	0.005 * 0.00	2.77 0.14	0.005 * 0.00	0.52 0.03	33.02 1.73	1.58 (A) 0.08	0.95 0.05	0.19 (A) 0.01			
24 h	Mean S.D.	0.005 * 0.00	3.23 0.17	0.005 * 0.00	0.63 0.03	33.33 1.75	1.47 (A) 0.08	0.85 0.04	0.17 (A) 0.01			
48 h	Mean S.D.	0.005 * 0.00	3.67 0.19	0.005 * 0.00	0.87 0.05	34.95 1.83	1.63 (AB) 0.09	0.96 0.05	0.13 (A) 0.01			
72 h	Mean S.D.	0.06 0.00	3.58 0.19	0.16 0.01	0.74 0.04	36.06 1.89	2.95 (AB) 0.15	1.03 0.05	0.33 (AB) 0.02			
96 h	Mean S.D.	0.06 0.00	3.33 0.17	0.14 0.01	0.65 0.03	38.12 2.00	3.77 (B) 0.20	1.01 0.05	0.40 (B) 0.02			
120 h	Mean S.D.	0.07 0.00	3.38 0.18	0.15 0.01	0.69 0.04	38.39 2.01	3.94 (B) 0.21	1.02 0.05	0.55 (B) 0.03			
		C18:2n6t	C18:2n6c	C18:3n6	C18:3n3	C20:2n6	C20:3n6	C20:3n3	C20:4n6	C20:5n3	C22:2	C22:6 n3
H statistic Asymp. Sign.		14.427 0.013	14.184 **0.004**	16.064 0.017	11.854 0.037	15.503 **0.008**	15.592 **0.008**	14.868 0.011	16.155 **0.006**	16.251 **0.006**	15.827 **0.007**	15.082 0.011
0 h	Mean S.D.	0.27 0.01	20.49 (A) 1.07	0.29 0.02	4.71 0.25	1.44 (AB) 0.08	0.26 (B) 0.01	0.18 0.01	0.60 (B) 0.03	0.16 (B) 0.01	0.39 (B) 0.02	2.93 0.15
24 h	Mean S.D.	0.26 0.01	20.50 (A) 1.07	0.22 0.01	3.70 0.19	1.12 (A) 0.06	0.10 (A) 0.01	0.28 0.01	0.75 (B) 0.04	0.15 (B) 0.01	0.38 (B) 0.02	1.97 0.10
48 h	Mean S.D.	0.21 0.01	19.07 (A) 1.00	0.20 0.01	4.25 0.22	1.96 (B) 0.10	0.09 (A) 0.00	0.23 0.01	0.09 (A) 0.00	0.04 (AB) 0.00	0.06 (A) 0.00	1.87 0.10
72 h	Mean S.D.	0.29 0.02	16.98 (AB) 0.89	0.33 0.02	4.36 0.23	1.83 (B) 0.10	0.08 (A) 0.00	0.12 0.01	0.10 (A) 0.01	0.02 (A) 0.00	0.05 (A) 0.00	2.26 0.12
96 h	Mean S.D.	0.25 0.01	15.47 (B) 0.81	0.25 0.01	4.06 0.21	1.73 (AB) 0.09	0.10 (A) 0.01	0.12 0.01	0.08 (A) 0.00	0.05 (AB) 0.00	0.06 (A) 0.00	2.45 0.13
120 h	Mean S.D.	0.22 0.01	15.59 (B) 0.82	0.26 0.01	4.07 0.21	1.52 (AB) 0.08	0.11 (A) 0.01	0.12 0.01	0.10 (A) 0.01	0.03 (AB) 0.00	0.07 (A) 0.00	2.43 0.13

* Values corresponding to the half of the determination limit; values in bold are significant at $p < 0.01$. A and B indicate homogeneous groups at $\alpha = 0.01$: fermentation times that do not differ from each other are designated by the same letter.

Previous studies carried out by Fickers et al. [43] and Yano et al. [44] reported similar fatty acid behavior. This trend can be ascribed to the yeast and lactic bacteria activities that degrade fats for single-cell protein production [44] and to obtain the energy necessary for metabolic activities during the fermentation process [45]. The fatty acids resulting from the degradation of lipids are subsequently degraded through the β-oxidation system in the yeast cells [44], resulting in a reduction in polyunsaturated fatty acids.

4. Conclusions

This study demonstrated an effective approach to utilizing the demonstrated fermented substrate for aquafeed, starting with fish and lemon peel wastes, allowing the conversion of both animal and vegetable food wastes into an added-value product.

The final fermented product is low in spoilage microorganisms and rich in healthy microorganisms, representing a healthy final substrate enriched by added value.

The microorganisms' ability to feed on fermenting fish waste that is supplemented by lemon peel was confirmed by the protein level's increasing during the process, up to 48.55%, making these wastes an excellent raw material for aquafeed production via *Lactobacillus reuteri* and *Saccharomyces cerevisiae*. In fact, the final protein and lipid contents represent adequate levels in the resulting aquafeed, reducing the problem of a lack of protein sources for aquaculture by encouraging the conversion of fish waste and lemon peel into feed.

This study pointed out the possibility of setting up a fermentation process based on the simultaneous addition of two different microorganisms, reaching a plateau after 96 h. Further studies are in progress for converting the resulting fermentation product into pellets and for testing the effect of the final product on the growth and immune response of fish from aquaculture and, consequently, in human consumers.

Finally, additional work will be needed to further optimize production to facilitate future larger-scale production, also evaluating it from an economic point of view.

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