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Biotransformation of tuna waste by co-fermentation into an aquafeed ingredient

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Abstract

Dried skipjack tuna (*Katsuwonus pelamis)* waste (red meat, gills, viscera, fins, etc.) were mixed with 25% wheat flour and inoculated with a starter culture of *Lactobacillus plantarum* National Collection of Industrial Microorganisms (NCIM) 2912 (10^8–10^9 cells mL^{-1}) and *Bacillus licheniformis* MTCC 6824 (10^7–10^8 cells mL^{-1}). Changes in the nutritional quality (crude protein, crude fat, crude ash, crude fibre and nitrogen-free extract and aminoacids) were monitored during a fermentation period of 14 days. The proximate analysis showed significant changes in the composition of *L. plantarum*-fermented tuna (LPFT) and *B. licheniformis*-fermented tuna (BLFT) from the unfermented raw materials. Fermentation of tuna waste has resulted in a significant ($P < 0.05$) increase in the protein content of tuna waste between days 6 and 12. All the amino acid contents in BLFT increased during fermentation, whereas, in LPFT the levels of serine, histidine, tyrosine, methionine, cystine and phenylalanine contents were decreased. A marginal increase in calcium and phosphorus levels was recorded in the fermented products. The results of the study suggest that LPFT or BLFT can be used as a novel aquafeed ingredient for different fish species.

Keywords: biotransformation, tuna waste, co-fermentation, aquafeed

Introduction

Large quantities of fish waste are generated from seafood processing in the canning industry and from cleaning the fish at the market. These wastes are either transformed into fish meal using costly procedures, dumped back into the ocean or disposed with the associated pollution problems. The tuna canning industry produces a large quantity of byproducts, forming about 45% of the total raw material weight. This includes the head, gills, gut, red meat, fins and so on, which have the same nutritive value as fish. These byproducts have a low storage quality if not frozen or preserved. One of the methods available for byproduct utilization is fish meal production, which requires a huge capital investment. The use of relatively simpler technologies, like ensiling or fermentation of fish processing wastes, is more suitable and convenient for small industries and farmers (Faid, Zouiten & Achkari-begdouri 1997). Effective utilization of fishery wastes has great potential as protein supplements in aquaculture feeds (Vidotti, Viegas & Carneiro 2003). In 2001, around 30 million metric tonnes (MT) of waste fish product, including trimmings and other smaller bony fish, were used in aquafeeds (IFFO 2001). Normally, fish wastes are ensiled by biological fermentation with lactic acid bacteria or by chemical acidification using inorganic and/or organic acids (Tatterson 1982; Raa, Gildberg & Strom 1983). The experimental usage of fish silage as an alternative protein ingredient in aquafeeds has been widely reported (Raa & Gildberg 1982; Hardy, Shearer & Spinelli 1984; Arason 1994; Faid *et al.* 1997). Even though the technique for the production of silage is simple, its usage is limited in tropical aquaculture. This results from the failure to optimize methods of manufacture, the use of spoiled raw materials or poor storage conditions (Goddard & Perret 2005). Co-drying of silage with wheat bran based on chemical and nutritional characteristics has been reported to improve storage conditions (Goddard & Al-Yahyai 2001; Goddard, Mclean & Wille 2003; Goddard & Perret 2005). In a developing country

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such as India, the inclusion of co-fermented fish waste as an ingredient in aquafeeds for fish or crustaceans could reduce dependence on expensive, imported fish meal. It would also present an opportunity to utilize fishery waste, including bycatch and processing waste through aquaculture. In the present study, fish waste was processed by co-fermentation using two different bacterial strains: *Lactobacillus plantarum* National Collection of Industrial Microorganisms (NCIM) 2912 and *Bacillus licheniformis* MTCC 6824. Two different experiments were performed and the changes in the nutritional profile were monitored for a period of 14 days.

Materials and methods

Bacterial strains

Lactobacillus plantarum NCIM 2912 was obtained from the NCIM, Pune, India and *B. licheniformis* MTCC 6824 was isolated from Mangalavanam, a local mangrove swamp. *Lactobacillus plantarum* was maintained in Lactobacilli Man, Rogosa and Sharpe (MRS) broth [composition: (10.0 g L^{-1} protease peptone, g L^{-1} yeast extract, 10.0 g L^{-1} beef extract, 20.0 g L^{-1} dextrose, 1.0 g L^{-1} tween 80, 2.0 g L^{-1} ammonium citrate, 5.0 g L^{-1} sodium acetate, 0.1 g L^{-1} magnesium sulphate, 0.05 g L^{-1} manganese sulphate, and 2.0 g L^{-1} dipotassium phosphate), the medium was sterilized at 121 °C under 15 lb for 15 min)] (De Man, Rogosa & Sharpe 1972) with monthly sub culturing and *B. licheniformis* was maintained in wheat bran agar [WBA, composition: wheat bran extract — 100 mL (100 g wheat bran in 1000 mL distilled water autoclaved for 1 h and filtered), 0.04 g L^{-1} $(NH_4)_2SO_4$, 0.02 g L^{-1} $MgSO_4 \cdot 7H_2O$, 1.0 g L^{-1} casein, 0.05 g L^{-1} KH_2PO_4, 0.04 g L^{-1} K_2HPO_4, 2.0 g L^{-1} agar, 6.0–6.2 pH; the medium was sterilized at 121 °C15 lb for 15 min and 0.2 mL $CaCl_2$ from a sterile 2% stock solution was added].

Co-fermentation of tuna waste

The tuna waste was obtained from Integrated Fisheries Project, Cochin, India, which included red meat, gills, viscera, fins, etc. It was dried at 70 °C and ground into a fine powder (400 μm) for further use. The substrate used for fermentation was skipjack tuna (*Katsuwonus pelamis)* waste mixed with 25% wheat flour. For the two different experiments using *L. plantarum* and *B. licheniformis*, the basal medium for fermentation containing 50 g substrate with 50% moisture (adjusted with distilled water) was autoclaved at 121 °C for 15 min and inoculated with 5 mL of *L. plantarum* (10^8–10^9 cells mL^{-1}) and *B. licheniformis* (10^7–10^8 cells mL^{-1}) respectively. Triplicate samples in 500 mL conical flasks were incubated at 37 °C for 14 days under a static condition. Sampling in triplicate was performed every 48 h for both the trials.

Analyses of nutritional profile

The products obtained after fermentation were dried to a constant moisture level in a hot air oven at 65–70 °C and proximate composition analyses were carried out (AOAC 1990). All analyses were performed in triplicate. The phosphorus and calcium contents in the fermented products were determined using the titrimetric method using the residue from ash, as described by AOAC (1990). Amino acid analysis of the fermented samples was performed after acid hydrolysis using 6 N HCl by reverse-phase high-performance liquid chromatography (HPLC) after pre-column derivatization by phenyl isothiocyanate by a modified method adapted from Fierabracci *et al.* (1991). HPLC was performed using a Waters 1525 Binary HPLC pump and Waters 2487 Dual Absorbance Detector Data were processed and analysed using WATERS BREEZE software. The operating conditions were: column temperature 38 °C, column, pico-tag (waters, pico tag system); absorbance, 254 μm; and pump pressure, 1500–1700 psi. HPLC was performed using a Waters 1525 Binary HPLC pump and Waters 2487 Dual Absorbance Detector. Data were processed and analysed using WATERS BREEZE software. The tryptophan content in the samples was determined after alkaline hydrolysis by spectrophotometry (AOAC 1990).

Statistical analyses

Standardization of the fermentation process was statistically analysed using one-way analysis of variance. Significant differences among means ($P < 0.05$) (between days or between organisms as the case may be) were tested by Duncan's multiple-range tests. All statistical analyses were conducted using SPSS for Windows (Statistical Package for Social Sciences, Windows Version, Chicago, IL, USA) and MICROSOFT EXCEL.

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Results and discussion

The present study reports a practical method for co-fermentation of tuna waste from the canning industry using *L. plantarum* NCIM 2912 and *B. licheniformis* MTCC 6824. These resources, often discarded at sea, represent a potential global resource exceeding 30 million tonnes each year (New & Csavas 1995). This is equivalent to the stock of fish currently targeted for fishmeal production (Barlow 2000). Fish processing wastes are found to be the only raw material that is regularly available but currently unutilized (Raghunath & Gopakumar 2002). The preservation of fish waste generated from seafood processing offers a huge potential for effective utilization these wastes as an alternative protein source (Raa & Gildberg 1982; Hardy *et al.* 1984; Arason 1994; Faid *et al.* 1997). Even though the dry fish waste contains $\geq$58% protein, it was presumed that more than protein-level enhancement, a kind of value addition is essential for it, which would convert the product that is more acceptable as a feed ingredient.

Dry waste was used in the present study because the product expected to obtain after fermentation was a low-moisture product, which is easy to handle, dry, transport and store, unlike the usual fish silage, which is bulky and difficult to handle, store and transport. Drying of fish waste was performed in hot air oven and the incubation was carried out in air-controlled incubator, because its research requires sophistication. However, when large quantities are used, simpler techniques would definitely be requried, which is not difficult in this case because the sun drying at $>35\,^{\circ}$C and incubation at 37 $^{\circ}$C are quite easy in many places because it is closer to the atmospheric temperature in tropical countries at least during certain months. For fermentation involving bacteria at controlled moisture levels (40–70%), the microbial action would be ideal if the substrate particle size is 400–600 μm and that is why it was dried and ground.

The methodology followed for co-fermentation is viable because, a dried co-fermented product has a better shelf-life (because it is a dried product), is more balanced nutritionally (because no acid is added like for silage preparation) and is easy to transport to different places (it can be properly packed and sent). Fish silage is normally acidic in nature and semi liquid in condition, which is difficult to store on many occasions. Also, its use at the place of production is rarely possible, whereas the dry co-fermented product can reduce transport cost and optimize the storage and shelf-life, which is desirable for any feed ingredient. Studies have proven *L. plantarum* as one of the effective starter cultures (Bello, Gutierre, Ottati & Martinez 1992), but the use of *B. licheniformis* for fermentation of fishery wastes has not been reported so far.

The proximate composition data of ingredient mix after fermentation using *L. plantarum* and *B. licheniformis* at different time intervals are given in Tables 1 and 2. With the progress of fermentation, an increase in the moisture content was observed for both *Lactobacillus plantarum*-fermented tuna (LPFT) and *Bacillus licheniformis*-fermented tuna (BLFT). For LPFT and BLFT, significant ($P<0.05$) increase in the moisture content (48.1% and 54% respectively) was observed on day 14 of fermentation. Fermentation of tuna waste resulted in a significant ($P<0.05$) increase in the protein content of tuna waste between days 6 and 12. The increase observed in the crude protein content may be due to the bioconversion of soluble carbohydrates in the substrate (wheat flour) to bacterial protein. It has been demonstrated that

Table 1 Proximate composition of LPFT (as % dry matter)

Day	Moisture	Crude protein	Crude fat	Crude ash	NFE
0	3.15 ± 0.49^{a}	58.51 ± 0.30^{a}	15.18 ± 0.14	5.30 ± 0.22	21.01 ± 0.55^{a}
2	2.81 ± 0.55^{b}	59.59 ± 0.37^{b}	15.50 ± 0.18	5.28 ± 0.23	19.63 ± 0.26^{a}
4	3.80 ± 0.17^{c}	59.21 ± 0.39^{c}	15.38 ± 0.17	5.09 ± 0.44	20.31 ± 0.89^{b}
6	4.00 ± 0.17^{d}	60.62 ± 0.86^{d}	15.47 ± 0.08	5.49 ± 0.07	18.42 ± 0.97^{c}
8	4.23 ± 0.25^{e}	59.82 ± 0.66^{e}	15.37 ± 0.33	5.46 ± 0.07	19.35 ± 0.31^{d}
10	4.70 ± 0.10^{f}	60.31 ± 0.75 ^{f}	15.70 ± 0.11	5.42 ± 0.06	18.56 ± 0.90^{e}
12	4.55 ± 0.49^{g}	60.16 ± 0.19^{g}	15.72 ± 0.03	5.50 ± 0.05	18.62 ± 0.27^{f}
14	6.07 ± 1.63^{h}	61.83 ± 1.12^{h}	15.79 ± 0.48	5.46 ± 0.09	16.92 ± 1.55^{g}

All values are average of triplicates ± SE; means within the same columns with different superscript letters are significantly different ($P<0.05$).

LPFT, *Lactobacillus plantarum*-fermented tuna; NFE, Nitrogen free extract.

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Table 2 Proximate composition of BLFT (as % dry matter)

Day	Moisture	Crude protein	Crude fat	Crude ash	NFE
0	1.98 ± 0.33[a]	60.25 ± 0.67	14.89 ± 0.30	5.51 ± 0.10	19.34 ± 0.81[a]
2	1.80 ± 0.10[b]	60.87 ± 0.64	14.97 ± 0.90	5.50 ± 0.10	18.66 ± 1.34[b]
4	2.30 ± 0.40 [c]	60.96 ± 0.15	15.83 ± 0.66	5.25 ± 0.46	17.96 ± 0.48[c]
6	3.73 ± 0.25 [d]	60.83 ± 0.69	15.50 ± 0.07	5.57 ± 0.11	18.10 ± 0.64[d]
8	3.60 ± 0.36[e]	59.62 ± 0.25	15.66 ± 0.85	5.67 ± 0.08	19.05 ± 1.03[a]
10	3.70 ± 0.61 [f]	60.05 ± 0.70	15.42 ± 0.92	5.64 ± 0.08	18.89 ± 1.63[f]
12	4.30 ± 0.44 [g]	59.95 ± 0.41	15.98 ± 0.22	5.64 ± 0.08	18.43 ± 0.40[g]
14	3.97 ± 0.64[h]	59.64 ± 0.38	15.73 ± 0.25	5.73 ± 0.04	18.90 ± 0.61[a]

All values are average of triplicates ± SE; means within the same columns with different superscript letters are significantly different ($P < 0.05$).

BLFT, *Bacillus licheniformis*-fermented tuna; NFE, Nitrogen free extract.

L. plantarum and *B. licheniformis* produce different enzymes and biomolecules, which are proteinaceous in nature during the course of fermentation (Hassan 2003; Nwanna 2003). The possibility of the production of non-protein nitrogen compounds-like ammonia, amines, amino acids and peptides due to autolysis during the process of fermentation also cannot be ignored (Haard, Kariel, Herzberg, Feltham & Winter 1985). The crude fat content also showed a marginal increase in LPFT and BLFT (4% and 7.3% respectively), which can be attributed to the production of fatty acids by the bacterial strains. The increase in the crude fat content could be correlated with the increase in the protein content as reported by Arbogast and Henderson (1975), in that the polar lipid synthesis is associated with the protein synthesis because the enzymes required for the lipid synthesis are to be replaced by *de novo* protein synthesis. As such, fish wastes have a very low fibre content and it requires no further discussion, for the low crude fibre content in both LPFT and BLFT. The marginal increase in the ash content observed in both LPFT and BLFT may be attributed to the loss of dry matter during the process of fermentation (Puniya & Singh 1995). The calcium and phosphorus contents also showed a slight increase up to day 10 in LPFT and BLFT and then a marginal reduction. The variations in the calcium and phosphorus levels LPFT and BLFT are given in Table 3.

Table 3 Calcium and Phosphorus content in LPFT and BLFT (expressed in % dry matter) at different durations of fermentation

Sample (Day)	LPFT		BLFT	
	Calcium	Phosphorus	Calcium	Phosphorus
0	0.73	1.24	0.44	1.12
2	0.86	1.24	0.50	1.20
4	0.88	1.27	0.56	1.31
6	0.95	1.33	0.60	1.33
8	1.36	1.34	0.67	1.36
10	1.63	1.33	0.73	1.47
12	1.04	1.28	0.66	1.27
14	0.93	1.09	0.46	1.27

LPFT, *Lactobacillus plantarum*-fermented tuna; BLFT, *Bacillus licheniformis*-fermented tuna.

The amino acid profiles of LPFT and BLFT are presented in Tables 4 and 5 respectively. For LPFT, a 24% increase in the total amino acid content was observed on day 10. The best duration for fermentation based on the amino acid profile was between days 8 and 12 and a slight change was observed on day 8, which requires further detailed analysis. The breakdown of proteins into free amino acids during silage production has already been reported (Tatterson & Windsor 1974; Backhoff 1976). An increase in the levels of essential amino acids-like arginine, threonine, valine, isoleucine, leucine, lysine and tryptophan was observed in LPFT during the course of fermentation. A significant increase in the levels of aspartic acid, glutamic acid, glycine, alanine, proline and tyrosine was also observed in LPFT. A high correlation between glutathione dehydrogenase activity in lactic acid bacteria and their ability to catabolize amino acids in the presence of glutamine has been demonstrated in *L. plantarum* and *Lactobacillus lactis* (Tanous, Kieronczyk, Helinck, Chambellon & Yvon 2002). The high negative values for the glutamine concentration correlated quite well with the increase in the glutamic acid concentration as a result of their possible interconversion, which may depend on the balance of ammonium, glutamate and glutamine concentrations (Chopin 1993). The increase in proteolytic activity shown for fish proteins might have caused maximal

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Table 4 Amino acid profile of LPFT at different durations of fermentation (expressed in g100 g^{-1} sample)

Amino acids	Day 0	Day 2	Day 4	Day 6	Day 8	Day 10	Day 12	Day 14
Aspartic acid	4.00 ± 0.15	4.22 ± 0.04	4.02 ± 0.01	4.23 ± 0.04	4.82 ± 0.00	4.84 ± 0.89	4.78 ± 0.04	4.37 ± 0.15
Glutamic acid	6.67 ± 2.04	8.54 ± 0.00	8.15 ± 0.05	8.40 ± 0.01	9.47 ± 0.02	8.91 ± 0.26	8.23 ± 0.10	8.63 ± 0.35
Serine	3.18 ± 1.77	2.07 ± 0.01	2.14 ± 0.01	2.17 ± 0.00	2.35 ± 0.01	2.62 ± 0.06	2.59 ± 0.05	2.11 ± 0.07
Glycine	1.73 ± 1.07	2.54 ± 0.01	3.18 ± 0.15	3.23 ± 0.05	3.18 ± 0.01	3.35 ± 0.14	3.61 ± 0.01	3.18 ± 0.10
Histidine	3.98 ± 2.51	2.62 ± 0.08	2.87 ± 0.34	2.69 ± 0.03	2.77 ± 0.02	2.95 ± 0.12	2.89 ± 0.23	2.91 ± 0.09
Arginine	3.37 ± 0.37	3.97 ± 0.16	4.02 ± 0.05	4.33 ± 0.03	4.43 ± 0.23	4.73 ± 0.40	3.91 ± 0.13	4.40 ± 0.10
Threonine	2.30 ± 0.32	2.43 ± 0.10	2.36 ± 0.04	2.30 ± 0.01	2.60 ± 0.16	2.75 ± 0.12	3.20 ± 0.06	2.39 ± 0.11
Alanine	2.03 ± 1.04	2.80 ± 0.03	3.03 ± 0.05	3.11 ± 0.02	3.33 ± 0.02	3.44 ± 0.30	3.29 ± 0.07	3.14 ± 0.12
Proline	2.42 ± 0.47	2.24 ± 0.02	2.81 ± 0.02	2.88 ± 0.03	2.75 ± 0.01	3.49 ± 0.24	4.62 ± 0.00	2.81 ± 0.08
Tyrosine	2.33 ± 1.43	1.56 ± 0.01	1.61 ± 0.01	1.73 ± 0.03	1.78 ± 0.01	2.13 ± 0.58	1.91 ± 0.04	1.81 ± 0.06
Valine	1.77 ± 0.86	2.68 ± 0.06	2.61 ± 0.02	2.69 ± 0.03	3.02 ± 0.02	3.28 ± 0.20	3.18 ± 0.12	2.83 ± 0.10
Methionine	2.14 ± 1.34	1.58 ± 0.06	1.48 ± 0.00	1.66 ± 0.05	1.75 ± 0.00	2.05 ± 0.19	2.09 ± 0.02	1.68 ± 0.05
Cystine	1.01 ± 1.29	0.14 ± 0.10	0.22 ± 0.01	0.31 ± 0.02	0.32 ± 0.00	0.27 ± 0.06	0.40 ± 0.39	0.34 ± 0.01
Isoleucine	1.14 ± 1.19	2.09 ± 0.04	2.24 ± 0.02	2.20 ± 0.03	2.47 ± 0.02	2.41 ± 0.21	2.16 ± 0.00	2.37 ± 0.04
Leucine	2.68 ± 0.83	3.51 ± 0.01	3.69 ± 0.04	3.62 ± 0.01	4.11 ± 0.04	3.92 ± 0.25	3.48 ± 0.02	3.91 ± 0.16
Phenylalanine	2.68 ± 1.40	1.81 ± 0.04	2.10 ± 0.01	2.06 ± 0.03	2.17 ± 0.01	2.19 ± 0.18	2.17 ± 0.04	2.19 ± 0.07
Lysine	2.06 ± 1.51	3.45 ± 0.02	3.13 ± 0.00	3.20 ± 0.07	3.78 ± 0.00	2.98 ± 0.97	3.91 ± 0.18	3.59 ± 0.13
Tryptophan	0.98 ± 0.00	0.11 ± 0.00	0.94 ± 0.00	0.98 ± 0.00	1.17 ± 0.00	1.28 ± 0.00	1.12 ± 0.00	0.92 ± 0.00

All values are average of triplicates ± SE.
LPFT, *Lactobacillus plantarum*-fermented tuna.

Table 5 Amino acid profile of BLFT fermented tuna (expressed in g100 g^{-1} sample)

Amino acids	Day 0	Day 2	Day 4	Day 6	Day 8	Day 10	Day 12	Day 14
Aspartic acid	3.08 ± 0.03	3.93 ± 0.14	3.69 ± 0.08	3.90 ± 0.01	4.61 ± 0.28	4.65 ± 0.14	3.06 ± 0.02	4.48 ± 0.04
Glutamic acid	6.35 ± 0.02	7.99 ± 0.34	7.58 ± 0.17	8.07 ± 0.01	9.20 ± 0.54	9.15 ± 0.25	8.05 ± 0.04	8.95 ± 0.04
Serine	1.72 ± 0.01	1.92 ± 0.18	1.87 ± 0.08	1.89 ± 0.01	2.05 ± 0.13	2.07 ± 0.01	2.43 ± 0.01	1.99 ± 0.00
Glycine	2.50 ± 0.01	2.84 ± 0.04	2.86 ± 0.07	3.17 ± 0.01	3.05 ± 0.21	3.28 ± 0.12	3.31 ± 0.01	2.96 ± 0.00
Histidine	2.31 ± 0.03	2.90 ± 0.27	3.09 ± 0.19	2.58 ± 0.06	2.92 ± 0.03	2.98 ± 0.07	2.78 ± 0.24	2.80 ± 0.00
Arginine	3.37 ± 0.05	3.99 ± 0.29	3.75 ± 0.07	4.14 ± 0.10	3.89 ± 1.03	4.37 ± 0.13	5.50 ± 0.12	4.27 ± 0.10
Threonine	1.97 ± 0.01	2.28 ± 0.17	2.17 ± 0.18	2.28 ± 0.04	2.42 ± 0.03	2.61 ± 0.04	3.04 ± 0.07	2.43 ± 0.04
Alanine	2.31 ± 0.03	2.83 ± 0.13	2.83 ± 0.05	3.09 ± 0.04	3.24 ± 0.05	3.31 ± 0.12	3.30 ± 0.02	3.09 ± 0.00
Proline	2.26 ± 0.04	2.60 ± 0.10	2.79 ± 0.18	3.13 ± 0.05	2.71 ± 0.06	2.90 ± 0.01	4.09 ± 0.10	2.58 ± 0.03
Tyrosine	1.71 ± 0.04	1.62 ± 0.03	1.64 ± 0.20	1.70 ± 0.02	2.10 ± 0.56	1.79 ± 0.05	1.77 ± 0.01	1.71 ± 0.01
Valine	2.24 ± 0.04	2.56 ± 0.05	2.53 ± 0.20	2.61 ± 0.03	3.44 ± 0.65	2.95 ± 0.01	3.41 ± 0.13	2.87 ± 0.00
Methionine	1.35 ± 0.04	1.51 ± 0.05	1.48 ± 0.22	1.51 ± 0.03	1.69 ± 0.08	1.73 ± 0.07	2.04 ± 0.07	1.61 ± 0.00
Cystine	0.27 ± 0.06	0.14 ± 0.01	0.30 ± 0.13	0.14 ± 0.03	0.35 ± 0.12	0.33 ± 0.09	0.59 ± 0.00	0.33 ± 0.02
Isoleucine	1.84 ± 0.07	2.16 ± 0.05	2.10 ± 0.07	2.22 ± 0.04	3.00 ± 0.72	2.47 ± 0.03	2.19 ± 0.01	2.32 ± 0.03
Leucine	3.00 ± 0.03	3.53 ± 0.02	3.46 ± 0.10	3.64 ± 0.05	4.20 ± 0.10	4.09 ± 0.14	3.57 ± 0.05	3.91 ± 0.02
Phenylalanine	1.76 ± 0.04	2.01 ± 0.12	2.08 ± 0.17	2.15 ± 0.00	2.16 ± 0.02	2.21 ± 0.04	2.11 ± 0.02	2.06 ± 0.00
Lysine	2.85 ± 0.04	3.28 ± 0.28	2.90 ± 0.11	2.93 ± 0.00	4.00 ± 0.15	3.80 ± 0.12	4.10 ± 0.15	3.65 ± 0.00
Tryptophan	1.36 ± 0.00	1.44 ± 0.00	1.24 ± 0.00	1.16 ± 0.00	1.30 ± 0.00	1.18 ± 0.00	0.87 ± 0.00	1.61 ± 0.00

All values are average of triplicates ± SE.
BLFT, *Bacillus licheniformis*-fermented tuna.

increases in lysine, and arginine levels, according to the specificity of *L. plantarum* for the substrate. A similar observation was made by Silvina, Sauz, Vignolo, Aristoy, Oliver and Toldra (1999) in terms of an increase in lysine and arginine levels after fermentation. Yeast and lactic acid bacteria convert free amino acids by Erlich's mechanism to flavour compounds such as alcohols. These alcohols have one carbon less than the corresponding amino acids. For example, valine, leucine and phenylalanine are converted, respectively, to isobutanol, 3-methylbutanol and 2-phenylethanol (Molard 1994). Histidine, phenylalanine and methionine have been reduced in LPFT compared with the unfermented mix. A reduction in serine and cysteine was also observed in the present study. Various amino acids, vitamins

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and minerals are essential for the growth and metabolism of lactic acid bacteria (Kandler & Weiss 1986). Predominant utilization of serine by homo-fermentative lactobacilli like *L. plantarum* for their growth was reported by Liu, Holland and Crow (2003). Another reason for the decrease in the amino acid content may be due to the chemical reactions between α-amino acids and aldehyde groups present as a result of a Maillard reaction (Johnson, Brown, Eason & Sumner 1985; Fagbenro & Jauncey 1995) or due to the deamination of certain amino acids (Dapkevicius, Robert Nout, Rombouts, Houben & Wymenga 2000).

For BLFT, except tryptophan (it increased on day 2 and then showed a reduction), all other amino acids increased during the course of fermentation (Table 5). When present in proteins, tryptophan is stable at a low pH, but is labile when free (Jackson, Kerr & Cowey 1984). The loss of tryptophan was reported as one of the most serious effects of long-term storage of silage (Jensen & Schmidtsdorff 1977; Kompiang, Yushadi & Creswell 1980). An increase in the levels of histidine, threonine and serine for biological and acid silage production has been reported by Vidotti *et al.* (2003). Higher levels of histidine were reported in both fresh sprat and resulting silages by Jackson *et al.* (1984). Leucine is a stimulatory precursor, which is also an inducer for bacitracin synthetase in the production of bacitracin (an antibiotic) by *B. licheniformis* (Haavik & Froyshov 1982). It has been demonstrated that during fermentation, certain amino acids are produced, with the improved availability of vitamins (Nout & Motarjemi 1997). The increase in the amino acid content in the BLFT may be due to the hydrolysis of protein to amino acid fractions as well as synthesis by the bacteria (Espe, Raa & Njaa 1989; Hassan 2003; Lee, Kim & Kim 2004).

A reduction in tryptophan is common in acid medium, especially in the case of acid ensilage process, but in the present study, a neutral pH was provided for fermentation and no acid was added. It is the bacteria that acted upon the substrate for fermentation, and a reduction in tryptophan in BLFT and not in LPFT was observed. In the BLFT, *B. licheniformes* would have reduced the substrate pH during fermentation, thereby resulting in tryptophan reduction and *B. plantarum* would have maintained the pH in the substrate during fermentation without affecting the level of tryptophan.

In the present study, the ratio of non-essential amino acids to essential amino acids was observed to be 2:1 throughout the fermentation process. The maximum values for amino acids were obtained between days 8 and 12, indicating it to be the peak phase of proteinaceous microbial metabolite production.

The results of the present study suggest the use of co-fermented tuna waste as a novel ingredient, which has the potential to be used as an alternative to fishmeal in aquafeeds.

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