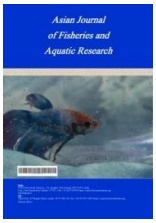

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Production of Fish Silages from Fish Entrails and Its Nutritional Evaluation

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Authors' contributions

This work was carried out in collaboration among all authors. Authors MFT and AS mainly collected the data and performed all the laboratory procedures under the supervision. Authors MNI, MRP, AM and AAM helped in data analysis. All authors read and approved the final manuscript.

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ABSTRACT

The study was undertaken to investigate fish entrails (heads, bones, viscera, fins) suitability as raw materials for fish silage production for the fish feed supplement. Two types of fish silages were prepared using pure culture of *Lactobacillus* as lactic fermentation starter and formic acid as acid fermenter. Lactobacilli was isolated and identified for the preparation of fermentation starter from fresh milk. The starter bacteria and molasses were added to minced raw materials in three different compositions to produce three fermented silages and 3% formic acid was used for acid silage production. Biochemical changes were monitored continuously during the fermentation of the silages. Non-protein nitrogen concentration increased from an initial value of 19.56% to 42%. The proximate composition of the final products after 80 days of fermentation on dry matter basis showed that acid silage contained higher crude protein (31.25±0.75%) than fermented silages (21±0.54% - 28±1.11%). Crude lipid content didn't show any significant differences among silages prepared (P>0.5). Most of the essential amino acids were present in fairly good concentration in all

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the silages which are comparable to those of the FAO/WHO requirement. Protein content and the amino acid profile of the silages suggest that it should be possible to partially replace with fish meal in feeds for fish and animal.

Keywords: Amino acid profile; fermentation; fish feed supplement; lactic acid bacteria.

1. INTRODUCTION

Developing a practical diet for the rearing of fish is a major constituent in aquaculture. The technological advancement in aquaculture makes it more dependent on the exogenous feed supply, and natural supply becomes less significant. Feeds contribute over 50% of operational cost in intensive aquaculture where protein sources are the most expensive ingredient [1]. High-quality protein concentrates have a high demand in poultry, livestock and fish feed formulation. Fish meal are widely used as a protein source for its amino acid balance and high digestibility. However, due to the high value and limited supply of fish meal, it requires identifying a suitable alternative source of protein in the animal feed industry.

The by-products from agro-industries of plant and animal origin have been considered potential substitutes for fish meal in animal feeds. Plant residues often contain anti-nutritional factors and lack of some essential amino acids and thus unlikely used in animal feed. On the other hand, animal by-products are free from such toxic compounds and require less effort in processing to preserve and upgrade their nutritive value [2]. The two most important techniques to preserve/upgrade the nutritional value of animal by-products/wastes are: (a) Fermented fish silage by chemical acidification or Microbial fermentation and (b) protein hydrolysate by hydrolysis using selected exogenous enzymes [3].

The present study was undertaken to find a suitable methodology for transforming fish waste into liquid fish silages and whether their nutritional quality supports to use in fish feed formulation.

2. MATERIALS AND METHODS

2.1 Preparation of Silages

Fish wastes such as heads, bones, viscera, fins, tails produced in large quantities during filleting process were used as protein substrates for this experiment. Substrates were collected from different freshwater fish market of Mymensingh town. Substrate samples were then sorted for the removal of fish scales and all other unnecessary materials. Then the samples were kept at freezing temperature (-20^0C) for silage preparation later on. Frozen samples (fish entrails) were thawed at room temperature. Then the samples were chopped and minced using a sharp knife. Minced samples were then weighed and divided equally into 4 cylindrical containers with a radius of 7 cm and height 25cm. The additional carbohydrate sources (molasses) along with various fermentation starters were added into different compositions (Table 1) into the three containers for fermented silage preparation. On the other hand, 3% formic acid was added into 97% minced samples for acid silage preparation. These three fermented silage treatments and one acid silage treatment were designated as FS-1, FS-2, FS-3 and AS-1 respectively.

Then all the containers were sealed and kept at room temperature for fermentation. During fermentation period pH, lactic acid bacterial growth (LAB) and non-protein nitrogen concentration (NPN) were estimated at regular intervals. On the 6^{th} day of fermentation all the silages pH were measured around 4 which is desirable for the fermentation. NPN content was determined by the trichloroacetic acid (TCA) precipitation technique [4].

Table 1. Percentage composition of materials used for the production of silage

Sample Treatments	Fish by-product	Molasses	Fermentation starter (*Lactobacillus*- 10^{8-9}/ml
Fermented silage(FS-1)	80%	15%	5%
Fermented silage(FS-2)	80%	12.5%	7.5%
Fermented silage(FS-3)	80%	10%	10%
Acid silage (AS-1)	97%	3% (formic acid)	

2.2 Preparation of Fermentation Starter (Isolation and Identification of *Lactobacillus* from Different Sources)

For the isolation of *Lactobacillus* fresh milk, yogurt and fish sauce were used as source sample. One milliliter of each liquid product was inoculated into MRS (de Man Rogosa Sharpe) broth (Oxoid) of various pH values (6.2, and 4.3). The broths were incubated at 30°C for 24 hours. Isolated colonies were obtained by streaking on MRS agar (Oxoid) plates and the plates were incubated anaerobically at 30°C for 48 hours. Predominant and typical colonies were picked from cultures isolated from the broth of pH 3.5.

All the isolates were then tested for gram staining, microscopic morphology, spore formation, Hugh and Leifson's Oxidative/Fermentative test (O/F test), motility and catalase test, respectively by the method described by Ismail and Goyal [5,6]. The identified isolates were found at low pH MRS broth, anaerobic, gram-positive, rod-shaped, endospore negative, fermentative in O/F test, non-motile and catalase-negative. Selected identified isolates were then inoculated into the MRS broth for fermented starter production and incubated at 35°C for 24 hours. Isolates were incubated until the *Lactobacillus* growth reached to 10^{8-9} bacteria/ml in the broth.

2.3 Analytical Procedures

Proximate composition analysis for moisture, crude protein, lipid and ash contents were carried out according to the methods of AOAC [7]. Therefore, Moisture content was determined by a thermostat oven (Gallenkamp, Hotbox Model Ovb) at 105°C for 24 hours until a constant weight was obtained. Micro-Kjeldahl method was used to estimate the total nitrogen content, then crude protein was identified by multiplying 6.25 the conversion factor, methods described by Zotti [8] , lipid content was determined by solvent extraction method using a ground joint Soxhlet Apparatus (AOAC, 1990) [7], ash content was estimated by heating the sample in a muffle furnace at a temperature of 550°C, crude fiber was estimated by the loss on ignition of dried lipid-free deposits after digestion with 1.25% H_2S0_4 and 1.25% NaOH by the method applied by Asowata [9].

2.4 Amino Acid Analysis

Samples were quantified based on total nitrogen content by hydrolysing with 6N HCL in an evacuated heat-sealed glass hydrolysis ampoule over an aluminium block heater at 110°C for 24 hours as the methodology followed by Moore and Stein [10]. Amino acids were determined by Liquid Chromatography using a cationic exchange column to retain the amino acids from the sample then the column was switched in-line with gradient pump and separator column by the methods described by Zandik [11].

2.5 Data Analysis

All the data obtained from this experiment were analysed in triplicates in each treatment. Results of triplicates were analysed using single factor ANOVA to identify the significant differences at $P<0.05$ followed by Tukey-HSD. The data were illustrated as mean ± standard deviation. Graphical representation of the data was performed by the software Sigmaplot 14.

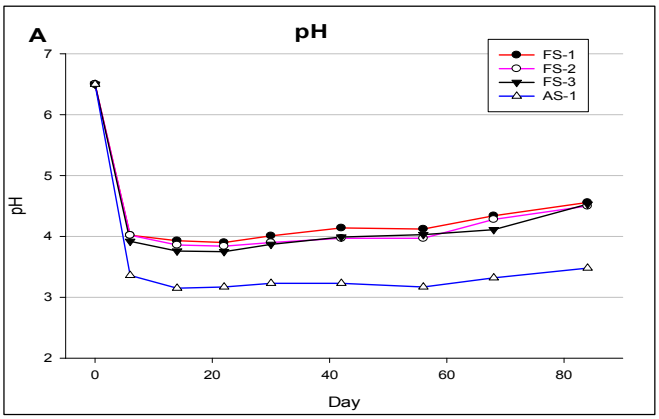

Fig. 1. Graphical illustration of pH value changes in fish silages during fermentation period

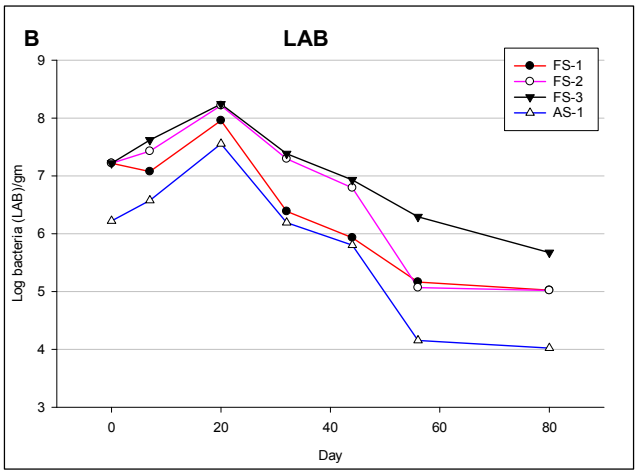

Fig. 2. Graphical illustration of lactic acid bacterial growth changes in fish silages during fermentation period

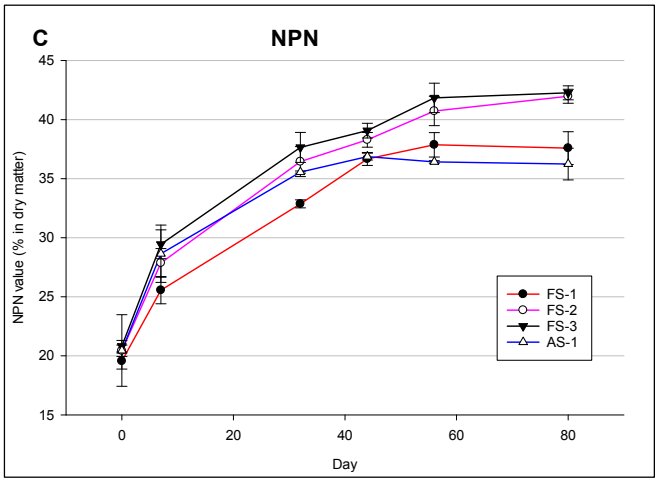

Fig. 3. Graphical illustration of NPN (Non-protein Nitrogen value changes in fish silages during fermentation period

3. RESULTS

3.1 Biochemical Changes during Silage Fermentation

Changes in pH, lactic acid bacteria (LAB) count and non-protein nitrogen (NPN) concentration were estimated at times interval. pH values indicated the amount of lactic acid production during fermentation by microorganisms, and successful fermentation of fish silage depends on lactic acid production. NPN values increased as days of fermentation progresses due to peptide bonds' breakdown and reduction of free amino acids from protein. Changes in pH values, LAB count, and NPN concentration during the fermentation period are illustrated in the Figs. 1-3.

A three -month-long study of biochemical changes in four different fish silages given in Figs. 1-3. The initial pH of all the samples was around 6.5. Then pH values began to decrease as fermentation progress. The pH value decreased to around 4 within 6 days of fermentation and then remained around 4.5 throughout the storage period with slight ups and downs. Lactic acid produced by microorganisms in all fermented silages (FS) decreased the pH of the silages and it decreased to a minimum of 3.75 within 42 days of fermentation in FS-3. Then

the pH began to increase slightly in the fermented silages but not more than above 4.5 until the end of the experimental period. The addition of 3% formic acid into the acid silage (AS-1) lowered the silage's pH quickly to under 4 within 6 days of fermentation. The minimum pH value in the acid silage (AS) was 3.17 at 22 days of fermentation. Then it began to increase but not gone above 4. It is due to the lactic acid bacterial growth was the maximum in 20 days of fermentation which indicates the highest amount of lactic acid production. Then the lactic acid bacterial growth decreases as a result pH drops. The result showed that the minimum pH value was in the acid silage (AS-1) and then the fermented silages FS-1, FS-2 and FS-3, respectively, throughout the fermentation period.

LAB counts increased considerably up to 7 days of fermentation, and the counts reached the maximum number within 20 days of fermentation. Maximum LAB counts were found in FS1, FS2, FS3 and AS1 were 7.959, 8.217, 8.243 and 7.556 log bacteria/g, respectively. The counts then decreased gradually to around log 5.00 bacteria/g at the end of the study. LAB counts were lower in acid silage compared to fermented silages throughout the storage period. This is due to the addition of bacterial fermentation starter *Lactobacillus* in fermented silage production.

NPN concentration changed with fermentation progress and showed significant differences among different silages ($P<0.05$). “NPN material as a significant chemical compound for fish silage since the most obvious changes during fermentation are autolysis of tissues and release of nitrogenous compounds” [12]. Changes of NPN concentration in % dry matter basis in different silages prepared in this experiment are given in Table 2. The initial NPN concentration of all the samples was around 20% of the dry matter then NPN values began to increase. NPN production among silages was rapid within 40 days of fermentation, and then the production slowed down a little bit. NPN values increased in FS-1 from an initial value of 19.56±0.68% to almost double 36.67±0.55% within 44 days of fermentation. The rest of the silages also showed a similar kind of increasing trend in NPN concentration. Initial values of NPN among four different fish silages do not have shown major significant differences ($P > 0.05$). As fermentation progress, NPN concentration varied significantly among the silages (Table 2).

3.2 Proximate Composition of Silages

For the Nutritional evaluation of silages, proximate composition was determined after the fermentation period. Fermented silages (FS-1, FS-2 and FS-3) contained less amount of dry matter than the acid silages. The proximate composition of silages on dry matter basis is given in Table 3.

Proximate composition of silages after fermentation showed some variation. Acid silage contained the highest amount of crude protein than any other fermented silages 31.25±0.75%. Protein content in three bacterial fermented silages has shown an increasing trend of FS-1<FS-2<FS-3 and contained the highest amount of protein (28.35±1.11%) in FS-3. All the silages have shown significant differences ($P<0.05$) of crude protein values among each other. The proximate composition report about the lipid content did not significantly differ in the silages as it varies from 23.45±0.63% to 24.32±0.50%. The final products lipid content was normally very high due to the substrate used for silage production: fish entrails that normally contain a high amount of lipid. Acid silage and fermented silage (FS-1) have shown good ash content, while the fermented silage with less molasses added has the lowest ash value. Crude fibre content was highest in fermented silage-1 (16.21±0.92%) and showed a significant difference ($P<0.05$) among the rest. The findings also indicated that all the silages contained a significant percentage of nitrogen-free extract (23.99 to 34.24%).

3.3 Amino Acid Profile

Amino acid composition of fish silages prepared are given below in table 4.

The amino acid profile of the fish silages indicated that all the essential amino acids except tryptophan (tryptophan values were not estimated) were present in all the silages. It also indicated that isoleucine, leucine, lysine and phenylalanine were present in high concentration among the rest of the amino acids. The Essential amino acids of the standard protein should have the composition: tryptophan 1.00, lysine 5.50, histidine 2.00, arginine 5.00, threonine 4.00, valine 5.00, methionine 3.50, isoleucine 4.00, leucine 7.00 and phenylalanine + tyrosine 4.29 [13].

Table 2. Changes of NPN concentration in % dry matter basis in different silages prepared

Treatments	NPN concentration in % dry matter basis					
	0 (Day)	7 (Day)	32 (Day)	44 (Days)	56 (Day)	80 (Day)
FS- 1	19.56±0.68^{a}	25.56±1.15^{a}	32.87±0.34^{a}	36.67±0.55^{a}	37.87±1.03^{a}	37.59±1.39^{a}
FS- 2	20.43±0.48^{a}	27.87±1.22^{ab}	36.45±1.27^{b}	38.29±0.62^{b}	40.73±1.24^{b}	41.98±0.59^{b}
FS- 3	20.82±0.67^{a}	29.45±0.3^{b}	37.65±1.25^{b}	39.07±0.75^{bc}	41.84±0.62^{b}	42.27±1.98^{b}
AS- 1	20.45±3.03^{a}	28.65±2.43^{ab}	35.56±0.09^{b}	36.87±0.29^{ab}	36.42±0.11^{a}	36.24±1.34^{a}

- *All data represented are Mean ± Standard deviation with analysed with three determinations.*
- *Means in the same column with different superscript are significantly different (P < 0.05), (ANOVA, Tukey HSD)*

Table 3. Proximate composition of the silages in dry matter basis after 80 days of fermentation period

Silage	Dry matter	(% dry matter)				
		Crude protein	Crude Lipid	Ash	Crude fiber	Nitrogen-free Extract
FS-1	19.49±1.10^{a}	21.61±0.54^{a}	24.32±0.50^{a}	10.45±1.62^{a}	16.21±0.92^{a}	27.41±1.55^{a}
FS-2	23.51±1.09^{b}	25.23±0.54^{b}	23.27±0.54^{a}	8.59±0.24^{ab}	8.67±1.26^{b}	34.24±0.77^{b}
FS-3	25.26±1.12^{b}	28.35±1.11^{c}	23.85±0.93^{a}	6.82±0.74^{b}	7.99±1.12^{b}	32.99±0.97^{b}
AS-1	31.55±1.05^{c}	31.25±0.75^{d}	23.45±0.63^{a}	10.75±0.54^{a}	10.77±0.39^{c}	23.99±1.03^{c}

- *Means in the same column with different superscript are significantly different (P < 0.05)*
- *FS-1 fermented silage-1 (n = 3)*
- *FS-2 fermented silage-1 (n = 3)*
- *FS-3 fermented silage-1 (n = 3)*
- *AS-1 fermented silage-1 (n = 3)*

Table 4. Amino acid profile of fish silages prepared

Amino acid	Amino acid composition (g/100 g of protein) in different silages				
	FS- 1	FS- 2	FS- 3	AS- 1	FAO/WHO (1991) requirement pattern [13]
Arginine	3.14	3.08	2.84	2.92	
Histidine	1.64	1.61	1.58	1.73	
Isoleucine	3.96	3.73	3.71	3.81	2.8
Leucine	4.97	4.89	4.99	4.95	6.6
Lysine	10.36	9.80	9.33	9.35	5.8
Methionine	2.67	2.55	2.52	2.61	2.5[a]
Phenylalanine	4.62	4.96	4.89	4.87	6.3[b]
Threonine	4.00	3.92	3.66	3.99	3.4
Tryptophan	NE	NE	NE	NE	1.1
Valine	4.97	4.53	4.81	4.88	3.5
Tyrosine	1.06	1.00	0.96	0.94	
Cystine	0.95	0.96	0.94	0.98	
Alanine	7.80	7.50	7.59	7.91	
Aspartic acid	10.61	10.34	10.04	10.49	
Glutamic acid	16.29	15.29	16.01	15.15	
Glycine	4.40	4.32	4.04	3.82	
Proline	4.92	4.50	4.69	4.79	
Serine	4.04	4.14	4.43	4.34	
EAA:NAA	0.81:1	0.81:1	0.79:1	0.81:1	

- *[a]: cystine+methionine*
- *[b]: tyrosine+phenylalanine*
- *NE: not estimated*
- *EAA:NAA : Essential amino acid: Non-essential amino acid*

Table 5. Chemical score of limiting amino acid of the silages according to FAO/WHO standard 1985 [12]

Silage	Tryptophan	Arginine	Valine	Methionine	Isoleucine	Lysine	Leucine
FS-1	NE	0.56	1.00	0.76	0.99	1.88	0.71
FS-2	NE	0.55	0.91	0.73	0.93	1.78	0.70
FS-3	NE	0.50	0.97	0.72	0.93	1.69	0.71
AS-1	NE	0.52	0.98	0.75	0.95	1.70	0.71

NE: Not estimated

To evaluate the nutritive value of the protein of different silages prepared, chemical score of the amino acids was calculated by previously described scientific method [14]. The findings of chemical score analysis of the present study is given in Table 5. The above table suggests that the arginine value is the lowest in all the silages and is considered a limiting amino acid.

4. DISCUSSION

The silages pH and lactic acid bacterial growth changes during fermentation period showed similar results with Fagbenro and Sotolu [14,15]. The research findings of Fagbenro [14] found a positive relationship between temperature with pH of fish silages. Temperature range 25-35^{o}C gave the best result for lowering the pH quickly as this is within the range of optimal lactic acid bacterial growth. Our experiment silages were kept at normal temperature (28-30^{o}C) and we achieved our desirable pH level (below 4.5) within 6 days of fermentation. Acid silage's pH was the lowest among all the silages during the fermentation period due to the addition of 3% formic acid.

LAB counts increased significantly up to 7 days of fermentation, and the counts reached the maximum number within 20 days of fermentation. Maximum LAB counts were found in FS1, FS2, FS3 and AS1 were 7.959, 8.217, 8.243 and 7.556 (log bacteria/g). The counts then

decreased gradually to around log 5.00 bacteria/g at the end of storage. LAB counts were lowered in the acid silage than in the other fermented silages throughout the storage time. This finding coincides with Hasan [16], although he reported log 10.45 bacteria/g as maximum LAB count.

Non-protein nitrogen values of all the silages were found very high after fermentation, and there was a little significant difference ($P<0.05$) between the acid silage and fermented silages. NPN concentration of the acid silage was comparatively lower than fermented silage. An increase in NPN value during fermentation was also reported by Hasan [16]. He found an increase in NPN from 18.37% to 60% during fermentation. However, our findings coincide with the findings of Fagbenro [16]. FS-1 produced the lowest NPN (37.56±1.39) among fermented silages, and FS-3 silage produced the highest NPN (42.27±1.98). The higher production of NPN in FS-3 silage may be due to the more readily active bacterial starter.

The proximate composition of the silages after fermentation have shown some variation. Moisture content was low in the acid silage than in fermented silages. Acid silage also contained the highest amount of crude protein (31.25±0.75%) and showed significant differences ($P<0.05$) with other fermented silages. FS-3 contained the highest amount of protein (28.35±1.11%) among the fermented silages. We have found the amount of crude protein is (21-32%) in dry matter that means three to four times more silage required than fish meal in feeds for equivalent protein contents. While, Fagbenro [14] suggested that four to five times more fish silage would be required.

The proximate composition report did not show any significant difference ($P>0.05$) in lipid content. Lipid content of the final products were normally very high which is different from the findings of Fagbenro and Hasan [14,16]. The differences are mainly due to the differences in fermented substrates. Most of the researchers used low fatty fish for silage production to avoid rancidity.

Our result on the silages amino acid profile showed that Methionine content was low around 2.52 to 2.67. Leucine and Phenylalanine content was also low, which is similar to the findings of Dong [17] in salmon viscera silage and Åsgård [18] in dogfish silage. They reported that this limitation is due to enzyme autolysis during storage. Alanine, lysine and glutamic acid content were very high in the present study. However, the study of Jackson and Fagbenro [19,14] reported a limitation in lysine and threonine content which is not shown in our study. Histidine content in ours is low, which is similar to the findings of Hasan [16]. Vidotti [20] reported high histidine, methionine, and tyrosine concentrations in his silages while in our silages contain those amino acids in low concentration. According to Tacon [20], thirty percent minimum essential amino acids are required for fish diet to FAO standard level of amino acids. All our silages fulfil these minimum amino acids requirement.

5. CONCLUSION

The raw materials used in this experiment is mainly treated as waste and discarded without further use. The methodology applied in this experiment is to utilise the nutritional value of the discarded fish waste. After analysing the crude protein content and amino acid profile of the silages, it is possible for the partial replacement of fish meal with fish silages produced by the methodology applied in this experiment. Although the silages nutritional quality supports to use it as animal feed substrate, it needs to pass animal feeding trial test.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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