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Biochemical and Microbiological Quality of Formic Acid Silage and Lactobacillus Fermented Silage

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Acid silages (AS) were prepared by mixing formic acid with silver belly (*Leiognathus sp.*) mince at 2%, 2.5% and 3% (v/w) and fermented silages (FS) were prepared by mixing *Lactobacillus plantarum* culture with fish mince at 5% (v/w) and molasses at 10% and 12% (v/w). Sodium benzoate was added at 0.5% (w/w) level to FS to inhibit mould growth. pH of 2.5%AS and 3%AS fell below 4.5 within 2 days and stabilized at 4.24 and 4.01, respectively. pH of 2%AS reached a minimum value of 4.68. In 10%FS and 12%FS, the pH dropped to less than 4.5 by the end of 1st day indicating good lactic acid fermentation by *Lactobacillus plantarum*. Crude protein of the silages ranged between 18.22% and 19.17%. Fat was lower in FS (1.0 – 1.14%) than in AS (3.67 – 5.13%) on wet basis. NPN of FS was found to be lower than that in AS which indicates lesser protein breakdown. α-amino nitrogen in FS changed to 21% of TN in 10% FS and 12%FS from an initial concentration of 11% of TN, which was lower than that in AS. PV was lower in AS than in FS. Microbiological quality of AS and FS was found to be good as indicated by the absence of total coliforms, faecal coliforms, *E. coli, Salmonella, Vibrio cholerae*, coagulase positive *Staphylococci* and H_2S producing bacteria. Total yeast mould count was highest in 2% AS (1600/g).

Key words: Fish silage, formic acid, *Lactobacillus plantarum*, biochemical changes, microbiological quality, proximate composition

Trawl bycatch is a major contributor to the total amount of fish caught in shrimp trawls and discarded at sea (Clucas & Teutscher, 1998). Most of the discards on the east coast of India come from Visakhapatnam based trawlers (Gordon, 1991). 224 species of fish belonging to 69 families representing the economically low value bycatch were observed off Visakhapatnam, of which 66.6% to 94.2% constituted of juveniles (Sujatha, 1996). These underutilized fish can be preserved conveniently for use as a source of protein by ensilation with acid or by microbial fermentation methods. Fish ensilage is a potential source of protein for livestock nutrition. The present work was taken up to study the biochemical changes that take place during the conversion of small sized low value fish, silver belly (*Leiognathus sp.*) into silage by formic acid treatment and lactobacillus fermentation methods and also to assess the microbiological quality of the silages.

Materials and Methods

Freshly landed silver bellies (*Leiognathus sp*) from Visakhapatnam fisheries harbour were brought to the laboratory in iced condition. The fish were washed in clean water and were chopped thoroughly in a mechanical chopper for 2 – 3 min to yield mince. 5 kg of fish mince was used for each

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treatment (Table 1). 3 batches of acid silage (AS) were prepared by mixing formic acid with fish mince at 3 levels viz., 2%, 2.5% and 3% (v/w). Two batches of fermented silage (FS) were prepared by mixing *Lactobacillus plantarum* (NCIM 2083, National Chemical Laboratory, Pune) culture (grown in MRS broth; 37°C/24h) at 5% (v/w) with fish mince and molasses at 2 different levels viz., 10% and 12% (v/w). Sodium benzoate was added at 0.5% (w/w) level to FS batches. Ensilation was done in airtight plastic containers kept at ambient temperature. The silages were stirred, daily at 9.30am and 4.30pm and at the time of drawing the sample for analysis.

Analysis of non-protein nitrogen (NPN), α-amino nitrogen, peroxide value (PV) and proximate composition was done as per standard methods (AOAC, 1990). Total volatile base nitrogen (TVN) was determined by the Conway micro diffusion method (Conway, 1947). pH was determined by homogenizing 5g samples in 100 ml distilled water and observing the pH using pH meter (Gri pH meter, Systronics) or by directly dipping the electrode into the ensilage after mixing thoroughly.

Aerobic plate count (APC), total yeast mould count (TYM), MPN total coliforms, MPN faecal coliforms, MPN *Escherichia coli*, *Salmonella*, *Vibrio cholerae*, coagulase positive Staphylococci were determined as per Bacteriological Analytical Manual, 1995. Lactic acid bacteria (LAB) counts were obtained using de Man Regossa Sharpe (MRS) agar (Speck, M.L.1978) and H_2S producing bacterial counts were determined employing peptone iron agar (Gram *et al.*, 1987).

Results and Discussion

Silver bellies (*Leiognathus sp.*), are available in large quantities along Visakhapatnam coast (CMFRI, 2002, 2003). They are lean fish with low fat content (Chakrabarti and Khasim, 1987), have smaller visceral portions and can be easily hydrolyzed using either formic acid or by lactobacillus fermentation. Whole silver bellies were washed and minced in a mechanical cutter. Chopping helps in utilizing the entire fish and also increases the surface area for the acid and bacteria to exert their action thoroughly. Three batches of AS were prepared using formic acid at 2%, 2.5% and 3% volume by weight of fish mince in order to ascertain the minimum level of formic acid required to obtain silage from silver bellies. Two batches of FS were made using molasses as carbohydrate source at 10% (10%FS) and 12% (12%FS) levels in order to obtain stable silage having low molasses odour. Low natural LAB counts and low level of free sugar in fishes necessitates external inoculation of LAB and addition of carbohydrate source for stable fish silage production. *L. plantarum*, which is homofermentative, has been shown to be very effective starter culture (Bello *et al.*, 1992). Different workers have used molasses at 10-15% level (Fagbenro and

Table 1. Ingredients for acid silage and fermented silages

Method	Treatment	Composition
Acid silage (AS)	2%AS	5 kg fish mince + 100ml Formic acid
	2.5%AS	5 kg fish mince + 125ml Formic acid
	3%AS	5 kg fish mince + 150ml Formic acid
Fermented Silage (FS)	10%FS	5 kg fish mince + 250ml *LP** culture + 500ml Molasses + 25g Sodium benzoate
	12%FS	5 kg fish mince + 250ml *LP** culture + 600ml Molasses + 25g Sodium benzoate

**LP -Lactobacillus plantarum*

Jauncey 1994; Suchindra *et al.*, 1994). Molasses was preferred, as it was relatively cheaper. Silver belly mince had a LAB count of only 30cfu/g. *Lactobacillus plantarum* (NCIM 2083) was used as starter culture (37°C/24h) at 5% level (v/w). Sodium benzoate was used at 0.5% level in FS batches to inhibit mould growth.

The odour of AS and FS was agreeable. 2%AS gave a distinct fish odour but 2.5%AS and 3%AS had relatively less fish odour. FS had predominantly molasses odour. Fish odour of FS was less than AS. The colour of AS is light brown with grayish tinge whereas FS was dark brown in unstirred condition. The silage, for first 24 h, appeared as thick solid mass but afterwards as the liquefaction proceeded, it gradually became semisolid and finally thick liquid. 2%AS gave a good consistency than 2.5%AS and 3%AS. 10%FS and 12%FS were similar in consistency.

Initial pH of raw silver belly fish mince was 5.9. The changes in the pH of different AS and FS are given in Table 2. The pH of stable silage should be less than 4.5 (Fagbenro and Olusoji, 1997). pH of 2.5%AS and 3%AS reached below 4.5 within 2 days and the pH of 2.5%AS stabilised at 4.24 while the pH of 3%AS stabilised at 4.01. In the case of 2%AS the pH reached a minimum value of 4.68 by the end of 4th day and remained constant thereafter. In FS batches, the pH dropped to < 4.5 by the end of 1st day in 10% FS and 12%FS. This decrease in pH to less than 4.5 in 10%FS and 12%FS indicates successful lactic acid fermentation by *Lactobaillus plantarum* culture (5% inoculum level). By the end of 9th day, the pH stabilised at 4.04 in 10%FS and 3.94 in 12%FS.

Liquefaction of fish is due to the action of wide variety of endogenous proteinases present in the fish (Raghunath & Gopakumar, 2002). During ensilation autolysis takes place and the fish muscle gradually liquefies as the acid or the microbial action breaks the cell wall due to their higher pKa value (Formic acid 3.75, Lactic acid 3.86) and hydrolytic

Table 2. pH values of acid silages and fermented silages over a period of 21 days

	1st day	3rd day	6th day	9th day	12th day	15th day	18th day	21st day
2%AS	5.00	4.82	4.68	4.68	4.68	4.68	4.68	4.68
2.5%AS	4.68	4.52	4.24	4.24	4.24	4.24	4.24	4.24
3%AS	4.34	4.21	4.01	4.01	4.01	4.01	4.01	4.01
10%FS	4.54	4.33	4.14	4.04	4.04	4.04	4.04	4.04
12%FS	4.34	4.23	4.12	3.94	3.94	3.94	3.94	3.94

Table 3. Changes in NPN (g/100g TN) of acid silages and fermented silages

DAY	1st day	3rd day	6th day	9th day	12th day	15th day	18th day	21st day
2%AS	28.56	40.80	42.16	65.28	70.72	70.72	70.72	70.72
2.5%AS	21.39	36.09	56.14	56.14	56.14	56.14	56.14	56.14
3%AS	27.44	43.90	43.80	43.80	50.76	50.76	50.76	50.76
10%FS	10.43	43.68	58.68	58.68	61.29	61.29	61.29	61.29
12%FS	13.36	45.45	53.47	53.47	61.49	61.49	61.49	61.49

enzymes are released which act in concert to bring about the autolytic activity (Raa and Gildberg, 1982). In 2%AS, NPN levels reached a maximum of 70.72% of TN by the end of 12 days (Table 3), which may be attributed to the proteolytic activity of microorganisms as the pH was above 4.5. By 6th day only 42.16% of total NPN value was observed which indicates a continuation of proteolytic activity and liquefaction. The values of NPN in fish silage may vary depending on the extent of protein breakdown in which pH plays a major role (Raghunath & McCurdy, 1990). The initial NPN levels for 10%FS and 12%FS were at 10.43% of TN and 13.36% of TN, respectively. This may be attributed to the adsorption of enzymes by the carbohydrates, thereby restricting their interactions (Raa and Gildberg, 1982). Fagbenro and Jauncey (1993) observed that NPN of tilapia FS increased gradually from 16% and attained a maximum of 46.5% after 30 days which was lower than that obtained in acid preserved silage. Faid *et al.*, (1997) noted an increase in NPN for 11 days and thereafter remained constant in fermented *Sardina pilchardus* silage.

Changes in TVN of AS and FS are given in Table 4. The initial TVN values were lower in 3% AS in comparison to 2.5%AS and 2%AS. 10%FS showed a relatively lower increase than 12%FS. This indicates the continuous production of volatile bases due to the breakdown of protein by the action of microorganisms more in 12% FS than 10% FS. Faid *et al.*, (1997) reported that TVN pattern showed slight increase during fermentation and observed an increase in TVN from 71.26 mg/100g to reach 95.03mg/100g after 1 day and remained constant at 132mg/100g after 15 days of fermentation at 22°C.

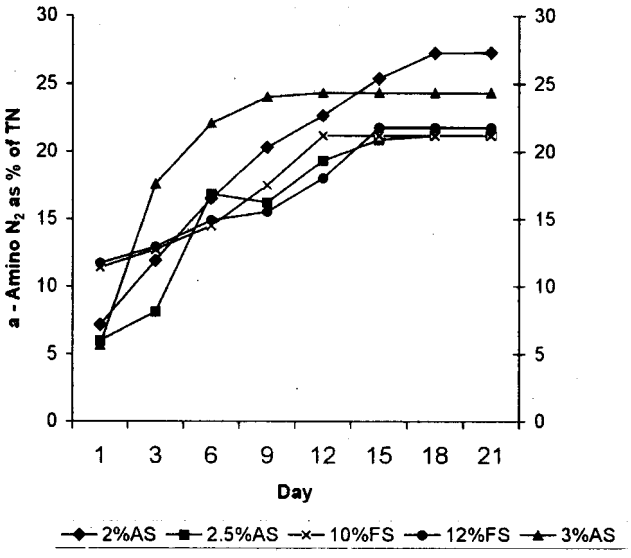

Fig. 1. Alpha-amino Nitrogen as percentage of Total Nitrogen

The differences in α-amino nitrogen levels of AS and FS are given in Fig 1. It was observed that the maximum α-amino nitrogen value (as % of TN) obtained was lower in 2.5% AS (21.21% of TN) than in 3%AS (24.33% of TN) and 2% AS (27.30% of TN). The α-amino nitrogen values in FS were constant at 21% of TN by 15th day in both the concentrations (10%FS and 12% FS) from an initial concentration of 11% of TN. The maximum α-amino nitrogen levels as % of TN were almost identical in 2.5% AS, 10%

Table 4. Changes in TVN (mg%) values of acid silages and fermented silages

	1st day	3rd day	6th day	9th day	12th day	15th day	18th day	21st day
2%AS	124	172	202	232	236	236	236	236
2.5%AS	72	116	168	170	180	180	180	180
3%AS	52	68	96	100	116	116	116	116
10%FS	40	ND	92	150	208	208	208	208
12%FS	44	ND	132	174	208	208	208	208

Table 5. Microbiological quality of acid silages and fermented silages

Parameter	2%AS	2.5%AS	3%AS	10%FS	12%FS
Aerobic Plate Count (APC), cfu/g	1.2×10^5	3.7×10^3	3.2×10^3	8.4×10^6	8.5×10^6
Total coliforms, MPN/g	0	0	0	0	0
Faecal coliforms, MPN/g	0	0	0	0	0
Escherichia coli, MPN/g	0	0	0	0	0
Coagulase positive *Staphylococci*, cfu/g	0	0	0	0	0
Salmonella /25g	Absent	Absent	Absent	Absent	Absent
Vibrio cholerae /25g	Absent	Absent	Absent	Absent	Absent
H_2S producing bacteria, cfu/g	0	0	0	0	0
Lactic Acid Bacteria (LAB) count, cfu/g	0	0	0	9.6×10^6	9.5×10^6
Total Yeast Mould count (TYM) cfu/g	1600	20	20	10	10

FS and 12% FS, indicating the extent of clevage of peptide bonds. By the sixth day itself appreciable production of á-amino nitrogen was observed indicating strong proteolysis.

The peroxide value of AS and FS silage were found to be within desirable level which indicates a low level of lipid oxidation as silver bellies had low fat. 3% AS had the lowest PV of 2.32 milliequivalents/Kg of oil followed by 2.5% AS with 4.68 milliequivalents/Kg of oil. 10% FS and 12% FS had PV of 13 milliequivalents/Kg of oil and 12.5 millimoles/Kg of oil.

Microbiological analysis of AS and FS was carried out after the stabilisation of pH of AS and FS and the results are given in Table 5. The utilization of AS and FS in livestock feed depends on quick reduction of pH in AS and rapid growth and acid production by LAB in FS eliminating harmful pathogens and arresting the growth of spoilage microbes. Microbiological quality of silages was good since AS and FS were found to be free from total coliforms, faecal coliforms, *E.coli* and harmful pathogens viz., *Salmonella*, *Vibrio cholerae*, coagulase positive *Staphylococci*. Acid sensitive typhoid and cholera bacteria are completely destroyed in acid silage (Raa & Gildberg, 1982). APC of 2% AS was higher than in 2.5% AS and 3%AS. Higher APC in FS was mainly due to the growth of LAB. 10%FS and 12%FS showed almost similar LAB counts of 9.6 x 10^6cfu/g and 9.5 x 10^6 cfu/g, respectively after stabilization of pH. The addition of *L. plantarum* as starter culture at (5% v/w) gave initial LAB concentration of 9 x 10^7 cfu per gram of silver belly mince. In the first two days of lactobacillus fermentation the LAB counts increased to 10^9 cfu per gram of FS and later decreased to 10^6 cfu per gram of FS after the stabilization of pH of FS. The minimum pH for survival of *L. plantarum* is 3.3 (Jackson, 2003). The continued presence of LAB in FS can be attributed to the higher pH as 10%FS and 12%FS stabilised at pH 4.04 and 3.94, respectively. LAB is the dominant bacterium in FS and so higher counts are obtained on LAB specific MRS agar than on ordinary plate count media. LAB were not detected in AS. H_2S producing bacteria were not detected in AS or FS. Spoilage in silages was detected at pH > 4.9 (Ariyani & Buckle, 1991). Low microbial counts in AS is mainly due to acidic pH whereas in FS the absence of indicator,

Table 6. Proximate composition of acid silages and fermented silages of silver bellies

	Moisture, %	Crude Protein, %	Fat, %	Ash, %
2%AS	74.33	18.38	3.67	4.1
2.5%AS	68.4	18.70	3.78	4.34
3%AS	70	18.22	5.13	4.5
10%FS	70.5	19.17	1.0	5.16
12%FS	69	18.64	1.14	5.68

pathogenic and spoilage bacteria can be attributed apart from low pH to other antimicrobial factors such as organic acids, hydrogen peroxide and bacteriocins produced by LAB (Earnshaw, 1992). AS had relatively higher fungal counts than FS. TYM count was highest in 2%AS (1600/g). Low TYM counts in FS might be attributed to the antifungal action of sodium benzoate. The addition of sodium benzoate at 0.5% level had not interfered with the fermentation process as evinced by good growth of *Lactobacillus plantarum* with associated drop in pH to < 4.5. Formic acid was unable to prevent the growth of the mould *Aspergillus flavus* at pH > 4.0 (Strom *et al.*, 1980).

Proximate composition of AS and FS is shown in Table 6. Moisture levels of AS ranged between 68.4% and 74.3% while that of FS ranged between 69% and 70.5%. Crude protein (CP) ranged between 18.22% (3%AS) and 19.17% (10%FS). Fat was distinctly lower in FS (1.0 – 1.14%) than in AS (3.67– 5.13%). Oil is efficiently trapped in the microbial silage because of the high binding ability of the added polysaccharide and the slow autolysis of the fish proteins (Raa and Gildberg, 1982). Ash content was higher in FS than in AS.

Feed using fermented silage as animal protein source was made by mixing liquid 10%FS (19.17%CP) with solid rice bran (16.7%CP) and solid sago processing waste (9.17%CP) to obtain feed with an expected CP of 15% after drying. Addition of liquid fish ensilage was done after taking into account the moisture content of liquid silage. The feed mix was dried, pulverized, packed and stored at ambient temperature. The dried feed had 4.4% moisture, 15.31%CP, 1.34% fat and 10.97% ash. There was little change in crude protein level and there was slight increase in moisture content from 4.4% to 6.7% by the end of 5th month. TVN showed no significant variations and was 64mg/100g by the 5th month. PV showed an increasing trend from 8.42 milliequivalents/Kg of oil at the end of first month to 21.81 milliequivalents/Kg of oil at the end of 5th month of storage.

Silver bellies can be efficiently transformed into silage either by formic acid treatment or lactobacillus fermentation and can be incorporated in livestock feeds as animal protein source. Formic acid level of 2.5% (v/w) gave better acid silage than 2% and 3% levels. Protein break down was higher in 3%AS while 2% had higher fungal contamination. Fermented silage was successfully prepared employing *Lactobacillus plantarum* at 5% (v/w) at both 10% and 12% level of molasses (v/w). 10%FS can be preferred as it had lesser molasses odour. Fish silage yields microbiologically good quality product that can be incorporated into feeds as animal protein source and can be prepared depending on the availability of low value underutilized fish even during cloudy or rainy days.

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References

AOAC (1990) Official Methods of Analysis, 15th edn., Association of Official Analytical Chemists, Washington, DC, USA

Ariyani, F. and Buckle, K. (1991) Ensiling of prawn Heads. *Asean Food J.* **6**, pp 58-63

Bacteriological Analytical Manual (1995) 8th edn., AOAC International, Gaithersburg, USA

Bello, R., Gutierrez, M., Ottati, M. and Martinez, A. (1992) *Study on the processing of fish silage microbiologically in Venezuela.* Paper presented at the Second Experts Consultation on Fisheries Products Technology in Latin America, Montevideo,11-15 December, 1989, **441**, 7p, FAO, Rome, Italy

Chakrabarti, R. and Khasim, I. (1987) Value added silver bellies : a boon. *Fishing Chimes.* **7** pp 71-74

Clucas, I. and Teutscher, F. (1998) *Report and proceedings of FAO/DFID expert consultation on by-catch utilization in tropical fisheries*, 21-28 September, 1998, Beijing, China.

CMFRI (2002) *CMFRI Annual Report* 2001-2002, 20p

CMFRI (2003) *CMFRI Annual Report* 2002 – 2003, 18p

Conway, F. (1947) *Microdiffusion and Analysis and Volumetric Error*, Crosby Lockwood, London, pp 157-159

Earnshaw, R.G. (1992) The antimicrobial action of Lactic Acid Bacteria: Natural food presevation system. In : *The Lactic Acid Bacteria in Health and Disease*, Vol 1, (Wood, J.B.A. Ed), pp 211-232, Elsevier Science Publishers, England

Faid, M., Zoiten, A., Elammarrakchi, A. and Achkari-Begdouri, A. (1997) Biotransformation of fish waste into a stable feed ingredient. *Food Chem.* **60**, pp 13-18

Fagbenro, O. and Jauncey, K. (1993) Chemical and Nutritional quality of raw, cooked and salted fish silages. *Food Chem.* **48**, pp 331-335

Fagbenro, O. and Jauncey, K. (1994) Chemical and Nutritional quality of fermented fish silage containing potato extracts, formalin or ginger extracts. *Food Chem.* **50**, pp 383-388

Fagbenro, O. and Olusoji, O.B. (1997) Preparation, nutrient composition and digestability of fermented shrimp head silage. *Food chem.* **60**, pp 489-493

Gram, L., Trolle, G. and Huss, H.H. (1987) Detection of specific spoilage bacteria from fish stored at low (0°C) and high (20°C) temperatures. *Intl. J. Food Microbiol.* **4**, pp 65-72

Gordon, A. (1991) The bycatch from Indian shrimp trawlers in the Bay of Bengal- the potential for its improved utilization. *Bay of Bengal Programme.* WP **68**

Jackson, L. (2003) Food Hazards : Biological. In: *Food safety Handbook* (Schmidt, R.H. and Rodrick, G.E. Eds.) 139p, Wiley Interscience, John Wiley & Sons Inc, Hoboken, New Jersey.

Raa, J. and Gildberg, A. (1982) Fish Silage: A review, *CRC Critical Reviews in Food Science and Nutrition.* **16**, pp 383-419

Raghunath, M.R. and Gopakumar, K. (2002) Trends in production and utilization of

fish silage. *J. Food Sci. Technol.* **39**, pp 103-110

Raghunath, M.R. and McCurdy, A.R. (1990) Influence of pH on the proteinase component and proteolytic products in rainbow trout viscera silage. *J. Agri. Food Chem.* **38**, pp 45-50

Speck, M.L. (1978) *Compendium of methods for microbiological examination of foods*, American Public Health Association, Washington DC, USA

Strom, T., Gildberg, A., Stromo, B. and Raa, J. (1980) In : *Advances in Fish Science and Technology*, (Connel J.J., Ed) 352p, Fishing News Books, Farnham, Surrey, England

Sujatha, K. (1996) Trash fish catch of the trawl fishery off Visakhapatnam. *J. Aqua. Biol.* **11**, pp 17-23

Suchindra, N.M., Lalitha, K., Rao, S. and Sripathy, N.V. (1994) Ensiling prawn carapace by lactic acid. *Seafood Export Journal*, March 1994, pp 13-16