BIOCHEMICAL, MICROBIOLOGICAL AND NUTRITIONAL EVALUATION OF FERMENTED FISH SILAGE

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CERTIFICATE

Certified that the thesis entitled **"BIOCHEMICAL, MICROBIOLOGICAL AND NUTRITIONAL EVALUATION OF FERMENTED FISH SILAGE"** is a record of independent bonafide research work carried out by **Mr. ZYNUDHEEN. A.A.** during the period of study from September 2003 to August 2006 under our supervision and guidance for the degree of **Doctor of Philosophy (Post Harvest Technology)** and that the thesis has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or any other similar title.

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सारांश

वांछित परिवर्तन के लिए किण्वन द्वारा मत्स्य संसाधन, सारे विश्व में प्रचलित पुरानी प्रथा है, खासकर दक्षिणी-पूर्वी देशों में। ये बहुत प्रचलित और अत्यधिक स्वादिष्ट उत्पन्न है। किसी भी मात्स्यकी संसाधन के लिए मत्स्य साइलेज की तैयारी सरलतम तरीका दिखाई पडा । किसी भी मात्स्यकी रद्दी कितनी परिणाम की हो कहीं भी हो बिना यंत्रों से संरक्षित किया जा सकता है। मक्खी कीडाणुबाधा, दुर्गन्ध आदि पर्यावरणीय समस्याओं को दूर करने पर यह प्रक्रिया सभी पौष्टिकों को बनाए रखता है। किण्वन द्वारा एनसाइलेज की तैयारी के लिए कम समय की आवश्यकता है और अंतिम उत्पन्न गुणवत्ता मानकीय व्यवस्थाओं के अधीन भविष्य कथित किया जा सकता है। खाद्य पूरक के रूप में अम्ल साइलेज की तुलना में किण्वित मत्स्य सइलेज कई पहलुओं पर अधिक पौष्टिक है। पौष्टिक गुणों के अलावा किण्वित मत्स्य साइलेज में लैक्टो बैसिलस जीवाणु निहित है जो पशुधन के लिए आंतीय फ्लोरा को सुधारकर प्रोबयोटिक का काम करता है। लैक्टाबैसिलस प्लान्टारम के साथ विभिन्न स्तरों पर कार्बोहाइड्रेट प्रयुक्त करके तैयारित किण्वित मत्स्य साइलेज सूचित किया गया है कि 13 दिनों के संपूर्ण किण्वन के लिए 10% स्तरों पर गुड पर्याप्त है। प्रसाधित मत्स्य साइलेज की तुलना में पूरे मत्स्य से लिए गए किण्वित मत्स्य साइलेज में उन्नत स्तर पर जल अपघटन दिखाई पडा है। किण्वित मत्स्य साइलेज में वाष्पित मूल संयुक्त और जैव-जन्य अमीन बहुत कम दिखाई पडा। सूखे किण्वित साइलेज को उपवेशी तापमान में 10 महीनों से अधिक बिना परिवर्तन से संग्रहित किया जा सकता है। सूखे किण्वित मत्स्य साइलेज खिलाए गए आलबिनो मूषिकों पर संचालित पौष्टिक अध्ययन ने नियंत्रण ग्रूप की तुलना में औसतन बढ़ती दर में हुए परिवर्तन को सूचित किया गया। साइलेज खिलाए मूषिकों के हृदय एवं जिगर ऊतक में निम्न कोलेस्ट्रॉल एवं निम्न लिपिड घटक थे। साइलेज खिलाए गए मूषिकों पर सीरम लिपिड घटक खासकर सीरम HDL बहुत निम्न दिखाई पडा। सुरुमी संसाधन रद्दी से किण्वित मत्स्य साइलेज तैयारित है और पौष्टिक मूल्यांकन अम्ल मत्स्य साइलेज एवं जापानिस बटेरों के बीच किया गया है। अध्ययन ने सूचित किया है कि किण्वित मत्स्य रद्दी साइलेज खिलायी गयी पक्षियाँ उन्नत संख्या में अण्डे देती हैं और अण्डों की गुणवत्ता हीग यूनिट जैसा है और छिल्कों की मोटापन नियंत्रण नमूनों से भी श्रेष्ठ दिखाई पडा। लैक्टो बैसिलस प्रयुक्त करके खानेयोग्य माँस से तैयारित किण्वित मत्स्य पाउडर कुकीस की तैयारी में विभिन्न स्तरों पर समावेशित करता है और देख लिया गया है कि 2% स्तर पर पाउडर प्रयुक्त करने पर भी सुवास में कोई अंतर नहीं दिखाई पडता। उत्पन्न को अच्छी इन्द्रियग्राही स्वीकार्यता होती है। अम्ल साइलेज की तुलना में किण्वित मत्स्य साइलेज, पौष्टिक एवं इन्द्रियग्राही तौर पर श्रेष्ठ गुणवत्ता उत्पन्न को प्रदान करता है।

ABSTRACT

Preservation of fish by fermentation for desirable changes is an age old practice throughout the world especially in the South East Asian countries. These products are very popular and highly relished. Preparation of silage is found to be the easiest method for preservation of fishery waste at any place; and any quantity can be preserved with no major machinery involved. Environmental problems like fly infestation and smell can be eliminated while the process retains all the nutrients. Preparation of ensilage by fermentation takes less time and the end product quality can be predicted under standard conditions. As feed supplement, fermented fish silage is found to be more nutritious compared to acid silage in many aspects. In addition to the nutritive value fermented fish silage contains lactobacillus bacteria which act as a probiotic for the livestock by improving the intestinal flora. Fermented fish silage prepared with *Lactobacillus plantarum* at different levels of jaggery indicated that 10% levels of jaggery is sufficient for complete fermentation in 13 days. Fermented fish silage from whole fish is found to have better degree of hydrolysis when compared to dressed fish silage. The production of volatile compounds and biogenic amines were found to be minimum in fermented fish silage. The dried fermented silage could be stored for more than 10 months in ambient condition without much change. Nutritional studies carried out in albino rats by supplementing dried fermented fish silage indicated better growth rate compared to control group. The silage fed rats had less cholesterol and less lipid components in their heart and liver tissue. The serum lipid components, especially serum HDL was also found to significantly less in silage fed rats. The fermented fish silage was prepared from surimi processing waste obtained from commercial factory. Nutritional evaluation of silage was carried out along with acid fish silage and unsalted dried fish as control in Japanese quails. The study indicated that fermented fish waste silage is fed birds laid significantly higher number of eggs and the egg quality like Heauge unit and better shell thickness indicating that fermented fish waste silage can be supplemented in layer birds. Fermented fish powder prepared from edible meat by using lactobacillus was incorporated in the preparation of cookies in different levels and it was observed that

incorporation of the powder at 2% levels does not result in flavour difference. The product had good acceptability when sensory analyses was carried out. Fermentation of fish with lactobacillus can result in products with modified quality characteristics and improved shelf life.

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LIST OF ABBREVIATIONS

AAN	-	Alpha amino nitrogen
ADP	-	Adenosine diphosphate
ANSA	-	Aminonaphthosulfonic acid
APS	-	Ammonium per sulphate
ATP	-	Adenosine triphosphate
BF_3	-	Boron trifluoride
BSA	-	Bovine serum albumin
BP	-	Boiling point
^0C	-	Degree celsius
cm	-	Centimeter
CFU	-	Colony forming Units
CPCSEA	-	Control and supervision of experiments on Animals
CuSO	-	Copper sulphate
DDC	-	Diethyldithiocarbomate
DHA	-	Docosahexaenoic acid
dl	-	Decilitre
DMA	-	Dimethyl amine
DTNB	-	5,5'-Dithiobis(2-nitrobenzoic acid)
EDTA.	-	Ethelene diamine tetraacetic acid
EPA	-	Eicosapentaenoic acid
FAME	-	Fatty acid methyl ester
FFA	-	Free fatty acid
Fig	-	Figure
g	-	grams
HDL	-	High density lipoprotein
HPLC	-	High performance liquid chromatography
h	-	Hours

IAEC	-	Institutional Animal Ethics Committee
IU	-	International unit
Kg	-	Kilogram
L	-	Litre
LAB	-	Lactic Acid Bacteria
LDL	-	Low density lipoprotein
M	-	Molar
mg	-	Milligram
μ moles	-	Micromoles
μg	-	Microgram
μl	-	Microlitre
min	-	Minutes
ml	-	Millilitre
mM	-	Millimolar
MRS	-	Man Rosoga Sharpe
N	-	Normal
NAD+	-	Nicotinamide adenine dinucleotide
NADH	-	Reduced nicotinamide adenine dinucleotide
NADP	-	Nicotinamide adenine dinucleotide phosphate
$NaHCO_3$	-	Sodium bi carbonate
NaOH	-	Sodium hydroxide
NPN	-	Non protein nitrogen
nm	-	Nanometer
OD	-	Optical density
OPA	-	Ortho phthalaldehyde
PE	-	Petroleum ether
Pi	-	Inorganic phosphorus
TN	-	Total nitrogen
PUFA	-	Poly unsaturated fatty acids
rpm	-	Revolution per minute
SD	-	Standard deviation
SDS-PAGE	-	Sodium dodecil sulphate – polyacrylamide gel electrophoresis

TBA	-	Thiobarbituric acid
TCA cycle	-	Tri carboxylic acid cycle
TCA	-	Trichloroacetic acid
TG	-	Triglyceride
TGA	-	Trypton glucose agar
TVBN	-	Total volatile base nitrogen
UV	-	Ultra violet
v/v	-	Volume / Volume
VLDL	-	Very low-density lipoprotein
WHO	-	World health organization
w/v	-	Weight / Volume

Introduction...

1. Introduction

The fisheries sector has been recognized as a powerful income and employment generator as it stimulates growth of a number of subsidiary industries and is a source of cheap and nutritious food. At the same time it provides livelihood for a large section of economically backward population of many of the developing nations. Of the total world fish production of 132.9 million tons, more than 75 % is utilized for human consumption and the rest is used for other purposes (FAO 2002). Out of the 32.25 million tons used for other purposes, 78% is used for reduction and remaining for miscellaneous purposes. The fish landing in India is around 6 million tons in 2005 of which the marine sector is contributing about 2.9 million tons, against the estimated potential of 3.9 million tons.

The fish processing industry in the country by and large depends on the shrimp landings which constitutes about 20% of the total landings. The trawling operations for prawn results in the landings of many low value varieties of fish most of which is thrown back to the sea. The by catch from Indian seas is mostly composed of Jew fish, perches, sole, barracuda, lizard fish, anchovies, lactarius, crab, bulls eye, threadfin breams etc. Industrial fish processing for human consumption yields only 40% edible flesh and the remaining 60% is thrown away as waste (Raa and Gildberg, 1982). The world export trade of fish waste is 6,75,970 tons in different forms worth 205.4 million US dollars. The import figures are 12,33,602 tons (value 328.1 million dollars). In India the export figure of the fish waste for the year 2002 is 2,016 tons worth 11.03 million US dollars (FAO 2002). With a view to utilize the by catch and processing waste, efforts have been made to develop methods for converting them into products for human consumption, animal nutrients or products of commercial importance. The wastage occurs mostly during fishing, handling and collection activities due to high ambient temperature, handling practices and the lack of ice and other

infrastructure facilities. In many countries of the world, some of the catch, which is not worth landing, is thrown back to the sea. In the shore, a significant quantity of the fish is wasted due to spoilage or damage during handling, storage and distribution. Additional wastage also occurs during fish processing operations.

Normally fishmeal production is considered as the best and first option for the utilization of fish waste or excess catch of fish during glut seasons as it is considered to be essential ingredient in live stock feeds, especially poultry. The world fishmeal production for the year 2002 is 6.4 million tons. Use of fishmeal as the major ingredient in commercial fish feeds is a common practice, despite extensive research into the possibility of replacing it with cheaper plant proteins. However fishmeal production is both capital and energy intensive, as it requires 60-70 kg fuel per ton of raw fish, mainly due to the necessity of separating the material into the three fractions viz., oil, protein and water. In contrast, the manufacture of commercial fish feeds often requires the recombining of fish protein with fish oil (Windsor and Barlow, 1981).

Some of the features of fish meal manufacturing like, huge capital investment, involvement of technical personal, and the environmental problems like smell during production are some of the disadvantages of fish meal production. In this respect, making the assumption that the both silage and fishmeal are nutritionally more or less equivalent, the production of fish silage gains significance. Additionally, the production of fermented fish silage has other advantages like, low cost of production, utilization of locally available jaggery sources, comparatively better nutritional qualities, the probiotic effects etc make it a more attractive option for the utilization of trash fish and fishery wastes from processing centres and market places compared to fish meal.

Fish silage is a liquid product that can be prepared from whole fish or parts of fish (fish waste) that are liquefied by the action of endogenous gut enzymes of the fish in presence of added acid or the *in-situ* production of the acid

by the microorganisms added along with some carbohydrate source. The action of the enzymes breaks down the fish protein into smaller units of increased solubility, whereas the addition of acid increases the activity of the enzymes and lowers the pH of the final material. It also inhibits the growth of spoilage organisms and thereby ensures the stability of the silage during storage. The process is neither capital nor energy intensive and the product possesses good storage characteristics if properly made (Jackson *et. al.,* 1984). However it is widely used as a dietary ingredient for poultry and pigs. The suitability of fish silage as a feed ingredient for poultry, cattle and fish has been well recognized (Asgard and Austreng, 1981).

Lactic acid fermented products can be prepared in a shorter period (and hence more cheaply) than the traditional fermented fish salt products which depend primarily on autolytic process. Their lower salt content also permits them to be consumed as main course rather than the condiment role of high salt fish sauce and pastes. Lactic acid bacteria are known to possess anti bacterial properties attributed to major end products of their metabolism such as lactic acid, acetic acid, hydrogen peroxide and peptide compounds termed bacteriocins.

The use of LAB fermentation of crustacean wastes was proposed for the deproteinisation and the recovery of proteins and pigments during the production of chitin. The ensilation involves the addition of fermentable sugar and inoculum of LAB for liquefaction and release of protein and calcium. The recent trends in utilization of fermentation technology includes the crustacean waste ensilation with cassava for use as poultry feed, aquaculture feed etc. Attempts have been made to apply LAB to whole fish or fillets to be eaten as such. Since the traditional means of fish preservation like, salting /smoking/pickling have fallen in popularity due to use of 'chemicals' such as vinegar and smoke components for preservation, the use of LAB could be a suitable choice for some of these preservative effects. The use of LAB fermentation in combination with other

preservative techniques like hurdle technology or MAP can contribute to the microbial safety and quality of fishery products (Hall, 2002). A new processing technique was attempted by inoculating LAB in mackerel mince for depression of main micro flora; decline in pH and to improve the sensory qualities (Yin *et. al.*, 2002). Similarly, intermediate moisture food was prepared by mincing followed by fermentation and extrusion to develop snack foods with better textural properties (Karmas and Lauber 1987).

With the development of probiotic feeds with inoculum of LAB it was found that certain strains have adaptive response for the adverse conditions of the gastrointestinal tract of animals. With advanced biotechnological tools it is possible to reduce the fermentation time of traditional products like fish sauce, and for the development of new semi preserved products.

1.1. Objectives of the Study:

- To optimize the jaggery level required for the production of fermented fish silage
- To assess the optimum period required for ensilation using *Lactobacillus plantarum.*
- To standardize of the process of ensilation by fermentation using dressed fish and whole fish.
- To assess the fermentation characteristics of silage from different species of dressed fish.
- To study the shelf life of fermented fish silage in ambient conditions.

- To assess the pattern of changes of biogenic amines in fermented fish silage.

- To study the nutritional quality of fermented fish silage prepared from different source by in vitro studies in albino rats.

- To explore the possibilities of the utilization of surimi processing waste as a source for the production of fermented silage.

- To assess the effect of supplementation of layer mash with fermented fish waste silage on Japanese quails.

- To develop high quality fermented fish powder for edible purpose

- To optimize the levels of incorporation of edible fermented fish powder in snack products and to evaluate its nutritional quality.

Review of Literature...

2.0 Review of Literature

2.1. Fish silage

Fish silage is the product of the process of preserving and storing wet biological material with the help of acid in a silo (Ockerman, 1992). The production of fish silage was started in Sweden in 1936 to utilize fish waste for feeding purpose of livestock. Fish silage may be defined as a liquid product, made from whole or parts of fish, to which no material has been added other than acid and in which liquefaction is carried out by enzymes already present in the fish (Gopakumar, 1997). Silage production is considered as one of the best ways of preserving agro and animal waste. Fish ensilaging is a method for utilizing trash fish, bycatch and processing wastes whilst supplying high quality protein for animals such as poultry, pigs, calves and other species such as mink. It is a stable liquid with a malty odour containing all the water present in the original material. Because of the increasing use of low-value fish for human food, production of fishmeal has decreased latterly and interest in production of fish silage has grown (Raghunath & Gopakumar, 2002)

Silage production could have many advantages for the maritime countries as it can make use of trash fish, bycatch fish and fish waste from processing industries, which are currently wasted. The scale of operation can be easily varied depending on the supply of fish. The conversion of fish waste to silage has the advantage of being an inexpensive supplement for animal feeds while at the same time reducing problems due to waste and environmental pollution

2.1.1. Nutritional characteristics of fish silage

Fish and marine invertebrates are important sources for nutrients for the world population, and many species have exceptionally high market value. The utilization of fish in different forms depends upon the quality and availability. Fish is an easily perishable commodity and the information on the biochemical

constituents will help a processing technologist to define the optimum processing and storage conditions, so that the quality is preserved to the maximum extent (Nair, 2002). The nutritional quality of fish varies according to the age, season of the catch, sex, the geographical location and the method of catch. The important components of nutritional significance in fish are protein, lipids, minerals and vitamins. Fish is a good source of animal protein and fish supplies 35-60% of the animal protein requirement of many Asian countries. On an average fish contain 10-22% protein, 1-20% fat and 0.5-5% minerals and small amount of jaggery. Fish protein is considered superior to protein from other sources (Diwan, 2002).

Fish silage is a nutritionally balanced diet extensively used in feeds in combination with other ingredients. It is found to be superior to other protein diet of plant or animal origin. Simple method of preparation and long shelf life make it a preferred choice of preservation of trash fish and fish waste. The nutritional qualities of fish are preserved in silage and in case of fermented silage it is enhanced. The nutritional composition of fish silage is almost similar to fish except a slight increase in moisture content. In many countries, seafood constitutes the main source of animal protein in human diet (Sikorski. *et. al.*, 1994). The protein content of the silage is in the range of 16-19% (Rattagool *et.al.*, 1977). Neethiselvan *et.al*, (2001) also reported the protein content of different types of fermented silages more or less same as that of fresh fish (Sidwell, 1981). The fat content of fish silage varies according to the fat content of the species used. Babu *et al.*, (2005) reported a higher level of fat (3.6% - 5.1%) in acid silage and nearly 1% fat in fermented silage. The presence of oil with high levels of polyunsaturated fatty acids (PUFA), thus very prone to oxidation is one of the constraints for its broad use in animal feeding. (Maria *et.al.*, 1998). The ash content of silage also depends on the nature of the raw material used; a higher level of ash can be expected if fish waste is used for ensilation. Generally acid silage contains less ash compared to fermented silage when the same raw material (Babu *et. al.*, 2005), probably due to the minerals present in the added molasses.

2.1.2. Acid fish silage

Ensiling can be achieved either by treating the fish directly with a mineral acid (sulphuric acid) or organic acid (formic or propionic acid) or by lactic acid produced *in situ* by fermentation. The fish is partially digested and preserved by the acid (James 1996). Among mineral acids, sulphuric acid or a mixture of sulphuric and hydrochloric acids is used to produce silage. The most commonly used organic acids are propionic, acetic and formic acids (Tatterson & Windsor, 1974; Disney *et. al.*, 1978). A 3% by weight of 98% formic acid is added to the well ground fish mince and mixed well ensuring a pH around 4 to prepare acid fish silage using organic acid. The whole fish is comminuted in a mechanical mincer and the required quantity of acid or acid mixture is added and the slurry is mixed well. After this process the whole material becomes a good paste that can be stored in tanks with daily stirring. Within 15-20 days the silage is ready for use.

For the successful production of acid silage, the following precautions are recommended (Disney *et. al.*, 1977):

- The material should be reduced in size, preferably to pieces of size 3-4 mm.
- Acid should be thoroughly dispersed throughout the minced fish to avoid air pockets of untreated material where bacterial spoilage can continue.
- Periodic agitation is necessary to bring about rapid liquefaction.
- Temperature of at least 20 ° C is desirable, since below this temperature, liquefaction is rather slow.

But for fish with a high mineral content, even 8% (v/w) of a 1:1 v/v mixture of formic and propionic acids may be necessary (Ariyani & Buckle 1991). Silage preserved with formic acid has a shelf life upto one year in tropical conditions of storage. Since organic acids are more expensive, a combination of organic and inorganic acid is recommended. Cheap mineral acids like sulphuric acid or

hydrochloric acid are used to lower the pH and organic acids like propionic or formic acids are added to it for antimicrobial activity (Gopakumar, 1997). Phosphoric acid also has been used for making silage, but has to be combined with potassium sorbate (0.1%) as preservative to prevent mold and yeast growth (Levin *et. al.*, 1989). Acid preserved fish silage liquefies rather quickly and after about a week in tropics, upto 80% of proteins become solubilised. The liquefied silage separates into 3-4 layers; an oily layer at the top, sometimes with an underlying emulsified layer, an aqueous middle layer containing the liquefied proteins and a sediment or sludge containing the undigested protein, scales, bones etc (Raghunath & McCurdy, 1987). The liquefaction is due to a wide variety of endogenous proteases present in the fish. Although a high conversion of proteins to small peptides and free amino acids promotes better liquefaction and separation but disadvantageous because. (a) The aromatic amino acids separate from the aqueous phase due to their low solubility (Raa & Gildberg, 1982) may even crystallize out (Espe *et.al*, 1991) (b) Higher leaching losses can occur when such silages are incorporated into feeds due tot their greater solubility (Lopez & Viana, 1995) and (c) Intact proteins appear to be utilised better in feeds especially by fish (Stone *et.al*, 1989). To curtail the excessive hydrolysis different methods like heating the mixture to inactivate the proteinases (Viana *et.al*, 1993; Lopez & Viana, 1995), addition of formalin (Husain & Offer, 1987) or addition of ginger and potato extracts have been tried out (Fagbenro and Jauncey, 1994).

The degree of autolysis and protein solubilisation in silage varied with the nature of raw material ranging from 80% in temperate fishes to 40-45% in tropical fishes like silver bellies (Gildberg & Raa, 1977). The undigested proteins appear to be peptide aggregates held together by non-covalent forces (Hall *et. al* 1985). Proteolytic breakdown of the silage does not proceed to completion and the presence of residue resistant to proteolysis has been reported (Tatterson and Windsor, 1974, Backhoff, 1976). The exact reason for the incomplete proteolysis is not fully understood so far, but pH, temperature, duration of ensilation and

nature of raw materials appear to play an important role for this phenomenon. The autolysis resistant sediment of fish silage consists of a large portion (about 50%) is insoluble in water and is a true insoluble portion. Non-polar, ionic and other association forces seem to be responsible for the insolubility of the rest of the sediment. According to Olley (1976), the sludge is highly nutritive but it is not suitable for animal feeding because of its high lipid content, which may lead to carcass tainting (Raa & Gildberg, 1982) and impaired performance of the animals.

2.1.3. Acid fish silage Vs fishmeal

In acid fish silage the preservative effect is due to the added acid, which will destroy all the microorganisms where as in fishmeal, the reduction in moisture content affects the preservation. The advantages of acid fish silage over fish meal includes the easiness of operation without any sophisticated machinery, the flexibility of production for any quantity of raw material in any location and the reduction of environmental problems like smell, fly infestation etc. The absence of any pathogenic microorganisms and storage at normal conditions are other advantages of fish silage. But fish silage is a liquid product, which requires drying with binder before transportation if the animal farms are at distant places.

2.1.4. Fermented fish silage

The principle of fermented silage is similar to that of acid silage; preservation is the result of acidity arising from the growth of lactic acid-producing bacteria. The technology relies on the production of lactic acid at a rapid rate in sufficient concentrations by fermentation, which suppresses spoilage organism and preserves the feed until it is needed (McDonald *et. al.*, 1991).

The production of fermented fish silages depends on *in situ* production of lactic acid by LAB added to the fish with a fermentable jaggery source. Since the

natural LAB in fish is limited, an external inoculum of LAB is necessary (Nair *et.al*, 1997).

Lactic acid fermentation represents a low- cost method for the preparation of food and feed products characterized by high hygienic quality and improved shelf life (Frazier & Westhoff, 1988; McDonald *et. al.*, 1991). Good quality silages were found to have the following characteristics: (Yeoh, 1979 a).

- Rapid drop in pH from about 6.0 or 6.5 to below pH 5.0 the more successful the fermentation, the more the production of lactic acid and the lower the final pH value.
- High lactic acid content. The level usually increases sharply during the first few days, and remains fairly constant for the rest of the fermentation.
- The ammoniacal nitrogen content is low.
- Low anaerobic spores former and coliform count.
- No pathogens such as Salmonellae spp. or Streptococcus spp.
- An acceptable fishy smell
- The volume of gas generated during fermentation is relatively small
- Remains stable for more than six months in the wet form and for more than one year in dehydrated form.

The autolytic activity occurring during the ensilage of fish leads to an increase in the concentration of ammonia, amines, amino acids and peptides. Up to 80% of the organic nitrogen becomes solubilised in acid preserved fish silages (Haard and Arcilla, 1985) where as ensiling by the biological methods yields solubilisation values of around 60% (Hassan and Heath, 1986)

2.1.5. Fermented fish silage Vs Acid silage

In acid silage the acids used exert their preservative action by the passage of the undissociated acid molecule into the bacterial cell where it dissociates and lowers the pH to kill the organism instantly. Thus organic acids

with higher pKa like propionic and formic (4.86 & 3.75) are more effective because a greater proportion of their molecules are undissociated at higher pH. The liquefaction or digestion of fish is due to the action of a wide variety of indigenous proteases present in the fish. (Raghunath & Gopakumar, 2002)

Acid preserved fish silages liquefy in a week's time and upto 80% gets solubilised in tropical conditions (Raa and Gildberg, 1982), and separates in to 3-4 layers; an oily layer at the top, sometimes an underlying emulsified layer, an aqueous middle layer containing the liquefied proteins and the sediment of sludge containing undigested protein, scales and bones (Raghunath & Mc Curdy, 1987)

The production of fermented silage depends on the *in-situ* production of lactic acid by the added bacterial culture and the time taken in fermented silage to produce a stable and desired pH is usually longer than acid silage but optimal conditions for fermentation can reduce the period. The extent of protein breakdown is normally lesser in fermented silage when compared to acid silage. Fagbenro and Jauncey (1993 a) observed 51 % hydrolysis in fermented tilapia silage.

According to Kompiag *et.al,* (1979) the difference of microbial and chemical silage on the ammoniacal nitrogen content might be at least partly due to the different method of storage. Microbial silage is stored in a closed container, thus ammonia being produced has no chance to escape while the chemical silage is stored in open or lightly closed containers. Compared with conventional acid silage the tryptophan content of naturally fermented silage is increased by a factor of 2 (Vam & Hey, 1985)

2.1.6. Fermented fish silage Vs Fishmeal

Silage is the preferred option for small scale waste utilization in isolated places as well as on large or small fishing vessels. The scale of its operation and

production can be varied without affecting the economy of the process and the energy requirement is low with minimal equipment when compared to fishmeal (Villela *et.al*, 1992)

Fishmeal is always a suspected source of pathogens especially of salmonella when used in feeds. But pathogens like *Aeromonas salmonicida, Mycobacterium chelonei, Yersenia ruckeri, Renibacterium salmonorium* etc., are rapidly destroyed in silage conditions. According to Hoq *et al.*, (1995) silages either wet or dried have lower bacterial counts than freshly prepared fishmeal, but dried fish silage upon storage reported to have higher counts. The infectious pancreatic necrosis virus that may survive can be inactivated by pasteurization or by virucidal agents (Smail. *et. al.*, 1990).

Fish silage either acid or fermented can be co-dried with other feed ingredients like soy meal, poultry by product meal (Fagbenro & Jauncy, 1994) or jaggery fillers and sun dried openly without fly infestation (Raa & Gildberg, 1982) which is not possible in case of fish meal. Such co dried products were found to have low aerobic counts and low water activity (Dong *et. al.*, 1993).

2.1.7. Fermented fish silage Vs Hydrolysate

Fish hydrolysate is normally prepared by the use of peptic or tryptic enzymes. Plant enzymes like bromolein can also be used. The choice of the enzyme for use depends on many factors like cost of the enzyme, end use of the product, type of raw material etc. but silage is prepared either by adding acid or by adding bacteria and fermentable jaggery.

In hydrolysate the resultant product contains only the breakdown products of protein such as peptides, amino acids etc. but fish silage is a rich source of minerals and essential fatty acids in addition to the protein breakdown products.

2.1.8. Fermented fish silage Vs Traditional fermented products

Fermented fish pastes and sauces are relished as a condiment (flavoured salt) along with cooked rice in many of the South East Asian countries. Traditional fermented fish products are basically salt fermented products. Depending on the proportion of salt added, the products can also be classified into high salt (more than 20% of total weight), low salt (6 to 8%) and no salt products. Subba Rao (1961) distinguished three types of traditional fermented fish products. (a) Products in which the fish retain substantially their original form, (b) Products in which fish are reduced to a paste and (c) Products in which fish are reduced to a liquid.

In traditional fermented fish products, fermentation takes place as a result of exogenic and endogenic enzymes which occur in guts and intestine of fish. Neither the microorganism nor the enzymes of microbial origin are involved in this process (Saisithi *et. al.*, 1966; Bedows *et. al.*, 1979). Orejana and Liston (1981) showed that living bacteria are unlikely to play more than a minor role since they rapidly die off in high salt medium and are not replaced by large populations of halophiles. According to Steinkaraus (1982) traditional fermented products are resulted by spontaneous direct fermentation and takes more time.

Lactic acid fermented products can be prepared in a shorter time (and hence more cheaply) than the fish salt products, which depend primarily on autolytic processes. Their lower salt contents also permit them to be consumed as a main course, rather than in the condiment role of the high-salt fish sauces and pastes. This suggests that the lactic fermented products offer greater scope for low cost fish preservation in South East Asia than the simple low water activity products (Adams *et. al.*, 1987).

2.1.9. Advantages of fishmeal production compared with fish silage

Fish silage is more bulky and more expensive to transport. Concentration of silage or co drying is the suggested remedial measures for this problem (Skrede and Nes, 1988). The thiaminase activity in fish can persist in silage leading to loss of thiamine, but by mixing the silage with a dry binder meal into a moist pellet is shown to suspend thiamine destruction (Anglesea and Jackson, 1985).

Production of fishmeal from fatty fish requires more capital-intensive equipments for cooking, pressing and oil separation, but fish offal, fatty fish or lean fish can all be ensiled and the oil layer easily separated by decanting or by centrifugation. The oil from fish silage is rich in poly unsaturated fatty acids (Jl. *et.al.*, 1996). Acid silages made with inorganic acids are to be neutralized before using as feed. Direct use may affect the growth performance of animals.

2.2. Fermentation

Fermentation not only aid in the preservation of food but they also imparts certain distinctive characteristics which appeal to the consumer attained through the complex changes during fermentation. While the changes known to occur among the carbohydrates are complex, a knowledge of the nature of the changes in the proteins, lipids, vitamins and other essentials of fermentation are as a whole inadequate.

The temperature of fermentation, the salt concentration, oxygen supply and freedom from other contaminating bacteria are the main controlling factor for governing the course of fermentation. The lactic acid bacteria are carbohydrate fermenting. The species have adapted themselves to the environment to carry out their metabolic processes without using free oxygen. Since lactic acid has a higher dissociation constant than acetic acid, a lower pH value is obtained even though less acid is present (Pederson, 1979).

The major metabolic pathways of carbohydrate fermentation are given in the Fig. 2.1

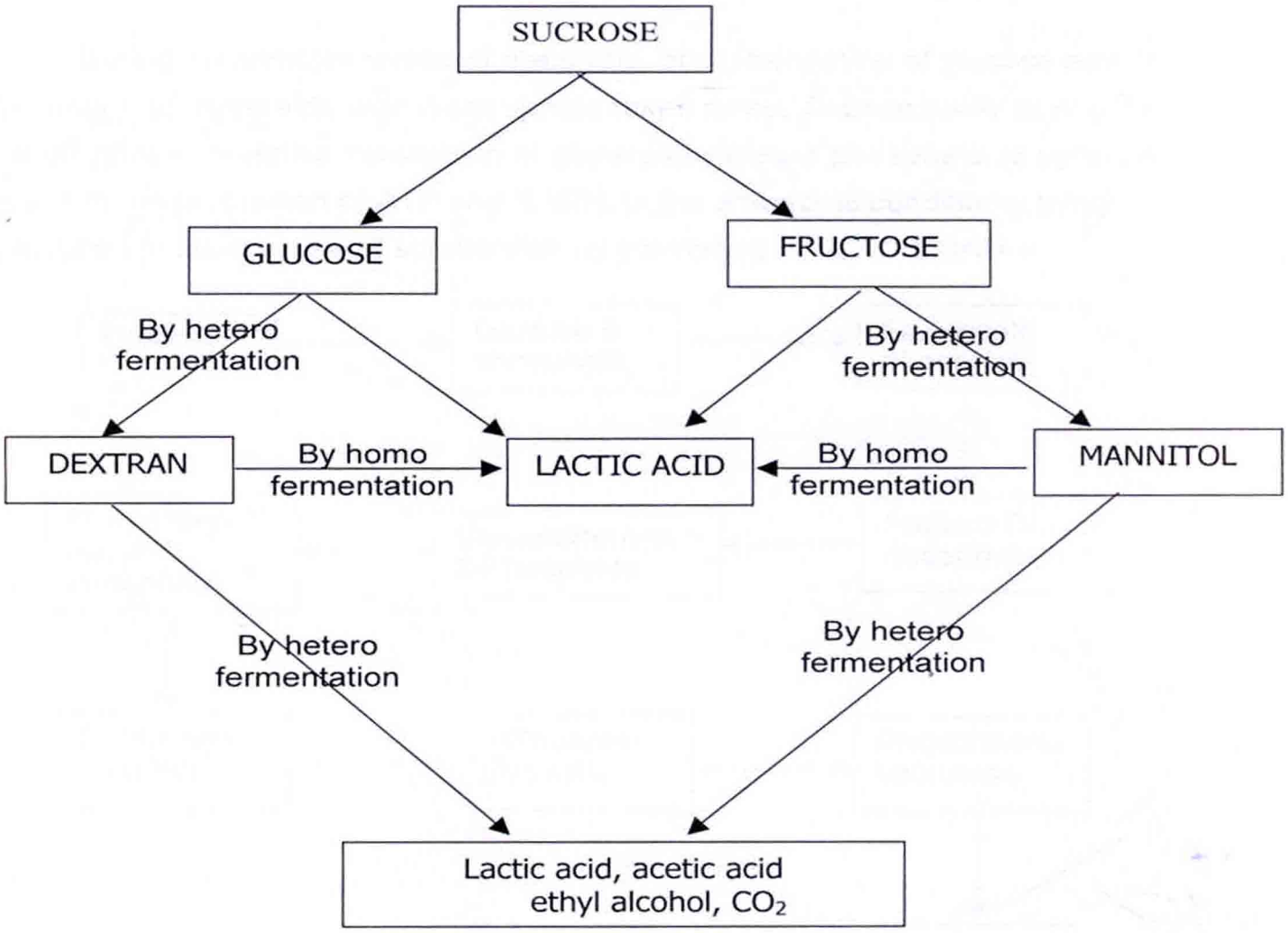


Fig. 2.1. Major metabolic pathways of carbohydrate fermentation.

Complete fermentation pathway begins with a substrate, includes glycolysis, and terminates with the formation of end products like lactic acid, ethanol, CO_2 etc. In lactic acid fermentation pathway, pyruvate is reduced to lactic acid, coupled with the reoxidation of NADH to NAD+. Bacteria that carry out this fermentation pathway, are called lactic acid producing bacteria (LAB). When the Embden-Meyerhof pathway of glycolysis is used in the lactic acid

fermentation pathway, the overall pathway is homolactic fermentation (Fig 2.2) because the only end product formed is lactic acid.

During preparatory phase of glycolysis, phosphorylation of glucose and its conversion to glyceraldehyde-3-phosphate takes place. Subsequently during the pay off phase, oxidative conversion of glyceraldehydes-3-phosphate to pyruvate results in the production of ATP and NADH. In the anaerobic conditions, pyruvate is reduced to lactic acid by Lactobacillus by converting NADH to NAD +.

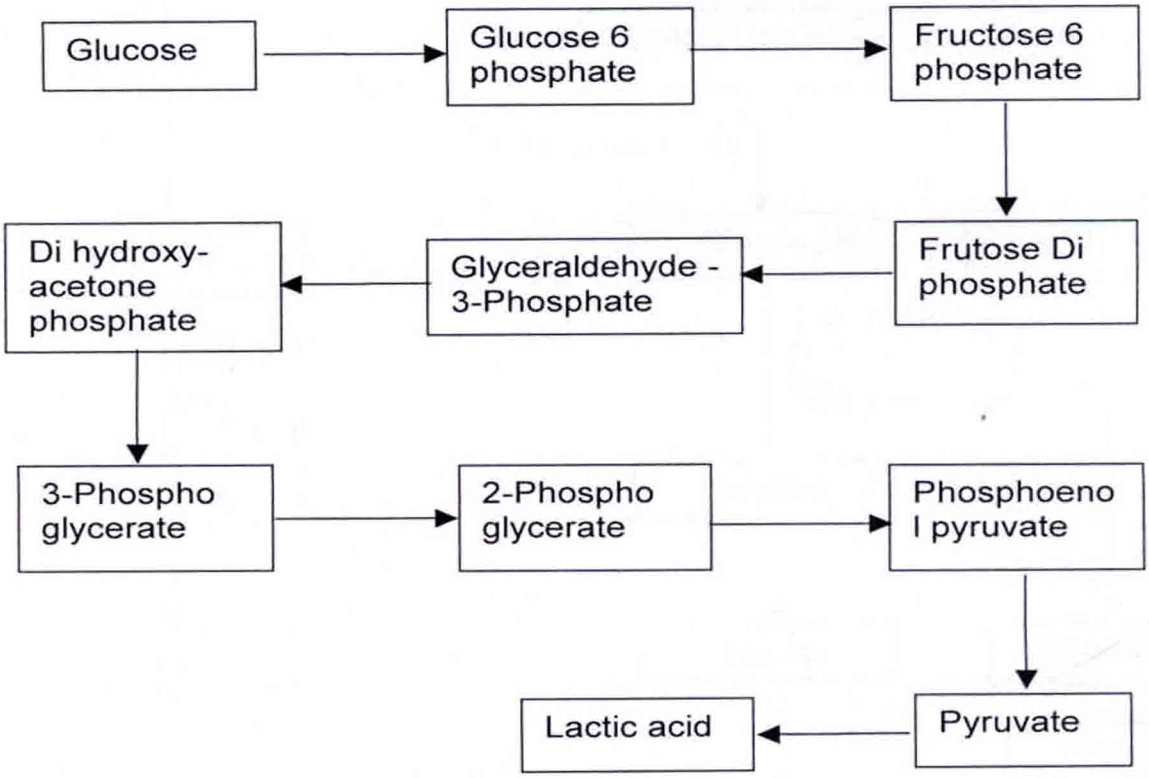


Fig. 2.2 Homolactic fermentation of glucose to lactic acid by LAB.

The overall lactic acid fermentation pathway can be expressed as follows.

Glucose + 2 ADP + 2 P ⟶ 2 lactic acid + 2 ATP.

Some microorganisms carry out a heterolactic acid fermentation (Fig 2.3) using the pentose phosphate pathway in which ethanol and carbon dioxide is produced in addition to lactic acid.

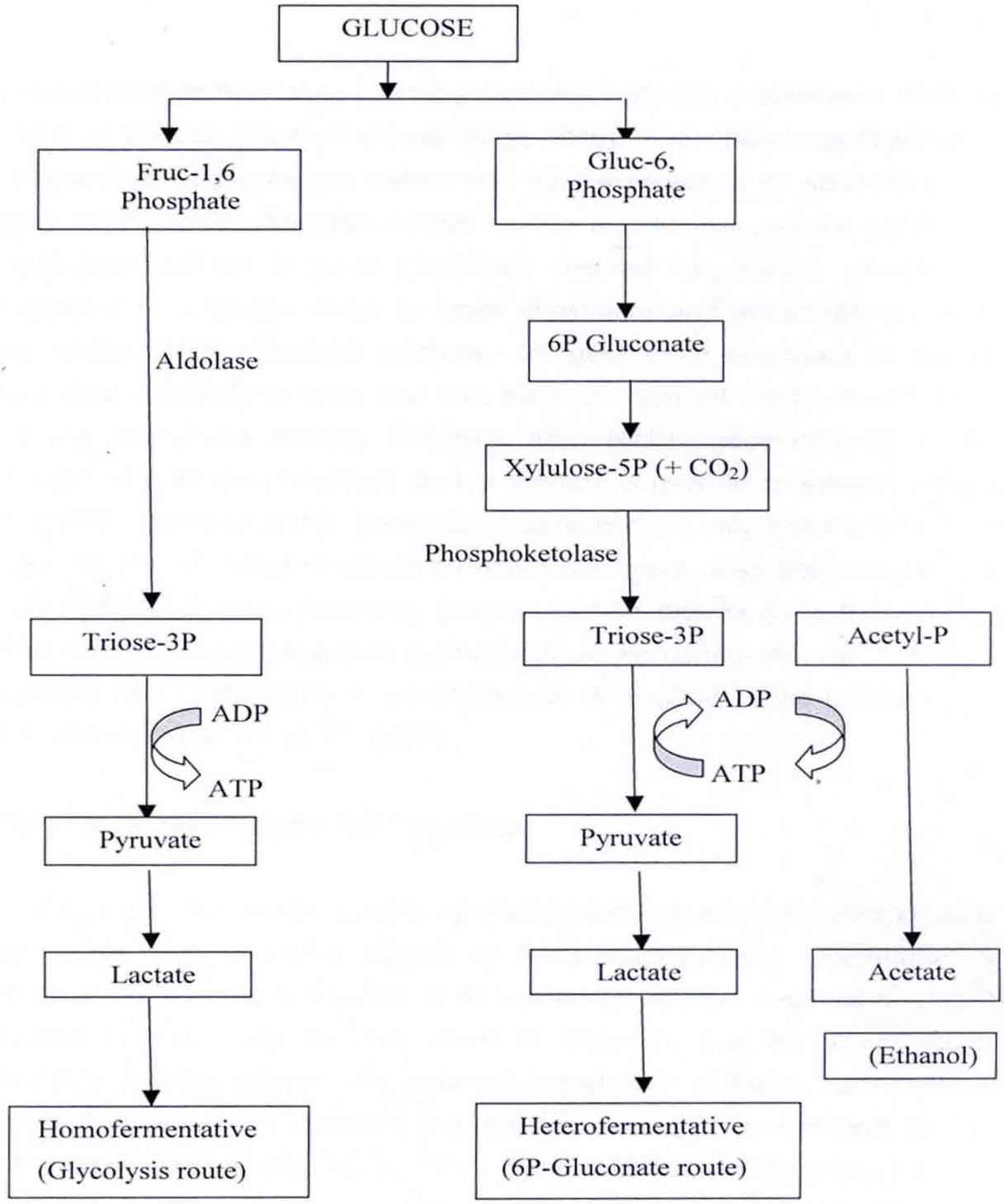


Fig. 2.3.Heterolactic fermentation of glucose

The overall reaction of the heterolactic fermentation can be expressed as follows.

Glucose + ADP + P $\longrightarrow$ Lactic acid + ethanol + CO_2 + ATP.

The heterolactic fermentation pathway produces only one molecule of ATP per molecule of glucose substrate utilized (Atlas 1995). The differences observed in the fermentation patterns are determined by the presence or absence of the enzyme aldoadolase. Homofermenters lacking aldoadolase cannot breakdown fructose bisphosphate to triose phosphate. Instead they oxidize glucose –6-phosphate that is broken down to triose phosphate and acetyl phosphate by means of the enzyme phospho ketolase. In heterofermenters triose phosphate is converted ultimately to lactic acid with the production of 1 mol of ATP, while the acetyl phosphate accepts electrons from NADH generated during the production of pentose phosphate and is thereby converted to ethanol without yielding ATP. Because of this, heterofermenters produce only 1 mol of ATP from glucose instead of 2 mol produced by homofermenters. Also heterofermenters decarboxylate 6-phosphogluconate, produce carbon dioxide as a fermentation product whereas homofermenters produce little or no carbon dioxide. Therefore one simple way of detecting a homofermenter is to observe the production of carbon dioxide (Madigan *et. al.*, 2000)

2.2.1. Preparation of fermented fish silage

Fermented fish silage is made by mixing fish/fish waste with a fermentable sugar source and a starter culture of lactic acid bacteria (Raghunath & Gopakumar, 2002). The production of fermented fish silages depends on *in-situ* production of lactic acid by LAB, which is added to the fish along with a fermentable jaggery source. An external inoculation of lactic acid bacteria becomes necessary as the bacteria naturally present are in few numbers, to start a fermentation process (Nair *et. al.*, 1997). Various species of lactic acid bacteria used in fish silage are *Lactobacillus plantarum* (Viete and Bello,1992; Ottati and Bello 1992a,b; Fagbenro and Jauncey, 1993 a, 1993 b, 1994; Lassen, 1995), Yogurt bacteria like *L. bulgaricus* and *Streptococcus thermophilus* (Areche *et. al.*, 1992, Yoon *et al.*, 1997), *L. delbruecki spp bulgaricus* and *S. salivarius spp thermophilus* (Martinez-Valdivieso *et. al.*, 1996), a marine yeast, *Hansenula*

montevideo (Bertullo, 1992) and natural inocula like sauerkraut (fermented cabbage) (Dhatemwa, 1989). *Lactobacillus plantarum* appears to be the most effective starter culture as per a comparative trial conducted to evaluate three different lactis by Bello *et. al.*, (1992).

By-catch or fish wastes, preferably chopped or minced, are placed in non-metallic vats and mixed with a single jaggery source, such as cassava, sweet potato or molasses or a mixture of these, and stored airtight. In order to start fermentation almost immediately, the addition of 20 to 30 percent of molasses has been recommended (Domínguez, 1988). According to Green *et. al.*, (1983) periodic agitation and temperatures of at least 20°C tend to induce rapid liquefaction of the raw material

In Latin America the silage is prepared using a 5 percent lactobacillus culture mixed with 60 percent by-catch, 30 percent ground maize and 5 percent molasses (Tibbetts *et. al.*, 1981). Later, Domínguez (1988) suggested that if the objective was to prepare a complete fish silage ration, using roots as the principal source of jaggery, then the following general formula of (percentage air-dried): roots 30 to 50 percent; molasses 10 percent; and fish wastes 40 to 60 percent might be used.

In Viet Nam, fish silage made from shrimp heads, fresh blood (abattoir) and molasses in proportions of 5:3:2, respectively, and fermented for a period of ten days reportedly had a pH of between 4.3 and 4.5 as well as a "pinkish colour, nice flavour and soft texture" (AHRI, 1993).

In Morocco, a mixture (50:50 w/w) of molasses and drained ground fish wastes, left uncovered and stirred daily, required a period of ten days to produce a stable final product with a pH of 4.5. The fish silage was then formed into blocks in order to have solid feeding material for sheep, goats, horses and camels (IAV , 1994).

Sodium chloride (2% w/w) could be of value as an additive for fermented fish silage production. It inhibits growth by the studied biogenic amine producing bacteria, but starter strains should be found that are not affected by the addition of NaCl. It additionally decreased NPN and TVBN values in biological fish silage (Fagbenro & Jauncey 1993, a).

2.2.2. Principle of preservation

Fish silage is preserved against microbial spoilage mainly by the lowered pH, obtained by the added or *in-situ* produced acid. Specifically, the unionized acid molecules are able to cross the cytoplasmic membrane barrier of the microbial cell while protons (H+) and acid anions cannot. But once inside the cell, the acid mole can ionize and since the membrane traps the ions, the pH gradually comes down killing the cell. Thus it is the unionized acid molecules that are responsible for the preservative action rather than the total acid concentration. At equal concentrations, organic acids are weakly ionized in solution when compared to inorganic acids, thus contain greater amount of unionized (free acid) molecule making them more effective in preservation. In case of fermented silage, preservation occurs by several means. The presence of fermentable sugar is the beginning of the ensilation process; prevents immediate deamination of amino acids by bacteria that would lead to ammonia production and foul smell. Later as the fermentation by lactic acid bacteria becomes dominant, spoilage bacteria are suppressed or killed by the increasing concentration of lactic acid, lowered pH and the production of several antibiotic substances called bacteriocins by the lactic acid bacteria. (Raghunath, 2002)

2. 2.3. Raw material

Fish of all species and fish wastes can be converted into silage. Silage production is an economical way of using by-catches from shrimp trawlers, fresh fish and fish offal (Gopakumar, 1997). Annual discard from the world fisheries

were estimated to be approximately 20 million tonnes (25%) per year. This includes "waste" or byproducts also. Only 36,000 tons of the by-products were used for human consumption, which amounts to about 15.5 % of the total (RUBIN; 2001). The rest is used for the production of fishmeal, silage and animal feed. Therefore fish silage has a great potential for the fishing industry to utilize more of what is landed.

2.2.4. Surimi processing waste

Surimi is a wet frozen concentrate of myofibrillar proteins of fish muscle (Lanier, 1986). It is deboned, washed and stabilized fish mince. The overall surimi manufacturing process is reported to be quite inefficient resulting in 12-20% yield from round fish to finished product. The bulk of the remaining solid waste (65-70%) ends up as fishmeal (French and Pederson, 1990). Perigreen *et.al* (1979) studied the filleting yield and wastage therein and observed that 10-18 % filleting waste meal could be produced from different marine fish species.

Yield of surimi or wastage during processing vary with species, size and season as well with the type of processing machinery used so that fluctuations in yield is to be expected. Careful control of the filleting and meat separation steps is required to reduce the wastage (Toyeda *et.al.*, 1993). Lee (1986) summarized the material balance during different steps of surimi processing. In the filleting machine 67.1% waste is generated and during deboning 1% waste is generated. If the unused sarcoplasmic protein, which comes to 7.5%, is included, 75.5 % by weight of round fish generated as waste which can be used for silage preparation. The surimi production in India is increasing every year and the export of surimi and fish fillet for the year 2004 is 31,509.5 tons and 421 tons respectively (Anon 2004). During the processing of the above quantity about 95,000 tons of wastes could have been generated, which can be effectively used for the production of fish silage.

2.2.5 Starter culture

The natural involvement of LAB in the silage process has been known since early this century (McDonald *et. al.*, 1991). According to Nair *et. al.*, (1997), lactic acid bacteria are naturally present in fish but their numbers are very few to compete against other micro flora and start lactic fermentation. Hence, an external inoculum of lactic acid bacteria is necessary (Raghunath & Gopakumar, 2002).

2.2.6. Lactic acid bacteria (LAB)

One large group of bacteria that play an important role in the process of fermentation is those, which produce lactic acid. They carry out essential metabolic biological processes without oxygen by means of a complex series of intra-molecular oxidations and reductions. Since they cannot utilise oxygen, the changes they accomplish do not decompose the matrix to basic components, rather the most commonly recognized end products of their metabolism is lactic acid derived from sugar. They also produce other product from sugar and alter food components (Pederson, 1979). It is this ability to convert carbohydrate to lactic acid, acetic acid, alcohol and carbon dioxide, which made this group so important to mankind in the preservation of edible and nutritive qualities in food. There is little caloric change in the conversion of carbohydrates to lactic acid.

Different species of lactic acid bacteria have adapted to grow under widely different environmental conditions, and they are widespread in nature. Lactic acid bacteria are commonly found in the gastrointestinal tract of various endothermic animals, mice, rats, pigs, fowl, humans and in seafood products (Mauguin & Novel, 1994).

The lactic acid bacteria are a group of bacteria composed of several genera with a number of morphological, physiological and metabolic characteristics in common. These characteristics include; Gram positive

staining, anaerobic, micro aerophilic or aero-tolerant and catalase negative. Members of this group include both rods and cocci. They are generally catalase negative and they usually lack cytochromes. Lactic acid bacteria are nutritionally fastidious, requiring jaggerys, amino acids, peptides, nucleic acid derivates and vitamins. Also, by definition they produce lactic acid as sole or main product of their metabolism. They are generally perceived as benign organisms with the ability to preserve various food products usually associated with favorable changes in texture, flavor and the probiotic effect (Hall, 2002). They destroy the spoilage organisms by competitive inhibition, also by producing antibiotics and other antimicrobial chemicals. The preservative action of lactic acid bacteria has been attributed to different causes. It may be due to the lowering of pH, production of H_2O_2, (Dahiya and Speck, 1968, Gilland and Speck, 1975) antibiotics produced, (Baribo and Foster, 1951), changes in the oxidation-reduction potential of environment (Barber and Deibel, 1972), or bacteriocin (Graham & Mickey, 1985).

Lactic acid bacteria are tolerant to low pH and this can be á key factor in the competition with spoilers. Eventhough, chemical modification of pH of the substrate is recommended for the initial pH of the fermented meat products, along with the spoiler growth rate, lactobacillus growth rate also inhibited with these modifiers (Adams *et. al.*, 1987).

The natural content of LAB in different products varies greatly. Vegetable materials usually contain high levels of LAB originating from the plant, which accumulates during processing. Fermentation of manure, slaughter house wastes and fish byproducts low in LAB require the addition of a fermentation flora. Cereals rich in LAB have been used for this purpose (Nilsson & Rydin, 1963).

2.2.6.1. *Lactobacillus plantarum*

Lactobacillus is a genus divided into three broad groups based on the fermentative characteristics. Group I &II are homofermentative, with over 85%

conversion of glucose to lactic acid, group III 50% conversion to lactate occurs with acetic acid, ethanol and carbon dioxide is also characteristically produced. *Lactobacillus plantarum* is included into group II and is distinguished from Group I by growth of many species at 15 ° C as well as by biochemical tests.

The strains used in the processes have been mainly *L. plantarum. L. acidophilus, Pedaucoccus halophilus* and *P. acidilactici.* The pH of stable silage should be less than 4.5 (Fagbenro & Olusoji, 1998) and a concentration of bacteria of 10 8 cells/g causes a decrease of the pH to 4.5 by the end of first day (Babu. *et. al.*, 2005). It is observed that *L. plantarum* produced about 4.5% lactic acid in fish silage (Lindgren, 1992). The normal level of lactobacillus used for fermentation is at 5% (v/w) level of 10 8/ ml cfu. But addition of inoculum at 10^6 / ml was found satisfactory with *L. fermentum* (Mathew & Nair, 2006).

Lactobacillus have complex nutritional requirements in terms of amino acid and vitamins and are also acidophilic or aciduric with pH growth optima in the range of 4-6, though growth may occur at a higher pH. Media used for isolation take account of this and the habitat under study. The fermentation caused by LAB, usually *L. plantarum* proceeds until a concentration of around 100milli mol/litre of undissociated lactic acid is reached. Autolytic activities and ammonia production during storage of silage increase the buffering potential with a concomitant pH raise (Lindgren & Pleje, 1983). This reduces the amount of undissociated lactic acid, which forces the fermentation flora to continue its fermentative activity in order to keep this amount constant. Production of lactic acid to levels favoring conservation properties varies in respect of buffering capacity.

Of the various starters *L. plantarum* appears to be the most effective starter culture (Bello *et.al.*, 1992). They can operate over a wide range and have

an extremely important effect in very modified products such as silage or to a lesser extend fish sauces and pastes (Han-Ching *et. al.*, 1994).

2.2.6.2. Other natural sources of starter culture:

The use of bacterial cultures would obviously be a deterrent for low technology processing by small holders. It is therefore interesting to note that although raw materials low in lactic acid bacteria content generally benefit from the use of suitable inoculants, it is not always essential that they be included (Martin & Bozoglu, 1995). Urlings *et al.* (1993) have observed that the small holders could well be able to produce fermented silages without the need to produce or purchase starter cultures provided that appropriate mixtures of fermentable and non-fermentable materials are selected. Natural fermentation without the inoculation of starter culture was reported by Zahar *et. al* (2002) but took comparatively long period to get stable pH and required 40% molasses.

2.2.6.2.1. Papaya latex

Under an All India Coordinated Research Project Programme, papaya latex fermentation technique was developed for fish silage preparation, in which 5% level of papaya skin peel was added with molasses to the cooked and cooled fish and the pH changed to 4.0 in 72 hours. It is likely that the microorganisms present in the skin of papaya initiated the production of acid by microbial fermentation (Gopakumar, 1997).

2.2.6.2.2. Fermented cabbage

Neethiselvan *et.al* (2001) used one day old fermented cabbage at 5% level as bacterial inoculum. It was added to the cooked fish and minced well which attained the required pH of 4.4 in 48 hours when kept in airtight plastic containers.

2.2.6.2.3. Buttermilk

Durairaj *et al.*, (1985), demonstrated the use of buttermilk at 5% level as inoculum instead of LAB starter and it was observed that the pH dropped to 4.5

in 24 hours and the silage so obtained almost had similar odor, flavor and composition as that of LAB fermented silage.

2.2.6.2.4. Pineapple & pappaya

Bello *et.al*, (1993) have prepared fermented fish silage from under-utilized fish mixed with juice and waste fruits (pineapple and papaya) at 35^{0}C. Process development was evaluated by measuring pH, acidity, non-protein nitrogen, consistency and exudate liquid. Results indicated a slow decrease in pH value and production of acidity during 20 days storage and observed that pineapple juice did not contribute to hydrolysis process.

2.2.7. Fermentation characteristics

The fermentation caused by LAB, usually *L. plantarum* proceeds until a concentration of around 100 millimol / litre of undissociated lactic acid is reached. Autolytic activities and ammonia production during storage of silage increase the buffering potential with a concomitant pH raise (Lindgren & Pleje 1983). This reduces the amount of undissociated lactic acid, which forces the fermentation flora to continue its fermentative activity in order to keep this amount constant. Production of lactic acid to levels favoring conservation properties varies in respect of buffering capacity. Thus Lindgren (1992) has observed that L. plantarum produce about 4.5 % lactic acid in fish silage.

2.2.8. Fermentable carbohydrate

The complex nutritional requirements of LAB and the lack of suitable nutrients in the raw material used for some fermentations has called for the addition of stimulants such as jaggery, amino acids, fatty acids, nucleic acids derivatives, vegetable extracts, minerals and vitamins (Beck, 1978). The successful preservation of fish by biological fermentation methods is dependent on the production of lactic acid by bacteria from fermentable jaggery. Since the fish contain only small amounts of fermentable jaggery,

addition of molasses, tapioca meal or cereal meal improves fermentation. In India molasses is a cheap jaggery source abundantly available for preparation of biological silage (Durairaj *et. al.*, 1985). As fish contains only small quantity of jaggery, they are added in silage production to facilitate microbial fermentation.

2.2.8.1. Molasses

The fermentable jaggery source mostly used in fish silage is molasses because of its low cost and ready availability of fermentable sugars. Molasses are also used regularly in feed manufacture (Raghunath, 2002). Fish : molasses ratio of 100:5 (Kompiang *et.al.*, 1979) gave a silage stable only up to a few days and ratios 100:10 or above gave stable silages having a typical acid smell. Molasses and apple pulp were successfully used to produce good quality silage from minced hake frames in South Africa (Wessel & Labuschange, 1974). The use of molasses or tapioca is ideally suited to tropical countries as they are abundantly available (Gopakumar, 1997).

2.2.8.2. Other sources

Even though molasses is the most commonly used fermentable jaggery source, different workers have used cereal meal, sugar cane, sucrose, tapioca meal, ragi etc in silage production. (Ali. *et. al* 1985, Areche *et. al*, 1992.; Nilsson & Rydin, 1963, Nilsson, 1970). But LAB cannot utilize complex jaggerys like starch or cereal meals. Hence, starch when used in the form of cereal flours like oats meal, should be accompanied by an enzyme source such as malt of fungal amylase to breakdown the complex jaggerys into simples sugars (Raghunath, 2002). According to Fagbenro and Jauncey (1993 a), Ahmed and Mahendrakar (1996), the use of 5% salt in fermented silages will reduce protein hydrolysis and swelling of the mass.

2.2.9. Advantages of lactic fermentation

LAB are known to possess antibacterial properties, attributed to the major end products of their metabolism, such as lactic acid, acetic acid, hydrogen peroxide and peptide compounds termed bacteriocins (Klaenhammer, 1982). Production of lactic acid, the major metabolite of LAB, is responsible for the associated decrease in pH, which may be sufficient to antagonize many microorganisms. The undissociated acid can cause the collapse of the electrochemical proton gradient of susceptible bacteria, leading to bacteriostasis and eventual death (Soomro *et al.*, 2002).

According to Bhunia *et. al.*, (1987) bacteriocins from food grade lactic acid bacteria are bactericidal to many bacteria associated with food spoilage. *L. plantarum* effectively and quickly inhibited *S. aureus* in 36 h as compared to *B. cereus, L. monocytogenes* and *Cl. Perfringens* in 48 h. *S. aureus* was inhibited by *L. plantarum* in 36 h of storage by 3.9 log reduction as compared to control.

About 4% of the total LAB isolates were producers of bacteriocin like substances, active against the indicator strains used by Sarkar & Banerjee (1996). Daeschel *et.al* (1986) initially observed that *L. plantarum c-11* isolated from cucumber fermentation produced a protenaceous inhibitory compound designated as plantaricin A. It is active against some strains of *L. plantarum, L. acidophilus* etc. Plantaricin A was purified (Nissen-Meyer *et. al.*, 1993) to homogeneity and found out two peptides of 2426 and 2497 Da. Both peptides may form amphiphilic helices, suggesting that they are pore-forming peptides that create cell membrane channels.

Wirahadikusumah *et. al.*, (1971) isolated some strains able to produce high molecular weight polypeptide fractions active against *P. aerogenosa* from fermented fish silage. Certain bacteriocins produced by LAB could inhibit some food borne pathogens, including *Bacillus cereus, Cl. Perfringens,*

Listeria spp (Carminati *et.al*, 1989, Harris *et.al* 1989, Schillinger and Luke, 1989). These results suggest that bacteriocins producing LAB has good potential for use as natural preservatives. The utilization of fish silage as a substrate for fermentation might offer an inexpensive alternative for microbial methods of metabolite production, such as amino acids, if proven to provide efficient utilization. With this in mind it would be useful to estimate kinetic fermentation data in the physiological state of metabolite production, such as specific rate of L-lysine production (Qp, g Lysine/g cell h) and specific production (g Lysine/g cell), besides yield product (g Lysine/g glucose consumed) and productivity (g Lysine/l h).

2.3. Changes During Fermentation

2.3.1. pH.

The most important factor to control in the bio transformation is the pH decrease which must be achieved as quickly as possible in order to inhibit the growth of spoilage microorganisms in the product. Moreover the lactic acid fermentation is usually accompanied by some metabolites (Bacteriocins), which may help in preservation of fermented foods.(Faid *et.al.* 1997). Durairaj *et.al* (1985) reported that the pH of the bacterial silage prepared from silver belly stored in jerry cans was stable at 4.0 through out the storage period of 12 months and even beyond.

2.3.2. Carbohydrate

The optimum concentrations of the carbohydrate to prepare fermented fish silage with required pH were 7% for dextrose and 10% for molasses on the basis of total weights of skipjack tuna viscera. The pH of fermented skipjack tuna viscera silages by the use of lactic acid bacteria was significantly declined from 5.9 to about 4.0 after 42 days of storage at 35 ° C (Yoon *et.al.*, 1997).

The stability of the silage depends upon the fish starch ratio. A ratio of fish to molasses 100:5 was stable for only a few days giving off odour after the 10 th day. At the higher ratios 100: 10, 100:15 and 100:20, the silage was stable for at least twenty one days and had a fresh acidic smell (Kompiang *et.al*, 1979). Studies with *Lactobacillus plantarum* indicated that fermentation rates increase in the range 0-5% w/w of glucose or sucrose, whereas increasing the salt concentration from 0-6% slows the rate of pH decrease.

2.3.3. Non-Protein Nitrogenous Compounds (NPN).

NPN compounds are of major importance and are used as quality parameters for fish and fish products (Iida *et.al* 1992). NPN accounts for 9 to 18% of the total nitrogen in teleosts, from 33 to 38% in elasmobranches and 20-25 % of the total nitrogen in crustaceans and molluscs (Belitz & Grosch, 1987). The non-protein nitrogen compounds are mostly from the sarcoplasm and include peptides, amino acids, amines, amine oxides, guanidine compounds, quaternary ammonium compounds, purines and urea (Haard, 1995).

The autolytic activity occurring during the ensilage of fish leads to an increase in the concentration of ammonia, amino acids and peptides. Up to 80% of the organic nitrogen becomes solubilised in acid preserved silage (Haard *et.al.*, 1985) whereas ensiling by the biological methods yields NPN values around 60% (Hassan & Heath, 1986). Fagbenro & Jauncey (1993 a) observed that NPN of fermented tilapia silage increased gradually from 16% and attained a maximum of 46.5% after 30 days, which was lower than that obtained in acid silage. Faid *et al.*, (1997) noted an increase in NPN for 11 days and thereafter remained constant in fermented *Sardina pilchardus* silage. The NPN content of fresh water fish viscera during fermentation has increased to 80% in 4 days from the initial value of 33.9% (Ahamed and

Mahendrakar 1996). Backhoff (1976) has reported a greater value of NPN (83.5%) in cod viscera compared to fish flesh. But Zuberi *et. al.*, (1993) observed only 45-50 % NPN from the initial value of 0% for fermented fish silage held for 7 days.

2.3.4. Total volatile basic nitrogen (TVBN)

TVBN measures the amount of volatile bases formed from solubilised nitrogen derivatives. TVB is a general term and it includes trimethyl amine (TMA), dimethyl amine (DMA), ammonia and other volatile basic nitrogenous compounds associated with seafood spoilage (Gill, 1990). It is a measure of decomposition of proteins. The formation of higher content of TVBN may lead to a reduction in the content of amino acids that could have negative consequences on the nutritive value of silage (Maria *et al.*, 1998). A number of volatile bases like ammonia are released during spoilage of fish. For good quality fish, less than 35-40 mg of TVBN/100g is recommended.

Faid *et al.*, (1997) reported that TVBN pattern showed slight increase during fermentation and observed an increase in TVBN from 71.26 mg/100g to reach 95.03 mg/100g after 1 day and remained constant at 132 mg/100g after 15 days of fermentation at 22°C. Durairaj *et al.*, (1985) observed that the TVBN content of the silage prepared from silver belly showed a steady increase during the storage period of one year. Maria (1998) observed that the TVBN content of fermented fish silage is much lower when compared to acid silage. According to Ahamed and Mahendrakar (1996) during fermentation of fresh water fish viscera, the TVBN values increased to 8% of the total nitrogen from the initial 1.3%. According to Neethiselvan *et. al.* (2001), the TVBN values increased with the increase in storage period and ranged between 28-98 mg% when lactobacillus was used for fish fermentation whereas the same was between 35-155mg% when curd was used as starter culture

Since the TVBN levels in fermented silage is lower, the biological silages can be considered as advantageous when compared with the acid silage. The formation of higher levels of TVBN may lead to a reduction in the content of amino acids that could have negative consequences on the nutritive value of the silage (Maria *et.al.*, 1998). Rose *et. al.*, (1987) observed that the presence of *L. plantarum* enabled the TMA production at a reduced level. But the nature of the inhibition was not determined. According to Orejana & Liston (1981) the autolysis occurs due to trypsin like enzymes.

2.3.5. α - amino nitrogen (α AN)

Alpha amino nitrogen gives the extent of hydrolysis of a product. It gives an estimation of total free amino acids (Finne, 1992; McCoid *et al.*, 1984) During hydrolysis the polypeptide chains will break down to simple peptides and amino acids. Raghunath and McCurdy (1987) reported that AAN increased with the progress of autolysis in formic acid silage (pH=4). This may be due to the initial generation of oligopeptide by endopeptidase action, which were further broken down to short peptides and free amino acids by the exopeptidases. The AAN content of fermented fresh water fish viscera silage increased from the initial value of 12% of TN to 17-18% of TN after a period of 4 days (Ahamed & Mahendrakar, 1996) which was subsequently stabilized. Mathew and Nair (2006) reported a lower value of AAN when shrimp waste was ensiled with *L. fermentum*. But according to Babu *et.al.*, (2005) a higher values of above 20 % of TN was noticed in both acid and fermented fish silage.

2.3.6. Fatty acid composition

The major chemical unit in most lipid molecules is fatty acids. Fish fat is different from both vegetable and animal fats in their fatty acid constitution. In fish

fat the degree of unsaturation is greater than in vegetable fat. In fish the essential fatty acids (EFA) constitute 2% of the total lipids. The nature of the fatty acids present in fish lipids is very complex. Fatty acids with carbon chains varying from 10 to 22 and unsaturation from 0 to 6 double bonds are common. Majority of the fatty acids in fish lipids, whether saturated or unsaturated, have an even number of carbon atoms in the molecules. Odd numbered acids are quantity–wise insignificant. In the unsaturated fatty acids, which have more than one double bond (polyunsaturated), the double bonds are separated by a methylene group and have cis- configuration. Thermodynamically more stable trans- isomers are very negligible in fish fatty acids. High degree of unsaturation, with 5 or 6 double bonds per molecule is common in the fatty acids of fish, which is seldom observed in the lipids of other animals or plants of terrestrial origin. These features make the fatty acids of the fish unique. An indisputable clinical effect of a high intake of long chain n-3 polyunsaturated fatty acids such as 20:5n-3 and 22:6n-3 is reduction of serum triglycerides (Harris, 1989; Colinglio, 1992)

Among the saturated fatty acids, myristic, palmitic and stearic are the important ones present in the fish from Indian waters. Palmitoleic and oleic acids are the important monounsaturated fatty acids. Arachidonic acid, eicosapentanoic acid (EPA) and docosahexanoic acid (DHA) are the major components of polyunsaturated fatty acids of which EPA and DHA account for about 90%.

Feeding of rats with fish oils derived from definite fish species having a high EPA and DHA content causes a predominant incorporation of these acids into cardiac phospholipids. Studies on rat myocardium phospholipids have demonstrated a predominant accumulation of DHA (compared to EPA) following the administration of DHA rich tunny fish oil (Charnock, *et. al.*, 1986). Also the results of animal studies carried out by different workers have shown that increasing the concentration of dietary PUFA derived from sea animal lipids or vegetable fats promotes lipid peroxidation activation in the liver (Hortog *et. al*

1987), heart (Nalbone *et. al.,* 1988) kidney (Hu, *et. al.*, 1989), plasma and other blood constituents (Garrido *et.al*, 1989; Lenz, *et.al*, 1991)

Lee et.al, (2004) reported a high level of (42%) of saturates, 23% monoenes and 10% of 18:1 fatty acids in tuna viscera while the values for the same in the fermented silage were 35.0%, 23%. But the level of C 22:6 was reduced during ensilation in the same study. Fatty acid profile of crude oil extracted from shrimp waste and shrimp waste silage prepared by using acid was carried out by Guillou *et.al.*, (1995). It was observed that, fatty acid profiles were similar for both the samples. These workers have observed presence of relatively large quantities of EPA and DHA.

2.3.7. Peroxide Value (PV)

Oxidative rancidity was one of the major causes of flavour deterioration in fish. One of the first tests devised to measure it was the Peroxide Value (PV). The unsaturated fish oils are particularly susceptible to oxidation and form peroxides (Liljemark *et al.*, 1959)

The decrease in peroxide value observed during ensilation and storage of silage probably reflects the degradation of part of the hydro peroxides to form secondary breakdown products such as aldehydes (Lundberg and Jarvi, 1968). Ahamed and Mahendrakar (1996) observed an increase in the PV for the initial 4 days and then decreased to low levels due to breakdown. The oil retained in fish silage can become oxidized rendering the feed unpalatable or unsafe to livestock (Haard *et. al.*, 1985). But according to Raa & Gildberg (1982), the lactic acid fermentation has a beneficial effect on the lipids in fish silage, stabilizing the oil and improving its acceptability in animal diet. Use of antioxidants (Machin 1990, Espe *et. al.*, 1992) and formalin have been tried out to inhibit the oxidation of lipid in acid silage (Haard *et. al.*, 1985). But in spite of adding formaldehyde Maria *et. al.*, (1998) have observed an increased peroxide value for blue whiting samples.

2.3.8. Free fatty acids (FFA)

Fish muscle contains lipase, which is able to catalyse the hydrolysis of short chain triglycerides. Free fatty acids are suspected of deriving primarily from phospholipids, as the latter disappear with time of storage (Lovern *et al.*, 1958), which can be affected by the action of bacteria, enzymes or non-enzymic catalysis (Dyer, 1957 and Lovern *et al.*, 1958). Fagbenro and Jauncey (1998) have observed an increase in the levels of FFA during the production and subsequent storage of tilapia silage in the control samples as well as samples added with antioxidants. According to Ahamed and Mahendrakar (1996) the time elapsed for processing resulted in initial high FFA value which on ensilation increased further for 4 days. A similar increase was observed by Tatterson and Windsor (1974) in fish offal silage during 40 days of fermentation.

2.3.9. Thiobarbituric acid value (TBA)

A major cause for food deterioration during storage is oxidative rancidity. This is due to the oxidation of unsaturated fatty acids, particularly the polyunsaturated fatty acid (Allen and Foegeding, 1981). The peroxides formed may breakdown to carbonyls, form polymers, or react with protein, vitamins, pigments etc (Gray, 1978.; Karel, 1973). TBA index is the most used indicator for advanced lipid oxidation (Nishimoto *et.al,* 1985). Highly significant correlations have been obtained between the TBA numbers and taste panel results in various oxidized foods. (Tarladgis *et.al*, 1960)

According to Fagbenro and Jauncey (1994) the TBA value increased in raw silage during fermentation and storage, while the same decreased in silage added with ethoxyquin and onion extract in 180 days of storage. They have observed that the lipid stability was improved during ensilation by the addition of onion extract by lowering the TBA value. Ahamed and Mahendrakar (1996)

observed a linier increase in TBA value during fermentation of fish viscera for 8 days.

2.3.10. Free amino acid (FAA)

The major protein degradation products are free amino acids, which are further catabolised to amines, ammonia and carboxylic acids (Oshima and McDonald 1978). The FAAs have been implicated to be responsible for the characteristic taste of seafood (Fuke, 1994). The free amino acids in the muscle of fish and shellfish are similar to the amino acids in the muscle. The free amino acid content in muscle of aquatic organisms ranges from about 0.5- 2% of muscle weight. Free amino acids apparently contribute to osmoregulation (Finne, 1992) and are depleted in the fish muscle during starvation (Love, 1988). Crustacean muscles contain a higher content of free amino acids than fin fish muscles.

Some unique non-protein free amino acids found in seafood are taurine, sarcosine, β- alanine, methyl- histidine, and α- amino-η-butyric acid. Taurine, a sulphonic acid is common and abundant in marine invertebrates. Taurine can function in osmoregulation, but can also serve as a food reserve (Nair & Mathew, 2000). Taurine is very active in the Maillard browning reaction and cause discolouration in dried squid (Hard & Arcilla, 1985). Histidine is the predominant amino acid in fish of Scombridae family, which on decarboxylation give rise to histamine. Putrescine and cadaverine are the biologically active amines present in spoiled fish.

Free amino acids and free fatty acids content of fish silage constantly increased for 15 days to 111.30-mg/100 g and 20.10-g/100 g respectively. Histamine content increased and reached a maximum of 37.5 mg/100 g the ninth day, then decreased significantly during the remaining days (Syaefudin. *et. al.*, 1991).

2.3.11. Amino acid profile

The amino acid profile of fermented silage prepared from different raw material have been studied by Durairaj *et. al.*, (1985). It was shown that the composition was almost similar for all the raw materials and the buttermilk fermented silage had highest value of lysine content.

Vidotti *et.al* (2003) prepared silage by adding acid and by lactobacillus fermentation with commercial marine fish waste, fresh water fish waste and with tilapia filleting waste. The comparison of amino acid composition of silages and raw materials showed an increase in histidine, threonine and serine levels for both acid and fermented silages whereas the levels of valine, isoleucine and leucine decreased in all the products. This could be due to chemical reactions between alpha amino acids and aldehydes present in amino acids (Johnson *et. al* 1985). Comparatively higher levels of leucine, glutamic acid and aspartic acid content in the free amino acid was reported by Lee *et. al.*, (2005) in skipjack tuna viscera fermented silage.

2.3.12. Biogenic amines

Biogenic amines are basic nitrogenous compounds formed mainly by decarboxylation of amino acids or by amination and transaminaton of aldehydes and ketones (Santos, 1996). Biogenic amines in food and beverages are formed by decarboxylation of amino acids by the enzymes generated by microbial action of the raw material (Hala'sz *et al.*, 1994). But it has been found that some of the aliphatic amines can be formed *in-vivo* by amination from corresponding aldehydes (Maijala *et al.*, 1993). Biogenic amines are formed by decarboxylation of their precursor amino acids, as a result of the action of either endogenous amino acid decarboxylase activity (Hala'sz *et. al.*, 1994) or by the growth of decarboxylase positive microorganisms (Santos, 1996). They are of importance

from the point of view of food poisoning and also as chemical indicators of spoilage (Shakila & Vasundhara, 2001).

Amines are used as a freshness index in fish and shellfish along with other biological indicators like TMA, DMA, TVBN and K value. Yamanaka reported that formation of putrescine, cadaverine and histamine and loss of spermidine and spermine were observed as decomposition of tuna progressed. Shakila and Vasundhara (2001) reviewed and found that histamine alone is not considered as a reliable indicator of decomposition as concentration of its precursor histidine vary greatly in scombroid and non scombroid fish. For these reason, Meitz & Karmas (1977) suggested a freshness index using different amines instead of taking only histamine.

$$\text{Freshness index} = \frac{\text{ppm histamine + ppm putrescine +ppm cadaverine}}{\text{1+ ppm spermidine + ppm spermine}}$$

The formula was based on the general observation that histamine, putrescine and cadaverine rise in their values, while spermidine and spermine fall as decomposition progresses.

Later it was slightly modified and gave the term as Quality Index (QI) or Biogenic Amine Index (BAI)

$$\text{So, QI or BAI} = \frac{\text{Putrescine(ppm)+ cadaverine(ppm)+histamine(ppm)}}{\text{Spermine (ppm)+ spermidine}}$$

Ensiled fish contains a considerable amount of the free amino acids that constitute the precursors for biogenic amines such as histamine, tyramine, putrescence and cadaverine (Haard *et. al.*, 1985). Decreased pH values and low oxygen concentrations within the silage facilitate decarboxylase activity. Biogenic

amines may constitute a potential risk in this type of product since their precursor amino acids are present in fish silage. (Maria *et.al., 2000*)

The low pH (below 4.5 after 2-4 days of fermentation) and the physical characteristics of fish silage that lead to a low oxygen concentration within the ensiled fish are favorable for the action of amino acid decarboxylases (Beautling, 1992). Biogenic amines may pose a potential risk in fish silage. These compounds are toxic for livestock, causing liver damage and decreasing the performance of the animals (Krizek, 1991). In susceptible humans, they can lead to a variety of cutaneous, gastrointestinal, haemodynamic and neurological symptoms (Taylor, 1986)

Little is known about histamine degradation by LAB, but Voigt and Eitenmiller (1978) and Leuschner *et. al.*, (1998) found diamine oxidase activity among some dairy and meat isolates, respectively. According to Eskeland & Nordal (1980) some lactic acid bacteria have lower deaminase activity than other bacteria and can prevent the formation of biogenic amines during the fermentation of sausage. According to (Maria *et.al.*, 2000) several potential starter strains were found to degrade histamine as single strain cultures and could find application in fish silage and other fish products in which histamine build-up poses a potential risk.

2.4. Changes During Storage of Silage

Espe & Lied (1999) studied the effects of storage (0-48 days) at different temperatures (4, 20 and 50°C) on physicochemical properties of fish silage. Raw fish silages were prepared from whole herring, whole mackerel, offal from cod and saithe, and cod viscera; silages were also manufactured from cooked raw material, with the exception of cod viscera silages. Composition of fish silage reflected that of the raw materials used for silage manufacture, while degree of hydrolysis of samples was dependent upon storage time and temperature;

together with the raw material used for silage production. Cooked silages did not show any alteration in degree of hydrolysis, while levels of dry matter, crude protein and total fat were unaffected by storage temperature or time.

The papaya latex fermented silage when kept stored in polythene containers remained in good condition up to three years (Gopakumar, 1997). Syaefudin *et. al.*, (1991) observed that during fermentation of *Rastrelliger brachysoma* total plate count increased for the first three days to 2.9 x 10 5 and then decreased until 15 days of fermentation.

2.5. Nutritional Study

The degree of hydrolysis of the protein in fish silage is likely to affect the nutritive value for ruminant livestock. The small peptides and amino acids formed during ensilage are more readily available to the ruminal micro flora. When metabolized by the micro flora in the rumen, the efficiency of nitrogen utilization by the animals can decrease and toxicity problems can occur, particularly when the animals is on a low energy diet (Orskov, 1977). The lower degree of hydrolysis in biological fish silage can be thus, regarded as advantageous from the nutritional point of view for these animals.

Fagbenro & Jauncey (1993 b) conducted storage and feeding study of co-dried fermented silage from Tilapia for catfish (*Clarias gariepinus*). It was observed that the protein quality of wet tilapia silage was reduced during storage; there were no differences ($P > 0.05$) in the apparent protein digestibility coefficients. It is suggested that autolysis in the stored tilapia silages had little effect on protein digestibility in the catfish. However no decrease in weight gains were observed when chicken were fed diets in which autolysed fish silages contributed up to 400g Kg -1 of the total dietary protein (Espe. *et. al.*, 1992).

Hammoumi. *et. al.*, (1998) studied the nutritional assays for broiler which showed a net increase in the weight relatively to the control diet. All the formula made with the combinations of different proportions of silage and ingredients resulted in similar growth of broilers compared with the commercial control feed formula. Hassan & Heath (1987) fed broiler chicks with a ration containing 5% and 10% fish silage and reported better feed efficiency than did birds fed a ration with no silage. Results indicate that up to 10% fish silage could be included in broiler rations without adversely affecting feed efficiency or body weight.

Feeding trials performed on several species of monogastric animals showed that it might be advantageous to have some pre digested protein in the diet, but there is a limit over which the animals would have difficulties in using the absorbed protein for metabolic purposes (Espe *et. al.*, 1992).

The nutritional evaluation conducted by Kompiang *et. al,* (1980) with chicken showed that feed containing upto 8% silage showed similar results as control but a high levels (29%) had shown a depression in growth. Feeding trials carried out with mice and chickens (Yeoh, 1979 b) showed that the fish silage was suitable for use either as a complete or partial replacement in the diet of young animals. Acceptability by the trial animals was good and no ill effects were observed.

2.5.1. Studies on fish

With a few exceptions acid or fermented fish silage showed better performance compared to control (fish meal or soy meal) in fish and shellfish. A variety of fresh water and marine fish have been raised with fish silage with no apparent ill effects. The use of fish ensilage has been proved to be effective in the feeds of salmon (Crampton *et.al.*, 1982., Jackson *et. al.*, 1984) and in Indian major carps (Ali. *et.al.*, 1985). It has been observed that the simpler peptides present in ensilage are easily assimilable by fish (Stone &Hardy, 1986). Equal to or better growth than control for African catfish was reported when fed with 38%

of fermented sardine silage (Cisse et. al., 1995). Rao et. al., (1996) reported better growth performance of common carp when fed with 30.8% protein as fish silage. It was observed that when Atlantic salmon fed with fermented silage showed no difference in fillet flavors between silage and control diets (Parrish. *et. al.*, 1991). Fish silage diets seem to advantages in the crucial early stages of aquaculture as better larval growth and survival rate have been reported with hybrid stripped bass and sea bass respectively (Gallagher, 1993).

2.5.2 Protein Efficiency ratio (PER):

Protein efficiency ratio is defined as the weight gain per gram of protein consumed and is usually done with feeding 3-4 weeks old albino rats. The test protein is fed to the rats as the sole source of protein in the diet with carbohydrates, fat, minerals etc and standardized vitamins etc., under carefully controlled conditions. Osborne *et.al,* (1919) observed that young rats fed with certain proteins gained little weight and ate little protein whereas those which were fed with better quality proteins gained more weight and consumed more protein. In an attempt to compensate for the difference in food intake, they calculated the gain in weight per gram of protein eaten and this has been called PER. Mathew *et. al.,* (1982) evaluated the PER for the squilla protein powder and found that a PER of 2.83 was obtained for the sample when compared with 2.92 of the control with casein, which was higher when compared with protein from threadfin bream (Nair & Gopakumar, 1982). According to Wibowo *et.al,* (2005), the PER and feed efficiency showed no significant difference when rats were fed with a diet containing the protein recovered from surimi wash water using chitosan with casein as control. Neethiselvan *et.al,* (2001) reported a higher PER for *E. suratensis* fed with fermented fish silage based diets than the control diets based on plant protein. According to Gonclaves. *et. al.,* (1989) eel fingerlings showed better FCR and PER with silage diets compared to control.

2.5.3 Net Protein Utilisation (NPU)

NPU is a well-known protein quality index. it can be described as the ratio of nitrogen retained in the body to the nitrogen intake. For correcting the nitrogen values for endogenous nitrogen a group of rats fed on nitrogen free diet is also taken along with the test group. It can be represented in the form an equation, as NPU = nitrogen retained/ nitrogen intake. Miller (1963) proposed a procedure which involved replicate groups of 4 weanling rats housed in group cages which were fed either the "protein-free" or the "test" diet for 10 days. These conditions were chosen empirically and the particular merits of these conditions remain to be demonstrated. Flores (1973) found that the net protein utilisation value for fish silage is higher 36.9 as compared to the control value of 33.3 for fishmeal probably because the silage undergoes a digestive process Canonisado (1977).

2.5.4. Feed Conversion Ratio

Feed conversion ratio is the ratio of feed intake to the gain in weight when fed on a particular diet. In most of the studies, feed conversion ratio of silage is found to be higher when compared with fishmeal as control. Kompiang *et.al (1977) reported a better feed conversion ratio in all the five* silage incorporated feeds at different levels with the highest value for 29% *sil*age in the feed. A significantly higher value of feed conversion ratio was reported by Kompiang *et.al* (1977) for formic acid silage than control and fermented fish silage (with 20% molasses). Rattagool *et. al.*, (1977) reported a better feed conversion ratio for fish silages of different qualities in broiler chicken. But on further studies of Rattagool *et. al.*, (1977) no significant different in feed conversion ratio of fish silage was noticed.

2.6. Recent Developments in LAB Fermentation for Food Applications

Fermentation technology has adapted itself to the demands of modern society in providing probiotic food and functional food to a great extent. The

fermentation technology has been used as a technique of food preservation and production of condiments in the earlier days which has been changed to the modern needs of the society such as specific food for the old people, patients suffering from digestive disorders and infants. Though the technology was highly localized in the earlier days, now the technique has wide application worldwide and more and more products are beings made. This includes the modernization of the existing ethnic technologies and invention of new ones with the help of modern biotechnological tools. Fermentation of fish and fishery products with LAB have also emerged as a promising field for the production of various products of commercial and pharmacological significance. The development of a process for the convenient production of hygienic fish sauce by lactic acid fermentation of shark meat (Sreerekha *et al.*, 2002), and the improvement of colour and acceptability of minced mackerel meat after fermentation for 72 hours with Lactobacillus indicates that new products with better property and quality can be made by applying this technique (Yin *et. al.*, 2002). The antimicrobial activity of some LAB against pathogens like *Listeria spp*, *Helicobactor pylori*, *Staphylococcus aureus* and the anti-diarrhea effect of *Lactobacillus spp* are well known. So the incorporation of the fermented products is of significance on health point of view. A new array of products can be developed incorporating the fermented fish to improve the utility and health of the consumers.

New processing technique was developed by Yin *et. al.*, (2000) with mackerel mince with different levels of protein concentration by adding lactic acid bacteria and fermenting it for 48 hours. Rapid growth of LAB, decline in pH, suppression of main micro flora, and increase in whiteness and sensory quality were observed and the VBN of the fermented samples were 25mg/100g. Various lab fermented fish products with high commercial potential can be prepared. The fermented mackerel mince were analyzed for organoleptic quality on a 9-point hedonic scale and found that the score increased during the course of fermentation i.e., 48 hours. However, higher score was recorded after 24 hours of fermentation.

According to Yin *et.al.*, (2002) fermentation with *L. plantarum CCRC 10069, Lactococcus lactis CCRC 12315, L. helvcticus CCRC 14092* and their combination could substantially suppress the development of VBN, growth of main micro flora, hydrolyze the muscle protein and accumulate free amino acids during fermentation. According to Karmas& Lauber (1987) Mincing or surimi processing, followed by fermentation, and extrusion can be combined to develop intermediate moisture food to create fish protein based snack foods. A dough of fermented fish (minced fish or surimi), wheat flour, corn starch, and water was extruded and subsequently dried resulting in an intermediate moisture food product at pH 5.2, water activity 0.90 and moisture about 30%. The products had a chewy texture, were shelf-stable and could be processed into flavored, high-protein snack foods

2.7. Application of LAB Fermentation in Probiotic Feed

As probiotic feed lactobacillus culture can be sprayed on the feed and dried and stored without affecting the viability of the culture. The resistance of the culture against the intestinal conditions of the hosts have been studied and it was found that the adaptive response of certain types of lactobacillus is quite satisfactory for making such probiotic feeds (Linders *et al.*, 1997). Certain strain of lactobacillus had a high likelihood to survive stress as in the gastrointestinal tract like low pH and pepsin, and bile presence and pancreatin.

The recent research work on fermented sea food has two main objectives.

1. : The reduction of fermentation time of traditional products
2. : The development of new semi preserved products for the increase of shelf life of fresh or processed fish.

With more biotechnological research into feed products, new techniques may improve fish waste silage by monitoring fermentation with an appropriate microbial starter. These would play a role in the preservation of fish waste by fermentation, removal of fish-odor; and probiotic effect by inhibiting pathogens (Hammoumi. *et.al.*, 1998).

Materials and Methods...

3.0 MATERIALS AND METHODS

3.1 Materials

3.1.1.Chemicals

Analytical reagents supplied by different companies as detailed below were used for the experiments.

Qualigens

Potassium sulphate
Sodium hydroxide
Boric acid
Petroleum ether
Trichloro acetic Acid
Potassium iodide
Sulphuric acid
Sodium meta bisulphate
Glacial acetic acid
Sodium chloride
Sodium sulphate
Chloroform
Sucrose
Cholesterol
Isopropanol
Acetyl acetone
Heptane
Ascorbic acid
Butanol
Ammonium molybdate

Merck

Boron trifluoride

Copper sulphate

Sodium dodecyl sulphate

Potassium carbonate

Hydrochloric acid

Perchloric acid

Acetonitrile

Thio barbituric acid reagent

Phenolphthalein

Potassium dihydrogen phosphate

Dipotassium hydrogen phosphate

Anthrone's reagent

Benzoyl chloride

Standards for potassium, calcium and sodium.

Copper-triethanolamine

Diethyldithiocarbomate

Sisco Research Laboratories (SRL)

L-Leucine

Tryptophan

Ferric chloride

Methanol

SD. Fine chemicals

Silicic acid

Tri sodium citrate

Sigma chemicals

Amino acid standards

Fatty acid standards

Histamine dihydrochloride

Putrescine dihydrochloride Cadaverine (free base)

Agmatine sulphate

Tyramine hydrochloride

Spermine tetra hydrochloride

Spermidine trihydrochloride

Standard-triolein

SD. Fine chemicals

Silicic acid

Tri sodium citrate

Oxoid Ltd, UK

Man Rosoga Sharpe Agar

3.1.2. Equipment and glass wares

Homogenizer : Euro Turrax, T20 b IKA Labortechnik, Germany

Centrifuge : REMI Cooling centrifuge, CPR 24, Remi Instruments, Mumbai, India

Flake ice machine : F90 compact electronic flake ice unit, Icematic, Italy

Balance: Sartorius Electronic Balance, Germany

Hot air oven: Beston hot air oven

Glass wares: Borosil Glass wares

pH meter: Cyberscan 510 pH meter. Eutech Instruments, Singapore

Spectrophotometer: Spectronic Genesys 5, Spectronic Instruments, Inc., USA

GC: Varian Inc. Lake Forest USA

HPLC: Jasco PU 2089 plus Quaternary gradient pump with C-18 column and multi wavelength UV detector.

Atomic Absorption Spectrophotometer: Varian AA 420 USA.

Flame photometer: TMF 45 Toshniwal instruments Ajmer- India

Bowl Chopper Tecator 1094

Deionised water used for the experiments: (ELGA, UK.)

3.1.3. Fish

Tilapia (*Oreochromis mossambicus*) of size 10-18 cm and weight 40-75 g were collected from brackish water farms near Cochin, Kerala for the studies and brought to the laboratory in ice. The washed whole fish in rigor or early stages of post rigor was homogenized in Euro turrax homogeniser. From another set of tilapia, viscera and gills were removed, washed thoroughly and homogenized.

Silver carp (*Ctenopharyngodon idella*) of size 15-20 cm and weight 200-250 g were collected from a fresh water farm near Kottayam, Kerala and brought to the laborotary in ice in rigor condition. They were dressed by removing viscera, gills and scales, washed thoroughly and homogenized.

Fresh Jew fish (*Johnius dussumerie*) of size 15-20 cm and weight 100-200 g in the early stages of post rigor were collected from local markets in Cochin. They were dressed by removing viscera, gills and scales, washed thoroughly and homogenized

Fresh Jew fish (*Johnius dussumerie*) of size 15-20 cm and weight 100-200 g in the early stages of post rigor were collected from local markets in Cochin. They were dressed by removing viscera, gills, scales and entrails, washed thoroughly and homogenized and used for the preparation of fermented fish powder.

Fish waste, from surimi-processing, viz., skin, scale and bones of *Nemepterus japonicus* was collected from a surimi processing plant near Cochin and mixed thoroughly. The above four homogenates and surimi processing waste were used for the preparation of samples.

3.1.4. Bacterial culture

Lactobacillus plantarum type culture (No.1425) procured from Microbial Type Culture Centre, Institute of Microbial Technology, Chandigargh, revived and repeatedly sub- cultured in MRS broth were used as the bacterial culture for the fermentation of fish waste for silage.

3.1.5. Jaggery

Jaggery as source of carbohydrate for the preparation of fermented silage was procured from the local market.

3.1.6. Experimental animals

3.1.6.1. Rats

Wistar strain male albino rats, weighing 60-62g, were selected for the study.

3.1.6.2. Japanese Quail

Japanese quail *(Coturnix Coromandelica)* was procured from Kerala Agricultural University, Mannuthy, Kerala, India. Two weeks old birds were procured and were acclimatized with the feed for one week. The experimental birds were maintained under hygienic condition and provided food and water *ad-libitum*. For the feeding experiment, the birds were divided into three groups consisted of 48 quails in each group.

For Japanese quail feeding study the following protocol was followed. The ingredients were powdered in a grinder and mixed together as given below.

Group 1. Control ration with 10% unsalted whole dried fish (sardine)

Group 2. Control ration in which unsalted dried fish was replaced by fermented silage prepared from surimi processing waste, in terms of crude protein content.

Group 3. Control ration in which unsalted dried fish was replaced by dried surimi processing fish waste in terms of crude protein content.

3.2 Methods

3.2.1. Preparation of fermented silage.

The fish (3.1.3) was homogenized in a Bowl Chopper (Tecator 1094) for 5-10 minutes and cooked in a steel vessel for 30 minutes with jaggery. It was cooled to room temperature and *Lactobacillus plantarum* culture containing 10^9 CFU/ml was added @ 5% (W/V) of weight of homogenate (Fagbenro and

Olusoji, 1998). The whole mass was mixed thoroughly and transferred into beaker and tightly covered with plastic sheet. The samples were stirred twice daily with sterile glass rod for proper mixing. Samples were drawn aseptically at different intervals for various analysis.

Standardization.

Silage was prepared with both whole and dressed Tilapia with different levels (5%, 10%, 15% and 20% of the homogenate) of jaggery and the changes during the period of ensilation were assessed. From the results obtained, it was found that jaggery at 10% level (W/W) was sufficient for successful fermentation. So further studies were conducted by adding jaggery at 10% (W/W) of the fish weight.

3.2.2. Preparation of acid silage

The dressing waste of fresh water fish (Rohu) was homogenized as such and formic acid was added @ 3.5%, and mixed well. The pH was checked and confirmed nearly 4. The whole mass was mixed daily and kept closed for hydrolysis for 7-10 days.

3.2.3. Preparation of surimi waste silage

The surimi processing waste from *Nemipterus japonicus* was homogenized for 10 min and steam cooked for 30 min with 10% by weight of jaggery. It was cooled and inoculated with *Lactobacillus plantarum* culture at 5% (w/v) level containing 10^9 CFU/ml. The whole mass was mixed thoroughly, transferred into plastic buckets and covered tightly with lid and allowed to ferment for a period of 10 days with daily stirring.

3.2.4. Preparation of fermented fish powder

For the preparation of fermented fish powder, dressed Jew fish was used. Fermentation was carried out as in the case of silage except that the period of ensilation was for 7 days only. After fermentation, the whole mass was vacuum dried, powdered and packed in polypropylene bags.

The same methodology was followed for the preparation of dehydrated fermented silage after a period of 13 days of fermentation.

3.2.5. Preparation of diets for feeding experimental rats

Control feed Basal feed supplied by M/s Sai Feeds, Bangalore, India was used for the study Table 3.1. The basal feed as such was used as control diet.

Fermented Jew fish silage supplemented feed: Dried fermented fish silage made from dressed Jew fish was added to the basal diet at 10% level.

Fermented tilapia silage supplemented feed: Dried fermented fish silage made from dressed tilapia was added to the basal diet at 10% level.

Fermented silver carp silage supplemented feed: Dried fermented fish silage made from dressed silver carp was added to the basal diet at 10% level.

Acid silage supplemented feed: Dried fermented acid silage made from rohu waste was added to the basal diet at 10% level.

3.2.6. Preparation of cookies

For preparation of cookies the fermented fish powder was incorporated in different proportions with ingredients of commercial formulations (Table 3.2.1) for making cookies. It was made into dough with sufficient quantity of water and kept for 10-15 minutes. It was shaped into cookies and baked at 170-180 $^{\circ}$ C for 20 minutes and packed after cooling.

3.2.7. Preparation of diets for feeding Japanese quails

The ingredients used for the preparation of feed is given in table 3.3. The feed ingredients were ground individually except gingelly oil. It was mixed together thoroughly in a pounder for 5 minutes.

3.3. Collection and Determination of Egg Quality of Japanese Quail.

Eggs were collected daily and quality determination was conducted once in a week (one week interval of time). Soft-shelled, cracked and small eggs have not

been used. The egg weight was noted one by one and they have been stored at 13°C. The egg shape index was measured using an electronic digital caliper. After measuring the diameters of eggs, they have been broken under well-arranged glass and five minutes later, long and short diameters and height of both albumen and yolk were measured with electronic caliper. Separated yolks have been weighed and recorded. Shells of broken eggs were washed with water for separating of albumen and air-dried. The shape index, albumen index, yolk index, shell thickness and Hauge unit of eggs collected were determined.

Shape index =short border/long border x 100

Albumen index =albumen height /albumen diameter x 100

Yolk index =yolk height /(long diameter of yolk + short diameter of yolk/2)x 100

Shell thickness = (sharp point thickness + equator thickness + stubby thickness)/3

Haugh Unit =100 x log (Albumen weight + 7.57 - 1.7 x egg weight x 0.37)

3.4. Rat Feeding Studies for Nutritional Evaluation of Silage.

Wistar strain male albino rats were housed in polyurethane cages under hygienic conditions and maintained at normal room temperature. The animals were allowed food and water *ad-libitum*. The experiments were carried out according to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India and approved by the Institutional Animal Ethics Committee (IAEC).

Seven days after acclimatization with the experimental food, the animals were further divided into five groups of five animals each. Group 1 fed with control diet, Group 2

fed with fermented Jew fish silage supplemented feed Group 3 fed with fermented Tilapia silage, group 4 fed with fermented silver carp silage and Group 5 fed with acid fish silage. Food and water for the animals were provided *ad-libitum*. The experiment was carried out for 35 days.

After the experimental period, the experimental animals were sacrificed, blood was collected using sodium citrate as anticoagulant and the plasma separated was used for assay of various biochemical parameters. The heart and liver was excised immediately and washed with chilled isotonic saline. The total weight of the organs were observed and accurately weighed heart and liver tissue homogenates were used for the extraction of lipid and for further biochemical analyses.

3.4.1. Experimental protocol for rats

Average weekly weight gain, Net protein ratio, Apparent nitrogen digestibility, True digestibility, Protein efficiency ratio were calculated from the observations on rat feeding study. On completion of the experimental period the rats were sacrificed and Total cholesterol, triglycerides, free fatty acids, phospholipids, low-density lipoproteins, inorganic phosphorus was determined for serum, heart tissue and liver tissue of the experimental rats.

3.4.2. Sampling procedure

Silage samples were drawn on 1^{st}, 2^{nd}, 4^{th}, 7^{th}, 10^{th} and 13th days and analysis were carried out as detailed below.

Moisture, crude protein, crude fat, ash content and jaggery were analysed for the initial samples at different levels of jaggery for whole tilapia silage and for dressed tilapia silage, initial samples of dressed silage of Jew fish and silver carp, dried silage of tilapia, silver carp and Jew fish, dried surimi waste silage, cookies developed.

Titrable Acidity, pH, TVBN, alpha amino nitrogen, Non protein nitrogen, Jaggery, peroxide value, free fatty acids, TBA, were determined for whole tilapia silage, dressed tilapia silage, dressed silver carp silage and Jew fish silage.

Biogenic amines, fatty acid composition, Amino Acids and tryptophan were determined for dressed tilapia silage, dressed silver carp silage, Jew fish silage at 10% level of jaggery. Biogenic amines were also assessed for whole tilapia silage at 10% level of jaggery.

Sodium, potassium, calcium and iron were determined for fermented fish powder and cookies.

Total plate Count, *Lactobacillus plantarum E. coli, Staphylococcus* and *salmonella* were determined for dressed fish silage at different levels of jaggery.

Table 3.1 Composition of control feed

Components	Percentage
Moisture	10.20%
Jaggery	54.20%
Crude protein	22.10%
Crude fat	4.03%
Crude fibre	3.12%
Ash	5.10%
Sand silica	1.11%

Table 3.2.1 Ingredients used for the preparation of cookies with different levels of fermented dried fish powder (g/kg)

Ingredients	Control	2%	5%	10%
Maida	400	380	350	300
Fermented dried fish powder	...	20	50	100
Dalda	200	200	200	200
Sugar	200	200	200	200
Ginger	20	20	20	20
Chilly	20	20	20	20
Curry leaves	10	10	10	10
Masala	30	30	30	30
Essence	2	2	2	2
Baking powder	4	4	4	4
Cashew nut	60	60	60	60
Sesame	40	40	40	40
Egg (Nos.)	3	3	3	3
Salt	10	10	10	10

Table 3.2.3 .Feed composition for Japanese Quail for nutritional evaluation of fermented silage (g)

Ingredients	Control	Fermented silage	Dried fish waste
Yellow maize	50	50	42
Gingelly oil cake	02	05	02
Soya bean	27	24	26
Unsalted dried fish	8.0	--	--
Fermented fish waste	--	10	--
Dried fish waste	--	--	8.5
Rice polish	6.0	4.0	10.5
Shell grit	5.0	5.0	5.0
Mineral mix	1.75	1.75	1.75
Salt	0.25	0.25	0.25
Wheat bran	---	---	4.0
Total	100	100	100

3.5. Analytical Methods

3.51. Determination of moisture (AOAC, 2000)

5-10 g sample was weighed into pre-weighed clean petri dishes. Dishes were placed in a hot air oven at 100±1° C for 6 hours. Dishes were cooled in a desiccators and weighed to a constant weight. Moisture loss was calculated as

$$\% \text{ Moisture} = \frac{\text{Loss in weight x 100}}{\text{Weight of the sample}}$$

3.5.2. Determination of crude protein (AOAC, 2000)

0.3 to 0.5 g of the moisture free fish sample was transferred into a digestion flask of 50 ml capacity. A few glass beads, a pinch of digestion mixture (8 parts K_2SO_4 & 1 part $CuSO_4$) and 10 ml concentrated sulphuric acid were added to the flask. It was digested over a burner until the solution turns colorless. The digest was transferred quantitatively into a 100 ml standard flask and made up to the mark. The 2 ml of well-mixed made-up solution was transferred to the reaction chamber of the Micro-Kjeldahl distillation apparatus, 2 drops of phenolphthalein indicator and 40% sodium hydroxide were added till the indicator changes to pink. Distillation was done for 4 minutes and ammonia liberated was absorbed into 2% boric acid containing a drop of Tashiro's indicator. The amount of ammonia liberated was determined by titration with N/50 sulphuric acid. Percentage Crude protein was determined as:

% Crude protein = nitrogen content x 6.25

3.5.3. Determination of crude fat (AOAC, 2000)

About 1-2 g of accurately weighed moisture free sample was taken in a thimble plugged with cotton and was extracted with petroleum ether (40-60°C BP) in a Soxhlet apparatus for about 10 hrs, at a condensation rate of 5-6 drops per min. Excess solvent was evaporated and the fat was dried at 100°C to a constant weight. The crude fat was determined as

$$\% \text{ Crude fat} = \frac{\text{Weight of fat x 100}}{\text{Weight of the sample}}$$

3. 5.4. Determination of ash content (AOAC, 2000)

About 2-3 g of the moisture free sample was transferred into a previously heated, cooled and weighed silica crucible. The sample was charred at low red heat. Then the crucible was placed in a muffle furnace at 550° C for about 6 hours until a white ash was obtained. Crucible was cooled in a desiccator and weighed. Ash content was calculated as

$$\% \text{ Ash} = \frac{\text{Weight of residue x 100}}{\text{Weight of the sample}}$$

3. 5. 5 Titrable Acidity IS 13844: (2003)

1gm sample was weighed and diluted with distilled water. One drop phenolphthalein indicator was added to it and titrated with 0.01N NaOH till end point.

Calculation

Eq. wt of lactic acid = 90

1 ml of 0.01N NaOH = 0.0009g lactic acid in 1 L

If the titre value is 'a', then

% Lactic acid = 'a' x 0.0009

3.5. 6 Determination of pH

For the determination of pH, direct measurement was carried out using a Cyberscan 510 pH meter.

3. 5.7. Preparation of Trichloroacetic Acid (TCA) extract.

About 10g of accurately weighed sample were extracted with 10% trichloro acetic acid by grinding in a mortar and pestle. Then the content was filtered quantitatively through what man filter paper No.1. Filter paper was thoroughly washed with TCA and filtrate was made up to 100ml. The TCA extract was used

to measure non-protein nitrogen (NPN), total volatile bases (TVN) and alpha amino nitrogen (AAN).

3. 5.8. Determination of TVBN (Conway, 1962)

Conway units were cleaned in chromic acid for 24 hours, soaked in water, washed and dried. Cover plates were coated on underside with wax-grease. The units were kept ready before preparing the extract.

One ml of the supernatant was taken from the TCA extract prepared and was put in the outer chamber of Conway micro diffusion units, spreading it around the chamber as much as possible. One ml of the standard acid is taken in the central chamber and one ml. saturated potassium carbonate solution in the outer chamber. Cover plate was put on ensuring that no leak occurs. Solutions in the outer chamber were gently swirled with great care to mix them. Unit was left overnight. Acid in the central chamber was titrated against 0.01N NaOH using 2 drops of Tashiro's indicator (B). A reagent blank is also titrated by taking standard acid at central chamber (A). Value (A-B) is volume of N/100 acid used up by volatile base. TVBN was expressed as mg%.

Calculations

mg TVBN / 100 g = (Blank – Titer value) x 0.14 x (100/10) x 100.

3.5.9. Determination of alpha amino nitrogen (TNBS method: Adler-Nissen Jens, 1979)

1ml of 10% TCA extract was neutralized with 1.5ml 5% NaOH and 0.1ml 10% SDS was added to it (i.e., total volume is 1.6ml). From this 0.25ml was taken in a test tube and 2ml phosphate buffer was added. This was vortexed thoroughly and incubated at 50°C in a water bath for 1 hour. 4ml of 0.1N HCl was added, vortexed and kept at room temperature for 30 minutes. This was then read at 340nm using a spectrophotometer.

Preparation of standard solution

0.0015M L-Leucine was prepared. 0.05, 0.1, 0.15, 0.2 and 0.25mlof standard solution was taken in duplicate.

3. 5.10. Estimation of Non protein nitrogen (NPN) (AOAC, 2000)

Digestion of sample

10ml of TCA extract was transferred to a clean, dry, kjeldahl digestion flask and about a pinch of digestion mixture. (8 parts K_2SO_4 and 1 part of $CUSO_4$) and 15 ml of concentrated H_2SO_4 was added. Glass beads were added to avoid bumping. The flask was heated on micro digestion unit till it became clear and colour less. The flask was cooled and volume was made up to 100 ml. with distilled water.

Distillation

The distillation was done as described in the section 3.2.2.

Non-protein nitrogen is calculated and expressed as percentage.

Calculation

If the titre value for the sample minus titre value of blank is 'x'

Then NPN mg / 100g = 0.28 x R / Volume pipetted x (100 / 10) x (100 / 10) x 100

3. 5.11. Determination of peroxide value (AOCS 1989)

About 10 ml of the aliquot of the fat in chloroform was used directly. The fat content in the chloroform was determined by evaporation and drying of another aliquot from the same solution. 15ml of glacial acetic acid was poured into the aliquot in an iodine flask and a pinch of potassium iodide was added. The flask is closed and sealed with the solution of KI. It is shaken well and kept in dark for 30 minutes. Then the flask is taken out. Sides were washed with distilled water. A few drops of starch indicator was added to it. This was then titrated immediately against N/100 $Na_2S_2O_3$. The end point is denoted by the disappearance of blue colour. Chemical reaction involved in PV determination is as follows.

$$2\ ROOH + 2H^+ + 2\ KI \longrightarrow I_2 + 2ROH + H_2O + K_2O$$

$$I_2 + 2\ Na_2S_2O_3 \longrightarrow Na_2S_4O_6 + 2\ NaI$$

Calculation

PV is denoted as milli equivalent of peroxide per 1000 gm of sample

$$PV = \frac{(\text{Titre value} - \text{Blank}) \times (\text{Normality} \times 100)}{\text{Weight of fat}}$$

3. 5.12. Determination of free fatty acids (FFA) AOAC (2000)

About 10 ml of the chloroform extract was taken into a clean beaker. Chloroform was evaporated off on a water bath and weight of fat determined. Another 10 ml of extract was transferred in to a conical flask. Chloroform was evaporated off. To this 5 ml of neutral alcohol was added and warmed. It was titrated against 0.01N NaOH using phenolphthalein as indicator. FFA was calculated as percentage of oleic acid.

Calculation

Equivalent weight of oleic acid = 280

1 ml of 1N NaOH = 0.28 gm of oleic acid in 1 Litre.

$$\% \text{ FFA} = \frac{\text{volume of NaOH used up} \times 0.01 \times 0.28 \times 100}{\text{Weight of fat in 10 ml of Chloroform extract}}$$

3. 5.13. Determination of Thiobarbituric Acid value (Tarladgis et al., 1960),

10g minced sample was mascerated with 100 ml of 2% concentrated HCl. It was transferred to a flat bottom flask, and distilled. 50 ml of the distilled solution was collected in a measuring cylinder.

Reagent

0.144 mg of TBA reagent in 50 ml of glacial acetic acid

Blank: 5 ml distilled water + 5 ml reagent

Test: 5 ml distillate + 5 ml reagent

Mixed properly and boiled in a water bath for 30 minutes and read in spectrophotometer at 538 nm.

Calculation:

$$\text{OD} \times 7.8 \text{ if 5 ml is taken, otherwise } \frac{\text{OD} \times 7.8 \times 5}{\text{ml taken}}$$

3. 5.14. Determination of fatty acid composition

Fatty acids were analyzed according to the method of AOAC (2000). Lipid content of mince and surimi was estimated by the method of Folch *et al* (1957). Methyl esters of fatty acids were separated and detected by gas chromatography.

Reagents

1. Boron trifluoride - methanol reagent.
2. Methanolic sodium hydroxide solution.
3. Petroleum ether.
4. Sodium sulphate.
5. Saturated NaCl

Procedure

Lipid extracted was added to a round bottom flask followed by methanolic NaOH (6 ml) and boiling chip. Condenser was attached and samples were refluxed until fat globules disappear. BF_3 methanol solution (5 ml) was added through the condenser and boiling was continued for 2 minutes. After removing from heat 5 ml saturated NaCl solution was added and the contents were transferred to a 250ml separator. 30 ml H_2O was added and extracted with two 25ml portions of petroleum ether (BP 60-80°C). Extract was dried over anhydrous Na_2SO_4, filtered and solvent was evaporated under a stream of nitrogen on steam bath.

Methyl esters of the fatty acid thus obtained were separated by gas liquid chromatography (Varian CP 3800. U.S.A) equipped with a capillary column (30 m long and 0.54 mm diameter) and a flame ionization detector in the presence of hydrogen and air. The carrier gas was nitrogen and the flow rate was 4ml/min. The chromatograph temperature started at 120°C and was increased 4°C/min until a temperature of 250°C was obtained. Fatty acids separated were identified by the comparison of retention times with those obtained by the separation of a mixture of standard fatty acids. Measurement of peak areas and data processing were carried

out by electronic integrator. Individual fatty acids were expressed as a percentage of total fatty acids.

3. 5.15. Estimation of Amino Acids

Total amino acids in silages prepared were determined as per the procedure of Ishada *et al* (1981).

Reagents

1. HCl : 6N
2. HCl : 0.05M
3. Buffer A: Dissolve tri sodium citrate (58.8g) in 2 L of double distilled water. add 210 ml ethanol of 99.5%, adjust the pH to 3.2 by adding 60% perchloric acid and make up to 3 L using double distilled water.
4. Buffer B: Dissolve tri sodium citrate, 58.8 g and boric acid, 12.4 g in double distilled water, adjust the pH to 10 by adding 4N NaOH, and make up the volume to 1L using double distilled water.
5. O-Phthaladehyde (OPA) Buffer: Dissolve 122.1 g of Na_2CO_3, 40.7 g of H_3BO_3 and 56.4 g of K_2SO_4 in double distilled water and make up the volume to 3L.
6. O-Phthalaldehyde solution (OPA): Dissolve 400 mg OPA, 7 ml ethanol, 1 ml of 2- Mercaptoethanol and 2ml of 30% w/v Brij-35 in 500ml OPA buffer.
5. Sodium hypochlorite solution: 4% w/v Sodium hypochlorite in OPA buffer. ie., 0.3ml Sodium hypochlorite in100ml OPA buffer.

3.5.16. Total amino acids

Principle

The amount of each amino acid present within a given protein does not vary from molecule and can provide useful information about the nature of the protein molecule. In a typical analysis of the amino acid content of a protein, peptide bonds are broken by acid hydrolysis with 6N HCl at 110^0 C (24h) so that

the released amino acids can be assayed. The amino acid tryptophan is not stable to acid digestion in the presence of even trace amounts of oxygen and is estimated separately by alkali digestion.

Sample preparation

Take the sample (100 mg tissue) in a heat sealable test tube. Add 10ml 6 N HCl and heat seal the tube after filling with pure nitrogen gas. Carry out the hydrolysis at 110^{0}C for 24 hrs. After the hydrolysis is over, open the test tube. Remove the contents quantitatively and filter into a round bottom flask through Whatman No 42 filter paper and wash the filter paper 2-3 times with distilled water. Flash evaporate the contents of the flask to remove all traces of HCl, the process should be repeated for 2-3 times with distilled water. Dissolve the residue and make up to 10 ml with 0.05 M HCl.

HPLC Analysis

Filter the sample thus prepared again through a membrane filter of 0.45µm and inject 20µl of this to an amino acid analyzer (HPLC- LC 10 AS) equipped with cation exchange column packed with a strongly acidic cation exchange resin i.e., styrene di vinyl benzene co polymer with sulphinic group. The column used was Na type i.e., ISC- 07/S 1504 Na having a length of 19 cm and diameter 5mm.

The instrument was equipped with Shimadzu FL 6A fluorescence detector and Shimadzu CR 6A Chrompac recorder. The mobile phase of the system consists of two buffers, Buffer A and buffer B. A gradient system can be followed for the effective separation of amino acids. The oven temperature can be maintained at 60^0 C. The total run was programmed for 62 minutes. The amino acid analysis can be done with non-switching flow method and fluorescence detection after post–column derivatization with o-phthalaldehyde.In the case of proline and hydroxyl proline, imino group is converted to amino group with hypochlorite.

Run an amino acid standards (Sigma chemical Co., St. Lousis, USA) also to calculate the concentration of amino acids in the sample. Calibration of equipment using standards needs to be done before the start of analysis.

Quantification of amino acids

The standard and the sample were analyzed under identical conditions. The elution time of the amino acids of the sample was compared and identified with those of the standard. Quantification of amino acid was done by comparing the respective peak areas in the chromatogram of the sample and the standard. The amino acid content was calculated as follows,

$$\text{mg amino acid/gm tissue} = \frac{\mu\text{mol}*\text{mol.wt}*\text{volume made up}*1000*100}{1000*1000*20*\text{wt. of sample}}$$

The amount of each amino acid is expressed as mg amino acid/ gm tissue and mg amino acid/ ml serum.

3. 5.17. Estimation of tryptophan

Tryptophan was estimated as per the method of Sastry and Tummuru (1985) after alkali hydrolysis of the sample using 5% sodium hydroxide at 110^{0}C for 24 hours. The 5-hydroxy furfural resulting from sucrose under acidic conditions formed a pale green coloured condensation product with thioglycolic acid, which on treatment with tryptophan formed a pink coloured complex. The colour intensity is measured at 500nm.

Reagents

1. NaOH : 5%
2. HCl : 6N
3. Sucrose :2.5%
4. Thioglycolic acid : 0.6%
5. H_2SO_4 :50%
6. HCl : 0.1N
7. Tryptophan Standard: 10μg/ ml solution

Procedure

Sample was hydrolyzed with 10ml of 5% NaOH at 110^0C for 24 hours in a sealed tube with pure nitrogen. The hydrolysate was neutralized to pH 7.0 with 6N HCl using phenolphthalein indicator. The volume was made up to 25 ml with distilled water. The solution was then filtered through Whatman No.1 filter paper and filtrate was used for estimation. The test tube containing 4ml of 50% H_2SO_4, 0.1mlk of 2.5% sucrose and 0.1ml of 0.6% thioglycolic acid were added. These tubes were kept for 5minutes in water bath at 45-50^0C and cooled. The sample was then added to the test tubes. A set of (0.1 to 0.8ml) standard tryptophan (10μg/ ml solution) was run in a similar way. The volume was made up to 5ml with 0.1N HCl and allowed to stand for 5 minutes for the development of colour. The absorbance was measured against a reagent blank at 500 nm in a spectrophotometer.

3. 5.18. Determination of sodium and potassium. (AOAC, 2000)

Sodium and Potassium were estimated by flame photometry. An atomized mist of the solution to be analysed is introduced to a flame photometer. The characteristic radiation for each representative element is filtered out using a suitable optical interference. Filter and intensity measured by a photo detector, displays it as an electrical signal, which under standard condition is proportional to the concentration of Sodium or Potassium in the solution. The sample was ashed and dissolved in 1:1 HCl and the filtrate is made upto 100 ml.

Determination of Sodium:- Calibrate the flame photometer using distilled water for zero reading and 10ppm sodium solution for 100 reading. Dilute samples if necessary to bring readings within the range of working standards. A few ml of the diluted solution was charged into the photometer and the reading was noted.

Determination of Potassium:- A 5ppm solution of Potassium salt is prepared and using this solution the flame photometer is calibrated. Reading of the pointer is noted. The made up solution of ash is diluted appropriately and a few ml of this solution is charged into the photometer. The reading is noted.

Calculation

$$\text{Concentration of sodium} = \frac{C_s \times R_1 \times 100 \times 100\text{mg\%}}{R_2 \times 10 \times W \times D}$$

$$\text{Concentration of potassium} = \frac{C_s \times R_1 \times 100 \times 100\text{mg\%}}{R_2 \times 10 \times W \times D}$$

C_s = Concentration of standard solution.

R_1 = Reading of sample solution.

R_2 = Reading of standard solution of sodium or potassium.

W = Weight of sample ashed.

D = Dilution Factor.

3.5.19. Estimation of calcium and iron using Atomic Absorption Spectrophotometer **(AOAC, 2000)**

Reagents

1. Nitric acid
2. Perchloric acid
3. 1&2 in 9:4
4. Stock solutions of calcium and Iron prepared by diluting concentrated solution of 1000mg/L (SRL)

Procedure

1 g homogenised sample was used for the experiment. To the sample containing flask, 7 ml of nitric acid and perchloric acid (9:4) mixture was added, covered with a watch glass and left at room temperature overnight. The samples were then digested using a microwave digester (Anton Paar). The completely digested samples were allowed to cool at room temperature, filtered using glass and carefully transferred and made up into a clean 50 ml volumetric standard flask. The samples were analysed using Varian spectra AA 220, AAS equipped with deuterium background corrector, for the determination of calcium and iron.

3. 5.20. Extraction of total lipids

The total lipid content of the tissues was estimated by the method of Folch *et al.*, (1957).

Reagents

1. Chloroform-methanol mixture (2:1 v/v)

Procedure

A weighed amount of the tissue was subjected to lipid extraction using chloroform-methanol mixture (2:1). The extraction was repeated twice with fresh aliquot of chloroform-methanol mixture. The lipid extracts were transferred to a separating funnel and added 20% of water into it and left overnight. Next day the lipid extracts were drained through filter paper containing anhydrous sodium sulphate and was collected in round bottom flask and was evaporated to dryness in a flash evaporator. The lipid in the round bottom flask was made up to 10 ml by using chloroform. From this 1.0 ml was taken into a pre-weighed vial and allowed to dry in warm temperature to constant weight and total lipid content were calculated from the difference in weight. Sample made up to 10 ml was used for the estimation of various lipid components viz., cholesterol (total and free), triglycerides, free fatty acids and phospholipids after evaporating the solvent in air at room temperature.

3. 5.21. Estimation of total cholesterol

The total cholesterol present in plasma and heart was estimated according to method of Parekh and Jung (1970) with slight modifications.

Reagents

1. Standard cholesterol solution (stock): 1mg /ml in chloroform
2. Working standard: 1.0 ml of the stock was diluted to 10 ml with chloroform.
3. $FeCl_3$ stock solution: 10g $FeCl_3$ in 100 ml acetic acid.
4. $FeCl_3$ - H_2SO_4 reagent: 2.0 ml of $FeCl_3$ stock solution was diluted to 200 ml with conc. H_2SO_4.
5. 33% KOH (w / v): 10g of KOH was dissolved in 20 ml distilled water.
6. Alcoholic KOH solution: 6.0 ml of 33% KOH was made up to 100 ml with distilled ethanol. This solution was prepared fresh before use.

Procedure

1.0 ml of the lipid sample was taken into a 25 ml glass stoppered tube and evaporated off the chloroform. Added 5 ml of freshly prepared alcoholic KOH solution. The tubes were shaken well and incubated in a water bath at 37^0C for 55 min. After cooling to room temperature, added 10 ml of petroleum ether and inverted the tubes once to mix the contents. Then added 5.0 ml of distilled water and shaken the tubes vigorously for 1 min. Took 0.5-2 ml aliquots from the supernatant (petroleum ether) into test tubes. Evaporated the petroleum ether extract under nitrogen. To each of the sample as well as the standard tubes including the blank, added 3.0 ml of glacial acetic acid followed by 0.1ml -distilled water. Mixed the tubes thoroughly and added 2 ml of the $FeCl_3$ - H_2SO_4 reagent to the sides of the test tubes. A brown ring was formed at the interface; tap the bottom of the tubes well to effect mixing and a light colour appeared which changed to an immense purple colour, which was measured in UV spectrophotometer at 560nm.

The amount of total cholesterol was expressed as mg/dl (plasma); mg/g (heart).

3. 5.22. Estimation of triglycerides

The level of triglycerides in plasma and heart were determined by the method of Rice (1970) with slight modifications.

Reagents

1. Activated silicic acid.
2. Saponification reagent: 5.0g of potassium hydroxide was dissolved in 60 ml distilled water and 4.0 ml isopropanol.
3. Sodium metaperiodate reagent: To 77g of anhydrous Sodium metaperiodate in 700 ml distilled water, added 60 ml glacial acetic acid and 650 mg of sodium metaperiodate and was dissolved and diluted to 1 litre with distilled water.
4. Acetyl acetone reagent: To 0.75 ml of acetyl acetone, 20 ml of isopropanol was added and mixed well.
5. Stock solution: 400mg of triolein was dissolved in 100 ml chloroform.
6. Working standard: 1.0 ml of the stock solution was diluted to 10 ml.

Procedure

0.2 ml of the lipid sample was taken into a test tube and evaporated off the chloroform, added 4.0 ml isopropanol. It was mixed well and added 0.4g of activated silicic acid. It was shaken in a vortex mixer for 15 min and centrifuged at 4000rpm for 5 min. To 2.0 ml of the supernatant and standards ranging from 20-100mg made up 2.0 ml with isopropanol, 0.6 ml of saponifying reagent was added and incubated at 60-70^{0}C for 15 min. After cooling, 1.0 ml sodium metaperiodate solution was added and mixed. To this, 5ml acetyl acetone was added, mixed and incubated at 50°C for 30 min. After cooling, the colour was estimated by measuring OD at 405nm in UV spectrophotometer.

The value of triglyceride in plasma was expressed as mg per dl and in tissue as mg per gm.

3. 5.23. Estimation of free fatty acids

Free fatty acids in plasma and heart were estimated by the modified method of Horn & Menahan (1981).

Reagents

1. Activated silicic acid
2. Chloroform, heptane, methanol (CHM) solvent mixture: It was prepared by mixing chloroform, heptane and methanol in the ratio of 200:150:7(v/v)
3. Copper-triethanolamine solution: 50 ml of 0.1M copper nitrate and 50 ml of 2M triethanolamine were mixed with 33 g of sodium chloride. The pH of the solution was adjusted exactly to 8.1.
4. Diethyldithiocarbomate (DDC) solution: 0.1% DDC in butanol was prepared.
5. Standard Stock: A solution containing 2 mg per ml of palmitic acid was prepared in CHM solvent. For working standard, the stock was diluted 1:10 in CHM to give a concentration of 200μg per ml.

Procedure

To 1.0 ml of the lipid sample, 6.0 ml of CHM solvent and 200mg of silicic acid were added. The mixture was shaken well, centrifuged at 4000rpm for 5 min and 3.0 ml of the supernatant taken. Standard solution in the range of 25-100 μg were taken and made up to 3.0 ml with CHM solvent. The blank contained 3.0 ml of CHM solvent. To all these samples, 2.0 ml of copper triethanolamine solution was added and then mixed on a mechanical shaker for 10 min. The tubes were centrifuged at 4000rpm for 5 min. To the 2.0 ml of the supernatant taken, 1.0 ml of DDC solution was added and shaken well. The colour intensity was read immediately at 430nm in spectrophotometer.

Values were expressed as mg/dl plasma and mg/g wet tissue.

3. 5.24. Estimation of phospholipids

Phospholipid content of plasma and heart was estimated by the method of Fiske & Subbarow (1925) as inorganic phosphorus liberated after Bartlette's perchloric acid digestion (1959).

Reagents

1. Ammonium molybdate reagent: 2.5g of ammonium molybdate was dissolved in 100 ml of water.
2. Aminonaptholsulfonic acid (ANSA): 0.5g of 1,2,4 aminonaptholsulfonic acid was dissolved in 195 ml of 15% sodium metabisulfite and 50 ml of 20% sodium sulfite was added for complete solubilisation. The solution was filtered and stored in a brown bottle.
3. Stock standard solution: 351mg of potassium dihydrogen phosphate was accurately weighed, dissolved and made upto 100 ml with double distilled water to give a final concentration of 80 mg phosphorus per ml.
4. Working standard: 1ml of the stock was diluted to 10 ml to give a conc. of 80μg phosphorus per ml.

Procedure

1 ml of the lipid sample was taken into a test tube and evaporated off chloroform. Added 0.5 ml of perchloric acid, the tubes were made up to 3.0 ml with double distilled water, and 1.0 ml of aliquot was taken. The tubes were made up to 4.0 ml with double distilled water. To all the tubes, 0.5 ml of ammonium molybdate reagent was added. After 10 min, added 0.5 ml of ANSA to all tubes. Aliquots of the standards and blank were carried through the same procedure. The blue colour developed was read after 20 min, at 620nm in UV spectrophotometer.

The phospholipid content of plasma was expressed as mg per dl serum and same for liver and heart as mg per gm tissue.

3. 5.25. Lipoprotein fractionation

Addition of heparin-manganous chloride to plasma caused the precipitation of VLDL and LDL. The supernatant represented the HDL fraction. in another aliquot of plasma, addition of sodium dodecyl sulphate resulted in aggregation of VLDL. The cholesterol content of each fraction was carried out in the following manner.

Total plasma cholesterol – (HDL+LDL) cholesterol = VLDL cholesterol

(HDL+LDL)- HDL = LDL

3. 5.26. Estimation of High density lipoprotein fraction

Total HDL was separated by the method of Burstein and Scholnick (1972).

Reagents

1. Heparin-Manganous chloride reagent: 3.167gm of manganous chloride was added to 1.0 ml of heparin containing 20,000 units/ml. This was made up to 8.0 ml with water.

Procedure

2.0 ml of plasma was added to 0.09 ml of heparin-manganous chloride reagent and mixed well. The solution was allowed to stand at 4°C for 30 min. The supernatant represented HDL fraction. Aliquots were taken from HDL fraction for the estimation of cholesterol.

3. 5.27. Estimation of low density lipoproteins

This differential analysis was made by the method of Brustein and Scholink (1972) using sodium dodecyl sulphate.

Reagent

Sodium dodecyl sulphate: 10% in 0.15M sodium chloride pH 9.0

Procedure

To 1.0 ml of plasma, 0.75 ml of sodium dodecyl sulphate solution was added, which was taken in a poly carbonate centrifuge tube. The contents were swirled briefly and packed for 2 hrs in a water bath at 35°C. The contents were centrifuged in a refrigerated centrifuge at 10,000g for 30 min. VLDL got aggregated as a pellicle at the top. The supernatant was a mixture containing HDL and LDL cholesterol was estimated in 0.05 ml aliquot of the supernatant as described above.

3. 5.28. Estimation of inorganic phosphorus

Inorganic Phosphorus was estimated by the method of Fiske & Subbarow (1925). The method is based on the formation of phosho-molybdic acid by the reaction between a phosphate and molybdic acid and its subsequent reduction to a dark blue phosphomolybdic acid, the intensity of which is proportional to the phosphate ion concentration.

Reagents

1. Ammonium molybdate reagent: 2.5g of ammonium molybdate was dissolved in 100 ml of 3N sulphuric acid.
2. Amino naphthol sulphonic acid (ANSA): 0.5g of ANSA was dissolved in 195 ml of 15% sodium metabisulphite and 5.0 ml of 20% sodium sulphate was added for complete solubilization. The solution was filtered and stored in a brown bottle.
3. Standard Phosphorus: 35.1mg of potassium dihydrogen phosphate was accurately weighed, dissolved and made up to 100 ml with distilled water.

Procedure

To suitable aliquots of the supernatant, 1.0 ml of ammonium molybdate reagent was added 0.4 ml of ANSA was added after 10 min incubation at room temperature, standards and blank were also treated in the above manner. The blue colour developed was read after 20 min at 640nm in UV Spectrophotometer.

The values were expressed as μg per mg protein.

3. 5.29. Determination of biogenic amines

The biogenic amines content in fish was determined in HPLC by pre-column derivatisation with benzoyl chloride as described by Redmond and Tseng (1979) with modification in gradient elution system as per the method of Ozogul *et al.*, (2002) using acetonitrile and water. The gradient system and the flow rate were modified depending on the retention time of the standard amine solution to get good resolution within a short time.

Preparation of standard amines solution

The standard amine solution was prepared so as to give a 10mg free base each amine per ml. For this the following amines were dissolved in 10ml HPLC grade Millipore water.

Histamine dihydrochloride	-165.7 mg
Putrescine dihydrochloride	-182.9 mg
Cadaverine (free base)	-10 mg
Agmatine sulphate	-175.2 mg
Tyramine hydrochloride	-126.7 mg
Spermine tetra hydrochloride	-172.0 mg
Spermidine trihydrochloride	-175.3 mg

Derivatisation of standard amine solution with benzoyl chloride

The benzoyl derivative of the Biogenic amines were done by following the Schotten- Baumann benzoylation reaction under alkaline condition as described by Redmond & Tseng (1979) but with little modification of the sample. For enhancing the reaction of amines, 2% benzoyl chloride in acetonitrile was prepared. (Ozogul *et al*, 2002).

For Derivatisation of standard amine solution, 50 microlitre of standard amine solution (10mg/ml) was added with 1ml of 2M NaOH followed by addition of 1ml of 2% benzoyl chloride (in acetonitrile). Then it was mixed thoroughly in a vortex mixture for 1 minute. The reaction mixture was left at room temperature (25°C) for 30 minutes to complete the benzoylation reaction. The reaction was stopped by the addition of 2ml of saturated NaCl solution. After that it was extracted with 4ml of di-ethyl ether by centrifugation at 3000rpm for 5 minutes.

Thereafter the upper organic layer was transferred to a clean tube and evaporated to dryness in a stream of nitrogen. The residue was dissolved in 0.5ml of acetonitrile and 20 µl aliquots were injected for HPLC analysis.

Preparation of sample (Yen & Hseih, 1991)

10 g of the silage was homogenized with 6% TCA and filtered through Whatman No.1 filter paper. The filtrate was made up to 100 ml with TCA. 2ml of this extract was derivatised with benzoyl chloride by the same procedure as described in derivatisation of standard amine solution.

Chromatographic condition

Chromatographic separation was done by continuous gradient elution with acetonitrile (solvent A) and HPLC grade Millipore water (solvent B) as described in Ozogul *et al* (2002). A gradient started with 50% acetonitrile and increased to 80% in the 6^{th} minute. The pressure was maintained between 48-52 Pascal throughout the separation period. Total separation of seven amines was completed within 9 minutes. Detection was done using Photo Diode Array (PDA) detector.

For calibration curve, 5 standard concentrations of amines mixtures were prepared and injected in a series comprising 10mg/ml, 5mg/ml, 1mg/ml, 0.1mg/ml and 0.01mg/ml standard concentration. The standard curve was prepared corresponding to Hitachi-Merck HPLC System M Anager Software. 20 µl sample was injected for analysis.

3. 5.30 Carbohydrate (Cleg, 1956)

About 1g of silage was weighed in a test tube to which 10ml 1N HCl was added. This was hydrolysed for 30minutes in a water condenser. It was neutralized with alkali to neutral pH. This was filtered through Whatman No.1 filter paper and made upto 50ml with distilled water. 1.5ml of the made up sample was mixed with 3ml Anthrone's reagent (200mg% in 95% H_2SO4). This was thoroughly boiled for 3 minutes, cooled and read at 620nm in a spectrophotometer.

Preparation of standard

100 mg glucose was dissolved in 100 ml distilled water to get 1mg/ml standard solution. 0.1 ml of this standard was diluted to 10ml, so as to get standard of 0.01mg/ml concentration. 0.5, 0.75, 1.0, 1.25 and 1.5 ml of the standard solution was mixed with 3 ml Anthrone's reagent. This was vortexed thoroughly and boiled for about 3 minutes, cooled and read at 620nm in a spectrophotometer.

3. 5.31. Bacteriological estimation and identification

For quantitative methods of microbiological analysis USFDA (2001) methodology was followed for Total plate Count, *Lactobacillus plantarum E. coli Staphylococcus and Salmonella* . For qualitative estimation, the colonies were isolated from TGA, identified as per Bergy's Manual (2001) and identified as *Bacillus sp.* (Surendran *et. al.*, 2003).

Lactic Acid Bacteria **(LAB)** identified in MRS agar plates as catalase negative , gram positive, opaque, white colonies were counted as lactic acid bacteria.

3.6. Statistical Analysis

Results expressed as mean or mean log ± SD for biochemical & microbiological parameters. Statistical analysis between the means using ANOVA and Dunkens's multiple test were carried out to test the significance of variance. Statistical package used in the study is SPSS, 10.

Results and Discussion....

4.0. Results and Discussion

Fish waste can be advantageously upgraded into animal feed by fermentation with lactic acid bacteria along with some locally available cheap source of carbohydrate. This procedure is safe, economically advantageous and environment friendly. Silage is a promising product of many industrial applications. Processing of fish in organized industries and stray dressing from markets in India produce about 300000 metric tons of waste (Ahmed & Mahendrakar, 1995). In addition, other wastes from unorganized sectors like discards from fishing vessels constitute the potential raw material for fish silage.

4.1 Studies on the Biochemical Alterations in the Fermented Silage Prepared from whole tilapia

4.1.1 Proximate composition

Proximate composition of homogenised whole tilapia after adding jaggery at different levels is given in Table 4.1.1. The moisture content was found to have a slight decreasing trend with increasing jaggery levels, which might be due to the jaggery added. Consequently the levels of protein and fat were found to decrease with the increase in the quantity of added jaggery. The slight increase in ash noticed could be from the jaggery added. Maria. *et. al* (1998) reported 14% and 10% of crude protein levels for silage prepared by adding molasses at 10% level and 20% levels respectively. Yeoh (1979) observed that the crude protein content of biological fish silage was not very much less than fishmeal.

4.1.2 pH

Table 4.1.2 depicts alterations in pH during ensilation of whole tilapia using different levels of jaggery. It is noticed that the pH reduced to near 4.2 on 4^{th} day

in all the 4 cases from the initial value of 6.97 to 7.48. The sample with 5% jaggery showed an increase in pH after 4th day. Ahmed and Mahendrakar (1996) also reported almost the same results during fermentation of fresh water fish viscera. In silage prepared with 5% jaggery the pH decreased to 4.7 on fourth day and increased thereafter. This could be due to the depletion of jaggery and consequently the pH increased and the samples were spoiled. The fermented fish silage prepared by Kompiang *et. al* (1979) with 5% molasses had a pH of 5 after three days and got spoiled after 10 days. The samples with 10% and 15% jaggery, the pH reached 3.97 and 4.16 respectively on 10th day and then decreased slowly. The sample with 20% jaggery, the pH showed a decease through out the study. During ensilation the jaggery was fermented by the bacteria and resulted in the production of lactic acid. As a result, the pH of the samples decreased from the initial value. The rate of decrease was faster during the first 2 days and then the change was slow. In silage samples prepared with 10% jaggery, the increase in pH after 10th day might probably due to the decrease in the level of jaggery, reduction in the rate of bacteria and neutralization of lactic acid with the basic nitrogenous compounds produced. The same trend was noticed in samples with 15% jaggery. But in case of silage with 20% jaggery the pH showed the decreasing trend throughout the experiment which could be due the presence of excess of sugar. It is observed that the addition of jaggery at higher concentrations viz., with 15% and 20%, does not have any significant effect on the pH change. Neethiselvan *et. al.* (2001) prepared fermented fish silage using fermented cabbage and curd as the inoculum and the pH of the silage reached 4.6 and 4.9 respectively

4.1.3 Titrable acidity

Changes in titrable acidity during ensilation of whole tilapia at different levels of jaggery is given in Table 4.1.3. The titrable acidity expressed as percentage lactic acid showed an increasing trend in all the samples upto 4 days. The acidity reduced in on further storage in the sample with 5% jaggery. In all the

other samples the acidity continued to increase upto the 10^{th} day. In the sample with 20% jaggery the acidity continued to increase on further storage. The changes pH showed agreement with the changes in acidity. A clear negative correlation between the lactic acid formation and sugar utilization was observed during fish silage fermentation using three different types of starter culture by Neethiselvan *et.al* (2002). Adams *et.al.* (1989) demonstrated the inverse relation between pH and titrable acidity using 4% glucose and 1% salt in fermenting minced whiting.

4.1.4 Carbohydrate

Table 4.1.4 gives the changes in carbohydrate levels during fermentation of minced whole tilapia with jaggery. At 5% level the jaggery was fully utilized by 4 days without producing sufficient acid needed for ensilation. The pH attained was not sufficient to prevent spoilage. It was noticed that the samples got spoiled with a resultant increase in pH. At 10% level the carbohydrate was almost completely utilized during the period of ensilation of thirteen days. But in the case of samples with 15% and 20% jaggery, the carbohydrate was not fully utilized leaving about 3% and 6% respectively after a period of 13 days. Different workers tried the production of fermented silage using molasses, cereal meal, sugar cane, sucrose, tapioca meal and ragi as source of carbohydrate and obtained satisfactory results (Ali. *et. al.,* 1985, Areche *et. al*, 1992.; Nilsson & Rydin 1963, Nilsson, 1970). But LAB cannot utilize complex carbohydrates like starch or cereal meals (Hall, 2002). Zahar *et. al.,* (2002) tried natural fermentation of sardine and sardine waste in sugar cane molasses at 60:40 ratio without inoculating any bacterial culture with satisfactory results. A similar result was obtained when waste from several species were mixed with 50% molasses (IAV, 1994). But Kompiang *et. al* (1980) successfully fermented fish with molasses at the ratio 80:20.

4.1.5. Degree of hydrolysis (DH)

The progress of hydrolysis (DH) of silage prepared from whole fish with different concentrations of jaggery is given in Table 4.1.5. Degree of hydrolysis indicates the breakdown of protein due to the action of acid. It is proportional to the acid produced during ensilation. Even though at 5% level of jaggery the degree of hydrolysis was progressing initially, but due to the depletion of sugar, the lactic acid concentration was not sufficient to prevent spoilage. Hence the sample got spoiled. Lactic acid bacteria ferment the sugar present to organic acid mostly lactic acid, thus lowering the pH. When the pH falls sufficiently low (4.5) growth of putrefactive organisms and pathogens is inhibited. Kompiang *et. al* (1979), observed that fermented fish silage prepared with 5% molasses was stable only for 7 days and got spoiled afterwards indicating insufficient production of acid.. But at 10% jaggery, the DH progressed faster initially and slowed down after 4 days. This could be due to the decreased activity of microbes and depletion of sugar. At 15%, DH was almost same as that at 10% and at 20% the trend was almost similar except an increased hydrolysis throughout the period. Increase of non protein nitrogen was observed when pilchard waste was fermented with 25% molasses throughout for the period of 11 days by Faid *et. al* (1997). The degree of hydrolysis of protein was 55 to 60% in samples with 10, 15 and 20% jaggery. It is noticed that 60% hydrolysis took place only in sample with 20% jaggery, which is due to the increased production of acid. A similar observation was made in blue whiting ensilage by Maria *et. al* (1998). Since the DH of the three sets of samples were almost same, the levels of jaggery could be limited to 10%.

4.1.6. α- amino nitrogen (α-AN)

Table 4.1.6 shows the changes in α-amino nitrogen content during silage production with homogenized whole tilapia having different levels of jaggery. α-amino nitrogen gives an indication of the extent of hydrolysis of the silage produced and of the total free amino acids (Finne, 1992) During hydrolysis, the

polypeptide chains are broken down into simple peptides and amino acids . In all the samples the α-AN showed a steady increase. The production of αAN was faster at 5% level of jaggery. At higher jaggery concentration, viz., 10, 15, and 20%, the rate of α-AN production was slower and showed almost the same trend. According to Ahamed & Mahendrakar (1996) the α-AN value increased to 18% from the initial value of 12 % of the TN in a period of 4 days of ensilation of fresh water fish viscera. The α-AN value stabilized near 140 mg/100 g at higher jaggery levels. Mathew & Nair (2006) reported a lower value of α-AN when shrimp waste was ensiled with *L. fermentum*. But Babu *et.al* (2005) observed higher values of α-AN above 20 % of TN in both acid and fermented fish silage.

4.1.7. Total volatile base nitrogen (TVBN)

Table 4.1.7. shows the changes in total volatile base nitrogen during ensilation of whole tilapia with different levels of jaggery. The total volatile base nitrogen content of a product is a measure of all volatile amines and ammonia which might have resulted from nucleotide breakdown or by the action of proteolytic enzymes (Jones *et.al.*, 1964). The TVBN content increased to 35.7 mg/100g on 7^{th} day indicating spoilage in the sample with 5% jaggery. This could be due to the depletion of sugar and consequent multiplication of spoilage bacteria. But in the case of other three samples, the TVBN levels were low at the end of fermentation period. The values increased to 14 mg/ 100g only indicating the prevention of the growth of spoilage bacteria. The formation of higher content of TVBN may lead to a reduction in the content of amino acids that could have negative impact on the nutritive value of silage (Maria *et al.*, 1998). Faid *et al.*, (1997) reported that TVBN pattern showed slight increase during fermentation and observed an increase in TVBN from 71.26 mg/100g to reach 95.03 mg/100g after 1 day and remained constant at 132 mg/100g after 15 days of fermentation of uncooked samples at 22°C using both lactobacillus and Sacharomyces sp. Durairaj *et. al.*, (1985) observed that the TVBN content of the silage prepared from silver belly showed a steady increase during storage for

one year. Maria (1998) observed that the TVBN content of fermented fish silage was much lower compared to acid silage. According to Ahamed and Mahendrakar (1996) during fermentation of fresh water fish viscera, the TVBN values increased to 8% of the total nitrogen from the initial 1.3%. According to Neethiselvan *et. al.*, (2001), the TVBN values increased with the increase in storage period and ranged between 28 and 98 mg% when lactobacillus was used for fermentation, whereas the same was between 35 and 155mg% when curd was used as starter culture. According to Yin *et.al.*, (2002), fermentation with *Lactobacillus plantarum CCRC 10096*, *Lactococcus lactis subs. Lactis CCRC 12315*, *Lactobacillus helveticus CCRC 14092*, and their combination could substantially inhibit the development of volatile bases, suppress the growth of main micro flora, hydrolyze the muscle proteins and accumulate free amino acids during fermentation.

4.1.8. Peroxide value (PV)

Changes in peroxide value during ensilation of whole tilapia at different levels of jaggery are given in Table 4.1.8. The levels of PV showed an increasing trend for 7 days in all the samples and showed a decrease afterwards in samples with 10 and 20% jaggery. The decrease in peroxide value observed during ensilation and storage (Fagbenro & Jauncey 1994) reflects the degradation of part of the hydro peroxides to form secondary breakdown products such as aldehydes (Lundberg & Jarvi, 1968). Ahamed & Mahendrakar (1996) observed an increase in the PV for the initial 4 days and then decreased to low levels due to breakdown. They also observed that oil was effectively trapped in microbial silage because of the high binding ability of the added polysaccharides. According to Tibbets *et. al* (1987) slow autolysis and fermentation processes seem to stabilize the oil. The low levels of peroxide value could also be due to the antioxidant effect of the released free amino acids (Castell *et.al.* 1966). But in case of sample with 5% jaggery the PV showed increasing trend up to 7 days and the samples were found spoiled by that time.

4.1.9 Free fatty acids (FFA)

Table 4.1.9 depicts the hydrolysed lipid and lactic acid formed during ensilation of whole tilapia at different levels of jaggery. The free fatty acids are derived from phospholipids by hydrolysis. In lactic acid fermentation, the lactic acid formed also contributes to the free fatty acid content of the product. At 5% levels of jaggery, the FFA content showed an increasing trend during ensilation. In all other cases a steady rise in FFA value was noticed contributed mainly by lactic acid as indicated by the increasing titrable acidity. Fagbenro & Jauncey (1994) have observed an increase in the levels of FFA during the production and subsequent storage of tilapia silage in the control samples as well as samples added with antioxidants. According to Ahamed and Mahendrakar (1996) the time elapsed for processing resulted in initial high FFA value, which on ensilation increased further for 4 days. They have also observed a steadily increasing level of lactic acid during fermentation of fish viscera. Increase in FFA was observed by Tatterson & Windsor (1974) in fish offal silage during 40 days of fermentation.

4.1.10. Thiobarbituric acid value (TBA)

Alterations in TBA during ensilation of whole Tilapia at different levels of jaggery is given in Table 4.1.10. The TBA value showed a sharp increase in samples with 5% jaggery whereas in all other samples the value increased slowly for first 7 days thereafter the values shot up in all the samples. Ahamed and Mahendrakar (1996) observed a linier increase in TBA value during fermentation of fish viscera and reported a TBA value of 1.3. In the present study, fermentation for a period of 7 days showed TBA value of nearly 1.2 in all the samples except in case of samples containing 5% jaggery. The higher TBA value noted in this case could be due to the increasing numbers of spoilage bacteria and suppression of lactobacillus count. The TBA values showed a sudden increase in all the three types of samples afterwards. Scavenging of

malonaldehyde by different strains of *Lactobacillus sp.* was proved by Lin & Yen (1999). According to Fagbenro & Jauncey (1994) the TBA value increased in raw silage during fermentation and storage, while the same decreased in silage added with ethoxyquin and onion extract in 180 days of storage. They have observed that the lipid stability was improved during ensilation by the addition of onion extract by lowering the TBA value. The sharp increase after 7 days indicates that, there could be lowering of lactobacillus count after 7th day of ensilation.

4.1.11. Fatty acids

The changes in fatty acid composition of whole tilapia silage during fermentation is given in Table 4.1.11. The saturated fatty acid content of whole tilapia was found to be higher during the initial periods of fermentation. A declining trend in saturated fatty acid of whole tilapia silage was noted as fermentation progressed, from the initial value of 35 % to 19 % after 13 days. This could be due to the action of the microbial flora of the product. The levels of mono unsaturated fatty acid remained almost same throughout the period of fermentation. But the quantity of PUFA showed an initial decrease for 2 days and it increased on subsequent days to reach 11%.

Table. 4.1.1 Proximate composition of minced whole tilapia after adding jaggery at different levels.

	Level of jaggery			
	5 %	10 %	15 %	20%
Moisture (%)	80±0.8^{d}	79.07±0.5^{c}	78.17±0.8^{b}	77.6±0.4^{a}
Protein (%)	13.67±0.4^{d}	12.4±0.7^{c}	11.8±0.3^{b}	10.57±0.4^{a}
Fat (%)	1.7±0.2^{a}	1.49±01	1.42±0.2	1.2±0.1
Ash (%)	1.66±0.1^{a}	2.3±0.1	2.41±0.2	2.45±0.1
Carbohydrate (%)	3.21± 0.28^{a}	4.9±0.15^{b}	6.01±0.5^{c}	7.31±0.62^{d}

Results are presented as mean ± standard deviation (SD) of 3 replications. ^{a,b,c,d,e}, mean in a row with the same superscript values are not significantly different ($P<0.05$).

Table 4.1.2. Changes in pH during ensilation of whole tilapia with different levels of jaggery.

Days	Level of jaggery			
	5 %	10 %	15 %	20%
1	6.97 ± .06	6.9 ± .03	7.0 ± .15	7.48 ± .16^{a}
2	5.1 ± .10	5.23 ± .04	5.1± .1	5.07 ± .05
4	4.2 ± .10	4.21 ± .03	4.2 ± .1	4.26 ± .15
7	4.9 ± .05	4.02 ± .04	4.1 ± .05	3.93 ± .10^{a}
10	5.6 ± .05^{c}	3.97 ± .06^{bc}	3.91 ± .05^{b}	3. 83 ± .02^{a}
13	---	4.12 ± .01^{b}	4.06 ± .15^{c}	3. 76± .05^{a}

Results are presented as mean ± standard deviation (SD) of 3 replications ^{a,b,c,d,e}, mean in a row with the same superscript values are not significantly different ($P<0.05$).

Table. 4.1.3.Changes in Titrable acidity (% lactic acid) during ensilation of whole tilapia with different levels of jaggery.

Days	Level of jaggery			
	5 %	10 %	15 %	20%
2	$1.82 \pm .02^{b}$	$1.64 \pm .04^{a}$	$1.81 \pm .05^{b}$	$1.86 \pm .02^{c}$
4	$3.71 \pm .07^{a}$	$3.25 \pm .04^{a}$	$3.48 \pm .12^{b}$	$3.70 \pm .01^{c}$
7	$3.23 \pm .20^{a}$	$3.49 \pm .06^{b}$	$3.90 \pm .01^{c}$	$4.12 \pm .2^{d}$
10	---	$4.21 \pm .03$	$4.13 \pm .11$	$4.26 \pm .11$
13	---	$4.23 \pm .15^{ac}$	$4.16 \pm .15^{b}$	$4.38 \pm .05^{c}$

Results are presented as mean ± standard deviation (SD) of 3 replications. ^{a,b,c,d,e}, mean in a row with the same superscript values are not significantly different ($P<0.05$).

4.1.4 Changes in residual carbohydrate level (%) during ensilation of whole tilapia with different levels of jaggery.

Days	Level of jaggery			
	5 %	10 %	15 %	20%
1	$3.67 \pm .20^{a}$	$6.53 \pm .25^{b}$	$12.03 \pm .25^{c}$	$13.86 \pm .36^{d}$
2	$1.33 \pm .11^{a}$	$4.80 \pm .26^{b}$	$9.30 \pm .17^{c}$	$9.54 \pm .25^{d}$
4	$0.32 \pm .03^{a}$	$2.22 \pm .01^{b}$	$8.56 \pm .11^{c}$	$8.13 \pm .21^{d}$
7	ND	$1.07 \pm .11^{a}$	$6.46 \pm .05^{b}$	$7.99 \pm .03^{c}$
10	---	$0.86 \pm .03^{a}$	$4.56 \pm .05^{b}$	$7.66 \pm .32^{c}$
13	---	$.67 \pm .06^{a}$	$3.23 \pm .20^{b}$	$6.25 \pm .08^{c}$

Results are presented as mean ± standard deviation (SD) of 3 replications. ^{a,b,c,d,e}, mean in a row with the same superscript values are not significantly different ($P<0.05$).

Table 4.1.5. Changes in the degree of hydrolysis of protein during ensilation of whole tilapia with different levels of jaggery.

Days	Level of jaggery			
	5 %	10 %	15 %	20%
1	14.93±0.51	15.40±0.12	17.20±0.7	18.46±0.4
2	32.01±0.23^{a}	33.52±0.23 ^{b}	36.47±1.4^{c}	43.39±0.8^{d}
4	37.38±0.51^{a}	50.15±1.1^{b}	48.53±0.9^{c}	55.71±1.1^{d}
7	44.86±0.21	53.681±0.9	55.44±1.4	60.84±1.8
10	---	55.92±1.7^{a}	56.28±0.8^{ab}	60.02±1.5^{b}
13	---	55.81±2.4	57.27±1.4	59.69±2.1

Results are presented as mean ± standard deviation (SD) of 3 replications. ^{a,b,c,d,e}, mean in a row with the same superscript values are not significantly different (P<0.05).

4.1.6. Changes in AAN (mg /100g) during ensilation of whole tilapia with different levels of jaggery.

Days	Level of jaggery			
	5 %	10 %	15 %	20%
1	84.0 ± 1.0^{ab}	78.3 ± .25^{ab}	70.33 ±1.5^{a}	93.00 ± 1.7^{b}
2	97.33 ± 1.1^{a}	89.2 ±.67 ^{b}	79.33 ± 1.5^{c}	116.00 ±1.3^{d}
4	110.3 ±1.5^{a}	98.50 ± .26^{b}	84.33 ± 1.1^{c}	125.00 ± 1.7^{d}
7	135.3 ± .57^{a}	121.4 ± .23^{b}	104.33 ± 0 .5^{c}	137.00 ±1.7^{d}
10	----	134.80 ± .45	120.33 ± 1.5	136.0 ±2.0
13	----	141.06 ± .26^{a}	137.6 ± 1.5^{b}	139.0 ±1.0^{c}

Results are presented as mean ± standard deviation (SD) of 3 replications. ^{a,b,c,d,e}, mean in a row with the same superscript values are not significantly different (P<0.05).

4.1.7. Changes in TVBN (mg/100g) during ensilation of whole tilapia with different levels of jaggery.

Days	Level of jaggery			
	5 %	10 %	15 %	20%
1	9.53 ± 0.05^{a}	9.66 ± 0.26^{ad}	7.73 ± 0.11^{c}	9.8 ± 0.15^{d}
2	14.35 ± 0.12^{a}	10.53 ± 0.32^{b}	11.33 ± 0.15^{c}	10.12 ± 0.52^{d}
4	20.36 ± 0.11^{a}	10.60 ± 0.1^{b}	11.76 ± 0.05^{d}	11.36 ± 0.30^{c}
7	35.74 ± 0.48^{a}	11.63 ± 0.12^{b}	12.16 ± 0.05^{c}	13.2 ± 0.24^{d}
10	----	12.60 ± 0.23^{a}	13.56 ± 0.15^{b}	14.78 ± 0.23^{c}
13	----	13.26 ± 0.15^{a}	13.63 ± 0.05^{b}	14.98 ± 0.37^{c}

Results are presented as mean ± standard deviation (SD) of 3 replications. ^{a,b,c,d,e}, mean in a row with the same superscript values are not significantly different ($P<0.05$).

Table. 4.1.8. Changes in PV (meq O_2/Kg fat) during ensilation of whole tilapia with different levels of jaggery.

Days	Level of jaggery			
	5 %	10 %	15 %	20%
1	8.23 ± 0.05^{a}	7.46 ± 2.0^{b}	$6.86 \pm .08^{c}$	8.4 ± 0.11^{a}
2	12.20 ± 0.10^{a}	8.40 ± 1.0^{b}	9.20 ± 0.1^{c}	10.66 ± 0.23^{d}
4	15.96 ± 0.20^{a}	8.53 ± 6.3^{b}	13.3 ± 0.15^{c}	16.43 ± 0.11^{d}
7	18.06 ± 0.25^{a}	12.26 ± 5.5^{b}	16.4 ± 0.11^{c}	17.18 ± 0.12^{d}
10	----	11.9 ± 1.0^{a}	17.3 ± 0.15^{c}	14.40 ± 0.34^{b}
13	----	10.26 ± 2.5^{a}	15.4 ± 0.05^{c}	11.46 ± 0.15^{b}

Results are presented as mean ± standard deviation (SD) of 3 replications ^{a,b,c,d,e}, mean in a row with the same superscript values are not significantly different ($P<0.05$).

Table. 4.1.9. Changes in FFA (% oleic acid) during ensilation of whole tilapia with different levels of jaggery.

Days	Level of jaggery			
	5 %	10 %	15 %	20%
1	2.76 ± 0.15	2.59 ± 0.25	2.32 ± 0.01	2.57 ± 0.05
2	6.43 ± 0.20^{a}	8.73 ± 0.47^{b}	8.46 ± 0.05^{b}	8.07 ± 0.14^{b}
4	9.16 ± 0.05^{a}	11.93 ± 0.45^{b}	11.10 ± 0.01^{b}	12.43 ± 0.05^{c}
7	11.46 ± 0.05^{a}	14.60 ± 0.34^{b}	15.63 ± 0.05^{c}	18.40 ± 0.17^{d}
10	----	18.10 ± 0.26^{a}	19.70 ± 0.20^{b}	22.66 ± 0.15^{c}
13	----	17.10 ± 0.43^{a}	25.05 ± 0.04^{b}	28.50 ± 0.51^{c}

Results are presented as mean ± standard deviation (SD) of 3 replications. $^{a.b.c.d.e}$, mean in a row with the same superscript values are not significantly different ($P<0.05$).

Table 4.1.10. Changes in TBA during ensilation of whole tilapia with different levels of jaggery.

Days	Level of jaggery			
	5 %	10 %	15 %	20%
1	0.61 ± .02^{a}	0.78 ± .03^{b}	0.64 ± .01^{ab}	0.68 ±.01^{ab}
2	0.75 ±.05^{a}	0.93 ± 0.06^{b}	0.78 ± .005^{a}	0.84 ±.03^{ab}
4	0.87 ± .05^{a}	1.09 ± .03^{b}	0.92 ± .005^{a}	1.06 ± .01^{b}
7	1.46 ± .03^{c}	1.29 ± .02^{b}	1.22 ± .01^{a}	1.23 ± .005^{b}
10	----	2.03 ± .02^{c}	1.54 ± .02^{a}	1.86 ± .30^{b}
13	----	2.10 ± .12^{b}	1.95 ± .05^{a}	1.90 ± .05^{a}

Results are presented as mean ± standard deviation (SD) of 3 replications $^{a.b.c.d.e}$, mean in a row with the same superscript values are not significantly different ($P<0.05$).

Table 4.1.11 Changes (%) in fatty acid composition of whole tilapia silage during fermentation with different levels of jaggery.

Days	1	2	4	10	13
Saturated fatty acids	35.03	33.77	28.12	29.68	26.25
Mono unsaturated fatty acids	32.58	35.66	25.74	33.71	34.74
Poly unsaturated fatty acids	9.69	8.03	9.03	10.50	11.19
Others	22.15	21.54	33.36	27.15	26.87

4.2. Changes in the Characteristics of Fermented Silage from Dressed Tilapia.

4.2.1. Proximate composition

Table 4.2.1. shows the proximate composition of dressed tilapia at different levels of jaggery is given in Table. 4.2.1. The levels of protein in all the samples were found to be higher when compared to whole fish silage. The dressed fish silage was having low levels of fat, which could be due to the removal of internal parts, which contain depot fat. Due to removal of scales the ash content was also found to be less and consequently the protein content of dressed fish homogenized with jaggery was found to be higher than that of whole fish. At higher levels jaggery a proportionate increase in ash content observed.

4.2.2. pH

Table 4.2.2 depicts the alteration in pH during ensilation of dressed tilapia at different levels of jaggery. As in the case of whole fish silage, the pH values decreased in all the samples during the period of ensilation except that of 5% jaggery sample which showed a higher pH value after four days. This could be due to depletion of jaggery as in the other case. The decrease in pH in the case of dressed fish was marginally higher compared to the whole fish with the same level of added jaggery. Due to the presence of higher levels of sugar in samples containing 20% jaggery, the pH value was very less in both whole and dressed samples indicating a higher stability of the samples. According to Petaja *et.al.*, (2000) pH of the LAB fermented cold-smoked fish products were reduced from 6.4 to 5.0-5.3 during fermentation with lactobacillus for a period of 7 days.

4.2.3. Titrable acidity

Changes in titrable acidity (as % lactic acid) during ensilation of dressed tilapia at different levels of jaggery are given Table 4.2.3. In dressed tilapia the

titrable acidity showed increasing trend in all the samples throughout the period of fermentation. As observed in case of pH the increase titrable acidity in dressed fish showed a marginally low value compared to whole fish. The change was significant in sample containing 20% jaggery during the final days of fermentation.

4.2.4. Carbohydrate

Alterations in carbohydrate levels during ensilation of dressed tilapia are given in Table 4.2.4. In case of dressed fish also the pattern of sugar utilization was of the similar in nature as that of whole fish. But in the case of whole fish samples, the sugar level was found to be less indicating more utilization. In case of whole fish the soft tissues and water-soluble proteins might have utilized by the microbes lending to increased utilization or the cathepsins of muscle on cooking might have resulted in rapid hydrolysis. In case of dressed fish also unused sugar was present after a period of 13 days of ensilation.

4.2.5. Degree of hydrolysis (DH)

Table 4.2.5.1 depicts the changes in NPN and Table 4.2.5.2. depicts degree of hydrolysis during ensilation of dressed tilapia at different levels of jaggery. In case of dressed fish also the DH was found to be increasing during the course of fermentation. The hydrolysis was faster during the initial 4 days and after 7 days the hydrolysis was slow up to 13 days which could be due to the presence of complex proteins difficult to break. But compared to whole fish silage, the DH was less in all levels of jaggery. The final observation of less hydrolysis could be due to utilization of hydrolyzed protein by the microbes on depletion of sugar. Backhoff (1976) has reported a greater value of NPN (83.5%) in cod viscera compared to fish flesh. Significant difference was noted between all the samples during the entire period of fermentation, which could be due to difference in the

concentration of sugar in the medium. Degree of hydrolysis was found to be higher in sample containing 20% jaggery.

4.2.6. Alpha amino nitrogen (AAN)

Alterations in AAN during ensilation of dressed Tilapia at different levels of jaggery are given in Table 4.2.6. In the case of dressed fish there was significant difference in the levels of AAN during ensilation. At all levels of jaggery, the AAN values increased throughout the period of fermentation.

4.2.7. Total Volatile Base Nitrogen

Changes in TVBN during ensilation of dressed Tilapia at different levels of jaggery are given Table 4.2.7. The change in levels of TVBN in dressed tilapia was similar to whole tilapia silage samples. In 5% level jaggery samples, there was sharp increase indicating the presence of spoilage organisms due to depletion of sugar. But in other samples, the change was very less and after 7 days the values stabilized at 20% level jaggery. The TVBN values in all cases were well below 35ppt. The slight increase in TVBN in dressed fish silage indicate the presence of proteolytic microorganisms and consequent breakdown of nitrogen as indicated by the changes in titrable acidity and pH.

4.2.8. Peroxide Value

Changes in PV during ensilation of dressed tilapia at different levels of jaggery are given in Table 4.2.8. In dressed fish silage the PV values showed an increasing trend till 7 days and showed a decreasing trend which could be due to further breakdown. A comparatively low levels of PV observed during ensilation could be due to removal of unsaturated fatty acids which might be present in internal organs while dressing of the fish. The observed value of below 10 meq O_2 /Kg fat is below the specified limit for edible fishery products.

4.2.9. Free Fatty Acids

Changes in FFA during ensilation of dressed Tilapia at different levels of jaggery are given in Table 4.2.9. In all samples the FFA values increased due to

breakdown of lipid and due to accumulation of lactic acid formed. At higher levels of jaggery added, a proportionately higher FFA values were observed, which could be due to the accumulation of lactic acid.

4.2.10. Thiobarbituric Acid value

Changes in TBA during ensilation of dressed Tilapia at different levels of jaggery are given in Table 4.2.10. The TBA values in the case of dressed fish showed increasing trend during ensilation. But the significantly lower levels of TBA values in the samples could be due to lower levels of unsaturated fatty acids in dressed fish. In the case of samples containing 10% jaggery the TBA values were significantly lower which could be due to the protective effect of lactobacillus in other samples in which the microbes are present in higher levels.

4.2.11. Amino acids

The changes in amino acid profile of dressed tilapia during fermentation are given in Table 4.2.11. The result showed that there was no significant change in the profile of amino acid during the course of fermentation. The important amino acids present in tilapia silage are found to be Aspartate glutamate, glycine alanine and leucine in higher concentration and other amino acids in lower concentration. The results are in agreement with those reported by Luiz *et.al* (2006). According to them the amino acid profile of fermented tilapia filleting waste had different amino acid particularly, lysine, valine, glycine, aspartica acid alanine, and glutamine in higher concentration. Fermentation is found to have only marginal effects on amino acid composition of fermented fish (Vanbelle *et.al.*, 1982).

4.2.12. Fatty acid profile

The changes in fatty acid composition of dressed tilapia silage during fermentation are given in Table 4.2.12. The saturated fatty acids were found to

be higher in dressed fish when compared to whole fish. As in the case of whole fish silage, there was a reduction in the saturated fatty acid during fermentation process. Since the levels of saturated fatty acids are found to be higher in dressed fish, it indicates that, the body oil of tilapia contains more saturated and the fat attached to visceral parts contains more of unsaturated fatty acids. This is evident from the fact that, the mono unsaturated fatty acids and poly unsaturated fatty acids of dressed fish are found to be less than that in whole fish. The mono unsaturated fatty acids remained without much change in quantity during fermentation while the polyunsaturated fatty acids levels were found to increase during the fermentation process. From the initial 3.3% it rose to 10.66% after fermentation indicating the beneficial effects of fermentation on nutritional point of view.

Table 4.2.1. Proximate composition (%) of dressed tilapia at different levels of jaggery

	Level of jaggery			
	5 %	10 %	15 %	20%
Moisture	79.27±0.7	79.07±1.2	78.17±0.9	77.6 ± 1.1^{a}
Protein	14.2 ± 0.2^{d}	12.4 ± 0.4^{c}	11.8 ± 0.6^{b}	10.57 ± 0.4^{a}
Fat	1.24 ± 0.11^{ab}	1.49 ± 0.1^{c}	1.42 ± 0.11^{ba}	1.2 ± 0.11^{a}
Ash	$1.52\pm.12^{a}$	2.3±0.21	2.41±0.14	2.45±0.13

Results are presented as mean ± standard deviation (SD) of 3 replications ^{a,b,c,d,e}, mean in a row with the same superscript values are not significantly different ($P<0.05$).

Table 4.2.2. Changes in pH during ensilation of dressed tilapia with different levels of jaggery.

Days	Level of jaggery			
	5 %	10 %	15 %	20%
1	$6.94 \pm .06^{a}$	$6.96 \pm .05^{a}$	$7.13 \pm .11^{a}$	$7.48 \pm .16^{b}$
2	5.40 ± 0.1^{ab}	$5.6 \pm .05^{b}$	$5.30 \pm .10^{a}$	$5.27 \pm .05^{a}$
4	4.38 ±.09	4.31 ± .01	4.38 ± .32	4.46 ± .15
7	5.20 ± 0.1^{a}	4.22 ± .005	4.14 ± .04	4.10 ± .10
10	---	$4.07 \pm .01^{b}$	$4.12 \pm .005^{c}$	$3.96 \pm .02^{a}$
13	---	$4.03 \pm .11^{a}$	$4.03 \pm .05^{a}$	$3.83 \pm .05^{b}$

Results are presented as mean ± standard deviation (SD) of 3 replications ^{a,b,c,d,e}, mean in a row with the same superscript values are not significantly different ($P<0.05$).

Table 4.2.3. Changes in Titrable acidity (% lactic acid) during ensilation of dressed tilapia with different levels of jaggery.

Days	Level of jaggery			
	5 %	10 %	15 %	20%
2	1.83 ± .01a	1.13 ± .05b	1.26 ± .05b	1.70 ± .02a
4	3.16 ± .09a	3.16 ± 3.6a	3.17 ± .11a	3.54 ± .01b
7	3.36 ± .05a	3.81 ± .02c	3.59 ± .04b	3.70 ± .20bc
10	---	4.17 ± .04	4.03 ± .05	4.16 ± .11
13	---	4.15 ± .01	4.06 ± .12	4.21 ± .05 a

Results are presented as mean ± standard deviation (SD) of 3 replications. $^{a.b.c.d.e}$, mean in a row with the same superscript values are not significantly different ($P<0.05$).

Table 4.2.4.Changes in residual carbohydrate (%) content during ensilation of dressed tilapia with different levels of jaggery

Days	Level of jaggery			
	5 %	10 %	15 %	20%
1	3.2 ± 0.1^{a}	6.52 ± 0.7^{b}	11.51 ± 0.7^{c}	13.9 ± 0.8^{d}
2	1.46 ± 0.13^{a}	4.65 ± 0.5^{b}	9.6 ± 0.08^{c}	8.53 ± 0.6^{d}
4	0.63 ± 0.02^{a}	2.79 ± 0.21^{b}	5.43 ± 0.06^{c}	7.43 ± 0.42^{d}
7	ND	1.96 ± 0.013^{a}	3.7 ± 0.02^{b}	6.69 ± 0.46^{c}
10	---	1.56 ± 0.02^{a}	3.16 ± 0.03^{b}	6.46 ± 0.21^{c}
13	---	0.87 ± 0.04^{a}	2.93 ± 0.01^{b}	6.2 ± 0.12^{c}

Results are presented as mean ± standard deviation (SD) of 3 replications. $^{a.b.c.d.e}$, mean in a row with the same superscript values are not significantly different ($P<0.05$).

Table 4.2.5.1.Changes in NPN (mg/ 100g) during ensilation of dressed tilapia with different levels of jaggery.

Days	Level of jaggery			
	5 %	10 %	15 %	20%
1	275.0 ± 4.1[a]	280.6 ±3.57[b]	311.33 ± 2.0[c]	311.0 ± 3.6[c]
2	521.6 ± 4.0[a]	692.0 ± 3.4[b]	697.6 ± 2.0[c]	731.0 ± 5.5[d]
4	732.0 ± 6.3[a]	786.0 ± 6.4[b]	894.6 ± 2.5[c]	908.6 ± 3.0[d]
7	767.0 ± 6.0[a]	980.6 ± 4.0[b]	1008.0 ± 6.7[c]	1025.0 ± 2.6[d]
10	---	1084.3 ± 6.3[a]	1029.0 ± 6.9[b]	1041.3 ± 1.1[c]
13	---	1078.6 ± 4.0[a]	1034.6 ± 3.7[b]	1005.6 ± 4.0[c]

Results are presented as mean ± standard deviation (SD) of 3 replications. [a,b,c,d,e], mean in a row with the same superscript values are not significantly different (P<0.05).

Table 4.2.5.2.Changes in Degree of hydrolysis (% of total nitrogen) during ensilation of dressed tilapia with different levels of jaggery

Days	Level of jaggery			
	5 %	10 %	15 %	20%
1	12.57±0.37 [a]	14.14± 0.4[b]	16.49±0.93 [c]	18.39± 1.1[d]
2	21.50± 1.1[a]	28.50±0.87 [b]	32.50± 0.92[c]	38.20±0.85 [d]
4	31.20±1.8 [a]	40.20± 1.1[b]	42.30±1.2 [c]	46.50± 1.3[d]
7	35.07±0.9 [a]	45.60±1.3 [b]	53.39± 1.01[c]	55.40±0.65 [d]
10	---	54.65± 0.9[a]	55.50±2.3 [b]	58.50±1.2 [c]
13	---	54.36± 2.1[a]	54.80±2.2 [b]	57.60± 3.1[c]

Results are presented as mean ± standard deviation (SD) of 3 replications. [a,b,c,d,e], mean in a row with the same superscript values are not significantly different (P<0.05).

Table 4.2.6. Changes in AAN (mg/100g) during ensilation of dressed tilapia with different levels of jaggery.

Days	Level of jaggery			
	5 %	10 %	15 %	20%
1	76.33 ± 2.0^{a}	64.00 ± 1.0^{b}	66.00 ± 2.5^{c}	93.0 ± 1.7^{d}
2	86.00 ± 1.7^{c}	$82.66 \pm .57^{b}$	74.66 ± 3.4^{a}	105.0 ± 1.7^{d}
4	105.66 ± 3.2^{c}	$95.33 \pm .57^{b}$	91.33 ± 1.1^{a}	120.0 ± 1.7^{d}
7	125.00 ± 1.7^{c}	$115.33 \pm .57^{a}$	120.66 ± 3.5^{b}	127.0 ± 1.7^{d}
10	---	134.33 ± 3.5^{b}	123.66 ± 3.0^{a}	136.0 ± 2.0^{c}
13	---	155.00 ± 1.7^{c}	142.21 ± 2.3^{b}	137.0 ± 1.0^{a}

Results are presented as mean ± standard deviation (SD) of 3 replications. ^{a,b,c,d,e}, mean in a row with the same superscript values are not significantly different ($P<0.05$).

Table 4.2.7. Changes in TVBN (mg/100g) during ensilation of dressed tilapia with different levels of jaggery.

Days	Level of jaggery			
	5 %	10 %	15 %	20%
1	$8.86 \pm .25^{b}$	$8.43 \pm .25^{a}$	$9.73 \pm .11^{c}$	$9.87 \pm .15^{c}$
2	$15.20 \pm .26^{d}$	$10.8 \pm .3^{b}$	$10.23 \pm .20^{a}$	$11.36 \pm .52^{c}$
4	29.66 ± 1.5^{d}	$11.40 \pm .26^{a}$	$12.33 \pm .30^{b}$	$13.21 \pm .30^{c}$
7	65.33 ± 1.1^{d}	$14.10 \pm .2^{b}$	$12.43 \pm .20^{a}$	$15.1 \pm .24^{c}$
10	---	$15.5 \pm .45$	$15.13 \pm .15$	$16.3 \pm .23$
13	---	$16.93 \pm .26^{b}$	$17.26 \pm .15^{c}$	$16.5 \pm .37^{a}$

Results are presented as mean ± standard deviation (SD) of 3 replications. ^{a,b,c,d,e}, mean in a row with the same superscript values are not significantly different ($P<0.05$).

Table 4.2.8. Changes in PV (meq O_2 /Kg fat) during ensilation of dressed tilapia with different levels of jaggery.

Days	Level of jaggery			
	5 %	10 %	15 %	20%
1	$8.23 \pm .05^{c}$	7.46 ± 2.0^{b}	$6.86 \pm .08^{a}$	8.4 ± .11d
2	$12.20 \pm .10^{d}$	8.40 ± 1.0^{a}	$9.20 \pm .1^{b}$	$10.66 \pm .23^{c}$
4	$15.96 \pm .20^{c}$	8.53 ± 6.3^{a}	$13.3 \pm .15^{b}$	$16.43 \pm .11^{d}$
7	$18.06 \pm .25^{d}$	12.26 ± 5.5^{a}	$16.4 \pm .11^{b}$	$17.18 \pm .12^{c}$
10	---	11.9 ± 1.0^{a}	$17.3 \pm .15^{c}$	$14.40 \pm .34^{b}$
13	---	10.26 ± 2.5^{a}	$15.4 \pm .05^{c}$	$11.46 \pm .15^{b}$

Results are presented as mean ± standard deviation (SD) of 3 replications. ^{a,b,c,d,e}, mean in a row with the same superscript values are not significantly different ($P<0.05$).

Table 4.2.9. Changes in FFA (% oleic acid) during ensilation of dressed tilapia with different levels of jaggery.

Days	Level of jaggery			
	5 %	10 %	15 %	20%
1	$0.06 \pm .04^{b}$	$.02 \pm .005^{a}$	$0.25 \pm .01^{c}$	$.26 \pm .05^{c}$
2	$2.46 \pm .05^{b}$	$0.63 \pm .01^{a}$	$4.10 \pm .09^{d}$	$3.56 \pm .14^{c}$
4	$3.66 \pm .23^{b}$	$1.21 \pm .02^{a}$	$4.88 \pm .02^{d}$	$4.32 \pm .05^{c}$
7	$3.73 \pm .28^{a}$	$4.49 \pm .04^{b}$	$7.65 \pm .12^{d}$	$6.75 \pm .17^{c}$
10	---	$6.30 \pm .1^{a}$	$8.86 \pm .07^{c}$	$8.07 \pm .15^{b}$
13	---	$7.50 \pm .1^{a}$	$13.01 \pm .21^{b}$	$12.43 \pm .51^{b}$

Results are presented as mean ± standard deviation (SD) of 3 replications. ^{a,b,c,d,e}, mean in a row with the same superscript values are not significantly different ($P<0.05$).

Table 4.2.10. Changes in TBA during ensilation of dressed tilapia with different levels of jaggery.

Days	Level of jaggery			
	5 %	10%	15 %	20%
1	$0.69 \pm .12^{a}$	$0.45 \pm .03^{c}$	$0.65 \pm .05^{b}$	$0.68 \pm .01^{ab}$
2	$1.30 \pm .1^{a}$	$0.65 \pm .005^{c}$	$0.75 \pm .05^{b}$	$0.84 \pm .03^{b}$
4	$1.54 \pm .06^{a}$	$0.86 \pm .05^{b}$	$0.92 \pm .05^{c}$	$0.96 \pm .01^{d}$
7	$1.67 \pm .04^{a}$	$0.96 \pm .02^{b}$	$1.07 \pm .21^{c}$	$1.03 \pm .005^{c}$
10	---	$1.20 \pm .02^{a}$	$1.92 \pm .21^{b}$	$1.26 \pm .30^{a}$
13	---	$1.82 \pm .02^{a}$	$2.20 \pm .21^{b}$	$1.30 \pm .05^{c}$

Results are presented as mean ± standard deviation (SD) of 3 replications. ^{a,b,c,d,e}, mean in a row with the same superscript values are not significantly different ($P<0.05$).

Table 4.2.11.Changes in Amino acid profile (g/100g) of dressed tilapia during fermentation

Days	1	2	4	7	10	13
Asp	10.39	10.09	10.07	9.32	10.90	9.23
Thr	4.25	3.78	3.82	4.32	4.48	4.17
Ser	6.23	6.26	6.05	5.73	5.82	5.52
Glu	13.06	12.17	11.87	11.91	13.74	11.74
Pro	0.91	1.10	1.09	0.98	1.21	1.21
Gly	12.74	15.30	15.73	15.67	15.44	14.73
Ala	8.95	9.10	9.11	9.83	10.07	8.97
Cys	0.33	0.00	0.21	0.41	0.00	0.41
Val	5.00	4.35	4.66	5.85	2.72	6.44
Met	2.32	2.30	2.01	2.33	2.83	2.32
Ile	4.91	4.78	4.42	4.00	5.56	4.43
Leu	7.68	6.86	6.73	6.65	7.93	6.69
Tyr	1.54	1.35	1.23	1.33	1.24	1.37
Phe	4.03	4.05	3.69	3.88	3.17	3.72
Try	2.50	2.63	4.75	3.35	3.15	2.97
His	2.98	3.52	3.31	2.85	2.92	3.07
Lys	1.16	1.34	1.32	0.67	0.90	0.91
Arg	5.50	5.67	5.69	5.27	1.07	5.16

Table 4.2.12. Changes in fatty acid composition (%) of dressed tilapia silage during fermentation

Days	1	2	4	7	10	13
Mono unsaturated fatty acid	21.06	37.17	28.92	26.16	26.73	26.46
Poly unsaturated fatty acid	3.31	6.11	8.05	9.23	10.48	10.66
Saturated fatty acid	45.61	35.32	43.08	41.53	44.76	43.72
Others	30.21	22.01	19.58	23.85	18.72	19.39

4.3. Changes in the Characteristics of Fermented Silage Prepared from Different Species of Dressed Fish

4.3.1. Proximate composition

Proximate composition of silage from different species of dressed fish is given in Table 4.3.1. The initial composition of the silages from different fishes does not vary much. Slight variations in the composition could be due to the species specificity and nature of habitat or feeding. The percentage of protein in silver carp is high which could be due to low mineral content in it. Similarly the higher levels of fat in tilapia is due to the seasonal conditions or the feeding habit of the fish.

4.3.2. pH

Table 4.2.3. Shows the variations in pH during ensilation of dressed fish with 10% jaggery. The pH of the mixtures decreased through out the period of ensilation in all the samples. 10 % of jaggery was found to be sufficient for complete ensilation in dressed tilapia silage; similarly 10% jaggery was used for other samples also. Since the decrease in pH was similar as that of tilapia 10% jaggery was found to be sufficient. The pH in all the samples decreased to the desired levels of around 4.5 by second day, but in case of tilapia pH on second day was found to be 5.2, which further reduced to 4.2 on next observation. The reduction in pH continued till the end of the period. A slight increase in pH on last day could be due to the production of compounds, which might have neutralized the acid, and also due to the low production of acid towards the end of fermentation. There was no significant variation in the pH changes among the samples.

4.3.3. Titrable acidity

Alterations in titrable acidity of fish silage prepared from different species of cleaned fish are given in Table 4.3.3. The titrable acidity of samples prepared

from different fishes increased from the initial value of 1.04 to 4 towards the end of the ensilation. The increase was almost uniform in all the samples without any significant variation among the samples. At the end of the period all the samples were having a value above 4 indicating sufficient acid production. Neethiselvan *et. al.*, (2001) observed a similar levels of lactic acid production in silver belly silage fermented with *Lactobacillus spp.* and fermented cabbage as inoculum for fermentation. But when curd was used as the inoculum in the same study, the acid production was found to be less.

4.3.4. Carbohydrate

Differences in carbohydrate levels during ensilation of dressed fish with 10% jaggery in Table 4.3.4. The carbohydrate levels decreased in all the samples due utilization by the microbes indicating the decrease in pH. The decrease was rapid during the initial days and on 7^{th} day around 1% of sugar was remaining in all the samples. Neethiselvan *et. al.*, (2001) reported a relatively higher amount of sugar (3.5%) in silage with fermented cabbage, compared to Lactobacillus silage (0.51%) indicating higher utilization by the strain when 15% molasses was used as sugar source. Ahmed and Mahendrakar (1996) reported that the sugar utilization by Lactobacillus was completed in 11 days when 15% molasses was used as jaggery source for fermentation of fish viscera.

4.3.5. Peroxide Value

Changes in PV during ensilation of dressed fish with 10% jaggery in Table 4.3.5. The peroxide value, indicator of the level of oxidation, is showing an increasing trend in all the samples initially up to 7 days except in silver carp samples where the increase was up to 10 days. Due to further breakdown of the compounds the PV levels decreased afterwards. All the three samples showed significant variation at the final stage of fermentation which indicates that the

nature of breakdown compounds in each sample was different. Dapkevicius *et. al* (1998) observed the increasing trend in peroxide value for the initial 6 days and then decreased during ensilaging blue whiting with 10 % and 20% molasses. The authors did not find any difference in oxidation of fat when formaldehyde was added.

4.3.6. Free fatty acid

Table 4.3.6. shows the changes in FFA during ensilation of dressed fish with 10% jaggery. The FFA levels in all the three samples were found to be increasing during the period of ensilation. This is due to the breakdown of lipids and also due to the production of lactic acid during the fermentation process. As observed in other indices the production of FFA was faster during initial days and the increase was marginal during the end of the process which could be due to the further breakdown of the acid into other compounds. In tilapia silage the production of FFA was found to be higher which could be due to the specific nature of the fish lipid.

4.3.7. Thiobarbituric acid value

Variations in TBA during ensilation of dressed fish with 10% jaggery is given in Table 4.3.7. As in the case free fatty acid, TBA was also found to increase during the course of fermentation due to breakdown of the lipid into the final product malonaldehyde. Significantly higher levels of TBA was noticed in silver carp during the course of fermentation. Ahamed and Mahendrakar (1996) observed a linear increase in TBA value during fermentation of fish viscera for 8 days. According to Fagbenro & Jauncey (1994) the TBA value increased in raw silage during fermentation and storage. In the case of tilapia samples, the TBA value was higher indicating the comparatively faster decomposition of lipid in this species during the fermentation process.

4.3.8. Degree of hydrolysis

Degree of hydrolysis during ensilation of dressed fish with 10% jaggery is given in Table 4.3.8. The patterns of hydrolysis of protein as calculated based on the production of non protein nitrogenous compounds during the period of fermentation, the species under study almost the same pattern. The hydrolysis rate was higher initially and the rate decreased during the final stage. In all the samples the DH was nearly 60% with Jew fish giving the higher value of 58.7 % on 10^{th} day of the process. The slight decrease of DH during last days could be due to the utilization of the amino acids formed by the microbes.

4.3.9. α- Amino nitrogen

Alterations in AAN during ensilation of dressed fish with 10% jaggery are given in Table 4.3.9. The changes in AAN levels were almost uniform in all the three samples. But tilapia samples showed a higher level of AAN compared to the other two. From the initial value of 60 mg/ 100g, the AAN value reached around 150 mg/100 g in 13 days ensilation indicating a better hydrolysis. In the case of silver carp the AAN level was less, which is also having a lesser ratio of hydrolysis of protein. This could be due to the specific proteins of the fish which is not easily amenable to breakage.

4.3.10. Total volatile base nitrogen

Changes in TVBN during ensilation of dressed fish with 10% jaggery are given in Table 4.3.10. The TVBN levels increased during the first two days in the case of silver carp and Jew fish during fermentation; which subsequently decreased during 4^{th} day and remained more or less constant. This could be due to the neutralization of the volatile bases that might have formed by the acid produced. But in the case of tilapia there was slow increase of TVBN throughout the period of fermentation. Since the degree of hydrolysis in all the three samples

were less, the formation of TVBN could also be less. The utilization of the nitrogenous compounds by the lactic acid bacteria could be one of the reasons for slow increase in TVBN. Maria *et. al.*, (1998) had observed that the TVBN content of fermented fish silage is much lower when compared to acid silage.

4.3.11. Amino acids changes

4.3.11.1. Amino acids changes of Jew fish silage

The changes in amino acid profile of dressed Jew fish during fermentation is given in Table 4.3.11.1. In case of Jew fish threonine aspartic acid, glutamaic acid, glycine, alanine and leucine were found in higher levels. A drastic reduction in threonine was noted during fermentation while the levels of aspartic acid, glycine, alanine and valine levels increased marginally during the same period. The levels of tryphtophan was found to decrease during fermentation. The changes in the amino acid levels could be due to the activity of bacterial enzymes which could result in conversion of amino acids to other compounds.

4.3.11. 2. Amino acids changes of silver carp silage

Table 4.3.11.2. shows the profile change of amino acid of dressed silver carp during fermentation. The amino acid profile of silver carp also does not showed significant changes during fermentation. The important amino acids present in silver carp silage were found to be Aspartic acid, glutamic acid, glycine alanine and leucine in higher concentration and other amino acids in lower concentration. Tryptophan levels showed a decreasing trend during the period, which could be due to the reduction occurred during ensiling due to chemical reactions between amino and aldehyde groups present in amino acids (Johnson *et al.*, 1985). According to Santana *et. al.*, (in press) tryptophan was the amino acid most affected by the silage process. Its concentration in the silage was decreased by 54% compared to that of fishmeal.

During fermentation of fish silage the histidine content did not change significantly in any of the three samples, indicating that decarboxylation of the amino acid could not have taken place. The histidine content remained more or less same around 3.5 % in both silver carp and Jew fish, but tilapia was showing lower levels of histidine during fermentation. According to Green *et. al.* (1983), the amino acid composition of fish silage had Lysine, threonine and sulphur containing amino acids in high levels and fish silage would appear to be an excellent protein supplement for non-conventional livestock feeding systems. During storage of silage, endogenous, proteolytic enzymes break down the tissue protein to low molecular weight peptides and amino acids that remain soluble and stable (Green *et.al.,* 1983). Apparently, under normal storage conditions, degradation of the amino acids is not of great importance. In fact, Gildberg and Raa (1977) showed that, in fish silage stored for up to 220 days, less than 8 percent of amino nitrogen is released as ammonia. Nevertheless, at temperatures exceeding 30°C, tryptophan, methionine and histidine tend to decompose (Machin, 1990). According to Luiz *et al.*, (2006), the fermented silage of tilapia filleting residue showed higher concentration of aspartic acid, glycine, methionine, threonine and serine, than tilapia filleting residue of acid silage. However, the contents of leucine, lysine, phenylalanine and histidine were lower. This reduction might have occurred during ensiling due to chemical reactions between amino and aldehyde groups present in amino acids (Johnson *et al.*, 1985).

4.3.12. Fatty acid composition

4.3.12.1. Fatty acid composition of Jew fish

The changes in fatty acid composition of silage from dressed Jew fish during fermentation is given in Table 4.3.12.1. In Jew fish the saturated fatty acid content was less when compared to tilapia and silver carp. There were not much

change in the levels of saturated fatty acids in Jew fish during the period of fermentation process. But the mono unsaturated fatty acid levels found to increase during the initial period up to 4 days and showed a declining trend up to 13 days. The PUFA content was found to be initially higher and decreased during fermentation up to 7 days, which further showed decreasing trend, which could have been utilized by the microbes.

4.3.12.2. Fatty acid composition of silver carp

The changes in fatty acid composition of dressed silver carp during silage fermentation are given in Table 4.3.12.2. A slight increase in the saturated fatty acid content was noted during fermentation of dressed silver carp, whereas the monounsaturated fatty acids contents does not showed much variation during the fermentation period. Santana *et. al* (in press) observed that most of the fatty acids were protected by the antioxidant and the ratio of n-3/n-6 PUFA (poly unsaturated fatty acid) in fishmeal and silage was 4.7 and 4.9, respectively. The PUFA content in silver carp levels showed a decline trend towards the end of the fermentation period as noted in case of Jew fish silage. From the initial value of 24 % the content decreased to 11% at the end of fermentation.

Table. 4.3.1. Proximate composition of silage prepared from different fishes

	Jew fish	Tilapia	Silver carp
Moisture (%)	80.4± 0.28[a]	79.6±0.54	79.8±0.51
Protein (%)	11.3± 0.21	11.5±0.14	11.5± 0.11
Fat (%)	1.4± 0.1 [a]	1.8±0.12 [b]	1.8± 0.11[b]
Ash (%)	1.7±0.08 [a]	1.9±0.07 [b]	1.9±0.02 [b]

Results are presented as mean ± standard deviation (SD) of 3 replications .[a,b,c,d,e], mean in a row with the same superscript values are not significantly different (P<0.05).

Table 4..3.2. Changes in pH during ensilation of different species of dressed fish with 10% jaggery.

Days	Jew fish	Tilapia	Silver carp
1	6.83 ± 0.06[a]	6.96 ± .05[a]	6.82 ± 0.14[a]
2	4.69 ± 0.08[a]	5.23 ± .05[b]	4.64 ± 0.05[a]
4	4.26 ± 0.05[a]	4.21 ± .01[a]	4.37 ± 0.03[a]
7	4.07 ± 0.11[a]	4.02 ± .005[a]	4.20 ± 0.01[a]
10	4.02 ± 0.02[a]	3.97 ± .01[a]	4.16 ± 0.05[a]
13	3.93 ± 0.03[a]	4.03 ± .11[a]	4.12 ± 0.01[a]

Results are presented as mean ± standard deviation (SD) of 3 replications .[a,b,c,d,e], mean in a row with the same superscript values are not significantly different (P<0.05).

Table 4.3.3. Changes in titrable acidity (as % lactic acid) during ensilation of different species of dressed fish with 10% jaggery.

Days	Jew fish	Tilapia	Silver carp
2	1.23 ± 0.03	1.13 ± .05	1.04 ± 0.03
4	2.25 ± 0.04	2.16 ± 3.6	2.05 ± 0.05
7	3.69 ± 0.14	3.01 ± .02	3.22 ± 0.08
10	4.01 ± 0.01^{b}	4.17 ± .04^{c}	3.75 ± 0.07^{a}
13	4.20 ± 0.10	4.05 ± .01	4.01 ± 0.01

Results are presented as mean ± standard deviation (SD) of 3 replications ^{a,b,c,d,e}, mean in a row with the same superscript values are not significantly different ($P<0.05$).

Table 4.3.4. Residual carbohydrate (g/100g) during ensilation of different species of dressed fish with 10% Jaggery

Days	Jew fish	Tilapia	Silver carp
1	5.85 ± 0.15^{a}	6.53 ± 0.25^{c}	6.30 ± 0.10^{b}
2	4.41± 0.09^{a}	4.8 ± 0.26^{b}	5.03 ± 0.15^{c}
4	3.30 ± 0.17^{b}	2.22 ± 0.01^{a}	3.50 ± 0.26^{c}
7	1.89 ± 0.01^{c}	1.07 ± 0.11^{a}	1.41 ± 0.06^{b}
10	0.65 ± 0.09^{a}	0.86 ± 0.03^{b}	1.24 ± 0.07^{c}
13	0.13 ± 0.01^{a}	0.67 ± 0.06^{c}	0.45 ± 0.05^{b}

Results are presented as mean ± standard deviation (SD) of 3 replications ^{a,b,c,d,e}, mean in a row with the same superscript values are not significantly different ($P<0.05$).

Table 4.3.5. Changes in PV (meq O_2/ kg fat) during ensilation of different species of dressed fish with 10% jaggery

Days	Jew fish	Tilapia	Silver carp
1	2.30 ± 0.10b	5.36 ± .20 c	1.53 ± 0.06a
2	7.61 ± 0.16^{b}	6.96 ± .28^{b}	4.53 ± 0.15^{a}
4	13.17 ± 0.25^{c}	7.50 ± .1^{a}	9.89 ± 0.06^{b}
7	13.43 ± 0.06^{b}	13.66 ± .23^{b}	10.30 ± 0.10^{a}
10	10.30 ± 0.10^{a}	11.36 ± .15^{b}	12.47 ± 0.21^{c}
13	8.68 ± 0.13^{a}	10.33 ± .11^{b}	11.53 ± 0.15^{c}

Results are presented as mean ± standard deviation (SD) of 3 replications ^{a,b,c,d,e}, mean in a row with the same superscript values are not significantly different ($P<0.05$).

Table 4.3.6. Changes in FFA (% Oliec acid) during ensilation of different species of dressed fish with 10% jaggery

Days	Jew fish	Tilapia	Silver carp
1	0.22 ± 0.01^{b}	0.03 ± 0.01^{a}	0.02 ± 0.01^{c}
2	2.47 ± 0.13^{b}	0.63 ± 0.01^{a}	3.20 ± 0.10^{c}
4	3.79 ± 0.14^{b}	1.22 ± 0.03^{a}	5.47 ± 0.06^{c}
7	5.67 ± 0.12^{b}	1.50 ± 0.05^{a}	7.73 ± 0.12^{c}
10	5.63 ± 0.21^{b}	2.30 ± 0.10^{a}	7.47 ± 0.06^{c}
13	8.47 ± 0.06^{b}	2.50 ± 0.10^{a}	12.60 ± 0.17^{c}

Results are presented as mean ± standard deviation (SD) of 3 replications ^{a,b,c,d,e}, mean in a row with the same superscript values are not significantly different ($P<0.05$).

Table 4.3.7. Changes in TBA during ensilation of different species of dressed fish with 10% jaggery

Days	Jew fish	Tilapia	Silver carp
1	0.66± 0.01^{b}	0.45± 0.03^{a}	0.81± 0.02^{c}
2	0.79± 0.01^{b}	0.65± 0.01^{a}	0.92± 0.03^{c}
4	0.99± 0.02^{b}	0.86± 0.06^{a}	1.20± 0.01^{c}
7	1.02± 0.01^{b}	0.96± 0.02^{a}	2.31± 0.03^{c}
10	1.87± 0.06^{b}	1.21± 0.03^{a}	2.76± 0.07^{c}
13	2.14± 0.05^{b}	1.82± 0.02^{a}	3.00± 0.03^{c}

Results are presented as mean ± standard deviation (SD) of 3 replications ^{a,b,c,d,e}, mean in a row with the same superscript values are not significantly different ($P<0.05$).

Table 4.3.8. Changes in Degree of hydrolysis (% of total nitrogen) during ensilation of different species of dressed fish with 10% jaggery

Days	Jew fish	Tilapia	Silver carp
1	19.3±0.54^{c}	14.1±0.8^{a}	16.9±0.8^{a}
2	19.7±0.33^{c}	34.9±1.1^{a}	32.1±1.1^{a}
4	42.4±0.84^{b}	39.6±1.2^{a}	47.0±1.1^{b}
7	54.4±1.1^{c}	49.4±2.1^{a}	52.2±2.1^{a}
10	58.7±2.1^{c}	54.7±2.1^{a}	53.8±1.4^{a}
13	57.1±2.4^{c}	54.4±1.2^{a}	55.6±2.4^{a}

Results are presented as mean ± standard deviation (SD) of 3 replications ^{a,b,c,d,e}, mean in a row with the same superscript values are not significantly different ($P<0.05$).

Table 4.3.9. Changes in AAN (mg/100g) during ensilation of different species of dressed fish with 10% jaggery

Days	Jew fish	Tilapia	Silver carp
1	59.80 ± 0.69^{b}	64 ± 1^{c}	51.67 ± 2.52^{b}
2	73.17 ± 1.05^{b}	82.66 ± 0.57^{c}	66.67 ± 1.53^{b}
4	86.03 ± 0.35^{a}	95.33 ± 0.57^{c}	92.33 ± 1.53^{a}
7	108.83 ± 1.19^{b}	115.33 ± 0.57^{c}	106.00 ± 1.73^{b}
10	124.50 ± 0.62^{b}	134.33 ± 3.5^{c}	122.33 ± 1.53^{b}
13	150.57 ± 2.84^{b}	155 ± 1.7^{c}	127.33 ± 0.58^{b}

Results are presented as mean ± standard deviation (SD) of 3 replications ^{a,b,c,d,e}, mean in a row with the same superscript values are not significantly different ($P<0.05$).

Table 4.3.10 Changes in TVBN (mg/100g) during ensilation of different species of dressed fish with 10% jaggery

Days	Jew fish	Tilapia	Silver carp
1	16.00 ± 0.53^{c}	9.66 ± 0.26^{a}	11.33 ± 0.15^{b}
2	25.13 ± 0.21^{c}	10.53 ± 0.32^{a}	19.23 ± 0.64^{b}
4	20.37 ± 0.12^{c}	10.6 ± 0.1^{a}	19.07 ± 0.25^{b}
7	20.32 ± 0.33^{c}	11.63 ± 0.12^{a}	18.57 ± 0.06^{b}
10	19.74 ± 0.41^{c}	12.6 ± 0.23^{a}	17.47 ± 0.12^{b}
13	18.03 ± 0.51^{c}	13.26 ± 0.15^{a}	17.43 ± 0.06^{b}

Results are presented as mean ± standard deviation (SD) of 3 replications ^{a,b,c,d,e}, mean in a row with the same superscript values are not significantly different ($P<0.05$).

Table. 4.3.11.1.Changes in amino acid composition (mg/100g) during ensilation of Jew fish

Days	1	2	4	7	10
Asp	7.34	11.30	11.80	10.49	10.71
Thr	31.42	5.08	4.72	5.24	5.29
Ser	4.17	6.75	5.60	6.06	5.72
Glu	9.55	14.61	13.49	13.90	13.86
Pro	0.58	1.22	1.12	0.73	0.77
Gly	10.54	15.28	12.66	12.66	11.70
Ala	5.99	9.52	7.96	9.09	9.61
Cys	0.00	0.21	0.00	0.34	0.36
Val	3.49	7.03	4.36	5.82	5.81
Met	1.51	0.25	1.56	1.70	2.39
Ile	3.27	5.83	5.29	5.04	5.23
Leu	5.70	0.95	7.93	8.21	8.07
Tyr	1.04	2.57	2.25	1.92	1.76
Phe	2.89	4.56	3.96	3.50	3.37
Try	3.48	3.12	2.93	1.70	1.53
His	2.74	4.11	4.57	2.76	3.06
Lys	0.81	1.35	1.63	1.10	1.14
Arg	0.97	1.38	2.14	1.48	1.17

Table 4.3.11.2. Changes in Amino acid profile (mg/100g) of silver carp during fermentation

Days	1	2	4	7	10	13
Asp	10.28	10.56	10.64	9.74	10.22	10.46
Thr	4.58	4.38	4.67	4.04	4.20	4.71
Ser	5.83	4.89	5.46	5.03	4.89	6.17
Glu	13.22	12.93	13.60	12.66	13.22	13.63
Pro	0.68	0.93	0.82	1.04	1.08	1.15
Gly	16.55	16.40	16.90	14.60	14.29	13.58
Ala	9.66	8.96	9.76	8.63	9.26	9.54
Cys	0.16	0.00	0.00	0.00	0.00	0.00
Val	5.61	4.48	5.99	5.89	4.95	4.51
Met	1.83	1.92	1.85	2.35	1.98	3.14
Ile	4.58	4.97	5.17	4.96	5.12	4.42
Leu	7.78	7.57	7.17	7.74	7.96	7.72
Tyr	1.68	1.78	1.05	2.17	1.93	2.21
Phe	3.34	3.84	2.64	3.95	3.89	3.62
Try	2.59	2.12	1.19	1.32	1.23	0.98
His	3.49	4.30	2.80	3.97	3.84	2.91
Lys	1.23	1.57	0.92	1.31	1.22	1.02
Arg	1.53	2.52	1.60	2.94	1.94	1.21

Table 4.3.12.1. Changes in Fatty Acid Composition (%) during Jew Fish silage fermentation

Fatty acid	1	2	4	7	10	13
Saturated fatty acid	13.03	20.11	19.54	17.53	24.88	19.55
Mono unsaturated fatty acid	45.11	45.82	50.17	47.01	50.59	44.96
Poly unsaturated fatty acid	33.34	29.33	25.87	26.39	19.37	18.42
Others	8.52	5.34	4.42	9.48	5.34	16.24

Table 4.3.12.2. Changes in fatty acid composition (%) of dressed silver carp ensilation

Fatty acid	1	2	4	7	10	13
Saturated fatty acid	25.06	27.16	20.72	33.83	34.66	28.9
Mono unsaturated fatty acid	35.14	33.92	29.69	34.67	36.39	39.55
Poly unsaturated fatty acid	24.94	24.85	27.87	16.08	12.20	11.56
Others	14.62	14.29	23.13	16.03	17.91	21.41

4.4. Changes in Biogenic Amines of Fermented Silage

Biogenic amines are basic nitrogenous compounds formed mainly by decarboxylation of amino acids or by amination and trasamination of aldehydes and ketones (Santos, 1996). Biogenic amines in food and beverages are formed by the enzymes of the raw material or generated by microbial decarboxylation of amino acids (Hala's *et.al.*, 1994), but it has been found that some of the aliphatic amines can be formed "*in vivo*" by amination from corresponding aldehydes (Maijala *et.al.*, 1993). Decreased pH values and relatively low oxygen concentrations within the silage facilitate decarboxylase activity (Maria *et.al.*, 2000). Fermentation with LAB is found to reduce the formation of biogenic amine in some products as per study carried out by Petaja *et.al.*, (2000). They have observed that the highest concentration of biogenic amines were in the control products without any LAB inoculation.

4.4.1. Putrescine

Alterations in putrescine levels during fermentation of silage with different species of dressed fish are given in Fig 4.4.1. In all the three samples, the initial levels of putrescine was found to be low and in the case of silver carp the initial value was as low as 0.1ppm and in other two samples it was 40ppm and 120 ppm for Jew fish silage and tilapia respectively on the first day of ensilation. The Putrescine content in silver carp does not change during the initial ten days of ensilation while the value increased drastically during the observation on 13^{th} day. The increasing of amine could be due to the proliferation of spoilage bacteria due to suppression of lactobacillus count. But in case of tilapia silage the contents declined throughout the period indicating that no putrescine was produced during the period of fermentation and amine already present was neutralized by the acid formed. The increase in the values during initial period could be due to the growth of gram negative bacteria which could have been destroyed during later stages. Consequently the values declined towards the end of ensilation. Hala'sz *et. al* (1994) reported that production of putrescine by

Enterobacter cloacae was reduced to half in anaerobic condition compared to aerobic condition Meanwhile the putrescine content in silver carp slowly increased during 10 days and there after showed a sharp increase resulting in 25ppm of putrescine.

4.4.2. Cadaverine

Variations in the Cadaverine content during fermentation of silage with different species of fish is given in Fig. 4.4.2. The levels of cadaverine during the fermentation does not show much changes. In case of tilapia silage the initial concentration was 110 ppm which was reduced 55 ppm after 13 days. The higher concentration of the amine could have been present in the raw material and the reduction during fermentation could due to the neutralization of the amines with the acid produced during fermentation. The precursor amino acid of cadaverine i.e, lysine does not change much during fermentation indicating that the activity of lysine decarboxylase is minimum in the samples. According to Petaja *et.al.*, (2000) Fish, raw material and fermented products contained low amounts of biogenic amines with one exception, cadaverine.

4.4.3. Spermidine

Changes in spermidine levels during fermentation of silage with different species of fish is given in Fig 4.4.3. The spermidine content in all the samples were found to be very less. In tilapia samples the spermidine levels increased after the initial 4 days of initial ensilation which could have formed as a result of breakdown of putrescine. But in both the other samples, the spermidine levels remained very low levels. This is in agreement with the general observation that when there is a rise the cadaverine content, corresponding decrease in spermidine and spermine contents as decomposition progresses (Meitz and Karmas, 1977).

4.4.4. Spermine

Variations in the levels of spermine during fermentation of silage with different species of fish is given in Fig. 4.4.4. the Spermine content in all the samples were comparatively low and their changes in levels during fermentation in all the three species showed a reverse relationship with that of spermidine, the precursor of spermine. In case of Jew fish and silver carp the contents increased after 10 days while in tilapia it showed decline during the same period, which could be due to the further conversion of the compounds to other metabolites by acetyl transferase into acetyl Spermine (Halasz, *et. al.*, 1994).

4.4.5. Tyramine

Changes in tyramine levels during fermentation of silage with different species of fish is given in Fig. 4.4.5. The tyramine levels during the fermentation does not show much change and remained at low levels during ensilation except in case of silver carp in which the amine increased after 10 days. However, the tyrosine levels in Jew fish during ensilation does not show any reduction, rather a slight increase was noted towards the end of ensilation. During fermentation, the majority of the gram negative bacteria disappear due to microbial competition and acidity. In silages that reached a pH below 4.5, such bacteria would not contribute to biogenic amine formation (Maria *et.al.*, 2000). But Santose *et. al.*, (1996) found a higher tyramine level in mackerel when pH is low.

4.4.6. Agmatine

Fig. 4.4.6. depicts the changes in Agmatine content during fermentation of silage with different species of fish. The agmatine levels in both tilapia was found to decline through out the period of fermentation where in the other two samples the contents remained more or less stable during the initial period. After 7 days the agmatine levels drastically in Jew fish silage and the same condition was noted in silver carp after 120 days. However, the values in case of silver

carp showed an increasing trend after 7 days. It has been noted that there was an inverse relation between the arginine (the precursor of agmatine) levels in the samples during the fermentation process. In ice stored tilapia also increasing of agmatine was observed by Nelson & Cox (2002).

4.4.7. Histamine

Fig. 4.4.7. shows the changes in histamine levels during fermentation of silage using different species of fish. In all the three samples, the levels of histamine decreased during the course of fermentation indicating the strain does not produce histamine. The decreasing trend could be due to the neutralization of the amines by the acid formed or by the action of the enzyme Di Amine Oxidase (DAO) which degrade the histamine formed. Maria *et.al* (2000) demonstrated the capacity of different strains of Lactobacillus to degrade histamine in a model system at pH near 4.5. Even though histidine decarboxylase activity was detected in *Enterobactor, Pseudomonas, Proteus* and some strains of *Lactobacillus,* the strain used under study i.e,., *Lactobacillus plantarum* is not reported to produce histamine during fermentation. Since the histamine levels in all the samples were reduced considerably towards the end of ensilation, the strains used does not pose any threat of histamine production.

The lower levels of histamine noticed in tilapia during fermentation when compared to other two groups could be due to the low concentrations of histidine in the fish. In all the three samples the histamine levels have come down during fermentation with initial lagging in all the cases which could be due to the degradation of histamine formed initially by the microbes i.e., *Lactobacillus plantarum.* During the period of fermentation of 13 days., the histamine levels were reduced to one fourth of the initial levels in all the samples. The amino acid profile of the same sample also reveals that the histidine content of the sample had shown an increase for the initial two days and gradually decreased. Since the histamine content was within the maximum

permitted levels, the presence of very low level does not make any potential health hazard on ingestion.

Different amines such as putrescine, cadaverine spermidine, spermine histamine, tyramine and agmatine were all produced in insignificant concentration in tilapia both in iced storage and ambient temperature storage. It was observed (Maria *et.al.*, 2000) that one half of the histamine added to a model system of ensilage production was degraded by the starter microbes probably due to the presence of minor quantity of oxygen. The histamine concentration remained stable for first hours and decreased to 80% of the initial concentration after 6 hours. In DAO (Diamine Oxidase) added sample histamine was degraded to about 20% of the initial concentration. This pattern in change of biogenic amine profile in the fish is almost same in all amines except spermidine and spermine. It was observed that the concentration of all the amines except spermidine and spermine were significantly low at the very fresh sample used for both the experiments. Putrescine, cadaverine, histamine, tyramine and agmatine were increasing with keeping time, whereas spermidine and spermine were decreased. It is probably because these two amine are more systemic amines as produced in living system. Among the other amines putrescine and cadaverine showed the lowest value in fresh fish.

Fig 4.4.1 Changes in putrescence levels during fermentation of silage with different fishes

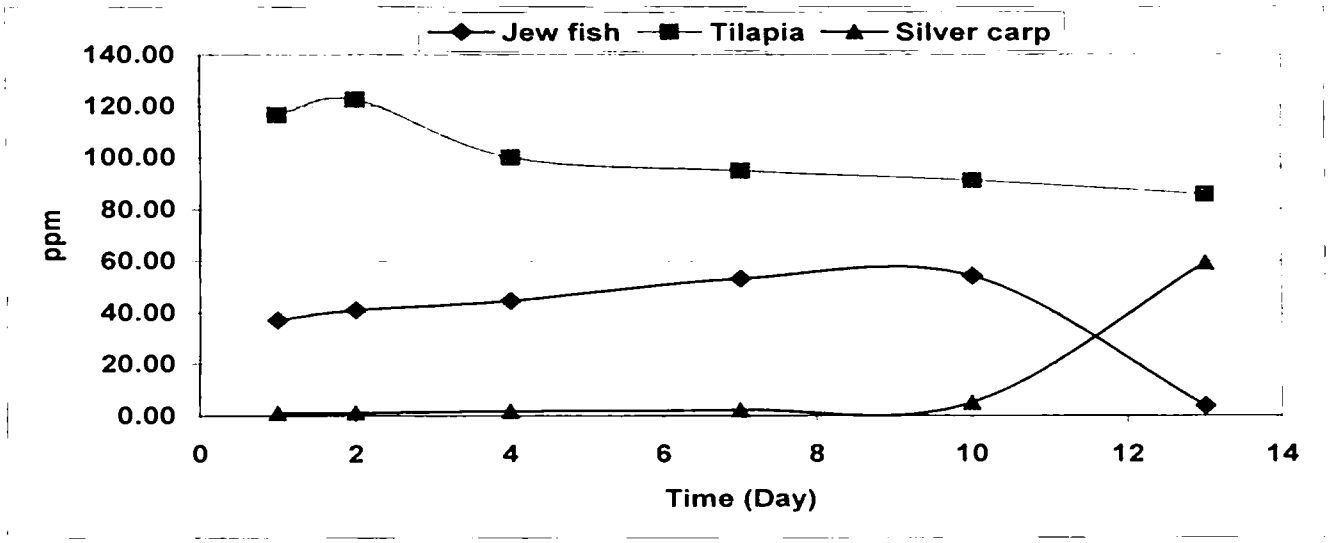


Fig 4.4.2. Changes in cadaverine levels during fermentation of silage with different fishes

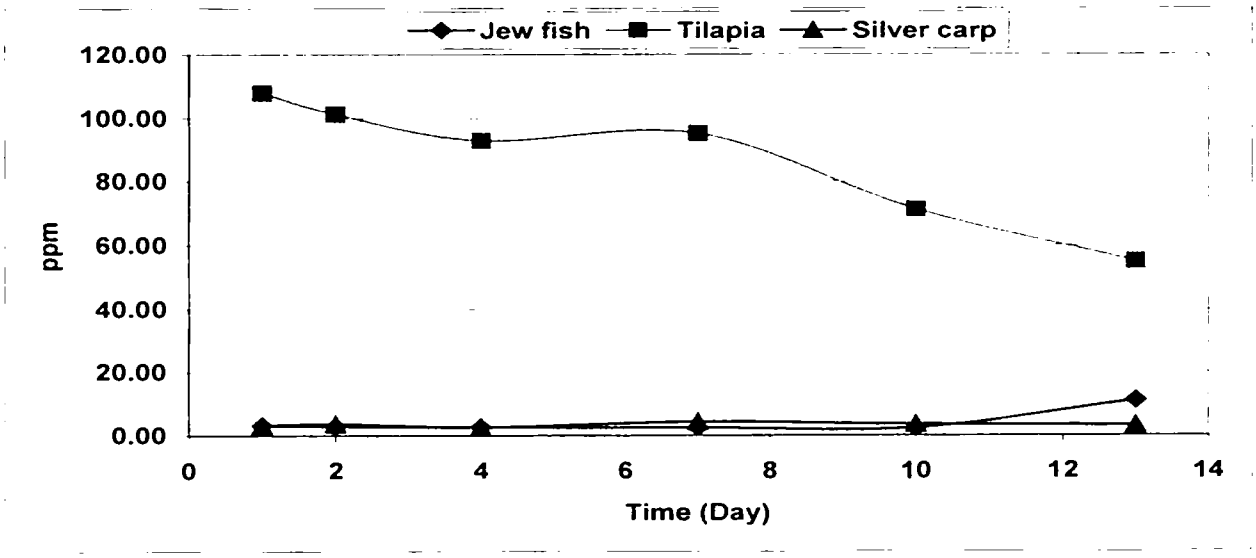


Fig 4.4.3. Changes in spermidine levels during fermentation of silage with different fishes

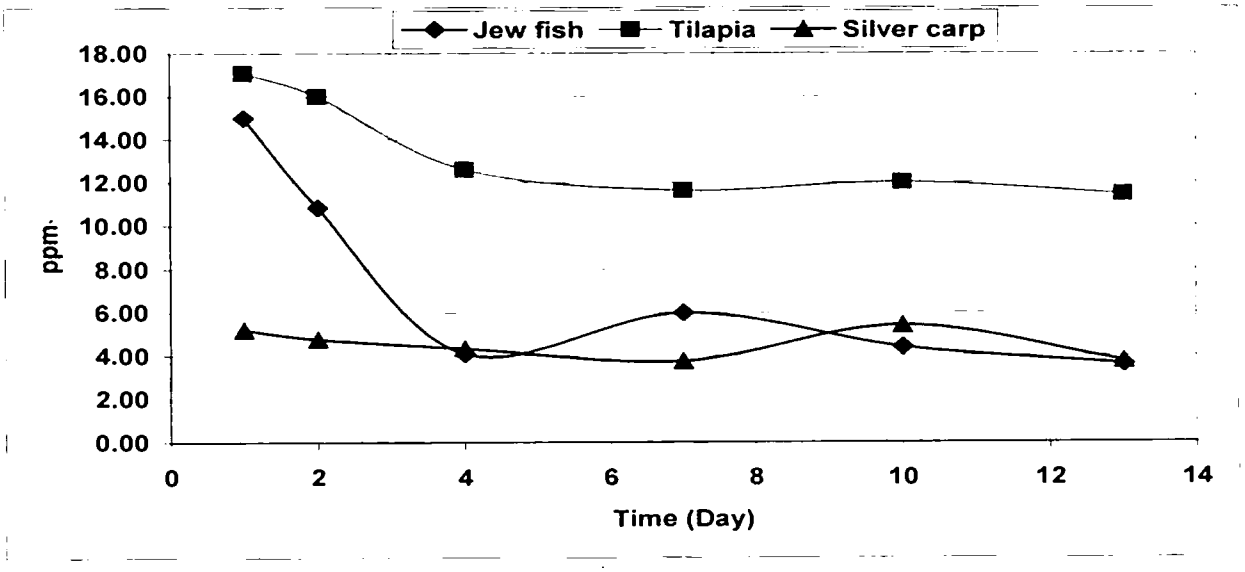


Fig. 4.4.4. Changes in spermine levels during fermentation of silage with different fishes

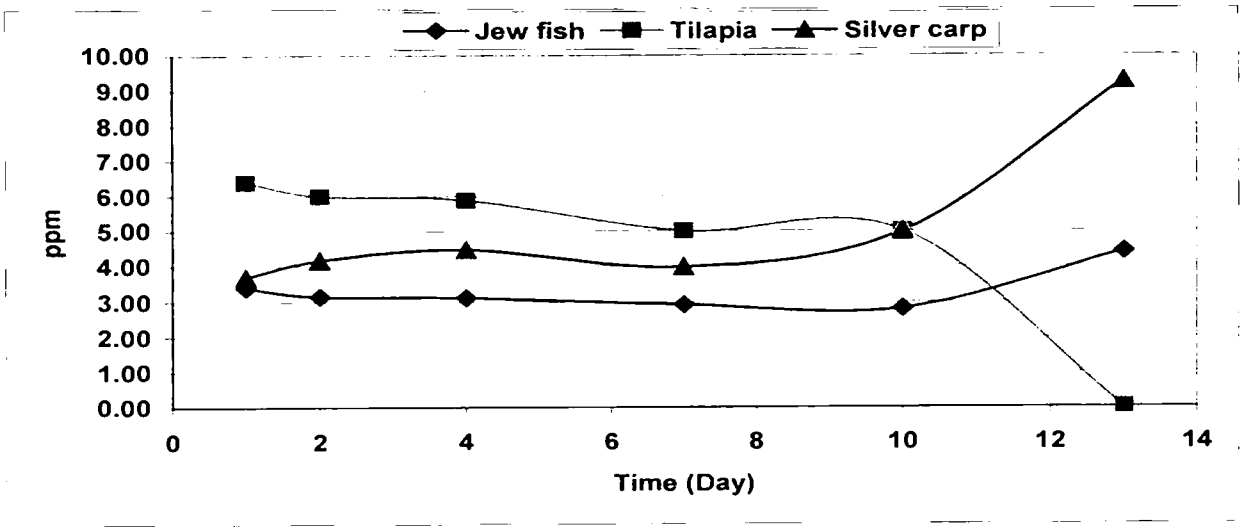


Fig. 4.4.5. Changes in tyramine levels during fermentation of silage with different fishes.

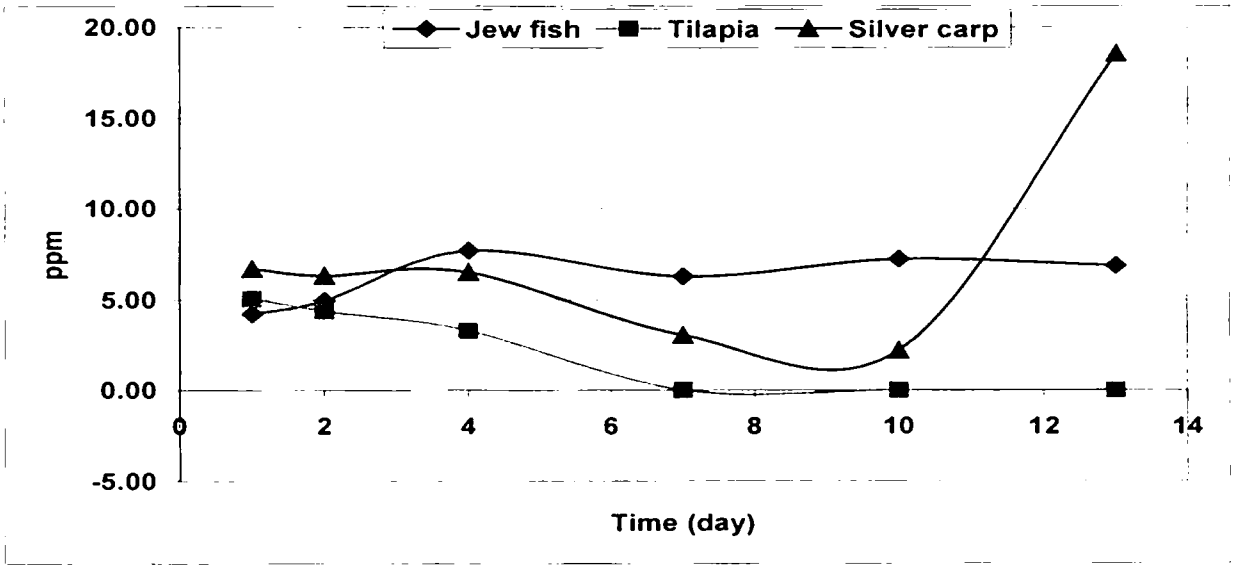


Fig. 4.4.6. Changes in Agmatine levels during fermentation of silage with different fishes.

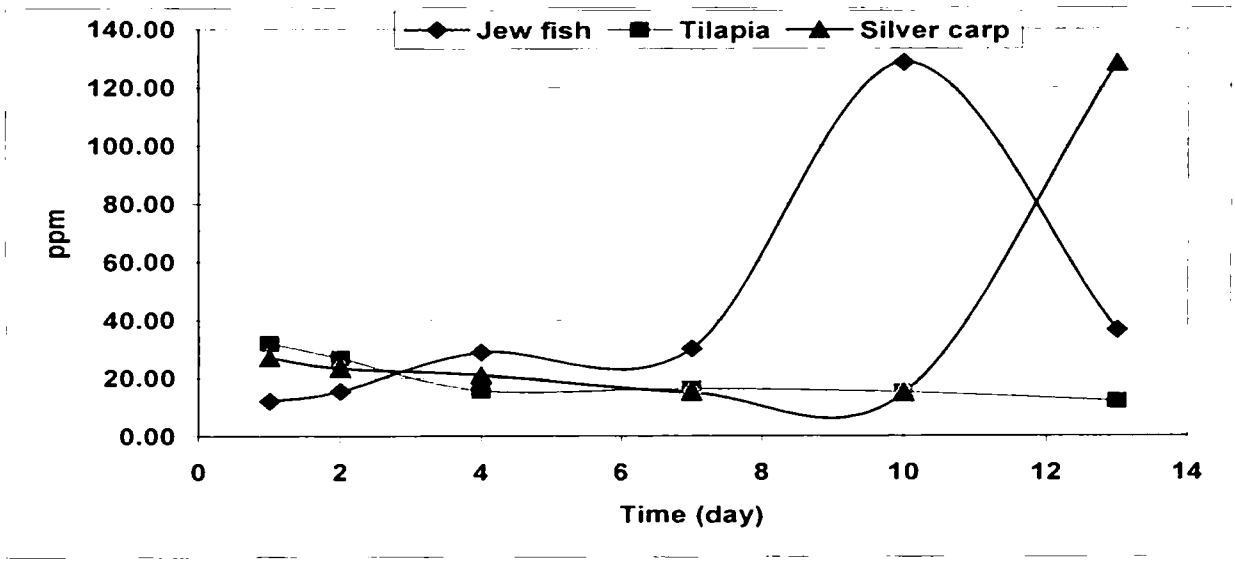
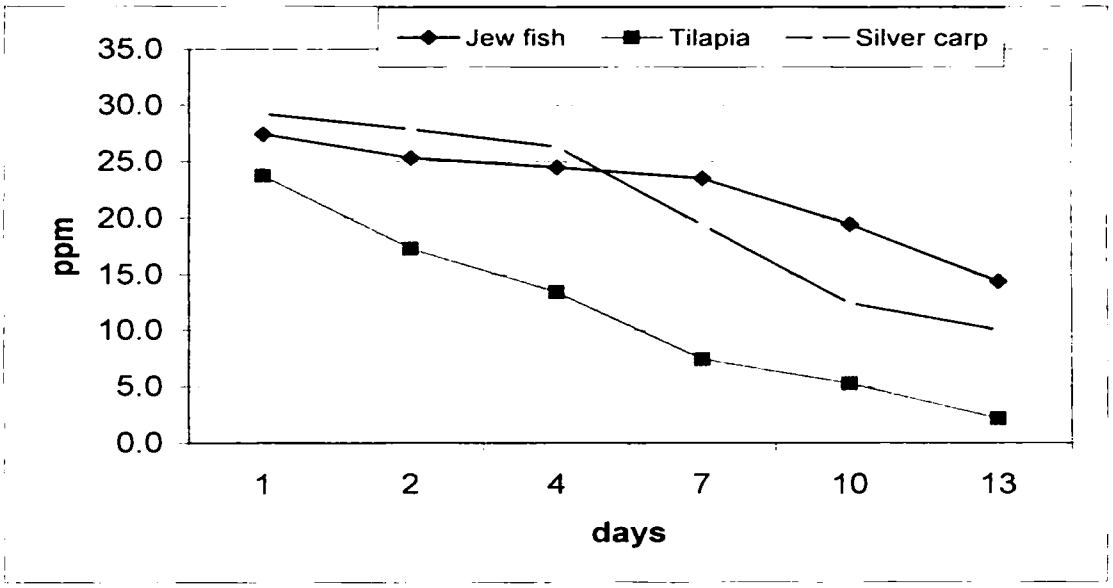


Fig. 4.4.7. Changes in histamine levels during fermentation of silage with different fishes



4.5 Changes in the Characteristics of Dehydrated Fermented Silage Prepared from Different Species of Dressed Fish

4.5.1. Proximate composition

Proximate composition of dehydrated silage samples is given in Table 4.5.1. The protein content was highest in silver carp, which showed the lowest moisture and fat content. In case of dried tilapia silage, protein content was lowest (59.1%) with highest content of fat and ash. The higher levels of ash could be due to more bones of the fish. In Jew fish silage the moisture content was highest (9.96%) with 67.6% crude protein.

4.5.2. Non protein nitrogen

Alterations in NPN during storage of dried silage are given in Table 4.5.2. The NPN during storage was found to be increasing in all the samples. During 10 months storage NPN content in all samples showed an initial slow increase up to 4 months and afterwards a marked increase was noted. However, the highest level of NPN was noted in tilapia silage from the initial value of 3257 mg/100g to 4474 mg/100g during the 10 months of storage while in the case of silver carp silage it 3248 mg/100g and 4149 mg/100g respectively. Even though the silage was dried and stored the increase in NPN noticed could be due to the fragmentation of the protein component. According to Gopakumar (1997), silage fermented by using papaya latex when stored in polythene containers remained in good condition up to three years.

4.5.3. Total volatile base nitrogen

Changes in TVBN during storage of dried silage are given in Table 4.5.3. As in case of NPN, the volatile base nitrogen content of the samples is found to be increasing during storage in all the samples. Up to 7 months the increase was very slow and the values were around 15mg/100g. Even though the rate of

increase was more after 7 months, the final values after 10 months were below 35mg/100 indicating that the products are more or less stable on storage for 10 months. A higher value of TVBN was noted in dried tilapia silage as in the case of NPN.

4.5.4. Peroxide Value

Changes in PV during storage of dried silage are given in Table 4.5.4. The PV during storage showed a marginal increase during the initial five months in all the samples thereafter PV showed an upward trend for 10 months. Although oxidation in silver carp silage was less compared to the other groups during storage; the initial value of 6.63 meq/kg fat was higher when compared to the other two species. According to Fagbenro & Jauncey (1994) the decrease in peroxide value observed during ensilation and storage of silage probably reflects the degradation of part of the hydro peroxides to form secondary breakdown products such as aldehydes. Haard *et. al.*, (1985) have observed that the oil retained in fish silage can be oxidized rendering the feed unpalatable or unsafe to livestock

4.5.5. Free fatty acids

FFA Changes during storage of dried silage are in Table 4.5.5. As in the case of PV, the FFA also increased during storage. The rate of increase was comparatively less during the initial four months and later the rate of increase was substantial. In tilapia silage the increase in FFA was sharp showing more than double the value of other samples. In silver carp and Jew fish the FFA value was within 30mg/100 g in 9 months storage. Increase in the levels of FFA during the production and subsequent storage of tilapia silage in control samples as well as in samples in which antioxidants were added was observed by Fagbenro & Jauncey (1994). According to Ahamed and Mahendrakar (1996) the time elapsed for processing resulted in initial high FFA value which on ensilation increased further for 4 days after ensilation.

4.5.6. Thiobarbituric acid value

Changes in TBA during storage of dried silage are in Table 4.5.6. Significant change was observed in TBA values during storage. Jew fish silage showed the lowest levels of TBA followed by silver and tilapia. After 10 months storage Tilapia silage sample had TBA content of 51.79. The values slightly increased after five months and rate of increase was less up to 7 months after which there was drastic increase in the TBA value of tilapia and silver carp silage. Tilapia silage had a very high TBA value after 10 months where as Jew fish silage was showing the lowest value among the three groups. TBA value increased in raw silage during fermentation and storage, while the same decreased in silage added with ethoxyquin and onion extract in 180 days of storage Fagbenro & Jauncey (1994). They have observed that the lipid stability was improved during ensilation by the addition of onion extract by lowering the TBA value.

Table 4.5.1. Proximate composition of dried silages from different species

	Jew fish	Tilapia	Silver carp
Moisture (%)	9.86±0.2^{c}	7.8±.021^{b}	6.41±0.04^{a}
Protein (%)	67.6±1.42^{b}	59.12±0.46^{a}	73.74±2.4^{c}
Fat (%)	11.96±0.84^{b}	18.27±0.6^{c}	8.534±0.85^{a}
Ash (%)	10.25±0.12	14.72±0.8	12.83±0.12

Results are presented as mean ± standard deviation (SD) of 3 replications. ^{a,b,c,d,e}, mean in a row with the same superscript values are not significantly different ($P<0.05$).

Table 4.5.2. Changes in NPN (mg/ 100g) during storage of dried silage prepared from different species of fish

Months	Jew fish	Tilapia	Silver carp
1	3228.7 ± 15.69^{a}	3257 ± 27^{c}	3248 ± 23.61^{b}
2	3256.3 ±18.04^{a}	3268 ± 15.3^{b}	3314 ± 17.21^{c}
3	3348.7 ± 11.53^{b}	3291 ± 32.0^{a}	3430.3 ± 25.13^{c}
4	3453.7 ± 12.89^{a}	3467.3 ± 24.0^{b}	3493.3 ± 34.62^{c}
5	3689.7 ± 22.52^{c}	3640.7 ± 27.0^{b}	3550.7 ± 26.07^{a}
6	3844.0 ± 23.61^{a}	3879.3 ± 33.8^{b}	3701.3 ± 23.51^{c}
7	3984.0 ± 37.94^{c}	3892.3 ± 25.9^{b}	3816.7 ± 27.23^{a}
8	4156.0 ± 29.54^{c}	4018.3 ± 21.5^{b}	3922.7 ± 24.16^{a}
9	4251.0 ± 33.61^{b}	4321.0 ± 36.0^{c}	4058.0 ± 33.61^{a}
10	4312.0 ± 26.24^{b}	4474.3 ± 25.7^{c}	4147.3 ± 26.03^{a}

Results are presented as mean ± standard deviation (SD) of 3 replications. ^{a,b,c,d,e}, mean in a row with the same superscript values are not significantly different ($P<0.05$).

Table 4.5.3. Changes in TVBN (mg/ 100g) during storage of dried silage prepared from different species of fish

Months	Jew fish	Tilapia	Silver carp
1	5.43 ± 0.47^{b}	8.17 ± 0.25^{c}	4.33 ± 0.15^{a}
2	11.27 ± 0.12^{c}	8.6 ± 0.17^{b}	7.03 ± 0.25^{a}
3	11.4 ± 0.26^{b}	9.73 ± 0.12^{a}	12.27 ± 0.06^{c}
4	13.23 ± 1.5^{a}	14.07 ± 0.4^{b}	14.7 ± 0.1^{c}
5	15.43 ± 1.3^{b}	14.6 ± 0.56^{a}	14.63 ± 0.57^{a}
6	14.93 ± 0.25^{a}	16.77 ± 0.25^{b}	15.4 ± 0.31^{a}
7	18.3 ± 0.1^{c}	17.03 ± 0.23^{b}	15.7 ± 0.26^{a}
8	25.5 ± 0.36^{c}	21.2 ± 0.53^{b}	16.63 ± 0.29^{a}
9	26.43 ± 0.47^{c}	23.83 ± 0.25^{a}	24.8 ± 0.3^{b}
10	32.3 ± 0.36^{c}	28.37 ± 0.4^{a}	29.83 ± 0.35^{b}

Results are presented as mean ± standard deviation (SD) of 3 replications. ^{a,b,c,d,e}, mean in a row with the same superscript values are not significantly different ($P<0.05$).

Table 4.5.4. Changes in PV (meq O_2/ kg fat) during storage of dried silage prepared from different species of fish

Months	Jew fish	Tilapia	Silver carp
1	2.41 ±0.02a	2.2 ± 0.1a	6.63 ± 0.04b
2	6.54 ±0.01b	4.55 ± 0.05a	15.57 ± 0.15c
3	15.57 ±0.21b	8.63 ± 0.31a	18.3 ± 0.1c
4	16.36 ±0.02b	14.3 ± 0.2a	18.97 ± 0.21c
5	19.5 ±0.02b	15.4 ± 0.26a	22.5 ± 0.3c
6	33.31 ±0.03c	22.33 ± 0.4a	24.8 ± 0.3b
7	40.91 ±0.03c	36.47 ± 0.06b	27.7 ± 0.35a
8	41.4 ±1.66b	46.77 ± 1.1c	33.1 ± 0.6a
9	66 ±1.91b	87.2 ± 4.25c	47.1 ± 0.26a
10	175.1 ±6.01c	140.6 ± 2.44b	56.5 ± 0.36a

Results are presented as mean ± standard deviation (SD) of 3 replications. ^{a,b,c,d,e}, mean in a row with the same superscript values are not significantly different ($P<0.05$).

Table 4.5.5. Changes in FFA (% oleic acid) during storage of dried silage prepared from different species of fish

Months	Jew fish	Tilapia	Silver carp
1	0.74 ± 0.16^{a}	1.44 ± 0.16^{b}	2.29 ± 0.07^{c}
2	2.45 ± 0.05^{a}	5.93 ± 0.25^{b}	6.54 ± 0.01^{c}
3	6.48 ± 0.08^{a}	11.17 ± 0.42^{b}	11.46 ± 0.37^{c}
4	12.37 ± 0.42^{a}	12.35 ± 0.39^{a}	12.63 ± 0.08^{b}
5	illegible	illegible	illegible
6	18.12 ± 0.51^{a}	36.81 ± 0.53^{c}	20.77 ± 0.19^{b}
7	22.5 ± 0.61^{a}	41.15 ± 1.03^{b}	22.37 ± 0.4^{a}
8	24.34 ± 0.31^{a}	42.63 ± 0.38^{c}	26.25 ± 1.88^{b}
9	25.78 ± 0.32^{a}	45.44 ± 0.36^{c}	27.32 ± 2.37^{b}
10	29.2 ± 0.51^{a}	48.24 ± 0.71^{b}	48.16 ± 0.96^{b}

Results are presented as mean ± standard deviation (SD) of 3 replications. ^{a,b,c,d,e}, mean in a row with the same superscript values are not significantly different ($P<0.05$).

Table 4.5.6. Changes in TBA during storage of dried silage prepared from different species of fish

Months	Jew fish	Tilapia	Silver carp
1	1.3 ± 0.06^{a}	4.52 ± 1.65^{c}	3.07 ± 0.15^{b}
2	2.13 ± 0.02^{a}	3.49 ± 0.03^{b}	4.56 ± 0.05^{c}
3	2.54 ± 0.09^{a}	4.77 ± 0.18^{b}	4.93 ± 0.05^{c}
4	2.7 ± 0.05^{a}	5.24 ± 0.01^{b}	5.56 ± 0.1^{c}
5	2.54 ± 0.04^{a}	5.36 ± 0.02^{b}	7.49 ± 0.4^{c}
6	4.54 ± 0.18^{a}	7.09 ± 0.12^{b}	8.2 ± 0.1^{c}
7	5.48 ± 0.11^{a}	12.5 ± 0.52^{c}	10.13 ± 0.13^{b}
8	5.23 ± 0.16^{a}	25 ± 0.62^{c}	15.5 ± 0.17^{b}
9	7.39 ± 0.13^{a}	36.51 ± 1.66^{c}	16.27 ± 0.32^{b}
10	7.63 ± 0.02^{a}	51.79 ± 1.14^{c}	25.1 ± 0.95^{b}

Results are presented as mean ± standard deviation (SD) of 3 replications ^{a,b,c,d,e}, mean in a row with the same superscript values are not significantly different ($P<0.05$).

4.6. Changes in the Bacterial Count of Fermented Silage.

4.6.1. Whole Tilapia Silage

4.6.1.1. Lactobacillus count

Changes in Lactobacillus count during ensilation of whole tilapia at different levels of jaggery is given in Fig 4.6.1.1. At 5% level of jaggery, there was an initial lagging till 2nd day of fermentation. The pH of this set showed the highest value of 5.26 on 2nd day. But on 4th day of inoculation, the counts increased to log 8.3 and remained more or less same during the next observation. The count showed a drastic reduction on next observation, which could be due to the depletion of jaggery and consequent growth of spoilage bacteria. But in case of samples with 10% and 15% jaggery levels, the LAB count increased initially upto 6th day of fermentation and thereafter showed a declining trend indicating the depletion of carbohydrate source. As a result during the next observation on 9th day and 14th day the lactobacillus count was reduced to log 6.3 in the case of 10% jaggery and log 7.4 in the case of 15% jaggery. A higher count in 15% jaggery is due to the presence of additional sugar. Fadda *et. al., (2002)* observed that, the Lactobacillus count remained at 10^8 CFU/ g through out the course of fermentation in a model sausage fermentation system. Whereas, according to Neethiselvan *et. al.,* (2001) the LAB count increased from the initial 1.6 X 10^6 /g on 5th day and it increased to reach a maximum count of 1.9 X10^7 /g on 70th day. According to Petaja *et.al.*, (2000) counts of Pseudomonas, which were the predominant bacteria of fish raw material, were completely suppressed by fermentation with lactobacillus for a period of 7 days. Whereas Kim *et.al.*, (1999)observed that LAB (*Lactococcus lactis*) is able to produce a bacteriocin capable to reduce the total bacterial load.

4.6.1.2. Total Plate Count

Changes in Total Plate Count (log cfu/ml) of whole tilapia during ensilation at different levels of jaggery is given in Table 4.6.1.2. The TPC levels during ensilation is showing an inverse relation as that of LAB count. For the initial 4 days, no TPC was detected indicating the inhibition of spoilers by

Lactobacillus bacteria. In sample with 5% jaggery, the total plate count observed on 6th day was 2.4 CFU/ml which increased to 10 CFU/ ml on 9th day and sample got spoiled. Depletion of jaggery and consequent decrease in lactobacillus count and increased pH caused the growth of spore forming spoilage bacteria, which might have caused the spoilage of samples. In samples with 10% and 15% jaggery, TPC was not detected till 10th day. This could be due to the inhibition of normal bacterial population by lactobacillus. According to Kannappan & Manja (2004), *Lactobacillus plantarum* showed the highest inhibition on *Bacillus cereus*. But since the jaggery supplied is limited by the time of 12 days, and due to release of other volatile compounds, the pH started increasing which consequently resulted in the growth of other spoilage organisms as evidenced by the increase in count of TPC.

4.6.2. Dressed Tilapia Silage

4.6.2.1. Lactobacillus count

Alterations in Lactobacillus count during ensilation of dressed tilapia at different levels of jaggery are given in Fig. 4.6.2.1. The LAB count of all the three samples increased initially indicating the utilization of jaggery by the starter culture. In 5% sample, the count increased up to four days and showed decreasing trend. But in whole tilapia the count increased up to 7 days. This could be due to the presence of partially digested proteins in whole samples by the action of intestinal cathepsins during the initial cooking. The lactobacillus count in dressed fish silage was found to be higher, which could be due to the nature of sample. The lactobacillus count declined in case of samples with 10% and 15% jaggery after 6 days. But the reduction was very less which could be due to the presence of sufficient quantity of sugar for the substrate. Through out the period of fermentation, the count of lactobacillus remained in the range of 7.4 log and 10.0 log CFU/ ml indicating satisfactory conditions of fermentation. According to Bello *et.al* (1993), raw material showed high counts of aerobic mesophilic and psychotropic organisms, in addition to Pseudomonas, coliform

and *S. aureus* when dressed fish is used for fermentation. However silage showed only a few aerobic mesophilic organisms due to low pH values and development of lactic acid bacteria.

4.6.2.2. Total Plate Count

Table 4.6.2.2. shows the total plate count (log cfu/ml) of dressed tilapia during ensilation at different levels of jaggery. During the initial 4 days no TPC was detected in any of the samples. On 7th day at 5% level, TPC to the tune of 6 log CFU/ ml was detected. But on 7^{th} day no TPC was noted in other two samples, which could be due to the low pH and the presence of higher levels of lactobacillus count. On 9th day the TPC increased to 7.3 CFU/ ml indicating the success of spoilage flora over lactobacillus due to the depletion of sugar. The plate count was detected in other two groups on 9th day of fermentation and a slow increase in count was observed. The spore formers that might have survived the initial heat treatment have germinated and started multiplying. But According to Petaja *et.al.*, (2000) counts of Pseudomonas, which were the predominant bacteria of fish raw material, were completely suppressed by fermentation with lactobacillus for a period of 7 days. The initial reduction in total plate could be also due to the production of bacteriocin by LAB capable to reduce the total bacterial load as reported by Kim *et.al.* (1999).

The TPC colonies were isolated, recultured and was identified to be *Bacillus sp.* Bello *et.al* (1993) observed that silage dehydration reduces possibilities of microbial growth, and only spores of Bacillus were observed. According to Zuberi *et.al* (1988) Bacilli predominated in the aerobic plate count of fermented fish sauce prepared from sardine after a particular period of fermentation. Pathogens like *staphylococcus* and *salmonella* were not detected during fermentation. This could be due to the low pH of the medium.

Fig. 4.6.1.1 Changes in Lactobacillus count (log cfu/ml) during ensilation of whole tilapia at different levels of jaggery

Days	Level of jaggery		
	5%	10%	15%
1	7.54±0.22	7.54±0.23	7.54±0.21
2	7.34±0.84[a]	8.40±0.52[b]	9.69±0.18[c]
4	8.33±0.23[a]	9.69±0.32[b]	9.89±0.8[b]
6	8.42±0.21[a]	9.68±0.35[b]	9.82±0.12[c]
9	4.21±0.11[a]	8.42±0.21[b]	9.35±0.27[c]
13	ND	6.39±0.14[a]	7.43±0.34[b]

Results are presented as mean ± standard deviation (SD) of 3 replications [a,b,c,d,e], mean in a row with the same superscript values are not significantly different (P<0.05).

4.6.1.2. Changes in Total Plate Count (log cfu/ml) of Whole Tilapia During Ensilation *at different levels of jaggery*

Days	Level of jaggery		
	5 %	10 %	15 %
2 day	Not detected	Not detected	Not detected
4 day	Not detected	Not detected	Not detected
6 day	2.49 ± 0.14	Not detected	Not detected
9 day	Not done	2.72 ± 0.21[a]	2.66 ± 0.19[a]
13 day	Not done	5.68 ± 0.20[b]	5.19 ± 0.06[a]

Results are presented as mean ± standard deviation (SD) of 3 replications [a,b,c,d,e], mean in a row with the same superscript values are not significantly different (P<0.05).

Fig. 4.6.2.1.Changes in Lactobacillus count (log cfu/ml) during ensilation of dressed tilapia at different levels of jaggery

Days	Level of jaggery		
	5 %	10 %	15 %
1	7.54±0.55	7.54±0.34	7.54±0.45
2	8.48±0.41	9.51±0.11	8.71±0.23
4	9.65±0.56	9.84±0.24	9.77±0.32
6	7.40±0.38[a]	9.51±0.21[b]	10.05±0.46[b]
9	3.24±0.12[a]	8.72±0.14[b]	9.69±0.57[c]
13	---	8.29±0.22[a]	8.57±0.52[b]

Results are presented as mean ± standard deviation (SD) of 3 replications .[a,b,c,d,e], mean in a row with the same superscript values are not significantly different (P<0.05).

Table 4.6.2.2 Changes in Total Plate Count (log cfu/ml) of Dressed Tilapia during Ensilation at Different Levels of jaggery

Days	Level of jaggery		
	5 %	10 %	15 %
2 day	Not detected	Not detected	Not detected
4 day	2.67±0.16	Not detected	Not detected
6 day	6.63±0.17	Not detected	Not detected
9 day	Not done	4.23±0.09a	3.68±0.12b
13 day	Not done	4.99±0.12	5.12±0.21

Results are presented as mean ± standard deviation (SD) of 3 replications .[a,b,c,d,e], mean in a row with the same superscript values are not significantly different (P<0.05).

4.7. Nutritional Evaluation of Fermented Fish Silage by Rat Feeding Studies

4.7.1. Proximate composition

Proximate composition of feed used for rat feeding study is given in Table 4.7.1. The control feed which was containing casein as protein source was having 54% protein and nearly the same levels of protein were there in other samples also. But in case of acid silage the protein content was found to be 43.1%. This could be due to higher moisture levels and ash content in acid silage. The crude fat content in all the samples were higher than that of the control group.

4.7.2. Amino acid profile of dried fish silage

The amino acid profile of the dried fermented fish silages incorporated in feed is given in Table 4.7.2., which indicates that no significant difference was noticed in the amino acid profile of the samples used. However, the histidine content of Jew fish samples were higher when compared to other groups and silver carp had the lowest levels of histidine (2.8 mg/100g). But in silver carp silage, arginine content was found to be higher and the same was lower in case of tilapia. The difference in the quantity of amino acids could be due to the species difference.

4.7.3. The amino acid profile of feed

The amino acid profile of feed used for nutrition study in rats is given in Table 4.7.3. and chemical score is given in Table 4.7.3.1 In all the compounded samples including acid silage amino acids like Aspartic acid, threonine, serine, glycine and alanine were found to be in higher levels than the control feed; whereas valine, histidine and agrinine were in lower levels. The differences in amino acid levels might have affected the growth rate of different groups. The chemical score of the important amino acids indicate that in all the samples the chemical score was found to be less in case of all amino acids except

tryptophan. The reduction in amino acid score in different types of silages prepared has been reported by Vidotti *et.al.*, (2003).

4.7.4. Protein Efficiency Ratio (PER)

The Protein Efficiency Ratio of rats supplemented with different silages prepared is given in Fig. 4.7.4. The PER of all the samples showed almost the similar trend silver carp silage supplemented groups which showed significantly low PER. This could be due to the nutritional peculiarity of the species. Neethiselvan *et.al.*, (2001) reported a higher PER for *E. suratensis* fed with fermented fish silage based diets than the control diets based on plant protein. According to Gonclaves *et. al.*, (1989), eel fingerlings showed better FCR and PER with silage diets compared to control. Rodriquez *et.al* (1990) similar PER in rats fed with acid fish silage when compared to casein diet. Kompiang *et.al* (1977) reported a better feed conversion ratio in all the five silage incorporated feeds at different levels with the highest value for 29% silage in the feed.

4.7.5. True digestibility (TD)

True digestibility of feeds supplemented with different silages prepared is given in Fig 4.7.4. True digestibility of all the experimental samples showed significantly higher values when compared to the control. Even though all the samples showed higher values; acid fish silage showed the highest value of more than 65%, which could be due to the presence of higher levels of hydrolysed proteins in the acid silage. Among the fermented silages, Jew fish silage supplemented rats showed higher value indicating that the protein of this species is of better quality. According to Marit *et.al* (1989) True Digestibility of acid silages stored for different period does not show significant difference even after storage for 180 days. However, decreased utilization of protein in stored silage has been reported in experiments with rats by Stone & Hardy (1986).

4.7.6. Apparent Nitrogen Digestibility (AND)

Apparent Nitrogen Digestibility of rats supplemented with different feeds studied is given in Fig 4.7.4. Apparent Nitrogen Digestibility of all the

experimental groups fed with silage were found to be significantly less than the control groups. This could be due to absorption of excess of free amino acid present in the silage samples by the animals, thus directing more of the amino acids from the diet into the catabolic pathway (Yamada *et. al.*, 1981). But according to Raa & Gildberg (1982), the nutritional difference of different fish silage products reported may be related to the quality of the raw material used. Luiz *et. al.*, (2006) suggested that the use of fermented fish silages incorporated feeds is linked to the characteristics of its protein fractions and may be supplied as a source of less soluble proteins.

4.7.7. Net Protein Ratio (NPR)

Net Protein Ratio of rats supplemented with different feeds studied is given in Fig. 4.7.4. The silver carp silage was found to have significantly lower NPR indicating the poor quality of the protein when compared to other groups. But the Jew fish silage fed groups were having significantly higher levels NPR, which is confirming the high quality of the protein which can be confirmed from other indices studied.

4.7.8. Percentage of weight gain

Figure 4.7.8. shows the percentage of weight gain in normal and experimental groups of rats. There was no significant change observed in the body weight gain in fermented fish silage supplemented groups as compared to that of control and acid silage fed rats. It is interesting to note that among the different groups, fermented Jew fish silage administered rats exhibited a comparatively high body weight gain. Silver carp fermented silage supplemented animals was found to possess less body weight gain as compared to that of other groups.

4.7.9. Weekly average weight gain

Table 4.7.5 depicts the weekly average weight gain in different groups of rats. The weight gain pattern of the groups was almost similar and, no significant difference was observed among the groups. However, rats fed with Jew fish

silage showed better weight gain indicating better quality of the silage produced from the species. The control group and tilapia silage fed group showed similar weight gain and acid silage and silver carp silage showed a marginally less weight gain. According to Yone *et.al,* (1986), and Baraquet & Lindo (1985) the microbial fermentation alters the composition of crude protein and dry matter and increases volatile nitrogen, which could have resulted in the difference of weight gain in different experimental groups. Fagbenro and Jauncey (1994) observed that the weight gain of *Clarius garipinus* fed with fermented whole tilapia silage in combination with other fillers like soy bean meal, feather meal, poultry waste meal etc, was not significantly different.

4.7.10. Hepato-somatic and cardio-somatic index

Figure 4.7.10 and 4.7.10.1 depict the Hepato-somatic and cardio-somatic index of normal and experimental groups of rats respectively. Though the overall body weight gain of the animals not showed any significant difference, there were significant changes observed in the hepatosomatic and cardio somatic indices. Significant reduction in the hepatosomatic index was noticed in silver carp fermented silage fed rats compared to that of other groups of rats indicating the beneficial nature of silver carp silage supplementation. However, in the case of cardio somatic index, acid silage fed animals showed significantly high value as compared to that of other groups showing the adverse nature of acid silage supplementation. According to Fagbenro and Jauncy (1994) the Hepato- somatic index of Clarius fed with a combination of fermented fish silage and soybean meal showed similar values as that of hydrolysed poultry byproducts meal or feather meal. A slight increase in the total cholesterol levels was noted in rats fed with tilapia silage and silver carp silage when compared to control. According to Kissler *et.al.,* (2005), the increased cholesterol levels in the liver might be due to increased uptake of LDL from the blood by the tissue.

4.7.11. Total lipid content in heart and liver

Total lipid content in heart and liver of rats fed with fermented silage prepared from different species of fish is given in Fig. 4.7.11. Significantly higher

levels of lipid accumulation was noted in the liver and heart of rats fed with fermented tilapia silage. In all heart samples, the lipid accumulation was found to be more than the control group of rats. But in case of fermented tilapia silage supplemented groups, the accumulation was found to be very high, which could be due to the presence of some specific metabolites found in tilapia silage which is indicating that, the lipid content in tilapia could have some toxic substances accumulated from the environment.

4.7.12. Total cholesterol in the heart and liver

Total cholesterol content in the heart and liver of experimental groups of rats is given in figure 4.7.12. The total cholesterol content of heart in all the experimental groups were found to be higher when compared to control group. But significantly higher levels of cholesterol was noted in tilapia silage and acid silage. Hypercholesterolemia noted in tilapia silage fed animals might be either due to its high lipid content or high level of pollutant accumulation such as pesticide residues and/or heavy metal content. In case of acid silage administered group, it might be due to its toxic nature. Hypercholesterolemia has long been recognized as one of the major risk factors responsible for hepatic and cardiac dysfunction. High level of circulating cholesterol and its accumulation in these tissues are well associated with vascular endothelial aberrations (Joan *et al.*, 1984). The level of total lipid in liver and heart tissue also confirms with the above findings. No significant alteration observed in the cholesterol content in serum, liver and heart tissue of silver carp and Jew fish silage supplemented groups of rats as compared to that of normal control animals, indicating the normo-lipidemic nature of these silage preparations. Wergedahl *et.al,* (2004) observed that in Zucker rats both FPH and soy protein treatment reduced the plasma cholesterol level. Further more, the HDL cholesterol: total cholesterol ratio was greater in these rats and in the Wistar rats fed with FPH and soy protein compared with those fed casein. But according to Kissler *et.al.,* (2005), the increased cholesterol levels in the liver might be due to increased uptake of LDL from the blood by the tissue.

4.7.13. Triglycerides content in liver and heart

Fig. 4.7.13 show the level of triglycerides content in liver and heart tissue of normal and experimental groups of rats. The level of triglycerides was significantly high in serum and heart tissue of tilapia silage supplemented rats, while it was significantly low in liver. Acid silage fed group registered increase in triglycerides content in liver and heart tissue of experimental rats. The abnormal accumulation of triglycerides may lead to structural alterations and functional disorders in the liver and heart tissue. Moreover, higher triglycerides accumulation in tissues also attributes to the increased formation of lipid peroxides, which is one of the major causative factors involved in the necrotic damage to the cellular and subcellular membranes. Hypertriglyceridemia has been reported to be associated with structural and functional aberrations in the liver and heart tissue (Freedman *et al.*, 1988). No significant variation observed in the triglycerides content in Jew fish silage fed rats as compared to normal control rats. However, a slight reduction in triglycerides content was noticed in the heart tissue of silver carp silage administered rats, showing the hypotriglyceridemic property of silver carp silage.

The triglyceride levels in the liver were higher when compared to triglycerides in the heart. Increased lipolysis of depot triglycerides liberates free fatty acids from adipose tissue stores (Kruger *et al.*, 1967: Stenberg, 1976) and the free fatty acids liberated by the adipose tissue are taken up by the liver tissue leading to hypertriglyceridemic condition. However, in case of tilapia silage supplemented rats, the triglyceride levels were found to be less in liver tissue than heart tissue. In Zucker rats Wergedahl. *et.al.*, (2004) observed that hydrolysed fish protein (FPH) treatment affected the fatty acid composition in liver, plasma, and triacylglycerol-rich lipoproteins.

4.7.14. Free fatty acids in liver and heart

Figure 4.7.14. show the levels of free fatty acids in liver and heart tissue of normal and experimental groups of rats. The levels of free fatty acids

were significantly high in liver and heart tissue of tilapia silage supplemented rats as compared to the control and other groups of rats. The levels of free fatty acids were also registered at significantly higher in acid silage supplemented groups of rats, but it was comparatively lesser to that of fermented tilapia silage. The free fatty acids content in the serum was significantly high in acid silage supplemented rats. A slight increase in free fatty acids content in serum of silver carp silage administered groups of rats as compared to that of normal control and Jew fish silage fed rats. In general, the free fatty acids liberated from adipose tissue stores enter into the hepatic and cardiac tissues and this process is proportional to the free fatty acid concentration in the sinus. Though these organs can utilize free fatty acids for their energy requirements, the excess free fatty acid may be used for the synthesis of triglycerides, which in turn results in hypertriglyceridemic condition, as observed in the present study. It is very much interesting to note that there were no significant variations observed in the free fatty acids content in liver and heart tissue of Jew fish and silver carp silage administered animals. However, the free fatty acid content was found to be slightly higher than that of control rats.

4.7.15. Phospholipids in liver and heart

Figure 4.7.15 shows the levels of phospholipids in liver and heart tissue of normal and experimental groups of rats. The levels of phospholipids were significantly higher in liver and heart tissue of tilapia silage supplemented rats as compared to the control and other groups of rats. However, there was no significant change observed in the phospholipids concentration in serum of fermented tilapia silage fed rats, indicating a phospholipidosis condition. There was a significant reduction noticed in the levels of phospholipids content in serum of acid silage administered rats compared to that of other groups of rats. The presence of high content of fat in the tilapia might have led to the increased accumulation of phospholipids in this group. Since cell membranes are rich sources of phospholipids rich in polyunsaturated fatty acids, they are easily

susceptible to free radical mediated oxidative damage. The results of the present investigation show that fermented fish silage mediated phospholipidosis may favor the cellular and sub cellular membranes to peroxidative damages. As the oxidative destruction of polyunsaturated fatty acids in the membrane phospholipids proceeds as a self-perpetual chain reaction, the accumulation of phospholipids in the liver and heart tissue makes cell membranes more easily susceptible to oxidative deterioration.

Table. 4.7.1. Proximate composition of silage incorporated feed used for rat feeding study (values in %).

	Moisture	Crude Protein	Carbohydrate	Crude fat	Ash
Control	9.56	54.07	28.21	3.18	5.80
Jew fish	8.51	51.16	27.5	5.19	7.82
Tilapia	8.27	50.02	27.3	6.42	8.46
Silver carp	7.66	52.88	26.6	4.36	8.73
Acid silage	12.48	43.1	28.6	6.01	9.84

Table 4.7.2. Amino acid profile (mg/ 100g) of dried silage prepared from different species used for rat feeding study

	Jew fish	Tilapia	Silver carp
Asp	10.69	11.00	10.12
Thr	4.76	4.58	4.02
Ser	5.77	6.63	5.32
Glu	12.67	12.95	13.53
Pro	1.14	1.29	0.81
Gly	10.47	13.84	13.00
Ala	7.59	9.11	8.19
Cys	0.00	0.00	0.00
Val	5.23	4.00	6.24
Met	3.07	3.06	2.12
Ile	4.91	4.41	5.21
Leu	8.35	7.74	7.47
Tyr	3.05	2.25	1.40
Phe	4.27	3.76	4.15
Try	2.16	1.15	1.70
His	4.49	3.64	2.80
Lys	1.87	1.31	1.12
Arg	2.65	1.53	5.51

Table. 4.7.3. Amino acid composition (mg/100g) of feed used for rat study *prepared from silage of different species.*

	Control	Jew fish	Tilapia	Silver carp	Acid silage
Asp	7.07	9.81	9.33	10.06	8.82
Thr	3.73	4.17	4.21	4.47	4.32
Ser	7.28	7.28	5.91	6.51	6.21
Glu	18.92	18.40	17.02	17.37	17.08
Pro	0.00	1.30	1.53	1.42	1.41
Gly	4.10	9.45	8.60	10.97	10.15
Ala	3.03	6.93	5.77	7.36	5.98
Cys	0.00	0.61	0.00	0.36	0.39
Val	*7.29*	*5.85*	*4.62*	*5.03*	*7.44*
Met	1.80	2.86	1.67	1.60	1.73
Ile	5.07	5.52	5.88	4.89	5.43
Leu	8.50	8.37	8.70	8.48	8.63
Tyr	1.26	1.25	3.27	1.91	1.65
Phe	3.64	3.36	4.97	4.04	3.52
Try	1.61	2.38	1.83	1.06	0.98
His	12.84	3.87	4.76	3.16	4.44
Lys	1.62	1.15	1.13	1.23	1.05
Arg	5.87	1.87	3.64	2.14	1.77

Table 4.7.3.1. Chemical score for important amino acid in silages prepared from different raw materials.

Amino acid	FAO/WHO (1985) standards	Tilapia silage	Silver carp silage	Jew fish silage
Tryptophan	1	3.15	1.23	1.53
Lysine	5.5	0.16	0.22	0.21
Arginine	5	1.03	0.39	0.23
Valine	5	0.54	0.99	1.16
Methionine	3.5	0.81	0.57	0.68
Isoleucine	4	1.39	1.29	1.31
Leucine	7	1.13	1.14	1.15

Chemical score = (g amino acid/100 g test protein)/(g amino acid/100 g standard protein).

Table. 4.7.4. Nutritional studies of control feed and fish silage prepared from different types of fish

	NPR	PER	TD	AND
Control	0.52 ±0.02^{b}	1.63 ±0.07^{b}	48.40 ±1.8^{a}	51.59 ±3.2^{e}
Jew fish	0.61 ±0.04^{c}	1.78 ±0.06^{c}	64.09 ±1.4^{c}	35.91 ±1.8^{b}
Tilapia	0.52 ±0.1^{ab}	1.72 ±0.21^{c}	56.47 ± 2.1^{b}	43.53 ±2.4^{d}
Silver Carp	0.49 ±0.15^{a}	1.38 ±.01^{a}	63.19 ±2.4^{c}	36.81±1.4^{c}
Acid silage	0.50ab	1.85^{d}	65.88^{e}	34.12 ^{a}

Results are presented as mean ± standard deviation (SD) of 3 replications ^{a,b,c,d,e}, mean in a row with the same superscript values are not significantly different (P<0.05).

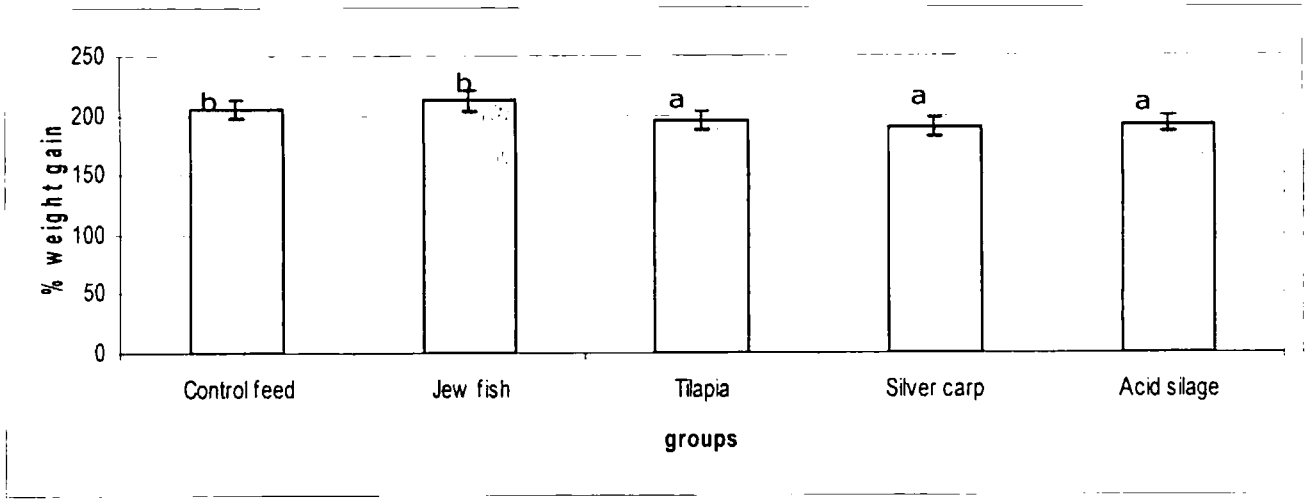


Fig. 4.7.8. Weight gain (%) in normal and experimental groups of rats

Results are presented as mean ± standard deviation (SD) of 3 replications. ^{a,b,c,d,e}, mean in a row with the same superscript values are not significantly different (P<0.05).

Table 4.7.5. Weekly average weight gain (g) in different groups of rats

Weeks	Control diet	Jew fish silage	Tilapia silage	Silver carp silage	Acid silage
I	59.4±1.1	59.4±1.7	60.4±1.6	59.4±2.2	59.6±.8
II	70.6±1.8	71.2±2.4	69.8±3.2	68.8±4.2	69.6±1.4
III	105.4±2.1	106.4±2.2	105±3.4	102.4±3.7	102.2±2.1
IV	130.8±2.8	135.8±3.4	130.6±5.4	127.2±5.1	128.6±3.6
V	152.4±3.8bc	56±1.6c	150.4±7.6ab	146.4±2.8a	46.4±2.1a
VI	181.2±4.1bc	185.6±3.8c	178.2±5.6ab	172.2±6.7a	174.2±11.0ab

Results are presented as mean ± standard deviation (SD) of 3 replications. ^{a,b,c,d,e}, mean in a row with the same superscript values are not significantly different ($P<0.05$).

4.7.16. Serum

The results of analysis of Serum cholesterol, LDL cholesterol, HDL cholesterol, free fatty acids and phospholipids is given in Figures 4.7.3.1. - 4.7.3.5.

4.7.16.1. Serum cholesterol

Serum cholesterol of rats fed with different types of silages is given in Fig. 4.7.3.1. The serum cholesterol levels of the control group, Jew fish silage fed group and silver carp silage fed groups does not show significant difference, but at the same time the levels of cholesterol in serum of tilapia silage fed group and acid silage fed group showed significantly higher levels of cholesterol in the serum. The significant reduction in the serum cholesterol could be due to fermentation since, the lactic acid fermentation has a beneficial effect on the lipids in fish silage stabilizing the oil and improving its acceptability in animal diet Raa & Gildberg (1982). But the higher levels of serum cholesterol in Tilapia silage fed rats could be those present in the fish used for silage preparation. According to Santhosh (2007) animals with induced stress could contain higher levels of serum cholesterol.

4.7.16.2. LDL cholesterol and HDL

Figure 4.7.16 and Fig. 4.7.16.1. illustrate the levels of LDL cholesterol and HDL cholesterol in serum of normal and experimental groups of rats. There were significant rise observed in the level of total cholesterol of fermented tilapia silage and acid silage administered group of rats compared to the control and other groups. These groups also registered significant rise in the levels of LDL cholesterol in serum with concomitant decline in HDL cholesterol level. Significantly high level of tissue cholesterol content was observed in liver and heart tissue of these groups of animals. The abnormal cholesterol deposition is favored by the dangerous tendency of cholesterol to passive exchange between the plasma lipoproteins and the cell membranes (Brown and Goldstein, 1986).

The serum LDL cholesterol levels of tilapia silage fed groups and acid silage fed rats were found to be higher when compared to other groups. Consequently, the HDL cholesterol levels were found to have an inverse relationship with LDL and HDL cholesterol was found to be significantly higher in both the groups of rats. The lower levels of HDL in tilapia silage fed groups could be due to specific nature of the lipid content of tilapia used.

4.7.16.3. Serum triglycerides

Serum triglycerides of rats fed with different types of silages is given in Fig. 4.7.3.3. As in the case of serum cholesterol, the serum triglycerides in tilapia silage fed groups of rats were found to significantly higher than other groups including the control group which could be due to increased lipolysis of depot triglycerides.

4.7.16.4. Free Fatty Acids and phospholipids

Serum Free Fatty Acids of rats fed with different types of silages is given in Fig. 4.7.16.4. The serum free fatty acids of silver carp silage fed rats and acid silage supplemented rats were found to be significantly higher when compared to other three groups. The significantly higher levels of free fatty acids in acid silage fed groups could be due to the quality of raw material used, which included the processing waste of fresh water fish. This could have higher levels of toxigenic material accumulated in their liver which was ensilaged and used for the study. *Serum phospholipids of rats fed with different types of* silages is given in Fig. 4.7.16.5. the serum phospholipids of rats supplemented with acid fish silage was significantly low, indicating that the phospholipids were converted to free fatty acids.

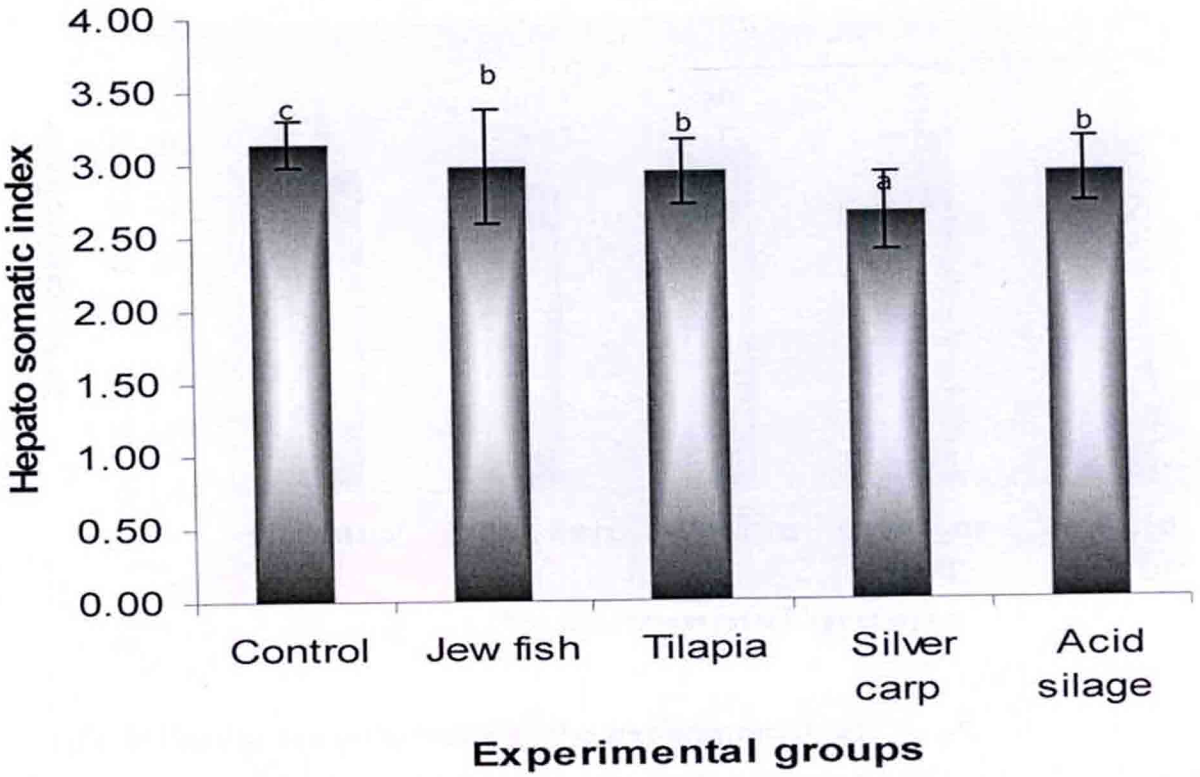


Figure 4.7.10. Hepato somatic index of the experimental rats

Results are presented as mean ± standard deviation (SD) of 3 replications ^{a,b,c,d,e}, mean in a column with the same superscript letters are not significantly different ($P<0.05$)

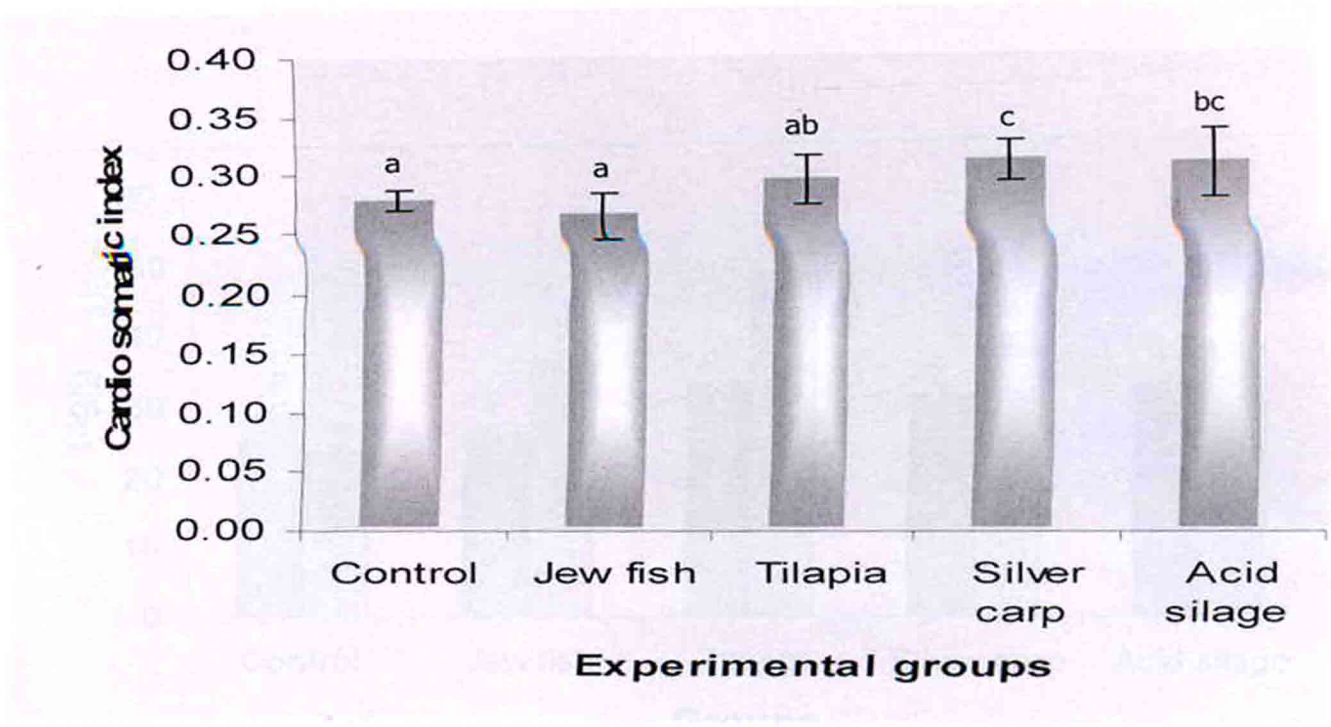


Fig. 4.7.10.1 Cardio-somatic index of the experimental rats

Results are presented as mean ± standard deviation (SD) of 3 replications ^{a,b,c,d,e}, mean in a column with the same superscript letters are not significantly different ($P<0.05$)

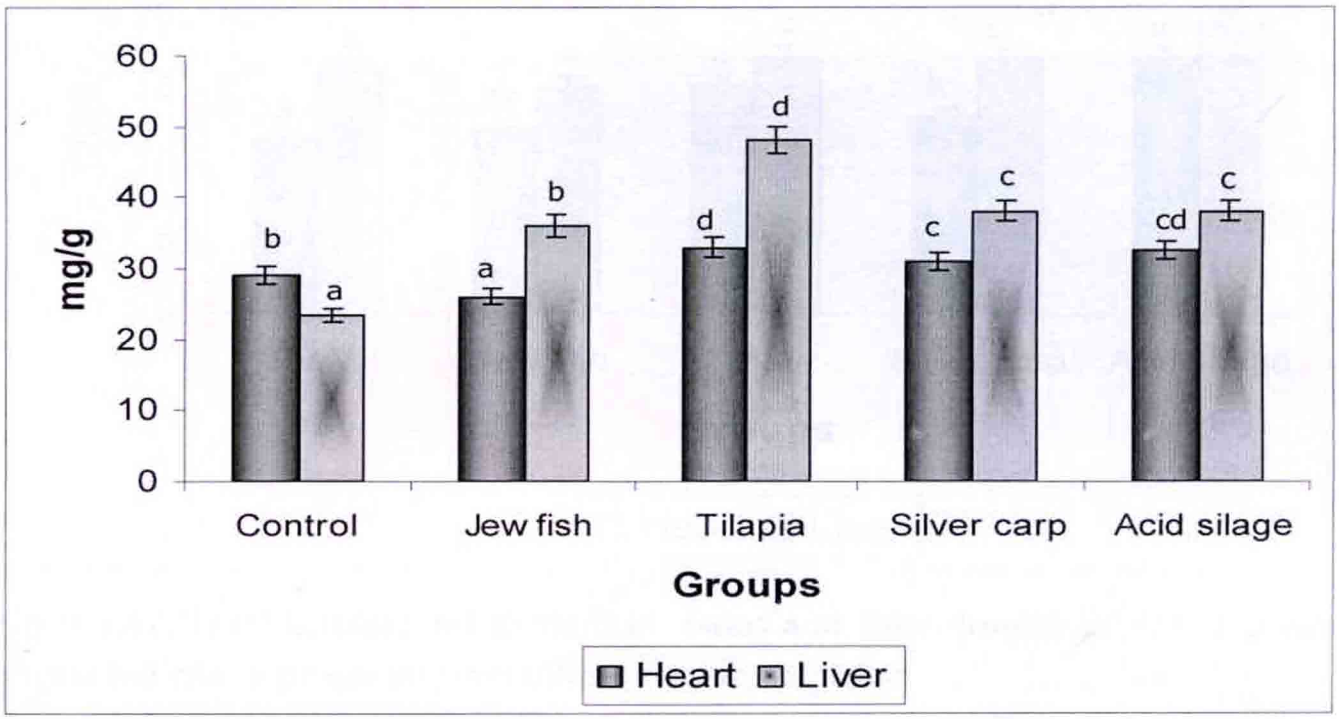


Fig. 4.7.11. Total lipid content in heart and liver of rats (mg/g) fed with fermented silage prepared from different species of fish

Results are presented as mean ± standard deviation (SD) of 3 replications ^{a,b,c,d,e}, mean in a column with the same superscript letters are not significantly different ($P<0.05$)

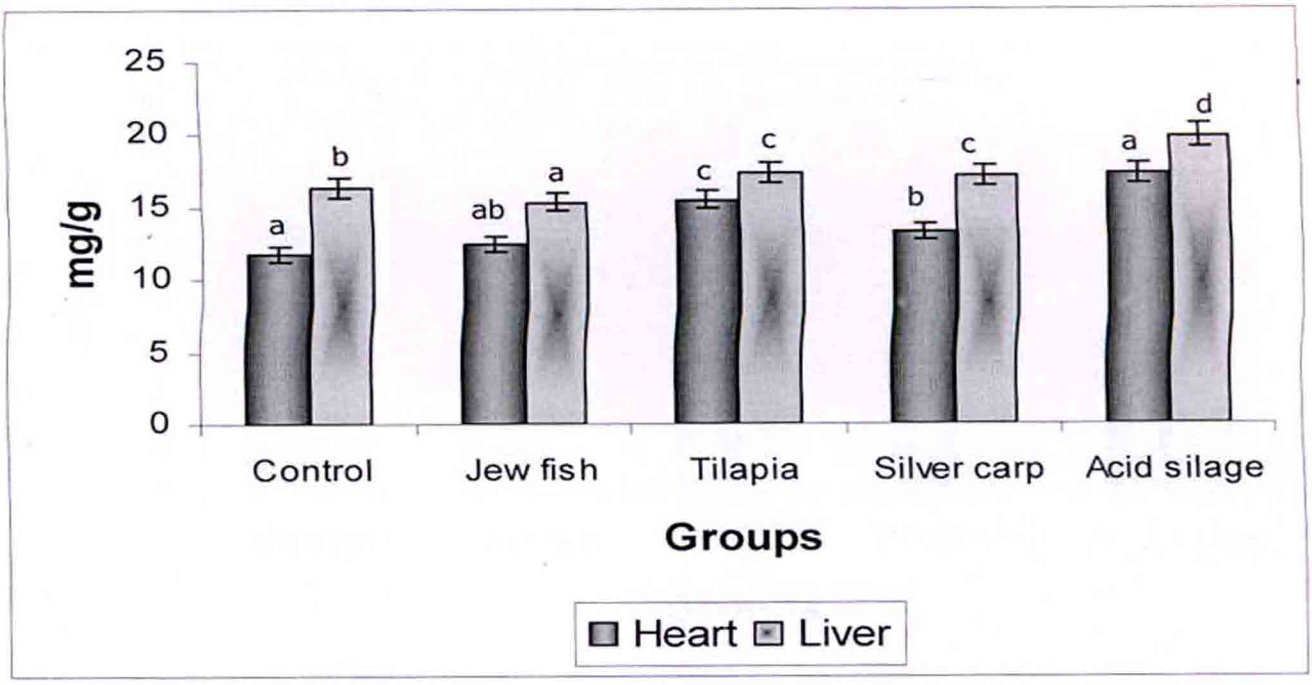


Fig. 4.7.12. Total Cholesterol content in heart and liver (mg/g) of rats fed with fermented silage prepared from different species of fish

Results are presented as mean ± standard deviation (SD) of 3 replications ^{a,b,c,d,e}, mean in a column with the same superscript letters are not significantly different ($P<0.05$)

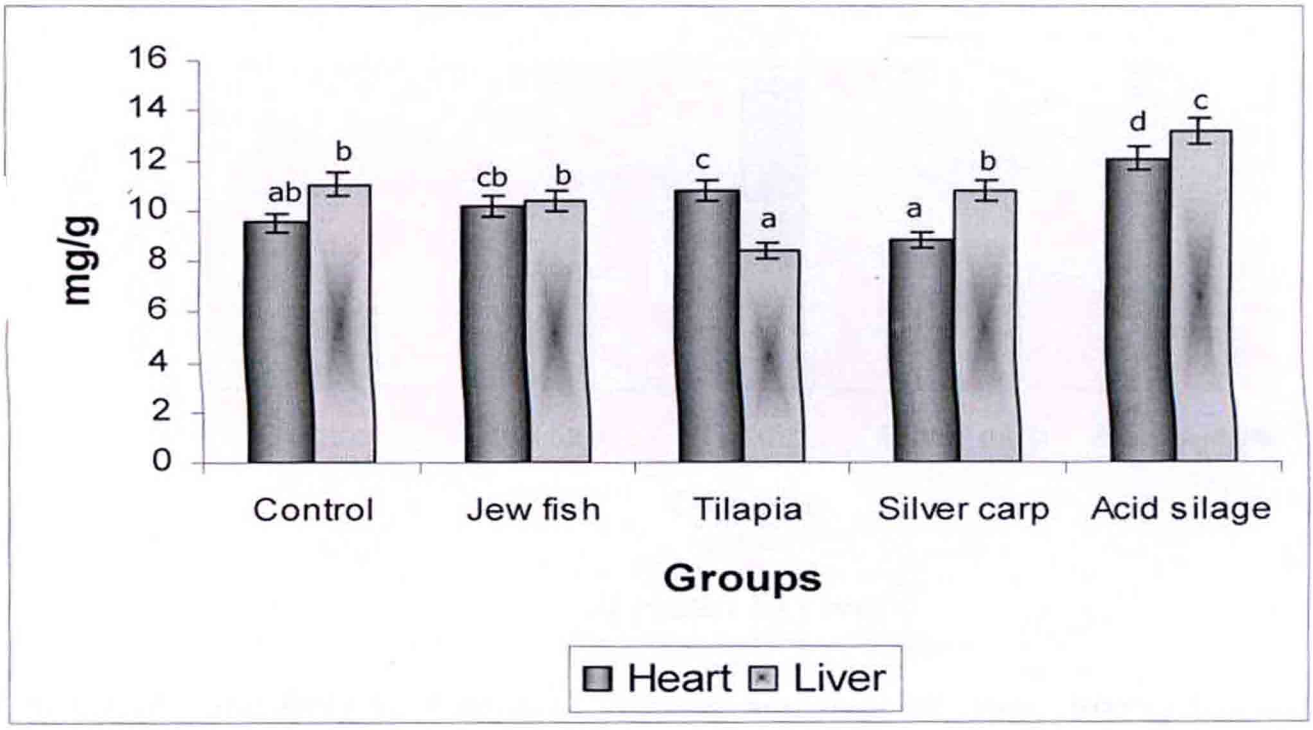


Fig. 4.7.13. Triglycerides content in liver and heart tissue (mg/g) of normal and experimental groups of rats

Results are presented as mean ± standard deviation (SD) of 3 replications ^{a,b,c,d,e}, mean in a column with the same superscript letters are not significantly *different (P<0.05)*

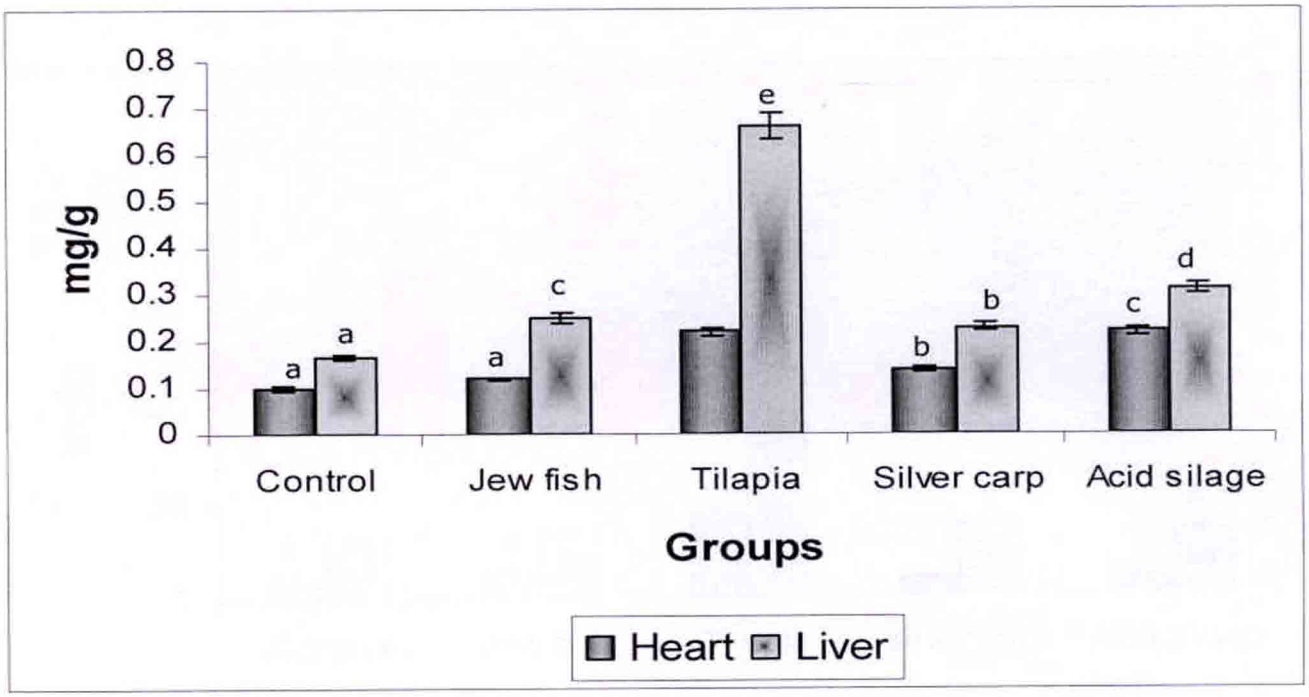


Fig. 4.7.14. Free fatty acid content in heart and liver of rats (mg/g) fed with fermented silage prepared from different species of fish

Results are presented as mean ± standard deviation (SD) of 3 replications ^{a,b,c,d,e}, mean in a column with the same superscript letters are not significantly different ($P<0.05$)

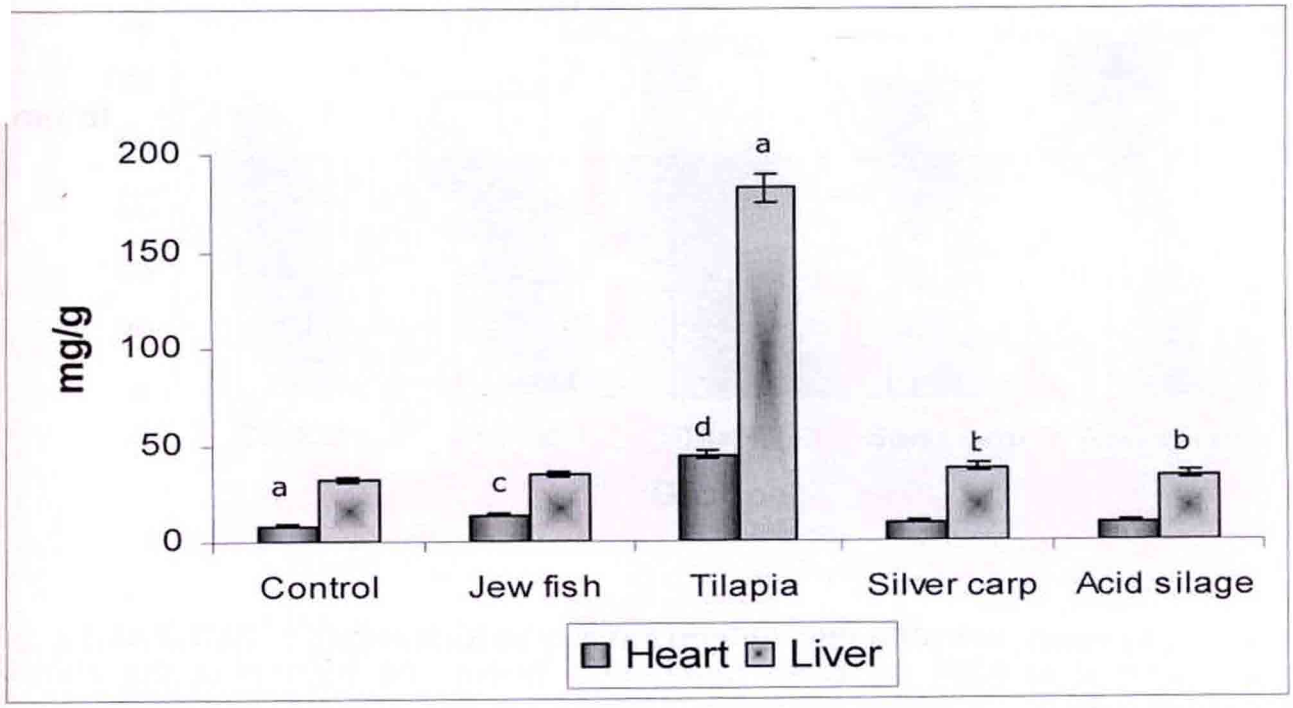


Fig. 4.7.15. Phospholipid content in heart and liver of rats (mg/g) fed with fermented silage prepared from different species of fish

Results are presented as mean ± standard deviation (SD) of 3 replications ^{a,b,c,d,e}, mean in a column with the same superscript letters are not significantly different ($P<0.05$)

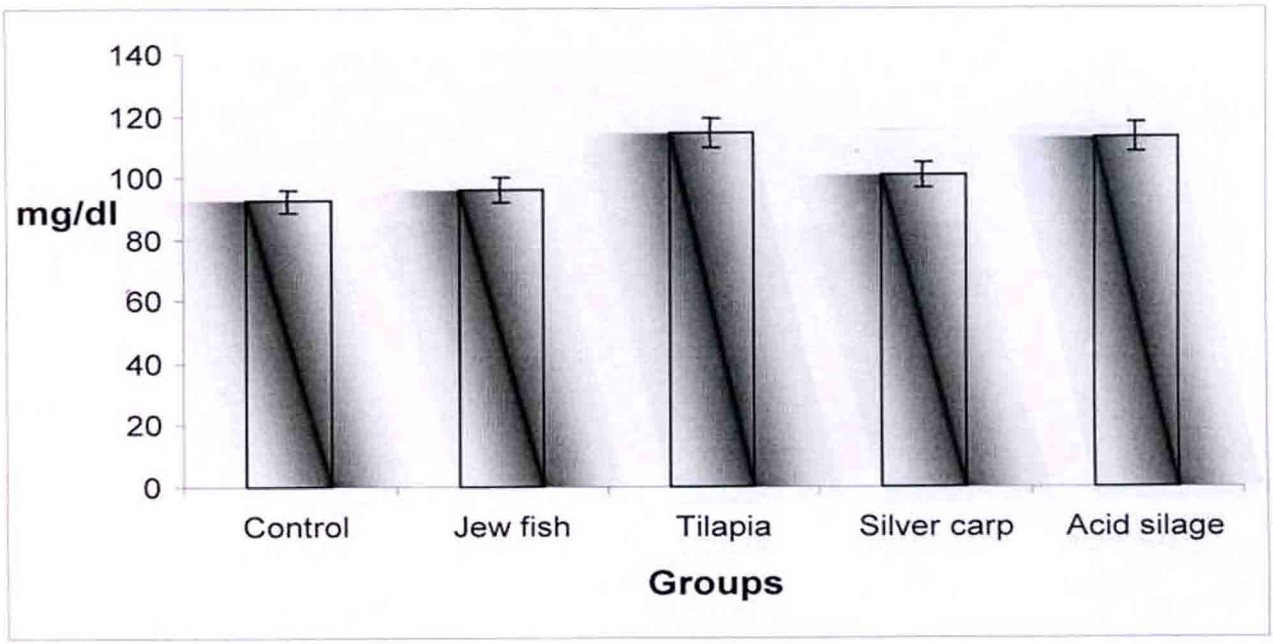


Fig. 4.7.16.1. Serum cholesterol of rats fed (mg/dl) with different types of silages. Results are presented as mean ± standard deviation (SD) of 3 replications ^{a,b,c,d,e}, mean in a column with the same superscript letters are not significantly different ($P<0.05$)

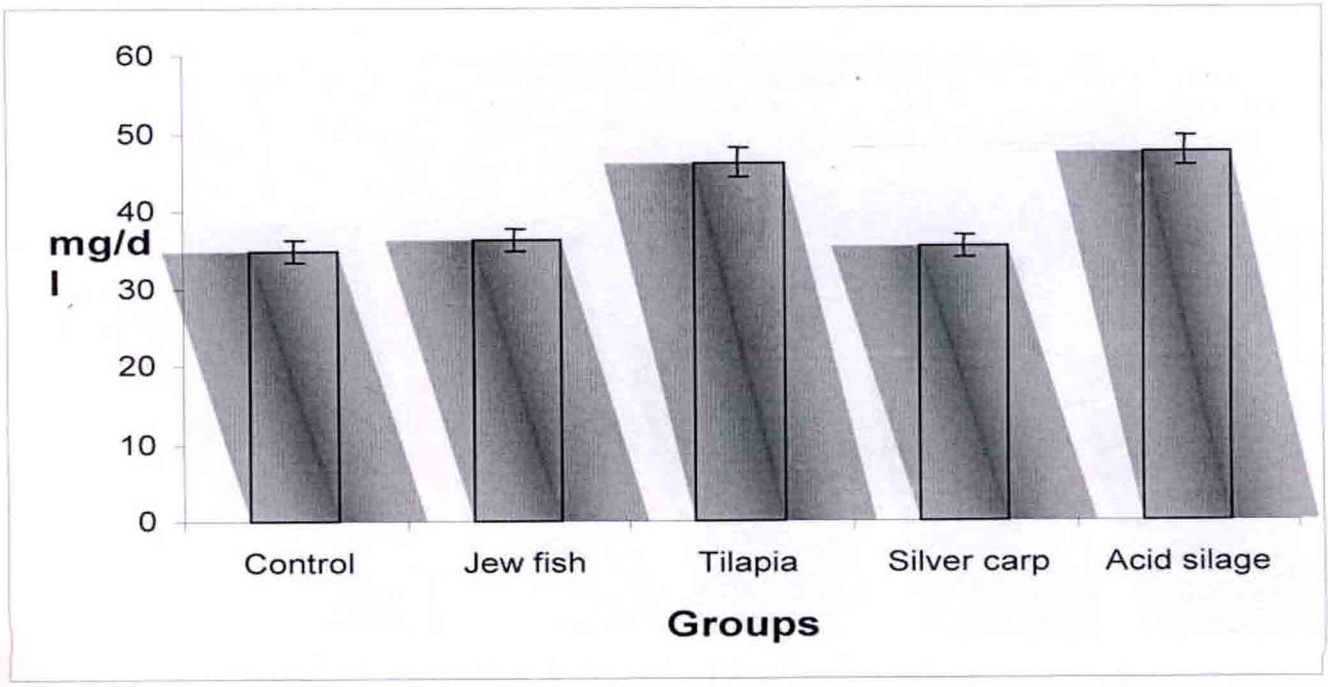


Fig. 4.7.16.2. Serum LDL of rats (mg/dl) fed with different types of silages

Results are presented as mean ± standard deviation (SD) of 3 replications ^{a,b,c,d,e}, mean in a column with the same superscript letters are not significantly different ($P<0.05$)

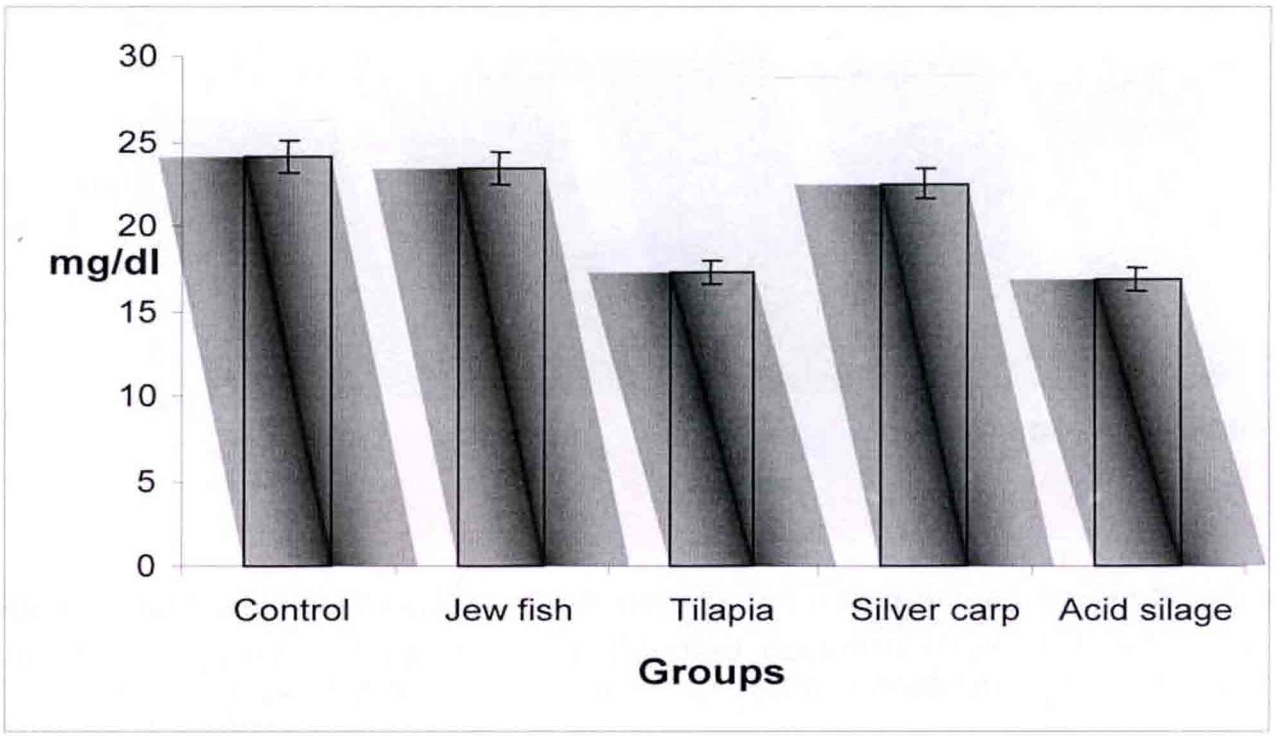


Fig. 4.7.16.2.1. Serum HDL of rats (mg/dl) fed with different types of silages
Results are presented as mean ± standard deviation (SD) of 3 replications ^{a,b,c,d,e}, mean in a column with the same superscript letters are not significantly different ($P<0.05$)

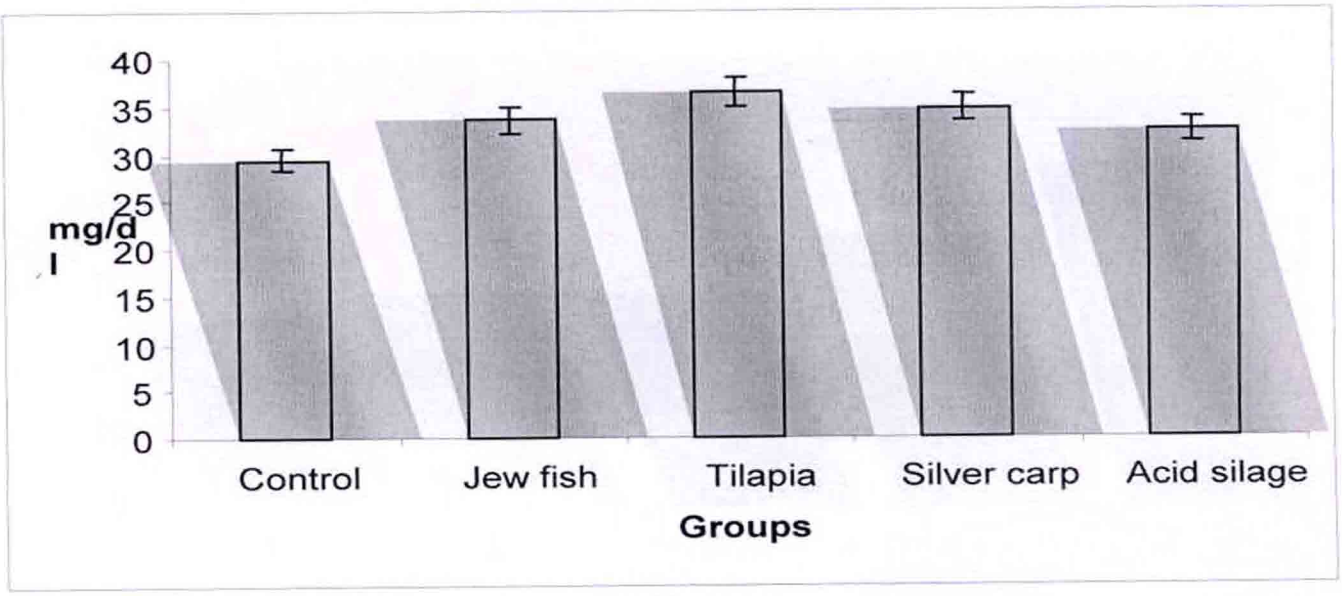


Fig. 4.7.16.3 Serum triglycerides of rats (mg/dl) fed with different types of silages

Results are presented as mean ± standard deviation (SD) of 3 replications ^{a,b,c,d,e}, mean in a column with the same superscript letters are not significantly different ($P<0.05$)

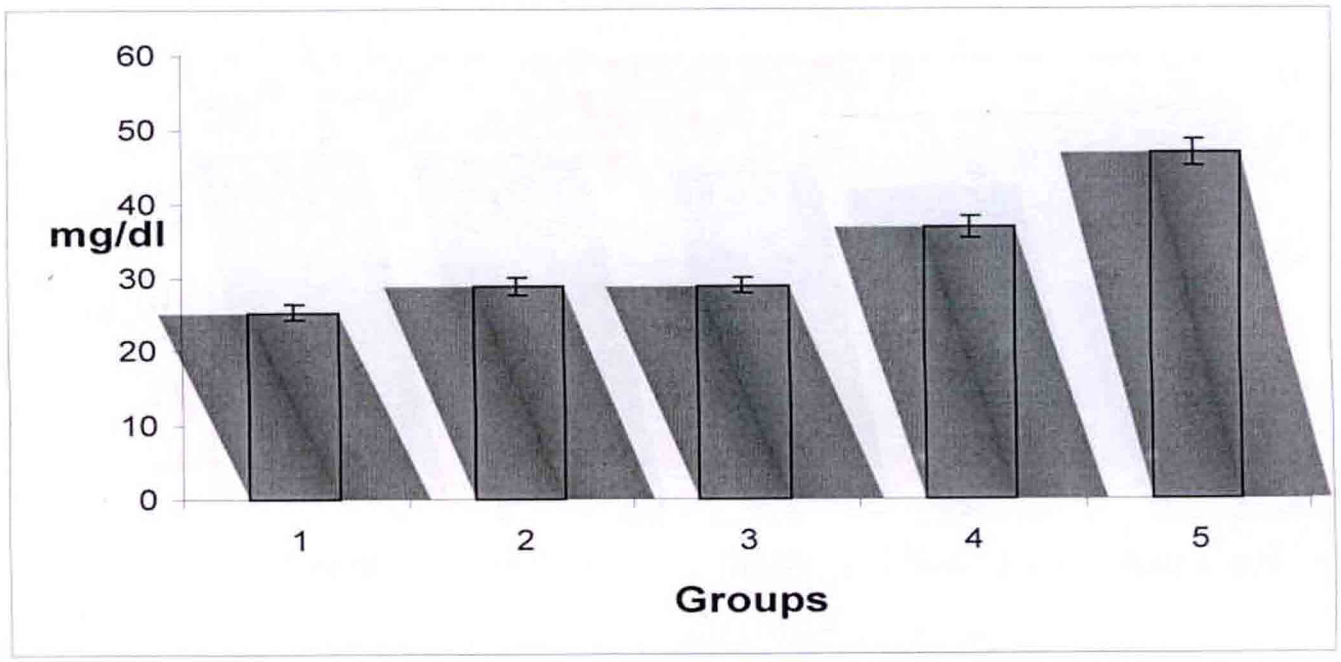


Fig. 4.7.16.4. Serum free fatty acids of rats (mg/dl) fed with different types of silages

Results are presented as mean ± standard deviation (SD) of 3 replications ^{a,b,c,d,e}, mean in a column with the same superscript letters are not significantly different ($P<0.05$)

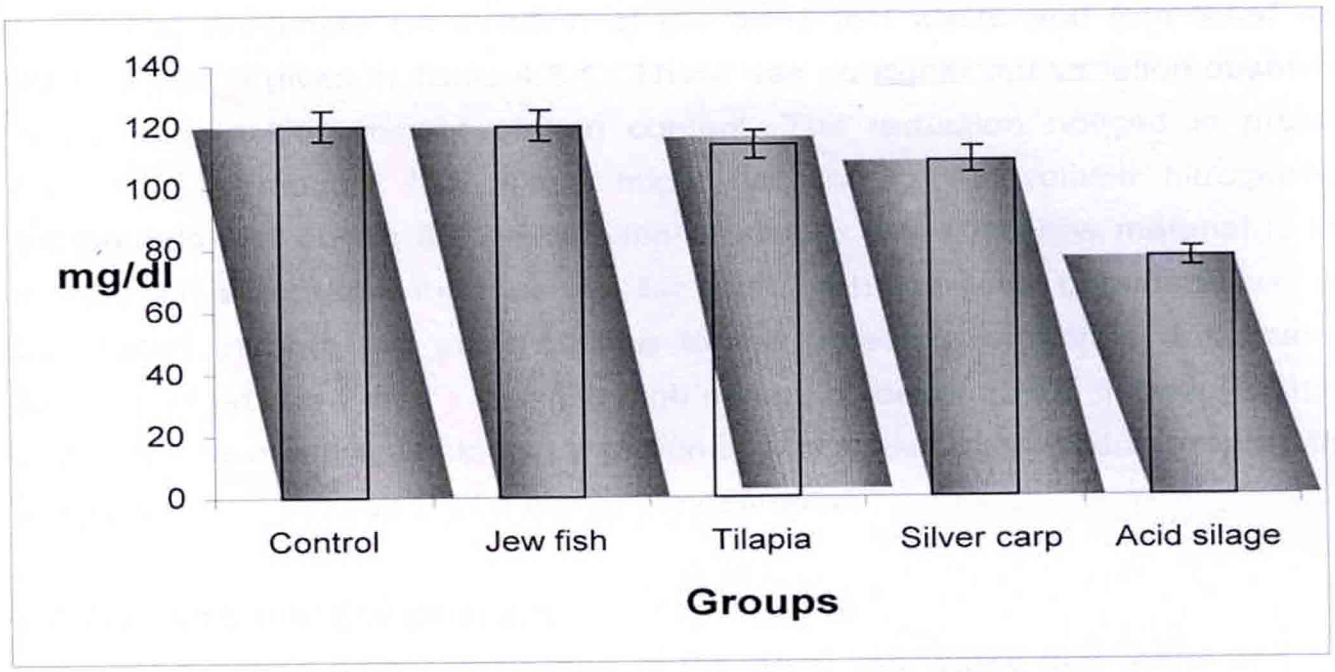


Fig. 4.7.16.5. Serum phospholipids in rats (mg/dl) fed with different types of silages.

Results are presented as mean ± standard deviation (SD) of 3 replications ^{a,b,c,d,e}, mean in a column with the same superscript letters are not significantly different ($P<0.05$)

4.8. Quail Feeding Studies

4.8.1. Proximate composition

The proximate composition of the dried fish waste and fermented fish waste silage is given in Table 4.8.1. There was no significant variation observed in the composition except protein content. The reduction noticed in protein content in fermented fish silage might be due to the volatile nitrogenous compounds lost during the preparation of silage. Since the raw material is the residue remaining after the muscles for surimi, a higher level of mineral content was noted in both the samples due to the presence of bones and scales. Santana *et. al.*, (in Press) reported high minerals concentration in both fishmeal and silage, since the whole fish with bones and scales was used to prepare the silage, showing Ca and P at a higher concentration.

4.8.2. Amino acid composition

The amino acid composition of the dried fish waste and fermented fish waste silage is given Table 4.8.2. Significant variation in the amino acid composition was observed between dried fish waste and fermented fish silage. The percentage of aspartate, threonine, tyrosine and valine was significantly higher in fermented fish silage. Reduction noted in other amino acids might be due to the utilization of amino acids by Lactobacillus during fermentation process.

4.8.3. Egg production and quality

The changes in the body weight, egg production, egg shape index, egg albumen index, egg yolk index and egg Hauge unit of experimental groups of quails showed in Table 4.8.3. Among the groups dried fish waste fed animals showed better body weight index. There were significant variation observed in the body weight gain among the group 3 experimental birds, which indicates that no adverse effect of dietary supplementation of fermented fish silage in quails. However, the egg production in Group II birds fed with fermented fish waste silage was significantly higher when compared to Group I unsalted fish and Group III dried fish waste fed birds. Since the birds under study were meant

mainly for egg laying purpose, the enhanced egg production indicates the superiority of the fermented silage over the other groups. However, there was no significant variation noted in the egg weight among the different groups. Similar observations were reported by Vali *et al.*, (2006) while comparing the egg weight of two different strains of quails. The eggs of Group II fermented silage fed birds exhibited maximum of the major egg quality index with an IQU of 75.44, which was significantly higher than the other two groups. This is in corroboration with an earlier reported study (Ihekoronye and Ngoddy, 1985), which showed that high quality egg generally had IQU of 70 and above. Studies conducted by Kirikçi *et.al.*, (2003) also showed similar results (75.5%) in Fulani-ecotype chicken. Other egg quality parameters like albumen index, shape index and shell thickness were also significantly better in group II birds. The yolk index was showing any significant difference; but the over all quality of eggs and enhanced production indicated that the fermented silage feeding in Japanese quails were highly desirable when compared with other two feeds. IQU and yolk index are the best indicators of internal egg quality (Kirikci *et al.*, 2003). In the feeding trial by Santana *et. al.*, (in press), no differences were found between the weight gain and feed conversion of the control diet and the four diets prepared with increasing amounts of silage

Table 4.8.1. Proximate composition of surimi waste silage

	Fermented surimi waste silage	Dried surimi waste
Moisture (%)	8.55 ± 0.5	7.22 ± 0.4
Protein (%)	51.04 ± 1.2	55.49 ± 1.7
Fat (%)	4.98 ± 0.4	5.13 ± 0.3
Ash (%)	33.32 ± 0.5	35.68 ± 0.4

Results are presented as mean ± standard deviation (SD) of 3 replications

Table .4.8.2. Amino acid profile (mg/100g) of dried surimi waste silage used for quail study

Amino acid	Dried surimi waste	Fermented surimi waste silage
Taurine	1.41	0.63
Asp	8.65	9.76
Thr	3.40	4.39
Ser	6.06	5.81
Glu	10.37	9.96
Pro	1.34	1.43
Gly	30.35	29.07
Ala	14.05	13.14
Cys	0.00	0.00
Val	3.22	4.25
Met	3.14	2.88
Ile	2.68	0.50
Leu	4.79	3.35
Tyr	1.54	5.65
Phe	3.02	2.19
Try	1.54	1.65
His	3.14	3.51
Lys	1.09	1.32
Arg	1.74	2.17

Table 4.8.3.Changes in the body weight, egg production, egg shape index, egg albumen index, egg yolk index and egg Hauge unit of experimental groups of quails

	Group 1	Group 2	Group 3
Body weight at 6 week	187.36±5.2[a]	188.00±4.7[a]	193.04±2.6[b]
Egg production	30.85±1.5[a]	45.75±2.1[b]	36.45±1.8[c]
Mean egg weight	*11.01±0.8*	*10.96±0.3*	*11.67±0.4*
Mean HQU (Hauge unit)	88.71±3.1[a]	75.44±2.7[b]	78.81±1.7[a]
Mean yolk index	0.471±0.02	0.471±0.1	0.472±0.07
Mean albumen index	0.138±0.01[a]	0.137±0.02[a]	0.127±0.04[b]
Shape index	75.74±2.1[a]	78.81±4.1[b]	84.28±2.7[c]
Mean shell thickness	0.194±0.006[b]	0.192±0.02[b]	0.181±0.02[a]

Group 1 Control (Unsalted dried fish) Group 2: Fermented silage and Group 3: Dried fish waste (unfermented)

Results are presented as mean ± standard deviation (SD) of 3 replications [a,b,c,d,e], mean in a row with the same superscript values are not significantly different (P<0.05).

4.9. Fermented Fish Powder

4.9.1. Composition of fermented fish powder

The chemical composition of fermented fish powder and cookies prepared is given in Table 4.9.1. The crude protein content of the powder was 59.4% and ash 14.6%. the protein content is higher than the dried surimi waste silage and dried surimi waste whereas the ash content is less, which is due to dressing of fish. In case of cookies when compared to control samples, the protein content and mineral content enhanced by incorporation of 2% level of fermented powder.

4.9.2. Mineral composition

The mineral composition of fermented fish powder and cookies developed is given in Table 4.9.2. the fermented fish powder was found to be rich in Na, K and Ca. the iron content was found to be less to the range of 1.08 + 0.04.in case of cookies the Na and K levels were found to be high (1241mg/100g and 2023mg/100g respectively) which is due to addition of salt while preparing cookies. In case of fermented fish powder supplemented cookies the potassium and iron content was found to be marginally increased whereas the calcium content increased significantly.

4.9.3. Sensory analysis

Table 4.9.3. shows results of the sensory analyses of cookies prepared by incorporating fermented fish powder. The appearance, colour and texture of the control and sample 1(2%) were not significantly different. Organoleptic quality assessment of the prepared cookies by a trained panel of experts indicated that the cookies prepared by incorporating 2% levels of fermented powder did not show any fishy smell or flavour of the powder and found to be acceptable when compared to higher levels (5% and 10%). Samples 3 and 4 were found to have fishy odour and flavour and acceptability was poor.

Table 4.9.1.Proximate composition of Fermented fish powder and cookies developed

	Fermented powder	Cookies	
		Control	2%
Moisture (%)	8.3	2.25	2.21
Protein (%)	59.4	9.56	10.42
Fat (%)	10.1	18.57	18.23
Ash (%)	14.6	0.065	0.32
Carbohydrate (%)	5.2	70.02	68.16

Table 4.9.2. Mineral composition of fermented fish powder and cookies (mg/100g)

	Na	**K**	**Ca**	**Fe**
Dried fermented powder	405±3.2	1305.6±11.2	9180±34	1.08±0.03
Cookies-control	1241±9.5	2023±14	2023±31	0.4±0.01
Cookies- 2% level powder	1066.75±22.4	469.2±7.6	2816.05±12.5	0.59±0.01

Results are presented as mean ± standard deviation (SD) of 3 replications

Table 4.9.3. Results or sensory analysis of cookies incorporated with fermented fish powder.

	Control	Fermented fish powder		
		2%	5%	10%
Appearance	8.2 ± 0.5^{b}	8.3 ± 0.4^{b}	7.4 ± 0.3^{a}	7.5 ± 0.6^{a}
Colour	8.1 ± 0.5^{b}	8.1 ± 0.6^{b}	7.4 ± 0.5^{a}	7.4 ± 0.4^{a}
Texture	8.3 ± 0.4^{b}	7.9 ± 0.7^{b}	7.5 ± 0.4^{a}	7.4 ± 0.5^{a}
Flavour	7.5 ± 0.6^{c}	7.4 ± 0.5^{c}	6.1 ± 0.2^{b}	4.1 ± 0.3^{a}
Odour	8 ± 0.77^{d}	6.5 ± 0.4^{c}	5.3 ± 0.5^{b}	3.7 ± 0.4^{a}
Overall acceptability	8 ± 0.5^{c}	7.6 ± 0.5^{c}	6 ± 0.4^{b}	3.9 ± 0.5^{a}

Results are presented as mean ± standard deviation (SD) of 3 replications ^{a,b,c,d,e}, mean in a row with the same superscript values are not significantly different ($P<0.05$).

Summary and Conclusion....

5.0 Summary and Conclusion

Preparation of fish silage is found to be the easiest method for preservation any fishery waste and any quantity can be preserved with no machinery involved at any place. Environmental problems like fly infestation, smell, presence of pathogens etc. can be eliminated while retaining all the nutritional qualities. Fermented fish silage has the advantages of low cost of production, utilization of locally available jaggery sources, comparatively better nutritional qualities and the probiotic effects which make it a more attractive option for the utilization of trash fish and fishery wastes from processing centres and market places. Its nutritional quality is comparable to fish meal and has the added advantage of reducing environmental pollution which invariably occurs during the production of fishmeal. Preparation of ensilage by fermentation takes less time and the end product quality can be predicted under standard conditions. As feed supplement, fermented fish silage is found to be more nutritious when compared to acid silage in many aspects. In addition to the nutritive value, fermented fish silage contains lactobacillus bacteria which can act as a probiotic for the livestock, improving the intestinal flora. From this study it can be concluded that :

☼ Ensilation was attained using 10%, 15%, 20% jaggery without significant variations in quality in the final product irrespective of the raw material used. So in order to minimize th cost of production, addition of 10% levels of jaggery is found to be sufficient for complete fermentation. At ambient condition the process of ensilation by fermentation was almost completed in seven days and further rate of increase was found to be low.

☼ The dehydrated silage prepared from different fish species was found to have 7 months of storage life in ambient condition. This indicates that the product if packed in proper packaging or under vacuum condition; can be stored

for more time which enables the transportation of the product and utilization of the same at a later stage.

☼ The problems of biogenic amine production is found to be minimum during fermentation of silage which could be due to the inhibition of amine producing bacteria by the added lactobacillus culture. The histamine levels were found to get reduced during the process. This ensures the safety of the product for indented use.

☼ The nutritional studies carried out in albino rats indicated that there are beneficial effects in reducing the LDL cholesterol of silage supplemented groups of rats especially in case of marine and fresh water fish species. But in case of tilapia silage this does not hold true. Rats fed with Jew fish silage had better Protein Efficiency Ratio, Total Digestibility, and Net Protein Ratio than other groups.

☼ For the utilization of surimi processing waste, fermented silage was prepared from this waste which is generated in huge quantities. The feeding studies conducted in Japanese quails indicated that the fish waste fermented silage is superior in quality when compared to acid silage and the standard feed. Increased egg production and improved egg quality was noticed without any significant difference in body weight. The study indicated that the surimi processing waste can be advantageously utilized for the production of ration to egg laying poultry birds.

☼ The cookies prepared by incorporating with fermented fish powder was found have good acceptability when 2% levels powder was incorporated. The incorporation of the powder enhanced the protein and mineral contents of the product developed.

Fermentation of fish products is gaining more significance in the food industry since the consumers have developed aversion of synthetic products or products containing added chemicals. Fermentation is the suitable technology for the development of minimally processed products. An array of product can be

developed to modify the sensory parameters of the fishery products, for extended shelf of the products. The combined technique like fermentation and hurdle technology can result in products with better sensory attributes and long storage life. Moreover, the utilization of fermentation technology for developing probiotic food for any specific group of consumers will have great potential in future.

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Biochemical changes during bacteriological ensilation of Tilapia

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Abstract

Fresh whole tilapia and eviscerated tilapia were fermented after cooking in water with jaggery. On cooling, the starter culture organism Lactobacillus plantarum *@ 5 % (w/v) was added. Changes in biochemical indices like pH, titrable acidity, non-protein nitrogen, alpha amino nitrogen, total volatile basic nitrogen and glucose was assessed during ensilation. The rate of liquefaction and drop in pH was initially fast and on third day the desired pH level of around 4 was attained. The maximum liquefaction was obtained by 13 days of ensilation. The degree of liquefaction was more in case of silage prepared from whole fish when compared to eviscerated fish while the level of total volatile nitrogen was less in eviscerated fish silage.*

Keywords: *Tilapia, Ensilage, Biological, Biochemical changes*

Introduction

Fish waste is a potential source of protein for animal nutrition. dustrial processing of fish for human consumption yields around 60 % l-products and only 40 % edible flesh (Raa and Gildberg, 1982). Fish sidues can be advantageously upgraded by conversion into fish silage id this approach is more environment-friendly, safer, technologically ore flexible and economically more efficient than manufacturing fish eal. Fish silage is an excellent protein product of high biological value for imal feeding which can be produced from, unutilized species such as by tches from marine fishing, fish processing waste and industrial sidues, which may cause environmental, health and economic problems. reful control of the degree of proteolysis and lipid oxidation is required produce silages of high nutritional value. Fish silage may be defined as liquid product, made from whole or parts of fish to which no material d been added other than an acid in which liquefaction is carried out by zymes already present in the fish. The use of lactic acid bacteria (LAB) uld have a natural inhibiting activity on the undesirable microflora in stes, which would result in a natural preservation. Fermentation of fish-rbohydrate mixture by LAB offers scope for the development of a variety products (Lee, 1989). Lactobacillus plantarum, *one of the highest acid oducing bacteria (Steinkraus, 1983) had been used in the preparation of mented fish by many workers (N. Neethiselvan* et al., *2002; Carl, 1953; euzer, 1953 and James 1966)*

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The degree of hydrolysis of the protein in fish silage is likely to result in a lower nutritive value for ruminant livestock. The small peptides and amino acids formed during ensilage are more readily available to the ruminal micro flora. (Hall *et al.*, 1985) reported that limited autolysis in fermented fish ensilages may be beneficial in restricting the release of free amino acids which are capable of reacting with lipid oxidation products resulting in the reduction of nutritional value of fermented minced meat based diets. Biological fish silage has been shown to have significantly better nutritional value than the acid fish silage (Kompaing *et al.*, 1980). Fermentation ensiling seems to be a more economical process, compared to acid ensiling as the latter requires expensive acids (Javed Ahmed and Mahendrakar 1996). The present work is carried out to compare the biochemical changes during ensilation of whole and eviscerated tilapia by using lactobacillus.

2. Materials and methods

2.1 Preparation of silage

Tilapia (*Oreochromis mossambicus*) in very fresh condition was obtained from the local fish market and brought to the laboratory. It was washed thoroughly and divided into two lots. One lot (Lot A) was minced as such and used for the preparation of silage and the other lot (Lot B) was minced after removing viscera and gills. Both lots were cooked for 30 min with 30 % by weight of water and 20 % jaggery (W/W). It was cooled and a starter culture of Lactobacillus plantarum was added @ 5 % (W/V). The silage was stored in glass bottles at ambient temperature and stirred daily during the period of experiment. Periodic samples were drawn and analysed for various biochemical parameters to assess the changes during fermentation. The jaggery used for the study was obtained from the local market and the bacterial strain used (*Lactobacillus plantarum*) was obtained from MTCC, Institute of Microbial Technology, Chandigarh.

2.2 Biochemical analysis

Moisture, crude protein, crude fat and ash were determined by standard procedures (AOAC, 2000). Total Volatile Nitrogen (TVN) was determined by the micro diffusion method of Conway (1962). For the determination of pH, direct measurement was carried out using a Cyberscan 510 pH meter. The titrable acidity of the sample was measured by titrating aliquot against standard NaOH using phenolphthalein as indicator and expressed as percent lactic acid. The non-protein nitrogen (NPN) was determined by the micro Kjeldahl method of trichloroacetic acid (10 %) extract. Glucose level was estimated by the method described by (Morales *et al.*, 1973) with anthrone as coloring reagent. The tyrosine was estimated with Folin-ciocalteau reagent by the method of Anson (1938).

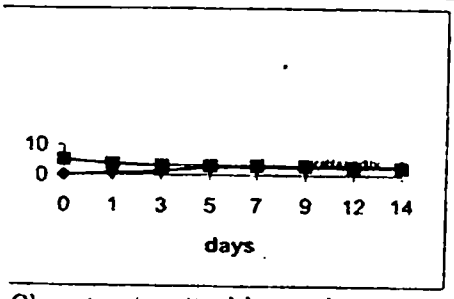
Table 1. Proximate composition of whole and eviscerated fish **UNITS++

	Whole fish	Eviscerated fish
Moisture	74.4	72.8
Fat	2.68	2.35
Total nitrogen	2.50	2.81
Crude protein	15.62	17.56
Ash	3.85	4.10
Non protein nitrogen	210	231
TVBN	11	8
Tyrosine	14.1	19.92

Results and discussion

Table 2. Biochemical changes during biological ensilation of eviscerated apia

ays of rmentation	PH	Non protein nitrogen	Total volatile basic nitrogen (mg %)	Degree of hydrolysis %
	6.9	371	10	22.1
	5.9	588	14	35.0
	4.3	714	32	42.5
	4.4	714	52	42.5
	4.5	735	63	43.8
	4.5	760	60	45.2
	4.4	777	-	46.3
	4.9	763	64	45.4



Changes in titrable acidity and glucose in eviscerated Tilapia during ensilation

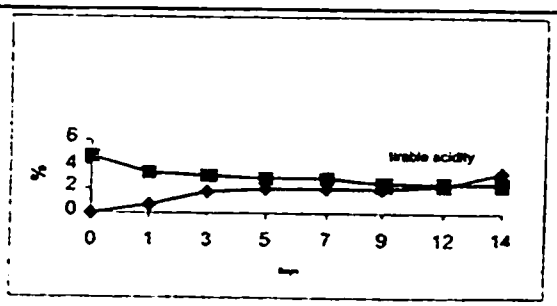


Fig. 2 Change in titrable acidity and and glucose in whole Tilapia during ensilation

The chemical changes in the eviscerated tilapia samples during ation are given in Table 2 and Fig. 1. The pH of the samples ıed 4.3 by third day indicating sufficient fermentation and acid ıction. During the same period the titrable acidity was 1.4 % acid. The degree of hydrolysis of the samples during the same

period was 42 %, which has increased to 46 % by the end of fermentation. The total volatile basic nitrogen content has increased from the initial 10 to 52 mg % by 5th day of ensilation and further increase to 64 mg % has been observed by the end of fermentation, which indicates that the breakdown of protein by microbes has intensified after 5 days. The titrable acidity of the sample has increased from 0.65 % to 3.2 % lactic acid by 14th day and a corresponding decrease in the glucose levels was noticed which reduced to 2.54 % by the same period. The tyrosine level, which also indicates the degree of protein hydrolysis, has shown an increasing trend (Fig. 3).

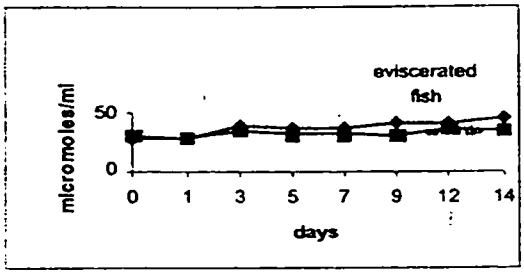


Fig. 3 Changes in tyrosine levels during ensilation

The chemical changes during ensilation of whole tilapia are given in Table 3 and Fig 2. The initial pH of 6.7 reduced to the desired level of 4.3 on 3rd day of fermentation and a marginal increase was noticed during the remaining days. This could be due to the production of various volatile amines. The TVBN value showed a steady increase from an initial value of 14 to 64 mg 100 g^{-1} during the course of fermentation. (Neethiselvan *et al*., 2002) have observed a TVBN range of 28 - 98 mg % for lactic acid fermented fish silage. The degree of hydrolysis was 43 % by one day fermentation and has increased to 62 % by the end of ensilation. The titrable acidity level has increased with a corresponding decrease in glucose level showing the utilization of glucose by the bacteria.

Table 3. Biochemical changes during biological ensilation of whole Tilapia

Days of fermentation	pH	Non protein nitrogen	Total volatile basic nitrogen (mg %)	Degree of hydrolysis %
0	6.74	380	14	28.5
1	5.64	574	32	43.0
3	4.23	714	41	53.5
5	4.4	756	55	56.6
7	4.4	672	52	50.3
9	4.6	637	60	47.7
12	5.3	784	63	58.7
14	5.3	833	74	62.4

Days of fermentation	Non protein nitrogen		Degree of hydrolysis %	
	Eviscerated	Whole	Eviscerated	Whole
1	588	574	35.0	43.0
3	714	714	42.5	53.5
5	714	756	42.5	56.6
7	735	672	43.8	50.3
9	760	637	45.2	47.7
12	777	784	46.3	58.7
14	763	833	45.4	62.4

4. Conclusion

A higher level of hydrolysis during ensilation noticed in the whole fish samples as indicated by the increase in pH. A corresponding increase in the degree of hydrolysis is noticed during the period of ensilation in whole fish samples. Since cooking has already destroyed the enzymes present in both the samples, an increased level of hydrolysis in whole sample could be due to the presence of soft tissues of viscera and gills, which have acted as an easy substrate for the growth of the microbes.

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Full Length Research Paper

Effect of dietary supplementation of fermented fish silage on egg production in Japanese quail (*Coturnix coromandelica*)

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The effect of dietary supplementation of fermented fish silage on egg production in Japanese quail was investigated. Body weight gain, egg production and egg quality parameters (shape index, albumen index, yolk index, shell thickness and Hauge unit) were determined. There was no significant difference observed in the body weight gain in fermented fish silage supplemented birds as compared to that of dried fish waste and unsalted dried fish supplemented groups of birds. The total egg production and egg Internal Quality Unit (Haugh unit) of fermented fish silage fed birds was significantly higher compared to the other groups of birds. The results of the study indicate that incorporation of fermented fish waste silage in the poultry feed formulation can increase the egg production in Japanese quails.

Key word: fermented silage, surimi, Japanese quail, egg production, egg quality, fish waste

INTRODUCTION

Silage production is considered as one of the best ways of preserving agro and animal waste. Ensilaging is an important method for utilizing trash fish, by catch and processing wastes which provide high quality protein for livestock such as poultry, pigs, calves and other species such as mink. By-products from fishing industry have great potential to be used as protein supplement in feeds (Rose et al., 2003). The conversion of fish waste to silage has the advantage of being an inexpensive supplement for animal feed, while reducing waste and environmental contamination. Fermented fish silage is prepared by mixing fish/fish waste with a fermentable sugar source and a starter culture of lactic acid bacteria (Raghunath and Gopakumar, 2002).

Surimi is a wet frozen concentrate of myofibrillar proteins of fish muscle (Rajalekshmi, 2004). It is deboned, washed and stabilized fish mince. The overall surimi manufacturing process is reported to be quite inefficient resulting in 12 - 20% yield from round fish to finished product. The bulk of the remaining solid waste (65 - 70%) ends up as fishmeal (French and Pederson, 1990). Muraleedharan et al. (1996) and Sankar (2000) studied the filleting yield and wastage from different marine fish species and Indian major carps, respectively. The surimi production in India is increasing every year; the export of surimi and fish fillet from the country for the year 2004 is 31509.5 and 421 tons, respectively (Anon, 2004). In the filleting machine, 67.1% waste is generated and additional 1% waste is resulted during deboning. If the unused sarcoplasmic protein which comes to 7.5% is included, 75.5% by weight of round fish becomes waste which can be used for silage preparation. Hence, during the processing of the above quantity of surimi about 94, 528 metric tons of waste is being generated which can be effectively used for the production of fish silage.

The production of fermented silage from the fish processing waste is not only an effective way to convert the waste into beneficial products but also minimize the environmental problems arising from the waste. In the present study, an attempt has been done to assess the

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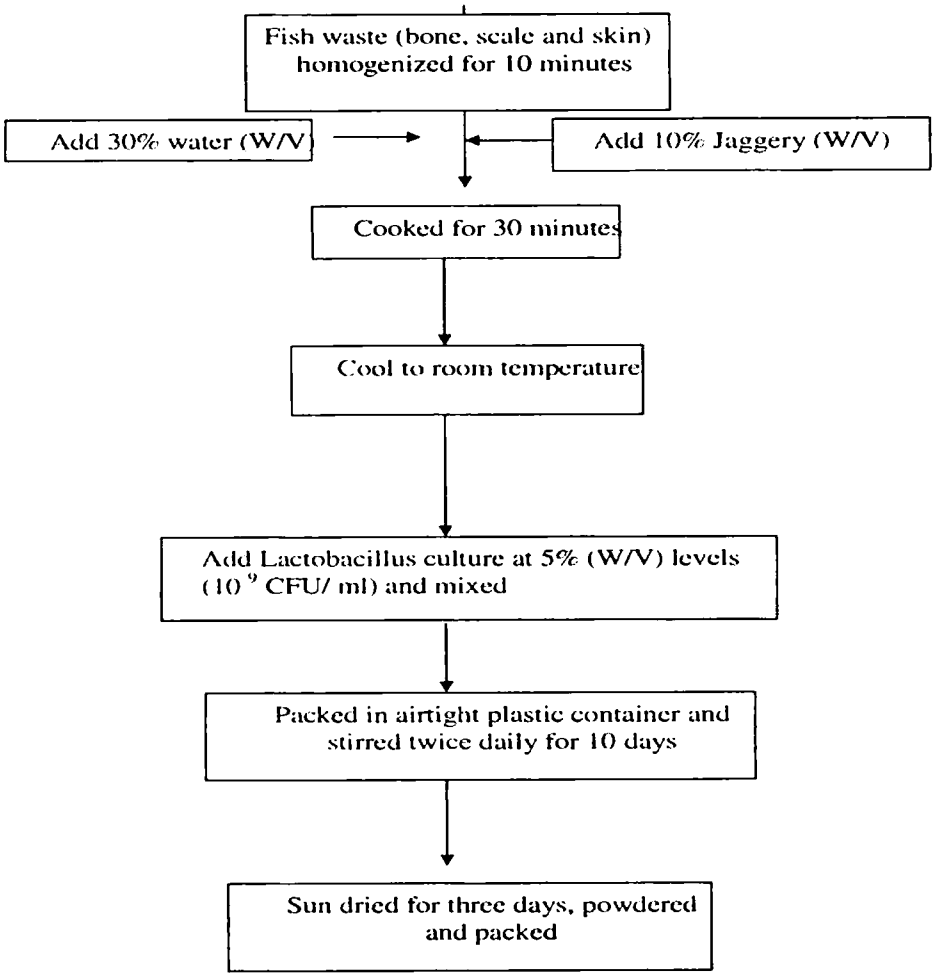

Figure 1. Schematic diagram in the preparation of fermented silage from surimi waste of *N. japonicus*.

effect of dietary supplementation of fermented fish silage prepared from surimi waste on egg production in Japanese quails.

Materials and Methods

The present study was conducted during March 2006 at Department of Poultry Science, Kerala Agricultural University, Mannuthy, Kerala, India. The experiment was carried out based on the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India.

Japanese quails (Coturnix coromandelica)

The quails (6 weeks old) were procured from Veterinary College, Kerala Agricultural University, Mannuthy, Kerala, India and acclimatized with the experimental feed for one week before the start of the study.

Chemicals and culture

Amino acid standards and tryptophan were obtained from M/s. Sigma Chemical Company, St. Louis. MO. USA. All the other chemicals used were of analytical grade. *Lactobacillus plantarum* culture was procured from MTCC 1425 IMTECH Chandigargh, revived and repeatedly sub cultured in MRS broth and used. Jaggery was obtained from local market.

Preparation of fermented fish silage

The surimi processing waste obtained from *Nemipterus japonicus* was homogenized for 10 min and cooked for 30 min with 10% Molasses (w/w). It was cooled and then inoculated with *L. plantarum* culture at 5% (w/v) level containing 10^9 CFU/ml. The whole mass was mixed thoroughly, transferred into plastic buckets and covered tightly with lid and allowed to ferment for a period of 10 days with occasional stirring. (Figure 1)

Table 1. Composition of feeds used for feeding Japanese quail.

	Ingredients (%)	Group 1	Group 2	Group 3
1	Yellow maize	50.0	50.0	42.0
2	Gingili oil cake	5.0	2.0	5.0
3	Soy bean	24.0	27.0	23.0
4	Unsalted dried fish	8.0	-	-
5	Fermented fish silage	-	10.0	
6	Dried fish waste	-	-	8.5
7	Wheat bran	-	-	4.0
8	Rice polish	6.0	4.0	10.5
9	Shell grit	5.0	5.0	5.0
10	Vitamin mineral mixture	1.75	1.75	1.75
11	Salt	0.25	0.25	0.25

Experimental protocol

Feed

Feed was prepared by mixing the ingredients to produce a total 2700 Kcal/Kg weight of feed. The supplemented silage and dried fish waste were adjusted by nitrogen content in dry weight basis so as to get equal quantity of protein nitrogen in all the groups. The feed components (Table 1) were powdered, mixed properly and provided to different groups as given below:

- Group 1. Control ration with 10% unsalted dried fish
- Group 2. Control ration in which unsalted dried fish was replaced by fermented fish waste silage in terms of crude protein content.
- Group 3. Control ration in which unsalted dried fish was replaced by dried fish waste in terms of crude protein content.

The birds were divided at random into three groups consisted of 48 quails in each group. The experimental birds were maintained under hygienic condition and provided with food and water *ad libitum*.

The experiment was carried out in triplicates for a period of 28 days. Eggs were collected daily and quality determination was conducted once a week (one week interval of time). Soft-shelled, cracked and small eggs were not used. The egg weight was noted one by one and stored at 13°C. The egg shape index was measured using an electronic digital caliper. After measuring the diameter of the eggs, these were broken under well-arranged glass and five minutes later, long and short diameter and height of both albumen and yolk were measured with electronic caliper. Separated yolks were weighed and recorded. Shells of broken eggs were washed with water to separate the albumen and air-dried. The shape index, albumen index, yolk index, shell thickness and Hauge unit of eggs collected were determined using the method of Kemal et al. (2003).

Shape index = short border/long border x 100; Albumen index = albumen height /albumen diameter x 100; Yolk index = yolk height /(long diameter of yolk + short diameter of yolk/2)x 100; Shell thickness = (sharp point thickness + equator thickness + stubby thickness)/3; Haugh Unit = 100 x log (Albumen weight + 7.57 - 1.7 x egg weight x 0.37).

Determination of proximate and amino acid composition analyses

The moisture, crude protein, crude fat and ash content of fish silages used were determined as per AOAC (2000). Total amino acid composition was estimated by the method of Ishida et al. (1981) using Shimadzu 10AS Amino Acid Analyzer. Tryptophan content was determined spectrophotometrically using the method of Sastry and Tummuru (1985).

Statistical analysis

Results are expressed as mean ± SD and Student *t*-test was used to determine the statistical significance.

RESULTS AND DISCUSSION

The proximate composition of the dried fish waste and fermented fish waste silage is presented in Table 2. There is no significant variation observed in the composition except that of the protein content. The reduction noticed in protein content in fermented fish silage might be due to the volatile nitrogenous compounds lost during the preparation of silage. Table 3 shows the amino acid composition of the dried fish waste and fermented fish waste silage. Significant variation in the amino acid composition was observed between dried fish waste and fermented fish silage. The percentage of aspartate, threonine, tyrosine and valine was significantly higher in fermented fish silage. Reduction noted in other amino acids might be due to the utilization of amino acids by *Lactobacillus* during the fermentation process.

Changes in the body weight, egg production, egg shape index, egg albumen index, egg yolk index and egg Hauge unit of experimental groups of quails are shown in Table 4. Results revealed that there were no significant variations observed in the body weight gain among the experimental groups of birds. These indicate that there were no adverse effect of dietary supplementation of dried fish waste, unsalted dried fish and fermented fish silage in quails. However, among the groups dried fish waste fed animals showed better body weight index.

The egg production in Group II birds fed with fermented

Table 2. Proximate composition of surimi waste silage and fish waste (%).

	Fermented surimi waste silage	Dried surimi waste
Moisture	8.55 ± 0.52	7.22 ± 0.41
Crude Protein	51.04 ± 1.25	53.49 ± 1.72
Crude fat	4.98 ± 0.42	5.13 ± 0.37
Ash	33.32 ± 0.51	35.68 ± 0.43

Table 3. Amino acid composition of dried fish waste and fermented surimi waste silage.

	Dried fish waste	Fermented fish waste silage
Taurine	1.41	0.63
Aspartic acid	8.65	9.76
Threonine	3.4	4.39
Serine	6.06	5.81
Glutamic acid	10.37	9.96
Proline	1.34	1.43
Glycine	30.35	29.07
Alanine	14.05	13.14
Cysteine	-	-
Valine	3.22	4.25
Methionine	3.14	2.88
Isoleucine	2.68	0.5
Leucine	4.79	3.35
Tyrosine	1.54	5.65
Histidine	3.14	3.51
Lysine	1.09	1.32
Arginine	1.74	2.17

Table 4. Changes in the body weight, egg production, egg shape index, egg albumen index, egg yolk index and egg Hague unit of experimental groups of quails.

	Group 1	Group 2	Group 3
Body weight at 6 week	187.36	188.00	193.04
Egg production	30.85	45.75	36.45
Mean egg weight	11.01	10.96	11.67
Mean IQU (Hague unit)	69.71	75.44	70.91
Mean yolk index	0.471	0.471	0.472
Mean albumen index	0.138	0.137	0.127
Shape index	75.74	78.81	84.28
Mean shell thickness	0.194	0.192	0.181

fish waste silage was significantly the highest compared to Group I unsalted fish and Group III dried fish waste fed birds. Since the birds under study were meant mainly for egg laying purpose, the enhanced egg production indicates the superiority of the fermented silage over the other groups. However, there was no significant variation noted in the egg weight among the different groups. Similar observations were reported by Vali et al. (2006) while comparing the egg weight of two different strains of quails.

In the present study, the eggs of Group II fermented silage fed birds exhibited maximum of the major egg quality index with an IQU of 75.44. This is in corroboration with an earlier reported study (Ihekoronye and Ngoddy, 1985) which showed that high quality egg generally had IQU of 70 and above. Studies conducted by Kirikçi et al. (2003) also showed similar results (75.5%) in Fulani-ecotype chicken. Other egg quality parameters like albumen index, shape index ad shell

thickness did not show much significant variation among the groups. The yolk index also suggested that eggs from the fermented silage fed quails were highly desirable when compared with the other two groups. IQU and yolk index are the best indicators of internal egg quality (Kirikci et al., 2007).

The results of the present study indicate that fermented fish silage prepared from waste of surimi production can be used as an imperative ingredient in the dietary feed formulation of Japanese quails to enhance good quality egg production. Since higher egg production with superior quality is an essential and preferable criterion in poultry, the incorporation of fermented fish silage in dietary feed formulation in poultry may be used as an effective, suitable and cheaper protein source.

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Effect of Different Levels of Fermentable Carbohydrate on the Degree of Hydrolysis of Fish Silage

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Fermented silage production by Lactic Acid Bacteria (LAB) represents a low cost method for the preparation of food and feed products characterized by hygienic quality and improved shelf life. The present study aimed to compare the effect of different levels of fermentable carbohydrate on the microbial level and the degree of hydrolysis in fermented fish silage from whole fish and dressed fish. Fermented fish silage was prepared with different levels of jaggery (w/w) i.e., 5%, 10% and 15% viz., type I type II and type III respectively. The study indicated that pH values decreased in all the samples during the period of ensilation except in type I sample which showed a higher pH after four days. Since the type I silage was spoiled at an early stage it is evident that 5% jaggery is not sufficient for proper preservation through fermentation. But in type II and type III, the changes in pH and degree of hydrolysis do not show significant difference during ensilation. The bacteriological results also indicate that the fermentation patterns of both type II and type III are almost same. Hence it is evident that for successful Lactobacillus fermentation of tilapia 10%, jaggery is sufficient either in whole condition or in dressed condition.

Key words: Lactic Acid Bacteria, Fish Silage, Fermentable carbohydrate

Lactic acid fermentation represents a low cost method for the preparation of food and feed products characterized by hygienic quality and improved shelf life (Frazier & Westhoff, 1988; McDonald *et. al.*, 1991). Fermentation of fish silages depends on *in situ* production of lactic acid by Lactic Acid Bacteria (LAB) added to the fish with a fermentable carbohydrate source. According to Nilsson & Rydin, (1965) fermentation of manure, slaughterhouse wastes and fish byproducts low in LAB require the addition of a fermentation flora. The technology of fermented silage production relies on the production of lactic acid at a rapid rate in sufficient concentrations by fermentation, which suppress spoilage organisms and preserve the feed until it is needed (McDonald et. al., 1991).

Lactic acid bacteria produce acids, reduce the pH and are tolerant to low pH and this can be the key factor in the competition with spoilers. Of the various starters like *L. plantarum. L. acidophilus, Pediococcus halophilus and P. acidilacti; L. plantarum* appears to be the most effective starter culture (Bello *et.al.*, 1992). They can operate over a wide range and have an extremely important effect in modified product such as silage and to a lesser extend in fish sauces and pastes (Han-Ching *et al.*, 1994). The present study aims to compare the effect of different levels of fermentable carbohydrate on the microbial level and the degree of hydrolysis in fermented fish silage from whole fish and dressed fish.

Materials and methods

Lactobacillus plantarum culture was procured from MTCC 1425 IMTECH Chandigargh, revived, repeatedly sub cultured in MRS broth. All the chemicals used were of analytical grade supplied by Merck, SRL, Sigma and Oxoid. Tilapia (*Orechromis mossambicus*) collected in fresh condition from local market, Cochin was used for the study. Fermented fish silage was prepared with different levels of jaggery (w/w) i.e.,

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5%, 10% and 15% viz. type I type II and type III respectively.

To prepare silage from whole fish, the fish was washed with potable water, homogenized in Tecator, 1094 Homogeniser for 5-8 minutes and cooked for 30 minutes with 30% of water and jaggery in a beaker. It was cooled and inoculated with *Lactobacillus plantarum* culture at 5% (W/V) level containing 10^9CFU / ml. The whole mass was mixed thoroughly, and covered tightly with polythene paper.

Preparation of silage from dressed fish was done by washing with potable water, followed by removal of gills and all internal organs and a second washing. It was thoroughly homogenized for 5-8 minutes, cooked and packed as above. Aliquots of samples were drawn at intervals and analysed for chemical and microbiological parameters.

For quantitative methods of microbiological analysis, USFDA (2001) methodology was followed for Total plate Count, Lactic acid bacteria (LAB) *E. coli*, *Staphylococcus* and *Salmonella*. For qualitative estimation, the colonies were isolated from TGA and identified by the method of Surendran *et. al.* (2003).

pH of the samples were directly measured using a Cyberscan 510 pH meter. Nitrogen content and Non Protein Nitrogen content were determined by the method of AOAC (2000). Carbohydrate content was determined by the method of Cleg (1956).

Statistical analysis of the results was expressed as mean or mean log ± SD for biochemical & microbiological parameters. Statistical analysis between the means using ANOVA and Tukey's test. Statistical package used in the study was SPSS, 10.

Results and Discussion

Changes in pH during ensilation of whole tilapia with different levels of carbohydrate are given in Table 1. During ensilation the carbohydrate added was fermented by the bacteria, which resulted in the production of lactic acid. As a result the pH of the samples decreased. The rate of decrease was faster during first 2 days followed by a slow decrease. The rapid growth of lactic acid bacteria during the early stages of fermentation resulted in the production of lactic acid with a concomitant reduction in pH. Ahmed & Mahendrakar (1996) also reported almost the same results during the fermentation of fresh water fish viscera. In type I (with 5% jaggery) the pH decreased to 4.7 on fourth day and increased thereafter. This could be due to the depletion of sugar and consequently the pH increased and the samples were spoiled. The fermented fish silage prepared by Kompiang *et. al* (1979) with 5% molasses had a pH of 5.0 by three days and got spoiled by 10 days. The pH of fermented fish silage prepared using fermented cabbage and curd as the inoculum reached 4.6 and 4.9 respectively (Neethiselvan *et. al.* 2002). In type II, the pH decreased to 3.9 on 10^{th} day and then started to increase probably due to the neutralization of lactic acid with the nitrogenous compounds produced. But in the case of type III, the pH showed the decreasing trend throughout the experiment due the presence of excess sugar. It is observed that the addition of sugar at higher concentrations does not have significance on the pH change. Durairaj *et. al.* (1985) reported that the pH of the bacterial silage prepared from silver belly stored in jerry cans was stable at 4.0 through out the storage period of 12 months and even beyond.

Table 1. Changes in pH during ensilation of whole tilapia at different levels of carbohydrate

Days	5%	10%	15%
1	6.97 ± .06 ^{a}	6.9 ± 0.03 ^{a}	7.0 ± 0.15 ^{a}
2	5.26 ± 0.1 ^{a}	5.23 ± 0.04 ^{a}	5.1 ± 0.1 ^{a}
4	4.7 ± 0.1 ^{a}	4.21 ± 0.03 ^{b}	4.2 ± 0.1 ^{b}
6	4.9 ± 0.05 ^{b}	4.02 ± 0.04 ^{a}	4.1 ± 0.05 ^{a}
10	-	3.97 ± 0.06 ^{a}	4.06 ± 0.05 ^{a}
14	-	4.12 ± 0.01 ^{b}	4.06 ± 0.15 ^{a}

Results are presented as mean ± standard deviation (SD) of 3 replications ^{a,b,c,d,e}, mean in a row with the same superscript values are not significantly different ($P<0.05$).

Changes in pH during ensilation of dressed tilapia at different levels of carbohydrate is given in Table 2. As in the case of whole fish silage, the pH values decreased in all the samples during the period of ensilation except in type I sample which showed a higher pH after four days. This could be due to depletion of carbohydrate as in the case of whole tilapia. The decrease in the case of whole fish was marginally higher compared to the dressed fish with the same level of sugar. This could be due to the action of intestinal cathepsins which could result in partial hydrolysis of the muscle during initial cooking and a consequent enhanced activity of the added bacteria. According to Babu *et. al.*, (2005), a concentration of bacteria of 10^8 cells/g caused a decrease of the pH to 4.5 by the end of first day. Lindgren (1992) observed that *L. plantarum* produced about 4.5% lactic acid in fish silage.

Table 2. Changes in pH during ensilation of dressed tilapia at different levels of carbohydrate

Days	5%	10%	15%
1	6.94 ± .06^a	6.96 ± .05^a	7.13 ± 0.11^b
2	5.40 ± 0.1^a	5.6 ± .05^a	5.30 ±0.10^a
4	4.09 ±.09^a	4.31 ± .01^b	4.38 ± 0.32^b
6	5.20 ± 0.1^b	4.22 ± .005^a	4.14 ± 0.04^a
10	5.60 ± 0.26^b	4.07 ± .01^a	4.12 ± 0.005^a
14		4.03 ± 0.11^b	4.03 ± 0.05^b

Results are presented as mean ± standard deviation (SD) of 3 replications. ^{a,b,c,d,e}, mean in a row with the same superscript values are not significantly different (P<0.05).

Changes in LAB count during ensilation of whole tilapia at different levels of jaggerry are given in Fig 1. The pH of this set showed the highest value of 5.26 on 2nd day. But on 4th day of inoculation, the counts increased to log 8.3cfu/ml and remained more or less same upto 6th day. Due to spoilage, no further observation was possible for this lot. But in type II and type III samples LAB count increased initially upto 6th day of fermentation and thereafter showed a declining trend indicating the depletion of carbohydrate source. As a consequence during the next observation on 9th day and 14th day the LAB count was reduced to log 6.3 cfu/ml in type II and log 7.4 cfu/ml in type III respectively. Fadda *et. al* (2002) observed that in a model sausage system, the LAB count remained at 10^8 cfu/g through out the course of fermentation whereas, according to Neethiselavan et. al (2002) the LAB count increased from the initial 1.6 X 10^6 cfu/ g on 5th day and further increased to reach a maximum count of 1.9 X10^7 /g on 7th day.

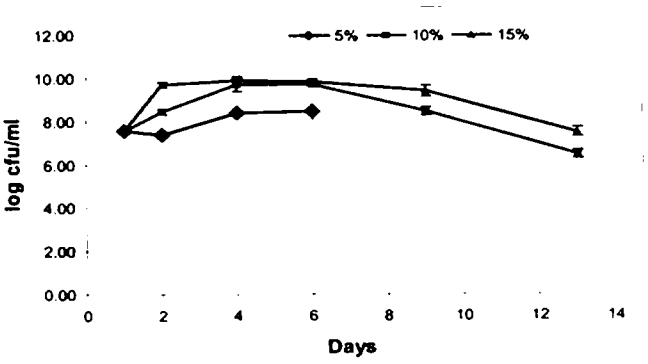

Fig. 1. Changes in Lactobacillus count during ensilation of whole tilapia at different levels of carbohydrate

Changes in LAB count during ensilation of dressed tilapia at different levels of carbohydrate are given in Fig. 2. The LAB count of all the three samples increased initially indicating the utilization of carbohydrate by the starter culture. In type I, the count increased up to four days and showed decreasing trend. The LAB count in dressed fish silage was found to be higher. This

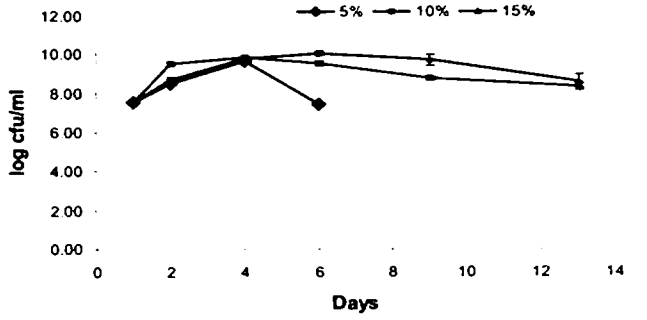

Fig. 2. Changes in LAB count during ensilation of dressed tilapia at different levels of carbohydrate

value declined in case of type II and type III after 6 days. But the rate of reduction was very low which could be due to the presence of sufficient quantity of sugar in the substrate. Through out the period of fermentation, the count of LAB remained in the range of 7.4 log and 10.0 log CFU/ ml indicating satisfactory conditions for fermentation.

Table 3 depicts the changes in Total Plate Count (log cfu/ml) of whole tilapia during ensilation at different levels of carbohydrate. The TPC levels during ensilation showed an inverse relation as that of LAB count. For the initial 4 days, no TPC was detected indicating the inhibition of spoilers by *Lactobacillus plantarum*. In type I, the total plate count observed on 6th day was 2.4 CFU/ ml and since sample got spoiled no further observation was made. Depletion of carbohydrate (Fig.3) and consequent decrease in LAB count and increasing pH caused the growth of spore forming spoilage bacteria which might have caused the spoilage of samples. In type II and type III samples, TPC was not detected till 9th day. This could be due to the inhibition of normal bacterial population by *L. plantarum*. According to Faid *et.al.* (1997) the lactic acid fermentation is usually accompanied by some metabolites (Bacteriocins), which may help the preservation of fermented foods by way of suppressing the spoilage organisms Kannappan & Manja (2004). Since the carbohydrate supplied is limited by 12 days, and due to release of other volatile compounds, the pH started

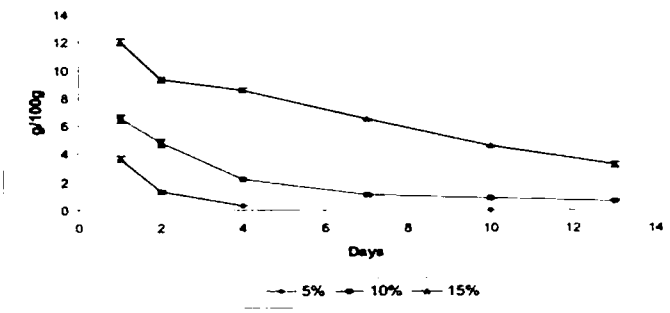

Fig. 3. Changes in the levels of carbohydrate during ensilation of whole tilapia

increasing which consequently resulted in the growth of other spoilage organisms as evidenced by the increase in total plate count. All the colonies were of same physical appearance. Typical colonies were selected and the isolated colonies were identified as *Bacillus* sps. (Surendran *et. al.* 2003). *E. coli, Staphylococcus and* Salmonella were not detected in the samples.

Total Plate Count of dressed tilapia during ensilation at different levels of carbohydrate is given in Table 4. During the initial 4 days TPC was not detected except in sample type I which increased on 6th day, to the tune of 6 log CFU/ ml. Aerobic plate count was not detected in other two samples on the same day, which could be due to the low pH and higher lactobacillus count. Colonies were detected in other two groups on 9th day of fermentation and a slow increase in count was observed which might be due to the germination of spores that survived the initial heat treatment and its multiplication.

Table 3. Total Plate Count (log cfu/ml) of Whole Tilapia During Ensilation at different levels of carbohydrate

	5%	10%	15%
2 day	Not detected	Not detected	Not detected
4 day	Not detected	Not detected	Not detected
6 day	2.49 ± 0.14	Not detected	Not detected
9 day	Not done	2.72 ± 0.21	2.66 ± 0.19
13 day	Not done	5.68 ± 0.20	5.19 ± 0.06

Results are presented as mean ± standard deviation (SD) of 3 replications.

Table 4. Total Plate Count (log cfu/ml) of Dressed Tilapia During Ensilation at Different Levels of Carbohydrate

	5%	10%	15%
2 day	Not detected	Not detected	Not detected
4 day	2.67±0.16	Not detected	Not detected
6 day	6.63±0.17	Not detected	Not detected
9 day	Not done	4.23±0.09	3.68±0.12
13 day	Not done	4.99±0.12	5.12±0.21

Results are presented as mean ± standard deviation (SD) of 3 replications

The degree of hydrolysis (DH) during ensilation of whole tilapia is given in Fig 3. Even though in type I the degree of hydrolysis was progressing initially, due to depletion of sugar, the sample was spoiled. Lactic acid bacteria ferment the sugar present to organic acid, thus lowering the pH. If the pH falls sufficiently low (4.5) growth of putrefactive organisms and pathogens are inhibited. But in type II, the DH progressed at a faster rate initially and slowed during the end of ensilation, which could be due to the decreased activity of microbes due to depletion of sugar. In type III, the degree of hydrolysis was similar to that in type II. Increase of non protein nitrogen was observed when pilchard waste was fermented with 25% molasses throughout for the period of 11 days by Faid et. al (1997). In the present study, about 60% hydrolysis of the protein was observed in all the samples. A similar observation was noted in blue whiting ensilage by Maria et. al (1998). Since the DH of the three sets of samples was almost same, the levels of sugar could be limited to 10%.

Degree of hydrolysis (ratio of total nitrogen to non protein nitrogen) during ensilation of dressed Tilapia at different levels of carbohydrate is given in Fig 4. In case of dressed fish also, the DH was found to be increasing during the course of fermentation. After 7 days, the rate of hydrolysis was less up to 13 days, which could be due to the presence of complex proteins difficult to break. But compared to whole fish silage, the DH was less in all sets of samples. The final observation of less DH could be due to utilisation of hydrolyzed protein by the microbes on depletion of sugar. Backhof (1976) has reported a greater value of NPN (83.5%) in cod viscera compared to fish flesh.

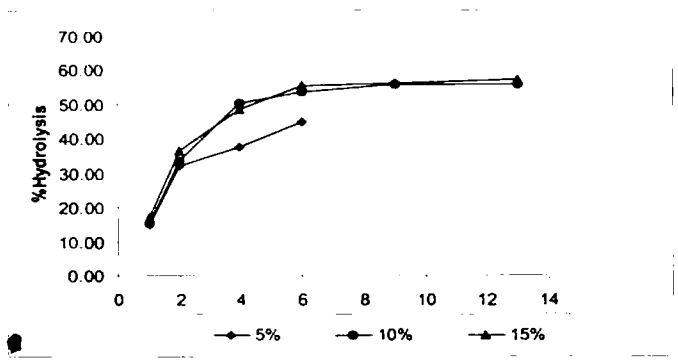

Fig. 4. Degree of hydrolysis during ensilation of whole Tilapia at different levels of carbohydrate

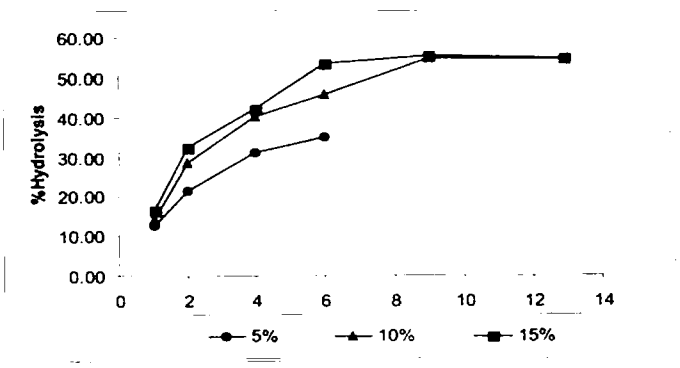

Fig. 5. Degree of hydrolysis during ensilation of dressed Tilapia at different levels of carbohydrate

Since the type I silage was spoiled at an early stage, it is evident that 5% jaggery was not sufficient for proper preservation through fermentation. But in type II and type III, the changes in pH and degree of hydrolysis do not show significant difference during ensilation. The bacteriological results also indicate that the fermentation patterns of both type II and type III are almost same. Hence it is evident that for successful Lactobacillus fermentation of tilapia 10%, jaggery is sufficient either in whole condition or in dressed condition.

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