GROWTH AND PROTEIN DIGESTIBILITY OF TAMBAQUI, COLOSSOMA MACROPOMUM, CUVIER 1818, FED DIETS BASED ON FISH SILAGE

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by

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Abstract

It is recognized that the cost of artificial fish feed makes a significant contribution to the costs of an aquaculture operation. In order to decrease the dietary costs an alternative protein source to traditional fish meal is needed. Silage is often used, however there is a lack of research on the utilization of silage as a main source of protein in fish diets in the Northeast of Brazil. Hence, this thesis was undertaken to determine the growth and apparent protein digestibility of tambaqui, *Colossoma macropomun*, when fed diets based on formic acid and biological fermented silage compared to a control fishmeal diet.

Fish silage was obtained by the addition of formic acid to minced tilapia, *Oreochromis niloticus*. *Lactobacillus plantarum* was used as inoculum and molasses as the carbohydrate source. Chemical composition, pH, total and free amino acid of the silages were determined. Formic acid and biologically fermented silage stored for 4 and 30 days were co-dried with wheat bran and incorporated into the diet of juvenile tambaqui as the main source of protein. The control diet was formulated using fishmeal as the main source of protein. The 5 experimental diets and the control diet were isocaloric and isonitrogenous.

The growth experiment used fifteen round concrete tanks (3 for each diet) with 15 fish per tank. Fish were fed twice a day at a rate of 3% of body weight. The fish were weighed every 15 days to determine the amount of food to be given, and to calculate

growth parameters such as; average daily gain, specific growth rate, feed conversion rate and protein efficiency rate. Fifteen aquaria were used for the digestibility experiment. Each aquarium was stocked with 5 juvenile tambaqui. Chromic oxide was used as a marker and feces were collected from the bottom of the water column twice daily.

Results showed that both acid and biological silages had good storage properties. The variations in moisture, protein, lipid and ash between the four silages (formic acid silage stored for 4 and 30 days and biological silage stored for 4 and 30 days), that were included in the fish diets, did not induce any significant change in the amino acid profile of silage diets and the amino acids requirements for tambaqui were met. Although formic acid silage, stored for 4 and 30 days, showed higher protein solubilization and higher levels of free amino acid at the end of the 30 days storage period compared to the biologically fermented silage, there was no significant difference in growth and digestibility between treatments.

Based on the excellent acceptability of all diets tested by juvenile tambaqui, good protein digestibility and the satisfactory growth performance obtained in this study, we concluded that co-dried tilapia fish silage could be successfully utilized as an alternative protein source for fishmeal in fish diets. In this way, it is possible to utilize processing wastes for producing valuable animal protein and, furthermore, make a contribution to the protection of the environment.

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CHAPTER I

1.0 Introduction

Tambaqui, *Colossoma macropomum*, is one of the most promising candidates for fish culture in fresh waters of Latin America because of its complimentary characteristics, such as its omnivorous feeding habit when juvenile, excellent growth food conversion, high degree of adaptation to adverse factors such as oxygen depletion and high population density, resistance to disease and good acceptance for human consumption. Some aspects of its potential have been studied (Saint-Paul, 1986), including feeding and growth (da Silva *et al.*, 1984; Eckman, 1987; Merola & Pagan-Font, 1988).

C. macropomum (Figure 1) is the second largest scaled fish (after *Arapaima gigas*, Osteoglossidae) in the Amazon basin and reaches at least one meter in total length and 30 kg in weight. Adult tambaqui are oval-shaped fishes, being usually black ventrally and golden to olive dorsally. Tambaqui has a unique dentition, which consists of a combination of molariform-like teeth adapted for crushing nuts, on which it feeds heavily, and long and fine gillrakers that are used, especially in young fish, to capture zooplankton.

Tambaqui, was introduced into the Northeast Brazil in 1966 by the "Departamento Nacional de Obras Contra as Secas" (DNOCS). In 1977 artificial reproduction was satisfactorily achieved, and since then several studies on its performance in monoculture and polyculture have been developed (Silva, 1981; Silva *et al.*, 1984; Silva *et al.*, 1986; Silva *et al.*, 1987; Silva and Pinheiro, 1989; Melo *et al.*, 1987 and Mavignier *et al.*, 1995).

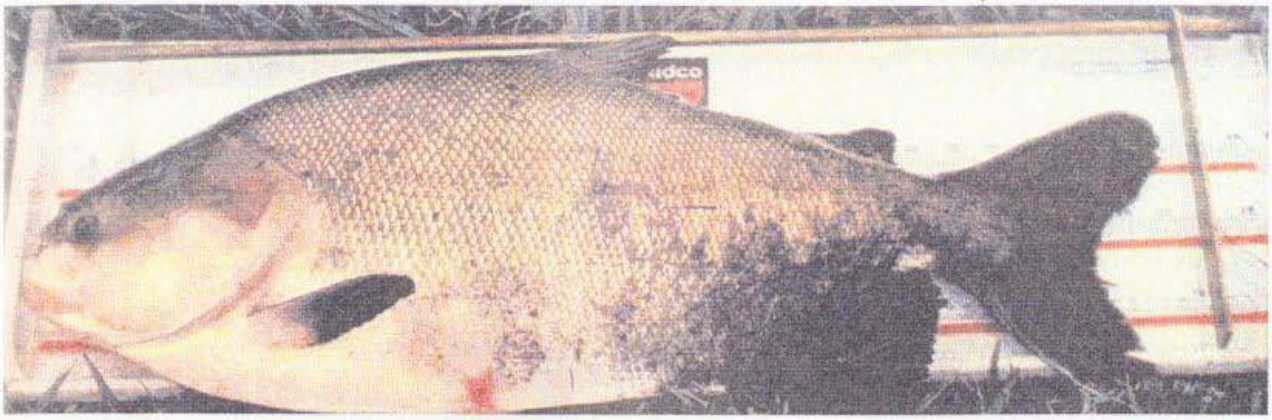


Figure 1 - Taxonomy of Tambaqui, *Colossoma macropomum*

Phylum Chordata
Subphylum Vertebrata
Class Osteichthyes
Subclass Actinopterygii
Order Cypriniformes
Suborder Characoidei
Family Characidae
Genus Colossoma
Species Colossoma macropomum

The first cultivation trials of *C. macropomum* in Latin America began in the 1980's. In 1988 fry production in Latin American was over 9.8 million specimens annually (more than 90% in Brazil); and were mainly distributed to re-stock reservoirs and used in small to medium scale culture systems (Hernandez 1989). However, this production was considered insufficient to meet regional needs. Some of the many factors limiting the culture of tambaqui, are: scarce fry supplies, high price of compound feeds and weak extension programs for the farmers.

Considerable interest has developed in the formulation of inexpensive and

nutritionally efficient feeds for the culture of *Colossoma macropomum*. The potential use of residues and by-products from agriculture, fisheries and food industries are being considered. Several by-products from those industries contain proteins of good biological value, which have the potential to partially replace animal protein from fishmeal in fish feeds. Such substitution is of major importance in developing countries, due to the high costs and low nutritional quality of locally produced fishmeal.

It is known that fish diets contribute largely to the increase in costs in an aquaculture operation. In order to decrease these costs to aquaculture, an alternative protein source to traditional fish meal is needed. The fish meal industry needs a regular supply of fish waste to operate economically. For this reason, in the industrialized countries, fish meal is concentrated in areas were these supplies are regular. This allows the economy of scale to overcome the high costs associated with fish meal production. The problems with raw material supply, energy costs, pollution control and process control faced by the fish meal industry are not easily overcome in developing countries and so large scale fish meal production is often infeasible.

The future of the fish meal industry is not promising, because of fluctuation in the amount of raw material landed and the increasing costs of production. It is becoming extremely necessary to search for an alternative technology by which fish protein can be preserved, and thus the high quality protein source present in fish waste can be recycled. The alternative process should be inexpensive and easily adjusted to small and large scale production.

Waste, such as fillet scrap (undersized fish and by-catch that may be generated) represents disposal problems for the fishing industry. The waste produced by this industry is commonly dumped into the sea. The biological costs are significant as is the

potential for pollution, and a waste of a valuable resource which could be utilized for the aquaculture industry. Furthermore, processing companies can increase their yield and optimize profits from trimmings that would normally be wasted.

Processing this waste into fish meal may be difficult because the supply is highly variable and fish meal plants require a relatively constant supply of raw material to operate economically. Considerable interest has developed during past few years in the preparation of a liquid fish product for animal feeds.

Ensilage was achieved on a large scale in some countries by using mineral or organic acid and by biological fermentation with lactic acid bacteria. The use of biological fermented or acid preserved silage could make a useful contribution to waste savings and nutritional improvement in low income societies.

The fish silage process requires very little capital outlay and the scale of the operation is easily adjusted to suit the supply of raw material. This makes the process particularly attractive in areas far removed from fish meal plants. Usually, wastes from these areas have to be transported, often over considerable distances, to the nearest fish meal factory. Ensilaging is also a process that is easily adaptable for use on board ships where by-catch and offal could be ensiled relatively cheap and efficiently.

Fish is a major source of animal protein for the people in developing countries. After reviewing the literature concerning the production of fish in the state of Ceará - Brazil, it was clear that there is a need to increase the production of cultured fish in order to expand the demand for cheap protein sources. By using indigenous species, such as, tambaqui, an alternative source of protein can be made available.

The lack of research work in the field of silage utilization as a main source of protein in fish diets in the Northeast of Brazil is noticeable. Important deficiencies in our present knowledge of the process include identification of the type of silage (acid preserved or biologically fermented) that is most suitable for the species of fish cultivated in the region, and its nutritive value. The investigation described in this thesis is designed to answer some of these questions and remove deficiencies in knowledge of the process.

The study is organized into the following phases:

I) Storage study:

The objectives of this study were to identify chemical changes that occur during the liquefaction and storage of a biological fermented silage and an acid preserved silage. To test the stability of the final product by storing it at ambient temperature for 30 days and record the quality changes. The changes in the quality of the products were determined at intervals by measuring the following:

A) Proximate analysis of the products at zero time and at intervals of 2, 4, 7, 14, and 30 days.

B) Amino acid analysis at the beginning and at the end of the storage period.

C) Changes in soluble nitrogen.

D) Changes in pH of the product during storage time.

II) Feeding trials:

The objectives of these trials were to test the nutritive value of the product by incorporating it in a basal diet to be fed to tambaqui. Evaluate the growth and digestibility of crude protein of the silage in juvenile tambaqui and compare with a control diet based on fishmeal.

CHAPTER II

2.0 Literature review

2.1. Nutrient requirements of fish

All animals require protein, lipids, carbohydrates, vitamins and minerals in their diets. The type and quantity of each one of these ingredients varies between species and within each species, depending on age, productivity function and environmental conditions. As an example, the rapid growth of a juvenile fish requires a higher level of protein than an adult fish. Therefore at each phase of the growth process, each species requires a different ration to satisfy its nutritional requirements. It has been postulated that reproductive fish will have nutritional requirements that are different from those of sexually immature fish. However these requirements have not been established for the great majority of the species which are potentially important for aquaculture.

2.2. Protein and amino acid requirements of fish

Protein are essential components that play a central role in the structure and functioning of all living organisms, making up 65% to 75% of their dry matter. Animals must consume protein to maintain a continuous supply of amino acids. Proteins are digested upon absorption or hydrolyzed to liberate free amino acids which are absorbed through the animal's intestinal wall and distributed to the various organs, via the blood stream, where they are used to synthesize new proteins during growth and reproduction, or to repair tissue. Therefore if the fish is not ingesting a sufficient quantity of protein, a rapid decrease of growth or loss of weight will occur, because the animal will retrieve protein of its own body in order to maintain vital functions.

Generally, fish require a higher percentage of protein in the diet than other vertebrate animals (Cowey and Sargent, 1979; Tacon and Cowey, 1985). Moreover, it has been suggested that the efficiency of protein utilization by fish is lower than that of other animals (Rumsey, 1981).

Protein requirement and utilization by the fish itself are largely influenced by such factors as: species and fish size; physiological phase; environmental conditions, such as temperature, salinity and water quality; diet composition, especially protein quality, availability of essential amino acids, energy level and feeding rate (Garling and Wilson, 1976; Cowey and Sargent, 1979; Lovell, 1980; Steffens, 1981; NAS, 1983).

Delong *et al.* (1958) observed that the protein demand for young *Oncorhynchus tshawytscha* at a water temperature of 8°C is about 40%. Water temperature of 14.5°C, however, necessitates a protein content of 55% to ensure rapid growth. Analogous observations on the impact of water temperature on protein requirements for maximum growth of channel catfish, *Ictalurus punctatus*, were suggest by Hastings (1973). Experiments have also shown that the protein requirements increased in salmonids as salinity increased. According to Zeitoun *et al.* (1973), for salinity of 10% and 20% the minimum protein demand of rainbow trout, was 40% and 43.5% respectively when casein and gelatin were used as a protein sources. Archdekin *et al.* (1988) concluded that post-juvenile chinook salmon maintained in seawater demanded a minimum of 44% protein in their diet to achieve maximum growth.

Page and Andrews (1973) reported that channel catfish with an average weight of 114-500g had a protein requirement of 25%, while fish with an average weight of 14-100g required 35% of protein in the diet.

Macedo *et al.* (1981), found that 22% of crude protein was satisfactory in the early fingerling stage of tambaqui (5 to 30 g) and 18% crude protein was satisfactory for the juvenile stages in rearing ponds.

Ramos and Grajales (1989) studied fingerling tambaqui, stocked in ponds at the rate of one fish per square meter and fed with diets containing 20 - 25 and 30% crude protein. They reported that the best growth performance was obtained by tambaqui fed diets containing 31.12% protein and 3800kcal/kg total energy.

Hastings (1973) found that at temperatures below 24°C, channel catfish, *Ictalurus punctatus*, grew no better on 35% protein than on 25%, but when water temperature exceeded 24°C the fish gained more on the 30% and 35% protein diet.

Dietary protein levels of 26 to 30% have been shown to provide the best growth performance in pacu, *Piaractus mesopotamicus*, fed isocaloric diets containing 3200 kcal/kg total energy (Carneiro, 1990; Carneiro *et al.*, 1984).

Every fish requires the same ten amino acids (Cowey & Sargent, 1972), which cannot be synthesized within the body and must exist in the supplied diet. Estimates of the nutritional balance of a protein are made by comparing the amino acid balance of the ingredient with the amino acids present in fish egg protein and in chicken egg protein (Halver 1957). This nutritional balance can also be found by experimentally determining the essential amino acid requirement for some other fish species (Tacon 1987), or the relative proportion of essential amino acids in fish muscle (Tacon and Cowey 1985) and expressed as a chemical score with 100% being the values found for fish egg and chicken egg.

Wilson and Cowey (1985), analyzed the amino acid composition of rainbow trout and Atlantic Salmon tissue and found that there was no significant difference between the two species. The authors showed that amino acid composition of these tissues was also similar to that of tissue from coho salmon and channel catfish. The authors also suggested that diets for fish may be improved by formulating them to simulate the amino acid balance of the whole body tissue of the fish being studied.

2.3. Energy balance in fish

Fish requires energy for growth, activity and reproduction. The biological processes involved in the utilization of the energy is defined as metabolism. The rate at which utilization of energy occurs is called the metabolic rate. Metabolic rate in fish is affected by temperature, species, age, physiological conditions, body functions and chemical conditions of water, such as oxygen, carbon dioxide saturation, pH and salinity.

Animals use energy not only to sustain the life process but also to support physical activity. Energy is necessary in all metabolic phases. According to Phillips (1972) fish are among the most efficient animals in converting feed into body protein. The great advantage to their high energetic efficiency is that fish are aquatic ectotherms and consequently do not have to use energy in maintaining body temperature.

Fish also have a very efficient mechanism for the excretion of waste nitrogen. According to Goldstein and Forster (1970), the formation and elimination of ammonia as a nitrogenous end product has many biological advantages. There is no expenditure of

energy involved in the conversion of the nitrogen in proteins to ammonia. Since ammonia is toxic even at very low concentrations and thus must be rapidly converted to less toxic compounds (urea and uric acid) by terrestrial homeotherms. Fish excrete approximately 85% of the waste nitrogen as ammonia (Randall and Wright, 1987) while the remnant is excreted as urea, uric acid, trimethylamine oxide, creatinine and creatin (Watts and Watts, 1974). According to Goldstein and Forster (1970) fish may use two mechanisms for excreting ammonia, simple diffusion as well as an exchange component in the gills.

According to Page and Andrews (1973), an optimal dietary protein concentration for fish is determined by a delicate balance of the dietary energy to protein ratio. Excessive nonprotein energy (lipids and carbohydrates) intake resulting from high digestible energy-to-dietary protein ratios, causes appetite or demand to be satisfied before a sufficient quantity of protein is ingested to satisfy demand or maximal growth rates. Moreover, a diet with a high level of energy to protein ratio can lead to deposition of large amounts of body fat.

The utilization of part of the protein through catabolism for the supply of energy is well known when a diet is poor in energy from other sources. Fish may also utilize protein more efficiently since the energetic value of protein for fish is higher. However, it is also known that the cost of protein is higher than other energy sources such as carbohydrates and lipids. Thus, the caloric/proteic relationship is one of the items of fundamental importance for animal nutrition. The ideal is to have a diet with a protein-energy balance in which minimum protein is used as a energy source by the fish, consequently showing higher protein efficiency (Castagnolli, 1979).

2.4. Sources of energy for fish

The three major sources of energy for animals, including fish, are carbohydrates, lipids and protein. These three components must be balanced in the diets in order that the fish can find the nutrients and energy necessary for its optimal development.

2.4.1. Protein as a dietary energy source

Generally, fish require a higher percentage of dietary protein than poultry and mammals. A possible reason for this can be that fish utilize carbohydrates less efficiently and some protein is metabolized for energy. Protein will be used for energy if insufficient energy is available from other sources, if protein is fed in excess of minimal requirements or if the protein is of poor quality. The ideal level of protein for fish is highly influenced by an optimal dietary protein to energy balance, amino acid composition and digestibility of the test protein and the amount of energy originated from the nonprotein sources in the test diet.

The protein requirements of fish are also influenced by fish size and age. According to Hilton and Slinger (1981) optimal dietary protein level for very young salmonids is 45 to 50% of their diet, while juveniles require 40% and yearlings require about 35% of dietary protein. Studies on tilapia protein requirement were developed by Balarin and Haller (1982) and the authors found that tilapia of less than 1g need 35 to 50% protein, tilapia from 1 to 5g need 30 to 40% protein , from 5 to 25g require 25 to 30% while tilapia weighing more than 25g require a level of protein around 20 to 25%.

Water temperature also plays an important role in the protein requirements of some fish. Chinook salmon were found to require 40% protein at 8°C and 55% protein

at 15°C (DeLong *et al.*, 1958). In contrast, according to the NRC (1981) no differences in protein requirements were found for rainbow trout when fed diets containing 35, 40 and 45% protein at temperatures ranging from 9° to 18°C.

2.4.2. Carbohydrate as a dietary energy source

Carbohydrates are considered the cheapest form of energy in the diet of humans and domestic animals. Carbohydrates constitute a group of the most controversial nutrients within the field of fish nutrition, especially the utilization of dietary carbohydrates by carnivorous fish such as salmonids. Carbohydrate utilization is generally poor in fish and results from a deficiency in digestive and metabolic capacity.

While carnivorous fish show little or no ability for carbohydrate assimilation, the herbivorous, omnivorous and planctophagus fish which synthesize the cellulase and amylase enzymes in their digestive tract easily hydrolyze the carbohydrates contained in natural vegetal foods or ingredients from plant origins integrated into their rations.

Carbohydrate levels ranging from 30% (Buhler and Halver, 1961) to as high as 38% (Kaushick *et al.*, 1989) may be used efficiently by salmonids. However according to Hilton and Atckinson, 1982 and Hilton *et al.*, 1982, digestible carbohydrate levels should not exceed 14% of the diet.

2.4.3. Lipids as a dietary energy source

Lipids are important sources of energy and the fatty acids are essential for the normal growth and survival of fish. Lipids are the vehicles for the absorption of the fat

soluble vitamins and other components such as steroids. The lipids, especially the phospholipds and the steroid esther, play an important role in cellular and subcelullar membrane structure and are also involved in many other aspects of metabolism, such as many hormones and steroids. Also, the long chains of polyunsaturated fatty acids are the precursors of prostaglandin in fish.

Lipids are an important energy source for carnivorous fish since carbohydrates are poorly utilized (Hilton and Atkinson, 1982), most likely because these fish encounter little carbohydrate in their diets (Walton and Cowey, 1982). Lipids contain over 1.5 times as much energy per unit weight relative to proteins and nearly 2.5 times as much as carbohydrates (Lehninger, 1982). An adequate level of lipids can minimize the cost of a diet by decreasing the protein level, which is a more expensive energy source (Cowey and Sargent, 1979; Watanabe, 1982).

According to Millikin (1982), the ideal lipid level to be included in a diet depends mainly on factors such as: the minimum level of lipid which will maximize the use of protein and promote a good growth rate; the possibility of obtaining a fish with low fat content for human consumption and an optimal lipid level that will not contribute to the oxidative rancidity of the diet.

Dupree (1969) working with channel catfish with an initial weight of 1.25g fed diets of 35% protein and 8% and 16% lipid (originating from equivalent parts of animal and plant fat), found that fish which received diet containing 16% fat showed a reduced growth and protein retention.

In order to evaluate the protein utilization related to lipid content, Lee and Putnan (1973) worked with fingerlings of rainbow trout with an initial weight of 4.8g, fed diets

with 35%, 44% and 53% protein, 8%, 16% and 24% lipid and a common average level of 6.5% crude fiber in all diets. The authors found that the best protein utilization was given by the fish fed diets with 24% lipid content.

Dupree *et al.* (1979) studied the optimum lipid level on fingerlings of channel catfish with an initial weight of 7 - 7.5g, fed diets containing 25% protein and 5%, 8%, 12% and 20% lipid. The author found that maximum growth gain and higher protein retention were showed by fish fed diets containing 12% lipid content.

Pezzato (1990) evaluated three levels of fat (8%,16% and 24%) as either plant (soybean) or animal (swine fat) origin which were supplemented to a diet containing corn, fish meal, soybean, vitamin and mineral premixes. The additional fat was added by replacing corn meal. The author reported that pacu, *Piaractus mesopotamicus*, can utilize high levels of either animal and plant fat. The higher dietary lipid intake resulted in higher body fat levels. Whole body fat content increased 38.1% and 27.6% in fish fed the higher animal and plant lipid sources, respectively.

According to Reinitz and Yu (1981), the replacement of soybean and fish oil with animal oil on diets fed to rainbow trout fingerlings, showed a positive result at the end of the 182 days long experiment. This confirmed the results obtained by Leatherland *et al.* (1979) and Cowey *et al.* (1979). These authors observed that by replacing oil from vegetal origin to animal origin, an increase in growth rate was observed with no significant changes in protein and lipid in the carcass composition. The authors also found a positive correlation between the fatty acid composition of the diet and that of the carcasses.

2.5. Digestibility measurements in fish

The nutritional value of food is based not only on its chemical composition but also by the ability of the fish to digest and absorb the nutrients. There are also considerable variations in the ingredient digestibility within the species and with different environmental conditions (Lovell, 1977).

Acquiring knowledge of the digestibility of nutrients from various feed ingredients is essential in preparing fish diets and can be done through the substitution of one ingredient by another, using an experimental and a control diet. The substitution of an expensive ingredient by a cheaper and satisfactory one can be important in the development of a minimum cost diet.

The percentage of a nutrient consumed, which did not appear in the feces, is defined as the digestion coefficient. The undigested portions of feed are excreted in the feces. By determining the amount of feed which is rejected in the feces, the digestibility of that specific feed by the animal can be calculated.

In order to conduct a digestibility experiment it is very important to determine the amount of each nutrient in both feed and feces. The earliest method used for determining digestibility coefficients for domestic animals was the direct or total collection method. This method consisted of determining the amount of protein, fat, carbohydrate, energy and total dry matter eaten, minus the amount found in the feces (Hepher, 1988). However the direct method has its drawbacks. One inconvenience is that the amount of food eaten by the fish has to be monitored and closely controlled. Since the fish are fed in water and feces are excreted into the water, nutrient leaching will occur in both food and feces. Thus the digestibility coefficient can be overestimated

according to the loss of nutrient in the water (Hepher, 1988).

The other method used to calculate digestibility is the indirect method. In this method, in addition to the chemical analysis for the usual proximate nutrients, the content in the feed and in the feces of a very indigestible reference substance is determined. The substance may be a natural constituent of the feed or be added to it, or both. The apparent digestibility coefficient (ADC) of the nutrient based on the indirect method is determined according to Maynard & Loosli (1969).

$$ADC\ (\%) = 100 - \frac{\%\ \text{indicator in diet}}{\%\ \text{indicator in feces}} \times \frac{\%\ \text{nutrient in feces}}{\%\ \text{nutrient in diet}} \times 100$$

The equation shown above is used to calculate apparent digestibility, since feces contain nitrogen of body origin as well as undigested feed nitrogen.

Markers or indicators used in this method can be of two types; artificial foreign substances introduced into the feed, such as Cr_2O_3 (Furukawa & Tsukahara, 1966) or substances that are a natural component of the feed itself, such as, cellulose (Buddington, 1980) and acid insoluble ash (Atkinson *et al.*, 1984; Bowen 1981; De Silva e Pereira, 1983). In either case, to be an effective marker, a substance must not be absorbed through the lumen or be toxic, move along the gut at the same velocity from the rest of the food material, and must not interfere with the digestive metabolism of the animal (De Silva, 1989).

Although nutrient leaching may still occur, the convenience of this indirect method over the direct, is that there is no need to determine the amount of food ingested nor the amount of feces egested (Maynard & Loosli, 1969). The major

disadvantages of this method are in the variety of techniques used to collect the fecal samples without leaching of nutrients into the water.

Many techniques have been used to collect feces. Several authors choose to collect the feces directly from the posterior region of the intestine (Smith & Lovell, 1973; Windell *et al.*, 1978). However, this technique requires killing the fish and using a large number of fish. Other authors have used the stripping technique (Austreng 1978; Tacon *et al.*, 1984). Wherein the fish is anesthetized and pressure placed on the fish's abdomen. The presence of milt, ova, blood and body slime with the striped feces has been suggested as a disadvantage of this technique. Also, the need to anesthetize the fish prior to handling may induce defecation and acceleration of intestinal transit (Spyridarkis *et al.*, 1989). Anal suction has also been used as a feces collection technique (Windell *et al.*, 1978), but is also known to cause high levels of stress.

In order to overcome the drawbacks of the fecal collection methods shown above, many authors have collected the feces as soon as they are naturally released into the water. However, according to Windell *et al.* (1978), this can result in overestimation of the digestibility values, since nutrient leaching may occur. Several devices for the separation and recovery of feces have been proposed. They vary from a fine mesh net to more complex mechanical techniques. Among them, the "Guelph system" is suggested to minimize fish handling and nutrient losses (Cho *et al.*, 1975). The advantage of feces collection from the water column is that repeated samplings can be done and growth experiments can be monitored at the same time.

2.6. Selection of dietary ingredients for fish diets

The ingredients used in animal feeds are basically composed of natural products such as the majority of by-products of the milling industry.

In the daily processing of diet formulation, ingredients are generally substituted for one another depending on the local market price. Fish meal is a product commonly used as the major protein source in formulated fish diets. The variable supply and quality of fish meal, and especially its high cost have been identified as the primary drawback of using it in fish feeds (Higgs et al., 1982). It is predicted that alternatives to traditional fish meal will be needed in fish feeds in the near future (Hardy & Masumoto, 1990). By the early years of the 21st century the use of fish meal by the aquaculture industry is expected to double while world production is expected to remain constant (Barlow 1989). Consequently, various attempts have been made to replace fish meal, partially, if not all with other animal or plant protein sources.

Waste (fillet scrap, undersized fish, and by-catch) that may be generated represents a disposal problem to the fishing industry and at the same time represents a potential resource to the animal feed industry. Processing this waste into fish meal may be difficult because the supply is highly variable and fish meal plants require a relatively constant supply of raw material to operate economically. Ensilaging is an inexpensive way to store the raw material until it can be processed into a useful by-product (Hardy *et al.*, 1983).

2.7. Fish silage

Fish silage is a liquefied product made from whole or parts of fish to which only an acid has been added, and in which liquefaction of the fish mass is carried out by enzymes already present in the fish (Tatterson and Windsor, 1974).

Historically, the practice of using acids for preservation was first explored in the 1920s in Finland by A.I. Virtanen who used mixtures of hydrochloric and sulphuric acid to preserve green fodder (Tatterson and Windsor, 1974). In 1936, in Sweden, Professor Edin used a similar acid mixture to liquefy and preserve fish and fish waste. In 1940 Edin quantified and calculated the acid requirements based on the pH, protein and ash measurements for minced fish (Tatterson and Windsor, 1974). Production of fish silage on a commercial scale started in Denmark in 1948. Tatterson and Wigall (1976) mentioned that Denmark produced between 50,000 and 100,000 metric tons of fish silage per year.

Generally one of two processes are employed in the production of fish silage, namely acid preservation or lactic acid fermentation. Acid preservation is accomplished by adding enough acid to minced fish or fish offal to lower the pH to a point in which the silage is stable to bacterial degradation. For production of lactic acid fermented silage also known as biologically fermented silage, bacterial fermentation is initiated by the addition of lactic acid bacteria and fermentable carbohydrates to minced fish. The carbohydrate source will then be progressively converted to acid, which lowers the pH of the silage and ultimately imparts spoilage resistance to the final liquid product. This procedure is preferred in developing countries because it is cheaper to do, involves simple technology that is adaptable to the cottage level, and the product has good storage properties (Dong *et al.*, 1993).

2.7.1. Fish silage by acid preservation

Acid preserved silage is produced by adding an organic or inorganic acid to the minced fish. The principle involved in this technique is that the enzymes already present in the fish break down the protein and liquefy the fish while the acid prevents microbial spoilage.

A number of acids have been used to produce fish silage. Organic acids are more expensive than inorganic. A mixture of inorganic and organic acid, using the cheap inorganic acid to lower the pH sufficiently for the organic acid to become antimicrobial was recommended by Raa & Gildberg, 1982.

Disney and Hoffmans (1976) combined formic and sulphuric acid to decrease the pH and increase the bacterial activity. Rattagol *et al.* (1979) using the same combination of the two acids above mentioned, reported that sulphuric acid is not only cheaper than formic acid but may also cause less damage to vitamin B_{12} in the diet.

Gildberg and Raa (1977) found that a silage produced by a combination of propionic acid and formic acid was more resistant to microbial deterioration even with moist mixtures containing straw meal.

The use of formic acid alone is particularly suitable, even though it is more expensive than inorganic acids. Formic acid does not require neutralization before feeding to animals. Corrosion of equipment and containers is also considerably reduced. It is noticed that when formic acid is used, a pH of 4 is sufficient to produce a stable product because of its antibacterial action. Formic acid is currently preferred as a preservative and extensive research has been done using this acid (Espe *et al.*,1990;

Haaland and Njaa, 1990; Haaland *et al.*, 1990; and Nicholson and Johnson, 1991).

2.7.2. Fish silage by biological fermentation

The principle in the ensiling process by biological fermentation is to add fermentable carbohydrate to the fish offal where the lactic acid produced preserves the product. Even though fish contain a natural flora of lactic acid bacteria, a starter culture can also be added which rapidly converts the sugar to acid and preserves the whole mass. Lindgren and Clevstrom (1978) reported that in addition to the acid, some lactic acid bacteria produce antibiotics which contribute to their preservative action. Twiddy *et al.* (1987) verified that common food pathogens *Staphylococcus aureus, Salmonella typhimurium, Clostridium sporogenes* and *Escherichia colii* rapidly disappear during fermentation.

It is generally acknowledged that biological fermentation of fish silage started in Scandinavia. Petersen (1953) described two methods for producing fermented fish silage. Sulphuric acid and molasses were used in the first method whereas the second method involved the utilization of molasses and lactic acid bacteria.

Nilsson and Rydin (1965) proposed that fish can be fermented along with cereal meal as a starch source and malt meal as an amylase source. A mixture of small Baltic herring with 20% barley meal and 2% malt meal, ensiled at 28^{o}C for 4 weeks, yielded a silage at pH 4.3 containing 5% acid as lactic acid. Staton and Yeoh (1977) studied the substitution of Malaysian ingredients namely tapioca starch instead of cereal, and ragi (a mixed culture of amylotic moulds grown on rice) instead of malt. They found that in the absence of salt, it was necessary to use fish:tapioca ratios of 3:2 or less to obtain

satisfactory results.

Dong *et al.* (1993) evaluated the suitability of converting salmon viscera into a co-dried product which could consequently be the major protein ingredient for salmonid diets. Silages were prepared by adding a dry culture of *Lactobacillus plantarum* and *Streptococcus faecium*, and alternatively by the addition of an organic acid or sulphuric acid. According to the authors, there were many advantages gained by using bacteria rather than acid to prepare fish silage. The bacteria were easy to maintain and to reproduce, thus the cost of purchasing acid was eliminated. Another advantage was that the handling of acid was avoided. The lactic acid procedure also allowed preparation of silage with a low level of moisture, which would have had to be evaporated later in the drying process.

Although lactic acid bacteria are natural inhabitants of fish, they are present in low numbers: 10^{1} - 10^{4} /g. Fish also contain only small amounts of free sugar, which is the essential substrate for growth of such bacteria (Mackie, 1971; Raa *et al.* 1983). Therefore, to preserve fish or animal waste products by fermentation, it is essential to add a sugar source and a starter culture of suitable lactic acid bacteria. Both spoilage bacteria and lactic acid bacteria will contribute to the initial acid production, since the conditions are anaerobic and sugars are available. However, growth of the lactic acid bacteria will be favored as the silage becomes more acidic. If the pH falls below 4 in such a silage, lactobacilli will become the predominant organism and harmful bacteria such as *coliforms*, *enterococci* and even *Clostridium botulinum* spores will be destroyed (Raa *et al.*, 1983).

Kompiang *et al.* (1980) investigated the preparation of biological silage using molasses as a carbohydrate source to study the ratio of fish to molasses. The results

showed that silage prepared with a fish:molasses ratio of 100:15 had a fresh, strong acidic smell up to 21 days of storage.

Lindgren and Pleje (1983) investigated silage storage stability related to temperature and the use of antifungal additives. Storage stability at 5°C, 12°C and 24°C was studied in fish silage after an initial fermentation period of four days at 20 - 24°C. It was found that during storage at 12°C and 24°C, lactic acid was produced at a level around 40 g kg^{-1}. A pH below 4 was obtained within 30 h at 24°C. At 5°C, a production of lactic acid to 37.5 g kg^{-1} was observed after 33 days, but the content declined to 32.5 g kg^{-1} after 54 days of storage. The pH increased slightly, independently of storage temperature and additives. After 33 days, no difference was observed for the nine treatments (*i.e.* stored at 5°C, 12°C and 24°C, with added propionic acid, sorbic acid and a control). After 54 days of storage, changes were observed and the pH had increased to 5.0 in the sample stored at 24°C without any antifungal additives. With sorbic acid or propionic acid added, at a storage temperature of 24°C, the pH was 4.7. The samples stored at 5°C and 12°C had pH values around 4.8 after 54 days of storage.

Twiddy *et al.* (1987) evaluated the use of rice cassava as a carbohydrate source with and without a one day pre-fermentation prior to mixing with the minced fish. Also, different fish/carbohydrate proportions were studied in the range of 20 - 100% w/w of minced fish. The authors found that the use of pre-fermented cassava (20% w/w) resulted in rapid fermentation. The pH declined to less than 4.5. The ratio of lactic acid bacteria/spoilage bacteria exceeded four log cycles of growth within 48 h. The use of added sugar (2% w/w glucose) was found to be necessary to prevent an undesirable pH increase after the first 2 days of fermentation.

Martin and Bemister (1994) evaluated the use of peat extract as a carbohydrate source for the fermentation of lactic acid bacteria in fish offal silage. The authors also compared the use of formic acid with peat extract as an acid source for the ensiling process. The effects of pre-heating and pre-freezing the raw material prior to ensiling were also investigated. The authors found that peat extract was an effective carbohydrate source for fermentation of fish offal, but that it was inadequate as an acid source for fish offal silage. The authors concluded that fermentation by lactic acid bacteria was the most efficient method of ensiling with either peat extract or glucose as the carbohydrate source. Also, heating or freezing the raw material prior to ensiling was found to have no effect on the preservation effectiveness.

2.8. Nutritive value of silage and its use

Fish silage has been used in animal feed with varying success, depending upon the animal species, the type of acid used (in the case of acid-preserved silage), and the method of processing. In some cases the animals grew poorly on fish silage diets. Reduced nutritional value due to high level of hydrolyzed protein has been suggested as a cause of this (Hardy *et al.*, 1983; Wood *et al.*, 1985: Stone and Hardy, 1986).

During liquefaction, connective protein tissues are broken down by the enzymes in the fish. The enzymes of importance in the silage are proteinases that break down proteins into peptides and individual amino acids, as well as lipases that break down fats into free fatty acids (Jangaard, 1987). During liquefaction, the majority of proteins are converted to short peptides, a portion of which may be further hydrolyzed to free amino acids. When autolysis is allowed to continue, the percentage of nitrogen as free amino acids increases while the percentage as polypeptides decreases. Nevertheless,

there is always a portion of the protein that is not broken down by enzymes.

Hardy *et al.* (1983) found that the length of storage of fish silage influence its nutritional value for trout, and suggested that the total replacement of fish meal by fish silage should not be employed if the liquefied fish silage was stored for a long period before being dried and used in trout diets. However, by heating the silage, the liquefaction process is discontinued, and a higher quantity of silage can be used in the diets without affecting fish growth or nutrient utilization (Gildberg *et al.* 1984; Fagbenro and Jauncey, 1993a). In practical terms, the reaction can be stopped much earlier than the point of maximum protein solubility and still yield a liquid product.

Wee *et al.* (1986) investigated the utilization of fish silage preserved either by acid or lactic acid bacteria. The biological fermented silage was formulated using *Lactobacillus casei*, cassava or molasses as the carbohydrate source, and with or without the antioxidant butylated hydroxytoluene. The silages were stored for 12 weeks and their nutritional and chemical characteristics were studied. The authors found that the proximate composition of the silage did not change with the storage period. Diets for catfish, *Clarias batrachus,* were formulated, and a control diet based on fish meal was used as a comparison. The fish grew well with all diets.

Fagbenro and Jauncey (1993a) investigated the nutritional value of co-dried fermented tilapia silage as a dietary protein supplement for catfish, *Clarias gariepinus*, in digestibility experiments. They found that a storage period of 180 days resulted in an increase in non-protein nitrogen content and a slight increase in free fatty acids content. However, this seems to have had an insignificant effect on the apparent protein digestibility by catfish of co-dried tilapia silage diets. The results showed that fermented tilapia silage proved to have good storage properties.

Extensive research has also confirmed that fish silage is a useful protein source for swine (Green *et al.*, 1988), poultry (Krogdahl, 1985 and Ologhobo *et al.*, 1988), cattle (Nicholson and Johnson, 1991) and fish (Wood *et al.*, 1985; Wee *et al.*, 1986; Rungruangsak and Uthe, 1992; Fagbenro and Jauncey, 1993a, 1994; Heras *et al.*, 1994 and Ali *et al.*, 1994).

CHAPTER III

3.0 Material and methods

3.1. Silage preparation

Mixed sex tilapia, *Oreochromis niloticus*, culled as a result of routine husbandry techniques, were obtained at the Rodolph Von Ihering Ictiological Research Center in Pentecoste, Ceará, Brazil. The fish were kept frozen (-20°C) and latter thawed at room temperature before use. Thawed whole tilapias were minced and passed through a die with 0.5 cm diameter holes using a Siemsem mill model PSL 291. Three samples were obtained for chemical analyses.

Acidification of silages was accomplished by two methods: by addition of lactic acid bacteria and subsequent fermentation (bacterial fermented silage) and by addition of formic acid (formic acid silage).

Biologically fermented silage, which was labeled silage BFS, was prepared by adding to the minced tilapia: 5% *Lactobacillus plantarum* ATCC 8014, 15% molasses, as a carbohydrate source, and 1% potassium sorbate to inhibit the growth of mould. *Lactobacillus plantarum* (ATCC 8014) was previously cultivated in MRS broth (Man Rogosa Sharpe) for 48 hours before the preparation of the silage and incubated for 24 hours at 30°C (Lamas *et al.*, 1992).

Formic acid silage, which was labeled silage FAS, was prepared by adding to the minced tilapia 3% (w/w) of formic acid (85%), and 1% of potassium sorbate to prevent mould growth.

Three batches of both silages were mixed in 20-l plastic buckets (12 kg/bucket), loosely covered with plastic lids and incubated at room temperature (28 - 32°C). The silages were stirred daily with a wooden paddle by hand for approximately 1 minute.

3.2. Analytical Methods

Samples from each batch were heated in a water bath for 30 minutes in 90°C to cease autolysis. The samples were taken for chemical analysis at the beginning of the experiment (minced tilapia) and after 2,4,7,14 and 30 days. Silages stored for 4 and 30 days from both, biological fermented silage (BFS) and formic acid silages (FAS), were subsequently co-dried with wheat bran, sun dried for approximately 20 hours in steel trays 1 cm in depth and then used in the feeding trial.

3.2.1. pH

The pH of the minced fish waste and of the silages were determined with a portable digital pH meter (DIGI-SENSE Model 5038-10) by inserting the pH probe into the plastic buckets containing well mixed samples of material.

3.2.2. Moisture Content

Moisture content was determined by oven-drying at 105°C to constant weight (A.O.A.C., 1990, Method 950.01).

3.2.3. Ash Content

Ash content was determined from weighed samples in a porcelain crucible by heating the dried sample in a muffle furnace at 550°C (A.O.A.C., 1990, Method 938.08).

3.2.4. Lipid Content

Crude lipid was determined after Soxhlet extraction of dried samples with petroleum ether (A.O.A.C., 1990).

3.2.5. Total Nitrogen

Total Nitrogen was determined using the Kjeldahl method described by A.O.A.C., 1990, Method 955.04. The content of crude protein in samples was calculated by multiplying the percentage of nitrogen by 6.25.

3.2.6. Soluble protein

Non-protein nitrogen was determined as the percent fraction of the total Kjeldahl nitrogen that was not precipitated in a 10% trichloroacetic acid (TCA) solution. Non-protein nitrogen was determined as follows: to 50g of sample, 100 mL of 10% trichloroacetic acid solution was added and blended for 1 hour at 20°C using a commercial Waring blender. After homogenization, the insoluble residue was removed by centrifugation at 2000 x g for 15 minutes at 5°C. The supernatant was collected and an aliquot of 10 ml was taken for determination of soluble nitrogen using the Kjeldahl method.

3.2.7. Amino acids analysis

Formic acid and biologically fermented silages stored for 4 and 30 days were used as ingredients for the formulation of the diets used during the growth and digestibility trials. Total amino acids and free amino acids analyses were performed only on the silages cited above.

3.2.7.1. Total amino acids

Amino acid analysis was performed using a 121 MB Amino Acid Analyzer (Beckman Instruments Inc, Palo Alto, CA), on the sun-dried fish silage samples. The samples were digested in 6N HCl under vacuum at 110°C (Blackburn, 1978). The analyzer was interfaced with a model HP 3395 integrator (Hewlett-Packard, Boise, Idaho) enabling accurate peak area analysis in the nanomole range.

The sulphur-containing amino acids, methionine and cysteine, were measured separately as methionine sulphone and cysteic acid, after perfomic acid oxidation and 6N HCl hydrolysis as described by Blackburn (1978).

Tryptophan was determined by UV absorption after hydrolysis with 3N mercaptoethanesulfonic acid at 110°C according to the method of Penke *et al.*, (1974).

3.2.7.2. Free amino acids and ammonia

Free amino acids and ammonia were determined according to Stone and Hardy (1989) using a Beckman Amino Acid Analyzer Model 121 MB after extracting with 4% sulphosalicylic acid (SSA) and filtering through a 0.45mm filter (excluded MW > 500).

3.3. Growth and digestibility trials

3.3.1. Preparation of diets

Five isocaloric and isonitrogenous diets were formulated using both silages (biological fermented and formic acid) with 4 and 30 days of storage and a control diet containing fish meal as the main protein source.

The diets were calculated using the LINDO (Linear Interactive and Discrete Optimizer, 1987) computer program. The diets were denominated as follows: Diet 1 (FAS4) - using formic acid silage with 4 days of storage as the main source of protein; Diet 2 (FAS30) - using formic acid silage with 30 days of storage as the main source of protein; Diet 3 (BFS4) - using biological fermented silage with 4 days of storage as the main source of protein; Diet 4 (BFS30) - using biological fermented silage with 30 days of storage as the main source of protein; and Diet 5 (control) - using fish meal as the main source of protein.

The liquefied silages used in the diet preparation were made into a dry product by mixing with wheat bran in the ratio of 3:1 (silage:wheat bran, w/w) and then sun-dried in steel trays with 1 cm of depth. All foodstuffs with the exception of the silages were obtained commercially. Chromic oxide (Cr_2O_3) was ground in a mortar and pestle until all lumps had disintegrated and then it was combined with the ingredients. Vitamin and mineral pre-mixes were added to all 5 diets and tapioca starch was used as a binder. The diets were prepared by mixing the ingredients homogeneously. Distilled water was slowly added while mixing until a sticky pulp was obtained. Mixed feeds were then passed in a household meat mincer with a 2mm diameter and then dried for 24 hours at 50^{o}C in a drying oven. In order to obtain pellets the dried diet was chopped manually. All diets were kept in plastic containers in a refrigerator throughout the experiment.

Chemical analysis of the diets were performed as described in section 3.2.

3.3.2. Experimental design

Growth and digestibility trials were conducted at the Rodolph Von Ihering Icthiological Research Center in Pentecoste, Ceara, Brazil. The trial started March 1st, 1995 and lasted 90 days. Fifteen circular cement tanks, each measuring 140 cm in diameter, with a volume of 650 l each and constant water exchange at a rate of 6 l/minutes were used in the experiment (Figure 2).

Figure 2 - Cement tanks used during growth trial.

Juvenile tambaqui, *Colossoma macropomum*, were obtained in February, 1995 from the Research Center. Fifteen days prior to the beginning of the experiment, the fish

were selected randomly and placed on an absorbent cloth to remove excess moisture, and individually weighed. At the end of a one-week acclimation period, all fish were removed from the tanks and re-weighed. Each tank was stocked with 15 fish. The tanks were randomly arranged into 5 treatments with three replications.

3.3.3. Growth trial

The fish were fed in two equal portions twice daily (0800 and 1600 hours) at a rate of 3% of fish body weight. Every 2 weeks the fish were removed and measurements of weight and length were performed. The fish length was determined by using an ictiometer rule and the weight recorded by using a commercial scale. Daily feed allowances were adjusted biweekly after weight determination, and recalculated as a percentage of the mean dry weight during every two week interval, taking into account any mortalities (Silva *et al.,* 1984; Silva *et al.,* 1987; Hancz, 1993).

After weighing the fish, each tank was cleaned to prevent accumulation of fecal materials and to reduce algae growth to a minimum. The same source and amount of water were used to refill each tank before the weighed fish were returned to their respective tanks. Water quality of the tanks were monitored once a week using an automatic water quality meter (grant/YSI 3800 Water Quality logging system; YSI inc, Yellow Springs, OH.)

Specific growth rate (SGR), average daily weight gain (ADG), protein efficiency ratio (PER) and feed conversion ratio (FCR) were calculated according to Tacon (1987). At the end of the 90 days growth trial, the final weight of the fish in each tank was measured, and the overall weight gain and survival rate determined.

Specific Growth Rate (SGR)

$$SGR = \frac{[(\ln \text{final body weight} - \ln \text{initial body weight})]}{\text{time period (in days)}} \times 100$$

Average Daily Weight Gain (ADG)

ADG = Final weight - initial weight / time period (in days)

Protein Efficiency Ratio (PER)

PER = weight gain (g) / protein fed (g)

Feed Conversion Ratio (FCR)

FCR = Feed fed (g) / weight gain (g)

3.3.4. Digestibility trial

Fish were acclimated in 15 aerated 110 l aquaria. A three-day starvation period was imposed prior to experimental feeding to assure empty alimentary canals. Each aquarium was stocked with 5 juvenile fish. The water was partially changed twice a week. After each feeding, the excess feed was removed and the bottom of the tank siphoned to flush out any uneaten food that had sunk to the bottom of the aquarium.

3.3.4.1. Fecal collection procedure

Feces were collected from the bottom of all fifteen aquaria twice daily (0730 hours and 1530 hours) over a period of four weeks. Feeding begun immediately after

each collection. The fecal collection times (morning and afternoon) were kept together. The collected feces were centrifuged at 10,000 x g for 30 minutes at approximately 5°C and the supernatant discarded. They were then stored at -40°C and subsequently freeze dried using a System Freezer Drier (Labconco, LYPH LOCK 6).

3.3.4.2. Chromic Oxide determination

Chromic oxide (Cr_2O_3) is commonly used as a reference substance in digestibility experiments. Chromic oxide was determined using the wet-acid digestion technique of Furukawa and Tsukuhara (1966). A mother-solution containing 10 mg of Cr_2O_3 in 100 ml of distilled water was diluted in different standard concentrations from 0.5 to 4.0 mg in 100 ml, in intervals of 0.5 mg each. A standard curve relating the absorbance readings with those of the solutions whose concentrations were previously known was determined. Using the method of orthogonal polynomials, a linear equation was developed which allowed the determination of Chromic oxide in the feces using an spectrophotometer (Spectronic 88, Bausch & Lomb), which read optical density at 350m.

3.3.4.3. Apparent protein digestibility

Apparent protein digestibility (APD), based on the indirect method, was determined according to the following formula (Maynard and Loosli, 1969).

$$\text{ADC (\%)} = 100 - \frac{\text{\% indicator in diet}}{\text{\% indicator in feces}} \times \frac{\text{\% nutrient in feces}}{\text{\% nutrient in diet}} \times 100$$

3.4 Proximate analysis of fish carcass

At the beginning and at the end of the feeding trial, several fish (10 fish/tank) were sacrificed, homogenized in a blender and frozen (-20 ^{o}C) for further analyses of moisture, protein, lipid and ash, as described in section 3.2.

3.5. Statistical analysis

Data on weight gain, SGR, AFCR, PER and APD for each treatment were analyzed by one way ANOVA ($p = 0.05$). The mean values were compared between diets according to t test for paired comparisons. Correlation analyses was also used to measure the relationship between the storage days and the amount of amino acids of the silages.

CHAPTER IV

4.0 Results and Discussion

4.1. Nutritional and storage characteristics of fish silage

4.1.1. pH

The evolution of pH values for both silages is shown in Figure 3. At the beginning of the experiment the pH (an indicator of the ensiling process) of minced tilapia, acid silage and biological silage were 6.5, 3.8 and 5.8, respectively. At the end of the first week the pH of the formic acid silage and the biological fermented silage decreased to 3.6 and 4.0, respectively, and remained constant until the end of the 30 days storage.

4.1.2. Proximate composition of fish silage

4.1.2.1. Protein content

The crude protein content of formic acid silage was 14.78% (wet weight basis) while the biological fermented silage contained 12.72% (Table 1). A lower value in the protein content of the biological fermented silage was also reported by James *et al.*, (1976); Fagbenro and Jauncey (1993b), Batista (1987) and Wee *et al.*, (1986). According to Batista (1987) the lower protein content in the biological fermented silage was due to the addition of carbohydrate and slight dilution effect by the acid produced.

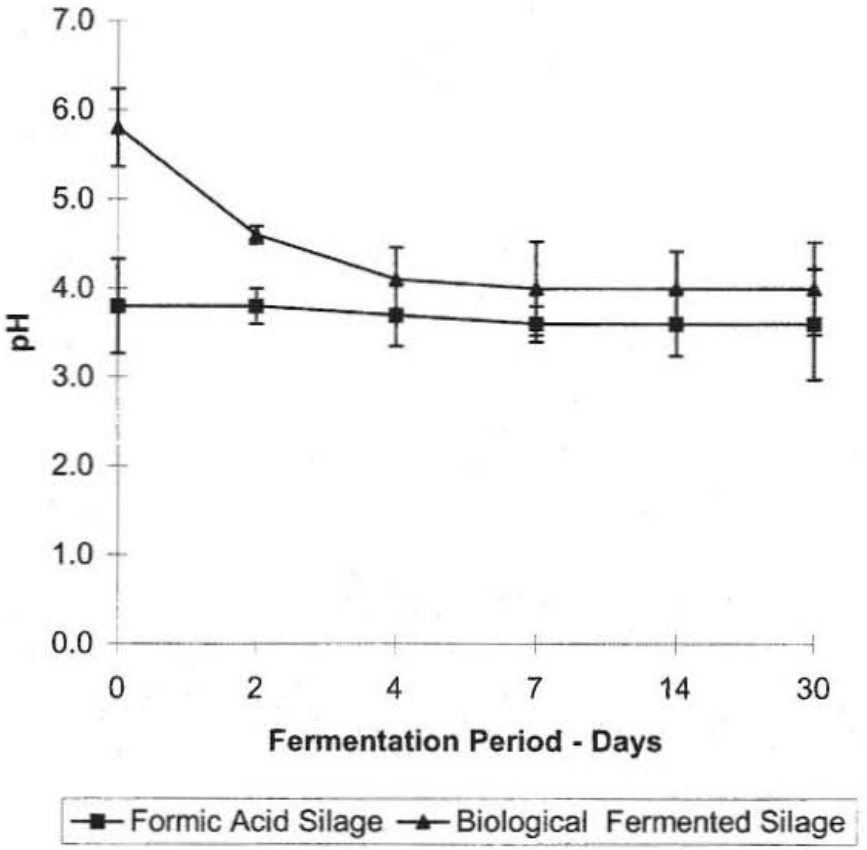


Figure 3 - pH of the tilapia silage during fermentation at 30° C

4.1.2.2. Moisture content

Moisture content in the biological fermented silage was lower than in the minced tilapia and in the formic acid silage (Table 1). An increase in dry matter in the biological fermented silage was also reported by Jackson *et al.* (1984); Wee *et al.* (1986); Espe *et al.* (1989) and Fagbenro & Jauncey (1993b). Espe *et al.* (1989) suggested that biding of water during proteolysis might be responsible. According to Dong *et al.* (1993), by preparing a silage using a lactic acid bacteria a low level of moisture in a final product is obtained. Thus it is an advantage to use the lactic acid bacteria procedure since the excess moisture would have to be evaporated during the drying process.

4.1.2.3. Lipid content

The lipid content of both silages were higher than in the minced fish (Table 1). Similar results were found by Fagbenro & Jauncey (1993b) who reported an increase in the lipid content of the lactic acid fermented silage. According to these authors this increase in lipid can be due to the fact that lactic acid is soluble in ether and therefore extractable with ether during lipid determination. In contrast, Wee *et al.* (1986) reported that lipid contents of both acid and biological fermented silages were similar to the minced tilapia.

Figure 4 presents the composition of minced tilapia and finished fish silage prepared by biological fermentation using molasses as the carbohydrate source, and that of silage prepared using formic acid.

Table 1- Proximate analyses of minced tilapia and tilapia silage during storage for 30 days at 30°C (all values represent means of three replicates).

	Minced Tilapia	Formic Acid Silage					Biological Fermented Silage				
Storage days	-	2	4	7	14	30	2	4	7	14	30
Moisture	73.10	72.80	72.20	72.45	72.23	72.00	67.74	67.69	68.22	68.88	68.70
Protein	16.19	14.38	14.70	14.18	14.22	14.78	12.03	12.96	12.82	12.71	12.72
Lipid	2.24	2.86	2.96	3.33	3.82	3.64	2.67	3.30	3.57	3.95	5.25
Ash	6.91	6.60	6.13	6.62	6.44	6.61	5.40	5.81	5.87	4.70	5.30
NFE*	1.56	3.36	4.01	3.42	3.29	2.97	12.16	10.24	9.52	9.76	8.03
pH	6.50	3.80	3.70	3.60	3.60	3.60	4.60	4.10	4.00	4.00	4.00

*NFE = 100 – (% moisture + % crude protein + % crude lipid + % ash)

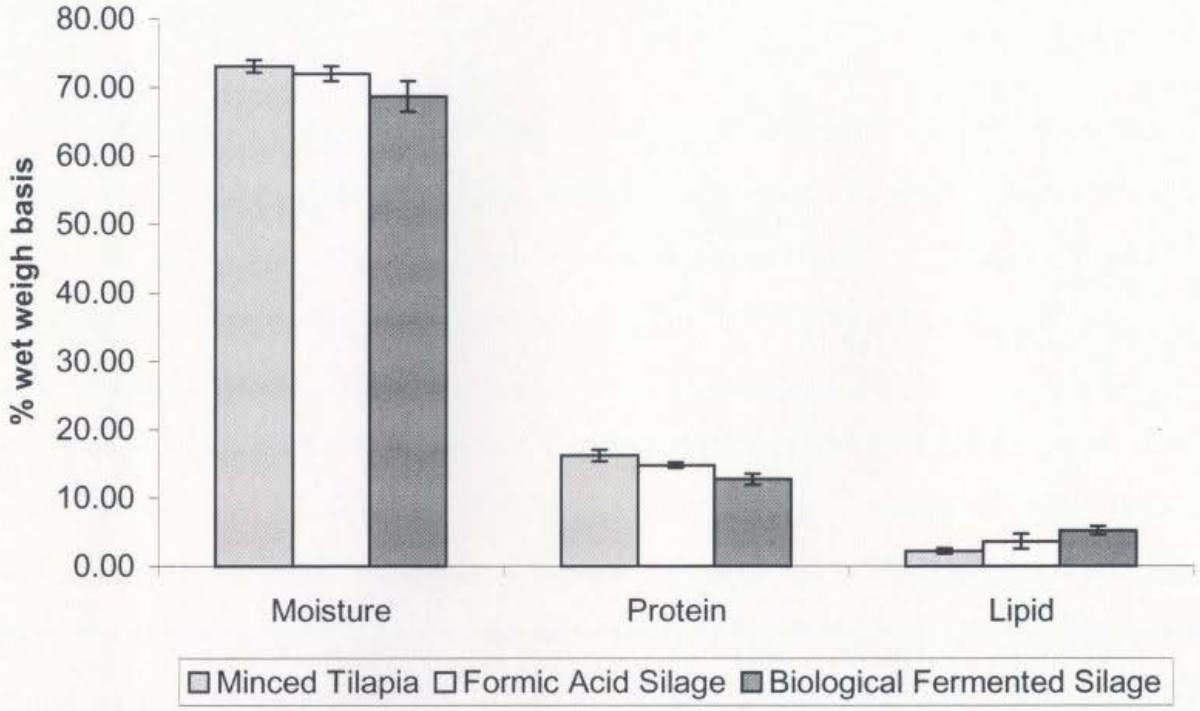


Figure 4- Composition of tilapia, *Oreochromis niloticus*, minced and ensiled by acid and biological methods stored for 30 days

4.1.3. Changes in the Protein fraction during fermentation of tilapia

4.1.3.1. Soluble protein

During the ensiling process, the protein is broken down by enzymes and the nitrogen in the silage becomes more soluble. The rates of formation of non-protein nitrogen for the formic acid silage and for the bacterial fermented silage are shown in Figure 5. The soluble nitrogen is plotted as a percentage of total nitrogen, and in the freshly prepared formic acid silage amounted to approximately 17%. At the end of the 30 days storage about 82% of the protein became soluble in the formic acid silage. Similar results were found by Windsor (1973); Backhoff (1976); Gildberg (1982); Tatterson (1982); Stone *et al.* (1989); Espe *et al.* (1992); Alwan *et al.* (1993) and Lo *et al.* (1993). The bacterial fermented silage had approximately 16% of protein as soluble protein at the beginning of the experiment. At the end of the storage period this value increased to 53%. A low value of soluble protein in the biological fermented silage was also reported by James *et al.* (1976); Raa *et al.* (1983); Batista *et al.* (1987) and Fagbenro & Jauncey (1995). According to James *et al.* (1976) partial preservation by the lactic acid production is the reason for the lower level of protein solubilization in the biological fermented silage. Raa *et al.* (1983) suggested that this low value of soluble nitrogen in biological fermented fish silage can be attributed to the adsorption of enzymes by molasses.

The increase in soluble nitrogen was associated with a change in consistency. The silage became more liquefied as storage time proceeded. A relationship was observed between the percentage soluble nitrogen and the consistency of the minced fish mass. The greater the amount of soluble nitrogen present in the mass the more liquid it became.

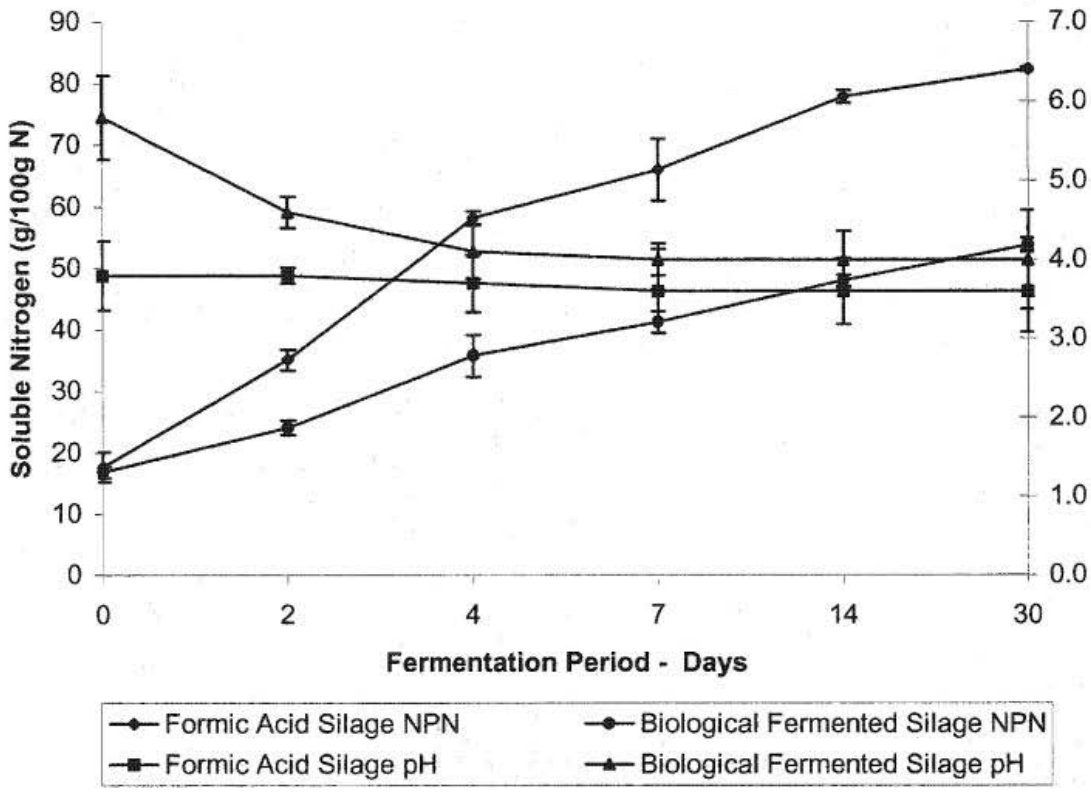


Figure 5– Soluble nitrogen (g/100g N) of the tilapia silage during fermentation at 30°C

4.1.3.2 Total amino acids

When autolysis occurs in fish silage, the percentage of nitrogen as free amino acids increases while the percentage of nitrogen as polypeptides decreases. According to Mathews (1972), high levels of free amino acids in the diet interfere with the mechanism for both amino acid and peptide absorption. The explanation may be that absorption of amino acids is faster when a hydrolyzed protein is ingested and that the influx of amino acids exceeds the capacity of the liver to synthesize proteins. While small-scale deamination of the amino acids is imminent and may not affect the nutritive value of the silage, heavy losses in one or a few essential amino acids will substantially change the composition of the silage and compromise its nutritional value.

Total amino acid composition in tilapia, *Oreochromis niloticus*, minced and ensiled by acid and biological methods are shown in Table 2. The correlation between the storage days and the amount of amino acids was determined. At the end of 30 days storage, the formic acid silage showed a slight decrease in the amount of arginine, aspartic acid, histidine, isoleucine, lysine, proline, threonine and tryptophan, while the biological fermented silage showed a decrease in isoleucine, serine, proline and tyrosine (Appendix I).

Although the loss of tryptophan is known to be one of the most serious effects of long term storage of silage (Backhoff, 1976; Kompiang *et al.*, 1980), in the present study, this decrease was not significant in either silage (Appendix I). The tryptophan levels observed showed that a loss of 41.98% and 49.62% of tryptophan occurred in the formic acid silage with 4 and 30 days of storage respectively. For the bacterial fermented silage those values were 9.92% and 16.03% respectively for silages with 4 and 30 days of storage. Jackson *et al.* (1984) reported a loss of 43.6% tryptophan in a 3% (w/w) acid

silage (1.5% sulphuric/1.5% formic acid combination) stored at 20°C for 8 weeks. Van Wyk and Heydenrych (1985) found a loss of approximately 58% in the tryptophan level of formic acid silage stored for one month at pH 3.69, while the tryptophan loss in the naturally fermented silage was around 13% when the silage was stored for one month at pH 4.09. Wessels *et al.* (1980) also reported a similar trend of higher tryptophan levels in fermented silage as compared to acid treated silages. Backhoff (1976) demonstrated that in herring silage (pH 3.7) there was a rapid loss of tryptophan when stored at 30°C, amounting to approximately 45% after 6 weeks, but after 3 years at room temperature the loss amounted to 50%. Since tryptophan is labile in acid conditions when present in the free form, a higher loss of tryptophan in the formic acid silage could be associated with the lower pH achieved and a higher level of protein solubilisation, which indicates protein breakdown leading to release of amino acids and other metabolites originating from protein.

The results obtained in the ratio between essential and nonessential amino acid and percentage of essential amino acids which are nutritive parameters calculated on the basis of the amino acid composition, show that the amino acid composition in all silages are similar to the minced fish thus, indicating that the amino acids appeared to be very stable in the silages.

Table 2 - Total amino acid composition (g/100g protein) in tilapia, *Oreochromis niloticus*, minced and ensiled by acid and biological methods (all values represent means of two replicates)

	Minced tilapia	st. dev.[c]	Formic acid silage	st. dev.	Formic acid silage	st. dev.	Biological fermented silage	st. dev.	Biological fermented silage	st. dev.
Storage days			4 days		30 days		4 days		30 days	
Amino Acid										
Alanine	6.33	0.02	6.21	0.02	6.58	0.02	6.04	0.02	6.28	0.02
Arginine*	6.58	0.02	6.40	0.02	6.10	0.02	6.13	0.02	6.93	0.02
Aspartic acid	8.21	0.02	8.14	0.02	7.99	0.02	8.31	0.01	8.73	0.02
Cysteine	0.91	0.02	1.17	0.02	1.32	0.02	1.01	0.02	0.99	0.02
Glutamic acid	14.04	0.03	14.56	0.03	14.40	0.02	14.14	0.03	14.52	0.02
Glycine	6.83	0.03	6.49	0.02	6.54	0.02	6.45	0.03	6.72	0.01
Histidine*	2.20	0.02	2.08	0.03	2.00	0.02	2.17	0.02	2.26	0.02
Isoleucine*	4.78	0.01	4.75	0.02	4.58	0.02	4.58	0.02	4.51	0.02
Leucine*	8.33	0.02	8.26	0.02	8.29	0.02	8.18	0.02	8.21	0.02
Lysine*	8.85	0.02	8.61	0.02	8.37	0.02	8.03	0.03	8.00	0.02
Methionine*	2.53	0.02	2.40	0.02	2.41	0.02	2.16	0.02	2.51	0.02
Phenylalanine*	3.42	0.02	3.30	0.02	3.39	0.02	3.14	0.02	3.11	0.02
Proline	4.59	0.02	4.39	0.02	4.18	0.02	4.39	0.02	4.01	0.02
Serine	3.81	0.02	3.61	0.02	3.64	0.02	2.78	0.02	2.67	0.02
Threonine*	4.80	0.02	4.62	0.02	4.49	0.02	4.67	0.02	4.72	0.02
Tryptophan*	1.32	0.02	0.76	0.02	0.66	0.02	1.18	0.03	1.10	0.02
Tyrosine	3.05	0.02	2.79	0.02	2.84	0.02	3.01	0.02	2.51	0.02
Valine*	4.09	0.03	4.01	0.02	4.00	0.02	3.87	0.02	3.97	0.02
Total	94.60		92.47		91.69		90.18		91.67	
EAA/NE[a]	0.98		0.95		0.93		0.95		0.97	
EAA/TAA[b]	49.57		48.86		48.30		48.91		49.43	

* Essential Amino Acids

[a]EAA/NE = Ratio between essential and nonessential amino acids

[b] EAA/TAA = (Essential Amino Acid/Total Amino Acid) x 100

[c] Standard deviation

4.1.3.3. Free amino acids

Free amino acid increased in both silages throughout the storage period (Table 3). A correlation statistical test was employed to check any loses in free amino acid of the silages compared to the minced tilapia. The amino acids breakdown, in both silages, occurred in an almost linear relationship, with Tyrosine being the least linear among all amino acids (Appendix II). A higher level of free amino acid in the formic acid silage (Figure 6) compared to the biological fermented silage (Figure 8) was observed. In the present study, at the end of the 30 day fermentation, 22.84% of the nitrogen was in the free amino acid form in the biological fermented silage compared to 27.48% in the formic acid silage. As suggested by Raghunath and McCurdy (1990) the differences in pH achieved in the biological fermented silage and in the acid preserved silage probably affect the type and rate of enzymatic activity. Dong *et al.* (1993) also reports that a 14 day old sulfuric acid silage had a higher percentage of lower molecular weight peptides and free amino acids when compared to a lactic acid silage.

During silage storage, soluble protein degraded to peptides and amino acids, and amino acids decomposition manifests as ammonia formation. Ammonia content was found to be higher in the biological fermented silage compared to the formic acid silage. An ammonia level of 2.3% was found for the formic acid silage (Figure 6), compared to a level of 11.7 % on the biological fermented silage (Figure 7), both stored for 30 days. The same results were reported by Nilsson and Rydin (1965) and by Batista (1987) who found that when autolysis was allowed to continue, the production of ammonia was higher in fermented fish silage than in acid silage resulting from a degradation of free amino acids. Kompiang *et al.* (1979) found that the ammonia in biological fermented silage was much higher (11-16%) compared to acid preserved silage (1.3-1.8%). According to the author,

the difference between biological and acid preserved silage on the ammonia content might be, at least partly, due to the different methods of storage. The biological fermented silage is usually stored in a closed container, thus the ammonia produced has difficulty escaping, whilst the acid preserved silage is stored in an open or slightly closed container. In the present study, both silages were stored in plastic buckets loosely covered with plastic lids and incubated at room temperature (30°C).

4.1.3.4. Protein autolysis in tilapia silage

The Nitrogen peptide was determined according to Stone and Hardy (1986) by subtracting free amino acid and ammonia nitrogen from the TCA soluble NPN. The autolysis of protein to TCA soluble peptides, amino acids and ammonia was reported as the percentage of each fraction to total Kjeldah nitrogen. According to Backhoff (1976) The amount of nitrogen from other sources is relatively low and thus, too small to cause errors in these values. As the percent of free amino acids increased in both tilapia silages the percent of nitrogen as polypeptides decreased (Figure 6 and 7). The majority of proteins were converted to peptides a portion of which were further hydrolyzed to free amino acids.

Table 3- Free amino acid composition (g/100g protein) in tilapia, *Oreochromis niloticus*, minced and ensiled by acid and biological methods (all values represents means of two replicates)

	Minced Tilapia	st. dev.[c]	Formic Acid Silage	st. dev.	Formic acid silage	st. dev.	Biological fermented silage	st. dev.	Biological fermented silage	st. dev.
Storage days			4 days		30 days		4 days		30 days	
Amino Acid										
Alanine	0.16	0.02	0.63	0.02	2.30	0.02	0.31	0.02	1.84	0.02
Arginine*	0.02	0.01	0.27	0.02	0.92	0.02	0.06	0.02	0.77	0.02
Aspartic acid	0.06	0.02	0.20	0.02	0.96	0.02	0.05	0.02	0.61	0.02
Cysteine	ND[b]		ND[b]		ND[b]		ND[b]		ND[b]	
Glutamic acid	0.29	0.02	0.91	0.02	2.88	0.02	0.18	0.02	2.19	0.02
Glycine	0.16	0.02	0.28	0.02	1.01	0.03	0.05	0.02	0.68	0.02
Histidine*	0.02	0.01	0.27	0.02	0.81	0.02	0.16	0.02	0.79	0.02
Isoleucine*	0.07	0.02	0.74	0.02	2.52	0.02	0.46	0.02	2.26	0.02
Leucine*	0.09	0.02	0.75	0.02	2.49	0.02	0.25	0.02	2.01	0.02
Lysine*	0.13	0.02	0.74	0.02	2.93	0.03	0.25	0.02	2.40	0.02
Methionine*	0.03	0.02	0.38	0.02	1.21	0.02	0.21	0.03	1.13	0.02
Phenylananine*	0.04	0.02	0.67	0.02	1.70	0.02	0.48	0.02	1.41	0.02
Proline	0.07	0.02	0.41	0.02	1.26	0.02	0.18	0.02	0.97	0.02
Serine	0.11	0.02	0.56	0.02	1.82	0.02	0.38	0.02	1.65	0.02
Threonine*	0.08	0.02	0.64	0.02	2.16	0.02	0.38	0.02	2.02	0.03
Tryptophan*	ND		ND		ND		ND		ND	
Tyrosine	0.05	0.02	0.67	0.02	0.91	0.02	0.58	0.02	0.75	0.02
Valine*	0.06	0.02	0.54	0.02	1.74	0.02	0.32	0.02	1.51	0.02

* Essential Amino Acids

[b] ND = Not Determined

[c] Standard deviation

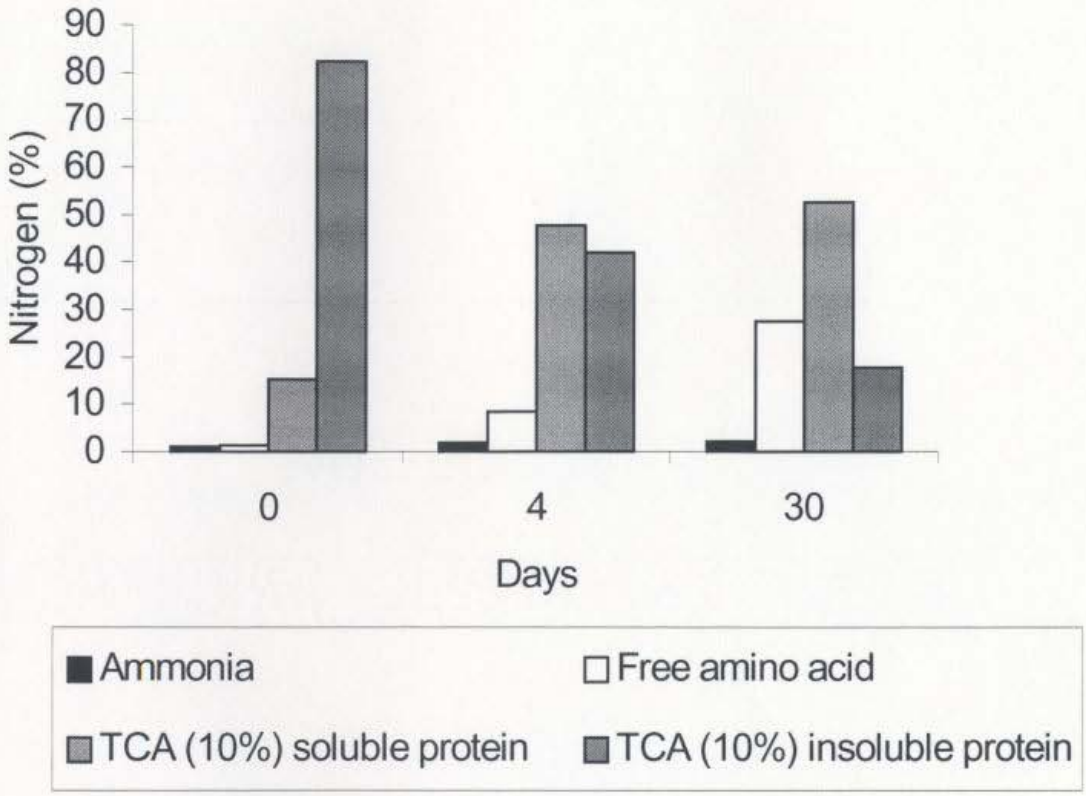


Figure 6– Protein autolysis of formic acid tilapia silage stored for 30 days at 30°C

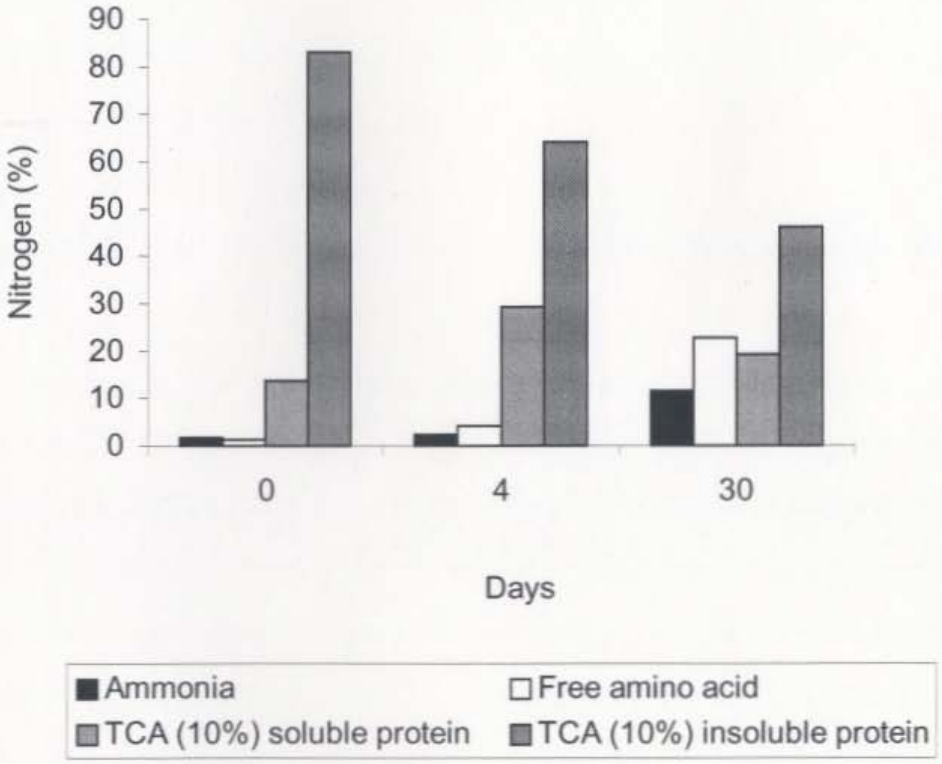


Figure 7 - Protein autolysis of biological fermented silage stored for 30 days at 30

4.2. Proximate analysis of the ingredients used in the diets

The proximate composition of the ingredients used in the diets formulation are listed in Table 4. The chemical analyses on the silages was done after the samples were co-dried with wheat bran and sun dried for approximately 20 hours in steel trays with 1 cm of depth. All ingredients used in the diet formulation, with the exception of the silages, were obtained commercially.

4.3. Formulation and proximate analysis of the experimental diets

The formulation and proximate analysis of the experimental diets are listed in Table 5. The crude protein level in the diets was maintained at 30% and the energy at about 370-380 Kcal/100g DM. The essential amino acid compositions of the diets are shown in Table 6. According to Tacon (1990) the essential amino acid requirement for omnivorous species are: Isoleucine – 1.09; Arginine – 1.68; Histidine – 0.71; Leucine – 1.99; Lysine – 2.31; Methionine – 0.75; Phenyalanine – 1.13; Threonine – 1.26; Tryptophan – 0.23 and Valine – 1.30. All diets formulated for the growth trial exceed the amount of amino acid required. At the end of the first week all fish became accustomed to the diets.

Table 4- Proximate analyses (%DM basis) of the ingredients used in the preparation of experimental diets (data are means of 3 replicates)

Ingredients	Dry matter	st. dev.	Crude protein	st. dev.	Crude lipid	st. dev.	Crude fiber	st. dev.	Ash	st. dev.	NFE[5]	Gross energy[6]
Corn meal	87.59	2.42	14.33	1.12	8.29	0.48	2.88	0.46	1.39	0.34	73.11	458.75
Wheat flour	86.71	1.21	19.95	1.29	1.76	0.23	1.62	0.32	9.21	0.45	67.46	405.03
Fish meal	88.57	1.96	55.21	1.48	15.81	2.92	1.10	0.19	22.00	1.21	4.88	488.88
FAS (4)[1]	89.50	0.85	53.41	1.41	13.00	1.93	6.80	1.02	13.52	0.54	13.27	477.00
FAS (30)[2]	88.91	1.57	52.81	1.72	14.12	0.17	5.98	0.20	14.10	0.18	12.99	483.13
BFS (4)[3]	91.30	2.36	46.65	0.57	12.78	1.66	5.71	0.29	12.64	0.33	22.22	473.75
BFS (30)[4]	91.12	2.04	45.86	0.66	13.36	0.54	6.03	0.59	11.87	1.28	22.88	477.54
Soybean meal	88.73	2.24	49.74	0.62	1.72	0.83	6.33	0.32	8.24	0.68	33.97	434.16
Manioc hay	87.59	2.42	14.33	1.12	8.29	0.48	2.88	0.46	1.39	0.34	40.69	293.81

[1]FAS(4) : WB - Formic acid silage stored for 4 days and co-dried with wheat bran
[2]FAS(30) : WB - Formic acid silage stored for 30 days and co-dried with wheat bran
[3]BFS(4) : WB - Biological fermented silage stored for 4 days and co-dried with wheat bran
[4]BFS(30) : WB - Biological fermented silage stored for 30 days and co-dried with wheat bran
[5]NFE = 100 – (% moisture + % crude protein + % crude lipid + % ash)
[6]Gross energy (Kcal/100g DM) = 4.1 Kcal g^{-1} for carbohydrate; 9.51 Kcal g^{-1} for lipid and 5.6 Kcal g^{-1} for protein (Fagbenro and Jauncey, 1994a)

Table 5- Formulation and proximate analyses[1] of experimental diets (g/100g dry weight) for juvenile tambaqui, *Colossoma macropomum*

	Diets[2]				
Ingredients	1	2	3	4	5 (control)
Fish meal	-	-	-	-	25.14
Wheat flour	47.66	44.19	44.32	45.73	17.88
Soybean meal	4.00	4.00	4.53	4.08	12.13
Manioc flour	17.80	20.73	12.15	11.20	8.46
Corn meal	-	-	-	-	32.39
FAS (4)	26.54	-	-	-	-
FAS (30)	-	27.08	-	-	-
BFS (4)	-	-	35.00	-	-
BFS (30)	-	-	-	35.00	-
Vitamin premix[3]	1.00	1.00	1.00	1.00	1.00
Mineral premix[4]	1.00	1.00	1.00	1.00	1.00
Tapioca starch[5]	1.00	1.00	1.00	1.00	1.00
Chromic oxide[6]	1.00	1.00	1.00	1.00	1.00
			Proximate analyses		
Moisture	8.20	8.00	8.90	7.80	9.00
Crude protein	30.81	30.76	30.25	30.00	31.00
Crude lipid	6.23	6.00	6.18	6.33	5.63
Crude fibre	4.10	4.40	4.90	4.20	4.60
Ash	14.29	14.73	15.12	15.23	13.04
NFE[7]	36.37	36.11	34.65	36.44	36.73
Gross Energy[8]	380.90	377.36	370.23	377.60	377.73
Calcium	1.33	1.34	1.41	1.43	2.20
Phosphorus	0.66	0.67	0.71	0.72	0.66

[1]All values represent means of three replicates

[2]Diets designated numbers corresponds to the type of silage used (see Table 4)

[3]The vitamin supplement provided the following (mg/100g diet): Thiamine (B1) 2.5mg; Riboflavin (B2) 2.5mg; Pyridoxine (B6) 2.0mg; Pantothenic acid 5.0mg; Inositol 100.0mg; Biofin 0.3mg; Folic acid 0.75mg; Para amino benzoic acid 2.5mg; Choline 200.0mg; Niacin (B3) 10.0mg; Cyanocobalamin (B12) 5mg; Ascorbic acid © 50.0mg; Menadione (K) 2.0mg.

[4]The mineral supplement provided the following amount per 100g of diet: $CaHPO_4 . H_2O$ 727.8mg; $MgSO_4 . 7H_2O$ 127.5mg; NaCl 60.0mg; KCl 50.0mg; $FeSO_4 . 7H_2O$ 25.0mg; $ZnSO_4 . 7H_2O$ 0.48mg; $CaIO_3 . 6H_2O$ 0.3mg; CrCl . 6H2O 0.13mg

[5]Binder

[6]Inert marker for the determination of apparent digestibility coefficient

[7]NFE (Nitrogen Free Extract) = 100 – (% moisture + % crude protein + % crude lipid + % ash)

[8]Gross energy (Kcal/100g DM) = 4.1 Kcal g^{-1} for carbohydrate; 9.51 Kcal g^{-1} for lipid and 5.6 Kcal g^{-1} for protein (Fagbenro and Jauncey, 1994a)

Table 6- Essential amino acid composition (g/100g protein) of experimental diets (data are means of two replicates)

Amino acid	Diets[a]									
	1	st. dev.[b] +/-	2	st. dev. +/-	3	st. dev. +/-	4	st. dev. +/-	5 (control)	st. dev. +/-
Isoleucine	4.73	0.15	4.55	0.40	4.26	0.36	4.29	0.45	4.28	0.01
Arginine	7.21	0.13	7.44	0.07	6.93	0.15	7.25	0.22	7.07	0.17
Histidine	2.52	0.30	2.21	0.25	2.36	0.40	2.37	0.06	2.51	0.66
Leucine	6.96	0.35	7.01	0.16	7.25	0.13	6.97	0.21	7.15	0.21
Lysine	5.51	0.55	5.25	0.13	5.71	0.14	5.36	0.04	5.40	0.66
Methionine	2.51	0.29	2.28	0.40	2.06	0.08	2.30	0.19	2.44	0.08
Phenyalanine	4.28	0.08	4.51	0.36	4.27	0.47	4.35	0.52	4.35	0.49
Threonine	4.28	0.17	4.13	0.18	4.26	0.42	4.35	0.49	4.24	0.48
Tryptophan	1.31	0.01	1.06	0.08	1.36	0.36	1.21	0.30	1.33	0.33
Valine	5.51	0.28	5.55	0.25	5.28	0.49	5.06	0.08	5.28	0.57

[b] Diets designated numbers corresponds to the type of silage used (see Table 4)
[a] Standard deviation

4.4. Water Quality

The water quality parameters in the experimental tanks remained relatively constant during the 90 days growth trial. Tambaqui, *Colossoma macropomum*, can tolerate very low oxygen levels in the water being able to survive in waters with an oxygen level of 0.5mg/l. In this case, a morphological adaptation occurs in which a lip protrusion develops allowing the absorption of oxygen from the surface by the fish. Water temperature and dissolved oxygen varied from 28 -29.5^{o}C and 4.0-7.0 mg/l respectively (Figure 8).

The exact concentration at which ammonia becomes toxic to fish varies among species. In addition other factors like water temperature and pH play a significant role. Ammonia in water occurs in two forms: toxic un-ionized ammonia (NH_3) and the relatively non toxic ionized ammonium (NH_4+). Both types of ammonia are measured to determine its total concentration in water. The mean concentration of NH_3 and NH_4+ ranged between 0.001-0.005 ppm and 0.03-0.07 mg/l, respectively. The pH values ranged from 6.9 -7.8 (Table7).

4.5. Growth response and feed utilization

4.5.1. Growth

Growth response for fish fed on the various diets is shown in Figure 9. No mortality occurred during the growth trial, which indicates proper water quality and handling of the fish. A paired sample test was used to compare the means of initial and final weight for each diet. According to the results obtained there was very little range in the weight changes in all 5 diets respectively (Appendix III). The paired sample correlation coefficient showed that within diets all fish grew proportionally to their original weight (Appendix IV).

Jackson *et al.* (1984) found no significant difference in final weight for salmon fed diets based on fish silage when compared to a commercial fish meal diet. Wee *et al.* (1986) compared the growth of *Clarias batrachus* L. fed diets based on tilapia fish silage and fish meal. The authors found no significant differences in final weight among diets.

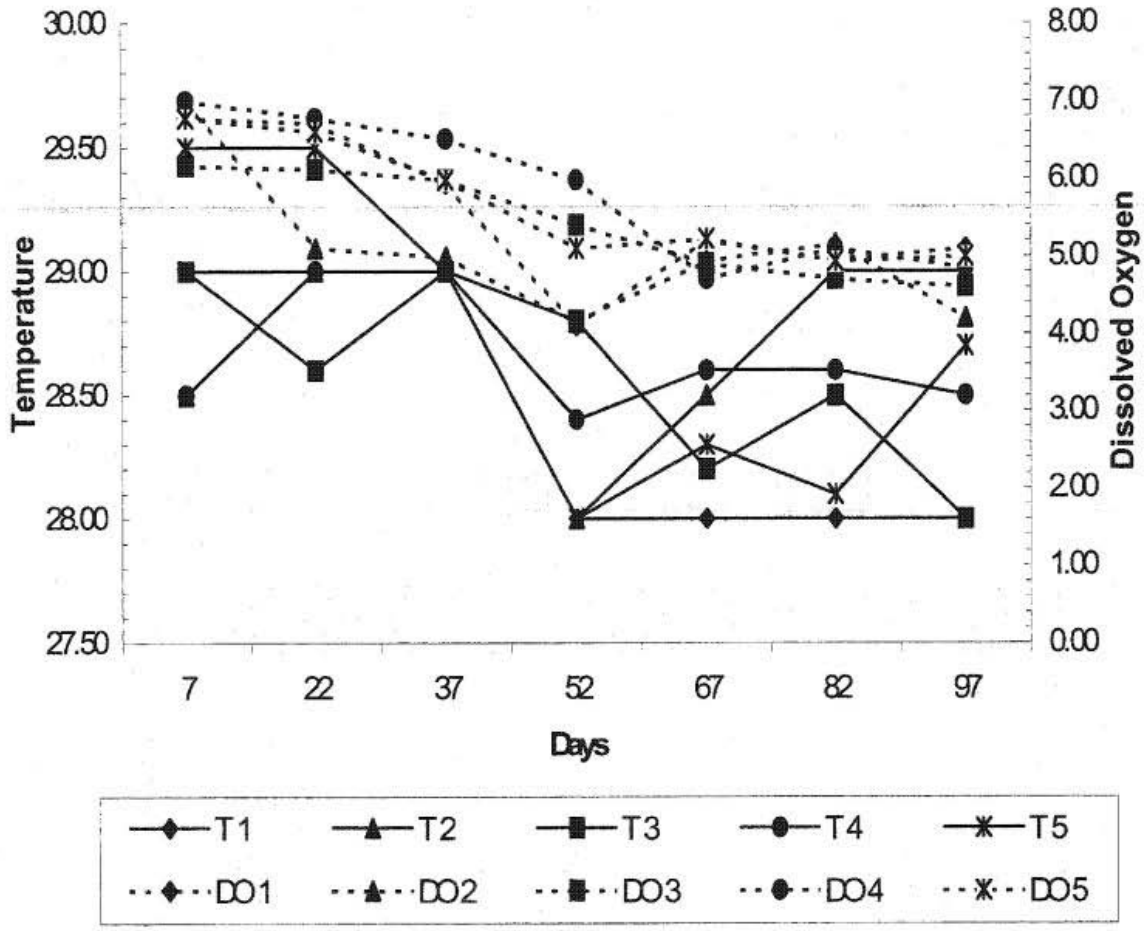


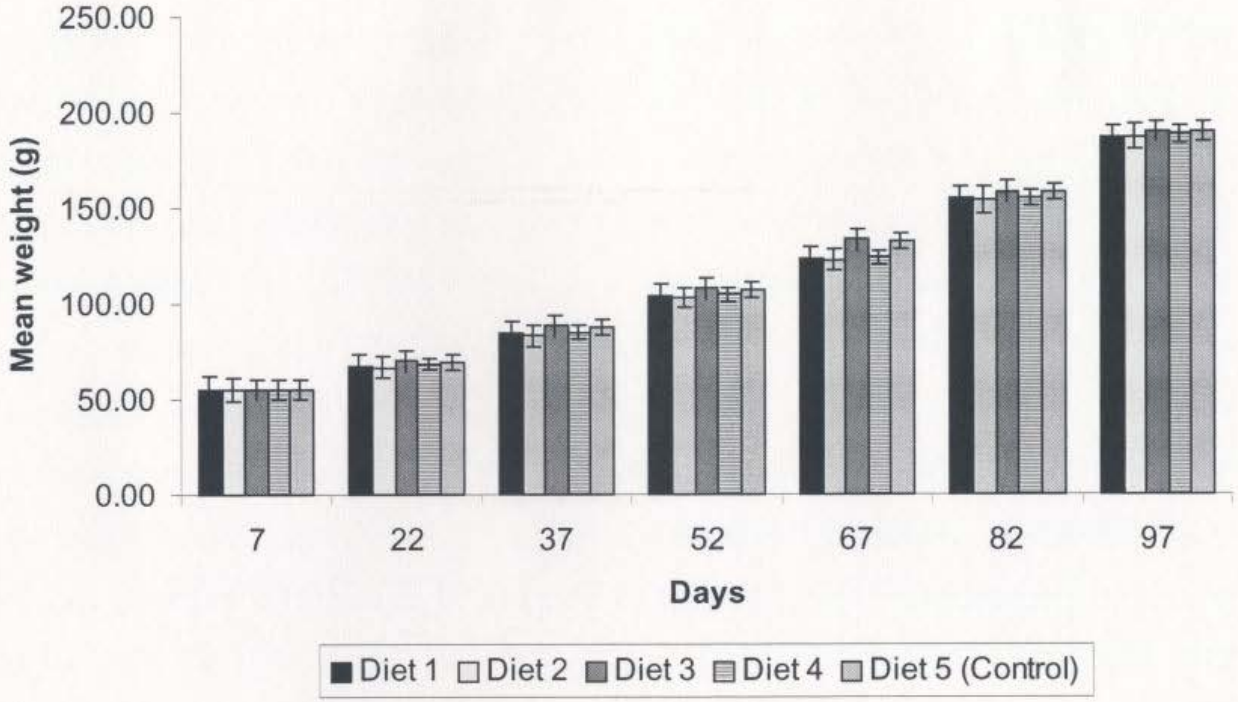
Figure 8– Water Temperature and Dissolved Oxygen (DO) of the experimental tanks used in the 90 days growth trial

Table 7– pH, NH_3 and NH_4 of the experimental tanks used in the 90 days growth trial

	Treatments														
	1			2			3			4			5		
Days	pH	NH_3	NH_4	pH	NH_3	NH_4	pH	NH_3	NH_4	pH	NH_3	NH_4	pH	NH_3	NH_4
7	7.40	0.01	0.05	7.00	0.02	0.05	7.60	0.01	0.04	7.00	0.07	0.04	7.02	0.01	0.03
22	7.87	0.09	0.04	7.35	0.15	0.05	7.12	0.15	0.04	7.30	0.01	0.04	7.00	0.03	0.04
37	7.12	0.03	0.05	7.25	0.08	0.04	7.06	0.09	0.05	7.16	0.12	0.05	7.12	0.11	0.05
52	7.82	0.16	0.03	7.30	0.12	0.05	7.33	0.01	0.05	7.06	0.09	0.05	7.25	0.02	0.05
67	7.06	0.08	0.04	7.20	0.20	0.05	6.99	0.13	0.06	7.10	0.01	0.05	7.06	0.16	0.06
82	7.70	0.03	0.03	7.30	0.17	0.06	7.06	0.08	0.04	6.98	0.16	0.05	7.00	0.13	0.03
97	7.00	0.08	0.06	7.07	0.19	0.05	7.03	0.08	0.06	7.00	0.10	0.07	6.90	0.09	0.05

Average daily weight gain (ADG) values found in this study ranged between 1.41-1.50 g/day (Table 8). These results were superior than those reported by Merola and Cantelmo (1987). These authors found values of 1.32 g/day for tambaqui fed diets containing 30% crude protein. Merola and De Sousa (1988) obtained values of 1.07 g/day. Ramos *et al.* (1992) fed a 25% crude protein diet to tambaqui and reported an average daily gain of 1.03 g/day. Eckmann (1983) (cited in Saint-Paul 1986), obtained an ADG of 0.8 g/day when feeding a diet containing 30% crude protein to juvenile tambaqui with a feeding rate of 3% body weight. The values found in the present study were higher than those found by the above authors. This may possibly be explained by the higher stocking density of 82500 fish/ha used in this trial compared to 24350 fish/ha used by the authors mentioned above, since the protein level in the diet and the feeding rates were similar.

The specific growth rate (SGR) values ranged between 1.34-1.40 (Table8). Martins and Guzman (1994) obtained a specific growth rate of 1.39 for tambaqui fed an experimental diet of 32.77% protein. Nunez and Salaya (1984) raising tambaqui in cages obtained values of specific growth rate ranging between 1.3-2.2 when a diet with 50% protein was used. Prada (1982) obtained higher values of SGR which ranged from 1.7-2.2 for tambaqui fed diets containing 23% protein using cement tanks at a stocking density of 20000 fish/ha.



Diets designated number corresponds to the type of silage used (see Table 4)

Figure 9- Growth response of tambaqui, *Colossoma macropomum* juvenile fed experimental diets

The highest average daily gain (ADG) was obtained with diets 3 and 5 and the lowest with diet 1. For specific growth rate (SGR), diet 5 gave the highest result followed by diet 3 and the lowest value was obtained in diet 1. The values for average daily gain (ADG) and specific growth rate (SGR) showed no significant differences between diets ($p<0.05$) (Appendix V). Fagbenro and Jauncey (1994) also found no significant difference in ADG and SGR for juvenile catfish, *Clarias gariepinus*, fed co-dried biological fish silage stored for 30 days when compared to a control fish meal diet. The authors also found that silage stored for 15 days gave better ADG and SGR values than a fish meal diet and a diet prepared using silage stored for 30 days. Ali *et al.* (1994) fed a diet containing 35% protein to major carp, *Cirrinus mrigala*, and found values of ADG and SGR significantly higher than those obtained by a control fish meal diet. The silage diet had sulphuric acid fish silage, fermented for 3 days, as the main source of protein (100%). Wood *et al.* (1985) reported a lower value of 1.25 for SGR in common carp fed a formic acid silage diet stored for 6 months compared to a 1.57 SGR when fish were fed a fish meal diet. In the present study SGR for fish fed the control diet based on fishmeal was 1.40 which was higher than the other diets. The reason for lower SGR of fish fed silage diets could possibly be explained by the length of silage storage prior to being used in the diet. The longer the autolysis is allowed to last, the higher the number of amino acids in the free form are presented in the diet . The amino acids may be absorbed faster in a diet contained hydrolyzed protein than in a diet containing intact proteins. This may result in an "overflow" of the anabolic pathways in the liver and more amino acids may enter the catabolic pathways (Espe *et al.* 1992). A reduced utilization of amino acids for the synthesis of protein by the liver occurs when a hydrolyzed protein is ingested. By limiting the autolysis period, a higher amount of silage could be included in the diet without compromising fish growth.

Table 8- Growth performance, protein utilization and protein digestibility of tambaqui, *Colossoma macropomum*, fed silage diets and fish meal diet for 90 days

Diet	Initial Weight (g)	st. dev[1] (+/-)	Final Weight (g)	st. dev (+/-)	ADG[2] (g/day)	st. dev (+/-)	SGR[3] (%/day)	st. dev (+/-)	AFCR[4]	st. dev (+/-)	PER[5]	st. dev (+/-)	APD[6]	st. dev (+/-)
1	55.01	6.91	187.04	6.14	1.41	0.44	1.36	0.21	2.05	0.41	1.63	0.27	80.39	3.00
2	54.69	6.18	187.14	6.38	1.47	0.57	1.37	0.31	2.13	0.88	1.66	0.41	79.13	7.20
3	55.19	5.08	190.20	5.00	1.50	0.41	1.38	0.28	2.03	0.40	1.69	0.40	82.00	6.15
4	55.23	5.06	188.27	5.02	1.49	0.54	1.37	0.29	2.08	0.61	1.69	0.40	82.00	3.36
5 (control)	54.71	5.05	190.02	4.93	1.50	0.39	1.40	0.20	1.98	0.28	1.66	0.24	81.64	1.87

[1] Standard deviation
[2] Average daily weight gain = (wt - wo)/t
[3] Specific growth rate = 100 x (ln wt-ln wo)/t
[4] Apparent Feed conversion Ratio = dry feed fed/wet weight gain
[5] Protein Efficiency Ratio = wet weight gain/protein fed
[6] Apparent Protein Digestibility = 100 - (% Cr_2O_3 in feed/%Cr_2O_3 in feces) x (% protein in feces/% protein in feed) x 100

4.5.2. Dietary protein utilization

Data on dietary protein utilization is shown in Table 8. The protein efficiency ratio (PER), which quantifies the efficiency of dietary protein in promoting growth, is defined as the gain in fish weight per gram of crude protein. In the present study, PER ranged from 1.63-1.69. Carneiro and Eross (1991) feeding fingerlings tambaqui, *Colossoma macropomum*, at 7% of the body weight using a diet with 27% of protein, in which the main source of protein originated from biological fermented silage stored for 7 days, reported a value of 1.98 for PER. Martins and Guzman (1994) and Merola and Cantelmo (1987) obtained a PER of 2.11 and 1.83 for juvenile tambaqui, using diets with 32.77 and 30% of protein respectively. The values for PER found in the present feeding trial were lower than those found by the authors mentioned above. Diets 3 and 4, which had biological fermented silage stored for 4 and 30 days as the main protein source, gave the best results for protein efficiency rate while diet 1 (acid silage stored for 4 days as the main source of protein) gave the lowest result.

The PER values obtained from the fish fed all 5 diets were not significantly different (Appendix V). Fagbenro *et al.* (1994) replacing 75% of fishmeal with lactic acid fish silage and feeding to juvenile tilapia, *Oreochromis niloticus*, found no significant difference for PER values when compared to a fish meal diet. Ali *et al.* (1994) obtained a PER value of 1.25 for major carp, *Cirrinus mrigala*, fed diets containing sulphuric acid silage as the main source of protein in contrast of a value of 0.83 for a fish meal control diet.

4.5.3. Apparent feed conversion ratio

Values on feed conversion ratio (FCR) are listed in Table 8. Results on feed conversion ratio obtained in the present feeding trial ranged between 1.98-2.13. These results were similar to those obtained by Carneiro and Eross (1991) who reported values of 2.11 for FCR for tambaqui fed a biological silage diet containing 27% protein and to those obtained by Eckman (1987) who reported a FCR of 1.9 for tambaqui fed diet with 30% protein. This study yielded lower results when compared to the values obtained by Merola and Cantelmo (1987) who obtained values of 1.63 for juvenile tambaqui fed diets with 30% protein. Werder and Saint-Paul (1978) obtained a higher FCR of 2.3 for tambaqui fed diets containing 30% protein. Although diet 5, using fishmeal as the main source of protein, gave the best result for apparent feed conversion while diet 2 (formic acid silage stored for 30 days as the main source of protein) gave the lowest value there was no significant difference for AFCR among diets (Appendix V).

4.6. Protein digestibility

4.6.1. Chromic oxide content in feed and feces

For the determination of chromic oxide (Cr_2O_3) content in the fish feces, it was necessary to construct a standard curve from the solutions with known Cr_2O_3 concentrations (Figure 10). A mother-solution containing 10 mg of Cr_2O_3 in 100ml of distilled water was diluted in different standard concentrations from 0.5 to 4.0 mg in 100 ml, in intervals of 0.5 mg each. Then a standard curve relating the absorbance readings with those of the solutions whose concentrations were previously known was determined.

Using the method of orthogonal polynomials the linear regression equation was determined as:

$$Y = - 0.0016 + 0.0196 X$$

where Y is the estimate for Cr_2O_3 in the feces samples (collected in the different treatments) and X is the absorbance reading.

Chromic Oxide concentration in the fish feces determined by wet-acid digestion (Furukawa and Tsukahara, 1966) based in the equation above, showed the following averages results for treatments 1, 2, 3, 4 and 5 respectively 2.258, 2.292, 2.6496, 2.6837 and 2.531 (Appendix VI). Protein content in feces samples were determined as described in Section 3.2. The average crude protein value in the feces samples for treatments 1, 2, 3, 4 and 5 were respectively; 13.07, 13.31, 13.76, 13.91 and 14.12.

With the values of crude protein obtained by the proximate analyses of the diets, and the results obtained for crude protein and Cr_2O_3 levels on diets and fish feces samples (Appendices VI and VII), the apparent protein coefficient was determined using the formula of Maynard and Loosli (1969) according to section 3.3.4.3. The results for Apparent Protein Digestibility are shown in Table 8.

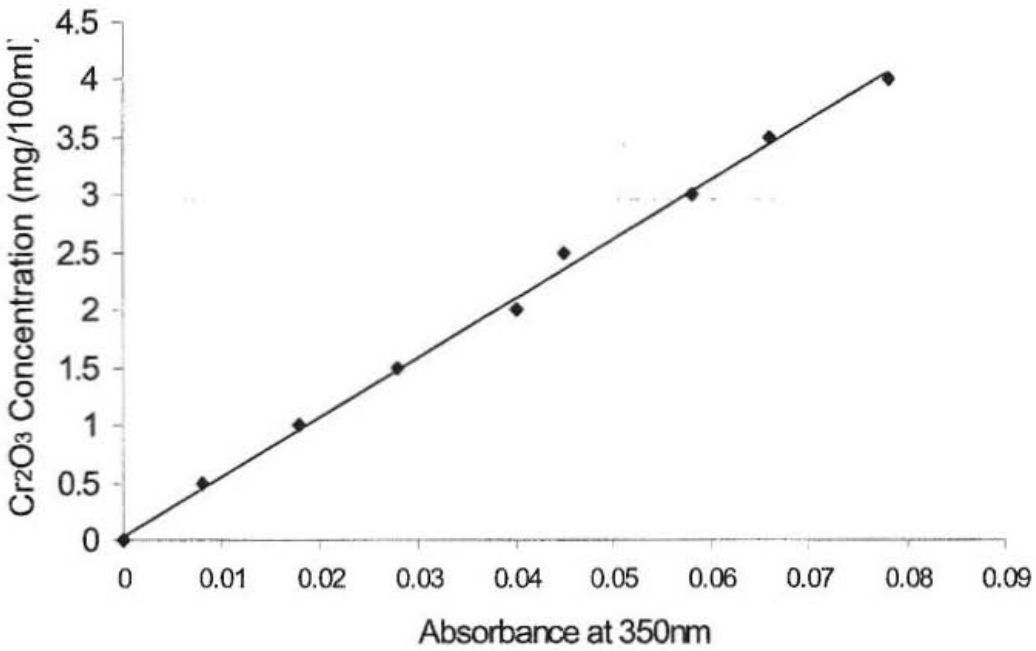


Figure 10– Absorbance readings for the determination of Cr_2O_3

4.6.2. Apparent protein digestibility

Data on apparent protein digestibility (APD) is presented in Table 8. In the present study the digestibility values ranged between 79 to 82%. Similar results were found by Hardy *et al.* (1984) for rainbow trout fed dry fish silage diets. Hossain *et al.* (1992), on the other hand, reported higher values for APD ranging between 88.23% to 88.85% for tilapia fed diets containing fish silage as the sole source of protein.

All silages gave high digestibility values for protein, the highest value being those of diet 3 and 4 and the lowest being that of diet 2. Both diets 3 and 4, which gave the highest protein digestibility, were based on biological fermented silage stored for 4 and 30 days respectively. Although diet 4 had silage stored for 30 days as the main source of protein, in this silage only 53% of the protein was in the soluble form compared to the silage used in diet 2, in which 82% of the protein became soluble at the end of the 30 day storage period.

The lower values for protein digestibility for diet 2, which was based on formic acid silage stored for 30 days as the main source of protein, may be attributed to the fact that the silage had a higher level of non-protein nitrogen (free amino acids and peptides) which may interfere with the mechanism for peptide and amino acid absorption. The analysis of variance could not be applied to testing APD, since a few replicates in each treatment are necessary in order to generate variation in within groups.

According to the NRC (1977) carp can digest 95% of fishmeal protein. However, the value can decrease to 80-85% (Ogino and Chen, 1973). The lower APD value of fishmeal in this study (81.63%) might be related to the origin of the product, which was

obtained in the local market.

4.8. Fish carcass composition

The carcass composition of fish at the beginning and end of the feeding trial is shown in Table 9. After feeding, protein, lipid and ash concentration in fish carcass increased while moisture decreased in all 5 groups. An increase in protein and lipid in fish carcass was also obtained by Lie et al. (1988), Fagbenro and Jauncey (1994a,), Fagbenro *et al.* (1994b) and Ali *et al.* (1994). A higher deposiition of protein at the cost of moisture was obtained on diets 3 and 4, were co-dried biological fermented silage was used to replace fishmeal in a rate of 35%. Thus, the diets containing higher levels of fish silage produced higher levels of carcass protein and lower levels of moisture. This increase in protein content may be due to higher apparent protein digestibility obtained by fish fed diets 3 and 4. According to Fagbenro *et al.* (1994b), the increase in lipid content of fish carcass of fish fed co -dried fish silage could be due to the occurrence of reduction in moisture content. The increase in protein and lipid content of fish carcass were highly statistically significant while the increase in ash content was not significant (Appendix IX).

Table 9- Carcass composition of tambaqui, *Colossoma macropomum*, at the beginning and at the end of the feeding trial [1]

Diet	Moisture	sd. dev.	Crude Protein	sd. dev.	Crude Lipid	sd. dev.	Ash	sd. dev.
Initial	76.77	1.23	13.59	0.63	5.61	0.42	2.26	0.37
1	71.58	1.03	15.67	1.36	7.52	0.46	4.30	0.63
2	73.53	1.13	15.37	1.90	6.34	0.39	4.39	0.48
3	68.55	1.33	18.32	0.60	8.47	0.50	4.43	0.43
4	68.45	0.57	18.31	0.61	7.68	0.69	4.29	1.00
5 (control)	70.43	1.96	17.93	0.45	8.13	0.40	3.44	0.41

1 All values represents means of three replicates

CHAPTER V

5.0 Conclusion

The present study with tilapia silage has successfully demonstrated that tilapia can be preserved by using formic acid or a lactic acid bacteria and molasses as a carbohydrate source. These products showed to be stable during the 30 days fermentation period at ambient temperature (30°C). The suitability of this technique at ambient temperature is expected to minimize operation costs.

The results showed that fish fed some of the dietary protein as partly autolysed protein, utilized protein at about the same efficiency as fish fed non-autolysed protein. Since tambaqui, *Colossoma macropomun*, grew well in all 5 diets, the increase in protein solubilisation seems to have an insignificant effect on the fish growth performance.

Feedstuffs that are poorly digested would be of limited nutritional value to an animal. Thus, digestibility values reported in this study suggested that tilapia silage meal represent an alternative form of a protein feed source in aquaculture.

Based on the reasonable acceptability of all diets tested by juvenile tambaqui, *Colossoma macropomum*, the good protein digestibility and the satisfactory growth performance obtained in this study, it can be concluded that co-dried tilapia fish silage can be successfully utilized as an alternative protein source for fishmeal in fish diets. In this way, it is possible to utilize processing wastes for producing valuable animal protein and, furthermore, make a contribution to the protection of nature and the environment.

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Appendix I - Correlation coefficient of the storage days and the amount of total amino acid in formic acid and biological fermented silage.

	Minced Tilapia	Formic Acid Silage		Correlation coefficient	Biological Fermented Silage		Correlation coefficient
Storage days		4 days	30 days		4 days	30 days	
Amino Acid							
Alanine	6.33	6.21	6.58	0.90	6.04	6.28	0.24
Arginine*	6.58	6.40	6.10	-0.97	6.13	6.93	0.75
Aspartic acid	8.21	8.14	7.99	-0.98	8.31	8.73	1.00
Cysteine	0.91	1.17	1.32	0.85	1.01	0.99	0.44
Glutamic acid	14.04	14.56	14.40	0.31	14.14	14.52	1.00
Glycine	6.83	6.49	6.54	-0.49	6.45	6.72	0.12
Histidine*	2.20	2.08	2.00	-0.90	2.17	2.26	0.90
Isoleucine*	4.78	4.75	4.58	-1.00	4.58	4.51	-0.78
Leucine*	8.33	8.26	8.29	-0.20	8.18	8.21	-0.44
Lysine*	8.85	8.61	8.37	-0.92	8.03	8.00	-0.64
Methionine*	2.53	2.40	2.41	-0.55	2.16	2.51	0.35
Phenylananine*	3.42	3.30	3.39	0.16	3.14	3.11	-0.67
Proline	4.59	4.39	4.18	-0.93	4.39	4.01	-0.98
Serine	3.81	3.61	3.64	-0.49	2.78	2.67	-0.67
Threonine*	4.80	4.62	4.49	-0.88	4.67	4.72	-0.25
Tryptophan*	1.32	0.76	0.66	-0.71	1.18	1.10	-0.40
Tyrosine	3.05	2.79	2.84	-0.45	3.01	2.51	-1.00
Valine*	4.09	4.01	4.00	-0.67	3.87	3.97	0.20

* Essential Amino Acids

Appendix II - Correlation coefficient of the storage days and the amount of free amino acid in formic acid and biological fermented silage.

	Minced Tilapia	Formic Acid Silage		Correlation coefficient	Biological Fermented Silage		Correlation coefficient
Storage days Amino Acid		4 days	30 days		4 days	30 days	
Alanine	0.16	0.63	2.30	0.99	0.31	1.84	0.99
Arginine*	0.02	0.27	0.92	0.99	0.06	0.77	0.99
Aspartic acid	0.06	0.20	0.96	0.99	0.05	0.61	0.99
Cysteine	ND[b]	ND	ND	ND	ND	ND	ND
Glutamic acid	0.29	0.91	2.88	0.99	0.18	2.19	0.98
Glycine	0.16	0.28	1.01	0.99	0.05	0.68	0.95
Histidine*	0.02	0.27	0.81	0.98	0.16	0.79	0.99
Isoleucine*	0.07	0.74	2.52	0.98	0.46	2.26	0.99
Leucine*	0.09	0.75	2.49	0.98	0.25	2.01	0.99
Lysine*	0.13	0.74	2.93	0.99	0.25	2.40	0.99
Methionine*	0.03	0.38	1.21	0.98	0.21	1.13	0.99
Phenylananine*	0.04	0.67	1.70	0.96	0.48	1.41	0.98
Proline	0.07	0.41	1.26	0.98	0.18	0.97	0.99
Serine	0.11	0.56	1.82	0.99	0.38	1.65	0.99
Threonine*	0.08	0.64	2.16	0.99	0.38	2.02	0.99
Tryptophan*	ND	ND	ND	ND	ND	ND	ND
Tyrosine	0.05	0.67	0.91	0.79	0.58	0.75	0.77
Valine*	0.06	0.54	1.74	0.98	0.32	1.51	0.99

* Essential Amino Acids

[b] ND = Not Determinated

Appendix III – Frequency data for initial and final fish weight in the 5 diets

Diets	Paired Differences			
	Mean (final weight - initial weight)	Std. Deviation	95% Confidence Interval of the Difference Lower	Upper
1	132.0267	3.0313	132.9374	131.1160
2	132.4533	1.3188	132.8496	132.0571
3	135.0067	0.9633	135.2961	134.7173
4	133.0400	0.9091	133.3131	132.7669
5	135.3156	1.6864	135.8222	134.8089

Appendix IV - Paired sample correlation for initial and final fish weight in the 5 diets

Diets	N	Correlation	Significance
1	45	0.899	Sig[1]
2	45	0.978	Sig
3	45	0.982	Sig
4	45	0.984	Sig
5	45	0.943	Sig

[1] Sig - Significant

Appendix V - Analysis of variance (one-way anova) for the effect of each diet on growth parameters

		Sum of Squares	Degrees of freedom	Mean square	F	Significance
ADG[2]	Between Groups	0.103	4	0.02581	0.114	ns[1]
	Within Groups	19.309	85	0.227		
	Total	19.412	89			
SGR[3]	Between Groups	0.0191	4	0.00478	0.070	ns
	Within Groups	5.782	85	0.06802		
	Total	5.801	89			
AFCR[4]	Between Groups	0.0487	4	0.01218	0.099	ns
	Within Groups	10.475	85	0.123		
	Total	10.525	89			
PER[5]	Between Groups	0.242	4	0.06056	0.195	ns
	Within Groups	26.401	85	0.311		
	Total	26.643	89			

[1] ns - Not significant at 5 % level

[2] ADG - Average Daily Weight Gain

[3] SGR - Specific Growth Rate

[4] AFCR - Apparent Food Conversion Ratio

[5] PER - Protein Efficiency Ratio

Appendix VI - Cr_2O_3 content in fish feces

Diets	Absorbance readings	Cr_2O_3	Mean
1	0.050	2.633	2.258
	0.040	2.123	
	0.038	2.020	
2	0.065	3.398	2.292
	0.030	1.612	
	0.035	1.867	
3	0.033	1.765	2.650
	0.065	3.398	
	0.053	2.786	
4	0.061	3.194	2.684
	0.042	2.225	
	0.050	2.633	
5	0.052	2.735	2.531
	0.050	2.6327	
	0.042	2.2245	

Appendix VII - Average protein content of fish feces and experimental diets used in each treatment

Diets	Crude protein in feces	Crude protein in diet
1	13.07	30.81
2	13.31	30.76
3	13.76	30.25
4	13.91	30.00
5	14.12	31.00

Appendix VIII - Analysis of variance (one-way anova) for the effect of each diet on fish carcass composition

		Sum of squares	Degrees of freedom	Mean square	F	Significance
Moisture	Between Groups	55.15100	4	13.78775		
	Within Groups	1.71233	10	0.17123	80.52025	0.000000145
	Total	56.86333	14			Sig*
Protein	Between Groups	26.05044	4	6.51261		
	Within Groups	1.15540	10	0.11554	56.36671	0.0000008
	Total	27.20584	14			Sig
Lipid	Between Groups	7.92976	4	1.98244		
	Within Groups	2.06573	10	0.20657	9.59679	0.001872
	Total	9.99549	14			Sig
Ash	Between Groups	2.02073	4	0.50518		
	Within Groups	2.30820	10	0.23082	2.18865	0.143690
	Total	4.32893	14			ns**

**ns – not significant

*sig- significant different