MONITORING THE SARDINE (*Sardinella brasiliensis*) FERMENTATION PROCESS TO OBTAIN ANCHOVIES

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ABSTRACT: Anchovies are traditional fish preserves, prepared from fermented fish of the engraulidae family, mainly in European countries. In Brazil, sardines (*Sardinella brasiliensis*) are an alternative fish for preparing these types of preserves, provided that the preservation process results in a high quality product. In this research, sardines were prepared for preservation and physicochemical, microbiological and sensory analyses were carried out during the preservation process. Whole or eviscerated sardines, with or without condiments/preservatives and with 20% of salt (w/w) were used. Sardines were analyzed fresh, and at 1, 15, 30, 45 and 60 days along the preservation process. The use of whole sardines, with or without condiments/preservatives, presented best results, with increased non-proteic nitrogen in the dry matter, higher levels of total volatile bases and higher contents of lactic acid and sodium chloride. The higher acidity observed in the whole sardine treatments resulted in better control of halophylic mesophilic microorganisms, which were kept under 1.4 x 10^3 CFU g^{-1} in both treatments. Total coliforms and *Staphylococcus aureus* reached 21 and 3.0 x 10^2 CFU g^{-1}, respectively. *Escherichia coli* and *Salmonella spp* were not present in the fresh sardines or in any of the four treatments, indicating that the concentration of salt used was appropriate to maintain the product under adequate microbiological control. Both whole or eviscerated sardines under the conditions of this experiment were appropriate in terms of the microbiological safety of the preserves. Treatments using whole fish, either with or without condiments/ preservatives, also presented better sensorial properties such as color, flavor, taste and texture, as compared to the eviscerated fish treatments. Whole sardines produced good quality, anchovy-type preserves, which can be used for consumption and marketing purposes.
Key words: seafood, fermented fish, cured process

MONITORAMENTO DO PROCESSO DE FERMENTAÇÃO DA SARDINHA, *Sardinella brasiliensis,* PARA OBTENÇÃO DE ANCHOVAS

RESUMO: As sardinhas brasileiras podem ser utilizadas para o preparo de pescado fermentado, à semelhança do que é feito com as anchovas na Europa, desde que o processamento permita a obtenção de um produto com qualidade. O objetivo desta pesquisa foi monitorar o processamento de fermentação de sardinhas, *Sardinella brasiliensis*, utilizando 4 tratamentos, a saber: peixes inteiros e eviscerados, ambos com ou sem condimentos, e 20% de sal. As sardinhas foram analisadas *in natura* e nos períodos de 1; 15; 30; 45 e 60 dias de fermentação. O pescado mantido com vísceras apresentou maior facilidade para fermentação e revelou maiores teores de bases voláteis, nitrogênio não protéico e acidez em ácido lático (19,82 mg 100 g^{-1}). A contagem total de mesófilos se manteve na faixa de 10^3 UFC g^{-1}. Coliformes totais e *Staphilococcus aureus* apresentaram baixas contagens. *Escherichia coli* e *Salmonella* não foram detectadas. O processamento do pescado com vísceras, não interferiu na segurança microbiológica e propiciou os melhores resultados para cor, aroma, sabor e textura do produto final.
Palavras-chave: pescado, peixe fermentado, processo de cura

INTRODUCTION

Certain fish species have intrinsic characteristics that can be of great value for the production of fermented preserves. The most important for obtaining a high quality final product are the sensory characteristics, which will produce specific color, flavor, taste and texture. These characteristics, intrinsic to certain species, are related to the fish muscles, viscera, microorganisms and enzymes (Oetterer, 2001). Additionally, fish used for fermentation are usually small, with low commercial value and seasonally abundant.

The most common fermented fish products are the anchovies, traditionally prepared from fish belonging to the engraulidae family (*Engraulis* spp). However, other fish species present the necessary characteristics for the preparation of good quality, fermented fish. Sardines (*Sardinella brasiliensis*) are small fish, containing all the biological components to provide the adequate flavor, taste, color and texture that are particular to anchovies (Beirão, 1979). This species has been used in Brazil for decades for the preparation of preserved fish such as canned sardines and slated-pressed sardines. The term "preserved fish" refers to fish prepared by enzymatic curing, or maturation, in which salt is added and acts on muscles, viscera, microorganisms and enzymes, developing microorganism which produces lactic acid, lowering the pH and making the product resistant to the development of putrefying bacteria (Oetterer, 2001).

The explanation that maturation results from enzymes such as cathepsins in muscles of fish, may not be quite satisfactory (Zaitsev et al., 1969). Eviscerated herings tested before the addition of salt have not matured satisfactorily. Sanchez (1977) concluded that enzymes of the digestive system of the red tail "lambari", *Astyanax fasciatus,* acted on the proteins decomposing them. In eviscerated fish, this fact did not occur, since the enzymes were not present. Lessi (1995) reports that pathogenic bacteria, including the *Clostridium botulinum,* do not develop at concentrations of 6.5% salt. The muscle of the salted fish has 0.75 water activity, thus it is not expected that pathogenic bacteria will develop under this low water content.

Sardines are highly consumed in the Brazilian market, being well accepted and having a steady consumption market. Specific legislation on the commercialization of this product is not available in Brazil. The closest product to the preserved fish dealt in this research is the cured fish, ruled by the Brazilian Government Decree number 1255, issued on June 25th, 1962, which specifies that cured fish should be made from whole fish and should be treated by special processes such as salted fish, pressed fish, smoked fish and dried fish (Brasil, 1962). The same decree makes it mandatory that the fish should be cleaned and eviscerated before processing for human consumption, whether the fish is canned or cured, independently of the type of processing it is submitted to.

Considering that evisceration of the fish is of critical concern in its processing and that the presence of viscera can contribute to the maturation of cured fish, this study focus on whether evisceration should be mandatory, provided that microbiological quality is achieved during fish processing. Thus, the main objective of this study was to monitor the sardine fermentation process for 60 days, in terms of physical, chemical, microbiological and sensory characteristics.

MATERIAL AND METHODS

Approximately 30 kg of fresh whole, ice chilled sardines (*Sardinella brasiliensis*), were obtained for the experiment and for analysis of fish *in natura.* 19.89 ± 1.22 cm in length and weighed 60.04 ±10.14 g each. The experiment consisted of four treatments, i.e., whole sardine (WS), whole sardine with condiments and preservatives (WC), eviscerated sardine (ES) and eviscerated sardine with condiments and preservatives (EC).

All treatments received 20% of granulated salt (w/w). The diameter of salt granules averaged 1.48 ± 0.76 mm. Condiments and preservatives added were prepared according to Ferreira & Andrade (1992), and consisted of a mixture of refined sugar (8.5 g), black pepper (8.5 g), white pepper (1.4 g), nutmeg (1.4 g), paprika (8.5 g), cloves (1.4 g), bay leaves (5 units), benzoic acid (0.5 g) and sodium nitrate (0.5 g) per kilogram of fish. Experiment was carried at 21.82 ± 1.31°C.

For the whole sardine treatments, WS and WC, 3 kg of whole sardines were washed and drained. Sardines were then divided into two sub-lots and placed in two, 8-L plastic trays, alternating layers of fish and salt (WS). In treatment WC, the formulation of condiments and preservatives was added with the salt. For the eviscerated sardine treatments, ES and EC, the sardines were eviscerated, beheaded, washed and drained, and then divided into two lots and prepared as described for the whole sardines, with ES consisting of alternating layers of eviscerated fish and salt, and EC of alternating layers of eviscerated fish and a mixture of salt and the formulation of condiments and preservatives. After the preparation in trays, a two-kg weight was placed over the fish to accelerate the formation of brine and, consequently, keeping fish immersed in liquid, providing the appropriate conditions for anaerobic processing (Oetterer 1999).

Analyses were carried out in samples of raw material, before the experiments were installed, and for all treatments after 24 hours, 15, 30, 45 and 60 days during the preservation process.

For preservation process, total nitrogen (TN) was determined according to AOAC (1990) and non-protein nitrogen (NPN) by precipitation of the muscle proteins with tri-chloride acetic acid (TCA), followed by analysis by the micro Kjeldhal method. The nitrogen fraction corresponding to the total volatile bases (N-TVB) was determined according to the methodology described by Pregnolatto & Pregnolatto (1985). Crude protein content by multiplying the total nitrogen (TN) by the factor 6.5 and lipids by the Soxhlet, method according to Pregnolatto & Pregnolatto (1985).

Total moisture content was obtained by the difference between fresh and dry weight of samples, dried at 105 ± 1°C until constant weight (AOAC, 1990). The ash fraction was obtained by incineration of the organic

matter at 550°C (AOAC, 1990); NaCl content was determined according to AOAC (1990); lactic acid acidity according to methodology described by Pregnolatto & Pregnolatto (1985), and pH by a digital potentiometer, according to Instituto Adolfo Lutz (1976).

Microbiological analyses were made following the superficial washing technique, described by Silva et al (1997). Dilutions of 1:10, $1:10^2$, $1:10^3$ and $1:10^4$ were used to determine the number of mesophilic aerobic microorganisms using the "Simplate TM Test Procedures", AOAC 970301 Approval Certificate (March 5, 1997) and DIPOA no. 29/97. The number of halophilic mesophilic microorganisms was obtained as suggested by Vanderzant (1992), and the number of coliforms and *Escherichia coli* using by "Simplate TM Test Procedures" (AOAC 970301 Approval Certificate, March 5, 1997; DIPOA no. 29/97). *Staphylococcus aureus* was determined by the method described by Vanderzant (1992) and *Salmonella spp* by the "Oxoid Salmonella Rapid Test" (FT 201-A from Oxoid), which has a Certificate of Approval – AOAC 960902 (Dec 11, 1996). Sensory analyses were made on the 60th day of the preservation process for all treatments. The final product was placed in hard plastic cups and covered with edible soybean oil. Fillets were then cut into 5 cm x 0.5 cm pieces. Two fillets of each of the four treatments were placed at random in porcelain dishes and served at room temperature to volunteer testers. Water with drops of lemon and were served to each tester after each sample to minimize possible residual effects of the previous samples. To define the intensity of each parameter, the testers graded the product according to a scale ranging from one to seven, one being "very poor", and seven, "excellent", on cards and reference material defining each parameter and establishing extremes of the grading scale provided to the testers. Data were subject to the F test ($P \geq 0.05$) with means compared by Tuckey test ($P > 0.05$)

The physicochemical analysis followed a 4x6 factorial statistical design, with four treatments (WS, WC, ES, EC) and six periods of preservation (0, 1, 15, 30, 45 and 60 days) (n = 9). The microbiological analysis followed a 2 x 6 factorial statistical design, with two treatments (WS and ES) and six periods of preservation (0, 1, 15, 30, 45 and 60 days) (n = 3).

RESULTS AND DISCUSSION

Chemical Analyses

The moisture content of sardines *in natura* was 74.27 g 100 g^{-1}, decreasing during the preservation period in all treatments, reaching 52 g 100 g^{-1}. The chemical constituents of Ghanaian fermented fish condiment obtained from retail outlets were : moisture content 50 g 100 g^{-1}, protein value 16.80 – 21.90 g 100 g^{-1} and pH [3] 6.0 (Sanni et al, 2002).

Protein levels in the dry matter presented a tendency of stabilization after the 30th day of preservation, especially for treatment WS (Figure 1). Fish proteins are broken into smaller nitrogen components by proteolysis, mediated by enzymes present either in the fish tissue, or in microorganisms. These nitrogen compounds migrated from the sardine tissues into the brine solution during the process, and fraction fraction of the decomposed proteins remained in the fish muscle as free amino acids and peptides (Alm, 1965).

The protein levels stabilized in a shorter period of time in treatment WS, as compared to the other three processes (Figure 1). This confirms the findings of Sanchez (1977), who reported that digestive enzymes and microorganisms act in muscle proteins, decomposing them faster than when eviscerated fish are used for curing. During the whole experiment period, the levels of proteins in the four processes had no difference between sardine *in natura* and the final processed product.

The average fat content in sardine was 1.97 g 100 g^{-1}. Such level of fat can improve the sensory qualities of anchovies (Alm, 1965). An increase in the levels of lipids was observed 24 hours after the experiment was installed, possibly resulting from dehydration. The level of lipids remained basically stabilized until the 60th day of the fish processing (3.64 g 100 g^{-1} for WS, 2.90 g 100 g^{-1} for ES, 3.95 g 100 g^{-1} for WC and 4.01 g 100 g^{-1} for EC). This was expected, since the enzymatic curing process acts mainly on proteins rather than in lipids (Chang et al; 1992).

The ash fraction observed in the muscle of sardines *in natura* was 1.42g 100 g^{-1}, increasing approximately 18% at the 60th day of the experiment in all processes (a tendency towards stabilization occurred after the 30th day of processing). This increase is directly related to the presence of NaCl in the sardine muscle. The levels of the ash fraction observed in all processes were similar to those reported by Beirão (1979) for sardine (*Sardinella brasiliensis*) and Sanchez (1981) for some Brazilian species of freshwater fish.

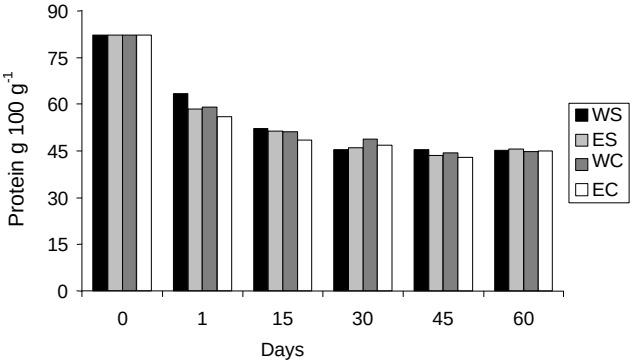

Figure 1 - Protein in dry matter of sardines *in natura (*day 0) and during the preservation processes (WS= whole sardine; ES = eviscerated sardine; WC= whole sardine with condiments and preservatives; EC= eviscerated sardine with condiments and preservatives).

Levels of non-protein nitrogen (NPN) increased during the 60 days in all processes (Figure 2). Higher levels were observed for treatments with whole sardines, probably because of the enzymes present in the digestive system of the sardines. The levels of NPN for solid matter increased for the two processes using whole sardines.The muscular enzymes, cathepsins, decompose the fish protein slowly, hence eviscerated sardines presented less protein decomposition, liberating less NPN. (Amano, 1962)

Fish by- products, Arctic capelin and Atlantic cod intestines, can be utilized as raw materials for the production of high value fish sauce for human consumption (Gildberg, 2001). The proteases present in cod pylorus caeca are cold adapted enzimes, that accelerate tissue solubilization and give hight fish sauce recovery at storage at 26°C.

The level of N-TVB observed in the sardines *in natura* (Figure 3) was 19.32 mg 100 g^{-1}, which is below the maximum limit determined by Decree No. 1255, which requires N-TVB levels for fresh fish lower than 30 mg 100 g^{-1} (Brasil, 1962). The processed fish presented higher levels of N-TVB (Figure 3) since the beginning of the processing, which is in accordance with Connel (1975), who reported a maximum limit of 200 mg 100 g^{-1} for levels of N-TVB in fish processed with salt, according to the legislation of certain countries. Beirão (1979) obtained levels of N-TVB around 70 mg 100 g^{-1} for whole preserved fish, *Sardinella brasiliensis.*

Processed of whole, sardines presented higher N-TVB levels in comparison to eviscerated sardines. The highest level of N-TVB during the 60 days of preservation was found in the WS treatment (56.79 mg 100 g^{-1}). The limit level allowed by many countries is 60 mg 100 g^{-1} for fish processed with salt. Brazilian legislation does not specify maximum limits for N-TVB in salt-preserved or cured fish Beirão (1979).

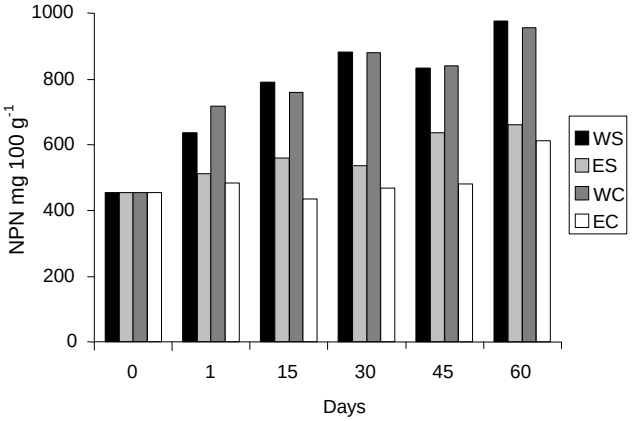

Figure 2 - Non-protein nitrogen (NPN) of sardines *in natura* (day 0) and during the preservation processes (WS = whole sardine; ES = eviscerated sardine; WC = whole sardine with condiments and preservatives; EC = eviscerated sardine with condiments and preservatives).

Acidity, calculated by acid lactic, was higher in the treatments with condiments and in whole sardines (Figure 4). The results from the WS and WC processes were different from those obtained with ES and EC, presenting higher levels of acidity during the 60 days of preservation. Sanchez (1977), in an experiment with "lambaris" (*Astyanax fasciatus*), concluded that the acidity increase was directly related to protein decomposition. In the present experiment higher protein decomposition occurred in the processes with whole sardines, possibly because of the presence of enzymes of the fish digestive system, such as trypsin and chymotrypsin. The levels of acidity observed are consistent with those obtained by Lessi (1995), who mentions preservation by *Lactobacillus* spp. in the anaerobic medium, during the production of canned, salted anchovies.

The pH measured in processes with condiments (WC and EC) was lower than in WS and ES processes (Figure 5). This was probably caused by the ingredients added to treatments WC and EC, especially sugar, which could have been used by the microorganisms to produce

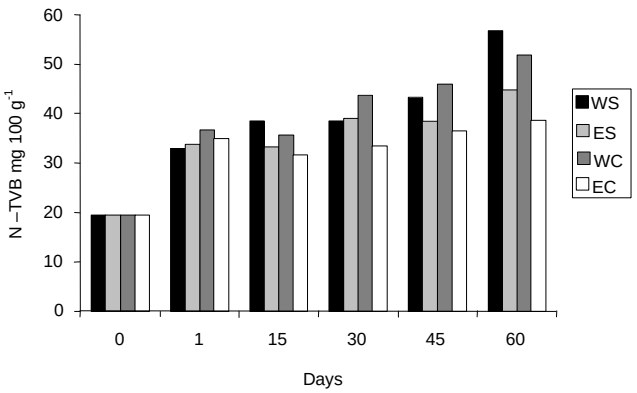

Figure 3 - Nitrogen total volatile bases (N-TVB) of sardine *in natura* (day 0) and during the fermentation processes (WS = whole sardine; ES = eviscerated sardine; WC = whole sardine with condiments and preservatives; EC = eviscerated sardine with condiments and preservatives).

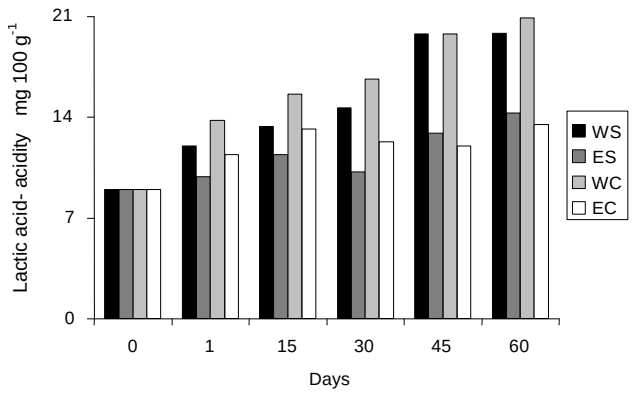

Figure 4 - Relative acidity in lactic acid of sardine *in natura* (day 0) and during the fermentation processes. (WS = whole sardine; ES = eviscerated sardine; WC = whole sardine with condiments and preservatives; EC = eviscerated sardine with condiments and preservatives).

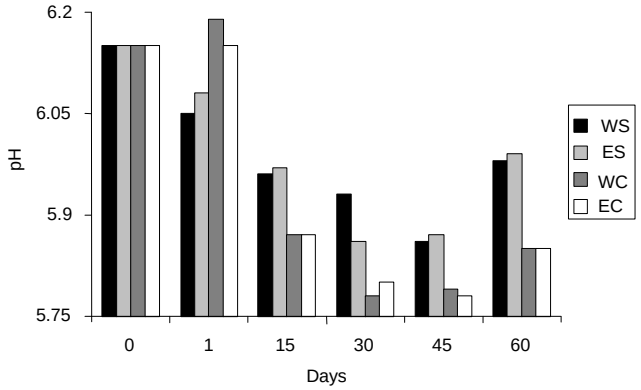

Figure 5 - pH variation of sardine *in natura* (day 0) and during the fermentation processes (WS = whole sardine; ES = eviscerated sardine; WC = whole sardine with condiments and preservatives; EC = eviscerated sardine with condiments and preservatives).

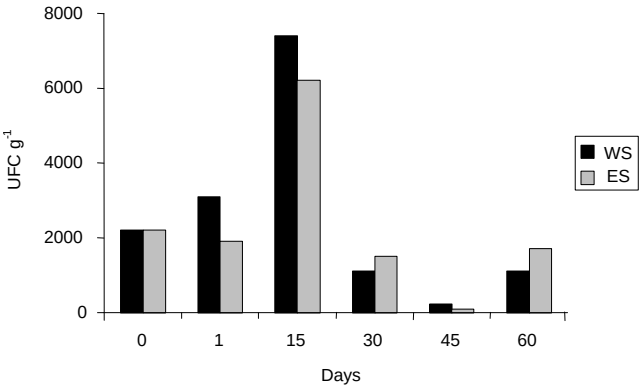

Figure 6 - Number of mesophilic microorganisms in sardine *in natura* (day 0) and during the 60-day-preservation processes WS and ES (WS = whole sardine; ES = eviscerated sardine; WC = whole sardine with condiments and preservatives; EC = eviscerated sardine with condiments and preservatives).

lactic acid, consequently lowering the pH. The pH in the four preservation processes was not affected by the use of eviscerated or whole fish. On the other hand, whole fish generated more lactic acid than eviscerated fish. A consistent decrease in pH from 6.5 to 4.3 and a corresponding increase in titratable acidity was observed during fermentation of sardinella (*Sardinella* sp.) to be used as soup flavourer or as protein source in infant foods. Fermentation caused a slight decrease in total viable count of microorganisms, which ranged from 250 to 380 CFU g^{-1} and increase in protein from 16 to 18 g 100 g^{-1} (Achinewhee & Oboh, 2002).

Microbiological Analyses

Brazilian legislation limits bacterial count of sardines *in natura* to 1.0 x 10^6 CFU g^{-1} (Brasil, 1989). The level obtained in the present study 12.2 x 10^3 CFU g^{-1} (Figure 6) - is under this legal limit. After 24 hours of preservation, the number of mesophilic microorganisms for the WS process (3.1 x 10^3 CFU g^{-1}) was higher than for ES (1.9 x 10^3 CFU g^{-1}), which might be accounted to the initially higher microbial level of the WS process. After 30 days of preservation, the number of mesophilic microorganisms in both processes decreased, being lower than the initial levels. This decrease probably resulted from lower availability of nutrients, increase in acidity with a consequent decrease in pH and competition with restrictive halophilic microorganisms.

For all analyses made up to the 45th day, the ES process presented higher numbers of bacteria, reaching the highest levels on the 15th day (3.3 x 10^3 CFU g^{-1}) (Figure 7). For the WS process, the highest number of bacteria was also observed on the 15th day (2.0 x 10^3 CFU g^{-1}), which is in accordance with the results reported by Thongthai & Siriwongpairat (1978) in an experiment with salt preservation of "Nam pla", in which

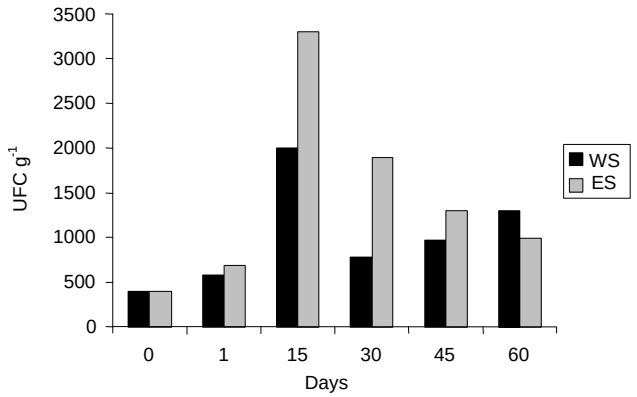

Figure 7 - Number of halophilic microorganisms of sardine *in natura* and during the 60-day WS and ES processes (WS = whole sardine; ES = eviscerated sardine; WC = whole sardine with condiments and preservatives; EC = eviscerated sardine with condiments and preservatives).

bacteria that survived 20% NaCl increased gradually to 9.5 x 10^5 CFU mL^{-1}) after 14 days. Asiedu & Sanni (2002) found predominance of latic acid bacteria from 48h of fermentation (5.8 x 10^8 CFU g^{-1}) in a fermented fish- carbohydrate dish made from minced fish flesh (75%), yam (20.5%), onions (1%), ginger (1%) and salt (2.5%).

For the WS process, the bacterial growth was not as high as for the ES process, probably because of limiting factors, such as the increase in acidity and the presence of some substances unfavorable to halophilic microorganisms. Coliforms, from secondary contamination, can be pathogenic. The Brazilian legislation determines the limit of 1.0 x 10^2 CFU g^{-1} for fecal coliforms (Brasil, 1989). In this experiment with sardine *in natura*, the number of coliforms was below this limit. The number of coliforms in sardine during the 60 days of the experiment, for the WS and ES processes, was significantly higher for

WS, probably because of the presence of the viscera. The decrease in the number of coliforms after 15 days of preservation can probably be related not only to the presence of salt but also to many other factors. These could be the increase in acidity, a decrease in pH, the probable presence of substances unfavorable to the survival of bacteria of this group and competition for nutrients by other microorganisms.

The presumptive test of *S. aureus* (3.0 x 10 CFU g^{-1}) was below the maximum limit determined by the Brazilian legislation. After 24 hs, for treatments WS and ES, the count increased, mainly in WS, reaching 3.0 x 10^2 CFU g^{-1}. The count of *S. aureus* was higher for WS probably because of the presence of the viscera.

S. aureus is quite resistant to high salinity (salt-tolerant), surviving in media with up to 20% of salt (Oetterer, 2001). The decrease in *S. aureus* in the WS and ES processes, reaching zero, could be attributed to other factors besides salt, such as the increase in acidity, decrease in pH, the lack of specific nutrients, the probable presence of substances that would inhibit its growth, or to competition with other microorganisms, otherwise the number of *S. aureus* would have decreased in the first 24 hs. This agrees with Bertullo (1975), who reports that *S. aureus* is not a strong competitor and that its growth is inhibited by microorganisms in most *in natura* or preserved food.

Escherichia coli, a fecal coliform, was not detected in any of the two processes nor in the sardines *in natura*. *Ecoli* does not resist saline concentrations of 6.5% - Aw 0.96 - and the saturated salt solutions or the muscles of salted fish which have 0.75 Aw (Lessi, 1995). Inhibitory effects of ingredients (mustard, black pepper, dried lemon) added during processing of mehiawah (fermented fish sauce) on pathogens (*S. aureus, Salmonella and E. coli)* were investigated by Al-Jedah et al (2000). The authors considered that lemon had the most inhibitory impact on *E. coli,* making the organism undetectable after 3 days. The presence of *Salmonella* spp was not detected in sardines *in natura,* nor during the 60 days in both processes. This is in agreement with Lessi (1995), who reported that common and pathogenic bacteria do not develop in NaCl concentrations higher than 16% (Aw 0.93).

Sensory Analysis for characteristics of color, aroma, taste and texture

Samples from the WS an WC processes presented higher average rates when compared to samples from ES and EC processes for all analysed sensory characteristics, i.e. color, aroma, taste and texture. There were no statistical differences between the WS and WC processes nor between the ES and EC processes. The standard deviation was lower for samples from the WS process, which indicates a consensus among the testers in evaluating the characteristics of aroma, taste and texture (Table 1 and Figure 8). Samples from the WS process, were considered superior for color, aroma and taste, reaching close to grade 6 for these characteristics. Samples from the WS and WC processes had the same evaluation for texture. The grades ranged from one (very bad) to seven (excellent). The samples from the WS and WC processes were in the "very good" range for all characteristics, whereas samples from the ES and EC processes were in the "good" range. Comparing the four processes it can be concluded that the use of condiments had little influence on the evaluation of the sensory characteristics. The use of whole or eviscerated fish in the preservation process was decisive in the evaluation of the final product – the use of whole fish was significantly more advantageous.

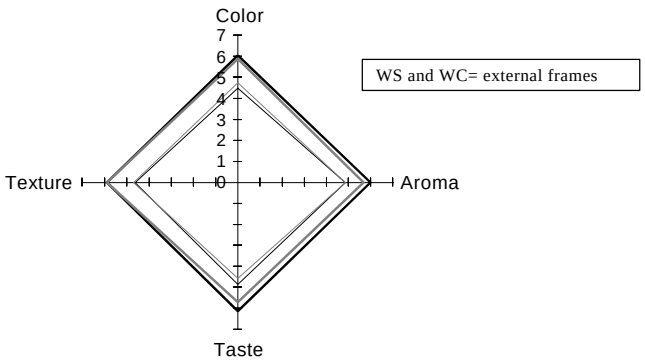

Figure 8 - Average grades for the characteristics of color, aroma, taste and texture for the WS, WC, ES and EC sardines preserving processes. (WS = whole sardine; ES = eviscerated sardine; WC = whole sardine with condiments and preservatives; EC = eviscerated sardine with condiments and preservatives). It is recommended that whole fish should be used for the production of anchovies from sardines.

Table 1 - Averages and standard deviations for the characteristics of color, aroma, taste and texture for the whole sardine (WS), whole sardine with condiments and preservatives (WC), eviscerated sardine (ES) and eviscerated sardine with condiments and preservatives (EC) processes in preserving sardines.

	WS	WC	ES	EC
Color	6.03 ± 1.08 a	5.87 ± 1.02 a	4.48 ± 0.99 b	4.71 ± 0.90 b
Aroma	5.97 ± 0.98 a	5.68 ± 1.04 a	4.81 ± 1.05 b	4.84 ± 1.03 b
Taste	6.13 ± 0.76 a	5.68 ± 1.01 a	4.84 ± 0.86 b	4.58 ± 0.99 b
Texture	5.87 ± 0.92 a	5.87 ± 1.17 a	4.60 ± 1.10 b	4.71 ± 0.94 b

*Averages with the same letters do not differ, according to the Tukey test ($P < 0.05$).

Beirão (1979) reported similar results in preserving *Sardinella brasiliensis*. The author conducted studies with whole fish, eviscerated fish, eviscerated fish with bromelin and eviscerated fish with papain and better sensory characteristics of color, taste and aroma were observed whole fish.

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