Physical-chemical and microbiological characteristics of acid silage of fish subjected to two processes of acidification and different storage periods

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ABSTRACT

This research aimed to evaluate the physical-chemical and microbiological characteristics of acid silage from whole fish submitted to two acidification processes and five periods of storage, in a completely randomized design. The acidification processes were acetic acid (5 %.biomass^{-1}) and lactic acid (5 %.biomass^{-1}) and the storage periods were 1, 7, 14, 21 and 28 days. The silage materials were stored in the BOD incubator (biological oxygen demand incubator) at 37°C for 28 days. The culture, quantification, and isolation of Enterobacteriaceae, *Staphylococcus* spp., *Lactobacillus* spp., filamentous fungi, and yeasts were performed. After 28 days the chemical analyzes of both silages were performed and compared with the raw material. The pH of the silage with lactic acid was significantly lower (P<0.05) than that produced with acetic acid. Enterobacteriaceae were only detected for raw material samples. There was a development of filamentous and yeasts up to seven days for both silages. Regression analysis estimated that the optimal time for stabilization of lactic silage acid is an average of 7.6 days, showing to be efficient for the reduction of *Staphylococcus* spp. population. Both silages had high protein contents (45-56 %) and would be a good alternative for feeding non-ruminants. This work represents the first study that evaluates the production of fish silage with lactic acid and the results indicate lower pH (3.7) and faster reduction of *Staphylococcus* spp., when compared to fish silage with acetic acid.

Key words: acidification, silage, bacteria, fungi, *Oreochromis niloticus*.

Características fisicoquímicas y microbiológicas del ensilaje ácido de pescado sometido a dos procesos de acidificación y diferentes períodos de almacenamiento

RESUMEN

El objetivo de esta investigación fue evaluar las características físico-químicas y microbiológicas del ensilaje ácido de pescados enteros sometidos a dos procesos de acidificación y cinco períodos de almacenamiento, en un diseño completamente aleatorizado. Los procesos de acidificación fueron ácido acético (5 %.biomasa^{-1}) y ácido láctico (5 %.biomasa^{-1}) y los períodos de almacenamiento fueron 1, 7, 14, 21 y 28 días. Los materiales de ensilaje se almacenaron en una incubadora BOD (incubadora de demanda biológica de oxígeno) a 37°C durante 28 días. Se realizó el cultivo, cuantificación y aislamiento de Enterobacteriaceae, *Staphylococcus* spp*., Lactobacillus* spp*.,* hongos filamentosos y levaduras. Después de 28 días se realizaron los análisis químicos de ambos ensilajes y se compararon con la materia prima. El pH del ensilaje con ácido láctico fue significativamente menor (P<0,05) que el producido con ácido acético. Se detectaron enterobacterias únicamente en muestras de materia prima. El desarrollo de filamentosos y levaduras ocurrió después de siete días para ambos ensilajes. El análisis de regresión estimó que el momento óptimo para la estabilización del ensilaje con ácido láctico es de 7,6 días, que mostró una reducción eficiente de *Staphylococcus* spp. Ambos ensilajes mostraron un alto contenido de proteína (45-56 %) y podría ser buena alternativa para la alimentación de no rumiantes. Este es el primer estudio que evalúa la producción de ensilado láctico de pescado y los resultados indican un pH más bajo (3,7) y una reducción más rápida de *Staphylococcus* spp., comparado con el ensilado de pescado elaborado con ácido acético.

Palabras claves: acidificación, ensilaje, bacterias, hongos, *Oreochromis niloticus*.

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INTRODUCTION

One of the most important components of animal production is food, since it represents a high percentage of production costs; on the other hand, the provision of protein can be a problem due to its limited availability and high cost (Berenz 1997). Among various alternative sources for food destined to the fish or other animal creation, the fish silage has elevated nutritional quality and high potential for the sustainable animal production (FAO 2010).

Fish silage can be obtained by acid addition or with microbial fermentation of fish waste or the entire fish not processing. The silage appearance is liquid and is unfit for human consumption. Different fish species can be used for the production of fish silage, however *Oreochromis niloticus* has been the most promising and frequent (Ucci 2004).

Different studies have indicated the favorable characteristics of the silage, as good quality, low cost and high digestibility (Vidotti and Goncalves 2006, Borghesi *et al*. 2008, Ramírez *et al*. 2013). However there are few reports of the microbial profile features in fish acid silage. Research on the microbial population involved in the fermentation process of the fish silage, will allow to detect microorganisms with biotechnological potential as inoculants.

Similarly, microbiological evaluation of the final products would reveal an alternative control of pathogenic or spoilage agents in silage. Therefore, the objective of the present study was to evaluate the physical and microbiological properties of fish acid silage subjected to two processes of acidification and different storage periods.

MATERIAL AND METHODS

The experiment was conducted at the Laboratory of Food Technology and Microscopy of the Institute of Agricultural Sciences, Federal University of Minas Gerais (UFMG), Regional Campus of Montes Claros, Brazil. Two fish silages (S1 y S2) and five times evaluation (T1, T7, T14, T21, T28), with three replicas each, were assigned to a completely randomized experimental design. S1 corresponding acetic acid addition, and S2 corresponding lactic acid addition; T1 silage evaluation on the first day of storage, T7 silage evaluation on the seventh day of storage, T14 silage evaluation at the fourteenth day of storage, T21 silage evaluation on the twenty-first day of storage, T28 silage evaluation on the twenty-eighth day of storage. A sample of raw material was also evaluated (T0).

The fish used in this study was Nile tilapia (*Oreochromis niloticus*), with approximately six months old and weighing ± 700 g; this fish was donated by the State University of Montes Claros (UNIMONTES) Janaúba campus. At zero time (T0), the fish waste used for the silage preparation were analyzed before the acidification process.

For silage preparation, whole fish passed through the cooking process (heat treatment by cooking) for 15 minutes and then was ground in multiprocessor electric. The processed material was homogenized and stored initially in two sterile beakers. A subsample of this raw material was reserved for dry matter (MS), crude protein (CP), ether extract (EE), calcium (Ca) and phosphorus (P) analysis, according to the AOAC (1995) protocols, in the Laboratory of Animal Nutrition, Federal University of Minas Gerais.

The processed material was weighed (500 g) on analytical balance and placed in beakers for addition of 5 % pure glacial acetic acid or 5 % pure lactic acid. After acidification, both silages were homogenized and packaged in sealed test tubes and covered with sterile foil. For each treatment and for each estimated time of storage, were prepared three replicates (do Carmo *et al*. 2008). The silages were stored in BOD chamber at 37°C and homogenized daily during one week with sterile glass rod.

The hydrogen potential (pH) was weekly estimated on digital potentiometer, and macroscopic characteristics such as odor, liquefaction time, oil production time and color were evaluated with the same periodicity.

Dry smears were made from the silages and were fixed and stained by the Gram method (Quin *et al*. 2005), to observe the micro morphological

characteristics and profile of predominant bacterial groups in the silages, in all storage periods.

The prepared slides were read using the cross method described by Dirksen (1993), in which, the crosses represented the bacterial density found in the view fields. One cross (+) represents few cells (0 to 50 per field was visualized), two crosses (++) indicates moderate occurrence (50 to 100 cells per field was visualized) and three crosses (+++) high microbial population density (more than 100 cells per field).

For analysis and quantification of the microbial populations, two serial decimal dilutions for each material sampled were prepared in tubes containing sterile saline; after dilutions, the tubes were homogenized in vortex during two minutes. Swabs sterile from silage samples and aliquots of 100 µL of the dilutions were inoculated into sterile Petri plates containing different culture media and incubated in a Biochemical Oxygen Demand (BOD) chamber at 37°C during 21 days, in which they were monitored for growth of microbial colonies (Lacaz *et al*. 2002).

For Enterobacteriaceae isolation was used MacConkey Agar culture medium, and for filamentous fungi and yeast were used the Sabouraud dextrose Agar plus chloramphenicol (300mg.L^{-1}). *Staphylococcus* spp., and *Lactobacillus* spp., were evaluated on plates containing Mannitol Salt Agar and MRS Agar in anaerobic jar, respectively (Lacaz *et al*. 2002, Quin *et al*. 2005).

Reisolation and culture were performed in tubes containing MacConkey agar in an oven at 37°C for 24 hours, to identify the genus of Enterobacteriaceae. After the exponential growth, each isolate was inoculated into tubes containing medium Rugai and Araujo, modified by Pessoa and Silva (1972). The tubes were incubated at 37°C for 24 hours and then analyzed using the identification key for Enterobacteriaceae, according to Pessoa and Silva (1972).

The pH averages were evaluated in Split-Plot with variance analysis (2 acids x 5 storage periods). However, as no significant interaction was observed between the independent variables, the averages of the silages were compared by ANOVA. Different storage periods for each silage were compared by Tukey test (5 % significance).

The data for the microorganisms quantification did not present a normal distribution, so a $\log_{10}$ (X + 1) logarithmic transformation of the values was performed. The above allowed the normal distribution of *Staphylococcus* spp. However, the filamentous fungi and yeast data quantification did not show normal distribution even after transformation.

To verify statistical differences of *Staphylococcus* spp., ANOVA and Tukey (5 % significance level) was carried out in Split-Plot analysis (2 acids x 5 times the storage). To determine the optimal acidification time of each silage to reduce the population of these microorganisms, a polynomial regression was performed.

For filamentous fungi and yeast, the means were evaluated by nonparametric Wilcoxon test with a significance level of 5 % (Sampaio 2010). The positivity rates of micro-organisms were evaluated with the Chi-square test ($P<0.05$). All statistical analyzes were processed in the statistical package SAEG® - System for Genetic Analysis and Statistics, version 9.1 (2007).

RESULTS AND DISCUSSION

The average pH of lactic silage (3.71) was significantly lower when compared to acetic silage (4.03), with 5.25 of variation coefficient. Mean pH at each storage time are arranged in Table 1. These results showed an acidification more efficient for fish silage prepared with the addition of 5 % lactic acid. Likewise, a significant interaction was observed between independent variables “silage” and “shelf life”.

The results indicate that the pH does not differ in function of time after addition of organic acids ($P>0.05$). A similar result was reported by do Carmo *et al*. (2008). These authors observed pH 4.37 in silage from tilapia waste produced with 5% acetic acid, after 20 days of storage. In addition to this, the low pH observed contributed to the microbiological quality of the silage.

According to Bello (1994), pH values close to 4 reduce food spoilage and promote greater activity of proteolytic enzymes present in fish. According

Table 1. pH averages of fish silage acidified with lactic acid or acetic acid at different storage times

Times evaluated	Lactic silage	Acetic silage
T0	6.55	6.55
T1	3.64	3.99
T7	3.59	4.15
T14	3.73	4.11
T21	3.80	3.93
T28	3.80	3.94
$\bar{X}$ (T1 at T28)	3.71a	4.03b

T = retention times (0, 1, 7, 14, 21 and 28 days). Means followed by different letters differ in the ANOVA ($P < 0.001$).

to the above, in the present study both silages presented a pH within the ideal range after 28 days of storage.

Macroscopic evaluations revealed the liquefaction of both silages in the first week of storage. The acid odor prevailed for both silages during the entire storage period. Oil production was observed after seven days for both treatments, more clearly (crystalline) for acetic silage. Vidotti and Gonçalves (2006) reported the acidification of conventional silage in the pH range between 3.9 to 4.2 and liquefaction at three days, with the establishment of a fat layer that preserves the enzymatic activity for many months.

According to Perez (1997), the fatty acids in fish oil are unsaturated and therefore oxidize easily. Oxidation can reduce nutritional quality and reduce the availability of proteins and amino acids or promote an unpleasant taste.

The oils produced in the acetic and lactic silage showed a brown-orange and brown-green coloration, respectively. No reports were found in the scientific literature describing the differences in color or composition of the oil produced in the fish acid silage. Future studies should assess the quality and profile of fatty acids in the lipid fraction of fish silages.

Gram staining revealed the predominance of Gram negative (++) bacteria in T1 for both silages. After seven days of storage, a large proportion of Gram-positive bacterial cells (+++) were detected in acetic silage compared to lactic silage (++). After 14 days of fermentation, the micromorphological profile for both silages was similar to those observed in T7, with a lower incidence of microbial cells in both treatments (+). No yeast cells were detected on direct exams.

There were no reports in the scientific literature describing the direct microscopic examination. However, a better quality of lactic fish silage is inferred, by promoting a greater reduction in the number of bacteria per field, in a shorter storage period. This quick and inexpensive analysis can be an indicator of the quality parameters of fish silage.

The Enterobacteria culture obtained in T0, showed an average of 1.6×10^4 colony forming units (CFU) per gram of raw material, 56.25 % of lactase producing bacteria (Lac +) and 43.75 % of non-producing bacteria (Lac -). At storage times T1, T7, T14, T21 and T28, these bacteria were not observed in any of the silages, indicating an efficient reducing effect after the addition of the organic acids used in this study.

From the gram-negative bacilli or cocobastonetes isolated in T0 (n = 8), *Escherichia coli* was identified in 50 %, *Enterobacter* spp., in 25 %, *Klebsiella* spp., in 12.5 % and *Pseudomonas* spp., in 12.5 %. The presence of these bacteria in the raw material was probably caused by contamination with intestinal contents during whole fish processing. However, the acids tested were effective in reducing these bacterial groups.

Studies conducted by de Oliveira *et al.* (2006), in which they evaluated Enterobacteria present in the Nile tilapia acid silage made with formic acid (3 %), observed the presence of total coliforms in 98 %, the first day of storage (2.4×10^2 CFU.g^{-1}) . However, coliforms were absent between 15 to 30 days of storage. According to Bolosco *et al.* (2010), fish silage made with 5 % acetic acid, could be stored for 201 days without a proliferation of *Salmonella* spp. and other coliforms.

In this study, the development of fungi and yeasts was observed until storage day seven, in both silages (Table 2). No fungal isolates were detected on storage days 14, 21 and 28.

The addition of antifungal agents in fish silage has been recommended, such as 0.25 % ascorbic acid (Machado 2010). However, it was not necessary under the conditions of the present study, because the acidification process was effective in controlling aerobic fungi, after storage fourteen days

The *Staphylococcus* spp. quantification is showed in Table 3. This bacterial group was isolated from samples T0, T1 and T7, for both silages. The observed concentrations of these bacteria were relatively high when compared to other groups of micro-organisms. As of day 14 of storage, this bacterial group was not observed in the silage with lactic acid; however, it was present in the silage with acetic acid. Probably, the low pH observed in lactic silage inhibited the development of these microorganisms.

The quantification analysis of *Staphylococcus* spp., (Table 3) indicated a significant interaction between the types of silage (plot) and the different storage times evaluated (subplots). Significant differences

Table 2. Mean colony forming units per mL of fungal in fish silage with lactic acid or acetic acid, at different times of storage

Time	Lactic silage		Acetic silage	
	Filamentous	Yeast	Filamentous	Yeast
T0	2×10^3	1×10^3	2×10^3	1×10^3
T1	1×10^3	4×10^3	1×10^3	1×10^3
T7	1×10^3	1×10^3	1×10^3	1×10^3
T14	0	0	0	0
T21	0	0	0	0
T28	0	0	0	0
$\bar{X}$ (T1 at T28)	$4x10^2a$	$1x10^3A$	$4x10^2a$	$4x10^2A$

Means for the times T1 to T28, followed by the same letters, uppercase for filamentous fungi and tiny letter for yeast, do not differ by nonparametric Wilcoxon test ($P<0.05$).

Table 3. Positivity and quantification of *Staphylococcus* spp. in fish silage acidified with lactic acid or acetic acid at different times of storage

Time	Acetic silage		Lactic silage	
	Positivity	(CFU/mL)	Positivity	(CFU/mL)
T0	+	1.1×10^9	+	1.1×10^9
T1	+	5.4×10^6a	+	4.1×10^6a
T7	+	1.0×10^5a	+	4.0×10^5b
T14	+	7.3×10^3b	-	0a
T21	+	0a	-	0a
T28	+	0a	-	0a

Means followed by different letters in the line differ by Tukey test at 5 % significance level (CV = 7.9 %). ($P<0.05$).

(P<0.05) were observed in the bacterial concentration at seven and fourteen days of acidification, however, after 21 days, there were no changes (P>0.05) in the occurrence of these microorganisms (P>0.05).

The production of lactic acid in biological silage is essential because it promotes reduction of pH (around 4.0), inhibiting the growth of bacteria of the *Staphylococcus*, *Escherichia, Serratia, Enterobacter, Citrosactu*, *Achromobacter* and *Pseudomonas* genera (Vidotti and Gonçalves 2006).

Simões *et al.* (2007) evaluated the ocurrence of *Staplylococcus* spp., positive coagulase in tilapia *in natura* and observed adequate concentrations of these microorganisms (less than 102 CFU.g^{-1}), established by the Health Surveillance Agency (ANVISA). However, other studies indicate that fish handlers can transport *Escherichia coli* and *Staphylococcus aureus* to farmed fish and also facilitate the conditions for the development of these bacteria (Muratori *et al*. 2007). The above may explain the presence of *Staphylococcus* spp., in both silages evaluated in this study, since the fish waste used was highly manipulated by the workers in the slaughter process.

The regression analysis determined the optimum point based on the storage time (independent variable) for each silage evaluated, in which the presence of *Staphylococcus* spp., decreased, and therefore, increased potential food safety. For silage with acetic acid, an optimal incubation time of 29.6 days was estimated for the reduction of these bacteria (Figure 1). The value was determined according to the equation: Y = 6.627-0.2238X (determination coefficient R^2 = 0.93), where Y is *Staphylococcus* spp., concentration and X represents the storage time of the silage (days). However, the estimated time exceeded the limits tested in this experiment (28 days), so it is advisable in future studies to perform the analysis with a longer storage period to confirm these results.

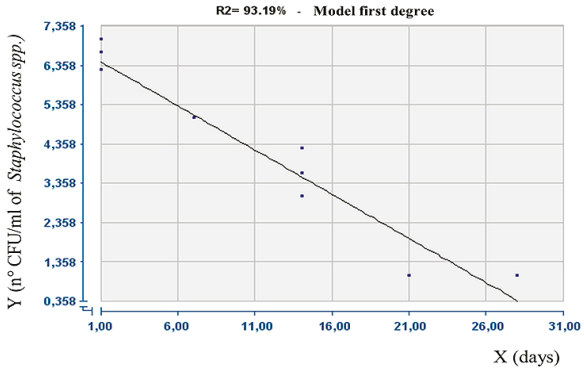

Figure 1. Graph of *Staphylococcus* spp. versus time of storage of the acetic silage: Y= 6.627-0.2238X (R^2= 0.93)

The CFU/ml of *Staphylococcus* spp., in the silage with lactic acid, varies according to equation Y = 4.7167-0.617X (determination coefficient R^2 = 0.90), where Y is *Staphylococcus* CFU/ml and X represents the silage storage time (days). The optimal time to stabilize the silage, obtained by the first derivative equal to zero Y with respect to X would be approximately 8 days (7.6 days; Figure 5).

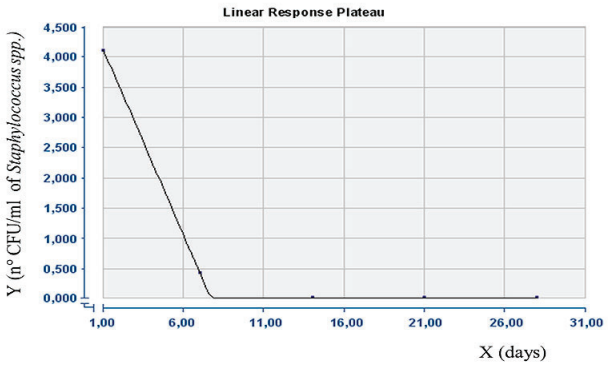

Figure 2. Graph of *Staphylococcus* spp. versus time of storage of the lactic silage. Y= 4.7167–0.617X (R^2 = 0.90)

Lactobacillus spp. were absent at all storage times for both silages. According to Vidotti and Gonçalves (2006), the quality of the silage is related to capacity of *Lactobacillus* spp., to promote stability, as well as the quantity and shelf life of fish. However, because the process was performed with the administration of 5 % acetic or lactic acid, it is possible that bacterial inhibition has occurred due to the inclusion of the acids or has been reduced by the presence of other microorganisms.

Another relevant factor and poorly reported in the scientific literature is the chemical composition of acid silage. The analysis of the nutritional characteristics of acid silage allows a better inference in animal diet formulations. Table 4 shows the values of humidity (A), crude protein (CP), ether extract (EE), calcium (Ca) and phosphorus (P) of acid silages and raw material composed of crushed whole fish.

The humidity values measured in both silages were close to those described in the literature and within the normal range for fish silage. Bolosco *et al.* (2010) reported humidity values for acetic silage produced with tilapia waste, between 67.4 and 73.0 %. These authors indicated no influence of storage time on dry matter, ashes and ether extract. However, according to the microbiological results of this study, high humidity did not provide the conditions for the proliferation and increase of the microbial population in acidified silage, after 28 days. Probably the most important regulatory factor to inhibit food contamination was the pH.

Both silages had high CP values (Table 4), which probably contributed to the growth of *Staphylococcus* spp., (Table 3). The CP value found for acetic silage in this study is less than 67.4 % DM reported by do Carmo *et al.* (2008) and higher than 48.3 % DM found by de Oliveira *et al.* (2006). Lactic silage showed a lower content of CP compared to acetic silage, however, the average was similar to that found in silage with 3 % formic acid, described by Pimenta *et al.* (2008), with CP values ranging between 39 and 48 % of MS (1 to 30 days).

In acidified silage, alterations in protein levels have been reported based on storage time; this can be justified by the endogenous action of the fish tissues proteases, which increases the solubility of the protein (Pimenta *et al.* 2008, Al Abri *et al.* 2014).

In this research a high level of lipids in acetic silage was observed (Table 4). This result is in agreement with do Carmo *et al.* (2008), who found 14.2 % ether extract in dry silage with acetic acid. It also coincides with Vasconcelos *et al.* (2011), who detected 13.3 % of lipids in tilapia silage made with 1 % citric acid and 6 % acetic acid.

Contrasting the results of this study with those found by Vasconcelos *et al*. (2011), the level of calcium in both silages evaluated was higher numerically compared to the raw material (Table 4). The high values observed in this research could be due by the greater solubility of the Ca^{++} in the carcasses, promoted by the acidification process (Kompiang *et al*. 1981).

Phosphorus levels in both acid silages were also higher numerically than those found in the raw material used in this study (Table 4). Future studies are necessary to elucidate the mechanism that promotes the greater availability of these minerals during the silage process. Phosphorus is essential for the bone structure of fish, and the lack of this mineral can cause reduced growth, reduced use of food and cause alterations in bone development in these animals (Pezzato *et al.* 2004, Witten *et al.* 2016).

Table 4. Compositions of two silages nutritive value of fish, with 28 days of storage and the raw material, consisting of residues of tilapia

Treatments	A(%)	CP(%)		EE(%)		Ca(%)		P(%)	
		DM	FM	DM	FM	DM	FM	DM	FM
Raw material	80.58	60.38	11.73	16.16	3.14	4.32	0.84	2.49	0.48
Acetic Silage	78.31	56.62	12.28	12.65	2.74	5.53	1.20	3.50	0.76
Lactic Silage	75.20	45.34	11.24	7.56	1.87	4.53	1.12	2.65	0.66

Variables: humidity (A), crude protein (CP), ether extract (EE), calcium (Ca), phosphorus (P), based on dry matter (DM) and fresh matter (FM) (AOAC, 1995).

CONCLUSIONS

Lactic and acetic acids were effective to reduce and maintain the low pH of fish silage until 28 days of storage. However, lactic silage had a better pH reduction and after 8 days of storage, it showed an optimal capacity to reduce the population of microorganisms pathogenic and spoilage, being more efficient in reducing contamination by *Staphylococcus* spp., compared to acetic silage. Likewise, high levels of protein were observed in both silages.

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