Bacteriocinogenic Effect of *Lactobacillus sakei* 2a on Microbiological Quality of Fermented *Sardinella Brasiliensis*

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ABSTRACT

Lactobacillus sakei 2a is a bacteriocin producer strain and, in this work, it's effects as a starter culture in the fermentation process of sardine (Sardinella brasiliensis) fillets were observed at different concentrations of NaCl (2, 4 and 6%) and glucose (2 and 4%), to determine it's ability to produce organic acids and consequent pH reduction. Experiments were carried out independently, with only one parameter (NaCl or glucose) varying at a time. After 21 days of fermentation the deteriorative bacteria concentration reached 9.7 Log_{10} CFU. g^{-1} corresponding to 6% NaCl and 4% glucose. Little differences were observed in lactic acid production when 2 and 4% glucose were added, since total acidity was 1.32 and 1.34% respectively, the experiments with 6% NaCl presented the best results. Initial pH of sardine fillets was 6 and after 21 days pH values were 3.8, 3.9 and 4 for the experiments with 2, 4 and 6% NaCl. This may have been due to the inhibitory properties of NaCl over the deteriorative bacteria. After 21 days of the fermentation process lactic acid bacteria concentrations were 14.5 Log_{10} $CFU.g^{-1}$. The ratio protein nitrogen and total soluble nitrogen was typical of a cured fish.

Key words*: Sardinella brasiliensis, Lactobacillus sakei 2a, fermented fish*

INTRODUCTION

Despite the recent progress in food biotechnology with the introduction of modern technologies and safety concepts (e.g. HACCP), the problem of food safety and security remains to be solved. On the other hand, consumer trends lead to a loss of intrinsic preservation and to a potential loss of protection from processing, since consumers prefer more stable and safer products with a longer shelf life and without chemical preservatives, as well as mild and light products with a low acid, sugar, salt or fat content. The manufacturers strategies to increase the safety of fish and fish products consist of developing new technologies to reduce the number of microorganisms in fish,

and new products minimally processed which could represent new hazards for health because of the pathogen growth (Hugas, 1998; Aymerich et al., 1998). LAB have been playing an important role in food fermented causing flavor and texture changes together with a preservative effect resulting in increase in the shelf life of the transformed product (Paludan-Müller et al., 2002;Reid, 1999; Zhang and Holley, 1999;). The present study examined the effect of *Lactobacillus sakei* 2a on the fermentation of sardine fillets. Fish-NaCl-glucose system was used to evaluate the factors that favor a rapid lactic fermentation.

MATERIALS AND METHODS

Fermentation of fish: Fresh sardine were deheaded, degutted, filleted and stored at 30^0C until processed. The fillet was then divided into 300g portions in sterile flasks, and various amounts of NaCl and glucose (2-4%) were added. The influence of NaCl and glucose were determined, using *Lactobacillus sakei* 2a as starter culture. Two independent experiments were performed in duplicate, in which only 1 parameter (NaCl or glucose) varied. Concentrations tested (expressed on total weight of sardine and water mixture) were 0, 2, 4 and 6% NaCl; 0, 2 and 4% glucose. The system was inoculated with single strains at a level of 10^8 CFU.g^{-1} and fermented at 23-24^{0}C for 21 days. The speed and efficiency of the lactic fermentation was monitored by the rate of pH decrease and the balance between lactic acid bacteria (LAB) counts and total spoiler counts on plate count agar (PCA). The slower grower LAB on PCA is easily excluded from the total spoiler count. The competition between LAB and spoilers is expressed as the log ratio between the counts on MRS and PCA media (Zhang and Holley, 1999; Gonzáles-Fernandez et al., 1997).

Bacterial strain: *Lactobacillus sakei* 2a, isolated from "lingüiça" (a typical Brazilian meat product), were kindly provided by Faculdade de Ciências Farmacêuticas, Universidade de São Paulo (De Martinis and Franco, 1997; Gonzáles-Fernandez et al., 1997).

Morphophysiological and biochemical characteristics of strain: The strain (*Latobacillus sakei* 2a) were characterized by Gram-reaction, morphology (phase-contrast microscopy), growth at 8, 15, 45 ^{0}C and pH 3.9, halophilism at 6.5, 7 and 10% NaCl, motility, catalase test (20% H_2O_2), Voges-Proskauer reaction (MR-VP medium), methyl red test, formation of H_2S, gas from glucose, lysine decarboxylase, indole test, gelatin liquefaction, bacteriocins production and carbohydrates fermentation (acid production). Glucose fermentation and gas production were tested in MRS broth with 1% glucose and Durham tubes. The detection of bacteriocins produced was examined using the *well - diffusion assay* (Lewus et al., 1991). A modification of the *well - diffusion assay* method was employed as follow. Cell-free supernatant from MRS broth culture of putative bacteriocin producer were collected by centrifugation at 9.77 x g for 10 min. The supernatant were neutralized to pH 7 with 1 N NaOH and sterilized by filtration (membrane GV Millipore - 0.22-*m*m). Pour plate were prepared from BHI containing 1% agar seeded with 10^6 UFC.mL^{-1} of *Listeria monocytogenes* Scott A. Wells cut into the pour plates with sterile straws were filled with 40 *m*L of the culture supernatant. The plate was incubated anaerobically overnight at 30^0C. Inhibition was detected by a zone of clearing round the supernatant well (Lewus and Montville, 1991).

Growth of *Lactobacillus sakei* 2a: Viable counts of *Lactobacillus sakei* 2a were determined by plating (aliquots of 1 mL) on MRS agar (Oxoid CM 361).
Culture was grown in MRS for 48 h at 30 ^{0}C to reach the stationary phase. The broth was centrifuged and the cell pellet was resuspended in diluent containing 1% (w/v) peptone in sterile deionized water. Serial dilutions were made and for each dilution, the optical density was measured at $OD_{600\ nm}$ using an MC 0141/visible spectrophotometer and the cell suspension was surface plated on MRS agar for enumeration. A linear relationship between the cell concentrations and optical densities was obtained around $10^8 - 10^9$ CFU. mL^{-1} levels (Lewus and Montville, 1991; De Man et al., 1960).

Microbiological analyses of sardine fillets: Samples of sardine fillets (10g) were aseptically removed and homogenized for 3 min in peptone water (90 mL). The homogenate was serially diluted and used for enumeration of microorganisms. Total bacteria counts were determined on Plate Count Agar (PCA, Oxoid CM 463) after incubation at 30^0C for 48 h for enumeration of *Enterococcus*, *Staphylococcus positive coagulase* were enumerated on Baird-Parker Medium (Oxoid CM 275) and coliforms were enumerated on Violet Red Bile Agar (Oxoid CM 107). Presence of *Salmonella* was assessed following the procedure described in APHA (1992); after enrichment in peptone water overnight and in Rappaport-Vassiliadis broth (Oxoid CM 669) at 37^{0}C for 24 h, samples were streaked on Brilliant Green Agar (Oxoid CM 263) and plates were incubated at 37^{0}C for 24 h.

Chemical analysis: The proximate composition (moisture, protein, fat and ash) of the raw sardines sample used in the present experiment were determined following the standard methods described by AOAC (1995).

Total titratable acidity (TTA): Using the same homogenate prepared for the determination of pH, the TTA was measure by titration with 0.1N sodium hydroxide to a final pH of 8. The % w/w lactic acid in the sample was calculated by multiplying the volume of alkali (mL) by the factor 0.09 (AOAC, 1995). This assumes that all the acid present in the sample is lactic acid.

Total soluble nitrogen and free amino nitrogen: The samples were analyzed for total soluble nitrogen (TSN), protein (total N x 6.25) and free amino nitrogen, FAN (α-amino nitrogen) content using methods AOAC (1995).

Physical analysis: 10 g of each sample was then taken and blended with 90 mL deionised water. The pH of the homogenized samples was measured with a pH meter (Corning pH meter 240, Corning, New York, USA). Samples were analyzed on a wet weight (as is) basis (MA, 1982).

Data analysis: The significance of effects of different process parameters and combinations of conditions in simulation assays were determined by 1- way analysis of variance.

RESULTS AND DISCUSSION

Raw material

The approximate composition of the raw sardines sample used in the experiment was: protein (19%), fat (3.1%), moisture (73.4%) and ash (1.9%) (Table 1).

Table 1 - Proximate composition of sardine fillets

	R$_1$	R$_2$	R$_3$	X	Dp
Moisture	72,9	73,5	73,7	73,4	± 0.39
Fat	2.0	3.0	4.2	3.1	+ 0.89
Protein	19,7	19,6	17,7	19,0	± 0,88
Ash	2,0	1,9	1,9	1,9	± 0,01

R$_{1, 2,3}$: samples.
X: means, Dp: standard deviation.

The chemistry composition of fish (*Sardinella* sp.) is complex; traditionally had a range of fat values from 2% (spring) to 8.6% (autumn (and moisture values from 66 to 84% (result were similar to the marine fish) (Badolato et al., 1994). The microbial analysis of raw is shown in Table 2.

Morphophysiological and biochemical characteristics of *L. sakei* 2a

The strain was identified as cocci Gram-positive, rods in short chains (1-7 cell units), nonsporing and no motile. The colonies showed: white to cream in color, circular shape, smooth, brilliant, convex, creamy consistency and diameter between 0,5 and 1mm. L. sakei 2a is aerotolerant and grow at 7 and 10% NaCl, at pH 3.9 and temperature at 8^0C and 15 ^{0}C but not at 45 ^{0}C (Table 3). The biochemical characteristics of the L. sakei 2a are presented in Table 4.

Table 2 - Microbiological characteristics of raw material

	R$_1$	R$_2$	R$_3$
Aerobic mesophilic bacteria (CFU. g^{-1})	3.5 x 10^2	1.1x 10^4	4.4x 10^3
Coliforms (MPN. g^{-1})	< 3	< 3	< 3
Fecal coliforms (MPN. g^{-1})	< 3	< 3	< 3
Staphylococcus positive coagulase (CFU. g^{-1})	< 10^2	< 10^2	< 10^2
Enterococcus (CFU. g^{-1})	3.5 x 10^2	3.5x 10^2	3.5 x10^2
Salmonella sp.	Absence	Absence	Absence

R$_{1, 2,3}$: means of three samples.

Table 3 - Physiological characteristics of the *Lactobacillus sakei* 2a

pH	Halofilismo (% NaCl)			Growth (°C)			Gelatin liquefaction
3.9	6.5	7	10	8	15	45	
+	+	+	W	+	+	-	-

Symbols: (+) positive reaction, (-) negative reaction and (w) slight reaction.

Table 4 - Biochemical characteristics of the *Lactobacillus sakei* 2a

Production	Reactions
Catalase	-
Voges-Proskauer	+
Gas from glucose	-
H_2S	-
Indole	-
Lysine decarboxylase	+
Methyl red	+
Bacteriocins	+
Fermentation	
Lactose	-
Sucrose	+
Glucose	+
Rhamnose	-
Xylose	-
Arabinose	+
Raffinose	-
Galactose	+
Maltose	-
Trehalose	-
Sorbitol	-

The microbiological quality of the raw material was good. Numbers of aerobic mesophilic bacteria (PCA) in the raw material ranged from 3.5×10^2 to 1.1×10^4 $CFU.g^{-1}$. *Enterococcus* counts were below Log_{10} 4×10^2 $CFU.g^{-1}$. *Staphylococcus positive coagulase* counts were $< 10^2$ $CFU. g^{-1}$. *Salmonella* were not detected.

Production of bacteriocin in broth
The Fig. 1 shown the growth of the *L.sakei* 2a in MRS broth at 30^0C.
Culture of *L.sakei* 2a was grown to 19 hours. The bacteriocins production only began after 4 hours of incubation (Fig. 2) when the bacterial amount was 9.0×10^6 $CFU.mL^{-1}$. In function of the inhibition zone observed and using *L. monocytogenes* Scott A as sensitive microorganism, the bacteriocins production obtained its maximum value after 7 hours. After this time there was not more bacteriocins production.
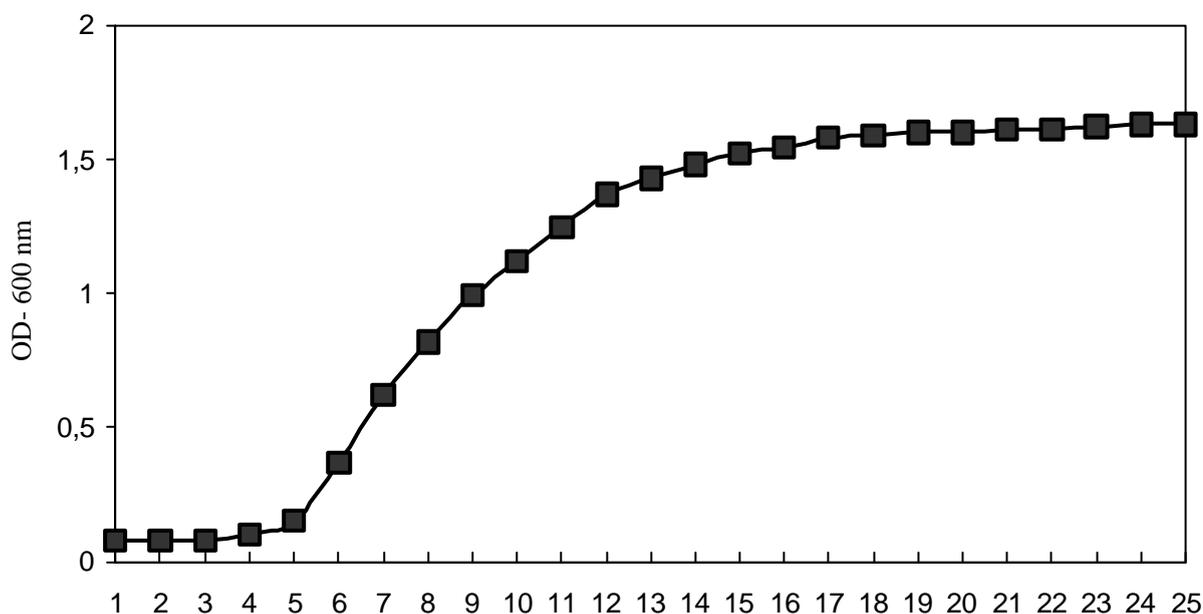

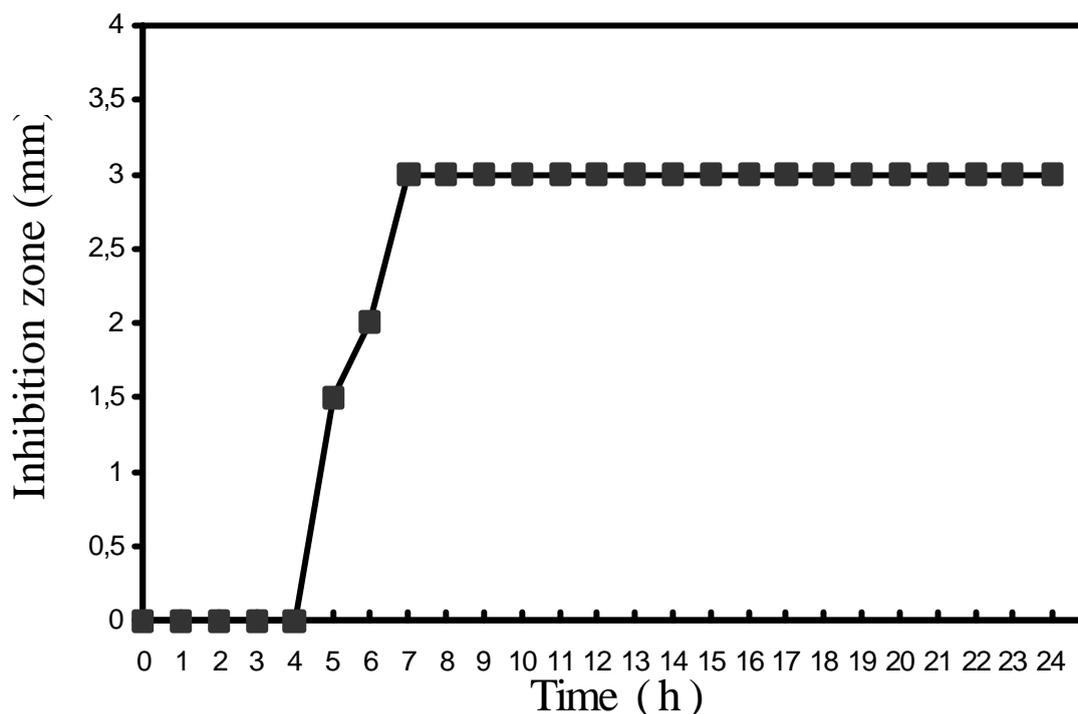**Figure 1 -** Growth of *L sakei* 2a in MRS incubated at 30^0C for 24 hours.

Figure 2 - Detection of bacteriocins produced by *L. sakei* 2a in MRS, incubated at 30 ^{0}C for 24 hours.

Optimization of fermentation parameters using the model system

The effect of NaCl on the pH of sardine without starter and glucose was presented in Fig.3. The pH values increased in all samples. The addition of 6.0% w/w NaCl caused the pH to remain above 6,6 throughout the 5 day incubation period.

Table 5 - Effect of the NaCl and glucose on the acidity (%) during sardine fermentation

| Time (days) | 2% Glucose | | | 4% Glucose | | |
| | NaCl | | | NaCl | | |
	2%	4%	6%	2%	4%	6%
0	0.48	0.48	0.48	0.48	0.48	0.48
7	1.21	1.12	0.74	1.77	1.42	0.79
14	1.73	1.60	1.22	2.55	2.23	1.32
21	2.55	2.23	1.32	2.76	2.64	1.34

The addition of carbohydrate is necessary to promote a satisfactory fermentation since fish flesh is very low in free carbohydrate. The speed and efficiency of the lactic fermentation was monitored by the rate of pH decrease and the balance between lactic acid bacterial (LAB counts and total spoiler (bacterial) counts on plate count agar (PCA). Results of the chemical analysis showed that acidity increases as fermentation progresses, as shown in Table 5.

Table 6 - Effect of the NaCl and glucose on the pH during sardine fermentation

| Time (days) | 2% Glucose | | | 4% Glucose | | |
| | NaCl | | | NaCl | | |
	2%	4%	6%	2%	4%	6%
0	6.0	6.0	6.0	6.0	6.0	6.0
7	4.1	4.2	4.3	4.0	4.1	4.2
14	4.1	4.2	4.3	4.0	4.0	4.1
21	4.0	4.1	4.2	3.8	3.9	4.0

The pH values decreased in all samples (Table 6). Final pH and % titrable acidity obtained after 21 days of fermentation were pH 4 and 2.55% (2% NaCl and 2% glucose). The pH decrease with glucose content (2% w/w), whereas increasing the NaCl concentration from 2-6% slows the fermentation rate. Using 2% w/w NaCl with 2% w/w glucose resulted in a acidity increase to approximately 1.21% after 7 days and 2.55% after

21 days. Incorporating 6% w/w NaCl resulted in an acidity increase to 0.74% after 7 days and 1.32% after 21 days. However, the reproducibility of fermentation rates between different batches of whiting was quite variable in the all-important first 2-day period. One explanation is that this reflects the differing degrees of freshness of the commercially obtained fish and its effect on the LAB: spoiler's competition.

Table 7 - Effect of the NaCl and glucose on the growth of lactic acid bacteria (Log_{10} $CFU.g^{-1}$) during sardine fermentation

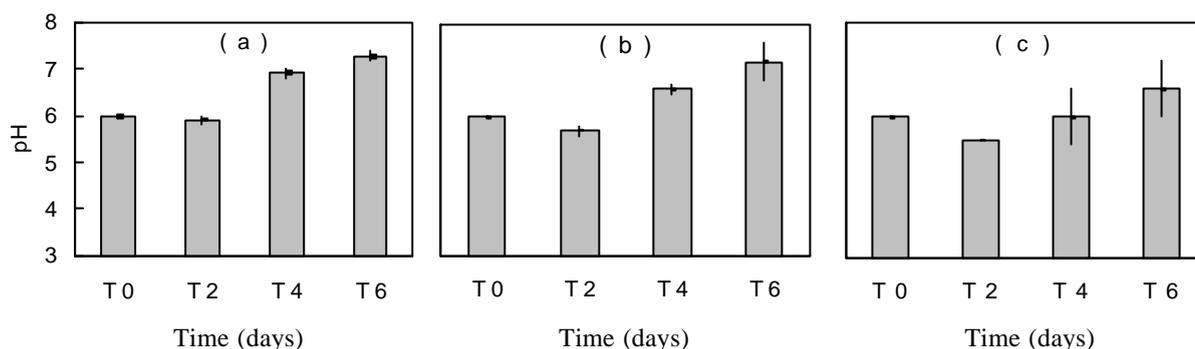| Time (days)	2% Glucose			4% Glucose		
	NaCl			NaCl		
	2%	4%	6%	2%	4%	6%
0	5.2	5.2	5.2	5.2	5.2	5.2
7	1.6	9.9	8.9	11.6	11.0	9.8
14	1.6	1.2	1.9	14.6	13.6	12.9
21	1.2	1.0	1.9	15.3	15.0	14.5

Figure 3 - Changes in the pH during sardine fermentation (without starter and glucose) with 2 w/w% NaCl (a), 4 w/w% NaCl (b) and 6 w/w % NaCl (c).

Results showed that the full potential of acid production was obtained with a glucose level of 4%. It was observed that NaCl has a negative effect on both bacterial growth and pH drop, by reducing the A_w and creating conditions unfavorable for LAB. The effect was observed even at the lowest concentration (2%). However, NaCl presents other desirable properties such as flavor enhancement and preservative effect. Figure 4 shows the trend in the total plate count (TPC). A sharp increase was observed on the first day, after which increase was more gradual up to the fifth day. The sharp increase could be due to the presence of non-acid producing colonies that gradually disappeared as the environment becomes more acidic. The sharp increase could be due to the presence of non-acid producing colonies that gradually disappeared as the environment becomes more acidic. The results of the determination of the non-acid formers (NAF) and acid formers (AF), given in Fig.4 and 5, show that AF

predominated almost throughout the seven-day fermentation period while NAF were present only at the early stages of the fermentation. The viability of non-acid formers existed only for a short period of time since the environment would be inhibitory to them as the fermentation progressed due to the production of lactic acid.

The inoculation with starter culture significantly inhibited the growth of spoilage microorganisms, maintaining the samples in relatively good microbiological quality throughout the study. In the inoculated samples, LAB counts increased at 5.0 Log_{10} $CFU.g^{-1}$ during the 21td day (Fig. 5). The evidence for autolysis in this experiment was provided by changes in the ratio of free amino nitrogen to total soluble nitrogen (Fig. 6).

This ratio increase slightly during the fermentation period, indicating the occurrence of autólise. Samples produced with NaCl at 6% showed less autolysis than samples with 2%.
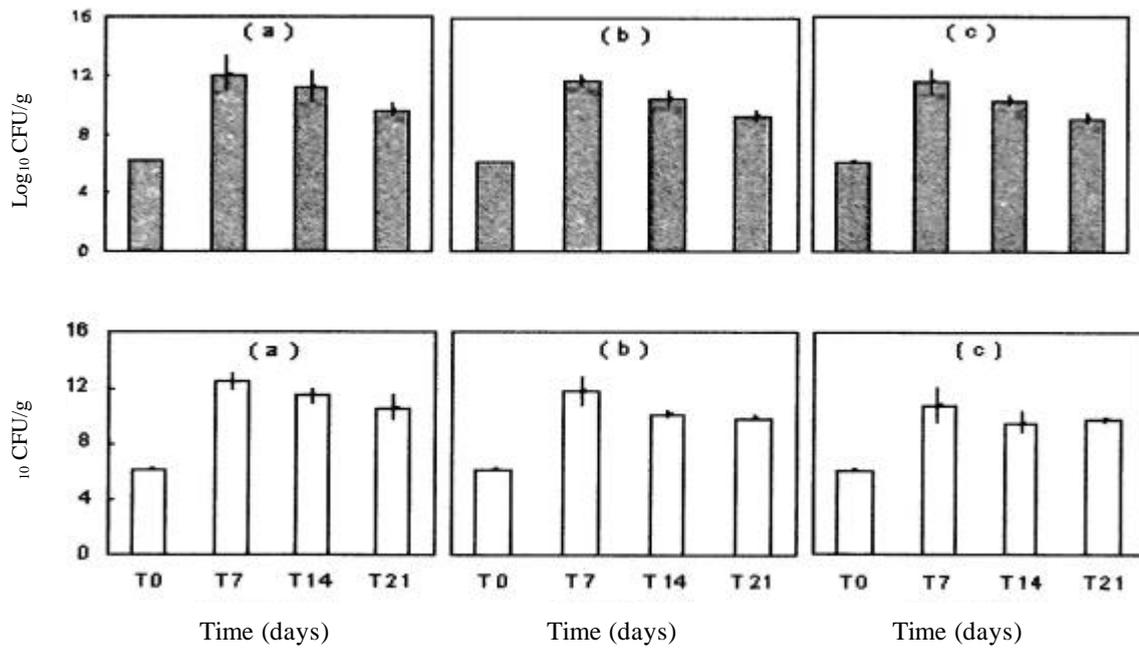
Figure 4 - Changes in the total plate count on nutrient agar during sardine fermentation. Samples were formulated with combinations of various proportions of NaCl; (a) 2%, (b) 4%, (c) 6% and glucose; 2% ■ , 4% □ .
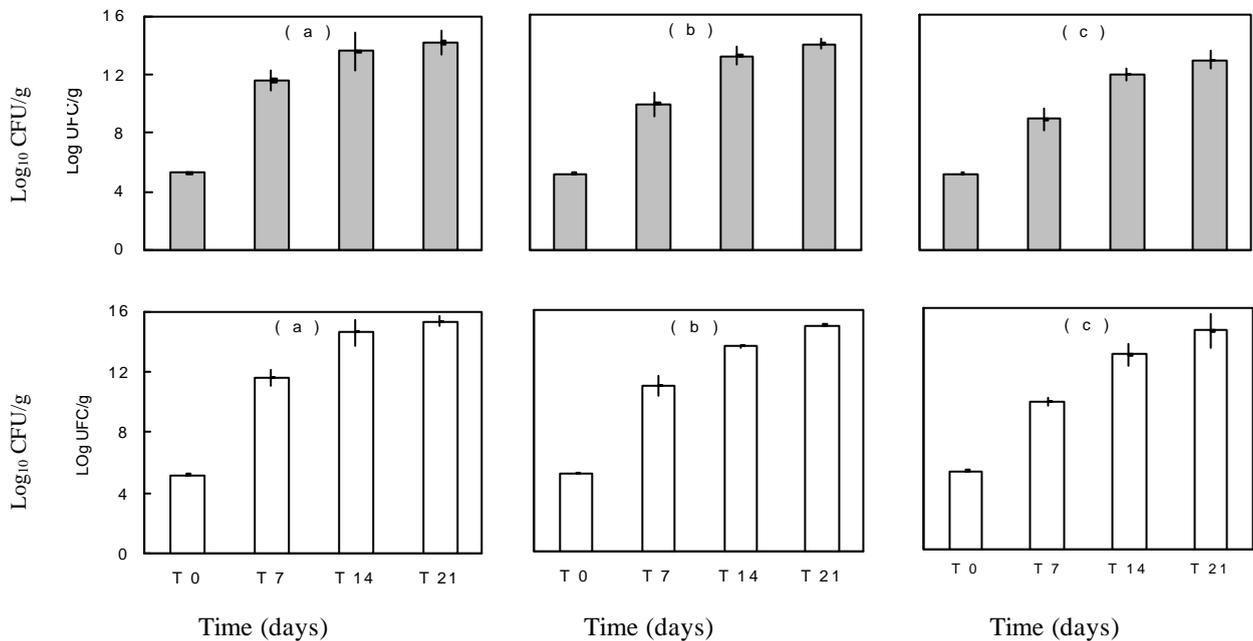

Figure 5 - Changes in the total plate count on MRS agar during sardine fermentation. Samples were formulated with combinations of various proportions of NaCl; (a) 2%, (b) 4%, (c) 6% and glucose; 2% ■ , 4% □ .
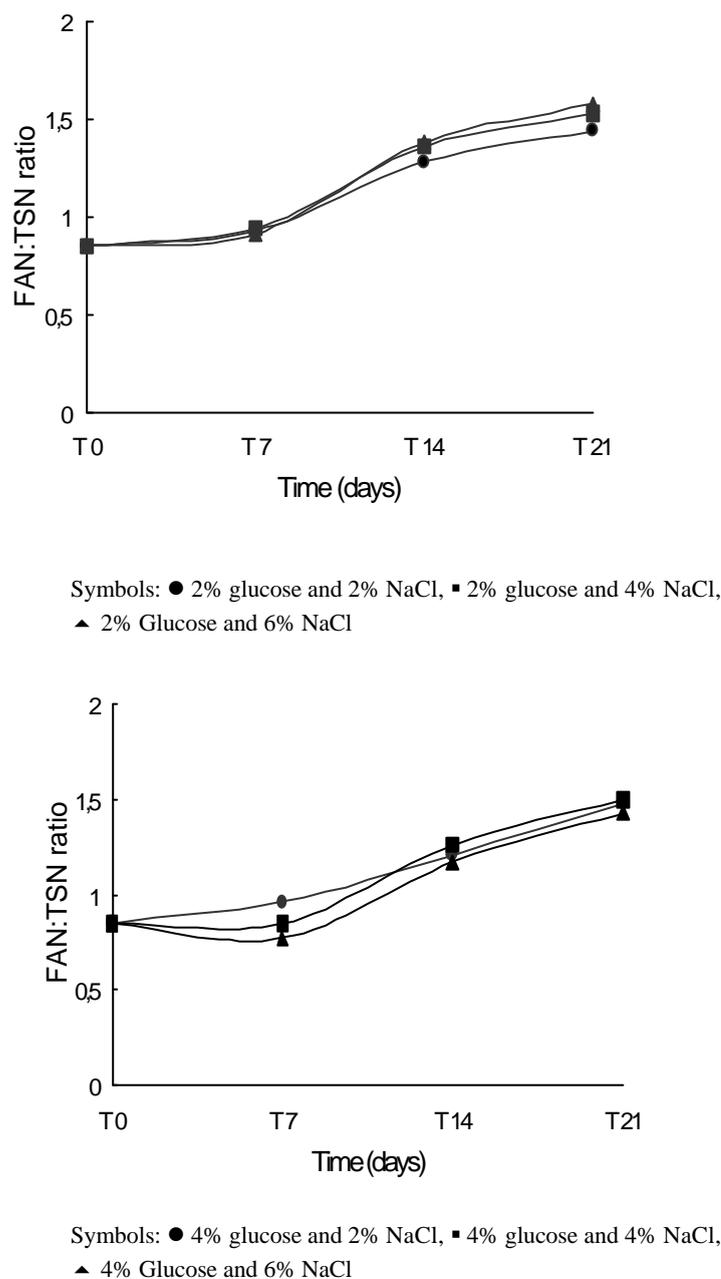
Symbols: ● 2% glucose and 2% NaCl, ▪ 2% glucose and 4% NaCl,
▲ 2% Glucose and 6% NaCl

Symbols: ● 4% glucose and 2% NaCl, ▪ 4% glucose and 4% NaCl,
▲ 4% Glucose and 6% NaCl

Figure 6 - Effect of glucose (2 and 4%) and NaCl (2, 4 and 6%) on sardine autolysis (Free amino
nitrogen/ total soluble nitrogen).

Addition of the higher amount of NaCl could have slowed down the breakdown of the fish meat by autolysis or microbial activities (Ijong and Ohta, 1996).Control of natural fermentation by addition of amine-negative starter cultures has been suggested to prevent amine formation (fish fermented) (Roi-Sagués and Eerola, 1997). One of the most widely studied starter cultures is

Pediococcus cerevisae. Inoculation with amine-negative *P. cerevisae* starter cultures reduces the amount of biogenic amines formed. The research trends towards food preservation focus on mild, physical preservation techniques and the use of natural antimicrobial compounds because of consumer attitude in the last years towards chemical, unnatural preservatives and the demand

for "natural" and fresher foods. (Hugas, 1998; Zhang and Holley, 1999). The production of competitive and bacteriocinogenic lactic acid bacteria may well provide an additional hurdle to improve fish preservation by natural means.

RESUMO

Lactobacillus sakei é comprovadamente uma cepa produtora de bacteriocinas e, neste trabalho procurou-se observar seus efeitos como cultivo iniciador, na fermentação de filés de sardinha-verdadeira (*Sardinella brasiliensis*) em diferentes concentrações de NaCl (2, 4 e 6%) e glicose (2 e 4%), observando-se sua capacidade para produzir ácidos orgânicos e conseqüente redução do pH e microrganismos deterioradores. Os tratamentos foram desenvolvidos de forma independente, variando apenas um dos parâmetros operacionais (NaCl ou glicose). Ao término de 21 dias de fermentação, a concentração de microrganismos deterioradores atingiu 9,7 Log_{10} $UFC.g^{-1}$, correspondente a 6% de NaCl e 4% de glicose. Poucas diferenças foram observadas na produção de ácido lático quando se adicionou 2 e 4% de glicose; a acidez foi 1,32 e 1,34%, respectivamente, para os experimentos com 6% NaCl, os quais apresentaram os melhores resultados. O pH inicial dos filés de sardinha foi seis e, ao término de 21 dias, 3,8, 3,9 e 4, equivalente aos experimentos com 2, 4 e 6% NaCl. Este comportamento pode ser atribuído ao poder inibidor do NaCl sobre a microbiota deterioradora. Ao término de 21 dias de fermentação a concentração de bactérias ácido láticas foi 14,5 Log_{10} $UFC.g^{-1}$.

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