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Effect of Different Levels of Sodium Chloride and Glucose on Fermentation of Sardines (*Sardinella brasiliensis*) by *Lactobacillus sakei* 2a

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ABSTRACT

Lactobacillus sakei 2a is a bacteriocin producer strain. In this study, it's effects as a starter culture in the curing process of sardine (Sardinella brasiliensis) fillets were studied at different concentrations of NaCl (2, 4, 6%) and glucose (2, 4%). After 21 days of fermentation, the spoilage microorganisms population reached 9.7 Log_{10} CFU g^{-1} corresponding to 6% NaCl and 4% glucose. With no addition of glucose and starter culture, sardine fillets began spoilage 72 hours after fermentation, even when 6% NaCl was used. 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 6.0 and after 21 days pH values were 3.8, 3.9 and 4.0 for the experiments with 2, 4 and 6% NaCl respectively. This could have been due to the inhibitory properties of NaCl over the spoilage microorganisms. After 21 days of the fermentation, the levels of lactic acid bacteria (LAB) were 14.5 Log_{10} CFU.g^{-1}.

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

INTRODUCTION

Fish fermentation is widely used for production of fish sauces and fish pastes (Olympia et al., 1992 and Ostergaard et al., 1998). Sensory attributes of the product undergo major changes during fermentation, due to the high level of salt involved (addition of up to 30%) and the high rate of proteolysis which is enhanced by high fermentation temperatures (Twddy et al., 1987). A key factor limiting fish utilization is its extreme perishability. In tropical countries the problem posed by the intrinsic suitability of fish flesh as a medium for microbial growth is further compounded by a high ambient temperature. (Morzel et al., 1997; Virulhakul, 2000 and Gory et al., 2001). The present study examined the use of *Lactobacillus sakei* 2a on the fermentation of sardine fillets. Fish-NaCl-glucose system was used to evaluate the factors that favoured a fast lactic fermentation.

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MATERIALS AND METHODS

Formulation of sardine model system

Fresh sardine were deheaded, degutted, filleted and stored at 30 ^{0}C. The fillets were then divided into 300g portions in sterile flasks, and different concentration of NaCl and glucose were added. Concentrations tested (expressed on total weight of sardine and water mixture) were 2, 4 and 6% NaCl; 2 and 4% glucose. Two independent experiments were performed in duplicate, in which only one parameter (NaCl, glucose) was varied. The system inoculated with the bacterial strain 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 aerobic counts using Plate Count Agar (PCA, Oxoid CM 463). The slow growing LAB on PCA was 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).

Bacterial strain

Lactobacillus sakei 2a, isolated from "lingüiça" (a typical Brazilian meat product), was 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 (*L. sakei* 2a) was characterized by Gram-reaction, morphology (phase-contrast microscopy), growth at 8, 15 and 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. The detection of bacteriocins produced by *L. sakei* 2a 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 was collected by centrifugation at 9.77 x g for 10 min. The supernatant was neutralized to pH 7 with 1 N NaOH and sterilized by filtration (membrane GV Millipore - 0.22-μm). Pour plates 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 μL of the culture supernatant. The plates were incubated anaerobically overnight at 30^{0}C. Inhibition was detected by a zone of clearing around the supernatant well (De Man et al., 1960 and Lewus et al., 1991).

Growth of *L. sakei* 2a

Viable counts of *L. sakei* 2a were determined by plating on MRS agar (Oxoid CM 361). Culture was grown in MRS for 48 h at 30 ^{0}C. 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 the optical density was measured at $OD_{520\ nm}$ using spectrophotometer and the cell suspension was spread-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 et al., 1991).

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 bacterial counts were determined by spread-plating on to Plate Count Agar (PCA) and incubating at 30^{0}C for 48 h. *Enterococcus* was determined by spread plating on to KF agar (Merck 10707), *Staphylococcus aureus* was enumerated by spread-plating on to Baird-Parker Medium (Oxoid CM 275) and coliforms on to Violet Red Bile agar (Oxoid CM 107). Presence of *Salmonella* was assessed following the procedure described in APHA (1992): after enrichment in 0.1% 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 was determined using standard methods (AOAC, 1995).

Total titratable acidity (TTA)
Using the same homogenate prepared for the determination of pH, the TTA was measured by titrating against 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 assumed that all the acid present in the sample was 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 standard methods (AOAC, 1995).

Physical analysis
Each sample (10 g) was blended with 90 mL deionised water. The pH of the homogenized samples was measured according to a direct method, using a pH meter model 240 (Corning, New York, USA). Each measurement represented the mean of three readings. Samples were analyzed on a wet weight (as) basis. Average pH drop between t=21 days and t=0 days were calculated.

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 ($p<0.01$). Data were expressed as means of three replicates and standard errors of means (Statsoft Inc./Computer Program Manual).

RESULTS AND DISCUSSION

Raw material
The proximate 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). *Sardinella* sp. traditionally had fat values from 2% (spring) to 8.6% (autumn) and moisture from 66 to 84% (similar to the marine fish) (Badolato et al., 1994).

Table 1 - 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: average, Dp: standard deviation.

The microbial analysis of the raw sardines is shown in Table 2. The total aerobic counts (PCA) in the raw material ranged from $3.5x10^2$ to $1.1x10^4$ CFU.g^{-1}. *Enterococcus* counts were below Log_{10} $4x10^2$ CFU.g^{-1}. *Staphylococcus aureus* counts were < 10^2 CFU. g^{-1}. *Salmonella* were not detected.

Morphophysiological and biochemical characteristics of *L. sakei* 2a
The strain was characterized as cocci Gram-positive, rods in short chains (1-7 cell units), nonsporing and nonmotile. The colonies showed white to cream in color, circular shape, smooth, brilliant, convex, creamy consistency and diameter between 0.5 and 1.0mm. *L. sakei* 2a was aerotolerant and grew at 7 and 10% NaCl, at pH 3.9 and 8 and 15 ^{0}C but not at 45 ^{0}C. The biochemical characteristics of the *L.sakei* 2a are shown in Table 4.

Optimization of fermentation parameters
Results of the chemical analysis, as expected, showed that acidity increased as fermentation progressed (Table 4). The pH values decreased in all samples. Final pH and % titrable acidity obtained after 21 days of fermentation were pH 4 and 2.55% respectively (2% NaCl and 2% glucose). The results showed that the pH decreased with the glucose content (2% w/w), nevertheless when the concentration of NaCl was increased from 2 to 6%, the fermentation rate

reduced. Using 2% NaCl with 2% glucose resulted in an acidity increase to approximately 1.21% after 7 days and 2.55% after 21 days. Addition of 6% 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 was quite variable in the all-important first 2-day period. This fact could be attributed to differing degrees of freshness of the commercially obtained fish and its effect on the LAB. The total soluble nitrogen content of all the samples increased slightly with increasing fermentation time. A similar observation was made during a study on "Bakasang"; a traditional Indonesian fermented fish sauce (Ijong and Ohta, 1996).

Table 2 - Microbiological characteristics of raw material

	R_1	R_2	R_3	X
Aerobic mesophilic bacteria (CFU. g^{-1})	$3.5x10^2$	$1.1x10^4$	$4.4x10^3$	$5.2x10^3$
Coliforms (MPN. g-1)	< 3	< 3	< 3	< 3
Fecal coliforms (MPN. g-1)	< 3	< 3	< 3	< 3
St. aureus (CFU. g-1)	$< 10^2$	$< 10^2$	$< 10^2$	$< 10^2$
Enterococcus (CFU. g^{-1})	$3.5x10^2$	$3.5x10^2$	$3.5x10^2$	$3.5x10^2$
Salmonella sp.	Absence	Absence	Absence	Absence

$R_{1, 2, 3}$: means of three samples.
X: average

Table 3 - 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 changes of total free amino nitrogen were similar to the changes in total soluble nitrogen (Roig – Sagués and Eerola, 1997). Samples containing 2% NaCl showed higher values of both total soluble nitrogen and free amino nitrogen than those containing NaCl at 6% regardless of the glucose concentration in the samples. The increases of both total soluble nitrogen and total free amino nitrogen during processing of sardine could be attributed to the combined effects of autolysis and microbial degradation of the fish muscle. The evidence for autolysis in this experiment was provided by changes in the ratio of free amino nitrogen to total soluble nitrogen (Fig.1). This ratio increased during the fermentation period, indicating the occurrence of autolysis. Samples produced with 6% NaCl showed less autolysis than samples with 2% NaCl. During the ripening of fermented sardines, biogenic amines may be formed by the action of bacterial descarboxylases of microorganisms originating from raw material (Silva et al., 1998). The inoculation with starter culture significantly inhibited the growth of spoilage microorganisms, maintaining the samples in relatively good microbiological quality throughout the study (Fig.2). In the inoculated samples, LAB counts increased to 5.0 Log_{10} $CFU.g^{-1}$ during the 21 days. The results showed that LAB predominated almost throughout the seven-day fermentation period while spoilage microorganisms were present only at the early stages of the fermentation. The viability of non-acid formers (NAF) 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.

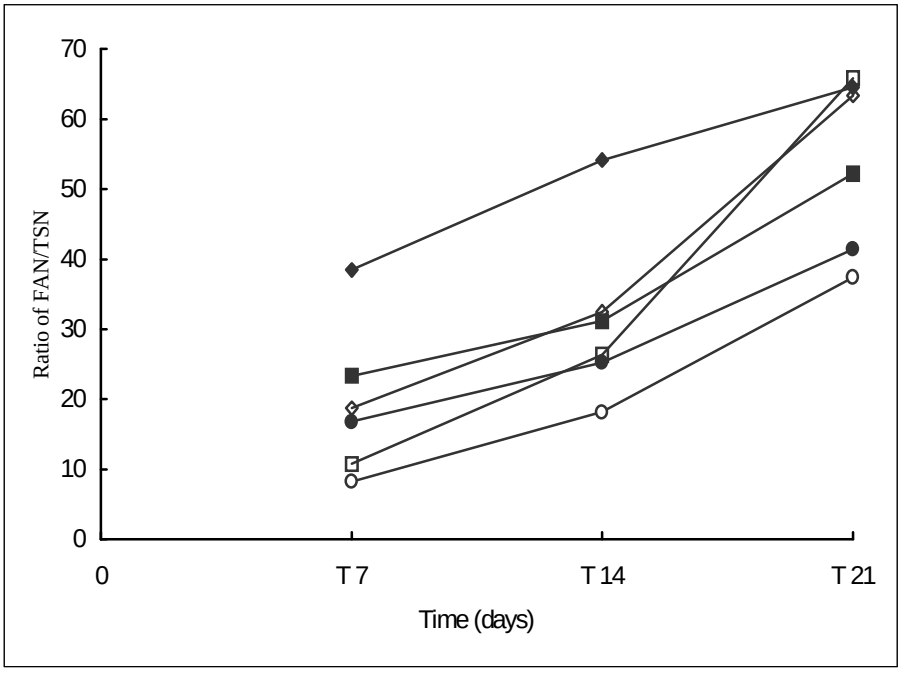

Figure 1 - Ratio of free amino nitrogen and total soluble nitrogen during fermentation of sardine.
Simbols: ♦ 2% glucose-2% NaCl, ▪ 2% glucose-4% NaCl, ● 2% glucose-6% NaCl, ◊ 4% glucose-2% NaCl, □ 4% glucose-4% NaCl, ○ 4% glucose-6% NaCl

Table 4 - Changes in acidity (% lactic acid) during fermentation of sardine

Time	2% glucose			4% glucose		
(days)	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

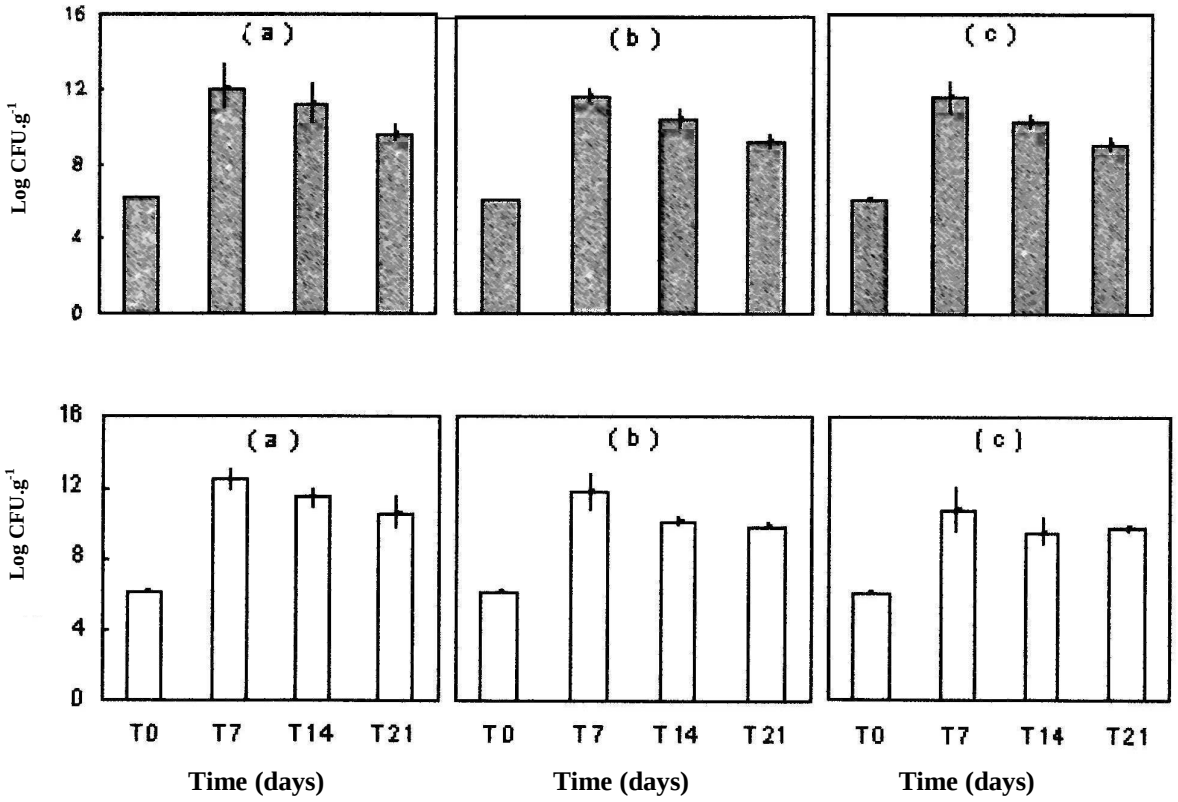

Figure 2 - Changes in plate count agar (PCA) during growth of spoilage microorganisms in fermented sardine. Samples were formulated with combinations of various proportions of NaCl; (A) 2%, (B) 4%, (C) 6% and glucose; 2% , 4% .

CONCLUSIONS

The inoculation with *Lactobacillus sakei* 2a significantly inhibited the growth of spoilage microorganisms, maintaining the samples in relatively good microbiological quality throughout the study. The production of competitive and bacteriocinogenic lactic acid bacteria may well provide an additional hurdle to improve fish preservation by natural means.

The implementation of biopreservation in a certain food system depends on the influence of formula and technology on the performance of bacteriocin-producing cultures as well as the adaptation of the

culture to the specific ecological habitat of the food.

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RESUMO

Lactobacillus sakei 2a é uma cepa produtora de bacteriocinas e, neste estudo, procurou-se observar seus efeitos como cultivo iniciador na fermentação de filés de sardinha (*Sardinella brasiliensis*) em diferentes concentrações de NaCl (2, 4, 6%) e glicose (2, 4%). Com 21 dias (fermentação), os microrganismos deterioradores atingiram 9,7 Log_{10} UFC.g^{-1}, correspondente a 6% NaCl e 4% de glicose. Sem a adição do *starter* e da glicose, a deterioração (filés) iniciou a partir de 72 horas, mesmo quando foi utilizado 6% NaCl. Pouca diferença foi observada na produção de ácido lático quando se adicionou 2 e 4% de glicose, já que a acidez atingiu 1,32 e 1,34%, respectivamente (6% NaCl), os quais apresentaram os melhores resultados. O pH inicial dos filés foi 6 e, ao término de 21 dias, atingiu 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}.

NOTATIONS

AOAC - Association of Official Analytical Chemists
APHA - American Public Healt Association
BHI - Brain Heart Infusion
CFU - Colony Forming Units
FAN - Free Amino Nitrogen
LAB - Lactic Acid Bacteria
MRS - Man, Rogosa and Sharpe
MR-VP - Methyl Red – Voges Proskauer
NaCl - Sodium Chloride
NAF - Non-acid Formers
PCA - Plate Count Agar
TSN - Total Soluble Nitrogen
TTA -Total Titratable Acidity
μm - Micrometer
μL - Microlitre
w/w - weight/weight

REFERENCES

Association of Official Analytical Chemists (1995), *Official Methods of Analysis*. 16th ed. W. Horwitz (Ed.), Washington.

Badolato, E. S. G.; Carvalho, J. B.; Amaral Mello, M. R. P.; Tavares, M.; Campos, N. C.; Aued-Pimentel, S. and Morais, C. (1994), Composição centesimal de ácidos graxos e valor calórico de cinco espécies de peixes marinhos nas diferentes estações do ano. *Rev. Inst. Adolfo Lutz*, **54**, 27-35.

De Man, J. C.; Rogosa, M. and Sharp, M. E. (1960), A medium for the cultivation of lactobacilli. *J. Appl. Bacteriol*., **23**, 130-135.

De Martinis, E. C. P. and Franco, B. D. G. M. (1997), Inhibition of foodborne by bacteriocin-producing *Leuconostoc* spand *Lactobacillus sake* isolated from "lingüiça frescal". *Rev. Microbiol*., **28**, 284-287.

Gonzáles-Fernandez, C.; Santos, E. M.; Jaime, I. and Rovira, J. (1997), Utilización de cultivos iniciadores en la elaboración de chorizo y su influencia en las propriedades sensoriales. *Food Sci. Tech. Int.*, **3**, 31-42.

Gory, L.; Montel, M. C. and Zagorec, M. (2001), Use of green fluorescent protein to monitor *Lactobacillus sakei* in fermented meat products. *Microbiol. Lett*., **194**, 127-133.

Ijong, F. G. and Ohta, Y. (1996), Physicochemical and microbiological changes associated with Bakasang processing - A traditional Indonesian fermented fish sauce. *J. Sci. Food Agric*., **71** : (1), 69-74.

Lewus, C. B.; Kaiser, A. and Montville, T. J. (1991), Inhibition of food-borne bacterial pathogens by bacteriocins from lactic acid bacteria isolated from meat. *Appl. Environ. Microbiol*., **57**, 1683-1688.

Morzel, M.; Fransen, N.G. and Elke, K.A. (1997). Defined starter cultures used for fermentation of salmon fillets. *J. Food Sci.*, **62** : (6), 1214-1218.

Olympia, M.; Ono, H.; Shinmyo, A. and Takano, M. (1992). Lactic acid bacteria in a fermented fishery product, "Burong Bangus". *J. Ferment. Bioengin*., **73** : (3), 193-197.

Ostergaard, A.; Embarek, P. K. B.; Wedell-Neergaard, C.; Huss H. H. and Gram, L. (1998). Characterization of anti-listerial lactic acid bacteria isolated from Thai fermented fish products. *Food Microbiol*., **15** : (2), 223-233.

Twddy, D. R.; Cross, S. J. and Cooke, R. D. (1987). Parameters involved in the production of lactic acid preserved fish-starchy substrate combinations. *Int. J. Food Technol.*, **22**, 115-121.

Virulhakul, P. (2000). The processing Thai fish sauce. *Inf. Int.*, **5** : (2), 49-53.

Zhang, G. and Holley, R. A. (1999). Development and PFGE monitoring of dominance among spoilage lactic acid bacteria from cured meats. *Food Microbiol.*, **16**, 633-644.

Roig-Sagués, A. and Eerola, S. (1997), Biogenic amines in meat inoculated with *Lactobacillus sake* starter strains and an amine-positive lactic acido bacterium. *Food Res. Technol.*, **205** : (3), 227-231.

Silva, C. C. G.; Ponte, J. B. and Dapkevicius, M. L. N. E. (1998), Storage temperature effect on histamine formation in big eye tuna and skipjack. *J. Food Sci.*, **63** : (4), 644-647.

Statsoft Inc. Stat. For Windows [Computer Program Manual] Tulsa, OK : Statsoft Inc.www.statsoft.com.

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