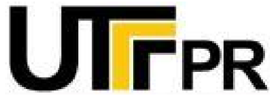

UNIVERSIDADE TECNOLÓGICA FEDERAL DO PARANÁ

Programa de Pós-Graduação em Tecnologia de Alimentos

Identificação de bactérias láticas deteriorantes e avaliação do efeito inibitório de nisina e pediocina em presunto cozido fatiado embalado a vácuo

Daneysa Lahis Kalschne

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IDENTIFICAÇÃO DE BACTÉRIAS LÁTICAS DETERIORANTES E AVALIAÇÃO DO EFEITO INIBITÓRIO DE NISINA E PEDIOCINA EM PRESUNTO COZIDO FATIADO EMBALADO A VÁCUO

Por

DANEYSA LAHIS KALSCHNE

Essa dissertação foi apresentada às 09 horas e 30 minutos do dia 02 de abril de 2013, como requisito parcial para obtenção do Título de Mestre em Tecnologia de Alimentos, Linha de Pesquisa Ciência e Tecnologia de Produtos Alimentícios, Programa de Pós-Graduação em Tecnologia de Alimentos, da Universidade Tecnológica Federal do Paraná. A candidata foi arguida pela Banca Examinadora composta pelos professores abaixo assinados. Após deliberação, a Banca Examinadora considerou o trabalho aprovado.

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GENERAL ABSTRACT

INTRODUCTION AND AIMS – In processed cooked meat products most of the microbiota is inactivated by heat application process which simulate a pasteurization. These products also receive additives in composition that generate inhibitory effect on the certain microorganisms groups growth. With the reduce of pathogenic microorganisms on vacuum packed or modified atmosphere samples, conditions are created for the lactic acid bacteria (LAB) growth, as described Gram positive, non-spore forming, catalase negative, devoid of cytochromes, facultative anaerobic, fastidious, mesophilic, acid tolerant, strictly fermentative metabolism that produce lactic acid as the major end product of fermentation of carbohydrates. The development of these microorganisms causes aroma, flavour and appearance undesirable in meat products. In view of these considerations, the aim of this work was to evaluate the LAB inhibition growth in samples of sliced vacuum-packed cooked ham, by the application of bacteriocins. Specific aims included: the quantification and identification of species of predominate LAB in the samples of sliced vacuum-packed cooked ham; the study of the ingredients of the formulation of cooked ham combined with nisin and pediocin conducted in Man Rogosa and Sharp (MRS) broth using a strategy of experimental design combined with predictive microbiology on evaluation of *Lactobacillus sakei* growth; and a validation of results obtained in MRS broths on food matrix by the elaboration of three formulations involving the use of nisin and a control formulation, which were submitted for microbiological and physico-chemical analysis during the shelf life of the product.

METHODS – Initially, the quantification of total LAB present was performed for 4 weekly batches of sliced vacuum-packed cooked ham at time 1 and 45 days of shelf life storage at 4 °C and 8 °C. Simultaneously was performed phenotypic identification of the genera present; additionally, at the time of 45 days of storage, was carried out a molecular analyze of 16S rRNA gene for identification of the predominant species, whereby selected the specie *Lactobacillus sakei*. Subsequently, a strategy of sequential experimental design was performed to evaluate the *Lactobacillus sakei* cell growth inhibition in MRS broth, applying a Fractional Factorial Design 2^{6-2} with the variables sodium chloride, sodium tripolyphosphate, sodium lactate, sodium erythorbate and the bacteriocins nisin and pediocin. The statistically significant variables in relation to *Lactobacillus sakei* growth inhibition were selected and a Central Composite Rotatable Design (CCRD) 2^2 was performed to optimize the application of nisin and pediocin by reducing the concentration applied to present similar efficiency, considering the high cost of these ingredients. The responses studied in both experimental design were the parameters logarithmic increase of population (A), maximum specific growth rate (μ) and lag phase duration (λ), obtained by the adjustment of Modified Gompertz predictive model. For validation of the results obtained in MRS broth, three test formulations were prepared (with different concentrations of nisin) and a control (without addition of nisin) for monitoring the shelf life of the product by physico-chemical and microbiological analyzes.

MAIN RESULTS – Mean counts around log 1.98 $CFU.g^{-1}$ at time 1 day, log 7.59 $CFU.g^{-1}$ at 4°C, and log 8.25 $CFU.g^{-1}$ at 8°C on time 45 days, was detected for the total LAB. The species predominantly identified on time 45 days were *Lactobacillus curvatus* and *Lactobacillus sakei*. In step of study in MRS broth, the results of the Fractional Factorial Design showed the sodium tripolyphosphate, nisin and pediocin like variables with statistically significant effects in *Lactobacillus sakei* growth inhibition. In the Central Composite Rotatable Design, nisin was the variable that had a significant effect in the *Lactobacillus sakei* growth inhibition, indicating that it may be reduced to smaller amounts than indicated by Brazilian law (12.5 mg/Kg) with similar effect on inhibition. The validation of

MRS broth studies for applying the reduced amounts of nisin (indicated by the CCRD) in the food matrix, showed that the minimum amount applied (0.001%) presented a similar effect on LAB growth inhibition related to the maximum quantity applied (0.013%); being efficient in both cases when compared to the control sample.

DISCUSSION AND CONCLUSION – From the results obtained, was possible to conclude that the LAB comprise the microbiota of sliced vacuum-packed cooked ham and the *Lactobacillus curvatus* and *Lactobacillus sakei* were the major species that cause deterioration on the samples stored at 4 °C and 8 °C for 45 days. Total LAB counts after 45 days was significantly affected by storage temperature, because the counts were higher for samples stored at 8 °C compared to the samples stored at 4 °C. Among the ingredients added in the formulation of cooked ham, sodium tripolyphosphate showed significant effect in *Lactobacillus sakei* inhibition in MRS broth. Similarly, nisin and pediocin showed significant effects on microorganism reducing, evaluated by applying a Fractional Factorial Design. The Central Composite Rotatable Design involving the variables nisin and pediocin with redefined ranges of concentrations showed that nisin presented significant effect on the *Lactobacillus sakei* growth inhibition, when applied in smaller amounts in comparison to the stipulated by Brazilian law. The results obtained in Central Composite Rotatable Design was validated by applying the optimized quantities of nisin in food matrix, that even when applying minimal concentration (0.001%) showed a similar effect to that obtained by applying the maximum concentration (0.013%). The count of LAB and mesophilic aerobic bacteria (MAB) total were lower for the samples containing nisin than control sample, approximately 2 to 3 log cycles for LAB, and 3 or 4 log cycles for MAB, while the pH was maintained until the end of shelf life (60 days); at the same time the pH of the control sample had a significant decrease. Altogether the sliced vacuum-packed cooked ham is a favorable product to the LAB spoilage development, principally to the species *Lactobacillus curvatus* and *Lactobacillus sakei*, and that among the studied bacteriocins against the inhibition of LAB, nisin showed effect on the *Lactobacillus sakei* growth inhibition by studies in MRS broth, and also on the LAB spoilage growth inhibition in sliced vacuum-packed cooked ham, even in smaller quantities than indicated by Brazilian law.

RESUMO GERAL

INTRODUÇÃO E OBJETIVOS – Nos produtos cárneos industrializados cozidos a maior parte da microbiota é inativada pela aplicação de calor em processo que simula uma pasteurização. Esses produtos também recebem aditivos em sua composição que geram efeito inibidor no crescimento de determinados grupos de microrganismos. Com a microbiota patogênica reduzida a níveis aceitáveis, em amostras embaladas a vácuo ou em atmosfera modificada, criam-se condições para o crescimento de bactérias ácido láticas (BAL), descritas como microrganismos Gram positivos, não formadores de esporos, catalase negativa, desprovidos de citocromos, anaeróbios facultativos, fastidiosos, mesofílicos, ácidos tolerante, com metabolismo estritamente fermentativo, que produzem ácido láctico como o principal produto final da fermentação de carboidratos. O desenvolvimento desses microrganismos gera aroma, sabor e aspecto indesejável nos produtos cárneos. Tendo em vista estas considerações, o objetivo geral desse trabalho foi avaliar a possibilidade da inibição do crescimento de BAL em amostras de presunto fatiado embalado a vácuo, pela aplicação de bacteriocinas. Os objetivos específicos incluíram: a quantificação e identificação das espécies de BAL predominantes nas amostras de presunto cozido fatiado embalado a vácuo; o estudo dos ingredientes que compõem a formulação do presunto cozido combinados com nisina e pediocina, realizados em caldo *Man Rogosa* e *Sharp* (MRS) pela aplicação da estratégia de planejamento experimental aliada à microbiologia preditiva na avaliação do crescimento do *Lactobacillus sakei*; a validação dos resultados obtidos nos caldos MRS na matriz alimentar a partir da elaboração de três formulações envolvendo a utilização da nisina e uma formulação controle, submetidas a análises físico-químicas e microbiológicas durante a vida de prateleira do produto.

MÉTODOS – Inicialmente foi realizada a quantificação das BAL totais presentes, para 4 lotes semanais de presunto cozido fatiado embalado a vácuo, nos tempos de 1 e 45 dias de armazenamento a 4 °C e 8 °C. Paralelamente foi realizada a identificação fenotípica dos gêneros presentes; adicionalmente, no tempo de 45 dias foram realizadas análises moleculares do gene 16S rRNA para a identificação das espécies predominantes, por meio da qual selecionou-se a espécie *Lactobacillus sakei*. Posteriormente, utilizou-se uma estratégia sequencial de planejamento experimental, que objetivou estudar a inibição do crescimento celular da espécie *Lactobacillus sakei* em caldo MRS, aplicando-se um Planejamento Fatorial Fracionário 2^{6-2} com as variáveis cloreto de sódio, tripolifosfato de sódio, lactato de sódio, eritorbato de sódio e as bacteriocinas nisina e pediocina. Adicionalmente foi realizado um Delineamento Composto Central Rotacional (DCCR) 2^2 objetivando otimizar a aplicação de nisina e pediocina por meio da redução das concentrações aplicadas que apresentassem eficiência semelhante, considerando os altos custos desses ingredientes. As respostas estudadas em ambos delineamentos experimentais foram os parâmetros preditos pelos ajuste das curvas de crescimento ao Modelo de Gompertz Modificado, a citar a população máxima atingida (*A*), velocidade específica máxima de crescimento (μ) e a duração da fase *lag* (λ). Para a validação dos resultados obtidos na etapa de estudo em caldo MRS, foram elaboradas três formulações teste (com diferentes concentrações de nisina) e uma controle (sem adição de nisina) para o acompanhamento da vida de prateleira do produto por meio de análises físico-químicas e microbiológicas.

PRINCIPAIS RESULTADOS – Foram observadas contagens médias de 1,98 log UFC.g^{-1} no tempo 1 dia; 7,59 log UFC.g^{-1} a 4 °C e 8,25 log UFC.g^{-1} a 8 °C no tempo 45 dias para a contagem total de BAL. As espécies predominantemente identificadas no tempo de 45 dias foram o *Lactobacillus curvatus* e o *Lactobacillus sakei*. Na etapa de estudo em caldo MRS, os resultados do Planejamento Fatorial Fracionário evidenciaram que o tripolifosfato de sódio, a nisina e a pediocina, foram as variáveis com efeito estatisticamente significativo (p ≤

0,05) na inibição do crescimento do *Lactobacillus sakei*. No Delineamento Composto Central Rotacional, a nisina foi a variável que apresentou efeito significativo na inibição do crescimento do *Lactobacillus sakei*, indicando a possibilidade de redução na concentração aplicada para níveis inferiores ao indicado pela legislação brasileira (12,5 mg/Kg), com efeito semelhante na inibição. A validação dos estudos em caldo MRS pela aplicação das quantidades reduzidas de nisina (indicadas pelo DCCR) na matriz alimentar, mostrou que a quantidade mínima aplicada (0,001%) apresentou um efeito semelhante na inibição das BAL em relação à quantidade máxima aplicada (0,013%); sendo em ambos os casos eficiente quando comparada à amostra controle.

DISCUSSÃO E CONCLUSÃO – De acordo com os resultados obtidos, verificou-se que os principais gêneros de BAL envolvidos na deterioração das amostras de presunto cozido fatiado embalado a vácuo, armazenadas durante 45 dias a 4 °C e 8 °C, foram o *Lactobacillus curvatus* e *Lactobacillus sakei*. A contagem de BAL totais após 45 dias mostrou-se significativamente influenciada pela temperatura de armazenamento, pois as contagens foram maiores para as amostras armazenadas a 8 °C quando comparadas às armazenadas a 4 °C. Dentre os ingredientes comumente adicionados na formulação do presunto cozido, o tripolifosfato de sódio mostrou efeito significativo na inibição do *Lactobacillus sakei* nos estudos em caldo MRS. De forma semelhante, a nisina e a pediocina mostraram efeito significativo na redução das contagens do microrganismo estudado quando avaliados pela aplicação de um Planejamento Fatorial Fracionário. No Delineamento Composto Central Rotacional envolvendo as variáveis nisina e pediocina com faixas de estudos redefinidas, a nisina apresentou efeito significativo na inibição do *Lactobacillus sakei*, quando aplicada em concentrações inferiores ao estipulado pela legislação brasileira. Os resultados obtidos no Delineamento Composto Central Rotacional foram validados pela aplicação das quantidades otimizadas de nisina na matriz alimentar, que mesmo na concentração mínima (0,001%) mostrou um efeito similar ao obtido pela aplicação da concentração máxima (0,013%). A contagem de BAL e bactérias aeróbias mesófilas (BAM) totais mostrou-se inferior para as amostras contendo nisina quando comparada com a amostra controle, em torno de 2 a 3 ciclos logarítmicos para as BAL, e 3 ou 4 ciclos logarítmicos para as BAM; em relação ao pH, o mesmo manteve-se praticamente inalterado até o final da vida de prateleira (60 dias), enquanto o pH da amostra controle diminuiu de forma significativa. De um modo geral constatou-se que o presunto cozido fatiado embalado a vácuo é um produto com condições favoráveis ao desenvolvimento de BAL deteriorantes, a citar as espécies *Lactobacillus curvatus* e *Lactobacillus sakei*, e que dentre as bacteriocinas estudadas, a nisina apresentou efeito na inibição do *Lactobacillus sakei* nos estudos em caldo MRS e também na inibição das BAL deteriorantes totais no presunto cozido fatiado embalado a vácuo, mesmo em concentrações inferiores ao indicado pela legislação brasileira.

APRESENTAÇÃO

Esta dissertação é composta por 2 artigos científicos.

1. Daneysa Lahis Kalschne; Rute Womer; Ademir Mattana; Cleonice Mendes Pereira Sarmento; Luciane Maria Colla; Eliane Colla. Characterization of the spoilage lactic acid bacteria in "sliced vacuum-packed cooked ham". Brazilian Journal of Microbiology.

2. Daneysa Lahis Kalschne; Simone Geitenes; Marilei Veit; Cleonice Mendes Pereira Sarmento; Eliane Colla. Growth inhibition of lactic acid bacteria in cooked ham by bacteriocins. Food and Bioprocess Technology.

Characterization of the spoilage lactic acid bacteria in "sliced vacuum-packed cooked ham"

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Abstract

The lactic acid bacteria are involved with food fermentation and in such cases with food spoilage. Considering the need to reduce the lactic acid bacteria growth in meat products, the aim of this work was to enumerated and investigated the lactic acid bacteria present on sliced vacuum-packed cooked ham stored at 4 °C and 8 °C during 45 days for the predominant genera by phenotypic, and specie by molecular techniques. The quantification showed that the lactic acid bacteria were present from the first day with mean count of 1.98 log CFU.g^{-1} for the four batches analyzed. The lactic acid bacteria grew rapidly on the samples, and plate counts around 7.59 log CFU.g^{-1} and 8.25 log CFU.g^{-1} were detected after 45 days of storage at 4 °C and 8 °C, respectively; storage temperatures studied showed significant influence on the microorganism in study growth. The predominant lactic acid bacteria associated with the spoilage samples at one day of storage includes *Lactobacillus* sp., the phenotypic overlap *Leuconostoc/Weissella* sp. and *Enterococcus* sp. At 45 days of storage at 4 °C and 8 °C the mainly specie was *Lactobacillus curvatus*, following by *Lactobacillus sakei* and *Leuconostoc mesentereoides*; the *Enterococcus* sp. was not present in the samples.

Key-words: *Lactobacillus curvatus, Lactobacillus sakei, Leuconostoc mesenteroides*, spoilage.

INTRODUCTION

Cooked meat products are economically important chilling products with a high consumption in world. The shelf-life of cooked and sliced meat products, like cooked ham, is limited mainly because of microbiological safety and spoilage issues (43).

The meat and meat products are highly perishable, so special care should be applied during all operations, to minimize deterioration and extend the shelf life. The meat shelf life is strictly depending on the number and type of bacteria initially present and their further growth in the ecological conditions applied during storage, particularly temperature, pH and gaseous atmosphere (35). The exclusion or reduction of oxygen in modified atmosphere packaging products using a barrier film prolongs the shelf life of meat by reducing oxidative rancidity and microbial growth (3). Whereas the combination of microaerophilic conditions, presence of sodium chloride (NaCl), sodium nitrite $(NaNO_2)$ and a reduced water activity inhibits growth of Gram-negative spoilage microbiota, favors lactic acid bacteria (LAB) growth (2, 3, 19).

Anyway, deterioration caused by LAB is primarily due to production of metabolites that cause unwanted changes in appearance, texture and flavor of the substrate (28).

As a result of LAB activities, acid off-flavors and off-odors, decrease in pH, milky exudates, gas production, swelling of the pack, discoloration and/or greenish color can be observed (3, 19, 21, 46).

LAB contribute actively in the spoilage of sliced cooked ham, where they have been identified as the main microbial group involved in the spoilage, especially in vacuum and modified atmosphere packaging (19, 23, 39, 43, 46).

Originally, the LAB group included four kinds of great importance in food: *Lactobacillus, Leuconostoc, Pediococcus* and *Streptococcus* (16, 38). Currently, this group consists on 15 genera: *Aerococcus*, *Atopobium*, *Bifidobacterium*, *Brochothrix*, *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus* and *Weissella* (21, 24, 38).

New tools for classification and identification of LAB are currently replacing and/or complementing the traditional phenotype-based methodologies. The most promising for routine use are 16S rRNA gene sequencing, PCR-based fingerprinting techniques and soluble protein patterns (4). Although the

classical approach to bacterial identification based on morphological, physiological and biochemical features provides reasonable results and is easy to perform, in general these techniques are not always reliable for the identification of LAB (42).

According to this, the aim of this work was quantify and identify the predominant LAB from sliced vacuum-packed cooked ham at time 1 day and 45 days at 4 °C and 8 °C. The isolates were initially quantified by plates count, following classified according phenotypic and molecular characteristics.

MATERIAL AND METHODS

Collection and storage of samples

The samples of sliced vacuum-packed cooked ham were prepared in a slaughterhouse company with conventional techniques and good manufacturing practices. The production was performed weekly during four weeks. A total of nine packages with 200 g of samples were collected for each batch. A microbiological analysis was performed in triplicate (A, B and C) on the time 1 day after the slicing and packaging of the product, and triplicates were performed on the time 45 days of shelf life, one with storage at 4 °C and another at 8 °C. In total, 36 samples were collected. The storage of the samples with 45 days of shelf life occurred in refrigerating chamber (Totaline, Rio de Janeiro, Brazil) and the mean plate counts obtained for samples storage during 45 days were analyzed statistically using the Tukey test ($p \leq 0.05$).

Isolation and enumeration of total LAB

Quantification of LAB in the samples was realized by Man Rogosa and Sharp (MRS) agar plates (Himedia, Mumbai, India). At each sampling date, three packets of sliced cooked ham per batch were used for microbiological analysis. For each package, 25 g portion was aseptically weighed and added 225 mL of sterile peptone water (0.1%) (MicroMed, produced by Isofar, Rio de Janeiro, Brazil) in a sterile plastic bag and blended with stomacher for 2 minutes. Aliquots of 1 mL of the dilutions of the samples (up to 10^{-7}) were inoculated and mixed with MRS agar, and poured an overlayer of this. The inverted plates were incubated at 30 °C for 48 hours (Novaética, model 403-3D, São Paulo, Brazil) in normal atmosphere (35, 38).

Phenotypic analysis

For the LAB identification by phenotypic characteristics, information from various researchers were collected and listed in the Table 1, and used as a basis for this differentiation. The group of LAB is relatively heterogeneous; some genera have differences in molecular levels that express single phenotypic characteristics.

Table 1. Key phenotypic characteristics of LAB.

Genera	Morphology	CO_2 from glucose	Lactic acid isomer	Growth at 10ºC	Growth at 45ºC	Growth in 18% NaCl	Growth at pH 4.4	Growth at pH 4.5	Catalase activity
Aerococcus	C[a] (4) C[a] (20) C[a] (44)	- (4) - (20) - (44)	L (4) L (20) L (44)	+ (4) + (20) + (44)	- (4) - (20) - (44)	- (4) - (20) - (44)	- (4) - (20) - (44)		- (1)
Atopobium	C/R (12) C/R (26)								- (12)
Bifidobacterium	R (5) R (8) R (29)	+[b] (8) +[b] (29)	L (29)	- (8) - (29)	+ (8) + (29)		- (8) - (29)	- (8) - (29)	- (8) - (29)
Brochothrix	C/R[d] (22) R[d] (34) R (41)	- (32) - (42)	L (32) L (40) L (41)		-[f] (40) -[f] (41)				+ (40) + (41) + (42)
Carnobacterium	R (4) R (38) R (44)	+[e] (4) -[e] (6) + (38)	L (4) L (20) L (44)	+ (4) + (38) + (44)	- (20) - (38) - (44)	- (4) - (20) - (44)		- (38)	- (10) - (38)
Enterococcus	C (4) C (33) C (38)	- (4) - (33) - (38)	L (4) L (6) L (33)	+ (4) + (20) + (33)	+ (4) + (20) + (37)	- (4) - (20) - (44)	+ (4) + (20) + (44)		- (33) - (38)
Lactobacillus	R (4) R (38) R (44)	± (4) ± (37) ± (38)	D, L, DL (4) D, L, DL (20) D, L, DL (44)	± (4) ± (20) ± (44)	± (4) ± (38) ± (44)	- (4) - (20) - (44)	± (4) ± (20) ± (44)	+ (38)	- (8) - (33) - (38)
Lactococcus	C (37) C (38) C (44)	- (37) - (38) - (44)	L (33) L (42) L (44)	+ (20) + (37) + (44)	- (20) - (37) - (44)	- (20) - (44)	± (20) ± (44)		- (8) - (33) - (38)
Leuconostoc	C (4) C (38) C (44)	+ (4) + (38) + (44)	D (4) D (37) D (44)	+ (4) + (38) + (44)	- (4) ± (38) - (44)	- (4) - (20) - (44)	± (4) ± (20) ± (44)		- (6) - (13) - (38)
Oenococcus	C (4) C (20) C (38)	+ (4) + (38) + (44)	D (4) D (6) D (44)	+ (4) + (38) + (44)	- (4) ± (38) - (44)	- (4) - (20) - (44)	± (4) ± (20) ± (44)		- (6) - (30)
Pediococcus	C[a] (37) C[a] (38) C[a] (44)	- (4) - (20) - (44)	L, DL (4) L, DL (6) L, DL (44)	± (4) ± (38) ± (44)	± (4) ± (33) ± (44)	- (4) - (20) - (44)	+ (4) + (20) + (44)		- (8) - (33) - (38)
Streptococcus	C (4) C (20) C (38)	- (4) - (38) - (44)	L (4) L (20) L (44)	- (4) - (38) - (44)	± (4) ± (38) ± (44)	- (4) - (20) - (44)	- (4) - (20) - (44)		- (8) - (38)
Tetragenococcus	C[a] (4) C[a] (38) C[a] (44)	- (4) - (20) - (38)	L (4) L (20)	+ (4) + (20) + (44)	- (4) - (20) - (44)	+ (4) + (20) + (44)	- (4) - (20) -[c] (38)	-[c] (38)	- (38)
Vagococcus	C (4) C (38) C (44)	- (4) - (20) - (38)	L (4) L (20)	+ (4) + (38) + (44)	- (4) - (38) - (44)	- (4) - (20) - (44)	± (4) ± (20)		- (38)
Weissella	C/R (4) C/R (13) C/R (20)	+ (4) + (13) + (44)	D, DL (4) D, DL (20) D, DL (44)	+ (4) + (20) + (44)	- (4) - (20) - (44)	- (4) - (20) - (44)	± (4) ± (20) ± (44)		- (13) - (33) - (38)

C: cocci; R: rod-shape; L, D and LD: optical isomers; +: positive; -: negative; a: cocci may also be tetrad

formation; b: differ from bacterial homo and heterofermentatives on fermentation of glucose, which occurs via fructose-6-phosphate; c: grow not occur at pH 5.0 or lower; d: in older cultures the rod may give rise to coccoid forms, which develop into rod forms when subcultured onto a suitable medium; e: small amounts of CO_2 from glucose can be produced; f: growth not occur at 37 ºC.

Among the 15 genera listed in Table 1, this study involved the identification of 12 genera. The genera *Atopobium*, *Bifidobacterium* and *Oenococcus* were not included in this study because their peculiar characteristics. The genus *Atopobium* is related with human's infections, especially women vaginal infections and is uncommon in food microbiology (25). The genus *Bifidobacterium* is grouped with the LAB and shares a few characteristics however is phylogenetically unrelated (4, 16). This genus differs from LAB on the mode of glucose fermentation, which occurs via fructose-6-phosphate, because the presence of the enzyme fructose-6-phosphate phosphoketolase (29). The genus *Oenococcus*, which consists an only one specie, *Oenococcus oeni*, is easily distinguished from other genera because their glycosylated derivative of pantothenic acid requirement, found in tomato juice (38). The Flowchart showed in Figure 1, adapted from Schillinger and Lücke (37), was used for the sequential analysis of phenotypic characteristics of the 12 LAB genera studied in this work.

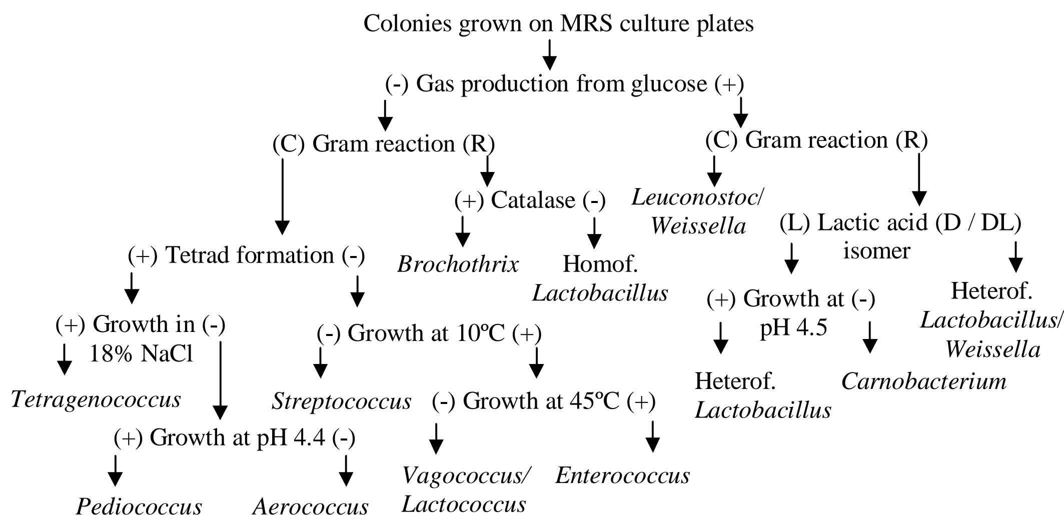

Figure 1. Flowchart for identification of LAB genera by phenotypic characteristics. C: cocci; R: rods; (-): negative; (+): positive; L, D and DL: optical isomers. The flowchart was adapted from Schillinger and Lücke (37).

Triplicate plates with typical LAB growth colonies were randomly selected and five colonies of each were tested by phenotypic characteristics. The proposed flowchart (Fig. 1) is able to identify the most of 12 genera listed, but there are overlaps between the genera and exceptions to rules can be found, especially when dealing with the genera *Lactobacillus* (heterofermentatives), *Leuconostoc* and *Weissella,* but also with *Lactococcus* and *Vagococcus*. Because this, molecular analyses were performed in parallel to identify the genus and species at time 45 days.

Phenotypic characteristics were tested as follows: gas production from glucose tested in MRS broth (Himedia, Mumbai, India) supplemented with 5% of glucose and an inverted Durham tube at 30 ºC for 48 hours (33, 45); Gram reaction (31, 38); catalase activity (27, 31); growth at 10 ºC for 7 days, and 45 ºC for 48 hours, tested in MRS broth with 0.02 g/L of bromocresol purple (16, 33, 45); growth in 18% NaCl added bromocresol purple 0.02 g/L at 30 ºC for 48 hours (16, 45); growth at pH 4.4 e 4.5 in MRS broth adjusted to correct pH by the addition 1 M HCl (37) at 30 °C for 48 hours; determination of lactic acid isomer formed (L, D or DL) performed using an enzymatic-colorimetric kit (R-Biopharm, 11112821035, Darmstadt, Germany). The reagents used were of analytical reagent grade.

Molecular analysis

The investigation of the spoilage bacteria of cooked cured meat products has been carried out, mostly depended on traditional microbiological methods, based on plate counts, isolation and biochemical identification (19). Phenotypic characterization based on sugar fermentation pattern and conventional phenotypic properties may not always provide sufficient basis for the reliable identification of LAB, although it is a useful tool for presumptive classification (36).

The differentiation between the genera *Leuconostoc* and *Weissella*, and heterofermentative *Lactobacillus* and *Weissella* could not possible by means of phenotypic methods. After Collins *et al.* (13) subdivided the genera *Leuconostoc* into a new genus called *Weissella*, the phenotypic methodologies can only describe the *Leuconostoc* family, that include the genera *Leuconostoc*, *Oenococcus* and *Weissella* (9). Similarly overlaps occurred with the genera *Lactococcus* and *Vagococcus*. The genera *Vagococcus* was created by Collins *et al.* (11) to accommodate new specie with all the features of *Lactococcus* but also peritrichous flagella (38). In such cases the molecular

analysis allows identification of the genus and even species level. Because this, identification of the LAB species selected was performed by DNA sequences of the 16S rRNA. The samples tested for the production of gas from glucose were streaked on MRS agar slants, incubated at 30 °C for 48 hours into anaerobic jars (Permution, Paraná, Brazil). LAB DNA was extracted from the colonies grown on the MRS slants. The nucleotide sequences were compared with the nucleotide sequence of GenBank database of National Center for Biotechnology Information (NCBI).

RESULTS

Isolation and enumeration of total LAB

The results of plate counts on MRS agar showed that the LAB was present in sliced vacuum-packed cooked ham. The typical colonies grown on MRS agar plate were white, circular and slightly convex, with small diameter (0.5 - 2.0 mm). They are white because do not produce pigments and are small, because the fermentation of carbohydrates does not release much energy (28). In most ways, they use up the sugars and produce too much acid, which inhibits their growth before the colonies become large. The results of the LAB counting for the 4 batches analyzed are detailed in Table 2.

Table 2. Results of plate count for LAB at the time 1 day and 45 days of storage at 4 °C and 8 °C.

Batch	Time 1 day (log $CFU.g^{-1}$)	Time 45 days (log $CFU.g^{-1}$) - 4 ºC	Time 45 days (log $CFU.g^{-1}$) - 8 ºC
1-A	1.48	8.00	8.59
1-B	2.15	7.85	8.54
1-C	1.70	8.00	8.63
2-A	1.70	7.45	8.20
2-B	1.30	7.23	8.04
2-C	2.00	7.41	8.11
3-A	2.00	4.85	7.67
3-B	2.11	7.72	8.20
3-C	2.11	7.30	7.34
4-A	2.46	8.51	8.40
4-B	2.48	8.49	8.63
4-C	2.30	8.32	8.57
Mean *	1.98 ± 0.36	7.59 ± 0.90^{a}	8.25 ± 0.38^{b}

A, B and C corresponds a triplicate; * Means with different letters are statistically different ($p \leq 0.05$) by the Tukey test.

Phenotypic analysis

Through the phenotypic characterization of the LAB present in samples of sliced vacuum-packed cooked ham, it was verified that the microbiota present were heterogeneous. Of the 15 colonies subcultured to each batch, four distinct genera were observed at time 1 day of storage: *Leuconostoc/Weissella* sp., *Enterococcus* sp. and homofermentative *Lactobacillus* sp. (Table 3).

Table 3. Results of phenotypic characteristics of LAB isolated in samples of sliced vacuum-packed cooked ham and related genera identified on the time 1 day.

Batch	Number of colonies tested	CO_2	Morphology	Catalase activity	Growth at 10 ºC	Growth at 45 ºC	Genera identified
1-A*	3	+	Cocci	NT	NT	NT	*Leuconostoc/Weissella* sp.
1-B	5	-	Rods	-	NT	NT	Homofermentative *Lactobacillus* sp.
1-C	5	+	Cocci	NT	NT	NT	*Leuconostoc/Weissella* sp.
2-A	2	-	Cocci	NT	+	+	*Enterococcus* sp.
2-A	3	+	Cocci	NT	NT	NT	*Leuconostoc/Weissella* sp.
2-B*	1	-	Rods	-	NT	NT	Homofermentative *Lactobacillus* sp.
2-B	1	+	Cocci	NT	NT	NT	*Leuconostoc/Weissella* sp.
2-C	5	+	Cocci	NT	NT	NT	*Leuconostoc/Weissella* sp.
3-A	4	-	Cocci	NT	+	+	*Enterococcus* sp.
3-A	1	+	Cocci	NT	NT	NT	*Leuconostoc/Weissella* sp.
3-B	4	-	Cocci	NT	+	+	*Enterococcus* sp.
3-B	1	+	Cocci	NT	NT	NT	*Leuconostoc/Weissella* sp.
3-C	1	+	Cocci	NT	NT	NT	*Leuconostoc/Weissella* sp.
3-C	1	-	Rods	-	NT	NT	Homofermentative *Lactobacillus* sp.
3-C	3	-	Cocci	NT	+	+	*Enterococcus* sp.
4-A	2	-	Rods	-	NT	NT	Homofermentative *Lactobacillus* sp.
4-A	3	+	Cocci	NT	NT	NT	*Leuconostoc/Weissella* sp.
4-B	5	+	Cocci	NT	NT	NT	*Leuconostoc/Weissella* sp.
4-C	5	+	Cocci	NT	NT	NT	*Leuconostoc/Weissella* sp.

A, B and C corresponds a triplicate; +: positive; -: negative; NT: not tested; * The number of colonies grown in plates was lower than 5.

By the results shown in Table 3, it can be verified that at 1 day of storage, the *Leuconostoc/Weissella* sp. showed to be the predominant genera in batch 1, 2 and 4, and in the batch 3, the *Enterococcus* sp. was the predominant LAB.

However, with the advance in storage time, the homofermentative *Lactobacillus* sp. showed greater

abilities to develop, being the predominant genera at 45 days of storage at 4 °C and 8 ºC in batches 1 and 4, and *Leuconostoc/Weissella* sp. in batches 2 and 3 (Table 4).

Table 4. Results of phenotypic characteristics of LAB isolated in samples of sliced vacuum-packed cooked ham and related genera identified on the time 45 day.

Batch	Number of colonies tested	CO_2	Morphology	Catalase activity	Genera identified
			Time 45 days at 4 °C		
1-A	5	+	Cocci	NT	*Leuconostoc/Weissella* sp.
1-B	5	+	Cocci	NT	*Leuconostoc/Weissella* sp.
1-C	1	-	Rods	-	Homofermentative *Lactobacillus* sp.
1-C	4	+	Cocci	NT	*Leuconostoc/Weissella* sp.
2-A	5	-	Rods	-	Homofermentative *Lactobacillus* sp.
2-B	5	-	Rods	-	Homofermentative *Lactobacillus* sp.
2-C	5	-	Rods	-	Homofermentative *Lactobacillus* sp.
3-A	5	-	Rods	-	Homofermentative *Lactobacillus* sp.
3-B	5	-	Rods	-	Homofermentative *Lactobacillus* sp.
3-C	5	-	Rods	-	Homofermentative *Lactobacillus* sp.
4-A	5	-	Rods	-	Homofermentative *Lactobacillus* sp.
4-B	4	-	Rods	-	Homofermentative *Lactobacillus* sp.
4-B	1	+	Cocci	NT	*Leuconostoc/Weissella* sp.
4-C	4	-	Rods	-	Homofermentative *Lactobacillus* sp.
4-C	1	+	Cocci	NT	*Leuconostoc/Weissella* sp.
			Time 45 days at 8 °C		
1-A	4	+	Cocci	NT	*Leuconostoc/Weissella* sp.
1-A	1	-	Rods	-	Homofermentative *Lactobacillus* sp.
1-B	5	+	Cocci	NT	*Leuconostoc/Weissella* sp.
1-C	4	+	Cocci	NT	*Leuconostoc/Weissella* sp.
1-C	1	-	Rods	-	Homofermentative *Lactobacillus* sp.
2-A	5	-	Rods	-	Homofermentative *Lactobacillus* sp.
2-B	5	-	Rods	-	Homofermentative *Lactobacillus* sp.
2-C	5	-	Rods	-	Homofermentative *Lactobacillus* sp.
3-A	5	-	Rods	-	Homofermentative *Lactobacillus* sp.
3-B	5	-	Rods	-	Homofermentative *Lactobacillus* sp.
3-C	5	-	Rods	-	Homofermentative *Lactobacillus* sp.
4-A	4	+	Cocci	NT	*Leuconostoc/Weissella* sp.
4-A	1	-	Rods	-	Homofermentative *Lactobacillus* sp.
4-B	4	-	Rods	-	Homofermentative *Lactobacillus* sp.
4-B	1	+	Cocci	NT	*Leuconostoc/Weissella* sp.
4-C	1	-	Rods	-	Homofermentative *Lactobacillus* sp.
4-C	4	+	Cocci	NT	*Leuconostoc/Weissella* sp.

A, B and C corresponds a triplicate; +: positive; -: negative; NT: not tested.

It was found that the batches 1 and 4 showed loss of vacuum-packaging, and the predominantly genera

belong to *Leuconostoc/Weissella* sp. For batches 2 and 3 that did not present loss of vacuum, homofermentative *Lactobacillus* sp. were predominantly identified. Both genera showed the production of milky exudates and after 45 days of storage, this change shows economical importance in the spoilage of vacuum-packaged sliced cooked ham, mainly due to changes that cause in the substrate.

Molecular analysis

The molecular analysis showed that the predominant *Lactobacillus* species present in sliced cooked ham samples at 45 days of storage, at 4 °C and 8 °C, were *Lactobacillus curvatus* (strain CTSPL4, access number EU855223 and strain MFPA15D06-03, access number JF756088) corresponding to 67% of the total *Lactobacillus* genus at 4 °C and 8 °C, followed by 33% of *Lactobacillus sakei* (strain Moo1, access number GU591801 and strain HS-1, access number AB124845). The genus *Leuconostoc/Weissella* were the second predominant genus of LAB, in this case the main specie was *Leuconostoc mesenteroides* (strain NCBF 529, access number AB023244).

DISCUSSION

The mean counts of the initial population of LAB present in the samples (1.98 log CFU.g^{-1}) were significant influenced by storage temperature; growth values around 7.59 log CFU.g^{-1} at 4 °C and 8.25 log CFU.g^{-1} at 8 °C were detected (Table 2) whose values were statistically different ($p \leq 0.05$, by the Tukey test). The temperature of 8 °C is closer to the optimum growth temperature of LAB, who are mesophilic. The growth of LAB reaches up to approximately 8 or 9 log CFU.g^{-1}, since at this point, their growth is eventually inhibited by the amount of acid produced or lack of nutrients, mainly in cooked ham, which has a lower carbohydrate concentration in the formulation. Hu et al. (2009) related that samples of sliced cooked ham stored at 4 °C, at times 0, 3, 7, 15, 25 and 35 days, showed respective LAB plates counts of 0.00; 6.04; 7.18; 8.57; 8.59; and 8.52 log CFU.g^{-1}, which demonstrate the rapid development of LAB (19). The LAB dominates the total viable microbiota in sliced cooked ham after a short storage time, independent of the storage temperature (assessed from 2 to 15 ºC), and

when plate counts was around 7 log $CFU.g^{-1}$, the product reaches the end of shelf life, which was indicated by changes in sensory quality and pH decrease (23). Sliced cooked ham at time 0, 30 and 90 days of storage at 4 ºC showed plate counts around 4.65, 8.72 and 7.27 log $CFU.g^{-1}$, respectively (17). Similar results were found after 45 days of storage for the quantification of LAB on four batches of sliced vacuum-packaged cooked ham.

Bacteria associated with the spoilage of refrigerated meat products causing defects such as off-odors, off-flavors, discoloration, gas production, slime production and decrease in pH, consist of *B. thermosphacta*, *Carnobacterium* spp., *Lactobacillus* spp., *Leuconostoc* spp. and *Weissella* spp. (7). In the four batches studied in this work, the formation of milky exudates was observed at 45 days of storage; for the batches 1 and 4 was also observed carbon dioxide (CO_2) gas production from the fermentation of carbohydrates by heterofermentative LAB, in addition to the loss of vacuum packaging. The differences between homofermentative and heterofermentative LAB have a genetic and physiological basis; the homofermentative have aldolase and hexose isomerase enzymes, but no exhibit the phosphoketolase and use the Embden-Meyerhof-Parnas pathway to produce two molecules of lactic acid from a glucose molecule, while the heterofermentative have the phosphoketolase, but not aldolase and hexose isomerase enzyme, so they use hexose-monophosphate pathway or pentose degradation of glucose to produce lactic acid, ethanol and carbon dioxide (4, 24).

In the industrial practice of vacuum-packed meat products is common to observe that when the LAB counts exceeds 7 log $CFU.g^{-1}$ the alterations become perceptive on substrate, among which the slightly acid flavor, the presence of milky exudates and loss of vacuum (only when refers to heterofermentative LAB). These changes were perceptible in samples of sliced vacuum-packed cooked ham, and the vacuum loss was only noted in samples with a predominance of *Leuconostoc/Weissella* sp. The milky exudates, loss of vacuum, and discoloration are the most important changes in the purchase decision of a consumer because affecting negatively the appearance of product.

According to Table 3, the samples of vacuum-packaged sliced cooked ham were colonized initially by *Leuconostoc/Weissella* sp. (60%), *Enterococcus* sp. (24%) and homofermentative *Lactobacillus* sp. (16%). At 45 days of storage at 4 °C and 8 °C, the predominant LAB were homofermentative

Lactobacillus sp. (73% at 4 °C and 63% at 8 °C) followed by *Leuconostoc/Weissella* sp. (27% at 4 °C and 37% at 8 °C), according Table 4. Among then, the predominant species were *Lactobacillus curvatus* (strain CTSPL4, and MFPA15D06-03), *Lactobacillus sakei* (strain Moo1 and HS-1) and *Leuconostoc mesenteroides* (strain NCBF 529).

The *Enterococcus* sp. was present in two samples at the beginning of the shelf life but no showed growth after 45 days of storage at 4 °C and 8 ºC. This genus is widely distributed in the environment, especially inhabiting the human and animal gastrointestinal tract (14). The results observed in this work are similar to those found by Ammor *et al.* (2) whose showed that *Lactobacillus sakei* was the predominant LAB present in traditional fermented dry sausage at one and nine weeks of storage; however, also *Enterococcus faecium* and *Enterococcus* spp. were present at the first time, but absent at 9 weeks. Similarly, Marty *et al.* (27) analyzed 21 samples of spontaneously fermented Swiss meat products, founding that *Lactobacillus* accounted for 76% of the LAB present, being the *Lactobacillus sakei* and *Lactobacillus curvatus* the predominant species, followed by 18.3% of the genus *Enterococcus* sp., and 2.9% of both *Pediococcus* sp. and *Streptococcus* sp. The *Enterococcus* resistance to pasteurization temperatures, and their adaptability to different substrates and growth conditions (low and high temperature, extreme pH, and salinity) implies that they can be found either in food products manufactured from raw materials (milk or meat) and in heat-treated food products, because the heating of processed meats during production may confer a selective advantage to *Enterococcus*, since this bacteria are the most thermotolerant among of the nonsporulating bacteria (14).

The *Lactobacillus* genus is a heterogeneous group of LAB with important implications in food fermentation. The ability to colonize a variety of habitats is a direct consequence of the wide metabolic versatility of this group of LAB (4, 15). This genus is one of the original LAB genera and several species of importance in foods have been reclassified in new genera, among: *Carnobacterium* (10); *Weissella* (13); and *Atopobium* (12). Members of the genus were subdivided in three groups: group I - the obligatory homofermentative who include species like *Lactobacillus acidophilus, Lactobacillus delbrückii, Lactobacillus helveticus* and *Lactobacillus salivarius*; group II – the facultative heterofermentative that include species like *Lactobacillus casei, Lactobacillus curvatus,*

Lactobacillus plantarum and *Lactobacillus sakei*; and the group III – the obligatory heterofermentative who include the species like *Lactobacillus brevis, Lactobacillus buchneri, Lactobacillus fermentum* and *Lactobacillus reuteri* (4). In this work the genera *Lactobacillus curvatus* (strains CTSPL4 and MFPA15D06-03) and *Lactobacillus sakei* (strains Moo1 and HS-1) were the predominant spoilage LAB, and according to Axelsson (4) they are facultative heterofermentative, who were identified like homofermentative *Lactobacillus* by the phenotypic identification. These genera normally no produce gas from glucose; however in singular conditions they can produce it. Commonly occur confound on phenotypic characteristics among *Leuconostoc, Weissella*, facultative heterofermentative and obligatory heterofermentative *Lactobacillus.* In this work, phenotypic analysis did not allow the identification of the genera *Weissella* and *Leuconostoc*, due to overlap; in this case the molecular analysis allowed the identification of the correctly specie, classified by phenotypic analyses like *Leuconostoc/Weissella* sp., and identified as *Leuconostoc mesenteroides* (strain NCBF 529) by molecular analyses. In this way, molecular analyses were really useful for the identification of LAB from sliced vacuum-packed cooked ham. These results are similar to those previously reported by Hu *et al.* (19). *Leuconostoc* were able to growth at 8 °C (18), but *Leuconostoc mesenteroides* is also able to growth at 4 °C. As *Lactobacillus*, the genus *Leuconostoc* is linked to a few negative aspects including spoilage in meat products. The presence of LAB in vacuum packaged meats and similar products such as sausages; among them, the genera *Lactobacillus* and *Leuconostoc* were cited as participants in the deterioration of meat packed in vacuum or modified atmosphere, causing surface slime and aroma of fermented (24, 38). Strains of LAB generally regarded as natural in meat and meat products are *Lactobacillus sakei, Lactobacillus curvatus, Lactobacillus plantarum, Carnobacterium divergens, Carnobacterium maltaromaticum* and *Weissella viridescens* (2). Similarly to the results founded in this work, Hu *et al.* (19) showed that the dominant spoilage bacteria in sliced vacuum-packed cooked ham were *Lactobacillus sakei* and *Lactobacillus curvatus*, and *Leuconostoc* genus were minor components. During the chilling storage, development of *Leuconostoc* species is favored in vacuum packed samples, while *Weissella viridescens* is predominant when the products is pasteurized after packing (36). The sliced vacuum-packed cooked ham is a meat product who pass by

a thermal process like a pasteurization, but after cooking it is manipulated and a cross contamination may be possible.

CONCLUSIONS

The LAB initially present in the samples showed mean plate counts around 1.98 log $CFU.g^{-1}$, and after 45 days of storage were influenced by storage temperature, rising to values of 7.59 log $CFU.g^{-1}$ at 4 °C and 8.25 log $CFU.g^{-1}$ at 8 °C. The dominant spoilage bacteria of sliced vacuum-packed cooked ham on time 45 day of storage are *Lactobacillus curvatus* (strains CTSPL4 and MFPA15D06-03) and *Lactobacillus sakei* (strains Moo1 and HS-1), and the *Leuconostoc mesenteroides* (strain NCBF 529) was a minor component. The quantification and phenotypic methodologies combined with molecular methodologies of LAB identification are helpful to better understand the growth and activity of spoilage microorganisms of sliced vacuum-packed cooked ham. The evolution of sliced vacuum-packed cooked ham microbiota may be important to select the main deteriorating LAB aiming inhibit these microorganisms by future studies.

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Running title: Lactic acid bacteria inhibition

Title: Growth inhibition of lactic acid bacteria in cooked ham by bacteriocins

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ABSTRACT

The aim of this work was to investigate the possibility of controlling spoilage lactic acid bacteria (LAB) growth in sliced vacuum-packed cooked ham following a sequential strategy of experimental design. The *Lactobacillus sakei* was previously identified by molecular techniques as a LAB that causes spoilage in sliced vacuum-packed cooked ham; based on this, it was selected for conducting studies in Man Rogosa and Sharp (MRS) broth. A Fractional Factorial Design (FFD) was carried out on simulate MRS broth containing the cooked ham formulations ingredients with possible inhibitory effect on *L. sakei* growth, quote sodium chloride, sodium lactate, sodium tripolyphosphate and sodium erythorbate combined with the bacteriocins nisin and pediocin. According to the FFD results, *L. sakei* growth showed to be influenced by the sodium tripolyphosphate, nisin and pediocin. In the sequence of the study, a Central Composite Rotatable Design (CCRD) was applied to optimize the amounts of nisin and pediocin. The validation of the observed results in the step of study in MRS broth was performed by tests in food matrix, by the cooked ham elaboration and monitoring of the shelf life by the count of total LAB, total mesophilic aerobic bacteria (MAB) and pH. Four formulations were prepared, one designed as control and three tests with different concentrations of nisin (0.001%, 0.007% and 0.013%); all the formulations with nisin showed around 2 or 3 log lesser for the LAB mean count, and 3 or 4 log lesser for MAB mean count. Additionally, the four samples attended the microbial and physical chemical criteria required by current Brazilian legislation.

KEY-WORDS: lactic acid spoilage bacteria, *Lactobacillus sakei*, preservation, bacteriocins, experimental design strategy.

1) INTRODUCTION

The characteristic microbial populations that develop in meat and meat products are the result of the effect of the prevailing environmental conditions on the growth of the type of microbes initially present in the raw material or introduced by cross-contamination (Castellano et al., 2008). In the majority of modified atmosphere packaging and vacuum packaging meat products, lactic acid bacteria (LAB) predominate (Arvanitoyannis & Stratakos, 2012). The *Lactobacillus sakei* has been described by many researchers as part of the microbiota deteriorating in meat products, specially sliced cooked ham (Hu et al., 2009; Han et al., 2011). The intrinsic and extrinsic factors governing microbial growth will determine the type and number of bacteria present in meat. Intrinsic factors are predominantly chemical (concentration and availability of nutrients, pH, redox potential, buffering capacity, water activity, meat structure) while extrinsic ones are concerned with storage and processing conditions (Castellano et al., 2008).

The formulation of meat products contain components that generate sensory characteristics in food such texture, juiciness, flavor, color and appearance, which additionally can generate positive, negative or neutral effects on microbial growth by interfering in intrinsic characteristics. Additionally, biopreservation systems, such as bacteriocinogenic LAB cultures and /or their bacteriocins, have received increasing attention and new approaches to control pathogenic and spoilage microorganisms have been developed (Mattila, Saris & Työppönen, 2003).

Bacteriocins can be defined as bacterially produced, small, heat-stable peptides that are active against other bacteria and to which the producer has a specific immunity mechanism (Cotter, Hill & Ross, 2005). The nisin is produced by *Lactococcus lactis* subsp. *lactis,* and is active against Gram positive organisms including bacterial spores, but no generally active against

Gram negative bacteria, yeasts and fungi (Economou et al., 2009). Nisin is essentially nontoxic to humans, leading to no cross-resistance with medical antibiotics, being degraded without damage into the intestinal tract (Lindsay, 2010).

Like nisin, the pediocin is produced by a LAB, the *Pediococcus acidilactici* and is the more searched bacteriocin after nisin, due to the antimicrobial activity against *Listeria* strains, however the pediocin has been inadvertently or empirically used for many years as starter culture in a variety of food fermentations (Díez et al., 2012), which justifies further research and involving its effect against different strains of microorganisms.

Additionally, natural preservatives has piqued the interest of consumers, who increasingly seek healthy and natural characteristics in all types of food, especially in the cases that already contains considerable amounts of chemical additives such as processed meat products.

This context creates a highly favorable scenario for the redesign of this category of meat products with a focus on extended durability front the growth spoilage bacteria, with primary purpose increasing the lag phase. Parallel is very important to study the possible synergistic effects among the ingredients and additives in the formulation of ham, with the most innovative ingredients, quote nisin and pediocin.

The aim of this work was to investigate the possibility of controlling *L. sakei* growth on Man Rogosa and Sharp (MRS) broth by the investigation of sliced vacuum-packed cooked ham formulation's ingredients with possible inhibitory effect combined with the bacteriocins nisin and pediocin, by a strategy of experimental design. The second step involved a validation on food matrix applying the result obtained on broth study by shelf life accompaniment. The *L. sakei* was identified as one of the main spoilage LAB of sliced vacuum-packed cooked ham in a previous work of identification of LAB isolated from the product (results not published).

2) MATERIAL AND METHODS

2.1) Tests of *L. sakei* growth in MRS broth

2.1.1) Fractional factorial design (FFD)

Aiming the minimization of cell growth, a FFD 2^{6-2} (5 central points, total of 21 trials) was initially used to evaluate the effect of six variables on the parameters of growth of *L. sakei*, quote: sodium chloride (Diana), sodium lactate (Purac syntheses), sodium tripolyphosphate (BKG), sodium erythorbate (Ashland), nisin (Global Foods) and pediocin (ALTA 2345 - Kerry). The statistical design and the coded and real values of the variables are show in Table 1. For comparison, along with the 21 trials of FFD were performed four tests called "control treatments", namely CT_1, CT_2, CT_3 and CT_4.

The responses of the FFD were obtained by the parameters of logarithmic increase of population (A), exponential microbial growth rate (μ) and lag phase extension (λ), which were obtained from the fit of the Modified Gompertz predictive model (MGM) (Eq. 1) (Zwietering et al., 1991).

$$y = A.\exp\left[-\exp\left(\frac{\mu^{e}}{A}.(\lambda - t) + 1\right)\right] \qquad (1)$$

Where: $y = \log N/N_0$; $e = 2.7182$; A = logarithmic increase of population; μ = maximum specific growth rate (h^{-1}); λ = duration of the lag phase (h); t = time (h).

2.1.2 Central composite rotatable design (CCRD)

The preliminary FFD allowed the selection of statistically significant variables in relation to *L. sakei* growth, evaluated by the parameters of *A*, μ and λ, adjusted to MGM. The variables without significant effect (sodium chloride, sodium lactate and sodium erythorbate) were fixed and maintained at the level -1 of FFD, whereas the variable sodium tripolyphosphate (with significant effect) was set and maintained at level 0 of FFD. With the variables nisin and pediocin (both with significant effects), a central composite rotatable design (CCRD) with five replicates at the central point and four axial points (2^2 plus star configuration, totaling 13 trials) was performed aiming to reduce the concentrations of nisin and pediocin applied for *L. sakei* growth inhibition. The statistical design and the coded and real values of the variables are shown in Table 2. Four control treatments were performed in parallel for comparison, namely CT_1, CT_2, CT_3, and CT_4.

2.1.3 Conduction of runs and statistical analysis

All the runs of the FFD and the CCRD, described in Tables 1 and 2 respectively, were conducted in 250 mL Erlenmeyer flasks by adding 1% (v/v) of *Lb. sakei* (ATCC 15521) inoculum (10^7 CFU.g^{-1}) acquired from André Tosello collection of cultures, and filled with a total volume of medium (MRS broth). The pH of the runs was adjusted in the range from 6.0 - 6.3 by adding 0.1 mol.L^{-1} NaOH or 0.1 mol.L^{-1} HCl. Microaerophilic cultivation was conducted at 30 ºC in incubator without shaking (Novaética, model 403-3D, São Paulo, Brazil). The monitoring of microbial growth experiments was performed by absorbance measurements (optical density) in a spectrophotometer (Perkin Elmer, Lambda XLS, Beaconsfield, UK) at predetermined intervals, 1 or 2 hours, calibrated for a wavelength at 600

nm. The growth time was different for each test, because they were followed until the stationary phase of cell growth, however the tests of FFD remained in the range 27 up to 49 hours and tests of the CCRD remained in the range 27 up to 55 hours.

The software STATISTICA 8.0 (Statsoft Inc., Tulsa, OK, USA) was used to obtain the growth responses (*A*, μ and λ) generated by MGM adjusting, and the adequacy of the MGM to predict the *L. sakei* growth in the experiments was assessed by the statistical indices: mean square error – MSE (Eq. 2); *Bias* factor (Eq. 3); accuracy factor (Eq. 4) (Ross, 1996); and correlation coefficient - R^2 (Eq. 5) (Rodrigues & Iemma, 2010).

$$\mathrm{MSE} = \frac{\sum (\mathrm{OV} - \mathrm{PV})^2}{\mathrm{n}} \qquad (2)$$

$$\mathrm{BiasFactor} = 10^{\left(\sum \frac{\log(\mathrm{OV}/\mathrm{PV})}{\mathrm{n}}\right)} \qquad (3)$$

$$\mathrm{AccurancyFactor} = 10^{\left(\sum \frac{|\log(\mathrm{OV}/\mathrm{PV})|}{\mathrm{n}}\right)} \qquad (4)$$

$$R^2 = \frac{\mathrm{QSLR}}{\mathrm{TQSC}} \qquad (5)$$

Where: MSE = mean square error; OV = observed value; PV = predicted value; n= number of degrees of freedom (number of experimental points - number of model parameters); QSLR = quadratic sum of linear regression; TQSC = total quadratic sum corrected.

The results of FFD and CCRD were also treated with the aid of the software STATISTICA 8.0. The adequacy of the models (generated by CCRD) was assessed using analysis of variance (ANOVA) ($p \leq 0.05$).

2.2) Tests in sliced vacuum-packed cooked ham

Aiming to validate the results obtained in the step of studies in broth, mainly a reduction in the amount of nisin applied (variable that showed significant effects in FFD and CCRD), three test formulations of cooked ham were prepared with addition of nisin, and a control formulation (Table 3).

The samples were prepared on a pilot scale in a slaughterhouse of the South of Brazil. The pork ham was injected with the brine indicated in Table 3, followed by a tumbling for 2 h and a cure for 8 h. Then the samples were embedded in cook-in packaging, cooked in tanks with water at 85 °C until the temperature of 72 °C in the geometric center of the product. The samples were chilled to 0 °C. After the hams were removed from the waterproof container and sliced to a thickness of 1.6 ± 0.2 mm in slicer and packaged ten slices totaling approximately 200 g per vacuum sealed package. The samples remained stored at 8 °C for 60 days and analyzed during the shelf life for total count of LAB (Russo et al. 2006; Silva et al., 2010), total count of mesophilic aerobic bacteria (MAB) (Silva et al., 2010) and pH (Brazil, 1999). The evaluation of LAB and MAB were adjusted to the Modified Gompertz predictive model and the adjustment was available by the statistics indices. Additionally, at the time of 1 day of storage the samples were analyzed for *Salmonella* spp. research (AFNOR-*Association Française de Normalisation* BIO-12/16-09/05, Vidas, BioMérieux, Marcy l'Etoile, France), *Listeria monocytogenes* research (AFNOR BIO-12/9-07/02, Vidas, BioMérieux, Marcy l'Etoile, France), count of Coagulase-positive *Staphylococcus* (Brazil, 2003; Silva et al.,

2010), count of Sulfite reducing *Clostridium* (Brazil, 2003; Silva et al., 2010), count of Coliforms at 45 °C (AFNOR 3M-01/2-09/89/C, Petrifilm, 3M, Minnesota, USA), and count of molds and yeasts (Brazil, 2003). The samples were characterized by physico-chemical analyses (moisture, protein, lipids, chloride and total carbohydrate) by official methodologies (Brazil, 1999). The nitrite and nitrate content (Adolfo Lutz, 2005) were analyzed and expressed as residual nitrite content and the water activity was analyzed by a mensuration equipment at 25 ℃ (4TE, Aqualab Decagon, Washington, USA).

3) RESULTS AND DISCUSSION

3.1) Tests of *L. sakei* growth in MRS broth

3.1.1) Fractional factorial design

The matrix of tests performed on FFD with real and coded values of the variables studied, and responses, as well the results obtained for the control treatments, are presented in Table 1. Comparing the responses of FFD with the results obtained for the control treatments, it was verified that run 1 and CT_1 were the medium who showed the largest *A* (1.384 and 1.381 log Do/Do_{zero}, respectively), the highest μ (0.111 and 0.158 h^{-1}) and smaller λ (3.403 and 3.799 h). The CT_2 and CT_3, as some runs showed no growth during 49 h of monitoring, so the *A* and μ were considered equal to zero (0.000 log Do/Do_{zero} and 0.000 h^{-1}, respectively) and the λ lasted 49.000 h. The CT_4 showed a slight increase in growth, the *A* achieved was small (0.267) like the μ (0.023 h^{-1}), while the λ was extensive (34.963 h).

Analyzing the results describe in Table 1 it was possible to calculate the effects of the six variables studied, which are presented in Table 4 for the three responses evaluated.

For the response of *A*, the factors nisin and pediocin were the only ones that had a significant negative effect ($p \leq 0.05$) (Table 4), consequently, a decrease in *A* values was observed with increase in bacteriocins concentration in the range studied; the other variables showed no statistically significant effect for this response.

Only the variable nisin showed significant effect ($p \leq 0.05$) on the response of μ, which was a negative effect; consequently, an increase in nisin concentration in the range studied tends to slow the growth of the LAB in study.

The response λ was significantly affected ($p \leq 0.05$) by sodium tripolyphosphate and nisin. Both showed positive effects in the range studied, therefore, an increase in the concentration of these variables promoted an increase in the lag phase. The other variables showed no significant effect for this response. The lag and exponential phase are of greatest interest to the food microbiologists, because the spoilage occurs before the microorganisms reaching the stationary phase (Nakashima, André & Franco, 2000).

Sarmento (2006) studied the influence of sodium polyphosphate (in the range of 0.1 - 0.5%) in MRS broth containing *L. sakei* by a FFD, together with sodium chloride, sodium lactate, sodium nitrite/sodium nitrate and garlic. Similarly with the results founded in this work, the author observed significant negative effect of sodium polyphosphate under the responses of *A* and μ, while significant positive effect was observed for λ; for NaCl, it was observed no significant negative effect for *A* and μ, and significant positive effect for λ.

Ingredients as sodium chloride and sodium tripolyphosphate are used together to obtain a synergistic effect and to increase the water retention capacity of the meat (particularly in brines). Thus, according to Strasburg, Xiong & Chiang (2010) injected fresh meat often contain salt (0.5 - 2.0%) as phosphate (0.25 - 0.40%), and when alkali phosphate is used, like sodium tripolyphosphate (pH 9.5 - 10.2) it benefit the meat, increasing the pH from 5.5 - 5.6 (close to the isoelectric point of myosin) to 5.8 - 6.0, causing the myosin and other muscle

proteins retain water more strongly, due to increased net charges. With equal importance, chelators, like hexametaphosphates, can destabilize the cell membranes of bacteria by complexing the divalent cations which act as salt bridges between membrane macromolecules such as lipopolysaccharides (Vaara, 1992).

The sodium lactate, used as acidity regulator/humectant to inhibit microorganism growth due to the decrease in water activity (Sarmento, 2006), shown no significance on the responses studied, which can be explained by the high inhibitory effect of nisin, showing an effect much higher than the other variables studied (Table 4) in the *L. sakei* growth inhibition, as in CT_3 where nisin alone was able to inhibit growth by 49.000 h (Table 1). In contrast Devlieghere et al. (2000) developed a predictive model to evaluate the effect of temperature, water activity and adding different concentration of sodium lactate in the *L. sakei* growth in meat products; these authors founded a significant shelf life extending effect of sodium lactate by predicted model, which was more pronounced at low refrigerated temperatures (rate of 4 - 12 °C). Treatment of ground beef with sodium lactate alone or in combination with sodium chloride was effective against the proliferation of MAB, psychrotrophic bacteria, LAB and *Enterobacteriaceae* (Sallam & Samejima, 2004).

The sodium erythorbate is the reduction compound more used in the world. It is added to the mixture of cured meat in order to accelerate color development, converting nitrite to nitric oxide, and iron ion in the heme ferrous ion (Strasburg, Xiong & Chiang, 2010). Barringer, Abu-Ali & Chung (2005) showed a synergistic effect between glucono-delta-lactone and sodium erythorbate for the improvement in color in meat, but not for the microbial inhibition. Additionally, the quantities applied are lower, around 0.15% (Hsu & Sun, 2006).

The behavior observed in this work for the significant inhibitory effects of the variables nisin and pediocin on the responses studied on the FFD was similar to the results observed by Coventry, Muirhead & Hickey (1995), whose showed that both bacteriocins demonstrated

inhibitory activity against *Lactobacillus curvatus* in a model system.

In general, considering the three responses studied, the variables sodium tripolyphosphate, nisin and pediocin showed significant effects, promoting a decrease in *L. sakei* growth. From these results, and with knowledge of the process as well, the next step of this work aimed to optimize the use of nisin and pediocin in less quantities than indicated by the Brazilian legislation (maximum of 12.5 mg nisin/kg or 0.0125% for milk products) and the manufacturer (0.05% to 1.00% of pediocin) that could give the same inhibitory effect in the *L. sakei* growth. In this way, a CCRD was performed with the redefinition of ranges studied for the variables nisin and pediocin, in comparison to the ranges studied in the FFD; the concentration of nisin was reduced to 0.0000% up to 0.0070% and pediocin to 0.000% up to 0.500%; the sodium tripolyphosphate was fixed in the CCRD (was not included as a variable) at the central point of the FFD (0.3%) because the functionality that carries the myofibrils meat products in specific dosages. The other ingredients (FFD variables), like sodium chloride, sodium lactate and sodium erythorbate were kept at level -1 of the FFD, because no significant effect was observed for these variables on *L. sakei* reducing growth by FFD.

3.1.2) Central composite rotatable design

The matrix of tests performed on CCRD with real and coded values of the variables studied, and responses, as well the results obtained for the control treatments, is presented in Table 2. Comparing the responses of CCRD with the results obtained for the control treatments, it could be seen that the CT_1 presented the highest growth, followed by CT_4 and run 5. Respectively, the responses were 1.210, 0.490 and 0.750 log Do/Do_{zero} for *A*; 0.114, 0.074 and 0.061 h^{-1} for μ, and 3.415, 39.091 and 46.317 h for λ. The CT_1 was the most favorable medium for LAB development, because has no ingredient except the MRS broth. The CT_2,

CT_3 and all others runs of the CCRD showed no growth during 55 h of monitoring, so the responses *A* and μ were considered equal to zero (0.000 log Do/Do_{zero} and 0.000 h^{-1}) and the λ lasted 55.000 h.

Analyzing the results of the Table 2 was possible to calculate regression coefficients of the two variables studied, which are presented in Table 5.

From the Table 5, it was verified that the variables studied showed no significant effect ($p \leq 0.05$), however, nisin show *p values* close to 0.05 (linear and quadratic terms). Thus, the coefficients of pediocin (L), pediocin (Q) and the interaction effect between nisin and pediocin were incorporated in residue, getting a new table for the regression coefficients (Table 6); from these values, was calculated the analysis of variance (ANOVA), shown in Table 7.

Using regression analysis, a second-order model equations (Eq. 6, 7 and 8) was obtained for *A*, μ and λ as a function of the statistically significant parameters ($p \leq 0.05$), aiming to predict the *L. sakei* growth on the conditions studied. The $F_{calculated}$ for the regression was significant for the three responses studied ($p \leq 0.005$), however the percentage of variance explained by the model was low ($R^2 \approx 56$ %; R-adjusted ≈ 47 %) for all parameters studied. $F_{calculated}$ was greater than $F_{tabulated}$, under this condition the response surface can be generated, considering the inherent variability of bioprocesses (Rodrigues & Iemma, 2010). However, the R^2 and R-adjusted showed a very low value to represent a considerable adjustment to the model. Thus, the surface response was not generated.

$$A = -0.033 - 0.133x_1 + 0.147x_1^2 \qquad (6)$$

$$\mu = -0.003 - 0.011x_1 + 0.012x_1^2 \qquad (7)$$

$$\lambda = 55.377 + 1.535x_1 - 1.702x_1^2 \quad (8)$$

Where: x_1 = nisin.

Nisin was the variable with more influence on *L. sakei* growth inhibition. The three responses studied were significantly influenced by nisin. The reduction in the amounts proposed in CCRD showed that in the MRS broth, minimal amount of 0.001% was efficiently along with the other ingredients which comprise naturally formulation of cooked ham. The nisin was demonstrated for various researches as a good LAB inhibitory (Samelis et al., 2005; Economou et al., 2009; Ercolini et al., 2010), but also mesophilic bacteria (Gögüs, Bozoglu & Yurdugul, 2004). Nisin is the only bacteriocin that has been officially employed in the food industry and its use has been approved worldwide (Zacharof & Lovitt, 2012). On Brazil nisin has only permission by use on milk products.

The bacteriocins mechanism of action is not yet fully elucidated, but it is known that the protein portion is essential for activity. It is believed that the action depends on the receptor binding bacterial cell surface, with permeabilization of the cytoplasmic membrane and form ion channels that cause the rapid flow of cellular components with low molecular weight, causing an ion imbalance and ion flux (Franco, 2008).

Nevertheless, nisin is not widely used in poultry meat because it activity is limited to Gram positives inhibition (Gögüs, Bozoglu & Yurdugul, 2004), but in industrialized meat cooked product the microbiota remaining are basically composed of gram positives, considering than the gram negatives whereas generally inactivated by heat treatment or preservatives such as sodium nitrite. In this case nisin can be promoting a good naturally preservative effect.

The pediocin has shown antimicrobial activity against *Listeria* strains (Díez et al., 2012), however, the results of the CCRD obtained in this work showed no significant effects of

pediocin, in the range studied, on the *L. sakei* growth inhibition, when combined and compared with nisin, whereas the nisin amounts applied are lower than pediocin.

3.1.3) Statistical indices to evaluate the fit of the FFD and CCRD growth curves to MGM

The adjustment of the MGM to the experimental data curves generated in FFD and CCRD were analyzed by statistical indices, described in Table 8.

The MSE provides a baseline for the error that takes into account the residual sum of squares and degrees of freedom, and the proximity of its values to zero indicates the best model fit. The results described in Table 8 shows the MSE for a good adjustment.

The *Bia*s factor is an estimate of the difference between the predicted and observed values. It is actually an average relative deviation, when the *Bias* factor is greater than 1 (one) the predicted value is greater than observed, whereas when the *Bias* factor is smaller than 1 (one) the predicted value is smaller than the observed. The *Bias* factor described a good adjustment for all runs.

The accuracy factor is a measure for the average absolute difference between the predicted and observed values. As the accuracy factor increases, the model is less accurate on average, just as it is estimated an average of the values, positive and negative values signs tend to cancel one another. The accuracy factor calculation, because it is absolute, is always greater than 1 (one), so how much higher the value, lower the accuracy of the estimate of the mean. The accuracy factor described a good adjustment for all runs.

The R^2 is a measure of the proportion of variation explained by the regression equation in relation to the total variation of responses (Rodrigues & Iemma, 2009). The closer to 1 (one) is considered the best value of R^2, and indicates that the observed values are very close to the predicted values. All runs shown good adjust for R^2 (Table 8).

3.2) Tests in sliced vacuum-packed cooked ham

3.2.1) Microbial characterization of the formulations

The four formulations elaborated are in accordance with Brazilian Legislation on time 1 day of storage for *Salmonella* ssp. research, *Listeria monocytogenes* research, count of Coagulase-positive *Staphylococcus*, count of Sulfite reducing *Clostridium* and count of Coliforms at 45 °C (Brazil, 2001; Brazil, 2009), as shown in Table 9. The molds and yeasts were not a preconized analyses in the Brazilian Legislation (Brazil, 2001) for this meat product, however, all samples showed low results for these microorganisms.

Ensuring safe food for human consumption depends of the application of different technologies that eliminate the risks associated with spoilage, extend shelf life and preserve the sensory qualities (González, Suárez & Martínez, 2010). Nisin has effect only against Gram positive bacteria (Economou et al., 2009; Lindsay, 2010), while Gram negative are usually affected by cooking temperature applied (72 ° C). The group of *Salmonella*, *Listeria monocytogenes* and Coliforms at 45 °C are Gram negative, that show no influence by nisin, but can be reduced by the heat application on cooked step and by efficient hygiene conditions of the manufacturing processes, especially the Coliforms group who is easily inactivated by sanitizers (Silva et al., 2010). These factors associated could be explain the results observed for these microbiological analysis.

The Coagulase positive *Staphylococcus* and Sulfite reducing *Clostridium* are Gram positive food pathogens. The Sulfite reducing *Clostridium* is specially inhibited by sodium nitrite application, but according to Lindsay (2010) nisin has been used in processed cheese with high moisture to prevent the potential growth of *Clostridium botulinum* at United States.

During the slicing step, products come in contact with equipment, utensils and handlers; these can carry cross-contamination in food. Thus, the application of preservatives like nisin has a synergistic effect with the implementation of good manufacturing practices and hazard analysis critical control points on the inhibition of Gram positive microbiota.

3.2.2) Physico-chemical characterization of the formulations

The results of the physical chemical characterization on time 1 day of storage (Table 10) indicated that moisture (1 = 72.4%; 2 = 74.0%; 3 = 73.0%; and 4 = 73.7%) and water activity (0.97 for all formulations) of the cooked ham formulations were high. These results are in accordance with previously reported by Zurera-Cosano et al. (2005) who found a moisture content and water activity around 72,10% and 0.983, respectively, for samples of ham. According to Massaguer (2006) the *Lactobacillus* group can grow up 0.91 water activity. The four samples attended the criteria for protein and for the relation between the moisture and protein stipulated by Brazilian legislation (Brazil, 2000). The lipid content was found to be low because of the use of pork leg, a cut with little intramuscular fat (bellow 5%), comprising in greatest proportion of water and protein. Similar results were found by Zell et al. (2012) for protein and fat, around 16% and 1.5%, respectively. The chloride content was around 2% in the samples, indicating that addition of 1% sodium chloride, and other ingredients, such as curing salt and flavoring who also possess sodium chloride. González, Suárez & Martínez (2010) reported the cooked ham is a food with low salt content (2%), with pH around 6.0 and water activity above 0.95, factors that are unable to inhibit the microorganisms associated with contamination post process.

The values for residual nitrite were similar for the four formulations, and in accordance with the 150 ppm stipulated by Brazilian legislation (Brazil, 1998; Brazil, 2006). The 0.2% of

curing salt added in all formulations is equivalent to 150 ppm, and after cook the amount reached down, because the cooked conditions cause the nitrite degradation. The nitrite show no significance on the LAB development, because the absence of the ferredoxin enzyme, which react with the nitric oxide (Terra, Fries & Terra, 2004).

The total carbohydrate content, depend of type of carbohydrate, acts as substrate for the LAB development. The amounts added of cooked ham are lower but combined with the microaerophilic conditions allow the development of this microorganism group, reason for the LAB reaches up around 7 log CFU.g^{-1}.

3.2.3) Shelf life of the formulations

3.2.3.1) Enumeration of total lactic acid bacteria

The count of LAB showed rapid increase in the control formulation, while the proliferation in test formulations 1, 2 and 3 were 2 or 3 log cycles slower than control. Test 1, 2 and 3 showed a lesser A, lower μ and more extensive λ when compared to control formulation, as shown in Table 11. The Figure 1 shows the curves of LAB growth. The addition of nisin showed significant influence on the LAB growth decrease, regardless of the amount applied (0.013, 0.007, and 0.001% for formulations 1, 2 and 3 respectively). These results could be confirm the previous results observed in MRS broth study by the DCCR, which indicated that a minimum amount of nisin between the range studied (0.001%), would result in inhibition of LAB. Thus the results obtained in the DCCR were validated in the food matrix.

3.2.3.2) Enumeration of total mesophilic aerobic bacteria

The MAB count showed that the control formulation was the first to leave the lag phase and

reach stationary phase. Test formulations 1, 2 and 3 showed 3 or 4 log cycles lower when compared with the control formulation. Additionally, tests 1, 2 and 3 showed lower *A*, lesser μ and longer λ than the control formulation, as shown in Table 11. The Figure 2 shows the curves of MAB growth, which indicates acceptable counts for formulations 1, 2 and 3, and unacceptable count for control, considering the limit of acceptability equal 7 log CFU.g^{-1} (Ntzimani, Giatrakou & Savvaidis, 2010). The addition of nisin showed significant influence on the MAB growth decrease, regardless of the amount applied (0.013%; 0.007%; 0.001%).

3.2.3.3) Evaluation of pH

The pH does not have a direct correlation with the growth of LAB, however brings indicative of their proliferation. The decrease in pH occurs due to the production of lactic acid generated by LAB, which ferment carbohydrates and produce lactic acid as metabolite. Because the main catabolite is lactic acid, the genus *Lactobacillus* prefers relatively acidic conditions, with pH 5.5 up to 6.5 (Giraffa, Chanishvili & Widyastuti, 2010).

The analysis of pH formulations during the shelf life indicates that the pH of formulations 1, 2 and 3 no decrease, while the control reached down at least mean of 5.94 (Figure 3). The average of pH of the formulations with the nisin addition remained around 6.35 - 6.40, results very similar with previously reported by Zurera-Cosano et al. (2005) (around 6.32).

The addition of nisin showed statistical significant influence ($p \leq 0.05$, by the Tukey test) on pH maintenance when compared with the control formulation, regardless of the amount applied (0.013%; 0.007%; 0.001%).

According to Kreyenschmidt et al. (2010) when LAB plate counts reaches 7 log CFU.g^{-1}, the product reaches the end of shelf life, which was indicated by changes in sensory quality and pH decrease.

3.2.3.4) Statistical indices to evaluate the fit of the LAB and MAB growth curves to MGM

The adjustment of the MGM to the experimental data curves generated in the step of LAB growth reduction in sliced vacuum-packed cooked ham was analyzed by statistical indices, described in Table 12. The MSE indicates good fit for LAB and MAB curves for all samples. Similarly occurred for the *Bia*s factor and accuracy factor, which were situated nearly 1 (one). The *Bias* factor showed the majority predicted values are lesser than experimental values, except for the control LAB curve and Test 3 MAB curve. The R^2 also indicates that the observed values are close to the predicted values. All curves also showed good fit for R^2 (Table 12).

4) CONCLUSIONS

Based on the results obtained was concluded that among the variables studied of the ham formulation, the most important variable with significant effect on *L. sakei* growth inhibition was the nisin. The sodium tripolyphosphate, nisin and pediocin showed significant effects when studied by the FFD, though in a CCRD (applied to reduce the amount of these bacteriocins), only nisin had a significant effect on reducing the *L. sakei* growth. The validation by nisin application in three concentrations in sliced vacuum-package cooked ham showed a reduction of total LAB around 2 or 3 log cycles and around 3 or 4 log cycles for total MAB during the shelf life storage at 8 °C during 60 days compared with the control formulation. The quality control microorganisms like *Salmonella* spp., *Listeria monocytogenes*, Coagulase-positive *Staphylococcus,* Sulfite reducing *Clostridium,* Coliforms at 45 °C and molds and yeasts showed satisfactory results, that indicate the application of good manufacture techniques during processing were effective. The physical chemical and

microbiological analysis indicated that the formulations were in accordance with the parameters required by Brazilian Legislation. The shelf life monitoring showed a significant influence on LAB and MAB decrease counts, and also maintenance of pH for the formulations 1, 2 and 3, added by 0.013%, 0.007% and 0.001% of nisin, when compared with control. The smaller amount of nisin added (0.001%) shown similar results that the intermediate (0.007%) and the greater amount tested (0.013%) in the extension of the sliced vacuum-package cooked ham shelf life.

These results are satisfactory from the technological and economic standpoints, because the consumers seek food products with smaller amounts of preservatives, and with preference for natural preservatives, and the companies seek smaller cost, considering the high price of nisin.

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TABLES

Table 1 - FFD (2^{6-2}) matrix with coded and real values for the variables and responses.

Run	x_1[a]	x_2[b]	x_3[c]	x_4[d]	x_5[e]	x_6[f]	A[g]	μ[h]	λ[i]
1	-1 (1.0)	-1 (1.0)	-1 (0.1)	-1 (0.05)	-1 (0.000)	-1 (0.0)	1.384	0.111	3.403
2	+1 (3.0)	-1 (1.0)	-1 (0.1)	-1 (0.05)	+1 (0.013)	-1 (0.0)	0.000	0.000	49.000
3	-1 (1.0)	+1 (3.0)	-1 (0.1)	-1 (0.05)	+1 (0.013)	+1 (1.0)	0.000	0.000	49.000
4	+1 (3.0)	+1 (3.0)	-1 (0.1)	-1 (0.05)	-1 (0.000)	+1 (1.0)	0.000	0.000	49.000
5	-1 (1.0)	-1 (1.0)	+1 (0.5)	-1 (0.05)	+1 (0.013)	+1 (1.0)	0.000	0.000	49.000
6	+1 (3.0)	-1 (1.0)	+1 (0.5)	-1 (0.05)	-1 (0.000)	+1 (1.0)	0.000	0.000	49.000
7	-1 (1.0)	+1 (3.0)	+1 (0.5)	-1 (0.05)	-1 (0.000)	-1 (0.0)	1.298	0.010	48.075
8	+1 (3.0)	+1 (3.0)	+1 (0.5)	-1 (0.05)	+1 (0.013)	-1 (0.0)	0.000	0.000	49.000
9	-1 (1.0)	-1 (1.0)	-1 (0.1)	+1 (0.15)	-1 (0.000)	+1 (1.0)	0.363	0.038	31.302
10	+1 (3.0)	-1 (1.0)	-1 (0.1)	+1 (0.15)	+1 (0.013)	+1 (1.0)	0.000	0.000	49.000
11	-1 (1.0)	+1 (3.0)	-1 (0.1)	+1 (0.15)	+1 (0.013)	-1 (0.0)	0.000	0.000	49.000
12	+1 (3.0)	+1 (3.0)	-1 (0.1)	+1 (0.15)	-1 (0.000)	-1 (0.0)	2.289	0.038	17.702
13	-1 (1.0)	-1 (1.0)	+1 (0.5)	+1 (0.15)	+1 (0.013)	-1 (0.0)	0.000	0.000	49.000
14	+1 (3.0)	-1 (1.0)	+1 (0.5)	+1 (0.15)	-1 (0.000)	-1 (0.0)	0.000	0.000	49.000
15	-1 (1.0)	+1 (3.0)	+1 (0.5)	+1 (0.15)	-1 (0.000)	+1 (1.0)	0.000	0.000	49.000
16	+1 (3.0)	+1 (3.0)	+1 (0.5)	+1 (0.15)	+1 (0.013)	+1 (1.0)	0.000	0.000	49.000
17	0 (2.0)	0 (2.0)	0 (0.3)	0 (0.10)	0 (0.007)	0 (0.5)	0.000	0.000	49.000
18	0 (2.0)	0 (2.0)	0 (0.3)	0 (0.10)	0 (0.007)	0 (0.5)	0.000	0.000	49.000
19	0 (2.0)	0 (2.0)	0 (0.3)	0 (0.10)	0 (0.007)	0 (0.5)	0.000	0.000	49.000
20	0 (2.0)	0 (2.0)	0 (0.3)	0 (0.10)	0 (0.007)	0 (0.5)	0.000	0.000	49.000
21	0 (2.0)	0 (2.0)	0 (0.3)	0 (0.10)	0 (0.007)	0 (0.5)	0.000	0.000	49.000
CT_1	-	-	-	-	-	-	1.381	0.158	3.799
CT_2	-	-	-	-	+1 (0.013)	+1 (1.0)	0.000	0.000	49.000
CT_3	-	-	-	-	+1 (0.013)	-	0.000	0.000	49.000
CT_4	-	-	-	-	-	+1 (1.0)	0.267	0.023	34.963

a: sodium chloride (%); b: sodium lactate (%); c: sodium tripolyphosphate (%); d: sodium erythorbate (%); e: nisin (%); f: pediocin (%); g: A - logarithmic increase of population (log Do/Do_{zero}); h: μ – maximum specific growth rate (h^{-1}); i: λ - duration of the lag phase (h).

Table 2 – CCRD 2^2 matrix with coded and real values for the variables and responses.

Run	x_1[a]	x_2[b]	A[c]	μ[d]	λ[e]
1	-1 (0.0010)	-1 (0.073)	0.000	0.000	55.000
2	+1 (0.0060)	-1 (0.073)	0.000	0.000	55.000
3	-1 (0.0010)	+1 (0.430)	0.000	0.000	55.000
4	+1 (0.0060)	+1 (0.430)	0.000	0.000	55.000
5	-1.41 (0.0000)	0 (0.250)	0.750	0.061	46.317
6	+1.41 (0.0070)	0 (0.250)	0.000	0.000	55.000
7	0 (0.0035)	-1.41 (0.000)	0.000	0.000	55.000
8	0 (0.0035)	1.41 (0.500)	0.000	0.000	55.000
9	0 (0.0035)	0 (0.250)	0.000	0.000	55.000
10	0 (0.0035)	0 (0.250)	0.000	0.000	55.000
11	0 (0.0035)	0 (0.250)	0.000	0.000	55.000
12	0 (0.0035)	0 (0.250)	0.000	0.000	55.000
13	0 (0.0035)	0 (0.250)	0.000	0.000	55.000
CT_1	-	-	1.210	0.114	3.415
CT_2	+1.41 (0.0070)	+1.41 (0.500)	0.000	0.000	55.000
CT_3	+1.41 (0.0070)	-	0.000	0.000	55.000
CT_4	-	+1.41 (0.500)	0.490	0.074	39.091

a: nisin (%); b: pediocin (%); c: A - logarithmic increase of population (log Do/Do_{zero}); d: μ – maximum specific growth rate (h^{-1}); e: λ - duration of the lag phase (h).

Table 3 – Formulations test (with nisin) and control (without nisin) of cooked ham for monitoring the shelf-life.

Raw materials / Ingredients (%)	F_1[a]	F_2[b]	F_3[c]	Control
Pork leg	74.628	74.633	74.639	74.640
Sodium chloride	1.000	1.000	1.000	1.000
Sodium lactate	1.000	1.000	1.000	1.000
Sodium erythorbate	0.050	0.050	0.050	0.050
Sodium tripolyphosphate	0.300	0.300	0.300	0.300
Nisin	0.0130	0.0070	0.0010	-
Water	18.000	18.000	18.000	18.000
Isolated soy protein	2.000	2.000	2.000	2.000
Curing salt[d]	0.200	0.200	0.200	0.200
Malto-dextrin	1.500	1.500	1.500	1.500
Condiment	0.800	0.800	0.800	0.800
Cochineal carmine	0.010	0.010	0.010	0.010
Carrageenan	0.500	0.500	0.500	0.500
Total	100.000	100.000	100.000	100.000

a: formulation 1; b: formulation 2; c: formulation 3; d: curing salt containing 2.0% of nitrite, 5.5% of nitrate and 92.5% of sodium chloride.

Table 4 - Effects of the factors studied on FFD 2^{6-2} for the responses of A, μ and λ.

Factor	Effect[g]	SE[h]	t (14)	p value	Effect[g]	SE[h]	t (14)	p value	Effect[g]	She	t (14)	p value
		A				μ				λ		
Mean	0.254	0.114	2.234	0.042*	0.009	0.005	2.010	0.064	44.452	2.232	19.911	<0.000*
x_1[a]	-0.094	0.260	-0.363	0.722	-0.015	0.011	-1.420	0.179	4.115	5.115	0.804	0.435
x_2[b]	0.230	0.260	0.883	0.392	-0.013	0.011	-1.174	0.260	3.884	5.115	0.759	0.460
x_3[c]	-0.342	0.260	-1.315	0.210	-0.022	0.011	-2.060	0.058	11.709	5.115	2.289	0.038*
x_4[d]	-0.004	0.260	-0.014	0.989	-0.006	0.011	-0.520	0.611	-0.309	5.115	-0.060	0.953
x_5[e]	-0.667	0.260	-2.561	0.023*	-0.025	0.011	-2.302	0.037*	11.940	5.115	2.334	0.035*
x_6[f]	-0.576	0.260	-2.212	0.044*	-0.015	0.011	-1.406	0.182	7.515	5.115	1.469	0.164

a: sodium chloride (%); b: sodium lactate (%); c: sodium tripolyphosphate (%); d: sodium erythorbate (%); e: nisin (%); f: pediocin (%); g: effects (%); h: standard error; * p≤0.05.

Table 5 - Regression coefficients for the responses of A, μ and λ obtained by CCRD.

	RC[c]	SE[d]	t (7)	p value	RC[c]	SE[d]	t (7)	p value	CR[c]	SE[d]	t (7)	P value
		A				μ				λ		
Mean	0.000	0.078	0.006	0.996	<0.000	0.006	0.006	0.996	54.995	0.901	61.066	<0.000*
x_1[a] (L)[e]	-0.133	0.062	-2.153	0.068	-0.011	0.005	-2.153	0.068	1.535	0.713	2.153	0.068
x_1[a] (Q)[f]	0.141	0.066	2.128	0.071	0.012	0.005	2.128	0.071	-1.632	0.767	-2.128	0.071
x_2[b] (L)[e]	<0.000	0.062	<0.000	1.000	<0.000	0.005	<0.000	1.000	<0.000	0.713	<0.000	1.000
x_2[b] (Q)[f]	-0.048	0.066	-0.720	0.495	-0.004	0.005	-0.720	0.495	0.552	0.767	0.720	0.495
x_1[a] X x_2[b]	<0.000	0.087	<0.000	1.000	<0.000	0.007	<0.000	1.000	<0.000	1.007	<0.000	1.000

a: nisin (%); b: pediocin (%); c: RC - regression coefficients; d: SE - standard error; e: L - linear term; f: Q - quadratic term; * p≤0.05.

Table 6 - Regression coefficients for the responses of A, μ and λ obtained by CCRD - only considering the effects of nisin (L) and nisin (Q).

	RC[b]	SE[c]	t (10)	p value	RC[b]	SE[c]	t (10)	p value	RC[b]	SE[c]	t (10)	p value
		A				μ				λ		
Mean	-0.033	0.055	-0.597	0.564	-0.003	0.004	-0.597	0.564	55.377	0.631	87.754	<0.000*
x_1[a] (L)[d]	-0.133	0.053	-2.483	0.032*	-0.011	0.004	-2.483	0.032*	1.535	0.618	2.483	0.032*
x_1[a] (Q)[e]	0.147	0.057	2.582	0.027*	0.012	0.005	2.582	0.027*	-1.702	0.659	-2.582	0.027*

a: nisin (%); b: RC - regression coefficients; c: SE - standard error; d: L - linear term; e: Q - quadratic term; * p≤0.05.

Table 7 - ANOVA of the quadratic model for prediction of *A*, μ and λ.

Response		Sum of square	Degrees of freedom	Mean square	$F_{calculated}$	$F_{tabulated}$	p value
A	Regression	0.292	2	0.146	6.636	4.103	0.015*
	Residual	0.228	10	0.022			
	Total	0.520	12				
μ	Regression	0.002	2	0.001	10.000	4.103	0.004*
	Residual	0.002	10	<0.000			
	Total	0.003	12				
λ	Regression	39.112	2	19.556	6.414	4.103	0.016*
	Residual	30.487	10	3.049			
	Total	69.600	12				

* p≤0.05.

Table 8 - Statistical indices to evaluate the MGM adjust to the experimental data obtained in the runs of FFD and CCRD.

Run	MSE[a]	*Bias* factor	Accuracy factor	R^2[b]
1 – FFD	0.001	1.039	1.079	0.999
7 – FFD	0.129	1.332	1.530	0.961
9 – FFD	0.139	1.009	1.061	0.992
12 – FFD	0.115	1.129	1.427	0.985
CT_1 – FFD	0.040	1.136	1.174	0.998
CT_4 – FFD	0.148	0.954	1.141	0.990
5 – CCRD	0.001	1.323	1.380	0.993
CT_1 – CCRD	<0.000	0.990	1.155	>0.999
CT_4 – CCRD	0.120	1.159	1.187	0.995

a: mean square error; b: correlation coefficient.

Table 9 – Results of microbiological analysis of the formulations at the time of 1 day of storage.

Sample	*Salmonella* spp. (in 25 g)	*Listeria monocytogenes* (in 25 g)	Coagulase-positive *Staphylococcus* ($CFU.g^{-1}$)	Sulfite reducing *Clostridium* ($CFU.g^{-1}$)	Coliforms at 45 °C ($CFU.g^{-1}$)	Molds and Yeasts ($CFU.g^{-1}$)
Test 1[a]	Absence	Absence	$<10^2$	$<10^1$	$<10^1$	$<10^1$
Test 2[a]	Absence	Absence	$<10^2$	$<10^1$	$<10^1$	$<10^1$
Test 3[a]	Absence	Absence	$<10^2$	$<10^1$	$<10^1$	$1.0x10^1$
Control[a]	Absence	Absence	$<10^2$	$<10^1$	$<10^1$	$<10^1$
Limit[b]	Absence	Absence[c]	$5x10^3$	$5x10^2$	10^3	-

a: average of triplicate measurements; b: maximum preconized in Brazilian legislation (Brazil, 2001); c: preconized in Brazilian legislation (Brazil, 2009).

Table 10 – Results of the physical chemical analysis of the formulations samples at the time of 1 day of storage.

Sample	Moisture (%)	Protein (%)	Relation Moisture/ Protein	Lipids (%)	Chlorides (%)	Total carbohydrate (%)	Residual nitrite (ppm)[a]	Water activity
Test 1	72.4 ± 1.2	18.9 ± 0.2	3.8 ± 0.0	2.2 ± 0.2	1.9 ± 0.2	1.3 ± 0.2	94.0 ± 5.0	0.97± 0.01
Test 2	74.0 ± 1.0	18.5 ± 0.5	4.0 ± 0.1	2.1 ± 0.1	2.0 ± 0.1	1.1 ± 0.1	76.0 ± 10.1	0.97± 0.00
Test 3	73.0 ± 1.0	19.0 ± 0.7	3.9 ± 0.2	2.0 ± 0.1	2.0 ± 0.2	1.3 ± 0.7	81.0 ± 27.7	0.97± 0.00
Control	73.7 ± 0.6	18.4 ± 0.2	4.0 ± 0.0	2.1 ± 0.2	1.9 ± 0.1	1.1 ± 0.6	78.0 ± 6.9	0.97± 0.00
Limit[b]	-	≥ 14%	≤ 5.35	-	-	≤ 2.0%	≤ 150 ppm[c]	-

a: part per million; b: limits preconized in Brazilian legislation (Brazil, 2000); c: Brazil (1998) and Brazil (2006).

Table 11 - Model parameters for the lactic acid bacteria and mesophilic aerobic bacteria growth for 60 days of monitoring the shelf life.

Sample	*A*[a]	μ[b]	λ[c]	*A*[a]	μ[b]	λ[c]
	Lactic acid bacteria			*Mesophilic aerobic bacteria*		
Test 1	3.897	0.496	14.704	4.060	0.374	9.740
Test 2	3.473	0.589	10.582	3.591	0.328	9.661
Test 3	4.075	0.392	13.661	4.225	0.375	9.652
Control	6.576	0.649	6.462	7.347	0.583	4.171

a: *A* - logarithmic increase of population; b: μ - maximum specific growth rate ($days^{-1}$); c: λ - duration of the lag phase (days).

Table 12 - Statistical indices to evaluate the MGM adjust to the experimental data obtained in validation.

Sample	MSE[a]	*Bias* factor	Accuracy factor	R²[b]	MSE[a]	*Bias* factor	Accuracy factor	R²[b]
	Lactic acid bacteria				*Mesophilic aerobic bacteria*			
Test 1	0.048	0.995	1.050	0.995	0.035	0.994	1.039	0.996
Test 2	0.154	0.982	1.111	0.979	0.173	0.949	1.166	0.976
Test 3	0.028	0.996	1.034	0.997	0.100	1.008	1.078	0.990
Control	0.232	1.109	1.216	0.989	0.406	0.956	1.144	0.984

a: mean square error; b: correlation coefficient.

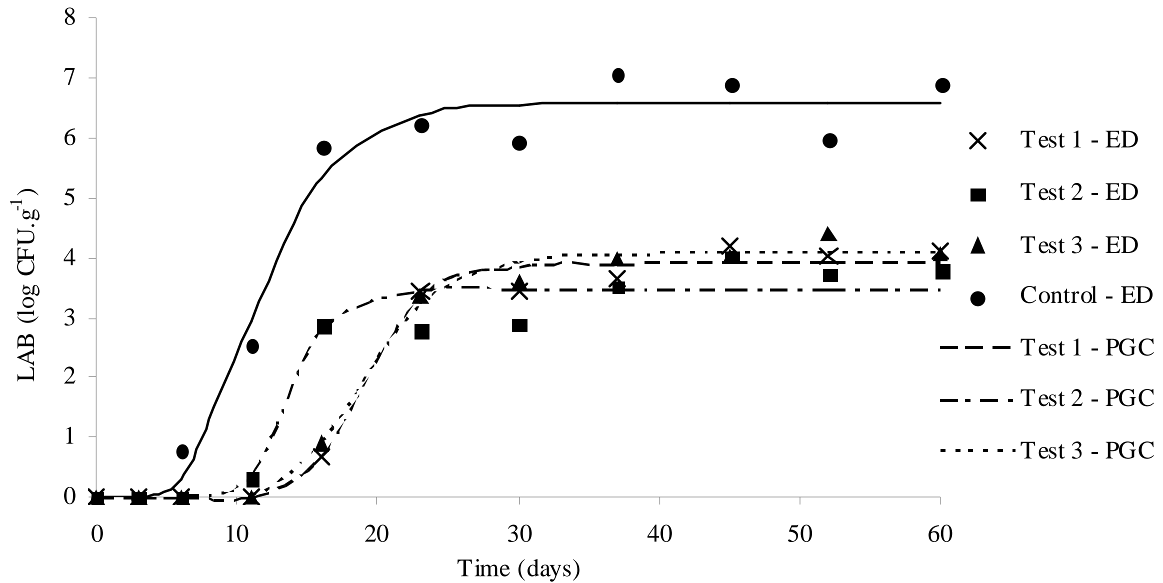

Figure 1 - Mathematical modeling of the growth of lactic acid bacteria in samples of sliced vacuum-packed cooked ham. ED: experimental data; PGC: predicted growth curve adjusted by MGM.

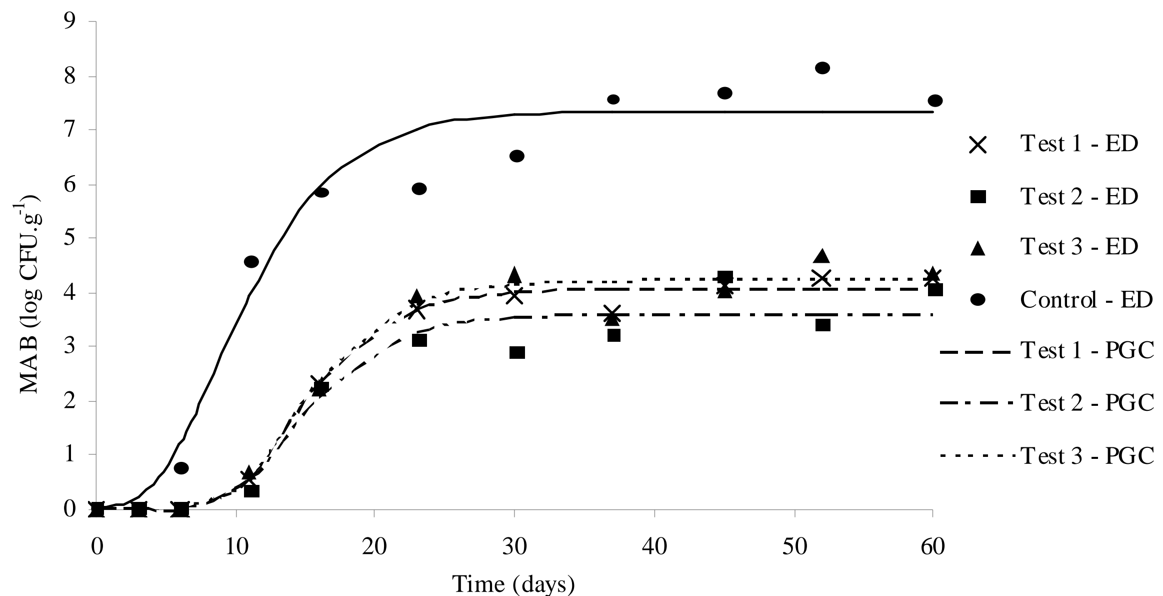

Figure 2 - Mathematical modeling of the growth of mesophilic aerobic bacteria in samples of sliced vacuum-packed cooked ham. ED: experimental data; PGC: predicted growth curve adjusted by MGM.

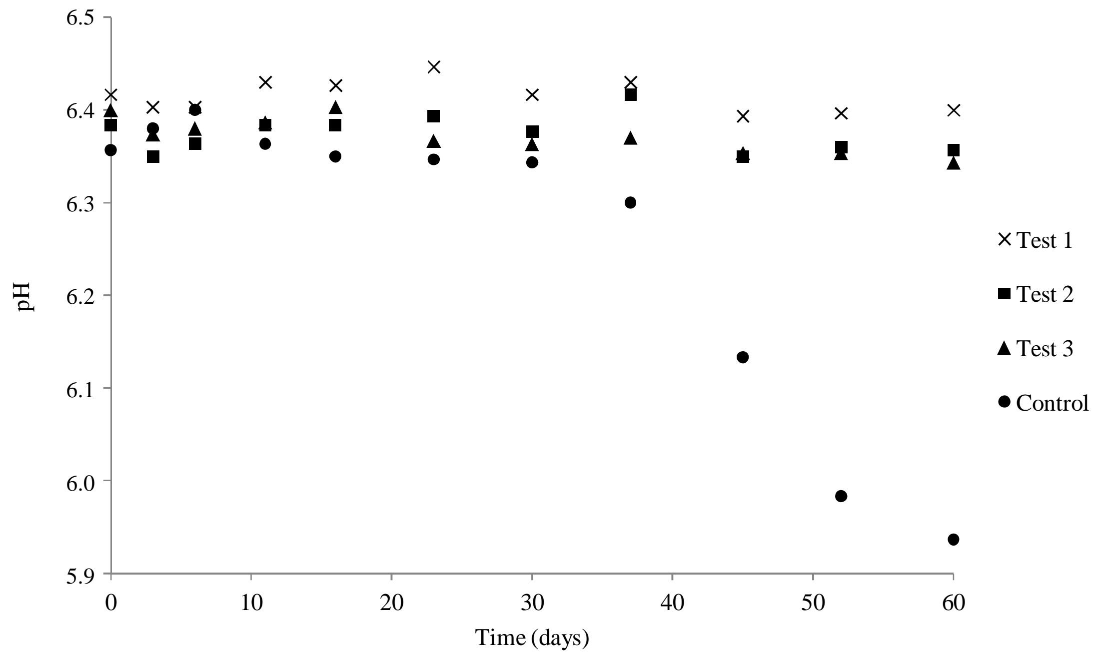

Figure 3 – Evaluation of the pH in samples of sliced vacuum-packed cooked ham.