Brazilian Journal of Microbiology Online Ahead of Print
ISSN 1678-4405
DOI: http://dx.doi.org/10.1590/S1517-838246120130019
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Characterization of the spoilage lactic acid bacteria in "sliced vacuum-packed cooked ham"

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Submitted: January 7, 2013; Approved: June 9, 2014.

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 for 45 days by phenotypic and 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 for the four batches analyzed. The lactic acid bacteria grew rapidly on the samples, and plate counts around 7.59 log cfu/g and 8.25 log cfu/g 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 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, as cooked ham, is limited mainly because of microbiological safety and spoilage issues (Vercammen *et al*., 2011).

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 (Russo *et al.*, 2006). 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 (Audenaert *et al.*, 2010). Whereas the combination of microaerophilic conditions, presence of NaCl, $NaNO_2$ and a reduced water activity inhibits growth of Gram-negative spoilage microbiota, favors growth of lactic acid bacteria (LAB) (Ammor *et al.*, 2005; Hu *et al.*, 2009; Audenaert *et al.*, 2010).

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 (Jay, 2005; Hu *et al.*, 2009; Zhang *et al.*, 2009; Audenaert *et al.*, 2010). Anyway, deterioration

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caused by LAB is primarily due to production of metabolites that cause unwanted changes in appearance, texture and flavor of the substrate (Massaguer, 2006).

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 (Hu *et al.*, 2009; Slongo *et al.*, 2009; Zhang *et al.*, 2009; Kreyenschmidt *et al.*, 2010; Vercammen *et al.*, 2011).

Originally, the group of LAB included four kinds of great importance in food: *Lactobacillus, Leuconostoc, Pediococcus* and *Streptococcus* (Hall *et al.*, 2001; Silva *et al.*, 2010). Currently, this group consists on 15 genera (Jay, 2005; Landgraf, 2008; Silva *et al.*, 2010).

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 (Axelsson, 2004). 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 (Stiles and Holzapfel, 1997).

Analysis of LAB is not required by Brazilian legislation for meat products, just some pathogens such as *Salmonella*, sulfite reducing *Clostridium*, Coagulase positive *Staphylococcus* and Coliforms at 45 °C have stipulated standards (Brazil, 2001). Although the analysis for LAB quantification and identification are of great importance for this kind of meat product, because the LAB represent a group of spoilage that cause economic losses during the shelf life of sliced vacuum-packed meat products, especially considering the shelf life of commercial brands of sliced vacuum-packed cooked ham available at supermarkets, which ranging from 30 to 60 days of storage at temperatures of 0 °C to 8 °C.

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.

Materials 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 of samples were collected for each batch. A microbiological analysis was performed in triplicate on the time 1 day after the slicing and packaging of the product, and two 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).

Isolation and enumeration of total LAB

Quantification of LAB in the samples was realized by plating in Man Rogosa and Sharp (MRS) agar (Himedia, Mumbai, India). For all samples, three packets of sliced cooked ham per batch were used for microbiological analysis. For each package, 25 g portion was aseptically weighed and pooled in 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 min. 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 h (Novaética, model 403-3D, São Paulo, Brazil) in normal atmosphere (Russo *et al.*, 2006; Silva *et al.*, 2010).

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.

The Flowchart showed in Figure 1, adapted from Schillinger and Lücke (1989), was used for the sequential analysis of phenotypic characteristics of the 12 LAB genera studied in this work.

Triplicate plates with typical LAB growth colonies were randomly selected and five colonies of each were tested by phenotypic characteristics. The proposed flowchart (Figure 1) identifies the most of genera 12 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, Lactobacillus* (homofermentatives) and *Brochothrix*, and also with *Lactococcus* and *Vagococcus*. Because this, molecular analyzes were performed in parallel to identify the genus and species at time 45 days.

Phenotypic characteristics were tested as follows: each colony was aseptically transferred to a tube with MRS broth (Himedia, Mumbai, India) supplemented with 5% of glucose and an inverted Durham tube incubated at 30 °C for 48 h for test the gas production from glucose (Potes and Marinho, 2007; Wu *et al.*, 2012); after aliquots of the tube of gas production from glucose were tested for Gram reaction (Oliveira *et al.*, 2008; Silva *et al.*, 2010). From this point the tests were performed following the sequence of classification according to the flowchart proposed (Figure 1) and all the test were performed by aliquots of the test

Table 1 - Key phenotypic characteristics of LAB.

Genera	Morphology	CO_2 from glucose	Lactic acid isomer	Growth at 10 °C	Growth at 45 °C	Growth in 18% NaCl	Growth at pH 4.4	Growth at pH 4.5	Catalase activity
Aerococcus	C[a] (2)	-(2)	L (2)	+(2)	-(2)	-(2)	-(2)		-(1)
	C[a] (10)	-(10)	L (10)	+(10)	-(10)	-(10)	-(10)		
	C[a] (23)	-(23)	L (23)	+(23)	-(23)	-(23)	-(23)		
Atopobium	C/R (7)								-(7)
	C/R (12)								
Bifidobacterium	R (3)	+[b] (5)	L (13)	-(5)	+(5)		-(5)	-(5)	-(5)
	R (5)	+[b] (13)		-(13)	+(13)		-(13)	-(13)	-(13)
	R (13)								
Brochothrix	C/R[d] (11)	- (15)	L (15)		-[f] (20)				+(20)
	R[d] (17)	- (22)	L (20)		-[f] (21)				+(21)
	R (21)	-	L (21)						+(22)
Carnobacterium	R (2)	+[e] (2)	L (2)	+(2)	-(10)	-(2)		-(19)	-(6)
	R (19)	-[e] (4)	L (10)	+(19)	-(19)	-(10)			-(19)
	R (23)	+(19)	L (23)	+(23)	-(23)	-(23)			
Enterococcus	C (2)	-(2)	L (2)	+(2)	+(2)	-(2)	+(2)		-(16)
	C (16)	-(16)	L (4)	+(10)	+(10)	-(10)	+(10)		-(19)
	C (19)	-(19)	L (16)	+(16)	+(18)	-(23)	+(23)		
Lactobacillus	R (2)	± (2)	D, L, DL (2)	± (2)	± (2)	-(2)	± (2)	+(19)	-(9)[g]
	R (19)	± (18)	D, L, DL (10)	± (10)	± (19)	-(10)	± (10)		-(19)[g]
	R (23)	± (19)	D, L, DL (23)	± (23)	± (23)	-(23)	± (23)		
Lactococcus	C (18)	-(18)	L (16)	+(10)	-(10)	-(10)	± (10)		-(5)
	C (19)	-(19)	L (22)	+(18)	-(18)	-(23)	± (23)		-(16)
	C (23)	-(23)	L (23)	+(23)	-(23)				-(19)
Leuconostoc	C (2)	+(2)	D (2)	+(2)	-(2)	-(2)	± (2)		-(4)
	C (19)	+(19)	D (18)	+(19)	± (19)	-(10)	± (10)		-(8)
	C (23)	+(23)	D (23)	+(23)	-(23)	-(23)	± (23)		-(19)
Oenococcus	C (2)	+(2)	D (2)	+(2)	-(2)	-(2)	± (2)		-(4)
	C (10)	+(19)	D (4)	+(19)	± (19)	-(10)	± (10)		-(14)
	C (19)	+(23)	D (23)	+(23)	-(23)	-(23)	± (23)		
Pediococcus	C[a] (18)	-(2)	L, DL (2)	± (2)	± (2)	-(2)	+(2)		-(5)
	C[a] (19)	-(10)	L, DL (4)	± (19)	± (16)	-(10)	+(10)		-(16)
	C[a] (23)	-(23)	L, DL (23)	± (23)	± (23)	-(23)	+(23)		-(19)
Streptococcus	C (2)	-(2)	L (2)	-(2)	± (2)	-(2)	-(2)		-(5)
	C (10)	-(19)	L (10)	-(19)	± (19)	-(10)	-(10)		-(19)
	C (19)	-(23)	L (23)	-(23)	± (23)	-(23)	-(23)		
Tetragenococcus	C[a] (2)	-(2)	L (2)	+(2)	-(2)	+(2)	-(2)	-[c] (19)	-(19)
	C[a] (19)	-(10)	L (10)	+(10)	-(10)	+(10)	-(10)		
	C[a] (23)	-(19)		+(23)	-(23)	+(23)	-[c] (19)		
Vagococcus	C (2)	-(2)	L (2)	+(2)	-(2)	-(2)	± (2)		-(19)
	C (19)	-(10)	L (10)	+(19)	-(19)	-(10)	± (10)		
	C (23)	-(19)		+(23)	-(23)	-(23)			
Weissella	C/R (2)	+(2)	D, DL (2)	+(2)	-(2)	-(2)	± (2)		-(8)
	C/R (8)	+(8)	D, DL (10)	+(10)	-(10)	-(10)	± (10)		-(16)
	C/R (10)	+(23)	D, DL (23)	+(23)	-(23)	-(23)	± (23)		-(19)

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 heterofermentadoras 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 situable medium; e: small amounts of CO_2 from glucose can be produced; f: growth not occur at 37 °C; g: can be present pseudocatalse activity.

References: (1) Aguirre and Collins, 1992; (2) Axelsson, 2004; (3) Ballongue, 2004; (4) Björkroth and Holzapfel, 2006; (5) Botelho, 2005; (6) Collins *et al.*, 1987; (7) Collins and Wallbanks, 1992; (8) Collins *et al.*, 1993; (9) Engesser and Hammes, 1994; (10) Inês *et al.*, 2008; (11) Kilcher *et al.*, 2010; (12) Linhares *et al.*, 2010; (13) Mazo *et al.*, 2009; (14) Mills *et al.*, 2005; (15) Pin *et al.*, 2002; (16) Potes and Marinho, 2007; (17) Rattanasomboon *et al.* (1999); (18) Schillinger and Lücke, 1989; (19) Silva *et al.*, 2010; (20) Sneath and Jones, 1976; (21) Sneath, 2009; (22) Stiles and Holzapfel, 1997; (23) Wright and Axelsson, 2011.

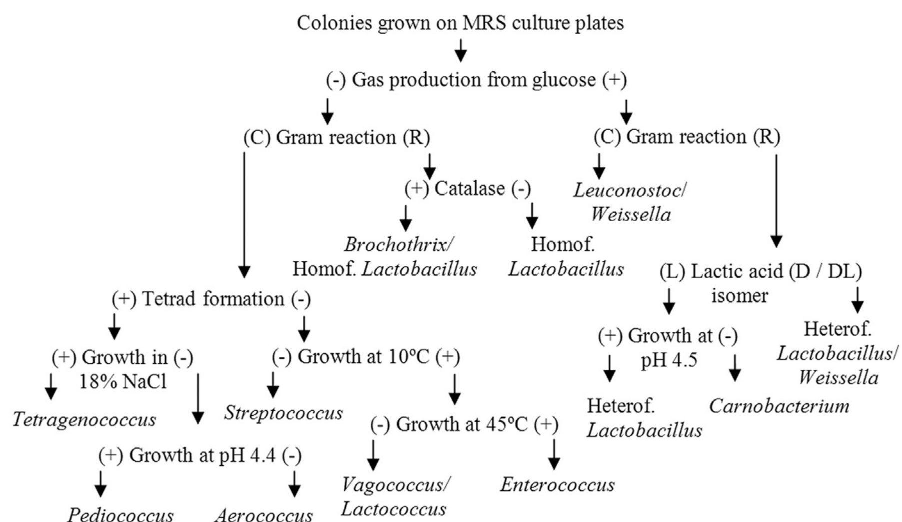

Figure 1 - Flowchart for identification of LAB genera by phenotypic characteristics. C: cocci; R: rods; (-): negative; (+): positive; L, D and DL: optical isomers. The flowchart was adapted from Schillinger and Lücke (1989).

of gas production from glucose: catalase activity was tested by addition of hydrogen peroxide 3% (Oliveira *et al.*, 2008; Marty *et al.*, 2012); growth at 10 °C for 7 days, and at 45 °C for 48 h, were tested in MRS broth with 0.02 g/L of bromocresol purple (Hall *et al.*, 2001; Potes and Marinho, 2007; Wu *et al.*, 2012); growth in 18% NaCl added bromocresol purple 0.02 g/L at MRS broth were tested at 30 °C for 48 h (Hall *et al.*, 2001; Wu *et al.*, 2012; growth at pH 4.4 e 4.5 in MRS broth adjusted to correct pH by the addition 1 M HCl (Schillinger and Lücke, 1989) were incubated at 30 °C for 48 h; determination of lactic acid isomer formed (L, D or DL) was performed using an enzymatic-colorimetric kit (R-Biopharm, 11112821035, Darmstadt, Germany) according to the indication of the supplier. 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 (Hu *et al.*, 2009).

The samples tested for the production of gas from glucose were streaked on MRS agar slants, incubated at 30 °C for 48 h into anaerobic jars (Permution, Paraná, Brazil). From this point the molecular analysis followed the method previously developed by Kullen *et al.* (2000). LAB DNA was extracted from the colonies grown on the MRS slants, and was deposited onto the base of a sterile microcentrifuge tube and microwaved for 6 min at 900 W. Immediately following PCR reagents were added directly to the microcentrifuge tube containing the colony and thermal cycling proceeded. The Primers plb16 (5' AGAGTTTGATCCTGGCTCAG 3') and mlb16 (5' GGCTGCTGGCACGTAGTTAG 3') were used to direct PCR amplification of a 500 bp portion of the 16S rRNA gene. Amplification of DNA was performed in a GeneAmp® PCR System 2400 thermal cycler (Perkin Elmer, Foster City, USA) programmed for 5 min at 94 °C (initial denaturation) and 35 cycles of 15 s at 94 °C (denaturation), 15 s at 55 °C (annealing), 1 min at 72 °C (extension) and 10 min at 72 °C (final extension). Reaction products were resolved by electrophoresis in 1.0% (w/v) agarose gels and visualized by ethidium bromide staining. PCR products were sent to a commercial sequencing facility (Center of Nuclear Energy in Agriculture). Bacterial sequences were compared to the sequences reported in GenBank, using the BLAST (Basic Local alignment Search Tool) algorithm. The nucleotide sequences determined in this study have been assigned Genbank Accession Numbers EU855223, JF756088, GU591801, AB124845 and AB023244.

Results

Isolation and enumeration of total LAB

The results of plate counts on MRS agar showed that the LAB was present in high counts 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). 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.

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 presumptive genera were observed at one day of storage:

Leuconostoc/Weissella sp., *Enterococcus* sp. and homofermentative *Lactobacillus* sp. (Table 3).

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. *H*owever, 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 (batches 2, 3 and 4) and 8 °C (batch 2 and 3), followed by *Leuconostoc/Weissella* sp., predominant at 4 °C in batch 1 and at 8 °C in batches 1 and 4 (Table 4).

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.

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)	Time 45 days (log cfu/g) - 4 °C	Time 45 days (log cfu/g) - 8 °C
1	1.77 ± 0.34	7.95 ± 0.09	8.59 ± 0.04
2	1.67 ± 0.35	7.36 ± 0.12	8.12 ± 0.08
3	2.08 ± 0.07	6.62 ± 1.55	7.74 ± 0.43
4	2.41 ± 0.10	8.44 ± 0.10	8.53 ± 0.12
Mean*	1.98 ± 0.37[a]	7.59 ± 0.97[b]	8.25 ± 0.41[c]

*Means with different letters are statistically different ($p \leq 0.05$) by the Tukey test; n=3.

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 13% 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 count of the initial population of LAB present in the samples (1.98 log cfu/g) were significant influenced by storage temperature; growth values around 7.59 log cfu/g at 4 °C and 8.25 log cfu/g at 8 °C were detected (Table 2) whose values were statistically different ($p \leq 0.05$, by the Tukey test). The colonies present in the plates are white because do not produce pigments and are small, because the fermentation of carbohydrates does not release much energy (Massaguer, 2006). The growth of LAB reaches up to approximately 8 or 9 log cfu/g, 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. Samples of sliced cooked ham stored at 4 °C, at times 0, 3, 7, 15, 25 and 35 days, showed respective LAB plates counts: not detected; 6.04; 6.68; 8.63; 8.67; and 8.51 log cfu/g, which demonstrate the rapid development of LAB (Hu *et al.*, 2009). 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

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 °C	Growth at 45 °C	Genera identified
1*	5	-	Rods	-	NT	NT	Homofermentative *Lactobacillus* sp.
	8	+	Cocci	NT	NT	NT	*Leuconostoc/Weissella* sp.
2*	2	-	Cocci	NT	+0	+	*Enterococcus* sp.
	1	-	Rods	-	NT	NT	Homofermentative *Lactobacillus* sp.
	9	+	Cocci	NT	NT	NT	*Leuconostoc/Weissella* sp.
3	11	-	Cocci	NT	+	+	*Enterococcus* sp.
	1	-	Rods	-	NT	NT	Homofermentative *Lactobacillus* sp.
	3	+	Cocci	NT	NT	NT	*Leuconostoc/Weissella* sp.
4	2	-	Rods	-	NT	NT	Homofermentative *Lactobacillus* sp.
	13	+	Cocci	NT	NT	NT	*Leuconostoc/Weissella* sp.

+: positive; -: negative; NT: not tested; * The number of colonies grown in each plate of triplicate was lower than 5.

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	1	-	Rods	-	Homofermentative *Lactobacillus* sp.
	14	+	Cocci	NT	*Leuconostoc/Weissella* sp.
2	15	-	Rods	-	Homofermentative *Lactobacillus* sp.
3	15	-	Rods	-	Homofermentative *Lactobacillus* sp.
4	13	-	Rods	-	Homofermentative *Lactobacillus* sp.
	2	+	Cocci	NT	*Leuconostoc/Weissella* sp.
Time 45 days at 8 °C					
1	2	-	Rods	-	Homofermentative *Lactobacillus* sp.
	13	+	Cocci	NT	*Leuconostoc/Weissella* sp.
2	15	-	Rods	-	Homofermentative Lactobacillus sp.
3	15	-	Rods	-	Homofermentative *Lactobacillus* sp.
4	6	-	Rods	-	Homofermentative *Lactobacillus* sp.
	9	+	Cocci	NT	*Leuconostoc/Weissella* sp.

+: positive; -: negative; NT: not tested.

15 °C), and when plate counts was around 7 log cfu/g, the product reaches the end of shelf life, which was indicated by changes in sensory quality and pH decrease (Kreyenschmidt *et al.*, 2010). Sliced cooked ham at time 0, 30 and 90 days of storage at 4 °C showed plate counts around 4.65, 8.72 and 7.27 log cfu/g, respectively (Han *et al.*, 2011). Similar results were found 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. (Borch *et al.*, 1996). 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 gas production (CO_2) 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 do not 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 (Axelsson, 2004; Landgraf, 2008).

In the industrial practice of vacuum-packed meat products is common to observe that when the LAB counts exceeds 7 log cfu/g 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 provided by LAB that affect the purchase decision of a consumer, because reach the appearance of product negatively.

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 (Libby *et al.*, 2008). The genus *Bifidobacterium* is grouped with the LAB and shares a few characteristics, however is phylogenetically unrelated (Hall *et al.*, 2001; Axelsson, 2004). 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 (Mazo *et al.*, 2009). 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; this substance can found in tomato juice (Silva et al., 2010).

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 showed no growth after 45 days of storage at 4 °C and 8 °C. This genus is widely distributed in the environment, especially inhabiting the human and animal gastrointestinal tract (Foulquié-Moreno *et al.*, 2006). The results observed in this work are similar to those found by Ammor *et al.* (2005) 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.* (2102) 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 (Foulquié-Moreno *et al.*, 2006).

The LAB are devoid of a “true” catalase and cytochromes when grown in laboratory growth media, which lack heme or related compounds (Axelsson, 2004), but some species of *Lactobacillus* can present a pseudocatalase activity (Engesser and Hammes, 1994). Since this, the proposed classification to separate the genus homofermentative *Lactobacillus* and *Brochothrix* from the catalase test may result an overlap in the case of positive reaction. It is known that the species of *Brochothrix* produce catalase, but a few *Lactobacillus* can also produce it. If a negative reaction of catalase it is found can be conclude that *Lactobacillus* is the only genera present, like as the result showed.

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 (Axelsson, 2004; Giraffa *et al.*, 2010). This genus is one of the original LAB genera and several species of importance in foods have been reclassified in new genera, among: *Carnobacterium* (Collins *et al.*, 1987); *Weissella* (Collins *et al.*, 1993); and *Atopobium* (Collins and Walbanks, 1992). 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* (Axelsson, 2004). 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 (Axelsson, 2004) they are facultative heterofermentative, who were identified like homofermentative *Lactobacillus* by the phenotypic identification. These genera normally do not 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 analysis was 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.* (2009). *Leuconostoc* were able to growth at 8 °C (Hemme and Foucaud-Scheunemann, 2004), 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 (Landgraf, 2008; Silva *et al.*, 2010). 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* (Ammor *et al.*, 2005). Similarly to the results founded in this work, Hu *et al.* (2009) 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 cold storage, development of *Leuconostoc* species is favored in vacuum packed samples, while *Weissella viridescens* is predominant when

the products is pasteurized after packing (Santos *et al.*, 2005). The sliced vacuum-packed cooked ham is not a pasteurized meat product after packing, is only a vacuum-packed, in this way, the results observed in this work are in agreement with the results founded by these authors.

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 (Santos *et al.*, 2005).

The differentiation between the genera *Leuconostoc* and *Weissella*, and heterofermentative *Lactobacillus* and *Weissella* are not possible by means of phenotypic methods. After Collins *et al.* (1993) subdivided the genera *Leuconostoc* into a new genus called *Weissella*, the phenotypic methodologies can only describe the *Leuconostoc* family, than include the genera *Leuconostoc*, *Oenococcus* and *Weissella* (Chelo *et al.*, 2007). Similarly overlaps occurred with the genera *Lactococcus* and *Vagococcus*. The genera *Vagococcus* was created by Collins *et al.* (1989) to accommodate new specie with all the features of *Lactococcus* but also peritrichous flagella (Silva *et al.*, 2010). 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.

Conclusions

The LAB initially present in the samples showed plate counts around 1.98 log cfu/g, and after 45 days of storage were influenced by storage temperature, rising to values of 7.59 log cfu/g at 4 °C and 8.25 log cfu/g 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 for select the main deteriorating LAB aiming inhibit these microorganisms by future studies.

Acknowledgments

The authors would like to thank Center for Nuclear Energy in Agriculture/USP and especially Dr. Siu Mui Tsai of the Laboratory of Molecular Biology for the identification of the LAB species described in this work.

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Associate Editor: Eduardo Cesar Tondo

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