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ORIGINAL ARTICLE

Characterization of the bacterial spoilage flora in marinated pork products

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Abstract

Aims: To investigate the microbiota in marinated, vacuum-packed pork and to characterize isolated bacteria with regard to their spoilage potential.
Methods and Results: Laboratory marinated pork meat and commercial products from three Norwegian producers were examined. Lactic acid bacteria dominated in all products at the expiration date. The flora in marinated products was similar only for products from the same plant. Strains of *Lactobacillus algidus*, *Lactobacillus sakei*, *Lactobacillus curvatus*, *Carnobacterium divergens*, *Carnobacterium maltaromaticum*, *Leuconostoc mesenteroides*, *Leuconostoc carnosum* and *Leuconostoc* sp. were isolated and tested for their spoilage potential. Samples inoculated with *Lact. algidus* or *Leuc. mesenteroides* were rated as most unpleasant by randomly selected people. A sensory panel scored samples with *Lact. algidus* highest for sour and intense odour. *Lactobacillus algidus* was found in products from two out of three production plants. Culture-independent DNA isolation confirmed that cultivation on Blood agar at 20°C yielded a representative picture of the total flora in marinated flintsteak.
Conclusions: *Lactobacillus algidus* may be an important, but underestimated, spoilage organism that needs to be focused on more when spoilage of vacuum-packed meat is considered.
Significance and Impact of the Study: Routine microbial testing may have to be revised in order to detect spoilage LAB that are unable to grow under currently used conditions.

Introduction

Spoilage has always been, and still is a major concern in the food industry. In meat, spoilage becomes apparent in forms of colour changes, off-odours and -flavours, as well as textural changes. In uncooked, vacuum- (VP) or modified atmosphere-packed (MAP) meat, microbial growth is the main cause of meat deterioration (Borch *et al.* 1996; Gram *et al.* 2002).

An increasing amount of meat is today sold as marinated meat products. In northern Europe, industrial marination is a process where meat products are injected with brine containing salt and phosphates before being packed in a flavouring sauce containing salt, sugar, organic acids and herbs or spices in the production plant. According to calculations from the industry, the total market for industrially marinated products in Norway is in the range 2500–3000 tonnes per year with a potential for increase of 10–15% per year the coming years. So far, marination has mainly been used on poultry, but an expansion of the product specter, including other meats, has been observed in the past years.

Extension of the shelf-life of fresh meat is often achieved by packaging products under vacuum or modified-atmosphere conditions, in addition to chill storage (Branen 1983; Stanbridge and Davies 1998; Tsigarida *et al.* 2000). Even though antioxidants have sometimes been added, marination has so far mainly been associated

with taste. Spices and herbs are added to the marinade to enhance the sensory properties of the product. Studies have reported that marination of poultry introduces or selects for new lactic acid bacteria (LAB) in the product and has no positive effects on the microbial quality (Bjorkroth 2005). However, it was shown that the marinade itself is not likely to be the source of specific spoilage LAB (Lundstrom and Bjorkroth 2007). Also the injection of meat with brine before marination has been shown to contribute to the total bacterial load of the products (Bohaychuk and Greer 2003; Greer *et al.* 2004).

Little research has been done to describe the development and final composition of the bacterial flora in marinated meat. Bjørkroth (2005) reviewed the effect of marination on the bacterial composition in marinated poultry, but no corresponding investigation has been done on pork. The microbiota in vacuum-packed and MAP pork has been shown to be dominated by LAB and some other bacterial groups like *Brochotrix thermosphacta* and Enterobacteriaceae (Shaw and Harding 1984; Mcmullen and Stiles 1993; Sakala *et al.* 2002; Holley *et al.* 2004). It is of interest to see whether marinating pork may have an impact on the development of the bacterial composition and whether this influences the microbial and thereby organoleptic quality of the product.

The aim of this study was to determine the microbial flora in fresh, vacuum-packed, marinated pork steak during storage and to identify important spoilage bacteria. The development of the microbial flora was examined and its composition at later storage phases was determined. Fresh meat marinated in the laboratory was used, as well as several commercial Norwegian products. Dominating strains with spoilage potential were used in further inoculation experiments to determine their effect on the development of off-odour in marinated pork.

Materials and methods

Bacterial growth in marinated products

Pork marinated in the laboratory

Fresh, brine injected bone-in pork steaks (flintsteak) were purchased directly from plant B. The steaks were marinated in the laboratory with 6% (w/w) marinade 2b (pH 4·5, Table 1) and vacuum-packed using a Multivac A300/16 (Multivac A/S, Wolfertschwenden, Germany) and vacuum bags (PA/PE 20/70; S-Gruppen ASA, Vinterbro, Norway). All steaks were stored at 4°C for 9 days, followed by storage at 8°C until the end of the storage period. These storage conditions were chosen to simulate a typical scenario where the product is stored under controlled temperature of 4°C at the production plants for 9 days before being distributed to grocery stores and further to consumers' refrigerators where temperatures may not be as closely monitored. The experiment was carried out twice, with independent batches of meat (batches 1 and 2). Samples for microbial examination were taken after 5, 9 and 19 days (also after 12, 16 and 23 days for the second batch, two steaks at each point).

Pre-experiments were conducted to compare different sampling techniques. Results showed that sampling of the marinade and sampling of homogenized meat together with the marinade yielded identical results both for total counts and for bacterial composition (data not shown). As a consequence, sampling of the marinade was chosen as the method to determine bacterial counts and composition.

Bacterial numbers (CFU ml^{-1} marinade) were determined by serial dilution of the marinade in peptone water (0·1% peptone, 0·85% NaCl) and plating on Standard plate count agar (PCA; Oxoid Ltd, Basingstoke, UK), de Man Rogosa Sharpe agar (MRSA; Oxoid), and Blood agar (BL; Oxoid). PCA and MRSA plates were incubated aerobically at 30°C for 3 and 2 days respectively. Two blood plates were incubated for each sample; one for aerobic counts (one week at 15°C) and one for anaerobic counts [stored anaerobically for one week at 15°C, using the AnaeroGen Atmosphere Generation System (Oxoid)]. Colonies were counted on all media and 20 colonies were picked randomly from each countable (25–250 colonies) BL plate. Each colony was placed directly into a 20% glycerol solution, mixed thoroughly and frozen at −80°C for later 16S-rDNA analyses.

In addition, a 1/10 dilution in peptone water of each marinade sample was frozen at −20°C for later analyses.

Commercial marinated pork products

Information on commercially marinated flintsteaks and marinades used in the study is given in Table 1. Commercially marinated, vacuum-packed pork flintsteaks were examined in 2005 (four different products) and 2007 (two products). Products from three different Norwegian production plants (A, B and C) were chosen. Four different marinades (1, 2a, 2b and 3) were used in the products. Marinades 2a and 2b were marinades with similar ingredients (one dry, the other containing water) and from the same producer. All samples were stored at 4°C until 9 days after indicated production day, and then at 8°C until labelled date of expiration. Three parallels of A1, A2a and B2b each and two parallels of C3 were examined in 2005; two parallels of each sample were analysed in 2007.

On the date of expiration bacterial numbers (CFU ml^{-1} marinade) were determined by serial dilution of the marinade in peptone water and plating on PCA, MRSA and BL as in the growth experiment. In addition, samples were analysed on Pseudomonas selective agar with Pseudomonas

Table 1 Commercial flintsteak marinades used in experiments

Production plant	Marinade type	Year	Marinade components*
A	1	2005	Water, salt, glucose, black pepper, paprika, garlic, basil
	2a	2005	Salt, paprika, black pepper, dextrose, white pepper, onion, allspice, curry, celery seed, caraway seed
B	2b	2005 + 2007	Water, salt, glucose, paprika, pepper, onion, allspice, curry, celery seed, caraway seed
C	3	2005 + 2007	Water, salt, vegetable oil, vegetable fat, coriander, pepper, paprika, onion, chilli pepper, caraway, yeast extract

*According to package labelling.

C-F-C supplement SR103 (Oxoid). The results in 2005 showed that the microbiota in marinated products could be isolated from BL incubated aerobically. Therefore, commercial products in 2007 were plated on BL and analysed after 5 days incubation in aerobic environments. Colonies were counted on all media and 20 colonies were picked randomly from each countable BL plate and frozen in 20% glycerol at −80°C for later 16S-rDNA analyses as described for samples marinated in the lab.

All bacterial counts are presented with standard error of the mean (SEM), based $\log_{10}$values.

Culture-dependent identification of bacterial flora

DNA isolation

All frozen colonies were thawed and cultivated on BL for 3 days at 20°C. From each plate, 1–15 colonies, the latter for small colonies, were picked and mixed with Tryptone Soy Broth (TSB, Oxoid CM0129; 45 μl), Guanidine Thiocyanate (Sigma, 4 mol l^{-1}, 135 μl) and Mag Prep Silica Particles (10 μl, Merck, Darmstadt, Germany) in wells of a 96-well Greiner U-plate (Greiner bio-one, Frickenhausen, Germany). DNA was isolated on the Biomek 2000 Workstation (Beckman Coulter, Fullerton, CA, USA) using magnetic Silica particles and Sarkosyl as described by Skanseng *et al.* (2006).

PCR

The 50 μl PCR mix contained DNA (5 μl), primers 8F and 1492r (Turner *et al.* 1999, 10 pmol μl^{-1}, 1 μl each), 10 × PCR buffer F-511 (Finnzymes, Espoo, Finland; 5 μl), dNTP (10 mmol l^{-1}, 2 μl), DyNAzym F-501-L (Finnzymes; 2 μl) and water (34 μl). The PCR conditions were as follows: 4 min of denaturation at 94°C followed by 25 cycles of 95°C for 30 s, 50°C for 30 s and 72°C for 90 s and a final extension step at 72°C for seven minutes. All PCRs were carried out on a GeneAmp® PCR system 9700 (Applied Biosystems, Foster City, CA, USA).

All PCR products were purified on the Biomek using Millipore Multiscreen PCRμ96 filter plates (Millipore Corp., Billerica, MA, USA) according to the producer's instructions.

Sequencing

Sequencing was carried out using the Big Dye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) according to the manufacturer's instructions with primer 534r (5′-ATT ACC GCG GCT GG-3′; 10 pmol μl^{-1}, 0·5 μl).

Resulting DNA was prepared for sequencing on the Biomek using a Montage SEQ96 Sequencing Reaction Cleanup Kit (Millipore), precipitated in formamide (1 : 1 v/v) and sequenced by a 3100 Genetic Analyzer (Applied Biosystems). For commercial samples taken in 2007, the samples were prepared for sequencing by using the Applied Biosystems BigDye® XTerminator™ Purification Kit (Applied Biosystems) according to the producer's manual.

Acquired sequences were compared to known sequences using Blast (http://www.ncbi.nlm.nih.gov/BLAST/, 11/05 and 07/07). *Lactobacillus sakei* and *Lactobacillus curvatus* as well as *Staphylococcus pasteuri* and *Staphylococcus warnerii* are very similar in sequence, and in some cases, Blast searches did not yield unambiguous results. In these cases strains are denoted as *Lact. sakei/curvatus* and *S. pasteuri/warnerii* respectively.

Culture-independent identification of bacterial flora

DNA isolation

Total bacterial DNA was isolated from the meat juice before marination and the marinade after 19 days of storage from meat marinated in the laboratory (batch 2). The frozen meat juice or marinade was mixed with peptone water (500 μl, 37°C) on a whirl mixer for one minute. The mixture was centrifuged at 300 ***g*** for 30 s in a Biofuge fresco (Heraeus Instruments, Osterode, Germany) and 800 μl were removed from the supernatant. The remaining 200 μl were remixed with 600 μl of peptone water. Both the removed 800 μl from the first step and the remixed precipitate were then centrifuged at 13 000 ***g*** for 15 min. The pellets were resuspended in 500 μl TES (10 mmol l^{-1} Tris–HCl pH 8·0, 1 mmol l^{-1} EDTA pH 8·0, 100 mmol l^{-1} NaCl) and the mixtures were centrifuged separately at 13 000 ***g*** for 15 min. The pellets were resuspended in a mixture of Tris–HCl (80 μl,

10 mmol l^{-1}), Lysozyme (90 μl, 40 mg ml^{-1}) and Mutanolysine (10 μl, 5000 U ml^{-1}) and the mixtures were incubated at 37°C for 30 min. Proteinase K (25 μl, 20 mg ml^{-1}) and buffer AL from the Qiagen DNeasy Tissue Kit (Qiagen, Hilden, Germany; 200 μl) were added and the mixtures were incubated at 70°C for 30 min. DNA was then isolated with the DNeasy Tissue Kit (Qiagen, starting at step 4 of the DNeasy Protocol for Animal Tissues) following the producers instructions.

PCR, cloning and sequencing

The PCR was performed using the Qiagen Multiplex kit (Qiagen) with primers 8F and 1492r in a total volume of 25 μl. The cycling conditions were as follows: 95°C for 15 min, 45 cycles of 94°C for 30 s, 50°C for 90 s and 72°C for 90 s and finally an extension step at 72°C for 10 min.

The PCR products were cloned into competent *E. coli* using the TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA) for chemically competent *E. coli*. Ten white colonies were picked from each LB-plate (Oxoid; two plates each from meat juice and 19 days) and each suspended in water (30 μl). All colonies were also replated on new plates of LB medium containing Ampicillin (Sigma-Aldrich, St Louis, MO, USA, 50 μg ml^{-1}). The resuspended colonies were heat shocked at 99°C for 5 min, put on ice for two minutes and centrifuged at 13 000 ***g*** for 1 min.

The supernatant from the heat shocked *E. coli* clones was used as template in a PCR reaction containing the primers HU and HR (Rudi *et al.* 2004; 10 pmol μl^{-1}, 0,5 μl each), 10x PCR buffer F-511 (5 μl), dNTP (10 mmol l^{-1}, 1 μl), DyNAzym F-501-L (1 μl), water (18·5 μl) and template (1 μl). The PCR conditions were as follows: Four minutes of denaturation at 94°C followed by 35 cycles of 95°C for 30 s, 65°C for 30 s and 72°C for 45 s and a final extension step at 72°C for seven minutes. The PCR product was run on an agarose gel (1·5%) at 100V for 30 min. Vector containing the desired insert showed a band at 2000 bp, whereas vector without the desired insert showed a band at 700 bp.

All PCR products were purified on the Biomek using Millipore Multiscreen PCRμ96 filter plates (Millipore) according to the producer's instructions.

Sequencing was performed as described for the culture-dependent method.

Sensory analyses of meat inoculated with LAB

Inoculation of samples

Pork filet (longissimus dorsi) was delivered from plant B and cut to pieces of 10 (±1) g. The samples were placed in vacuum bags (PA/PE 20/70; S-Gruppen ASA) and each sample was inoculated with one single strain of LAB. An overview of the strains used in inoculation experiments is given in Table 2. Strains were selected following a pulse field gel electrophoresis in a way that provided that genetically different strains of all species were chosen. All strains were cultured in Anaerob Basal Broth (Oxoid) supplemented with 2% (w/v) glucose and 0·1% (v/v) Tween 80 at 20°C. Several Eppendorff tubes of each culture were stored at −80°C in 20% (v/v) glycerol. Bacterial cultures of 10^6 CFU ml^{-1} of each strain were prepared in either a brine solution with NaCl (4% w/w) and glucose (2% w/w) or a commercial marinade 2b (Table 1). These cultures (600 μl) were added directly to the samples in the bag with a sterile pipette and distributed evenly on the meat. Finally the samples were vacuum-packed with a Multivac A300/16 (Multivac A/S) and stored at 4°C for 11 days, after that at 8°C until the end of the experiment. Three consecutive experiments (Run 1, 2, 3) were conducted; all runs included cultures in a brine solution, only runs 1 and 2 included cultures

Table 2 Overview of strains used in inoculation experiments

Species	Strain	Source
Lact. algidus	O2 ‡‡	Marinated flintsteak*
	P4	Marinated flintsteak*
	C3-15 ††,‡‡	Marinated flintsteak B2b†
	D1-8 ††,‡‡	Marinated flintsteak C3†
Lact. curvatus	C1-11	Marinated flintsteak B2b†
	C2-9	Marinated flintsteak B2b†
Lact. sakei	C3-10	Marinated flintsteak B2b†
	D2-20 ††	Marinated flintsteak C3†
	18·8	Norwegian fermented fish‡
	Th1	Cooked ham‡ (Bredholt, Nesbakken and Holck, 1999)
Leuc. mesenteroides	G16	Marinated flintsteak*
	M11 ‡‡	Marinated flintsteak*
	C2-7	Marinated flintsteak B2b†
Leuc. carnosum	A2-7	Marinated flintsteak A1†
	B1-12 ††	Marinated flintsteak A2a†
	B-SF-43 ††,‡‡	Commercial protective culture§
Leuconstoc sp.	N8	Marinated flintsteak*
	O10	Marinated flintsteak*
Lact. plantarum	Alc01	Muenstercheese‡
C. divergens	9/1-1	Marinated flintsteak**
	9/1-2	Marinated flintsteak**
C. maltaromaticum	3/5-1	Marinated flintsteak**
	3/5-3	Marinated flintsteak**

*Isolated from flintsteak marinated at Matforsk 2005.
†Isolated from commercial flintsteak 2005 (type indicated).
‡Matforsk's strain collection.
§Dezlivered by Christian Hansen (Protective culture Safe Pro B-SF-43, Hørsholm, Denmark).
**Isolated at Matforsk from quality control samples from plant 2.
††Samples from run 2 were analysed by trained sensory panel.
‡‡Samples from run 3 were analysed by trained sensory panel.

in a commercial marinade. The total bacterial counts of the meat before marination were between $2{\cdot}5 \times 10^3$ and 4×10^4 CFU ml^{-1}.

Odour evaluation by randomly chosen persons

Randomly chosen persons (six persons on each occasion, partly overlapping) evaluated the odour of the samples on a scale from one (unpleasant, sour odour) to nine (pleasant, fresh) after 11, 18, 25 and 29 days of storage. The meat samples were removed from the bags and placed on a Petri dish with a lid. The test persons were given two sets of samples, each set containing one sample for each bacterial strain and one control without added bacteria in random order. One common control was used to calibrate the test persons before they were given the samples, and was given grade six as a starting point. The resulting data were analysed using PROC GLM in SAS 9.1.3 (SAS Institute, Cary, NC, USA). Bacterial strain (2–4 strains of each type), bacterial type, assessors, parallels (two parallels of each sample) and experiment (three consecutive experiments) were used as class variables. Data from day 25 (experiments 1–3) and day 29 (experiment 3) were used.

Odour evaluation by trained panel

In a second set of experiments, selected samples (Table 2) from run 2 and 3 were evaluated by a trained sensory panel (11 persons) using a scoring method with a scale from one to nine, one being not detected, nine being the highest value. Odour intensity, meat odour, sourish odour, sour odour, fresh odour, metal odour, paprika odour and rancid odour were used as attributes. In addition yeast odour was evaluated in run 3. Data from the sensory analyses were evaluated by analysis of variance, using STATISTIX 8.1 (Analytical Software, Tallahassee, FL, USA) and PROC GLM in SAS 9.1.3 (SAS Institute, Cary, NC, USA). The assessor and parallel effects and any interactions involving them, were considered as random effects in the models.

For both sets of odour evaluation experiments, Tukey's pairwise comparison test was used to decide which bacterial types were significantly different from the others at the 0·05 level. An approximate version was used in the more complicated unbalanced model with parallels. The approximate test utilizes the Satterthwaite approximation (Satterthwaite 1946) to estimate the error term and degrees of freedom.

In all sensory experiments, meat juice was isolated from all the vacuum bags at sampling time, diluted and plated on BL. The plates were incubated aerobically at 20°C for 2 (samples inoculated with *Lact. algidus* for seven) days and the total CFU ml^{-1} was determined. Colonies of the dominating species were picked and a selection of strains was sequenced to verify that the added strain was dominating in the final product.

Results

Bacterial counts and microbiota of pork, marinated in the laboratory

Two separate batches of pork meat were marinated with a commercial marinade in the laboratory. Figure 1 shows the development of the bacterial numbers in the marinated product from batch 2, obtained on various cultivating media. Curves from batch 1 followed the same pattern, but indicated lower total numbers (1 log lower than batch 2, results not shown). The anaerobic bacterial count on blood agar was low at day 0 but increased exponentially for 13 days. The number of aerobes did not increase over time until day 9, and after that the increase on aerobically stored plates corresponded to the increase of anaerobic counts. The number of aerobes detected was higher on BL than on MRSA or PCA. The composition of the microbiota in batch one and two of the marinated pork product during the storage period is shown in Fig. 2. Similarity to known sequences in BLAST ranged from 98% to 100%. Meat samples from batch 2 showed a greater diversity in the flora, even before storage. Still, anaerobic, facultative aerobic LAB dominated after day 9.

To rule out the possibility of overlooking dominant bacteria that are unable to grow on the selected media and under the chosen incubation conditions, total DNA was isolated directly from the marinade from batch 2 and cloned into plasmids in competent *E. coli*. Results from DNA sequencing of clones and BLAST-searches are shown in Fig. 3. The results generally confirmed the results

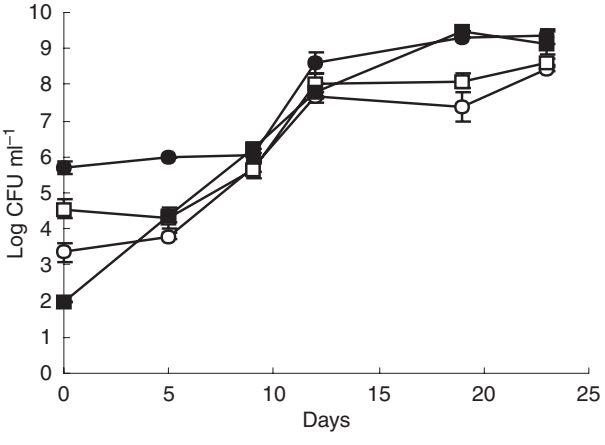

Figure 1 Total bacterial counts on de Man Rogosa Sharpe agar (open circles), Plate Count agar (open squares), Blood agar incubated aerobically (filled circles) and Blood agar incubated anaerobically (filled squares), samples from vacuum-packed pork, batch 2, marinated in the laboratory.

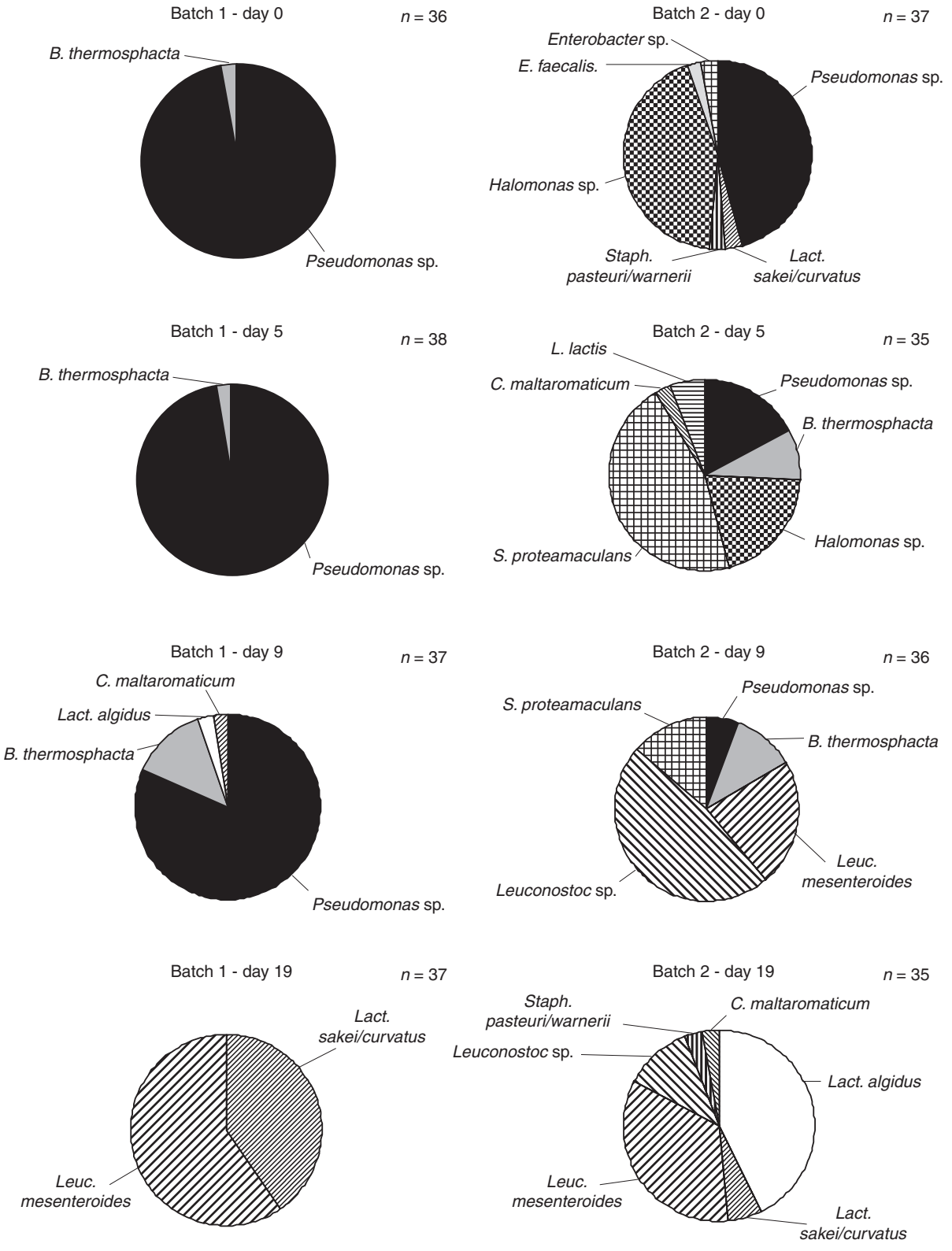

Figure 2 Development of the microbial flora in marinated pork during storage period. *n* = number of sequenced isolates.

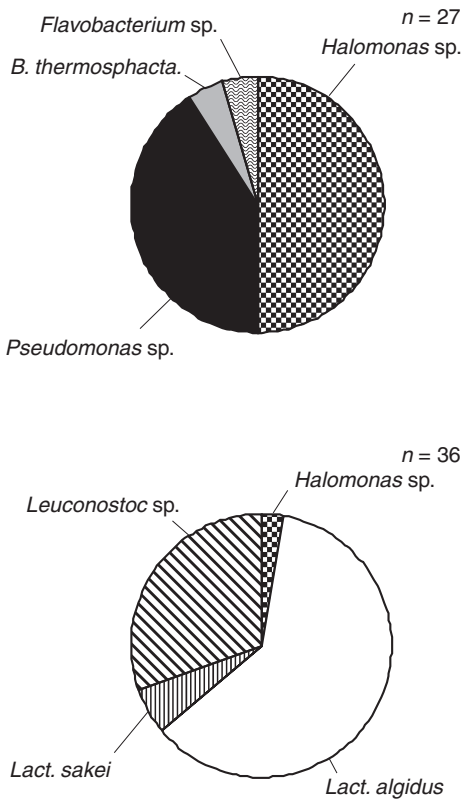

Figure 3 Microbial flora in pork meat, marinated in the laboratory (batch 2), before marination (top) and after marination and 19 days of storage (bottom). DNA was isolated directly from marinade without a cultivating step. *n* = number of sequenced isolates.

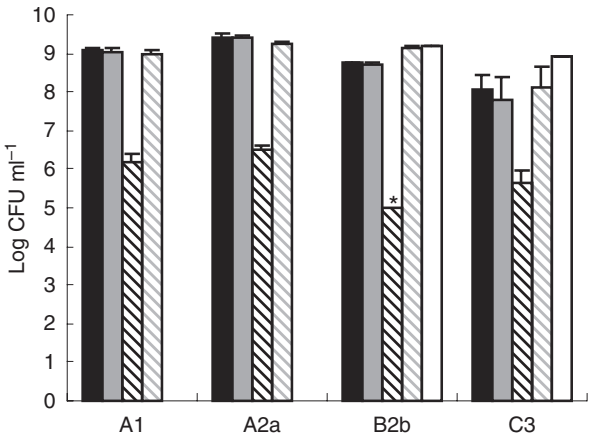

Figure 4 Total bacterial counts (with SEM) in commercial, marinated pork at date of expiration on different media in 2005. A, B, C: Producer; 1, 2a, 2b, 3: Type of marinade. (black: Standard Plate Count Agar; grey: de Man–Rogosa–Sharpe agar; black stripes: Pseudomonas specific agar; grey stripes: Blood agar; white: Blood agar, samples from 2007). *Below detection limit.

obtained by isolating colonies from BL. With the exception of one strain of *Flavobacterium* sp. all detected species had been isolated from the selected growth media.

Microbial level and composition of commercial products

Four different commercial products, two of them in two different years, were examined at the expiration date to see whether production site or type of marinade had an effect on total counts and bacterial composition and whether the bacterial flora was similar from one season to another.

The total number of bacteria on BL at the expiry varied between 6×10^8 and 2×10^9 in all products with the exception of the products from plant C in 2005 (Fig. 4). The composition of the microbial flora at indicated date of expiration is shown in Fig. 5. Results showed that LAB dominated in all products, but *Leuc. carnosum* dominated in both types of products from plant A while *Lact. algidus* dominated in the products from plant B. The experiment was repeated in 2007 for products B2b and C. Results are shown in Fig. 6. As in 2005, *Lact. algidus* was found to be the dominant species in product B2b. Products from plant C did not contain singly dominating strains, either in 2005 or 2007.

Identification of specific spoilage bacteria

Meat samples were inoculated with selected strains suspended in brine or marinade 2b (Table 1) and sensory changes were measured to identify strains contributing to spoilage. Figure 7 shows the results from evaluations of meat odour in the laboratory on a scale from one (sour, unpleasant smell) to nine (fresh, pleasant smell) after 25 and 29 days of storage (for samples inoculated with brine). The graph also presents the total bacterial number, indicating no correlation between bacterial counts and odour evaluations.

Samples inoculated with *Lact. algidus* yielded a significantly lower average score by Tukey's all pairwise comparison test compared to control samples and all other inoculated samples, except samples inoculated with *Leuc. mesenteroides*. Similar results were obtained with meat marinated with a commercial marinade, but in these experiments no overall significance was found (results not shown). Some additional strains that were not isolated from marinated products (Th1, 18.8, B-SF-43 and Alc01, Table 2) were also tested, but did not score significantly different from the control samples.

Evaluation of inoculated meat on the last day of storage by a trained sensory panel showed few significant differences. Selected attributes from one of the two experiments are shown in Table 3. Tukey's all pairwise

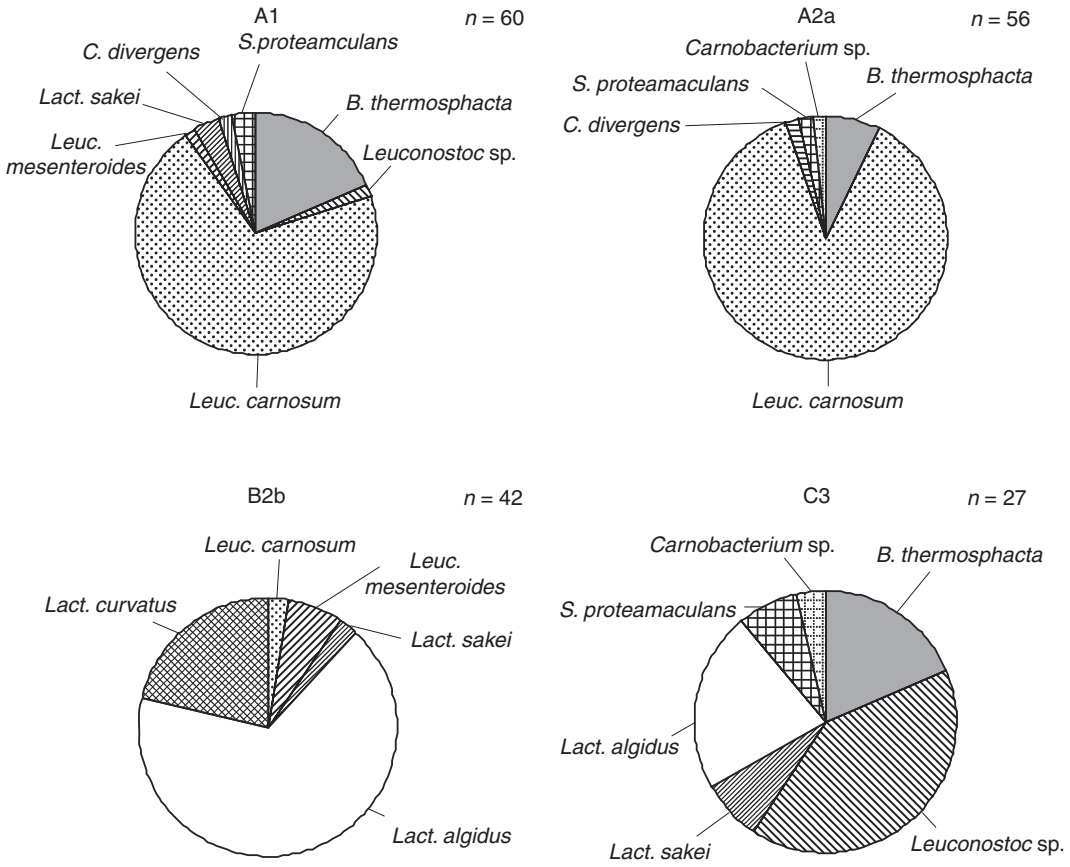

Figure 5 Microbial flora in commercially marinated pork from 2005 at date of expiration. A, B, C: producer; 1, 2a, 2b, 3: type of marinade. *n* = number of sequenced isolates.

comparison test showed that *Lact. algidus* C3-15 had a significantly higher sour smell than the control sample. No other significant differences were found, however trends could be observed: *Lact. algidus* strains scored lowest for meat, sourish and fresh odour, parameters that are perceived as positive by the consumer, while they scored highest for intensity and sour odour. In the replicate experiment, *Lact. algidus* and *Leuc. mesenteroides* strains scored higher than control for sour odour and samples with *Lact. algidus* strains were the only ones to yield any rancid odour (results not shown). No significant differences or trends were found by the sensory panel for the samples containing a commercial marinade.

To verify that inoculated strains were still dominant after storage, 96 colonies were picked after each run and 16S-rDNA analyses were conducted (results not shown). *Lactobacillus algidus* strain D1-8 was not detected after storage in run 3 and results for this strain were hence not considered in the calculations. All other strains used as inocula were found in high numbers at the end of the storage period.

Discussion

The aim of this study was to determine the microbial flora in marinated, vacuum-packed pork products and to identify important spoilage bacteria.

As expected, the examination of the development of the microbial flora in pork, marinated and vacuum-packed in the laboratory, showed a shift from aerobic, Gram-negative *Pseudomonas* to Gram positive LAB. At the end of expiry, LAB dominated the flora in all commercial products. Our results were in agreement with earlier studies that examined the bacterial flora in vacuum- and MA-packed and marinated meat products and found that LAB, especially lactobacilli, *Leuconostoc* sp. and carnobacteria, dominated in late storage phases (Susiluoto *et al.* 2003; Bjorkroth *et al.* 2005; Koort *et al.* 2005). There has so far been no study available that describes in detail the microbial flora in marinated, vacuum packed pork. *Leuc. carnosum* dominated all samples from plant A while *Lact. algidus* dominated samples from plant B. These results suggested that production site has an impact on the development of the bacterial flora

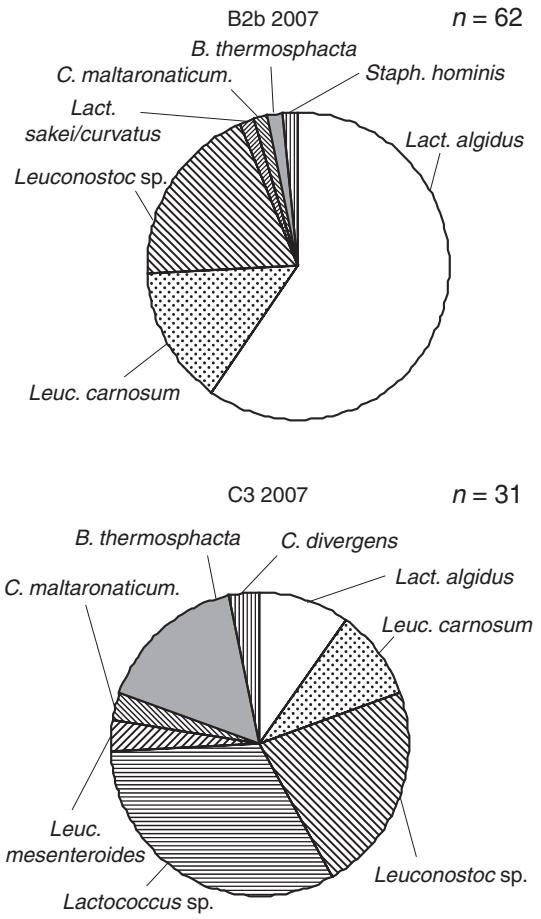

Figure 6 Microbial flora in marinated pork from 2007 at date of expiration. B, C: producer; 2b, 3: type of marinade. *n* = number of sequenced isolates.

while the type of marinade has less effect. Whether this is caused by the presence of a contaminating flora at the production site or the microbial flora of the meat before processing remains an open question. Comparison of the microbial flora on corresponding products in two different years showed that there were slight differences in the flora from one year to the other, but most dominating bacteria were isolated in both years. This further indicated that production site influences the final bacterial composition in the products. Further studies are needed to understand how processing conditions affect the presence and growth of potential spoilage bacteria in marinated meat products.

The storage conditions in this experiment were chosen to represent a realistic scenario with a storage period of 9 days at 4°C followed by 8°C. One cannot rule out that the microbiota would develop otherwise at other storage temperatures.

Sensory analyses in the laboratory and sensory analyses with a trained panel showed that *Lact. algidus* had the most negative impact on the odour of pork in inoculation experiments. It may hence be an important spoilage organism in marinated, vacuum-packed pork. Samples, each inoculated with one of three genetically different strains of *Lact. algidus* were rated significantly lower than control samples and other strains in odour evaluations in the laboratory. They also, together with samples inoculated with *Leuc. mesenteroides*, received highest scores for sour and intense odour and they received lowest scores for fresh and sourish odour by a trained sensory panel. It was confirmed that the differences found in the sensory analyses were due to inoculated strains and not to differences in total bacterial counts. *Lactobacillus algidus* was shown to be the dominant species isolated from marinated pork from plant B and was also present in products from plant C in both 2005 and 2007. It was also isolated as the dominant species from the raw material in one experiment (run 2) where fresh pork meat from plant B was inoculated with LAB, indicating that *Lact. algidus* is not only present in marinated pork products. The flora in samples from plant A was dominated by *Leuc. carnosum*, strains of which did not show any negative impact on the odour of inoculated meat.

For samples that were packed with a commercial marinade, *Leuc. mesenteroides* and *Lact. algidus* were rated

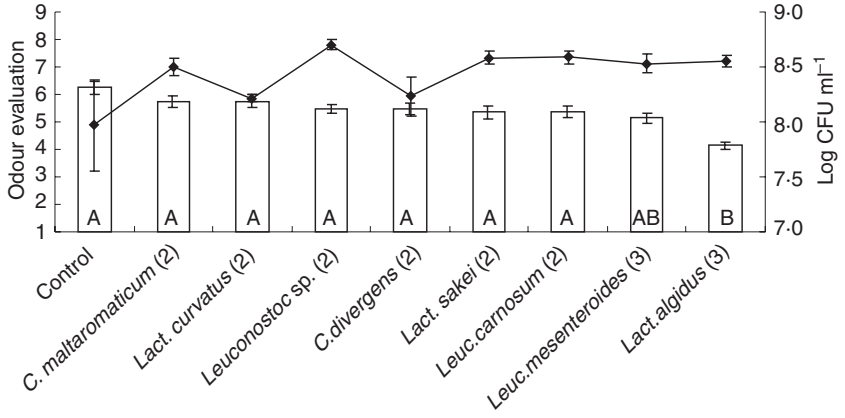

Figure 7 Consumer evaluation of odour of meat samples after 25–29 days of storage, each inoculated with one strain of lactic acid bacteria (bars) and total bacterial counts determined on Blood agar (line), presented as average with SE. Bars bearing different letters are significantly different ($P < 0{\cdot}05$). Number in brackets signifies the number of strains of each species that were tested separately in each experiment. Each bar represents the average of the results from three separate experiments.

Table 3 Sensory evaluation of pork meat, after 26 days of storage, run 2, marinated in brine

Species	Strain	Intensity	Meat	Sourish	Sour	Fresh
Lact. algidus	C3-15	**7·46***	**1·95**	**1·00**	**7·38 (A)†**	**1·05**
Lact. algidus	DI-8	**7·44**	**1·78**	**1·25**	**7·20 (AB)**	**1·10**
Leuc. carnosum	B1-12	6·72	2·42	1·33	6·22 (AB)	1·23
Lact. sakei	D2-20	7·17	2·15	1·34	6·44 (AB)	1·15
None	None	6·85	2·43	1·44	5·96 (B)	1·21
Leuc. carnosum	B-SF-43	7·00	**1·95**	1·40	6·52 (AB)	1·23

*Highest (intensity, sour) or lowest (meat, sourish, fresh) values in bold letters.

†Classification by Tukey's all pairwise comparison test, strains with common letters are not significantly different.

most unpleasant in odour in the laboratory, however no overall statistical significance was obtained. Evaluations of samples with *Leuc. mesenteroides* differed widely between the two experiments and between the three strains that were applied separately. This suggested that different strains of the same species may display different properties, and that differences in meat and storage conditions may affect spoilage properties of various strains.

As far as we know, *Lact. algidus* has been isolated from beef (Sakala *et al.* 2002), but it has not been described as a spoilage organism of meat. *Lactobacillus algidus* has been described as lactic-acid producing, homofermentative rod with a growth optimum between 15 and 25°C, growth at 0°C, but no growth at 30°C (Kato *et al.* 2000). In our experiments, *Lact. algidus* did not grow at 30°C on any tested growth media (MRS, PCA, Blood Agar). It may hence not be detected in industrial quality controls which often include only total aerobic bacterial counts on petri film or on PCA at 30°C as described in NordVal No. 12 (NordVal, 2008) and NMKL method no. 86 (Nordic committee on food analyses, 2006). Since it is a very slow growing organism in air, colonies are even difficult to see on BL the first 2 days of aerobic storage at 20°C. This might result in low bacterial counts and in false conclusions when determining the dominating flora in meat products. Holley *et al.* (2004) described the flora in vacuum-packed, fresh pork; however data were based on colonies from plates that were incubated at 35°C, conditions that would not allow for growth of *Lact. algidus*.

Leuc. mesenteroides has been reported to spoil pork in earlier reports. Vermeiren *et al.* (2005) inoculated cooked pork in a model system with *Leuc. mesenteroides*, *Lact. sakei*, *Leuc. carnosum* and *B. thermosphacta* strains. *Lact. algidus* was not included in the experiment. They showed that *Leuc. mesenteroides* subsp. *mesenteroides* and *B. thermosphacta* gave products with shorter time to rejection compared to control samples while *Leuc. carnosum* and *Lact. sakei* had no negative impact on the shelf-life. These results were in agreement with our results.

A culture-independent method similar to those previously described by Cambon-Bonavita *et al.* (2001), Rudi *et al.* (2002, 2004) was employed to confirm that cultivating the marinade aerobically on BL at 20°C yielded a representative picture of the total flora in marinated pork products. The detection limit was equal in both methods and depending on the number of isolates.

Results showed that *Lact. algidus* may be an important spoilage bacterium that is not only present in marinated pork, but also in other fresh meat products. Its lacking ability to grow at high temperatures and on commonly used growth media and its slow growth in air make it difficult to detect in quality controls. On the other hand, the need for instant results and cost restrictions make it difficult to employ new methods for quality controls with a wider variety of growth media and new growth conditions – lower temperatures, longer incubation time and aerobic and anaerobic incubation. Still the presence of this bacterium must be recognized and detection methods must be evaluated. This study further showed that there was no direct correlation between total bacterial counts and the sensory properties of a product. Even if a method is used that enables the detection of all present microorganisms, further characterization is necessary to make reliable assumptions about the quality of the product.

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