Leuconostoc gelidum and *Leuconostoc gasicomitatum* strains dominated the lactic acid bacterium population associated with strong slime formation in an acetic-acid herring preserve

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Abstract

Spoilage characterised by strong slime and gas formation affected some manufacture lots of an acetic-acid Baltic herring (*Culpea haerengus membras*) preserve after few weeks' storage at 0-6°C. The product consisted of herring filets in acetic acid marinade containing sugar, salt, allspice and carrot slices. Microbiological analyses of the spoiled product showed high lactic acid bacterium (LAB) levels ranging from 4.5×10^8 to 2.4×10^9 CFU/g. Yeasts were not detected in any of the herring samples. Since LAB contaminants seldom are associated with fresh fish, LAB populations associated with marinade ingredients (carrots, allspice) were also analysed. The highest LAB levels exceeding 10^7 CFU/g were detected in equilibrium modified atmosphere packaged baby carrots whereas the levels detected in the allspice samples did nor exceed 4.3×10^5. A total of 176 randomly selected LAB isolates originating from herring, carrot and allspice samples were further identified to species level using a 16 and 23S rRNA gene RFLP (ribotyping) database. *Leuconostoc gelidum* and *Leuconostoc gasicomitatum* strains dominated both in the spoiled herring and carrot samples. These species are heterofermentative producing CO_2 from glucose and they also produce dextran from sucrose. Inoculation of some commercial herring products with spoilage-associated *L. gelidum* and *L. gasicomitatum* strains verified that these strains have the capability of producing slime and gas in herring preserves although slime formation was not as strong as in the original samples. Since *L. gelidum* and *L. gasicomitatum* strains were commonly detected in carrots, carrot slices used for the fish marinade were considered to be the probable source of these specific spoilage organisms.

1 Introduction

Spoilage of semi-preserved, marinated fish products occurs usually due to the growth of non-putrefactive organisms, such as acetic acid-tolerant lactic acid bacteria (LAB) (Blood, 1975). The marinating process applied, i.e. the treatment of the fish with acetic acid and sodium chloride, is responsible for the microbial selection (Blood, 1975; Sharpe and Pettipher, 1983). Spoilage organisms must tolerate low pH (< 5) together with high NaCl concentrations (usually from 2.5 to 5%). Gaseous spoilage type, manifested by bulging of the lids of the jars after some storage weeks, has been associated with acetic acid fish preserves (Blood, 1975; Sharpe and Pettipher, 1983; Lyhs et al., 2001). *Lactobacillus* spp. (Meyer, 1956b; Kreuzer, 1957; Lerche, 1960; Reuter, 1965; Erichsen, 1967; Sharpe and Pettipher, 1983; Lyhs et al., 2001) or yeasts (Somners, 1975) have been the specific spoilage organisms detected in these herring marinades. Limited information exists about the microbial ecology associated with other types of spoilage in marinated fish products. Borgström (1953) reported upon some cases of sliminess occurring in the brine of sugar-salted herring. *Pseudomonas* spp. and less frequently *Leuconostoc* spp. were considered to cause this slime formation.

In this study, an unusual spoilage phenomenon affecting an acetic-acid Baltic herring (*Culpea haerengus membras*) preserve is described. During a problematic manufacturing period, some plastic containers started to show bulging due to gas formation after 2 to 3 storage weeks. At the same time, extremely strong slime and gas formation was observed in the marinade. The products were manufactured in one processing plant and they were expected to maintain good quality during a shelf-life of 6 months at the recommended storage temperature between 0°C to 6°C. Spoilage had affected only some production lots and occurred from time to time. According to the manufacturer, fresh, good quality raw fish had always been used for the product but the quality of some other ingredients, such as carrot slices and spices used in the marinade, had been called in question. This was considered meaningful, since only certain LAB species have been found in low numbers in the normal microflora of healthy fish (Ringø et al., 1998) and it has been suggested that bacterial strains thriving from other sources may contaminate herring products (Borgström, 1953; Lerche, 1960; Krüger, 1973; Lyhs et al., 2001).

The aim of this study was to identify the organisms associated with this unusual spoilage case. Microbiological analyses enumerating LAB and yeasts were performed and a 16 and 23S rRNA gene RFLP (ribotyping) database was used for the identification of the spoilage LAB. In order to evaluate the contamination risk associated with the non-fish ingredients used for the fish marinade, we identified LAB populations in carrot and allspice samples. Finally, the spoilage potential of the dominating herring strains was verified by inoculation tests in some commercial herring products.

2 Materials and methods

2.1 Acetic-acid herring preserve samples associated with spoilage

A total of 7 containers of marinated herring were studied. Five of the containers showed strong slime and gas formation indicating spoilage (Fig. 1), and two had a normal appearance. The total weight of a container was 2400 g, of which 1420 g were fish, 80 g ingredients used for spicing and decoration, and the rest consisted of water and acetic acid. All containers held herring cut into pieces, onions, carrots, water, sugar, salt (NaCl), acetic acid and allspice. According to the manufacturer, the salt and sugar concentrations of the product were 2.4% and 18% (w/w), respectively. The recommended storage temperature was from 0 to 6°C. At the time of the study, only few weeks less than the 6 months expected shelf-life (at the recommended temperature) were still remaining.

2.2 Carrot and allspice samples

A total of 9 packages of different types of carrots were analyzed for spoilage LAB. Unfortunately the ingredients used for the spoiled lots were not anymore available but the handling of the carrots mimicked the protocols used for the herring manufacture. Equilibrium modified atmosphere (EMA) packaged carrots were also studied because sometimes carrots sliced and packaged elsewhere might be used. Five of the carrot samples consisted of 200 g EMA-packaged baby carrots, 2 were 500 g packages of washed common carrots and the last 2 were 500 g packages of washed and organically-grown carrots. EMA-packaged baby carrots and the peels of the other carrot types were analyzed immediately. After peeling, the common and "organic" carrots were sliced and stored in plastic bags at 4°C up to 4 weeks in order to simulate the circumstances associated with the herring manufacture. During the storage period, the slices were analyzed once a week.

A total of 8 packs of commercial allspice from 5 different Finnish companies were analyzed for spoilage LAB. Five of the packs contained whole and 3 grind allspice.

2.3 Sensory evaluation of the acetic-acid herring preserves and determination of the swell type

Evaluation of color, odor, appearance and texture of the spoiled herring products was performed by five trained judges as described by Korkeala and Lindroth (1987).

CO_2 swell was distinguished from hydrogen or H_2S swell using KOH as described by Jay (2000).

2.4 Microbiological analyses and pH measurement

LAB in all samples were enumerated from 10-fold serial dilutions on MRS agar (Oxoid, Basingstoke, UK) as described by Lyhs et al. (1999). The plates were incubated in an anaerobic jar with a H_2+CO_2 generating kit (Oxoid) at 25°C for 5 days. The allspice samples were also enriched in MRS broth (Difco, Detroit, Michigan, USA) containing 1% sorbic acid as a yeast inhibitor (MRS-S broth) at 25°C for 3-4 days. For enrichment, 1 g of allspice was weighed into 9 ml of MRS-S broth and if growth was detected, a loop-full of the broth was spread onto MRS agar to provide colonies.

Yeasts were determined by the method of the Nordic Committee on Food Analysis (1993) using OGYE agar (Oxoid). The plates were incubated aerobically at 25ºC for 3 days.

The pH was determined from the first sample dilution by a WTW-530 Digital-pH-meter (Wissenschaftliche-Technische Werkstätten, Weilheim, Germany).

2.5 Selection the LAB strains for species identification

Altogether 176 colonies were picked randomly from the MRS plates (Oxoid) and cultured pure using MRS broth (Difco) and MRS agar (Oxoid) as described by Lyhs et al. (2002). A total of 76 colonies were selected from the herring samples, of which 10 colonies originated from a container possessing a normal appearance and 66 were from the ones showing spoilage. From the carrot samples, a total of 63 colonies were picked. These included 19, 10 and 34 colonies from the baby, common, and organically-grown carrots, respectively. From the allspice, 12 colonies were selected from the plates without enrichment and seven strains were cultured pure from the enrichment broths. All 176 isolates were subjected to species identification and they were stored, if needed, in MRS broth (Difco) at -70°C.

2.6 Isolation of DNA, restriction endonuclease analysis (REA) and 16 and 23S rRNA RFLP (ribotyping)

Cells harvested from 1 to 2 ml (depending on the growth) of MRS broth (Difco) culture were used for DNA analyses. DNA was isolated by the guanidium thiocyanate method of Pitcher et al. (1989) as modified by Björkroth and Korkeala (1996a) by the combined lysozyme and mutanolysin (Sigma, St. Louis, Missouri) treatment. Restriction endonuclease treatment of 3µg of DNA was done using *Hin*dIII restriction enzyme as specified by the manufacturer (New England Biolabs, Beverly, Mass.). The rDNA probe was labeled for ribotyping by reverse transcription (AMV-RT, Promega, Madison, Wisconsin and Dig DNA Labeling Kit, Roche Molecular Biochemicals, Mannheim, Germany) as described by Blumberg et al. (1991). REA, genomic blots and hybridization of the membranes were done as described (Björkroth and Korkeala, 1996a). *Hin*dIII enzyme was chosen because it has been found to provide species-specific patterns for various LAB (Björkroth and Korkeala, 1996b; Björkroth and Korkeala, 1997; Björkroth et al., 1998; Björkroth et al., 2000; Björkroth et al., 2001).

2.7 Pattern analysis

The *Hin*dIII ribopatterns were compared with the corresponding patterns in the previously established LAB database of the Department of Food and Environmental Hygiene, University of Helsinki. This database comprises patterns of all relevant spoilage LAB in the genera of *Carnobacteria*, *Enterococcus, Lactobacillus*, *Leuconostoc*, *Pediococcus* and *Weissella* (Björkroth and Korkeala, 1996b; Björkroth and Korkeala, 1997; Björkroth et al., 1998; Björkroth et al., 2000; Björkroth et al., 2001). For numerical analysis, ribopatterns were scanned using a Hewlet Packard (Boise, Idaho) ScanJet 4c/T scanner and analyzed using the BioNumerics 2.5 software package (Applied Maths, Sint-Martens-Latem, Belgium). The similarity between all pairs was expressed by Dice coefficient correlation and UPGMA (unweighed pair group method using arithmetic averages) clustering was used for the construction of the dendrograms. Based on the use of internal controls, pattern optimization and band position tolerances of 1.0 and 1.5%, respectively, were allowed. Species identification was based on the locations of the type strains in the clusters formed.

2.8 Verification of the spoilage potential by inoculation tests

Four specific spoilage strains originating from the product possessing slimy appearance were used for the inoculation test. The strains were selected based on their ribopatterns and they represented the major spoilage-associated bacterial types: 2 *L gasicomitatum* strains KSL 3-8 and KSL 3-15 possessing ribotypes IIId and IIIb and 2 *L. gelidum* strains KSL 3-13 and KSL 3-14 possessing ribotypes IIb and IIa, respectively. Three different strain combinations A, B and C were used for the inoculations. Combination A contained cells from all four strains, combination B the two *L. gasicomitatum* strains and combination C the two *L. gelidum* strains. Four different (Table 4) commercial herring products possessing initial LAB levels <100 CFU/mg and pH values from 4.3 to 4.5 were used as the test material. LAB were enumerated as described in the section 2.4. NaCl content of the products varied from 2.5 to 3.5% (w/w) and the highest sugar contents, 28 to 30% (w/w) were in the tomato and sugar-spice marinated products whereas 19 to 20% (w/w) had been used in acetic acid and acid marinated products. Combinations A, B and C were made using MRS broth culture mixes adjusted according their OD_{600} values to the final cell density of approximately 5×10^5 CFU/ml. One ml of the strain combination was added into 100 g of commercial herring product placed in a low oxygen-permeable plastic pack. This resulted in the initial level of approximately 5000 CFU/g. The packs were vacuum sealed and kept at 6ºC for 2 weeks. During the storage, gas and slime formation was observed and finally the packs were opened and the slime and gas formation were judged (3 judges) from none to strong using a score from 0 to 5, respectively. Table 4 shows the product and strain combinations studied.

3 Results

3.1 Microbiological, pH, sensory and swell type analyses

Table 1 shows the LAB counts on MRS agar and the corresponding pH values obtained from the 7 herring containers. The spoiled samples showed LAB growth up to 2.4×10^9 CFU/g whereas one of the unspoiled samples showed no growth and the other 9.8×10^5 CFU/g. Yeasts were detected neither in the unspoiled nor in the spoiled samples. The pH of the spoiled samples ranged from 4.7 to 4.8 whereas pH from 4.3 to 4.4 was detected in the unspoiled samples. Visual examination of the 5 containers showed clear bulging of lids, significant slime formation, and gas bubbles in the marinade (Fig. 1). All judges deemed the contents unfit for human consumption and also detected an off-odor described as buttery, butyrate-like or curd cheese-like. However, the texture of the fish was found normal. No negative remarks were associated with the two normal-looking contents. The swell type detected was a CO_2-swell.

Table 2 shows the results from the microbial enumeration on MRS agar and the corresponding pH values in all carrot samples. LAB counts up to 2.3×10^7 CFU/g were observed in the EMA-packaged baby carrots. The peels of carrots showed growth from 7×10^4 to 2.1×10^6 CFU/g. In the slices of the common carrots, the LAB counts increased from 10^3 CFU/g of the first storage week up to 6.0×10^5 CFU/g in the fourth week. In the organically-grown carrots, counts ranged from the initial 1.2×10^4 CFU/g to 8.7×10^6 CFU/g at the end of the 4 weeks storage. The pH values in the carrot samples varied between 5.4 and 6.6 showing reduction parallel to the increasing LAB levels.

In the allspice, levels of LAB between 1.1×10^4 CFU/g and 4.3×10^5 CFU/g and pH values from 5.2 to 5.6 were detected.

3.2 LAB associated with the herring product

Table 3 shows the division of the *Hin*dIII ribotypes of LAB strains from the spoiled and unspoiled herring samples. The different patterns obtained and a dendrogram based on the pattern similarity are seen in Fig. 2. Three main clusters (cluster I-III) were formed at the similarity level of 56% (Fig. 2). Cluster I contained the type strain of *Leuconostoc mesenteroides* (CCUG 21965), cluster II of *L. gelidum* (NCFB 2775) and cluster III of *L. gasicomitatum* (LMG 18811). The herring isolates in these 3 clusters either shared identical pattern with the type strain or showed high (83-90%) similarity with them (Fig. 2) and were thus identified according the position of the type strain in the cluster. In all spoiled samples, an even distribution of *L. gelidum* and *L. gasicomitatum* strains was observed types IIa, IIb, IIIb and IIId being the most prevalent. Also in the one sample looking normal but already showing LAB growth of 9.8×10^5 CFU/g these strains were detected.

3.3 LAB associated with carrots and allspice

In Fig. 3, the different patterns and the clustering based on the similarity of the patterns is shown. Ten main clusters (cluster I-X) were formed at the similarity level of 75%. Seventy-four isolates from the different carrot products and their peels possessed the ribotypes Ic, IIb, IIc, IIIc, IIId, IVb and IVc and were assigned to the genus *Leuconostoc*. Forty isolates were identified as *L. gelidum*, of which 35 isolates possessed type IIc and 5 isolates had patterns identical with the pattern (IIb) of the *L. gelidum* type strain. From the total of 22 isolates, 21 isolates possessed ribotype IIIc being identical with the ribotype of *L. gasicomitatum* (LMG 18811) type strain. The ribotype IIId (one isolate) differed from the pattern of the type strain by one fragment only. In the organic carrots and their peels more *L. gelidum* strains than *L. gasicomitatum* were found (Table 3). Cluster IV contained the different patterns (IVa-IVc) gained from 11 isolates together with the *Leuconostoc citreum* type and reference strains (LMG 11417 and LMG 9824). The reference strains shared an unique pattern, ribotype IVa. The patterns of the five isolates possessing ribotype IVb had one band difference compared to ribotype IVc possessed by six isolates. Four isolates and the type strain of *Enterococcus faecium* (LMG 11423) shared identical patterns (Cluster VI). Patterns in the cluster V were also considered to belong to the genus *Enterococcus* but they did not match any reference strains closely. Cluster VIII, divided into eight subclusters (VIIIa-VIIIh) contained the different ribotypes gained from seven isolates together with the type strains of *Lactococcus lactis* subsp. *hordniae* (LMG 8520), *L. lactis* subsp. *lactis* (LMG 6890) and *L. lactis* subsp. *cremoris* (LMG 6897). Due to the adjacent *Lactococcus* cluster, stains in the cluster IX were also considered as lactococci but species-level identification was not obtained. The 4 strains possessing types Xa and Xb were not identified by this database. Table 3 shows the amount of the isolates possessing the different *Hin*dIII ribotypes and the corresponding LAB species detected in the carrot and allspice samples.

3.4 Verification of spoilage potential by strain inoculation

Table 4 shows the EPS and gas formation in 4 commercial herring products. Gas production was seen in all products but the strongest EPS production was detected in the tomato and sugar-spice marinated herring products. Already after 2 days marinades turned cloudy (could not be observed in the not-transparent tomato marinade) and gas formation was visible in all products in few days. pH of the products remained unchanged or were slightly reduced (by 0.1 to 0.2).

4 Discussion

Leuconostoc spp. were the specific spoilage organisms (SSO) in this acetic-acid herring preserve; 50% of all isolates were identified as *L. gelidum* and 48% as *L. gasicomitatum* species. They are unusual LAB species for this type of a fish preserve. *L. gelidum* species was described by Shaw and Harding (1989) and it has been reported to occur in vacuum or modified atmosphere-packed (MAP) meat products stored at chilled temperatures (Williamson, 1959; Leisner et al., 1995). *L. gasicomitatum* had previously been associated only with MAP poultry products (Björkroth et al., 2000; Susiluoto et al., 2002) and fresh cut produce (Jacxsens et al., 2001). This species was described by Björkroth et al. (2000) and it was then considered to be a SSO in MAP, tomato-marinated broiler meat strips showing gaseous spoilage. This finding was supported recently by Susiluoto et al. (2002) revealing that *L. gasicomitatum* is dominating the developing spoilage LAB population in retail, MAP, marinated broiler meat strips.

Slime formation is a rare spoilage type in fish products. It has been reported twice affecting the brine of sugar-salted herring (Borgström, 1953; Magnússon and Möller, 1985). *Pseudomonas* spp., *Leuconostoc* spp. and halophilic Gram-negative, oxidase-positive, aerobic rods were then considered to be responsible for the sliminess. Some LAB are able to produce exopolysaccharides (EPS), which can be secreted into the extra cellular environment (De Vuyst and Degeest, 1999). The formation of EPS can be advantageous to some products serving as viscosifying, stabilizing or gelling agents (Cerning, 1990; van den Berg et al., 1993; Stingele et al., 1996; De Vuyst and Degeest, 1999; Duboc and Mollet, 2001). On the other hand, EPS from *Pedicoccus* spp. and heterofermentative lactobacilli may spoil alcoholic beverages such as beers, ciders and wines (Williamson, 1959; Llaubères et al., 1990; Lonraud-Funel et al., 1993; Back, 1994; Duenas et al., 1995; Manca de Nadra and Strasser de Saad, 1995; Fernandez et al., 1996; Satokari et al., 2000). Also in vacuum-packaged, cooked meat products the formation of ropy-slime is a known spoilage sign and in these products it has mainly been associated with *L. sakei* species (Korkeala and Lindroth, 1987; Mäkelä et al., 1992b; Korkeala and Björkroth, 1997).

The slime in the acetic acid preserve was resisting deformation and had a thick, clumpy texture resembling wall paper paste (Fig. 1). This confers to the viscosity of dextran, a homopolysaccharide produced by the action of dextran-sucrase of *Leuconostoc* spp. on sucrose. *L. carnosum*, *L. gasicomitatum* and *L. mesenteroides* species detected in this acetic acid preserve are all able to produce dextran from sucrose (Björkroth et al., 2000; Garvie, 1979; Garvie, 1983; Shaw and Harding, 1989) and also the inoculation test verified the slime-production in two of the herring products tested. *L. gasicomitatum* and *L. carnosum* strains have not been reported to produce slime on ham or broiler products (Björkroth et al. 1998, 2000). This is probably due to the lack of sucrose in these meat products. On the contrary to leuconostocs, the ropy-slime producing *L. sakei* strains cause slimy spoilage also in sucrose-devoid meat products (Korkeala et al., 1988). The EPS produced by *L. sakei* are, however, usually very viscous glucose and galactose-containing heteropolysaccharides (Duboc and Mollet, 2001) and their formation is very different from homopolysaccharides like dextran (Monsan et al, 2001).

Since fish barely contains carbohydrates, the dominating *Leuconostoc* spp. in the present case must have used the sucrose added by the manufacturer as crystal sugar and carrots in the marinade. Also in our inoculation test, the strongest EPS production was observed in the two herring products containing approximately 30% (w/w) sugar. When Magnússon and Möller (1985) studied the ability of the slime-producing bacteria associated with the brine of sugar-salted herring, they did not detect ropiness in brine where sucrose had been substituted by glucose. In order to avoid sliminess Magnússon and Möller (1985) recommended the use of glucose instead of sucrose. However, obligatory heterofermentative LAB produce gas during glucose fermentation and substituting sucrose with glucose may therefore lead to gaseous spoilage type. The butter-like off-odor detected in the spoiled product was likely associated with diacetyl formation. Because the fish muscle is not rich in citrate, another precursor producing pyruvate may have triggered diacetyl formation.

The slime formation in the acetic acid preserve was accompanied with bulging of the containers and a slight increase in the pH. Meyer (1956a) was the first to report this type of LAB spoilage and he called it as “protein swell”. It has been suggested that the acetic acid provides an environment suitable for the action of proteolytic enzymes present in the fish muscle. The products of this proteolysis, i.e. amino acids, provide an energy source for the acetic acid-tolerant LAB (Meyer, 1962b; Stamner, 1976). Usually in the case of LAB spoilage, product pH falls due to the formation of lactic acid but in “protein swell” pH is elevated. This has been attributed to the production of ammonia by deamination of amino acids. In “protein swell”, CO_2 production in the product may be due to the decarboxylation of amino acids being independent from the heterofermentative glucose utilization. This type of LAB spoilage has been caused by *Lactobacillus* spp. in marinated herring products (Meyer, 1956b; Kreuzer, 1957; Lerche, 1960; Meyer, 1962a; Reuter, 1965; Erichsen, 1967; Sharpe and Pettipher, 1983; Lyhs et al., 2001). It has also been associated with anchovy-stuffed olives (Harmon et al., 1987) and it was suspected to cause gaseous spoilage in MAP, raw, tomato-marinated broiler meat strips (Björkroth et al., 2000). Leuconostocs are, however, obligatory heterofermentative LAB and produce gas (CO_2) also during the fermentation of glucose. This was probably the reason for gas formation in the inoculated herring products. Since the pH of these products did not rise, we were not able to repeat the decarboxylation reaction considered to have happened in the spontaneously spoiled acetic acid herring preserve. These results emphasize how the type of the spoilage reaction varies between the different products. Future studies are warranted in order to clarify the metabolism associated with these bacterial strains in herring products.

During this study, EMA-packaged baby carrots and carrots stored up to four weeks at 4°C showed higher numbers of LAB (Table 2) than the levels of 10^3 to 10^4 CFU/g reported previously (Carlin et al., 1989; Garg et al., 1990; Liao and Fett, 2001). An increase of LAB counts up to 10^5 CFU/g was observed during the 4-weeks cold storage of carrot slices. LAB have also previously been associated with cold-stored carrot products (Kakiomenour et al., 1995; Barry-Ryan and O'Beirne, 2000). In our study, the majority of the strains isolated from EMA-packaged baby and organically-grown carrots and their peels were identified as *L. gelidum* and *L. gasicomitatum*. In the common carrots and their peels, mostly *L. citreum* strains were found. The occurrence of leuconostocs in vegetable products has also been reported previously (Garg et al., 1990; Garcia-Gimeno and Zurera-Cosano, 1997) but the species were not identified.

We considered the carrots as a risk for *L. gelidum* and *L. gasicomitatum* contamination even there were some differences between the ribotypes associated with the spoiled herring preserve and carrot samples. In the allspice samples, mostly strains of *E. faecium* and *L. lactis* subsp. *hordniae* were identified and these species did not play any apparent role in the spoilage process. After this study, the manufacturer started to pay closer attention to the carrot quality and storage times for sliced carrots. It is now already a year since the last spoiled lot was detected. These results emphasize the fact that all ingredients, even used only as small amounts for decoration and spicing, play an important role in the hygiene of food manufacture.

5 Conclusion

Leuconostoc spp. were the specific spoilage organisms (SSO) in an acetic-acid herring preserve shoving slimy spoilage type. Fifty-% of all isolates were identified as *L. gelidum* and 48% as *L. gasicomitatum* species. These same species were also commonly detected in carrot samples and during cold storage their levels were increased. Cold-stored carrots must therefore be considered as a risk for *L. gelidum* and *L. gasicomitatum* contamination.

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Table 1. Growth on MRS agar and pH values in an acetic-acid herring preserve possessing either normal (samples 1 and 2) appearance or slimy spoilage type

Sample no.	Bacterial counts (CFU/g)	pH
1[a]	< 100	4.3
2[a]	9.8×10^5	4.4
3	6.8×10^8	4.8
4	9.9×10^8	4.7
5	4.5×10^8	4.7
6	2.4×10^9	4.7
7	1.5×10^9	4.7

[a] Sample of normal appearance.

Table 2. Growth on MRS agar and pH values detected in equilibrium modified atmosphere-packaged baby carrots, sliced common and organic carrots stored at +4°C up to 4 weeks and their peels at the beginning of the storage

Product	Time of analysis	Package No.	Bacterial counts (CFU/g)	pH
Common carrots, slices	Week 1	1	1.6×10^4	6.4
		2	10^3	6.4
	Week 2	1	3.0×10^4	6.6
		2	10^4	6.5
	Week 3	1	-	6.3
		2	3.6×10^4	6.5
	Week 4	1	6.0×10^5	6.5
		2	1.2×10^5	6.4
Organic carrots, slices	Week 1	1	1.2×10^4	6.5
		2	1.4×10^4	6.4
	Week 2	1	3.6×10^5	6.4
		2	2.3×10^5	6.3
	Week 3	1	2.0×10^6	6.2
		2	4.5×10^5	6.4
	Week 4	1	8.7×10^6	5.4
		2	1.1×10^6	5.5

Table 2 continues.

Product	Time of analysis	Package No.	Bacterial counts (CFU/g)	pH
Peels of common carrots		1	8.6×10^5	6.3
		2	2.1×10^6	6.2
Peels of organic carrots		1	7.0×10^4	6.4
		2	1.5×10^5	6.3
Baby carrots		1	1.9×10^6	5.7
		2	2.3×10^7	5.4
		3	4.2×10^6	5.7
		4	4.3×10^6	5.4
		5	5.0×10^6	5.4

Table 3. Lactic acid bacterium species distribution, ribotypes and numbers of the strains detected in the herring,, carrot and allspice samples

Species	Ribotype	Number of strains in sources							
		Herring product with normal appearance	Herring product with slimy spoilage	Baby carrots	Organic carrots	Peels of organic carrots	Common carrots	Peels of common carrots	Allspice
Leuconostoc mesenteroides subsp. *mesenteroides*	Ia		1						
	Ic							1	
Leuconostoc gelidum	IIa	7	17						
	IIb		15	1	2	2			
	IIc			8	21	6			
Leuconostoc gasicomitatum	IIIa		2						
	IIIb	2	5						
	IIIc			9	10	2	1		
	IIId	1	26						
Leuconostoc citreum	IVb						1	4	
	IVc			1			2	3	
Enterococcus sp.	Va								5
	Vb								1

Table 3 continues.

Species	Ribotype	Number of strains in sources							
		Herring product with normal appearance	Herring product with slimy spoilage	Baby carrots	Organic carrots	Peels of organic carrots	Common carrots	Peels of common carrots	Allspice
Enterococcus faecium	VI								4
Lactobacillus curvatus subsp. *curvatus*	VII				1				
Lactococcus lactis subsp. *hordniae*	VIIIa								2
	VIIIc								1
	VIIId								2
	VIIIe						2		
Lactococcus sp.	IXa								1
	IXb								1
	IXc								1
	IXd								1
Not identified	Xa						3		
	Xb						1		
Total		10	66	19	34	10	10	8	19

Table 4. Exopolysaccharide (EPS) and gas formation in 4 commercial herring products after 2 weeks storage at 6ºC. Inoculations were made using 2 *L. gasicomitatum* strains and 2 *L. gelidum* strains as 3 combinations A, B and C containing all four strains, the two *L. gasicomitatum* strains or the two *L. gelidum* strains, respectively. Inoculum cell density of 5 × 10^5 CFU/ml into 100g of herring was used in all tests and the severities of slime and gas formation were judged by scoring from none (0) to severe (5).

LAB strain combination	Herring filets in acetic acid		Herring in acid marinade		Herring in tomato marinade		Herring in sugar and spice marinade	
	Gas	EPS	Gas	EPS	Gas	EPS	Gas	EPS
A	5	1	3	0	4	4	4	3
B	5	1	3	0	5	4	5	2
C	5	1	3	0	ND[a]	ND	ND	ND

[a]ND, not determined

LEGENDS TO THE FIGURES

Fig. 1. Strong slime formation in an acetic acid herring preserve after 2 to 3 weeks storage at 0 to 6°C.

Fig. 2. *Hin*dIII 16 and 23S RFLP patterns and numerical analysis of the patterns presented as a dendrogram. Patterns obtained from lactic acid bacterium strains detected in an acetic acid herring preserve showing slimy spoilage. Left side of the banding patterns possesses high molecular weights, < 23 kbp, and right side >1000 bp.

Fig. 3. *Hin*dIII 16 and 23S RFLP patterns and numerical analysis of the patterns presented as a dendrogram. Patterns obtained from lactic acid bacterium strains detected in carrot and allspice samples. Left side of the banding patterns possesses high molecular weights, < 23 kbp, and right side >1000 bp.

Fig. 1 Lyhs et al.

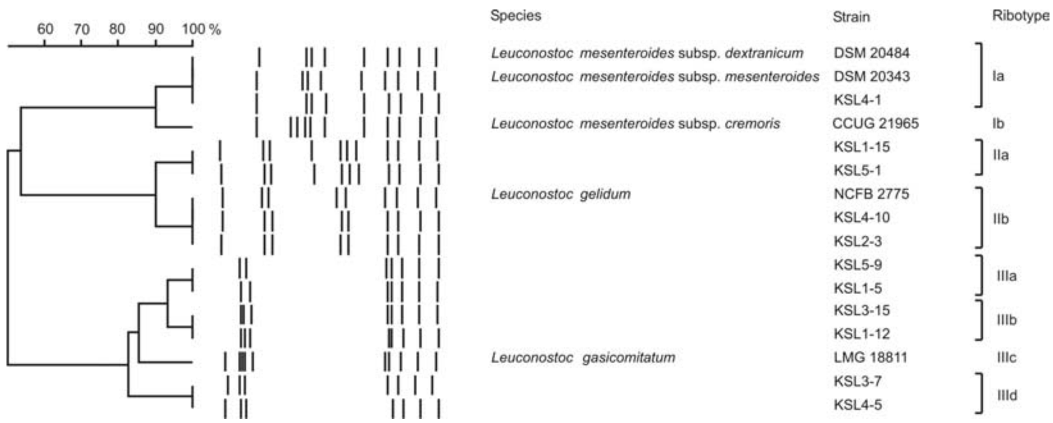

Fig. 2. Lyhs et al.

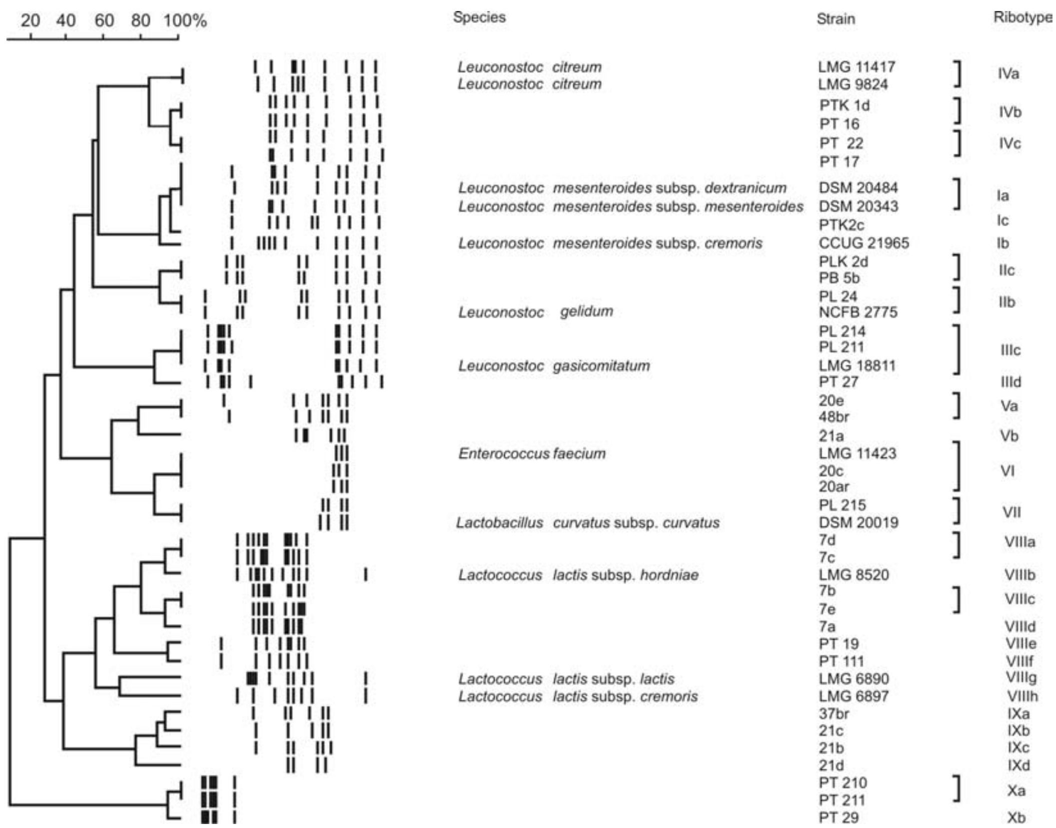

Fig. 3. Lyhs et al.