Lactobacillus alimentarius – a specific spoilage organism in marinated herring

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Abstract

Spoilage characterised by bulging of lids and gas formation affected various product lots of different marinated herring types. Microbiological analyses resulted in growth on MRS and Rogosa SL agar. Altogether, 206 randomly selected colonies from two unspoiled and ten spoiled samples were characterised using phenotypical key tests and a 16+23S rRNA gene-based RFLP identification database. *L. alimentarius* was found to be the specific spoilage organism in all samples. All isolates obtained from the different product types were of the same clonal type. The slight rise in pH value together with marked gas production suggested a rare lactic acid bacteria spoilage type called ‘protein swell’. *L. alimentarius* has not been previously associated with herring spoilage.

Key words: Herring; Spoilage; *Lactobacillus alimentarius*

1. Introduction

In marinated herring products, the raw or cooked herring is preserved in vinegar and salt to inhibit spoilage caused by salt-and acid-sensitive putrefactive bacteria. Bacterial growth and spoilage may still occur due to the growth of non-putrefactive organisms which can survive in acidic and high-salt environments. The growth of acetic acid-tolerant lactic acid bacteria (LAB) may form this spoilage flora in marinated herring. As the first sign of spoilage after some storage weeks, gas formation may occur due to the growth of these organisms (Blood, 1975).

Lactobacillus spp. have been identified as the specific spoilage organisms in marinated herring (Meyer, 1956b, 1962c; Kreuzer, 1957; Lerche, 1960; Reuter, 1965; Erichsen, 1967; Sharpe and Pettipher, 1983). In different kinds of spoiled herring types heterofermentative *Lactobacillus* spp. as *L. brevis*, *L. buchneri* and *L. fermentum* have been predominating (Meyer, 1956b; Kreuzer, 1957; Lerche, 1960; Meyer, 1962c; Reuter, 1965; Erichsen, 1967; Sharpe and Pettipher, 1983). Moreover, *L. plantarum* and *L. delbruckii* subsp. *lactis* (*L. leichmannii*), possessing homofermentative glucose metabolism, have been detected in some spoilage cases (Lerche, 1960; Meyer, 1962c; Sharpe and Pettipher, 1983). In unspoiled marinades, *L. buchneri* and *L. delbruckii* subsp. *lactis* have been identified (Reuter, 1965).

During this study, spoilage of marinated herring products was assessed. Clear bulging of lids and gas formation indicated spoilage after a few storage weeks of various marinated herring types from different lots produced by one company. At the time of the study, seven months of the expected shelf-lives were still remaining. The aim of this work was to characterise and identify the main spoilage LAB causing the gas production and spoilage. The species identification in the previous studies had been performed by means of phenotypical testing. Since these tests have been found

insufficient in the identification of many LAB (Björkroth et al., 1998, 2000; Lyhs et al., 1998) a 16+23 rRNA gene RFLP (ribotyping) database was used. This has been shown to be an excellent tool for LAB species identification (Björkroth and Korkeala, 1997; Lyhs et al., 1999).

2. Material and methods

2.1. Product description and sampling

A total of twelve glass jars containing marinated herring were sampled; ten of these showed clear bulging of lids and gas formation indicating spoilage, and two jars had a normal appearance. All jars contained herring cut into pieces, water, sugar (saccharose), salt (NaCl), onions, vinegar, spices and sodium benzoic acid as a preservative. The two normal (nos. 1 and 2) and six of the spoiled (nos. 3 to 8) samples contained only the basic ingredients described above. Three spoiled samples (nos. 9 to 11) also contained garlic and one spoiled sample (no. 12) additional onion to the base marinade. According to the manufacturer, the salt concentration of each sample was 3%. The recommended storage temperature was 4°C to 8°C.

2.2. Microbiological analyses and selection of the strains for further characterisation

A 10 g portion of herring in marinade was aseptically weighed into 90 ml of 0.9% NaCl (w/v) and 0.1% (w/v) peptone water in a sterile plastic bag, and then blended in a Stomacher 400 Lab Blender (Seward Medical, London, UK) for 30 seconds. Ten-fold serial dilutions were used for microbiological analyses. LAB were determined by the method of the Nordic Committee on Food Analysis (1991) using MRS agar (Oxoid, Basingstoke, UK). Rogosa SL agar (Orion Diagnostica, Espoo,

Finland) was cultured parallel to MRS agar (Oxoid). All plates were incubated in an anaerobic jar with a H_2+CO_2 generating kit (Oxoid) at 25°C for 5 days. The pH was determined from the first dilution by a WTW-530 Digital-pH-meter (Wissenschaftliche-Technische Werkstätten, Weilheim, Germany).

A total of 206 colonies were cultured pure. All six isolates growing on MRS agar from the unspoiled samples were included. With respect to all spoiled samples, half of the isolates analysed originated from MRS agar, and the other half from Rogosa SL agar. All 206 isolates were subjected to basic phenotyping and *Hin*dIII REA analyses. The isolates were maintained in MRS broth (Difco, Detroit, Michigan, USA) at -70°C and cultured using MRS broth (Difco) or MRS agar (Oxoid).

2.3. Phenotypic characterisation

All isolates were Gram-stained and catalase-tested. Production of gas from glucose was studied using the method of Schillinger and Lücke (1987).

2.4. Isolation of DNA, restriction endonuclease analysis (REA) and determination of 16+23S rRNA gene restriction patterns (ribotyping)

The similarity between isolates was initially checked using *Hin*dIII REA. Two isolates from each jar of herring were further subjected to ribotyping. *Hin*dIII was chosen because it has been found to provide species-specific patterns for spoilage LAB (Björkroth and Korkeala, 1996; Björkroth et al., 1998, 2000).

DNA was isolated according to the guanidium thiocyanate method of Pitcher et al. (1989), modified by Björkroth and Korkeala (1996) with combined mutanolysin (Sigma Chemical Company, St. Louis, MO, USA) and lysozyme (Sigma) treatment. Restriction endonuclease digestion of 6 μg of DNA was done according to

manufacturer instructions with *Hin*dIII enzyme (New England Biolabs, Beverly, MA, USA). REA, southern transfer, hybridisation and the cDNA probe for rRNA gene restriction patterns (ribotypes) were prepared as described by Björkroth and Korkeala (1996).

2.5. Ribotyping data management

The membranes were scanned with a Hewlett-Packard ScanJet 4c/T tabletop scanner (Boise, Idaho, USA). Numerical analysis of the ribopatterns was performed using the Gelcompar II software package (Applied Maths, Kortrijk, Belgium). A 1-% position tolerance was allowed for the bands. The similarity between all pairs was expressed by Dice coefficient correlation, and the unweighted pair-group method with arithmetic averages (UPGMA) was used for the construction of the dendrogram. The ribopatterns were compared with the corresponding patterns in the LAB database at the Department of Food and Environmental Hygiene, University of Helsinki, Finland, which comprises patterns for all relevant spoilage LAB in the genera of *Carnobacterium*, *Lactobacillus*, *Leuconostoc*, *Enterococcus* and *Weissella* (Björkroth and Korkeala, 1996, 1997; Björkroth et al., 1998, 2000; Lyhs et al., 1999). For the dendrogram, heterofermentative reference strains and the *L. alimentarius* type strain were included (Fig. 2).

3. Results

Visual examination of the herring from the bulging jars showed soft herring flesh, cloudy marinade and strong gas production. These changes were not seen in the unspoiled samples. pH values in the spoiled samples ranged from pH 3.7 to 3.9, as compared with a pH of 3.6 to 3.7 in the samples showing no gas formation. LAB

counts in the unspoiled samples were clearly lower than in the spoiled samples (Table 1). Counts in all herring samples were very similar on parallel MRS and Rogosa SL media, suggesting *Lactobacillus* spp. growth. All isolates were Gram-positive, catalase-negative, short, thick or long, filamentous rods. A total 139 of the 206 isolates showed gas production in the media used (Schillinger and Lücke, 1987).

All 206 LAB isolates originating from different jars of different marinated herring types shared the same *Hin*dIII restriction endonuclease type (Fig. 1). Identical patterns were also obtained when two isolates from each herring jar were further ribotyped. The *L. alimentarius* type strain possessed very similar *Hin*dIII ribotypes to the pattern obtained from the herring isolates (Fig. 2), merging in the dendrogram at a similarity level of 89%. The *L. alimentarius* cluster was clearly distinct from the other LAB species.

4. Discussion

L. alimentarius has not been previously reported as a predominant spoilage organism in gaseous spoilage of herring. Reuter (1983) has detected *L. alimentarius* in marinated fish products, meat products (fermented sausages, sliced prepackaged sausages) and sour dough. Usually, in the case of LAB spoilage, the pH of the product decreases due to lactic acid formation. However, in our study, a slight pH rise was observed. Meyer (1956a) reported this type of spoilage in canned semi-preserved fish and named it ‘protein swell’. He distinguished it from ‘carbohydrate swell’, where increased acidity and CO_2 formation result from heterofermentative utilisation of glucose. In contrast to ‘protein swell’, ‘carbohydrate swell’ can result in very low pH values. In a case of *Lactobacillus fructivorans* spoilage in tomato ketchup, pH values as low as 3.3 to 3.4 were detected (Björkroth and Korkeala, 1997).

The decrease in acidity related to ‘protein swell’ has been attributed to production of ammonia by bacterial deamination of amino acids. Later, Meyer (1962b) suggested that the acetic acid provides an acidic environment appropriate for the action of protoelytic enzymes present in fish muscle. The products of proteolysis, i.e. amino acids, provide an energy source for the growth of the acetic acid-tolerant LAB, leading to carbon dioxide production. While LAB activity has been associated only with degradation of oligopeptides and free amino acids (Verplaetse, 1994; Molly et al., 1997), it may also cause formation of biogene amines. However, the product studied here was in such an advanced stage of spoilage that its consumption would be very improbable. To restrict the growth of LAB, Meyer (1956b) recommended for the marinating process a pH value no higher than 4.0, a salt concentration of the marinade not much under 6% and a storage temperature as low as possible. ‘Protein swell’ has also been associated with anchovy-stuffed olives (Harmon et al., 1987). *L. plantarum* and *L. brevis* growth have been observed, and enzymes originating from the anchovy were considered to be the reason for initial proteolysis.

If enhanced CO_2 production is caused by decarboxylation of amino acids, the LAB having an effect on gas production may possess homofermentative glucose metabolism, similar to *L. alimentarius*. Facultatively heterofermentative *Lactobacillus* spp. may produce CO_2 while utilising gluconate or citrate. In food containing many substrates, *L. alimentarius* strains may have utilised substrates leading to CO_2 formation without amino acid decarboxylation. Thus, the gaseous spoilage described here might not represent a typical form of ‘protein swell’. The nominal change in pH values between spoiled and unspoiled samples increases this doubt. However, facultative heterofermentative organisms show no gas production in the test determining the glucose utilisation type; yet, 139 of the 206 isolates did show gas

production when tested (Schillinger and Lücke, 1987). These controversial reactions caused by facultatively heterofermentative *Lactobacillus* spp. have also been reported earlier. In a study of LAB from a sorghum-based fermented weaning food, Kunene et al. (2000) described 25 strains from the facultative heterofermentative *L. sakei/curvatus*-group as gas producing. They used the same test (Schillinger and Lücke, 1987) for determination of glucose fermentation type as in the present study. Because this medium contains no gluconate or citrate, the reason for gas production is unclear. However, false results from glucose fermentation type determination will lead to false species identification if only phenotypic characteristics are used.

In our study, all isolates originating from various lots of different marinated herring types were of the same clonal type. This suggests contamination of the processing facilities with an organism thriving at the processing environment and possessing strong spoilage potential. In fish originating from temperate waters, lactobacilli can be found among the dominant psychrotrophic Gram-negative bacteria in only very low numbers (Huss, 1995). Krüger (1973) suggested that LAB contaminate herring products during the processing as a secondary contamination. However, since salt concentrations lower than 6% are commonly used in herring today, it is difficult to prevent the contaminating *Lactobacillus* spp. from growing.

5. Conclusion

A single *L. alimentarius* clone was found to be the specific spoilage organism in different marinated herring types. Since the gas production test yielded misleading results, ribotyping was very useful for the identification of the specific spoilage organisms. In order to prevent gaseous spoilage of herring with LAB, good manufacturing hygiene should be maintained.

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Table 1

Growth on MRS and Rogosa SL agar and pH values analysed from marinated herring jars of normal appearance and those with bulging lids indicating spoilage

Sample no.	Bacterial counts (cfu/g)		pH
	MRS agar	Rogosa SL agar	
1[a]	3×10^{2}	< 100	3.6
2[a]	< 100	< 100	3.7
3	1.2×10^{7}	6×10^{6}	3.7
4	2.5×10^{6}	1.6×10^{6}	3.8
5	1.7×10^{7}	1.3×10^{7}	3.7
6	8×10^{6}	6.1×10^{6}	3.8
7	1.9×10^{6}	2.8×10^{6}	3.9
8	5.6×10^{6}	3.9×10^{6}	3.7
9	2.5×10^{7}	1.1×10^{7}	3.7
10	1.3×10^{7}	1.2×10^{7}	3.7
11	9.2×10^{6}	8.9×10^{6}	3.7
12	1.7×10^{6}	1.6×10^{6}	3.8

[a] Samples of normal appearance.

Legends to the figures:

Fig. 1. Restriction endonuclease patterns obtained in the *Hin*dIII restriction endonuclease analysis of the herring spoilage isolates. Lanes 1a – 2b, strains isolated from the two unspoiled samples; lanes 3a – 10a, strains isolated from the ten spoiled samples. Lambda DNA cleaved by *Hin*dIII was used as a molecular weight marker.

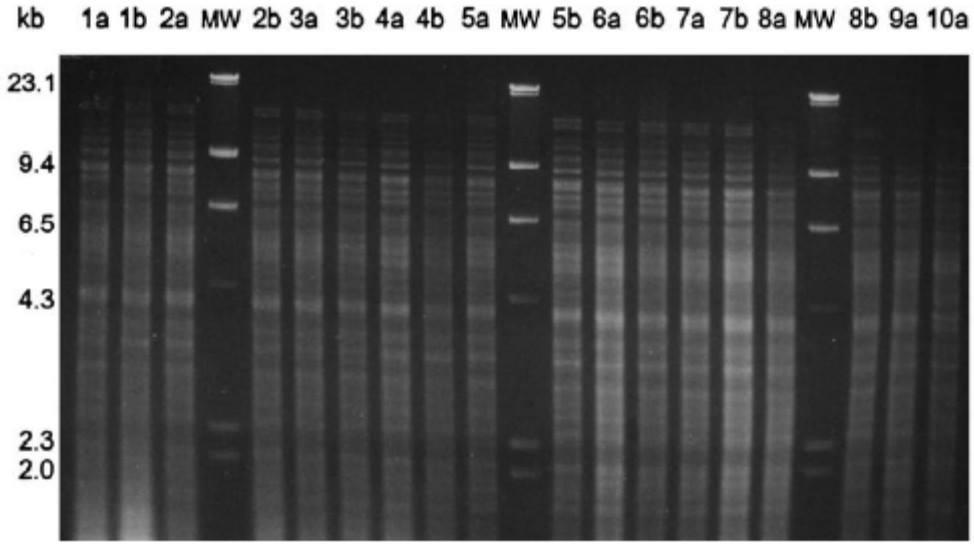

Fig. 2. Dendrogram and schematic banding patterns based on *Hin*dIII ribopatterns. Nos. 179, 192 and 200 represent herring spoilage strains.