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# Diversity of *Leuconostoc gasicomitatum* associated with meat spoilage

Elina J. Vihavainen *, K. Johanna Björkroth

*Department of Food and Environmental Hygiene, Faculty of Veterinary Medicine, University of Helsinki, P.O. Box 66, 00014 University of Helsinki, Finland*

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ABSTRACT

*Leuconostoc gasicomitatum* isolates ($n=384$) associated with spoilage of meat and vegetable-based foods were characterised by pulsed-field gel electrophoresis (PFGE) typing. Our aim was to evaluate the diversity and distribution of spoilage-associated *L. gasicomitatum* isolates from meat products, and to determine whether the PFGE genotypes are specific to product, producer, or isolation year (1997-2007). PFGE typing differentiated the isolates into 68 genotypes, and revealed that none one of the 54 genotypes associated with meat products was recovered from vegetable-based foods. Generally, the meat-derived genotypes were not specific to meat animal species, and many genotypes included isolates from products of different types or processors, as well as isolates collected in different years. Furthermore, certain genotypes were repeatedly identified from products of the same processing plant suggesting that the processing environment may have an impact on *L. gasicomitatum* contamination of meat products.



## 1. Introduction

Psychrotrophic lactic acid bacteria (LAB) constitute a major part of microbial population in modified atmosphere packaged (MAP) meat and meat products. If allowed to grow to high levels in packaged meats, LAB may produce adverse sensory changes such as sour off-odours (Borch et al., 1996). Among the LAB associated with meat spoilage, *L. gasicomitatum* has gained attention as a spoilage organism in MAP, marinated meat products (Björkroth et al., 2000; Susiluoto et al., 2003; Björkroth, 2005; Vihavainen and Björkroth, 2007). In these products, spoilage by *L. gasicomitatum* is typically characterised by gas formation, buttery or sour off-odours, and green discolouration on red meat. Additionally to meat, *L. gasicomitatum* may contribute to spoilage of other chilled, nutrient-rich foods. For example, in a pickled fish and a vegetable sausage products formation of slime, gas and off-odours were associated with *L. gasicomitatum* (Lyhs et al., 2004; Vihavainen et al., 2008).

The previous studies reporting the spoilage role of *L. gasicomitatum* in various foods have focused mainly on the characterisation of the spoilage microbial populations (Björkroth et al., 2000; Susiluoto et al., 2003; Lyhs et al., 2004; Vihavainen and Björkroth, 2007). During these studies, *L. gasicomitatum* isolated were identified by a numerical by numerical taxonomy approach utilizing *Hin*dIII ribotypes as operational taxonomy units. This approach, however, lacks the discriminatory power needed for assessing the genetic diversity of *L. gasicomitatum* isolates. Concerning molecular typing techniques for lactic acid bacteria (LAB), PFGE typing has been shown to provide the high resolution necessary to differentiate closely related LAB strains (Tenreiro et al., 1994; Björkroth et al., 1996). Furthermore, authors of previous studies have concluded that PFGE is suitable for subtyping of *Leuconostoc* species, particularly with *Sma*I as the restriction endonucleases (Villani et al., 1997; Björkroth et al., 1998; Sánchez et al., 2005).

To date, *L. gasicomitatum* is a concern to the meat industry, since its growth may lead to early spoilage thus shortening the shelf life of MAP products. Nevertheless, to prevent meat contamination and spoilage by *L. gasicomitatum*, further knowledge is required about its distribution and diversity. To address these issues, we applied *Sma*I PFGE typing to characterise *L. gasicomitatum* isolates collected during 1997-2008 from meat and vegetable-based retail products. The specific aims were to identify the major genotypes associated with meat spoilage, and to determine whether these genotypes are specific to product or processor.

## 2. Materials and methods

### 2.1. L. gasicomitatum isolates and culture conditions

For this study, we selected 395 *L. gasicomitatum* isolates from the culture collection of our laboratory. The isolates were recovered during several independent surveys between 1997 and 2008 from retail products analysed at the end of shelf life or at the onset of spoilage. Usually in these products, *L. gasicomitatum* was present at levels of $10^6$ to $10^8$ cfu/g. Product sampling, enumeration of LAB, and identification of isolates were conducted as described in our previous reports (Vihavainen and Björkroth, 2007; Vihavainen et al., 2008).

To maximize the potential diversity of the isolates, we chose them to represent different products, processing plants, and sampling years. When available, two to five isolates per food sample were included to

* Corresponding author. Tel.: +358 9 19157118; fax: +358 9 19157101.
*E-mail address:* elina.vihavainen@helsinki.fi (E.J. Vihavainen).

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determine the genotypic diversity of isolates originating from the same sample. In total, 77 isolates were from samples of beef products ($n=32$), 145 from samples of broiler products ($n=55$), 51 from samples of pork products ($n=32$), 27 from samples of turkey products ($n=14$), and 18 from samples raw meat products ($n=13$) containing both beef and pork. Isolates were chosen to represent both unprocessed meats ($n=55$), and marinated raw meat products ($n=80$). Additionally, 66 isolates originating from vegetables ($n=12$) or vegetable-based products ($n=8$) were included. A complete list of isolates, including sources and isolation years, is presented in the Table S1 (see supplementary material in the Appendix).

For this study, isolates preserved as frozen stocks in MRS broth (Difco, Le Pont de Claix, France) at -70 °C were revitalized in MRS broth at 25 °C for 48 h, and cultured anaerobically on MRS agar (Oxoid, Basingstoke, UK) at 25 °C for 5 days.

### 2.2. In situ cell lysis, and isolation and restriction digestion of DNA in agarose plugs

Agarose-embedded DNA was prepared as described by Björkroth et al. (1998). Briefly, cells were harvested from MRS broth and embedded in agarose plugs prepared in 2% (w/v) low-gelling-temperature agarose (Lonza Rockland, Rockland, ME, USA). For *in situ* isolation of DNA from agarose-embedded cells, lysozyme (1 mg/ml; Sigma, St. Louis, MI, USA), mutanolysin (10 U/ml; Sigma), and ribonuclease A (20 µg/ml; Sigma) were added to the lysis solution (6 mM Tris-HCl, 1 M NaCl, 100 mM EDTA, 0.5% Brij-58, 0.2% sodium deoxycholate, 0.5% sodium lauroyl sarcosine). After this, plugs were treated with a solution containing 0.5 M EDTA (pH 9.0), 1% sodium lauroyl sarcosine, and proteinase K (100 mg/ml; Finnzymes, Espoo, Finland).

For *in situ* digestion of DNA with *Sma*I endonuclease (New England Biolabs, Ipswich, MA, USA), a slice (1 mm) was cut from a plug, and following the manufacturer's recommendation for reaction conditions, 25 to 35 U of *Sma*I in a final volume of 100 µl per slice was used for digestion of DNA.

### 2.3. PFGE and PFGE patterns analysis

Restriction fragments were separated on 1% SeaKem Gold agarose (Lonza Rockland) in 0.5× solution of Tris–borate–EDTA at 14 °C using a contour-clamped homogeneous electric fields PFGE apparatus (Bio-Rad CHEF-DRIII system; Hercules, CA, USA) and the following PFGE parameters: initial switching time 0.5 s; final switching time 25 s; at 6 V/cm for 20 h. Tagged image file format images of ethidium bromide-stained gels were captured, and imported into BioNumerics (version 5.1; Applied Maths, Sint-Martens-Latem, Belgium). Using the BioNumerics software, PFGE patterns were analysed after normalisation of each gel against lambda ladder PFG markers (New England Biolabs). A dendrogram of PFGE patterns was constructed using the unweighted-pair group method with arithmetic averages (UPGMA) and the band-based Dice similarity coefficient with a position tolerance of 1.8%.

## 3. Results

### 3.1. PFGE typing and genotypes

Of the 395 *L. gasicomitatum* isolates from meat and vegetable products, 384 isolates were successfully genotyped. For each isolate, PFGE of *Sma*I digested DNA yielded 8 to 16 visible fragments ranging in size from approximately 30 to 500 kb. Among these 384 *L. gasicomitatum* isolates genotyped, we identified 68 indistinguishable *Sma*I PFGE patterns (genotypes), each representing between 1 and 40 isolates. Fig. 1 shows these 68 *Sma*I patterns, genotype designations and source(s) for each genotype. Table S1 lists the genotypes of all *L. gasicomitatum* isolates analysed in this study.

Additionally, despite repeated trials, nine isolates (2.3% of all isolates) yielded unreliable PFGE patterns due to incomplete digestion with *Sma*I or its isochizomer *Xma*I. The untypable isolates originated from vegetables, and are not discussed further.

### 3.2. Genotypic diversity of isolates from meat

For the 326 meat-derived *L. gasicomitatum* isolates, 53 *Sma*I genotypes were identified. Of these genotypes, 33 contained isolates from at least two meat samples; together these genotypes accounted for 93% of the meat-derived isolates. Table 1 shows the distribution of the meat-derived isolates assigned to these 33 *L. gasicomitatum* genotypes according to the meat species (beef, pork, broiler or turkey), and product type.

Of these 33 genotypes, 15 (45%) included isolates only from products of a certain meat species whereas 7 (21%) genotypes (genotypes 30, 34, 35, 39, 47, 54 and 68) were associated with the products of both red meat (beef, pork) and poultry (broiler, turkey). Overall, the genotypes identified from two or more meat animal species included 189 isolates, accounting for 58% of all meat-derived isolates. Additionally, most of the genotypes (67%) contained isolates from both unprocessed and marinated meat products.

Comparison of *Sma*I genotypes of isolates originating from the same meat sample revealed that in 70% of meat samples two or more genotypes were identified (data provided in Table S1). In the rest of the samples with several isolates genotyped the isolates showed indistinguishable PFGE patterns.

A total of 15 genotypes included isolates from a single meat sample (see Fig. 1.). As these genotypes were found only in a single sample each, they were not useful for the analysis of potential source-specificity, and thus, these isolates are not discussed further.

### 3.3. Distribution of genotypes among poultry products of unrelated processing plants

Among the isolates genotyped in this study, 150 originated from retail poultry products manufactured at three plants that had no association with each other. PFGE typing differentiated these isolates into 24 genotypes of which 10 were encountered in two or more samples that were either from the products of more than one poultry plant, or collected in different years. Table 2 summarises the distribution of these 10 genotypes according to the processing plant and the year of isolation. For example, genotype 4 included isolates from poultry products of plant A sampled in 1997, 2000 and 2006, as well as isolates from products of plant B sampled in 2000 and 2005 (Table 2).

## 4. Discussion

*Sma*I PFGE typing differentiated the *L. gasicomitatum* isolates into 68 genotypes allowing us to identify the major genotypes associated with spoilage of meat and vegetable-based foods, as well as to view the relationship between the strains originating from different products.

In the dendrogram (Fig. 1) depicting the pattern similarities of these 68 *Sma*I genotypes, many genotypes encountered in vegetables (such as genotypes 8, 27, 28 and 59) clustered close to meat-derived genotypes. However, although some isolates from vegetable and meat sources had closely related genotypes, none of the genotypes was common to both of these food categories. Thus, it seems that these vegetable-derived genotypes were not disseminated into the meat processing chain, or alternatively, these genotypes lack the ability to develop to high numbers in MAP, meat systems. Nevertheless, further data are needed to conclude whether vegetable and meat products harbour or support the growth of different *L. gasicomitatum* genotypes.