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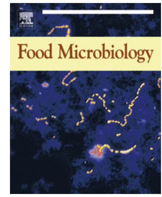

Biodiversity of spoilage lactobacilli: Phenotypic characterisation

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ABSTRACT

Preventing food spoilage is a challenge for the food industry, especially when applying mild preservation methods and when avoiding the use of preservatives. Therefore, it is essential to explore the boundaries of preservation by better understanding the causative microbes, their phenotypic behaviour and their genetic makeup. Traditionally in food microbiology, single strains or small sets of selected strains are studied. Here a collection of 120 strains of 6 different spoilage related *Lactobacillus* species and a multitude of sources was prepared and their growth characteristics determined in 384-well plates by optical density measurements (OD) over 20 days, for 20 carbon source-related phenotypic parameters and 25 preservation-related phenotypic parameters. Growth under all conditions was highly strain specific and there was no correlation of phenotypes at the species level. On average *Lactobacillus brevis* strains were amongst the most robust whereas *Lactobacillus fructivorans* strains had a much narrower growth range. The biodiversity data allowed the definition of preservation boundaries on the basis of the number of *Lactobacillus* strains that reached a threshold OD, which is different from current methods that are based on growth ability or growth rate of a few selected strains. Genetic information on these microbes and a correlation study will improve the mechanistic understanding of preservation resistance and this will support the future development of superior screening and preservation methods.

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1. Introduction

Microbial food spoilage is characterised by random contamination with microbes which may lead to uncontrolled changes to the food product. The nature and character of a contaminant are not known before the food-microbe contact happens. Consequently, in food preservation and the prevention of spoilage, it is desired to account for "all" possible contaminants. In practice this is not feasible as many different species and strains from very different sources may cause issues, even in a selective environment based on a defined food formulation. This problem drives the need to better characterise possible contaminants and to describe the microbial biodiversity with respect to geographical and food source origins. Such knowledge may help to define the boundaries better for preservation of a particular food category, which is of relevance to consumers demanding minimally preserved, high quality and yet reliable foods. The importance of assessing biodiversity has been well appreciated for food pathogens (Lianou and Koutsoumanis, 2013) but not much yet for food spoilage microbes. Traditionally, preservation boundaries are defined on the basis of challenge testing, assessing a large set of different food compositions and exposing these to a limited number of microbes, isolated from spoilt foods (latest guidelines for challenge testing have been provided by the US National Advisory Committee on Microbiological Criteria for Foods, (NACMCF, 2010)). This provides a detailed picture from the perspective of the food composition. However, the influence of microbial biodiversity is largely ignored in this approach. With the advance of analytical power, it is now straightforward to characterise large sets of microbes, both phenotypically and genetically, on their spoilage behaviour.

Lactobacilli are associated with the spoilage of various foods. *Lactobacillus* species such as *Lactobacillus brevis*, *Lactobacillus buchneri*, *Lactobacillus curvatus*, *Lactobacillus hilgardii*, and *Lactobacillus plantarum* occur frequently in must or wine and are mainly responsible for the formation of biogenic amines. Some spoilage strains of these species produce exopolysaccharide slimes, acetic acid, diacetyl and other off-flavours (Petri et al., 2013). In beer, only certain isolates of lactic acid bacteria (LAB) can grow e.g. *L. brevis* strains (Bergsveinson et al., 2012). Lactate metabolism by *L. buchneri* was indicated as the source of pH rise in fermented cucumber spoilage (Johanningsmeier and McFeeters, 2013). Spoilage outbreaks in pickled cucumber products characterized by the

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development of red colour on the surface of the fruits were attributed to *Lactobacillus casei* and *Lactobacillus paracasei* in 2 cases (Pérez-Díaz et al., 2007). Acidified speciality products or condiments, such as ketchups, salad dressings and mayonnaise-like products, are amongst the most microbiologically stable food products. These have a long ambient shelf life when unopened. However, many products are designed for repeated use, which makes them vulnerable to contamination, despite a very low pH (Sperber, 2010). Spoilage of acidified products is caused by acid resistant yeast and by lactic acid bacteria, particularly *Lactobacillus fructivorans*, *L. brevis*, *L. buchneri*, and *L. plantarum* (Sharpe and Pettipher, 1983). Gas (CO_2) formation in glass bottles is a potential danger. This spoilage defect has been associated with heterofermentative lactobacilli such as *L. brevis* which was isolated from Ranch dressing and also caused product acidification (Waite et al., 2009) and with *L. fructivorans,* which was involved in spoilage of bottled ketchup (Bjorkroth and Korkeala, 1997).

The recent development and accessibility of high throughput phenotyping and genome analysis methods now allows the assessment of much larger sets of strains. This will provide insight into the species and strains that are involved in spoilage, their phenotypic properties and ultimately specific genes that may be attributed to spoilage incidents. In this work, the ability to grow in two different types of phenotypic assays, i.e. preservation conditions and sugar utilization, was tested on a total of 120 *Lactobacillus* strains of 6 different species. The different preservation conditions were defined on the basis of past experience with the spoilage behaviour of lactobacilli. These spoilage-related conditions include resistance to low pH, weak acids and high salt concentrations. The evaluation of sugar consumption phenotypes was similar to the experiments performed by Siezen et al. (Siezen et al., 2010) in which it was shown that carbohydrate metabolism could be used to differentiate *L. plantarum* strains phenotypically. The phenotypic variation of the total dataset was then explored statistically.

The high throughput phenotyping approach presented here may allow the definition of food preservation boundaries on the basis of the number of spoilage strains that is able to grow, rather than on the basis of the growth rate or growth/no-growth data for one or a limited number of strains. The use of this new definition may improve the reliability of predictive models for microbiological food spoilage.

2. Materials and methods

2.1. Strains and fermentation conditions

In the present study, the phenotypic diversity of 90 spoilage lactobacilli combined with 31 NIZO benchmark lactobacilli was tested (Table 1). Of the 31 NIZO strains, 22 were of the species *L. plantarum*. *L. plantarum* strains were selected on the basis of two criteria: i) the availability of the genome sequences, and ii) the distribution of the strains in the phenotypic tree, as determined by Siezen et al. (Siezen et al., 2010). Strains of the five other species (*L. brevis, L. buchneri, L. (para)casei, Lactobacillus fermentum,* and *L. fructivorans*) were selected primarily on the basis of different source of isolation (as far as known). Strains were stored at −80 °C. The identity of the spoilage strains was confirmed by species specific PCR (using previously described methods (Schmidt et al., 2008; Settanni et al., 2005; Song et al., 2000; Stevenson et al., 2006; Walter et al., 2000)), partial 16S rRNA gene sequencing or full genome sequencing. For this work the taxonomy of the genus *Lactobacillus* as defined by Salvetti et al. was used (Salvetti et al., 2012). This is based on 16S rRNA gene sequences and covers all the *Lactobacillus* species of interest here. All strains were transferred in triplicate into a single 384-well plate, called the mother plate, distributing all 121 strains (120 strains, of which 1 in duplicate, *L. casei* ATCC334) to different positions on the 384-well plates to avoid location-bias. Growth of the strains was in MRS medium (De Man et al., 1960) supplemented with 2% glucose at 30 °C. The fully grown cultures in the 384-well plate were mixed with 1 volume of 60% glycerol and stored at −80 °C.

2.2. Phenotypic studies of carbon source usage

The carbon consumption capacity of the complete *Lactobacillus* collection was tested on a wide range of different sugars, adopted from Siezen et al. (Siezen et al., 2010). Using replicate pin-plating, a fraction (approximately 1–2 μl, or 1–2%) of the 384-well mother plate (containing about 1×10^9 cfu/ml) was transferred to a carbon-depletion plate (MRS without a carbon source) and incubated overnight to consume any traces of intra- and extracellular glucose. No growth was expected during this incubation. Next, a similar size fraction of these depleted cells was transferred, by pin-plating, to 23 different receiver plates (final inoculation level was approximately 1×10^5 cfu/ml) containing 80 μl per well of MRS with a specific carbon source. The capability of each strain to grow on the carbon sources was determined over a 21 day time span at 30 °C by optical density (OD) measurements at 600 nm in the 384 well plates using a Spectramax spectrophotometer on days 1, 2, 3, 4, 5, 7, 11, 14, 18 and 21. Evaporation and cross-contamination was prevented by sealing the 384-well plates with transparent sterile adhesive PCR foil seals.

MRS without carbon source was supplemented with 2% of the carbon source of interest. The ability to grow on the following carbon sources (>99% purity) was studied: D-xylose, amygdalin, glycerol, methyl-αD-glucopyranoside, methyl-αD-mannopyranoside, D-melibiose, dulcitol, D-melezitose, D-raffinose, D-lactose, L-rhamnose, starch, D-sorbitol, D-turanose, L-arabinose, potassium gluconate and D-trehalose. In addition, the ability to grow at 45 °C, in the presence of 6% w/v NaCl and in the presence of 1 μg/ml nisin, all with glucose as the main carbon source was determined. The ability of strains to grow in MRS with or without glucose, at 30 °C was included as a positive and negative control, respectively. Finally, an un-inoculated plate was maintained as a sterility control.

2.3. Phenotypic studies of preservation-specific conditions

The ability of the *Lactobacillus* strains to grow under several preservation (stress) conditions was tested in a medium lacking protein, to avoid precipitation of protein at the low pHs of some of the preservation conditions, which could interfere with the growth (OD) measurements. Double strength MRS (2× concentrated to allow for later additions) was made by dissolving 20 g Bacto proteose peptone nr. 3, 20 g Lemco Powder (from which 1.1% salt), 10 g Bacto yeast extract, 40 g dextrose, 2 g Tween 80, 0.4 g magnesium sulphate 7 H_2O, 0.12 g manganese sulphate 1 H_2O and 4 g dipotassium hydrogen phosphate in 650 ml deionised water. The magnesium sulphate and manganese sulphate were dissolved separately in a small quantity of the water before use in the medium. The pH was adjusted to 2.6 with HCl and demineralised water was added to 800 g. The medium was brought to the boil and incubated in a water bath at 100 °C for 30 min. It was then cooled in ice water and stored overnight at 4 °C, allowing proteins to precipitate. The medium was filtered over a 240 mm diameter Schleicher & Schuell foldfilter nr 595. Next, the medium was filtered successively over 0.8 μm, 0.45 μm and a 0.22 μm filters. Proteins still left after filtering were expected to precipitate during storage of the filter sterilised basic media at 4 °C (for 48–72 h). This precipitate was removed by filter sterilisation (0.2 μm filter). This double concentrated, protein depleted, MRS was mixed with NaOH, HCl, NaCl, lactic acid, acetic acid, sorbic acid or sucrose to create the preservation related conditions as listed in Table 2. The

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