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Short communication

Use of M17 and a milk-based medium enables isolation of two distinct and diverse populations of *Lactococcus lactis* strains from undefined mesophilic starter cultures

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ABSTRACT

Dairy starter culture development and bacteriophage research depends on adequate growth media for isolation of relevant starter culture strains and their bacteriophages. We show that the use of two growth media, M17 and a milk-based medium (GMA), enables isolation of two distinct subpopulations of *Lactococcus lactis* ssp. from undefined mesophilic starter cultures. Phage typing revealed large diversity within each subpopulation, and interestingly, that there was very little overlap in phage sensitivity between the two sets of bacteria. Acidification activity tests performed in the presence of M17-and GMA-derived bacteriophages, both separately and in combination, demonstrated the relevance of both bacterial subpopulations in the acidification process. Use of both media enables higher diversity sampling of *Lactococcus* ssp., and their bacteriophages.

1. Introduction

Mesophilic mixed (LD-type) starter cultures are used in the production of Dutch-type cheeses. These starter cultures contain undefined mixtures of *Lactococcus lactis* and *Leuconostoc* strains, where the lactococci are the major contributors in the acidification process. Bacteriophages infecting *L. lactis* are ubiquitous in dairies and can negatively affect the production process and quality of the final product (Kleppen, Bang, Nes, & Holo, 2011; Rousseau & Moineau, 2009).

Lactococcal phages are classified into ten groups, where the most frequent groups found in dairy environments are the 936-, c2-, and P335-like bacteriophages (Deveau, Labrie, Chopin, & Moineau, 2006). In an earlier study by our group, no c2-like bacteriophages were found in Norwegian dairies and P335-like bacteriophages were found in titres too low to be of significant consequence in fermentations. However, the strictly lytic 936-like bacteriophages were found in high titres in all bulk starters regardless of fermentation activity, emphasising the relevance of bacteriophage diversity (host range) in disrupting fermentation (Kleppen et al., 2011).

Undefined starter cultures gain their robustness against phage attack from their large number of strains with diverse phage sensitivity (Boucher & Moineau, 2001). To keep up production and maintain reproducibility, the cheese industry prefer frozen batches to back slopping. However, the use of frozen starter culture batches effectively halts lactococcal evolution, whilst giving phages the advantage to evolve freely (Rousseau & Moineau, 2009). The composition of starter cultures is often unknown, and knowledge about the individual strains and their phages is of great importance to the dairy industry. Starter culture lactococci are commonly cultivated on M17, a medium also well suited for isolation of lactococcal phages (Johansen, Øregaard, Sørensen, & Derkx, 2015; Terzaghi & Sandine, 1975). However, working with a Dutch mixed starter culture, Erkus et al. (2013) reported that the majority of lactococci could only be isolated on Reddy's medium (Reddy, Vedamuthu, Washam, & Reinbold, 1972), a medium with milk as one of its ingredients, and not on M17. This prompted us to compare strains and bacteriophages isolated using M17 and the milk-based medium GMA (Hugenholtz, Splint, Konings, & Veldkamp, 1987) from two commonly used undefined mesophilic starter cultures. We show that different phagovars can be isolated on the two

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media. Thus, combined use of M17 and GMA enables isolation of a wider diversity of lactococci and their bacteriophages.

2. Materials and methods

2.1. Growth of bacteria and bacteriophages

The media used for cultivation of *Lactococcus* sp. were M17 (Oxoid, Hampshire, UK) (Terzaghi & Sandine, 1975) supplemented with 0.5% (w/v) lactose (Merck, Oslo, Norway), or 10% (w/v) skimmed milk (TINE SA, Oslo, Norway) supplemented with 50 mM β-glycerophosphate (Sigma–Aldrich, Munich, Germany) (GMA; Hugenholtz et al., 1987). Commercial starter cultures were suspended in Luria–Bertani broth (Bertani, 1951) to an optical density (OD) at 600 nm of 1.0, serially diluted and spread plated on M17 and GMA agar plates in triplicate. Plates were incubated at 22 °C for 5 days, after which the number of colonies on each plate was counted. Isolates were transferred into the same media without agar, and cultivated in 22 °C with daily transfers until they would grow in M17 (a minimum of two days). Finally, all cultures were transferred to M17, grown for two passages before aliquots were stored frozen at −70 °C in M17 supplemented with 15% (w/v) glycerol (Sigma–Aldrich). Bacteriophages were isolated by plaque assays (Lillehaug, 1997). When propagating phages, M17 was supplemented with 5 mM calcium chloride (Merck, Oslo, Norway) (M17-c). Filtered phage lysates and bacteriophages isolated from plaques were stored at 4 °C for up to 12 months or at −20 °C with 15% (w/v) glycerol.

2.2. Dairy sample collection

Bulk starter samples were collected from several cheese plants and treated as described by Kleppen et al. (2011).

2.3. Bacteriophage inhibition array

Bacterial isolates were cultivated in M17-c in 96-well microtitre plates and growth inhibition by dairy samples or bacteriophage was assayed essentially as described by Kleppen et al. (2011) with the following modifications; each well contained 150 μL M17-c and 50 μL dairy sample or phage suspension. Controls contained 200 μL M17-c. The microtitre plates were inoculated with arrays of lactococcal isolates using a stainless steel 48-pin replicator (Sigma–Aldrich, Munich, Germany), incubated over night at 22 °C and the OD at 620 nm was measured for each well using a SPECTROstar Nano microtiter plate-reader (BMG LABTECH, Ortenberg, Germany). All analyses were performed in triplicate. Isolates showing OD less than 50% of the control in at least one of the three replicates were considered inhibited.

2.4. Phage typing

Bacteriophage isolates were distributed into M17-and GMA-collections in microtiter plates and used to phage type isolates that were inhibited in the bacteriophage inhibition arrays. Indicator bacteria were grown in M17-c broth to an OD at 620 nm of about 0.2, and cast in 0.8% (w/v) M17-c soft agar over 1.2% (w/v) M17-c bottom agar. Phage suspensions from the microtitre plates were spotted on top using a stainless steel 48-pin replicator delivering approximately 5 μL per pin. Assays were performed in triplicate. Results were logged as sensitive when clear plaque was observed, or insensitive when no plaque was observed. No turbid plaques were observed. Hierarchal clustering of the results was performed in R (www.r-project.org) with RStudio (RStudio, 2012; Version 0.98.1103; RStudio, Boston, Massachusetts, US) using complete-linkage UPGMA using Manhattan distances. The resulting distance-matrix was used to construct a heatmap with dendrograms using the heatmap.2 function from the Gplots package (Version 2.16; Warnes et al., 2015) supplemented by the Dendextend package (Version 0.18.3; Galili, 2015). A cut-off for the number of clusters was determined using the knee of the curve according to Salvador and Chan (2004) to establish robust clusters.

2.5. Genotyping

Bacterial isolates were typed by Polymerase Chain Reaction (PCR) as described by Mahony et al. (2013) for CWPS-genotyping. Random samples from both bacterial collections were analysed by PCR as described by Erkus et al. (2013) to identify TIFN7-genotype bacterial isolates.

2.6. Starter culture acidification activity tests

Starter culture acidification activity tests were performed in triplicate largely as previously described (IDF, 2009) using reconstituted skim milk 10% (w/v) (RSM), heated to 95 °C for 45 min and cooled to room temperature prior to use. A pre-culture in RSM inoculated with 0.1% (v/v) frozen starter culture and grown overnight at room temperature was diluted 33 times in 10 mL RSM aliquots and incubated at 30 °C for 4 h before pH was measured. The effects of phages were studied by including at least 10^7 plaque-forming-units (pfu) mL^{-1} in each assay.

3. Results and discussion

3.1. Bacterial counts on M17 and GMA

Two starter cultures (A and B) commonly used in the production of Dutch-type cheese were used to compare bacterial counts on M17 and a milk-based medium. For the latter we chose GMA, which yielded the same counts for culture A as the more complex milk-based medium of Nickels and Leesment (1964) in earlier experiments (data not shown). For culture A, the counts on GMA were three times higher than on M17 (2.1×10^9 versus 7×10^8 cfu mL^{-1}), while counts on the two media both gave 1.3×10^9 cfu mL^{-1} for culture B. The difference in counts for culture A was similar to the results described by Erkus et al. (2013). For this reason, the focus of diversity analyses in this study was on culture A.

3.2. Transfer of GMA-isolates to M17 for phage studies

The bacterial counts suggested that culture A, but not culture B, had a bacterial subpopulation that would only grow on milk-based media. However, most of the GMA isolates from both culture A and B would not grow when inoculated directly from the GMA-plate into M17. This demonstrates that for both cultures, many of the strains isolated using GMA (GMA-population), have properties different from those isolated using M17 (M17-population). Phage assays often rely on visual measurements and are difficult to perform using opaque media. Therefore, transfer of the GMA-isolates to M17 prior to phage studies was important. The GMA-isolates were successfully grown in M17 after cultivation in GMA without agar for two to four passages. After successful transfer, isolates would readily grow in M17.

3.3. Bacteriophage isolation

From three major cheese plants producing Dutch-type cheese in Norway, bulk starter samples produced using culture A were collected over a period of two months. The bulk starter samples