J. Dairy Sci. 98:1–10
http://dx.doi.org/10.3168/jds.2015-9535

Strain diversity and phage resistance in complex dairy starter cultures

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ABSTRACT

The compositional stability of the complex Gouda cheese starter culture Ur is thought to be influenced by diversity in phage resistance of highly related strains that co-exist together with bacteriophages. To analyze the role of bacteriophages in maintaining culture diversity at the level of genetic lineages, simple blends of *Lactococcus lactis* strains were made and subsequently propagated for 152 generations in the absence and presence of selected bacteriophages. We first screened 102 single-colony isolates (strains) from the complex cheese starter for resistance to bacteriophages isolated from this starter. The collection of isolates represents all lactococcal genetic lineages present in the culture. Large differences were found in bacteriophage resistance among strains belonging to the same genetic lineage and among strains from different lineages. The blends of strains were designed such that 3 genetic lineages were represented by strains with different levels of phage resistance. The relative abundance of the lineages in blends with phages was not stable throughout propagation, leading to continuous changes in composition up to 152 generations. The individual resistance of strains to phage predation was confirmed as one of the factors influencing starter culture diversity. Furthermore, loss of proteolytic activity of initially proteolytic strains was found. Reconstituted blends with only 4 strains with a variable degree of phage resistance showed complex behavior during prolonged propagation.
Key words: starter culture, bacteriophage, diversity, proteolytic activity

INTRODUCTION

In cheese production, 2 types of starter cultures are used: undefined and defined. Undefined starters originate from successful artisanal cheese production processes and were traditionally propagated in milk by back-slopping (Stadhouders and Leenders, 1984; Wouters et al., 2002); with back-slopping, the milk is inoculated with a small portion of a previously performed successful fermentation. Generally, the composition of undefined starters is unknown, which leads to uncertainty in terms of their performance in milk (Daly et al., 1996). Defined starters are manufactured blends of two or more strains, which ensures consistency in product quality. Nevertheless, defined starter cultures are more susceptible to bacteriophage predation when used in cheese manufacturing, and bacteriophages are still a major cause of fermentation failures in the dairy industry, leading to substantial raw material losses (McGrath et al., 2007). The limited number of strains used in such cultures explains the higher bacteriophage sensitivity of defined blends compared with more complex undefined starters. On the other hand, complex starter cultures containing highly related strains with variable levels of phage sensitivity are expected to be more resistant to phage attack because the phage-sensitive strains in these cultures will be replaced by their phage-resistant counterparts upon phage attack (Erkus et al., 2013).

Previously, an undefined complex Gouda type cheese starter culture called Ur was characterized in detail (Erkus et al., 2013). This starter culture consists of only 2 lactic acid bacteria: *Lactococcus lactis* and *Leuconostoc mesenteroides*, encompassing in total 8 genetic lineages as determined by a high-resolution amplified fragment length polymorphism (**AFLP**)-based method (Kütahya et al., 2011). Complex culture isolates (strains) belonging to the same genetic lineage possess similar functionally relevant phenotypic characteristics. For instance, all strains belonging to lineages 1, 3, and 5 (all *Lactococcus lactis* ssp. *cremoris*) possess proteolytic activity, and all lactococcal strains with the ability to utilize citrate belong to lineages 2 and 4 (both *Lactococcus lactis* ssp. *lactis* biovar *diacetylactis*). Isolates from the same genetic lineage are expected to show similar behavior during cheese production. Based on the analysis of only a limited number of strains, Erkus and co-workers (2013) showed that different strains belonging to the same genetic lineage display a large variation in bacteriophage resistance when challenged

Received March 5, 2015.
Accepted April 11, 2015.
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with phages originating from the same complex starter culture. Moreover, individual strains isolated from the starter culture were found to differ in plasmid content (Erkus et al., 2013).

The compositional stability of the Ur starter culture is thought to be partially based on the diversity in phage resistance of highly related strains that co-exist with bacteriophages (Erkus et al., 2013). It has been suggested that bacteriophage predation can prevent domination of a microbial community by a single variant. This mechanism provides the basis of the constant-diversity (**CD**) dynamics model, described by Rodriguez-Valera et al. (2009). The CD model explains the maintenance of microbial community diversity through bacteriophage predation based on a "kill-the-winner" mechanism (Thingstad, 2000; Winter et al., 2010). This mechanism prevents domination of the community by the fittest variant, because abundance of this variant will be eventually reduced by the bacteriophage.

We hypothesize that when the microbial community of a starter culture, stratified in genetic lineages, possesses diversity at the strain level, the diversity at the level of genetic lineages will be maintained by bacteriophage predation. To investigate if this phenomenon also occurs in simple blends of strains, such reconstituted cultures were sequentially propagated in the absence and presence of selected bacteriophages. In our study, diversity is a function of the number of genetic lineages present in the blend and their relative abundance (Haegeman et al., 2013). Compared with the nonchallenged control blends, relative abundance of different genetic lineages was not stable in blends challenged with bacteriophages, demonstrating the effect of phages on diversity in simple blends.

MATERIALS AND METHODS

Complex Starter Culture Isolates

Single-colony isolates of the complex starter culture Ur used in this study were collected from LM17 agar plates (Oxoid, Basingstoke UK; 1.5% wt/vol) supplemented with lactose (Oxoid; 0.5% wt/vol; Terzaghi and Sandine, 1975) and Reddy agar plates as described previously by Erkus et al. (2013). Individual isolates were characterized and classified into 8 genetic lineages by AFLP typing (Kütahya et al., 2011): *Lactococcus lactis* ssp. *cremoris* (lineages 1, 3, 5, 6, and 7), *Lactococcus lactis* ssp. *lactis* biovar *diacetylactis* (lineages 2 and 4), and *Leuconostoc mesenteroides* ssp. *cremoris* (lineage 8). Similarity of the genetic profiles of <90% was considered as the cut-off for defining separate genetic lineages. Isolates were maintained as glycerol stocks at −80°C and reactivated, in the case of lactococci, in LM17 broth (Oxoid) with addition of 0.5% (wt/vol) lactose (Oxoid) and, for *Leuconostoc* strains, in de Man, Rogosa, and Sharpe broth (Merck, Schiphol-Rij, the Netherlands).

Determination of Growth Rate of Representative Complex Starter Isolates

Complex culture isolates *L. lactis* TIFN1 (lineage 1), *L. lactis* TIFN2 (lineage 2), *L. lactis* TIFN3 (lineage 3), *L. lactis* TIFN4 (lineage 4), *L. lactis* TIFN5 (lineage 5), *L. lactis* TIFN6 (lineage 6), *L. lactis* TIFN7 (lineage 7), and *Leu. mesenteroides* TIFN8 (lineage 8) were used to determine specific growth rates in skim milk supplemented with 1% (wt/vol) casiton (Oxoid). Overnight cultures were washed by centrifugation at 5,000 × *g* for 10 min. The supernatant was discarded and pellets were resuspended in the same volume of magnesium sulfate (5 m*M*)-potassium phosphate (15 m*M*) buffer (pH = 6.5). Washed cells were inoculated (1% vol/vol) into skim milk (Friesche Vlag Lang Lekker, non-fat, UHT, Friesland Campina, Amersfoort, the Netherlands) for growth experiments. Optical density at 600 nm ($\mathbf{OD_{600}}$) was measured hourly in a 1.5-mL semi-micro cuvette (light path length = 1cm) using a spectrophotometer (Novaspec Plus, Biochrom, Cambridge, UK). Before measurement, milk samples were cleared with 0.2% (wt/vol) NaOH and 0.2% (wt/vol) Titriplex III (Merck) solution. Cleared skim milk was used as a reference. The specific growth rate was then calculated by plotting semi-logarithmically the OD_{600} values of exponential growth phase (logarithmic scale) versus time (linear scale); specific growth rates were expressed as h^{-1}. Because of the clarification step, plate counting was used to verify the accuracy of the growth curve based on OD_{600} data. One milliliter of milk sample was added to 9 mL of peptone physiological salt solution containing 0.85% (wt/vol) NaCl and 0.1% (wt/vol) neutralized bacteriological peptone (Oxoid) to make the first dilution. Serial dilutions were made and 50 μL of diluted sample was spread onto an LM17 agar plate or, for strain TIFN8, an MRSV agar plate (MRS supplemented with 30 mg/mL of vancomycin; Duchefa Biochemie, Haarlem, the Netherlands) using a spiral plater. Plates were then incubated at 30°C (LM17) or 25°C (MRSV) for 48 h and colonies were counted.

Sequential Propagation of Simple Defined Blends

Blends used as an inoculum for sequential propagation were prepared from overnight cultures of selected isolates (for details, see Figure 1B). Optical density of the overnight isolates cultures was measured and