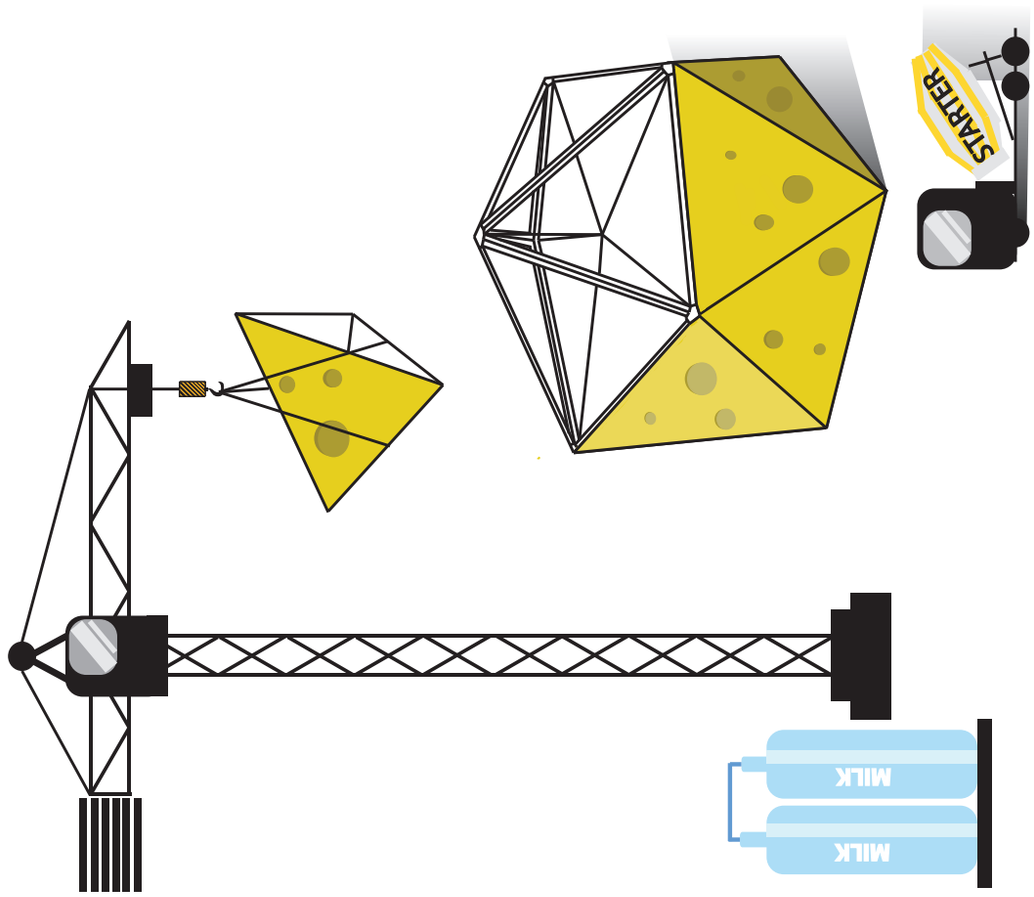
MIXED CULTURE ENGINEERING FOR STEERING STARTER FUNCTIONALITY

Maciej Spuś



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Mixed culture engineering for steering starter functionality

Maciej Spuś

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Nothing in biology makes sense except in the light of evolution

CH Dobzhansky

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Chapter 1

General introduction and outline of the thesis

INTRODUCTION

Humans have been crafting fermented foods for millennia, but only in the second half of 19th century (Pasteur, 1857), have they come to realize that microbes drive production of cheese, yoghurt, beer, wine or sourdough. This knowledge triggered further efforts towards assuring consistent quality of fermented foods by developing industrial-scale processes of manufacturing and selection of most suitable microbial 'workhorses'. These carefully selected microbes are called (fermentation) starter cultures or simply starters and their production became an activity of commercial companies. The dairy industry capitalizes on the development of starter cultures and has a strong interest in studying the behavior of starter communities, with the aim to control and steer their performance in a required direction. Additionally, starter cultures are attractive models to study ecology of microbes, evolution, microbe-microbe interactions and the impact of bacteriophage predation on population dynamics in microbial communities.

Dairy producers seek for consistently good quality in their products. Product quality is defined in terms of taste, aroma, texture and appearance and is, apart from the optimized technological process, assured by the performance of the starters. However, the performance of starters in the actual food matrix remains understudied. To control the process of dairy fermentation, knowledge of the composition, microbe-matrix and microbe-microbe interactions as well as ecology of the starter culture is necessary (Fleet, 1999). Only with the expansion of knowledge can one build a means to steer the processes of milk fermentation in the desired directions (Steele et al., 2013).

From traditional fermentations to modern industrial processes

The history of dairying and production of fermented milk products begins ca. 10,500 years ago together with the change of human lifestyle from hunting and gathering to farming and animal husbandry (cattle domestication; Vigne, 2008). All classic milk fermentation processes relied on spontaneous acidification by the microbes present in raw milk. Cheese production was no exception and traditional or artisanal cheese fermentations were and still are driven by natural milk cultures (NMCs) – mainly lactic acid bacteria (LAB) obtained by incubation of milk in specific conditions (Wouters et al., 2002). Such NMCs are being used, for instance, in production of Italian Traditional Specialty Guarantied (TSG) and Protected Designation of Origin (PDO) cheeses (Parente et al., 2016).

Nevertheless, relying on natural raw milk flora can lead to unpredicted results in terms of product quality (Parente and Cogan, 2004). To assure reproducibility of subsequent batches, part of the previous day's product is used as an inoculum to start a new fermentation. This way of maintenance of fermentation called back-slopping (Nout, 1994) was practiced for centuries even before it was known that bacteria are involved in the process at all. Back-slopping is considered to be the first technique which improved the success rate of natural fermentations. Practicing the technique of back-slopping for many years at farm level, allowed the propagated microbial community to be shaped towards an optimal composition. The factors responsible for shaping the culture's composition are: 1) population dynamics, 2) evolution, 3) influx of new biological material (including "new" bacterial strains and bacterial viruses = bacteriophages), 4) environmental conditions and 5) propagation regime. All these factors are addressed in this thesis.

LAB strains which ended up in the milk environment can have diverse origins, for instance, strains with the potential to be used in dairy applications were recently isolated from grass, vegetables and bovine rumen by Cavanagh and co-workers (2015). Moreover, signatures of adaptation to milk environment was found by Bachmann and colleagues (2012) in a plant-derived *Lactococcus lactis* strain after only 1000-generation-long sequential propagation in milk, suggesting much faster adaptation to a food-related niche than anticipated. Some LAB strains adapted to the conditions present in milk to such an extent that they are not anymore able to grow outside of milk (Kelly et al., 2010). Such adaptation to milk resulted in the loss of certain functions related to growth on plant substrate, reduction of genomes size as well as acquisition of new traits (mainly plasmid-encoded) allowing growth in milk (lactose fermentation, proteolytic activity; Siezen et al., 2011).

The role of starter cultures

The discovery of the role of lactococci in milk fermentations allowed further improvement of such processes (Lister, 1878). Isolation and characterization of LAB strains led to the development of starter cultures. The use of starter cultures assured production of microbiologically safe products with reproducible sensory and structural characteristics (Bassi et al., 2015). Nowadays, fermented dairy manufacturers rely on starter cultures produced on industrial-scale by a limited number of starter producers.

Dairy starters are usually mixtures of LAB strains (Leroy and De Vuyst, 2004), which originate from successful batches of fermented dairy products or from so-called whey-starters (i.e. used in a back-slopping procedure as described above). The principle role of the starter cultures is milk acidification, which directly contributes, via production of lactic acid from lactose, to the shelf life, safety and digestibility of the product. Moreover, especially in cheese, starters substantially contribute to the flavor development during the ripening process (Smit et al., 2005). In cheese, the complex flavor is formed through the combined effect of milk proteins degradation, including metabolism of amino acids, utilization of lactose and citrate and finally the degradation of lipids. The variety of metabolites released in all of these processes act as precursors for aroma formation (McSweeney and Sousa, 2000).

In cheese production one can classify starters into two groups: 1) thermophilic and 2) mesophilic. Thermophilic starters are mainly used in production of Italian and Swiss-type cheeses where a high temperature (37-52 °C) of manufacture is used. On the other hand, mesophilic starters are used in production of cheeses, such as Gouda, where temperature does not exceed 40 °C (Weimer, 2007).

Nowadays, in industrial Dutch-type cheese fermentations, two types of mesophilic starters are used (Lodics and Steenson, 1990): 1) defined and 2) complex undefined. Defined starters consist of up to several strains of known characteristics. Complex undefined starters comprise of many undefined LAB strains. Previous studies (Weimer, 2007) demonstrated that Gouda cheese starters contain strains of *Lactococcus lactis* and of *Leuconostoc mesenteroides* (in low abundance). Moreover, several different functional subspecies and variants of *L. lactis* were found in Gouda starters such as: caseinolytic and non-caseinolytic variants of *Lactococcus lactis* ssp. *cremoris* or citrate-fermenting *Lactococcus lactis* ssp. *lactis* biovar *diacetylactis.* However, it was indicated in recent studies (Erkus et al., 2013; Chapter 2 of this thesis) that undefined starter culture diversity reaches beyond the sub-species level. The investigation of the diversity of a culture beyond the level of (sub-)species is one of the principle aims of this thesis.

The study of Erkus and colleagues (2013) aimed to describe the clonal diversity of an undefined mixed Gouda starter culture to better understand the impact of this clonal diversity phenomenon on the functionality of the culture. Their results laid foundations for the great part of work described in this thesis. Erkus identified the players involved in the model undefined mixed

complex starter called "Ur" and delivered tools used in population dynamics studies included in this thesis. In particular, one of the main themes (bacteriophage predation) of the present study was based on the indication that heterogeneity in bacteriophage resistance among strains present in Ur culture is a basis for community resilience. The Ur starter culture and its phages emerged as an ideal model system to investigate the role of bacteriophage predation in microbial community diversity.

Many single colony isolates were collected after plating the Ur culture on different types of growth media to cover all possible diversity of strains typically present in undefined complex mixed starters (Erkus et al., 2013). These isolates were further ascribed to genetic lineages using high throughput amplified fragment length polymorphism (AFLP) typing (Kütahya et al., 2011). Eight genetic lineages were established and these were used as operational units in population dynamics when describing the Ur community during the cheese production process (Erkus et al., 2013; Erkus et al., 2016).

In the work presented in various chapters of this thesis, genetic lineages of Ur were used extensively as community population units to describe behavior of the Ur starter and defined multi-strain starters designed as blends of Ur isolates. In Figure 1 we present an overview of the Ur starter genetic lineages together with their most pronounced characteristics: phage resistance or sensitivity, caseinolytic activity, citrate utilization ability (leading to specific aroma formation) or oxygen sensitivity. These characteristics determine the main role of a particular lineage in Ur starter community as well as its behavior in terms of population dynamics both in the cheese production process and during sequential propagation in milk.

The role of bacteriophages

The role of bacteriophages in the ecology of microbial communities was addressed broadly throughout the history of microbiology, beginning with work of d'Herelle (1917). Different ecological niches such as horse gut, aquatic environment and human gut, were investigated with the focus on bacteriophage interactions and to unravel the role of viral particles (Golomidova et al., 2007; Rodriguez-Brito et al., 2010; Reyes et al., 2012). In the context of fermented dairy products, bacteriophages were mentioned for the first time by Whitehead and Cox (1935). Since that early work, bacteriophages have been considered as a threat to dairy fermentations (Daly et al., 1996).

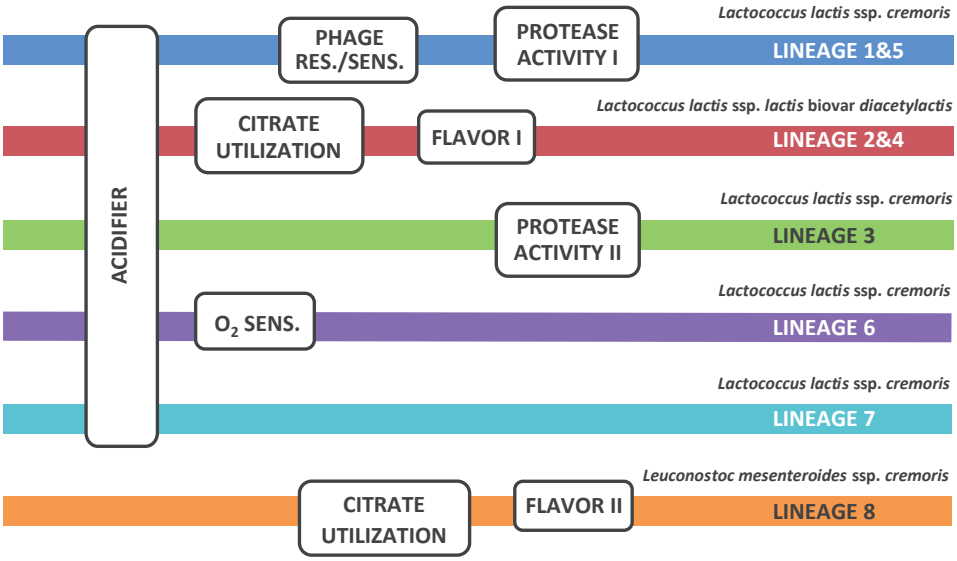

Figure 1. Overview of genetic lineages identified by Erkus et al. (2013) in Ur starter culture. Main characteristics impacting cultures overall functionality are given in boxes. O_2 SENS. – oxygen sensitivity; PHAGE RES./SENS. – diversity in terms of bacteriophage sensitivity profiles against phages isolated from Ur culture.

The dairy industry is heavily capitalizing on microbial communities to produce, for instance, cheese and yoghurt. For both cheese and yoghurt production, bacteriophages were found to be detrimental by causing acidification failures (Emond and Moineau, 2007). Since the bacteriophages are ubiquitously present in the cheese production environment (Verreault et al., 2011) perhaps the quotation from the paper of Lawrence et al. (1978) best explains the need for expanding knowledge on dairy starter culture's relations with phages: "To put a strain into a plant and hope for the best reminds one of Johnson's comment about a friend of his who, after an unhappy first marriage, was going to get married for a second time. It represented - he said; a triumph of hope over experience." Strong motivation to prevent detrimental effect of bacteriophage predation on the performance of LAB in dairy fermentations led both to investigation of viruses and their hosts. In the case of bacteriophages, more efforts were put into the improvement of process design and production hygiene (Daly, 1983; Mc Grath et al., 2007; Geagea et al., 2015)

as well as investigation into the mode of attachment of viral particles to bacterial cell walls (Klaenhammer and Fitzgerald, 1994; Kelly et al., 2013; Mahony et al., 2013; Farenc et al., 2014) and possible prevention of this event (Mahony et al., 2016). On the other hand, in case of bacteria, interim solutions to phage predation such as strain-rotation systems (Daly, 1983) and isolation of bacteriophage-insensitive mutants (BIMs) (Mills et al., 2007) were proposed. Moreover, studies on LAB led to discovery of several bacteriophage resistance mechanisms (Labrie et al., 2010): adsorption inhibition (Akçelik et al., 2000; Coffey and Ross, 2002), injection blocking, restriction/modification (Durmaz and Klaenhammer, 1995), abortive infection (Chopin et al., 2005) and as a hot topic of current molecular microbiology – CRISPR/Cas systems (Millen et al., 2012). Despite all these efforts, bacteriophage predation remains one of the biggest contemporary issues in dairy industry (Kleppen et al., 2011). Interestingly, in cheese production this issue concerns mostly defined starters not undefined complex starters, which naturally contain high numbers of bacteriophages (up to 10^8 pfu/mL; Cogan and Hill, 1993). This is possibly due to the presence of bacteriophages in undefined complex starters like Ur which plays a role in inherent phage resistance of such cultures (Stadhouders and Leenders, 1984; Stadhouders, 1986).

In contrast to studies emphasizing the negative aspects of phages in dairy industry, studies of other ecosystems claim an important role of bacteriophages in the generation of diversity of various microbial communities. This specific role of bacteriophage predation is based on the "kill the winner" principle, described by Thingstad (2000). This principle is analogous to classical Lotka-Volterra model explaining prey-predator relationships (Yorke and Anderson, 1973). In case of bacteria-bacteriophage dynamics, the increase in the abundance of host (the winner) is followed by the increase in the abundance of its bacteriophage predator, which eventually results in accelerated killing of the population of host cells. This principle is incorporated into a constant diversity (CD) dynamics model described by Rodriguez-Valera and colleagues (2009). The authors compare two opposite but not mutually excluding models of microbial community dynamics: periodic selection (PS) and CD. PS dynamics predicts that lineages with higher fitness would expand and replace other types, resulting in a clonal sweep (Rodriguez-Valera et al., 2009; Bendall et al., 2016). As a consequence, the ecosystem efficiency will reduce together with the reduction of abundance of the specialist lineages exploiting low abundant substrates. Such a situation

could take place also in case of the CD dynamics, although there it would be only a transient state since the expanding lineage would be selected against in accordance to the "kill the winner" principle. Eventually, the high diversity, leading to better exploitation of the resources, would be maintained and ecosystem efficiency in CD model would be higher than in PS model.

One could consider complex undefined mixed starters as microbial communities in which phage presence results in better distribution of diversity at genetic lineage level preventing the fittest strain from domination (Erkus et al., 2013). Bacteriophage predation in mixed starter cultures for cheese production can lead to more effective utilization of substrates by all members of the community preventing the risk of the culture becoming homogeneous. As such, bacteriophages can be considered as diversity drivers and eventually functionality guardians. This role of bacteriophages is questioned throughout the work described in this thesis.

A study of Ur starter community stability was described in the above-mentioned paper of Erkus et al. (2013), and it is important to introduce this work in the context of the central theme of this thesis. As stressed in the paper of Erkus et al. (2013), the Ur starter has a history of back-slopping. This propagation process led to continuous diversification of community members, which resulted in their heterogeneity and eventually in a culture resilient towards environmental fluctuations. This diversification sustains genetic potential of the entire population.

As mentioned before, heterogeneous phage sensitivity of strains within and between the genetic lineages may also play a role in sustaining the resilience of the culture. Due to the heterogeneous phage sensitivity profile, predation by phages will only lead to eradication of the sensitive cells but will fail to kill an entire lineage. This process was shown to be operational in the Ur starter (Erkus et al., 2013). Erkus and co-workers propagated the Ur starter daily for several weeks in milk and the abundance of genetic lineages of the starter was monitored continuously. In addition, a particular strain representing genetic lineages 1&5 (*L. lactis* TIFN1) was used as an indicator for monitoring the abundance of lytic phages predating on that specific strain. The results showed that despite dramatic changes in the abundance of phages throughout the propagation experiment (Fig. 2a, adapted from Erkus et al., 2013), which suggests changes in abundance of sensitive strain(s), strains belonging to the genetic lineages 1&5 maintained their position in the community (Fig. 2b and 2c). This experiment illustrates that phage predation can lead to

eradication of particular sensitive strains but due to heterogeneous phage sensitivity this will have only small effect on the relative abundance of the lineage to which these strains belong.

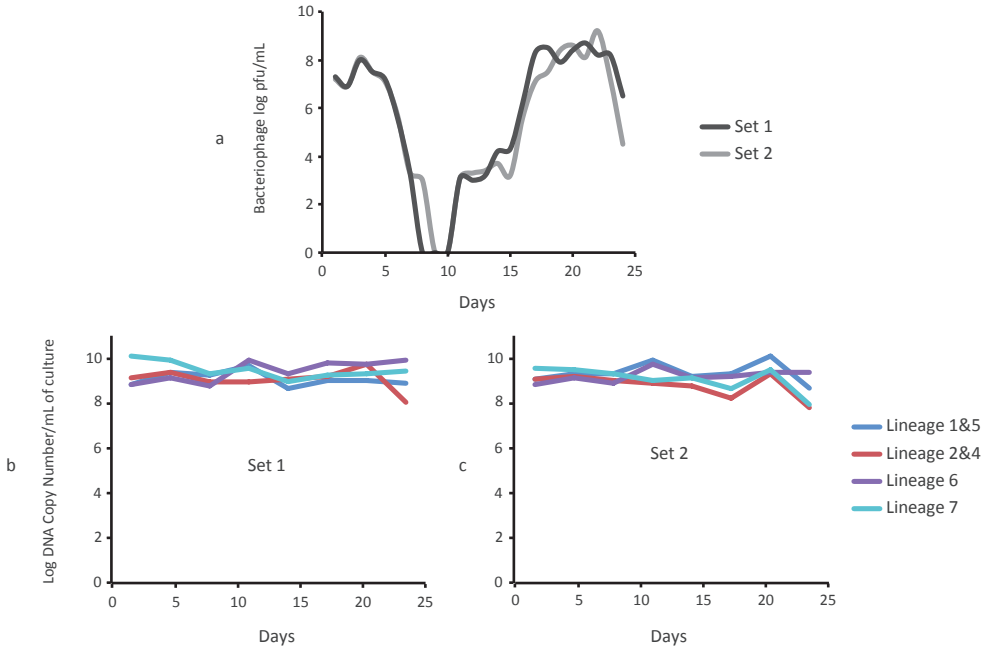

Figure 2. Bacteriophage abundance and Ur starter community dynamics during a back-slopping propagation regime. (a) Bacteriophage titer dynamics when *L. lactis* TIFN1 was used as an indicator organism for 25-day sequential propagation of Ur culture in milk. (b and c) Absolute abundance of the genetic lineages 1&5, 2&4, 6 and 7 during propagation as determined by genetic lineage-specific QPCR in two independent sets (adapted from Erkus *et al.*, 2013).

Trends in dairy research

Constantly changing consumer demand pushes the food industry to develop new products with novel properties. In case of dairy industry, which strongly capitalizes on milk fermentation processes, the development of new starter cultures is constantly required. A lack of consumer acceptance and tight regulation of recombinant DNA technology requires from starter producers the use of classical approaches for starter improvement including isolation, selection and characterization of new LAB strains or adaptive evolution (Derkx et al., 2014).

Finding new strains with interesting functionality

A challenge for the dairy industry and applied research is to develop new products with novel and desirable characteristics such as new flavors, aromas, vitamins and textures for which specific starters are responsible. One possible strategy to meet this challenge is to find and develop new strains. Some of the approaches focus on isolation and characterization of 'wild strains' suitable for industrial fermentations (Randazzo et al., 2008). Another approach relies on knowledge of abilities of already known strains to be able to adapt them to specific conditions and steer their performance in new directions.

One can discriminate two trends in dairy fermentation studies concerning the discovery of new strains or starter cultures. The first trend relates to the traditional fermentation processes of local products, for example Mabisi in Zambia (Schoustra et al., 2013) or Oscypek in Poland (Alegría et al., 2012). These traditional processes rely on the performance of an unknown collection of microbial species/strains, mostly LAB. Here the focus is on the characterization of the unknown microbes to the extent that it will be possible to develop more stable and efficient starter cultures for more robust and predictable (industrial) production, which can result in higher profits for producers. The second trend capitalizes on the same traditional food fermentation processes as a source of new and interesting strains (Cafara et al., 2015). Both trends are complementary and together aim towards better understanding of the function of microbes and microbial communities in dairy fermentation as such, and pave the way towards novel industrial applications.

Evolutionary engineering

Another approach is described by the term 'evolutionary engineering' (Sauer et al., 2001; Bachmann et al., 2015). This approach utilizes laboratory (experimental) evolution and further characterization of strains towards improvement and better understanding of the phenomena occurring in fermentation processes. One example is the study by Van Bokhorst-Van de Veen and co-workers (2013) who demonstrated the feasibility of experimental evolution for the enhancement of the functionality of a probiotic strain. *Lactobacillus plantarum* WCFS1 was sequentially passed through the digestive tract of a mouse, resulting in an intestine-adapted isolate, which could reside for longer in the mouse gut. Adaptation of a strain to specific controlled conditions might cause mutations, which can result in the required

phenotype. The conditions can be maintained in, for instance, a continued fed-batch bioreactor.

Adaptive evolution approaches can also be used to improve the performance of aroma adjunct strains such as *Lactobacillus helveticus* DSM 20075 used in cheese manufacturing fermentation processes. In particular, *Lb. helveticus* is known to possess a broad enzymatic potential (Griffiths and Tellez, 2013). Upon lysis of the cells during the ripening process intracellular enzymes are released into the cheese matrix and contribute to the aroma formation in cheese (Valence et al., 2000; Kenny et al., 2006; Broadbent et al., 2011). One of the goals for this strain improvement would be to obtain a variant of *Lb. helveticus* with increased lysis capacity. The challenge is to select the right set of cultivation conditions to enrich mutants with the desired properties. One example described in this thesis (Chapter 5) as well as others (Teusink et al., 2009; Zhang et al., 2012) demonstrate the power of adaptive evolution approaches to select industrial strains with specific characteristics (Portnoy et al., 2011).

Sequential propagation of a particular microbial strain under defined laboratory conditions is often used as a tool to study the genetic adaptation to particular environmental constraints (Teusink et al., 2009; Kawecki et al., 2012). Laboratory evolution experiments with microbial communities (not single strains) are rarely described in literature with only a few examples so far (Bessmeltseva et al., 2014; Fiegna et al., 2015). The experimental evolution with microbial communities can be used to study population dynamics of different lineages in response to environmental factors as well as to investigate the evolutionary processes resulting in changes of microbial interactions. A study described in Chapter 4 of this thesis demonstrates that experimental evolution of a complex starter culture leads to enrichment of a strain initially present at low abundance and undetectable for previously used analysis techniques such as metagenomics.

Summary

The role of bacteriophage predation in ecology of food fermentation microbial communities did not receive sufficient attention in empirical studies so far. Furthermore, the perception of bacteriophages in the dairy industry is certainly negative. This thesis demonstrates a beneficial role of bacteriophages in complex starter cultures. Moreover, from the perspective of

microbial ecology, such complex dairy starter cultures provide an excellent model to study microbe-phage relationships.

Experimental evolution using complex microbial communities of the starter culture can teach us more about population dynamics, ecology, evolution and factors impacting these phenomena. In addition, adaptive or experimental evolution approaches also provide tools to steer functionality of microbial cultures towards industrially-relevant directions.

Outline of the thesis

In *Chapter 2* the focus is put on the characterization of a collection of strains, isolated from the complex starter culture Ur, in terms of their bacteriophage resistance to three lytic phages isolated from the same culture. We demonstrate a high degree of variation in phage resistance among the Ur strains. Furthermore, population dynamics of blends of strains - representing three genetic lineages - were monitored during prolonged sequential propagation in milk in the presence and absence of bacteriophages. The result of this experiment confirmed complex behavior of blends of strains during propagation and the impact of phage predation on the strain composition.

In *Chapter 3*, the complexity of the defined blends of strains was increased by using a 24-strain blend to investigate the impact of bacteriophage predation on the population dynamics during prolonged sequential propagation in milk. The results presented confirm the impact of phage predation at the level of genetic lineage diversity of the starter community but also suggest that other factors such as microbe-microbe interactions play role in establishing the composition and functionality of the starter culture.

A Long-term experimental evolution experiment using an Undefined Mixed Starter Culture (LUMSC) is the central theme of *Chapter 4*. The tools for determining the relative abundance of genetic lineages were used to monitor the composition of the complex culture throughout a 1000-generation propagation experiment in milk. The LUMSC experiment confirmed the important role of factors such as temperature of incubation and propagation regime on the culture composition. Moreover, the LUMSC experiment uncovered under the radar complexity of the starter with the discovery of a previously non-identified *Lactococcus* species reaching a substantial abundance after 1000 generations.

Chapter 5 describes the efforts to obtain a mutant *Lactobacillus helveticus* with increased autolytic capacity. Inspired by the work done with *Lactococcus lactis* by others (Smith *et al.*, 2012), we used suboptimal elevated incubation temperatures to select for survivors *Lb. helveticus* DSM20075 and screen them for increased autolytic capacity. We found a mutant with increased lysis capacity compared to wild-type strain in a low pH lactate buffer with added NaCl. We further confirmed that the same mutant (variant V50) had a defined impact on the aroma profile of a model lab-scale cheese.

Finally, in *Chapter 6 – General Discussion,* the factors for establishing starter microbial communities are discussed. Here a reflection on studies performed in the context of this doctoral research project is presented together with generic conclusions, case studies and terms that shed light on better control over the performance of the dairy starter cultures.

Chapter 2

Strain diversity and phage resistance in complex dairy starter cultures

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Judith CM Wolkers-Rooijackers, Marcel H Zwietering,
Tjakko Abee, Eddy J Smid

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.

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 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 *Le. 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 (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 cultures were diluted further to obtain the same cell density: OD_{600} = 1.1. Bacterial cells were centrifuged (10,000 × g, 3 min) and resuspended in magnesium sulfate (5 m*M*)-potassium phosphate (15 m*M*) buffer (pH 6.5). Next, cells were centrifuged again and resuspended in 10 times less buffer. Defined blends of strains were inoculated (1%) in 10 mL of skim milk (Friesche Vlag Lang Lekker, non-fat, UHT milk, Friesland Campina). Each 24 h, 0.5% (vol/vol) of the previous day's milk culture was used as an inoculum for propagation in fresh milk, obtaining 7.6 generations after each transfer. Blends were incubated at 20°C. Samples of the blends were taken each 48 h and kept frozen at −20°C until further analysis. Where relevant, bacteriophages were added to the blends at a concentration of 10^8 pfu/mL.

Bacteriophages

Bacteriophages used in this study were isolated as described in Erkus et al. (2013). Three phages found in the supernatant of the undefined starter culture were labeled as ΦTIFN1, ΦTIFN5, and ΦTIFN7. Based on genome sequence information, ΦTIFN1 and ΦTIFN7 belong to the lactococcal phage type P335, whereas ΦTIFN5 is classified as a type 936 lactococcal phage (Mahony and van Sinderen, 2014).

Viable Plate Counting

Propagated blends of *L. lactis* strains were plated on β-glycerolphosphate-buffered milk agar (GMA) plates supplemented with bromocresol purple (Huggins and Sandine, 1984). Samples of cultured blends were diluted and plated in duplicate. The differential plating was used to estimate the loss of protease activity in the sequentially propagated blends. Protease-positive colonies are larger and are surrounded by a yellow zone compared with the small, translucent colonies of protease-negative variants.

Quantitative PCR

As described by Erkus et al. (2013), representative strains TIFN1, TIFN2, TIFN3, TIFN4, TIFN5, TIFN6, TIFN7, and TIFN8 of the 8 genetic lineages were selected and their genomes sequenced. Primer sets were developed based on sequences of unique genes of each representative strain (Supplemental Table S1) to obtain amplicon lengths of 60 to 70 bp. Primers were optimized for consistent annealing at 60°C for similar reaction conditions. Pairwise comparison of the genomes of strains TIFN1 and TIFN5 and of strains TIFN2 and TIFN4 showed only a limited number of strain-specific unique genes. None of those unique genes met all the criteria set for designing specific primers. To enumerate lineage 1 and 5 strains, probes were designed that reacted specifically with strain TIFN1 as well as strain TIFN5 (hereafter, lineage 1–5). To enumerate lineage 2 and 4 strains, probes were designed that reacted specifically with strain TIFN2 as well as strain TIFN4. Samples of the propagated blends were collected after 24 h and subsequently each 48 h (every 2 transfers), and lineage-specific quantitative (q)PCR was performed to determine the relative abundance of copy numbers of each lineage in the blend.

Colony PCR

Primers for colony PCR (Supplemental Table S3) were designed based on the same collection of unique genes as described in the qPCR approach. Primers were designed so that the obtained PCR products would differ by approximately 80 bp to allow distinction by gel electrophoresis. A colony was picked from the GMA plate using a sterile loop and directly transferred to the PCR mix. PCR mix contained 5 µL of dNTP (2 m*M*), 2 µL of forward primer (10 µ*M*), 2 µL of reverse primer (10 µ*M*), 5 µL of 10 × DreamTaq buffer (Thermo Fisher Scientific, Waltham, MA), 3 µL of $MgCl_2$ (25 m*M*), and 0.4 µL of DreamTaq DNA Polymerase (Thermo Fisher Scientific; 5 U/µL). The program of the PCR run contained the following steps: 10 min at 95°C for cell lysis and activation of DNA polymerase, 25 cycles of 30 s at 95°C for DNA melting, 20 s at 60°C for primer annealing, and 30 s at 72°C for elongation. The PCR reaction finished with 4 min at 72°C for final elongation.

Phage Resistance by Spot Assay

Phage resistance of 102 single-colony isolates of the undefined complex starter culture Ur was determined using a qualitative spot assay (Sanders and Klaenhammer, 1983). Modifications of the method included smaller Petri dish plates containing LM17 agar [35 mm in diameter, 1.5% (wt/vol) agar, supplemented with 0.5% (wt/vol) lactose and 10 m*M* $CaCl_2$; Greiner BioOne , Alphen aan den Rijn, the Netherlands], a lesser volume of soft layer agar (1 mL), and a lesser volume of highly concentrated (10^{10} pfu/mL) phage solution (5 μL).

Plaque Assay

The plaque assay used in this study was a slight modification of the method described by Lillehaug (1997). One hundred microliters of phage solution diluted in a phage buffer [Tris-HCl pH 7.5 (final concentration 50 m*M*), NaCl (final concentration 100 m*M*), $CaCl_2$ (final concentration 5 m*M*), $MgSO_4$ (final concentration 1 m*M*), gelatin (final concentration 0.01% (wt/vol)] and sterile water was added to 100 μL of the overnight culture of a single-colony isolate of a query strain. The mixture was kept for 10 min at 37°C in a water bath, mixed with 3 mL of top soft agar (0.75% wt/vol), and finally poured on LM17 agar plates. After 24-h incubation at 30°C, plaques were counted and plaque-forming units per milliliter values were calculated.

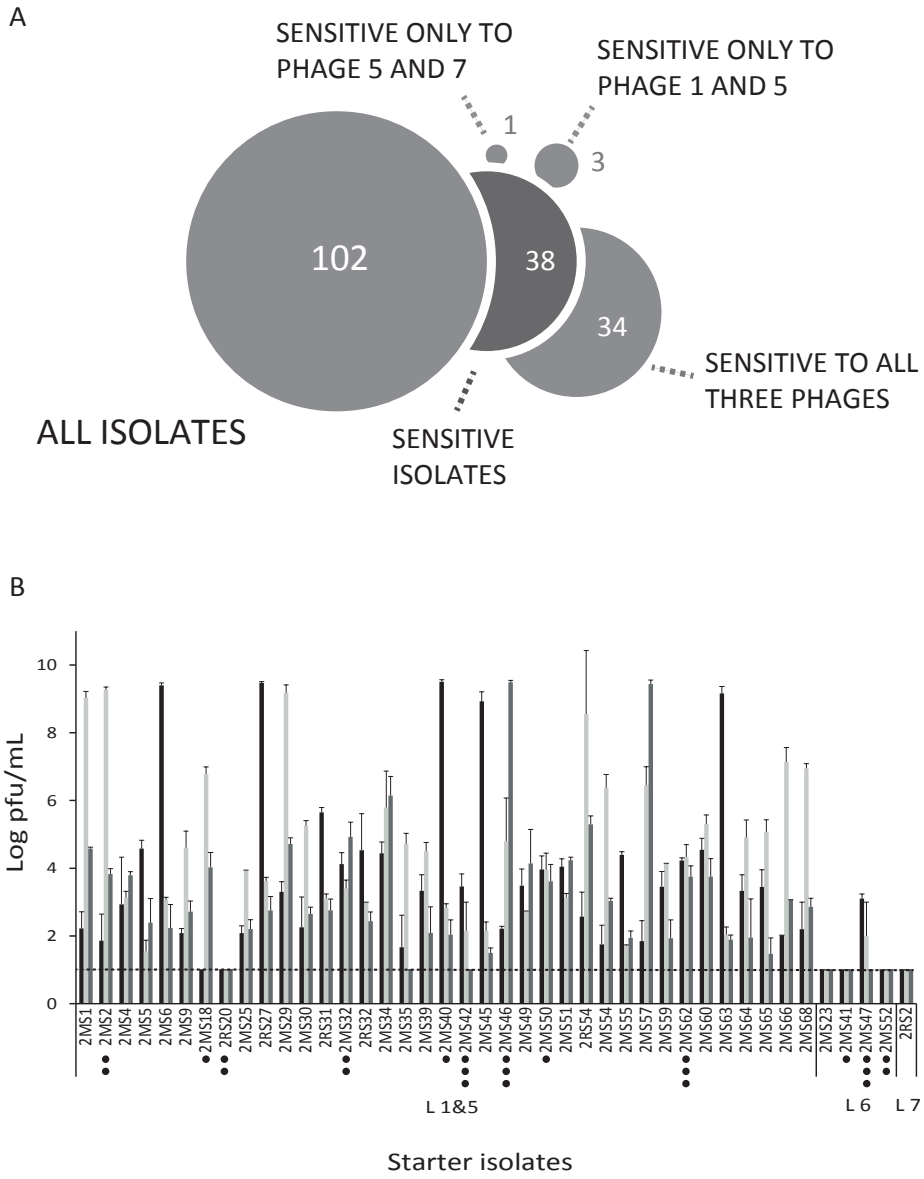

Figure 1. (A) Summary of bacteriophage resistance survey of 102 Ur starter isolates based on spot assay results. (B) Phage titer determination of single-colony isolates of Ur starter culture. All strains that were sensitive in the spot assay were tested plus 4 strains that showed complete resistance in the spot assay (in total 42 strains). L 1-5 = lineage 1-5; L 6 = lineage 6; L 7 = lineage 7. Strains used in blend 1 = 1 black dot; strains used in blend 2 = 2 black dots; strains used in blend 3 = 3 black dots. Dashed line represents detection limit for phage titer determination method (1 log pfu/mL). Black bars = results for phage ΦTIFN1; light gray bars = results for phage ΦTIFN5; = dark gray bars = results for phage ΦTIFN7.

RESULTS

Growth Rates of Individual Strains of Complex Starter

Individual strains isolated from the complex starter culture and representing all genetic lineages present in the culture were found to differ in their maximum growth rates (μmax) in casiton-fortified milk. Strain TIFN5 (representative of lineage 5) had the highest growth rate (μmax = 0.67 h^{-1}), followed by strain TIFN1 (lineage 1; μmax = 0.58 h^{-1}), strain TIFN8 (lineage 8; μmax = 0.5 h^{-1}), strain TIFN3 (lineage 3; μmax = 0.49 h^{-1}), strain TIFN4 (lineage 4; μmax = 0.41 h^{-1}), strain TIFN7 (lineage 7; μmax = 0.31 h^{-1}), strain TIFN2 (lineage 2; μmax = 0.29 h^{-1}) and, finally, strain TIFN6 (lineage 6; μmax = 0.24 h^{-1}). Based solely on these measured growth rate differences, the slow-growing lineage 6, for instance, is expected to disappear from the culture after 5 transfers (46 generations; see Supplemental Figure S2). The co-existence of different strains in the starter despite differences in individual growth rates suggests the influence of other factors in maintaining strain diversity, such as microbe–microbe interactions (Smid and Lacroix, 2013), selective conditions in acidifying milk, or phage predation.

In-Depth Analysis of Phage Resistance Profiles of 102 Individual Isolates

To obtain a detailed overview of the level of bacteriophage resistance of members of the complex starter culture, we determined the bacteriophage resistance of 102 strains (single-colony isolates), representing the 7 lactococcal genetic lineages of the starter culture, was determined using a spot test assay using the 3 phages (ΦTIFN1, ΦTIFN5, and ΦTIFN7) previously isolated from Ur (Erkus et al., 2013; Supplemental Figure S1). This screening demonstrated that 38 out of 102 strains tested were sensitive to at least 1 of the 3 bacteriophages used in this study (Figure 1A). These 38 sensitive strains together with 4 resistant strains (in total 42) were subsequently used for quantitative phage titer determination. A large variation was found in bacteriophage resistance among the 42 isolates (Figure 1B). First, differences in resistance for one particular phage were found between isolates belonging to different genetic lineages. Interestingly, very large differences in resistance were detected between isolates belonging to the same genetic lineage, ranging from values below the detection limit (10 pfu/mL) to 10^9 pfu/mL. Large differences in phage resistance of closely related *L. lactis* strains have been

reported previously (Ward et al., 2004). Based on results of the quantitative phage resistance assay, the collection of strains was classified into 4 resistance groups: (1) sensitive (S), >6 log pfu/mL; (2) moderately resistant (MR), between 3 and 6 log pfu/mL; (3) resistant (R), between 1 and 3 log pfu/mL; and (4) no detectable susceptibility (ND), <1 log pfu/mL (detection limit). Results of the phage resistance analysis can be used to design blends of strains with a known degree of phage resistance. This also allows us to determine experimentally if relatively simple blends (composed of 4 strains representing 3 lineages) meet the required level of culture diversity for stabilizing the community diversity driven by a CD-like mechanism.

Design of Defined Blends of Strains with Variation in Phage Resistance

The next step was to determine the effect of bacteriophage predation on the maintenance of diversity of simple blends of starter isolates upon sequential propagation. For that reason, we designed simple blends of strains from lineage 1–5 and lineage 6 with a known degree of phage resistance (Supplemental Table S2). In the experimental design (Figure 2), we (1) reduced the complexity of the starter culture by limiting the number of strains used in blends, and (2) mimicked the variation in resistance to phages found in the starter culture. These 2 requirements were met by using only 4 strains inoculated in equal amounts: 3 representatives from lineage 1–5 and 1 representative from lineage 6. Three strains from lineage 1–5 differ in their degree of resistance to phages: S, MR, and R. No detectable sensitivity (ND) was found for the strain belonging to lineage 6. The strains from lineage 1–5 possessed caseinolytic activity (protease positive, prt^+) and the strains from lineage 6 were protease negative (prt^-). In total, 3 blends (blends 1, 2, and 3) were prepared that were challenged at the onset of the experiment with phage ΦTIFN1, ΦTIFN5, or ΦTIFN7, respectively (Supplemental Table S2). Relative abundance at the level of genetic lineages was monitored throughout the propagation experiment using a qPCR-based method (see Materials and Methods). Control blends were not challenged with phages.

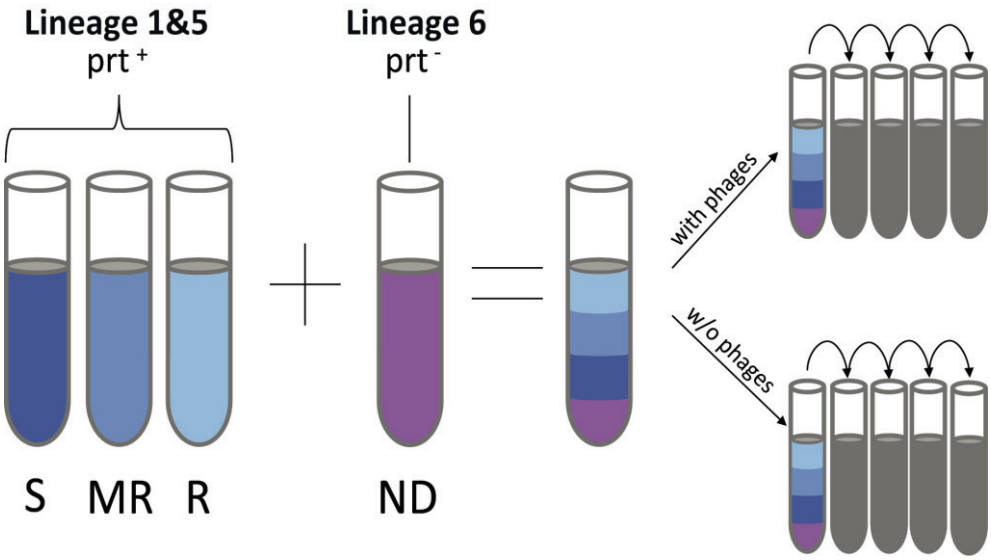

Figure 2. Experimental design of simple blends. S = sensitive isolate (>6 log pfu/mL); MR = moderately resistant isolate (>3, <6 log pfu/ mL); R = resistant isolate (>1, <3 log pfu/mL); ND = no detectable susceptibility (<1 log pfu/mL; detection limit); prt^+ = protease positive isolates; prt^- = protease negative isolate.

Population Dynamics in Defined Blends

All blends described in the previous paragraph were propagated in milk for 152 generations and the relative abundance of lineage 1–5 and lineage 6 was determined during the course of the experiment (Figure 3).

In all designed blends, the initial distribution of lineages (75% lineage 1–5; 25% lineage 6) changed after 1 propagation cycle and, in all cases, one of the lineages became dominant. In blends without phage addition, lineage 1–5 became dominant, with relative abundances ranging from 79% (blend 3) to 99% (blend 1). In the phage-challenged blend 2 and blend 3, lineage 6 developed dominance with more than 99% relative abundance in the culture. One exception was the phage-challenged blend 1, in which lineage 1–5 was detected at a relative abundance of 97%. In nonchallenged blends, lineage 1–5 retained dominance throughout the entire propagation experiment, with only one deviating observation—in blend 2 after 91 generations.

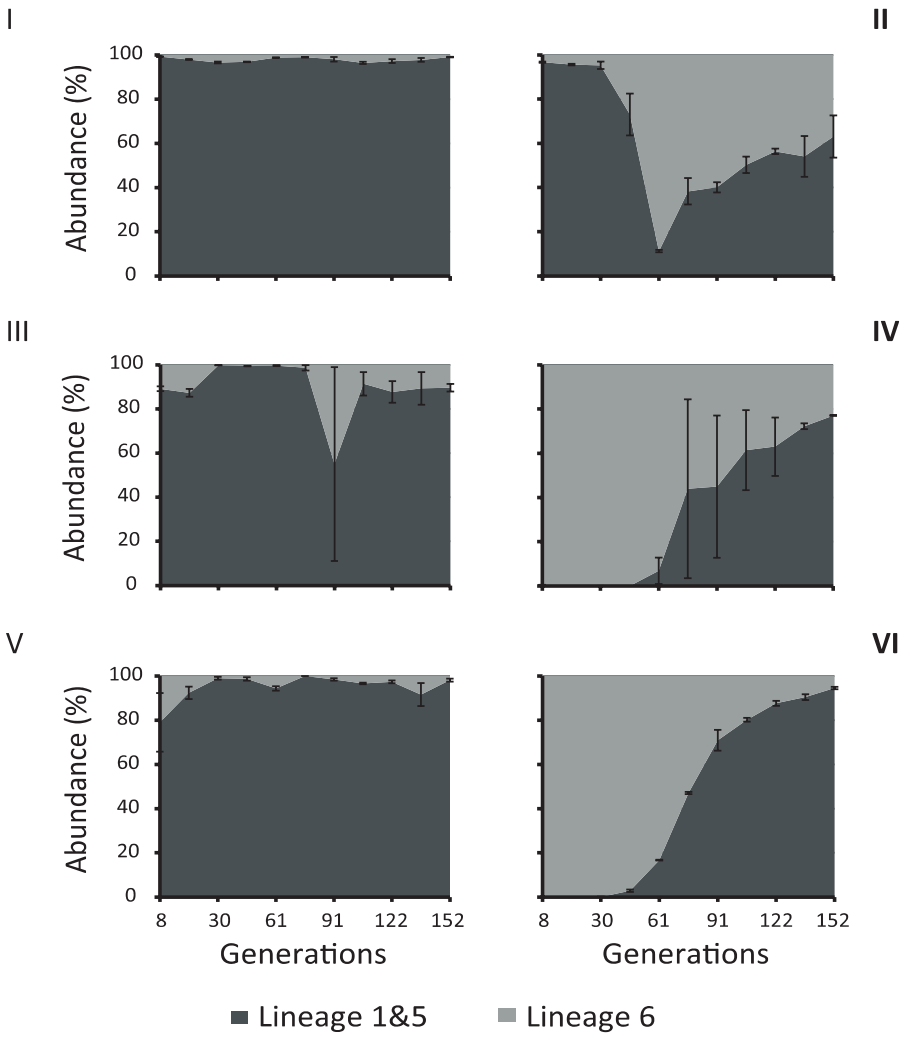

Figure 3. Relative abundance of lineages in blends during propagation experiment. Dark gray = lineage 1-5; light gray = lineage 6; I = blend 1 without phage; II = blend 1 with ΦTIFN1; III = blend 2 without phage; IV = blend 2 with ΦTIFN5; V = blend 3 without phage; VI = blend 3 with ΦTIFN7. Black bars represent standard deviation of 2 independent biological replicates.

Phage-challenged blends were overall more dynamic in composition compared with the nonchallenged blends. In blend 1, lineage 1–5 decreased and then increased, whereas in blends 2 and 3, initial abundance of lineage 6 was reversed after about 40 generations.

Prt⁺/Prt⁻ Shifts Can Be Explained by Competition and Loss of Plasmid Encoding prtP Gene

The initial percentage of protease-positive strains in all 3 blends was set at 75% (contributed by 3 lineage 1–5 strains). At the end of the propagation experiment, we could still detect protease-positive colony forming units but the initial percentage was reduced substantially. For blend 1, challenged with phage ΦTIFN1, prt⁺ colonies averaged 8% (while 60% lineage 1–5) and, without phage challenge, averaged 69% (99% lineage 1–5). For blend 2, challenged with phage ΦTIFN5, prt⁺ colonies averaged 65% (77% lineage 1–5) and without challenge, averaged 51% (90% lineage 1–5). For blend 3, challenged with phage ΦTIFN7, prt⁺ colonies averaged 27% (95% lineage 1–5) and, without phage challenge, averaged 32% (98% lineage 1–5; Supplemental Table S4).

After 152 generations (end-point of sequential propagation), we isolated 10 prt⁺ colonies and 10 prt⁻ colonies from blends challenged with bacteriophages and performed colony PCR with lineage-specific primer sets. Interestingly, in blends 2 and 3, all prt⁻ colonies were found to belong to lineage 1–5 (data not shown). It is known that upon propagation in milk, prt⁺ variants can lose the plasmid that carries the gene encoding the caseinolytic protease (*prtP*; McKay and Baldwin, 1975; Laan et al., 1989). The loss of plasmids can give a growth advantage to the host (higher growth rate) in competition for niche domination (Hugenholtz and Veldkamp, 1985; Bachmann et al., 2011).

Phage Sensitivity After Sequential Propagation

We exposed the 60 single-colony isolates described previously (originating from the end-point of sequential propagation; 20 per blend) to the filtered supernatant collected from the blends at the same time point as well as to the original bacteriophages ΦTIFN1, ΦTIFN5 and ΦTIFN7 (Supplemental Figure S3). Only 2 isolates were sensitive to the phages present in the supernatant; these came from blend 1. These 2 isolates were also sensitive to ΦTIFN1. Although none of the single-colony isolates from blends 2 and 3 showed plaques when exposed to the corresponding end-point supernatants, they were all sensitive towards the original bacteriophages (ΦTIFN5 and ΦTIFN7, respectively).

DISCUSSION

In this study, we demonstrated in detail the large variation in the degree of phage resistance among strains isolated from a complex cheese starter culture representing different lineages and, more importantly, among strains originating from the same lineage. This variation is thought to be instrumental for phage- mediated compositional stability of microbial communities because it theoretically prevents clonal sweeps of genetic lineages. Similar observations were reported in a study of *Cellulophaga baltica* in the marine environments by Holmfeldt et al. (2007), who showed that large differences in phage resistance are present among highly related strains of *C. baltica*, indicating the highly complex character of phage–host interactions in this environment. Holmfeldt et al. (2007) suggested that high variation in resistance to phages assures continuous succession of less-susceptible strains and supports the hypothesized role of phages as drivers of strain diversity and their impact on population dynamics.

In this study, the genetic lineage diversity of a complex starter culture was investigated by designing blends of well-characterized culture isolates. This approach enabled us to assess the effect of bacteriophages on diversity at the level of genetic lineages in simple blends of well-characterized *L. lactis* strains.

In blends without phage addition (i.e., control blends), lineage 1–5 (phage sensitive and protease positive) dominated the community, most likely due to higher growth rate in milk compared with that of the strain representing lineage 6. This observation is in accordance with our predictions based on the specific growth rates of the representative strains (Supplemental Figure S2). After 1 propagation cycle of conjoint growth of strain TIFN1 with strain TIFN6, TIFN1 would reach 9 log (94% total abundance) and TIFN6 would reach 7.8 log (6% total abundance). Based on the observed growth rates, when strains TIFN5 and TIFN6 are co-cultured, they would reach 9 log (96% total abundance) and 7.7 log (4% total abundance), respectively. These theoretical calculations are comparable to the experimental results, where after 1 propagation cycle, the measured abundance of lineage 1–5 ranged from 79% (blend 3) to 99% (blend 1). It has been reported previously that prt^+ strains of *L. lactis* have higher growth rates compared with prt^- strains at pH values <6 (Hugenholtz and Veldkamp, 1985), a pH value typical of acidifying milk. Moreover, the phenomenon of high local substrate availability stabilizing

cooperative traits such as the expression of an extracellular protease (Bachmann et al., 2011) conceivably contributes to the dominance of lineage 1–5 in blends without phage. In this model, the outcome of the competition between prt^+ and prt^- is density dependent. Protease-negative strains can only outcompete prt^+ strains at high cell densities when high global concentrations of peptides prevail. After 152 generations of sequential propagation (final time point in our experiment), we found prt^- variants belonging to lineage 1–5. Apparently, a fraction of the prt^+ cells of lineage 1–5 lost the plasmid carrying the protease-encoding gene (McKay and Baldwin, 1975). The newly emerging prt^- variants substituted part of nonproteolytic lineage 6 variants in the control blends, leading to stable cooperation between the prt^+ fraction of lineage 1–5, the prt^- fraction of lineage 1–5, and the prt^- lineage 6 strains. In blend 1 without phage, 99% of the abundance was ascribed to strains of lineage 1–5 but we found only 69% of the colonies to be prt^+. For blend 2, strains of lineage 1–5 comprised 90% of total abundance but only 51% of all colonies were prt+, and for blend 3, lineage 1–5 dominated with 98% abundance but we found only 32% prt+ colonies.

In the phage-challenged blends, we observed 2 trends in the population dynamics: (1) initial dominance of lineage 1–5 followed by a decrease in its abundance and finally a steady increase (Figure 3, panel II) and (2) initial dominance of lineage 6 through the first 46 generations followed by a steady increase in abundance of lineage 1–5 (Figure 3, panels IV and VI). Both phenomena are probably influenced by phage predation because we did not observe them in the control blends. In the phage-challenged blend 1, most likely the phage-resistant strain (R) of lineage 1–5 dominated the blend until 30 generations, followed by a decrease until 61 generations. The latter might be explained by the occurrence of (evolved) variants of phage ΦTIFN1 with increased lytic activity towards strain 2MS18 (the R variant of lineage 1–5). It is known that phages can adapt quickly to new potential hosts (Gómez and Buckling, 2011). Because many prophage sequences have been found in the genomes of sequenced Ur isolates (Erkus et al., 2013), an alternative explanation might be the expression of prophages during the propagation experiment, potentially releasing phages that predate on the resistant variants in the blend. Moreover, individual strains isolated from Ur and belonging to genetic lineage 1–5 were found to contain inducible prophages (our unpublished results). Finally, the subsequent increase in abundance of lineage

1–5 variants might reflect an increase in numbers of MR or S strains due to inability of the expressed prophage to predate on these strains.

When we challenged blends 2 and 3 with ΦTIFN5 and ΦTIFN7, respectively (Figure 3, panels IV and VI), initially only a small fraction of lineage 1–5 variants (<1%) survived predation by these phages. Both blends were initially dominated by the prt$^-$ lineage 6 strain, feeding on the peptides and amino acids released by the large number of lysed cells or by the minor fraction of proteolytic survivors belonging to lineage 1–5. The subsequent increase in abundance of lineage 1–5 strain(s) observed after 46 generations can be ascribed to the occurrence of variants with increased phage resistance or to "wash out" of phages after disappearance of phage-sensitive host cells in the culture. In blends 2 and 3, lineage 1–5 variants gradually increased in abundance after 46 generations (i.e., 5 transfers), with each transfer (0.5% inoculum) diluting the phage fraction 200 times, giving <0.1 pfu/mL after 4 transfers. A wash-out of phage after 46 generations could explain the observed increase in the abundance of lineage 1–5. Moreover, in the supernatant of blends 2 and 3, we did not find phages predating on the end-point isolates (Supplemental Figure S3). The new fraction of strains of lineage 1–5 slowly recovered and gradually increased up to 77% (blend 2) and 95% (blend 3) after 152 generations.

In both observed phenomena—initial dominance of lineage 1–5 and initial dominance of lineage 6—phages seem to affect blend diversity, but after 46 (blends 2 and 3) or 61 (blend 1) generations, the effects of phages weakened and other factors, such as growth rate in milk and the action of cooperative traits (prt$^-$ benefiting from prt$^+$), influence the relative abundance of the lineages. In all 3 phage-challenged blends after 5 to 7 transfers (46 to 61 generations), we observed a trend of increasing abundance of lineage 1–5 variants. When there were no phages present, this simple culture gravitated toward stable domination of lineage 1–5. The results obtained in phage-challenged blends suggest that eventually phage-resistant variants survive in these simple communities, as witnessed by their gradual increase in relative abundance, finally leading to a stable situation, as found in blends without phage addition. Our results on phage susceptibility of isolates from the end-point of propagation (152 generations) support this conclusion. Only two strains (from blend 1) out of 60 strains isolated from end-point (20 strains per blend) showed sensitivity to the supernatant obtained from the sample at the end-point (Supplemental Figure S3).

Bacteriophage predation is not the only factor affecting the composition of microbial communities in general and that of complex dairy starters in particular. Many other factors, such as the genetic repertoire of the strains, interactions between strains, competition, and environmental fluctuations, are thought to be important (Smid and Lacroix, 2013), although the relative contribution of each of these factors is difficult to quantify. Yogurt starter cultures represent another example of simple community of 2 interacting species of lactic acid bacteria: *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*. The interaction between these 2 yogurt bacteria, known as proto-cooperation, is crucial for optimal culture functionality and final product characteristics (Sieuwerts et al., 2008; Settachaimongkon et al., 2014). From earlier studies (Hugenholtz et al., 1987; Bachmann et al., 2011), we know that the ratio of protease-positive to protease-negative variants is crucial for the acidifying activity of the cheese starter culture. Analysis of the protease activity of the isolates used for blend preparation and of the isolates picked up after 152 generations demonstrated that some of the protease-positive strains lost their prt^+ phenotype upon propagation in milk. Our results for blends with phages showed that the niche occupied initially by the lineage 6 strain was partially taken over by prt^- variants of lineage 1–5 strain(s). Hugenholtz and Veldkamp (1985) recognized the presence of protease-negative variants in mixed starter cultures as a separate factor contributing to culture complexity.

The complex cheese starter culture Ur consists of multiple highly related strains representing different co-existing genetic lineages, and the current study demonstrated that strains belonging to the same genetic lineage differ to a large extent in bacteriophage sensitivity. The approach of designing simple blends (i.e., low strain number) was chosen to investigate the influence of phages on culture diversity. The relatively low lineage diversity in these simple blends and exposure to one of the selected phages from the original starter was not enough to sustain maximal diversity at the level of lineages. Further research with defined blends encompassing a larger collection of strains and more diversity resembling the original complex starter culture, combined with predation by multiple types of phages, would allow evaluation and grading of the role of phage predation and other factors in sustaining diversity of the culture upon propagation in milk and its effect on starter functionality (Smid et al., 2014; Smid and Kleerebezem, 2014).

SUPPLEMENTARY MATERIAL

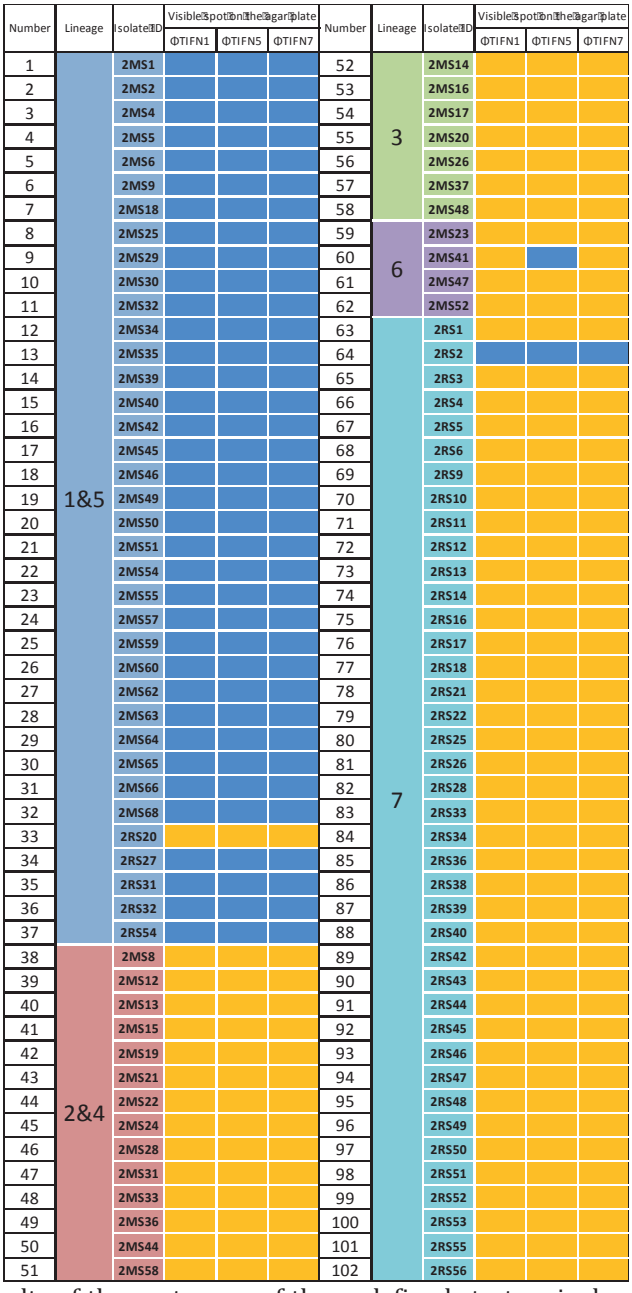

Figure A1. Results of the spot assay of the undefined starter single colony isolates. 5 µL of bacteriophage solutions (ΦTIFN1, ΦTIFN5 and ΦTIFN7) was put on the bacterial cell lawn of each of the isolates. Blue – visible spot; yellow – no visible spot.

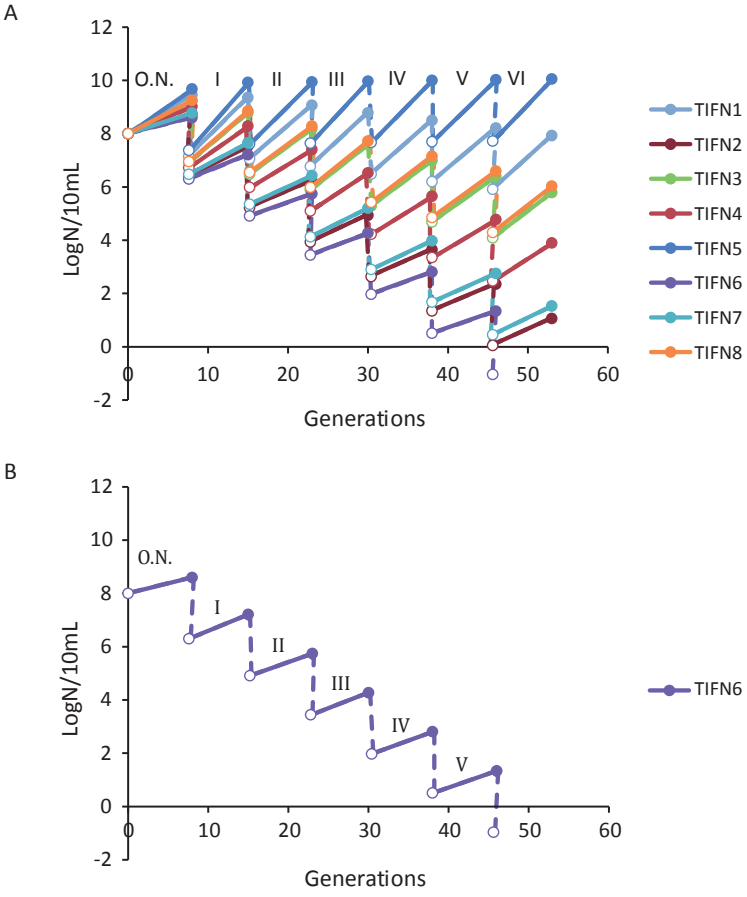

Figure A2. Panel A: Theoretical cell number at inoculation (open circles) and after each 24 hours (filled circles) (7.6 generations) of individual representatives of Ur culture lineages: lineage 1 - strain TIFN1, lineage 2 - strain TIFN2, lineage 3 - strain TIFN3, lineage 4 - strain TIFN4, lineage 5 - strain TIFN5, lineage 6 - strain TIFN6, lineage 7 - strain TIFN7, lineage 8 - strain TIFN8. Assumptions made to develop this model: starting point cell numbers is logN/10 mL = 8, initial inoculum 1%, inoculum at each transfer 0.5%, 24 hours of incubation after each transfer to fresh milk, maximum cell number set at logN/10 mL = 10. Growth rates used in this model were obtained by growing individual representatives of each complex starter culture genetic lineage in skim milk with 1% addition of casiton. Growth rates used: for TIFN1 $\mu_1 = 0.58\ h^{-1}$; for TIFN2 $\mu_2 = 0.29\ h^{-1}$; for TIFN3 $\mu_3 = 0.49\ h^{-1}$; for TIFN4 $\mu_4 = 0.41\ h^{-1}$; for TIFN5 $\mu_5 = 0.67\ h^{-1}$; for TIFN6 $\mu_6 = 0.24\ h^{-1}$; for TIFN7 $\mu_7 = 0.31\ h^{-1}$; for TIFN8 $\mu_8 = 0.5\ h^{-1}$. Panel B: Slow-grower strain TIFN6 is "washed-out" after the fifth transfer (46 generations). O.N. – over-night culture. I-VI – transfers. Dashed lines indicate 200x dilution at each transfer.

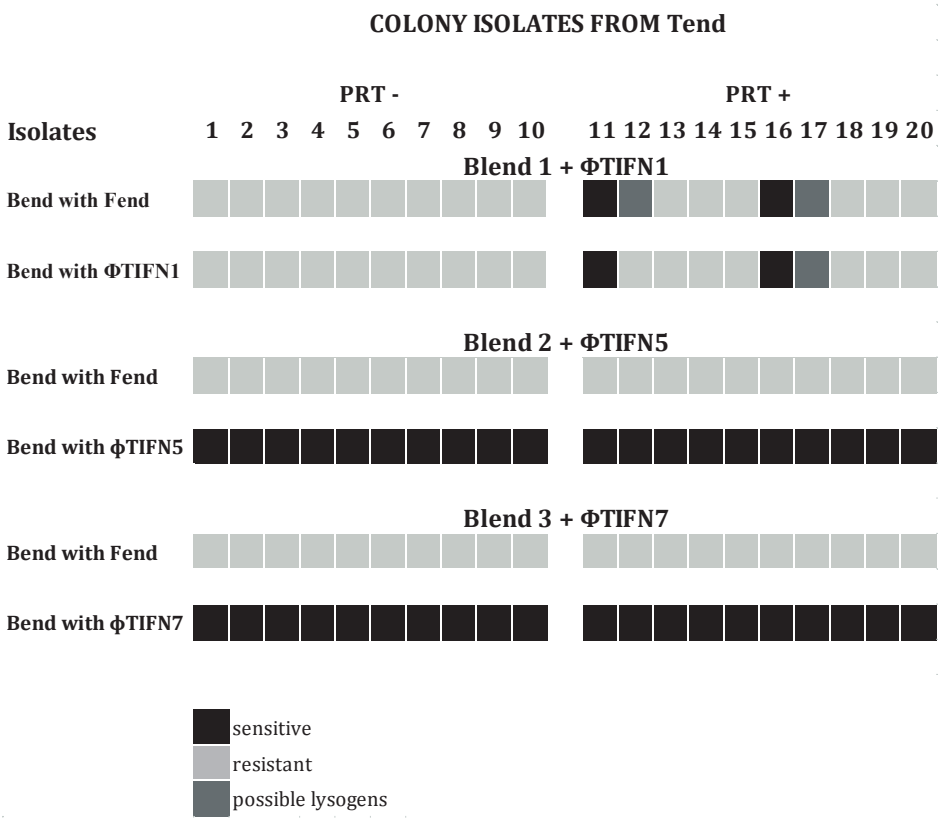

Figure A3. Results of a spot test of 60 strains (20 isolates per blend) isolated at the end time point of the propagation experiment (152 generations). Bend – bacterial isolates from end time point; Fend – filtrate from end time point of the blends at 152 generations; PRT - : protease negative strains; PRT+ : protease positive strains.

Table A1. Sequence of the primers used in unique gene-based qPCR for discrimination and population dynamics measurements of genetic lineages of complex starter culture.

Genetic lineage	Sequence of lineage-specific primers for qPCR
1&5	Forward primer: AAAAGAAAAAGGCTGGCTGAAA Reverse primer: GCACCGAGGTCAGACCAAGA
2&4	Forward primer: GCTTGTGCTGCCTCTTTAATTTC Reverse primer: TTTACCCGAGCGTCTATTAGCAA
3	Forward primer: CACGTCGTCAAACTGGTTACTCA Reverse primer: TGAACAGGCTTGCTCTTATCTATGA
6	Forward primer: TCAGGCAAGGCGAGCAA Reverse primer: GTATGCGGAAGAAAATTCATGGA
7	Forward primer: GGGAGCAAGCCTATCCTCACT Reverse primer: ACTGCCATCTTTGGTGATTTCTAAA
8	Forward primer: AATGAGCGACATCAACAACAAGTAA Reverse primer: ATGGCGGAACGATAGCAACT

Table A2. Strains and bacteriophages used for defined simple blends design.

	Lineage 1&5 isolates			**Lineage 6 isolates**	**Bacteriophage**
	Resistant (R)	Moderately Resistant (MR)	Sensitive (S)	No detectable susceptibility (ND)	
Blend 1	2MS18	2MS50	2MS40	2MS41	ΦTIFN1
Blend 2	2RS20	2MS32	2MS2	2MS52	ΦTIFN5
Blend 3	2MS42	2MS62	2MS46	2MS47	ΦTIFN7

Table A3. Sequence of lineage-specific primers used in colony PCR.

Genetic lineage	Sequence of lineage-specific primers for colony PCR	Size of the amplicon (bp)
1&5	Forward primer: tcgctgtcattggtatcagc Reverse primer: ccaaattccgcagtgttttc	172
2&4	Forward primer: tggcttagtattggcacctca Reverse primer: agctgttcgaccgacacttt	340
3	Forward primer: catcactcgctcattccatc Reverse primer: tccatctgccttgaaatagaca	263
6	Forward primer: ggcttcaaagataaagcgatg Reverse primer: caccgttggcagcaataagt	444
7	Forward primer: tagaaatcaccaaagatggcagt Reverse primer: cagacaggttcggtacgcttagt	90
8	Forward primer: tacgcgacagatatggtgga Reverse primer: tctgcggatacttcttgtgct	539

Table A4. LogN values calculated based of cfu/mL counts of both protease positive and protease negative colonies found on GMA plates at the end time point of propagation experiment of simple defined blends. Percentages of protease positive colonies are presented in the table as well to illustrate the differences between blends challenged and not challenged by bacteriophages.

Blend	logN protease positive colonies	logN protease negative colonies	Percentage of protease positive colonies [%]
Blend 1 with ΦTIFN1	5.8	6.6	14
Blend 1 with ΦTIFN1 (replicate)	5.4	7	2.6
Blend 1 without phage	6.6	6.2	72
Blend 1 without phage (replicate)	6.6	6.3	66
Blend 2 with ΦTIFN5	7.3	6.6	81
Blend 2 with ΦTIFN5 (replicate)	6.9	6.9	48
Blend 2 without phage	7.3	7.3	53
Blend 2 without phage (replicate)	6.9	6.9	49
Blend 3 with ΦTIFN7	6.3	6.6	29
Blend 3 with ΦTIFN7 (replicate)	6.0	6.5	24
Blend 3 without phage	6.2	6.6	31
Blend 3 without phage (replicate)	6.2	6.5	32

Chapter 3

How lytic bacteriophages affect the population dynamics of multi-strain microbial communities

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Judith CM Wolkers-Rooijackers, Tjakko Abee, Eddy J Smid

ABSTRACT

Lytic bacteriophages inactivate bacteria, and as a by-product potentially affect diversity in microbial communities through predation on the fittest strains. We used our knowledge of the complex dairy starter Ur to investigate *Lactococcus lactis* and *Leuconostoc mesenteroides* population dynamics in terms of constant-diversity and periodic-selection models. We designed blends of 24 strains to mimic this dairy starter and we propagated them by daily transfers in milk for over 500 generations in the presence and absence of a cocktail of bacteriophages. The abundance of strains representing specific genetic lineages of *L. lactis* and *Le. mesenteroides* and phages was monitored. In phage-challenged blends, predation initially caused a catastrophic shift in the microbial composition by killing the fittest and sensitive strains. In line with the constant-diversity model, phage-challenged blends maintained their diversity at the level of genetic lineages, while control blends showed decreased diversity leading to a stable state due to domination of the fittest strain(s) (periodic-selection dynamics).

INTRODUCTION

In natural environments bacteria rarely or never exist as a homogeneous single strain culture but rather as microbial consortia encompassing many strains of a variety of different species (Acinas et al., 2004). This species richness and abundance, is a function of the physico-chemical and biological conditions found in a given habitat. Bacteria not only interact with each other and their environment but also are constantly exposed to bacteriophage predation.

Recently, Erkus and co-workers (2013) characterized in detail an industrially relevant microbial community of a complex cheese starter culture Ur. As mentioned by Smid et al. (2014), the history of use of the Ur starter led to establishment of, at first glance, a simple two-species (*Lactococcus lactis* and *Leuconostoc mesenteroides*) culture. Further analysis of the Ur starter demonstrated a substantial degree of diversity beyond sub-species level. In fact, seven genetic lineages of *L. lactis* could be distinguished within a representative collection of single strain isolates. Bacteriophage resistance tests with individual strains (Spus et al., 2015) uncovered another level of diversity among isolated strains of the Ur starter belonging to the same genetic lineage.

In general, bacteriophages are thought to play a crucial role in controlling the abundance of bacteria in the environment (Suttle et al., 2007; Fuhrman, 1999). A theoretical model explaining the role of bacteriophages in microbial communities was discussed before by Thingstad (2000) who suggested that "kill-the-winner principles" apply in aquatic microbial systems. These ecological principles explain the prevention of niche domination by the best competitors and thus the maintenance of community diversity. Along with the principles set by Thingstad, Rodriguez-Valera and co-workers (2009) introduced the constant-diversity (CD) dynamics model, where phage predation is a driver of microbial communities' diversity. The CD dynamics model is opposite to periodic-selection (PS) dynamics, although they are not mutually exclusive. In case of PS dynamics the fittest strain eventually dominates the niche, which leads to a clonal sweep of other less fit strains. The clonal sweep results in reduction in ecosystem efficiency as indicated by Rodriguez-Valera and colleagues (2009). If PS dynamics would take place in the community of a complex starter culture it would affect the starter's functionality. The CD model explains the generation and maintenance

of microbial diversity in natural ecosystems where bacterial populations can interact. In such habitats, nutrients are dissolved and bacterial populations are characterized by large diversity in bacteriophage resistance. Diversity in bacteriophage resistance prevents complete lysis of the bacterial population caused by bacteriophages due to the presence and emergence of resistant variants. According to Rodriguez-Valera and co-workers (2009) not only bacterial populations in aquatic environments could follow CD dynamics but also other communities of interacting microbes found in nature.

All natural complex systems are exposed to gradual changes in abiotic and biotic conditions. A smooth response to such changes can be disrupted by a sudden catastrophic shift event, resulting in an alternative state. Such shifts were observed in natural complex systems such as lakes, coral reefs and oceans (Scheffer et al., 1997; Knowlton, 1992; Hare and Mantua, 2000). Various triggers can cause these shifts and eventual loss of resilience (capacity to respond to a change) which is a factor pushing a microbial community towards a tipping point of the shift. The shift leads to an alternative state that is difficult to reverse. Often, catastrophic shifts are caused by a stochastic event, which can lead e.g. to a wipe out of a part of a population (Scheffer et al., 2001). In the particular case of a complex starter culture, such a population wipe out can be caused by bacteriophage predation. The negative impact of phage predation on acidification during the production of dairy products was addressed in literature before (Samson and Moineau, 2013; Mc Grath et al., 2007). Nevertheless, complex starter cultures in general present better resilience to phage predation as compared to defined starters (Stadhouders, 1986). Many researchers studied the impact of bacteriophages on individual bacterial strains (Pal et al., 2007; Mizoguchi et al., 2002; Chao et al., 1977), but in the case of complex (multi-strain) microbial communities, empirical data are lacking.

In this study we used complex blends of well-characterized strains from the Ur starter as model system to investigate the role of bacteriophages in population dynamics. These blends were designed according to the following criteria. Firstly, all eight genetic lineages of Ur starter (Erkus et al., 2013) were represented by multiple strains. Secondly, all strains were mixed at equal initial relative abundance. Thirdly, we deliberately included strains, which differ in their bacteriophage resistance (Spus et al., 2015). Consequently, we obtained a blend of strains, which resembles the diversity of the natural complex starter culture: (i) at the level of genetic lineages, (ii) at the strain

level and (iii) at the level of bacteriophage resistance. Our blends were sequentially propagated in milk for more than 500 generations without (control) and with the addition of a phage cocktail composed of three lytic phages isolated previously from the Ur culture (Erkus et al., 2013). Throughout the propagation experiment we monitored the abundance of genetic lineages as well as the presence of bacteriophages. We hypothesize that in control blends, without phage addition, diversity at the level of genetic lineages will be reduced due to an increased abundance of the fittest strain(s).

MATERIALS AND METHODS

Preparation of the multi-strain blend

The starter culture Ur (obtained from foundation BOZ, Ede, The Netherlands) comprises of two species of lactic acid bacteria (LAB): *L. lactis* and *Le. mesenteroides*. Many single colony isolates of the Ur culture were previously characterized using AFLP-typing (Kütahya et al., 2011) and ascribed to eight genetic lineages (Erkus et al., 2013). Five genetic lineages (1, 3, 5, 6 and 7) encompass *L. lactis* ssp. *cremoris* strains, two (2 and 4) *L. lactis* ssp. *lactis* biovar *diacetylactis* strains and one (lineage 8) *Le. mesenteroides* ssp. *cremoris* strains. Genetic lineages of the Ur starter possess certain traits relevant for their function in converting milk into cheese. For instance, lineages 1, 3 and 5 have caseinolytic activity linked to a functional extracellular protease, which cleaves caseins into peptides (prt^+) (Smid et al., 1991; Thomas and Pritchard, 1987). Moreover, lineages 2, 4 and 8 are able to degrade citrate (cit^+) into acetoin and/or diacetyl (Starrenburg and Hugenholtz, 1991), two compounds that have a profound impact on cheese aroma. The main function of the remaining two lineages 6 and 7 is acidification.

When the genomes of representative strains of lineages 1 and 5 as well as 2 and 4 were compared, only a limited number of unique gene sequences was found between the strains in these two genome pairs. None of these unique genes met the criteria for specific primers design thus lineages 1 and 5 (1&5) as well as lineages 2 and 4 (2&4) were further enumerated together.

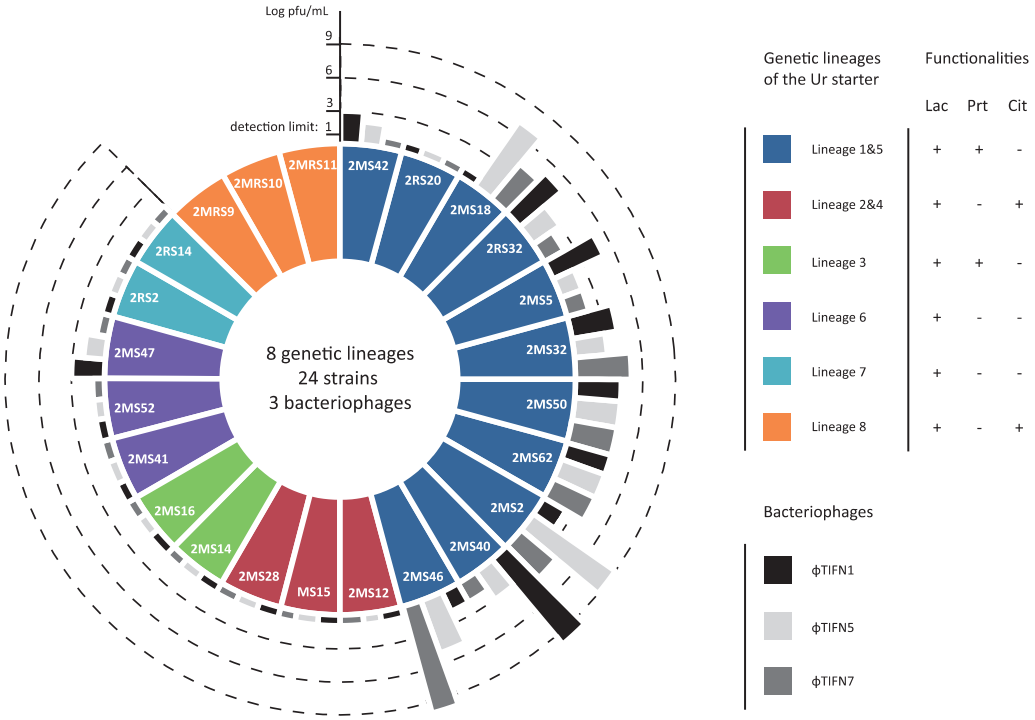


Figure 1. Design of the multi-strain blend used in the propagation experiment. *L. lactis* and *Le. mesenteroides* were represented with 24 strains from eight genetic lineages of the Ur starter culture. For bacteriophage challenge, a cocktail of three bacteriophages was used including ϕTIFN1, ϕTIFN5 and ϕTIFN7. Strains in the blend differ among each other in terms of resistance to phages present in the cocktail – illustrated by black, dark grey and light grey columns. Lineage 8 encompasses *Le. mesenteroides* ssp. *cremoris* strains, which are resistant to lactococcal phages used in this study. Lac – lactose utilization ability; Prt – caseinolytic protease activity; Cit – citrate degradation ability.

The blend design included 24 strains representing all eight genetic lineages of the Ur starter: eleven strains belonging to lineages 1&5, three strains to lineages 2&4, two strains to lineage 3, three to lineage 6, two to lineage 7 and three to lineage 8 (Fig. 1). To prepare the strains for the blend preparation, frozen cultures of *L. lactis* were streaked on M17 agar (OXOID, Basingstoke, UK) plates supplemented with 0.5% (wt/vol) lactose (LM17) and *Le. mesenteroides* strains were streaked on MRS agar plates (1.5% agar, Merck KGaA, Darmstadt, Germany) supplemented with vancomycin (final concentration of 30μg/mL). Next, single colonies were picked and LM17 broth (in case of *L. lactis* strains) and MRS broth (in case of *Le. mesenteroides*

strains) were inoculated. Cultures of *L. lactis* strains were grown at 30 °C and *Le. mesenteroides* strains were grown at 25 °C overnight. Cells were washed twice by removing the supernatant after centrifugation at 2500 × g for 30 min and exchanging it with peptone physiological saline (PPS). Optical density of washed cell preparations was determined at 600nm (OD_{600}) (Novaspec II, Pharmacia Biotech, Pharmacia LKB, Montreal, Canada). The cell preparations were diluted to OD_{600} = 0.4 (st. dev. ± 0.02) and 100 µL of each suspension was then mixed to obtain the blend to inoculate milk for the propagation experiment. After the blend preparation the relative abundance of each genetic lineage was determined using a qPCR approach (described below). The initial ratio of genetic lineages was as follows: 49.5% of lineage 1&5, 12.5% of lineage 2&4, 8% of lineage 3, 15.5% of lineage 6, 12.5% of lineage 7 and 2% of lineage 8. Strains used to create blends were previously characterized in terms of their bacteriophage resistance (Spus et al., 2015). The levels of resistance to three bacteriophages (ϕTIFN1, ϕTIFN5 and ϕTIFN7) used in the cocktail to challenge the blends are indicated in the Fig. 1. Eleven strains included in the blend showed diverse susceptibility to at least one of the three phages and ten out of these eleven strains were ascribed to lineage 1&5 with the exception of strain 2MS47 (lineage 6). In total four blends (two sets of two biological replicates) were prepared: A-1 and A-2; control, no phage cocktail added, B-1 and B-2; treatment, cocktail of three phages added. At the onset of the propagation experiment a cocktail of three phages: ϕTIFN1, ϕTIFN5 and ϕTIFN7 was added to two (treatments) out of four replicates (final concentration: 10^8 pfu/mL per phage type).

Propagation regime of the blends

The blends were cultivated in skim milk ('Friesche Vlag Lang Lekker', non-fat, UHT, Friesland Campina, The Netherlands) for 24h. After 24h 1% (0.1 mL) of the culture was transferred to fresh milk (9.9 mL). Consequently, each transfer cycle delivered approximately 6.6 generations. The experiment was performed continuously for 81 days reaching approximately 538 generations. Samples of propagated blends were collected after one day (6.6 generations) and then after each 20 days (each 132.8 generations).

Unique-gene based qPCR for genetic lineage relative abundance monitoring

The unique-gene based qPCR method developed by Erkus and co-workers (2013) and described in Spus et al. (2015) was used to monitor the relative abundance of the genetic lineages. The relative abundances of lineages 1&5, 2&4, 3, 6, 7 and 8 were determined after 6.6, 139, 272, 405 and 538 generations. The Shannon diversity index (Haegeman et al., 2013) at the level of genetic lineage was calculated using the following equation: $-\Sigma_i(n_i/N * \ln(n_i/N))$, with: n_i – abundance of lineage i and N - the total lineages abundance. In this case, the higher the Shannon diversity index is, the more equally the relative abundance among genetic lineages is distributed.

Phage resistant assay

A qualitative spot assay (Sanders and Klaenhammer, 1983) was used to determine the resistance of strains used for the blend preparation to phages present in the supernatants of the blends. The supernatants of the blends at different time points (after 6.6, 139, 272, 405 and 538 generations) were used as a possible source of phages and spotted onto the bacterial cell lawn of each of the strains used in the blend preparation. In detail, 10 µL of filtered supernatant (0.2 µm pore size sterile Minisart® filters, Sartorius Stendim Biotech, Göttingen, Germany) was used to spot each indicator culture in the soft agar layer (0.75% agar w/v; 0.5% lactose; 10mM $CaCl_2$).

The same procedure was performed with the end time point (after 538 generations) isolates (200 single colony isolates, 50 isolates per replicate) using the filtered supernatant as well as the solution (10^{10} pfu/mL) of all three bacteriophages used in the cocktail (ϕTIFN1, ϕTIFN5 and ϕTIFN7). To obtain the isolates, all four blends A-1, A-2, B-1 and B-2 were diluted in PPS and plated onto LM17 agar. After 48h of incubation at 30 °C, 50 single colonies of each blend were picked from the plates and transferred to LM17 broth and incubated for 24h. These new single strain isolates were preserved at -80 °C in glycerol (20% v/v) for further use.

RESULTS

Population dynamics in the multi-strain blends

Strains of genetic lineage 1&5 used for the blend preparation were chosen for their variation in terms of resistance to bacteriophages (Fig. 1). For strains of other lineages used in the blend, with one exception of strain 2MS47 (lineage 6), we did not detect susceptibility to any of the three phages (ϕTIFN1, ϕTIFN5 and ϕTIFN7) used in the cocktail.

To evaluate the impact of the phage presence/absence we monitored the abundance of all genetic lineages in both control (A-1, A-2) and phage-challenged blends (B-1, B-2) throughout the propagation experiment (Fig. 2). At the start of the experiment the distribution of abundance of genetic lineages was the same in all four cultures. This initial composition was not stable throughout the course of the propagation experiment; in control blends we observed a gradual increase in abundance of lineage 1&5 strains. At the end time point of the experiment this gradual increase resulted in dominance of the lineage 1&5 strains in both control blends with a relative abundance of 97% in A-1 and 98% in A-2.

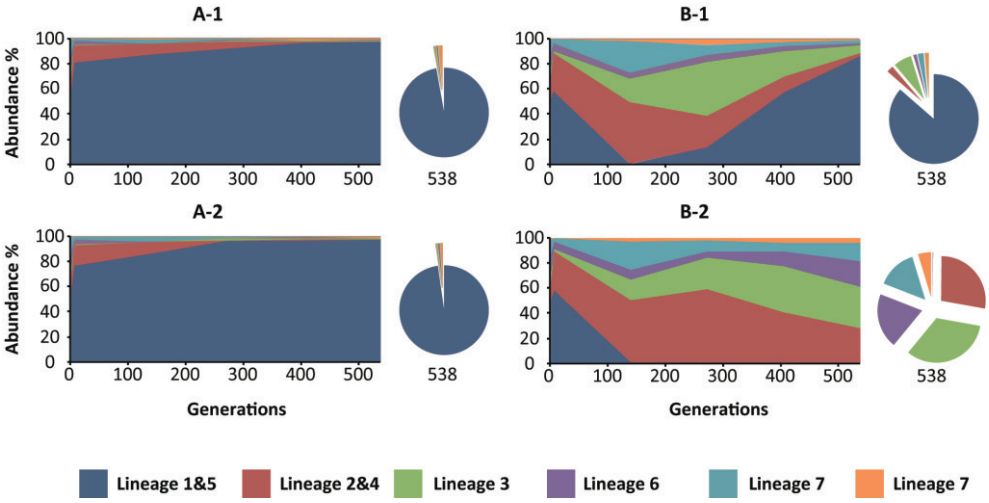


Figure 2. Relative abundance in percentage of lineages in blends during the propagation experiment based on DNA copy number values obtained with unique-gene-based qPCR. Panel A-1 represents the 1st replicate of the blend without phage challenge; Panel A-2 represents the 2nd replicate of the blend without phage challenge; Panel B-1 represents the 1st replicate of the blend with phage challenge; Panel B-2 represents the 2nd replicate of the blend with phage challenge. At the starting point the abundances of genetic lineages were equal in all four replicates.

In the phage-challenged blends we observed a far more complex behavior in terms of dynamics of relative abundances of all genetic lineages. Until 139 generations, both replicates B-1 and B-2 behaved in a similar way - the abundance of lineage 1&5 strains decreased to the level below 1%. Most (10/11) of the strains of lineage 1&5 are susceptible to at least one of the bacteriophages used in the cocktail. After 139 generations of propagation, the two phage-challenged replicates started to deviate, especially in terms of the abundance of lineage 1&5. At the next sampling point (272 generations) the abundance of lineage 1&5 in B-2 was still very low, below 0.5% and lineage 2&4 (cit^+) was found to be the most dominant with 59% relative abundance, with lineage 3 (prt^+) as the second most abundant (25%) followed by lineage 7 (prt^- and cit^-) with 9% and lineage 6 (prt^- and cit^-) with 4.5% abundance. Finally, lineage 8 (prt^- and cit^+) occupied 2.5% of the total population. However, in the other replicate B-1 at 272 generations, lineage 1&5 increased in abundance to 13%, with the other prt^+ lineage 3 as the most dominant at this point with 44% followed by lineage 2&4 with 25% and lineage 7 with 8% relative abundance. Previous experiments (Spus et al., 2015) showed that strain(s) of lineage 1&5 dominated the 4-strain blends during sequential propagation in milk, without phage predation, suggesting that these strains are the "winners" – the fittest strains for the imposed propagation regime. The fittest strains are potential targets for phage predation in accordance to above mentioned kill-the-winner mechanism. Lineages 6 and 8 were the least abundant with 5% both. At later sampling points the relative abundance of genetic lineages changed further. In B-1 we observed a clear pattern of a gradual increase in abundance of prt^+ lineage 1&5 reaching 57.5% and 86.5% at 405 and 538 generations, respectively. Consequently, all other lineages decreased in abundance with the biggest drop of prt^+ lineage 3 by 37 percentage points between 272 and 538 generations. The prolonged propagation experiment led to two different final results (i.e. alternative states) in the treated blends. After 538 generations, blend B-1 was dominated by lineage 1&5 and resembled in composition the control blends. In case of replicate B-2 however, genetic lineage 1&5 remained at extremely low levels in the community and lineage 3, the other prt^+ lineage in the culture, became the most abundant. These results show the enormous impact of lytic bacteriophages on population dynamics at the level of genetic lineages in defined multi-strain blends.

Bacteriophage resistance before, during and after propagation

All 24 strains included in the blend design were challenged with the supernatant of the propagated blends collected at different time points of propagation to monitor the presence of the bacteriophages during the course of the experiment (Fig. 3). In the supernatant after 6.6 generations we found bacteriophages predating on 10 different lineage 1&5 strains. The two strains, which previously showed susceptibility to phages used in the cocktail, namely 2MS2 (lineage 1&5) and 2MS47 (lineage 6), did not show susceptibility to phages from the supernatants of the phage-challenged blends after 6.6 generations. Strain RS20 for which initially no detectable susceptibility was found to any of the three phages, showed sensitivity to phages from the supernatant of both phage-challenged blends after 6.6 generations. Supernatants collected from the phage-challenged cultures after prolonged propagation (272 gen. – only replicate B-1; and 405 gen. – both replicates) showed lytic activity on one and two of the original isolates, respectively (Fig. 3). None of supernatants collected after 139 and 538 generations caused lysis of any of the original strains.

Next, we isolated single colonies (50 for each blend, in total 200 colony isolates) from the end time point of the propagation experiment to check if any of the previously resistant strains became sensitive to phages used in the cocktail or to the ones possibly present in the supernatant of the cultures at the end time point. First, we determined for these 200 strains the genetic lineage identity, using colony PCR (Spus et al, 2015). Next, all these single colony isolates were challenged in a spot assay with individual phages used in the cocktail as well as with the supernatants of the end time point (538 generations) blends (Fig. 4). We did not find any susceptibility of strains isolated from all four blends A-1, A - 2, B-1 and B-2 when the supernatants of the end time point were used in the spot assay. However, 18 out of 50 strains isolated from blend B-1 at the end time point were susceptible to at least one of the three phages used in the cocktail at the onset of the propagation. Most of these susceptible strains belong to lineage 1&5 (16/18). Two isolated strains, namely 4.30 (ascribed to lineage 3) and 4.46 (ascribed to lineage 7) were found to be susceptible to phage ϕTIFN5 and ϕTIFN7 (strain 4.30) and phage ϕTIFN5 (strain 4.46). At the onset of the experiment the strains of lineages 3 and 7 used for the blend design were not susceptible to any of the phages used in the cocktail.

Figure 3. Susceptibility of the original strains used in the blend to phages present in the supernatants at different time points (after 6.6, 139, 272, 405 and 538 generations) of the propagation experiment. Blends were sequentially propagated in milk for up to 538 generations. Replicates A-1 and A-2 were used as control where no phage cocktail consisting of ϕTIFN1, ϕTIFN5 and ϕTIFN7 was added. Replicates B-1 and B-2 were challenged at the onset of the experiment with the cocktail of three phages.

Strains isolated from blend B-2 at the end time point were all resistant to the three phages. None of the strains isolated from B-2 belonged to lineage 1&5 and most of them were ascribed to lineage 3, and showed no detectable susceptibility.

In terms of the strains isolated from the control blends most of them belong to lineage 1&5, which in case of blend A-1 was 43/50 strains and for blend A-2 42/50. Those 85 out of 100 strains were susceptible to all three phages used in the cocktail at the onset of the propagation.

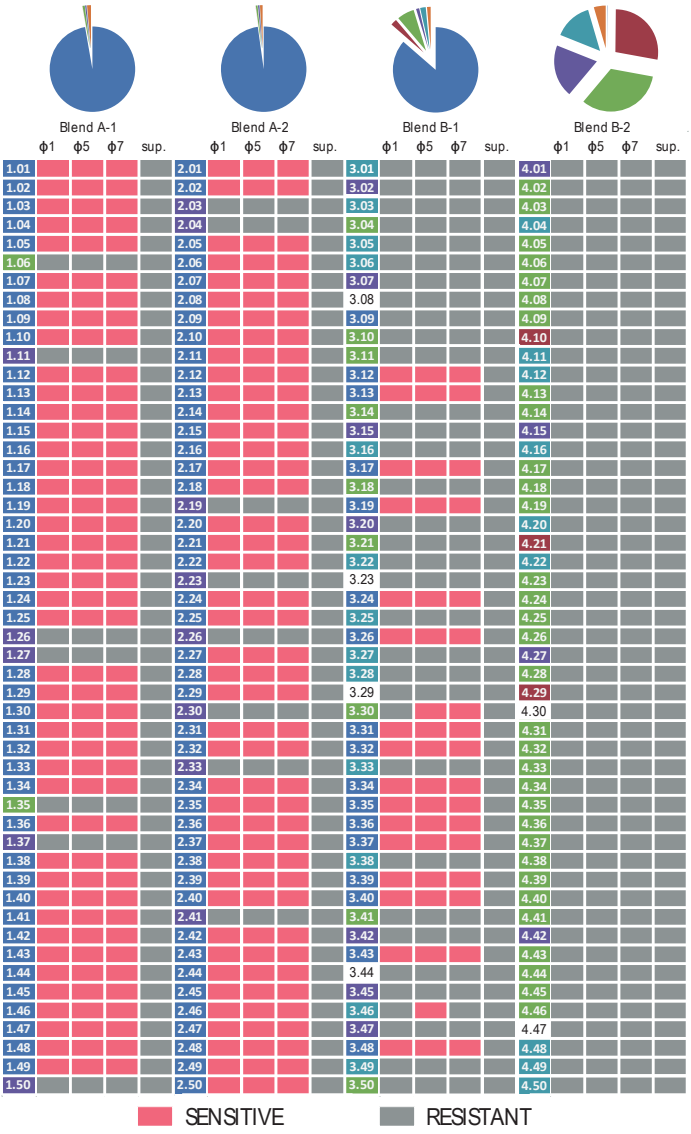


Figure 4. Susceptibility of the single colony isolates from the end time point of the propagation experiment to the three phages used in the cocktail: ϕTIFN1 (ϕ1), ϕTIFN5 (ϕ5), ϕTIFN7 (ϕ7) and to the phages present in the supernatant of the blends at the end time point (538 generations). Replicates A-1 and A-2 were used as control – no phage cocktail was added. Replicates B-1 and B-2 were challenged at the onset of the experiment with a cocktail of three phages: ϕTIFN1, ϕTIFN5 and ϕTIFN7. The strains 1.01 to 1.50 were isolated from blend A-1, strains 2.01 to 2.50 were isolated from blend A-2, strains 3.01 to 3.50 were isolated from blend B-1 and strains 4.01 to 4.50 were isolated from blend B-2 (for detail description see Materials and Methods section).

These results confirm the dynamic character of interactions of strains in blends with bacteriophages from the cocktail. In case of control blends, strains present after 538 generations were still susceptible to the three phages used in the cocktail. Susceptible strains can thrive in the community in the absence of phage predation pressure. In contrast, in case of phage-challenged blend B-1, after long propagation most of the isolated strains were resistant to phages used in the cocktail at the onset of the experiment (Fig. 4) suggesting important competitive advantage of phage resistant strains in the culture. In blend B-2 no susceptibility to phages used in the cocktail was found among strains isolated at the end time point.

DISCUSSION

As indicated by Smid et al. (2014) the relative abundance of the individual strains belonging to a specific genetic lineage of the starter culture may vary due to changes in the environment (temperature, pH) and propagation regime. In the present study we investigated the impact of bacteriophage predation on the culture's population dynamics at the level of co-existing genetic lineages. To reach that goal we used our current knowledge of the structure and properties of a complex dairy starter culture (named Ur) to design a defined multi-strain starter culture (blend) and sequentially propagated it in milk for an extended period of time (538 generations). The blend included all eight genetic lineages of the Ur starter represented by 24 strains, which extends our previous studies in which blends with three lineages represented by four strains were used (Spus et al., 2015). We purposely selected strains belonging to lineage 1&5 –containing the "winner" (the fittest) strains, and which express a diverse susceptibility to the three phages used in the cocktail. We did not observe detectable susceptibility (except strain 2MS47, lineage 6) to the three phages for the remaining strains representing lineages 2, 3, 4, 6, 7 and 8 (Fig. 1).

Rodriguez-Valera and colleagues (2009) suggested that: "it would be interesting to measure experimentally the individual fitness of different bacterial strains isolated from a natural habitat to determine whether fitter variants are selected against in environments under phage pressure". According to our previous work (Spus et al., 2015) prt^+ lineage 1&5 includes the fittest strains (highest growth rate in milk supplemented with casiton) and at the same time covers a diverse sensitivity profile to phage predation. Our present results on the population dynamics in phage-challenged blends

confirm the prediction that the fittest strains were selected against under bacteriophage predation pressure (in B-1 until 139 generations, in B-2 throughout the experiment).

Several possible events could take place during the propagation of the blend: i) a prophage induction, ii) a resistant variant could emerge and gradually increase in abundance, iii) bacteriophages without a host would be "washed out" from the culture due to daily dilution and iv) phages could adapt to predate on previously resistant strain(s). All of these four events would introduce new dynamics to the system and change the "rules of the game", which impact the diversity of such a community. Based on the results obtained in the current study, we will discuss the role of phage predation as the trigger causing a catastrophic shift in the culture's community that may finally lead to two alternative stable states.

Even though evidence exists that strains of the Ur starter contain inducible prophages (Alexeeva et al., 2016, submitted for publication), we did not observe susceptibility to the supernatants of control blends (throughout the propagation experiment) and phage-treated blends (at the end time point of propagation). Based on this, we exclude the induction of an active phage crop as one of the major events impacting the population dynamics in our blends.

In the phage-challenged blends the final outcome of the propagation experiment was very different in each of the two replicates. In case of B-1, strain(s) of lineage 1&5 recovered after the initial drop after 139 generations, presumably caused by phages, and gradually increased in abundance. This increase can be explained by the emergence of a phage resistant strain(s) and/or by the "wash out" of phages due to the serial dilution during sequential propagation leading to increase of abundance of the faster growing strain(s). We found phages in the supernatant of blend B-1 after 405 generations confirming the presence of susceptible strain(s) at that point of the experiment. Despite the presence of predators the abundance of lineage 1&5 still increased between 405 and 538 generations and at the end time point we did not observe bacteriophage predation on the strains present in the blend (Fig. 4). The absence of bacteriophage predation after 538 generations, conceivably due to "wash out", suggests that there was no phage host present anymore in the culture allowing the population of the fastest multiplying strains of lineage 1&5 to increase their abundance and thus gradually become the dominant in the culture, similar to the control blends. The B-1 blend (and both control blends A -1 and A-2) showed a behavior characteristic to the PS

dynamics (Rodriguez-Valera et al, 2009). In the conditions of absence of bacteriophage predation the fittest strain(s) became dominant resulting in the reduction of the genetic lineage level diversity (Fig. 5).

In case of blend B-2, strains belonging to lineage 1&5 did not recover from the phage attack and after 139 generations their relative abundance was still lower than 1%. Despite our previous observations of relatively low growth rate of strains representing lineage 2&4 (Spus et al., 2015), lineage 2&4 was found to be the most abundant in the B-2 blend between 139 and 405 generations. After 538 generations, the dominant prt$^+$ genetic lineage in the blend (lineage 3, fourth highest growth rate for the representative strain, according to Spus et al., 2015) became the most abundant.

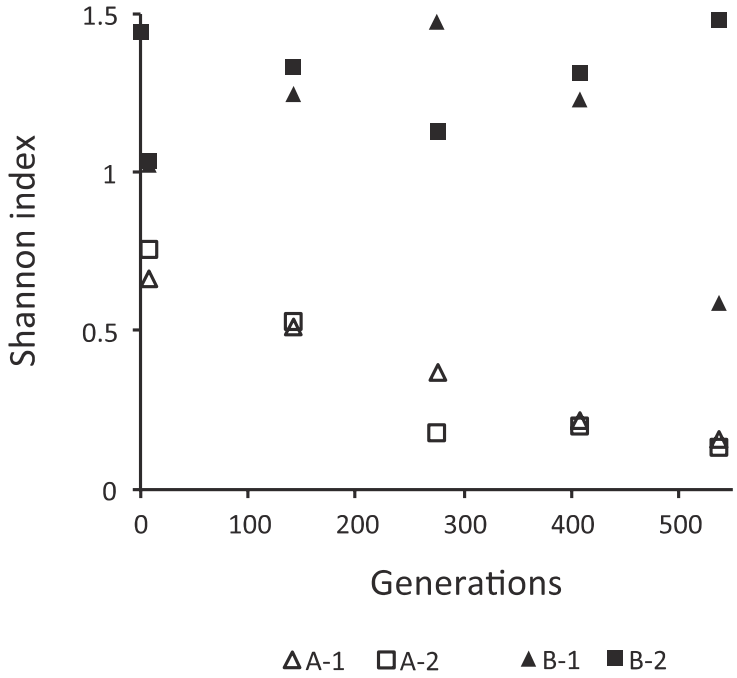


Figure 5. Shannon diversity index at different time points of propagation. Shannon index was calculated based on the relative abundance of genetic lineages. Open triangles – A-1 - blend without phage cocktail at 538 generations (first biological replicate); open squares – B-1 - blend with phage cocktail at 538 generations (first biological replicate); filled triangles – A-2 - blend without phage cocktail at 538 generations (second biological replicate); filled squares – B-2 - blend with phage cocktail at 538 generations (second biological replicate).

In blend B-2, culture diversity at the level of genetic lineages was always higher than in the controls (Fig. 5) and we suggest that this was due to the initial phage predation event resulting in decrease of the abundance of the fittest lineage 1&5, which remained at low relative abundance level, rather than a CD-like mechanism (Rodriguez-Valera et al, 2009). Our phage resistance (spot) tests showed absence of phages in the supernatant after 538 generations predating on the contemporary strains in the phage-challenged blends, which is in contrast to the assumption of the CD dynamics model of the constant bacteriophage predation pressure.

Although many strains were included in our model and a cocktail of three phages was used, the multi-strain blend does not represent the entire complexity of the Ur starter culture in which genetic lineages are assumed to be stabilized by "kill-the-winner" dynamics (Erkus et al., 2013). Within the time frame of the propagation experiment, the system of the multi-strain blend was not stable in terms of the relative abundance of the genetic lineages. To solve this instability one could i) develop a more complex model similar to the Ur starter, ii) use a different initial abundance of genetic lineages or iii) change the propagation regime.

Despite this relative instability, it is important to note that under the given conditions (defined propagation regime; presence/absence of phages; prolonged sequential propagation in milk), none of the genetic lineages was lost (Fig. 6). This observation confirms inherent dependencies between strains in the community – microbe-microbe interactions as discussed before by Smid and Lacroix (2013). For example, as was suggested earlier (Spus et al., 2015), the plasmid-encoded protease activity can be lost upon propagation resulting in a fraction of prt^- 'cheaters' (Bachmann et al., 2011). The sub-population of 'cheaters' can benefit from the peptides released due to action of the prt^+ fraction without carrying the burden of expressing a protease, which allows them to persist in the community. Another example of dependencies between strains is the conversion of glutamate to succinic acid, which is predicted to be a result of combined activities of *L. lactis* and *Le. mesenteroides* (Erkus et al, 2013).

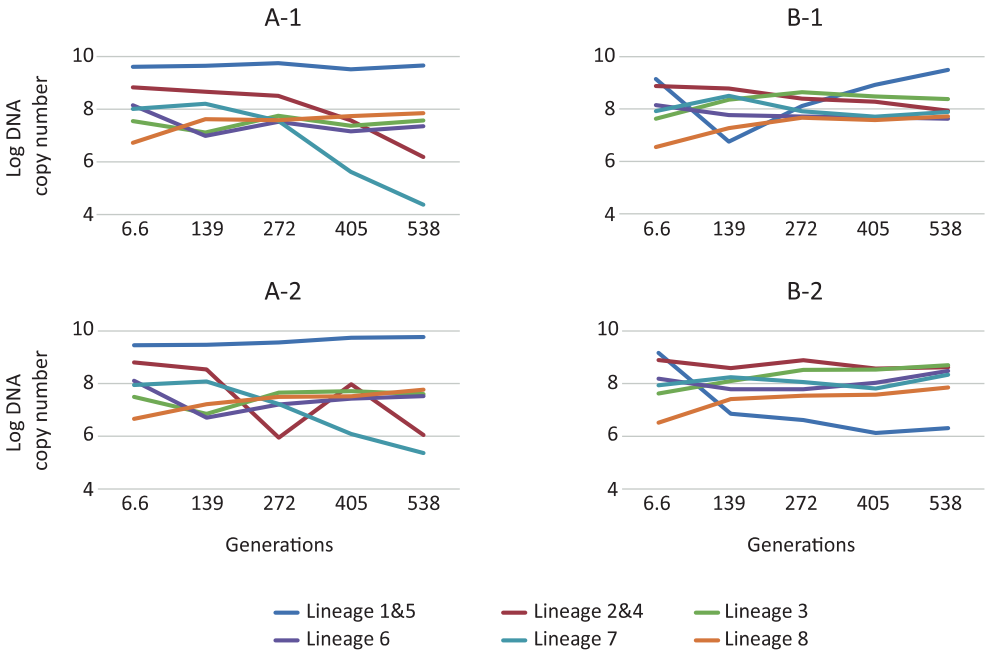


Figure 6. Log transformed DNA copy numbers of genetic lineages of Ur starter in the multi-strain blend throughout the propagation experiment. Replicates A-1 and A-2 were used as control – no phage cocktail was added. Replicates B-1 and B-2 were challenged at the onset of the experiment with the cocktail of three phages.

It was noted in other ecosystems that the decrease in diversity reduces the optimal utilization of resources present (Cardinale et al., 2006). The absence of bacteriophage predation pressure in the blends led to domination by strains of one particular genetic lineage (the fittest strains) without a complete clonal sweep of other lineages (Fig. 6). Possibly these low abundant strains, as specialists with e.g. citrate degradation ability, remained in the blend by exploiting different (micro)niches. In such case, a culture dominated by a single lineage not necessarily has lower ecosystem efficiency but due to division of labor exploits the resources optimally. It would be of interest to investigate the parameters of the blend efficiency (acidification, aroma formation) to define the effect of reduction in genetic lineage diversity on the blend's functionality.

In our propagation experiment we observed two contrasting stable states. In the case of absence of bacteriophage predation, the fittest lineage 1&5 dominated the blend throughout the entire time span of the propagation. Without bacteriophage predation pressure "the winner" strain's abundance was not kept in control.

Phage predation appeared to be the trigger for a catastrophic shift in culture community leading to a decrease in the abundance of the fittest (prt^+) lineage 1&5. This catastrophic shift resulted in a stable state with extremely low abundance of the fittest lineage, which niche was subsequently occupied by the alternative prt^+ lineage 3. Due to a stochastic event in one of the two replicates of phage-challenged blends, the fittest strain(s) recovered from the catastrophe and the stable state emerging from the recovery resembled the one described for the blends without phage predation.

In conclusion, phage predation, among other factors impacting complex microbial communities, can trigger catastrophic shifts in the community population dynamics. Nevertheless, as shown with our lineage level culture composition results, recovery to an alternative stable state (demonstrating PS dynamics) can take place even after a catastrophic shift. Empirical data presented in this study on the impact of bacteriophage predation on multi-strain community diversity add a further level of detail to the PS versus CD dynamics models (Rodriguez-Valera et al, 2009) and stress the role of microbe-microbe interactions and (micro)niche adaptation in preventing clonal sweeps.

Chapter 4

Under the radar - experimental evolution of complex dairy starter culture leads to discovery of *Lactococcus laudensis*

Maciej Spus, Yasmeen Qazi, Irma MH van Rijswijck, Judith CM Wolkers-Rooijackers, Michiel Wels, Tjakko Abee, Eddy J Smid

ABSTRACT

The compositional and functional stability of a complex mixed Gouda cheese starter culture Ur is thought to be sustained by density-dependent phage predation in accordance with the constant-diversity dynamics model. To investigate this stability *in situ* we performed a 1000-generation long-term experimental evolution experiment with Ur by daily propagation in milk. Surprisingly, the conditions in our experiment (temperature of incubation, propagation regime) favoured enrichment of a recently described new species *Lactococcus laudensis. L. laudensis* on its own shows poor growth in milk (no caseinolytic activity), poor survival in acidic conditions but can grow on D-mannitol as an additional carbon and energy source. Abilities of *L. laudensis* to (i) use oligopeptides produced by caseinolytic *Lactococcus lactis* subsp. *cremoris* and (ii) utilize D-mannitol secreted by *Leuconostoc mesenteroides* subsp. *cremoris* are possibly advantageous for thriving in the enforced propagation regime conditions. Prior to its enrichment *L. laudensis* was under the radar of detection methods and after increasing in abundance was under the radar of detected throughout the experiment bacteriophage predation. This observation sheds new light on the possibility of how a strain can become abundant in transiently enclosed microbial community operating in accordance with constant-diversity dynamics model.

INTRODUCTION

Written history of use of a complex cheese starter culture Ur prior to its isolation in the 1950's does not exist. Although, we can fairly assume that Ur was maintained at farm level using a traditional back-slopping technique (Nout, 1994). Back-slopping, repeated for generations allowed selection and adaptation of certain lactic acid bacteria (LAB) to establish a stable functional cheese starter culture. Moreover, this unrestricted propagation of the culture allowed influx of strains of LAB from different sources, including raw milk, cattle, farmer/cheese producer, plant material, etc. Evolutionary studies using a *Lactococcus lactis* strain originating from a plant source, showed that certain traits useful in a milk environment can relatively quickly evolve (Bachmann et al., 2012), confirming that at least part of the strains used in dairy find their origin in plant material.

Another consequence of propagation by back-slopping is the influx of bacteriophages, both from the environment and released by new incoming strains through the expression of prophages. We demonstrated recently that bacteriophages have an impact on the diversity of simple microbial communities (Spus et al., 2015) and affect the diversity in complex blends of lactococci and leuconostocs (Spus et al., unpublished results).

Previous studies (Erkus et al., 2013) showed that the complex cheese starter culture Ur comprises only two species of lactic acid bacteria, namely, *Lactococcus lactis* and *Leuconostoc mesenteroides.* Erkus and co-workers (2013) identified eight genetic lineages in Ur: lineages 1, 3, 5, 6 and 7 encompassing strains of *L. lactis* ssp. *cremoris*; lineages 2 and 4 encompassing strains of *L. lactis* ssp. *lactis* biovar *diacetylactis*; lineage 8 encompassing strains of *Le. mesenteroides* ssp. *cremoris*. Strains in the starter are constantly exposed to phage predation and their level of resistance is variable (Spus et al., 2015). Also the presence of plasmids encoding starter functionality-related genes is a variable trait among the strains found in Ur. Lineage diversity, plasmid content and phage resistance collectively describe the culture's multifactorial diversity. This multifactorial diversity is thought to sustain the stability of the microbial community of the starter culture (Erkus et al., 2013).

Based on the outcome of recent work, but also taking into account the tradition of back-slopping, we set out to execute a long term experimental evolution experiment using the undefined mixed starter culture. As a tribute to the Long Term Evolution Experiment (LTEE) still being executed with

Escherichia coli we based our design on Lenski's experiment (Lenski et al., 1991) and called it Long term experimental evolution of Undefined Mixed Starter Culture (LUMSC). The aim was to demonstrate and understand the compositional and functional stability of the complex starter culture upon prolonged sequential propagation.

Twelve replicates of Ur starter were propagated in milk for 1000 generations. Bacteriophages are claimed to be an integral part of the community of Ur culture stabilizing the diversity and preventing domination of the culture by the fittest variant in accordance with "kill the winner"-based constant-diversity dynamics model (Erkus et al., 2013; Rodriguez-Valera et al., 2009; Thingstadt, 2000). By propagation of the culture in a closed environment we prevented the influx of new DNA. The abundance of culture's genetic lineages was determined after 500 and 1000 generations using a metagenomics approach and a unique-gene based qPCR method. Additionally, we monitored the presence of lytic phages in the supernatant of the LUMSC cultures by performing a spot assay with Ur strains as indicators.

METHODS

Propagation and sampling of undefined mixed starter culture

The complex starter culture called Ur (provided by Stichting BOZ, Ede, The Netherlands) was used as our model cheese starter culture in the long-term propagation (experimental evolution) study. Based on the design of the famous single-strain long-term evolution experiment of *Escherichia coli* (Fox and Lenski, 2015), we decided to perform our propagation in twelve replicates (LUMSC 1-12). Twelve tubes with 9.9 mL of skim milk ('Friesche Vlag Lang Lekker', non-fat, UHT, Friesland Campina) were inoculated with 100 µL of original Ur starter culture (1% v/v) at the onset of the experiment and incubated at 20 °C. Every 24h, 100 µL of each culture was transferred to fresh 9.9 mL of skim milk giving 6.64 generations (G) per transfer. Propagation was continued until 1000G. Samples for determination of genetic lineage composition were collected both at 500 and 1000G. Samples for determining functional characteristics (acidification, acid formation and volatile compounds in cheese model system) of the LUMSC cultures were collected at 1000G.

Bacteriophage abundance determination in the LUMSC cultures

Strains previously isolated from the original Ur starter culture (Spus et al., 2015) were used as indicator strains when exposed to phages from LUMSC cultures both at 500 and 1000G, as well as from Ur starter (control). In detail, each culture was centrifuged at 10,000 × g for 10 min to obtain supernatant. Next, the supernatants were filtered (0.2 µm pore size sterile Minisart® filters, Sartorius Stendim Biotech, Göttingen, Germany) and 10 µL was used to spot each indicator strains in the soft agar layer (0.75% agar w/v; 0.5% lactose; 10mM $CaCl_2$). Individual plaque forming units were counted (when a complete clearance spot was not observed) or the number of pfu's in the spot was estimated with a maximum of 100 pfu/complete clearance spot. Maximum of 100 pfu/spot was calculated with the following assumptions: 1) one spot (diameter of 1 cm) has a surface area of 0.785 cm^2 and an average surface area of one plaque (diameter of 1 mm) is 0.00785 cm^2.

Unique-gene based qPCR for genetic lineage relative abundance monitoring

A method developed by Erkus (2013) was used to monitor abundance of strains at the level of genetic lineages throughout the propagation experiment. A randomly chosen representative strain of each genetic lineage present in the Ur culture was sequenced: TIFN1 – strain of genetic lineage 1; TIFN2 – strain of genetic lineage 2; TIFN3 – strain of genetic lineage 3; TIFN4 –strain of genetic lineage 4; TIFN5 – strain of genetic lineage 5; TIFN6 – strain of genetic lineage 6; TIFN7 – strain of genetic lineage 7; TIFN8 – strain of genetic lineage 8. Conserved unique genes were identified for each representative strain and used to design primer pairs. When the genomes of representative strains of lineages 1 and 5 as well as 2 and 4 were compared, only a limited number of unique gene sequences was found between the strains in these two genome pairs. None of these unique genes met the criteria for specific primers design. For that reason, lineages 1 and 5 (1&5) as well as lineages 2 and 4 (2&4) were further enumerated together. The primer pairs were subsequently used in qPCR run to determine the specific gene copy number, which then determines each genetic lineage relative abundance in the given sample (see more details in Erkus et al., 2013).

Sequencing and characterization of isolated Lactococcus laudensis strain

A single colony isolate from LUMSC line 8 was grown for 48h in M17 broth (0.5% lactose w/v). 5mL of a fully-grown culture was harvested by centrifugation at 3,000 × g for 10 minutes and the DNA was extracted using DNeasy Blood and Tissue Kit according to procedure mentioned in Spus et al. (2015). Commercially available (BaseClear B.V., Leiden, The Netherlands) hybrid strategy was used combining Illumina HiSeq2500 technology (http://www.illumina.com/systems/hiseq_2500_1500.html) to generate short reads and PacBio RS (http://www.pacificbiosciences.com) to generate long reads. This hybrid strategy yielded with only 4 contigs: 2,234 kbp; 6.2 kbp; 55.4 kbp and 7.8 kbp. *De novo* assembly analysis was performed in CLC Genomics Workbench version 8.0 (QIAGEN, Germany). The optimal k-mer size was automatically determined using KmerGenie (Chikhi and Medvedev, 2014). Alignment of the contigs has been performed with BLASR (Chaisson and Tesler, 2012). The orientation, order and distance between the contigs were estimated from the alignment using SSPACE-LongRead scaffolder version 1.0 (Boetzer and Pirovano, 2014). Genome annotation was performed using BaseClear's inhouse annotation pipeline based on Prokka Prokaryotic Genome Annotation System (http://www.vicbioinformatics.com).

Api50 CHL (bioMérieux, USA) test was used for determination of carbohydrates utilization capacity of the *L. laudensis* strain and the representative sequenced strains of all 8 genetic lineages of Ur starter (TIFN1, TIFN2, TIFN3, TIFN4, TIFN5, TIFN6, TIFN7 and TIFN8).

Enrichment procedure of Lactococcus laudensis strain from original Ur starter

M17 broth without an added carbohydrate source was inoculated with the original Ur starter and incubated for three days at 20 °C to exhaust all the nutrients required for its growth. Next, the culture was centrifuged at 12,000 × g for 10 min to precipitate the biomass, then pH was adjusted to 7 and the supernatant was filter-sterilized (0.2 μm pore size) to obtain the spent medium. D-mannitol up to the level of 0.5% was added to the spent medium and next the medium was inoculated with a loop of original Ur starter or of *L. laudensis* YQ01 (positive control) and incubated at 20 °C for 48 h.

Generation of the metagenome sequences

Paired-end sequence reads were generated using the Illumina HiSeq2500 system. FASTQ sequence files were generated using the Illumina Casava pipeline version 1.8.3 (http://www.illumina.com). Initial quality assessment was based on data passing the Illumina Chastity filtering (http://www.illumina.com). Subsequently, reads containing adapters and/or PhiX control signal (http://www.illumina.com) were removed using BaseClear's in-house filtering protocol. The second quality assessment was based on the remaining reads using the FASTQC quality control tool version 0.10.0 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc /). Sequencing yielded more than 44 million reads (11,142 in MB) for 1000G LUSMC 4 sample, more than 42 million reads (10,658) for LUMSC 8 and more than 36 million reads (9,098) for the original Ur sample, with the average quality Phred scores of 33.77, 35.2 and 35.25, respectively.

Mapping of metagenomes of two LUMSC lines against the representative 16S rRNA sequences

The coverage of the perfect matching reads per position of the 16S rRNA fragment for four strains: *L. laudensis, L. lactis* ssp. cremoris, *L. lactis* ssp. *lactis* biovar *diacetylactis* and *Le. mesenteroides* ssp. *cremoris* was determined using breseq (Deatherage and Barrick, 2014). As assumed for large number of positions the output coverage was actually "0", which means that these fragments have multiple matches in different 16S rRNA sequences. In practice it means that only variable regions provide an accurate prediction of the presence of the specific species. To get a good overview of the presence of different types of strains, the median of the coverage excluding the "0" cases was taken. These coverage values were turned into relative values per sample by dividing over the total coverage of the reads per sample. These results are presented in the Fig. 1 in the Appendix.

Acidification profile

Acidification profiles of Ur starter and all 12 LUMSC cultures were determined by classical pH measurement. In detail, a -80 °C-kept stock sample of each culture was thawed to room temperature. Next, 9.9 mL of skim milk was inoculated with the 100 µL of thawed culture and grown at 20 °C for 24h to reach stationary phase. Further, 100 µL of fully-grown culture was inoculated

into 9.9 mL of skim milk. The first pH measurement was taken just after milk inoculation. The pH of each culture was subsequently measured in two hours intervals up to 10h and at time end of the measurement after 24h.

HPLC for determination of fermentation products formed

Reversed phase–high performance liquid chromatography (RP-HPLC) was used to analyze sugar consumption and acid production during the fermentation process. The calibration curves were determined for the compounds of interest in order to determine their retention times and response factors. Calibration curves of lactic acid, acetic acid and ethanol were prepared with varying concentrations. The Ur starter cultures as well as the evolved cultures were grown overnight (1% inoculum) in skim milk ("Friesche Vlag", 0% fat, UHT, Friesland Campina, The Netherlands). Further, the samples were deproteinized using Carrez reagents. 0.25mL of Carrez A solution (42.2 g of $K_4Fe(CN)_6*3H_2O$ /L of H_2O) was added to 0.5mL of overnight culture. After this step, sample was mixed by vortexing, following which 0.25mL of Carrez B solution (57.5 g of $ZnSO_4.7H_2O$ /L of H_2O) was added and mixed again. The sample was then centrifuged for 5 minutes at 15,000 × g. Finally, 200µL of the clear supernatant was transferred to an HPLC vial.

Samples were analyzed in an Ultimate 3000 (Dionex, Thermo Scientific) HPLC system, using Aminex HPX-87H column of 300 × 7.8mm (Bio-Rad) along with the pre-column. Sulphuric acid (5mM concentration) was used as the eluent, which was operated at a flow rate of 0.6 mL/min at a temperature of 40 °C. Detection was achieved by a refractive index (Shodex RI 101). For HPLC data analysis the Chromeleon software (version 7.0) was used.

Milli-cheese preparation

The MicroCheese model system developed by Bachmann and co-workers (2009) was adapted to a 24-deep well plate format and further referred to as the milli-cheese system. Each batch of milli-cheese was prepared in a separate 24-deep well plate and using a specific starter culture (either the original Ur starter or one of the twelve LUMSC cultures from 1000G point). 120 mL of pasteurized full fat milk (Friesland Campina, The Netherlands; content: 3.5% of protein, 4.7% of carbohydrate, and 3.6% of fat) was pre-heated at 30.5 °C for 30 min. To pre-heat the deep-well plate, the sterile 24 deep-well plate covered with an adhesive seal (Microseal®, BIO-RAD, CA, USA) was placed in

the oven at 37 °C for approximately 30 min. Subsequently, 30 µL of rennet, 48 µL of 33% (w/v) of $CaCl_2$, 1% (v/v) of starter culture was added to the milk and mixed thoroughly. Furthermore, the 24-deep-well plate was filled with 5 mL of milk mixture per well. The plate was sealed with the adhesive seal and incubated in a thermomixer (Comfort, Eppendorf, USA) at 32.5 °C. After 40 min, the coagulated milk (curd) was cut with a sterile, custom-made handheld, 24-pins stainless steel stirring device. This stirring device was made from stainless steel, consisted of a plate with a handle at the top and 24 pins attached to the bottom. The pins were aligned accordingly to the shape of the 24 deep-well plate. Manual stirring was carried out for 20 min with certain intervals (20s stirring and 3 min rest). The curd was rested for 5 min and the plate was sealed again for centrifugation step (Centrifuge 5804 R, Eppendorf, USA) at 500 × g for 30 min at 30 °C. Subsequently, 1.9 mL of whey was removed and replaced with 1.8 mL of sterile demi water (pre-heated at 45 °C). After addition of water, the plate was placed in the thermomixer at 35.5 °C and the matrix was cut as described above, for 40 min followed by 20 min resting. To remove the remaining whey and liquid, the plate was centrifuged at 2250 × g for 2 hours at 30 °C. The supernatant was discarded by carefully pipetting out the liquid from each well. Finally, the plate was sealed with a gas-permeable seal (BREATHseal™, Greiner Bio One, Frickenhausen, Germany) and incubated overnight at 30 °C. After overnight incubation, 50 µL of a sterile 19% (w/v) sodium chloride solution was added to each well, followed by a centrifugation step at 500 × g for 5 min. Each batch was sealed with the gas-permeable seal, placed in a jar under anaerobic conditions and incubated at 12 °C. Finally; the milli-cheeses were ripened for six weeks.

Volatile compounds analysis of milli-cheeses

Milli-cheeses were prepared using original Ur starter culture and twelve 1000G LUMSC cultures as starters. Headspace Solid-Phase Micro-Extraction Gas Chromatography – Mass Spectrometry (HS SPME GC-MS) was used to analyze the samples collected at 6 weeks of ripening. Vials were stored at -20 °C until GC-MS analysis was performed. Volatile organic compounds (VOCs) were extracted with a HS- SPME fiber (divinylbenzene /carboxen /polydimethylsiloxane, DVB/CAR/PDMS, Supelco Inc. USA; Gamero, et al., 2013). Samples were pre-incubated at 60 °C for 5 minutes with agitation, followed by exposure of the fiber to the sample headspace for 40 minutes at 60 °C. Volatile compounds were injected into the GC column by desorption of the fiber for 10 minutes. The Finnigan Trace GC Ultra (Thermo Fisher

Scientific, USA) equipped with a Stabilwax® -DA-Crossband® -Carbowax® -polyethylene-glycol column (30 m length, 0.32 mm internal diameter, 1µm internal thickness; Restek, Bellefonte, PA, USA) was used for GC-MS analysis. The injection device was a TriPlusTM autosampler (Thermo Fisher Scientific, USA) in PTV Split-less mode (5 minutes) at 250 ºC. Helium was used as carrier gas at a constant flow of 10 ml/min. The GC oven was kept initially at 40 ºC for 2 minutes, and then the temperature was raised to 250 ºC (10 ºC /min) and kept at 250 ºC for 5 minutes. Mass spectral data were collected over a range of m/z 33-250 in full scan mode, scan time 0.3 seconds. Chromatograms were manually scanned for VOCs in AMDIS (NIST, U.S.) to build up a retentions list of the identified compounds. Further, automated analyses were performed using MetAlign and MetAlignID (Lommen, 2009; Lommen and Kools, 2012). The obtained peak areas of identified compounds were used to calculate their relative abundance in percentage. A two-sample assuming equal variance *t* test was used to calculate the p values.

RESULTS

Under the radar strain characterization and sequencing

After plating on M17 agar (0.5% lactose) of the 12 sequentially propagated parallel lines of Ur, for 1000 generations (LUMSC), we observed rigid and translucent colonies contrasting with the typical round and opaque *L. lactis* colonies. We determined the sequence of the 16*S* rRNA gene of the suspicious colony isolated from LUMSC line 8, and after performing a nucleotide BLAST (Altschul et al., 1990), the isolate resembled *Lactococcus raffinolactis* (97% identity on the 16*S* rRNA gene sequence level). Next, the API CHL50 test was performed to determine the capacity for the isolated strain to utilize different carbon sources. The strain was not able to utilize raffinose, which suggested that we were not dealing with a *L. raffinolactis* strain. Indeed, after running the 16*S* rRNA comparison of our isolated strain against (i) representative Ur strains (TIFN1-8), (ii) the *L. raffinolactis* type strain, (iii) *Lactococcus laudensis* and (iv) *Lactococcus hircilactis* published by Meucci et al. (2015), the published *L. laudensis* and our strain clustered together (Fig. 1).

Full genome sequencing of our *L. laudensis* strain (designated as YQ01) resulted in four contigs. The biggest contig of 2.3 Mbp (2233 genes) – chromosomal DNA, and three smaller ones representing plasmids, of: 55 kb (52 genes), 7.7 kb (9 genes) and 7 kb (6 genes). The biggest plasmid contains

genes of a complete lactose operon, including *lacA*, *lacB*, *lacC_2*, *lacD* (van Rooijen, 1991), *lacE*, *lacF_2*, *lacX_3* (de Vos et al., 1990) and *lacG* (de Vos and Gasson, 1989) as well as two copies of the complete gene operon for oligopeptides transport: *oppDFBCA* and *pepO* (Tynkkynen, 1993). The results of sequencing clearly confirms the ability of the strain to grow on lactose but suggesting its dependence on other (caseinolytic) strains i.e. *L. lactis* ssp. *cremoris*, since the serine protease genes were not found on the *L. laudensis* genome.

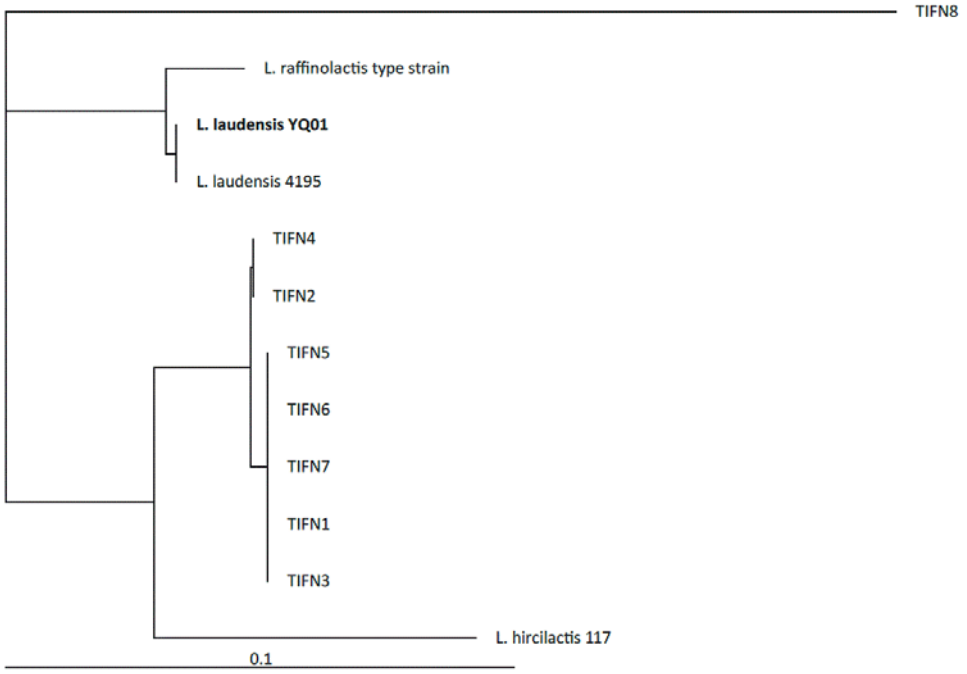


Figure 1. Phylogenetic tree (maximum likelihood) of sequenced representative strains present in Ur starter culture. Tree was constructed using 16S rRNA gene sequences. TIFN1 – sequenced strain of genetic lineage 1; TIFN2 – sequenced strain of genetic lineage 2; TIFN4 – sequenced strain of genetic lineage 4; TIFN5 – sequenced strain of genetic lineage 5; TIFN6 – sequenced strain of genetic lineage 6; TIFN7 – sequenced strain of genetic lineage 7; TIFN8 – sequenced strain of genetic lineage 8; *L. laudensis* YQ01 in bold– strain isolated from Ur culture after 1000-generation sequential propagation; *L. laudensis* 4195T and *L. hircilactis* 117T– strains published in the paper of Meucci et al., 2015.

The results of the API50 CHL test (Appendix, Table 1) showed that *L. laudensis*, as the only one among the tested strains, could utilize D-mannitol.

We confirmed the presence of *L. laudensis* in the original Ur culture using an enrichment medium with D-mannitol as the sole fermentable substrate. Two flasks with spent medium, inoculated with either YQ01 or with Ur starter, became both turbid after two days incubation at 20 ºC. This procedure allowed for direct isolation of *L. laudensis* from original Ur starter as confirmed by 16S rRNA sequencing (data not shown).

Taking into account that after each 24h we transfer 100 µL of culture to fresh milk the minimal possible abundance of *L. laudensis* strain could be 10 cfu/mL (one cell in 100 µL). According to the calculations based on 16S rRNA gene sequence in the metagenome of the original Ur starter fraction of *L. laudensis* stands for 9 x 10^3 of the total of 1 x 10^8, which gives 9000 cfu/mL. Based on these calculations the range of the initial number of *L. laudensis* in the original Ur starter is estimated to be between 10 and 9000 cfu/mL.

Composition of the LUMSC cultures at 500 and 1000 generations

We monitored the composition of the 12 parallel LUMSC cultures (LUMSC 1-12) after 500 and 1000 generations (referred to as 500G and 1000G respectively) of propagation. Using the unique-gene-based method introduced earlier by Erkus and co-workers (2013) we were able to determine the abundance of Ur genetic lineages: 1&5, 2&4, 3, 6, 7 and 8 (Fig. 2). Having the full genome sequence of the newly discovered *L. laudensis* YQ01 strain, we were able to identify unique genes and successfully designed primers to determine the abundance of *L. laudensis* strain(s) at 500G and 1000G. At 500G, the abundance of *L. laudensis* strain(s) was between 2% (LUMSC 1) and 34% (LUMSC 5) and at 1000G its abundance increased in all lines and was between 8% (LUMSC 1) and 76% (LUMSC 12). Analysis of the data revealed a weak anticorrelation between the abundance of lineages 1&5 and the abundance of *L. laudensis* (R^2 = 0.43 for 500G and R^2 = 0.66 for 1000G; Figure 2, Appendix). Furthermore, the combined abundance of prt^+ lineages 1&5 and 3 was never lower than 5% both at 500G and 1000G time points. Interestingly, at 500G in LUMSC 10 the abundance of lineages 1&5 was 0.5% but at the same time abundance of another prt^+ lineage 3 reached 12%. Throughout the time course of the experiment all cultures maintained their key-property of fast acidification of milk (see Fig. 3) confirming the crucial role of the prt^+ lineages in the functionality of the mixed culture. The relative abundance of genetic lineages was highly dynamic when we compare the compositions of 500G and 1000G LUMSC samples (Fig. 3), although, a general picture emerges that

representatives of three lineages dominated the LUMSC cultures after 1000G: (i) lineage 1&5 (*L. lactis* ssp. *cremoris*), (ii) lineage 8 (*Le. mesenteroides* spp. *cremoris*) and (iii) *L. laudensis*.

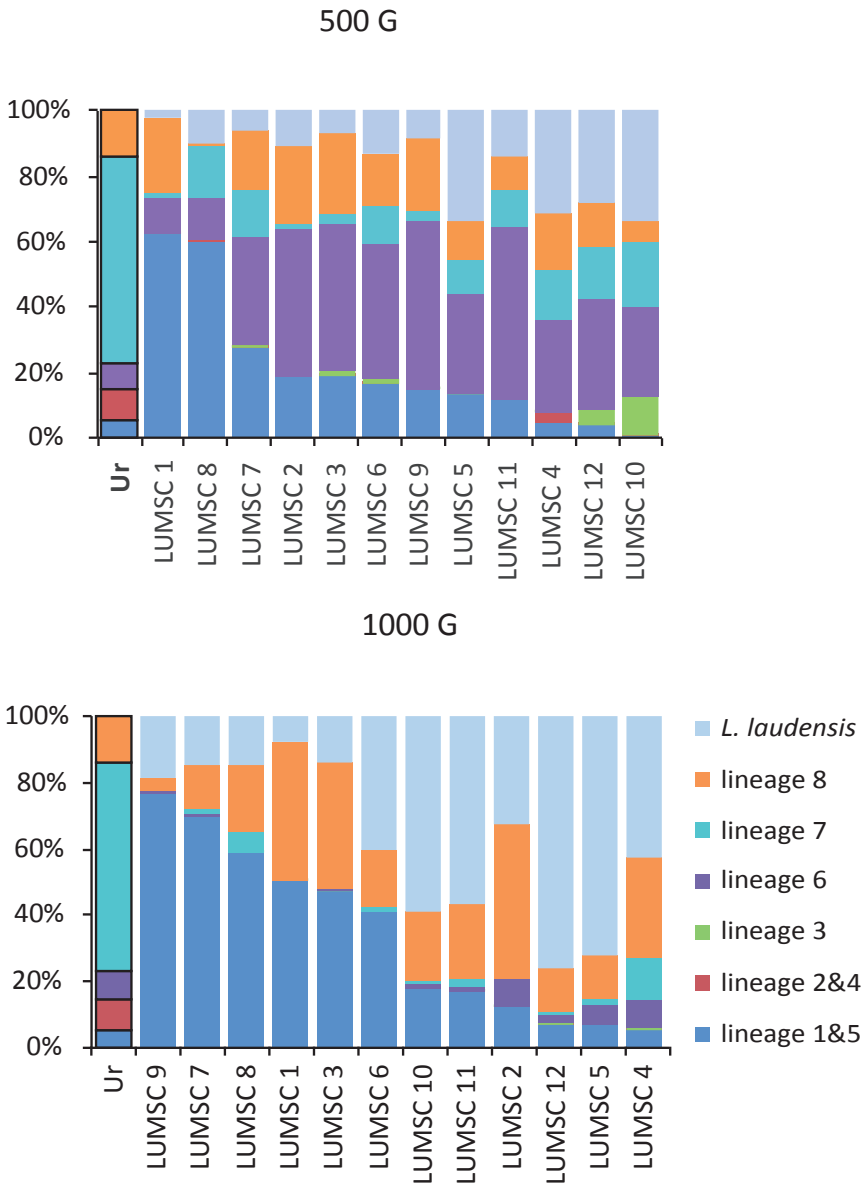


Figure 2. Relative abundance of genetic lineages and *L. laudensis* in original Ur starter and in twelve lines of LUMSC cultures at 1000G. Results of LUMSC 1-12 were sorted from the highest to the lowest abundance of lineage 1&5 to illustrate the anticorrelation between the abundance of lineage 1&5 and *L. laudensis*.

Acidification profiles of Ur starter and LUMSC cultures

Fast acidification is one of the most important attributes of a functional starter culture. Acidification is usually monitored as time dependent change of pH after inoculation of cheese milk with the starter resulting in a characteristic acidification profile.

We determined the acidification profiles of LUMSC cultures and compared them to that of the original Ur starter (Fig. 3). Clearly, the acidification profiles obtained with the evolved LUMSC cultures differ from the acidification profile obtained with Ur starter. For all 12 LUMSC cultures, the profiles are steeper in the period from 2 to 8 h. All 12 LUMSC cultures reached lower pH values at time point of 10 h compared to the original Ur starter (between 4.39 for LUMSC 9 and 4.51 for LUMSC 6, 4.73 for Ur). Already at time point 8 h, the pH values reached by LUMSC cultures were close to the final pH reading after 24 h, confirming the higher acidification rate of the evolved LUMSC cultures in comparison to Ur. Nevertheless, after 24 h the pH values of Ur starter and some of LUMSC samples were comparable (e.g. 4.48 both for LUMSC 1 and Ur).

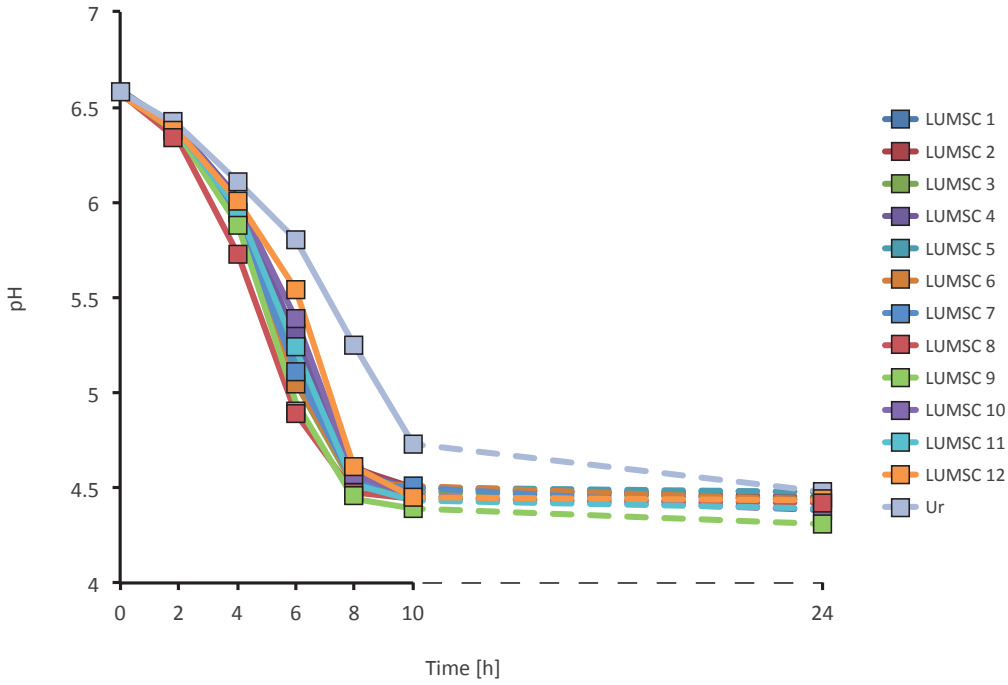


Figure 3. Acidification profiles of Ur starter and twelve 1000 generations-evolved LUMSC samples.

Fermentation products formation in Ur and LUMSC cultures

The production of organic acids (lactic acid, acetic acid) and ethanol in the Ur starter as well as in the evolved LUMSC cultures were determined by RP-HPLC. In all twelve evolved LUMSC cultures as well as in the original Ur lactic acid and acetic acid were found (Fig. 4). The highest concentration of the monitored compounds in all samples was found for the lactic acid (between 5.8 g/L for LUMSC 2 to 7.2 g/L for LUMSC 7). Second most abundant product for which we determined the concentrations in our cultures was ethanol (between 1.4 g/L for LUMSC 1 and 1.5 g/L for LUMSC 9). Third most abundant fermentation product for all cultures was acetic acid (between 0.06 g/L for LUMSC 9 and 0.4 g/L for Ur).

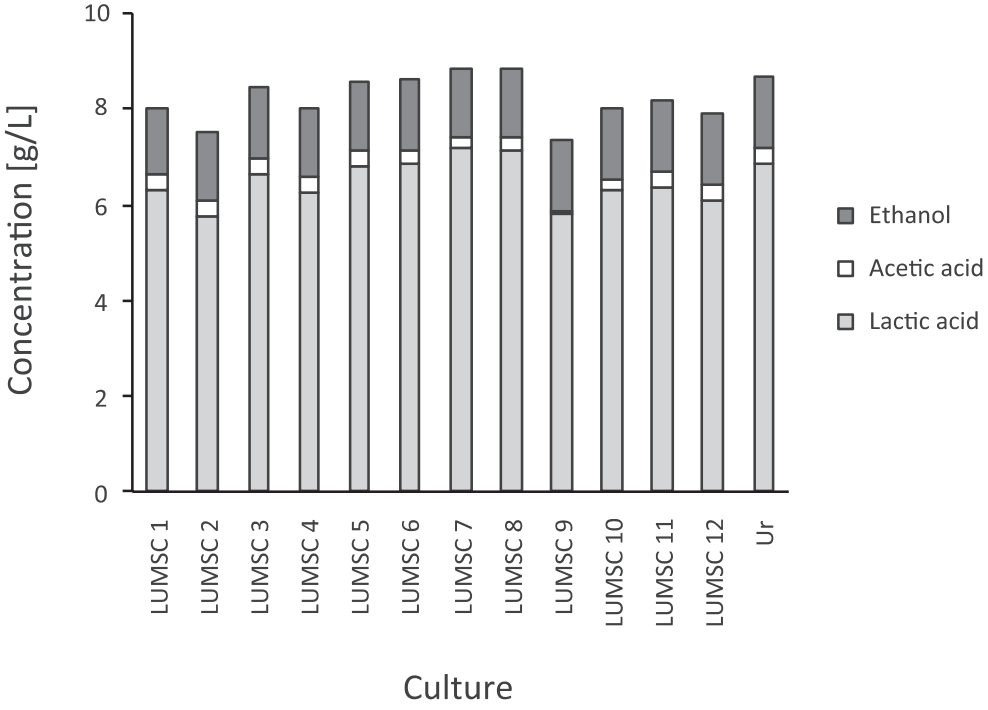


Figure 4. Formation of three basic fermentation products in Ur starter and twelve 1000-generation-evolved LUMSC cultures as determined by the RP-HPLC.

Aroma formation in milli-cheeses made with LUMSC cultures

All 12 parallel LUMSC cultures obtained after 1000 generations of sequential propagation were used as starters for the production of milli-cheeses with the objective to determine the formation of volatile aroma compounds during cheese ripening. As a benchmark, cheeses were produced with the original Ur

starter. After 6 weeks of ripening, we identified 43 compounds in Ur cheeses and 36 compounds in LUMSC cheeses (Table 1, Appendix). All compounds detected were divided into the following categories: alcohols, aldehydes, alkanes, alkenes, carboxylic acids, esters, ketones, sulphur compounds, pyrazine-derived compounds and butanimidamide (in case of Ur culture) or 1, 3-bis(1, 1-dimethylethyl)-benzene (in case of LUMSC cultures). Three most abundant categories of compounds found in the 6 weeks ripened milli-cheeses were esters, alcohols and ketones (Fig. 5). In all objects where LUMSC starters were used, the contribution of esters in the aroma profiles was lower than control cheeses made with the original Ur starter (p=1.13E-6). In contrast, in all LUMSC cheeses the relative abundance of alcohols was higher than in the Ur cheese (p=0.007). For ketones, no significant difference in the abundance between cheese made with the Ur starter and cheese made with LUMSC cultures was observed (p=0.094).

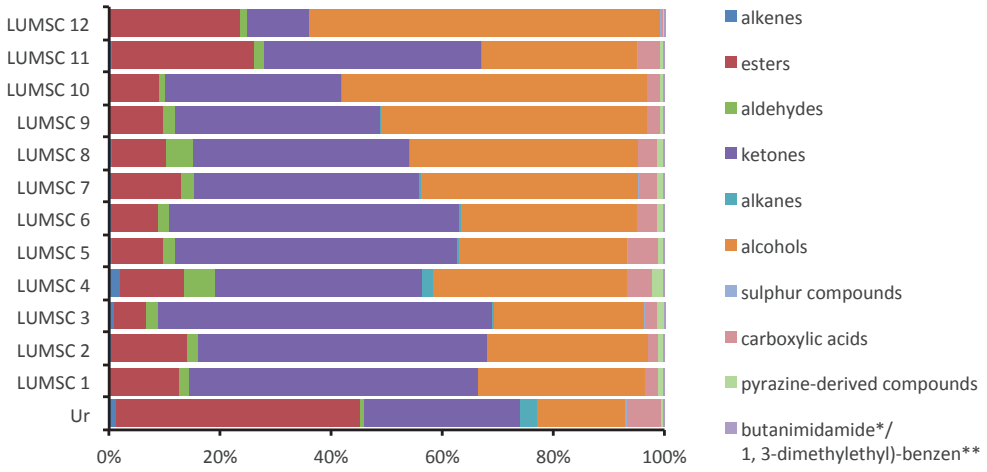


Figure 5. Relative abundance of VOCs detected at 6 weeks of ripening in milli-cheese samples prepared with the use of different cultures as starters, including original Ur starter and all twelve lines of LUMSC 1000G cultures. * - found exclusively in Ur samples; ** - found exclusively in LUMSC samples

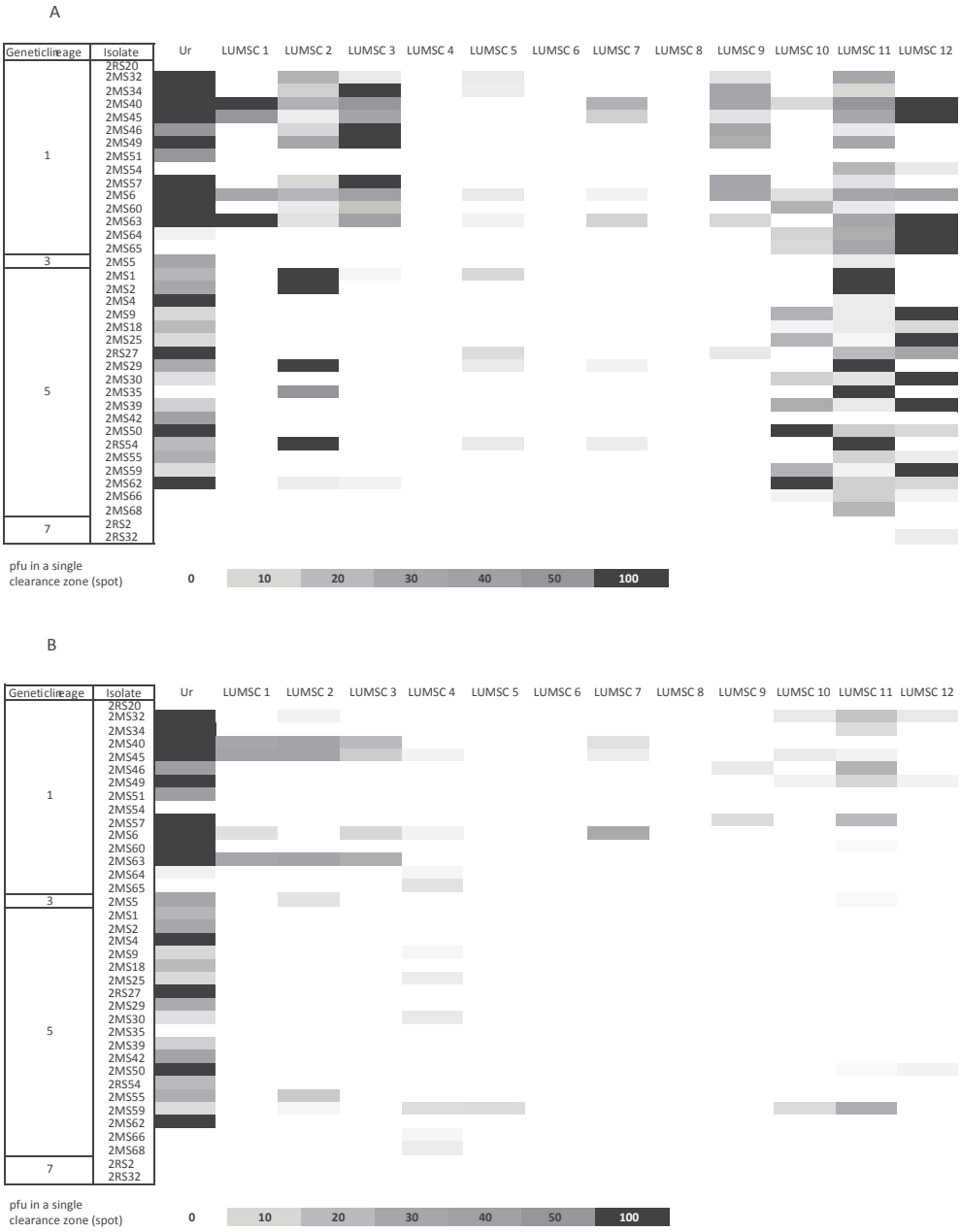


Figure 6A and 6B. Plaque assay of strains isolated from Ur starter cultures challenged by the phages from the supernatant of the LUMSC cultures at 500-generation time point (A) and 1000-generation time point (B). Grey scale indicates the intensity of clearance zone and corresponds to plaque forming units counted in the clearance zone (spot).

Bacteriophages in LUMSC cultures

The presence and activity of lytic bacteriophages in the LUMSC was monitored during the long-term propagation experiment at two time points: 500G and 1000G. A representative collection of strains previously isolated from the Ur starter (Spus et al., 2015) was used for the detection of active bacteriophages in the supernatants using a spot assay. The phage abundance was determined by visual inspection of clearance zones in the bacterial cells lawns (See Fig. 6A and 6B). For both time points (500G and 1000G) the phage presence was diverse among LUMSC cultures. In some of the LUMSC cultures (4, 6 and 8) at 500 G we did not find any phages predating on the indicator strains (Fig. 6A). Interestingly, at 1000 G time point we detected some susceptibility for phages present in the LUMSC 4. In general, at 500G we detected more phage activity than at 1000G. Furthermore, the phage titers were higher at 500G compared to what was found in the 1000G supernatants (indicated by the intensity of the shades of grey Fig. 6A and Fig. 6B).

DISCUSSION

To our knowledge, this study is the first record of a long-term evolution experiment carried out with a complex culture of known composition. Previous attempts discussed in the literature focus on single strain evolution experiments (Blount et al., 2008; Barret et al., 2005), pair of strains (Pekkonen et al., 2013) or defined mixtures of single strains (Celiker and Gore, 2014; Fiegna et al., 2015; Spus et al., 2015).

The most unexpected outcome of our long-term experimental evolution study with the undefined mixed starter culture was the enrichment of a new *Lactococcus* species, recently described as *L. laudensis* (Meucci et al., 2015). We demonstrated that this species was present in the original complex starter culture at extremely low abundance (between 10 and 9000 cfu/mL) and its presence has never been noticed before. Further analysis of the metagenome of the original Ur starter confirmed presence of 16S rRNA sequences unique for *L. laudensis* strain but comprising small fraction of the total 16S rRNA abundance (Fig. 1, Appendix). In two samples collected at the 1000-generation time point (LUMSC 4 and 8) we found a dramatic increase in abundance of *L. laudensis* unique sequences supporting the observed enrichment of this species in the population (Fig. 1, Appendix). Evidently the chosen propagation regime (non-fat, UHT milk, 20 °C, daily propagation of 1%) favored fast

growth of *L. laudensis*. The incubation temperature of 20 °C is neither optimal for growth of *L. lactis*, which grows best at 30 °C nor for *Le. mesenteroides*, which grows best at 25 °C. Preliminary studies in our laboratory showed that prolonged sequential propagation of the Ur culture at 30 °C caused gradual 'wash out' of lineage 8 strains (*Le. mesenteroides*; data not shown).

The second condition impacting enrichment of *L. laudensis* is the propagation regime of transferring the culture each 24h. At this point in time, all of the strains representing all lineages in the culture are already in the stationary phase of growth and the exposure to low pH is apparently not long enough to impact *L. laudensis* survival. We performed a follow-up experiment with all 12 LUMSC lines 1000G where the propagation regime was changed from every 24h to every seven days. Within three weekly transfers, the relative abundance of *L. laudensis* dropped from between 8% (LUMSC 1) and 76% (LUMSC 12) to the level below the detection limit (Fig. 7). This experiment shows that *L. laudensis* is a poor survivor in harsh acidic conditions suggesting its possible high autolytic capacity.

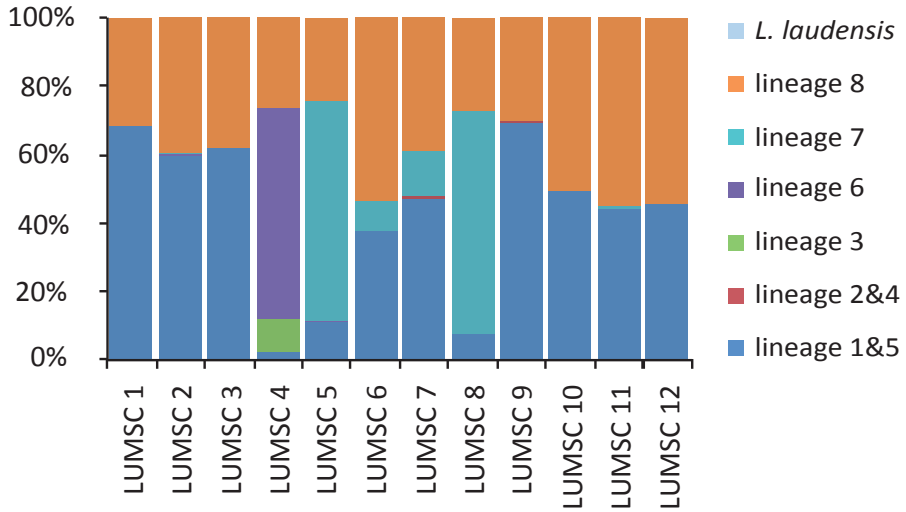


Figure 7. Relative abundance of genetic lineages and *L. laudensis* in twelve lines of 1000-generation LUMSC cultures after three weekly transfers in milk. *L. laudensis* abundance was found to be below detection limit (3 log DNA copy numbers) of unique-gene based qPCR.

We confirmed bacteriophage predation to be present throughout the propagation experiment. Bacteriophages as an integral part of the microbial community such as an undefined mixed starter can play a role in its inherent compositional and eventual functional stability (Erkus et al., 2013). Along with the principles of CD dynamics model (Rodriguez-Valera et al., 2009) prey density dependent bacteriophage predation would keep in control the most abundant strains preventing their domination in the community and at the same time sustain community's functional versatility.

Apart from environmental conditions, the interactions between strains in the culture potentially played a role in *L. laudensis* enrichment. We demonstrated that *L. laudensis* is the only member of the microbial culture that can utilize D-mannitol (see Table 1 in Appendix). From previous work it is known that *Le. mesenteroides* secretes D-mannitol (Wisselink et al., 2002) and our study showed that *L. laudensis* could consume D-mannitol. Interestingly, the abundance of *Le. mesenteroides* gradually increased throughout the prolonged propagation experiment. This leads to a predicted increase of D-mannitol secretion in the medium making the environment of milk even more favorable for *L. laudensis.* Traces of D-mannitol were indeed found in the fermented milk samples (data not shown), supporting the model of cross-feeding between *Le. mesenteroides* and *L. laudensis*. Here, we suggest commensal relationship between *L. laudensis* and *Le. mesenteroides* (Fig. 8).

Additionally, considering the anticorrelation between the abundance of lineage 1&5 (prt^+) and *L. laudensis* we suggest that *L. laudensis* is a good scavenger of peptides released by the prt^+ strains (lineage 1, 3 and 5). *L. laudensis* possesses multiple plasmid bound gene clusters encoding oligopeptides transport systems. Perhaps the conditions present in the experiment resulted in higher expression of genes coding for oligopeptides transporters in *L. laudensis* making it more competitive. Not only the interactions among *L. laudensis*, *Le. mesenteroides* and prt^+ *L. lactis* ssp. *cremoris* (Fig. 8) are responsible for compositional stability of the LUMSC cultures after 1000 generations. None of the lineages present in original Ur was lost during the prolonged propagation confirming presence of other strong microbe-microbe interactions (Smid and Lacroix, 2013).

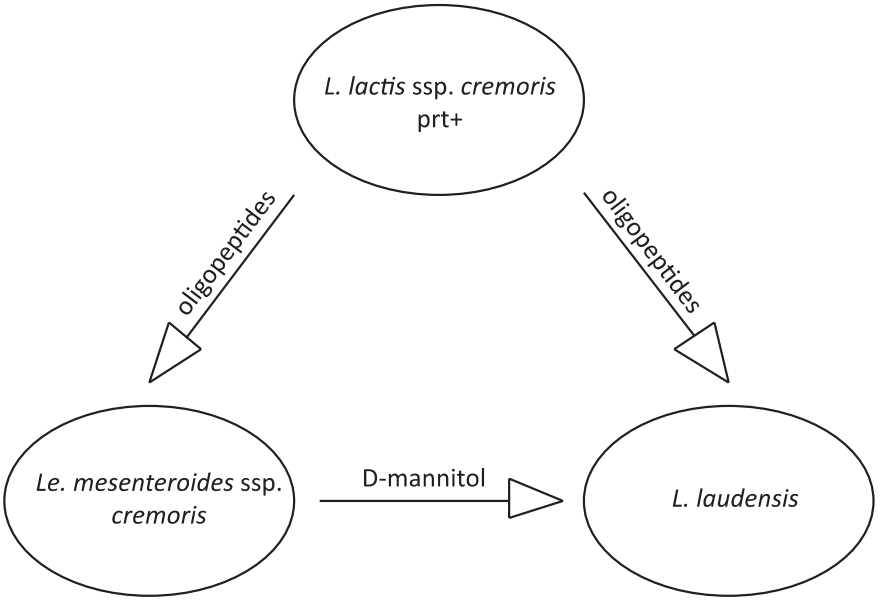


Figure 8. New hypothesized interaction between *Le. mesenteroides* ssp. *cremoris* and *L. laudensis* found in evolved Ur starter culture. D-mannitol is secreted by *Le. mesenteroides* ssp. *cremoris* strain and *L. laudensis* strain can utilize it as a carbon source giving it an competitive advantage in the complex microbial community.

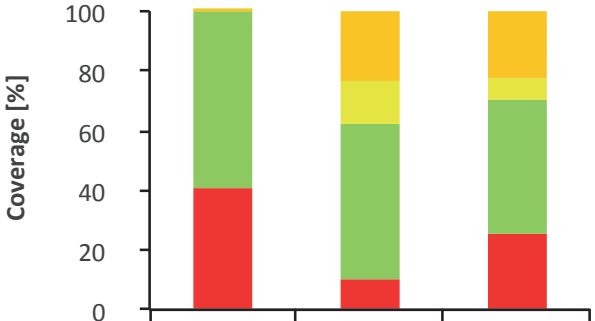
For a given starter culture, any aberration from the characteristic acidification profile (slow acidification) is an indicator of sub-optimal starter performance caused by e.g. bacteriophage attack (Mc Grath et al., 2007) or too low numbers of caseinolytic lactic acid bacteria. From the industrial point of view, it is crucial that the acidification rate of the starter culture is consistently high, since the change in rate can cause delays in production and eventual financial losses. Acidification was not affected in case of 1000G LUMSC cultures and the acidification rate even increased when compared to original Ur starter culture.

Despite differences found in concentrations of fermentation end-products in twelve LUMSC cultures and original Ur starter, all cultures presented a profile of fermentation products indicative for combined homo- (lactococci) and heterofermentative (leuconostocs) metabolism. This result underpins the inherent stability of complex undefined mixed starter cultures, which, in the particular case of our LUMSC cultures, even after substantial increase in relative abundance of particular community members, sustains in its basic

functionalities. We observed significant differences in the aroma profiles for the milli-cheeses made with 1000-generation-evolved LUMSC cultures in comparison to milli-cheeses made with the original Ur starter. We identified a trend of increased abundance of compounds grouped in the category of alcohols in all LUMSC cultures when compared to Ur. This group of compounds was mainly comprised of branched-chain alcohols, which can be formed through Strecker degradation of amino acids (Fox, 2000). On the other hand, the group of compounds comprised of esters was lower in abundance in all milli-cheeses made with LUMSC cultures when compared to Ur milli-cheeses. Esters are formed when free fatty acids (derived from lipolysis) react with alcohols (products of lactose fermentation and amino acid degradation) or when they are hydrolyzed by bacterial esterases. Majority of the esters found in Gouda cheese by Alewijn and co-workers (2013) were ethyl esters, which contrasts with our results. We did not find any ethyl esters in our milli-cheeses samples and majority of esters were actually methyl esters (Table 2, Appendix). The presence of *L. laudensis* significantly impacted the aroma profiles of manufactured lab-scale cheeses, although these results require follow-up sensory studies for confirmation.

In conclusion, we provide experimental evidence for extreme robustness and versatility of a microbial community despite phage predation and differences in growth rates among strains. The particular competitive success of the new enriched *L. laudensis* strain emerged from the combination of favorable conditions, including the subjected propagation regime (daily transfer and specific temperature of incubation) and the putative commensal interaction with the prt$^+$ *L. lactis* ssp. *cremoris* and *Le. mesenteroides* allowing both scavenging essential for growth oligopeptides and utilizing additional C-source, D-mannitol. In consequence, as predicted in a microbial community functioning according to CD model (Rodriguez-Valera et al., 2009), evolved cultures were more efficient in resources exploitation (consumption of alternative C-source in D-mannitol) and better in functioning of the ecosystem (faster acidification). New enriched *L. laudensis* strain was not only under the radar for the previously used culture-dependent (differential plating) and culture-independent (metagenomic sequencing) methods (Erkus et al., 2013), but also, under the radar for the bacteriophage predation contributing to its competitive edge. However, we cannot exclude that further propagation of LUMSC cultures might result in possible evolution of *L. laudensis* predator, which yet again would change the balance among lineages.

SUPPLEMENTARY MATERIAL



	Ur	LUMSC 4	LUMSC 8
laudensis	**0.00009**	0.24	0.23
mesenteroides	0.003	0.14	0.07
cremoris	0.59	0.52	0.45
diacetylactis	0.41	0.1	0.25

Figure 1. The coverage of the perfect matching reads per position of the 16S rRNA fragment based on metagenome sequencing results for four strains present in three analyzed samples: Ur – original Ur starter; LUMSC 4 – 1000-generation-evolved Ur starter 4th replicate; LUMSC 8 - 1000-generation-evolved Ur starter 8th replicate.

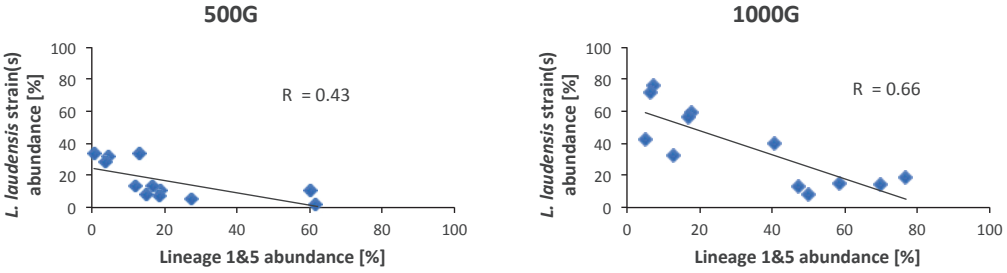


Figure 2. Anti-correlation between the abundance of *L. laudensis* strain(s) and lineage 1&5 in LUMSC samples at 500 and 1000 generations.

Table 1. Results of the Api50 CHL test. Eight sequenced representative strains of genetic lineages of Ur starter and the isolated *L. laudensis* (L.l.) strain were used here to test the ability of these strains for diverse carbohydrates utilization. In this table "+" indicates that the carbohydrate was utilized, "-" indicates lack of ability to utilize a particulate substrate.

Carbon source	T1	T2	T3	T4	T5	T6	T7	T8	L. l.
Control	-	-	-	-	-	-	-	-	-
Glycerol	-	-	-	-	-	-	-	-	-
Erythritol	-	-	-	-	-	-	-	-	-
D-Arabinose	-	-	-	-	-	-	-	-	-
L-Arabinose	-	-	-	-	-	-	-	-	-
D-Ribose	-	+	-	+	+	-	-	-	+
D-Xylose	-	-	-	-	-	-	-	-	-
L-Xylose	-	-	-	-	-	-	-	-	-
D-Adonitol	-	-	-	-	-	-	-	-	-
Methyl-βD-Xylopyranoside	-	-	-	-	-	-	-	-	-
D-Galactose	+	+	+	+	+	+	+	-	+
D-Glucose	+	+	+	+	+	+	+	+	+
D-Fructose	+	+	+	+	+	+	-	-	+
D-Mannose	+	+	+	+	+	+	+	-	+
L-Sorbose	-	-	-	-	-	-	-	-	-
L-Rhamnose	-	-	-	-	-	-	-	-	-
Dulcitol	-	-	-	-	-	-	-	-	-
Inositol	-	-	-	-	-	-	-	-	-
D-Mannitol	-	-	-	-	-	-	-	-	+
D-Sorbitol	-	-	-	-	-	-	-	-	-
Methyl-αD-Mannopyranoside	-	-	-	-	-	-	-	-	-
Methyl-αD-Glucopyranoside	-	-	-	-	-	-	-	-	-
N-Acetylglucosamine	+	+	+	+	+	+	+	+	+
Amygdalin	-	-	-	+	-	-	-	-	-
Arbutin	-	+	-	+	+	-	-	-	-
Esculin ferric citrate	+	+	+	+	+	+	+	+	+
Salicin	-	+	-	+	+	+	-	-	+
D-Cellobiose	-	+	-	+	+	-	-	-	+
D-Maltose	-	+	-	+	+	+	-	-	+
D-Lactose	+	+	+	+	+	+	+	+	+
D-Melibiose	-	-	-	-	-	-	-	-	-

D-Saccarose	-	-	-	-	-	-	-	+	+
D-Trehalose	-	+	-	+	+	+	-	+	+
Inulin	-	-	-	-	-	-	-	-	-
D-Melezitose	-	-	-	-	-	-	-	-	-
D-Raffinose	-	-	-	-	-	-	-	-	-
Amidon	-	+	-	+	+	-	-	-	-
Glycogen	-	-	-	-	-	-	-	-	-
Xylitol	-	-	-	-	-	-	-	-	-
Gentiobiose	-	+	-	+	-	+	-	-	-
D-Turanose	-	-	-	-	-	-	-	-	-
D-Lyxose	-	-	-	-	-	-	-	-	-
D-Tagatose	-	-	-	-	-	-	-	-	-
D-Fucose	-	-	-	-	-	-	-	-	-
L-Fucose	-	-	-	-	-	-	-	-	-
D-Arabitol	-	-	-	-	-	-	-	-	-
L-Arabitol	-	-	-	-	-	-	-	-	-
Potassium Gluconate	-	-	-	-	-	-	-	-	-
Potassium 2-Keto-Gluconate	-	-	-	-	-	-	-	-	-
Potassium 5-Keto-Gluconate	-	-	-	-	-	-	-	-	-

Table 2. Aroma compounds identified in our milli-cheeses samples at 6 weeks of ripening.

Alkenes	1	2,4-Dimethyl-1-heptene
	2	D-Limonene
	3	trans-4a-Methyl-decahydronaphthalene
	4	1-Pentene
Esters	5	Methyl propionate
	6	Butanoic acid, methyl ester
	7	2-Propenoic acid, 2-methyl-, methyl ester
	8	Propanoic acid, ethenyl ester
	9	2-Propenoic acid, butyl ester
	10	Hexanoic acid, methyl ester
	11	Butanoic acid, butyl ester
	12	Propanoic acid, 2-oxo-, methyl ester
	13	Acetic acid, 2-ethylhexyl ester
	14	Octanoic acid, methyl ester
	15	2-Propenoic acid, 2-methylpropyl ester

	16	Decanoic acid, methyl ester
	17	Dodecanoic acid, methyl ester
Aldehydes	18	Butanal, 3-methyl-
	19	Benzaldehyde
Ketones	20	2,3-Butanedione
	21	2-Butanone, 3-hydroxy-
	22	1-Octen-3-one
	23	Acetophenone
Alkanes	24	Undecane
	25	Dodecane
	26	Butane, 1-methoxy-
	27	Cyclohexane, isothiocyanato-
Alcohols	28	3-Buten-1-ol, 3-methyl-
	29	2-Propyn-1-ol, acetate
	30	1-Pentanol, 2-ethyl-4-methyl-
	31	1-Heptanol, 6-methyl-
	32	1-Heptanol, 3-methyl-
	33	Cyclohexanol, 4-(1,1-dimethylethyl)-
	34	Benzyl Alcohol
	35	Phenylethyl Alcohol
Sulphur compounds	36	Dimethyl trisulfide
	37	Dimethyl sulfone
Carboxylic acid	38	Acetic acid
	39	Butanoic acid
	41	Hexanoic acid
Pyrazine-derived compounds	42	Pyrazine, 2,5-dimethyl-
	43	Pyrazine, trimethyl-
Others	44	Butanimidamide
	45	1, 3-bis(1, 1-dimethylethyl)-benzen

Chapter 5

Isolation and characterization of *Lactobacillus helveticus* DSM 20075 variants with improved autolytic capacity

Maciej Spus, Hua Liu, Michiel Wels, Tjakko Abee, Eddy J Smid

ABSTRACT

Lactobacillus helveticus is widely used in dairy fermentations and produces a range of enzymes, which upon cell lysis can be released into the cheese matrix and impact degradation of proteins, peptides and lipids. In our study we set out to explore the potential of *Lb. helveticus* DSM 20075 for increased autolytic capacity triggered by conditions such as low pH and high salt concentrations encountered in cheese environments. *Lb. helveticus* DSM 20075 was subjected to varied incubation temperatures (ranging from 37 to 50 °C). High-temperature incubation (in the range of 45 to 50 °C) allowed us to obtain a collection of six variant strains (V45-V50), which in comparison to the wild-type strain, showed higher growth rates at elevated temperatures (42 °C – 45 °C). Moreover, variant strain V50 showed a 4-fold higher, in comparison to wild type, autolytic capacity in cheese-like conditions. Next, strain V50 was used as an adjunct in lab-scale cheese making trials to measure its impact on aroma formation during ripening. Specifically, in cheeses made with strain V50, the relative abundance of benzaldehyde increased 3-fold compared to cheeses made with the wild-type strain. Analysis of the genome sequence of strain V50 revealed multiple mutations in comparison to the wild-type strain DSM 20075 including a mutation found in a gene coding for a metal ion transporter, which can potentially be linked to intracellular accumulation of Mn^{2+} and benzaldehyde formation. The approach of high-temperature incubation can be applied in dairy industry for the selection of (adjunct) cultures targeted at accelerated cheese ripening and aroma formation.

INTRODUCTION

Adjunct cultures are used in cheese production to accelerate ripening and support specific flavor development (Fox et al., 1996; 1998). Both the acceleration of ripening and flavor development can be affected by the addition of an adjunct culture due to its enzymatic activity (Khalid and Marth, 1990). Adjunct cultures are usually inoculated at high cell counts together with the starter culture and they can survive and sometimes even grow in cheese (Antonson et al., 2002; Briggiler-Marcó et al., 2007). Despite this fact, improvement of adjunct cultures is often focused on obtaining strains with increased autolytic capacity. Autolysis of adjunct cultures leads to the release of intracellular enzymes, which can improve the formation of specific aroma compounds. Previous attempts to modify adjunct cultures of *Lactobacillus helveticus* I or *Lactobacillus casei* T included a variety of sublethal physical treatments such as freeze shock, heat shock or spray drying (Madkor et al., 2000).

Lb. helveticus strains are widely used as adjuncts for accelerated ripening in different types of cheeses (Cheddar, Swiss-type). This application of *Lb. helveticus* is based on the fact that this lactic acid bacterium has a potent collection of enzymes including cell-envelope bound proteinases (CEPs) and intracellular peptidases (Griffiths and Tellez, 2013), which upon cell lysis can be released into the cheese matrix and impact proteolysis, as previously shown in Cheddar cheese (Hannon et al., 2003; 2007).

Recently, Smith and colleagues (2012) isolated heat-resistant variants of *Lactococcus lactis* MG1363 with increased autolytic capacity. The observed autolysis was found to correlate with salt hypersensitivity. Genome analysis of these variants of *L. lactis* MG1363 revealed mutations in gene *llmg_1816* encoding a membrane-bound stress signaling protein of the GdpP family.

In our study we used high-temperature incubation of *Lb. helveticus* DSM 20075 to select naturally occurring heat-tolerant and salt sensitive variants with higher autolytic capacity compared to the wild type (WT) in cheese-like conditions. We demonstrated that application of the variants in a cheese ripening model leads to a significant increase of one specific aroma compound (benzaldehyde). Finally, the complete genomes of the WT and of one the heat-tolerant variants were compared to find explanations for the industrially relevant phenotype.

MATERIALS AND METHODS

Strains, growth conditions and growth rate determination

Lactobacillus helveticus DSM 20075 (WT) was incubated at temperatures ranging from 37 °C to 50 °C. Incubation of the cultures was performed in test tubes placed in a PCR machine (Veriti® Thermal Cycler, Applied Biosystems, Bleiswijk, The Netherlands), which allowed six different temperatures to be set up in the same run – an increasing temperature gradient every two lanes. The cultures were incubated in MRS broth (0,5% (w/v) lactose) and at different time points during the incubation (2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24 and 36 h) samples were taken, diluted in phosphate buffered saline (PBS) and plated on MRS agar (1.5%) supplemented with 0,5% (w/v) lactose.

After a certain period of exposure to elevated incubation temperatures, non-diluted cultures were plated (with the exception of variant 45 (V45) which was isolated from 100 times diluted culture) and colonies of survivors with increased heat resistance were collected (for details see Table A.2, Supplementary material). The collected isolates were subsequently grown in MRS broth (0,5% lactose) and preserved in glycerol (20% (v/v)) at -80 °C for further analysis. Specific growth rates of six isolates (designated as variants V45, V46, V47, V48, V49 and V50) were determined using modified Gompertz model (Fig. A.1 in Supplementary material; Zwietering et al., 1990). Strain V50, showed the highest growth rate at elevated temperature of 45 °C and was chosen for further analysis of autolytic capacity and the milli-cheese trial (see below).

The caseinolytic *Lactococcus lactis* subsp. *cremoris* TIFN1 (Erkus et al., 2013) was used as a starter during milli-cheese preparation (see below). *L. lactis* TIFN1 was plated on LM17 (Oxoid, Landsmeer, The Netherlands) agar supplemented with 0,5% (w/v) lactose and incubated at 30 °C for 24h to further pick a single colony for inoculation of LM17 broth (0,5% (w/v) lactose). The culture was incubated for 24h at 30 °C before use in milli-cheese preparation.

Lactate dehydrogenase activity measurement

The lytic capacity of *Lb. helveticus* strains was determined by measuring the activity of lactate dehydrogenase (LDH) released from cells suspended in a lactate buffer (100 mM sodium lactate, 10 mM KCl, 1 mM $MgSO_4 \times 7H_2O$, 5 mM KH_2PO_4, pH 5) in the absence (control conditions) and presence of 0.5 M

NaCl. LDH activity in the supernatant was determined using the LDH cytotoxicity test (Cytotoxicity Detection Kit, Roche, USA) executed according to the manufacturers' protocol. The low pH and high NaCl concentration were chosen to mimic the cheese environment (Weimer, 2007). Cells grown at 37 °C in MRS broth (0.5% lactose w/v) were harvested, collected by centrifugation at 6,000 × g for 10 min, washed twice and finally re-suspended in the lactate buffer. Optical density (determined at 600 nm - OD_{600}, path length 1 cm) of cell suspensions was adjusted to 0.35 ± 0.05 resulting in approximately 10^8 cfu/mL. Samples of 1mL were collected during the salt stress intervention at the following time points: 0, 4, 6, 9, 24, 48, 72, 96 and 120 h. After centrifugation at 13,000 × g at 4 °C the supernatant was used directly in LDH activity test. The measurement of each sample was performed in triplicate using absorbance microplate reader (SpectraMax® Plus384, Molecular Devices, CA, USA). Cell lysis was expressed in percentage as relative cell lysis. The maximum cell disruption percentage was determined using the homogenizer (FastPrep®-24, MP BIOMEDICALS, CA, USA) and 2mL tubes with 0.1mm silica spheres (Lysis Matrix B, MP BIOMEDICALS, CA, USA) at varied cycles of cell disruption. Generally, the amount of active LDH released from cells of V50 and the WT into lactate buffer (pH 5) was growing with increasing cycle numbers until a plateau level was reached followed by a drop in LDH activity with further increase in the disruption cycle count. Results of cycle three were used to ensure the maximal release of LDH from both V50 and the WT strain. Viable plate count data confirmed the cell disruption efficiency at cycle three to be 98% (see Fig. A.2, Supplementary material).

Milli-cheese model system

The MicroCheese model system for starter bacteria screening developed by Bachmann et al. (2009) was adapted to a 24-deep well plate format and will be further referred to as the milli-cheese model system. 40 mL of full-fat pasteurized milk (Friesland Campina, The Netherlands) was used to manufacture each of the control milli-cheeses (without the adjunct) and the milli-cheeses with adjunct. The sterile 24 deep-well plate was covered with an adhesive seal (Microseal®, BIO-RAD, CA, USA) and placed in an incubator at 37 °C for approximately 30 min (pre-heating). Subsequently, 10 µL of rennet, 16 µL of 33% (w/v) of $CaCl_2$, 400 µL (8.7 log cfu/mL) of the starter (TINF1 strain) and 400 µL of the adjunct (8.6 log cfu/mL) were added to 40 mL of milk and mixed thoroughly. Furthermore, the 24-deep well plate was filled with 5 mL per well of the milk-rennet-$CaCl_2$-starter/adjunct mixture. The plate

was sealed with the adhesive seal and incubated in a thermomixer (Eppendorf, USA) at 32.5 °C. After 40 min, the coagulated milk (curd) was cut with a custom-made sterile stirring device. This stirring device is made from stainless steel, and consists of a plate with a handle at the top and 24 pins attached to the bottom. The pins are aligned according to the shape of the 24-deep well plate. Manual stirring was carried out with horizontal and vertical movements for 20 min at defined intervals (20 s stirring and 3 min rest). Next, the curd was kept untouched for 5 min and the plate was sealed again for a centrifugation step (Centrifuge 5804 R, Eppendorf, USA) at 500 × g for 30 min at 30 °C. Subsequently, 1.9 mL of whey was removed and replaced with 1.8 mL of sterile demi water (pre-heated at 45 °C). After addition of water, the plate was placed in thermomixer at 35.5 °C, followed by manual cutting and stirring, as described above, for 40 min. Then, the plate was rested at the same temperature for 20 min without stirring. To remove the remaining whey and liquid, the plate was centrifuged at 2250 × g for 2 hours at 30 °C. The supernatant was discarded well-by-well using a pipet. Finally, the plate was sealed with a gas-permeable seal (BREATHseal™, Greiner Bio One, Frickenhausen, Germany) and incubated overnight at 30 °C. After overnight incubation, 50 µL of a sterile 19 % (w/v) sodium chloride solution was added to each well, followed by a centrifugation step at 75 × g for 5 min. Each batch was sealed with gas-permeable seal, placed in a jar under anaerobic conditions and incubated at 12 °C. Finally, the milli-cheeses were ripened for 2 and 6 weeks.

Volatile compounds analysis

Samples of milli-cheeses at different ripening time points (2 and 6 weeks) were collected and transferred to GC-MS vials. Vials were sealed and kept at -20 °C until the GC-MS analysis was performed. Volatile organic compounds (VOCs) were extracted and detected using Head Space Solid-Phase Micro-Extraction Gas Chromatography-Mass Spectrometry (HS-SPME GC-MS) with fiber (divinylbenzene/carboxen/polydimethylsiloxane, DVB/CAR/PDMS, Supelco Inc. USA; Gamero et al., 2013). The procedure entails pre-incubation for 2 minutes at 60 °C without agitation. Then the fiber was exposed to the sample headspace for 5 minutes at 60 °C. Volatiles were injected into the GC column by desorption of the fiber for 10 minutes. For GC-MS analysis we used the Finnigan Trace GC Ultra (Thermo Fisher Scientific, USA) with a Stabilwax®-DA Crossband® acid deactivated Carbowax® polyethylene-glycol column (30 m length, 0.25 mm internal diameter, 0.5 µm internal

thickness; Restek, Bellefonte, PA, USA). As an injection device we used a TriPlusTM autosampler (Thermo Fischer Scientific, USA) in PTV Split-less mode for 5 minutes at 250 °C. Helium was used as mobile phase flowing at a constant rate of 10 mL/min. First, the temperature in the GC oven was set at 40 °C for 2 minutes, then it was gradually raised to 250 °C (10 °C/min) and then kept for 5 minutes at the final temperature of 250 °C. We collected the mass spectral data over a range of m/z 33-250 in full scan mode with 0.5 seconds of scan time.

HS-SPME GC-MS data analysis

Chromatograms were analyzed first manually in AMDIS tool integrated in NIST MS Search Program (http://chemdata.nist.gov/mass spc/ms search/;10.03.2016) to determine a retention times list of VOCs found in milli-cheeses. Then the mass spectral data were processed in MetAlign (Lommen, 2009; Lommen and Kools, 2012) using parameters optimized for the Finnigan Trace GC Ultra. The resulting dataset was subjected to VOCs identification in MetAlignID (Lommen et al., 2012) using the prepared retention times list and an in-house compounds library. Final dataset contained total ion counts (TICs) of 23 compounds found in 2-week ripened milli-cheese samples and 27 compounds found in 6-week ripened milli-cheese samples. TIC of each compound in each sample was presented as percentage of the total TICs found (Fig. 1).

Sequencing and genome data analysis

Wild-type strain of *Lb. helveticus* DSM 20075 and the heat resistant variant V50 were both sequenced using Illumina HiSeq 2500 technology. The Illumina reads were assembled using Ray assembler, using standard settings (Boisvert et al., 2010). The assembled sequences were annotated using RAST (Aziz et al., 2008). Then, the reads of the WT and The V50 strains were mapped onto the annotated contigs of the WT using breseq (Deatherage and Barrick, 2014). This resulted in two SNP/INDEL analyses: i) WT reads against WT annotated contigs and ii) V50 reads against WT annotated contigs.

RESULTS

Selection of heat-resistant variants

Smith and co-workers (2012) obtained stable heat-resistant and salt hypersensitive variants of *L. lactis* MG1363 after a high-temperature incubation step. We adapted this approach in an attempt to isolate *Lb. helveticus* DSM 20075 variants with increased heat resistance. *Lb. helveticus* DSM 20075 cultures were incubated at different high (non-optimal) temperatures ranging between 38 °C and 50 °C with 1 °C intervals for up to 36 h. Afterwards, the incubated cultures were plated to isolate survivors. In total six single colony isolates designated as V45, V46, V47, V48, V49 and V50 were obtained from cultures exposed to 45, 46, 47, 48, 49 and 50 °C, respectively. The probability of occurrence of such heat resistant variants at a given temperature was estimated based on the initial cell number, plate counts after the incubation and dilution used (for details see Table A.2, Supplementary material). We calculated that the frequency of occurrence of surviving variants ranges between 2.3×10^{-6} and 7×10^{-7} at temperatures between 46 °C to 50 °C and approximates 1.1×10^{-4} at 45 °C.

Growth characteristics of the wild type and the heat-resistant variants

The specific growth rates of WT and heat-resistant variants were determined at optimal growth temperature for *Lb. helveticus* DSM 20075 (37 °C) as well as at elevated temperatures ranging from 38 °C to 45 °C with 1 °C intervals (Fig. A.1, Supplementary material). As expected, the WT strain showed the highest growth rate at the optimal 37 °C (μ_{max} = 1.22 1/h). Compared with the WT, four variants, namely V45 (μ = 0.53 1/h), V46 (μ = 0.55 1/h), V47 (μ = 0.45 1/h) and V48 (μ = 0.59 1/h), showed significantly reduced growth rates at 37 °C. On the other hand, variants V49 (μ = 1.23 1/h) and V50 (μ = 1.25 1/h) had comparable (p=0.88 and p=0.54, respectively) growth rates to the WT at 37 °C.

As expected, the growth rate of the WT culture decreased with increasing temperature. This was not the case for the heat-resistant variants, which showed higher growth rates at elevated temperatures, especially above 41 °C. Variant V50 showed the highest growth rate at 45 °C among the examined strains (Fig. A.1, Supplementary material). These results show that, compared to the WT, heat resistant variants grow better at elevated temperatures while variants V45, V46, V47 and V48, but not V49 and V50, show lower growth

rates at 37 °C. Based on these results we selected variant V50 for further characterization.

Lytic behavior of the heat-resistant variant

To test the hypothesis that heat resistant variants of *Lb. helveticus* DSM 20075 also show more cell lysis in cheese-like conditions we determined the relative lysis of the selected variant V50 at pH 5 and in the presence of 0.5 M NaCl (Fig. 1). Already after 2 hrs of incubation in lactate buffer with salt, the relative cell lysis of strain V50 was 4.5-fold higher compared to the WT. Cell lysis of the suspensions of WT and V50 progressed during incubation but was always at least 4-fold higher for strain V50 (Fig. 1). In control suspensions without salt addition, we also observed higher lysis of V50 (up to 2.9-fold at 120 h) compared to the WT, although the values were lower both for the WT (between 1.7% at 2 hrs and 15.5% at 120 h) and V50 (between 4.5% at 2 h and 40.8% at 120 h) (Fig. 1). These results illustrate that, under conditions mimicking the cheese matrix, strain V50 has a higher capacity to lyse compared to the WT.

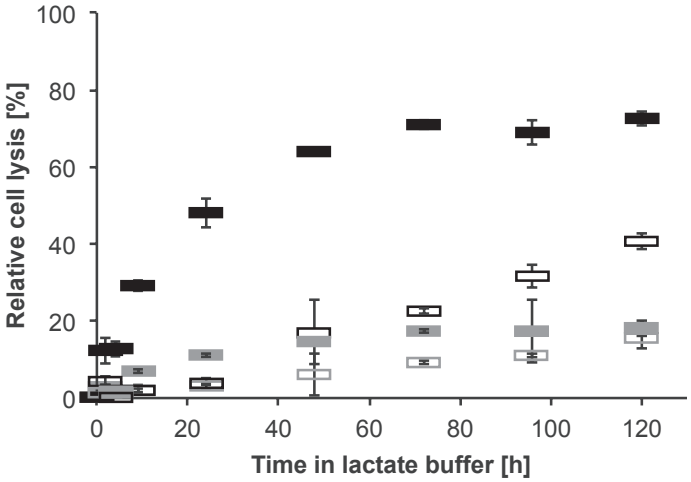


Figure 1. Relative cell lysis in lactate buffer (pH 5). *Lactobacillus helveticus* DSM 20075 (wild type; grey boxes) and *Lactobacillus helveticus* DSM 20075 variant strain V50 (black boxes) with (filled boxes) or without (open boxes) 0.5 M NaCl addition. Error bars indicate the standard deviations of three independent replicates.

Specific mutations found in V50

Using comparative genome analysis, we identified seven mutations present in V50 but absent in WT (Table A.1, Supplementary material). Four SNPs were present in the annotated genes: glycerol kinase (EC 2.7.1.30) – resulting in G151D substitution; site-specific recombinase (DNA invertase Pin related protein) – mutation in stop codon (TAA -> GAA); a silent mutation in a putative pheromone cAM373 precursor lipoprotein CamS; and in a gene encoding multi-domain ZnuB, an ABC-type Mn^{2+}/Zn^{2+} transport system – resulting in I102L substitution. The other two SNPs were identified in an intergenic region in a close vicinity (40 bp distance) to each other. We studied the position of these two intergenic region mutations on the genome to determine if they possibly can impact neighboring genes. In fact, these mutations are located in the upstream region of a gene encoding for glycerophosphoryl diester phosphodiesterase (E.C. 3.1.4.46), an enzyme involved in converting glycerol 1-phosphate into glycerylphosphocholine. Prior to that glycerol is phosphorylated to glycerol 1-phosphate by the glycerol kinase. Interestingly, as described above, the gene encoding a glycerol kinase in strain V50 also carries a mutation. To predict any possible promoter elements and regulons we applied the PePPER online tool (de Jong et al., 2012) using as input the sequence of a scaffold in which the two mutations were identified. However, this analysis did not predict any promoter elements or regulons in the query sequence suggesting no impact of these two mutations on the phenotype.
The last identified mutation was a 664bp deletion in an intergenic region, although this deletion was not confirmed by PCR suggesting a sequencing artifact (data not shown).

Impact of Lb. helveticus adjuncts on volatile compounds formation in milli-cheese

To compare the aroma forming capacity of the heat resistant variant *Lb. helveticus* V50 with that of WT *Lb. helveticus* DSM 20075, we produced milli-cheeses with the starter culture (*L. lactis* TIFN1) and the *Lb. helveticus* strains as adjuncts and analyzed aroma formation after 2 and 6 weeks of ripening. In addition, control cheeses were made without an adjunct using only the single strain caseinolytic starter culture (*L. lactis* TIFN1). We found 23 VOCs in 2-week and 27 VOCs in 6-week ripened (Fig. 3) milli-cheeses confirming the impact of the maturation time on the development of a more complex aroma

profile. The same volatiles were detected in control samples and samples of cheeses made with adjunct cultures, indicating that addition of *Lb. helveticus* DSM 20075 as adjunct does not lead to the formation of additional compounds. The most dominant compound found in both 2- and 6-week ripened cheeses was 3-hydroxy-2-butanone (acetoin) (Fig. 2). Similarly as described in a previous study by Van Leuven et al. (2008), we detected a range of free fatty acids including butanoic acid, hexanoic acid, octanoic acid and n-decanoic acid in all cheeses. In addition, fatty acid methyl esters such as 2-methyl-2-propenoic acid-methyl ester, butanoic acid-methyl ester, 2-propenoic acid-methyl ester, hexanoic acid-methyl ester, acetic acid-2-ethylhexyl ester, octanoic acid-methyl ester and decanoic acid-methyl ester, were also detected. In both milli-cheeses samples with WT and V50 2-methyl-2-propenoic acid-methyl ester was identified to be present at higher levels compared to the other fatty acid methyl esters. Moreover, a variety of alcohols was detected in our milli-cheeses, including: iso-octanol, 2-ethyl-1-hexanol, (S)-3-ethyl-4-methylheptanol, 3-methyl-1-heptanol, 1-octanol, 6-methyl-1-heptanol and trans-4-(1, 1-dimethylethyl)-cyclohexanol.

However, the most distinguishing difference in aroma formation in the cheeses with only starter and the ones supplemented with adjunct strains was found to be the abundance of benzaldehyde (Fig. 3). The relative abundance of benzaldehyde in 2-week ripened samples with WT as adjunct increased in abundance 12-fold (Fig. 3), when compared with the control without adjunct addition. A slightly more pronounced increase in benzaldehyde relative abundance (15-fold) was found in the samples of 2-week ripened milli-cheese prepared with V50 as adjunct (Fig. 3) compared with the control. Although, the difference in benzaldehyde abundance between 2-week milli-cheese samples with WT and V50 was not significant (p=0.45), we found significant differences (p=0.02) in benzaldehyde levels in samples of 6-week ripened cheeses. The abundance of benzaldehyde in 6-week ripened milli-cheeses made with either WT or V50 compared to the control was 16-, and 45-fold higher, respectively (Fig. 3).

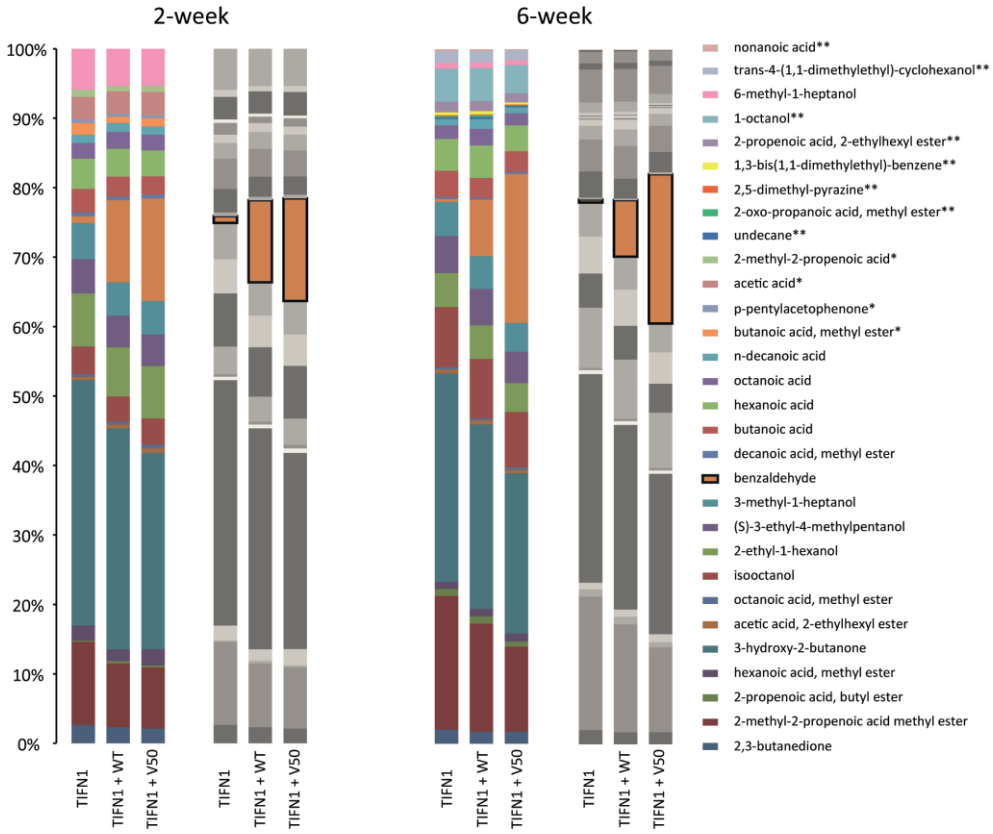


Figure 2. Relative abundance of VOCs identified in milli-cheese samples at 2 and 6 weeks of ripening. The percentage was calculated using total ion counts (TICs) obtained from 6 replicates (in case of samples without the adjunct, TIFN1), 3 replicates (in case of samples with WT, TIFN1 + WT) and 9 replicates (in case of samples with V50, TIFN1 + V50). Legend: * - compounds found exclusively in 2-week ripened samples; ** - compounds found exclusively in 6-week ripened samples.

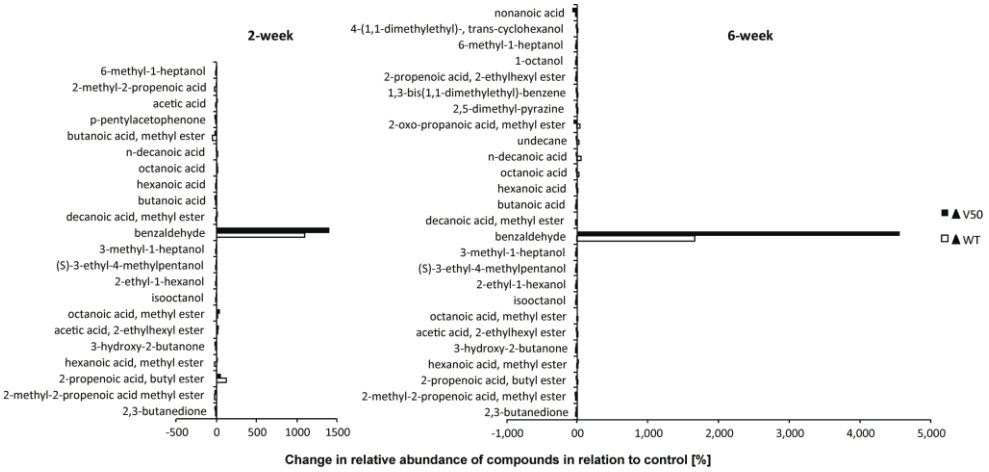


Figure 3. Change in abundance of VOCs relative to the control (milli-cheese without adjunct addition). The percentage was calculated using total ion counts (TICs) of each compound identified in GC-MS run in milli-cheese model samples at 2- and 6-week ripening time points. Black bars – samples where V50 was used as an adjunct; white bars – samples where WT was used as an adjunct.

DISCUSSION

This study describes the successful isolation of variants of *Lactobacillus helveticus* DSM 20075 with increased autolytic capacity triggered by conditions resembling the conditions in cheese ripening (low pH and high salt concentration). As described in other studies, autolysis of lactic acid bacteria (LAB) can be induced by various environmental triggers such as nutrient depletion, high NaCl concentration and heat shock (Ardö and Petterssen, 1988; Lortal and Chapot-Chartier, 2005; Riepe et al., 1997). Dako and co-workers (1995) considered NaCl to be one of the key factors triggering cell autolysis and Wilkinson and colleagues (1994) noticed that the increase of salt in cheese was accompanied by an increase of LDH activity in the cheese matrix. The autolysis of different strains of *Lb. helveticus* was documented before by Valence et al. (2000) and they concluded that a decrease in viability in cheese is not a reliable indicator of lysis due to the possible fraction of dead but not lysed cells or non-culturable cells which could not form colonies. Measurement of the activity of released intracellular enzymes e.g. LDH is regarded as a good indicator of bacterial lysis (Hannon et al., 2003). Our results line-up with the observation of Smith et al. (2012) who used incubation at elevated temperatures to isolate variants of *Lactococcus lactis*

MG1363 that showed increased cell lysis in selected conditions. Smith and colleagues suggested that the heat-resistant and salt-hypersensitive phenotype obtained by high-temperature incubation is not a strain-specific phenomenon, which indeed was confirmed in the case of our *Lb. helveticus* DSM 20075 strain.

In *L. lactis* MG1363, high-temperature incubation resulted in spontaneous mutations in *llmg_1816* gene, which encodes for a membrane-bound stress signaling protein of the GdpP family. In our study using *Lb. helveticus* DSM20075, comparative genome sequencing identified seven mutations in variant strain V50. We calculated the frequency of occurrence of the survivor for the variant V50, which was approximately 2.3×10^{-6}. In practice it means that at least two cells per million in the culture of wild-type *Lb. helveticus* DSM20075 were able to survive prolonged incubation at 50 °C. In different studies, Curragh and Collins (1992) described a high frequency of spontaneous nitrofurazone-resistant cells in lactobacilli (10^{-5} to 10^{-4}), which suggests relatively high potential heterogeneity in the population of *Lb. helveticus* strains. The possible impact of the identified mutations on the phenotype of V50 (heat-resistance and salt induced lysis) will be discussed below.

One of the detected SNPs was located in the gene encoding for a glycerol kinase, an enzyme, which plays a role in degradation of sugar alcohols in *Corynebacterium glutamicum* (BRENDA pathway), glycerol degradation (MetaCyc) in enterococci (Charrier et al., 1997) and in glycerolipid metabolism (KEGG). According to the BRENDA database a mutation found in glycerol kinase was not previously investigated and the effect on the functionality cannot be determined without use of genetic tools for *Lb. helveticus*, which was out of the scope of present work.

We found another SNP in the homologue of *znuB* in *E. coli* (Patzer and Hantke, 1998), encoding the membrane-embedded protein of an ABC-type Mn^{2+}/Zn^{2+} transport system. It is well documented that metal ions are essential for the growth of LAB, probably because of their role as catalytic centers of many enzymes (Boyaval, 1989). Moreover, Mn^{2+} was found to be accumulated by *Lb. plantarum* cells as a defense mechanism against oxygen toxicity (Archibald and Fridovich, 1981). The putative role of Mn^{2+} in aroma formation is discussed below.

The comparative genome analysis provided leads for further investigation to explain and understand the phenotype of the strain V50. To confirm the impact of the identified mutations on the phenotype further studies (i.e. making gene deletion mutants) are required.

Our study demonstrated the impact of the wild-type *Lb. helveticus* DSM 20075 and variant strain V50 on aroma formation in a lab-scale cheese model system. We confirmed the relevance of milli-scale model system adapted from Bachmann et al. (2009) by finding aroma compounds common for Gouda cheese, such as acetoin (Bintsis and Robinson, 2004; Dirinck and De Winne, 1999; Van Leuven et al., 2008), 2, 3-butandione (diacetyl), a variety of alcohols (products of amino acid metabolism (Smit et al., 2005; Engels et al., 1997) and esters.

We found that the presence of *Lb. helveticus* DSM 20075 leads to significantly higher abundance of benzaldehyde in our model milli-cheeses. Moreover, in 6-week ripened cheeses made with variant V50, the abundance of benzaldehyde was significantly higher than in cheeses made with the WT. Benzaldehyde has a characteristic pleasant almond-like odor and is commonly found in dairy products, including Swiss cheeses. It was also detected in Gouda-type cheeses ripened for six weeks (Van Leuven et al., 2008). Benzaldehyde can be formed from phenylpyruvic acid, which is enzymatically derived from phenylalanine (McSweeney and Sousa, 2000). Alpha-keto acids such as phenylpyruvic acid, can be formed from amino acids by various enzymes, including amino acid oxidases, aminotransferases, and dehydrogenases (Nierop Groot and de Bont, 1998). Gummalla and Broadbent (2001) and Nierop Groot and de Bont (1999) further demonstrated that production of benzaldehyde in LAB is associated with the conversion of phenylalanine to phenylpyruvic acid by aminotransferase and is stimulated by the accumulation of Mn^{2+} in the cells. Biological effects of Mn^{2+} on LAB were comprehensively studied by Raccach (1985), who concluded its association with structure/activation of enzymes, detoxification of the harmful superoxide radicals and stabilization in subcellular entities. Archibald and Fridovich (1981) further showed that manganese accumulation in LAB provided the cells with a defense mechanism against the toxic effects of oxygen. Oxidation of phenylpyruvic acid to benzaldehyde was reported by Pitt (1962), who observed the oxidation of enol tautomer of alpha-keto acid instead of the alpha-keto acid itself, and bivalent metal ions such as Mn^{2+} can accelerate this tautomerization. Based on the studies in *Lactobacillus plantarum*, Nierop Groot and de Bont (1999)

proposed a mechanism for benzaldehyde formation from phenylalanine by a combination of enzymatic and chemical steps, and emphasized the required presence of an active uptake system for Mn^{2+} in this bacterium. In that enzymatic-chemical pathway, phenylpyruvic acid is formed enzymatically from the phenylalanine; and the catalysing metal ions can enhance the presence of the enol tautomer of the alpha-keto acid, which is chemically converted to benzaldehyde in the presence of oxygen. Notably, Klein and co-workers (2001) used non-treated and boiled cell free extracts of *Lactobacillus helveticus* strains LRTL 735 and LBLH2 to find that benzaldehyde was produced in both cases providing more evidence for the conclusions of Nierop Groot and de Bont (1999) and Gummalla and Broadbent (2001) for a non-enzymatic reaction (see above) involved in benzaldehyde production after the transamination of phenylalanine.

As mentioned above, we found a mutation in the gene encoding for the homologue of ZnuB, a component of an ABC-type Mn^{2+}/Zn^{2+} transport system. Such putative manganese transport systems were found before in *L. plantarum* (Nierop Groot et al., 2005). Whether the higher levels of benzaldehyde in cheese with V50 can be explained by the mutation in the gene encoding ZnuB remains to be determined. As reported by Nierop Groot et al. (1999) metal ions, and Mn^{2+} in particular, are essential for the formation of benzaldehyde and we suggest here that enhanced lysis of the adjunct strain leads to locally released Mn^{2+} that stimulates the production of benzaldehyde in the cheese matrix.

With improved autolytic capacity of *Lb. helveticus* strain V50 derived from DSM 20075, two previously identified key factors in benzaldehyde formation, namely aminotransferase activity and intracellular Mn^{2+} ions are expected to be released more rapidly, which conceivably accelerates production of benzaldehyde. Our approach of high-temperature incubation inspired by the work of Smith et al. (2012), led to the isolation of *Lb. helveticus* variants with improved growth rates at elevated temperatures combined with - in case of variant V50 - a higher autolytic capacity at low pH in the presence of NaCl. This approach can be applied in dairy industry for the selection of (adjunct) cultures targeted at accelerated cheese ripening and aroma formation.

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SUPPLEMENTARY MATERIAL

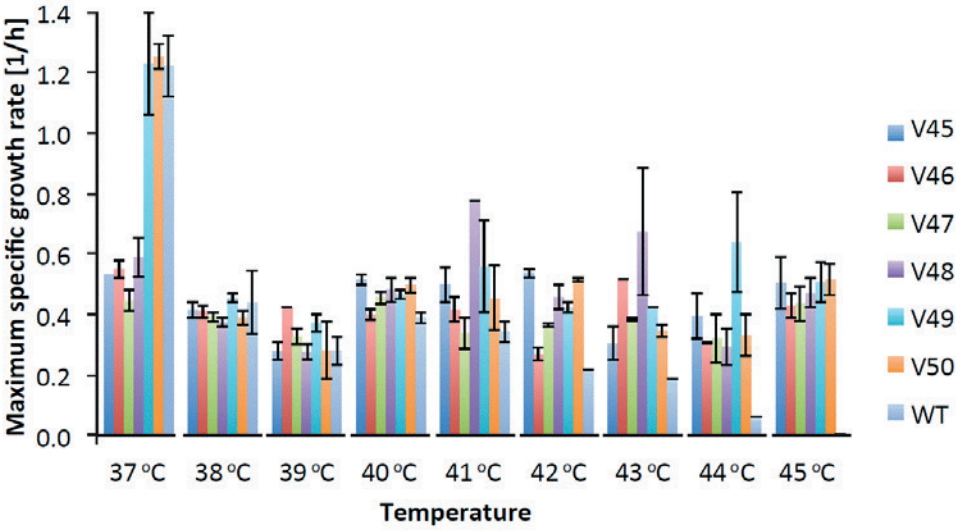

Figure A.1 Maximum specific growth rates (μ_{max}) of wild-type *Lactobacillus helveticus* DSM 20075 and obtained variants (V45-V50) at different elevated suboptimal temperatures in MRS broth (0.5% lactose). Error bars indicate the standard deviations of the twelve independent replicates.

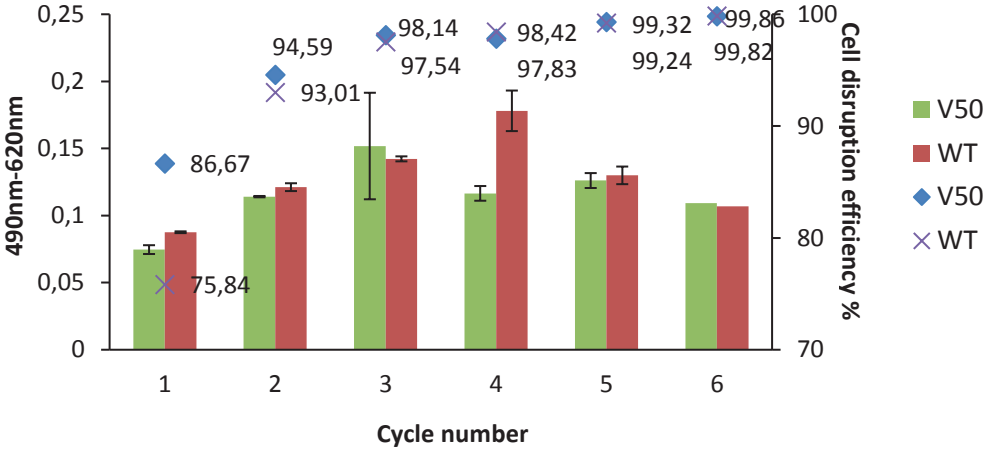

Figure A.2 Release of LDH from cells of WT and V50 measured with the colorimetric method after the cell disruption using homogeniser and silica beads. An increase in dead or membrane-damaged cells results in an increase of LDH enzyme activity in the culture supernatant. The increase in the amount of enzyme activity in the supernatant is directly correlated to the amount of formazan (red) formed during a limited time period. Therefore the amount of color formed in assay is proportional to the number of lysed cells, acquired by the difference between OD_{490nm} and OD_{620nm}. Cell lysis with maximally released active LDH was studied by the colorimetric measurement at varied cycles of cell disruption. Generally, the amount of active LDH released from cells of V50 and the WT into lactate buffer (pH 5) was growing as the cycle number increased until certain point where was followed by a drop in LDH amount. Specifically for the WT strain, amount of released LDH from cell disruption reached a peak at the fourth cycle, while for the V50 strain cells lysed maximally one cycle earlier, presumably due to its more sensitive lytic property, a decrease in LDH amount was also noticed after the summit. Decline in LDH abundance was considered to be the indication of enzyme activity loss caused by mechanical force of the homogenizer, which may have led to denaturation of the enzymes. Since maximally released LDH was applied later as high control for cell autolysis measurement, results of cycle three were used to ensure the maximal release of LDH from both V50 and the WT strain. Data of plate counting illustrated the cell disruption efficiency at cycle three to be 98%.

Table A.1 Summary of SNPs identification in the variant V50. SNPs were found by mapping the reads onto the annotated contigs of the wild-type *Lb. helveticus* DSM 20075.

seq id	position	mutation	annotation	gene	description
scaffold103_ size5580	2466	G→A	G151D (GGT→GAT)	→	Glycerol kinase (EC 2.7.1.30)
scaffold135_ size2853	2547	G→A	intergenic (–/–)	– / –	–/–
scaffold135_ size2853	2587	C→T	intergenic (–/–)	– / –	–/–
scaffold157_ size2826	1755	A→C	*43E (TAA→GAA)	←	Site-specific recombinase, DNA invertase Pin related protein
scaffold178_ size664	1	Δ664 bp	intergenic (–/–)	– / –	–/–
scaffold17_ size31141	18400	A→G	T305T (ACT→ACC)	←	Putative pheromone cAM373 precursor lipoprotein CamS
scaffold85_ size7630	2709	T→G	I102L (ATT→CTT)	←	Zinc ABC transporter, inner membrane permease protein ZnuB

Table A.2 Summary of the elevated incubation temperature experiment for six variants of the *Lactobacillus helveticus* DSM 20075 strain.

Variant code	Incubation time [h]	Dilution used for plating	CFU/mL	Initial cell number (N)	Survivor occurrence frequency ((CFU/mL)/ N)
V45	12	-2	6000	5.7E+7	1.1E-4
V46	14	0	20	5.7E+7	3.5E-7
V47	18	0	40	5.7E+7	7E-7
V48	18	0	360	5.7E+7	6.3E-6
V49	14	0	30	5.7E+7	5.3E-7
V50	10	0	130	5.7E+7	2.3E-6

Chapter 9

General discussion

INTRODUCTION

Spontaneously occurring fermentations are driven by microbes naturally present in raw milk (Wouters et al., 2002). From the initial diverse species/strains composition (Vithanage et al., 2016; Vacheyrou et al., 2011) in raw milk, LAB are often the ones that end up dominating this food niche (Schoustra et al., 2013). Rapid consumption of fermentable sugars and simultaneous acidification leads to quick formation of organic acids, mainly lactic, but also acetic and formic acid as well as CO_2 and ethanol. Based on the studies with *Lactobacillus plantarum* it was suggested that fast consumption of nutrients, related to high growth rates and fast acidification of medium, is an alternative strategy for fitness enhancement in LAB (Teusink et al., 2009). The fittest LAB strains end up in the final fermented food product contributing to its textural, sensorial and nutritional characteristics (Bassi et al., 2015). The notion to preserve these attractive characteristics led to continuous controlled repetition of fermentation processes and resulted in established fermentation practices (Douglas and Klaenhammer, 2010).

The modern industrialized dairy fermentation processes rely on microbial communities of starter cultures, which have their origin in traditional spontaneous milk fermentations and were preserved and continuously used to initialize new rounds of fermentation in man-driven back-slopping manner (Leroy and De Vuyst, 2004). This long-term intentional selection for flavor, texture, quality and safety is the key to understand what impact the transition from traditional spontaneous fermentations to modern industrialized cheese production had on the microbial communities responsible for these processes.

The inoculation of milk with a small portion of previously performed successful fermentation (back-slopping) had important consequences for shaping the composition of the microbial community of the starter culture. In fact, long-term back-slopping represents execution of an accelerated adaptive evolution of microbial community or 'community breeding'. Each cycle of back-slopping can be considered as a pre-conditioning step (Pagaling et al., 2014) in adaptation to milk environment during which selected bacterial strains are gaining fitness. During this men-driven 'community breeding' microbes evolved and further adapted to the nutrient-rich milk environment through gene loss and gain via horizontal gene transfer (HGT), genome decay and beneficial mutations (Cavanagh et al., 2015; Bachmann et al., 2012; Siezen et al., 2011; Douglas and Klaenhammer, 2010; Makarova et al., 2006).

It is fair to assume that all complex undefined starter cultures currently in use by the Dutch dairy industry for production of Gouda cheese originate from artisanal practice of farm level cheese making. Therefore, these starters can be seen as domesticated microbial communities shaped by 'community breeding' processes of evolution and population dynamics. After the isolation of these cultures in the mid-20th century focus was put on limiting compositional shifts by reduction of propagation cycles and preparation of sufficient frozen aliquots (Smid et al., 2014).

In case of our model cheese starter Ur the 'community breeding' resulted in a simple composition of only two LAB species: *Lactococcus lactis* and *Leuconostoc mesenteroides*. However, milk environment adaptation and HGT processes led to a gene-content diversification of Ur strains. This intraspecies diversity was suspected due to reported large size difference between the core-genome and the pan-genome of Ur strains (Erkus et al., 2013). Eventually, diversity in Ur population was detected at multiple levels: i) at the sub-species level – Ur contains functional variants of *L. lactis* including *L. lactis* ssp. *lactis* biovar *diacetylactis* and *L. lactis* ssp. *cremoris*; ii) at the genetic lineage level that differ in gene content; iii) at the level of plasmid content, which appears to be partially independent of the differentiation in genetic lineages; and (iv) at the level of phage resistance within and between genetic lineages (Spus et al., 2015; Smid et al., 2014; Erkus et al., 2013).

This demonstrated multifactorial diversity of Ur was suggested to sustain this microbial community stability (Erkus et al., 2013). This thesis focuses on the aspect of bacteriophage predation to challenge its role in the microbial community of complex starter cultures and potentially use the gathered knowledge in designing new robust and versatile starters. Further, an approach was utilized which resembles the traditional back-slopping procedure not only to investigate the impact of phage predation presence on population dynamics of defined multi-strain cultures (*Chapter 2* and *Chapter 3*) but also to study evolution in the context of ecology of microbial communities upon prolonged propagation in rich medium (*Chapter 4*). Additionally, temperature of incubation was a factor studied in the context of adaptation to sub-optimal conditions of an adjunct strain of *Lb. helveticus* DSM20075 and the role of this adaptation in the formation of aroma compounds in a model cheese system (*Chapter* 5).

Eco-evolutionary processes and their consequences for starter community

The LAB population present in the fermentable raw material (i.e. milk) strongly interacts with its environment. The above-mentioned evolution towards effective acidification as well as the ability of LAB strains to degrade caseins in milk (via cell-wall-bound proteinase PrtP; Steele at al., 2013) and active transport of di- (Smid et al., 1989) and oligo-peptides (Tynkkynen et al., 1993) confirms the capacity of the population to evolve in response to changes in its environment. Our Long-term experimental evolution experiment with Undefined Mixed Starter Culture (LUMSC) described in *Chapter 4* demonstrates that the original Ur starter evolved towards even higher acidification rates. Moreover, in the response to environmental conditions and presence of other microbes, the process of adaptation can result in microbe-microbe interactions (Andrade-Dominguez et al., 2014). Microbes of complex microbial communities exemplify all known types of microbial interactions: competition, mutualism, commensalism, amensalism and parasitism (Smid and Lacroix, 2013).

In the model starter culture community, strains are competing for available milk nutrients (growth factors) and this can result in differences in relative abundance among them. A serial transfer of bacterial cells in suspension (i.e. back-slopping) will lead to enrichment of the fastest growing and thus acidifying variants (Teuskink et al., 2009). In theory, in a starter culture community, such a procedure would eventually lead to a clonal sweep; an event in which only the fittest (fastest acidifying) strain persists. However, in practice, as confirmed by experimental studies described in *Chapter 4* of this thesis, a culture clonal sweep event did not occur even after 1000-generation-long back-slopping. This observation confirms two phenomena present in the starter community: 1) strong (commensal) microbe-microbe interactions and 2) division of labor among generalist and specialist strains.

It is known that strains of *Le. mesenteroides*, which are present in Ur starter as well, are able to produce diacetyl and acetoin from citrate only at acidic pH (Cogan and Jordan, 1994). In cheese starter cultures lactococci are principally responsible for milk acidification (Mc Sweeney and Sousa, 2000) creating conditions for *Le. mesenteroides* to metabolize citrate. The work performed before by Erkus and co-workers (2013), as well as the study described in *Chapter 4* of this thesis, shows several other examples of interactions between

Ur culture strains, which collectively have impact on the culture's composition and final outcome of the fermentation. The first example, described in *Chapter 2*, is the simultaneous presence of two types of *L. lactis* ssp. *cremoris* strains: caseinolytic (prt^+) and non-caseinolytic (prt^-) (Spus et al., 2015). The latter group benefits from the action of the caseinolytic strains which results in a release of peptides from milk proteins supporting growth of non-caseinolytic 'cheater' strains (Bachmann et al., 2011). This interaction is an example of commensalism, where one group receives benefits from the other without harming it. Interestingly, as previously described by Hugenholtz et al. (1987), prt^- population can invade prt^+ and lead to its extinction, which changes the commensal interaction into a parasitic one, suggesting a dynamic character of microbial interactions in starter culture communities. Another example of interactions found in the Ur starter is connected to stress response-related excretion of γ-amino-butyric acid by *L. lactis* (Cotter and Hill, 2003) and further, as suggested by Erkus and colleagues (2013), active uptake of this compound by *Le. mesenteroides* for the formation of succinate. Further on, In *Chapter 4* we proposed a newly emerged putative interaction in a propagated culture between *Le. mesenteroides* and *L. laudensis* based on the D-mannitol production by the first strain and its consumption by the latter. Likely, all these mentioned interactions played an important role in establishment of Ur starter composition and in particular in the case of *L. laudensis* strain in its enrichment in evolved LUMSC cultures.

Not only microbe-microbe interactions are important in shaping the starter communities but the fermentable substrate composition plays a role as well. Substrates for fermentation processes are heterogeneous in terms of their physicochemical composition. This allows specialist strains to occupy multiple niches by utilization of different substrates. For instance, milk contains between 8 and 10 mM citrate (Fox and Wallace, 1997), which as mentioned above, can be metabolized by *Le. mesenteroides* and *L. lactis* ssp. *lactis* biovar *diacetylactis*. According to the buttom-up model of niche-based mechanisms for biodiversity of prokaryotes (Sandaa et al, 2009) each species persists in the community due to its specialization for a given substrate – '(food)-specialist' model. The ability of some of the starter community strains to quickly convert lactose and acidify milk and other strains to uniquely metabolize nutrients, such as citrate by *Le. mesenteroides* and *L. lactis* ssp. *lactis* biovar *diacetylactis* or D-mannitol by *L. laudensis*, confirms division of labor among starter community strains. Such a division of metabolic labor in

microbial communities was previously confirmed advantageous leading to higher growth rate (Pande et al., 2014) or higher biomass yield.

At the same time, the action of individual players in a given community can result in creation of a new niche. Our LUMSC experiment shows a possible niche construction event (Laland et al., 1999), where propagated strains modified the resource distribution allowing "new" strain(s) to emerge from under the radar levels. Such niche construction events alter ecology of the undefined mixed starter and might even lead to evolutionary changes.

Strains vs genes

Nowadays, high-throughput ~omics technologies like microarrays, next-generation (metagenomic) sequencing, RNA-seq, proteomics and metabolomics are used to characterize phenotypes of individual strains (Bayjanov et al., 2012) as well as compositions and functionality of microbial communities (Wolfe et al., 2014). This molecular biology information has a potential to identify industrially-relevant traits such as active enzymes (Cantarel et al., 2009) and predict development of flavor compounds (van Kranenburg et al., 2002). However, these ~omics technologies will have to be used in concert (Alkema et al., 2016) due to reasons such as environmental conditions-dependent variation in gene expression (Bachmann, 2009b) and the presence of nonfunctional genes (pseudogenes). In addition, microbial interactions are not only important for the composition of the starter communities, as stressed in the previous section, but can also impact a culture's functionality. Thus, to be able to steer fermentations or to be able to develop new functional starter cultures the knowledge of microbe-microbe and microbe-environment interactions is crucial. In result, the concept of strain collections should be replaced by the concept of genotype/transcript ome/phenotype collections. This collective information obtained in high-throughput functionality screening experiments such as MicroCheese (Bachmann et al, 2009) could help develop new prediction strategies (Alkema et al., 2016) for starter culture development and steering.

Aroma formation in dairy products from the perspective of starter microbes

As mentioned in the introduction, starter cultures were selected based on their specific traits impacting the texture, safety, quality and flavor of the final product. Our perception of dairy product flavor is influenced by the formation

of aroma compounds during fermentation. Simultaneously, these aroma compounds are expected to have a biological meaning and consequences for microbes. Alternatively, aroma compounds formation could be a side effect of phenomena in which microbes are not directly involved (non-enzymatic reaction) or involved only at a certain stage (enzymatic-chemical reaction).

In a protein-rich environment such as milk, and assuming the presence of sufficient proteolytic activity, the amount of available amino acids exceeds the requirements of the starter culture strains. Such an excess of amino acids leads to their conversion into an array of aroma precursors or directly to formation of aroma compounds such as: α-ketoacids, aldehydes, carboxylic acids, alcohols or esters (Smid and Kleerebezem, 2014).

In various LAB, the production of aroma compounds can be related to the response to specific environmental conditions such as low pH encountered in acidified milk. In particular, *L. lactis* co-metabolizes pyruvate, glucose and citrate in acidic conditions to produce neutral compounds such as diacetyl, acetoin or 2, 3-butanediol. These compounds are important contributors to flavor of dairy fermented products, but at the same time are used by bacteria to control their internal pH by prevention of pyruvate accumulation during sugar fermentation (Zuljan et al., 2014).

Some of the aroma compounds found in dairy products are formed via non-enzymatic chemical reactions (Smit et al., 2005). For instance, as described by Nierop Groot and De Bont (1998) and demonstrated in *Chapter 5*, *Lb. helveticus* forms phenylpyruvic acid from phenylalanine via activity of its aminotransferase. Next, a Mn^{2+}-dependent chemical oxidation (upon cell lysis) of phenylpyruvic acid results in production of a specific aroma compound – benzaldehyde (Nierop Groot and De Bont, 1999). In this particular case production of benzaldehyde can be considered as an indirect effect of amino acid catabolism (Yvon and Rijnen, 2001).

Moreover, not always functional pathways are present in individual strains but often strains complement each other's pathways. In a study described by Ayad et al. (2001) pairs of different *L. lactis* strains were mixed to prepare defined starters and subsequently investigated for their potential to develop volatile compounds in milk. One of the used strain pairs was able to develop strong chocolate-like flavor (branched-chain aldehydes derived from branched-chain amino acids), which was only moderately present or absent

when individual strains were used. Further investigation of these two strains confirmed a complementary effect in flavor-formation pathway.

Practical aspects of starter culture use in context of bacteriophage predation

Established dairy fermentation processes leave little space to adjust parameters (temperature, pH, redox potential, osmolarity, salinity and nutrient composition), which can affect metabolic response of starter bacteria and change their impact on the characteristics of the final product (Smid and Kleerebezem, 2014). Additionally, due to the lack of acceptance for the use of genetically modified microorganisms in food applications (Sybesma et al., 2006), there are essentially two ways of expanding the possible application of LAB in generation of products with new characteristics. The first is the exploration of natural biodiversity among LAB strains and screening for interesting characteristics. The possibility of screening for natural biodiversity of LAB, was described already in the *Introduction Chapter* of this thesis and will not be covered in this section. The second possibility is to impact the relative abundance of aroma-producing strains at different stages of the fermentation by changing the composition of defined or complex starter cultures.

It is possible to adjust the composition and thus the functionality of the starter via 1) changing the sub-cultivation regime or the temperature of incubation during the sequential propagation (applicable for complex undefined starters) or 2) rational design of robust multi-strain starters. The first possibility is exemplified in an experiment described by Erkus (2014), which shows change in relative abundance of starter lineages in a culture sub-cultivated from the late stationary phase of growth versus the exponential phase of growth. These cultures behaved opposite in terms of the abundance of prt^+ lineages, stationary phase-sub-cultivated culture had been dominated by the prt^+ lineages and exponential phase-sub-cultivated culture had only a small fraction of prt^+ lineages. Another example of the effect of changing the sub-cultivation regime is described in *Chapter 4* where daily-propagated 1000-generation evolved LUMSC cultures were subjected to weekly propagation. This change from daily to weekly regime resulted in 'wash-out' of the new *L. laudensis* strain from the community. As mentioned before temperature of incubation during serial propagation can have an effect on the culture composition. This effect is shown in an experiment where low (20 °C) versus

high (30 °C) incubation temperature during sequential daily propagation (1% inoculum) of Ur starter was used (Figure 1). In this case, *Le. mesenteroides* lineage was 'washed-out' in high temperature-propagated culture before the 66 generations time point. Conversely, in low temperature-propagated culture *Le. mesenteroides* lineage was still present after 160 generations. Incubation at high temperature pushed the *Le. mesenteroides* lineage beyond the physiological boundaries allowing competitive growth. Such a compositional change has obvious consequences for the functionality of the microbial community, for instance, in a cheese made with the culture without *Le. mesenteroides* one would expect less products of citrate metabolism like diacetyl and acetoin and no "eye formation" due to the absence of CO_2 production.

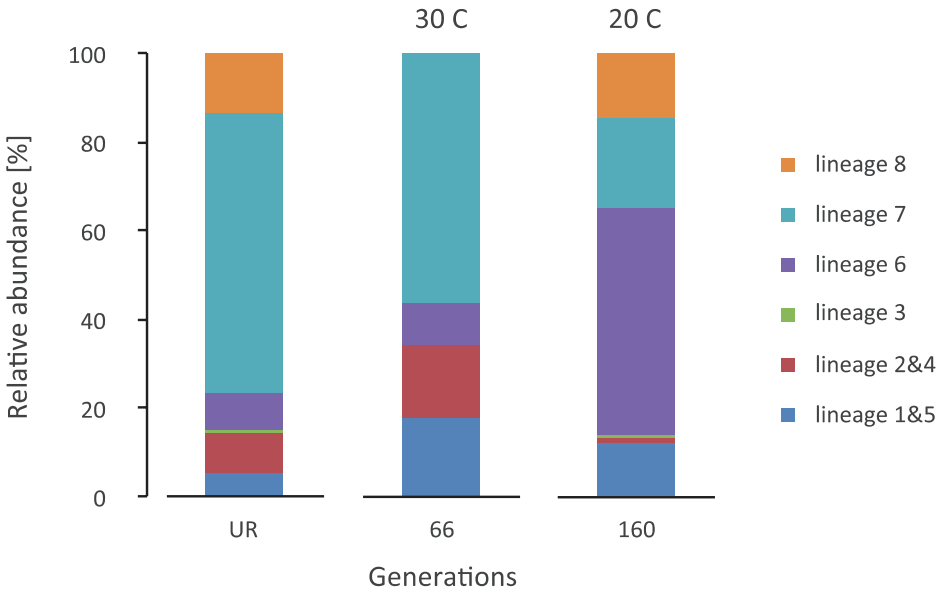

Figure 1. Relative abundance of Ur starter culture genetic lineages incubated at different temperatures during the sequential propagation experiment. UR – original Ur starter composition; 30 C – Ur culture incubated at 30 °C degrees; 20 C – Ur culture incubated at 20 °C degrees.

Another approach would be to design defined robust multi-strain starters with predictable characteristics from the available strain collection. This approach was validated when individual representatives of each genetic lineage of Ur (Ur starter culture consists of eight genetic lineages including seven lineages of *L. lactis* and one of *Le. mesenteroides*) were used to prepare

reconstituted culture and produce lab-scale model cheese (Smid et al., 2014). Aroma profiles of this reconstituted culture cheese and cheese made with the original Ur starter were similar. However, this approach has several challenges addressed below.

When such reconstituted starters contain limited number of functional strains a phage attack can have a detrimental impact on the functionality of the culture. To prevent this, such a reconstituted culture should have one well-known characteristic of undefined mixed starter cultures – their robustness towards bacteriophage predation (Stadthouders and Leenders, 1984; Stadthouders, 1986). This bacteriophage robustness of undefined mixed starters is a result of two particular traits. Firstly, as mentioned before, highly related strains (i. e. belonging to the same genetic lineage) of undefined mixed starters possess diversity at the level of phage resistance. This diversity assures continuous presence of an alternative resistant strain if the phage attack eradicates a sensitive related strain. Secondly, undefined starters are known to naturally contain high titers of bacteriophages (Cogan and Hill, 1993) and our model starter culture Ur is no exception to this rule (see *Chapter 4*). However, this natural phage-carrier state, explained by de Vos (1989) as 'a meta-stable balance between curing of plasmids which carry genes encoding phage resistance mechanisms and multiplication of phages on sensitive strains', does not seem to affect culture's functionality.

The combination of both defined starter culture functionality - controllability – 'you know what you put in, you know what comes out' and undefined mixed starter's inherent robustness to bacteriophage predation would be desired in new starters. To achieve this goal the design of multi-strain starters should include a sufficient intraspecies diversity to cope with the environmental fluctuations and phage attack. Moreover, such approach would have a practical advantage of simply growing blends of strains together and not, as it is done now, separately to mix them at the later stage of production. Our systematic approach in increasing the strain-level diversity in defined multi-strain starter described in *Chapter 2* and *Chapter 3* led to relevant design conclusions for the concept of multi-strain robust cultures. In particular, with the introduction of higher strain-diversity (four strains, three genetic lineages in *Chapter 2* to 24 strains and eight genetic lineages in *Chapter 3*) defined starter culture is becoming more robust towards phage predation in terms of preservation of higher level of genetic lineage diversity for a longer period of sequential propagation in milk. On the other hand, without phage predation,

genetic lineage diversity is quickly lost upon sequential propagation. These results support the deliberate use of phage predation to sustain starter community genetic lineage diversity. However, especially in the multi-strain blends from *Chapter* 3, it was clear that the composition might be affected by a stochastic event. This generates a follow up question: how stable should the composition of the starter be to assure a stable functionality? It is possible that the genetic lineage composition of a given microbial community is only loosely connected to its basic functionalities such as acidification of milk or aroma formation in cheese. As indicated by the study of microbial community dynamics of methanogenic reactor (Fernández et al., 1999), despite the extremely dynamic changes in the microbial community population, the ecosystem function was stable. If the crucial functionality is present in one particular starter strain, then it is essential that this strain is present in the community, but if the functionality is there due to the presence of multiple interacting microbes their particular relative abundance becomes less important. An example illustrating this hypothesis is described in *Chapter 3*. In one of the replicates of the propagated multi-strain starter the principal caseinolytic lineage 1&5 was eradicated from the community by a phage attack. However, this culture did not lose the ability to acidify milk due to the presence of an alternative caseinolytic lineage 3, which filled the 'empty' niche and helped in maintaining this basic functionality. In this particular case the relatively high diversity beyond the level of genetic lineages, so the complexity of the starter community, assured the basic functionality. This result is in agreement with the statement of Smid and Lacroix (2013) that complex microbial communities in comparison to pure cultures are more versatile, since they perform more complex activities, and are more robust - compositionally stable - even under phage predation pressure.

Concluding remarks

This thesis describes results of experimental evolution studies using a complex mixed dairy starter culture to investigate the impact of prolonged sequential propagation on its composition and functionality. The strains of a model complex mixed starter culture (Ur) were characterized in terms of their growth in milk and bacteriophage resistance. Further, the strategy of rational reconstitution of the starter culture with increasing level of strain and genetic lineage diversity was employed to investigate the aspects of compositional and functional stability in the context of bacteriophage predation pressure.

Finally, a specific adjunct strain for accelerated aroma formation was developed and analyzed for functionality in lab-scale cheese model.

By the employment of tools for monitoring population dynamics, it was possible to analyse the genetic lineage level composition of both the original Ur starter and of the reconstituted multi-strain blends of strains originating from Ur (with or without bacteriophage predation pressure) during the prolonged sequential propagation (back-slopping) in milk. The studies of the reconstituted blends of strains confirmed the important role of phage predation in impacting composition of the starter cultures. Reconstituted cultures without phage predation were quickly losing their genetic lineage level diversity upon propagation. However, even with the presence of phage predation pressure the composition of the reconstituted cultures was not stable. On the other hand, in the 1000-generation-long experimental evolution of undefined mixed starter culture experiment, even with phage predation pressure, none of the genetic lineages was lost confirming culture's compositional robustness. This robustness stems from the multi-level intraspecies diversity present in Ur, which is far beyond the one introduced in reconstituted cultures, and from the specific microbe-microbe interactions discovered in the culture.

Surprisingly, enclosed propagation under enforced conditions of temperature and propagation regime of the LUMSC experiment without a possible influx of new strains or bacteriophages led to enrichment of an "under the radar" strain. This new *L. laudensis* strain was not only under the radar of the culture-dependent (differential plating) and culture-independent (previously performed metagenome sequencing) methods for community studies, but also under the radar of the bacteriophage predation impacting the starter culture diversity. Prolonged propagation of the Ur starter confirmed its inherent compositional and functional stability, but at the same time, opened space for eco-evolutionary feedback mechanisms (Post et al., 2009) resulting in emergence of new players in the culture's composition (Figure 2). This result underlines the importance of phenomena such as microbe-microbe interactions and niche construction in complex starter culture composition.

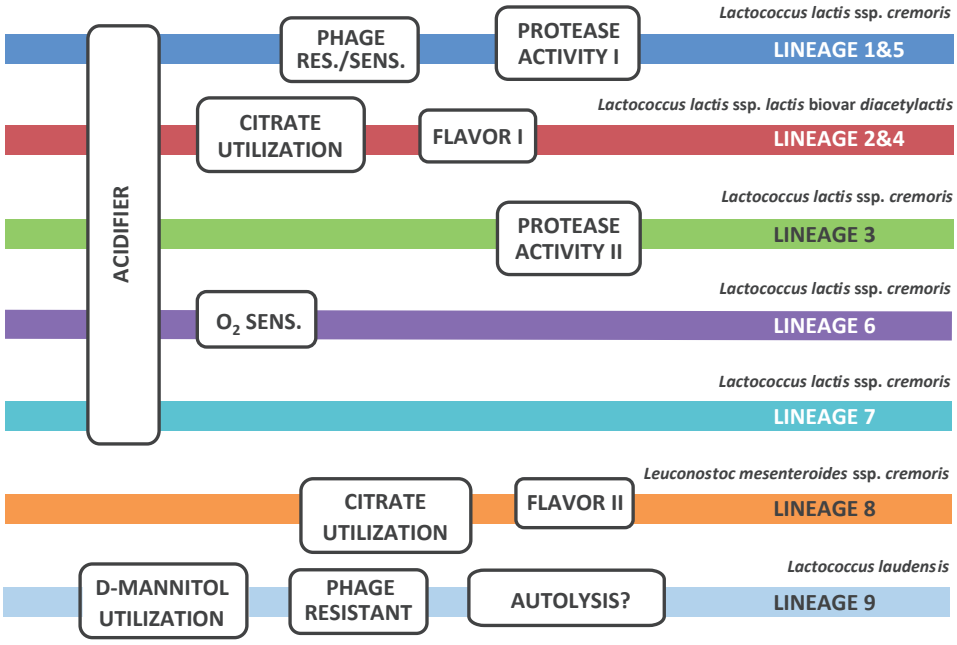

Figure 2. Overview of genetic lineages present in the evolved LUMSC cultures, including the new *Lactococcus laudensis* Lineage 9. Main characteristics impacting cultures overall functionality are given in boxes. O_2 SENS. – oxygen sensitivity; PHAGE RES./SENS. – diversity in terms of bacteriophage sensitivity profiles against phages isolated from Ur culture.

Despite all these results, the main question of dairy producers is 'Will the culture have the functionality we require?' To address such an industrially relevant question directly we employed the strategy of elevated temperature of incubation in an attempt to develop an adjunct strain for acceleration of cheese ripening. We successfully obtained a mutant strain with increased autolytic capacity, which resulted in increase of a specific aroma compound in lab-scale model cheese.

In conclusion, the knowledge of microbial interactions in a starter community, the response of the community to phage predation and changes in environmental conditions, the role of phage predation in stabilizing the composition and functionality is crucial for designing robust multi-strain starters. Further development of gene content-based prediction of culture functionality with inclusion of the knowledge of above-mentioned aspects will support rational design of new starter cultures in the future.

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Summary

Summary

Undefined mixed complex starter cultures are broadly used in Gouda-type cheese production due to their robustness to phage predation, resilience for changes in environmental conditions and aroma compounds production ability during ripening. These microbial communities of lactic acid bacteria prior their isolation and deposition in starter culture collections were continuously used at the farm-level production facilities. Thus, one can consider undefined mixed complex starters as domesticated microbial communities. The process of domestication was facilitated by humans who have been continuously repeating successful fermentations using part of previous batch as inoculum (i.e. back-slopping). Therefore, a term 'community breeding' can describe this human-driven domestication of microbial communities. Community breeding of a model complex starter Ur led to establishment of a simple two-species composition of *Lactococcus lactis* and *Leuconostoc mesenteroides* represented by, in total, 8 genetic lineages. At the same time, this simple microbial community displays a high degree of intraspecies diversity, presumably caused by evolutionary processes of horizontal gene transfer, genome decay and mutations. Such diversity at strain level is particularly interesting in the context of continuous bacteriophage predation pressure present in this microbial community. It is thought that constant-diversity (CD) dynamics, based on the 'kill-the-winner' principles, is operational in Ur starter at the strain level. According to CD model, the fittest strain(s), which feed on the most abundant substrate, will be selected against due to density-dependent phage predation. The control of the fittest strain abundance by bacteriophages opens space for differentiation of strains via eco-evolutionary feedbacks. In particular, strains of complex starter culture not only adapted to quickly acidify milk (via efficient consumption of lactose and protein to peptides degradation), but concurrently, to consume other substrates present in milk. In addition, throughout the process of community breeding microbe-microbe interactions between community members have evolved. These interactions have led to division of metabolic labor among strains present in the culture, and eventually to better starter microbial community functioning.

The aim of this thesis was to investigate the factors impacting the formation of compositionally and functionally stable undefined mixed complex starter cultures to further use this knowledge in steering its functionality, and potentially in developing new strategies for robust starter culture design.

To facilitate this study, well-characterized Ur culture strain isolates were used to systematically reconstitute the starter culture into multi-strain blends with increasing level of strain and genetic lineage diversity. The investigation of factors such as phage predation, level of strain and genetic lineage diversity as well as environmental conditions, was performed during experimental evolution studies in milk. The functionality of the (evolved) starter cultures was tested in an adapted lab-scale MicroCheese model system. The specific approach used in each of the research chapters is described below in more detail.

Strains isolated from Ur starter culture were characterized in terms of their resistance against bacteriophages isolated from the same starter (*Chapter 2*). This test confirmed high diversity in phage resistance among strains belonging to different genetic lineages as well as among strains of the same lineage. Next, selected strains, which represented different levels of bacteriophage predation: resistant, moderately resistant, sensitive and no detectable sensitivity, were mixed in simple blends containing 4 strains representing 3 genetic lineages of Ur starter (3 such blends were designed). These blends were exposed to phage predation (one phage per blend) at the onset of prolonged sequential propagation experiment or propagated without phage addition (control). Throughout the serial propagation the genetic lineage composition was monitored. During the propagation of control blends we detected quick domination of a single lineage. This dominating lineage contained strains sensitive to phages. Genetic lineage level composition of the phage-challenged blends was much more dynamic suggesting the impact of phage predation. The relatively low strain diversity introduced in these blends was not high enough to sustain maximal diversity at the level of lineages.

Chapter 3 describes a study using defined blends with higher complexity by extending the number of strains used. In total, 24 strains representing all 8 Ur starter lineages were exposed in sequential propagation experiment to a cocktail of 3 phages isolated from Ur starter. The propagation in milk of this multi-strain blend was executed for more than 500 generations and the abundance of genetic lineages was monitored throughout. Similarly as in the simple blends experiment, control blends were not exposed to bacteriophages. In control blends we observed a domination of one genetic lineage upon serial propagation, which resembles a periodic-selection-like (PS) behavior, where the fittest strains are dominating the microbial community and in result genetic-lineage diversity is being substantially reduced. In contrast, the

composition of phage-challenged blends was again more dynamic than in control blends. In one of the phage-challenged blends behavior characteristic for a constant-diversity dynamics model was observed; throughout the serial transfer experiment, genetic lineage diversity was maintained by the presence of phage predation at relatively high level. In case of the second phage-challenged blend, due to a stochastic event, which likely caused a reduction in phage pressure, we observed a gradual recovery of the fittest strains, which again resembled a periodic-selection behavior. Therefore, phage predation, among other factors, can lead to shifts in microbial community population dynamics resulting in alternative stable states.

The experimental evolution approach, resembling traditional process of back-slopping, was used in a Long-term experimental evolution of Undefined Mixed Starter Culture (LUMSC) study described in *Chapter 4*. The aim of this study was to investigate the compositional and functional stability ascribed to the undefined mixed Ur starter during enclosed prolonged propagation without any possible external influx of bacterial or phage material. Surprisingly, during this 1000-generation long experiment the enforced conditions of specific incubation temperature and propagation regime resulted in enrichment of previously not detected strain of *Lactococcus laudensis*. This strain was found to consume a by-product of metabolism of another strain present in the community, in particular, D-mannitol produced by *Le. mesenteroides*. Thus, a new putative interaction in the microbial community of the complex starter culture was found. This new interaction and the possible ability of *L. laudensis* to efficiently use peptides released by caseinolytic *L. lactis* ssp. *cremoris* resulted in a relatively high abundance of *L. laudensis* in all evolved LUMSC cultures. The high abundance of *L. laudensis* had a certain effect on the functionality of the cultures. The aroma profiles of model lab-scale milli-cheeses manufactured with LUMSC cultures, showed significant differences in formation of esters and alcohols when compared to cheeses produced with the original Ur starter. Moreover, *L. laudensis* strain was not only under the radar of previously used culture-dependent and culture-independent methods, but as well, under the radar of phage predation continuously present throughout the LUMSC experiment. This observation sheds new light on the possibility of how a strain can emerge to relatively high abundance in an enclosed serially propagated microbial community operating in accordance with CD dynamics model.

Finally, the aspect of adaptation to environmental conditions was addressed by the study of an adjunct strain of *Lactobacillus helveticus* DSM 20075 described in *Chapter 5*. The aim was to develop a strain with increased autolytic capacity in conditions resembling the cheese matrix to possibly improve cheese ripening. The approach used here was based on a previously reported study, where the incubation of *Lactococcus lactis* MG1363 at high temperature resulted in spontaneous mutations causing stable heat-resistant and, in some cases, salt-hypersensitive phenotypes. In present study, after incubation of the *Lb. helveticus* DSM 20075 adjunct at different high temperatures (45-50 °C), heat-sensitive variants were recovered from plates. These variants were further characterized in terms of their growth rates at elevated temperatures (42-45 °C) and their autolytic capacity in low pH buffer with addition of NaCl. One of the variants (V50) showed substantially increased intracellular lactate dehydrogenase enzyme activity in the buffer suggesting its increased autolytic capacity. Next, both wild type and variant V50 were tested as adjuncts in lab-scale model milli-cheeses to determine their possible impact on the cheese aroma profiles. Indeed, adjunct strains, both WT and the variant, impacted the aroma profiles by producing benzaldehyde. In case of the variant strain the relative abundance of this compound was 3-fold higher. The applied strategy of incubating *Lb. helveticus* DSM20075 at high temperature resulted in specific, but different than in case of *L.* lactis MG1363, mutations suggesting another, yet to be elucidated, mechanisms for increasing the autolytic capacity of industrially-relevant strains. The approach of high-temperature incubation can be applied in dairy industry for the selection of (adjunct) cultures targeted at accelerated cheese ripening and aroma formation.

In conclusion, the work presented in this thesis highlights the importance of co-evolution of strains in compositional and functional stability of the complex undefined mixed starter culture. In particular, the factors such as heterogeneity of bacteriophage resistance among highly related strains, microbe-microbe interactions and division of metabolic labor are crucial for optimal functioning of a complex starter microbial community. Further investigation of the factors impacting the composition of starter cultures is crucial to steer the functionality in a desired direction. With straightforward methods, such as changing the incubation temperature or the propagation regime it is possible to induce shifts in strain composition and thereby obtain cultures with new characteristics. Moreover, experimental evolution studies

with microbial communities used in food fermentation can lead to the discovery of new strains with potentially new characteristics. Additionally, the study of microbial communities of starter cultures not only delivers industrially applicable knowledge but also reveals the action of basic principles in microbial ecology and evolution.

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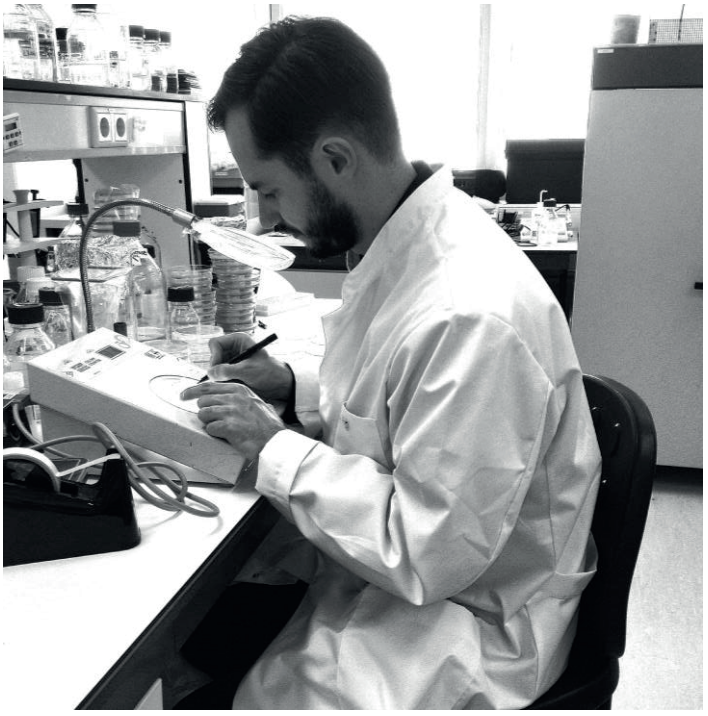
Maciek

About the author

Maciej Spuś was born on 3rd of January 1987 in Łódź, Poland. In 2006 he was accepted to the degree programme of Biotechnology at the Faculty of Biotechnology and Food Sciences at the Technical University of Łódź (now Lodz University of Technology). Last year of his studies Maciej has spent at the Wageningen University in the Netherlands, where under the supervision of Prof. Eddy J Smid he finalized his MSc thesis. His master thesis project dealt with the role of bacteriophages as a driver for diversity meintanance in an undefined complex starter culture. Just after graduating from the Technical University of Łódź in September 2011, Maciej began his PhD studies in the Laboratory of Food Microbiology, again under the supervision of Prof. Smid. The PhD project entitled 'Mixed culture engineering for steering starter functionality' was a part of a Functional Fermentation theme of TI Food and Nutrition led by Prof. Dr Michiel Kleerebezem. The results of this work are described in this thesis. Currently, Maciej is working as a posdoctoral scientist at Aalto University in Espoo, Finland. His postdoctoral project is partially performed at the R&D department of Valio Ltd. – dairy company located in Helsinki, Finland.



List of publications

O Erkus, VCL de Jager, M Spus, IJ van Alen-Boerrigter, IMH van Rijswijck, L Hazelwood, PWM Janssen, SAFT van Hijum, M Kleerebezem, EJ Smid (2013). Multifactorial diversity sustains microbial community stability. *The ISME Journal 7*: 2126–36.

EJ Smid, O Erkus, M Spus, JCM Wolkers-Rooijackers, S Alexeeva, M Kleerebezem (2014). Functional implications of the microbial community structure of undefined mesophilic starter cultures. *Microbial Cell Factories* S2.

M Spus, M Li, S Alexeeva, JCM Wolkers-Rooijackers, MH Zwietering, T Abee, EJ Smid (2015). Strain diversity and phage resistance in complex dairy starter cultures. *Journal of Dairy Science*, *98*: 5173–82.

Overview of training activities

Discipline specific activities

Courses

Advanced Course on Food Fermentation	VLAG, Wageningen	2012
Application of Genomics in Industrial Fermentation	TU DELFT/VLAG, Wageningen	2012
BioIT Workshop	CMBI, Nijmegen	2012
Genetics and Physiology of Food-associated Microorganisms	VLAG, Wageningen	2013

Meetings and conferences

Annual Spring Meeting of KNVM/NVMM, oral presentation	Arnhem, The Netherlands	2012
Annual Spring Meeting of KNVM/NVMM	Arnhem, The Netherlands	2013
SfAM Summer Conference, poster	Cardiff, Wales	2013
LAB11, KNVM/FEMS, poster and oral presentation	Egmond aan Zee, The Netherlands	2014
Phages 2014, LipPubMedia, oral presentation	Oxford, United Kingdom	2014

General courses

Philosophy and Ethics of Food Science & Technology	WGS, Wageningen	2011
VLAG PhD week	VLAG, Wageningen	2012
Project and time management	WGS, Wageningen	2012
Techniques for Writing and Presenting a Scientific Paper	WGS, Wageningen	2013
Basic Statistics	PE&RC, Wageningen	2013

Optional activities

Preparation of research proposal	FHM, Wageningen	2011
PhD trip 2012 Japan	FHM, Wageningen	2012
Organizing PhD trip 2012 Japan	FHM, Wageningen	2012
PhD trip 2014 Republic of Ireland	FHM, Wageningen	2014
Food Microbiology department seminars	FHM, Wageningen	2011-2015
TIFN expert meetings	TIFN, Wageningen	2011-2015

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