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# The Microbial Community of Natural Whey Starter: Why Is It a Driver for the Production of the Most Famous Italian Long-Ripened Cheeses?

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**Abstract:** The remarkable global diversity in long-ripened cheese production can be attributed to the adaptability of the cheese microbiota. Most cheese types involve intricate microbial ecosystems, primarily represented by lactic acid bacteria (LAB). The present study aims to review the microbial community's diversity in dairy fermentation processes, focusing on two famous Italian cheeses, Grana Padano and Parmigiano Reggiano, produced using natural whey starter (NWS). NWS, created by retaining whey from the previous day's cheese batches, forms a microbiological connection between daily cheese productions. Through this technique, a dynamic microbiota colonizes the curd and influences cheese ripening. The back-slopping method in NWS preparation ensures the survival of diverse biotypes, providing a complex microbial community in which interactions among microorganisms are critical to ensuring its technological functionality. As highlighted in this review, the presence of microbial cells alone does not guarantee technological relevance. Critical microorganisms can grow and colonize the curd and cheese. This complexity enables NWS to adapt to artisanal production technologies while considering variations in raw milk microbiota, inhibitory compounds, and manufacturing conditions. This critical review aims to discuss NWS as a key factor in cheese making, considering microbial communities' ability to evolve under different selective pressures and biotic and abiotic stresses.

**Keywords:** lactic acid bacteria; cheese fermentation; Italian cheeses; natural whey starter; raw milk cheese; dairy microbial ecology



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## 1. Premise: Cheeses and Their Microbiota

Cheese is a product obtained via the acidic or enzymatic destabilization of casein, or more commonly, through a combination of both processes [1,2]. The earliest historical documentation of cheese production was found in ancient texts recovered in Iraq, dating back to about 3200 BC. In truth, the cheese transformation of milk may have spread to different people and places in the world even earlier as a result of random experiences. Subsequently, the relevant procedures would have been empirically reproduced until they became an industrial process through the development of scientific and technological knowledge that forms the basis of modern cheese processing [3–5].

Most modern cheese types are produced using only milk, lactic acid bacteria (LAB), rennet, and often sodium chloride [1,4]. Moreover, the different microbial communities harbored by different types of cheeses arise from raw milk, starter cultures, and adventitious microorganisms that originate from the equipment and cheese-making plant environment [4].

Raw milk is a rich and very attractive substrate for different microbial species that use lactose as a carbon source. Environmental factors play crucial roles in shaping the composition of the raw milk microbiota and in defining its evolution in cheese during ripening [1,4,6–13].

Thus, the manufacture of most cheese types involves a complex and dynamic microbial ecosystem in which several biochemical reactions occur, largely based on lactic acid fermentation by LAB [1,4,6,7,10,14,15]. However, not only the variability but also the versatility of this microbiota define the great differences that can be obtained in the fermentation of curds to produce very different cheeses.

In summary, the microbial evolution in cheese production is a dynamic process that encompasses adverse and fluctuating conditions for fermenting or not fermenting microorganisms that reach the curd alive in different ways.

The contribution of the cheese microbiota to flavor development characterizes the quality and recognition of the cheese and is thus of critical significance. The final cheese flavor, as with many of the final characteristics of cheese, is due to the interactions between the cheese microorganisms, the growth substrates, proteins in the milk, and the cheese environment [14,16]. During cheese manufacturing, environmental parameters such as temperature, pH, osmolarity, and lactose concentration change significantly. These parameters, particularly LAB, can be stressful to the microbiota of all cheeses. Rapid environmental changes impose limitations on the adaptation and cellular duplication of cheese through alternative secondary metabolism, leading to the production of metabolites with impacts on taste and aroma [17].

The process of transforming milk into cheese can be schematically divided into three blocks of operations (Figure 1). The first block of operations takes place before the milk is placed in the coagulation tank (vat) and corresponds to the milk preparation phase, including any refrigeration, possible skimming and pre-maturation, and any heat treatments. The second block of operations occurs in the vat and consists of the stages of milk processing that lead to the production of the curd. The third part consists of a series of operations that transform the curd into cheese. All these procedures together lead to the formation of the peculiar structures, flavors, and aromas of the different types of products.

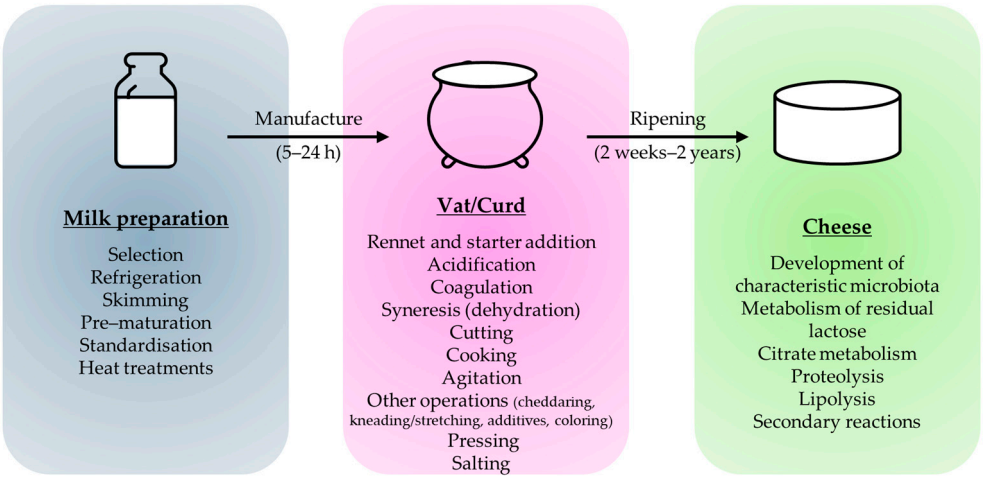


**Figure 1.** A simplified outline of the general flowsheet for the manufacture of rennet coagulated cheese. Adapted from ref. [4].

Starter LAB first produce mild acidification of the milk, followed, more importantly, by acidification of the curd. These changes define the main characteristics of the three blocks of operations during the first part of the cheese-making process (Figure 1) [4,6,10,18]. The factors related to lactic fermentation and multiplication of the acidifying LAB are critical in transforming the curdled milk during the first hours of maturation. For this reason, these LAB are called starter LAB. Acidification and lactose depletion are the first steps in curd formation. In addition to making the substrate less hospitable to most other microbial species, the acidification resulting from homolactic fermentation induces changes in casein hydration and its ability to remain in a colloidal suspension. In addition to coagulation, cheese curd fermentation plays a central role in defining the rheological

structure of the future cheese. Lactic acid is the primary metabolite produced by this type of lactose fermentation. The speed and intensity of acidification induce dramatic changes in the destabilization and structure of casein micelles. Much of the casein is found as a colloidal suspension in raw milk. The stability of this colloidal state is deeply connected to the presence of saline bridges and the availability of calcium phosphate in the state. The acidification caused by the fermentation by LAB modifies this physical–chemical equilibrium, thereby increasing the permeability of the curd [1,4,7,19].

After lactose depletion, bacterial cell death begins. Then, the lysis of the starter LAB, together with other bacteria sensitive to these new adverse conditions, induces the release of a significant amount of intracellular proteolytic enzymes, which constitute the heritage of the cheese microbiota and can participate in the ripening of the cheese. It is well established that cell lysis is a key event for the release of cytoplasmic enzymes into the cheese matrix and is crucial for understanding the contribution of different microbial cells to cheese ripening [17,20–30]. In this way, the proteolysis of caseins should be considered a crucial event in determining the outcome of the process [1,7,16,31].

The number of starter LAB cells in the acidified curd amounts to almost one billion per gram of cheese. This high quantity represents a huge reservoir of enzymatic activity [32]. These enzymes, or at least some of them, can remain active in the curd for very long periods of time and contribute to the different stages of cheese ripening [1,4,7].

From this perspective, starter LAB could be considered responsible for cheese modifications—firstly, as a well-defined cellular entity and secondly, as enzymes released after cell lysis. However, it was recently proposed that cell lysis alone cannot explain the long-term enzymatic activity observed during cheese ripening. From this perspective, bacterial cells that derive from the starter cultures could undergo permeabilization events, allowing for intracellular enzyme activity that might be relevant for prolonged metabolic conversions and thus flavor compound synthesis [17].

In this context, choosing the type of starter LAB for cheese making involves determining, in detail, the microbiota of the vat milk and how it will develop, first in the curd and then in the final cheese. Cheese technology leads to the selection of different bacterial populations. In addition, the complexity of the cheese-making parameters represents an aid or a tool to manage the versatility of the different LAB biotypes. Critically, not all the microorganisms present in the milk and curd that are considered cultivable or detectable based on DNA must fully participate in the dairy transformation. Some microorganisms may simply be present but could be of little interest in cheese production and ripening [17].

The presence of a microbial cell (or, worse, microbial DNA) is not enough to be considered technologically relevant. The most important microorganisms are those capable of multiplying, growing, and colonizing the curd and cheese. Indeed, microorganisms that are technologically relevant and increase the "quality" of the resulting cheese can be relatively rare in the composition of the raw milk and starter microbiome.

## 2. The Main Factors of the Microbial Ecosystem Involved in Long-Ripened Cheese, Such as Grana Padano and Parmigiano Reggiano

The most commonly studied and famous long-ripened Italian cheeses are Grana Padano (GP) and Parmigiano Reggiano (PR). These two varieties are traditional and long-ripened hard-cooked cheeses produced with raw milk in restricted geographical areas of Northern Italy, delimited by official regulations ("https://www.granapadano.it/wpcontent/uploads/2023/02/SpecificationsGBOct2022-50252.pdf (accessed on 11 January 2024)", "https://www.parmigianoreggiano.com/consortium-specifications-and-legislation/ (accessed on 11 January 2024)") [4,24,33,34]. Both varieties are "Grana cheeses", which refers to a cheese with a grainy structure. Such cheeses have been produced in the Po Valley since the 13th century.

GP and PR cheeses are made from partially skimmed raw milk through lactic acid fermentation and subjected to slow and long ripening for at least 9 and 12 months. LAB from raw milk, commonly called non-starter LAB (NSLAB) and starter LAB, plays a fundamental role in achieving the typical sensory characteristics of these cheeses [4,24].

GP and PR cheeses have many common characteristics and some distinct properties. Although the similarities between these varieties are defined by their cheese-making technologies, the differences are largely determined by the methods of raw milk collection, milk management before coagulation, and ripening conditions. Briefly, to produce PR, the milk is not refrigerated and should be maintained at a temperature no lower than 18 °C. The evening milk is partially skimmed after overnight creaming at about 20 °C in special tanks called "bacinelle". For GP, the feeding of high-quality silage fodder is allowed, and the cheese is produced from two consecutive rounds of milking. This milk is stored at a temperature no lower than 8 °C on the farm. To inhibit the late blowing of cheese, the addition of lysozyme to the vat milk (20–25 ppm) is allowed, as the use of silage favors the contamination of raw milk by spore-forming clostridia. The milk is skimmed via creaming in "bacinelle" for about 12 h at 8–20 °C [24,35].

For both cheeses, the slight microbial acidification that occurs during creaming favors rennet activity in the milk vat. At the same time, slight proteolysis produces short peptides that may favor further growth of the LAB in the natural whey culture (NWS). In both cheeses, calf rennet (powder preparation) is used.

A large amount of the NWS, about 3% (*v*/*v*), is added to the vat milk, yielding a total titratable acidity of ca. 28–32 °SH/50 mL [18]. As the use of commercial/selected starters is not allowed, NWS is prepared using whey from the previous cheese-making process. This whey is held under a temperature gradient (from about 50–54 °C to about 30–34 °C for 12–16 h) to reach a final titratable acidity close to about 20–32 °SH/50 mL. It is well known that the microbial composition of the NWS is dominated by thermophilic LAB (about $10^9$ CFU/mL), such as *Lactobacillus helveticus* and *Lactobacillus delbrueckii* subsp. *lactis*. [24,26,36,37]. The relative abundance of the species *L. helveticus* and *L. delbrueckii* subsp. *lactis* in the NWS varies according to the dairy used. Recently, Sola et al. [37] proposed a distinction between NWS dominated by the species *L. helveticus* (NWS type-H) and that dominated by the species *L. delbrueckii* (NWS type-D), noting that these ecological differences in starter cultures can influence the early stages of curd acidification [38].

After coagulation, the curd is broken into particles the size of rice grains and cooked at 52–56 °C for 5–15 min under stirring. The time from rennet addition at 32–34 °C to the end of cooking is close to 23 min. The combination of heating and acidifying activity by the NWS allows the formation of curd grains with the right texture along with whey drainage. After cooking, curd grains settle to the bottom of the vat for about 30–50 min with a whey temperature not exceeding 53–55 °C. Then, the curd is removed, cut into two parts, molded, acidified for about 48 h, salted in brine, and ripened for at least 12 months.

The use of the NWS is a practice that started at the beginning of 1900. This process aims to reduce microbiological defects in cheese and has consolidated over time. Acid production at the appropriate rate and time is a key step in the manufacture of high-quality cheese [24,39].

The method used to produce NWS by retaining some of the whey drained from the cheese vat at the end of cheese making leads to the selection of a characteristic microbiota [36]. The different treatments used during cheese making, starting from the addition of NWS to curd removal and the collection of "sweet whey", promote the selection of thermophilic and acid-tolerant lactic bacteria in the acidified whey [18,24]. The most influential parameters are the temperature of curd cooking, the management of the gradient temperature during whey fermentation, and the increase in acidity. Changing one or more of these parameters can lead to the selection of a characteristic microbiota mainly consisting of thermophilic, aciduric, and moderately heat-resistant LAB [24,36,40].

The NWSs obtained in this way demonstrate how the inhabitants of these specific geographical areas empirically learned to use their fermentation capabilities for dairy purposes by exploiting the abilities of specific microbial ecosystems to adapt and evolve. The curd structure in the vat and after breaking and extraction is defined by the acidification of the curd and, consequently, by LAB activity. During the first few hours of cheese making, the thermophilic LAB present in NWS quickly grow in the curd, but the correctness of curd

acidification depends on the residual availability of sugar, the pH, the residual moisture, and the temperature. It is known that the LAB in NWS mainly develop in the molded curd within 12 to 24 h and that the growth of these bacteria is coupled with lactic acid production and a decrease in pH to approximately 5.10–5.25. During the first 24 h of cheese molding, the conversion of lactose into lactic acid is the main biochemical process that occurs in cheese [4,24,41]. Moreover, due to the large size of the cheese (a diameter of 35 to 45 cm and a side height of 18 to 25 cm), all these parameters can differ in the different areas of the curd, creating differences in the acidification activity between the central and the external parts of the cheese. Within 48 h, the total LAB count starts to decrease.

The performance of NWS during cheese ripening can also be influenced by its cultivation history, modulating the proteome allocation and metabolic stability in starter cultures, thereby providing novel approaches to influence flavor formation [17,42,43]. Additionally, the presence of NSLAB in raw milk [44,45] can influence starter LAB's growth capacity in vat milk. During the first stage of cheese making, the weak proteolytic activity of NSLAB favors an increase in free amino acids in the milk. These free amino acids, or little peptides, allow the fast growth of starter LAB and, consequently, facilitate acidification kinetics [46]. The metabolic interactions between NSLAB and starter LAB affect the inhibition of spoilage bacteria and curd structuring [1,4,7,19].

The aim for the remainder of this critical review is to discuss the NWS as the driver of the cheese-making process, considering microbial communities and their capability to evolve under the different ecosystems that change during the production cycle of the NWS itself and during that of long-ripened cheeses.

## 3. Natural Whey Starter—Peculiarly Complex Microbial Ecosystems

According to PDO regulations, to prepare NWS for cheese making the following day, the whey remaining after curd separation, i.e., whey that is cooked and not already acidified (cooked non-acidified whey, often simply called "sweet whey"), is recovered, usually from one vat, and incubated at a decreasing temperature. This back-slopping procedure establishes a microbiological connection between subsequent batches of production.

The composition of these undefined multiple-strain cultures is the sum of the LAB obtained from raw milk and the LAB introduced in the previous batch of cheese with the previous NWS [24,36,39,47,48]. Under this traditional protocol, whey represents the link between these cheeses, which are manufactured each subsequent day. One of the peculiarities of using NWS for cheese making is that it forms a "microbiological bond", which is transferred through whey from one day's milk to that of the following day. In this way, NWS serves as a link between the dairy products manufactured each day. For this reason, following production regulations, cheese must be produced every day.

The success of the NWS is linked to its ability to adapt to the different technological parameters encountered in the cheese production process, thereby maintaining a high level of resilience among thermophilic LAB species. The peculiar adaptive features of the microbiota of NWS allow this undefined culture to retain its technological functions by adapting to a cyclical production process based on back-slopping [24,36,39].

However, this modality of preparation based on the back-slopping principle also enables the survival of many different biotypes, some of which are likely useful for the development of the whey ecosystem itself. A mixture of strains of the same species facilitates the development of a natural starter with a poorly defined composition but a strong ability to self-adapt to variable technological performance, as required by non-standardized cheese-making operations [36,39,49]. Small changes in technological parameters such as the temperature of curd cooking, the temperature and modality of NWS cooling, and differences in the final acidity and pH reached can affect the bacterial consortium present in NWS [24,36,38,39].

Notably, natural whey cultures experience two different thermal gradients. After inoculation into the milk vat, the temperature increases up to about 53–55 °C, exposing the bacteria cells to thermal stress for about one hour, while after separation of the whey

from the curd, the temperature decreases, alleviating the severe thermal conditions. It was observed that the two main thermophilic species present in whey, *L. helveticus* and *L. delbrueckii* subsp. *lactis*, respond differently to the different gradients and composition of the environment during the production phases [37]. Indeed, *L. helveticus* is regarded as a more acid-tolerant strain, which might explain its increase in quantity despite the low pH values reached in NWS [36]. Conversely, the inoculated vat milk had higher pH values that, in combination with a possibly higher tolerance to the thermal stress of *L. delbrueckii* subsp. *lactis*, could explain the numerical increase observed after the phase of cooking. This evidence suggests the resilience of this peculiar ecosystem in adapting itself to different stress conditions [36].

Other studies have focused on the intraspecies (i.e., strain) characterization of the most abundant species isolated from NWS, showing how these cultures vary not only by species but also, and primarily, by strains within species, as observed in undefined cultures used for the production of other cheeses [50,51]. Because it is the dominant species in NWS, *L. helveticus* has been the focus of many studies on its phenotypic and genotypic diversity [52].

According to ecological principles, Giraffa et al. evaluated different bacterial interactions involving either stimulatory or inhibitory effects for *L. helveticus*, *L. delbrueckii* ssp. *lactis*, and *L. fermentum* [53]. Certainly, future studies are needed to better understand the role of microbial interactions in the stability and functionality of the NWS ecosystem.

Comparing culture-independent (i.e., microscopy) and culture-dependent (i.e., plate count) quantification [26,39,54] indicates that bacterial viability in NWS cannot be evaluated only based on LAB's capacity to form colonies on MRS or a whey agar medium. The number of total cells, particularly viable cells, is often higher (up to 1 log unit) than the number of colony-forming units. Questions remain about the roles of cells that are viable but not cultivable, which often represent most of the culture. Because these bacteria cannot be cultivated, the role of this population in the whey culture during cheese curd fermentation is not currently known.

Recently, it was demonstrated that *Lactococcus lactis* can form persistent and viable but not culturable (VBNC) cells when exposed to antimicrobial agents [55]. Other reports on the same species suggest that bacteria that enter dormant, low-growth states could be relevant in the microbial ecology of dairy products since they are metabolically active [56]. However, such bacterial strains are challenging to isolate from complex environments such as the NWS.

In general, the molecular mechanisms of nonculturable cells are perplexing, and the condition of VBNC is controversial [57,58].

However, the greatest and most well-known advantage of NWS's biological systems is undoubtedly their wide resistance to lytic bacteriophage attacks [59]. Natural starters are widely considered highly tolerant to phage infection because they are grown in the presence of phages, which leads to the dominance of resistant or tolerant strains [18,60–62]. Although Carminati et al. [62] found that lysogeny occurred in *L. helveticus* cultures isolated from GP NWS, these cultures were found to carry defective phages or killer particles when induced by mitomycin. More recently, a study by Mancini et al. [63] confirmed the prevalence of bacteriophages in NWS cultures used to produce Trentin Grana cheese, despite showing the limited capability of the isolated bacteriophages to form lysis plaques on cultures of *L. helveticus*. The consistent presence of lytic phages in the NWS did not impair their performance. This result could be related to the presence of various bacterial strains of the *L. helveticus* species, each with different phage sensitivity profiles, allowing the species to effectively counteract phage predation [64]. However, when the concentration of bacteriophages increases, adverse effects could be encountered in the sensory profiles of cheeses resulting from such a production process [65]. The presence of phages in cheese might select for resistance traits among bacteria, especially if the bacteria and phage association persists between different production cycles [65].

## 4. An Ecological Perspective on Natural Whey Starters

Over time, microorganisms have been mutating and evolving to adapt to an ever-changing ecosystem [66]. Microbial populations adapt rapidly when they are introduced to a new environment. However, at the same time, microbial populations could continue to improve indefinitely, albeit slowly, even in a constant environment, through the contributions of individual mutations to fitness improvement [67].

NWS can be considered one complex microbial community among many such communities in nature. The production of fermented products such as NWS is the result of activities not by an individual but by a group of microorganisms. For example, most food fermentation processes depend on mixtures of microorganisms (species and biotypes), which act in concert to produce the desired product characteristics. All fermentation processes are often characterized by the presence of a complex microbiota. Notably, the LAB community of NWS can be discussed while considering the scenario of complex microbial communities.

About 35 years have passed since multicellularity was proposed as a possibility for understanding the growth and development of complex prokaryotic ecosystems. Indeed, the hypothesis that complex microbial ecosystems act like multicellular organisms whose individual components interact and condition each other remains intriguing [68,69].

Intercellular communication and multicellular coordination are known to be widespread among prokaryotes and influence the expression and intensity of multiple phenotypes. Following this approach, the interactions between microorganisms that comprise complex ecosystems represent the decisive factor that influences the development of different microbial cultures [70]. Beyond microbial quantity or the presence of different species, biotypes, and variants, the interactions between microorganisms represent a key factor for understanding the biological functionality of complex microbial ecosystems and their ability to adapt to stress, survive, evolve, and express different phenotypes [68,69].

This evidence highlights the need to deeply explore the diversity of the microbial community involved in natural food fermentation processes and the links between their technological capabilities and product quality [71–74]. The back-slopping principle applied to an environment such as non-thermally treated milk brings the results of NWS very close to those of natural fermentation. Thus, we should explore how the technological choices made by humans can direct growth and microbial metabolism under conditions of stress. It is well known that the technological processes used to produce fermented foods usually involve process conditions that guide fermentation through the imposition of differently selective or elective conditions on the microbiota present. The ability of microorganisms to resist different stress factors enables their resilience under conditions that are hostile to growth and metabolism [75–79]. Microbial selection guides the fermentation process [71,72,80–82].

Concerning this microbial adaptative capacity, Charles Darwin wrote in a letter to Asa Gray, "What a trifling difference must often determine which shall survive, and which perish!" [71]. Therefore, the colonization of food by different microorganisms may also be studied in terms of both ecological strategy and community development [18,83–85].

In natural food systems, the stimulatory and inhibitory effects among microorganisms could support the possibility of maintaining the viability of a crucial part of the microbial population (population stability), even in the presence of continuous changes in the food environment, including those resulting from the metabolic activity of the microorganisms themselves [18,29,53,83–87].

In general, it can be stated that the NWS bacterial consortium is more versatile and robust than pure cultures used in cheese production because it performs more complex activities and can tolerate more variation in the environment. This deduction is based on the concept that bacteria benefit from multicellular cooperation by using the cellular division of labor, accessing resources that cannot effectively be utilized by single cells, collectively defending against antagonists, and optimizing population survival by differentiating into distinct cell types [68]. Even micro-interactions with the environment matrix, including

milk, whey, curd, and cheese, can be crucial in defining cellular cooperation and the evolution of microbial communities [69,88–90].

In complex ecosystems, even population heterogeneity can be a determinant in defining resilience against environmental uncertainty [91]. Intraspecies diversity among closely related strains is commonly linked to functionally adaptive traits encoded on genomic islands that are acquired by horizontal gene transfer [92]. The generation of subpopulations with varying plasmid content in natural communities yields selective advantages in the face of environmental uncertainty [50,82,93,94]. Analogously, bacteriophages play a regulatory role in population dynamics through density-dependent predation [50,95].

The presence of isogenic bacteria, populations that are traditionally considered to be composed of identical cells, can also contribute to the survival and evolution of complex microbial ecosystems [96]. Despite containing the same genetic material, protein levels between cells can vary due to stochastic events associated with gene expression and regulation. Thus, cell-to-cell heterogeneity has important implications for allowing populations of cells to diversify and thus survive environmental stress [50,71,82,94,97,98].

It was verified that microbial communities in sourdough microbiota undergo changes in composition that threaten their resilience. To support resilience and good performance, the sourdough metacommunity includes dominant, subdominant, and satellite players, which together ensure gene and transcript redundancy [86].

The microbial consortia offer several advantages for the survival aptitude of the microbiota. These benefits include the increased quality and safety of several food systems, flavor development, and increased stability to improve shelf life and consumer safety. In summary, the interactions that occur within the food ecosystem can play a decisive role in the evolution of all players present in the ecosystem itself. This evidence has increased interest in studying the diversity of the community of fermenting microorganisms and linking the evolution of microbiota to their properties in adapting to technological processes and product quality.

For GP and PR cheese production, NWS represents a large component of the future microbiota of curd and, in collaboration with the raw milk microbiota, the "engine" of the metabolism involved in cheese making and cheese ripening [13,45]. LAB species/biotypes present in vat milk (from NWS and raw milk) can grow, survive, decline, and even become dominant during cheese manufacture. The outcomes depend on metabolic potential, which is species- and even biotype-specific. The environmental conditions that species encounter are first the biochemical composition of the vat milk, followed by the curd matrix modified by acidification and technological parameters.

The large number of NWS LAB cells that develop in the curd, thereby acidifying it, also represent the most important source of the large pool of proteolytic enzymes released after cell lysis; these enzymes are significantly involved in cheese ripening. Enzyme activities are regulated by cheese composition, moisture, NaCl concentration, pH, and temperature, which change not only over time but also according to the different cheese zones [22,25,32,99]. Interactions between SLAB and NSLAB occur starting from the earliest stages of cheese manufacture through cheese ripening. One of the most well-accepted theories indicates that SLAB lysates provide energy sources for NSLAB [18,24,28–30,100–104].

It was previously observed that the bacterial consortia of NWS coevolved during adaptation to whey and curd acidification [36]. The management of this dynamic ecosystem could be considered a superorganism, consisting of the sums of microbial metabolism and the interactions between individual microbes [18,72,105]. This super-organism activity recalls the multicellularity proposed by Shapiro [68,69] as the key to understanding the growth and development of complex prokaryotic ecosystems, whose individual components interact and condition each other.

For simplicity, the different microorganisms of the cheese microbiota can be arbitrarily clustered into three parts (Figure 2). Many of these parts are only occasionally present, likely without any (or minimal) technological significance, or are not yet understood (supporting actors) (Figure 2, area A). The size of this area can vary according to the type of milk used to

produce the cheeses. As in the case of raw milk, the more complex the milk microbiota, the larger the area. Another area (Figure 2, area B) includes all microorganisms (biotypes and variants) that are functional in the survival of the whey starter's ecosystem itself and its ability to adapt and survive/evolve. This part of the microbiota determines the resilience of the NWS ecosystem [82]. As suggested by Charles Darwin, this component determines the continuity and survival of the ecosystem [71]. The third part (Figure 2, area C) represents a core microbiota that is not needed for natural whey starter fermentation but remains necessary for curd fermentation. This part represents the microbial core responsible for the cheese-making process and can change during the different moments (or zones) of the cheese-making process. Unlike part B, part C does not represent the biological element of stability and continuity in the whey starter ecosystem itself but only the part that is functional for the transformation of the cheese. This component is capable of adapting to stress factors induced by technology. Starting from this part of the microbiota alone, unlike with part B, there is no certainty that the NWS ecosystem will survive. Therefore, it cannot be excluded that even small variations in the preparation of the starter cultures can significantly influence the dominance of otherwise minority bacterial populations that influence the functional properties of the NWS ecosystem [63].

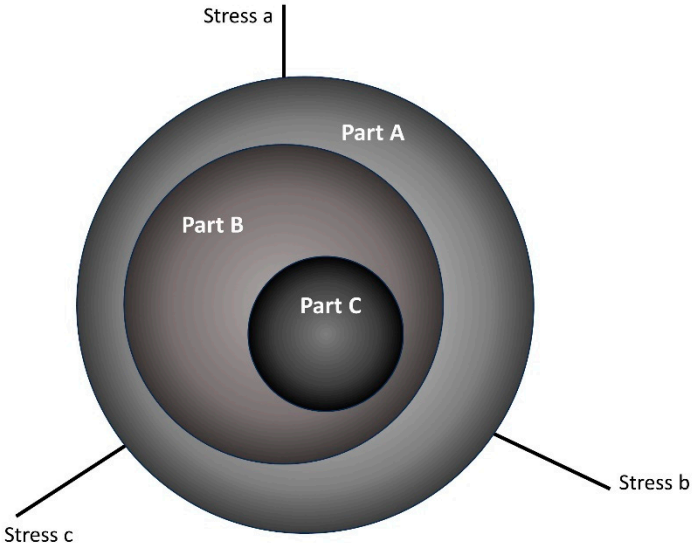


**Figure 2.** Schematic representation of the microbial complexity observed in the NWS ecosystem. Assuming an environment where different abiotic or biotic stresses (Stress a, b, c) are faced by the NWS microbial community, it is likely that the majority of components (Part A) are superfluous and not relevant to the functionality of the microbial community itself, while different subsets (Part B, Part C) could better adapt to different steps in the cheese-making process. The existence of microbial interactions, within-species biodiversity, and phenotypic variability makes it difficult to disentangle and characterize the contribution of each microbial population to the complex NWS microbiome.

Studying NWS to produce Parmigiano Reggiano cheese, Bertani et al. [36] observed a large number of microorganisms present in the natural culture mixed with raw milk (part A). These microorganisms mainly came from raw milk, a core microbiota useful for the persistence of the ecosystem, and adapted, in turn, to a mixture of raw milk and whey or non-acidified/acidified whey (part B). A minority of the microorganisms adapted to the curd ecosystem (part C). This minority component of the natural whey microbiota (part C) could be considered essential for the cheese-making process. Bertani et al. [36] also showed that it is not possible to develop a natural whey starter useful for cheese making with only part C because part B is able to maintain the complex culture, adapt to vat milk, and produce the natural whey starter fermentation. Even if these bacteria (species and/or biotypes) had found ideal conditions to grow in the NWS, only a minority of them could

have better adapted to the curd ecosystem compared to cooked non-acidified whey and the NWS ecosystem [29,38,53,106].

In part A, there are other microorganisms not involved in the persistence or resilience of NWS. Such microorganisms likely come from environmental raw milk contamination. We believe that the interactions between microorganisms belonging to these different parts of the microbiota, not only their presence and amount, are key factors in understanding their technological functionality, ability to adapt to stress, ability to survive, and ability to evolve and express phenotypes in the final cheese.

It can be assumed that a core microbiota would be useful for the persistence of the ecosystem. This microbiota could adapt to the mixture of raw milk and NWS in the vat, to sweet whey after curd separation, or to acidified whey after preparation of the NWS. The majority of LAB present in acidified whey is necessarily adapted to this substrate and capable of reaching the desired level of acidification.

This factor is likely related to the difficulties sometimes encountered when selecting starter cultures with technological and aromatic performance like that achieved using natural starters. Indeed, isolating and using mixtures of strains obtained from milk or a natural starter seems insufficient to obtain starters with good dairy performance. Rather, it is necessary to identify the biotypes, which may represent a minority in the natural starter, with the ability to develop into the curd. Consequently, minority populations or even apparently non-viable strains could be necessary to maintain cell interactions.

## 5. Conclusions

The biological complexity of the LAB consortium characterizing the NWS is both a strength and a weakness of these natural cultures. Indeed, this biological complexity and biodiversity guarantee the LAB consortium's ability to adapt to artisanal production technologies based on its intrinsic ability to evolve in response to external factors such as the microbiota of raw milk, the presence of any compounds that might inhibit bacterial growth, and the manufacturing conditions. On the other hand, the presence of different species and numerous biotypes makes it more difficult to standardize the daily propagation of the starter. Consequently, the activity of this consortium can vary slightly from day to day.

Despite the findings provided by previous studies, the composition of these natural microbial cultures adapted to the selective pressure of dairy processing is not yet fully understood. It will be necessary for future studies to explore individual players and, especially, the relevant complex ecosystem communities and bacterial interactions.

The ability of all living species to colonize an ecosystem includes their interactions with other species living in the ecosystem. Biotic and abiotic effects determine the evolution of this interaction. NWS seems to represent a good example of this complexity and functionality.

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## References

1. Alais, C. *Science du Lait: Principes des Techniques Laitières*, 4th ed.; Société D'édition et de Promotion Agro-Alimentaires, Industrielles et Commerciales: Paris, France, 1984.

2. Fox, P.F.; Mcsweeney, P.L.H. *Rennets: Their Role in Milk Coagulation and Cheese Ripening*; Springer: Berlin/Heidelberg, Germany, 1997.
3. Douillard, F.P.; Ribbera, A.; Kant, R.; Pietilä, T.E.; Järvinen, H.M.; Messing, M.; Randazzo, C.L.; Paulin, L.; Laine, P.; Ritari, J.; et al. Comparative Genomic and Functional Analysis of 100 Lactobacillus rhamnosus Strains and Their Comparison with Strain GG. *PLoS Genet.* **2013**, *9*, e1003683. [CrossRef] [PubMed]
4. Gobbetti, M.; Neviani, E.; Fox, P. *The Cheeses of Italy: Science and Technology*; Springer International Publishing: Berlin/Heidelberg, Germany, 2018. [CrossRef]
5. McClure, S.B.; Magill, C.; Podrug, E.; Moore, A.M.T.; Harper, T.K.; Culleton, B.J.; Kennett, D.J.; Freeman, K.H. Fatty acid specific $\delta^{13}C$ values reveal earliest Mediterranean cheese production 7200 years ago. *PLoS ONE* **2018**, *13*, e0202807. [CrossRef] [PubMed]
6. Ercolini, D. Secrets of the cheese microbiome. *Nat. Food* **2020**, *1*, 466–467. [CrossRef] [PubMed]
7. Fox, P.F.; Guinee, T.P.; Cogan, T.M.; McSweeney, P.L.H. *Fundamentals of Cheese Science*; Springer: Boston, MA, USA, 2017. [CrossRef]
8. Fox, P.F.; McSweeney, P.L.H. Methods used to study non-starter microorganisms in cheese: A review. *Int. J. Dairy Technol.* **2000**, *53*, 113–119. [CrossRef]
9. Li, N.; Wang, Y.; You, C.; Ren, J.; Chen, W.; Zheng, H.; Liu, Z. Variation in Raw Milk Microbiota Throughout 12 Months and the Impact of Weather Conditions. *Sci. Rep.* **2018**, *8*, 2371. [CrossRef] [PubMed]
10. Parente, E.; Ricciardi, A.; Zotta, T. The microbiota of dairy milk: A review. *Int. Dairy J.* **2020**, *107*, 104714. [CrossRef]
11. Quigley, L.; O'Sullivan, O.; Stanton, C.; Beresford, T.P.; Ross, R.P.; Fitzgerald, G.F.; Cotter, P.D. The complex microbiota of raw milk. *FEMS Microbiol. Rev.* **2013**, *37*, 664–698. [CrossRef] [PubMed]
12. Renoldi, N.; Innocente, N.; Rossi, A.; Brasca, M.; Morandi, S.; Marino, M. Screening of Aroma-Producing Performance of Anticlostridial Lacticaseibacillus casei Strains. *Food Bioprocess Technol.* **2024**. [CrossRef]
13. Bettera, L.; Levante, A.; Bancalari, E.; Bottari, B.; Cirlini, M.; Neviani, E.; Gatti, M. Lacticaseibacillus Strains Isolated from Raw Milk: Screening Strategy for Their Qualification as Adjunct Culture in Cheesemaking. *Foods* **2023**, *12*, 3949. [CrossRef]
14. Afshari, R.; Pillidge, C.J.; Dias, D.A.; Osborn, A.M.; Gill, H. Cheesomics: The future pathway to understanding cheese flavour and quality. *Crit. Rev. Food Sci. Nutr.* **2020**, *60*, 33–47. [CrossRef]
15. Cocolin, L.; Gobbetti, M.; Neviani, E.; Daffonchio, D. Ensuring safety in artisanal food microbiology. *Nat. Microbiol.* **2016**, *1*, 16171. [CrossRef] [PubMed]
16. Christensen, L.F.; Høie, M.H.; Bang-Berthelsen, C.H.; Marcatili, P.; Hansen, E.B. Comparative Structure Analysis of the Multi-Domain, Cell Envelope Proteases of Lactic Acid Bacteria. *Microorganisms* **2023**, *11*, 2256. [CrossRef] [PubMed]
17. Nugroho, A.D.W.; Kleerebezem, M.; Bachmann, H. Growth, dormancy and lysis: The complex relation of starter culture physiology and cheese flavour formation. *Curr. Opin. Food Sci.* **2021**, *39*, 22–30. [CrossRef]
18. Gobbetti, M.; Di Cagno, R.; Calasso, M.; Neviani, E.; Fox, P.F.; De Angelis, M. Drivers that establish and assembly the lactic acid bacteria biota in cheeses. *Trends Food Sci. Technol.* **2018**, *78*, 244–254. [CrossRef]
19. McSweeney, P.L.H.; Fox, P.F.; Cotter, P.D.; Everett, D.W. *Cheese: Chemistry, Physics & Microbiology*; Academic Press: Cambridge, MA, USA, 2017.
20. Chapot-Chartier, M.-P.; Deniel, C.; Rousseau, M.; Vassal, L.; Gripon, J.-C. Autolysis of two strains of Lactococcus lactis during cheese ripening. *Int. Dairy J.* **1994**, *4*, 251–269. [CrossRef]
21. Crow, V.L.; Coolbear, T.; Gopal, P.K.; Martley, F.G.; McKay, L.L.; Riepe, H. The role of autolysis of lactic acid bacteria in the ripening of cheese. *Int. Dairy J.* **1995**, *5*, 855–875. [CrossRef]
22. De Dea Lindner, J.; Bernini, V.; De Lorentiis, A.; Pecorari, A.; Neviani, E.; Gatti, M. Parmigiano Reggiano cheese: Evolution of cultivable and total lactic microflora and peptidase activities during manufacture and ripening. *Dairy Sci. Technol.* **2008**, *88*, 511–523. [CrossRef]
23. El Soda, M.; Farkye, N.; Vuillemard, J.C.; Simard, R.E.; Olson, N.F.; El Kholy, W.; Dako, E.; Medrano, E.; Gaber, M.; Lim, L. Autolysis of Lactic Acid Bacteria: Impact on Flavour Development in Cheese. In *Developments in Food Science*; Elsevier: Amsterdam, The Netherlands, 1995; Volume 37, pp. 2205–2223. [CrossRef]
24. Gatti, M.; Bottari, B.; Lazzi, C.; Neviani, E.; Mucchetti, G. Invited review: Microbial evolution in raw-milk, long-ripened cheese produced using undefined natural whey starters. *J. Dairy Sci.* **2014**, *97*, 573–591. [CrossRef]
25. Gatti, M.; De Dea Lindner, J.; De Lorentiis, A.; Bottari, B.; Santarelli, M.; Bernini, V.; Neviani, E. Dynamics of whole and lysed bacterial cells during Parmigiano-Reggiano cheese production and ripening. *Appl. Environ. Microbiol.* **2008**, *74*, 6161–6167. [CrossRef]
26. Gatti, M.; Bernini, V.; Lazzi, C.; Neviani, E. Fluorescence microscopy for studying the viability of micro-organisms in natural whey starters. *Lett. Appl. Microbiol.* **2006**, *42*, 338–343. [CrossRef]
27. Levante, A.; De Filippis, F.; La Storia, A.; Gatti, M.; Neviani, E.; Ercolini, D.; Lazzi, C. Metabolic gene-targeted monitoring of non-starter lactic acid bacteria during cheese ripening. *Int. J. Food Microbiol.* **2017**, *257*, 276–284. [CrossRef] [PubMed]
28. Lortal, S.; Chapot-Chartier, M.P. Role, mechanisms and control of lactic acid bacteria lysis in cheese. *Int. Dairy J.* **2005**, *15*, 857–871. [CrossRef]
29. Sgarbi, E.; Bottari, B.; Gatti, M.; Neviani, E. Investigation of the ability of dairy nonstarter lactic acid bacteria to grow using cell lysates of other lactic acid bacteria as the exclusive source of nutrients. *Int. J. Dairy Technol.* **2014**, *67*, 342–347. [CrossRef]
30. Wilkinson, M.G.; Guinee, T.P.; O'Callaghan, D.M.; Fox, P.F. Autolysis and proteolysis in different strains of starter bacteria during Cheddar cheese ripening. *J. Dairy Res.* **1994**, *61*, 249–262. [CrossRef]

31. Savijoki, K.; Ingmer, H.; Varmanen, P. Proteolytic systems of lactic acid bacteria. *Appl. Microbiol. Biotechnol.* **2006**, *71*, 394–406. [CrossRef] [PubMed]
32. Gatti, M.; Fornasari, M.E.; Mucchetti, G.; Addeo, F.; Neviani, E. Presence of peptidase activities in different varieties of cheese. *Lett. Appl. Microbiol.* **1999**, *28*, 368–372. [CrossRef] [PubMed]
33. Giraffa, G. The Microbiota of Grana Padano Cheese. A Review. *Foods* **2021**, *10*, 2632. [CrossRef] [PubMed]
34. Zago, M.; Bardelli, T.; Rossetti, L.; Nazzicari, N.; Carminati, D.; Galli, A.; Giraffa, G. Evaluation of bacterial communities of Grana Padano cheese by DNA metabarcoding and DNA fingerprinting analysis. *Food Microbiol.* **2021**, *93*, 103613. [CrossRef]
35. Olivera Rodi, J.; Gonz´ález Ramos, M.J.; Díaz Gadea, P.; Reginensi, S.M. Study of the inhibitory effect of Lactobacillus strains and lysozyme on growth of *Clostridium* spp. responsible for cheese late blowing defect. *Nova Biotechnol. Chim.* **2022**, *22*, e1229. [CrossRef]
36. Bertani, G.; Levante, A.; Lazzi, C.; Bottari, B.; Gatti, M.; Neviani, E. Dynamics of a natural bacterial community under technological and environmental pressures: The case of natural whey starter for Parmigiano Reggiano cheese. *Food Res. Int.* **2020**, *129*, 108860. [CrossRef]
37. Sola, L.; Quadu, E.; Bortolazzo, E.; Bertoldi, L.; Randazzo, C.L.; Pizzamiglio, V.; Solieri, L. Insights on the bacterial composition of Parmigiano Reggiano Natural Whey Starter by a culture-dependent and 16S rRNA metabarcoding portrait. *Sci. Rep.* **2022**, *12*, 17322. [CrossRef] [PubMed]
38. Santarelli, M.; Bottari, B.; Malacarne, M.; Lazzi, C.; Sforza, S.; Summer, A.; Neviani, E.; Gatti, M. Variability of lactic acid production, chemical and microbiological characteristics in 24-hour Parmigiano Reggiano cheese. *Dairy Sci. Technol.* **2013**, *93*, 605–621. [CrossRef]
39. Bottari, B.; Santarelli, M.; Neviani, E.; Gatti, M. Natural whey starter for Parmigiano Reggiano: Culture-independent approach. *J. Appl. Microbiol.* **2010**, *108*, 1676–1684. [CrossRef]
40. Helal, A.; Nasuti, C.; Sola, L.; Sassi, G.; Tagliazucchi, D.; Solieri, L. Impact of Spontaneous Fermentation and Inoculum with Natural Whey Starter on Peptidomic Profile and Biological Activities of Cheese Whey: A Comparative Study. *Fermentation* **2023**, *9*, 270. [CrossRef]
41. Giraffa, G.; Rossetti, L.; Mucchetti, G. Influence of the Temperature Gradient on the Growth of Thermophilic Lactobacilli Used as Natural Starters in Grana Cheese. *J. Dairy Sci.* **1998**, *81*, 31–36. [CrossRef]
42. Teusink, B.; Molenaar, D. Systems biology of lactic acid bacteria: For food and thought. *Curr. Opin. Syst. Biol.* **2017**, *6*, 7–13. [CrossRef] [PubMed]
43. Wels, M.; Siezen, R.; Van Hijum, S.; Kelly, W.J.; Bachmann, H. Comparative Genome Analysis of Lactococcus lactis Indicates Niche Adaptation and Resolves Genotype/Phenotype Disparity. *Front. Microbiol.* **2019**, *10*, 4. [CrossRef]
44. Gobbetti, M.; De Angelis, M.; Di Cagno, R.; Mancini, L.; Fox, P.F. Pros and cons for using non-starter lactic acid bacteria (NSLAB) as secondary/adjunct starters for cheese ripening. *Trends Food Sci. Technol.* **2015**, *45*, 167–178. [CrossRef]
45. Bettera, L.; Levante, A.; Bancalari, E.; Bottari, B.; Gatti, M. Lactic acid bacteria in cow raw milk for cheese production: Which and how many? *Front. Microbiol.* **2023**, *13*, 1092224. [CrossRef]
46. Carminati, D.; Brizzi, A.; Giraffa, G.; Neviani, E. Effect of amino acids on S. salivarius subsp. thermophilus growth in modified milk deprived of non-protein nitrogen fraction. *Milchwissenschaft* **1994**, *49*, 481–540.
47. Rossetti, L.; Carminati, D.; Zago, M.; Giraffa, G. A Qualified Presumption of Safety approach for the safety assessment of Grana Padano whey starters. *Int. J. Food Microbiol.* **2009**, *130*, 70–73. [CrossRef] [PubMed]
48. Fornasari, M.E.; Rossetti, L.; Carminati, D.; Giraffa, G. Cultivability of *Streptococcus thermophilus* in Grana Padano cheese whey starters. *FEMS Microbiol. Lett.* **2006**, *257*, 139–144. [CrossRef] [PubMed]
49. Santarelli, M.; Gatti, M.; Lazzi, C.; Bernini, V.; Zapparoli, G.A.; Neviani, E. Whey Starter for Grana Padano Cheese: Effect of Technological Parameters on Viability and Composition of the Microbial Community. *J. Dairy Sci.* **2008**, *91*, 883–891. [CrossRef] [PubMed]
50. Erkus, O.; De Jager, V.C.L.; Spus, M.; van Alen-Boerrigter, I.J.; Van Rijswijck, I.M.H.; Hazelwood, L.; Janssen, P.W.M.; Hijum, S.A.F.T.v.; Kleerebezem, M.; Smid, E.J. Multifactorial diversity sustains microbial community stability. *ISME J.* **2013**, *7*, 2126–2136. [CrossRef] [PubMed]
51. Somerville, V.; Berthoud, H.; Schmidt, R.S.; Bachmann, H.P.; Meng, Y.H.; Fuchsmann, P.; von Ah, U.; Engel, P. Functional strain redundancy and persistent phage infection in Swiss hard cheese starter cultures. *ISME J.* **2022**, *16*, 388–399. [CrossRef] [PubMed]
52. Gatti, M.; Lazzi, C.; Rossetti, L.; Mucchetti, G.; Neviani, E. Biodiversity in Lactobacillus helveticus strains present in natural whey starter used for Parmigiano Reggiano cheese. *J. Appl. Microbiol.* **2003**, *95*, 463–470. [CrossRef] [PubMed]
53. Giraffa, G.; Mucchetti, G.; Neviani, E. Interactions among thermophilic lactobacilli during growth in cheese whey. *J. Appl. Bacteriol.* **1996**, *80*, 199–202. [CrossRef]
54. Bottari, B.; Ercolini, D.; Gatti, M.; Neviani, E. Application of FISH technology for microbiological analysis: Current state and prospects. *Appl. Microbiol. Biotechnol.* **2006**, *73*, 485–494. [CrossRef]
55. Tatenhove-pel, R.J.; Zwering, E.; Solopova, A.; Kuipers, O.P.; Bachmann, H. Ampicillin-treated Lactococcus lactis MG1363 populations contain persisters as well as viable but non-culturable cells. *Sci. Rep.* **2019**, *9*, 9867. [CrossRef] [PubMed]
56. van Mastrigt, O.; Abee, T.; Lillevang, S.K.; Smid, E.J. Quantitative physiology and aroma formation of a dairy Lactococcus lactis at near-zero growth rates. *Food Microbiol.* **2018**, *73*, 216–226. [CrossRef]

57. Babu, D.; Kushwaha, K.; Juneja, V.K. Viable but Nonculturable. In *Encyclopedia of Food Microbiology*; Elsevier: Amsterdam, The Netherlands, 2014; pp. 686–690. [CrossRef]
58. Emerson, J.B.; Adams, R.I.; Román, C.M.B.; Brooks, B.; Coil, D.A.; Dahlhausen, K.; Ganz, H.H.; Hartmann, E.M.; Hsu, T.; Justice, N.B.; et al. Schrödinger's microbes: Tools for distinguishing the living from the dead in microbial ecosystems. *Microbiome* **2017**, *5*, 86. [CrossRef] [PubMed]
59. Zago, M.; Bonvini, B.; Rossetti, L.; Meucci, A.; Giraffa, G.; Carminati, D. Biodiversity of Lactobacillus helveticus bacteriophages isolated from cheese whey starters. *J. Dairy Res.* **2015**, *82*, 242–247. [CrossRef]
60. Briggiler-Marcó, M.; Capra, M.L.; Quiberoni, A.; Vinderola, G.; Reinheimer, J.; Hynes, E. Nonstarter Lactobacillus strains as adjunct cultures for cheese making: In vitro characterization and performance in two model cheeses. *J. Dairy Sci.* **2007**, *90*, 4532–4542. [CrossRef] [PubMed]
61. Carminati, D.; Giraffa, G.; Quiberoni, A.; Binetti, A.; Suárez, V.; Reinheimer, J. Advances and Trends in Starter Cultures for Dairy Fermentations. In *Biotechnology of Lactic Acid Bacteria*, 1st ed.; Mozzi, F., Raya, R.R., Vignolo, G.M., Eds.; Wiley: Hoboken, NJ, USA, 2010; pp. 177–192. [CrossRef]
62. Carminati, D.; Mazzucotelli, L.; Giraffa, G.; Neviani, E. Incidence of Inducible Bacteriophage in Lactobacillus helveticus Strains Isolated from Natural Whey Starter Cultures. *J. Dairy Sci.* **1997**, *80*, 1505–1511. [CrossRef]
63. Mancini, A.; Rodriguez, M.C.; Zago, M.; Cologna, N.; Goss, A.; Carafa, I.; Tuohy, K.; Merz, A.; Franciosi, E. Massive Survey on Bacterial–Bacteriophages Biodiversity and Quality of Natural Whey Starter Cultures in Trentingrana Cheese Production. *Front. Microbiol.* **2021**, *12*, 678012. [CrossRef] [PubMed]
64. Spus, M.; Li, M.; Alexeeva, S.; Zwietering, M.H.; Abee, T.; Smid, E.J. Strain diversity and phage resistance in complex dairy starter cultures. *J. Dairy Sci.* **2015**, *98*, 5173–5182. [CrossRef] [PubMed]
65. Walsh, A.M.; Macori, G.; Kilcawley, K.N.; Cotter, P.D. Meta-analysis of cheese microbiomes highlights contributions to multiple aspects of quality. *Nat. Food* **2020**, *1*, 500–510. [CrossRef] [PubMed]
66. Bleuven, C.; Landry, C.R. Molecular and cellular bases of adaptation to a changing environment in microorganisms. *Proc. R. Soc. B* **2016**, *283*, 20161458. [CrossRef] [PubMed]
67. Elena, S.F.; Lenski, R.E. Evolution experiments with microorganisms: The dynamics and genetic bases of adaptation. *Nat. Rev. Genet.* **2003**, *4*, 457–469. [CrossRef]
68. Shapiro, J.A. Thinking about bacterial populations as multicellular organisms. *Annu. Rev. Microbiol.* **1998**, *52*, 81–104. [CrossRef]
69. Shapiro, J.A. Bacteria as Multicellular Organisms. *Sci. Am.* **1988**, *258*, 82–89. [CrossRef]
70. De Vos, M.G.J.; Schoustra, S.E.; De Visser, J.A.G.M. Ecology dictates evolution? About the importance of genetic and ecological constraints in adaptation. *EPL* **2018**, *122*, 58002. [CrossRef]
71. Booth, I.R. Stress and the single cell: Intrapopulation diversity is a mechanism to ensure survival upon exposure to stress. *Int. J. Food Microbiol.* **2002**, *78*, 19–30. [CrossRef] [PubMed]
72. Papadimitriou, K.; Pot, B.; Tsakalidou, E. How microbes adapt to a diversity of food niches. *Curr. Opin. Food Sci.* **2015**, *2*, 29–35. [CrossRef]
73. Smid, E.J.; Lacroix, C. Microbe-microbe interactions in mixed culture food fermentations. *Curr. Opin. Biotechnol.* **2013**, *24*, 148–154. [CrossRef]
74. Wolfe, B.E.; Dutton, R.J. Fermented Foods as Experimentally Tractable Microbial Ecosystems. *Cell* **2015**, *161*, 49–55. [CrossRef] [PubMed]
75. Berg, G.; Rybakova, D.; Fischer, D.; Cernava, T.; Vergès, M.-C.C.; Charles, T.; Chen, X.; Cocolin, L.; Eversole, K.; Corral, G.H.; et al. Microbiome definition re-visited: Old concepts and new challenges. *Microbiome* **2020**, *8*, 103. [CrossRef]
76. Jousset, A.; Schmid, B.; Scheu, S.; Eisenhauer, N. Genotypic richness and dissimilarity opposingly affect ecosystem functioning: Genotypic diversity and ecosystem functioning. *Ecol. Lett.* **2011**, *14*, 537–545. [CrossRef]
77. Konopka, A. What is microbial community ecology? *ISME J.* **2009**, *3*, 1223–1230. [CrossRef]
78. Levante, A.; Lazzi, C.; Vatsellas, G.; Chatzopoulos, D.; Dionellis, V.S. Genome Sequencing of five Lacticaseibacillus Strains and Analysis of Type I and II Toxin-Antitoxin System Distribution. *Microorganisms* **2021**, *9*, 648. [CrossRef]
79. Prosser, J.I.; Bohannan, B.J.M.; Curtis, T.P.; Ellis, R.J.; Firestone, M.K.; Freckleton, R.P.; Green, J.L.; Green, L.E.; Killham, K.; Lennon, J.J.; et al. The role of ecological theory in microbial ecology. *Nat. Rev. Microbiol.* **2007**, *5*, 384–392. [CrossRef]
80. De Angelis, M.; Gobbetti, M. Environmental stress responses in *Lactobacillus*: A review. *Proteomics* **2004**, *4*, 106–122. [CrossRef]
81. Hutkins, R.W. (Ed.) *Microbiology and Technology of Fermented Foods*, 1st ed.; Wiley: Hoboken, NJ, USA, 2006. [CrossRef]
82. Ryall, B.; Eydallin, G.; Ferenci, T. Culture History and Population Heterogeneity as Determinants of Bacterial Adaptation: The Adaptomics of a Single Environmental Transition. *Microbiol. Mol. Biol. Rev.* **2012**, *76*, 597–625. [CrossRef] [PubMed]
83. Boddy, L.; Wimpenny, J.W.T. Ecological concepts in food microbiology. *J. Appl. Bacteriol.* **1992**, *73*, 23s–38s. [CrossRef] [PubMed]
84. Juillard, V.; Spinnler, H.E.; Desmazeaud, M.J.; Boquien, C.Y. Phénomènes de coopération et d'inhibition entre les bactéries lactiques utilisées en industrie laitière. *Lait* **1987**, *67*, 149–172. [CrossRef]
85. Mossel, D.A.A.; Struijk, C.B. The contribution of microbial ecology to management and monitoring of the safety, quality and acceptability (SQA) of foods. *J. Appl. Bacteriol.* **1992**, *73*, 1s–22s. [CrossRef] [PubMed]
86. Calabrese, F.M.; Ameur, H.; Nikoloudaki, O.; Celano, G.; Vacca, M.; Junior, W.J.F.L.; Manzari, C.; Vertè, F.; Di Cagno, R.; Pesole, G.; et al. Metabolic framework of spontaneous and synthetic sourdough metacommunities to reveal microbial players responsible for resilience and performance. *Microbiome* **2022**, *10*, 148. [CrossRef]

87. Lynch, C.M.; McSweeney, P.L.H.; Fox, P.F.; Cogan, T.M.; Drinan, F.D. Manufacture of Cheddar cheese with and without adjunct lactobacilli under controlled microbiological conditions. *Int. Dairy J.* **1996**, *6*, 851–867. [CrossRef]
88. Jeanson, S.; Floury, J.; Gagnaire, V.; Lortal, S.; Thierry, A. Bacterial Colonies in Solid Media and Foods: A Review on Their Growth and Interactions with the Micro-Environment. *Front. Microbiol.* **2015**, *6*, 1284. [CrossRef]
89. Skandamis, P.N.; Jeanson, S. Colonial vs. planktonic type of growth: Mathematical modeling of microbial dynamics on surfaces and in liquid, semi-liquid and solid foods. *Front. Microbiol.* **2015**, *6*, 1178. [CrossRef]
90. Skandamis, P.N.; Nychas, G.-J.E. Quorum Sensing in the Context of Food Microbiology. *Appl. Environ. Microbiol.* **2012**, *78*, 5473–5482. [CrossRef] [PubMed]
91. Heuer, H.; Abdo, Z.; Smalla, K. Patchy distribution of flexible genetic elements in bacterial populations mediates robustness to environmental uncertainty: Population-level robustness through genome flexibility. *FEMS Microbiol. Ecol.* **2008**, *65*, 361–371. [CrossRef] [PubMed]
92. Penn, K.; Jenkins, C.; Nett, M.; Udwary, D.W.; Gontang, E.A.; McGlinchey, R.P.; Foster, B.; Lapidus, A.; Podell, S.; Allen, E.E.; et al. Genomic islands link secondary metabolism to functional adaptation in marine Actinobacteria. *ISME J.* **2009**, *3*, 1193–1203. [CrossRef] [PubMed]
93. Avery, S.V. Microbial cell individuality and the underlying sources of heterogeneity. *Nat. Rev. Microbiol.* **2006**, *4*, 577–587. [CrossRef] [PubMed]
94. Koutsoumanis, K.P.; Aspridou, Z. Individual cell heterogeneity in Predictive Food Microbiology: Challenges in predicting a "noisy" world. *Int. J. Food Microbiol.* **2017**, *240*, 3–10. [CrossRef] [PubMed]
95. Rodriguez-Valera, F.; Martin-Cuadrado, A.-B.; Rodriguez-Brito, B.; Pašić, L.; Thingstad, T.F.; Rohwer, F.; Mira, A. Explaining microbial population genomics through phage predation. *Nat. Rev. Microbiol.* **2009**, *7*, 828–836. [CrossRef] [PubMed]
96. Somerville, V.; Schowing, T.; Chabas, H.; Schmidt, R.S.; von Ah, U.; Bruggmann, R.; Engel, P. Extensive diversity and rapid turnover of phage defense repertoires in cheese-associated bacterial communities. *Microbiome* **2022**, *10*, 137. [CrossRef] [PubMed]
97. Meouche, I.E.; Siu, Y.; Dunlop, M.J. Stochastic expression of a multiple antibiotic resistance activator confers transient resistance in single cells. *Sci. Rep.* **2016**, *6*, 19538. [CrossRef]
98. Viney, M.; Reece, S.E. Adaptive noise. *Proc. R. Soc. B Biol. Sci.* **2013**, *280*, 20131104. [CrossRef]
99. Gatti, M.; De Dea Lindner, J.; Gardini, F.; Mucchetti, G.; Bevacqua, D.; Fornasari, M.E.; Neviani, E. A Model to Assess Lactic Acid Bacteria Aminopeptidase Activities in Parmigiano Reggiano Cheese During Ripening. *J. Dairy Sci.* **2008**, *91*, 4129–4137. [CrossRef]
100. Calasso, M.; Mancini, L.; Di Cagno, R.; Cardinali, G.; Gobbetti, M. Microbial cell-free extracts as sources of enzyme activities to be used for enhancement flavor development of ewe milk cheese. *J. Dairy Sci.* **2015**, *98*, 5874–5889. [CrossRef] [PubMed]
101. Lane, C.N.; Fox, P.F.; Walsh, E.M.; Folkertsma, B.; McSweeney, P.L.H. Effect of compositional and environmental factors on the growth of indigenous non-starter lactic acid bacteria in Cheddar cheese. *Lait* **1997**, *77*, 561–573. [CrossRef]
102. Montel, M.C.; Buchin, S.; Mallet, A.; Delbes-Paus, C.; Vuitton, D.A.; Desmasures, N.; Berthier, F. Traditional cheeses: Rich and diverse microbiota with associated benefits. *Int. J. Food Microbiol.* **2014**, *177*, 136–154. [CrossRef] [PubMed]
103. Visser, S. Proteolytic Enzymes and Their Relation to Cheese Ripening and Flavor: An Overview. *J. Dairy Sci.* **1993**, *76*, 329–350. [CrossRef]
104. Wilkinson, M.G.; Kilcawley, K.N. Mechanisms of incorporation and release of enzymes into cheese during ripening. *Int. Dairy J.* **2005**, *15*, 817–830. [CrossRef]
105. De Pasquale, I.; Calasso, M.; Mancini, L.; Ercolini, D.; Storia, A.L.; De Angelis, M.; Di Cagno, R.; Gobbetti, M. Causal Relationship between Microbial Ecology Dynamics and Proteolysis during Manufacture and Ripening of Protected Designation of Origin (PDO) Cheese Canestrato Pugliese. *Appl. Environ. Microbiol.* **2014**, *80*, 4085–4094. [CrossRef]
106. Giraffa, G.; Neviani, E. Different Lactobacillus helveticus strain populations dominate during Grana Padano cheesemaking. *Food Microbiol.* **1999**, *16*, 205–210. [CrossRef]