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Review

Challenging Sustainable and Innovative Technologies in Cheese Production: A Review

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Abstract: It is well known that cheese yield and quality are affected by animal genetics, milk quality (chemical, physical, and microbiological), production technology, and the type of rennet and dairy cultures used in production. Major differences in the same type of cheese (i.e., hard cheese) are caused by the rennet and dairy cultures, which affect the ripening process. This review aims to explore current technological advancements in animal genetics, methods for the isolation and production of rennet and dairy cultures, along with possible applications of microencapsulation in rennet and dairy culture production, as well as the challenge posed to current dairy technologies by the preservation of biodiversity. Based on the reviewed scientific literature, it can be concluded that innovative approaches and the described techniques can significantly improve cheese production.

Keywords: heredity; lactic acid bacteria; dairy cultures; cheese quality; rennet; chymosin; coagulation; biodiversity; sustainable; microencapsulation

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1. Introduction

Cheese production is a process dating back several thousands of years, and cheese, which is found in every part of the world, has found its place on the top of the foodstuffs chart thanks to its nutritional value and rich diversity. The earliest indication of a cheese-making process is in cave paintings around 5000 BC, representing the oldest technological application of enzymes [1]. This application has made possible the intentional conversion of milk into cheese, making it safer and longer-lasting.

Since its beginning, the technology of cheese production has changed, thanks to technological and scientific advancements in its materials and production process. Currently, cheese production is very high-level, thanks to modern materials and incorporated technology. Cheese quality is affected by the genotypic and phenotypic characteristics of animals, the chemical and microbiological properties of milk, and production technology [2–4]. An important factor in the diversity of milk from the same breed comes from dairy cultures and the type of rennet used in cheese production, because they affect chemical processes during production and ripening.

Currently, cheese quality is still affected by the same factors, but due to globalization and industrial advancements, originality and dissimilarities within certain regions have been lost. Large-scale cheese production is mainly based on the usage of pasteurized milk and cheesemakers are supplied by a few worldwide producers of dairy cultures and rennet. When considering the same type of cheese production (i.e., hard cheese), this means that

differences between producers are based solely on the quality of milk and the selection of rennet and dairy cultures.

The characteristics of milk affecting cheese quality are regulated by animal genetics, which creates the possibility of using quantitative genetics to preserve biodiversity and indigenous traits. Raw milk and cheeses from raw, unpasteurized milk present rich sources of beneficial microbes, such as lactic acid bacteria (LAB) with probiotic properties [5]. The isolation of indigenous LAB cultures and their use in cheese production can result in the preservation of biodiversity and better diversification between cheese producers globally and regionally.

Microencapsulation is seen as a novel approach to the preservation of biodiversity and to delivering important ingredients into cheese, although it should be borne in mind that the successful encapsulation of payload relevant to cheese production, such as microorganisms, enzymes, peptides, aromatic compounds, chemical agents (Ca^{2+}), and even essential oils, can be extremely challenging. Furthermore, combinations of more than one active ingredient can make the process of encapsulation even more complex [6–9].

Considering that little research on this topic has been undertaken, this paper aims to investigate current progress in DNA characterization and the use of quantitative genetics for improving desired traits in dairy animals, rennet production, and analysis, the isolation and production of lactic acid bacteria, and the possible applications of microencapsulation in the development of new, innovative, and sustainable technologies in cheese production, with an emphasis on indigenous forms and the preservation of biodiversity.

2. The Impact of Livestock Genetics on Milk as an Important Component in Cheese Production

The production of cheese is a complex process, whose quality and uniqueness depend on a variety of factors. The composition and characteristics of raw milk, which are largely determined by the genetics of the animals involved in its production, are certainly among the important factors that contribute to the uniqueness of cheese as a final product [10]. Therefore, the genetics of an individual or a specific population (breed) is one of the crucial elements for the successful production of indigenous dairy products, which are often commercially associated with the Protected Designation of Origin (PDO). At the same time, the influence of animal genetics on the quality and distinctiveness of cheese fits well with the concept of sustainable animal husbandry and the protection of the diversity of farm animals and dairy products.

2.1. Polygenic Inheritance

It was recognized very early that milk secretion is influenced by heredity [11]. Later, it was clearly defined that the composition and characteristics of raw milk are measured on a continuous scale and, like most economically relevant livestock production traits (phenotypes), are inherited as quantitative or complex genetic traits (for more detailed explanations, see [12]). For a long time, the inheritance of quantitative traits was successfully modeled by an infinitesimal model, in which an infinite number of loci, each with an infinitesimal effect (polygenic component), and the influence of the environment are responsible for measured (phenotypic) variations [13–15]. Therefore, in many cases, the inheritance of raw milk quantity and composition has been quantified by parameters such as heritability, more specifically heritability in the narrow sense (h^2), i.e., the proportion of phenotypic variation explained by additive gene effects [12]. Milk, milk fat yield, and later, protein and lactose yield, had the longest tradition of measurement and were recognized as the most important factors in milk production for a century [16]. It is therefore not surprising that the quantitative inheritance of these traits is among the best-studied, while a large number of studies provide estimates of heritability and genetic correlations. In Table 1, we present a representative overview of the estimated h^2 values for the most important traits related to milk production in cattle.

It is important to point out that the traditional traits of milk production (milk yield, fat yield, and protein yield) have been successfully incorporated into breeding programs because they fulfill the desirable characteristics of selection programs. According to Shook [17], successful breeding programs rely on preferred traits that should meet the following selection criteria: (1) they must have relatively high genetic variability and heritability, (2) they must have economic value that increases the profitability of production, (3) they must be positively correlated with other traits used in the breeding program, and, finally, (4) they must be clearly defined and measurable at low cost. Shortening the generation interval increases the annual genetic progress (response).

Table 1. Heritability estimates for milk production traits in cattle.

Species	Trait	Heritability	Reference
Cattle	Milk yield	0.34	[18]
		0.09	[19]
		0.10	[20]
		0.07	[21]
	Milk fat (%)	0.43	[18]
		0.11	[20]
		0.39	[19]
	Protein (%)	0.34	[18]
		0.29	[20]
		0.30	[19]
	Casein (%)	0.34	[21]
		0.35	[20]
		0.35	[19]

Therefore, all these traits should be measured at an early life stage. Furthermore, the reliable estimation of heritability and genetic correlations requires a large sample size, which is sometimes difficult to ensure, even in experimental research analyses.

Some other traits, such as saturated and unsaturated fatty acid content, mineral content (Ca, P, Mg, K, Se, and Zn), milk protein fraction content (casein and serum proteins), and milk coagulation properties (RCT—rennet coagulation time, min; k_{20}—the time needed for the curd to obtain firmness of 20 mm, min; a_{30}—curd firmness at the end of analysis, mm) have been of interest to researchers for a long time [22–27], but their implementation in the selection process has been difficult because their routine measurements were expensive and time-consuming, which prevented their implementation in breeding programs. The estimates of heritability for some important traits (variables) related to cheese production and manufacture in cattle, sheep, and goats are presented in Table 2, while a more detailed description of the majority of genetic factors that have a major influence on coagulation traits in cattle is provided by Bittante et al. [27].

Table 2. Heritability estimates for the milk production traits in cattle.

Species	Trait	Heritability	Reference
Cattle	RCT	0.35	[18]
		0.25	[19]
		0.29	[28]
		0.23	[20]

Table 2. *Cont.*

Species	Trait	Heritability	Reference
	k_{20}	0.66	[18]
		0.21	[20]
	a_{30}	0.57	[18]
		0.15	[19]
		0.12	[28]
		0.17	[20]

RCT—rennet coagulation time, min (time from rennet addition to the beginning of coagulation), k_{20}—time needed for the curd to achieve firmness of 20 mm (min), a_{30}—curd firmness at the end of analysis (mm).

However, Etzion et al. [29] used mid-infrared spectrometry (MIR) to predict milk protein content. Their publication seems to have stimulated a large number of studies on the use of the same methodology for different milk constituents. For example, Soyeurt et al. [30] showed that fatty acid content can be efficiently and inexpensively predicted from MIR signals, soon followed by the use of MIR to estimate the coagulation properties of milk [31–33], the mineral content (i.e., Ca and P) [34], and the content of milk protein fractions [35,36]. More detailed information on the use of MIR in the estimation of different variables (chemical components) from milk is described by Bittante and Cecchinato [37] and De Marchi et al. [38]. In this way, MIR phenotyping has enabled the estimation of genetic parameters for many chemical components associated with cheese production.

As might be noted from Table 2, the heritability estimates for almost all cheese production traits are within the range that would allow sufficiently large and profitable selection progress.

2.2. Candidate Genes

The remarkable improvements of domestic animals achieved by artificial selection in the 20th century were based exclusively on the infinitesimal model [39,40], while the idea that genes with large effects or candidate genes could influence continuously varying traits has been challenged sporadically [41–44]. Thus, the main objective of a series of studies conducted from 1970 to the end of the 20th century was to find significant genotype–phenotype associations that could be functionally explained in so-called candidate gene association studies (CGAS). In the absence of efficient molecular genetic methods and possibilities to determine the genotype of each functional gene on a large scale (population), the major ruminant milk proteins (in ruminants, *αs1*-CN, β-CN, *αs2*-CN, and γ-CN represent four caseins, which together with β -LA and β -LG are responsible for coding 95% of the proteins in milk) were among the best-studied candidate genes, since their polymorphism was discovered quite early [45,46]. They were quite polymorphic [47–50], their genotyping ("phenotyping" as it was based on electrophoretic migration of proteins) was simple and inexpensive [51], while their causal relationship with milk production and technological traits was biologically expected. A large number of significant associations in milk production traits and milk protein polymorphisms were found in different breeds of cattle [52–57], sheep [58–62], and goats [62].

We should mention that four casein genes are tightly linked in a physical map in the order CSNAS1–CSNB–CSNS2–CSN3 within a 250 kb region on chromosome 6 in cattle [63,64], goats [63], and sheep [65], so it is impossible to completely separate the effects of each casein gene. For example, Meier et al. [64] analyzed whole-genome sequences in 14 cattle breeds (1821 animals) and found 37 different haplotypes. Haplotype B-A2-A-A (CSNAS1–CSNB–CSNS2–CSN3) was predominant in Angus (0.64), Charolais (0.33), Flechvieh (0.42), Gelbvieh (0.49), Holstein (0.53), Herford (0.36), Limousin (0.42), and Simmental (0.45) cattle breeds, while other haplotypes were predominant in Brown Swiss

(B–A2–A–B = 0.50), Danish Red (B–A1–A–A = 0.44), DNS (B–A1–A–A = 0.72), Jersey (C–A2–A–B = 0.51), Normande (C–A2–A–B = 0.28), and Montebéliarde (B–A2–D–B = 0.36).

Therefore, it is quite reasonable to estimate the effects of segregating casein haplotypes (a combination of alleles at different loci on the same chromosome of an organism that are inherited together from one parent), as has been achieved with cattle [66,67], sheep [61], and goats [68,69]. For more details on the association of milk protein polymorphism with milk production and/or technological traits in cattle, sheep, and goats, see Buchberger and Dovč [70], Martin et al. [71], Caroli et al. [49], Bittante et al. [27], and Selvaggi et al. [50].

Using various sources of information, Ogorevc et al. [72] identified a large number of other potential candidate genes, some of which were identified in association studies that were likely to be associated with milk production traits. The most promising candidate genes are the following: ATP-binding cassette sub-family G—ABCG2 [73,74], Diacylglycerol acyltransferase—DGAT1 [75–78], growth hormone receptor—GRH [77], leptin—LEP [77–79], peroxisome proliferator-activated receptor-γ coactivator-1—PPRGC1 [80,81], prolactin—PRL [82–84], signal transducer and activator of transcription 5A—STAT5A [85], and insulin-like growth factor 1 -IGF-1 [86]. For more information on candidate genes with major effects on milk production, see Ibeagha-Awemu et al. [87] and Strucken et al. [88].

2.3. Genome-Wise Analyses and Genomic Selection

Recent developments in molecular genetics have enabled the genotyping of a large number of markers distributed across the genome, which has greatly improved our understanding of variation in complex traits [89–91]. Thus, based on new evidence from genome-wide association (GWA) studies, we have revised the concept of infinitesimal inheritance to a mixed inheritance model, in which variability in complex traits is caused by a polygenic component (many genes with small effects), as well as the existence of a set of candidate genes with medium-to-large effects that rarely explain more than 5% of phenotypic variation in humans [92–94].

In human populations, genes with moderate-to-large effects occur at low frequencies as rare or "private" mutations [95], whereas the occurrence of mutations with moderate-to-large effects at moderate or high frequencies has been documented more in domestic animal populations [95–97]. This difference is thought to be the result of artificial selection pressure and specific breeding goals. For good examples of candidate genes with strong effects on complex traits that segregate at moderate-to-high frequencies, see Kemper and Goddard [95] and Curik et al. [97].

Overall, the invention of the concept of genomic selection [98] and the availability of high-throughput genotyping data at low cost have revolutionized animal breeding in the last two decades. As a result, numerous GWA studies have been conducted on milk production [99–105] and technological traits [106,107] in cattle, sheep, and goat populations, pointing to a number of influential causative genes (mutations) and/or statistically associated markers. While some of these analyses have confirmed the results of the association studies, we have also been able to identify genes that have illuminated our biological understanding of milk as a raw material for good cheese production.

In general, genomic selection significantly accelerates annual genetic gain by shortening the generational interval and allowing the more efficient and rapid achievement of breeding goals [108–111].

2.4. Future Perspectives and Sustainable Development

Over the past two decades, our current technological knowledge of breeding for more specific milk composition and coagulation characteristics has improved considerably. This is primarily due to developments in and the availability of large-scale high-throughput genotyping [109,110,112], the phenotyping of new traits—previously measured at very high cost [113,114], the utilization of genomics in animal breeding [110,115], and our ability to analyze very large data sets [113,116]. Therefore, efficient breeding for more specialized dairy products is a very realistic option. At the same time, we are more

aware of the potential for the adaptation of some landscape breeds to harsh environmental conditions and the potential negative consequences that could result from current climate changes [117,118]. However, on a broader scale, we are still not bold enough to radically shift our breeding objectives focused on milk quantity and basic milk composition (fat and protein) to more specialized milk composition and dairy products (cheese). Fortunately, the new options available to us are recent and offer many opportunities and challenges to make more sustainable choices in the future.

3. Isolation and Techniques of Rennet Production

3.1. Nomenclature and Function of Rennet

Peptidases are a large and important group of enzymes, many of which have been applied in technological processes involving food, beverages, feed, medicines, detergents, the production of chemicals, leather, paper, and textile processing. The active milk-clotting enzymes in all preparations that are successful in cheesemaking are aspartic proteinases (EC 3.4.23), which are so named because aspartic (Asp) residues are ligands of the activated water molecule within their active sites, which mediates nucleophilic attacks on scissile peptide bonds [119]. In the great majority of known aspartic peptidases, two Asp residues act together to bind and activate catalytic water molecules but in some, the residues of other amino acids replace the second Asp. One notable characteristic of aspartic peptidases is that all the enzymes described so far are endopeptidases. Endopeptidases cleave peptide bonds in the inner parts of their polypeptide chains, away from the N- and the C-terminus.

Peptidases are distinguished by the functional group in their active site: (A) for aspartate, (C) for cysteine, (G) for glutamic, (M) for metallo, (P) for mixed, (S) for serine, (T) for threonine, (N) for asparagine lyase, and (U) for as-yet unclassified peptidases of unknown catalytic type. Based on statistically significant similarities in their primary structures, peptidases are classified into families (approximately 268), which are identified by a letter representing their catalytic type. Further, some families may contain subfamilies. Some families are divided into subfamilies based on evidence of ancient divergence within the family (e.g., S1A, S1B). Families are then grouped into about 62 clans according to similarities in their 3D structures. Some clans are divided into subclans based on evidence of ancient divergences within the clan. The name of the clan consists of two letters. The first letter represents the catalytic type and the second letter is assigned sequentially as each clan is identified. This method of classification is used in the MEROPS database [120] for peptidases and the proteins that inhibit them [121,122].

In addition to this classification, depending on the functional group in the active peptidase site, peptidases are additionally classified according to the specificity of certain amino acids ("sequence specificity"), which form sensitive peptide bonds. For example, aspartate endopeptidases from the pepsin family hydrolyze peptide bonds with large hydrophobic amino acids in P1 or P1′ [121,123,124].

Aspartic peptidases are assigned to clans AA, AC, AD, AE, and AF [121]. They are a group of peptidases in the pepsin family (A1), with the same catalytic mechanism, and usually function in acidic solutions, which is why they are called acid peptidases. Aspartic peptidases have a long history and are found in animals, fungi, plants, protozoa, bacteria, and viruses. The feature of action in specific acidic environments limits their functions in living organisms and they are less abundant than other groups of peptidases, but because of its great physiological and commercial importance, this class of peptidases is unique [125]. Most aspartic proteases show maximal activity at low pH (pH 3 to 4) and have isoelectric points in the range of pH 3.0 to 4.5. Their molecular masses are between 30 to 45 kDa [126].

According to its classification in the MEROPS databanks, chymosin (EC 3.4.23.4) is a member of clan AA, family (pepsin) A1, subfamily A1A peptidase within the third class of hydrolases [121,122,127,128]. Deschamps performed the first isolation of this enzyme in 1840 and suggested the name chymosin (Gr. chyme, "gastric juice") [126]. In 1890, Lea and Dickinson [129] suggested the name rennin (derived from rennet), but due to confusion with the related proteolytic enzyme, renin, from the kidneys, in 1970, Foltman suggested

a return to the first name, chymosin, which was accepted by the International Union of Biochemistry and Molecular Biology (IUBMB) [130]. In 1872, Olof Hammarsten showed that rennet (chymosin) was synthesized and stored in an inactive form and activated by contact with stomach acid; this was the first thorough study on the effects of rennet on casein [131].

According to the MEROPS databanks, pepsin A is a member of clan AA, family (pepsin) A1, subfamily A1A peptidase within the third class of hydrolases [127,132]. Pepsin A (EC 3.4.23.1), known simply as "pepsin", was discovered and recognized, in the 18th century, as the first enzyme that starts the digestion of food proteins in the stomach. Pepsin was originally named by Schwann in 1825. Pepsin is the predominant gastric peptidase in the fundus of adult mammals [121,131,132] and is exceptionally stable and active in acidic conditions. It is an endopeptidase characterized by lower specific activity, high general proteolytic activity, and a high pH dependency. Pepsin B (EC 3.4.23.2) is a minor proteinase found in porcine stomachs [1].

Plant aspartic peptidases are members of clans AA and AD. In clan AA, they are distributed among the families A1, A3, A11, and A12. The majority of plant aspartic peptidases, together with pepsin-like enzymes, belong to the A1 family [133].

Rennet (a mixture of chymosin and pepsin) is produced in the fourth stomach (the abomasum) of suckling ruminants (calves, lambs, kids/caprine, etc.). Chymosin is a neonatal peptidase that has a postnatal uptake of immunoglobulins [1] and can be found in fetuses from as early as the sixth month of gestation, with the biggest increase between the ninth month and the third or fourth day post-partum [134]. The reason for the secretion of chymosin in the stomach of newborn ruminants is the coagulation of milk to increase the nutritional value during retention in the intestine, allowing young animals to utilize more nutrients. Both peptidases (chymosin and pepsin) are secreted in their inactive form as zymogens (prochymosin and pepsinogen) into the channel with a direct connection to the lumen of the stomach, and they are activated by the low pH in the abomasum to chymosin and pepsin by the removal of the N-terminal pro-segment. The pro-segment is responsible for the stability of the inactive forms of zymogens and prevents the binding of the substrate into the active site. Chymosin is a polypeptide built from 323 amino acids, with a molecular weight of 35.6 kDa [119,135]. Generally, chymosin has low proteolytic activity but high milk-clotting activity. Calf chymosin has a narrow substrate specificity and cleaves the specific single-peptide bond in κ-casein between Phe 105 and Met 106, converting it into an insoluble form of curd with calcium para-κ-casein [136]. According to Harboe et al. [1], chymosin has been found to have high activity against milk of its own species. Calf chymosin is found in three allelic forms: A, B, and C. Chymosin B is the most abundant. The main difference between the A and B variants is that variant A has an aspartic acid (Asp) in position 244 and variant B has a glycine (Gly) in the same position. Variant A has 50% more proteolytic activity than the B form [126]. Variant C is genetically distinct and is a product of a different allele [137]. Thanks to its use in the cheese industry, calf chymosin is well investigated and characterized at enzymatic and molecular levels [133].

3.2. Types of Rennets and Most Commonly Used Coagulants in Cheese Production

The most important enzyme preparation in cheese production is rennet. It is a mixture of chymosin and pepsin, which is responsible for milk coagulation, but also proteolytic processes, during cheese ripening. Traditionally, rennet was produced by extracting the gastric juices of suckling mammals (calves, lambs, or kids) to obtain an animal rennet. Until the 19th century, the cheese was produced directly on farms and producers used homemade extracts from dried abomasa to coagulate the milk. Starting in the 1850s, cooperative dairies were established, which increased the demand for the production of larger amounts of rennet [126]. The industrial production of calf rennet (chymosin) dates back to 1874, in Denmark, when Christian Hansen started with the extraction of chymosin from calves' abomasa for cheese production. Rennet was the first industrially

produced enzyme preparation sold with standardized enzyme activity. The worldwide increase in cheese production during the 20th century led to a rennet shortage because the demand began to exceed the available supply around the 1950s and 1960s [138]. This problem was solved with the introduction of new types of milk-clotting agent, such as animal rennets (e.g., bovine pepsin) [139], microbial rennets (*Rhizomucor miehei*, *Rhizomucor pussillus*, *Parasitica coagulans*) [140], fermentation-produced chymosin (FPC) produced from *Kluyveromyces marxianus var. lactis* and *Escherichia coli K-12* [141] or plant origins (*Calotropis procera*, *Cynara cardunculus*) [142,143].

Calf chymosin is one of the most frequently studied animal enzymes, and it was one of the first mammalian enzymes to have been successfully cloned, in the 19th century [144,145]. Because of its commercial importance, the gene for chymosin is the most commonly used and has been cloned and expressed at a higher level in various microbial hosts, such as: (1) bacteria *Escherichia coli* (Pfizer) [145–147], (2) mold *Aspergillus niger* (C. Hansen) [1], and (3) yeast *Kluyveromyces marxianus var. lactis* (Gist-brocades) [1,148]. Bovine chymosin is also successfully cloned in other microbial hosts, such as *A. nidulans* [149] and *Pichia pastoris* [150–153]. There is a significant volume of scientific literature about the production and biochemical properties of recombinant chymosin from other mammalian species: (1) camel [133,154–157], (2) lamb/ovine [158], (3) kid/caprine [155,159–161], (4) buffalo [155,162,163], (5) yak [164,165], and (6) alpaca [166].

According to Jacob et al. [167], of all coagulants or rennet substitutes, recombinant *Bos Taurus* chymosin is by far the most prominent genetically engineered clotting enzyme. The downside regarding FPC is its production through the fermentation of genetically modified organisms, which may be the reason why consumers prefer different products. Microbial rennets, on the other hand, are not products of GMO; they are easily produced in unlimited quantities, and their characteristics can be improved using biotechnology tools [168]. Since they are not produced from animal tissue (i.e., calf, lamb, kid) there is no possibility of transferring bovine spongiform encephalopathy (BSE) or scrapie [167]. Microbial coagulants, however, show higher proteolytic activity compared to rennet or FPC, which can cause lower cheese yield during production and the development of bitterness during the ripening process [167,168]. Plant or vegetable coagulants are rarely used for commercial production [133]. Ben Amira et al. [169] state that the use of vegetable coagulants is very limited for large-scale production because of their negative effect on cheese yield and the development of bitterness. Garcia et al. [170] reported that dry matter content, hardness, gumminess, and chewiness showed significant differences in cheeses made with vegetable coagulants and calf rennet, with higher values being obtained in cheeses made with vegetable coagulants. Manuelian et al. [171], in their study on mozzarella made from buffalo milk, reported slower coagulation after the addition of vegetable coagulants than with rennet. They concluded that longer coagulation times could be overcome by adding more coagulant, but further studies are needed to assess the sensory properties and textural characteristics of the product. Peptidases extracted from the cardoon flowers of various species of the genus Cynara have been used to make artisanal sheep cheeses in Portugal and Spain, especially Serra da Estrela cheese [143].

The original rennet preparation, commonly called animal rennet, is an extract from ruminant abomasum. According to this definition, it is agreed that the name "rennet" should be used for enzyme preparations from ruminant stomachs, and that other milk-clotting enzymes should be named "coagulants". Rennets and coagulants are mostly categorized according to their source: (1) animal rennet and coagulants, (2) microbial coagulants, (3) fermentation-produced chymosin, and (4) vegetable coagulants. Chymosin produced by a genetically modified organism, as in, *A. niger* and *K. lactis*, is named fermentation-produced chymosin (FPC) [1,133,172]. According to the literature review, the known fermentation-produced chymosins (FPCs) from mammals are recombinant bovine chymosin [155], ovine prochymosin [158], caprine prochymosin [159,173,174], water buffalo chymosin [162], and camel chymosin [154].

Rennet, obtained from the gastric juices of suckling mammals (lambs, calves, kids) is produced mostly in liquid, solid, and paste form. Rennets in the form of pastes are traditionally used in countries in the Mediterranean region at the artisanal level and contain variable amounts of lipolytic activity with chymosin and pepsin [119,175,176]. Lamb and kid rennets are used in Greece for the production of Feta and Ketalotyri cheese from sheep and goats' milk [175].

Due to its high content of chymosin, calf rennet is regarded as the ideal enzyme product for cheesemaking [1]. In the abomasum and extracts from its tissues, the proportion varies between chymosin and pepsin. The ratio between these two enzymes depends on the age and the feeding regime of the animal, as shown in Table 3 [126,177]. The most desirable proportion of chymosin to pepsin in preparation for cheese-making in regular calf rennets is 80:20 [119,126,177]. Generally, extracts from younger and milk-fed animals are higher in chymosin, while extracts from adult animals are higher in pepsin. In the world production of rennet, animals are slaughtered at different ages, and extracts feature different kinds of enzyme mixture.

Table 3. The ratio between chymosin and pepsin in the bovine abomasal mucosa, depending on the age and feeding regime.

Breeding Stage	Age (Months)	Feeding	Chymosin (Weight %)	Pepsin (Weight %)	Reference
Suckling calf	<3	Milk	80–90	10–20	[126,177]
Milk-fed calf	3–6	Milk Suckled and pasture-fed	70–75	25–30	
Concentrate fed calf	6	Cereals and hay	12	88	
Heifer	12–24	Cereals and hay	2	98	
Cow	>24	Cereals and hay	Traces	100	

Although calf chymosin is the industrial gold standard for cheese manufacturing, chymosin from other animal sources (e.g., lamb, kid, buffalo, and camel) has also been studied as an alternative [119,133]. Several products of lamb/ovine and kid/caprine origin exist, but they are best suited to clotting milk of their species [1,178]. The most commonly used alternative to calf rennet is rennet of adult bovine origin. The most common commercially available recombinant chymosins are Chy-max, obtained by fermentation in *E. coli K-12*; Chymogen and ChymoStar, produced by *Aspergillus niger var awamori*; and Maxiren, generated in *K. marxianus var lactis*. A proteinase from *R. miehei* has been cloned and expressed in *A. oryzae* by Novo Nordisk A/S (Denmark) [1].

3.3. Rennet Production Methods

Procedures for industrial rennet production are very scarce and little is known about them. Although the procedures used by producers vary, the production of animal rennet involves the following commonly used steps: (1) production (abomasa preparation, extraction, activation), (2) the purification and determination of the chymosin: pepsin ratio, (3) standardization, and (4) quality control of the enzyme product (chemical and microbiological). In the past, animal rennet was produced from dried or fresh abomasa; however, currently, it is mainly produced from frozen abomasa [1]. The cleaned enzyme-containing tissues are preserved by salting, freezing, or drying in the slaughterhouse. The abomasa may be washed, dried, and salted before extraction. They are cut into small pieces and minced; the enzymes are then extracted, usually with a 5–10% NaCl and acid solution with or without preservatives, to produce single-strength rennet. The single-strength rennet is then concentrated using ultrafiltration to produce double- or triple-strength rennet [179]. Foltmann [180] used 1/3 M $Al_2(SO_4)$ for the simultaneous clarification and activation of raw extract followed by the addition of 1 M Na_2HPO_4 to adjust pH to final 5.6. After the removal of tissue by filtration, NaCl is usually adjusted to 20% [126,181]. Anifantakis and

Green [175] experimented by changing solutions four times on the same tissue in four days and reported that most enzymes are extracted in the first 24 h, and the rennet is most stable at a pH value of 5.5. The use of ultrasound resulted in an increase in activity and a yield of chymosin with a significantly shorter extraction time (80 min) [182–184]. The crude extract is then activated by lowering the pH to around 2.0 for a certain amount of time. Subsequently, the suspension is often clarified by another centrifugation, filtration, or purified by ion-exchange chromatography to obtain a product enriched with chymosin. The final product is then standardized for strength and enzyme composition (chymosin to pepsin), and subjected to quality control according to specifications. Commercial rennets are found on the market in liquid, powder, or tablet form.

3.4. Determination of Rennet Strength

The most important parameters of rennets are strength (enzyme activity), enzyme composition, identity, and purity. There are many methods to measure the strength (enzyme activity) of rennets, several of which have been inspired by Soxhlet and Berridge. In 1877, Von Soxhlet defined Soxhlet's units (SU) as the volume of raw milk, which one volume of enzyme preparation can clot in 40 min at 35 °C. The strength is expressed as the clotting ratio, e.g., 1:10,000 (1 mL of rennet can clot 10,000 mL of raw milk in 40 min at 35 °C). The SU is easy for cheesemakers to understand, but it depends on both the pH and the milk quality, which causes variations because no reference standards are used; therefore, SU should be considered only as an indicative measurement of milk-clotting activity [1,185]. In 1952, an English scientist, N. J. Berridge [186], introduced standardized milk powder reconstituted in 0.1 M $CaCl_2$ instead of raw milk. The Berridge, or Rennin unit (RU), was created and was widely used until the 1990s by the British Standard Institution (BSI). One RU is defined as the activity that can clot 10 mL of standardized milk in 100 s at 30 °C. The major drawbacks of RU units are that the pH value of the standardized substrate is 6.3, which is lower than in any milk used in cheesemaking (i.e., pH 6.4–6.6), and the very high calcium content of Berridge milk, which gives unreliable results because of calcium ions' effects on cheese curd.

In general, bovine rennets contain both the main milk-clotting enzymes, chymosin and pepsin. Each of these two enzymes has its physicochemical characteristics, which influence the milk-clotting activity and cheese-making properties. The major difference between these two milk-clotting enzymes is in the stronger pH dependence and general proteolytic activity of the pepsin. Thus, for both economic and processing reasons, it is very important to determine the total milk-clotting activity of a certain rennet type in comparison with an internationally recognized reference standard of known composition and milk-clotting activity. The current standard method for bovine rennets and measures of milk-clotting activity is IDF Standard 157:2007/ISO 11815, developed in 1997 by the International Dairy Federation (IDF), known as the "relative milk-clotting activity test" or REMCAT [1,185,187]. The IDF has developed methods for the determination of the milk-clotting activity of microbial enzymes [188] and ovine and caprine rennets [189]. The strength of the enzyme activity i.e., the milk-clotting activity of rennets, is expressed in international milk-clotting units per gram or milliliter (IMCU/g or IMCU/mL). The Berridge clotting-time method is used in all the IDF Standards for the determination of the total milk-clotting activity as the indicator of milk flocculation appearance [185]. The internationally recognized reference standards for the known composition and milk-clotting activity for calf rennet reference standard powder (containing >98% chymosin and <2% bovine pepsin) and adult bovine rennet reference standard powder (containing <2% chymosin and >98% bovine pepsin) were produced by the Association of Manufacturers of Animal-Derived Food Enzymes (AMAFE), appointed by the IDF. Their milk-clotting activity was adjusted to be the same on a standardized milk substrate (0.05 g $CaCl_2$ per 100 mL) at pH 6.5 and 32 °C; it is set at 1000 IMCU/g [126,190].

4. Isolation, Determination, and Application of Indigenous Dairy Lactic Acid Bacteria

4.1. Indigenous Lactic Acid Bacteria (LAB) of Raw Milk and Cheese

The composition of raw milk microbiota is very complex and related to different intrinsic and extrinsic factors, such as udder health, general farm management, milking procedures, hygiene conditions, milk storage on farms, etc. From the public health point of view, the microbiological safety of milk should be always discussed through a tight connection with animal health and environmental hygiene. Our recent findings showed that raw milk from vending machines may be contaminated, although rarely, with foodborne pathogens, such as *L. monocytogenes* [191,192], or multi-resistant *Staphylococcus aureus* [193].

The LAB number in raw milk is very variable and likely related to the phase of the milk chain where and when it is collected. For instance, the LAB population in raw cows' milk at farm level range from 2.0 to 3.6 log CFU/mL [194], while at retail level (vending machines) it may exceed 5 log CFU/mL [192] due to the specific conditions of milk cooling that dictate the growth of psychotropic bacteria during storage, including LAB population. Concerning cheese production, LAB population counts are related to cheese type and storage conditions [195]. Therefore, in soft cheeses, LAB increases from 4 to 7 log CFU/g within a few days at cold storage, while during the ripening and storage of hard cheeses for 1 year, a reduction from 7 to 2 log CFU/g occurs [195].

Within the LAB group, enterococci are considered highly controversial bacteria, and usually referred to as being at the limits of food safety [196]. Enterococci are not necessarily indicators of faecal contamination, as they are ubiquitous bacteria. For example, multi-resistant enterococci strains were isolated from raw milk collected from the udders of healthy cows, which had never been treated with antibiotics but were kept with treated animals [197]. Currently, enterococci are considered beneficial microbes with probiotic properties, while, on the other hand, they are potentially virulent bacteria, producers of biogenic amines, or carriers of resistance genes [198]. Therefore, enterococci are bacteria with a dual role in dairy production, and their most frequently isolated species are *E. faecalis* and *E. faecium* [199].

Enterococci colonize raw materials, including milk, through the digestive system or contaminated environments, where they can survive and multiply during the fermentation process [200]. As fermentative bacteria, enterococci can be crucial for the development of specific sensorial properties in cheese; however, their resistance to high temperatures, salt, and low pH may lead to their excessive growth and cause spoilage [201].

Enterococci also demonstrate strong antimicrobial (antilisterial) activity by producing enterocins [202]. Many dairy-related enterococcal strains are characterized and intended for experimental applications as functional cultures in fermented food production [203]. Raw-milk-originated *Enterococcus faecalis* EF-101 was recently characterized as a novel bacteriocin-producer with inhibitory properties against the majority of food-related *Listeria* strains in vitro [204].

4.2. Determination of Lactic Acid Bacteria in Milk and Cheese

The determination of milk microbiota is often very complex and the reported results are very diverse, depending strongly on the methodology used [205]. Different methods that are used for its identification and detection can be culture-dependent and culture-independent, as shown in Table 4.

Table 4. The methods for the detection and analysis of microbiota.

Culture-Dependent Methods	Culture-Independent Methods	Reference
• Classical phenotypic tests • Biochemical identification kits (analytical profile index (API) systems, Vitek) • Ribosomal DNA analysis (ribotyping) • Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) • Pulsed-field gel electrophoresis (PFGE) • Random amplification of polymorphic DNA (RAPD) • Amplified fragment length polymorphism (AFLP) • Whole-genome sequencing (WGS) • 16S ribosomal RNA sequencing • Matrix-assisted laser desorption/ionization (MALDI) time of flight (TOF) mass spectrometry (MS)	• Denaturing gradient gel electrophoresis (DGGE) • Temporal temperature gradient electrophoresis (TTGE) • Terminal restriction fragment length polymorphism (T-RFLP) • Length heterogeneity polymerase chain reaction (LH-PCR) • Single-strand conformation polymorphism polymerase chain reaction (SSCP-PCR) • Reverse transcription RNA (RT-RNA) • Real-time PCR • Quantitative PCR (qPCR) • Fluorescence in situ hybridization (FISH) • Amplicon-based sequencing • Next-generation sequencing, (NGS) • Metagenomics	[206–208]

Traditional microbiological methods of isolation and identification are costly and time-consuming, with uncertain results for bacterial identification at the species level due to high biochemical (enzymatic) variability, especially in lactic acid bacteria, including enterococci [194]. The results of several studies dealing with LAB in raw milk and cheeses in Croatia are summarized in Table 5. The diversity of non-starter LAB (NSLAB) in autochthonous dairy products from Western Balkan Countries has been recently reviewed by Terzić-Vidojević et al. [209].

Table 5. Results of determination of indigenous microbiota lactic acid bacteria (LAB) of different types of milk and cheese in Croatia.

Strains Selected	Milk or Cheese Type	Methods of Characterization	Reference
E. faecium *E. faecalis* *E. durans* *E. casseliflavus* *Lb. plantarum* *Lb. brevis* *P. pentosaceus* *P. acidilactici* *Streptococcus* spp. *Ln. mesenteroides* *Lc. lactis*	Artisan Istrian Raw Milk Hard Cheese	PCR RAPD-PCR	[210]
E. faecium *E. faecalis* *E. durans* *S. parauberis* *Ln. mesenteroides* *Lc. lactis*	Traditional Istrian Ewe's Cheese	PCR DGGE	[211]
E. faecium *E. faecalis* *E. durans* *S. parauberis* *Ln. mesenteroides* *Lc. lactis*	Different Croatian cheese types derived from raw ewes' milk cheeses	T-RFLP WGS	[212]
E. faecalis *Ln. pseudomesenteroides* Cow cheese: *Lc. lactis* *Lb. paracasei* Ewe cheese: *Ln. mesenteroides* *Lb. plantarum*	Soft cow cheeses	16S rDNA sequencing, rep-PCR	[213]
E. faecalis *E. faecium* *E. durans* *Ln. mesenteroides* subsp. *cremoris* *Ln. mesenteroides* subsp. *dextranicum* *Lc. lactis* subsp. *lactis* *Lb. helveticus*	Cows' and Ewes' cheese maturing in lambskin sacks	API 50 CHL MALDI TOF MS	[214]
Ln. mesenteroides subsp. *cremoris* *Ln. mesenteroides* subsp. *mesenteroides*	raw cows' milk, farm-level	API STREP MALDI TOF-MS	[194]
	Soft cows' cheese	API 50 CHL	[215]
	Soft cows' cheese	API 50 CHL	[216]

Lb. = Lactobacillus; Ln. = Leuconostoc; S. = Streptococcus; P. = Pediococcus; Lc. = Lactococcus; E.= Enterococcus.

4.3. Selection of Indigenous LAB for Dairy Cultures

In general, food safety and quality depend on many specific factors, including favorable or harmful microbial properties. The upgrading of cheese safety and quality may be achieved by applying competitive starter cultures or their metabolites, which are capable of preventing cheese spoilage and extending shelf-life through microbial interactions, reducing or eliminating potential hazards (i.e., pathogens, spoilers, biogenic amine producers, mycotoxins producers, resistant bacteria), and exerting beneficial effects on consumers' health through their probiotic properties [217,218]. LAB strains with probiotic properties have been isolated from different dairy-related niches, including cows' or ewes' raw-milk

cheeses, and whey. Hence, many studies are focused on implementing probiotic strains in fermented milk beverages or cheeses to produce novel products with enhanced health benefits [5].

The most adequate strains for starter cultures are usually selected from autochthonous microbiota, since they are well adapted to the food environment and the specific manufacturing process [214]. The selection procedure for potential starters should involve all potentially "risky" criteria, such as toxigenicity, acquired transmissible antimicrobial resistance, or technologically unacceptable pathways [219]. The general criteria for the selection of starter cultures include safety, technological properties, and economic aspects [214]. Some of the specific criteria for their selection include: (1) the quick and adequate production of lactic acid, the production of L (+)—lactic acid; (2) rapid growth at different temperatures, salt concentrations, and pH; (3) homofermentative metabolism; (4) catalase activity and the hydrolysis of hydrogen peroxide; (5) proteolytic and lipolytic enzyme activity; (6) tolerance or synergism towards other microbial components of starter culture; (7) production of antimicrobial compounds; (8) antagonism towards pathogenic and technologically undesirable microorganisms; (9) lack of antimicrobial resistance, biogenic amines, and mucus production; and finally (10) probiotic properties (tolerance to low pH, tolerance to bile salts, adhesion to human intestinal cells) [214,220,221]. The sensory appearance of traditional cheeses can be achieved and preserved by using selected autochthonous strains as starter cultures in thermally treated milk. Another possible technique in cheese-making is the introduction of starter cultures in the form of whey collected the day before the cheese is made. In addition to quality characteristics, autochthonous microbiota in the form of protective cultures, usually added as adjunct cultures, can affect the hygienic and safety characteristics of cheese. Protective cultures do not alter the sensory properties of cheese, but, rather, the cheese safety by suppressing the pathogenic microbiota. In this sense, their antimicrobial properties in cheese production are directed to toxigenic molds, amine-producing microbiota, or foodborne pathogens, such as Listeria monocytogenes [203,215,222].

4.4. Application of Microencapsulated Bacteria Strains in Cheese Production

Despite their wide application in the pharmaceutical industry, microencapsulated products are still a rarity in the food industry. It is a well-established fact that the viability of probiotic strains in food products is often restricted by many factors. For this purpose, some alternative methods were established to protect microorganisms against undesirable environmental conditions. One of the most promising techniques for bacterial protection is microencapsulation [223]. Gomes da Cruz et al. [224] suggest it as a possible solution to losses in viability due to salting or extended storage.

Microencapsulation is defined as a process of coating small particles of solids, liquids, or gaseous components, with a protective coating material [225], thus providing numerous benefits to encapsulated materials. One of its main advantages is the controlled release mechanism in which the active ingredients are released at controlled rates over prolonged periods [226]. This improves the stability of starter and/or adjunct cultures with a reduction in ripening time. There are many extensive reviews on the microencapsulation techniques used in the food industry, mostly based on the different encapsulating bioactive ingredients and methods that are used. Spray-coating and gel-particle technologies are most often used for the microencapsulation of probiotics [227]. Different types of encapsulating material have been used to trap probiotic bacteria, but the most effective is alginate [228].

Goderska et al. [229] demonstrated that Lactobacillus rhamnosus microencapsulated in alginate matrix kept their viability up to 48 h, unlike free cells that were inactivated completely under the same conditions. Similarly, Abd-Elhamid [230] proved that microencapsulation significantly increased the survival of Bifidobacterium adolescentis in Kariesh cheese during cold storage. However, by contrast, Godward and Kailasapathy [231] claimed that the microencapsulation of probiotic cells in Feta cheese caused a higher microbial loss, probably by inhibiting the disposal of cell metabolites that may have accumulated inside the

encapsulated capsule. In addition to the increased microbial viability, microencapsulation can also affect the chemical and physical properties of foods containing microencapsulated bacteria. In a survey conducted by Ozer et al. [232], the authors noted that the development of proteolysis was more pronounced in cheeses containing probiotic bacteria in the encapsulated form.

Despite the aforementioned facts, microencapsulation technology is yet to become an everyday tool in the development of functional and sustainable food, which can only be achieved by comprehensive research.

5. Microencapsulation

Encapsulation in Cheese Production

The encapsulation process involves the entrapment of active substances into another substance wall material that produces particles on various scales [233–235]. In general, there are two main forms and structures (morphology) of encapsulation system: *matrix type (spheres)* and *core-shell type (capsules)* (Figure 1) [236]. The substance that is encapsulated is usually labeled as the *core*, *fill*, *active*, *internal*, or *payload phase*, while the wall material used for the encapsulation is labeled as *coating membrane*, *shell*, *capsule*, *carrier material*, *external phase*, or *matrix* [233,237,238].

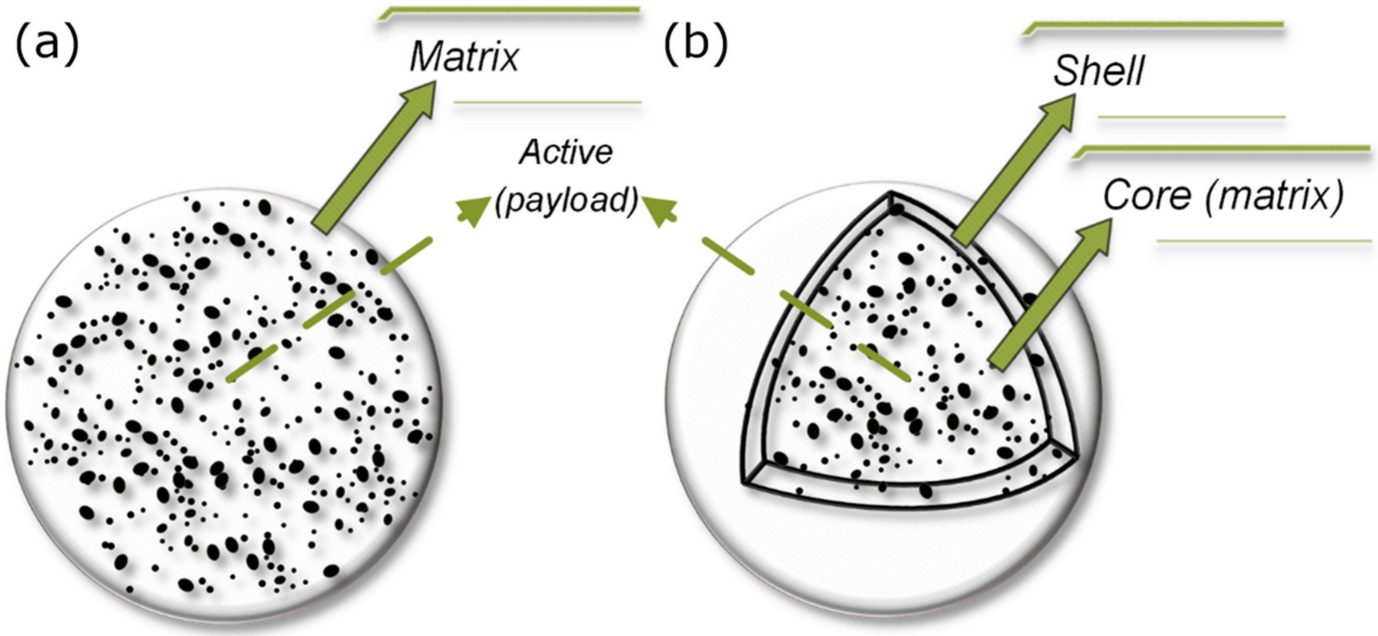

Figure 1. Schematic representation of main forms for active substance encapsulation: (**a**) *matrix type* (*sphere*) and (**b**) *core-shell type* (*capsule*).

Encapsulation technology can significantly contribute to the cheese production process, and it can protect encapsulated ingredients within an effective barrier against environmental factors such as oxygen, light, free radicals, etc. and enable their controlled delivery [239–241]. Due to the increasing popularity of the use of natural materials, encapsulation in biodegradable polymers is becoming versatile. Biopolymers are relatively easy to obtain from natural sources and they may also be prepared with the use of microorganisms or synthesized with precision and with predetermined properties [242]. Numerous techniques can be used for encapsulation in biopolymeric matrices, but the ionic gelation method is often utilized in production, mainly because it uses mild conditions throughout the encapsulation process [243–245]. Encapsulation in biopolymeric microparticles is continuously improving and mostly advancing in the direction of the improvement of physicochemical, functional, and release properties while keeping in mind

cost-effectiveness and the use of environmentally friendly and "green" material throughout the process [246].

The encapsulation process depends on the type of biopolymers used, since they vary in their composition and physicochemical properties. To achieve the successful encapsulation of active ingredients, the proper choice and understanding of biopolymer structure are necessary [247]. The encapsulation process and its effectiveness generally depend on the material that is to be encapsulated (*payload*), the encapsulation matrix material, and the encapsulation method/process. The proper selection of the encapsulation conditions may result in the desirable functionality and properties of the systems encapsulating the core [248].

Before proceeding with the encapsulation process, some issues that may arise should be addressed. The development of microparticles with more than one active ingredient starts with the determination of the conditions of preparation. It is important to observe the influence of each component used in the process of encapsulation. For example, the nature of microorganisms is to interact with their environment; thus, it is important to determine molecular interactions with other components before the encapsulation process [247]. Encapsulation in biopolymeric matrices is suitable for the formulation of particles loaded with more than one ingredient, such as lactic acid bacteria, enzymes, and chemical agents (relevant to the cheese production process, i.e., Ca^{2+}) [249,250]. What makes this challenging is the simultaneous encapsulation of more than one ingredient. These types of particle can be created so that the inner core contains bacterial culture, while the outer shell contains enzymes. Depending on the fundamental desirability, the active ingredient in the outer shell releases faster, while the core material releases at a significantly slower speed. Remarkably, research concerning the development and implementation of these types of microparticle in the cheese production process is non-existent. The proper optimization of particles simultaneously loaded with multiple ingredients may result in a product that can be easily implemented in cheese production, making the entire process simpler from the technological point of view.

Throughout cheese production, there are processes, such as cheese ripening, that are not fully controllable. This is not only time-consuming but also a very complex process that involves the enzymatic breakdown of the protein, carbohydrate, and lipid contents of curd [251,252]. The use of encapsulation technology enables the acceleration of cheese ripening via encapsulated flavourzyme [253]. This is important because the conventional addition of the "free" enzyme is generally not recommended due to the negative influence on the flavor and texture [254–256]. Furthermore, the conveniently encapsulated enzyme can be physically separated from the curd mixture during cheese-making and may be released into the matrix during ripening [257]. This is only one example that shows how encapsulation technology can be implemented in the process of cheese production. In addition to accelerating cheese ripening, encapsulation technology is mainly used to fortify cheese with bioactive compounds or to increase the shelf-life of cheese products [258]. In Table 6, we present recent examples of the employment of the encapsulation method that may aid in the process of cheese production, increasing its shelf-life and adding value.

It is well known that, if present in an appropriate amount of food, probiotics can withstand hostile conditions in the gastrointestinal tract, adhere to cells, and promote positive effects [259]. The sustainability of probiotic microorganisms in any food depends on not only the storage of food but on the food matrix itself, as well as its components (fat, protein, moisture content). However, more importantly, probiotic bacteria must be stable during food processing and storage conditions. However, microorganisms, such as lactic acid bacteria, show low survivability in food products, as well as during food production, while their encapsulation leads to their preservation [260]. For example, Afzaal et al. [261] encapsulated probiotic bacteria, which resulted in a significantly higher survival rate with improved stability and protection during cheese production. Furthermore, Ningtyas et al. [262] encapsulated probiotics with the addition of β-glucan and phytosterol emulsion, which maintained viable cell count throughout the storage of cream cheese. The encapsulation of

probiotic bacteria can be useful for the cheese production process and, when considering its survival and protection, there is the possibility of including natural additives. This may further improve bacterial survivability and even enhance its abilities. With this in mind, the proper selection of natural encapsulation material, with an economically viable and mild production process and the selection of compatible co-encapsulants, is of crucial importance. When considering the implementation of "unconventional" technologies in conventional cheese production processes, encapsulation in biopolymeric microparticles offers numerous benefits. However, some sensory issues may still arise. For example, microparticles are generally in the 1–1000 μm size range [263], and this may influence consumers' appreciation of the product. When considering the application of microparticles into real food products, i.e., cheese, in addition to the enhancement of encapsulated components' properties, protection, and sustained/controlled release, microparticles may offer benefits in terms of sensory characteristics. According to some reports, consumers showed a preference for products with particle sizes in the range of 250–500 μm, and this was associated with attractive qualities such as crispiness and granularity, which were not perceived in lower size ranges [264–267]. Furthermore, there are some reports in which sizes from 620–980 μm had no negative influence on the sensory acceptability of the real food product [268]. With this in mind, in addition to the numerous benefits that may be achieved via the microencapsulation of ingredients relevant to cheese production, additional desirable sensory characteristics can be obtained for cheese products.

Modern encapsulation science moves forward because it takes into account materials that can add desired functionality to particles, making them more than just carriers. Even though encapsulation science has helped to produce highly sophisticated systems, the gold standard for the encapsulation of microorganisms remains calcium alginate particles. Simultaneous encapsulation (more than one "active" ingredient) enhances its efficiency and may even have a synergistic effect. It is expected that soon, microencapsulated formulations with multiple ingredients will not only have a smooth introduction into the conventional production of cheese, but into food production in general [247,249].

Table 6. Most recent examples of the encapsulation of various active agents (functional components) to aid in cheese production and increase its bioactive value and shelf-life.

Encapsulated Agents	Fabrication Method/Carrier	Particle Size	Results/Outcomes	Reference
Bifidobacterium bifidum ATTC-29521	• internal gelation method • k-carrageenan and sodium alginate	n.s. *	• encapsulation of probiotic bacteria resulted in better survival with improved protection and stability in cheddar cheese	[261]
nisin from *Lactococcus lactis* (n.s. *)	• electrospinning method • amaranth protein isolate/pullulan	120 nm	• nanofibers protected nisin, decreasing its interaction with food components and maintaining its antimicrobial activity in fresh cheese	[269]

Table 6. *Cont.*

Encapsulated Agents	Fabrication Method/Carrier	Particle Size	Results/Outcomes	Reference
Lactobacillus rhamnosus (n.s. *)	• spray aerosol method • sodium alginate and $CaCl_2$	n.s. *	• encapsulated probiotic significantly altered the firmness of functional reduced-fat cream cheese • β-glucan and phytosterol emulsion aids in maintaining the viable cell count during storage • encapsulation was able to maintain the viable cells with less reduction during 35 days of storage	[262]
mandarin peel extract	• multilayered nanoliposomes • lecithin/chitosan/ maltodextrin	250–450 nm	• processed cheese supplemented with nanoliposome-containing extracts remained unaffected during cold storage for 3 months	[270]
Lactobacillus paracasei LAFTI® L26	• enzymatic gelation • skim milk powder/rennet • skim milk powder and sodium caseinate/ transglutaminase	n.s. *	• incorporation of microparticles influenced cheese textural characteristics, such as hardness and stringiness, and resulted in a significant increment of antioxidative capacity of Iranian UF Feta cheese	[271]

Table 6. *Cont.*

Encapsulated Agents	Fabrication Method/Carrier	Particle Size	Results/Outcomes	Reference
oregano essential oil (*Origanum vulgare L.*)	• nanoemulsions prepared via phase inversion temperature method • sunflower oil and surfactants (PEG-40 hydroxylated castor oil, polyoxyethylene 4-lauryl ether, and sorbitan monooleate)	n.s. *	• antifungal activity of nanoemulsions against the growth of Cladosporium sp., Fusarium sp., and Penicillium sp. on Minas Padrão cheese slices inoculated with the same fungal genera	[272]
Lactobacillus acidophilus Chr. Hansen A/S (Denmark)	• emulsification, ionic gelation methods • sodium alginate and $CaCl_2$	60–500 µm	• edible films containing encapsulated L. acidophilus control mesophilic aerobic bacteria during storage of Manaba cheese at refrigeration conditions	[260]
aromatic compounds-3-methylbutanal (from *Carnobacterium maltaromaticum* LMA 28)	• ionic gelation method • sodium alginate and $CaCl_2$ • cheeses were coated with paraffin with or without beads	1.69 ± 0.15 mm	• bacterial encapsulation superior to direct encapsulation of volatile compounds for imparting 3-methylbutanal olfactory notes to cheese	[273]
Lactobacillus rhamnosus 6134	• cold gelation within an emulsification system • Maillard reaction products of whey proteins and isomaltooligosaccharides	n.s. *	• encapsulation had a protective effect on bacteria in white brined cheese with no significant effect on the sensory properties	[274]
flavourzyme®	• liposome produced by heating method • soy lecithin/cholesterol	180 nm	• increase in the overall impression and a decrease in texture score of brined cheese	[253]
catechin and epigallocatechin gallate	• liposomes via mechanical agitation • soy lecithin	133 nm	• enhanced stability of low-fat hard cheese	[275]

n.s. *—not specified.

6. Conclusions and Prospects

Recent developments in and the availability of the genotyping and phenotyping of desired traits combined with advancements in the statistical analysis of large data sets offer

a good foundation for specialized dairy products studies (i.e., cheese). A new approach with the use of genetics in the production of rennet and dairy cultures should be used to challenge conventional production.

The production of animal rennets has been thoroughly researched in the 20th century. Because of the constant growth in cheese production and the shortage of this type of rennet, producers have turned to other coagulants, which can be produced in desired quantities. According to the literature, rennet has the best influence on the milk of the same species, but only calf rennet and bovine substitutes are produced. The production and use of rennet produced for a specific type of cheese or region are currently possible, and we suggest that more studies on this topic are needed.

The isolation and determination of indigenous lactic acid bacteria for use in dairy cultures are possible, and their use in a given cheese or region should maximize desirable characteristics.

Numerous benefits may be achieved with the microencapsulation of ingredients relevant to cheese production, including additional desirable sensory characteristics. Modern encapsulation techniques can be used to add the desired functionality to the product. Despite high technological advancements, the gold standard for the encapsulation of microorganisms remains calcium alginate particles. It is expected that microencapsulated formulations with multiple ingredients (simultaneous encapsulation) might have a synergistic effect, along with the enhancement of efficiency.

Technological and scientific advancements in animal science, chemistry, microbiology, and dairy science have enabled the presentation of the described topics in a single review to stimulate the application of sustainable and innovative technologies in cheese production. New options that are currently available should be used to develop sustainable production systems, with an emphasis on the preservation of biodiversity and the introduction of new technologies into conventional cheese production. This approach could enable producers to create new dairy products, emphasize differences between regions, or highlight the different ways in which they produce the same product with the preservation of biodiversity on a broader scale.

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