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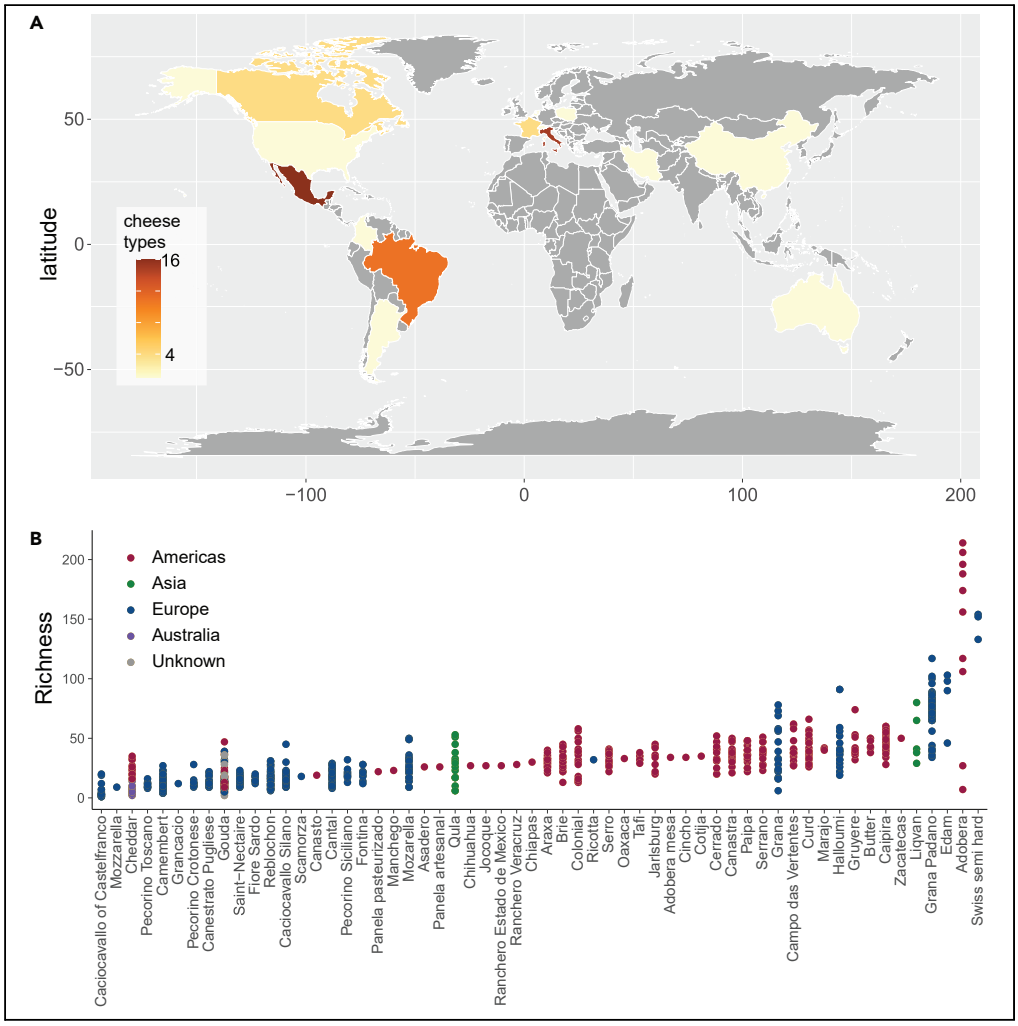

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Highlights

Most microbiology-centered cheese studies (75%) focus on lactic acid bacteria

Cheese microbial diversity is driven by characteristics of the production process

Globally, cheese microbiomes cluster into four groups

These groups have distinct, dominant taxa, and exhibit geographic signatures

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Universal drivers of cheese microbiomes

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SUMMARY

The culinary value, quality, and safety of cheese are largely driven by the resident bacteria, but comparative analyses of the cheese microbiota across cheese types are scarce. We present the first global synthesis of cheese microbiomes. Following a systematic literature review of cheese microbiology research, we collected 16S rRNA gene amplicon sequence data from 824 cheese samples spanning 58 cheese types and 16 countries. We found a consistent, positive relationship between microbiome richness and pH, and a higher microbial richness in cheeses derived from goat milk. In contrast, we found no relationship between pasteurization, geographic location, or salinity and richness. Milk and cheese type, geographic location, and pasteurization collectively explained 65% of the variation in microbial community composition. Importantly, we identified four universal cheese microbiome types, driven by distinct dominant taxa. Our study reveals notable diversity patterns among the cheese microbiota, which are driven by geography and local environmental variables.

INTRODUCTION

Cheese consumption and production are on the rise. The world cheese production reached 21 million metric tons in 2014 and is expected to grow to a global market value of 106 billion U.S dollars by 2026.[1] Generally, cheese is produced through the coagulation of milk protein (casein), which is separated from the milk's whey. Cheese varieties depend on the geographic region of production, the processing method, and the components used, which include milk, a coagulating agent, and in some cases, microbial starter cultures. The cheese microbiome is inextricably linked to the cheese matrix where it resides, driving the complex biochemical changes that underlie the ripening process.[2–6]

The cheese microbiome is dominated by lactic acid bacteria (LAB), which are members of the order Lactobacillales that produce lactic acid as an end product of carbohydrate fermentation.[7,8] Depending on the cheese type, specific microbes or consortia can be inoculated into milk or acquired from the environment to begin the ripening process. These microbes are adapted to different abiotic stresses including changes in pH, salinity, temperature, or moisture alongside biotic stresses such as competition and invasion resistance both at the individual or community levels.[9] During the ripening process, the cheese microbiota are influenced by the quality of the raw milk, the starter cultures added, and the ripening conditions.[10,11] These bacteria, and especially LAB, modulate cheese appearance, texture, aroma, nutrient composition, quality, and shelf-life,[12,13] while undesirable microorganisms may adversely affect cheese quality and safety.[14]

Much like the rest of microbiology, cheese microbiome research has undergone a revolution over the past two decades, shifting from culture-dependent to culture-independent methods (i.e., sequencing) to identify microorganisms in cheese, providing a more complete view of the microbes involved in cheese ripening.[11,15] For example, LAB in starter cultures are generally culturable and abundant in the earlier stages of cheese ripening, while non-starter LAB are dominant and essential to later stages of ripening but are less easily cultured.[10,16] In recent years, sequence-based research has been used to characterize the microbiomes of a wide range of cheeses, spanning a variety of milk types, geographic locations, and production styles.[2,17–19] Due to the ease of comparability among sequence data, the increasing popularity of sequencing-based approaches also facilitate synthesis research.[18,20]

Understanding how abiotic factors (geography, cheese pH, ripening conditions, milk type, and so forth) drive microbial diversity across cheese types can offer insights into improving the ripening process, reveal how domestication by different cultures results in different microbial consortia, and shed light on universal patterns of ecological assembly in cheese microbiomes. To this end, we conducted the first systematic

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review and synthesis of cheese microbiomes at a global scale. From the available literature, we selected publicly available cheese microbiome 16S rRNA gene amplicon sequencing datasets and examined how abiotic factors shape the cheese microbiome. In addition to identifying gaps in the current cheese microbiome research, this study provides the first global survey across cheese types, identifying the drivers of their diversity.

RESULTS

Trends in cheese microbiome research

Over the period studied, we found no trends in the number of microbiology-oriented cheese studies published over time (correlation test, $p = 0.66$) or in the proportion of those studies performing amplicon sequencing ($X^2 = 2.3572$, $p = 0.12$; Figures 1 and 2). Most studies (75%) focused on LAB rather than the whole cheese microbiome (Table S1).

Across the 120 studies reviewed, 71.7% reportedly sampled cheeses directly at the point of production, 27.5% sampled commercially available cheeses, and 0.8% did not disclose the source of their samples (Figure 3A). Most studies sampled ripened cheeses (55.8%; Figure 3B) and did not report the use or composition of starter cultures (51.7%). Among the studies which did report on starter culture use, 13.3% did not use any starter culture, while 20.8%, 12.5%, and 1.7% focused on cheeses made with commercial, local, and unspecified starter cultures, respectively (Figure 3C). Only 2.5% of the studies[2,19,21] sequenced the starter cultures used in the production of the cheeses using 16S rRNA gene amplicon sequencing (Table S1). Physicochemical parameters were reported for the minority of studies: on average, salinity was $2.5 \pm 1.1\%$ (reported for 11% of the studies), pH was 5.3 ± 0.5 (reported for 18% of studies), and cheeses were ripened at $10.7 \pm 5.5°C$ (reported for 26% of the studies, Figure 4). Cheese were ripened for 14 to 720 days (reported for 46% of the studies).

The global cheese microbiome

To further delve into the relationship between a cheese's location, ripening conditions, and its resident microbiome, we obtained publicly available sequences from 27 studies that performed 16S rRNA gene or transcript amplicon sequencing (Table 1). This dataset included sequences from 58 cheese types, spanning 16 countries (Figure 5), and distributed across 824 samples. In total, we detected 5,521 distinct ASVs.

Across all samples, an average richness was 29 ± 25 ASVs, but varied between cheese types. Caciocavallo of Castelfranco (Italy) cheese had the lowest (5 ± 6 ASVs), and Swiss semi-hard cheese had the highest (146 ± 12 ASVs) average richness (Figure 5). The cheeses' microbiomes were dominated by members of the Firmicutes and Proteobacteria phyla. Notably, whereas the microbiomes of Caciocavallo of Castelfranco, Cheddar, Chiapas, Chihuahua, Gruyere, and Jarlsburg cheeses were dominated by Firmicutes (>85% of the community), Adobera mesa, Canasto and Ranchero Veracruz were dominated by Proteobacteria (>85% of the community). Furthermore, the microbiomes of Halloumi, Grana, and Edam cheeses were found to contain <5% of Cyanobacteria, a phylum not often reported in cheeses.

Among the 4 studies which reported salinity (84 samples), we found no consistent relationship between richness and salinity (slope estimate 0.04 ± 0.08, posterior probability of 0.69). In contrast, among the 7 studies which reported pH (119 samples), we found a strong, positive relationship between richness and pH (posterior probability of 1), with a slope of 7.87 ± 2.59 and an intercept of -24.23 ± 14.81 (Figure 4).

We found no difference in richness between pasteurized and unpasteurized cheeses (posterior probability of differences = 0.51, Figure S2A). On average, cheeses made from goat's milk had the highest richness (44 ± 1 ASVs) and were consistently richer than cheeses from cow milk and sheep milk. Cheeses made from sheep's milk had the lowest richness (23 ± 5 ASVs), and were consistently less rich than those from cow milk or milk mixtures (posterior probabilities of 1 for all comparisons, Figure S2B). We also found no differences between cheeses of different geographic regions (posterior probability of differences <0.79 for all comparisons, Figure S2C).

To assess whether the proportion of LAB in the community affected community richness, we examined the relationship between the relative abundance of members of the order Lactobacillales and richness in the whole community. We found a strong, negative relationship between richness and LAB abundance (posterior probability of 1) with a slope of -18.13 ± 1.28 and an intercept of 49.07 ± 6.81 (Figure S3).

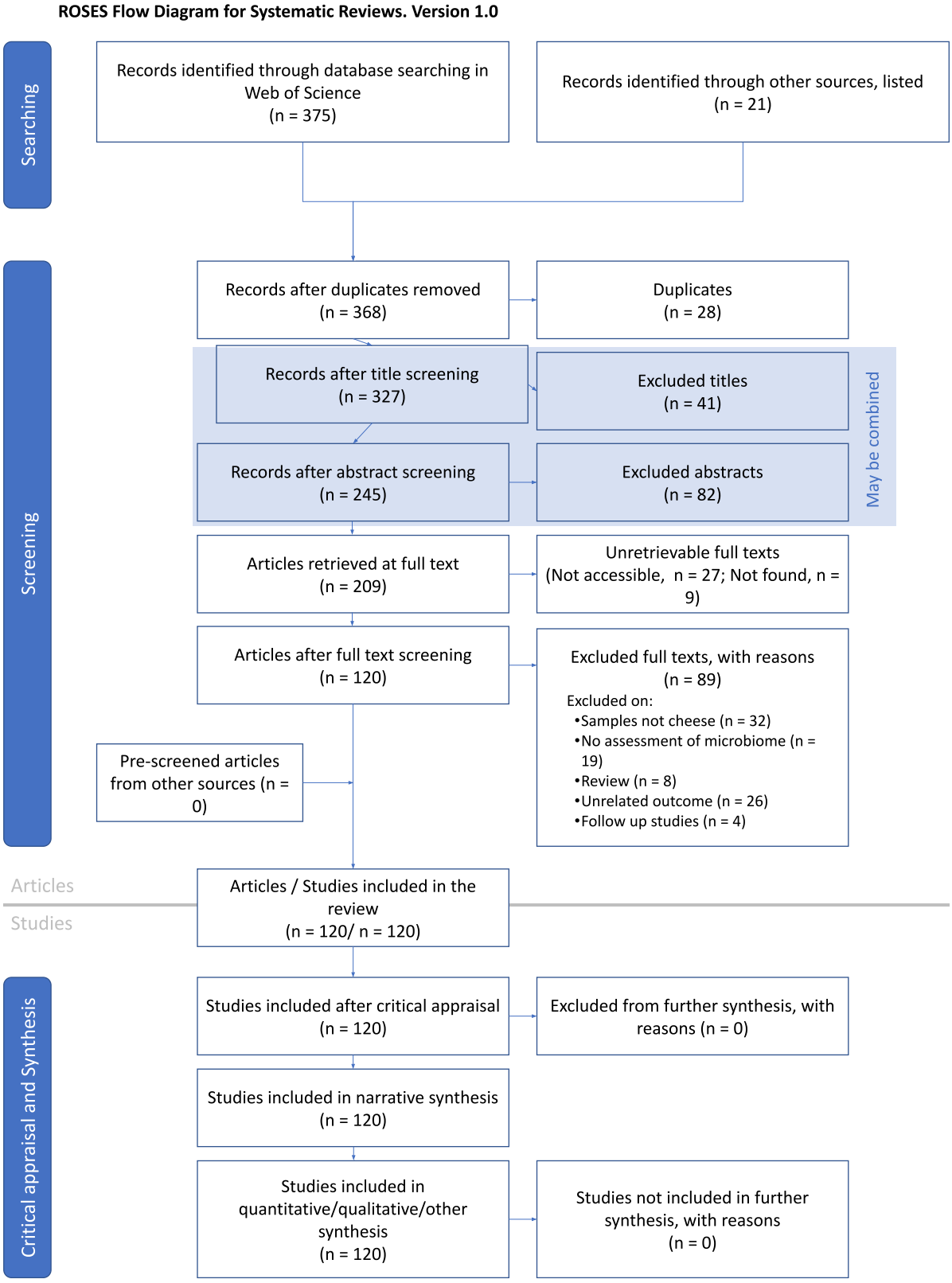

Figure 1. ROSES flowchart illustrating systematic search, identification, screening, and final selection of articles

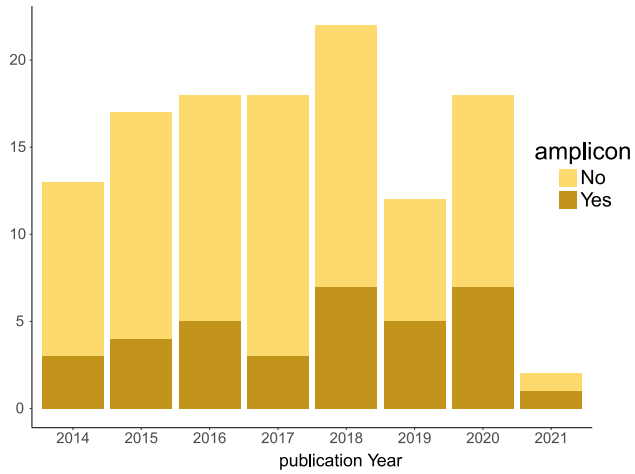

Figure 2. Cheese microbiome-related publications with (deep brown) and without (light brown) 16S rRNA gene amplicon sequencing (n = 120) over time

There was little overlap in cheese types across studies, and only three cheese types (Adobera, Cheddar, and Grana Padano) were sequenced by more than a single study. A distance-based variance partitioning of experimental variables (extraction kit used, study identity, type of sequencer used, and whether DNA or RNA amplicons were sequenced) explained 55.6% of the variability in community composition at the genus level (Figure S4A); however, of this variation, 48.5% was shared by more than one variable. Notably, study alone explained 7.1%, while the combination of study and extraction kit used explained 32.7% of the variation in community composition.

A distance-based variance partitioning of cheese ripening conditions (cheese type, pasteurization, country of origin, and milk source) explained 65.5% of the variation in community composition (Figure S4B). As expected, all of this variation was nested within cheese type, which alone explained 29.9% of the variation in community composition. Importantly, the combination of the country of origin and cheese type explained an additional 25.7% of the variation in community composition, while pasteurization only explained a modest portion (1.8%) of the variation in community composition. While the significance of these nested variance fractions is not testable, the whole model was statistically significant, suggesting that these ripening conditions have consistent and relevant effects on the microbiome.

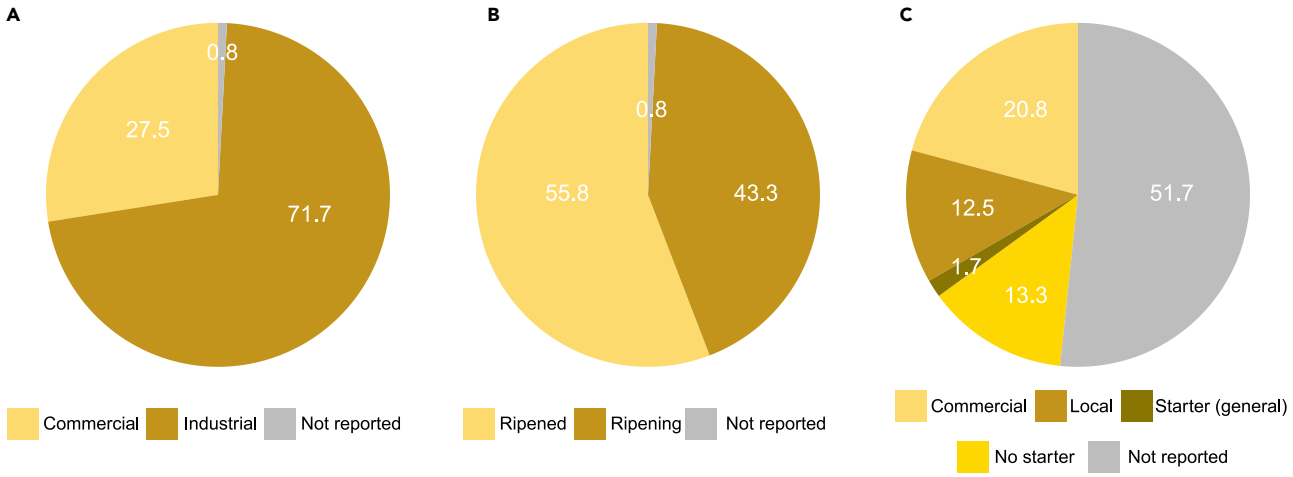

Figure 3. Features of cheese samples included in this study

Features of cheese samples reflecting (A) cheese source, (B) cheese state, and (C) starter culture use (n = 120).

Table 1. Metadata of the studies which performed 16S rRNA gene or transcript amplicon sequencing

NCBI SRA Accession	# Samples	Original Reads	Filtered reads	Final reads	Molecule	Pasteurized	pH	NaCl	Mean Richness	Cheeses	Animal species	Countries of origin	Starter culture	Reference
PRJNA283170	4	4076	3875	3557.5	DNA	No	NA	NA	17.7	Grancacio, Mozzarella, Ricotta, Scamorza	Cow, Sheep	Italy	Yes	Consonni and Cagliani[22]
PRJNA637891	4	442,000	442,000	400,602	DNA	Yes	NA	1.5	84.2	Edam	Cow	Poland	Yes	Ritschard et al.[23]
PRJEB24792	6	80,642	80,601	79,068	DNA	Yes	5.2	NA	33.5	Tafi	Cow	Argentina	No	Murugesan et al.[24]
PRJEB36556	22	189,222	188,652	183,195	DNA	Yes	NA	NA	37.4	Paipa	Cow	Colombia	NA	Ramezani et al.[25]
PRJNA230456	9	5541	5387	4982	RNA	Yes	NA	NA	20.4	Fontina	Cow	Italy	Yes	De Pasquale et al.[26]
PRJNA238397	16	5401	5375	2155	RNA	Yes	5.2	3.275	13.9	Canestrato Pugliese	Sheep	Italy	No	Delcenserie et al.[27]
PRJNA255096	18	8735	6759	6476	RNA	Yes	NA	NA	38.6	Grana	Cow	Italy	Yes	Alessandria et al.[2]
PRJNA272374	29	5849	5630	5434	DNA	Yes	NA	NA	25.9	Mozarella	Cow	Italy	NA	Salazar et al.[28]
PRJNA277133	37	133,106	133,100	126,003	DNA	Yes	NA	NA	78.6	Grana Padano	Cow	Italy	NA	Zhu et al.[29]
PRJNA286758	29	6265	5816	5178	RNA	No	5.8	4.052	15.7	Pecorino Toscano, Pecorino Siciliano, Fiore Sardo	Sheep	Italy	Yes	Kamimura et al.[30]
PRJNA290349	40	5781	5473	5372	RNA	Yes	NA	NA	16.7	Caciocavallo Silano	Cow	Italy	Yes	Falardeau et al.[31]
PRJNA294953	6	12,685	12,663	10,588	RNA	Yes	NA	NA	39	Grana Padano	Cow	Italy	Yes	Dugat-Bony et al.[32]
PRJNA295825	3	13,529	13,493	11,698	DNA	Yes	NA	NA	146.3	Swiss semi hard	Cow	Switzerland	NA	Frétin et al.[33]
PRJNA311540	18	3997	3871	3737	DNA	Yes	5.3	NA	5.4	Caciocavallo of Castelfranco	Cow	Italy	Yes	Giello et al.[18]

(*Continued on next page*)

Table 1. *Continued*

NCBI SRA Accession	# Samples	Original Reads	Filtered reads	Final reads	Molecule	Pasteurized	pH	NaCl	Mean Richness	Cheeses	Animal species	Countries of origin	Starter culture	Reference
PRJNA316626	17	43,316	42,829	42,265	DNA	No	NA	NA	27.9	Adobera, Zacatecas, Chihuahua, Cincho, Oaxaca, Canasto, Asadero, Manchego, Adobera mesa, Cotija, Panela pasteurizado, Panela artesanal, Jocoque, Chiapas, Ranchero Estado de Mexico, Ranchero Veracruz	Cow	Mexico	NA	Kamilari et al.[34]
PRJNA319425	5	71,453	65,868	65,128	RNA	Yes	5.0	NA	50.6	Liqvan	Sheep	Iran	NA	Ruvalcaba-Gómez et al.[35]
PRJNA379167	15	35,083	35,039	33,302	DNA	Yes	NA	NA	12.3	Pecorino Crotonese	Sheep	Italy	Yes	Haddaway et al.[36]
PRJNA382370	89	109,117	104,938	102,021	DNA	NA	5.4	1.491	14.5	Gouda	Cow, Goat	USA, Netherlands, Unknown	NA	Bunn and Korpela[37]
PRJNA413466	15	34,232	34,167	32,655	DNA	Yes	NA	NA	26.8	Qula	Yak	China	NA	Quast et al.[38]
PRJNA421256	24	60,069	58,602	57,947	DNA	Yes	NA	NA	19.1	Cantal	Cow	France	Yes	Turnbaugh et al.[39]

(*Continued on next page*)

Table 1. *Continued*

NCBI SRA Accession	# Samples	Original Reads	Filtered reads	Final reads	Molecule	Pasteurized	pH	NaCl	Mean Richness	Cheeses	Animal species	Countries of origin	Starter culture	Reference
PRJNA476316	180	82,455	81,258	75,293	DNA	Yes	NA	NA	38.2	Serrano, Cerrado, Araxa, Colonial, Curd, Campodas Vertentes, Serro, Canastra, Cerrado, Caipira, Campodas Vertentes, Cerrado, Caipira, Butter, Marajo, Curd,	Cow	Brazil	Yes	Engel et al.[40]
PRJNA499132	43	278,760	278,576	233,882	DNA	No	NA	NA	33.1	Brie, Cheddar, Jarlsburg, Gruyere	Cow	Canada	Yes	Chao et al.[41]
PRJNA523139	97	24,883	24,056	24,003	DNA	Yes	5.2	NA	13.6	Camembert; Reblochon	Cow	France	Yes	McMurdie et al.[42]
PRJNA578621	32	31,487	24,489	24,239	DNA	Yes	NA	NA	14.9	Saint-Nectaire	Cow	France	Yes	Oksanen et al.[43]
PRJNA598815	18	141,051	140,017	137,710	DNA	Yes	NA	NA	43.7	Halloumi	Goat, Sheep, Mixed	Cyprus	NA	Morgan[44]
PRJNA673975	40	63,315	63,035	61,401	DNA	Yes	NA	NA	6.5	Cheddar	Cow	Australia	NA	Afshari et al.[19]
PRJNA681198	8	144,153	137,781	132,221	DNA	Yes	NA	NA	169.6	Adobera	Cow	Mexico	No	Kolde et al.[45]

The number of original reads, filtered reads, and final reads is shown as an average per study. NA, not available; DNA, deoxyribonucleic acid; RNA, ribonucleic acid.

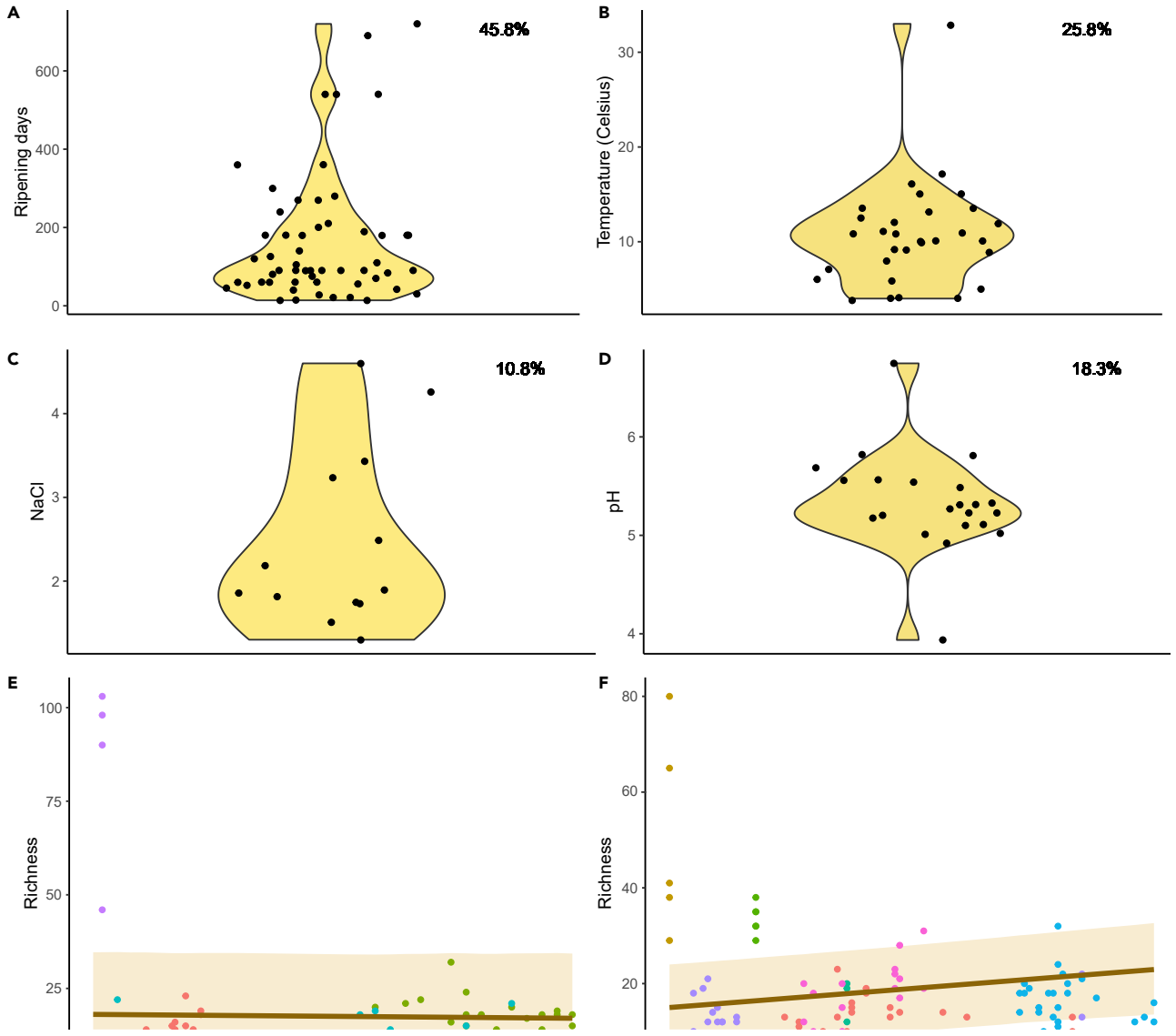

Figure 4. Ripening parameters of cheese samples included in this study

(A–F) Ripening parameters including ripening duration (A), ripening temperature (B), salinity (C and E), and pH (D and F) varied among cheese types. While salinity did not affect the richness of the resident microbiota (E), pH was positively related to richness. In (A–D), the percentage of studies (out of 120) reporting each parameter is shown on the top right of each panel. Data in panels (E and F) was obtained from 5 to 7 microbiome datasets which reported salinity and pH values, respectively. Regression lines indicate the mean response across cheese types and studies. The shaded ribbon indicates the 95% CI of the overall response, and data points are colored according to their study membership. In f, the overall trend diverges from zero with a probability of 1.

To investigate similarities among cheeses, we performed a cluster analysis across all cheese types for which triplicate samples were available (n = 805). We found the strongest support for four clusters of cheeses, which exhibited distinct dominant communities (Figure 6). Cheese microbiomes in *Group 1* included 280 samples from 23 cheese types and were dominated by *Lactococcus*. Similarly, microbiomes in *Group 2* included 236 samples from 12 cheese types and were dominated by *Lactococcus*, but had lower evenness and richness than those in *Group 1* ($p < 0.05$ for all Wilcoxon tests, Figure S5). Microbiomes in *Group 3* included 230 samples from 18 cheese types and were dominated by *Streptococcus*, and microbiomes in *Group 4* included 59 samples from 8 cheese types that were dominated by members of the genus *Lactobacillus*. *Group 4* had the highest richness ($p < 0.05$ for all Wilcoxon pairwise tests, Figure S5). Of the 39 cheese types included in the cluster analysis, samples from 17 cheese types were classified into multiple groups. Instead, groups seemed to reflect geographic origin: *Group 1* was predominantly composed of samples of North- and South American origin

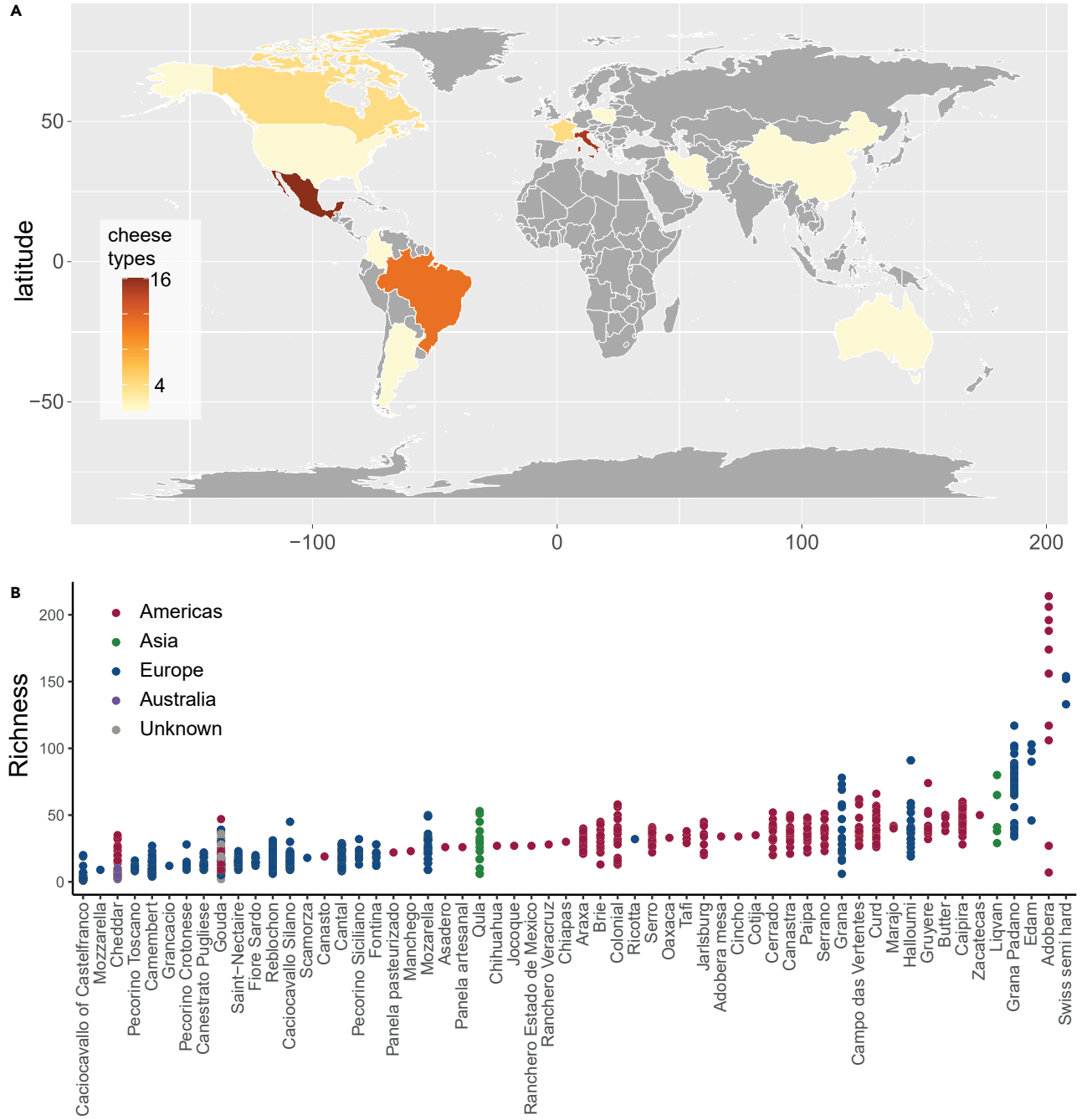

Figure 5. Geographic distribution and richness of cheese samples included in this study

(A and B) Geographic distribution of cheese samples included in this study (A and B) and their richness (B). In (B), each point represents a sample.

(88.6% of samples), while *Groups 3* and *4* were dominated by samples of European origin (95.2 and 91.5% of samples, respectively, Figure 6). *Group 2* had samples of diverse origins, including 19.9% from North and South America, 55.5% from Europe, and 16.9% from Australia.

DISCUSSION

As global cheese consumption and production continue to rise,[1,46] understanding the drivers of microbial diversity in cheese is crucial to determining the final product's value, consumers' enjoyment, quality and safety, and shelf-life. From an ecological perspective, the cheese microbiome is the product of centuries of

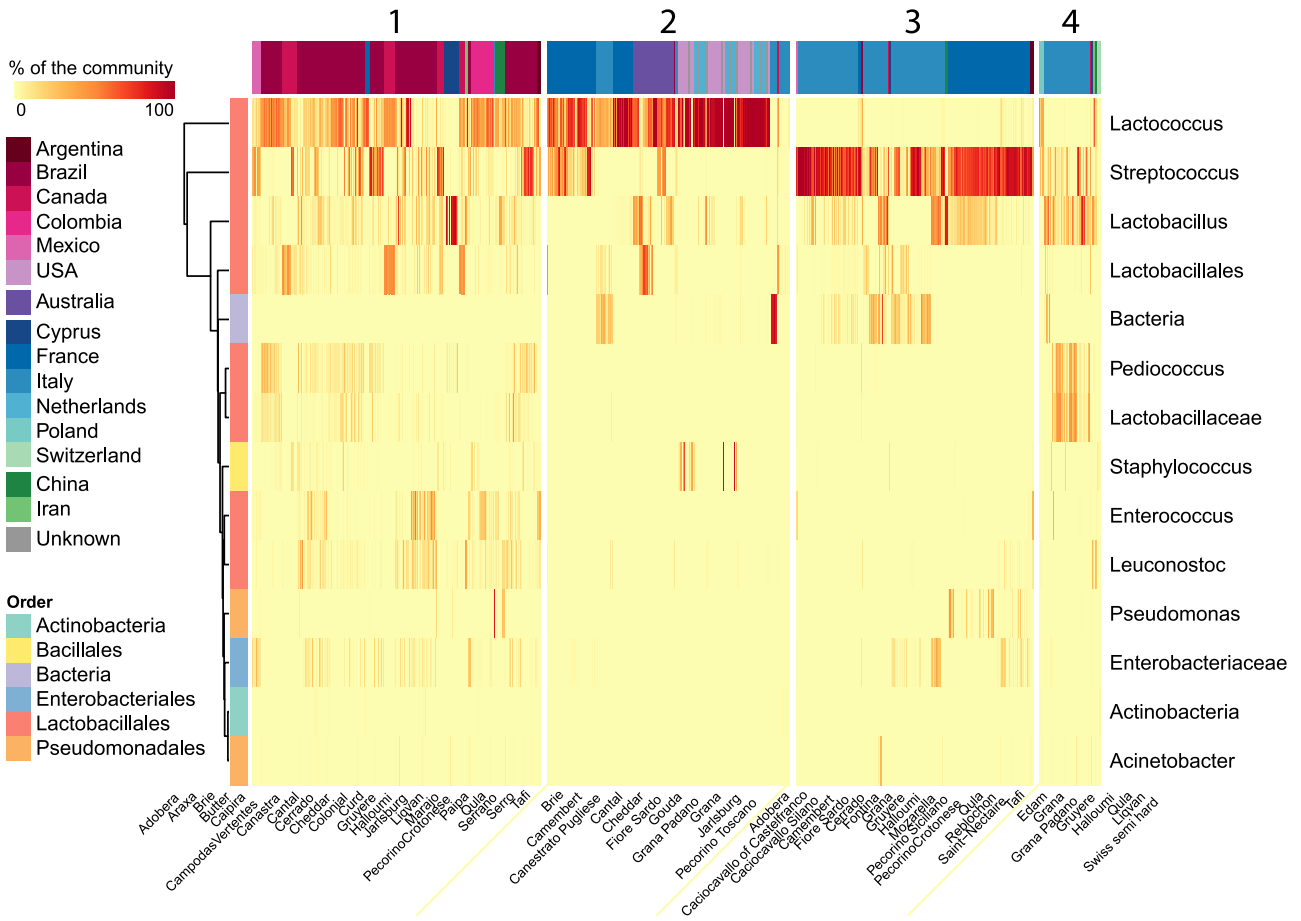

Figure 6. Cheese microbiomes clustered into four main groups

Clusters were determined using Dirichlet multinomial mixture models, and are labeled on the top margin. The most abundant or prevalent genera are displayed. These 14 genera comprised 83.5 ± 21.9% of the community on average, across all samples, and order membership is displayed on the left margin. The tree on the left reflects similarity in abundance profiles among genera. Within each cluster, cheeses were ordered by type and cheese types in each cluster are indicated in the lower margin. Each sample's geographic origin is shown on the top margin.

domestication by different human cultures,[47] and thus may serve as a simplified model system for the study of microbial ecology.[15] We characterized the global distribution of cheese microbiomes through synthesis.

While the use of sequencing technologies in the food sciences is expected to increase in the future[14,48]; we found that the number of cheese microbiome studies did not increase over the short period studied. We also found that cheeses are most often sampled at the point of production. Information regarding the use and composition of starter cultures, and the abiotic factors including cheese salinity and pH were seldom reported; however, our study highlights how these variables influence the diversity and composition of the cheese microbiome[11,49] Among the 16S rRNA gene amplicon sequencing studies in which pH was reported, we found a consistent, positive relationship between microbiome richness and pH. Low pH exerts a selective pressure that likely favors the survival and dominance of a few bacterial species within the cheese microbiome.[50] In contrast, we found no relationship between cheese salinity and richness. This is contrary to extant literature[51,52]; however, most available studies examine a specific cheese type and thus focus on specific cheese microbiomes. Indeed, increasing salinity influences cheese microbial composition and growth, biochemical changes and enzymatic activities, microbial succession, and cheese quality.[11,51,53] Our findings suggest that while salinity can modulate a specific microbiome, it does not explain the different microbial community compositions found across cheese types. Nevertheless, our analyses are limited by the small number of microbiome studies that reported abiotic parameters.

Of the available cheese microbiome literature, 75% focused on LAB rather than the entire microbiome, likely due to their central role in cheese ripening, and also, the health benefits they exert.[54–57] Interestingly,

we found a negative relationship between the relative abundance of Lactobacillales and community richness across cheese samples, suggesting that few, specialized LAB strains outcompete other resident microbes in ripened cheese over time. Within the cheese matrix, LAB are known to competitively exclude and counter the development of a range of co-existing microorganisms, especially potential spoilers which may contaminate and negatively impact the cheese quality and safety.[58]

Depending on cheese type and safety concerns due to the possible presence of pathogens, milk collected for cheese production is often pasteurized. Pasteurization can impact autochthonous non-pathogenic milk microbiota and also inactivates some microbial-secreted antimicrobial compounds.[46,59,60] Nevertheless, we found no difference in richness between pasteurized and unpasteurized cheeses, which aligns with a previous study of the microbiome of Herve cheese, where the lack of difference between pasteurized and unpasteurized cheese microbiomes was attributed to the similarities in their manufacturing process.[27] Cheese quality and safety are ultimately impacted by bacteria in milk and especially those that withstand pasteurization.[52,61] Our study suggests that this process does not affect the cheese microbiome's richness, and only modestly, but significantly, affects composition.

We found that the origin of milk has a great influence on the cheese microbiome's richness and composition. The composition of milk microbiota is dynamic and has been linked to the animal's physiological state, presence and activity of endogenous enzymes, number of milking sessions,[2,16,46,62] which are species-specific and influenced by animal husbandry and farm management, the teat microbiome, and hygiene practices related to the milking equipment and storage vessels.[11] For example, 27% of bacteria detected in ripened raw milk cheeses were also found on the teat surface.[63] Furthermore, some of these bacteria including LAB (*Lactobacillus casei/paracasei*, *Lactococcus chungangensis/raffinolactis*, and *Lactococcus lactis*), *Brevibacterium linens*, and *Staphylococcus equorum* are known to be involved in the development of different cheese organoleptic properties and the metabolism of fat and protein.[11,63]

Our study reveals strong geographic signatures in the cheese microbiome, independent of cheese types and their associated production processes. We identified four main compositional profiles across cheese types. Interestingly, samples from 17 cheese types (out of the 39 that were analyzed for clusters) were present in more than one cluster, suggesting that the clusters were not driven by differences in cheese types. Differences among cheeses in groups 2 and 3 were driven by the dominance of *Lactococcus* and *Streptococcus*, respectively. In contrast, differences between cheeses in groups 1 and 2 were driven by dominance patterns: both groups were dominated by *Lactococcus*, but microbiomes in group 1 had a higher evenness and richness than those in group 2, suggesting more complex communities. Cheeses in these two groups exhibited marked differences in geographic origin, and cheeses with richer, more even communities were mostly manufactured in North and South America, while those with lower richness and evenness were predominantly European. Similarly, the microbiomes of cheeses in Group 4 had higher richness and evenness than Groups 2 and 3. These clusters could be driven by local differences in domestication and quality regulations across the globe. For instance, the legal somatic cell counts (SCC) threshold level for milk acceptance differs across countries, and until now, there is yet to be a global consensus on SCC-acceptable limits in the dairy industry. Whereas the European Union (EU), Australia, New Zealand, Norway, and Switzerland have an SCC limit of <400,000 cells/mL, the United States, South Africa, and Brazil have a limit of 750,000, 500,000, and 1,000,000 cells/mL, respectively.[46,64–66] Still yet, several countries have no legal SSC limit for milk acceptance in their dairy industries, and this may not only impact cheese microbiome variability but the overall safety and quality of dairy products.

Within the last few years, several varieties of cheese obtained the protected designation of origin (PDO) status. The increasing application of PDO on cheeses identifies not just their specific geographical origin but also helps ensure cheese quality and protect consumers from frauds.[22,67] Since cheese with a PDO trademark must be produced with milk obtained from animals bred strictly within the PDO area, its organoleptic and microbiome-associated properties are not easily reproducible in other geographical areas. The environmental conditions associated with a specific geographical area/region are responsible for the unique characteristics in both the milk as well the cheese, becoming primary determinants of cheese's nature, quality, and safety.[22] Ultimately, understanding the drivers of regional differences in cheese microbiomes may provide insights into the functional potentials of the abundant microbiome member(s), their diversity, and beneficial roles in cheese typical characteristics. Further research is necessary to determine whether these differences in dominance patterns are due to regional differences in manufacturing processes, ripening practices, animal husbandry, and food safety regulations, or the local environmental microbiome.

The microbiome of cheese and other fermented foods has been repeatedly proposed as a model microbial system for understanding and managing microbiome diversity.[14,68] However, our study shows that cheese microbiomes vary globally, and are driven by the cheese production process, as well as by the environment (i.e., pH). The insights provided by this study may serve to and situate new findings with a global context of all cheese microbiomes, rather than remain limited to a localized single cheese type. This could greatly help improve current knowledge regarding microbial diversity and patterns associated with different cheese types as well as their quality and sensorial characteristics, and regional production practices, as research in these areas continues to develop (e.g.,[69]). Our study may thus serve as a universal reference point for future cheese microbiome research and aid in the optimization of food production while highlighting the drivers of microbial diversity in the cheese microbiome. Future works may, for example, leverage the processed sequence data created for this study (available at https://github.com/drcarrot/Cheese_synthesis/), to compare the microbiome in their cheeses to those in the same region, made from the same milk, or with similar abiotic parameters.

Developing consensus workflow methodologies from cheese sampling to molecular techniques, sequencing and bioinformatics may greatly accelerate research into the microbiome of cheeses. Similar approaches have proven invaluable in human and environmental microbiome research,[39,70–72] and could foster interdisciplinary collaborations in cheese research, leading to better global microbiome diagnostics, the optimization of monitoring and cheese production, quality, and safety strategies. In particular, consistent adoption of a single hypervariable region of the 16S rRNA gene, metadata standards (i.e., technical metadata as well as key abiotic parameters such as salinity and pH) and consistent methodological reporting (i.e., the region of the cheese sampled) may greatly improve comparability among studies without affecting the labor or resources required to produce microbiome data. A global perspective on the cheese microbiome and its drivers may aid researchers (across multiple disciplines), producers, food regulatory agencies, and policymakers in understanding and effectively tracing regional diversity, peculiarities, and trends in cheese-associated microbiomes.

Limitations of the study

Our study stresses the need for standardized reporting and, ideally, unified data collection methods in cheese microbiome research. Despite our robust synthesis, our analyses were limited by the different experimental approaches, the unavailability of deposited (sequence) data, and a lack of reporting, consistent with previous reports.[73] Accordingly, we employed conservative bioinformatics approaches and statistics that accounted for the random effect of study-specific techniques, at the cost of discriminatory power in these analyses, particularly with respect to low-abundance taxa. While our study reveals broad-scale (i.e., global) spatial patterns in cheese microbiomes, further research is needed to determine the contribution of smaller-scale parameters (i.e., cheese shape, exposed surface area, or sampling location within the cheese wheel) to the cheese microbiome.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2022.105744.

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AUTHOR CONTRIBUTIONS

R.C.R carried out the literature search, reviewed and selected relevant literature, organized the tables, and wrote the article. D.L extracted and assembled all the 16S rRNA metadata. N.E supervised the study and organized the presentation of the results. SD.J. conceptualized the study, performed the bioinformatics and statistical analyses, and created all figures. All authors contributed substantially to subsequent revisions.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Software and algorithms		
R V. 4.0.2	Bunn and Korpela,[37]	https://cran.microsoft.com/snapshot/2019-09-22/web/packages/dplR/vignettes/chron-dplR.pdf
SILVA V.132	Quast et al.[38]	https://doi.org/10.1093/nar/gks1219
Other		
Sequence data and analysis pipelines	This study	https://github.com/drcarrot/Cheese_synthesis

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources should be directed to the lead contacts, Dr. Rine Christopher Reuben (reubenrine@yahoo.com).

Materials availability

This study did not generate new unique reagents.

Data and code availability

All accession numbers and code supporting this study are available in this paper's supplemental information, and https://github.com/drcarrot/Cheese_synthesis, respectively.

METHODS DETAILS

Literature search, systematic review, and data extraction

In March 2021, we performed a literature search in Web of Science (www.webofscience.com) to assess the global state of cheese microbiome research using the RepOrting standards for Systematic Evidence Syntheses (ROSES) guidelines[36] (Figure 1). Our keyword search included the terms 'cheese microbiome' OR 'cheese microbiota' OR 'cheese microbial flora' OR 'cheese microbial community' OR 'cheese 16S rRNA sequencing', and was restricted to studies published between 2014 to March 2021. Of the resulting 396 studies, we selected 120 studies that used 1) 16S rRNA gene or transcript amplicon sequencing, 2) other culture-independent techniques (e.g., shotgun metagenomics), 3) culture-dependent techniques to characterize the whole cheese microbial community, or 4) both culture-dependent and independent techniques to characterize multiple LAB in cheese by reading their titles and abstracts. Studies that focused on other fermented foods, the cheese processing environment, commentaries, editorials, reviews, systematic reviews, and meta-analyses were excluded.

From each study, we collected information about cheese type, cheese state (whether commercial or industrial, ripened or under ripening), duration of ripening, physicochemical parameters (pH, salinity, and ripening temperature), whether starter cultures were used, whether temporal or spatial gradients were used, and sampling location (Table S1). Additionally, for studies which performed 16S rRNA gene amplicon sequencing, we recorded technical variables including the sequencing platform used, the DNA extraction method, the target molecule (DNA or RNA), and the 16S rRNA gene region amplified (Table S1).

Bioinformatics

We downloaded sequence data and metadata for studies that performed 16S rRNA gene amplicon sequencing and had accessible and reusable sequences from NCBI's Sequence Read Archives. We excluded samples from milk, unripened cheese, and technical controls. Sequence processing was performed in R[37] with the *dada2* package.[5] For each study, reads were first inspected with the *plotQualityProfile*, and trimmed to 90 base pairs with the *filterAndTrim* function, as recommended by the Earth Microbiome Project.[72] Further trimming parameters were selected for each study (Table 1. Reads were assigned a taxonomy using SILVA V.132 [76]. The proportion of reads lost at each processing step for

each study are detailed in Figure S1. Prior to analyses, all samples were standardized to 1500 reads per sample using the *rarefy_even_depth* function, which led to a loss of 7 samples. Coverage was estimated using the *coverage* function of the BetaC package[40] as previously described,[41] and was 0.99 ± 0.007% (mean ± SD) of the community across all samples, on average. The final dataset contained 824 samples from 27 studies.

QUANTIFICATION AND STATISTICAL ANALYSES

For data obtained from the systematic review, changes in the proportion of studies for which amplicon sequencing was performed over time were evaluated with a X^2-test for trends in proportions. All sequence-based analyses were performed using the *phyloseq*[42] and *vegan*[43] packages. Microbiome richness was measured as the number of bacterial ASVs (amplicon sequence variants) per sample. We used Bayesian statistics to assess the relationship between richness and cheese ripening conditions (e.g., whether the cheeses were pasteurized, the milk source, NaCl content, and pH, were available as well as the country of origin) using the *brms* package.[4] We used a poisson distribution with an identity link function, with default priors for each model's intercepts, standard deviations, and random effects. We computed posterior distributions using the MCMC No-U-Turn Sampler and ran 3 MCMC chains of 2000 iterations with a warm up phase of 1,000 samples. We verified convergence by Gelman-Rubin statistics (Rhat<1.01) and adequate effective sampling size (n_eff). To estimate richness per cheese, we used cheese as a fixed effect and study as a random effect to account for technical differences among studies. To determine whether richness varied among cheese types according to country, milk source, or pasteurization, we used cheese type nested in study as a random effect. To estimate the relationship between cheese richness and NaCl, pH, and the percent of Lactobacillales in the community, we included cheese type nested within in study as a random intercept. We report confidence in differences between means as posterior probabilities of differences, which were estimated using the brms *hypothesis* function. Richness values and model estimates are presented as *value* ± *SD*.

Differences in the composition of the microbial communities across samples were quantified using Bray-Curtis distances, and analyses were performed at the genus level by agglomerating ASVs with *phyloseq*'s *tax_glom* function. Prior to these analyses, cheese types with less than triplicate samples (n = 19) were removed. First, to determine the amount of compositional variance explained by technical parameters (molecule type, extraction kit, and sequencer used), we performed a distance-based variance partitioning using the *varpart* function in *vegan*. Then, to determine the role of environmental parameters (cheese type, country of origin, milk source, and pasteurization) on microbial composition, we performed a second distance-based variance partitioning. The significance of each model and of testable components of both variance partitions was tested using the *ANOVA.cca* function.

To determine whether the microbial communities of different cheeses clustered into groups, we used a Dirichlet multinomial mixture model (*dmn* function) (*DirichletMultinomial* package[44]), allowing up to 20 clusters (one for each cheese type). To determine the optimal number of clusters, we selected the cluster number with the lowest Laplace approximation. Group membership for all cheese samples and the relative abundance of most abundant taxa (i.e., those which appeared in at least 3% of the samples or represented at least 10% of the community in a sample) was depicted in a heatmap with the package *pheatmap*.[45] Differences in the richness and evenness between groups were tested with Kruskall-Wallis tests for overall differences among groups, and Wilcoxon tests for pairwise comparisons. The scripts used to produce these analyses are available in https://github.com/drcarrot/Cheese_synthesis.