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Review

A review of methods for the inference and experimental confirmation of microbial association networks in cheese

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ABSTRACT

Interactions among microorganisms deeply affect the dynamics of cheese microbial communities and, as a consequence, multiple aspects of cheese quality, from the production of metabolites affecting the taste, aroma and flavour, to body, texture and colour. Understanding and exploiting interactions among beneficial or detrimental microorganisms is therefore key to managing cheese quality. This is true for the simplest systems (fresh cheeses produced from pasteurized milk using defined starters) and the more so for complex, dynamic systems, like surface ripened cheese produced from raw milk, in which a dynamic succession of diverse microorganisms is essential for obtained the desired combination of sensory properties while guaranteeing safety. Positive (commensalism, protocooperation) and negative (competition, amensalism, predation and parasitism) interactions among members of the cheese biota have been reviewed multiple times. However, even if the complex, multidimensional datasets generated by multi-omic approaches to cheese microbiology and biochemistry are ideally suited for the representation of biotic and metabolic interactions as networks, network science concepts and approaches are rarely applied to cheese microbiology.

In this review we illustrate concepts relevant to the description of microbial interactions using a network science framework. Then, we briefly review methods used for the inference and analysis of microbial association networks (MAN) and their potential use in the interpretation of the cheese interactome. Finally, since these methods can only be used for mining microbial associations, we review the experimental methods used to confirm the nature of microbial interactions among cheese microbes.

1. Foreword

Cheeses, like all fermented foods, are man-made dynamic ecosystems, in which the environment is organic and the biota is made solely by microbes (bacteria, fungi and viruses; Gobbetti et al., 2018; Jonnala et al., 2018; Wolfe and Dutton, 2015), with a few exceptions in which arthropods play a more or less beneficial role (Carvalho et al., 2020; Marcellino and Benson, 2014). Microbial metabolism is among the main drivers of cheese sensory properties, and the dynamics of the microbiota strongly impacts cheese quality and safety (Gobbetti et al., 2018; Jonnala et al., 2018). Even when the complex microbiota of raw milk is drastically simplified by heat treatments and by the addition of defined starter cultures, and when ripening and storage are relatively short (like in fresh cheeses), microbial interactions are still important in determining the success of the fermentation. Obvious examples are parasitism, when lytic bacteriophages infect starter strains, or protocooperation among key starter species, like *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* or *lactis* (Blaya et al., 2017; Irlinger and Mounier, 2009). The other side of the spectrum is represented by raw milk cheeses produced with no starter or by using traditional undefined starters, and with longer ripening. In these cheese varieties, a complex pattern of microbial interactions and a succession of species and strains invariably develops and its control is key to cheese quality (Blaya et al., 2017; Gobbetti et al., 2018; Irlinger and Mounier, 2009; Jonnala et al., 2018; Mayo et al., 2021). The complexity of microbial successions and interactions in surface ripened cheeses is well known (Irlinger and Mounier, 2009), and has been demonstrated in an elegant and comprehensive way in a series of recent studies (Bonham et al., 2017; Cosetta et al., 2020; Kastman et al., 2016; Niccum et al., 2020; Wolfe et al., 2014; Zhang et al., 2018).

In all ecosystems, several types of positive (commensalism, protocooperation) and negative (competition, amensalism, parasitism) interactions are possible between couple of partners or among more complex modules and cliques, i.e., among groups of species which have

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more interactions among them that with other species in the network (see Canon et al., 2020; D'Souza et al., 2018 for recent reviews). Even if, ultimately, co-culturing in the laboratory and/or in appropriate model systems is the only way to obtain in-depth knowledge on the nature of interactions (Cosetta and Wolfe, 2019; D'Souza et al., 2018; Wolfe et al., 2014), the wealth of data provided by metataxonomic, metagenomic and metabolomics approaches provides ample opportunity to mine for microbial association networks (MAN) and metabolic networks (Layeghifard et al., 2017; Liu et al., 2020; Röttjers and Faust, 2018).

Microbial interactions in cheese and in other fermented foods have been the subject of recent comprehensive reviews (Blaya et al., 2017; Canon et al., 2020; Gobbetti et al., 2018; Mayo et al., 2021). A schematic representation of interactions (parasitism, commensalism, amensalism, competition, protocooperation) occurring in an idealized surface ripened cheese is shown in Fig. 1, with some of the interactions described in detail in Section 3 and Table 1.

The ensemble of microbial interactions in cheese is best described using network concepts. Surprisingly, while network science approaches are frequent in the study of host and environmental microbiomes, they are much less so in food and dairy microbiology (Parente et al., 2018). This is rather unfortunate, given the potential of the study of microbial interactions in food for the development of new processes and products and for the optimization of microbiome intervention strategies even in complex communities (Canon et al., 2020).

In this review, we will briefly illustrate network approaches to the study of microbial communities, related terminology and methods. We will then review recent literature using high-throughput sequencing or meta-omic approaches for the study of microbial interactions in cheese microbial communities. Finally, we will point out to the possibility of using the metataxonomic data stored in the DairyFMBN database (Parente et al., 2020) for the mining of microbial associations in cheese.

2. Network analysis concepts and approaches to the study of microbiota

2.1. Network science and microbial ecology

A network is, simply put, a collection of objects (nodes, vertices) connected by interactions (edges, arcs, links) (Newman, 2010): a schematic network representation of microbial interactions in cheese is shown in Fig. 1. Because of the flexibility and power of this concept, network science is used in representing and understanding interactions in very diverse fields (physics, social sciences, biology, etc.).

In the study of microbial associations, nodes can be of the same type (unipartite networks, as in microbial association networks, in which the nodes are microbial taxa, Operational Taxonomic Units, OTU, or Amplicon Sequence Variants, ASV) and the edges represent some sort of true or inferred association (positive or negative), which may or may not reflect a true biological interaction. Bipartite networks (i.e. networks with nodes belonging to two different types) are also of interest: networks of this type include Phage-Bacteria Interaction Networks (PBINs; Flores et al., 2011) and Food-Microbe interaction networks (which have

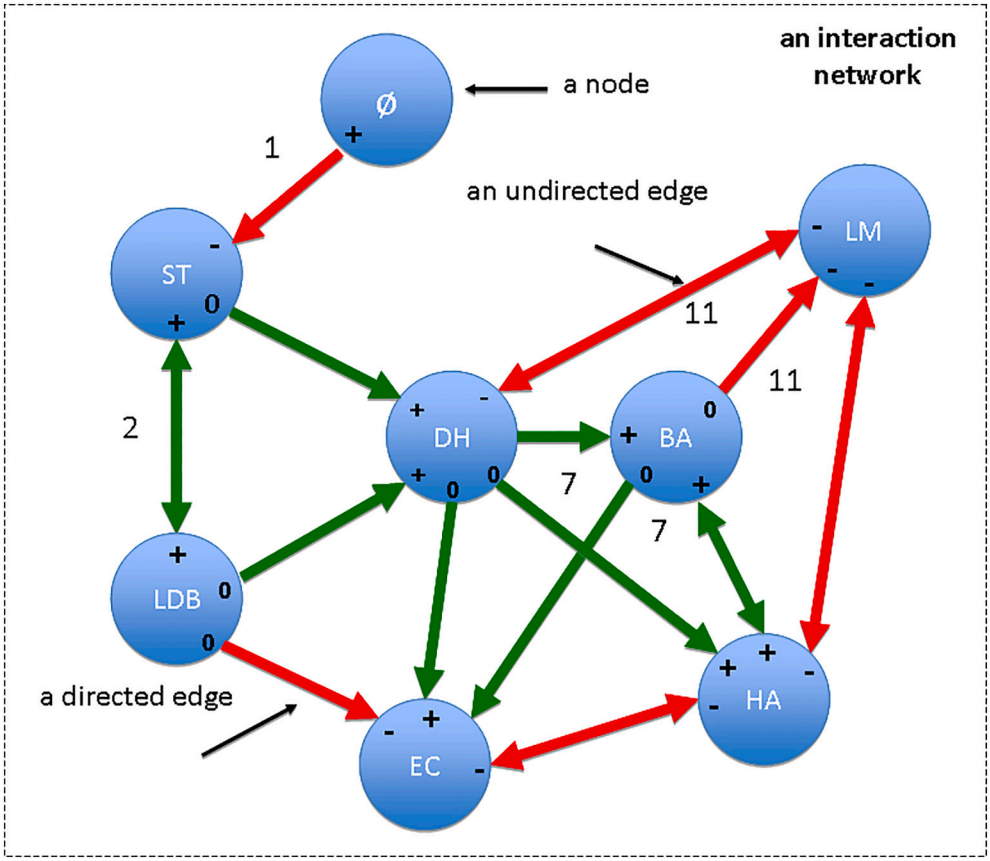

Fig. 1. A simplified representation of a potential interaction network in a surface ripened cheese. Red arrows indicate interactions which have a negative effect (often referred to as mutual exclusion interactions) on one or both partners. Green arrows indicate interactions which have a positive effect on one or both partners (often referred to as co-occurrence interactions). 0 indicates no effect, + a positive effect, − a negative effect. Numbers refer to examples in Table 1. The direction of the arrows may be used to indicate the direction of the interaction. Parasitism (1) due to bacteriophage (∅) infections of starter bacteria, like *Streptococcus thermophilus* (ST) is one of the most frequent, and technologically relevant interactions in cheesemaking, together with the protocooperation between starter species (2), like *S. thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* (LDB). Starter lactic acid bacteria (SLAB) frequently inhibit pathogenic or spoilage bacteria, like *Escherichia coli* (EC) by competition, amensalism or ecosystem conditioning (decrease of pH, which in turn, due to syneresis and loss of water during ripening, results in reduced a_W). The latter (decrease in pH due to production of lactic acid, increase in pH due to consumption of lactic acid or proteolysis) is a frequent, indirect type of interaction. In some commensalistic (7) relationships products of the metabolism of one microorganism may become substrate for another like the use of galactonate produced by the yeast *Debaryomyces hansenii* (DH, which is also responsible of increasing the pH) by *Brevibacterium aurantiacum* (*BA*) and *Hafnia alvei* (HA). In turn, *H. alvei* may develop commensalistic or proto-cooperative interactions, since siderophores produced by HA stimulate BA, which in turn releases energy compounds for HA from proteins and lipids. Complex cheese consortia are also implicated in the inhibition of *Listeria monocytogenes* (LM) by amensalism or competition (11). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Some examples of microbial interactions between cheese microorganisms and of methods used for their study in model systems or in cheese.

	Type of interaction	Cheese	Partner(s) (effect/cost)[a]	Mechanism	Time and space dependence	Methods	References
1	Parasitism	All varieties	Lytic bacteriophages (+/0) Several LAB (−/€)	Infection followed by lytic cycle	Same time. Contact required.	Traditional culture-based methods, molecular methods, lineage specific qPCR, metagenomic approaches, matching CRISPR arrays and targets	Dairy bacteriophages reviewed in (Pujato et al., 2018; Erkus et al., 2013; Somerville et al., 2019; Walsh et al., 2020)
2	Proto - cooperation	Fresh cheese, some semi-hard and cooked varieties, undefined starters	*S. thermophilus* (+/0) *L. delbrueckii* subsp. *bulgaricus* (+/0) or L. *delbrueckii* subsp. lactis	Cross-feeding (CO_2, formic acid, amino acids, vitamins, amines, fatty acids), modification of the environment (O_2 consumption)	Same time. No contact required (diffusible products)	Growth kinetics, metabolome and transcriptome analysis in monocultures and mixed cultures, metagenome analysis	Reviewed in (Sieuwerts et al., 2008; Herve-Jimenez et al., 2009; Sieuwerts et al., 2010; Somerville et al., 2021)
3	Commensalism	Surface ripened cheeses	*Brevibacterium linens/ aurantiacum* (producing hydroxamate siderophores) (=/€) *Brevibacterium linens/ aurantiacum* (siderophore negative strains) (+/0)	Siderophore negative strains are limited in the cheese environment by competition for Fe^{3+}; siderophores produced by other strains allow growth	Same time. No contact required (diffusible products)	Coculture experiments, Comparative genomics, metabolomic analysis	(Noordman et al., 2006; Pham et al., 2017)
4	Competition	Surface ripened cheese	*Penicillium* spp. (?/?) *Glutamicibacter arilaitensis* (−/€)	The presence of the mould generates competition for microelements, which induced production of a Zn^{2+} chelating agent (coproporphyrin III), which is also a pink pigment. At high concentration coproporphyrin III may inhibit *Penicillium*	Same time. No contact required (diffusible products)	Co-culture experiments, metabolome analysis, imaging mass spectrometry, RNA-seq	(Cleary et al., 2018)
5	Commensalism	Swiss-type cheeses	Starter Lactic Acid Bacteria (=/0) *Propionibacterium* (+/0)	SLAB produce lactic acid and release peptides and amino acids from caseins, which subsequently are used by *Propionibacterium*	Succession (SLAB grow first); no contact required (diffusible products)	Traditional co-culturing approaches	(Baer, 1995; Fröhlich-Wyder et al., 2002)
6	Commensalism	Undefined strain starters, many cheeses	Prt+ strains/species (=/€), Prt− strain/ species (+/0)	Co-existence of proteinase negative and positive strains/species, due to the release of peptides by the proteinase: several examples in mixed cultures as representative cases of an interaction with some cost for one of the partners	Same space and time (SLAB/ SLAB) same space, different time (SLAB/ NSLAB)	Traditional co-culturing approaches, qPCR, metagenomic analysis in starter cultures, cheese and model cheeses	(Desfossés-Foucault et al., 2014; Erkus et al., 2013; Juillard et al., 1996; Moser et al., 2018)
7	Commensalism	Surface - ripened cheeses	*Debaryomyces hansenii* (=/0), *Brevibacterium aurantiacum* (+/€), and *Hafnia alvei* (+/0)	*D. hansenii* provides ecosystem conditioning by removing lactic acid and increasing pH. Galactonate produced by *D. hansenii* is consumed by the two bacteria. A strong positive interaction exists between *B. aurantiacum* (which is stimulated by siderophores) and *H. alvei* (stimulated by glycerol and FAA)	Succession (yeast/others) or same time. Cheese surface. No contact required.	Co-culture experiments in mini-cheese model, RNA-seq analysis and soluble metabolome analysis by UHPLC-MS and HPLC-UV in mini-cheese models,	(Pham et al., 2019)
8	Commensalism	Natural rind cheeses	*Staphylococcus equorum* (+/0) *Scopulariopsis* or *Penicillium* (=/€)	Selected moulds stimulate the growth of *S. equorum* by modulating iron (siderophore production) and possibly amino acid availability.	Same time. No contact required.	Co-culture experiments. Comparative genomics, RNA-seq	(Kastman et al., 2016)
9	Other + competition	Bloomy rind cheese (Saint Nectaire)	*Serratia proteamaculans* (−/?) *Mucor lanceolatus* (−/?)	*Mucor* specifically facilitates the dispersal of motile *Serratia*	Same time. Contact required.	Co-culture experiments. Imaging, meta-transcriptomics, transposon mutagenesis, comparative genomics	(Zhang et al., 2018)
10	Other	Model surface ripened cheese		Volatiles (including acetic acid) produced by	Same time. No contact required		(Cosetta et al., 2020)

(*continued on next page*)

Table 1 (*continued*)

	Type of interaction	Cheese	Partner(s) (effect/cost)[a]	Mechanism	Time and space dependence	Methods	References
			Galactomyces geotrichum (?/?), *Vibrio casei* (+/−)	*G. geotrichum* strongly stimulate the growth of *V. casei*		Co-culture experiments on cheese agar, RNA-seq, meta-taxonomic analyses	
11	Amensalism, competition	Surface ripened cheese	Smear cheese consortia (0/€), *Listeria monocytogenes* (−/−)	Microbial consortia of surface ripened cheese inhibit *L. monocytogenes* by competition or amensalism (bacteriocin production)	Same time. No contact required	Co-culture experiments in vitro, simulated cheese environments, cheeses	(Callon et al., 2014; Eppert et al., 1997; Imran et al., 2010)

[a] Effect: + stimulation of growth, = no effect, − inhibition/death; cost: 0 no cost, € metabolic cost, ? unclear.

been frequently used as a descriptive tool for the structure of microbial communities in foods: see Parente et al., 2018 for a review). In this case, edges represent simply the occurrence of a relationship (a bacteriophage infecting a bacterial strain, the presence of a given taxon in a given sample), which can be sometimes weighted (see below). More complex networks (sample-OTU-metabolite) can be built using multi-omic data (Liu et al., 2020).

In most cases, networks used for representing microbial associations are undirected (i.e., the edge does not point from one of the nodes to another, because the relationship is considered to be reciprocal). On the other hand, parasite-host networks are usually represented as directed networks, with edges going from phages to bacteria. Directed networks can be used to represent in a more meaningful way ecological relationships like commensalism, amensalism or parasitism, while mutualistic relationships and competition are typically undirected (Canon et al., 2020; D'Souza et al., 2018).

Networks can be weighted if the edges have some sort of integer or real number associated to them, representing the "strength" of the association (i.e., a correlation or distance value for association networks, abundance of a taxon in a given sample, some measure of the virulence of a phage toward a host, etc.).

Networks can be characterized by many network- and node- or edge-level indices, whose analysis may provide insights in the structure of the microbial community, allow the quantitative comparison of networks observed under different circumstances or assist in the identification of microorganisms which may have a prominent role in the structure and functioning of the microbial community (see Sections 2.1.1 and 2.1.2). A detailed review of these indices is beyond the scope of this work, and interested readers are referred to comprehensive reviews on microbial interaction networks (Layeghifard et al., 2017; Liu et al., 2020). Here we will present a simplified description of the most important indices, which is essential for the interpretation of the information provided in the following sections.

2.1.1. Global network properties

Several network level indices allow to typify network properties, to evaluate robustness of the microbial community toward disturbances and to compare networks occurring in different biomes or in the same biome under different conditions (Layeghifard et al., 2017; Peschel et al., 2020; Röttjers and Faust, 2018). In host biomes, disease is known to modify microbial association network structure (Ma, 2018; Röttjers and Faust, 2018). In a similar way, comparison of global network properties may provide insights on the effect of technological interventions (i.e. effect of starter addition and/or heat treatment), bacteriophage infection or spoilage on the structure of cheese microbial association networks.

The number of nodes and edges, the average degree, the connectance, the average path length (the average distance between pair of nodes), the node degree distribution and the global clustering coefficient are all important measures of network structure. The average degree, is, simply, the average number of edges per node. Connectance is defined as the ratio between the actual number of interactions with the potential number of interactions (Delmas et al., 2019; Dunne et al., 2002). The node degree distribution is the distribution of the probability that a node has a given degree. The clustering coefficient is a measure of the organization of the network in modules or cliques with a higher average degree among them than with other nodes. Microbial association networks in host and environmental biomes have been found to differ significantly from random networks and to have a scale-free structure. While in random networks each node has an equal probability of having an edge with another node, with a node degree distribution that follows a Poisson distribution, microbial interaction networks tend to have a power law node degree distribution, in which most nodes have a small degree while some, the hubs, have a large number of edges. This, in turn, results in the so-called small-world properties (low average path length, highly modular structure). The occurrence of highly interconnected hubs and of densely connected modules of nodes should result in resistance to disturbance (i.e., removal of random nodes or edges should not significantly change the structure of the network). On the other hand, removal of hub species or of edges connecting different modules can result in significant disruptions. Food MAN have been found to be simpler, to lack the small-world, scale free structure of environmental MAN, and to have at most a truncated power-law distribution of node degrees (Layeghifard et al., 2017; Parente et al., 2018; Röttjers and Faust, 2018). Since MAN can include both positive and negative associations, another important network property is the proportion of positive edges (PEP) (Faust et al., 2015). Changes in the ratio of positive to negative interactions may be considered markers for shift from a "healthy" to a "diseased" microbiome (Ma, 2018).

2.1.2. Node and edge properties

Several individual node properties contribute to the identification of keystone taxa in microbial association networks. Keystone taxa have been defined as "highly connected taxa that individually or in a guild exert a considerable influence on microbiome structure and functioning irrespective of their abundance across space and time. These taxa have a unique and crucial role in microbial communities, and their removal can cause a dramatic shift in microbiome structure and functioning" (Banerjee et al., 2018). Properties which have been used to measure the centrality of a taxa or its importance in an ecosystem are the degree, the weighted degree, other measures of how central is a node in the network (closeness, betweenness or eigenvector centrality) and the clustering coefficient). Their definition and significance have been reviewed recently (Layeghifard et al., 2017; Liu et al., 2020; Röttjers and Faust, 2018).

Hub species are characterized by high values of centrality measures (degree, closeness and eigenvector centrality) while bottlenecks are characterized by high values of betweenness centrality (the number of shortest paths passing through a node) and both may have key roles in ecosystem functioning and stability.

The detection of modules of highly interconnected nodes (with a high clustering coefficient) may help in the identification of groups of taxa which share the same niche or may have strong metabolic interactions.

It is worth noting that betweenness can be also calculated for edges: edges which have high edge betweenness are important for the structure

of the network, because their removal disrupts the network.

2.2. Methods for the inference and visualization of networks in microbiome studies

Because of the importance of network approaches in the description and understanding of microbial communities, a large variety of methods have been developed for the representation and inference of microbial association networks. We will briefly describe the tools used for network visualization and those used for the inference and analysis of microbial association networks and of bipartite networks.

2.2.1. Network visualization tools

Visual representation and analysis of bipartite sample-OTU networks (in which edges simply represent the occurrence of an OTU in a sample, possibly weighted by the relative abundance) can be used as a descriptive tool for the identification of clusters of samples or taxa and for the identification of the core and accessory microbiota (Parente et al., 2016). Node and edge tables derived from metataxonomic data (like those generated using QIIME's make_otu_network.py script) can be imported in graph visualization software (like Gephi, https://gephi.org/, or Cytoscape https://cytoscape.org/; Shannon et al., 2003). The ShinyFMBN app also provides a simple way of exporting data extracted from the FoodMicrobionet database (Parente et al., 2019) to Gephi and Cytoscape. These tools allow to filter nodes, calculate network and node statistics, use them or node or edge metadata to apply styles (shapes, sizes, colours, etc.) to subsets of nodes and edges, and to rearrange the graph using layout algorithms (which rearrange the position of node and edges in a 2-D or 3-D space) with the purpose of identifying and visualizing modules or hubs. As a result, the core microbiota, including taxa which are consistently present in a large number of samples, usually appears in the centre of the graph, while members of accessory microbiota, which is are only occasionally present, appear at the periphery. The interested readers are referred to De Filippis et al. (2014) for the first application of this approach in foods and Parente et al. (2016) for further examples on how application of styles (colour, size, thickness) to nodes and edges can enhance the visualization of bipartite food-microbe networks.

While both Cytoscape and Gephi provide tools for obtaining network and node statistics, they are most frequently used using menu driven interfaces and visualizations are hardly reproducible. R packages (including igraph and GGraph, among others) are more difficult to use and less flexible in terms of visualization options but have a significant advantage in terms of reproducibility and transparency of documentation.

Graph visualizations are visually pleasant and may provide some insight on the structure of microbial communities, but their interpretation is highly subjective and, due to the large number of options of layout algorithms, may be misleading and unreproducible if not properly documented.

2.2.2. Inference of microbial associations

While simple network visualizations are relatively straightforward, the inference of microbial associations (or of more complex associations in meta-omic data) requires specialized tools (Liu et al., 2020; Peschel et al., 2020; Röttjers and Faust, 2018). The most frequently used data are 16S rRNA marker gene data, which are affected by sparsity and compositionality.

Sparsity is related to the occurrence of a large number of zeros in OTU or ASV abundance tables. It is usually difficult to establish if the zeros are simply missing data due to insufficient sequence depth or if they are structural zeroes, reflecting the absence of a given feature. Compositionality is due to the fact that the true abundance of the target gene is rarely measured and relative frequencies are often used in OTU or ASV abundance tables. Even when absolute abundances are used, they suffer from a compositional bias, since, when the increase in abundance of a given taxon must be accompanied by the decrease in abundance of less abundant taxa.

In addition, in several cases, indirect correlations may be detected. Indirect correlations occur when an association between taxa A and B is inferred if both are associated with taxon C or with a common environmental condition.

Further difficulties ensue from the limitations of amplicon targeted approaches, whose resolution is often limited to the genus level. Finally, time-series data with enough time points to use model-based approaches for the inference of microbial interactions are relatively rare (Röttjers and Faust, 2018), and methods developed for cross-sectional data (i.e., data obtained for a collection of related samples which do not represent a time series) are more common.

These difficulties prevent the use of simple correlation measures between the abundance of different OTU/ASVs to infer the occurrence of an association, which, in turn, may represent a true biological interaction, and have led to the development of specific methods for the transformation of data prior to analysis or for the calculation of alternative association measures. A complete review of the methods and algorithms used to infer microbial association networks for cross-sectional data, of their strengths and weaknesses is beyond the scope of this paper: this is an active area of research and scores of papers have been published in recent years. The interested readers are encouraged to peruse one of the many recent reviews on the subject (Jiang et al., 2019; Liu et al., 2020; Röttjers and Faust, 2018).

Here we will briefly describe a few approaches which have been used more frequently and tested in comparative studies.

SparCC (Sparse Correlations for Compositional data; Friedman and Alm, 2012) infers networks based on Pearson correlations on log-ratio transformed data, thus addressing, at least in part, the issue of compositionality, and has been frequently used. CCREPE (Compositionality Corrected by REnormalization and Permutation; Faust et al., 2012), also known as ReBoot, uses permutations and renormalization to remove correlations due to compositionality alone and is implemented in the CoNet app (Faust and Raes, 2016) with a variety of correlation, similarity- and dissimilarity-based measures, which can be used in an ensemble approach. SPIEC-EASI (SParse InversE Covariance Estimation for Ecological Association Inference; Kurtz et al., 2015) is based on estimation of conditional covariances, is robust to both compositionality and indirect correlations, and has performed well in benchmarking, especially when using the neighbourhood selection method (also known as the MB method (Meinshausen and Bühlmann, 2006). Another recent method based on estimation of semi-parametric correlation and inference of conditional dependence is SPRING (SemiParametric Rank-based approach for INference in Graphical model; Yoon et al., 2019).

Some of the methods mentioned above (SPIEC-EASI, SPRING) have been reported to be robust toward indirect associations, but more direct methods to remove and/or identify the effect of environmental variables (pH, a_W, salt in moisture, temperature, etc.) exists. The CoNet app in Cytoscape (Faust and Raes, 2016) does allow to process metadata and infer associations between OTU/ASV and environmental variables. FlashWeave, a high throughput method for large scale network inference is also able to remove indirect correlations by including information on sample properties and is an extremely promising tool, given its ability to handle large composite datasets (Tackmann et al., 2019). Furthermore, an approach based on Joint Species Distribution Models and Poisson-Lognormal models has been claimed to be able to remove edges due to association between OTU/ASV and environmental variables, but has not been tested on microbial communities (Chiquet et al., 2021). While there is clearly a need to explicitly model the effect of covariate on microbial association networks, this has only rarely been done and, rather unfortunately, covariates are very rarely deposited as sample metadata in sequence repositories.

Finally, it is well known that network inference is method specific, and specific interactions, like amensalism, may be undetectable for cross-sectional studies (Weiss et al., 2016), but we have recently shown

that combining several methods may help in detecting stable and scientifically reasonable associations in food microbiomes (Parente et al., 2018, 2022). In fact, using a combination of three approaches (CoNet, SparCC and SPIEC-EASI) we were able to detect >40 co-presence and >40 mutual exclusion relationships in association networks for a variety of foods which were detected by at least 2 methods. The associations usually included known co-presence relationship between beneficial bacteria or between spoilage bacteria and mutual exclusion relationships between beneficial and spoilage organisms. More recently (Parente et al., 2022), we inferred microbial association networks for 34 studies on cheese microbiota using two correlation-based methods (SparCC and CCREPE) and two conditional independence methods (SPIEC-EASI and SparCC) and detected several stable (i.e. association detected by more than one method in more than one study) associations at the genus and species level, including both well-known associations and novel associations which need experimental confirmation.

2.2.3. *Software for the inference of microbial association networks*

The scarcity of studies on the inference of microbial association networks in cheese (and in food in general) may have been, at least in part, due to the delay with which approaches base on high throughput sequencing methods have been used in foods compared to other biomes and to the lack of user-friendly software for network inference, characterization and comparison.

The CoNet app in Cytoscape has been for a long time the only user friendly, menu driven tool for network inference and analysis (Faust and Raes, 2016). Although it does provide different methods for network inference, it does not allow network comparisons.

A number of R packages allow the inference of microbial associations, including SpieacEasi, (Kurtz et al., 2015), which implements both the SparCC and SPIEC-EASI methods, and SPRING (Yoon et al., 2019). A comprehensive and relatively user-friendly R package (NetCoMi, Peschel et al., 2020) for network inference with a large variety of methods, which also includes tools for estimation of network and node statistics, network visualization and formal network comparison, has recently become available.

Web based platforms, like MetagenoNets (Nagpal et al., 2020) are also available, thus empowering users who lack coding abilities.

3. Experimental approaches for the validation of interactions predicted by microbial association networks inference

Computational tools for the inference of microbial association networks are essentially explorative tools: although they provide a variety of information related to the structure of the interaction network, on the occurrence of potentially novel interactions and on keystone “species”, the interactions must be confirmed by in-vitro or in-vivo experiments. In a recent review Cosetta and Wolfe (2019) exemplified this approach in the sequence pattern – process – mechanism. In the pattern detection approach network inference tools can be used to identify statistically robust interactions. These are then tested in the process phase in experimental microbial communities, possibly in model systems. Finally, in the “mechanism” phase, metabolomic and transcriptomic approaches are used to identify the genetic and metabolic bases for the interaction. There are several examples for experimental approaches validating predictions based on microbial association network inference for environmental and host biomes (see Röttjers and Faust, 2018 for a review). As to cheese microbial communities, potential interactions are usually inferred by more heuristic approaches based on simple observations on the composition of microbial communities rather than on formal criteria.

Interactions in cheese are frequently mediated by diffusible or volatile metabolites (including volatile organic compounds) and/or by physical contact, and different approaches are used to de-construct interactions in vivo or in vitro.

With the exception of experiments performed in-vivo (i.e., in real cheese systems), simplified communities (2–4 members) are most frequently used, even if much larger assemblages have been tested in some cases (Callon et al., 2011, 2014; Imran et al., 2010). Simplified communities grown in liquid media are clearly the easiest way to clarify the mechanisms of microbial interactions, but they may be insufficient to resolve the complex and dynamic interplay in cheese, especially in cheeses produced with undefined starters, raw milk varieties or surface ripened cheeses.

In fact, several commensalistic interactions are, in fact, indirect: they are due to niche conditioning (change of pH, production/consumption of substrates, release of nutrients) and most microbial growth occurs in different life stages of the cheese (curd manufacturing, ripening) or at different locations (core, surface). Examples are the commensalistic interactions between starter lactic acid bacteria (SLAB) and non-starter lactic acid bacteria (NSLAB) in cheeses ripened internally by bacteria; lactic acid bacteria (LAB) - propionibacteria in Swiss-type cheeses; LAB - fungi - surface flora in surface ripened cheeses; (Blaya et al., 2017; Gobbetti et al., 2018; Mayo et al., 2021; Sieuwerts et al., 2008; Smid and Lacroix, 2013). The interested reader is referred to the many excellent reviews (Blaya et al., 2017; Gobbetti et al., 2018; Mayo et al., 2021; Sieuwerts et al., 2008; Smid and Lacroix, 2013) which have been published on this subject. Here we will concentrate on more direct interactions, based on physical contact or exchange of soluble or volatile metabolites.

Table 1 summarizes some representative interactions, together with the experimental approaches used for their study, ranging from simple growth experiments, occasionally with modelling of growth kinetics, to meta-transcriptomic and metabolomic approaches in cheese or model systems. Most studies analyse commensalistic relationships, either due to ecosystem conditioning or to exchange of metabolites, and the majority is focused on surface ripened cheeses and bloomy rind cheeses, in which the development of desired sensory properties relies on complex interactions between LAB, *Proteobacteria*, *Actinobacteria*, *Staphylococcaceae*, yeasts and moulds.

Negative interactions due to bacteriophages (parasitism) are relatively simple to study and have a profound impact on the structure and dynamics of microbial communities in cheese (Erkus et al., 2013; Pujato et al., 2018; Zotta et al., 2021). Because of their importance, bacteriophage - host interaction networks are reviewed in a separate paragraph (see Section 4).

Amensalism due to production of bacteriocins is also important, both because it might affect the stability of mixed strain starters and because of potential uses in bio-preservation for the control of pathogenic and spoilage microorganisms (Silva et al., 2018). Furthermore, this phenomenon is relatively easy to study in vitro (see Favaro et al., 2015; Lozo et al., 2021 for recent reviews). In addition, mining metagenomes for bacteriocin genes (Escobar-Zepeda et al., 2016; Lozo et al., 2021; Walsh et al., 2020), and using targeted methods for studying their expression in cheese is relatively easy (Trmčić et al., 2011). To our knowledge, there is no meta-transcriptomic data on the expression of bacteriocin genes in cheese during ripening.

Complex interactions are responsible of anti-listerial activity of beneficial microorganisms which develop on surface-ripened cheeses. The inhibitory activity may be due to factors other than bacteriocin production, including competition, and studies in model systems (cheese agar) have shown that complex consortia are needed, and that activity varies significantly with their composition and complexity (Callon et al., 2014; Imran et al., 2010).

Amensalism can be strongly affected by the solid nature of the cheese matrix: cheese moisture and even the internal environment of colonies may affect the diffusion of relatively large molecules (Floury et al., 2015; Guitián et al., 2019) like bacteriocins but also, in the short term, of smaller molecules. In fact, spatial distribution of colonies and diffusion of molecules within and between colonies in or on the cheese matrix may significantly affect cheese quality for several reasons. First of all,

except when cells or microcolonies are in close proximity, interactions are probably not relevant. Gradients within a single colony may be important in affecting the growth in different locations of a colony (Malakar et al., 2000, 2003), although the buffered environment of cheese may prevent the existence of pH microgradients, at least in microcolonies (Jeanson et al., 2013). Inoculum level may affect size and distribution of microcolonies and this, in turn, has been shown to affect cheese composition (Boucher et al., 2015a,b; Jeanson et al., 2010), although, in the long term, it might not affect the distribution of small soluble metabolites implicated in some cross-feeding relationships between SLAB and LAB (Czárán et al., 2018), at least in cheeses internally ripened by bacteria.

The situation is quite different in mould ripened cheeses and surface ripened cheeses. In fact, the composition of the microbiota on the surface and core of several cheeses (including cheese internally ripened by bacteria) is significantly different (see Jonnala et al., 2018 for a review) and differences in ecological conditions may strongly affect interactions between microorganisms and vice-versa. In Stilton cheese, the occurrence of mixed micro-colonies close to the internal veins formed by the mould was shown by Fluorescence In Situ Hybridization (Ercolini et al., 2003) and this was hypothesized to be due to commensalism. However, in the same cheese, *Lactococcus* and *Leuconostoc* formed in other parts of the curd pure culture microcolonies, and microcolonies identified as *Lactiplantibacillus plantarum* or tentatively identified as *Latilactobacillus curvatus* appeared in different location (underneath the crust or close to the veins).

In conclusion, imaging techniques are certainly most useful in studying the spatial organization of interacting microorganisms in cheese (Hickey et al., 2015), but mass-spectrometry techniques may also be of great importance in evaluating the importance of compounds like siderophores (Cleary et al., 2018) or possibly bacteriocins (Hindré et al., 2003).

Microbial interactions on cheese rinds may affect in a complex way the survival and growth of *Listeria monocytogenes* and *Escherichia coli*, two pathogens which have been associated with foodborne outbreaks due to the consumption of surface-ripened cheeses (Fusco et al., 2020). Co-cultivation with bacteria found on the surface of cheese (*Brevibacterium*, *Psychrobacter*) has been found to affect the expression of genes whose transcription is regulated by the global stress regulator σ_B (Anast and Schmitz-Esser, 2020) and the expression of a small non coding RNA, thus suggesting that stress due to competition is involved in this interaction. Random Barcode Transposon Sequencing and RNA-Seq have recently been used to identify genes which are related to the survival and growth in a cheese model of *Escherichia coli*, when grown alone or in binary and multiple associations with cheese-rind microorganisms like *Geotrichum candidum*, *Hafnia alvei* and *Penicillium camemberti* (Morin et al., 2018). Important genes were related to amino acid synthesis or metabolism, iron acquisition or response to toxic compounds and oxidative stress, thus confirming once again the importance of these factors in fitness and interactions in cheese. In addition, growth in co-culture demonstrated that the cheese species might exert both positive effects (by providing free amino acids) and negative effects (by amensalism through the production of toxic compounds or oxidative stress). Incidentally, this experiment also showed that pairwise interactions may be not representative of the growth in more complex (4 members) communities. Although this highlights the limitations of experiments in model systems, teasing out interactions in real systems may prove exceedingly difficult, due to variability in time and space and to the complex and dynamic nature of the microbial communities, especially in surface ripened cheeses.

The collection and interpretation of multi-omic data is probably the most promising approach for the study of microbial interactions in cheese during curd production and ripening, although cost and computational power issues may still limit the collection of large longitudinal data sets needed to use model-based approaches for inferring interactions. The most extensive demonstration of the value of complex, integrated approaches, for finely dissecting the complex interactions in cheese microbial communities is probably the on-going work on surface ripened cheeses (Bonham et al., 2017; Cleary et al., 2018; Cosetta and Wolfe, 2020; Cosetta et al., 2020; Kastman et al., 2016; Niccum et al., 2020; Wolfe et al., 2014; Zhang et al., 2018). Starting from an extensive metataxonomic and metagenomic characterization of the rind microbial communities of bloomy surface, washed rind and natural dry rind (Wolfe et al., 2014) from all over the world, and from the development of a detailed set of protocols for dissecting interactions in model cheeses (Cosetta and Wolfe, 2020), this group was able to provide detailed evidence for the drivers which determine the diversity and functionality of microbial communities on the surface of cheeses.

In fact, surface ripened cheeses provide an excellent model for the study of microbial interactions in an environment which, at least in part, can be mimicked in the laboratory under the controlled conditions which are needed to dissect the nature of the interactions and establish cause-effect relationships.

First of all, cheeses which are surface ripened by filamentous fungi (thus resulting in a bloomy rind, like Camembert, Brie, Saint Nectaire; Spinnler, 2017) or by complex consortia of yeasts, moulds and bacteria (washed rind cheeses, like Limburger, Tilsit, Port du Salut, Taleggio, etc.; Mounier et al., 2017) are economically important worldwide. They include both traditional varieties (which are still produced by using raw milk and rely on natural contamination from the environment, with very limited use of starter cultures) and industrial varieties, which are produced using pasteurized milk inoculated with defined or undefined starters and in which the development of the surface microbiota is promoted by the addition of specific combinations of fungi and bacteria. The composition of the rind microbiota of these cheeses is significantly more complex that those of cheeses with hard, dry rind (Wolfe et al., 2014), and the mature microbiota is the result of a complex succession: growth of halophilic, acid sensitive bacteria is made possible by the consumption of lactic acid by yeasts and by the increase in pH due to proteolysis caused by yeasts, moulds and, to a lesser extent, by LAB. Recent research using high-throughput sequencing approaches has shown that, beyond the complex assemblages of *Actinobacteria* (*Brevibacterium*, *Microbacterium*, *Arthrobacter*, *Corynebacterium*) and *Firmicutes* (*Staphylococcus*) which have been traditionally been associated with the pigmentation and aroma of these varieties, several *Proteobacteria*, including *Pseudoalteromonas*, *Hafnia*, *Vibrio*, *Halomonas*, and *Psychrobacter*, may contribute with their metabolic activities (Afshari et al., 2018; Jonnala et al., 2018; Wolfe et al., 2014). In addition, the increase in pH of the rind during ripening makes the growth and survival of pathogenic microorganisms easier, including *Listeria monocytogenes* and Shiga-toxin producing *Escherichia coli*. Understanding how these species are controlled by amensalism and competition is a key factor for the safety of these cheeses. Finally, several subdominant species which are prevalent on the surface of these cheeses are not deliberately inoculated (as part of the primary starter or the secondary ripening cultures). The role of cheesemaking environments, including different areas in the cheese plant and ripening shelves in the dispersal of these species and in the maintenance of a continuous inoculation source is therefore important in both providing beneficial and spoilage microbes (Bokulich and Mills, 2013; Guzzon et al., 2017).

While environmental and technological conditions may determine some of the co-occurrence and mutual exclusion patterns among bacteria and fungi, microbial interactions (both trophic and non-trophic) do explain why some of these relationships systematically occur across cheeses from different geographical areas (Wolfe et al., 2014). Apart from indirect interactions (de-acidification of the cheese surface due to growth of yeasts or moulds), several direct interactions are known to exist (Mayo et al., 2021; Mounier et al., 2008). Competition for micro-elements, with interactions mediated by exchange of siderophores or other metal chelators is frequent in surface ripened cheeses as is competition for folic acid, which can be alleviated by the exchange of corrinoids (modified tetrapyrroles with a cobalt centre, which include

vitamin B12), a frequent mechanism for microbial interactions (see Table 1; Abreu and Taga, 2016). Below, two examples from recent studies are described in more detail.

Clear-cut classification of microbial interactions in the framework of those listed in Fig. 1 and Table 1 may be difficult. Competitive relationships may hide more subtle relationships between partners: on the surface of bloomy rind cheeses motile proteobacteria like *Serratia proteamaculans* can exploit the network of hyphae formed by fungi like *Mucor lanceolatus* for dispersal: motile cells move along the water film on the lax hyphal growth formed by the fungus to disperse on the surface of the cheese, thus colonizing a larger area (Pujato et al., 2018). The relationship is somewhat specific and no dispersal or significantly lower dispersal is observed with other cheese fungi forming denser hyphal networks. Dispersal ability also varies among strains of *Serratia*. On the other hand, as in many other cases, some competition is observed between the two partners. RNA-seq analysis of *Serratia* grown in co-culture with *Mucor* showed that co-growth alters the supply of nutrients and metabolites and the metabolism of *Serratia*, but no significant difference in the expression of genes related to motility and quorum-sensing was found. Both transposon mutagenesis and comparative genomics supported the fact that genes related to flagellin production (and hence to motility) are essential for dispersal, but also provided proof that other genes are implicated in the relationship between these two species: a few mutants were able to kill *Mucor*, while others showed similar dispersal but altered colony morphology. In the same paper the authors elegantly demonstrated that fungal networks dramatically affect the structure of the microbial community and that dispersal, as well as other interactions (competition, amensalism) is implicated. In fact, several motile *Proteobacteria* could disperse on fungal networks, while motile and non-motile species of *Firmicutes* and *Actinobacteria* showed limited dispersal or no dispersal. Fungal growth may be therefore responsible for better growth of *Proteobacteria* on the surface of bloomy rind cheeses.

While many interactions may require a more or less close contact between partners, some are mediated by volatile compounds. Recently (Cosetta et al., 2020) it has been shown that volatile compounds produced by moulds isolated from bloomy or washed rind cheeses (*Galactomyces geotrichum*, *Debaryomyces hansenii*, *Penicillium* sp., *Scopulariopsis* sp., *Fusarium domesticum*) strongly stimulate or inhibit the growth of selected *Proteobacteria* (with *Vibrio* being strongly stimulated, and *Pseudomonas* often inhibited), and contribute to explaining the abundance of these species on cheese rinds. In the case of *Vibrio casei*, stimulation was related to significant changes in the transcriptome, and free fatty acids and esters of free fatty acids produced by the fungal partners were implicated in the relationship.

4. Future prospects

4.1. Does inference of microbial association networks provide useful information?

Even with all limitations related to the inference of association networks from metataxonomic data (see Section 2, and Röttjers and Faust, 2018 for a review), microbial association network inference offers invaluable information which may then be used to guide experiments to confirm the nature of the associations inferred in silico. The availability of simple to use workflows to calculate network properties and to compare networks (Peschel et al., 2020) can be used to formally test hypotheses on the effect of operational parameters (heat treatment, spoilage, use of starter cultures, etc.) on the structure of association networks. The networks structure of host microbiomes is known to be dramatically affected by disease (Layeghifard et al., 2017; Liu et al., 2020; Ma, 2018) and this has been proven to be true for the effect of mastitis on microbial communities in milk (see Parente et al., 2020, for a review). It is tempting to speculate that a given set of network properties may be associated to "healthy" cheese microbiomes in high quality cheeses, and that spoilage or sensory profiles deviating from those which are optimal for a given cheese may be associated, beyond the simple identification of biomarkers, to systematic changes in the structure of microbial association networks.

Finally, analysis of microbial association networks may help to mine for previously unknown interactions among microorganisms relevant for cheese ripening and provide the basis for the design of microbiome-based starter cultures (Canon et al., 2020; Mayo et al., 2021).

While a naïve use of microbial association network inference tools should be discouraged, their availability, in conjunction with complex metataxonomic databases as FoodMicrobionet and DairyFMBN (Parente et al., 2019, 2020) should significantly facilitate the mining of existing and new data for the inference of microbial associations.

4.2. Down to the strain level

One limitation of HTS targeting 16S rRNA gene is that, at the very best, they can provide taxonomic resolution at the genus and, sometimes, at the species level. Inference of Amplicon Sequence Variants and/or use of improved taxonomic databases may improve taxonomic resolution. On the other hand, biological interactions in cheese happen at the strain level, not at the species level (i.e., different strains of the same species can show a different interaction pattern). This is certainly true for bacteriophage-host interactions (Erkus et al., 2013, 2016) and has also been recently demonstrated for surface ripened cheeses (Niccum et al., 2020). Bacteriophages strongly affect the structure and microdiversity of mixed strain starters (see Section 4.4 and Zotta et al., 2021, for a review). Strain specific behaviours and response to positive (indirect effects due to change in pH; direct effects due to cross-feeding relationships possibly related to the availability of iron, vitamins, amino acids) and negative (amensalism, competition) relationships in simplified communities including combinations of different strains of *Staphylococcus equorum*, *Brachybacterium alimentarium*, *Brevibacterium aurantiacum* and a *Penicillium* strain, in simulated cheese-rind experiments, result in different community assemblies, which are reflected by changes in quality relevant feature (volatile compounds, colour). Strain specific relationships were also found in interactions mediated by volatile compounds (Cosetta et al., 2020).

Although HTS methods targeting protein-coding genes have been used to study the structure of population of key bacterial species (see Bertuzzi et al., 2018; Moser et al., 2018; Walsh et al., 2020 for recent reviews), they can only detect sequence variants of selected genes, not strains. Shotgun metagenomic approaches can provide full resolution for both bacterial strains and bacteriophages (Afshari et al., 2018, 2020; Somerville et al., 2019, 2021; Walsh et al., 2020). However, the cost and the need for computing resources is still significantly higher compared to amplicon targeted approaches, and, as a result, shotgun metagenomic studies typically have a lower number of samples. In addition, the relatively low diversity of some cheese microbial communities may prevent the detection of the less abundant members of the microbiota: in fact, using a combination of short-reads and long-read de novo assemblies, Somerville et al. (2019, 2021) only found a very low number of strains in Emmental and Gruyere cheese and related starter cultures. This low diversity may be due to population bottlenecks caused by repeated propagation under selective conditions, and may be common to several traditional cheese types (Erkus et al., 2013, 2016; Zotta et al., 2021). Improvements in sequencing technology and in bioinformatic pipelines (Hildebrand, 2021) and integration between metagenomics, metatranscriptomics and metabolomics, and custom designed qPCR methods to quantitatively monitor single strains or lineages may offer great potential for the study of ecological and metabolic interaction in cheese (Erkus et al., 2013; Niccum et al., 2020).

4.3. Model-based approaches and effect of environmental variables

Correlation or conditional dependence tools used for the inference of microbial association networks may be unable to detect some negative

interactions (like amensalism; Weiss et al., 2016), especially in cross sectional studies based on compositional data. In addition, they may be unable to properly separate the effect of habitat filtering if environmental variables are not included (see Section 2.2.2). The number of time series studies using metataxonomic or meta-omic approaches for the study of the dynamics of cheese microbiota is increasing (Choi et al., 2020; De Filippis et al., 2016; De Pasquale et al., 2014), and the lack of quantitative measurements of the target genes and the lack of metadata related to significant environmental variables (temperature, pH, a_W, salt in moisture) prevents the use of model-based approaches for the inference of microbial interactions. In principle, using absolute quantification of the total amount of target (16S RNA or RNA gene) is possible, and has been attempted in a few studies (Cauchie et al., 2017; Fougy et al., 2016; Rouger et al., 2018; Zotta et al., 2019). However, only rarely have attempts been made to use these data to study the dynamics of members of the bacterial community (Zotta et al., 2019). In addition, when DNA is used as a target the question of the viability of the detected taxa remains open, and qPCR of 16S rRNA or rRNA gene can only provide partial indications of the real abundance of a given taxon, given the differences in copy numbers of these targets (Větrovský and Baldrian, 2013). On the other hand, lineage specific qPCR methods have successfully been used to study the dynamics of individual strains or groups of strains in cheese (Erkus et al., 2013, 2016). Viability PCR with ethidium monoazide (EMA) or propidium monoazide (PMA) is one method for inferring which portion of a microbial community is active (Emerson et al., 2017) and has been used in cheese (Erkus et al., 2016; Porcellato and Skeie, 2016) and milk and milk environments (Kable et al., 2019). Although the technique needs to carefully standardized and validated, because of potential biases (Emerson et al., 2017) it is certainly a promising approach for the detection of the dynamics of the viable portion of cheese microbial communities.

Therefore, in principle, use of time series metataxonomic data, possibly coupled to viability PCR and model-based inference methods may be feasible and may be used to test in cheese model complex interactions after their preliminary evaluation in simplified laboratory models. MetaMis is one example of software packages using model-based inference (Shaw et al., 2016). More study with properly designed experiments is definitely needed in this area.

4.4. Phage-bacteria interaction networks

Bacteriophage infections of starter bacteria are the main cause of failure of cheese fermentations when defined starter cultures are used (Erkus et al., 2013; Zotta et al., 2021). Even when the more phage-tolerant undefined starter cultures are used, bacteriophages have a dramatic impact on the structure and dynamics of bacterial populations and communities (Erkus et al., 2013). In the latter case they have been found to be important in maintaining the equilibrium in complex association by a "kill the winner" mechanism (Erkus et al., 2013; Flores et al., 2011): bacteriophages killing of the most competitive strains prevent the elimination of the slow-growing strains from cultures reproduced by back-slopping. Even if bacteriophages of starter species are more frequently studies, several non-starter bacteria which are important in surface ripened cheeses (*Brevibacterium*, *Glutamicibacter*, *Microbacterium*, etc.: Jacobs-Sera et al., 2020; Klyczek et al., 2017; de Melo et al., 2020) or Swiss-type cheese (*Propionibacterium:* Cheng et al., 2018) are also known and, in some cases might be responsible of defects in cheese. More recently, metavirome studies are being carried out in starter cultures and in cheeses (Colombo et al., 2018; Dugat-Bony et al., 2020; Frantzen and Holo, 2019; Muhammed et al., 2017; Queiroz et al., 2021) and are allowing the discovery of novel bacteriophage-host interactions relevant to cheese ecology and quality.

Phage-Bacteria Interaction Networks (PBINs) are typically represented as bipartite networks (see Section 2.1). Their statistical structure has been elucidated (Flores et al., 2011; Weitz et al., 2013), and differences between the structure of PBINs for *Lactococcus* (which tend to show nestedness) and *S. thermophilus* (which tend to be modular) have been attributed to the differences in phage resistance mechanisms in these species.

To the best of our knowledge, approaches based on bipartite network analysis have not been recently used to elucidate the structure of PBINs of dairy microorganisms. The development of new high-throughput approaches for the study of the metavirome in dairy products (see Zotta et al., 2021 for a recent review) combined with network science approaches is certainly promising in studying the evolution of bacteriophage-host relationships in natural starters and cheese in self-assembled communities (Canon et al., 2020) in cheese manufacture. In fact, many traditional cheeses are still produced by using undefined starters reproduced by back-slopping (Zotta et al., 2021), and dispersal, diversification, evolution and drift (Nemergut et al., 2013) all play a role in shaping the structure, dynamics and function of microbial communities, at least in early stages of cheese-making.

Finally, beyond their role in regulating the structure of bacterial populations, bacteriophages (and prophages) may have other beneficial effects in microbial communities (induction of lysis, with release on nutrients for other members of the community; providing mechanisms for recombination and gene exchange; Paillet and Dugat-Bony, 2021).

Use of the concepts and computational approaches of bipartite network analysis (available, for example in the R bipartite package; Dormann et al., 2009) may be of assistance to both scientists interested in studying the structure and evolution of PBINs in cheese and to starter companies seeking to develop phage rotation schemes by facilitating the identification of potential hub strains and the identification of modules of virulent phages and susceptible strains.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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