Omics Approaches to Assess Flavor Development in Cheese

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Cheese is characterized by a rich and complex microbiota that plays a vital role during both production and ripening, contributing significantly to the safety, quality, and sensory characteristics of the final product. In this context, it is vital to explore the microbiota composition and understand its dynamics and evolution during cheese manufacturing and ripening. Application of high-throughput DNA sequencing technologies have facilitated the more accurate identification of the cheese microbiome, detailed study of its potential functionality, and its contribution to the development of specific organoleptic properties. These technologies include amplicon sequencing, whole-metagenome shotgun sequencing, metatranscriptomics, and, most recently, metabolomics. In recent years, however, the application of multiple meta-omics approaches along with data integration analysis, which was enabled by advanced computational and bioinformatics tools, paved the way to better comprehension of the cheese ripening process, revealing significant associations between the cheese microbiota and metabolites, as well as their impact on cheese flavor and quality.

cheese flavor omics lactic acid bacteria yeasts cheese microbiome metagenomics metatranscriptomics metabolomics

1. Omics Insights into Flavor Formation in Cheese

1.1. Genomics

The first omics technique to appear, genomics, focused on sequencing and annotation of the complete genome of an organism in order to reveal the genetic structure and to predict gene functions that are encoded within the genome. Since the publication of the long-awaited whole genome sequence of the first LAB strain in 2001, namely *L. lactis* subsp. *lactis* IL1403 [1], the number of sequenced LAB genomes has grown exponentially, due to the advent of HTS, with the genome sequences of thousands of LAB species and strains currently being deposited in public databases [2].

The study of Bolotin et al. [1] was the first genomic milestone in LAB research and laid the groundwork for a better understanding of the many aspects of bacterial physiology, metabolic pathways, and regulatory mechanisms, as well as how phenotypes are affected by genetic variations. In the following years, numerous genomics studies were conducted regarding strains of the “laboratory workhorse” species *L. lactis* [3][4][5][6][7]. As mentioned before, *S. thermophilus*, *L. bulgaricus,* and *L. helveticus* are also the main SLAB used in cheese production along with *L.*

lactis. Therefore, due to their industrial and economic importance, efforts are constantly being made to fully sequence and annotate genomes of these taxa [8][9][10][11]. In recent years though, genomics studies included NSLAB as well, due to their considerable impact on flavor development in cheese [12][13][14][15][16][17][18][19][20][21][22][23].

Proteins Associated with Flavor Formation in Cheese

Proteolysis, lipolysis, and AAs/FAs catabolism have all been studied in detail before whole genome sequencing (WGS) appeared. However, the available genome sequences allowed an in-depth analysis of the respective genes, so as to understand the genetic instability of several traits and unravel strain-specific differences. From this perspective, a comparative genomics analysis of all the proteins involved in the proteolytic system of 22 completely sequenced LAB strains was performed, including the cell-wall-bound proteinase, peptide transporters, and peptidases [24]. Based on the results, the genomes of the *Lactobacillus acidophilus*, *Lactobacillus johnsonii*, *Lactobacillus gasseri*, *L. bulgaricus*, and *L. helveticus* strains studied encoded a relatively higher number of proteolysis-related genes. Furthermore, the cell-wall-bound proteinase PrtP was solely identified in the chromosomes of *L. acidophilus*, *L. johnsonii*, *L. bulgaricus*, *L. casei*, *L. rhamnosus*, and *S. thermophilus* strains, as well as in the plasmid of *L. lactis* subsp. *cremoris* SK11. On the other hand, endopeptidases PepE/PepG and proline peptidases PepI, PepR, and PepL were absent in *Lactococcus* and *Streptococcus* strains, while aminopeptidases PepC, PepN, and PepM and proline peptidases PepX and PepQ were present in all genomes analyzed [24]. More recently, comparative genomics analysis among 213 assemblies, of which 175 belonged to *Lactobacillus* species and 38 to associated genera, was performed [25]. Regarding the metabolic potential of the 213 strains analyzed, the authors found genes for 60 cell envelope proteinases, which are important for cleaving casein during growth in milk and thus, contribute to cheese flavor, ranging in length from 1097 to 2270 amino acids. In addition, a broad repertoire of glycoside hydrolases and glycosyltransferases was identified, which are both important in carbohydrate metabolism [25]. Therefore, genome-scale metabolic models have been constructed and applied for the in silico prediction of the metabolic patterns of LAB strains under various conditions [26][27][28][29][30][31]. It should be noted, however, that the accuracy of these models depends on the quality of the genome sequencing and the correct annotation.

Furthermore, genomes of the non-LAB genera *Propionibacterium* and *Brevibacterium* have also been sequenced and annotated, as they also contribute to the flavor of certain types of cheeses. Genomics analysis of *Propionibacterium* assemblies mainly focuses on the identification of genes that are involved in the two key metabolic pathways for the propionate production, i.e., the Wood–Werkman and the tricarboxylic acid cycles, the amino acid catabolic pathways, which result in the formation of volatile compounds, and the detection of esterases involved in the formation of FFAs and esters [32][33][34]. Similarly, WGS studies of *Brevibacterium* assemblies reported, among others, the gene repertoire responsible for the catabolism of lactose, galactose, citrate, lipids, proteins, and amino acids, which contribute to the flavor, texture, and appearance of cheeses [35][36][37][38].

Apart from bacteria, several fungal species are also used as starters in internally and surface-ripened cheeses, such as Roquefort and Camembert, respectively, with a vital role in the flavor formation of the final product. Albeit

their importance, there is still a limited number of sequenced genomes in the NCBI database, e.g., only five and six partially sequenced assemblies for *P. roqueforti* and *P. camemberti*, respectively, which are two of the main fungal species used in cheese production. However, most of the WGS studies performed focused on the phylogeny and not the metabolic pathways regarding flavor formation [39][40][41][42][43].

Linking genotype to phenotype is of paramount importance to an in-depth understanding of the technological potential of a strain and therefore, to a better selection of candidates with flavor-forming metabolic potential to be used as starter or adjunct cultures in cheese production. However, as cheese ripening depends on a complex microbial community, the need for metagenomics analysis quickly arose.

1.2. Metagenomics

Metagenomics encompasses two different HTS approaches, namely amplicon sequencing and shotgun metagenome sequencing. In amplicon sequencing, a highly conserved marker gene or genome's region of DNA directly extracted from a food microbial community, e.g., that of cheese, is amplified and sequenced. These markers are of taxonomic relevance, with the 16S rRNA gene being used for the identification of bacteria taxa and the 18S rRNA gene together with the ITS DNA region for the yeasts/fungal taxa. On the other hand, in shotgun metagenomics, the extracted DNA is fully sequenced in a non-targeted manner, thus providing not only taxonomical identification results, but also information on the metabolic potential of the microbial community by reconstructing metabolic pathways [44].

From this perspective, a shotgun metagenomics study was recently performed in 25 Cotija cheese samples [45]. This artisanal Mexican cheese is produced by raw milk without the addition of starter cultures and is ripened in an open environment. Therefore, the organoleptic characteristics of Cotija cheese depend on a range of biotic and abiotic factors. Taxonomic identification revealed that the bacterial microbiota is mainly composed of Firmicutes, followed by Actinobacteria and Proteobacteria. The authors were able to reconstruct the genome assemblies of the three dominant species detected, i.e., *L. plantarum, Leuc. mesenteroides,* and *W. paramesenteroides*, which accounted for more than 80% of the total bacterial sequences. Gene functional annotation related to Cotija cheese flavor resulted in the identification of genes involved in the catabolism of phenylalanine, branched-chain amino acids, and fatty acids [45].

1.3. Transcriptomics

Genomics is widely applied to study the technological potential of a strain; however, it can only in silico predict its functional potential. Therefore, transcriptomics (RNA-seq), although a relatively new field of research, has gained much attention as it provides an accurate profile of a strain's functional activity at a given time point.

Regarding cheese microbiota, the majority of the transcriptomic studies have been performed on *L. lactis*, due to its importance in cheese production [2][46][47][48][49][50][51][52][53][54]. In particular, the transcriptional responses of *L. lactis* have been investigated during different stresses, such as cold, heat, acid, osmotic, oxidative, and starvation [55][56][57][58][59][60][61], or during growth in media with different carbon sources [62][63][64]. Apart from *L. lactis*,

transcriptomics studies have also been performed in other important cheese bacterial [48][65][66][67][68][69][70][71], yeasts [72][73][74], and fungal species [75][76][77][78][79][80][81]. Most interestingly, Dalmasso et al. [82] reported that *P. freudenreichii* CIRM-BIA1^T adapted from warm (28 °C) to cold storage (4 °C) was able to express genes involved in the formation of important cheese flavor compounds through the catabolism of branched-chain amino acids. Similarly, the transcriptome profile of *P. freudenreichii* CIRM-BIA138 was analyzed during the adaptation of the strain to starvation [83], while that of *P. freudenreichii* ITG P14 during different stresses, i.e., cold, heat, and starvation [84]. Moreover, the transcriptome profile of *L. rhamnosus* PR1019 was recently evaluated in a cheese-like medium during carbon source starvation [85]. The analysis revealed that the strain was able to adapt under these conditions using alternative metabolic pathways, such as pyruvate degradation and ribose catabolism.

1.4. Metatranscriptomics

In contrast to the plethora of transcriptomics studies in single microbes, a limited number of metatranscriptomics projects have been performed to assess the entire gene expression of a food microbial community, such as that of cheese. Among the key problems are the short half-life of mRNA, the large amount of mRNA needed for analysis, the experimental design, and, most importantly, the selection of the proper sampling points and some technology-specific limitations, including the available bioinformatics tools and workflows for data processing and analysis [86]. The first comprehensive metatranscriptomics study was performed by Lessard et al. [87]. The authors monitored the metatranscriptome profiles of *G. candidum* and *P. camemberti* in the rind of an industrial Canadian Camembert-type cheese. Based on the functional annotation performed, transcripts related to energy metabolism, such as glycolysis/gluconeogenesis, the pentose phosphate pathway, the tricarboxylic acid cycle, and oxidative phosphorylation, were identified. Furthermore, lyases involved in the production of volatile sulfur compounds during methionine catabolism as well as transcripts related to cabbage sulfur aroma development and ammonia production were also detected [87].

More recently, Monnet et al. [88] evaluated the functional activity of the microbiome in the rind of a French Reblochon-type cheese, which was produced by *S. thermophilus, L. bulgaricus, G. candidum*, and *D. hansenii*. Metatranscriptomics analysis revealed that during ripening, only minor changes occurred in the LAB, such as an upregulation of genes involved in lipid and carbohydrate metabolism, while in yeasts, a significant upregulation of genes related to amino acid catabolism occurred from day 14 to day 35 (end of ripening), suggesting their contribution to flavor formation [88].

However, discrepancies observed between mRNA levels and protein abundance, which have been attributed to the variable half-lives of mRNA, variable rates of protein synthesis, and possible post-translational modifications of proteins [89], make the analysis of expressed proteins a valuable tool for investigating the functional activity of microbiota.

1.5. Metaproteomics

Metaproteomics is the large-scale characterization of the entire protein complement of microbial communities in a biological system of increased complexity at a given time point [90]. The progress of proteomics has been driven by

the development of new technologies for peptide/protein separation, isotope labeling for quantification, and bioinformatics data analysis, while the analysis and quantification of proteins has been revolutionized by MS-based methods [91]. There are several challenges that must be overcome to address a metaproteomic study in animal food products. Among them are the presence of raw material proteins that interfere with the detection of microbial proteins, the complexity of the microbiota-expressed proteins in a fermented food present at concentration ranges that may vary dramatically, and finally, the presence of certain highly abundant proteins that are often not interesting for metaproteomic analysis, while the microbiota proteins may be several orders of magnitude less abundant. For these reasons, large-scale metaproteomic studies are limited. However, they are gradually gaining attention in the field of fermented food research, including cheese [92][93], to assess the functional diversity of microbial communities. To our knowledge, there are no metaproteomics studies on cheese flavor formation and development. However, the proteomic approach applied in a survey of bacterial proteins released in Emmental cheese revealed functional groups of proteins involved in proteolysis and glycolysis, pathways that influence the organoleptic characteristics of cheese [94].

1.6. Metabolomics

1.6.1. Analytical Techniques

Metabolomics is a relatively new field of omics research dealing with the simultaneous and high-throughput identification and quantification of low molecular mass (<1500 Da) metabolites that are not genetically encoded and are produced and modified by the metabolism of living organisms, such as organic acids, carbohydrates, amino acids, peptides, nucleic acids, vitamins, polyphenols, alkaloids, and minerals [95]. Metabolomic analyses have been classified as targeted (specific) or untargeted (non-selective) analyses.

The vast chemical diversity of the compounds in the metabolome requires efficient metabolite extraction, chromatographic separation, mass spectral detection, identification, quantification, and multivariate data analysis. Various analytical techniques are used for separation, such as liquid chromatography (LC), including high-performance LC (HPLC) or ultra-performance LC (UPLC), gas chromatography (GC), and capillary electrophoresis (CE), coupled to mass spectrometry (MS). MS-based platforms predominate because of both their ability to identify a wide range of compounds and their high throughput capacity. Apart from MS, other commonly used detection techniques are nuclear magnetic resonance (NMR) and near infrared spectrometry (NIR). However, none of the individual analytical methods are capable of effectively analyzing all the metabolites of a sample.

1.6.2. Metabolomics to Assess Flavor Formation in Cheese

Metabolomic studies in cheese provide insights on specific metabolites produced by cheese microbiota, with special interest in flavor compounds, and have significantly enabled characterization of the metabolite diversity in cheese, as well as the factors affecting it. According to Ochi et al. [96], GC/TOF-MS fingerprinting of hydrophilic low-molecular-mass compounds can be the basis of prediction models for sensory attributes, such as “rich flavor” and “sour flavor” in ripened Cheddar and Gouda, while specific amino acids [97] or volatile compounds, such as

hexanoic acid, heptanoic acid, octanoic acid, 2-decenal, and acetoin [98], may be used as ripening markers in Cheddar cheese.

The cheese metabolome is affected by the degree of LAB autolysis. Grana-Padano cheeses produced in two different dairies and with different volatile profiles, as shown by solid phase micro-extraction (SPME) GC-MS, were analyzed with respect to their total culturable lactic microbiota and starter lysis. Remarkably, it was shown that increased complexity of microbial origin volatiles, such as ketones, alcohols, hydrocarbons, acetic acid, and propionic acid, was associated with the complex microbiota composition, with NSLAB (mainly *L. rhamnosus*/*L. casei*) being dominant. On the other hand, intracellular enzymes released due to SLAB cell lysis were involved in a higher content of FFAs, benzaldehyde, and organic acids, such as pyroglutamic and citric acid [99].

LC high-resolution MS (LC–HRMS) metabolic fingerprinting in model cheese revealed that the cheese metabolome is influenced by the spatial distribution of the starter *L. lactis* colonies throughout ripening [100]. By varying the time of renneting (at 0 h, e.g., simultaneously, or after 8 h from starter inoculation), they generated two different spatial distributions of immobilized bacterial colonies (few big colonies spread away from each other, or numerous small colonies close to each other, respectively), and identified 26 metabolites, including amino acids, organic acids, vitamins, nucleotides, and proteolysis products, being more abundant in small-colony cheeses. Moreover, according to Le Boucher et al., bacterial cells forming small colonies use the same metabolic pathways but display higher metabolic activity than big colonies, resulting in higher concentrations of metabolites being accumulated due to proteolysis or carbohydrate catabolism [101].

Investigation of the flavor compounds produced by the whole cheese microbiota or the individual contribution of SLAB or NSLAB strains is of great interest for cheese producers. Studies of this type are often performed in a cheese-based medium, mimicking the cheese environment, thus providing the nutrients and precursors present in cheese during ripening.

Sgarbi et al. explored the volatile compounds produced by different *L. casei* and *L. rhamnosus* strains grown on either a cheese-based medium (CBM) or on a LAB cell lysate-based medium (LCM). Volatile analysis by SPME GC-MS showed differences between NSLAB strains grown in CBM (where pyruvate could be a common precursor for all compounds produced) and LCM (where in the absence of lactate and citrate, NSLAB strains used mainly FFAs and FAAs), thus providing a better understanding of how NSLAB-produced volatile flavor compounds contribute to the development of cheese flavor during ripening, a first step toward the selection of wild NSLAB possessing a specific aromatic profile, for use as adjunct culture [102].

Similarly, Pogačić et al. evaluated the potential of *Lactobacillus* spp. and *Leuconostoc* spp. in the production of aroma compounds by incubating single strains in a curd-based slurry medium [103]. Analysis of volatiles by GC–MS revealed strain to strain variation. Acetoin, diacetyl, acids, and esters were mainly produced by *L. rhamnosus* and *L. paracasei*, while *Leuconostoc* spp. were major producers of alcohols and esters. The same curd-based slurry medium has been used by Pogačić et al. to screen LAB, Actinobacteria, *P. freudenreichii*, and *Hafnia alvei* strains

for their ability to produce aroma compounds. Forty-nine out of 52 aroma compounds identified differed in their abundance among the bacteria [104].

A cheese-based medium was also used by Guarrasi et al. to grow SLAB and NSLAB strains isolated from Caciocavallo Palermitano cheese to assess their contribution to the volatile organic compounds (VOCs) production in ripened cheese [105]. By applying head space (HS)-SPME GC-MS to the fermented substrates, they found that strains of *L. delbrueckii*, *L. casei*, *L. paracasei*, *L. rhamnosus*, and *Enterococcus gallinarum* mainly influenced the development of the characteristic ripened-cheese aromatic compounds.

Furthermore, according to Yee et al., strains of dairy propionibacteria exhibited large inter-species and intra-species diversity in their ability to produce different aroma compounds when grown in a cheese curd-based medium [106]. GC-MS data differentiated *P. freudenreichii* strains from each other, as well as from *Propionibacterium acidipropionici* strains. For the same compound, differences between strains of the same species were as high as ~500-fold, with *P. freudenreichii* strains harboring the widest potential to produce cheese aroma compounds, making it a potential candidate to modulate cheese flavor.

Except cheese-based medium, cheese model systems are important tools that facilitate the study of the impact of microbial metabolism on cheese sensorial characteristics formation since they can be prepared under controlled microbiological conditions, are more economical, reproducible, and easier to obtain.

Miniature cheeses were prepared by Ruggirello et al. [107] to evaluate *L. lactis* commercial starter cultures' viability and ability to produce aroma compounds during ripening. Culture-independent and -dependent approaches, as well as HS-SPME GC-MS, revealed the persistence of *L. lactis* in cheese throughout cheese making and ripening, although in a metabolically active hypothetical viable but not culturable (VBNC) state, since the expression of *L. lactis* cystathionine β-lyase (*metC*) and α-acetolactate synthase (*als*) genes, involved in the biosynthesis of sulfur aroma compounds and diacetyl/acetoin, respectively, was partially associated with acetoin, diacetyl, 2,3-butanediol, and dimethyl disulfide production in ripened cheeses.

Irlinger et al. [108] applied GC-MS for metabolic profiling of the Gram-negative bacteria *Psychrobacter celer* and *H. alvei* when grown on a mini smear cheese. Both bacteria were able to colonize the cheese surface and compete in the microbial community, modifying at the same time the aromatic content of cheeses, highlighting the fact that less abundant microorganisms can have a significant impact on cheese flavor.

In a recent study, Suzuki-Iwashima et al. [109] investigated the combined effects of LAB starters and the white fungus *P. camemberti* on the production of volatile compounds during the ripening of a model white mold surface-ripened cheese. Metabolomics analysis by GC-MS showed that the early ripening period was characterized by metabolites, such as lactose, galactose, lactic acid, ethanol, diacetyl, acetoin, ethyl acetate, and sulfur compounds (dimethyl sulfide and dimethyl disulfide), that derived from carbohydrate metabolism of LAB, while fungal metabolism of proteins (with amino acid-derived compounds including branched aldehydes, such as 3-methyl

butanal), and of fatty acids and proteins (with methyl ketones, fatty acids and amino acids) characterized the intermediate- and the late-ripening stages, respectively.

1.6.3. Metabolomics Shed Light on the Ability of Adjunct Cultures to Produce Flavor Compounds

Starter bacteria combined with adjunct LAB strains selected for desirable metabolic potential is a common tool used to control and accelerate cheese ripening [110], enhance and improve flavor intensity [111], especially in cheeses made with pasteurized milk [112], and accelerate flavor development [113]. Metabolomics approaches have been a valuable tool to assess the contribution of the potential adjuncts on cheese flavor development [89][114][115].

So far, several studies have been performed evaluating not only the contribution of potential adjunct cultures to cheese flavor development, but also their persistence, technological performance, together with interactions with the starter cultures in cheese making experiments. In a recent study, Stefanovic et al. [21], selected three *L. paracasei* strains based on their proteolytic enzyme activities and ability to produce flavor compounds in cheese model systems [116] to be used as adjunct cultures in Cheddar cheese manufacture. The authors found that the *L. paracasei* strains contributed to the development and diversification of flavor-related compounds in short-aged cheeses. Different adjunct cultures did not influence the gross cheese composition, nor primary or secondary proteolysis or lipolysis. However, cheese volatile analysis by GC-MS showed variation in long-chain aldehydes, acids, and esters that originated from the metabolism of FFAs, suggesting that starter lipolytic activity produced the primary metabolites, which were further metabolized by the adjuncts into flavor-contributing compounds.

Bancalari et al. [117] evaluated the use of a wild *L. paracasei* strain, selected for its ability to produce in vitro acetoin and diacetyl, as adjunct culture to enhance the flavor formation in Caciotta-type cheese. Indeed, the adjunct strain was able to develop in curd and cheese, producing higher amounts of volatile compounds and organic acids as monitored through SPME GC-MS, thus differentiating the experimental Caciotta with respect to the control cheese. Moreover, Belkheir et al. [118] prepared a Tetilla-type cheese using as adjunct cultures a high-diacetyl producer *L. plantarum* strain together with a peptidolytic *L. brevis* strain producing volatile sulfur compounds, to study the volatile profile and sensory characteristics of the model cheese and to compare it with the PDO Tetilla cheese. Volatile analysis with SPME GC-MS showed an increased abundance of acetic acid, hexanoic acid, ethyl butanoate, and ethyl hexanoate, as well as higher scores for flavor preference in the cheese made with the two adjuncts than in the control cheese, highlighting the fact that the use of selected adjunct strains would differentiate the cheese sensory properties. Interestingly, when *Kocuria varians* (*Micrococcaceae*) and *Y. lipolytica*, selected for their proteolytic and lipolytic activities, were used as adjunct cultures, for the manufacture of experimental Tetilla cheese from pasteurized cow's milk, Centeno et al. [119] were able to recover the traditional flavor and sensory characteristics of raw-milk PDO Tetilla cheese. The volatile profiles of cheese manufactured with both adjuncts, detected by GC-MS, showed enhanced formation of fatty acids, esters, and sulfur compounds, thus the modifying the flavor profile of the experimental Tetilla cheese, which was considered very similar to good-quality artisanal raw-milk cheese.

In a potentially probiotic Caciotta cheese, industrially produced with autochthonous putative probiotic *Lactobacillus* and *Kluyveromyces* strains as adjunct cultures, Pisano et al. [120] investigated the adjunct's influence on the cheese chemical and microbiological composition and sensory properties. Cheese metabolome characterization by means of ^{1}H NMR spectroscopy (for amino acids, organic acids, and carbohydrates) together with HPLC-diode array detector/evaporative light scattering detector (DAD/ELSD) for the cholesterol, α-tocopherol, and fatty acid composition, highlighted significant variations in the cheese metabolome both in terms of the ripening time and strain combination, with *Kluyveromyces* and *Lactobacillus* strains surviving the manufacturing process and retaining their viability till the end of ripening, suggesting that Caciotta cheese can be used as a carrier for probiotic bacteria delivery.

1.6.4. Metabolomics as a Means of Cheese Authentication

Effective and reliable analytical methods are of paramount importance to securing the authenticity of PDO cheeses, aiming to protect both the product value and consumers. To this end, metabolomics-based approaches represent a powerful method to discriminate fraudulent varieties of a given food product [121][122][123].

Pisano et al. [124] analyzed the polar metabolite profiles by GC-MS, together with the predominant cultivable microbiota from buffalo and cow Mozzarella, in order to discriminate them. PDO buffalo Mozzarella exhibited a higher microbial diversity together with less psychrotrophic bacteria, while cow Mozzarella showed the highest counts of *S. thermophilus*, originating from the commercial starter culture. Furthermore, the polar metabolites reflected differences in the production protocols and microbiota complexity of theses cheeses, suggesting that the polar metabolite profile can be a promising tool to characterize and verify the authenticity of Italian buffalo Mozzarella. Moreover, Rocchetti et al. [125] characterized low-molecular-mass metabolites based on ultra-high-pressure liquid chromatography coupled with quadrupole time-of-flight MS (UHPLC/QTOF-MS), aiming to reveal differences between genuine PDO and non-PDO Grana Padano cheeses. Amino acids, oligopeptides, and fatty acids were the biomarkers with the highest discriminatory power.

Another authenticity problem involves adulteration related to non-declared processing methods, such as in the case of Fiore Sardo (FS) cheese, where the use of raw ovine milk is mandatory. Caboni et al. studied the polar low-molecular-mass metabolites, by GC-MS, aiming to discriminate FS cheese produced from raw or thermized milk. FAAs and saccharides were the metabolites that mostly changed, suggesting the polar low-molecular-mass metabolites as a potential biomarker for detecting milk thermization in ovine PDO cheeses [126].

1.7. Integration

Studies combining multiple-omics approaches, integrating genomics, transcriptomics, together with metabolomics, that is, a systems biology approach, provide a detailed picture of the cheese microbiota dynamics, as well as information on potential microbial interactions and their contribution with respect to cheese sensorial characteristics development. An overview of the most comprehensive studies combining multiple omics approaches to study flavor development in cheese can be found in Supplementary Table S1. In this section, integrating omics studies, which

have been performed not only in mature cheese, but also during cheese ripening, are discussed, to gain deeper insights on microbial succession and metabolite production.

1.7.1. Amplicon Metagenomics-Shotgun Metagenomics

The first pioneering study incorporating different -omics techniques was performed by Wolfe et al. [127]. The authors analyzed the rind microbial communities of 137 cheese samples, including 61 natural cheeses that were left undisturbed during the aging process, 52 washed with 20% *w/w* NaCl, and 24 bloomy cheese rinds, from different geographic regions, milk types, and milk treatments. Using amplicon-based metagenomics analysis, only 14 bacterial and 10 fungal genera were identified at higher than 1% average abundance. Interestingly, the microbiota was found to be associated with the rind type (natural, washed, and bloomy) and moisture instead of the geographic origin. Additionally, shotgun metagenomics was performed to assess the functional potential of the microbiota based on the different rind types. In particular, shotgun data identified genes involved in several metabolic pathways associated with flavor formation, such as the catabolism of cysteine and methionine, which are known to contribute to the production of volatile sulfur compounds, and that of valine, leucine, and isoleucine, which provide sweaty and putrid aromas. It should be noted that the majority of these genes were detected in the washed rind cheeses. Furthermore, the halotolerant γ-proteobacteria genus *Pseudoalteromonas*, originally associated with marine environments, was found for the first time in cheese microbiota and, in particular, in the natural and bloomy cheese rinds. The shotgun metagenomics results identified a few cold-adapted enzymes produced by *Pseudoalteromonas* spp. that participate in lipolysis and proteolysis. Therefore, the presence of *Pseudoalteromonas,* as part of the cheese microbial community, could be considered beneficial, as it can contribute to the development of flavor compounds in cheeses during ripening and storage at low temperatures [127].

1.7.2. Amplicon Metagenomics-Metabolomics-Metatranscriptomics

De Pasquale et al. [128] used Fiore Sardo, Pecorino Siciliano, and Pecorino Toscano cheeses as hard cheese model systems to study the spatial distribution of metabolically active microbiota and its effect on secondary proteolysis and VOC production, since these cheeses present a decreasing NaCl gradient from the surface to the center and an opposite moisture trend, properties that affect the microbiota distribution. By combining 16S rRNA gene pyrosequencing (targeting RNA) and Purge and Trap coupled with GC-MS (PT GC-MS), they found that in all cheese varieties, the poorest VOC profile was detected in the core region, due to the low oxygen availability, with high levels of alcohols originating from aldehydes and methyl-ketones reduction. Mesophilic lactobacilli (predominantly *L. plantarum*) positively correlated to alcohols, aldehydes, methyl and branched esters, and sulfur compounds. Thermophilic LAB in Pecorino Siciliano (including *L. delbrueckii* and *S. thermophilus*) positively correlated with the total concentration of FAAs, in different cheese regions, as well as with alcohols and related esters, while *Brevibacterium* sp. present on the surface of Pecorino Toscano correlated with alcohols, aldehydes, ketones, esters, and sulfur compounds.

Recently, Turri et al. [129] characterized the mature Historic Rebel (HR) cheese, an Italian heritage cheese produced from raw cow milk in the Alps. Microbiota diversity, assessed by 16S rRNA gene amplicon sequencing,

revealed that the core microbiota comprising *Streptococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, and *Pediococcus* genera correlated positively with the VOCs hexanal, 2-heptanal, 3-hydroxybutan-2-one (or acetoin), and ethanol respectively, as determined by SPME GC-MS. Moreover, a lipidomics approach was included by applying Dynamic Headspace (DHS) GC-MS to analyze the terpene fraction and the polyunsaturated fatty acids composition of HR cheese, parameters that were closely related with pasture vegetation and feeding, respectively, and contribute to the richness of cheese flavor.

In a sophisticated study [73], metabolite analysis by Ultra HPLC-MS (UHPLC-MS) and HPLC-UV in combination with dual RNA-seq analysis were applied in ripened lab-scale cheeses to provide insight into the metabolic interactions in a simple synthetic community composed of three species commonly used for the production of smear-ripened cheese. A strong mutualistic interaction between *Brevibacterium aurantiacum* and *H. alvei* was proposed, according to which, proteases and lipases secreted by *B. aurantiacum* liberate energy compounds from caseins and triglycerides that stimulate *H. alvei*, which in turn produces siderophore that increases iron availability for *B. aurantiacum*. Furthermore, the proteolytic activity of *B. aurantiacum* led to increased methionine catabolism in *H. alvei* producing methanethiol, a precursor for a wide variety of volatile sulfur compounds that contribute to cheese flavor.

1.7.3. Shotgun Metagenomics-Metatranscriptomics

Recently, Duru et al. [130] and DeFilippis et al. [131] studied the effect of modifying the cheese ripening temperature on microbial community structure and function. More specifically, in a thorough study, the metagenome and metatranscriptome profiles of the semi-hard Swiss-type Maasdam cheese were studied during warm (20 °C) and cold (5 °C) room ripening, using *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* as starter strains and as adjunct cultures strains of *P. freudenreichii* subsp. *shermanii*, *L. rhamnosus*, and *L. helveticus* [130]. The authors constructed four genomes (one genome per species) from the shotgun data to near completeness (higher than 97%), and based on the mean DNA read coverage, *L. lactis* was found to be the dominant species. Annotation of genome assemblies and pathways reconstruction identified genes required for FFAs biosynthesis in all genomes, proteolytic enzymes only in the LAB, and lipolytic enzymes in the genomes of *L. lactis, L. rhamnosus,* and *P. freudenreichii*. Furthermore, genes for valine catabolism were found in *L. lactis* and *P. freudenreichii*, while those for methionine and cysteine catabolism in *L. lactis, L. rhamnosus,* and *L. helveticus* genomes. Therefore, all species are important for the flavor formation of Maasdam cheese to a different extent. RNA-seq analysis confirmed the dominance of *L. lactis*, as more than 85% of the transcript reads mapped uniquely to this species. Moreover, metatranscriptomic data showed that *L. lactis* was metabolically active despite the ripening temperature. However, this was not the case for the other species, as genes related to the central metabolism were downregulated during cold room ripening, suggesting that fewer flavor compounds were produced. On the other hand, according to De Filippis et al. [131], elevation of the ripening temperature of Caciocavallo Silano cheese from standard (16 °C) to experimental (20 °C) temperatures directly affected microbiota diversity and metabolism. More specifically, 16S rRNA amplicon and shotgun metatranscriptome sequencing revealed an increased relative abundance of NSLAB in cheese ripened at higher temperatures, together with differential expression of 651 genes. Furthermore, overexpression of proteolysis, lipolysis, amino, and fatty acid catabolism-related genes at 20 °C

correlated with increased production of cheese VOCs, as found by SPME GC-MS, and significantly increased the cheese maturation rate.

The functional potential of the microbiome in an experimental surface-ripened cheese was recently assessed by Dugat-Bony et al. [132]. Using reference genomes, the assembly of the shotgun metagenomics data resulted in the genome construction of the nine microbes used for the production of the cheese, i.e., six bacteria species (*L. lactis*, *Staphylococcus equorum*, *Corynebacterium casei*, *H. alvei*, *B. aurantiacum*, and *Arthrobacter arilaitensis*), and three yeast species (*D. hansenii*, *G. candidum*, and *K. lactis*). In addition to the shotgun metagenomics, the authors also employed metatranscriptomics to link metabolically related transcript reads to the microbiome at specific time points during cheese ripening. RNA-seq analysis revealed that enzymes involved in lactose fermentation were found to be expressed by *L. lactis* and *K. lactis*. Moreover, lactate degradation was attributed to *D. hansenii* and *G. candidum*, due to the high levels of lactate dehydrogenase transcripts detected in these species. Furthermore, although several transcript reads associated with protein and lipid metabolism were mapped to *L. lactis*, it was found that *G. candidum* was the main contributor to proteolysis and lipolysis. In addition, *G. candidum* was also found to be a key microbe regarding the catabolism of amino acids and thus, the production of flavor compounds throughout ripening. However, it should be noted that transcripts related to amino acid catabolism also mapped to the *L. lactis* genome, mainly at the early stage of ripening, as well as to the *C. casei* and *H. alvei* genomes at the end of ripening [132].

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