Research Reflection

Developments in effective use of volatile organic compound analysis to assess flavour formation during cheese ripening

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Abstract

In the burgeoning demand for optimization of cheese production, ascertaining cheese flavour formation during the cheese making process has been the focal point of determining cheese quality. In this research reflection, we have highlighted how valuable volatile organic compound (VOC) analysis has been in assessing contingent cheese flavour compounds arising from non-starter lactic acid bacteria (NSLAB) along with starter lactic acid bacteria (SLAB), and whether VOC analysis associated with other high-throughput data might help provide a better understanding the cheese flavour formation during cheese process. It is widely known that there is a keen interest to merge all omics data to find specific biomarkers and/or to assess aroma formation of cheese. Towards that end, results of VOC analysis have provided valuable insights into the cheese flavour profile. In this review, we are pinpointing the effective use of flavour compound analysis to perceive flavour-forming ability of microbial strains that are convenient for dairy production, intertwining microbiome and metabolome to unveil potential biomarkers that occur during cheese ripening. In doing so, we summarised the functionality and integration of aromatic compound analysis in cheese making and gave reflections on reconsidering what the role of flavour-based analysis might have in the future.

Cheese maturation is a process where microbial fermentation is accompanied by a series of enzymatic and chemical reactions that form the final textural and flavour traits that appeal to the consumer's overall sensorial perception. Even though both artisanal and industrial cheese manufacturing encompass a ripening period, sensorial discrepancies might be found due to the nature of controlled and uncontrolled cheese maturation processes that are carried out (Afshari *et al.*, 2020*b*). Probing differences and separating out all parameters that affect ripening have attracted enormous research interest in order to improve cheese production (Madkor *et al.*, 2000; Sgarbi *et al.*, 2013; Gobbetti *et al.*, 2015; Pogačić *et al.*, 2015, 2016; Bancalari *et al.*, 2017; Calasso *et al.*, 2020; Levante *et al.*, 2020; Štefániková *et al.*, 2020). To that end, the complex relationship between microorganisms in cheese and substrates from milk has been investigated to unravel many unknowns of cheese ripening. The ever-increasing amount of information obtained from metabolomics, genomics and proteomics sheds light on the elusive relationship between microbes and substrates within the cheese matrix (Khattab *et al.*, 2019; Afshari *et al.*, 2020*a*). Starter and non-starter lactic acid bacteria (SLAB and NSLAB, respectively) contribute to cheese flavour formation through the assimilation of substrates that are present and/or produced during cheese making. Fermentable sugars in milk, citrate, glycomacropeptides, glycoconjugates of milk-fat globule membranes, N-acetylglucosamine, sialic acid, cell lysate, free amino acids are assimilated from SLAB and NSLAB as carbon sources during the maturation process (Ercan *et al.*, 2015; Gobbetti *et al.*, 2015; van Mastrigt, *et al.*, 2018*b*, 2019; de Møller *et al.*, 2021). Considering microbial flavour formation by utilization of substrates in milk during cheese ripening, it is crucial to acknowledge two major points: (i) the viability of starter cultures during the ripening and (ii) adequate cell density to form flavour compounds by cellular activity of starter cultures. It was postulated that adjunct culture and/or starter culture can help form flavour compounds during maturation only if they are adequately alive at a certain cell density to perform microbial metabolism through the uptake of substrates in the curd (Nugroho *et al.*, 2020). Since almost all carbon sources are depleted during cheese maturation, the cell viability of SLAB and NSLAB comes into question as does whether dead/alive/injured cells in cheese can still contribute to the flavour formation during ripening (Nugroho *et al.*, 2020). Even though SLAB cell lysis during cheese ripening leads to the release of intracellular enzymes that may catalyse the conversion of amino acids into flavour compounds, it is known that volatile compounds can also be generated through metabolic pathways in which multiple intracellular co-factors must be present. Since these co-factors cannot be supplied by dead cells, prolonged survival of SLAB might
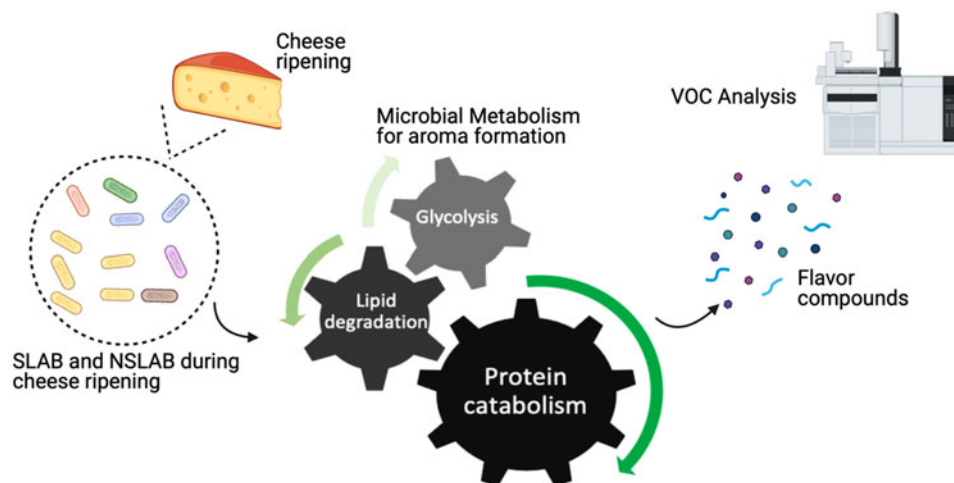
Fig. 1. Schematic representation of aroma formation during cheese ripening.

more effectively generate volatile compounds. Moreover, flavour-producing enzymes are more prone to rapid denaturation in food matrix compared to those residing in the cell (Wilkinson and LaPointe, 2020; Nugroho *et al.*, 2021).

Throughout cheese ripening, microbial utilization of substrates generates cheese taste and aroma. SLAB and NSLAB exploit three major metabolisms for flavour formation: sugar metabolism, proteolysis, and lipolysis (Fig. 1). As a principal carbohydrate for microbial metabolism, lactose is metabolized to lactate, acetate, formate, acetaldehyde and others. In addition to that, citrate-positive lactic acid bacteria (LAB) can produce flavour compounds such as acetoin, 2,3-butanediol, di-acetyl and 2-butanone (Bertuzzi *et al.*, 2018*b*). It is known that proteolytic activity is one of the main triggers to form aroma compounds due to the fact that aromatic amino acids are sources of flavour compounds developed during cheese maturation. As a starting point of proteolysis, proteinases and peptidases from residual coagulant (usually chymosin) and endogenous milk enzymes as well as those from starter and non-starter cultures, degrade milk casein to peptides, which in turn produce free amino acids in the curd as substrates for the subsequent steps. Aminotransferases convert these free amino acids into their corresponding α-keto acids that are further decarboxylated or deaminated into volatile organic compounds (VOCs). When there is a surplus of amino acids within the cell, flavour compounds can be generated by glutamate dehydrogenase in the presence of a receptor, α-ketoglutarate (Smid and Lacroix, 2013; Lazzi *et al.*, 2016; Stefanovic *et al.*, 2017*a*). Likewise, flavour compounds can also be produced *via* lipolysis where milk triglycerides are hydrolysed through lipases and esterases into free fatty acids (FFA), mono- and di-glycerides and glycerol. While short-chain fatty acids directly contribute to flavour, FFAs also serve as precursors to a wide range of other compounds, such as esters, lactones, methyl ketones and secondary alcohols that are associated with diverse cheese flavours (Khattab *et al.*, 2019).

Acids, alcohols, ketones, sulphur compounds, aldehydes, secondary alcohols, and lactones are among volatile compounds contributing to the final aroma of cheese. Gas chromatography mass spectrometry (GC-MS) has been a prevalent method for monitoring volatile compounds in cheese ripening. The conventional VOC analysis in cheese is a complex procedure mainly comprising of extraction/concentration techniques, gas chromatographic

separation of the peaks followed by their identification and quantification (Marilley and Casey, 2004; Bertuzzi *et al.*, 2018*b*; Terpou *et al.*, 2018). Sensitivity and reliability of GC-MS depends on many factors such as the extraction technique, the nature of the extraction polymeric material, the GC column, and the detector used and currently employed extraction methods are quite error-prone, leading to confounding results. Bertuzzi *et al.* (2018*b*) recently reviewed the analytical methodologies used for VOC analysis by highlighting the advantages and potential shortcomings of each technique.

In improving cheese quality through the addition of novel non-industrial microbial strains, VOC measurements have highlighted the variation of cheese flavour composition. Hence for adjunct culture selection, alterations in the cheese volatilome due to NSLAB addition were monitored through VOC analysis (Van Hoorde *et al.*, 2010; Stefanovic *et al.*, 2017*b*). Moreover, VOC analysis was also carried out for SLAB with constrained cellular activity (van Mastrigt *et al.*, 2018*a*, 2018*b*, 2019) and attenuated NSLAB (Di Cagno *et al.*, 2011) to test their aroma-forming potential (Table 1). With the increasing quest to discover biomarkers of cheese quality, the identification of biomarker compounds detected by GC-MS has gained more attention, especially to determine which compounds might serve as indicators for optimum cheese quality (Afshari *et al.*, 2021). This research reflection is intended to update recent developments of cheese ripening as determined by volatile profile analysis and to summarize recent efforts in operable aroma formation analysis for the cheese making process.

VOC analysis to select potential starter cultures for improving cheese flavour

LAB are innate resident microorganisms of cheese. SLAB and NSLAB interactions during cheese ripening may also lead to desired or undesired volatile compounds (van Hoorde *et al.*, 2010; Sahingil *et al.*, 2014). Our group investigated whether the metabolic behaviour of a cheese microbial community could be explained by the characterized individual organisms that constituted the culture. To address this question, the dairy-origin LAB *Lactococcus lactis subsp. cremoris*, *Lactococcus lactis subsp. lactis*, *Streptococcus thermophilus* and *Leuconostoc mesenteroides*, commonly used in cheese starter cultures, were grown in pure

Table 1. Functional utilization of aroma formation in cheese ripening

Cheese/Medium	Tested strains	Aroma attributes	Reference
NSLAB selection process for aroma formation			
Gouda-type cheese	Lacticaseibacillus paracasei	Only low cell density inoculated *L. paracasei* produced branched-chain acids.	van Hoorde *et al.* (2010)
Cheese-based medium and Lactic acid bacteria lysed cell medium	*Lacticaseibacillus casei* and *Lacticaseibacillus rhamnosus*	While 2-decenal was only produced by *L. rhamnosus*, 2,2-dimethyl-propanoic acid was only produced by *L. casei*.	Sgarbi *et al.* (2013)
Curd-based medium	*Leuconostoc lactis, Lactobacillus sakei, Lactocaseibacillus paracasei, Lactobacillus fermentum, Lactobacillus helveticus, Bra-chybacterium articum, Brachybacterium tyrofermentans, Brevibacterium aurantiacum, and Microbacterium gubbeenense, Propionibacterium freudenreichii, and Hafnia alvei*	The most abundant acetoin was found in *L. sakei*-cultured medium	Pogačić *et al.* (2015)
Caciocavallo Palermitano cheese, Cheese-based medium, and Curd-based medium	*Lactobacillus delbrueckii, Streptococcus thermophilus, Enterococcus casselliflavus, Enterococcus faecalis, Enterococcus durans, Enterococcus gallinarum, Lactocaseibacillus casei, Lactocaseibacillus paracasei, Lactocaseibacillus rhamnosus, Pediococcus acidilactici and Pediococcus pentosaceus*	*Pediococcus acidilactici* generated significant amount of aldehyde as compared to other strains did	Guarrasi *et al.* (2017)
Amino acid rich medium and Cheese curd medium	*Lactocaseibacillus casei* and *Lactocaseibacillus paracasei*	Of all VOCs detected in the curd-based model medium, ten VOCs were distinguished in terms of their abundances in. 2,3-butanedione was the most abundant VOC when compared to uninoculated culture.	Stefanovic *et al.* (2017a)
Caciovallo cheese	*Macrococccus caseolyticus subsp. caseolyticus*	Out of six strains, only three of them were able to produce free fatty acid-derived VOCs, even though six strains showed esterase activity.	Mazhar *et al.* (2020)
Attenuation of NSLAB for aroma formation			
Caciotta-type cheese	*Lactocaseibacillus casei, Lactocaseibacillus paracasei, Lactocaseibacillus curvatus*	Attenuated strains contributed to sulphur compounds of the cheese. Especially, they produced more carbon disulphide and methanethiol	Di Cagno *et al.* (2011)
Caciocavallo cheese	*Lactocaseibacillus casei, Lactocaseibacillus paracasei, Lactocaseibacillus plantarum*	Low aminopeptidase type N and cystathionine lyase activity of attenuated adjunct cultures did not negatively affect sensorial acepptance	Di Cagno *et al.* (2012)
Increasing aroma formation capacity of SLAB			
Caciotta cheese	*Lactococcus lactis*	Attenuated *L. lactis* improved cheese flavour, which was confirmed by sensorial analysis	Calasso *et al.* (2020)
CDM, Hydrolysed Micellar Casein Isolate, and Milk supplemented with 1% Bacto-tryptone	*Lactococcus lactis*	The limiting VOC in 8 week-ripened cheese was ethyl ester	van Mastrigt *et al.* (2018b)
CDM	*Lactococcus lactis*	Within the cell, fat-derived VOC octanoic acid that was previously detected in cheese was abundant in retentostat culture	van Mastrigt *et al.* (2018a)
Indicators of cheese quality and ripening			
Cheddar cheese		In the abundance of *Streptococcus thermophilus*, the cholestrol level in cheese decreased, ripening time was monitored	Afshari *et al.* (2020b)
Cheddar cheese		3-hydroxy propanoic acid and O-methoxycatechol-O-sulphate were detected in artisanal cheese	Afshari *et al.* (2020c)
Cheddar cheese		High-quality cheese was high for proline, histidine, aspartic acid, and isoleucine	Afshari *et al.* (2021)
Raclette-Type Cheese, Reconstituted Milk, and MRS		In addition to 3-methylbutanal and 3- methylbutan-1-ol, there might be another malt flavour indicator that has not been discovered yet	Meng *et al.* (2021)
Kazak artisanal cheese		Twenty-three VOCs were positively correlated with *Macroccocus* and *Anoxybacillus*	Zheng *et al.* (2021)

culture and as co-cultures. Dynamic metabolic modelling based on the integration of the genome-scale metabolic networks of the involved organisms was used to simulate the co-cultures and it was shown that potential metabolic interactions in co-cultures of SLAB and NSLAB may produce flavour compounds in the ripening period, possibly explaining improvements in cheese quality by regulating metabolic fluxes among microorganisms (Özcan *et al.*, 2019; Özcan *et al.*, 2021). Even though NSLAB can boost aroma of the cheese, directly adding adjunct may lead to off flavour formation in cheese (van Hoorde *et al.*, 2010; Sahingil *et al.*, 2014). Hence, adjunct culture selection for cheese manufacture is important in relation to the presence of potential conflicting contributions of NSLAB, suggesting that complimentary experimental-based evaluation is necessary to determine which NSLAB could be the best. Of these experimental evaluations, VOC analysis has been the workhorse of observing aroma and taste changes by adding potential adjunct cultures in cheese manufacturing (Marilley and Casey, 2004; Di Cagno *et al.*, 2014; Bertuzzi *et al.*, 2018*b*; Khattab *et al.*, 2019). van Hoorde *et al.* (2010) evaluated NSLAB *Lacticaseibacillus paracasei* strains for their lipolytic and proteolytic activities as well as VOC profiles in model cheese by steam-distillation extraction coupled with GC-MS. Upon adding *L. paracasei* strains, overall cheese flavour composition was altered by increasing aldehydes, sulphur compounds, alcohols, secondary alcohols and also by decreasing ketones and lipid-derived volatile compounds. Principal component analysis (PCA) demonstrated the obvious difference between non-adjunct culture-added cheese volatilome and adjunct culture added cheese volatilome (van Hoorde *et al.*, 2010). A similar approach was undertaken to assess the aroma-producing capabilities of 11 candidate microbial strains for their potential use in cheese processing (Pogačić *et al.*, 2015). Volatile compounds were detected by headspace (HS)-trap GC-MS system, and the strain selection process was carried out in a curd-based medium mimicking cheese-like conditions. Overall volatilomes of cheese produced by these strains were assessed, and PCA revealed differences among strains, heralding that utilization of different microbial strains for cheese manufacturing can lead to different volatile profiles (Pogačić *et al.*, 2015). VOC analysis combined with sensorial analysis of cheeses made with attenuated adjunct cultures enabled the evaluation of the flavour-producing performance of different strains. There are, however, confounding results that were not correlated with VOC analysis, which showed quantitative alterations of cheese aroma compounds during the study. Due to higher amino acid content of cheese made with attenuated adjunct cultures compared to non-attenuated ones, 1-propanol, 1-hexanol, 3-methyl-2-butanol, 2-methyl-1-butanol, and 2-methyl-propanol of the control group made without adjunct culture were lower than that of the cheese made with attenuated adjunct starter (Di Cagno *et al.*, 2011). Branched chain amino acids such as leucine and valine were less abundant in cheese made without adjunct culture, resulting in more VOCs produced by attenuated starter cultures than that of non-attenuated starter cultures. In this study, 10 untrained panelists evaluated cheese quality with a rubric on flavour and aroma intensity, texture and overall acceptability (Di Cagno *et al.*, 2011). Without employing VOC analysis for assessing flavour-producing performances of attenuated adjunct starter cultures, the same research group used only a sensorial analysis, in which 42 panelists participated using forced-choice protocol with random distribution of cheese samples. Results showed that 45-d ripened cheese with attenuated adjunct culture reached the same sensorial level of 60-d ripened cheese that did not contain attenuated adjunct culture. Even though the activities of amino peptidase type N, cystathionine lyase were weakened due to the application of the attenuation process, the overall acceptance of cheese made with attenuated adjunct cultures was similar to the cheese made with non-attenutaed adjunct cultures (Di Cagno *et al.*, 2012). In another study, without VOC analysis, the effects of attenuated *L. lactis, Leuconostoc lactis, Vibrio casei, Staphylococcus equorum, Brochothrix thermosphacta* on cheese ripening were tested *via* sensorial evaluation in which 11 trained panelists attended three consecutive tasting sessions where all cheese samples were randomly subjected to the sensorial analysis. Cheese made with attenuated *L. lactis* had the best score in terms of overall sensorial acceptance (Calasso *et al.*, 2020). It is interesting to consider to what extent VOC analysis could corroborate/associate sensorial evaluations in these studies.

To observe VOCs-producing capabilities of NSLAB and SLAB, VOCs analyses were performed in a model medium. VOCs profiles of isolated NSLAB strains *L. casei* and *L. rhamnosus* were detected in lactose-free cheese-based medium for simulating cheese-like environment (Sgarbi *et al.*, 2013). Also, 11 LAB strains were compared based on generating VOCs in a cheese-based medium to find and correlations between Caciocavallo Palermitano cheese volatile compounds and the LAB strains. Five LAB strains showed similar VOC profiles to the Caciocavallo Palermitano cheese VOC profile (Guarrasi *et al.*, 2017), although the presence of these strains was not demonstrated in the cheese.

Stefanovic *et al.* (2017*a*) reported that *L. casei* and *L. paracasei,* known as *casei* group members, had assocations with flavour formation during cheese ripening. The activity of enzymes involved in the proteolytic processes for flavour development (cell envelope protease, aromatic aminotransferase, and glutamate dehydrogenase) and VOC analyses in medium enriched separately with phenylalanine (as an aromatic amino acid), methionine (as a sulphur amino acid) and leucine (as a branched chain amino acid) were carried out to distinguish 10 *casei* group microorganisms based on their aroma-producing capability. Of all the 10 strains evaluated for flavour-forming performances in enriched medium, DPC 4206 strain was the most distinct as evident by PCA plots of VOCs for each strain in enriched medium (Stefanovic *et al.*, 2017*a*). It is interesting to note that the DPC 4206 strain did not have distinct enzymatic activities amongst all 10 strains, indicating that enzymatic activity assays cannot help fully delineate aroma-producing potential. In parallel with that, flavour-forming abilities of *Macrococcus caseolyticus subsp. caseolyticus* strains were measured in a similar way. Isolated *M. caseolyticus subsp. caseolyticus* strains had high proteolytic and esterase activity, indicating that *M. caseolyticus subsp. caseolyticus* strains can contribute to flavour formation during cheese making process. Cultivated into lactose free milk, *M. caseolyticus subsp. caseolyticus* strains liberated more amino acids from peptides as compared to low proteolytic activity strains. Having proven high proteolytic and lipolytic activity of tested strains, VOC analysis was undertaken. As compared to the control group which was uninoculated lactose free medium, 23 VOCs out of 74 VOCs detected by headspace solid phase microextraction (HS SPME) were only produced by *M. caseolyticus subsp. caseolyticus* strains used in the study. Of 23 volatiles, methylhexanoate, ethyl-decanoate, 2,3-heptanedione, phenylethyl alcohol were not produced by all isolated *M. caseolyticus subsp. caseolyticus* strains. But, hexyl-butanoate, 2-undecanone, nonanal and benzeneacetaldehyde were produced by some isolated *M. caseolyticus subsp. caseolyticus* strains. For instance, benzeneacetaldehyde was

produced by only two *M. caseolyticus subsp. caseolyticus* strains out of three *M. caseolyticus subsp. caseolyticus* strains. Even though isolated *M. caseolyticus subsp. caseolyticus* strains provided high amino acid content as well as high proteolytic and esterase activity, not all-mof the distinctive volatile compounds detected in HS SPME GC-MS were the same for all *M. caseolyticus* subsp. *caseolyticus* strains (Mazhar *et al.*, 2020). Results of proteolytic and lipolytic enzyme activities were not sufficient to explain irregular presence of volatile compounds produced by isolated *M. caseolyticus* subsp. *caseolyticus* strains. In this study, results of enzymatic activity assays were not fully in agreement with data presented by VOC analysis.

Cheese ripening can take from two weeks up to two years. Depending on *L. lactis* physiological state during cheese ripening, the development of volatile compounds is endowed by metabolic pathways of aroma formation. Therefore, to analyse the aroma-producing ability, *L. lactis* with near-zero growth rate can be used to understand how flavour production occurs during the cheese ripening. van Mastrigt *et al.* (2018*a*) utilized retentostat cultures, in which supernatants of nutrient-poor media are continuously refreshed while keeping biomass by means of a filter in the bioreactor. This can help *L. lactis* reach its slow-growing physiological state, simulating that found during cheese ripening. When cultivated in chemically defined medium (CDM) containing lactose, ammonium citrate, and bacto-tryptone, slow-growing *L. lactis* produced 62 VOCs detected in HS GC-MS. In this study, VOC analysis was conceptually carried out in a different way. Intracellularly and extracellularly-present VOCs were identified, with the result that intracellular flavour compounds contributed to 16 VOCs produced at near-zero growth rate in CDM (van Mastrigt, *et al.*, 2018*a*). It can be inferred that not only would cheese microbiota contribute to the cheese flavour profile, but also cheese microbiota itself could improve cheese flavour composition through VOCs produced/remained within cells.

Another functional use of VOC analysis was performed in appraising *L. lactis subsp. lactis biovar diacetylactis* aroma-forming potential for 8-week slow growth in milli-model cheese. Imitated by retentostat culture, slow growth of *L. lactis* during cheese ripening was ensured by growing it in three different media, namely, CDM, hydrolysed micellar casein isolate and milk supplemented with 1% bacto-tryptone. In batch and retentostat cultures, 82 aroma compounds were generated. Of these 82, only 10 aroma compounds were specifically formed by retentostat cultures of *L. lactis*. Extensive VOC analysis of *L. lactis* retentostat cultures indicated that *L. lactis* cultivated in CDM showed an increase in some fatty acid-derived VOCs such as methyl ketones, primary and secondary alcohols, even though CDM did not contain fatty acids. When quantitively compared to milli-cheese model system, *L. lactis* produced more ethanol and acetaldehyde in rentensostat cultures. 3-methyl-1-butanol and 2-methyl-1-butanol as branched-chain alcohols were low in retentostat cultures, suggesting that branched-chain amino acids liberated by *L. lactis* could be low as found in the milli-cheese model system (van Mastrigt, *et al.*, 2018*b*).

Implementation of VOC analysis for identifying biomarkers of cheese ripening

Activity of the cheese microbiota influences the cheese metabolome during cheese ripening. Since an obvious relationship between VOCs and microbiome exists, intertwining microbiome and metabolome would explain which species and/or strains can contribute most to flavour (or even specific VOCs) during cheese ripening (Bertuzzi *et al.*, 2018*a*; Afshari *et al.*, 2020*a*). Effects on microbiome and VOCs production by two different mixed industrial starter cultures harbouring yeast and bacteria for surface-ripened cheese were studied for cheddar cheese ripening. Also, the intertwining of microbiome and VOCs was executed to elucidate which strains of starter cultures were associated with certain VOCs (Bertuzzi *et al.*, 2018*a*). Overall, 53 VOCs were detected in the cheese made with industrial starters. The number of esters and aldehydes of surface-ripened cheddar cheese were less than other VOCs detected in HS SPME GC-MS. Sulphur compounds that were not detected in the 30-d ripened cheese without starter culture were only produced in the ripened cheese made with industrial starter cultures. Also, pentanoic acid production was observed only in one starter culture in which *Glutamicibacter arilaitensis* was allegedly responsible for pentanoic acid production as revealed through integration of microbiome and VOC analysis (Bertuzzi *et al.*, 2018*a*). A similar approach was undertaken to investigate Kazak cheese microbiome and metabolome connection. In this study, microbiome and VOCs of Kazak cheese were monitored at 3-d intervals during ripening. Species-level microbiome analysis combined with VOCs analysis showed that 8 bacterial genera played significant roles to form VOCs during Kazak cheese ripening (Zheng *et al.*, 2021). Metabolomics data obtained by untargeted GC-MS were intercorrelated with microbiome to show differences of artisanal and industrial cheese (Afshari *et al.*, 2020*c*), and to demonstrate different cheese produced by different manufacturers with different cheese ripening periods (Afshari *et al.*, 2020*b*). Further applications of correlation analysis were performed to define biomarkers of cheese ripening. With the power of untargeted cheese metabolome data of GC-MS, cheese quality was evaluated to find biomarkers during cheese ripening. Afshari *et al.* (2021) developed a different point of view to unveil specific biomarkers of high-quality cheese. A two-step process was undertaken to find biomarkers of high-quality cheese: classifying quality of cheddar cheese by sensorial evaluations of professional cheese graders and applying data integration analysis for biomarker discovery using latent components (DIABLO). Having determined the cheese quality by professional cheese graders, microbiome and metabolome data were integrated by DIABLO. It was interesting to note that *St. thermophilus* was abundant in high-quality cheddar cheese while *L. lactis* was abundant in low-quality cheddar cheese. With respect to biomarker compounds detected in GC-MS, low-quality cheddar cheese was low in proline, histidine, aspartic acid and isoleucine and high in stearic acid and octadecanol as compared to high-quality cheddar cheese (Afshari *et al.*, 2021).

Malty aroma formation in raclette-type cheese is an off-flavour defect that is supposedly produced by isoleucine, valine, and leucine amino acids conversion into malty flavour compounds. Through GC-olfactometry, 3-methyl-butan-1-ol and 3-methyl-butanal were found as main indicators for formation of malty aroma in raclette-type cheese. To observe malty aroma formation, *L. lactis* subsp. *cremoris* was grown in skim milk (SM) and de Man, Rogosa and Sharpe (MRS) medium supplemented with leucine, α-ketoglutaric acid and α-ketoisocaproic acid, which are known to participate in the formation pathway of 3-methyl-butanal. Interestingly, it was shown that 3-methyl-butan-1-ol and 3-methyl-butanal production in spiked MRS and SM media were observed already during the first hours of cheese manufacture with the latter compound formed even before microbial activity when spiked with α-ketoisocaproic acid. The
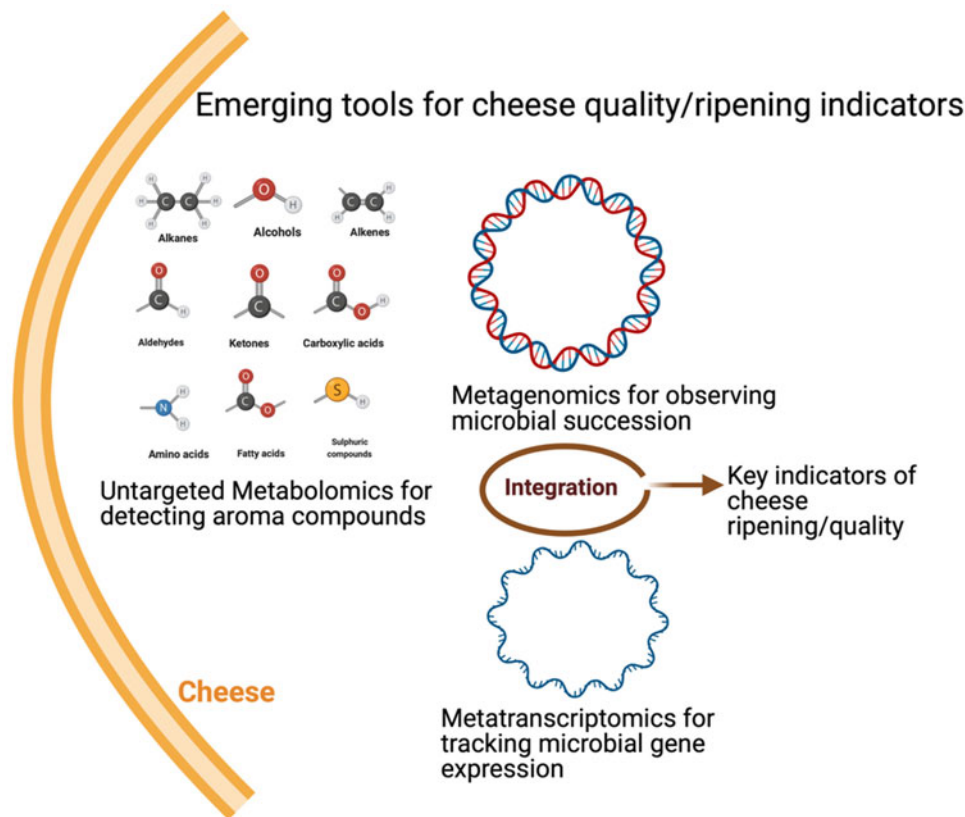
Fig. 2. Emerging tools to identify indicators of cheese quality and cheese ripening.

discrapancies between the two media pointed to the presence of other unknown reactants that might be responsible for formation of malty aroma in raclette-type cheese (Meng *et al.*, 2021).

In conclusion, aroma and taste in cheese are achieved during cheese ripening. To elucidate flavour compounds produced during cheese ripening, VOC analysis has been utilized extensively for many years. Corroborating VOC analysis results with enzymatic assays and sensorial evaluations can be useful but is cumbersome. It should also be noted that enzymatic assays in isolation maybe misleading since enzymatic activities are measured under optimum conditions for the enzyme rather than in a real cheese-like conditions. On the other hand, metabolomic data from newly applied tools such as novel liquid chromatography-based MS, nuclear magnetic resonance and untargeted GC-MS would certainly add to the understanding of the flavour formation during cheese ripening (Afshari *et al.*, 2020c; Pisano e*t al.*, 2020). Since metabolomics provides higher numbers of metabolites as compared to conventional VOC analysis, the integrated metabolome and microbiome might provide more possible biomarkers associated with specific cheese flavour traits (Fig. 2).

GC-MS based metabolomics approaches are becoming more common with greater sensitivity to detect a wide range of metabolites. The foremost advantage of untargeted GC-MS analysis comes from the ability to detect VOCs and non volatile compounds through derivatization (Afshari *et al.*, 2021).

It is our opinion that simply inputting enormous quantities of high-throughput data into understanding cheese ripening may not clear up all of the confounding results obtained by omics technology. Questions such as how to decide when cheese ripening is sensorially at its optimum and which biomarkers at what concentration indicate successful and adequate cheese ripening are still requring answers. Even though VOC analysis reflects quantities of numerous compounds for cheese ripening, there is no unanimous theory that a certain VOC profile at a certain concentration is a sign or indicator of cheese ripening for specific cheese types. Considering the wide variety of cheese types, determining indicators of the completion of cheese ripening will be a quite arduous and laborious task to achieve.

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