Flavour formation by lactic acid bacteria and biochemical flavour profiling of cheese products

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Abstract

Flavour development in dairy fermentations, most notably cheeses, results from a series of (bio)chemical processes in which the starter cultures provide the enzymes. Particularly the enzymatic degradation of proteins (caseins) leads to the formation of key-flavour components, which contribute to the sensory perception of dairy products. More specifically, caseins are degraded into peptides and amino acids and the latter are major precursors for volatile aroma compounds. In particular, the conversion of methionine, the aromatic and the branched-chain amino acids are crucial. A lot of research has focused on the degradation of caseins into peptides and free amino acids, and more recently, enzymes involved in the conversion of amino acids were identified. Most data are generated on Lactococcus lactis, which is the predominant organism in starter cultures used for cheese-making, but also Lactobacillus, Streptococcus, Propionibacterium and species used for surface ripening of cheeses are characterised in their flavour-forming capacity. In this paper, various enzymes and pathways involved in flavour formation will be highlighted and the impact of these findings for the development of industrial starter cultures will be discussed.

Keywords: Fermentative flavour formation; Lactic acid bacteria; Dairy products; Amino acid converting enzymes

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1. Introduction to flavour

The quality of fermented (dairy) products is largely determined by the sensory perception. Sensory perception is a complex process, which is influenced by many factors, such as the content of flavour components, texture, and appearance. Flavour perception is defined as the sensation arising from the integration or interplay of signals produced as a consequence of sensing chemical substances by smell, taste and irritation stimuli from food or beverage [1]. Regarding flavour, in the mouth five basic differences like sweet, bitter, salt, acid and umami are sensed by taste-receptor cells as well as cold and hot sensations [2]. In the nose, many different receptors (3–400) are present and able to respond to a large variety of volatile flavour components [1,3]. These receptors involved in odour detection have been identified and characterised (see Buck and Axel [4], for an excellent overview for which they were rewarded with the Nobel Prize in 2004). However, the integration and interpretation of taste and smell stimuli in the brain, leading to the ultimate liking or disliking of a food product is still poorly understood.

The balance of flavour components in a given food product determines largely whether that food product is liked or disliked. It is good to realise that many flavour molecules can be found in a fermented food product such as cheese [5–7]. In order to control the liking of (fermented) food products it is highly important to understand which of these flavour compounds are determining the overall quality the most.

A commonly used step in the determination of the dominant compounds, the so-called key-flavour components, can be done by GC-O, which is a combination of separation of compounds by gas chromatography (GC) and analytical identification by the human nose (olfactometry). Similarly, for tastants a combination between separation (LC) and tasting of fractions (LC-taste) is applied.

For GC-O analysis of volatile compounds, a number of methods have been described. Generally, various dilutions of an extract or a product are prepared, analysed on a GC and detected by a number of subjects. The compounds which are still sensed in the highest dilution (=FD-factor) of the product are regarded as key-flavour components [8–10]. In order to also include the importance of the product matrix in sensory perception, Grosch and co-workers [11] introduced the term odour activity value (OAV). The OAV is the ratio between the concentration of a compound in a product and the nasal or retro nasal threshold of the compound, as it is present in the matrix of the product or a matrix that closely resembles this product. In this way, also the matrix interactions are included in determining the key-flavour compounds. This approach does not yet take into account the possible interactions between volatiles that might affect their perception also in concentrations below their threshold [12]. Although the determination of key-flavours has some flaws, recombination of the key-flavour compounds usually gives a good imitation of the product studied, although a mix of a limited set of compounds might require some alterations in the concentration of the individual concentration of components in order to match the original product [10,12].

Several important flavour compounds of different types of cheeses are shown in Table 1. Since not all products were analysed by GC-O, not all flavour components may be called key-flavours. The flavour compounds are categorised by the metabolic pathway/substrate they are most likely derived from, as will be discussed in the following sections. As an indication the following references are given: Limburger [13–15], Gruyère [16,17], Gorgonzola [18], Mozzarella [19], Parmigiano [5,20], Grana Padano [21], Mahón, Fontina, Comté, Beaufort and Appenzeller [5].

In Table 2, a number of descriptions are given of some important flavour components and their thresholds in water and oil (if known). Not only the amount of the individual components, but also the balance between these components is very important in the ultimate perception by the consumers. This has important implications for the work as described in the following parts of this paper. The work on controlling the flavour formation in dairy products, such as cheese, can focus on two aspects: an overall increase in all important key flavour components (improved/faster cheese ripening) or an increase in some target flavours. The latter might result in a new cheese variety with an appreciated flavour diversification, but may also result in a perceived unbalance of the flavour, a so-called off-flavour [22,23]. To control the desired flavour formation, the pathways leading to flavour have to be known. In the next sections of this paper, we will report our current knowledge of the (bio)chemical pathways and also some examples for application of this knowledge will be discussed.
In previous sections, flavour has been introduced as an important product characteristic of fermented dairy products, e.g., cheese. Flavour compounds in cheese arise from the action of enzymes from rennet, milk, the (secondary) starter and non-starter bacteria, together with non-enzymatic conversions \[24–27\]. In case of cheese ripening, the formation of flavours is a rather slow process involving various chemical and biochemical conversions of milk components. Three main pathways can be identified: the conversions of lactose (glycolysis), fat (lipolysis), and caseins (proteolysis). The starter cultures used in these fermentations are the main source of the enzymes involved in these pathways.

The predominant organisms in these starters are lactic acid bacteria (LAB), e.g., *Lactococcus lactis*, *Lactobacillus species*, *Streptococcus thermophilus*, *Leuconostoc mesenteroides*. However, also additional cultures are used, such as *Propionibacterium* in the case of Swiss-type and Maasdammer-type of cheeses, and various aerobic cultures (e.g., *Brevibacterium*, *Arthrobacter*, *Staphylococcus*, *Penicillium*, *Debaromyces*) for surface-ripened cheeses \[28–30\]. Apart from these starter organisms, also mesophilic lactobacilli, originating from the milk environment might grow in the dairy products and thereby be a source of enzyme activities leading to the formation of flavours.

In the case of the lactose fermentation, the main conversion obviously leads to the formation of lactate by

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**Table 1**

Examples of key- and other important flavour components in four types of cheese

<table>
<thead>
<tr>
<th>Metabolism</th>
<th>Gouda</th>
<th>Cheddar</th>
<th>Camembert</th>
<th>Swiss-type (and Maasdam)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amino acid</td>
<td>3-Methylbutanal</td>
<td>3-Methylbutanal</td>
<td>3-Methylbutyrate</td>
<td>Methional</td>
</tr>
<tr>
<td>Butanol</td>
<td>3-Methylbutanol</td>
<td>Isovaleric acid</td>
<td>3-Methylbutanal</td>
<td>3-Methylbutanol</td>
</tr>
<tr>
<td>Methionol</td>
<td>Methionol</td>
<td>Methionol</td>
<td>Methional</td>
<td>Skatole</td>
</tr>
<tr>
<td>Dimethylsulphide (DMS)</td>
<td>Methionol</td>
<td>DMDS</td>
<td>DMS</td>
<td>Phenylethylacetdehyde</td>
</tr>
<tr>
<td>2-Methylpropanol</td>
<td>DMTS</td>
<td>Butyric acid</td>
<td>1-Octen-3-one</td>
<td>Ethyl butyrate</td>
</tr>
<tr>
<td>Dimethyltrisulphide (DMTS)</td>
<td>Butanone</td>
<td>Acetic acid</td>
<td>1-Octen-3-one</td>
<td>Ethyl hexanoate</td>
</tr>
<tr>
<td>Sugar</td>
<td>Diacetyl</td>
<td>Butyric acid</td>
<td>2-Undecalectone</td>
<td>Phenylethyl acetate</td>
</tr>
<tr>
<td>Fat</td>
<td>Butyric acid</td>
<td>Acetic acid</td>
<td>γ-Decalactone</td>
<td>Ethyl butyrate</td>
</tr>
<tr>
<td>Butanon</td>
<td>Butyric acid</td>
<td>Acetic acid</td>
<td>γ-Decalactone</td>
<td>Ethyl hexanoate</td>
</tr>
<tr>
<td>Hexanone</td>
<td>1-Octen-3-one</td>
<td>Butyric acid</td>
<td>γ-Decalactone</td>
<td>Ethyl-3-methylbutyrate</td>
</tr>
<tr>
<td>Pentanone</td>
<td>Butanone</td>
<td>1-Octen-3-one</td>
<td>γ-Decalactone</td>
<td>Phenylethyl acetate</td>
</tr>
<tr>
<td>Rest and combined pathways</td>
<td>Ethyl butyrate</td>
<td>Ethyl butyrate</td>
<td>Phenylethyl acetate</td>
<td>Ethyl butyrate</td>
</tr>
<tr>
<td>Ethylacetate</td>
<td>Ethyl hexanoate</td>
<td>Ethyl hexanoate</td>
<td>Ethyl butyrate</td>
<td>Ethyl hexanoate</td>
</tr>
<tr>
<td>References</td>
<td>[77,161]</td>
<td>[164–166]</td>
<td>[96]</td>
<td>[123,166,167]</td>
</tr>
</tbody>
</table>

**Table 2**

Description of some important key-flavours and their odour thresholds \[22,168–171\]

<table>
<thead>
<tr>
<th>Flavour compound</th>
<th>Description</th>
<th>Odour threshold in ppb (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Water</td>
</tr>
<tr>
<td>2-Methylpropanal</td>
<td>Banana, malty, chocolate-like</td>
<td>0.1–2.3 (0.002–0.03)</td>
</tr>
<tr>
<td>3-Methylbutanal</td>
<td>Malty, powerful, cheese</td>
<td>1 (0.01)</td>
</tr>
<tr>
<td>3-Methylbutanol</td>
<td>Fresh cheese, breathtaking, alcoholic</td>
<td>250 (2.8)</td>
</tr>
<tr>
<td>3-Methylbutyric acid</td>
<td>Rancid, sweat, cheese, putrid</td>
<td>120–700 (1.2–6.9)</td>
</tr>
<tr>
<td>Butyric acid</td>
<td>Sweaty, butter, cheese, strong, acid</td>
<td>240 (2.7)</td>
</tr>
<tr>
<td>Propionic acid</td>
<td>Pungent, sour milk, cheese</td>
<td>2 × 10^4 (270)</td>
</tr>
<tr>
<td>Ethylbutyrate</td>
<td>Fruity, buttery, ripe fruit</td>
<td>1 (0.008)</td>
</tr>
<tr>
<td>Diacetyl</td>
<td>Buttery, strong</td>
<td>2.3 (0.026)</td>
</tr>
<tr>
<td>Acetaldehyde</td>
<td>Yoghurt, green, nutty, pungent</td>
<td>15 (0.34)</td>
</tr>
<tr>
<td>Methional</td>
<td>Cooked potato, meat like, sulphur</td>
<td>0.05–10 (1 × 10^−4–0.9)</td>
</tr>
<tr>
<td>Methanethiol</td>
<td>“Rotting” cabbage, cheese, vegetative, sulphur</td>
<td>0.02–2 (3 × 10^−4–0.3)</td>
</tr>
<tr>
<td>Benzaldehyde</td>
<td>Bitter almond oil, character, sweet cherry</td>
<td>350 (3.3)</td>
</tr>
<tr>
<td>Phenyl acetate</td>
<td>Rough, lily-jasmine with metallic note</td>
<td>–</td>
</tr>
</tbody>
</table>

---

2. (Bio-)chemical routes leading from proteins to flavour

In previous sections, flavour has been introduced as an important product characteristic of fermented dairy products, e.g., cheese. Flavour compounds in cheese arise from the action of enzymes from rennet, milk, the (secondary) starter and non-starter bacteria, together with non-enzymatic conversions \[24–27\]. In case of cheese ripening, the formation of flavours is a rather slow process involving various chemical and biochemical conversions of milk components. Three main pathways can be identified: the conversions of lactose (glycolysis), fat (lipolysis), and caseins (proteolysis). The starter cultures used in these fermentations are the main source of the enzymes involved in these pathways.
LAB, but a fraction of the intermediate pyruvate can alternatively be converted to various flavour compounds such as diacetyl, acetoin, acetaldehyde, or acetic acid, some of which contribute to typical yoghurt flavours.

Lipolysis results in the formation of free fatty acids, which can be precursors of flavour compounds such as methylketones, secondary alcohols, esters and lactones. LAB, in general, contribute relatively little to lipolysis, but additional cultures, e.g., moulds in the case of surface-ripened cheeses [30] often have high activities in fat conversion. Flavours derived from the conversion of fat are particularly important in soft cheeses like Camembert and Roquefort.

The conversion of caseins is undoubtedly the most important biochemical pathway for flavour formation in hard-type and semi-hard-type cheeses. Degradation of caseins by the activities of rennet enzymes, and the cell-envelope proteinase and peptidases from LAB yields small peptides and free amino acids. For specific flavour development, further conversion of amino acids to various alcohols, aldehydes, acids, esters and sulphur compounds is required. This paper focuses the main pathways involved in flavour formation from amino acids.

Flavour forming pathways originating from proteins are combined in Fig. 1. After a short description of this figure, each part of the route will be reviewed separately.

Amino acids are primarily needed for protein synthesis, but Lactococcus lactis is not able to produce all amino acids from the central metabolism [31,32]. Certain genes coding for enzymes involved in the amino acid biosynthesis seem to be disrupted [33,34]. To obtain all essential amino acids, L. lactis is able to take up small peptides and to a lesser extend amino acids from the environment. This uptake can be preceded by extracellular degradation of proteins (proteolysis and peptidolysis, Fig. 1(A)–(D)) [35,36]. Intracellularly, most amino acids can be converted at first by aminotransferases (Fig. 1(1)) to their corresponding α-keto acids. Other types of deaminating enzymes have not been found in LAB. α-Keto acids are central intermediates, and can be converted to hydroxy acids (Fig. 1(2)), aldehydes (Fig. 1(3)) and CoA-esters (Fig. 1(8)). These reactions are mostly enzymatic, but some chemical conversion steps have also been described, like the formation of benzaldehyde from phenylpyruvic acid [37,38]. The aldehydes formed can generally be dehydrogenated (Fig. 1(4)), or hydrogenated (Fig. 1(5)) to their corresponding alcohols or organic acids, which are in their turn substrates for esterases and acyltransferases (Fig. 1(7)), leading to (thio)esters. One of the biological roles of these amino acid degrading pathways is the generation of precursors, which are needed for example in the sterol and branched-chain fatty acids

Fig. 1. Overview of general protein conversion pathways relevant for flavour formation in dairy fermentations. Numbers and letters besides reactions are used for pointing out these reactions in the text. This figure is adapted from [34,113].
synthesis [39]. On the other hand, the hydrogenation of the \( \alpha \)-keto acids may act as sink for excessive redox potential (NADH). The conversion of amino acids to alcohols via \( \alpha \)-keto acids as described above was first identified for the formation of so-called fusel alcohols (short-[branched-]chain alcohols) in yeast, where it is called the Ehrlich’s pathway [40].

Another important conversion route of amino acids is initiated by lyases (Fig. 1(10)), like cystathionine \( \beta \)-lyase (EC 4.4.1.8), which is able to convert methionine to methanethiol [41–43]. Threonine aldolase (EC 4.1.2.5) (Fig. 1(9)) belongs to another class of lyases, and is able to convert threonine directly to acetaldehyde [44–48].

A third conversion pathway for amino acids is the deamination/decarboxylation to amines. These reactions have been studied, in regard to the health risk of biogenic amines [49–51]. The direct decarboxylation of amino acids explains the presence of most of the amines found, but not the formation of secondary and tertiary amines [52].

Chemical conversion reactions are discussed as last category of flavour forming reactions in this review. Although enzymes are needed to catalyse many reactions, in order to shorten the timescale of processes, still a variety of chemical reactions proceed at the mild conditions of the cheese ripening process, and thereby contribute to flavour formation. The conversions by LAB often provide substrates, or the LAB might influence the chemical reactions by influencing environmental characteristics such as pH, cofactor and oxygen availability.

The most potent flavour compounds in Fig. 1 are the aldehydes, alcohols, carboxylic acids and esters (Tables 1 and 2). Especially important are the aldehydes, alcohols, carboxylic acids and esters derived from the amino acids methionine, phenylalanine, threonine and the branched-chain amino acids. The importance of these amino acids for the cheese flavour is a combination of their abundance, their conversion rates, and the odour threshold of the compounds derived from them. Fig. 1 will be discussed in detail in the next subsections.

2.1. Proteolysis and peptidolysis

Since the concentrations of free amino acids and peptides are very low in milk, the starter cultures depend for growth in milk heavily on their proteolytic systems. The degradation of milk proteins (Fig. 1(A)-(D)) leads to peptides and free amino acids, which can subsequently be taken up by the cells. Proteolysis is initiated by a single cell-wall-bound extracellular proteinase [53,54]. While many dairy LAB strains contain such an extracellular proteinase, several do not and these are mainly dependent on other strains in the starter culture for the production of peptides and amino acids. Such dependency of strains is rather common in starter cultures, and indicates the relevance of knowledge on the population dynamics between strains in order to be able to develop stable starter cultures.

Peptide uptake occurs via oligopeptide transport systems (OPP system), and di-/tri-peptide transporters. In addition, various amino acid transport systems have been identified with a high specificity for structurally similar amino acids. Selected references [35,53,55–59] give further information.

Following uptake, the peptides are degraded intracellularly by a variety of peptidases, which have been extensively studied in both lactococci and lactobacilli. These peptidases of LAB can be divided into endopeptidases, aminopeptidases, di-/tri-peptidases, and proline-specific peptidases (reviews by Kunji and Christensen [53,54]).

Although peptides and amino acids do have specific flavour characteristics, like sweet, bitter, or malty, [7,60–62], it is generally believed that they only add to the basic taste of cheese. Stimulation or over-expressing of several proteolytic enzymes and also the addition of amino acids to cheese did hardly influence the positive flavour perception of the product [63]. However, unbalanced proteolysis might lead to excess of bitter peptides, which can lead to decreased cheese flavour perception [64–67]. Specific cultures have been selected with high bitter-tasting-peptide degrading abilities [64] and such cultures are nowadays frequently used in the preparation of various types of cheese. Free amino acids though can be substrates for successive flavour forming reactions. All together, the conclusion might be drawn that although proteolysis and peptidolysis are important for flavour formation, they are generally not rate-controlling in flavour formation from proteins.

2.2. Transaminase-pathway

Methional, 3-methylbutanal, isovaleric acid, and benzaldehyde are examples of key flavour compounds which are formed by a (initially) similar pathway, which is initiated by a transaminase (AT = aminotransferase). In Table 3, specific flavour compounds derived from some relevant amino acids are specified, corresponding to the class of chemical compounds used in Fig. 1. The AT-pathway is very important for the formation of many flavours, and it is interesting to know which step in the pathway is rate-controlling in relation to the formation of flavours by dairy related microorganisms. In the next subsections, individual reactions of this pathway are reviewed.

2.2.1. Transaminases

Transaminases (Fig. 1(1)) are widely distributed among micro-organisms, and use pyridoxal-5’-phosphate as cofactor for catalysis. Transaminases catalyse the conversion of an amino acid to its corresponding
-keto acid, and as such transamination is the first step of the amino acid catabolism. The enzymes are also able to catalyse the reversed reaction, and in that role they are the last enzyme in the amino acid anabolism. For the conversion of several types of amino acids, such as the branched-chain amino acids (BcAA) and aromatic amino acids (ArAA), specific transaminases have been identified and characterised [68–73]. Rijnen et al. [74] showed with mutants lacking either BcAA or ArAA that these enzymes are essential for flavour formation from amino acids. In addition, a double BcAA or ArAA mutant showed that another pathway or another aminotransferase appears also to be weakly involved in the formation of volatile sulphur compounds [74]. The enzymes have overlapping substrate specificities, which, e.g., also leads to the conversion of methionine, an amino acid for which no specific transaminase has been identified [75–77]. Because of their overlapping specificities, the activity of the branched chain transaminases is probably not limiting in the pathway to the production of branched chain fatty acids [78]. The BcAA transaminase identified by Engels was stable and active under cheese ripening conditions [77]. Knocking out the aromatic amino acid transaminase (ArAA-TA) gene, resulted in a lack of phenylalanine-derived flavour compounds in semi-hard cheese, which proved that transamination is essential for the formation of phenylalanine-derived flavours, and also that the aromatic amino acid transaminase is the major phenylalanine-transaminating enzyme [79] (see Fig. 2).

- Ketoglutarate is generally the preferred amino group acceptor (co-substrate) for transamination reactions. Improving the availability of this co-substrate leads to increased conversion of amino acids. This was shown in situ by increased amino acid conversion in several types of cheese, when adding -ketoglutarate to the curd [80,81]. This increased conversion led to increased concentrations of the -keto acids, -hydroxy acids, and carboxylic acids. Besides externally adding the -ketoglutarate, the availability of -ketoglutarate could also be increased by introduction of a glutamate dehydrogenase gene from Peptostreptococcus in L. lactis.

### Table 3

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Keto acid</th>
<th>Aldehyde</th>
<th>Organic acid</th>
<th>Alcohol (thiol)</th>
<th>Esters (example)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ile</td>
<td>-Keto-3-methyl-pentanoic acid</td>
<td>2-Methylbutanal</td>
<td>2-Methyl butyric acid</td>
<td>2-Methylbutanol</td>
<td>Ethyl-3-methylbutanoate</td>
</tr>
<tr>
<td>Leu</td>
<td>-Ketoisocaproic acid</td>
<td>3-Methylbutanal</td>
<td>3-Methylbutyric acid</td>
<td>3-Methylbutanol</td>
<td>Ethylisobutanoate</td>
</tr>
<tr>
<td></td>
<td>-Ketoisocaproic acid</td>
<td>2-Methylpropional</td>
<td>2-Methylpropionic acid</td>
<td>2-Methylpropional</td>
<td>Ethyl benzoate</td>
</tr>
<tr>
<td>Val</td>
<td>Phenyl pyruvate</td>
<td>Phenylacetaldehyde</td>
<td>Phenylacetic acid</td>
<td>Phenylethanol</td>
<td>Phenylethyl acetate</td>
</tr>
<tr>
<td>Phe</td>
<td>Phenyl pyruvate</td>
<td>Phenylacetaldehyde</td>
<td>Phenylacetic acid</td>
<td>Phenylethanol</td>
<td>Phenylethyl acetate</td>
</tr>
<tr>
<td>Trp</td>
<td>Indole-3-pyruvate</td>
<td>Indole-3-acetaldehyde</td>
<td>Indole-3-acetic acid</td>
<td>Methionol</td>
<td>Ethyl-3-methylthio propionate</td>
</tr>
<tr>
<td>Met</td>
<td>-Keto methylthio butyrate</td>
<td>Methional</td>
<td>Methylthiobutyric acid</td>
<td>Methanethiol</td>
<td>Methylthioacetate</td>
</tr>
</tbody>
</table>

Compounds in italic are formed by non-enzymatic reactions (adapted from [113]).

Fig. 2. Transaminase activities on leucine of tested strains grouped by (sub)species. Error bars indicate the standard deviation of three independent measurements (adapted from Smit et al. [110]).
Towards various amino acids. Production depends on the relative AT activities -ketoglutarate produced by GDH and thus aroma compounds were produced from BcAAs, while aroma compounds from aspartic acid were produced [84,85]. This means that there is competition of AT/C213.

The AT-catabolic pathway, and can be hydrogenated to the corresponding AT-hydroxy acid, decarboxylated to the corresponding aldehyde, converted chemically, or dehydrogenated/oxidative decarboxylated by a dehydrogenase complex, resulting in the corresponding CoA-ester. The CoA ester can in many organisms be used as building block for several compounds, but can also be converted to the flavour compound isovaleric acid. These conversions are discussed next.

2.2.2. Hydroxy acid dehydrogenase

Hydroxy acids are not major flavour compounds, and are not known as precursors of flavour compounds [76]. The decarboxylation of keto acids to hydroxy acids (Fig. 1(2)), can lead to low AT-keto acid concentrations, thereby negatively affecting the flux towards flavour compounds such as aldehydes. The hydroxy acids derived from BcAAs, ArAAs and methionine have been observed in many dairy fermentations [86–88]. Several 2-hydroxy acid dehydrogenases, such as lactate dehydrogenase (LDH), hydroxy isocaproate dehydrogenase (HicDH), mandelate dehydrogenase, have been identified and characterised from several LAB [88–92]. The best known enzyme is LDH, which substrate specificity is mainly restricted to pyruvate. Several other enzymes are collectively called hydroxy isocaproate dehydrogenase (HicDH), since AT-ketoisocaproate is often their preferred substrate. These enzymes have thus a broad substrate specificity [76], and catalyse the stereo specific hydrogenation of AT-keto acids, using NADH as hydrogen donor. HicDH enzymes appear in two forms, the α and β form, with the latter being rather unusual. In LAB, hydroxy acid dehydrogenases play an important role in maintaining the intracellular redox balance, by converting the excess NADH from the glycolysis. Tokuda et al. [93] were able to convert a LDH of Lactobacillus pentosus into a highly active HicDH by the replacement of Tyr52 with Leu.

Since hydroxy acids are the major amino acid degradation products found in semi hard cheeses made with lactococci [80], inactivation of the genes encoding for some of these dehydrogenases could allow a better use of AT-keto acids for producing aroma compounds. Over expression of the AT-hydroxyisocapric acid dehydrogenase yielded decreased non-enzymatic AT-keto acid degradation products in 3 months old low fat cheeses, and a retarded flavour formation in these cheeses [94].

2.2.3. Esterases and acyltransferases

Esters, such as ethylbutyrate, contribute to Cheddar and Gouda flavour, although, an excess of esters in proportion to other flavour compounds could be responsible for the fruity defect of Cheddar [95]. In Camembert, phenylacetaldehyde, 2-phenylethanol and the ester phenylethyl acetate, which all result from phenylalanine degradation, are identified in fractions with floral rose-like odour [96], and could cause the pleasant floral note of this cheese [97]. Esters are formed in a reaction between an alcohol and an organic acid [7,8], which also might be activated by coupling to CoA. Besides amino acid metabolism, also sugar and fat metabolism provide substrates for ester formation [30,76]. Although ester formation is generally considered to be an enzymatic catalysed reaction, the reaction between acetyl-CoA and methanethiol is spontaneous [98]. Esterases and lipases are serine hydrolases capable of synthesising or hydrolysing esters, depending on the environmental conditions, while alcohol acetyltransferases only catalyse ester synthesis. By knocking out the esterase gene (estA) in L. lactis, Fernandez et al. [99] showed that all ester hydrolysing activity in L. lactis was lost and that this organism most probably had only one enzyme with esterase activity. Later, this EstA-enzyme has been reported to be responsible for the formation of short chain fatty acid esters in vitro [100]. The extrapolation of these data to cheese is not directly possible, since the reaction equilibrium for these kind of esterifications depends strongly on environmental parameters like water activity. However, recent work by Wouters et al. [101] with an EstA overproducing L. lactis strain, showed that the EstA activity resulted in a strong increase in degradation of both thio-esters and ethyl esters. The degradation of thio-esters and ethyl esters by EstA was also observed at conditions resembling the cheese matrix indicating that EstA is capable of degrading thio-esters and ethyl esters at cheese-like conditions. Liu et al. [102] described an alternative reaction for formation of esters by dairy lactic acid bacteria. The process, alcoholysis, is essentially a transferase reaction in which fatty acyl groups and acyl groups from acyl-CoA derivatives are directly transferred to alcohols.

2.2.4. Decarboxylase

The decarboxylation (Fig. 1(3)) of the branched-chain keto acids results in aldehydes with a malty/chocolate-like flavour and a very low odour threshold.
The production of these characteristic malty flavour compounds has only been shown in few strains belonging to the following species: *Carnobacterium piscicola*, *Lactobacillus casei*, *L. lactis maltigenes* and “wild” *L. lactis* [103–109]. Most lactococci produce only small amounts of these aldehydes, suggesting that these strains weakly express the decarboxylase gene, that in these strains a less efficient pathway may exist, or that the aldehyde is converted relatively fast to other compounds [76].

Recently, Smit et al. [38,109–111] studied the α-keto acid decarboxylase enzyme and the pathway leading to 3-methylbutanal in more detail (Fig. 3). By screening enzyme activities in various dairy related micro-organisms it was found that 3-methylbutanal was only formed in considerable amounts in just the few of the strains studied (Fig. 4). In contrast, the flavour compound 3-methylbutyric acid, which can, amongst others, be produced by subsequent oxidation of the aldehyde, has been

![Fig. 3. Reaction scheme of simplified leucine degradation pathway. TA = transaminase, HaDH = hydroxy acid dehydrogenase, DC = keto acid decarboxylase, ADH = alcohol dehydrogenase, AIDH = aldehyde dehydrogenase and KaDH = keto acid dehydrogenase (adapted from [110]).](image-url)

![Fig. 4. Specific keto acid decarboxylase activity of various dairy related micro-organisms. Error bars indicate the standard deviation of three independent measurements (adapted from Smit et al. [110]).](image-url)
found in many LAB. The absence of aldehyde, but presence of the organic acid, must be explained by action of a ketoacid dehydrogenase complex [76,112] rather than by rapid conversion of the aldehyde into the organic acid.

In order to identify the gene coding for the decarboxylase in *L. lactis* B1157 a high throughput screening was developed based on measuring volatile (aldehyde) metabolites by direct-inlet mass spectroscopy (DI-MS) followed by screening a knock-out mutant library of a strain which possessed the enzyme activity [109,111]. The screening resulted in the identification of the gene coding for the decarboxylase (*kdcA*) [113]. The major part of the gene was identical to the as *ipd* annotated gene of *L. lactis* IL1403. Compared to *kdcA* the *ipd* gene missed a large c-terminal part [114]. Over expressing *kdcA* resulted in a 30-fold increased decarboxylating activity compared to the wild type B1157, while over expression of *ipd* did not result in an active decarboxylase indicating that the absence of decarboxylase activity in IL1404, is due to a truncated gene [111]. Modelling of the KdcA enzyme suggested that larger substrates than pyruvate would fit in the KdcA, and confirmed that the open reading frame annotated as *ipd* in *L. lactis* IL1403 is probably an inactive variant of the KdcA [111]. Over expression of *kdcA* in *L. lactis* NZ9000 resulted in a 3-fold increase in 3-methylbutanal production from leucine. This increase was much lower than the increased specific enzyme activity mentioned above and suggests a second rate-limiting step in the conversion route from leucine to the aldehyde. Most probably the availability of the substrate α-keto acid, produced via transamination is rate limiting in this case [80,81].

De la Plaza et al. [115] identified a similar decarboxylating enzyme, using N-terminal peptide sequencing of bands from a SDS-gel of a partial purified protein sample followed by the identification of the *kivd* (keto isovaleric acid decarboxylase) gene using the genome sequence of *L. lactis* IL1403 [114]. Over expression of the gene was achieved in *E. coli* [115].

Characterisation of the decarboxylating enzyme revealed that the enzyme can be active under cheese ripening conditions [111,115]. The highest activity was measured on branched- and straight-chain α-keto acids with 4–6 carbon atoms, but also the keto acids of methionine, phenylalanine and tryptophan were converted, though with a relatively low conversion rate (Table 4) [111,115,116]. Comparison of the enzyme with other keto acid decarboxylases revealed that the sequence and molecular mass are very similar to IPD and PDC, but not to the branched-chain α-keto acid decarboxylase from *Bacillus* described by Oku [39] and the molecular mass reported by Amarita [116].

For selecting strains to control of aldehyde formation in dairy fermentations the knowledge regarding the activity of α-keto acid decarboxylating enzymes is of major relevance as the aldehydes produced are very potent flavour compounds.

### 2.2.5. Alcohol, aldehyde and keto acid dehydrogenases

Oxidation of a branched-chain aldehyde by aldehyde dehydrogenase (Fig. 1(5)) leads to the corresponding branched-chain organic acid. The reaction equilibrium is close to this organic acid, and the enzyme uses NAD⁺ as hydrogen acceptor [117]. Branched chain organic acids are generally believed to be the substrates for the formation of (longer) branched-chain fatty acids. In addition to the decarboxylase in combination with the aldehyde dehydrogenase, a keto acid dehydrogenase complex (Fig. 1(8)) is most probably present in LAB.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Corresponding amino acid</th>
<th>Enzymatic conversion rate</th>
<th>Chemical conversion rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-Methyl-2-oxobutanoic acid</td>
<td>Val</td>
<td>100%</td>
<td>3%</td>
</tr>
<tr>
<td>4-Methyl-2-oxopentanoic acid</td>
<td>Leu</td>
<td>31%</td>
<td>29%</td>
</tr>
<tr>
<td>3-Methyl-2-oxopentanoic acid</td>
<td>He</td>
<td>28%</td>
<td>2%</td>
</tr>
<tr>
<td>4-Methylthio-2-oxobutanoic acid</td>
<td>Met</td>
<td>8%</td>
<td>18%</td>
</tr>
<tr>
<td>2-Oxo-3-phenylpropanoic acid</td>
<td>Phe</td>
<td>7%</td>
<td>100%</td>
</tr>
<tr>
<td>3-Indol-3-yl-2-oxopropanoic acid</td>
<td>Trp</td>
<td>3%</td>
<td>n.d.</td>
</tr>
<tr>
<td>3-(4-Hydroxyphenyl)-2-oxopropanoic acid</td>
<td>Tyr</td>
<td>1%</td>
<td>54%</td>
</tr>
<tr>
<td>2-Oxopentanedioic acid</td>
<td>Glu</td>
<td>0%</td>
<td>32%</td>
</tr>
<tr>
<td>2-Oxohexanoic acid</td>
<td></td>
<td>25%</td>
<td>44%</td>
</tr>
<tr>
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<td>2-Oxopropanoic acid</td>
<td></td>
<td>1%</td>
<td>5%</td>
</tr>
<tr>
<td>Isohexanoic acid</td>
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</tr>
<tr>
<td>Isopentanoic acid</td>
<td></td>
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<td>0%</td>
</tr>
<tr>
<td>Isobutanoic acid</td>
<td></td>
<td>0%</td>
<td>0%</td>
</tr>
</tbody>
</table>

n.d. = not determined.

Table 4
Substrate specificity of the branched chain α-keto acid decarboxylase compared to chemical conversion of keto-acids to aldehydes in the presence of manganese ions [38,111]
which is able to convert the α-keto acid directly to the corresponding organic acid. Although both routes might be active under other conditions, with different rates, the existence of two routes indicates that one could be missed, without affecting growth or survival. This would explain the absence of decarboxylating activity in many LAB. The dehydrogenase enzyme complex performs the oxidative decarboxylation of α-keto acids resulting in the formation of organic acids, without transitory formation of aldehydes. Although this dehydrogenase complex has not been identified or characterised in LAB yet, the reaction proceeds in lactococci [80,118], but also in propionibacteria and micrococci [76]. A similar dehydrogenase complex for branched-chain α-keto acids has been characterised in Bacillus subtilis [119]. This enzyme complex clearly differs from pyruvate dehydrogenase (PDH). The dehydrogenase complex consists of three catalytic components, being a keto acid dehydrogenase, dihydrolipoyl transacylase and a lipamide dehydrogenase. The oxidative decarboxylation of α-keto acids by this complex in LAB is also relevant for flavour formation in cheese, since carboxylic acids like isovaleric acid are important flavour compounds. Furthermore, these carboxylic acids are precursors for other aroma compounds, such as esters, thioesters, cresol and skatole. Cresol and skatole can chemically as well as enzymatically be formed from the amino acids, tyrosine and tryptophan [6,120].

Alcohol dehydrogenase (Fig. 1(4)) is identified in most LAB [107,117,121–124]. Although the reaction equilibrium of this reaction is far to the side of the alcohol, in many fermented dairy products, aldehyde concentrations are stable at relatively high concentrations. This might be explained by the relatively low activity of this enzyme in LAB. The flavour intensity of aldehydes is higher than that of their corresponding alcohols (Table 2), and therefore this conversion to alcohols might not be favourable, when maximal flavour intensity is desired.

2.3. Lyase pathway

Besides the transaminase pathway, also the activity of several lyases results in flavour compounds. These pathways are usually shorter, because the type of splicing of the substrates leads directly to smaller and volatile compounds. The conversion of methionine by LAB for example, can occur in several steps via the aminotransferase-initiated pathway as described above, but also via a direct α,γ-elimination of methionine to methanethiol by lyase activities (Fig. 5).

Cystathionine β-lyase (CBL, EC 4.4.1.8) and cystathionine γ-lyase (CGL, EC 4.4.1.1) posses this activity and are found in and isolated from several LAB [42,125–129]. However, the main physiological function of these enzymes is the α–β and α–γ elimination of cystathionine in homoserine and cysteine in the anabolism (Fig. 8). All cystathionine lyases are able to convert several substrates, use PLP as cofactor and have a pH optimum close to 8. The relative activity towards methionine is below 10% compared to the rate for cystathionine [126]. Although cystathionine lyases are active under cheese-ripening conditions [42,129,130], their activity towards methionine could not be detected in lactococci using 13C nuclear magnetic resonance [86]. With this technique, only the aminotransferase-initiated pathway was observed suggesting that this pathway is most prominent in methionin a catabolism to produce

![Fig. 5. Conversion of methionine to volatile sulphur compounds.](https://academic.oup.com/femsre/article-abstract/29/3/591/546865)
known, this lyase pathway contributes largely to the acetaldehyde pool. The reaction has intensively been studied and references suggested for reading are: [44–48,123,124,136–138].

2.4. Non-enzymatic conversions

Although most of the flavour forming reactions during cheese production and ripening are enzymatic, e.g., proteolysis, transaminase- and lyase pathways, the α-keto acids of phenylalanine (phenylpyruvic acid) and methionine (KMBA) can (also) non-enzymatically be converted into flavour compounds such as benzaldehyde and methylthioacetaldehyde. The existence of the chemical conversion of phenylpyruvic acid was demonstrated by Villablanca et al. [139] and by Nierop Groet et al. [37], the conversion of indole-3-pyruvate by Gao et al. [118], the conversion of α-ketoisocaproic acid by Smit et al. [38] and the conversion of KMBA to methylthioacetaldehyde by Bonnarme et al. [134]. All these conversions seem to proceed via a similar reaction mechanism [113], Table 4. The spontaneous degradation of hydroxyphenylpyruvate to hydroxybenzaldehyde also occurs under simulated Cheddar cheese conditions [86], and both benzaldehyde and hydroxybenzaldehyde were found in significant amounts in semi-hard cheeses [80]. The reaction is catalysed by several divalent metal ions [37]. The conversion products of indole-3-pyruvate (indole acetic acid, indol-3-acetaldehyde and skatole) have been identified as off-flavours in Cheddar cheese [118].

This chemical conversion of α-keto acids, especially the α-keto acid of leucine, was studied in more detail by Smit et al. [38]. Fig. 7 shows that the component...
2-methylpropanal thus can be formed by two mechanisms: an enzymatic conversion originating from valine and a non-enzymatic chemical conversion with leucine as the substrate. The essence of the reaction mechanism is most probably the formation of a reactive peroxide as a result of a reaction between oxygen and the enol tautomer of the α-keto acid. The reaction is strongly influenced by the availability of substrates (α-keto acid and oxygen), manganese and the pH. Only in the presence of MnCl₂ and MnSO₄ (both at 10 mM) chemical conversion occurred. The simultaneous presence of 20 mM EDTA effectively suppressed the catalytic effect of manganese ions [51]. Although the pH of cheese ripening seems to be rather optimal, the manganese, oxygen and α-keto acid concentration are most probably not [38]. Increasing these concentrations (locally) might therefore be an obvious way for controlling this chemical reaction. High local manganese concentrations might be obtained by selecting strains, which are able to accumulate manganese and lyse later in the ripening process [140,141]. Since only an effect of Mn²⁺ was observed, it would be relevant to know in which form the manganese in these bacteria will be present, and what happens with the manganese if these bacteria lyse. Oxygen is consumed quickly in the fermentation process in semi-hard cheeses, thereafter being almost absent, which is a major difference with the laboratory experiments described in the paper by Smit et al. [38], although only low concentrations of oxygen were needed for the non-enzymatic conversion to proceed. On the other hand, on the surface of smear- and fungal-ripened cheeses the situation might be more in favour of such conversions.

Another important chemical reaction leading from amino acids to flavour compounds is the Strecker degradation. Generally the Strecker degradation is described as the reaction of the amino group of an amino acid with an α-dicarbonyl like a reducing sugar, and it is an important step in the Maillard reaction [142,143]. However, at high temperatures also direct oxidative decarboxylation of amino acids can lead to the same aldehydes [144,145]. These reactions are especially intense at high temperatures, and therefore contribute largely to the flavour of baked products. Nevertheless, at lower temperatures the reactions have also been shown to proceed, e.g., in the case of cheese and beer production [22,146]. Strecker degradation of leucine can result in 3-methylbutanal, but it has also been suggested that the conversion of valine results in this aldehyde as well [146].

Taken together, also certain non-enzymatic conversions result in flavour active compounds and therefore these reactions might also be relevant for the flavour formation in cheese. However, more research on the conversions under cheese ripening conditions is desired.

### 2.5. Regulation of AACEs and role of lysis

Apart from being present or not, activity of amino acid converting enzymes can also be affected by the growth and culture conditions of the bacteria. This is highly relevant, because it also offers opportunities for practical implication and moreover, it is of importance for screening strategies (see below).

As reviewed by Kranenburg et al. [34] and Smit et al. [147], the biosynthesis and degradation of some amino acids are intricately coupled pathways as exemplified in Fig. 8 for serine, cysteine and methionine. During cheese ripening, cystathionine β-lyase can convert methionine to various volatile flavour compounds.

The physiological role of CBL is the conversion of cystathionine to homocysteine, which is the penultimate step in the biosynthesis of methionine. This indicates that amino acid converting enzymes can in fact be involved in the biosynthesis of amino acids rather than catabolism only. It is well known that biosynthesis of amino acids is highly regulated, and therefore the growth conditions of the starter cultures may affect their flavour forming capacities. For instance, in L. lactis the gene coding for cystathionine β-lyase (metC) is clustered together with a gene coding for cysteine synthase (cysK) [128], thus genetically linking the methionine and cysteine biosynthesis pathways. The expression of the metC-cysK gene cluster is strongly influenced by the amounts of methionine and cysteine in the culture medium [127]. High concentrations of these amino acids completely abolish transcription and result in L. lactis cells almost deficient of cystathionine β-lyase activity. These regulatory aspects are most likely very important in the control of flavour forming enzymes in starter cultures and adjunct cultures.

![Cysteine and methionine biosynthesis pathways in E. coli and S. enterica serovar typhimurium](https://academic.oup.com/femsre/article-abstract/29/3/591/546865)

**Fig. 8.** Cysteine and methionine biosynthesis pathways in E. coli and S. enterica serovar typhimurium and the responsible genes. The conversion of cystathionine to cysteine is not described for E. coli. Cystathionine β-lyase from L. lactis is able to catalyse this reaction in vitro ([42,172]).
The MGL activity of *Brevibacterium linens* is highly induced by the presence of methionine or methionine containing peptides in the culture medium [41,148].

Other lactococcal enzymes involved in flavour generation that are reported to be regulated are the amino-transferases AraT and BcaT. Chambellon and Yvon [149] reported that these enzymes, which physiological role is to catalyse the last step in the biosynthesis of branched-chain or aromatic amino acids, are regulated by CodY. CodY has also been identified as a regulator of several genes encoding proteo- and peptidolytic enzymes, sensing the nutritional supply as a function of the branched chain amino acid pool in the cell [150,151]. Den Hengst et al. [151] demonstrated that *L. lactis* CodY interacts directly with a region upstream of the promoter of its major target known so far, the oligopeptide transport system (OPP system). It is suggested that multiple molecules of CodY interact with this promoter and that the amount of bound CodY molecules is affected by the presence of branched-chain amino acids.

Inactivation of both the AraT and BcaT strongly reduces the growth of *L. lactis* in milk, which was found to result in excess in intracellular pool of isoleucine. This in turn likely represses the proteolytic system of the cells, due to distortion of the CodY regulation [149]. Several enzymes can thus been considered as being involved in both biosynthesis and degradation of amino acids, and α-keto acids are intermediates in both directions. Expression of the *bcaT* gene is repressed by high concentrations of branched-chain amino acids or methionine [71]. These examples illustrate that the selection of culture conditions can strongly influence the flavour-forming capacities of *L. lactis*.

Until now, not many reports deal with the role of lysis for the activity of AACEs in cheese. Dias and Weimar indicated that in addition to the presence of the metabolic pathway, methionine degradation in Cheddar cheese also depends on the organism used in production, the amount of enzyme released during aging, and the amount of methionine in the matrix [41]. While studying the effect of lysis on proteolysis and peptidolysis Meijer et al. [141] and Lepeuple et al. [152], showed that the mechanism behind the differences between ‘bitter’ or ‘non-bitter’ cultures was strongly correlated with the sensitivity of the cells to lysis. The introduction of a transposon for nisin immunity (it encodes for immunity to nisin, a lanthionine-containing peptide with antimicrobial activity, and the capacity to utilize sucrose via a phosphotransferase system) in *L. lactis* SK110 by Meijer et al. [141] and the introduction of a prophage Phi AM2 in *L. lactis* AM2 [152] resulted in cultures with decreased an increased sensitivity to lysis, respectively. Development of bitterness in cheeses made with these cultures was linked to the sensitivity to lysis. These results indicate that the cell membrane can be a barrier between the enzymes, located intracellularly, and the peptide substrates present in the cheese matrix. Apparently, there is not enough active transport anymore by the starter cultures, for taking-up the peptides, once they are present in the cheese matrix and lysis then is essential for enhancing enzyme-substrate interaction. Bourdat-Deschamps et al. [153] showed that the conversion of phenylalanine to flavour compounds in cheese models was enhanced by autolysis, when α-ketoglutarate concentration was not limiting. In a study by De Palencia [154], bacteriocin sensitive strains of *L. lactis*, with BcAA activity and α-keto acid decarboxylase activity, were used as adjunct together with a bacteriocin-producing (Lacticin 3147) *L. lactis* strain in cheese making. In control cheese making, a non-bacteriocin producing strain was used. The bacteriocin produced enhanced lysis of the adjunct strains, which led to an increase in isoleucine transamination. The concentration of the flavour compound 2-methylbutanal was about doubled again indicating that increased aldehyde formation can be obtained due to lysis.

In contrast to the activity of peptidases, where lysis generally enhances the activity enzymes, enzymes that require cofactors or co-substrates (e.g., PLP, NAD, and NADP), might be negatively affected by lysis of the cells. It likely depends on the type of enzyme (system) whether lysis will improve the activity (and formation of flavour) or not. The examples above show that the delicate balance in the whole set of conversions involved in flavour formation can be affected by lysis and therefore the role of lysis deserves to be further studied. The effect of lysis on formation of various flavour compounds then could become much clearer.

### 2.6. Natural biodiversity

It has already been mentioned that various LAB strains differ in amino acid converting abilities and that these activities are in fact linked to the ability to synthesise amino acids. The KdCA activity studies by Smit et al. [38,109,111] give a good example of such a diversity (Fig. 4). The strains that were found to have the highest activity were *L. lactis* strains isolated from natural sources and non-dairy environments, the so-called ‘wild lactococci’. Ayad et al. [23,104] focused on the ability of these strains and they were found to have unique and diverse properties, when compared to commercially available starter strains. For instance, many of these strains do not degrade caseins, produce antimicrobial compounds and/or have low acidifying activity. However, when the dependency of these strains for amino acids in the growth medium was determined using the single omission technique [155], it was found that these strains had a much larger potential to synthesise their own amino acids as compared to industrial strains. Lactococci used in dairy fermentations are known for their limited capacity for biosynthesis of amino acids, which
explains their complex nutritional requirements. Most of these strains require at least glutamate, valine, methionine, histidine, serine, leucine and isoleucine for growth, and the number of essential amino acids is strain-dependent [31,32,104,156,157]. Industrial *L. lactis* subsp. *cremoris* strains require even more different amino acids for growth [104]. Wild *L. lactis* subsp. *cremoris* strains generally require two to three amino acids while some *L. lactis* subsp. *lactis* strains only need one or two amino acids. The absence of some amino acid biosynthetic pathways in dairy lactococci might be a consequence of their adaptation to dairy products, since in milk, the amino acids are readily available from the proteolytic degradation of caseins. Wild strains are not naturally associated with a rich environment such as milk, which makes them more dependent on their own biosynthesis of amino acids compared to industrial strains. Interestingly, lactococci isolated from natural niches were not only found to have a larger potential in amino acid production, but concomitantly, also found to be able to produce rather unusual flavour components and/or flavour profiles [104]. This natural biodiversity could offer new possibilities when explored and applied in practice.

### 2.7. Application of strains with selected enzyme activities for improving cheese flavour

For the application of selected lactococci, it was found that strains possessing a specific flavour-forming enzyme do not necessarily possess other enzymatic activities of the complete pathway. In addition, strains might lack other characteristics for application as cheese starter (e.g., fast acidification). In order to be able to use such strains and to overcome problems, it is required to combine selected strains with industrial strains in order to obtain a starter with both good flavour generating potential as well as good acidifying and proteolytic activities [23,158].

It was found by Ayad et al. [159] that different strains could influence each other in formation of flavour components. Strains, which each had only a limited set of enzymes in a certain pathway could complement each other. For instance, the combination of *L. lactis* B1157 and SK110 strains in milk resulted in the formation of high levels of 3-methylbutanal. In SK110, a highly proteolytic strain from industrial origin, the complete pathway from casein via leucine to 3-methylbutanal cannot proceed due to the lack of a decarboxylating enzyme (Fig. 9). *L. lactis* strain B1157 on the other hand is a non-proteolytic wild strain and thus unable to produce enough free amino acids that can serve as substrate for the subsequent transamination and decarboxylation steps. However, when B1157 and SK110 are cultivated together, the strains complement each other with regard to their enzyme activities resulting in a high production of the chocolate flavour component 3-methylbutanal (Fig. 9). This proto-cooperation between strains as it is called offers new possibilities for the construction of tailor-made starter cultures, because it makes it clear that not all the desired enzyme activities in a certain flavour pathway leading to flavour need to be present in one strain.

An example of the application of knowledge of proto-cooperation, but also of population dynamics of starter cultures for the optimisation of a cheese flavour is given by Ayad et al. [160]. A selected *L. lactis* strain (strain B851) with high (in vitro) activity to form 3-methylbutanal was used to improve the taste of Proosdij cheese. Proosdij cheese is a Gouda-type cheese, prepared with a mesophilic starter culture in combination with a thermophilic adjunct culture. This cheese has a flavour profile, which has characteristics between Gouda and Parmesan cheese. One of the key flavour components in this type of cheese is 3-methylbutanal [77,161]. The selected *L. lactis* strain B851 was used in combination with the regular cultures used for this type of cheese. The cheeses made with and without the selected adjunct strain were analysed for the production of 3-methylbutanal by headspace gas chromatography [159] and graded by an expert panel [162]. It was found that the use of the selected adjunct strain in cheese resulted in both an increase in the key-flavour production as well as in the intensity of the Proosdij cheese flavour (Fig. 10). This is a first example on how fundamental knowledge can lead to the development of new starter cultures in a directive manner.

In order to control the flavour intensity, the selected strain was first tested at different levels in a defined strain starter (DSS) culture as well as in combination with a mixed strain starter (MSS) culture. The latter is generally used for Gouda and Proosdij-type cheese production. The results of population dynamics, sensory evaluation and analysis of volatile compounds pointed to the possibility of controlling both the cell numbers of strain B851 as well as the flavour intensity resulting

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**Fig. 9. Proto-cooperation between different *L. lactis* strains in the formation of 3-methylbutanal.** The thickness of the arrows indicates the relative enzymatic activities of the individual strains and a combination thereof. A, the proposed conversion pathway from casein to 3-methylbutanal; B, relative activities found for strain SK110; C, relative activities for strain B1157; and D, relative activity of a mixed culture of both strains (adapted from Ayad et al. [159]).
from this strain in cheese. Based on these results, B851 was used to enhance the flavour development of a Proosdij-type cheese made with a new thermophilic culture B1138. This culture had previously been developed to prevent crack formation in Proosdij cheese. In this cheese, the addition of culture B851 led to an increase in the overall flavour intensity, indicating that it is possible to tailor the flavour of cheese by using specifically selected cultures, even in combination with complex starter cultures.

3. Conclusions

In fermented dairy products, most notably cheese, the degradation of caseins is the main pathway to flavour formation. Proteolysis and peptidolysis are a prerequisite to generate free amino acids. A balanced degradation of caseins, i.e., formation and breakdown, is important in order to prevent accumulation of bitter-tasting peptides. The volatile flavour components, which predominantly determine the typical flavour, are subsequently derived from the activity of amino acid converting enzymes. In LAB strains the physiological functions of these enzymes are most likely the biosynthesis of amino acids. Based on this, a strong regulation is anticipated and found for these enzymes. This characteristic is very important for practical applications, since it means that the activity of starter cultures can be influenced by the cultivation conditions. Moreover, a large natural biodiversity is found within LAB species, which also offers good possibilities for flavour enhancement and diversification of dairy products.

The increasing knowledge on the amino acid converting enzymes, together with genome data, which will become available for various LAB, will expand our knowledge of flavour-forming pathways and mechanisms in different bacteria even faster. Obviously, one should also focus on other pathways (e.g., leading to flavours originating from lactose and fat), which play a role in the liking of the products, as well as on the role of the interaction of the various taste, aroma and structure components in the cheese, again also a rapidly expanding field of (flavour) research.

Whole genome sequences of various lactic acid bacteria are available, e.g., *L. lactis* [114] and *L. plantarum* [163] and more are being sequenced (for example http://genome.jgi-psf.org/draft_microbes). It will soon allow prediction of the flavour-forming capacity of various lactic acid bacteria, and secondly lead to the design of probes for high-throughput screening and strain selection in the future. Probes will become available to rapidly screen for various enzymes by which screening for the presence of whole pathways will become possible. On the other hand, it appears that not all enzymes have to be present in one strain, making it possible to combine a number of strains in defined starter systems. One should, however, be cautious that not only the presence of certain enzymes, but also the proper level of these enzymes will have an impact on the overall flavour perception. A careful balance is needed and in order to know this balance it might be required to determine not only the compounds required for a certain flavour impact, but also their concentration. It the light of this, it would be very powerful if fast screening of total aroma profiles could be realised in order to match the desired profile.

Apart from that, one has to realise that most screening systems are based on laboratory media. Obviously, the activity of the various enzymes will change in the actual food matrices (e.g., cheese). Good predictable model systems (like the so-called Cheasy model) should therefore be linked to rapid screening and analysis methods. The basic insight into the flavour formation pathways is essential for all of this, but further knowledge on the balance of key-flavour components, on the screening methods for desired enzyme activities as well as on the regulation of these enzymes in product matrices is all needed at the same time to make this possible.

Genomics might assist us in getting a fast insight whether other enzymes or pathways exist in the broad variety of lactic acid bacteria, thereby enabling a faster selection of interesting strains. The long-term endeavour
of generating a consumer preferred cheese flavour, should however not prevent us from applying already the various (smaller) steps towards new and improved cheese production by the selection of new or adaptation of existing cheese starter cultures. A very interesting and tasty future lies ahead of us.

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