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Cheese flavour formation by amino acid catabolism

Mireille Yvon*, Liesbeth Rijnen

INRA, Unité de Biochimie et Structure des Protéines, 78352 Jouy-en-Josas, France

Abstract

Amino acid catabolism is a major process for flavour formation in cheese. The ability of lactic acid bacteria (LAB) and other cheese micro-organisms to degrade amino acids to aroma compounds is highly strain dependent. Generally, amino acid catabolism proceeds by 2 different pathways. The first one, mainly observed for methionine, is initiated by elimination reaction and leads to major sulphur aroma compounds. The second pathway is generally initiated by a transamination reaction and is the main pathway for degradation of all amino acids by LAB. The resulting α-keto acids are then degraded to various aroma compounds via 1 or 2 additional steps. The lactococcal enzymes initiating both pathways have been well characterised, and their importance in the formation of aroma compounds has been demonstrated by using isogenic strains lacking each enzyme. From the new knowledge several applications have been successfully developed, especially for intensifying or diversifying cheese flavour by controlling amino acid transamination. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: Amino acid catabolism; Cheese micro-organisms; Flavour; Cheese

1. Introduction

Accelerating or diversifying flavour development in cheese is of major economical interest since final flavour of cheeses partly determines consumer choice and because flavour development is a time consuming and expensive process that is still not well mastered. Flavour formation occurs during cheese ripening. It is a complex process in which three major catabolic pathways are involved: glycolysis, lipolysis and proteolysis. Proteolysis was supposed to be rate limiting in the maturation of many cheeses, especially in semi-hard cheeses, and hence has been the focus of most research on the acceleration of ripening. However, recent results show that enhancing the free amino acid release by intensifying peptidolysis by lactic acid bacteria or adding free amino acids did not affect aroma formation in cheese (Christensen, Johnson, & Steele, 1995; Wallace & Fox, 1997), suggesting that the rate-limiting factor was not the release of free amino acids but the conversion of amino acids to aroma compounds.

Therefore, over the past five years, several research groups have focused on amino acid catabolism by cheese micro-organisms, especially by lactic acid bacteria and *Brevibacterium linens*, which is used as surface flora in many cheeses. These studies have provided new insights into amino acid catabolism, which offer new prospects for controlling aroma formation in cheese at the level of amino acid catabolism. In this review, firstly we will examine the importance of amino acid catabolism in flavour formation in cheese and the ability of cheese micro-organisms to generate aroma compounds from amino acids. Then we will review new knowledge on the enzymes and metabolic pathways involved in the conversion of amino acids to aroma compounds in cheese micro-organisms and we will finish by looking at some examples of controlling aroma formation in cheese at the level of amino acid catabolism.

2. Cheese aroma compounds resulting from amino acid degradation

Many studies have used GC/MS to analyse the aroma of cheeses. However this method measures all the volatile compounds while only a small fraction of volatiles are odour-active. In order to identify the odour-important compounds in cheese, analytical methods that combine gas chromatography and olfactometry have been developed. Recently, by using these methods the potent odorants of various cheeses have been identified (Table 1). Aroma profiles of Cheddar (Christensen & Reineccius, 1995; Milo & Reineccius, 1997)

*Corresponding author.
E-mail address: mireille.yvon@diamant.jouy.inra.fr (M. Yvon).

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and Camembert (Kubickova & Grosch, 1997) share many similarities. Half of the potent odorants result mainly from lactose fermentation or citrate degradation and a few from lipolysis while the other half result from leucine and methionine degradation. The major aroma compounds produced from methionine are methional, methanethiol and its oxidation products, dimethyldisulphide (DMDS) and dimethyltrisulphide (DMTS) while isovaleric acid and 3-methylbutanal result from leucine degradation. In Emmental cheese, methional and 3-methylbutanal were also among the most potent aroma compounds although the typical sweet caramel aroma of Emmental is mainly due to furaneol and homofuraneol.

According to Dacremont and Vickers (1994), methional, which has a boiled potato-like aroma, is an important contributor of good (mild) Cheddar aroma and methanethiol, DMDS and DMTS likely contribute to the desirable garlic note of the cheeses (Manning, 1974). These sulphur compounds are very volatile and consequently they are mainly found in the headspace of cheese (Kubickova & Grosch, 1997; Milo & Reineccius, 1997). 3-methylbutanal has a green malty odour (acrid-pungent) but in low concentrations the odour becomes fruity, rather pleasant while when it was present in amount higher than 200 ppb in Cheddar, it caused unclean-harsh and dulling flavour sensations (Dunn & Lindsay, 1985). Isovaleric acid, the acid derived from leucine, appears to be more prevalent in Camembert than in Cheddar and it was also found in other cheeses such as Swiss cheese (Bosset, Collomb, & Sieber, 1993). It has a rancid, cheesy, sweaty and putrid odour that probably highly contributes to the very-ripened-cheese aroma. However, the most potent odorant in Camembert and Cheddar is butyric acid, which has a cheesy, sweaty and sour odour that contributes to the pleasant sweet flavour of Cheddar (Dacremont & Vickers, 1994). Although butyric acid is a short-chain fatty acid arising mainly from lipolysis, it is interesting to note that a very similar aroma could be given by isobutyric acid which is the carboxylic acid derived from valine (Arctander, 1994). The derived ester, ethylbutyrate also participates in the cheddar flavour, however, an excess of esters in proportion to other flavour components would be responsible for the fruity defect of Cheddar (Bills, Morgan, Libbey, & Day, 1965).

In Camembert, phenyl acetaldehyde, 2-phenylethanol and the derived ester phenethyl acetate which all result from phenylalanine degradation are identified in fractions with floral rose-like odour (Kubickova & Grosch, 1997). These compounds have been previously assumed to cause the pleasant floral note of Camembert (Dumont, Roger, & Adda, 1974; Roger, Degas, & Gripon, 1988). In contrast, the same degradation products of phenylalanine and some other aromatic amino acid metabolites such as *p*-cresol, indole and skatole were identified as responsible for unclean-utensil, rose-like off-flavours in Cheddar (Dunn & Lindsay, 1985).

The identification of the key aroma compounds of various cheeses shows that amino acid degradation is a

Table 1
Major odorants of Cheddar, Camembert and Emmental cheeses. Compounds are listed in the order of their importance in odour and compounds resulting from amino acid degradation appear in bold characters

Cheddar cheese[a]	Camembert cheese[b]	Emmental cheese[c]
Butyric acid	Butyric acid	Propionic acid
Ethyl butyrate	**Isovaleric acid**	**Methional**
3-Methylbutanal	**3-Methylbutanal**	Furaneol[d]
Propionic acid	**Methional**	Homofuraneol[e]
Ethyl caproate	2,3-Butanedione	Diacetyl
Isovaleric acid	1-Octen-3-ol	**3-Methylbutanal**
Acetic acid	1-Octen-3-one	Ethyl butyrate
Methional	**Phenethyl acetate**	
1-Octen-3-one	2-Undecanone	
Methanethiol	γ-Decalactone	
DMDS	**Methanethiol**	
DMTS	**DMTS**	
Diacetyl	**DMS**	
Furaneol/homofuraneol	**Phenylacetaldehyde**	

[a] Christensen and Reineccius (1995) and Milo and Reineccius (1997).
[b] Kubickova and Grosch (1997).
[c] Preininger and Grosch (1994).
[d] 4-Hydroxy-2,5-dimethyl-3(2 H)-furanone.
[e] 2-Ethyl-4-hydroxy-5-methyl-3(2 H)-furanone.

Table 2
Name and chemical nature of the major aroma compounds derived from branched-chain and aromatic amino acids and methionine

Amino acids	Aldehydes	Alcohols	Carboxylic acids	Thiol/divers
Leucine	3-Methylbutanal or isovaleraldehyde	3-Methylbutanol	3-Methylbutanoïc acid or isovaleric acid	
Isoleucine	2-Methylbutanal	2-Methylbutanol	2-Methylbutanoïc acid	
Valine	2-Methylpropanal or isobutyraldehyde	2-Methylpropanol	2-Methylpropanoïc acid or isobutyric acid	
Phenylalanine	Phenylacetaldehyde, benzaldehyde (-2C)	Phenylethanol	Phenylacetic acid	
Tyrosine	OH-Phenylacetaldehyde, OH-benzaldehyde (-2C)	OH-Phenylethanol	OH-Phenylacetic acid	*p*-Cresol, phenol
Tryptophane	Indol-3-acetaldehyde, indol-3-aldehyde	Tryptophol	Indol-3-acetic acid	Skatole, indole
Methionine	3-Methylthiopropanal, or methional	3-Methylthiopropanol	3-Methylthiopropionic acid	Methanethiol

major process for aroma formation in cheese and that aromatic amino acids (phenylalanine, tyrosine tryptophan), branched-chain amino acids (leucine, isoleucine and valine) and methionine are the major precursors of these aroma compounds (Table 2). Since the final flavour of cheeses depends on the respective concentrations of the different key aroma compounds, the control of amino acid degradation during cheese ripening appears to be a good means of controlling aroma development in cheese.

3. Ability of cheese micro-organisms to produce aroma compounds from amino acids

A number of different LAB and other cheese micro-organisms have been evaluated for their ability to degrade amino acids to aroma compounds. The ability has been determined by incubating resting cells or cellular extracts in cheese models or in synthetic media containing casein or free amino acids and analysing products formed either by GC/MS or by HPLC. Many cheese micro-organisms, including lactic acid bacteria (LAB), coryneform bacteria, yeasts and *Geotrichum candidum,* are capable of producing aroma compounds from amino acids, but the ability is highly strain dependent.

The major LAB associated with the production of aldehydes and alcohols from branched-chain amino acids (BcAAs) is *Lactococcus lactis* var. *maltigenes* (MacLeod & Morgan, 1958; Morgan, 1976; Morgan, Lindsay, & Libbey, 1966). This *maltigenes* variant of *L. lactis* is also capable of converting phenylalanine and methionine to phenylacetaldehyde and methional (Morgan, 1976). However, recently, several groups observed that a few wild-type strains of *L. lactis*, not classified as *maltigenes*, produced also large amount of aldehydes and alcohols from BcAAs and methionine (Ayad, Verheul, deJong, Wouters, & Smit, 1999; Weerkamp, Klijn, Neeter, & Smit, 1996; Pelaez, personal communication; Tammam, personal communication). These compounds are also formed in large amounts by another genus of LAB, *Carnobacterium piscicola* that was previously classified as *Lactobacillus maltaromicus* (Larrouture, Ardaillon, & Montel, 1999; Miller, Morgan, & Libbey, 1974). Propionibacteria such as *Propionibacterium freudenreichii* or *Propionibacterium shermanii* have been clearly associated with the production of carboxylic acids from BcAAs (Biede, Paulsen, Hammond, & Glatz, 1979; Paulsen, Kowalewska, Hammond, & Glatz, 1980; Thierry, Maillard, & Maubois, 2000) and are probably mainly responsible for the formation of these compounds in Swiss-type cheeses. Furthermore, recently, non-starter LAB and especially some strains of *Lactobacillus paracasei* were shown to generate low amounts of aldehydes, alcohols and acids from BcAAs, phenylalanine and methionine when grown in medium containing casamino acids or lactalbumin hydrolysate (Tammam, Williams, Noble, & Lloyd, 2000).

Several LAB such as *L. lactis* subsp. *lactis* and *cremoris*, *Lactobacillus lactis*, *Lactobacillus helveticus*, *Lactobacillus bulgaricus* and *Lactobacillus casei*, are also capable of degrading methionine to methanethiol, DMDS and DMTS (Dias & Weimer, 1998a; Imhof, Glättli, & Bosset, 1995; Law & Sharpe, 1978; Manning, 1974). However, *Micrococcaceae* and coryneform bacteria and especially *Brevibacterium linens* that are used as surface flora in various cheeses, are much better producers of methanethiol and DMDS than LAB (Bloes-Breton & Bergère, 1997; Dias & Weimer, 1998a; Ferchichi, Hemme, Nardi, & Pamboukdjian, 1985). Moreover, these bacteria are capable of producing *S*-methylthioesters from methanethiol and different carboxylic acids such as acetic, propionic, isobutyric or isovaleric acids (Bloes-Breton & Bergère, 1997; Jollivet, Bézenger, Vayssier, & Belin, 1992; Lamberet, Auberger, & Bergère, 1997a, b). All these volatile sulphur compounds: methanethiol, DMDS, DMTS and methylthioesters, are also produced in significant quantities by *Geotrichum candidum* that is commonly present in ripening cultures used in the dairy industry especially for Camembert cheese. However, the amount of methanethiol and the type of thioester produced are dependent on and specific to the strain of *G. candidum* (Berger, Khan, Molimard, Martin, & Spinnler, 1999; Jollivet, Chataud, Vayssier, Bensoussan, & Belin, 1994). Moreover, most of the *G. candidum* strains produced alcohols and carboxylic acids from Leu, Ile, Val and Phe (Jollivet et al., 1994) as well as yeasts isolated from Camembert (Lee & Richard, 1984) which explains the presence of these compounds in Camembert.

Since ability of micro-organisms to generate aroma compounds from amino acids is highly strain dependent, knowledge of the amino acid catabolic pathways and enzymes playing a key role in aroma formation would make it possible to develop rapid screening tests for selecting strain with interesting catabolic activities.

4. Catabolic pathways involved in amino acid conversion to aroma compounds

The enzymatic reactions involved in amino acid conversion to aroma compounds by cheese micro-organisms remain only partially characterised. They had been partly studied in yeast, *Brevibacterium linens* and some LAB but, over the past five years, knowledge was detailed especially in *L. lactis* and in some other LAB, and several enzymes involved in these reactions have been biochemically and genetically characterised. Regulation of gene expression has also been partially studied.

Generally, amino acid conversion to aroma compounds proceeds by 2 different pathways (Fig. 1). The first one is initiated by elimination reactions catalysed by amino acid lyases which cleave the side chain of amino acids. This pathway has been observed for aromatic amino acids (ArAAs) and methionine and leads by a single step to phenol, indol and methanethiol, respectively. The second pathway goes through α-keto acid intermediates, it is mainly initiated by a transamination reaction catalysed by amino acid aminotransferases and has been observed for ArAAs, BcAAs and methionine. The resulting α-keto acids are then degraded to aldehydes, alcohols, carboxylic acids, hydoxy acids or methanethiol for methionine via 1 or 2 additional steps.

4.1. Pathway initiated by elimination reactions

The pathway initiated by elimination reaction is not a major pathway used by cheese micro-organisms for the catabolism of aromatic amino acids. However, yeast, micrococci and *Brevibacterium linens* are capable of cleaving the side chain of tyrosine and tryptophan, releasing phenol and indol respectively (Jollivet et al., 1992; Parliment, Kolor & Rizzo, 1982). Such activities have never been detected in any LAB (Gummalla & Broadbent, 1999). The enzymes involved in this β-elimination of tyrosine and tryptophan are tyrosine–phenol lyase and tryptophan–indol lyase respectively. They produce, in addition to phenol and indol, ammonia and pyruvate. In general, they are pyridoxal-phosphate- and Mg^{2+}-dependent enzymes but they have never been isolated from cheese micro-organisms.

Methionine γ-elimination produces methanethiol which can be further chemically oxidised to DMDS and DMTS. This pathway seems to be a major pathway for methionine degradation by some cheese micro-organisms such as brevibacteria. *L. lactis* is also capable of cleaving the side chain of methionine, producing methanethiol directly, but this pathway does not seem to be its major catabolic pathway. In fact the enzymes involved in γ-elimination of methionine in both micro-organisms are different. In *B. linens*, the enzyme is a methionine γ-lyase which specifically acts on methionine while in lactococci the enzymes are cystathionine β-lyase and cystathionine γ-lyase, which are not specific to methionine.

Methionine γ-lyase (MGL) (EC 4.4.1.11) also known as methionase or L-methionine γ-demethiolase is a PLP-dependent enzyme that catalyses the α–γ elimination of methionine and its derivatives. It is widely distributed in bacteria but has never been identified in any LAB. The only one isolated from a cheese micro-organism is that

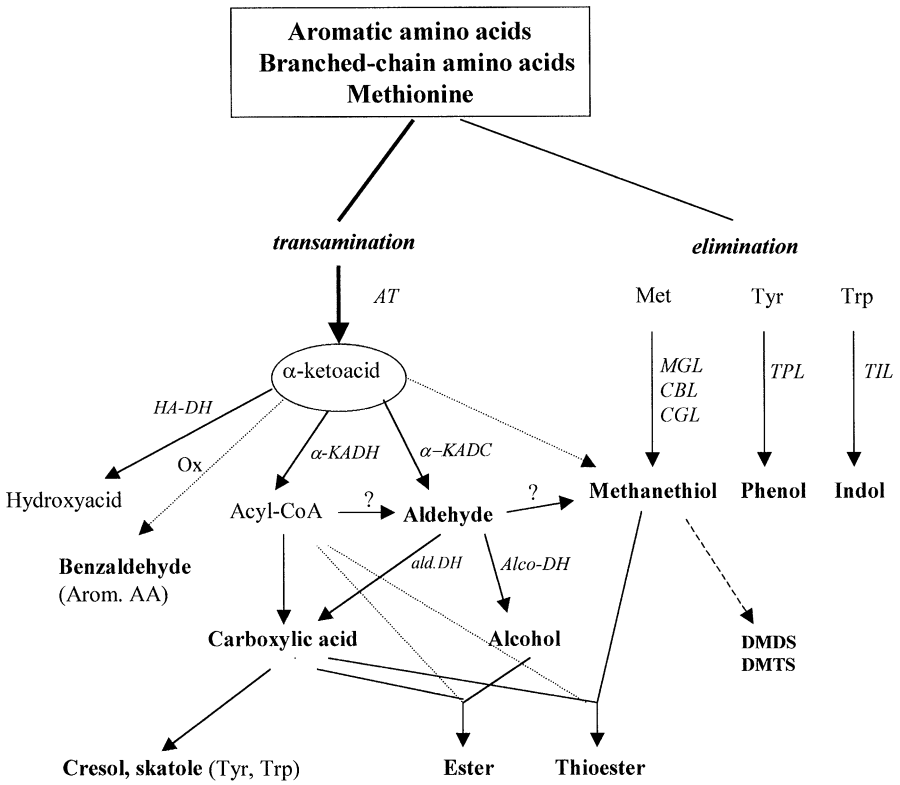

Fig. 1. Amino acid catabolism pathways found in different cheese micro-organisms, and some chemical reactions occurring in cheese (dotted line). DMDS: dimethyl disulphide; DMTS: dimethyl trisulphide; *AT*: aminotransferase; *HA-DH*: hydroxyacid dehydrogenase; α-*KADH*: α-keto acid dehydrogenase; α-*KADC*: α-ketoacid decarboxylase; *aldDH*: aldehyde dehydrogenase; *alcoDH*: alcohol dehydrogenase; *MGL*: methionine γ-lyase; *CGL*: cystathionine γ-lyase; *CBL*: cystathionine β-lyase; *TPL*: tyrosine–phenol lyase; *TIL* tryptophan–indol lyase; ox: oxidation.

of *B. linens* (Collin & Law, 1989; Dias & Weimer, 1998b). MGL of *B. linens* has optimal activity at pH 7.5 and at 25°C but is still active at the pH, temperature and salt concentration of cheese ripening, although the residual activity should not exceed 0.6% of the activity under optimum conditions (Dias & Weimer, 1998b). Moreover, the enzyme is susceptible to proteolysis suggesting that this activity may be lost with cell lysis. Indeed no residual activity was detected in cell-free extract of *B. linens* at pH 5.2 and with 5% NaCl (Dias & Weimer, 1998a). However addition of MGL to cheese slurries increased the production of methanethiol and DMTS (Dias & Weimer, 1999).

The gene encoding MGL in *B. linens* has not been sequenced yet but its expression is probably regulated since MGL activity is highly induced by the presence of methionine (Ferchichi et al., 1985; Dias & Weimer, 1998a) or methionine-containing peptides in the growth medium (Weimer et al., 1997). The concentration of free methionine in milk seems to be sufficient to highly stimulate methionine γ-lyase activity (Dias & Weimer, 1998a).

Since the enzyme is susceptible to proteolysis and its activity is highly decreased by the physicochemical conditions of cheese, adding *B. linens* cells as adjunct will probably be more efficient than adding free enzyme (Weimer et al., 1997) for intensifying flavour development in cheese.

Cystathionine β-lyase (CBL) (EC 4.4.1.8) and cystathionine γ-lyase (CGL) (EC 4.4.1.1) catalyse the $\alpha-\beta$ elimination and $\alpha-\gamma$ elimination of cystathionine, producing homocysteine and cysteine respectively plus α-ketobutyrate and ammonia. CBL or CGL activity has been detected in several LAB such as *L. lactis* (Alting, Engels, van, & Exterkate, 1995), *Lactobacillus fermentum* (Smacchi & Gobbetti, 1998), *Lb. casei* and *Lb. helveticus* and in some strains of *B. linens* (Dias & Weimer, 1998a), but only the enzymes of *L. lactis* and *Lb. fermentum* have been isolated. The enzyme isolated from *L. lactis* B78 (Alting et al., 1995) is a CBL, which preferably catalyses $\alpha-\beta$ elimination of cystathionine but is capable of catalysing $\alpha-\gamma$ elimination reaction as well. The two enzymes isolated from *L. lactis* SK11 (Bruinenberg, Roo, & Limsowtin, 1997) and *Lb. fermentum* (Smacchi & Gobbetti, 1998) are CGL since they produce only cysteine and not homocysteine from cystathionine. The primary function of these enzymes is not related to amino acid catabolism but rather to methionine biosynthesis. However all three are capable of catalysing γ-elimination of methionine producing methanethiol, although the demethiolation activity of these enzymes on methionine is about 10–100 fold less than on cystathionine. The three enzymes show similar broad substrate specificity but the relative activities towards the substrates are slightly different. They also share many properties: They are pyridoxal-5′-phosphate (PLP)-dependent enzymes with an optimum pH for activity around pH 8 but are still active under the pH and salt concentration existing during cheese ripening. However, the residual activity of lactococcal enzymes under cheese-like conditions (pH 5, 4% NaCl) is highly strain dependent, varying from 1% to 25% of optimal activity (Dias & Weimer, 1998a). In similar conditions the cystathionine lyase activity of *B. linens* and *Lb. helveticus* was completely lost while that of *L. casei* was only slightly decreased.

Recently, the *metC* gene, which encodes CBL, has been cloned from 2 *L. lactis* strains (Fernandez et al., 2000). The gene is cotranscribed with a downstream *cysK* gene, which encodes a putative cysteine synthase, confirming the probable role of CBL in methionine biosynthesis. Inactivation of *metC* inhibits the activity of the strain on cystathionine by 90% but does not affect the activity on methionine, indicating that other enzymes are involved in cystathionine and methionine degradation and that CBL is not essential for the conversion of methionine to volatile products by *L. lactis* (Fernandez et al., 2000). Moreover *metCcysK* gene expression is probably repressed by the presence of methionine and cysteine since cystathionine lyase activity of *L. lactis* was highly decreased by increasing the concentration of methionine and cysteine in the growth medium and the methionine concentration in milk is sufficient to decrease the activity by at least 50% (Dias & Weimer, 1998a). Therefore, CBL of *L. lactis* probably does not play a key role in the formation of volatile sulphur compounds in cheese. However, overproduction of metC gene resulted in an increase in the enzyme activity of 25–100 fold towards both cystathionine and methionine in vitro. These results suggest that although CBL is not essential for volatile sulphur compound formation by *L. lactis*, its overproduction may increase flavour compound formation from methionine in cheese. However, this should be confirmed by studies in cheese model since the effect was only observed in vitro under optimal conditions for CBL activity.

4.2. Pathway initiated by a transamination reaction: α-keto acid pathway

The second pathway for the conversion of amino acids to aroma compounds by cheese micro-organisms is a multi-step pathway that is initiated by a transamination reaction. This reaction is catalysed by aminotransferases that degrade amino acids to α-keto acids. Amino acid deamination to α-keto acids might also be catalysed by either dehydrogenases or oxidases. But such activities towards aromatic and branched-chain amino acids and methionine have never been detected in cheese micro-organisms (Gao, Broadbent, Johnson, Weimer, & Steele, 1997; Lee & Desmazeaud, 1985b; Lee, Lucas, &

Desmazeaud, 1985; Schmidt & Lenoir, 1974; Tammam et al., 2000; Thirouin et al., 1995). In contrast, aminotransferase activities have been detected in most cheese micro-organisms.

4.2.1. Amino acid transamination

Amino acid transamination is a key step in the amino acid conversion to aroma compounds by cheese micro-organisms. Indeed, in LAB catabolism of ArAAs, BcAAs and Met is essentially initiated by a transamination reaction since the degradation occurs only in presence of an α-keto acid which is used as amino group acceptor. This was demonstrated in lactococci (Gao et al., 1997; Gao, Mooberry, & Steele, 1998; Yvon, Thirouin, Rijnen, Fromentier, & Gripon, 1997), in mesophilic lactobacilli such as *Lb. paracasei*, *Lb. casei*, *Lb. plantarum*, *Lb. rhamnosus* (Gummalla & Braodbent, 1996; Tammam et al., 2000; Yvon, unpublished results) and also in thermophilic lactobacilli such as *Lb. helveticus*, *Lb. delbrueckii lactis*, *Lb. delbrueckii bulgaricus*, *Streptococcus thermophilus* or *Propionibacterium freudenreicheii* (Gummalla & Broadbent, 1999; Thierry et al., 2000; Yvon, unpublished results). Transamination reactions are also essential for ArAA catabolism by cheese coryneform bacteria (Lee et al., 1985) and especially by *B. linens* (Lee & Desmazeaud, 1985b) and initiate the Ehrlich–Neubauer pathway of alcohol formation from ArAAs and BcAAs by yeasts (Lee & Richard, 1984; Roger et al., 1988).

Amino acid transamination is catalysed by aminotransferases and results in the formation of α-keto acids while the α-keto acid acceptor, often α-ketoglutarate, is transformed to the corresponding amino acid: glutamate.

Aminotransferases are pyridoxal-5′-phosphate-dependent enzymes and are widely distributed in micro-organisms. Generally, they are more or less specific for one amino acid group (e.g. ArAAs, BcAAs) but have broadly overlapping substrate specificities. They can catalyse reversible transamination reactions and can consequently catalyse in vivo either the first step of amino acid catabolism or the last step of amino acid biosynthesis, their in vivo function usually being identified by enzyme synthesis regulation. Synthesis of catabolic enzymes is, in general, induced by the substrate amino acids while synthesis of biosynthetic enzymes is rather repressed by the substrate amino acids or is constitutive.

Despite their importance in amino acid conversion to aroma compounds, aminotransferases of cheese micro-organisms have been poorly studied. Only ArAA aminotransferases of *B. linens* were partially purified 15 years ago, and recently the aminotransferases active on ArAAs, BcAAs and Met were purified and characterised from *L. lactis*.

Three aminotransferases active on ArAAs were detected in *B. linens* and two of them have been partially purified (Lee & Desmazeaud, 1985a). Both enzymes are also active on aspartate but differ substantially in substrate preference. Based on the kinetic parameters, one was classified as L-ArAA aminotransferase (AraT) (EC 2.6.1.57) and the other as L-aspartate aminotransferase (AspAT) (EC 2.6.1.1). Both have similar optimal pH for activity around pH 8.5–9 and an optimal temperature around 37–40°C but they are still active at 13°C. The AraT synthesis was 5–10 fold induced by the growth of cells on L-Phe as the sole nitrogen source, suggesting a catabolic role of the enzyme while the AspAT seemed to be constitutive. The genes encoding these aminotransferases have not been studied.

From *L. lactis*, an AraT and a BcaT (BcAA aminotransferase) (EC 2.6.1.42) were purified and characterised. The AraT was purified from both *L. lactis* ssp. *cremoris* NCDO763 (Yvon et al., 1997) and *L. lactis* ssp. *lactis* S3 (Gao & Steele, 1998) (Table 3). The AraT of NCDO763 appeared only in homodimeric form while both homodimeric and tetrameric forms of the same enzyme were present in *L. lactis* S3, the homodimer being however much more abundant than the tetramer. The three AraTs are very similar. They have similar subunit size of around 42–43.5 kDa and have identical N-terminal sequences. All three enzymes are active on ArAAs, Leu and Met utilising α-ketoglutarate as the amino group acceptor, but the relative activities towards ArAAs differ slightly, Phe being the best aromatic substrate for the AraT of NCDO763 instead of Trp for the AraT of S3. Based on kinetic parameters, the tetrameric enzyme appears to be more catalytically efficient on the ArAAs than the dimeric form, suggesting that the cell may adjust its aminotransferase activity by regulating the ratio of dimeric and tetrameric forms of the enzyme in response to growth conditions (Gao & Steele, 1998). AraTs have optimal activity at pH 6.5–8 and at temperature 35–45°C for NCDO 763 and 55–65°C for S3. However they are still active under the pH, temperature and salt concentration existing in cheese. Indeed, AraT activity was recovered in lactococcal cells incubated for 3 weeks in cheese model system (Gao et al., 1997) and was also detected in experimental semi-hard cheese (Yvon, Berthelot, & Gripon, 1998).

Recently, a BcaT was also purified from *L. lactis* NCDO763 (Yvon, Chambellon, Sorokine, & Roudot-Algaron, 2000). The BcaT is mainly active with the three BcAAs and methionine utilising α-ketoglutarate as amino group acceptor. Its best substrate is Ile but it has overlapping substrate specificity with AraT especially for Leu and Met. The enzyme is homodimeric with a molecular mass of the subunit of 38 kDa. Like AraT, the optimal pH for activity is around pH 7.5 and the optimal temperature around 35–40°C. Previously two BcaTs were partially purified from *L. lactis* B78 (Engels, 1997). Both enzymes significantly differ from BcaT of

Table 3
Enzymes involved in the amino acid catabolism of cheese micro-organisms

Name	Abrev.	Substrate	Strain	MW[a] (kDa)	Type[b]	pH optim.	Gene seq.[c]	References
Methionine-γ-lyase	MGL	Met	*Brevibacterium linens* NCDO739	175	PLP	8	No	Collin & Law (1989)
		Met	*Brevibacterium linens* BL2	43×4	PLP	7.5	No	Dias & Weimer (1998a)
Cystathionine-β-lyase	CBL	Cystathionine, Cys, Met	*Lactococcus lactis* B78	$(35–40) \times 4$	PLP	8	Yes	Alting et al. (1995) and Fernandez et al. (2000)
Cystathionine-γ-lyase	CGL	Cystathionine, Cys, Met	*Lactococcus lactis* SK11	40×4	PLP	8	No	Bruinenberg et al. (1997)
			Lactobacillus fermentum DT41	35×4	PLP	8	No	Smacchi & Gobetti (1998)
L-aromatic amino acid aminotransferase	AraT	Phe, Trp, Asp, Tyr	*Brevibacterium linens* 47	126	PLP	8.5–9	No	Lee & Desmazeaud (1985a)
		Leu, Tyr, Phe, Trp, Met	*L. lactis* ssp. *cremoris* NCDO763	43.5×2	PLP	6.5–8	Yes	Yvon et al. (1997) and Rijnen et al. (2000)
		Leu, Trp, Tyr, Phe, Met	*L. lactis* ssp. *lactis* S3	42×2	PLP	6.5–7.5	No	Gao & Steele (1998)
		Leu, Trp, Tyr, Phe, Met	*L. lactis* ssp. *lactis* S3	42×4	PLP	6.5–8	No	Gao & Steele (1998)
L-aspartate aminotransferase	AspAT	Asp, Phe, Trp, Tyr	*Brevibacterium linens* 47	81	PLP	8.5–9	No	Lee & Desmazeaud (1985a)
L-branched-chain amino acid aminotransferase	BcaT	Ile, Leu, Val, Met	*L. lactis* ssp.*cremoris* NCDO763	38×2	PLP	7.5	Yes	Yvon et al. (2000)
D-2-hydroxyisocaproate dehydrogenase or D-mandelate dehydrogenase	D-HicDH	Ketoacids from Leu, Phe,	*Lactobacillus casei* DSM20008	38×2	NAD	5.5–7[d]	Yes	Hummel et al. (1985) and Lerch et al. (1989a)
		Leu, Phe, Met	*Lb. delb. bulgaricus* NCBI11778	$34 \times ?$	NAD		Yes	Bernard et al. (1994)
		Leu, Ile, Val, Phe, Tyr, Met	*Lactobacillus curvatus* DSM20019	30×2	NAD	6[d]	No	Hummel et al. (1988)
		Val, Ile, Phe	*Enterococcus fae*calis IFO12964	34×2	NAD	4.5[d]	No	Yamazaki & Maeda (1986)
L-2-hydroxyisocaproate dehydrogenase	L-HicDH	Leu, Val, Ile, Phe, Met	*Lactobacillus confusus* DSM20196	33×4	NAD	7[d]	Yes	Schütte et al. (1988) and Lerch et al. (1989b)

[a] MW as determined by SDS-PAGE $\times$ number of subunits.
[b] Type of enzyme: PLP, pyridoxal-5′-phosphate-dependent enzyme; NAD, NAD-dependent enzyme.
[c] Indicates if gene sequence is available or not available.
[d] pH optimum of activity for α-ketoacid reduction.

L. lactis NCDO763 with regard to molecular mass, substrate specificity and relative activity towards BcAAs and N-terminal sequence. However, since final preparations were not pure, it is not possible to conclude whether different BcaTs exist or not in *L. lactis* B78.

The genes encoding AraT and BcaT in *L. lactis* NCDO763 have been cloned and sequenced (Rijnen, Bonneau, & Yvon, 1999a; Yvon et al., 2000). A single copy of each gene is present in the chromosome and both genes are monocistronically transcribed. Transcription of the araT gene does not seem to be regulated (Rijnen et al., 1999a) while that of BcaT is repressed by the presence of free amino acids, especially by Ile in the growth medium, suggesting an original biosynthetic function of the enzyme (Yvon et al., 2000). However AraT and BcaT play a key role in the catabolism of ArAAs, BcAAs and methionine by *L. lactis*. They are the only two enzymes catalysing transamination of these amino acids and they are complementary in this role, AraT being essentially responsible for ArAAs degradation, BcaT essentially responsible for Ile and Val degradation and both enzymes participating in Leu and Met degradation. This was demonstrated by using derivative strains of *L. lactis* NCDO 763 in which *araT* and *bcaT* genes were inactivated separately (Rijnen et al., 1999a; Yvon et al., 2000) or together (unpublished results) (Fig. 2). Inactivation of *araT* led to a 90–95% decrease in aminotransferase activity on ArAAs and to 50% and 25% decreases in activity on Met and Leu respectively and did not affect aminotransferase activity with Ile and Val. In contrast, inactivation of *bcaT* gene reduced the activity on Ile and Val by more than 90% and the activity on Leu and Met by 50% and 40% respectively while it only scarcely altered activity with ArAAs. Recently, inactivation of both genes together allowed us to conclude that no other aminotransferase is active on these seven amino acids, since no residual aminotransferase activity was found in the double mutant, even after incubation of double mutant cells for 40 h under optimal conditions (Yvon, unpublished results).

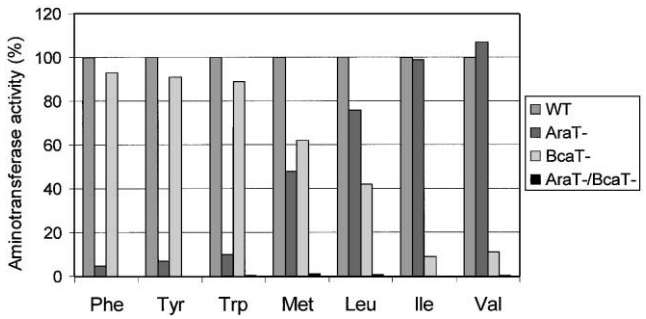

Fig. 2. Effect of inactivation of the aminotransferase genes *araT* and *bcaT* of *L. lactis* NCDO763 on aminotransferase activities of the strain. Activities are expressed as percents of the activities of the wild-type strain (WT) and were determined by using phenlyalanine (Phe), tyrosine (Tyr), tryptophan (Trp), methionine (Met), leucine (Leu), isoleucine (Ile) and valine (Val) as substrate and α-ketoglutarate as cosubstrate. WT: wild-type strain; AraT-: the derivative AraT mutant; BcaT-: the derivative BcaT mutant; AraT-/BcaT-: the double AraT/BcaT mutant.

Considering the key role of transamination reactions in amino acid catabolism by most cheese micro-organisms, controlling this step appears to be an ideal means of controlling aroma formation from amino acids. Indeed, on the one hand, a general intensification of amino acid transamination may globally enhance aroma formation from all amino acids. On the other hand, controlling activities of each aminotransferase may favour or prevent the degradation of an amino acid group, i.e. ArAAs or BcAAs, and consequently may favour or prevent the formation of an aroma type, i.e. floral or animal.

4.2.2. α-Keto acid degradation

α-Keto acids resulting from ArAA-, BcAA- and methionine transamination are further degraded to various compounds either by enzymatic reactions or by chemical reactions. Four major α-keto acid degradation reactions have been revealed in cheese micro-organisms. They lead to hydroxy acids, carboxylic acids, aldehydes with 1 carbon missing, or aldehydes with 2 carbons missing. Moreover, α-ketomethylthiobutyrate (KMBA), the methionine α-ketoacid, can be degraded to methanethiol.

All these compounds except hydroxyacids are major aroma compounds. However, these reactions and the enzymes involved have been only partially elucidated.

4.2.3. α-Keto acid reduction to hydroxy acids

Although hydroxyacids are not major aroma compounds and are not known as precursors of aroma compounds, it is interesting to know the enzymes involved in their formation since α-keto acid reduction to hydroxyacids may cause a leakage for the α-keto acid conversion to aroma compounds.

The reduction of α-keto acids derived from BcAAs, ArAAs and methionine to hydroxyacids has been observed in many LAB but to our knowledge, it has never been reported in other cheese micro-organisms. This reduction has been observed in lactococci (Gao et al., 1998; Roudot-Algaron & Yvon, 1998) and in many lactobacilli (Gummalla & Broadbent, 1999; Hummel, Schütte, & Kula, 1985; Yvon, unpublished results) and occurs in semi-hard cheese made with lactococci (Yvon et al., 1998). The α-keto acid reduction by resting cells of *L. lactis* was mainly observed at pH 7–8 when glucose or fructose diphosphate was present in the reaction medium (Roudot-Algaron & Yvon, 1998), probably because glycolysis provides NADH required for the reaction.

Indeed, this α-ketoacid reduction is catalysed by NAD(H)-dependent 2-hydroxyacid dehydrogenases

(HA-DHs). The HA-DHs active on α-keto acids derived from amino acids have a broad substrate specificity contrary to lactate dehydrogenase (LDH), which strongly prefers pyruvate. They catalyse the stereospecific and reversible reduction of various aliphatic α-keto acids, especially branched-chain aliphatic α-keto acids, and aromatic α-keto acids to the corresponding hydroxy acids. They have been named hydroxyisocaproate dehydrogenase (HicDH) since ketoisocaproate (KIC) is often their best substrate. However a similar enzyme has been named mandelate dehydrogenase although KIC was also its best substrate. These enzymes have been divided into two groups according to the D- or L-stereoisomer produced. D-HicDH and/or D-mandelateDH appear to be widely distributed in LAB. D-HicDH activity has been found in *Lb. casei* (Hummel et al., 1985), *Lb. curvatus* (Hummel, Schütte, & Kula, 1988), *Lb. delbrueckii bulgaricus* (Bernard et al., 1994), *Lb. confusus*, *Enterococcus faecalis* (Yamazaki & Maeda, 1986), *Pediococcus acidilactici* (Delcour, Bernard, Garmyn, Ferain, & Hols, 1993) and in leuconostoc (Hummel et al., 1985), and D-mandelateDH activity has also been detected in various lactobacilli (Hummel et al., 1988). In contrast, L-HicDH is much more unusual. It was only detected in *Lb. confusus* out of 45 tested strains of lactobacillus and leuconostoc (Schütte, Hummel, & Kula, 1984).

Several of these enzymes have been isolated and characterised. D-HicDH and D-mandelateDH have been purified from *Lb. casei* (Hummel et al., 1985), *Lb. bulgaricus* (Bernard et al., 1994), *E. faecalis* (Yamazaki & Maeda, 1986) and *Lb. curvatus* (Hummel et al., 1988), and L-HicDH has been isolated from *Lb. confusus* (Schütte et al., 1984).

D-HicDH and/or D-mandelateDH are homodimers with a subunit size of around 30,000–38,000 Da. They are active with straight-chain aliphatic α-keto acids, with the three α-keto acids derived from BcAAs, with phenylpyruvate (PPA) derived from Phe and with KMBA derived from Met, but their preferred substrate is KIC derived from Leu. Moreover, D-HicDH of *E. faecalis* and D-mandelateDH of *Lb. curvatus* are also active with benzoyl formate, producing mandelate, but this substrate has not been tested with the other D-HicDHs. The optimal pH is around 5.5–7 for the reduction reaction and around 9 for the reverse reaction, and the optimal temperature for activity is around 50°C.

L-HicDH from *Lb. confusus* is very similar to D-HicDHs with regard to α-keto acid substrates, optimal pH and temperature for activity, but the hydroxy acids produced are L-stereoisomers and the enzyme is constituted of 4 subunits of 33,000 Da.

In addition to HicDHs, LDHs, that are generally specific for pyruvate, might also be active towards some α-keto acids derived from amino acids. For example, D-LDH isolated from *Lb. confusus* catalyses the reduction of phenylpyruvate.

The gene encoding D-HicDH has been cloned from *Lb. casei* (Lerch et al., 1989a) and from *Lb. bulgaricus* (Bernard et al., 1994) and the L-HicDH gene has been cloned from *Lb. confusus* (Lerch, Frank, & Collins, 1989b). Comparison of deduced amino acid sequences of HicDHs and LDHs indicates that L-Hic and L-LDH as well as D-Hic and D-LDH are structurally related, while L-Hic and D-Hic as well as L-LDH and D-LDH are unrelated.

Finally, recently, a gene homologous to L-HicDH of *Lb. confusus* and several other genes homologous to L-LDH have been found in the *L. lactis* genome (Bolotin, Mauger, Malarme, Ehrlich, & Sorokin, 1999). One of them may encode the enzyme responsible for α-keto acid reduction observed in *L. lactis*.

Since hydroxy acids are the major amino acid degradation products found in semihard cheese made with lactococci (Yvon et al., 1998), the inactivation of the gene encoding HA-DH could allow a better use of α-keto acids for producing aroma compounds.

4.2.4. α-Keto acid decarboxylation to aldehydes

Decarboxylation of α-keto acids derived from BcAAs, ArAAs and methionine to corresponding aldehydes has mainly been reported in yeasts. This transformation is one step of the Ehrlich–Neubauer pathway that leads to "fusel" alcohol production from amino acids. This activity appears to be rather unusual in other cheese micro-organisms. However, it has been detected in some LAB such as C*arnobacterium piscicola* (Larrouture et al., 1999; Miller et al., 1974), in some strains of *Lb. ca*sei (Hickey et al., 1983) and more frequently in *L. lactis* var. *maltigenes* (Morgan, 1976; Tucker & Morgan, 1967) or in wild-type strains of *L. lactis* not classified as *maltigenes* although they produce malty aroma (Ayad et al., 1999; Weerkamp et al., 1996). It should be pointed out that resting cells of *L. lactis* var. *maltigenes* are capable of converting Phe and Met as well as BcAAs to corresponding aldehydes (MacLeod & Morgan, 1958). Most other lactococci produce only small amounts of aldehyde (MacLeod & Morgan, 1958; Rijnen, Courtin, Gripon, & Yvon, 2000) suggesting that this activity may be weakly expressed in *L. lactis* outside of the *maltigenes* biovariant cluster or that a much less efficient pathway may exist in lactococci. However, Gao et al. observed a significant decarboxylation of indole-3-pyruvate (IPA) (from Trp) to indole-3-aldehyde by two *L. lactis* strains (Gao et al., 1997).

Despite the interest of many research groups in the α-keto acid decarboxylating activity in LAB the enzyme involved in the conversion has never been isolated from any LAB. In the yeast *Saccharomyces cerevisiae*, the decarboxylation of α-keto acids derived from Val is mainly catalysed by pyruvate decarboxylase (PDC)

which can act on α-keto acids other than pyruvate, while a PDC-like enzyme, different from PDC is mainly responsible for the decarboxylation of the Leu-derived α-keto acid. Another α-keto acid decarboxylase (IPA-DC) highly specific to indole-3-pyruvate, the Trp-derived α-keto acid, was purified from *Enterobacter cloacae* (Koga, Adachi, & Hidaka, 1992).

The physical characteristics of the IPA-DC from *E. cloacae* are very similar to those of PDC from yeast. They are holoenzymes composed of four monomers that require TPP and Mg^{2+} as cofactors (Koga et al., 1992) and TPP was found to stabilise the enzyme. However, the substrate specificity and affinity of both enzymes are different. PDC of *Streptomyces cerevisiae* has low affinity and specificity for pyruvate, while IPA-DC has high specificity and affinity for IPA. Moreover IPA-DC is also able to catalyse decarboxylation of pyruvate although it has a much lower affinity for pyruvate than for IPA and is not active with other α-keto acids.

It is interesting to note that a gene that shares 41% homology with the gene encoding IPA-DC of *E. cloacae* exists in the *L. lactis* IL1403 genome (Bolotin et al., 1999). The corresponding enzyme may be responsible for the IPA decarboxylating activity found by Gao et al. in *L. lactis* (Gao et al., 1997).

In yeast, as well as in LAB, aldehydes resulting from α-keto acid decarboxylation can be further reduced to alcohol by alcohol dehydrogenases (Morgan et al., 1966; Sheldon, Lindsay, Libbey, & Morgan, 1971), or might be oxidised to carboxylic acids by aldehyde dehydrogenases (Sugawara & Sasaki, 1977). Furthermore, methional, the aldehyde derived from methionine can be degraded to methanethiol by either decarboxylation reaction or spontaneous degradation (Ballance, 1961)

This pathway and the enzymes involved really need to be extensively studied since they produce major aroma compounds such as methional or 3-methylbutanal and corresponding alcohols which are often desired in cheese, but which can also generate off-flavours such as the malty or floral off-flavours when their concentration is too high. A better knowledge of the enzymes may make it possible to control their activity to obtain the desired compounds at the correct concentration.

4.2.5. Oxidative decarboxylation of α-keto acids to carboxylic acids and further reactions

The oxidative decarboxylation of α-keto acids derived from amino acids leads to the formation of carboxylic acids without transitory formation of aldehydes. The reaction is catalysed by α-keto acid dehydrogenases and generates acyl-CoAs, which are further hydrolysed, releasing carboxylic acids.

The production of carboxylic acids from amino acids by cheese micro-organisms does not appear to be common. However, yeasts, some LAB such as lactococci (Gao et al., 1997; Roudot-Algaron & Yvon, 1998; Yvon et al., 1997), propionibacteria (Thierry, personal communication) and some surface bacteria such as microbacterium (Jollivet et al., 1992) produce, under certain conditions, carboxylic acids from BcAAs, ArAAs or methionine, but little is known about the formation pathway. α-keto acid oxidative decarboxylation has been demonstrated in yeast, especially in *S. cerevisiae* (Dickinson & Dawes, 1992; Sinclair, Dawes, & Dickinson, 1993) and in the non-dairy LAB: *Enterococcus faecalis* (Ward, Claiborne, Kok, & Westphal, 1997). These micro-organisms possess, in addition to pyruvate dehydrogenase (PDH), a branched-chain α-keto acid dehydrogenase (BKDH) (EC 1.2.4.4), but in some bacteria such as *Bacillus subtilis* the PDH also catalyses the decarboxylation of α-keto acids derived from Leu, Ile and Val (Lowe, Hodgson, & Perham, 1983).

Recently, we have shown that *L. lactis* also degraded α-keto acids derived from amino acids *via* an oxidative decarboxylation. Indeed, the conversion occurs mainly at pH 5.5 and is inhibited by trivalent arsenicals, which are known inhibitors of α-keto acid dehydrogenase complexes (Webb, 1966). Moreover, cells grown in medium lacking lipoïc acid, an essential cofactor of α-ketoacid dehydrogenase complexes, are unable to produce carboxylic acids (Yvon, unpublished results). Although the lactococcal PDH has been isolated (Snoep, Mattos, Starrenburg, & Hugenholz, 1992) we do not know whether it is active on α-keto acids other than pyruvate or if another α-keto acid dehydrogenase exists in *L. lactis*.

PDH and BKDH are multienzyme complexes. They consist of 3 catalytic components: α-keto acid dehydrogenase (E1), dihydrolipoyl transacylase (E2) and lipoamide dehydrogenase (E3), E1 being generally composed of 2 subunits: E1α and E1β. E1 catalyses both the decarboxylation of α-keto acid and the oxidative transfer of an acyl group to the lipoyl moiety of E2, using thiamine pyrophosphate (TPP) and Mg^{2+} as cofactors, with the formation of a hydroxyacyl-TPP intermediate. E2 catalyses the acyl group transfer to CoA, generating an Acyl-CoA. Finally, the resulting dihydrolipoamide is oxidised back to the disulphide form by the E3 component at the expense of NAD and utilising FAD as cofactor (Reed, 1974).

α-Keto acid dehydrogenases are generally active at pH 5.5–6.5. They are more or less inhibited by NADH but PDH of *L. lactis* is highly sensitive to NADH (Snoep et al., 1993, 1992). Moreover, the E3 component is only weakly synthesised in *L. lactis*, which explains why no PDH activity is observed in vivo anaerobically in *L. lactis*. In some bacteria, PDH and BKDH and perhaps α-ketoglutarate dehydrogenase may share the same E3 component (Lowe et al., 1983). This explains why genes encoding E1 components are sometimes not clustered with those encoding E2 and E3 components

(Inoue et al., 1997) while generally all the genes encoding BCDH or PDH complex are in the same operon. The sequence of the entire *L. lactis* IL1403 genome reveals only one cluster with 4 genes encoding proteins homologous to E1α, E1β, E2 and E3 components of PDH or α-ketoglutarate dehydrogenase (Bolotin et al., 1999), suggesting that no other α-keto acid dehydrogenase exists in *L. lactis*.

The acyl-CoAs resulting from oxidative decarboxylation of α-ketoacids can be further hydrolysed to carboxylic acids either by an acid: acyl-CoA hydrolase such as in yeast (Dickinson, Harrison, & Hewlins, 1998), or by 2 successive steps catalysed by phosphate butyrate-CoA transferase and butyrate kinase such as in *E. faecalis* (Ward et al., 1997) or in *B. subtilis* (Debarbouille, Gardan, & Arnaud, 1999).

This oxidative decarboxylation pathway is of major interest since carboxylic acids and especially branched-chain fatty acids are major cheese aroma compounds. Moreover, carboxylic acids and acyl-CoAs are precursors of many aroma compounds found in cheese such as esters, thioesters, aldehydes, cresol and skatole. Firstly, acyl-CoAs and carboxylic acids could be reduced to aldehydes by an acyl-CoA reductase and by a fatty acid reductase respectively. Such enzymes have been isolated from green alga (Wang & Kolattukudy, 1995) or from bacteria (Engebrecht, Nealson, & Silverman, 1983; Riendeau, Rodriguez, & Meighem, 1982).

Indolacetic and hydroxyphenylacetic acids derived from Trp and Tyr respectively can be non-enzymatically (Urbach, 1995) or enzymatically (Barker, 1981; Elsden, Hilton, & Waller, 1976; Honeyfield & Carlson, 1990; Yokoyama & Carlson, 1981) degraded to skatole and *p*-cresol, which are major contributors to off-flavour in Cheddar cheese, and methylthiopropionic acid derived from Met might also be subsequently degraded to methanethiol as is the case in a variety of mammals (Scislowski, Bermer, Thienen, & Davis, 1989).

Moreover, various cheese micro-organisms are capable of generating esters from carboxylic acids (or acyl-CoAs) and alcohols. This esterification reaction has been observed in yeast, in brevibacteria (Molimard & Spinnler, 1996) and in various LAB including lactococci, lactobacilli, *Streptococcus thermophilus*, leuconostocs and pediococci (Liu, Holland, & Crow, 1998), but this ability is highly strain dependent. The enzymes involved could be carboxyl-esterases, which have broad substrate specificity, aryl esterases or alcohol acyltransferase which have been identified in yeast (Molimard & Spinnler, 1996). Esterase, that is widely distributed in LAB (Chich, Marchesseau, & Gripon, 1997; Hosono, 1974) might also participate in the ester synthesis although its primary function is in ester hydrolysis. The reaction may also be spontaneous but may require the activation of carboxylic acids to acyl-CoAs.

S-methyl thioesters can also be generated from acyl-CoA including acyl-CoA derived from amino acids, and methanethiol. This reaction is essentially spontaneous (Bonnarme, Helinck, & Spinnler, 2000). It is optimal at pH 7 and at 56°C but it still occurs under the pH and temperature of cheese ripening. Moreover some cheese micro-organisms such as *Geotrichum candidum* and maybe *B. Linens* (Lamberet et al., 1997b) possess enzymes capable of catalysing the synthesis of methylthioacetate from acetyl-CoA and methanethiol. However the enzyme is not active at pH below 6 and at the temperature of cheese ripening. Therefore, in cheese, thioesters synthesis is probably essentially spontaneous (Bonnarme et al., 2000). In fact, the ability of the strains to produce thioesters as well as esters may be essentially related to their ability to generate acyl-CoA.

4.2.6. Chemical degradation of α-keto acids

Various chemical degradations of α-keto acids have been reported in cheese. The two main reactions are the conversion of aromatic α-keto acids to aldehydes with two carbons missing and the conversion of KMBA to methanethiol. Moreover the α-keto acid derived from tryptophan is very unstable and generates various compounds, which have often been associated with off-flavours in cheddar cheese.

The chemical oxidation of phenylpyruvate (PPA) and hydroxyphenylpyruvate (HPPA) to benzaldehyde and hydroxybenzaldehyde had been reported by Pitt and Doy respectively (Doy, 1960; Pitt, 1960) as early as 1960. Pitt observed that it was not the keto acid itself but the enol tautomer that was oxidised. The reaction occurs rapidly at alkaline pH (Doy, 1960) and is favoured by heating (Casey & Dobb, 1992). At neutral pH, the reaction occurs at a negligeable rate but is highly accelerated by the presence of bivalent cations such as Cu^{++} or Mn^{++} (Pitt, 1960). Recently, this conversion has been observed by incubation of resting cells of *Lb. plantarum* with PPA, and the ability of various lactic acid bacteria to produce benzaldehyde from phenylalanine has been related to their capacity to accumulate Mn^{++} inside the cells (Nierop-Groot & Bont, 1998, 1999). The spontaneous degradation of hydroxyphenylpyruvate to hydroxybenzaldehyde also occurs under simulated Cheddar cheese conditions (Gao et al., 1998) and both benzaldehyde and hydroxybenzaldehyde were found in significant amounts in semihard cheeses (Yvon et al., 1998). These compounds are major flavour compounds. Benzaldehyde has the typical aroma of the bitter almond and the taste of hydroxybenzaldehyde is sweet (Arctander, 1994).

Indole-3-pyruvate (IPA), is much more unstable than the other aromatic α-keto acids. It is spontaneously degraded to indole acetic acid, indol-3-aldehyde and skatole, which have been identified as being responsible

for off-flavours in Cheddar cheese (Gao et al., 1997). Total degradation of IPA has been observed after storage for 2 h at 37°C or 30 min at 0°C or overnight at −20°C. In similar conditions PPA and HPPA are not degraded (unpublished results).

Non-enzymatic degradation of KMBA to methanethiol has also been reported (Gao et al., 1998; Manning, 1979; Soda, 1987). However, according to Gao et al. (1998) significantly greater quantities of methanethiol were produced when whole cells of *L. lactis* were incubated with KMBA under cheese-like conditions, indicating that an enzyme may also be involved in this reaction. However, the activity was not retrieved in the cell extract suggesting that the enzyme is inactivated or removed during cell preparation or that the conversion is not enzymatic but catalysed by some components present in cells. According to these authors, this pathway will be the major pathway for the formation of methanethiol by lactococci.

While numerous enzymatic reactions are involved in the conversion of amino acids to aroma compounds by cheese micro-organisms, only a few of them have been well characterised. However, transamination and elimination reactions have been identified as key reactions since they initiate the two pathways leading to the formation of aroma compounds from amino acids. The enzymes involved in both steps are now well known. Therefore, controlling these reactions appears to be an ideal way to control aroma formation in cheese.

5. Intensification and diversification of cheese aroma formation by controlling amino acid catabolism

New knowledge on amino acid catabolism by cheese micro-organisms has offered new insights into controlling aroma formation at the level of amino acid catabolism. Especially, control at the level of the transamination and elimination reactions has been evaluated to intensify or diversify aroma formation in semihard cheese.

5.1. Controlling aroma formation at the level of methionine elimination reaction

To intensify sulphur aroma formation in Cheddar cheese, and especially in low-fat cheeses, several groups have tried to specifically intensify methionine degradation to methanethiol. First, Law (1987) added cell-free extracts of brevibacteria, which produces large amounts of methanethiol, to Cheddar cheese. This addition led to an accelerated formation of the flavour typical of aged cheddar. However, taking into account that the methanethiol-producing capacity occurred under cheese-like conditions in whole cells but not in free extracts, Weimer (Weimer et al., 1997) successfully used *B. linens* as an adjunct to improve the flavour of low-fat Cheddar cheese, but he did not demonstrate that this improvement was due to higher level of volatile sulphur compounds. This was demonstrated by Dias and Weimer (1999) who recently showed that the addition of *B. linens* or pure methionine γ-lyase in slurry containing *L. lactis,* and added methionine, effectively increase the formation of these sulphur compounds.

5.2. Controlling aroma formation at the level of transamination reaction

Based on the conclusion that the transamination reaction is a key step in the conversion of amino acids to aroma compounds, we imagined that aroma formation could be controlled at this level. Two possibilities of controlling aroma formation at this level were demonstrated. The first one consists in a global enhancement of aroma formation by a global intensification of amino acid transamination, and the second consists in preventing or favouring the formation of an aroma type, i.e. floral or animal, by preventing or favouring the transamination of an amino acid group, i.e. ArAAs or BcAAs.

5.2.1. Global enhancement of cheese aroma

Based on the fact that only very low amino acid degradation was observed in semihard cheese made with lactococci, although lactococci have high aminotransferase activities, we suspected that the α-keto acid required for transamination could be the limiting factor for the amino acid degradation. We therefore proposed to add exogenous α-ketoglutarate to the cheese curd or to use a strain capable of producing α-ketoglutarate to intensify amino acid degradation during ripening.

Adding α-ketoglutarate to St Paulin cheese made with various starters highly increased the conversion of ArAAs, BcAAs and methionine to potent aroma compounds which enhanced the intensity of cheese aroma (Rijnen et al., 1999b; Yvon et al., 1998). These results were recently confirmed by Banks et al. (2001) who added α-ketoglutarate to cheddar cheese. Additionally, they identified enhanced production of 3-hydroxy-2-butanone or acetoin, a precursor of diacetyl. Moreover this study has specified the effect on aroma character of cheese. Addition of 6 g of α-ketoglutarate per kg of cheese increased aroma intensity, creamy and fruity aromas. Results clearly indicate that aroma intensity in six (or 12) weeks cheddar containing α-ketoglutarate was equivalent to the aroma intensity in a 6-month cheddar.

As an alternative to adding exogenous α-ketoglutarate to the cheese, we proposed to use, as starter, a strain capable of producing α-ketoglutarate from the glutamate that is always found in large quantities in cheese. To try this, we expressed a gene encoding a catabolic

glutamate dehydrogenase in *L. lactis* (Rijnen et al., 2000). This enzyme catalyses the deamination of glutamate to α-ketoglutarate. The GDH-producing lactococcal strain degraded amino acids without added α-ketoglutarate in a cheese model to the same extent that the wild-type strain degraded amino acids with added α-ketoglutarate (Fig. 3). Interestingly, the GDH-producing strain produced a higher proportion of carboxylic acids, which are major aroma compounds. These results suggest a high enhancement of cheese aroma, which was not evaluated in the study.

5.2.2. Diversification of cheese aroma

Taking into account the difference in the substrate specificity of the two lactococcal aminotransferases which initiate the conversion of amino acids to aroma compounds, we imagined that the cheese aroma could be diversified or directed by controlling the activities of these enzymes in the starter used for cheese making. As a demonstration, we used a *L. lactis* strain in which the gene encoding AraT was inactivated to prevent the degradation of ArAAs, which are precursors of floral aroma compounds. As expected, the gene inactivation greatly prevented formation of floral aroma compounds in St Paulin cheese while it did not affect the formation of volatile aroma compounds from BcAAs and methionine (Rijnen et al., 1999b). However, the sensorial analysis performed by sniffing did not reveal any significant effect probably because floral aroma was masked by the very potent odorant resulting from BcAA degradation. However, we can assume that in cheese in which an undesirable rose-like flavour sometimes appears, the use of a starter with low or no AraT activity may prevent the off-flavour formation from aromatic amino acids.

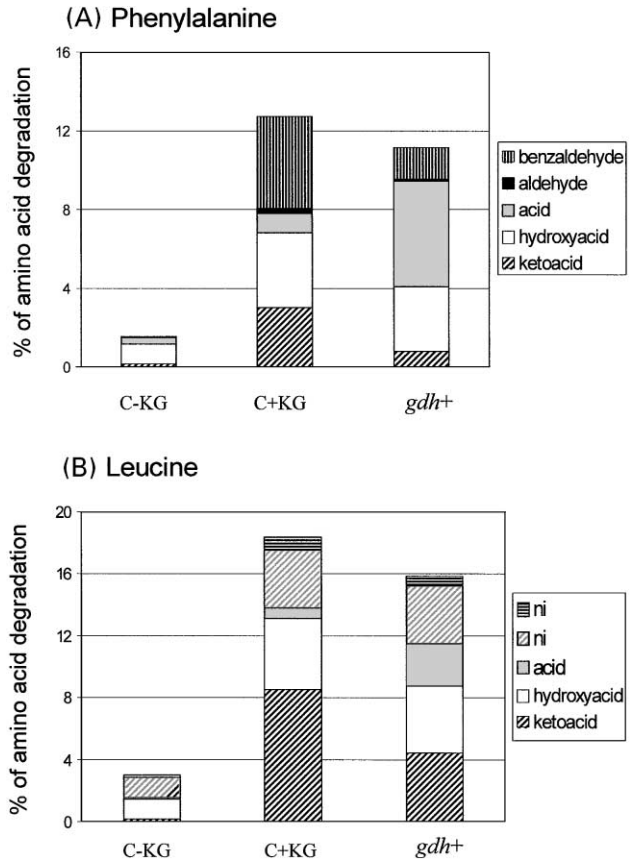

Fig. 3. Amino acid degradation in the cheese model by the control strain without added α-ketoglutarate in the cheese paste (C-KG) or with added α-ketoglutarate (C + KG) and by the *gdh* + strain. (A) Metabolites produced from phenylalanine. (B) Metabolites produced from leucine. ni, non-identified compounds. The data are means of results from two trials. The percentages of carboxylic acids, keto acids, and benzaldehyde produced by the *gdh* + strain were significantly different from the percentages produced by the control strain in the presence of α-ketoglutarate. Reproduced from Rijnen et al. (2000).

6. Conclusion

Controlling amino acid catabolism by cheese microorganisms appears to be a promising way to control aroma formation in cheese. Indeed, amino acid catabolism leads to different compounds with various aromas, which are major aroma compounds in most cheeses. Moreover, we have seen that intensifying amino acid catabolism results in a clear enhancement of cheese aroma.

While numerous enzymatic and non-enzymatic reactions are involved in the conversion of amino acids to aroma compounds, only a few of them have been well characterised. However, elimination and transamination reactions have been identified as key reactions since they initiate the two pathways of amino acid conversion to aroma compounds, and the enzymes involved are now well known.

The new basic knowledge on amino acid catabolism has led to new approaches for controlling aroma formation in cheese, and recent results show that it is possible to intensify or diversify aroma formation in cheese by controlling the first step of amino acid catabolism, i.e. elimination and transamination reactions. However, many reactions remain to be elucidated, especially at the level of α-keto acid degradation. The knowledge of enzymes playing a key role in the conversion of α-keto acid to aroma compounds will probably offer more possibilities in controlling aroma formation in cheese. For example, we could envisage directing α-keto acid degradation towards the formation of interesting aroma compounds, in order to create new products (cheese varieties) with new aromas or to produce a specific aroma for aromatic bases. This control could be carried out either by metabolic engineering or by selecting strains with interesting catabolic activities since these catabolic activities seem to be highly strain dependent.

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