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Review

Production of cheese flavour compounds derived from amino acid catabolism by *Propionibacterium freudenreichii*

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Abstract — The catabolism of amino acids by cheese micro-organisms results in the production of various volatile flavour compounds. It was recently shown to be a rate-limiting factor in the formation of cheese flavour, leading to an increased interest in elucidating the pathways and the flora involved. This paper reviews the ability of propionibacteria (PAB) to produce flavour compounds deriving from branched-chain, aromatic and sulphur-containing amino acids. In culture media, PAB produced volatile compounds derived from Leu, Ile, Met and Phe. In cheese, the presence of PAB is positively correlated to the amount of acids, alcohols and/or aldehydes derived from Leu or Ile. The metabolic pathways of amino acid conversion to flavour compounds by PAB have been only partly elucidated. Aminotransferase(s) catalyse the first step of conversion of branched-chain, aromatic amino acids and methionine, with a higher activity for branched-chain amino acids. The α-keto acids resulting from transamination are further degraded to various compounds by resting cells of PAB. So α-ketoisocaproic acid, derived from Leu, is essentially converted to isovaleric acid by a ketoacid dehydrogenase complex; phenylpyruvic acid, derived from Phe, is converted to phenyllactic acid, phenylacetic acid, benzoic acid and benzaldehyde. Methionine can also be directly degraded by α, γ -elimination, leading to methanethiol. The amino acid catabolism pathways in PAB share similarities with those of lactic acid bacteria but PAB seem to produce higher amounts of branched-chain acids, which are important flavour compounds in cheese.

propionibacteria / flavour compound / amino acid / catabolism / cheese ripening

Résumé — **Production de composés d'arôme du fromage issus du catabolisme des acides aminés par *Propionibacterium freudenreichii*.** Le catabolisme des acides aminés par les micro-organismes du fromage entraîne la formation de composés volatils variés. C'est une étape limitante de la formation de la flaveur du fromage, et les recherches visant à déterminer les voies métaboliques et les flores impliquées se sont récemment multipliées. Cette revue fait le point sur le catabolisme des

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acides aminés en composés d'arôme chez les bactéries propioniques (PAB). Les PAB sont impliquées dans la formation des composés volatils dérivant de Leu, Ile, Phe et Met, par des voies métaboliques qui ne sont que partiellement connues. La première étape de conversion des acides aminés ramifiés, aromatiques et de la méthionine est catalysée par une(des) aminotransférase(s). Les cétoacides résultant de la transamination sont ensuite dégradés en différents composés. Ainsi, l'acide α-cétoisocaproïque, issu de Leu, est pour l'essentiel converti en acide isovalérique par un complexe cétoacide déshydrogénase ; l'acide phénylpyruvique, issu de Phe, est converti en acide phényllactique, acide phénylacétique, acide benzoïque et benzaldéhyde. La méthionine peut également être directement dégradée par α, γ-élimination, formant du méthanethiol. Ces voies cataboliques présentent des similarités avec celles existant chez les bactéries lactiques, mais les PAB produisent plus d'acides ramifiés, qui sont des composés importants dans la flaveur du fromage.

propionibactéries / composé d'arôme / acide aminé / catabolisme / affinage

1. INTRODUCTION

Numerous volatile compounds are formed during cheese ripening, which is a complex phenomenon in which glycolysis, proteolysis and lipolysis are involved [38]. Only a few of them would be actually involved in cheese flavour, according to recent studies combining gas chromatography, olfactometry and sensory analysis [21]. Some flavour-active compounds in Swiss-type cheeses are given as an example, with their possible origin, in Table I. For many of them, we have only a fragmentary knowledge of the flora and the pathways involved in their formation. Most cheese flavour compounds are common to very different cheese varieties, suggesting that different flora are able to produce them [10, 20, 37]. They can be involved either in positive cheese aroma or in flavour defects, depending on their amount in cheese and on the type of cheese [39].

Many flavour compounds have structures which indicate that they could originate from branched-chain, aromatic and sulphur-containing amino acids. Proteolysis has been known for a long time to be an essential event in cheese ripening. Recent results, however, showed that the content of free amino acids in cheese could be increased, either by adding amino acids or by enhancing peptidolysis, without any concomitant increase in flavour, suggesting that the rate-limiting factor in the formation of cheese flavour is the degradation of amino acids rather than their release [67]. Several groups have therefore recently directed their research into the conversion of amino acids to flavour compounds by some cheese-related bacteria. Most of the efforts have focused on *Lactococcus lactis*, *Brevibacterium linens* and, to a lesser extent, some other lactic species. These results have been recently reviewed [67].

Our team has recently focused on the conversion of amino acids to volatile compounds by propionibacteria (PAB). We have demonstrated the ability of PAB to convert branched-chain, aromatic and sulphur-containing amino acids to volatile compounds. This paper reviews our current knowledge of this subject and includes some new data that we recently obtained.

2. ROLE OF PROPIONIBACTERIA IN THE FORMATION OF CHEESE FLAVOUR

PAB are used as secondary starters mainly in Swiss cheese manufacture and in other "cheeses with eyes" like Maasdamer (The Netherlands) or Jarlsberg (Norway). The main species isolated from Emmental cheese and added as starter is *Propionibacterium freudenreichii*. Three other dairy species, *P. jensenii*, *P. thoenii*, and *P. acidipropionici* can also be isolated

from milk or cheese [43]. Indigenous PAB, present in raw milk at levels ranging from 5 to more than 10^5 colony-forming units (cfu) mL^{-1}, can also grow during cheese ripening. They can reach counts as high as 10^8 cfu·g^{-1} of Swiss-type cheeses [7]. In addition, PAB have also been proposed by starter manufacturers as adjunct starter to enhance or diversify the flavour of some semi-hard cheeses.

PAB are involved in the formation of cheese eyes and are important for proper development of Swiss cheese characteristic flavour, described as "sweet" and "nutty" [36, 43, 46]. Indigenous PAB can also influence the formation of cheese flavour. Their presence, in association with those of other milk microflora, was positively correlated to aroma notes described as "pungent" in experimental mini Swiss-type cheeses made from raw milk [7]. They were also shown to be responsible for an atypical flavour in raw milk Raclette cheese [33].

Although it is beyond doubt that PAB can influence cheese flavour, most of the knowledge on PAB metabolism concerns the fermentation of lactic acid to acetic acid, propionic acid and CO_2 (for reviews, see [6, 26, 47]). Their ability to produce other volatile flavour compounds is comparatively poorly documented. We will successively present the data showing the ability of PAB to produce volatile compounds derived from branched-chain amino acids, aromatic amino acids and methionine, and then our knowledge of the metabolic pathways involved in these conversions.

3. ABILITY OF PROPIONIBACTERIA TO PRODUCE VOLATILE COMPOUNDS DERIVED FROM AMINO ACIDS

Branched-chain, aromatic and sulphur-containing volatile compounds are formed during cheese ripening. Taking into account their analogy of structure with amino acids and the knowledge of amino acid catabolism pathways in various micro-organisms [67], we can consider these volatile compounds as deriving from the corresponding amino acids. The main compounds formed in cheese are given in Table I with their possible origin and the associated aroma descriptors. This section will summarise the results showing the ability of PAB to produce such compounds in culture medium or in cheese.

3.1. Production of volatile compounds in culture medium

Several volatile branched-chain acids are produced in minor amounts by PAB besides the main fermentation products (acetic and propionic acids): isobutyric acid (= 2-methylpropanoic acid), isovaleric acid (= 3-methylbutanoic acid, but which represents in many studies the sum of 2-methylbutanoic acid and 3-methylbutanoic acid), and isocaproic acid (4-methylpentanoic acid) [13]. These acids are described as sweaty, fruity, sweet, cheesy and fatty acid-like (Tab. I). The production of branched-chain acids seems to be a common feature in PAB and was observed in different culture media. Most strains of the four PAB dairy species (32 of 37) produced isobutyric acid and/or isovaleric acid in yeast-extract-lactate (YEL) medium, at amounts ranging from 0.3% to 3.3% of total volatile fatty acids, i.e. from ~15 to 110 mg·L^{-1}, depending on the strains [13]. High amounts of isocaproic acid were also produced by half of the tested strains of *P. thoenii*, *P. jensenii* and *P. acidipropionici*. The kinetics of isovaleric acid production was determined in YEL medium for two *P. freudenreichii* strains: it was produced throughout growth, concomitantly with lactate fermentation [59]. After lactate exhaustion, growth stopped while isovaleric acid further increased. Under modified YEL culture conditions carried out to mimic Swiss cheese

Table I. Origin and characteristics of volatile compounds of Swiss-type cheeses derived from amino acid catabolism.

Volatile compounds[1]	Origin	Aroma descriptors[2]	Flora potentially involved[3]	References
Acids				
Isobutyric acid (2-methylpropanoic acid)	Val	sweet, apple-like, sweaty	PAB	[13, 42]
Isovaleric acid * (3-methylbutanoic acid)	Leu	rancid, cheese, sweat socks, faecal, rotten fruit	PAB, *Lactobacillus* sp.	[8, 13, 42, 46, 59]
2-Methylbutanoic acid*	Ile	fruity, waxy, sweaty-fatty acid	PAB, *Lactobacillus* sp.	[42, 59]
Isocaproic acid (4-methylpentanoic acid)	Leu	cheese-like, sweet, dirty sweat socks	Clostridia	[3, 59]
Aldehydes				
2-Methylpropanal*	Val		unk	
3-Methylbutanal*	Leu	green, malty	unk	
2-Methylbutanal*	Ile	green, malty	unk	
Benzaldehyde	Phe	bitter almond	*Lb. helv.*	[34]
Phenylacetaldehyde	Phe	rosy, violet-like	unk	
Alcohols				
2-Methylpropanol	Val	alcohol	unk	
3-Methylbutanol	Leu	fruity, alcohol, solvent-like, grainy	unk	
2-Methylbutanol	Ile		unk	
Phenylethanol	Phe	rosy, violet-like, floral	unk	
Sulphur compounds				
Methanethiol	Met	cooked cabbage	unk	
Methylthiopropanal* (methional)	Met	boiled potato-like, cheese-cracker-like	unk	
Dimethylsulfide (DMS)*	Met	Intense, boiled cabbage	*Lb. helv., Lb. lactis, Lb. casei,* PAB	[27] [19]
Dimethyldisulfide (DMDS)	Met	cauliflower, garlic, very ripe cheese	unk	
Dimethyltrisulfide (DMTS)	Met	cabbage, cheesy	unk	
S-methylthioacetate	Met	cauliflower, garlic, cheesy	unk	

Table I *(suite)*

Volatile compounds[1]	Origin	Aroma descriptors[2]	Flora potentially involved[3]	References
Esters				
Ethyl 3methylbutanoate*	Leu + ethanol		unk	
3-Methylbutyl acetate	Leu + acid	pear, banana	unk	
3-Methylbutyl propanoate	Leu + acid	apricot, pineapple	unk	
Other compounds				
Furaneol[4], Homofuraneol[5]	amino acids + carbonyl compounds	caramel-like	non enzymatic reactions	[23]

[1] Compounds marked with * are flavour-active in Swiss cheese, according to [48–50, 54].
[2] From [12, 18, 40, 57, 64].
[3] LAB: lactic acid bacteria. PAB: propionic acid bacteria. *Lb.: Lactobacillus. Lb helv.: Lb. helveticus. Lb. lactis: Lb. delbrueckii* subsp. *lactis*. unk: unknown.
[4] 4-hydroxy-2,5-dimethyl-3(2H)-furanone.
[5] 5-ethyl-4-hydroxy-2-methyl-3(2H)-furanone.

conditions (21 g·L^{-1} added NaCl, pH 5.4 and incubation at 24 °C), the specific isovaleric acid production by *P. freudenreichii* showed a three to four-fold increase [59]. The production of isovaleric acid by *P. freudenreichii* was also observed in milk cream, at a concentration of ~40 mg·L^{-1} [8]. Interestingly, only *P. freudenreichi* produced isovaleric acid under these conditions, out of the three main species used in Swiss cheese manufacture, *Streptococcus thermophilus*, *Lactobacillus helveticus*, and *P. freudenreichi* [8].

PAB are also capable of producing volatile sulphur compounds. This ability is strain-dependent [30, 51]. *P. freudenreichii* subsp. *shermanii* ATCC 9617 produced ~500 μg·L^{-1} dimethylsulfide (DMS) in milk cultures but not in YEL medium. DMS probably came from sulphur-containing amino acids in peptide linkage, but not directly from sulphur-containing amino acids, since the addition of L-Met, D-Met, L-Cys or D-Cys did not enhance the production of DMS in milk culture and did not induce it in YEL culture [19]. Other volatile sulphur compounds are also produced from Met degradation by PAB. Methanethiol (20–140 μg·L^{-1}), dimethyldisulfide (DMDS, 600–1 800 μg·L^{-1}) and dimethyltrisulfide (DMTS, 50–250 μg·L^{-1}) were produced by resting cells of four *P. freudenreichii* strains incubated in the presence of Met and cofactors [51]. Lower amounts of methanethiol and DMTS were also produced by *P. freudenreichii* TL 34 grown in YEL supplemented with 1 g·L^{-1} of L-Met [51]. DMDS and DMTS can be formed by auto-oxidation of methanethiol [64].

3.2. Production of volatile compounds in cheese

In order to evaluate the potential of PAB to produce flavour compounds in cheese, we compared the profile of volatile compounds of experimental cheeses manufactured with or without addition of PAB starters. Only the compounds which probably derive from amino acids are reported here.

Microbiologically-controlled mini Swiss cheeses were manufactured from microfiltered milk by the Institut Technique Français des Fromages according to their standardised process [52]. PAB reached 6×10^9 cfu·g^{-1} in the inoculated cheeses, whereas indigenous PAB remained under 5×10^6 cfu·g^{-1} at the end of ripening in control ones [25]. Several volatile branched-chain compounds were found at a significantly higher level in the presence of PAB: 3-methylbutanol, 2-methylbutanol, 2-methylbutanal, two branched-chain esters and isovaleric acid (Tab. II). In contrast, volatile sulphur compounds and benzaldehyde were not significantly affected by PAB, except DMTS which was found at a lower concentration in cheese containing PAB.

The effect of PAB on the profile of volatile compounds was also investigated in Morbier cheese made from raw milk. This type of cheese is usually manufactured without addition of PAB starters. The two PAB strains used as starters reached 10^8 cfu·g^{-1} at the end of ripening in the inoculated cheeses, whereas indigenous PAB reached 5×10^6 cfu·g^{-1} in control ones. The level of 2-methylbutanol was significantly higher in the presence of both strains, and that of 2-methylbutanal with the strain ITGP20 (Tab. III). In addition, higher amounts of DMDS and thioesters were also

Table II. Volatile compounds derived from amino acid catabolism in mini Swiss cheeses manufactured with or without addition of *P. freudenreichii* ITGP22. [25] Values are means of duplicate cheeses made from the same microfiltered milk.

Volatile compounds[1]	Without added propionibacteria	With *P. freudenreichii* ITGP22
2-Methylpropanal	2.4 [a]	2.9 [a]
2-Methylpropanol	24.3 [a]	10.5 [a]
3-Methylbutanal	7.3 [a]	6.7 [a]
3-Methylbutanol	9.6 [b]	16.1 [a]
3-Methylbutyl acetate	1.7 [a]	1.7 [a]
3-Methylbutyl propanoate	< 0.1 [b]	1.4 [a]
2-Methylbutanal	5.5 [b]	46.8 [a]
2-Methylbutanol	1.2 [b]	35.0 [a]
2-Methylbutyl acetate	0.5 [b]	1.0 [a]
Benzaldehyde	4.4 [a]	2.0 [a]
Dimethyldisulfide	1.9 [a]	2.5 [a]
Dimethyltrisulfide	0.3 [a]	< 0.1 [b]
Isovaleric acid (mg·kg^{-1})[2]	6.7 [b]	45.3 [a]

[1] Results are expressed in arbitrary area units, obtained from total ion chromatograms. They were extracted by head space and analysed by gas chromatography-mass spectrometry [58], except isovaleric acid, separately analysed as described by Berdagué [5].

[2] Corresponds to the sum of 2-methylbutanoic and 3-methylbutanoic acids, co-eluted under the chromatographic conditions used.

[a, b] For each compound, values without a common superscript letter were significantly different by least significant difference test : $P < 0.05$.

Table III. Volatile compounds derived from amino acid catabolism in Morbier cheeses manufactured from raw milk inoculated or not with propionibacteria.

Volatile compounds[1]	Without added propionibacteria[2]	With *P. freudenreichii*	
		ITGP10[2]	ITGP20[2]
2-Methylpropanol	7.0 [a]	4.9 [b]	3.5 [b]
3-Methylbutanal	3.1 [a]	1.3 [a]	3.1 [a]
3-Methylbutanol	22.4 [a]	20.9 [a]	18.8 [a]
2-Methylbutanal	1.2 [b]	1.9 [b]	7.4 [a]
2-Methylbutanol	3.0 [b]	13.0 [a]	11.9 [a]
Benzaldehyde	2.7 [a]	0.6 [a]	0.3 [a]
Dimethyldisulfide	21.5 [b]	14.5 [b]	61.9 [a]
Dimethyltrisulfide	1.3 [a]	0.4 [b]	1.2 [ab]
S-methylthioacetate	2.4 [b]	2.7 [b]	8.7 [a]
S-methylthiopropanoate	0.4 [b]	0.7 [b]	4.0 [a]
Isovaleric acid ($mg \cdot kg^{-1}$)[3]	42.5 [a]	37.0 [a]	33.0 [a]

[1] Results are expressed in arbitrary area units, obtained from total ion chromatograms. They were extracted by head space and analysed by gas chromatography-mass spectrometry [58], except isovaleric acid, separately analysed as described by Berdagué [5].
[2] Values are means of two separate cheese experiments, analysed in duplicate.
[3] corresponds to the sum of 2-methyl- and 3-methylbutanoic acids, coeluted under the chromatographic conditions used.
[a, b, c] For each compound, values without a common superscript letter were significantly different by least significant difference test: $P < 0.05$.

observed with this strain. Neither isovaleric acid, DMTS or benzaldehyde were significantly affected by PAB. Taken as a whole, these data show that PAB had a lesser effect on the volatile profile of Morbier cheese than on that of Swiss cheese (Tabs. II and III). It could be explained by at least two factors. First, PAB reached 60-times lower counts in Morbier cheeses than in Swiss cheeses. Secondly, Morbier cheeses contained a more complex ecosystem including a smear flora and non-starter lactic acid bacteria (which reached 2×10^8 $cfu \cdot g^{-1}$ in these cheeses). These flora also have the ability to produce volatile compounds [67], and could therefore interfere with PAB activity.

These data show that the main volatile compounds affected by the presence of PAB were those deriving from Ile, and, to a lesser extent, Leu (Tabs. II and III). Accordingly, the comparison of the respective amounts of volatile compounds arising from Ile and Leu in different types of cheese with or without PAB suggests that PAB could convert Ile more efficiently than Leu. In Swiss cheese, 2-methylbutyric acid (from Ile) appeared at a higher concentration and/or was detected in a higher number of samples than 3-methybutyric acid (from Leu) [35, 60], whereas it was the opposite in Parmesan cheese [2]. In Maasdamer cheese, a semi-hard cheese displaying a propionic fermentation, 2-methylbutanol and 2-methylbutanal (from Ile) were found in large amounts whereas, in a range of semi-hard cheeses without propionic

fermentation, 3-methylbutanol and 3-methylbutanal (from Leu) were more prevalent [20, 28, 62].

The approximate concentrations of branched-chain neutral compounds were calculated from the data in Table II by using a standard addition method as previously described [58]. The concentration of 3-methylbutanol, 3-methylbutanal, 2-methylbutanol and 2-methylbutanal were ~0.13, 0.02, 0.29 and 0.11 $mg \cdot kg^{-1}$, respectively, i.e. ~1% of the volatile branched-chain compounds, isovaleric acid amounting to the remaining 99%. The high content of isovaleric acid in Swiss cheese (~30 $mg \cdot kg^{-1}$ on average, [54, 61]), compared to Cheddar, had early been observed [45]. In general, branched-chain acids appear to be more prevalent in Swiss-type cheeses and some Italian cheeses than in semi-hard cheese like Cheddar, Edam and Gouda [9, 14, 17, 55]. Branched-chain acids can however be produced by other flora, as observed in Morbier cheese (Tab. III). Some variations in the production of isovaleric acid in cheese could also be explained by the strain-dependence of the ability of PAB to produce branched-chain acids [13].

3.3. Conclusions

In vitro and cheese experiments showed that PAB have a markedly high ability to convert branched-chain amino acids to acids. The compounds which are most likely involved in flavour are those having the highest number of odour units, i.e. their concentration divided by their odour threshold in the same medium [38]. These numbers were calculated for the compounds whose content was increased in Swiss cheese in the presence of PAB. These data show that isovaleric acid was the most potential branched-chain aroma in Swiss cheese, with a concentration ~600-times higher than its odour threshold in water (0.07 $mg \cdot L^{-1}$, [12]). 2-Methylbutanal was at a concentration close to its odour threshold in milk (0.13 $mg \cdot L^{-1}$, [57]), whereas branched-chain alcohols were at concentrations markedly lower than their odour thresholds.

The in vitro ability of PAB resting cells to produce DMDS and DMTS was not confirmed in Swiss cheese, to our knowledge. In Morbier cheese, however, higher levels of DMDS and sulphur esters were found with one PAB strain (Tab. III). S-methylthioacetate reached a concentration around 0.03 $mg \cdot kg^{-1}$, i.e. ~7 times higher than its odour threshold in cheese (0.005 $mg \cdot L^{-1}$, [64]. The role of PAB in the formation of this compound remains to be clarified. More work is required to investigate the factors affecting the production of flavour compounds derived from aromatic amino acids and Met by PAB.

4. PATHWAYS OF CONVERSION OF AMINO ACIDS TO FLAVOUR COMPOUNDS BY PROPIONIBACTERIA

4.1. First step of amino acid conversion

Amino acids can be catabolised by several reactions: deamination, decarboxylation, oxidative desamination, transamination and elimination [14, 55, 67]. However, only transamination and elimination initiate the conversion of branched-chain amino acids, aromatic amino acids and Met by cheese-related bacteria [67]. The known pathways of catabolism of these amino acids are schematised in Figure 1, and detailed in this section.

4.1.1. Decarboxylation and deamination

Most studies on the ability of PAB to catabolise amino acids, until recently, were not directly related to flavour formation. They mainly aimed at determining the effect of amino acid catabolism on the

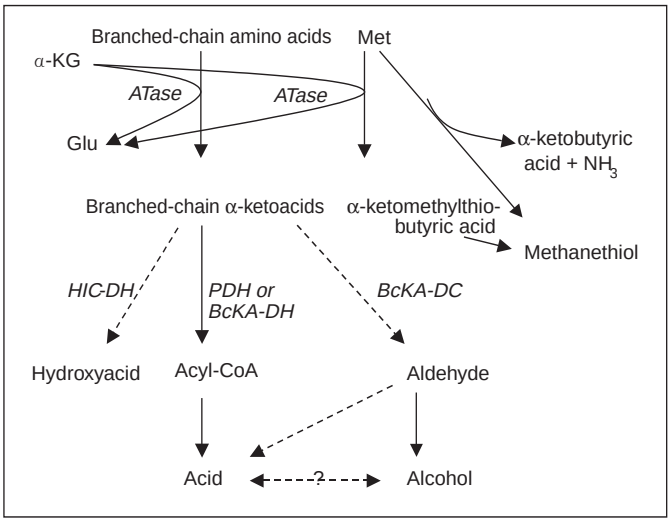

Figure 1. Known pathways of catabolism of branched-chain amino acids and methionine in *P. freudenreichii* (thick arrows). Dashed arrows represent other pathways described in cheese-related bacteria. ATase: aminotransferase; α-KG: α-ketoglutarate; HIC-DH: hydroxyisocaproate dehydrogenase. BcKA-DH: branched-chain ketoacid dehydrogenase complex; PDH: pyruvate dehydrogenase complex; BcKA-DC: branched-chain ketoacid decarboxylase (from [59, 67]).

production of carbon dioxide and/or ammonia and on lactate metabolism [1, 11, 15, 16, 22, 32]. They showed that Asp, Ala and Ser are the most degraded amino acids by both resting cells and growing cells of PAB. Two enzymes have been partially purified: aspartase and alanine dehydrogenase, respectively involved in the formation of fumarate from Asp and pyruvate from Ala [16]. Other amino acids are degraded to a lesser extent: Gly, Arg, Cys, Lys, Tyr and Val, with variations in amounts depending on the PAB strain and the experimental conditions. In addition, a glutamate decarboxylase activity, converting Glu to γ-aminobutyric acid and CO_2, has been found in *P. freudenreichii* [4].

The end products of these amino acid degradations are acetate, propionate and succinate (from Asp), in addition to carbon dioxide and/or ammonia [11, 15]. Their catabolism could therefore have an indirect effect on cheese flavour by modifying the ratio of acids produced from lactate fermentation and by inducing pH variations.

4.1.2. Transamination

Aminotransferases are pyridoxal 5'-phosphate (PLP)-dependant enzymes widely distributed in bacteria, where they are involved in the first step of catabolism of some amino acids and also in the last step of their synthesis. Therefore, the presence of aminotransferase activities was expected in PAB, at least for the strains able to synthesise amino acids. The ability to use ammonium as sole nitrogen source depends on the strain and on the conditions of incubation, as shown by early observations showing that "*P. shermanii*" required strictly anaerobic conditions to grow in such a medium [56].

The first step of conversion of branched-chain amino acids by *P. freudenreichii* is catalysed by aminotransferase(s), since

these amino acids, either by cell-free extracts or resting cells, are degraded only in the presence of an acceptor of an amino group like α-ketoglutaric acid (α-KG) [59]. Aminotransferase activity on branched-chain amino acids was also found in *P. jensenii* [31]. α-Ketoglutaric acid was the most efficient amino group acceptor for PAB aminotransferases, in agreement with the results obtained in *L. lactis* [66, 68]: the replacement of α-KG with phenylpyruvic acid or pyruvic acid as amino group acceptor drastically reduced Leu aminotransferase activity, which was respectively only ~20% and 4% of that observed with α-KG in the two tested strains of *P. freudenreichii* [59]. Leu aminotransferase activity of PAB was enhanced at pH 8.0, as generally observed for these enzymes [67]. The addition of α-KG in YEL cultures induced an increase by more than 80% in the amount of isovaleric acid [59].

The conversion of aromatic amino acids and Met is also initiated by transamination in PAB. The aminotransferase activity was found, however, to be lower for these amino acids than for branched-chain amino acids, by using the cell-free extracts of two strains of *P. freudenreichii* (Fig. 2).

These results are in accordance with the results of previous experiments carried out with resting cells of PAB without addition of an amino group acceptor, which showed no or little degradation of Leu, Ile, Phe and Trp [1, 11, 32]. The low amounts of Ile and Phe degraded in the absence of added α-KG [11] could be explained by the use as amino group acceptors of α-KG or other keto acids present in the cells [32]. The formation of an odour described as "old Emmental cheese", arising from Leu and Ile degradation, was however early noticed by Kiuru [32], who observed this phenomenon after 8 d incubation of resting cells of PAB. This odour was probably due to the formation of branched-chain acids.

PAB aminotransferase(s) have not been yet been purified, to our knowledge. Aminotransferases appear in bacteria as homodimers, homotetramers or homohexamers, with subunits of a molecular mass of 32 to 44 kg·mol^{-1} [24]. By incubation of SDS-PAGE gel slices in transamination mixture, an aminotransferase

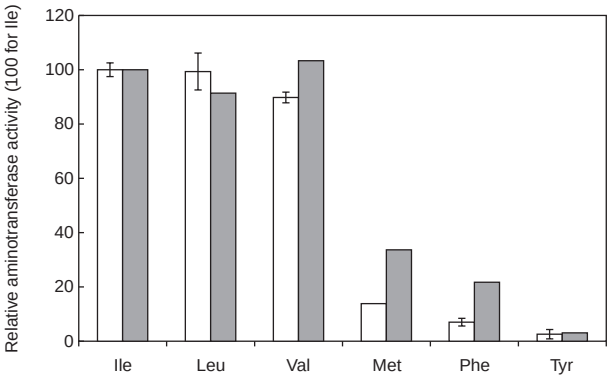

Figure 2. Comparison of aminotransferase activity of two strains of *P. freudenreichii* for several amino acids. □: ITGP23; ■: TL 34. Cell-free extracts were incubated in 60 mmol·L^{-1} phosphate, pH 6.8, containing 5 mmol·L^{-1} L-leucine, 10 mmol·L^{-1} α-KG, 50 μmol·L^{-1} PLP and 50 μmol·L^{-1} TPP, for 2 h at 30 °C. Activity was measured by enzymatic determination of the glutamate according to Yvon [68].

activity was revealed for a band at 42 ± 4 kg·mol^{-1} (unpublished results).

4.1.3. Elimination of methionine

Some preliminary results indicate that Met could be degraded by α-, γ-elimination in PAB besides its conversion by transamination. This one-step degradation leads to methanethiol, ammonia and α-keto-butyric acid. The production of methanethiol was shown for the cell-free extracts of 4 strains of *P. freudenreichii* incubated in a reaction mixture containing either Met or α-keto-γ-methylthiobutyric acid, the α-keto-acid formed from Met transamination [51]. The addition of α-KG as an amino group acceptor did not increase the amount of methanethiol produced, suggesting that Met conversion was preferably initiated by elimination rather than transamination under these conditions [51]. The enzyme involved has not been characterised in PAB. Elimination of methionine can be catalysed by a PLP-dependent enzyme, methionine γ-lyase (EC. 4.4.1.11, also referred to as methionine γ-demethiolase). Methionine γ-lyase has not been found in lactic acid bacteria, but was recently purified and characterised in *Brevibacterium linens*, where it is active under pH, salt and temperature conditions which exist during the ripening of Cheddar cheese [64]. Elimination of methionine can also be catalysed by non-specific enzymes: cystathionine β-lyase or cystathionine γ-lyase. These enzymes were found in *Lactococcus lactis* and several lactobacillus species: *Lb. fermentum*, *Lb. helveticus* and *Lb. casei* [67].

4.2. Degradation of -ketoacids

The α-keto acids derived from amino acids can be further converted by enzymatic or non-enzymatic reactions by cheese micro-organisms. These reactions lead to the corresponding hydroxy acids, carboxylic acids with one carbon atom less, and aldehydes with one or two carbon atoms less [67].

In *P. freudenreichii*, the branched-chain α-keto acids derived from Val, Leu and Ile, α-ketoisovaleric acid, α-ketoisocaproic acid, and α-keto-β-methylvaleric acid, respectively, were mainly converted by resting cells of PAB to the corresponding acids, isobutyric acid, 3-methylbutyric acid and 2-methylbutyric acid, respectively [59]. Low amounts of branched-chain alcohols (< 2 mg·L^{-1}) were also produced by PAB, but branched-chain aldehydes and α-hydroxy-acids were not detected under the conditions used [59]. The proportion of end products differed from that observed in lactic acid bacteria, where α-hydroxy acids were generally the main products found (Fig. 1) [67].

Two pathways can lead to the production of branched-chain acid from branched-chain α-ketoacids. The first is oxidative decarboxylation via a branched-chain α-ketoacid dehydrogenase complex (EC 1.2.4.4), yielding branched-chain acyl-CoA, which can then be converted to the acid. The second is non-oxidative decarboxylation by pyruvate decarboxylase (EC 4.1.1.1) or a pyruvate decarboxylase-like enzyme to yield aldehyde, which could be further converted to the acid. In *P. freudenreichii*, an α-ketoacid dehydrogenase complex is involved in the second step of leucine conversion since arsenicals, which are known inhibitors of α-ketoacid dehydrogenase complex [63], caused a drastic reduction in the conversion of α-ketoisocaproic acid to 3-methylbutyric acid [59]. Pyruvate dehydrogenase activity has been found in PAB but it is not known whether this complex is active on other α-ketoacids [4]. α-ketoacid dehydrogenase complexes have been found in numerous organisms, including *L. lactis* [67]. It has not yet been described in other cheese-related bacteria, but several other species are capable of producing acids from amino acids like *Lactobacillus* sp. [42] and *Microbacterium* [29].

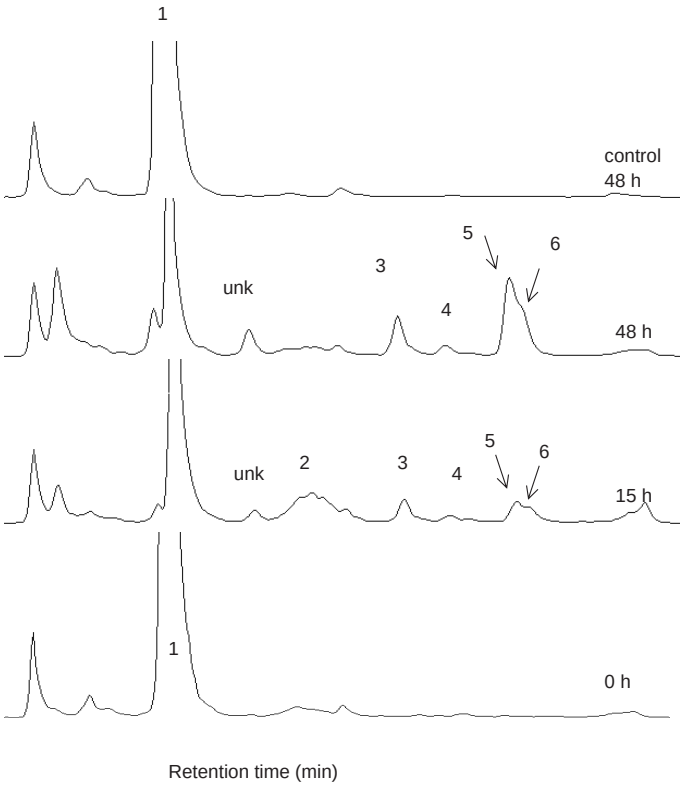

Figure 3. Catabolism of phenylalanine by resting cells of *P. freudenreichii* TL34 incubated in the presence of L-[2,3,4,5,6-^{3}H]phenylalanine as tracer. The incubation mixture contained 2 mmol·L^{-1} of non-labelled L-phenylalanine, 2% NaCl, 10 mmol·L^{-1} α-KG, 50 µmol·L^{-1} PLP and 50 µmol·L^{-1} TPP in 60 mmol·L^{-1} Tris-HCl, pH 8. Radioactive products were analysed by radio-HPLC on a Novapack C_{18} column as described by Yvon [68]. "Control": incubated without addition of resting cells. 1: phenylalanine, 2: phenylpyruvic acid, 3: phenyllactic acid, 4: benzaldehyde, 5: benzoic acid, 6: phenylacetic acid, unk: unknown.

P. freudenreichii also produces various aromatic compounds from phenylpyruvic acid, (the α-ketoacid of Phe): phenyllactic acid, phenylacetic acid, benzaldehyde and benzoic acid (Fig. 3). The part of enzymatic reactions and the enzymes involved in these reactions have however not yet been determined. Phenyllactic acid is probably formed by reduction of phenylpyruvic acid by hydroxyacid dehydrogenase, as observed in lactic acid bacteria [67]. Phenylacetic acid and benzaldehyde can originate from non-enzymatic degradation of phenylpyruvic acid, whereas benzoic acid can result from further oxidisation of benzaldehyde [67].

5. CONCLUSION

These data show that PAB have the ability to produce various volatile flavour compounds from amino acids. Most of these compounds are found in very different cheeses, suggesting that they can be produced by various flora [20, 37].

Compared with lactic acid bacteria, PAB showed similarities and differences in their in vitro ability to convert amino acids to flavour compounds. As in lactococci and lactobacilli, the first step of conversion of branched-chain and aromatic amino acids is catalysed by aminotransferases. The occurrence of transamination in cheese was shown early [55]. The content of α-ketoacid in cheese was recently demonstrated to be the rate-limiting factor of amino acid conversion by lactococci [53, 65, 67]. It could be the same for PAB, since it was shown that the addition of α-KG to YEL cultures enhanced the production of isovaleric acid by PAB [59]. The conversion of α-ketoacids formed by transamination differs in PAB and in lactic acid bacteria. They appear, at least for those of branched-chain amino acids, to be mainly converted into acids by PAB, whereas higher proportions of hydroxyacids are produced by lactococci. More work is however needed to investigate more thoroughly the pathways of conversion of α-ketoacids by cheese-related bacteria.

The main ability demonstrated in PAB is the conversion of Ile/Leu to isovaleric acid, which was observed in medium culture and in cheese without addition of α-KG. The physiological role of the production of isovaleric acid by *P. freudenreichii* may be related to the biosynthesis of the cell membrane, since PAB membrane, like that of *Bacillus*, contains high levels of branched-chain fatty acids [41]. The synthesis of these fatty acids in *B. subtilis* requires the synthesis of primers which can be either a branched-chain acyl-CoA or an aldehyde derivative [44]. The predominant fatty acid in *P. freudenreichii* is *anteiso* C_{15} (12-methyltetradecanoic acid), which is structurally related to Ile and whose synthesis is enhanced by addition of Ile to culture medium.

As it is the case with many bacteria [67], the ability to convert amino acid to flavour compounds in PAB seems to be very strain-dependent [13]. Therefore, there is a need to develop appropriate screening tools and to validate the results of screening by cheese experiments.

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