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Review article

Flavours of cheese products: metabolic pathways, analytical tools and identification of producing strains

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Abstract

Aroma development in cheese products results from the metabolic activities of cheese bacteria, by glycolysis, lipolysis and proteolysis. To respond to the increasing demand for products with improved aroma characteristics, the use of bacterial strains for cheese ripening with enhanced flavour production is seen as promising. In this review, the catabolism of amino acids, presumably the origin of some major aroma compounds, is discussed. The techniques of detection of flavour-producing strains are then presented. Their detection may be achieved either by genotyping, by enzymatic analysis, or by physico-chemical analysis such as HPLC, TLC, GC, and electronic nose.

Keywords: Cheese; Flavour; Lactic acid bacteria; Metabolic pathways; Catabolism of amino acids; Analysis; Electronic nose; Molecular profiling; Enzymatic activity; Review

1. Introduction

During cheese manufacture, the initiation of the fermentation process begins with the addition of starter cultures to milk. Mesophilic and thermophilic starters, with optimal growth temperatures of about 30 and 45 °C, respectively, are used. Thermophilic starters are added for the production of semi-hard and hard cheeses, typical of Italian and Swiss varieties. Thermophilic lactic acid bacteria found in starter cultures are *Streptococcus salivarius* subsp. *thermophilus*, *Lactobacillus helveticus*, *Lb. delbrueckii* subsp. *lactis*, while *Lactococcus lactis* subsp. *cremoris*, *Lc. lactis* subsp. *lactis*, *Leuconostoc lactis* and *Ln. mesenteroides* subsp. *cremoris* are used as mesophilic starters (Cogan and Hill, 1993). Semi-hard and hard cheeses are usually manufactured with a combination of *S. thermophilus* and a thermophilic lactobacillus.

However, the presence of starter cultures is not sufficient to explain the flavour formation in raw milk cheeses. The native microflora may play an important role. Indeed, facultatively heterofermentative lactobacilli are regularly found at low concentrations in milk, but reach concentrations of about 10^8 cfu/g in Swiss-type cheeses (Weinrichter et al., 2001). In semi-hard goat's milk cheese, a mixed adjunct culture containing *Lc. lactis* subsp. *lactis*, *Lb. casei* subsp. *casei*, *Lb. plantarum*, *Ln. mesenteroides* subsp. *dextranicum* and *Ln. paramesenteroides* provided the cheeses with the best scores for aroma and flavour development (Rodríguez et al., 1996, 1997). The flavour of cheddar

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cheese was also improved by addition of non-starter lactobacilli, an improvement likely due to increased formation of amino acids (Lynch et al., 1996). The addition of *Lb. paracasei* subsp. *paracasei* and *Lb. plantarum* to cheddar cheese influenced cheese aroma in a similar manner and suggests a slight acceleration of ripening (Lynch et al., 1999).

Surveys on the potential of cheese native microflora isolates to produce cheese flavour remain scarce. Wild lactococci isolated from dairy and non-dairy environments were assessed (Weerkamp et al., 1996; Ayad et al., 1999). Ayad et al. (1999) emphasized that wild lactococci isolated from dairy and non-dairy origin produced specific flavours distinct from those produced by industrial strains. Wouters et al. (2002) showed that several lactococci exhibited more pronounced sensory characteristics than the reference industrial starter strains. Drake et al. (1999) showed that five cheese lactobacilli adjuncts varied widely in volatile and acid production, and in proteolytic activity. Seefeldt and Weimer (2000) examined the levels and types of volatile sulfur compounds produced from methionine by 13 lactococci and 24 lactobacilli strains and showed that there was a wide variation between the species and the subspecies tested. Williams et al. (2001) selected 29 lactic acid bacteria from among 152 strains isolated and analysed the production of volatiles from valine, leucine, isoleucine, phenylalanine and sulphur-containing amino acids. The profiles of volatile metabolites were shown to be isolate-specific. Lactococci, lactobacilli, leuconostocs, enterococci and streptococci were compared on the basis of their production of neutral volatile compounds in whey (Mauriello et al., 2001). These authors showed that the production of volatile flavours varied between *Lactococcus*, *Streptococcus*, *Enterococcus*, mesophilic and thermophilic *Lactobacillus*, and in some cases between strains. Ayad et al. (1999) emphasized that wild lactococci isolated from dairy and non-dairy origin produced specific flavours distinct from those produced by industrial strains. The utilisation in the dairy industry of new isolates with specific flavour-forming abilities is considered as a promising tool to respond to the increasing demand for products with improved organoleptic properties.

The aims of this article are (i) to discuss in more detail some main metabolic routes such as the catabolism of amino acids, which produce the major aroma compounds but which can also generate off-flavours (Fox and Wallace, 1997; Christensen et al., 1999; Yvon and Rijnen, 2001), (ii) to focus on the analytical tools that can be applied to the analysis of the role of microorganisms in the development of flavours or off-flavours in cheese, and (iii) to review the molecular tools that can be applied to the detection and identification of flavourflavour-producing strains. Indeed, flavour-producing capabilities can be strain-specific or related to taxonomic affiliation, requiring the use of chemical and molecular analysis. Techniques presented here focus on lactic acid bacteria in the cheese environment, but can also be applied to the other fields of food microbiology.

2. Metabolic pathways

During cheese ripening, biochemical reactions (Fig. 1) lead to the formation of cheese aroma. Flavour compounds are produced from three major milk constituents: lactose, lipids and proteins (for a review, see Dumont and Adda, 1979; Fox et al., 1995; McSweeney and Sousa, 2000; Smit et al., 2002).

2.1. Lactose and citrate

Lactose is hydrolysed by starter cultures which produce glucose and galactose (galactose-6-P for lactococci). Glucose is then oxidised to pyruvate by the Emden-Meyerhof pathway of glycolysis. Galactose is converted by galactose-positive starter bacteria and leuconostocs through the Leloir pathway to glucose-6-P and by lactococci through the tagatose pathway to glyceraldehyde-3-P (Cogan and Hill, 1993). Pyruvate is a starting material for the formation of short-chain flavour compounds such as diacetyl, acetoin, acetate, acetaldehyde and ethanol (Cogan and Hill, 1993; Escamilla-Hurtado et al., 1996; Henriksen and Nilsson, 2001; Syu, 2001; Melchiorsen et al., 2002). Citrate is metabolised to produce acetolactate, diacetyl and acetoin (Cogan and Hill, 1993; de Figueroa et al., 2000, 2001). However, thermophilic starter bacteria are usually citrate-negative (Cogan and Hill, 1993).

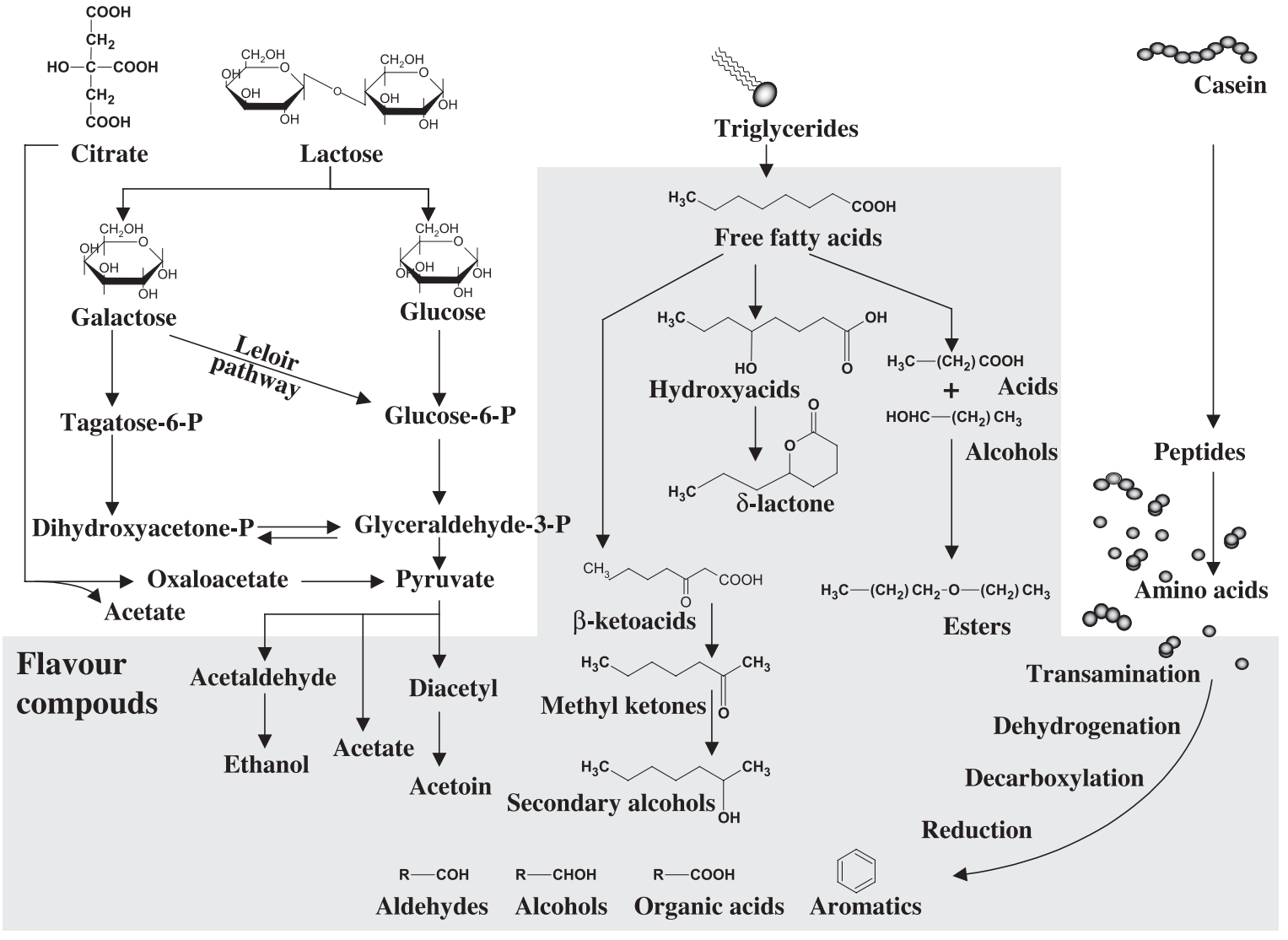

Fig. 1. Biochemical pathways leading to the formation of flavour compounds. The gray surface indicated compounds with a flavor note.

2.2. Milk fat

The replacement of whole milk by skim milk showed that milk fat is an essential prerequisite to flavour development (Foda et al., 1974). Cheese lipids can be oxidised or hydrolysed. However, oxidation is probably limited because of the low redox potential in cheeses. Lipolysis of milk triglycerides releases high concentrations of short- and intermediate-chain fatty acids (Bills and Day, 1964). Short-chain fatty acids have a considerable flavour impact, but intensive lipolysis is undesirable in most cheese varieties because of the development of rancidity. Free fatty acids must be counter-balanced with other flavour compounds to develop an appreciated aroma (Bosset and Gauch, 1993; Fox et al., 1995). Free fatty acids are substrates of enzymatic reactions yielding flavours. β-oxidation and decarboxylation yield methyl ketones and secondary alcohols, and esterification of hydroxy fatty acids produces lactones. Fatty acids react with alcohol groups to form esters, such as ethyl butanoate, ethyl hexanoate, ethyl acetate, ethyl octanoate, ethyl decanoate, and methyl hexanoate (McSweeney et al., 1997). Butyric acid concentrations found in cheeses are in part due to the hydrolytic activities of lipases (Dumont and Adda, 1979; Fox et al., 1995).

2.3. Proteolysis and catabolism of amino acids

Proteolysis directly contributes to cheese flavours by releasing peptides and amino acids. Amino acids are substrates for transamination, dehydrogenation, decarboxylation and reduction, producing a wide variety of flavour compounds such as phenylacetic acid, phenethanol, *p*-cresol, methane thiol, dimethyl disulphide, 3-methyl butyrate, 3-methyl butanal, 3-methyl butanol, 3-methyl-2-butanone, 2-methyl propionate, 2-methyl-1-propanal, 2-methyl butyrate, and 2-methyl butanal. The main flavour compounds identified in cheeses are displayed in Table 1 (for a more exhaustive list, see Curioni and Bosset, 2002).

2.3.1. Branched-chain amino acids

The catabolism of branched-chain amino acids is initiated by an aminotransferase (Fig. 2), forming α-ketoisocaproate, α-keto-β-methyl valerate and α-keto-isovalerate from leucine, isoleucine and valine, respectively. Two branched-chain aminotransferases, one from *Lc. lactis* subsp. *cremoris* NCDO763 (Yvon et al., 2000), with activities on isoleucine, leucine, valine and methionine, and one from *Lc. lactis* LM0230 (Atiles et al., 2000) and *Lc. lactis* subsp. *cremoris* B78 (Engels, 1997) with activities on isoleucine, leucine, valine, methionine and phenylalanine have been characterised. A third aminotransferase with activities on leucine was described in *Lc. lactis* (Yvon et al., 1997; Gao and Steele, 1998; Rijnen et al., 1999, 2000). This enzyme differs from the above-mentioned aminotransferases by its specificity. It is also active on tryptophan, tyrosine, phenylalanine and methionine, and is therefore called aromatic amino acid aminotransferase. The transamination abilities of lactobacilli are not as well documented and seem to be a more complicated feature. For example, the production of 2- and 3-methyl butanal by *Lb. paracasei* subsp. *paracasei* and *Lb. casei* was not attributed exclusively to the corresponding amino acids (Kieronczyk et al., 2001). Aminotransferase activity specific for isoleucine, valine and leucine was detected in *Lb. paracasei* (Hansen et al., 2001) and Williams et al. (2001) measured activities upon leucine, phenylalanine and methionine with strains of *Lb. paracasei*, *Lb. curvatus*, *Lb. brevis*, *Lb. plantarum* and *Lb. lactis* isolated from Cheddar cheese.

The pathways responsible for the conversion of branched-chain α-keto acids in lactic acid bacteria have been only partially elucidated. Three biochemical reactions may be involved (Fig. 2): the oxidative decarboxylation to carboxylic acids, the decarboxylation to aldehydes and the reduction to hydroxyacids. With the exception of hydroxyacids, all of these products have strong flavour notes. Oxidative decarboxylation of branched-chain keto acids to carboxylic acids appears uncommon in lactic acid bacteria (Christensen et al., 1999; Yvon and Rijnen, 2001), but was reported for lactococci (Nakae and Elliott, 1965; Gao et al., 1997; Yvon et al., 1997; Roudot-Algaron and Yvon, 1998), propionibacteria (Thierry and Maillard, 2002), corynebacteria (Jollivet et al., 1992) and for an unspecified lactobacillus (Nakae and Elliott, 1965).

The decarboxylation reaction of branched-chain keto acids to aldehydes is not well documented and is therefore apparently uncommon in cheese bacteria. This reaction was first detected in *Lc. lactis* var. *maltigenes* (Tucker and Morgan, 1967; Morgan,

Table 1
Compounds isolated as flavours in various cheeses[a]

Alcohols	isohexanal	**Esters**	methylene bis(methylsulphide)
1,2-butanediol	2-methylbutanal	ethyl acetate	hexanethiol
2-butanol	3-methylbutanal	ethyl benzoate	***N*-compounds**
ethanol	2-methylpropanal	ethyl butyrate	2-acetyl-1-pyrroline
2-ethylbutanol	nonanal	ethyl hexanoate	**Pyrazines**
2-ethylhexanol	(*E*,*E*)-2,4-nonadienal	ethyl isobutanoate	2,3-diethyl-5-methylpyrazine
2-heptanol	(*Z*)-2-nonenal	ethyl octanoate	2-ethyl,3-5-dimethyl-pyrazine
hexanol	(*E*)-2-nonenal	ethyl 2-methylbutanoate	2-methoxy-3-isopropylpyrazine
isobutanol	octanal	ethyl 3-methylbutanoate	**Furans**
2-methylbutanol	pentanal	isobutyl butanoate	2-ethyl-4-hydroxy-5-methyl-3-(2*H*)furanone
3-methylbutanol	propanal	3-methylbutyl acetate	3-hydroxy-4,5-dimethyl-2-(5*H*)furanone
2-methylpropanol	propenal	methyl-2-methylbutanoate	4-hydroxy-2,5-dimethyl-3-(2*H*)furanone
2-nonanol	thiophen-2-aldehyde	3-octyl acetate	tetrahydrofuran
(*Z*)-1,5-octadien-3-ol	**Ketones**	pentyl acetate	**Phenolic compounds**
2-octanol	acetoin	phenethyl acetate	*p*-cresol
1-octen-3-ol	acetone	propyl butyrate	**Fatty acids**
2-pentanol	2,3-butanedione (diacetyl)	**Lactones**	acetate
phenylethanol	2-butanone	δ-decalactone	butyrate
2-phenylethanol	*b*-damaescenone	γ-decalactone	caproate
1-propanol	2-heptanone	δ-dodecalactone	decanoate
2-propanol	2-hexanone	δ-octalactone	isobutyrate
Aldehydes	3-methyl-2-butanone	(*Z*)-6-dodecen-γ-lactone	methyl acetate
acetaldehyde	2-nonanone	***S*-compounds**	2-methylbutyric acid
decanal	3-octanone	dimethyl disulphide	3-methylbutyric acid
heptanal	1-octen-3-one	dimethyl sulphide	octanoate
(*Z*)-4-heptenal	2-pentanone	dimethyl trisulphide	phenylacetate
hexanal	2-tridecanone	methanethiol	propionate
2-hexenal	2-undecanone	methional	valerate

[a] Arora et al., 1995; McSweeney and Sousa, 2000; Kubickova and Grosch, 1997; Milo and Reineccius, 1997; Moio et al., 1993; Moio et al., 2000; Rychlik and Bosset, 2001a,b; Urbach, 1993.

1976), in *Lb. casei* (Hickey et al., 1983) and in wild-type strains of *Lc. lactis* (Weerkamp et al., 1996; Ayad et al., 1999). The enzyme involved in the reaction is related to pyruvate-decarboxylase. The production of branched-chain amino acid-derived aldehydes have been reported for lactococci and for lactobacilli (McLeod and Morgan, 1958; Ayad et al., 1999; Drake et al., 1999; Rijnen et al., 2000; Kieronczyk et al., 2001; Mauriello et al., 2001). The aldehydes can be then reduced to alcohols by an alcohol dehydrogenases or oxidised to carboxylic acids by an aldehyde dehydrogenase (Yvon and Rijnen, 2001).

Two enzymes catalysing the reduction of α-keto acids to hydroxyacids, L-2-hydroxyisocaproate dehydrogenase (Schütte et al., 1984) and D-2-hydroxyisocaproate dehydrogenase (Hummel et al., 1984) have been characterised. L-2-hydroxyisocaproate dehydrogenase has been found only in *Lb. confusus* (Schütte et al., 1984), while D-2-hydroxyisocaproate appears to be widespread. It was found in lactobacilli (Hummel et al., 1985, 1988; Yamazaki and Maeda, 1986; Bernard et al., 1994) and in leuconostocs (Hummel et al., 1985). A third enzyme, D-mandelate dehydrogenase, showing similarities to D-2-hydroxyisocaproate dehydrogenase was found in several lactobacilli and showed a higher activity with α-ketoisoglutarate than with mandelic acid (Hummel et al., 1988).

The physiological significance of branched-chain amino acid degradation is not well established. The pathways may be implicated in the catabolism with the production of cellular energy and in the preservation of the $NAD^+/NADH + H^+$ ratio. Acyl-CoAs produced by oxidative decarboxylation may serve as energy source for the production of ATP by two successive enzymatic reactions catalysed by a phosphotransferase and a kinase. *Enterococcus faecalis* has a branched-chain α-keto acid dehydrogenase residing in a gene cluster containing *ptb* and *buk*, which exhibit significant homologies with phosphotransbutyrylase and butyrate kinase, respectively (Ward et al., 1999,

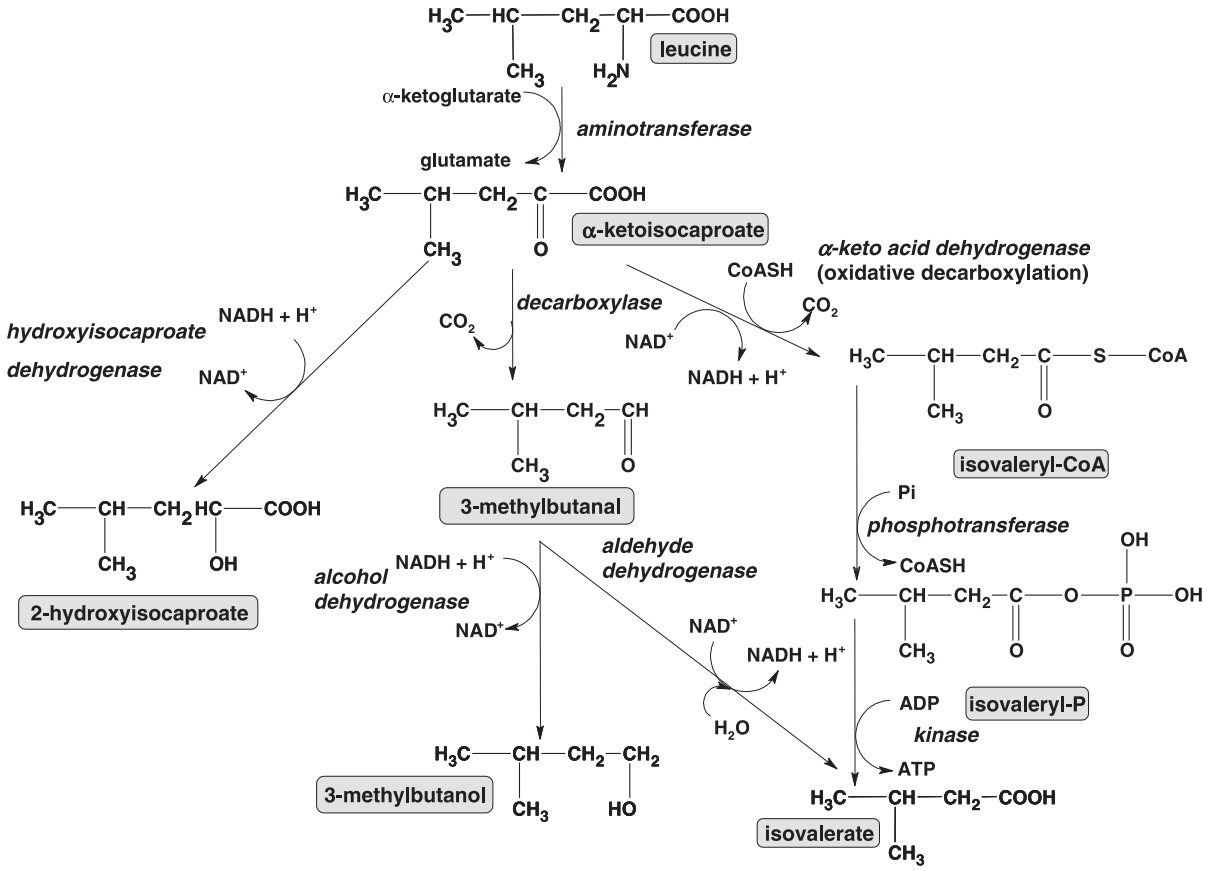

Fig. 2. Catabolism of branched-chain amino acids with leucine as example.

2000). These authors showed that this pathway was implicated in the production of ATP.

3-Methyl butanal, 2-methyl butanal and 2-methyl propanal are characterised by a malty flavour (Griffith and Hammond, 1989; Kubickova and Grosch, 1997; Friedrich and Acree, 1998; Rychlik and Bosset, 2001b; Thierry and Maillard, 2002). 3-Methyl butanal from Gorgonzola cheese and from milk was also described as unripe, apple-like and cheesy (Moio et al., 2000), and sweet fruity (Karagül-Yüceer et al., 2002). The corresponding alcohols 3-methyl butanol, 2-methyl butanol and 2-methyl propanol produce alcoholic and fruity odours (Thierry and Maillard, 2002). The fatty acids derived from branched-chain amino-acids (isovaleric, 2-methylbutyric and isobutyric acids) produce sweaty, rancid, fecal, putrid, estery and rotten fruit-like flavours (Brennand et al., 1989; Karagül-Yüceer et al., 2002; Moio et al., 2000; Rychlik and Bosset 2001a; Thierry and Maillard, 2002). These compounds might have detrimental effects on cheese aroma in function of their concentration. In Cheddar cheese, unclean, harsh and dulling flavour sensations were associated with the presence of 3-methyl butanal and 2-methyl butanal (Dunn and Lindsay, 1985). In cheeses, the role of the matrix and the combined effects of other flavour compounds should also be considered. Indeed, the perception threshold of a compound is strongly dependent on the matrix and other cheese components can modify the quality of the organoleptic perception of a given compound (Curioni and Bosset, 2002). For example, 2-methyl propanal and 3-methyl butanol have detection thresholds of 100 and 3200 μg/kg in water, and 180 and 4750 μg/kg in skim milk, respectively (Dunn and Lindsay, 1985). In Emmentaler cheese, 3-methyl butanol may play a role in suppressing the sweaty

odour of butyric acid originating from lipolysis (Preininger et al., 1996). The exact role of the degradation of branched-chain amino acids in flavour development needs to be better understood. Banks et al. (2001) reported that Cheddar cheeses develop better aroma characteristics when the production of volatile compounds from branched-chain and aromatic amino acids is enhanced.

2.3.2. Aromatic amino acids

The catabolism of aromatic amino acids begins with a transamination step which produces indole pyruvate, phenyl pyruvate and *p*-hydroxy-phenyl pyruvate from tryptophan, phenylalanine and tyrosine, respectively. Aromatic amino acid aminotransferases have been isolated from *Lc. lactis* subsp. *cremoris* NCDO763 (Yvon et al., 1997) and *Lc. lactis* subsp. *lactis* S3 (Gao and Steele, 1998). The reduction of indole pyruvate and phenyl pyruvate to the corresponding hydroxy acids indole-3-lactate and phenyl lactate has been described for *Lc. lactis* (Yvon et al., 1997) and for an unspecified *Brevibacterium* (Hummel et al., 1984, 1986a,b) and *Lactobacillus* (Gummalla and Broadbent, 1999). Products probably linked to an oxidative decarboxylation step yielding indole-3-acetate, phenyl acetate and 4-hydroxyphenyl acetate have been detected in lactococci (Gao et al., 1997; Yvon et al., 1997). These findings suggest that the degradation of aromatic amino acids is also linked to energetic metabolism and that NAD^+ must be regenerated, either by reduction to hydroxyacids, or by decarboxylation–dehydrogenation. However, the mechanisms of conversion remain unknown and therefore still need to be studied. Indole aldehyde and 4-hydroxyl-benzaldehyde are formed by lactococci (Gao et al., 1997). The conversion of indole acetate and *p*-hydroxy-phenyl acetate to skatole and *p*-cresol by non-specified strains of lactobacilli was reported (Yokoyama and Carlson, 1981; Honeyfield and Carlson, 1990). Non-enzymatic conversions producing *p*-cresol, skatole, benzaldehyde, phenylethanol from *p*-hydroxy-phenyl pyruvate, indole acetate, indole pyruvate or phenyl pyruvate have also been reported (McSweeney and Sousa, 2000). Several lactic acid bacteria are known to produce, by enzymatic decarboxylation of tryptophan and tyrosine, the biogenic compounds tryptamine and tyramine (Christensen et al., 1999; McSweeney and Sousa, 2000).

Compounds derived from aromatic amino acids have an impact on cheese aroma. Benzaldehyde is characterised by a bitter almond, phenyl acetaldehyde by a honey-like, floral, rosy and violet-like, phenyl ethanol by a rosy, violet-like and floral, phenyl acetate by a honey-like, and phenyl propanoate by a flowery odour (Kubickova and Grosch, 1997; Friedrich and Acree, 1998; Moio et al., 2000; Rychlik and Bosset, 2001a; Thierry and Maillard, 2002; Curioni and Bosset, 2002). Indole and skatole produce a fecal, putrid, musty odour, even if they have a floral note at higher dilutions. *p*-Cresol has a phenolic, medicinal flavour. These products are often associated with the formation of unclean flavours in dairy products.

2.3.3. Methionine

Sulphur compounds originating from methionine are responsible for the garlic (methanethiol, dimethyl disulphide, dimethyl trisulphide, *S*-methyl thioacetate), boiled potato-like (methional) and cooked cabbage (methanethiol) flavours (McSweeney and Sousa, 2000; Yvon and Rijnen, 2001; Thierry and Maillard, 2002). *S*-compounds are major contributors to Cheddar cheese aroma and contribute to the garlic notes of well-ripened Camembert cheese (McSweeney and Sousa, 2000). The catabolism of methionine is also initiated by a transamination step, yielding 4-methylthio-2-ketobutyric acid (Fig. 3). Oxidative deamination of methionine to 4-methylthio-2-ketobutyrate has been reported only for *Proteus rettgeri* (Chen et al., 1971), but it is likely that transamination is the principal pathway in cheese bacteria (Seefeldt and Weimer, 2000; Amárita et al., 2001a). This activity is higher than demethiolation, which converts methionine to methanethiol (Gao et al., 1998), and as well branched-chain as aromatic amino acid aminotransferases are active on methionine.

The demethiolation of methionine requires pyridoxal phosphate-dependent lyases. Cystathionine-β-lyase has been found in *Lc. lactis* B78 (Alting et al., 1995; Fernandez et al., 2000), cystathionine-γ-lyase in *Lc. lactis* SK11 (Bruinenberg et al., 1997), in *Lb. fermentum* DT41 (Smacchi and Gobbetti, 1998), and in *Brevibacterium linens* BL2 (Dias and Weimer, 1998). While methionine-γ-lyase from *B. linens* BL2 is active under salt and pH conditions found in cheeses, cystathionine-lyases are only slightly active, and therefore probably contribute only weakly to the

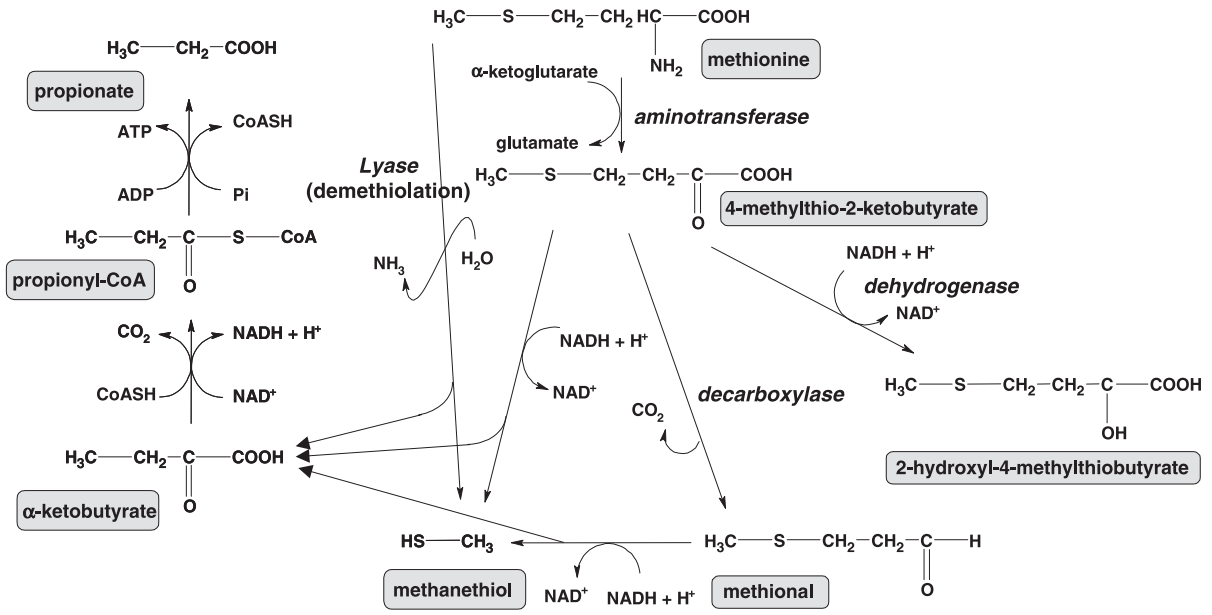

Fig. 3. Catabolism of methionine and generation of volatile sulphur compounds.

production of volatile sulphur compounds in cheeses. Indeed, cystathionine-lyase activity in lactobacilli is low and is inhibited in lactococci by the methionine concentrations found in cheeses (Seefeldt and Weimer, 2000). Demethiolation of methionine liberates methanethiol, α-ketobutyrate and ammonia. Methanethiol is then auto-oxidised to dimethyl sulphide, dimethyl disulphide and dimethyl trisulphide (Bonnarme et al., 2000; McSweeney and Sousa, 2000).

Decarboxylation of 4-methylthio-2-ketobutyrate yields methional, which has been found to be produced by one strain of *Lc. lactis* (Amárita et al., 2001a). The activity is probably strain specific. Gao et al. (1998) showed for several strains of lactococci that 4-methylthio-2-ketobutyrate is also enzymatically converted to 2-hydroxyl-4-methylthiobutyric acid and to methanethiol.

The physiological role of these reactions remain unclear. ATP may be produced by phosphorylation at the substrate level from α-ketobutyric acid, yielding propionic acid as the final product (Fig. 3). α-Ketobutyrate may also be produced by demethiolation of 4-methylthio-2-ketobutyrate or methional. These reactions may also be used for the regeneration of NAD^+, which, like 2-hydroxy-4-methylthiobutyrate, may be used as a temporary redox sink. However, this catabolic scheme still needs to be experimentally proved.

2.4. Impact of genomics

Our current knowledge of metabolic pathways can be significantly improved by genomics. To date, seven genomes of lactic acid bacteria are publicly available and two are still incomplete www.ncbi.nlm.nih.gov/sutils/genom_table.cgi; www.biol.ucl.ac.be/gene/genome). Analysis of whole genome sequences includes the prediction of open reading frames (ORFs) to which putative functions are assigned after searches for homology or for conserved motives, and finally experimental confirmation. The genes are often organized in operons, facilitating the prediction of metabolic pathways. Prediction of flavour formation from amino acids was discussed by van Kranenburg et al. (2002). In the genome of *Lb. plantarum* WCFS1, 19 genes encoding intracellular aminopeptidases and enzymes catalysing the formation of formate, acetate, ethanol, acetoin and 2,3-butanediol from pyruvate were predicted (Kleerebezem et al., 2003). The genome of *Lc. lactis* JL1403 was screened for genes encoding aminotransferases and at least 12 were found, cataly-

sing the transamination of aromatic, branched-chain and sulphur-containing amino acids (Bolotin et al., 2001). Complete genomic sequences also reveal the presence of a large percentage of genes whose function is unknown. For example, Kleerebezem et al. (2003) identified 3052 protein-encoding genes on the genome of *Lb. plantarum* WCFS1, 2120 of them were assigned to biological functions, 588 to conserved proteins of unknown function but 344 ORFs could not be assigned to database matches. This new genetic information concerning metabolic pathways involved in the flavour formation capabilities of lactic acid bacteria can be used to design molecular probes, thus providing a tool for rapid screening of strains.

3. Analytical tools

3.1. Enzymatic analysis

Measurements of enzyme activity (Table 2) involved in the metabolic pathways leading to the production of flavours are an indication of the ability to produce flavours. Although these methods are limited by the time-consuming preparation of cell-free extracts, studies comparing enzymatic activities of lactic acid bacteria showed that these techniques allow clear differentiation between strains, and consequently between their ability to produce flavours. Cystathionine-lyase activity was shown to vary greatly between

Table 2
Enzymatic assays performed to assess the activity of enzymes involved in the production of flavours by lactic acid bacteria

Reaction	Enzyme	Spectrometric detection	Reference
Catabolism of amino acids			
Methionine/methanethiol	Cystathionine lyase	Reaction thiol-DTNB	1, 2, 6, 17, 18, 21
Amino acid/keto acid	Aminotransferase	Formation of glutamate	6, 21, 22
Acyl-CoA/acyl-P	Phosphotransferase	Reaction CoASH-DTNB	19, 20
4-Methylthio-2-ketobutyrate/ methanethiol	Demethiolase	Reaction thiol-DTNB	1
Tryptophan/indole pyruvate	Aminotransferase	Formation of indole pyruvate	10, 11, 12
Phenylalanine/phenyl pyruvate	Aminotransferase	Formation of phenyl pyruvate	11, 13
Tyrosine/*p*-hydroxy phenyl pyruvate	Aminotransferase	Formation of *p*-hydroxy phenyl pyruvate	11, 13
α-Keto acid/hydroxy acid	Branched-chain α-keto acid dehydrogenase	NAD oxidoreduction	14
Indolepyruvate/indolelactate	Indolelactate dehydrogenase	NAD oxidoreduction	13, 15
Tryptophan/Tryptamine	Trp decarboxylase	Formation of tryptamine	13, 16
p-Hydroxy phenyl acetate/ *p*-hydroxyphenyl lactate	*p*-hydroxyphenyl lactate dehydrogenase	NAD oxidoreduction	13, 15
Phenyl acetate/phenyl lactate	Phenyl lactate dehydrogenase	NAD oxidoreduction	13, 15
Lactose and citrate			
Diacetyl/acetoin	Diacetyl reductase		3, 5
Acetoin/2,3-butanediol	Acetoin reductase		3, 5
Metabolism of lipids			
Acyl-CoA/β-ketoacyl-CoA	L-3-Hydroxyacyl-CoA dehydrogenase	NAD oxidoreduction	7
Acyl-CoA/β-ketoacyl-CoA	L-3-Hydroxyacyl-CoA dehydrogenase	Reaction DTNB-CoASH	4, 7
β-Ketoacyl-CoA/β-ketoacid	Thioesterase	Reaction DTNB-CoASH. CoASH is formed by condensation of acyl-CoA and oxaloacetate in presence of citrate synthase.	7
Methyl ketone/secondary alcohol	Alcohol dehydrogenase	NADPH oxidoreduction	8, 9

(1) Bonnarme et al., 2000; (2) Bonnarme et al., 2001; (3) Boumerdassi et al., 1997; (4) Broadway et al., 1992; (5) Cogan, 1981; (6) Curtin et al., 2001; (7) Engelvin et al., 2000; (8) Fadda et al., 2002a; (9) Fadda et al., 2002b; (10) Frankenberger and Poth, 1988; (11) Gao et al., 1997; (12) Gummalla and Broadbent, 1999; (13) Gummalla and Broadbent, 2001; (14) Hawes et al., 1995; (15) Hummel et al., 1986a,b; (16) Nakazawa et al., 1977; (17) Seefeldt and Weimer, 2000; (18) Smacchi and Gobbetti, 1998; (19) Ward et al., 1999; (20) Wiesenborn et al., 1989; (21) Williams et al., 2001; (22) Yvon et al., 1997; (23) Zhao et al., 1994.

several cheese-related strains in genera including *Lactobacillus*, *Lactococcus*, *Streptococcus*, *Brevibacterium*, *Corynebacterium*, *Arthrobacter* and *Brachybacterium* (Curtin et al., 2001). Under simulated Cheddar cheese ripening conditions tyrosine and phenylalanine aminotransferase activities of two strains of *Lb. casei* and two strains of *Lb. helveticus* did not differ, while *p*-hydroxy phenyl lactate and phenyl lactate dehydrogenase activities of *Lb. casei* were significantly greater than those of *Lb. helveticus* (Gummalla and Broadbent, 2001). Tryptophan aminotransferase, indole lactate dehydrogenase and tryptophan decarboxylase were also shown to vary between strains of *Lb. casei* and *Lb. helveticus* (Gummalla and Broadbent, 1999). From a Cheddar cheese, Williams et al. (2001) isolated 29 lactobacilli belonging to the species *Lb. paracasei*, *Lb. curvatus*, *Lb. brevis*, *Lb. plantarum*, *Lb. rhamnosus* and *Lb. lactis* and measured the aminotransferase activity with leucine, phenylalanine and methionine. These authors detected both intra- and inter-species differences, with *Lb. paracasei* tending to have the highest activity. Two *Lb. paracasei* failed to produce thiols from methionine. Aromatic aminotransferase activities were also shown to vary between strains of lactococci (Gao et al., 1997).

Studies dealing with peptidolytic activities are abundant and have already been extensively reviewed (Fox et al., 1995; Gonzales and Robert-Baudouy, 1996; Christensen et al., 1999; Gagnaire et al., 1999). Peptidase activities varied between lactic acid bacteria, which have therefore more or less enzymic capacity to contribute to amino acid generation and flavour formation during cheese maturation (Arora and Lee, 1989; Lemée et al., 1998; de Palencia et al., 2000). Peptidase activities were measured with *p*-nitroanilide derivatives, that releases a chromogen, after hydrolysis of the peptidic bond, which can easily be spectrometrically measured (Arora and Lee, 1989; Williams et al., 1998; de Palencia et al., 2000; Joutsjoki et al., 2002). A method for screening diacetyl- and acetoin-producing bacteria was described by Phalip et al. (1994). The screening was performed on agar plates by visualizing, in presence of creatine, the formation of a red insoluble complex resulting from the reaction between acetoin and/or diacetyl and α-naphthol. A 96-well plate spectrophotometric assay for branched-chain amino acid aminotransferase was developed by Cooper et al. (2002). The aminotransferase activity was analysed by enzymatic determination of the concentration of keto acids. The assay was based on the decrease of absorbance at 340 nm due to the NADH oxidation in presence of leucine dehydrogenase and ammonia. The interference of glutamate dehydrogenase (GDH) was minimised by the use of a limited amount of ammonium and a GDH inhibitor.

The time-consuming preparation of cell-free extracts can be significantly reduced by using high-throughput mixers for disrupting biological materials, although the yield is reduced in comparison with traditional procedures. Cell extracts can be used for strain identification by SDS-PAGE electrophoresis and for screening of enzyme activities, as shown by Tsakalidou et al. (1994) for strains isolated from traditional Greek-dairy products. In some cases, the enzyme activity can be directly determined on non-denaturing gels. Zymograms were reported for the detection of aminotransferase activities (Gao et al., 1997; Thierry et al., 2002). Moreover, the use of cell permeabilisation techniques offers alternatives to the preparation of extracts. Toluene was used as permeabilising agent to measure the activity of fumarate hydratase of *Lactobacillus*, *Enterococcus*, *Streptococcus*, *Lactococcus* and *Leuconostoc* species (Jung et al., 1993). The efficiency of the permeabilisation of *S. thermophilus* with several detergents (Somkuti and Steinberg, 1994), and of *S. thermophilus* and *Lb. delbruckii* ssp. *bulgaricus* with ethanol (Somkuti et al., 1998) was studied, showing under optimal treatment a 15-fold increase in β-glactosidase activity.

3.2. Physico-chemical analysis

3.2.1. Liquid chromatography

Liquid chromatography can be used to simultaneously monitor the concentrations of organic acids, diacetyl and acetoin (Guerrant et al., 1982; Amárita et al., 2001b; Klein et al., 2001; Sarantinopoulos et al., 2001; Zeppa et al., 2001). High-pressure liquid chromatography (HPLC) is well adapted to the study of glycolysis and fermentative pathways, as well as to the study of the first intermediates in the degradation of amino acids. Indeed, α-ketoisocaproate, 3-methyl-2-oxovalerate, 3-methyl-2-oxobutyrate, 4-methylthio-2-oxobutyrate, β-phenyl pyruvate, *p*-hydroxyphenyl pyruvate can be detected after chromatographic sep-

aration with ion exchange or reverse-phase chromatographic separations. The production of these keto acids is related to the aminotransferase activities, and, consequently, to the production of flavours such as aldehydes, alcohols and carboxylic acids. The carboxylic acids derived from keto acids can also be measured by HPLC.

Automation of sample preparation procedure can be performed in a 96-well plate format (Jemal, 2000) and several instrumental channels can be used in parallel (Feng et al., 2001). Nevertheless, the risk of spoiling the chromatography column is relatively high with complex microbiological samples. Therefore, it is generally necessary to extract the sample and to perform long runs, limiting the extent of the screening capabilities.

3.2.2. Thin layer chromatography

Thin layer chromatography (TLC) can be applied to the detection of taste components such as organic acids and sugars. TLC is a tool used mainly to study glycolysis, fermentations and catabolism of amino acids. TLC has been used to study the production of indole pyruvate, indole acetate, indole-3-aldehyde and 4-hydroxylbenzaldehyde by lactococci (Gao et al., 1997), and to identify bifidobacteria at the genus level based on their organic acid production (Lee et al., 2001). Planar chromatography is highly flexible, allowing the simultaneous analysis of compounds of different chemical nature, as well as the parallel analysis of many samples. This technique reduces the time-consuming preparation of samples because the unique usage of the stationary phases. Chromatogram development allows the quantitative analysis of the compounds detected on plates.

3.2.3. Gas chromatography (GC)

Several methods, each with their advantages and drawbacks, have been developed for extracting, concentrating and injecting volatile compounds into gas chromatograms (for a review, see Mariaca and Bosset, 1997). Steam, high vacuum and molecular distillation were applied, but these procedures are time-consuming. Moreover, unstable compounds can decompose thermally if the distillation is not performed under reduced pressure. Distillation techniques are more adapted to the study of low-volatile components such as phenols, free fatty acids, ketones, and long-chain aldehydes, ketones, alcohols and esters.

Static headspace analysis by direct injection of the headspace into the GC column is restricted to the analysis of the most volatile and concentrated components, or to the analysis of compounds that elicit strong detector response. This technique considerably reduces the time of analysis and sample preparation. Static headspace analysis has often shorter analysis times than HPLC techniques and presents good reproducibility of quantitative results. However, this technique fails to detect poorly volatile compounds that could have a high aroma value.

Dynamic headspace techniques concentrate the volatiles by purging the sample with a carrier gas (stripping techniques). With the purge and trap procedure, the purged volatiles are first adsorbed onto an inert support (usually a Tenax column), and then thermally desorbed and concentrated once again by crystallisation in a cold trap (cryofocusing) before thermic injection onto the GC-column. This method is very sensitive and is therefore subject to severe contamination from the culture medium, chemicals and water. Many peaks are thus produced. It is therefore necessary to work in parallel with blanks, using a mass spectrometer to identify the numerous unknown peaks and also to thoroughly wash the cultures with an appropriate buffer and to resuspend the cells in a minimal medium containing high-pure reagents. Volatile metabolites of *Pseudomonas aeruginosa* were analysed with a purge and trap procedure, confirming the production of the flavours 2-heptanone, 2-nonanone, 2-undecanone (Labows et al., 1980; Zechman and Labows, 1985). Lactic acid bacteria were compared on the basis of neutral volatile compounds produced in whey using a distillation technique (Mauriello et al., 2001). Based on the production of flavour volatiles, strains could be differentiated at two taxonomic levels: between species and within species.

Solid-phase microextraction (SPME) is a more recent alternative to extract the volatile fraction. The selectivity of SPME fibres towards chemicals and the lack of robustness are the limiting factors of this method. This method has been used to evaluate the volatile metabolites produced by *Staphylococcus xylosus* and *S. carnosus* (Vergnais et al., 1998). The procedure used by these authors allowed the detection

of ethyl esters, 3-methyl butanal, 3-methyl butanol, 3-methyl butanoate, phenyl pyruvate, phenyl acetaldehyde, phenethanol, 2-pentanone, hexanol, hexanoate and alcanes from 1.5 ml of resting cells at a final optical density of 1.0, demonstrating the sensitivity of SPME fibers, which can be applied to liquid and gas samples. SPME drastically simplifies the sample preparation procedure, and therefore seems to be well adapted to screening purposes. However, the reproducibility from one sample to an another due to the ageing of the fiber and from one fiber to the another one for quantitative measurements remains nevertheless a well-known problem.

3.2.4. Electronic nose

Electronic noses are not used to analyse individual compounds after chromatographic separation, but to analyse the sum of volatiles by injecting them together into a mass spectrometer (for a review, see Hodgins and Simmonds, 1995; Schaller et al., 1998). The instrument mimics the functionalities of the biological nose, which integrates the sum of total volatile compounds to an aroma perception. The main advantage of this technique is its speed. The signals produced from each sample are statistically interpreted by multivariate analysis, grouping samples that have similar profiles of volatile production. Appenzell, Tomme vaudoise, Tête de Moine, Tilsit and Gruyère cheese samples were clustered separately on the basis of their content of volatile compounds (Mariaca and Bosset, 1997). Arnold and Senter (1998) compared *Salmonella enteritidis*, *Escherichia coli*, *Enterobacter cloacae*, and *P. aeruginosa* by means of an electronic nose and found that these species formed distinct clusters.

This method could be adapted to the clustering of a large number of bacterial strains, but, to our knowledge, has never been used for such a purpose. However, this technique does not identify compounds nor provide quantitative information.

4. Selection and identification of flavour-producing strains

4.1. Selection of flavour-producing strains

Strains should be selected from a cheese with high organoleptic properties. Indeed, it is possible that a product with a high-quality aroma houses bacterial strains of great interest in comparison with standard quality products of the same age. Fitzsimons et al. (1999) showed by molecular profiling that the majority of isolates from premium-quality Cheddar cheese clustered together and apart from groups of strains from defective-quality cheeses. It is therefore important that the cheese samples are organoleptically assessed before selection. Moreover, chemical analysis of cheeses may help to determine the chemical compounds responsible for the cheese aroma. These data may help to choose the selective growth medium. However, the lack of data dealing with selective enrichments shows the difficulty of this approach.

The ability to utilize a given compound as energy source, and therefore to acquire growth competitive advantages, constitutes a good selection factor. Candidates are the above-mentioned amino acids and their corresponding keto acids, whose catabolism produces aroma compounds. However, this approach is not very promising because of the complex growth requirements of lactic acid bacteria. Therefore, three stages are usually necessary: (i) isolation of strains with specific media, (ii) preselection with molecular tools of strains having genotypes related to those of known aroma producers, and (iii) analysis of their activity.

4.2. Molecular tools

4.2.1. Molecular profiling

4.2.1.1. ARDRA. Amplified ribosomal DNA restriction analysis (ARDRA) is a rapid technique based on the polymerase chain reaction (PCR) amplification of the gene encoding 16S rRNA with universal primers and restriction of the amplicon, usually tetrameric endonucleases to generate multiple restriction fragments. Moyer et al. (1996) used a computer-simulated restriction profile analysis to determine the number of taxa which could be differentiated among a selection of 106 bacterial 16S rDNA sequences deposited in the Ribosomal Database Project (Maidak et al., 1994). The combination of two tetrameric endonucleases allowed the detection of 96% of the taxa. The mean sequence identity of the undiscriminated taxa was 95,6%, which is below the percentage used as criterion to define species. Indeed, strains of the same species have usually more than 70% of DNA–DNA

reassociation values, corresponding to more than 97% 16S rDNA sequence identity (Stackebrandt and Goebel, 1994). The discriminative power of ARDRA is therefore limited because it is close to the genus-species level. ARDRA patterns using four restriction endonucleases were successfully used to identify strains of the *Lb. acidophilus* and *Lb. casei* complex, and a single restriction enzyme was found to discriminate *Lb. delbrueckii* ssp. *bulgaricus* and *lactis* (Roy et al., 2001).

4.2.1.2. ISR. The use of the 16S–23S rRNA intergenic spacer region ISR (for a review, see Gürtler and Stanisich, 1996) is an alternative to ARDRA for detecting heterogeneity between and within species. Heterogeneity has been found in terms of both the number and the length of the spacers. For example, three ISR alleles, each of them with various copy numbers, were found in *Enterococcus faecalis* (Gürtler et al., 1999). At least six ribosomal RNA loci belonging to two ISR alleles are present in the chromosome of *Lb. delbrueckii* (Moschetti et al., 1997). The size of the spacers were shown to vary between species and subspecies of *Propionibacterium* (Tilsala-Timisjärvi and Alatossava, 2001). This approach has therefore broad applicability as a rapid, automatable method for bacterial identification and typing. The spacer is amplified by PCR with primers designed to anneal to conserved regions flanking the 16S–23S rRNA ISR, usually located at the 3′-end and 5′-end of 16S and 23S rDNA, respectively.

The discriminative power of this method was shown to be at the species level (Jensen et al., 1993). These authors performed a clustering of PCR-produced 16S–23S spacer patterns of over 300 strains belonging to *Listeria*, *Staphylococcus*, *Salmonella* and additional related strains. Drake et al. (1996) showed that 9 of 16 *Lb. helveticus* strains were differentiated by ISR PCR amplification. Because of the relatively small size of the amplicons, restriction analysis does not significantly improve the differentiation, as shown with propionibacteria of dairy origin (Rossi et al., 1997) and with streptococci (Moschetti et al., 1998).

4.2.1.3. Ribotyping. Ribotypes are obtained after restriction of genomic DNA by an endonuclease (generally a 6-bp recognizing enzyme), separation of the restriction fragments by agarose gel electrophoresis, southern transfer to a membrane and finally hybridisation with a labelled 5S, 16S or 23S rDNA probe. The patterns depend on the copy number and on the position of the rRNA operons on the genome. The number of rRNA operon copies is related to the ecological strategies of bacteria, those growing rapidly having a higher number of copies (Klappenbach et al., 2000). Hybridisation with rDNA probes generates between four and seven bands for *Lc. lactis* ssp. *lactis* and *cremoris* (Basaran et al., 2001), and between two and eight bands for *Lactobacillus* species and subspecies (Ferrero et al., 1996; Zhong et al., 1998). Commercial automated systems are now available, reducing the intensive bench work. Although ribotyping was used to differentiate *Lb. helveticus* retrieved from Grana and Provolone cheeses (Giraffa et al., 2000) and to cluster *Lactobacillus* species and subspecies (Moschetti et al., 1997; Zhong et al., 1998; Ryu et al., 2001), this method is not highly discriminative but is highly reproducible.

The discriminative power can be greatly enhanced by using insertion sequence (IS) as a DNA probe. IS*1201* was used to analyse the genotypic diversity of 74 *Lb. helveticus* strains isolated from Italian cheeses, resulting in good strain differentiation (Giraffa et al., 2000).

4.2.1.4. RAPD. Genotyping at the strain level has been performed by arbitrary primed PCR (AP-PCR) or random amplified polymorphism DNA (RAPD). RAPD was successfully used to differentiate *Lb. helveticus* (Drake et al., 1996), *Lb. plantarum* (Johansson et al., 1995), *Pediococcus* (Simpson et al., 2002), *Lb. rhamnosus* and *casei* (Tynkkynen et al., 1999) strains. Streptococci from different geographical and dairy origins were also differentiated (Moschetti et al., 1998). In this method, PCR amplification is realized with only one short primer, usually 10–12 bp long. The number of DNA fragments generated is suitable to perform similarity analysis and to build a dendrogram. This technique is discriminative and rapid, but is subject to poor reproducibility due to subtle changes in reaction conditions as *Taq* polymerase, primers, Mg^{2+}, buffer concentration, efficiency of cell lysis and DNA extraction, and in the amount of exopolysaccharides produced by the culture. To overcome

these limitations, Cusick and O'Sullivan (2000) performed AP-PCR in triplicate.

4.2.1.5. rep-PCR. Repetitive sequences are interspersed extragenic elements found in several regions of bacterial chromosome. PCR amplification with primers annealing to these repetitive elements generates profiles specific for the organism studied (Versalovic et al., 1994). REP, ERIC and BOX sequences have been found in genomes of *E. coli* and *Salmonella typhimurium*, of enterobacteria, and of *Streptococcus pneumoniae*, respectively. REP and ERIC primers are generally ineffective for fingerprinting Gram-positive bacteria (Versalovic et al., 1991; Richard et al., 2001). Nevertheless, they have been used by several authors. Enterobacterial repetitive intergenic consensus (ERIC) sequences were used as primer binding sites to genotype *Bifidobacterium* (Shuhaimi et al., 2001) and *Listeria monocytogenes* (Sciacchitano, 1998; Jersek et al., 1999). Repetitive extragenic palindromic (REP) elements were also widely used to achieve molecular profiling of lactic acid bacteria. The population structure of lactobacilli in Comté cheese was determined with ERIC and REP-PCR fingerprinting of isolates. Mesophilic lactobacilli (488) originating from raw milk were assigned to 44 different strains and 3 different species (Berthier et al., 2001).

BOX elements are composed of three different subunits, namely boxA (57 bp), boxB (43 bp), and boxC (50 bp). BoxA unit is conserved and has been PCR amplified from many bacterial species (Koeuth et al., 1995). BOX-PCR was shown to differentiate at the subspecies level *Enterococcus faecalis* (Malathum et al., 1998), *Lb. delbrueckii* ssp. *lactis* and *bulgaricus*, and *S. thermophilus* (de Urraza et al., 2000).

Urbach et al. (1998) designed an alternative LL-Rep1 primer to complement repeated sequence found in lactococci, as well as in low % G + C Gram-positive bacteria and showed that this technique can detect genetic differences among closely related strains. Gevers et al. (2001) analysed 30 lactobacilli strains and found that the number of DNA fragments produced by BOX and REP-PCR was comprised between 0 and 6, and between 1 and 10, respectively. These authors increased the average number of bands per PCR profile to 16.5 by using the primer $(GTG)_5$. The *rep*-PCR fingerprinting technique using $(GTG)_5$ primer was shown to be a rapid and reproducible tool to differentiate lactobacilli at the species, subspecies, and potentially the strain level. *rep*-PCR is more reproducible than RAPD profiling (Gao et al., 1996).

4.2.1.6. PFGE. Pulsed-field gel electrophoresis is considered to have both good reproducibility and resolving power for typing bacterial strains. In this technique, the bacterial chromosome is digested with a statistically rare-cutting endonuclease, generating large DNA fragments, which are separated with specifically orientated electric pulses. To avoid shearing of the fragments, all manipulations must be performed in agarose plugs. This technique has the disadvantage of being labor intensive. Its discriminative power was demonstrated for streptococci (Moschetti et al., 1998; O'Sullivan and Fitzgerald, 1998), for *Lb. rhamnosus* and *casei* (Ferrero et al., 1996; Tynkkynen et al., 1999), for *Lb. delbrueckii* subsp. *bulgaricus* (Moschetti et al., 1997), for *Enterococcus faecium* (Bedendo and Pignatari, 2000), for *Lb. helveticus* (Lortal et al., 1997), and for *Pediococcus* strains (Simpson et al., 2002). This technique was shown to be effective in studies dealing with the determination of lactobacilli and bifidobacteria in human feces (McCartney et al., 1996; Kimura et al., 1997).

4.2.1.7. Discriminatory power. The discriminatory power of molecular profiling methods has been compared. In general, PFGE and *rep*-PCR are the most sensitive methods, followed by ribotyping and RAPD, ISR, and ARDRA (Chachaty et al., 1994; Moschetti et al., 1997, 1998; Malathum et al., 1998; Tynkkynen et al., 1999; Bedendo and Pignatari, 2000; Simpson et al., 2002). Phylogenetically, ISR sequences used in ribosomal intergenic spacer analysis was shown to distinguish closely related strains where 16S rDNA lacks resolution (Tilsala-Timisjärvi and Alatossava, 2001). PFGE is considered as an excellent method for genotyping of bacteria at the subspecies level, but is time-consuming and labor intensive. Malathum et al. (1998) did not find significant differences in the discriminatory power between PFGE and *rep*-PCR with *Enterococcus faecalis* strains, while more *Enterococcus faecium* genotypes were identified with REP-PCR than with PFGE (Bedendo and Pignatari, 2000). Although *rep*-PCR is less time-consuming, results are more difficult to interpret because some products are inconsistently seen and multiple weak

bands are present on gel (Malathum et al., 1998; Bedendo and Pignatari, 2000). In Comté cheese, changes in the population of *Lb. helveticus* and *Lb. delbrueckii* subsp. *lactis* during ripening were followed by RAPD, REP-PCR and PFGE (Bouton et al., 2002). The different methods generated slight differences in the composition of molecular clusters.

4.2.2. Hybridisation and PCR

Hybridisation and PCR are rapid means to simultaneously analyse a high number of strains. However, these techniques are subjected to limitations by the difficult-to-lyse nature of the lactic acid bacterial cell wall. To our knowledge, probes directed at genetic targets concerned with the production of flavours have never been reported. However, the occurrence of certain metabolic activities in only a few strains, particularly those involved in the catabolism of amino acids and in the production of methyl ketones, strongly suggest the possibility to detect specific sequences by hybridisation or PCR. Moreover, the increasing amount of available genome sequences should facilitate the design of specific probes.

5. Future developments

Until recently, the classification of cheeses was based mainly on the absence of faults, and was therefore referred to a negative classification. The aspect, the presence of potentially harmful bacteria and the divergence from a previously defined standard were parameters used to qualify a cheese. At present, cheese manufacturing is perfectly controlled and consequently deviation in the overall quality is very low. The research of faults is well adapted for the quality control of industrial productions, but is not satisfying for products with local or artisanal specificities, for which a positive classification, emphasizing the character and the originality of the product, is desired. The utilization of bacteria with enhanced capabilities to produce a pleasant flavour is therefore promising and will become increasingly important in the near future.

Until recently, dairy microbiologists focused mainly on molecular profiling tools to produce a detailed description of the structure of the bacterial populations in dairy products, but failed to assess in parallel their potentialities for flavour development. For this reason, it is now necessary to investigate the relationship between the genotypes and the capability of the corresponding strains to generate aroma, and thus to utilise this molecular information for the detection of new isolates with potential use for cheese manufacture.

New perspectives arise from genome-sequencing programmes. Data derived from whole genome sequences together with the numerous information already available for lactic acid bacteria open opportunities to the understanding of aroma formation. Functions can be attributed to open reading frames (ORFs) either experimentally or by bioinformatic comparison tools such as BLAST (Altschul et al., 1990) or FASTA (Pearson and Lipman, 1988; Pearson, 1990). Proteins showing 25% or more sequence identity are likely to have a common ancestor and therefore to perform similar functions (Wipat and Harwood, 1999). These data can be used as reference material in a gene expression profiling assay, such as microarrays, which allow the assessment of transcription profile of a given organism on the genomic scale.

The discovery of new information concerning genes involved in the production of flavours will not only bring a detailed understanding of their expression and function, but will also facilitate the extension of techniques used to identify the flavour-producing strains and the design of expression systems devoted to the elaboration of new strains by recombinant technology. *Lactococcus* starters recombined with peptidases from *Lactobacillus* have been used to increase the peptidolytic activity during cheese ripening (Courtin et al., 2002; Joutsjoki et al., 2002).

An alternative to the time-consuming and labor intensive screening procedures is the development of programs of strain improvement in order to modify their metabolic capabilities. While yields of primary metabolites can now be readily improved by metabolic engineering, changes in the biosynthesis of secondary metabolites are more difficult because the complexity of the corresponding metabolic networks. The classical strain improvement techniques have relied on mutation and random screening of strains, but recently promising techniques, such as genome shuffling, which can lead to rapid phenotypic improvements in bacteria, are emerging.

The current review highlights the great diversity of approaches which can be applied to the detection and

ultimately to the isolation of better aroma producers. Genotypic approaches could have the potential of high-throughput screening procedures but there is a need to determine the relationship between the phenotypes, assessed by chemical methods, and the genotypes. Gene expression profiling will certainly play an increasing part in screening strategies as they become accessible both in terms of price and of availability of genome sequences. Strain improvement will certainly also become an increasingly important tool to engineer bacterial strains with the required characteristics.

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