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Conversion of amino acids into aroma compounds by cell-free extracts of *Lactobacillus helveticus*

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Aims: *Lactobacillus helveticus* is an essential starter in Swiss-type cheeses such as Emmental. This study was to determine whether cell-free extracts of *Lact. helveticus* were able to convert free amino acids into neutral volatile aroma compounds at the pH and temperature occurring in cheese.

Methods and Results: A mix of branched-chain (Leu, Ile, Val), aromatic (Tyr, Phe) and sulphur (Met) amino acids was incubated for 7 days, at pH 5·7 and 24°C, with cell-free extracts of six strains. The amino acids were all transaminated into the corresponding keto acids when an amino group acceptor (α-ketoglutaric acid) was provided. Phe and Tyr were transaminated the most efficiently, followed by Leu, Met, Ile and Val. Three major volatile compounds were detected by GC-MS: benzaldehyde, dimethyl disulphide and 2-methyl propanol. Whatever the strain, benzaldehyde was produced in the highest quantity (0·25–1 μmol l^{-1} mg^{-1} protein).

Conclusions, Significance and Impact of the Study: *Lactobacillus helveticus* intracellular enzymes could significantly contribute to the production of aroma compounds from amino acid catabolism.

INTRODUCTION

Lactobacillus helveticus is an essential starter in Swiss-type cheeses such as Emmental (400 000 tons per year produced worldwide). This species contributes to milk and curd acidification by consuming galactose, to proteolysis by its cell-wall-located proteases, and to the production of free amino acids by the activity of its intracellular peptidases released after lysis (Valence *et al.* 2000). Free amino acids can then be converted into cheese aroma compounds or, sometimes, into off-flavour compounds, by bacterial enzymes and/or non-enzymatic reactions (Keeney and Day 1957; Christensen *et al.* 1999). Decarboxylation, deamination and transamination have frequently been cited as possible mechanisms. Recently, the conversion of branched-chain, sulphur and aromatic amino acids by lactococci was shown to be essential in cheese flavour development (Yvon *et al.* 1997, 1998; Gao *et al.* 1997; Rijnen *et al.* 1999b). Several aminotransferases of cheese-related micro-organisms, such as lactococci, *Brevibacterium linens* and mesophilic lactobacilli have been characterized (Lee and Desmazeaud 1985; Yvon *et al.* 1997, 2000; Gao *et al.* 1998; Rijnen *et al.* 1999a). In contrast, the potential contribution of *Lact. helveticus* to the production of flavour compounds has rarely been studied. Some authors reported that *Lact. helveticus* contributed particularly to the burned and nutty flavours of Swiss cheeses (Biede *et al.* 1979; Paulsen *et al.* 1980; Scolari *et al.* 1985; Kowalewska *et al.* 1985). When cultured on milk, *Lact. helveticus* can produce high quantities of various ketones, aldehydes and sulphur compounds, but the metabolic pathways remain unknown (Imhof *et al.* 1995). Regarding amino acid conversion by *Lact. helveticus*, the production of acetaldehyde from threonine has been described previously (Hickey *et al.* 1983), as the production of methanethiol from methionine (Dias and Weimer 1998). The formation of potential off-flavours from tryptophan catabolism by this species was recently highlighted (Gummala and Broadbent 1999).

As lysis of *Lact. helveticus* in Swiss cheeses has been demonstrated previously (Valence *et al.* 1998, 2000), the aim

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of the present study was to investigate whether cell-free extracts of *Lact. helveticus* were able to convert branched-chain (Leu, Ile, Val), aromatic (Tyr, Phe) and sulphur (Met) amino acids into aroma compounds at the pH and temperature occurring during cheese ripening.

MATERIALS AND METHODS

Chemicals

Amino acids, ketoacids, hydroxyacids, branched-chain acids, branched-chain aldehydes and alcohols, pyridoxal 5′-phosphate (PLP) and thiamine pyrophosphate chloride (TPP) were obtained from Sigma-Aldrich; oxalic acid was obtained from Prolabo.

Bacterial strains and growth conditions

Six strains of *Lact. helveticus* were used in this study (Table 1). They were stored at −80°C in MRS broth (Difco) containing 15% (v/v) glycerol. Three transfers at 1% (v/v) in MRS broth during 15 h at 37°C were performed before the cells were grown statically in 1 litre MRS broth at 37°C.

Table 1 Origin of the *Lactobacillus helveticus* strains

Strain name	Source
LRTL* 735	Isolated from an industrial lactic starter, France
LBLH2	DSM Gist-brocades Food Specialties, Australia
ISLC† 5	Isolated from Grana serum, Italy
CP 615	Provided by Pr. Yamamoto, Japan
CNRZ‡ 32	Artisanal Comté lactic starter, France
L§ 112	Artisanal Comté lactic starter, France

*Collection of this laboratory.
†Collection of the Instituto Latterio Caesario, Lodi, Italy.
‡CNRZ collection, INRA Jouy en Josas.
§Collection of INRA Poligny.

Growth was monitored by measuring absorbance at 650 nm ($O.D._{650}$) using a model DU 7400 spectrophotometer (Beckman Instrument Inc., Fullerton, CA, USA).

Preparation of cell-free extracts (CFE) and incubation conditions

Cells harvested in the exponential growth phase (at an $O.D._{650}$ of 1·0) were washed twice with cold, sterile distilled water, and recovered by centrifugation for 20 min at 18 900 *g* at 4°C. The cell pellets were stored at −20°C overnight in order to fragilize the cells. They were then suspended in 50 ml cold, sterile distilled water before mechanical disruption, at 106 MPa, in a refrigerated French pressure cell (SLM-Aminco, Urbana, IL, USA) under the conditions described by Valence and Lortal (1995). Undisrupted cells and cell debris were removed by centrifugation at 39 200 *g* for 30 min at 4°C, and the collected supernatant fluid was filtered (0·45 μm then 0·20 μm, Sartorius, Laboratoires Humeau, La Chapelle S/Erdre, France), distributed in sterile tubes (1 ml), and stored at −20°C until use. For all the strains, the protein content of CFE was determined according to the method of Lowry *et al*. (1951), using bovine serum albumin (Sigma) as standard.

Preparation of the mix of amino acids

A mix of different amino acids was prepared in 50 mmol l^{-1} potassium phosphate buffer pH 5·7. The concentration of each amino acid was fixed according to the content and the existing ratio of free amino acids in Emmental cheese juice, i.e. Met/Ileu/Leu/Val/Phe/Tyr: 1/2/4/4/1·5/0·8 (unpublished data). The methionine content at entry into the warm room was 3·5 mmol l^{-1}; the concentrations of the other amino acids in the mix are reported in Table 2. A volume of 100 mmol l^{-1} α-ketoglutaric acid was added as an acceptor of amino groups for transamination; PLP and TPP were also added at 50 μmol l^{-1} each.

Table 2 Decrease in free amino acid and α-ketoglutaric acid content of the mix after 168 h of incubation at 24°C incubation with cell-free extracts (CFE) of two *Lactobacillus helveticus* strains (LRTL 735 and LBLH2), and concomitant formation of glutamic acid

	Valine	Isoleucine	Leucine	Methionine	Tyrosine	Phenyl-alanine	Total	α-Ketoglutaric acid	Glutamic acid
Initial content (mmol l^{-1})	13·5	7·2	13	3·5	2·8	5·3	45·3	100	nd
Consumption (mmol l^{-1}) by									
CFE LRTL 735 at 168 h	0·8	0·4	3·5	1·0	1·9	3·5	11·1	12·8	10·9
(% decrease)	6	5·5	27	28·5	68	66	24·5		
CFE LBLH2 at 168 h	0·5	1·3	4·0	0·4	1·9	3·7	11·8	15·8	11·4
(% decrease)	4	18	31	11·4	68	70	26		
Blank at 168 h (without CFE)	0·2	0·1	0·1	0·1	nd	nd	0·5	0	nd

nd, not detectable.

Conversion of amino acids by CFE of *Lact. helveticus*

The amino acid mix was incubated at 24°C with the CFE of each of the six strains. The cytoplasmic protein concentration of the mixture was 125 μg ml^{-1}, corresponding to about 5×10^8 cells. Samples were analysed at zero time, 24 h (day 1) and 168 h (day 7). Controls with boiled CFE (40 min at 100°C) and without α-ketoglutaric acid were included, as well as a blank without CFE. Two strains (LBLH2 and LRTL 735) were tested in three separated assays.

Conversion of β-phenylpyruvic acid into benzaldehyde

A volume of 10 mmol l^{-1} β-phenylpyruvic acid in 50 mmol l^{-1} potassium phosphate buffer pH 5·7 was incubated with three quantities of CFE: 125 μg ml^{-1} protein, 350 μg ml^{-1} and 475 μg ml^{-1} (final concentrations in the buffer). The same experiment was carried out with identical amounts of boiled CFE (40 min at 100°C). The benzaldehyde produced was quantified by GC-MS as described below.

α-Ketoacid and amino acid analyses

α-ketoglutaric acid and the transamination products of Ile, Leu, Val, Phe and Tyr (α-keto-methylvaleric acid, α-ketoisocaproic acid, α-ketoisovaleric acid, β-phenylpyruvic acid and p-hydroxyphenylpyruvic acid, respectively), were determined using a Beckman HPLC (Beckman Coulter Corp., Fullerton, CA, USA). They were separated using an ion exchange column packed with Aminex A-6 resin (Bio-Rad, Hercules, CA, USA) kept at 55°C, with 0·01N H_2SO_4 as eluent at a flow rate of 1·0 ml min^{-1}. Ultraviolet detection was performed at 210 nm. Amino acids were determined using an amino acid analyser (AlphaPlus serie2, Pharmacia, Uppsala, Sweden). In addition, glutamic acid was enzymatically determined with a Boerhinger kit (R-Biopharm, Darmstadt, Germany) to assay the kinetics of transamination.

Volatile compound analyses

Samples were thawed just before analysis, homogenized and diluted if necessary. Neutral volatile compounds were analysed by head-space GC-MS as previously described (Thierry *et al*. 1999). A 3 g aliquot of sample solution was weighed in a 35 ml non-fritted sparger. Briefly, compounds were trapped on a Vocarb 3000 trap (Supelco, Bella Fonte, PA, USA), thermally desorbed using helium as vector gas (1·4 ml min^{-1}) at 250°C (35°C for 5 min, then 5°C min^{-1} until 140°C, then 15°C min^{-1} until 250°C), and cryofocused at −100°C before being injected and separated on an HP5 capillary column (60 m × 0·32 mm, 1 μm film thickness) (Agilent Technologies, Palo Alto, CA, USA). They were detected by an HP7972A quadrupole mass spectrometer (Agilent Technologies) after ionization by electronic impact, and identified by comparison of spectra and retention times with those of reference compounds. Quantification was performed by regression analysis of the peak areas of total ion chromatograms and known concentrations of standard compounds.

Branched-chain volatile acids were analysed with a Varian 3800 gas chromatograph equipped with a flame ionization detector and a capillary column (25 m × 0·53 mm; 0·5 μm film thickness) coated with modified polyethylene glycol (BP21, SGE, Ringwood, Victoria, Australia) under the following conditions: cold on-column injection of 0·5 μl; detector temperature, 250°C; carrier gas, hydrogen; 30 ml min^{-1}; initial temperature programme, 50°C to 135°C at 6°C min^{-1}.

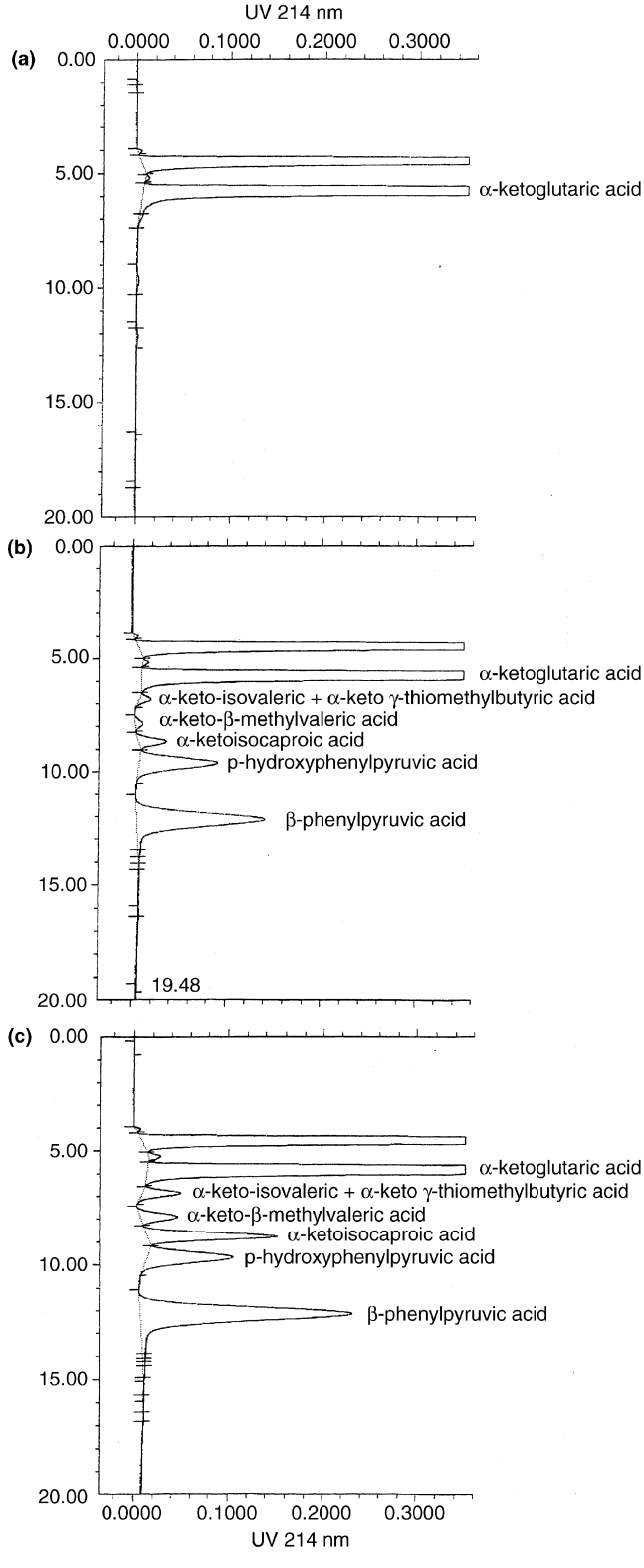
Sensory analysis of odour (sniffing)

All tubes containing the mixture with CFE or boiled CFE of *Lact. helveticus* LBLH2 were evaluated by sniffing after 24 h and 168 h of incubation. The sensory panel consisted of 15 assessors from the institute staff. The panel was asked to score the intensity of flavour using a 3-level scale (1 = weak, 2 = moderate, 3 = strong) and to describe it in free terms.

RESULTS

Amino acid conversion by CFE

The decrease in amino acid content, reflecting their conversion into other compounds, by two *Lact. helveticus* strains, LBLH2 and LRTL 735, is shown in Table 2. Phe, Leu, Tyr and, to a lesser extent, Met, Ile and Val, decreased for both strains. The amount of α-ketoglutaric acid also decreased and proportional amounts of glutamic acid were produced (Table 2), indicating a transamination reaction. Using boiled CFE, no decrease in amino acid was observed, showing the absence of any significant non-enzymatic conversion. Various α-keto acids were detected by HPLC after 24 and 168 h, including β-phenylpyruvic acid, α-keto-isocaproic acid and, in lower amounts, α-keto-isovaleric acid, α-keto-β-methylvaleric acid and p-hydroxyphenylpyruvic acid (Fig. 1). The keto acid corresponding to the transamination of methionine (α-keto methylthiobutyric acid) was co-eluted with the α-keto methylvaleric acid. For that reason, methionine transamination was further demonstrated by incubating methionine alone (3·5 mmol l^{-1}, in the same buffer as in the mix and in the presence of α-ketoglutaric acid) with LBLH2 CFE, and quantifying the glutamic acid produced (42 μmol l^{-1} after 24 h and 75 μmol l^{-1} after 168 h; data not shown). Thus, it can be



concluded that transamination of each of the six amino acids occurred. Since, in the absence of α-ketoglutaric acid, no significant decrease in amino acids was detected under the conditions used, transamination was the first and main step of amino acid conversion by *Lact. helveticus* CFE.

◀

Fig. 1 HPLC profiles of the keto acids produced at (a) 0, (b) 24 and (c) 168 h of incubation, reflecting amino acid transamination of Phe, Tyr, Leu, Ile and Val by cell-free extracts of *Lactobacillus helveticus* LRTL 735

Kinetics of transamination

The decrease in α-ketoglutaric acid and the increase in glutamic acid were quantified during incubation with six strains of *Lact. helveticus* (Fig. 2). Two phases of kinetics were observed: an initial high rate of transamination during the first day of incubation, which then slowed down until the end of the reaction. In the curves, the decrease in α-ketoglutaric acid and the increase in glutamic acid were obviously symmetric. Depending on the strain, 10–16 mmol l^{-1} glutamic acid were produced, whereas 10–18 mmol l^{-1} α-ketoglutaric acid were consumed. After 120 h, the rate of the reaction was greatly reduced for LBLH2, LRTL 735, CNRZ 32 and L 112. Two strains, however, still exhibited some significant aminotransferase activity after this time (ISLC5 and CP 615).

Volatile compounds produced

The profiles of neutral volatile compounds obtained at the end of incubation with the six strains were compared with the profiles obtained at zero time (Table 3). Among the compounds identified, five increased in concentration during the incubation: benzaldehyde (produced from Phe), dimethyl-disulphide (from Met), 2-methyl-propanol (from Val), 2-methyl-butanal (from Ile) and 3-methyl-butanal (from Leu).

Benzaldehyde was the main compound found and increased only in the presence of non-boiled CFE (data not shown), demonstrating that at least one step of phenylalanine degradation was enzymatic. All the tested strains produced high amounts of benzaldehyde, with variations of 1·5 between strains within the same experiment. However, between one experiment and another for the same strain, inexplicable variations of the same order were found. Dimethyl-disulphide and 2-methylpropanol increased during incubation only in the presence of non-boiled CFE, demonstrating that at least one step of Met and Val degradation was enzymatic. Both aldehydes (2- and 3-methylbutanal) showed a fivefold increase during incubation, independent of the presence of CFE, showing that they were produced from amino acids through a non-enzymatic mechanism.

Under the conditions used, no other volatile compound was produced from branched-chain and aromatic amino acids

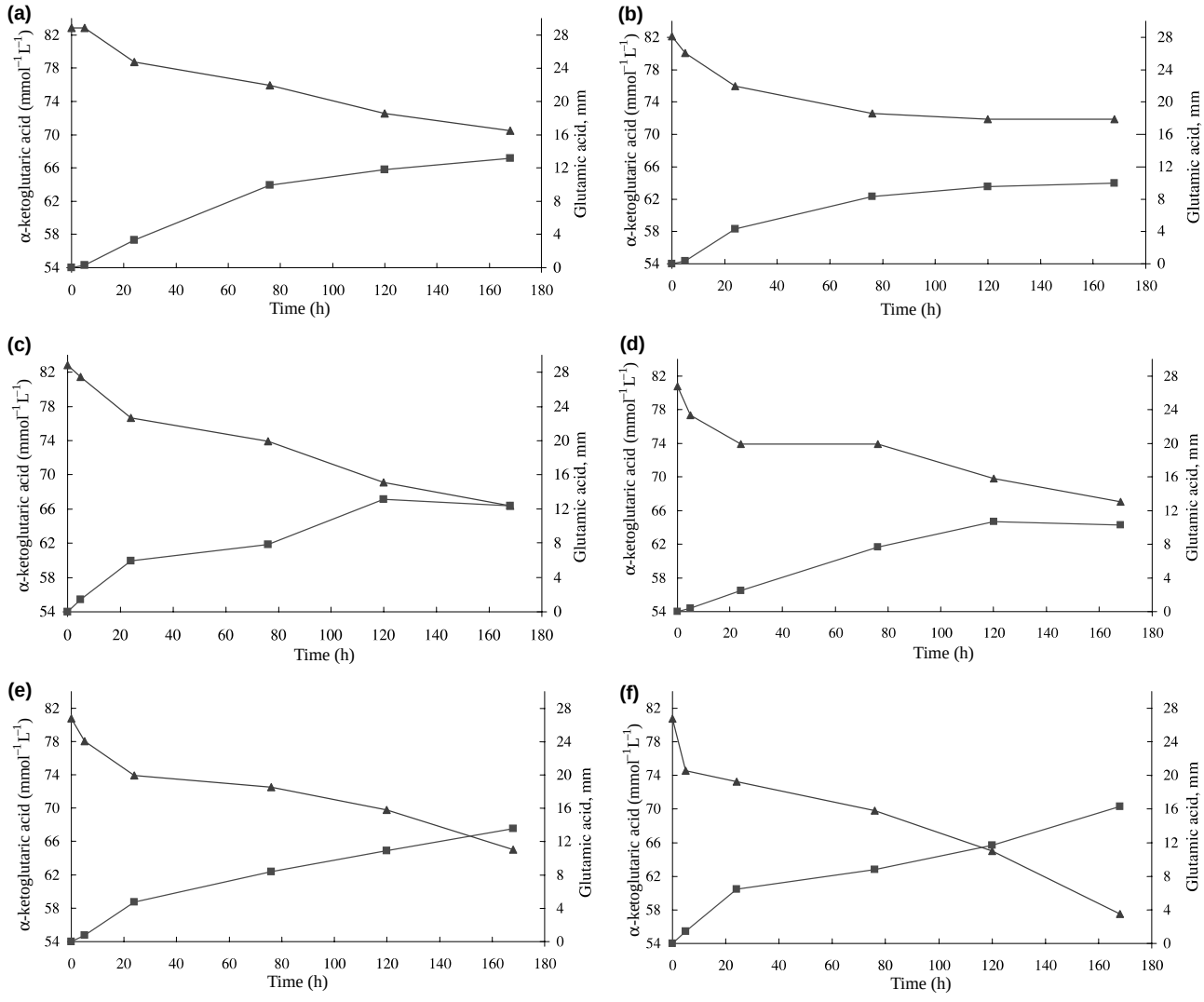


Fig. 2 Kinetics of transamination of a mixture of amino acids by cell-free extracts of six strains of *Lactobacillus helveticus*. Release of glutamic acid (■) through transamination and concomitant decrease of α-ketoglutaric acid (▲). (a) LRTL 735; (b) LBLH2; (c) CNRZ 32; (d) L 112; (e) ISLC 5; (f) CP 615

such as branched-chain acids (isobutyric, isovaleric, 2-methylbutyric, phenylacetic and hydroxy-phenylacetic acids), α-hydroxyacids and alcohols (2- and 3-methylbutanol).

Sniffing assay

The sniffing assay was carried out for the strain *Lact. helveticus* LBLH2. After 24 h of incubation, nine persons out of 14 detected an odour (weak intensity) which was qualified as 'light cheese, acid, yoghurt, honey, cooked sugar' by the different panellists. After 168 h, 13 persons out of 14 detected an odour (weak or moderate intensity for 11 persons; strong intensity for two persons). The odour was mainly qualified as 'bitter almond, solvent, acid, cooked sugar, yeast extract or bacterial culture'.

Production of benzaldehyde from β-phenylpyruvic acid

Various quantities of CFE from strains LBLH2 and LRTL 735, either boiled or non-boiled, were added to a solution of β-phenylpyruvic acid in order to follow the production of benzaldehyde. In the blank without CFE, no spontaneous degradation of β-phenylpyruvic acid into benzaldehyde occurred. Using boiled or non-boiled CFE, the quantities of benzaldehyde obtained were similar, indicating that the conversion of β-phenylpyruvic acid was very likely a non-enzymatic reaction (Fig. 3). However, the amount produced clearly increased with the quantity of CFE, which suggests that an intracellular component was essential for this chemical conversion. The amount of benzaldehyde differed signifi-

Table 3 Neutral volatile compounds produced from an amino acid mix incubated for 7 days at 24°C in the presence of the cell-free extracts of six *Lactobacillus helveticus* strains

Compound (μmol l^{-1})	Incubation time (h)	CFE*	Blank without CFE
Benzaldehyde	0	0·7 ± 0·4	< 0·01
	168	151 ± 23	0·4
Dimethyl-disulphide	0	0·02 ± 0·01†	< 0·01
	168	0·18 ± 0·13†	< 0·01
2-methyl-propanol	0	< 0·02	< 0·01
	168	1·5 ± 0·6‡	< 0·02
2-methyl-butanal	0	0·1 ± 0·01	0·1
	168	0·7 ± 0·03	0·4
3-methyl-butanal	0	0·2 ± 0·05	0·2
	168	1·1 ± 0·2	1·0

*Mean ± standard deviation of the values obtained for the six strains.
†Mean ± standard deviation of the values obtained for strains 735 and LBLH2.
‡Mean ± standard deviation of the values obtained for only five strains (no 2-methyl-propanol produced by FTL112).

cantly for the two tested strains, with a ratio of benzaldehyde to CFE proteins of 0·25 ± 0·05 and 0·48 ± 0·11 μmol l^{-1} mg^{-1} for LRTL 735 and LBLH2, respectively.

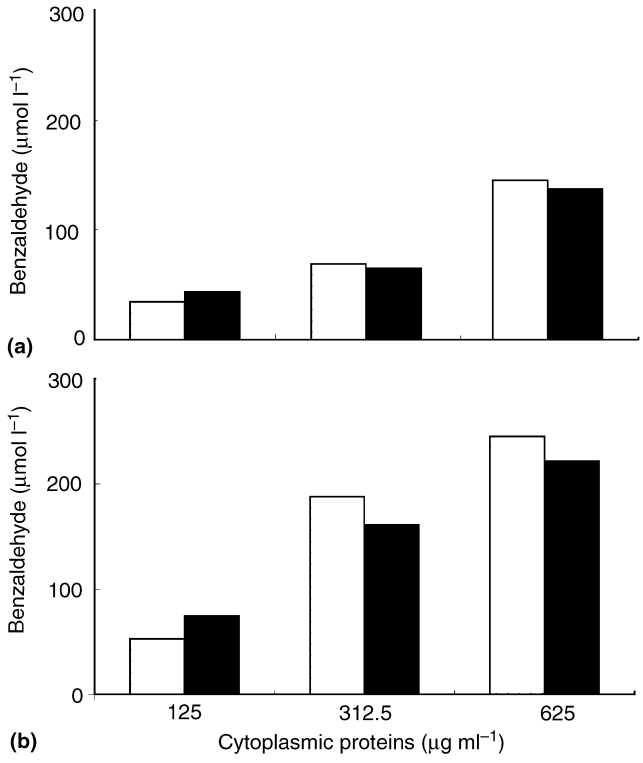


Fig. 3 Production of benzaldehyde from β-phenylpyruvic acid following the addition of various amounts of boiled (■) or non-boiled (□) cell-free extracts of two strains of *Lactobacillus helveticus*; incubation for 168 h at 24°C. (a) LRTL 735; (b) LBLH 2

DISCUSSION

In this work, the transamination of aromatic, branched and sulphur amino acids by cell-free extracts of *Lact. helveticus* under the pH and temperature for cheese ripening was demonstrated for the first time. Only methionine aminotransferase activity had previously been reported in *Lact. helveticus* CNRZ 32 (Dias and Weimer 1998). From a mixture of amino acids (Phe, Tyr, Met, Leu, Val, Ile) in molar ratios similar to those found in Emmental cheese, Phe and Tyr were the most efficiently converted (about 70% of the initial content) by aminotransferase activity, followed by Leu and Met. Among lactic acid bacteria, the aminotransferase activities of lactococci have been the most extensively studied; two aminotransferases have been characterized, AraT and BcaT, the first being active mainly on aromatic amino acids and on Leu, and the second on branched-chain amino acids, both being slightly active on Met (Yvon *et al.* 1997, 2000; Rijnen *et al.* 1999a). The present results could thus indicate the presence of an efficient aromatic aminotransferase in *Lact. helveticus.* The addition of PLP, a co-factor of aminotransferase, was not necessary, which would indicate either that this co-factor is tightly bound to the aminotransferase(s) (Kanda *et al.* 1995), or that it is present in sufficient amounts in the cell-free extracts. The kinetics of transamination over 7 days of incubation were similar for the six strains of *Lact. helveticus* from various countries and currently used in different cheese technologies (Emmental, Comté, Grana etc.). Only slight differences in the rate and total duration of the reaction were found. In all cases, the transamination rate decreased with time, although α-ketoglutaric acid and free amino acids were not in limiting amounts, indicating a progressive inhibition/denaturation of the transaminase(s) involved. However, for two strains of *Lact. helveticus*, CP 615 and ISLC5, the reaction was not finished after a week of incubation. Using standardized protein contents of the cell-free extracts, a 1·5-fold variation in the glutamic acid produced was observed between strains. This extent of variation is in agreement with previous work comparing the transaminase activity of various lactococci strains (Gao *et al.* 1997). As far as is known, the various keto-acids produced through transamination do not contribute much to cheese flavour, but could participate in the basic taste of cheese. Their degradation, however, led to the production of volatile compounds which are often involved in flavour.

The same volatile aroma compounds were found with the six strains: 2-methyl propanol, dimethyldisulphide and large amounts of benzaldehyde. Benzaldehyde and sulphur compounds, including DMDS, have already been observed by Imhof *et al.* (1995) with cells of *Lact. helveticus* grown in milk. Except for low amounts of 2-methyl propanol, no branched-chain acids and alcohols were found. These

compounds are produced during cheese ripening by lactic acid bacteria (*Lactococcus lactis*), but their formation probably requires the regeneration of co-factors such as NAD/$NADH^+$ (which were not added in our experiments), and/or may require a functional enzymatic complex such as α-keto acid dehydrogenase which would be inactive in our CFE. Benzaldehyde, dimethyl-disulphide and 2-methyl-propanol are commonly found in dairy products, including Swiss cheeses (Bosset and Liardon 1984; Maarse *et al*. 1994; Engels *et al*. 1997). Nevertheless, the fact that aminotransferase activities of *Lact. helveticus* were able to initiate, *in vitro*, the conversion of amino acids into these flavour compounds at a pH and temperature comparable with those of cheese ripening, does not prove that it occurs in cheese. This would require at least the presence of one amine acceptor for the transamination step. Further experiments are needed to correlate the presence of *Lact. helveticus* cells, either whole or lysed, with the *in situ* production of the compounds found in this work. To determine what compound(s) are still produced in cheese by enzymes liberated through lysis, and what compound(s) require active whole cells, is of high technological relevance when defining the right live/lysed ratio. Indeed, *Lact. helveticus* is used not only in Swiss cheese but also, widely, as an adjunct or attenuated starter in numerous other cheeses (Klein and Lortal 1999). It was selected primarily because of its high peptidasic activity for reducing cheese bitterness leading, in general, to higher flavour scores, but very little is presently known about the contribution of this adjunct in terms of aroma compounds (Wijesundera *et al*. 1997).

Benzaldehyde was produced by CFE of *Lact. helveticus* in quantities 50–100-fold higher than the other volatile compounds under our conditions. Its concentration was 50–5000-fold higher than its perception threshold in water (0·03–3·3 μmol l^{-1}; Sablé and Cottenceau 1999) and was very likely responsible for the 'solvent' and 'bitter almond' odours detected in the sniffing assay. As yet, the ability of lactobacilli to produce benzaldehyde from Phe has only been described in *Lactobacillus plantarum* (Nierop Groot and de Bont 1998). As in *Lact. plantarum*, even boiled CFE of *Lact. helveticus* led to benzaldehyde when added to phenylpyruvic acid. Moreover, the quantity of benzaldehyde produced was directly related to the amount of CFE added. Thus, this work shows that after the transamination of Phe, a non-enzymatic reaction is involved in benzaldehyde production. This reaction can be a chemical oxidation requiring the presence of at least one intracellular component, such as manganese proposed for *Lact. plantarum* and for other lactic acid bacteria (Nierop Groot and de Bont 1999). Interestingly, benzaldehyde is used as a flavouring compound in numerous products, giving a specific 'bitter almond' taste. In terms of quantity, it's industrial production worldwide is second after vanillin. The industrial process currently involves either chemical production or a direct extraction from almonds, leading to toxic by-products. As they are food grade and able to grow in whey, the ability of various lactobacilli to produce benzaldehyde from Phe may be an interesting biotechnological alternative.

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