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ORIGINAL ARTICLE

Ability of meat starter cultures to catabolize leucine and evaluation of the degradation products by using an HPLC method

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The degradation of leucine generates aroma compounds involved in the typical flavour of dry fermented sausage. The ability of lactic acid bacteria and staphylococci of meat origin to produce aromatic compounds from leucine was studied. Whole cells were incubated with ^{3}*H-labelled leucine under different conditions. The radioactive metabolites produced were analysed by high performance liquid chromatography. All the strains studied were able to catabolise leucine but catabolic profiles were quantitatively and qualitatively different. The catabolism of leucine by* Lactobacillus sakei*,* Lactobacillus curvatus *and* Pediococcus acidilactici *was very low and required* α*-ketoglutarate, which indicates that an aminotransferase was involved in the first step of leucine catabolism.* Carnobacterium piscicola *produced a large amount of 3-methyl butanal, whereas* Staphylococcus carnosus *mainly produced 3-methyl butanoic acid. Leucine was mainly degraded into* α*-ketoisocaproic acid by* Lactobacillus plantarum *and* Carnobacterium divergens*. Nitrate affected leucine catabolism only in* S. carnosus*.*

Introduction

Dry sausage mixture is naturally contaminated by different micro-organisms derived from raw materials and the environment (Buckenhüskes 1993). Because these bacterial flora are variable, the industrial manufacturing of dry fermented sausage involves the use of starter cultures (combination of lactic acid bacteria and Micrococcaceae) to ensure products with reproducible hygienic and organoleptic qualities (Liepe 1983).

Lactic acid bacteria play an important role in the acidification of sausage by producing lactic acid from carbohydrate fermentation (Hammes 1990). Lactic acid inhibits pathogenic flora and is necessary for the appropriate texture of the product. Micrococcaceae participate in colour development through nitrate reductase activity. Starter cultures could also be involved in flavour formation. Flavour results from a subtle balance between non-volatile and volatile compounds. According to Meynier et al. (1999), volatile compounds arise from spices (60%), lipid oxidation (19%), amino acid catabolism (12%) and fermentation processes (5%).

The effect of starter cultures on the production of volatile compounds has been studied in model systems. Sausages inoculated with *Staphylococcus carnosus* were characterized by a

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high content of branched aldehydes (3-methyl butanal, 2-methyl butanal) and their corresponding acids (3-methyl butanoic acid, 2-methyl butanoic acid) (Berdagué et al. 1993, Montel et al. 1996). Volatiles, 3-methyl butanal and 3-methyl butanoic acid derived from leucine catabolism have a strong effect on the sensorial qualities of sausages (Berdagué et al. 1993, Stahnke 1995, Schmidt and Berger 1998). Relatively little information is available concerning the role of bacteria isolated from meat in the production of these molecules. Nevertheless, Stahnke (1995) attributed to 3-methyl butanal an important role in the aroma of dry sausage inoculated with *Staphylococcus xylosus*. Hinrichsen and Andersen (1994) associated 3-methyl butanal with the presence of *Vibrio* sp., *Staphylococcus warneri, Staphylococcus saprophyticus,* and *S. xylosus* in bacon. To a lesser extent, 3-methyl butanol was associated with the presence of *S. carnosus* and *Corynebacterium callunae* in a 'bacon model' (Pedersen and Hinrichsen 1996). Moller et al. (1998) attributed the production of this molecule in a cured meat model system to *Moraxella phenylpyruvica*. It has been reported that 3-methyl butanal was generated by leucine catabolism in *Streptococcus lactis* var. *maltigenes* (MacLeod and Morgan 1955). Stahnke (1999) demonstrated that *S. xylosus* produced 2-methyl propanal and 2- and 3-methyl butanal in a minimal medium with different amino acids, and their corresponding acids and alcohols were degradation products of valine, isoleucine and leucine, respectively. The same relationship has been shown for *S. carnosus* for leucine and 3-methyl butanal/3-methyl butanoic acid production (Masson et al. 1999). The aim of this study was to investigate the ability of lactic acid bacteria and staphylococci to catabolize leucine and to evaluate the degradation products by an HPLC (High Performance Liquid Chromatograph) method.

Materials and Methods

Chemicals

Leucine, α-ketoglutaric acid, pyridoxal-5 phosphate, thiamine pyrophosphate, α-ketoisocaproic acid, hydroxy α-ketoisocaproic acid, 3-methyl butanal, 3-methyl butanol and 3-methyl butanoic acid were obtained from Sigma Chemical Co., (St. Louis, Missouri, USA). Na_2HPO_4 and KH_2PO_4 were from Merck KGaA (Darmstadt, Germany) and L-(4-5 ^{3}H) leucine from Amersham (Little Chalfont, UK).

Standards

Because the metabolites studied were volatile, it was necessary to control the stability during all steps of the procedure: incubation at 30°C, freezing and waiting in the autosampler before chromatographic analysis. α-ketoglutaric acid, α-ketoisocaproic acid, hydroxy α-ketoisocaproic acid, 3-methyl butanal, 3-methyl butanol and 3-methyl butanoic acid (1, 5, 10, 50, 100, 250, 500, 1000 ppm) were prepared in water. Each standard preparation was aliquoted in four fractions. The first one was incubated at 30°C for 22 h, the second and the third aliquots were frozen at −20°C for 24 h and 7 days respectively, the fourth one was kept at room temperature for 12 h until chromatographic analysis. A 10% loss was observed for 3-methyl butanol stored for 7 days at −20°C and for 3-methyl butanal stored 12 h at room temperature (data not shown). Therefore, precautions were taken to reduce the time before analysis, and the preparations were stored for less than 7 days at −20°C and less than 12 h at room temperature (La Ferté sous Jouarre, France).

Bacterial strains

Lactobacillus plantarum Lpl, *Lactobacillus sakei* 205 and 23K, *Lactobacillus curvatus* 411, *Carnobacterium piscicola* 545, *Carnobacterium divergens* 210, *S. carnosus* 833, *S. saprophyticus* 852, *S. xylosus* 16 and *S. warneri* 863 strains were obtained from our laboratory collection. *Pediococcus acidilactici* 716 strain was obtained from SKW Biosystems (La Ferté sous Jouarre, France).

Lactobacillus plantarum Lpl, *L. sakei* 23K and 205, *L. curvatus* 411, *C. piscicola* 545, *C. divergens* 210 and *P. acidilactici* 716 strains were cultivated in Niven medium (Hitchener et al. 1982). Staphylococci were grown with shaking in a medium described by Hussain et al. (1991). After 16 h of growth at 30°C, cells

were centrifuged (10 000 *g* for 10 min at 4°C), washed twice with saline solution (8% NaCl) and suspended in a KH_2PO_4/Na_2HPO_4 buffer (0·067 M, pH 7). The optical density of the cell suspension was measured at 600 nm and adjusted between 10 and 15 in the reaction mixture.

Reaction mixture

The resting cells were incubated for 22 h at 30°C under static conditions in KH_2PO_4/Na_2HPO_4 buffer (0·067 M pH 5·4) according to three conditions: (i) with leucine (2 mM) and ^3H leucine (5 µCi/ml), (ii) with leucine (2 mM), ^3H leucine (5 µCi/ml), α-ketoglutaric acid (10 mM) and pyridoxal-5 phosphate (2 mM), (iii) with leucine (2 mM), ^3H leucine (5 µCi/ml), α-ketoglutaric acid (10 mM), pyridoxal-5 phosphate (2 mM) and nitrate (0·01%). Each experiment was repeated twice. The reactions were stopped after 22 h of incubation by the addition of $HClO_4$ (0·4 M). The reaction mixture was centrifuged for 7 min at 4000 *g* at 4°C, the supernatant was stored at −20°C to avoid loss of metabolites before analysis.

Apparatus

Analysis of the metabolites from leucine catabolism was carried out by HPLC using the method described by Fernandez-Garcia and MacGregor (1994). The HPLC equipment consisted of a Kontron system (Saint-Quentin en Yvelines, France) with pump system 32X, autosampler 360, a computer for data treatment and automatic driving of chromatography. The metabolites from the leucine catabolism were detected with three systems. The first one was a visible-UV detector (430 Kontron), the second one was a differential refractive detector (Precision instruments, Marseille, France) and the last one was a radiometric Flo-one/Beta A-515TR radio-HPLC detector (Packard Instrument Co., Meriden, Connecticut, USA). The metabolites were separated with an Aminex HPX 87H ion exchange column (300 x 7.8 mm) with a microguard column cation H+ cartridge (Biorad Labs, Richmond, California, USA).

Analytical method

Each sample (100 µl) was injected into the column, which was thermostatically controlled at 55°C. The mobile phase consisted of an isocratic gradient of H_2SO_4 (0·0075 N) with a flow rate of 1 ml min^{-1}. This solvent was filtered through a 0·45 µm filter. The metabolites were detected by refractometry and by measurements of absorbance at 210 nm and of radioactivity. They were identified by comparison of retention times with those of the appropriate standards.

Results

Detection of metabolites

The metabolites resulting from the degradation of leucine could be separated on an Aminex HP87 column in 30 min. The measurement of absorbance at 210 nm allowed quantification of the acids only. As shown in Table 1, α-ketoglutaric acid, α-ketoisocaproic acid and hydroxy α-ketoisocaproic acid were correctly quantified between 1 and 1000 ppm by measurement of absorbance at 210 nm. 3-methyl butanoic acid was better detected by refractometry (threshold detection: 1 ppm) than by measurement of absorbance at 210 nm (threshold detection: 5 ppm). So, α-ketoglutaric acid, α-ketoisocaproic acid, hydroxy α-ketoisocaproic acid were detected by measurement of absorbance and 3-methyl butanoic acid by refractometry. Aldehydes and alcohols were not detected by absorbance at 210 nm, so 3-methyl butanal and 3-methyl butanol were only quantified by refractometry. The sensitivity of detection ranged from 5 to 500 ppm.

In the 66 experiments performed (11 strains studied under three conditions, repeated twice) a good correlation was shown between quantification by measurement of absorbance or refractometry and quantification by measurement of radioactivity for three metabolites: α-ketoisocaproic acid ($r^2 = 0·96$), 3-methyl butanoic acid ($r^2 = 0·97$) and 3-methyl butanal ($r^2 = 0·98$). No correlation was found for the hydroxy α-ketoisocaproic acid ($r = -0·15$). By the absorbance method, the peak corresponding to hydroxy α-ketoisocaproic acid was almost merged with peaks from other molecules having similar retention times.

Table 1. Detection of metabolites from leucine catabolism by refractometry and measurement of absorbance at 210 nm

	Threshold of detection (ppm)		Linearity (ppm)	
	Absorbance at 210 nm	Refractometry	Absorbance at 210 nm	Refractometry
α-ketoglutaric acid	1	5	1–1000	5–100
α-ketoisocaproic acid	1	1	1–1000	1–100
Hydroxy α-ketoisocaproic acid	1	1	1–1000	1–500
3-methyl butanal	ND*	5	ND	5–500
3-methyl butanoic acid	5	1	5–1000	1–500
3-methyl butanol	ND	5	ND	5–500

*ND = not detected.

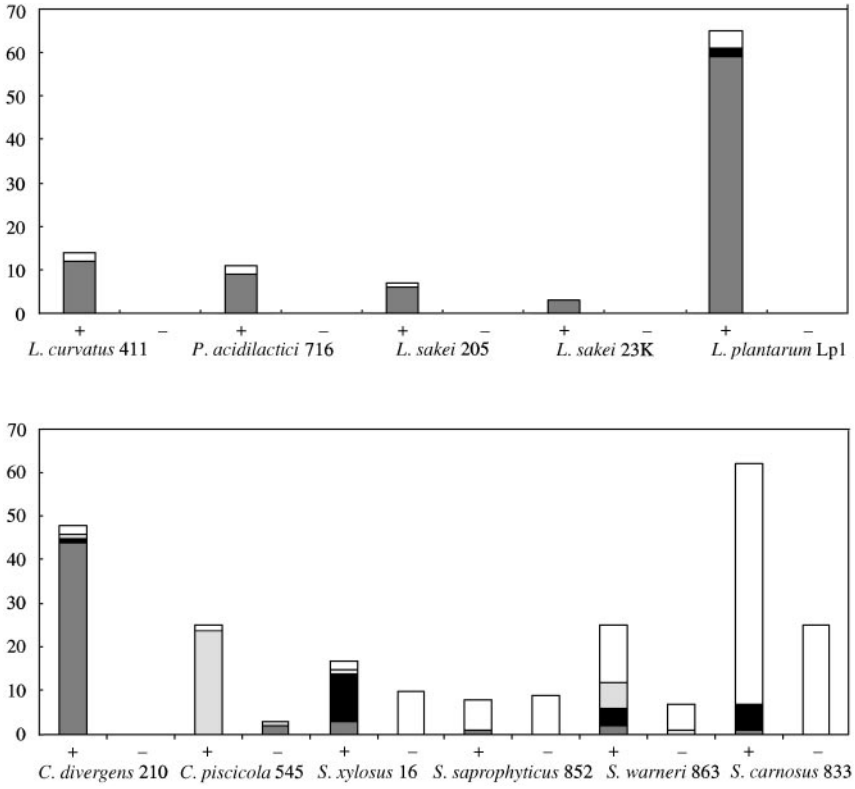

Figure 1. Leucine catabolism with (+) or without (−) cofactors (α-ketoglutaric acid 10 mM and pyridoxal 5-phosphate 2 mM) for the strains studied:
(a) *L. curvatus*, *P. acidilactici*, *L. sakei* and *L. plantarum*
(b) *C. divergens*, *C. piscicola*, *S. xylosus*, *S. saprophyticus*, *S. warneri* and *S. carnosus* ▩ α-ketoisocaproic acid, ■ hydroxy α-ketoisocaproic acid, □ 3-methyl butanoic acid, ▨ 3-methyl butanal
Results were expressed in percentage of radioactive leucine degraded.

Screening

The results of screening will take into account only the radioactive detection data. In the first series of experiments, the ability of strains to catabolize leucine was tested by incubating cells with radioactive leucine in a buffer solution (Fig. 1). Under these conditions, leucine was degraded only by *S. carnosus*, *S. xylosus*, *S.*

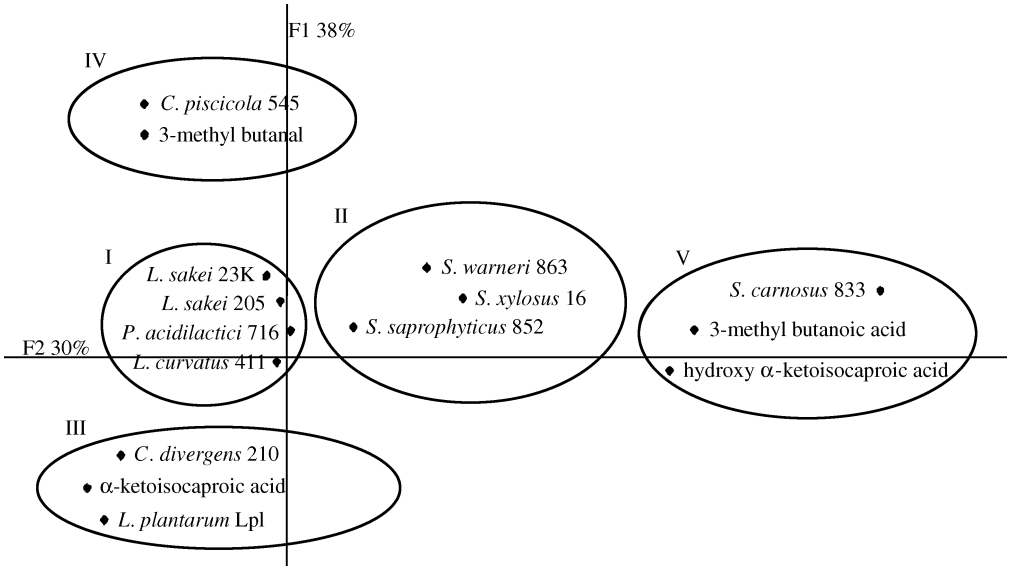

Figure 2. The principal component analysis taking into account metabolites production from leucine by lactic acid bacteria and *Staphylococcus* species in the presence of α-ketoglutaric acid and pyridoxal-5 phosphate.

saprophyticus and *S. warneri* strains. There was no degradation by strains of lactic acid bacteria. Then, in the second series of experiments, cells were incubated with leucine, α-ketoglutaric acid and pyridoxal 5-phosphate. These two compounds were required for the first step of leucine degradation (transamination reaction) described by Yvon et al. (1997) for *Lactococcus lactis*. In presence of α-ketoglutaric acid and pyridoxal 5-phosphate, lactic acid bacteria were able to produce metabolites from leucine. The degradation of leucine by the strains of staphylococci was higher with the addition of α-ketoglutaric acid and pyridoxal 5-phosphate.

On the basis of the main metabolites produced in the medium, the different species of lactic acid bacteria and staphylococci could be separated into five catabolic groups as shown in Fig. 2. Group I corresponded to *L. curvatus, L. sakei, P. acidilactici* strains and was characterised by a very low production of metabolites. In our conditions, they only degraded leucine into α-ketoisocaproic acid and 3-methyl butanoic acid, and the amounts produced were low. Group II, which consisted of *S. saprophyticus*, *S. xylosus* and *S. warneri* strains, was mainly characterized by a low production of 3-methyl butanoic acid. Group III included *L. plantarum* and *C. divergens* strains. These bacteria produced the highest amount of α-ketoisocaproic acid from leucine, but in our experimental set-up they were unable to degrade this keto acid into aromatic compounds. *Carnobacterium piscicola* strain (group IV) differed from the other strains by its very strong production of 3-methyl butanal. It degraded 25% of labelled substrate and produced 150 μg of 3-methyl butanal per ml of reaction mixture. Finally, *S. carnosus* 833 strain (group V) was distinguished by its high ability to catabolise leucine into 3-methyl butanoic acid.

Different effects of nitrate have been described in the literature. Nitrate was described by Masson et al. (1999) as a negative factor on the aroma producing capacity of *S. carnosus*. The production of 3-methyl butanoic acid was 20-fold higher without nitrate than with nitrate (0·03%). However, Stahnke (1999) showed that nitrate had a positive effect on the production of 3-methyl butanoic acid from leucine by *S. carnosus*. Therefore, the effect of nitrate was tested in this study. It had no effect on metabolite production by lactic acid bacteria species (*Lactobacillus*, *Carnobacterium*, *Pediococcus*)

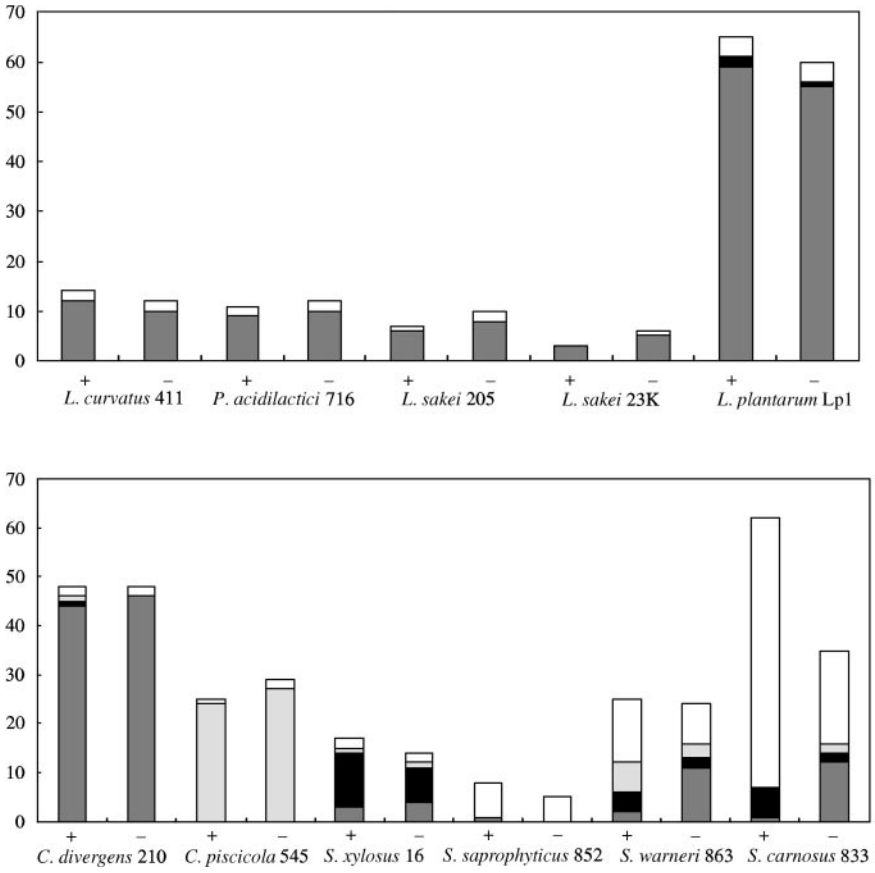

Figure 3. Effect of nitrate on leucine catabolism for the strains studied:
(a) *L. curvatus, P. acidilactici, L. sakei* and *L. plantarum*
(b) *C. divergens, C. piscicola, S. xylosus, S. saprophyticus, S. warneri* and *S. carnosus* ■ α-ketoisocaproic acid, ■ hydroxy α-ketoisocaproic acid, □ 3-methyl butanoic acid, □ 3-methyl butanal Results were expressed in percentage of radioactive leucine degraded.

but inhibited the production by *S. carnosus* and *S. warneri* strains (Fig. 3). The production of 3-methyl butanoic acid was significantly reduced (threefold) by the presence of nitrate.

Discussion

For the evaluation of the aromatic potential of starter cultures used in meat fermentation, rapid methods for qualitative and quantitative screening are essential. The method using radioactive substrate and HPLC is simple, rapid and sensitive. It constitutes an efficient method for the analysis of volatile compounds and should therefore facilitate the selection of starter cultures.

This study has shown that strains isolated from meat products presented a diversity of aromatic profiles. Among the strains studied, some of them had high aromatic potential from leucine catabolism. *Carnobacterium piscicola* produced high amounts of 3-methyl butanal. This high production was never observed from other species. *Staphylococcus carnosus* mainly produced 3-methyl butanoic acid. This was consistent with the studies of Montel et al. (1996) and Stahnke (1999) which pointed out that inoculation of *S. carnosus* in the dry sausage model led mainly to the production of 3-methyl butanoic acid. Among all the strains tested, we have found five catabolic profiles which could correspond to different enzymatic pathways involved in leucine catabolism. Three

enzymes were able to produce α-ketoisocaproic acid from leucine: leucine dehydrogenase and leucine oxidase by a oxidative deamination reaction (Massey et al. 1976) and aminotransferase by a transamination reaction which transfers the α amino group of amino acid to a keto acid acceptor (α-ketoglutaric acid) in the presence of pyridoxal 5-phosphate (Yvon et al. 1997, Gao et al. 1997). For lactic acid bacteria, transamination seemed to be the only enzymatic system involved in the first step of leucine degradation since no metabolite was detected in the absence of α-ketoglutaric acid and pyridoxal 5-phosphate. On the contrary, the degradation of leucine by staphylococci is just reduced in the absence of α-ketoglutaric acid and pyridoxal 5-phosphate. Therefore, two catabolic pathways may be involved: transamination and oxidative deamination.

The production of 3-methyl butanal from leucine observed in *C. piscicola* could correspond to a decarboxylation of α-ketoisocaproic acid by a ketoacid decarboxylase as described by Hickey et al. (1983). The detection of 3-methyl butanoic acid by all the staphylococci strains tested might result from two possible catabolic pathways. The first one could be a decarboxylation of α-ketoisocaproic acid into 3-methyl butanal which could be immediately dehydrogenized into 3-methyl butanoic acid by an aldehyde dehydrogenase (Sugawara and Sasaki 1977). The second one could be a dehydrogenation of α-ketoisocaproic acid by a multi-enzymatic complex leading to a acyl-CoA which could be hydrolysed into 3-methyl butanoic acid. This complex involves decarboxylase, transacylase and lipoamide oxidoreductase as described for *Bacillus subtilis* (Namba et al. 1969), and *Enterococcus faecalis* (Rüdiger et al. 1972, Ward et al. 1999).

Further studies are underway to identify the different catabolic pathways involved in leucine catabolism. A better understanding of the synthesis of aromatic compounds would lead to a better control of their production in dry sausage fermentation.

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