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Production of volatile compounds by *Lactobacillus sakei* from branched chain α-keto acids

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ABSTRACT

Lactobacillus sakei belongs to the main flora of raw fermented sausages and is used as starter culture. Bacterial starter cultures can convert amino acids to α-keto acids by aminotransferases. These α-keto acids are the precursors of aroma active aldehydes, alcohols and carboxylic acids. In this study the formation of aldehydes, alcohols and carboxylic acids from leucine, isoleucine, valine and the corresponding α-keto acids are analysed in model fermentations with two different strains of *L. sakei*. In the absence and upon addition of leucine, isoleucine and valine they produced 1 μg/ml 3-methylbutanoic, 0.2 μg/ml 2-methylbutanoic acid and 3 μg/ml 2-methylpropanoic acid, respectively. Upon addition of α-ketoisocaproic acid, α-keto-3-methyl-pentanoic acid or α-ketoisovaleric acid the amount of the corresponding carboxylic acid was increased to 40 μg/ml 3-methylbutanoic acid, 20 μg/ml 2-methylbutanoic acid and 35 μg/ml 2-methylpropanoic acid. The response patterns of the strains and amounts of carboxylic acids produced were similar. This behaviour was typical when compared with other strains of *L. sakei* and suggests general lack of transaminase activity and a limit in the transport of branched chain amino acids and their conversion to volatiles, some of which can contribute to the aroma of fermented sausages.

1. Introduction

Lactic acid bacteria (LAB), mainly the species *Lactobacillus sakei*, *Lactobacillus curvatus*, *Staphylococcus carnosus*, *Staphylococcus xylosus* and *Staphylococcus saprophyticus* belong to the main flora of naturally raw fermented sausages (Hugas et al., 1993; Coppola et al., 2000; Papamanouli et al., 2002). Strains of this species are therefore often used as starter cultures in industrial meat fermentation (Hugas and Monfort, 1997; Leroy et al., 2006). They contribute to the typical aroma of these products apart from lactic acid by the degradation of branched chain amino acids to e.g. 3-methylbutanol, 2- and 3-methylbutanal, 3-methylbutanoic acid and 2-methylpropanoic acid, which belong to the important aroma compounds of dry fermented sausages (Berdagué et al., 1993; Schmidt and Berger, 1998).

Amino acids are transported into the cell as such or as peptides, which are subsequent cleaved by peptidases. In this respect, lactobacilli generally prefer transport of peptide via the oligopeptide transporter Opp over amino acids, whose transport is restricted by a mostly reduced set of amino acid transporters. We have characterised the respective situation in strains of *L. sakei* demonstrating that there all investigated strains possess Opp and a full set of peptidases (Freiding et al., 2011a). The first intracellular step in the formation of aroma compounds from amino acids is the conversion to α-keto acids by aminotransferases. In the case of the branched chain amino acids leucine, isoleucine and valine these are α-ketoisocaproic acid, α-keto-3-methyl-pentanoic acid and α-ketoisovaleric acid, respectively. An aminotransferase specific for branched chain amino acids is e.g. described for a *Lactococcus lactis* strain used in cheese fermentations (Yvon et al., 2000). However screenings with different LAB showed that the transaminase activity is very low in most LAB (Smit et al., 2004). Very recently we have demonstrated that the lack of IlvE is a major bottleneck in conversion of amino acids to aroma compounds (Freiding et al., this issue).

The α-keto acids can then further be converted to aldehydes by a decarboxylase. The corresponding aldehydes of the branched chain α-keto acids are 3-methylbutanal, 2-methylbutanal and 2-methylpropanal. Production of these aldehydes is only described for some strains of LAB (Smit et al., 2004; Ayad et al., 1999). It seems that the aldehydes are used as electron acceptors and to enable NAD recycling needed for the glycolysis and immediately converted to the corresponding acids, or the α-keto acids are converted directly into the carboxylic acids by a ketoacid dehydrogenase via

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acyl-CoAs (Smit et al., 2005a,b). It is reported that lactobacilli produce 2- and 3-methyl-1-butanol, 2- and 3-methylbutanoic acid and 2-methylpropanoic acid in model fermentations (Freiding et al., this issue). The formation of 2- and 3-methylbutanoic acid delivers three or one molecules of ATP and therefore conversion reactions of leucine can contribute to the competitiveness of LAB in a specific habitat (Ganesan et al., 2006). It has been demonstrated for *Lactobacillus sanfranciscensis* that their formation is enhanced under acidic stress conditions (Serrazanetti et al., 2011). The production of these compounds can also be observed in LAB isolated from Cheddar cheese and in sausages manufactured with *S. xylosus* and *S. carnosus* (Williams et al., 2001; Olesen et al., 2004).

Alcohols and carboxylic acids can be connected to esters via acyltransferases or esterases. An esterification activity was found in various LAB isolated from cheese including lactococci, lactobacilli, *Streptococcus thermophilus*, Leuconostocs and Pediococci (Liu et al., 1998). Also staphylococci used in meat fermentations showed a strong esterification activity (Talon et al., 1998; Casaburi et al., 2006).

The aim of this work was to investigate the production of volatile compounds of two different *L. sakei* strains in model fermentations inoculated with branched chain amino acids and the corresponding α-keto acid, in order to understand their amino acid catabolism and their role in the flavour formation of sausage fermentation.

2. Materials and methods

2.1. Bacteria, media, growth

The *L. sakei* strains TMW 1.1383 and 1.1393 were used. Bacteria were routinely grown at 30 °C in mMRS-medium containing 1.5% glucose. Model fermentations for a subsequent volatilome analyses were performed in mMRS-medium containing 0.3% glucose derived from conditions in sausage fermentation. The medium was supplemented with 25 mM α-ketoisocaproic acid, α-keto-3-methyl-pentanoic acid, α-ketoisovaleric acid, leucine, isoleucine, valine or used without supplement (α-ketoisocaproic acid α-ketoisovaleric acid, α-keto-3-methyl-pentanoic acid were purchased from Sigma–Aldrich, USA; α-ketoisovaleric acid from Fluka, USA; leucine from Merck, Germany; isoleucine and valine from Serva, Germany). Over night cultures were washed twice with the corresponding medium. The GC vials (20 ml; VWR, Germany) were filled with 10 ml medium and inoculated with washed cells to a density of 10^6 cells/ml. The fermentations were done in four parallels and performed for 5 days at 30 °C. Samples were analysed at day 0 and 5. Controls were done with medium without inoculation.

2.2. Analysis and quantification of the volatile compounds by SPME-GC-MS

Analysis of volatile compounds in the headspaces of the GC vials were determined with a 75 μm SPME fibre type carboxen/polydimethylsiloxane (CAR/PDMS), (Supelco, USA) coupled with GC–MS. Before the analysis, the fibres were preconditioned in the injection port of the GC as indicated by the manufacturer. The fibre was exposed to the samples for 30 min at 30 °C. The adsorbed compounds were thermally desorbed at 250 °C for 10 min and injected onto the column of an Agilent 7890 A gas chromatograph equipped with a ZB-Wax capillary column (60 m × 0.25 mm × 0.25 μm film thickness); (Zebron, Phenomenex, USA). Volatile compounds were separated under the following conditions: carrier gas helium 1.03 ml/min, initial column temperature was 30 °C for 15 min, heated to 50 °C at 30 °C min^{-1}, followed by heating to 110 °C at 4 °C min^{-1}, heating to 150 °C at 5 °C min^{-1}, heating to 250 °C at 10 °C min^{-1} and holding for 10 min for a total run time of 64.67 min. The GC column was connected without splitting to the ion source of an Agilent 5975C mass spectrometer operating in the scan mode within a mass range of *m/z* 29–150. Ionisation was performed by electronic impact at 70 eV; calibration was performed by autotuning.

Compounds were identified by comparison of the mass spectral data with those of the library Nist 2002 Mass Spectral Database and comparison of retention times of reference compounds (2-methylbutanoic acid, 3-methylbutanoic acid, 2-methylpropanoic acid, 2-methylbutanal, 3-methylbutanal, 2-methylpropanal, 2-methyl-1-butanol, 3-methyl-1-butanol, 2-methylpropanol, were purchased from Sigma–Aldrich, USA; diacetyl, acetoin from Fluka, USA; 2,3-hexanedione, 2,3-heptanedione from SAFC, USA). An estimation of the quantities of the compounds was achieved along a calibration curve based on a series of measurements with different quantities of the compounds. As internal standard 1,2-dimethoxy-ethane (Sigma–Aldrich, USA) was added to each sample at a concentration of 19.24 nM.

3. Results

In an extensive screening of 51 *L. sakei* strains very similar patterns of volatiles were observed in SPME/GC–MS analyses (Freiding et al., 2011a). From these experiments two representative strains were selected for a more detailed analysis towards the conversion of branched chain amino acids and α-keto acids to volatile alcohols, aldehydes and carboxylic acids. The strains were cultivated in mMRS-medium supplemented with α-ketoisocaproic acid, α-keto-3-methyl-pentanoic acid, α-ketoisovaleric acid, leucine, isoleucine, valine or without supplement. 2-methylbutanoic acid, 3-methylbutanoic acid, 2-methylpropanoic acid, 2-methylbutanal, 3-methylbutanal, 2-methylpropanal, 2-methyl-1-butanol, 3-methyl-1-butanol, 2-methylpropanol, diacetyl, acetoin, 2,3-hexanedione and 2,3-heptanedione were quantified as described in the methods section after 5 days of fermentation. Both strains showed no or marginal increases in the formation of the respective alcohols, aldehydes or carboxylic acids upon addition of leucine, isoleucine or valine. However, upon addition of α-ketoisocaproic acid, α-keto-3-methyl-pentanoic acid or α-ketoisovaleric acid the amount of the corresponding carboxylic acid was increased. Under these conditions both *L. sakei* strains produced similar amounts in the range of 40 μg/ml 3-methylbutanoic acid, 20 μg 2-methylbutanoic acid and 35 μg/ml 2-methylpropanoic acid (Fig. 1A–C). Without addition of α-keto acids the *L. sakei* strains produced only 1 μg/ml 3-methylbutanoic, 0.2 μg/ml 2-methylbutanoic acid and 3 μg/ml 2-methylpropanoic acid. The amount of the corresponding aldehydes and alcohols were not or marginally increased through the addition of α-keto acids (data not shown). Both strains produced also more diacetyl, acetoin and 2,3-heptanedione upon addition of α-ketoisocaproic acid (Fig. 2A–C), and 2,3-hexanedione with addition of α-ketoisovaleric acid (Fig. 2D).

4. Discussion

The metabolism of branched chain amino acids to volatiles, some of which can contribute to the aroma of fermented sausages, appears limited by transamination and transport of amino acids. The data suggest that *L. sakei* is a minimalist, which is highly adapted to meat (Nyquist et al., 2011). In this environment peptides provide the major source of nitrogen, which are readily transported into the cell by Opp and cleaved to amino acids by a large set of peptidases (Freiding et al., 2011a). The amino acids resulting from this intracellular peptidolysis appear to be mainly used for the