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Metabolism of amino acids, dipeptides and tetrapeptides by *Lactobacillus sakei*

Quirin Sinz, Wilfried Schwab*

Technische Universität München, Biotechnology of Natural Products, Liesel-Beckmann-Straße 1, D- 85354 Freising, Germany

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ABSTRACT

The microbial degradation of proteins, peptides and amino acids generates volatiles involved in the typical flavor of dry fermented sausage. The ability of three *Lactobacillus sakei* strains to form aroma compounds was investigated. Whole resting cells were fermented in phosphate buffer with equimolar amounts of substrates consisting of dipeptides, tetrapeptides and free amino acids, respectively.

Dipeptides disappeared quickly from the solutions whereas tetrapeptides were only partially degraded. In both approaches the concentration of free amino acids increased in the reaction mixture but did not reach the equimolar amount of the initial substrates. When free amino acids were fed to the bacteria their levels decreased only slightly. Although peptides were more rapidly degraded and/or transported into the cells, free amino acids produced higher amounts of volatiles.

It is suggested, that after transport into the cell peptides are only partially hydrolyzed to their amino acids, while the rest is metabolized via alternative metabolic pathways. The three *L. sakei* strains differed to some extend in their ability to metabolize the substrates to volatile compounds. In a few cases this was due to the position of the amino acids within the peptides. Compared to other starter cultures used for the production of dry fermented sausages, the metabolic impact of the *L. sakei* strains on the formation of volatiles was very low.

1. Introduction

The flavor of dry fermented sausages derives from the ingredients (meat, spices and smoke) and the chemical changes occurring during the fermentation and drying process. Fermentative flavor formation occurs by bacterial ferments and by meat enzymes which are responsible for the metabolism of fats, carbohydrates and proteins (Dainty and Blom, 1995; Ordóñez et al., 1999). The inside bacterial flora of fermented meat products is dominated by lactic acid bacteria (LAB), mainly *Lactobacillus sakei*, *Lactobacillus curvatus* and *Lactobacillus plantarum* (Hugas et al., 1993; Montel et al., 1998). By lowering the pH, they contribute to the improvement of food safety and stability of fermented foods (Ross et al., 2002). Since the flavor of fermented products could be improved by increasing levels of proteins, peptides and free amino acids, the proteolytic system of several LABs has been investigated (Kunji et al., 1996; Ordóñez et al., 1999; Vermeulen et al., 2005; Savijoki et al., 2006).

Peptides are transported into LAB cells by three known systems. An Opp system is capable to transport oligopeptides consisting of up to 35 amino acids (Doeven et al., 2005). A Dpp (previously referred to as DtpP) system delivers di-, tri-, and tetrapeptides with relatively hydrophobic branched-chain amino acids (BCAA) into the cell. This system displays the highest affinity for tripeptides (Foucaud et al., 1995; Sanz et al., 2003). Finally, a DtpT system prefers more hydrophilic and charged di- and tripeptides (Hagting et al., 1994). Free amino acids incorporated into LAB cells by amino acid transporters or released intracellular by peptidase activity can be converted, among other metabolic pathways, to various volatile compounds by transamination and decarboxylation (Christensen et al., 1999; van Kranenburg et al., 2002; Ordóñez et al., 1999; Smit et al., 2009). The transformation of the branched chain amino acids (BCAAs) valine, leucine and isoleucine, the aromatic amino acids tyrosine, tryptophan and phenylalanine and the sulfur-containing amino acids methionine and cysteine by transamination and decarboxylation leads to volatile compounds which contribute to the sensory perception of fermented food (Ardö, 2006; Smit et al., 2005, 2009; Yvon and Rijnen, 2001). Some have been identified as major flavor contributor in Hungarian salami, such as 3-methylbutanal, 3-methylbutanoic acid, 3-(methylthio)-propanal (methional) and phenylacetaldehyde that can result from the degradation of leucine, methionine and phenylalanine, respectively (Söllner and Schieberle, 2009). The metabolites 2-methylpropanal, 2-methylpropanol and 2-methylpropanoic acid formed by valine

* Corresponding author. Tel.: +49 8161 712912; fax: +49 8161 712950.
E-mail address: schwab@wzw.tum.de (W. Schwab).

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degradation as well as 3-methylbutanol produced by leucine catabolism were also identified in high concentrations in dry fermented sausages (Flores et al., 2004; Marco et al., 2008; Partidário et al., 2006). It has been calculated that 11.8% of the volatile compounds in Milano salami probably originate from amino acid catabolism (Meynier et al., 1999). The lyase-mediated metabolism of cysteine and methionine also results in desired sulfur-containing flavor molecules in cheese (Bruinenberg et al., 1997; Dias and Weimer, 1998; Weimer et al., 1999). However, genes encoding enzymes involved in methionine and cysteine metabolism could not be identified in the genome sequence of *L. sakei* 23K (Liu et al., 2008). Studies with several *Lactococcus lactis* strains showed that an aminotransferase initiates the formation of methanethiol from methionine, although specific genes coding lyases (e.g. cystathionine γ-lyase) were identified in these bacteria (Gao et al., 1998; Liu et al., 2008).

L. sakei, formerly known as *Lactobacillus sake*, is one of the dominating LAB in dry spontaneously fermented sausages (Hugas et al., 1993; Trüpler and De'Clari, 1997). Therefore, it is commonly used in starter cultures for the production of dry fermented sausages, as it ensures the dominance of the starter during the whole ripening process (Hammes et al., 1990). Proteinase activities of whole cells and cell free extracts from some *L. sakei* strains have been studied by monitoring the hydrolysis of muscle myofibrillar and pork muscle sarcoplasmic proteins (Toldrá et al., 1992; Sanz et al., 1999; Fadda et al., 1999). Besides, several peptidases have been purified and studied in this species (Montel et al., 1995; Sanz and Toldrá, 1997; Sanz et al., 1998; Sanz and Toldrá, 2001, 2002). The analysis of the genome sequence of *L. sakei* 23K delivered insights into the strain's potential concerning transport and proteolysis as well as aroma formation from amino acid substrates (Chaillou et al., 2005; Freiding et al., 2011; Liu et al., 2008, 2010). The studies showed that *L. sakei* strains possess a putative transport system for oligopeptides (Opp) as well as a di/tripeptides ABC transport system consisting of five subunits (DppA/P, DppB, DppC, DppD and DppF) and a di/tripeptides ion-linked transport system (DtpT). Furthermore, 18 peptidases with different specificities (unique aminopeptidases, endopeptidases, di/tripeptidases and proline peptidases) could be found. Although genes coding typical aminotransferases (*araT* und *bcaT*) could not be found in the genome sequence of more than 50 *L sakei* strains (Freiding et al., 2011; Liu et al., 2008), the formation of volatile amino acid derived metabolites could be demonstrated for *L. sakei* 23K (Larrouture et al., 2000). Pulsed-field gel electrophoresis analysis and DNA–DNA reassociation analysis showed high genetic heterogeneity (Chaillou et al., 2009; Champomier et al., 1987) whereas biochemical and physiological studies uncovered wide phenotypic heterogeneity within *L. sakei* strains (Champomier-Vergès et al., 2001). This suggests that the metabolic potential of these strains can be as diverse. Metabolic studies to investigate the formation of 3-methylbutanal from leucine also showed a large variation between and within species and that intra-species variation is in many cases larger than that between species (Brandsma et al., 2008; Smit et al., 2004). Contrarily, a recent screening with 51 *L sakei* strains from different origins showed that all strains were nearly uniform in the genes forming the peptidolytic system (Freiding et al., 2011).

Several studies with LAB showed a preferential uptake of peptides and an associated better growth of the microorganisms. (Foucaud et al., 2001; Kunji et al., 1996; Saguir et al., 2008; Smit and Konings, 1990). However, most studies on amino acid metabolism by lactobacilli have employed amino acids rather than peptides as substrates. The possibility to increase amino acid turnover by an optimized supply of peptide substrates has not been analyzed explicitly. Only one study showed that the metabolization of phenylalanine in *L. plantarum* and *L. sanfranciscensis* could be enhanced by applying peptides compared to free amino acids (Vermeulen et al., 2006).

In this study *L. sakei*'s potential to form volatile compounds from peptides and amino acids was investigated. Three strains were selected for a more detailed analysis on peptide metabolism by *L. sakei* (Freiding et al., 2011). Furthermore, two other bacteria commonly employed in starter cultures, *L. plantarum* and *Staphylococcus carnosus* (Hammes and Hertel, 1998), were chosen for comparison of the metabolic capacities. Resting cells were incubated in phosphate buffer with equimolar amounts of substrates consisting of dipeptides, tetrapeptides and free amino acids, respectively. Specific peptides were selected to analyze the preference of the cellular transport systems and the specificity of peptidases and enzymes responsible for the metabolism of amino acids. The degradation of peptides and amino acids was monitored by LC-UV/ESI-MS^n and GC/MS analysis, the formation of volatile compounds was determined by SPME-GC/MS analysis. The aim of this study was to analyze the metabolic capacity of *L. sakei* strains to improve their flavor formation potential.

2. Materials and methods

2.1. Chemicals

L-Leucine, L-valine, L-phenylalanine, L-methionine, peptone from casein, yeast extract, meat extract, ammonium chloride, cysteine–HCl, Tween® 80, D-maltose, D-glucose, $K_2HPO_4 \times 3H_2O$, KH_2PO_4, Na_2HPO_4 and NaH_2PO_4 were obtained from Carl Roth GmbH & Co. KG (Karlsruhe, Germany). 3-Methylbutanal, 3-methylbutanol, 3-methylbutanoic acid, 2-methylpropanal, 2-methylpropanoic acid, phenylacetaldehyde, benzaldehyde, methional (3-(methylthio)-propanal), dimethyldisulfide, pyridoxal-5-phosphate, 1,2-dimethoxy- ethane, α-ketoglutaric acid and vitamins and metals for mMRS medium were purchased from Sigma Chemical Co. (St. Louis, Missouri, USA). Peptides were ordered from GenScript USA Inc. (Piscataway, New Jersey, USA).

2.2. Bacterial strains, media and growth

The strains used in this study were *L. sakei* TMW 1.1322, *L. sakei* TMW 1.1383, *L. sakei* TMW 1.1393 and *L. plantarum* TMW 1.708 as well as *S. carnosus* TMW 2.801 for comparison. They were all obtained from the laboratory collection of Technische Universität München, Lehrstuhl für Technische Mikrobiologie, Freising, Germany. *L. sakei* TMW 1.1322 and *S. carnosus* TMW 2.801 are isogenic clones of the genome sequenced strains *L. sakei* 23K (Chaillou et al., 2005) and *S. carnosus* TM300, which is phenotypically indistinguishable from the type strain DSM20501 (Rosenstein et al., 2009). In this communication the TMW clone numbers are used for correctness, as strains may change upon prolonged lab propagation. *L. sakei* TMW 1.1383 and TMW 1.1393 have been isolated from dry fermented sausages with a typical and poor aroma of dry fermented salami, respectively. The sausages have been evaluated by a sensory panel but the strains are probably not to blame for the different aromas (data not shown).

The organisms were pre-cultured overnight at 30 °C in 2 mL modified De Man Rogosa Sharpe medium (mMRS) (De Man et al., 1960; Stolz et al., 1995). A 20 mL aliquot of mMRS containing 0.3% D-glucose and no D-maltose to mimic conditions prevailing in fermenting sausage were inoculated with the overnight cultures and incubated for 5–7 h at 30 °C. Cells were harvested by centrifugation (3000× g for 5 min) when reaching the stationary phase of growth, which was determined by measuring the optical density at 600 nm ($O.D._{600}$); 2.3–2.6 for *L. sakei* TMW 1.1322; 2.7–2.9 for *L. sakei* TMW 1.1383 and 2.0–2.2 for *L. sakei* TMW 1.1393.