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Identification of genetic markers and bottlenecks in *Lactobacillus sakei* constituting safety and quality determinants of fermented sausages

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Habe Geduld,

alle Dinge sind schwierig, bevor sie leicht werden.

(Persisches Sprichwort)

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Abbrevations

°C	degree Celcius
µg	micorgram
µl	microlitre
Ω	ohm
%	percent
αKGA	α-keto glutarate
aa	amino acid
ACK	acyl kinase
AlcDH	alcohol dehydrogenase
AAA	aromatic amino acid
AraT	aromatic amino acid aminotransferase
amp	ampicillin
Ap^r	ampicillin resistence
APS	ammonium persulfate
BA	biogenic amine
BCAA	branched-chain amino acid
BcaT	branched-chain amino acid aminotransferase
BHI	Brain Heart Infusion Broth
BLAST	basic local alignment search tool
bp	base pair(s)
BSA	bovine serum albumin
CDM	chemically defined medium
CoA	coenzyme A
coPCR	cross-over PCR
Da	Dalton
DNA	desoxyribo nucleic acid
dNTP	desosy nucleotide triphosphate
DTT	dithiothreitol
EDTA	ethylendiaminetetraacetic acid
Em^r	erythromycin resistance
Erm/erm	erythromycin
FPLC	free presure liquid chromatography
g	gram
GC	gas chormatograph(y)
GDH	glutamate dehydrogenase
GFP	green fluorescent protein
GRAS	generally regarded as safe
h	hours
INRA	Institut National de la Recherche

	Agronomique
k	kilo
KaDH	α-keto acid dehydrogenase complex
KIV	α-keto acids α-ketoisovalerate
KIC	α-ketoisocaproate
KMV	α-keto-β-methylvalerate
l	litre
LAB	lactic acid bacteria
LB	lysogeny broth
M	molar, mol per litre
mA	milliampere
mg	milligram
min	minutes
ml	milliliter
mM	millimolar, millimol per litre
MRS	`de Man, Rogosa and Sharpe
mMRS	modified MRS medium
nm	nano meter
NO	nitric oxide
OD	optical density
OD_{590}	optical density at 590 nm wavelength
ori	origin of replication
PAGE	polyacrylamide gelelectrophoresis
PCR	polymerase chain reaction
PLP	pyridoxal-5-phosphate
RAPD	random amplified polymorphic DNA
rpm	rounds per minute
sec	second
SDS	sodium dodecyl sulfate
TAE	Tris-Acetat-EDTA
TBE	Tris-Borat-EDTA
TEMED	N,N,N´,N´-tetramethyl-ethylendiamine
TLC	thinlayer chromatography
TMW	Technische Mikrobiologie Weihenstephan
Tris	Tris-(hydroxymethyl-) aminomethane
U	units
UV	ultra violet
V	Volt
v	volume
X-Gal	5-Bromo-4-chloro-3-indolyl-ß-D-galactopyranoside

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1 Introduction

1.1 Meat Fermentations

In Germany the turnover in meat industry was 16.1 billion Euros in 2009 and is particularly powerful by European comparison. A recent market study has shown (Figure 1 left) that 26 % of meat products in Europe were produced in Germany followed by Spain and Italy with 11 % each and France and Poland with 9 % each (Pressemitteilung Bundesverband der deutschen Fleischwarenindustrie e.V., 2009).

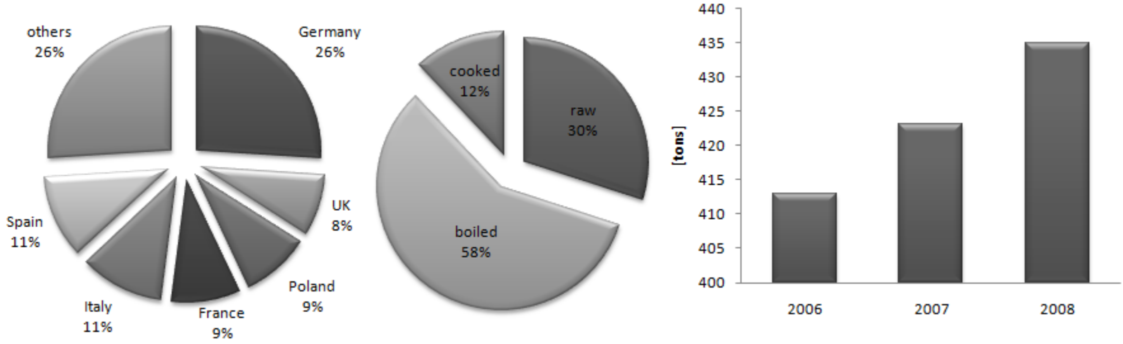


Figure 1: Left: Distribution of sausage production in Europe. Middle: Proportions of cooked, boiled and raw fermented sausages in Germany in 2008. Right: Production figures of raw fermented sausages in Germany from 2006 to 2008. Figures are adapted from Pressemitteilung Bundesverband der deutschen Fleischwarenindustrie e.V. (2009).

Meat is an important part of our diet and its storage requires special measures since it is highly sensitive to microbial spoilage. Factors contributing to this feature are: high water activity (a_w = 0.995 - 0.985); a favourable pH (5.4 - 5.6), and the availability of all nutrients, growth factors, and minerals required for optimal microbial growth (Weber, 2004). Traditional methods for preservation of meat are drying, salting and fermentation. These are ancient processes. First evidence of sausage production dates back to the period of the Roman Empire (Lücke, 1985). From Mediterranean area sausage fermentation spread and developed into a huge variety of types. Especially in Germany an immense diversity of fermented sausages can be found on the market. In 2008, production of raw sausages accounts for 30 % of the overall sausage production in Germany and a steady increase in their production from 413 kilotons in 2006 to 435 kilotons in 2008 could have been observed during the last few years (Figure 1 middle/right) (Bundesverband der Deutschen Fleischwarenindustrie e. V., 2007; Bundesverband der Deutschen Fleischwarenindustrie e. V., 2009).

To all of these products the following definition can be applied: Fermented sausages are cured meat products that are shelf stable (without cooling; above +10 °C) and are commonly consumed without the application of any heating process. Fermented sausages

are spreadable or have become sliceable during a ripening process that involves fermentation and reduction of water content by drying. Sugars can be added at an amount of not more than 2 % (Leitsätze für Fleisch und Fleischerzeugnisse. Deutsches Lebensmittelbuch, 2010).

Dry fermented sausages can be classified according to a range of criteria, such as acidity, mincing size of the ingredients, addition of some ingredients and spices, diameter and type of casings used, the application of smoke, etc. (Toldra, 2010). All of these criteria have influence on the fermentative transformation of raw materials into the desired end product during the ripening process.

In principle, fermented sausage production proceeds as follows: Mincing of the meat and fat is performed at low temperatures (-5 - 0 °C) to achieve a clean cut. Once meat and fat have been comminuted, the starter culture (not necessarily), curing salts (salt, nitrates, and nitrites), additives (colorants, ascorbic acid, etc.), and other ingredients (sugars, herbs, and spices) were added. All ingredients were mixed and natural or synthetic casings were filled with the meat batter avoiding oxygen introduction which could lead to the development of undesirable colours and flavours. Subsequently sausages are placed in ripening rooms under controlled temperature, relative humidity and air movement. Depending on the type of fermented sausage, various different ripening procedures are applied. In general, temperature is decreased, from 25 - 20 °C to 15 °C, relative humidity from 95 - 90 % to 75 % and air movement from about 1.0 to 0.2 m/s. Dry fermented sausages commonly have a moisture content of about 35 % when ripening has finished (Stiebing, 1995; Toldra, 2010).

1.2 Microbiota of sausage fermentations

The microbiology of fermented sausages is diverse and complex. The type of microflora that develops is often closely associated to the ripening technique applicated. Traditional dry fermented sausages are manufactured without addition of starter cultures in small-scale processing units. Thus, fermentation in traditional dry sausages relies on the indigenous bacterial flora (Aymerich *et al.*, 2003). Two groups of microorganisms are found in fermented sausages as being the main organisms responsible for the transformations occurring during fermentation and ripening of sausage: lactic acid bacteria (LAB) and Gram-positive, catalase-positive cocci (GCC+, mostly *Staphylococcus* and *Kocuria* species). Less important, yeasts and moulds are involved in fermentation of dry sausages (Aymerich *et al.*, 2003; Hugas *et al.*, 1993; Toldra, 2008). *L. sakei* and/or *L. curvatus* generally dominate the fermentation process (Aymerich *et al.*, 2003; Coppola *et al.*, 1998; Coppola *et al.*, 2000; Hugas *et al.*, 1993; Papamanoli *et al.*, 2003; Rantsiou *et*

al., 2005; Rebecchi *et al.*, 1998; Santos *et al.*, 1998). *L. sakei* appears to be the most competitive of both strains, since it frequently represents half to two thirds of all LAB isolates from spontaneously fermented sausage, whereas *L. curvatus* often accounts for one fourth of all LAB isolates. Other lactobacilli that may be found in minor counts include *L. plantarum, L. brevis, L. buchneri*, and *L. paracasei* (Aymerich *et al.*, 2003; Hugas *et al.*, 1993; Papamanoli *et al.*, 2003). Pediococci occasionally occur in European fermented sausages (Papamanoli *et al.*, 2003; Santos *et al.*, 1998) and sometimes enterococci are associated with fermented meat, especially in artisan products from Southern Europe, where they increase during early fermentation stages and can be detected at levels of 10^2 - 10^5 cfu/g in the end product (Aymerich *et al.*, 2003; Papamanoli *et al.*, 2003; Rebecchi *et al.*, 1998). To ensure the sensory quality of fermented sausages, the contribution of GCC+ is necessary (Hugas and Monfort, 1997). Dominating species are *Staphylococccus carnosus*, and *Staphylococcus xylosus* (Coppola *et al.*, 1997; Papamanoli *et al.*, 2002; Rebecchi *et al.*, 1998).

1.3 Meat starter cultures

Dominating strains of sausage fermentations have been isolated and have been partly used in starter cultures to ensure an optimal fermentation process and a reproducible quality of the products (Hammes and Hertel, 1998; Leroy *et al.*, 2006). According to the definition of Hammes *et al.*, meat starter cultures are preparations that contain active or dormant microorganisms that develop the desired metabolic activity in the meat. They are, by definition, used to change the sensory properties of the raw product (Hammes *et al.*, 1990). Strains used as starter cultures must be “generally regarded as safe” (GRAS). Some further requirements starter cultures have to meet are: good stability under the processing conditions (resistance to acid pH, low water activity, tolerance to salt), resistance to phage infections, strong growth at fermentation temperatures (e.g., 18 - 25 °C in Europe), generation of products of technological and sensorial interest (e. g., lactic acid for pH drop, volatile compounds for aroma, nitrate reduction, secretion of bacteriocins, etc.), and a lack of undesirable enzymes (e.g., decarboxylases responsible for amine formation)(Smith and Hui, 2004).

Most commercially available meat starter cultures contain mixtures of LAB and GCC+ (Ammor *et al.*, 2005). These bacterial groups are responsible for the basic microbial reactions that occur during fermentation: the decrease of pH values via glycolysis mainly by LAB, the reduction of nitrate, and the development of aroma primarily by GCC+. The most important starter strains used for meat fermentations and their main contribution to sausage fermentation are listed in Table 1 (Hui *et al.*, 2001; Hutkins, 2006).

Table 1: Important starter organisms for meat fermentations. Table was obtained and adapted from Hui *et al.* and Hutkins (Hui *et al.*, 2001; Hutkins, 2006).

Organism	Acid from glucose	Nitrate reductase	Primary function
Lactobacillus sakei	+	-	acid formation
Lactobacillus curvatus	+	-	acid formation
Lactobacillus plantarum	+	-	acid formation
Pediococcus pentosaceus	+	-	acid formation
Pediococcus acidilactici	+	-	acid formation
Kocuria varians	-	+	flavour, aroma
Staphylococcus carnosus	-	+	flavour, aroma
Staphylococcus xylosus	-	+	flavour, aroma
Debaryomyces hansenii	-	-	flavour, aroma
Penicillium nalgiovense	-	-	flavour, aroma
Penicillium chrysogenum	-	-	flavour, aroma

The major role of LAB in sausage fermentation is the production of organic acids (mainly lactic acid) from carbohydrates and a subsequent lowering of the pH value (5.9 - 4.6). Thereby, the muscle protein coagulates, resulting in the sliceability, firmness, and cohesiveness of the final product. Moreover, low pH values enhance the reduction of nitrites to nitric oxide (NO), and thereby the reddening process (Hugas and Monfort, 1997). Accumulation of lactic acid, acetic acid, formic acid, ethanol, ammonium, fatty acids, hydrogen peroxide, acetaldehyde, antibiotics and bacteriocins, formed by LAB, lead to an inhibition of pathogenic and spoilage flora (Hugas and Monfort, 1997) and therefore enhances the product safety and shelf-life. Studies of Hammes *et al.* have shown that a mixed strain culture with several nitrate-reducing lactobacilli achieved the reduction of nitrate and nitrite. However, the abilities to reduce nitrate and nitrite are rare traits amongst lactobacilli and nitrate reduction was, slow compared to activities of staphylococci, and final product showed defects in flavour and colour (Hammes *et al.*, 1990). GCC+ play a major role in the development of sensory properties of fermented sausages by nitrate reductase activity, by preventing rancidity through peroxide decomposition, and by producing flavour and aroma compounds through lipolysis but mainly through proteolysis (Hammes and Hertel, 1998; Søndergaard and Stahnke, 2002). High nitrate reductase activity allows an effective reduction of nitrate into nitrite which is further reduced to nitric oxide (NO). NO subsequently reacts with myoglobin to nitrosyl myoglobin that is responsible for the typical pinky-red colour of cured meat products. This reaction is favoured at low pH values (Hutkins, 2006) provided by lactic acid production of

LAB. Amongst other volatiles, several aromatic methyl-branched aldehydes, alcohols, and acids are generated by species of the genus *Staphylococcus* (Beck *et al.*, 2002; Larrouture *et al.*, 2000; Stahnke, 1994). These are mainly derived from branched-chain amino acids and enhance cured meat aroma (Ardo, 2006; Beck *et al.*, 2002; Montel *et al.*, 1996; Stahnke, 2002).

Yeasts (*Debaryomyces* species) and moulds (mainly *Penicillium* species) contribute to product quality by protective effects against discoloration and rancidity and aroma formation by lipolytic and proteolytic activities (Hui *et al.*, 2001).

1.4 *Lactobacillus sakei*

1.4.1 Origin

Lactobacillus sakei, first described as *Lactobacillus sake*, a spoilage microorganism in the fermented beverage sake, in 1934, was renamed following recommendations of Trüper and De Clari (Trüper and De'clari, 1997). *L. sakei* is a facultative heterofermentative bacillus that groups by pairs or short chains (Figure 2).

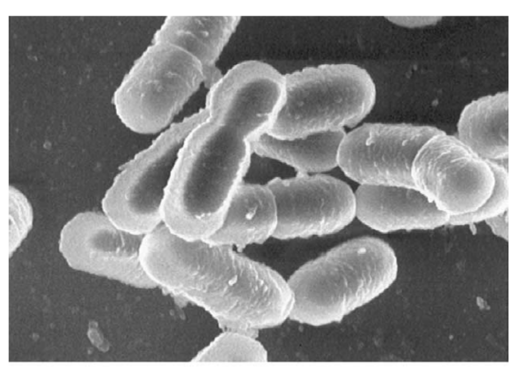


Figure 2: Scanning electron microscopy of *L. sakei* strain 23K grown at 30 °C. This figure is obtained from Champonmier-Verges *et al.* (Champomier-Verges *et al.*, 2001).

Later, *L. sakei* has found to be a ubiquitous lactic acid bacterium commonly associated with food environments. It has been isolated from several raw fermented products of plant and animal origin. It can be detected in kimchi (Choi *et al.*, 2003) silage, sauerkraut (Vogel *et al.*, 1993), sourdough, and fish products (Najjari *et al.*, 2008), but is, as mentioned above, the dominating *Lactobacillus* species in fermented meat products (Fontana *et al.*, 2005; Hammes *et al.*, 1990; Hugas *et al.*, 1993).

1.4.2 Meat adaption properties of *L. sakei*

The high competitiveness of *Lactobacillus sakei* in the meat environment can be explained by its distinct meat adaption properties. *L. sakei* differs from many LAB in being

tolerant to various adverse conditions such as low temperature, high salt concentrations and varying oxygen levels (Chaillou *et al.*, 2005; Champomier-Verges *et al.*, 2001). The analysis of *L. sakei* 23K´s genome revealed potential survival strategies as well as metabolic properties enabling effective competition in the raw-meat environment (Chaillou *et al.*, 2005).

Since meat is a protein-rich substrate and amino acids and peptides are mainly released from meat proteins upon proteolysis by meat proteases (cathepsins), *L. sakei* is auxotrophic for most amino acids (except aspartic and glutamic acid)(Champomier-Verges *et al.*, 2001). Genome analysis has indicated that these two amino acids could potentially be produced by deamination of asparagine and glutamine respectively (Chaillou *et al.*, 2005). Genes coding for enzymes, likely responsible for interconversion of asparagine and aspartate as well as glutamine and glutamate, could be *glnA* (Lsa1321; glutamate-ammonia ligase, EC: 6.3.1.2), *asnA1* (Ls0347; L-asparaginase EC: 3.5.1.1), *asnA2* (Lsa1693; asparaginase EC: 3.5.1.1) and *asnB* (Ls0636, asparagine synthetase; EC: 6.3.5.4)(Chaillou *et al.*, 2005). Reaction equations of these enzymes are shown in Table 2.

Table 2: Reaction equations for glutamate-ammonia ligase EC 6.3.1.2 (*glnA*), asparagines synthetase EC 6.3.5.4 (*asnB*), and L-asparaginase EC 3.5.1.1 (*asnA1, asnA2*)

EC	Reaction
6.3.1.2	Glutamate + ATP + NH_3 → Glutamine + ADP + Phosphate + H_2O
6.3.5.4	ATP + L-Aspartate + L-Glutamine + H_2O ↔ AMP + Pyrophosphate + L-Asparagine + L-Glutamate
3.5.1.1	L-Asparagine + H_2O ↔ L-Aspartate + NH_3

Furthermore, *L. sakei* does not possess extracellular proteinases, like PrtP. (Chaillou *et al.*, 2005). However, *L. sakei*´s enzyme systems for peptide and amino acid uptake and peptide degradation to free amino acids seem to be well developed (Chaillou *et al.*, 2005; Liu *et al.*, 2010).

Glucose is the preferred carbon source for *L. sakei*. It originates from glycogen or is exogenous added to accelerate and improve the ripening process of fermented meat products. In *L. sakei*, hexose fermentation is homolactic and proceeds via anaerobic glycolysis. The resulting formation of lactic acid and the subsequent decrease in pH is of major importance for the hygienic safety and quality of the fermented product (Champomier-Verges *et al.*, 2001). Although *L. sakei* 23K is not a bacteriocin producer, the species *L. sakei* is generally well known for its ability to produce bacteriocins

(Champomier-Verges *et al.*, 2001), e. g. the antilisterial peptide sakacin P, inhibitory against *Listeria monocytogenes,* and therefore contributing to food safety.

Since carbohydrates are quickly used up in meat fermentations, *L. sakei* additionally uses arginine as energy source. Arginine degradation in *L. sakei* is governed by the arginine deiminase pathway which leads to NH_3 and ATP production (Zuniga *et al.*, 1998). *L. sakei* is able to grow on meat under refrigeration temperatures and in the presence of curing salts (3 - 9 % NaCl). Accumulation of osmo- and cryoprotective solutes such as betaine and carnitine, driven by ABC uptake systems together with a Na+-dependent symporter could be a key factor for *L. sakei*´s adaption to cold and salt (Chaillou *et al.*, 2005). All those properties contribute to the robustness and competitiveness of *L. sakei* at harsh sausage fermentation conditions.

1.4.3 *L. sakei* – food spoiler and probiotic

L. sakei was also found to be associated with spoilage of meat and fish products. Some isolated *L. sakei* strains have been shown to be responsible for meat spoilage through ropy slime production (Mäkelä *et al.*, 1992). *L. sakei* and *L. curvatus* were found to dominate the spoilage of vacuum packaged Frankfurter, Vienna and related sausage types (German: “Brühwurst”). Association with smoked salmon (Joffraud *et al.*, 2001) and maatjes herring spoilage (Lyhs and Björkroth, 2008) has also been reported. However, recently probiotic properties of some *L. sakei* strains have been described in animal and humans: Several *L. sakei* stains have been observed as transient inhabitants of the human gastrointestinal tract (Chiaramonte *et al.*, 2009; Dal Bello *et al.*, 2003; Heilig *et al.*, 2002). Mice triggered by an allergen showed a more rapid recovery if they received *L. sakei* probio 65 compared to control mice, as assessed by visual evaluation of the severity of allergic dermatitis and levels of IgE and IL-4 (Park *et al.*, 2008). *L. sakei* BK19 enriched diet enhances the immunity status and disesase resistance to streptococcosis infection in kelp grouper, *Epinephelus bruneus* (Harikrishnan *et al.*, 2010). Supplementation of *L. sakei* KCTC 10755BP in children with atopic eczema-dermatitis syndrome (AEDS) was associated with a substantial clinical improvement and a significant decrease in chemokine levels, reflecting the severity of AEDS (Woo *et al.*, 2010).

1.4.4 Genetic heterogeneity of *L. sakei*

Wide phenotypic heterogeneity within *L. sakei* strains has been demonstrated in many studies based on biochemical and physiological features, especially in sugar fermentation (McLeod *et al.*, 2008). Genetic diversity between *Lactobacillus sakei* strains is also relatively high. Thus, PFGE analysis demonstrated a 25 % variation in genome sizes of

different *L. sakei* strains from 1,815 kb to 2,320 kb (Chaillou *et al.*, 2009). DNA-DNA reassociation analysis have revealed low levels of relatedness (as low as 72 %) between *L. sakei* strains, indicating that this species exhibits important elements of genetic heterogeneity (Champomier *et al.*, 1987).

On the basis of phenotypic and genetic properties, *L. sakei* has been divided into two sub-groups, mainly based on results from numerical analysis of whole-cell protein and RAPD patterns (Berthier and Ehrlich, 1999; Klein *et al.*, 1996; Torriani *et al.*, 1996). In several publications, these sub-groups are described as sub-species: *L. sakei* subsp. *sakei* and *L. sakei* subsp. *carnosus* (Champomier-Verges *et al.*, 2002; Koort *et al.*, 2004; Torriani *et al.*, 1996).

With *L. sakei* 23K´s (originally isolated from a French sausage and plasmid cured) genome now available (Chaillou *et al.*, 2005), it has become possible to study *L. sakei* strain diversity at a deeper genomic level, as well as performing wider searches for differences between *L. sakei* strains isolated naturally from various products. Unfortunately, *L. sakei* 23K has one of the smallest genomes (1,884 kb) of the strains investigated up to now (Chaillou *et al.*, 2009; McLeod *et al.*, 2008). That makes only a one-way comparison possible to discover the genes deleted or divergent in other strains compared to the sequenced strain, but not a detection of genes not present in the reference strain but maybe in others. In spite of a high phenotypic relatedness, the species *L. sakei* and *L. curvatus* are clearly separated at the genomic level (40 % to 50 % identity) (Kagermeier-Callaway and Lauer, 1995; Kandler, 1986).

1.5 Aroma in sausage fermentations

Odour is defined as volatiles with an ability to reach and generate response in the receptors of the olfactory epithelium (Tjener and Stahnke, 2008). Aroma is any odour that is additionally perceived orthonasal, e. g. during swallowing (odorants related to food) (Schieberle, 2011). Besides taste and texture, aroma is a very important characteristic for the overall quality of dry fermented sausages.

The addition of spices is influencing the content of volatiles, as well as factors of the production process, such as curing agents, fermentation stage or smoking (Marco *et al.*, 2008). Furthermore, aroma results from carbohydrate, protein and lipid degradation (Figure 3). These processes are linked to endogenous and microbial enzyme activities and also to chemical reactions dependent on the technological processing. According to Meynier *et al.* (Meynier *et al.*, 1999), 60 % of volatiles arise from spices, 19 % from lipid oxidation, 12 % from amino acid metabolism and 5 % from fermentation process (carbohydrate metabolism).

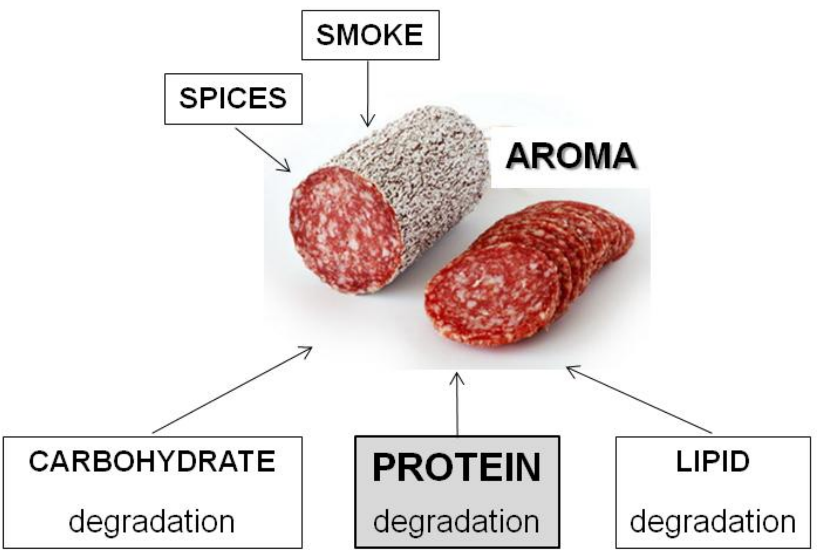


Figure 3: Aroma origins in sausage fermentations.

The unique aroma of fermented sausages is not due to one substance, but usually due to many compounds of diverse structures, blended in a specific ratio. The volatile profile of dry fermented sausage comprises a wide variety of compounds such as hydrocarbons, aldhydes, acids, ketons, alcohols, esters, sulphides, nitriles, furanes, and so on (Berdagué *et al.*, 1993; Berger *et al.*, 1990; Johansson *et al.*, 1994; Stahnke, 1994; Stahnke, 1999). In analogy to widely spread terms as genome, proteome or metabolome, we speak of volatilome refering to the entirety of volatile compounds. Up to 400 volatile compounds have been identified (Beck *et al.*, 2002; Stahnke, 2002) in fermented sausages. However, many of these volatiles do not contribute to the aroma because of their high sensory threshold values. Identification of the important aroma compounds requires a combination of analytical and sensory methods. Gas chromatography-olfactometry (GC-O), combining classical chromatography with the detection system of the human nose, is one of the preferred approaches. Sollner and Schieberle determined about 50 main aroma-active compounds in Hungarian Salami with a flavour dilution (FD) factor ≥ 16 and succeeded in aroma reconstitution (Sollner and Schieberle, 2009).

Volatiles originating from microbial breakdown of amino acids are of major importance for the overall flavour of fermented sausages. The branched-chain amino acids (valine, leucine, and isoleucine), the aromatic amino acids (tyrosine, tryptophan, and phenylalanine), and the sulphur containing amino acids (methionine and cysteine) are the main amino acid sources for aroma compounds in fermented food (Ardo, 2006; Smit *et al.*, 2005b; Yvon and Rijnen, 2001). Furthermore, especially the breakdown of leucine, isoleucine and valine via the corresponding α-keto acid into methyl-branched aldehydes, alcohols and acids (Table 3) has been shown to be strongly linked to dry sausage odour (Olivares *et al.*, 2009; Sollner and Schieberle, 2009; Stahnke *et al.*, 1999).

Table 3: Branched amino acids and corresponding metabolites

Amino Acid	α-keto acid	Aldehyde	Alcohol	Carboxylic Acid
Leucine	α-ketoisocaproate (KIC)	3-methylbutanal	3-methylbutanol	3-methylbutanoic acid
Isoleucine	α-keto-ß-methylvalerate (KMV)	2-methylbutanal	2-methylbutanol	2-methylbutanoic acid
Valine	α-ketoisovalerate (KIV)	2-methylpropanal	2-methylpropanol	2-methlypropanoic acid

1.6 Protein, peptide and amino acid metabolism in fermented sausages

Figure 4 gives an overview of general protein conversion pathways relevant for aroma formation in meat fermentations. Briefly, meat proteins are degraded into oligopeptides. After uptake of peptides (and amino acids) via various transport systems, peptide degradation by peptidases with various specificities leads to an intracellular pool of free amino acids. Free amino acids can then either be decarboxylated into undesirable biogenic amines (BA) or can be supplied to one of the pathways resulting in volatiles. The latter usually start with transamination of amino acids to their corresponding α-keto acid followed by decarboxylation and dehydrogenation steps leading to aldehydes, alcohols and carboxylic acids that can contribute to aroma profiles of fermented meat products.

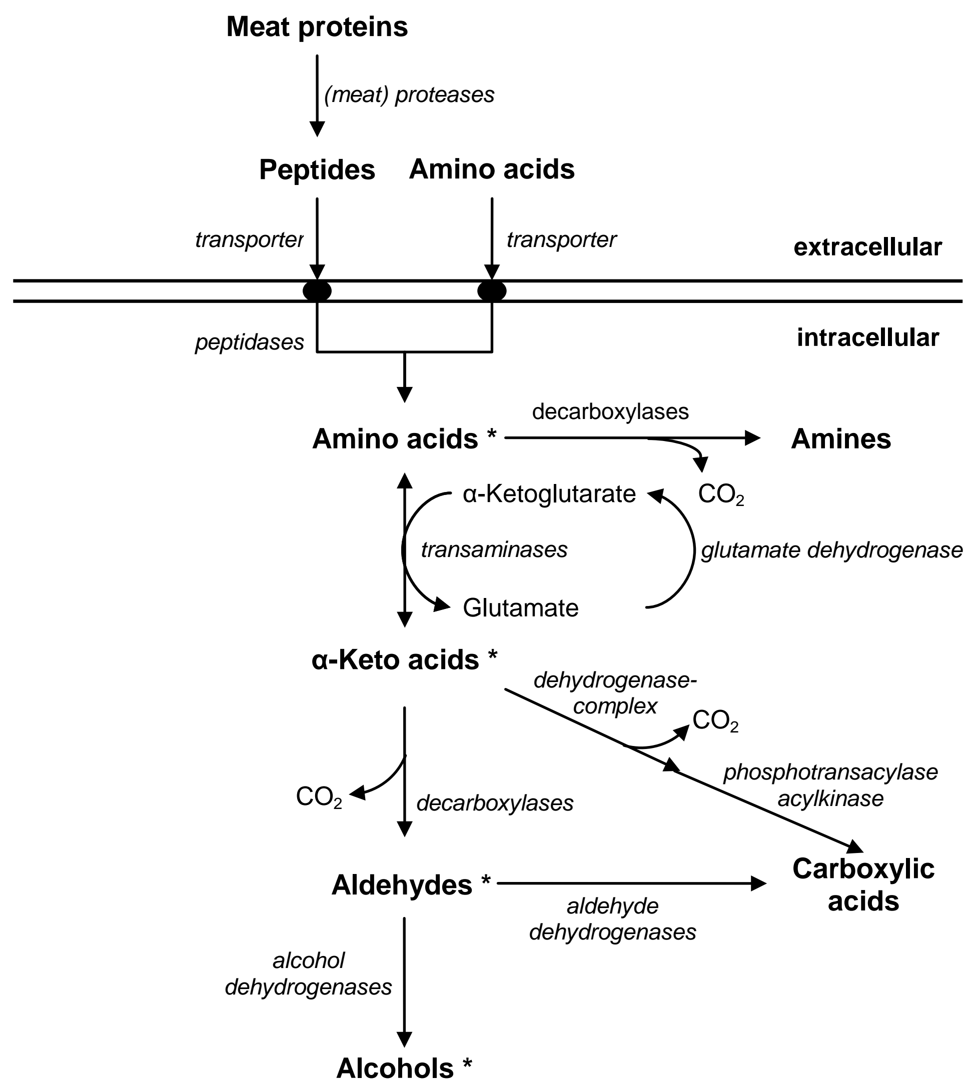


Figure 4: Overview of main protein conversion pathways relevant for aroma formation in meat fermentations. *marks metabolites of the Ehrlich pathway. This figure was adapted from van Kranenburg *et al.* (van Kranenburg *et al.*, 2002).

1.6.1 Proteolytic system

Proteolytic activities of lactic acid bacteria (LAB) play a major role in growth and also in the development of flavour and texture of fermented products. In general, the proteolytic system of LAB comprises three major components: cell-wall bound proteinases like PrtP that initiate the degradation of extracellular protein into oligopeptides, peptide transporters for peptide uptaking into the cell, and various intracellular peptidases that degrade oligopeptides into shorter peptides and amino acids. For dairy LAB these activities are generally well documented, and there are also studies about LAB in sourdough (Ganzle *et al.*, 2007; Vermeulen *et al.*, 2005) while insight in the proteolytic system on meat lactobacilli like *L. sakei* and *L. curvatus* is rather limited. Extracellular proteases appear to be ubiquitous in dairy lactococci and lactobacilli to initiate the degradation of milk caseins into oligopeptides (Kunji *et al.*, 1996). In meat fermentations, the initial hydrolysis of

muscle proteins has been mainly attributed to endogenous cathepsins and bacterial enzymes being active for the degradation of oligopeptides into small peptides and free amino acids (Verplaetse, 1994). This fits with the lack of data about extracellular proteases from meat-born lactobacilli in general and the fact that *L. sakei* 23K does not possess any genes coding for an extracellular protease in particular (Chaillou *et al.*, 2005).

The release of the genome sequence of *L. sakei* 23K as well as a study that compared the proteolytic system of LAB provides *in silico* information about the proteolytic potential of *L. sakei* 23K. According to these, *L. sakei* possesses an oligopeptide ABC transport system (Opp), a di/tripeptide ionlinked transporter (DtpT), a putative oligopeptide transporter (Puopt) and several amino acid transporters, as well as a set of 19 peptidases with different specificities (Chaillou *et al.*, 2005; Liu *et al.*, 2010). However, only four peptidases have been purified and studied: a general aminopeptidase with broad specificity (Sanz and Toldra, 1997), a dipeptidase with main specificity towards Ala-X peptides and neutral amino acids (Montel *et al.*, 1995), a X-prolyl-dipeptidyl-aminotransferase (Sanz *et al.*, 1998), and a tripeptidase with broad specificity against di- or tripeptides (Sanz *et al.*, 1998). Therefore, the involvement of peptidases in growth and physiology or their possible impact on meat fermentations is not established yet.

Proteolysis does not only contribute to aroma development but also plays a part in taste formation (by amino acids, small peptides). However, among such peptides, hydrophobic fragments are known to be the main bitter-taste factor in cheese (Lindsay, 2000). Generation and degradation of “bitter-peptides” and their effects on product quality has been well described for cheese products, where bitterness is a common problem. In cheddar cheese, for instance, bitterness occurs, when 2 to 23 residue-long hydrophobic peptides are formed. Proline-containing peptides are hydrophobic and exhibit a bitter taste (Singh *et al.*, 2003) whereas the pure amino acid L-proline is sweet (Ishibashi N., 1988). Only some peptidases have been found to act specifically on the hydrolysis of these peptides: Proline-specific peptidases PepI, PepR, XPDAP (PepX), and PepQ recognize proline containing peptide substrates. XPDAP (PepX) participates in a unique reaction among LAB peptidases: The release of N-terminal Xaa-Pro dipeptides from 3 to 7 residue-length peptides (Lloyd and Pritchard, 1991; Tsakalidou *et al.*, 1998). The generated dipeptides are subsequently exclusively digested by the prolidase PepQ, also characterizing it as a unique and important enzyme. PepI and PepR however, cleave proline form di- or tripeptides at the N-terminus (Baankreis, 1991) (Shao *et al.*, 1997). Unless bitterness is mainly described for cheese products, bitter off-tastes are also known in fermented sausages whereby activities of PepX, PepQ, PepR encoded by genes found in *L. sakei* 23K might play a role.

1.6.2 Biogenic Amines (BA)

Biogenic amines (BA) are organic bases with low molecular weight and have been reported in different kinds of food, such as fishery products, cheese, wine, beer, dry sausages and other fermented foods. These amines are designated as biogenic amines because they are formed by living organisms. Amino acid decarboxylation is the most common mode of synthesis of biogenic amines in food. Amino acid decarboxylation takes place by removal of the α-carobxyl group to give the corresponding amine (Figure 5) (Shalaby, 1996).

Figure 5: General amino acid decarboxylase reaction

The accumulation of biogenic amines in foods requires a pool of amino acid precursors, microorganisms with amino acid decarboxylase activity, and favourable conditions (temperature and pH) for growth and decarboxylation. Proteolytic activities during meat fermentation and ripening provides sufficient amounts of precursor amino acids for later decarboxylation by both starter cultures and wild microbiota (Suzzi and Gardini, 2003).

In general, histamine, tyramine, tryptamine, 2-phenylethylamine (monoamines), putrescine, cadaverine (diamines), spermine and spermidine (polyamines) are the most important BA in foods. The consumption of food containing high amounts of BA is responsible for various pharmalogical effects, which lead to several types of food-born disease, including histamine poisoning (scombroid poisoning) and tyramine toxicity (cheese reaction) (Shalaby, 1996).

These problems may be even more severe in persons with insufficient mono- and diamine oxidase activity, the enzymes responsible for BA detoxification (Bodmer *et al.*, 1999). Histamine from histidine and, to a lesser extent, tyramine from tyrosine are the most toxic biogenic amines (BAs) affecting human health (Masson *et al.*, 1996). Histamine can cause headaches, low blood pressure, heart palpitations, edema, vomiting, diarrhea, etc. Tyramine leads to hypertensive resoponses especially in patients treated with monoamine oxidase inhibitors. The diamines putrescine and cadaverine, although not toxic themselves, may potentiate the toxic effects, by inhibiting the detoxification enzymes (Bardócz, 1995; Straub *et al.*, 1995; ten Brink *et al.*, 1990). Furthermore, putrescine and cadaverine may cause off-flavours (Wang *et al.*, 1975) and form mutagenic N-nitrosamines after conversion into secondary amines (Yamamoto *et al.*, 1982).

High levels of tyramine but also histamine and the diamines putrescine and cadaverine could be found in fermented sausages (Eerola *et al.*, 1998; Shalaby, 1993; Trevino *et al.*, 1997). Many LAB from meat and meat products showed decarboxylase activities against amino acids. In several studies it has been observed that *L. curvatus* appears to be the main BA producer, but also some strains of *Lactobacillus brevis, Lactobacillus paracasei*, and *L. sakei* produced tyramine (Bover-Cid and Holzapfel, 1999; Bover-Cid *et al.*, 2001a; Latorre-Moratalla *et al.*, 2010). *L. sakei* LTH 2076 was identified as histidine decarboxylase positive strain (HDC+) (Coton and Coton, 2005). Rosenstein *et al.* (Rosenstein *et al.*, 2009) reported that *S. carnosus* encodes an ornithine decarboxylase (Sca0122) that could convert ornithine to putrescine. Moreover, enterococcal strains, found in dry fermented sausages, had a high potential for production of biogenic amines. All enterococcus strains tested by Latorre-Moratalle *et al.* were decarboxylase positive and produced high amounts of tyramine and considerable amounts of beta-phenylethylamine (Latorre-Moratalla *et al.*, 2010).

Final BA concentrations in fermented sausages depend on the microbial composition, the quality of the meat used as raw material and the activities of the starter culture applied. The use of decarboxylase negative strains prevented biogenic amine formation in meat products. A mixed starter culture consisting of *L. sakei* and *Staphylococcus* spp, drastically reduced tyramine, cadaverine, and putrescine accumulation and prevented formation of histamine, phenylethylamine or tryptamine (Bover-Cid *et al.*, 2000). The decarboxylase negative strain *L. sakei* CTC494 was solely able to reduce and even inhibit biogenic amine accumulation during sausage fermentation (Bover-Cid *et al.*, 2001b). However, a prerequisite of such a successful prevention of BA accumulation in meat fermentations is the use of raw materials with good hygienic quality (Bover-Cid *et al.*, 2000; Bover-Cid *et al.*, 2001b).

Since knowledge of the decarboxylation potential of potential starter cultures is essential, several molecular methods (including PCR and DNA hybridization methods) have been developed for the detection of BA-producing bacteria. They were reviewed by Landete *et al.* (Landete *et al.*, 2007).

1.6.3 Formation of volatiles from amino acids

1.6.3.1 Transamination

As mentioned above, branched-chain amino acids, aromatic amino acids, and sulfur-containing amino acids are the main amino acid sources for flavour compounds in fermented products (Ardo, 2006; Smit *et al.*, 2005b; Yvon and Rijnen, 2001). Especially the breakdown of leucine, isoleucine and valine into methyl-branched aldehydes, acids

and alcohols has been linked to dry sausage aroma (Montel *et al.*, 1996; Stahnke, 1995b; Stahnke *et al.*, 2002).

The initial step in the degradation of e. g. L-leucine is its conversion to the corresponding α-keto acid. Generally, this could occur by one of three enzymatic reactions: an oxidative deamination of L-amino acids, catalyzed by a leucine dehydrogenase (Nagata *et al.*, 1995) or a leucine oxidase (Brearley *et al.*, 1994) or a transamination, catalyzed by a leucine transaminase (Yvon *et al.*, 2000).

Aminotransferases (transaminases) are enzymes that catalyze the transfer of an amino group from a donor molecule (Figure 6) to a recipient molecule, using pyridoxal-5´-phosphate (PLP) as cofactor. PLP is tightly bound to the enzyme and carries the amino group from the donating amino acid. The enzyme uses a specific α-keto acid that is commonly α-ketoglutaric acid (αKGA), which is transformed into glutamic acid by accepting the amino group carried by PLP for regeneration (Voet *et al.*, 2002).

Figure 6: General aminotransferase reaction with α-ketoglutarate as acceptor molecule

For degradation of BCAA in staphylococci, two catabolic pathways may be involved (transamination and oxidative deamination), since degradation of leucine to α-ketoisocaproic acid is only reduced in the absence of α-ketogluaric acid and PLP, the acceptor molecule and cofactor in transaminase reaction (Larrouture *et al.*, 2000). However, only a branched-chain aminotransferase of *S. carnosus* has been characterized genetically and biochemically and was shown to catalyze the transamination of branched-chain amino acids and, to some extent, methionine. An *ilvE* deletion mutant revealed that IlvE is the most important BCAA transamination enzyme in *S. carnosus*, since the mutant degraded less than 5 % of the BCAA, while the wild-type strain degraded 75 to 95 %. Moreover, since addition of free amino acids leads only to a minor increase of total BCAA transamination whereas addition of α-ketoglutarate highly enhances conversion of amino acids into aroma compounds, glutamate dehydrogenase (GDH), the enzyme catalyzing the regeneration of α-ketoglutarate, was pointed out as rate-limiting enzyme in transamination of amino acids under sausage fermentation conditions (Tjener *et al.*, 2004b). Since no metabolite was detected in the absence of α-ketogluatric acid and PLP, transamination seems to be the only enzymatic system involved in the first step of amino acid degradation in lactic acid bacteria (Larrouture *et al.*, 2000). Indeed, it has been

proven that transaminases are the only enzymes in *L. lactis*, which are responsible for AAA and BCAA deamination (Atiles *et al.*, 2000; Rijnen *et al.*, 1999; Yvon *et al.*, 1997; Yvon *et al.*, 2000). A knock-out of *ilvE*, coding for a branched-chain aminotransferase (BcaT), resulted in approximately 90 % reduction of total transamination activity against branched-chain amino acids. Residual activity was caused by the aspecificity of other tranaminases (Yvon *et al.*, 2000). Transaminases of *L. lactis* have been described comprehensively, including aromatic amino acid (Gao and Steele, 1998; Rijnen *et al.*, 1999) and branched-chain amino acid transaminases (Yvon *et al.*, 2000).

L. paracasei is the only meat-relevant lactobacillus for which a transaminase has been characterized yet. *L. paracasei* is the dominant species of non-starter LAB (NSLAB) in several semi-hard cheese varieties but has also been isolated from meat products (Coppola *et al.*, 1998; Coppola *et al.*, 2000; Jahreis *et al.*, 2002). It belongs to the *L. casei* group and is known to have probiotic properties (Jahreis *et al.*, 2002; Klein *et al.*, 1998). Furthermore, it was demonstrated that permeabilized cells of *L. paracasei* show a large variation in activities and specificities of aminotransferase among strains (Thage *et al.*, 2004a). BcaT of the strain with highest activity on BCAAs has been previously isolated and characterized and has been shown to have almost the same affinity for all three BCAA (Thage *et al.*, 2004b).

Genetic approaches did not reveal aminotransferases specific for BCAA or AAA in the meat-born lactic acid bacterium *Lactobacillus sakei* 23K (Chaillou *et al.*, 2005; Liu *et al.*, 2008). However, several studies have shown that presence and activities of transaminases vary largely among bacterial species and strains (Fernandez and Zuniga, 2006; Liu *et al.*, 2008; Smit *et al.*, 2004b).

1.6.3.2 Decarboxylation and dehydrogenation

Branched-chain keto acid decarboxylase (KdcA) is an enzyme that catalyzes the decarboxylation of an α-keto acid (e. g. α-keto isocaproic acid) to the corresponding aldehyde (e. g. 3-methylbutanal). KdcA activity has been found in a limited number of *L. lactis* strains and could be characterized in *L. lactis* B1157. Highest activities were observed with branched-chain α-keto acids (Smit *et al.*, 2005a). The gene coding for KdcA occurs only rarely in the sequenced LAB genomes (Liu *et al.*, 2008), and *kdcA* is not present in the genome of *S. carnosus* TM300 (Rosenstein *et al.*, 2009). Besides KdcA activity, a chemical conversion of α-keto acid to corresponding aldehydes has been described under conditions derived from cheese fermentations. This reaction is catalyzed by manganese and it can be modulated by Mn^{2+}, oxygen and redox potential (Kieronczyk *et al.*, 2006; Smit *et al.*, 2004a). Since, variation in the formation of volatiles in a meat model system was mainly determined by pH and bacterial species and, only to a lesser

extent, by the manganese concentration (Thonning Olesen and Stahnke, 2004; Tjener *et al.*, 2004a), chemical conversion of α-keto acid is probably not dominant in this system (Smit *et al.*, 2009).

Alcohol dehydrogenases can catalyze the reduction of methyl-aldehydes (e. g. 3-methylbutanal) to methyl-alcohols (e. g. 3-methybutanol). Alcohol dehydrogenases (AlcDH) are present in nearly all genome sequenced LAB. In *Lactobacillus sakei* 23K even six putative AlcDHs have been detected (Chaillou *et al.*, 2005; Liu *et al.*, 2008).

Beck *et al.* (Beck *et al.*, 2002) found out by deuterium-labelling experiments that in *S. xylosus* 3-methylbutanoic acid is mainly formed by decarboxylation of an α-keto acid followed by a rapid oxidation of the aldehyde by an aldehyde dehydrogenase. The pathway from leucine via the corresponding α-keto acid and aldehyde to the alcohol is referred to as Ehrlich pathway, which was identified in yeast as main route for fusel alcohol formation (Ehrlich, 1907).

Besides the Ehrlich pathway, α-keto acids can be converted directly to carboxylic acids via oxidative decarboxylation. This pathway does not lead to the formation of 3-methylbutanal or the corresponding alcohol from α-ketoisocaproate but forms the corresponding organic acids. The first step is the substitution of CO_2 by the cofactor CoA, while reducing NAD^+, and results in acyl coenzyme A (acyl-CoA). The reaction is catalyzed by an α-keto acid dehydrogenase complex (KaDH), composed of four subunits: E1α, E1β, E2, and E3. KaDH is known to be active in yeasts, bacilli, propioni bacteria, *Enterococcus faecalis* and *Lactococcus lactis* (Richard Dickinson and John R. Sokatch, 2000; Sokatch *et al.*, 1981; Ward *et al.*, 1999; Zhu *et al.*, 2005). The CoA coupled acid can be used in fatty acid biosynthesis or can be hydrolyzed to the branched-chain organic acid. The latter reaction is catalyzed by a phosphotransacylase and an acyl kinase (ACK) that further converts acyl-CoA into the corresponding carboxylic acid by a CoA-ester intermediate. The oxidative decarboxylation pathway was characterized in *E. faecalis* and a phosphotransacylase (ACK), as well as a KaDH complex with specific activity against the branched-chain α-keto acids α-ketoisovalerate (KIV), α-ketoisocaproate (KIC) and α-keto-β-methylvalerate (KMV) were found encoded in the gene cluster *ptb-buk-bkdDABC*. Furthermore, it is proposed that the conversion of the branched-chain α-keto acids to the corresponding free acids results in the formation of ATP via substrate level phosphorylation (Rüdiger *et al.*, 1972; Ward *et al.*, 1999; Ward *et al.*, 2000). Although among genome sequenced LAB a similar orthologous operon could only be found in *L. casei*, homologs of the *ptb* gene, *buk* gene, and *bkdDABC* genes were found to be encoded separately in various other LAB. For instance, in *L. sakei* genes encoding for a KaDH complex and a (branched chain) phosphotransacylase could be found in different regions of the chromosome. Caution is required, however, since the best homologs of

KaDH in many LAB are annotated as either pyruvate or acetoin dehydrogenase complex, and it is unclear whether these complexes have overlapping substrate specificities (Liu *et al.*, 2008). In the genome of *S. carnosus* TM300 an operon containing *ptb-buk-bkdDABC* could be found (Rosenstein *et al.*, 2009) but, so far there is no experimental data available about the activity of enzymes encoded by genes belonging to this operon.

1.7 The aim of this study

During the last years culture collections were established in industry and research laboratories. However, for a lack of clear selection criteria and rapid valuation methods, these collections often remain unused and therefore starter strains utilized in meat industry originate from the 1960s-1980s. On the one hand starter cultures, like *Lactobacillus sakei*, have to be safe for human consumption and on the other hand they should positively contribute to texture, colour and flavour of fermented sausages.

In terms of safety and aroma formation metabolism of peptides and amino acids is of particular importance, since toxicological critical biogenic amines and sensory relevant volatiles are formed from these precursors.

Therefore, the aim of this study was to characterize the genetic composition of various *Lactobacillus sakei* strains concerning genes of the peptidolytic system and involved in amino acid degradation in order to facilitate and accelerate the screening and valuation of starter lactobacilli for sausage fermentations along their genetic potential.

2 Material and Methods

2.1 Materials

2.1.1 Devices

Major devices used in this work are listed in Table 4 in alphabetical order.

Table 4: Devices used in this study

Device	Model	Manufacturer
Agarose gel chamber 25 x 20 cm	Easy Cast electrophoresis system	Owl Separation Systems, Portsmouth, NH, USA
Agarose gel chamber 13.8 x 12 cm	Easy Cast electrophoresis system	Owl Separation Systems, Portsmouth, NH, USA
Autoclaves	2540 ELV	Systec GmbH, Wettenberg, Germany
	Varioklav	H + P Labortechnik, Oberschleißheim, Germany
Blotting oven	MINI 10	MWG Biotech AG, Ebersberg, Germany
Breeding/incubation	Certomat BS-1	B. Braun Biotech International, Melsungen, Germany
	Hereaus B5042E	Hereaus Instruments, Hanau, Germany
	Memmert INB series	Memmert GmbH & Co. KG, Schwabach, Germany
	WiseCube®WIS-ML02	Witeg Labortechnik GmbH, Wertheim, Germany
Centrifuges	Sigma 1 K 15	Sigma Labortechnik, Osterrode am Harz, Germany
	Sigma 6-16K	Sigma Labortechnik, Osterrode am Harz, Germany
	J-6	Beckman, Palo alto, CA, USA
	J-2	Beckman, Palo alto, CA, USA
	Hermle Z383 K	Hermle Labortechnik, Wehningen, Germany
	Hermle Z382 K	Hermle Labortechnik, Wehningen, Germany
Electroporation system	Bio-Rad Gene pulser device	Bio-Rad Laboratories, Hercules, CA, USA
FPLC system	Biologic HR Controller	Bio-Rad Laboratories, Hercules, CA, USA
	Biologic HR Workstation	Bio-Rad Laboratories, Hercules, CA, USA
	Modell 2128 Fraction Collector	Bio-Rad Laboratories, Hercules, CA, USA
Fluorescence microscopy	SteREO Discovery Stereomikroskop	Carl Zeiss MicroImaging GmbH, Germany
	HBO50 Microscope llumination device	
	AxiocamICc1	

Device	Model	Manufacturer
GC equipment	SPME device	Supelco, Bellefonte, Pennsylvania, USA
	carboxen/polydimethylsiloxane (CAR/PDMS) fiber	
	gas chromatograph Agilent 7890A	
	ZB-Wax capillary column 60 m, 0.25 mm i.d., film thickness 0.25 µm	Zebron, Phenomenex, Torrance, CA, USA
	mass selective detector Agilent 5975C	Agilent Technologies Inc., Santa Clara, CA, USA
	CTC CombiPAL autosampler	CTC Analytics AG, Zwingen, Switzerland
Incubation hood	Certomat H	B. Braun Biotech International, Melsungen, Germany
Laminar flow sterile work bench	HERA safe	Heraeus Instruments, Hanau, Germany
Microscope	Axiolab	Carl Zeiss MicroImaging GmbH, Germany
Nanodrop	Nanodrop1000	Peqlab Biotechnologie GmbH, Erlangen, Germany
PCR-Cycler	Primus 96 plus	MWG Biotech, AG, Ebersberg, Germany
	Mastercycler gradient	Eppendorf AG, Hamburg, Germany
pH determination (electrode)	InLab 412, pH 0-14	Mettler-Toledo, Gießen, Germany
pH determination (measuring device)	Knick pH 761 Calimatic	Knick elektronische Geräte, Berlin, Germany
Photometer	NovaspelIq	Pharmacia Biotech, Cambridge, England
Pipettes	Pipetman	Gilson-Abomed, Langenfeld, Germany
Plate readers	TECAN SPECTRAFluor	TECAN Deutschlan GmbH, Crailsheim, Germany
	TECAN SUNRISE	TECAN Deutschlan GmbH, Crailsheim, Germany
Power supplies	MPP 2 x 3000 Power Supply	MWG Biotech AG, Ebersberg, Germany
	Electroophoresis Power Supply EPS 3000	Pharmacia Biotech, Cambridge, England
	2197 Supply PPS 200-1D	MWG Biotech AG, Ebersberg, Germany
Pure water	Euro 25 and RS 90-4/UF pure water system	SG Wasseraufbereitung GmbH, Barsbüttel, Germany
SDS-PAGE	Mini Protean III-System	Bio-Rad Laboratories, Hercules, CA, USA
Shaking	Certomat R	B. Braun Biotech International, Melsungen, Germany
	Vortex 2 Genie	Scientific Industries Inc., Bohemia, NY, USA

Device	Model	Manufacturer
Stirring	RCT-Basic	Mettler-Toledo, Gießen, Germany
Thermo block	Techne DRI-Block DB3	Thermo-Dux Gesellschaft für Laborgerätebau mbH, Wertheim, Germany
Ultra sonic water bath	Sonorex Super RK 103H	Bandelin electronic, Berlin, Germany
Ultra sonification	UP 200S	Dr. Hielscher GmbH, Teltow, Germany
	SONOPLUS/SH70G	Bandelin electronic, Berlin, Germany
UV table	Herolab UVT 28M	Herlab GmbH Laborgeräte, Wiesloch, Germany
Water bath	Lauda BD	LAUDA Dr. D. Wobser GmbH & Co., Lauda-Königshofen, Germany

2.1.2 Chemicals

Chemicals used in this work are listed in Table 5 in alphabetical order

Table 5: Chemicals used in this study

Chemicals	Purity	Manufacturer
1,2 dimethoxy ethan	p.a.	SIGMA-Aldrich, Steinheim, Germ
6 x DNA loading dye	-	Fermentas GmbH, St. Leon-Rot, Germany
α-keto-glutaric acid disodium salt	>97 %	SIGMA-Aldrich, Steinheim, Germany
Acetic acid	99 - 100 % (glacial)	Merck, Darmstadt, Germany
Acetone	for HPLC	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Acrylamid-Bis solution	(19:1); 30 % (w/v)	SERVA, Heidelberg, Germany
Adenine	p.a.	SERVA, Heidelberg, Germany
Agar	european agar	Difco, BD Sciences, Heidelberg
Agarose	for electrophoresis	Biozym Scientific GmbH, Oldendorf, Germany
Amino acids	research grade	SERVA, Heidelberg, Germany
Ampicillin sodium salt	93.3 %	Gerbu Biotechnik GmbH, Gaiberg, Germany
Ammonium chloride	≥99.5 % p.a.	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Ammonium persulfate (APS)	electrophoresis grade	SERVA, Heidelberg, Germany
Anaerocult C mini	-	Merck, Darmstadt, Germany
Arabinose	>98 %	SIGMA-Aldrich, Steinheim, Germany
Bio-Rad Protein assay	-	Bio Rad Laboratories GmbH, München, Germany
Biotin	~98 %	SIGMA-Aldrich, Steinheim, Germany

Chemicals	Purity	Manufacturer
Boric acid	≥99.5 %	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Brain Heart Infusion Broth (BHI)	for microbiology	SIGMA-Aldrich, Steinheim, Germany
Bromcresol purple	p.a.	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Bromphenol blue	for electrophoresis	SIGMA-Aldrich, Steinheim, Germany
BSA	fraction V for biochemical use	Merck, Darmstadt, Germany
$CaCl_2$ * 2 H_2O	p.a.	Merck, Darmstadt, Germany
(1S)-(+)-10-Camphorsulfonic acid	99 %	SIGMA-Aldrich, Steinheim, Germany
Cobalamine	p.a.	SIGMA-Aldrich, Steinheim, Germany
Cyanocobalamine	p.a.	SIGMA-Aldrich, Steinheim, Germany
Cysteine hydrochloride * H_2O	p.a.	Merck, Darmstadt, Germany
Dimidium bromide	≥98 %	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
DMSO (dimethyl sulfoxide)	≥99.5 % p. a.	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
DTT (1,4 Dithio-D,L-Threitol)	high purity	GERBU Biotechnik, GmbH, Gaiberg, Germany
EDTA	for molecular biology	SIGMA-Aldrich, Steinheim, Germany
Erythromycin	-	SIGMA-Aldrich, Steinheim, Germany
Ethanol, denatured	99 % with 1 % methylethylketone	Chemikalien und Laborbedarf Nierle, Freising, Germany
Ethanol, absolute	≥99,8 %	VWR, Prolabo, Foutenay-sous-Bois, France
Ethidium bromide	1 % in H_2O for electrophoresis	Merck, Darmstadt, Germany
Fast-AP	-	Fermentas GmbH, St. Leon-Rot, Germany
FD restriction buffer	-	Fermentas GmbH, St. Leon-Rot, Germany
FD restriction enzymes	-	Fermentas GmbH, St. Leon-Rot, Germany
Folic acid	p. a.	SIGMA-Aldrich, Steinheim, Germany
Glucose	for biochemical use	Merck, Darmstadt, Germany
Glycerol	99.5 %, high purity	GERBU Biotechnik, GmbH, Gaiberg, Germany
Glycine	p. a.	Merck, Darmstadt, Germany
Guanine	≥99,8 %	Merck, Darmstadt, Germany
HCl 37 %	p.a.	Merck, Darmstadt, Germany

Chemicals	Purity	Manufacturer
Imidazole	for biochemical use	SIGMA-Aldrich, Steinheim, Germany
Inosine	p.a.	SIGMA-Aldrich, Steinheim, Germany
IPTG	p.a.	GERBU Biotechnik, GmbH, Gaiberg, Germany
Isoamyl alcohol	>98.5 %	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Isopropanol	p.a.	Scharlau Chemi S. A., Sentmenat, Spain
KH_2PO_4	p. a.	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
K_2HPO_4 * 3 H_2O	p.a.	Merck, Darmstadt, Germany
Kalium acetate	p. a.	Merck, Darmstadt, Germany
KCl	p. a.	Merck, Darmstadt, Germany
L-amino acids	p.a.	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Lysozyme	-	SERVA, Heidelberg, Germany
Meat extract	for microbiology	Merck, Darmstadt, Germany
Methanol	HPLC-grade	Mallinkrodt Baker B. V., Deventer, NL
$MgCl_2$ * 6 H_2O	p.a.	Merck, Darmstadt, Germany
$MgSO_4$ * 7 H_2O	p.a.	Merck, Darmstadt, Germany
$MnCl_2$	p.a.	Merck, Darmstadt, Germany
$MnSO_4$ * 4 H_2O	p.a.	Merck, Darmstadt, Germany
NaCl	p.a.	Merck, Darmstadt, Germany
NaH_2PO_4	p.a.	Merck, Darmstadt, Germany
NaOH	p.a.	Merck, Darmstadt, Germany
NH_4Cl	p.a.	Merck, Darmstadt, Germany
Nicotinic acid	p.a.	SIGMA-Aldrich, Steinheim, Germany
Ninhydrin	p.a.	Merck, Darmstadt, Germany
Orotic acid	p.a.	SIGMA-Aldrich, Steinheim, Germany
p-amino benzoic acid	≥99,8 %	SIGMA-Aldrich, Steinheim, Germany
Panthothenic acid	p.a.	SIGMA-Aldrich, Steinheim, Germany
Paq5000™ polymerase	-	Stratagene, La Jolla, USA
Paraffin oil	-	SIGMA-Aldrich, Steinheim, Germany
Pepton from casein	for microbiology	Merck, Darmstadt, Germany
PhatGel™BlueR (Coomassie)	-	Amersham Biosciences, Uppsala, Sweden
Pyridoxal 5´-phosphate hydrate	-	SIGMA-Aldrich, Steinheim, Germany
Primer	-	MWG-BiotechAG, Ebersberg, Germany
1-propanol	>99.5 %, for synthesis	Carl Roth GmbH + Co. KG, Karlsruhe
Pyridoxal-HCl	p.a.	SIGMA-Aldrich, Steinheim, Germany

Chemicals	Purity	Manufacturer
SDS	research grade	SERVA, Heidelberg, Germany
Sodium acetate * 3 H_2O	p.a.	Merck, Darmstadt, Germany
Sodium azide	p.a.	SIGMA-Aldrich, Steinheim, Germany
Sodium citrate	p.a.	Merck, Darmstadt, Germany
Sodium phosphate	p.a.	Merck, Darmstadt, Germany
Sucrose	HPLC-grade	Gerbu Biotechnik GmbH, Gaiberg, Germany
Sulfuric acid	p.a.	Merck, Darmstadt, Germany
T4 DNA ligase	-	Fermentas GmbH, St. Leon-Rot, Germany
Taq Core Kit	-	MP Biomedicals Solon, Ohio, USA
TEMED	p.a.	Merck, Darmstadt, Germany
Thiamine HCl	-	SIGMA-Aldrich, Steinheim, Germany
Thymine	p.a.	SIGMA-Aldrich, Steinheim, Germany
Tris	ultra pure	MP Biomedicals Solon, Ohio, USA
Tris base	ultra pure	ICN Biomedicals, Inc., Ohio, USA
Tris-HCl	p.a.	Merck, Darmstadt, Germany
Tween 80	-	Mallinkrodt Baker B. v., Deventer, NL
Uracil	p.a.	SIGMA-Aldrich, Steinheim, Germany
Xanthine	p.a.	SIGMA-Aldrich, Steinheim, Germany
X-Gal (5-Bromo-4-chloro-3-indolyl-ß-D-galactobpyranoside)	p.a.	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Yeast extract	for microbiology	Merck, Darmstadt, Germany

2.1.3 Expendable materials

Other materials used in this work are listed in Table 6 in alphabetical order.

Table 6: Further materials used in this work.

Material	Type	Manufacturer
Electroporation cuvettes	-	Biozym scientific GmbH, Oldendorf, Germany
Microtiter plates	multi well plate 96-well flat bottom with lid	Sarstedt, Nümbrecht, Germany
Reaction tubes	2 ml, 1.5 ml, 200 µl	Eppendorf, Hamburg, Germany
Sterile ml tubes	5 ml, 15 ml, 50 ml	Sarstedt, Nümbrecht, Germany
TLC Silica gel 60 WF_{254S}	-	Merck, Darmstadt, Germany
VISKING® dialysis tubing 20/32	-	SERVA, Heidelberg, Germany
HisTrap™HP-column	-	Amersham Biosciences, Uppsala, Sweden

Material	Type	Manufacturer
Sterile filter	Filtropur S 0.2 (0.2 µm)	Sarstedt, Nümbrecht, Germany
GC vials	20 ml, 75.5 x 22.5 mm	VWR, international
Magnetic crim cap	UltraClean™, 1.6 mm	VWR, international
Septum	Butyl/PTFE 1.6 mm	VWR, international
Anaerocult	-	Merck, Darmstadt, Germany

2.1.4 Kits

Kits used in this work are listed in Table 7 in alphabetical order.

Table 7: Kits used in this study

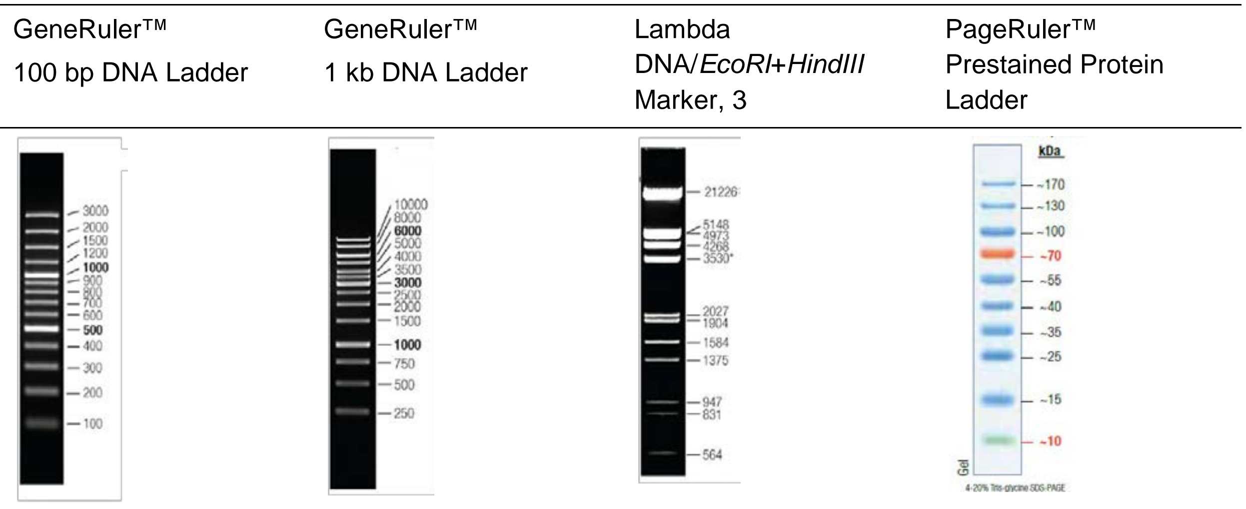
Kit	Type	Manufacturer
E.Z.N.A. Bacterial DNA Kit	DNA isolation	Omega Bio-Tek Inc., Norcross, GA, USA
EZ:faast™	Free Amino Acid Analysis by GC-MS	Phenomenex, Torrance, CA, USA
KOD hot start DNA polymerase	DNA polymerase	Novagen, EMD chemicals Inc., San Diego, CA, USA
L-Glutamic acid	L-glutamic acid	Boehringer, Mannheim, Germany
peqGOLD plasmid miniprep kit	Plasmid miniprep kit	PEQLAB Biotechnologie GmbH, Erlangen, Germany
peqGOLD Gelextraction Kit	Gel extraction	PEQLAB Biotechnologie GmbH, Erlangen, Germany
pSTBlue1 AccepTor™ Vector Cloning kit	Cloning kit	Merck, Darmstadt, Germany
Pure Yield plasmid midiprep system	Plasmid midiprep system	Promega, Madison, WI, USA
QIAquick PCR purification Kit	PCR purification Kit	Qiagen GmbH, Hilden, Germany
Taq Core Kit	DNA polymerase	MP Biomedicals Solon, Ohio, USA

2.1.5 DNA and protein markers

DNA and protein markers used for size comparison were purchased from Fermentas GmbH, St. Leon-Rot and are listed in Table 8.

Table 8: DNA and protein markers

GeneRuler™ 100 bp DNA Ladder	GeneRuler™ 1 kb DNA Ladder	Lambda DNA/*EcoRI+HindIII* Marker, 3	PageRuler™ Prestained Protein Ladder



2.1.6 Bacterial strains

All strains used in this work were obtained from TMW culture collection. For subcloning and knock-out experiments, *E. coli* K12 DH5α was used. For heterologous expressions, expression strain *E. coli* TOP 10 was used.

All lactic acid bacteria strains and one *Staphylococcus carnosus* strain used in this study are listed in Table 9.

Table 9: Lactic acid bacteria and staphylococci used in this study

Species	Strain	Source	Synonyms/comments
L. sakei	TMW 1.2	Sausage (Spain)	
L. sakei	TMW 1.3	Sausage (Spain)	
L. sakei	TMW 1.4	Sausage (Spain)	
L. sakei	TMW 1.13	Starter preparation	
L. sakei	TMW 1.22	Starter preparation	LTH 677
L. sakei	TMW 1.23	Sausage	LTH 673
L. sakei	TMW 1.30	Unknown	
L. sakei	TMW 1.46	Starter preparation	
L. sakei	TMW 1.114	Starter preparation	
L. sakei	TMW 1.147	Sausage	CTC 335
L. sakei	TMW 1.148	Unknown	
L. sakei	TMW 1.149	Unknown	
L. sakei	TMW 1.150	Unknown	
L. sakei	TMW 1.151	Unknown	
L. sakei	TMW 1.152	Unknown	
L. sakei	TMW 1.153	Unknown	

Species	Strain	Source	Synonyms/comments
L. sakei	TMW 1.154	Unknown	
L. sakei	TMW 1.155	Unknown	
L. sakei	TMW 1.161	Starter preparation	
L. sakei	TMW 1.162	Starter preparation	
L. sakei	TMW 1.163	Starter preparation	
L. sakei	TMW 1.165	Unknown	
L. sakei	TMW 1.402	Sauerkraut	LTH 2068
L. sakei	TMW 1.411	Sauerkraut	
L. sakei	TMW 1.412	Sauerkraut	
L. sakei	TMW 1.417	Starter preparation	
L. sakei	TMW 1.454	Sausage	LTH 673
L. sakei	TMW 1.578	Starter preparation	
L. sakei	TMW 1.579	Starter preparation	
L. sakei	TMW 1.587	Starter preparation	
L. sakei	TMW 1.588	Starter preparation	
L. sakei	TMW 1.589	Starter preparation	
L. sakei	TMW 1.1189	DSM 20017 T	type strain: *L. sakei* subspecies *sakei*
L. sakei	TMW 1.1239	Sourdough	
L. sakei	TMW 1.1240	Sourdough	
L. sakei	TMW 1.1290	Sausage	
L. sakei	TMW 1.1322	Meat	*L. sakei* 23K INRA, France
L. sakei	TMW 1.1366	Starter preparation	
L. sakei	TMW 1.1383	Starter preparation	
L. sakei	TMW 1.1385	Starter preparation	
L. sakei	TMW 1.1386	Starter preparation	
L. sakei	TMW 1.1388	Starter preparation	bitter aroma
L. sakei	TMW 1.1392	Starter preparation	mild aroma
L. sakei	TMW 1.1393	Starter preparation	
L. pentosus /plantarum	TMW 1.1394	Starter preparation	amended, before *L. sakei*
L. sakei	TMW 1.1395	Starter preparation	
L. sakei	TMW 1.1396	Starter preparation	
L. sakei	TMW 1.1397	Starter preparation	
L. sakei	TMW 1.1398	Starter preparation	bitter aroma
L. sakei	TMW 1.1399	Starter preparation	mild aroma
L. sakei	TMW 1.1474	Sauerkraut	LTH 2076
L. sakei	TMW 1.1407	Fermented fish	
L. curvatus	TMW 1.7	DSM 20019 T	type strain *Lactobacillus curvatus*
L. curvatus	TMW 1.17	Sausage	
L. curvatus	TMW 1.27	Unknown	

Species	Strain	Source	Synonyms/comments
L. curvatus	TMW 1.48	Starter preparation	
L. curvatus	TMW 1.49	Starter preparation	
L. curvatus	TMW 1.50	Starter preparation	
L. curvatus	TMW 1.51	Starter preparation	
L. curvatus	TMW 1.158	Starter preparation	amended, before *L. sakei*
L. curvatus	TMW 1.166	Unknown	amended, before *L. sakei*
L. curvatus	TMW 1.167	Unknown	
L. curvatus	TMW 1.401	Sauerkraut	LTH 2053
L. curvatus	TMW 1.407	Sauerkraut	
L. curvatus	TMW 1.408	Sauerkraut	
L. curvatus	TMW 1.421	Sausage material	
L. curvatus	TMW 1.439	Sausage	
L. curvatus	TMW 1.440	Hungarian salami	
L. curvatus	TMW 1.591	Unknown	
L. curvatus	TMW 1.593	Starter preparation	
L. curvatus	TMW 1.594	Starter preparation	
L. curvatus	TMW 1.595	Starter preparation	
L. curvatus	TMW 1.596	Starter preparation	
L. curvatus	TMW 1.624	Italian sausage	
L. curvatus	TMW 1.1291	Sausage	
L. curvatus	TMW 1.1365	Unknown	
L. curvatus	TMW 1.1381	Starter preparation	
L. curvatus	TMW 1.1382	Starter preparation	
L. curvatus	TMW 1.1384	Starter preparation	
L. curvatus	TMW 1.1389	Starter preparation	
L. curvatus	TMW 1.1390	Starter preparation	
L. curvatus	TMW 1.1391	Starter preparation	
L. curvatus	TMW1.1408	Fermented fish	
L. paracasei	TMW 1.1434	Probiotic starter	
E. faecalis	TMW 2.852	Clinical isolate	V583
S. carnosus	TMW 2.801	Starter preparation	

2.1.7 Primer

Oligonucleotides for screening, cloning and sequencing purposes were purchased from MWG Biotech AG, Ebersberg, Germany. All primers used are listed in Table 10. Specific primers were designed from single nucleotide sequences. Degenerated primers were designed from nucleotide sequence alignments of several organisms.

Table 10: Primer used in this study. * was obtained from Coton and Coton, 2005 (Coton and Coton, 2005). Recognition sites of restriction enzymes are underlined.

Primer	Primer sequence: 5´->3´	Use	Species/plasmid considered
glnA-Flanke-for	GGAAGAAATGCTTTCAGTCGG	Sequencing	*L. sakei* 23K
glnA-Flanke-rev	CAATCACTAATGTTTTCATACAC	Sequencing	
asnB-F	AGTAACAGCCGCTCATGCTT	Screening	*L. sakei* 23K
asnB-R	TGCTGGCATCTTTTTGACTG	Screening	
asnA1-F	TGCGATGCGTTCTTCTAATG	Screening	*L. sakei* 23K
asnA1-R	CACCAAGAGCTTCGATCACA	Screening	
asnA2-F	GCAGGAACTTCGACTTCTGG	Screening	*L. sakei* 23K
asnA2-R	TGCGGTGTCTGTTCATCATT	Screening	
HDC-for	TGGTATTGTTTCGTATGACCG	Screening	*L. sakei* LTH 2076 *L.. buchneri* DSM 5987 *L. hilgardii* IOEB 0006
HDC-rev	GGCTTCATCATTGCATGTGC	Screening	
HDC3*	GATGGTATTGTTTCKTATGA	Screening	*T. muriaticus. LMG 18498* *L. sakei.* *O. oeni.* *L. buchneri L 30a* *C. perfringens*
HDC4*	CAAACACCAGCATCTTC	Screening	
Tdc-for	ATGTTATGGAATGGTAATAACG	Screening	*L. curvatus* HSCC1737
Tdc-rev	TACCATAGCCAGTAACGTTC	Screening	
TDC1	ATGAGTAACACTAGTTTTAGTGC	Screening	*L. curvatus* HSCC1737
TDC2	TTATTTACGATCTTCGTAAATTGC	Screening	
ARA-deg-for3	CCNGAYTTYAAYACNCCN	Screening	*L. delbrueckii ATCC11842* *L. helveticus DPC4571* *L. helveticus CNRZ32* *L. acidophilus NCFM*
ARA-deg-rev3+4	NGCRAADATRTARAANGC	Screening	
ARA-deg-for4	GTHCANGTHGGNGCNACN	Screening	*L. salivarius UCC118_1*
ARA-deg-rev3+4	NGCRAADATRTARAANGC	Screening	*L. salivarius UCC118_2* *L. plantarum WCFS1_1*
Bcat-deg-for A	TRCCAACWGGMRTDGCAAARA	Screening	*L.salivarius UCC118* *L.plantarum WCFS1* *L. delbrueckii subsp. bulgaricus ATCC 11842*
Bcat-deg-revA	CAAGHYTTTGARGGBWTRAARG	Screening	
Bcat-deg-forB	AARGCTTATCGSASAAAAGAYGG	Screening	*L. acidophilus NCFM* *L. helveticusCNRZ32* *Lactococcus lactis subsp. cremoris*
Bcat-deg-revB	CCTTTAAHATAAGMRCCAACWGG	Screening	
oppA-forward	TACCAAGTSGTTAARGATCCTTC	Screening	*L. plantarum* WCFS1 *L. sakei* 23K
oppA-reverse	TTTCTTASCAGYATCCGTATCATC	Screening	
oppB-sakei-F	TTGTCGCTTCTGTGACGTTC	Screening	*L. sakei* 23K
oppB-sakei-R	GACGAAGTTGGGAATCGAAA	Screening	*L. sakei* 23K
oppC-deg-for	ACGATCGCYCGRCTGAYTCGGGC	Screening	*L. plantarum* WCFS1 *L. sakei* 23K
oppC-deg-rev	CAATCCCGATAWAACTCAAGAAYGCTTCG	Screening	

Primer	Primer sequence: 5´->3´	Use	Species/plasmid considered
oppD-forward	GGCAACGGATTGTGATTG	Screening	*L. plantarum* WCFS1 *L. sakei* 23K
oppD-reverse	GGTGGWTCCAACAAATCTGG	Screening	
oppF-forward	AAGGTGAAACATTYGGGTTAGTTGG	Screening	*L. plantarum* WCFS1 *L. sakei* 23K
oppF-reverse	CGGGCAATYCCRATCCGTTG	Screening	
Dtpt-deg-for	TYGGGATYAACTTVGGDTC	Screening	*L. sakei* 23K
Dtpt-deg-rev	TARGAACCACATRCTCATCAT	Screening	*L. plantarum* WCFS1 *L. casei* BL23
OPT-for	GGRATGATGCTTTGTGGWGGT	Screening	*Cl. butyricum E4*
OPT-rev	ATTGTCATMCCTGATACACAGG	Screening	*str. BoNT E BL5262* *Clostridium perfringens B* *str.* ATCC 3626 *L. sakei* 23K
pepN-F	CTCAGCAACATGCCTGAAAA	Screening	*L. sakei* 23K
pepN-R	ACTAAATCGCCGAACCATTG	Screening	
PepS-F	AACGTCCAAGATGGCGATAC	Screening	*L. sakei* 23K
PepS-R	TCAACACGGGTTGTCTTGAA	Screening	
PepC2-F	GCCACAGCTTACAAGCACAA	Screening	*L. sakei* 23K
pepC2-R	TCCGGTAAACTTCGGTCAAC	Screening	
pepO-F	AAAGAAGCGTTGGCTTTTGA	Screening	*L. sakei* 23K
pepO-R	TAACGGCGGCCATAGTAATC	Screening	
pepR-F	GTGACCAAACGTGAATGTGG	Screening	*L. sakei* 23K
pepR-R	ATACGGCCAACACTTGAAGG	Screening	
pepR-sequi-for	GAAACCCGCAACTAATCCTTG	Sequencing	*L. sakei* 23K
pepR-sequi-rev	GGCGTAATAATTTCCGCTTAATC	Sequencing	
PepC1-F	TACTCAAGTCTCTTCAGTATTG	Screening	*L. sakei* 23K
PepC1-R	TTGATGATTGAATATATAACTTGG	Screening	
PepC1-fs-for	TACTCAAGTCTCTTCAGTATTG	Sequencing	*L. sakei* 23K
PepC1-fs-rev	TTGATGATTGAATATATAACTTGG	Sequencing	
pepD1-F	TTCAATCGACGGTTCAACAA	Screening	*L. sakei* 23K
pepD1-R	GTTTCCAGCCACCAAACACT	Screening	
pepD2-F	AAACCAAGTGGCGATTCAAC	Screening	*L. sakei* 23K
pepD2-R	ACATCGTTGCGCAATTGTAA	Screening	
pepD2-F	CAGCCGAAGGTAATGGTGTT	Screening	*L. sakei* 23K
pepD3-R	TCTTCCGTCCCATTACCAAG	Screening	
pepD4-F	ATTCCGCGTTATTCAACCAG	Screening	*L. sakei* 23K
pepD4-R	ATTAGGCGCAACAACAAAGG	Screening	
pepD5-F	AACCGCCTGTACCAGTGTTC	Screening	*L. sakei* 23K

Primer	Primer sequence: 5´->3´	Use	Species/plasmid considered
pepD5-R	CGGTTGTGCCATATTCACTG	Screening	
pepF1-F	ATCAAGCTCAGGTCGATGCT	Screening	*L. sakei* 23K
pepF1-R	ATCTCAGTCGCAAGGGTTGT	Screening	
pepF2-F	AGCAAATGGGCAGTTTTGTC	Screening	*L. sakei* 23K
pepF2-R	AACGATCCCAAGTTTGTTGC	Screening	
pepQ-F	CTAACGCAACATTCCCTGGT	Screening	*L. sakei* 23K
pepQ-R	CCGGGTTTAACAGCATCTTG	Screening	
pepQ-sequi-for2	AGGCAAATGCAAGCGGTTTAC	Sequencing	*L. sakei* 23K
pepQ-sequi-rev2	CTTCAGCAGGCATATTTCTGC	Sequencing	
pepV-1-F	CAAGTCGGTGATTGTGTTGG	Screening	*L. sakei* 23K
pepV-1-R	CCCGTCAATCGCTGTAATTT	Screening	
pepV-F	TCGGGACTGACGAAGAAAGT	Screening	*L. sakei* 23K
pepV-R	CCGATGTAGTTGCCACCTTT	Screening	
pepX-F	CCTAACTTGGCGGATTTGAA	Screening	*L. sakei* 23K
pepX-R	CAAGCCGTTTTCACGGTAGT	Screening	
pepX-sequi-for	GGTTATTGCTTTTAGAATATCAAG	Sequencing	*L. sakei* 23K
pepX-sequi-rev	TCGCAAACACTCGATGATTGC	Sequencing	
pepX-sequi-rev	AGTTGGTATGATTACTACCGTG	Sequencing	*L. sakei* 23K
pepT-F	ATCAAACATGGCGACATCAA	Screening	*L. sakei* 23K
pepT-R	GTTGCTTTAACGCGACCTTC	Screening	
pepM-bF	GGTGTTCATCGTGGTTTGC	Screening	*L. sakei* 23K
pepM-bR	GTTGGTTGGATACCGTGACC	Screening	
brnQ-deg-for1	GCTSYTTAGCYTTTGGBGT	Screening	*L. buchneri* ATCC11577 *L. brevis* ATCC27305 *L. casei* BL23 *L. rhamnosus* Lc705
brnQ-deg-for1	GAAAGGATAKARGAACATCAGC	Sequencing	
brnQ-S5-for	TTAGCGGTCAAGAATGATGC	Scre+Sequ	*L. sakei* TMW 1.22
brnQ-S5-rev	GTTCGCACCTTAGGCAAAAA	Scre+Sequ	
AspAT-Ls-klon-for	TATACCATGGTTATGGATAAAATTGATGTAACAAAATTAAC	Cloning	*L. sakei* 23K
AspAT-Ls-klon-rev	TATACTGCAGTTGTGCTTTAGATTGCAAGTATTC	Cloning	
AT-Ls-klon-for	TATACCATGGTTATGGAAATTGCAAATTTCG	Cloning	*L. sakei* 23K
AT-Ls-klon-rev	TATAAAGCTTATAGGTTCTGAGCGCTTTTG	Cloning	
pBAD-for	CTACTGTTTCTCCATACCCG	Sequencing	pBAD-Myc/HisB
pBAD-rev	CTGATTTAATCTGTATCA	Sequencing	

Primer	Primer sequence: 5´->3´	Use	Species/plasmid considered
ldh1a	TGCGGTACCTACTGAGAAGTTGCTCTC	Cloning	*L. sakei* 23K
ldh1	ATGCGAATTCTACTGAGAAGTTGCTCTC	Cloning	*L. sakei* 23K
ldh4	TATAATGACGTCCTTTCTGTAAAA	Cloning	*L. sakei* 23K
ilvE-X-co			
ilvE-carn-co	AAGGACGTCATTATAATGTCAGAAAAAGT AAAATT	Cloning	*S. carnosus* BioCarna Ferment S1
ilvE-faec-co	AAGGACGTCATTATAATGGAAAAAGCCAA TCTTGA	Cloning	*E. faecalis* V583
ilvE-para-co	AAGGACGTCATTATAATGAGTGTCAATATT GATTG	Cloning	*L. paracasei subsp. paracasei* 8700:2
ilvE-X-re			
ilvE-carnosus-re	TATAGAATTCTTAATATTCTGGTACTACGA	Cloning	*S. carnosus* BioCarna Ferment S1
ilvE-faecalis-re	TATAGAATTCTTAAACTTTTACAATCCAGC	Cloning	*E. faecalis* V583
ilvE-paracasei-re	TATAGAATTCTTAATTAAACGGGACGTCA	Cloning	*L. paracasei subsp. paracasei* 8700:2
ilvE-X-re2			
ilvE-carn-re2	TTAATATTCTGGTACTACGACT	Cloning	*S. carnosus* BioCarna Ferment S1
ilvE-faec-re2	TTAAACTTTTACAATCCAGCCTTC	Cloning	*E. faecalis* V583
ilvE-para-re2	TTAATTAAACGGGACGTCAACG	Cloning	*L. paracasei subsp. paracasei* 8700:2
mcherry-co-X-for:			
mcherry-co-carn-for	GTACCAGAATATTAAgcatAGGAGGGAAcTC ATGGTGTCGAA	Cloning	pSTBlueScript-mcherry
mcherry-co-faec-for	ATTGTAAAAGTTTAAgcatAGGAGGGAAcTC ATGGTGTCGAAG	Cloning	pSTBlueScript-mcherry
mcherry-co-para-for	GTCCCGTTTAATTAAgcatAGGAGGGAAcTC ATGGTGTCGAAG	Cloning	pSTBlueScript-mcherry
mcherry-rev	ATGCGAATTCTTACTTGTACAGTTCGTCC	Cloning	pSTBlueScript-mcherry
616V	AGAGTTTGATYMTGGCTCAG	Sequencing	universal primer
609R	ACTACYYGGGTATCTAAK	Sequencing	universal primer
M13V	GTTTTCCCAGTCACGAC	RAPD	random primer
pG+host5-for	CGTTGTAAAACGACGGCCAG	Sequencing	pG+host5
pG+host5-rev	ATTAACCCTCACTAAAGGGAAC	Sequencing	pG+host5
pMG36e-for	CGGAGGAATTTTGAAATGGC	Sequencing	pMG36e
pMG36e-rev	AACTGTCTTGGCCGCTTCAA	Sequencing	pMG36e

Primer	Primer sequence: 5´->3´	Use	Species/plasmid considered
pRV86-for	CGAATTGGCGGGTTATGAGC	Sequencing	pRV86
pRV86-rev	GCAAACATGAAAAGGCAAATGG	Sequencing	pRV86

2.1.8 Restriction enzymes

All restriction enzymes used in this work were provided by MBI Fermentas GmbH, St. Leon-Rot, Germany and applied as recommended in manufacturer's instructions. To reduce incubation times, FastDigest enzymes were used.

2.1.9 Plasmids

2.1.9.1 pBAD-Myc/His vector

Heterologous expression of proteins in *E. coli* was performed with the pBAD/*Myc*-His expression vector (Figure 7). pBAD/*Myc*-His plasmids are pBR322-derived expression vectors designed for regulated, dose-dependent recombinant protein expression in *E. coli* and a subsequent purification. Optimum levels of soluble, recombinant protein are possible using the *araBAD* promoter (P_{BAD}) from *E. coli*. The regulatory protein, AraC, is provided on the pBAD/His and pBAD/*Myc*-His vectors allowing regulation of P_{BAD}. In the presence of L-arabinose, expression from P_{BAD} is turned on while the absence of L-arabinose produces very low levels of transcription from P_{BAD} (Lee, 1980; Lee *et al.*, 1987). The C-terminal polyhistidine region (331-348) forms a metal-binding site for affinity purification of recombinant fusion protein on metal-chelating resin. In addition, it allows detection of the recombinant protein with an Anti-His (C-term) or by an Anti-*Myc* Antibody (Evan *et al.*, 1985). An ampicillin resistance gene coding for a β-lactamase (989 – 1849) allows the selection of the plasmid in *E. coli*.

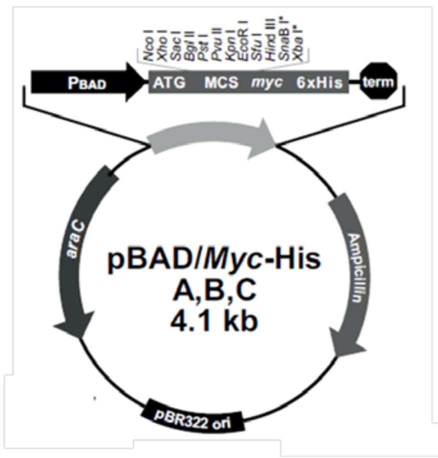


Figure 7: Vectormap of pBAD-Myc/His vectors

Further features of pBAD/*Myc*-His plasmids are the pBR322 origin (1994 – 2667) for low copy replication and growth in *E. coli*, an optimized ribosome binding site that increases efficiency of recombinant fusion protein expression, the initiation ATG (319 – 321 bp) that provides a translational initiation site for the fusion protein, a *rrn*B transcription termination region (553 – 710), and a multiple cloning site (MCS) (430 – 470 bp) that allows insertion of the gene for expression.

2.1.9.2 pSTBlue-1 AccepTor™ Vector Cloning Kit

The AccepTor™ Vector Cloning Kit was used to for rapid cloning of PCR products for sequencing purposes. PCR amplification was performed by Taq polymerase, which leaves single 3´-dA overhangs on the reaction. The linearized AccepTor vector contains single 3´-dU DNA ends that anneal efficiently with the 3´-dA extensions on PCR products. The dU residues are converted to dT residues in vivo following transformation. pSTBlue-1 AccepTor vector provides easy visualization of recombinants by blue/white screening using lacZ α-complementation (Cloning was performed as described by the manufacturer).

2.1.9.3 pRV85 and pRV86

pRV85 and pRV86 were obtained from Monique Zagorec (INRA, France). Figure 8 shows the construction of vectors pRV85 and pRV86 (Gory, 2001). pRV85 is based on the replicative plasmid pG+host5 with a temperature sensitive origin Ts, based on the replicon of pWV01, a cryptic plasmid originally obtained from a *L. lactis* subsp. *cremoris* Wg2 plasmid (Biswas *et al.*, 1993; Maguin *et al.*, 1992; van de Guchte *et al.*, 1989) but carrying four mutations (Maguin *et al.*, 1992). Additionally, pRV85 possess the ori pBR322 for low copy replication and growth in *E. coli* and an erythromycin resistance cassette (Em^r) (Biswas *et al.*, 1993).A *pldhL::*gfp_{uv} fusion was cloned at the *EcoRI* site of pG+host5, leading to pRV85. pRV86 was derived from pRV300 (Leloup *et al.*, 1997) and pRV80 (Stentz *et al.*, 2000), two plasmids designed for integration into the *L. sakei* chromosome. Plasmid pRV80 was used for a stable chromosomal integration of the *pldhL*::*gfpuv* fusion. pRV80 is an integrative plasmid containing the 5´-end of *lacL* and the 3´-end of *lacM*, genes encoding the ß-galactosidase of *L. sakei* (Obst *et al.*, 1995). This plasmid can be integrated by two successive crossovers at the *lacLM* locus allowing gene replacement (Stentz *et al.*, 2000). Therefore, pRV80 (carrying an erythromcycin Em^r and an ampicillin Ap^r resistance cassette) was used to replace the *lacLM* operon by the *pldhL*::*gfpuv* fusion in the chromosome of *L. sakei* 23K.

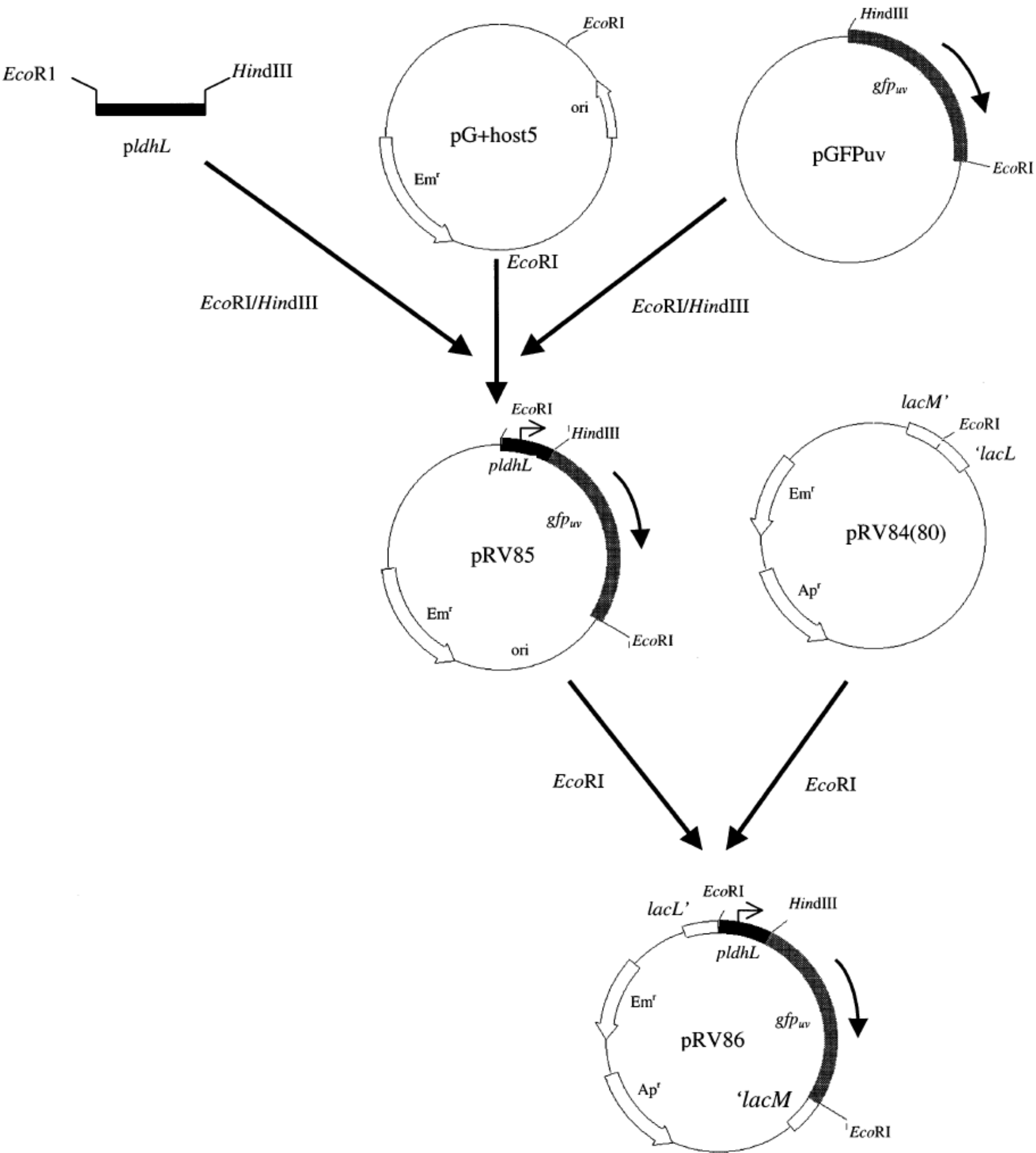


Figure 8: Schematic illustration of pRV85 and pRV86. Em^r, Ap^r: resistance against erythromycin and ampicillin, respectively. This figure was reproduced from Gory (2001).

First, the multiple cloning site upstream of *lacL*, was deleted in pRV80 by digestion with Bsp120-I and *HindII*I and subsequent self ligation. The resulting plasmid pRV84 presented a single *EcoRI* site between the *lacL* and *lacM* parts. The *pldhL*::*gfpuv* fusion of pRV85 was cloned at the *EcoRI* site of pRV84, leading to pRV86 (Gory, 2001).

2.1.9.4 pMG36e

pMG36e vector was originally constructed for expression of heterologous genes in *Lactococcus lactis*. In addition to an origin of replication (pGK11-derived part containing the pWVO1 origin of replication), pMG36e contains a multiple cloning site flanked by gene expression signals originating from *L. lactis subsp. cremoris* Wg2. pMG36e is about 3.7 kilobase pairs in size and carries an erythromycin resistance marker (from

*Staphylococcus aur*eus plasmid pE194) instead of a kanamycin resistance gene as the related plasmid pMG36 shown in Figure 9 (van de Guchte *et al.*, 1989).

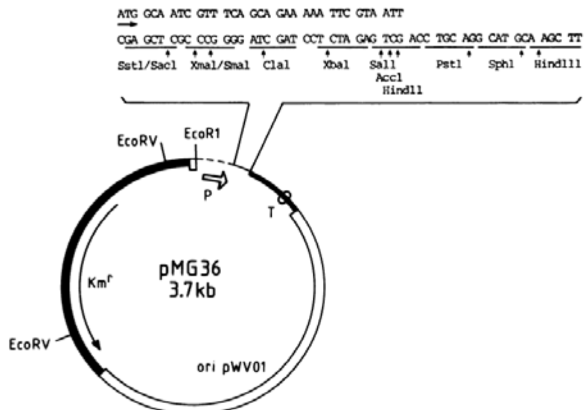


Figure 9: Vector map of pMG36 (van de Guchte *et al.*, 1989), a vector identical to pMG36e except for the antibiotic resistance.

2.1.9.5 pBlueScript-mcherry

pBlueScript-mcherry was obtained from Dipl. Biol. Marina Lamparter (TMW, TU München) and was used as template DNA for amplification of the *mCherry* sequence. *mCherry* sequence of that vector was originally obtained from Shaner *et al.* (Shaner *et al.*, 2004). Subsequently sequence was optimized for Gram+ bacteria and synthesized (Caliper LifeSciences, Alameda, CA, USA).

2.2 Methods

2.2.1 Microbiological Methods

2.2.1.1 Media and growth conditions

All lactobacilli were cultivated in modified MRS (mMRS) medium (Stolz, 1995). Basic medium contained 10 g/l pepton from casein, 5 g/l meat extract, 5 g/l yeast extract, 4.0 g/l KH_2PO4, 2.6 g/l K_2HPO_4 * 3 H_2O, 3.0 g/l NH_4Cl, 0.5 g/l cysteine hydrochlorid, and 1.0 ml/l Tween 80. For agar plates, 1.5 % agar was additionally added. The components were dissolved in 800 ml H_2O_{dest} resulting in a pH of 6.2 (if necessary, pH was adjusted by adding HCl). Sugars were dissolved separately in 200 ml H_2O_{dest}. For routinely growth, 15 g/l glucose was used (mMRS G15). For experiments imitating sausage fermentations mMRS with 3 g/l glucose was utilized (mMRS G3). To avoid the formation of Maillard products, basic medium and sugar solutions were autoclaved (121 °C, 20 min) separately and mixed after cooling. 1 ml sterile filtrated (pore size Ø 0.2 µm) magnesium/manganese 1000-fold stock solution ($MgSO_4$ * 7 H_2O 100 g/l, $MnSO_4$ * 4 H_2O 50 g/l) and 1 ml of a vitamin mix solution (0.2 g/l of cobalamine, 0.2 g/l folic acid, 0.2 g/l nicotinic acid, 0.2 g/l

panthotenic acid, 0.2 g/l pyridoxal-HCl, and 0.2 g/l thiamine (stored at –20 °C)) was added. Recombinant *Lactobacillus* strains were cultivated in mMRS medium containing 5 µg/ml erythromycin (erm). X-Gal (5-bromo-4-chloro-3-indolyl-L-D-galactopyranoside) was added to MRS G15 agar at 32 mg/l for selection of *L. sakei* double crossover mutants transformed with pRV84-pil-X-constructs (see below). *Lactobacillus sakei* and *Lactobacillus curvatus* strains were cultivated at 30 °C, *Lactobacillus paracasei* strains at 37 °C. Liquid cultures were incubated in tight closed bottles or plastic tubes without shaking. Agar plates were incubated anaerobically using Anaerocult systems in heat-sealed plastic bags or airtight incubation containers.

Enterococcus faecalis strains were cultivated anaerobically in Brain Heart Infusion (BHI) broth or on BHI agar plates at 37 °C.

Staphylococcus carnosus strains were cultivated like lactobacilli.

Escherichia coli strains were grown in LB medium (Luria *et al.*, 1960) containing 10 g/l pepton, 5 g/l yeast extract and 5 g/l NaCl. Components were dissolved in H_2O_{dest} and pH was adjusted to 7.2 with NaOH. Medium was sterilized by autoclaving (121 °C, 20 min). For agar plates, 1.5 % agar was added. When appropriate, ampicillin (amp) or erythromycin (erm) was added to an end concentration of 100 µg/ml (amp) or 150 µg/ml (erm) respectively.

E. coli strains were usually cultivated at 37 °C. Liquid cultures were incubated in Erlenmeyer flasks at 180 rpm and plates aerobically in an incubator.

2.2.1.2 Screening for amino acid requirements

Determination of amino acid requirements of several *L. sakei* and *L. curvatus* was performed in a chemically defined medium (CDM) originally developed by Morishita *et al.* (Morishita *et al.*, 1981) and subsequently adapted by Hebert et al. (Hebert *et al.*, 2004). CDM consisted of a basal medium containing 10 g/l glucose, 5 g/l sodium acetate ($NaC_2H_3O_2$ * 3 H_2O), 3 g/l KH_2PO_4, 3 g/l K_2HPO_4 and 1 g/l Tween 80, a metal mix containing 0.2 g/l MgSO4 * 7 H_2O, 0.05 g/l $MnSO_4$ * 4 H_2O, and 0.02 g/l $FeSO_4$ * 7 H_2O, 0.1 g/l of L-alanine, 0.1 g/l L-arginine, 0.1 g/l L-glycine, 0.1 g/l L-hidstidine HCl, 0.1 g/l L-isoleucine, 0.1 g/l L-leucine, 0.1 g/l L-ysine, 0.1 g/l L-methionine, 0.1 g/l L-phenylalanine, 0.1 g/l L-proline, 0.1 g/l L-serine, 0.1 g/l L-threonine, 0.1 g/l L-tryptophan, 0.1 g/l L-tyrosine and 0.1 g/l L-valine, 0.2 g/l of L-aspartic acid, 0.2 g/l L-asparagine, 0.2 g/l L-cysteine-HCl, 0.2 g/l L-glutamic acid and 0.2 g/l L-glutamine, a vitamine mix containing 0.001 g/l nicotinic acid, 0.001 g/l pantothenic acid, 0.001 g/l riboflavin, 0.001 g/l cyanocobalamin, and 0.001 g/l thiamine, 0.002 g/l pyridoxal-HCl, and 0.01 g/l p-aminobenzoic acid, inosine and orotic acid, a bases-mix consisting of 0.01 g of each of

the bases adenine, guanine, xanthine, uracil and thymine. Finally, the CDM contained 0.01 g/l biotin. CDM was prepared from concentrated individual stock solutions (basal medium 2 x, metal mix 500 x, each amino acid 200 x, vitamine mix 100 x, biotin 100 x, 100 x bases) which were stored at 4 °C after sterile filtration through a sterile filter (pore size Ø 2 µm). L-cysteine-HCl solution was freshly prepared for each experiment. pH 6.5 was adjusted by adding NaOH and before use medium was again sterile filtrated. A full CDM (containing all amino acids) was used for positive controls ensuring that the respective strain is in principle able to grow in CDM. Subsequently, a series of deletion experiments were performed to determine the requirement of amino acids using different variations of CDM each lacking a single amino acid. A preparatory culture was obtained by inoculating 1 ml mMRS G15 medium with a single colony of the respective strain and incubated over night at 30 °C. To eliminate carryover of any essential nutrients, cells were washed twice with 500 µl 1 x basal medium and were subsequently resuspended in 1 ml 1x basal medium. This suspension was incubated for 2 h at 30 °C to deplete amino acids potentially available at internal pools. Then, a 2.5 %-dilution of the cells was prepared in 200 µl of each CDM variation. Growth in each medium was determined by observing the absorbance at 590 nm (OD_{590}) for 20 h at 30 °C spectrophotometrically by a microtiter plate reader. Auxotrophy experiments were performed at least in duplicate.

2.2.1.3 Decarboxylase assay

Amino acid decarboxylase activity was tested using a slightly modified procedure, originally described by Bover-Cid *et al.* (Bover-Cid and Holzapfel, 1999): Liquid medium was used instead of agar plates. 1 ml of the respective decarboxylase medium was inoculated with a single colony of the strain to be checked and incubated at 30 °C for 48 – 36 h. A positive result was indicated by a colour change of the medium from yellow to purple. This change was due to the response of pH-indicator bromcresol purple to a pH shift caused by formation of the more alkaline biogenic amines (BA) from the precursor amino acids initially added to the medium by decarboxylase activity.

2.2.1.4 Preparation of chemical competent *E. coli* cells and transformation conditions

100 ml LB medium were inoculated with 1 % of an overnight culture of *E. coli* DH5α or *E. coli* TOP10 and incubated at 37 °C/180 rpm till an OD_{590} = 0.5 - 0.6 is reached. Cells were harvested by centrifugation (5000 rpm/5 min/ 4 °C) and washed with 30 ml ice-cold 0.1 M $CaCl_2$. After centrifugation (5000 rpm/4 °C/5 min), pellet was resuspended in 2 ml ice-cold 0.1 M $CaCl_2$ containing 15 % glycerol. Aliquots of 200 µl were directly used for transformation or stored at –80 °C.

200 µl competent *E. coli* cells were thawed on ice, mixed with respective amounts of DNA (plasmid/ligation mix), and incubated for 30 min on ice. After heat shock at 42 °C for 90 s, cells were incubated for another 2 min on ice. 1 ml LB medium was added and after incubation for 30 min at 37 °C (180 rpm), cells were plated on LB agar plates supplemented with the respective antibiotic.

2.2.1.5 Transformation efficiency of *L. sakei* 23K

All washing solutions used were ice-cooled and electroporation was carried out in prechilled cuvettes with a Bio-Rad gene pulser apparatus.

Two methods for preparing electrocompetent *L. sakei* 23K cells were applied and the resulting transformation efficiencies were subsequently compared. The first method was obtained from Zagorec (Zagorec, 2009): A 1 % inoculated culture was grown in mMRS G15 medium. Cells were harvested by centrifugation (5000 rpm/4 °C/5 min) at an OD_{590nm} of 0.4 - 0.5, washed twice with 10 mM $MgCl_2$, once with a glycerol-sucrose solution (10 % v/v, 0.5 M) and were subsequently resuspended in 250 µl of the latter solution. 50 µl aliquots were stored at –80 °C. Cells were transformed with vector pRV85 carrying a *pldhl*::gfp_{uv} fusion. Electroporation was carried out at 1.8 kV, 25 µF and 600 Ω (11.8 ms). Immediately after electroporation, 450 µl fresh mMRS G15 medium containing 80 mM $MgCl_2$ and 1 % glucose were added.

The second method was obtained from a transformation protocol used by default in our lab for transformation of LAB, but slightly modified. A 1 % inoculated culture was grown in mMRS G15 medium supplemented with 1.5 % glycine. Cells were harvested by centrifugation (5000 rpm/4 °C/5 min) at an OD_{590nm} of 0.5, washed twice with 10 mM $MgCl_2$, once with glycerol (10 % v/v), once with a glycerol-sucrose solution (10 % v/v, 0.5 M) and were subsequently resuspended in 1 ml of the latter solution. 100 µl aliquots were stored at – 80 °C.

Cells were transformed with vector pRV85 carrying a *pldhl*::gfp_{uv} fusion. Electroporation was carried out at 1.8 kV, 25 µF and 1000 Ω (>18 ms). After electric shock, 500 µl mMRS G15 medium were added.

In both cases cells were incubated for 2 h at 30 °C and dilutions were plated on mMRS agar medium containing 5 µg/ml erythromycin. After 24 - 48 h of anaerobic incubation, transformants were counted. Same counts of transformants were picked randomly from plates of each approach and analyzed for fluorescence (GFP) and plasmid DNA content. The transformation efficiencies were expressed as the number of transformants (CFU) per microgram of plasmid DNA. The method with higher transformation efficiencies was used as standard method for preparing and transforming electrocompetent *L. sakei* cells.

2.2.2 Molecular Biological Methods

2.2.2.1 Sequence analysis and bioinformatics

Nucleotide and amino acid sequence alignments were performed with an online version of ClustalW software, available on e.g. http://www.ebi.ac.uk/Tools/clustalw2/index.html. Phylogenetic trees could be calculated with ClustalW software directly.

NCBI portal, available on http://www.ncbi.nlm.nih.gov, was used for access to BLAST algorithms for search of homologous nucleotide and amino acid sequences, and searches for genes and proteins. Analysis of endonuclease restriction sites within DNA sequences, DNA conversions into its complementary sequence, and translation of DNA sequences to amino acid sequences was performed by a free online tool, namely “DNA tools”, accessible via http://biology.semo.edu/cgi-bin/dnatools.pl. Cloning experiments were planned using Clone Manager 5.0 software. Grouping of strains was performed by Pearson correlation coefficient and cluster analysis by UPGMA of Bionumerics software (Applied Maths, Belgium).

2.2.2.2 Agarose gel electrophoresis

Visualization of DNA was performed by agarose gel electrophoresis. 1 - 1.3 % agarose gels were prepared with 0.5 x TBE buffer (stored as 10 x TBE buffer: 150 g/l Tris, 26.2 g/l boric acid, 9.0 g/l EDTA; pH 8.9) for analytical gels and with 1 x TAE buffer (stored as 50 x TAE buffer: 0.1 M EDTA, 1 M acetic acid (100 %), 2 M Tris, pH 8.2) for preparative gels. DNA samples were mixed with loading dye in ratio 6:1 (sample : loading dye). Separation was routinely performed in electrophoresis chambers (12 x 13.8 cm) at 90 – 120 V for 1 - 1.5 h. Electrophoresis of RAPD patterns was carried out in a electrophoresis chamber (20 x 25 cm) with a 1.3 % 0.5 x TBE gel (220 ml) at 170 V for 3 h. Several ready-to-use DNA size standards were applied (10 µl in small cavities, 15 µl in large cavities) to determine DNA fragment sizes by comparison (Table 8). Gels were stained with ethidium bromide/dimidium bromide and the banding profiles were visualized under UV light (wavelength 320 nm) and digitalized by a gel documentation system from INTAS-science imaging instruments GmbH.

2.2.2.3 Nanodrop analysis

Besides the estimation of DNA amounts by agarose gel electrophoresis, the quantity of DNA was also determined by analysis with a Nanodrop 1000 device according to manufacturer´s instructions.

2.2.2.4 Isolation of chromosomal DNA

Chromosomal DNA was isolated, using the E.Z.N.A. Bacterial DNA kit (Omega bio-tek) according to the manufacturer´s instructions. The result of DNA isolation was monitored by analytical agarose gel electrophoresis and the amount of DNA was determined by nanodrop analysis.

2.2.2.5 Plasmid isolation

E. coli plasmid DNA was isolated with the peqGOLD plasmid miniprep kit or the Pure Yield plasmid midiprep system according to the manufacturer´s instructions. Plasmid DNA from lactobacilli was obtained by the same procedure but prior to the first step described in the kit, cells were incubated for 30 min at 37 °C in TE buffer containing 10 mg/ml lysozyme.

2.2.2.6 General DNA amplifications

DNA was amplified with a Thermo Cycler (PRIMUS 96 plus, MWG-Biotech AG, Ebersberg, Germany) or an Eppendorf Gradient Cycler (Eppendorf, Hamburg, Germany). For routine amplifications the Taq Core Kit was used. In general, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 µM primer and 1.5 U Taq were used for each reaction unless stated otherwise. Standard cycling conditions for amplification with Taq polymerase were as follows: In a first step, genomic DNA was denatured (94 °C/2 min). Then an amplification cycle consisting of a denaturation step (94 °C/40 s), primer annealing step (melting temperature of primer –2 to 3 °C/40 s) and an elongation step (72 °C, depending on fragment length – extension rate of Taq: 1 kb/min) was repeated for 32 times. Eventually a final elongation step (72 °C/5 min) finished PCR reactions.

DNA amplifications for cloning or sequencing purpose were performed using the KOD Hot Start DNA Polymerase with 3′→5′ exonuclease activity. According to manufacturer´s instructions, a reaction setup was prepared containing a final concentration of 1 x buffer for KOD Hot Start DNA Polymerase, 1.5 mM $MgSO_4$, 0.2 mM (each) dNTPs, 0.3 µM of sense (5´) primer and 0.3 µM of anti-sense (3´) primer, and 0.02 U/ml KOD Hot Start DNA Polymerase. Standard cycling conditions for amplification with KOD Hot Start DNA polymerase were as follows: In a first step, genomic DNA was denatured (95 °C/2 min). Then an amplification cycle consisting of a denaturation step (95 °C/20 s), primer annealing step (lowest primer Tm °C for 10 s) and an elongation step (70 °C, depending on fragment length – extension rate of KOD: 10 s/kb for targets < 500 bp, 15 s/kb for targets of 500 – 1000 bp, 20 s/kb for targets of 1000 – 3000 bp and 25 s/kb for targets > 3000 bp) was repeated 32 times.

2.2.2.7 Screening PCR

Screening PCR experiments with negative or indistinct results were conducted at least twice to get clear results. In several cases, additional primer sets were designed to verify the absence/presence of genes. Very weak bands were assessed as negative. Paq5000™ DNA Polymerase was used according to manufacturer´s recommendations for screening PCR reactions. The general amplification program used was: 95 °C for 2 min, 32 cycles of 95 C for 20 s, (primer melting temperature –5 °C) °C for 20 s, 72 °C (extension rate of Paq5000™: 2 kb/min) with a final extension at 72 °C for 5 min.

All screening PCRs were performed either in a Primus 96 cycler (MWG-Biotech, Ebersberg, Germany) or in an Eppendorf Gradient Cycler (Eppendorf, Hamburg, Germany).

2.2.2.8 PCR purifications

PCR products amplified for sequencing or cloning purposes were purified using the QIAquick PCR purification kit or were isolated from preparative agarose gels using the peqGOLD Gelextraction Kit following the instructions of the suppliers.

2.2.2.9 Strain typing by RAPD analysis

Strain typing was assessed by RAPD-PCR analysis that was carried out with the Taq Core Kit using the random primer M13V primer. Each 50 µl PCR mix contained 5 µl 10 x PCR buffer without $MgCl_2$, 7 µl $MgCl_2$ (25 mM), 2 µl dNTPs (10 mM each), 0.5 µl Primer M13V (100 pmol/µl), 0.3 µl Taq polymerase (5 U/µl) and 0,5 µl (~50 – 100 ng) extracted DNA. To increase reproducibility, amplification was always performed in the same Primus 96 cycler (MWG-Biotech, Ebersberg). The amplification program started with 3 cycles consisting of 3 min at 94 °C, 5 min at 40 °C and 5 min at 72 °C followed by 32 cycles consisting of 1 min denaturation at 94 °C, 2 min annealing at 60 °C and 3 min elongation at 72 °C. 10 µl of the amplification products were electrophoresed and geles were stained and documented as described above. Electrophoretic profiles were analyzed with the software Bionumerics (Applied Maths, Belgium). The analysis included the registration of the electrophretic patterns normalization of the densitometric traces and substraction of background noise, grouping of strains by Pearson correlation coefficient and cluster analysis by UPGMA. The identities of strains with untypical fragment profiles and representatives of each group were checked by partial sequencing of the 16S rRNA gene.

2.2.2.10 Strain identification

Strain identification was performed by partial sequencing of the 16S rRNA gene, amplified with primers 616V and 609R. Sequencing was performed by GATC Biotech GmbH (Konstanz, Germany).

2.2.2.11 Sequencing

Sequencing of PCR products or plasmids was performed by GATC Biotech GmbH (Konstanz, Germany).

2.2.2.12 Restrictions and ligations

Restriction enzyme digestions and ligations with T4-DNA ligase were performed following the recommendations of the supplier (MBI Fermentas GmbH, St. Leon-Rot, Germany).

2.2.2.13 Cloning of *aspD* and *arcT* in pBAD-*Myc*/HisA

The gene coding for the aspartate-4-decarboxylase (*aspD*) was amplified by PCR using the primer pair AspAT-Ls-klon-for/rev containing *NcoI* or *PstI* recognition sites respectively and genomic DNA of *L. sakei* 23K as template. The amplified PCR product was digested by *NcoI* and *PstI*, and subsequently ligated in similarly digested pBAD/*Myc*-HisA vector. The *arcT* gene was amplified with primers AT-Ls-klon-for/rev containing *NcoI* or *HindIII* recognition sites respectively. The amplified PCR fragment was digested by *NcoI* and *HindIII* and ligated into a similar digested pBAD/*Myc*-HisA vector. The resulting constructs were introduced into chemical competent *E. coli* TOP10 cells. Pure plasmid DNA was isolated from positive clones and the correctness of constructs was verified by sequencing (GATC Biotech GmbH, Konstanz).

2.2.2.14 Reconstruction of pG+host5 and pRV84

Vectors pRV85 and pRV86 are derivates of pG+host5 and pRV84 respectively. They were constructed by introducing a *pldhL::gfp*$_{uv}$ fusion via *EcoRI* restriction sites. For reconstruction of pG+host5 and pRV84, pRV85 and pRV86 were restricted with *EcoRI* and subsequently religated.

2.2.2.15 Construction of integration vectors pRV84-pil-X

The inserts for cloning into pRV84 vector comprised the constitutive *L. sakei* promoter for lactate dehydrogenase (*pldhL*), and an *ilvE* gene coding for a branched-chain amino acid aminotransferase. The complete insert fragment was obtained by crossover PCR. The crossover PCR strategy consisted of two rounds of PCR amplifications shown in Figure 10. First, fragments *pldhL* and *ilvE* were amplified separately and subsequently linked to

each other by a crossover PCR reaction. The *ldhL*-promotor fragment was obtained by amplifications with primers ldh1 and ldh4 (PCR1) using DNA of *L. sakei* 23K as template. Genomic DNA of *Lactobacillus paracasei* TMW 1.1434, *Enterococcus faecalis* TMW 2.852 (V583) and *Staphylococcus carnosus* TMW 2.801 was used for the amplification of the *ilvE* genes by ilvE-X-co and ilvE-X-re (PCR2). Primers ilvE-X-co contained, in 5´ to 3´direction a 15-base sequence complementary to the 3´-end of the *pldhL* sequence. Crossover-PCR with ldh1 and ilvE-X-re linked products of PCR1 and PCR2. Primers ldh1 and ilvE-X-re were designed with a restriction site for *EcoRI* (underlined) at the 5´-end.

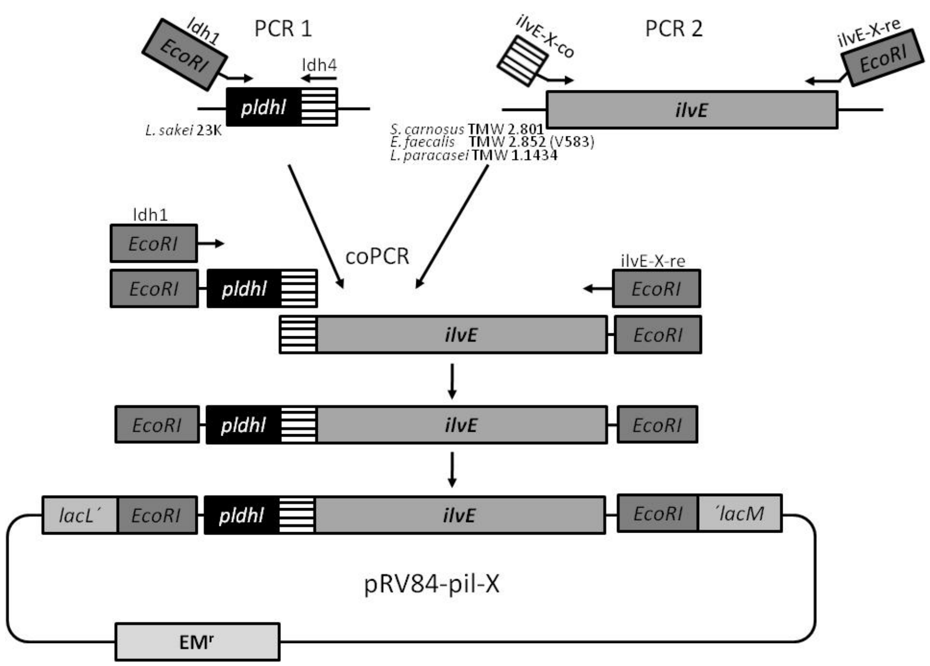


Figure 10: Construction of pRV84-pil-X integration vector.

The generated fusions *plhdL*::*ilvE-X* (*pldhL*::*ilvE-Lp, pldhL*::*ilvE-Ef1, pldhL*::*ilvE-Sc*) were digested with *EcoRI* and ligated into similarly digested pRV84 to obtain constructs pRV84-pil-X (pRV84-pil-Lp, pRV84-pil-Ef1, pRV84-pil-Sc). pRV84 contains 513 bp of the 5´-end of *lacL* and 522 bp of the 3´-end of *lacM*. pRV84 derivates pRV84-pil-Lp, pRV84-pil-Ef1, pRV84-pil-Sc can be integrated into the chromosome of *L. sakei* by homologous recombination either in *lacL* or in *lacM*. Recombination at the *ldhL* locus with the 234-bp *pldhL* fragment was not expected as the minimum size of homology required for recombination was estimated to be 300 bp (Leloup *et al.*, 1997).

2.2.2.16 Genome integration of pRV84-pil-X

L. sakei 23K was transformed with pRV84-pil-Lp, pRV84-pil-Ef1, pRV84-pil-Sc for erythromycin resistance. Single crossover integration of pRV84-pil-Lp, pRV84-pil-Ef1, pRV84-pil-Sc, at the *lacLM* locus, was checked by PCR on chromosomal DNA extracted from transformants. Single crossover transformants were grown without erythromycin in

order to allow excision of the plasmid by a second crossover, leading either to gene replacement or to the wild-type genotype. Possible crossing over events are shown in Figure 11. Every 24 h single crossover transformants were transferred into fresh mMRS 15G medium and diluted culture aliquots were plated on mMRS 15G medium containing X-Gal. White clones suggested that the *lacLM* operon of *L. sakei* was replaced by the *pldhL*::*ilvE-X* fusion.

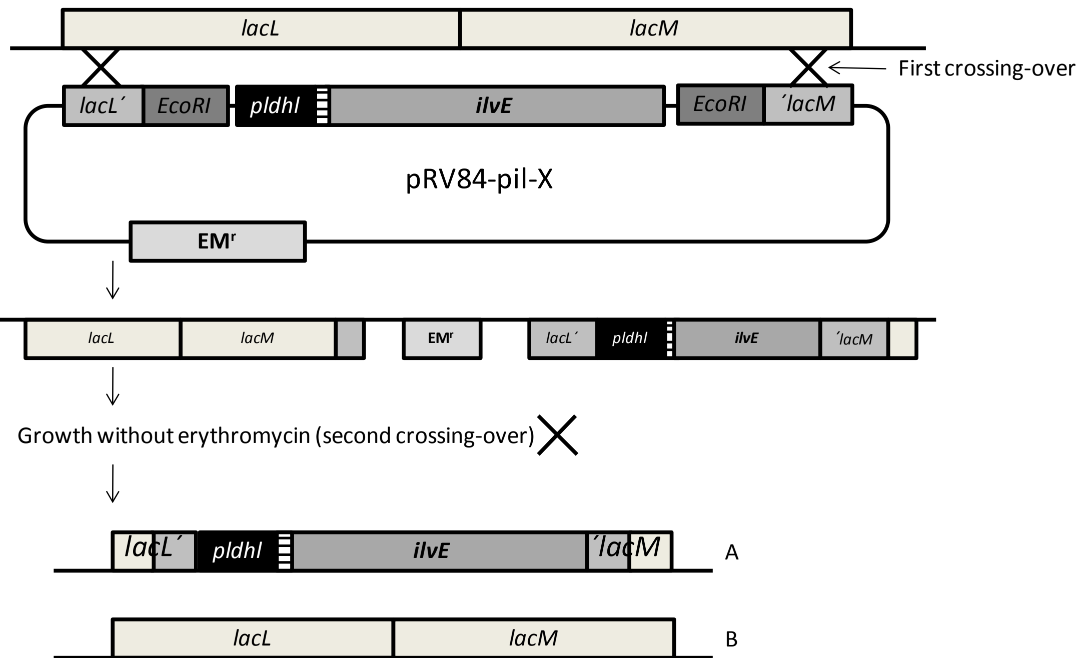


Figure 11: Schematic overview of integration events by homologous recombinations. Second crossover can (A) lead to gene replacement (*L. sakei* 23K-pil-Sc) or (B) to the wildtype genotype (*L. sakei* 23K). Figure was adapted from Gory (2001).

2.2.2.17 Construction of pMG36e-pldhl-ilvE-X-mcherry

The inserts for cloning into pMG36e vector consisted of the *L. sakei* promoter for lactate dehydrogenase (*pldhL*), an *ilvE* gene coding for a branched-chain aminotransferase and *mCherry* coding for a red fluorescent protein. The complete insert fragment was obtained by crossover PCR. The crossover PCR strategy (Figure 12) comprised three rounds of PCR amplification. First, fragments *pldhL, ilvE* and *mCherry* were amplified separately and were subsequently linked to each other by two consecutive crossover PCR reactions. The *pldhL*-promotor fragment was obtained by amplification with primers ldh1a and ldh4 (PCR1) on genomic DNA of *L. sakei* 23K. Genomic DNA of *Lactobacillus paracasei* TMW 1.1434, *Enterococcus faecalis* V583 and *Staphylococcus carnosus* TMW 2.801 was used for the amplification of the *ilvE* genes by ilvE-X-co and ilvE-X-re2 (PCR2). The *mCherry* fragment was obtained with primers mcherry-co-X-for and mcherry-rev with pSTBluescript-mcherry as template DNA (PCR3). Primers ilvE-X-co and mcherry-co-X contained, in 5´ to 3´ direction, a 15-base sequence complementary to the 3´-end of the

pldhl-fragment or the *ilvE-X*-fragments respectively. Moreover mcherry-co-X contained downstream of the complementary sequence a 4-base spacer sequence before the Shine-Dalgarno sequence of *mCherry* started. Crossover-PCR1 (coPCR1) with ldh1a + ilvE-Xre2 linked products of PCR1+PCR2, whereas the following second crossover PCR (coPCR2), performed with ldh1a+mcherry-rev linked products of coPCR1 with the *mCherry*-fragment from PCR3. Primers ldh1a and mcherry-rev were designed with a restriction site for *KpnI* or *EcoRI* respectively at the 5´-end.

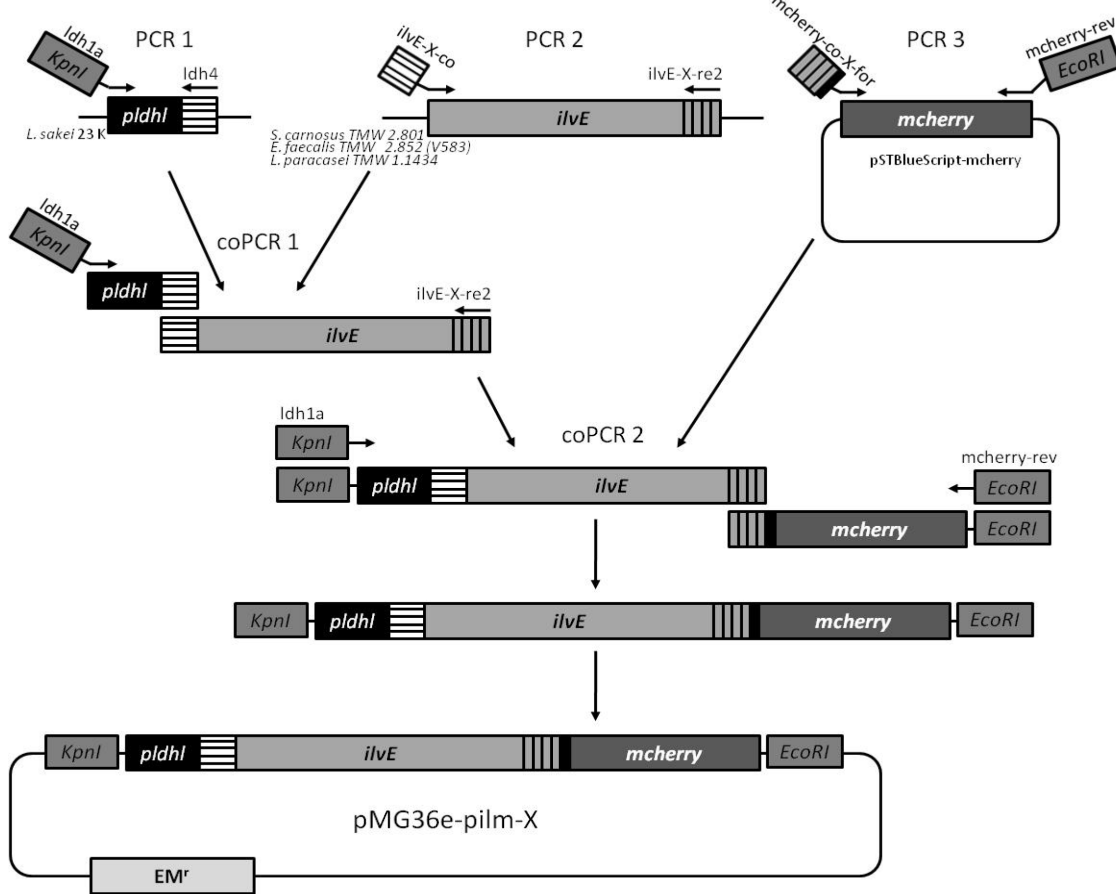


Figure 12: Construction of pMG36e-pilm-X

The generated fusions *plhdL*::*ilvE-X::mcherry* (*pldhL*::*ilvE-Lp::mcherry, pldhL*::*ilvE-Ef::mcherry, pldhL*::*ilvE-Sc::,mcherry*) were digested with *KpnI* and *EcoRI* and ligated into similarly digested pMG36e to obtain pMG36e-pilm-X (pMG36e-pilm-Lp, pMG36e-pilm-Ef, pMG36e-pilm-Sc).

2.2.2.18 Construction of pG+host5-pldhl-ilvE-X

The inserts for cloning into pG+host5 were obtained as described for pMG36e. However, instead of primer ldh1a containing a *KpnI* restriction site, ldh1 with an *EcoRI* restriction site, was used. Digestion of fusions *plhdL*::*ilvE-X::mcherry* and pG+host5 was carried out with *EcoRI*. Fusions and pG+host5 were ligated to obtain pG+host5-pilm-X.

2.2.3 Fluorescence Microscopy

Fluorescence of cells was examined by a SteREO Discovery Stereomikroskop (Zeiss) equipped with respective filter sets. To visualize mCherry positive cells, either cell material from picked colonies grown on selective agar plates directly or liquid cultures inoculated with these colonies, were used for fluorescence microscopy with filter set 31 (mCherry: 587 nm excitation, 610 nm emission). Cells expressing GFP (489 nm excitation, 509 nm emission) were examined with filter set 44.

2.2.4 Protein chemical methods

2.2.4.1 Expression of recombinant proteins by *E. coli* TOP10 and generation of cell free extracts (CFE)

1 l LB medium, containing 0.3 M sorbitol and 100 µg/ml ampicillin was inoculated with 10 ml of an overnight culture of *E. coli* TOP10 transformed with pBAD/*Myc*-HisA-aspD or pBAD/*Myc*-HisA-arcT respectively. Incubation at 37 °C/220 rpm lasted till OD_{590} reached 0.6 - 0.8. Then, 0.05 mM of cofactor PLP was added. After cooling down to 30 °C, culture was further incubated till OD_{590} reached ~1 (adaption phase). Expression was subsequently induced by adding 100 µM arabinose and was performed over night (16 – 18 h) at 30 °C/120 rpm. Cells were harvested by centrifugation (5000 rpm/10 min/4 °C). Supernatant was discarded. Cells were washed once with precooled buffer A (application buffer) (20 mM NaH_2PO_4, 500 mM NaCl, 50 mM imidazole, pH 7.4) and resuspended in 10 ml of buffer A. Cell extract was obtained by sonification (SONOPLUS/SH70G Bandelin electronic, Berlin) of cell suspensions on ice (cycle 0.5/90 %/20 s) in three repeats with breaks lasting at least one minute. Cell debris was separated from crude cell extract by centrifugation (45 min/14000 rpm/4 °C). The clear supernatant was pooled and used as CFE. Overview of expression and generation of CFE is depicted in Figure 13.

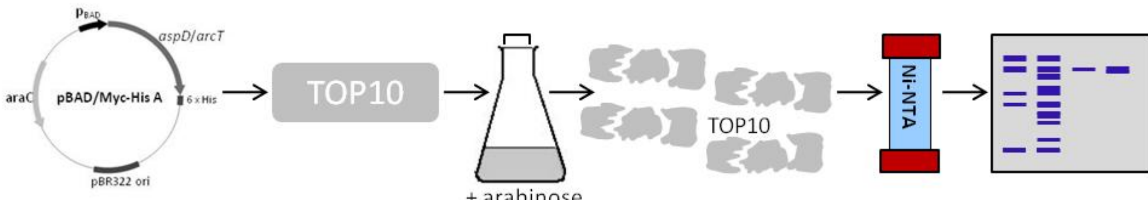


Figure 13: ArcT/AspD expression strategy (from left to right): Plasmids pBAD/*Myc*-HisA-aspD/arcT were introduced into chemical competent *E.coli* TOP10 cells. These transformants were cultivated in 1 l liquid medium; expression was induced by addition of arabinose and cells were harvested by centrifugation. Cell extract was obtained by breaking washed cells using ultrasonification and His-tagged target protein was separated by Ni-NTA affinity FPLC. Success of purification was monitored by SDS-PAGE and coomassie staining.

2.2.4.2 Generation of cell free extracts of Gram+ organisms

350 ml mMRS G15 medium was inoculated with 10 ml of an overnight culture of the respective organism and incubated for 16 - 18 h. Cells were harvested by centrifugation (5000 rpm/30 min/4 °C) and supernatant was discarded. Cells were washed with precooled 50 mM sodium phosphate buffer, pH 6.5. Cell extract was obtained by ultrasonification of cell suspensions on ice (cycle 0.5/90 %/20 s) (UP 200S Dr. Hielscher GmbH, Teltow, SONOPLUS/SH70G Bandelin electronic, Berlin). Disruption process was checked by mixing one drop of cell suspension with one drop SDS 25 %. Sonification was repeated till SDS test mixture become slimy. Cell debris was removed by centrifugation (45 min/14000 rpm/4 °C) and supernatant was used as cell free extract (CFE).

2.2.4.3 FPLC

For FPLC (free pressure liquid chromatography) all buffers and solutions were degassed and sterilized by filtration (pore Ø 0.2 µm) using a vacuum pump. A 1 ml HisTrap HP affinity column (Amersham Biosciences) was equilibrated with at least 10 column volumes of buffer A (application buffer) (20 mM NaH_2PO_4, 500 mM NaCl, 50 mM imidazole, pH 7.4). Cell extract was manually applied. While target protein was bound to column due to the His-tag, residual proteins were flushed out in a wash step of ten column volumes of buffer A (2 ml/min). A gradient from 100% buffer A and 0% buffer B to 0% buffer A to 100% buffer B (elution buffer) (20 mM NaH_2PO_4, 500 mM NaCl, 500 mM imidazole, pH 7.4) (four column volumes, 2 ml/min) released the target protein of the His-Trap column. A washing step of ten column volumes buffer B (2 ml/min) regenerated the column by removing any residual protein. A steep gradient from 0 % buffer A/100 % buffer B to 100 % buffer A/0 % buffer B with 2 ml at 2 ml/min finished the elution. In the end, the column was equilibrated again by washing with six column volumes of buffer A. To store the HisTrap columns buffer was replaced by 20 % ethanol. During the FPLC process, eluate was collected in 1 ml fractions and process was monitored by UV detection.

2.2.4.4 Dialysis

To increase temperature stability of the purified His-tag-enzymes, fractions containing protein were dialyzed against 75 mM acetate buffer, pH 5.0 containing 0.5 mM PLP and 1 mM α-ketoglutaric acid. Dialysis was performed over night at 4 °C under stirring.

2.2.4.5 Determination of Protein concentration

The Bio-Rad Protein Assay, based on the method of Bradford (Bradford, 1976) was used for determining protein concentrations. The “Standard Procedure for Microtiter Plates” was used following manufacturer´s instructions:

Dye reagent was prepared by diluting 1 part Dye Reagent Concentrate with 4 parts H_2O_{dest} and filtrating through a filter to remove particles. A dilution series of a BSA standard was prepared. The linear range of this microtiter plate assay is 0.05 mg/ml to approximately 0.5 mg/ml. 10 µl of each standard and sample solution were transferred into separate microtiter plate wells and 200 µl of diluted dye reagent were added. After incubation at room temperature for at least 5 minutes, absorbance was measured at 595 nm in a microtiter plate reader.

2.2.4.6 SDS-PAGE

Protein size and pureness were checked by one dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). 12 % polyacrylamide gels were prepared as follows. For the separating gel, 3.35 ml H_2O_{dest}, 2.5 ml Tris-HCl (1.5 M, pH 8.8), 40 µl of 25 % SDS solution and 4 ml of acrylamide/bis 30 % were mixed. For stacking gel 3.05 ml H_2O_{dest}, 1.25 ml Tris-HCl (0.5 M, pH 6.8), 20 µl of 25 % SDS solution and 665 µl of acrylamide/bis 30 % were mixed separately. To start cross-linking of the gels, 50 µl ammonium persulfate (APS) 10 % and 12 µl tetramethylethylendiamine (TEMED) were added to the gel mixtures. After gelling, 10 µl of protein samples were mixed with 10 µl of Laemmli buffer (4.58 ml H_2O_{dest}, 1 ml Tris (0.5 M, pH 6.8), 920 µl glycerol 87 %, 700 µl SDS 25 %, 400 µl bromphenol blue 1 % and 1.5 g dithiothreitol (DTT)) and boiled for 20 min. Separation was performed in a 1 x electrophoresis buffer (5 x concentrated stock solution: 9 g Tris base, 43.2 g glycine, 3 g SDS disodium salt in 600 ml H_2O_{dest}, pH adjusted to 8.3). 10 to 15 µl of samples were applied in the gel cavities. For protein size determination 10 µl PAGERuler™ Protein Ladder was used. Separation was started at 60 V for 15 min, then voltage was increased to 120 V and gel was run for 90 to 120 min. Proteins were visualized by a rapid Coomassie staining method. Gel was covered with staining solution (filtrated mixture of 80 ml H_2O_{dest}, 120 ml ethanol, 100 ml 20 % acetic acid, 1 tablet PhatGel™BlueR) and heated on maximum in a microwave oven for approximately one minute. Then gel was gently shaken for at least 10 min. Staining solution was discarded an de-staining solution (40 ml ethanol, 20 ml 100 % acetic acid, 140 ml H_2O) was applied and heated again in the microwave oven for one minute. For complete de-staining, de-staining solution was exchanged and gel was shaken for some hours. Finally, gel was washed with H_2O_{dest} and digitalized by scanning.

2.2.4.7 Aspartate decarboxylase activity

The standard AspD activity assay was started by adding 5 µl enzyme solution to a reaction mixture buffer (75 mM sodium acetate buffer, pH 5.0) containing 20 mM neutralized L-aspartate, 0.5 mM PLP and 1 mM α-ketoglutarate. After 30 min at 37 °C, reaction was terminated by boiling for 5 min.

To examine the influence of pH on AspD activity, different buffers were used. Each buffering substance was only applied in its useful buffering range. The buffers were 75 mM sodium citrate (pH 3.5; 4.0; 4.5), 75 mM sodium acetate (pH 3.5; 4.0; 4.5; 5.0; 5.5) and 75 mM sodium phosphate (pH 6; 6.5; 7; 7.5; 8). For determination of the temperature optimum, standard buffer (sodium acetate pH 5.0) was used and reactions were performed at 15, 30, 35, 40, 45, 50, and 60 °C.

A thermostability experiment on AspD was performed by incubating enzyme samples in 75 mM acetate buffer (pH 5.0) at temperatures between 30 and 60 °C for 5 to 60 min. After incubation, the residual activity was determined by standard AspD activity assay.

2.2.4.8 Thinlayer Chromatography (TLC)

Qualitative detection of amino acids and estimation of aspartate decarboxylase activity was performed by thin-layer chromatography following the method of Laskar *et al.* (Laskar *et al.*, 1991). Aliquots of AspD activity assays and amino acid standard solutions were spotted onto the TLC plates. After drying, plates were developed in a propan-1-ol and water mixture (70 + 30 v/v). Plates were dried and sprayed with a 2 % solution of the spray reagent D-camphor-10-sulphonic acid in a mixture of absolute ethanol and 1 M NaOH solutions (3 + 1 v/v). Plates were dried and heated for 10 min at 110 °C and after cooling sprayed with 0.25 % ninhydrin in acetone. Plates were air-dried and subsequently heated at 110 °C for 10 min. Colours were determined and relative amounts of amino acids could be estimated. For each run standard solutions of the expected amino acids were also applied on TLC plates.

2.2.4.9 Analysis of amino acids by GC/MS

The concentration of free amino acids in reaction mixtures was measured by GC/MS after precolumn derivatization using the EZ:faast™-kit (Phenomenex, Torrance, California, USA). After separation from cells by centrifugation and dilution, 100 µl of the supernatant were used for derivatization as indicated by the manufacturer. The system used for GC/MS analysis was an Agilent 6890N gas chromatograph equipped with an Agilent 7683B injector, an Agilent 7683 autosampler and an Agilent 5975 mass selective detector (Agilent Technologies Inc., Santa Clara, California, USA). 2 µl derivatized sample was injected into the injection port of the gas chromatograph at 250 °C with the purge valve on (split mode), split ratio 15:1 and split flow 16.5 ml/min. The compounds were separated in a ZB-AAA Zebron Amino Acid column delivered with the EZ:faast™-kit (10 m, 0.25 µm i.d.). Helium was used as carrier gas with a constant flow of 1.1 m/min and an average velocity of 68 cm/s. The initial GC oven temperature was 110 °C, ramped to 320 °C at 30 °C/min and held at 320 °C for 5 min. The total run time with a post run time of 5 min at

320 °C was 12 min and the GC-mass spectrometer interface was maintained at 310 °C. Mass spectra were obtained in the scan mode within a mass range of *m/z* 45-450 with a threshold of 150 and gain factor of 1. Ionisation was performed by electronic impact at 70 eV, calibration was performed by autotuning. The amino acids were identified by comparison with the EZ:faast™ database. Quantification was done by calibration of each amino acid with the delivered standards as indicated by the manufacturer. Data analysis was performed using the MSD ChemStation E.02.00.493 software (Agilent Technologies Inc., Santa Clara, California, USA).

2.2.4.10 Aminotransferase activity assay of purified enzymes AspD and ArcT

Aminotransferase activity tests of purified enzymes were performed in 250 µl of a reaction mixture containing 50 mM potassium phosphate buffer pH 6.5, 6 mM α-ketoglutaric acid, 50 mM PLP, 5 mM of the respective amino acid and 20 µl crude extract. For microbial stabilization 0.15 % sodium azide, 10 µg/ml erythromycin and 200 µg/ml ampicillin were added. Samples were incubated at 37 °C for 8.5 h. Enzyme reaction was stopped by heating at 90 °C for 20 min. L-glutamic acid formed by aminotransferase reaction was determined by the L-Glutamic acid kit (see below). Therefore 100 µl of reaction mixture were used.

2.2.4.11 Aminotransferase activity of cell-free extracts (CFE)

Aminotransferase activity of cell-free extracts were performed in 250 µl of a reaction mixture containing 50 mM potassium phosphate buffer pH 6.5, 6 mM α-ketoglutaric acid, 50 mM PLP, 5 mM of the respective amino acid and 50 µl crude extract. For microbial stabilization 0.15 % sodium azide, 10 µg/ml erythromycin and 200 µg/ml ampicillin were added. Samples were incubated at 37 °C. After 1 h, 12 h and 24 h samples were taken and enzyme reaction was stopped by heating at 90 °C for 20 min. After centrifugation (14000 rpm/30 min/4 °C) supernatants were stored at -20 °C for further analysis. Blanks were performed without the addition of enzyme to the reaction. Assays were made in triplicates. L-glutamic acid formed by aminotransferase reaction was determined by the L-Glutamic acid kit (see below). Therefore 100 µl of reaction mixture were used.

2.2.4.12 Determination of L-glutamic acid by L-Glutamic acid kit

Aminotransferase activity was determined by the L-Glutamic acid kit (Boehringer Mannheim). This method is based on the determination of L-glutamic acid formed from α-ketoglutaric acid by aminotransferase activity. Determination was performed following the manufacturer´s instructions. However, the assay was down-scaled for microtiter plates and final volume was reduced from 3.030 ml to 0.303 ml.

2.2.5 Detection of volatiles

2.2.5.1 Assay for detecting volatile compounds with whole resting cells

5 ml of an overnight culture were taken to inoculate 45 ml mMRS G3 medium containing 5 µg/ml erythromycin. Cells were statically incubated at 30 °C till stationary phase has just reached (OD_{590} ~ 2). After harvesting cells were washed twice with 50 mM sodiumphosphate buffer pH 6.5 containing 3 g/l glucose. Reaction mixture with a final volume of 5 ml containing 10 mM amino acid (L-leucine or L-valine), 2 mM PLP, 10 mM α-ketoglutaric acid and 50 mM sodiumphosphate buffer pH 6.5 and washed cells at an OD_{590} = 1 were placed in a 20 ml GC vial. After adding the internal standard 1,2 dimethoxyethan to a final concentration of 19.24 nmol/ml, vials were immediately capped with a magnetic crimp cap (VWR, international) containing a septum (Butyl/PTFE 1.6 mm, VWR, international) and incubated at 30 °C for 5 days. Blank assays without cells were treated like vials with cells.

2.2.5.2 Analysis of volatile compounds by SPME-GC/MS

The extraction of headspace volatile compounds was performed by using a SPME device (Supelco, Bellefonte, Pennsylvania, USA), equipped with a 75 µm carboxen/polydimethylsiloxane (CAR/PDMS) fiber and operated by the autosampler of the GC/MS system. Before analysis the fibre was preconditioned in the injection port of the GC as indicated by the manufacturer. The SPME fiber was exposed to the headspace while maintaining the sample at 30 °C for 30 min. The compounds adsorbed by the fiber were subsequently identified and quantified by GC/MS.

The compounds adsorbed by the fiber were desorbed from the injection port of the gas chromatograph (Agilent 7890A) for 10 min at 250 °C with the purge valve off (splitless mode). The compounds were separated in a ZB-Wax capillary collumn (60 m, 0.25 mm i.d., film thickness 0.25 µm; Zebron, Phenomenex, Torrance, California, USA). The GC was equipped with an Agilent 5975C mass selective detector (Agilent Technologies Inc., Santa Clara, California, USA) and a CTC CombiPAL autosampler (CTC Analytics AG, Zwingen, Switzerland) for SPME sample collection. As carrier gas helium was used with a constant flow of 1.03 ml/min and an average velocity of 25.88 cm/sec. The GC oven temperature program started when the fiber was inserted and held at 30 °C for 15 min, ramped to 50 °C at 3 °C/min, then to 110 °C at 4 °C/min, to 150 °C at 5 °C/min, and to 250 °C at 10 °C/min and finally, held at 250 °C for 10 min. Total run time was 64.67 min and the GC-mass spectrometer interface was maintained at 250 °C. Mass spectra were obtained in the scan mode within a mass range of *m/z* 29-150 with a threshold of 150 and gain factor of 0.15. Ionisation was performed by electronic impact at 70 eV and calibration

was done by autotuning. The volatile compounds were identified by comparison with mass spectra from a library database (NIST MS Search 2.0; FairCom Corporation, Columbia, Missouri, USA) and by comparison with the authentic standards. Data analysis was performed using the MSD ChemStation E.02.00.493 software (Agilent Technologies Inc., Santa Clara, California, USA).

The quantification of volatile compounds was performed by calibration of the authentic standards with the method described for the determination of volatile metabolites. Because of the varying sensitivity of SPME-GC/MS, the internal standard 1,2-dimethoxy ethane was added in all measurements (19.24 µmol/l). By using the SPME phase for sampling, calibration of relative response factors (RRF) was not possible. Therefore, the calibration was performed by calibration curves of the corresponding relative concentrations of the compounds to the internal standard 1,2-dimethoxy ethane. The calibration curves could be described by logarithmical equations. For each substance, the calibration was performed in triplicate and the average was formed.

3 Results

3.1 Strain typing

RAPD-PCR profiles were obtained for all 51 *Lactobacillus sakei* and 28 *Lactobacillus curvatus* strains present in the TMW culture collection at that time. RAPD-patterns were compared using UPGMA (Unweighted Pair Group Method with Arithmetic Mean) provided by BioNumerics Software. Strains designated as *L. sakei* or *L. curvatus* clustered in two distinct groups with three exceptions (Figure 14). Strains TMW 1.158 and TMW 1.166 supposed as *L. sakei* species clustered within the *L. curvatus* group and assumed as *L. sakei* TMW 1.1394 did not group to any of the main clusters but grouped separately. Subsequent sequencing of 16S rDNA identified TMW 1.158 and TMW 1.166 as *L. curvatus* species and TMW 1.1394 as *L. plantarum/L. penosus* species. Consequently, strain descriptions were amended. Similiarity values indicated that the *L. curvatus* strains were genetically more diverse than *L. sakei* strains. Lowest similiarity values among the *L. sakei* and *L. curvatus* isolates were 70 % and 44 % respectively (Figure 15 and Figure 16). The RAPD analysis of the *L. sakei* group (Figure 15) resulted in roughly 10 major bands per pattern and clustering showed a clear separation in two main groups. *L. sakei* TMW 1.1239 and *L. sakei* TMW 1.1240 clustered together and formed one group (A) whereas the remaining strains clustered in a second group (B). The latter group again split up in two distinct subgroups (a + b), in which the three strains TMW 1.46, 1.22, and 1.13 formed one group (a) that separated clearly from the other strains (b). Again, the cluster of these remaining strains (b) was formed by two sub-subgroups (I + II). Sub-subgroup I comprised with 29 strains the majority of *L. sakei* strains including the genome sequenced strain *L. sakei* 23K (TMW 1.1322) and *L. sakei* supspecies *sakei* type strain TMW 1.1189 (DSM20017). Sub-subgroup II contained the bacteriocin forming isogenic strains *L. sakei* TMW 1.454/1.23 (LTH 673) as well as 12 additional strains. All patterns of sub-subgroup II showed a unique band with about 1200 bp. RAPD analysis of *L. curvatus* strains revealed approximately 17 major bands per pattern and grouping into two RAPD-PCR clusters. Group C formed the smaller group with 7 *L. curvatus* strains and the pattern of this group showed roughly 16 bands whereas group D formed the major cluster with a mean of 18 bands and contained *L. curvatus* type strain TMW 1.7 (DSM 20019). These two groups could be distinguished by an additional band in the size of about 400 bp within group C. 16S rDNA sequencing of a representative of each group and subgroup confirmed the identity of the strains.

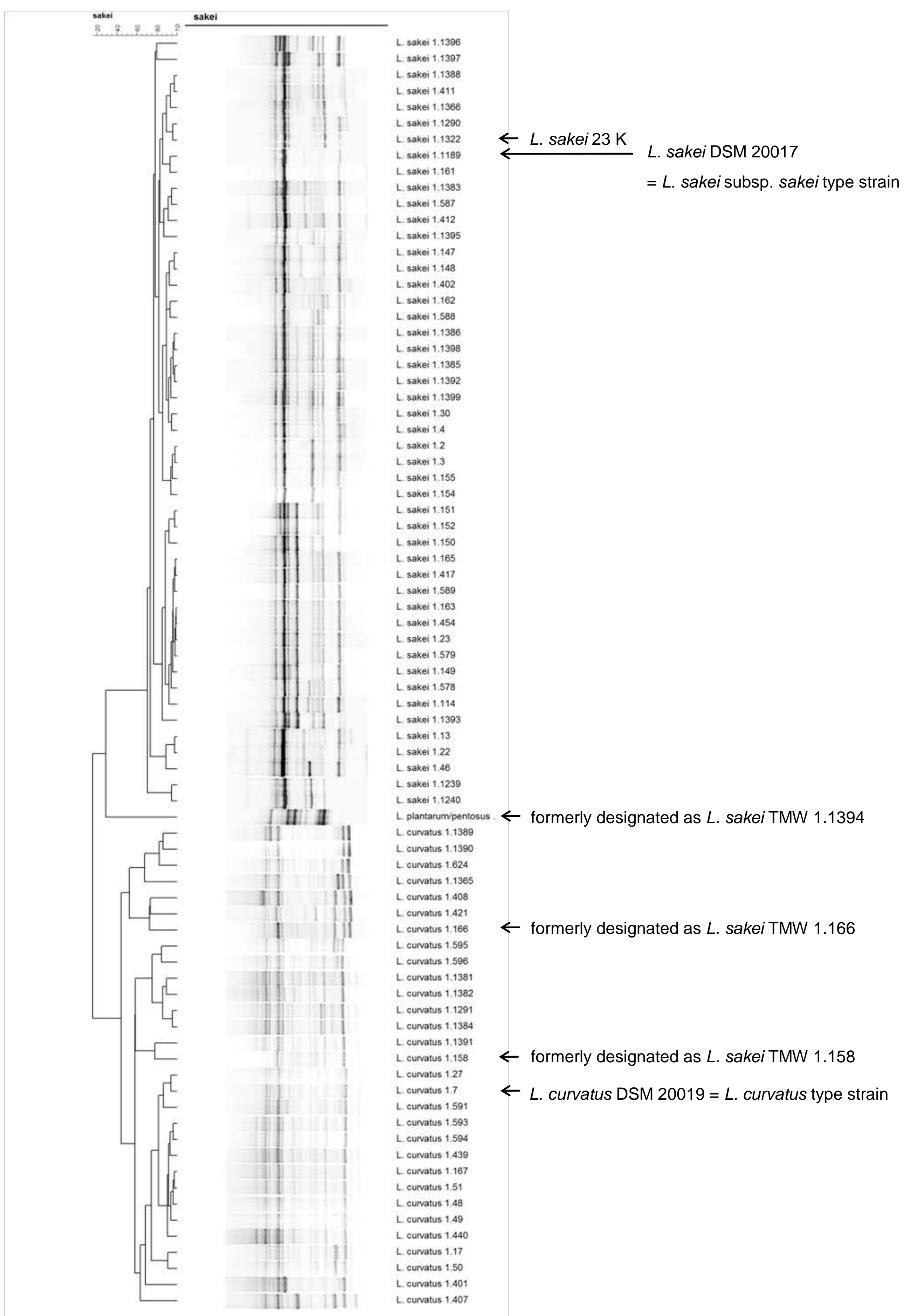


Figure 14: Clustering of RAPD patterns obtained from *L. sakei* and *L. curvatus.*

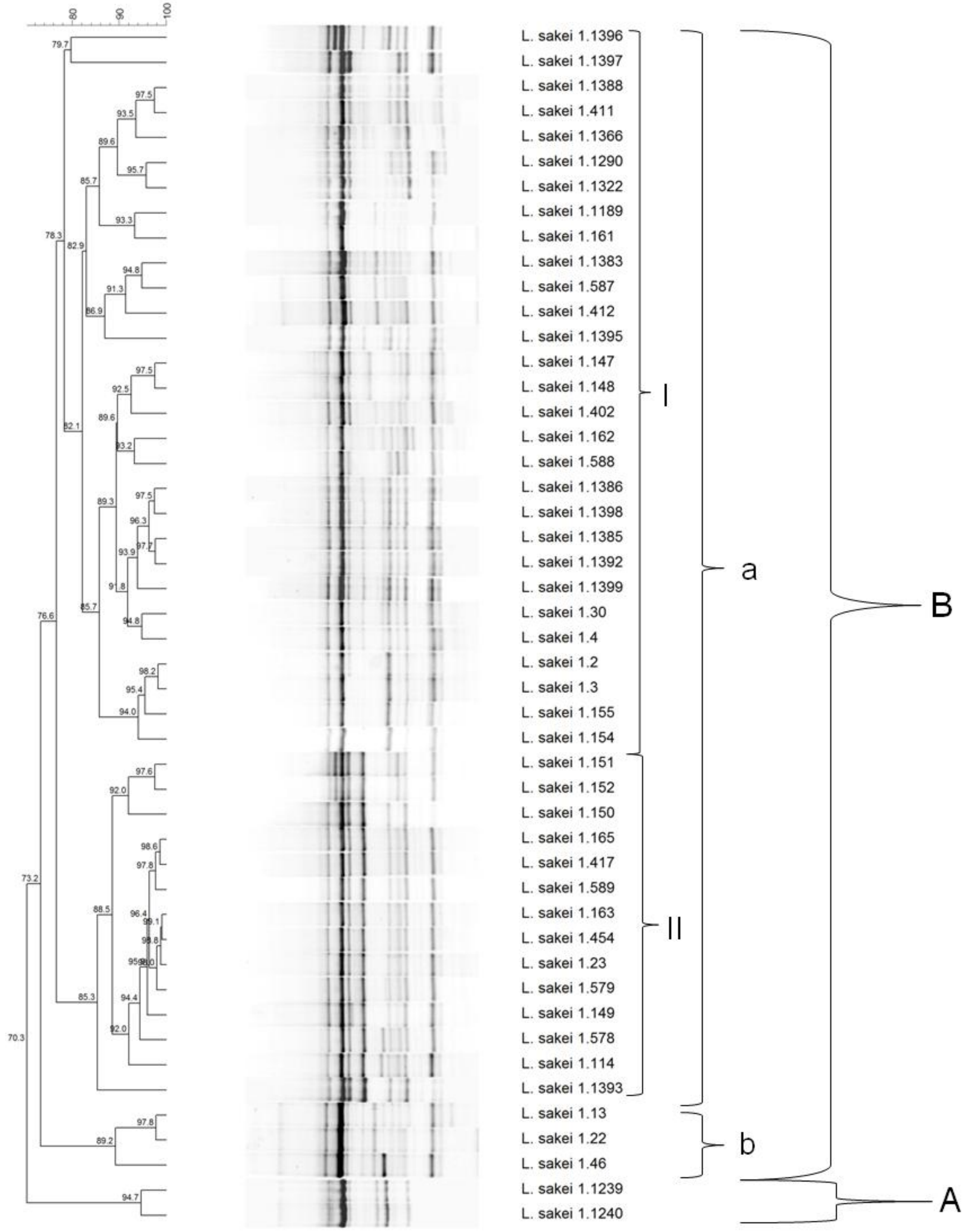


Figure 15: Clustering of RAPD patterns obtained from *L. sakei.* A and B show two groups of *L. sakei* at which group B can be divided in two subgroups (a and b). Subgroup a splits up into sub-subgroups I and II. *L. sakei* subsp. *sakei* type strain TMW 1.1189 (DSM 20017) and genome sequenced strain *L. sakei* 23K (TMW 1.1322) group together in sub-subgroup I.

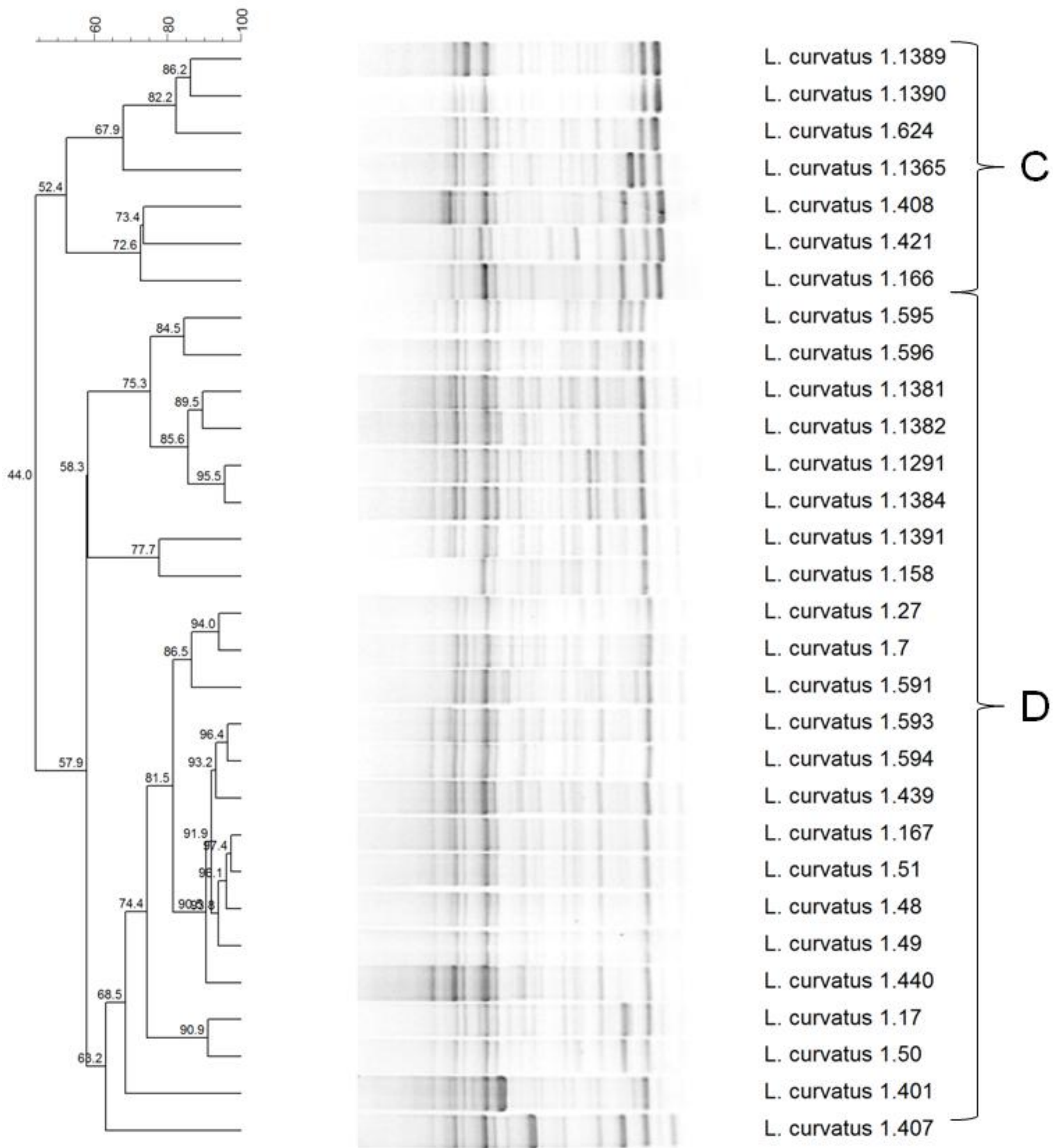


Figure 16: Clustering of RAPD patterns obtained from *L. curvatus. L. curvatus* type strain DSM 20019 (TMW 1.7) grouped in D.

3.2 Screening for amino acid requirements of *L. sakei* and *L. curvatus* strains

The amino acid requirements of 20 *L. sakei* and 3 *L. curvatus* strains were analyzed by growth studies in a chemically defined medium (CDM) with single amino acid omissions. Growth was observed at OD_{590} in a microtiter plate reader (examples in Figure 17). Control experiments in complete CDM supplemented with all 20 proteinogenic amino acids (A, C, D, E, F, G, H, I, K, L, M, N, P, Q, R, S, T, V, W, Y) revealed significant growth of all strains tested. This confirmed the chemically defined medium (CDM) as suitable for auxotrophy experiments with *L. sakei* and *L. curvatus*. Significant growth was defined as an increase of OD_{590} of at least 0.2 units.

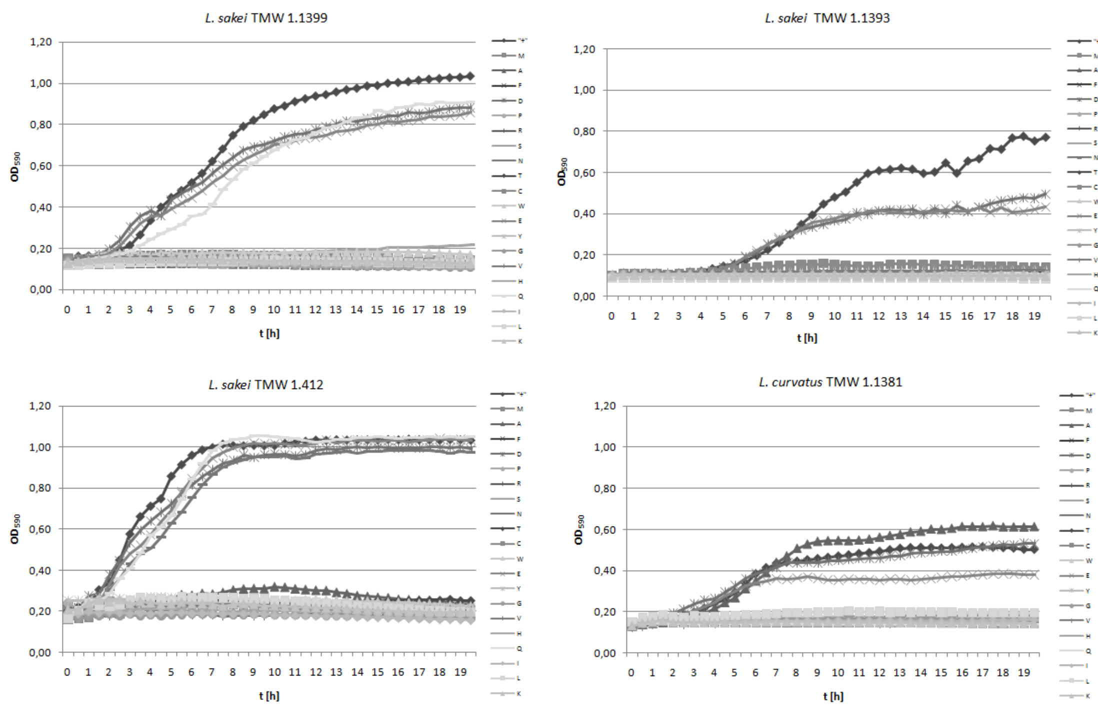


Figure 17: Growth curves of selected *L. sak*ei (TMW 1.1399, TMW 1.1393, and TMW 1.412) and *L. curvatus* (TMW 1.1381) strains in CDM medium. Letters in legend stand for amino acids omissions, + stands for CDM containing all 20 amino acids.

Results of auxotrophy tests are summarized in Figure 18. All *L. sakei* strains tested were prototrophic for 2 amino acids (L-aspartate and L-glutamate). Most of the *L. sakei* strains were also able to grow without L-glutamine. Only *L. sakei* TMW 1.1393 and TMW 1.1397 could not grow in CDM without L-glutamine. *L. sakei* TMW 1.148 and TMW 1.1322 (23K) had, with about 10 h, a longer lag-phase in CDM without L-glutamine, compared with other prototrophic strains. Additionally to growth abilitiy in medium without L-aspartate, L-glutamate or L-glutamine, *L. sakei* strains TMW 1.22, TMW 1.412, and TMW 1.1474 were also able to grow in CDM lacking L-asparagine. In general, final OD_{590} of *L. curvatus* strains were lower than those of *L. sakei* strains in all CDM variations. All three *L. curvatus* strains tested were auxotrophic for all proteinogenic amino acids except for L-alanine, L-aspartate and L-glutamate.

3.3 Screening/sequencing for/of *glnA, asnA1* and *asnA2*

Primer pair glnA-Flanke-for/Flanke-rev was used to amplify a 1510 bp DNA-fragment consisting of the *glnA* gene of *L. sakei* 23K (1341 bp) coding for a glutamate-ammonia ligase (glutamine synthetase) [EC: 6.3.1.2] and approximately 80 bp of its flanking regions. Genomic DNA of *L. sakei* strains, auxotrophic for L-glutamine (TMW 1.1393 and TMW 1.1397) or showing a long lag-phase in medium without L-glutamine (*L. sakei* 23K, TMW 1.148) were used as templates for the PCR. Sequencing of these fragments did not

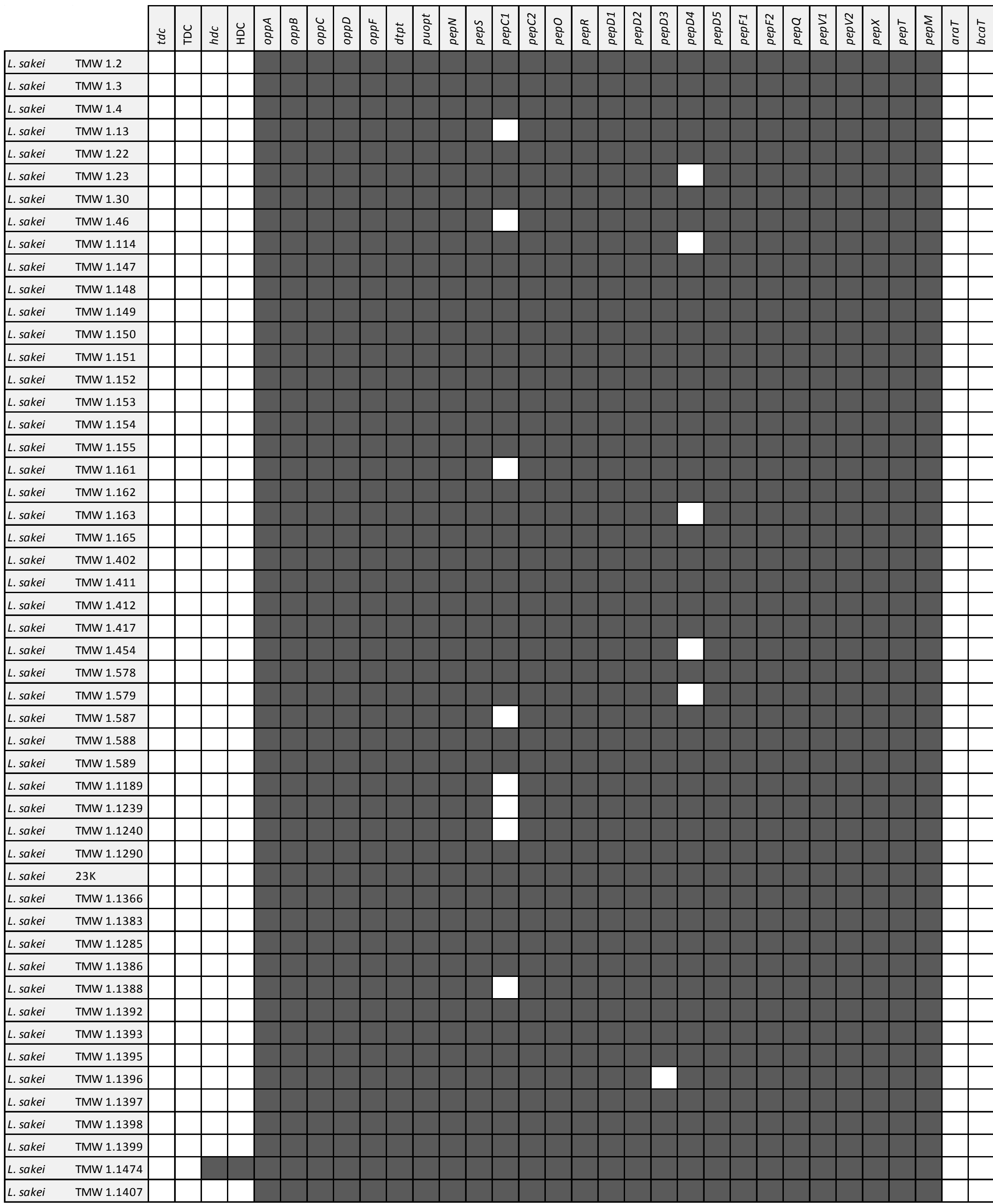
show any mutations that led to a frame shift and subsequently to an auxotrophy for L-glutamine.

Species	Strain	+	L-Methionine	L-Alanine	L-Phenylalanine	L-Aspartate	L-Proline	L-Arginine	L-Serine	L-Asparagine	L-Tryptophane	L-Cysteine	L-Tryptophane	L-Glutamate	L-Tyrosine	L-Glycine	L-Valine	L-Histidine	L-Glutamine	L-Isoleucine	L-Leucine	L-Lysine	*glnA*	*asnA1*	*asnA2*	*asnB*
L. sakei	TMW 1.2					■								■					■				■	■	■	■
L. sakei	TMW 1.3					■								■					■				■	■	■	■
L. sakei	TMW 1.4					■								■					■				■	■	■	■
L. sakei	TMW 1.13					■								■					■				■	■	■	■
L. sakei	TMW 1.22					■				■				■					■				■	■	■	■
L. sakei	TMW 1.23					■								■					■				■	■	■	■
L. sakei	TMW 1.148					■								■					■				■	■	■	■
L. sakei	TMW 1.155					■								■					■				■	■	■	■
L. sakei	TMW 1.412					■				■				■					■				■	■	■	■
L. sakei	TMW 1.454					■								■					■				■	■	■	■
L. sakei	TMW 1.1239					■								■					■				■	■	■	■
L. sakei	23 K					■								■					■				■	■	■	■
L. sakei	TMW 1.1383					■								■					■				■	■	■	■
L. sakei	TMW 1.1388					■								■					■				■	■	■	■
L. sakei	TMW 1.1393					■								■									■	■	■	■
L. sakei	TMW 1.1395					■								■					■				■	■	■	■
L. sakei	TMW 1.1397					■								■									■	■	■	■
L. sakei	TMW 1.1398					■								■					■				■	■	■	■
L. sakei	TMW 1.1399					■								■					■				■	■	■	■
L. sakei	TMW 1.1474					■				■				■					■				■	■	■	■
L. curvatus	TMW 1.7			■		■								■									■	■	■	■
L. curvatus	TMW 1.51			■		■								■									■	■	■	■
L. curvatus	TMW 1.1381			■		■								■									■	■	■	■

Figure 18: Summarized results of auxotrophy screening (left) and screening for presence or absence of genes (*glnA, asnA1, asnA2, asnB*) likely involved in amino acid interconversions. Filled boxes indicate positive results, empty boxes negative results.

Sequencing of *glnA* from *L. sakei* 23K obtained from TMW culture collection did not reveal any differences to the *glnA* sequence found in the database (GI:81428932). Between *glnA* sequences of *L. sakei* TMW 1.148, TMW 1.1393 and TMW 1.1397 few minor base substitutions within nucleotide sequences could be detected. None of them led to an amino acid substitution. Comparison of *glnA* sequences of *L. sakei* TMW 1.148, TMW 1.1393 and TMW 1.1397 with the sequence of *L. sakei* 23K showed more nucleotide substitutions (similiarity 98 %), whereas only a single amino acid was affected. Presence of *glnA* was demonstrated for all 20 *L. sakei* strains by PCR screening with primer pair glnA-Flanke-for/Flanke rev (Figure 18). *L. sakei* 23K specific primers were used to screen for the absence or presence of *asnB* (asnB-F/R), *asnA1* (asnA1-F/R) and *asnA2* (asnA2-F/R). All three genes could be detected in all 20 *L. sakei* strains tested for amino acid requirements (Figure 18).

3.4 PCR-Screening for peptide transport genes



		tdc	TDC	*hdc*	HDC	*oppA*	*oppB*	*oppC*	*oppD*	*oppF*	*dtpt*	*puopt*	*pepN*	*pepS*	*pepC1*	*pepC2*	*pepO*	*pepR*	*pepD1*	*pepD2*	*pepD3*	*pepD4*	*pepD5*	*pepF1*	*pepF2*	*pepQ*	*pepV1*	*pepV2*	*pepX*	*pepT*	*pepM*	*araT*	*bcaT*
L. sakei	TMW 1.2					■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		
L. sakei	TMW 1.3					■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		
L. sakei	TMW 1.4					■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		
L. sakei	TMW 1.13					■	■	■	■	■	■	■	■	■		■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		
L. sakei	TMW 1.22					■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		
L. sakei	TMW 1.23					■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		■	■	■	■	■	■	■	■	■		
L. sakei	TMW 1.30					■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		
L. sakei	TMW 1.46					■	■	■	■	■	■	■	■	■		■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		
L. sakei	TMW 1.114					■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		■	■	■	■	■	■	■	■	■		
L. sakei	TMW 1.147					■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		
L. sakei	TMW 1.148					■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		
L. sakei	TMW 1.149					■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		
L. sakei	TMW 1.150					■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		
L. sakei	TMW 1.151					■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		
L. sakei	TMW 1.152					■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		
L. sakei	TMW 1.153					■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		
L. sakei	TMW 1.154					■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		
L. sakei	TMW 1.155					■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		
L. sakei	TMW 1.161					■	■	■	■	■	■	■	■	■		■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		
L. sakei	TMW 1.162					■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		
L. sakei	TMW 1.163					■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		■	■	■	■	■	■	■	■	■		
L. sakei	TMW 1.165					■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		
L. sakei	TMW 1.402					■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		
L. sakei	TMW 1.411					■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		
L. sakei	TMW 1.412					■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		
L. sakei	TMW 1.417					■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		
L. sakei	TMW 1.454					■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		■	■	■	■	■	■	■	■	■		
L. sakei	TMW 1.578					■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		
L. sakei	TMW 1.579					■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		■	■	■	■	■	■	■	■	■		
L. sakei	TMW 1.587					■	■	■	■	■	■	■	■	■		■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		
L. sakei	TMW 1.588					■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		
L. sakei	TMW 1.589					■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		
L. sakei	TMW 1.1189					■	■	■	■	■	■	■	■	■		■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		
L. sakei	TMW 1.1239					■	■	■	■	■	■	■	■	■		■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		
L. sakei	TMW 1.1240					■	■	■	■	■	■	■	■	■		■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		
L. sakei	TMW 1.1290					■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		
L. sakei	23K					■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		
L. sakei	TMW 1.1366					■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		
L. sakei	TMW 1.1383					■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		
L. sakei	TMW 1.1285					■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		
L. sakei	TMW 1.1386					■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		
L. sakei	TMW 1.1388					■	■	■	■	■	■	■	■	■		■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		
L. sakei	TMW 1.1392					■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		
L. sakei	TMW 1.1393					■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		
L. sakei	TMW 1.1395					■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		
L. sakei	TMW 1.1396					■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		■	■	■	■	■	■	■	■	■	■		
L. sakei	TMW 1.1397					■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		
L. sakei	TMW 1.1398					■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		
L. sakei	TMW 1.1399					■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		
L. sakei	TMW 1.1474			■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		
L. sakei	TMW 1.1407					■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		

Figure 19: Results of screening-PCRs (*L. sakei*) for genes coding for peptide transporters (*opp, dtpt, puopt*), peptidases (*pep*), aminotransferases (*araT, bcaT*), decarboxylases (*tdc, hdc*) and results for decarboxylase activities (TDC, HDC); filled boxes indicate positive results, empty boxes negative results.

The results of PCR screenings of 51 *L. sakei* strains and of 28 *L. curvatus* strains are depicted in Figure 19 and in Figure 20. The genome of *L. sakei* 23K contains five *opp*-genes (*oppABCDF*) each coding for a subunit of the oligopeptide ABC transport system Opp, one gene for a di/tripeptide ionlinked transporter (*dtpt*) and one gene for a putative

oligopeptide transporter (*puopt*) that shows homology to the oligopeptide transporter (OPT family) of *Clostridium perfringens* B str. ATCC 3626 and *Clostridium butyricum* 5521 (64 % similiarity in nucleotide sequence). Screening primers were either specific for *L. sakei* or were designed from nucleotide sequences obtained from several organisms (Table 10).

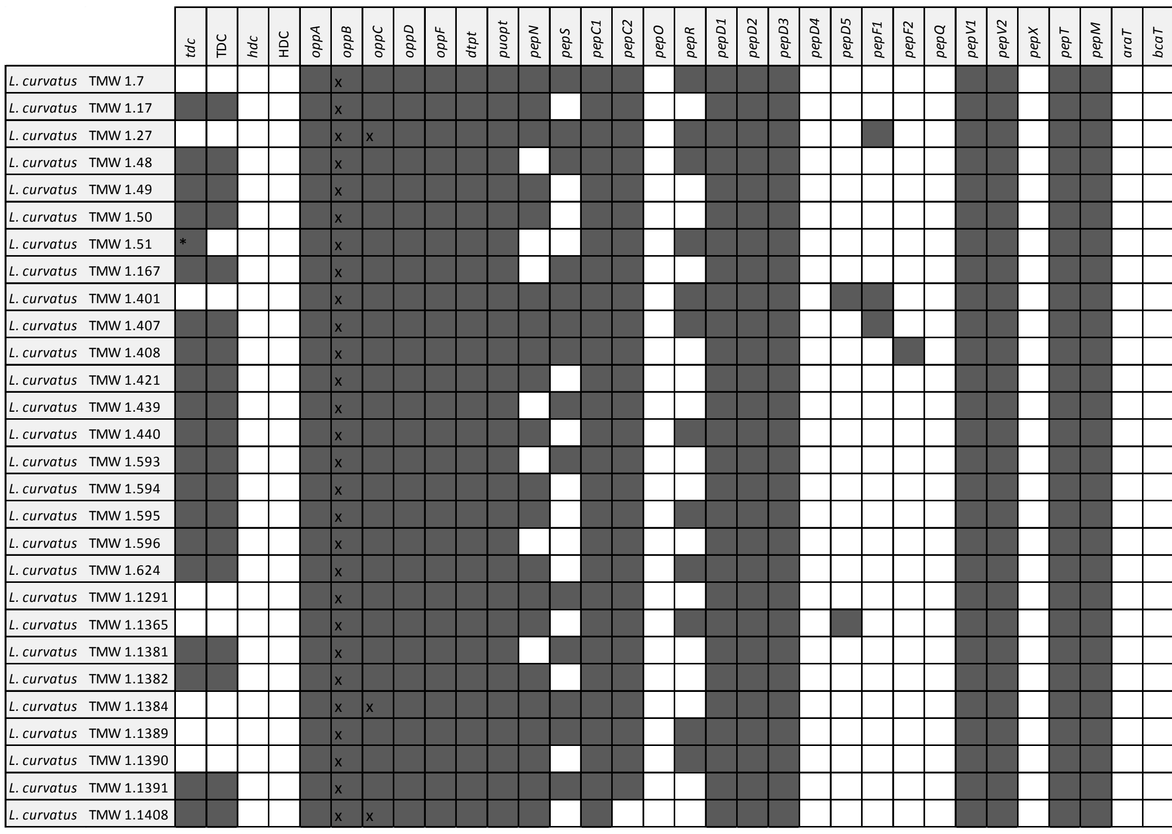


Figure 20: Results of screening-PCRs (*L. curvatus*) for genes coding for peptide transporters (*opp*, *dtpt*, *puopt*), peptidases (*pep*), aminotransferases (*araT*, *bcaT*), decarboxylases (*tdc*, *hdc*) and results for decarboxylase activities (TDC, HDC); filled boxes indicate positive results, empty boxes negative results; (x = indirect positive result by primer pair oppA-forward/oppC-deg-rev; * frame shift mutation in *tdc*-sequence).

All *L. sakei* strains tested showed amplificates for each of the seven transporter genes. Also most of the *L. curvatus* strains gave PCR products for the peptide transport genes. The primers specific for *oppB* did not reveal positive results for any of the *L. curvatus* strains, whereas amplificates of *oppADF* fragments could be amplified for all *L. curvatus* strains tested. *oppC* could not be obtained for three *L. curvatus* strains (TMW 1.27, TMW 1.1384 and TMW 1.1408). PCR amplification with oppA-forward and oppC-deg-rev showed amplificates with the expected size of 2761 bp for all *L. curvatus* strains. Screening PCRs for *dtpt* and *puopt* were positive for all *L. curvatus* strains.

3.5 PCR-Screening for peptidases

19 peptidase genes are annotated for *L. sakei* 23K. There are genes coding for four aminopeptidases *pepN, pepS, pepC2* and *pepM*, for four endopeptidases *pepC1*, *pepO*, *pepF1* and *pepF2*, for seven dipeptidases *pepD1-pepD5*, *pepV1* and *pepV2*, for three proline peptidases *pepR, pepX* and *pepQ* and for a tripeptidase *pepT*. The distribution of these peptidase genes within the *L. sakei* species appeared very uniform. *PepN, pepS, pepC2, pepO, pepR, pepD1, pepD2, pepD3, pepD5, pepF1+2, pepQ, pepV1+2, pepX*, *pepM* and *pepT* could be detected in all *L. sakei* strains by PCR amplification with *L. sakei* specific primer pairs. There were only some differences regarding the peptidases *pepC1, pepD3* and *pepD4*. *PepC1* could not be found in *L. sakei* strains TMW 1.13, TWM 1.46, TMW 1.161, TMW 1.587, TMW 1.1189, TMW 1.1239, TMW 1.1240 and TMW 1.1388, *pepD3* was not detectable in TMW 1.1396, and TMW 1.23, TMW 1.114, TMW 1.163, TMW 1.454, and TMW 1.579 did not give an amplificate for *pepD4*. All together, there was no strain of *L. sakei,* which lacked more than one peptidase.

PepC1 of *L. sakei* 23K contained a point mutation on base position 1209, which leads to the stop codon TGA (Chaillou *et al.*, 2005). Sequencing data of four further *L. sakei* strains (*L. sakei* TMW 1.2, TMW 1.412 and TMW 1.1399), shown in Figure 21, indicated that this frameshift mutation is not widely distributed among *L. sakei* strains, since it could be only found in this single strain.

```
L. sakei 23K          GACGCTTGATTTGATGACTATGTATACGAAGTGGTTATTAAGAAGGAATA   1250
L. sakei TMW 1.2      GACGCTTGGTTTGATGACTATGTATACGAAGTGGTTATTAAGAAGGAATA   1250
L. sakei TMW 1.22     GACGCTTGGTTTGATGACTATGTATACGAAGTGGTTATTAAGAAGGAATA   1250
L. sakei TMW 1.412    GACGCTTGGTTTGATGACTATGTATACGAAGTGGTTATTAAGAAGGAATA   1250
L. sakei TMW 1.1399   GACGCTTGGTTTGATGACTATGTATACGAAGTGGTTATTAAGAAGGAATA   1250
                      ******** *****************************************
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Figure 21: Part of sequence alignment of five *L. sakei* strains regarding *pepC1* frame shift mutation (shaded).

To some extent, *L. sakei* specific primers for several peptidases did also give positive results for *L. curvatus* strains. Amplification of p*epC1, pepD1, pepD2, pepD3, pepV1, pepV2, pepM* and *pepT* was successfull in all *L. curvatus* strains. Only *L. curvatus* TMW 1.1408 lacks *pepC2*. Amplificates for *pepN*, *pepR* and *pepS* were strain dependent in *L. curvatus: pepR* could be detected in 45 %, *pepS* in 52 % and *pepN* in 76 % of the *L. curvatus* strains. No amplificates could be obtained for *pepX, pepQ, pepD4,* and *pepO*. Primers for *pepD5*, *pepF1, pepF2* and *pepM* gave only positive PCR results for single *L. curvatus* strains.

3.6 Comparison of peptidase sequences

PepX, pepQ and *pepR* are genes coding for peptidases specific for proline containing peptides (Lloyd and Pritchard, 1991; Shao *et al.*, 1997; Tsakalidou *et al.*, 1998). *PepR* and

pepQ were amplified from *L. sakei* starter strains TMW 1.1392 and 1.1399 that led to mild aroma and TMW 1.1388 and 1.1398 that led to bitter aroma in fermented sausages. *PepX* was amplified with genomic DNA of *L. sakei* strains TMW 1.1399 and TMW 1.1398 as template.

PepR-sequi-for/pepR-sequi-rev was used to amplify a 1093 bp fragment that contains *pepR* (903 bp) and its flanking regions of 60 - 80 bp in upstream and downstream direction. *PepX* (2412 bp) and its surrounding region (81 - 93 bp) was amplified by pepX-sequi-for/rev to get a 2586 bp DNA-fragment and *pepQ* with its surrounding regions was amplified with pepQ-sequi-for2/pepQ-sequi-rev2 to get a 1333 bp amplificate. Sequencing of *pepX* was performed by pepX-sequi-for/rev and pepX-mifo and pepX-mifo2 which lie within the *pepX* sequence. Only two nucleotide substitutions could be detected at the very end of the gene between *pepX* sequences of *L. sakei* TMW 1.1388 and TMW 1.1398 that led to a change into similar amino acids. Comparison of *pepX* from TMW 1.1388, TMW 1.1398 and 23K revealed more differences but similiarity of nucleotide sequences was still 97 %. Comparison of *pepQ* nucleotide sequences from *L. sakei* TMW 1.1392, TMW 1.1399, TMW 1.1388 and 1.1398 showed 100 % identity. If compared with *pepQ* of *L. sakei* 23K four different bases could be detected, that led to the substitution of a single amino acid. Comparison of the four *pepR* sequences did not reveal any differences. Only single base substitutions could be observed, when compared to *pepR* of the genome sequenced *L. sakei* 23K. However, these substitutions did not led to a change in amino acid sequence.

3.7 Screening for amino acid transporter genes specific for branched-chain amino acids

Several genes for amino acid transporters could be found within the genome of *L. sakei* 23K but there are no annotations of genes coding for transporters specific for branched-chain amino acids whereas many other lactobacilli carry one or even more genes encoding these kinds of transporters. Sequence alignment of *brnQ* genes from *L. buchneri* ATCC11577, *L. brevis* ATCC27305, *L. casei* BL23 and *L. rhamnosus* Lc705 gave sequence identities between 60 and 97 %. Based on this alignment, degenerated primers brnQ-deg-1-for/rev were designed for amplification of a 453 bp *brnQ* fragment and ten *L. sakei* strains were initially screened by PCR using this primer pair. A PCR product of the correct size could be detected for *L. sakei* TMW 1.22 (Figure 22).

Figure 22: Screening of *L. sakei* strains for a 453 bp fragment of *brnQ*. 1 = TMW 1.2, 2 = TMW 1.1397, 3 = TMW 1.3, 4 = TMW 1.4, 5 = TMW 1.13, 6 = TMW 1.22, 7 = 23K, 8 = TMW 1.1366, 9 = TMW 1.1393, 10 = TMW 1.1395, + = positive control *L. rhamnosus* TMW 1.1330 - = neg.

Sequencing of this fragment with brnQ-deg-1-for and a subsequent BLAST search revealed best consensus with the *brnQ* genes of *L. salivarius* CECT 5713 and *L. salivarius* UCC118 (coverage of 98 % and 69 % homology). Also, significant similiarities to branched-chain transporter genes of several other lactobacilli could be observed. Based on the sequenced PCR product, *L. sakei* TMW 1.22-specific primers (brnQ-S5-for/rev) were designed and applied for a screening of 51 *L. sakei* strains. Four strains could amplify the expected 356 bp fragment. *BrnQ* fragments of *L. sakei* TMW 1.22, TMW 1.114, TMW 1.578 and TMW 1.1290 were sequenced. Alignment of the five sequenced *brnQ*-fragments showed high similiarities. Only differences at two single nucleotide sites could be detected.

3.8 Screenings for decarboxylase genes and activites

PCR screening for the presence of a tyrosine decarboxylase gene was performed by two *L. curvatus* HSCC1737 specific primer pairs (Tdc-for/rev and TDC1/2). The primer pair HDC3/4 for histidine decarboxylase genes was obtained from Coton and Coton (2005) and in addition, another degenerated primer pair was designed after alignment of *hdc* genes of *L. sakei* LTH 2076, *L. buchneri* DSM 5987 and *L. hilgardii* IOEB 0006 (Table 10). Results of PCR and the physiological screening for decarboxylase activity in medium supplemented with precursor amino acids for tyramine and histamine are summarized in Figure 19 (*tdc*, *hdc*, TDC, HDC). Neither by PCR screening procedures nor by physiological tests a tyrosine (TDC^+) or a histidine decarboxylase positive (HDC^+) *L. sakei* strain could be detected with one exception: Strain *L. sakei* LTH 2076 (TMW 1.1474) showed amplification of the 594 bp and the 437 bp fragments with primer pairs HDCfor/rev and HDC3/4 respectively, corresponding to the *hdc* gene, and was also positive in the physiological histamine screening (Figure 23).

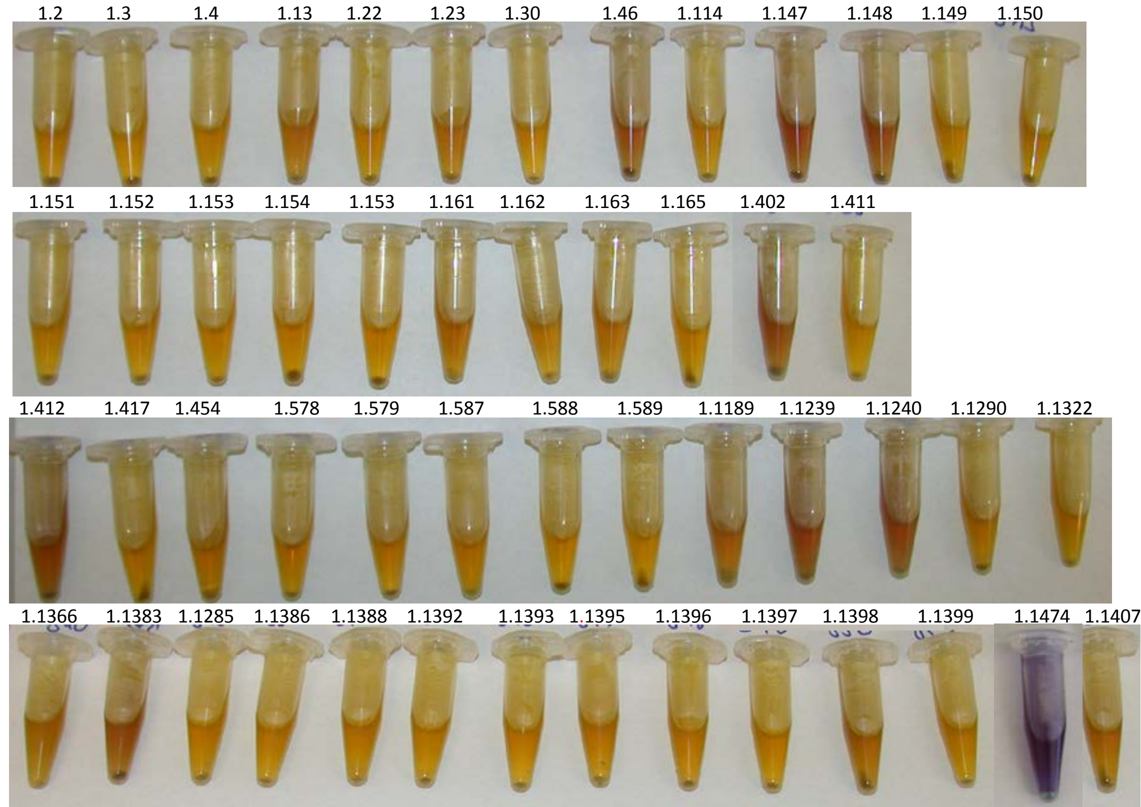


Figure 23: Physiological screening of *L. sakei* strains (figures are TMW numbers) for histidine decarboxylase activity by change of colour. yellow = HDC-, purple = HDC+.

None of the *L. curvatus* strains was positive in the HDC-screenings (PCR and physiological), whereas the screening for a tyrosine decarboxylase showed strain dependence within *L. curvatus* species. 20 of the tested 29 strains gave a PCR product for the 1013 bp *tdc* gene fragment. In contrary to this result, only 19 of the PCR positive strains induced a colour change of the tyramine test medium (Figure 25). DNA of *L. curvatus* TMW 1.51 amplified the *tdc* gene fragment but was not positive in the test medium.

```
L. curvatus HSCC1737    GGATGGCGTCAAAACTATATGCCACAAGACAAACCAATGATTTCAGCTGAACAACAAACA 180
L. curvatus TMW 1.51    GGATGGCGTCAAAACTATATGCCACAAGACAAACCAATGATTTCAGCTGAACAACAAACA 180
                        ************************************************************

L. curvatus HSCC1737    TCACCTGAGTTCCGAGGAACAGTCAACAATATGAAAGACGTTTTAGATGAACTTTCATCA 240
L. curvatus TMW 1.51    TCACCTGXXXXXCGAGGAACAGTCAACAATATGAAAGACGTTTTAGATGAACTTTCATCA 235
                        *******     ************************************************
```

Figure 24: Part of the sequence alignment of *L. curvatus* HSCC1737 and *L. curvatus* TMW 1.51 containing *tdc* region with frame shift mutation (shaded).

To check these ambiguous results the complete *tdc*-gene of *L. curvatus* TMW 1.51 was amplified using the primer set TDC1/TDC2 and was consequently sequenced. Sequence data (Figure 24) were aligned against the published *tdc*-sequence of *L. curvatus* HSCC1737. The alignment revealed a deletion of 5 basepairs at positions 188 – 192 that led to a frame shift within the *tdc*-gene sequence (Figure 24) of *L. curvatus* strain TMW 1.51 and subsequently to a non-functional decarboxylase.

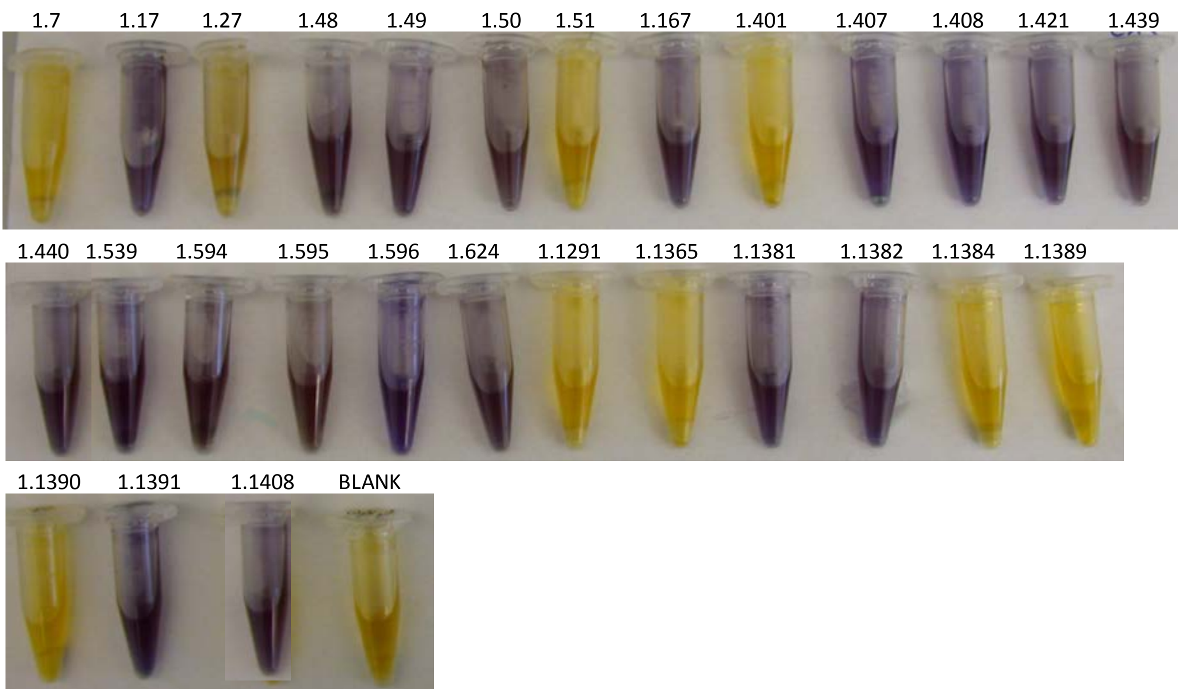


Figure 25: Physiological screening of *L. curvatus* strains (figures are TMW numbers) for tyrosine decarboxylase activity by change of colour. yellow = TDC-, purple = TDC+.

3.9 PCR screening for *araT* and *bcaT*

Genes coding for aminotransferases specific for the transamination of branched-chain amino acids and aromatic amino acids could not be found in the genome of *L. sakei* 23K. Thus, degenerated primers were designed obtaining aminotransferase sequences from several lactobacilli. But with none of the primer combinations (ARA-deg-for3/ARA-deg-rev3+4; ARA-deg-for4/ARA-deg-rev3+4; Bcat-deg-for A/Bcat-deg-revA; Bcat-deg-forB/Bcat-deg-revB) amplification of either a *bcat* or an *araT* fragment of the expected size was possible. Only positive controls showed the expected PCR products.

3.10 Search for putative aminotransferase genes involved in aroma formation in *L. sakei*

L. sakei 23K does not possess genes coding for aminotransferases specific for branched-chain amino acids (BcaT) or aromatic amino acids (AraT). Moreover, as mentioned above, no PCR amplificates could be generated with degenerated primers obtained from sequence alignments of several lactobacillus-*bcaT* genes.

Only two annotations for aminotransferases associated with amino acid metabolism could be found. *ArcT* (YP_394986) lies in between a gene cluster (*arcABCTDR*) encoding the enzymes of arginine deiminase pathway of *L. sakei*. Database search among lactobacilli and lactococci revealed only few further *arcT* sequences. Four nearly identical sequences are available for different *L. plantarum* strains, and another *arcT* sequence could be found

in *L. lactis* Il1403. Sequence alignment showed similiarities between 53 and 59 % and within all three sequences the consensus pattern ([GS]-[LIVMFYTAC]-[GSTA]-K-x-(2)-[GSALVN]-[LIVMFA]-x-[GNAR]-(V)-R-[LIVMA]-[GA]) for aminotransferases class-I pyridoxal-phosphate attachment site K could be detected (Figure 26).

```
L. plantarum ST-III        ETP-FVSIADLYERGIAVNSLSKTYSAPGIRIGWTATPSQAIADIFRKYR 249
L. sakei 23K               EGQQTTSIADLYELGIATNSLSKTYSVPGIRVGWLVAN-ETLTDLFRKYR 248
L. lactis Il1403           EETPYSPIADLYEKGISTNSISKTYSVPGIRVGWVATQDRDLCNEFRKIR 249
                           :************  .:::*::*.****:***************:*::.*
```

Figure 26: Part of alignment of ArcT amino acid sequences from *L. plantarum* ST-III, *L. sakei* 23K and *L. lactis* Il1403; light grey: aminotransferase class-I consensus pattern, dark grey: aminotransferase class-I pyridoxal-phosphate attachment site K.

Beyond that, a gene *aspD*, annotated as coding for an aspartate amiotransferase (YP_394920) and a L-aspartate-beta-decarboxylase (CAI54608) could be found. Two further lactobacillus-*aspD*-genes were available for *Lactobacillus oris* PB013-T2-3 and *Lactobacillus antri* DSM 16041. Sequence alignment showed relatively varying similiarities between 47 and 95 %. Motif search in AspD-sequences resulted in the same aminotransferase class-I pyridoxal-phosphate attachment site as for ArcT (Figure 27).

```
L. oris PB013-T2-3         DLVIITDDVYGTFSPSYQSIFAVAPHNTILVYSFSKLYGATGQRLGVVCM 336
L. antri DSM 16041         DLVIITDDVYGTFSPSYQSIFAVAPHNTILVYSFSKLYGATGQRLGVVCM 348
L. sakei 23 K              NLVIITDDVYGTFVDDFKTIYSVVPHNTLLVYSFSKLYGATGQRIGLIAM 327
                           :************  .:::*::*.****:***************:*::.*
```

Figure 27: Part of alignment of AspD amino acid sequences from *L. oris* PB013-T2-3, *L. sakei* 23K and *L. antri* DSM 16041; light grey: aminotransferase class-I consensus pattern, dark grey: aminotransferase class-I pyridoxal-phosphate attachment site K.

PCR screening of 51 *L. sakei* strains for *aspD* and *arcT* revealed that theses genes are ubiquitary in this species (Figure 19).

3.11 Cloning and heterologous expression of putative aminotransferase genes *aspD* and *arcT* of *L. sakei* 23K

AspD and *arcT* genes of *L. sakei* 23K were amplified with specific primers (AspAT-Ls-klon-for/rev or AT-Ls-klon-for/rev) to obtain PCR products of 1627 bp and 1135 bp respectively. Purified PCR products were cloned into a pBAD/*Myc*-HisA vector expression system using *NcoI* and *PstI* (*aspD*) or *NcoI* and *HindIII* (*arcT*) restriction sites. The pBAD/*Myc*-HisA vector system provided codons for a His-tag at the 3´ end of the genes.

Correct insertion of the fragments and fidelity of PCR was proven by digestion of isolated vector DNA with respective restriction enzymes and sequencing with pBAD-for and pBAD-rev primers located on the pBAD/Myc-HisA plasmid. Expression of genes in *E. coli*

TOP 10 expression strain resulted in a peak at the UV detector, when target protein was eluted from the His-Trap column (Figure 28) with buffer B.

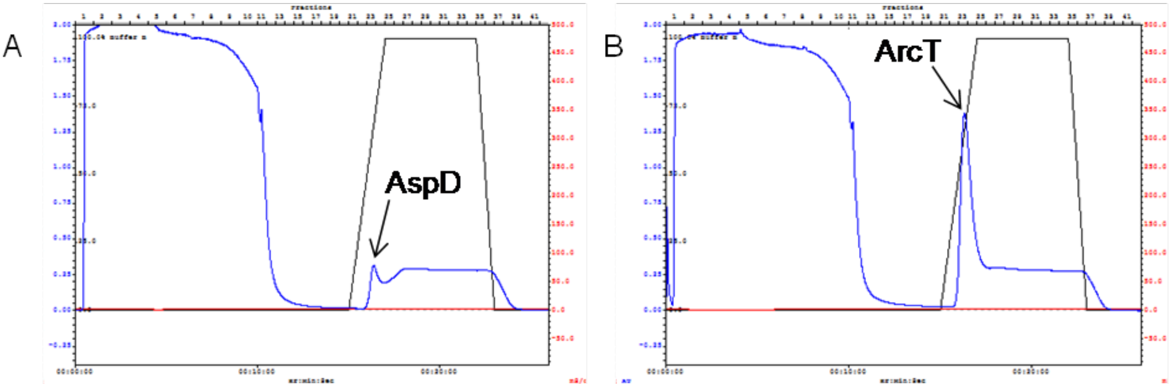


Figure 28: Elution profiles of AspD (A) and ArcT (B) from His-trap column (FPLC).

Coomassie stained SDS-PAGE of eluted fractions (Figure 29) revealed significant amounts of nearly pure protein at the expected molecular weights (AspD 60.8 kDa; ArcT 41.3 kDa). Only some weak bands of other proteins could be detected in AspD and ArcT fractions.

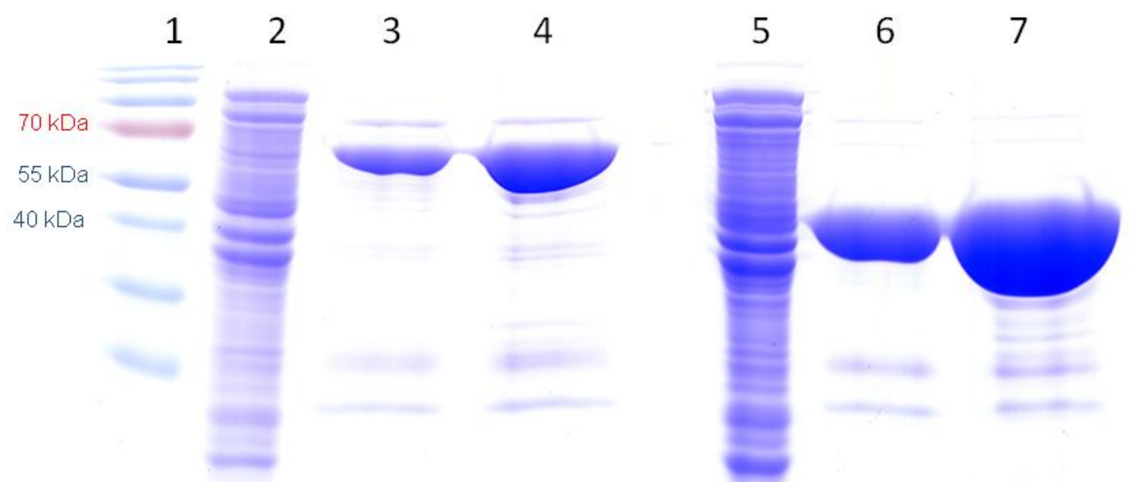


Figure 29: SDS-PAGE of AspD and ArcT expressions. 1 = PageRuler™ Prestained Protein Ladder, 2 = crude extract AspD, 3+4 = fractions 23 + 24 eluted rom His-trap column (FPLC) at AspD run (expected size: 60.8 kDa), 5 = crude extract ArcT, 6+7 = fractions 23+24 eluted from His-trap column at ArcT run (expected size: 41.3 kDa).

3.12 Determination of aminotransferase activities of AspD and ArcT

Aminotransferase activities of purified and dialyzed protein fractions were determined by the L-Glutamic acid kit. Ten L-amino acids (L-leucine, L-soleucine, L-valine, L-tyrosine, L-phenylalanine, L-aspartate, L-asparagine, L-alanine, L-glutamine, L-methionine), partly relevant for aroma formation in sausage fermentations were used as substrates for the transaminase activity assay for putative aminotransferases AspD and ArcT.

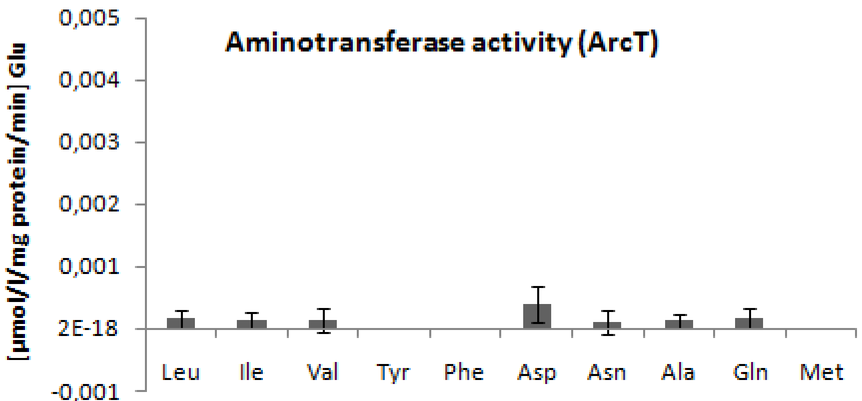


Figure 30: Aminotransferase activities of ArcT against ten amino acids (Leu, Ile, Val, Tyr, Phe, Asp, Asn, Ala, Gln, Met) in [µmol/l/mg/protein/min] formed L-glutamate.

The amount of L-glutamate, formed by AspD (Figure 30) and ArcT (Figure 31) were below the detection limit of the L-Glutamic acid test kit. For AspD a slight transaminase activity against L-tyrosine could be supposed.

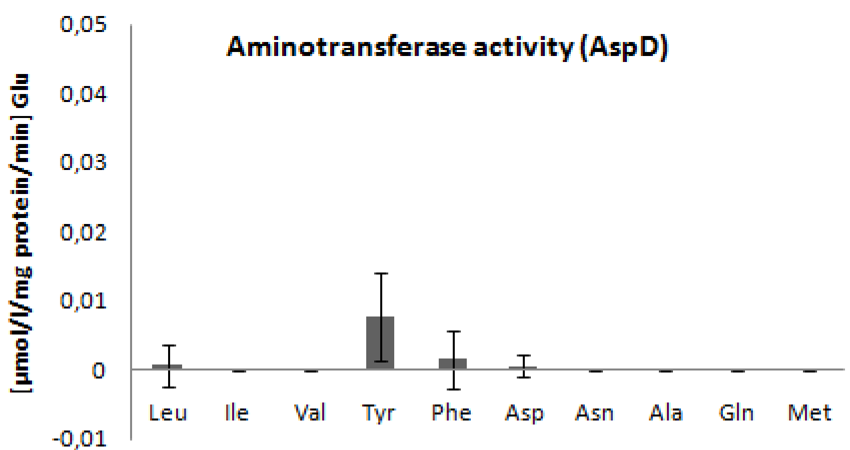


Figure 31: Aminotransferase activities of AspD against ten amino acids (Leu, Ile, Val, Tyr, Phe, Asp, Asn, Ala, Gln, Met) in [µmol/l/mg/protein/min] formed L-glutamate.

3.13 Partially characterization of aspartate-beta-decarboxylase (AspD)

Aspartate-beta-decarboxylase activity could be detected for AspD. Estimation of AspD activity was performed by a TLC method and confirmation of TLC results and quantitative analysis of AspD acitivity was partly performed by sample preparation with the EZ:faast kit and subsequent GC/MS analysis. Thermoinstability of AspD could be observed when pooled enzyme fractions were used directly after elution with buffer B from His-Trap column by FPLC. Pre-incubation of the enzyme for 20 min at 30 °C led to a decrease of approximately 50 % transaminase activity in a subsequently performed aminotransferase assay and pre-incubation at 40 °C led to almost complete loss of activity (data not shown).

Therefore, an effort was done to increase thermostability of AspD. After FPLC one aliquot of the pooled enzyme fractions was supplemented with 0.5 mM PLP, a second aliquot was dialyzed against 75 mM acetate buffer, pH 5.0 containing 0.5 mM PLP and 1 mM α-ketoglutaric acid and the third aliquot remained untreated. Subsequent comparison of decarboxylase activities of the aliquots by TLC showed a strong increase of thermostability when AspD was dialyzed (Figure 32). The untreated enzyme in buffer B showed a decrease of activity after 5 min at 40 °C, after 10 min only weak activity was left and after 20 min no activity could be detected any more. The enzyme fraction in buffer B (FPLC) supplemented with 0.5 mM PLP showed loss of activity after 30 min at 40 °C, after 45 min approximately 50 % of activity was lost and after 60 min only weak activity was detectable. The dialyzed enzyme fraction showed unaltered activity even after 60 min at 40 °C.

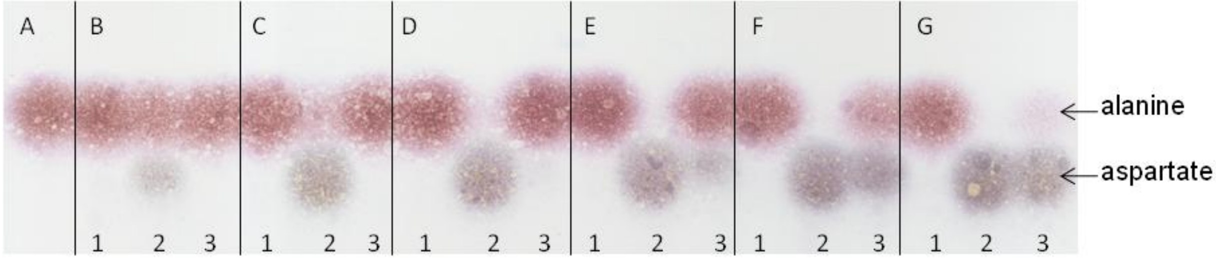


Figure 32: Decarboxylase activity of AspD after incubation in different buffers (1 = dialyzed against 75 mM acetate buffer pH 5.0 containing 0.5 mM PLP and 1 mM a-ketoglutaric acid, 2 = Buffer B (FPLC), 3 = Buffer B (FPLC) supplemented with 0.5 mM PLP at 40 °C for several durations (A = without heat treatment, B = 40 °C for 5 min, C =40 °C for 10 min, D = 40 °C for 20 min, E = 40 °C for 30 min, F = 40 °C for 45 min, G = 40 °C for 60 min). Pink dots display alanine formed from aspartate (purple).

For further experiments the dialyzed enzyme fractions were used. Thermostability analysis of dialyzed enzyme AspD revealed no obvious loss of activity during 60 min incubation at 40 °C. The enzyme showed still considerable activity after 10 min at 50 °C. Longer incubations at 50 °C and for 5 min and longer at 60 °C led to a complete loss of activity (Figure 33).

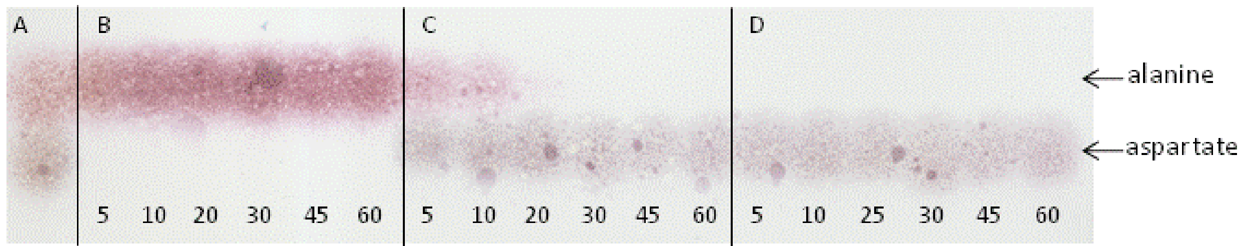
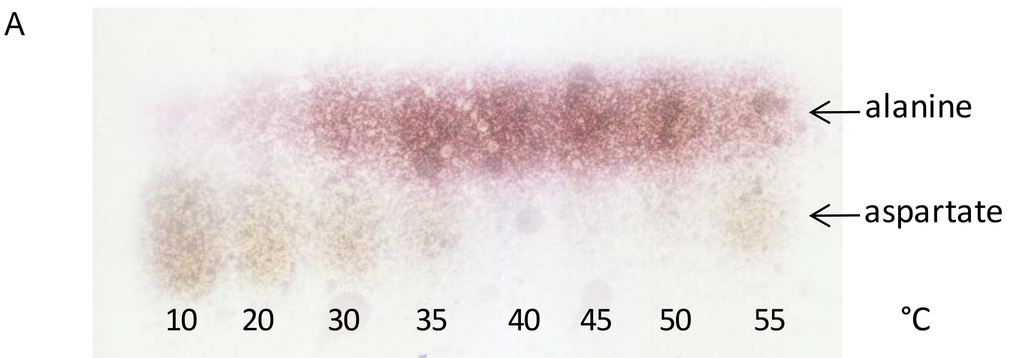


Figure 33: Thermostability of dialyzed AspD. A = standard solution alanine and aspartate (1:1), B = 40 °C, C = 50 °C, D = 60 °C. Pink dots display alanine formed from aspartate (purple).

The optimum temperature for AspD was determined by performing the aminotransferase assay at temperatures between 15 °C and 60 °C. Temperature optimum was found between 35 and 45 °C by TLC and GC-MS analysis (Figure 34).

A



B

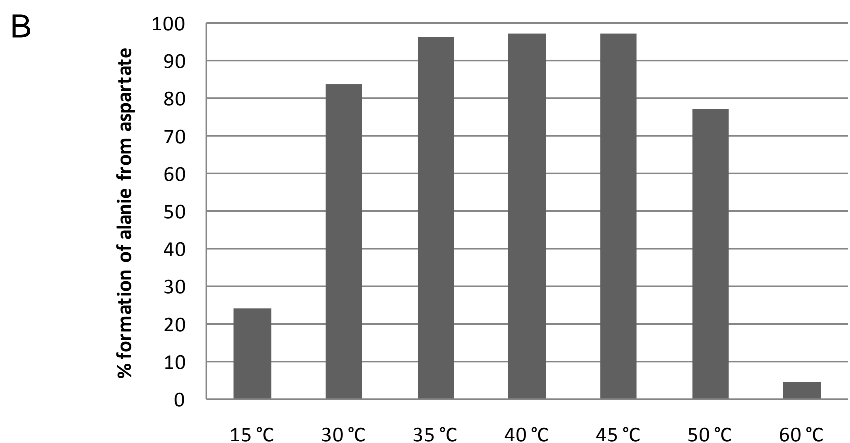


Figure 34: Effect of temperature on AspD activity. A = TLC, B = Analysis by GC-MS [% formation of alanine from aspartate]. Pink dots display alanine formed from aspartate (purple).

To determine the pH-dependence of recombinant AspD, the aminotransferase assay was performed in different buffers.

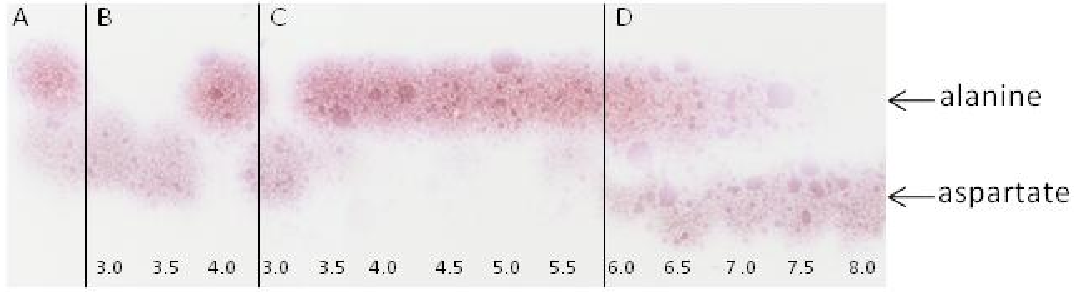


Figure 35: Determination of pH optimum of AspD on TLC plates. A = standard solution alanine and aspartate (1:1), B = citrate buffer (pH 3 – 4.0), C = acetate buffer (pH 3.0 – 5.5), D = phosphate buffer (pH 6.0 – 8.0). Pink dots display alanine formed from aspartate (purple).

pH range between pH 3 and pH 4.0 was performed in citrate buffer, pH range between pH 3 and pH 5.5 in acetate buffer and the range between pH 6 and pH 8 was performed in phosphate buffer. TLC showed the maximum activity in acetate buffer between pH 4 and pH 5.5 (Figure 35).

3.14 Transformation efficiency of *L. sakei* 23K

Two methods for generating competent *L. sakei* 23K cells were compared. The method obtained from Zagorec (Zagorec, 2009) did not result in any erythromycin resistant clones whereas a slightly modified method from our lab revealed transformation rates from 0.5×10^4 transformants per µg DNA when 85 ng DNA were used for transformation to 3.1×10^4 transformants per µg DNA when 170 ng of plasmid-DNA were used for transformations. All colonies picked for plasmid preparation and fluorescence analysis (Figure 36) revealed positive results. For further transformations 85 - 170 ng plasmid-DNA were used.

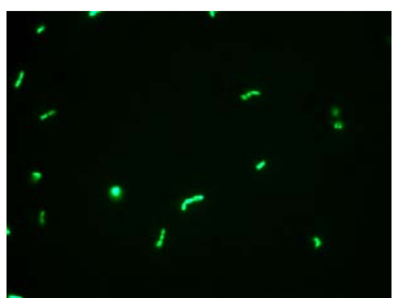


Figure 36: Fluorescence microscopy of *L. sakei* 23K carrying pRV85

3.15 Chromosomal integration of *pldhL::ilvE* in *L. sakei* 23K

The reconstructed integrative vector pRV84 was used to generate integration mutants of *L. sakei* containing *ilvE* genes under control of promoter *pldhL*. Primers for *ilvE*-amplification were designed from published *ilvE* sequences of *L. paracasei* subsp. *paracasei* 8700:2, *E. faecalis* V583 and *S. carnosus* BioCarna Ferment S1 (Wisby Starter Cultures and Media, Niebüll, Germany) and used for PCR reactions with genomic DNA of *L. paracasei* TMW 1.1434, *E. faecalis* V583 and *S. carnosus* TMW 2.801 as templates. Amplificates of 1035 bp (*ilvE* of *E. faecalis*), 1047 bp (*ilvE of L. paracasei*) and 1095 bp (*ilvE* of *S. carnosus*) could be obtained. A lactate dehydrogenase promoter fragment (*pldhL*), 198 bp in size, was also amplified successfully. *pldhL*::*ilvE-X* fusions were obtained by a cross-over PCR that connected the promoter sequence *pldhL* of *L. sakei* with the *ilvE* genes of *L. paracasei*, *E. faecalis* and *S. carnosus* respectively. PCR fusions at the expected sizes of 1288 bp for *pldhl*::*ilvE-Sc*, 1240 bp for *pldhl*::*ilvE-Lp* and 1228 bp for *pldhl*::*ilvE-Ef* were obtained and subsequently cloned into the reconstructed, integrative vector pRV84 to generate recombinant plasmids pRV84-pil-Sc, pRV84-pil-Lp

and pRV84-pil-Ef (pRV84-pil-X). Plasmid DNA of ampicillin resistant DH5α clones was used for sequencing of the *ilvE* genes. Sequencing of *ilvE* genes showed high similarities to the published gene sequences. *IlvE* genes of published and used *E. faecalis* V583 were identical. Sequences of the two *S. carnosus* strains differed in a single nucleotide but this did not lead to an amino acid substitution. *L. paracasei* sequences differed at 13 nucleotide sites but only in a single amino acid. Comparison of the three amplified *ilvE* nucleotide sequences revealed homologies of 46 to 63 %. Amino acid sequences showed similiarities of 35 to 61 % (Figure 37).

```
TMW1.1434       ------------MSVNIDWNNLGFDYMQLPYRYVAHWKD--GAWDEGKLSTDPNLTMNEG 46
V583            -----------MEKANLDWNNLGFSYIKTPFRYISYWRD--GKWEEGTLTDNNQLTISEG 47
TMW2.801        MSEKVKFEKREDLKQKPDPKNLGFGQYFTDYMLSYDYDSEKGGWHDLKITPYAPIELDPA 60
                             . : * :****.     :     : .  * *.: .::    : :. .

TMW1.1434       SPILHYGQGAFEGMKAYRTKSGKIQLFRPDQNAHRLHNSADKLLMPPSPEDRFIDAVKQV 106
V583            SPALHYGQQCFEGLKAYQCADGSVNLFRPDENAKRLQKSCARLLMPQVPVETFVSACQEV 107
TMW2.801        AQGLHYGQLVFEGLKAYKHN-GEVVLFRPDQNFARINQSLDRLEMPQIDEEELLEGLKQL 119
                :  *****  ***:***:   *.: *****:*  *:::*  :* **    : ::.. :::

TMW1.1434       VAANHEYVPPYGTGATLYLRPILIGVGPNIGVAPAKEYIFDVFAMPVGPYFKG-GMVPTK 165
V583            VKANLAYLPPYGTGGTLYLRPYMIGVGDNIGVAPAKEYIFSIFCVPVGSYFKN-GLAPTN 166
TMW2.801        VDVERDWVP-EGEGQSLYIRPFVFATEAGLGVHPAHNYKLLIILSPSGSYYGGDSLKPTR 178
                * .:  ::*  * * :**:** ::..  .:** **::* : ::  * *.*: . .: **.

TMW1.1434       FIVADQFDRAAHYGTGQSKVGGNYAASLQAGKFAHEHGYGDAIYLDPIEHKYIEEVGSAN 225
V583            FIVS-EYDRAAGRGTGAAKVGGNYAASLLPGAEAHEKEFSDCIYLDPYTHTKIEEVGAAN 225
TMW2.801        IYVEDEYVRAVRGGVGFAKVAGNYAASLLSQSNANEQGYDQVLWLDGVERKYIEEVGSMN 238
                : *  :: **.  *.* :**.******* .   *:*: :.: ::**   :. *****: *

TMW1.1434       FFGISKDGKTLKTPKS-PSILPSITKYSILALAHDRFGMTTEETKIAITDLDQFGEAG-- 282
V583            FFGITKDG-TFITPKS-ASILPSITKYSLLTLAKERLGMTALEGDVYIDRLADFSEAG-- 281
TMW2.801        IF-FVENG-KLVTPKLNGSILPGITRKTVIALAKE-LGYEVEERHISIDELLESYDKGEL 295
                :* : ::* .: ***   ****.**: :::**:: :*  . * .: *  * :  : *

TMW1.1434       ----ACGTAAVITPIASITYEDHEHVFYSETKVGPYTQKLYDELTGIQFGDVPAPEGWVV 338
V583            ----ACGTAAIISPIGGIQNGTDFHVFYSETEVGPVTKQLYDELVGIQFGDKEAPEGWIV 337
TMW2.801        EEVFGTGTAAVISPVGTLKYEDRE-ITINNNETGPITQRLYDEYTGIQSGKLDDPQGWRV 354
                    . ****:*:*:. :       :  .:.:.** *::**** .*** *.   *:** *

TMW1.1434       DVPFN 343
V583            KV--- 339
TMW2.801        VVPEY 359
                 *
```

Figure 37: Alignment of IlvE amino acid sequences from *L. paracasei* TMW 1.1434, *E. faecalis* V583 and *S. carnosus* TMW 2.801, introduced into *L. sakei* 23K.

L. sakei was transformed with pRV84-pil-X plasmids for erythromycin resistance. Single crossover integration of pRV84-pil-X was checked by PCR on chromosomal DNA extracted from transformants. One transformant containing a single integrated copy of pRV84-pil-Sc, pRV84-pil-Lp or pRV84-pil-Ef respectively was selected and cultured without erythromycin in order to allow excision of the plasmid by a second crossover, leading either to gene replacement or to the wild-type genotype. Every 24 h the single crossover transformants were transferred into fresh mMRS 15G medium and diluted culture aliquots were plated on mMRS 15G medium containing X-Gal. During two weeks of cultivation and plating, almost all colonies grown were blue, suggesting that a second

crossover had occurred and led to the wild-type genotype. Only one colony was white (Figure 38), suggesting that the *lacLM* operon was replaced by the *pldhL*::*ilvE-Sc* fusion. As expected, this clone, designated as *L. sakei* 23K-pil-Sc, was erythromycin sensitive. Chromosomal DNA from *L. sakei* 23K-pil-Sc was extracted and the structure of the integrated *pldhL*::*ilvE-Sc* fragment was verified by PCR. As expected, the *lacLM* operon had been replaced by the *pldhL*::*ilvE-Sc* fusion.

Figure 38: *L. sakei* 23K single crossover mutant with integrated pRV84-pil-Sc (blue) and double crossover mutant *L. sakei* 23K-ilvE-Sc with replaced *lacZ* gene (white).

3.16 Construction of replicative vectors for the expression of *ilvE* and *mCherry*

pMG36e and pG+host5 were used to introduce *ilvE* and *mCherry* into *L. sakei* on replicative plasmids. Both genes were under control of the constitutive *L. sakei* promoter of the lactate dehydrogenase (*pldhL*). As for the construction of pRV84-constructs, *ilvE* genes of *L. paracasei* TMW 1.1434, *E. faecalis* V583 and *S. carnosus* TMW 2.801 were amplified with primers designed from published *ilvE* sequences of *L. paracasei* subsp. *paracasei* 8700:2, *E. faecalis* V583 and *S. carnosus* BioCarna Ferment S1 (Wisby Starter Cultures and Media, Niebüll, Germany) and PCR products with the expected sizes were obtained as well as a PCR product for *pldhL* (198 bp). Additionally a *mCherry* sequence with 752 bp was amplified successfully from pBlueScript-mcherry as template. *pldhL::ilvE-X::mcherry* (*pilm-X*) fusions were obtained by two consecutive cross-over PCR reactions that connected the promoter sequence *pldhL* of *L. sakei*, the *ilvE* genes of *L. paracasei*, *E. faecalis* or *S. carnosus* respectively and *mCherry* of pBlueScript-mcherry. These resulted in PCR fusions in size of 2045 bp for *pilm-Sc*, 1997 bp for *pilm-Lp* and 1985 bp for *pilm-Ef* which were subsequently cloned into the shuttle vectors pMG36e and pG+host5.

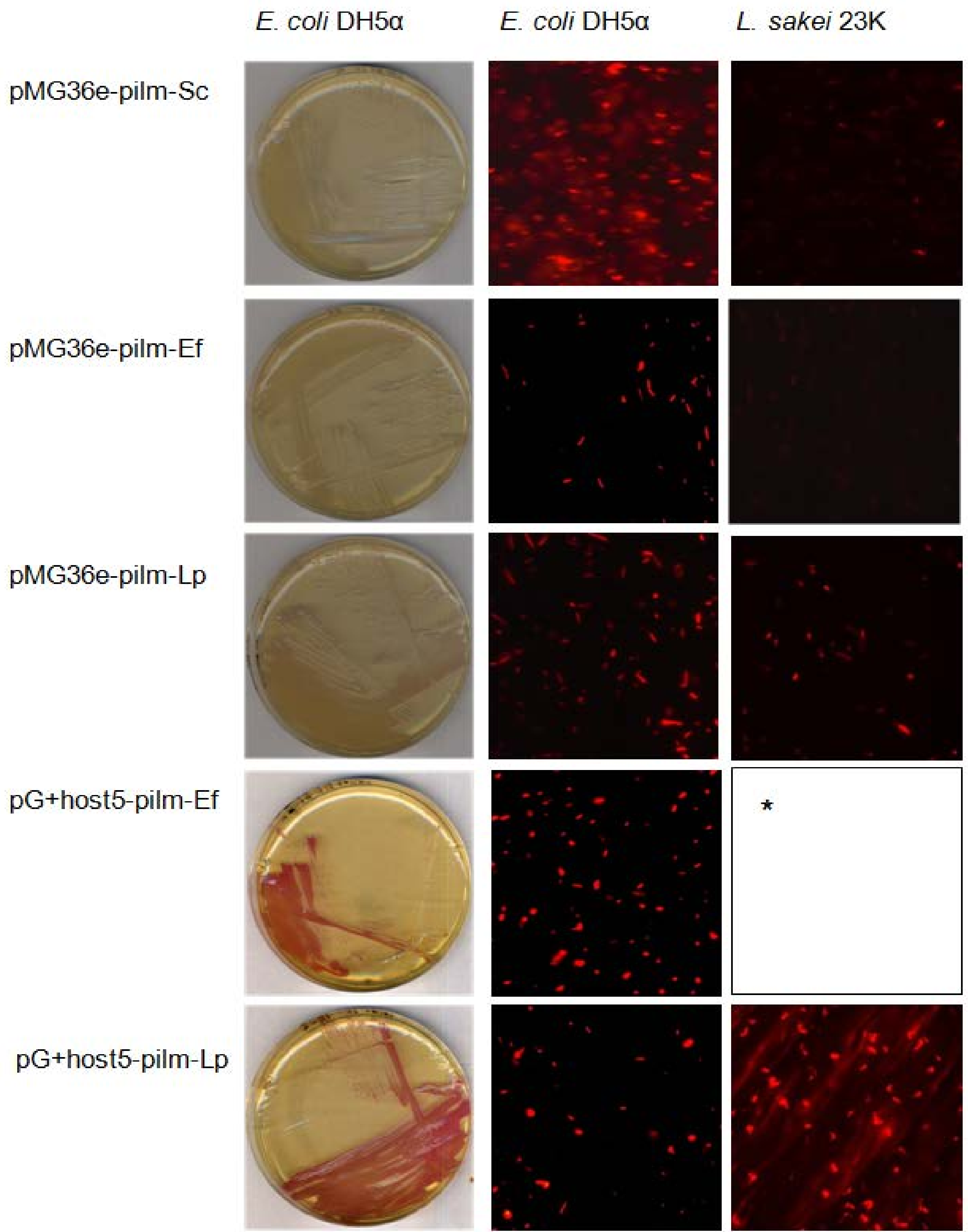


Figure 39: First column: *MCherry*-carrying *E. coli* DH5α recombinants streaked on LB plates (150 µg/ml erm). Second column: Fluorescence microscopy of *mCherry*-carrying *E. coli* DH5α recombinants; third column: Fluorescence microscopy of *mCherry*-carrying *L. sakei* 23K (* Transformation in *L. sakei* was not successful). Constructs, cells were transformed with, are shown on the left.

Ligation products were introduced into DH5α for subcloning. Colonies that were able to grow on LB plates with erythromycin (150 µg/ml) carrying pG+host5-pilm-Lp and pG+host5-pilm-Ef as well as pMG36-pilm-Sc, pMG36e-pilm-Ef and pMG36e-pilm-Lp

appeared pink (Figure 39 first column) and cells were bright red fluorescent as observed by fluorescence microscopy (Figure 39 second column). The colour intensity of the colonies varied significantly. Plasmid DNAs of positive *E. coli* DHα clones were sequenced to ensure PCR fidelity. Sequencing data revealed for all five plasmids two intact open reading frames, one for *mCherry*, one for the respective *ilvE* genes, both under control of *pldhl*. *L. sakei* 23K was transformed with the replicative plasmid-constructs pG+host5-pilm-Ef, pG+host5-pilm-Lp and pMG36e-pilmX for erythromycin resistance. Colonies able to grow on MRS G15 plates (containing 5 µg/ml erm) carried pG+host5-pilm-Lp, pMG36e-pilm-Sc, pMG36e-pilm-Ef or pMG36e-pilm-Lp respectively. Transformation of *L. sakei* 23K with pG+host5-pilm-Ef was not successful. *L. sakei* colonies carrying any of the constructs appeared white. Observation of recombinant cells by fluorescence microscopy showed slight red fluorescence (Figure 39 third column). Bleaching effects could be observed within seconds. All four plasmids could be re-isolated from *L. sakei* and their structures were verified by restriction analysis.

3.17 Aminotransferase acitivity

Cell free extracts of *L. sakei* 23K wildtype and recombinant *L. sakei* strains carrying different *ilvE*-constructs were obtained from overnight cultures. Determination of protein contents by Bradford microtiter plate assay revealed similar protein concentrations in the CFEs, suggesting that cell disruption was similarly successful for all cultures. After incubation for one hour in the aminotransferase reaction mix, enzyme reaction was stopped by heating and aminotransferase activity was determined by the L-Glutamic acid kit (Boehringer, Mannheim). Specific activities were calculated as micromoles of formazan (L-glutamate) formed per min and per mg of protein (µmol/min/mg of protein) (Figure 40, Table 11). In general, a significant increase of specific aminotransferase activity against all branched-chain amino acids could be revealed for each of the recombinant *L. sakei* strains. Relative activities of recombinant strains in comparison to the wild type strain (relative activity = 1) are shown in Figure 41. CFE of *L. sakei* strain carrying pMG36e-pilm-Lp showed a 25-fold higher transaminase activity compared to the wildtype strain and CFE of strains with pM36e-pilm-Ef and pM36e-pilm-Sc still a 15 and 13-fold increase respectively.

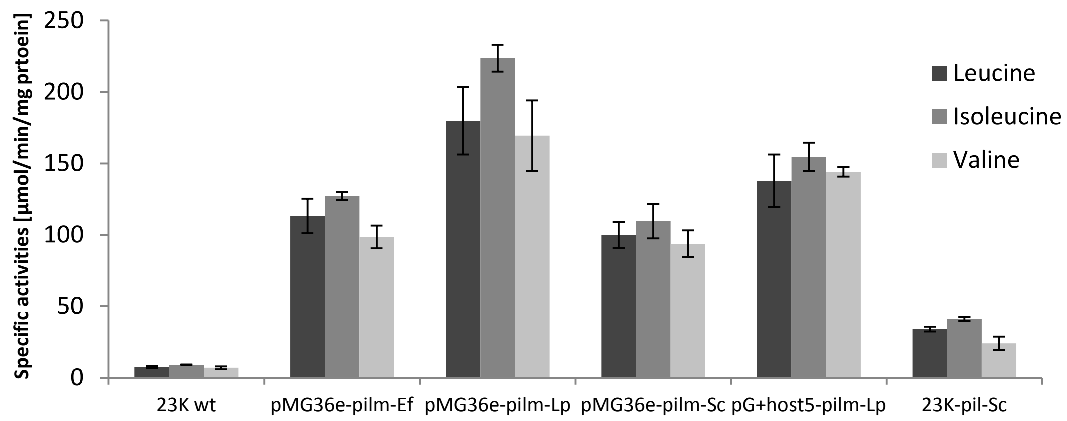


Figure 40: Specific aminotransferase acitivites of CFEs from *L. sakei* 23K wildtype and *ilvE*-carrying recombinants. Substrates were L-leucine, L-isoleucine and L-valine.

Transaminase activity of the recombinant strain with pG+host5-pilm-Lp was nearly 20 fold higher as that of *L. sakei* wildtype and CFE of *L. sakei* 23K-ilvE-Sc carrying an intragenomic *ilvE* gene revealed at least about 5-fold higher activities than the wild type strain.

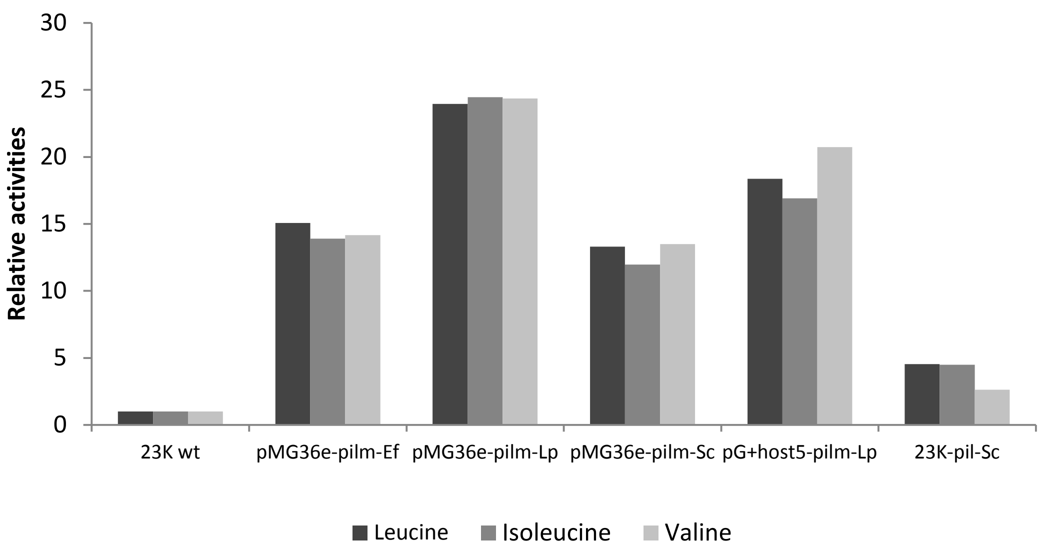


Figure 41: Relative activites of *L. sakei* 23K and *ilvE*-carrying recombinants. Activities of *L. sakei* 23K are considered as 1.

All recombinant strains showed significant transaminase activities against all branched-chain amino acids and only minor differences in substrate specificities towards L-leucine, L-isoleucine and L-valine could be detected. The specific activity of IlvEs against L-isoleucine was always highest. Activity against branched-chain amino acid

substrates was mostly in descending order: Ile > Leu > Val. Relative substrate activities are shown in Table 11.

Table 11: Specific activities and relative substrate specificities of *L. sakei* 23K wildtype and *ilvE*-carrying recombinants.

	Specific activity [µmol/min/mg]			Relative substrate activity [%]		
Organism	Ile	Leu	Val	Ile	Leu	Val
23 K wildtype	9.1 ± 0.24	7.5 ± 0.6	7.0 ± 1	100	82	76
23 K pMG36e-pilmEf	172.2 ± 2.8	113.1 ± 12.1	98.6 ± 7.9	100	89	78
23 K pMG36e-pilm-Lp	223.7 ± 9.4	179.8 ± 23.6	166.4 ± 24.6	100	80	76
23 K pMG36e-pilm-Sc	109.6 ± 12.1	99.9 ± 9.1	93.8 ± 9.3	100	91	86
23 K pG+host5-pilm-Lp	154.7 ± 9.8	137.9 ± 18.3	144.2 ± 3.36	100	89	93
23 K-pil-Sc	41.2 ± 1.5	34.1 ± 1.7	24.1 ± 4.7	100	83	49

Considering the amount of glutamic acid (is equivalent to the amount of formazan formed) in reaction vials formed from α-keto glutaric acid during transaminase reaction, an increase of glutamic acid could be detected when reaction time was prolonged from 1 h to 12 h. This applies to the wildtype strain *L. sakei* 23K and all recombinant strains with one exception. *L. sakei* 23K pMG36-pilm-Lp has already reached the highest concentration of glutamic acid during the first hour of reaction. A further extension of reaction time to 24 h did not lead to a general increase of glutamic acid concentrations. Only in the reaction mix with CFEs of *L. sakei* 23K wildtype and *L. sakei* 23K ilvE-Sc a slight further increase of glutamic acid accumulation could be detected. In contrast, glutamic acid concentrations in enzyme reaction mix with CFEs of all other strains stagnated or even decreased slightly when incubated for 24 h (Figure 42).

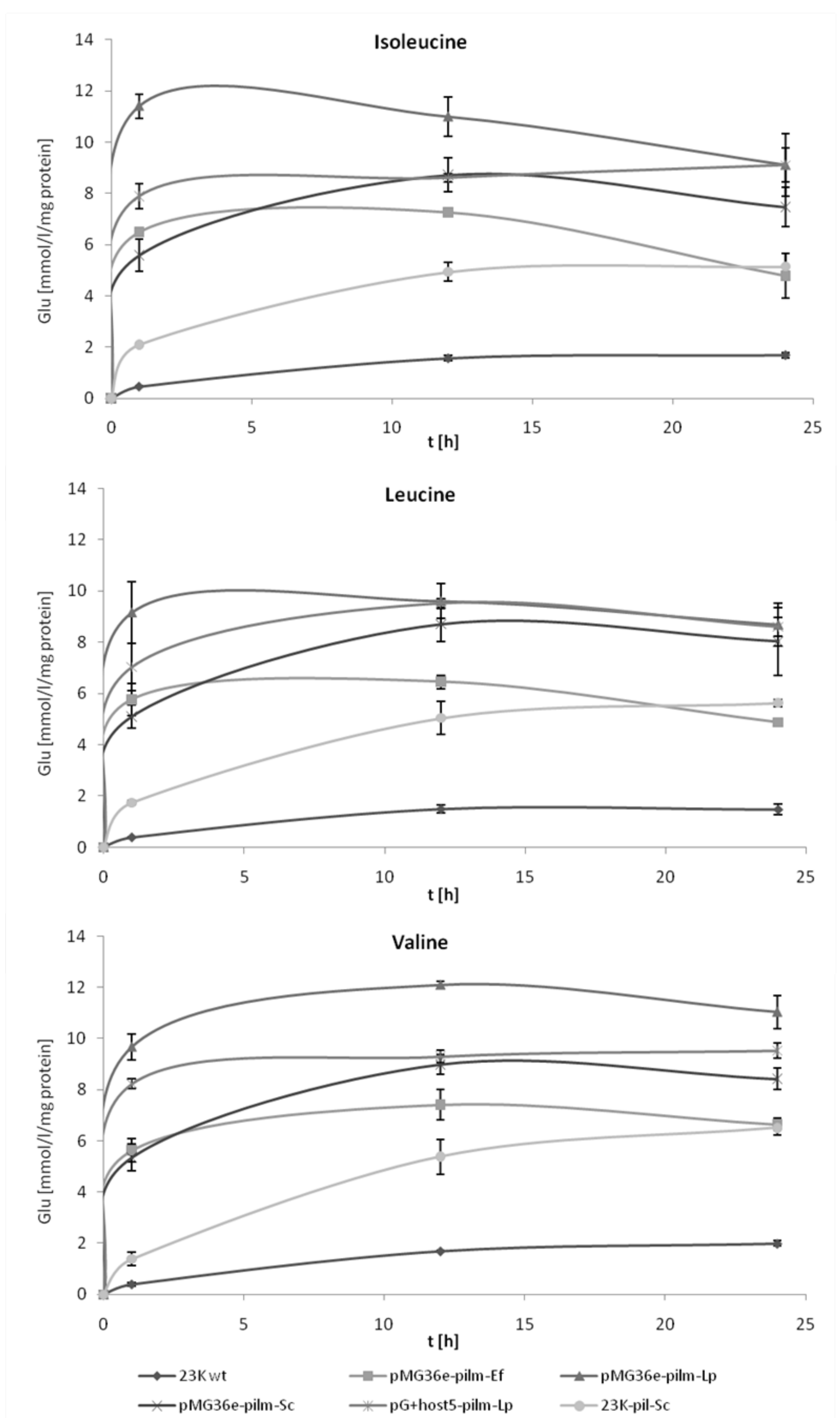


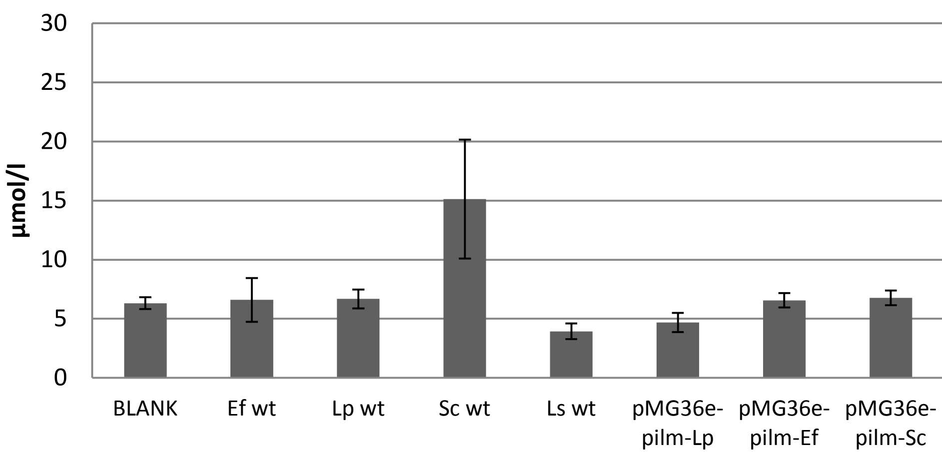
Figure 42: Time course of L-glutamic acid formation by aminotransferase activity of *L. sakei* 23K wildtype (23K wt) and recombinant *L. sakei* strains with isoleucine (top), leucine (middle) and valine (bottom) as substrates.

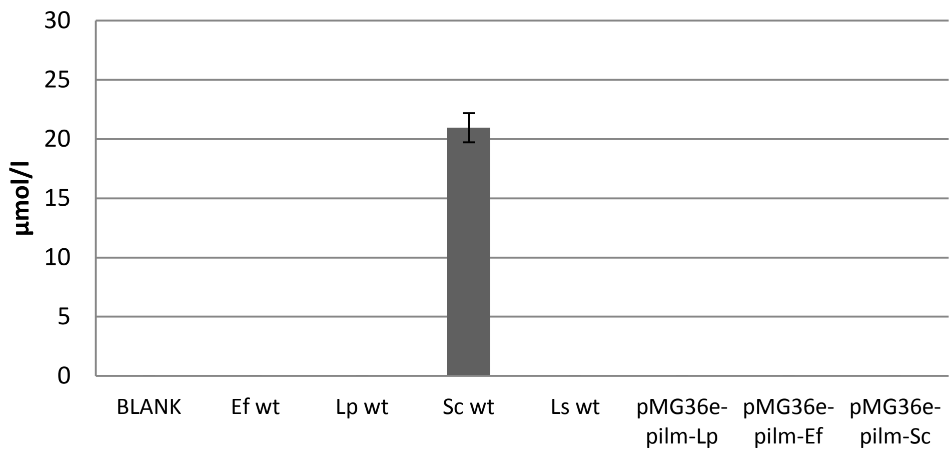
3.18 Formation of volatiles from Leucine and Valine

Cells of wildtype strains *L. sakei* 23K, *E. faecalis* V583, *L. paracasei* TMW 1.1434 and *S. carnosus* TMW 2.801 and cells of recombinant strains *L. sakei* 23K carrying pMG36e-pilm-X were washed and subsequently incubated for five days in a buffer system with α-ketoglutaric acid, PLP and L-leucine or L-valine respectively. Subsequently, volatiles formed from L-leucine (3-methylbutanal, 3-methylbutanol and 3-methylbutanoic acid) and L-valine (2-methylbutanal, 2-methylbutanol and 2-methylpropanoic acid) were measured by SPME-GC/MS analysis.

SPME-GC/MS analysis allowed detection of 3-methylbutanal, 3-methylbutanol and 3-methylbutyric acid, volatiles derived from L-leucine (Figure 43) and 2-methylpropanal and 2-methylpropanoic acid derived from L-valine (Figure 44). Furthermore, low amounts of few further volatiles, like acetic acid, could be detected, but since they are not related to amino acid metabolism, they were not further mentioned. Generally, amounts of carboxylic acids were about 1000-fold higher than amounts of corresponding aldehydes and alcohols. Blank vials already contained a significant amount of the respective aldehyde, however no alcohol or carboxylic acid derived from L-leucine or L-valine could be detected in blanks. In L-leucine containing blank vials 3-methylbutanal could be determined in concentrations of about 6 µmol/l which is approximately as high as in vials supplemented with *E. faecalis, L. paracasei* and *L. sakei* wildtype and recombinant *L. sakei* 23K cells (carrying pMG36e-pilm-X). Only in vials with *S. carnosus* TMW 2.801 cells, significant higher 3-methylbutanal concentrations could be detected compared to the blank vial. 15 µmol/l 3-methylbutanal corresponded to a 3-fold increase of the basic aldehyde content in the buffer solution. The corresponding alcohol 3-methylbutanol was only formed by *S. carnosus* TMW 2.801 and could be detected in a concentration of about 21 µmol/l. 3-methylbutyric acid concentration was again highest in vials with *S. carnosus* TMW 2.801, where nearly 25 mmol/l could be measured. Concentrations of 9 to 13 mmol/l were formed by *L. sakei* 23K wildtype and the recombinant strains carrying pMG36e-pilm-X. Less than 5 µmol/l were formed by *L. paracasei* TMW 1.1434 and no 3-methylbutyric acid could be detected in vials with *E. faecalis* V583 cells.

Generally, the amount of volatiles derived from L-valine was at least 10-fold lower, compared to volatiles formed from L-leucine. 2-methylpropanal content in blank vials was about 0.7 µmol/l.





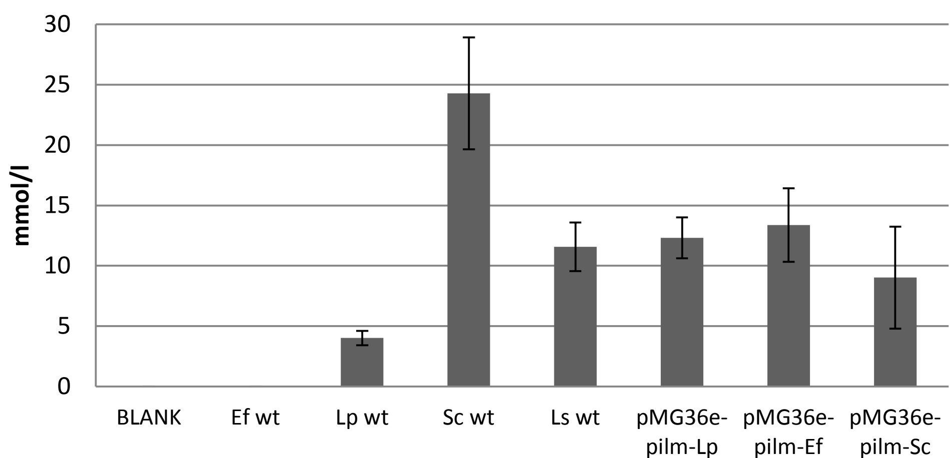


Figure 43: Volatiles derived from L-leucine. BLANK = vial without cells; Ef/Lp/Sc wt= vials with wildtype cells of *E. faecalis* V583, *L. paracasei* TMW 1.1434, *S. carnosus* TMW 2.801 or *L. sakei* 23K; pMG36e-pilm-Lp/Ef/Sc = vials with recombinant *L. sakei* 23K cells carrying the respective pMG36e-pilmX construct.

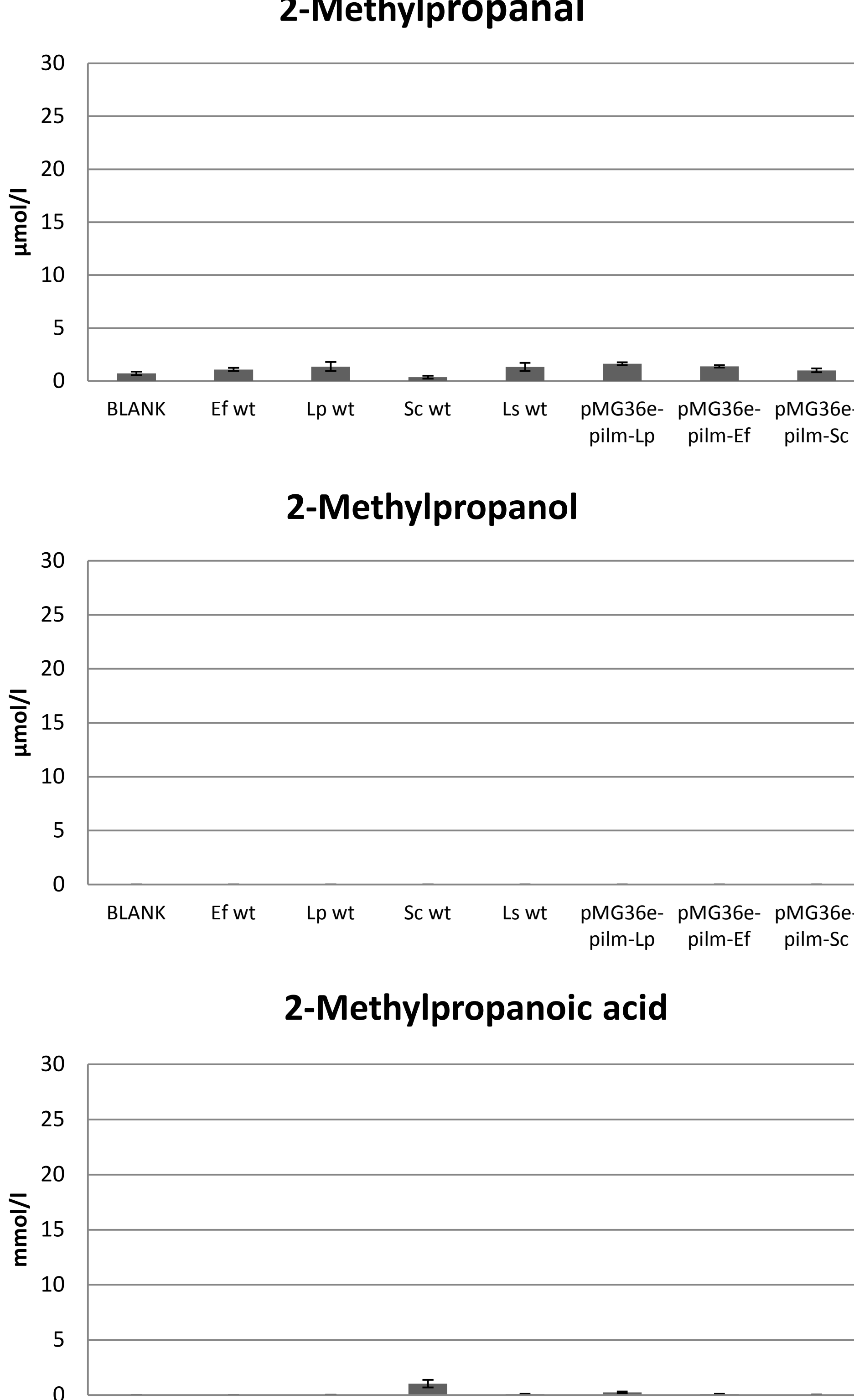


Figure 44: Volatiles derived from L-valine. BLANK = vial without cells; Ef/Lp/Sc wt= vials with wildtype cells of *E. faecalis* V583, *L. paracasei* TMW 1.1434, *S. carnosus* TMW 2.801 or *L. sakei* 23K; pMG36e-pilm-Lp/Ef/Sc = vials with recombinant *L. sakei* 23K cells carrying the respective pMG36e-pilmX construct.

Vials with wildtype strains *E. faecalis* V583, *L. paracasei* TMW 1.1434 and *L. sakei* 23K, as well as recombinant *L. sakei* 23K strains with pMG36e-pilm-X showed a slight increase of 2-methylpropanal concentrations (0.4 - 1.2 µmol/l) whereas *S. carnosus* TMW 2.801 showed just half of the concentration present in the blank vial. 2-methylpropanol could not be detected in any of the vials. The highest concentration of 2-methylpropanoic acid could be determined in vials with *S. carnosus* TMW 2.801, which produced about 1 mmol/l. An approximately 5 to 10-fold lower concentration could be detected in vials with *L. sakei* strains. *L. paracasei* produced even less 2-methylpropanoic acid compared to *L. sakei* strains and no formation of this carboxylic acid could be detected in vials with *E. faecalis* V583.

4 Discussion

4.1 Strain typing by RAPD analysis

Molecular techniques with high discriminatory power are essential to distinguish between bacterial isolates. In this study *L. sakei* and *L. curvatus* strains were analyzed by RAPD (random amplification of polymorphic DNA) analysis, reported as one of the most discriminatory techniques applied in strain typing (Speijer *et al.*, 1999). RAPD analysis uses low-stringency hybridization conditions with a single random oligonucleotide primer. As shown before (Berthier and Ehrlich, 1999) RAPD analysis allowed a clear separation of *L. sakei* and *L curvatus*. A single strain, previously affiliated with *L. sakei*, clustered separately from all other strains. 16S rDNA analysis of this strain identified it as *L. pentosus/L. plantarum* species. Two other strains, formerly designated as *L. sakei* TMW 1.166 and *L. sakei* TMW 1.158 grouped clearly with *L. curvatus* strains and sequencing of their 16S rDNA revealed them as *L. curvatus* species. For these isolates it is not known whether the cultures we received were named incorrectly or whether they became contaminated during storage and cultivation in our lab. They were consequently renamed as *L. curvatus* TMW 1.166, *L. curvatus* TMW 1.158 and *L. pentosus*/*L. plantarum* TMW 1.1394.

Earlier studies have demonstrated that the application of RAPD could discriminate two genetic groups within *L. sakei* (Berthier and Ehrlich, 1999; Klein *et al.*, 1996; McLeod *et al.*, 2008; Torriani *et al.*, 1996). The present study confirmed this, since clustering of RAPD patterns revealed two major groups. Strains TMW 1.1239 and TMW 1.1240 grouped separately from remaining *L. sakei* strains and could be defined as one genetic group. Consistent with a former clustering (Berthier and Ehrlich, 1999) the major *L. sakei* group contained strains TMW 1.1189 (DSM 20017) TMW *L. sakei* 1.147 (CTC 335), 1.402 (LTH 2068), and 1.22 (LTH 677). Consequently, this major group may be described as *L. sakei* subsp. *sakei* as it contains the type strain DSM 20017 and the minor group as *L. sakei* subsp. *carnosus*. Due to the fact that the type strain for the *L. sakei* subsp. *carnosus* subspecies (CCUG41580) was not available for these experiments, this classification remains to a certain degree speculative. Since strains *L. sakei* TMW 1.1239 and TMW 1.1240 were the only isolates from sourdough whereas all other isolates of the present and former studies were obtained from meat or meat products, sake, sauerkraut and fish products (Berthier and Ehrlich, 1999; Klein *et al.*, 1996; McLeod *et al.*, 2008; Torriani *et al.*, 1996). One could moreover speculate that they possibly form a further, up to date not described, subgroup of *L. sakei*. Therefore, the separate clustering could be caused by genetic differences of those two strains gained by the unusual ecological niche

the *L. sakei* isolates originated from. However, further genetic and phenotypic investigations would be necessary to substantiate this assumption. According to Torriani *et al.* (Torriani *et al.*, 1996), two subgroups within *L. curvatus* and *L. sakei* were revealed, with one *L curvatus* sub-group more closely linked to one of the *L. sakei* than to the other *L. curvatus* group. In this study, as well as reported by Berthier *et al.* (Berthier and Ehrlich, 1999), *L. curvatus* pattern could be clearly separated from *L. sakei*. However, two distinct groups have been formed within *L. curvatus* (C+D). In accordance to subgroup A1 (Berthier and Ehrlich, 1999), the *L. curvatus* type strain TMW 1.7 (DSM 20019) and strain TMW 1.401 (LTH 2053) clustered together in group C, depicted in Figure 16. Taken together, RAPD analysis allows separation of *L. sakei* and *L. curvatus* species from each other and from other species and clustering of subgroups within the species was largely in accordance with former studies.

4.2 Amino acid requirements of *L. sakei* and *L. curvatus* strains

Meat is a protein-rich substrate and the protein fraction in meat accounts for 20 - 25 %. Therefore it constitutes the second major component in meat after water (Ebermann R., 2008). *L. curvatus* and *L. sakei* has shown to exhibit only low proteolytic activities against meat proteins (Sanz *et al.*, 1999) and in general, bacterial enzymes have been reported to have a minor participation in initial breakdown of meat proteins while the later decomposition of peptides into free amino acids by peptidases is due to bacterial enzymes (Molly *et al.*, 1997; Verplaetse, 1994). Consistent with life in such an environment, *L. sakei* is auxotrophic for most amino acids. A chemically defined medium suitable for various *L. sakei* strains has been described by Lauret *et al.* and must contain all proteinogenic amino acids, except glutamic acid and aspartic acid (Lauret *et al.*, 1996). This is consistent with results of this present study where 20 *L. sakei* strains could be cultivated in a chemically defined medium originally developed for *Lactobacillus delbrueckii* subsp. *lactis* without L-glutamic acid or L-aspartic acid (Hebert *et al.*, 2004). Genome analysis by Chaillou *et al.* has indicated that these two amino acids could potentially be produced by deamination of asparagines and glutamine respectively (Chaillou *et al.*, 2005). Two genes, *asnA1* and *asnA2* are coding for a L-asparaginase [EC: 3.5.1.1] that converts L-asparagine and water to L-aspartate and NH_3 (Youssef and Al-Omair, 2008). However, database search did not reveal a L-glutaminase gene in *L. sakei* that could, analogous to L-asparaginase form L-glutamate from L-glutamine. However, another possible reaction for L-glutamate formation and at the same time synthesis of L-asparagine could be catalyzed by an asparagine synthetase [EC: 6.3.5.4] (glutamine-hydrolyzing) encoded by *asn.* Asparagine synthetase catalyzes the synthesis of L-asparagine from L-aspartic acid, in an ATP dependent reaction for which the nitrogen

source can be either L-glutamine or free ammonia (Boehlein *et al.*, 2001). When L-glutamine is utilized, L-glutamate will be released. Moreover glutamate-ammonia ligase [EC: 6.3.1.2] encoded by *glnA* catalyzes the ATP driven conversion of L-glutamate and NH_3 to L-glutamine and H_2O (Rhee and Chock, 1976). Most of the *L. sakei* strains were also able to grow without L-glutamine, probably due to glutamate-ammonia ligase activity. Only strains *L. sakei* TMW 1.1393 and TMW 1.1397 were auxotrophic for L-glutamine. Furthermore, three strains, TMW 1.22, TMW 1.412 and TMW 1.1474 were prototrophic for L-asparagine. Strain dependence of absence or presence of *glnA, asnA1*, *asnA2* and *asnB* is unlikely as an explanation for different amino acid requirements, since PCR screenings revealed the presence of all four genes in all *L. sakei* strains tested. Moreover, mutations within *glnA*, that could lead to a non-functional enzyme are unlikely, since sequence comparison of *glnA* from strains TMW 1.1397 and TMW 1.1393, auxotrophic for L-glutamine, and a subset of strains, prototrophic for that amino acid, revealed none or only minor differences. However, mutations within *asnA1*, *asnA2* and *asnB* genes cannot be excluded. By means of the results from these experiments, the cause of different amino acid requirements could not be clarified. One could suggest that specific regulatory mechanisms could differ withinin the strains. Strains TMW 1.22, TMW 1.412 and TMW 1.1474, auxotrophic for L-asparagine, possibly possess an aspartate-ammonia ligase which catalyzes the conversion of L-aspartate to L-asparagine analogous to a glutamate-ammonia ligase.

Earlier, strain *L. sakei* 23, from which *L. sakei* 23K was obtained by plasmid curing, was described as auxotrophic for L-glutamine (Lauret *et al.*, 1996) but in this present study *L. sakei* 23K as well as *L sakei* TMW 1.148 were able to grow without L-glutamine. However, the lag phase was much longer compared to the other strains and lasted roughly 10 h. This discrepancy can maybe be attributed to the use of different chemically defined media as it is known that the relative concentrations of amino acids may affect growth rates (Poolman and Konings, 1988). It is also sometimes difficult to distinguish between poor growth and no growth. For instance, it has been observed that arginine and methionine highly stimulate the growth of 36 *L. lactis* subsp. *lactis* strains while their omission still allows growth at a very low rate, indicating that *L. lactis* subsp, *lactis* possesses the genes for the biosynthesis of these amino acids (Chopin, 1993). Furthermore, the involvement of regulatory mechanisms in amino acid requirements has also been demonstrated in *L. lactis* subsp. *lactis*. It has been observed that the capacity of *L. lactis* IL1403 to grow in the absence of glutamine was affected by the ammonium ion concentration in the medium (Chopin, 1993). This is consistent with the observation that in *E. coli* expression of enzymes for interconversion of glutamate and glutamine is regulated by the balance between ammonium, glutamate and glutamine concentrations (Reitzer,

1987). This might explain the discrepancies in the reported requirements for glutamine or glutamate for lactobacilli.

The amino acid requirements of the *L. curvatus* strains tested in this study differed slightly from those of *L. sakei* strains. As in the case of *L. sakei*, all strains were auxotrophic for L-aspartate and L-glutamate, but they could also grow without L-alanine. If this difference between the species could be confirmed in an extended screening, it could possibly be used as a further distinctive feature for the phenotypical closely related species as they typically only differ in the fermentation of a few carbohydrates (Kandler, 1986; Klein *et al.*, 1996) and hydrolysis of arginine (Berthier and Ehrlich, 1999).

4.3 Proteolytic system of *L. sakei* and *L. curvatus* is genetically homogenous

Within the genome sequenced LAB cell-wall bound proteinases (PrtP) have only been found on the chromosomes of dairy LAB, e. g. *L. acidophilus, L johnsonii, L. bulgaricus, L. casei, L rhamnosus and S. thermophilus strain* LMD9 (Liu *et al.*, 2010). Extracellular proteases seem to be ubiquitous in dairy *lactococci* and *lactobacilli* to initiate the degradation of milk caseins into oligopeptides (Kunji *et al.*, 1996). Due to low amounts of free amino acids and peptides and the absence of proteolytic activities in the milk, dairy LAB are dependent on a proteolytic system that allows degradation of milk proteins (caseins) (Mills & Thomas, 1981; Juillard et al., 1995b). In this study all *L. curvatus* and *L. sakei* strains tested formed weak degradation halos of casein in an agar plate assay (mMRS with 10 % skim milk), but no significant strain-dependence in proteolytic activity was detectable (data not shown). An earlier study confirmed this weak protein hydrolysis potential of *L. curvatus* and one *L. sakei* in an approach using muscle myofibrillar proteins instead of casein (Sanz *et al.*, 1999). Bacterial enzymes have been reported to have only a minor participation in protein breakdown in meat products and the initial degradation of myosin and actin into peptides is due to cathepsin D, while the later decomposition of peptides into free amino acids by peptidases is due to bacterial enzymes (Molly *et al.*, 1997; Verplaetse, 1994). This is further supported by investigations of Fadda *et al.* (Fadda *et al.*, 2010) that demonstrated a maximum expression level of two peptidases when sarcoplasmic protein hydrolysis due to muscle endogenous enzymes was high. Present results support this suggestion. Peptide and amino acid transport systems have been studied extensively in lactococci. Peptide uptake occurs via oligopeptide transport systems (Opp, Opt) and di-/tri-peptide transporters (DtpT, DtpP) (reviewed by Kunji *et al.*1996). However, much less is known about peptide and amino acid transporters in lactobacilli so far.

Oligopeptide transport systems of sequenced LAB were listed by Liu *et al*. (2010). A di/tripeptide ionlinked transporter (Dtpt), one Di/Ttripeptide ABC transport system (DppA/PBCDF) (Liu *et al.*, 2010) which was originally annotated as oligopeptide ABC transport system (*oppABCDF*) and a putative oligopeptide transporter (Puopt) (Chaillou *et al.*, 2005) were present in all *L. sakei* strains tested. In the same way all *L. curvatus* strains showed amplificates for *dtpt* and *puopt*. However, it was not possible to get any amplificate for *oppB* for any of the *L. curvatus* strains. Since primers were primarily designed for the *L. sakei* screening, lacking amplificates for *oppB* could be explained by non-matching primers. That is further supported by the fact, that PCR reactions with primers oppA-forward and oppC-deg-rev showed amplificates with the expected size of 2761 bp for all *L. curvatus* strains. Moreover, nearly all *L. curvatus* strains showed positive results for the other *opp* subunits *oppACDF* and it seems most unlikely that *L. curvatus* lost only one of the *opp* subunits.

Subsequently to proteolysis in the meat matrix and peptide uptake by respective transporters, degradation of peptides to free amino acids was described to be mainly performed by bacterial peptidases (Molly *et al.*, 1997; Verplaetse, 1994). The number of peptidases within the genome sequenced LAB varies between 12 peptidases in *Streptococcus thermophilus* and 27 peptidases in *L. helveticus. L. sakei* possess 19 peptidases and represents therefore approximately the average (Chaillou *et al.*, 2005; Liu *et al.*, 2010). The distribution of peptidases among the tested *L. sakei* strains appeared to be very homogenous. Nearly all strains possessed all peptidases, whereas only a few strains were lacking a single peptidase. Many of the peptidases, like PepC2, PepN, and PepM, and proline peptidases PepX and PepQ, seem to be essential for bacterial growth or survival, since they are encoded on all LAB genomes (Liu *et al.*, 2010). Results of the present study suggest that distribution of peptidases is in general not strain-dependent in *L. sakei*. Nevertheless, slight differences in the distribution of *pepC1*, *pepD3* and *pepD4* within the strains could be found.

Earlier, five major PepD subfamilies (PepD1-D5) have been be clearly distinguished based on a multiple sequence alignment and PepD1-4 are assigned according to the four *pepD* genes from *L. helveticus* (Smeianov *et al.*, 2007). Due to the lack of experimental evidence, it is still unclear whether the substrate specificities vary between those subfamilies. But if one suppose that substrate specificity correlates with the classification of the PepD subfamilies, the absence of PepD4 in some *L. sakei* strains should not be of great relevance for their physiology, since pepD1 and pepD4 belong to the same subfamily PepD1 (Liu *et al.*, 2010) and could therefore be redundant.

Distribution of peptidase genes among *L. curvatus* strains appears for many of them to be similar to the situation in *L. sakei*. *PepV, pepV2*, *pepT, pepM* and *pepD1-D4* could be

detected for all *L. curvatus* strains tested. For *pepN, peps, pepC2* and *pepR,* strain dependence could be presumed and *pepO, pepD4, pepD5, pepF1, pepF2* and *pepQ* could be only found in single *L. curvatus* strains. One explanation would be the absence of these peptidases in *L. curvatus.* As only PepR was purified and characterized for *L. curvatus* (Magboul and McSweeney, 1999) very little is known about their physiological requirement in this species and this option cannot be further discussed. On the other hand, primers specifically designed for *L. sakei* may just not have matched the respective *L. curvatus* genes. Therefore, results for PCR screenings among *L. curvatus* strains should be treated with caution.

4.4 Bitter flavour due to hydrophobic peptides

For some *L. sakei* strains used in this study sensory information was available (Table 9). When strain *L. sakei* TMW 1.1388 or TMW 1.1398 was used in sausage fermentations, the resulting product had a bitter flavour, whereas application of strain TMW 1.1392 or TMW 1.1399 led to sausages with a mild flavour. In cheese, proline-containing peptides are responsible for bitterness (Singh *et al.*, 2003) and peptidases, degrading these peptides, are important for avoiding bitterness (Baankreis, 1991; Lloyd and Pritchard, 1991; Shao *et al.*, 1997; Tsakalidou *et al.*, 1998). Sequence analysis of *pepQ, pepR* and *pepX* of the “mild” or “bitter” *L. sakei* strains was performed but did not reveal any mutations that would lead to a frameshift mutation or to stop codons which consequently would have resulted in non-functional enzymes. Moreover, sequence comparisons of strains leading to mild or bitter taste respectively resulted only in minor differences, suggesting that enzymes could have similar activities. Based on this, enzyme activities of PepX, PepQ and the so called bitter-taste remover PepQ are probably not responsible for the sensory discrepancies of sausages fermented with “mild” or “bitter” *L. sakei* strains. Therefore, sausage fermentation and subsequent sensory evaluation should be repeated with these strains and possible microbial contaminations that could have led to those off-flavours should be excluded.

4.5 Distribution of biogenic amine (BA) forming potential

The screening for presence of decarboxylase genes and for the ability of BA formation showed, that neither *L. curvatus* nor *L. sakei* possess a distinct potential for histamine production. Only strain *L. sakei* LTH 2076 gave positive results in the PCR screening and in screening medium with histidine as precursor. This strain, known as HDC^+ (Coton and Coton, 2005), was used as positive control for experiments regarding histidine decarboxylase acitivity.

Tyramine production was only associated with *L. curvatus* strains. Nearly two thirds of the *L. curvatus* strains tested showed TDC activity and a PCR product for the *tdc* fragment, whereas none of the *L. sakei* strains induced a pH shift in the screening medium containing tyrosine as precursor and gave an amplificate for *tdc*. These results matches with other studies describing *L. curvatus* as main producer of tyramine within LABs found in dry fermented sausages (Aymerich *et al.*, 2006; Bover-Cid *et al.*, 2001a). Therefore, *Lactobacillus sakei* would be, on the basis of competitiveness and hygienic aspects such as biogenic amine production, the species of choice for further use as starter culture in fermented sausage production. Still, some strains of *L. curvatus*, which prove negative in tyramine production, may form safe alternatives.

4.6 Aminotransferases in *L. sakei* 23K

Catabolism of aromatic amino acids (AAA), branched-chain amino acids (BCAA), and methionine is believed to play a major role in the formation of aroma compounds in fermented meat as well as in fermented dairy products (Montel *et al.*, 1998; Ordonez *et al.*, 1999; Yvon and Rijnen, 2001). In *L. lactis* aminotransferases seem to be the only enzymes, which are responsible for AAA and BCAA deamination (Atiles *et al.*, 2000; Rijnen *et al.*, 1999; Yvon *et al.*, 1997; Yvon *et al.*, 2000) the initial step for aroma formation from amino acids. *L. sakei* species obviously lack AraT and BcaT, since, genome search did not reveal genes coding for an AraT or a BcaT (Chaillou *et al.*, 2005). Furthermore, an approach to amplify aminotransferase fragments with degenerated primers obtained from sequence alignments of several LAB species was not successful. However, two putative aminotransferase genes, namely *arcT* and *aspD,* were annotated in *L. sakei*´s *genome* (Chaillou *et al.*, 2005). *ArcT* (YP_394986) lies in between a gene cluster encoding the enzymes of arginine deiminase pathway of *L. sakei.* This cluster, structurally and functionally analyzed by Zungia *et al.* (Zuniga *et al.*, 1998), contains ADI (*arcA*), OTC (*arcB*), carbamate kinase (*arcC*), a putative carrier (*arcD*) and finally the putative transaminase-encoding gene (*arcT*). However, the latter has not been characterized yet. Within the genera lactobacillus and lactococcus few further *arcT*-sequences are available but there is no experimental data about function and substrate specificity of the corresponding enzyme ArcT. At amino acid level similiarities between 53 and 59 % were found and the consensus pattern for aminotransferases class-I pyridoxal-phosphate attachment site K could be detected in all sequences. Generally, aminotransferases (EC 2.6.1.0) can be grouped on the basis of sequence similarity (Sung *et al.*, 1991). Class-I contains aspartate aminotransferases (AAT) (EC 2.6.1.1), tyrosine aminotransferases (EC 2.6.1.5) and aromatic aminotransferases (EC 2.6.1.57) among others (McHardy *et al.*, 2003). Therefore, it could be supposed that ArcT is maybe

involved in transamination of aromatic amino acids. To this end, ArcT was heterologous expressed in the present study and transaminase activity of the purified enzyme was measured. However, even after incubation for 8.5 h in buffers each containing another L-amino acid (L-leucine, L-isoleucine, L-valine, L-tyrosine, L-phenylalanine, L-aspartate, L-asparagine, L-alanine, L-glutamine, L-methionine), amount of L-glutamic acid formed by aminotransferase acitivity from α-ketoglutarate was below the detection limit of the glutamic acid test kit used. Hence, the actual function of ArcT remains still unclear but it can be suggest that ArcT is not relevant for transamination of L-amino aicds involved for aroma formation in sausage fermentations.

Further database search for AspD, the second putative aminotransferase in *L. sakei* 23K genome, revealed a second annotation, namely L-aspartate-beta-decarboxylase, a synonyme for L-aspartate 4-decarboxylase. *AspD* genes are not widely distributed among bacteria (Wang N., 2006) and only two further lactobacillus-*aspD* genes could be found for *Lactobacillus oris* PB013-T2-3 and *Lactobacillus antri* DSM 16041. Sequence alignment showed varying homologies between 47 and 95 % but motif search resulted in the same aminotransferase class-I pyridoxal-phosphate attachment site as for ArcT, suggesting that the enzyme could have aminotransferase activity.

Heterologously expressed and purified AspD showed significant L-aspartate 4-decarboxylase activity. A comprehensive characterization of function, structure and mechanisms of L-aspartate 4-decarboxylases has only been done for *Pseudomonas* species (Chen *et al.*, 2009; Lima *et al.*, 2009; Phillips *et al.*, 2010; Wang N., 2006; Wang *et al.*, 2008). In the present study the his-tagged recombinant AspD of *L. sakei* exhibited optimal pH values and optimal reaction temperatures similar to the recombinant L-aspartate 4-decarboxylase from *Pseudomas sp.* ATCC 19121 (Wang and Lee, 2006). Furthermore, recombinant L-aspartate 4-decarboxylase from *Pseudomonas* sp. ATCC 19121 has shown to be a bifunctional enzyme that exhibits additionally aminotransferase activity apart from decarboxylase activity when D,L-aspartate, L-glutamate, L-glutamine, and L-alanine were utilized as substrates. However, the decarboxylase activity against L-aspartate was about 2.5 times higher than its aminotransferase activity (Wang N., 2006). Despite similar decarboxylase acitivities and at least 37 % amino acid identity between Asd of *Pseudomonas* sp ATCC 19121 and AspD of *L. sakei* 23K, a similar bifunctional feature could not be found for the latter one. No aminotransferase activities could be detected when L-leucine, L-isoleucine, L-valine, L-tyrosine, L-phenylalanine, L-aspartate, L-asparagine, L-alanine, L-glutamine and L-methionine respectively were used as substrate.

Taken together, *in silico* analysis of *L. sakei´s* genome and PCR approaches with degenerated primers could not detect any specific aminotransferases against branched

chain amino acids and aromatic amino acids. Additionally, genes annotated as putative aminotransferases, namely *arcT* and *aspD*, seem not to be involved in transamination of amino acids relevant for aroma formation. Furthermore, transamination activities of cell-free extracts obtained from *L. sakei* 23K wildtype against the branched-chain amino acids L-leucine, L-isoleucine and L-valine were very weak. This is consistent with an earlier study that has revealed very low catabolism of L-leucine by *L. sakei* and *L. curvatus* in contrast to other meat relevant organisms like staphylococci (Larrouture *et al.*, 2000) for which a BcaT has been characterized (Madsen *et al.*, 2002). Since the enormous importance of AraT and BcaT for aroma formation has been demonstrated in LAB (Rijnen *et al.*, 2003) it is obvious that the presence of *araT* and *bcaT* in the genome of LAB and their functional expression represent at least one bottleneck in the formation of aromatic compounds from amino acids.

4.7 Choice of *ilvE* sequences for heterologous expression

4.7.1 *IlvE* of *S. carnosus*

Stapylococcus carnosus, a member of the Gram+ Staphylococcaceae family, is widely used in combination with lactic acid bacteria as starter culture in the production of dry fermented sausages. Besides *S. carnosus´* contribution to colour development by nitrate reductase activity (Geisen *et al.*, 1992), *S. carnosus* is known to contribute to the all over flavour of fermented sausages. Several studies revealed a high aroma producing capacity of *S. carnosus* especially regarding aromatic compounds derived from degradation of BCAA (Berdagué *et al.*, 1993; Masson *et al.*, 1999; Montel *et al.*, 1996; Stahnke, 1999). Additionally, the aminotransferase gene *ilvE* from *S. carnosus* BioCarna Ferment S1 has been cloned and inactivated and characterization of the enzyme was performed (Madsen *et al.*, 2002). Based on this, *ilvE* from another *S. carnosus* starter strain TMW 2.801 was chosen for introduction into *L. sakei* 23K. Since *ilvE* amino acid sequences of the two *S. carnosus* strains are identical, characteristics of aminotransferases should be comparable.

4.7.2 *IlvE* of *L. paracasei*

L. paracasei belongs to the *L. casei* group, comprising *L. casei, L. paracasei, L. rhamnosus* and *L. zeae*. The first three are used as probiotics (Klein *et al.*, 1998). The *L. casei/paracasei/rhamnosus* (LCR) group accounts for a large part of nonstarter lactic acid bacteria (NSLAB) in fermented foods. Such microorganisms are believed to contribute, together with the biochemical activities of starter bacteria, to the quality of final products. *L. paracasei* is commonly found on the oral and rectal mucosae of healthy

subjects (Ahrné *et al.*, 1998). *L. paracasei* is the dominant species of non-starter LAB (NSLAB) in several semi-hard cheese varieties but has also been isolated from meat products and has already been used as probiotic in sausages (Jahreis *et al.*, 2002). Furthermore, it was demonstrated that permeabilized cells of *L. paracasei* show a large variation in activities and specificities of aminotransferase among strains (Thage *et al.*, 2004a). Cheese production with these strains revealed different aroma profiles of the products (Thage *et al.*, 2005) but interestingly, the strain that had considerably highest aminotransferase activity did not show the largest increase in content of aroma compounds derived from the BCAA in cheese (Thage *et al.*, 2005). The aminotransferase of the strain with the highest activity on BCAA (CHCC2115) has been previously isolated and characterized and shown to have almost the same affinity for all the three BCAA (Thage *et al.*, 2004b). Due to its meat relevance, the use as probiotic and the fact that its BcaT has already been characterized, the *ilvE* sequence of *L. paracasei* was used in this study for heterologous expression in *L. sakei* 23K. Since, *L. paracasei* CHCC2115 was not available in our lab, DNA of another probiotic strain, *L. paracasei* TMW 1.1434, was used as template for amplifying *ilvE*. Sequencing revealed that *ilvE* amino acid sequences of *L. paracasei* CHCC2115 and TMW 1.1434 differed only in one amino acid residue, suggesting similar properties of both IlvEs.

4.7.3 *IlvE* of *E. faecalis*

Enterococci represent one of the LAB species that can be found in relatively high numbers during meat fermentations. Therefore, they may constitute an important part of the natural microbiota of fermented sausages. About 10^3 – 10^5 CFU/g of these bacteria have been reported in different European meat products (Aymerich *et al.*, 2003; Marchesini *et al.*, 1992; Montel *et al.*, 1998). Enterococcus strains have already been used as adjunct culture in combination with LAB for different European cheeses (Centeno *et al.*, 1999; Sarantinopoulos *et al.*, 2002) and positively affected taste, aroma, colour, structure as well as the overall sensory profile of the full-ripened cheeses. At the time we chose *ilvE* sequences, no data was available about the contribution of enterococci to volatile profiles of sausages. Due to the promising features of enterococci regarding the future application as (adjunct) starter culture in meat products, *ilvE* of *E. faecalis* was chosen for transformation into *L sakei* 23K.

4.8 Introduction of *ilvE* of different origins into *L. sakei* 23K

The lack of a BcaT, presumed as a bottleneck for aroma formation, has been compensated in *L. sakei* by integration and expression of *ilvE* genes from three different origins using two replicative plasmids and an integrative vector. Chromosomal integration

of all three *ilvE*-constructs by a single crossover was successful but efforts to promote the excision of the plasmid by a second crossover, leading to a gene replacement turned out to be very time consuming and succeeded only in one white clone indicating the designated result. In all other erythromycin sensitive clones, the second crossover had led to the wild-type genotype again, indicated by blue colonies. A low success rate for a gene replacement has already been described for the application of pRV86 in *L. sakei* 23K (Gory, 2001). These results showed that if *L. sakei* is not any longer under selective pressure exerted by antibiotics it rather tries to get rid of external DNA than keep it integrated in its chromosome. On the contrary, introduction of replicative plasmids pG+host5 and pMG36e with *ilvE* sequences worked out quickly.

4.9 *MCherry* as reporter gene for protein expression in *L. sakei*

Among the "mFruits" (Shaner *et al.*, 2004), developed from the first monomeric red fluorescent protein (mRFP1), mCherry is described as the most promising red fluorescent protein in terms of photostability, maturation, pH stability and tolerance for tagging (Shaner *et al.*, 2005). In contrast to *lacZ* which has already been used as an efficient reporter gene in *Lactobacillus sakei* (Stentz *et al.*, 2000), a great advantage of fluorescent proteins is that they can be detected directly on complex matrices without addition of chromogenic or specific substrates and does not require prior extraction or plating of bacteria. Hence, a gfp_{uv} gene coding for the green fluorescent protein (GFP) was successfully integrated into a replicative plasmid and into the chromosome of *L. sakei* in order to specifically monitor GFP+ strains (Gory, 2001). In the present study *mCherry* was chosen for generating a *pldhL::ilvE-X::mcherry* fusion for cloning into pG+host5 and pMG36e.

The main purpose of these transcriptionally coupled constructs was to control effective expression of *ilvE*. Red fluorescent cells expressing the fluorescent protein mCherry should also express the gene of interest *ilvE*. In this study, *E. coli* DH5α clones, carrying a *pldhL*::*ilvE-X::mcherry* fusion could be detected and selected due to their reddish colonies. Colouring of colonies has not been developed until at least 48 h at 37 °C. However, since colonies showed a variety of shades of red, expression of mCherry seemed to be diverse in different clones. Since, sequencing of *mCherry* from different coloured clones did not reveal any differences (data not shown) and streaking of a single clone, again led to different coloured colonies, it could be suggested that mCherry is not very stable in *E. coli*. Contrary to *E. coli*, colonies of *L. sakei* carrying an *pldhL::ilvE-X::mcherry* fusions were not visible to the naked eye. The colonies remained white even after long incubation times. However, observation of erythromycin resistant *L. sakei* cells by fluorescence microscopy revealed red fluorescence of the cells. This indicates that the *ldhL*-promoter obtained from

a Gram+ organism is also working in Gram- *E. coli* and exhibit even stronger expression rates than in *L. sakei*. Although mCherry was described as the best general-purpose red monomer because of to its superior photostability (Shaner *et al.*, 2005) in this study photobleaching let disappear red fluorescence of *L. sakei* cells very quickly, compared to green fluorescent *L. sakei* cells carrying pRV85 (Gory, 2001). Nevertheless, there was a correlation between expression of *mCherry* confirmed by red fluorescent cells and expression of *ilvE* which was verified by measuring aminotransferase activity. Taken together, mCherry could be used for an indirect expression proof of *ilvE* but GFP seems to be suited better for fluorescence monitoring in *L. sakei* because of its brighter fluorescence and higher photostability.

4.10 Aminotransferase activity of recombinant *L. sakei* 23K

While conversion of branched-chain amino acids to their corresponding α-ketoacids was low in the *L. sakei* 23K wildtype, it was significantly increased in all transformants expressing *ilvE* genes. Promotor *pldhL* has already been described as suitable for effective protein expressions in *L. sakei* 23K (Gory, 2001). *L. sakei* 23K-pil-Sc with a single chromosomal integrated copy of the *pldhL*::*ilvE-Sc* fusion exhibited less aminotransferase activity than mutants with *ilvE*-sequences on replicative plasmids. This might be due to a higher copy number of the *pldhL::ilvE-X::mcherry* fusions in these strains. It has already been shown that *L. sakei* mutants with a higher copy number of a *pldhL::gfp*$_{uv}$ fusion exhibited higher fluorescence intensity than *L. sakei* mutants with a single copy on their chromosomes (Gory, 2001).

Comparison of transaminase activities induced by introduction of the replicative plasmids pMG36e-pilm-Lp or pG+host5-pilm-Lp respectively revealed a 25-fold increase of activity for *L. sakei* pMG36e-pilm-Lp but only a 20-fold increase for pG+host5-pilm-Lp compared to the wildtype strain. Vectors pMG36e and pG+host5 are both originally based on the replicon of pWV01 (low copy numbers), a cryptic plasmid originally obtained from a *L. lactis subsp. cremoris* Wg2 plasmid (Biswas *et al.*, 1993; Maguin *et al.*, 1992; van de Guchte *et al.*, 1989) and should therefore show similar copy numbers. However, in case of pG+host5 the origin of replication is a temperature sensitive mutant of pWV01 that carries four mutations. However, it has been shown that at 30 °C, the favoured growth temperature of *L. sakei*, copy numbers of plasmids with native pWV01 origin and the Ts derivate were similar (Maguin *et al.*, 1992). The influence of different copy numbers on different transaminase activities could not be definitely excluded, since no explicit analysis has been performed in this study. Furthermore one could not be sure that during years of propagation and rearrangement of plasmids the origins have remained absolutely conserved.

Despite the lower transamination rate of pG+host5-pilm-Lp compared to pMG36e-pilm-Lp, it was still higher than transamination activities of pMG36e-pilm-Sc and pMG36e-pilm-Ef which showed only a 13 - 15 fold increase of aminotransferase activity compared to the wild type. IlvE of *L. paracasei* seems to exhibit highest transaminase activity of heterologous expressed IlvE proteins in the background of *Lactobacillus sakei*. Only minor differences in substrate specificities of IlvEs could be detected in this study. This is consistent with data obtained from characterization of a branched-chain aminotransferase from *L. paracasei* subsp. *paracasei* DHCC 2115 that only differs in one amino acid residue from IlvE-Lp examined in this study (Thage *et al.*, 2004b). Furthermore, characterization of aminotransferase activity against BCAA of whole *Staphylococcus carnosus* cells showed also only minor differences in substrate specificity (Madsen *et al.*, 2002).

Observation of transaminase activity by measurement of L-glutamic acid has revealed that maximal L-glutamic acid concentrations have already been reached after 1 h of incubation in most approaches with CFE from recombinant *L. sakei* strains. Only for *L. sakei* wildtype that showed a very low transaminase activity could be suggested that activity has slightly increased beyond the 24 h examined in this study. Despite the fact that this experiment was performed with CFE and not with whole cells where additionally transport effects have to be considered, it could be suggested that under sausage fermentation conditions even *L. sakei* wildtype can, to a certain degree, contribute to conversion of BCAA to α-keto acids with regard to the long fermentation and ripening times of sausages.

4.11 Volatiles formed by whole cells in buffer system

SPME-GC/MS analysis of wildtypes (*E. faecalis* V583, *S. carnosus* TMW 2.801, *L. paracasei* TMW 1.1434 and *L. sakei* 23K) and recombinant strains after 5 days of fermentation in a buffer model system revealed 5 volatiles derived from breakdown of branched-chain amino acids which are strongly linked to dry sausage odour (Montel *et al.*, 1996; Stahnke, 1995a; Stahnke *et al.*, 2002): 3-methylbutanal, 3-methylbutanol and 3-methylbutyric acid in approaches containing L-leucine and 2-methylpropanol and 2-methylpropanoic acid in buffer systems with L-valine as precursor amino acid (other substances have not been taken into account). Taken as single compounds, odour of 3-methylbutanal is described as sour, cheesy, nailpolish-like (Stahnke, 1994) and malty (Sollner and Schieberle, 2009), 3-methylbutanoic acid like smells like sweaty socks (Stahnke, 1994), odour of 2-methylpropanoic acid is fatty and like savory snacks (Marco *et al.*, 2007), 2-methylpropanol has an alcoholic odour and 3-methylbutanol exhibits a fruity flavour note (reviewed by (Montel *et al.*, 1998)). However, together with volatiles

from lipid degradation, fermentation, and from spices and derived from smoking they contribute to the unique aroma of fermented sausages.

Generally, amounts of branched-chain acids were about 1000fold higher as amounts of the respective aldehyde or alcohol. According to experiences made in our lab, affinity of the used fibre (CAR/PDMS) against volatile acids was low and therefore the detection limit was respectively high. This means, once a peak could be detected, even if the peak area was very small, concentration of the detected acid was considerably high.

Remarkably, significant concentrations of the respective aldehyde could even be measured in blank vials without cells. Possibly, the non-enzymatic Strecker degradation of L-valine and L-leucine could be responsible for this phenomenon. It has often been suggested that branched-chain aldehydes could originate from Strecker degradation of the respective amino acids (Barbieri *et al.*, 1992; Berdagué *et al.*, 1993; Halvarson, 1973). In fact, a former study showed that the amounts of those branched-aldehydes were of the same magnitude in sausages with added staphylococci as in sausages without any microbial growth (Stahnke, 1994). However, test conditions were not optimal for Strecker degradation which usually needs high temperatures and low water activity. Considering these amounts of aldhydes in blank vials, only *S. carnosus* wild type cells produced considerably amounts of 3-methylbutanal. Comparing wildtypes, 3-methylbutanol and 3-methylbutyric acid from L-eucine were also mainly formed by *S. carnosus*. However, the major volatile derived from L-leucine was 3-methylbutyric acid.

This is consistent with former studies, which pointed out that *Staphylococcus carnosus* mainly produced 3-methylbutyric acid in a buffer model (Larrouture *et al.*, 2000) as well as in a dry sausage model system (Montel *et al.*, 1996; Stahnke *et al.*, 1999). *L. sakei* 23K wildtype and the *L. sakei* recombinants carrying pMG36e-pilm-X produced similar amounts of 3-methylbutyric acid but only about half of the amount formed by *S. carnosus*. Low production of metabolites from leucine degradation, like 3-methylbutyric acid, has already been described for *L. sakei* (Larrouture *et al.*, 2000).

Volatile formation from L-valine was at least 10-fold lower compared to volatiles derived from L-leucine. Since transamination activities for L-leucine and L-valine were similar, one can suggest that decarboxylases and dehydrogenases forming volatiles from 3-methyl-2-oxobutanoic acid (α-ketoisovaleric acid) are not as active as those specific for 4-methyl-2-oxopentanoic acid (α-ketoisocaproic acid). Assuming that the same decarboxylases and dehydrogenases are responsible for conversion of α-ketoisovaleric acid and α-ketoisocaproic acid, specificity of the enzymes against α-ketoisocaproic acid should be higher.

All strains formed approximately the same low amount of 2-methyl-1-propanal except *S. carnosus* which oxidized a large part of the aldehyde to 2-methyl propanoic acid. That is consistent with kinetic studies of resting cells performed by Beck *et al.* (Beck *et al.*, 2002) which revealed that *S. xylosus* DD-34 quickly oxidized α-ketoacids (generated by transamination of the branched-chain amino acids and decarboxylated into the corresponding branched-chain aldehydes) into the corresponding acids.

L. paracasei TMW 1.1434 exhibited only low aroma forming capacity, which is not consistent with earlier studies that has described *L. paracasei* as potent producer of volatiles derived from BCAA in cheese (Thage *et al.*, 2004a). However, it is also known that aminotransferase activity and aroma formation capacity is strongly strain-dependent in *L. paracasei* (Thage *et al.*, 2004a; Thage *et al.*, 2005). Furthermore, no data is available about volatile formation experiments with *L. paracasei* in buffer systems.

Since, enterococci have been detected at levels of 10^2 - 10^5 cfu/g especially in artisan products from Southern Europe it is supposed that these bacteria may also constitute an important part of the natural microbiota and may impact on the aroma profile of dry fermented products. However, data about contribution of enterococci to the volatile profile of fermented sausages is rare but a very recent study was addressed to this topic. Latorre-Moratalla *et al.* found out that different bacteriocinic enterococci strains did not alter the volatile profile of slightly-fermented sausage whereas quantitative differences between the batches containing different enterococci strains could be detected. However, no significant differences in volatiles derived from branched-chain amino acids could be revealed between batches inoculated with enterococci and the non-inoculated control batch. The authors presume that contribution of methyl-branched volatiles from enterococci is negligible compared to the production by indigenous staphylococci present in the batches (Latorre-Moratalla *et al.*, 2011). Since, Latorre-Moratalla *et al.* examined several enterococcus strains the low volatile formation capacity from BCAAs is probably not strain-dependent. Thus, the present study supports Latorre-Moratalla´s presumption that enterococci´s contribution to volatiles derived from amino-acid metabolism is rather low. None of the recombinant *L. sakei* strains, expressing BcaT, showed significant increased amounts of aroma relevant volatiles compared to the wildtype. Since *E. faecalis* and *L. paracasei* did not produce more volatiles than *L sakei* wildtype, it is not surprising that the corresponding recombinant strains (*L. sakei* 23K pMG36e-pilm-Ef, pMG36e-pilm-Sc and pG+host5-pilm-Lp) did not show enhanced volatile formation features. However, *L. sakei*-pMG36e-Sc, carrying the BcaT of *S. carnosus*, known as a potent volatile forming meat organism, also failed in terms of increasing volatile formation in the background of *Lactobacillus sakei* 23K. Therefore, it may be presumed that a functional BcaT is one

bottleneck for an effective formation of aromatic compounds from BCAAs but it is not the only one.

As further bottlenecks, transport of amino acids into the cell and further conversion of α-keto acids to corresponding aldheydes, alcohols and acids are worth considering.

Due to the experimental design with whole cells and free amino acids, additionally to intracellular enzymatic activities, amino acid transport processes must be taken into consideration. A study about the sourdough organism *Lactobacillus sanfranciscensis* suggested that peptide uptake is more effective than amino acid uptake in this organism. Since, both lactobacillus species possess at least the same transport systems for oligopeptides Opp and DtpT, one can presume that uptake efficiencies of peptides and maybe also amino acids could be similar in *L. sakei*. Possibly, aroma forming capacity of *L. sakei*-pMG36e-Sc could be increased by providing peptides as substrate. It is not much experimental data available about specific transport of branched-chain amino acids into cells. However, genome search of *S. carnosus* TM300 revealed three putative transporters for branched-chain amino acids (Rosenstein *et al.*, 2009), whereas no such specific transporters could be found in *L. sake*i 23K (Chaillou *et al.*, 2005). Characterization of the *brnQ* gene product of *Corynebacterium glutamicum* ATCC 13032 showed that the protein is probably solely responsible for isoleucine uptake (only isoleucine was tested) (Tauch *et al.*, 1998). Furthermore, BrnQ of *L. delbrueckii* subsp. *lactis* DSM7290 was characterized as proton motive force (pmf) driven transporter for valine, leucine and isoleucine (Stucky *et al.*, 1995). Although experimental data is lacking for BrnQ of *S. carnosus*, it can be suggested that the presence of these transporters leads to a more efficient uptake of branched-chain amino acids into the cell. *BrnQ* sequences are available for several lactobacilli. PCR reactions with a degenerated primer pair, designed from alignment of *brnQ* sequences from *L. rhamnosus*, *L. casei, L. buchneri and L. brevis,* were positive for four of 51 *L. sakei* strains. A BLAST-search with sequenced *brnQ*-fragments revealed about 70 % sequence identity with branched-chain amino acid transport proteins of various lactobacilli. This let suggest that presence of *brnQ* could be strain-dependent among *L. sakei* and therefore transformation of one of the *brnQ*-carrying *L. sakei* strains (*L. sakei* TMW 1.22, TMW 1.114, TMW 1.578 and TMW 1.1290) could lead to an increased level of volatiles, provided that *bcaT* and *brnQ* are the only two bottlenecks for an effective formation of volatiles from amino acids. Although *L. sakei* 23K does not possess any specific transporters for branched-chain amino acids, uptake of these amino acids at least to a certain degree must occur via general amino acid transporters present in *L. sakei* 23K otherwise *L. sakei* 23K would be not possible to grow in the chemically defined medium used for auxotrophy tests in this study.

The conversion of α-keto acids to corresponding aldheydes, alcohols and acids should be considered as a third possible bottleneck in aroma formation from BCAA. Two distinct ways have been described for the formation of volatiles from α-keto acids. Firstly, a part of the Ehrlich-pathway which includes the decarboxylation (maybe via KdcA) of the α-keto acids to the corresponding aldehyde and the subsequent dehydrogenation to the corresponding alcohol (Ehrlich, 1907) and secondly, the direct conversion of α-keto acids to carboxylic acid by an α-keto acid dehydrogenase complex (KaDH) and subsequent phosphotransferase and kinase reactions (Ward *et al.*, 1999).
Since, *L. sakei* strains did not produce 3-methyl-butanol and 3-methyl-butanal but at least low amounts of 3-methyl-butanoic acid, the pathway via KaDH could be supposed. Furthermore, the genome of *L. sakei* 23K encodes at least a putative KaDH complex and a putative (branched-chain) phosphotransacylase but does not encode a KdcA. However, no acyl kinase gene, homologous to *buk* gene of *E. faecalis* could be detected in *L. sakei* 23K (Liu *et al.*, 2008). Genome analysis of *S. carnosus* TM300 however, revealed an operon (Sca1142 – Sca1137) homologous to *ptb-buk-bkdDABC* of *E. faecalis* containing four genes coding for the KaDH complex, a phophotransacylase gene and a butyrate kinase gene whereas no *kdcA* gene could be found (Rosenstein *et al.*, 2009). This could explain the preferred formation of methyl-branched acids by *S. carnosus*, demonstrated in the present and in earlier studies (Larrouture *et al.*, 2000; Montel *et al.*, 1996; Stahnke, 1999).

5 Summary

Lactobacillus sakei is one of the dominating *Lactobacillus* species in European fermented sausages. *L. sakei* is well adapted to the meat environment and is regularly used as starter culture for industrial sausage production, mainly in combination with a representative from Gram positive, catalase positive cocci (GCC+). The pH drop by lactic acid formation inhibits pathogenic flora and makes *L. sakei* essential for the food safety of the fermented meat products. Furthermore, low pH values lead to sliceable products and enhances the reddening of meat, mainly induced by nitrate reductase activity of GCC+. Since, LAB are auxotrophic for most amino acids, proteolytic activities are important for their growth. Furthermore, peptides and amino acids are precursors for volatiles contributing to the aroma of fermented sausages but also precursors for biogenic amines that can be harmful to health. Therefore, the genetic composition of *Lactobacillus sakei* concerning genes of the peptidolytic system and genes involved in amino acid metabolism was characterized in this study to facilitate and accelerate the screening and valuation of starter lactobacilli for sausage fermentations.

Lactobacillus sakei generally considered as genetically diverse species proved in this study to be very homogenous regarding genes involved in peptide uptake as well as peptide and amino acid degradation. Peptide transporters and peptidases were nearly ubiquitary in the 51 *L. sakei* strains screened, and only few strains lacked single peptidases. Consistent with the protein-rich meat environment, the presence of several peptide transporters and a variety of peptidases, *L. sakei* strains are auxotrophic for most amino acids. All strains were prototrophic for L-glutamic acid and L-aspartic acid. Some strains were additionally able to grow without L-glutamine or L-asparagine. However, this variability could not be reduced to differences in the absence or presence of specific genes involved in amino acid biosynthesis.

Tyramine-forming potential was detected only within *L. curvatus* species and histamine decarboxylase (HDC) activity could only be found in one *L. sakei* strain, previously described as histidine decarboxylase positive, indicating that *L. sakei* is a safe species regarding to biogenic amine formation. In accordance with the weak aminotransferase activities towards branched-chain amino acids (leucine, isoleucine, and valine) of cell-free extracts (CFE), all *L. sakei* strains tested lacked *ilvE* and also *araT* homologs coding for aminotransferases specific for branched-chain and aromatic amino acids respectively. *IlvE* sequences obtained from three meat-associated microorganisms (*L. paracasei, E. faecalis, and S. carnosus*) were successfully introduced into *L. sakei* 23K by replicative plasmids and chromosomal integration (only *ilvE* of *S. carnosus*). All *ilvE*-carrying recombinants revealed significant increased aminotransferase activities towards

branched-chain amino acids compared to the wildtype *L. sakei* 23K. However, copy numbers influenced transaminase activities of CFE. The mutant *L. sakei* 23K-pil-Sc with a chromosomal integrated single copy of *ilvE* showed only a 5-fold increase of aminotransferase activity, whereas the recombinant strain with pMG36e-pilm-Sc showed 13-fold enhanced activity compared with *L. sakei* 23K wildtype. The activity of IlvE obtained from *L. paracasei* was highest in the background of the *ilvE*-negative *L. sakei* 23K. Only minor differences in substrate specificities against branched-chain amino acids could be determined. This is in accordance to IlvE of *S. carnosus* and *L. paracasei* purified and characterized in earlier studies.

However, since increased transaminase activities did not increase formation of the respective methyl-branched volatiles by recombinant *L. sakei* strains, presence of *ilvE* cannot be the only bottleneck in aroma formation from amino acids. Amino acid or peptide uptake into the cell via specific transport systems and the conversion of α-keto acids to the corresponding aldehydes, alcohols and carboxylic acids must be considered as further limiting steps.

6 Zusammenfassung

In Europa ist *Lactobacillus sakei* die am häufigsten vorkommende Laktobazillenspezies in Rohwurstprodukten. *L. sakei* ist sehr gut an den Lebensraum Fleisch angepasst und wird, meist in Kombination mit einem Vertreter der Grampositiven, katalase-positiven Kokken (GCC+), als Starterkultur in der industriellen Rohwurstproduktion eingesetzt. Die Anwesenheit von *L. sakei* ist essentiell für die Produktsicherheit, da die gebildete Milchsäure eine möglicherweise pathogene Begleitflora hemmt. Außerdem führen niedrige pH-Werte zu einem schnittfähigen Endprodukt und fördern den Umrötungsprozess, der durch die Nitratreduktaseaktivität der GCC+ induziert wird. Da Laktobazillen auxotroph für die meisten Aminosäuren sind, sind peptidolytische Aktivitäten besonders wichtig für ihr Wachstum. Desweiteren sind Peptide und Aminosäuren einerseits Vorstufen flüchtiger Substanzen, die zum Rohwurstaroma beitragen, gleichzeitig aber auch Vorläufer toxikologisch bedenklicher biogener Amine.

Daher wurde in dieser Arbeit die genetische Ausstattung von *Lactobacillus sakei*, im Bezug auf Gene des peptidolytischen Systems und im Bezug auf Gene, die am Aminosäureabbau beteiligt sind, mit dem Ziel charakterisiert, das Screening und die Bewertung von möglichen Rohwurststarter-Laktobazillen zu erleichtern und zu beschleunigen.

Lactobacillus sakei, eine Spezies, die im Allgemeinen als genetisch sehr divers gilt, erwies sich in dieser Arbeit als äußerst homogen bezüglich Genen, die an der Peptidaufnahme sowie dem Peptid- und Aminosäureabbau beteiligt sind. Peptidtransporter und Peptidasen waren in fast allen gescreenten *L. sakei* Stämmen zu finden und nur einigen wenigen Stämmen fehlte eine einzelne Peptidase. Passend zum Wachstum im proteinreichen Medium Fleisch und der Anwesenheit mehrerer Peptidtransporter sowie einer Vielzahl von Peptidasen, waren *L. sakei* Stämme auxotroph für die meisten Aminosäuren. Alle Stämme waren prototroph für L- Glutaminsäure und L-Asparaginsäure und einige Stämme konnten zudem auch ohne L-Glutamin oder L-Asparagin wachsen. Die unterschiedlichen Aminosäureabedürfnisse konnten allerdings nicht auf die An- oder Abwesenheit bestimmter Gene des Aminosäure-biosynthesestoffwechsels zurückgeführt werden.

Das Potential Tyramin zu bilden war nur für einige *L. curvatus*-Stämme, nicht aber für *L. sakei* nachweisbar. Nur ein *L. sakei* Stamm, der bereits als HDC+ beschrieben war, zeigte Histidindecarboxylaseaktivität. Diese Ergebnisse weisen *L. sakei* bezüglich der Bildung von biogenen Aminen als sicheren Starterorganismus aus. Entsprechend der schwachen Aminotransferaseaktivitäten des zellfreien Extrakts von *L. sakei* 23K gegenüber verzweigten Aminosäuren (Leucin, Isoleucin und Valin), konnten in keinem der

getesteten *L. sakei* Stämme Gene gefunden werden, die für Aminotransferasen kodieren, die spezifisch verzweigte oder aromatische Aminosäuren abbauen (IlvE bzw. AraT). *IlvE*-Sequenzen von drei fleischrelevanten Mikrooorganismen (*L. paracasei, E. faecalis* und *S. carnosus*) wurden erfolgreich in *L. sakei* 23K eingebracht (in replikativen Plasmiden und chromosomal integriert). Alle *ilvE*-tragenden Rekombinanten zeigten, im Vergleich zum Wildtyp, signifikant gesteigerte Aminotransferaseaktivitäten gegenüber verzweigten Aminosäuren. Allerdings ergaben sich Aktivitätsunterschiede, die sich auf die jeweilige Kopienzahl der *ilvE*-Konstrukte in der Zelle zurückführen ließen. So war das zellfreie Extrakt von *L. sakei* 23k-pil-Sc mit einer einzelnen, chromosomal integrierten *ilvE*-Sequenz nur 5-mal aktiver als das Wildtypextrakt, wohingegen die Rekombinante mit pMG36e-pilm-Sc eine Aktivitätssteigerung um das 13-fache erreichte. Die Substratspezifitäten gegenüber verzweigten Aminosäuren unterschieden sich nur geringfügig, was frühere Studien über IlvEs von *S. carnosus* und *L. paracasei* bestätigt. Da eine gesteigerte Transaminaseaktivität nicht zu einer vermehrten Bildung von methylverzweigten flüchtigen Substanzen führte, kann das Vorhandensein einer aktiven IlvE nicht das einzige „Bottleneck“ bei der Aromabildung aus Aminosäuren darstellen. Aminosäure- und Peptidaufnahme in die Zelle über spezielle Transportsysteme, sowie die Umsetzung von α-Ketosäuren zu den korrespondierenden Aldehyden, Alkoholen und Carbonsäuren müssen als zusätzlich limitierende Schritte in Betracht gezogen werden.

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8 Appendix

Alignment of GlnA amino acid sequences, obtained by sequencing of *glnA* genes of four *L. sakei* strains and subsequent translation.

```
L. sakei TMW 1.1393          MARKMLTAEAIKQLVDQENVKFLRLMFTDINGIIKNVEVPISQLDKVLSN 50
L. sakei TMW 1.1397          MARKMLTAEAIKQLVDQENVKFLRLMFTDINGIIKNVEVPISQLDKVLSN 50
L. sakei TMW 1.148           MARKMLTAEAIKQLVDQENVKFLRLMFTDINGIIKNVEVPISQLDKVLSN 50
L. sakei 23K                 MARKMLTAEAIKQLVDQENVKFLRLMFTDINGIIKNVEVPISQLDKVLSN 50
                             **************************************************

L. sakei TMW 1.1393          KMMFDGSSIDGFVRIEESDMYLRPDLSTWLIFPWEAEHGKVARLICSVYT 100
L. sakei TMW 1.1397          KMMFDGSSIDGFVRIEESDMYLRPDLSTWLIFPWEAEHGKVARLICSVYT 100
L. sakei TMW 1.148           KMMFDGSSIDGFVRIEESDMYLRPDLSTWLIFPWEAEHGKVARLICSVYT 100
L. sakei 23K                 KMMFDGSSIDGFVRIEESDMYLRPDLSTWLIFPWEAEHGKVARLICSVYT 100
                             **************************************************

L. sakei TMW 1.1393          ADGEPFLGDPRNNLKKMVREMQDKGFKDFNIGPEPEFFLFKLDEIGKPTL 150
L. sakei TMW 1.1397          ADGEPFLGDPRNNLKKMVREMQDKGFKDFNIGPEPEFFLFKLDEIGKPTL 150
L. sakei TMW 1.148           ADGEPFLGDPRNNLKKMVREMQDKGFKDFNIGPEPEFFLFKLDEIGKPTL 150
L. sakei 23K                 ADGEPFLGDPRNNLKKMVREMQDKGFKDFNIGPEPEFFLFKLDEIGKPTL 150
                             **************************************************

L. sakei TMW 1.1393          KLNDQGGYFDFAPVDLGENCRRDIVLELEKMGFEVEASHHEVAPGQHEID 200
L. sakei TMW 1.1397          KLNDQGGYFDFAPVDLGENCRRDIVLELEKMGFEVEASHHEVAPGQHEID 200
L. sakei TMW 1.148           KLNDQGGYFDFAPVDLGENCRRDIVLELEKMGFEVEASHHEVAPGQHEID 200
L. sakei 23K                 KLNDQGGYFDFAPVDLGENCRRDIVLELEKMGFEVEASHHEVAPGQHEID 200
                             **************************************************

L. sakei TMW 1.1393          FKYADAVDAADNIQTFKLVVKTIARKHGLHATFMPKPLHGVNGSGMHINM 250
L. sakei TMW 1.1397          FKYADAVDAADNIQTFKLVVKTIARKHGLHATFMPKPLHGVNGSGMHINM 250
L. sakei TMW 1.148           FKYADAVDAADNIQTFKLVVKTIARKHGLHATFMPKPLHGVNGSGMHINM 250
L. sakei 23K                 FKYADAVDAADNIQTFKLVVKTIARKHGLHATFMPKPLHGVNGSGMHINM 250
                             **************************************************

L. sakei TMW 1.1393          SLFNQDGTNAFFDENGKEQLSETAYHFLAGLLRHARAITAINNPTVNSYK 300
L. sakei TMW 1.1397          SLFNQDGTNAFFDENGKEQLSETAYHFLAGLLRHARAITAINNPTVNSYK 300
L. sakei TMW 1.148           SLFNQDGTNAFFDENGKEQLSETAYHFLAGLLRHARAITAINNPTVNSYK 300
L. sakei 23K                 SLFNQDGTNAFFDENGKEQLSETVYHFLAGLLRHARAITAINNPTVNSYK 300
                             ***********************.**************************

L. sakei TMW 1.1393          RLVPGFEAPVYVAWSGHNRSPLIRVPQSRGLSTRLELRSVDPSANPYLAI 350
L. sakei TMW 1.1397          RLVPGFEAPVYVAWSGHNRSPLIRVPQSRGLSTRLELRSVDPSANPYLAI 350
L. sakei TMW 1.148           RLVPGFEAPVYVAWSGHNRSPLIRVPQSRGLSTRLELRSVDPSANPYLAI 350
L. sakei 23K                 RLVPGFEAPVYVAWSGHNRSPLIRVPQSRGLSTRLELRSVDPSANPYLAI 350
                             **************************************************

L. sakei TMW 1.1393          SSILAAGLSGLEQGLSPEAGVDRNIYSMDETERKENHITDLPSTLHNALK 400
L. sakei TMW 1.1397          SSILAAGLSGLEQGLSPEAGVDRNIYSMDETERKENHITDLPSTLHNALK 400
L. sakei TMW 1.148           SSILAAGLSGLEQGLSPEAGVDRNIYSMDETERKENHITDLPSTLHNALK 400
L. sakei 23K                 SSILAAGLSGLEQGLSPEAGVDRNIYSMDETERKENHITDLPSTLHNALK 400
                             **************************************************

L. sakei TMW 1.1393          ELAKDDIIKDSMGTYLYQSFMDSKSLEWAAYRQQVSEWEREQYLELY 447
L. sakei TMW 1.1397          ELAKDDIIKDSMGTYLYQSFMDSKSLEWAAYRQQVSEWEREQYLELY 447
L. sakei TMW 1.148           ELAKDDIIKDSMGTYLYQSFMDSKSLEWAAYRQQVSEWEREQYLELY 447
L. sakei 23K                 ELAKDDIIKDSMGTYLYQSFMDSKSLEWAAYRQQVSEWEREQYLELY 447
                             ***********************************************
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Alignment of PepX amino acid sequences, obtained by sequencing of *pepX* genes of two *L. sakei* strains (*L. sakei* TMW 1.1398 and TMW 1.1399) and subsequent translation (*L. sakei* 23K sequence was obtained from database)

```
L. sakei TMW 1.1398      MKLNQFARLTTTYSEQIKALQRIKLLDEGYEALSVQALAQQIFARFFPEAHSKTAQNEQM 60
L. sakei TMW 1.1399      MKLNQFARLTTTYSEQIKALQRIKLLDEGYEALSVQALAQQIFARFFPEAHSKTAQNEQM 60
L. sakei     23K         MKLNQFARLTTTYSEQIKALQRIKLLDEGYEALSVQALAQQIFARFFPEAHSKTAQNEQM 60
                         ************************************************************

L. sakei TMW 1.1398      QKIQATASLNLADYLAGISTSFDQRTFYNIALQLLGFKVTTDFQFNHPRRFMAKVGIPYV 120
L. sakei TMW 1.1399      QKIQATASLNLADYLAGISTSFDQRTFYNIALQLLGFKVTTDFQFNHPRRFMAKVGIPYV 120
L. sakei 23K             QKIQATASLNLADYLAGISTSFDQRTFYNIALQLLGFKVTTDFQFNHPRRFMAKVGIPYV 120
                         ************************************************************

L. sakei TMW 1.1398      DQPVLTQELFLEAVYLLLTTRSQNGLLYLDCLANRGFFAHWQKATAPEFLIFNGKTQPVF 180
L. sakei TMW 1.1399      DQPVLTQELFLEAVYLLLTTRSQNGLLYLDCLANRGFFAHWQKATAPEFLIFNGKTQPVF 180
L. sakei 23K             DQPVLTQELFLEAVYLLLTTRSQNGLLYLDCLANRGFFAHWQKATAPEFLIFNGKTQPVF 180
                         ************************************************************

L. sakei TMW 1.1398      DTQNFIREVVYVESSLDTDLDGHLDLLETTIFRPKETEKGLRVPVLYTASPYYKGTNDVD 240
L. sakei TMW 1.1399      DTQNFIREVVYVESSLDTDLDGHLDLLETTIFRPKETEKGLRVPVLYTASPYYKGTNDVD 240
L. sakei 23K             DTQNFIREVVYVESSLDTDLDGHLDLLETTIFRPKETEKGLRVPALYTASPYYKGTNDVD 240
                         ********************************************.***************

L. sakei TMW 1.1398      ADLHNVDVPIQAKAAIQPNLADLKTGTNQSVPAARKPLGETTETELEAADDSNYLLNDYF 300
L. sakei TMW 1.1399      ADLHNVDVPIQAKAAIQPNLADLKTGTNQSVPAARKPLGETTETELEAADDSNYLLNDYF 300
L. sakei 23K             ADLHNVDVPIQAKAAIQPNLADLKTGTNQSVPAAREPLGETTEPELEAADDSNYLLNDYF 300
                         ***********************************:*******.****************

L. sakei TMW 1.1398      LARGFATVYAGGIGTRGSDGMRTCGSPEETASTTAIIEWLAGNRRAYTNKTDRIEIKAWW 360
L. sakei TMW 1.1399      LARGFATVYAGGIGTRGSDGMRTCGSPEETASTTAIIEWLAGNRRAYTNKTDRIEIKAWW 360
L. sakei 23K             LARGFATVYAGGIGTRGSDGMRTCGSPEETASTTAIIEWLAGNRRAYTNKTDRIEIKAWW 360
                         ************************************************************

L. sakei TMW 1.1398      CNQKVAMTGKSYLGTLATAAATTGVEGLKTVIAEAAISSWYDYYRENGLVVAPVDCQGED 420
L. sakei TMW 1.1399      CNQKVAMTGKSYLGTLATAAATTGVEGLKTVIAEAAISSWYDYYRENGLVVAPVDCQGED 420
L. sakei 23K             CNQKVAMTGKSYLGTLATAAATTGVEGLKTVIAEAAISSWYDYYRENGLVVAPVDCQGED 420
                         ************************************************************

L. sakei TMW 1.1398      ADVLAKLCQTREMDAADHAKSGALFEEQLTALREGQDRITGNYNAFWAERNYRDNVQKIN 480
L. sakei TMW 1.1399      ADVLAKLCQTREMDAADHAKSGALFEEQLTALREGQDRITGNYNAFWAERNYRDNVQKIN 480
L. sakei 23K             ADVLAKLCQTRQMDAADHTKSGALFEEQLAALREGQDRITGNYNAFWAERNYRDNVQKIN 480
                         ***********:******:**********:******************************

L. sakei TMW 1.1398      CDVVLVHGLNDWNVKLQNAGALWDDLRQLPIEKKLFLHQGQHIYMNNIQSIDFTDMMNLW 540
L. sakei TMW 1.1399      CDVVLVHGLNDWNVKLQNAGALWDDLRQLPIEKKLFLHQGQHIYMNNIQSIDFTDMMNLW 540
L. sakei 23K             CDVVLVHGLNDWNVKLQNAGALWDDLRQLPIEKKLFLHQGQHIYMNNIQSIDFTDMMNLW 540
                         ************************************************************

L. sakei TMW 1.1398      LSYQLLDIDNHAPEILPTVTIQDNTQEATWHTQDDWLNPKNPRQTYFLNDPEHLGLDQTP 600
L. sakei TMW 1.1399      LSYQLLDIDNHAPEILPTVTIQDNTQEATWHTQDDWLNPKNPRQTYFLNDPEHLGLDQTP 600
L. sakei 23K             LSYQLLDIDNHASEILPTVTIQDNTQEATWHTQDDWLNPKNPRQTYFLNDPEHLGLDQTP 600
                         ************.***********************************************

L. sakei TMW 1.1398      TTPVADFSDDGVAMFKKQHLSEAAWQDQLLAPQSDFTQNRLLLLSPAQSKQLVIDGRVQL 660
L. sakei TMW 1.1399      TTPVADFSDDGVAMFKKQHLSEAAWQDQLLAPQSDFTQNRLLLLSPAQSKQLVIDGRVQL 660
L. sakei 23K             TTPVADFSDDGVAMFKKQHLSEAAWQDQLLAPQSDFTQNRLLLLSQAQSKQLVIDGRVQL 660
                         ********************************************* **************

L. sakei TMW 1.1398      KTKVAVNTDRGLLSVMLVDYGLFSRLGTTPAILAAKGQQLGYHWRYDDLKEFKLGALSPY 720
L. sakei TMW 1.1399      KTKVAVNTDRGLLSVMLVDYGLFSRLGTTPAILAAKGQQLGYHWRYDDLKEFKLGALSPY 720
L. sakei 23K             KTKVAVNTDRGLLSVMLVDYGLFSRLGTTPAILAAKGQQLGYHWRYDDLKEFKLGALSPY 720
                         ************************************************************

L. sakei TMW 1.1398      QLITKGHLNLQNRHNSYQTETVDAGTFYEVQLDLQPTHYHLAAGHQLGLVIYATDMGMTL 780
L. sakei TMW 1.1399      QLITKGHLNLQNRHNSYQTETVDAGTFYEVQLDLQPTHYHLAAGHQLGLVIYATDMGMTL 780
L. sakei 23K             QLITKGHLNLQNRHNSYQTETVDAGTFYEVQLDLQPTHYHLAAGHQLGLVIYATDMEMTL 780
                         ******************************************************** ***

L. sakei TMW 1.1398      REEQQNQYQVDLGANRLVIPTLD 803
L. sakei TMW 1.1399      REEQQNQYQVDLGASRLIIPTLD 803
L. sakei 23K             REEQQNQYQVDLGASRLVIPTLD 803
                         **************.**:*****
```

Alignment of PepR amino acid sequences, obtained by sequencing of *pepR* genes of four *L. sakei* strains (*L. sakei* TMW 1.1398 and TMW 1.1399, TMW 1.1392 and TMW 1.1388) and subsequent translation (*L. sakei* 23K sequence was obtained from database).

```
L. sakei 23K          MKQGTTILTLDNGYHLWTNTQGTGDIHLLCLHGGPGGNHEYWENFGKELADLGVQVHMYD 60
L. sakei TMW1.1388    MKQGTTILTLDNGYHLWTNTQGTGDIHLLCLHGGPGGNHEYWENFGKELADLGVQVHMYD 60
L. sakei TMW1.1392    MKQGTTILTLDNGYHLWTNTQGTGDIHLLCLHGGPGGNHEYWENFGKELADLGVQVHMYD 60
L. sakei TMW1.1399    MKQGTTILTLDNGYHLWTNTQGTGDIHLLCLHGGPGGNHEYWENFGKELADLGVQVHMYD 60
L. sakei TMW1.1398    MKQGTTILTLDNGYHLWTNTQGTGDIHLLCLHGGPGGNHEYWENFGKELADLGVQVHMYD 60
                      ************************************************************

L. sakei 23K          QLGSFYSDQPDYSKPGNDQLLTYDYFLDEVEEVRQKLGIDNFYLIGQSWGGALVQMYAAK 120
L. sakei TMW1.1388    QLGSFYSDQPDYSKPGNDQLLTYDYFLDEVEEVRQKLGIDNFYLIGQSWGGALVQMYAAK 120
L. sakei TMW1.1392    QLGSFYSDQPDYSKPGNDQLLTYDYFLDEVEEVRQKLGIDNFYLIGQSWGGALVQMYAAK 120
L. sakei TMW1.1399    QLGSFYSDQPDYSKPGNDQLLTYDYFLDEVEEVRQKLGIDNFYLIGQSWGGALVQMYAAK 120
L. sakei TMW1.1398    QLGSFYSDQPDYSKPGNDQLLTYDYFLDEVEEVRQKLGIDNFYLIGQSWGGALVQMYAAK 120
                      ************************************************************

L. sakei 23K          YGQHLKGAIISSMVDEIDEYVTNINKIREDIMTPEQLKFMQDCEAKNDYDNDEYQALVDK 180
L. sakei TMW1.1388    YGQHLKGAIISSMVDEIDEYVTNINKIREDIMTPEQLKFMQDCEAKNDYDNDEYQALVDK 180
L. sakei TMW1.1392    YGQHLKGAIISSMVDEIDEYVTNINKIREDIMTPEQLKFMQDCEAKNDYDNDEYQALVDK 180
L. sakei TMW1.1399    YGQHLKGAIISSMVDEIDEYVTNINKIREDIMTPEQLKFMQDCEAKNDYDNDEYQALVDK 180
L. sakei TMW1.1398    YGQHLKGAIISSMVDEIDEYVTNINKIREDIMTPEQLKFMQDCEAKNDYDNDEYQALVDK 180
                      ************************************************************

L. sakei 23K          LNAGYVDRKQPLAISHLIPTMATDVYGVFQGDNEFVVTGKLKDWHFRDQLHKITVPTLIT 240
L. sakei TMW1.1388    LNAGYVDRKQPLAISHLIPTMATDVYGVFQGDNEFVVTGKLKDWHFRDQLHKITVPTLIT 240
L. sakei TMW1.1392    LNAGYVDRKQPLAISHLIPTMATDVYGVFQGDNEFVVTGKLKDWHFRDQLHKITVPTLIT 240
L. sakei TMW1.1399    LNAGYVDRKQPLAISHLIPTMATDVYGVFQGDNEFVVTGKLKDWHFRDQLHKITVPTLIT 240
L. sakei TMW1.1398    LNAGYVDRKQPLAISHLIPTMATDVYGVFQGDNEFVVTGKLKDWHFRDQLHKITVPTLIT 240
                      ************************************************************

L. sakei 23K          FGEHETMPIATAKIMAEKIPHSRLVTTPNGGHHHMIDNAPVYFDHLKTFIKDVESGNFKD 300
L. sakei TMW1.1388    FGEHETMPIATAKIMAEKIPHSRLVTTPNGGHHHMIDNAPVYFDHLKTFIKDVESGNFKD 300
L. sakei TMW1.1392    FGEHETMPIATAKIMAEKIPHSRLVTTPNGGHHHMIDNAPVYFDHLKTFIKDVESGNFKD 300
L. sakei TMW1.1399    FGEHETMPIATAKIMAEKIPHSRLVTTPNGGHHHMIDNAPVYFDHLKTFIKDVESGNFKD 300
L. sakei TMW1.1398    FGEHETMPIATAKIMAEKIPHSRLVTTPNGGHHHMIDNAPVYFDHLKTFIKDVESGNFKD 300
```

Alignment of PepQ amino acid sequences, obtained by sequencing of *pepQ* genes of four *L. sakei* strains (*L. sakei* TMW 1.1398 and TMW 1.1399, TMW 1.1392 and TMW 1.1388) and subsequent translation (*L. sakei* 23K sequence was obtained from database).

```
L. sakei TMW 1.1392    MNNQLAQLQNWLVENNMDVAYISNPTNILYFTGFESDPAERVLALFVFADQDPFLFTPQL 60
L. sakei TMW 1.1399    MNNQLAQLQNWLVENNMDVAYISNPTNILYFTGFESDPAERVLALFVFADQDPFLFTPQL 60
L. sakei TMW 1.1388    MNNQLAQLQNWLVENNMDVAYISNPTNILYFTGFESDPAERVLALFVFADQDPFLFTPQL 60
L. sakei TMW 1.1398    MNNQLAQLQNWLVENNMDVAYISNPTNILYFTGFESDPAERVLALFVFADQDPFLFTPQL 60
L. sakei 23K           MNNQLAQLQNWLVENNMDVAYISNPTNILYFTGFESDPAERVLALFVFADQDPFLFTPQL 60
                       ************************************************************

L. sakei TMW 1.1392    EVESAKKAGWKLDVYGYLDHEDPYAIIADQIKKRMANPTRWALEKDDLPVQRYEAILKQF 120
L. sakei TMW 1.1399    EVESAKKAGWKLDVYGYLDHEDPYAIIADQIKKRMANPTRWALEKDDLPVQRYEAILKQF 120
L. sakei TMW 1.1388    EVESAKKAGWKLDVYGYLDHEDPYAIIADQIKKRMANPTRWALEKDDLPVQRYEAILKQF 120
L. sakei TMW 1.1398    EVESAKKAGWKLDVYGYLDHEDPYAIIADQIKKRMANPTRWALEKDDLPVQRYEAILKQF 120
L. sakei 23K           EVGSAKKAGWKLDVYGYLDHEDPYAIIADQIKKRMANPTRWALEKDDLPVQRYEAILKQF 120
                       ** *********************************************************

L. sakei TMW 1.1392    PNATFPGDASRFMENLKLIKTPEEIALMEAAGREADYAFEVGFNALKAGKTEQDIVAEIE 180
L. sakei TMW 1.1399    PNATFPGDASRFMENLKLIKTPEEIALMEAAGREADYAFEVGFNALKAGKTEQDIVAEIE 180
L. sakei TMW 1.1388    PNATFPGDASRFMENLKLIKTPEEIALMEAAGREADYAFEVGFNALKAGKTEQDIVAEIE 180
L. sakei TMW 1.1398    PNATFPGDASRFMENLKLIKTPEEIALMEAAGREADYAFEVGFNALKAGKTEQDIVAEIE 180
L. sakei 23K           PNATFPGDASRFMENLKLIKTPEEIALMEAAGREADYAFEVGFNALKAGKTEQDIVAEIE 180
                       ************************************************************

L. sakei TMW 1.1392    YALMRKGVMHMSFDTIVQSGINAANPHGGPEANILTPDALVLFDLGTLHKGYMSDATRTV 240
L. sakei TMW 1.1399    YALMRKGVMHMSFDTIVQSGINAANPHGGPEANILTPDALVLFDLGTLHKGYMSDATRTV 240
L. sakei TMW 1.1388    YALMRKGVMHMSFDTIVQSGINAANPHGGPEANILTPDALVLFDLGTLHKGYMSDATRTV 240
L. sakei TMW 1.1398    YALMRKGVMHMSFDTIVQSGINAANPHGGPEANILTPDALVLFDLGTLHKGYMSDATRTV 240
L. sakei 23K           YALMRKGVMHMSFDTIVQSGINAANPHGGPEANILTPDALVLFDLGTLHKGYMSDATRTV 240
                       ************************************************************
L. sakei TMW 1.1392    AFGKPDAKSLEIHKVCLEANLAAQDAVKPGITAAELDKIARDVITKAGYGEYFIHRLGHG 300
```

```
L. sakei TMW 1.1399        AFGKPDAKSLEIHKVCLEANLAAQDAVKPGITAAELDKIARDVITKAGYGEYFIHRLGHG 300
L. sakei TMW 1.1388        AFGKPDAKSLEIHKVCLEANLAAQDAVKPGITAAELDKIARDVITKAGYGEYFIHRLGHG 300
L. sakei TMW 1.1398        AFGKPDAKSLEIHKVCLEANLAAQDAVKPGITAAELDKIARDVITKAGYGEYFIHRLGHG 300
L. sakei 23K               AFGKPDAKSLEIHKVCLEANLAAQDAVKPGITAAELDKIARDVITKAGYGEYFIHRLGHG 300
                           ************************************************************

L. sakei TMW 1.1392        IGTSEHEFPSIMEGNDMIIKPGMCFSIEPGIYIPDVAGVRIEDCVHVTETGAESFTHMTK 360
L. sakei TMW 1.1399        IGTSEHEFPSIMEGNDMIIKPGMCFSIEPGIYIPDVAGVRIEDCVHVTETGAESFTHMTK 360
L. sakei TMW 1.1388        IGTSEHEFPSIMEGNDMIIKPGMCFSIEPGIYIPDVAGVRIEDCVHVTETGAESFTHMTK 360
L. sakei TMW 1.1398        IGTSEHEFPSIMEGNDMIIKPGMCFSIEPGIYIPDVAGVRIEDCVHVTETGAESFTHMTK 360
L. sakei 23K               IGTSEHEFPSIMEGNDMIIKPGMCFSIEPGIYIPDVAGVRIEDCVHVTETGAESFTHMTK 360
                           ************************************************************

L. sakei TMW 1.1392        ELQTFX 366
L. sakei TMW 1.1399        ELQTFX 366
L. sakei TMW 1.1388        ELQTFX 366
L. sakei TMW 1.1398        ELQTFX 366
L. sakei 23K               ELQTFX 366
                           ******
```

Alignment of *brnQ*-fragments obtained from four *L. sakei* strains

```
L. sakei TMW 1.1290        TTGGGCAAAAGCCGCAACTAAACCGATCGCCGTGGTTAAGCAAGTGACGGTGATCAAAGT 60
L. sakei TMW 1.22          TTGGGCAAAAGCCGCAACTAAACCGATCGCCGTGGTTAAGCAAGTGACGGTGATCAAAGT 60
L. sakei TMW 1.114         TTGGGCAAAAGCCGCAACTAAACCGATCGCCGTGGTTAAGCAAGTGACGATGATCAAAGT 60
L. sakei TMW 1.578         TTGGGCAAAAGCCGCAACTAAACCGATCGCCGTGGTTAAGCAAGTGACGATGATCAAAGT 60
                           ************************************************* **********

L. sakei TMW 1.1290        GGCTAAGATGGCCTGACCAGCGACGCCCATATAATGATTGACAATTTGGTCAAAAGCGAT 120
L. sakei TMW 1.22          GGCTAAGATGGCCTGACCAGCGACGCCCATATAATGATTGACAATTTGGTCAAAAGCGAT 120
L. sakei TMW 1.114         GGCTAAGATGGCCTGACCAGCGACGCCCATATAATGATTGACAATTTGGTCAAAAGCGAT 120
L. sakei TMW 1.578         GGCTAAGATGGCCTGACCAGCGACGCCCATATAATGATTGACAATTTGGTCAAAAGCGAT 120
                           ************************************************************

L. sakei TMW 1.1290        GCCGCCATTAGCGGAAACTTTGAATTTACCTAAGGACATCGCGCCAACGAAGATCAAACC 180
L. sakei TMW 1.22          GCCGCCATTAGCGGAAACTTTGAGTTTACCTAAGGACATCGCGCCAACGAAGATCAAACC 180
L. sakei TMW 1.114         GCCGCCATTAGCGGAAACTTTGAGTTTACCTAAGGACATCGCGCCAACGAAGATCAAACC 180
L. sakei TMW 1.578         GCCGCCATTAGCGGAAACTTTGAGTTTACCTAAGGACATCGCGCCAACGAAGATCAAACC 180
                           ********************** *************************************

L. sakei TMW 1.1290        AACGTAGATGACGGCGATCATTGCCATTGCGAAGACGCCGGCACGGGCAGTGACCAGCGC 240
L. sakei TMW 1.22          AACGTAGATGACGGCGATCATTGCCATTGCGAAGACGCCGGCACGGGCAGTGACCAGCGC 240
L. sakei TMW 1.114         AACGTAGATGACGGCGATCATTGCCATTGCGAAGACGCCGGCACGGGCAGTGACCAGCGC 240
L. sakei TMW 1.578         AACGTAGATGACGGCGATCATTGCCATTGCGAAGACGCCGGCACGGGCAGTGACCAGCGC 240
                           ************************************************************

L. sakei TMW 1.1290        GACACTGTTAGCGCGTTTTTTGC 263
L. sakei TMW 1.22          GACACTGTTAGCGCGTTTTTTGC 263
L. sakei TMW 1.114         GACACTGTTAGCGCGTTTTTTGC 263
L. sakei TMW 1.578         GACACTGTTAGCGCGTTTTTTGC 263
                           ***********************
```

Alignment of ArcT amino acid sequences obtained from *L. plantarum* ST-III, *L. sakei* 23K, and *L. lactis* Il1403.

```
L. plantarum ST-III.     MKIAGFGVEAWLNAHEREATTDISQSSIAALTMAEIAALDDQQAPQDFYE 50
L. sakei 23K             MEIANFGVEEWLNVYETQATLDIAQSTIASMTMSELMALSPDNGTQ-FYQ 49
L. lactis Il1403         MELVQFGCEDWLNVWEKSATIDIAQSTIDSLSLEEVLAFEEDNGEA-FMS 49
                         *::. ** * ***. * .** **:**:* :::: *: *:. ::.   * .

L. plantarum ST-III.     ELGAARLDYGWIEGSPRFKELVSQLYEQVPAANVLQTNGATGANHLAIYS 100
L. sakei 23K             DLAQQKMNYGAIEGSEAFKQAVSELYQTVNSNQVLQTNGATGANLLALYA 99
L. lactis Il1403         QMMKEKFSYGWIEGSPAFKSEVAKLYKRVPEDNILSTNGATGANFLTILG 99
                         ::   ::.** ****  **. *::**: *   ::*.******** *:: .

L. plantarum ST-III.     LVEPGDHVIALYPSYQQLYDIPKSLGATVDYWHIHESAGWLPDIMELQRL 150
L. sakei 23K             LVKPGDHVISMFPTYQQLYEIPISIGATVSYWQLDEANNWVPDIAELKKL 149
L. lactis Il1403         LIGQGDHVIAQYPSYQQLYDWPKTLGAQVDYWHIKEENNWLPQIEELRRL 149
                         *:  *****: :*:*****: * ::** *.**::.*  .*:*:* **::*

L. plantarum ST-III.     IRPETKMILLNNAINPTGSLLDRTLLEQVVTLARSVGAYVLADEVYEPLD 200
L. sakei 23K             IRPETKLICLNNANNPTGTVISTELMQAIVEVARTVGAYVLVDEVYLPLG 199
```

```
L. lactis Il1403          VKSNTKLICLNNAAQPTGAIMSPKFLSEVVEIARSVDAYILCDEVYLPLD 199
                          ::.:**:* **** :***:::.  ::. :* :**:*.**:* **** **.

L. plantarum ST-III.      ETP-FVSIADLYERGIAVNSLSKTYSAPGIRIGWTATPSQAIADIFRKYR 249
L. sakei 23K              EGQQTTSIADLYELGIATNSLSKTYSVPGIRVGWLVAN-ETLTDLFRKYR 248
L. lactis Il1403          EETPYSPIADLYEKGISTNSISKTYSVPGIRVGWVATQDRDLCNEFRKIR 249
                          *     .****** **:.**:*****.****:** .:  . : : *** *

L. plantarum ST-III.      DYTMICGGVLNDQLAVRILAHRQRVLARNRELVSRNLKILTEWVAQEPRV 299
L. sakei 23K              DYTMICAGVFSDQLAVYVLQHRKQVLARNRALVQRNLKIFKAWVAQEPLV 298
L. lactis Il1403          DYTLICTGVFDDAVAALVLKHKDKVLERARKIVKGNLSILKEWVENEPLV 299
                          ***:** **:.* :*. :* *:.:** * * :*. **.*:. ** :** *

L. plantarum ST-III.      ELITPHGISVSCIKLIVPI--DDETFCQQLLRDTGVLLVPGSRFDIPGHA 347
L. sakei 23K              DVVYPESVSTSFIHFKEIE--DDEAFCKYLLKEYGVLLVPGKRFEIPGHA 346
L. lactis Il1403          SMVYPNAVSVSFVKFEELDPTKTEDFAIQLLREKGVLIIPGNRFDLSGYA 349
                          .:: *..:*.* :::      . * *.  **:: ***::**.**::.*:*

L. plantarum ST-III.      RLGYCTDTPTLQRGLDLLGQYLRRFD- 373
L. sakei 23K|             RLGYCAPEATLKKGLAELSKALRTY-- 371
L. lactis Il1403          RIGYCTDETTLRQGLKLLSEFLREYQV 376
                          *:***:  .**::**  *.: ** :
```

Alignment of AspD amino acid sequences obtained from *L. oris* PB013-T2-3, *L. sakei* 23K, and *L. antri* DSM 16041

```
L. oris PB013-T2-3        ------------MTADMNIFNQVDDSQVSNLDQLSNFEVAAIFNKYAQHN 38
L. antri DSM 16041        MGIVVCDRRDKTMTADMNIFEQVDDSKVSSLDQLSNFEVAAIFNKYAQHN 50
L. sakei 23 K             ----------------------MDKIDVTKLTQMSNFEVAALFYKYALTN 28
                                                :*. .*:.* *:*******:* ***  *

L. oris PB013-T2-3        LRGNEVVNGGRGNPNWIATTARLAYSRLLEFGVTEAERTY-FDPRGMAGD 87
L. antri DSM 16041        LRGNEVVNGGRGNPNWIATTARLAYSRLLEFGVTEAERTY-FDPRGMAGD 99
L. sakei 23 K             SRGLRAINVGRGNPNWINTQARFAFNRIVEFGMKESLQTINLDDGNLAGY 78
                           ** ..:* ******** * **:*:.*::***:.*: :*  :*  .:**

L. oris PB013-T2-3       VQKEGIYQRLMIALK-SSRRDIFLRTVIDAAISQLAIKDKDAFVYELVDG 136
L. antri DSM 16041       VQKAGIYQRLMIALK-ESRRDIFLRTAIDAAVSQLAIKDKDAFVYELVDG 148
L. sakei 23 K            TDQTGIAERFNQFLDGDNPVDAFLKQALAYTRDVMHIN-QDELVFEWVDG 127
                         .:: ** :*:   *. ..  * **: .:  : . : *: :* :*:* ***

L. oris PB013-T2-3        ALGDHYPYPPRCLTYTEKVLQQYLQKVCFKDVQMAQDVDIFPTEGGTAAM 186
L. antri DSM 16041        ALSDHYPYPPRCLTYTEQVLQQYLQKVCFKDVQLAADVDVFPTEGGTAAM 198
L. sakei 23 K             VIGNHYPEPSRSLVNVEKILNRYLEVNLYRGEHLSDKTKVFPTEGGTAAM 177
                          .:.:*** *.*.*. .*::*::**:   ::. ::: ...:**********

L. oris PB013-T2-3        VYIFQELHYAHVLYPGDTVVVNSSIFTPYLQIPELSEYNLRIKTVTTKRE 236
L. antri DSM 16041        VYIFQELHYAHILYPGDTVVVNSSIFTPYLQIPELSEYNLRIKTVTTKRE 248
L. sakei 23 K             VYLFNELKVSHILEAGDTIAINTPIFTPYLQIPELNEFKLQEFNVSSDES 227
                          **:*:**: :*:* .***:.:*:.***********.*::*:   .*::...

L. oris PB013-T2-3        NNWQMTDEQFEQLKDPRVKAFFAVNPTNPTARAFTPERLAKFKEVIKANP 286
L. antri DSM 16041        DNWQMTDEQFEELKDPRVKAFFAVNPTNPTARAFTPERLAKFKEVVKANP 298
L. sakei 23 K             DDWQLVDHKFEELKDPNVKAFFVVNPTNPTSRAFSDHALDKLKEVVEANP 277
                          ::**:.*.:**:****.*****.*******:***: . * *:***:***

L. oris PB013-T2-3        DLVIITDDVYGTFSPSYQSIFAVAPHNTILVYSFSKLYGATGQRLGVVCM 336
L. antri DSM 16041        DLVIITDDVYGTFSPSYQSIFAVAPHNTILVYSFSKLYGATGQRLGVVCM 348
L. sakei 23 K             NLVIITDDVYGTFVDDFKTIYSVVPHNTLLVYSFSKLYGATGQRIGLIAM 327
                          :************  .:::*::*.****:****************:*::.*

L. oris PB013-T2-3        HHQNVCDRIIQEN-LQNRRLKELDERRYSIVVPDPHKMKFIDRMVADSRA 385
L. antri DSM 16041        HHQNVCDRIIQEN-LQNRRLKELDERRYSIVVPNPHKMKFIDRMVADSRA 397
L. sakei 23 K             HDDNIFDKLIEEMTAEDPVIREAFRKRYSYVTNKPHEMSFIDRTVADSRN 377
                          *.:*: *::*:*   ::  ::*  .:*** *. .**:*.**** *****

L. oris PB013-T2-3        IGLYHTAGLSSPQQVMMDMFALSNLKDAG-LSPYVQLSREVVAARYHEFW 434
L. antri DSM 16041        IGLYHTAGLSSAQQVMMDMFALSNLKDAG-LSPYVKLSREVVADRYHEFW 446
L. sakei 23 K             IGLYHAAGLSTPQQITMALFSLTNLIYEGREDPYVTASKEIVSHRYKAFW 427
                          *****:****:.**: * :*:*:**   *  .***  *:*:*: **: **
```

```
L. oris PB013-T2-3         HGLGIQPDETPENTRYYTLVDIFDLMRQRHGKEFCQYFKDNYNYLDFTYR 484
L. antri DSM 16041         HGLGILPDETPENTRYYTLVDIFDLMRQRHGKEFCQYFKDNYNYLDFTYR 496
L. sakei 23 K              ESLGLVVKTTEENAEYYTVFSIYKLAESKYSKDFRAYLEQNCNHLAFEWR 477
                           ..**:  . * **:.***:..*:.* ..::.*:*  *:::* *:* * :*

L. oris PB013-T2-3         LATEFGAVVMDATAFGAEKGNVRVSLANLEKADYRKLARAILDLVDEYYQ 534
L. antri DSM 16041         LATEFGAVVMDATAFGAEKGNVRVSLANLKKADYRKLAQAILDLVDEYYQ 546
L. sakei 23 K              LAAEYGVVVMDGAGMGTKAGYLRISLANRPDKDYETVGGRISDLLASYYT 527
                           **:*:*.****.:.:*:: * :*:****  . **..:.  * **: .**

L. oris PB013-T2-3         VFKKKNKK 542
L. antri DSM 16041         VFKKKSKK 554
L. sakei 23 K              EYLQSKAQ 535
                            : :.. :
```

Mcherry-sequence

ATGGTGTCGAAGGGCGAGGAGGACAACATGGCGATCATCAAGGAGTTCATGCGCTTCAAGGTGCAC
ATGGAGGGCTCGGTGAACGGCCACGAGTTCGAGATCGAGGGCGAGGGCGAGGGTCGGCCGTACGAG
GGCACGCAGACCGCCAAGCTGAAGGTCACCAAGGGCGGTCCGCTGCCGTTCGCCTGGGACATCCTG
TCCCCGCAGTTCATGTACGGTAGCAAGGCCTACGTCAAGCACCCCGCCGACATCCCCGACTACCTG
AAGCTCTCGTTCCCGGAGGGCTTCAAGTGGGAGCGCGTGATGAACTTCGAGGACGGCGGTGTGGTC
ACCGTCACCCAGGACTCGTCCCTCCAGGACGGTGAGTTCATCTACAAGGTGAAGCTGCGCGGCACC
AACTTCCCGTCCGACGGTCCGGTCATGCAGAAGAAGACCATGGGCTGGGAGGCCTCGTCGGAGCGC
ATGTACCCCGAGGACGGCGCGCTGAAGGGCGAGATCAAGCAGCGCCTGAAGCTGAAGGACGGCGGC
CACTACGACGCCGAGGTCAAGACGACCTACAAGGCGAAGAAGCCGGTGCAACTGCCGGGCGCCTAC
AACGTGAACATCAAGCTGGACATCACGAGCCACAACGAAGACTACACCATCGTCGAGCAGTACGAA
CGGGCCGAGGGCCGCCACTCGACCGGCGGTATGGACGAACTGTACAAGTAA

ilvE-sequence of *S. carnosus* TMW 2.801

ATGTCAGAAAAAGTAAAATTTGAAAAACGTGAAGACTTAAAACAAAAACCAGATCCAAAAAATTTA
GGGTTTGGTCAATACTTCACAGATTACATGTTAAGTTATGACTACGATAGCGAAAAAGGCGGTTGG
CATGATTTGAAGATTACACCTTATGCACCGATCGAACTAGATCCAGCTGCACAAGGACTTCATTAT
GGTCAACTTGTATTCGAAGGATTAAAAGCTTATAAACATAACGGTGAAGTGGTTTTATTCAGACCA
GACCAAAACTTCGCACGTATCAATCAATCACTTGATCGTTTGGAAATGCCTCAGATTGATGAAGAA
GAGCTATTAGAAGGCTTGAAACAATTAGTAGATGTAGAACGTGATTGGGTGCCTGAAGGCGAAGGA
CAATCTTTATATATTCGTCCATTTGTATTTGCAACAGAAGCAGGATTAGGTGTTCACCCTGCACAT
AACTATAAATTATTAATTATCTTATCTCCTTCTGGTTCTTACTATGGTGGAGATTCATTGAAACCA
ACACGTATTTATGTTGAAGACGAATACGTTCGTGCTGTACGCGGTGGTGTAGGTTTTGCAAAAGTT
GCAGGTAACTATGCAGCTAGTTTACTATCACAATCTAATGCTAATGAACAAGGCTATGACCAAGTA
TTATGGTTAGATGGTGTAGAACGCAAATATATTGAAGAAGTGGGCAGTATGAATATCTTCTTCGTA
GAAAATGGTAAACTTGTAACACCTAAATTAAACGGTAGTATCTTACCAGGTATTACACGTAAGACT
GTTATCGCTTTAGCAAAAGAATTAGGATATGAAGTAGAAGAACGTCATATCTCTATCGATGAATTA
CTAGAGTCTTATGATAAAGGCGAATTAGAAGAAGTATTTGGAACAGGTACTGCTGCGGTTATTTCA
CCAGTAGGCACTTTAAAATATGAAGATCGTGAAATCACAATCAACAATAATGAAACTGGTCCAATT
ACACAACGCTTATATGATGAATATACAGGTATTCAAAGCGGTAAATTAGACGATCCACAAGGTTGG
AGAGTCGTAGTACCAGAATATTAA

ilvE-sequence of *L. paracasei* TMW 1.1434

ATGAGTGTCAATATTGATTGGAACAATCTAGGCTTCGATTATATGCAACTGCCATACCGTTATGTT
GCCCACTGGAAAGACGGGGCATGGGATGAAGGTAAGCTGTCCACCGATCCCAACCTGACGATGAAT
GAGGGCTCCCCGATTTTGCATTATGGTCAAGGCGCTTTTGAAGGTATGAAGGCTTACCGGACCAAA
TCAGGAAAAATCCAACTTTTCCGGCCTGACCAAAATGCCCATCGACTGCACAATTCTGCTGACAAA
CTACTGATGCCGCCATCTCCTGAAGATCGCTTTATTGATGCGGTCAAGCAAGTGGTTGCCGCTAAC
CATGAATACGTACCGCCATATGGCACCGGTGCTACCTTATACTTACGCCCGATTTTGATTGGTGTC
GGTCCCAATATCGGGGTTGCTCCGGCTAAGGAATACATTTTCGATGTCTTCGCCATGCCAGTCGGC
CCCTACTTCAAAGGCGGCATGGTGCCAACCAAGTTCATCGTTGCCGATCAATTCGACCGAGCGGCG
CATTACGGCACTGGCCAATCGAAAGTCGGAGGGAACTACGCCGCATCCTTGCAAGCCGGCAAATTC
GCCCACGAGCACGGTTATGGCGATGCAATCTATCTGGACCCAATTGAACATAAATATATTGAAGAA
GTTGGGTCAGCTAACTTCTTCGGCATTAGCAAAGATGGTAAAACTTTGAAAACACCGAAGTCACCA
TCCATCCTGCCAAGTATCACCAAGTATTCGATTTTGGCACTGGCGCATGATCGGTTTGGCATGACC
ACTGAAGAAACTAAGATTGCGATTACCGATTTGGACCAATTTGGTGAAGCCGGGGCCTGTGGCACT
GCGGCGGTCATCACCCCGATTGCCAGCATCACCTACGAAGACCACGAACATGTCTTTTATTCGGAA
ACCAAAGTCGGTCCATATACACAGAAACTGTATGATGAACTGACGGGTATCCAGTTTGGCGATGTA
CCGGCACCTGAAGGCTGGGTCGTTGACGTCCCGTTTAATTAAGAATTCCGGTGCTGGATAA

ilvE-sequence of *E. faecalis V583*

ATGGAAAAAGCCAATCTTGATTGGAATAATTTAGGATTTTCTTACATTAAAACGCCCTTTCGTTATATTAGTT
ATTGGCGAGATGGCAAGTGGGAAGAAGGCACGCTAACAGATAACAATCAGTTAACGATTAGTGAAGGTTCGCC
TGCTCTACATTATGGCCAACAATGTTTTGAAGGATTAAAAGCGTATCAATGTGCAGATGGTTCTGTCAATTTG
TTTCGTCCAGATGAAAATGCCAAACGGCTACAAAAAAGTTGTGCGCGTCTACTGATGCCACAAGTGCCAGTAG
AAACCTTTGTTTCTGCATGCCAAGAAGTGGTGAAAGCCAACCTTGCTTATTTACCGCCATATGGAACGGGCGG
TACGTTGTATCTTCGCCCTTATATGATTGGTGTCGGCGATAATATTGGCGTAGCACCTGCCAAAGAATACATT
TTTTCTATTTTTTGCGTGCCGGTTGGTTCGTATTTTAAAAATGGTTTAGCACCAACCAACTTTATCGTTTCTG
AATATGATCGAGCAGCGGGTCGAGGAACTGGGGCAGCTAAAGTGGGGGGCAATTATGCAGCTAGTCTTTTACC
AGGTGCAGAAGCACATGAGAAAGAATTTAGTGATTGCATTTATTTAGATCCGTATACGCATACGAAAATTGAA
GAAGTTGGTGCAGCCAATTTCTTTGGCATAACCAAAGATGGTACTTTTATTACGCCGAAATCAGCGTCTATTT
TGCCAAGTATTACTAAATATTCATTATTGACGCTAGCAAAAGAACGTTTAGGGATGACGGCGCTTGAAGGAGA
CGTTTACATTGATCGATTAGCGGACTTCTCCGAAGCGGGTGCTTGTGGCACAGCGGCGATTATTTCTCCTATT
GGTGGGATTCAAAACGGCACAGATTTCCATGTATTTTATAGTGAAACGGAAGTTGGACCAGTGACTAAGCAAC
TATATGATGAACTGGTTGGCATACAGTTTGGTGACAAAGAAGCGCCAGAAGGCTGGATTGTAAAAGTTTAA

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08.2005 – 09.2005	**Industriepraktikum** CRELUX GmbH, Martinsried