Microbiological Research 221 (2019) 1–9

Contents lists available at ScienceDirect

Microbiological Research

journal homepage: www.elsevier.com/locate/micres

Comparative genomics of *Lactobacillus sakei* supports the development of starter strain combinations

Lara Eisenbach, Andreas J. Geissler, Matthias A. Ehrmann, Rudi F. Vogel*

Lehrstuhl für Technische Mikrobiologie, Technische Universität München, Freising, Germany

ARTICLE INFO

Keywords:
Lactic acid bacteria
Lactobacillus sakei
Pan-
Accessory-
Core-genome
Comparative genomics
Assertiveness
Competiveness
Raw fermented sausages

ABSTRACT

Strains of *Lactobacillus sakei* can be isolated from a variety of sources including meat, fermented sausages, sake, sourdough, sauerkraut or kimchi. Selected strains are widely used as starter cultures for sausage fermentation. Recently we have demonstrated that control about the lactic microbiota in fermenting sausages is achieved rather by pairs or strain sets than by single strains. In this work we characterized the pan genome of *L. sakei* to enable exploitation of the genomic diversity of *L. sakei* for the establishment of assertive starter strain sets. We have established the full genome sequences of nine *L. sakei* strains from different sources of isolation and included in the analysis the genome of *L. sakei* 23K. Comparative genomics revealed an accessory genome comprising about 50% of the pan genome and different lineages of strains with no relation to their source of isolation. Group and strain specific differences could be found, which namely referred to agmatine and citrate metabolism. The presence of genes encoding metabolic pathways for fructose, sucrose and trehalose as well as gluconate in all strains suggests a general adaptation to plant/sugary environments and a life in communities with other genera. Analysis of the plasmidome did not reveal any specific mechanisms of adaptation to a habitat. The predicted differences of metabolic settings enable prediction of partner strains, which can occupy the meat environment to a large extent and establish competitive exclusion of autochthonous microbiota. This may assist the development of a new generation of meat starter cultures containing *L. sakei* strains.

1. Introduction

Strains of *Lactobacillus (L.) sakei* can gain dominance in a variety of spontaneously fermented foods, representing different environments, e.g. sake, sauerkraut, sourdough and meat (Champomier-Verges et al., 2002). Due to its high competitiveness in meat fermentations, selected strains of *L. sakei* are commercially used as starter cultures (Hammes et al., 1990; Hammes and Hertel, 1996). Additionally, *L. sakei* increasingly attracts attention as probiotic and protective culture (Zagorec and hampomier-Verges, 2017; Zhang et al., 2018).

The ability to successfully occupy such a broad range of habitats is reflected in a correspondingly high intraspecies heterogeneity with respect to key phenotypic properties. Genomic variety has been indicated upon DNA-DNA reassociation analysis that revealed very low levels of relatedness between strains. (Chaillou et al., 2009). Moreover, Chaillou et al. show that strains of *L. sakei* species grouped into three lineages with distinct evolutionary histories based on genetic analysis (Chaillou et al., 2013).

Different metabolic and genomic studies reflected that *L. sakei* strains are well adapted to the meat environment. A proteomic approach identified down-regulation of the glycolytic pathway more efficiently in meat-starter and probiotic strains than in other strains in presence of ribose as single carbon source (McLeod et al., 2010). This may indicate some adaptation to RNA (ribose)-rich environments. The first full genome sequence of *L. sakei* 23K published by Chaillou et al., revealed an adaptation to meat at the metabolic level (Chaillou et al., 2005), like a highly efficient purine nucleoside metabolism. Moreover, different genes for stress response are encoded as well as genes, which are involved in biofilm formation and cellular aggregation (Chaillou et al., 2005). A microarray-based comparative genome hybridization of additional strains by Nyquist et al. (Nyquist et al., 2011) revealed that the majority of the conserved genes correlates with adaptation to meat, like ability to flexibly use of meat components, surviving during ripening and storage, and the ability to overcome changes in redox potential and oxygen level. The draft genome sequence of *L. sakei* LS25 (2.0 Mb) was published by McLeod et al. (McLeod et al., 2013). *L. sakei* LS25 encoded a citrate cluster, which is not orthologous to the one encoded in strain 23K. Furthermore, 250 protein-encoding genes were

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* Corresponding author at: Technische Universität München, Lehrstuhl für Technische Mikrobiologie, Gregor-Mendel-Straße 4, 85354, Freising, Germany.
E-mail addresses: lara.eisenbach@wzw.tum.de (L. Eisenbach), a-geissler@hotmail.de (A.J. Geissler), m.ehrmann@wzw.tum.de (M.A. Ehrmann), rudi.vogel@wzw.tum.de (R.F. Vogel).

https://doi.org/10.1016/j.micres.2019.01.001
Received 19 November 2018; Received in revised form 7 January 2019; Accepted 10 January 2019

found to be unique to LS25 (McLeod et al., 2013).

Recently it has been demonstrated that assertiveness of *L. sakei,* and its close relative *L. curvatus,* in a competitive setting in a fermenting sausage model is mostly achieved by the use of strain combinations rather than single strains (Janßen et al., 2018a, b). The background of assertive strain combinations supposedly is the occupation of multiple options of the rich ecological niche provided in the fermenting sausage by metabolic complementarity with subsequent competitive exclusion of contaminants originating from the raw meat. Comparative genomics of *L. curvatus* further revealed that the selection of assertive partner strains may even be predicted from their complementary accessory genomes (Eisenbach et al., 2018).

To further elucidate key features delineating strategies of adaptation, assertiveness, and survival in meat fermentations and possibly exploit these for assertive strain combinations we sequenced and compared genomes of additional nine *L. sakei* strains isolated from different sources like meat, starter cultures and sourdough.

2. Material and methods

2.1. Bacterial strains and cultivation

Bacterial strains of *L. sakei* were taken from the strain collection of Technische Mikrobiologie Weihenstephan (TMW) and are listed in Table 1. The complex medium MRS (20 g L^{-1} glucose, 10 g L^{-1} casein pepton, 10 g L^{-1} meat extract, 5 g L^{-1} yeast extract, 1 g L^{-1} Tween 80, 2 g L^{-1} K_2HPO_4, 5 g L^{-1} Na-Acetate, 2 g L^{-1} $(NH_4)_2$ citrate, 0.2 g L^{-1} $MgSO_4*7H_2O$, 0.05 g L^{-1} $MnSO_4*H_2O$) (de Man et al., 1960) was used for cultivation of strains. The cultures were grown at 30 °C under anaerobic conditions. For DNA isolation a preculture was inoculated. After 14 h of incubation 2.5 ml were inoculated in a main culture of 45 ml MRS and incubated for 8 h at 30 °C. Cells were harvested in the late exponential growth phase.

2.2. Genomic analysis

The genomic analysis was performed like described in detail by Eisenbach et al (Eisenbach et al., 2018).

2.3. DNA isolation and genome analysis

High molecular DNA was isolated by using Qiagen Genomic-tip 100/G (Qiagen, Germany) and Qiagen Genomic DNA kit (Qiagen, Germany). After precipitation of the DNA, it was dissolved in 200 µl TE.

A library with an insert size of 8–12 kb were constructed and Single-molecule real-time sequencing (SMRT) (PacBio RS II) resulting in at least 200 Mb of raw data from one to two SMRT cells (1 × 120-min movies) applying P4-C2 chemistry was carried out at GATC Biotech (Konstanz, Germany). The draft genome assembly was performed by SMRT® Portal.

The DNA sequences were annotated by NCBI under Bioproject ID: PRJNA317414. In addition, the sequences were annotated with Rapid Annotation Subsystem Technology (RAST) server to generate lists of E.C. numbers for metabolic analysis (Aziz et al., 2008). The relationship between sequenced strains was determined by Average Nucleotid Identity (ANI) calculation with web tool ANI calculator (http://enve-omics.ce.gatech.edu/ani/). Moreover, R-Studio Version 3.2.1 was used to create dendograms by package pvclust. PHASTER (Arndt et al., 2016) was used to identify gene clusters of prophages. CRISPRdb (http://crispr.i2bc.paris-saclay.fr) was used for identification of CRISPR/Cas gene clusters (Grissa et al., 2007). BAGEL (de Jong et al., 2006) was used to determine putative gene clusters, which encoded genes for the production of bacteriocins.

For identification of pan and core genomes the BlAst Diagnostic Gene findEr (BADGE) (Behr et al., 2016) was used with default settings, but “megablast percent identity cut” and “megablast within group qscov” were changed to 90 or 0.90 and “min DMG occurrence” was set at 0.1. Blast Ring Image Generator (BRIG) (Alikhan et al., 2011) was used to visualized the genomic comparison of *L. sakei* strains, in which annotated ORFs of pan genome was used as reference.

Metabolic predictions were identified along paralougs of each step, and every sequence of a predicted enzyme was additionally controlled by smart BLAST (https://blast.ncbi.nlm.nih.gov/smartblast/).

2.4. Detection of bacteriocin/antimicrobial peptide production

Production of bacteriocins were tested by an inhibition test. All strains used are listed in table S1. Predicted producers were incubated in precultures with MRS. Afterwards, 5 µl of the cell suspension was dropped at MRS-Agar with 0.2% glucose and incubated over night at 30 °C under aerobic conditions. The colonies were overlaid with an overlay-agar inoculated with 200 µl of a preculture of different indicator strains of *L. sakei* and *L. curvatus*. The plates were incubated for 2 days at 30 °C under aerobic conditions. Inhibition zones indicated the production of antimicrobial peptides. As positive control *L. curvatus* TMW 1.624 was used because the production of at least one bacteriocin by this strain was demonstrated by Janßen et al. (Janßen et al., 2018b).

2.5. HPLC analyses of metabolites

Two biological replicates were analysed for each strain as well as a non-inoculated control. For precipitation of proteins and acid analytics 50 µl 70% (v:v) perchloric acid (Sigma-Aldrich, St. Louis, USA) were added to 1 ml cell suspension and incubated overnight at 4 °C. After incubation the sample was centrifuged 10 min at maximum speed (13,000*g*) to remove the precipitate. Analysis was carried out by using a Rezex ROA-Organic AcidH+ (Phenomenex, Aschaffenburg, Germany) at 85 °C in combination with a Dionex UltiMate 3000 Pump (Dionex,

Table 1

Summary of the general features of the sequenced genomes of *L. sakei*.

	L. sakei									
Genome	TMW 1.3	23K	TMW 1.46	TMW 1.114	TMW 1.417	TMW 1.578	TMW 1.1396	TMW 1.1398	TMW 1.1239	DSM 20017^T
number of Contigs	2	1	2	2	2	2	2	4	1	1
Genome size (bp)	1,930,337	1,884,661	2,079,718	1,976,338	1,980,463	1,976,312	1,935,698	2,066,572	1,975,742	1,942,056
GC content (%)	41.12	41.26	41.1	41.19	41.18	41.18	41.28	41.17	41.2	41.18
Number of CDS	1,861	1,844	2,03	1,901	1,887	1,898	1,873	2,015	1,880	1,863
Total CDS length (bp)	1,669,711	1,627,920	1,816,491	1,713,165	1,714,895	1,712,580	1,680,893	1,791,826	1,717,616	1,691,532
coding density (%)	86.50	86.38	87.34	86.68	86.59	86.66	86.84	86.71	86.94	87.10
number of plasmids	1	0	1	1	1	1	1	3	0	0
origin	sausage (spain)	meat	starter culture	starter culture	starter culture	starter culture	starter culture	starter culture	sourdough	type strain
accession number (chr)	CP016465	NC_007576	CP015487	CP017566	CP017568	CP017570	CP017273	CP017275	CP017272	CP017271

Idstein, Germany) and a Shodex RI-71 detector (Showa Denko Shodex, Munich, Germany). Quantification was done by using external standards of lactate (Sigma Aldrich, St. Louis, USA), acetate (Carl Roth, Karlsruhe, Germany), citrate (Carl Roth, Karlsruhe, Germany) and glucose (Merck, Darmstadt, Germany) and the Chromeleon evaluation software version 6.80 (Dionex, Idstein, Germany). Glucose was detected like described by Eisenbach et al. (Eisenbach et al., 2018).

2.6. Decarboxylation test medium (DCM)

The utilization of arginine, agmatine, and ornithine by *L. sakei* were tested by using decarboxylation test medium (DCM) like described by Bover-Cid et al. (Bover-Cid et al., 2001). 1 ml of liquid DCM was inoculated with one colony of each strain in triplicates. Cell suspensions were inoculated at 30 °C under anaerobic conditions. The colour was checked at the beginning and after 24 h, 48 h, 72 h 96 h and after 7 days. A positive result was indicated by a colour, which changed from yellow to purple as a result of pH neutralization caused by the decarboxylation or desamination.

2.7. Accession numbers of sequenced genomes

Following accession numbers are part of this study: NC_007576, CP015487, CP015488, CP016465, CP016466, CP017271, CP017272, CP017273, CP017274, CP017275, CP017276, CP017277, CP017278, CP017566, CP017567, CP017568, CP017569, CP017570, CP017571.

3. Results

3.1. Analysis of genomic sequences suggest that species L. sakei is genetically diverse

Strains were compared based on genomic statistics listed in Table 1, and Average Nucleotide Identity (ANI), which is depicted in a dendogram in Fig. S1. All tested and sequenced strains were isolated from different sources like meat, starter cultures or sourdough. The genome sizes of *L. sakei* strains range from 1.8 Mb up to 2.0 Mb. However, the gene content (number of CDS) ranged from 1844 up to 2030. Most strains have one up to three plasmids. Indeed, there are strains without any detected plasmids. The GC-content of sequenced strains is around 41% or 42% (Table 1). The coding density on the chromosomes ranged from 86.38% to 87.34%, whereas the coding density on the plasmids ranged from 75.79% to 86.89% as shown in Table 2.

The ANI of the whole genome was calculated to define the relation of the strains within the species. It suggests that there are two main groups within the species (Fig. S1), which clustered into subgroups. The intra species ANI diversity was 100%–97.05%. All meat associated strains are in one group with the exception of *L. sakei* TMW 1.46, which clustered in a group with *L. sakei* DSM 20017^{T} and TMW 1.1239 both isolated from cereal environments, i.e. Sake-starter (Moto), or sourdough.

3.2. Plasmidome reflects genetically heterogeneity

The plasmidome of *L. sakei* is heterogenous (Table 2). Nevertheless, comparison of the plasmids reveals that pL146-1, pL1114-1, pL11396-1, pL11398-2, pL11398-3 and pL1578-1 are closely similar. Still, within this group pL11396-1, pL11398-2 and pL11398-3 formed a sub group as well as pL1114-1 and pL1578-1. All these plasmids encoded the gene cluster of an electron transfer flavoprotein and a ribonucleotide-diphosphate reductase. Interestingly, both genes are encoded in the chromosome of *L. sakei* DSM 20017^{T} and TMW 1.417, which did not carry any such genes on plasmids. But for both strains could be observe that the gene cluster is flanked by IS30 family transposase. Furthermore, plasmid pL13-1 encoded the electron transfer flavoprotein as well, but no cluster of ribonucleotide-diphosphate reductase is encoded either at a plasmid or at the chromosome.

3.3. A few strains encoded at least one gene cluster for bacteriocin production

The web-based tool BAGEL was used to identify bacteriocin gene clusters within the whole genomes. The results suggest that a few strains of *L. sakei* carry gene clusters, which are correlated with genes of bacteriocin production like it is shown in Table S2. Indeed, *L. sakei* TMW 1.46 has a gene cluster, which encodes proteins homologous to those involved in production of carnocin, and *L. sakei* TMW 1.3 encodes a putative protein, which was homologous with lactocin-S. Additionally, the results suggest that *L. sakei* TMW 1.114 and TMW 1.578 are able to produce peptides belonging to COMC family (Table S2). The expression of the antimicrobial activities was tested. *L. sakei* TMW 1.3, TMW 1.46, TMW 1.114 and TMW 1.578 carrying predicted bacteriocin gene clusters did not show any inhibitory effect against any of the indicator strains. Still, the antimicrobial activity of *L. curvatus* TMW 1.624 could be corroborated against all indicator strains (Table S1).

3.4. Prophage gene clusters are detectable within genomes

All genomes were screened for predicted/putative prophage gene clusters like it is shown in Table S3. At least one incomplete and maximally six putative prophage gene clusters, which are questionable, incomplete or intact, were identified. Moreover, the predicted clusters are carried on plasmids or chromosomes. Interestingly, all strains carried at least one putative prophage.

CRISPR/Cas-systems are part of the adaptation and result in a better immunity against prophages and extracellular plasmids

In *L. sakei* four different CRISPR-clusters were detected presented in Table 3 (presence absence) and Table S4 (spacer sequences). CRISPR-1 to CRISPR-3 are identified in different strains but not in all, whereas, *L. sakei* TMW 1.417 has a CRISPR-cluster (CRISPR-4), which cannot be detected in one of the other 10 strains. Interestingly, *L. sakei* TMW 1.1398 has no detectable CRISPR-Cluster.

Table 2
General features of *L. sakei* plasmids.

plasmids	pL1114-1	pL11396-1	pL11398-1	pL11398-2	pL11398-3	pL13-1	pL1417-1	pL146-1	pL1578-1
Contigs	1	1	1	1	1	1	1	1	1
Plasmid length (bp)	34,136	40,197	27,120	40,209	40,107	35,983	64,141	68,079	34,136
GC content (%)	41.69	39.75	40.16	39.74	39.62	36.66	38.54	40.48	41.69
Number of CDS	37	43	27	44	40	36	69	69	37
Total CDS length (bp)	26,373	34,926	20,555	34,746	33,770	28,727	53,162	58,470	26,373
coding density (%)	77.26	86.89	75.79	86.41	84.20	79.83	82.88	85.89	77.26
accession number	CP017567	CP017274	CP017276	CP017277	CP017278	CP016466	CP017569	CP015488	CP017571

Table 3
List of detected CRISPR/Cas cluster in *L. sakei.* + marks the identification of the specific CRISPR/Cas cluster; - markes the missing of a cluster. All defined clusters were numbered consecutively.

CRISPR	TMW 1.3	TMW 1.46	TMW 1.114	TMW 1.417	TMW 1.578	DSM 20017^T	TMW 1.1239	23K	TMW 1.1396	TMW 1.1398
CRISPR_1	+	–	–	–	–	–	+	–	+	–
CRISPR_2	–	+	–	–	–	+	+	+	–	–
CRISPR_3	–	–	+	–	+	–	–	–	–	–
CRISPR_4	–	–	–	+	–	–	–	–	–	–

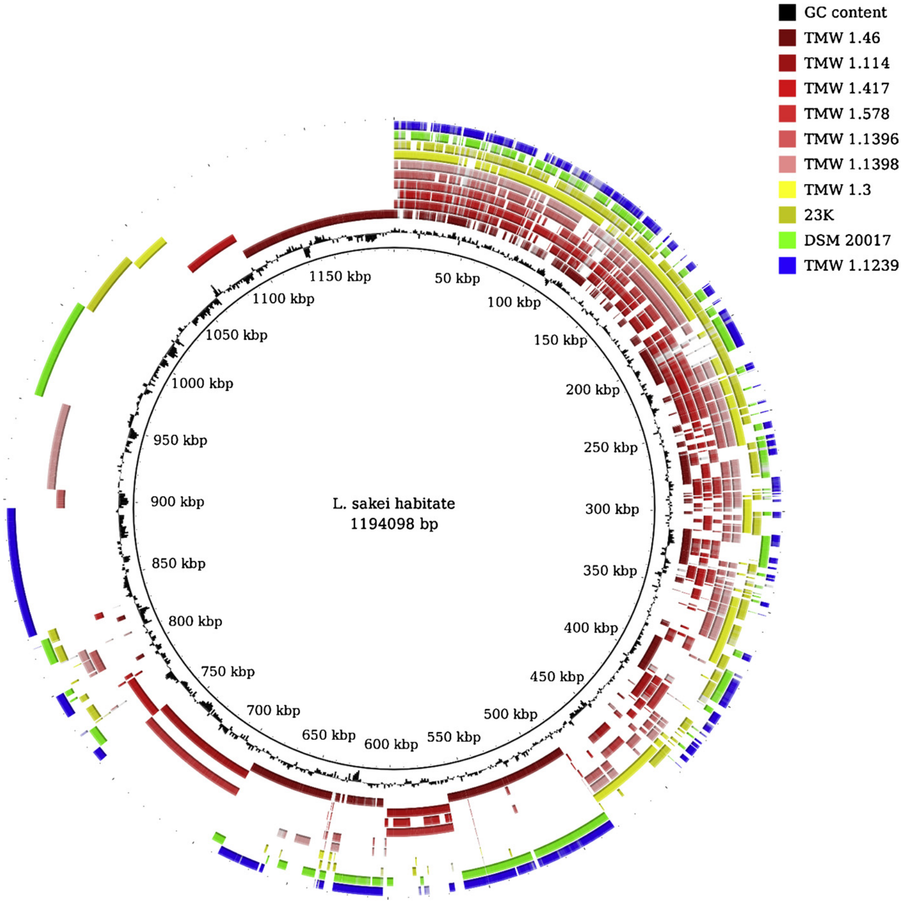


Fig. 1. Genomes of 10 sequenced strains of *L. sakei* were mapped against the accessory genome of *L. sakei* based on these 10 sequenced genomes. The structures of the accessory genome and the genomes did not reflect the physical structure of the chromosomes and plasmids. The strains are coloured based on the source of isolation: red starter preparation; yellow meat or raw sausage; blue sourdough and green sake starter strain.

3.5. *Core genome is half of pan genome indicating genomic diversity within the species*

The pan- and core genomes of the strains were determined to show the genetic diversity within the species as well as similarity within the strains. It was shown that the core genome is approximately half of the pan genome like it is shown in Fig. S2. The accessory genome reflects the diversity and the adaptation of the strains based on the genetic level presented in Fig. 1, and strain specific genes were identified. These genes are chromosome as well as plasmid encoded. Moreover, within the accessory genome, a higher variability of the GC-content was detected, whereas the GC content is highly conserved within the core genome. The genome comparison shows a close relationship between *L. sakei* TMW 1.1396 and TMW 1.1398, as well as the high similarity of *L. sakei* TMW 1.46, TMW 1.1239 and DSM 20017^T, and *L. sakei* TMW 1.114 and TMW 1.578.

At the depicted regions, which are not correlate with the regions of the chromosoms of the bacteria, of approximately 500 kbp and 600 kbp of the accessory genome, the strains *L. sakei* TMW 1.46, DSM 20017^T and TMW 1.1239 are closely similar, but significantly different to the other strains (Fig. 1). At around 400 kbp *L. sakei* TMW 1.46, 23K, DSM 20017^T and TMW 1.1239 encoded a similar set of genes.

3.6. *Carbohydrate utilization reflects the similarity as well as diversity of the species*

Genomic analysis predicted that all sequenced strains are able to ferment melibiose like it is listed in Table S5). However, *L. sakei* TMW 1.1398 encodes a glycerol-3-phosphate dehydrogenase, which carries a mutation resulting in an introduction of a stop codon at amino acid position 38 of the protein. This enzyme catalyses the reaction of glycerol-3-phosphate to dihydroxyacetone-phosphate which is a step in the glycerol-lipid-metabolism. Moreover, the *araA* gene encodes L-arabinose-isomerase and is encoded within all sequenced genomes, but the nucleotide sequence is not highly conserved. De facto, *L. sakei* TMW 1.417 encoded *araA* gene, but the gene alignment revealed that the first 304 bp are missing compared to the other sequences of the other strains (Table S5). Nevertheless, the amino acid sequences suggest that the functional part of the gene is intact and putatively functional based on the sequence alignment as well as control by smart BLAST.

All sequenced strains encoded a trehalose specific IIA component of a PTS system, which catalyzes the reaction of extracellular trehalose to

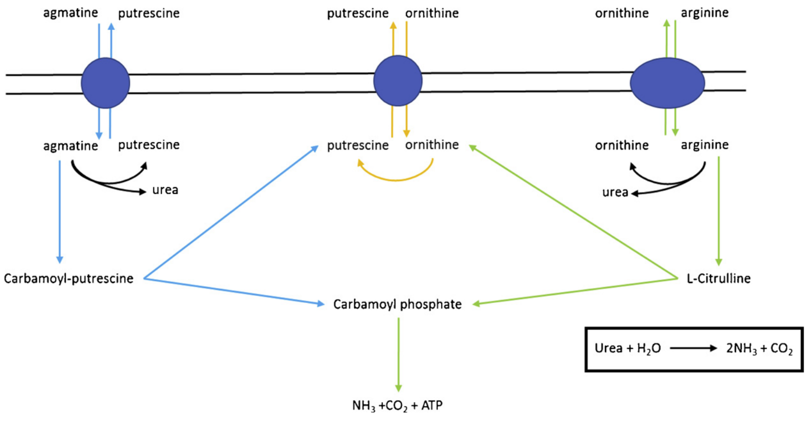


Fig. 2. Agmatine and ADI-pathway are encoded within the genomes. Interestingly, ADI-pathway ist part of the core genome of *L. sakei*, whereas, agmatine pathway is only encoded by *L. sakei* TMW 1.3, TMW 1.114, TMW 1.578 and 23K. Moreover, no strain of sequenced *L. curvatus* strains published by Eisenbach et al. (Eisenbach et al., 2018) encoded one of both pathways, but *L. curvatus* TMW 1.407, TMW 1.421, TMW 1.595 and TMW 1.624 encoded a ornithine/putrescine antiporter and ornithine decarboxylase.

trehalose-6-phosphate (Table S5).

A sucrose operon is part of the core genome. All strains are able to take up sucrose by a PTS like it is illustrated in Fig. S3 (Table S5).

The core and accessory genome reflect differences and similarities based on Agmatine and ADI-pathway.

A main difference between *L. sakei* and *L. curvatus* is the ADI-pathway (Champomier Verges et al., 1999). The genomic analysis reveals that *L. curvatus* encoded no agmatine and ADI pathway (Eisenbach et al., 2018). All sequenced strains of *L. sakei* encoded the whole ADI-pathway like it is described by Rimaux et al. (Rimaux et al., 2012) and shown in Fig. 2. Furthermore, there are 4 strains (*L. sakei* TMW 1.114, TMW 1.3, TMW 1.578 and 23K), whose genomes encoded the agmatine metabolism. However, the agmatine deiminase gene cluster revealed differences based on the genetic level. *L. sakei* TMW 1.578 and TMW 1.114 encoded an identical gene cluster, and the structure of this cluster could also be detected within the genome of *L. sakei* TMW 1.3 and 23K. Detailed analysis showed that both *L. sakei* TMW 1.3 and 23K carried mutations, which could influence the functionality of parts of the gene clusters. On the one hand, *L. sakei* 23K has a mutation within the *aguD* which encoded an agmatine-putrescine antiporter (AgmP). The transmembrane structure was controlled by web-based tool TMHMM, and the results suggest that the structure is changed. Furthermore, *L. sakei* TMW 1.3 has a mutation within the gene *aguC*, which encoded carbamate kinase (CK). This enzyme catalyses the reaction of carbamoyl phosphate to NH_3 and CO_2 under the generation of ATP.

The activity of agmatine and ADI pathway was tested by using DCM, and after 5 days the reached pH was determined as illustrated in Fig. 3. Interestingly, all strains were able to degrade arginine, but the pH of the cultures differed in presence of arginine. Both strains *L. sakei* TMW 1.114 and TMW 1.578, which encoded the agmatine deiminase gene cluster increased the pH upon growth in presence of agmatine, whereas *L. sakei* TMW 1.3 and 23K, which encoded the cluster as well, decreased the pH in presence of agmatine.

3.7. Two different citrate clusters are encoded in a few strains of L. sakei

Interestingly, the use of malate and citrate are not part of the core genome. *L. sakei* TMW 1.46, DSM 20017^T and TMW 1.1239 encoded a malate dehydrogenase, which catalyzes the reaction of L-malate to oxaloacetate, whereas no other sequenced strains encoded this enzyme. Moreover, genomic comparison of *L. sakei* strains reveals two different citrate clusters like it is shown in Fig. 4. First, *L. sakei* TMW 1.114, TMW 1.417 and TMW 1.578 do not have any citrate gene cluster. All other sequenced strains encoded one citrate cluster, and they could be grouped based on these both clusters. Basically, the nucleotide sequence of the gene clusters is completely different. Nevertheless, both citrate clusters encoded protein homologous of all relevant enzymes and transporters involved in citrate metabolism. Furthermore, the clusters could be differentiated based on different transporters.

Group I conclude *L. sakei* TMW 1.3, TMW 1.1396, TMW 1.1398 and 23K encoded an Mg^{2+}/citrate complex transporter (CitM). Moreover, the cluster encoded CitC ([citrate [pro-3S]-lyase] ligase), CitD (citrate lyase ɣ-chain), CitE (citrate lyase ß-chain), CitF (citrate lyase α-chain) and CitG (ATP: dephospho-CoA 5′-triphosphoribosyl transferase; EC 2.7.5.25). Downstream of CitF and upstream of CitG the genes CitT (Apo-citrate lyase phosphoribosyl-dephospho CoA transferase), oadA (Oxaloacetate decarboxylase) and the transcriptional regulator GntR are located. Between the CitC and the transporter CitM, BCCP (Biotin carboxyl carrier protein), oadB (oxaloacetate decarboxylase ß-subunit) and oadC (oxaloacetate decarboxylase ɣ-subunit) are encoded. Within group I, genome analysis revealed that *citF* gene of *L. sakei* TMW 1.3 is mutated. The sequence of the gene was controlled by using Smart Blast, and the result revealed that the enzyme is not functional based on this *in silico* prediction. Moreover, *L. sakei* TMW 1.1396 and TMW 1.1398 carried a mutation within the sequence of the transporter which results in an earlier stop codon (Fig. 5B). Furthermore, the sequence of the transporter of *L. sakei* TMW 1.1396 is shorter than the sequence of the other transporters (Fig. 5A). TMHMM was used to analysis the structure of the transporters (Fig. S4). The structure of *L. sakei* TMW 1.3 and 23K are identical and the structure of the transporter of *L. sakei* TMW 1.1398 differs at amino acid position 266. The structure of *L. sakei* TMW 1.1396 is different at the first 100 amino acids and the sequence is shorter than the other three ones. Nevertheless, the results suggest functional transporters, but their efficiency could be different.

Group II, concluding *L. sakei* TMW 1.46, DSM 20017^T, TMW 1.1239, encoded a citrate gene cluster as well, and the transporter system is a citrate permease (CimH). Downstream of the transporter the gene cluster encoded *citCDEFG*. This structure is one main difference to the cluster of group I. Furthermore, upstream of the transporter CitT, CitI (citrate lyase transcriptional regulator) and oadA are located. The sequence comparison of this cluster revealed differences at the sequence of *citI* gene. *L. sakei* TMW 1.1239 carried a gene, which is homologous to *citI* gene, but the sequence is shorter compared to the other strains.

3.8. Growth experiments reveals different efficiency of citrate utilization based on genetic differences

The described citrate cluster is involved in the citrate metabolism like it is shown in Fig. 6.

HPLC analysis revealed differences based on the uptake of citrate and glucose under different conditions, as well as based on the secretion of lactate and acetate, which are both products of glycolysis and citrate metabolism. The results are presented in Table S5.

If strains are incubated in presence of citrate as single energy source no significant differences or changes were detectable. No uptake of citrate was detectable for all tested strains. Moreover, the glucose uptake was not different if glucose was present as single carbon source compared to growth in presence of glucose and citrate as listed in Table

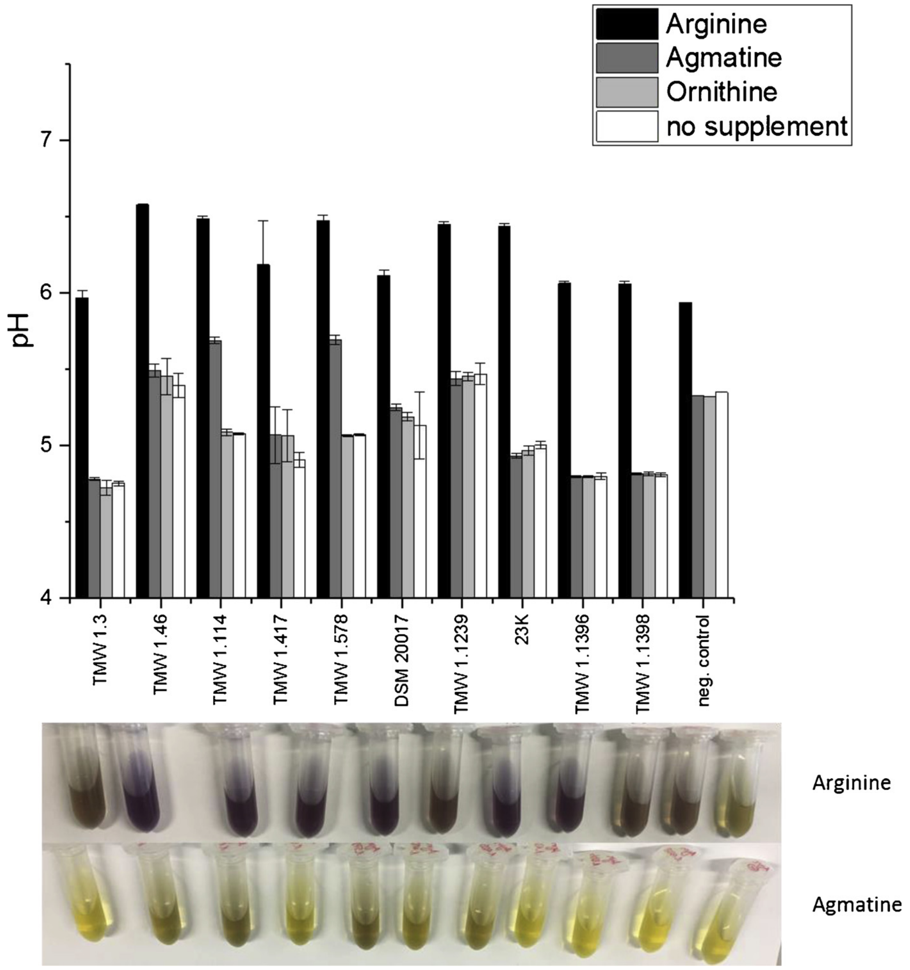


Fig. 3. The pH was measured after growth of 5 days on DCM in presence of arginine, agmatine, ornithine or no supplement. It was shown, that all strains are able to use arginine to decrease the pH, whereas only a few strains are able to use agmatine for the same reason.

S6.

Within the group of cluster I, differences of acetate concentration were detected. Firstly, more acetate was produced in presence of citrate and glucose than with glucose alone; with exception of *L. sakei* TMW 1.3, which takes up 9.72% of citrate, but no significant acetate production was detectable. *L. sakei* TMW 1.1396 had the highest citrate uptake (30.32%) as compared to the other strains of the group. Strains with cluster II are not highly diverse based on acetate and lactate production, but *L. sakei* TMW 1.1239 took up citrate faster than the other strains, after 48 h no citrate was detectable. Moreover, *L. sakei* DSM 20017^T took up less citrate and produced less lactate compared to the other two strains of the same group.

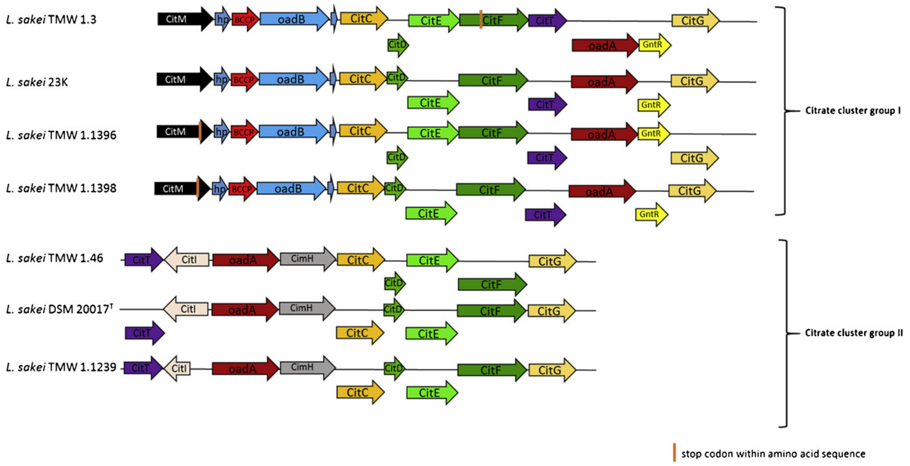
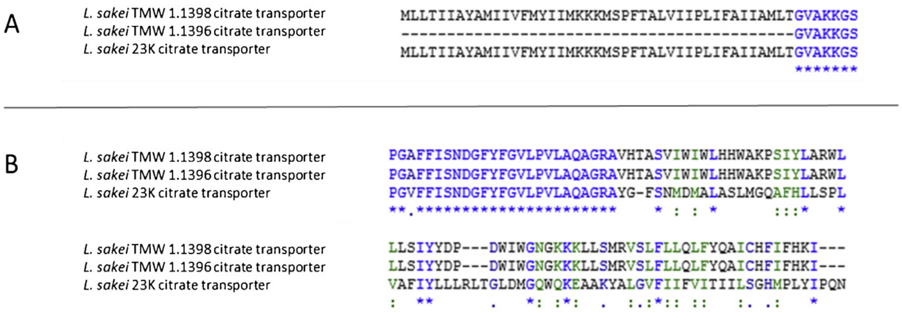


Fig. 4. citrate cluster which was found at genomes of *L. sakei*. Strains of *L. sakei* encoded different citrate clusters. The structure of the clusters is different as well as the nucleotide sequences are different based on the selected setting during BADGE.



```
A  L. sakei TMW 1.1398 citrate transporter   MLLTIIAYAMIIVFMYIIMKKKMSPFTALVIIPLIFAIIAMLTGVAKKGS
   L. sakei TMW 1.1396 citrate transporter   -------------------------------------------GVAKKGS
   L. sakei 23K citrate transporter          MLLTIIAYAMIIVFMYIIMKKKMSPFTALVIIPLIFAIIAMLTGVAKKGS
                                                                                        *******

B  L. sakei TMW 1.1398 citrate transporter   PGAFFISNDGFYFGVLPVLAQAGRAVHTASVIWIWLHHWAKPSIYLARWL
   L. sakei TMW 1.1396 citrate transporter   PGAFFISNDGFYFGVLPVLAQAGRAVHTASVIWIWLHHWAKPSIYLARWL
   L. sakei 23K citrate transporter          PGVFFISNDGFYFGVLPVLAQAGRAYG-FSNMDMALASLMGQAFHLLSPL
                                             **.**********************    * : : *      :::*   *

   L. sakei TMW 1.1398 citrate transporter   LLSIYYDP---DWIWGNGKKKLLSMRVSLFLLQLFYQAICHFIFHKI---
   L. sakei TMW 1.1396 citrate transporter   LLSIYYDP---DWIWGNGKKKLLSMRVSLFLLQLFYQAICHFIFHKI---
   L. sakei 23K citrate transporter          VAFIYLLLRLTGLDMGQWQKEAAKYALGVFIIFVITIILSGHMPLYIPQN
                                             :  **      .   *: :*:  .  :.:*:: ::   :. .:   *
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Fig. 5. citrate transporter CitM is different within *L. sakei*. (A) *L. sakei* TMW 1.1396 has a shorter amino acid sequence (first 33 amino acids or 99 bp are missing). (B) The end of the amino acid sequence is different between *L. sakei* 23K and TMW 1.1398 respectively TMW 1.1396 caused by an insertion and transition mutation.

4. Discussion

Strains of *L. sakei* can be isolated from different sources including meat, fermented sausages, Moto, sourdough, sauerkraut, and kimchi. For most isolates from these sources it remains unclear whether these formed true habitats of *L. sakei*, i.e. environments, in which it reached high cell numbers and outcompeted other strains indicating its adaptation to the respective physical and chemical conditions. Specifically, for strains isolated from starter preparations their original habitat remains mostly unclear. Therefore, any correlation of genomic settings to their source of isolation cannot be expected. As a typical example for this constellation it was found that *L. sakei* TMW 1.46 isolated from meat is genetically closely related to *L. sakei* DSM 20017^T and TMW 1.1239, which were isolated from Sake starter Moto or sourdough (i.e. cereal sources), respectively (Fig. 1).

Still, our study revealed that specific genomic lineages of *L. sakei* exist within a large accessory genome comprising half of the pangenome, which suggests different environmental preferences of such groups. Apart from a general diversity within the accessory genome referring to CRISPR and prophage determinants, metabolic differences within these lineages can be identified namely in citrate utilization and agmatine metabolism. On the other hand, the presence of fructose, sucrose and trehalose metabolism corroborates their general adaptation to plant materials.

Mechanisms of adaptation to a specific environment include the uptake of exogenous DNA, like plasmids, which could carry genes, which promote the assertiveness in a special habitat. This has been exemplarily demonstrated for beer spoiling lactobacilli, which gain most of their tolerance to environmental growth restrictions in beer from plasmid encoded traits (Behr et al., 2015, 2016; Fraunhofer, 2018). In *L. sakei* we could not find any plasmid-encoded traits related to (known) determinants to cope with the conditions of sausage fermentation or a plant-adapted lifestyle. If cells take up exogenous DNA, they have to differentiate between self and foreign DNA. Therefore, a microbial adaptive immune system has developed, which is called CRISPR/Cas system (Horvath and Barrangou, 2010). These clusters are influenced by the contact with prophages and other bacteria. An overview on CRISPR/Cas systems in *L. sakei* is provided by Schuster et al. (Schuster et al., 2019). While differences in CRISPR/Cas settings were detected, we could not figure out any correlation to a respective strain behaviour in a competitive setting as performed by Janßen et al. (Janßen et al., 2018b). The results suggest, that the presence/absence of CRISPR/Cas did not correlate with physiological observations. Moreover, the CRISPR/Cas clusters did not influence the competitiveness of strains in raw sausage fermentation.

The ability to overcome the growth of the competitors is decisive for the occupation of an ecological niche to form habitat. At the same time this is exactly what is expected from an assertive starter strain, in this case for sausage fermentation, which ensures a repeatable process and safe, sensorically attractive product. The production of antimicrobial peptides is one industrially exploitable strategy of single strain assertiveness in the complex meat environment (Tichaczek et al., 1992). Still, it needs to be validated if the bacteriocins are produced and indeed are the functional agent against the competing microbiota (Tichaczek et al., 1994). *L. curvatus* TMW 1.624 encoded several bacteriocin gene clusters, and proved assertive in a competitive setting with other strains in a fermenting sausage model (Eisenbach et al., 2018; Janßen et al., 2018b) as well as an industrial ring trail (Janßen et al., 2018a). As this strain exhibited antimicrobial activity in a plate assay against all tested *L. curvatus* and *L. sakei* strains, it is likely that at least one of its bacteriocin gene clusters is functionally expressed. In this case bacteriocin production could explain the assertiveness of this

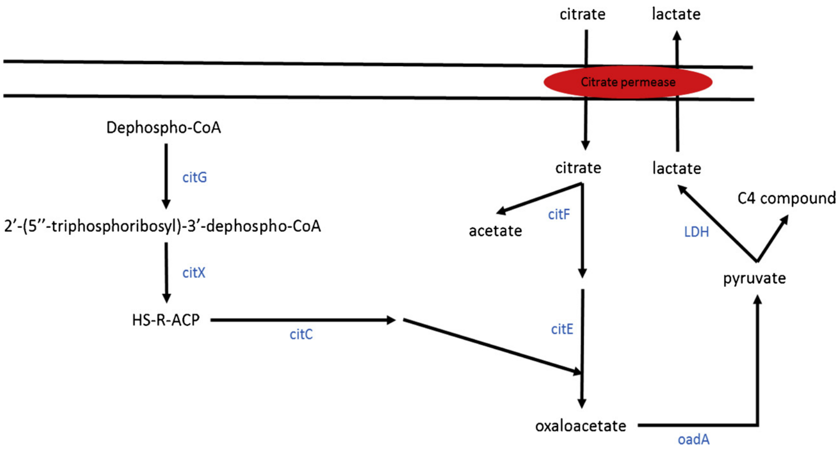

Fig. 6. citrate metabolism which is encoded within the described citrate gene clusters (Fig. 4) citrate can be degraded to acetate and oxaloacetate which can be degraded to pyruvate.

strain. On the other hand, *L. sakei* strains used in this work encoded either none or only one predicted bacteriocin gene cluster. However, none of the tested *L. sakei* strains expressed antimicrobial activity in the plate assay (Table S1). This suggests that the bacteriocin gene clusters in *L. sakei* TMW 1.3, 1.46, 1.114 and 1.578 are not functionally expressed under the conditions tested. Remarkably, *L. sakei* TMW1.1396 proved assertive in the competitive setting of Janßen et al. (Janßen et al., 2018b) despite it did not harbour a predicted bacteriocin gene cluster in its genome, nor expressed antimicrobial activity in the plate assay (Table S2). Consequently, the explanation for assertiveness of *L. sakei* TMW1.1396 cannot parallel the assertiveness of *L. curvatus* TMW 1.624, but supposedly resides in other traits than being caused by bacteriocin production. Nevertheless, the clusters may confer resistance against other bacteriocins of related bacteria (Claesson et al., 2007), which could be seen as a mostly neglected competitive trait for its own.

Another strategy to achieve competitive exclusion has been demonstrated as cooperation of strains in the sausage environment (Janßen et al., 2018a, b). As a consequence, metabolically complementary strains, which are able to use a variety of substrates with limited competition should be able to conduct a (sausage) fermentation process. Actually, the life in communities is a general principle of microbial life beyond single species or strains. In *L. sakei*, complementary strains of different genomic lineages can be identified, and markers for the selection of partner strains can be derived from the following paragraphs.

The metabolic properties and adaptation to meat has been intensively studied in the last years. Based on the genome sequence of *L. sakei* 23K Chaillou et al. predicted the putatively important metabolic pathways upon growth in meat (Chaillou et al., 2005). Genomic prediction reveals that *L. sakei* 23K is auxotrophic for all amino acids except of aspartic and glutamic acid (Chaillou et al., 2005). This was also metabolically corroborated by Freiding et al. (Freiding et al., 2011). Different transporter systems are encoded, which are associated with sugar uptake. Additionally, genome analysis suggested catabolic pathways of melibiose, gluconate, galactose, arabinose, ribose and, glycerol, malate and citrate (McLeod et al., 2010, 2011) are part of the metabolic properties of *L. sakei*.; Nyquist et al. (Nyquist et al., 2011) has shown that all strains, which were tested, fermented glucose, mannose, fructose, *N*-acetylglucosamine, and sucrose, while not all strains are able to ferment cellobiose and trehalose. Our genomic comparison revealed that all strains encoded a PTS sugar specific IIC component for cellobiose as well as trehalose, but 6-phospho-ß-glucosidase, which catalyses the reaction of cellobiose-6-phosphate to D-glucose-6-phosphate and D-glucose, is not part of the core genome (Table S5). It is likely that the cellobiose metabolism is important for adaptation to plant related habitats. Also, 6-phospho-ß-glucosidase is encoded within the genomes of *L. sakei* TMW 1.46, DSM 20017^T and TMW 1.1239. *L. sakei* TMW 1.1239, *L. sakei* TMW 1.1396 and TMW 1.1398, which further suggests their plant adaptation, irrespective of their source of isolation. Furthermore, trehalose-6-phosphate hydrolase, which catalyzes the reaction of trehalose-6-phosphate to D-glucose-6-phosphate and D-glucose is part of the core genome (Table S5). The genomic comparison therefore suggests that all strains are able to ferment trehalose, whereas only 50% of the sequenced strains are able to ferment cellobiose.

Furthermore, for a few strains it was shown that they fermented melibiose, gluconate, arabinose, malate and glycerol (Nyquist et al., 2011). The pan and core genome analysis revealed that all strains encoded a gluconokinase and gluconate-6-phosphate-dehydrogenase, which catalyzes the reaction of D-gluconate, intermediate G-gluconate-6-phosphate to ribulose-5-phosphate, which could be converted either to ribose-5-phosphate by pentose-5-phosphate-isomerase or to xylulose-5-phosphate-epimerase by pentose-5-phosphate-epimerase. Genomic analysis suggests that the catabolism of gluconate is encoded within the core genome, and no specific point mutations or differences were detected. This may indicate a lifestyle of *L. sakei* in co-existence of acetic acid bacteria sharing sucrose rich environments producing gluconate. Indeed, pathways for effective sucrose metabolism were predicted from the core genome (Fig. S3).

In all strains a ribose operon, which encodes genes involved in ribose uptake and catabolism was detected by genomic comparison in this study. Several studies (Nyquist et al., 2011) revealed a divergent region within this operon, but it was shown in this study, by the genomic analysis of 10 strains concluding *L. sakei* 23K, that this operon is highly conserved (Table S5). Moreover, other sequenced strains were included in this genomic comparison. The ribose operon comprising *rbsU, rbsD, rbsK,* and *rbsR* are highly conserved within all available genome sequences (data not shown). But, there are differences within the sequences of transcriptional regulator *padR*, and an MFS transporter downstream of the operon.

Another important metabolic property is the utilization of purines and pyrimidines like inosine and adenosine, which might improve the survival on meat (Chaillou et al., 2005). Inosine is generated by the hydration of ATP and adenosine. The purine nucleoside metabolism can use for energy production by *L. sakei* (Claesson et al., 2007). These metabolic properties could be detected in the genome of the strains of *L. sakei*, but there are differences based on the nucleotide sequences. This may result in differences of the effectivity of this metabolism, which has been considered as a competitive trait in meat environments.

The catabolism of arginine is induced when sugar concentration is low. So, the pathway should be induced upon growth in meat, when the residual sugar is depleted. Moreover, *L. sakei* is known to use arginine by arginine deiminase (ADI) pathway (Champomier Verges et al., 1999; Rimaux et al., 2012). Moreover, the genomic analysis revealed that *L. sakei* TMW 1.3, TMW 1.114, TMW 1.578 and 23K encoded genes, which encoded homologous proteins of agmatine metabolism (Fig. 2). Compared to the results of pH-neutralization reaction, *L. sakei* TMW 1.3 and 23K are not able to metabolize agmatine which would result in a pH increase, whereas cultures of *L. sakei* TMW 1.114 and TMW 1.578 show an increased pH (Fig. 3). Indeed, genomic analysis revealed that *L. sakei* TMW 1.114 and TMW 1.578 encoded an agmatine deiminase gene cluster which is functional, but *L. sakei* TMW 1.3 and 23K encoded the same gene cluster which has different mutations. *L. sakei* 23K has a mutation within the *aguD* gene which encoded a homologous protein of the agmatine/putrescine antiporter (data not shown), and *L. sakei* TMW 1.3 encoded a mutated gene homologous of *aguC*, which encoded the carbamate kinase. So, the physiological results compared to the genomic prediction suggest that detected mutations result in non-functional transporter or enzymes. Interestingly *L. sakei* TMW 1.3 encoded the well conserved cluster carried the ADI pathway like every sequenced *L. sakei* strain. Part of this gene cluster is a carbamate kinase as well. Within this gene cluster no mutations are detectable. The results suggest that both clusters are regulated separately, and presence of arginine seems to induce expression of ADI-gene cluster, whereas, agmatine did not induced the expression of it. Putatively, agmatine induced the expression of the agmatine deiminase gene cluster.

A divergence of the citrate catabolic genes was described by Nyquist et al (Nyquist et al., 2011), and the comparison of *L.* sakei LS25 and 23K suggest that this gene cluster is divers within *L. sakei* species. *L. sakei* LS25 encoded a citrate cluster which is not orthologous to the one encoded in 23K. Furthermore, 250 protein-encoding genes were unique to LS25 (McLeod et al., 2013). In agreement, the genomic comparison revealed that there are two different citrate gene clusters detectable in most strains. But there are also strains, which encoded no citrate gene cluster. It is described that a citrate permease (CitP) is essential for citrate metabolism. This permease shows higher efficiency at defined pH. In *Lactococcus lactis* an optimum of pH 5.0 and 6.0 was observed, whereas the optimum in *L. plantarum* is pH 4.0. But, at these low pH stages citrate ($pK_a = 3.14, 4.77, 5.40$) is present in uncharged and acidic form (Hugenholtz, 1993). So, it can easily diffuse through the membrane. Within the gene cluster II a citrate permease was described, whereas citrate cluster I encoded an Mg^{2+}/citrate complex transporter (CitM). This transporter is known from *Bacillus subtilis* and it was shown the citrate uptake is induced by Mg^{2+} ions (Boorsma et al., 1996).

The initial citrate breakdown is characterized by the initial conversion of citrate to oxaloacetate and acetate by citrate lyase. So, acetate can be used as indicator of an active citrate metabolism. The HPLC results revealed a higher acetate production in presence of citrate if the strain encoded a putative citrate gene cluster (Table S6). Citrate lyase is unique for citrate utilizers and encoded by all detected citrate gene clusters within the genomes of *L. sakei* strains. Interestingly, sequence analysis of citrate lyase of *L. sakei* TMW 1.3 suggest that the enzyme is not functional because a point mutation at the nucleotide sequence results in an earlier stop codon. Compared to the results of HPLC analysis this strain produces no significant acetate amount (1.18%), although, this strain takes up 9.72% of available citrate. The results suggest that the mutation within the citrate lyase results in a non-functional citrate lyse. Interestingly, *L. sakei* TMW 1.1396 encoded a mutated transporter within the citrate gene cluster. Uptake of citrate revealed that this strain has the highest citrate uptake (30.32%) in comparison to the other strain of the same group. So, the mutated transporter is functional and the results of HPLC suggest a higher efficiency of citrate uptake. *L. sakei* TMW 1.1239, which encoded a divers *citI* gene has a higher citrate uptake rate as well. This result suggests that this regulator influenced the citrate uptake.

Comparison between both clusters revealed that strains with cluster II takes up citrate faster during the first 48 h. No differences of produced acetate could be detected.

Such differences resulting from citrate and agmatine metabolism and possible other traits described here could be used to establish assertive starter strain pairs/sets with complementary metabolic traits. Thus, they could occupy an environmental niche with little competition and establish competitive exclusion of autochthonous meat strains. For example *L. sakei* TMW 1.417 and TMW 1.1398 differ in citrate, while TMW 1.46 and TMW 1.578 differ in agmatine metabolism and both formed assertive pairs in a competitive sausage fermentation model (Janßen et al., 2018a, b).

Taken together, the results suggest that marker genes can be found for the prediction of suitable strain combinations to be used as starter strains for sausage fermentation. One single locus in the genomes promoting assertiveness of an *L. sakei* strain over other strains cannot be identified. As a consequence, and in contrast to the general strategy of single strain starter development, an approach is enabled to develop assertive strain combinations of *L. sakei* and even between *L. sakei* and *L. curvatus*. This can be based on predictions derived from the complementary accessory genome of *L. sakei* and as already demonstrated for *L. curvatus* (Eisenbach et al., 2018; Janßen et al., 2018a, b).

Acknowledgements

Part of this work was funded via AiF within the program for promotion of Industrial Collective Research (IGF) of the German Ministry of Economic Affairs and Energy (BMWi), and the research association of the German food industry (FEI) in project 18552 N.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.micres.2019.01.001.

References

Alikhan, N.-F., Petty, N.K., Zakour, N.L.B., Beatson, S.A., 2011. BLAST Ring Image Generator (BRIG): simple prokaryote genome comparisons. BMC Genomics 12 (402).

Arndt, D., Grant, J.R., Marcu, A., Sajed, T., Pon, A., Liang, Y., Wishart, D.S., 2016. PHASTER: a better, faster version of the PHAST phage search tool. Nucleic Acids Res. 44.

Aziz, R.K., Bartels, D., Best, A.A., DeJongh, M., Disz, T., Edwards, R.A., Formsma, K., Gerdes, S., Glass, E.M., Kubal, M., Meyer, F., Olsen, G.J., Olson, R., Osterman, A.L., Overbeek, R.A., McNeil, L.K., Paarmann, D., Paczian, T., Parrello, B., Pusch, G.D., Reich, C., Stevens, R., Vassieva, O., Vonstein, V., Wilke, A., Zagnitko, O., 2008. The RAST Server: rapid annotations using subsystems technology. BMC Genomics 9, 75.

Behr, J., Geissler, A.J., Preissler, P., Ehrenreich, A., Angelov, A., Vogel, R.F., 2015. Identification of ecotype-specific marker genes for categorization of beer-spoiling Lactobacillus brevis. Food Microbiol. 51, 130–138.

Behr, J., Geissler, A.J., Schmid, J., Zehe, A., Vogel, R.F., 2016. The identification of novel diagnostic marker genes for the detection of beer spoiling *Pediococcus damnosus* strains using the BlAst Diagnostic Gene findEr. PLoS One 11 (3), e0152747.

Boorsma, A., van der Rest, M.E., Lolkema, J.S., Konings, W.N., 1996. Secondary transporters for citrate and the Mg2+ - citrate complex in *Bacillus subtilis* are homologous proteins. J. Bacteriol. 178 (21), 6216–6222.

Bover-Cid, S., Hugas, M., Izquierdo-Pulido, M., Vidal-Carou, M.C., 2001. Amino acid-decarboxylase activity of bacteria isolated from fermented pork sausages. Int. J. Food Microbiol. 66 (3), 185–189.

Chaillou, S., Champomier-Verges, M.C., Cornet, M., Crutz-Le Coq, A.M., Dudez, A.M., Martin, V., Beaufils, S., Darbon-Rongere, E., Bossy, R., Loux, V., Zagorec, M., 2005. The complete genome sequence of the meat-borne lactic acid bacterium *Lactobacillus sakei* 23K. Nat. Biotechnol. 23 (12), 1527–1533.

Chaillou, S., Daty, M., Baraige, F., Dudez, A.M., Anglade, P., Jones, R., Alpert, C.A., Champomier-Verges, M.C., Zagorec, M., 2009. Intraspecies genomic diversity and natural population structure of the meat-borne lactic acid bacterium *Lactobacillus sakei*. Appl. Environ. Microbiol. 75 (4), 970–980.

Chaillou, S., Lucquin, I., Najjari, A., Zagorec, M., Champomier-Verges, M.C., 2013. Population genetics of *Lactobacillus sakei* reveals three lineages with distinct evolutionary histories. PLoS One 8 (9), e73253.

Champomier Verges, M.C., Zuniga, M., Morel-Deville, F., Perez-Martinez, G., Zagorec, M., Ehrlich, S.D., 1999. Relationships between arginine degradation, pH and survival in *Lactobacillus sakei*. FEMS Microbiol. Lett. 180 (2), 297–304.

Champomier-Verges, M.C., Chaillou, S., Cornet, M., Zagorec, M., 2002. Erratum to "*Lactobacillus sakei*: recent developments and future prospects" [Research in Microbiology 152 (2001) 839]. Res. Microbiol. 153 (2), 115–123.

Claesson, M.J., van Sinderen, D., O'Toole, P.W., 2007. The genus *Lactobacillus*–a genomic basis for understanding its diversity. FEMS Microbiol. Lett. 269 (1), 22–28.

de Jong, A., van Hijum, S.A., Bijlsma, J.J., Kok, J., Kuipers, O.P., 2006. BAGEL: a web-based bacteriocin genome mining tool. Nucleic Acids Res. 34, W273–W279 Web Server issue.

de Man, J.D.R., Rogosa, M., Sharpe, M.E., 1960. A medium for the cultivation of lactobacilli. J. Appl. Bact. 23, 130–135.

Eisenbach, L., Janßen, D., Ehrmann, M.A., Vogel, R.F., 2018. Comparative genomics of *Lactobacillus curvatus* enables prediction of traits relating to adaptation and strategies of assertiveness in sausage fermentation. Int. J. Food Microbiol. 286, 37–47.

Fraunhofer, M.E., 2018. Characterization of EPS-Producing Brewery-Associated Lactobacilli. Technische Universität München, Germany.

Freiding, S., Gutsche, K.A., Ehrmann, M.A., Vogel, R.F., 2011. Genetic screening of *Lactobacillus sakei* and *Lactobacillus curvatus* strains for their peptidolytic system and amino acid metabolism, and comparison of their volatilomes in a model system. Syst. Appl. Microbiol. 34 (5), 311–320.

Grissa, I., Vergnaud, G., Pourcel, C., 2007. The CRISPRdb database and tools to display CRISPRs and to generate dictionaries of spacers and repeats. BMC Bioinformatics 8, 172.

Hammes, W.P., Hertel, C., 1996. Selection and Improvement of Lactic Acid Bacteria Used in Meat and Sausage Fermentation. Le Lait, INRA Editions Vol. 76. pp. 159–168.

Hammes, W.P., Bantleon, A., Min, S., 1990. Lactic acid bacteria in meat fermentation. FEMS Microbiol. Rev. 87, 165–174.

Horvath, P., Barrangou, R., 2010. CRISPR/CAS, the immune system of bacteria and archaea. Sci. Aliment 327.

Hugenholtz, J., 1993. Citrate metabolism in lactic acid bacteria. FEMS Microbiol. Rev. 12, 165–178.

Janßen, D., Ehrmann, M.A., Vogel, R.F., 2018a. Monitoring of assertive Lactobacillus sakei and Lactobacillus curvatus strains in raw fermented sausage using MALDI-TOF MS. J. Appl. Microbiol. https://doi.org/10.1111/jam.14144. [Epub ahead of print].

Janßen, D., Eisenbach, L., Ehrmann, M.A., Vogel, R.F., 2018b. Assertiveness of Lactobacillus sakei and *Lactobacillus curvatus* in a fermented sausage model. Int. J. Food Microbiol. 285, 188–197.

McLeod, A., Zagorec, M., Champomier-Verges, M.C., Naterstad, K., Axelsson, L., 2010. Primary metabolism in *Lactobacillus sakei* food isolates by proteomic analysis. BMC Microbiol. 10, 120.

McLeod, A., Snipen, L., Naterstad, K., Axelsson, L., 2011. Global transcriptome response in *Lactobacillus sakei* during growth on ribose. BMC Microbiol. 11, 145.

McLeod, A., Brede, D.A., Rud, I., Axelsson, L., 2013. Genome sequence of *Lactobacillus sakei* subsp. *sakei* LS25, a commercial starter culture strain for fermented sausage. Genome Announc. 1 (4).

Nyquist, O.L., McLeod, A., Brede, D.A., Snipen, L., Aakra, A., Nes, I.F., 2011. Comparative genomics of *Lactobacillus sakei* with emphasis on strains from meat. Mol. Genet. Genomics: MGG 285 (4), 297–311.

Rimaux, T., Riviere, A., Illeghems, K., Weckx, S., De Vuyst, L., Leroy, F., 2012. Expression of the arginine deiminase pathway genes in *Lactobacillus sakei* is strain dependent and is affected by the environmental pH. Appl. Environ. Microbiol. 78 (14), 4874–4883.

Schuster, J., Eisenbach, L., Vogel, R.F., Ehrmann, M., 2019. Characterization and distribution of CRISPR-Cas systems in *Lactobacillus sakei*. Curr. Microbiol in press.

Tichaczek, P.S., Nissen-Meyer, J., Nes, I.F., Vogel, R.F., Hammes, W.P., 1992. Characterization of the Bacteriocins Curvacin A from *Lactobacillus curvatus* LTH1174 and Sakacin P from *L. sake* LTH673. Syst. Appl. Microbiol. 15, 460–468.

Tichaczek, P.S., Vogel, R.F., Hammes, W.P., 1994. Cloning and sequencing of sakP encoding sakacin P, the bacteriocin produced by *Lactobacillus sake* LTH 673. Microbiology 140 (Pt 2), 361–367.

Zagorec, M.C., hampomier-Verges, M.C., 2017. *Lactobacillus sakei*: a starter for sausage fermentation, a protective culture for meat products. Microorganisms 5 (3).

Zhang, Y., Zhu, L., Dong, P., Liang, R., Mao, Y., Qiu, S., Luo, X., 2018. Bio-protective potential of lactic acid bacteria: effect of *Lactobacillus sakei* and *Lactobacillus curvatus* on changes of the microbial community in vacuum-packaged chilled beef. Asian-Australas. J. Anim. Sci. 31 (4), 585–594.