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COMBINED PHENOTYPIC-GENOTYPIC ANALYSES OF THE GENUS *LACTOBACILLUS* AND SELECTION OF CULTURES FOR BIOPRESERVATION OF FERMENTED FOOD

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Invictus

William Ernest Henley 1875

Out of the night that covers me,
black as the pit from pole to pole,
I thank whatever gods may be
for my unconquerable soul.
In the fell clutch of circumstance
I have not winced nor cried aloud.
Under the bludgeonings of chance
my head is bloody, but unbowed.
Beyond this place of wrath and tears
looms but the horror of the shade,
And yet the menace of the years
finds and shall find me unafraid.
It matters not how strait the gate,
how charged with punishments the scroll,
I am the master of my fate,
I am the captain of my soul.

Contents

Abbreviations

%G+C	Guanine-cytosine percentage
3-HPA	3-hydroxypropionaldehyde
ANI	Average nucleotide identity
BHI	Brain heart infusion
Bp	Base pair
CDS	Coding DNA sequence
Cfu	Colony forming unit
CRISPR	Clustered regularly interspaced short palindromic repeats
COG	Cluster of orthologous groups of proteins or genes
DDH	DNA-DNA hybridization
DNA	Deoxyribonucleic acid
ecoSNP	Ecological SNP
EFSA	European Food Safety Authority
EMP	Embden-Meyerhof-Parnas pathway
ESI-LC/MS	Electrospray ionization liquid chromatography – mass spectrometry
HGT	Horizontal gene transfer
HPLC	High performance liquid chromatography
HSA	High-throughput screening assay
HSA-B	Antibacterial high-throughput assay
HSA-F	Antifungal high-throughput assay
IMG	Infinitely many genes model
KEGG	Kyoto Encyclopedia of Genes and Genomes
kDa	Kilo Dalton
KO	KEGG ontology
LAB	Lactic acid bacteria
LaCOG	*Lactobacillales* COG
Mb	Mega base
mRNA	Messenger RNA

MIC	Minimal inhibitory concentration
MLST	Multilocus sequence typing
Mn(II)	Manganese
MRS	Man Rogosa Sharpe broth
NCBI	National Center for Biotechnology Information
OD	Optical density
OMCL	Ortho Markov Cluster algorithm
PC	Protective culture
PLA	Phenyllactic acid
PP	Pentose phosphate pathway
PTM	Post translational modification
QPS	Qualified presumption of safety
rRNA	Ribosomal RNA
SMRT	Single molecule real time sequencing
SNP	Single nucleotide polymorphism
SOD	Superoxide dismutase
SPP	Sum polyvariable positions
TSY broth	Trypticase soy yeast broth
WGS	Whole genome sequencing
WHO	World Health Organization
YM broth	Yeast mold broth

Summary

Food waste is an economic and ethical issue and can be reduced for fermented food products with a biopreservation approach to increase shelf-life and avoid the outgrowth of spoilage microorganisms. Thereby protective cultures are applied to produce antimicrobial compounds which inhibit these spoilage organisms. *Lactobacillus* species occur in various fermented food products and many strains were detected to supply antimicrobial activity against *Listeria* and other Gram-positive bacteria and a broad spectrum of fungi. Nevertheless, screening procedures and applications in individual foods is not optimized yet.

The aim of this thesis was to develop an approach to screen and select tailor-made protective cultures from a strain collection to implement protective cultures in industrial-scale food fermentations and to understand mechanisms contributing to biopreservation activity to increase food product safety.

Therefore, 504 *Lactobacillus* isolates were screened with a novel developed high-throughput antibacterial and antifungal screening approach (Chapter 2). This novel approach is based on microtiter plates and allows to determine 2000 – 5000 antimicrobial interactions per day. A total of 65 antibacterial and 154 antifungal isolates were detected by this novel approach.

To better understand antifungal activity in lactobacilli, the complete genome of the salami isolate *Lactobacillus plantarum* RI-113 was determined using single-molecule real time sequencing (Chapter 3). The strain showed antifungal activity against *Trichosporon* spp. and *Rhodotorula mucilaginosa* LME. Additionally, the genomes of 43 *Lactobacillus* strains belonging to the species *L. curvatus, L. fermentum, L. paracasei, L. plantarum, L. rhamnosus,* and *L. sakei* were determined using Illumina MiSeq (Chapter 4). These strains were selected in a phenotypic screening and exhibited an uncommon or unique physiological properties or were regarded as candidate protective cultures.

The phenotype of the 504 *Lactobacillus* isolates was further characterized by a screening for growth limits in modified MRS to mimic conditions in food matrices (Chapter 5). Antibacterial activity based on proteinaceous compounds was determined for 22 isolates with protease digestion of heat-treated supernatant. The in silico screening with the BAGEL3 and antiSMASH3.0 analysis tools proposed potential genes encoding bacteriocins suppressing bacterial growth. Antifungal activity of tested lactobacilli in MRS was determined to be based on organic acid concentration up to 210 mM lactic acid and 78 mM acetic acid. The power of antifungal activity was demonstrated for *L. plantarum* RI-162. A minimal amount of 1-2 cfu/ml of this strain was able to decrease 5 x 10^5 cfu/ml *Rhodotorula mucilaginosa* LME below the detection limit of 100 cfu/ml in a 1-ml co-culture assay within 48 hours. Based on the combination of phenotypic and genotypic screening potential protective cultures for salami and raw milk cheese fermentations were selected. In a small-scale salami fermentation 1 *Lactobacillus sakei* and *5 Lactobacillus plantarum* strains were tested in a laboratory environment. 4

of these 6 cultures reduced the initial *Listeria ivanovii* DSM 12491^T concentration of 10^5 cfu/g below the detection limit of 100 cfu/g at 18 - 24 °C within 5 days of incubation and all 6 cultures lowered the pH below 5.0 within 2 days. In an industrial-scale salami fermentation, the protective potential of *L. sakei* RI-409 was tested in a working environment. Strain RI-409 was able to reduce *in situ* the initial concentration of 6.33 x 10^5 cfu/g of *L. ivanovii* DSM 12491^T concentration by 96% to 4.33 x 10^4 cfu/g within incubation for 5 days. In the absence of any starter and the protective culture, the *L. ivanovii* DSM 12491^T concentration increased in the same time to 6.33 x 10^6 cfu/g. In another approach, we tested *Lactobacillus* strains to reduce undesired enterococci proliferating in dairy fermentations. In a 1000-L raw milk soft cheese industrial-scale fermentation, *L. plantarum* strain RI-271 at initial concentrations of 10^5 cfu/ml in raw milk decreased resident enterococci in the final cheese product by 96% to 2.20 x 10^5 cfu/g compared to the non-treated cheese reaching up to 6.50 x 10^6 cfu/g enterococci after 8 days of ripening.

Based on our genome sequencing data, the genomic variation within 5 lactobacilli species and the genus *Lactobacillus* was investigated (Chapter 6). The core- and pan-genome of 98 completely sequenced genomes of the genus *Lactobacillus* and of 234 whole genome datasets from the *Lactobacillus* species *L. delbrueckii, L. helveticus, L. reuterin, L. rhamnosus* and *L. plantarum* were calculated. The core-genome of the *Lactobacillus* genus contained 266 genes and the non-closed pan-genome 20'800 genes. The core-genome of the 5 *Lactobacillus* species ranged between 756 and 1037 genes and the pan-genomes between 3350 and 7610 genes. The heterogeneity of *L. plantarum* was visible in the genomic variation since it is the only species with a non-closed pan-genome. Clustering according to core- and pan-genome showed similar phylogenetic trees with species clustering together in general. Outliers were analyzed in detail. We revealed that *L. casei* type strain ATCC 393 (DSM 20011^T) clustered next to *L. zeae* DSM 20178 instead next to the other *L. casei* and *L. paracasei* strains, which confirmed other studies defining the phylogenetic outlier position of the *L. casei* type strain by using phenotypic approaches. Analysis of the genetic functions of the core gene revealed that genes involved in "genetic information processing" are conserved in the core-genome, whereas genes involved in "signaling and cellular processes" are not conserved in the core-genome. Twenty genomes of the type-species *Lactobacillus delbrueckii* clustered according to the core-genome in three major clades including one clade solely for the subspecies *Lactobacillus delbrueckii* subsp. *bulgaricus* and two other mixed subspecies clades. No clade specific ecological single nucleotide polymorphisms (ecoSNPs) were detected. A total of 57 genes affected by horizontal exchange were found in *L. delbrueckii* clades. We illustrated an approach to implement whole genome sequencing data into a polyphasic approach to classify bacteria.

Conclusively, a new approach was established to select protective cultures for fermentation processes to inhibit spoilage outgrowth by biopreservation including a high-throughput antimicrobial

screening approach, a phenotypic growth limit screening assay to mimic culture conditions and selection of strains which can grow in specific food matrices and a genotypic screening to detect bacteriocin encoding genes. Based on whole genome analysis the strains can be further characterized and classified and their exchange of genetic material with other strains can be detected. Our approach, successfully applied with *Lactobacillus* strains, can now be extended to other genera in order to assign tailor-made protective strains for industrial food fermentations to increase food safety.

Zusammenfassung

Lebensmittelverschwendung ist ein ökonomisches und ethisches Problem und kann in fermentierten Lebensmitteln durch Biopräservation verhindert werden. Dadurch wird die Haltbarkeitsdauer verlängert und das Wachstum von Kontaminanten reduziert. Hierfür werden Schutzkulturen verwendet, welche mittels antimikrobieller Komponenten Kontaminanten hemmen. *Lactobacillus* Spezies kommen in diversen fermentierten Lebensmitteln vor. Für verschiedene Stämme wurde antimikrobielle Aktivität gegen Listerien und andere Gram-positive Bakterien sowie ein breites Spektrum von Pilzen dokumentiert. Trotzdem wurden die Charakterisierung sowie die Anwendung dieser *Lactobacillus*-Schutzkulturen in Lebensmitteln noch nicht optimiert.

Das Ziel dieser Thesis war die Entwicklung einer Methode zur Charakterisierung und Selektion von massgeschneiderten Schutzkulturen aus einer Stamm-Sammlung und deren Implementierung in industrielle Fermentationen. Zudem wollten wir die Mechanismen, welche zur Biopräservation und zur Erhöhung der Lebensmittelsicherheit beitragen, erforschen.

Dafür wurde bei 504 *Lactobacillus* Stämmen die antibakterielle und antifungale Aktivität mit einer neu entwickelten Hochdurchsatz-Methode bestimmt (Kapitel 2). Diese neue Methode basiert auf Mikrotiterplatten und erlaubt es uns 2000 – 5000 antimikrobielle Interaktionen pro Tag zu messen. Insgesamt wurden durch diese neue Methode 65 antibakterielle und 154 antifungale Isolate entdeckt.

Um die antifungale Aktivität in Lactobacillen besser zu verstehen, wurde das Genom von *Lactobacillus plantarum* RI-113 analysiert (Kapitel 3). Mit der Methode „single-molecule real time sequencing“ wurde ein geschlossenes Genom sequenziert. Der Stamm RI-113 zeigte antifungale Aktivität gegen eine *Trichosporon* Spezies und gegen *Rhodothorula mucilaginosa* LME. Zusätzlich wurden die Genome von 43 weiteren Lactobacillen der Spezies *L. curvatus, L. fermentum, L. paracasei, L. plantarum, L. rhamnosus* und *L. sakei* vollständig sequenziert (Kapitel 4). Diese Stämme wurden mittels einer phänotypischen Charakterisierung selektioniert und zeigten atypische oder einzigartige physiologische Eigenschaften oder wurden als potentielle Schutzkulturen betrachtet.

Das Wachstum im modifizierten MRS-Medium, welches Bedingungen in Lebensmitteln simulierte, wurde für 504 Lactobacillen bestimmt, um die Isolate besser zu charakterisieren (Kapitel 5). Proteinöse antibakterielle Aktivität wurde für 22 Isolate durch Protease-Verdau von Hitze-inaktiviertem Überstand nachgewiesen. Mittels einer in silico Analyse mit den Online-Tools BAGEL3 und antiSMASH3.0 wurden Bakteriozin-codierende Gene bestimmt, welche das Wachstum von Bakterien hemmen. Antifungale Aktivität in Lactobacillen basiert auf der Produktion von organischen Säuren wie Milchsäure oder Essigsäure, welche in Konzentrationen bis zu 210 mM oder 78 mM vorkommen. Die Stärke der antifungalen Aktivität von *Lactobacillus plantarum* RI-162 wurde bestimmt. In einem Co-Kultur-Experiment reduzierten 1-2 Kolonie-bildende Einheiten (kbe) / ml die

Anfangskonzentration von 5 x 10^5 kbe/ml *Rhodotorula mucilaginosa* LME unter das Detektionslimit von 100 kbe/ml innert 48 Stunden in einer 1-ml Co-Kultur. Basierend auf der Kombination von phänotypischer und genotypischer Analyse, wurden potentielle Schutzkulturen für die Fermentation von Rohmilchweichkäse und Salami selektioniert. In einer Salami-Fermentation unter Laborbedingungen wurden 1 *Lactobacillus sakei* und 5 *Lactobacillus plantarum* getestet. 4 von 6 Kulturen reduzierten eine Anfangskonzentration von 10^5 kbe/g *Listeria ivanovii* DSM 12491^T unter das Detektionslimit von 100 kbe/g bei einer Reifungstemperatur von 24 - 18°C innert 5 Tagen. Alle 6 Kulturen reduzierten den pH unter 5.0 innert 48 Stunden. In einer anschliessenden industriellen Salami-Fermentation wurde die Schutzkultur *Lactobacillus sakei* RI-409 auf ihre Praktikabilität getestet. Stamm RI-409 reduzierte *in situ* eine Anfangskonzentration von 6.33 x 10^5 kbe/g von *L. ivanovii* DSM 12491^T um 96% auf 4.33 x 10^4 kbe/g innert 5 Tagen. In Abwesenheit von Starter- und Schutzkultur wuchs *L. ivanovii* DSM 12491^T bis zu einer Konzentration von 6.33 x 10^6 kbe/g in der gleichen Zeit. In einem anderen Ansatz wurde die Hemmung von unerwünschten Enterokokken durch Zugabe von Lactobacillen in Milchprodukten getestet. In einer 1000-L industriellen Rohmilchweichkäse-Fermentation wurde *Lactobacillus plantarum* RI-271 in einer Konzentration von 10^5 kbe/ml inokuliert, um die natürlich vorkommenden Enterkokken zu hemmen. Nach 8 Tagen Reifung wurde im fertigen Rohmilchweichkäse mit Schutzkultur eine Reduktion um 96% auf 2.20 x 10^5 kbe/g erzielt. Gleichzeitig stieg die Enterokokken-Konzentration in unbehandeltem Rohmilchweichkäse auf 6.50 x 10^6 kbe/g.

Die genetische Variation in 5 *Lactobacillus* Spezies und im Genus *Lactobacillus* wurde anhand unserer sequenzierten Genome analysiert (Kapitel 6). Das Kern- und Pan-Genom wurde für 98 geschlossene Genome des Genus *Lactobacillus* und für 234 vollständige Genome der *Lactobacillus* Spezies *L. delbrueckii, L. helveticus, L. reuteri, L. rhamnosus* und *L. plantarum* bestimmt. Das Kern-Genome des Genus *Lactobacillus* enthält 266 und das Pan-Genom 20'800 Gene. Das Kern-Genom der 5 *Lactobacillus* Spezies enthält zwischen 756 und 1037 und das Pan-Genome zwischen 3350 und 7610 Gene. Aufgrund der Heterogenität in der Spezies *L. plantarum* hat ebendiese als einzige Spezies ein nicht-geschlossenes Pan-Genom. Clustering aufgrund der Kern- und Pan-Genome produziert phylogenetische Bäume, in welchen die Spezies generell zusammen clustern. Ausreisser wurden detailliert analysiert. Wir zeigten, dass der Typ-Stamm *L. casei* ATCC 393 (DSM 20011^T) mit *L. zeae* DSM 20178 zusammen clustert und nicht wie erwartet mit anderen Stämmen der Spezies *L. casei* und *L. paracasei.* Dies bestätigt andere Studien, welche ähnliche phylogenetische Resultate mit phänotypischer Klassifizierung erhielten. Genetische Funktionen der Kategorie „Gentische Informationsverarbeitung“ sind im Kern-Genom konserviert, während genetische Funktionen der Kategorie „Signalisierung und zelluläre Prozesse“ nicht im Kern-Genom konserviert sind. Die 20 Genome der Typ-Spezies *L. delbrueckii* wurden gemäss ihrem Kern-Genom in drei Gruppen

geclustert. Eine Gruppe enthält nur Genome der Subspezies *L. delbrueckii* subsp. *bulgaricus,* während die anderen beiden Gruppen verschiedenen Spezien enthalten. In keiner der Gruppen wurden ökologische „single nucleotide polymorphisms“ (ecoSNPs) gefunden. In den drei Gruppen wurden insgesamt 57 Gene entdeckt, welche mit horizontalem Gentransfer assoziiert sind. Wir konnten aufzeigen, wie vollständig sequenzierte Genome in einem polyphasischen Ansatz zur Bakterien-Nomenklatur implementiert werden können.

Abschliessend lässt sich sagen, dass wir einen neuen Ansatz entwickelt haben, um Schutzkulturen zu selektionieren und diese zur Biopräservation in Lebensmitteln einzusetzen. Dieser Ansatz beinhaltet ein Hochdurchsatzverfahren zur Bestimmung der antimikrobiellen Aktivität, die Charakterisierung der Wachstumslimiten, welche Bedingungen im Lebensmittel simulieren und eine genotypische Analyse zu Bestimmung von Bakteriozin-codierenden Genen. Mittels der vollständig sequenzierten Genome können die Stämme charakterisiert und klassifiziert werden, sowie deren Genaustausch mit anderen Stämmen bestimmt werden. Unser Ansatz hat erfolgreich *Lactobacillen* Stämme in Lebensmitteln implementiert und kann nun auf andere Genera expandiert werden, um massgeschneiderte Schutzkulturen für industrielle Lebensmittel zu entwickeln und die Lebensmittelsicherheit zu erhöhen.

Chapter 1

General introduction

1 Background

Food waste is an economic and ethical problem and can be reduced by increasing the shelf-life of fermented food products. Therefore, selected bacterial cultures are applied to produce components which inhibit spoilage organisms. These bacteria, later termed protective cultures, since they protect the food product, have to be selected for each food product individually, since they affect specific spoilage organisms, starter cultures, texture, flavor and odor of the product.

In the following chapter, the main pillars of the research in this thesis are introduced: the description of the genus *Lactobacillus* and its role in food fermentation; the mechanisms of antimicrobial activity in lactobacilli; the application of protective cultures; and an approach to cluster lactobacilli with comparative genomics.

2 Lactic acid bacteria

Lactic acid bacteria (LAB) are Gram-positive, acid-tolerant, non-sporulating bacteria with a low %G+C content in their genome which produce primarily lactic acid from hexose sugar fermentation. LAB are a heterogeneous, non-monophyletic group due to their biological definition (Makarova and Koonin, 2007) and most of them belong to the order Lactobacillales (Wood and Holzapfel, 1995). The order Lactobacillales contains 6 families: Streptococcaceae (3 genera including *Streptococcus and Lactococcus*), Enterococcaceae (7 genera including *Enterococcus* and *Vagococcus*), Carnobacteriaceae (16 genera including *Carnobacterium*), Lactobacillaceae (2 genera: *Lactobacillus* and *Pediococcus*), Leuconostoccaceae (4 genera including *Weissella, Leuconostoc* and *Oenococcus*) and Aerococcaceae (7 genera including *Aerococcus*) (Holzapfel and Wood, 2014).

The family Lactobacillaceae is dominated by the genera *Lactobacillus* with over 170 species, whereas the genus *Pediococcus* contains only 11 species (Goldstein et al., 2015). Until 2011 the family Lactobacillaceae also contained the genus *Paralactobacillus*. This genus was created for the species *Paralactobacillus selangorensis* and later integrated into the genera *Lactobacillus* with the novel species *Lactobacillus selangorensis* (Haakensen et al., 2011; Leisner et al., 2000).

Lactobacillales have a small genome with on average around 2 Mb and approximately 2000 genes (Makarova and Koonin, 2007). The variation in gene numbers, ranging from ~1600 to ~3000 genes, suggest that their evolution is based on acquisition, duplication and gene loss (Makarova and Koonin, 2007). Evolutionary-genomic analysis is based on robust identification of sets of orthologues (Koonin, 2005). *Lactobacillales*-specific clusters of orthologous protein coding genes (LaCOGs) were used to

demonstrate the evolution from a bacilli ancestor via a Lactobacillales ancestor to various species of the genera *Oenococcus, Leuconostoc, Lactobacillus, Pediococcus, Lactococcus* and *Streptococcus* (Makarova and Koonin, 2007). Reconstruction of the ancestor of Lactobacillales shows a major gene loss between ~600 and ~1200 genes and only gaining <100 genes while diverging from the bacilli ancestor (Figure 1.1). This reconstructed evolutionary tree parallels the finding of reconstructed trees with either 42 ribosomal proteins or 7 genes in the Embden-Meyerhof-Parnas pathway and pentose phosphate pathway (Salvetti et al., 2013). However, a reconstruction according to 16S rRNA shows differences in the organization of *L. brevis, L. plantarum* and isolates from *Pediococcus* (Salvetti et al., 2012).

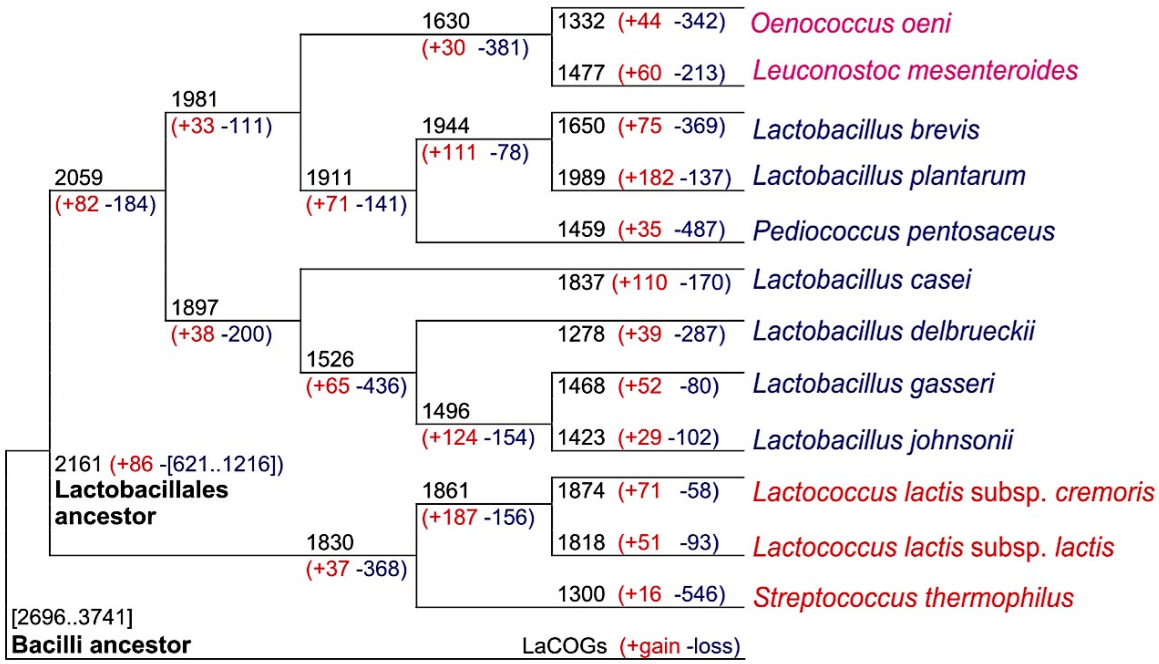

Figure 1.1 Reconstruction of gene content evolution in *Lactobacillales*. Lost (blue) and gained (red) LaCOGs for each node indicating the evolution from a bacilli ancestor via a *Lactobacillales* ancestors to different species of *Lactobacillus, Streptococcus, Pediococcus, Lactococcus, Leuconostoc* and *Oenococcus* (Makarova and Koonin, 2007).

They are associated with the gastrointestinal (GI) tract and mucosal surfaces, with decaying plant material and with various food-related environments like meat, milk, wine and plant based materials (Wood and Holzapfel, 1995; Wood and Warner, 2003). Historically, their presence in fermented food products and the good processability were key factors for their selection as starter cultures in industrial fermentations for dairy products, meat, vegetables, cocoa beans and many more. Nowadays, the acidification mainly due to lactic acid production and the property of many strains to produce antimicrobial substances (e.g. bacteriocins) are added to the selection criteria. Strains with antimicrobial activity can be used as protective cultures to inhibit the outgrowth of spoilage

organisms in food and feed. Some LAB strains are beneficial for human health and are selected for the use as probiotics (Klaenhammer et al., 2008; Makarova et al., 2006).

3 Genus *Lactobacillus*

Lactobacillus is the predominant genus of Lactobacillales with over 170 species and lactobacilli are isolated from different fermented food products (Goldstein et al., 2015). Isolates of the genus *Lactobacillus* are non-sporeforming rods with a low %G+C content in the genome and a catalase-negative phenotype (Salvetti et al., 2012). Lactobacilli can grow at temperatures of 2 to 53 °C with best growth between 30 and 40°C and within a pH range from 3 to 8 in aerobic and anaerobic conditions (Holzapfel and Wood, 2014). In general, they are fermentative, with a homofermentative or heterofermentative metabolism (Giraffa et al., 2010; Tannock, 2004). Lactobacilli have been isolated mainly from dairy products, fermented meat products and fermented plant products such as sourdough, beer, wine, silage, sauerkraut and olives (Wood and Holzapfel, 1995). Historically, lactobacilli have been used for biopreservation of food products due to fast acidification by production of organic acids, their fast substrate utilization, the production of antimicrobial peptides and metabolites and their enhancement of texture and flavor (Stiles, 1996). They are further commonly found as a functional part of the human and animal microflora (Mujagic et al., 2017; van Baarlen et al., 2013). The Qualified Presumption of Safety (QPS) status from the European Food Safety Agency (EFSA) facilitates commercial use and acceptance of certain *Lactobacillus* species and makes them ideal candidates for the use as protective and starter cultures (EFSA - NDA Panel, 2015). Protective cultures against *Listeria monocytogenes* in fermented meat and against fungal spoilage in dairy products are only two examples of various products that are on the market (Barbosa et al., 2014; Miescher Schwenninger and Meile, 2004). Aside from their preserving qualities, some *Lactobacillus* species such as *L. acidophilus, L. casei, L. gasseri, L. johnsonii, L. reuteri and L. salivarius* are also exploited for their health promoting potential as probiotics and vaccine carriers (Goh and Klaenhammer, 2009; Saito, 2004).

The genus *Lactobacillus* is very heterogeneous with a genome size range of 1.23 Mb for *L. sanfranciscensis* to 4.87 Mb for *L. parakefiri* (NCBI Resource Coordinators, 2016). Initially, *Lactobacillus* taxonomy was based on phenotypic markers like temperature and pH growth range, carbohydrate fermentation type and cell wall composition (Klein et al., 1998). Nowadays, lactobacilli are amongst other criteria classified according to their ability to ferment different hexoses and their hetero- or homofermentative metabolism (Carr et al., 2002; Hammes and Hertel, 2009). Homofermentative lactobacilli (group A) ferment hexoses to lactic acid via the Embden-Meyerhof-

Parnas pathway (EMP) or the glycolysis, whereas gluconate and pentoses are not fermented. Facultative heterofermentative species (group B) can ferment pentoses and gluconate with a phosphoketolase in the pentose phosphate pathway (PP) producing ethanol, formic acid and acetic acid when glucose is limited. They can also ferment hexose via EMP to lactic acid. Obligate heterofermentative species (group C) use fructose 1,6-bisphosphate aldolase instead of phosphoketolase to ferment pentoses and hexoses via phosphogluconate pathway to lactic acid, CO_2 and ethanol or acetic acid (Salvetti et al., 2012). There are species with unclear or mixed fermentation patterns, suggesting that taxonomy based only on fermentation patters can be misleading (Hammes and Hertel, 2009).

Based on 16S rRNA gene sequence comparison, the genus *Lactobacillus* and related genera were initially clustered into three subgroups: the *Lactobacillus delbrueckii* group, the *Lactobacillus casei-Pediococcus* group and the *Leuconostoc* group (Collins et al., 1991). New species were described over the past years, which led to a reorganization based on 16S rRNA gene sequences. Only little correlation was detected between 16S rRNA gene sequences clustering and traditional clustering based on fermentation type and metabolic properties (Felis and Dellaglio, 2007). Salvetti et al. (2012) clustered the 16S rRNA gene of each type strain for 152 *Lactobacillus* species which resulted in a phylogenetic tree with 15 major groups containing up to 27 species per group, 4 couples and 10 single species.

Taken together, taxonomic classification of lactobacilli has undergone many changes and a useful grouping based on true evolutionary events is still under discussion.

4 Interaction of lactobacilli with other organisms

Lactobacilli colonize nutrient-rich environments with frequently a high microbial density such as fermenting food products. Adaptation to other microorganisms in the environment is essential to survive and compete for resources. Detection of population density and the associated modification of gene expression is called quorum sensing (QS). A bacterium is using signal molecules concentration such as lactic acid, in the environment to estimate the cell density in its environment (Popat et al., 2014). Autolysis of *Lactobacillus delbrueckii* subsp. *bulgaricus* ATCC BAA-365 is regulated by quorum sensing (Pang et al., 2014) via a two-component (Pang et al., 2016). A 21-amino acid peptide is the QS-signal molecule to regulate autolysis in *L. delbrueckii* subsp. *bulgaricus* ATCC BAA-365. Beside self-regulating processes like autolysis, also defense mechanisms such as bacteriocin production are regulated by QS (Rizzello et al., 2014).

4.1 Antibacterial activity in *Lactobacillus*

Antibacterial activity in lactobacilli occurs over a wide range of species and is based on unspecific factors such as substrate utilization, acid formation and decrease of pH or specific inhibitors such as bacteriocins, reuterin, reutericyclin, fatty acids or peroxide (Gänzle, 2009).

4.1.1 Bacteriocins

Bacteriocins are ribosomally encoded antimicrobial proteinaceous compounds produced by bacteria to inhibit the growth of other bacteria which are closely related or a broad spectrum across genera (Cotter et al., 2013; Klaenhammer, 1988). Self-killing of the producer strain by its own bacteriocin is inhibited by an immunity gene (Kristiansen et al., 2016). Bacteriocin production can be induced by co-cultivation with live cultures, heat treated cells or supernatant (Chanos and Mygind, 2016). In general, bacteriocins are not harmful for humans, due to their high specificity for bacterial cell membranes. An exception is the bacteriocin cytolysin produced by *Enterococcus faecalis,* which is active against a broad range of cell types including Gram-positive bacteria, eukaryotic cells as well as horse, bovine and human enterocytes, retina cells and human intestinal epithelial cells and can lead to terminal infections in humans (Cox et al., 2005). The cytotoxicity of a purified bacteriocin and its interaction with drugs has to be determined to evaluate the application potential of the bacteriocin (Todorov et al., 2017). The bacteriocin nisin, initially isolated from cheddar cheese, was the first well-documented peptide (Mattick and Hirsch, 1947; Whitehead, 1933). Nisin was first applied in 1951 in Swiss-type cheese to prevent spoilage of anaerobic spore-forming bacteria (Hirsch et al., 1951) and is until today the only bacteriocin approved as a food additive (E234) (European Commission, 2010).

Bacteriocins have been grouped in various schemes ranging from 3 to 5 classes (Kemperman et al., 2003; Klaenhammer, 1993; Nes et al., 1996). In 2005 a classification was proposed with 3 classes: lanthionine-containing lanthibiotics (class I), non-lanthionine-containing bacteriocins (class II), and a group for bacteriolysins and non-lytic bacteriocins (class III) (Cotter et al., 2005). This classification received only minor adjustments since then. In silico detection and annotation is done today with online tools such as Bagel 3 (van Heel et al., 2013) and antiSMASH 3.0 (Weber et al., 2015). These tools compare the submitted gene sequence with an existing database of described bacteriocins to detect potential candidate genes.

Bacteriocins or bacteriocin like inhibitory substances (BLIS) were detected in 26 *Lactobacillus* species (Table 1.1). So far, no class I bacteriocin is documented within the genus *Lactobacillus.* The wide spread of bacteriocin genes in the genus *Lactobacillus* might result in a larger list in the future.

Table 1.1 Detected bacteriocins in *Lactobacillus* species.

Species	Class II	Class III	unclear	Reference
L. acidophilus	Lactacin B/F	Acidophilucin A		Barefoot and Klaenhammer, 1983; Muriana and Klaenhammer, 1991; Toba et al., 1991
L. amylovorus	Amylovorin L471			Callewaert et al., 1999
L. animalis			BLIS	Chen and Yanagida, 2006
L. bavaricus	Bavaricin A			Larsen et al., 1993
L. brevis			Bacteriocin	Ogunbanwo et al., 2003
L. buchneri			Buchnericin LB	Yildirim et al., 1999
L. casei	Lactacin B, lactocin 705	Caseicin 80		Barefoot and Klaenhammer, 1983; Rammelsberg et al., 1990; Vignolo et al., 1995
L. coryniformis	Lactocin MXJ 32A			Lü et al., 2014
L. crispatus	Crispaticin A			Thara and Kanatani, 1997
L. crustorum	Bacteriocin MN047 A			Yi et al., 2016
L. curvatus	Curvaticin A			Tichaczek et al., 1992
L. delbrueckii	Lacticin			Toba et al., 1991c
L. fermentum			Fermenticin B	Yan and Lee, 1997
L. gasseri	Gassericin A			Pandey et al., 2013
L. helveticus		Helveticin J/V		Joerger and Klaenhammer, 1990; Vaughan et al., 1992
L. hordei			BLIS	Rouse et al., 2008
L. johnsonii	Lactacin F			Abee et al., 1994)
L. murinus		Bacteriocin		Elayaraja et al., 2014
L. paracasei			Bacteriocin 217	Lozo et al., 2004
L. paraplantarum		Paraplantaricin C7		Lee et al., 2007
L. pentosus	Pentocin TV35b			Okkers et al., 1999
L. plantarum	Plantaricin E/F/J/K/S/T/W			Zacharof and Lovitt, 2012
L. reuteri			Reutericin 6	Toba et al., 1991a
L. rhamnosus			Lactocin 160	Li et al., 2005
L. sakei	Sakacin A/G/P			Barbosa et al., 2014
L. salivarius	Salivaricin T/L/P			Messaoudi et al., 2013

BLIS = Bacteriocin-like inhibitory substance

4.1.2 Other antimicrobial substances such as reutericyclin and reuterin

Reutericyclin is a low-molecular-weight tetramic acid with antimicrobial activity produced by some *Lactobacillus reuteri* strains (Lin et al., 2015). The broad range of inhibition of reutericyclin includes amongst others *Bacillus cereus*, *Bacillus subtilis, Escherichia coli, Staphylococcus aureus, Candida krusei, Saccharomyces cerevisiae* and various *Lactobacillus* species (Gänzle et al., 2000).

Reuterin is a multi-component system consisting of 3-hydroxypropionaldehyde (3-HPA), 3-HPA dimer and 3-HPA hydrate (Engels et al., 2016b) with a broad unspecific antimicrobial activity including the inhibition of *Listeria innocua* and *Escherichia coli* (Cleusix et al., 2007). Strains from the genera *Enterobacter*, *Lactobacillus, Clostridium, Klebsiella* and *Citrobacter* (Vollenweider and Lacroix, 2004) as well as the species *Eubacterium hallii* (Engels et al., 2016a) convert glycerol via glycerol dehydratase to 3-HPA. In *L. reuteri* and *E. hallii* 3-HPA is released into the environment where it acts as an antimicrobial compound (Engels et al., 2016a; Vollenweider and Lacroix, 2004).

4.2 Antifungal activity in *Lactobacillus*

Organic acids such as lactic acid, acetic acid and phenyllactic acid are end-products of the carbohydrate metabolism of lactobacilli. These weak acids lower the pH to a level for which metabolism and growth of bacteria and fungi is inhibited (Batish et al., 1997). However the exact mechanism how organic acid inhibit microbial growth is not fully understood (Crowley et al., 2013a). In theory, non-dissociated acids diffuse into the cytoplasm where protons are released, which results in the acidification of the cytoplasm and a dissipation of the pH gradient over the membrane causing microbial growth inhibition (Piard and Desmazeaud, 1991). Antifungal activity in lactobacilli is mainly caused by lactic acid and to a smaller part by acetic acid (Dang et al., 2009). Acetic acid works synergistically with lactic acid when exerting antifungal activity. Phenyllactic acid (PLA) is one of the most studied antifungal organic acids. PLA is produced from phenylpyruvate via hydroxyphenylpyruvate reductase (EC: 1.1.1.237) or (R)-4-hydroxyphenyllactate dehydrogenase (EC: 1.1.1.222). However, little is known about the mechanism of antifungal inhibition. PLA works pH-dependent and its production in lactobacilli can be induced by adding phenylalanine to a particular growth medium (Cortés-Zavaleta et al., 2014; Svanström et al., 2013). PLA is produced by various lactobacilli including strains from the species *L. acidophilus, L. casei, L. fermentum, L. rhamnosus, L. reuteri, L. sakei, L. plantarum, L. paracasei* and *L. brevis* and has a broad inhibition spectrum including *Aspergillus niger, Asperillus flavus, Penicillium roqueforti, Penicillium expansum, Ebdinyces fibuliger, Botrytis cinerea* and *Colletorichum gloeospoioides* (Cortés-Zavaleta et al., 2014; Prema et al., 2010; Valerio et al., 2016). Further antifungal acids such as formic acid, propionic acid, butyric acid, caproic acid and fatty acids are described. Those acids are not produced or only produced in low concentrations in lactobacilli and are not discussed further here.

Antifungal peptides produced by lactobacilli are rarely described and the mechanism of inhibition is unknown. *Lactobacillus pentosus* TV35b isolated from the vaginal secretion of a prenatal patient produces a bacteriocin-like peptide, pentocin TV35b with a size between 2.3 and 3.4 kDa (Okkers et al., 1999). This peptide inhibits the growth of strains from different species of *Clostridium, Lactobacillus*, *Propionibacterium* and the yeast *Candida albicans.* Pentocin TV35b is heat-stable for 30 min at 100 °C and can be inactivated with proteinase treatment. Antifungal peptides isolated from lactobacilli associated with plants are more common than from lactobacilli from meat or dairy. Proteinaceous antifungal activity was detected in *L. coryniformis* subsp. *coryniformis* Si3 (Magnusson and Schnürer, 2001) isolated from grass silage and *L. brevis* AM7 (Coda et al., 2008), *L. plantarum* LB1 and *L. rossiae* LB5 (Rizzello et al., 2011) all isolated from sourdough. Antifungal peptides from these strains were shown to inhibit the growth of a wide range of yeasts and molds.

Cyclic dipeptides are very short peptides minimally containing only 2 amino acids and possess antifungal activity (Crowley et al., 2013a). While peptide diversity and inhibition range is well documented, less is known about production and the *modus operandi* (Ryan et al., 2011; Ström et al., 2002; Yang and Chang, 2010). The concentration of cyclic dipeptide which is necessary to inhibit fungal growth is also responsible for bitter flavor and taste. Therefore, the application potential in food products is limited (Da Costa et al., 2010).

5 *Lactobacillus* applications in food

Lactobacilli were isolated from a broad range of food products, as already mentioned. This makes them suitable for potential food-associated applications such as starter, protective or probiotic cultures.

5.1 Starter, protective and probiotic cultures

An annual food waste of 88 million tonnes is estimated in the European Union, resulting in associated costs of 143 billion Euros. Beside these economical issues, wasting food is also an ethical issue due to the limited ressources on our planet. Food waste occurs along the entire supply chain with a major part of 53% occuring in the households (Stenmarck et al., 2016). Appropriate preservation of food leads to longer shelf life which contributes significantly to reduction of food waste (Martindale, 2017). The consumer trend for minimally processed food shifts the focus of the industry away from chemical preservatives towards more natural preservatives techniques like biopreservation (Crowley et al., 2013b). Biopreservation is an approach were natural or controlled microbiota and/or antimicrobial compounds to increase shelf life and food safety (Ananou et al., 2007). The key components of biopreservation are starter and protective cultures. Starter cultures can be defined as naturally occuring or intentionally added cultures to control a fermentation process. These cultures are selected for criteria such as fast acidifaction and contribution to desired flavor and texture (Campbell et al., 2011). Protective cultures are used to inhibit the outgrowth of specifically targeted food spoilage microorganisms. These cultures are defined as strains which contribute to the food safety by production of antimicrobial substances like bacteriocins, low molecular-weight substances and/or various metabolites that reduce spoilage (Anacarso et al., 2014). Protective cultures are selected based on the targeted spoilage microorganism. While starter and protective cultures benefit taste, sensory aspects and safety of a food product, probiotic cultures are supposed to benefit the health of the food consumer. Probiotic cultures can be defined as viable microorganisms that colonize compartment of the gastrointestinal tract and benefit the health of the host such as *Lactobacillus plantarum* and *Lactobacillus acidophilus* (Parvez et al., 2006).

5.2 Properties of a selected protective culture

Protective cultures in food applications should fulfil the following criteria (Holzapfel et al., 1995): (1) they should possess QPS status; (2) produce heat-stable antimicrobial active compounds; (3) not be associated with health risks; (4) benefit the quality, texture and flavor of the food product; (5) not affect the starter culture or any other intentionally added culture in the food product; (6) the inhibitory activity should be stable at least as long as the eat-by date of the product; (7) the economical balance should be positive.

The selection of an appropriate protective culture for a targeted food product is based on the above mentioned criteria. Protective culture with a narrow-activity spectrum can be used in food products with a specific spoilage organism, e.g *Listeria monocytogenes* in meat products (Chapter 5). Protective cultures against Gram-negative bacteria often use an additional treatment to attack the outer cell membrane in combination with bacteriocin activity that targets the inner membrane. These treatments can be amongst others high pressure (Alpas et al., 1999), temperature shock (Boziaris and Adams, 2001) and eukaryotic antimicrobial peptides (Lüders et al., 2003) or a combination of them (Kalchayanand et al., 1998).

A major problem of bacteriocin-producing protective cultures is the maintenance of the *in situ* activity of the bacteriocin. Bacteriocins are absorbed by proteins (Aasen et al., 2003) or fat in the food matrix (Settanni et al., 2005b), resulting in decreased activity. The *in situ* production of bacteriocin is another challenge since it's not necessarily as efficient as the *in vitro* production (Settanni et al., 2005a). Nisin is the only bacteriocin licensed as food additive in Switzerland today (EDI, 2017). Aside from nisin, other bacteriocins have to be produced *in situ* for application.

In general, bacteriocins target spoilage organisms that reduced the shelf-life of a food product and/or are pathogenic for the consumer. Therefore, most of the protective cultures inhibiting spoilage organisms such *Listeria monocytogenes, Salmonella enterica* or *Escherichia coli* O157:H7 (Chaillou et al., 2014; Katla et al., 2001). However, spoilage organisms don't necessarily impact the shelf-life. Enterococci are viewed critical since they have a high prevalence of antibiotic resistance and can transfer the respective genes by horizontal gene transfer (HGT) to other bacteria within or across genus border (Haug et al., 2010; Leisibach, 2004). Therefore, the intake of acquired antibiotic resistances in starter and protective and probiotic cultures should be monitored closely (Kastner et al., 2006). Since those cultures occur in high concentrations in the product, it's crucial to select cultures without antibiotic resistance genes (Marty et al., 2012).

The selection process of a protective culture generally starts with a preferably large set of strains ideally isolated from a latter targeted food product. These isolates are then phenotypically characterized depending on their application range (Marty, 2011). The following patterns are

evaluated amongst others: acid tolerance, salt tolerance, ability to ferment different sugars, ability to grow in different low and high temperature conditions, antimicrobial activity against selected spoilage and/or pathogen indicator, ability to grow in food matrix and antibiotic resistance characterization. Those parameters can be used for a funnel-shaped selection. First cultures with "no-go" attributes are excluded. Classical no-go attributes are transferable antibiotic resistance genes, no antimicrobial activity against targeted spoilage organism and inability to grow or to be metabolic active in a targeted food matrix. Remaining isolates are further tested for their ability to grow in the targeted food matrix. Testing of each isolate in a small-scale fermentation would be too expensive. Therefore, food matrix conditions are classically mimicked with modified growth media such as addition of 10% NaCl. The most promising cultures are selected and tested *in situ* to evaluate: antimicrobial activity in food matrix, impact on flavor, odor and texture of the product, stability of antimicrobial activity and stability of the food product.

5.3 Application of protective cultures in food products

The properties of protective cultures vary for each product. The following protective cultures are exemplary for their food product category.

Meat products – Fermented meat products such as salami are typically contaminated with strains from the genus *Listeria, Staphylococcus* and *Clostridium*. *L. monocytogenes* can cause listeriosis (Maertens de Noordhout et al., 2014), *S. aureus* can produce toxins which affects the human body (Bosi et al., 2016) and *C. botulinum* is responsible for botulism, a fatal infection which blocks signal transmission in nerves and muscles (Proverbio et al., 2016). *L. plantarum* PCS20 was tested in combination with nitrate for its activity against *Clostridium perfringens* DSM 756 and *Clostridium* sp. DSM 1985, both closely related to *Clostridium botulinum* (Di Gioia et al., 2016). Salami batches were produced with either i) protective culture, ii) 150 mg/kg nitrate or iii) a combination of both and *Clostridium* strains as spoilage organisms. 10^4 cfu / g *C. perfringens* DSM 756 and 10^3 cfu/ g *C*. sp. DSM 1985 were reduced to at least 10^2 cfu/g and 50 cfu/g, respectively after 9 days. The treatment with nitrate was slightly better than the combination of nitrate and protective culture or the protective culture only. Nevertheless, adding a protective culture enables the reduction of nitrate in meat fermentation due to the proteinaceous antibacterial activity.

Dairy products – Fermented dairy products such as yoghurt typically have a low pH and are therefore susceptible for spoilage by acid-tolerant fungi, such as *Candida parapsilosis, Candida diffluens, Debaryomyces hansenii, Klyveromyces marxianus,* and *Rhodotorula mucilaginosa* (Mayoral et al., 2005). Most fungal spoilage is harmless and only creates an unsavory biofilm, but spoilage with *Candida parapsilosis* can lead to acute health problems (Trofa et al., 2008). Several lactobacilli from

the species *L. harbinensis, L. zeae, L. paracasei* and *L. rhamnosus* were used in challenge tests in yoghurt production at 42°C (Delavenne et al., 2013). The isolate *L. harbinensis* K.V9.3.1Np completely prevented fungal growth despite its slow growth rate at 42°C. The synthesis or the activity of the antifungal metabolite was potentiated by the yoghurt starter culture (Delavenne et al., 2013). Another example of a protective culture application in dairy product is Holdbac YM-C, a commercially available application of a *Lactobacillus-Propionibacterium* co-culture that prevents fungal spoilage in fresh fermented products and white cheese (Miescher Schwenninger et al., 2008; Miescher Schwenninger and Meile, 2004).

Seafood – Seafood products, such as cold smoked salmon contaminated with *L. monocytogenes* are a risk for the consumers. Cold-smoked salmon is a ready-to-eat product that is stored at low temperature at which *L. monocytogenes* is able to grow. *L. sakei* Lb790, a sakacin P producing strains, was used as a protective culture to control the outgrowth of *L. monocytogenes* in cold smoked salmon (Katla et al., 2001). The application with sakacin P or the strain Lb790 controlled the *L. monocytogenes* concentration at the inoculation concentration level of 10^4 cfu/g, compared to 10^8 cfu/g in the control. The combination of the strain *L. sakei* Lb790 and sakacin P reduced the *L. monocytogenes* concentration even below 10^2 cfu/g.

Plants – Pest management in agriculture is a key factor for stable food production and reduction of food waste. Fire blight, a wide-spread disease in pome fruits and rosaceaous plants, is caused by the Gram-negative pathogen *Erwinia amylovora*. The antibiotic streptomycin protects against fire blight, but usage is prohibited in Switzerland since 2016 (BLW, 2016). Therefore, the control of fire blight relies nowadays on copper compounds. As an alternative, a protective culture with different *Lactobacillus plantarum* strains was developed and patented (Montesinos Segui et al., 2014). The culture showed similar reduction rates as streptomycin (Roselló et al., 2013).

Non-food – Lactobacilli are also used in protective culture application to prevent non-food products from bacterial or fungal spoilage. As an example, *L. plantarum* is used against *Pseudomonas aeruginosa* and *Bacillus putrefacines* in the industrial production of leather (Kanagaraj et al., 2014) or in several medical applications e.g. as vaginal application to prevent listeriosis in pregnant women (Borges et al., 2013). Especially the medical application as an alternative for antibiotics is discussed frequently (Cotter et al., 2013; Joerger, 2001; Pieterse and Todorov, 2010). For example, nisin F was intranasally administered to control the concentration of *S. aureus* in mice in a preclinical study (De Kwaadsteniet et al., 2009).

More examples of bacteriocin and protective *Lactobacillus* culture applications in food products is listed in Table 1.2.

Table 1.2 Applications of lactobacilli protective cultures and lactobacilli bacteriocins for food products.

Antimicrobial compound	Producer	PC	BA	Application in	Inhibition of	Reference
Sakacin P	*L. sakei*		x	Cold smoked salmon	*Listeria monocytogenes*	Katla et al., 2001
Paraplantaracin L-ZB1	*L. paraplantarum* L-ZB1		x	Rainbow trout fillets stored at 4°C	Enterobacteriaceae, *Pseudomonas,* spore-forming bacteria	Gui et al., 2014
Bacteriocin-like substance	*L. pentosus* 39	x		Salmon fillets	*Aeromonas hydrophila* ATCC 14715, *Listeria monocytogenes* ATCC 19117	Anacarso et al., 2014
Bacteriocin RC20975	purified bacteriocin from *L. rhamnosus*		x	Apple juice	*Alicyclobacillus acidoterrestris*	Pei et al., 2017
Lactic acid, acetic acid, phenyllactic acid	*L. plantarum*	x		Orange juice	*Rhodotorula mucilaginosa*	Crowley et al., 2012
Plantaricin	*L. plantarum*	x		Pome fruits and ornamental rosaceous plants	*Erwinia amylovora* (fire blight)	Roselló et al., 2013
Bacteriocin mixture	*L. curvatus, Pediococcus acidilactici, Enterococcus faecalis*		x	Hot dogs	*Listeria monocytogenes*	Vijayakumar and Muriana, 2017
Bacteriocin	*L. fermentum* R6		x	Chicken breast meat	*Clostridium perfringens*	Li et al., 2017
Bacteriocins	*L. sakei, L. curvatus*	x		Beef	*Enterobacteriaceae, Pseudomonsa spp., Brochothriy thermospacta*	Katikou et al., 2005
Sakei N1 cocktail	*L. sakei*	x		Ground beef	*Salmonella enterica Typhimurium, Escherichia coli* O157:H7	Chaillou et al., 2014
Bacteriocin	*L. acidophilus NCDC 291*	x		Raw poultry meat	*Alternaria alternata*	Garcha and Natt, 2012
Propionic acid, lactic acid, acetic acid	*Lactobacillus paracasei, Propionibacterium jensenii*	x		Yoghurt	*Candida pulcherrima, Rhodotorula mucilaginosa*	Miescher Schwenninger and Meile, 2004
Antimicrobial peptides	*L. plantarum LR/14*		x	Wheat grain	*iAspergillus niger, Rhizopus Stolonifer, Mucor racemosus, Penicillium chrysogenum*	Gupta and Srivastava, 2014
3,6-bis(2-methylpropyl)-2,5-piperazinedion	*L. plantarum* AF1	x		Soybean	*Aspergillus flavus*	Yang and Chang, 2010
Peptides	*L. rossiae, L. paralimentarius*	x		Pantone	*Aspergillus japonicus*	Garofalo et al., 2012
Metabolites	*L. buchneri*	x		Corn silage	yeasts	Tabacco et al., 2011
Coriolic acid	*L. hammesii*	x		Sourdough	*Aspergillus niger, Penicillium roqueforti*	Black et al., 2013

PC = protective culture; BA = bacteriocin application

6 Comparative genomics

Selection of potential protective cultures is based on a phenotypic and genotypic screening of strains. The created datasets can be used for polyphasic taxonomy and in silico studies. Evolutionary history of a certain isolate and horizontal gene transfer can be detected. In combination with comparative genomics, genetic differences behind phenotypic adaptations can be detected.

6.1 Polyphasic approach

Bacterial taxonomy is based on a polyphasic approach to delineate taxa on all levels. The term "polyphasic" was introduced by Colwell (1970) to describe the taxonomy of the genus *Vibrio*. A polyphasic approach includes phenotypic, genotypic and chemotaxonomic information to characterize and classify a microorganism (Vandamme and Peeters, 2014). The non-standardized approach is adapted for each microorganism according to the predicted genus or species. For each species, a type strain is defined and a new isolate is either closely related to an existing type strain or serves as a new type strain for a novel species (Kyrpides et al., 2014).

Phenotypic classification for bacterial taxonomy includes morphological, physiological and biochemical information and requires consistency to create a useful taxonomy (Vandamme et al., 1996) (Figure 1.2). Morphological features are: bacterial shape, endospore formation, presence and numbers of flagella, forming of inclusion bodies, Gram-staining. The physiological and biochemical features include: temperature range for growth, pH range, salt concentration, ability to grow on different substrates. Genotypic classification is based on: %G+C content in the genome, DNA-DNA hybridization (DDH), rRNA sequence homology, multilocus sequence typing (MLST), whole genome sequence. Chemotaxonomic classification includes: cell wall composition, fatty acid spectra or whole cell protein analysis. Since the approach is constantly evolving new "all in one" methods such as MALDI BioTyper may get integrated soon (Ramasamy et al., 2014; Sogawa et al., 2011). These approaches generally require an already existing structure to rely on while a standardized library based on existing taxonomic books is not yet available (Zhi et al., 2012).

6.1.1 Polyphasic taxonomy of lactobacilli

As already mentioned, the taxonomy of isolates in the genus *Lactobacillus* was based for years on different phenotypic attributes (Klein et al., 1998) and carbohydrate fermentation pattern (Hammes and Hertel, 2009). A more recent clustering based on the 16S rRNA sequence grouped lactobacilli in 15 major clusters (Salvetti et al., 2012). However, certain species couples are not distinguishable

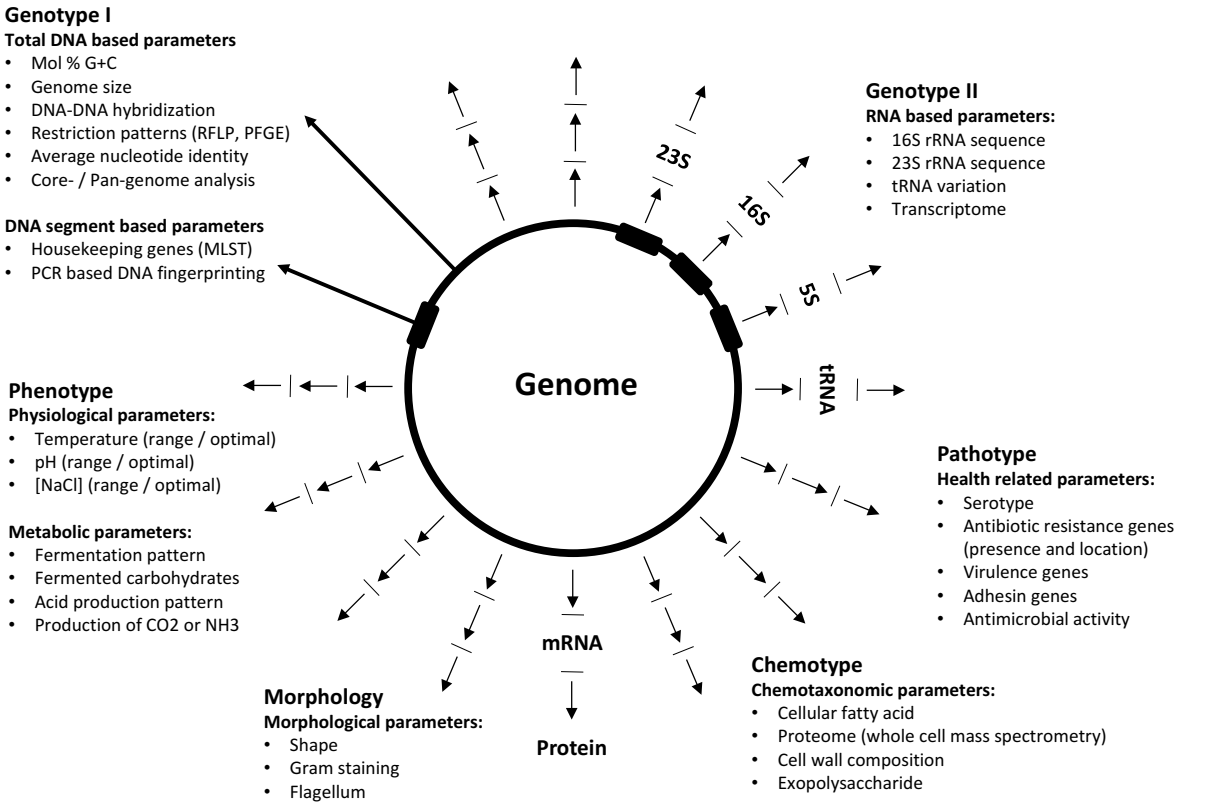

Figure 1.2 Phenotypic, chemotypic and genotypic criteria for a polyphasic approach adapted from Vandamme et al. (1996) for bacterial classification

with 16S rRNA sequence comparison such as *L. sakei / L. curvatus* and *L. helveticus / L. acidophilus* (O'Sullivan et al., 2009). Multilocus sequence typing (MLST) was established to cluster below species level and distinguish between closely related species. MLST compares the sequence of several conserved genes in the genome and clusters the isolates accordingly. *Lactobacillus delbrueckii* was clustered with MLST according to 7 genes into 4 main clusters and 4 subclusters (Tanigawa and Watanabe, 2011). Depending on the *modus operandi* of MLST, the algorithm uses either sequences or profiles based on sequences which leads to different phylogenetic clusters (Jans et al., 2016). Clustering based on the complete genomic content instead of only selected parts, can be achieved by the implementation of whole genome sequencing (WGS) datasets. Since the sequencing costs of WGS decreased drastically, these datasets are available for every new sequenced bacterium (Ott et al., 2015). An initial implementation of WGS datasets into *Lactobacillus* taxonomy was based on the *Lactobacillales*-specific clusters of orthologous protein coding genes (LaCOG) model (Makarova and Koonin, 2007). LaCOGs were defined based on 12 sequenced *Lactobacillales* genomes according to the COG procedure (Tatusov et al., 1997). However, a major disadvantage of the LaCOG method is the sensitivity due to calculation based on genes affected by horizontal gene transfer (HGT) or paralogs. Therefore, lactobacilli were clustered in this study (Chapter 6) according to the genus-

independent approach of average nucleotide identity (ANI). The approach to cluster genomes according to their pairwise calculated ANI uses the entire WGS dataset and not only parts of it (Arahal, 2014). ANI calculates the evolutionary distance between two strains according to the identity of all conserved genes with the BLAST algorithm. A major advantage of ANI is its robustness to horizontal gene transfer events (Arahal, 2014). An ANI score of >94% is comparable with >70% DDH and can be used to cluster species according to Konstantinidis and Tiedje (2005). The calculation of ANI was implemented in the clustering according to the core- and pan-genome of a dataset. The core-genome is defined as all homologous genes present in each genome of the dataset, whereas the pan-genome is defined as all homologous genes present in all genomes (Tettelin et al., 2005). Clustering of the family Lactobacillales according to the core-genome was demonstrated with non-closed genomes (Sun et al., 2015). A comparative genomics approach to cluster *Lactobacillus* genomes according to their core- and pan-genome is described in chapter 6.

6.2 Comparative genomics study designs

Studies in comparative genomics can be classified into five categories (1) comparing of a single genome with other genomes; (2) comparing genomes from a single species in a single habitat; (3) comparing multiple species from a single habitat; (4) comparing a single species from various habitats; (5) comparing multiple species from various habitats. The study design is depending on the aim of a particular study.

6.2.1 Single genome comparison

Studies focusing on a single genome are used for species with little available genome sequences. The genome is compared with isolates from the same habitat or with closely related species. *Lactobacillus iners* AB-1, isolated from human vagina and one of the lactobacilli with the smallest genome (1.3 Mb), was compared with isolates from the species *L. johnsonii, L. gasseri, L. delbrueckii, L. acidophilus* and *L. crispatus* (Macklaim et al., 2011). The predicted core-genome of those 6 species contained 766 genes, which is 64% of the *L. iners* genome content. 65 of 1180 genes were acquired by HGT and 26 of those share >80% amino acid sequence identity with non-*Lactobacillus* organisms. This study demonstrated, how a relatively unknown bacterium can be characterized at genotypic level with comparative genomics.

6.2.2 Single species / single habitat

Studies focusing on genomes from single species in a single habitat are rare and focus on differentiation of the genomes on subspecies level, horizontal gene transfer between the strains, evolution of core- and pan-genome and strain specific genes. Since all included genomes originate

from the same habitat, genetic variation should not be driven by environmental adaptation. The concept of a pan-genome was first established in a study using 8 *Streptococcus agalactiae* genomes from the same habitat (Tettelin et al., 2005). Within those 8 isolates, 69 genomic islands were found. Each of those genomic islands was absent in at least one of those 8 genomes. An atypical nucleotide composition was detected in some of those genomic islands indicating a potential horizontal gene transfer in an early ancestor. A closed core-genome with 1806 genes was calculated, whereas the pan-genome remained open, gaining 33 genes per added genome and a total of 358 genes were found present in only one of the 8 genomes. The open pan-genome indicates, that the analysis requires more than the 8 included genomes to represent the genomic variability.

The serotype clustering showed significant differences with the clustering according to the core-genome (Tettelin et al., 2005). This indicated that serotypes are not adequate indicators for taxonomy based on evolutionary events. Another study in *Streptococcus agalactiae* demonstrated that the exchange of large chromosomal fragments up to 334 kb, are part of evolutionary history (Brochet et al., 2008). Similar content were demonstrated for *Streptococcus pneumoniae* indicating that the presence of a distribute genome is bacterial strategy for host interaction (Hiller et al., 2007).

6.2.3 Multiple species / single habitat

Environmental adaptations can be studied with comparing multiple isolates from the different species in the same habitat. A significantly lower %G+C content in the genome was detected in vaginal isolates compared to non-vaginal isolates in a study comparing 25 species of *Lactobacillus* (Mendes-Soares et al., 2014). COGs exclusively detected in vaginal lactobacilli were related to phosphate transport system, indicating that phosphate acquisition is necessary in the vaginal environment. Adaptations to plant habitat such as degradation of xylan, arabinan, glucans and fructans as well as degradation of typical plant cell wall products was demonstrated in a study comparing two *Lactococcus lactis* isolates (Siezen et al., 2008). Neither of those adaptations were present in the dairy isolated control group. 6 dairy specific genes, encoding for proteins in the proteolytic system and restriction modification systems were detected in a study with 11 lactic acid bacteria (O'Sullivan et al., 2009). These differences indicate the adaptation of strains to their corresponding environment.

6.2.4 Single species / multiple habitats

Studies comparing a single species in multiple habitats generally demonstrate the biotechnological potential and diversity of the species while gaining insights into the genomic complexity of the population. Marker genes for clades within the species such as subspecies or environmental specific genetic adaptations to a specific habitat are another output of these studies. Subspecies

differentiation was demonstrated in the heterogenic species *L. plantarum*, where 22 genes were detected that were absent in *L. plantarum* subsp. *argentoratensis* and present in *L. plantarum* subsp. *plantarum* (Siezen et al., 2010). The pan-genome of 17 *L. casei* isolates revealed genetic exchange between plant fermenting *L. casei* and *L. coryniformis* and *L. casei* from dairy fermentations and *L. fermentum* (Broadbent et al., 2012). Accessory genes with high homology to genes in *Listeria* species were found in *L. casei* strains isolated from dairy and humans but not from plants even *Listeria* is commonly present in plant environment (Broadbent et al., 2012). The population of 100 *L. rhamnosus* isolates could be split into two clades based on their relative shared gene content (Douillard et al., 2013). One clade included isolates from stable, nutrient rich niches such as dairy products and the other isolates from variable environments such as intestinal tracts. The phenotypic and genotypic data also revealed that those *L. rhamnosus* isolates could reside in multiple niches. Acid resistance in *L. plantarum* was linked to the heat-shock protein GrpE, the methionine synthase and a 30S ribosomal protein, while acid sensitivity was linked to a phosphotransacetylase and a adenylosuccinate synthase (Hamon et al., 2014). Studies with a single species allows to determine the genomic variation within a species, which improves the species description.

6.2.5 Multiple species / various habitats.

Comparison of related bacteria from multiple species and habitats with each other is by far the most used study design. This approach determines amongst others the biotechnological potential of the strains, HGT between resident strains, phylogenic classification or variation of modules such as CRISPR. Clustering with 16S rRNA gene sequences or the core-genome is present in almost every study. Phylogenetic trees were constructed based on smaller genus datasets (Bottacini et al., 2016; Kant et al., 2011; Suzuki et al., 2012), hundreds of genomes from closely related lactobacilli (Lukjancenko et al., 2012; Sun et al., 2015) or from not related bacteria with different lifestyles (Merhej et al., 2009). A wide range of genomes allows to cluster the strains for several adaptations or lifestyles. Evolutionary history and the importance of HGT in lactic acid bacteria was illustrated by defining homologous genes for different species and genera (Makarova et al., 2006; Makarova and Koonin, 2007). Co-localization of genetic loci (synteny) was demonstrated for *L. johnsonii* with *L. acidophilus* and *L. gasseri* with an *in silico* comparative genomic approach (Berger et al., 2007). CRISPR sequences of lactobacilli and associated genera were clustered in 3 clades (Sun et al., 2015).

7 Aim of the thesis

7.1 Background and objectives for this thesis

Fermented food products are part of the daily diet of most humans. The fermentation process is controlled by microorganisms such as starter and protective cultures which modify the food product to achieve desired flavor, texture and odor. Additionally, these microorganisms produce compounds which prevents the outgrowth of spoilage organisms. The preservation of food products by intentionally added microorganisms is called biopreservation. Biopreservation can be used to increase the shelf-life and safety of fermented food products. Food waste due to spoilage occurs mainly at the households. Food spoilage is frequently harmless but creates an unsavory odor, taste or texture. On the other hand, contamination with pathogenic microorganisms can lead to severe health problems in humans. The outgrowth of pathogenic spoilage organisms was initially controlled with chemical preservatives. Consumers these days are objective to these chemical preservatives and prefer natural products that are minimally processed. Those products have a higher risk for contamination with spoilage organisms. Therefore, biopreservation with starter and protective cultures gained more attention recently. The application of selected cultures to preserve our food products from spoilage, could reduce food waste and food related diseases.

Traditionally fermented food products contain a high variety of naturally occurring *Lactobacillus* isolates. Strains from the genus *Lactobacillus* are known for antimicrobial activity inhibiting pathogenic bacteria such as *Listeria* and *Enterococcus* as well as a broad range of fungi. The combination of natural occurrence in fermented food products and antimicrobial activity makes them potential candidates for starter and protective cultures.

In this study, *Lactobacillus* isolates were investigated to inhibit food associated spoilage microorganisms such as yeast, molds and bacteria. The antimicrobial activity was characterized and potential protective cultures were selected according to a phenotypic and genotypic screening. Selected cultures were tested in food fermentation to inhibit the outgrowth of spoilage indicators.

7.2 General objectives

The general objective of this thesis was to further characterize lactobacilli in the Food Biotechnology ETH strain collection and unravel the hidden potential of those cultures for biotechnological applications. Interactions of microorganisms in fermentation processes and biopreservation was investigated and potential protective cultures were selected. A safety analysis of the sequenced genomes of these potential cultures was performed.

7.3 Specific objectives

The following specific objectives were defined for this work:

- Phenotypic characterization of lactobacilli such as determination of growth limits in conditions mimicking food samples and antimicrobial activity screening.
- Genotypic characterization of sequenced genomes of lactobacilli to determine genes encoding for potential bacteriocins
- Safety analyses of sequenced genomes for the presence of antibiotic resistance genes, virulence factors and phage insertions.
- Selection of potential protective cultures according to phenotypic and genotypic data.
- Application of protective cultures in small-scale and industrial-scale food fermentations to inhibit potential spoilage organisms and improve product safety.
- Analysis of genomic variation in sequenced lactobacilli to better understand genomic adaptation to environments, horizontal gene transfer and shared gene content.

Chapter 2

High-throughput screening assays for antibacterial and antifungal activity of *Lactobacillus* species

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Abstract

We describe high-throughput screening techniques to rapidly detect either antimicrobial activity, using an agar-well diffusion assay in microtiter plates, or antifungal activity using an agar-spot assay in 24-well plates. 504 *Lactobacillus* isolates were screened with minimal laboratory equipment and screening rates of 2'000 – 5'000 individual antimicrobial interactions.

Scientific work

Fermented food products are part of the daily diet and of high economic importance. The spoilage of such products by microorganisms is a major problem in industry (Ross et al., 2002). Lactic acid bacteria (LAB) like lactobacilli can be isolated from many fermented food products and selected strains exhibit antibacterial or antifungal activity (Barbosa et al., 2014; Gaggia et al., 2011; Jiménez et al., 2013). Therefore, lactobacilli are applied in starter and in protective cultures for fermented products (Delavenne et al., 2013). LAB inhibit growth of spoilage organism via general mechanism such as organic acid production and environmental pH decrease. In addition, strain-specific inhibition occurs via production of for example ribosomally encoded bacteriocins or low-molecular weight compounds (Castellano et al., 2010; Nes et al., 2007). Bacteriocin-related inhibition is associated with blocking of the cell wall biosynthesis or with formation of pores in the cell membrane resulting in leakage of the cell (O'Sullivan et al., 2002). Low-molecular weight compounds, mainly acids, inhibit via various mechanisms including pH reduction and inhibition of metabolic reactions (Batish et al., 1997; Crowley et al., 2013b). The application of protective cultures containing natural occurring strains with antifungal or antibacterial activity is a growing trend in food preservation. Strains with antimicrobial or antifungal activity can be detected by testing spoilage-free food isolates in traditional antimicrobial assays. However, screening antimicrobial activity with such assays, often neglecting pH effects, is cumbersome (Bao et al., 2010; Zhu et al., 2014). Moreover, if individual pH adjustment and microfiltration of supernatant is included in such assays, labor increases rapidly when the number of isolates is increased (Maragkoudakis et al., 2009). Recently, an ESI-LC/MS based high-throughput assay to detect novel bacteriocins was developed but this method requires expensive equipment available in only few laboratories (Perez et al., 2014).

Here we present a rapid and easy-to-handle screening assay for antimicrobial strains. We integrated an agar-well diffusion assay (Grinstead and Barefoot, 1992) and an agar-spot assay (Miescher Schwenninger and Meile, 2004) into a high-throughput screening assay (HSA). A standardized

methodology in multi-well plates using common laboratory equipment enables rapid screening for antimicrobial *Lactobacillus* strains.

Lactobacillus strains (Table 2.1) were incubated anaerobically in MRS broth (BioLife, Switzerland) at 30 °C using the oxygen scavenging system AnaeroGen (Thermo Scientific, Switzerland). Growth conditions of the indicator strains used in this study are listed in Table 2.2.

Lactobacillus supernatant for antibacterial assays (HSA-B) was obtained from outgrown cultures in 1.8-ml 96-deep-well plates (Life Systems Design, Switzerland) inoculated from cryo-stocks using a replicator pin. The plate was centrifuged (6000 x g, 15 min) and aliquots of the supernatant were transferred to a 96-well PCR plate and pasteurized at 75 °C for 3 min. The indicator strain media (Table 2.2) were supplemented with 0.5% agar and 0.1 M K_2HPO_4. After sterilization, the media were tempered to 50 °C and inoculated with 0.5% of an overnight-culture of the indicator strain. 50 µl of the inoculated agar was transferred to a 200-µl clear-glass-flat-bottom 96-well microtiter plate (Sigma) using a multichannel pipette and air-dried for 30 min. 30 µl *Lactobacillus* supernatant was transferred to each well and air-dried for 15 min. The optical density at 600 nm (OD_{600}) was measured (time = t_0) using a plate reader (PowerWave XS) and the plate was subsequently incubated at conditions preferable for the indicator strain. The OD_{600} was determined after 24 h and 48 h and growth in wells with OD values below a threshold of 1.5 x OD_{600} at t_0 were classified as inhibited (Figure 2.1A).

The antifungal screening assay (HSA-F) was performed in a 24-well cell-culture plate (Sigma) containing 300 µl of 1.5% MRS agar and 0.1 M K_2HPO_4. *Lactobacillus* strains were spotted at the center of a well with 0.75 µl of an outgrown culture and incubated for 48h. Thereafter wells were overlaid with 100 µl of 0.5% YM soft-agar supplemented with 0.1 M K_2HPO_4 and inoculated either with 10^3-10^4 fungal spores/ml or with 1% of a yeast overnight-culture. The plates were incubated for 24-48 h at conditions preferable for the indicator strain. The inhibition areas were visually recorded daily (Figure 1B). Both assays were performed in a sterile bench to avoid contamination when handling a high amount of plates simultaneously. Initially, all experiments were performed as triplicates. Due to high reproducibility, the following experiments were performed as single screening and only the positives were then confirmed by an agar-well diffusion assay. Antimicrobial activity was tested in agar-well diffusion assay with and without protease XIV (Sigma, Switzerland) digestion to assess proteinaceous characteristics of inhibitory compounds. The HSA-B was also performed in broth instead of soft agar for comparison. The HSA-F was compared to the modified agar-spot assay where colonies were poured onto an agar plate and overlaid with indicator strain inoculated soft-agar (Miescher Schwenninger and Meile, 2004).

Table 2.1: *Lactobacillus* strains used for the antimicrobial assay (HSA)

Identification code	species	Number of Isolates[a]	Antimicrobial activity[b]	Antifungal activity[c]
ace	*L. acetotolerans*	1	0	0
aci	*L. acidipiscis*	1	0	0
aco	*L. acidophilus*	7	0	4
ani	*L. animalis*	1	0	0
bre	*L. brevis*	12	2	5
buc	*L. buchneri*	6	0	1
cas	*L. casei*	23	2	7
cet	*L. ceti*	1	0	0
cor	*L. coryniformis*	1	0	1
cris	*L. crispatus*	1	1	1
cru	*L. crustorum*	1	0	1
cur	*L. curvatus*	26	2	6
del	*L. delbrueckii*	49	11	11
fab	*L. fabifermentans*	1	0	0
far	*L. farciminis*	1	0	0
fer	*L. fermentum*	58	6	15
dru	*L. fructivorans*	4	0	1
gas	*L. gasseri*	1	0	0
har	*L. harbinensis*	1	0	0
hel	*L. helveticus*	12	2	3
hom	*L. hominis*	1	0	0
joh	*L. johnsonii*	2	0	1
lac	*L. lactis*	1	0	0
lin	*L. lindneri*	3	1	2
mal	*L. mali*	1	0	0
mur	*L. murinus*	1	1	0
ota	*L. otakiensis*	1	0	0
prc	*L. paracasei*	18	3	3
prp	*L. paraplantarum*	8	0	2
pen	*L. pentosus*	3	2	1
pla	*L. plantarum*	67	9	31
pon	*L. pontis*	1	0	0
reu	*L. reuteri*	6	1	0
rha	*L. rhamnosus*	21	2	6
sak	*L. sakei*	25	4	10
skc	*L. sakei-curvatus*[d]	35	2	3
sal	*L. salivarius*	1	0	1
san	*L. sanfranciscensis*	1	0	1
ult	*L. ultunensis*	1	0	0
zea	*L. zeae*	1	0	0
spp	*L. spp.*[e]	98	14	37
Total		**504**	**65**	**154**

[a] from each species; [b] with antibacterial activity; [c] with antifungal activity. [d] The species *L. sakei-curvatus* contains isolates that belong to either one of these species. [e] Species *L.* spp. contains all *Lactobacillus* with no further species classification.

Table 2.2: Indicator strains, their culture conditions and their inhibition by lactobacilli

Indicator strains			Cultivation conditions[a]	Inhibitory lactobacilli
Genus	**Species**	**Strain**		**Identification code[b]**
Enterococcus	*avium*	DSM 20679T	BHI / 37°C / aerobic	bre, cas, del, prc, pen, pla, sak, skc, spp
	casseliflavus	DSM 20680T		cas, cur, del, fer, hel, pla, rha, skc, spp
	cecorum	DSM 20682T		prc, pen, sak, spp
	durans	DSM 20633T		bre, cas, del, prc, pla, rha, sak, spp
	faecalis	DSM 20478T*		cas, del, fer, prc, pla, spp
		DSM 2570		cas, del, mur, prc, pla, sak, spp
		DSM 2981		cas, del, mur, prc, pla, sak, spp
	faecium	DSM 20477T*		cas, del, fer, prc, pla, spp
		SL1.1		del, mur, prc, pla, spp
		SL.10.9		del, mur
	gallinarum	DSM 20628T		cas, cur, del, fer, hel, prc, pen, pla, sak, spp
	hirae	DSM 20160T		bre, del, mur, prc, pla, skc, spp
	saccharolyticus	DSM 20726T		bre, cas, cri, cur, fer, hel, lin, pla, rha, sak, skc, spp
	sulfureus	DSM 6905T		bre, cas, cur, del, hel, prc, pen, pla, reu, spp
Escherichia	*coli*	DH5α	BHI / 37°C / aerobic	no inhibition
		K12		no inhibition
Lactobacillus	*curvatus*	RI-504		del, mur, prc, pla, sak, skc, spp
Lactococcus	*lactis*	MG 1363	MRS / 30°C / anaerobic	no inhibition
Leuconostoc	*mesenteroides*	Y105	BHI / 25°C / aerobic	no inhibition
Listeria	*innocua*	HPB13*	BHI / 37°C / aerobic	prc, pla, spp
		DSM 20649T		mur, pla, spp
		L17		pla
		L19		cas, del, mur, prc, pla, spp
	ivanovii	HPB28*		cas, del, fer, hel, prc, pen, pla, sak, skc, spp
		DSM 20750T		cas, del, hel, mur, prc, pen, pla, sak, skc, spp
		DSM 12491T		del, hel, mur, prc, pen, pla, sak, skc, spp
	monocytogenes	ATCC 19114		del, hel, mur, prc, pla, sak, spp
		10403S		del, mur, prc, pla, sak, spp
		H90 2008		del, mur, prc, pla, sak, spp
		F95 2008		del, mur, prc, pla, sak, spp
		Lm15		del, mur, prc, pla, sak, spp
Staphylococcus	*aureus*	DSM1104	BHI / 37°C / aerobic	no inhibition
		463		no inhibition
		DSM 2569		no inhibition
Streptococcus	*mutans*	DSM 20523T	BHI / 37°C / aerobic	no inhibition
	salivarius	OMZ513	TSY / 37°C / aerobic	no inhibition
		ATCC 9759		no inhibition
	thermophilus	DSM 20617T	BHI / 44°C / aerobic	no inhibition
	vestibularis	CCUG 24686	BHI / 37°C / aerobic	no inhibition
		DSM 5636T		no inhibition
Aspergillus	*tamarii*	S078*	YM / 25°C / aerobic	no inhibition
Candida	*krusei*	3-69/2*	YM / 25°C / aerobic	fer, rha
Kluyveromyces	*marxianus*	LME*	YM / 25°C / aerobic	fer, rha
Rhodotorula	*mucilaginosa*	LME*	YM / 25°C / aerobic	aci, bre, buc, cas, har, cri, cru, cur, del, fer, fru, hel, joh, lin, prc, prp, pen, pla, rha, sak, skc, sal, san, spp

[a] BHI broth (Labo-Life Sàrl, Switzerland); TSY (Jans et al., 2012); YM broth (Becton Dickinson AG, Switzerland); [b] Identification code from Table 2.1 that indicates that isolates from *Lactobacillus* species were able to inhibit the indicator strain. * Indicator strains that were tested as triplicates.

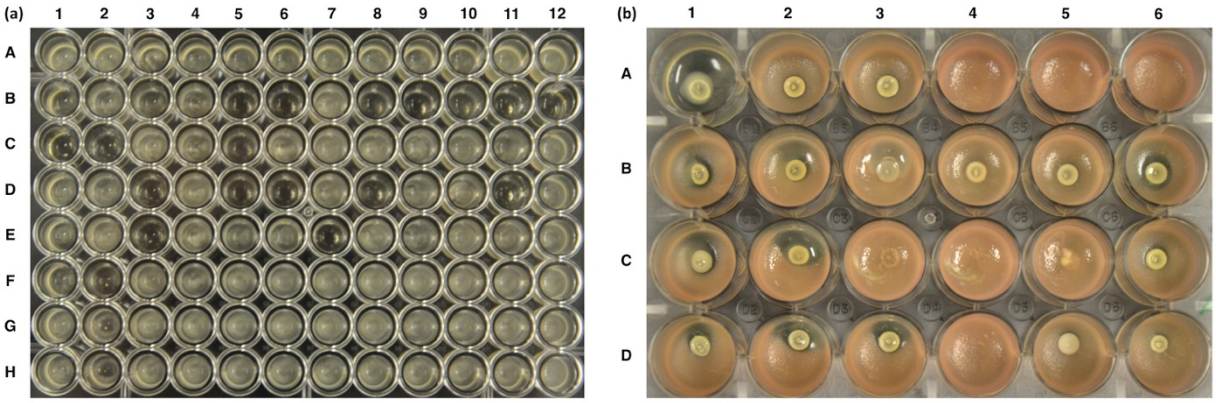

Figure 2.1: Demonstration of the antibacterial (HSA-B) (a) and antifungal (HSA-F) (b) high-throughput screening assay. The HSA-B (a) with 92 *Lactobacillus* strains and 4 negative control samples against *Lactobacillus curvatus* RI-504, a commercial salami starter culture, as an indicator strain shows clear inhibition in wells B5, B6, B8 – B12, C1, C2, C5, D3, D5, D6, D8, D11, E3, E7 and F2; 4 negative control samples for intact growth of RI-504 are gained from supernatants from MRS incubated without lactobacilli in wells A1, A12, H1 and H12. The HSA-F (b) from 22 *Lactobacillus* stains against *Rhodotorula mucilaginosa* LME as an indicator strain. Complete inhibition is detected in well A1. Medium inhibition is detected in the wells B1, B6, C2, D2 and D3. Weak inhibition is detected in wells B1, C1 and D1; negative controls with no lactobacilli colonies in the wells A5 and A6 are completely grown.

We screened 504 *Lactobacillus* strains from 39 different species mainly isolated from food-products for their activity against potential spoilage organisms (Table 2.2). The pH in inhibition zones was checked and ranged between 6.5 – 6.8, showing that low-pH inhibitory effect can be excluded.

The HSA-B revealed 65 strains active against all *Enterococcus* (n=14) and *Listeria* species (n=13). Among these 65 there was *L. plantarum* 12 BH, previously described to inhibit these species and used as positive control (Wullschleger, 2009). The validation of these 65 strains using agar-well diffusion assay (Grinstead and Barefoot, 1992) revealed no false-positive and false-negative strains. Further, 24 of the 65 inhibiting strains exhibited protease-sensitive activity. No qualitative differences were detected between broth- and soft-agar approaches. However, the HSA-B soft-agar assay was easier to handle, and less water condensed in the wells.

Aspergillus tamari, *Kluyveromyces marxianus*, *Candida krusei* and *Rhodotorula mucilaginosa* strains were tested in the HSA-F. The HSA-F revealed 154 strains, including the antifungal strain *L. plantarum* DSM 20205 as positive control (Miescher, 1999), that inhibited one of the four tested indicator strains with *Rhodotorula mucilaginosa* being most frequently inhibited (Table 2.2). *K. marxianus* and *C. krusei* could be inhibited by 5 isolates and *A. tamarii* was not inhibited. In comparison to the traditional agar-spot assay, HSA-F has the advantage that no overlapping inhibition zones and no mixing of strains occurs because of separation of the tests.

The use of buffered media to, avoiding pH adjustment, pasteurization to avoid filtration of supernatants and use of multichannel pipettes enables screening of 2'000 antifungal and 5'000 antibacterial individual interactions per day e.g. 500 lactobacilli with 4 - 10 indicator strains. The classical antibacterial agar-well diffusion and the antifungal agar-spot assays allow screening of approximately 300 interactions per day.

Our new HSA, based on a soft-agar microtiter plate assay, is suggested as fast and accurate primary qualitative screening approach in combination with traditional quantitative methods to detect individual antimicrobial interactions. The screening rate and the low equipment cost make this approach suitable for every laboratory and it can be extended to other bacteria and fungi, bringing phenotypic analyses in pace with next generation sequencing.

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Conflict of Interest

The authors declare no conflict of interest.

Chapter 3

Complete and Assembled Genome Sequence of *Lactobacillus plantarum* RI-113 Isolated from Salami

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Abstract

The complete genome sequence of *Lactobacillus plantarum* RI-113, a strain isolate from salami was determined using single-molecule real-time sequencing (SMRT).

Scientific work

Lactobacillus plantarum strains have been isolated from a broad spectrum of ecosystems, such as silage, olives, sourdough, sauerkraut, cheese and fermented sausages (Rizzello et al., 2011; Siezen and van Hylckama Vlieg, 2011). This habitat diversity of *Lactobacillus plantarum* might be related to abundant gene functions resulting in a genome size which is one the largest among lactobacilli (Bringel et al., 2001; Kant et al., 2011). *Lactobacillus plantarum* RI-113 is a single colony isolate from salami and grows at pH of 3.5, at 7.5% of NaCl and at 5% ethanol and at a temperature range of 14°C to 43°C in Man-Rogosa-Sharpe (MRS) medium. The strain shows antifungal activity against *Trichosporon* sp. and *Rhodothorula mucilaginosa* as detected in a high-throughput screening (Inglin et al., 2015). Genomic DNA was isolated using a lysozyme-based cell-wall digestion prior to the isolation using the Wizard genomic DNA purification kit (Promega, Dübendorf, Switzerland). The genome was sequenced using single–molecule real-time sequencing (SMRT) cells on a PacBio RS II (Pacific Biosciences, Menlo Park, CA, USA) at the Functional Genomics Center Zurich (Zurich, Switzerland). In total, 94'382 reads with a mean length of 12,974 basepairs (bp) resulting in 370x coverage, were assembled into a single contig and 6 plasmids using the hierarchical genome-assembly process (Chin et al., 2013). The genome was automatically annotated using the NCBI Prokaryotic Genomes Automatic Annotation Pipeline. The genome of *L. plantarum* RI-113 consists of a 3'462'990 bp circular molecule and comprises 67 tRNA genes and 16 rRNA genes. The G+C content of the genome is 44.34% and a total of 3'361 protein coding sequences (CDS) were predicted.

Accession number(s). Sequence and annotation data of the complete *Lactobacillus plantarum* RI-113 strain are deposited in the GenBank database under the accession number CP017406.1 for the genome and CP017407.1 – CP017412.1 for the 6 plasmids.

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Chapter 4

Draft Genome Sequences of 43 *Lactobacillus* Strains from the Species *L. curvatus, L. fermentum, L. paracasei, L. plantarum, L. rhamnosus, L. sakei,* Isolated from Food Products

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Abstract

The genome sequences of 43 *Lactobacillus* strains from the species *L. curvatus, L. fermentum, L. paracasei, L. plantarum, L. rhamnosus,* and *L. sakei* were determined using Illumina MiSeq.

Scientific work

Lactobacillus strains have been isolated from a broad spectrum of food products, such as salami type sausages, meat, dairy products, sauerkraut and fermented vegetables (Kant et al., 2011; Settanni and Corsetti, 2008). Lactobacilli are used as starter-, and protective cultures in industrial fermentations to control the fermentation process, extend the shelf-life of the fermented product and increase its safety. In addition, some strains are marketed as probiotic and benefit the health of the consumer (Klaenhammer et al., 2008; Makarova et al., 2006). Here, the sequenced genomes of 4 *L. curvatus*, 1 *L. fermentum*, 3 *L. paracasei,* 28 *L. plantarum*, 1 *L. rhamnosus*, and 6 *L. sakei* strains are presented. These strains were selected from a phenotypic screening and exhibited an atypical phenotype, or were selected as potential protective cultures in a high-throughput screening assay (Inglin et al., 2015). Genomic DNA was isolated by using a lysozyme-based cell wall digestion step and subsequently a Wizard genomic DNA purification kit (Promega, Dübendorf, Switzerland). The genomes were sequenced with Illumina MiSeq, pairwise reads of 150 bp, 30-fold coverage at the Functional Genomic Center Zurich (Zurich, Switzerland). Potential functions of predicted genes were automatically annotated using the NCBI Prokaryotic Genome Annotation Pipeline.

Accession number(s). Sequence and annotation data of the *Lactobacillus* strains are deposited as BioProject PRJNA343164 in the GenBank database and corresponding accession numbers listed in Table 4.1.

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Table 4.1 Sequenced genomes of *Lactobacillus* in NCBI BioProject PRJNA343164

Strains	Accession	Genome size (Mb)	No. of contigs	% G+C	No. of CDS[a]
Lactobacillus curvatus RI-124	MKDR00000000	1.81	77	42.0	1838
Lactobacillus curvatus RI-193	MKGD00000000	1.81	82	42.0	1862
Lactobacillus curvatus RI-198	MKGC00000000	1.80	77	42.0	1848
Lactobacillus curvatus RI-406	MKDG00000000	2.01	52	41.7	2020
Lactobacillus fermentum RI-508	MKGE00000000	1.92	74	52.2	1959
Lactobacillus paracasei RI-194	MKFZ00000000	3.06	86	46.3	3197
Lactobacillus paracasei RI-195	MKGA00000000	3.03	125	46.3	3170
Lactobacillus paracasei RI-210	MKFY00000000	3.06	58	46.1	3164
Lactobacillus plantarum RI-011	MJHC00000000	3.17	33	44.6	3063
Lactobacillus plantarum RI-012	MJHD00000000	3.22	101	44.4	3175
Lactobacillus plantarum RI-048	MJHG00000000	3.19	94	44.5	3150
Lactobacillus plantarum RI-086	MKDP00000000	3.08	90	44.6	3003
Lactobacillus plantarum RI-123	MKDQ00000000	3.32	60	44.3	3253
Lactobacillus plantarum RI-139	MKDS00000000	3.33	78	44.4	3264
Lactobacillus plantarum RI-140	MKDT00000000	3.31	85	44.3	3241
Lactobacillus plantarum RI-146	MKDU00000000	3.37	61	44.3	3304
Lactobacillus plantarum RI-147	MKDV00000000	3.32	100	44.4	3253
Lactobacillus plantarum RI-162	MJHH00000000	3.32	41	44.6	3053
Lactobacillus plantarum RI-165	MJHI00000000	3.30	101	44.3	3212
Lactobacillus plantarum RI-189	MJHJ00000000	3.10	56	44.5	3012
Lactobacillus plantarum RI-190	MJHK00000000	3.10	58	44.5	2996
Lactobacillus plantarum RI-208	MKFX00000000	3.14	82	44.5	3042
Lactobacillus plantarum RI-266	MKDY00000000	3.47	71	44.2	3412
Lactobacillus plantarum RI-405	MKDF00000000	3.32	72	44.3	3273
Lactobacillus plantarum RI-408	MKDH00000000	3.07	123	44.6	2988
Lactobacillus plantarum RI-422	MKDK00000000	3.33	55	44.3	3274
Lactobacillus plantarum RI-505	MKDZ00000000	3.10	52	44.7	3018
Lactobacillus plantarum RI-506	MKEA00000000	3.39	76	44.2	3344
Lactobacillus plantarum RI-507	MKEB00000000	3.53	126	44.1	3499
Lactobacillus plantarum RI-509	MKEC00000000	3.32	66	44.4	3296
Lactobacillus plantarum RI-510	MKED00000000	3.37	110	44.2	3353
Lactobacillus plantarum RI-511	MKEE00000000	3.33	105	44.2	3300
Lactobacillus plantarum RI-512	MKEF00000000	3.30	151	44.3	3284
Lactobacillus plantarum RI-513	MKEG00000000	3.28	71	44.4	3199
Lactobacillus plantarum RI-514	MKEH00000000	3.23	61	44.5	3134
Lactobacillus plantarum RI-515	MKGF00000000	3.32	94	44.4	3258
Lactobacillus rhamnosus RI-004	MJHB00000000	2.92	72	46.6	2993
Lactobacillus sakei RI-394	MKDC00000000	1.94	44	41.0	1963
Lactobacillus sakei RI-403	MKDD00000000	2.00	29	41.0	2032
Lactobacillus sakei RI-404	MKDE00000000	1.95	28	41.0	1977
Lactobacillus sakei RI-409	MKGB00000000	1.99	67	40.9	2022
Lactobacillus sakei RI-410	MKDI00000000	1.93	73	41.1	1949
Lactobacillus sakei RI-412	MKDJ00000000	1.92	32	41.1	1934

[a] CDS, coding sequence

Chapter 5

An Approach to select *Lactobacillus* isolates as Protective Cultures for Food Fermentations

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Abstract

Food waste reduction can be achieved by applying protective cultures to avoid spoilage of fermented food products. In this study, we present an approach to screen large numbers of strains for potential use as protective cultures in food. A phenotypic screening of 504 *Lactobacillus* strains for 27 food relevant growth conditions revealed variations and physiological limits for the genus. Previously, the strains were tested for their antibacterial activity in a high-throughput-assay. Here, the activity of 22 positive strains from that screening was assessed in more detail, mainly against *Listeria, Enterococcus, Rhodotorula* and *Candida* species. The proteinaceous nature of inhibiting substances was confirmed by protease digestion. 22 antibacterial and 42 antifungal strains were detected. In a co-culture competition-assay 1-2 cfu/ml of *Lactobacillus plantarum* RI-162 were able to inhibit the outgrowth of *Rhodotorula mucilaginosa* LME and reduce the cell number below the detection limit of 50 cfu/ml within 48 hours.

Practical application

To translate the result to industrial application, the potential protective culture *L. sakei* RI-409 reduced the initial *Listeria ivanovii* DSM 12491^T concentration in an industrial-scale salami fermentation by 1.4 log within 5 days. In a further small-scale salami fermentation 1 *Lactobacillus sakei* and 5 *Lactobacillus plantarum* strains were tested as protective cultures. Four of them strains reduced the spiked counts of *L. ivanovii* DSM 12491^T from 10^5 cfu/g at the start of fermentation to below the detection limit of 100 cfu/g within 2 days. In a 1000-L small-scale raw milk soft cheese fermentation, the potential protective culture *L. plantarum* RI-271 reduced the endogenous enterococci concentration with 1.5 log compared to untreated raw milk. In conclusion, we have developed an approach to select tailor-made antimicrobial protective cultures for biopreservation in fermented food products.

Introduction

Approximately 88 million tons food waste is produced in the European Union annually. This corresponds to an economic loss of 143 billion euros and occurs over the entire supply chain (Stenmarck et al., 2016). Food waste could be reduced with appropriate preservation (Martindale, 2017). Consumers are however skeptical against chemical preservatives and expect minimally processed food and biological preservation (Crowley et al., 2013b). Biopreservation is an approach where natural or controlled microbiota are used in combination with *in situ* produced antimicrobial compounds to increase the shelf life and food safety (Ananou et al., 2007). A key component in biopreservation of food products is the application of protective cultures. These cultures occur naturally in food or are added to prevent the outgrowth of spoilage or pathogenic microorganisms. Spoilage of food products can occur by harmless microorganisms that putrefy the food product or by pathogenic microorganisms which might produce toxins or which are invasive such as *Listeria monocytogenes.* This bacterium is a potential meat spoilage organism which causes listeriosis, in humans after the consumption of such meat (Maertens de Noordhout et al., 2014). A recall of *Listeria*-contaminated products causes reputational and financial damage for producers. Therefore, the development of highly specialized protective cultures that inhibited outgrowth of spoilage organisms in food products is interesting for the industry. So far only a few studies demonstrated the potential of lactobacilli as protective cultures on cheese surfaces (Loessner et al., 2003) or the application of purified bacteriocins to inhibit *L. monocytogenes* in fish and meat products (Katla et al., 2001; Vijayakumar and Muriana, 2017). Enterococci can be naturally present in fermented food and their presence does not lead directly to recalls or health risks. However, enterococci frequently carry transferable antibiotic resistances and a reduction of enterococci in food product is desirable. By our knowledge, protective cultures against enterococci are not developed yet.

Protective cultures inhibit other microorganisms by strain-specific mechanisms such as excretion of bacteriocins and low-weight molecular substances. Bacteriocins are genetically encoded peptides of 20-40 amino acids that are active, mostly against closely-related bacteria. Most bacteriocins and antimicrobial metabolites such as lactic and acetic acid are heat resistant. They withstand pasteurization and are useful active compounds of protective cultures in food products (Gaggia et al., 2011).

To select protective cultures, strains are phenotypically screened for desired attributes such as inhibition of targeted spoilage microorganisms and *in situ* bacteriocin production without negatively affecting the starter culture or taste, odor and texture of the product. This latter property is difficult to test for a large set of strains. Therefore, candidate strains are initially tested in conditions that

mimic a particular food matrix such as fermentation of a different carbon sources, low pH resistance, salt tolerance, and the ability to grow in low or high temperatures. Additionally, a genotypic screening should be performed to access the safety of the strains. The presence of transferable antibiotic resistance genes, prophages and genes encoding virulence factors are undesired for safety and production reasons (Alkema et al., 2016).

Lactobacilli are lactic acid bacteria that occur in a wide range of fermented food products. Antimicrobial activity of *Lactobacillus* is well documented in specific strains and can be divided in two categories, antibacterial and antifungal activity. Antibacterial activity is mostly based on unspecific mechanisms such as lowering pH via lactic acid production or specific mechanisms such as the production antimicrobial metabolites such as reuterin or reutericyclin and the production antimicrobial peptides or proteins such as bacteriocins (Gänzle, 2009). In the genus *Lactobacillus* several strains produce bacteriocins mostly inhibiting species from the genus *Listeria* and *Enterococcus* (Casaburi et al., 2016; Maldonado-Barragán et al., 2013). Antifungal activity in lactobacilli is mainly based on metabolites such as lactic acid, acetic acid, phenyllactic acid and cyclic dipeptides (Ryan et al., 2011). Strains of the species *L. pentosus*, *L. coryniformis* subsp. *coryniformis, L. brevis*, *L. plantarum* and *L. rossiae* also produce antifungal peptides such as subtilisin-like proteases (Fan et al., 2014; Rizzello et al., 2011). However, the mechanisms behind antifungal activity remain mostly unclear (Crowley et al., 2013b). Specific antibacterial activity in lactobacilli is widely distributed but only a few isolates are able to maintain this activity in food products.

Therefore, the aim of this study was to establish a systematic procedure to select and test protective cultures. To understand the antimicrobial mechanisms, active compounds were isolated and characterized depending on their production, stability and activity range. We screened *Lactobacillus* isolates from an existing strain library for different phenotypical attributes as described above. Since isolates with antimicrobial activity were selected, their genome was sequenced and strains were tested *in situ* in small-scale food fermentation models. Candiate protective cultures were implemented into industrial-scale food fermentation applications to test their activity and robustness.

Material and Methods

Microbial strains, media and growth conditions

Lactobacillus strains in this study, originating from the laboratory strain collection, were identified by biochemical analysis, 16S rRNA gene sequence comparison, or whole genome sequencing (Table S5.1). Lactobacilli were routinely grown in MRS broth (BioLife, Switzerland) under anaerobic conditions at 30 °C for 24 h. Anaerobic conditions were achieved using the oxygen scavenging system AnaeroGen (Thermo Scientific, Switzerland). If applicable, the pH of MRS was adjusted using 3 M HCl. To increase the buffer capacity of MRS supplemented with acetate or lactate, 0.1 M potassium phosphate buffer at pH 6.5 was added. Indicator strains from *Listeria* and *Enterococcus* (Table S5.2) were grown in BHI broth (Labo-Life Sàrl, Pully, Switzerland) and aerobically incubated at 37 °C for 24 h. Indicator yeast (Table S5.2) were grown in YM broth (Becton Dickinson AG, Allschwil, Switzerland) at 25 °C under aerobic conditions for 48 h. Long-time storage of microorganisms was done by mixing an overnight culture 1:1 with 60% glycerol and storage at -80 °C. To compare the ability of the strains to grow in modified MRS, microtiter plates were used. Lactobacilli pre-cultures were inoculated from cryo stocks and grown in 96-deep-well plates for 48 hours. For the screening, 150 µl MRS modified for the tested condition was pipetted into a 200-µl clear-glass-flat-bottom 96-well microtiter plate (Sigma-Aldrich Chemie GmbH, Buchs, Switzerland) and inoculated with the pre-culture using a replicator pin. The optical density at 600 nm (OD_{600}) was measured in a plate reader (PowerWave XS, BioTek, Switzerland) after 24 and 48 h. Growth above $OD_{600} = 0.4$ was defined as threshold for growth - this corresponds to an OD_{600} of approximately 0.85 measured in a 1-cm cuvette. Three independent biological replicates were performed per condition and the median of each triplicate was integrated into the analysis if the variance of the triplicate was < 0.04.

Characterization of antibacterial activity

Antibacterial isolates from high-throughput screening (Inglin et al., 2015) were tested against five food-related indicator strains from the genus *Enterococcus* and *Listeria* using an agar well diffusion assay. 20 ml BHI agar supplemented with 0.1 M potassium phosphate buffer at pH 6.5 was tempered at 50 °C. Subsequently, 1% of an overnight culture of the respective indicator strain was added and the mixture rapidly poured into petri dishes. Holes with 6 mm diameter were cut and filled with 80 µl supernatant from a 24 h grown *Lactobacillus* culture. If needed, supernatants of lactobacilli were concentrated to 10% of the original volume by freeze drying (Virtis Dri-Block® DR-2D, Witec AG, Littau, Switzerland) and digested with 10 mg/ml protease XIV from *Streptomyces griseus* (Sigma-

Aldrich Chemie GmbH, Buchs, Switzerland) in THMS buffer containing 30 mM Tris-HCl (pH 8.0), 3 mM $MgCl_2$ (both from Sigma-Aldrich Chemie GmbH, Buchs, Switzerland) and 25% sucrose (AppliChem GmbH, Darmstadt, Germany) at 37 °C for 2 h. Digested and non-digested supernatants were diluted with sterile ddH_2O to initial volume and heat treated at 95 °C for 5 min to inactivate the protease. Bacteriocin activity was defined as proteinaceous antibacterial activity that was not affected by the heat treatment.

Analyses of genomes

The genome sequence of selected antibacterial strains (Inglin et al., 2017a, 2017b) were analyzed with the BAGEL3 (van Heel et al., 2013) and antiSMASH3.0 (Weber et al., 2015) software to identify potential bacteriocin-encoding genes.

Characterization of antifungal activity (AF)

AF1 - Overlay assay

Sequenced strains with antifungal activity were included in a standard non-buffered overlay assay (AF1). Therefore, 1 µl of a *Lactobacillus* spp. culture (approximately 10^6 cfu) was spotted on MRS plates and incubated anaerobically at 30 °C for 48 h. The plates were then overlaid with 6 ml YM soft agar (0.7%) at 50 °C containing approximately 10^4 cfu/ml *Rhodotorula mucilaginosa* LME cells and incubated aerobically at 25 °C. Inhibition zones were qualitatively assessed after 70 h and classified into no inhibition (-), weak inhibition (+), moderate inhibition (++) and strong inhibition (+++).

AF2 - Agar well diffusion assay

Antifungal activity of cell free supernatants was assessed using an agar well diffusion assay. 20 ml YM soft agar tempered to 50 °C was inoculated with 10^4 cfu/ml *R. mucilaginosa* LME cells and poured into plates. Holes with 6-mm diameter were cut and filled with 80 µl supernatant. Concentrated supernatants were produced as described above. Crude and 10-fold concentrated supernatants were used in this AF2 assay and agar plates were incubated at 25 °C for 70 h.

To investigate whether antifungal activity was proteinaceous, 10-fold concentrated supernatants were digested with 10 mg/ml of either proteinase K, proteinase E or trypsin (Sigma-Aldrich Chemie GmbH, Buchs, Switzerland) in THMS buffer at 37 °C for 2 h. Thereafter the enzymes were inactivated at 95 °C for 5 min and the supernatant was tested in assay AF2.

AF3 - Induction of antifungal activity with yeast-preparations

L. plantarum strains RI-162, RI-271, RI-422 and WCFS1 were used in AF3, where a potential induction of antifungal activity of lactobacilli by direct contact with yeast-preparations was tested. A culture of 10^7 cfu/ml *R. mucilaginosa* LME was heat-inactivated at 70 °C for 20 min, and subsequently mechanically disrupted (5 x 30 s at 5 m/s) using a FastPrep®-24 kit (MP Biomedicals, Illkirch, France). 1 µl of this yeast-preparation was spotted on a 1 µl spot of a *L. plantarum* overnight culture on an MRS agar plate. Plates were incubated anaerobically at 30 °C and after 48 h overlaid with YM soft agar containing 10^4 cfu/ml *R. mucilaginosa* and incubated at 25 °C for 70 h.

To exclude auto-induction effect by yeast extract from MRS medium the experiments were performed in standard MRS and MRS without added yeast extract.

AF4 - Competition assay with *L. plantarum* and *R. mucilaginosa*

An overnight culture of *L. plantarum* RI-162 was inoculated 1/500 in MRS medium and grown to early exponential phase at an OD_{600} = 0.4. *R. mucilaginosa* LME was inoculated 1/100 in YM medium and grown to early exponential phase at OD_{600} = 1. The lactobacilli culture was serially 10-fold diluted in MRS broth to a final volume of 500 µl. The yeast culture was adjusted to 5 x 10^5 cfu/ml with YM in a volume of 500 µl. A 1-ml co-cultures of different concentrations of the two cultures were mixed and incubated aerobically, shaking at 90 rpm at 30 °C.

After 48 h of co-cultivation, appropriate dilutions were plated on MRS and YM + 20 mg/L chloramphenicol agar plates and incubated under anaerobic and aerobic conditions, respectively, at 30 °C for 48 h. Each experiment was performed in 3 biological replicates.

AF5 – Yeast cell dependence for antifungal activity

To determinate whether lactobacilli produced antifungal substance regardless of the presence of yeast an overlay assay AF1 was modified. 10 µl of an overnight culture of *L. plantarum* strains were spot-inoculated on MRS agar plates and incubated for 48 hours. Formed colonies were removed with a swab and any remaining cells killed by UV light (UVC 451x28 mm TUV15 - 15W, Philips Lighting Holding B.V., at a distance of 15 cm) for 45 min. Plates were overlaid with YM soft agar containing 10^4 cfu/ml *R. mucilaginosa* and incubated at 25 °C for 70 h. To investigate the stability of inhibition the plates were incubated at 25 °C for an additional 2 days.

AF6 – Determination of metabolic antifungal activity

Antifungal metabolite production for lactic acid, acetic acid, 2-pyrrolidon-5-carboxylic acid, hydroxyl phenyllactic acid, propionic acid, phenyllactic acid and ethyl-L-lactic acid was determined from cell

free supernatant by HPLC (Hitachi LaChrome, Merck, Dietikon, Switzerland) on an Aminex HPX-87H column (300 x 7.8 mm, BioRad Reinach, Switzerland) and a refractive index detector (Merck, Dietlikon, Switzerland) with a mixture of 5% 10 mM formic acid + 95% 10 mM sulfuric acid as eluent and a flow rate of 0.6 ml min^{-1}.

Application of protective cultures in a salami application

The protective culture *L. sakei* RI-409 was tested in an industrial-scale fermentation. A *L. sakei* RI-409 pre-culture was incubated at 30 °C for 24 h, centrifuged (5000 g, 10 min, 4 °C), washed twice with peptone water and stored on ice for a maximum of 2 hours. 13 kg raw meat (4 kg beef, 6 kg pork, 3 kg back fat from pork), 52 g glucose, 330 g nitrite and 276 g of spices were inoculated with $5x10^5$ cfu/g from a *L. ivanovii* DSM 12491^T pre-culture and with either i) no additional culture; ii) starter culture (Bitec starter LK30, 2 x 10^{10} cfu/g); iii) 10^7 cfu/g protective culture *L. sakei* RI-409. All ingredients were mixed for 5 min and batches of 1200 g were packed into artificial sausage skin. The salamis were ripened at 23 °C with 88% humidity for 36 h and from than at 21 °C, 19 °C and 17 °C each for 24 h at 86% humidity. After ripening the salami were stored at 15 °C for 9 days. For analysis, 10 g samples were homogenized in 90 ml peptone water for 10 min. 0.1 ml of serial dilutions were plated on ALOA Agar (Labo-Life Sàrl, Pully, Switzerland). Samples were taken before ripening, after ripening and after storage. Plates were incubated at 37 °C for 48 – 72 h aerobically.

The 6 protective isolates *L. plantarum* RI-046, *L. plantarum* RI-208, *L. plantarum* RI-303, *L. sakei* RI-409, *L. plantarum* RI-460 and *L. plantarum* RI-461 were tested in a small-scale salami fermentation. *Lactobacillus* strains were grown in MRS at 30 °C for 24 h, centrifuged (5000 g, 10 min, 4 °C) and washed twice with peptone water. 6 batches were produced, each containing 1 kg frozen lean shoulder from pork, 600 g fresh pork minced to 2 mm pieces, 400 g frozen back fat without rind, 5 x 10^6 cfu/g of a *Lactobacillus* protective culture candidate and 5 x 10^6 cfu/g of a *Staphylococcus carnosus* strain, a starter culture from Frutarom (Holdorf, Germany). All ingredients were mixed and the meat batter was kneaded for 1 minute. 30 g/kg curing salt, 10 g/kg ripening mixture (Frutarom Savory Solutions GmbH, Holdorf, Germany) and 5 x 10^4 cfu/g *Listeria ivanovii* DSM 12491^T were added and the batter was kneaded for 90 seconds. With a piston sausage filler, 700-g portions were filled in 55-mm diameter Walsroder FR nature casings. The sausages were incubated at 24 °C at 75% humidity for 6 hours followed by 48 hours at 24 °C and 94% humidity, 48 hours at 22 °C and 92% humidity and 18 hours at 18 °C and 90% humidity. Cell count and pH was determined after 0, 1, 2 and 5 days. For analysis, 10 g samples were homogenized in 90 ml peptone water for 10 min (BagMixer 400, Huber & Co. AG, Reinach, Switzerland). 0.1 ml of serial dilutions were plated on List Agar for listerial growth (Oxoid, Pratteln, Switzerland) and aerobically incubated at 37 °C for 48 h.

Inhibition of enterococci in raw milk soft cheese

As a food model, we chose a mold-ripened cow raw milk soft cheese with a weight of 140 g and a diameter of 9 cm from a local organic producer. Protective culture of *L. plantarum* RI-271 was inoculated in MRS without glucose + 20mM lactose and incubated for 16 hours. *L. plantarum* RI-271 was diluted in peptone water to 5 x 10^9, 5 x 10^8 and 5 x 10^7 cfu/ml, centrifuged (5000 g, 4 °C, 10 min) and the supernatant was separated and heat-treated at 80 °C for 3 minutes. The cells were washed twice in peptone water, suspended in the same volume and stored on ice for a maximum of 2 hours. In a 1000-L small-scale fermentation, the fresh raw milk was inoculated with 5 x 10^5 cfu/ml starter culture (CHOOZIT STAM 3 *Streptococcus salivarius*, Danisco) and 5 x 10^5 cfu/ml a commercial strain of *Penicillium camemberti* and incubated at 36 °C for 1 hour. 0.1 ml of the raw milk was plated on KFS agar (Becton Dickinson AG, Allschwil, Switzerland) to determine the initial concentration of enterococci. 1-L batches were inoculated with 0.3 ml natural calf rennet (Winkler, Konolfingen, Switzerland) and 10 ml of *L. plantarum* RI-271 to final concentrations of 5 x 10^7, 5 x 10^6 and 5 x 10^5 cfu/ml or with the heat-treated supernatant (80 °C for 3 min), incubated at 37 °C for 30 min and cut with a cheese harp. The crude cheese was incubated at 37 °C for another 2 hours and filled into 500-ml forms. The cheeses were drained at 25 °C for 7 hours, incubated in a salt bath (22% NaCl) for 30 min and ripened at 14 °C and 94% humidity for 8 days. For analysis, 10 g cheese sample was homogenized in 90 ml peptone water for 10 min. 0.1 ml of serial dilutions were plated on KFS Agar plates and incubated at 43 °C for 48 – 72 h aerobically. 2 technical replicates were performed.

Results

Phenotypic analyses of 504 lactobacilli isolated from food

The phenotypical variation of 504 *Lactobacillus* strains were evaluated for 27 growth conditions using the growth monitoring assay in microtiter-plates. All strains grew within 24 hours in MRS under standard conditions (Figure 5.1Z2).

Bile salt affected the growth of lactobacilli. 81% of the strains grew in the presence 2% bile salt in 24 hours and 93% in 48 hours (Figure 5.1A). In the presence of 10% bile salt, 47% of the strains grew above the threshold in 24 hours and 71% in 48 hours (Figure 5.1B).

A similar growth reduction was observed in the presence of elevated NaCl levels. 86% and 99% of the strains grew at 4% NaCl after 24 and 48 hours, respectively. At 7.5% NaCl, the numbers reduced to 32% and 81% within 24 and 48 hours, respectively. At 10% NaCl only specialized strains such as *L. farciminis* RI-339 and *L. rhamnosus* RI-002 were able to grow within 48 hours (Figure 5.1C - E).

Low pH environments occur with accumulation of acidic metabolites from carbohydrate fermentation and lactobacilli are expected to be tolerant to low pH since they produce lactic acid. While at pH 5.0 99.8% of the strains grew within 48 hours, at pH 4.0 only 84% and at pH 3.5 only 49% were able to grow within 48 hours (Figure 5.1F - H). Only the dairy isolate *L. delbrueckii* RI-233, was sensitive to pH 5.0 and wasn't able to grow within 48 hours.

The ability to grow at low and high temperatures is essential for certain food fermentations e.g. salami. The high temperature screening at 43 °C was the only condition were the median at 24 hours with OD_{600} = 1.19 was higher than after 48 hours with OD_{600} = 0.97 (Figure 5.1I). Out of the 441 and 444 *Lactobacillus* strains with consistent growth behavior, 92% grew in 24 hours and 88% in 48 hours. At 47 °C only 34% and 33% grew within 24 hours and 48 hours, respectively (Figure 5.1J). Reduced growth was detected at 14 °C where 22% and 79% of the strains were able to grow after 24 and 48 hours (Figure 5.1K). At 8 °C, only psychrophilic strains grew and *L. sakei* RI-329 was the only *Lactobacillus* strain able to grow within 24 hours (Figure 5.1L). After 48 hours, 10% of the strains grew at 8 °C. Remarkably the 10 strains reaching the highest OD_{600} after 48 hours at 8 °C belonged all to the species *L. sakei.*

MRS without glucose was the only condition were none of the *Lactobacillus* strains was able to grow after 48 hours (Figure 5.1M). In MRS with mannose as carbohydrate source, 93% and 95% of the strains were able to grow within 24 and 48 hours, respectively (Figure 5.1N). 90% of the strains were able to grow MRS without acetate (Figure 5.1O) and 86% in MRS without magnesium (Figure 5.1P) within 48 hours. In MRS without manganese only 18% of the isolates grew within 48 hours (Figure

5.1Q). Accumulation of acid, even in a buffered environment, is stressful for bacteria. 88% of the strains were able to grow in MRS supplemented with 50 mM acetic acid in 48 hours. At higher acetic acid concentrations of 200 mM only 11% of the strains grew (Figure 5.1R - S). Remarkably, nine out of ten isolates with the highest OD_{600} after 48 hours in 200 mM acetic acid belonged to the species *L. sakei*, the other being *L. plantarum* RI-422. Growth in MRS with 50 mM lactic acid was comparable to MRS with 50 mM acetic acid with 88% growth within 48 hours (Figure 5.1T). 63% of the strains were able to grow in 100 mM lactic acid within 48 hours (Figure 5.1U).

10% hop extract in MRS inhibited 4 strains from the species *L. sakei* and *L. curvatus*, even for 48 hours (Figure 5.1V). Reuterin concentrations of 2 and 10 mM did not inhibit the growth of lactobacilli (Figure 5.1W - X). Reuterin concentration of 20 mM reduced the growth for 42% of the lactobacilli with only 9% of the isolates grew within 24 hours and 58% within 48 hours (Figure 5.1Y). The strains with a tolerance to 200 mM acetic acid showed also tolerance to 20 mM reuterin in 24 hours. At 5% ethanol, 67% of the strains grew in 24 hours, while in MRS with 15% ethanol only 3% of the isolates were able to grow above the threshold even after prolonged incubation for 96 hours (Figure 5.1Z – Z1). 13 of these 23 isolates belong to the species *L.* sakei and the same isolates that showed already a high tolerance to reuterin and acetic acid grew at high ethanol concentrations. Interestingly, *L. sakei* isolates belonged either to the psychrophilic or the acetic-acid-reuterin-ethanol tolerant group. Hence a trade-off between ability to grow at low temperature and resistance to acetic acid, reuterin or ethanol was observed. These growth condition profiles can be used in the selection process to align a potential protective or starter culture to its most suitable environment according to the screening data.

Characterization of antibacterial activity

Typical spoilage microorganisms associated with fermented meat and dairy products were selected as targets in antibacterial assays. Out of 65 antibacterial *Lactobacillus* strains 22 strains showed proteinaceous antibacterial activity against the indicator strains *Enterococcus faecalis* DSM 20478^T, *Enterococcus faecium* SL1.1, *Listera innocua* HPB13, *Listeria ivanovii* DSM 12491^T and *L. monocytogenes* ATCC 19114. In the qualitative antibacterial activity screening *L. plantarum* RI-080 and *L. murinus* RI-256 were solely active against *Listeria monocytogenes* ATCC 19114 (Table 5.1). The genomes of antibacterial strains were sequenced and revealed genes encoding bacteriocins such as plantaricin, sakacin, curvacin, helveticin, enterocin, pediocin and enterolysin for all sequenced antibacterial strains (Table 5.1). For multiple isolates the proteinaceous antibacterial activity was demonstrated *in vitro*, but the corresponding bacteriocin was not determined.

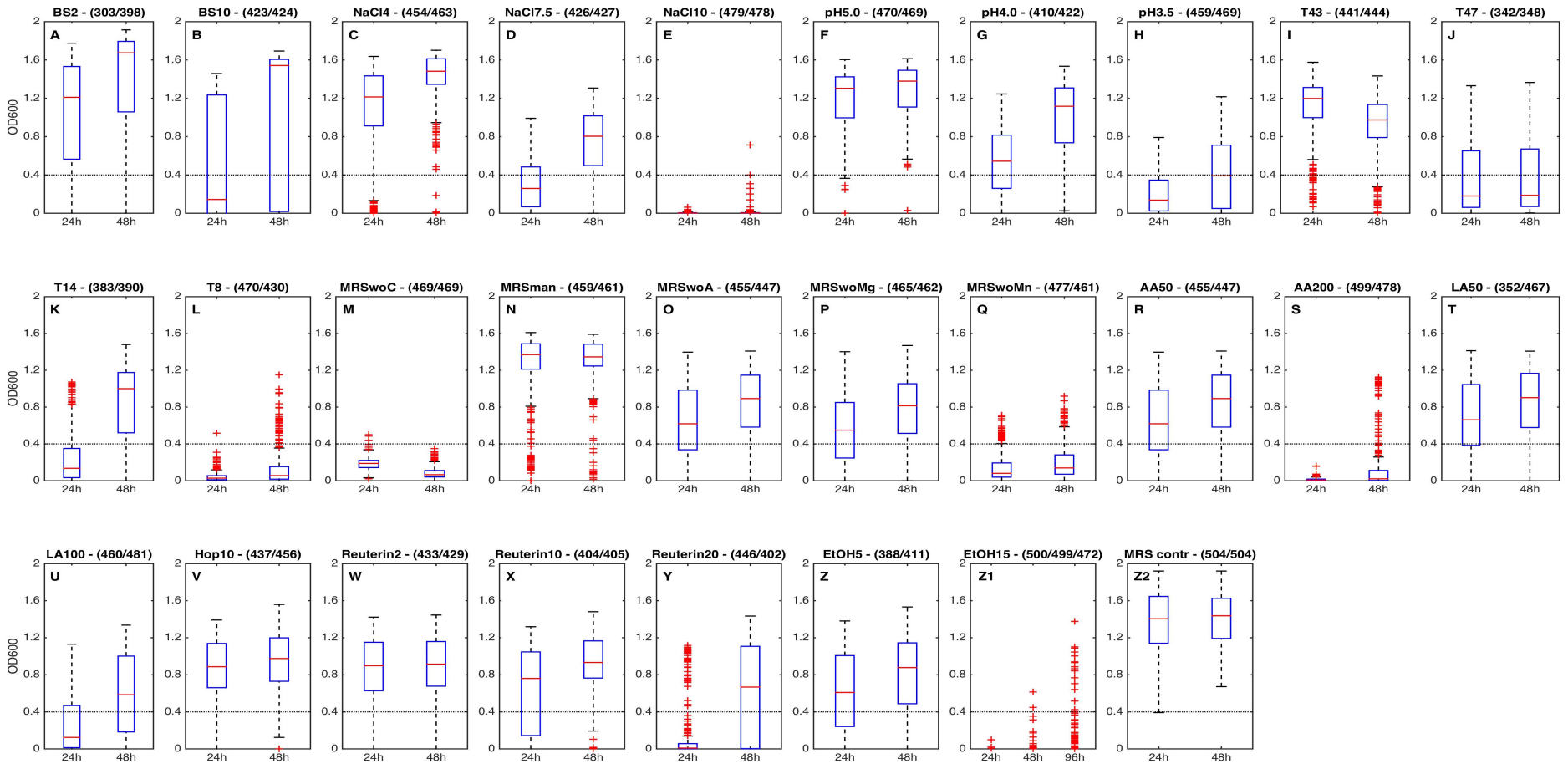

Figure 5.1 Phenotypic screening of 504 lactobacilli for 27 growth conditions. A MRS with 2% bile salt, **B** MRS with 10% bile salt, **C** MRS with 4% NaCl, **D** MRS with 7.5% NaCl, **E** MRS with 10% NaCl, **F** MRS at pH 5.0, **G** MRS at pH 4.0, **H** MRS at pH 3.5, **I** Growth at 43°C, **J** Growth at 47°C, **K** Growth at 14°C, **L** Growth at 8°C, **M** MRS without glucose, **N** MRS without mannose, **O** MRS without acetate, **P** MRS without magnesium, **Q** MRS without manganese, **R** MRS with 50 mM acetic acid, **S** MRS with 200 mM acetic acid, **T** MRS with 50 mM lactic acid, **U** MRS with 100 mM lactic acid, **V** MRS with 10% hop extract, **W** MRS with 2 mM reuterin, **X** MRS with 10 mM reuterin, **Y** MRS with 20 mM reuterin, **Z** MRS with 5% ethanol, **Z1** MRS with 15% ethanol, **Z2** MRS control. Numbers next to the title indicates how many datasets were included (24 h/48 h). Grey line indicates the growth threshold at OD_{600} = 0.4. Boxplot: box indicates 1. quantile (25%) and 3. quantile (75%), red line = median, wishker = 1 or 3 quantil ± 1.5 x inter-quantil-range or maximal/minimal value, red + = outlier above or below wishker.

Characterization of antifungal activity

Comparative genomics of antifungal and non-antifungal isolates revealed no clear evidence for antifungal related genes (data not shown). However, a subtilisin-like serine protease was detected in the antifungal strain *L. plantarum* RI-162 (WP_015825367.1) and the antifungal activity of a this gene was already described (Fan et al., 2014; Yu et al., 2012). An overexpression of the subtilisin-like serine protease with the NICE system in *Lactococcus lactis* NZ9000, showed no antifungal activity (data not shown). A high-throughput screening with supernatant revealed no strains with proteinaceous antifungal activity in our strain collection (data not shown). Based on these initial findings we focused on the mechanism of antifungal activity in lactobacilli. Therefore 45 lactobacilli were selected for the overlay assay AF1 and 33 strains showed strong inhibition against *R. mucilaginosa* LME and *C. parapsilosis* 4-5/1 (Table 5.1). These strains belong to *L. plantarum* (n=28), *L. rhamnosus* (n=1), *L. paracasei* (n=3) and *L. casei* (n=1). In contrast, the 5 tested *L. sakei* isolates were not active against these two fungal indicators. The 33 antifungal *Lactobacillus* strains from AF1 were tested in an agar well diffusion assay (AF2) to confirm their antifungal activity. 10-fold concentrated supernatants inhibited fungal growth whereas crude supernatant or the 10-fold concentrated MRS were not active. Digestion of the supernatants with proteases did not result in reduced antifungal activity suggesting that the inhibitory compounds are non-proteinaceous metabolites.

In follow-up experiments, *L. plantarum* RI-162 and *L. plantarum* RI-271, both dairy isolates *L. plantarum* RI-422, a salami isolate and *L. plantarum* WCFS1, a human saliva isolate, were used since they showed reliable antifungal activity and their genome sequence is available. Yeast-preparations achieved by heat and mechanical treatments were used to determine a potential induction of antifungal activity (AF3) by yeast components. Such a yeast-component mediated induction was absent, since no difference in antifungal activity was detected between *Lactobacillus* cells incubated with or without yeast-preparations.

The necessity of living yeast cells presence for antifungal activity was determined with the AF5 assay. For that, two colonies of the *L. plantarum* strains RI-162, RI-217, RI-422 and WCFS1 were overlaid with *R. mucilaginosa* LME. The inhibition zone around the position of the removed *L. plantarum* WCFS1 colony (Figure 5.2A – left side) was similar to that of the WCFS1 colony still present (Figure 5.2A – right side), suggesting that the simultaneous presence of living yeast is not necessary for antifungal activity by lactobacilli. The antifungal activity remained stable for 5 days.

To quantify antifungal activity *L. plantarum* RI-162 and *R. mucilaginosa* LME were mixed in a 1-ml competition co-culture assay (AF4). Different cell numbers of the two microorganisms were tested and the critical ratio where the lactobacilli were not able to inhibit the yeast was determined.

Table 5.1 *Lactobacillus* strains with antimicrobial activity

Genus	Species	Strain	*Listeria innocua* HPB13	*Listeria ivanovii* DSM 12491T	*Listeria monocytogenes* ATCC 19114	*Enterococcs faecalis* DSM 20478T	*Enterococcus faecium* SL1.1	*Rhodotorula mucilaginosa* LME	*Candida parapsilosis* 4-5/1	Bacteriocin predicted with BAGEL3 and antiSMASh3.0	Genome sequenced / Accession number of published genomes
Lactobacillus	*rhamnosus*	RI-004						+	nt	Enterocin X	MJHB00000000
Lactobacillus	*plantarum*	RI-009						+++	+++	Plantaricin E	no
Lactobacillus	*plantarum*	RI-011						+++	+++	Plantaricin E	MJHC00000000
Lactobacillus	*plantarum*	RI-012						+++	+++		MJHD00000000
Lactobacillus	*plantarum*	RI-029						+++	+++		yes
Lactobacillus	*plantarum*	RI-031						+++	+++		yes
Lactobacillus	*plantarum*	RI-046	+++	+++	+++	+	++	nt	nt		no
Lactobacillus	*plantarum*	RI-048						+++	+++	Plantaricin E/F	MJHG00000000
Lactobacillus	*paracasei*	RI-075			+++						no
Lactobacillus	*paracasei*	RI-076	+++	+++	nt	-	+				no
Lactobacillus	*paracasei*	RI-077	+++	+++	nt	-	-				no
Lactobacillus	*plantarum*	RI-080	-	-	+++	-	-	nt	nt		no
Lactobacillus	*plantarum*	RI-086						+++	+++		MKDP00000000
Lactobacillus	*plantarum*	RI-087						+++	+++		yes
Lactobacillus	*plantarum*	RI-113						+++	+++	Plantaricin A/E	CP017406-12
Lactobacillus	*plantarum*	RI-123						+++	+++	Plantaricin E	MKDQ00000000
Lactobacillus	*curvatus*	RI-124								Sakacin P/Q, Enterocin NRK-5-3-A	MKDR00000000
Lactobacillus	*plantarum*	RI-139						+++	+++	Plantaricin E	MKDS00000000
Lactobacillus	*plantarum*	RI-140						+++	+++	Plantaricin E, Enterocin NRK-5-3-A	MKDT00000000
Lactobacillus	*plantarum*	RI-146						+++	+++	Plantaricin A/E/F	MKDU00000000
Lactobacillus	*plantarum*	RI-147						+++	+++	Plantaricin E/K	MKDV00000000
Lactobacillus	*casei*	RI-149						+	nt		MJHH00000000
Lactobacillus	*plantarum*	RI-162						+++	+++	Plantaricin E/K	yes
Lactobacillus	*plantarum*	RI-164	+++	+++	+++	+	+	nt	nt		no
Lactobacillus	*plantarum*	RI-165	+++	+++	+++	+	+	+++	+++	Pediocin PA, Plantaricin A/J	yes
Lactobacillus	*plantarum*	RI-189						+++	+++	Enterocin X, Plantarcin A/J	MJHJ00000000
Lactobacillus	*plantarum*	RI-190						+++	+++	Enterocin X, Plantarcin J	MJHK00000000
Lactobacillus	*plantarum*	RI-191						+++	+++	Enterocin X, Plantarcin J	yes
Lactobacillus	*paracasei*	RI-194						++	nt	Enterocin X, Plantarcin A/J	MKFZ00000000
Lactobacillus	*paracasei*	RI-195						++	nt	Enterocin X, Plantarcin J	MKGA00000000
Lactobacillus	*plantarum*	RI-203						+++	+++	Plantaricin A/E/F	yes
Lactobacillus	*plantarum*	RI-208			++			+++	+++	Enterocin X, Plantarcin A/J	MKFX00000000
Lactobacillus	*paracasei*	RI-210						++	nt	Thermophilicin A	MKFY00000000
Lactobacillus	*delbrueckii*	RI-247	+++	+++	+++	++	++				no
Lactobacillus	*delbrueckii*	RI-248	+++	+++	+++	++	++				no
Lactobacillus	*delbrueckii*	RI-249	+++	+++	+++	++	++				no
Lactobacillus	*delbrueckii*	RI-250	+++	+++	+++	++	++				no
Lactobacillus	*delbrueckii*	RI-251	+++	+++	+++	++	++				no
Lactobacillus	*delbrueckii*	RI-252	+++	+++	+++	++	++				no
Lactobacillus	*delbrueckii*	RI-253	+++	+++	+++	++	++				no
Lactobacillus	*rhamnosus*	RI-255						++	nt		no
Lactobacillus	*murinus*	RI-256	-	-	+++	-	-	-	-		no
Lactobacillus	*plantarum*	RI-265						+++	+++	Plantaricin E	yes
Lactobacillus	*plantarum*	RI-266						+++	+++	Plantaricin A/E/F	MKDY00000000
Lactobacillus	*plantarum*	RI-271	-	-	+++	-	-	++	++	Plantaricin F/423	yes
Lactobacillus	*sakei*	RI-391	++	++	+++	+	-	-	-		no
Lactobacillus	*plantarum*	RI-393						+++	+++	Plantracin K/N	yes
Lactobacillus	*plantarum*	RI-405						+++	+++	Plantaricin E/F	MKDF00000000
Lactobacillus	*helveticus*	RI-407						++	nt		no
Lactobacillus	*plantarum*	RI-408						+++	+++	Helveticin J, Plantaricin E/F, Enterolysin A	MKDH00000000

Lactobacillus	*sakei*	RI-409	+++	+++	nt	++	++	-	nt		MKGB00000000
Lactobacillus	*sakei*	RI-410						-	nt		MKDI00000000
Lactobacillus	*sakei*	RI-412						-	nt	Sakacin G	MKDJ00000000
Lactobacillus	*plantarum*	RI-422						+++	+++	Plantracin K/N	MKDK00000000
Lactobacillus	*helveticus*	RI-434						++	nt		no
Lactobacillus	*helveticus*	RI-436						+	nt		no
Lactobacillus	*helveticus*	RI-440						+	nt		no
Lactobacillus	*spp*	RI-460	+++	+++	+++	-	+				no
Lactobacillus	*spp*	RI-461	+++	+++	+++	-	+				no
Lactobacillus	*sakei*	RI-493	-	-	nt	-	-	-	nt	Sakacin A, Plantaricin S-alpha	yes
Lactobacillus	*plantarum*	RI-498	-	++	nt	-	-				no
Lactobacillus	*plantarum*	WCFS1						+++	+++		yes

Antibacterial activity score according to halo size: +++ = ≥ 20 mm; ++ = 14-19 mm; + = 7-13 mm; +/- = unstable inhibition; - = no inhibition; nt = not tested. Antifungal activity was Inhibition zones were qualitatively assessed and classified into no inhibiton (-), weak inhibition (+), moderate inhibition (++) and strong inhibition (+++). Bacteriocin score: x = bacteriocin gene detected with BAGEL3 and antiSMASH3.0. Accession number: yes = genome of isolate is sequenced but not published, no = genome of isolate is not sequenced.

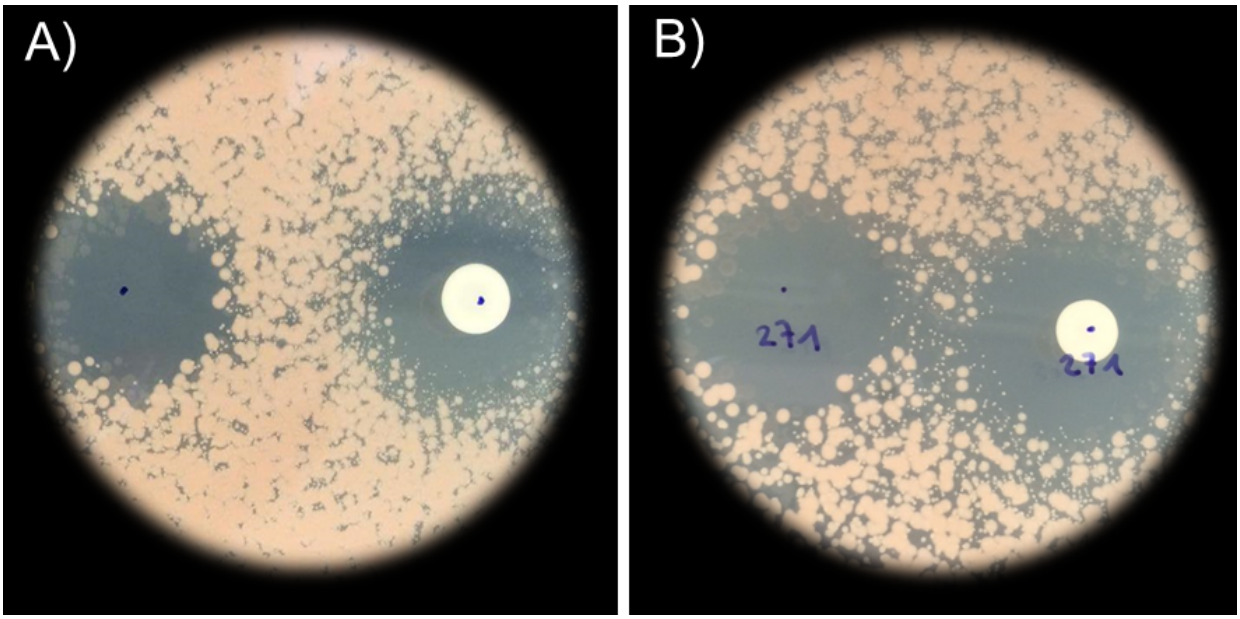

Figure 5.2 Antifungal assay AF5 with **A** *L. plantarum* WCFS1 and **B** *L. plantarum* RI-271. White *Lactobacillus* colony on the left side of the agar plate was removed before YM-soft-agar with *R. mucilaginosa* LME was overlaid. Inhibition zone after 5 days is similar with or without the *Lactobacillus* colony.

An amount of only 1-2 cfu/ml of *L. plantarum* RI-162 were able to reduce an initial *R. mucilaginosa* LME culture of (8.6±0.6) x 10^5 cfu/ml to a level of (4.6±3.6) x 10^2 cfu/ml within 48 hours, while the lactobacilli grew to a final concentration of (2.3±0.4) x 10^9 cfu/ml (Figure 5.3). A diluted concentration of <1 cfu/ml *L. plantarum* RI-162 did not inhibit *R. mucilaginosa* LME. The yeast grew to a final concentration of (3.6±0.3) x 10^6 cfu/ml while *L. plantarum* concentration remained below the detection limit after 48 h (Table S5.3).

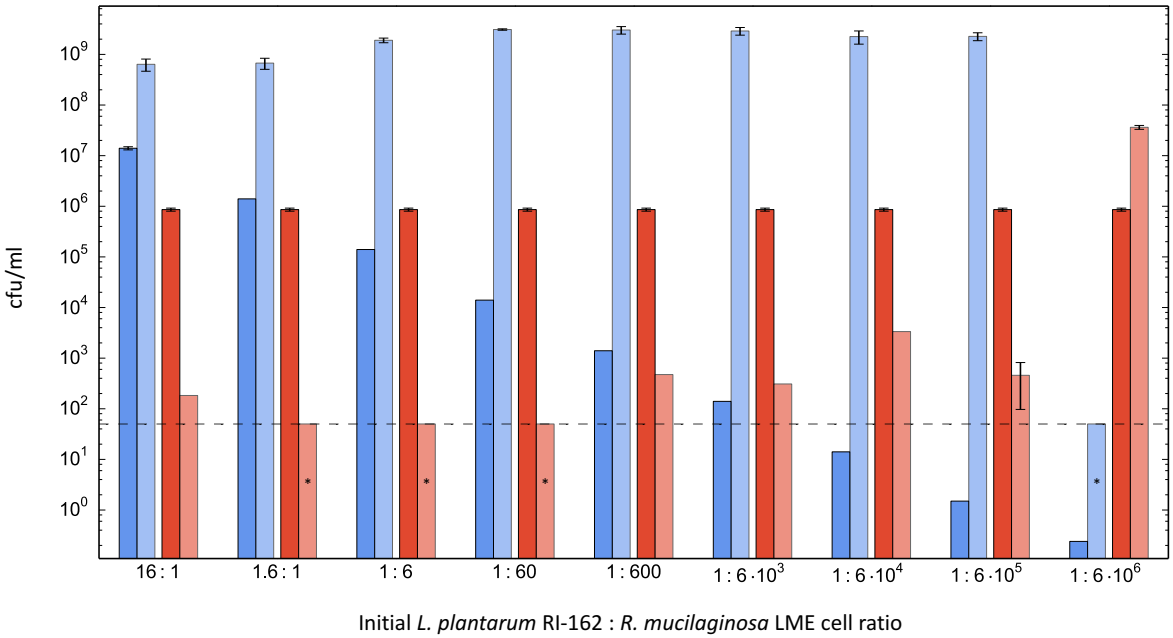

Figure 5.3 Competition co-culture assay with *L. plantarum* RI-162 and *R. mucilaginosa* LME at variable concentration ratios. Dark blue = *L. plantarum* RI-162 before co-cultivation, light blue = *L. plantarum* RI-162 after co-cultivation, dark red = *R. mucilaginosa* LME before co-cultivation, light red = *R. mucilaginosa* LME after co-cultivation. Liquid cultures were mixed in variable concentrations and incubated for 48 h at 30 °C. Dotted line indicates the detection limit of 50 cfu. * indicates isolates below the detection limit. Error bars based on 3 biological replicates.

The dependence of antifungal activity on the acidification rate was determined using *L. plantarum* NZ7306 *ΔrpoN,* a *L. plantarum* WCFS1 derivative with lower acidification rates (Stevens et al., 2010). The halo of the mutant strain was smaller than the halo of *L. plantarum* WCFS1 in an overlay assay (AF1). This suggests, that antifungal activity is affected by lactic acid production. The analysis of the antifungal metabolite production revealed that all 4 tested *L. plantarum* strains produce mainly lactic acid as well as small amounts acetic acid, propionic acid, ethyl-L-lactic acid and phenyllactic acid (Table 5.2).

Table 5.2 Acid analysis of culture supernatant from four *Lactobacillus plantarum* strains

Analyte [mM]	*L. plantarum* RI-162	*L. plantarum* RI-271	*L. plantarum* RI-422	*L. plantarum* WCFS1	MRS broth
Lactic acid	206. ± 5	184. ± 3	209. ± 2	210. ± 7	<0.44
Acetic acid	73. ± 6	78. ± 5	74. ± 3	76. ± 5	65. ± 3
PCA	3.02 ± 0.33	3. ± 0.32	2.85 ± 0.27	2.83 ± 0.33	2.92 ± 0.47
Propionic acid	4.08 ± 0.40	5.56 ± 0.38	1.61 ± 1.04	2.41 ± 0.10	<0.44
Ethyl-L-lactic acid	2.91 ± 1.98	4.02 ± 2.08	2.14 ± 0.87	2.51 ± 0.56	1.32 ± 0.55
OH-PLA	<0.15	<0.15	0.19 ± 0.02	0.21 ± 0.01	<0.11
PLA	0.36 ± 0.03	0.44 ± 0.09	0.51 ± 0.05	0.55 ± 0.05	<0.06

PCA = 2-Pyrrolidone-5-carboxylic acid; OH-PLA = hydroxyl phenyllactic acid; PLA = phenyllactic acid; ± standard deviation from biological triplicates.

Application of antibacterial strains in fermented sausage

Listeria contamination in fermented sausages like salami is a potential risk in industrial fermentations. The salami-isolate *L. sakei* RI-409 was selected for potential industrial application since it showed antimicrobial activity and a bacteriocin gene curvacin A (WP_011374266.1 / Table 5.1), growth at 8 °C and originates from salami. The in silico genome analysis revealed antibiotic resistance for teichoplanin/vancomycin with the gene *vanZ* (WP_056948756.1). 3 salami batches were prepared with an initial concentration of 6.33 x 10^5 cfu/g of *Listeria ivanovii* DSM 12491^T. The pH decreased from 5.8 to 5.2 in the spontaneous fermentation within 5 days, whereas in the starter culture and the protective culture batch a pH below 5.0 was reached (Figure 5.4). The listeria concentration increased from 9.6 x 10^4 cfu/g to 5.3 x 10^6 cfu/g in the spontaneous fermentation without starter or protective culture after 5 days of ripening (Figure 5.4A). In contrast, the listeria concentration decreased from 9.6 x 10^4 cfu/g to 4.3 x 10^4 cfu/g in the batch with starter culture (Figure 5.4B) and from 9.6 x 10^4 cfu/g to 2.5 x 10^4 cfu/g in the batch with *L. sakei* RI-409 after 5 days (Figure 5.4C). During the 9-days long storage phase, the listeria concentration decreased in all batches to a concentration of approximately 10^4 cfu/g.

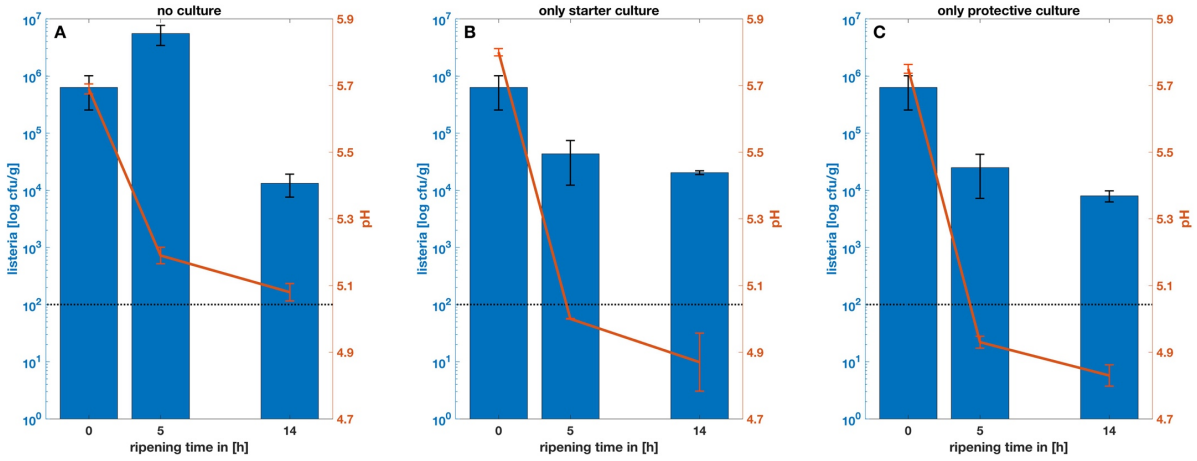

Figure 5.4 Survival of *Listeria ivanovii* DSM 12491^t during salami ripening and storage with *Lactobacillus* protective candidate culture. **A** batch produced with no additional culture. **B** batch produced with starter culture only. **C** batch produced with protective candidate culture only. Salamis were 5 days ripened at changing conditions and later stored for 9 days at 15 °C. Black dotted line indicated the detection limit of 100 cfu/g. Error bars based on 3 technical replicates.

Since strain RI-409 showed no significant improvement compared with the starter culture Bitec LK30, strain *L. sakei* RI-409 was tested with the 5 antibacterial strains *L. plantarum* RI-046, RI-208, RI-303, RI-460 and RI-461 in a small-scale industrial application. Therefore, raw meat was inoculated with *Listeria ivanovii* DSM 12491^T as a spoilage organism, an in-house starter culture and one of the tested

Lactobacillus culture. All cultures acidified the sausages to a pH below 5.0 within 48 hours, the standard for salami acidification. The addition of *L. plantarum* RI-046, RI-208, RI-460 and RI-461 resulting in a reduction of *Listeria ivanovii* DSM 12491^T. from the initial concentration of 10^5 cfu/g to below the detection limit of 100 cfu/g within 48 hours (Figure 5.5A-B / E-F). In contrast, the protective cultures *L. plantarum* RI-303 and *L. sakei* RI-409 reduced *Listeria ivanovii* DSM 12491^T concentration to 8.3 x 10^3 and 1.45 x 10^4 cfu/g within 48 hours, respectively (Figure 5.5C-D). After 120 h, still no listeria was detected in sausages with RI-046, RI-208, RI-460 and RI-461, whereas values of 2.6 x 10^3 and 400 cfu/g for RI-303 and RI-409 were detected, respectively. Hence, 4 protective cultures inhibited strongly and 2 moderately *Listeria* in small-scale sausages, which parallels the finding from the industrial-scale tested strain RI-409.

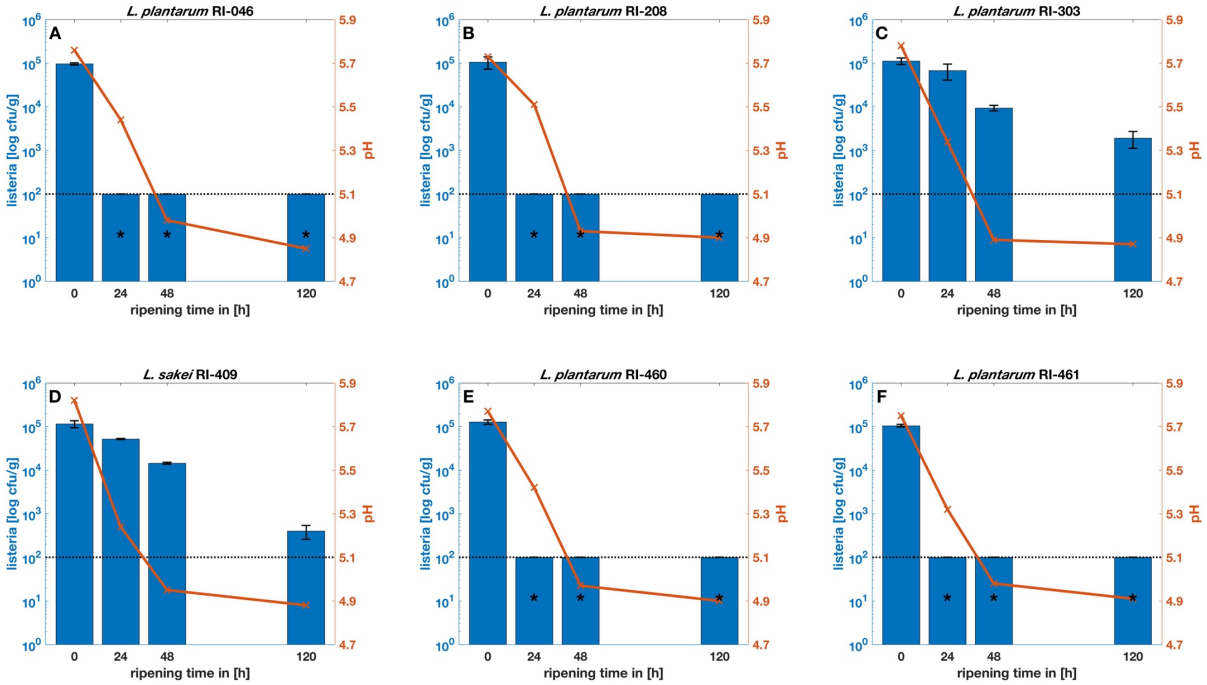

Figure 5.5 *Listeria ivanovii* DSM 12491^T concentration and pH during a small-scale salami ripening.
A-F 6 batches of salami inoculated with 10^5 cfu/g *Listeria ivanovii* DSM 12491^T and ripened with *Staphylococcus carnosus* (Frutarom starter culture) and different *Lactobacillus* strains as candidate protective culture were tested. Blue bars = *Listeria ivanovii* DSM 12491^T concentration (right axes), orange line = pH (left axes), black dotted line indicated the detection limit of 100 cfu/g, * indicates isolates below the detection limit. Error bars for *Listeria* concentration from 2 biological replicates.

Application of antibacterial protective culture in raw milk soft cheese

The protective culture *L. plantarum* RI-271 and its supernatant was tested for its capacity to reduce enterococci in a 1000-L raw milk soft cheese fermentation. The fresh raw milk contained approximately 400 cfu/ml indigenous enterococci. A number of 6.50 x 10^6 cfu/g enterococci were detected in the untreated control cheese after 8 days of ripening (Table 5.3). Treatment of the cheese curd with culture supernatant resulted in an enterococci reduction of 56 – 89% compared with untreated raw milk soft cheese. A correlation between the inhibition rate and the cell concentration of the culture was observed. In cheese samples treated with the active cells of *L. plantarum* RI-271, an enterococci reduction of 96-97% was measured, apparently independent of cell concentration since no decrease of inhibition was detected in lower concentrations.

Table 5.3 Growth inhibition of enterococci by *Lactobacillus plantarum* RI-271 in raw milk soft cheese

***L. plantarum* RI-271 [cfu/ml]**		***Enterococci* [cfu/g]**	
type	[pre-culture]	after 8 days	reduction
untreated		$6.50x10^6$	0%
SN	$5x10^7$	$7.00x10^5$	89%
	$5x10^6$	$1.88x10^6$	71%
	$5x10^5$	$2.88x10^6$	56%
live cells	$5x10^7$	$2.30x10^5$	96%
	$5x10^6$	$2.20x10^5$	97%
	$5x10^5$	$2.50x10^5$	96%

Detection limit at 10^2 cfu/ml, SN = supernatant of *L. plantarum* RI-217, live cells = active cells of L. plantarum RI-217

Discussion

The goal of this study was to establish an approach to select tailor-made protective cultures for fermented food products. Therefore, 504 *Lactobacillus* strains were tested for their growth ability under 27 different conditions to select potential protective cultures suitable in fermented food products. Tolerance to high bile salt concentrations was measured since bile salt can be regarded as a stress adaptor for lactobacilli (Ruiz et al., 2013). In general, lactobacilli grew up to 10% bile salt (Figure 5.1B) which is higher than the 0.05 – 2% bile salt concentration in human gut and also higher than the MIC of 0.5 – 1.8% bile salt for the species *L. rhamnosus* and *L. fermentum* (Bao et al., 2010; Douillard et al., 2013). The ability to grow in salt concentrations up to 5% is another criterion for protective culture selection as these conditions occur during ripening processes in many food products (De Almeida et al., 2016). Halotolerance in strains isolated from fermented meat would be expected as those products contain salt concentration of 3 - 5%. However, it is remarkable that none of our halo-tolerant lactobacilli belonged to the species *L. sakei,* although 35 out of 504 isolates are isolated from fermented salami. Halotolerance up to 10% NaCl in lactobacilli is documented for isolates of *L. acidipiscis, L. alimentarius, L. farciminis, L. sakei* subsp. *sakei* and *L. vermoldensis* (Hammes and Hertel, 2009; Phalakornkule and Tanasupawat, 2007) which parallels our findings for strain *L. farciminis* RI-339. Less than 50% of the *Lactobacillus* isolates in our collection were able to grow at pH 3.5 (Figure 5.1H) and none of them at pH 3.0 (data not shown), which is remarkable since growth of lactobacilli was detected at even lower pH in other studies. Strain *L. salivarius* UCO_979C-2, isolated from human stomach, grew at pH 2.6 within 48 hours (Sanhueza et al., 2015). Growth and activity at low temperatures is interesting since fermented food products are generally stored at low temperature conditions. 9 out of 60 tested *L. sakei* strains grew at 8 °C which parallels the finding that several strains of this species are able to grow at low temperatures (Hammes and Hertel, 2009). 18% of the tested lactobacilli grew in absence of manganese, although growth media supplementation with manganese was clamied to be essential for several lactobacilli species (Hammes and Hertel, 2009). The group of 83 “manganese-independent” lactobacilli contained 24 *L. sakei* strains. Neither of those had a superoxide dismutase (SOD) gene which is present in the type strain *L. sakei* K23. *L. plantarum* RI-409 was the only strain with a *sod* gene with 99% nucleotide identity with the *sod* gene from *L. sakei* K23 and a detected growth in the phenotypic screening in MRS without manganese (data not shown). There is a hypothesis that an Mn(II) concentration between 20 and 25 mM in *Lactobacillus* is as effective to reduce oxygen radicals as micromolar level of SOD in other bacteria (Archibald and Fridovich, 1981). Therefore, the activity of SOD in active strains under manganese starvation could be an explanation. According to our observations, growth under manganese starvation and presence of *sod* genes are not correlating.

All 28 tested *L. plantarum* strains in the screening inhibited *R. mucilaginosa* LME and *C. parapsilosis* 4-5/1. Other *Lactobacillus* species showed no or only weak inhibition (Table 5.1). Strong antifungal activity of *L. plantarum* isolates was already demonstrated *in vitro* (Broberg et al., 2007; Ndagano et al., 2011; Prema et al., 2010; Sjögren et al., 2003; Ström et al., 2002) and confirmed our observations. Whereas *L. plantarum* is commonly associated to antifungal activity, this phenotype is only rarely reported in *L. sakei* which parallels our findings (Crowley et al., 2013b). However, studies are difficult to compare with our experiment since different fungi indicators were used. In the current study, only non-buffered media was used to include antifungal metabolites with a low-pH dependent production such as cyclic dipeptides (Ryan et al., 2009). Antifungal activity based solely on low pH inhibitory effects can however, be excluded since the tested lactobacilli showed antifungal activity in a buffered assay (Inglin et al., 2015).

The supernatant of 4 *L. plantarum* strains was antifungal in an overlay assay (AF1) but not in an agar well diffusion assay (AF2). 10-fold concentrated supernatant showed antifungal activity in the agar well diffusion assay AF2. Such concentration dependent inhibition activity are in agreement with other studies (Coloretti et al., 2007; Saladino et al., 2016; Yang and Chang, 2010). Moreover, antifungal activity is based on non-proteinaceous compounds (AF2) which parallels findings of other studies (Crowley et al., 2013a; Ndagano et al., 2011; Russo et al., 2017). Antifungal substances are present in *L. plantarum* supernatant although no simultaneous contact with fungal cells occurred (AF3). The antifungal activity could not be increased by cell-to-cell contact of the lactobacilli with fungi, showing that antifungal activity is not a triggered response to fungi. In fact, we could demonstrate that the presence of a fungus is not required for an antifungal activity of lactobacilli. Antifungal activity of *L. plantarum* is, amongst others, based on organic acid production as demonstrated for the low glycolysis mutant strain. In theory, the highest achievable lactic acid concentration in MRS is 220 mM originating from 110 mM glucose. Since this is below the lactic acid MIC of >500 mM for *R. mucilaginosa* (Miescher Schwenninger et al., 2008), we assume a synergistic effect between lactic acid and other organic acids which confirms other studies (Dal Bello et al., 2007; Gerez et al., 2010; Rizzello et al., 2011). The power of antifungal activity in *L. plantarum* was demonstrated when a 1-2 cfu/ml of *L. plantarum* RI-162 was sufficient to inhibit of 8.6×10^5 cfu/ml *R. mucilaginosa* LME in a co-culture assay. The application of a *L. plantarum* strain could inhibit *R. mucilaginosa* by fungistatic activity in orange juice over 30 days (Crowley et al., 2012). In our co-culture experiment a reduction of *R. mucilaginosa* was detected and hence there was a fungicide effect, the difference that can be explained by the difference in strain, pH and initial yeast concentration.

In the antibacterial application project, we tested protective cultures against *Listeria* and *Enterococcus* in food models. The genome analysis of the protective strain *L. plantarum* RI-409 revealed resistances against the antibiotics teicoplanin/vancomycin. However, since lactobacilli are intrinsic vancomycin resistant and the resistance gene operon, including *vanZ* is not complete, we regarded RI-409 as safe for use in applications (Arthur et al., 1995; Maragkoudakis et al., 2006). Further the protective culture *L. plantarum* RI-409 was tested in large-scale industrial production, showing no significant improvement compared to the starter culture treatment. However, the listeria concentration was decreased compared to the non-treated batch after 5 days. The listeria reduction after 14 days might be related to the increased salt concentration during the drying, even if resistance to high salt concentration was demonstrated in listeria (Nightingale et al., 2006). Nevertheless, the addition of a starter culture and a protective culture is a necessity to reduce listeria concentration in short ripened salami.

In a preliminary experiment, we tested the ability of *L. plantarum* RI-271 and its bacteriocin plantaricin F, to reduce the outgrowth of enterococci in the 1000-L small-scale raw milk soft cheese fermentation. The inhibition with heat-treated supernatant was concentration dependent, whereas the inhibition in batches treated with live protective culture cells was not concentration dependent. This suggest that bacteriocins are produced *in situ*, as reported for other lactobacilli (Gálvez et al., 2010). Therefore, the application with the lowest concentration of protective culture cells has the best trade-off between additional costs and efficiency. The enterococci reduction of 97% between protective culture and the non-treated batch needs improvement before applying for larger industrial applications.

Conclusion

In this study, we demonstrated an approach to select and apply *Lactobacillus* isolates as protective culture for food fermentations to inhibit spoilage organisms such as *Listeria, Enterococcus* and fungi. The combination of phenotypic screening and whole genome sequencing reveals an optimal output of information to characterize and select bacteria. The screening of existing culture collection unraveled their biotechnological potential since protective cultures can also be expanded to non-food products such as microbial pest management. Since our approach is based on low cost equipment and the cost of genome sequencing reduced drastically over the past years, this approach is suitable for every laboratory.

Supporting information

Table S5.1 *Lactobacillus* strains used for phenotypic screening in chapter 5

Genus	Species	Subspecies	Strain	Source
Lactobacillus	*casei*		RI-001	Dairy
Lactobacillus	*rhamnosus*		RI-002	Unknown
Lactobacillus	*casei*		RI-003	Dairy
Lactobacillus	*reuteri*		RI-004	Dairy
Lactobacillus	*rhamnosus*		RI-005	Dairy
Lactobacillus	*delbrueckii*	*lactis*	RI-006	Dairy
Lactobacillus	*acidophilus*		RI-007	Dairy
Lactobacillus	*zeae*		RI-008	Dairy
Lactobacillus	*plantarum*		RI-009	Dairy
Lactobacillus	*paracasei*	*paracasei*	RI-010	Dairy
Lactobacillus	*reuteri*		RI-011	Dairy
Lactobacillus	*plantarum*		RI-012	Dairy
Lactobacillus	*rhamnosus*		RI-013	Dairy
Lactobacillus	*casei*		RI-014	Dairy
Lactobacillus	*casei*		RI-015	Dairy
Lactobacillus	*casei*		RI-016	Dairy
Lactobacillus	*casei*		RI-017	Dairy
Lactobacillus	*casei*		RI-018	Dairy
Lactobacillus	*rhamnosus*		RI-019	Dairy
Lactobacillus	*rhamnosus*		RI-020	Dairy
Lactobacillus	*rhamnosus*		RI-021	Dairy
Lactobacillus	*rhamnosus*		RI-022	Dairy
Lactobacillus	*rhamnosus*		RI-023	Dairy
Lactobacillus	*crispatus*		RI-024	Dairy
Lactobacillus	*rhamnosus*		RI-025	Dairy
Lactobacillus	*casei*		RI-026	Dairy
Lactobacillus	*casei*		RI-027	Dairy
Lactobacillus	*brevis*		RI-028	Dairy
Lactobacillus	*plantarum*		RI-029	Dairy
Lactobacillus	*curvatus*		RI-030	Dairy
Lactobacillus	*plantarum*		RI-031	Dairy
Lactobacillus	*delbrueckii*	*delbrueckii*	RI-032	Dairy
Lactobacillus	*acidophilus*		RI-033	Dairy
Lactobacillus	*acidophilus*		RI-034	Dairy
Lactobacillus	*coryniformis*	*coryniformis*	RI-035	Dairy
Lactobacillus	*casei*		RI-036	Dairy
Lactobacillus	*casei*		RI-037	Dairy
Lactobacillus	*casei*		RI-038	Dairy
Lactobacillus	*casei*		RI-039	Dairy
Lactobacillus	*casei*		RI-040	Dairy
Lactobacillus	*casei*		RI-041	Dairy
Lactobacillus	*rhamnosus*		RI-042	Dairy
Lactobacillus	*acidophilus*		RI-043	Dairy
Lactobacillus	*casei*		RI-044	Dairy
Lactobacillus	*salivarius*		RI-045	Dairy
Lactobacillus	*plantarum*		RI-046	Dairy
Lactobacillus	*curvatus*		RI-047	Dairy

Lactobacillus	*curvatus*		RI-048	Dairy
Lactobacillus	*curvatus*		RI-049	Dairy
Lactobacillus	*curvatus*		RI-050	Dairy
Lactobacillus	*curvatus*		RI-051	Dairy
Lactobacillus	*delbrueckii*	*bulgaricus*	RI-052	Dairy
Lactobacillus	*delbrueckii*	*bulgaricus*	RI-053	Dairy
Lactobacillus	*delbrueckii*	*bulgaricus*	RI-054	Dairy
Lactobacillus	*delbrueckii*	*lactis*	RI-055	Dairy
Lactobacillus	*delbrueckii*	*lactis*	RI-056	Dairy
Lactobacillus	*delbrueckii*	*lactis*	RI-057	Dairy
Lactobacillus	*delbrueckii*	*lactis*	RI-058	Dairy
Lactobacillus	*delbrueckii*	*lactis*	RI-059	Dairy
Lactobacillus	*delbrueckii*	*lactis*	RI-060	Dairy
Lactobacillus	*delbrueckii*	*lactis*	RI-061	Dairy
Lactobacillus	*fermentum*		RI-062	Dairy
Lactobacillus	*fermentum*		RI-063	Dairy
Lactobacillus	*fermentum*		RI-064	Dairy
Lactobacillus	*fermentum*		RI-065	Dairy
Lactobacillus	*fermentum*		RI-066	Dairy
Lactobacillus	*fermentum*		RI-067	Dairy
Lactobacillus	*fermentum*		RI-068	Dairy
Lactobacillus	*fermentum*		RI-069	Dairy
Lactobacillus	*fermentum*		RI-070	Dairy
Lactobacillus	*fermentum*		RI-071	Dairy
Lactobacillus	*fermentum*		RI-072	Dairy
Lactobacillus	*fermentum*		RI-073	Dairy
Lactobacillus	*fermentum*		RI-074	Dairy
Lactobacillus	*paracasei*	*paracasei*	RI-075	Dairy
Lactobacillus	*plantarum*		RI-076	Dairy
Lactobacillus	*plantarum*		RI-077	Dairy
Lactobacillus	*paracasei*	*paracasei*	RI-078	Dairy
Lactobacillus	*paracasei*	*paracasei*	RI-079	Dairy
Lactobacillus	*plantarum*		RI-080	Dairy
Lactobacillus	*plantarum*		RI-081	Dairy
Lactobacillus	*plantarum*		RI-082	Dairy
Lactobacillus	*plantarum*		RI-083	Dairy
Lactobacillus	*plantarum*		RI-084	Dairy
Lactobacillus	*plantarum*		RI-085	Dairy
Lactobacillus	*plantarum*		RI-086	Dairy
Lactobacillus	*plantarum*		RI-087	Dairy
Lactobacillus	*plantarum*		RI-088	Dairy
Lactobacillus	*rhamnosus*		RI-089	Dairy
Lactobacillus	*rhamnosus*		RI-090	Dairy
Lactobacillus	*paracasei*		RI-091	Dairy
Lactobacillus	*sp.*		RI-092	Dairy
Lactobacillus	*paracasei*	*paracasei*	RI-093	Dairy
Lactobacillus	*fermentum*		RI-094	Dairy
Lactobacillus	*fermentum*		RI-095	Dairy
Lactobacillus	*fermentum*		RI-096	Dairy
Lactobacillus	*plantarum*		RI-097	Dairy
Lactobacillus	*curvatus*		RI-098	Dairy
Lactobacillus	*acidophilus*		RI-099	Unknown
Lactobacillus	*casei*		RI-100	Unknown

Lactobacillus	*johnsonii*		RI-101	Unknown
Lactobacillus	*plantarum*		RI-102	Unknown
Lactobacillus	*brevis*		RI-103	Unknown
Lactobacillus	*casei*		RI-104	Unknown
Lactobacillus	*acetotolerans*		RI-105	Sourdough
Lactobacillus	*acidophilus*		RI-106	Sourdough
Lactobacillus	*brevis*		RI-107	Sauerkraut
Lactobacillus	*brevis*		RI-108	Sauerkraut
Lactobacillus	*brevis*		RI-109	Sauerkraut
Lactobacillus	*buchneri*		RI-110	Sauerkraut
Lactobacillus	*sp.*		RI-111	Fermented meat
Lactobacillus	*curvatus*		RI-112	Fermented meat
Lactobacillus	*plantarum*		RI-113	Fermented meat
Lactobacillus	*sp.*		RI-114	Unknown
Lactobacillus	*delbrueckii*	*delbrueckii*	RI-115	Fermented meat
Lactobacillus	*lindneri*		RI-116	Sauerkraut
Lactobacillus	*lindneri*		RI-117	Fermented meat
Lactobacillus	*plantarum*		RI-118	Fermented meat
Lactobacillus	*plantarum*		RI-119	Fermented meat
Lactobacillus	*plantarum*		RI-120	Fermented meat
Lactobacillus	*plantarum*		RI-121	Fermented meat
Lactobacillus	*plantarum*		RI-122	Sourdough
Lactobacillus	*plantarum*		RI-123	Sourdough
Lactobacillus	*sakei/curvatus*		RI-124	Sauerkraut
Lactobacillus	*sakei*		RI-125	Fermented meat
Lactobacillus	*brevis*		RI-126	Gras silage
Lactobacillus	*brevis*		RI-127	Gras silage
Lactobacillus	*brevis*		RI-128	Gras silage
Lactobacillus	*brevis*		RI-129	Gras silage
Lactobacillus	*buchneri*		RI-130	Maize silage
Lactobacillus	*delbrueckii*	*bulgaricus*	RI-131	Dairy
Lactobacillus	*fructivorans*		RI-132	Gras silage
Lactobacillus	*fructivorans*		RI-133	Gras silage
Lactobacillus	*fructivorans*		RI-134	Gras silage
Lactobacillus	*lindneri*		RI-135	Gras silage
Lactobacillus	*paracasei*	*paracasei*	RI-136	Fermented meat
Lactobacillus	*plantarum*		RI-137	Fermented meat
Lactobacillus	*plantarum*		RI-138	Fermented meat
Lactobacillus	*plantarum*		RI-139	Gras silage
Lactobacillus	*plantarum*		RI-140	Maize silage
Lactobacillus	*brevis*		RI-141	Maize silage
Lactobacillus	*fructivorans*		RI-142	Gras silage
Lactobacillus	*paracasei*	*paracasei*	RI-143	Fermented meat
Lactobacillus	*paracasei*	*paracasei*	RI-144	Fermented meat
Lactobacillus	*paracasei*	*paracasei*	RI-145	Fermented meat
Lactobacillus	*plantarum*		RI-146	Maize silage
Lactobacillus	*plantarum*		RI-147	Maize silage
Lactobacillus	*plantarum*		RI-148	Unknown
Lactobacillus	*plantarum*		RI-149	Unknown
Lactobacillus	*fermentum*		RI-150	Chicken intestine
Lactobacillus	*fermentum*		RI-151	Chicken intestine
Lactobacillus	*fermentum*		RI-152	Chicken intestine
Lactobacillus	*fermentum*		RI-153	Chicken intestine

Lactobacillus	*fermentum*		RI-154	Chicken intestine
Lactobacillus	*fermentum*		RI-155	Chicken intestine
Lactobacillus	*paracasei*	*paracasei*	RI-156	Dairy
Lactobacillus	*paracasei*	*paracasei*	RI-157	Dairy
Lactobacillus	*paracasei*	*paracasei*	RI-158	Dairy
Lactobacillus	*paracasei*	*paracasei*	RI-159	Dairy
Lactobacillus	*plantarum*		RI-160	Unknown
Lactobacillus	*rhamnosus*		RI-161	Dairy
Lactobacillus	*helveticus*		RI-162	Unknown
Lactobacillus	*rhamnosus*		RI-163	Dairy
Lactobacillus	*plantarum*		RI-164	Dairy
Lactobacillus	*plantarum*		RI-165	Unknown
Lactobacillus	*plantarum*		RI-166	Dairy
Lactobacillus	*plantarum*		RI-167	Dairy
Lactobacillus	*plantarum*		RI-168	Dairy
Lactobacillus	*plantarum*		RI-169	Dairy
Lactobacillus	*plantarum*		RI-170	Dairy
Lactobacillus	*plantarum*		RI-171	Dairy
Lactobacillus	*plantarum*		RI-172	Dairy
Lactobacillus	*plantarum*		RI-173	Dairy
Lactobacillus	*plantarum*		RI-174	Dairy
Lactobacillus	*plantarum*		RI-175	Dairy
Lactobacillus	*plantarum*		RI-176	Dairy
Lactobacillus	*plantarum*		RI-177	Dairy
Lactobacillus	*plantarum*		RI-178	Dairy
Lactobacillus	*plantarum*		RI-179	Dairy
Lactobacillus	*sp.*		RI-180	Dairy
Lactobacillus	*sp.*		RI-181	Dairy
Lactobacillus	*sp.*		RI-182	Dairy
Lactobacillus	*sp.*		RI-183	Dairy
Lactobacillus	*sp.*		RI-184	Dairy
Lactobacillus	*sp.*		RI-185	Dairy
Lactobacillus	*sp.*		RI-186	Dairy
Lactobacillus	*delbrueckii*	*lactis*	RI-187	Dairy
Lactobacillus	*paraplantarum*		RI-188	Unknown
Lactobacillus	*plantarum*		RI-189	Unknown
Lactobacillus	*reuteri*		RI-190	Human intestine
Lactobacillus	*reuteri*		RI-191	Dairy
Lactobacillus	*sp.*		RI-192	Unknown
Lactobacillus	*paracasei*	*paracasei*	RI-193	Unknown
Lactobacillus	*paracasei*	*paracasei*	RI-194	Unknown
Lactobacillus	*paracasei*	*paracasei*	RI-195	Unknown
Lactobacillus	*curvatus*		RI-196	Fermented plant
Lactobacillus	*curvatus*		RI-197	Fermented plant
Lactobacillus	*helveticus*		RI-198	Fermented plant
Lactobacillus	*helveticus*		RI-199	Fermented plant
Lactobacillus	*sp.*		RI-200	Fermented plant
Lactobacillus	*pentosus*		RI-201	Fermented plant
Lactobacillus	*delbrueckii*	*lactis*	RI-202	Fermented plant
Lactobacillus	*plantarum*		RI-203	Fermented plant
Lactobacillus	*sakei*		RI-204	Fermented plant
Lactobacillus	*rhamnosus*		RI-205	Dairy
Lactobacillus	*rhamnosus*		RI-206	Dairy

Lactobacillus	*acidophilus*		RI-207	Unknown
Lactobacillus	*plantarum*		RI-208	Unknown
Lactobacillus	*casei*	*casei*	RI-209	Unknown
Lactobacillus	*helveticus*		RI-210	Unknown
Lactobacillus	*fermentum*		RI-211	Dairy
Lactobacillus	*delbrueckii*	*lactis*	RI-212	Unknown
Lactobacillus	*delbrueckii*	*bulgaricus*	RI-213	Unknown
Lactobacillus	*delbrueckii*	*lactis*	RI-214	Unknown
Lactobacillus	*paracasei / casei*		RI-215	Unknown
Lactobacillus	*delbrueckii*	*lactis*	RI-216	Unknown
Lactobacillus	*delbrueckii*	*lactis*	RI-217	Unknown
Lactobacillus	*delbrueckii*	*lactis*	RI-218	Unknown
Lactobacillus	*delbrueckii*	*lactis*	RI-219	Unknown
Lactobacillus	*delbrueckii*	*lactis*	RI-220	Unknown
Lactobacillus	*sp.*		RI-221	Unknown
Lactobacillus	*delbrueckii*	*lactis*	RI-222	Unknown
Lactobacillus	*delbrueckii*	*lactis*	RI-223	Unknown
Lactobacillus	*delbrueckii*	*lactis*	RI-224	Unknown
Lactobacillus	*delbrueckii*	*lactis*	RI-225	Unknown
Lactobacillus	*delbrueckii*	*lactis*	RI-226	Unknown
Lactobacillus	*sp.*		RI-227	Unknown
Lactobacillus	*sp.*		RI-228	Unknown
Lactobacillus	*delbrueckii*	*lactis*	RI-229	Unknown
Lactobacillus	*delbrueckii*	*lactis*	RI-230	Unknown
Lactobacillus	*sp.*		RI-231	Unknown
Lactobacillus	*sp.*		RI-232	Unknown
Lactobacillus	*delbrueckii*	*lactis*	RI-233	Unknown
Lactobacillus	*delbrueckii*	*lactis*	RI-234	Unknown
Lactobacillus	*delbrueckii*	*lactis*	RI-235	Unknown
Lactobacillus	*sp.*		RI-236	Unknown
Lactobacillus	*sp.*		RI-237	Unknown
Lactobacillus	*sp.*		RI-238	Unknown
Lactobacillus	*delbrueckii*	*lactis*	RI-239	Unknown
Lactobacillus	*sp.*		RI-240	Unknown
Lactobacillus	*curvatus*		RI-241	Unknown
Lactobacillus	*sp.*		RI-242	Unknown
Lactobacillus	*sp.*		RI-243	Unknown
Lactobacillus	*fermentum*		RI-244	Unknown
Lactobacillus	*fermentum*		RI-245	Unknown
Lactobacillus	*plantarum*		RI-246	Unknown
Lactobacillus	*plantarum*		RI-247	Unknown
Lactobacillus	*plantarum*		RI-248	Unknown
Lactobacillus	*plantarum*		RI-249	Unknown
Lactobacillus	*plantarum*		RI-250	Unknown
Lactobacillus	*plantarum*		RI-251	Unknown
Lactobacillus	*plantarum*		RI-252	Unknown
Lactobacillus	*plantarum*		RI-253	Unknown
Lactobacillus	*johnsonii*		RI-254	Mouse
Lactobacillus	*reuteri*		RI-255	Mouse
Lactobacillus	*murinus*		RI-256	Mouse
Lactobacillus	*delbrueckii*		RI-257	Fermented plant
Lactobacillus	*delbrueckii*		RI-258	Fermented plant
Lactobacillus	*sp.*		RI-259	Unknown

Lactobacillus	*sp.*	RI-260	Unknown
Lactobacillus	*sp.*	RI-261	Unknown
Lactobacillus	*curvatus*	RI-262	Fermented plant
Lactobacillus	*curvatus*	RI-263	Fermented plant
Lactobacillus	*plantarum*	RI-264	Fermented plant
Lactobacillus	*plantarum*	RI-265	Fermented plant
Lactobacillus	*plantarum*	RI-266	Fermented plant
Lactobacillus	*lactis*	RI-267	Fermented plant
Lactobacillus	*fermentum*	RI-268	Dairy
Lactobacillus	*fermentum*	RI-269	Dairy
Lactobacillus	*plantarum*	RI-270	Dairy
Lactobacillus	*plantarum*	RI-271	Dairy
Lactobacillus	*fermentum*	RI-272	Dairy
Lactobacillus	*sp.*	RI-273	Unknown
Lactobacillus	*fermentum*	RI-274	Dairy
Lactobacillus	*sp.*	RI-275	Unknown
Lactobacillus	*sp.*	RI-276	Unknown
Lactobacillus	*fermentum*	RI-277	Dairy
Lactobacillus	*fermentum*	RI-278	Dairy
Lactobacillus	*fermentum*	RI-279	Dairy
Lactobacillus	*fermentum*	RI-280	Dairy
Lactobacillus	*fermentum*	RI-281	Dairy
Lactobacillus	*fermentum*	RI-282	Dairy
Lactobacillus	*fermentum*	RI-283	Dairy
Lactobacillus	*fermentum*	RI-284	Dairy
Lactobacillus	*fermentum*	RI-285	Dairy
Lactobacillus	*fermentum*	RI-286	Dairy
Lactobacillus	*sp.*	RI-287	Unknown
Lactobacillus	*fermentum*	RI-288	Dairy
Lactobacillus	*fermentum*	RI-289	Dairy
Lactobacillus	*fermentum*	RI-290	Dairy
Lactobacillus	*fermentum*	RI-291	Dairy
Lactobacillus	*fermentum*	RI-292	Dairy
Lactobacillus	*fermentum*	RI-293	Dairy
Lactobacillus	*fermentum*	RI-294	Dairy
Lactobacillus	*fermentum*	RI-295	Dairy
Lactobacillus	*fermentum*	RI-296	Dairy
Lactobacillus	*fermentum*	RI-297	Dairy
Lactobacillus	*fermentum*	RI-298	Dairy
Lactobacillus	*fermentum*	RI-299	Dairy
Lactobacillus	*fermentum*	RI-300	Dairy
Lactobacillus	*fermentum*	RI-301	Dairy
Lactobacillus	*fermentum*	RI-302	Dairy
Lactobacillus	*plantarum*	RI-303	Dairy
Lactobacillus	*plantarum*	RI-304	Dairy
Lactobacillus	*plantarum*	RI-305	Dairy
Lactobacillus	*plantarum*	RI-306	Dairy
Lactobacillus	*plantarum*	RI-307	Dairy
Lactobacillus	*plantarum*	RI-308	Dairy
Lactobacillus	*plantarum*	RI-309	Dairy
Lactobacillus	*pontis*	RI-310	Dairy
Lactobacillus	*sp.*	RI-311	Unknown
Lactobacillus	*sp.*	RI-312	Unknown

Lactobacillus	*sp.*	RI-313	Dairy
Lactobacillus	*sp.*	RI-314	Dairy
Lactobacillus	*sp.*	RI-315	Dairy
Lactobacillus	*sp.*	RI-316	Dairy
Lactobacillus	*sp.*	RI-317	Unknown
Lactobacillus	*sp.*	RI-318	Dairy
Lactobacillus	*sanfranciscensis*	RI-319	Sourdough
Lactobacillus	*reuteri*	RI-320	Animal
Lactobacillus	*pentosus*	RI-321	Unknown
Lactobacillus	*casei*	RI-322	Dairy
Lactobacillus	*buchneri*	RI-323	Fermented meat
Lactobacillus	*brevis*	RI-324	Fermented meat
Lactobacillus	*curvatus*	RI-325	Fermented meat
Lactobacillus	*paraplantarum*	RI-326	Fermented meat
Lactobacillus	*plantarum*	RI-327	Fermented meat
Lactobacillus	*plantarum*	RI-328	Fermented meat
Lactobacillus	*sakei*	RI-329	Fermented meat
Lactobacillus	*sakei*	RI-330	Fermented meat
Lactobacillus	*sakei*	RI-331	Fermented meat
Lactobacillus	*sp.*	RI-332	Unknown
Lactobacillus	*sp.*	RI-333	Unknown
Lactobacillus	*sp.*	RI-334	Unknown
Lactobacillus	*sp.*	RI-335	Unknown
Lactobacillus	*sp.*	RI-336	Unknown
Lactobacillus	*ultunensis*	RI-337	Human intestine
Lactobacillus	*harbinensis*	RI-338	Fermented plant
Lactobacillus	*farciminis*	RI-339	Fermented meat
Lactobacillus	*mali*	RI-340	Apple Juice
Lactobacillus	*sp.*	RI-341	Unknown
Lactobacillus	*fabifermentans*	RI-342	Cacao
Lactobacillus	*ceti*	RI-343	Lungs of a whale
Lactobacillus	*hominis*	RI-344	Human intestine
Lactobacillus	*acidipiscis*	RI-345	Dairy
Lactobacillus	*otakiensis*	RI-346	Fermented plant
Lactobacillus	*gasseri*	RI-347	Unknown
Lactobacillus	*animalis*	RI-348	Dental plaque of baboon
Lactobacillus	*sp.*	RI-349	Unknown
Lactobacillus	*sp.*	RI-350	Unknown
Lactobacillus	*sp.*	RI-351	Unknown
Lactobacillus	*sp.*	RI-352	Unknown
Lactobacillus	*curvatus*	RI-353	Fermented meat
Lactobacillus	*curvatus*	RI-354	Fermented meat
Lactobacillus	*sp.*	RI-355	Unknown
Lactobacillus	*curvatus*	RI-357	Fermented meat
Lactobacillus	*curvatus*	RI-358	Fermented meat
Lactobacillus	*curvatus*	RI-359	Fermented meat
Lactobacillus	*sp.*	RI-360	Fermented meat
Lactobacillus	*curvatus*	RI-361	Fermented meat
Lactobacillus	*curvatus*	RI-362	Fermented meat
Lactobacillus	*curvatus*	RI-363	Fermented meat
Lactobacillus	*curvatus*	RI-364	Fermented meat
Lactobacillus	*plantarum*	RI-365	Fermented meat
Lactobacillus	*paraplantarum*	RI-366	Fermented meat

Lactobacillus	*paraplantarum*	RI-367	Fermented meat
Lactobacillus	*paraplantarum*	RI-368	Fermented meat
Lactobacillus	*paraplantarum*	RI-369	Fermented meat
Lactobacillus	*sp.*	RI-370	Unknown
Lactobacillus	*paraplantarum*	RI-371	Fermented meat
Lactobacillus	*sp.*	RI-372	Unknown
Lactobacillus	*sp.*	RI-373	Unknown
Lactobacillus	*plantarum*	RI-374	Fermented meat
Lactobacillus	*sp.*	RI-375	Unknown
Lactobacillus	*plantarum*	RI-376	Fermented meat
Lactobacillus	*sp.*	RI-377	Unknown
Lactobacillus	*sakei*	RI-378	Fermented meat
Lactobacillus	*sp.*	RI-379	Unknown
Lactobacillus	*sakei*	RI-380	Fermented meat
Lactobacillus	*sakei*	RI-381	Fermented meat
Lactobacillus	*sp.*	RI-382	Unknown
Lactobacillus	*sp.*	RI-383	Unknown
Lactobacillus	*sakei*	RI-384	Fermented meat
Lactobacillus	*sakei*	RI-385	Fermented meat
Lactobacillus	*sakei*	RI-386	Fermented meat
Lactobacillus	*sakei*	RI-387	Fermented meat
Lactobacillus	*sakei*	RI-388	Fermented meat
Lactobacillus	*sakei*	RI-389	Fermented meat
Lactobacillus	*sakei*	RI-390	Fermented meat
Lactobacillus	*sakei*	RI-391	Fermented meat
Lactobacillus	*sakei*	RI-392	Fermented meat
Lactobacillus	*sakei*	RI-393	Fermented meat
Lactobacillus	*sakei-curvatus*	RI-394	Fermented meat
Lactobacillus	*sakei-curvatus*	RI-395	Fermented meat
Lactobacillus	*sakei-curvatus*	RI-396	Fermented meat
Lactobacillus	*sakei-curvatus*	RI-397	Fermented meat
Lactobacillus	*sakei-curvatus*	RI-398	Fermented meat
Lactobacillus	*sakei-curvatus*	RI-399	Fermented meat
Lactobacillus	*sakei-curvatus*	RI-400	Fermented meat
Lactobacillus	*sakei-curvatus*	RI-401	Fermented meat
Lactobacillus	*sakei-curvatus*	RI-402	Fermented meat
Lactobacillus	*sakei-curvatus*	RI-403	Fermented meat
Lactobacillus	*sakei-curvatus*	RI-404	Fermented meat
Lactobacillus	*sakei-curvatus*	RI-405	Fermented meat
Lactobacillus	*sakei-curvatus*	RI-406	Fermented meat
Lactobacillus	*sakei-curvatus*	RI-407	Fermented meat
Lactobacillus	*sakei-curvatus*	RI-408	Fermented meat
Lactobacillus	*sakei*	RI-409	Fermented meat
Lactobacillus	*sakei-curvatus*	RI-410	Fermented meat
Lactobacillus	*sakei-curvatus*	RI-411	Fermented meat
Lactobacillus	*sakei-curvatus*	RI-412	Fermented meat
Lactobacillus	*sakei-curvatus*	RI-413	Fermented meat
Lactobacillus	*sakei-curvatus*	RI-414	Fermented meat
Lactobacillus	*sakei-curvatus*	RI-415	Fermented meat
Lactobacillus	*sp.*	RI-416	Unknown
Lactobacillus	*sakei-curvatus*	RI-417	Fermented meat
Lactobacillus	*sakei-curvatus*	RI-418	Fermented meat
Lactobacillus	*sakei-curvatus*	RI-419	Fermented meat

Lactobacillus	*sakei-curvatus*	RI-420	Fermented meat
Lactobacillus	*sakei-curvatus*	RI-421	Fermented meat
Lactobacillus	*sakei-curvatus*	RI-422	Fermented meat
Lactobacillus	*sakei-curvatus*	RI-423	Fermented meat
Lactobacillus	*sakei-curvatus*	RI-424	Fermented meat
Lactobacillus	*sakei-curvatus*	RI-425	Fermented meat
Lactobacillus	*sakei-curvatus*	RI-426	Fermented meat
Lactobacillus	*sakei-curvatus*	RI-427	Fermented meat
Lactobacillus	*sakei-curvatus*	RI-428	Fermented meat
Lactobacillus	*sakei-curvatus*	RI-429	Fermented meat
Lactobacillus	*sp.*	RI-430	Fermented meat
Lactobacillus	*fermentum*	RI-431	Unknown
Lactobacillus	*fermentum*	RI-432	Unknown
Lactobacillus	*fermentum*	RI-433	Unknown
Lactobacillus	*helveticus*	RI-434	Unknown
Lactobacillus	*helveticus*	RI-435	Unknown
Lactobacillus	*helveticus*	RI-436	Unknown
Lactobacillus	*sp.*	RI-437	Unknown
Lactobacillus	*sp.*	RI-438	Unknown
Lactobacillus	*sp.*	RI-439	Unknown
Lactobacillus	*helveticus*	RI-440	Unknown
Lactobacillus	*helveticus*	RI-441	Unknown
Lactobacillus	*sp.*	RI-442	Unknown
Lactobacillus	*helveticus*	RI-443	Unknown
Lactobacillus	*sp.*	RI-444	Unknown
Lactobacillus	*sp.*	RI-445	Unknown
Lactobacillus	*sp.*	RI-446	Unknown
Lactobacillus	*sp.*	RI-447	Unknown
Lactobacillus	*sp.*	RI-448	Unknown
Lactobacillus	*sp.*	RI-449	Unknown
Lactobacillus	*sp.*	RI-450	Unknown
Lactobacillus	*sp.*	RI-451	Unknown
Lactobacillus	*sp.*	RI-452	Unknown
Lactobacillus	*sp.*	RI-453	Unknown
Lactobacillus	*sp.*	RI-454	Unknown
Lactobacillus	*sp.*	RI-455	Unknown
Lactobacillus	*sp.*	RI-456	Unknown
Lactobacillus	*sp.*	RI-457	Unknown
Lactobacillus	*sp.*	RI-458	Unknown
Lactobacillus	*sp.*	RI-459	Unknown
Lactobacillus	*plantarum*	RI-460	Unknown
Lactobacillus	*plantarum*	RI-461	Unknown
Lactobacillus	*sp.*	RI-462	Unknown
Lactobacillus	*sp.*	RI-463	Unknown
Lactobacillus	*sp.*	RI-464	Unknown
Lactobacillus	*sp.*	RI-465	Unknown
Lactobacillus	*sp.*	RI-466	Unknown
Lactobacillus	*sp.*	RI-467	Unknown
Lactobacillus	*sp.*	RI-468	Unknown
Lactobacillus	*sp.*	RI-469	Unknown
Lactobacillus	*sp.*	RI-470	Unknown
Lactobacillus	*sp.*	RI-471	Unknown
Lactobacillus	*sp.*	RI-472	Unknown

Lactobacillus	*sp.*	RI-473	Unknown
Lactobacillus	*sp.*	RI-474	Unknown
Lactobacillus	*sp.*	RI-475	Unknown
Lactobacillus	*sp.*	RI-476	Unknown
Lactobacillus	*sp.*	RI-477	Unknown
Lactobacillus	*sp.*	RI-478	Unknown
Lactobacillus	*sp.*	RI-479	Unknown
Lactobacillus	*brevis*	RI-480	Fermented plant
Lactobacillus	*buchneri*	RI-481	Fermented plant
Lactobacillus	*buchneri*	RI-482	Fermented plant
Lactobacillus	*buchneri*	RI-483	Fermented plant
Lactobacillus	*casei*	RI-484	Fermented plant
Lactobacillus	*casei*	RI-485	Fermented plant
Lactobacillus	*casei*	RI-486	Fermented plant
Lactobacillus	*crustorum*	RI-487	Fermented plant
Lactobacillus	*fermentum*	RI-488	Fermented plant
Lactobacillus	*plantarum*	RI-489	Fermented plant
Lactobacillus	*sakei*	RI-490	Fermented meat
Lactobacillus	*plantarum*	RI-491	Fermented meat
Lactobacillus	*plantarum*	RI-492	Fermented meat
Lactobacillus	*sakei*	RI-493	Fermented meat
Lactobacillus	*sakei*	RI-494	Fermented meat
Lactobacillus	*sakei*	RI-495	Fermented meat
Lactobacillus	*sakei*	RI-496	Fermented meat
Lactobacillus	*plantarum*	RI-497	Fermented meat
Lactobacillus	*plantarum*	RI-498	Fermented meat
Lactobacillus	*sakei*	RI-499	Fermented meat
Lactobacillus	*sakei*	RI-500	Fermented meat
Lactobacillus	*curvatus*	RI-502	Fermented meat
Lactobacillus	*curvatus*	RI-503	Fermented meat
Lactobacillus	*curvatus*	RI-504	Fermented meat
Lactobacillus	*plantarum*	RI-505	Fermented cacao bean
Lactobacillus	*plantarum*	RI-506	Fermented cacao bean
Lactobacillus	*plantarum*	RI-507	Fermented cacao bean
Lactobacillus	*plantarum*	RI-508	Fermented cacao bean
Lactobacillus	*plantarum*	RI-509	Fermented cacao bean
Lactobacillus	*plantarum*	RI-510	Fermented cacao bean
Lactobacillus	*plantarum*	RI-511	Fermented cacao bean
Lactobacillus	*plantarum*	RI-512	Fermented cacao bean
Lactobacillus	*plantarum*	RI-513	Fermented cacao bean
Lactobacillus	*plantarum*	RI-514	Fermented cacao bean
Lactobacillus	*plantarum*	RI-515	Unknown
Lactobacillus	*plantarum*	RI-516	Fermented meat
Lactobacillus	*plantarum*	RI-517	Fermented meat
Lactobacillus	*plantarum*	NZ7306 *ΔrpoN*	Unknown
Lactobacillus	*plantarum*	WCFS1	Human saliva

Table S5.2 Indicator strains for antibacterial screening

Genus	Species	Strain	Organism
Enterococcus	*faecalis*	DSM 20478^T	bacterium
Enterococcus	*faecium*	SL1.1	bacterium
Listeria	*innocua*	HPB13	bacterium
Listeria	*ivanovii*	DSM 12491T	bacterium
Listeria	*monocytogenes*	ATCC 19114	bacterium
Rhodotorula	*mucilaginosa*	LME	yeast
Candida	*parapsilosis*	4/5-1	yeast

Table S5.3 Cell counts in competition co-culture experiment

Initial concentration in cfu/ml		**Concentration in cfu/ml after co-culture**	
L. plantarum RI-162	*R. mucilaginosa* LME	*L. plantarum* RI-162	*R. mucilaginosa* LME
(1.4±0.1) x 10^7	(8.6±0.6) x 10^5	(6.4±1.7) x 10^8	<1.8 x 10^2
(1.4±0.1) x 10^6	(8.6±0.6) x 10^5	(6.7±1.7) x 10^8	ND
(1.4±0.1) x 10^5	(8.6±0.6) x 10^5	(1.9±0.2) x 10^9	ND
(1.4±0.1) x 10^4	(8.6±0.6) x 10^5	(3.1±0.1) x 10^9	ND
(1.4±0.1) x 10^3	(8.6±0.6) x 10^5	(3.0±0.5) x 10^9	<4.7 x 10^2
(1.4±0.1) x 10^2	(8.6±0.6) x 10^5	(2.9±0.5) x 10^9	<3.1 x 10^2
14	(8.6±0.6) x 10^5	(2.3±0.7) x 10^9	<3.4 x 10^2
1.4	(8.6±0.6) x 10^5	(2.3±0.4) x 10^9	(4.6±3.6) x 10^2
<1	(8.6±0.6) x 10^5	ND	(3.6±0.3) x 10^6

Data from Figure 5.3, AF4 assay has a detection limit of 50 cfu/ml. ND = not detected, ± SD from biological triplicates.

Chapter 6

Clustering of Pan- and Core-Genome of *Lactobacillus* Provides Novel Evolutionary Insights

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Abstract

Background: Bacterial taxonomy aims to classify bacteria based on true evolutionary events. It relies on a polyphasic approach including phenotypic, genotypic and chemotaxonomic parameters. Most studies focus on average nucleotide identity or index based distance of shared genes. The complete genome is ignored in such analyses although evolution occurs on the whole organism.

Results: we clustered 98 complete sequenced genomes of the genus *Lactobacillus* and 234 genomes of 5 different *Lactobacillus* species. i.e. *L. reuteri*, *L. delbrueckii*, *L. plantarum*, *L. rhsmnosus* and *L. helveticus.* The core-genome of the genus *Lactobacillus* contains 266 genes in a pan-genome of 20'800 genes. Clustering of the *Lactobacillus* pan- and core-genome resulted in highly similar trees. This shows that evolutionary history is traceable in the core-genome and that clustering of the core-genome is sufficient to explore relationships. Clustering of core a pan-genome at species' level resulted in similar trees as well. Detailed analyses of the core-genomes showed that the functional class "genetic information processing" is conserved in the core-genome but that "signaling and cellular processes" is not. The latter class encoded functions that direct with the environment. The type species *L. delbrueckii* was analyzed in detail and its pan-genome tree contained two major clades, which contain different genes yet identical function. This shows that convergent evolution appears in lactobacilli. In addition, evidence for horizontal gene transfer between strains of *L. delbrueckii, L. plantarum,* and *L. rhamnosus,* and between species in the genus *Lactobacillus* is presented. Our data provide evidence for evolution of lactobacilli according to the parapatric model for species differentiation.

Conclusions: Core-genome trees are useful to detect evolutionary relationships in lactobacilli. *Lactobacillus* evolution is directed by the environment, convergent evolution and HGT according to the parapatric model.

Background

Sequencing of complete genomes developed within 10 years from research that required a consortium to an effort that a single researcher can manage (Ekblom and Wolf, 2014). Bioinformatics tools and advances in next generation sequencing (NGS) technology developed rapidly, resulting in decreasing costs and increasing speed of complete genomes sequencing. These developments have led to an enormous amount of data of various quality and that is not completely analyzed yet (Goodwin et al., 2016). However, research on diseases or phenotypic variations that targets specific genes needs high quality genome sequences (Ott et al., 2015). Whole genome sequencing (WGS) generates completely assembled chromosome and plasmid sequences. Such genomes are pivotal for complex applications such as like sub-typing *Salmonella enterica* for monitoring outbreaks or calculating a bacterial pan-genome (Leekitcharoenphon et al., 2014; Tettelin et al., 2008). Until now, WGS is only poorly applied in bacterial classification and phylogenetic studies (Vandamme and Peeters, 2014). Evolutionary pressure works; however, on the complete organism and complete genomes are therefore most preferable for phylogenetic studies.

A polyphasic approach is used for bacterial classification and to analyze evolutionary relationships (Colwell, 1970; Murray et al., 1990). Polyphasic approaches are not standardized and include phenotypic, genotypic and chemotaxonomic parameters to determine whether a bacterial isolate belongs to an existing species or if a new species has to be defined (Vandamme et al., 1996; Vandamme and Peeters, 2014). Nowadays, assigning a bacterial strain to a species is based amongst others on two genotypic parameters: sequence similarity of more than 98.7% in the 16S rRNA gene and a DNA-DNA hybridization (DDH) degree of more than 70% (Stackebrandt and Ebers, 2006; Wayne et al., 1987). EcoSNPs are SNPs that are specific for a dimorphic nucleotide position in a clade. EcoSNPs are frequently used to determine relation and build phylogenetic trees such as in MLST approaches and 16S rRNA trees (Shapiro et al., 2012). To compare complete genome sequences to 70% DNA-DNA hybridization levels parameters are defined for conserved DNA regions and maximal unique matches (Deloger et al., 2009; Goris et al., 2007; Richter and Rosselló-Móra, 2009). Additionally, an average nucleotide identity (ANI) value of 94% corresponds to 70% DNA-DNA hybridization and is thus also usable parameter to define species.

The core-genome is the set of homologous genes that are present in all genomes of an analyzed dataset and the pan-genome is the set of all genes that are present in the analyzed dataset (Tettelin et al., 2005). In addition, the softcore-genome is the set of genes, present in ≥95% of the genomes (Kaas et al., 2012). The softcore-genome is useful, because it circumvents the absolute impact of poor quality genomes on the core-genome. An open pan-genome is increasing with every new genome included whereas a closed pan-genome remains on a constant gene number after a certain

number of genomes were included (Lefébure et al., 2010). The status of the core- and pan-genomes depends on number of analyzed genomes, on the ability of the species to integrate exogenous DNA and on the species' lifestyle and environment (Bosi et al., 2016; Georgiades and Raoult, 2011; Medini et al., 2005).

Classification of bacterial taxonomy using the core- and pan-genome might be a powerful extension of the polyphasic approach. In addition, pan-genome clustering of 29 *Geobacillus* genomes revealed horizontal gene transfer as a factor in evolution of *Geobacillus* and such transfer should be implemented in its taxonomy (Bezuidt et al., 2016). Horizontal gene transfer was also detected in a recently diverged *Vibrio* population, where ecological differentiation based on single nucleotide polymorphisms occurred (Shapiro et al., 2012).

The heterogeneous genus *Lactobacillus (L.)* contains 173 species not including 17 subspecies (Goldstein et al., 2015). Lactobacilli have been isolated from a whole range of fermented food products such as yoghurt, cheese, vegetables, wine and beer, sausages and sourdough. Further, lactobacilli are also found in the human and animal tracts (Claesson et al., 2007). The Qualified Presumption of Safety (QPS) status from the European Food Safety Agency (EFSA) facilitates commercial use and acceptance of most *Lactobacillus* species and makes them ideal candidates for the use as protective and starter cultures (EFSA - NDA Panel, 2015). Aside from their preserving qualities, some *Lactobacillus* species are also exploited for their health promoting potential as probiotics and vaccine carrier (Goh and Klaenhammer, 2009; Saito, 2004). In December 2016, a total of 121 completely sequenced and assembled genomes were available in public databases with sizes ranging from 1.37 Mpb for *L. sanfranciscensis* TMW 1.1 to 3.74 Mbp for *L. paracellinoides* TMW 1.1995 (NCBI Resource Coordinators, 2016). *Lactobacillus* and related genera were initially clustered into three subgroups based on 16S RNA gene comparison: the *Lactobacillus delbrueckii* group, the *Lactobacillus casei-Pediococcus* group and the *Leuconostoc* group (Collins et al., 1991; Felis and Dellaglio, 2007). A recent 16S rRNA gene based clustering of the *Lactobacillus* type strains species resulted in a phylogenetic tree with 15 major groups (Salvetti et al., 2012). There is; however, only moderate correlation between 16S rRNA gene sequence clustering and grouping based on fermentation type and metabolic properties.

The goal of this study was to analyze the phylogeny of the *Lactobacillus* genus and a dedicated set of species via core-, softcore- and pan-genome clustering. Such complete genome based clustering provides a detailed overview of gene contents of the core- and pan-genome and will provide insights on relationship of species and their gene exchange.

Material and Methods

Genome sequences

A total of 98 complete sequenced *Lactobacillus* genomes and 202 draft genomes belonging to the species *Lactobacillus plantarum*, *Lactobacillus helveticus*, *Lactobacillus delbrueckii*, *Lactobacillus reuteri* and *Lactobacillus rhamnosus* were obtained from public databases (Table S6.1). To prevent too high impact of poorly assembled genomes for the *Lactobacillus* species calculation, draft genomes were only used if they fell within a range of $\pm 2\sigma$ around the average gene and protein number of the species.

Calculation of core- and pan-genome

Orthologous cluster were created using the Perl script collection GET_HOMOLOGOUS (Contreras-Moreira and Vinuesa, 2013) applying the following for identification and clustering CDS into orthologous groups: *-E* < 1e-05 for blastp searches and *-C* 75% minimum alignment coverage. The core-genome was determined using the Ortho Markov Cluster algorithm (OMCL) (Li et al., 2003) and the pan-genome using the OMCL algorithm with *–t* 0; reporting all clusters in the pan-genome. A pan-genome matrix was created using the script compare_clusters with the settings: *-d* including only OMCL data, *-m* produce intersection in pan-genome matrix.

The core-genome was defined by genes present in all genomes, the softcore by genes present in 95 – 100% of the genomes, the shell by genes present in more than 2 genomes but less than 95% of the genomes and the cloud genes present in 2 or less of the genomes and calculated with the parse_pangenome_matrix script: *-s* report clusters.

The development-calculation of core- and pan-genome starts with comparing two genomes and including single genomes step-by-step until all genomes are integrated. The order of the included genome was randomized n-times (n=number of included genomes) and calculated with a home-made script in MATLAB R2014b based on the pangenome_matrix_t0.

Clustering and analyses of core- and pan-genome

Protein-based clustering was performed with GET_HOMOLOGOUS (Contreras-Moreira and Vinuesa, 2013) using the OMCL algorithm as follows: *-t* 0, *-t* all or *-t* n (n=0.95 x number of included genomes) for clustering the pan-, core- and softcore-genome, *-M;* with the OMCL algorithm and – A; to create an average identity matrix. The created average identity matrix of clustered sequences was visualized using the script hcluster_matrix with the option *–d* gower; for selecting the gower distance calculation for clustering (Gower, 1966). Core- and pan-genome (Table S6.1) were analyzed with the

metagenome analysis tool GhostKOALA against "genus_prokaryotes + family_eukaryotes" database using the Brite, Pathway and Module reconstruction algorithm (Kanehisa et al., 2016). Brite reconstruction uses KEGG Brite hierarchies with combined sets of K numbers. Pathway reconstruction aligns gene to the KEGG pathway map and Module reconstruction uses sets of K numbers to evaluate if a block (pathway or structural complex) is complete. Increase of entries from reconstruction results of core- and pan-genomes were calculated and analyzed with Fisher's exact test with a p-value of 0.01 in MATLAB R2014b.

Identification of clade specific genes.

Identification of clade specific genes in a set of bacterial isolate was performed using the parse_pangenome_matrix script of GET_HOMOLOGOUS (Contreras-Moreira and Vinuesa, 2013) with option; *-A* a list of genomes in one clade; option *–B* a list genomes of another clade to compare against; -g finding genes present in genomes of clade A and absent in genomes of clade B; -e find gene family expansions in A with respect to B. To determine if a gene encodes a unique function in a clade that is not compensated by isoenzymes in the other clade, the core-genome of the clade was compared with the pan-genome of all other clades using GhostKOALA (Kanehisa et al., 2016). The presence of isoenzymes was analyzed for each gene manually.

Identification of representative core genes for classification of the type species *Lactobacillus delbrueckii*

To identify which gene or set of genes represents most closely the pan-genome phylogenetic tree of *L. delbrueckii,* the tree of each core gene was compared to the tree of the pan genome of *L. delbrueckii* using TOPD/FMTS (Puigbò et al., 2007) and CLC Workbench 8 (CLC Genomic, Aarhus, Denmark). Each homologous gene set from the core genome was imported as multi-entry FASTA into CLC Genomic Workbench 8. The genes were as aligned using the "Create alignment" tool using standard parameters. Trees were created with the toolbox "Create Tree" using "Neighbor Joining" as tree construction method and "Junkes-Cantor" as nucleotide distance measure with a Bootstrap value of 100. Trees were exported as nexus files and compared to the pan-genome tree using TOPD/FMTS using the following parameters: *-m* nodal method of calculation; *-n* 10 number of random sequences; *-c* reference comparing all versus pan-genome tree. Identical trees have a nodal distance = 0. The higher the nodal distance is the less identical are the trees. The 5% and 95% percentile was calculated for all core gene nodal distances and the genes outside this range analyzed manually.

Identification of ecoSNPs in *Lactobacillus delbrueckii*

To analyze ecoSNP distribution, core gene alignments were exported as ClustalW files and import into MATLAB R2014b and consensus sequence was calculated. Each gene was compared with the consensus sequence and SNPs were determined and analyzed for its specificity to a clade in the pan-genome of *L. delbrueckii*.

Potential horizontal gene transfer within clades

For identification of potential horizontal gene transfer (HGT) events, genes with a 30 – 70% presence in all clades were selected. An absence-presence matrix for all genes and strains was constructed for each clade in MS Excel and genes within the 30-70% criterion selected.

Results

Calculation of core- and pan-genome of complete *Lactobacillus* genomes

To obtain a general view of *Lactobacillus* genome contents, the core- and pan-genome for 98 completely assembled *Lactobacillus* genomes were calculated. The pan-genome for *Lactobacillus* genus still increases with approximately 50 genes after addition of a 98th genome and thus can be considered as open (Figure 6.1A). The core- genome rapidly decreases with the first set of genomes, but stabilizes after the 70th is added, showing it's closed (Figure 6.1B). The core-genome contained 266 genes and the pan-genome 20'800 genes (Table 6.1). A core-genome based clustering revealed 4 major, mostly multiple-species clades: (A), a *reuteri-fermentum-salivarius* clade, (B), a *plantarum-paraplantarum* clade, (C) a *casei-paracasei-rhamnosus* clade and (D) a *helveticus-delbrueckii-johnsonii* clade (Figure 6.2). The softcore- and pan-genome were also clustered and the 4 clades appeared again as separate clusters and contained the same isolates (Figure 6.3). The highly similar pan and core genome clusters shows that evolutionary relationship appears already in the core genome. In general, species clustered together. However, some strains from the species *L. casei* / *L. paracasei* and *L. helveticus* / *L. gallinarum* did not.

Table 6.1 Size of core- and pan-genome of the genus *Lactobacillus* and the 5 species.

Genus	Species	Number of genomes	Genome size mean ± SD (Mb)	Genes mean ± SD (Mb)	core	softcore	shell	cloud	pan
Lactobacillus	*spp*	98	2.47±0.55	2274±528	266	594	7249	12957	20800
Lactobacillus	*helveticus*	19	2.02±0.13	2050±164	908	1062	1133	1155	3350
Lactobacillus	*reuteri*	25	2.10±0.12	2050±117	897	1306	1364	1290	3960
Lactobacillus	*rhamnosus*	51	2.97±0.08	2788±71	811	1920	1736	1233	4889
Lactobacillus	*plantarum*	122	3.27±0.13	3075±140	1037	2144	2826	2640	7610
Lactobacillus	*delbrueckii*	29	1.88±0.13	1873±93	756	1042	1336	1082	3460

Detailed analysis of strains with deviating cluster behavior

The *L. casei* type strain ATCC 393 did not clusters with other *L. casei* strains, but with the 6 *L. rhamnosus* strains (Figure 6.2 and Figure 6.3). The genome of strain ATCC 393 contains 213 KEGG orthology (KO) assignments that are not present in any other of the 21 a *L. casei*, *L. paracasei* and *L. rhamnosus* genomes. 11 of these 213 KOs are related to carbohydrate metabolism, 7 to environmental information processing and all other to hypothetical functions (Table 6.2). From 27 annotated KOs, 22 describe functions that are present in other *L. casei*, *L. paracasei* and *L. rhamnosus*

isolates but are encoded by isogenes. *L. casei* ATCC 393 contains 5 KOs with a unique function (Table 3). Beside 3 KOs located in metabolic pathways this strains also contains a catalase function (EC 1.11.1.6).

L. zeae was not included in the pan/core-genome analyses because a closed genome is not available for the species. If the incomplete genome of *L. zeae* DSM 20178 is included, its clusters together with *L. casei* ATCC 393 and next to the *rhamnosus* clade (Figure S6.1).

L. gallinarum HFD4 clusters in the core-genome cluster with the 8 *L. helveticus* strains in the *helveticus* clade (Figure 6.2 and Figure S6.2). In the pan-genome; however, it clusters outside of the *helveticus* clade (Figure 6.3). Analyses of the 16S rRNA sequence search of HDF4 revealed over 99% identity with the 16S rRNA sequence of various *L. helveticus* strains. *L. gallinarum* HFD4 contains 181

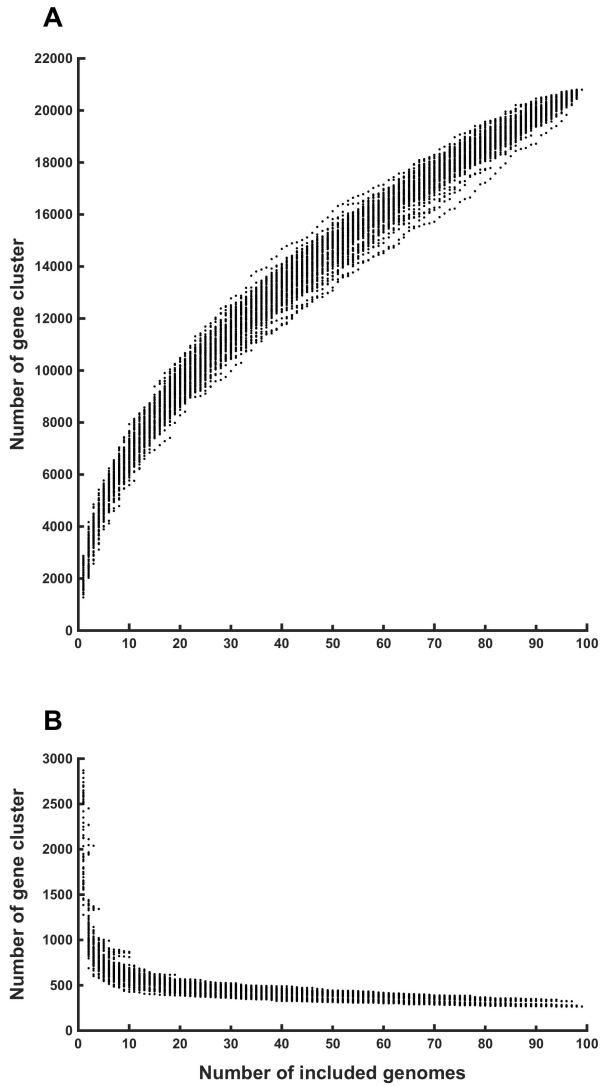

Figure 6.1 Pan- and core-genome evolution of *Lactobacillus.* **A** For every included genome the size of the pan-genome increases. **B** Evolution of core-genome of 98 complete *Lactobacillus* genomes. After 70 genomes, the size of the core-genome is only decreasing by a few genes per included genome. Order of calculation was randomized for 98 sets, each represented with a single point.

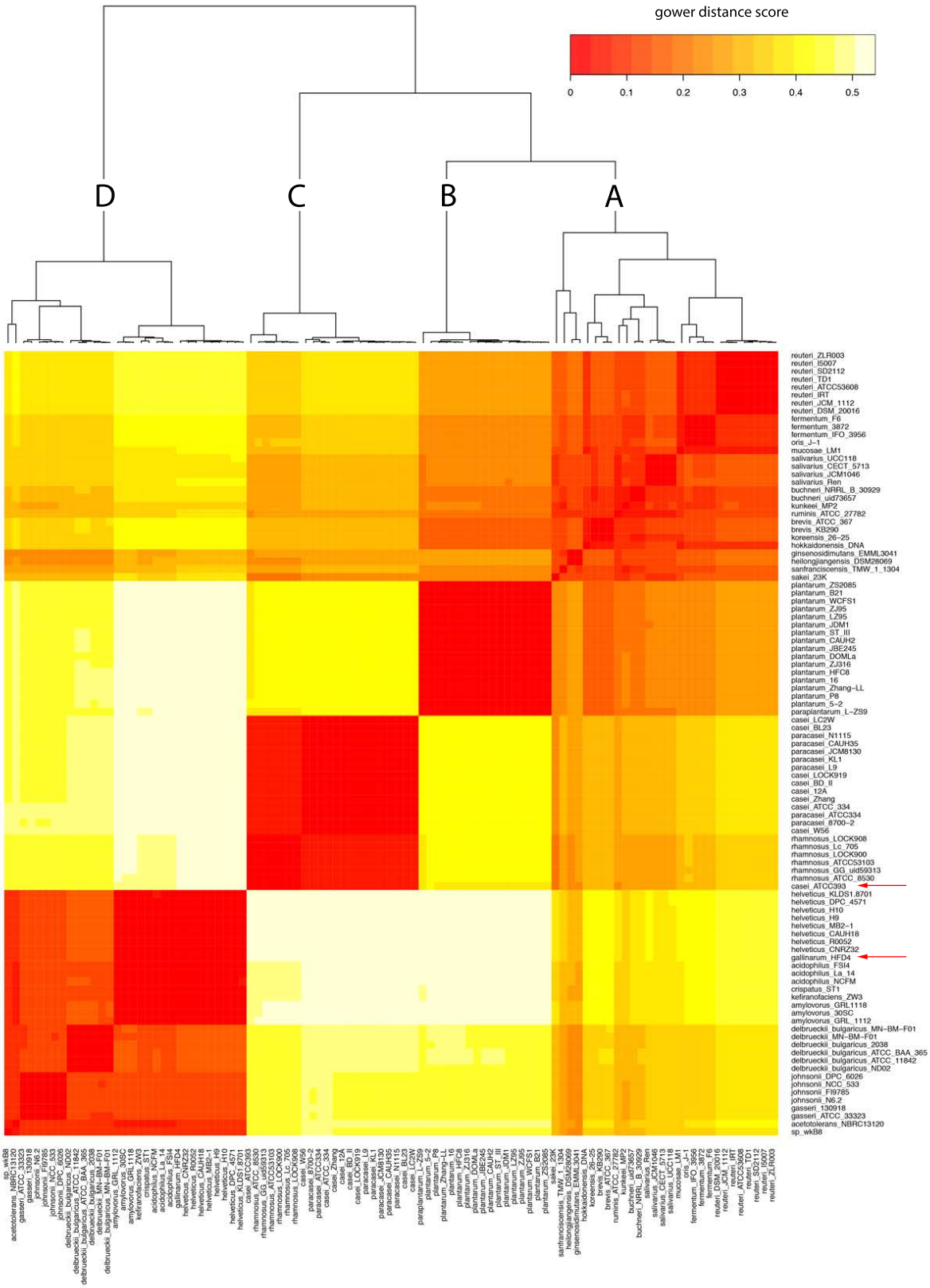

Figure 6.2 Core-genome clustering of genus *Lactobacillus*. Heatmap clustering according to 266 core genes from 98 *Lactobacillus* genomes. Gower distance score based on ANI: Red = more similar, white = less similar, Outlier marked with red arrows. (A) reuteri-fermentum-salivarius clade, (B) plantarum clade, (C) casei-paracasei-rhamnosus clade and (D) helveticus-delbrueckii-johnsonii clade.

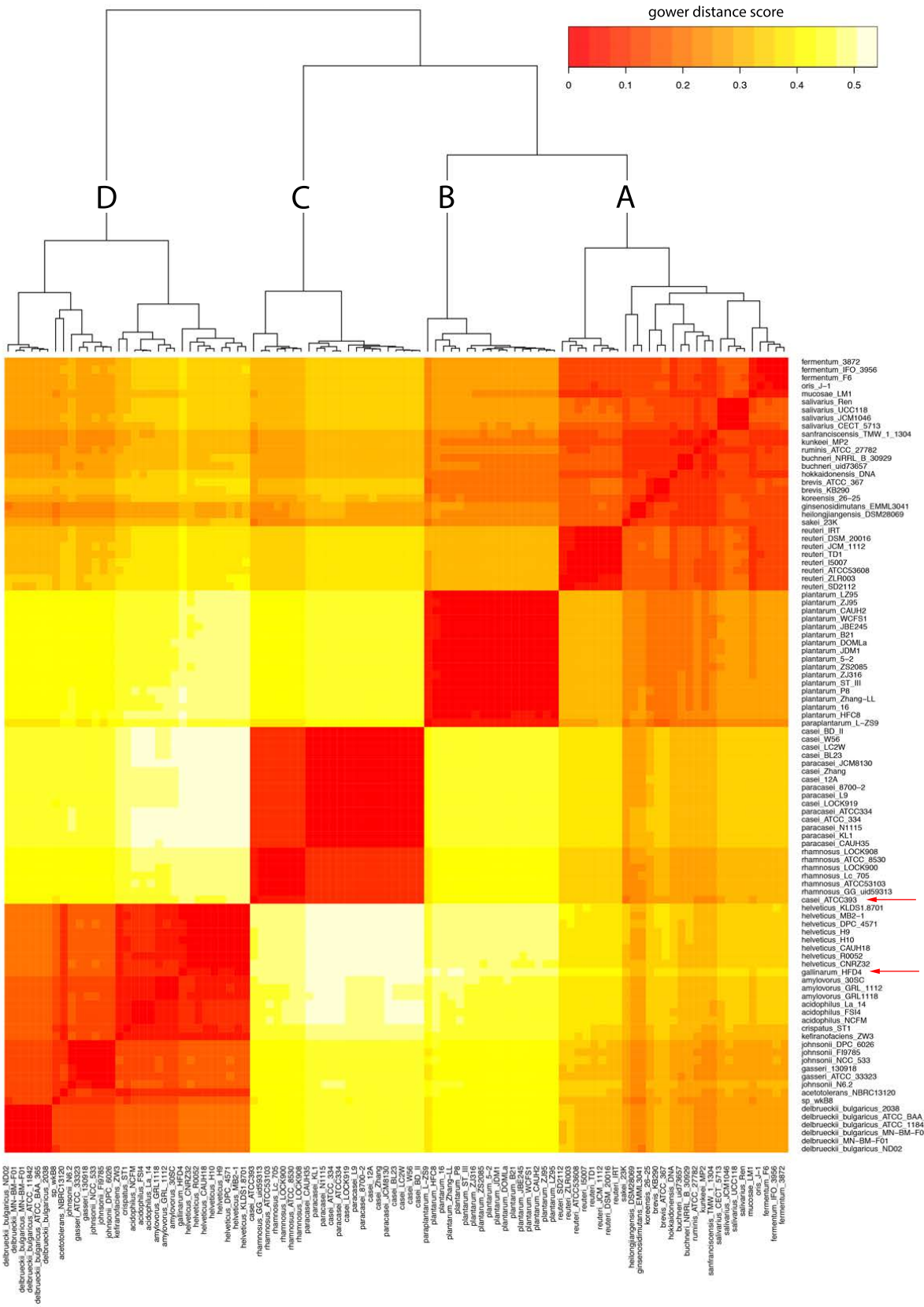

Figure 6.3 Pan- genome clustering of genus *Lactobacillus*. Heatmap clustering according to 20'800 pan-genome genes from 98 *Lactobacillus* genomes. Gower distance score based on ANI: Red = more similar, white = less similar, Outlier marked with red arrows. (A) reuteri-fermentum-salivarius clade, (B) plantarum clade, (C) casei-paracasei-rhamnosus clade and (D) helveticus-delbrueckii-johnsonii clade.

Table 6.2 Unique genes per isolate analyzed with GhostKOALA functional categories.

KEGG orthology	*L. casei* ATCC393	*L. gallinarum* HDF4	bulgaricus clade	diverse clade
Carbohydrate metabolism	11	5	7	1
Energy metabolism	0	0	2	1
Lipid metabolism	0	2	3	0
Nucleotide metabolism	1	0	0	1
Amino acid metabolism	1	3	3	3
Metabolism of other amino acid	1	0	0	0
Glycan biosynthesis and metabolism	2	0	0	0
Metabolism of cofactors and vitamins	0	3	0	1
Metabolism of terpenoids and polyketides	0	1	0	0
Biosynthesis of other secondary metabolites	0	1	0	0
Xenobiotics biodegradation and metabolism	0	0	3	0
Enzyme families	2	1	0	0
Genetic Information Processing	2	11	1	1
Environmental Information Processing	7	9	6	0
Cellular Processes	5	6	3	0
Organismal Systems	1	0	0	0
Human Diseases	1	2	2	0
Unclassified	5	9	0	1
Annotated KEGG onthologys	27	38	12	5
Hypothetical function	186	143	30	5
Query dataset	**213**	**181**	**42**	**10**

KOs that are not present in the 8 *L. helveticus* strains. Beside 135 hypotheticals, 10 KOs are associated with genetic information processing and 9 KOs with environmental information processing. Isolate HFD4 possesses an L-aspartate oxidase, an enzyme that converts L-aspartate to oxaloacetate and a DNA (cytoseine-5)-methyltransferase 1, which catalyses the conversion from L-aspartate-4-semialdehyde to L-homoserine. However, these two KOs do not allow the strain to produce additional amino acids compared to the 8 *L. helveticus* strains. Additionally, isolate HFD4 contains macrolide transport system ATP-binding/permease protein (Table 6.3).

Analysis of core- and pan-genome of the genus *Lactobacillus*

The metabolic capacity of the core- and pan-genome of the genus *Lactobacillus* was analyzed by using Brite protein family enrichment and pathway reconstruction in GhostKOALA. Reconstruction of protein families revealed a 6.1-fold increase from core- to pan-genome. A significant lower increase of 2.8-fold was observed in the class “genetic information processing” from core- to pan-genome and

a significant higher increase of 17.9 -fold in the "signalling and cellular processes" class (Table 6.4). The pathway reconstruction showed similar result with a 7.1-fold increase core- to pan-genome, a significant lower increase of 2.4-fold of genes in "genetic information processing" and a significant higher 24.9-fold increase for "Environmental information processing".

Table 6.3 Unique genes of strains from table 2 with no isoenzymes in the compared pan-genome which would comply the same function. K-number according to KEGG database.

Present in isolate	K-number	EC-number	Function
L. casei ATCC393	K01788	5.1.3.9	N-acylglucosamine-6-phosphate 2-epimerase
	K03781	1.11.1.6	Catalase
	K00681	2.3.2.2	gamma-glutamyltranspeptidase
	K00681	3.4.19.13	glutathione hydrolase
	K20997		polysaccharide biosynthesis protein (pslA)
L. gallinarium HFD4	K00278	1.4.3.16	L-aspartate oxidase (nadB)
	K00558	2.1.1.37	DNA (cytosine-5)-methyltransferase 1
	K03517	2.5.1.72	quinolinate synthase (nadA)
	K18231		Macrolide transporter symstem ATP-binding/permease protein (msrA)
diverse cluster	K00135	1.2.1.16	Succinate semialdehyde dehydrogenase
	K00926	2.7.2.2	carbamate kinase
	K00611	2.1.3.3	ortnithine carbamoyltransferase
	K02970		small subunit ribosomal protein S21

Table 6.4 Reconstruction of core-, softcore- and pan-genome of *Lactobacillus delbrueckii* species with Brite and Pathway algorithm of GhostKOALA.

Brite Reconstruction Result				**n-fold increase**	
	core	**softcore**	**pan**	**core-pan**	**softcore-pan**
Orthologs and modules	237	471	1650	7.0*	3.5*
Protein families: metabolism	180	320	1093	6.1	3.4
Protein families: genetic information processing	165	292	458	2.8*	1.6*
Protein families: signaling and cellular processes	27	73	484	17.9*	6.6*
Total	**609**	**1156**	**3685**	**6.1**	**3.2**

Pathway Reconstruction Result				**n-fold increase**	
	core	**softcore**	**pan**	**core to pan**	**softcore-pan**
Metabolism	303	502	2298	7.6*	4.6*
Genetic Information Processing	84	156	199	2.4*	1.3*
Environmental Information Processing	10	28	249	24.9*	8.9*
Cellular Processes	11	20	135	12.3	6.8
Organismal Systems	8	9	83	10.4	9.2
Human Diseases	18	33	109	6.1	3.3
Total	**434**	**748**	**3073**	**7.1**	**4.1**

* indicates p-value < 0.01

Core- and pan-genome of the type species *Lactobacillus delbrueckii*

To gain insight in the core- and pan-genome of a *Lactobacillus* species, similar analyses were performed with the type species of the genus *Lactobacillus delbrueckii.* The *L. delbrueckii* core-genome contained 756 genes, the softcore-genome 1042 genes and the pan-genome 3460 genes, with an average genome size of 1873±93 genes (Table 6.1). After 26 included genomes the pan-genome of *L. delbrueckii* is gaining only 4-5 genes per genome and can be considered as closed (Figure 6.4).

If *L. delbrueckii* MN-BM-F01, formerly *L. acidophilus* MN-BM-F01 (Yang et al., 2016), was excluded from the analyses, the core-genome increased only by 4 genes. This supports strongly the new classification of *L. delbrueckii* MN-BM-F01.

The quality criterion for genomes was set that the gene number should be within a range of $\pm 2\sigma$ around the average gene and protein number. If genomes that do not match this criterion were included, e.g. the genomes of *L. delbrueckii* JCM1002, *L. delbrueckii* JCM1012 and *L. delbrueckii* CRL871, the core-genome dropped dramatically from 756 to 302 core genes, showing clearly the sensibility of the core genome for low quality sequenced genomes.

The 29 *L. delbrueckii* strains are separated in 2 clades in both softcore- and pan-genome (Figure 6.5). In the core-genome a small third clade containing the 3 strains PB2003_004-T3-4, ND02 and JCM17838 occurs. One clade in the pan-genome contains 13 strains that belong all to *Lactobacillus delbrueckii* subsp. *bulgaricus* and was therefore designated "*bulgaricus*" clade. The second clade, contains 16 isolates of the subspecies *Lactobacillus delbrueckii* subsp. *delbrueckii*, *-lactis*, *-indicus*, *-sunkii, -jakobsenii* and *-bulgaricus*, was designated "diverse" clade.

The average ANI over all *L. delbrueckii* genomes was 96.58±0.93%. The average ANI in the *bulgaricus* clade was 98.05±0.23% and in the diverse clade 96.23±0.93%.

Core-genomes of both clades were constructed and the core genes were categorized with GhostKOALA. The *bulgaricus* clade core-genome contains 42 KO that are not found in the diverse core-genome, of which 30 are hypothetical KOs. The 12 functionally annotated KOs are associated with carbon metabolism and environmental information processing (Table 6.2), including a complete sucrose-specific type II PTS system. There were, however, no functional differences between the two clades. This shows that evolutionary distinct genes with identical functions were acquired by the strains in the clades, a process known as convergent evolution occurred.

The diverse clade core-genome contains 10 KOs that are not present in the *bulgaricus* clade. Of these KOs, 5 encoded for hypothetical KOs, 3 for amino acid metabolism KOs and one small subunit

ribosomal protein S21 (Table 6.3). An α-glucoside transport system is uniquely present in the diverse cluster. This ABC transporter transports, amongst others, maltose.

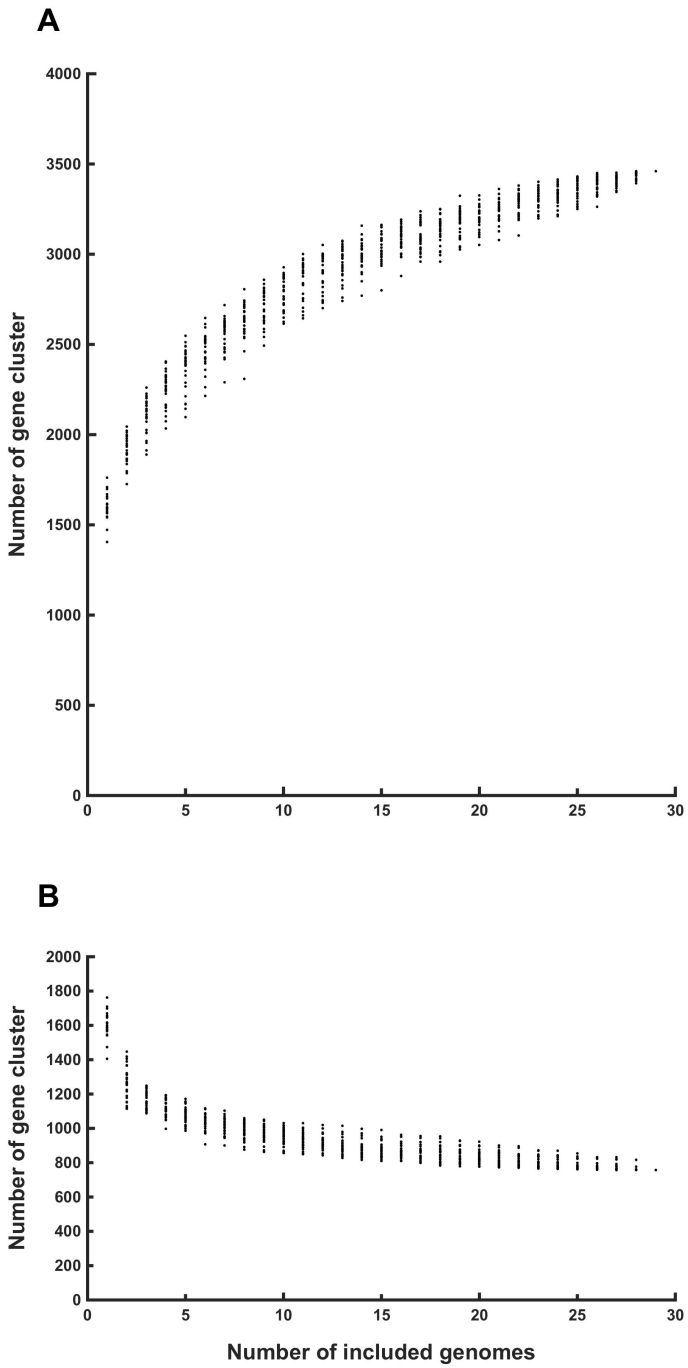

Figure 6.4 Pan- and core-genome evolution of *Lactobacillus delbrueckii*. **A** Evolution of the pan-genome for *L. delbrueckii*. After 20 included genomes, the pan-genome is closed. **B** Evolution of the core-genome for *L. delbrueckii*. Order of calculation was randomized for 29 sets, each represented with a single point.

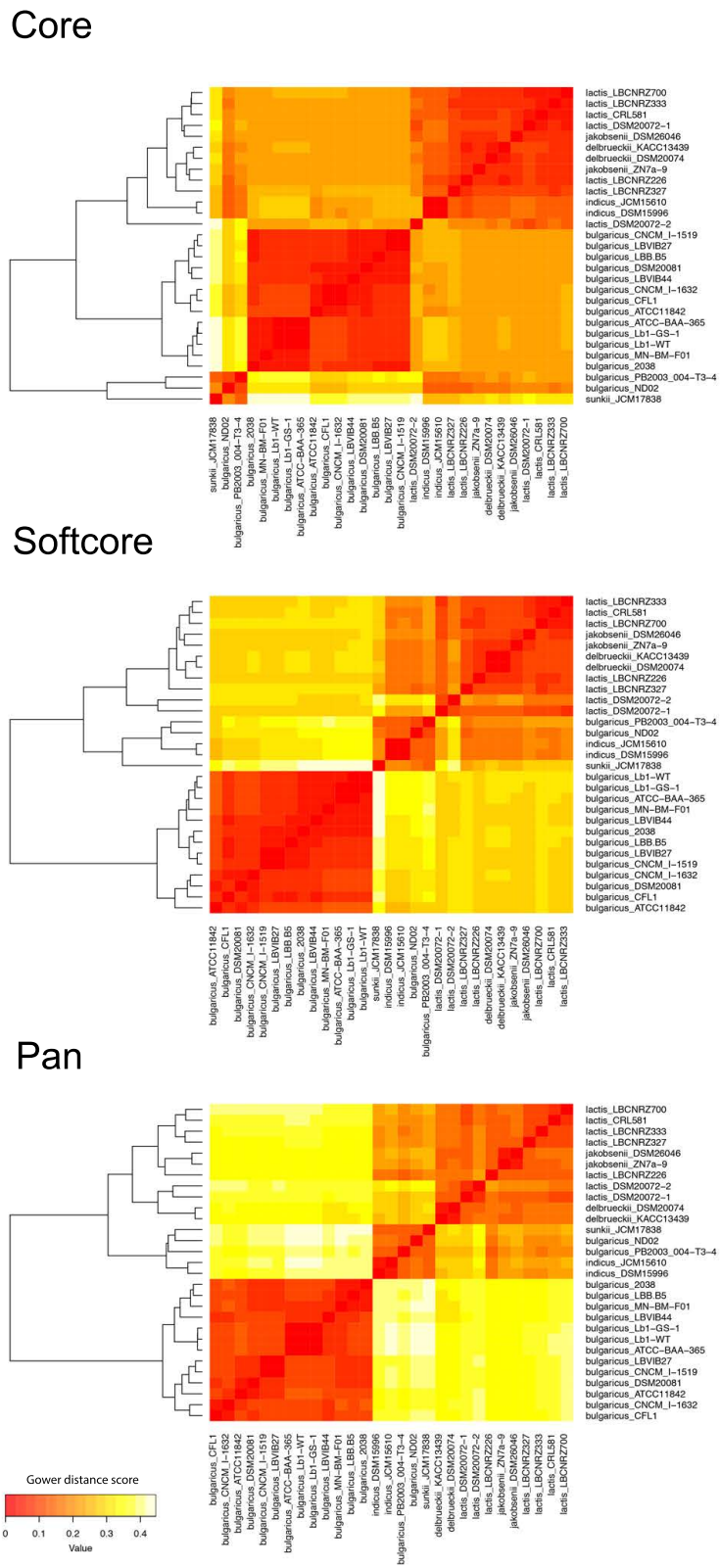

Figure 6.5 Heatmap of core-, softcore- and pan-genome for *L. delbrueckii.* In the core-genome heatmap the *bulgaricus* clade is embedded within the diverse clade. In the softcore- and pan-genome heatmap is the *bulgaricus* and the diverse clade are separated. Gower distance score based on ANI: Red = more similar, white = less similar.

Analysis of core- and pan-genome of *Lactobacillus delbrueckii*

The metabolic capacity of the core- and pan-genome of *L. delbrueckii* was analyzed using protein family enrichment and pathway reconstruction in GhostKOALA. An increase of 1.8-fold from core- to pan-genome was measured with a significant lower increase of 1.4-fold in the class "genetic information processing" from core- to pan-genome and a significant 2.6 -fold increase in the "signaling and cellular processes" class (Table S6.2). In the pathway reconstruction, a significant lower increase of genes in "genetic information processing" and a higher increase for "Environmental information processing" was measured. These findings parallel the previous analysis the core- and pan-genome of the genus *Lactobacillus* (Table 6.4).

The reconstruction according to manually defined functional units, KEGG modules, revealed that short pathways are completely present in the core-genome whereas longer pathways frequently 1 or 2 enzymes (Table S6.3). However, many of such longer pathways such as the glycolysis, purine ribonucleotide biosynthesis, RNA polymerase, aminoacyl-tRNA biosynthesis and the ribosome protein complex are complete in the softcore. Taken together, fundamental processes in the cell are conserved in the softcore-genome and processes involved in interactions with the environment are only complete in the pan-genome.

Analyses of core-genes of *L. delbrueckii*

To determine which SNPs in the core genes are responsible for the occurrence of the clades in the core-genome matrix, the consensus sequence for all 756 genes in the *L. delbrueckii* core-genome was calculated and SNPs in all 29 strains analysed. In total, 53'583 SNP were detected in all core genes. However, no cluster specific SNPs were detected, showing that the formation of 2 clades in the clustering is not dependent on a small set of SNPs.

To analyse if all genes in the core genome had a similar phylogenetic tree as the strains, the tree of every gene in the core-genome was compared to the tree from the pan-genome (Figure 6.5). The 38 genes (5%) with trees most similar to the strain tree had an average nodal distance score of 2.10±0.13 and an average gene size of 1424 bp. The consensus sequences of the genes had an SNP density of 75.3 SNPs/kb. Of these 38 genes, 9 genes were interacting with DNA or RNA and there were no hypothetical genes (Table S6.4). The genes least similar to the strain tree had an average nodal distance score of 6.47±0.78, an average gene size of 407 bp. Of the 38 genes, 16 are either annotated as 30S or 50S ribosomal proteins. The consensus sequences of the 38 genes had an SNP density of 26.54 SNPs/kb, a density that is clearly lower than the average SNP density of 70.25 SNPs/kb. The 38 genes are thus highly homologous. This shows that highly conserved genes have a

different phylogenetic tree than moderate conserved genes and such genes are not useful for phylogenetic reconstruction at species level.

Potential HGT in *Lactobacillus delbrueckii*

To detect whether gene transfer appeared between the two clades in *L. delbrueckii*, we screened for potential HGT-genes within the two clades. In the *L. delbrueckii* pan-genome, a total of 57 genes were detected that were present in a subset of strains in both clades and are therefore potentially involved in HGT. 42 of those 57 genes were encode for hypothetical proteins or are associated with phages or transposons (Table S6.5). Phages and transposons are commonly associated with HGT and their occurrence shows that our simple algorithm can detect HGT related genes.

Core- and pan-genome of other *Lactobacillus* species

To determine if the type species *L. delbrueckii* is representative for other *Lactobacillus* species, we calculated the core- and pan-genome for four other species selected from each of the four clades observed in the core-genome clustering (Figure 6.1); *L. helveticus*, *L. rhamnosus*, *L. reuteri* and *L. plantarum*. *L. helveticus* has a core-genome of 908 and pan-genome of 3350 genes with an average genome size of 2050±164 genes (Table 6.1). A similar ratio of core-genome to average genome size was calculated for *L. reuteri* with 897 core genes and 3960 pan genes on an average genome size of 2050±117 genes (Table 6.1). A lower ratio of core-genome to average genome size was calculated for *L. rhamnosus* with 811 core genes and 4889 pan genes on an average genome size of 2788±71 genes (Table 6.1). The core- and pan-genome for those three species are all closed (Figure S6.3 – S6.5). The biggest core-genome was calculated for the species *L. plantarum* with 1037 core-genes which is around 34% of the average genes in a *L. plantarum* genome (Table 6.1). Neither the core- nor the pan-genome of *L. plantarum* were closed even after 122 genomes were included (Figure S6.6).

Clustering of core- and pan-genome of other *Lactobacillus species*

L. rhamnosus and *L. plantarum* clustered in two clades (Figure S6.7 – S6.8), *L. reuteri* and *L. helveticus* in a number of minor clades (Figure S6.9 – S6.10). The smaller *L plantarum* clade contained the type strain *L. plantarum* subsp. *argentoratensis* DSM 16365 and was designated the *argentoratensis* clade whereas the bigger clade contained the type strain *L. plantarum* subsp. *plantarum* ATCC 14917 and was designated the *plantarum* clade.

Potential HGT in other *Lactobacillus* species

The species *L. plantarum* and *L. rhamnosus* cluster in 2 clearly separated clades and were used for HGT analyses. *L. plantarum* and *L. rhamnosus* possess 95 and 38 potential HGT genes in their pan-genome, respectively (Table 6.5). The majority of those genes encode hypothetical proteins. In *L. helveticus* only one gene was detected, a transposase, and none in *L. reuteri.*

Table 6.5 Gene annotation for potential HGT genes in *Lactobacillus* species.

			Genes related to			
Organism	**Clades**	**Mobile elements**	**phage**	**transposon**	**hypothetical**	**others**
L. delbrueckii	2	57	0	5	26	26
L. helveticus	3	1	0	1	0	0
L. plantarum	2	95	5	2	44	44
L. reuteri	2	0	0	0	0	0
L. rhamnosus	2	38	6	5	11	16

HGT between clades on genus level

Since we detected in four out of five analysed species potential genes related to HGT, also the pan-genome of *Lactobacillus* genus was analysed for HGT. From 20'800 pan genes for the genus *Lactobacillus* only 2 genes occur in all 4 clades with a probability of 30 – 70% (Table 6.6). Gene 1 is a type I restriction-modification system subunit M (ID= YP_004888889 in *L. plantarum* WCFS1) with a length of 539 aa. Gene 2 is a putative cell division protein (ADY84228 in *L. delbrueckii* 2038) with a length of 659 aa. Therefore, HGT occurs even between species.

Table 6.6 Gene annotation of potential HGT genes in the genus *Lactobacillus*. Mobile elements within clades are marked in green.

Unique identifyer	Presence in clade D	Presence in clade C	Presence in clade B	Presence in clade A	Mobile in all	Mobile in D, C and A	Mobile in D, B and A	Mobile in D, C and B	Mobile in C, B and A	Function
725_type_I_restriction-m...faa	0.32	0.59	0.35	0.55	YES	YES	YES	YES	YES	type I restr.-mod. system subunit M
222713_hypothetical_protein.faa	0.42	0.55	0.53	0.41	YES	YES	YES	YES	YES	putative cell divison protein
31_transposase.faa	0.35	0.55	0.06	0.48	NO	YES	NO	NO	NO	transposase
727_hypothetical_protein.faa	0.32	0.36	0.29	0.38	NO	YES	NO	NO	NO	hypothetical protein
2478_hydrophobic_protein.faa	0.39	0.59	0.06	0.31	NO	YES	NO	NO	NO	hypothetical protein
2798_transcriptional_regu...faa	0.32	0.68	1.00	0.41	NO	YES	NO	NO	NO	hydrophobic protein
3031_DNA-binding_response...faa	0.39	0.55	1.00	0.41	NO	YES	NO	NO	NO	DNA-binding response regulator
3032_two-component_sensor...faa	0.39	0.55	0.94	0.41	NO	YES	NO	NO	NO	two-component sensor histidine kinase
5428_MarR_family_transcri...faa	0.39	0.32	1.00	0.48	NO	YES	NO	NO	NO	MarR familify transcriction regulator
9953_Lj965_prophage_repre...faa	0.39	0.68	0.18	0.31	NO	YES	NO	NO	NO	phage related protein
222000_rpmG.faa	0.65	0.45	1.00	0.55	NO	YES	NO	NO	NO	50S ribosomal protein L33
222041_lmrA.faa	0.32	0.68	1.00	0.59	NO	YES	NO	NO	NO	ABC transporter permease
54_transcriptional_regu...faa	0.58	0.27	0.35	0.34	NO	NO	YES	NO	NO	transcription regulator
728_restriction_endonucl...faa	0.39	0.77	0.59	0.55	NO	NO	YES	NO	NO	type I site-specific restr.-mod. system R
1590_glycosyl_transferase.faa	0.42	0.18	0.35	0.41	NO	NO	YES	NO	NO	glycosyl transferase
222568_hypothetical_protein.faa	0.48	0.14	0.47	0.31	NO	NO	YES	NO	NO	hypothetical protein
222729_hypothetical_protein.faa	0.35	0.05	0.53	0.41	NO	NO	YES	NO	NO	hypothetical protein
222774_hypothetical_protein.faa	0.65	0.00	0.53	0.52	NO	NO	YES	NO	NO	hypothetical protein
2432_Trp_operon_repressor.faa	0.58	0.68	0.47	0.79	NO	NO	NO	YES	NO	flavodoxin
2550_macrolide_transporte...faa	0.35	0.68	0.65	0.17	NO	NO	NO	YES	NO	major facilitator superfamily permease
222722_glf.faa	0.52	0.32	0.59	0.79	NO	NO	NO	YES	NO	UDP galactopyranose mutase
18529_hypothetical_protein.faa	0.00	0.68	0.59	0.34	NO	NO	NO	NO	YES	hypothetical protein
20678_prophage_pi1_protein...faa	0.06	0.55	0.65	0.38	NO	NO	NO	NO	YES	phage related protein

Discussion

We clustered 98 complete sequenced genomes of 32 species of the genus *Lactobacillus* and calculated core- and pan-genome. The core-genome contained 266 genes. A core-genome of 175 *Lactobacillus* isolates and 26 strains from 8 *Lactobacillus*- calculated with similar parameters presented a core-genome of only 73 genes (Sun et al., 2015). The lower amount of core genes in the latter study might be due to the higher number of genomes in the dataset, the integration of genomes from other genera, and including draft genomes in the analysis (Lefébure et al., 2010). Especially incomplete or poorly assembled genomes have a large impact on the core-genome, as shown for core-genome of *L. delbrueckii* in this work. Since the core-genome is very sensitive to heterogeneous datasets and low sequence quality, a prior quality selection is necessary (Mendes-Soares et al., 2014; Tettelin et al., 2005). The minimum standards for submitting a prokaryotic genome to Genbank are, amongst others, at least one copy of 5S, 16S and 23S rRNA-operon, a tRNA gene for each amino acid, and a ratio of genes to genome length close to 1 (NCBI Genome Annotation Coordinators, 2017). However, we showed that those standards not restrictive enough for core-genome analysis and an additional selection of 2-fold the standard deviation of genes number was therefore used.

Another study using closed genomes revealed that the core-genome of 67 *Lactobacillus* strains from 25 species contained 311 genes (Mendes-Soares et al., 2014). The core-genome of *Lactobacillus* in our study was however, not closed after 67 genomes and between 290 and 406 genes (Figure 6.1). The difference in the core-genomes is likely due to the lower number of genomes in the previous study. The pan-genome of the *Lactobacillus* genus based on 67 strains contained 11'047 genes, clearly less than the pan-genome calculated in this study: 16148 – 18318 genes for 67 genomes and 20'800 genes for 98 genomes. The larger pan-genome in our study is likely due to the more heterogenic dataset containing 32 species. Remarkably, the pan-genome of *Lactobacillus* is 4 times larger than the combined pan-genome of the narrow range genera *Staphylococcus* and *Macrococcus*. This exemplifies the wide habitat range and versatility of the *Lactobacillus* genus compared to *Staphylococcus* and *Macrococcus* (Mendes-Soares et al., 2014; Suzuki et al., 2012). Moreover, the pan-genome of *Lactobacillus* was not closed after 98 genomes (Figure 6.1). A closed pan-genome is rapidly reached in species that occur in a few habitats only or have a low capacity to acquire genes, such as *Bacillus anthracis* (Rouli et al., 2014), and in this study *L. delbrueckii.* Non-closed pan-genomes are typical for heterogeneous datasets, like the *Lactobacillus* dataset in this study, for species with diverse habitats, like *L. plantarum,* and in species with high acquisition of genes, such as natural competent streptococci (Bosi et al., 2016; Tettelin et al., 2005). Further, HGT occurs in

lactobacilli, which parallels observations in another genus frequently associated with the human gut: Bifidobacteria (Vazquez-Gutierrez et al., 2017).

We analyzed the *Lactobacillus* type species *L. delbrueckii,* and the species *L. helveticus, L. reuteri, L. rhamnosus* and *L. plantarum* in more detail. The relative core-genome to average genome size was similar for all 5 species. In general, species with more genomes included in pan-genome analyses and higher genomic diversity, such as *L. plantarum,* have a lower core-genome than species with less included genomes and a lower genomic diversity such as *L. delbrueckii* (Siezen et al., 2010; Song et al., 2016). A previous study calculated a core-genome of 2164 genes for 40 *L. rhamnosus* genomes (Ceapa et al., 2016) which is lower than the 811 genes in our core-genome, yet close to soft-core genome of 1920 genes from 51 isolates (Table 6.1). Other studies revealed a *L. plantarum* core-genome of 1957 genes from 54 genomes and for *L. rhamnosus* a core-genome of 2419 genes from 100 included genomes (Douillard et al., 2013; Martino et al., 2016), again much higher compared to the core-genomes in this study (Table 6.1). These core-genomes were however, based on conserved function and not on sequence identity. The homologous based comparison in this study is however preferable, because it is based on true evolutionary events and are not affected by convergent evolution. The impact of convergent evolution was illustrated by the two clades in the core-genome of *L. delbrueckii*. The clades are clearly different from evolutionary view, but possess identical functional capacity (Table 6.3).

Analysis of the core- and pan-genome content revealed that fundamental processes like processing of genetic information and key metabolic pathways were conserved in the core-genome of *L. delbrueckii*, whereas environmental genes were not. These results are similar with compositions found in *S. aureus* (Bosi et al., 2016) and *P. aeruginosa* (Ozer et al., 2014), and parallels previous finding in lactobacilli (Mendes-Soares et al., 2014).

In general, the clustering of core- and pan-genome resulted in highly similar trees. Since the core genome contains the same genes for all isolates, the clustering has to be based on information in these sequences. The strains of *Lactobacillus* clustered in species specific clusters (Figure 6.1), with the exception of two strains: *L. casei* ATCC 393 and *L. gallinarum* HFD4. Differences of type strain *L. casei* ATCC 393 with other strains of *L. casei* are well documented (Acedo-Félix and Pérez-Martínez, 2003; Chen et al., 2000; Collins et al., 1989; Dellaglio et al., 1991, 1975; Dicks et al., 1996; Felis et al., 2001; Ferrero et al., 1996; Mills and Lessel, 1973; Mori et al., 1997). The clustering in this study shows that ATCC 393 is most closely related to *L. zeae* DSM 20178 (Figure S6.1), which confirms previous studies (Dicks et al., 1996; Toh et al., 2013). However, a reclassification of type strain ATCC 393 as *L. zeae* was rejected by the Judicial Commission of the International Committee on Systematics of Bacteria (Tindall, 2008). Strain *L. gallinarum* HFD4 clustered different in core- and pan-genome clustering (Figure 6.2 and Figure 6.3). Genotypic differentiation for *L. gallinarum* and *L.*

helveticus based on 16S rRNA sequence is not evident (Jebava et al., 2014). Initially, *L. gallinarum* and *L. helveticus* were differentiated based on their sugar fermentation pattern; *L. gallinarum* ferments amygdalin, cellobiose, salicin and sucrose, *L. helveticus* not (Hammes and Hertel, 2009). However, none of the 181 KOs uniquely present in HDF4 encodes for any of these carbon sources and the phylogenetic differentiation between *L. helveticus* and *L. gallinarum* remains therefore unclear.

A separation in subspecies in the clustering of *L. delbrueckii* was already detected in a previous study based on MLST (Tanigawa and Watanabe, 2011). The separation is also visible in the ANI values within the clades, which were higher in the subspecies *bulgaricus* clade was than in the mixed subspecies clade. Nevertheless, those ANI values were still above the cutoff value of 94% for different species (Konstantinidis and Tiedje, 2005) and all the analyses strains belong therefore to the same species.

Separation of populations into groups and further to species has been explained with several models. The infinitely many genes (IMG) model relates evolution and separation to all non-core-genome genes (Baumdicker et al., 2012). Since the *bulgaricus* clade separation already appears in the core-genome, the IMG model does not fit the evolution of *L. delbrueckii.* The ecotype model relates a mutation, identifiable as an ecoSNP, within a population to evolve into two subpopulations (Cohan and Perry, 2007; Fraser et al., 2009). EcoSNPs were not found in the *L. delbrueckii* analyses However, ecoSNPs are only visible in recently diverged populations (Shapiro et al., 2012) whereas the division of *L. delbrueckii* into subspecies might not be recent. Convergent evolution was suggested in the genus *Lactobacillus* (Makarova and Koonin, 2007), and we showed it here for *L. delbrueckii*. In addition, gene exchange between the *L. delbrueckii* subpopulations occurred.

The enrichment of environmental function in the accessory genome suggests that a *L. delbrueckii* population occupies a novel niche and then adapts via gene gain. Other populations can undergo same adaptation but via different genes, i.e. convergent evolution. The distribution of homologous genes shows that horizontal gene transfer between the populations is still possible. *L. delbrueckii* evolved therefore into subspecies according to the parapatric model: a novel niche is occupied by a subpopulation. These subpopulations differentiate, but gene exchange is still possible. The detection of HGT in *L. plantarum,* and *L. rhamnosus,* suggests they evolve similarly. Remarkably, *L. plantarum,* and *L. rhamnosus* were both considered as nomadic in a recent study (Duar et al., 2017) and such lifestyle provides opportunities for parapatric specification. *L. reuteri* and *L. helveticus* were not considered as nomadic (Duar et al., 2017) and indeed no evidence for parapatric differentiation in these species was found in our analyses.

Conclusion

The sequenced based core- and pan-genome analyses of *Lactobacillus* and are useful to cluster and classify lactobacilli. The core- and pan-genome clustering yield similar trees. However, core-genomes clustering does not respect environmental adaptations, convergent evolution or horizontal gene transfer. Pan-genome clustering was therefore necessary to show that *L. delbrueckii* evolved into subspecies via a sympatric model.

Supporting information

Table S6.1 All strains used for core- and pan-genome analysis study in chapter 6

Accession	Genus	species	subspecies	strain	sequence quality
AP014808	*Lactobacillus*	*acetotolerans*		NBRC 13120	complete
CP010432	*Lactobacillus*	*acidophilus*		FSI4	complete
NC_021181	*Lactobacillus*	*acidophilus*		La-14	complete
NC_006814	*Lactobacillus*	*acidophilus*		NCFM	complete
CP002559	*Lactobacillus*	*amylovorus*		30SC	complete
NC_014724	*Lactobacillus*	*amylovorus*		GRL 1112	complete
NC_017470	*Lactobacillus*	*amylovorus*		GRL 1118	complete
NC_008497	*Lactobacillus*	*brevis*		ATCC 367	complete
NC_020819	*Lactobacillus*	*brevis*		KB290	complete
NC_018610	*Lactobacillus*	*buchneri*		CD034	complete
NC_015428	*Lactobacillus*	*buchneri*		NRRL B-30929	complete
CP006690	*Lactobacillus*	*casei*		12A	complete
NC_008526	*Lactobacillus*	*casei*		ATCC 334	complete
AP012544	*Lactobacillus*	*casei*		ATCC 393	complete
NC_017474	*Lactobacillus*	*casei*		BD-II	complete
NC_010999	*Lactobacillus*	*casei*		BL23	complete
NC_017473	*Lactobacillus*	*casei*		LC2W	complete
NC_021721	*Lactobacillus*	*casei*		LOCK919	complete
NC_018641	*Lactobacillus*	*casei*		W56	complete
NC_014334	*Lactobacillus*	*casei*		Zhang	complete
NC_014106	*Lactobacillus*	*crispatus*		ST1	complete
NC_017469	*Lactobacillus*	*delbrueckii*	*bulgaricus*	2038	complete
NC_008054	*Lactobacillus*	*delbrueckii*	*bulgaricus*	ATCC 11842	complete
NC_008529	*Lactobacillus*	*delbrueckii*	*bulgaricus*	ATCC BAA-365	complete
CZPS00000000	*Lactobacillus*	*delbrueckii*	*bulgaricus*	CFL1	not complete
AGHW00000000	*Lactobacillus*	*delbrueckii*	*bulgaricus*	CNCM I-1519	not complete
AGFO00000000	*Lactobacillus*	*delbrueckii*	*bulgaricus*	CNCM I-1632	not complete
JQAV00000000	*Lactobacillus*	*delbrueckii*	*bulgaricus*	DSM 20081	not complete
LVWY00000000	*Lactobacillus*	*delbrueckii*	*bulgaricus*	Lb1-GS-1	not complete
LVWX00000000	*Lactobacillus*	*delbrueckii*	*bulgaricus*	Lb1-WT	not complete
LUGK00000000	*Lactobacillus*	*delbrueckii*	*bulgaricus*	LBB.B5	not complete
CCET00000000	*Lactobacillus*	*delbrueckii*	*bulgaricus*	LBVIB27	not complete
CCEU00000000	*Lactobacillus*	*delbrueckii*	*bulgaricus*	LBVIB44	not complete
CP013610	*Lactobacillus*	*delbrueckii*	*bulgaricus*	MN-BM-F01	complete
NC_014727	*Lactobacillus*	*delbrueckii*	*bulgaricus*	ND02	complete
AEAT00000000	*Lactobacillus*	*delbrueckii*	*bulgaricus*	PB2003/004-T3-4	not complete
AZCR00000000	*Lactobacillus*	*delbrueckii*	*delbrueckii*	DSM 20074	not complete
BALP00000000	*Lactobacillus*	*delbrueckii*	*delbrueckii*	JCM 1012	not complete
LHPL00000000	*Lactobacillus*	*delbrueckii*	*delbrueckii*	KACC 13439	not complete
AZFL00000000	*Lactobacillus*	*delbrueckii*	*indicus*	DSM 15996	not complete
LGAS00000000	*Lactobacillus*	*delbrueckii*	*indicus*	JCM 15610	not complete
JQCG00000000	*Lactobacillus*	*delbrueckii*	*jakobsenii*	DSM 26046	not complete
ALPY00000000	*Lactobacillus*	*delbrueckii*	*jakobsenii*	ZN7a-9	not complete
ATBQ00000000	*Lactobacillus*	*delbrueckii*	*lactis*	CRL581	not complete
AEXU00000000	*Lactobacillus*	*delbrueckii*	*lactis*	DSM 20072-1	not complete
AZDE00000000	*Lactobacillus*	*delbrueckii*	*lactis*	DSM 20072-2	not complete
CCDT00000000	*Lactobacillus*	*delbrueckii*	*lactis*	LBCNRZ226	not complete

CCDV00000000	*Lactobacillus*	*delbrueckii*	*lactis*	LBCNRZ327	not complete
CCDS00000000	*Lactobacillus*	*delbrueckii*	*lactis*	LBCNRZ333	not complete
CCDU00000000	*Lactobacillus*	*delbrueckii*	*lactis*	LBCNRZ700	not complete
LGHR00000000	*Lactobacillus*	*delbrueckii*	*sunkii*	JCM 17838	not complete
CP011536	*Lactobacillus*	*fermentum*		3872	complete
NC_021235	*Lactobacillus*	*fermentum*		F-6	complete
NC_010610	*Lactobacillus*	*fermentum*		IFO 3956	complete
CP012890	*Lactobacillus*	*gallinarum*		HFD4	complete
CP006809	*Lactobacillus*	*gasseri*		130918	complete
NC_008530	*Lactobacillus*	*gasseri*		ATCC 33323	complete
CP012034	*Lactobacillus*	*ginsenosidimutans*		EMML 3041	complete
CP012559	*Lactobacillus*	*heilongjiangensis*		DSM 28069	complete
JRQG00000000	*Lactobacillus*	*helveticus*		ATCC 10386	not complete
CP012381	*Lactobacillus*	*helveticus*		CAUH18	complete
AZEK00000000	*Lactobacillus*	*helveticus*		CGMCC 1.1877	not complete
CBUN000000000	*Lactobacillus*	*helveticus*		CIRM-BIA 101	not complete
CBUL010000000	*Lactobacillus*	*helveticus*		CIRM-BIA 104	not complete
CBUK010000000	*Lactobacillus*	*helveticus*		CIRM-BIA 951	not complete
NC_021744	*Lactobacillus*	*helveticus*		CNRZ32	complete
CBUM000000000	*Lactobacillus*	*helveticus*		CRIM-BIA 103	not complete
NC_010080	*Lactobacillus*	*helveticus*		DPC 4571	complete
ACLM00000000	*Lactobacillus*	*helveticus*		DSM 20075	not complete
NC_017467	*Lactobacillus*	*helveticus*		H10	complete
CP002427	*Lactobacillus*	*helveticus*		H9	complete
CP009907	*Lactobacillus*	*helveticus*		KLDS1.8701	complete
LSVI00000000	*Lactobacillus*	*helveticus*		Lh 12	not complete
LSVJ00000000	*Lactobacillus*	*helveticus*		Lh 23	not complete
JQCJ00000000	*Lactobacillus*	*helveticus*		LMG 22464	not complete
JRTS00000000	*Lactobacillus*	*helveticus*		M3	not complete
CP011386	*Lactobacillus*	*helveticus*		MB2-1	complete
NC_018528	*Lactobacillus*	*helveticus*		R0052	complete
AP014680	*Lactobacillus*	*hokkaidonensis*		DNA	complete
NC_017477	*Lactobacillus*	*johnsonii*		DPC 6026	complete
NC_013504	*Lactobacillus*	*johnsonii*		FI9785	complete
NC_022909	*Lactobacillus*	*johnsonii*		N6.2	complete
NC_005362	*Lactobacillus*	*johnsonii*		NCC 533	complete
NC_015602	*Lactobacillus*	*kefiranofaciens*		ZW3	complete
CP012033	*Lactobacillus*	*koreensis*		26-25	complete
CP012920	*Lactobacillus*	*kunkeei*		MP2	complete
CP011013	*Lactobacillus*	*mucosae*		LM1	complete
CP014787	*Lactobacillus*	*oris*		J-1	complete
NC_022112	*Lactobacillus*	*paracasei*		8700-2	complete
CP000423	*Lactobacillus*	*paracasei*		ATCC 334	complete
CP012187	*Lactobacillus*	*paracasei*		CAUH35	complete
AP012541	*Lactobacillus*	*paracasei*		JCM 8130	complete
CP013921	*Lactobacillus*	*paracasei*		KL1	complete
CP012148	*Lactobacillus*	*paracasei*		L9	complete
CP007122	*Lactobacillus*	*paracasei*		N1115	complete
CP013130	*Lactobacillus*	*paraplantarum*		L-ZS9	complete
CBZW000000000	*Lactobacillus*	*plantarum*		80	not complete
AVFJ00000000	*Lactobacillus*	*plantarum*		2025	not complete
NC_021514	*Lactobacillus*	*plantarum*		16	complete
AWTS00000000	*Lactobacillus*	*plantarum*		19L3	not complete

AYTU00000000	*Lactobacillus*	*plantarum*	4_3	not complete
LOMH00000000	*Lactobacillus*	*plantarum*	43-3	not complete
CP009236	*Lactobacillus*	*plantarum*	5-2	complete
LBDF00000000	*Lactobacillus*	*plantarum*	8 RA-3	not complete
JXAX00000000	*Lactobacillus*	*plantarum*	90sk	not complete
JHWA00000000	*Lactobacillus*	*plantarum*	AG30	not complete
ACGZ00000000	*Lactobacillus*	*plantarum*	ATCC 14917	not complete
CP010528	*Lactobacillus*	*plantarum*	B21	complete
CP015126	*Lactobacillus*	*plantarum*	CAUH2	complete
LIGM00000000	*Lactobacillus*	*plantarum*	CGMCC 1.557	not complete
AZEJ00000000	*Lactobacillus*	*plantarum*	CGMCC1.2437	not complete
JSUW00000000	*Lactobacillus*	*plantarum*	CIP104448	not complete
LUWN00000000	*Lactobacillus*	*plantarum*	CNW10	not complete
LNCP00000000	*Lactobacillus*	*plantarum*	CRL 1506	not complete
JOJT00000000	*Lactobacillus*	*plantarum*	DmCS_001	not complete
CP004406	*Lactobacillus*	*plantarum*	DOMLa	complete
JQAW00000000	*Lactobacillus*	*plantarum*	DSM 13273	not complete
AZFR00000000	*Lactobacillus*	*plantarum*	DSM 16365	not complete
LUXL00000000	*Lactobacillus*	*plantarum*	ER	not complete
LQHB00000000	*Lactobacillus*	*plantarum*	FBR4	not complete
LQHC00000000	*Lactobacillus*	*plantarum*	FBR5	not complete
LQHD00000000	*Lactobacillus*	*plantarum*	FBR6	not complete
JPSU00000000	*Lactobacillus*	*plantarum*	FMNP01	not complete
CP012650	*Lactobacillus*	*plantarum*	HFC8	complete
ASJE00000000	*Lactobacillus*	*plantarum*	IPLA88	not complete
CP014780	*Lactobacillus*	*plantarum*	JBE245	complete
NC_012984	*Lactobacillus*	*plantarum*	JDM1	complete
LEKW00000000	*Lactobacillus*	*plantarum*	L31-1	not complete
LDEL00000000	*Lactobacillus*	*plantarum*	Lp1610	not complete
LDEM00000000	*Lactobacillus*	*plantarum*	Lp1612	not complete
JIBX00000000	*Lactobacillus*	*plantarum*	Lp90	not complete
CP012122	*Lactobacillus*	*plantarum*	LZ95	complete
LUXN00000000	*Lactobacillus*	*plantarum*	NAB1	not complete
AGRI00000000	*Lactobacillus*	*plantarum*	NC8	not complete
LTAU00000000	*Lactobacillus*	*plantarum*	Nizo1837	not complete
LUWA00000000	*Lactobacillus*	*plantarum*	Nizo1838	not complete
LUWB00000000	*Lactobacillus*	*plantarum*	Nizo1839	not complete
LUWC00000000	*Lactobacillus*	*plantarum*	Nizo1840	not complete
LUWD00000000	*Lactobacillus*	*plantarum*	Nizo2029	not complete
LUWE00000000	*Lactobacillus*	*plantarum*	Nizo2256	not complete
LUWF00000000	*Lactobacillus*	*plantarum*	Nizo2257	not complete
LUWG00000000	*Lactobacillus*	*plantarum*	Nizo2258	not complete
LUWH00000000	*Lactobacillus*	*plantarum*	Nizo2259	not complete
LUWI00000000	*Lactobacillus*	*plantarum*	Nizo2260	not complete
LUWJ00000000	*Lactobacillus*	*plantarum*	Nizo2262	not complete
LUWK00000000	*Lactobacillus*	*plantarum*	Nizo2263	not complete
LUWL00000000	*Lactobacillus*	*plantarum*	Nizo2264	not complete
LUWM00000000	*Lactobacillus*	*plantarum*	Nizo2457	not complete
LUWO00000000	*Lactobacillus*	*plantarum*	Nizo2484	not complete
LUWP00000000	*Lactobacillus*	*plantarum*	Nizo2485	not complete
LUWQ00000000	*Lactobacillus*	*plantarum*	Nizo2494	not complete
LUWR00000000	*Lactobacillus*	*plantarum*	Nizo2535	not complete
LUWS00000000	*Lactobacillus*	*plantarum*	Nizo2726	not complete

LUWT00000000	*Lactobacillus*	*plantarum*	Nizo2741	not complete
LUWU00000000	*Lactobacillus*	*plantarum*	Nizo2753	not complete
LUWV00000000	*Lactobacillus*	*plantarum*	Nizo2757	not complete
LUWW00000000	*Lactobacillus*	*plantarum*	Nizo2766	not complete
LUWX00000000	*Lactobacillus*	*plantarum*	Nizo2776	not complete
LUWY00000000	*Lactobacillus*	*plantarum*	Nizo2801	not complete
LUWZ00000000	*Lactobacillus*	*plantarum*	Nizo2802	not complete
LUXA00000000	*Lactobacillus*	*plantarum*	Nizo2806	not complete
LUXB00000000	*Lactobacillus*	*plantarum*	Nizo2814	not complete
LUXC00000000	*Lactobacillus*	*plantarum*	Nizo2830	not complete
LUXD00000000	*Lactobacillus*	*plantarum*	Nizo2831	not complete
LUXE00000000	*Lactobacillus*	*plantarum*	Nizo2855	not complete
LKHZ00000000	*Lactobacillus*	*plantarum*	Nizo2877	not complete
LUXF00000000	*Lactobacillus*	*plantarum*	Nizo2889	not complete
LUXG00000000	*Lactobacillus*	*plantarum*	Nizo2891	not complete
LUXH00000000	*Lactobacillus*	*plantarum*	Nizo3400	not complete
LUXI00000000	*Lactobacillus*	*plantarum*	Nizo3892	not complete
LUXJ00000000	*Lactobacillus*	*plantarum*	Nizo3893	not complete
LUXK00000000	*Lactobacillus*	*plantarum*	Nizo3894	not complete
JZSB00000000	*Lactobacillus*	*plantarum*	NL42	not complete
LUSM00000000	*Lactobacillus*	*plantarum*	NRCC1	not complete
NC_021224	*Lactobacillus*	*plantarum*	P-8	complete
LBHS00000000	*Lactobacillus*	*plantarum*	PS128	not complete
MJHC00000000	*Lactobacillus*	*plantarum*	RI-011	not complete
MJHD00000000	*Lactobacillus*	*plantarum*	RI-012	not complete
MJHG00000000	*Lactobacillus*	*plantarum*	RI-048	not complete
MKDP00000000	*Lactobacillus*	*plantarum*	RI-086	not complete
CP017406	*Lactobacillus*	*plantarum*	RI-113	complete
MKDQ00000000	*Lactobacillus*	*plantarum*	RI-123	not complete
MKDS00000000	*Lactobacillus*	*plantarum*	RI-139	not complete
MKDT00000000	*Lactobacillus*	*plantarum*	RI-140	not complete
MKDU00000000	*Lactobacillus*	*plantarum*	RI-146	not complete
MKDV00000000	*Lactobacillus*	*plantarum*	RI-147	not complete
MJHH00000000	*Lactobacillus*	*plantarum*	RI-162	not complete
MJHI00000000	*Lactobacillus*	*plantarum*	RI-165	not complete
MJHJ00000000	*Lactobacillus*	*plantarum*	RI-189	not complete
MJHK00000000	*Lactobacillus*	*plantarum*	RI-190	not complete
MKFX00000000	*Lactobacillus*	*plantarum*	RI-208	not complete
MKDY00000000	*Lactobacillus*	*plantarum*	RI-266	not complete
MKDF00000000	*Lactobacillus*	*plantarum*	RI-405	not complete
MKDH00000000	*Lactobacillus*	*plantarum*	RI-408	not complete
MKDK00000000	*Lactobacillus*	*plantarum*	RI-422	not complete
MKDZ00000000	*Lactobacillus*	*plantarum*	RI-505	not complete
MKEA00000000	*Lactobacillus*	*plantarum*	RI-506	not complete
MKEB00000000	*Lactobacillus*	*plantarum*	RI-507	not complete
MKEC00000000	*Lactobacillus*	*plantarum*	RI-509	not complete
MKED00000000	*Lactobacillus*	*plantarum*	RI-510	not complete
MKEE00000000	*Lactobacillus*	*plantarum*	RI-511	not complete
MKEF00000000	*Lactobacillus*	*plantarum*	RI-512	not complete
MKEG00000000	*Lactobacillus*	*plantarum*	RI-513	not complete
MKEH00000000	*Lactobacillus*	*plantarum*	RI-514	not complete
MKGF00000000	*Lactobacillus*	*plantarum*	RI-515	not complete
LMVD00000000	*Lactobacillus*	*plantarum*	SF2A35B	not complete

LGIM00000000	*Lactobacillus*	*plantarum*	SNU.Lp177	not complete
NC_014554	*Lactobacillus*	*plantarum*	ST-III	complete
JSUX00000000	*Lactobacillus*	*plantarum*	TIFN101	not complete
LSND00000000	*Lactobacillus*	*plantarum*	UC8491	not complete
APHP00000000	*Lactobacillus*	*plantarum*	UCMA 3037	not complete
NC_004567	*Lactobacillus*	*plantarum*	WCFS1	complete
JMEL00000000	*Lactobacillus*	*plantarum*	wikim18	not complete
LKLZ00000000	*Lactobacillus*	*plantarum*	WJL	not complete
AUTE00000000	*Lactobacillus*	*plantarum*	WJL136	not complete
LKCO00000000	*Lactobacillus*	*plantarum*	WLPL04	not complete
CP011769	*Lactobacillus*	*plantarum*	Zhang-LL	complete
NC_020229	*Lactobacillus*	*plantarum*	ZJ316	complete
CP012122	*Lactobacillus*	*plantarum*	ZJ95	complete
CP012343	*Lactobacillus*	*plantarum*	ZS2085	complete
AAPZ00000000	*Lactobacillus*	*reuteri*	100-23	not complete
MBLQ00000000	*Lactobacillus*	*reuteri*	480_44	not complete
MBLR00000000	*Lactobacillus*	*reuteri*	482_46	not complete
MBLS00000000	*Lactobacillus*	*reuteri*	482_54	not complete
MBLU00000000	*Lactobacillus*	*reuteri*	484_39	not complete
FR854373	*Lactobacillus*	*reuteri*	ATCC 53609	not complete
ACHG00000000	*Lactobacillus*	*reuteri*	CF48-3A	not complete
LYWI00000000	*Lactobacillus*	*reuteri*	CRL 1098	not complete
NC_009513	*Lactobacillus*	*reuteri*	DSM 20016	complete
AZDD00000000	*Lactobacillus*	*reuteri*	DSM 20016-1	not complete
CP015408	*Lactobacillus*	*reuteri*	I49	complete
NC_021494	*Lactobacillus*	*reuteri*	I5007	complete
AEAX00000000	*Lactobacillus*	*reuteri*	Ipuph	not complete
CP011024	*Lactobacillus*	*reuteri*	IRT	complete
NC_010609	*Lactobacillus*	*reuteri*	JCM 1112	complete
JOSX00000000	*Lactobacillus*	*reuteri*	LTH2584	not complete
JOOG00000000	*Lactobacillus*	*reuteri*	LTH5448	not complete
AEAW00000000	*Lactobacillus*	*reuteri*	mlc3	not complete
ACLB00000000	*Lactobacillus*	*reuteri*	MM2-3	not complete
ACGX00000000	*Lactobacillus*	*reuteri*	MM4-1A	not complete
NC_015697	*Lactobacillus*	*reuteri*	SD2112	complete
NC_021872	*Lactobacillus*	*reuteri*	TD1	complete
JOKX00000000	*Lactobacillus*	*reuteri*	TMW1.112	not complete
JOSW00000000	*Lactobacillus*	*reuteri*	TMW1.656	not complete
CP014786	*Lactobacillus*	*reuteri*	ZLR003	complete
JPZB00000000	*Lactobacillus*	*rhamnosus*	24	not complete
JTDC00000000	*Lactobacillus*	*rhamnosus*	116	not complete
JWHC00000000	*Lactobacillus*	*rhamnosus*	308	not complete
LFNB00000000	*Lactobacillus*	*rhamnosus*	313	not complete
JVQV00000000	*Lactobacillus*	*rhamnosus*	186_LRHA	not complete
JVPR00000000	*Lactobacillus*	*rhamnosus*	214_LRHA	not complete
JVLT01000001	*Lactobacillus*	*rhamnosus*	319_LRHA	not complete
JVIZ00000000	*Lactobacillus*	*rhamnosus*	390_LRHA	not complete
LFNA00000000	*Lactobacillus*	*rhamnosus*	40f	not complete
JMSI00000000	*Lactobacillus*	*rhamnosus*	51B	not complete
JVDP00000000	*Lactobacillus*	*rhamnosus*	526_LRHA	not complete
JVCX00000000	*Lactobacillus*	*rhamnosus*	541_LRHA	not complete
JUWQ00000000	*Lactobacillus*	*rhamnosus*	699_LRHA	not complete
JUWG00000000	*Lactobacillus*	*rhamnosus*	708_LRHA	not complete

JUTS00000000	*Lactobacillus*	*rhamnosus*	769_LRHA	not complete
JUTB00000000	*Lactobacillus*	*rhamnosus*	784_LRHA	not complete
JUPX00000000	*Lactobacillus*	*rhamnosus*	870_LRHA	not complete
JUPA00000000	*Lactobacillus*	*rhamnosus*	893_LRHA	not complete
JUON00000000	*Lactobacillus*	*rhamnosus*	906_LRHA	not complete
JUMP00000000	*Lactobacillus*	*rhamnosus*	943_LRHA	not complete
JULF00000000	*Lactobacillus*	*rhamnosus*	979_LRHA	not complete
JUKV00000000	*Lactobacillus*	*rhamnosus*	988_LRHA	not complete
CP014645	*Lactobacillus*	*rhamnosus*	ASCC 290	complete
AFZY00000000	*Lactobacillus*	*rhamnosus*	ATCC 21052	not complete
NC_017482	*Lactobacillus*	*rhamnosus*	ATCC 53103	complete
NC_017491	*Lactobacillus*	*rhamnosus*	ATCC 8530	complete
CBZU000000000	*Lactobacillus*	*rhamnosus*	BPL15	not complete
LT220504	*Lactobacillus*	*rhamnosus*	BPL5	complete
LAZE00000000	*Lactobacillus*	*rhamnosus*	CNCM-I-3698	not complete
JYCS00000000	*Lactobacillus*	*rhamnosus*	CSL17	not complete
BALT00000000	*Lactobacillus*	*rhamnosus*	DSM 20021	not complete
JDRW00000000	*Lactobacillus*	*rhamnosus*	E800	not complete
NC_013198	*Lactobacillus*	*rhamnosus*	GG	complete
ABWJ00000000	*Lactobacillus*	*rhamnosus*	HN001	not complete
JNNV00000000	*Lactobacillus*	*rhamnosus*	K32	not complete
AYTR00000000	*Lactobacillus*	*rhamnosus*	L34	not complete
AYTP00000000	*Lactobacillus*	*rhamnosus*	L35	not complete
NC_013199	*Lactobacillus*	*rhamnosus*	Lc 705	complete
ACIZ00000000	*Lactobacillus*	*rhamnosus*	LMS2-1	not complete
NC_021723	*Lactobacillus*	*rhamnosus*	LOCK900	complete
NC_021725	*Lactobacillus*	*rhamnosus*	LOCk908	complete
JUIL00000000	*Lactobacillus*	*rhamnosus*	LR071	not complete
JUIK00000000	*Lactobacillus*	*rhamnosus*	LR073	not complete
JUIH00000000	*Lactobacillus*	*rhamnosus*	LR108	not complete
JUII00000000	*Lactobacillus*	*rhamnosus*	LR138	not complete
AMQW00000000	*Lactobacillus*	*rhamnosus*	LRHMPD2	not complete
AMQX00000000	*Lactobacillus*	*rhamnosus*	LRHMPD3	not complete
JDFQ00000000	*Lactobacillus*	*rhamnosus*	PEL5	not complete
JDFR00000000	*Lactobacillus*	*rhamnosus*	PEL6	not complete
AGKC00000000	*Lactobacillus*	*rhamnosus*	R0011	not complete
LWBT00000000	*Lactobacillus*	*rhamnosus*	R19-3	not complete
NC_015975	*Lactobacillus*	*ruminis*	ATCC 27782	complete
NC_007576	*Lactobacillus*	*sakei*	23K	complete
NC_017481	*Lactobacillus*	*salivarius*	CECT 5713	complete
CP007646	*Lactobacillus*	*salivarius*	JCM1046	complete
CP011403	*Lactobacillus*	*salivarius*	Ren	complete
NC_007929	*Lactobacillus*	*salivarius*	UCC118	complete
NC_015978	*Lactobacillus*	*sanfranciscensis*	TMW 1.1304	complete
CP009531	*Lactobacillus*	spp	wkB8	complete
AZCT00000000	*Lactobacillus*	*zeae*	DSM 20178	not complete

Table S6.2 Reconstruction of core, softcore- and pan-genome of *Lactobacillus delbrueckii* species with the Brite and pathway algorithm of GhostKOALA.

Brite Reconstruction Result				**n-fold increase**	
	core	**softcore**	**pan**	**core-pan**	**softcore-pan**
Orthologs and modules	498	623	933	1.8	1.5
Protein families: metabolism	328	426	617	1.9	1.5
Protein families: genetic information processing	251	315	342	1.4*	1.1*
Protein families: signaling and cellular processes	91	116	237	2.6*	2.0*
Total	**1168**	**1480**	**2129**	**1.8**	**1.4**

Pathway Reconstruction Result				**n-fold increase**	
	core	**softcore**	**pan**	**core to pan**	**softcore-pan**
Metabolism	611	792	1248	2.0	1.6
Genetic Information Processing	129	159	166	1.3*	1.0*
Environmental Information Processing	45	59	114	2.5	1.9
Cellular Processes	19	25	57	3.0	2.3
Organismal Systems	7	9	15	2.1	1.7
Human Diseases	27	40	54	2.0	1.4
Total	**838**	**1084**	**1654**	**2.0**	**1.5**

* indicates p-value < 0.01

Table S6.3 Reconstruction of core, softcore- and pan-genome of *Lactobacillus delbrueckii* species with the Module algorithm of GhostKOALA. Pathways are fractured in blocks (number). Green = pathway complete; yellow = 1 block is missing in the pathway; red = 2 or more blocks are missing in the pathway.

Pathway module		Block in pathway		
Energy metabolism		**core**	**softcore**	**pan**
Carbon fixation				
M00579	Phosphate acetyltransferase-acetate kinase pathway, acetyl-CoA => acetate	2	2	2
Carbohydrate and lipid metabolism				
Central carbohydrate metabolism				
M00001	Glycolysis (Embden-Meyerhof pathway), glucose => pyruvate	14	16	18
M00002	Glycolysis, core involving three-carbon compounds	10	12	14
M00006	Pentose phosphate pathway, oxidative phase, glucose 6P => ribulose 5P	3	3	3
M00005	PRPP biosynthesis, ribose 5P => PRPP	1	1	2
Other carbohydrate metabolism				
M00632	Galactose degradation, Leloir pathway, galactose => alpha-D-glucose-1P	0	0	6
M00549	Nucleotide sugar biosynthesis, glucose => UDP-glucose	3	3	4
M00554	Nucleotide sugar biosynthesis, galactose => UDP-galactose	0	0	3
M00793	dTDP-L-rhamnose biosynthesis	0	0	4
Fatty acid metabolism				
M00082	Fatty acid biosynthesis, initiation	3	6	6
M00083	Fatty acid biosynthesis, elongation	5	7	12
Terpenoid backbone biosynthesis				
M00364	C10-C20 isoprenoid biosynthesis, bacteria	2	2	2
Nucleotide and amino acid metabolism				
Purine metabolism				
M00048	Inosine monophosphate biosynthesis, PRPP + glutamine => IMP	0	11	13
M00049	Adenine ribonucleotide biosynthesis, IMP => ADP,ATP	3	4	4
M00050	Guanine ribonucleotide biosynthesis IMP => GDP,GTP	5	5	5
Serine and threonine metabolism				
M00018	Threonine biosynthesis, aspartate => homoserine => threonine	0	3	6
Cysteine and methionine metabolism				
M00021	Cysteine biosynthesis, serine => cysteine	0	0	3
M00017	Methionine biosynthesis, apartate => homoserine => methionine	0	0	10
Lysine metabolism				
M00525	Lysine biosynthesis, acetyl-DAP pathway, aspartate => lysine	0	0	12
Arginine and proline metabolism				
M00015	Proline biosynthesis, glutamate => proline	0	0	4
Cofactor and vitamin biosynthesis				
M00120	Coenzyme A biosynthesis, pantothenate => CoA	4	4	4
Structural complex				
Energy metabolism				
ATP synthesis				
M00157	F-type ATPase, prokaryotes and chloroplasts	8	8	8
Genetic information processing				
DNA polymerase				
M00260	DNA polymerase III complex, bacteria	5	7	9
RNA polymerase				
M00183	RNA polymerase, bacteria	3	4	5
Ribosome				
M00178	Ribosome, bacteria	43	47	50

Environmental information processing				
Mineral and organic ion transport system				
M00299	Spermidine/putrescine transport system	3	4	4
M00193	Putative spermidine/putrescine transport system	0	0	4
M00209	Osmoprotectant transport system	0	1	8
Saccharide, polyol, and lipid transport system				
M00491	arabinogalactan oligomer/maltooligosaccharide transport system	0	0	4
M00207	Putative multiple sugar transport system	0	3	5
M00211	Putative ABC transport system	1	1	2
Phosphate and amino acid transport system				
M00222	Phosphate transport system	2	4	4
M00223	Phosphonate transport system	4	4	4
M00234	Cystine transport system	0	0	3
M00589	Putative lysine transport system	2	3	3
M00237	Branched-chain amino acid transport system	0	0	5
M00238	D-Methionine transport system	2	3	4
M00228	Putative glutamine transport system	4	5	5
M00236	Putative polar amino acid transport system	6	6	14
Peptide and nickel transport system				
M00439	Oligopeptide transport system	4	5	32
M00239	Peptides/nickel transport system	0	1	4
Metallic cation, iron-siderophore and vitamin B12 transport system				
M00240	Iron complex transport system	0	0	3
M00582	Energy-coupling factor transport system	5	5	14
M00247	Putative ABC transport system	1	1	6
ABC-2 type and other transport systems				
M00298	Multidrug/hemolysin transport system	0	0	2
M00258	Putative ABC transport system	2	2	9
M00254	ABC-2 type transport system	1	3	10
Drug efflux transporter/pump				
M00707	Multidrug resistance, SmdAB/MdlAB transporter	2	2	2
M00708	Multidrug resistance, PatAB transporter	0	0	2
Phosphotransferase system (PTS)				
M00269	PTS system, sucrose-specific II component	0	0	3
M00271	PTS system, beta-glucosides-specific II component	0	0	9
M00273	PTS system, fructose-specific II component	0	0	3
M00274	PTS system, mannitol-specific II component	0	0	3
M00275	PTS system, cellobiose-specific II component	1	1	10
M00807	PTS system, galactose-specific II component	0	0	3
M00276	PTS system, mannose-specific II component	0	4	4
Functional set				
Metabolism				
Aminoacyl tRNA				
M00360	Aminoacyl-tRNA biosynthesis, prokaryotes	0	20	26
Environmental information processing				
Two-component regulatory system				
M00434	PhoR-PhoB (phosphate starvation response) two-component regulatory system	0	1	2
M00459	VicK-VicR (cell wall metabolism) two-component regulatory system	2	2	2

Table S6.4 TOPD/FMTS nodal distance scores and SNPs evaluation Genes are sorted according to the nodal distance scores compared with the pan-genome tree and the 5% and 95% quantile is listed. Sum SNP – The sum of all SNP according to the consensus sequence of the 29 homologous sequences; SNP/base – sum of SNP divided by length of consensus sequence in nucleotide; Sum polyvariable positions (SPP) – sum of all positions with 3 or more different nucleotides a specific position; Length in bp – length of consensus sequence.

	TOPD/FMTS Score						
Gene	core	softcore	pan	Sum SNP	SNP/Base	SPP	Length in bp
17524_aspS	2.431	2.359	1.778	94	0.051	0	1845
17294_phosphonate_ABC_tran..	2.412	2.031	1.818	44	0.055	0	801
17742_DNA_repair_protein_R..	2.614	2.518	1.825	79	0.057	0	1377
16818_oligoendopeptidase_F	2.325	2.240	1.894	104	0.058	1	1803
17523_histidine--tRNA_liga..	2.518	2.561	1.931	64	0.050	2	1287
16623_translation_initiati..	2.752	2.621	1.981	108	0.044	2	2478
17738_MFS_transporter	2.266	2.301	2.007	66	0.054	1	1221
17241_LytR_family_transcri..	2.570	2.360	2.012	175	0.153	4	1143
17199_two-component_sensor..	2.504	2.709	2.017	59	0.049	0	1209
17700_guanosine_monophosph..	2.674	2.368	2.029	53	0.053	2	993
17395_flavocytochrome_c	2.643	2.446	2.034	53	0.038	1	1398
17672_MFS_transporter	2.576	2.507	2.038	107	0.087	3	1236
16633_adenine_phosphoribos..	2.309	2.262	2.039	29	0.055	0	528
17312_acetylesterase	2.421	2.352	2.067	108	0.125	1	867
16543_amidophosphoribosylt..	2.433	2.242	2.077	149	0.101	5	1479
16462_DNA_primase	2.211	2.161	2.088	84	0.046	2	1839
16873_magnesium-translocat..	2.520	2.420	2.106	144	0.054	5	2691
17559_DNA-binding_protein_..	2.428	2.672	2.114	47	0.050	1	933
16888_GMP_synthetase	2.596	2.506	2.117	76	0.049	1	1554
16694_tig	2.553	2.563	2.121	59	0.044	2	1326
16765_cell_division_protei..	2.970	2.919	2.127	51	0.038	1	1353
17488_enoyl--acyl-carrier-..	2.716	2.677	2.131	56	0.073	1	765
16525_multidrug_MFS_transp..	2.690	2.719	2.159	63	0.043	1	1449
17121_GTP_pyrophosphokinas..	2.591	2.491	2.164	73	0.119	5	615
16927_phosphate_ABC_transp..	2.684	2.574	2.164	78	0.088	1	885
17608_DNA_polymerase_IV	2.639	2.527	2.164	45	0.040	0	1116
17419_glucan_modification_..	2.744	2.624	2.185	189	0.063	2	3003
16813_DNA_polymerase_III_s..	2.375	2.501	2.186	306	0.088	0	3480
17729_esterase	2.365	2.404	2.192	66	0.081	2	819
17214_helicase	2.662	2.649	2.199	141	0.051	3	2767
16700_amino_acid_permease	2.668	2.452	2.221	822	0.498	40	1649
16731_ATP_synthase_F0F1_su..	3.144	2.950	2.225	24	0.044	1	543
17668_phosphoglucomutase	2.720	2.612	2.226	91	0.053	0	1722
16440_ribonuclease_HII	3.019	2.803	2.232	49	0.064	2	771
17657_dipeptidase_PepV	2.726	2.459	2.236	94	0.067	4	1413
17165_formate--tetrahydrof..	2.281	2.181	2.239	63	0.038	2	1680
16824_glucosamine-6-phosph..	2.692	2.550	2.242	76	0.108	3	705
16880_bifunctional_N-acety..	2.568	2.571	2.250	50	0.036	2	1386
17803_S-ribosylhomocystein..	5.520	5.312	5.600	14	0.029	0	480
16607_hypothetical_protein	5.255	5.373	5.663	21	0.048	1	435
17297_universal_stress_pro..	4.910	5.050	5.703	23	0.049	1	474
17572_sigma-54_modulation_..	5.491	5.797	5.714	14	0.025	0	558
17602_hypothetical_protein	5.071	5.443	5.735	11	0.034	0	324
17131_DNA-binding_response..	5.365	5.593	5.753	24	0.034	1	714
16794_30S_ribosomal_protei..	5.974	5.796	5.773	7	0.026	0	270
17045_preprotein_transloca..	5.554	5.554	5.788	3	0.013	0	234
16651_rplL	5.477	5.708	5.813	4	0.011	0	366
16996_50S_ribosomal_protei..	5.220	5.380	5.816	12	0.019	1	618
16493_glnQ	5.569	5.731	5.848	18	0.024	0	741
17253_hypothetical_protein	5.772	6.010	5.869	23	0.068	0	339
16489_phosphopyruvate_hydr..	5.217	5.424	5.940	13	0.010	0	1278
16775_hypothetical_protein	5.456	5.741	5.942	12	0.035	0	342
17693_peptide_ABC_transpor..	5.770	5.794	5.956	50	0.048	0	1044
16556_50S_ribosomal_protei..	5.782	6.086	5.960	5	0.017	0	294
17341_acylphosphatase	5.547	5.778	6.036	14	0.051	0	273
17651_hypothetical_protein	5.707	5.842	6.110	6	0.032	0	186
16995_50S_ribosomal_protei..	5.727	5.695	6.138	10	0.034	0	297
16851_30S_ribosomal_protei..	5.968	5.920	6.217	5	0.013	0	396
16413_AsnC_family_transcri..	5.697	5.858	6.260	30	0.062	0	486
17282_phosphocarrier_prote..	5.888	6.058	6.296	7	0.026	1	267
16639_hypothetical_protein	6.230	6.244	6.339	9	0.042	0	216
17001_30S_ribosomal_protei..	6.017	6.022	6.369	7	0.017	1	408
16983_50S_ribosomal_protei..	6.435	6.109	6.468	17	0.032	0	531
16967_rpmE2	5.998	6.230	6.645	6	0.024	0	252
17604_hypothetical_protein	6.203	6.405	6.647	6	0.023	0	258
16852_50S_ribosomal_protei..	6.654	6.814	7.023	4	0.009	0	444
16793_30S_ribosomal_protei..	6.719	6.783	7.044	6	0.024	0	252
16642_50S_ribosomal_protei..	6.557	6.887	7.100	3	0.020	0	150
16770_cold-shock_protein	7.351	7.437	7.502	5	0.024	0	210
16976_infA	7.538	7.645	7.655	2	0.009	0	222
16645_50S_ribosomal_protei..	7.452	7.577	7.701	4	0.009	0	426
16555_50S_ribosomal_protei..	7.888	7.907	7.814	3	0.010	0	312
16987_50S_ribosomal_protei..	8.014	7.770	7.858	6	0.016	0	369
16972_50S_ribosomal_protei..	7.897	7.757	7.861	2	0.005	0	384
16992_50S_ribosomal_protei..	7.910	7.673	7.864	9	0.025	1	354
16988_30S_ribosomal_protei..	8.061	7.909	7.910	3	0.011	0	267

Table S6.5 Potential HGT in *Lactobacillus delbrueckii*. Clade B = bulgaricus clade, clade D = diverse clade, Number indicates copies of gene within the genome, presence = possibility of occurrence within the clade in percent.

Clade	B	B	B	B	B	B	B	B	B	B	B	B	B	B	D	D	D	D	D	D	D	D	D	D	D	D	D	D		
unique identifier	delbrueckii_DSM20081.gbk	delbrueckii_ATCC11842.gbk	delbrueckii_2038.gbk	delbrueckii_LBVIB44.gbk	delbrueckii_ATCC_BAA_365.gbk	delbrueckii_LBB.B5.gbk	delbrueckii_MN_BM_F01.gbk	delbrueckii_CFL1.gbk	delbrueckii_Lb1_GS_1.gbk	delbrueckii_Lb1_WT.gbk	delbrueckii_CNCM_I_1519.gbk	delbrueckii_CNCM_I_1632.gbk	delbrueckii_LBVIB27.gbk	delbrueckii_LBCNRZ327.gbk	delbrueckii_DSM20072_1.gbk	delbrueckii_ZN7a_9.gbk	delbrueckii_DSM20074.gbk	delbrueckii_DSM20072_2.gbk	delbrueckii_JCM17838.gbk	delbrueckii_DSM26046.gbk	delbrueckii_KACC13439.gbk	delbrueckii_LBCNRZ226.gbk	delbrueckii_NDO2.gbk	delbrueckii_LBCNRZ700.gbk	delbrueckii_LBCNRZ333.gbk	delbrueckii_PB2003_004_T3_4.gbk	delbrueckii_CRL581.gbk	delbrueckii_JCM15610.gbk	delbrueckii_DSM15996.gbk	Presence in calde *bulgaricus*	Presence in calde diverse
48_hypothetical_protein.faa	1	1	1	0	0	1	1	0	0	0	1	0	1	0	1	0	0	0	1	1	0	0	0	0	1	1	0	1	1	1	0.54	0.50
210_prolyl_tRNA_editing_...faa	1	1	1	0	0	1	1	0	0	0	1	0	1	1	1	0	0	1	1	0	0	0	0	0	0	1	1	1	1	1	0.54	0.56
348_carbamoyl_phosphate_...faa	1	1	1	0	0	1	1	0	0	0	1	0	1	1	1	0	0	1	0	0	0	0	1	1	1	0	1	1	1	0	0.54	0.56
639_transposase.faa	0	12	7	5	4	1	5	0	0	0	0	0	0	0	1	1	1	0	0	0	0	1	6	9	0	0	1	0	3	0	0.46	0.50
1533_hypothetical_protein.faa	1	1	1	0	0	1	1	0	0	0	1	0	1	0	1	0	0	1	1	1	0	0	0	0	0	1	1	1	1	0	0.54	0.44
1857_hypothetical_protein.faa	1	1	0	0	1	0	1	0	1	1	0	0	0	0	0	1	0	0	0	1	1	0	0	0	0	0	1	1	1	1	0.46	0.44
3257_hypothetical_protein.faa	1	1	0	0	1	0	0	0	1	2	1	0	1	2	2	0	0	2	1	0	0	0	0	2	1	0	1	1	1	1	0.54	0.56
4060_integrase.faa	1	1	0	1	0	1	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	0.54	0.50
4723_2_hydroxyacid_dehydr...faa	1	1	0	1	0	1	1	0	0	0	1	0	1	0	0	0	0	0	1	0	0	0	0	1	0	1	1	1	1	1	0.54	0.44
17105_hypothetical_protein.faa	1	1	1	0	1	0	1	0	1	1	0	0	0	1	1	0	1	1	0	0	1	0	0	1	1	0	1	0	0	0	0.54	0.50
17502_hypothetical_protein.faa	0	0	1	1	0	1	1	0	0	0	1	0	1	1	0	0	1	0	0	0	1	0	1	0	1	1	1	1	1	1	0.46	0.56
17812_NAD_dependent_dehydr...faa	0	0	1	0	1	1	0	0	1	1	1	0	1	0	0	0	1	0	1	0	1	1	1	1	1	1	1	1	0	0	0.54	0.56
17814_hypothetical_protein.faa	0	0	1	0	1	1	0	0	1	1	1	0	1	0	1	0	1	0	1	1	1	0	1	1	0	1	0	1	0	0	0.54	0.50
18_hemagglutinin.faa	0	0	1	1	1	1	0	0	0	0	0	0	0	1	0	1	0	0	1	1	0	0	0	0	1	0	1	0	0	0	0.31	0.38
49_transposase.faa	1	0	1	1	1	1	1	1	0	1	0	1	0	1	1	0	0	0	0	0	0	0	0	0	1	0	1	1	1	0	0.69	0.38
64_transposase.faa	0	4	4	0	0	1	4	0	0	0	0	0	1	6	0	1	0	0	1	1	0	1	8	0	0	1	1	1	0	0.38	0.56
89_hypothetical_protein.faa	1	1	1	1	0	1	1	1	0	0	0	1	0	1	1	0	0	1	1	0	0	0	1	1	1	0	1	1	1	0.62	0.63
390_hypothetical_protein.faa	0	0	1	1	1	1	0	1	1	1	0	1	0	1	1	0	0	1	0	0	0	0	1	0	1	0	1	0	0	0.62	0.38
473_hypothetical_protein.faa	0	0	1	0	0	1	1	0	1	1	0	0	0	1	1	1	0	1	1	1	0	1	0	0	0	0	1	0	1	1	0.38	0.63
784_serine_threonine_pro...faa	1	1	1	1	0	0	0	1	0	0	0	1	0	1	1	1	1	0	1	1	1	0	1	1	0	0	1	1	0	0	0.46	0.63
827_fumarate_reductase.faa	1	2	2	0	0	1	2	0	0	0	1	0	1	1	1	1	0	1	1	1	0	1	0	1	1	1	1	1	0	0	0.54	0.69
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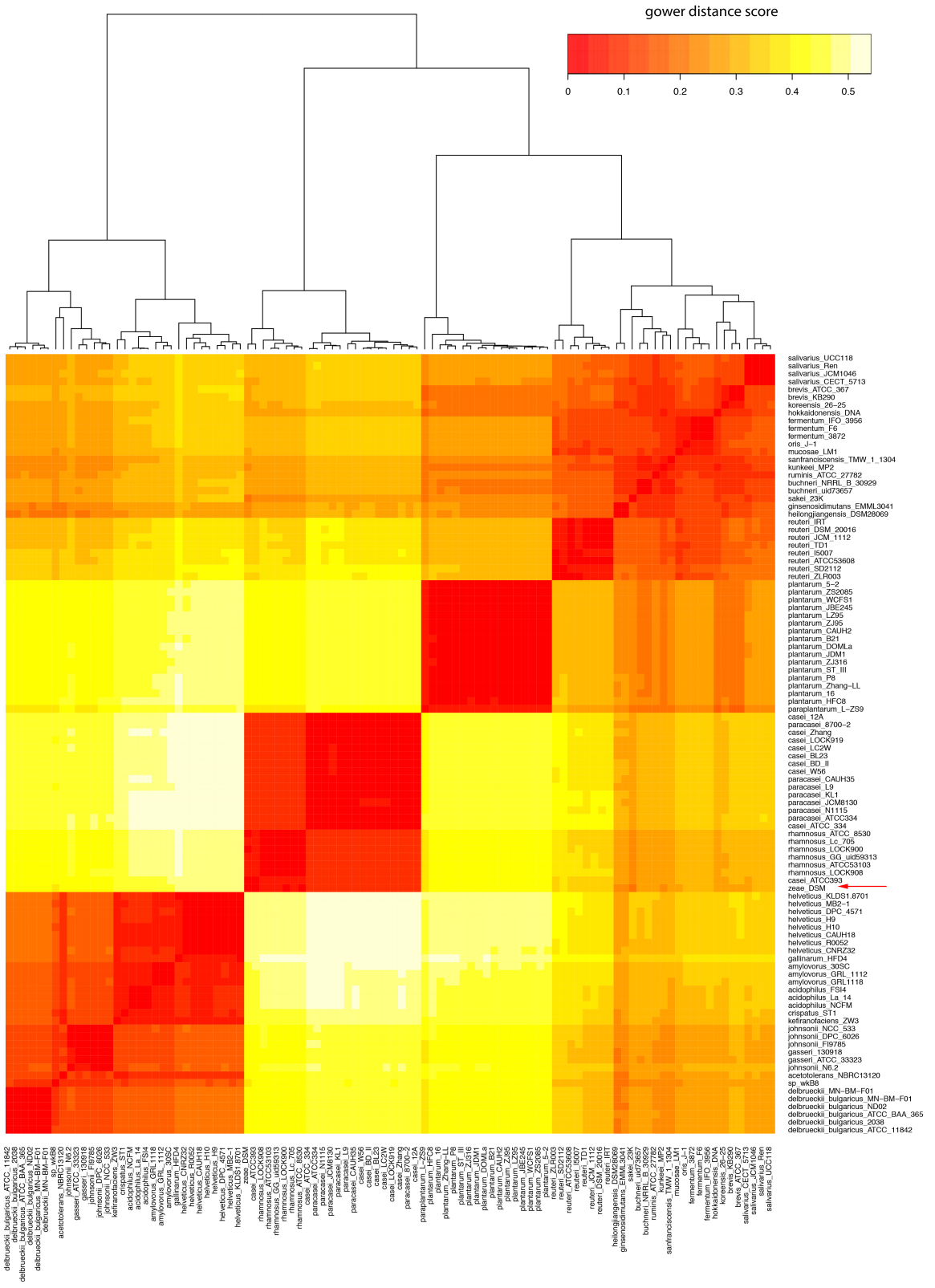

Figure S6.1 Heatmap clustering according to 20969 pan-genome genes from 99 *Lactobacillus* genomes including the non-complete genome of *L. zeae* DSM 20178. Gower distance score based on ANI: Red = more similar, white = less similar. *L. zeae* DSM 20178 marked with a ed arrow.

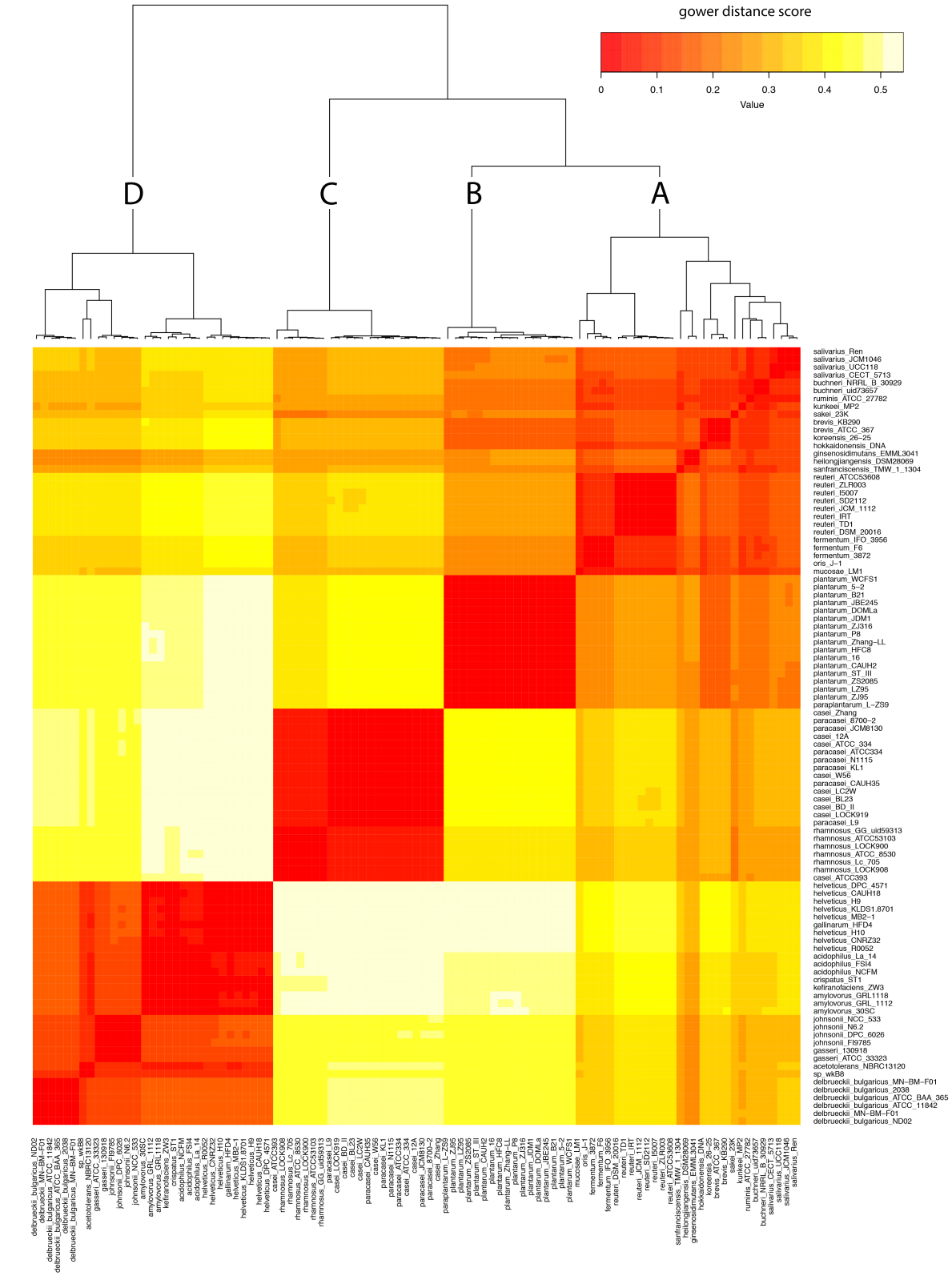

Figure S6.2 Heatmap clustering according to 594 softcore genes from 98 *Lactobacillus* genomes. Gower distance score based on ANI: Red = more similar, white = less similar.

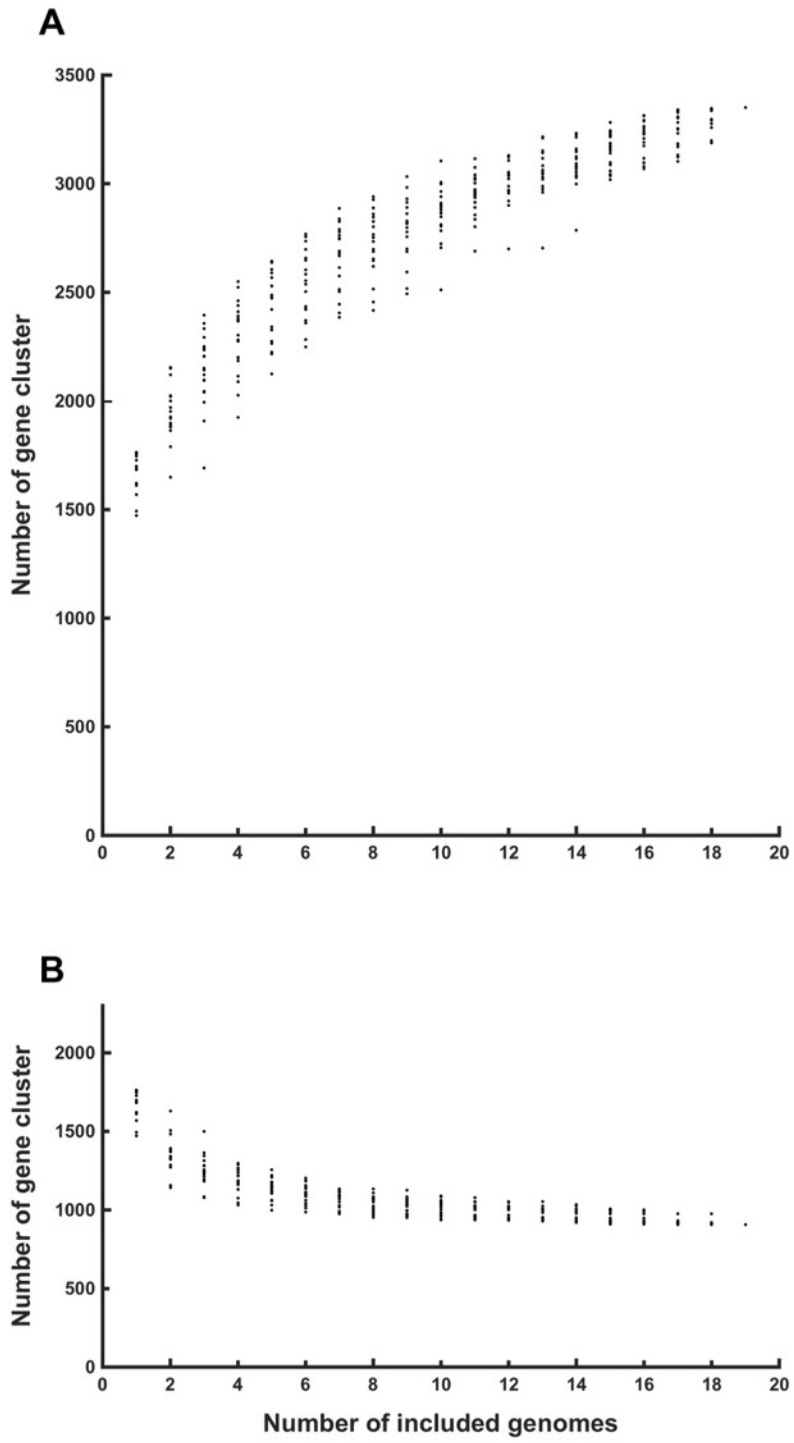

Figure S6.3 A Evolution of the pan-genome for *L. helveticus*. After 14 included genomes, the pan-genome is closed. **B** Evolution of the core-genome for *L. helveticus*. Order of calculation was randomized for 19 sets, each represented with a single point.

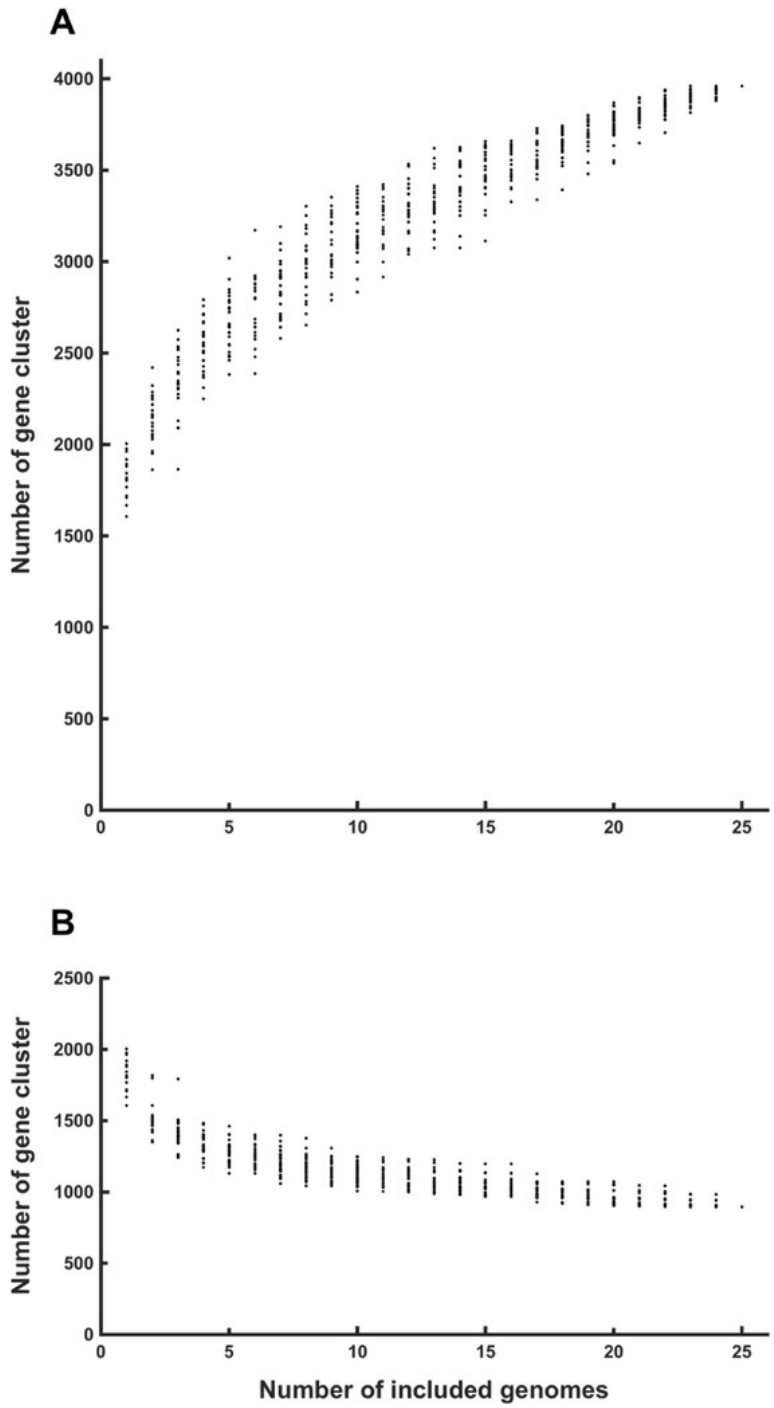

Figure S6.4 A Evolution of the pan-genome for *L. reuteri*. After 20 included genomes, the pan-genome is closed. **B** Evolution of the core-genome for *L. reuteri*. Order of calculation was randomized for 25 sets, each represented with a single point.

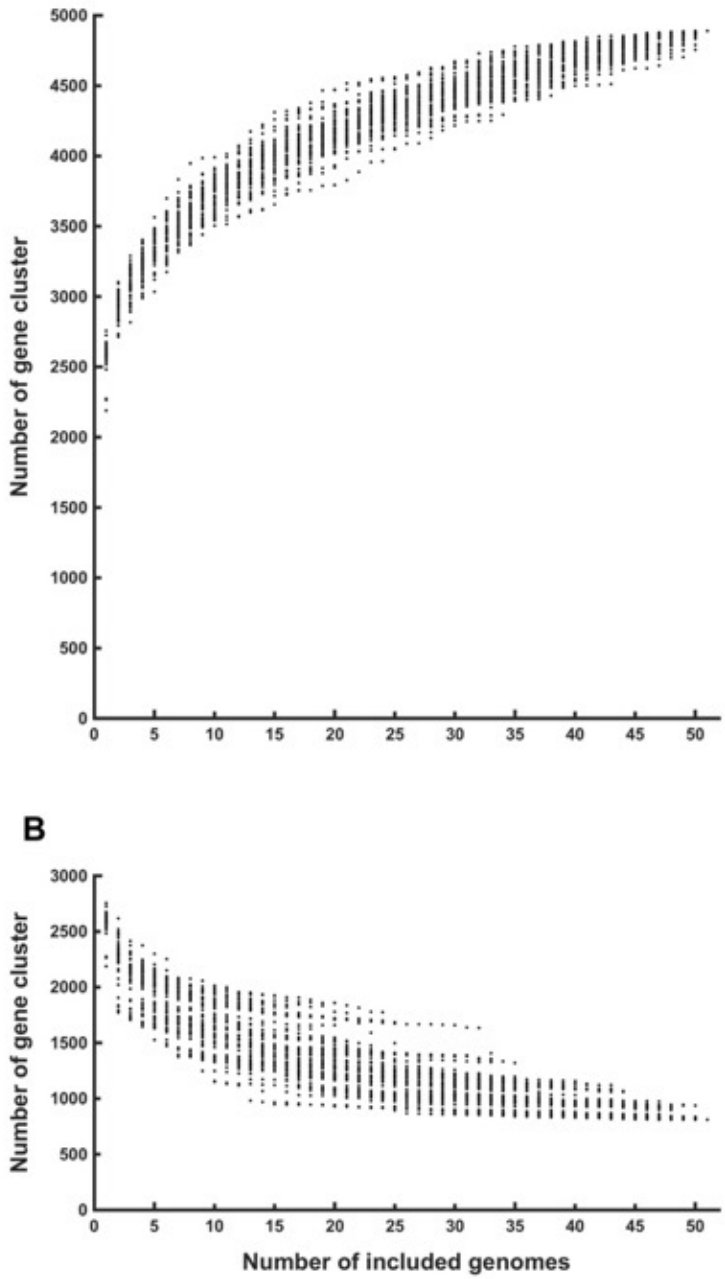

Figure S6.5 A Evolution of the pan-genome for *L. rhamnosus*. After 20 included genomes, the pan-genome is closed. **B** Evolution of the core-genome for *L. rhamnosus*. Order of calculation was randomized for 51 sets, each represented with a single point.

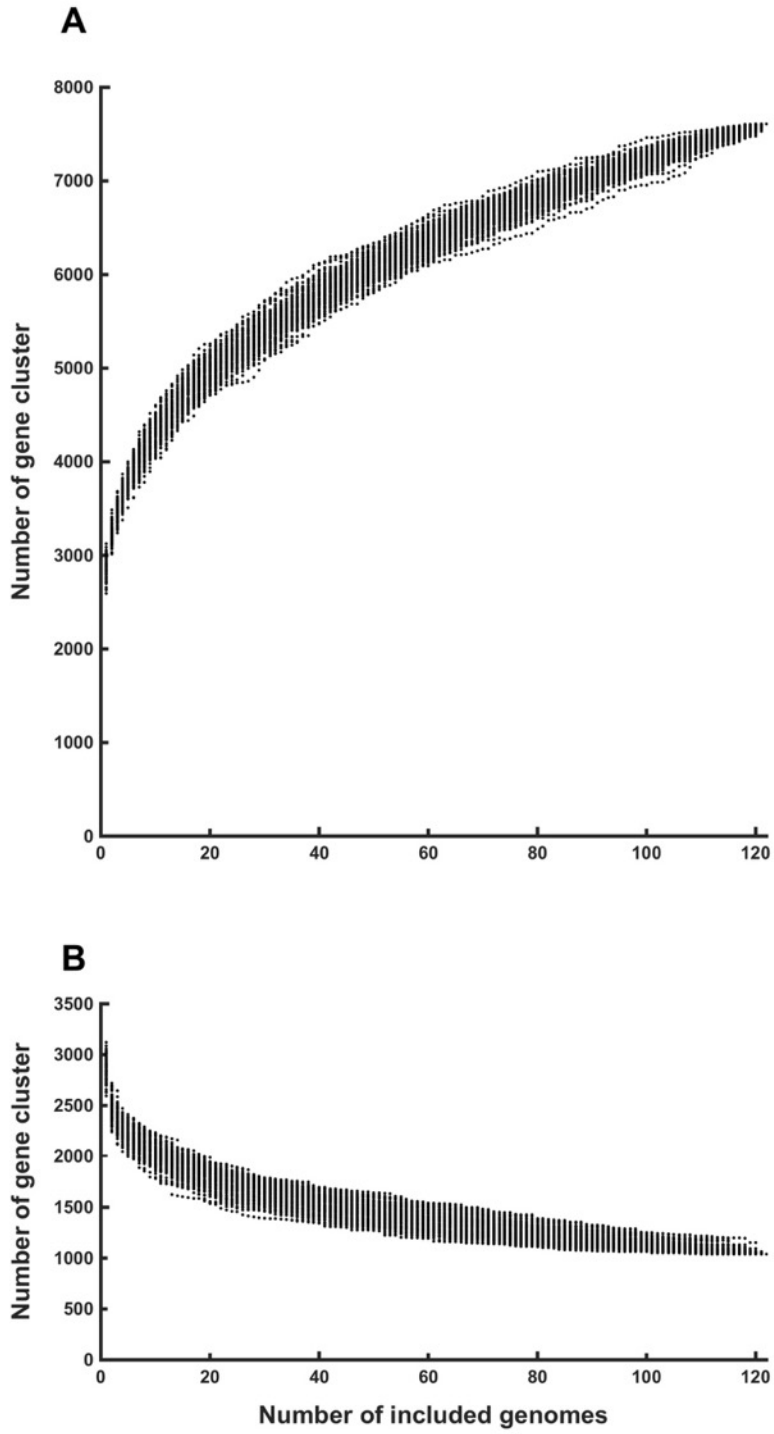

Figure S6.6 A Evolution of the pan-genome for *L. plantarum*. The pan-genome remains open even after 122 genomes were included. **B** Evolution of the core-genome for *L. plantarum*. Order of calculation was randomized for 122 sets, each represented with a single point.

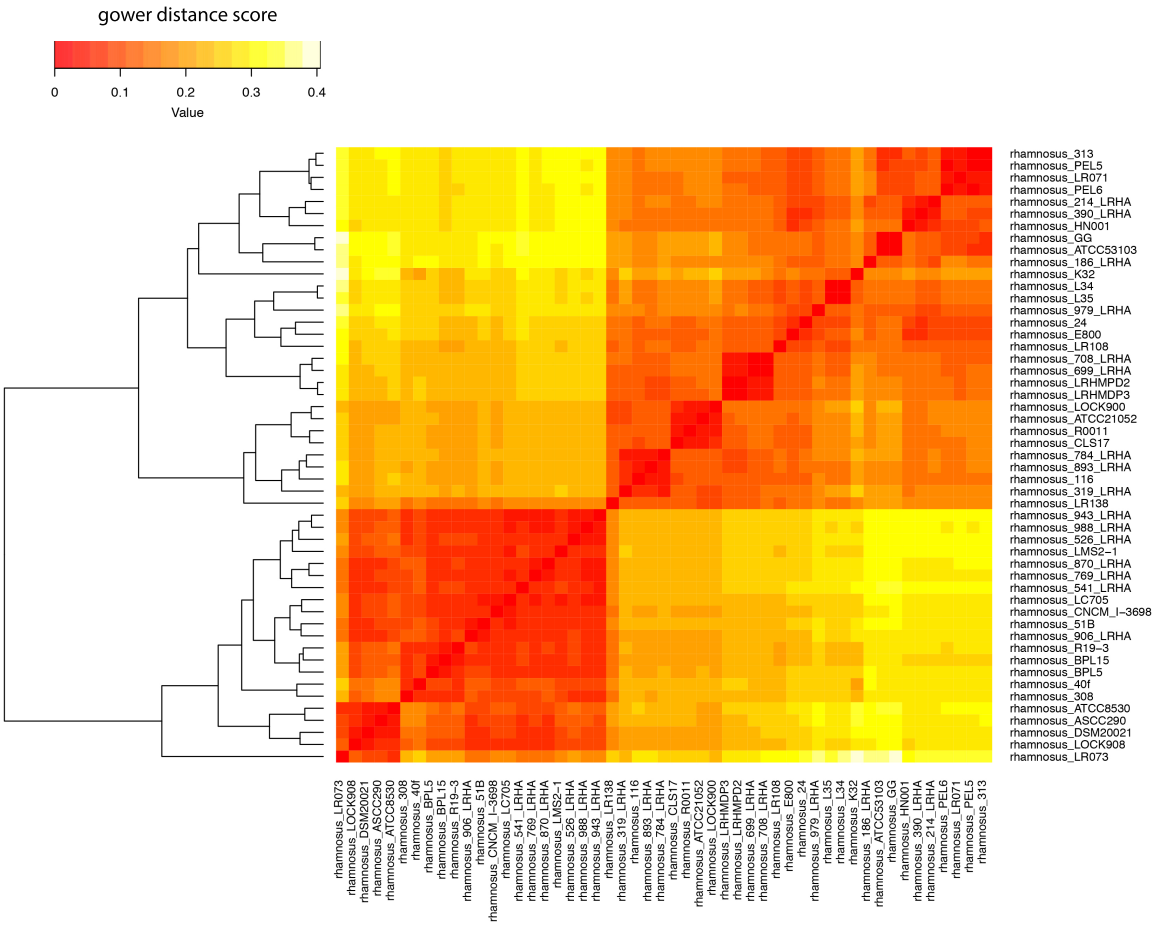

Figure S6.7 Pan-genome heatmap of *L. rhamnosus.* Heatmap clustering according to 4889 pan-genome genes from 51 *Lactobacillus rhamnosus* genomes. Gower distance score based on ANI: Red = more similar, white = less similar, strains with deviating cluster behavior marked with red arrows.

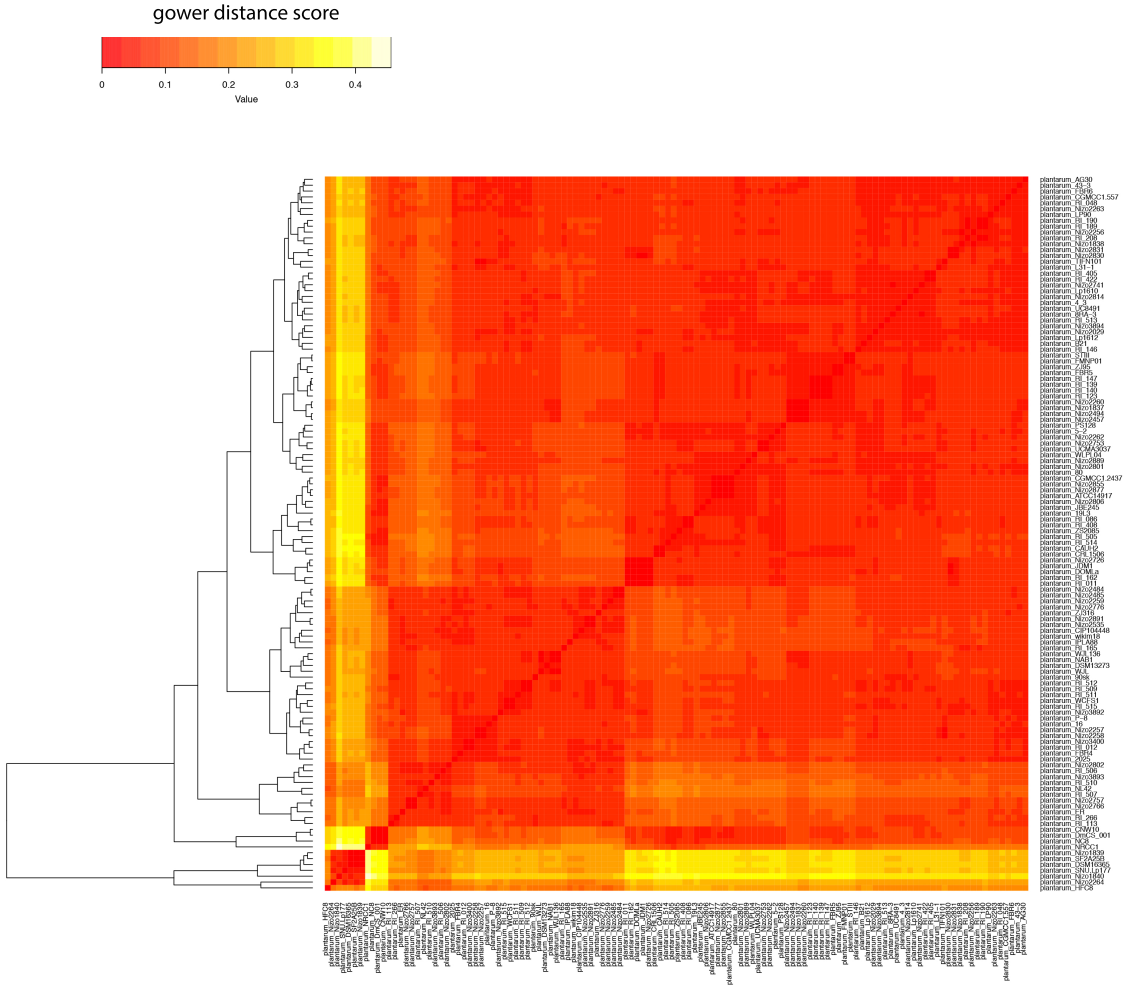

Figure S6.8 Pan-genome heatmap of *L. plantarum.* Heatmap clustering according to 7610 pan-genome genes from 122 *Lactobacillus plantarum* genomes. Gower distance score based on ANI: Red = more similar, white = less similar, strains with deviating cluster behaviour marked with red arrows.

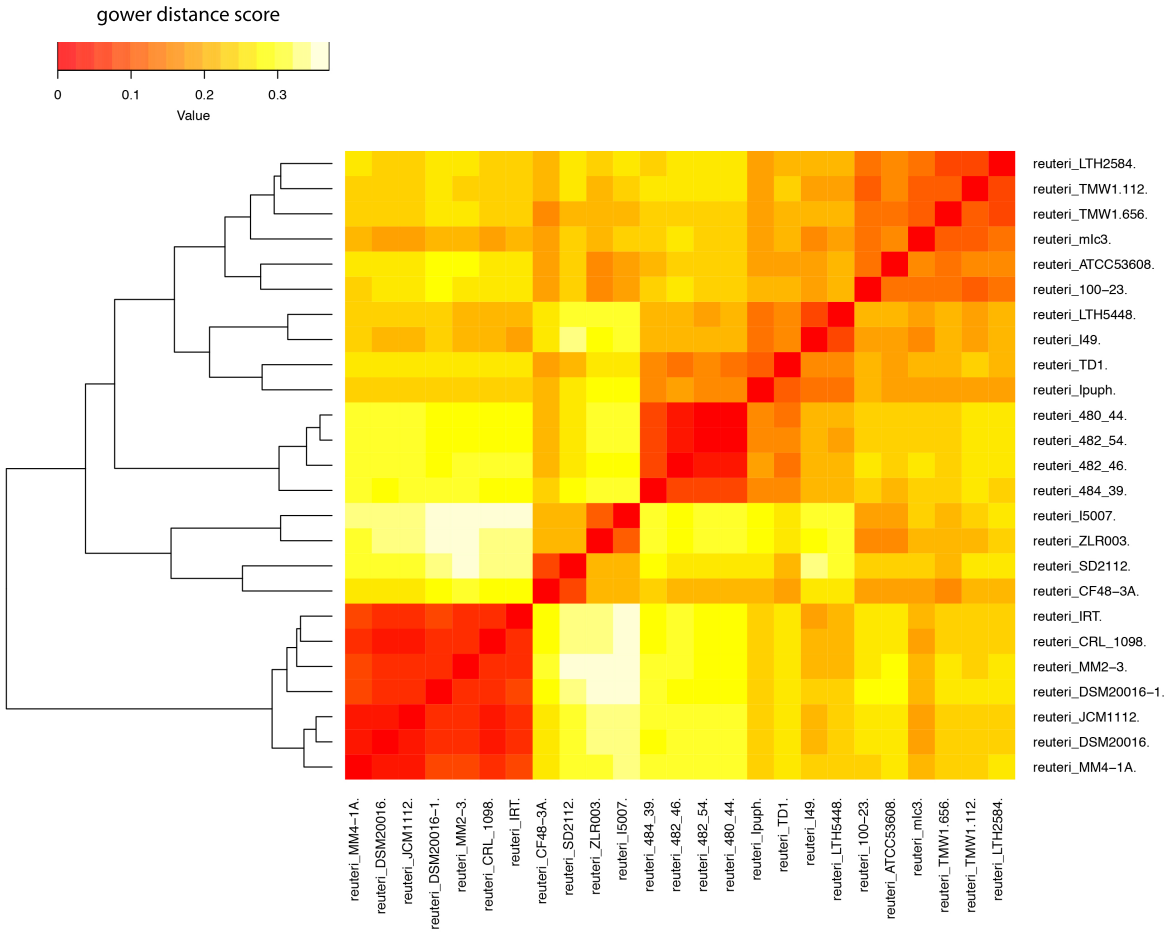

Figure S6.9 Pan-genome heatmap of *L. reuteri.* Heatmap clustering according to 3960 pan-genome genes from 25 *Lactobacillus reuteri* genomes. Gower distance score based on ANI: Red = more similar, white = less similar, strains with deviating cluster behavior marked with red arrows.

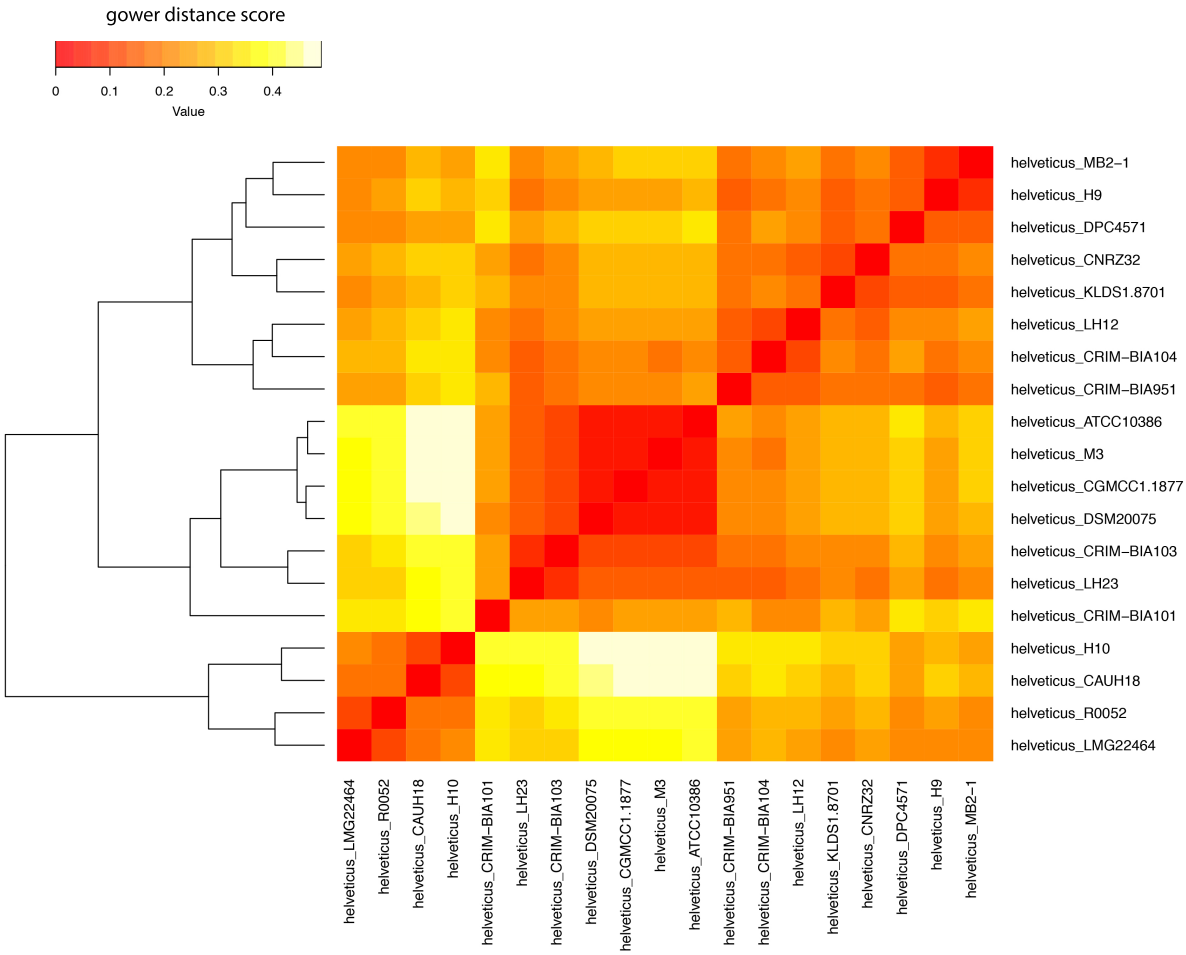

Figure S6.10 Pan-genome heatmap of *L. helveticus.* Heatmap clustering according to 3350 pan-genome genes from 19 *Lactobacillus helveticus* genomes. Gower distance score based on ANI: Red = more similar, white = less similar, strains with deviating cluster behavior marked with red arrows.

Chapter 7

General conclusions and perspectives

General conclusion

On this planet with limited resources and increasing population, food waste is a luxury we can't afford anymore. Food products have to be available these days in each location, minimally processed and ready-to-eat. There is a conflict of objects between food safety, reduction of food waste and consumer behaviour. Microbial spoilage of fermented food products can be reduced by the direct application of a protective culture and thereby offering a possible reduction of chemical preservatives.

In this work, it was demonstrated how protective cultures can be selected from existing strain collections and applied in industrial food fermentations. However, the approach is basic and can be expanded to describe the candidate bacterial strains at more detail. Through a combination of phenotypic-genotypic screening with a comparative genomics, related genes behind uncommon phenotypes can be determined. However, this approach ignores different activities of genes in different strains. A gene might be less active due to mutation in its promoter sequences, instability of its mRNA or mutations in active sites of the protein. Further, the impact of gene on the cell might be minimal due to absence of connecting reactions in a pathways or missing interactions with other genes.

These mechanisms are far more challenging to detect for an algorithm. There are programs available to identify SNPs in genomes (Gardner et al., 2015), but the analysis of these SNPs is complicated. In addition, the number of SNPs in two genomes are typically a few hundred (Chapter 5), making the interpretation off all SNPs highly labour intensive. Therefore, such analyses are more or less restricted to compare a wild type strain with its mutated derivative, for example in *L. plantarum* (van Bokhorst-van de Veen et al., 2013). The interaction between gene products are even more difficult to analyse. However, machine learning has come to a point where this should be possible in the near future (Okser et al., 2014).

The function of a single gene or a module of genes can be proved by creating deletion and overexpression mutants to minimize or maximize the phenotypic properties, respectively. The initial approach in this project was to identify genes responsible for proteinaceous antifungal activity by a combination of phenotypic screening and comparative genomics, followed by the creation and analyses of gene deletion mutants. The gene encoding a subtilisin-like serine protease in *L. plantarum* RI-162 (Chapter 5) was an ideal candidate since the antifungal activity of this gene was demonstrated by another study (Kotb, 2014). However, the knockout vector for target gene replacement via homologues recombination was presumably attacked in our target strain *L. plantarum* RI-162. Modification of the recombination inducing vector by a non-methylation strain

(Spath et al., 2012) didn't work either. We assume that the internal system to digest foreign DNA in the target strain was active against the recombination inducing plasmid (van Pijkeren and Britton, 2014). Since deletion of the target gene for proteinaceous antifungal activity didn't work, the focus was set on metabolic antifungal activity. *L. plantarum* RI-162 showed a tremendous inhibition potential in a 1-ml co-culture, with a single *Lactobacillus plantarum* cell could inhibit 6 x 10^5 cells of *Rhodotorula mucilaginosa* (Chapter 5). The potential of this *Lactobacillus* culture for protection of dairy products against other yeasts is of great interest. In our study, all tested *L. plantarum* strains showed strong antifungal activity, which points towards a strong role for lactate. Their application potential for fruit juices or silage should be tested as well (Crowley et al., 2013b).

Beside the potential antifungal protective cultures, we tested 6 potential antibacterial protective cultures in a salami fermentation project. The strains, that were sequenced and displayed antibacterial activity all harboured a potential bacteriocin encoding gene. Further, the antibacterial activity was protease sensitive. Therefore, is it likely that the antibacterial activity is caused by bacteriocin production. All of them were able to grow in a meat matrix and presumably produced bacteriocin *in situ*. Isolate *L. plantarum* RI-409 was selected as a potential protective culture for salami fermentation and was further tested in an industrial-scale fermentation. Since the *in situ* activity decreased in the industrial-scale fermentation compared to the small-scale fermentation, the protective culture needs further improvements. Optimization potential remains in terms of stability, efficiency and easy handling. The potential application of the 5 other tested strains for different meat products should be examined in combination with a fermentation-genomics platform (Bron et al., 2012).

The enterococci concentration reached up to 6.5 x 10^6 cfu/g in the small-scale fermentation of a raw milk soft cheese. A decrease of 97% was achieved with an application of a protective culture. For an initial approach this reduction leaves room for optimization such as that the protective culture supplementation could done at beginning of the raw milk incubation phase and not at the same time with the rennet.

Bacteriocin are bactericidal, therefore the use of protective cultures producing bacteriocins, or the bacteriocins themselves, can be expanded to non-food products. Fire blight is one of the most important pome fruit pathogens worldwide triggered by the Gram-negative bacterium *Erwinia amylovora* (Gusberti et al., 2015). Microbial pest management with lactobacilli was successfully demonstrated when fire blight was inhibited by a *L. plantarum* strain (Roselló et al., 2013) and confirms the potential of protective cultures in agriculture. Surface protection is another field for protective culture or bacteriocin application. Multi-drug resistant enterococci are frequently isolated from surfaces in hospitals or medical tools and are a major health problem nowadays (Arias et al.,

2010; Woods et al., 2017). Bacteriocin sprayed on medical surfaces could kill the enterococci and this be a potential tool to reduce hospital related enterococci infections.

Bacterial classification according to a phenotype dominated polyphasic approach creates minor problems as described in chapter 6. However, classification according to phenotypic criteria can be misleading, since a certain parameter can occur also in only 90% of the strains, and the phenotype is still used to determine the species or subspecies (Hammes and Hertel, 2009). This "phenotypic range" approach increases the robustness of the clusters, otherwise a single point mutation in a selected gene could change the annotation of the strain. On the other hand, phenotypes can be spread over population if a certain criterion is encoded on a plasmid and easily exchanged by HGT. Nevertheless, classification is only based on selected properties, which might be interesting for the researcher or easy to test in the laboratory. Bacteria are classified based on a combination of selected phenotypic parameters, instead of their actual "complete" phenotype or even their phenotypic potential. Finally, the determination of a phenotype requires the correct screening conditions which might not be the same for all strains. Therefore, a classification solely based on strict mathematical calculations such as ANI, core- or pan-genome clustering is more favourable. However, the presence of a gene with an annotated function doesn't mean that this gene is active, as we demonstrated for a presumed catalase gene in *L. casei* ATCC 393 (Acedo-Félix and Pérez-Martínez, 2003; Chapter 6). Therefore, classification according to the genome versus genome comparison would classify more on a potential phenotype instead of a measurable one.

Finally, pan- and core-genome clustering revealed a good overview about the shared genomic elements and functional categories within species or genera. In fact, clustering according to the core genome can be termed as a "perfect" MLST, since it implements all available data into the clustering. MLST is an accepted technique to cluster strains on species level. The cost for an MLST or a core-genome analysis are the same since whole genome sequencing cost decreased. However, the validity of the clustering increases since the clustering is based on several hundreds of genes instead of a dozen. The pan-genome clustering even includes more genes into the clustering and should result in an even better representation since the strain comparing is based on the entire genome instead only on the core-genome.

Perspectives

High-throughput screening of a culture collection for specific criteria is in most laboratories only limited by material costs and available full-time equivalents. Automation with pipetting robots drastically increases the speed of testing and reduces the tested sample volume and hence the screening costs for a strain or a function is reduced drastically. The future of phenotypic screening will be for sure a more automated screening approach. The approach for growth condition screening and antimicrobial activity, demonstrated in this study, can be easily adapted to an automated process structure. The same might be true for strain isolation approach. Environmental sampling and cultivation of collected isolates can be done systematically. Finally, whole genome sequencing completes the strain characterization approach and enhancing safety. Sequencing of genomes is already well established and might evolve to a more in-house application instead of external sequencing. Since the technique produces already high-quality data a further improvement might be the amount of necessary genetic material for sequencing. Environmental sampling significantly improved with the development of single-cell sequencing, an approach using the genetic content of only a few cells to determine the genome sequence (Gawad et al., 2016).

Based on this automated toolset, genotypic patterns for a certain phenotype can be proposed with comparative genomics. Therefore, a machine learning algorithm would crawl through the genomes and proposes candidate genes for phenotypes. However, since phenotype regulation is more complex than just the presence or absence of a gene or a genetic pattern, the detection algorithm would need some development. The following major criteria should be implemented into a future algorithm: (1) presence/absence of a gene or genetic pattern; (2) complete presence of pathways or patterns; (3) genetic regulation in promoters; (4) single nucleotide polymorphism; (5) allosteric regulation of genes; (6) and visualization of the results. This new developed set of tools would provide a systematic species description and gene annotation.

Finally, selected strains have to be tested in food fermentations. The more the initial screening represents conditions similar to the food product, the better the final selection of a few potential starter or protective strains fit the product. Such cultures can then be tested in an industrial-scale approach against the natural spoilage flora or an intentionally added spoilage organism.

This screening and selection approach can be extended to biotechnological challenges other than food applications such as environmental pollution. An oil spill, as happened on the Deepwater Horizon platform in the Gulf of Mexico, are ecological nightmares. Intoxication with crude oil leads to severe health problems like cardiotoxicity in sea animals (Incardona et al., 2014). Systematic screening for bacteria to help digesting crude oil into harmless products such as H_2O and CO_2 could

be a strategy to fight against this kind of pollution. There are bacteria that are able to digest components of crude oil (Golyshin et al., 2013). Soil contamination from dioxin straying during the Vietnam war is another biotechnological challenge. Potential dioxin digesting bacterial cultures are already described (Pöritz et al., 2015).

Based on the work of this study and other studies, a new possible model of bacterial classification could be applied in the near future. A genome-genome comparing model would compare a sequenced genome with a database of already calculated genomes. X-means clustering is an approach to cluster a dataset containing an unknown number of clusters (Pelleg and Moore, 2000). The genomes in the database would then rearrange themselves while the user only defines what kind of sequence identity within a cluster is necessary. Based on this approach only a threshold for species sequence identity would be defined to cluster bacteria. The advantages of this model would be a defined average nucleotide identity range within species. This eliminates fuzzy intermixes species such as *L. casei* and *L. paracasei* with more or less identical genomes or very narrow species. To establish this model, faster algorithms and more efficient data handling are a necessity.

No matter what kind of a model is chosen by the taxonomy consortium, adjustments in the next years have to include a better involvement of complete genome data. The sooner a standardized approach is defined, the fewer work has to be done in reannotation of strains and species.

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