CORA Cork Open Research Archive
Cartlann Taighde Oscailte Chorcaí

Title	Advances in the genomics and metabolomics of dairy lactobacilli: A review
Authors	Stefanovic, Ewelina;Fitzgerald, Gerald F.;McAuliffe, Olivia
Publication date	2016-08-31
Original Citation	Stefanovic, E., Fitzgerald, G. and McAuliffe, O. (2016) 'Advances in the genomics and metabolomics of dairy lactobacilli: A review', Food Microbiology, 61, pp. 33-49. doi:10.1016/j.fm.2016.08.009
Type of publication	Review
Link to publisher's version	10.1016/j.fm.2016.08.009
Rights	© 2016, Elsevier Ltd. All rights reserved. This manuscript version is made available under the CC-BY-NC-ND 4.0 license. - http://creativecommons.org/licenses/by-nc-nd/4.0/
Download date	2026-11-08 16:11:11
Item downloaded from	https://hdl.handle.net/10468/5004

UCC
University College Cork, Ireland
Coláiste na hOllscoile Corcaigh

Advances in the genomics and metabolomics of dairy lactobacilli: A review

Ewelina Stefanovic[a,b], Gerald Fitzgerald[b] and Olivia McAuliffe[a,*]

[a]Teagasc Food Research Centre, Moorepark, Fermoy, Ireland

[b]School of Microbiology, University College Cork, Cork, Ireland

*Corresponding author: Olivia McAuliffe, Teagasc Food Research Centre, Moorepark Fermoy, Ireland. Tel: +3532542609, Fax +3532542340, Email: olivia.mcauliffe@teagasc.ie

Abstract

The *Lactobacillus* genus represents the largest and most diverse genera of all the lactic acid bacteria (LAB), encompassing species with applications in industrial, biotechnological and medical fields. The increasing number of available *Lactobacillus* genome sequences has allowed understanding of genetic and metabolic potential of this LAB group. Pangenome and core genome studies are available for numerous species, demonstrating the plasticity of the *Lactobacillus* genomes and providing the evidence of niche adaptability. Advancements in the application of lactobacilli in the dairy industry lie in exploring the genetic background of their commercially important characteristics, such as flavour development potential or resistance to the phage attack. The integration of available genomic and metabolomic data through the generation of genome scale metabolic models has enabled the development of computational models that predict the behaviour of organisms under specific conditions and present a route to metabolic engineering. Lactobacilli are recognised as potential cell factories, confirmed by the successful production of many compounds. In this review, we discuss the current knowledge of genomics, metabolomics and metabolic engineering of the prevalent *Lactobacillus* species associated with the production of fermented dairy foods. In-depth understanding of their characteristics opens the possibilities for their future knowledge-based applications.

Keywords: *Lactobacillus*, dairy, genomic, metabolic engineering

1. Introduction

The lactic acid bacteria (LAB) are a group of Gram-positive, non-sporulating, aerotolerant bacteria, with a fermentative metabolism that has lactic acid as the principal final product. The LAB group comprises seven genera: *Lactococcus, Lactobacillus, Enterococcus, Pediococcus, Streptococcus, Leuconostoc and Oenococcus* (O'Sullivan et al., 2009). The practical importance of the organisms within this group is unquestionable as they find application in industry, food and health-related fields. In the food industry, LAB are widely used in the production of fermented dairy, meat and vegetable products as well as in wine and sourdough production (Pfeiler and Klaenhammer, 2007; O'Sullivan et al., 2009). In addition, the production of antimicrobials or bacteriocins by certain species of the LAB has prompted their use as biopreservative agents in foods (Cleveland et al., 2001; Cotter et al., 2005; De Vuyst and Leroy, 2007). Other members of the LAB group exhibit health benefits and are often used as probiotics in the treatment of intestinal infections, inflammatory bowel disease and allergy development (Ljungh and Wadstrom, 2006). Members of the LAB group have also been suggested for use in mucosal vaccines as delivery vehicles for vaccine antigens (Bermudez-Humaran et al., 2011; Villena et al., 2011; Wyszynska et al., 2015). The wide variety and number of applications of the LAB raises the need to correlate industrially and clinically important features with genomic information to examine the possibilities for exploitation of their metabolic potential, thus improving their use in biotechnological and health-related applications. The complete and draft genomes of many LAB species are available in online databases (Genome Online Database, https://gold.jgi.doe.gov/, NCBI database http://www.ncbi.nlm.nih.gov/genome/, Ensemble Genomes database http://ensemblgenomes.org/, etc.) and they present valuable sources of information regarding genetic diversity and the metabolic potential of strains. In addition, state-of-the-art

developments in genomics and metabolomics provide the tools for a more 'knowledge-based' approach to selection of desirable cultures for application in industry (McAuliffe, 2017). LAB are phylogenetically closely related, but the number of predicted protein-coding genes in the LAB varies between 1,700 and 2,800 (Makarova et al., 2006). Genomic studies of members of the LAB have confirmed the overall trend of minimisation of genomes, which is in close agreement with the transition to nutritionally rich environments. Nevertheless, some gene families were expanded by gene duplication or acquisition of paralogous genes via horizontal gene transfer (HGT) (Makarova et al., 2006). Based on the analysis of the genomes of 12 LAB species it was concluded that the core LAB genome, comprising orthologous genes conserved in all analysed genomes (Collins and Higgs, 2012), consists of 567 genes, mostly encoding translation, transcription and replication processes, but 41 of the genes were uncharacterized and 50 had only general functions predicted. This study also identified two core genes exclusive for LAB, the products of which are LysM (peptidoglycan-binding) domain and the highly conserved LaCOG01237 with no known domains, but based on its localisation, it is probably involved in modification of tRNA (Makarova et al., 2006).

The genus *Lactobacillus* comprises a diverse group of bacteria currently consisting of more than 200 species and subspecies (Sun et al., 2015a) that share the common features of other LAB, including low GC content, acid tolerance and conversion of sugars to lactic acid as one of the main end products of metabolism. Species of lactobacilli are present in various environments such as plants, fermented food products (dairy, meat, wine), and both the human and animal gastrointestinal tracts. Their ability to ferment milk, meat and plant material presents the basis for their artisanal and industrial usage (Sun et al., 2015a). Apart from this, strains of *Lactobacillus* are well known for their probiotic properties (Lebeer et al., 2008).

78 This review aims to present recent findings related to the genus *Lactobacillus*, with a
79 particular emphasis on strains commonly used in the production of fermented dairy foods.
80 Genomic features of the main dairy species will be discussed, including their remarkable
81 niche specialisation. Advancements in our knowledge through genomic analysis of key
82 attributes of dairy species will also be reviewed. Finally, innovations in the applications of
83 genome scale metabolic models and metabolic engineering, highlighting new possibilities in
84 exploitation of strains of *Lactobacillus,* are also discussed.

2. Genomics of the *Lactobacillus* genus

Due to their importance in various biotechnological and health-related applications, there has been a growing interest in exploring the genomic features of the genus *Lactobacillus,* which is the largest and most diverse genus of LAB (Broadbent et al., 2012). *Lactobacillus* genomes range in size from 1.23 Mbp (*Lb. sanfranciscensis)* to 4.91 Mbp (*Lb. parakefiri)* (Sun et al., 2015a). Species of this genus are present in dairy products *(Lb. delbrueckii* ssp. *bulgaricus, Lb. helveticus*), human and animal gastrointestinal tracts (*Lb. acidophilus* and *Lb. gasseri*) or in a variety of niches (*Lb. plantarum, Lb. pentosus, Lb. brevis,* and *Lb. paracasei*) (Smokvina et al., 2013). The first genome of the *Lactobacillus* genus sequenced was *Lb. plantarum* WCFS1 (Kleerebezem et al., 2003) followed by *Lb. johnsonii* NC533 (Pridmore et al., 2004) and *Lb. acidophilus* NCFM (Altermann et al., 2005). These studies revealed some interesting genomic features of the *Lactobacillus* genus, such as lifestyle adaptation islands in *Lb. plantarum* WCFS1, lack of general biosynthetic pathways in the probiotic strain *Lb. johnsonii* NC553 and unique structures called potential autonomous units (PAU) in *Lb. acidophilus* NCFM, all of which triggered further investigation and comparison with newly sequenced strains of the same species. Currently (July 2016), there are 214 *Lactobacillus* genome sequencing projects available in public databases (http://www.ncbi.nlm.nih.gov).

The pangenome (or supragenome) is considered as the full set of all genes within a selected genome set (species, genera or higher taxonomic groups) (Medini et al., 2005; Collins and Higgs, 2012). The size of the pangenome generated for *Lactobacillus* and associated genera of LAB reaches almost 45000 gene families, while 73 genes mainly responsible for cell growth and replication make up the core genome (Sun et al., 2015a). In a study based on the features of 20 complete *Lactobacillus* genomes representing 14 species whose genomes ranged from 1.8 to 3.3 Mbp, the number of proteins within these genomes was between 1721-3100 (Kant et al., 2011). The estimated size of the pangenome of the *Lactobacillus* genus

consists of almost 14000 proteins, while the core genome consists of 383 orthologs (Kant et al., 2011). This number is higher than the 141 core genes reported in the study of Claesson et al. (2008), who used more strict criteria and took into account only 12 completely sequenced *Lactobacillus* genomes. Over 100 out of 383 genes of the *Lactobacillus* core genome were organised in operon-like clusters that are conserved in other related Gram-positive bacteria (Kant et al., 2011). Among 41 genes specific for *Lactobacillus*, 13 were predicted to code for ribosomal proteins, and 13 were annotated as hypothetical (Kant et al., 2011). Taken together, comparative genomic studies of lactobacilli confirmed the overall trend observed in other LAB, which is loss of ancestral genes and minimisation of genomes, as well as acquisition of genes by HGT as a response to adaptation to the primary habitat of these bacteria (Makarova et al., 2006).

The main species of *Lactobacillus* used as starter cultures for the production of fermented dairy products are *Lb. delbrueckii* and *Lb. helveticus*, but more recently, a group of non-starter lactobacilli has attracted growing attention due to their contribution to the quality and characteristics of the final products. This group includes *Lb. casei*, *Lb. paracasei*, *Lb. rhamnosus* and less often *Lb. plantarum*. Additionally, dairy products can be used as "carriers" of probiotic strains, such as *Lb. acidophilus* and *Lb. rhamnosus*. Therefore, general information regarding genomics of these most important dairy-related lactobacilli is presented in Table 1, and specific genomic features of these species will be discussed in more detail.

2.1 *Lactobacillus delbrueckii*

From the perspective of the dairy industry, *Lactobacillus delbrueckii* contains two industrially important subspecies: subspecies *bulgaricus* and subspecies *lactis*. Of the 22 genome sequences available for these two subspecies, five are complete sequences. While *Lb. delbrueckii* ssp. *bulgaricus* is widely used in the production of yoghurt, subspecies *lactis* is

used primarily as a starter in the manufacture of cheeses like Emmental, Grana Padano and Parmigiano Reggiano (El Kafsi et al., 2014). The core genome of the three *Lb. delbrueckii* ssp. *bulgaricus* strains (2038, ATCC 11842 and ATCC BAA-365) consists of 1276 genes, with the genomes of strains 2038, ATCC 11842 and ATCC BAA-365 consists of 211, 150 and 166 unique genes, respectively (Hao et al., 2011). An alignment of the three genomes revealed two duplicated segments flanking the predicted replication terminus, but strain 2038 has a unique 8.5 kbp region between the duplication regions, which could be the reason for the bigger genome size (1.87 Mbp compared to 1.86 Mbp ATCC 11842 and ATCC BAA-365). This region is most likely inherited from an ancestor, but lost in the other two strains, probably due to their independent evolution from strain 2038 (Hao et al., 2011).

A genome analysis of sequenced *Lb. delbrueckii* strains showed that the average GC and GC3 content (GC at codon position 3) in coding sequences (CDSs) is approximately 52% and 65%, respectively (El Kafsi et al., 2014), which is in agreement with a previously reported higher GC content in *Lb. delbrueckii* ssp. *bulgaricus* compared to other lactobacilli (van de Guchte et al., 2006). Higher GC content is a sign of rapid ongoing evolution in these species (O'Sullivan et al., 2009). In both subspecies, decay and inactivation of superfluous genes was evident, indicating an evolutionary trend towards adaptation to the dairy environment. A deeper insight into the genomics of these subspecies revealed some interesting genetic differences. Firstly, it was shown that the size of the ssp. *bulgaricus* genomes is smaller compared to ssp. *lactis* (1.8 Mbp and 2 Mbp, respectively). However, the number of CDS did not differ considerably between the two subspecies, as it varied in range from 1333-1783 for subspecies *bulgaricus* to 1593-1721 for subspecies *lactis*. Comparison of the core proteomes of five ssp. *lactis* and five ssp. *bulgaricus* strains surprisingly revealed quite similar sizes of core proteomes and significant overlapping of these. The overall core proteome consists of 989 proteins, with 65 proteins specific for ssp. *lactis* and 25 proteins specific for ssp.

bulgaricus. The majority of the 65 specific ssp. *bulgaricus* proteins have unknown functions, while those of known function are mainly membrane transporter-associated proteins. The 25 specific ssp. *lactis* proteins have mainly known functions, involved in carbohydrate and amino acid metabolism. For both subspecies, fragments of other subspecies-specific genes could be found as pseudogenes, implying that differential loss of genes caused subspecies divergence. Another important finding of the extensive genomic analysis is re-classification of strain ND02, which was designated as ssp. *bulgaricus* but confirmed to be ssp. *lactis*, not only due to the larger genome but also due to the higher number of insertion sequences (IS). Besides that, it was previously shown that *Lb. delbrueckii* subspecies can be distinguished based on the number of *Eco*RI sites in their 16S rDNA sequences, where ssp. *lactis* possesses one, and ssp. *bulgaricus* has two restriction sites (Giraffa et al., 1998). The detailed analysis of 16S rRNA of strain ND02 showed it did not contain two specific restrictions sites, adding an argument to its re-classification as ssp. *lactis* (El Kafsi et al., 2014).

2.2 *Lactobacillus helveticus*

Lactobacillus helveticus represents an important starter for the production of Swiss-type and long-ripened Italian cheeses (Broadbent et al., 2011; Giraffa, 2014). Apart from the dairy environment, *Lb. helveticus* strains are present in fermented plant and meat materials as well as the gastrointestinal and urogenital tracts of humans and animals and their probiotic activity is confirmed (Strahinic et al., 2013; Taverniti and Guglielmetti, 2012). While the complete genome sequences of eight strains are currently available, a total comparative genomic study of this species has not been performed to date, and information regarding the core, pan and specific genomes is not currently available, to the best of our knowledge. Strains sequenced to date originate from various fermented dairy products, such as koumis, sour milk, kurut, or they were used as industrial starters. Genome sizes vary from 1.87 to 2.38 Mbp, with a GC content of 37%, and the number of genes ranges between 1743 - 2540.

2.3. The *Lactobacillus casei/paracasei* group

The taxonomic status of *Lb. casei* is still a matter of much debate (Smokvina et al., 2013) as molecular studies have implied that the majority of *Lb. casei* strains are more related to *Lb. casei* ATCC 334 (also named *Lb. paracasei*) than to the official type strain *Lb. casei* ATCC 393 (Dellaglio et al., 2002). Because of this uncertainty, the information available for both *Lb. casei* and *Lb. paracasei* will be reviewed together here. The members of this group have been isolated from dairy and plant materials (cheese, wine, pickle, silage) (Toh et al., 2013) and reproductive and gastrointestinal tracts of humans and animals (Cai et al., 2009). In the cheese industry, they are used as adjunct cultures for development of desired flavour (Milesi et al., 2010; Van Hoorde et al., 2010). Besides application in fermented food production, members of this group are well known for their probiotic characteristics (Herias et al., 2005; Ya et al., 2008). Such a diverse range of sources and broad ranging possible applications makes this group one of the best explored species within the *Lactobacillus* genus with eight and seven genome sequences completed for *Lb. casei* and *Lb.paracasei,* respectively, and 27 and 46 draft genome sequences available for *Lb. casei* and *Lb. paracasei,* respectively. Genome sizes range from 2.38 Mbp for *Lb. paracasei* ssp. *tolerans* DMS20258 and 3.27 Mbp for *Lb. casei* Lbs2, with an average GC content of 46.5%. Analysis of the draft sequences of 12 strains of *Lb. casei* of different origins (dairy, plant and human) along with five fully sequenced genomes have determined that the size of the *Lb. casei* pangenome is 3.2 X the average genome size, consisting of 1715 core and 4220 accessory genes (Broadbent et al., 2012). Another comparative study (Yu et al., 2015) performed on 12 draft *Lb. casei* genomes revealed 806 novel regions larger than 500 kbp harbouring both hypothetical proteins and mobile genetic elements in these strains compared to the seven complete genomes. This suggested that the *Lb. casei* pangenome expands with every new sequenced genome and potential for environmental adaptation within the species increases (Yu et al., 2015).

Similarly, when 37 genomes of *Lb. paracasei* were analysed, 1800 core and 4200 accessory genes were detected (Smokvina et al., 2013). A common feature of all 37 analysed genomes of *Lb. paracasei* is a cluster involved in the conversion of branched chain alpha-keto acids into branched chain fatty acids important for maintenance of the colonic epithelium. This gene cluster is unique for *Lb. paracasei*, implying its acquisition through HGT (Smokvina et al., 2013). Pangenome analysis revealed the ability of *Lb. paracasei* to utilise a broad range of carbohydrates. In total, 74 sugar utilisation cassettes were detected 15 of which belonged to the core genome. These cassettes were localised on two genomic islands (Smokvina et al., 2013), structures usually connected with the environmental adaptation (described in details below).

2.4 *Lactobacillus acidophilus*

Taxonomically, *Lactobacillus acidophilus* is part of a larger complex comprising several species: *Lb. acidophilus*, *Lb. amylovorus*, *Lb. crispatus*, *Lb. gallinarum*, *Lb. gasseri*, and *Lb. johnsonii* (Berger et al., 2007; Ramachandran et al., 2013). Strains of *Lb. acidophilus* are often used in dairy products as probiotics and as flavour contributing strain in certain dairy products, such as yoghurt, sweet acidophilus milk and cheese (Buriti et al., 2005; Ong et al., 2007; Ejtahed et al., 2011). The genome of *Lactobacillus acidophilus* NCFM was the first *Lb. acidophilus* to be sequenced (Altermann et al., 2005). Presently, 16 strains of this species have been sequenced, with three complete genomes available. Genomes range in size from 1.25 - 2.05 Mbp, with GC content of 34.7%. Although phenotypic and biochemical characterisation of strains show a certain level of diversity, genotypic analysis indicates less variation within genomes of this species (Ramachandran et al., 2013; Stahl and Barrangou, 2013; Bull et al., 2014). In a recent study reporting the genome sequences of *Lb. acidophilus* strains isolated from yoghurt (Iartchouk et al., 2015), the alignment of the three sequenced genomes (FSI4, NCFM, and La-14) confirmed a high level of genome similarity for these

strains at the DNA level. Similarly, alignment of La14 and NCFM showed extremely high similarity between these two strains and synteny with ATCC 4769 (Stahl and Barrangou, 2013). Strain 30SC was initially designated as *Lb. acidophilus*, but unlike other strains of this species, it possesses 2 plasmids and has higher GC content (38%) (Stahl and Barrangou, 2013). After detailed phylogenetic analysis of its genome, it was re-classified as *Lb. amylovorus* (Bull et al., 2014)

Intraspecific diversity of 33 *Lb. acidophilus* strains was examined by whole genome multi locus sequence typing (wgMLST), at 1864 loci defined in the *Lb. acidophilus* NCFM genome sequence (Bull et al., 2014). It was found that the core genome comprised 1815 genes, which makes up to 97.4% of *Lb. acidophilus* NCFM loci. A number of commercial strains analysed in this study showed a narrow window of variation, unlike the type strains analysed where a somewhat higher level of variation in loci was detected. When a pairwise comparison of selected isolate sequences was performed with the NCFM strain, it confirmed that the genetic variation in the core genome was predominantly the effect of single nucleotide polymorphism (SNP). Pairwise analysis also revealed partial evidence of gene decay, during which phage, mucus-binding and sugar metabolism genes were lost. Similar findings were observed at the phenotypic level where no significant differences between the commercial or culture collection strains was observed, following analysis by API 50CHL. An interesting finding of this study is that all investigated isolates showed no evidence of extrachromosomal DNA, such as plasmids, and no evidence of an active phage, again confirming the stability of *Lb. acidophilus* genomes. However, three prophage remnants termed Potentially Autonomic Units (PAU) discovered in NCFM genome (Altermann et al., 2005) and a novel region with phage related functions showed variable presence in other *Lb. acidophilus* isolates. While PAU1 was present in all analysed isolates, PAU2 and PAU3 were present in commercial isolates, but variably present in culture collection isolates (Bull et al., 2014).

2.5 *Lactobacillus rhamnosus*

Lactobacillus rhamnosus is present in various dairy products, such as cheese and yoghurt, but also in human cavities and gastrointestinal tract (GIT) (Douillard et al., 2013; Kant et al., 2014). In dairy products, it is mainly present as part of the non-starter LAB (NSLAB) in Italian cheese varieties (Gobbetti et al., 2015), and there is evidence of its positive effect on flavour development in these products (Sgarbi et al., 2013; Innocente et al., 2016). However, its main application is as probiotic cultures (Tuo et al., 2013), often administered through fermented dairy products. To date, 102 genome sequences have been elucidated, with the completed sequences of six strains available. The size of the genomes range from 2.52 Mbp for strain MTCC 5462 up to 3.41 Mbp for strain CRL1505, and the average GC content is 46.7%. General genomic features of this species were determined based on 100 sequenced strains of various origin (cheese, yoghurt, vaginal cavity, oral cavity, intestinal tract, abscess, blood, clinical isolates) mapped according to the reference strain *Lb. rhamnosus* GG. The number of shared genes between these 100 strains and strain GG ranged from 87-100% (Douillard et al., 2013). The pangenome analysis based on the complete or draft genomes of 13 strains, originating from various environments (milk, human airways, faeces, dairy starter, infected dental pulp, Cheddar cheese and gut biopsy), estimates a total of 4893 genes, 1.6 X the average size of a *Lb. rhamnosus* genome (Kant et al., 2014). Pangenome studies show that, in general, the rate of increase of the size of the pangenome slows down with every additional genome being sequenced (Kant et al., 2014). As the pangenome curve of *Lb. rhamnosus* reaches a plateau at about 5000 genes, it is predicted that with only a few more additional genomes of strains from different origins would be sufficient to reach total genome variability of the species (Kant et al., 2014). The core genome of *Lb. rhamnosus* is estimated to encode 2095 genes, or approximately 43% of the pangenome. There are at least 75 genes present only in *Lb. rhamnosus* species, and the majority of these are hypothetical proteins

followed by membrane transporters, transcriptional regulators and glycosyl-transferases. The dispensable genome, which contains genes present in two or more strains (Medini et al., 2005), of *Lb. rhamnosus* is estimated to contain 2798 genes, and the number of unique (strain-specific) genes is 855, which is approximately 30% of the dispensable genome. Most of the dispensable genes in the *Lb. rhamnosus* pangenome are annotated as hypothetical and it remains unknown what proportion of these would actually encode functional proteins (Kant et al., 2014).

2.6 *Lactobacillus plantarum*

Lactobacillus plantarum is present in many ecological niches ranging from vegetables, meat, dairy products and gastro-intestinal tract. Apart from a prominent role in fermentations such as sourdough (Corsetti and Settanni, 2007), strains of this species are present in dairy fermentations and non-starter flora (Settanni and Moschetti, 2010; Gobbetti et al., 2015). Besides that, they are well known for their probiotic characteristics (Siezen and van Hylckama Vlieg, 2011). To date, 114 genome sequences are publically available, with 18 completely sequenced genomes. The genome of this species is one of the largest in the *Lactobacillus* group, with a size of approximately 3.4 Mbp, and a GC content of 44.4%. In an extensive study, 185 isolates from different environments were phenotypically characterized, and based on the observed phenotypic diversity, a set of 42 candidates were selected for genomic analysis (Siezen et al., 2010). The core genome of *Lb. plantarum* was found to comprise 2050 - 2200 genes. Approximately 120 fully conserved genes were unique to *Lb. plantarum*. Many of the unique genes encode hypothetical proteins, while some genes encode functions that could be used for phenotyping. The two candidates are a conserved cluster for tartarate and sulfur uptake and metabolism, which are associated with plant habitats (Siezen et al., 2010). The reference genome WCFS1 itself has over 50 genes not found in any of the other selected strains isolated from different environments. Most notable are three gene

clusters encoding exopolysaccharide, a putative macrolide and a non-ribosomal synthesized hybrid peptide-polyketide, all of which take part in the interaction with environment. They were most likely acquired in a recent evolutionary event due to their GC content, suggesting adaptations necessary for survival in a specific niche (Siezen et al., 2010). Apart from these 50 genes, all other strains were estimated to lack between 9% and 20% of genes present in the reference genome, WCFS1. These genes are mainly organised in functional gene clusters, or cassettes as parts of operons and they encode prophages, restriction/modification systems, exopolysaccharide, bacteriocin and non-ribosomal peptide biosynthesis and carbohydrate utilisation components and are located on genomic islands (described in details in the next section) (Siezen et al., 2010; Siezen and van Hylckama Vlieg, 2011).

3. Niche adaptability of lactobacilli

The widespread dissemination of members of the lactobacilli in different environments testifies to their extraordinary niche adaptability. Lactobacilli are present in grass and on plant material, in dairy products, on human skin, in the mouth, intestine and in the female reproductive system (Claesson et al., 2007), habitats with many contrasting environmental conditions (temperature, pH value, available nutrients, and competing microorganisms). Comparative genomic analysis has revealed that adaptation to such highly variable environments is a result of genome evolution and the genetic basis for niche specialisation appears to be the result of eliminating anabolic systems that are not needed through adaptation to nutritionally rich habitats, such as milk. On the other hand, in all LAB, including lactobacilli, duplications of genes coding for transporters and metabolism of carbohydrates, amino acid transporters and peptidases occurred, further enhancing the ability of these species to live in nutrient-rich environments (Fig. 1a) (Douglas and Klaenhammer, 2010; Makarova and Koonin, 2007; Mayo et al., 2008).

3.1 Horizontal gene transfer (HGT) is the main pathway of niche adaptability in lactobacilli

Although gene loss and acquisition, which are the principal events resulting in niche adaptation, occur in different ways, HGT via bacteriophages, transposons and other mobile elements appears to be an especially dominant force of adaptation to novel environments in *Lactobacillus* species (Broadbent et al., 2012), and it is responsible for various genome rearrangements (Rossi et al., 2014). Such events have made the LAB amenable to adaption to different habitats, including milk and other food matrices, plant material, and GIT. Transposons and plasmids present the main mechanism of gene exchange that occurs amongst different taxonomic groups that do not possess strictly controlled restriction/modification systems (Rossi et al., 2014). Both niche specialists and generalists

have undergone multiple genetic changes which have led to restriction or broadening of the possible habitats in which these strains could survive.

Apart from the traditional classes of mobile genetic elements (plasmids and prophages), structures acquired by the host bacteria through HGT comprising mobile elements and genes contributing to the ability of the host to adapt to specific conditions of habitat, are known as genomic islands (GI) (Bellanger et al., 2014). The first record of "lifestyle adaptation" islands in *Lactobacillius* was in the genome of *Lb. plantarum* WCFS1, where numerous genes involved in sugar transportation and metabolism are grouped together in a region characterised by lower GC content (41.5%) than the rest of the genome (44.45%), suggesting recent acquisition by HGT (Kleerebezem et al., 2003). Apart from strong overrepresentation of genes involved in energy metabolism, regulatory proteins coordinating sugar metabolism are also present on GI (Molenaar et al., 2005). In strain *Lb. helveticus* DPC4571, a number of amino acid metabolism genes along with lipid biosynthesis genes were also identified in a region characterised with higher GC content (42% compared to 37% in the rest of the genome) and insertion sequences flanking this region suggest a recent transfer of this GI (Callanan et al., 2008). One of the GI of *Lb. casei* BL23 carries genes for catabolism of myoinosytol, a cyclic polyol not commonly metabolised by LAB and potentially present in degrading plant material (Yebra et al., 2007, Cai et al., 2009). Genomic islands of *Lb. casei* ATCC 334 encode hypothetical proteins and transcriptional regulators, sugar transporters and metabolic enzymes and are characterised by high prevalence of insertion sequences, recombinases, integrases with higher GC content supporting their recent acquisition and a heterologous origin (Cai et al., 2009). The 26 genomic islands of *Lb. rhamnosus* ATCC 53103, isolated from the human gut, include six carbohydrate utilisation gene clusters, which seem to have secured the survival of the strain in a less nutritionally rich environment, such

as the human intestine (Toh et al., 2013). These examples of different genes present in GI confirm their importance for adaptation and survival in specific environmental conditions.

3.2 Niche adaptation studies reveal lactobacilli as niche specialists

Niche specialists can be described as strains that are able to live in a limited number of habitats, while niche generalists have the capacity to populate various environments. Genome analysis of dairy specialists show that these strains have an abundance of sugar transportation, proteolysis and amino acid transportation encoding genes, some of which have undergone duplication as they enable the organism to uptake nutrients from the rich milk environment (Makarova et al., 2006). On the other hand, substantial gene decay has been confirmed in some lactobacilli, such as in the dairy *Lb. casei* strains, which have more than 120 CDS absent. As a result, these strains have improved their ability to survive in the dairy niche but have a reduced capacity for survival in other niches (Cai et al., 2009). In the genomes of dairy LAB, more than 10% of coding genes are present only as pseudogenes (Zhu et al., 2009), which are non-functional due to frameshifts, nonsense mutations, deletions or truncations (O'Sullivan et al., 2009). For example, the dairy isolate *Lb. helveticus* DPC4571 is reported to have 217 pseudogenes, while *Lb. bulgaricus* ATCC 11842 carries a staggering 533 pseudogenes coding for proteins involved in regulating amino acid and nucleotide metabolism and bile salt hydrolysis (Callanan et al., 2008; O'Sullivan et al., 2009). In contrast, species mainly present in the gut, such as *Lb. acidophilus, Lb. gasseri, Lb. reuteri* and *Lb. johnsonii* have either pseudogenes or a low abundance of pseudogenes, which is likely the genetic basis supporting survival of these species in the gut environment (O'Sullivan et al., 2009).

Efforts have been made to find at least a partial correlation between genome characteristics and niche for such a versatile group as *Lactobacillus*. The study of O'Sullivan et al. (2009) compared the genomes of 11 LAB (ten *Lactobacillus* and one *Streptococcus thermophilus*)

arising from different sources. In total, nine genes were identified as niche determinative as they insured survival in the gut or dairy environments. These genes were grouped into four classes that could be used as niche-specific genes for gut and dairy LAB: sugar metabolism, the proteolytic system, restriction/modification systems and bile salt hydrolysis. In contrast to this study, Kant et al. (2011) did not reveal any niche-specific genes in a study that analysed 20 genomes of 14 different *Lactobacillus* species. The possible cause of this observation is that the isolation source does not always correspond to the actual habitat, but rather a transient habitat (Fig. 1b), as some species, like *Lb. plantarum* can be found in various environments (Kant et al., 2011).

Correlation between gene loss and niche adaptation was examined by growing nine *Lb. casei* strains from various isolation sources in chemically defined amino acid media supplemented with one of the substrates representing plant, gut or dairy habitats (Broadbent et al., 2012). The two cheese specialists had the most restricted substrate profiles, with no genes for inulin, sucrose or cellobiose utilisation present in their genomes, while the other strains used a higher number of different substrates, with corn silage isolates growing on 26 different substrates (Broadbent et al., 2012). In the study of Smokvina et al. (2013), niche affinity of *Lb. paracasei* was examined through utilisation of carbon sources as growth factors for a set of strains with diverse origins: plant, mammalian and dairy. The analysis revealed the clustering of seven out of the 16 dairy isolates that could be considered as niche specialists, which had smaller genomes compared to the others (2.8 Mbp average), limited numbers of sugar cassettes and an absence of genes involved in utilisation of plant-derived sugars. This was expected, as the spectrum of sugars in the dairy environment is narrow with lactose dominating. On the other hand, no clear clustering pattern was revealed for plant and mammalian isolates. Plant isolates originate from a broad range of ecosystems that differ in environmental and nutritional conditions, while mammalian isolates come from the gut where

419 they are exposed to constantly changing surroundings due to the presence of food and other
420 microorganisms, and this complicates their precise grouping (Smokvina et al., 2013).
421 Lactobacilli occupy habitats that differ considerably in environmental conditions. The dairy
422 niche bacteria have to be robust enough to survive manufacture and storage conditions
423 encountered during industrial production. In the gut, strains need to be able to survive in the
424 presence of other intestinal microbiota and resist bile salts and other harsh conditions found
425 in the gut (Senan et al., 2014). A genome-scale study based on genes involved in stress
426 responses of the *Lb. helveticus* strains MTCC 5463 (probiotic strain isolated from a vaginal
427 swab of a healthy volunteer, Senan et al. (2015)) and DPC4571 (a dairy isolate, Callanan et
428 al. (2008)) gave an insight into genes responsible for adaptation to various environments
429 (Senan et al., 2014). When comparing these two genomes for the ability of the strains to
430 survive in a bile-rich environment, it was shown that the MTCC 5463 genome exhibited
431 multiple coding sequences for bile salt hydrolase (bsh). However, the cheese starter
432 DPC4571, adapted to a dairy niche, displayed a total lack of active *bsh* genes. The probiotic
433 strain is exposed to other gut microbiota and in constant competition for successful
434 colonisation and available nutrients. In order to survive in these conditions, it carries a higher
435 number of starvation-induced genes. By contrast, while the dairy strain possessed some genes
436 for starvation proteins, such as phosphate starvation inducible stress-related protein, it was
437 deficient in the gene for the carbon starvation protein CstA. Both strains carried a substantial
438 number of genes that allow response to heat and cold shock, but the molecular chaperones
439 were far more prevalent in the probiotic genome (Senan et al., 2014). Another study
440 performed on *Lb. helveticus* strains confirmed loss of genes encoding mucus-binding proteins
441 from strains adapted to the milk environment, but confirmed their maintenance in probiotic
442 strain R0052, where they are essential for survival and residence of the strain in the gut
443 (Cremonesi et al., 2012).

Another noteworthy conclusion regarding niche adaptability was made when genome sequences of two strains, *Lb. helveticus* DPC4571 and *Lb. acidophilus* NCFM, were compared. The remarkable level of identity of 98% for 16S rRNA sequences was observed. Additionally, 75% of ORFs in DPC4571 were found in NCFM, which confirmed a close relationship between the two strains that inhabit significantly different environments (milk and gut). The genetic differences between these two strains were examined and they explained the genetic basis for niche specialisation. It was shown that the dairy strain lacked many genes that were retained in the probiotic strain, such as PTS systems, cell-wall anchoring proteins and the already mentioned mucus binding proteins (Callanan et al., 2008). In the previously mentioned study that analysed 100 *Lb. rhamnosus* strains, interesting observations regarding niche adaptability and clustering were made. Most dairy isolates clustered together, while intestinal and probiotic strains shared similarities with other human isolates. When both the phenotypic and genomic data of each strain were joined, two geno-phenotypes were identified. Firstly, the strains in group A were characterised by the absence of SpaCBA pili, lactose, maltose and rhamnose metabolism all of which point to dairy adaptation. Secondly, group B strains were bile resistant, pili possessing and L-fucose utilising, all characteristics important for intestinal tract survival. Although isolates of the same origin could be found in both groups, cheese isolates mainly belonged to group A, while intestinal isolates belonged mainly to group B. Intestinal isolates in group A may have originated from the consumption of food and represent rather a transient flora, while isolates from group B represent typical GIT residents (Fig. 1b). Interestingly, vaginal and oral isolates shared geno-phenotype A, which suggests a connection with dairy isolates (Douillard et al., 2013). Another study attempted to link genotypes and carbohydrate utilisation profiles of 65 *Lb. rhamnosus* strains isolated from diverse habitats, such as human, baby and goat feces, cheese and fermented milk (Ceapa et al., 2015). Genomic fingerprinting was performed by

amplified fragment length polymorphism (AFLP) genotyping, and 11 genotypic groups were determined. Although not seen as a strict rule, strains of the same origin clustered together. Some clusters contained strains from various origins, indicating that these strains frequently change habitats (Fig. 1b). Conversely, some clusters had members of a single isolation niche, such as dairy. Following on from this, 25 isolates that represent all 11 clusters obtained by AFLP were tested for the carbon sources they could potentially use. Based on 72 carbon sources, three metabolic groups were determined, with group A including strains that could use plant derived carbohydrates, group B including strains with no ability to use lactose and group C containing strains that could use various carbohydrates. Although group B had no ability to use lactose, some strains isolated from cheese did belong to this group, where they were present as non-starter flora and had a role in proteolysis in the later stages of ripening. Interestingly, there was no direct correlation between metabolic groups and niche isolation, but strains coming from the same AFLP cluster appeared in the same metabolic group. This work again confirms that origin of isolation gives only an indication of potential metabolic capacity of the strain, but other approaches also have to be employed to fully understand strain fitness. For example, *Lb. rhamnosus* strain HN001 is present as a cheese isolate, but it has the ability to use 53 different carbon sources, which contradicts the general tendency of niche specialists to use a more narrow range of carbohydrates indicating that this strain was most probably very recently introduced into cheese environment. On the other hand, strain ATCC 53103 (GG) which originated from the intestine, belongs to a metabolically specialist group, possibly because it was transferred from dynamic environment such as GIT to more stable industrial habitat, which may have led to the metabolic simplification (Ceapa et al., 2015).

Finally, the effect of niche adaptation could be seen even within different dairy products. In the multi locus sequence typing (MLST) study of 11 housekeeping genes in 245 *Lb.*

494 *helveticus* isolates from natural fermented products, particular branches of isolates could be
495 associated with the dairy product from which they originated (koumiss group, tarag group
496 and coumiss-tarag group). These results suggest that even ecological niches representing
497 different dairy environments may impact evolution of *Lb. helveticus* strains because genetic
498 relationships are generally correlated with the ecological niches (Sun et al., 2015b).

4. A genomic perspective on key dairy traits: flavour formation and phage resistance

The successful application of lactobacilli in the industrial environment depends on the robustness of selected strains and their ability to contribute to the desirable properties of the final product. Apart from their metabolic potential which affects the technological and organoleptic characteristics of dairy products, the ability of dairy lactobacilli to combat phage attacks which are frequent in dairy plants also contributes to the overall quality of product. Thus, a genomic perspective of these two features of dairy related lactobacilli will be discussed in more details.

4.1 Diverse proteolytic and flavour formation abilities of dairy lactobacilli

Flavour formation in dairy products is the result of a complex network of processes which ends in specific combinations of flavour compounds and aroma development. Three major processes contribute to flavour development: glycolysis, lipolysis and proteolysis (van Kranenburg et al., 2002; Smit et al., 2005; Settanni and Moschetti, 2010). Glycolysis refers mainly to the metabolism of lactose and citrate. While lactose, the primary milk sugar, is mostly metabolised to lactic acid, a proportion of it can be converted to flavour compounds such as diacetyl, acetoin, acetaldehyde, or acetic acid, depending on the organism (van Kranenburg et al., 2002). Certain organisms also have the ability to metabolise citrate. Citrate is generally metabolised to pyruvate, which can be further metabolised to acetoin in the final product (Medina de Figueroa et al., 2001; Mortera et al., 2013). Lipolysis in fermented milk products arises mainly from the activity of microbial lipolytic enzymes (Collins et al., 2003). Esterases hydrolyse hydrosoluble ester chains between 2 and 8 C atoms, and lipases are more active on longer ester chains (10 C atoms). Free fatty acids contribute to cheese flavour, particularly short and intermediate chain fatty acids, which represent the starting molecules for catabolic reactions resulting in the production of numerous flavour and aromatic compounds (Collins et al., 2003). Of all the metabolic processes responsible for flavour

development in dairy products, proteolysis is considered the most important and complex one, affecting texture, hardness, elasticity and the overall flavour of the fermented product (Savijoki et al., 2006). The proteolysis cascade starts with casein degradation by cell-envelope proteinases (CEP, Prt). The peptides released in this processes are then transported in the cell, where peptidases with varying specificities cleave them, releasing amino acids. These amino acids are the substrates for various metabolic reactions, with aminotransferases being the first enzymes in the subsequent catabolic cascade. Diverse and numerous aromas are released in these reactions (aldehydes, ketones, carboxylic acids and volatile sulfur compounds) (Marilley and Casey, 2004). In this section, the genomics of the components of proteolytic system of *Lactobacillus* will be discussed, as proteolysis represents a critical process in flavour development in dairy products.

Cell envelope proteinases (CEPs) are multi-subunit, cell wall associated proteinases and their main role during growth in milk is degradation of casein into smaller peptides (Sun et al., 2015a). The importance of surface proteinases is made clear in studies that showed that knock-out strains lack the ability to grow in milk (Mayo et al., 2010).

In an extensive study performed on the genomes of 213 *Lactobacillus* and associated genera, intriguing diversity in CEP characteristics was revealed (Sun et al., 2015a). In total, genes for 60 CEPs were identified and presence of genes for CEPs was highly correlated with phylogenetic clades. Three different anchoring mechanisms were observed: a SLAP domain (S-layer anchoring domain) responsible for non-covalent interactions was present, particularly in the *Lb. delbrueckii* sub-clade; a LPXTG motif for covalent linkage to peptidoglycan and a derivative of the LPXTG motif. In thirteen cases, no anchoring domain for CEP was identified as sequences were terminated exactly before the typical start of the anchoring domain sequence. Multiple alignments indicated the sequences of these 13 CEPs differ from other CEPs along the entire length of the protein. Besides this, the possibilities of

549 various domain combinations in the CEPs enable a diversity of potential substrates to be
550 utilised, resulting in a range of final products, which could contribute to improvement of
551 dairy products flavour (Sun et al., 2015a).
552 The vast majority of LAB have only one CEP, but for certain strains of *Lb. helveticus*, it has
553 been confirmed through multiplex PCR analysis that at least four different proteinases exist
554 (Broadbent et al., 2011) and four *prt* genes were described in the genome of *Lb. helveticus*
555 CNRZ32 (Broadbent et al., 2013). The presence of a higher number of proteinases with
556 different substrate and cleavage specificities could explain the efficiency of the *Lb. helveticus*
557 proteolytic system. CEPs have different and complimentary properties and some strains could
558 have acquired additional genes because they provide an adaptive advantage regarding milk
559 protein hydrolysis (Genay et al., 2009). In the study by Broadbent et al. (2011), 51 *Lb.*
560 *helveticus* strains were tested for presence of *prt* paralogs. The distribution of *prt* genes
561 varied among *Lb. helveticus* strains and the most abundant gene was *prtH3*, which contradicts
562 the study by (Genay et al., 2009) who found that *prtH2* was in fact a ubiquitous gene in *Lb.*
563 *helveticus* strains. The reasons for this contradiction are that sequences for *prtH4* were not
564 available, and *prtH3* gene from DPC4571 strain was described as an allele of *prtH2*
565 (Broadbent et al., 2011). From the dairy industry perspective, the diverse proteinase gene
566 content in *Lb. helveticus* may be a crucial factor in determining the function and behaviour of
567 these strains with regard to desired flavour formation (Broadbent et al., 2011).
568 The correct maturation of CEP depends on the presence of the maturation proteins, PrtM. For
569 instance, while *Lb. helveticus* CNRZ32 has 2 *prtM* paralogs designated as *prtM* and *prtM2*, in
570 other analysed *Lb. helveticus* strains *prtM* was found only in strains that possessed *prtH*, and
571 *prtM2* was encoded in genomes of all tested strains. It has been proposed that *prtM* is needed
572 for activation of *prtH*, and *prtM2* is responsible for folding and activation of other *prt*
573 paralogs (Broadbent et al., 2011). On the other hand, no *prtM* gene for this protein was found

574 in any of the 4 completely sequenced *Lb. delbrueckii* strains (Liu et al., 2012). However, the
575 foldase protein (PrsA) involved in maturation of extracellular proteinase and folding and
576 stability of subtilisins in *Bacillus subtilis* was detected. PrsA might be involved in maturation
577 of PrtB, as PrsA from four *Lb. delbrueckii* strains were homologous with known PrtM
578 proteins (Liu et al., 2012).
579 Peptides released by the activity of CEP are transported by various transport systems inside
580 the cell, where they are cleaved by peptidases of different activities, releasing amino acids.
581 Several studies that took into consideration various LAB genomes concluded that the general
582 peptidases (PepN, PepC, PepX) were widely distributed among *Lactobacillus*, including
583 species of interest in dairy fermentation (Cai et al., 2009; Liu et al., 2010). A closer look
584 suggests that PepN and PepX are encoded by single genes, but genes for other peptidases,
585 such as PepC/E and PepO were detected as multiple copies in strains belonging to species
586 generally seen as important for dairy industry, enabling higher adaption in habitat abundant in
587 proteins and peptides (Cai et al., 2009).
588 The diversity in peptidase content is observed on the same species level, where strains differ
589 in numbers of peptidases and transport system components. Upon analysis of four fully
590 sequenced genomes of *Lactobacillus delbrueckii* (ATCC 11842, BAA-365, 2038 and ND02),
591 strain ND02 possessed the highest number of proteinase and peptidase genes, as well as the
592 highest number of peptidase and amino acid transport systems. Intracellular peptidases
593 showed some differences between the four strains, such as three unique peptidases in strain
594 ND02. In the case of strain 2038, two cell surface peptidases EnlA and Pep-D4 were present
595 as complete genes, indicating that this strain has a more powerful proteolytic capability and
596 potentially produces more free amino acids than the other strains (Liu et al., 2012). All four
597 sequenced strains possessed two complete Opp systems, but they differed in numbers and
598 organisation of substrate binding protein OppA. The highest number of OppA genes was

found in the industrial strain 2038 and their products enable transport of different oligopetides (Liu et al., 2012).

The next step in the protein degradation cascade is the metabolism of free amino acids, following which a large number of flavour compounds arise. Aminotransferases are the first enzymes in the cascade, transferring amino groups from amino acids to alpha-keto acids, most often alpha-keto glutarate. In a comparative study of enzymes involved in amino acid metabolism contributing to generation of flavour compounds in 21 genomes of different LAB species, (12 of which were lactobacilli), a homolog of the *bcaT* gene, coding for branched-chain aminotransferase activity, was present in all *Lactobacillus* strains considered as important in dairy production, while a larger number of homologs for the *araT* gene, coding for aromatic aminotransferase activity, were usually present (Liu et al., 2008). The distribution of amino acid metabolising enzymes amongst starter and NSLAB including the species discussed in this review, were compared by Gobbetti et al. (2015), and it confirmed the diversity of the metabolic capability of lactobacilli and underlined the importance of genomic analysis as part of a knowledge-based approach to strain selection.

Cysteine and methionine are precursors for the production of volatile sulfur compounds (VSCs) which are important flavour compounds that are found in many cheese varieties. The metabolism of sulfur containing amino acids is complex as multiple alternative metabolic pathways exist (Mayo et al., 2010). One of the enzymes involved in metabolism of methionine is cystathionine gamma lyase (CGL), which was found in several *Lb. casei* strains isolated from cheese and milk (Irmler et al., 2008). Two variants of the gene encoding CGL shared 81% of similarity and were named *ctl1* and *ctl2*. Homologs of *ctl1* and *ctl2* were found in other LAB: *Lb. helveticus, Lb. bulgaricus Lb. rhamnosus* and *S. thermophilus*, but they were not present in three publicly available genomes of *Lb. casei* (ATCC 334, Zhang and BL23) and it is likely that these strains uptake sulfur-containing peptides and amino acids

624 from the environment (Irmler et al., 2009). Analysis of nucleotides upstream from a *ctl* gene
625 cluster found an ORF encoding for a putative transposase, supporting the possibility of
626 horizontal transfer of the cluster to *Lb. casei* strains. The gene cluster forms an operon
627 important in cysteine biosynthesis, as its expression was downregulated when L-cysteine is
628 added to the medium (Bogicevic et al., 2012). Furthermore, when these strains were used in
629 cheese production, significantly higher levels of VSC were detected at the end of ripening
630 (Bogicevic et al., 2013).
631 Glutamate dehydrogenase (GDH) is an enzyme that acts as a cofactor for aminotransferase
632 function, as it enables recycling of alpha-ketoglutarate, the receptor of the amino group
633 during transamination. When genomes of 12 species of *Lactobacillus* were analysed, the
634 presence of a *gdh* gene was confirmed only in *Lb. plantarum* WCFS1 and *Lb. salivarius*
635 UCC118 (Liu et al., 2008), which agrees with the strain dependency of *gdh* presence and
636 higher prevalence in natural strains commonly found in cheese manufacture (Tanous et al.,
637 2002). However, the majority of *Lb. casei*, *Lb. rhamnosus* and *Lb. plantarum* genomes
638 possess the *gdh* gene (Gobbetti et al., 2015), but no *gdh* gene was found in any of the
639 sequenced *Lb. delbrueckii* strains (Liu et al., 2012; Gobbetti et al., 2015). Nevertheless, two
640 genes encoding proteins homologous to aspartate aminotransferase were found in *Lb.*
641 *delbrueckii* and which could potentially catalyse the formation of glutamate from 2-
642 oxoglutarate and L-aspartate (Liu et al., 2012).
643 Collective data from genomic analysis of dairy-related strains present a first step in
644 knowledge based strain selection. The insight into the number and characteristics of genes of
645 interest enables strategic choice of cultures for dairy manufacture. Besides that, selection of
646 strains with variable key enzyme presence and activities opens the possibilities for
647 development of products with diverse flavour and broadens the overall portfolio offered to
648 the final customer.

4.2 CRISPR regions of dairy-related lactobacilli

Bacteriophages present a serious problem in dairy industry affecting continuity of quality for the final product as they affect survival of starter and adjunct cultures in the fermentation process. Although huge efforts are made to prevent and control phage levels, phage infections regularly cause disruptions in production and product downgrading (Marco et al., 2012). Several mechanisms of phage resistance were previously described for lactic acid bacteria and they include prevention of phage adsorption, blocking the entry of phage DNA, cutting phage nucleic (restriction/modification systems) acid and abortive infection (Garneau and Moineau, 2011). However, recently, a new system that enables effective resistance to phage attacks was discovered, and it was shown that this system was almost universally present in bacteria, including LAB. CRISPR (clustered regularly interspaced short palindromic repeats), together with CRISPR-associated genes (*cas*) form a bacterial immune system against foreign DNA, such as phage or plasmids (Barrangou and Horvath, 2012). The typical CRISPR locus, located behind the leader sequence, contains a string of DNA repeats and spacers, which represent short sequences corresponding to foreign DNA inserted between two repeats (Deveau et al., 2010). The efficient defence from foreign DNA attack involves the incorporation of short sequences of foreign DNA in CRISPR loci (acquisition) (Fig. 2a). In the event of foreign DNA being present in the cell, these short sequences are transcribed into small interfering RNAs, called CRISPR RNA (crRNA), which guide multifunctional protein complexes to recognise and cleave matching foreign DNA (Fig. 2b) (Barrangou and Horvath, 2012).

Two genes, *cas1* and *cas2*, are regularly present in CRISPR-Cas systems, and they are involved in the acquisition process (Barrangou, 2013). Based on the signature genes which confer interference, three types of CRISPR-cas systems are well described. Type I systems have *cas3* as the signature gene, which encodes an endonuclease involved in the cleavage of

DNA. Another feature of this type is the Cascade complex, participating in processing of crRNA and recognition of target DNA. The signature gene of Type II systems is *cas9,* which encodes a protein important for the crRNA synthesis and target DNA cleavage. Specificity of Type II systems is trans-activating CRISPR RNA (tracrRNA) that hybridizes to crRNA and enables its maturation by endoribonuclease RNAse III. Type III systems are defined by the signature gene *cas10* and they are mechanistically diverse, with IIIA systems cleaving DNA and IIIB systems cleaving RNA molecules (Barrangou, 2013; Selle and Barrangou, 2015). Besides these three systems, novel types (IV, V and VI) were discovered more recently (Wright et al., 2016).

In LAB, eight different families of CRIPSR loci were found and these families did not correlate with phylogeny of LAB indicating their independent evolution from other elements on the chromosome. The analysis of CRISPR loci at the level of the LAB showed that highly similar loci were found in distant genera and species. This could be explained by HGT and indeed, these loci have different GC content compared to the rest of the host genome. Interestingly, the comparison of CRISPRs of two closely related species, *Lb. helveticus* and *Lb. casei*, showed that they belong to different families, once again confirming the high level of variability of these regions (Horvath et al., 2009).

In the analysis of 213 genomes of *Lactobacillus* and associated genera, 137 CRISPR loci were found in 63% of all analysed genomes. All three types of systems were found in *Lactobacillus* and the size of loci varied between 2 and 135 spacers. Type II systems were found to be the most prevalent (36% of analysed genomes). In addition, novel Type II systems with heterogeneous *cas9* sequences were detected, and their potential use could be as tool for specific DNA cleavage in genome editing in both prokaryotes and eukaryotes (Sun et al., 2015a).

CRISPR profiles of 100 *Lb. rhamnosus* strains were generated by spacer oligotyping, a method firstly described by Kamerbeek et al. (1997), and a considerable level of strain variety was revealed (Douillard et al., 2013). Additionally, in certain cases, correlation between CRISPR loci and specific niche was observed. In total, 24 spacers were identified from both plasmids and phage DNA. Spacers that corresponded to phages belonged *to Lb. rhamnosus* phages or *Lb. casei* phages. The study defined two general geno-phenotypes (discussed above) and the CRISPR locus profiles were substantially different in these two groups (Douillard et al., 2013). A comparative study of CRISPR in *Lactobacillus delbrueckii* ssp. *bulgaricus* that took into consideration 33 strains showed that these strains possessed either Type II or Type III CRISPR systems (Urshev and Ishlimova, 2015). However, in the genome of recently sequenced strain CFL1 both CRISPR types (II and III) were present simultaneously (Meneghel et al., 2016).

As described previously, *Lb. casei* represents a highly genomically diverse species of lactobacilli, while *Lb. acidophilus* is characterised by remarkable genome stability. These differences are also apparent in the comparison of CRISPR systems in the two species. The CRISPR spacers of *Lb. casei* show a high level of variability and homology to *Lactobacillus* phages and plasmids. It was noted that strains isolated from commercial cheeses possess higher numbers of spacer sequences highlighting potential interactions with phage in the dairy manufacturing environment (Broadbent et al., 2012). Conversely, CRISPR loci of *Lb. acidophilus* show striking stability. When CRISPRs of La-14 and NCFM were compared, a high level of identity was observed, and similar sequences were found in strain ATCC 4796 (Stahl and Barrangou, 2013). In addition, CRISPR loci of 20 *Lb. acidophilus* strains also showed stability and uniformity (Bull et al., 2014). This may suggest that *Lb. acidophilus* has not recently encountered phage attack, as this species does not encode for an active phage and there is no recent report of validated phages of this species. The fact that *Lb. acidophilus* is

resistant to phage attack supports its wide and successful commercial application (Bull et al., 2014).

4.2.1 Applications of CRISPR systems

Analysis of the CRISPR loci present in strains provides the evidence of previous phage interaction and opens possibilities for enhancing phage resistance of industrial strains. A potential strategy would be to improve the CRISPR systems both in resistance level and spectrum, which would contribute to the robustness of the industrial strains. This could be achieved by selecting CRISPR mutants after repeated exposure to different phages selected from a diverse collection. Mutants with novel spacers with high homology to conserved phage sequences could be used in culture rotation schemes of dairy strains. Another benefit of mutant selection, as described by Barrangou and Horvath (2012), is the development of tagging system for proprietary strains (Barrangou and Horvath, 2012).

Due to their hypervariability in spacer regions, CRISPR loci could be used in strain typing studies, as nearly identical strains could be distinguished, and this typing has already been performed for pathogens such as *Mycobacterium tuberculosis* or *Yersinia pestis*, as well as for industrially important LAB (Barrangou and Horvath, 2012). High level of diversity in CRISPR loci represents a basis for comparative analysis of strains originating from different habitats, and it may be used in phylogenetic relationship studies (Horvath et al., 2009).

Genome editing represents a novel and elegant approach that has revolutionised the idea of genetic engineering. This approach was inspired by the mechanism of action of Type II CRISPR systems, where crRNA introduces double-stranded DNA breaks (DBS) of invading DNA (Jiang and Marraffini, 2015). DBS and targeted genome editing was successfully performed by adapting the Type II CRISPR system from *Streptococcus pyogenes* (Jinek et al., 2012). For the genome engineering process, two components have to be present in the cell: Cas9 nuclease that makes the DBS and a guide RNA, a chimeric molecule combined of

748 crRNA and tracrRNA that leads the Cas9 to a specific DNA site (Fig. 2c). The DNA break
749 can be followed by non-homologous end joining which induces indels, or homology-directed
750 repair that introduces site-specific insertion from DNA donor templates (Sander and Joung,
751 2014). This simple and highly specific approach has moved the boundaries of genetic and
752 biochemical research, and it is almost ideal for genome editing applications due to its
753 efficiency and affordability (Selle and Barrangou, 2015).

5. Genome scale metabolic models and metabolic engineering of *Lactobacillus* species

While comparative genomic studies represent the starting point for advancing our understanding of the evolution, diversity and metabolism of LAB, systems biology approaches, which combine mathematical modelling with 'omics' information, can predict how cells will behave and what modifications could be made to improve their performance (King et al., 2015). An example of this are genome-scale metabolic models (GSMM), which represent a catalogue of all the metabolic reactions and their associations in a single organism from gene to final metabolic process based on merging information about gene functions, the biochemical reactions in which the product is involved and theoretical background (Teusink et al., 2011). GSMMs connect the genotypic and phenotypic data and combine with transcriptomic, proteomic and metabolomics data (Steele et al., 2013). Some of applications of GSMM constructed for LAB include design of metabolic engineering experiments, detection of differences between the strains and testing of characteristics of potential probiotic strains (Vinay-Lara et al., 2014). From the perspective of the dairy lactobacilli, the development of such models could be of immense importance for desired product design (Steele et al., 2013) and metabolic engineering projects (Gaspar et al., 2013) (Fig. 3).

The metabolic network of an organism is based on genomic information, and this network connects the information of genes and the metabolic reactions they are involved in (Lewis et al., 2012). After detailed revision and correction of the (genome-scale) metabolic model, it is then transformed to a stoichiometric matrix, which is a mathematical representation of metabolic reactions. The purpose of this step is to convert GSMM to a computational one (O'Brien et al., 2015). Constraint-based reconstruction and analysis (COBRA) models are the most widely used in GSMM analysis (Lewis et al., 2012). Flux Balance Analysis (FBA) is the oldest, most basic and commonly used COBRA method (Lewis et al., 2012; O'Brien et al., 2015; Orth et al., 2010) for simulating GSMM. Detailed explanation of how FBA

779 operates can be found in Orth et al. (2010). Flux variability analysis (FVA), introduced by
780 Mahadevan and Schilling (2003), modifies the FBA approach as it considers the effect of
781 metabolic uncoupling. FVA determines, for each reaction in the model, the range of possible
782 fluxes that correspond to experimental values of constraints (Smid and Hugenholtz, 2010).
783 *Lc. lactis* was the first LAB to have a genome-scale model constructed (Oliveira et al., 2005),
784 followed by *Lb. plantarum*, (Teusink et al., 2006) and *Streptococcus thermophilus* (Pastink et
785 al., 2009) and most recently, *Lb. casei* (Vinay-Lara et al., 2014; Xu et al., 2015). Here, we
786 will review the most important findings of models designed for some species of
787 *Lactobacillus*.
788 The GSMM of *Lb. plantarum* WCFS1 was used to compare a traditional view of ATP
789 production from lactate and acetate and ATP production based on the constraints approach
790 when experimental constraints were applied. The traditional approach has certain
791 disadvantages as it takes into account lactate and acetate production in other metabolic
792 processes which do not contribute to ATP yield, like amino acid or citrate metabolism. After
793 comparison of ATP production in both approaches, the same result was obtained in both
794 cases, meaning that the effects of amino acid and citrate metabolism were not crucial.
795 Additionally, the model identified catabolic reactions such as transamination of aromatic and
796 branch-chained amino acids to generate ATP. These reactions are seen as a major factor in
797 flavour development, but have not been previously connected with ATP production. Further
798 on, the model attempted to assess the effect of uncoupling on metabolic capacities. FVA was
799 used to calculate the spectrum of flux values consistent with the experimental constraints and
800 showed higher flexibility of the flux ranges for the uncoupled energy production and
801 consumption. However, FBA was not able to correctly predict *Lb. plantarum* biomass
802 production, as it did not take into account inefficient lactate production. FBA predicted
803 higher growth, as it detected lactate production as incompatible with optimised growth. In

804 reality though, *Lb. plantarum* produces lactate and tends to utilise a route that is less efficient
805 even under limited energy conditions, and this event cannot be predicted by FBA, which
806 proposed higher yield as a result of mixed acid fermentation (Teusink et al., 2006).
807 The study by Vinay-Lara et al. (2014) compared metabolic networks from two *Lb. casei*
808 strains that are fully sequenced, ATCC 334 and 12A. FBA was used to analyse the properties
809 and capabilities of both models. Both tested strains have similar amino acid requirements -
810 branched-chain and aromatic amino acids and arginine are essential. It is most likely that the
811 rich environment (cheese and corn silage) that these strains were isolated from reduced the
812 need for synthesising all amino acids. Although models initially did not predict glutamate as
813 an essential amino acid, excluding this amino acid from the culture medium significantly
814 reduced the growth of ATCC 334 and resulted in no growth for 12A. However, in both
815 metabolic models glutamine can be converted into glutamate, and the experimental studies
816 suggested that this interconversion of glutamate to glutamine results in low yields of
817 synthesised glutamate, thus explaining why glutamate is needed even in the presence of
818 glutamine. A correction of the metabolic pathway was possible in the case of ATCC 334, but
819 fixing the inconsistency in 12A was not successful, and the model was not unable to
820 determine the strain's requirements for glutamate. Carbohydrate utilisation analysis of these
821 strains once again confirmed the hypothesis of gene decay during adaptation to nutrient rich
822 environments. Strain 12A, isolated from corn silage (Cai et al., 2007) possesses an ABC
823 transporter for uptake of raffinose and enzymes needed for pullulan and panose degradation,
824 sugars frequently present in plant material. On the contrary, ATCC 334, a cheese isolate,
825 lacks these genes as they are most likely redundant in the dairy environment. Interestingly,
826 the metabolic model for strain 12A shows that all the genes for converting myoinositol to
827 glyceraldehyde-3-phosphate are present. Myoinositol can be used as phosphate storage
828 molecule in plants. Although the majority of LAB cannot use this sugar as carbon source,

strain 12A has all the genes needed for conversion of myoinositol, but this metabolic pathway is not active in 12A probably due to regulatory effects (Vinay-Lara et al., 2014). In other *Lb. casei* models it was shown that, *in silico* growth of *Lb. casei* LC2W was improved by myoinositol under aerobic conditions, suggesting that this strain could utilise energy sources that seemed inappropriate under anaerobic conditions (Xu et al., 2015).

A genome-scale metabolic model of *Lb. casei* LC2W was used for the analysis of the oxygen effect on flavour compound synthesis and three new *in silico* knockout targets were selected for acetoin production. In *Lb. casei* LC2W, the main precursor of flavour compounds is alpha-acetolactate. Acetoin and diacetyl are produced from alpha-acetolactate by acetolactate-decarboxylase or through non-enzymatic processes. Although acetoin could accumulate in LC2W in both aerobic and anaerobic conditions, production of diacetyl was dependent on oxygen and it was possible to maintain diacetyl production at a high level with the increase of oxygen uptake. Additionally, FBA suggested three new *in silico* knockout targets for acetoin production: dihydrofolat-reductase, methylen-tetrahydrofolate-dehydrogenase and glycerol-phospho-transferase (Xu et al., 2015).

Regarding the flavour potential of LAB, a completely different approach was recently proposed. As seen, GSMM contain numerous gaps which cannot always be completed. Although there are many known pathways involved in flavour formation, the overall process of flavour development is highly complex. Compounds that are often seen as flavour contributors are products of amino acid metabolism: alcohols, aldehydes and acids, and especially sulfur compounds, products of methionine metabolism (Curioni and Bosset, 2002; Smit et al., 2005; Yvon, 2006). Reverse pathway engineering (RPE) (Liu et al., 2014) takes small molecules as a starting point and looks for enzymatic or chemical reactions that can track these compounds back to the known precursors. This method was used in LAB to predict so far unknown reactions in metabolic pathways by combining retrosynthesis and

genomic information. To confirm that the proposed approach is correct, the relatively well-known pathway of leucine degradation in LAB was tested in the model. Not only were the main branches confirmed, but it also suggested a novel route of generating 3-methyl butanoic acid, one of the most important flavour compounds of leucine metabolism. This novel route starts with the transamination product of leucine, alpha-keto-isocaproat, which is further reduced to alpha-hydroxy-isocaproate. The second step suggests formation of 3-methyl butanoic acid from alpha-hydroxy-isocaproate, and the related reaction found in the database was a lactate oxidation reaction catalysed by lactate-2-monoxygenase (LOX), so it was assumed that LOX could possibly catalyse oxidation of alpha-hydroxy-isocaproate. Broader activity of LOX seems to be dependent on the amino acid at position 95 and it could be obtained if alanine in position 95 was mutated to glycine (detailed explanation in Liu et al. (2014)). The RPE method also revealed a non-enzymatic reaction of converting alpha-keto-isocaproate to 2-methyl propanal, and this reaction connects valine and leucine catabolism. Regarding the methionine degradation, RPE discovered an enzymatic reaction responsible for the conversion of methanethiol to dimethyl-sulfide (DMS), using DMS as an input. Enzymes homocystein-S-methyltransferase, methionine synthase and thiol-S-methyl-transferase were proposed using the bioinformatics approach. The prediction of novel reactions using RPE opens up new possibilities for metabolic engineering. For example, hydroxy-isocaproat is often seen as an off-flavour in cheese products, but the proposed conversion to the flavour compound 3-methyl butanoic acid could be implemented in novel strategies for production of flavour by utilising off-flavours as precursors (Liu et al., 2014).

5.1 Metabolic engineering as a future application of lactobacilli

A vast amount of knowledge on genetics and metabolism of LAB opened the door for implementation of LAB in novel biotechnological applications (Gaspar et al., 2013). Application of LAB is not limited only to classical food fermentation and the use of LAB as

879 cell factories is expected to increase (Gaspar et al., 2013). LAB are characterised by limited
880 biosynthetic capacity and metabolic versatility and their physiology is relatively simple. They
881 are characterised by relatively small genomes (2-3 Mbp), fast growth, high sugar uptake rates
882 and less high-level control systems, all of which make them suitable candidates for metabolic
883 engineering (Papagianni, 2012; Gaspar et al., 2013). Genetic engineering made possible the
884 production of molecules not natively present in the host, but also enabled engineering of
885 native genes (Keasling, 2012). Genetic engineering proved successful in the development of
886 strains producing recombinant proteins and small chemicals, but development of tools that
887 exceed genetic engineering is needed, as some molecules are synthesised in multiple
888 reactions (Bution et al., 2015). Metabolic engineering summarizes previous knowledge
889 regarding cell metabolic features and it uses molecular tools to deliberately change cellular
890 metabolism for the purpose of the efficient production of target molecules (Bution et al.,
891 2015) (Fig. 3). However, the host cell needs to meet several requirements to ensure efficient
892 metabolic engineering occurs. Host cells should be genetically stable, not interfering with
893 heterologous genes on the introduced vector, and have optimal traits for industrial
894 applications. Apart from these, genomic information can help in the choice of host, as new
895 pathways can induce stress response and impede gene expression (Keasling, 2012).
896 Metabolic engineering of lactic acid bacteria presents a novel approach for re-routing
897 metabolic reactions in LAB so specific and desired compounds are produced in higher
898 amounts. Several different types of molecules can be produced by LAB as cell-factories:
899 lactic acid, flavour compounds (diacetyl, acetaldehyde), sweeteners (L-alanine, mannitol,
900 sorbitol, xylitol), exopolysaccharide, vitamins etc. (Papagianni, 2012). Historically, the first
901 attempt of engineering of LAB was oriented towards improving production of the bitter
902 aroma compound diacetyl in *Lc. lactis*. Subsequently, many other studies expanded the
903 species of LAB that were subject to engineering as well as the types of molecules produced.

Several recent review articles (Papagianni, 2012; Gaspar et al., 2013; Mazzoli et al., 2014) give detailed information about achievements in production of industrially important compounds in LAB. Production of food ingredients, commodity compounds, vitamins and ethanol are thoroughly reviewed with methods of engineering and future perspectives anticipated. Besides this, metabolic engineering is used as a tool for improvement of adherence and immunomodulatory properties of probiotic strains (described and reviewed in Yebra et al. (2012)). While most of results come from *Lc. lactis* as most widely used LAB, novel information comes from *Lactobacillus* species as well. Here we review studies performed on strains of *Lactobacillus* spp. mainly associated with dairy food.

Lactic acid is used as a preservative and flavour enhancing agent by the food industry, and also in cosmetic and pharmaceutical industries (Papagianni, 2012). In addition, L-lactic acid is used as the starting material in the production of biopolymers (Gaspar et al., 2013). Unlike chemical synthesis, which often leads to racemic mixture of L- and D-lactic acid, microbial fermentation can be optimised for production of a single enantiomer (Gaspar et al., 2013). The L-isomer is a preferred for two reasons: D-isomer is not metabolised in humans and has a toxic effect and L-isomer polymerises which is important in polymers production (Kyla-Nikkila et al., 2000; Papagianni, 2012). The initial attempts to influence lactic acid production in lactobacilli date in 1990's, when the enhancement of L-lactic acid was achieved by the inactivation of *ldhD* in *Lb. helveticus* (Bhowmik and Steele, 1994), but the overexpression of *ldhL* in *Lb. plantarum* did not cause an increase of L-lactic acid synthesis, although increased activity of L-LDH was observed (Ferain et al., 1994). More recently, selective L-lactate production was tested in *Lb. helveticus* CNRZ32 and two approaches were used (Kyla-Nikkila et al., 2000). The promoter of the *ldhD* gene was deleted in the construct GRL86 while in the other construct, GRL89, the structural gene of *ldhD* was replaced with an additional copy of the structural gene of *ldhL*. Both constructs produced only L-lactic acid in

amounts that were on the level of total lactate produced by the wild type strain and no difference in growth profiles for either construct was observed compared to the wild strain. Additionally, the L-lactic acid production phase of mutant strains was prolonged compared to the wild strain (Kyla-Nikkila et al., 2000).

Ethanol represents an important biofuel and the high demand for renewable energy sources puts efficient ways of ethanol production in focus (Mazzoli et al., 2014). Although many bacteria have low ethanol tolerance, some species of LAB, especially lactobacilli are relatively tolerant to high concentration of alcohols (Mazzoli et al., 2014). Initial efforts to enhance ethanol production were focused on the overexpression of heterologous genes encoding pyruvate decarboxylase (*pdc*) and alcohol dehydrogenase (*adh*), the enzymes responsible for conversion of pyruvate to ethanol. When *pet* operon, which carries *pdc* and *adh* genes from *Zymomonas mobilis* (Gram-negative bacteria) was used for the transformation of *Lb. casei* 686, the recombinant strains showed more than a two-fold increase in ethanol production (Gold et al., 1996). In a later study (Nichols et al., 2003), the *pet* operon was modified for expression in Gram-positive bacteria and several strains *Lb. plantarum* and *Lb. casei* were transformed. After glucose fermentations were carried out, some engineered strains showed higher ethanol production compared to the parental strains, but lactic acid was detected as a major metabolic product (Nichols et al., 2003). In the study of Liu et al. (2006), *pdc* gene from Gram-positive bacteria *Sarcina ventriculi* (*Spdc*) was expressed in *ldh* deficient *Lb. plantarum* TF103, which accumulated pyruvate. Three different promoters and native *Spdc* 5' flanking sequences were fused with *Spdc* gene and introduced in T103. All constructs produced higher amounts of ethanol than the control carrying an empty vector, but they also produced significant amounts of lactate and the level was higher than in the control strain (Liu et al., 2006).

Sorbitol is a sugar alcohol largely used in the food industry as a sweetener (Gaspar et al., 2013). It is poorly absorbed in small intestine and as it has low calorie value, is used in diabetic appropriate foods (Ladero et al., 2007), but also as a softener and texturing agent (Yebra et al., 2012). An attempt to construct sorbitol-producing LAB was performed by introducing the *gutF* gene coding for sorbitol-6-phosphate-dehydrogenase, into the *lac* operon of *Lb. casei.* The strain with the integrated *gutF* was named BL232 and the expression was controlled as in other *lac* genes. Additionally, a L-lactate-dehydogenase (*ldhL)* knockout of BL232 was constructed, and designated as BL233. Resting cells of both of these strains produced sorbitol from glucose, and the *ldhL* knockout showed higher production of sorbitol compared to BL232. It was proposed that *ldhL* inactivation leads to a higher NADH/NAD+ ratio and the cell uses this for the sorbitol production (Nissen et al., 2005). In further studies, metabolic engineering of *Lb. casei* led to a strain that could produce sorbitol without consequent uptake after glucose exhaustion, by introducing a mutation in the sorbitol-specific phospho-transferase system. Sorbitol producing *Lb. casei* were constructed through a series of transformations of strain BL232: deletion of *ldh1* gene encoding the main lactate-dehydrogenase (BL251) followed by deletion of *gutB* gene (BL283) involved in transport of sorbitol and subsequent mutation of the mannitol-1-phosphate-dehydrogenase (*mtlD)* gene (BL300). While mutant BL251 used sorbitol after glucose consumption, BL283 was not able to transport sorbitol and levels of sorbitol did not drop after glucose exhaustion. To avoid synthesis of mixed polyols (sorbitol and mannitol, as occurred in the study of Nissen et al. (2005)), a gene encoding mannitol-1-phosphate dehydrogenase was inactivated (BL300) and this knockout strain did not produce mannitol, and sorbitol production was doubled compared to BL283. In addition, the resting cells of BL300 were able to produce sorbitol from lactose in 1% supplemented MRS, especially at pH 5.5 and 4.75, but this conversion was less efficient than the conversion of glucose. Additionally, BL300 cells were able to produce

978 sorbitol as a sole polyol from whey permeate, a by-product of the dairy industry (De Boeck et
979 al., 2010).
980 In order to obtain *Lb. plantarum* producing sorbitol, a different approach was used. In the
981 genome of *Lb. plantarum* NCIMB8826, two genes for the enzyme sorbitol-6-phosphate
982 dehydrogenase (*srlD1* and *srlD2*) were present. The two *srlD* coding regions were
983 overexpressed in transformed *Lb. plantarum* strain VL103 which is lactate-dehydrogenase
984 deficient. High sorbitol-6-phosphate-dehydrogenase activities as well as sorbitol levels were
985 detected in the overexpressing strains VL103, while no activity could be detected in the wild-
986 type and VL103 strains harbouring the empty vector, used as a control strain. The deficiency
987 in LDH was essential and LDH-positive control did not produce sorbitol under any of
988 conditions examined (Ladero et al., 2007).
989 Succinic acid is a starting block in synthesis of biodegradable plastic (Babu et al., 2013) and
990 can be used as a food additive (Beauprez et al., 2010). In a study by Tsuji et al. (2013),
991 production of succinic acid was examined in the previously described lactate-dehydrogenase
992 deficient strain *Lb. plantarum* VL103. Three enzymes involved in succinic acid production:
993 pyruvate-carboxylase (PC), phospho-enol-pyruvate (PEP) and malic enzyme (ME) were
994 overexpressed in this strain, and all transformants showed increased activity of the
995 corresponding enzyme, up to 2.4 fold in the case of PC. However, although PC
996 overexpression was the most effective for succinic acid production in *Lb. plantarum*, a
997 mutant with PEP enzyme overexpressed, exhibited a higher specific growth rate, compared to
998 the two others, and seemed a better candidate for LAB succinic acid production, as PC
999 overexpression was effective but slowed down the growth rate. Additionally, combined levels
1000 of succinic acid production were observed in mutants displaying overproduction of the two
1001 enzymes and the co-expression of PC and PEP increased succinic acid yield and biomass
1002 (Tsuji et al., 2013).

Engineered *Lb. casei* were used to increase the production of diacetyl and acetoin from whey permeate (Nadal et al., 2009). These two compounds have a buttery flavour and are used as additives in the food industry (Yebra et al., 2012). The presence of the lactococcal aceto-hydroxy-acid synthase (*ilvBN*) gene and deletion of lactate-dehydrogenase gene (*ldh*) resulted in an increase in diacetyl/acetoin synthesis from glucose, but strain with only *ldh* deletion showed a similar result. By contrast, when the bacterial cells were exposed to lactose, strains carrying the *ilvBN* gene showed four times higher production of the desired compounds. The strain containing *ilvBN* and *ldh* mutations and a strain with additional *pdhC* (gene coding the E2-dihydrolipoamide-acetyl-transferase, component of pyruvate-dehydrogenase complex Pdh) mutation were used for whey permeate fermentations. Having found the most suitable conditions for pH, the total amount of diacetyl/acetoin production was higher for the strain with the *pdhC* mutation. Fed batch experiments with this strain were done with the addition of whey permeate and yeast extract, but no further increase in diacetyl/acetoin concentrations was observed, and it was proposed that higher concentrations of product might have inhibitory effect. However, the amount of product obtained was still lower compared to engineered *Lc. lacits* (Nadal et al., 2009).

Exopolysaccharides (EPS) have been widely used in food industry, as they impact on the texture of food products, but they have also been shown to possess prebiotic characteristics (Papagianni, 2012). The EPS production levels in LAB are relatively low, and there have been several attempts to increase its production, mainly in *Lc. lactis* (for review see Gaspar et al. (2013)). In an attempt to increase EPS production in *Lb. casei*, the effects of cofactors involved in EPS biosynthesis were investigated. The gene encoding NADH-oxidase (*nox*), from *Streptococcus mutans*, was cloned and overexpressed in *Lb. casei* LC2W. The strain obtained grew slower than the wild type, but showed 46% increase in EPS production (Li et al., 2015b). Furthermore, several other genes believed to be involved in EPS production were

chosen from different *Lactobacillus* strains (*Lb. plantarum, Lb. casei* and *Lb. rhamnosus*) and their effect on EPS biosynthesis was tested. The genes *tga* (trans-glutaminase), *pfk* (phospho-fructokinase), *pgm* (phospho-glucomutase), *galtf* (galacto-transferase), *rhatf* (rhamnosyl-transferase), *rfbB* (dTDP-glucose-4,6-dehydratase) and *galT* (galactose-1-phosphate-urydil-transferase), and previously described *nox* (NADH-oxidase), all involved in various steps of EPS production were successfully cloned and overexpressed in *Lb. casei* LC2W. Although recombinant strains had slower growth rates, some of them showed the positive effect of overexpressed genes (*pfk, rfbB* and *galT*) on EPS production (Li et al., 2015a), but lower than for the previously described *nox* mutant. Besides that, the *nox*-mutant was shown to produce EPS in higher amounts in aerobic conditions, although growth was less than in anaerobic conditions. In aerobic conditions, the strain with overexpression of NADH oxidase reduced used more NADH and produced lower amounts of lactate, all of which led to the increased EPS production (Li et al., 2015a).

The question remains, however, would engineered bacteria be acceptable for direct use in food production. According to the current EU legislation (Directive 2009/41/EC of the European Parliament and of the Council), a genetically modified microorganism (GMM) is any microorganism that has foreign DNA introduced in a way that does not occur naturally. Many of these modified bacteria could potentially be used in dairy food production where they could contribute to flavour and texture or fermented products containing these LAB could be used as a vehicle for probiotic delivery. However, these foods would have GMO status and fall under specific legislation, and guidelines for their applications have been proposed (European Food Safety Authority, 2011). It also raises issues in applicability and market potential as well as consumer acceptance of the modified LAB and careful analysis of variations in legislatives as well as possibilities and limits in applying genetically modified LAB in food, mainly in regard to consumers risk and benefits, should be taken into

consideration (Pedersen et al., 2005; Sybesma et al., 2006). In addition, new approaches of genome editing with employment of CRISPR-cas system would not be seen as GMM-generating tools according to the current definition, as it was recently discussed in case of genetically edited crops (Kanchiswamy et al., 2015), as only oligonucleotides that correspond to native molecules are needed for this reaction and the complex that derives edition is further degraded in the cell. This opens questions about redefining GMM and their use in the food industry. One issue that has to be considered is the fact that although the CRISPR systems have a high specificity level, the problem of unexpected negative effects remains a possibility, which could have massive effect on global food market (Au, 2015).

On the other hand, less restriction embraces the usage of modified LAB as potential cell factories. The era of application of recombinant bacteria for molecules started with human insulin production by recombinant *E. coli* developed in late 1970's (Goeddel et al., 1979). In general, LAB are recognised as safe and non-pathogenic, which makes them suitable for engineering projects. Even though these cells are engineered, the final product is purified and separated from the bacterial producer and is used as a sole chemical in food or other industries. However, the disposal of GMM in these cases presents a challenge, and optimal destruction and prevention of environmental dissemination of engineered strains have to be implemented in industrial strategies (Gautier, 2008).

6. Concluding remarks

The *Lactobacillus* genus represents a versatile group of LAB that continues to intrigue scientists from different fields of microbiology. Their genetic characteristics are constantly being supplemented with new data. The rising number of available genomes provides greater opportunities for implementation of the data to give a better understanding of and improved application of these microorganisms. Construction of pangenomes reveals genetic and phenotypic diversity, and explains adaptability of lactobacilli to various habitats. Genetic data can be also used to anticipate the potential of strains for application in various industrial fields.

The construction of genome scale computational models gives an indication of a strains metabolic potential and facilitates identification of genes most suitable for engineering studies (Bution et al., 2015). The introduction of next generation sequencing (NGS) methods and metabolite profiling reveals new and unexpected features of LAB. The construction of metabolic models of industrial microorganisms is becoming an essential step in the development of fermented foods and food ingredients (Smid and Hugenholtz, 2010). The overall knowledge obtained after deployment of all approaches described in this review contributes to a better understanding of the physiology of *Lactobacillus* cultures during dairy production, which encourages the development of novel production technologies that will provide continuous product quality improvement (Steele et al., 2013).

Acknowledgments

The authors wish to thank to Dr Mary Rea and Dr Brian Healy for their critical reading of this manuscript. Ewelina Stefanovic is in a receipt of Teagasc Walsh Fellowship.

1093 References:

1094 Altermann, E., Russell, W.M., Azcarate-Peril, M.A., Barrangou, R., Buck, B.L., McAuliffe,
1095 O., Souther, N., Dobson, A., Duong, T., Callanan, M., Lick, S., Hamrick, A., Cano, R.,
1096 Klaenhammer, T.R., 2005. Complete genome sequence of the probiotic lactic acid bacterium
1097 *Lactobacillus acidophilus* NCFM. Proc. Natl. Acad. Sci. U S A 102, 3906-3912.
1098 Au, R., 2015. From genetic engineering to genome engineering: what impact has it made on
1099 science and society. Adv. Biol. Biotechnol. Genet. 2, 1-8.
1100 Babu, R.P., O'Connor, K., Seeram, R., 2013. Current progress on bio-based polymers and
1101 their future trends. Progr Biomater 2, 1-16.
1102 Barrangou, R., 2013. CRISPR-Cas systems and RNA-guided interference. Wiley Interdiscip.
1103 Rev. RNA 4, 267-278.
1104 Barrangou, R., Horvath, P., 2012. CRISPR: new horizons in phage resistance and strain
1105 identification. Annu. Rev. Food Sci. Technol. 3, 143-162.
1106 Beauprez, J.J., De Mey, M., Soetaert, W.K., 2010. Microbial succinic acid production:
1107 Natural versus metabolic engineered producers. Process. Biochem. 45, 1103-1114.
1108 Bellanger, X., Payot, S., Leblond-Bourget, N., Guedon, G., 2014. Conjugative and
1109 mobilizable genomic islands in bacteria: evolution and diversity. FEMS Microbiol. Rev. 38,
1110 720-760.
1111 Berger, B., Pridmore, R.D., Barretto, C., Delmas-Julien, F., Schreiber, K., Arigoni, F.,
1112 Brussow, H., 2007. Similarity and differences in the *Lactobacillus acidophilus* group
1113 identified by polyphasic analysis and comparative genomics. J. Bacteriol. 189, 1311-1321.
1114 Bermudez-Humaran, L.G., Kharrat, P., Chatel, J.M., Langella, P., 2011. Lactococci and
1115 lactobacilli as mucosal delivery vectors for therapeutic proteins and DNA vaccines. Microb.
1116 Cell Fact. 10 Suppl 1, S4.

1117 Bhowmik, T., Steele, J.L., 1994. Cloning, characterization and insertional inactivation of the
1118 *Lactobacillus helveticus* D(-) lactate dehydrogenase gene. Appl. Microbiol. Biotechnol. 41,
1119 432-439.
1120 Bogicevic, B., Fuchsmann, P., Breme, K., Portmann, R., Guggenbuhl, B., Irmler, S., 2013. A
1121 preliminary study on the effect of *Lactobacillus casei* expressing *cystathionine*
1122 *lyase1/cystathionine lyase2* on Cheddar cheese and the formation of sulphur-containing
1123 compounds. Int. Dairy J. 33, 97-103.
1124 Bogicevic, B., Irmler, S., Portmann, R., Meile, L., Berthoud, H., 2012. Characterization of
1125 the *cysK2-ctl1-cysE2* gene cluster involved in sulfur metabolism in *Lactobacillus casei*. Int. J.
1126 Food Microbiol. 152, 211-219.
1127 Broadbent, J.R., Cai, H., Larsen, R.L., Hughes, J.E., Welker, D.L., De Carvalho, V.G.,
1128 Tompkins, T.A., Ardo, Y., Vogensen, F., De Lorentiis, A., Gatti, M., Neviani, E., Steele, J.L.,
1129 2011. Genetic diversity in proteolytic enzymes and amino acid metabolism among
1130 *Lactobacillus helveticus* strains. J. Dairy. Sci. 94, 4313-4328.
1131 Broadbent, J.R., Hughes, J.E., Welker, D.L., Tompkins, T.A., Steele, J.L., 2013. Complete
1132 Genome Sequence for *Lactobacillus helveticus* CNRZ 32, an Industrial Cheese Starter and
1133 Cheese Flavor Adjunct. Genome Announc. 1.
1134 Broadbent, J.R., Neeno-Eckwall, E.C., Stahl, B., Tandee, K., Cai, H., Morovic, W., Horvath,
1135 P., Heidenreich, J., Perna, N.T., Barrangou, R., Steele, J.L., 2012. Analysis of the
1136 *Lactobacillus casei* supragenome and its influence in species evolution and lifestyle
1137 adaptation. BMC Genomics 13, 533.
1138 Bull, M.J., Jolley, K.A., Bray, J.E., Aerts, M., Vandamme, P., Maiden, M.C., Marchesi, J.R.,
1139 Mahenthiralingam, E., 2014. The domestication of the probiotic bacterium *Lactobacillus*
1140 *acidophilus*. Sci. Rep. 4, 7202.

1141 Buriti, F.C.A., da Rocha, J.S., Saad, S.M.I., 2005. Incorporation of *Lactobacillus acidophilus*
1142 in Minas fresh cheese and its implications for textural and sensorial properties during storage.
1143 Int. Dairy J. 15, 1279-1288.
1144 Bution, M.L., Molina, G., Abrahao, M.R., Pastore, G.M., 2015. Genetic and metabolic
1145 engineering of microorganisms for the development of new flavor compounds from terpenic
1146 substrates. Crit. Rev. Biotechnol. 35, 313-325.
1147 Cai, H., Rodriguez, B.T., Zhang, W., Broadbent, J.R., Steele, J.L., 2007. Genotypic and
1148 phenotypic characterization of *Lactobacillus casei* strains isolated from different ecological
1149 niches suggests frequent recombination and niche specificity. Microbiology 153, 2655-2665.
1150 Cai, H., Thompson, R., Budinich, M.F., Broadbent, J.R., Steele, J.L., 2009. Genome
1151 sequence and comparative genome analysis of *Lactobacillus casei*: insights into their niche-
1152 associated evolution. Genome Biol. Evol. 1, 239-257.
1153 Callanan, M., Kaleta, P., O'Callaghan, J., O'Sullivan, O., Jordan, K., McAuliffe, O.,
1154 Sangrador-Vegas, A., Slattery, L., Fitzgerald, G.F., Beresford, T., Ross, R.P., 2008. Genome
1155 sequence of *Lactobacillus helveticus*, an organism distinguished by selective gene loss and
1156 insertion sequence element expansion. J. Bacteriol. 190, 727-735.
1157 Ceapa, C., Lambert, J., van Limpt, K., Wels, M., Smokvina, T., Knol, J., Kleerebezem, M.,
1158 2015. Correlation of *Lactobacillus rhamnosus* Genotypes and Carbohydrate Utilization
1159 Signatures Determined by Phenotype Profiling. Appl. Environ. Microbiol. 81, 5458-5470.
1160 Claesson, M.J., van Sinderen, D., O'Toole, P.W., 2007. The genus *Lactobacillus* - a genomic
1161 basis for understanding its diversity. FEMS Microbiol. Lett. 269, 22-28.
1162 Claesson, M.J., van Sinderen, D., O'Toole, P.W., 2008. *Lactobacillus* phylogenomics-
1163 towards a reclassification of the genus. Int. J. Syst. Evol. Microbiol. 58, 2945-2954.
1164 Cleveland, J., Montville, T.J., Nes, I.F., Chikindas, M.L., 2001. Bacteriocins: safe, natural
1165 antimicrobials for food preservation. Int. J. Food Microbiol. 71, 1-20.

1166 Collins, R.E., Higgs, P.G., 2012. Testing the infinitely many genes model for the evolution of
1167 the bacterial core genome and pangenome. Mol. Biol. Evol. 29, 3413-3425.
1168 Collins, Y.F., McSweeney, P.L.H., Wilkinson, M.G., 2003. Lipolysis and free fatty acid
1169 catabolism in cheese: a review of current knowledge. Int. Dairy J. 13, 841-866.
1170 Corsetti, A., Settanni, L., 2007. Lactobacilli in sourdough fermentation. Food Res. Int. 40,
1171 539-558.
1172 Cotter, P.D., Hill, C., Ross, R.P., 2005. Bacteriocins: developing innate immunity for food.
1173 Nat. Rev. Microbiol. 3, 777-788.
1174 Cremonesi, P., Chessa, S., Castiglioni, B., 2012. Genome sequence and analysis of
1175 *Lactobacillus helveticus*. Front. Microbiol. 3, 435.
1176 De Boeck, R., Sarmiento-Rubiano, L.A., Nadal, I., Monedero, V., Perez-Martinez, G., Yebra,
1177 M.J., 2010. Sorbitol production from lactose by engineered *Lactobacillus casei* deficient in
1178 sorbitol transport system and mannitol-1-phosphate dehydrogenase. Appl. Microbiol.
1179 Biotechnol. 85, 1915-1922.
1180 De Vuyst, L., Leroy, F., 2007. Bacteriocins from lactic acid bacteria: production, purification,
1181 and food applications. J. Mol. Microbiol. Biotechnol. 13, 194-199.
1182 Dellaglio, F., Felis, G.E., Torriani, S., 2002. The status of the species *Lactobacillus casei*
1183 (Orla-Jensen 1916) Hansen and Lessel 1971 and *Lactobacillus paracasei* Collins et al. 1989.
1184 Request for an opinion. Int. J. Syst. Evol. Microbiol. 52, 285-287.
1185 Deveau, H., Garneau, J.E., Moineau, S., 2010. CRISPR/Cas system and its role in phage-
1186 bacteria interactions. Annu. Rev. Microbiol. 64, 475-493.
1187 Douglas, G.L., Klaenhammer, T.R., 2010. Genomic evolution of domesticated
1188 microorganisms. Annu. Rev. Food Sci. Technol. 1, 397-414.
1189 Douillard, F.P., Ribbera, A., Kant, R., Pietila, T.E., Jarvinen, H.M., Messing, M., Randazzo,
1190 C.L., Paulin, L., Laine, P., Ritari, J., Caggia, C., Lahteinen, T., Brouns, S.J., Satokari, R., von

1191 Ossowski, I., Reunanen, J., Palva, A., de Vos, W.M., 2013. Comparative genomic and
1192 functional analysis of 100 *Lactobacillus rhamnosus* strains and their comparison with strain
1193 GG. PLoS Genet 9, e1003683.
1194 European Food Saftey Authority, 2011. Guidance on the risk assessment of genetically
1195 modified microorganisms and their products intended for food and feed use. EFSA Journal 9,
1196 2193.
1197 Ejtahed, H.S., Mohtadi-Nia, J., Homayouni-Rad, A., Niafar, M., Asghari-Jafarabadi, M.,
1198 Mofid, V., Akbarian-Moghari, A., 2011. Effect of probiotic yogurt containing *Lactobacillus*
1199 *acidophilus* and *Bifidobacterium lactis* on lipid profile in individuals with type 2 diabetes
1200 mellitus. J. Dairy Sci. 94, 3288-3294.
1201 El Kafsi, H., Binesse, J., Loux, V., Buratti, J., Boudebbouze, S., Dervyn, R., Kennedy, S.,
1202 Galleron, N., Quinquis, B., Batto, J.M., Moumen, B., Maguin, E., van de Guchte, M., 2014.
1203 *Lactobacillus delbrueckii* ssp. *lactis* and ssp. *bulgaricus:* a chronicle of evolution in action.
1204 BMC Genomics 15, 407.
1205 Ferain, T., Garmyn, D., Bernard, N., Hols, P., Delcour, J., 1994. *Lactobacillus plantarum*
1206 *ldhL* gene: overexpression and deletion. J. Bacteriol. 176, 596-601.
1207 Garneau, J.E., Moineau, S., 2011. Bacteriophages of lactic acid bacteria and their impact on
1208 milk fermentations. Microb. Cell Fact .10 Suppl 1, S20.
1209 Gaspar, P., Carvalho, A.L., Vinga, S., Santos, H., Neves, A.R., 2013. From physiology to
1210 systems metabolic engineering for the production of biochemicals by lactic acid bacteria.
1211 Biotechnol. Adv. 31, 764-788.
1212 Gautier, M., 2008. Ethical issues raised by genetically modified microorganisms.
1213 http://bioethics.agrocampus-ouest.eu/infoglueDeliverLive/digitalAssets/57484_41EN-ethical-
1214 issues-ogm.pdf, Last accessed on 20th July 2016.

1215 Genay, M., Sadat, L., Gagnaire, V., Lortal, S., 2009. *prtH2*, not *prtH*, is the ubiquitous cell
1216 wall proteinase gene in *Lactobacillus helveticus*. Appl. Environ. Microbiol. 75, 3238-3249.
1217 Giraffa, G., 2014. *Lactobacillus helveticus*: importance in food and health. Front. Microbiol.
1218 5, 338.
1219 Giraffa, G., De Vecchi, P., Rossetti, L., 1998. Note: identification of *Lactobacillus*
1220 *delbrueckii* subspecies *bulgaricus* and subspecies *lactis* dairy isolates by amplified rDNA
1221 restriction analysis. J. Appl. Microbiol. 85, 918-924.
1222 Gobbetti, M., De Angelis, M., Di Cagno, R., Mancini, L., Fox, P.F., 2015. Pros and cons for
1223 using non-starter lactic acid bacteria (NSLAB) as secondary/adjunct starters for cheese
1224 ripening. Trends Food. Sci. Tech. 45, 167-178.
1225 Goeddel, D.V., Kleid, D.G., Bolivar, F., Heyneker, H.L., Yansura, D.G., Crea, R., Hirose, T.,
1226 Kraszewski, A., Itakura, K., Riggs, A.D., 1979. Expression in *Escherichia coli* of chemically
1227 synthesized genes for human insulin. Proc. Natl. Acad. Sci. U S A 76, 106-110.
1228 Gold, R.S., Meagher, M.M., Tong, S.X., Hutkins, R.W., Conway, T., 1996. Cloning and
1229 expression of the *Zymomonas mobilis* "production of ethanol" genes in *Lactobacillus casei*.
1230 Curr. Microbiol. 33, 256-260.
1231 Hao, P., Zheng, H., Yu, Y., Ding, G., Gu, W., Chen, S., Yu, Z., Ren, S., Oda, M., Konno, T.,
1232 Wang, S., Li, X., Ji, Z.S., Zhao, G., 2011. Complete sequencing and pan-genomic analysis of
1233 *Lactobacillus delbrueckii* subsp. *bulgaricus* reveal its genetic basis for industrial yogurt
1234 production. PLoS One 6, e15964.
1235 Herias, M.V., Koninkx, J.F., Vos, J.G., Huis in't Veld, J.H., van Dijk, J.E., 2005. Probiotic
1236 effects of *Lactobacillus casei* on DSS-induced ulcerative colitis in mice. Int. J. Food.
1237 Microbiol. 103, 143-155.

1238 Horvath, P., Coute-Monvoisin, A.C., Romero, D.A., Boyaval, P., Fremaux, C., Barrangou,
1239 R., 2009. Comparative analysis of CRISPR loci in lactic acid bacteria genomes. Int. J. Food
1240 Microbiol. 131, 62-70.
1241 Iartchouk, O., Kozyavkin, S., Karamychev, V., Slesarev, A., 2015. Complete Genome
1242 Sequence of *Lactobacillus acidophilus* FSI4, Isolated from Yogurt. Genome Announc 3.
1243 Innocente, N., Biasutti, M., Rita, F., Brichese, R., Comi, G., Iacumin, L., 2016. Effect of
1244 indigenous *Lactobacillus rhamnosus* isolated from bovine milk on microbiological
1245 characteristics and aromatic profile of traditional yogurt. LWT-Food Sci. Technol. 66, 158-
1246 164.
1247 Irmler, S., Raboud, S., Beisert, B., Rauhut, D., Berthoud, H., 2008. Cloning and
1248 characterization of two *Lactobacillus casei* genes encoding a cystathionine lyase. Appl.
1249 Environ. Microbiol. 74, 99-106.
1250 Irmler, S., Schafer, H., Beisert, B., Rauhut, D., Berthoud, H., 2009. Identification and
1251 characterization of a strain-dependent cystathionine beta/gamma-lyase in *Lactobacillus casei*
1252 potentially involved in cysteine biosynthesis. FEMS Microbiol. Lett 295, 67-76.
1253 Jiang, W., Marraffini, L.A., 2015. CRISPR-Cas: New Tools for Genetic Manipulations from
1254 Bacterial Immunity Systems. Annu. Rev. Microbiol. 69, 209-228.
1255 Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J.A., Charpentier, E., 2012. A
1256 programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. Science
1257 337, 816-821.
1258 Kamerbeek, J., Schouls, L., Kolk, A., van Agterveld, M., van Soolingen, D., Kuijper, S.,
1259 Bunschoten, A., Molhuizen, H., Shaw, R., Goyal, M., van Embden, J., 1997. Simultaneous
1260 detection and strain differentiation of *Mycobacterium tuberculosis* for diagnosis and
1261 epidemiology. J. Clin. Microbiol. 35, 907-914.

1262 Kanchiswamy, C.N., Malnoy, M., Velasco, R., Kim, J.S., Viola, R., 2015. Non-GMO
1263 genetically edited crop plants. Trends Biotechnol. 33, 489-491.
1264 Kant, R., Blom, J., Palva, A., Siezen, R.J., de Vos, W.M., 2011. Comparative genomics of
1265 *Lactobacillus*. Microb. Biotechnol. 4, 323-332.
1266 Kant, R., Rintahaka, J., Yu, X., Sigvart-Mattila, P., Paulin, L., Mecklin, J.P., Saarela, M.,
1267 Palva, A., von Ossowski, I., 2014. A comparative pan-genome perspective of niche-adaptable
1268 cell-surface protein phenotypes in *Lactobacillus rhamnosus*. PLoS One 9, e102762.
1269 Keasling, J.D., 2012. Synthetic biology and the development of tools for metabolic
1270 engineering. Metab. Eng. 14, 189-195.
1271 King, Z.A., Lloyd, C.J., Feist, A.M., Palsson, B.O., 2015. Next-generation genome-scale
1272 models for metabolic engineering. Curr. Opin. Biotechnol. 35, 23-29.
1273 Kleerebezem, M., Boekhorst, J., van Kranenburg, R., Molenaar, D., Kuipers, O.P., Leer, R.,
1274 Tarchini, R., Peters, S.A., Sandbrink, H.M., Fiers, M.W., Stiekema, W., Lankhorst, R.M.,
1275 Bron, P.A., Hoffer, S.M., Groot, M.N., Kerkhoven, R., de Vries, M., Ursing, B., de Vos,
1276 W.M., Siezen, R.J., 2003. Complete genome sequence of *Lactobacillus plantarum* WCFS1.
1277 Proc .Natl. Acad. Sci. U S A 100, 1990-1995.
1278 Kyla-Nikkila, K., Hujanen, M., Leisola, M., Palva, A., 2000. Metabolic engineering of
1279 *Lactobacillus helveticus* CNRZ32 for production of pure L-(+)-lactic acid. Appl. Environ.
1280 Microbiol. 66, 3835-3841.
1281 Ladero, V., Ramos, A., Wiersma, A., Goffin, P., Schanck, A., Kleerebezem, M., Hugenholtz,
1282 J., Smid, E.J., Hols, P., 2007. High-level production of the low-calorie sugar sorbitol by
1283 *Lactobacillus plantarum* through metabolic engineering. Appl. Environ. Microbiol. 73, 1864-
1284 1872.
1285 Lebeer, S., Vanderleyden, J., De Keersmaecker, S.C., 2008. Genes and molecules of
1286 lactobacilli supporting probiotic action. Microbiol Mol Biol Rev 72, 728-764.

1287 Lewis, N.E., Nagarajan, H., Palsson, B.O., 2012. Constraining the metabolic genotype-
1288 phenotype relationship using a phylogeny of *in silico* methods. Nat. Rev. Microbiol. 10, 291-
1289 305.
1290 Li, N., Huang, Y., Liu, Z., You, C., Guo, B., 2015a. Regulation of EPS production in
1291 *Lactobacillus casei* LC2W through metabolic engineering. Lett. Appl. Microbiol. 61, 555-
1292 561.
1293 Li, N., Wang, Y., Zhu, P., Liu, Z., Guo, B., Ren, J., 2015b. Improvement of
1294 exopolysaccharide production in *Lactobacillus casei* LC2W by overexpression of NADH
1295 oxidase gene. Microbiol. Res. 171, 73-77.
1296 Liu, E., Hao, P., Konno, T., Yu, Y., Oda, M., Zheng, H., Ji, Z.-S., 2012. Amino Acid
1297 Biosynthesis and Proteolysis in *Lactobacillus Bulgaricus* Revisited: A Genomic Comparison.
1298 Comput. Mol. Biosci. 02, 61-77.
1299 Liu, M., Bayjanov, J.R., Renckens, B., Nauta, A., Siezen, R.J., 2010. The proteolytic system
1300 of lactic acid bacteria revisited: a genomic comparison. BMC Genomics 11, 36.
1301 Liu, M., Bienfait, B., Sacher, O., Gasteiger, J., Siezen, R.J., Nauta, A., Geurts, J.M., 2014.
1302 Combining chemoinformatics with bioinformatics: *in silico* prediction of bacterial flavor-
1303 forming pathways by a chemical systems biology approach "reverse pathway engineering".
1304 PLoS One 9, e84769.
1305 Liu, M., Nauta, A., Francke, C., Siezen, R.J., 2008. Comparative genomics of enzymes in
1306 flavor-forming pathways from amino acids in lactic acid bacteria. Appl. Environ. Microbiol.
1307 74, 4590-4600.
1308 Liu, S., Nichols, N.N., Dien, B.S., Cotta, M.A., 2006. Metabolic engineering of a
1309 *Lactobacillus plantarum* double *ldh* knockout strain for enhanced ethanol production. J. Ind.
1310 Microbiol. Biotechnol. 33, 1-7.

1311 Ljungh, A., Wadstrom, T., 2006. Lactic acid bacteria as probiotics. Curr. Issues Intest.
1312 Microbiol. 7, 73-90.
1313 Mahadevan, R., Schilling, C.H., 2003. The effects of alternate optimal solutions in constraint-
1314 based genome-scale metabolic models. Metab. Eng. 5, 264-276.
1315 Makarova, K., Slesarev, A., Wolf, Y., Sorokin, A., Mirkin, B., Koonin, E., Pavlov, A.,
1316 Pavlova, N., Karamychev, V., Polouchine, N., Shakhova, V., Grigoriev, I., Lou, Y., Rohksar,
1317 D., Lucas, S., Huang, K., Goodstein, D.M., Hawkins, T., Plengvidhya, V., Welker, D.,
1318 Hughes, J., Goh, Y., Benson, A., Baldwin, K., Lee, J.H., Diaz-Muniz, I., Dosti, B., Smeianov,
1319 V., Wechter, W., Barabote, R., Lorca, G., Altermann, E., Barrangou, R., Ganesan, B., Xie,
1320 Y., Rawsthorne, H., Tamir, D., Parker, C., Breidt, F., Broadbent, J., Hutkins, R., O'Sullivan,
1321 D., Steele, J., Unlu, G., Saier, M., Klaenhammer, T., Richardson, P., Kozyavkin, S., Weimer,
1322 B., Mills, D., 2006. Comparative genomics of the lactic acid bacteria. Proc. Natl. Acad. Sci.
1323 U S A 103, 15611-15616.
1324 Makarova, K.S., Koonin, E.V., 2007. Evolutionary genomics of lactic acid bacteria. J.
1325 Bacteriol. 189, 1199-1208.
1326 Marco, M.B., Moineau, S., Quiberoni, A., 2012. Bacteriophages and dairy fermentations.
1327 Bacteriophage 2, 149-158.
1328 Marilley, L., Casey, M.G., 2004. Flavours of cheese products: metabolic pathways, analytical
1329 tools and identification of producing strains. Int. J. Food Microbiol. 90, 139-159.
1330 Mayo, B., Aleksandrzak-Piekarczyk, T., Fernández, M., Kowalczyk, M., Álvarez-Martín, P.,
1331 Bardowski, J., 2010. Updates in the Metabolism of Lactic Acid Bacteria, in: Mozzi, F., Raya,
1332 R., Vignolo, G. (Eds.), Biotechnology of Lactic Acid Bacteria. Wiley-Blackwell, pp. 3-33.
1333 Mayo, B., van Sinderen, D., Ventura, M., 2008. Genome analysis of food grade lactic Acid-
1334 producing bacteria: from basics to applications. Curr. Genomics 9, 169-183.

1335 Mazzoli, R., Bosco, F., Mizrahi, I., Bayer, E.A., Pessione, E., 2014. Towards lactic acid
1336 bacteria-based biorefineries. Biotechnol. Adv. 32, 1216-1236.
1337 McAuliffe, O. 2017. Genetics of Lactic Acid Bacteria, in: Fox, P.F., McSweeney, P.L.H.,
1338 Cotter, P., Everett, D.W. (Eds), Cheese: Chemistry, Physics And Microbiology, Fourth
1339 Edition, Elsevier, The Netherlands - Currently in press
1340 Medina de Figueroa, R., Oliver, G., Benito de Cádenas, I.L., 2001. Influence of temperature
1341 on flavour compound production from citrate by *Lactobacillus rhamnosus* ATCC 7469.
1342 Microbiol. Res. 155, 257-262.
1343 Medini, D., Donati, C., Tettelin, H., Masignani, V., Rappuoli, R., 2005. The microbial pan-
1344 genome. Curr. Opin. Genet. Dev. 15, 589-594.
1345 Meneghel, J., Dugat-Bony, E., Irlinger, F., Loux, V., Vidal, M., Passot, S., Beal, C., Layec,
1346 S., Fonseca, F., 2016. Draft Genome Sequence of *Lactobacillus delbrueckii* subsp. *bulgaricus*
1347 CFL1, a Lactic Acid Bacterium Isolated from French Handcrafted Fermented Milk. Genome
1348 Announc. 4.
1349 Milesi, M.M., Wolf, I.V., Bergamini, C.V., Hynes, E.R., 2010. Two strains of nonstarter
1350 lactobacilli increased the production of flavor compounds in soft cheeses. J. Dairy Sci. 93,
1351 5020-5031.
1352 Molenaar, D., Bringel, F., Schuren, F.H., de Vos, W.M., Siezen, R.J., Kleerebezem, M.,
1353 2005. Exploring *Lactobacillus plantarum* genome diversity by using microarrays. J.
1354 Bacteriol. 187, 6119-6127.
1355 Mortera, P., Pudlik, A., Magni, C., Alarcon, S., Lolkema, J.S., 2013. Ca^{2+}-citrate uptake and
1356 metabolism in *Lactobacillus casei* ATCC 334. Appl. Environ. Microbiol. 79, 4603-4612.
1357 Nadal, I., Rico, J., Perez-Martinez, G., Yebra, M.J., Monedero, V., 2009. Diacetyl and
1358 acetoin production from whey permeate using engineered *Lactobacillus casei*. J. Ind.
1359 Microbiol. Biotechnol. 36, 1233-1237.

Nichols, N.N., Dien, B.S., Bothast, R.J., 2003. Engineering lactic acid bacteria with pyruvate decarboxylase and alcohol dehydrogenase genes for ethanol production from *Zymomonas mobilis*. J. Ind. Microbiol. Biotechnol. 30, 315-321.

Nissen, L., Perez-Martinez, G., Yebra, M.J., 2005. Sorbitol synthesis by an engineered *Lactobacillus casei* strain expressing a sorbitol-6-phosphate dehydrogenase gene within the lactose operon. FEMS Microbiol. Lett. 249, 177-183.

O'Brien, E.J., Monk, J.M., Palsson, B.O., 2015. Using Genome-scale Models to Predict Biological Capabilities. Cell 161, 971-987.

O'Sullivan, O., O'Callaghan, J., Sangrador-Vegas, A., McAuliffe, O., Slattery, L., Kaleta, P., Callanan, M., Fitzgerald, G.F., Ross, R.P., Beresford, T., 2009. Comparative genomics of lactic acid bacteria reveals a niche-specific gene set. BMC Microbiol. 9, 50.

Oliveira, A.P., Nielsen, J., Forster, J., 2005. Modeling *Lactococcus lactis* using a genome-scale flux model. BMC Microbiol. 5, 39.

Ong, L., Henriksson, A., Shah, N.P., 2007. Chemical analysis and sensory evaluation of Cheddar cheese produced with *Lactobacillus acidophilus, Lb. casei, Lb. paracasei* or *Bifidobacterium* sp. Int. Dairy J. 17, 937-945.

Orth, J.D., Thiele, I., Palsson, B.O., 2010. What is flux balance analysis? Nat. Biotechnol. 28, 245-248.

Papagianni, M., 2012. Metabolic engineering of lactic acid bacteria for the production of industrially important compounds. Comput. Struct. Biotechnol. J 3, e201210003.

Pastink, M.I., Teusink, B., Hols, P., Visser, S., de Vos, W.M., Hugenholtz, J., 2009. Genome-scale model of *Streptococcus thermophilus* LMG18311 for metabolic comparison of lactic acid bacteria. Appl. Environ. Microbiol. 75, 3627-3633.

1383 Pedersen, M.B., Iversen, S.L., Sorensen, K.I., Johansen, E., 2005. The long and winding road
1384 from the research laboratory to industrial applications of lactic acid bacteria. FEMS
1385 Microbiol. Rev. 29, 611-624.
1386 Pfeiler, E.A., Klaenhammer, T.R., 2007. The genomics of lactic acid bacteria. Trends
1387 Microbiol. 15, 546-553.
1388 Pridmore, R.D., Berger, B., Desiere, F., Vilanova, D., Barretto, C., Pittet, A.C., Zwahlen,
1389 M.C., Rouvet, M., Altermann, E., Barrangou, R., Mollet, B., Mercenier, A., Klaenhammer,
1390 T., Arigoni, F., Schell, M.A., 2004. The genome sequence of the probiotic intestinal
1391 bacterium *Lactobacillus johnsonii* NCC 533. Proc. Natl. Acad. Sci. U S A 101, 2512-2517.
1392 Ramachandran, P., Lacher, D.W., Pfeiler, E.A., Elkins, C.A., 2013. Development of a tiered
1393 multilocus sequence typing scheme for members of the *Lactobacillus acidophilus* complex.
1394 Appl. Environ. Microbiol. 79, 7220-7228.
1395 Rossi, F., Rizzotti, L., Felis, G.E., Torriani, S., 2014. Horizontal gene transfer among
1396 microorganisms in food: current knowledge and future perspectives. Food Microbiol. 42,
1397 232-243.
1398 Sander, J.D., Joung, J.K., 2014. CRISPR-Cas systems for editing, regulating and targeting
1399 genomes. Nat. Biotechnol. 32, 347-355.
1400 Savijoki, K., Ingmer, H., Varmanen, P., 2006. Proteolytic systems of lactic acid bacteria.
1401 Appl. Microbiol. Biotechnol. 71, 394-406.
1402 Selle, K., Barrangou, R., 2015. Harnessing CRISPR-Cas systems for bacterial genome
1403 editing. Trends. Microbiol. 23, 225-232.
1404 Senan, S., Prajapati, J.B., Joshi, C.G., 2014. Comparative genome-scale analysis of niche-
1405 based stress-responsive genes in *Lactobacillus helveticus* strains. Genome 57, 185-192.

1406 Senan, S., Prajapati, J.B., Joshi, C.G., 2015. Whole-genome based validation of the adaptive
1407 properties of Indian origin probiotic *Lactobacillus helveticus* MTCC 5463. J. Sci. Food
1408 Agric. 95, 321-328.
1409 Settanni, L., Moschetti, G., 2010. Non-starter lactic acid bacteria used to improve cheese
1410 quality and provide health benefits. Food Microbiol. 27, 691-697.
1411 Sgarbi, E., Lazzi, C., Tabanelli, G., Gatti, M., Neviani, E., Gardini, F., 2013. Nonstarter lactic
1412 acid bacteria volatilomes produced using cheese components. J. Dairy Sci. 96, 4223-4234.
1413 Siezen, R.J., Tzeneva, V.A., Castioni, A., Wels, M., Phan, H.T., Rademaker, J.L.,
1414 Starrenburg, M.J., Kleerebezem, M., Molenaar, D., van Hylckama Vlieg, J.E., 2010.
1415 Phenotypic and genomic diversity of *Lactobacillus plantarum* strains isolated from various
1416 environmental niches. Environ. Microbiol. 12, 758-773.
1417 Siezen, R.J., van Hylckama Vlieg, J.E., 2011. Genomic diversity and versatility of
1418 *Lactobacillus plantarum*, a natural metabolic engineer. Microb. Cell Fact. 10 Suppl 1, S3.
1419 Smid, E.J., Hugenholtz, J., 2010. Functional genomics for food fermentation processes.
1420 Annu. Rev. Food. Sci. Technol. 1, 497-519.
1421 Smit, G., Smit, B.A., Engels, W.J., 2005. Flavour formation by lactic acid bacteria and
1422 biochemical flavour profiling of cheese products. FEMS Microbiol. Rev. 29, 591-610.
1423 Smokvina, T., Wels, M., Polka, J., Chervaux, C., Brisse, S., Boekhorst, J., van Hylckama
1424 Vlieg, J.E., Siezen, R.J., 2013. *Lactobacillus paracasei* comparative genomics: towards
1425 species pan-genome definition and exploitation of diversity. PLoS One 8, e68731.
1426 Stahl, B., Barrangou, R., 2013. Complete Genome Sequence of Probiotic Strain *Lactobacillus*
1427 *acidophilus* La-14. Genome Announc. 1, e00376-00313.
1428 Steele, J., Broadbent, J., Kok, J., 2013. Perspectives on the contribution of lactic acid bacteria
1429 to cheese flavor development. Curr. Opin. Biotechnol. 24, 135-141.

Strahinic, I., Lozo, J., Terzic-Vidojevic, A., Fira, D., Kojic, M., Golic, N., Begovic, J., Topisirovic, L., 2013. Technological and probiotic potential of BGRA43 a natural isolate of *Lactobacillus helveticus*. Front. Microbiol. 4, 2.

Sun, Z., Harris, H.M., McCann, A., Guo, C., Argimon, S., Zhang, W., Yang, X., Jeffery, I.B., Cooney, J.C., Kagawa, T.F., Liu, W., Song, Y., Salvetti, E., Wrobel, A., Rasinkangas, P., Parkhill, J., Rea, M.C., O'Sullivan, O., Ritari, J., Douillard, F.P., Paul Ross, R., Yang, R., Briner, A.E., Felis, G.E., de Vos, W.M., Barrangou, R., Klaenhammer, T.R., Caufield, P.W., Cui, Y., Zhang, H., O'Toole, P.W., 2015a. Expanding the biotechnology potential of lactobacilli through comparative genomics of 213 strains and associated genera. Nat. Commun. 6, 8322.

Sun, Z., Liu, W., Song, Y., Xu, H., Yu, J., Bilige, M., Zhang, H., Chen, Y., 2015b. Population structure of *Lactobacillus helveticus* isolates from naturally fermented dairy products based on multilocus sequence typing. J. Dairy Sci. 98, 2962-2972.

Sybesma, W., Hugenholtz, J., de Vos, W.M., Smid, E.J., 2006. Safe use of genetically modified lactic acid bacteria in food. Bridging the gap between consumers, green groups, and industry. Electron. J. Biotechn. 9, 424-448.

Tanous, C., Kieronczyk, A., Helinck, S., Chambellon, E., Yvon, M., 2002. Glutamate dehydrogenase activity: a major criterion for the selection of flavour-producing lactic acid bacteria strains. Antonie Van Leeuwenhoek 82, 271-278.

Taverniti, V., Guglielmetti, S., 2012. Health-Promoting Properties of *Lactobacillus helveticus*. Front. Microbiol. 3, 392.

Teusink, B., Bachmann, H., Molenaar, D., 2011. Systems biology of lactic acid bacteria: a critical review. Microb. Cell Fact. 10 Suppl 1, S11.

1453 Teusink, B., Wiersma, A., Molenaar, D., Francke, C., de Vos, W.M., Siezen, R.J., Smid, E.J.,
1454 2006. Analysis of growth of *Lactobacillus plantarum* WCFS1 on a complex medium using a
1455 genome-scale metabolic model. J. Biol. Chem. 281, 40041-40048.
1456 Toh, H., Oshima, K., Nakano, A., Takahata, M., Murakami, M., Takaki, T., Nishiyama, H.,
1457 Igimi, S., Hattori, M., Morita, H., 2013. Genomic adaptation of the *Lactobacillus casei* group.
1458 PLoS One 8, e75073.
1459 Tsuji, A., Okada, S., Hols, P., Satoh, E., 2013. Metabolic engineering of *Lactobacillus*
1460 *plantarum* for succinic acid production through activation of the reductive branch of the
1461 tricarboxylic acid cycle. Enzyme Microb. Technol. 53, 97-103.
1462 Tuo, Y., Zhang, W., Zhang, L., Ai, L., Zhang, Y., Han, X., Yi, H., 2013. Study of probiotic
1463 potential of four wild *Lactobacillus rhamnosus* strains. Anaerobe 21, 22-27.
1464 Urshev, Z., Ishlimova, D., 2015. Distribution of clustered regularly interspaced palindrome
1465 repeats CRISPR2 and CRISPR3 in *Lactobacillus delbrueckii* ssp. *bulgaricus* strains.
1466 Biotechnol. Biotec. Eq. 29, 541-546.
1467 Van de Guchte, M., Penaud, S., Grimaldi, C., Barbe, V., Bryson, K., Nicolas, P., Robert, C.,
1468 Oztas, S., Mangenot, S., Couloux, A., Loux, V., Dervyn, R., Bossy, R., Bolotin, A., Batto,
1469 J.M., Walunas, T., Gibrat, J.F., Bessieres, P., Weissenbach, J., Ehrlich, S.D., Maguin, E.,
1470 2006. The complete genome sequence of *Lactobacillus bulgaricus* reveals extensive and
1471 ongoing reductive evolution. Proc. Natl. Acad. Sci. U S A 103, 9274-9279.
1472 Van Hoorde, K., Van Leuven, I., Dirinck, P., Heyndrickx, M., Coudijzer, K., Vandamme, P.,
1473 Huys, G., 2010. Selection, application and monitoring of *Lactobacillus paracasei* strains as
1474 adjunct cultures in the production of Gouda-type cheeses. Int. J. Food Microbiol. 144, 226-
1475 235.

Van Kranenburg, R., Kleerebezem, M., van Hylckama Vlieg, J., Ursing, B.M., Boekhorst, J., Smit, B.A., Ayad, E.H.E., Smit, G., Siezen, R.J., 2002. Flavour formation from amino acids by lactic acid bacteria: predictions from genome sequence analysis. Int. Dairy J. 12, 111-121.

Villena, J., Oliveira, M.L., Ferreira, P.C., Salva, S., Alvarez, S., 2011. Lactic acid bacteria in the prevention of pneumococcal respiratory infection: future opportunities and challenges. Int. Immunopharmacol. 11, 1633-1645.

Vinay-Lara, E., Hamilton, J.J., Stahl, B., Broadbent, J.R., Reed, J.L., Steele, J.L., 2014. Genome-scale reconstruction of metabolic networks of *Lactobacillus casei* ATCC 334 and 12A. PLoS One 9, e110785.

Wright, A.V., Nunez, J.K., Doudna, J.A., 2016. Biology and Applications of CRISPR Systems: Harnessing Nature's Toolbox for Genome Engineering. Cell 164, 29-44.

Wyszynska, A., Kobierecka, P., Bardowski, J., Jagusztyn-Krynicka, E.K., 2015. Lactic acid bacteria-20 years exploring their potential as live vectors for mucosal vaccination. Appl. Microbiol. Biotechnol. 99, 2967-2977.

Xu, N., Liu, J., Ai, L., Liu, L., 2015. Reconstruction and analysis of the genome-scale metabolic model of *Lactobacillus casei* LC2W. Gene 554, 140-147.

Ya, T., Zhang, Q., Chu, F., Merritt, J., Bilige, M., Sun, T., Du, R., Zhang, H., 2008. Immunological evaluation of *Lactobacillus casei* Zhang: a newly isolated strain from koumiss in Inner Mongolia, China. BMC Immunol. 9, 68.

Yebra, M.J., Pérez-Martínez, G., Rodríguez-Díaz, J., Monedero, V., 2012. Genetically Engineered Lactobacilli for Technological and Functional Food Applications, in Valdez, B. (Ed), Food Industrial Processes –Methods and Equipment. InTech, Shanghai, China, pp. 143-168.

1499 Yebra, M.J., Zuniga, M., Beaufils, S., Perez-Martinez, G., Deutscher, J., Monedero, V., 2007.
1500 Identification of a gene cluster enabling *Lactobacillus casei* BL23 to utilize myo-inositol.
1501 Appl. Environ. Microbiol. 73, 3850-3858.
1502 Yu, S., Peng, Y., Zheng, Y., Chen, W., 2015. Comparative Genome Analysis of
1503 *Lactobacillus casei*: Insights into Genomic Diversification for Niche Expansion. Indian J.
1504 Microbiol. 55, 102-107.
1505 Zhu, Y., Zhang, Y., Li, Y., 2009. Understanding the industrial application potential of lactic
1506 acid bacteria through genomics. Appl. Microbiol. Biotechnol. 83, 597-610.

1507 Table 1: General genomic features of the most important dairy related *Lactobacillus* species.

1508 All data were obtained at http://www.ncbi.nlm.nih.gov/, last assessed in July 2016.

Species of *Lactobacillus*	Number of sequences available	Median total length (Mbp)	Median number of proteins	Median GC content (%)
Lb. delbrueckii	32	1.865	1637	49.8
Lb. helveticus	22	2.077	1784	36.8
Lb. casei	35	3.036	2736	46.4
Lb. paracasei	53	2.961	2749	46.3
Lb. acidophilus	16	1.979	1815	34.6
Lb. rhamnosus	102	2.937	2641	46.6
Lb. plantarum	114	3.275	2912	44.4

1509

Figures captions:

Figure 1: Process of niche adaptation. (a) Ancestor of *Lactobacillus* spp. had undergone multiple genome changes, such as decay of superfluous genes and acquisition of genes that support survival in specific environmental conditions, which all led to niche specialisation for various habitats, three of which have been depicted here (dairy, environment, human and animal GIT). However, strains of *Lactobacillus* could change their habitat (b), for instance during human consumption of dairy or plant food, and this is why isolation source does not always correspond to the strains' natural environment. This has to be kept in mind while analysing characteristics of strains isolated from different ecological niches, as origin of isolation gives only an indication of metabolic capacity of an organism.

Figure 2: (a) CRISPR-Cas system of bacteria enables efficient resistance to phage attack. For example, in case of dairy lactobacilli, when the cells encounter the dairy phage for the first time, its DNA is cleaved and a sequence that includes repeater (black box) and spacer (blue box) is integrated in CRISPR-cas locus, directly behind the leader sequence. (b) In the event of repeated attack by the same phage, its DNA sequence corresponding to an existing spacer induces transcription and maturation of CRISPR RNA (crRNA), which activates Cas complex and efficiently cleaves the foreign DNA. Further stages of phage reproduction are terminated, and there are no newly assembled phage particles. As the dairy strain combats the phage, normal fermentation process occurs. (c) CRISPR systems mechanism initiated development of genome editing tool. Here, Cas 9 nuclease interacts with chimeric guide RNA, that provides the enzyme to the specific site in DNA, after which precise double stranded break (DBS) occurs. After DBS, breaks can be either nonhomologously joined leading to an indel mutation, or, in presence of a donor DNA, this sequence is precisely inserted in a homology directed repair event.

Figure 3: Schematic view of range of applications of available genome sequences. The whole genome sequencing (WGS) data provides the basis for genomic characterisation of species or genera, as well as evolutionary studies, such as niche adaptability. Insight in genetic content of a strain can predict the presence of metabolic machinery that could generate flavour compounds. Additionally, they enable the construction of genome scale metabolic models, which coupled to genetic information and biochemical data lead to the development of metabolic engineering studies. Results of these studies reveal strains capacity for plausible industrial applications.