Ecological Fitness and Interspecies Interactions of Food-Spoilage-Associated Lactic Acid Bacteria: Insights from the Genome Analyses and Transcriptome Profiles

MARGARITA ANDREEVSKAYA

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Ecological Fitness and Interspecies Interactions of Food-Spoilage-Associated Lactic Acid Bacteria: Insights from the Genome Analyses and Transcriptome Profiles

Margarita Andreevskaya

ACADEMIC DISSERTATION

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AUTHOR’S CONTRIBUTION

I. MA performed functional annotation of the complete *Lc. piscium* genome, comparative analysis of *Lactococcus* genomes, RNA-seq data pre-processing and differential expression analysis, interpretation of the results, and wrote the manuscript.

II. MA performed functional annotation of the complete *Lb. oligofermentans* genome, carried out RNA-seq data pre-processing and differential expression analysis, search of transcription factor binding sites, interpretation of the results, and wrote the manuscript.

III. MA performed functional annotation of the complete *Le. gelidum* subsp. *gasicomitatum* KG16-1 genome, comparative analysis of 41 *Leuconostoc* genomes, interpretation of the results and wrote the manuscript.

IV. MA carried out RNA-seq data pre-processing and differential expression analysis, functional enrichment analysis, interpretation of the results, and wrote the manuscript.
ABSTRACT

Lactic acid bacteria (LAB) play a dual role in food manufacturing. While being indispensable for food fermentations and preservation, they are also involved in spoilage of foods and beverages, and some food-borne LAB are pathogens. Particularly, they became the main spoilage organisms in the cold-stored modified atmosphere packaged (MAP) foods. LAB species composition and their relative abundances depend on the nature of food products and preservation technology. However, two LAB species, *Leuconostoc gelidum* and *Lactococcus piscium*, have frequently been predominating at the end of shelf life in a variety of packaged and refrigerated foods of animal and plant origin. Besides the predominant species, spoilage LAB communities contain less abundant and slower growing species, such as *Lactobacillus oligofermentans*, the role of which in food spoilage is unclear. Taking into account the increased popularity of MAP technology combined with cold storage for preservation of minimally processed fresh foods, the need to obtain more information on the metabolism, genomics, ecology and interactions of psychrotrophic food-spoilage-associated LAB is clear. In this thesis a genomic approach was used to study these LAB.

In order to characterize spoilage community members, we sequenced and annotated genomes of *Lc. piscium* MKFS47 and *Lb. oligofermentans* LMG 22743\(^{T}\), both isolated from broiler meat, and seven strains of *Le. gelidum*, isolated from vegetable-based foods. The analysis of their gene contents and their comparison with gene repertoire of other close related species allowed us to predict putative factors that might facilitate their survival in their habitats and increase competitiveness in the spoilage microbial communities. No major differences in the gene contents of the “vegetable” and “meat” *Le. gelidum* strains were observed that would suggest niche-specificity, therefore, indicating that the absence of strain dissemination between vegetable- and meat-processing chains is a more likely factor responsible for the reported strain segregation between vegetable and meat-based products.

*Lc. piscium* MKFS47 was identified as an efficient producer of buttery off-odors compounds from glucose under aerobic conditions, which is in agreement with the previous inoculation studies. Time course glucose catabolism-based transcriptome profiles revealed the presence of classical carbon catabolite repression mechanism for the regulation of carbohydrate catabolism, which was relieved along with decreasing concentration of glucose. During the same time, the shift from homolactic to heterolactic fermentation mode was observed.

For *Lb. oligofermentans*, a pentose-preferring obligate heterofermentative LAB, the induction of efficient utilization of hexoses was confirmed indicating that it has flexible carbohydrate catabolism, which can be adjusted depending on the carbohydrate sources available in the environment. Unexpectedly, transcriptome responses of *Lb. oligofermentans* during growth on glucose and xylose were more alike than during fermentation of ribose in the early exponential growth phase. In addition, cross induction of glucose and xylose catabolic genes by either glucose or xylose was observed. These phenomena could be governed by the CcpA transcriptional regulator, the regulation mechanism of which remains to be determined.
Transcriptome-based study of interspecies interactions between three above mentioned LAB species revealed their different survival strategies to cope with competition for the common resources. *Le. gelidum* was shown to enhance its nutrient-(mainly carbohydrates) scavenging and growth capabilities under glucose limitation conditions when competing with the other LAB species, while the opposite was observed for *Lc. piscium* and *Lb. oligofermentans*. Such behavior might explain the competitive success and, hence, the predominance of *Le. gelidum* in spoilage microbial communities. Peculiarly, interspecies interactions activated expression of prophages and restriction modification systems in *Lc. piscium* and *Lb. oligofermentans*, but not in *Le. gelidum*. The downregulation of stress protection-related genes in all the LAB at the early growth stage was unexpected, and it requires further studies. Finally, overexpression of the numerous putative adhesins in *Lb. oligofermentans* during growth with other LAB could be one of the factors explaining its survival in actively growing communities in meat.
ABSTRAKTI


indusoitavissa sekä glukoosilla että ksyloosilla. Näitä ilmiöitä saattaa hallinnoida CcpA-säätelytekijä, jonka säätelymekanismi on toistaiseksi selvittämättä.

Transkriptomeihin perustuva tutkimus kolmen yllämainitun maitohappobakteerilajin vuorovaikutuksista paljasti niiden erilaiset selviytymisstrategiat yhteisistä resurssista kilpailtaessa. Le. gelidumin osoitettiin tehostavan kykyään hankkia ravinteita (lähinnä hiilihydraatteja) sekä kasvaa glukoosirajoitetuissa olosuhteissa, kun se kilpaili muiden maitohappobakteerilajien kanssa. Lc. piscium ja Lb. oligofermentans puolestaan toimivat päinvastoin. Tämä käytös saattaa selittää Le. gelidumin kilpailumenestystä, ja siten sen vallitsevuutta pilaajabakteeriyhteisöissä. Yllättävää kyllä lajienväliset vuorovaikutukset aktivoivat profaagien ja restriktiomodifikaatiosysteemien ekspresion Lc. pisciumilla ja Lb. oligofermentansilla, mutta ei Le. gelidumilla. Lisätutkimusta vaatisi myös se odottamaton havainto, että kaikilla maitohappobakteereilla esiintyi stressiltä suojaantumiseen liittyvien geenien vaimentumista varhaisessa kasvu vaiheessa. Lopuksi, lukuisen ennustetujen adhesiinien yleiespressio Lb. oligofermentansilla muiden maitohappobakteerien kanssa kasvatettaessa saattaa olla yksi selitys sille, miten bakteeri selviää aktiivisesti kasvavissa yhteisöissä lihassa.

Translated into Finnish by Velma Aho
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>CCA</td>
<td>carbon catabolite activation</td>
</tr>
<tr>
<td>CCC</td>
<td>carbon catabolite control</td>
</tr>
<tr>
<td>CCR</td>
<td>carbon catabolite repression</td>
</tr>
<tr>
<td>CDS</td>
<td>coding DNA sequence</td>
</tr>
<tr>
<td>CFU</td>
<td>colony-forming unit</td>
</tr>
<tr>
<td>COG</td>
<td>clusters of orthologous groups of proteins</td>
</tr>
<tr>
<td>DE analysis</td>
<td>differential expression analysis</td>
</tr>
<tr>
<td>DE genes</td>
<td>differentially expressed genes</td>
</tr>
<tr>
<td>DHA</td>
<td>dihydroxyacetone</td>
</tr>
<tr>
<td>FC</td>
<td>fold change</td>
</tr>
<tr>
<td>LAB</td>
<td>lactic acid bacterium/bacteria</td>
</tr>
<tr>
<td>MAP</td>
<td>modified atmosphere packaging/packaged</td>
</tr>
<tr>
<td>MRS</td>
<td>de Man-Rogosa-Sharpe</td>
</tr>
<tr>
<td>OD$_{600}$</td>
<td>optical density at 600 nm</td>
</tr>
<tr>
<td>PTS</td>
<td>phosphotransferase system</td>
</tr>
<tr>
<td>RM</td>
<td>restriction modification</td>
</tr>
<tr>
<td>RNA-seq</td>
<td>RNA sequencing</td>
</tr>
<tr>
<td>RPKM</td>
<td>reads per kilobase of gene per million mapped reads</td>
</tr>
<tr>
<td>TEM</td>
<td>transmission electron microscopy</td>
</tr>
<tr>
<td>TFBS</td>
<td>transcription factor binding site</td>
</tr>
</tbody>
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1 INTRODUCTION

1.1 LAB and their diverse roles

Lactic acid bacteria (LAB) constitute a well-known group of Gram-positive bacteria that are generally characterized as nonsporulating, nonrespiring, acid-tolerant, facultative anaerobic cocci and rods producing lactic acid as a main end product during carbohydrate fermentation (1). LAB belong to the order Lactobacillaceae, phylum Firmicutes. This order contains six families and 36 genera, the most known of which are Lactobacillus, Lactococcus, Leuconostoc, Enterococcus, Streptococcus and Carnobacteria. Historically, genus Bifidobacterium was also considered to belong to LAB due to their physiological resemblance of LAB. However, phylogenetically this genus is distant from other LAB and belongs to the phylum Actinobacteria (2). LAB are widespread in nature and occupy mainly nutritionally rich niches of animal and plant origin. Therefore, adaptation to these niches was accompanied by the gene loss and genome size reduction (3). LAB are best known for their use in preservation and fermentation of foods and beverages, such as dairy products, meats, vegetables, wine, silage, sourdough and others (3–6). Released antimicrobial compounds, such as lactic and acetic acids, bacteriocins, carbon dioxide and oxygen metabolites, inhibit pathogenic microbiota in foods and explain the preservative properties of LAB (4, 7, 8). In addition, end products of LAB metabolism improve the texture of food products, confer characteristic flavors and tastes, and increase nutritional quality of fermented foods (4, 5). Being part of normal microbiota of mucosal surfaces in animals and humans, LAB confer benefits on the host through the pathogenic microbiota exclusion (9). Besides playing positive roles in human life, some LAB are well-known pathogens causing different diseases, such as dental caries, infections of skin and mucosal surfaces, meningitis and other (10). Finally, growth and metabolic activities of some LAB cause spoilage of variety of food products and beverages, such as red meats, poultry, fish, vegetables, beer and wine (11–14).

1.2 The role of LAB in spoilage of MAP food products

Food spoilage is characterized by any alterations in food products that render them unacceptable for consumption (15). It can be caused by physico-chemical factors (physical damage, loss of texture, oxidation), endogenous enzyme-associated hydrolysis and growth of microorganisms, which is associated with production of off-odor compounds and toxins, increased acidification, discoloration, bulging and textural changes (15, 16). Microbial spoilage is the most common cause of food spoilage.

To inhibit growth of spoilage-causing microorganisms and prolong the shelf life of food products, different preservation techniques, such as drying, marination, salting,
cold storage, addition of preservatives, modified atmosphere packaging (MAP) and other are applied (16). Due to the increased demand on minimally processed fresh foods with minimal amount of preservatives, combination of MAP with chill storage became a common practice to prolong shelf life of foods during the last decades (16). In MAP technology a food product is packaged and the gas composition inside the package is modified to inhibit growth of spoilage and pathogenic bacteria. The gas composition is determined by the nature of a product and type of spoilage changes. Generally, modification process attempts to reduce oxygen levels and increase carbon dioxide concentration. Low oxygen concentration allows to diminish oxidation-related deterioration, such as rancidity and discoloration, and inhibit growth of aerobic microbiota (16, 17). The exceptions are red meats, which require high-oxygen atmosphere to maintain the bright red color due to oxidized myoglobin (18), or vegetables, which cannot be maintained anaerobically (17). Carbon dioxide exhibits antimicrobial properties and its high concentration allows to inhibit growth of Gram-negative spoilage bacteria (16, 19). While MAP creates a negative selective pressure towards aerobic Gram-negative bacteria, the members of which constitute the majority of rapidly-growing food spoilage organisms (20), modified atmosphere facilitates growth of carbon dioxide tolerant, facultative anaerobic and slower growing LAB (15, 19). In addition, LAB are salt tolerant (21) and nitrite resistant (22), which also facilitate their prevalence in cooked cured meats (19). The chill storage of MAP foods determines the psychrotrophic nature of spoilage LAB. Due to the traditional use of aerobic mesophilic counts instead of psychrotrophic counts as a parameter of microbial quality and reference shelf life of food products, LAB contamination levels were largely underestimated in chilled MAP foods for a long time (23). Even though the shift to LAB microbiota is preferable due to the general non-pathogenic nature of LAB, their slower growth rates and less offensive spoilage metabolites, their metabolic activities change organoleptic and textural characteristics of food products. These activities include production of gas, slime and biogenic amines, discoloration, off-odor formation, increased acidity (24). Processing facilities and food ingredients are assumed to be the main sources of LAB contamination in food products (25–27).

1.3 Spoilage-associated LAB in packaged foods

The most abundant LAB genera associated with spoilage of packaged and chilled-stored food products are *Leuconostoc, Lactobacillus, Lactococcus, Carnobacterium* and *Weissella* (12, 13) The LAB species composition and their relative abundances depend on the nature of food products (e.g. red meat, poultry, fish, vegetables), treatment procedures and additives (e.g. raw, marinated, salted, cooked) and the composition of modified atmosphere (e.g. high oxygen, vacuum, anaerobic) (13, 19). However, two species, *Leuconostoc gelidum* and *Lactococcus piscium* (12, 14, 28), were found to be predominant at the end of shelf life in a variety of cold-stored packaged foods of animal and plant origin.
1.4 *Leuconostoc gelidum*: a specific spoilage organism

The members of the genus *Leuconostoc* are characterized as ovoid cocci occurring in pairs or short chains, non-motile, vancomycin-resistant, psychrotrophic mesophiles with optimal growth temperature 14-30°C (29). The recent phylogenetic analysis of lactobacilli and other LAB (30), showed that species from *Leuconostoc* genus as well as from *Pediococcus* (31), *Weissella*, *Oenococcus* and *Fructobacillus* genera were grouped as subclades within *Lactobacillus* species, therefore all these species can be considered as members of a bigger group “Lactobacillus Genus Complex” (30). Leuconostocs are obligate heterofermentative LAB, which ferment carbohydrates only through the phosphoketolase pathway (32). Their habitats include decaying plant materials and fermented vegetable products, as well as food of animal origin, such as dairy products, meat, poultry and fish (33). Due to their presence in food products, fermentative metabolism and ability to produce antimicrobial substances, leuconostocs are used in production of fermented foods and as biopreservative cultures (33, 34). Nevertheless, several species, such as *Le. mesenteroides*, *Le. carnosum*, *Le. citreum*, *Le. lactis* and *Le. gelidum* were reported to be involved in food spoilage (35).

Among these species *Le. gelidum* has emerged as the most predominant MAP food spoilage causative species during the last two decades in the northern European countries (36) (Finland, Belgium and others). Therefore, large portion of studies on psychrotrophic food-spoilage-associated LAB have been done with this species. According to a recent reclassification study, *Le. gelidum* encompasses three subspecies: *Le. gelidum* subsp. *gelidum*, *Le. gelidum* subsp. *gasicomitatum* and *Le. gelidum* subsp. *aenigmaticum* (37). *Le. gelidum* was first isolated from chill-stored vacuum packaged beef (38). Later, two subspecies, *gelidum* and *gasicomitatum* (which were considered as separate species at that time), were reported numerous times in association with spoilage of meat and vegetable products (vacuum-packaged ham and vegetable sausages, MAP beef, pork and broiler meat, acetic-acid preserved herring, brined boiled eggs and minimally processed vegetable salads) (14, 28, 37, 39–44). Its spoilage activities depended on the product type and included bulging due to gas formation, production of slime, discoloration, sour and buttery off-odors. *Le. gelidum* subsp. *gelidum* has also been isolated from fermented kimchi, the Korean traditional fermented food (45, 46). The previous studies of the genetic diversity and population structure showed that *Le. gelidum* subsp. *gasicomitatum* strains isolated from vegetable products were almost not recovered from meat-based foods (42, 47). It was proposed that this phenomenon could be explained by either “niche-specificity” of the different strains due to the different gene contents or the absence of strain exchange between vegetable- and meat-processing units (42). Independently on the source of isolation *Le. gelidum* subsp. *gasicomitatum* strains exhibit high spoilage potential and are able to grow fast under variety of gas compositions (48). In addition, the ability to rapidly outgrow from the small initial counts and strong attachment capacity of some *Le. gelidum* subsp. *gasicomitatum* isolates to food contact surfaces were observed and suggested to contribute to the observed predominance and competitive success (27, 36).
Before, only one genome of *Le. gelidum* associated with food (broiler meat) spoilage was available (*Le. gelidum* subsp. *gasicomitatum* type strain LMG 18811^T^) (40, 49). In addition, two genomes were available for *Le. gelidum* strains recovered from kimchi (45) (46). The analysis of the complete genome of the strain 18811^T^ identified pathways/genes putatively associated with spoilage reactions, such as butyryl off odors, meat greening and slime formation. In addition, genes, facilitating better survival in meat environment, such as genes involved in nucleoside catabolism and adhesion (collagen- and platelet-binding like proteins) were discussed (49). The reported heme-induced oxygen respiration leading to growth promotion and extreme increase of butyryl off-odor compounds (acetoin and diacetyl) was suggested as a key factor explaining its good adaptation and predominance in high-oxygen packaged meat (49, 50).

Gene expression data (microarray-based) have been available so far only for one strain, *Le. gelidum* subsp. *gasicomitatum* LMG 18811^T^. In the first study, the respiration-induced transcriptional response showed no changes in acetoin/diacetyl production genes, suggesting that the observed increase in accumulation of these compounds is due to regulation at the metabolic level rather than at the transcriptional level (50). In the second study, the gene expression profiles during growth on different carbon sources (glucose, ribose and inosine) were compared (51). The observed upregulation of acetoin/diacetyl production pathways and downregulation of lactate and pyruvate dehydrogenases during growth on ribose and inosine in comparison with glucose were in agreement with the fact that acetoin/diacetyl were produced only when ribose or inosine were present in the medium, but not glucose.

1.5 *Lactococcus piscium*: roles in food spoilage and food biopreservation

Cells of *Lactococcus* species have a spherical or ovoid shape, occur singly or in chains, they are non-motile, facultative anaerobic, grow at 10°C but not at 45°C (52). They are, generally, considered homofermentative, although, under certain conditions (starvation, utilization of pentoses instead of hexoses, citrate co-fermentation etc.) they can shift to heterofermentative metabolism (1, 53). Lactococci occupy diverse range of niches, such as dairy environments, plants, animals and fermented foods (53), and their most probable original habitat has been suggested to be phyllosphere (54, 55). *Lc. lactis* strains are widely used in manufacturing fermented dairy products, and, therefore, it is the most extensively studied species in the genus and is the model organism for LAB (54). Other members of the genus are known to be pathogens (*Lc. garvieae*) (56), involved in food spoilage or used for food preservation (*Lc. piscium*) (57–59).

To date, *Lc. piscium* has been the only member of *Lactococcus* genus with reported spoilage activity (13, 57). Although, *Lc. raffinolactis* was found along with *Lc. piscium* in MAP meat products at the end of shelf life (57), its isolation frequency was much lower than for *Lc. piscium* and its role in food spoilage is unknown. Phylogenetically, the closest species to *Lc. piscium* are *Lc. raffinolactis*, *Lc. plantarum* and *Lc. chungangensis*, while *Lc. garvieae* and three subspecies of *Lc. lactis* form a distinct
branch (57). *Lc. piscium* was initially isolated from a diseased salmonid fish (60). Subsequently, it was detected in a variety of chilled vacuum-packaged or MAP meat products (beef, pork, poultry and fish) and vegetable salads, frequently as part of a dominant microbiota at the late shelf life or at the spoilage stage (12, 28, 57, 61–63). Investigation of *Lc. piscium* spoilage potential was performed in several studies by inoculating pork samples (57), raw salmon (64), and a bell pepper simulation medium (48). In these studies *Lc. piscium* was shown to produce buttery and sour off-odor compounds as its main spoilage activity. In addition, these studies pinpointed a significant variation of spoilage potential among *Lc. piscium* strains: some strains exhibit very offensive metabolism or were shown to shorten the sensory shelf life (48, 57), while other strains can be considered as light spoilers (48, 64). Moreover, one *Lc. piscium* strain, CNCM I-4031, is extensively studied for its bioprotective properties in seafood products due to the ability to inhibit growth of pathogenic and spoilage bacteria, such as *Brochothrix thermosphacta* and *Listeria monocytogenes*, and to prolong food shelf life (59, 65). The growth inhibition of *L. monocytogenes* is presumably mediated by cell-to-cell contacts (59). The proteomic study on cold response of this strain showed its very high promptness in cold adaptation putatively due to constitutive expression of the cryoprotective protein CspE and upregulation of glycolytic enzymes (66).

Despite the apparent relevance of *Lc. piscium* for food industry, no genome sequence and transcriptome information had been publicly available before our study.

1.6 *Lactobacillus oligofermentans*: spoilage co-occurring pentose-prefering bacterium

*Lactobacillus* is the largest and most heterogeneous genus among other LAB genera, which encompasses species with a variety of phenotypic and physiological properties (1). The common characteristic of this genus is a rod-like shape of cells. Lactobacilli have been divided into three groups based on the mode of carbohydrate catabolism: obligate homofermentative (e.g. *Lb. acidophilus*), facultative heterofermentative (e.g. *Lb. plantarum*) and obligate heterofermentative (e.g. *Lb. vaccinostercus*) (67). The genome size and GC content of lactobacilli range from 1.23 Mb to 4.91 Mb and from 31.93 to 57.02 %, respectively. Genetic diversity of the genus based on the average and total nucleotide identity is bigger than that for a typical family (30). As mentioned earlier, genus *Lactobacillus* is paraphyletic and intermixed with five other genera, which explains the observed diversity of lactobacilli (30). Lactobacilli are widely used in food fermentation (dairy, meat and vegetables), as probiotics, in chemical industry as producers of lactic acid and 1,3-propanediol, and considered as delivery systems for vaccines and therapeutics (30). However, some species have been associated with food spoilage, such as *Lb. algidus*, *Lb. fuchuensis*, *Lb. sakei*, *Lb. curvatus* and *Lb. oligofermentans*, with *Lb. algidus* being the most predominant or frequently reported species in chilled packaged foods (12, 13, 24, 28).

In turn, *Lb. oligofermentans* has never been reported to belong to the predominant microbiota in refrigerated spoiled or long stored MAP broiler products, from
which it has mainly been isolated (68, 69). In these products *Lb. oligofermentans* constituted 10-18 % of spoilage LAB; therefore its role in meat spoilage is unclear. In addition to poultry products, this bacterium was recently isolated from MAP ground beef (70) and fermented Chinese vegetables (71). *Lb. oligofermentans* belongs to the so-called *Lb. vaccinostercus* group of obligate heterofermentative lactobacilli, which includes *Lb. vaccinostercus*, *Lb. suebicus*, *Lb. hokkaidonensis*, *Lb. nenjiangensis* and *Lb. wasatchensis* (67, 72). The common characteristic of these species is the preference of pentoses (e.g. arabinose, ribose, xylose) to hexoses (e.g. glucose, fructose, mannose, N-acetylglucosamine), resulting in considerably higher growth rate and yield during the fermentation of pentoses than hexoses. This physiology is consistent with the fact that the primary ecological niche for the four species from the *Lb. vaccinostercus* group (excluding *Lb. wasatchensis* and *Lb. oligofermentans*) is plant material, which is abundant in pentoses (67, 73). So far, *Lb. oligofermentans* was the only bacterium among these species which has repeatedly been isolated from meat-derived products. The concentration of free pentoses in meat is much lower than in plant materials (74) and significantly lower than concentration of glucose, which is the main fermentable carbohydrate in fresh meat (75). Taking it into account together with the fact that growth rate of *Lb. oligofermentans* on glucose is considerably lower than that of other predominant LAB in chilled MAP foods (such as *Le. gelidum*), it is not clear how *Lb. oligofermentans* is able not only coexist and compete with the predominant microbiota, but also to grow to substantial amounts in some cases (68). However, the higher efficiency of hexose utilization was shown to be possible to be induced in *Lb. vaccinostercus* (76) and *Lb. oligofermentans* (77).

The draft genome of a type strain of *Lb. oligofermentans*, LMG 22743T, became recently available as part of the big sequencing project of lactobacilli genomes (30). Several comparative genomic studies were performed between the genomes of lactobacilli and associated genera, which included *Lb. oligofermentans* draft genome (30, 31, 67). According to one of them (67), *Lb. oligofermentans* lost many genes involved in amino acid biosynthesis in comparison with other members of the *Lb. vaccinostercus* group, probably, due to the adaptation to meat environment, which is rich in amino acids. To our knowledge, before the Study II no transcriptional data were available for any member of *Lb. vaccinostercus* group.

1.7 Catabolism of carbohydrates in LAB and its relation to food spoilage

Organoleptic and textural food spoilage changes caused by LAB are consequences of their metabolic activities, mainly fermentation of carbohydrates (11). Carbohydrates represent the main carbon and energy source for LAB. According to the mode of hexose and pentose catabolism, all LAB have been divided into three groups: obligate homofermentative (e.g. group I lactobacilli), facultative heterofermentative (e.g. lactococci, group II lactobacilli) and obligate heterofermentative (e.g. leuconostocs, weisselllas, group III lactobacilli) (1, 32).
1.7.1 Obligate homofermentative and facultative heterofermentative LAB:
fermentation of hexoses and pentoses

Obligate homofermentative and facultative heterofermentative LAB ferment hexoses through glycolysis (Figure 1). Separation between the two groups of LAB is based on the presence of the phosphoketolase pathway in facultative heterofermentative LAB, which is used for heterofermentation of pentoses (1, 11, 32). However, few obligate homofermentative and facultative heterofermentative LAB (some lactobacilli, enterococci and lactococci) are able to perform homofermentative catabolism of pentoses through the pentose phosphate pathway (11, 78). Taking it into account together with the fact that major metabolic differences arise from the mode of hexose fermentation (which is the same for the both groups), these two groups of LAB were suggested to be considered as one group of homofermentative LAB as opposed to the obligate heterofermentative LAB (11). Catabolism of hexoses through glycolysis results in formation of pyruvate, the fate of which is dependent on the availability of oxygen and substrates (Figure 2). When preferable hexoses (mainly glucose) are abundant, pyruvate is generally converted to lactate under anaerobic and in most cases in aerobic conditions (1, 11, 32). Under anaerobic conditions and when glycolytic rate is reduced due to substrate limitation or fermentation of less preferable carbohydrate, pyruvate is predominantly metabolized by pyruvate formate lyase (79, 80) (inactive in the presence of oxygen) (81) with the formation of acetate and/or ethanol as end products. This shift allows producing more ATP per hexose molecule compared to homolactic fermentation, which is important under nutrient limitation conditions. Starvation or reduced glycolytic rate under aerobic conditions, the presence of oxygen itself, which can act as an electron acceptor for NAD+ cofactor regeneration through functional respiration and direct oxidation of NADH by NADH oxidases, can cause a dramatic shift to heterolactic fermentation mediated by three pyruvate-utilization pathways: pyruvate oxidase, pyruvate dehydrogenase and acetoin/diacetyl production pathways (1, 11). Pyruvate oxidase requires oxygen, does not affect redox balance and produces hydrogen peroxide and acetyl-phosphate. The last metabolite is then converted to acetate with concurrent ATP production or ethanol to restore the redox balance. Therefore this pathway acts similarly to the pyruvate-formate lyase pathway, but under aerobic conditions (82). Pyruvate dehydrogenase pathway also leads to increased production of acetate and/or ethanol, however, since it proceeds with NADH formation, the prerequisite for this reaction is the presence of additional electron acceptors, such as oxygen (1). Lastly, acetoin/diacetyl produced from pyruvate do not cause acidification in comparison with lactate and acetate, therefore, acetoin/diacetyl production pathway can act as a pyruvate “sink” and contributes to pH homeostasis being active under low pH (83). In addition, produced diacetyl can act as an electron acceptor for NAD+ regeneration. Production of large amounts of acetoin/diacetyl occurs in the presence of electron acceptors or their precursors, such as oxygen and citrate (precursor of pyruvate, which is an electron acceptor) (Figure 3) (84, 85). Fermentation of pentoses in facultative heterofermentative LAB through the phosphoketolase pathway (Figure 1) proceeds in the same manner as in obligate heterofermentative LAB, which is described in the next chapter. During catabolism of pentoses through the pentose phosphate pathway, produced pyruvate is mainly converted to lactate and the net energy gain is slightly higher.
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comparing with pentose fermentation through the phosphoketolase pathway (11, 78). Among LAB investigated in our studies, *Lc. piscium* is assumed to belong to obligate homofermentative LAB (1, 32).

1.7.2 Obligate heterofermentative LAB: fermentation of hexoses and pentoses

Obligate heterofermentative LAB use the phosphoketolase pathway (Figure 1) for the catabolism of both, pentoses and hexoses, yielding pyruvate and acetyl phosphate (1, 32). Pyruvate is usually reduced to lactate, while acetyl phosphate is either converted to acetate during pentose catabolism or reduced to ethanol during hexose catabolism (Figure 2). The latter is required to re-oxidize NADH formed during hexose oxidation. Therefore, the yield of ATP is twice lower for hexoses versus pentoses fermentation. This explains generally poor growth of obligate heterofermentative LAB on hexoses (1, 11, 86). Some of these LAB do not grow at all on hexoses as a sole carbon source due to the low activity (87, 88) or absence of the enzyme aldehyde-alcohol dehydrogenase (89). Growth on hexoses becomes possible or improved in the presence of additional electron acceptors (e.g. oxygen, fructose, pyruvate) or their precursors (citrate, glycerol, amino acids) (Figure 2 and Figure 3), which allow acetyl phosphate to be channeled to the formation of acetate and ATP (1, 11). In addition, endogenous pathways for NADH re-oxidation exist in some of these LAB, such as formation of glycerol and erythritol (86). The use of external electron acceptors (especially during respiration) in co-fermentation with hexoses and pentoses can also spare some pyruvate from being reduced to lactate, and more acetoin/diacetyl and acetate can be formed through the acetoin/diacetyl production pathway, pyruvate oxidase and pyruvate dehydrogenase pathways (50, 90, 91). Pyruvate formate lyase is not found in obligate heterofermentative LAB (90). Another characteristic feature of this group of LAB is higher growth efficiency on disaccharides than on hexoses. Disaccharides are cleaved by phosphorylases and this eliminates the need of ATP-dependent phosphorylation to enter the phosphoketolase pathway (92). Two LAB investigated in our studies, *Le. gelidum* and *Lb. oligofermentans*, belong to obligate heterofermentative LAB. However, their growth on hexoses in comparison with pentoses is different. *Le. gelidum* grows very efficiently on glucose and significantly faster than on ribose (51) under nearly anaerobic conditions (no culture shaking). The phenomenon might be explained by a strong activity of the aldehyde-alcohol dehydrogenase in leuconostocs (89). In addition, the efficient use of electron acceptors or their precursors, available in growth media (citrate, residues of oxygen, amino acids) (Figure 3) and putative high activity of endogenous pathways for NADH reoxidation could be proposed as a possible reason for the efficient hexose utilization. As mentioned earlier, the opposite is true for *Lb. oligofermentans*.

The above described metabolic routes of pyruvate and acetyl-phosphate (Figure 2) are not necessarily present in all LAB and represent an overview of possible pathways occurring in LAB.
Figure 1. Central carbohydrate catabolic pathways in LAB. Glycolysis is utilized by obligate homofermentative and facultative heterofermentative LAB for hexose degradation. Phosphoketolase pathway is used by obligate heterofermentative LAB for hexose fermentation, and by both facultative and obligate heterofermentative LAB for pentose degradation. Selected enzymes are numbered: 1, fructose-1,6-diphosphate aldolase; 2, phosphoketolase. DHA, dihydroxyacetone phosphate.

1.7.3 Spoilage metabolites: the end products of carbohydrate catabolism

The main spoilage activities of food spoilage LAB are associated with the end products of carbohydrate fermentation (Figure 2). Acetate is responsible for the sour, pungent or vinegar taste and odor, while acetoin/diacetyl are associated with buttery off-odors and creamy taste in meat and vegetable products (13, 24). Hydrogen peroxide can cause meat greening, and excessive formation of carbon dioxide causes bulging of food packages (49). High concentration of both acetate and lactate leads to increased acidity in foods. Formation of slime (exopolysaccharides, particularly dextran) occurs during growth with sucrose (40), which is cleaved by the cell wall-associated dextranucrase into glucose and fructose, which are used as substrates for dextran synthesis and fermentation, respectively (1).
1.7.4 Carbon catabolite control in LAB

When several carbohydrates/carbon sources are present in the environment, many bacteria utilize them in sequential, hierarchical manner to ensure the optimal growth. At first, the preferred carbon source(s), allowing most efficient growth, is (are) utilized and catabolism of others is inhibited. Only after depletion of the preferred carbon source(s) other carbon sources are being fermented. This phenomenon and its regulation comprise the so-called carbon catabolite control (CCC), which is mediated by carbon catabolite repression (CCR) and activation (CCA) mechanisms. In Firmicutes (bacilli, staphylococci, LAB) these mechanisms were observed and studied in species that ferment preferred carbohydrates (mainly glucose) through glycolysis (in LAB these are homo- and
Figure 3. Fermentation of citrate, aspartate and alanine to pyruvate. These pathways create pyruvate surplus, which, in turn, favors formation of acetoin and acetate (heterofermentative metabolism) instead of lactate ( homofermentative metabolism). 2-Oxoglutarate is required for the transamination reactions during catabolism of aspartate and alanine. Selected enzymes are numbered: 1, citrate lyase complex; 2, oxaloacetate decarboxylase; 3, aspartate aminotransferase; 4, alanine aminotransferase; 5, glutamate dehydrogenase.

Facultative heterofermentative species). The mechanisms appear to be very conserved among Firmicutes and involve the phosphocarrier protein HPr, HPr kinase/phosphatase HPrK/P and catabolite control protein CcpA (93–96).

Consumption of the preferred, usually phosphotransferase system (PTS) transported carbon source(s), creates high concentration of glycolytic intermediates (e.g. fructose bisphosphate, ATP), which activate the kinase activity of HPrK/P leading to formation of the seryl-phosphorylated form of HPr (P-Ser-HPr) (Figure 4). Simultaneously, the efficiency of the PTS transport is decreasing, since it requires another form of HPr, histidyl-phosphorylated (P-His-HPr). P-Ser-HPr forms a complex with CcpA, which exerts global transcription control of carbon metabolism. This includes upregulation (as part of CCA) of genes involved glycolysis, acetate and acetoin formation from pyruvate; ammonium assimilation (glutamate synthase), branched-chain amino acids biosynthesis related genes required for rapid growth. On the other hand, it downregulates (as part of CCR) catabolic/transport genes for carbohydrates other than preferred, respiration genes and many other catabolic and anabolic genes involved in carbon and nitrogen metabolism (85, 93–96). Regulation occurs when the complex CcpA/P-Ser-HPr binds to cre-sites (consensus sequence of cre-sites in Bacillus subtilis: WTGAAARCGYTTWNN) located upstream the regulated operons genes (94). In B. subtilis the number of CcpA-regulated genes is roughly estimated to be 300 of which 75 % being repressed (97). Furthermore, P-Ser-HPr itself confers inducer exclusion (prevention of entry of less preferred carbohydrates that can induce corresponding catabolic genes) by inhibition of carbohydrate permeases (as part of CCR) (93, 98). When glycolytic rate decreases, HPrK/P catalyzes dephosphorylation of P-Ser-HPr, yielding HPr that can be phosphorylated by the PTS E1 enzyme leading to formation of P-His-HPr, acting as a phosphotransferase in the PTS-dependent transport of carbohydrates. Therefore, formation of P-His-HPr leads to enhancement of PTS transport of the preferred carbohydrates and, consequently, to the increased glycolytic rate, which leads again to CCR. However, when the preferred source is depleted, CCR stays relieved and alternative carbohydrates are being utilized. In addition, the level of P-His-HPr becomes higher and it activates carbon
Figure 4. Carbon catabolite control in *Firmicutes*. Catabolism of the preferred sugar, usually transported via PTS, through glycolysis increases concentration of FBP and ATP, which induce the kinase activity of HPrK/P. As a result, HPr is phosphorylated at Ser and forms a complex with CcpA regulator. The complex binds to *cre* sites of genes and either represses (e.g. catabolic genes of alternative carbohydrates) or activates (e.g. glycolytic enzymes) expression of these genes depending on the position of *cre* sites. It also inhibits the permeases of alternative carbohydrates. When glycolytic rate decreases, HPrK/P catalyzes dephosphorylation of P-Ser-HPr, and HPr is phosphorylated by the PTS EI enzyme at His. P-His-HPr enhances PTS transport, which leads again to CCR. However, when the preferred source is depleted, CCR stays relieved and alternative carbohydrates are being utilized. In addition, the high level of P-His-HPr activates carbon source-specific regulators, which activate catabolism of other carbon sources/carbohydrates. Designations used: PTS, phosphotransferase system dependent transporter; CSR, carbon source specific regulator; FBP, fructose-bis-phosphate; PEP, phosphoenol pyruvate; ATP, adenosine triphosphate; HPr, histidine-containing PTS phosphotransferase; EI, PTS phosphotransferase enzyme I; HPrK/P, HPr kinase/phosphatase; CcpA, catabolite control protein; *cre*, CcpA binding site; CCR, carbon catabolite repression; CCA, carbon catabolite repression. Dashed arrows indicate regulation: "+", activation and "−", repression.

Source-specific regulators leading to activation of catabolism of other carbon sources/carbohydrates (93–96, 98).

Hierarchical carbohydrate utilization is generally not observed in obligate heterofermentative LAB fermenting hexoses and pentoses by the phosphoketolase pathway. Instead, they are able to co-ferment glucose with other hexoses and pentoses (99) or prefer pentoses to hexoses in the absence of additional electron acceptors as mentioned...
earlier. Nevertheless, all the main components of CCC, such as HPr, HPrK/P, CcpA and EI seem to be present in these LAB ((31): Supplemental file 3), but their role in carbon metabolism regulation is not well characterized. For *Lb. brevis* the relaxed control of carbohydrate utilization was proposed to explain the co-fermentation of glucose with other carbohydrates with approximately equal efficiencies (99). The precise mechanism of the relaxed CCR have not yet been elucidated. Another study showed P-Ser-HPr-dependent expulsion of galactose analogue accumulated inside the cell in the presence of glucose indicating the presence of certain level of CCR in *Lb. brevis* (100).

### 1.8 Putative factors contributing to increased survival and predominance of LAB within the food spoilage microbial community.

#### 1.8.1 Catabolism of alternative carbon and energy sources

When the most abundant or preferred carbohydrate(s) is exhausted in the environment, the ability to utilize alternative niche-specific compounds as carbon and/or energy sources is crucial for survival and can offer a competitive advantage.

In meat the main fermentable carbohydrate is glucose (75). Upon its depletion substances naturally present and abundant in meat, such as amino acids (e.g. arginine, aspartate and alanine), nucleosides, glycogen and putatively glycerol, can be used as alternative carbon and/or energy sources. Deamination of arginine leads to formation of ATP and ammonia. Therefore, arginine can be used as energy source and contributes to pH homeostasis (together with histidine, tyrosine, glutamine and glutamate) (11). Arginine is abundant in meat and its degradation was proposed as one of the factors increasing competitiveness and survival of *Lb. sakei* in meat environment (101). Aspartate and alanine catabolism eventually creates pyruvate surplus (Figure 3) (102), which can be used for energy production and biosynthetic purposes as well as it favors formation of acetoin/diacetyl (1, 102), which are undesirable in meat. Catabolism of nucleosides, concentration of which can be twice more than that of glucose, provides a source of fermentable carbohydrate ribose. Meat-inhabiting bacteria were shown to contain nucleoside catabolic genes and efficiently grow on nucleosides as a sole carbon source (49, 75, 101). Glycerol is present in meat as a product of triacylglycerol degradation catalyzed by lipases present in meat (endogenous or bacterial) (75, 103) and can be utilized by some LAB as a sole carbon source (104, 105). Glycogen is the main storage carbohydrate in animal muscles and, therefore, abundant in meat. However, the role of glycogen catabolism in LAB adaptation to meat environment is not well studied.

Plant environment and, therefore, plant-derived foods are rich in complex polysaccharides constituting plant cell walls (e.g. cellulose, xylan, arabinan, galactan) and acting as storage components (e.g. starch and fructan). Therefore, the ability to utilize these
polysaccharides as well as their degradation products (e.g. xylose, arabinose, gluconate, uronic acids, beta-glucosides and alpha-galactosides) is beneficial for and characteristic of strains isolated from such products (54).

### 1.8.2 Adhesion

Adhesion along with biofilm formation allows to anchor bacteria on particular surfaces and, therefore, withstand shear-forces. Consequently, it might facilitate persistence of food spoilage bacteria in the food processing environment and quick colonization of food surfaces as a result of cross-contamination, which is a big problem for food manufacturers. Adhesion and biofilms mediate also cell-to-cell attachment, allowing better communication between bacterial cells, which might lead to a better survival under hostile or nutrient-limiting conditions (106). Bacterial adhesion is mediated by different surface molecular structures, including polysaccharides, teichoic acids/lipoteichoic acids and proteinaceous adhesins (107).

Prediction of proteinaceous adhesins is based on the presence of characteristic domains and motifs. Surface proteins, including adhesins, usually have the N-terminal signal peptide, which targets them for transport through cell membrane and is cleaved off after the export. Consequently they are either being covalently linked to the cell wall components or stay associated with the cell wall non-covalently. The majority of covalently linked proteins in Gram-positive bacteria contain the C-terminal motif LPxTG, which is recognized by the enzyme sortase that cleaves the motif and covalently links the protein to the peptidoglycan. Many known adhesins in LAB are sortase-dependent. In addition to the LPxTG motif, these proteins can have adhesion associated domains, such as mucus-, collagen- or fibronectin-binding domains. The example of non-covalently cell wall associated proteins, shown to be involved in adhesion in LAB, are the S-layer proteins that form sheets with paracrystalline structure. These proteins usually contain bacterial S-layer protein domain(s). Another proteinaceous structures involved in adhesion in LAB are pili, which are filamentous multi-subunit surface appendages. They are covalently linked to the cell wall by the sortase-dependent or independent mechanism. Recently another group of cell wall associated proteins was identified. These are well-known cytoplasmic metabolic enzymes (e.g. enolase, elongation factor EF-Tu, chaperone GroEL), which have “moonlighting” functions when they are exported to the surface. These functions include physical interactions with the extracellular matrix, virulence and adhesion (107, 108).

### 1.8.3 Release of antimicrobial compounds

LAB release a variety of antimicrobial substances that inhibit growth of other LAB and food pathogens. This ability allows them to gain a competitive advantage over the other members of the microbial community. Antimicrobial properties of LAB are also of interest due to the possibility to use these LAB as bioprotective cultures in food fermentations. Antimicrobial compounds include antimicrobial peptides (bacteriocins), as well as metabolites of primary and secondary metabolism (7, 8). Bacteriocins are produced
by the majority of LAB and their spectrum of activity is usually restricted to species which are phylogenetically close to the producer (8). Gene sequences, structures, biochemical characteristics and mode of action of different bacteriocins are highly variable, which impedes their prediction and identification. Positively charged bacteriocins permeabilize bacterial membranes disrupting membrane potential (109). The self-immunity to bacteriocins is achieved through either posttranslational modifications or expression of specific immunity proteins (110). Besides bacteriocins, LAB can produce non-peptide antimicrobial compounds as part of their secondary metabolism, such as reuterin and polyketides, which have broad activity spectrum (8, 111).

Oxygen metabolites (H$_2$O$_2$, superoxide and hydroxyl radical) are reported to inhibit growth of LAB (7), that are mainly adapted to anaerobic and microaerobic conditions. The aerobic growth of LAB results in production of H$_2$O$_2$ mediated by the oxygen-dependent redox enzymes, mainly NADH oxidases, or superoxide dismutase (7, 112). The presence of both H$_2$O$_2$ and superoxide simultaneously can lead to formation of the third oxygen metabolite, hydroxyl radical. The bacteriostatic and bactericidal effects of these metabolites are mediated through peroxidation of membrane lipids, oxidation of sulfhydryl compounds and DNA damage (7). The resistance to oxidative stress depends on the presence and activity of enzymes that eliminate oxygen metabolites (e.g. different peroxidases, superoxide dismutase and others) and restore damaged cell compounds (e.g. peptide methionine sulfoxide reductases, organic hydroperoxide-resistance protein, thioredoxins and glutathione reductases and others) (101). Therefore, LAB species that produce large amount of oxygen metabolites under aerobic conditions and have a good oxidative stress protection could inhibit growth of other bacteria, while less effecting their own growth.

Organic acids, such as lactic and acetic, increase acidity and cause formation of salts inside bacterial cells, which lead to inhibition of metabolic enzymes and, therefore, bacterial growth. High concentration of these acids is detrimental to many food spoilage and pathogenic Gram negative (e.g. E. coli, salmonellae) and Gram-positive (L. monocytogenes, clostridia) bacteria. However, LAB are generally resistant to high concentrations of lactic and acetic acids. Other end products of primary metabolism, such as diacetyl and acetaldehyde, could also contribute to antimicrobial properties (7).

1.8.4 Cold tolerance

Packaged food products are stored at chilled temperatures to prevent or limit growth of bacteria. This condition creates a selective pressure for cold-resistant, so called psychrotrophic, bacteria. Three LAB species that are within the scope of the study are psychrotrophic and able to grow at 4-15°C with an optimum growth temperature around 25°C and slow growth at 30°C (58, 68, 113). Cold temperature decreases membrane fluidity, therefore, affecting all membrane-associated cellular functions. It also stabilizes secondary structures of DNA and RNA affecting transcription, translation and replication (114). Cold shock protection is achieved by induction of several sets of genes, including those coding for cold-shock proteins (act as RNA chaperones), genes involved in biosynthesis of unsaturated fatty acids (to increase membrane fluidity), replication- and transcription-associated factors and general and oxidative stress protection factors (66, 114,
The observed high promptness of *Lc. piscium* CNCM I-4031 in cold adaptation was mentioned earlier (66).

### 1.9 Interspecies interactions between food spoilage LAB

In many cases LAB that are used in food fermentations, inhabiting animal mucosal surfaces and involved in food spoilage grow in microbial communities consisting of several bacterial and/or fungi species. Interactions between these species along with abiotic factors (e.g. nutrient content, temperature, atmosphere) determine the population structure (in terms of species composition and their relative abundances) and the global metabolic activity of the microbial community. Therefore, knowledge on interspecies interactions is of importance for developing stable starter cultures with desirable metabolic properties, designing composition of probiotics and developing strategies in order to extend shelf life of food products.

Generally, co-existence of different species (genotypes) in dense communities is predicted to create competition, while mutualistic relationships emerge much rarer (15, 116, 117). Competition can be classified as exploitative, when different species compete for the same resources (e.g. nutrients and space), and interference, when species directly harm/attack each other (e.g. release of bacteriocins, oxygen metabolites and contact-induced-killing) (7, 8, 59, 116, 117). In response to exploitative competition bacteria can increase efficiency of the nutrient uptake, while in response to attack from another species bacteria develop defense mechanisms (116). During mutualistic relationships species can create more favorable growth conditions and increase availability of nutrients (118, 119). Cultivation of LAB in mixed cultures with other species (LAB and non-LAB) results in different outcomes for LAB from growth inhibition (120–124) during competition to growth promotion during symbiotic relationships (122, 124–126). Nevertheless, the knowledge on genome-wide transcriptome responses of LAB during interspecies interactions is limited to only few studies focusing on species, involved in food fermentations, pathogenesis or considered to be probiotics (126–130). As far as I know, no studies have been conducted so far on transcriptional responses of food-spoilage-associated LAB during their co-cultivation in mixed cultures.

### 1.10 Annotation of bacterial genomes

Annotation of a sequenced and assembled genome can include several steps such as prediction of genes (protein-, tRNA-, rRNA- and other RNA-coding), characterization of their functions, metabolic reconstruction based on the predicted gene functions and prediction of other features and elements, such as frameshifts, pseudogenes, prophages, genomic islands, transposons, regulatory sites, CRISPR systems and operon structure.
(131). In practice, annotation of most of the genomes comes down to the first two steps: prediction of genes and their functions.

1.10.1 Gene prediction.

Identification of protein-coding gene’s coordinates in prokaryotes is rather well understood and easy process in comparison with eukaryotes due to the small size of prokaryotic genomes and absence of introns in prokaryotic genes. Generally, gene prediction starts with identification of all possible open reading frames (ORFs), which are long stretches of DNA with the start and stop codons in the same frame. Consequently, coding potential of the open reading frames should be assessed and truly protein-coding genes are determined. Methods for gene prediction can be divided into two classes: ab initio methods that rely on sequence statistical properties and presence of gene-associated signals, and evidence-based methods (131, 132). Ab initio tools distinguish true coding DNA sequence (CDSs) from non-coding ORFs using base composition, codon usage statistics and probabilistic models, usually hidden Markov models (HMM), that, in a simplistic view, assume that the probability of occurrence of a certain nucleotide in coding and non-coding sequences depends on the identity of several previous nucleotides (131, 133). To estimate these conditional probabilities (model parameters) the training set of coding sequences (long non-overlapping ORFs, ORFs sharing homology to experimentally verified genes and other most likely coding sequences) is required. Some tools can employ identification of the ribosomal–binding sites (Shine-Dalgarno sequences) to correctly determine the translation start sites (134). Different ab initio software tools can correctly identify 97-99 % of protein-coding genes (131, 134, 135). The problems with currently existing ab initio gene prediction methods are the prediction of long gene overlaps, short genes, false positives (especially in low quality draft genomes) and incorrect determination of translational start sites. The last two problems are particularly pronounced in the genomes with high GC content (131, 134, 135). Evidence-based methods make use of homology to experimentally verified proteins and protein family profiles, inter-genomic conservation (132) as well as RNA sequencing (RNA-seq) based gene expression data (136). Noteworthy, mapped RNA-seq data (total and enriched in primary transcripts) have numerous applications in genome annotation, such as identification of transcriptional start sites and untranslated regions of mRNA genes, prediction of novel small mRNA and ncRNAs, operon organization and alternative promoters (137). Shortcomings of the evidence-based methods are inability to identify unique/novel genes that are not present in the nucleotide/protein databases or not present/conserved in other genomes, and impossibility to predict genes that are not expressed under examined conditions. Comprehensive high-quality gene prediction is achieved through combination of ab initio and evidence-based methods followed by manual curation. Software solutions (designed mainly for eukaryotes) exist for integration of the results obtained from different gene prediction tools/methods (138, 139). Prediction of frameshifts before and during gene prediction phase with their concurrent classification into sequencing errors, mutations inside pseudogenes and programmed frameshifting events allows to prevent omission of genes and prediction of broken genes (140).
Ribosomal RNA and transport RNA genes are predicted based on sequence and structural homology to existed genes using similarity searches and probabilistic models built from structural alignments (141, 142). Other non-coding RNAs can be detected by screening complete genomes against RFAM database (143), which is a collection of non-coding RNA families, or using comparative RNA-seq (137).

1.10.2 Function assignment.

Following gene prediction, assignment of function to protein-coding genes is performed. Protein function can be expressed in various ways. Description line is a traditional, human-readable and the most common way to characterize functional properties. Nevertheless, due to ambiguity of description lines and the presence of synonyms, computational parsing and comparison of description lines are challenging. Therefore, more standardized and uniform schemes that are using controlled vocabularies have been developed for protein functional labeling. Among them, the most known and commonly used are clusters of orthologous groups of proteins (COGs) (144), Enzyme Classification (EC) (145), Transporter Classification (TC) (146) and Gene Ontology (GO) (147). COGs are manually curated orthology-based functional groups of proteins derived from complete genomes, where group’s function is assigned based on the experimentally verified function of its member(s). EC and TC are similar systems of nomenclature for enzymes and transporters, respectively, which have four and five levels of hierarchy, respectively. The most comprehensive classification scheme of protein functions is GO terms. They describe functions along three aspects, biological process, molecular function and cellular localization, and are arranged into a hierarchical structure. GO classification is extensively used for machine-readable functional annotations.

Conventionally, function is assigned to a newly predicted protein based on sequence homology to a protein with characterized function in a database (132), such as UniProtKB or Genbank. Hence, the quality of functional annotation depends on the quality of the database used with the best choice being manually curated databases of proteins with experimentally verified functions (e.g. UniProtKB/SwissProt). However, such databases are much smaller than those of computationally predicted/annotated proteins (e.g. UniProtKB/TrEMBL), which, in turn, contain high levels of misannotations (148). Another challenge is to define a threshold of sequence identity or similarity between proteins, which is enough to presume that they are homologous and retain the same function. For enzymes, different amino acid sequence identity levels, such as 30 %, 50 % and 70 %, were proposed to be sufficient to assume conservation of their function (149). During homology-based transfer of function, it is also useful to discriminate between two classes of homologs: orthologs and paralogs (150). Orthologs evolved from a single ancestral gene by speciation and normally retain the same function. Paralogs emerge by duplication within the same genome and often evolve new functions that are different from the original one. Therefore, ideally, function transfer should be performed between orthologs, and not between paralogs.

Homology-based transfer of functional annotation is carried out using various computational tools that perform similarity searches against protein databases. Basic Local Alignment Search Tool (BLAST) is the most widely used tool for such purpose, which
allows fast query times (151). BLAST search gives a list of hits that can be used to extract functional information. The most used and simple way for annotation transfer is to use the best (first) BLAST hit or best informative BLAST hit if the first hit is missing functional annotation (152). However, these methods are very error prone since they rely only on one hit. Alternately, information from the k-nearest neighbors can be used to assign a proper function (153, 154). This method is more reliable, but more sophisticated tools are required to resolve possible annotation conflicts. When regular BLAST does not give any close homologs, Position-Specific Iterated BLAST (PSI-BLAST) can be used to find distant homologs (151). In this algorithm, position-specific scoring matrix (PSSM) is built based on hits obtained during the first round of regular BLAST and this matrix is used to query a database in the second round. The newly found sequences are used to update the matrix. This procedure can be iterated several times.

Another approach for functional annotation is to search against databases containing profiles (in the form of PSSM or HMM) of protein families, domains and signatures. The most known such database is InterPro (155) that encompasses several databases, such as CDD, Pfam, TIGRFAM, PROSITE and others. This approach is also applied to assign genes to the COG functional groups using COG PSSMs. In addition to the general databases, more specific exist, containing either protein sequences or group profiles or both to search against. The examples include databases of carbohydrate-active enzymes (CAZy) (156), restriction modification (RM) enzymes (REBASE) (157), bacteriocins (158) and others. Recognition of functionally important and discriminating residues can be useful to distinguish between different families and to infer/confirm function of a protein in case of remote homology (159).

Context-based approaches for function assignment can be powerful when a function of the nearest homologs was not characterized. These methods make use of phylogenetic profiles, gene fusion events and gene neighborhood (160).

Ab initio methods that predict protein function solely based on sequence composition and physico-chemical characteristics of individual amino acids are limited to annotation of special classes of proteins, such as transmembrane, secreted, surface-associated and DNA-binding proteins (161).

Most complete and qualitative functional annotation of a genome is achieved through the combination of the above mentioned approaches (e.g. as implemented at the STRING’s website) (160) coupled with manual curation.

1.10.3 Metabolic pathway reconstruction

Knowledge about the presence of certain functions/enzymatic activities in the genome can be used to reconstruct metabolic pathways in the organism. This is usually done by mapping enzymes from the genome to the reactions in a reference database (162), such as MetaCyc or reference KEGG (163). For reaction matching, gene’s description lines, EC numbers or GO terms can be used. The set of obtained reactions is projected onto metabolic pathways in the reference databases. The probability of existence of each matched pathway in the organism is then being assessed both automatically and manually. As the last step, pathways holes (reactions in the detected metabolic pathways with no assigned gene product) are being filled using bioinformatic tools or manually (162).
The comprehensive lists of computational tools and various databases used for gene prediction, functional annotation and metabolism reconstruction can be found in Kankainen M., 2015 (161).

1.11 Comparative genomics

Availability of numerous bacterial genomes in the databases allows to make their interspecies and intraspecies comparisons. This powerful approach determines similarities (relatedness) and differences (genomic diversity) between genomes of different species and isolates. Comparing whole genomic sequences allows to detect the regions of synteny, genomic rearrangements (insertions, translocations and duplications), horizontal gene transfer events, the presence of prophages and other aspects (164, 165). The whole genome comparisons are usually performed using local alignment tools (e.g. nucleotide BLAST) (165, 166) and visualized afterwards using e.g. ACT, BRIG (166, 167).

Comparison of gene repertoire of different bacterial isolates and species is another approach (168, 169). This requires identification of orthologous genes between genomes of interest and discrimination them from paralogs. Several methods were suggested, the most commonly used and intuitive of which is bidirectional best-hit approach (168, 169).

Orthologs that are present in all compared genomes form their core genome (170, 171), which can be used for the inference of phylogenetic relationships between the compared organisms. To do this, multiple alignments of sequences constituting each orthologues group (selected for the analysis) are constructed. Subsequently two scenarios are possible (172). Multiple alignments can be concatenated into one super gene-alignment, which is then used for the construction of the phylogenetic tree using several methods (e.g. maximum parsimony, likelihood, distance and Bayesian approaches) (173). Alternatively, trees can be constructed for each orthologous group, followed by their reconciliation into a single consensus tree (172).

Orthologs that are present only in some genomes form so-called dispensable genome (170, 171). Analysis of these gene groups give opportunity to identify genes that are specific for certain (sub)species, strains isolated from particular environmental niches or exhibiting a particular phenotype (e.g. virulence, fermentation capabilities, antibiotic resistance etc.), thereby providing a link between their phenotype and genotype. Core and dispensable genomes form together pangenome, which represent genetic reservoir of the group of compared organisms (170, 171). Usually, pangenome size of a bacterial species correlates with the level of diversity of the environments inhabited by this species. A profile of presence/absence of orthologous groups in the compared genomes is used to build a pangenome tree, which reflects not only phylogenetic relationships, but also horizontal gene transfer and phenotypic similarities (174).
1.12 RNA-sequencing and differential gene expression analysis

RNA-seq technology (high-throughput cDNA sequencing) allows to take a snapshot of the entire organism’s transcriptome under certain conditions at a particular time both qualitatively and quantitatively with single-base resolution (175, 176). In comparison with hybridization-based DNA microarrays, widely used for gene expression profiling in the past, RNA-seq has several advantages, such as absence of necessity to have a known genomic sequence and, hence, ability to detect novel transcripts, lower background signal levels, larger dynamic range of expression level detection, higher accuracy and reproducibility of quantitative measurements (175, 177, 178). Briefly, RNA-seq experiment starts with total RNA extraction that can be followed by enrichment of certain types of transcripts, such as small RNAs, primary (unprocessed) mRNAs, poly(A)-containing mRNA, or omission of rRNA (137, 175). Consequently, RNA is converted to cDNA, which is then fragmented into short pieces (fragmentation can also come before the reverse transcription). cDNA fragments are ligated to adaptors with a possible subsequent amplification and sequenced in a high-throughput manner to obtain short reads, that are then mapped to a reference genome or assembled de novo. RNA-seq and its modifications have many applications, such as cataloging different kinds of RNA species (mRNAs, small and micro RNAs, antisense RNAs etc.), determination of the transcriptional structure (5’ and 3’ untranslated regions, location of primary and alternative promoters, splicing sites) and detection of changes in expression levels of genes/isoforms in response to certain conditions or occurring during development of an organism (137, 175, 176).

To estimate transcription levels of genes/isoforms, short RNA-seq reads are first mapped against a genome and then reads belonging to each gene/isoform are counted. The major problems arising at this step are mapping reads that span exon junctions and multi-matching reads (175, 178, 179). Several read aligners and counting methods exist that differ regarding their ability and strategy to deal with these reads (178, 179). In order to compare expression levels between genes and samples, read counts have to be properly normalized. There are two major sources of systemic variation: different sequencing depth of samples, resulting in different numbers of reads produced for each library (sample); and variation in gene lengths that results in more reads generated from longer transcripts than from shorter transcripts assuming that their abundances are the same (179, 180). The early used and most intuitive normalization method is the reads per kilobase of gene per million mapped reads (RPKM), which directly normalizes read counts by the total library size and gene length. Although useful for between-genes comparisons, this method is ineffective for differential expression analysis (DE analysis) between samples, since it causes a bias in the per-gene variances and does not account for other sources of systemic variation (differences in GC contents of different genes, differences in library composition, relative nature of compared transcript abundances) (180, 181). To account for these factors, more complex normalization methods were developed, of which trimmed mean of M-values (TMM) method and DESeq normalization method were shown to perform better than other methods (180). It is worth to note, that these two methods rely on the assumption that most of genes in the genome are not differentially expressed and that differentially expressed genes (DE genes) are distributed evenly between up- and downregulated categories (180). However, these assumptions do not always hold. At the last step of the DE analysis, the differences of normalized read counts between samples are statistically tested. Many
software packages based on different inferential approaches have been developed to date. Comparison of these methods identified significant differences in their results (177, 181, 182). No single method was found to be optimal under all circumstances and, therefore, the choice of the method should ideally depend on the experimental set up and, possibly, on the library and sequencing technology utilized. Alternatively, several methods can be applied, and the intersection of their results would present a more reliable list of DE genes (177). All methods were found to perform better with higher number of replicates and sequencing depth with the number of replicates having greater effect on the detection accuracy of differential expression than sequencing depth (182).

The obtained list of DE genes can be further analyzed by assigning these genes to different functional groups/pathways with subsequent functional/pathway enrichment analysis (183) or by extracting groups of co-regulated (e.g. up- or downregulated under certain conditions) genes, followed by the analysis of their upstream regions for the presence of common transcription factor binding sites (TFBS) (184).


2 AIMS OF THE STUDY

In the present study, I and my co-authors aimed at expanding knowledge on functional genomics/transcriptomics, physiology and interspecies interactions of food-spoilage-associated LAB using bioinformatic analyses of genomic and RNA-seq data as well as phenotypic analyses. The focus was on genes/pathways that are associated with food spoilage activities of LAB, facilitate their better survival and increase their ecological fitness in different food matrices, and give them competitive advantage during growth in spoilage microbial communities. On the other hand, the general/fundamental aspects of the metabolic pathway regulation were also of interest and, therefore, investigated.

More specific objectives included:

1. Functional annotation of genes and metabolic pathways of the complete genomes of three psychrotrophic food-spoilage-associated LAB: *Lc. piscium* MKFS47 isolated from MAP broiler meat (25) (I), *Lb. oligofermentans* LMG 22743T isolated from MAP broiler meat (68) (II) and *Le. gelidum* subsp. *gasicomitatum* KG16-1 isolated from vacuum-packaged vegetable sausages (44) (III).

2. Comparative genomic analyses within the genera *Lactococcus* (I) and *Leuconostoc* (III) to identify putative lineage- and niche-specific genes, as well as to characterize the predicted gene contents of the newly sequenced strains, *Lc. piscium* MKFS47 and seven strains of *Le. gelidum* subsp. *gasicomitatum*, including KG16-1.

3. Investigation of transcriptional regulation of carbohydrate catabolism and fermentation pathways in *Lc. piscium* MKFS47 during the time course coupled with depletion of glucose (I).

4. Comparison of transcriptomes of *Lb. oligofermentans* LMG 22743T obtained during growth on glucose, ribose and xylose to gain insights into the transcriptional regulation of carbohydrate catabolism in obligate heterofermentative LAB (II).

3 MATERIALS AND METHODS

3.1 Sequencing and assembly of the genomes (I, II and III).

**DNA isolation.** *Lc. piscium* MKFS47 (I), *Lb. oligofermentans* LMG 22743^T^ (II) (henceforth simply *Lc. piscium* and *Lb. oligofermentans*, respectively), and seven *Le. gelidum* subsp. *gasicomitatum* strains (KG16-1, C120c, C122c, KSL4-2, PB1a, PB1e, PL111) (III) were grown anaerobically in de Man-Rogosa-Charpe (MRS) liquid medium at 25 °C overnight. For *Lb. oligofermentans*, the medium contained xylose instead of glucose. DNA were extracted using a modified method (185) of Pitcher et al. (186), and the genomic DNA was mechanically sheared with a needle.

**Genome sequencing.** The genomes were sequenced using a 454-shotgun sequencer with GS Flx chemistry (Roche) and Illumina HiScanSQ platform (I), ABI 3730 sequencer with BigDye Chemistry (Applied Biosystems) for fosmid libraries and 454-shotgun sequencer GS20 (II), 454-shotgun sequencer with GS Flx chemistry (III, strain KG16-1) and Illumina HiSeq2500 platform (III, six other strains).

**Genome assembly.** Reads were assembled using Newbler (Roche) (I, II, III for strain KG16-1) and Velvet (187) (III for six other strains). For complete genomes (I, II and III), genome closure was done by using the Gap4 program from the Staden package (188). The order of contigs was determined by PCR, and Sanger sequencing was used to fill the gaps.

3.2 Functional annotation of the genomes (I, II and III)

**Frameshift prediction.** For the complete genomes of *Lc. piscium*, *Lb. oligofermentans* and *Le. gelidum* subsp. *gasicomitatum* KG16-1 (I, II and III) frameshift prediction was done using the GeneTack program (140) and similarity searches with proteomes of closely related species. To differentiate frameshifts caused by sequencing errors, insertion or deletion mutations and programed frameshifting events, all frameshift candidates were manually reviewed. Determined sequencing errors were corrected.

**Gene prediction.** Protein-coding genes in the complete genomes of *Lc. piscium*, *Lb. oligofermentans* and *Le. gelidum* subsp. *gasicomitatum* KG16-1 (I, II and III) were predicted by Prodigal (134) and either Glimmer3 (135) or EasyGene (133). The outputs of the two programs were manually compared and their discrepancies were resolved based on similarity searches, multiple sequence alignments, the presence of potential ribosomal binding sites and published data. To further improve the gene prediction, the GenePRIMP pipeline (189), which detects erroneously predicted translational start sites, broken and
missing genes, was applied. Non-coding RNA genes, rRNA, tRNA and tmRNA, were identified with RNAmmer (141), tRNAscan-SE (142) and ARAGORN (190), respectively. Other non-coding RNAs were predicted by scanning the genome sequences against the Rfam database (143).

**Gene function annotation.** Gene functions were predicted using two annotation tools: RAST (191) and PANNZER (154), followed by the comparison of their outputs. Predicted functions were manually reviewed by using similarity searches against public databases and literature data. BAGEL2 (158), CW-PRED (192), PHAST (193), CRISPRFinder (194), Expasy Compute pI/Mw tool (195) and APD3: Antimicrobial peptide calculator and predictor (196) were utilized to determine bacteriocins, LPxTG-motif-containing proteins, prophage regions, CRISPRs, peptide isoelectric point and antimicrobial peptides, respectively. Finally, all genes were classified into functional categories by an RPS-BLAST search against the COG database and checked for the presence of all core functions (197).

**Pathway prediction.** Databases of metabolic pathways were constructed using PathwayTools software (198). Following the automatic prediction, pathways that were in the focus of this study, have been manually reviewed.

The draft genomes of six *Le. gelidum* subsp. *gasicomitatum* strains (C120c, C122c, KSL4-2, PB1a, PB1e, PL111) (III) were annotated by RAST (191), which performed gene prediction and functional assignment.

### 3.3 Ortholog prediction and pangenome tree construction (I and III)

Prediction of orthologous groups of proteins for 30 *Lactococcus* genomes (I) and 41 *Leuconostoc* genomes (III) was performed using OrthoMCL (168) (I) and GET_HOMOLOGUES software package (169) (III) with OrthoMCL clustering algorithm, respectively. Based on the matrices of the presence and absence of orthologs in different genomes, pangenome trees of lactococci and leuconostocs were constructed using the PARS program from the PHYLYP package (199). Clade-specific genes for *Lactococcus* genomes were determined as genes that are present in 90 % of the genomes in the clade and absent in 90 % of the other genomes. Comparison of the whole *Leuconostoc* genome sequences was performed using BLASTN alignments and visualized using BRIG (166).

### 3.4 Phylogenetic tree construction (III)

The phylogenetic tree of leuconostocs was built using T-REX web server (200) based on MUSCLE (201) aligned concatenated nucleotide sequences of *atpA*, *pheS* and *rpoA* genes, which were shown to successfully discriminate between species of the genus *Leuconostoc* (202). Poorly aligned positions were removed using Gblocks (203) with
default parameters and the tree was inferred using RAxML program (204) with GTRCAT substitution model and *Bacillus subtilis* as an outgroup. Bootstrap analysis was performed with 500 replicates.

### 3.5 Phenotypic analyses (I, II and III)

**Carbohydrate/carbon source utilization profiles.** Carbohydrate fermentation of *Lc. piscium* strains (I), *Lb. oligofermentans* (II) and *Le. gelidum* subsp. *gasicomitatum* KG16-1 (III) was assessed by the API 50CH and API 20 Strep identification systems (bioMerieux, Marcy l'Etoile).

For *Lc. piscium* (I), glycerol utilization was tested in M17 broth with 1 % glycerol aerobically and anaerobically after 24 h. Citrate fermentation was assessed by using a plate assay screening (205) anaerobically and aerobically after 48 h.

For *Lb. oligofermentans* (II), PM1 and PM2A Phenotype MicroArrays (BIOLOG) and growth with selected carbon sources (glucose, N-acetylglucosamine, maltose, ribose, xylose, 2-deoxyribose, inosine, pyruvate, dihydroxyacetone (DHA) and glycerol) (75 mM) anaerobically and aerobically were used to confirm and extend carbohydrate utilization profiles. To test the utilization of carbon sources as electron acceptors, *Lb. oligofermentans* was grown with xylose (28 mM) as a primary carbon source, and pyruvate, DHA, glycerol or 2-deoxyribose (75 mM) as prospective electron acceptors or inosine (75 mM) as a secondary carbon source aerobically and anaerobically.

In these growth experiments MRS liquid medium without citrate and glucose substituted with the carbon sources of interest was used. Cultures were grown for 48 h with catalase (1000 U/ml).

**Acetoin/diacetyl production assay.** Detection of the total amount of diacetyl and acetoin (I and II) was performed according to the modified Voges-Proskauer assay (206). Before detection, bacteria were grown in MRS medium without acetate with 2 % glucose or glycerol aerobically (I) and in MRS medium without citrate, with glucose substituted with the carbon sources of interest and with catalase (1000 U/ml) aerobically and anaerobically (II).

**Other analyses.** The twitching motility of *Lc. piscium* (I) was tested under different conditions (different media, carbon sources, atmosphere) on 1 % agar on microscope slides and examined by phase microscope. For transmission electron microscopy (TEM), 20 μl droplets of an overnight culture of *Lc. piscium* were inoculated on M17 plates with 0.5 % glucose and 1 % agar. Cells from the outermost edge of the inoculum were suspended in a saline solution, stained with 3 % uranylacetate, and examined by TEM.

Production of hydrogen peroxide (I) was tested as described previously (207), except that M17 broth with 1 % glycerol or 1 % glucose was used instead of BHI broth. Plates were incubated for 48 h anaerobically and aerobically.

To test for respiration (II), *Lb. oligofermentans* was grown for 48 h with glucose, ribose or xylose (50 mM) only, and with the addition of heme (2.5 μg/ml) or menaquinone
MATERIALS AND METHODS

(1.0 μg/ml) or both, heme and menaquinone, aerobically. The medium used was MRS without citrate with catalase (1000 U/ml).

All the tests were done at 25°C with appropriate controls and in at least three replicates. The growth was determined by measuring sample optical density at 600 nm (OD$_{600}$).

3.6 RNA extraction and sequencing (I, II and IV)

LAB cultivation for transcriptome profiling. All cultures were grown at 25°C, under microaerobic conditions and in three replicates.

*Lc. piscium* MKFS47 (I) was grown in a modified MRS broth without acetate with 2% glucose during 11 h. Samples were taken at three time points, 3, 5, and 11 h.

*Lb. oligofermentans* LMG 22743 $^T$ (II) was grown in a modified malo-lactic differential (MLD) medium (208) without malic acid, cellulose and bromocresol green, and with the concentration of a sole carbon source (either glucose, ribose or xylose) being 50 mM for 30 h. Samples were taken at three time points, 20, 24 and 30 h.

Three LAB (IV), *Le. gelidum* subsp. *gasicomitatum* LMG 18811$^T$, *Lc. piscium* MKFS47 and *Lb. oligofermentans* LMG 22743 $^T$, were grown separately in individual cultures and in mixed cultures pairwise (three combinations) and all together (hereafter, triple culture). The experiments were done in two batches. At first, co-cultivation of *Le. gelidum* and *Lc. piscium* accompanied with their individual cultures was performed and processed. Later, two other pairwise cultures (*Le. gelidum* + *Lb. oligofermentans* and *Lc. piscium* + *Lb. oligofermentans*) and the triple culture were grown together with the individual cultures of all three LAB. For all the growth experiments each LAB was precultured separately in the MRS liquid medium without acetate to reach approximately equal OD$_{600}$ for all three LAB. The obtained pre-cultures were inoculated into 250 ml of fresh MRS broth without acetate individually or as mixtures with equal total amount of inoculum and equal ratios of each species in mixed cultures. The cultures obtained were grown for 11 h. Samples for RNA extraction were taken at three time points, 3, 5 and 11 h.

During growth of cultures in all the studies OD$_{600}$ values and total viable cell counts (plate cultivation for 5 days on MRS medium without acetate anaerobically) were measured once an hour or two hours. Selective media was used to determine species specific viable cell counts in co-cultures (IV): MRS medium with fusidic acid (30 μg/ml) for *Le. gelidum* subsp. *gasicomitatum* LMG 18811$^T$, GM17 medium with polymyxin (60 μg/ml) for *Lc. piscium* and MRS medium with kanamycin (85 μg/ml) and xylose instead of glucose for *Lb. oligofermentans*.

RNA extraction. Samples were treated with a cold 10:1 mixture of ethanol-phenol to inhibit cell metabolism and RNase activity, then centrifuged and immediately frozen. The cells were disrupted by the use of a mixer mill. RNA was using the RNeasy plant minikit (Qiagen) with DNase treatment according to the manufacturer’s instructions.
Ribosomal RNAs were omitted from the total RNA by magnetic beads (Ribo-Zero magnetic kit; Epicentre).

**RNA-seq library preparation and sequencing.** RNA-seq libraries were produced using the Ovation RNA-seq system V2 (NuGEN). The double-stranded DNA obtained was sheared by sonication. The obtained fragments were polished with T4 DNA polymerase, followed by the ligation of adapters for SOLiD sequencing. Libraries were size selected and thereafter sequenced in five or six lanes by using SOLiD 5500XL (Life Technologies) to produce 75-bp single-end reads.

### 3.7 RNA-seq data pre-processing and differential expression analysis (I, II and IV)

**RNA-seq read mapping and counting.** Lifescope software (Life Technologies) was used for mapping against corresponding genomes and counting the RNA-seq reads. In the interactome study (IV) RNA-seq reads obtained from all samples were mapped against all three genomes of the LAB at the same time to allow properly map reads that align to more than one genome. The overall mapping percentages varied for different samples and were more than 90 % (I), 23 – 87 % (II) and 69 – 95 % (IV). Approximately half (45 – 51 %) of the mapped reads in each sample belonged to the protein-coding genes, which were used for further analysis. The number of CDS-mapped reads varied for different samples: 4.1 - 8.1 (I), 0.76 - 13.84 (II) and 0.17 - 9.9 (IV) million per sample.

**Similarity-based clustering of samples.** To visually explore sample relationships and assess the quality of RNA-seq data, samples were hierarchically clustered based on RPKM values (I) and based on rlog-transformed data as described in the manual (209) for DESeq2 package (II and IV) using R functions. In addition, in the two studies (II and IV), principal component analysis (PCA) was performed for the samples.

**Differential expression analysis (DE analysis).** In the study I, DE analysis was performed between different time points by using EBSeq (210) and edgeR (211). The genes were considered to be differentially expressed (DE) if their false discovery rate (FDR) was ≤ 0.05 (for edgeR) or if the posterior probability (PP) was ≥ 0.95 (for EBSeq) and if the absolute fold change (FC) was ≥ 1.5. Genes that were found to be significantly differentially expressed by at least one program were taken for further analysis.

In the studies II and IV, DE analysis was performed using DESeq2 (209) for each time point independently. The genes were considered to be DE if their adjusted p-value (similar to FDR) was ≤ 0.05 and absolute FC ≥ 2 (II) or FC ≥ 1.5 (IV). For Lb. oligofermentans (II) three pairwise comparisons of transcriptomes, obtained during growth on different carbon sources (glucose-ribose, glucose-xylose and ribose-xylose), were made. For LAB interaction study (IV), hierarchical clustering revealed a strong batch affect, therefore, the DE analysis was performed independently for each batch. Comparisons were made for each LAB species between RNA-seq reads mapped to its
genes in the individual culture (served as a control) and in its mixed cultures (two pairwise and one triple cultures for each species).

3.8 Motif discovery and search (II)

Upstream regions (in relation to the translational start site) up to 300 nucleotides of *Lb. oligofermentans* genes were extracted. Motif discovery search, enrichment analysis and querying the databases of transcription factor binding sites (TFBS), were done using the tools *meme*, *ame* and *tomtom* from MEME suite (212). The database of TFBS comprised the combination of the PRODORIC (213) and RegTransBase (214) databases. For verification, *Lactobacillaceae*-specific TFBS profiles of CcpA, XylR, RbsR and Rex, as well as the *Bacillales*-specific Rex TFBS profile, were added to the database. These profiles were built using *sites2meme* script (MEME suite) based on motif sequences obtained from the RegPrecise database (215). In turn, *fimo* tool (MEME suite) was used to scan all the upstream regions in the genome for the presence of CcpA, XylR, RbsR and Rex TFBS with the adjusted *p*-value threshold of 0.05.

3.9 Functional enrichment analysis of DE genes (IV)

The DE genes for each pairwise comparison between individual and mixed cultures, as well as genes commonly DE in both pairwise cultures for each species were tested for the enrichment of the COG functional categories using one-tailed Fisher’s exact test. The number of expressed genes in the genome that belonged to different COG categories served as a background. The functional categories were considered to be enriched if their *p*-value of enrichment was ≤ 0.05.

More details about the methods used in the studies can be found in the corresponding articles.
4 RESULTS

4.1 The general characteristics of food-spoilage-associated LAB genomes (I, II and III)

Completely sequenced and assembled genomes of *Lc. piscium*, *Lb. oligofermentans* and *Le. gelidum* subsp. *gasicomitatum* KG16-1 were functionally annotated both automatically and manually leaving only ~ 15-16 % of their genes with completely unknown function (ENA accession numbers are LN774769 - LN774771 for *Lc. piscium*, LN898144 - LN898145 for *Lb. oligofermentans*, and LN890331- LN890334 for *Le. gelidum* subsp. *gasicomitatum* KG16-1.). Both protein-coding and non-coding RNA genes were predicted (Table 1). During the annotation process, detected sequencing errors were corrected or reported in the genome annotation files, pseudogenes and one programed frameshifting event (in *prfB* gene of *Lb. oligofermentans*) were predicted. All three genomes had low GC content 35-38 %, contained plasmid(s), bacteriophage(s) and RM systems (Table 1). The distributions of the protein-coding genes in the genomes among COG functional categories are illustrated in the figures of the included articles (Study I: Figure 1; Study I: Additional file 1: Figure S1; Study III: Figure 3). The minimal set of essential functions for a prokaryotic organism (61 core COGs) (197) was present in all the genomes.

In addition, six strains of *Le. gelidum* subsp. *gasicomitatum* (C120c, C122c, KSL4-2, PB1a, PB1e, PL111) associated with spoilage of vegetable-based food products (Study III: Table 6) were sequenced, partially assembled and automatically annotated (Study III: Additional file 1: Table S3 for ENA accession numbers).

4.2 Comparative genomic analyses (I and III)

For the newly sequenced genomes of *Lc. piscium* and seven *Le. gelidum* subsp. *gasicomitatum* strains including KG16-1, comparisons of their gene repertoire were made with the gene contents of other publicly available *Lactococcus* and *Leuconostoc* genomes, respectively. As for *Lb. oligofermentans* LMG 22743\textsuperscript{T}, several comparative genomic studies (30, 67, 31) of *Lactobacillus* genomes that included the draft genome of *Lb. oligofermentans* LMG 22743\textsuperscript{T} existed already. Therefore, we did not perform comparative genomic analysis for the complete genome of *Lb. oligofermentans* LMG 22743\textsuperscript{T}.

Comparison of *Lactococcus* genomes (I). As a result of ortholog prediction between 30 lactococci genomes, including *Lc. piscium*, 4938 orthologous groups (excluding singletons) were identified. Among them, 337 groups (11-19 % of the genomes) were shared by all lactococci genomes and, therefore, represented the core genes, sixteen of which had completely unknown functions. Based on similarity of
**Table 1. Genome statistics.**

<table>
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<tr>
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<th><em>Lc. piscium</em> MKFS47</th>
<th><em>Lb. oligofermentans</em> LMG 22743&lt;sup&gt;f&lt;/sup&gt;</th>
<th><em>Le. gelidum subsp. gasicomitatum</em> KG16-1</th>
</tr>
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RM – restriction modification

<sup>a</sup> Plasmid genes were not expressed according to the RNA-seq data
RESULTS

Ortholog presence/absence profiles of the genomes, pangenome tree of the Lactococcus genus was built (Study I: Figure 2). The tree had three major clades: two clades separated Lc. lactis subsp. lactis and Lc. lactis subsp. cremoris genomes, while the third included Lc. piscium, Lc. raffinolactis, Lc. chungangensis and Lc. garvieae strains. “Lc. lactis subsp. lactis,” “Lc. lactis subsp. cremoris”, and “Lc. garvieae,” clades were shown to have 33, 54, and 67 clade-specific genes, the interesting examples of which included phosphoketolase (ptk, an indicator of facultative heterofermentative metabolism in lactococci) in the Lc. lactis subsp. lactis clade, nitroreductase gene (can be involved in conversion of nitrocompounds into the toxic or carcinogenic derivatives) (216) in the Lc. lactis subsp. cremoris clade and phosphatidylglycerol lysyltransferase gene (mprF, mediates protection against cationic antimicrobial peptides) (217) in the Lc. garvieae clade. Lc. piscium and its most phylogenetically close species, Lc. raffinolactis and Lc. chungangensis, formed a separate branch inside the Lc. garvieae clade. Their core genome was more than three times bigger than the core genome of all lactococci analyzed and consisted of 1278 genes. The comparison also showed that Lc. piscium shared more orthologs with Lc. raffinolactis than with Lc. chungangensis (Study I: Figure 3). The interesting examples of the genes shared only by two species included pyruvate and lactate oxidase genes in Lc. raffinolactis and Lc. chungangensis, agmatine catabolic and malolactic operon in Lc. piscium and Lc. raffinolactis, and LPxTG-like pili in Lc. piscium and Lc. chungangensis.

Lc. piscium genome contained 395 (16% of the genome) unique genes that were not present in any other Lactococcus genomes analyzed. These genes included, for instance, type IV pili cluster (pilBTCA), L-ascorbate catabolic operon (ula) and three putative bacteriocins. In turn, Lc. piscium genome was lacking glucenate kinase, cytochrome and menaquinone biosynthesis genes, and arginine deiminase that were present in 29, 25 and 24 other lactococci genomes, respectively.

Comparative genomic analysis between Le. gelidum subsp. gasicomitatum strains and other Leuconostoc species (III). Comparison of gene contents was made for seven newly sequenced “vegetable” strains of Le. gelidum subsp. gasicomitatum (KG16-1, C120c, C122c, KS4L-2, PB1a, PB1e, PL111) and 34 publicly available Leuconostoc genomes, including the genome of a “meat” strain of Le. gelidum subsp. gasicomitatum, LMG 18811T. The number of orthologous groups determined was 6,248 (including singletons) with 406 clusters constituting the core genome of all leuconostocs analyzed. Based on the constructed pangenome tree (Study III: Figure 4) genomes belonging to the same species, generally, clustered together, except for the two genomes, Le. gelidum subsp. gasicomitatum 1301_LGAS and Le. citreum 1300_LCIT, obtained during the study of clinical isolates (218). The additional phylogenetic analysis (Study III: Additional file 1: Figure S1) based on the concatenated nucleotide sequences of atpA, pheS and rpoA genes confirmed that the first genome (1301_LGAS) actually belongs to Le. citreum, while the second genome (1300_LCIT) is a member of the Le. lactis group. In addition, the phylogenetic analysis showed that the genome assigned to Le. inhae LMG 22919 (= KCTC 3774) actually belongs to Le. gelidum subsp. gasicomitatum and very close to strain LMG 18811T. Its clustering together with Le. fallax on the pangenome tree (Study III: Figure 4) was probably a result of numerous frameshifts in the genome due to the sequencing errors and high genome fragmentation (~ 900 contigs). These might have caused many genes to
be missing or truncated. The clustering of *Le. carnosum* with *Le. kimchi* genomes on the pangenome tree was different from that on the phylogenetic tree (Study III: Figure 1), where it clustered together with *Le. citreum* and *Lc. lactis* strains. This might imply the presence of certain phenotypic traits, which are common for *Le. carnosum* and *Le. kimchi*, but not present in *Le. citreum* and *Lc. lactis*. Interestingly, no clear separation was observed between vegetable strains and the meat strain of *Le. gelidum* subsp. *gasicomitatum* based on their gene contents. Instead, only three vegetable strains formed a separate branch, while four other clustered together with the meat strain.

*Le. gelidum* subsp. *gasicomitatum* KG16-1 genome contained 75 unique genes (~4 %) that were not present in other Leuconostoc genomes. Most of them (77 %) were uncharacterized and phage related (Study III: Figure 5B). Strains KG16-1 and C120c contained lacticin-481 biosynthesis cluster, that is homologous to that in *Lc. lactis* subsp. *lactis* (219). Peculiarly, several functionally interesting genes that are present in all other *Le. gelidum* strains were absent in the KG16-1 genome (Study III: Figure 5A and Study III: Table 6). They included pyruvate oxidase (*poxB*), xylose transporter (*xylT*), collagen-binding protein (*can*) and biofilm formation genes (*icaAB*) that form together a single locus in LMG 18811^T^ genome. In addition, polyketide biosynthesis cluster was present in all but KG16-1 and PB1e *Le. gelidum* subsp. *gasicomitatum* strains. Beside *Le. gelidum* subsp. *gasicomitatum* strains, the same polyketide biosynthesis cluster was found only in *Streptococcus thermophilus* JIM 8232 (genes pig-1,2,3,4).

Mucus-binding protein containing LPxTG-like motif (putative adhesin) (LEGAS_0414) was found only in *Le. gelidum* subsp. *gasicomitatum* strains (however, frameshifted in KG16-1), and accessory Sec system was detected in all strains of *Le. gelidum* subsp. *gasicomitatum* and only in a few other Leuconostoc species (Study III: Figure 5 and Study III: Table 6). All *Le. gelidum* subsp. *gasicomitatum* strains contained serine-rich proteins (putative adhesins), which in the two complete genomes surrounded the accessory Sec system genes.

### 4.3 Metabolism of food-spoilage-related LAB: genome-based inference and phenotypical tests (I, II and III)

Based on the predicted metabolic pathways, the three LAB species possessed different central carbohydrate catabolic pathways: glycolysis in *Lc. piscium*, which classifies *Lc. piscium* as an obligate homofermentative LAB, and the phosphoketolase pathway in *Lb. oligofermentans* and *Le. gelidum* subsp. *gasicomitatum* KG16-1, which classifies these two species as obligate heterofermentative LAB. All three genomes contained three alternative pyruvate utilization pathways, including L- and/or D-lactate dehydrogenase, pyruvate dehydrogenase and acetoin/diacetyl pathways, and two acetyl-CoA utilization pathways, including acetate and ethanol formation pathways. In addition, pyruvate-formate lyase pathway was present in the *Lc. piscium* genome. Spoilage substances, such as acetoin/diacetyl, acetate and excessive amount of carbon dioxide, are produced as a result of these pathways (Study I: Table 3). Catabolic/transport pathways and/or utilization profiles for particular carbohydrates/carbon sources were predicted and
obtained (Study I: Table 4 for *Lc. piscium*, Study II: Table 1 and Additional file 1: Table S3 for *Lb. oligofermentans*, and Study III: Table 1 for *Le. gelidum* subsp. *gasicomitatum* KG16-1). Even though *Lc. piscium* and *Lb. oligofermentans* strains analyzed in this study were both isolated from meat products (25, 68) (protein-rich environment), they have different amino acid biosynthetic capabilities based on their gene contents. While *Lc. piscium* had all proteinogenic amino acid biosynthesis pathways except for phenylalanine, *Lb. oligofermentans* has only half of them. Respiratory electron-transport chain and menaquinone biosynthesis genes were found in *Lb. oligofermentans* and *Le. gelidum* subsp. *gasicomitatum* KG16-1, but not in *Lc. piscium* as already mentioned earlier. Typically for LAB, they miss a heme biosynthesis pathway (220). For *Lb. oligofermentans* no aerobic growth promotion was observed on any carbon source when menaquinone-containing medium was supplemented with heme, suggesting that *Lb. oligofermentans* did not respire under the examined conditions.

**Acetoin/diacetyl production by *Lc. piscium* and associated pathways (I).** Under aerobic conditions, *Lc. piscium* generated a significant amount of acetoin/diacetyl (8.5 mM) after 48 h of growth (final OD$_{600}$ ~ 2.2; MRS medium) on the medium containing glucose as main carbon source. The factors that create pyruvate surplus such as the presence of electron acceptors (e.g. oxygen) or catabolism of citrate, aspartate or alanine are known to enhance acetoin/diacetyl production in LAB (Figure 3) (1, 102).

*Lc. piscium* was found to possess a complete citrate catabolism/transport operon (*citMCDEFXG*), which is, otherwise, present only in four strains of *Lc. lactis* subsp. *lactis*: IL1403 and biovar *diacetylactis* LD61, TIFN2, TIFN4. However, *Lc. piscium* did not ferment citrate during 48 h both aerobically and anaerobically, and citrate catabolic genes had very low expression levels based on RNA-seq data, although, citrate was present in the growth medium.

Conversion of aspartate and alanine to pyruvate requires corresponding transaminases, which were present in the genome, and alpha-ketoglutarate, which can be produced from glutamate by glutamate dehydrogenase. This enzyme was present in *Lc. piscium* and its phylogenetically close lactococci: *Lc. raffinolactis* and *Lc. chungangensis*.

Oxygen-dependent oxidation of NADH could be mediated by two NADH oxidases, *noxE* and *noxC*, that could be identified in the genome. Furthermore, *Lc. piscium* possessed genes for oxygen-dependent glycerol catabolism (Study I: Figure 5), which was shown to contribute to increased production of acetoin/diacetyl in *Lb. rhamnosus* (104). We showed that *Lc. piscium* was able to grow with glycerol as a sole carbon source only in the presence of oxygen with the growth efficiency comparable to or lower than that of glucose, depending on the type of growth media used. The aerobic production of acetoin/diacetyl from glycerol amounted to 1.1 mM after 48 h of growth (final OD$_{600}$ ~ 1.0; MRS medium)

**Utilization of different carbohydrates/carbon sources by *Lb. oligofermentans* (II).** According to the API 50CH test results and actual growth experiments (Study II: Table 1), *Lb. oligofermentans* was able to efficiently utilize and grow not only on pentoses, as it was reported previously, but also on hexoses (glucose and N-acetylglucosamine), maltose and inosine. Presence of oxygen caused a significant decrease of the biomass on all the growth-supporting carbon sources (Study II: Figure 1A) impairing the growth on
pentoses (ribose and xylose) most of all. As a consequence, in anaerobic atmosphere the
most efficient growth was observed on maltose, ribose and xylose, while aerobically on
maltose and glucose.

Pyruvate could only slightly (FC ~ 1.7 in comparison with carbon source free
control) support anaerobic growth as a sole carbon source (Study II: Figure 1A). However,
its addition to growth medium containing xylose as a primary carbon source increased
biomass by ~ 50 % and acetoin/diacetyl production (Study II: Figure 1B). This indicates
that \textit{Lb. oligofermentans} can use pyruvate as an electron acceptor. Other putative electron
acceptor candidates (DHA, 2-deoxyribose and nucleosides), as determined by BIOLOG
phenotype microarrays, did not exhibit electron acceptor properties.

Inability of \textit{Le. gelidum} subsp. \textit{gasicomitatum} KG16-1 to utilize xylose (III).
According to the API 50CH test (Study III: Table 1), strain KG16-1 was able to utilize the
variety of carbohydrates similarly to other \textit{Le. gelidum} subsp. \textit{gasicomitatum} strains (37, 40). But unlike the majority of \textit{Le. gelidum} subsp. \textit{gasicomitatum} strains, KG16-1 did not
produce acid from D-xylose. Genome analysis showed that KG16-1 possessed the required
xylose catabolic genes (\textit{xylA} and \textit{xylB}) and putative xylose transporter (\textit{xylP}), which did
not have frameshifts or premature stop codons. Nevertheless, in KG16-1, genes \textit{xylA} and
\textit{xylB} carried two and four amino acid substitutions, respectively, in comparison with the
corresponding genes in xylose-fermenting \textit{Le. gelidum} subsp. \textit{gasicomitatum} strains with
available genome sequences (LMG 18811\textsuperscript{T}, C120c, KSL4-2 and PL111). In addition,
KG16-1 was lacking another xylose transporter \textit{xylT}, which was present in the xylose-fermenting
strains. These findings could explain the negative reaction of KG16-1 for D-
xylose utilization.

4.4 Predicted factors facilitating better survival in food environment
and increased competitiveness within microbial community (I, II
and III)

Survival factors could be divided into niche specific and non-specific. The
former include, for example, the ability to utilize alternative carbon and energy sources
available in the niche. Both meat-inhabiting bacteria, \textit{Lc. piscium} and \textit{Lb. oligofermentans},
contained all the genes required for purine/pyrimidine (deoxy)ribonucleoside transport and
catabolism. As already mentioned, \textit{Lb. oligofermentans} was shown to efficiently utilize
inosine as carbon source. As for arginine, both bacteria did not possess genes for arginine
degrading enzymes. Nevertheless, \textit{Lc. piscium} genome contained an operon for the
catabolism of agmatine, the derivative of arginine. Likewise, its degradation leads to
formation of ATP, putrescine and ammonia are produced (221). Interestingly, \textit{Lc. piscium}
had also a cluster for glycogen biosynthesis/degradation. Nevertheless, no extracellular
amylase was found in the genome and none of the tested \textit{Lc. piscium} strains produced acid
from glycogen. As for plant environment, the ability to ferment cell wall polysaccharides is
advantageous. Generally, “vegetable” \textit{Le. gelidum} subsp. \textit{gasicomitatum} strains contained
many plant-carbohydrate specific catabolic pathways. Interestingly, four “vegetable”
strains, KG16-1 (plasmid), KSL4-2, PL111 and PB1e, contained a putative type I galactan catabolic gene cluster, which was not present in the “meat” strain, LMG 18811T.

Other survival factors can include those mediating adhesion and antimicrobial properties. Scanning the genomes for the domains/motifs associated with adhesion in LAB (LPxTG-like motif, serine-rich domains, mucus-binding and fibronectin-binding domains) identified several candidates in each genome (for details see studies I, II and III). Particularly, *Lc. piscium* was found to possess gene clusters for two types of pili: LPxTG motif-containing pili, which were homologous to the adhesive pili *spaCED* in *Lactobacillus rhamnosus* (222), and type IV pili (*pilBTCA*), which were shown to be involved in twitching motility and adhesion (223). However, no pili were observed on the surface using TEM and their gene expression levels were very low based on the RNA-seq data. In addition, *Lc. piscium* did not exhibit twitching motility. *Lb. oligofermentans* was particularly enriched in putative adhesins, encoding eight LPxTG motif-containing proteins and 14 adhesin candidates, ten of which were serine-rich proteins. Putative adhesins predicted in the *Le. gelidum* subsp. *gascomitatum* strains were reported earlier. Bacteriocin-encoding genes, as putative antimicrobial factors, were found in *Lc. piscium* (three putative bacteriocins) and *Le. gelidum* subsp. *gascomitatum* KG16-1 (lacticin-481).

Hydrogen peroxide, a potential antimicrobial agent, was found to be released by *Lc. piscium* only from glycerol (not glucose) under aerobic conditions and by *Lb. oligofermentans* from a variety of carbon sources both aerobically and anaerobically.

### 4.5 Time dependent differential expression of *Lc. piscium* genes during glucose fermentation (I).

Individual cultures of *Lc. piscium*, used for the time course transcriptome profiling, reached the stationary phase after approximately 7 h of growth. Therefore, samples taken at 3, 5 and 11 h corresponded to the early exponential, middle exponential and stationary phases, respectively. Genes of the plasmid 1 were not expressed. Comparison of gene expression between the time points (3 h < – > 5 h and 5 h < – > 11 h) showed that many genes for carbohydrate transport and catabolism (fructose, mannose, mannitol, xylose, lactose/galactose, maltose and beta-glucosides as well as glycogen biosynthesis/degradation operon) were highly upregulated (FC > 4) during the second time period (5-11 h) or the whole time (3-11 h). Furthermore, expression of the universal ABC transporter ATP-binding protein (*msmK*), which energizes multiple carbohydrate ABC transporters (224), increased significantly (FC ~ 10) in the second time period. In addition, agmatine and glycerol catabolic operons were overexpressed at 11 h in comparison with the earlier time points. All (four) enzymes involved in pyruvate dissipating routes showed time course differential expression (Study I: Figure 4A). Pyruvate dehydrogenase complex (*pdhABCD*) had the highest expression levels at all time points in comparison with other three enzymes (encoded by *pfl, ldh* and *alsS*) and was highly upregulated after 5 h (FC ~ 4). During the same time, expression of pyruvate-formate lyase (*pfl*) and lactate dehydrogenase (*ldh*) genes were significantly increased (FC ~ 8) and decreased (FC ~ 16), respectively. Acetyl-CoA, produced by both pyruvate dehydrogenase and pyruvate-formate
lyase is further reduced to either ethanol by aldehyde-alcohol dehydrogenase \textit{adhE}, which was significantly upregulated (FC ~ 13) in the second period, or converted to acetyl-phosphate by phosphate acetyltransferase \textit{(pta)}, which had much higher expression levels than \textit{adhE} at any time without statistically significant changes across time points (Study I: Figure 4B). Acetolactate synthase \textit{(alsS)} was found to be upregulated only in the first time period (Study I: Figure 4A), while diacetyl reductases, chromosomal \textit{budC} and plasmid \textit{butA}, were both upregulated during the whole time. Interestingly, the expression level of \textit{butA} was extremely higher (34-72 times) than of \textit{budC}, indicating that \textit{butA} is the main diacetyl-reducing enzyme in \textit{Lc. piscium}.

4.6 \textit{Lb. oligofermentans} transcriptome responses during exponential growth on glucose, ribose and xylose (II).

When \textit{Lb. oligofermentans} was grown on the modified MLD medium with three different carbohydrates under microaerobic conditions, growth rate on xylose was slightly higher than that on glucose and ribose. Samples taken at 20, 24 and 30 h corresponded to the beginning, middle and end of the exponential growth phase, respectively (Study II: Figure 2). Plasmid-encoded genes were not expressed.

Comparison of transcriptome responses during exponential growth on glucose, ribose and xylose. Hierarchical clustering of samples based on the Euclidean distances and principal component analysis (Study II: Figure 3) revealed time-dependent changes in the relative clustering of transcriptomes obtained during growth with the different carbohydrates. Surprisingly, in the beginning and the middle of the exponential growth phase (20 and 24 h), xylose transcriptome showed higher similarity to glucose transcriptome, but at the end of this phase (30 h) it became more similar to ribose transcriptome. The total numbers of DE genes, identified in pairwise comparisons of three different transcriptomes at each time point independently, reflected the detected relationships between the different transcriptomes (Study II: Table 2).

Differential expression of carbohydrate/carbon source catabolic pathways and transporters. As expected, the growth on different carbohydrates (glucose, ribose and xylose) induced the expression of their corresponding catabolic pathways and transporters (Figure 5A, B and C). It is notable that some level of cross-induction was observed for hexose (e.g. glucose, mannose, fructose and gluconate) and xylose catabolic/transport genes by either glucose or xylose. Namely, hexose catabolism related genes (\textit{glcK, pmi, gmuE} and \textit{gntK}) and putative hexose PTS transporter (\textit{manXYZ}) were significantly overexpressed both on glucose and xylose in comparison with ribose at 20-24 h, while at the same time xylose transporter (\textit{xylT}) and xylose catabolic genes (\textit{xylAB}), although having the highest expression levels on xylose, were also induced by glucose in comparison with ribose. Interestingly, a maltose catabolic operon was highly induced by glucose during the whole time (Figure 5A).

The genome possessed two gene clusters for ribose catabolism/transport each
containing functional isoforms of three ribose catabolic enzymes (rbsK, rpiA, rbsD), but different ribose transporters. Although both clusters were clearly upregulated during growth on ribose in comparison with glucose and xylose during the whole time, the time course dynamics of their gene expression on ribose differed: while one cluster had relatively constant expression, another cluster was strongly downregulated with time.

Catabolic pathways for nucleoside and ribose degradation are linked. Nevertheless, no ribose-induced upregulation of nucleoside catabolic genes was observed. Moreover, one operon, deoDBC, involved in nucleoside degradation was even upregulated on glucose and xylose in comparison with ribose at 20 h.

**Differential expression of fermentation and NAD(P)H re-oxidation related genes.** Growth with pentoses (ribose or xylose) caused significant upregulation of genes involved in acetoin/diacetyl (alsSD) and acetate (ackA) formation (Figure 5). Expression of pyruvate dehydrogenase genes (pdhABCD) on different carbohydrates reflected the transcriptome clustering revealed previously. In the beginning of the exponential growth phase, the expression levels were similar on glucose and xylose, and higher than on ribose, whereas at the end of this phase they became similar for ribose and xylose, and lower than for glucose. The expression of two L- and D-lactate dehydrogenases genes (ldh and ldhD) were only mildly dependent on the fermented carbohydrates. Glucose catabolism led to the strong and continuous (20-30 h) upregulation of genes coding for NAD(P)H re-oxidizing enzymes (Figure 5D), such as aldehyde-alcohol dehydrogenase (adhE), other alcohol dehydrogenases (adhA and radh) that might be involved in ethanol production, NAD(P)H oxidase (nox), one of the three diacetyl reductases (butA2) and 1,3-propanediol dehydrogenase (dhaT).

**Transcriptional regulation of genes with similar differential expression profiles.** Genes, co-regulated based on the transcriptional profiles, were searched de novo for commonly occurring motifs that are enriched in their upstream regions. The identified motifs were searched against the databases of known prokaryotic TFBS (Study II: Table 3).

Genes upregulated during catabolism of either glucose, xylose or both in comparison with ribose in the beginning of the exponential growth phase (20 h) were found to be significantly enriched in a motif that had significant similarity to a CcpA-like binding motif of Lactobacillaceae. Genes containing this motif included, for instance, genes involved in catabolism of hexoses and xylose, as well as pyruvate dehydrogenase complex and central components of the PTS and the CCC systems (ptsH1). The genome-wide search identified 109 upstream regions containing Lactobacillaceae-specific CcpA-binding motif. Only half (55) of these genes showed differential expression in at least one pairwise comparison of the transcriptomes at 20 h. Yet, within CcpA-binding motif-containing DE genes, the prevailing groups of genes were those that were upregulated on glucose (30) or xylose (34) or both glucose and xylose (19) (Study II: Table 4). The expression of the CcpA encoding gene (ccpA) was not dependent on the fermented carbohydrate.

The upstream regions of genes overexpressed during growth on glucose in comparison with xylose at 20 h and 24 h were enriched in a motif that had significant similarity to an anaerobic regulator Fnr TFBS of B. subtilis (Study II: Table 3). However, the literature search identified another potentially matching transcription factor, redox-
Figure 5. (The legend is on the next page)
RESULTS

Figure 5. Differential expression of the genes involved in carbohydrate catabolism/transport, fermentation and NAD(P)H re-oxidation in *Lb. oligofermentans*. Colored areas: blue (A) - catabolism of hexoses and maltose, pink (B) – ribose catabolism, green (C) – xylose catabolism, grey (D) - NAD(P)H re-oxidation. Bold arrows indicate NAD(P)H-producing reactions, white arrows – NAD(P)H-re-oxidizing reactions, yellow boxes represent transporters and green arrows – transport across the cell membrane. Notation ‘G/R’ means the ratio of normalized read counts between glucose and ribose samples. The 3 × 3 matrices for each enzyme/transporter gene contain values log2FC for pairwise comparisons between three transcriptomes for three time points, as indicated in the legend. The log2FC values are shown in black if the change was statistically significant (adjusted p-value ≤ 0.05) and in gray if it was not statistically significant. The figure is from Study II.

sensing transcriptional repressor Rex (225). When *Lactobacillaceae* -specific Rex TFBS profile was built and included in the database, the enriched motif matched Rex TFBS with much higher similarity than Fnr TFBS. The genome-wide search with the identified Rex TFBS-like motif (Study II: Figure 5) revealed 20 genes containing such motif, 12 of which were upregulated on glucose in comparison with both xylose and ribose most of the time (Study II: Table 5). Six out of 12 genes are (putatively) involved in NAD(P)H re-oxidation (adhE, adhA, radh, nox, butA2 and dhaT). All alcohol dehydrogenases contained at least two such putative motifs in their upstream regions. Noteworthy, both lactate dehydrogenases did not contain the Rex TFBS-like motif. Rex- encoding gene (*rex*) was not DE in any pairwise comparisons at any time point.

The genome was also scanned for the presence of TFBS of ribose (RbsR) and xylose (XylR) catabolism regulators. The found motifs were mainly restricted to the upstream regions of ribose and xylose catabolic genes, respectively. In addition, these regions contained non-overlapping CcpA-binding sites. Peculiarly, the CcpA- binding motif, predicted in the promoter region of *ptsHI*, completely overlapped with the predicted RbsR- binding site.

4.7 Interspecies interactions between food-spoilage-associated *Leuconostoc, Lactococcus* and *Lactobacillus* species resolved by transcriptome profiling (IV)

**LAB growth in individual and mixed cultures.** The relation between growth rates of *Le. gelidum* subsp. *gasicomitatum* LMG 18811T, *Lc. piscium* and *Lb. oligofermentans* grown during 11 hrs in the individual cultures depended on the type of measurement used (optical density values OD600 or colony-forming units (CFUs) /ml representing viable cell counts). Based on the measurement of the overall cell abundance (OD600), *Le. gelidum* and *Lc. piscium* had significantly higher growth rates than *Lb. oligofermentans* (Study IV: Figure 1A-D). On the other hand, according to the concentration of viable cells (Study IV: Figure 1E-H), the growth rate of *Le. gelidum* was
considerably higher than that of *Lc. piscium* and *Lb. oligofermentans*, where last two species had similar growth rates. Co-cultivation of the LAB in different combinations did not cause any clear growth promotion or inhibition for the co-culture members (Study IV: Figure 2). *Le. gelidum* clearly dominated in its both pairwise co-cultures, whereas its domination in the triple culture was seen only during a short period of time. Co-cultivation of *Le. gelidum* with the other two LAB made it to enter the stationary phase earlier.

**General characteristics of the co-cultivation-induced transcriptome responses.** To detect changes in the gene expression in response to interspecies interactions, the transcriptome profiles of the LAB grown in individual cultures were compared with their corresponding profiles obtained during growth in their co-cultures (two pairwise and one triple for each species) independently for each LAB species and time point (3, 5 and 11 h) (Study IV: Figure 3). For all the LAB the number of co-cultivation-invoked DE genes varied significantly depending on the type of co-culture and time point, and constituted maximally 12 %, 16 % and 16 % of the genomes of *Le. gelidum*, *Lc. piscium* and *Lb. oligofermentans*, respectively. Genes with high FCs (≥ 4), were detected after 11 h in *Le. gelidum* and *Lc. piscium*, and at all the time points in *Lb. oligofermentans*. The number and proportion of DE genes that were common for the interactions with two different cohabiters in the pairwise interactions and had the same direction of changes were much higher for *Lb. oligofermentans* (19-34 %), than for *Le. gelidum* and *Lc. piscium* at all the time points. However, the number and proportion of such “overlapped” DE genes in *Le. gelidum* and *Lc. piscium* increased with time being maximum at 11 h (5-7 %). Wide array of functions were affected in the LAB by their co-culturing in these LAB were “Carbohydrate transport and metabolism” (“G”), “Energy production and conversion” (“C”) and “Posttranslational modification, protein turnover and chaperones” (“O”) and “Translation, ribosomal structure and biogenesis” (“J”) (Study IV: Table 1, 2, 3).

**Carbohydrate catabolism/transport and fermentation.** In *Le. gelidum* genes, differentially expressed in the triple culture at 11 h, were highly enriched in the “G” COG category (Study IV: Table 1). Among the genes from this category there were many upregulated carbohydrate-specific catabolic/transport genes (mannose, galactose, glucosamine, sucrose, trehalose, lactose, maltose, oligo-1,6-glucosides and beta-glucosides) and seven upregulated genes from the phosphoketolase pathway. In addition, genes involved in CCC (*ccpA, ptsH* and *hprK*) were overexpressed in the triple culture. Such significant upregulation of carbohydrate catabolism genes was not observed in the pairwise culture of *Le. gelidum*. Interestingly, strong transcriptional activation and inhibition of glucosamine-degrading (*nagB*) and -synthesizing (*glmS*) enzymes, respectively, were observed in *Le. gelidum* in the presence of *Lc. piscium*, indicating of redirection of glucosamine metabolism from its biosynthesis for the cell wall formation towards its catabolism. At the same time (11 h) and in the same co-culture (triple) two pyruvate-dissipating enzymes (*pdhABCD* and *poxB*) were also upregulated.

In *Lc.piscium* the “G” COG category was enriched in the DE genes during its growth pairwise with *Le. gelidum* and in the triple culture at 11 h (Study IV: Table 2). The major part of this category was consisted of downregulated genes involved in
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carbohydrate-specific catabolism/transport (maltodextrin/maltose, mannitol, fructose, beta-glucosides and unknown sugars) and glycogen biosynthesis/degradation. Such effect was not observed in the pairwise co-culture with *Lb. oligofermentans*. Three pyruvate-dissipating enzymes (encoded by *pdhABCD*, *alsS* and *pfl*) were downregulated in all the co-cultures, however, at different times. In addition, several ATP synthase subunits were downregulated in the pairwise culture with *Lb. oligofermentans* (3 h) and in the triple culture (11 h).

Interestingly, malolactic operon (*mleSP*) was downregulated in *Le. gelidum* and *Lc. piscium* at 11 h during their growth in more than one co-culture (Study IV: Figure 4). In *Lc. piscium* this operon was also upregulated at 3-5 h in all its co-cultures.

In *Lb. oligofermentans* genes differentially expressed in all its co-cultures were enriched in “G” COG category, but at different time points (3-11 h) (Study IV: Table 3), which consisted mainly of downregulated genes involved in carbohydrate-specific catabolism/transport (glucose, fructose, mannose, gluconate, maltose, N-acetylglucosamine and xylose), nucleoside catabolism, phosphoketolase pathway and CCC (*ccpA* and *ptsH*). On opposite, ribose catabolic/transport genes became upregulated in the co-cultures at 3-5 h. The downregulation of carbohydrate catabolism in the co-cultures was coupled with downregulation of the three pyruvate-dissipating enzymes (encoded by *pdhABCD*, *ldh* and *ldhD*) at 3-5 h, upregulation of acetoin producing enzymes (encoded by *alsS* and *alsD*) at 5 h, downregulation of six NAD(P)H re-oxidation genes at different time points and three ATP synthase subunits in the triple culture at 5 h. Many of these fermentation-related genes had the same changes in the pairwise co-cultures.

**Translation.** The enrichment of *Lb. oligofermentans* DE genes during interactions with other two LAB in the “J” COG category was mostly due to upregulation at 3 h and downregulation at 11 h of ribosomal proteins in all its co-cultures (Study IV: Table 3). Enrichment of the “J” category was not that prominent during interactions of *Le. gelidum* and *Lc. piscium*. Nevertheless, in *Le. gelidum* 16-35 % of its ribosomal proteins were mildly upregulated in its pairwise co-cultures at 11 h, while during growth of *Lc. piscium* in the triple culture 23 % of its ribosomal proteins were first mildly overexpressed at 5 h and then downregulated at 11 h.

**Fatty acid biosynthesis.** In *Lc. piscium*, fatty acid biosynthesis cluster (LACPI_0829-0839) was overexpressed in all its co-cultures at 11 h, which was reflected in the enrichment of the “Lipid transport and metabolism” (“I”) COG category during *Lc. piscium* interactions (Study IV: Table 2).

**Stress protection and protein turnover.** Many genes, involved in general and oxidative stress protection as well as in protein turnover were downregulated at 3-5 h in all the LAB in response to co-culturing with other species. In *Le. gelidum* this was observed in the pairwise co-culture with *Lc. piscium*, while in *Lc. piscium* and *Lb. oligofermentans* this was a response to growth in all their co-cultures. For *Lb. oligofermentans* the later (11 h) upregulation of such genes was observed in all the co-cultures. The observed phenomena were partly reflected in the enrichment of the “O” COG category within interaction-triggered DE genes in all the LAB (Study IV: Table 1, 2, 3), but especially in *Lc. piscium* and *Lb. oligofermentans*. Noteworthy, some proteins involved in the protein turnover and

Putative antibacterial peptides and bacteriocin immunity proteins. Genes, predicted previously to be involved in production of bacteriocins and other antimicrobial substances in Le. gelidum and Lc. piscium (Study I and III), did not show differential expression during their interactions with other species. Nevertheless, two candidates for antimicrobial cationic peptides, LEGAS_1650 in Le. gelidum and LACOL_0036 in Lb. oligofermentans, that were predicted to have a positive total network charge, form alpha helices and interact with membranes, were upregulated in Le. gelidum in response to co-cultivation with Lb. oligofermentans (11 h) and in Lb. oligofermentans in response to co-cultivation in all its co-cultures (11 h), respectively. LEGAS_1650 did not have any homologs in the UniProtKB database (March, 2016), while LACOL_0036 had more than 80 homologs with uncharacterized function found only in Lactobacillus species. As for bacteriocin-immunity, OmdA domain-containing protein (LACOL_1493) (226) was upregulated in Lb. oligofermentans during growth in all the co-cultures at 11 h. In addition, antiholin-like proteins (lrgAB) (227) were highly overexpressed only in the co-culture with Le. gelidum.

Horizontal gene transfer and protection against it. Prophage genes (structural, DNA regulation or lysis-related) and RM systems (as a whole or only their restriction enzymes) were transcriptionally induced in Lc. piscium and in Lb. oligofermentans during their growth in the triple culture (in case of Lc. piscium also in the pairwise culture with Lb. oligofermentans). Prophages were upregulated at the early hours (3-5 h) in both species. Genes, involved in transformation and conjugation, were not, generally, affected by the co-cultivation in the LAB studied.

Quorum sensing. The gene involved in the biosynthesis of an autoinducer-2 (luxS) was downregulated in Le. gelidum (5-11 h) and upregulated in Lb. oligofermentans (11 h) in response to the co-culture with Lc. piscium.

Adhesins. In Lb. oligofermentans, eleven out of 22 predicted adhesins (Study II) were upregulated at least in the two co-cultures. They included eight serine rich proteins, two mucus-binding domain-containing proteins (one with LPxTG motif) and fibronectin-binding protein. Co-cultivation did not affected expression of the predicted adhesins in Le. gelidum and Lc. piscium, even though their genomes contain the same types of predicted adhesins as Lb. oligofermentans genome.
5 DISCUSSION

Microbial food spoilage is still a serious problem in the modern world, which leads to huge loss of goods and, hence, severe economical issues for the food chain operators. It has been estimated that 25% of the world’s food supply is lost due to microbial spoilage (228). Application of the MAP and cold storage allowed to inhibit significantly the growth of Gram-negative bacteria. However, these conditions favor growth of psychrotrophic LAB, which become a prevailing group of spoilage microorganisms (15, 19). Taking it into account, the need to obtain more information on psychrotrophic food-spoilage-related LAB is clear. In addition, knowledge that can be obtained for these LAB is expected to be of importance and applicable to other biotechnologically relevant LAB.

In this project, sequencing and annotation of the genomes of three psychrotrophic food-spoilage-related LAB allowed us to reconstruct their metabolism, including spoilage-related reactions, compare their genomes with the genomes of their close related species and determine putative genetic determinants facilitating their survival and competitive success in their corresponding habitats. In addition, the availability of the complete genomes allowed more accurate investigation of their transcriptome profiles, used to characterize the changes in metabolic modes under different growth conditions and interspecies interactions during co-cultivation of these LAB.

5.1 Lc. piscium (I).

So far Lc. piscium has been the only Lactococcus species with reported food spoilage activity. Moreover, this bacterium was found to belong to predominant microbiota in a variety of MAP food products (28). This study delivers the first genome sequence of Lc. piscium. Based on the comparison of gene contents of different Lactococcus species, Lc. piscium is more similar to Lc. raffinolactis, Lc. chungangensis and Lc. garvieae strains, which altogether form a separate clade (“Lc. garvieae” clade) on the pangenome tree as opposed to other two clades consisting of Lc. lactis subsp. lactis and Lc. lactis subsp. cremoris strains, respectively. While Lc. raffinolactis and Lc. chungangensis are also phylogenetically the closest species to Lc. piscium, Lc. garvieae is more phylogenetically related to Lc. lactis strains than to Lc. piscium (57). The observed difference in tree topologies could be explained by the fact that pangenome trees reflect not only evolutionary relationships or vertical gene transfer, as in phylogenetic trees, but also the horizontal gene transfer and their phenotypic differences (174). Since most of the species in the “Lc. garvieae” clade (on the pangenome tree) were isolated from animal bodies or considered to be environmental, this clustering could be a reflection of their niche adaptation. Peculiarly, unlike the majority of lactococci, Lc. piscium is predicted to be unable to degrade arginine and to respire aerobically, the two features that could be beneficial in meat environment. The presence of numerous catabolic pathways and transporters for plant-specific carbohydrates suggests a plant origin of Lc. piscium. Even
though the genome contains the enzyme for glycogen degradation (glgP), which would be beneficial in meat environment, the absence of extracellular amylase in the genome and inability to grow on glycogen suggest that glgP is meant for degradation of intracellular synthesized glycogen.

Nevertheless, the presence of several pathways/genes in the genome could potentially contribute to increased survival of Lc. piscium and its predominance in meat-derived foods. These include i) catabolic pathways for alternative carbon and energy sources in meat, such as nucleosides, aspartate, alanine and agmatine, which can be used when glucose is depleted; ii) genes encoding putative adhesins (including pili) and iii) antimicrobial factors, such as bacteriocin-encoding genes and hydrogen peroxide production from glycerol. Similarly to arginine, agmatine can be used as an alternative energy source and contributes to pH homeostasis (221). It is produced from arginine (which is abundant in meat) by arginine decarboxylase-containing bacteria (229) and naturally present in meat (230, 231). Even though genes for two types of pili, LPxTG-like and type IV, were present in the genome, their low RPKM values and absence of pilus structures and twitching motility indicate that both types of pili are not expressed under the examined conditions. However, the result might be different for the “real” habitat of the isolate, which was broiler meat.

The main spoilage activity of Lc. piscium was described to be the production of buttery and sour off-odor compounds (diacetyl and, to a much lesser extent, acetoin) (57). The concentration of acetoin/diacetyl (8.5 mM) produced by Lc. piscium after 48 h of aerobic growth with glucose is comparable to that produced by Lc. lactis subsp. lactis bv. diacetylactis strains after 25 h of aerobic growth (232). The latter LAB are efficient producers of these aroma compounds such as diacetyl and acetoin and, therefore, are used in cheese manufacturing. Efficient acetoin/diacetyl production occurs when pyruvate excess is created due to either production of pyruvate without generation of reduced NADH (e.g., degradation of citrate, aspartate, or alanine) or sparing pyruvate from reduction to lactate in the presence of additional electron acceptors (e.g., oxygen) (1, 102). Interestingly, among lactococci genomes the citrate catabolic operon was present only in Lc. piscium and Lc. lactis subsp. lactis bv. diacetylactis strains (IL1403 is a plasmid-free derivative of a diacetylactis strain) (233). Fermentation of citrate was shown to increase production of buttery off-odor compounds in diacetylactis strains (232). Nevertheless, the absence of citrate utilization and the very low expression levels of the citrate catabolic operon indicate that citrate does not contribute to acetoin/diacetyl production in Lc. piscium. On the other hand, the presence of aspartate/alanine aminotransferases and glutamate dehydrogenase makes Lc. piscium (as well as Lc. chungangensis and Lc. raffinolactis) a potentially good producer of acetoin/diacetyl from these amino acids, which does not require exogenous alpha-ketoglutarate to be added to the medium whereas other lactococci do (234). Finally, oxygen dependent increase of acetoin/diacetyl production was previously reported in Lc. lactis (232, 235) and also observed in Lc. piscium. Such phenomenon could be mediated by two NADH oxidases found in the genome with NoxE assumed to be the major enzyme involved. We also showed that Lc. piscium can grow on glycerol as the sole carbon source with concomitant production of acetoin/diacetyl. Since glycerol is present in meat (75, 103) as a product of triacylglycerol degradation, we speculate that it might be utilized by Lc. piscium as an alternative carbon source in meat, which leads to the formation of buttery off-odors.
Comparison of *Lc. piscium* gene expression across three time points revealed upregulation of the carbohydrate catabolic pathways that can be explained by the relief of CCR (94) occurring as a result of decrease in glucose (preferred carbon source) concentration with time. Catabolic operons for agmatine and glycerol, which were shown previously to be subjects to CCR (236, 237), were also upregulated. Due to the absence of alternative carbohydrates in the medium (other than glucose) the upregulation of CCR-regulated genes seems to be caused solely by the low concentration of glucose. At the same time the gene expression changes of pyruvate dissipating enzymes indicate that pyruvate fermentation switched from the lactate dehydrogenase pathway to the acetyl-CoA generation pathways (pyruvate-formate lyase and pyruvate dehydrogenase pathways) in the late hours of growth. The switch to pyruvate formate lyase fermentation is known to occur in other homofermentative LAB under substrate limitation, since it results in higher yield of ATP per glucose molecule (1, 11). On the other hand, upregulation of the pyruvate dehydrogenase is peculiar, since it consumes NAD+ and, therefore, does not lead to increase of ATP yield in comparison with lactate dehydrogenase fermentation. The increased conversion of pyruvate to acetyl-CoA could possibly make bacterial metabolism more flexible and efficient under glucose limitation conditions since acetyl-CoA can be used not only for NADH reoxidation, but also for energy production and biosynthesis of amino acids and fatty acids. Consistent with the decrease of lactate dehydrogenase expression, aldehyde-alcohol dehydrogenase becomes upregulated during the late hours of growth, taking over the role of NAD+ regeneration. In addition, upregulation of both diacetyl reductases during the whole time would lead to additional NADH reoxidation (could possibly work in connection with pyruvate dehydrogenase to increase its ATP yield) and increased conversion of diacetyl to acetoin which has a significantly less powerful aroma than diacetyl and, hence, is more preferable (234).

5.2 *Lb. oligofermentans* (II).

*Lb. oligofermentans* was repeatedly isolated from MAP meat products at the late shelf life or spoilage stage (68–70). Nevertheless, its contribution to spoilage is unknown. Opposite to *Le. gelidum* and *Lc. piscium*, this bacterium was reported to grow poorly on glucose (68), which is the most abundant carbohydrate in meat (75). As other obligate heterofermentative LAB it prefers fermentation of pentoses (67, 86), which are scarce in meat (74). Therefore, it is not clear how *Lb. oligofermentans* can compete, for example, with the other two LAB. In addition, regulation of carbohydrate catabolism in obligate heterofermentative LAB is not well studied in general. This makes *Lb. oligofermentans* an interesting object to study its carbohydrate catabolic capabilities in more details as well as the regulation of carbohydrate catabolism. This study provides the second complete genome and the first analysis of transcriptome response to growth with different carbohydrates for a member of *Lb. vaccinostercus* group, complemented with the carbohydrate utilization profiles.

Even though on initial isolation *Lb. oligofermentans* utilized efficiently only pentoses (68), the genome was predicted to have catabolic pathways for multitude of
different carbohydrates (including several for hexoses), indicating that efficient catabolism of other carbohydrates might occur under certain conditions, such as presence of electron acceptors. In fact, our results and the previous study (77) showed that this bacterium has adapted with time to fermentation of a variety of carbon sources, including hexoses and maltose. This was, probably, induced by its long-term cultivation in the glucose-containing medium as it was shown for *Lb. vaccinostercus* (76) indicating that members of *Lb. vaccinostercus* have flexible carbohydrate catabolism, which can be adjusted, depending on the carbohydrates available in the environment. This ability can be beneficial in meat environment with limited amount and variety of carbohydrates. Among the tested candidates for electron acceptors, only pyruvate promoted significantly growth of *Lb. oligofermentans* on the primary carbon source (xylose) and increased production of acetoin/diacetyl, indicating that it might act as an electron acceptor. Therefore, it can be speculated, that pyruvate contained in meat could possibly facilitate catabolism of carbohydrates, present in meat (such as hexoses). Pyruvate can also be used as carbon source for growth by some meat spoilage bacteria (238). Nevertheless, our results show that pyruvate can only poorly support growth of *Lb. oligofermentans*. Potentially, oxygen could be used as an electron acceptor in the reactions mediated by the NADH oxidase or the respiratory electron transport chain. Nevertheless, aerobic atmosphere had an inhibitory effect on *Lb. oligofermentans* growth and no evidence was observed for the functional respiration ability in this bacterium. Peculiarly, the oxygen-dependent inhibitory effect was more pronounced during growth on pentoses than on glucose. This might possibly be explained by the glucose induced overexpression of the aldehyde-alcohol dehydrogenase gene *adhE* (and possibly of the other alcohol dehydrogenases), since AdhE was shown to possess an antioxidant activity in *Escherichia coli* (239). The ability to catabolize efficiently inosine (and possibly other nucleosides), which provide a source of carbon in the form of ribose, may also offer a competitive advantage to *Lb. oligofermentans* in meat environment as was proposed for *Lb. sakei* (75, 101). Nucleoside catabolic genes, including *deoDBC*, were induced by growth on ribose in comparison with glucose in facultative heterofermentative *Lb. sakei* (240). The opposite situation was observed for *Lb. oligofermentans*. The reason behind it is not clear.

Higher similarity of glucose and xylose transcriptome profiles in comparison with ribose transcriptome in the beginning of the exponential growth phase was unexpected due to higher biochemical similarity between xylose and ribose, which are both pentoses and enter the phosphoketolase pathway almost at the same level without additional oxidation steps, which are required for glucose. The observed cross-induction of hexose and xylose catabolic/transport genes by either glucose or xylose could be an ancient adaptation of *Lb. oligofermentans* to plant environment, which is assumed to be the original habitat for the *Lb. vaccinostercus* group. Plant cell walls contain 30-55 % of hemicellulose, which consists mainly of glucose and xylose moieties (241) and, hence, both glucose and xylose catabolic pathways are required for the hemicellulose utilization. Therefore, it can be speculated that synergistic induction of these pathways by both glucose and xylose would lead to more efficient fermentation of hemicellulose. The change in the similarity-based relationships of transcriptomes at the end of the exponential growth phase might be caused by the depletion of carbohydrates in the media. We did not observe glucose-induced CCR in *Lb. oligofermentans*, as seen, for instance, in *Lb. sakei* (240). Conversely, as already noted, catabolic pathways for hexoses and xylose were higher
expressed on glucose in comparison with ribose. A similar effect was observed for disaccharide/hexose transporters in *Le. gelidum* subsp. *gasicomitatum* (51), another obligate heterofermentative bacterium. These observations indicate that there is a significant difference in transcriptional regulation of carbohydrate catabolism in obligate and facultative heterofermentative bacteria. Taking into account the detected significant enrichment of CcpA-like TFBSs in front of the genes upregulated on both glucose and xylose at 20 h, the above mentioned similarity in glucose and xylose transcriptome responses could be governed by the CcpA regulator. However, since most of the genes, predicted to be regulated by CcpA, were not upregulated neither on glucose nor xylose, the exact mechanism of CcpA regulation in *Lb. oligofermentans* remains unclear and needs further studies. Nevertheless, the variety of observed expression patterns within CcpA-containing genes could possibly be explained by the interplay with other regulators as well as the dual activating/repressive nature of CcpA (94). The complete overlap of predicted CcpA- and RbsR-binding sites in front of the HPr gene is peculiar since both regulators, CcpA and RbsR, were shown to be able to interact with HPr-Ser46-P and the formed complexes can bind DNA, containing the corresponding binding sites (242). In this case, CcpA and RbsR would compete not only for HPr-Ser46-P, but also for the binding site upstream the HPr gene, suggesting the putative involvement of RbsR in CCC along with CcpA in *Lb. oligofermentans*.

The extreme upregulation of the alcohol dehydrogenases (including *adhE*) and other NAD(P)H re-oxidizing enzymes on the medium containing glucose instead of pentoses is explained by the need to restore cell redox balance in terms of NAD(P)+/NAD(P)H ratio, which is decreased during glucose catabolism through the phosphoketolase pathway that requires additional oxidation compared to pentose catabolism (1). This is supported by the finding that the upstream regions of these glucose-induced NAD(P)H re-oxidizing enzymes contained a motif similar to the binding site of a Rex transcriptional repressor, which is shown to negatively regulate the expression of genes, such as involved in respiration, fermentation and central carbohydrate metabolism, in response to high NAD(P)+/NAD(P)H levels (243–247). Instead, the decrease of NAD(P)+/NAD(P)H levels, created, for example, when glucose is fermented instead of pentoses, leads to derepression and, hence, upregulation of Rex-regulated genes, which eventually results in the restoration of the redox balance (243–245). Both lactate dehydrogenases did not show any significant upregulation in response to glucose fermentation and were not predicted to be Rex-regulated. This indicates that activation of the ethanol branch and other NAD(P)H re-oxidizing enzymes, and not lactate dehydrogenase pathway, is the main mechanism of adaptation to hexose fermentation in *Lb. oligofermentans*. In turn, in *Lb. sakei*, which catabolize glucose through glycolysis, no induction of *adhE* was observed during growth on glucose instead of ribose (240). The Rex regulation mechanism was experimentally confirmed only in few model species of Gram-positive bacteria (243–247). Nevertheless, Rex regulons were predicted to be widespread in six phyla of Gram-positive bacteria, including Firmicutes (225). Six genes that were significantly upregulated during growth on glucose and predicted to be Rex regulated in this study (*adhE*, *adhA*, *nox*, *butA2*, *dhaT* and *gldA*), were also predicted to be part of Rex regulons in other *Lactobacillaceae* (225). Therefore, our results implicitly confirm the previously computationally predicted Rex-dependent regulation of redox balance in *Lactobacillaceae*.
The absence of additional oxidative steps during pentose catabolism lessens the necessity of NAD(P)H re-oxidation, which results in creation of pyruvate and acetyl-phosphate surplus and, hence, increased generation of acetoin/diacetyl and acetate (1). This explains the higher expression levels of the acetoin/diacetyl and acetate production pathways during growth on pentoses comparing to glucose in *Lb. oligofermentans*. Similar results were shown for *Lb. sakei* (240).

5.3 “Vegetable” strains of *Le. gelidum* subsp. *gasicomitatum* (III)

*Le. gelidum* was found to be a predominant spoilage LAB in a variety of food products (28). Interestingly, strains isolated from meat- and vegetable derived foods did not almost overlap, which could be due to i) their niche specificity or ii) the absence of strain dissemination between meat- and vegetable-processing chains (42, 47). The study presents a comparison of seven newly sequenced genomes of “vegetable” strains of *Le. gelidum* subsp. *gasicomitatum* with publicly available genomes of other leuconostocs, including one “meat” strain of *Le. gelidum* subsp. *gasicomitatum* (49). No major differences were observed between the meat- and vegetable-isolated strains of *Le. gelidum* subsp. *gasicomitatum* based on their gene contents that would explain their niche specificity. Therefore, strain segregation between meat and vegetable-based food products is more likely to be explained by the absence of strain dissemination between meat- and vegetable-processing chains. Nevertheless, the galactan catabolic gene cluster found in four “vegetable” strains, but not in a meat strain, could provide a survival advantage to these strains in plant environment, since galactan is a structural polysaccharide constituting pectin, one of the major components of plant cell walls (241). Several genes/gene clusters found to be *Le. gelidum* (subsp. *gasicomitatum*) -specific (present in almost all *Le. gelidum* or *Le. gelidum* subsp. *gasicomitatum* genomes, but only in few genomes of other leuconostocs) could contribute to their survival and predominance in food microbial communities. These include a biosynthesis cluster for an unknown polyketide and putative adhesion-related genes for collagen-binding protein (*can*), polysaccharide adhesin biosynthesis (*icaAB*), mucus-binding protein and accessory Sec system, surrounded by serine-rich proteins. Polyketides are bioactive compounds that can exhibit antibacterial activity (248). Genes *can* and *icaAB* were speculated to mediate adhesion and better survival in meat environment of a “meat” strain LMG 18811\(^{T}\) (49). Our analysis shows that they are also present in “vegetable” strains. Accessory Sec system was shown to be involved in the export and glycosylation of serine-rich adhesins (249), therefore, serine-rich proteins found in the vicinity of this system are potential adhesins. Strain KG16-1 is peculiar in a sense that it misses several genes that are present in other *Le. gelidum* genomes, including *can*, *icaAB*, xylose transporter (*xylT*), pyruvate oxidase (*poxB*) and polyketide biosynthesis cluster. In turn, it harbors a biosynthesis cluster for lacticin-481 type bacteriocin, shown to be active against LAB (250), which is present only in one other *Le. gelidum* subsp. *gasicomitatum* strain.
Finally, the comparative genomic analysis with subsequent phylogenetic analysis allowed to revise a taxonomical status of three *Leuconostoc* genomes. This shows importance of a proper phylogenetic analyses that should accompany each genome submission/announcement to avoid assignment of the genomes to wrong species.

5.4 LAB interspecies interactions (IV)

The food-spoilage-associated LAB species investigated in the first three studies and described above constitute parts of MAP food spoilage microbial communities consisting of several bacterial (mostly LAB) species. Spoilage is caused by the activity of a whole microbial community, and this activity is determined by the species composition and interactions between different species. To get insights into system level interactions between food spoilage LAB and, potentially, to expand this knowledge for LAB in general, we chose to study transcriptome responses of the above described species, *Le. gelidum* subsp. *gasicomitatum* LMG 18811^T^, *Lc. piscium* MKFS47 and *Lb. oligofermentans* LMG 22743^T^, during their co-cultivation pairwise and in the triple culture. These species have different modes of carbohydrate catabolism as well as different roles and relative abundances in the spoilage microbial community (49) (Study I and II). The particular strains of these species were isolated from MAP broiler products (25, 40, 68) and, therefore, share the same ecological niche.

During growth of the LAB in individual and mixed cultures, *Le. gelidum* had the highest growth rate in terms of the viable cell counts than other two LAB, *Lc. piscium* and *Lb. oligofermentans*, which had comparable growth rates. However, based on measurements of OD\(_{600}\), growth of the total biomass was the highest for *Lc. piscium*. The difference in relative growth rates of *Lc. piscium* obtained using different measurements (OD\(_{600}\) values or viable cell counts CFUs/ml) might indicate that large proportion of its cell population is not dividing and can consist of dormant or dead cells. Alternatively, it could suggest that the relation between the sample optical density and overall cell concentration for *Lc. piscium* differ significanly from that in the other two LAB. It can be also speculated, that even though the growth rates of *Lc. piscium* and *Lb. oligofermentans* were comparable in terms of viable cell counts, the consumption of glucose could be higher for *Lc. piscium* taking into account its faster growth of the total biomass. Co-cultivation of the three LAB in the mixed cultures did not lead to their growth promotion or clear growth inhibition. Although no clear evidence of competition-like relationships between these LAB was observed, generally, different species are predicted to develop competition (both exploitative and interference) during co-cultivation, and much more rare mutualistic relationships (116).

Co-cultivation of the LAB significantly affected their gene expression with up to 12-16 % of their genes being DE. The higher number of the “overlapped” DE genes (DE genes that are common for both pairwise interactions) in *Lb. oligofermentans* than in *Le. gelidum* and *Lc. piscium* at all time points might be a consequence of higher consistency of *Lb. oligofermentans* data, obtained during one batch of experiments. Alternatively, this might indicate that *Lb. oligofermentans* responded more universally to the presence of two
different species *Le. gelidum* and *Lc. piscium*. The last two species seem to develop more uniform response with time. Co-cultivation of the LAB affected wide array of their functions, which can be explained by the fact that the presence of other species can create different kinds of stresses (nutrient limitation, acidification, oxidative stress etc.) through utilization of the same resources or release of different substances (116).

The interspecies interactions strongly affected the carbohydrate catabolism/transport and fermentation pathways in all three LAB (Study IV: Figure 4). Under glucose-limitation conditions (11 h) and in the presence of both other LAB in the culture *Le. gelidum* seemed to enhance its nutrient-scavenging and fermentation capabilities by upregulation of the carbohydrate catabolic/transport genes and two pyruvate-dissipating enzymes. Such response might give a competitive advantage to *Le. gelidum* under nutrient limitation conditions and explain its ability to outgrow other bacteria from the small initial cell counts and dominate in a variety of food products (27). The absence of such response in the pairwise cultures of *Le. gelidum* might indicate that the factors generated by both, *Lc. piscium* and *Lb. oligofermentans*, are required for induction of such response. The coordinated up- and downregulation of glucosamine-6-phosphate degradation and biosynthesis enzymes, respectively, in *Le. gelidum* during growth with *Lc. piscium* was similar to that obtained in *E. coli* in response to growth on amino sugars (251). This suggests that *Lc. piscium* might provide a source of amino sugars to *Le. gelidum*, which could possibly be a peptidoglycan of the non-dividing fraction of *Lc. piscium* cells that presumably can contain dead cells. Conversely, interspecies interactions caused downregulation of the carbohydrate catabolic/transport and fermentation/energy producing pathways in two slower growing LAB, *Lc. piscium* (3-11 h) and *Lb. oligofermentans* (3-5 h). The drop in glucose concentration in the presence of presumably faster glucose-consuming bacteria, *Le. gelidum* and *Lc. piscium*, could explain the downregulation of carbohydrate catabolism in *Lb. oligofermentans* in all its co-cultures. However, it would not explain the same phenomenon in *Lc. piscium* in the presence of *Le. gelidum*, since *Lc. piscium* possesses CCR regulation mechanism of carbohydrate catabolism, which would upregulate the catabolism of different carbohydrates with decrease of glucose concentration (Study I). Therefore, this effect could be a result of the other interaction mechanism, occurring between *Le. gelidum* and *Lc. piscium*.

Transcriptional regulation of the ribosomal proteins, the expression of which was shown to be positively correlated with growth rate in bacteria (252, 253), during interspecies interactions at 11 h was generally consistent with expression changes of carbohydrate catabolism and fermentation pathways in the LAB (Study IV: Figure 4). Therefore, it seems that at the late hours (glucose limitation condition) *Le. gelidum* attempted to enhance its growth capabilities in response to growth with other LAB, while opposite is true for *Lc. piscium* and *Lb. oligofermentans*.

The early (3-5 h) downregulation of stress (mainly oxidative) protection related and protein turnover genes in all the LAB in response to co-culturing (Study IV: Figure 4) is peculiar taking into account that the presence of different species usually creates different kinds of stresses. The possibility that co-cultivation leads to alleviation of certain stresses after a short time of interactions is interesting and it requires further studies. Interestingly, downregulation of stress related and protein turnover genes was observed in *Lb. casei* in response to acid stress (254). But its role was not clarified. Other expression changes in *Lc. piscium* in response to growth in all its co-cultures, such as upregulation of
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the malo-lactic operon (3-5 h) and fatty acid biosynthesis (11 h), were also similar to that obtained for \textit{Lb. casei} after its exposure to acid stress. Malo-lactic fermentation leads to increased pH and provides additional energy (254–256), while fatty acid biosynthesis allows to adjust membrane fluidity to acidic environment (254). Together these findings might suggest that two other LAB create an acid stress-like condition for \textit{Lc. piscium}.

To inhibit growth of competitors, LAB can release bacteriocins that are very diverse antimicrobial peptides. They share little homology, but often contain a positive network charge allowing them to permeabilize bacterial membranes and eventually disrupt them (109, 257). They are difficult to predict based on homology and \textit{ab initio}. Since they are released to harm competitors, investigation of bacterial transcriptional responses during their co-cultivation provides an opportunity to discover novel putative bacteriocins and to support computational prediction of bacteriocins. In this study two such bacteriocin candidates, LEGAS\_1650 in \textit{Le. gelidum} and LACOL\_0036 in \textit{Lb. oligofermentans}, were proposed based on their overexpression in the co-cultures and predicted physico-chemical properties. The presence of LACOL\_0036 in many \textit{Lactobacillus} species and its overexpression in all the co-cultures of \textit{Lb. oligofermentans} (11 h) suggest that this peptide can be a competitor non-specific bacteriocin of lactobacilli. In turn, the upregulation of LEGAS\_1650 was interaction specific (only pairwise with \textit{Lb. oligofermentans}). For self-protection, bacteriocin-producing bacteria express corresponding bacteriocin-immunity proteins (258). One such putative bacteriocin-immunity protein (LACOL\_1493) in \textit{Lb. oligofermentans} could possibly mediate self-protection from the above mentioned cationic peptide LACOL\_0036, since it was also upregulated in all the co-cultures at 11 h.

Finally, interspecies interactions of LAB affected expression of their autoinducer-2 biosynthetic enzyme (\textit{luxS}), putative adhesins and genes involved in HGT and protection against it. Quorum sensing is a mechanism of bacterial interaction and environmental sensing, mediated by the autoinducer-2, which is released and accumulated in the environment during bacterial growth (259). High concentrations of autoinducer-2 regulate many important processes (259, 260), of which spoilage activities and secretion of virulence factors are of interest for food preservation and safety. Our study suggests that bacterial interspecies interactions could affect the amount of autoinducer-2 produced by different species through transcriptional regulation of \textit{luxS} gene. In addition, the same species (\textit{Lc. piscium}) can cause opposite regulation (up- and down-) of \textit{luxS} depending on the species affected. Adhesion allows bacteria to persist in the environment, which could give a competitive advantage. Co-cultivation-induced upregulation of many putative adhesins in \textit{Lb. oligofermentans} (serine-rich, fibronectin-binding and mucus-binding domain-containing proteins) might indicate that adhesion could be an important survival mechanism for \textit{Lb. oligofermentans}, explaining its quite high abundancy (10-18 %) in some food spoilage microbial communities despite the low growth rate (68). Exchange of genetic information between different species occupying the same environment allows to increase ecological fitness of a bacterial community and is, therefore, beneficial for the community as a whole. However, it could be harmful for the individual bacteria (261). Therefore, both mechanisms, for HGT and protection against it, exist in bacteria. Co-cultivation of LAB (Study IV: Figure 4) induced prophages (one of the mechanisms of HGT) (262) and RM systems (innate immune system against foreign DNA entry) (262) in \textit{Lc. piscium} and \textit{Lb. oligofermentans}, but not in \textit{Le. gelidum}. Upregulation of prophages occurred in the two LAB only or more strongly in the triple culture, which might indicate
that induction of prophages in these two species is a cumulative effect invoked by the presence of more than one species. Prophages are known to be induced in response to different stresses (starvation, heat and oxidative stress, DNA damage) and their upregulation was also observed during co-cultivation of soil bacteria (263).
6 CONCLUSIONS

Studies included into the dissertation provide new genomic and transcriptomic information on the psychrotrophic food-spoilage-associated LAB. Sequenced and annotated genomes of *Lc. piscium*, *Lb. oligofermentans* and seven “vegetable” strains of *Le. gelidum* subsp. *gasicomitatum* provide a foundation for further “omics” studies of these species. The analysis of their gene contents allowed to predict factors (e.g. catabolism of niche-specific carbohydrates/carbon sources, adhesins and bacteriocins) that might facilitate their survival and give them competitive advantage in the spoilage microbial communities.

*Le. piscium* MKFS47 was identified as an efficient producer of acetoin/diacetyl from glucose under aerobic conditions, which is in agreement with the previously reported ability of this species to cause formation of buttery off-odors in food products. Aerobic glycerol-based growth of *Lc. piscium* was proposed to facilitate its better survival and contribute to formation of buttery off-odors and discoloration of meat. Time course glucose catabolism-based transcriptome profiles revealed the presence of classical carbon catabolite repression mechanism for the regulation of carbohydrate catabolism, which was relieved along with decreasing concentration of glucose. During the same time the shift from homolactic to heterolactic fermentation mode was observed.

For *Lb. oligofermentans* the induction of efficient utilization of hexoses was confirmed indicating that it has flexible carbohydrate catabolism that can be adjusted depending on the carbohydrate sources available in the environment. Unexpected higher similarity of glucose and xylose transcriptomes in comparison to ribose transcriptome in the early exponential growth phase as well as cross-induction of glucose and xylose catabolic genes by either glucose or xylose in this obligate heterofermentative LAB could be governed by the CcpA-dependent regulation mechanism. This phenomenon can be a result of its ancient adaptation to the original habitat for this bacterium (presumably plants), which is particularly rich with both glucose and xylose. Rex transcriptional regulator was indirectly confirmed to be involved in cell redox balance maintenance in *Lb. oligofermentans*, which is an agreement with the previous computational prediction for the *Lactobacillaceae* family. Generally, the transcriptome data suggested that there are considerable differences in carbohydrate catabolism regulation between the obligate and facultative heterofermentative LAB.

No major differences in gene contents between the “vegetable” and “meat” strains of *Le. gelidum* subsp. *gasicomitatum* were observed that would suggest their niche-specificity indicating that the absence of strain dissemination between vegetable- and meat-processing chains is a more likely factor explaining the strain segregation between vegetable and meat-based food products.

During co-cultivation, three above mentioned LAB species employed different strategies to cope with the competition. The fastest growing species, *Le. gelidum*, seemed to enhance its nutrient- (mainly carbohydrates) scavenging and growth capabilities (expression of ribosomal proteins) under glucose limitation conditions. The opposite was observed for the slower growing LAB, *Lc. piscium* and *Lb. oligofermentans*. Such behavior might explain the predominance of *Le. gelidum* in spoilage microbial communities in a variety of food products. The clear difference between the co-cultivation
induced transcriptome responses of *Le. gelidum* and the two other LAB is further emphasized by the fact that interspecies interactions induced expression of prophages and RM systems in *Lc. piscium* and *Lb. oligofermentans*, but not in *Le. gelidum*. Downregulation of stress protection-related genes in all the LAB at the early growth stage was unexpected, and it requires further studies to understand the role of such phenomenon during interspecies interactions. Finally, overexpression of the numerous putative adhesins in *Lb. oligofermentans* during growth with other LAB could be one of the factors explaining its survival in actively growing co-cultures in meat despite its relatively slow growth rate on glucose.

The extensive and comprehensive knowledge obtained during these studies on food-spoilage-related LAB would be expected to be of importance and applicable to other biotechnologically relevant LAB.
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