Characterization of lifestyle-associated metabolic traits of host-adapted lactobacilli that are relevant in food fermentations

by

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Abstract

Lactobacilli are present in a majority of food fermentations, and the fermentation organisms of different fermented foods are recruited from free-living, host-adapted, and nomadic lactobacilli. The metabolic traits of host-adapted lactobacilli relate to their ecological success in their vertebrate or insect hosts and also facilitate the ecological fitness of host-adapted lactobacilli in fermented foods. The corresponding metabolites contribute to the flavor, structure, and quality of fermented food. This dissertation aimed to characterize glutaminase, and extracellular fructanase and arabinanases that are lifestyle-associated and exclusively presence in host-adapted lactobacilli.

Acid resistance is essential for the competitiveness and persistence of host-adapted lactobacilli in fermented food and in host habitats. Glutaminase contributes to acid resistance of *Limosilactobacillus reuteri* at pH 2.5 but not pH 3.5 but its specific contribution to glutamate and GABA accumulation was not elucidated. Experimentation with *L. reuteri* derivative with deletions of three *gls* genes demonstrated that glutaminases are not required for deamidation of glutamine in cereal substrates. Moreover, the presence of *gls-gadB / C* operon is not a requirement for acid resistance mediated by glutaminase.

The sourdough isolate *Lactobacillus crispatus* DSM29598 harbors the putative extracellular fructosidase FruA and the putative arabinanases AbnA and AbnB. Surface layer protein domains at the C-terminor of FruA and AbnA indicate their association with the cell wall. FruA degraded inulin and levan to produce fructose as end products, and the activity was not repressed by glucose. The application of conventional sourdough in bread making substantially reduced fructan in bread, improving the tolerance in healthy individuals; sourdough fermentation with FruA-expressing *L. crispatus* reduced fructans in bread by more than 90% to produce a low-FODMAP bread.

Extracellular arabinanases allowed the utilization of linear arabinan as the carbon source for the growth of *L. crispatus.* Two arabinanases, AbnA and AbnB, are located at the same arabinan utilization operon encoding multiple arabinan and arabinose metabolic enzymes. Both arabinanases and the operon are exceptional in *L. crispatus* as strains of this species are generally unable to use arabinose as carbon source. The characterization of AbnA and AbnB demonstrated that both enzymes hydrolyzed linear arabinan. AbnA also degraded branched arabinan.

This dissertation provides basic knowledge on lifestyle-associated metabolic traits of host-adapted lactobacilli related to acid resistance and extracellular polysaccharide hydrolysis, which is essential to guide the application of lactobacilli in food fermentation or as probiotics, and to explore their impact on host health.

Preface

This thesis is an original work by Qing Li, written according to FGSR thesis formatting requirements.

Chapter 2 has been published as Qing Li and Michael G Gänzle (2020), "Host-adapted lactobacilli in food fermentations: Impact of metabolic traits of host-adapted lactobacilli on food quality and human health". *Current Opinion in Food Science*, 31, 71–80. I reviewed the literature and collected the related information. The manuscript was written by me with the guidance of Dr. Gänzle.

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Chapter 5 is submitted as Qing Li, Michael G. Gänzle, "Characterization of AbnA and AbnB, two extracellular arabinanases in *Lactobacillus crispatus*". *Applied Microbiology and biotechnology.* I designed the experiments with suggestions from Dr. Gänzle. I accomplished

all the laboratory work and data analysis, as well as phylogenetic analysis and manuscript writing with Dr. Gänzle.

Dedication

I would like to dedicate this Ph.D. thesis to my beloved family. Thanks for your love and support in my life.

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ADI	Arginine deiminase
BLASTp	Protein basic local alignment search tool
CE	Collision energy
DP	Degree of polymerization
DPt	Declustering potential
EPS	Extracellular polysaccharides
FODMAPs	Fermentable oligo-, di-, mono-saccharides and polyols
FOS	Fructooligosaccharides
GABA	γ-Aminobutyric acid
GadB	Glutamine decarboxylase
GadC	Glutamate / GABA antiporter
Gls	Glutaminase
HePS	Heteropolysaccharides
HoPS	Homopolysaccharides
HPAEC-PAD	High-performance anion-exchange chromatography with pulsed amperometric detection
HPLC	High performance liquid chromatograph
IBS	Irritable bowel syndrome
LB	Luria-Bertani
mMRS	Modified deMan-Rogosa-Sharpe
NCBI	National center for biotechnology information

ORF	Open reading frame
PCR	Polymerase chain reaction
ScrP	Sucrose phosphorylase
SLAP	Surface layer protein
RAST	Rapid annotations using subsystems technology

Chapter 1 General introduction and thesis objectives

1.1 Introduction

Lactic acid bacteria (LAB) describes a group of gram-positive, nonsporing, nonrespiring, rod or sphere-shaped bacteria that produce lactic acid as the major end metabolite of carbohydrate fermentation [1]. LAB generally populate habitats rich in nutrients, including foods, feeds, plants, soil or aqueous environments, and vertebrate and invertebrate hosts [2]. Food fermentation with LAB preserves fresh raw material via accumulation of antimicrobial metabolites which prevents food spoilage and the growth of pathogens, and improves desirable flavors, sensory attributes, and the beneficial effects on human health [3–7]. For example, *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* are persistently present in yogurt fermentation [8]. *Lactococcus*, *Leuconostoc*, *Weissella*, *Enterococcus*, and lactobacilli are generally present during vegetable fermentation processes [9]. Lactic fermentation of milk and vegetables achieves a longer storage life compared with raw materials. *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* in yogurt fermentation serve as an exogenous source of β-galactosidase for lactose-intolerant populations to hydrolyze lactose, which allows lactose-intolerant individuals to obtain calcium, vitamins, and high quality protein in milk without gastrointestinal symptoms [3,8]. *Lactobacillaceae* species are frequently associated with cereal fermentations including East-Asian liquor, vinegar fermentation, and sourdough fermentation [8,10]; these fermentation processes dramatically changed the limited functionality of cereal flour from making flat bread to developing diverse products [3,8].

The term “lactobacilli” designates all organisms that were classified as *Lactobacillaceae* until 2020, representing a major part of food fermenting lactic acid bacteria [11,12]. To

explore the evolutionary properties of lactobacilli, phylogenetic analysis and the average nucleotide identity have split all the species into 24 phylogenetic groups [13]. New taxonomy reclassified the genus *Lactobacillus* into 25 genera; comprising an emended genus *Lactobacillus* (previously: *Lactobacillus delbrueckii* group), *Paralactobacillus* and 23 new genera that previously included in the genus *Lactobacillus*. The emended description of the family *Lactobacillaceae* includes all genera that were previously included in families *Lactobacillaceae* and *Leuconostocaceae* [11]. Combined with isolation source, metabolic capabilities, growth temperature, and the resistance to environmental stressors of given habitats, lactobacilli have been assigned into three main lifestyle categories: free-living, host-adapted, or nomadic [2]. Free-living lactobacilli are associated with environmental and plant habitats, possessing large genome sizes that maintain their metabolic versatility and efficiency by encoding various enzymes to utilize a wide spectrum of substrates [13]. Host-adapted lactobacilli with small genome sizes are relevant to vertebrate and insect-host habitats, where they obtain stable food sources from the diet of their hosts, thereby they only maintain metabolic abilities that match their available nutrients [2]. The reduction of genome size as an evolutionary process reflects the adaptation of lactobacilli to host niches and is closely correlated with the degree of host specialization [14]. Nomadic lactobacilli possess large genomes as free-living lactobacilli without specialization to particular habitats and are found in vertebrate and invertebrate-hosts, and in multiple food materials [2]. The evolution model of lifestyles in lactobacilli revealed that, from free-living ancestry, host-adapted lactobacilli have experienced a substantial loss of functions in carbohydrate metabolism, amino acid, and cofactor biosynthesis via gene decay [2,15].

The metabolic pathways of host-adapted lactobacilli reflect their adaptation to host habitats and contribute to their ecological success by withstanding environmental stressors [2,10]. Acid resistance systems help host-adapted lactobacilli survive low pH habitats, such as the

gastric environment [16]. These systems modulate intracellular pH by reducing intracellular protons by ATPase and amino acid decarboxylation, production of alkali via amino acid deamination or hydrolysis of urea, as well as modification of cell membrane [17]. Cyclopropane-fatty-acyl-phospholipid synthase converts unsaturated fatty acids in phospholipids into cyclopropane fatty acids. Increased membrane cyclopropane fatty acid content may enhance the acid tolerance of *Escherichia coli* and *Levilactobacillus brevis* (previously *Lactobacillus brevis* group [11]) [18,19]. The arginine deiminase (ADI) pathway restores the optimal intracellular pH through ammonia production and proton consumption. In addition, the extra energy (ATP) produced via the ADI pathway enables the export of cytoplasmic protons by F_0F_1 ATPase [20,21]. The occurrence of agmatine deiminase pathway in lactobacilli is correlated with arginine deiminase and agmatine deiminase pathway contributes to acid resistance via ammonia and putrescine production [13,22,23]. Glutamate decarboxylase maintains pH homeostasis by consuming intercellular protons and electrogenic antiport of metabolites [21,24]. Glutamine deamidation also protects *Limosilactobacillus reuteri* from acidic stress [21,25], and the protective ability of glutaminase from extreme acid stress has been demonstrated in *E. coli* and *Brucella microti* [25,26]. Moreover, glutamine is the most abundant amino acid released from cereal proteins during sourdough fermentation [27,28], and glutamine deamidation accumulates glutamate, relevant to umami flavor [29]. However, the specific contribution of glutaminase to glutamine-mediated acid resistance and metabolite accumulation has not been described in lactobacilli.

Polysaccharide degradation by lactobacilli is limited by the degenerative ability of transport system towards substrates with a high degree of polymerization and rarely identified extracellular glycosidase [30]. Extracellular amylopullulanase (AmyX) has been characterized only in few lactobacilli, including *Amylolactobacillus amylophilus,*

Lactobacillus amylovorus, *Lactobacillus gasseri*, *Lactiplantibacillus plantarum*, *Lacticaseibacillus manihotivorans* and *Limosilactobacillus fermentum* [30–33]. The extracellular fructosidase FosE of *Lacticaseibacillus paracasei* is the first extracellular fructanase that has been characterized in lactobacilli [34]. The extracellular fructosidase FruA mediates fructan degradation in *Streptococcus mutans* [35]; in lactobacilli, it is found only in few strains of *Lactobacillus crispatus* and *L. amylovorus* from sourdough and the swine intestine [36,37]. Dietary fibers including resistant starch, hemicellulose, pectin, and fructans are accessible carbohydrate sources in both human and swine intestine; glucans and fructans also provide carbon source from biofilms when other fermentable carbohydrate sources are limited [10,38]. Therefore, extracellular glycosidase may contribute to the ecological fitness of lactobacilli in the intestinal tract.

1.2 Hypotheses

1) Lifestyle-associated metabolic traits of host-adapted lactobacilli used in food fermentation impact food quality and human health.

2) Glutaminase contributes to the acid resistance of *L. reuteri* and the accumulation of glutamine metabolites during sourdough fermentation.

3) FruA of *L. crispatus* is an extracellular cell fructosidase, subjected to catabolite repression and the activity is sufficient to produce low-FODMAP bread.

4) Putative arabinanases of *L. crispatus* are extracellular arabinanase.

5) Glutaminase, FruA, and extracellular arabinanases are exclusive present in host-adapted lactobacilli.

1.3 Objectives

1) Review the impact of metabolic traits of host-adapted lactobacilli on food quality and human health (Chapter 2).

2) Evaluate the contribution of glutaminases to glutamine metabolism and acid resistance in *L. reuteri* and other vertebrate-adapted lactobacilli (Chapter 3).

3) Characterize the extracellular fructosidase FruA in *L. crispatus* and its contribution to fructan hydrolysis in breadmaking (Chapter 4).

4) Explore the structure of arabinan utilization operon in *L. crispatus* and characterize two extracellular arabinanases on the operon (Chapter 5).

5) Investigate the presence and phylogenetic properties of glutaminase, FruA, and arabinanase in *Lactobacillaceae* species (Chapter 3, 4, and 5).

Chapter 2 Host-adapted lactobacilli in food fermentations: Impact of metabolic traits of host-adapted lactobacilli on food quality and human health

2.1 Introduction

A substantial proportion of the human diet consists of fermented foods, where the metabolic activity of fermentation micro-organisms determines and maintains the safety and quality of the products. Historically, non-alcoholic food fermentations aimed to improve the digestibility, nutritional value and / or the storage life of products [8]; their unique sensory properties maintained their popularity even when alternative processing methods become available. Fermented foods are not only a source of nutrients but also a major source of dietary micro-organisms if the fermentation organisms are not killed by a cooking or pasteurization step after the fermentation [39].

The microbiota of traditional food fermentation is controlled by the selection of raw materials, the product formula and the fermentation processes, and by back-slopping or the use of starter cultures. Back-slopping, the practice of inoculating a fermentation with a previous batch, profoundly alters the composition of fermentation microbiota when compared to spontaneous fermentations. In spontaneous fermentation, fermentation micro-organisms are selected from those organisms that are associated with the raw material or the processing environment [8,40,41]. In contrast, micro-organisms in back-slopped fermentations are challenged by microbiota of the raw materials in every new batch. Every time the raw material or the processing environment introduces a new strain that is more competitive than resident strains, the latter will be out-competed after a few fermentation cycles; a process that results eventually in the stabilization of fermentation microbiota after a sufficient number of fermentation cycles [42]. Once stabilization of fermentation microbiota is achieved, back-

slopping maintains undefined, mixed cultures over decades or centuries with remarkable stability at the species or even strain level [42,43].

2.2 Back-slopping of food fermentations recruits host-adapted fermentation organisms.

The origin of fermentation micro-organisms in back-slopped food fermentations and hence the source of "contamination" or inoculation with desirable fermentation organisms is in many cases enigmatic. For example, the microbial community of surface-ripened cheeses, which includes *Staphylococcus, Brevibacterium,* and *Corynebacterium* species, is independent of the geographic location but resembles human skin microbiota [8,44,45]; experimental evidence for a human origin of cheese rind microbiota, however, is lacking. As outlined below, increasing knowledge on the phylogeny and ecology of food fermenting lactic acid bacteria, particularly lactobacilli, supports the hypothesis that animal or human host-adapted lactic acid bacteria frequently dominate the microbiota of back-slopped food fermentations. Lactobacilli have free-living, nomadic, insect-adapted, or vertebrate host-adapted lifestyles [2]. Host-adapted lactobacilli have specialized to ecological niches that are associated with insect or vertebrate hosts. Some lactobacilli have specialized to very narrow ecological niches, e.g. *Lactobacillus iners*, which occurs only in the human vagina [2], or *Apilactobacillus* species (previously *Lactobacillus kunkeei* group) [11] which occur only in the intestinal tract of social bees [46]. *Limosilactobacillus reuteri* and *Ligilactobacillus ruminis* are examples of species that inhabit the intestinal tract of diverse vertebrate hosts; strains of these species diversified into intra-species phylogenetic lineages that adapted to specific hosts [47,48]. Other lactobacilli, such as *Ligilactobacillus salivarius* and *Lactobacillus gasseri*, appear not to be adapted to specific hosts but occur in multiple host species and several body sites [49]. The specialization of host-adapted lactobacilli resulted in a higher ecological fitness in their respective hosts at the expense of ecological fitness in

other habitats [50]. When conditions in the food fermentations match their niche conditions, host-adapted lactic acid bacteria outcompete less specialized competitors and dominate in the microbial community of those products. Experimental evidence for the animal origin of food fermenting lactobacilli was provided for sourdough isolates of *L. reuteri*, which retain all metabolic characteristics of rodent-lineage strains including the ability to colonise mice [51]. An overview of host-adapted lactobacilli in food fermentations is shown in Table 2.1. This chapter aims to explore whether host-adapted lactobacilli share "lifestyle-associated" metabolic traits and whether these metabolic traits are relevant for the safety and quality of fermented foods.

2.3 Species of host-adapted lactobacilli prevalent in fermented foods

Fermentation control by back-slopping is commonly used in dairy fermentations including cheese cultures, yogurt, kefir and other fermented milk beverages, and many cereal fermentations including sourdough fermentations, several African fermentations for production of porridges or beverages, and mash fermentations for production of vinegar or liquor in East Asia [8,52]. Owing to their importance in fermentation control, seed cultures that are used in back-slopped fermentations often have a designation that differentiates them from the corresponding fermented food products, e.g. kefir grain, mother of vinegar, and 'chef' or 'levain' for seed sourdoughs. Host-adapted lactobacilli associated with cereal fermentation include organisms from the vertebrate host-adapted *Limosilactobacillus* and *Lactobacillus*, and insect associated species of the *Fructilactobacillus* species (previously *Lactobacillus fructivorans* group [11]). In dairy fermentations, *Lactobacillus* and *Ligilactobacillu*s (part of previously *Lactobacillus salivarius* group [11]) species are frequently present (Table 2.1). The metabolic focus of *Lactobacillus delbrueckii* on lactose was explained by adaptation to dairy environments through reduction of genome size and

silencing of carbohydrate active enzymes other than β-galactosidase [53], however, the presence of *L. delbrueckii* in the intestine of suckling piglets demonstrates adaptation to the intestine of suckling mammals rather than dairy fermentations [37,54]. Host adapted lactic acid bacteria also include the oral streptococci *S. mutans*, a human adapted pathogen [55,56], and *S. salivarius,* a commensal inhabitant of the oral cavity [57] (Table 2.1). *Streptococcus thermophilus* was identified as a core member of human intestinal microbiota [58]; this organism is closely related to oral streptococci but lost virulence-related genes [59].

2.4 Metabolic properties in host-adapted lactobacilli associated with fermented food.

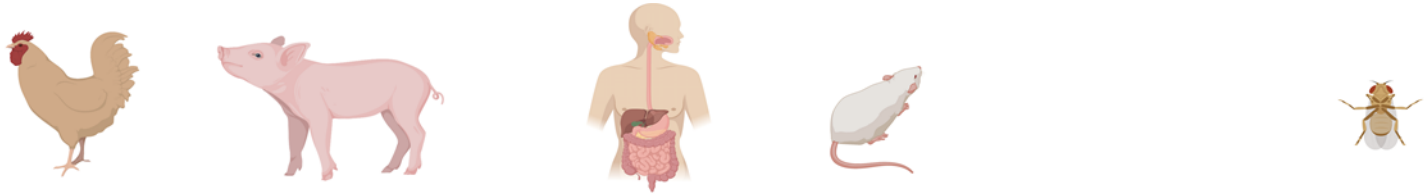
Host-adapted lactobacilli harbour lifestyle-associated metabolic traits, including acid resistance, biofilm formation, extracellular hydrolysis of polysaccharides, bacteriocin producing, and tetracycline resistance. An overview on metabolic properties of host-adapted lactobacilli that relate to their adaptation to the host is provided in Figure 2.1.

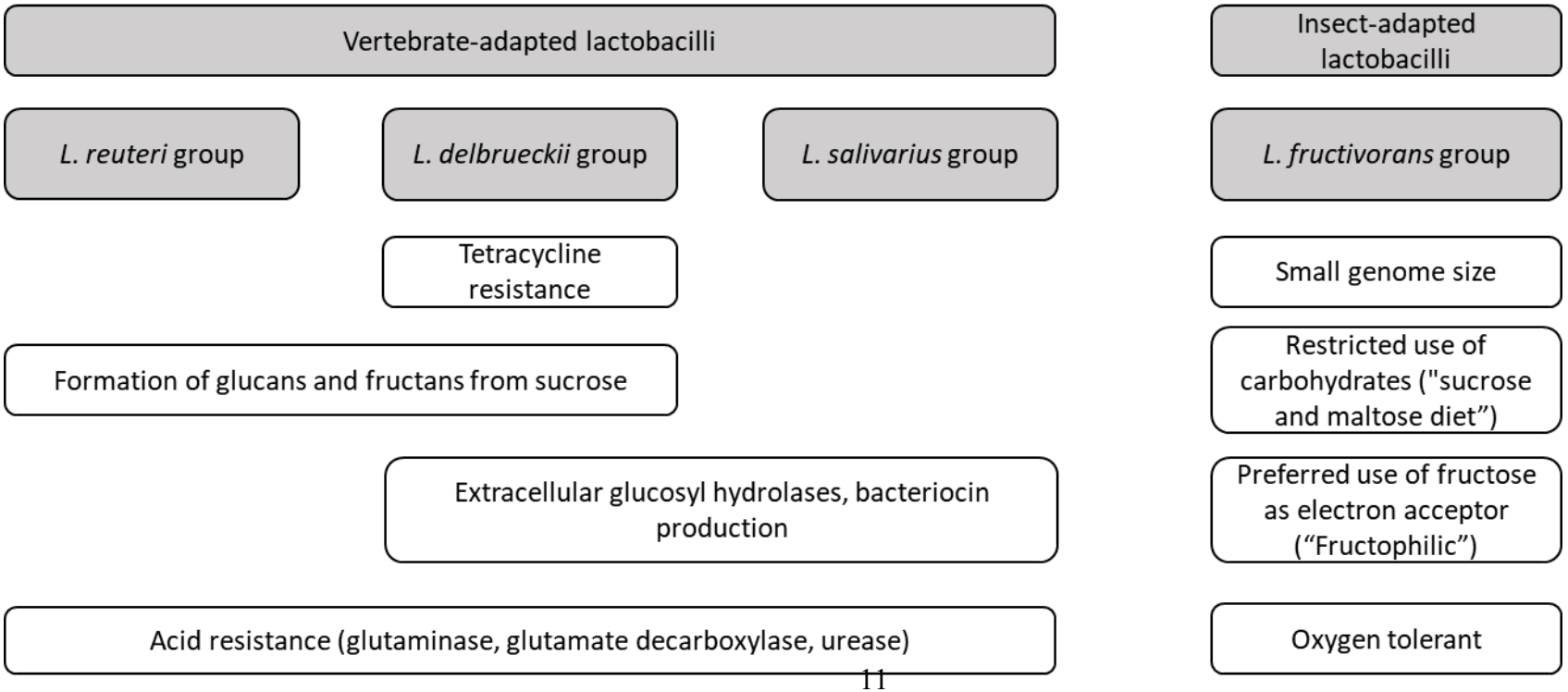
Table 2. 1 Host-adapted lactobacilli in food fermentation

	Products	Substrate	Host-adapted lactic acid bacteria	Stage of production	Reference
Cereal fermentations	Ting	Sorghum	*Limosilactobacillus reuteri*	Back-slopping	[60,61]
	Kisra	Sorghum	*L. reuteri, Lactobacillus amylovorus*	Back-slopping	[62]
	Mawe	Maize	*L. reuteri, Ligilactobacillus salivarius*	Back-slopping	[63,64]
	type I sourdough	Wheat or rye	*Fructilactobacillus sanfranciscensis, Limosilactobacillus. pontis, Limosilactobacillus panis*	Back-slopping	[65,66]
	type II sourdough	Wheat or rye	*L. pontis, L. amylovorus, L. reuteri, L. panis, Limosilactobacillus frumenti, L. crispatus, L. acidophilus*	Back-slopping	[65,66]
	vinegar	Barley, wheat, rice or sorghum	*Lactobacillus acetotolerans*	Back-slopped mash fermentation	[67,68]
	Baijiu		*L. acetotolerans, L. panis*	Back-slopped mash fermentation	[69,70]
	Chicha	Cassava	*Lactobacillus acidophilus, Lactobacillus delbrueckii, L. reuteri, Streptococcus salivarius, Streptococcus mutans*	Spontaneous, inoculation with human saliva	[71,72]
Dairy fermentations	Yoghurt	milk	*L. delbrueckii* subsp. *bulgaricus, Streptococcus thermophilus*		[8,52]
	Koumiss	Mare's milk	*Lactobacillus helveticus, L. delbrueckii* subsp. *bulgaricus, L. salivarius, L. acidophilus, Lactobacillus kefiranofaciens, Streptococcus thermophilus*	Back-slopping or starter cultures matching traditional back-slopped fermentations	[63,73]
	kefir	Milk and kefir grain	*L. kefiranofaciens, L. delbrueckii* subsp. *bulgaricus, S. thermophilus, L. helveticus*		[74]
	cheese	milk	*L. delbrueckii* subsp. *bulgaricus, L. helveticus, S. thermophilus*		[8,52]

Figure 2. 1 Representative characteristics of host-adapted lactobacilli associated with food fermentation

Current taxonomy classifies species in the *L. reuteri* group, *L. delbrueck*ii group, *L. salivari*us group and the *L. fructivoran*s group have been assigned in the genera *Limosilactobacillus*, *Lactobacillus*, *Ligilactobacillus*, and *Fructilactobacillus*, respectively according to the new nomenclature [11] (partially created with biorender.com).





The acid resistance system is essential for the competitiveness of vertebrate-host adapted organisms as the colonization of a new host by oral or intestinal lactobacilli depends on survival during gastric transit [2,16]. Urease is the most powerful bacterial mechanism against stomach acidity and is present in *Lactobacillus*, *Ligilactobacillus,* and *Limosilactobacillus* species [16,75]. Urease is also expressed in oral *S. thermophilus* and *S. salivarius* [76,77]; urease activity in *S. thermophilus* and *S. salivarius* is differentially regulated in response to the pH and the carbohydrate supply [77,78]. Glutaminase, which consumes intracellular protons by deamidation of glutamine, is almost exclusively present in host-adapted *Lactobacillus* and *Limosilactobacillus*[79]. Arginine deiminase (ADI) also contributes to acid resistance in lactobacilli and is expressed by host-adapted, nomadic, and free-living lactobacilli [13]. The genes of the ADI pathway were overexpressed in lactobacilli colonizing the stomach of mice [80] but did not enhance ecological fitness [16].

Extracellular polysaccharides (EPS) production in lactobacilli is mediated by extracellular fructansucrases or dextransucrases that use sucrose as substrate, or by intracellular glycosyltransferases [8]. Capsular EPS formation by pyogenic streptococci is a virulence factor to evade the host immune system. Expression of capsular EPS promoted invasive disease caused by *Streptococcus pneumoniae,* a colonizer and pathogen of the nasopharynx [81,82]. In *S. pneumoniae*, capsular EPS expression is regulated by Rgg / small hydrophobic peptide quorum-sensing system and has been inversely associated with biofilm formation, whereas other EPS seem to promote biofilm formation [82,83]. The inhibition of biofilm formation by the capsule is attributed to the capsule effect, which blocks the exposure of *S. pneumoniae* surface adhesins that promote attachment to epithelial cells [84]. Regulation of surface polysaccharide expression by quorum-sensing system may enable *S. pneumoniae* to adjust interactions with the host and other bacteria in response to environmental conditions [82]. Capsular EPS of commensal bacteria also contribute to their fitness in the host but

benefit health. Capsular EPS produced by *Bifidobacterium breve* aids in long-term *in viv*o persistence [85]. *S thermophilus* also produce capsular heteropolysaccharides (HePS) primarily consisting of glucose, galactose and rhamnose with glucuronic acid, similar to the capsule of S. *pneumoniae* [86]. Genes found in the *eps* cluster of *S. thermophilus* are related to those involved in capsule synthesis in *S. pneumoniae* [87] and were reported to increase acid and bile tolerance [88].

Homopolysaccharides (HoPS) produced from sucrose contribute to biofilm formation and thus support the colonization of host epithelia by lactic acid bacteria [89–91]. Fructansucrases or glucansucrases of *S. mutans* produce fructan or glucan, respectively, which form the biofilm matrix that is necessary to colonize the surface of teeth [91,92]. In lactobacilli, glucansucrase and fructansucrase activity are frequently found in the host-adapted genera *Lactobacillus* and *Limosilactobacillus*, and in *Liquorilactobacillus* (part of previously *Lactobacillus salivarius* group [11]) which predominantly has a free-living lifestyle [13]. In direct analogy to *S. mutans*, homopolysaccharides produced by *L. reuteri* are required for biofilm formation and cell aggregation and are essential for colonization of the mouse gastrointestinal tract [90,93]. Glucansucrases and fructansucrases also mediate metabolism of sucrose; in *Fructilactobacillus sanfranciscensis*, levansucrase is the only enzyme with activity on sucrose [94].

Only few lactobacilli express extracellular enzymes catalyzing the hydrolysis of polysaccharides [30]. Starch, pullulan, and fructans provide carbon source from polysaccharides or biofilms when other fermentable carbohydrate sources are limited. Extracellular amylopullulanase (AmyX) is present only in few lactobacilli; most of these are classified in the genera *Amylolactobacillus* (previously *Lactobacillus amylophilus* group [11])and *Lactobacillus* [30]. As resistant starch is a major carbohydrate source in the human and swine intestine, AmyX may contribute to the ecological fitness of lactobacilli in the

intestinal tract [30,95]. The extracellular fructosidase FruA mediates fructan degradation in *S. mutans* [35]; in lactobacilli, it is found only in few strains of *Lactobacillus crispatus* and *Lactobacillus amylovorus* from sourdough and the swine intestine [36,37].

Sucrose phosphorylase (ScrP) phosphorolyses sucrose into fructose and glucose-1-phosphate. Lactobacilli harboring sucrose phosphorylase belong to the vertebrate host-adapted genera *Lactobacillus*, *Ligilactobacillus,* and *Limosilactobacillus*, and the free-living *Lentilactobacillus* (previously *Lactobacillus buchneri* group [11]). Sucrose metabolism is repressed by glucose in homofermentative lactobacilli; in heterofermentative lactobacilli of the *L. reuteri* group, sucrose metabolism is preferred over glucose metabolism [96]. Phosphorolysis in combination with fructose reduction to mannitol increases the energy yield of the phosphoketolase pathway more than twofold [97] and increases the growth rate in cereal substrates [13,98]. Since sucrose is present only in the upper intestine, ScrP increases ecological fitness only of those lactic acid bacteria that inhabit the oral cavity, the crop, or (fore)-stomach epithelia.

Tetracycline resistance of lactobacilli is mediated by the ribosomal protection proteins Tet(M), Tet(S), Tet(Q), and Tet(W), and the efflux pumps [Tet(L) and Tet(P)]. Tet(W) is almost exclusively present in intestinal lactobacilli and was likely acquired by horizontal gene transfer [99]. Tet(M) is the most widespread in lactobacilli; this gene is present in the genera *Lactobacillus* and *Amylolactobacillus*, and in *Limosilactobacillus equigenerosi* [100]. The gene *tet*(M) is also the most widespread antibiotic resistance gene in food-associated lactobacilli, including *L. delbrueckii* supsb. *bulgaricus*, *L. salivarius*, and *L. reuteri* [101]. Tet(M) was shown to have ribosome-dependent GTPase activity. The energy from GTP hydrolysis by Tet(M) releases the tetracycline from the ribosome, thereby reduced the binding of tetracycline to the ribosomes. The distribution of *tet*(M) gene is generally associated with conjugative chromosomal transposons, which transfer mobile plasmids to

other species and even unlinked genomic DNA [102]. Tet(M) was also found in bifidobacteria, a commensal genus in the gastrointestinal tracts of humans and animals, and transferred between different *Bifidobacterium* species [103,104]. Therefore, the acquisition of *tet*(M) by intestinal lactobacilli likely occurred by lateral gene transfer from bifidobacteria or other intestinal organisms.

Bacteriocin production by lactobacilli is strain specific and not limited to host-adapted species. Bacteriocin production is often assumed to be a desirable trait of probiotic bacteria but experimental evidence that bacteriocins of intestinal or probiotic lactobacilli modulate intestinal microbiota remains elusive [105,106]. Analysis of the prevalence of bacteriocin-encoding genes in the metagenome of different human body sites, however, revealed that bacteriocin production is particularly frequent in oral and vaginal microbiota, which implies an ecological role of bacteriocin production by lactobacilli in these body sites [107]. Correspondingly, oral streptococci are prolific producers of bacteriocins [108] and also frequently harbor non-ribosomal peptide synthases with a putative function in the synthesis of antimicrobial compounds [109]. Also, multiple vaginal isolates produce bacteriocins with antimicrobial activity against vaginal pathogens [110,111].

Reuterin is a broad-spectrum antimicrobial compound, which is produced as intermediate of glycerol metabolism by strains of *L. reuteri* [112]. Glycerol metabolism in *L. reuteri* is encoded by the gene cluster *pdu-cbi-hem-cob* that contains the *pdu* genes encoding cobalamin-dependent glycerol/diol dehydratase PduCDE which utilizes glycerol or 1,2-propanediol [113]. The gene cluster is also present in intestinal microbes such as *Salmonella* and *Eubacterium hallii*. In the human colon, specific member of intestinal microbiota produce 1,2-propanediol from fucose or rhamnose [114]; 1,2-propanediol metabolism generates propionate and propanol [115]. Glycerol is available in cereals and other plant foods; glycerol metabolism by *L. reuteri* enhances its competitiveness in cereal substrates

[116]. In *L. reuteri*, glycerol / propanediol metabolism is frequent only in strains of the human-adapted lineage II, which colonizes the intestine of herbivores and humans, and in the poultry-adapted linage VI, which colonizes the crop of birds but also persists in humans [113]. The differential regulation of reuterin production in *L. reuteri* strains of different lineages may reflect the availability of glycerol and 1,2 propanediol in the upper and lower intestine, respectively [113].

Fructilactobacillus species, likely including *F. sanfranciscensis*, are associated with insect hosts and have distinctive metabolic properties when compared to vertebrate-host adapted lactobacilli. They utilize only a few carbohydrates and depend on the availability of fructose as an electron acceptor [117]. Their small genome size and restricted metabolic potential indicate specialization to very narrow ecological niches.

2.5 The contribution of metabolic traits in host-adapted lactobacilli to food quality.

The metabolic traits of host-adapted lactobacilli that contribute to the flavour, structure, and quality of fermented food are shown in Table 2.2. Glutamine and glutamate metabolism enhance bread quality by generating glutamate and γ-aminobutyric acid (GABA), respectively. The glutaminase mediated glutamate accumulation exceeds the taste threshold in bread and ripened cheese and thus contributes to the umami taste [29,118]. Dietary GABA has relaxing properties [119,120]. In baked goods, arginine conversion by sourdough lactic acid bacteria provides ornithine as a precursor to the character impact aroma compound of wheat bread crust, 2-acetyl-1-pyrroline [98]. During malolactic fermentation of wine, arginine deamidation by lactic acid bacteria may accumulate citrulline as an intermediate, which is a precursor for the formation of the carcinogen ethyl carbamate [121].

In the initial stages of yogurt fermentation, urease catalyzes the hydrolysis of urea into ammonia and CO_2. During co-culture of *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus*,

urease is essential for effective protocooperation and yogurt acidification of two species by providing ammonia nitrogen to support growth and acidification of *S. thermophilus*, and CO_2 for the CO_2-responsive *L. delbrueckii* subsp. *bulgaricus* [122].

EPS formation in cereal fermentations improves bread volume and texture and reduces bread staling; EPS also contributes to the texture of other fermented cereal foods or beverages [123,124]. Production of HePS in dairy fermentations affects the texture and rheology of the products [125]. The interaction of EPS and milk proteins influences protein gel formation and water-binding capacity. Free EPS typically leads to ropiness, while capsular EPS increases viscosity without causing ropiness. In milk products, EPS is located at the pore/protein network interface or located in the aqueous environment of the pores. Stirring redistributes EPS in the protein network; ropy EPS attached to protein maintains high viscosity and the firmness of the protein network after stirring while EPS in pores maintains only the high viscosity. The presence of capsular EPS leads to a higher firmness, viscosity, thickness, and creaminess [126]. The interplay of capsular and free EPS seems also relevant to product texture, influencing protein aggregation, pore size, and structure recovery of the network [127]. Kefiran is a water-soluble HePS, composed of glucose and galactose, exclusively produced by *Lactobacillus kefiranofaciens* during kefir fermentation and contributes to the formation of the kefir grain and the gel formation and viscosity of the finished product [126].

Table 2. 2 Impact of metabolisms in host-adapted lactobacilli on food quality and human health

Metabolic activity / metabolite	Food products	Impact on food quality	References
Glutamine deamidation / Glutamate accumulation	Bread, cheese	Umami taste, salt reduction	[29,118]
Glutamate decarboxylation / GABA accumulation	Bread, cheese, kimchi	Anti-hypertensive properties	[119,120]
Urea metabolism / Acidification	yoghurt	Symbiosis of *L. delbrueckii* and *S. thermophilus* results in stable fermentation culture	[122]
Arginine deamidation to ornithine / formation of 2-acetyl-1-pyrroline, the crust odor compound, from ornithine during baking	Bread	Flavor	[98]
Formation of homopolysaccharides and heteropolysaccharides	Bread; yoghurt	Improved texture and volume of bread; Improved texture and rheology of yoghurt; Prevent adhesion of pathogens; Stimulation of immunological defense mechanisms	[124,126–129]
Degradation of fructans or raffinose (FODMAPs); sugar reduction in wheat products	Bread, other cereal products	Increase the tolerance to rye bread of IBS patients; Increase of sweet taste	[36,130]
Production of bacteriocins or reuterin	Cheese	antimicrobial activity as bio-preservatives / “clean label” products	[8,131,132]
Delivery of dietary microbes	Probiotic	Probiotic activity	[61,74,133,134]

EPS formation by probiotic strains also contributes to human health. HoPS isolated from *L. reuteri* inhibited adhesion of enterotoxigenic *Escherichia coli* to the swine intestinal mucosa [128]. HePS produced by probiotic strains, lactic acid bacteria, and bifidobacteria, may modulate the immune system of the host. Capsular EPS produced by *B. breve* reduces the levels of colonization by intestinal pathogens [85]. EPS-deficient variants of *B. breve* strains elicited a strong immune response that was absent in the wild type strains producing capsular EPS, indicating that capsular EPS mediates immune evasion, especially avoiding B-cell responses [85]. HePS produced by *S. thermophilus* stimulated human gastric epithelial cell regeneration and immunological innate defense mechanisms [129].

The ability to degrade polysaccharides is rare in *Lactobacillus* species. Expression of extracellular fructanases by *L. crispatus* or *L. amylovorans* eliminated fructans from cereal during sourdough fermentation. A reduced fructan content of bread improved the tolerance of patients with irritable bowel syndrome (IBS) to rye bread with a high dietary fiber content [36,130].

Bacteriocins of lactobacilli find food applications to inhibit or to eliminate pathogens, particularly in ready-to-eat meat or fish products; none of the strains that find commercial applications, however, are of intestinal origin [135]. Reuterin is a highly reactive compound, the reactivity limits its application in food. It was demonstrated, however, that reuterin producing *L. reuteri* in combination with the addition of glycerol is an effective approach to prevent late blowing defect of cheese [131,132].

Food fermentations with probiotic fermentation organisms is increasingly recognized as a tool to deliver beneficial microbes to the human or animal intestinal tract [136,137]. Host-adapted lactobacilli show improved survival after gastrointestinal transit in swine and in humans [138,139], which may relate to their increased acid resistance, and enhance the

probiotic activity of host-adapted lactobacilli that are present in food fermentations. For example, several African non-alcoholic cereal beverages including *mawe* and *mahewu* contain viable fermentation organisms, were proposed as the route of delivery for probiotic bacteria [61]. *Koumiss* contains high cell counts of probiotic *Lactobacillus helveticus,* which contributes to anti-inflammatory attributes [133]. *L. kefiranofaciens* in kefir decreased inflammation in a mouse model of obesity [74]. Traditional kefir has also been proved to reduce weight gain, improving plasma and liver lipid profiles in a mouse model of obesity [134]. Fermented foods containing large numbers of live probiotic bacteria are also considered giving similar health benefits as intake of probiotic lactobacilli of the same species [137]. However, the tetracycline resistance of host-adapted lactic acid bacteria may limit their use as a starter or probiotic cultures [99,100,140].

In conclusion, fermentation micro-organisms in back-slopped food fermentations are often recruited from lactic acid bacteria that have evolved to form stable associations with insects or vertebrate hosts. The ecological fitness of host-adapted lactobacilli in host and food environments is dependent on lifestyle-associated metabolic traits. Some of these traits, including exopolysaccharide formation and bacteriocin production, are also present in free-living or nomadic lactic acid bacteria while other metabolic properties, for example, glutaminase- and urase mediated acid resistance, the extracellular fructanase FruA, and antibiotic synthesis by non-ribosomal peptide synthases are virtually exclusive to host-adapted lactobacilli. An improved understanding of the ecological origin of food fermenting lactic acid bacteria will facilitate the selection of starter cultures for food production and may support the simultaneous use of lactic acid bacteria as food-fermenting and probiotic cultures.

Chapter 3 Contribution of glutaminases to glutamine metabolism and acid resistance in *Limosilactobacillus reuteri* and other host-adapted lactobacilli

3.1 Introduction

The fermentation of sourdough confers characteristic taste and flavor to bread and steamed bread. Proteolysis and conversion of amino acids during sourdough fermentation provide taste compounds and precursor compounds for flavor volatiles [27]. Glutamine is the most abundant amino acid in wheat and rye proteins; cereal proteases and microbial peptidases release glutamine during sourdough fermentation. Microbial metabolism converts glutamine to glutamate, which imparts the umami taste of bread. Glutamate is a substrate for further conversion to γ-aminobutyrate (GABA) [24,141,142]. Dietary GABA may regulate immune responses and mediate anti-hypertensive effects [119,120]. Glutamate is also a substrate for the production of γ-glutamyl peptides [143], agonists of calcium-sensing receptor proteins in taste cells that enhance umami, sweet and salty tastes. This influence on taste perception has been termed “kokumi” [144,145], and described as mouthfulness, thickness, and continuity of taste [146].

Glutamine-amidotransferases or glutaminases [EC 3.5.1.2] catalyze the bacterial conversion of glutamine to glutamate [147]. *Limosilactobacillus reuteri* 100-23 harbours three glutaminases, one of these genes is part of a glutamine/glutamate operon that also codes for glutamate decarboxylase (GadB) and two putative glutamate/GABA antiporters (GadC) [21,24]. The contribution of these glutaminases on the formation of glutamine metabolites in sourdough, however, remains unclear.

The extended fermentation time of type II sourdoughs imposes acid stress on lactobacilli

[148,149]. Glutamate decarboxylation increases the acid resistance of lactobacilli including *L. reuteri* through intracellular decarboxylation, which consumes protons, and electrogenic antiport of glutamate and GABA [21,24]. Glutamate-mediated acid resistance increases the ecological fitness of *L. reuteri* in the rodent forestomach, and in type II sourdough fermentations [16,116]. Glutamine deamidation also increased acid resistance of *Escherichia coli* and *L. reuteri* [21,25,150]. In *L. reuteri* and *Brucella microti* CCM4915 but not in *E. coli*, glutaminase, glutamate decarboxylase, and a glutamate / GABA antiporter are arranged in a single operon [21,26,151]. It was the aim of this study to investigate the distribution of glutaminase in lactobacilli, to evaluate the contribution of glutaminase in *L. reuteri* to amino acid metabolism in sourdough, and to determine whether glutaminase contributes to acid resistance in lactobacilli. The study generated *L. reuteri* 100-23 Δ*gls1*Δ*gls2*Δ*gls3*, a derivative with deletion of all three *gls* in the genome of *L. reuteri* 100-23.

3.2 Materials and Methods

3.2.1 Strains, plasmids and growth conditions

Table 3.1 shows bacterial strains and plasmids used in this study. *L. reuteri* 100-23*, Lactobacillus taiwanensis* 107q*, Lactobacillus johnsonii* 117a and *Lactobacillus acidophilus* FUA3066 were cultivated in modified deMan-Rogosa-Sharpe (mMRS) medium at 37 °C [152]. *E. coli* JM 109 (Promega, Nepean, ON, Canada) was cultured in Luria-Bertani (LB) medium at 37 °C. The frozen stock cultures were inoculated on agar plates; single colonies were inoculated in 1 ml broth, subcultured with 1 % inoculum in broth. LB medium with ampicillin (100 mg L^{-1}) or erythromycin (500 mg L^{-1}) was used for antibiotic-resistant *E. coli* selection. Erythromycin-resistant *L. reuteri* was selected by adding erythromycin (5 mg L^{-1}) into mMRS medium.

3.2.2 Phylogenetic analysis of glutaminase in lactobacilli

Protein sequences of glutaminases in genomes of all lactobacilli were retrieved from the National Center for Biotechnology Information database using BLASTp [153] and the sequences of three glutaminases in *L. reuteri* 100-23 as query sequences. Protein sequences were retrieved with a cut-off of 30 % amino acid identity; sequences were discarded if an identical sequence from a second strain in the same species was available. The 21 remaining sequences were aligned by MEGA7 [154] using ClustalW method. The phylogenetic analysis of glutaminases was conducted by the Maximum Likelihood method based on the JTT matrix-based mode [155] and the bootstrap support values were calculated from 500 replicates by MEGA7.

3.2.3 Generation and confirmation of *L. reuteri* 100-23Δ*gls1*Δ*gls2*Δ*gls3*.

The genome of *L. reuteri* 100-23 encodes for three glutaminases, namely Gls1, Gls2, and Gls3 [21]. A triple mutant *L. reuteri* 100-23Δ*gls1*Δ*gls2*Δ*gls3* was constructed by truncating *gls1*, *gls2*, and *gls3* using pJRS233 [156] according to a double crossover mutagenesis method [157]. Table 3.1 lists plasmids used for the construction of mutant strains and Table 3.2 lists primers. PCR with primers *gls2*-KO1-BamHI and *gls2*-KO2-XbaI and *gls2*-KO3-XbaI and *gls2*-KO4-PstI amplified 5' and 3' flanking fragments of *gls2*, respectively, using genomic DNA from *L. reuteri* 100-23 as a template. The 5' and 3'-flanking fragments of *gls2* were ligated to pGEMTeasy vector (Promega) to generate pGLS2-AB. The DNA fragment in pGLS2-AB was cut with *Bam*HI and *Sal*I, purified and ligated into pJRS233 carrying an erythromycin resistance gene as a selective marker to generate pGLS2-KO-AB. The resulting plasmid pGLS2-KO-AB was electro-transformed in *L. reuteri* cells. Transformants were incubated in mMRS-erm broth at 42-44 °C for 80 generations to select for single crossover mutants. These single crossover mutants were subsequently incubated for 100 generations in

mMRS broth at 37 °C. Replica plating on mMRS and mMRS-erm agar identified erythromycin sensitive derivatives that lost the plasmid by a double-crossover event; *L. reuteri* 100-23Δ*gls2* were identified by PCR with primers *gls2*-5F and *gls2*-6R. The deletion was verified by amplification of the truncated *gls2* with primers *gls2*-F and *gls2*-R. Subsequently, *L. reuteri* 100-23 Δ*gls2*Δ*gls3* was generated by interrupting *gls3* in *L. reuteri* 100-23Δ*gls2* with the same protocol and plasmids and primers shown in Table 3.1 and 3.2. *L. reuteri* 100-23Δ*gls1*Δ*gls2*Δ*gls3* was constructed by disruption of *gls1* in *L. reuteri* 100-23Δ*gls2*Δ*gls3*. PCR analysis and DNA sequencing (MacrogenUSA, Rockville, MD, USA) verified the deletion regions using primers *gls1*-F and *gls1*-R, *gls2*-F and *gls2*-R, and *gls3*-F and *gls3*-R.

3.2.4 Accumulation of amino acids during buffer fermentation

Overnight cultures of *L. reuteri* 100-23, *L. reuteri* Δ*gadB*, and *L. reuteri* Δ*gls1*Δ*gls2*Δ*gls3* were harvested by centrifugation and washed twice with 50 mmol L^{-1} sodium acetate buffer (pH 4.5). Cells were re-suspended in 50 mM Sodium acetate buffer or buffers supplemented with 10 mmol L^{-1} glutamine or 10 mmol L^{-1} glutamate. Buffer without inoculum served as control. Samples were collected after 0 and 8 hours of incubation for monitoring bacterial survival and quantification of amino acids. Buffer fermentations were carried out in three biological replicates.

Table 3. 1 Bacterial strains and plasmids used in this study

Strains or plasmid	Genotype	Source or reference
Strains		
Limosilactobacillus reuteri 100-23	Rodent isolate; wild type strain	Wesney et. al.
Lactobacillus johnsonii 117a	Rodent isolate; wild type strain	[158]
Lactobacillus taiwanensis 107q	Rodent isolate; wild type strain	[158]
Lactobacillus acidophilus FUA3066	Isolate from commercial probiotic culture	This study
L. reuteri Δ*gls3*	*L. reuteri* 100-23 derivative with deletion of *gls3*	This study
L. reuteri Δ*gls2*Δ*gls3*	*L. reuteri* 100-23 derivative with deletions of *gls2* and *gls3*	This study
L. reuteri Δ*gls1*Δ*gls2*Δ*gls3*	*L. reuteri* 100-23 derivative with deletions of *gls1, gls2,* and *gls3*	This study
L. reuteri Δ*gadB*	*L. reuteri* 100-23 derivative with deletions of *gadB*	[24]
Escherichia coli JM109	Cloning host for pGEMTeasy- and pJRS233-deviative plasmids	Promega
Plasmids		
pGEMTeasy	Cloning vector used in *E. coli*; 3.0 kb; Amp^r	Promega
pGLS1-A	pGEMTeasy containing 1 kb of the DNA sequence upstream of *gls1*; 4.0 kb; Amp^r	This study
pGLS1-B	pGEMTeasy containing 1 kb of the DNA sequence downstream of *gls1*; 4.0 kb; Amp^r	This study
pGLS1-AB	pGEMTeasy containing upstream and downstream sequences of *gls1*; 5.0 kb; Amp^r	This study
pJRS233	Shuttle vector used in the hosts *E. coli* and *L. reuteri* 100-23, Erm^r	[156]
pGLS1-KO-AB	pJRS233 containing 2.0 kb of the flanking sequences of *gls1*; Erm^r	This study
pGLS2-A	pGEMTeasy containing 1 kb of the DNA sequence upstream of *gls2*; 4.0 kb; Amp^r	This study
pGLS2-B	pGEMTeasy containing 1 kb of the DNA sequence downstream of *gls2*; 4.0 kb; Amp^r	This study
pGLS2-AB	pGEMTeasy containing upstream and downstream sequences of *gls2*; 5.0 kb; Amp^r	This study
pGLS2-KO-AB	pJRS233 containing 2.0 kb of the flanking sequences of *gls2*; Erm^r	This study
pGLS3-A	pGEMTeasy containing 1 kb of the DNA sequence upstream of *gls3*; 4.0 kb; Amp^r	This study
pGLS3-B	pGEMTeasy containing 1 kb of the DNA sequence downstream of *gls3*; 4.0 kb; Amp^r	This study
pGLS3-AB	pGEMTeasy containing upstream and downstream sequences of *gls3*; 5.0 kb; Amp^r	This study
pGLS3-KO-AB	pJRS233 containing 2.0 kb of the flanking sequences of *gls3*; Erm^r	This study

Table 3. 2 Primers used in the construction of *L. reuteri* 100-23Δ*gls1*Δ*gls2*Δ*gls3*.

Distupted gene	Primers	Primer sequences (5'-3')
gls1	*gls1*-KO1-PstI	AACTGCAGGGGATTGTAACTTGAAATTAAC
	gls1-KO2-BglII	GAAGATCTCATTCTTGAATTGCGTCATTAAG
	gls1-KO3-BglII	GAAGATCT AGGTACTAGTTGCAAATATTCGC
	gls1-KO4-BamHI	CGGGATCC GATATTCAGCAGTCGAAAG
	gls1-5F	GCCAAATATCTGCTGATCG
	gls1-6R	AACAGCGTTTGTTCCAA
	gls1-F	TGGCTGATTCCAGTCACATTAG
	gls1-R	GAGTGGGAAGTAAGGGACAAAG
gls2	*gls2*-KO1-BamHI	CGGGATCCTTGCCGATGCATTAAC
	gls2-KO2-XbaI	GCTCTAGACTATTGCTCTAATTTTTGCATCGT
	gls2-KO3-XbaI	GCTCTAGATTAGAATTAGTAGTTTAATAAAAGCG
	gls2-KO4-PstI	AACTGCAGGGAAACGCAGATGAGAG1263-1297
	gls2-5F	AGAGCGGGGTATTTCG
	gls2-6R	GCTGGTTGGGTAAAAGTT
	gls2-F	ACAATACTCAAGCCGACCTAAC
	gls2-R	CTATACCCAGCGTGTGAAGAAA
gls3	*gls3*-KO1-PstI	AACTGCAGAAAAGCTTGGACAACCC
	gls3-KO2-EcoRI	GGAATTCTTATTTAAGATCCAAAGTAATCACCTC
	gls3-KO3-EcoRI	GGAATTC TTTCAGTACTAATAATTAAGGTCCAA
	gls3-KO4-BamHI	CGGGATCCGCATGTGCTGAAAATTG
	gls3-5F	CCTTTATCAACCATCAGCT
	gls3-6R	AGCTGGTGTGCTACTTT
	gls3-F	GATTCCAGCAAAGCCAAACC
	gls3-R	GCTGAAGATACCACCACCATTA

3.2.5 Accumulation of amino acids during sourdough fermentation

Sourdoughs were fermented with *L. reuteri* 100-23, *L. reuteri* 100-23Δ*gadB*, or *L. reuteri* 100-23Δ*gls1*Δ*gls2*Δ*gls3* respectively to determine cell counts, pH and amino acid accumulation during growth in sourdough. Two grams of all purpose wheat flour (Robin Hood, Markham, ON, Canada), 2 ml of sterilized tap water were incubated with an initial cell count of $1 \pm 0.5 \times 10^7$ CFU g^{-1}. Samples were collected after 0, 12, 24, and 48 hours of incubation. Sourdough fermentations were carried out in three biological replicates.

3.2.6 Quantification of amino acids by high performance liquid chromatography (HPLC)

Amino acids were quantified by HPLC after derivatization with o-phthaldialdehyde [159]. Buffer samples were centrifuged to remove cells; the supernatant was mixed with 5% w / v trichloroacetic acid and β-aminobutyric acid, which served as the internal standard. The mixture was centrifuged and the supernatant was then derivatized with ortho-phthalaldehyde (1 vol) with the addition of saturated potassium borate (5 vol). Sourdough samples were lyophilized and extracted with water at an extraction ratio of 1:6 (w / v); the supernatant was mixed with 5 % w / v trichloroacetic acid and β-aminobutyric acid. The mixture was then derivatized with o-phthaldialdehyde described above.

3.2.7 Synthesis of γ-glutamyl dipeptides during buffer fermentation and sourdough fermentation

Buffer fermentations were performed with *L. reuteri* 100-23 and *L. reuteri* 100-23Δ*gls1*Δ*gls2*Δ*gls3* as described with modifications [143]. Cells from overnight cultures of three strains were washed twice with autoclaved tap water, and re-suspended in 20 mmol L^{-1} phosphate buffer (pH 6.5) containing 5 g L^{-1} of maltose, 10 mmol L^{-1} of glutamine, and 10 mmol L^{-1} of lysine, glutamate, leucine, isoleucine, phenylalanine or valine as glutamate acceptors. Buffers were incubated at 37 °C for 24 h. Samples were collected before and after incubation for LC-MS / MS analysis of γ-glutamyl dipeptides in the culture supernatant. Buffer inoculated with strains and maltose but without the addition of amino acids, and uninoculated buffers served as controls. The cell count and pH were monitored on each sample. Buffer fermentations were carried out in two independent experiments, and samples were analyzed in duplicate.

Sourdoughs were fermented as described above with *L. reuteri* 100-23 or *L. reuteri* 100-23Δ*gls1*Δ*gls2*Δ*gls3*. Sourdough samples were collected at 0 h and 48 h and lyophilized. Then 0.5g lyophilized sourdough was mixed with1 ml of 0.1 % formic acid and the mixture was incubated at 25 °C for 1 h with 250 rpm shaking. The mixture was diluted at a ratio of 1: 10 into 30 % methanol and pellets in the dilution were collected by centrifuge. The supernatant was collected and filtered for LC-MS / MS analysis.

3.2.8 Quantification of γ-glutamyl dipeptides by LC-MS/MS

γ-Glutamyl dipeptides were quantified by LC-MS / MS as described [143]. In short, peptides were separated on a 1200 series HPLC unit with a diode array detector (Agilent Technologies, Palo Alto, CA, USA) connected to a 4000 Q TRAP LC-MS / MS system (MDS SCIEX, Applied Biosystems, Streetsville, ON, Canada). The mobile phase consisted of 0.1 % formic acid in Milli-Q water (solvent A) and 0.1 % formic acid in acetonitrile (solvent B). Samples were eluted from an Express C18 HPLC column (2.7 μm, 150 mm × 2.1 mm, Phenomenex, Torrance, CA, USA) at a flow of 0.2 ml min^{-1} with the following gradient: 0 min, 95 % A; 10 min, 75 % A; 15 min, 0 % A; followed by re-equilibration with 95 % A. LC-MS/MS parameters for quantitation of the six kokumi peptides are shown in Table 3.3. External calibration standards (0.1-100 μg L^{-1}) of γ-glutamyl dipeptides were prepared at 30 % (v / v) methanol in 0.1 % aqueous formic acid.

Table 3. 3 LC-MS/MS parameters for the determination of γ-glutamyl dipeptides in water-soluble extracts of sourdough

Peptide	Transition	Retention time (min)	DPt	CE
γ-Glutamyl glutamate	277.3 / 84.1	1.89	50	40
γ-Glutamyl cysteine	251.2 / 122.2	1.95	40	17
γ-Glutamyl valine	247.2 / 72.1	2.78	50	35
γ-Glutamyl isoleucine	261.2 / 132.5	8.40	50	19
γ-Glutamyl leucine	261.2 / 132.5	9.30	50	19

3.2.9 Survival of lactobacilli at pH 2.5 and 3.5

To determine the survival of lactobacilli at acid pH in presence or absence of glutamine, *L. reuteri* 100-23, *L. reuteri* 100-23Δ*gls1*Δ*gls2*Δ*gls3*, *L. reuteri* 100-23Δ*gadB, L. acidophilus* FUA3066, *L. johnsonii* 117a, and *L. taiwanensis* 107q were incubated at pH 2.5 or 3.5 with or without the addition of 10 mmol L^{-1} glutamine or glutamate. To detect the different effects of three *gls* on the survival of *L. reuteri* 100-23, *L. reuteri* 100-23Δ*gls3*, *L. reuteri* 100-23Δ*gls2*Δ*gls3*, *L. reuteri* 100-23Δ*gls1*Δ*gls2*Δ*gls3* were incubated at pH 2.5 with or without the addition of 10 mmol L^{-1} glutamine or glutamate for 24h. Cells from overnight cultures were washed in 50 mmol L^{-1} Na_2HPO_4 buffer (pH 7.0) and re-suspended in 50 mmol L^{-1} potassium phosphate buffer (pH 2.5) or in 50 mmol L^{-1} lactate buffer (pH 3.5). Viable plate counts were determined by surface plating on mMRS agar. Experiments were performed in three biological replicates. The absence of *gls* in *L. acidophilus* FUA3066 was checked by primers in Table 3.4.

Table 3. 4 Primers used to confirm the absence of *gls* in *L. acidophilus* FUA3066

Targeted strains	Primers	Primer sequences (5'-3')	Annealing temperature
L. gasseri, L. taiwanensis, L. johnsonii	Forward	CATGGGACAACAAACGCATTAT	62.8
	Reverse	GACATAAGACCACCACCAACA	
L. crispatus	Forward	ATGATGGTAACGGCACGTAG	62.9
	Reverse	CACCTCCAACACCACTCTTAG	
L. antri, L. oris	Forward	CCCGCACATTACCCTCAATAAT	62.9
	Reverse	AAATCAGTCGTTCCTGATCCC	

3.2.10 Statistical analysis

Data analysis was performed with IBM SPSS statistics 23, using one-way or two-way analysis of variance (ANOVA). A *P*-value of ≤ 0.05 was considered statistically significant.

3.2.11 Nucleotide sequence accession numbers

The genome sequences of *L. reuteri* 100-23 were obtained from the National Center for Biotechnology Information databases (GenBank: AAPZ02000001.1 and AAPZ02000002.1). Nucleotide sequences and annotations were retrieved from GenBank with accession numbers AAPZ02000001.1:333174-334094, AAPZ02000001.1:1455778-1456692, and AAPZ02000002.1:548219-549139 for genes *gls1*, *gls2*, and *gls3*, respectively. The sequences of the truncated *gls1*, *gls2*, and *gls3* in *L. reuteri* 100-23Δ*gls1*Δ*gls2*Δ*gls3* were deposited with accession number MN147878, MN147879, and MN147880, respectively.

3.3 Results

3.3.1 Phylogenetic analysis of glutaminases in *Lactobacillaceae* species

A phylogenetic analysis determined the frequency and distribution of glutaminases in *Lactobacillaceae* species (Figure 3.1A). Gls2 of *L. reuteri* 100-23 is least similar to other glutaminases and has no homologues in other lactobacilli. Three and two genomes of other *Limosilactobacillus* species (previously *L. reuteri* group [11]) harboured homologues of Gls1 and Gls3, respectively. Ten genomes of *Lactobacillus* species (previously *Lactobacillus*

delbrueckii group [11]), as well as *Fructilactobacillus lindneri* (previously *Lactobacillus fructivorans* group [11]) and *Ligilactobacillus aviarius* (previously *Lactobacillus salivarius* group [11]) harbored putative glutaminases that clustered separately from *L. reuteri* enzymes. All lactobacilli with glutaminases are adapted to animal hosts; with the exception of *L. lindneri*, glutaminases were present in organisms that are adapted to the intestine of vertebrate animals [2]. We additionally analyzed whether glutaminases in lactobacilli are part of operons that also include GadB and / or GadC. The genetic loci of glutaminases of lactobacilli that are part of an operon including GadC and / or GadB shown in Figure 3.1B. *L. aviarius* is the only other *Lactobacillaceae* that harbours an operon with glutaminase, GadB and GadC. *L. lindneri* encodes for a GadC / Gls operon. In other lactobacilli with glutaminase, GadC was present *in trans; Limosilactobacillus antri* and *Limosilactobacillus oris* encoded for a GadB / GadC operon distant from Gls (data not shown). None of *Lactobacillus* species with glutaminase encoded for GadB.

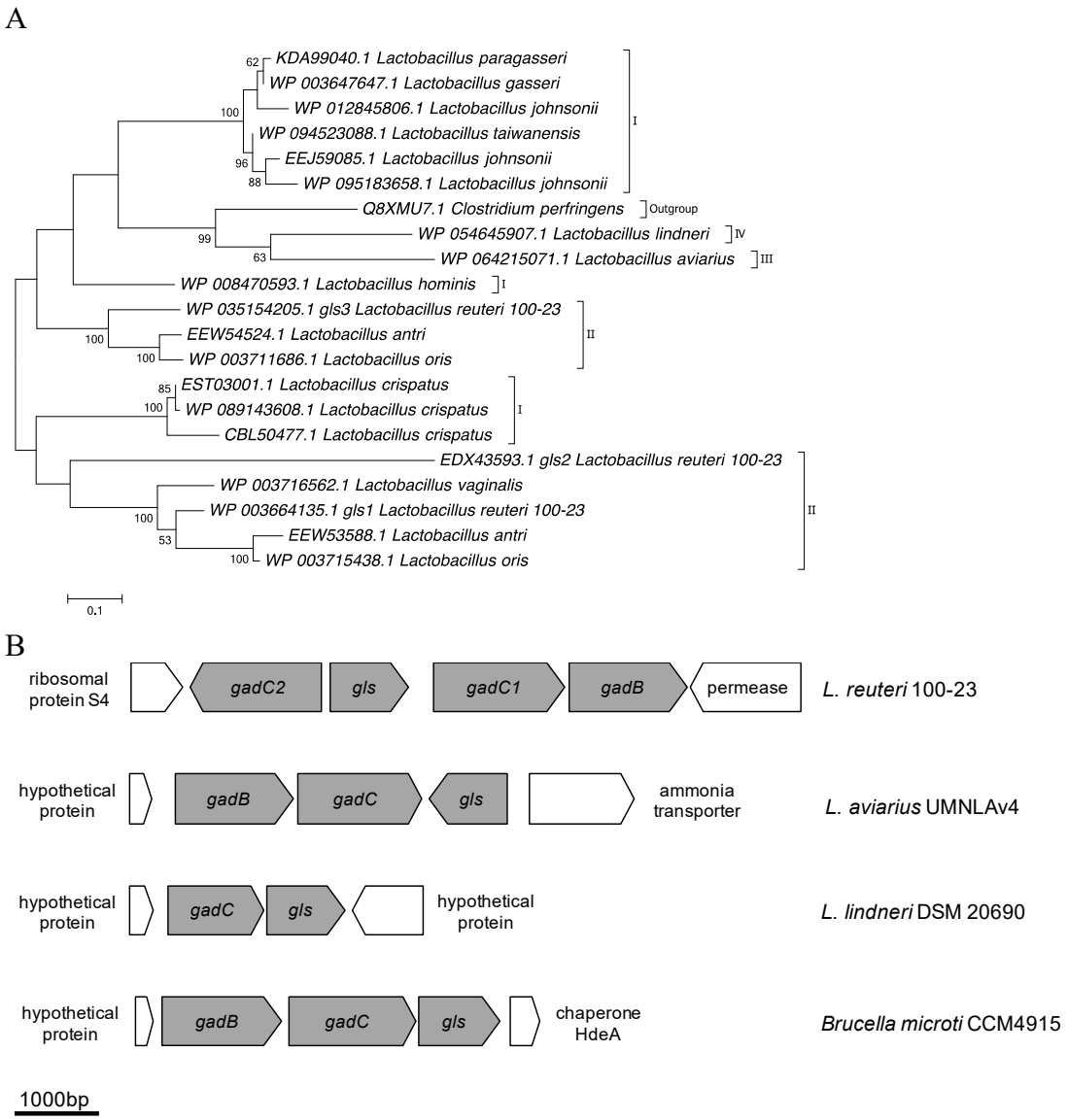


Figure 3. 1 Analysis of glutaminases in *Lactobacillaceae* species

Panel A. Phylogenetic analysis of glutaminases in *Lactobacillaceae* species. Sequences in all genomes of lactobacilli were identified by BLASTp using *gls1*, *gls2*, or *gls3* as query sequences. Identical sequences from the same species were discarded. Phylogenetic tree was constructed based on 21 glutaminase sequences from 12 *Lactobacillus* species by using the Maximum Likelihood method based on the JTT matrix-based model [155] and the bootstrap support values were calculated from 500 replicates by MEGA7 [154]. Bootstrap values are shown if they are higher than 50. Branch lengths were measured in the number of substitutions per site. Roman numerals designate the phylogenetic groups: *L. delbrueckii* (I), *L. reuteri* (II), *L. salivarius* (III), and *L. fructivorans* (IV)[13], that are different genera in new taxonomy: *Lactobacillus*, *Limosilactobacillus*, *Ligilactobacillus, Fructilactobacillus* [11].

Panel B. Genetic loci of those *gls* sequences that are adjacent to *gadB* or *gadC* in *Lactobacillaceae* species. *Brucella microti* CCM4915 is used for comparison. Nucleotide sequences and annotations were retrieved from GenBank with accession numbers AAPZ02000002.1: 548219-549139, LVKF01000063.1: 29436-30365, JQBT01000033.1: 133694-134626 and CP001579.1: 322403-323356 for glutaminases in *L. reuteri* 100-23, *L. aviarus* UMNLAv4, *L. lindneri* DSM20690, and *Brucella microti* CCM4915, respectively. Gene name: *gls* (glutaminase), *gadB* (glutamate decarboxylase), *gadC* (glutamate/GABA antiporter).

3.3.2 Glutamine metabolism of *L. reuteri* 100-23Δ*gls1*Δ*gls2*Δ*gls3* in sourdough.

To test the role of glutaminases of *L. reuteri* in glutamine metabolism in sourdough, the concentrations of glutamine, glutamate and GABA were measured during wheat sourdough fermentation with *L. reuteri* 100-23, 100-23Δ*gls1*Δ*gls2*Δ*gls3*, and 100-23Δ*gadB* respectively (Figure 3.2). The growth of strains and the pH of sourdough were also monitored (Figure 3.7). The growth of three strains in sourdough was identical throughout fermentation, the cell counts increased to the maximum after 12 h and maintained at ~10 log CFU g^{-1} during the following 24 h fermentation, indicating that the growth of strains during sourdough fermentation was not influenced by the truncation of *gadB* or three *gls*.

The disruption of *gls* genes or *gadB* had no influence on the concentration of (glutamine + glutamate + GABA) after 48 h of fermentation. Glutamine accumulated in chemically acidified dough but its concentration remained low in all sourdoughs. *L. reuteri* 100-23 converted glutamine to GABA and accumulated the highest level of GABA after 48 h of fermentation. The deletion of *gadB* resulted in glutamate accumulation and low GABA concentrations. GABA concentration in sourdough fermented with *L. reuteri* 100-23Δ*gls1*Δ*gls2*Δ*gls3* was higher than the concentration in sourdough fermented with *L. reuteri* 100-23Δ*gadB*.

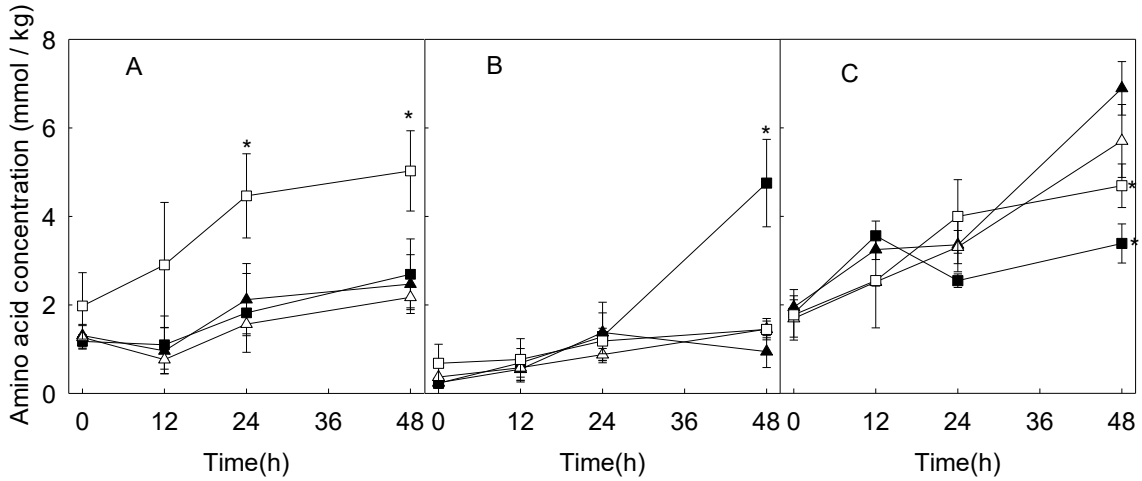


Figure 3. 2 Concentration of amino acids during sourdough fermentation

The concentration of glutamine (A), glutamate (B) and γ-aminobutyric acid (C) during sourdough fermentation with *L. reuteri* 100-23 (▲), *L. reuteri* Δ*gls1*Δ*gls2*Δ*gls3* (Δ) or *L. reuteri* Δ*gadB* (■) over 48 h were quantified. Chemically acidified dough fermented for 48 h served as control (□). Symbols indicate means ± standard deviation of three biological replicates. Values for chemically acidified sourdough or for sourdoughs fermented with isogenic mutant derivatives of *L. reuteri* 100-23 are marked with an asterisk if they differ significantly ($P < 0.05$) from values for sourdoughs fermented with *L. reuteri* 100-23.

3.3.3 Synthesis of γ-glutamyl dipeptides in buffer and sourdoughs

Glutamyl-cysteine ligases in *L. reuteri* LTH5448 synthesize γ-Glu-Ile and γ-Glu-Cys but deletion of both glutamyl-cysteine ligases did not fully eliminate synthesis of γ-glutamyl peptides in *L. reuteri* LTH5448 [160]. Bacterial glutaminases catalyze transglutamination to produce γ-glutamyl peptides [161,162], therefore, the γ-glutamyltransferase activity of glutaminase in *L. reuteri* 100-23 was assessed by monitoring the concentration of γ-glutamyl dipeptides after incubation of the strain and its mutants in buffers (Figure 3.3) and in sourdough (Table 3.5). The deletion of *gls* decreased the synthesis of γ-Glu-Glu in buffers, however, the formation of other γ-glutamyl peptides was not affected (Figure 3.3). The deletion of glutaminases in *L. reuteri* did not affect the synthesis of γ-glutamyl dipeptides in sourdoughs (Table 3.5).

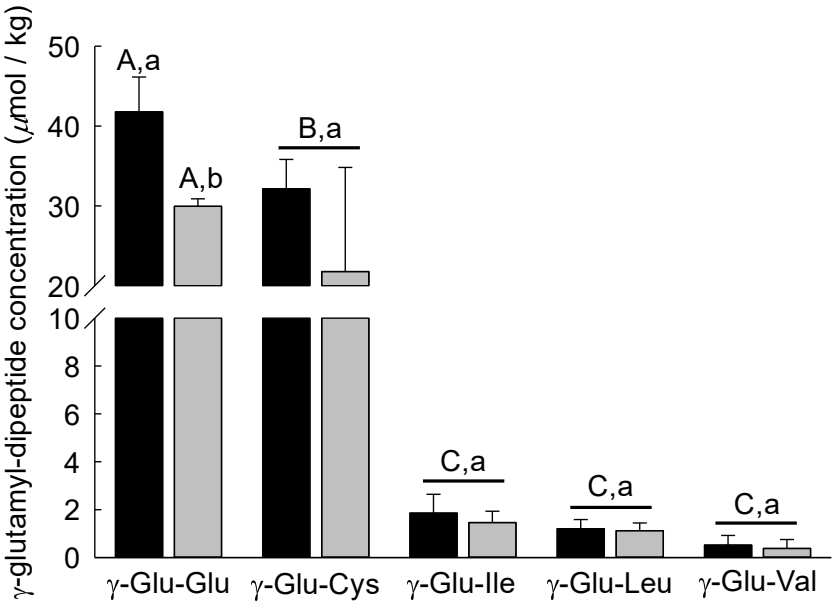


Figure 3. 3 Concentration of γ-glutamyl dipeptides in buffers

L. reuteri 100-23 (black columns) and *L. reuteri* 10-23Δ*gls1*Δ*gls2*Δ*gls3* (gray columns) were used to ferment buffers for 24h, separately. Symbols indicate means ± standard deviation from two biological replicates analyzed in duplicate. Values for different peptides that do not share a common uppercase superscript differ significantly ($P < 0.05$). Values for the same peptide that do not share a common lowercase superscript differ significantly between samples obtained after incubation with *L. reuteri* 100-23 and *L. reuteri* 10-23Δ*gls1*Δ*gls2*Δ*gls3* ($P < 0.05$).

Table 3. 5 Concentration of γ-glutamyl dipeptides in wheat sourdough fermentations

Dipeptides	γ-Glu-Glu	γ-Glu-Cys	γ-Glu-Ile	γ-Glu-Leu	γ-Glu-Val
	0 h				
L. reuteri 100-23	3.39 ± 1.46^{a}	1.46 ± 0.13^{b}	0.30 ± 0.05^{a}	0.51 ± 0.18^{a}	0.56 ± 0.05^{a}
100-23Δ*gls1*Δ*gls2*Δ*gls3*	4.56 ± 0.74^{a}	1.40 ± 0.17^{b}	0.27 ± 0.07^{a}	0.41 ± 0.11^{a}	0.58 ± 0.05^{a}
Chemically acidified	3.34 ± 1.06^{a}	2.11 ± 0.35^{a}	0.35 ± 0.10^{a}	0.47 ± 0.14^{a}	0.60 ± 0.08^{a}
	48 h				
L. reuteri 100-23	8.08 ± 2.19^{a}	1.07 ± 0.44^{a}	5.27 ± 0.09^{a}	0.91 ± 0.38^{a}	0.62 ± 0.10^{a}
100-23Δ*gls1*Δ*gls2*Δ*gls3*	5.74 ± 0.86^{ab}	1.28 ± 0.41^{a}	5.62 ± 0.58^{a}	1.08 ± 0.40^{a}	0.63 ± 0.07^{a}
Chemically acidified	3.37 ± 0.39^{b}	0.92 ± 0.17^{a}	0.58 ± 0.23^{b}	0.96 ± 0.16^{a}	0.58 ± 0.04^{a}

The concentration of γ-glutamyl dipeptides in sourdoughs fermented with different strains at the same incubation time that does not share a common lowercase superscript differ significantly ($P < 0.05$).

3.3.4 Effects of glutamine and glutamate on acid resistance of *L. reuteri* 100-23

The survival of *L. reuteri* 100-23Δ*gls1*Δ*gls2*Δ*gls3* and *L. reuteri* 100-23Δ*gadB* was compared with that of the wild type *L. reuteri* 100-23 in phosphate buffer at pH 2.5 and

lactate buffer at pH 3.5. Amino acid supplementation did not influence survival in lactate buffer at pH 3.5 and *L. reuteri* 100-23Δ*gadB* was the most sensitive strain (Figure 3.4). During incubation in phosphate buffer at pH 2.5, glutamate supplementation protected the wild type strain and *L. reuteri* 100-23Δ*gls1*Δ*gls2*Δ*gls3* but not *L. reuteri* 100-23Δ*gadB*. The effect of glutamine supplementation on the survival of *L. reuteri* 100-23 was comparable to the effect of glutamate. Glutamine supplementation increased survival of *L. reuteri* 100-23Δ*gadB* when compared to supplementation with glutamate, however, glutamine did not improve or decrease survival of *L. reuteri* 100-23Δ*gls1*Δ*gls2*Δ*gls3* when compared to glutamate supplementation (Figure 3.4).

Previous studies suggested that overexpression of *gls1*, *gls2*, and *gadB* compensates the disruption of *gls3* in *L. reuteri* 100-23 [163]. To assess the role of the three glutaminases in acid resistance, *L. reuteri* 100-23, 100-23Δ*gls3*, *L. reuteri* 100-23Δ*gls2*Δ*gls3*, and 100-23Δ*gls1*Δ*gls2*Δ*gls3* were incubated in phosphate buffer of phosphate buffer containing glutamate or glutamine (Figure 3.5). Glutamine protected all strains except *L. reuteri* 100-23Δ*gls1*Δ*gls2*Δ*gls3* against the acid challenge, indicating that Gls2 or Gls1 compensate for the loss of Gls3, or Gls2 and Gsl3.

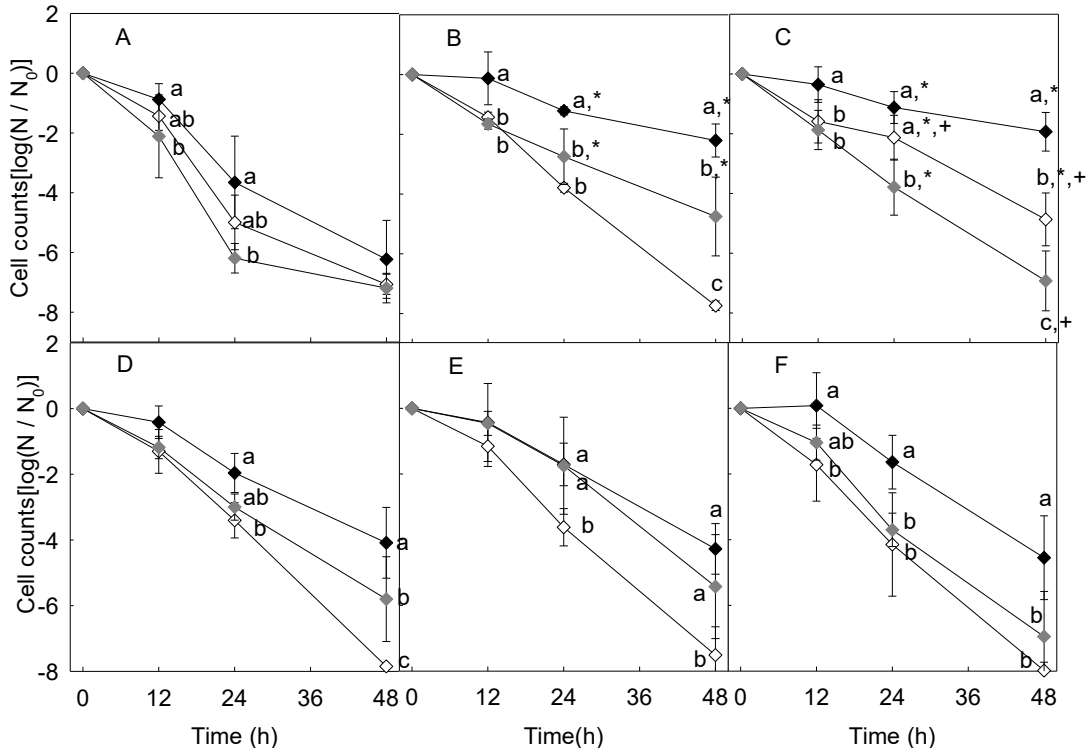


Figure 3. 4 Acid resistance of *L. reuteri* 100-23 and its isogenic mutants in buffers

Acid resistance of *L. reuteri* 100-23 (black symbols), *L. reuteri* Δ*gls1*Δ*gls2*Δ*gls3* (grey symbols), or *L. reuteri* Δ*gadB* (open symbols) in phosphate buffer (pH 2.5, A), in phosphate buffer with 10mM glutamate (B), or in phosphate buffer with 10 mM glutamine (C). Acid resistance of *L. reuteri* 100-23 (black symbols), *L. reuteri gls1*Δ*gls2*Δ*gls3* (grey symbols) or *L. reuteri* Δ*gadB* (open symbols) in lactate buffer (pH 3.5, D), in lactate buffer with 10mM glutamate (E), or in lactate buffer with 10 mM glutamine (F) were quantified. Symbols indicate means ± standard deviation from biological replicates. Values for different strains in the same buffer at the same incubation time do not share a common lowercase superscript differ significantly ($P < 0.05$). Values obtained in buffers with glutamate or glutamine are marked with an asterisk if they differ significantly ($P < 0.05$) from values obtained at the same incubation time with the same strain in control buffer. Values for the same strain in buffers with glutamine are marked with a plus sign if they differ significantly ($P < 0.05$) from values obtained with the same strain at the same incubation time in buffer with glutamate.

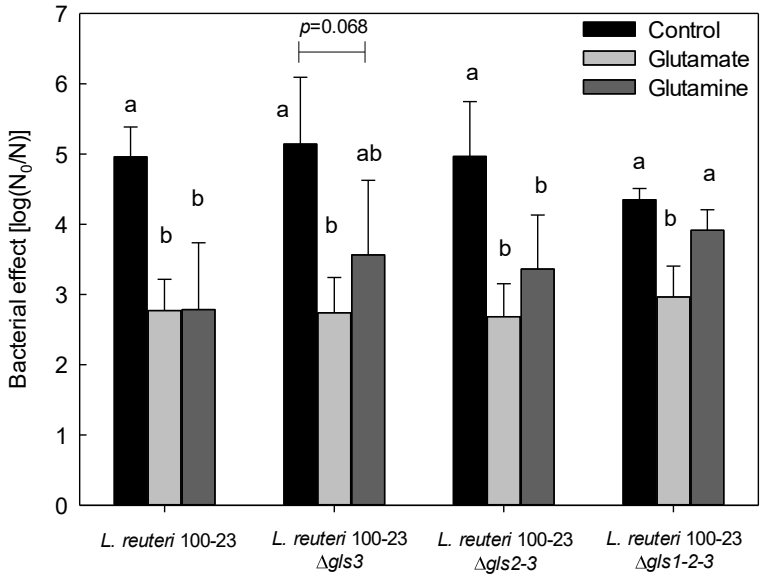


Figure 3. 5 Acid resistance of *L. reuteri* 100-23, 100-23Δ*gls3*, *L. reuteri* 100-23Δ*gls2*Δ*gls3* and 100-23Δ*gls1*Δ*gls2*Δ*gls3* in phosphate buffers

Three strains were inoculated in phosphate buffer (pH 2.5, black bars), in phosphate buffer with 10mM glutamate (light grey bars), or in phosphate buffer with 10 mM glutamine (dark grey bars) after 24h incubation. Data represent means ± standard deviation from biological replicates. Values for different strains in the same buffer at the same incubation time do not share a common uppercase superscript differ significantly ($P < 0.05$). Values obtained with the same strain in different buffers differ significantly ($P < 0.05$) if they do not share a common lowercase superscript.

3.3.5 Survival of host-adapted lactobacilli at pH 2.5 and pH 3.5

To confirm the contribution of glutamine metabolism to the acid resistance of other host-adapted lactobacilli, the survival of *L. johnsonii* 117a, *L. taiwanensis* 107q and one strain of *L. acidophilus* FUA3066 at pH 2.5 or 3.5 was determined. All three organisms are host-adapted organisms in the *L. delbrueckii* group; the genome sequences of *L. johnsonii* 117a and *L. taiwanensis* 107q contain a glutaminase gene [158] but none of the available genome sequences of *L. acidophilus* encompasses a glutaminase. The absence of glutaminases in *L. acidophilus* FUA3066 was confirmed with primers targeting the conserved regions of glutaminases in *L. taiwanensis, L. johnsonii,* and *L. gasseri*, and in *L. crispatus* (Table 3.4). At pH 2.5, supplementation of glutamine and glutamate improved survival of *L. taiwanensis* 107q but amino acid supplementation did not improve survival of *L. johnsonii* 117a or *L.*

acidophilus (Figure 3.6). The addition of glutamate or glutamine did not improve the survival of any of the strains in lactate buffer at pH 3.5.

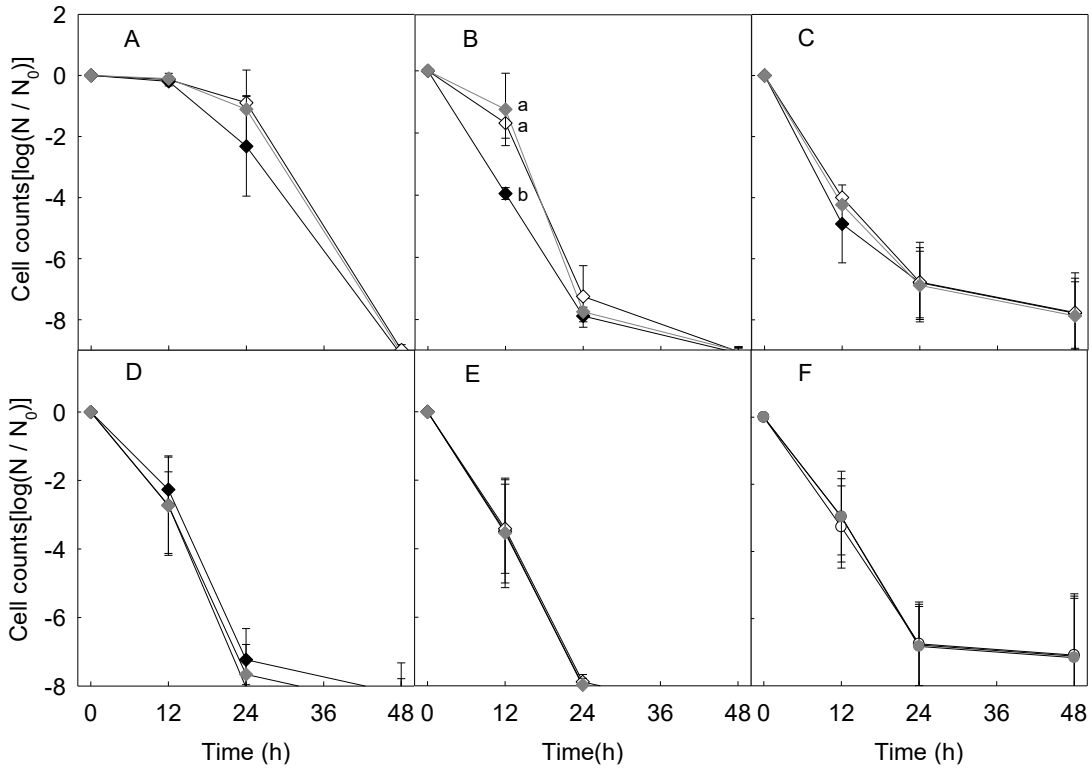


Figure 3. 6 Acid resistance of host-adapted lactobacilli in buffers

Acid resistance of *L. johnsonii* 117a (A), *L. taiwanensis* 107q (B) or *L. acidophilus* (C) in phosphate buffer (pH 2.5, black symbols), in phosphate buffer with 10mM glutamate (open symbols), or in phosphate buffer with 10 mM glutamine (grey symbols). Acid resistance of *L. johnsonii* 117a (D), *L. taiwanensis* 107q (E) or *L. acidophilus* (F) in lactate buffer (pH 3.5, black symbols), in phosphate buffer with 10 mmol / L glutamate (open symbols), or in phosphate buffer with 10 mmol / L glutamine (grey symbols). Symbols indicate means ± standard deviation from triplicates. Values for the same strain in different buffers at the same incubation time do not share a common lowercase superscript differ significantly ($P < 0.05$).

3.4 Discussion

This study investigated the contribution of glutaminase to glutamine metabolism in sourdough and to acid resistance of lactobacilli. Phylogenetic analysis of glutaminase sequences indicates glutaminase of lactobacilli is exclusively present in host-adapted lactobacilli. The deletion of glutaminases in *L. reuteri* did not influence glutamine and glutamate metabolism in sourdough but decreased the acid resistance of *L. reuteri* at pH 2.5. During sourdough fermentation, the most abundant amino acid released from wheat and rye proteins is glutamine. Microbial conversion of glutamine yields glutamate, an umami tastant,

GABA, or kokumi-active γ-glutamyl peptides. Glutaminase hydrolyzes γ-amino group of L-glutamine to produce L-glutamic acid [161,164]. Glutaminase activity in *L. reuteri* KCTC3594 was described as a salt- and thermotolerant enzyme with activity in the range of pH 5.0 – 11.0 [165,166]. The molecular weight of the partially purified enzymes (50 – 70 kDa), however, does not match the molecular weight of Gls1 (33.47 kDa), Gls2 (33.25 kDa) or Gsl3 (33.15 kDa) [21,165]. This suggests the presence of multiple enzymes in *L. reuteri* with activity on glutamine. Accordingly, the disruption of all three glutaminases in *L. reuteri* 100-23 had no effect on the glutamine metabolism by *L. reuteri* in sourdough (Figure 3.2). In buffer, the truncation of glutaminases reduced the synthesis of γ-glutamyl-glutamate (Figure 3.3) and GABA (data not shown), suggesting a reduced rate of glutamine conversion to glutamate.

Glutamine amidotransferases also catalyze the removal of the ammonia group from glutamine and subsequently transfer of ammonia to a specific substrate [167]. A wide range of biosynthetic enzymes, including carbamoyl-phosphate synthase, pyridoxal 5'-phosphate synthase, and guanosine monophosphate synthase, contain glutamine amidotransferase subunits with glutaminase activity [147,168,169]. Those genes that are encoded in the genome of *L. reuteri* 100-23 that relate to glutamine conversion to glutamate are glutamine amidotransferases including a GMP synthase [EDX43269.1], an asparagine synthase [EDX43329.1], a peptidase C26 [EDX43401.1], carbamoyl-phosphate synthase [EDX41777.1], and an isomerizing glutamine-fructose-6-phosphate aminotransferase [EDX41955.1]. The glutaminase activity of these biosynthetic enzymes likely accounts for glutaminase conversion in *L. reuteri* 100-23Δ*gls1*Δ*gls2*Δ*gls3*, for glutaminase activities of *Lacticaseibacillus rhamnosus* [170] and *Fructilactobacillus sanfranciscensis* DSM20451 [171], strains which harbour glutamine amidotransferases but not glutaminase. The γ-glutamyltranspeptidase from *Bacillus subtilis* also exhibits glutaminase activities at pH 8.0-

8.5, but is absent in the genome of *L. reuteri* 100-23 [24,172]. In conclusion, glutaminases are accessory enzymes that are present only in few lactobacilli and do not make a substantial contribution to glutamine metabolic phenotypes.

The deamidation of glutamine contributes to acid resistance of bacteria. Glutaminase Ybas in *E. coli* is active at acidic pH and protects *E. coli* at pH 2.5 via the release of ammonia [25]. A contribution of glutaminase to acid resistance of *L. reuteri* is documented by the loss of protective effect of glutamine on acid resistance of *L. reuteri* 100-23Δ*gls1*Δ*gls2*Δ*gls3* (this study), and by the demonstration that glutamine remains protective for *L. reuteri* 100-23Δ*gadB* (this study) [21].

The glutaminase *gls3* in *L. reuteri* 100-23 is part of an operon that also includes *gadB* and *gadC*; a comparable operon structure is found in *Brucella microti* [25,26]. The *gls-gadB / C* operon is prevalent in Gram-negative bacteria but rarely found in Gram-positive bacteria [151]. Among *Lactobacillus* species, the *gls-gadB / C* operon is present only in *L. reuteri* and *L. aviarius* subsp. *aviarius*; *L. lindneri* harbored a *gls-gadC* operon. The presence of glutaminase and glutamate decarboxylase in enteric bacteria and rodent-lineage strains of *L. reuteri* strongly indicates their role to protect bacteria against acid stress in the gut or the transit through the stomach (this study) [16,51,151,173]. Comparison of the glutamine-mediated acid resistance and the operon structure in lactobacilli and *E. coli* suggests that glutamine-mediated acid resistance requires GadC and Gls and that the system is functional even if the two genes are not part of the same operon. This is further supported by the acid resistance phenotype in *L. reuteri* 100-23Δ*gls3 and* 100-23Δ*gls2*Δ*gls3*, where the remaining glutaminase genes, *gls1* and *gls2*, or *gls1*, are located distant from the GadC / GadB operon (this study) [21]. *L. taiwanensis* 107q maintains glutaminase activity without GadB but not *L. johnsonii* 117a, however, glutamine addition increased acid resistance of this strain much less when compared to *L. reuteri* 100-23. Because glutamine and glutamate are alternative

substrates for this acid resistance mechanism, deletion of one or the other has a modest but significant impact on the acid resistance of *L. reuteri* 100-23 *in situ* and *in vivo* (this study) [16,116].

Type II sourdough microbiota are derived from vertebrate-adapted lactobacilli in the *L. delbrueckii* and *L. reuteri* groups [51,66,174]. Acid resistance is a physiological trait that increases competitiveness in type II sourdoughs as well as intestinal ecosystems [13,16,66,116]. This study demonstrated that glutaminase is exclusively present in lactobacilli adapted to vertebrate intestinal ecosystems. Among these, the glutaminase based acid resistance is predominantly but not exclusively maintained by *L. reuteri*, *L. taiwanensis,* and *L. johnsonii* that represent rodent forestomach microbiota [2,47,158]. The genomes of *L. taiwanensis* 107q and *L. johnsonii* 117a encode for glutaminase but not for GadB (this study) [158], suggesting that lactobacilli alternatively maintain glutamine or glutamate based mechanisms of acid resistance. Utilizing the concept of "lifestyle" in sourdough ecology thus helps to explain how specific metabolic traits in lactobacilli contribute to the competitiveness and bread quality during sourdough fermentation [66].

In conclusion, this study demonstrated the exclusive presence of glutaminase in host-adapted lactobacilli and the contribution of glutaminase to acid resistance of *L. reuteri* 100-23. Glutaminase-mediated acid resistance is thus a "lifestyle-associated" metabolic trait in lactobacilli that is shared with Gram-negative pathogens. The accumulation of GABA without glutaminase implies that an alternative pathway of glutamine deamidation exists in *L. reuteri* 100-23 to hydrolyze glutamine and produce glutamate as the precursor of GABA.

3.5 Supplementary materials

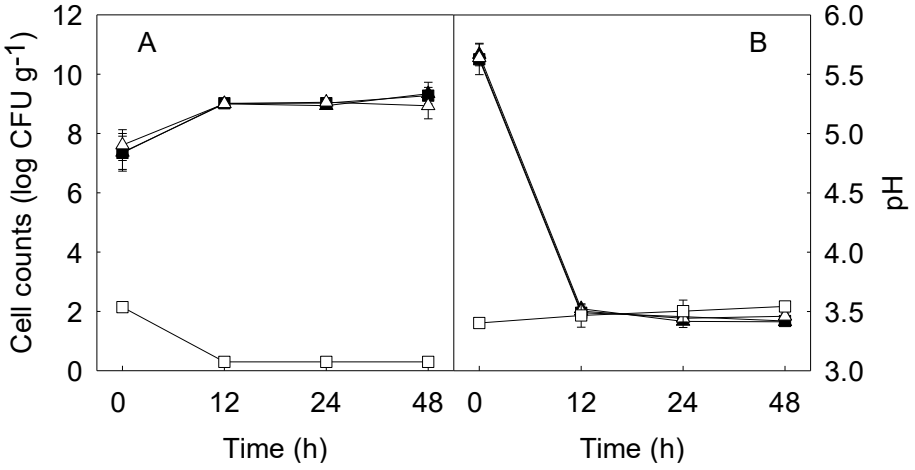


Figure 3. 7 Cell counts and pH during sourdough fermentation

Cell counts (A) and pH (B) during sourdough fermentation with *L. reuteri* 100-23 (▲), *L. reuteri* Δ*gls1*Δ*gls2*Δ*gls3* (Δ), or *L. reuteri* Δ*gadB* (■) over 48 h were quantified. Chemically acidified dough fermented for 48 h served as control (□). Symbols indicate means ± standard deviation of three biological replicates.

Chapter 4 Characterization of extracellular fructanase in *Lactobacillus crispatus* and its contribution to fructan hydrolysis in breadmaking

4.1 Introduction

Fermentable oligosaccharides, disaccharides, monosaccharides, and polyols (FODMAPs) trigger symptoms of the irritable bowel syndrome (IBS) [175–177], a gastrointestinal disorder affecting about 11 % of the population worldwide. FODMAPs are rapidly fermented by ileal and colonic microbiota to organic acids, H_2 and CO_2. The increase of luminal water and gas production can cause luminal distension, bloating, and osmotic diarrhea [177,178]. Sensitivity to FODMAPs is particularly frequent in fructose-malabsorbent individuals [179]; adverse symptoms are caused by the ingestion of FODMAPs exceeding about 15 g d^{-1}[180]. IBS significantly overlaps with non-celiac wheat intolerance [181]. Symptoms in many IBS patients and individuals with non-celiac wheat intolerance are improved by a low-FODMAP diet [182–185].

Whole grain products including bread contribute substantially to the dietary fiber intake [186] but wheat and rye also have a high content of FODMAPs with fructans as main contributors [36]. Rapidly-fermentable carbohydrates including fructans have prebiotic activity [187] and provide health benefits through short chain fatty acid production by intestinal bacteria including bifidobacteria and *Faecalibacterium prausnitzii* [188]. Low-FODMAP diets often reduce the overall dietary fibre intake and thereby also can reduce the intestinal abundance of bifidobacteria and the formation of short chain fatty acids [184,189].

Current strategies for the production of low-FODMAP bread without reducing the content of slowly-fermentable dietary fiber include fermentation with lactobacilli or yeasts that express extracellular fructanases [36,190,191]. The enzymatic degradation fermentation specifically reduces fructans; because the overall content of dietary fiber is not reduced substantially

[192], low-FODMAP bread may alleviate symptoms of IBS without the adverse consequences of a low fiber diet [130].

In straight dough, partial degradation of wheat or rye fructans, which have an average degree of polymerization (DP) of 5-6, is achieved by yeast invertase [36,193]. The activity of yeast invertase decreases with an increase in the molecular size of fructooligosaccharides because the dimeric enzymes do not accommodate long chain substrates [194,195]. Metabolism of fructan in most lactobacilli is mediated by the transport of low DP fructans followed by their intracellular hydrolysis [30], which thus provides only partial degradation of fructans in wheat and rye. Extracellular fructanases are rare in lactobacilli [30]. The extracellular β-fructosidase FosE in *Lacticaseibacillus paracasei* is induced by fructose, sucrose, or inulin but repressed by glucose [34,196]. FosE is also found in genomes of *Liquorilactobacillus* species. In *Lactobacillus* species, an extracellular fructosidase (FruA) has been identified in *Lactobacillus crispatus* DSM29598 [197]. However, the contribution of FruA in *L. crispatus* DSM29598 to fructan degradation in bread making is not well characterized, or compared to fructan degradation in conventional sourdoughs fermented with fructanases-negative lactobacilli. Therefore, this study explored the presence of FruA in lactic acid bacteria and characterized the location, substance specificity, and expression of FruA in *L. crispatus* DSM29598. Fructan degradation in bread produced with sourdough fermented with *L. crispatus* DSM29598 was compared to a straight dough process and conventional sourdough fermentation.

4.2 Materials and Methods

4.2.1. Strain and growth condition

L. crispatus DSM29598 and *Limosilactobacillus frumenti* FUA3675 were isolated from an industrially prepared rye sourdough; *Limosilactobacillus reuteri* 100-23 is a rodent isolate.

The strains were cultivated in modified deMan-Rogosa-Sharpe [152] (mMRS) medium at 37 °C. The frozen stock culture was inoculated on agar plates; single colonies were inoculated in 1 mL broth, subcultured with 1 % inoculum in broth. For induction and enzyme experiments, cells were grown in mMRS basal medium supplemented with filter-sterilized solutions of 1 % fructooligosaccharides (FOS with purify ≥ 90 %, Sigma-Aldrich, St. Louis, MO, USA), inulin from chicory (with purify ≥ 90 %, Sigma), levan (with purity > 98 %, Megazyme Inc, Bray, Ireland), glucose (Sigma), fructose (Sigma), or sucrose (Sigma).

4.2.2 Prediction of conserved proteins domains in FruA

Signal peptides were predicted by Signal 5.0 based on the amino acid sequences of FruA. The prediction of domains in FruA was conducted by comparison of amino acid (AA) sequences in the Conserved Domain Database on National Center for Biotechnology Information (NCBI) website.

4.2.3 Growth of *L. crispatus* with different carbon sources

L. crispatus was subcultured twice in mMRS basal broth containing 1 % inulin, mMRS or mMRS basal broth, respectively, and grown overnight. The cultures were subsequently used to inoculate (1-2 % inoculum) mMRS broth containing the sugars at the same concentrations. The optical density at 600 nm (OD_{600nm}) and pH of the culture were measured after overnight growth. Lactic acid production was quantified by HPLC as described [198].

4.2.4 Identification of FruA in *L. crispatus* and phylogenetic analysis of fructanases in lactobacilli

The genome of *L. crispatus* was sequenced by paired-end Illumina sequencing by service of Genome Quebec (Montreal, Quebec, Canada) and assembled with ABySS1.3.4 (Assembly By Short Sequence). After quality control with the FastQC tool and the removal of low quality reads by Quake [199], the genome was annotated by RAST. Protein sequences of

fructanases in lactic acid bacteria were retrieved from the National Center for Biotechnology Information database using BLASTp and FruA of *L. crispatus* DSM29598 or FosE of *Lacticaseibacillus paracasei* 1195 as query sequence. Fructanase sequences in lactic acid bacteria with more than 50 % coverage and more than 50 % amino acid identity to FruA were selected. The homologues with identity of 30 - 50 % to FruA were not from lactic acid bacteria. LevB of *Bacillus subtilis,* an extracellular fructanase from *Bacteroidales* was also selected for comparison. Fructanases identified in metagenomic sequences from piglets were included for comparison [37]. A phylogenetic tree was constructed using the Maximum Likelihood method based on the JTT matrix-based model [155] and the bootstrap support values were calculated from 100 replicates by MEGAX [200].

4.2.5 Hydrolysis of fructans by *L. crispatus*

L. crispatus was grown in mMRS broth containing 1 % designated carbon source and cells were harvested by centrifugation. Cells were fractionated at 4 °C to obtain concentrated culture supernatant, cell wall fraction, and concentrated cytoplasmic extract as described earlier [34].

For substrate specificity of FruA, *L. crispatus* was subcultured twice in mMRS basal broth containing 1 % FOS, inulin, or levan. The cultures were subsequently used to inoculate (1-2 % inoculum) mMRS broth containing the sugars at the same concentrations. When OD_{600nm} reached 1.0, the cells were collected by centrifugation and cell fractionation was performed as described above.

Hydrolysis of fructans was assayed by adding concentrated culture supernatant, cell wall fraction, or cytoplasmic extract to a 1 % FOS, inulin, or levan solution. The reaction mixtures were incubated at 37 °C for 3 h, and enzymes were inactivated by boiling for 2 min, and the enzyme activities were expressed as the amount of fructose released per minute per milligram of protein. Fructose concentrations were determined by high-performance liquid

chromatography (HPLC, 1200 series, Agilent Technologies, Santa Clara, CA, USA) using an Aminex HPX-87H column (Bio-Rad Laboratories, Mississauga, ON, Canada) that was eluted with 0.4 mL min^{-1} of 0.5mmol L^{-1} H_2SO_4 at 70 °C. Protein concentrations were determined with the Bradford reagent (Sigma), following the manufacturer's instruction. All experiments were done in triplicate.

4.2.6 Gene expression and activity of FruA in *L. crispatus* with multiple inducers

To determine *fruA* expression in *L. crispatus*, the strain was subcultured twice in mMRS basal broth containing 1 % FOS, sucrose, glucose, or fructose. The cultures were subsequently used to inoculate (1-2 % inoculum) mMRS broth containing the sugars at the same concentrations. When OD_{600nm} reached 0.4, the cells were collected by centrifugation. Cells were used for RNA extraction and cDNA construction, as well as cell fractionation. The hydrolysis of fructans by concentrated culture supernatant, cell wall fraction, or cytoplasmic extract were performed with 1 % FOS as substrate as described in 4.2.5.

4.2.7 Sourdough fermentation

Sourdoughs were fermented with *L. crispatus*, *L. frumenti*, *L. reuteri* and prepared with 100 g of steel-cut whole rye grain (Fazer Mills, Lahti, Finland) with 0.2 % of damaged starch or whole rye flour obtained at a local market in Edmonton with ~ 2.7 % damaged starch. Flour was mixed with 150 mL of sterilized tap water, incubated with an initial cell count of $1 \pm 0.5 \times 10^7$ CFU g^{-1}. Chemically acidified dough was prepared by adding acetic acid / lactic acid (1 : 4, v / v) to achieve a pH of 3.5 ± 0.25. All the sourdoughs were incubated at 37 °C. Samples were collected after 0, 12, 24, 48 and 72 h incubation and stored at -80 °C for RNA isolation and at -20 °C for the extraction of sugars and organic acids. All the fermentations were done in triplicate.

Sourdoughs fermented by cocktail of *L. crispatus* and *L. frumenti* were prepared as described above with equal initial cell counts of each strain of $1 \pm 0.5 \times 10^7$ CFU g^{-1}. Samples were collected after 0, 6, 12, 24, 48, and 72 h incubation and stored at -20 °C for extraction of sugars and organic acids. All the fermentations were done in duplicates.

4.2.8 RNA isolation and cDNA library construction of *L. crispatus*

To quantify the expression of FruA in *L. crispatus* in presence of 1 % fructose, glucose, sucrose, or FOS, samples were taken from cultures grown to the exponential phase (OD_{600nm} of 0.4). Cells were harvested by centrifuge at 4 ℃, then the supernatant was removed and cell pellets were resuspended into 1ml of fresh same media. Two volumes of RNA protect Bacteria Reagent (Qiagen, Mississauga, ON, Canada) were added to 1 mL of culture to maintain RNA integrity. After incubating 5 min at room temperature, cells were harvested by centrifugation and stored at -80 ℃.

For RNA extraction from *L. crispatus* in sourdough, 0.3-0.5 g sourdough was mixed with 3 mL of RNA protect bacterial reagent, incubated for 10 min at room temperature. Solids were removed by centrifugation at 400 g for 10 min. Cells in the supernatant were harvested by centrifugation and the cell pellets were stored at -80 ℃ prior to RNA isolation.

RNA was isolated from cell pellets using RNeasy Mini kit according to the manufacturer's instructions (Qiagen). Contaminant genomic DNA was digested by DNase treatment, and cDNA libraries were generated by Quantiscript Reverse Transcriptase as manufacturer's instructions (Qiagen). RNA samples were isolated from three biological replicates of exponentially growing cultures or fermented sourdoughs.

4.2.9 Relative quantification of gene expression by reverse-transcription quantitative PCR

Primers used for qPCR amplification targeting *fruA* and 16S rRNA of *L. crispatus* are listed in Table 4.1. The reactions were performed with QuantiFast SYBR green master mixture (Qiagen) in a 7500 Fast Real Time-PCR System (Applied Biosystems, Foster City, CA, USA. DNase-treated RNA samples and RNase-free water (Qiagen) were used as negative controls in all qPCR reactions. The efficiencies of the PCR reactions were determined in PCR reactions with serial 10-fold dilutions of chromosomal DNA of *L. crispatus* as a template. The efficiencies (E) of PCR reactions targeting *fruA* and 16S rRNA were 2.0 and 2.0, respectively. The relative expression ratio is calculated using 16S rRNA as a reference gene with 5 copies and exponential phase culture of *L. crispatus* in mMRS basal medium with 1 % glucose or FOS as reference condition[201]. The reaction for every sample was performed in duplicates.

Table 4. 1 Primers used to determine the expression of *fruA* in *L. crispatus* DSM29598

Targeted genes	Primers	Primer sequences(5'-3')	Annealing temperature (°C)	Amplicon size (bp)
fruA	FruA-F	CCACTAAAGCCGGAGAAGTAAA	63.1	105
	FruA-R	CTTGACCTCTGGCATCACTATG		
16S rRNA	*L. crispatus* 16s-F	GGTGGAGCATGTGGTTTAATTC	62.7	94
	L. crispatus 16s-R	CCCGAAGGGAACTTTGTATCT		

4.2.10 Bread baking

Sourdoughs were prepared with 40 g whole rye flour and 60 mL of cell suspension of *L. crispatus*, *L. reuteri* or cocktail of *L. crispatus* with *L. frumenti* to achieve an initial cell count of $1 \pm 0.5 \times 10^7$ CFU g^{-1}. Sourdoughs were incubated at 37 ℃ for 16h. Sourdough samples were collected after 0 and 16 h incubation and stored at -20 ℃ for the extraction of sugars and organic acids.

Bread dough was prepared by mixing sourdough prepared from 40 g whole rye flour with 60 g whole rye flour, 100 g wheat flour, 4 g yeast, 4 g salt, and 80 mL of water with a spiral kneader (Kitchen Aid K45SS, Hobart Co. Troy, OH, USA) for 9 min. The straight dough was prepared by mixing 100 g whole rye flour, 100 g wheat flour, 4 g yeast, 4 g salt, and 140 mL of water. Bread was also prepared by adding 2 g of FruA containing bread improver (Fazer LOFO™, Fazer Mills, Lahti, Finland) to the bread dough. After resting for 1.5 h at 32 °C, the bread was shaped by hand and placed for 1.5 h in an incubator at 37°C (Fisher Scientific, Waltham, MA, USA). Bread was baked in a multideck oven (Bakers Pride, Elgin, IL, USA) with forced air at a temperature of 177 °C for 20 min. The bread was cooled down for > 1 h at room temperature and cut into pieces. Bread dough samples were collected after proofing. The crust was cut off from bread, and bread crumb and crust were stored at -20 ℃ separately for sugar and organic acid analysis. All the bread baking was conducted in three independent experiments.

4.2.11 Quantification of fructan, fructose, and mannitol in sourdough and bread

Sugars and oligosaccharides from sourdough, bread dough, and bread samples were extracted by suspending 1 g samples into 4 mL of 0.1 mol L^{-1} sodium phosphate buffer (pH 8.0) and incubated at 80 ℃ for 2 h. Then the suspension was cooled and centrifuged to remove the solids. The supernatant was diluted in 10 fold with 18 MΩ water and filtered with 0.22 µm filters into vials for fructose analysis by HPLC as described in 4.2.5. Mannitol was quantified by HPLC as described [198].

Total fructans were quantified with the fructanases mixture in fructan assay kit (K-FRUC, Megazyme) and calculated referring to the equation in the kit. Samples were treated with fructanase by incubating 10-fold diluted extract with equal volumes of fructanase at 40 ℃ for 40 min, followed by boiling for 2 min. Controls were incubated in parallel with the addition of buffer instead of the enzyme. Fructose was quantified by HPLC as described in

4.2.5 and the fructan content was calculated as difference between samples incubated with or without fructanase. The concentration of fructan, fructose, and mannitol were expressed as mmol kg^{-1} based on dry mass.

4.2.12 Qualitative analysis of fructan in sourdough.

The qualification of fructan degradation during sourdough fermentation was performed with high-performance anion-exchange chromatography with pulsed ampero-metric detection (HPAEC-PAD). In brief, fructanase treated and untreated sourdough samples fermented with *L. crispatus* were separated on a Carbopac PA20 column coupled to an ED40 chemical detector (Dionex, Oakville, Canada). Water (A), 0.2 mol L^{-1} NaOH (B) and 1 mol L^{-1} NaAcetate (NaOAc) (C) were used as mobile phase with the following gradient: 0 min, 68.3 % A,30.4 % B and 1.3 % C; 30 min, 54.6 % A, 30.4 % B and 15.0 % C; 50 min, 46.6 % A, 30.4 % B and 23 % C; 95 min, 33.3 % A, 30.4 % B and 36.3 % C; 95.1 min, 63.7 % A and 36.3 % C; 100 min, 50 % A and 50 % C; 105min, 10 % A, 73 % B and 17 % C; 105.1 min, 33.3 % A, 30.4 % B and 36.3 % C; 111 min, 10 % A, 73 % B and 17 % C; followed by re-equilibration.

4.2.13 Statistical analysis

Data analysis was performed with IBM SPSS statistics 23, using one-way analysis of variance (ANOVA). A *P*-value of ≤ 0.05 was considered statistically significant.

4.2.14. Accession numbers

The genome sequences of *L. crispatus* DSM20598 and *L. frumenti* FUA3675 were deposited at Genebank with the accession numbers JAATOH000000000 and JAAUWX000000000, respectively.

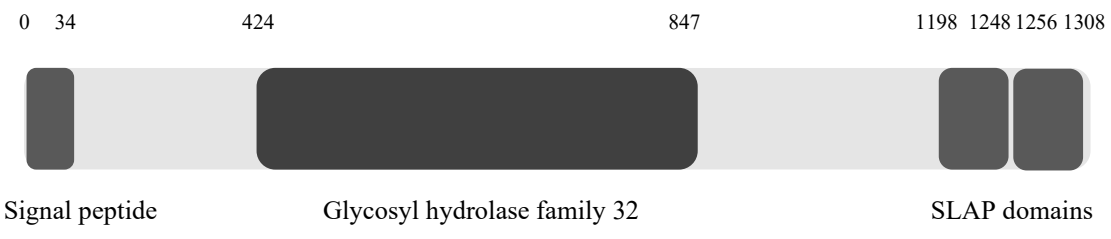
4.3 Results

4.3.1 Location of FruA in *L. crispatus* DSM29598

L. crispatus DSM29598 was isolated as a fructan-degrading organism from an industrial rye sourdough. Analysis of the genome sequence with FruA of *S. mutans*, LevB of *B. subtilis* and FosE of *Lacticaseibacillus paracasei* as query sequences demonstrated that *fruA* is the only gene in the genome of *L. crispatus* that encodes for a putative fructan hydrolase. Analysis of the protein sequence predicted an N-terminal sec-dependent signal peptide mediating protein export with a cleavage site between positiions 32 and 33 (VKA - DT) [202]. A predicted GH family 32 domain with 70.59 % amino acid identity to FruA of *S. mutans* is located between positions 424 and 847. The C-terminus of FruA contains two surface layer protein (SLAP) domains at positions 1198 to 1308 (Figure 4.1A). The presence of a signal peptide indicates that FruA is an extracellular fructosidase. The presence of a SLAP domain connected to a glycosyl hydrolase domain is unprecedented in lactic acid bacteria but may indicate that the enzyme is attached to surface layer proteins of *L. crispatus* [203].

The presence of an extracellular fructanase was confirmed by incubation of *L. crispatus* in media with FOS or inulin as sole carbon sources, which supported growth as well as glucose, maltose and fructose (Figure 4.9). The cellular location of FruA was assessed by determination of the fructanase activity of the supernatant, the cell wall fraction, and the cytoplasmic fraction of *L. crispatus* grown with FOS or inulin (Figure 4.1B). Fructanase activity was almost exclusively associated with the cell wall fraction.

A



B

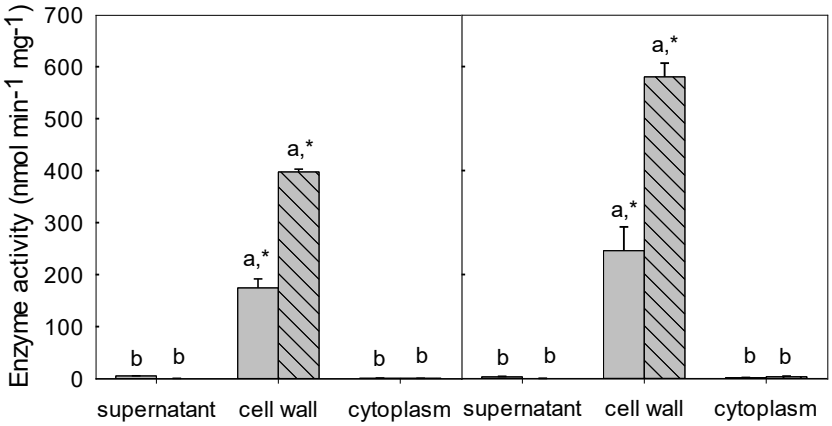


Figure 4. 1 Location of FruA in *L. crispatus* DSM29598

Panel A. Genetic indication of three domains in FruA. A signal peptide mediating protein secretion was predicted at the N-terminus; the C-terminus of the protein includes two predicted the S-layer domains which are unique to FruA in Lactobacillus species.
Panel B. Enzyme activities of supernatant, cell wall, and cytoplasm of *L. crispatus* DSM29598, respectively. *L. crispatus* DSM29598 was grown in mMRS with 1 %FOS (gray bars) or inulin (hatched bars) as sole carbon source. The enzyme activities were quantified with different cellular fractions and are expressed as the amount of fructose released from FOS (right) or inulin (left) per minute per milligram of protein. Values indicate mean ± standard deviation from three biological replicates. The activity of the different cellular fractions obtained from cells grown in the same medium differs significantly ($P < 0.05$) if bars do not share a common superscript. The activity of the same cellular fraction obtained from cells grown in different media differs significantly ($P < 0.05$) if bars are marked with an asterisk.

4.3.2 Phylogenetic analysis of fructanases in lactic acid bacteria

The phylogenetic analysis determined the distribution and frequency of fructanases in lactic acid bacteria (Figure 4.2). Fructanases were mainly identified in *Streptococcus* spp. and in few genomes of the genera *Enterococcus*, *Latilactobacillus* (previously *Lactobacillus sakei* group [11]), *Ligilactobacillus*, *Liquorilactobacillu*s, *Lacticaseibacillus* (previously *Lactobacillus casei* group [11]), *Pediococcus,* as well as *Lactobacillus*. Four of these fructanases have been characterized biochemically, including LevB in *Bacillus subtilis*, FosE in *L. paracasei,* FruA in *S. mutans*, and FruA in *L. crispatus* (this study)[197]. The homologues of FosE were mainly identified in *Lacticaseibacillus* and *Liquorilactobacillus*, as well as four genomes of *Bacteroidales* [37]. FruA in *L. crispatus* is most similar to FruA in *S. mutans*, but not homologous to LevB or FosE. Homologues of FruA were also identified

in *Ligilactobacillus equi*, *Lactobacillus amylovorus*, *Ligilactobacillus salivarius*, *Latilactobacillus curvatus*, as well as two genomes of *L. delbrueckii* and *L. amylovorus* from piglets [37]. With exception of *L. curvatus*, FruA was exclusively found in host-adapted lactobacilli.

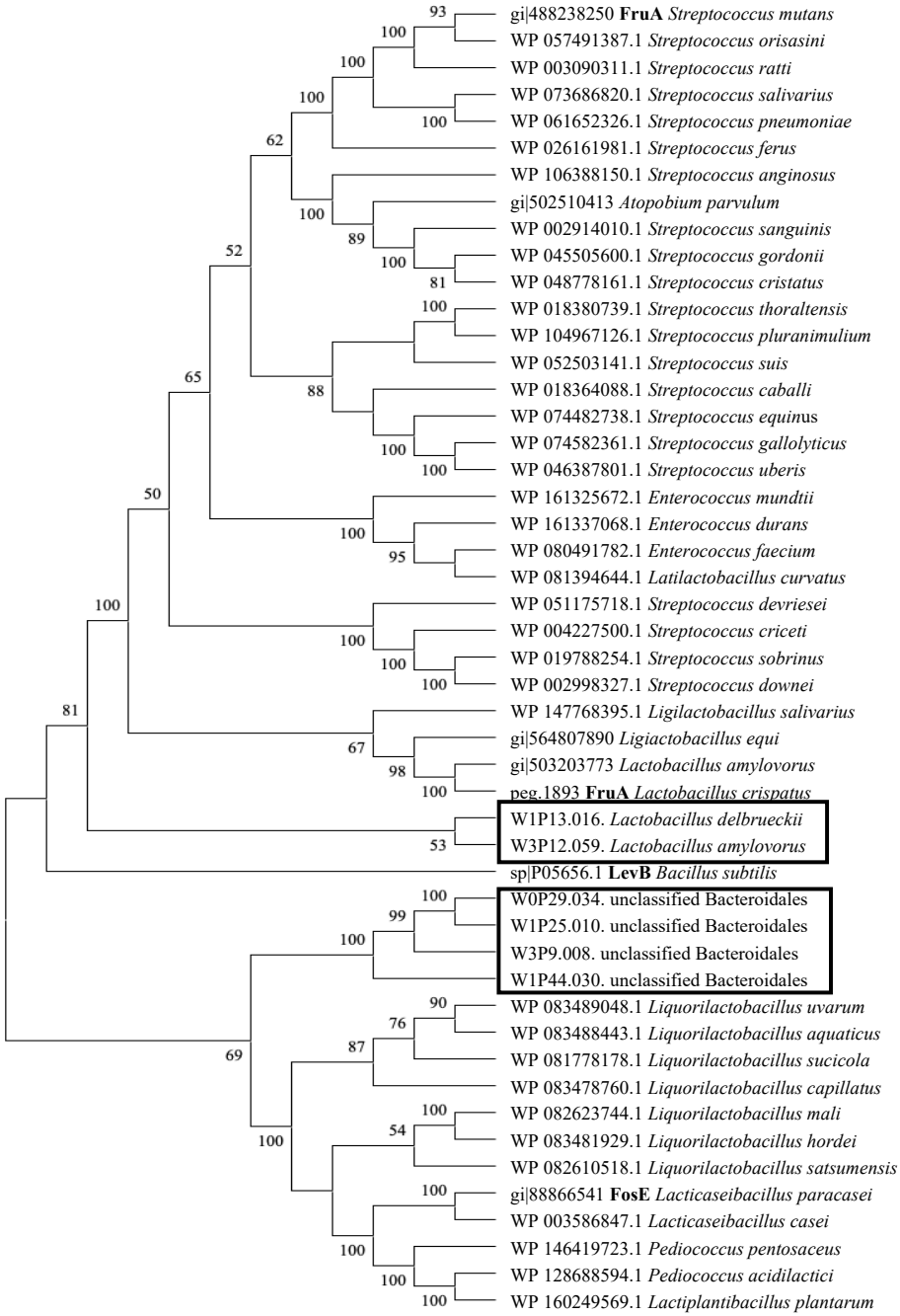


Figure 4. 2 Phylogenomic analysis of FruA in lactobacilli.

Sequences in all genomes were identified by BLASTp using FruA in *L. crispatus* DSM29598 as query sequences. Shown are all FruA sequences with more than 50 % coverage and more than 50% amino acid identity

in lactobacilli species and representative FruA sequences from every species of lactic acid bacteria in which the enzyme was identified. LevS of *Bacillus subtilis,* an extracellular fructanase from *Bacteroidales* and selected homologues of FosE in *Lacticaseibacillus* and *Liquorilactobacillus* are shown for comparison. The sequences highlighted by boxes were obtained from the intestine of piglets [37].

4.3.3 Substrate specificity of FruA in *L. crispatus*

The hydrolysis of FOS, inulin, and levan was evaluated with cell wall fractions of *L. crispatus* grown in FOS, inulin or levan as sole carbon sources (Figure 4.3). FruA activity of *L. crispatus* was highest with levan as substrate, followed by FOS and inulin (Figure 4.3). *L. crispatus* grow with inulin expressed higher fructanase activity when compared to cultures grown with FOS or levan (Figure 4.3). The enzyme activity of FruA in *L. crispatus* on FOS or inulin was comparable when induced by FOS or inulin, which is consistent with FosE in *L. paracasei* [34]. When induced by inulin or levan, FruA showed higher activity on hydrolysis of levan than FOS or inulin, consistent with FruA characterized in *S. mutans*, which exhibited the highest specificity for levan [204].

4.3.4 Gene expression and activity of FruA in *L. crispatus* with multiple inducers

In homofermentative lactobacilli, the utilization of carbohydrate sources other than glucose is generally repressed by carbon catabolite repression [96,205]. To determine the expression of FruA in *L. crispatus* in presence of glucose and fructans, gene expression and enzyme activity of FruA were quantified after the growth of *L. crispatus* to the exponential phase of growth with glucose, fructose, sucrose, or FOS as sole carbon sources (Figure 4.4). Gene expression of FruA was calculated relative to the expression in mMRS with 1 % glucose and comparable levels of expression were detected after incubation with all of the substrates (Figure 4.4A). Analysis of fructosidase activity at the same conditions provided similar results (Figure 4.4B). Both mRNA abundance and quantification of FruA activity thus demonstrated that FruA is not repressed by glucose.

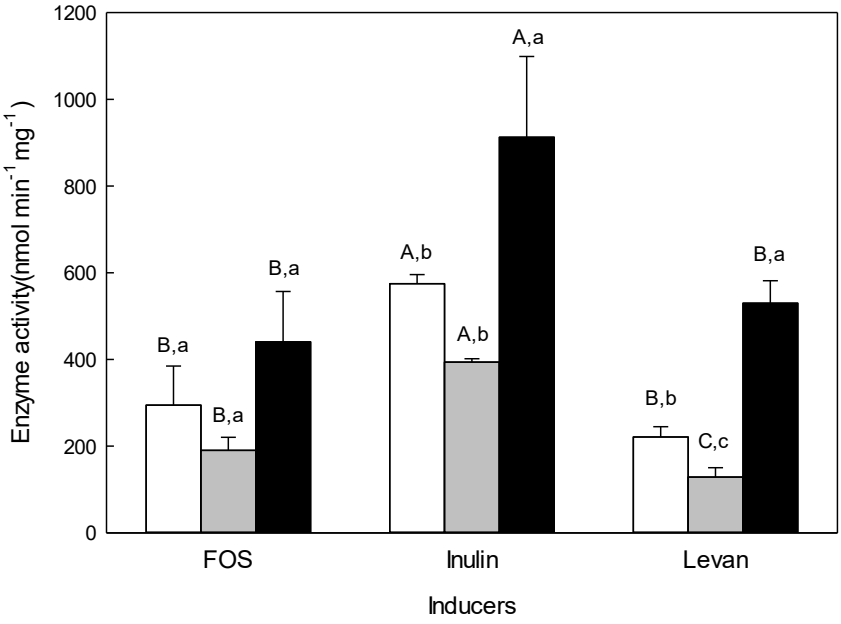


Figure 4. 3 Enzyme activity of FruA in *L. crispatus* DSM29598

L. crispatus DSM29598 was grown in mMRS containing FOS, inulin, or levan as sole carbohydrate sources as indicated on the x-axis. The activities were quantified with FOS (white bars), inulin (gray bars) or levan (black bars) as substrates. Values indicate mean ± standard deviation from three biological replicates. Significant differences ($P < 0.05$) between different inducers with the same substrate are indicated by uppercase letters; significant differences ($P < 0.05$) between different substrates with the same inducer are indicated by lowercase letters.

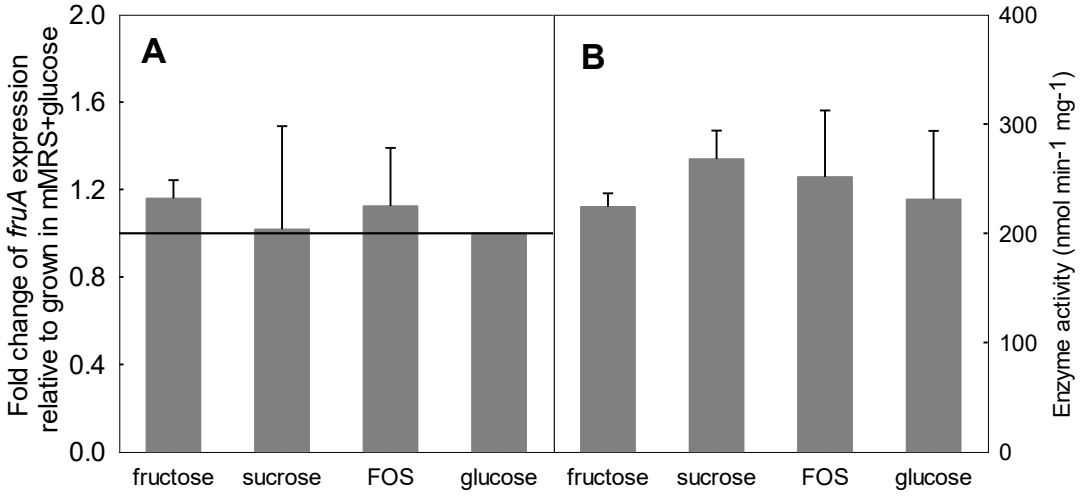


Figure 4. 4 The effects of substrates on the expression of FruA in *L. crispatus* DSM29598

Panel A. Fold change of *fruA* mRNA in cells grown with fructose, sucrose, FOS as sole carbon source relative to the mRNA level in cells grown with glucose.
Panel B. Enzyme activity of FruA induced by fructose, sucrose, FOS, or glucose with FOS as substrate. Values indicate mean ± standard deviation from three biological replicates. Values obtained with different substrates were not significantly different ($P > 0.05$).

4.3.5 Gene expression of FruA of *L. crispatus* during sourdough fermentation

Sourdoughs were prepared with steel-cut kernels of rye with 0.2 % damaged starch or rye flour with ~2.7 % damaged starch. Damaged starch is hydrolyzed by cereal amylases to generate maltose and maltodextrins which are utilized by intracellular glycosyl hydrolases of lactobacilli to generate glucose [30]. During 72 h sourdough fermentation, the expression of FruA in *L. crispatus* growing whole rye and rye flour sourdoughs was not different (Figure 4.5), which confirms that expression of FruA is not repressed by glucose. Compared to the expression in mMRS with FOS, FruA was overexpressed after fermented for 12 h in both sourdoughs.

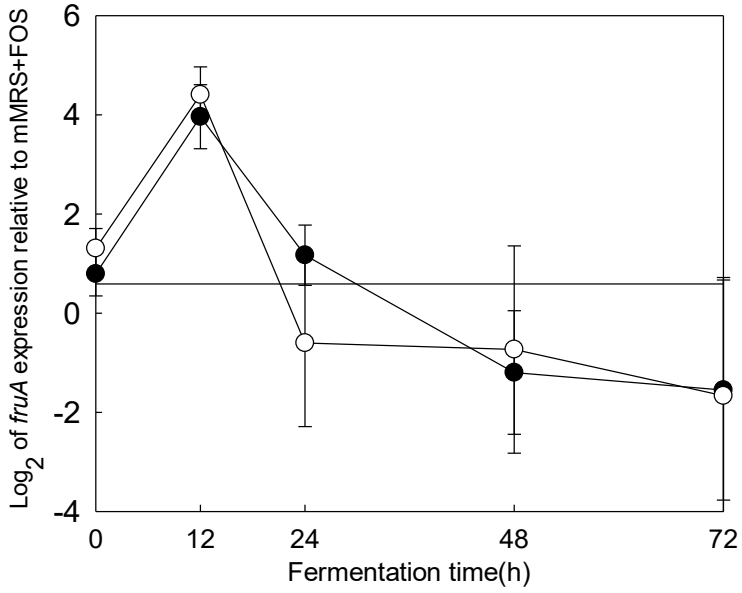


Figure 4. 5 Expression of *fruA* during sourdough fermentation

Gene expression was tested during whole rye (black symbols) and rye flour (open symbols) sourdough fermentation with *L. crispatus* DSM29598, and calculated relative to the expression of *fruA* during the growth of *L. crispatus* in mMRS with FOS as carbon source. Symbols indicate mean ± standard deviation from three independent fermentations.

4.3.6 Fructan degradation during sourdough fermentation

Fructan degradation in sourdough was determined by HPAEC-PAD analysis of sourdough extracts (Figure 4.6), and by enzymatic quantification of fructans (Figure 4.7). Sourdough

samples collected before fermentation (0 h) showed a series of peaks with a retention time of 17 - 40 min. These peaks were degraded by fructanase treatment of sourdough extracts and represent fructans with a DP of 3-10 according to the chromatograph of FOS (data not shown). These fructans were not present in sourdough fermented for 12h with *L. crispatus* (Figure 4.6).

The fructan content was also quantified enzymatically over 72 h fermentation (Figure 4.7). Fructan hydrolysis by *L. crispatus* was compared to *L. frumenti, L. reuteri,* and a chemically acidified dough. The genome of *L. reuteri* 100-23 does not contain extracellular fructanases [206]; a copy of *fruA* was identified in the genome of *L. frumenti* but the gene is not expressed and *L. frumenti* does not hydrolyze fructans (data not shown and Figure 4.10). Fructan content in sourdoughs fermented with *L. crispatus* decreased to less than 0.5 % after 12h fermentation. The fructan content of chemically acidified doughs remained essentially unchanged over the incubation period. Fermentation with the FruA-negative *L. reuteri* and *L. frumenti* reduced the fructan content to intermediate levels, about 1 % in whole rye sourdoughs (Figure 4.7A) and about 2 % in rye sourdoughs (Figure 4.7B). FruA is thus necessary to achieve rapid and complete hydrolysis of fructans.

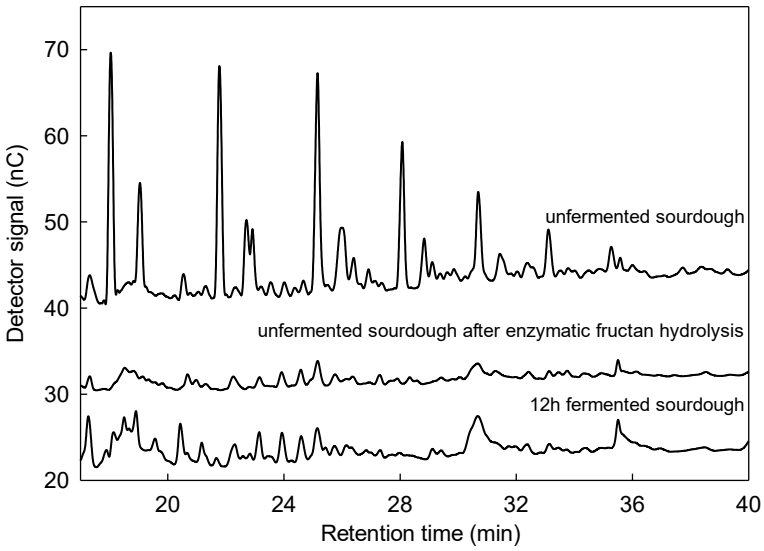


Figure 4. 6 Qualitative analysis of fructan hydrolysis by *L. crispatus* during growth in whole rye sourdough

Shown are chromatographic traces obtained with HPAEC-PAD analysis of unfermented sourdough, unfermented sourdough after enzymatic fructan hydrolysis, and sourdough fermented with *L. crispatus* after 12h of fermentation. Enzymatic fructan hydrolysis of the 12 h fermented *L. crispatus* sourdough did not alter the peak pattern (data not shown). Chromatograms are representative of two independent biological replicates.

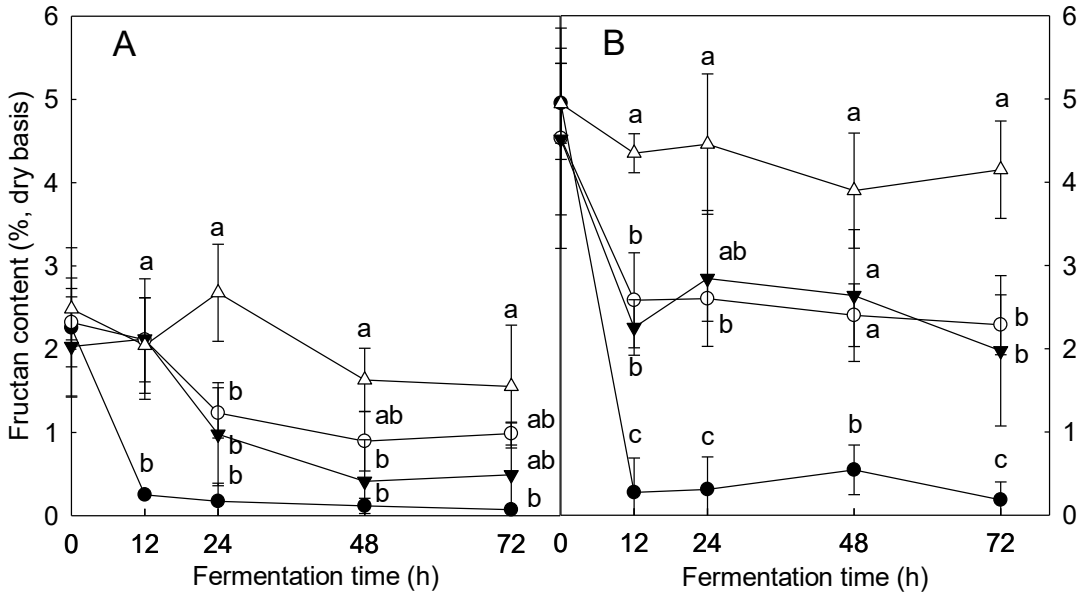


Figure 4. 7 Fructan content of whole rye and rye flour sourdoughs

Sourdoughs were prepared with whole rye (Panel A) or rye flour (Panel B) and fermented with *L. crispatus* DSM29598 (●), *L. frumenti* FUA3675 (○) or *L. reuteri* 100-23 (▼), and of chemically acidified dough (Δ). Values are expressed as mean ± standard deviation from three independent experiments. The fructan content of different sourdoughs after the same fermentation time differs significantly ($P < 0.05$) if symbols do not share a common superscript.

4.3.7 Dynamics of fructan, fructose, and mannitol content during sourdough fermentation with strain cocktails

Type II sourdoughs generally are dominated by homofermentative *Lactobacillus* species and heterofermentative *Limosilactobacillus* species. Heterofermentative lactobacilli convert fructose to mannitol, which is included in the definition of FODMAPs. To test fructan degradation and mannitol formation during co-fermentation of *L. crispatus* and *L. frumenti*, whole rye and rye flour sourdoughs were fermented with these two strains and fructan, fructose, and mannitol concentration were monitored (Figure 4.8). The fructan content in sourdoughs decreased after 12 h of fermentation, resulting in a temporary accumulation of fructose and, during later stages of fermentation, mannitol. In both sourdoughs, less than 50 % of the fructose released from fructans was converted to mannitol (Figure 4.8).

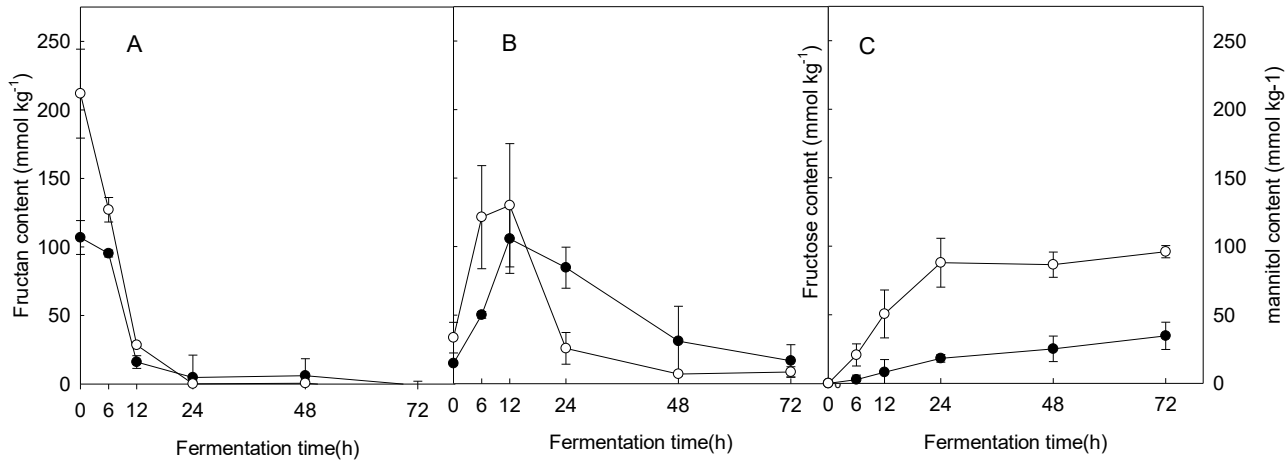


Figure 4. 8 Fructan, fructose and mannitol content of sourdoughs fermented with two strains

Fructan (Panel A), fructose (Panel B), and mannitol (Panel C) content of whole rye (●) and rye flour (○) sourdoughs after fermentation with *L. crispatus* DSM29598 and *L. frumenti* FUA3675. Values are expressed as mean ± standard deviation from two independent experiments and each sample was analyzed in duplicate.

4.3.8 Effect of *L. crispatus* and FruA on the content of fructan, fructose, and mannitol in bread

To assess if *L. crispatus* or FruA is sufficient to degrade majority of fructan in a bread baking process, sourdough bread were prepared with *L. crispatus*, a co-culture of *L. crispatus* and *L. frumenti*, or *L. reuteri* as FruA-negative control. Sourdough breads were compared to yeast-leavened bread (straight dough control) with or without addition of FruA, and to sourdough bread fermented with *L. reuteri* and addition of FruA (Table 4.2). The fructan content was highest in the straight dough control, here, the fructan content of bread was comparable to the fructan content of the wheat and rye flours used in bread making. FruA addition to the straight dough control reduced fructans to non-detectable levels. Sourdough fermented with *L. reuteri* reduced fructans to about 50 % of the initial levels; the concentration of mannitol in bread accounted for about 10% of the initial fructan. FruA addition to *L. reuteri* sourdough bread also substantially reduced the fructan content of bread to non-detectable levels but did not increase the content of mannitol. The use of sourdough fermented with *L. crispatus* reduced fructan levels in bread to about 5 % of the fructan content in flours (Table 4.2). Co-fermentation of *L. crispatus* and *L. frumenti* resulted in a comparable reduction of fructan in bread but accumulated 25 mmol kg^1 mannitol (Table 4.2).

Analysis of the fructan content throughout the bread-making process confirmed that fructans were completely (*L. crispatus*) or substantially (*L. reuteri*) degraded during sourdough fermentation (Table 4.2). Fructans were further degraded after mixing and proofing of the bread dough (Table 4.2) while the reduction of fructan levels after baking was not significant (Table 4.2). The degradation of fructan after 16 h of sourdough fermentation led to an increase of fructose in sourdough with only *L. crispatus*, and a higher level of mannitol content in sourdough with *L. crispatus* and *L. frumenti*. In bread doughs after proofing, the fructan contents of straight dough and sourdough fermented with *L. reuteri* were significantly

higher than doughs with either *L. crispatus* or FruA containing bread improver as complement, consistent with the fructan contents in bread. Addition of FruA also decreased the fructan content of bread produced with *L. reuteri* sourdough.

Table 4. 2 Fructan, fructose and mannitol content of sourdoughs, bread dough, and bread after fermentation with different *L. crispatus* DSM29598, *L. crispatus* DSM29598 and *L. frumenti* FUA3675, or *L. reuteri* 100-23

Strain	FruA addition		Sampling	Contents in dry basis (mmol kg^{-1})		
				Fructans	Fructose	Mannitol
Whole rye				242.8 ± 26.2	n.d.	n.d.
Whole wheat				120.4 ± 7.7	n.d.	n.d.
L. crispatus	N	Sourdough*	0h	$160.5 \pm 42.1^{A,a}$	$43.0 \pm 9.5^{B,a}$	$0.9 \pm 0.6^{A,a}$
			16h	$4.9 \pm 4.6^{B,b}$	$133.9 \pm 26.4^{A,a}$	$1.5 \pm 0.9^{A,c}$
		Bread dough*	Proofing	$26.4 \pm 17.5^{B,b}$	$42.4 \pm 13.92^{B,a}$	$2.3 \pm 1.6^{A,c}$
		Bread	Crumb	$10.9 \pm 3.5^{B,c}$	$9.7 \pm 1.3^{B,ab}$	$0.7 \pm 0.4^{A,c}$
			Crust	$20.8 \pm 5.6^{B,bc}$	$11.6 \pm 3.3^{B,ab}$	$1.3 \pm 1.0^{A,b}$
L. crispatus and *L. frumenti*	N	Sourdough	0h	$154.7 \pm 29.2^{A,a}$	$37.1 \pm 9.1^{A,a}$	$3.0 \pm 4.3^{C,a}$
			16h	$4.4 \pm 2.0^{B,b}$	$23.0 \pm 15.9^{AB,b}$	$63.2 \pm 9.6^{A,a}$
		Bread dough	Proofing	$4.3 \pm 1.0^{B,b}$	$10.4 \pm 3.5^{B,b}$	$22.1 \pm 7.5^{BC,a}$
		Bread	Crumb	$11.5 \pm 2.5^{B,c}$	$6.6 \pm 1.9^{B,ab}$	$15.5 \pm 5.7^{BC,a}$
			Crust	$13.7 \pm 9.0^{B,bc}$	$10.5 \pm 1.9^{B,ab}$	$25.7 \pm 7.8^{B,a}$
L. reuteri	N	Sourdough	0h	$103.3 \pm 13.6^{A,a}$	$25.3 \pm 1.1^{A,a}$	$4.9 \pm 1.8^{C,a}$
			16h	$89.8 \pm 6.0^{AB,a}$	$22.5 \pm 6.6^{A,b}$	$37.9 \pm 2.2^{A,b}$
		Bread dough	Proofing	$70.2 \pm 19.3^{ABC,a}$	$22.1 \pm 14.2^{A,ab}$	$17.2 \pm 3.7^{B,ab}$
		Bread	Crumb	$40.9 \pm 11.7^{C,b}$	$8.2 \pm 7.6^{A,ab}$	$8.9 \pm 4.5^{C,ab}$
			Crust	$50.2 \pm 28.9^{BC,b}$	$10.7 \pm 9.2^{A,ab}$	$19.3 \pm 2.0^{B,a}$
L. reuteri	Y	Bread dough	Proofing	$n.d.^{A,b}$	$2.7 \pm 1.9^{A,b}$	$21.3 \pm 0.7^{A,a}$
		Bread	Crumb	$n.d.^{A,c}$	$1.2 \pm 0.4^{A,b}$	$12.2 \pm 0.6^{B,ab}$
			Crust	$n.d.^{A,c}$	$1.0 \pm 0.8^{A,b}$	$24.3 \pm 2.2^{A,a}$
Straight dough	Y	Bread dough	Proofing	$n.d.^{A,b}$	$10.4 \pm 3.9^{A,b}$	$9.1 \pm 2.4^{A,bc}$
		Bread	Crumb	$n.d.^{A,c}$	$8.0 \pm 1.6^{A,ab}$	$3.5 \pm 2.4^{C,bc}$
			Crust	$n.d.^{A,c}$	$12.5 \pm 2.9^{A,ab}$	$8.5 \pm 0.7^{BC,b}$
Straight dough	N	Bread dough	Proofing	$101.2 \pm 12.9^{A,a}$	$24.7 \pm 8.9^{A,ab}$	$3.5 \pm 0.6^{B,c}$
		Bread	Crumb	$120.1 \pm 20.9^{A,a}$	$20.9 \pm 14.8^{A,a}$	$3.1 \pm 1.3^{B,c}$
			Crust	$154.7 \pm 30.8^{A,a}$	$20.1 \pm 14.5^{A,a}$	$6.1 \pm 0.1^{A,b}$

n.d., not detectable.

* Sourdough was prepared with whole rye flour; the bread dough was prepared with 100 g whole rye flour and 100 g whole wheat flour.

Data obtained from the same dough at different sampling times differ significantly ($P < 0.05$) if they do not share a common capital letter superscript. Data obtained with different doughs at the same sampling time differ significantly ($P < 0.05$) if they do not share a common lowercase letter superscript.

4.4 Discussion

This study investigated the location, induction, and substrate specificity of FruA in *L. crispatus* DSM29598. FruA is an extracellular surface protein in this strain, induced by all the carbon source used in this study and not repressed by the presence of glucose. FruA is an exo-β-fructosidase hydrolyzing terminal, non-reducing fructose moieties linked by β-(2→1) bonds in FOS or inulin, as well as β-(2→6) bonds in levan. The application of *L. crispatus* in sourdough fermentation hydrolyzed the majority of fructan in rye during the first 12 h fermentation. A combination of *L. crispatus* and *L. frumenti* in sourdough fermentation leads to dynamic changes of fructan, fructose, and mannitol. Bread dough prepared with *L. crispatus* fermented sourdough has a much lower content of fructan but the comparable amount of fructose and mannitol compared to straight dough; the same results can also be achieved by adding FruA to the bread dough. When *L. crispatus* or FruA was present, fermentation with heterofermentative *L. frumenti* or *L. reuteri* led to higher mannitol contents. Extracellular FruA is frequently present in oral streptococci. Oral streptococci convert sucrose to extracellular glucans and fructans; both polymers form part of the biofilm matrix on the tooth enamel. In addition, fructans are hydrolyzed for use as a carbon source if other sugars are not available [10,207]. Homologues of FruA are also present in the vertebrate-host adapted genera *Lactobacillus* and *Ligilactobacillus.* FruA in host-adapted lactic acid bacteria contributes to the degradation of fructans that are present in the diet, or fructans that are formed by *Limosilactobacillus* species as part of the biofilm matrix [10,37]. A second extracellular fructosidase in lactobacili, FosE, is present in few *Lacticaseibacillus* and several *Liquorilactobacillus* species [34][37]. *Liquorilactobacillus* species form biofilms in liquid and plant derived habitats [11]. In analogy to oral streptococci, the biofilm matrix is formed by glucans and fructans synthesized from sucrose and FosE likely serves a comparable role in *Liquorilactobacillus* species as FruA in oral streptococci [11].

FruA in *S. mutants* has an N-terminal export signal, a C-terminal LPXTGX motif which covalently links proteins in Gram-positive bacteria to the peptidoglycan [208], and was shown experimentally to be located at the cell surface [209]. Similar to streptococci, the FruA in *L. crispatus* has an N-terminal export signal. In contrast to FruA in streptococci, FruA in *L. crispatus* lacks the LPXTG motif but includes two C-terminal SLAP domains. The SLAP domain is found in multiple cell surface proteins of lactobacilli including a putative SLAP in *L. acidophilus* NCFM[210] and *L. crispatus* (WP_013086129.1), and surface layer associated protein in *Lactobacillus pasteurii* DSM 23907 (GenBank: CCI84788.1). Consistent with the organization of SLAPs in *L. acidophilus* ATCC4356 [211] and *L. crispatus* JCM5810 [212], the SLAP domains of FruA are at the C-terminus of the protein while surface-associated proteins in *Levilactobacillus brevis* [213] and *Lentilactobacillus hilgardii* [214] display the SLAP domain in the N-terminal region. We confirmed the association of FruA with the cell wall of *L. crispatus;* the SLAP domains of FruA are thus likely responsible for anchoring of the enzyme to the cell wall [215]. Surface layer protein is mostly present in the vertebrate-host adapted *Lactobacillus* species (previously *L. delbrueckii* group) and found only in few *Levilactobacillus* and *Lentilactobacillus* species [2]. BLASTp analysis and the identification of conserved SLAP domains on NCBI demonstrated that the SLAP domain in FruA and the combination of a GH32 catalytic domain with SLAP domain are exclusive to vertebrate-host adapted *Lactobacillus* species. S-layers composed of surface layer proteins promote cell adhesion [215], and most of the surface proteins in *L. reuteri* are predicted to be involved in epithelial adhesion and biofilm formation [47]. *Lactobacillus* species alone do not form intestinal biofilms but generally co-exist with biofilm-forming *Limosilactobacillus* species [2,158]. Taken together, the surface-associated FruA in *L. crispatus* DSM29598 and other swine-associated lactobacilli likely serves two ecological

functions, first, to attach to the biofilm matrix formed by *Limosilactobacillus* species, and second, to use extracellular fructans as carbon source.

In homofermentative lactobacilli including *Lactobacillus* species, the expression of glycosyl hydrolases is generally subject to carbon catabolite repression [30]. Carbon catabolite repression is regulated at the transcriptional level. The levanase operon in *B. subtilis* is repressed by catabolite control protein CcpA repressor in the presence of glucose and induced at the transcriptional level by fructose [216]. FruA expression in *S. mutans* was modulated by antitermination mechanism as well as CcpA binding [207]. The extracellular fructanases LevB in *L. casei* is repressed by the regulator LevR through phosphotransferase-system mediated catabolite repression [217]. The *fosE* operon in *L. paracasei* is likely controlled by similar mechanisms as a transcriptional regulator and a *cre* binding site was identified [34]. FruA in *L. crispatus* DSM29598 is a part of an operon and the promotor region does not include a *cre* binding site (Figure 4.11). Moreover, open reading frames with homology to transposases or mobile element proteins are located directly upstream and downstream of *fruA*, suggesting that *fruA* was acquired by lateral gene transfer (Figure 4.11). The differences in the transcriptional regulation may relate to the differences in protein structure and ecological function. In *Lactobacillus* species but not in oral streptococci, FruA includes SLAP domains which likely serve an important ecological function independent of the carbohydrate sources that are available. The differences between the regulation of FruA in *Lactobacillus* species and the regulation of FruA, FosE, and LevB in other homofermentative lactic acid bacteria have important repercussions for FruA expression in sourdough fermentation, where glucose and maltose are readily available.

FruA of *L. crispatus* was expressed in sourdough irrespective of the presence of glucose and maltose, and the use of FruA-positive *L. crispatus* allowed the production of low FODMAP bread. Remarkably, the FruA activity in sourdough was sufficient to degrade fructans during

mixing and proofing, which confirms and extends prior analyses of bread produced with sourdough including FruA-positive lactobacilli [192]. Fructan degradation during wheat baking was also achieved with *Kluyveromyces marxianus* producing an extracellular inulinase [191]. The co-culture of *K. marxianus* and *S. cerevisiae* in bread preparation resulted in fructan levels below 0.2 % dm [218]. The use of sourdough with FruA-expressing lactobacilli, however, not only reduced fructans and other FODMAPS but also has the advantage of using established fermentation processes, and to improve bread flavor.

Fructan degradation in a straight dough process with *Saccharomyces cerevisiae* degraded fructans by about 56 %. Yeast invertase has only limited activity towards fructan with high DP [218]. Remarkably, conventional sourdough fermentation reduced fructans in bread by 65 to 75 % (this study) [219]. Most lactobacilli are capable to hydrolyze fructan with a DP of 4 or less, which are transported into cells [13] and hydrolyzed by intracellular fructosidases or phospho-fructosidases [30]. In addition, raffinose-family oligosaccharides in wheat and rye sourdoughs are hydrolysed by α-galactosidase, levansucrase, and sucrose-phosphorylase activities [220]. However, the fructan levels are above the cut-off value for low FODMAP diet that was defined as less than 0.3 g FODMAPs per serving of grains or cereals that by Varney et al. for IBS patients [221]. Mannitol produced by heterofermentative lactobacilli adds to the FODMAP load [179]. When assuming that fructans, fructose and mannitol are the major FODMAPs in bread, a value of 0.3 g FODMAPs corresponds to 2.7 mmol [fructose + mannitol]. Consumption of more than 120 g (wet weight) of any bread produced with *L. crispatus* or FruA addition is required to exceed that dose, in contrast, 0.3 g of [fructose + mannitol] are contained in 70 g of conventional sourdough bread and in 28 g of straight dough bread. Because adverse or beneficial effects of FODMAPs are dose-dependent, the FODMAP reduction that was achieved by conventional sourdough fermentation may suffice to prevent adverse symptoms in many individuals [180,187].

In conclusion, extracellular FruA in *L. crispatus* DSM29598 hydrolyzed both inulin-type and levan-type fructans without subject to glucose repression. Phylogenetic analysis indicates a role of FruA in host-adapted lactic acid bacteria, which allows organisms to utilize fructans as carbon source, and to attach to biofilms. Analyzing the presence of FruA in organisms from an ecological perspective helps to explore its use in food production [10]. Conventional sourdough application in bread making likely improves tolerance in many individuals by substantial reduction of fructan levels (this study)[219] and by partial detoxification of offending proteins including the amylase trypsin inhibitor [222]. Including *L. crispatus* DSM29598 in sourdoughs reduced the fructan content in the final products by > 90 % and total FODMAPs by > 70 %, thereby produce a low FODMAP bread for IBS patients (this study)[130,192].

4.5 Supplementary materials

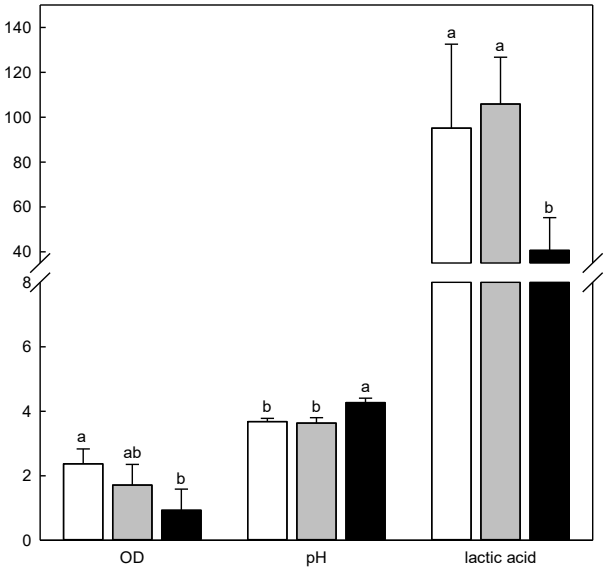


Figure 4. 9 Growth of *L. crispatus* DSM29598 in mMRS with inulin, mMRS or mMRS base

L. crispatus was incubated overnight in mMRS with 1 % of inulin (white bar), mMRS (gray bar), or mMRS base broth (black bar), respectively. OD_{600}, pH and lactic acid production of *L. crispatus* DSM29598 were quantified. Lactic acid was expressed as mmol kg^{-1}. Significant differences ($P < 0.05$) between different media are indicated by different letters.

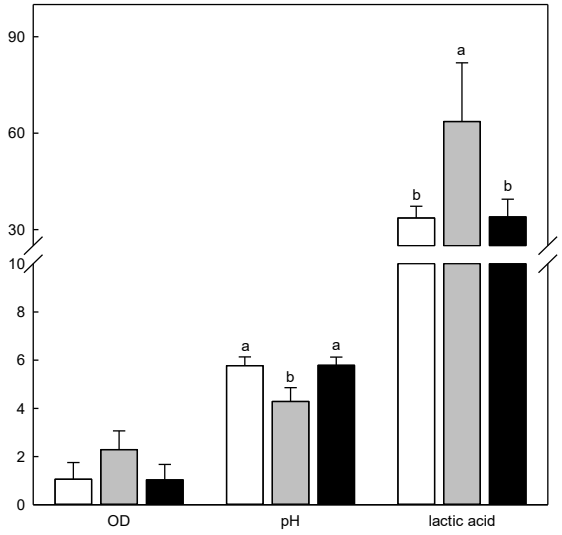


Figure 4. 10 Growth of *L. frumenti* FUA3675 in mMRS with inulin, mMRS or mMRS base.

L. frumenti FUA3675 was incubated overnight in mMRS with 1 % of inulin (white bar), mMRS (white bar), or mMRS base broth (black bar), respectively. Lactic acid was expressed as mmol kg^{-1}. Significant differences ($P < 0.05$) between different media are indicated by different letters.

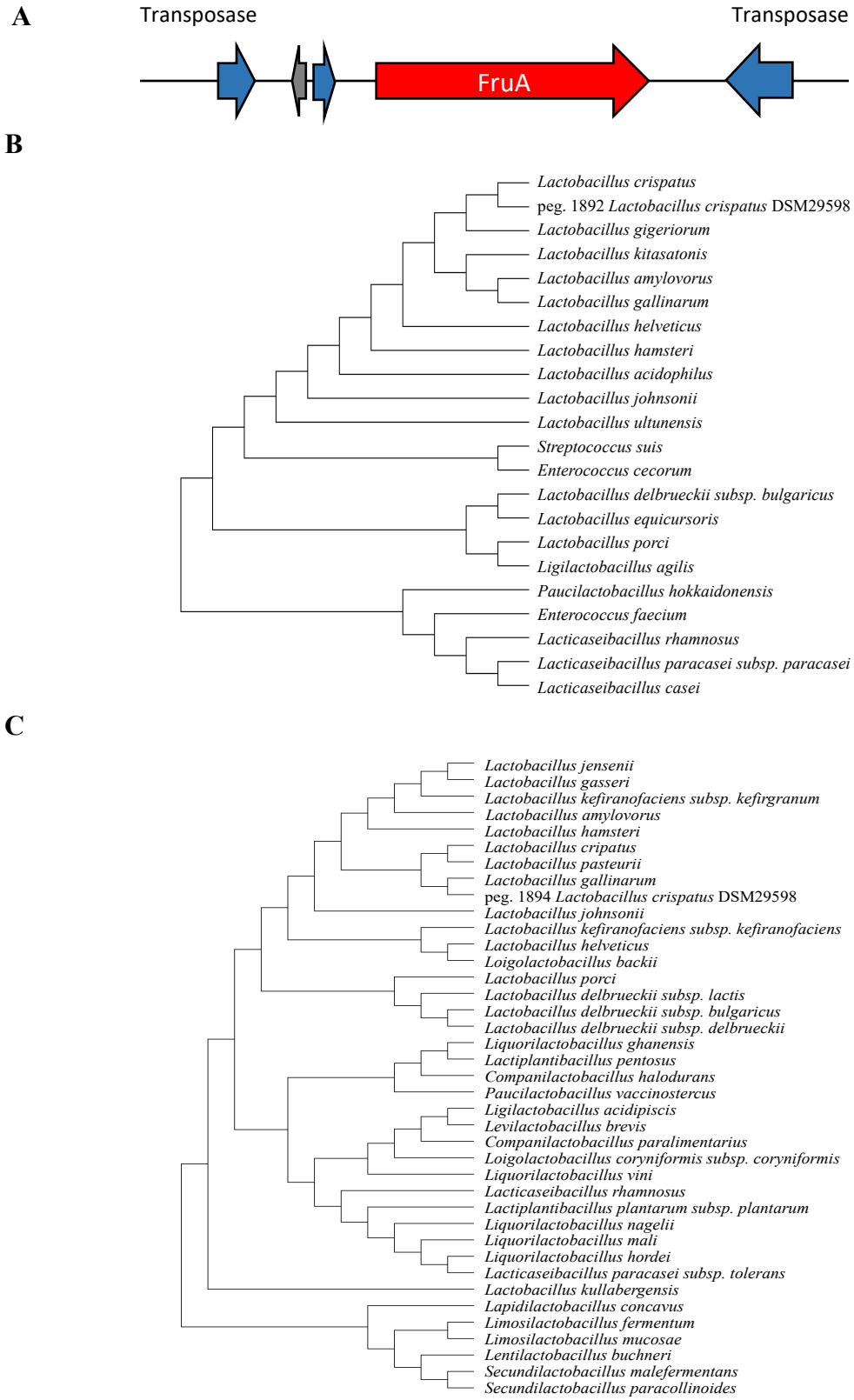


Figure 4. 11 Operon structure of FruA in *L. crispatus* and homology to mobile element proteins upstream and downstream of FruA to transposases of lactobacilli

Panel A. Operon structure of FruA in *L. crispatus.* From left to right: IS66 family transposase; hypothetical protein, mobile element protein, extracellular FruA, IS256 family transposase.
Panel B and C. Homology to mobile element proteins upstream and downstream of FruA to transposases of lactobacilli. Sequences in all genomes were identified by BLASTp using peg.1892 (B) or peg.1894 (C) in *L. crispatus* DSM29598 as query sequences. Shown are representative sequences from every species of lactic acid bacteria in which the protein was identified with more than 50 % coverage and more than 50 % amino acid identity.

Chapter 5 Characterization of AbnA and AbnB, two extracellular arabinanases in *Lactobacillus crispatus*

5.1 Introduction

Lactobacilli adapted to nutrient-rich environments including ecological niches in plants, humans, and animals but also are associated with food and feed fermentations [11]. Their competitiveness in nutrient-rich environments is based on the rapid conversion of abundant carbon sources rather than a high metabolic efficiency [2,13,96]. Carbohydrate metabolism of lactobacilli relies mainly on the intracellular conversion of mono-, and di- and trisaccharides. Extracellular glycosyl hydrolases are rarely present in lactobacilli and the utilization of oligosaccharides with a degree of polymerization of four or higher is limited by transport to the cytoplasm [30]. Extracellular amylases of lactobacilli support starch utilization by hydrolyzing amylose, amylopectin, or pullulan and were mostly identified in host-adapted *Lactobacillus* species [13,30]. Other extracellular glycosyl hydrolases in lactobacilli include the fructosidases FosE and FruA in *Lacticaseibacillus paracasei* and *Lactobacillus crispatus*, respectively [34,36], and glucansucrases and fructansucrases, which are particularly frequent in *Lactobacillus*, *Liquorilactobacillus* and *Limosilactobacillus* species [11,223].

Pentosans including xylan, arabinan, and arabinoxylan, are components of the plant cell wall and a major part of non-starch polysaccharides in many plant foods including cereal grains, vegetables, and legumes. Pentosans in foods are recognized as dietary fiber and are utilized by intestinal microbiota. The hydrolysis and metabolism of pentosans by colonic microbiota generates short chain fatty acids as the main mediators of health benefits [224,225]. Utilization of pentosans is mediated by extracellular glycosidase hydrolases and polysaccharide lyases of intestinal bacteria including *Bacteroides* species and *Roseburia*

species [38,226]. Analysis of pentosan degradation by *Bacteroides ovatus* identified two polysaccharide-utilization loci, which target branched arabinoxylan and includes six extracellular enzymes [38]. *Roseburia intestinalis* displays large cell-wall bound xylanase to degrade xylan into oligomers that are subsequently internalized with an ATP-binding cassette transporter[226]. Co-cultures of *R. intestinalis* and *B. ovatus* indicated a preference of *R. intestinalis* for oligomers of 4-5 units, while *B. ovatus* targets larger ligands [227]. Species in the genera *Lactobacillus, Lactilactobacillus*, and *Limosilactobacillus* are stable components of the intestinal microbiota of animals, however, lactobacilli are not known to utilize pentosans. *Furfurilactobacillus rossiae* utilizes xylooligosaccharides [228]. Two pentosanases were identified in the genome of *F. rossiae*, however, these proteins are intracellular oligosaccharide hydrolases as their sequences do not include a predicted signal peptide and extracellular hydrolysis of arabinoxylans was not verified experimentally [228,229].

The genome sequence of *Lactobacillus crispatus* DSM29598 indicates the presence of a pentosan utilization operon with mobile element protein (Chapter 4). This gene cluster is unusual in two respects. First, most of *Lactobacillus* species, including *L. crispatus*, do not ferment pentoses and thus lack the capability to utilize products of pentosan hydrolysis [11]. Second, extracellular pentosanases that facilitate growth with pentosans as a sole substrate have not been described in lactobacilli. Therefore, this study aimed to explore the structure and function of the pentosan utilization operon, as well as the presence of related pentosanases in lactic acid bacteria. Two extracellular pentosanases of *L. crispatus* DSM29598 were characterized by cloning and expression of their catalytic domain and testing their activities and functional properties.

5.2 Materials and Methods

5.2.1. Strain and growth condition

Strains and plasmids used in this study are shown in Table 5.1. *L. crispatus* DSM29598 was cultivated in modified deMan-Rogosa-Sharpe [152] (mMRS) medium at 37 °C. *Escherichia coli* BL21 was cultured in Luria-Bertani (LB) medium at 37 °C. *E. coli* DH5α with pET28a$^+$ was grown in LB medium with kanamycin (50 mg L^{-1}), which also used for antibiotic-resistant *E. coli* selection. The frozen stock culture was inoculated on agar plates; single colonies were inoculated in 1 mL broth, subcultured with 1 % inoculum in broth.

Table 5. 1 Bacteria strains and plasmids used in this study

Strains or plasmids	Genotype	Source or reference
	Strains	
Lactobacillus crispatus DSM29598	Rye sourdough isolate; wild type strain	[197]
Escherichia coli BL21 Star (DE3)	Cloning host for pET28a$^+$ and its derivative plasmids	Thermofisher
E. coli DH5α	Host for pET28a$^+$ empty plasmid	
	Plasmids	
pET28a$^+$	Cloning vector used in *E. coli* BL21, 5.4kb, Kan^r	Novagen
pET-abnA	pET28a$^+$ containing 1.9kb of the DNA sequence encoding 52-675aa of AbnA, 7.2kb, Kan^r	This study
pET-abnB	pET28a$^+$ containing 1.5kb of the DNA sequence encoding 45-554aa of AbnB, 6.9kb, Kan^r	This study

5.2.2 Identification of pentosan utilization operon in *L. crispatus* DSM29598 and analysis of the domain organization of putative arabinanases

The putative function of predicted open reading frames in the pentosan utilization operon in the genome sequences of *L. crispatus* DSM29598 (accession number: JAATOH000000000) were analyzed by using BLASTp tool of NCBI, searching against the UniProtKB / Swiss-Prot and the non-redundant protein sequence databases. Signal peptides of the putative

extracellular endo-arabinanases, AbnA and AbnB were predicted by SignalP 5.0 based on the amino acid sequences of AbnA and AbnB. The prediction of domains in AbnA and AbnB was conducted by comparison of amino acid sequences using the Conserved Domain Database on the NCBI website.

5.2.3 Phylogenetic analysis of arabinanases in bacteria

Bacterial protein sequences of pentosanases in bacteria were retrieved from the National Center for Biotechnology Information database using BLASTp and AbnA and AbnB of *L. crispatus* DSM29598 as query sequences. Representative sequences from every species of lactic acid bacteria and sequences from representative species of every other genus were selected with a cut-off value of 80 % coverage and 45 % amino acid identity to AbnA or AbnB to exclusive unrelated sequences. A phylogenetic tree was constructed using the Maximum Likelihood method based on the JTT matrix-based model [155] and the bootstrap support values were calculated from 100 replicates by MEGAX [200].

5.2.4 Growth of *L. crispatus* with different carbon sources

L. crispatus was subcultured twice in mMRS basal broth containing 1 % linear arabinan (with purity of > 95 %, Megazyme Inc, Bray, Ireland), pectin (Sigma-Aldrich, St. Louis, MO, USA), 0.5 % starch (Sigma), or mMRS, respectively and grown overnight. The cultures were subsequently used to inoculate (1 - 2 % inoculum) mMRS broth containing the sugars at the same concentrations. The optical density at 600 nm (OD_{600nm}) and pH of the culture were measured after overnight growth. Lactic acid and acetic acid production were quantified by HPLC as described [198]. All experiments were done in triplicate.

5.2.5 Hydrolysis of linear and branched arabinan by *L. crispatus*

L. crispatus was grown in mMRS broth containing 1 % linear arabinan and cells were harvested by centrifugation. Cells were fractionated at 4 °C to obtain concentrated culture

supernatant, cell wall fraction, and concentrated cytoplasmic extract as described [34]. Cell fractions were incubated with the same volume of 1% linear arabinan or branched arabinan from sweet beets (with purify of ~ 95 %, Megazyme) in 50 mM citrate phosphate buffer (pH 6.0) at 40 °C for 20 min. Protein concentrations were determined with the Bradford reagent (Sigma), using the manufacturer's instruction. The enzyme activities are expressed as the amount of reducing sugar released from linear-arabinan or arabinan per minute per mg of protein. The experiments were done in triplicate.

5.2.6 Cloning, expression, and purification of arabinanases

The sequences of AbnA and AbnB without the signal peptide and surface layer associated protein (SLAP) domain were amplified by PCR with AbnA-F and AbnA-R, and AbnB-F and AbnB-R (Table 5.2). The products of PCR were purified and ligated into pET28a^{+} containing a C-terminal His-tag for purification of AbnA and AbnB. The recombinant plasmid was transformed into *E. coli* BL21 and positive transformation was selected on LB agar with 50 mg L^{-1} of kanamycin and verified by DNA sequencing with primers shown in Table 5.2. Recombinant *E. coli* were cultivated in LB broth with kanamycin at 37 °C until the OD_{600} reached 0.5-0.8. Then 0.5 mM IPTG was added to the medium to induce enzyme expression and the culture was further incubated at 20 °C for 20 h.

Table 5. 2 Primers used in the cloning of catalytic domains in AbnA and AbnB

Location of target domain	Primers	Primer sequences(5'-3')	Annealing temperature (°C)	Amplicon size (bp)
AbnA	AbnA-F	TACTCGAATTCATGGAAGAACAAACAG	63.6	1872
	AbnA-R	TATATCTCGAGTTCAGTGTTGTTTGAA		
AbnB	AbnB-F	ATGCTGAATTCATGGAAAAACATCCTGC	63.6	1530
	AbnB-R	ATCGTCTCGAGTTTGAGGTCTTTGAATCC		
AbnA and AbnB	T7-F	TAATACGACTCACTATAGGG	55.9	AbnA: 2165 AbnB: 1820
	T7-R	GCTAGTTATTGCTCAGCGG		

The proteins were purified by using His-pure Ni-NTA column and His-pure Cobalt column (Thermo Scientific, Waltham, MA, the USA) following the suppliers' manuals. In brief, AbnA was purified with washing buffer with 25 mM and 50 mM of imidazole for both columns. AbnA was eluted from the Ni-NTA column with 250 mM imidazole, or eluted from the Cobalt column with a gradient from 100 mM to 250 mM imidazole. For the purification of AbnB, buffers were prepared with 50 mM sodium phosphate, 1 M sodium chloride, 0.1 % Triton-X100, 3 mM β-mercaptoethanol. The concentration of imidazole in buffers were: equilibration buffer 25 mM, washing buffer 25 mM or 35 mM, and elution buffers 150 mM, 200 mM or 250 mM. All the buffers were adjusted to pH 8.0. The concentrated active fractions were stored in PBS buffer (pH 7.4) at 4 °C or -80 °C. Purification steps were analyzed on 10% SDS-PAGE gels (Bio-Rad laboratories, Mississauga, ON, Canada). The degree of purification was determined by SDS - PAGE gels (Figure 5.8). The protein concentration was determined as described in 2.5. The protein purified by Ni-NTA column was used for chemical characterization and protein purified by Cobalt column was used for the qualitative and quantitative analysis of substrate specificity.

5.2.7 Substrate specificity of AbnA and AbnB

Purified AbnA and AbnB were mixed with the same volume of 0.5 % linear arabinan, branched arabinan, rye arabinoxylan (Megazyme), corn arabinoxylan (Agrifiber Holdings LLC, IL, USA), xylan (Megazyme), or arabinogalactan from larch wood (Megazyme) in 50 mM citrate phosphate buffer (pH 6.0) and incubated at 37 °C for 20 min. Qualitative determination of polysaccharide hydrolysis by purified AbnA and AbnB was performed with high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD). In brief, reaction mixtures were separated on a Carbopac PA20 column coupled to an ED40 chemical detector (Dionex, Sunnyvale, CA, USA). Water (A), 0.2 M NaOH (B) and 1 M NaAcetate (NaOAc) (C) were used as mobile phase with the following gradient: 0 min, 68.3 % A, 30.4 % B and 1.3 % C; 30 min, 54.6 % A, 30.4 % B and 15.0 % C; 50 min, 46.6 % A, 30.4 % B and 23 % C; 95 min, 33.3 % A, 30.4 % B and 36.3 % C; 95.1 min, 63.7 % A and 36.3 % C; 100min, 50 % A and 50 % C; 105min, 10 % A, 73 % B and 17 % C; 105.1 min, 33.3 % A, 30.4 % B and 36.3 % C; 111min, 10 % A, 73 % B and 17 % C; followed by re-equilibration. The enzyme activity was quantified by determination of reducing sugars with the 3,5 - dinitrosalicylic acid (DNS) method [230]. One unit of enzyme activity was defined as 1 μmol of reducing sugar as arabinose produced per minute.

5.2.8 Effect of pH and temperature on activity of AbnA and AbnB

The optimum pH for arabinanase activity was determined by incubating purified AbnA or AbnB with 0.5 % linear arabinan in the pH range from 2 to 10 at 37 °C for 20 min. The following buffers were used: 50 mM citrate phosphate buffer, pH 2.0 to 7.5; 50 mM potassium phosphate, pH 8.0; 50 mM Tris-HCl, pH 8.5 to 9.0; 50 mM glycine-NaOH, pH 10.0. The maximum temperature for enzymatic activity was determined at the optimum pH by using temperatures ranging from 20 °C to 80 °C. The results were expressed as percentages of the activity obtained at either the optimum pH or the optimum temperature.

The thermal stability of the purified enzyme was determined by incubating purified AbnA or AbnB at 40 °C, 50 °C, and 60 °C for 5 min, 10 min, and 30 min. Samples were withdrawn at the indicated times, cooled on an ice bath, and assayed for the residual enzyme activities at optimum pH and temperature.

5.2.9 Effects of additives on the activity of AbnA and AbnB

The effects of several metals on the enzyme activity were determined at optimum pH and temperature as described in 2.7. Several metals ($MnCl_2$, $MgCl_2$, $FeCl_2$, $FeCl_3$, $ZnCl_2$, $CaCl_2$, KCl_2, $BaCl_2$, each at 1 mmol L^{-1}) and chelating agent (EDTA, 1 mmol L^{-1}) were added into the reaction mixture. The results were expressed as percentages of the activity obtained in the reaction with 18 MΩ water in place of the compounds.

5.2.10 Substrate specificity of AbnA and AbnB

The substrate specificity of enzymes was tested using 0.5 % linear arabinan, branched arabinan, rye arabinoxylan, corn arabinoxylan, xylan, and arabinogalactan from larch wood at optimum pH and temperature as described in 2.7. Enzyme activity was expressed as unit per milligram protein.

5.2.11 Statistical analysis

Data analysis was performed with IBM SPSS statistics 23, using one-way analysis of variance (ANOVA). A *P*-value of ≤ 0.05 was considered statistically significant.

5.3 Results

5.3.1 Pentosan utilization operon of *L. crispatus* DSM29598

The annotation of the *L. crispatus* DSM29598 genome sequence identified an arabinan utilization operon (Figure 5.1). The operon starts with open reading frames with homology to transposases or mobile element proteins, suggesting that the operon was acquired by lateral

gene transfer. The operon also includes enzymes with activity on enzymes related to transport and metabolism of arabinan, arabinose, and galactose, several hypothetical proteins, one putative transcriptional regulator, and several glycosyl hydrolases with predicted activity on arabinoxylans or galactans (Table 5. 3). The extracellular enzymes AbnA and AbnB include a GH43 domain and are 35% and 36%, respectively, identical to an endo-arabinanase in *Thermotoga petrophila* (Table 3)[231]. AbfF was a predicted exo-active enzyme that hydrolyzes non-reducing α-L-arabinofuranoside residues in α-L-arabinosides. AraNPQ and MsmK were putative ABC-type transporters with predicted affinity to arabinose-oligosaccharides and α-galacto-oligosaccharides, respectively [232]. AbfA and AbfB were predicted intracellular and exo-active arabinan hydrolases. AbfH putatively hydrolysed β-arabinodisaccharides [233]. AraA, XylB, AraD, and XpkA were putative metabolic enzymes for the conversion of arabinose to acetyl-CoA and glyceraldehyde-phosphate via ribulose, L-ribulose-5-phosphate, and D-xylulose-5-phosphate [232]. Additional glycosyl hydrolases included an intracellular LacLM-type β-galactosidase, and the α-galactosidase Aga. GalM putatively converted α-aldoses to the β-anomers, and it is active on D-glucose, L-arabinose, D-xylose, D-galactose, maltose, and lactose. The operon is exceptional in two respects: first, because it encodes for several putative extracellular pentosanases, which have not been characterized in *Lactobacillaceae*; second, it encodes all genes for metabolism of pentoses, which is exceptional in *Lactobacillus* spp. and has not been described for *L. crispatus*.

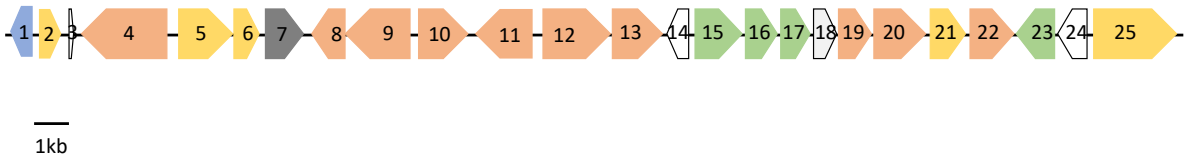


Figure 5. 1 Structure of pentosan utilization operon in *L. crispatus* DSM 29598

AbnA and AbnB are in the same operon and all the open reading frames (ORFs) are shown. Different colors refer to corresponding different protein function: orange, glycocyl hydrolase; green, transport protein; gray, regulatory protein; yellow, metabolic proteins; blue, mobile element proteins; white, hypothetical proteins. The

name of ORFs are from left to right: 1. *traA*, 2.*iep*, 3. *hypA*, 4. *abnA*, 5. *xylB*, 6. *araD*, 7. *araR* 8. *lacM*, 9. *lacL*, 10. *araA*, 11. *abnB*, 12. *abfH*, 13. *abfA*, 14. *hypB*, 15. *araN*, 16. *araP*, 17. *araQ*, 18. *hypC*, 19. *abfF*, 20. *abfB*, 21. *galM*, 22. *aga*, 23. *msmK*, 24.*yxkF*, 25. *xpkA*

5.3.2 Protein domains of AbnA and AbnB

Analysis of the protein sequence of AbnA and AbnB predicted an N-terminal sec-dependent signal peptide mediating protein export with a cleavage site between positions 42 and 43 (VMA-DT) of AbnA, and between positions 29 and 30 (LTS-CS) of AbnB [202]. The presence of a signal peptide indicates that AbnA and AbnB are extracellular enzymes. The lack of a signal peptide in AbfF indicates that it is an intracellular enzyme while the homologous enzyme Araf43A in *Streptomyces avermitilis* is extracellular and includes a signal peptide [234]. Further analyses focused on the extracellular AbnA and AbnB. A predicted GH family 43 domain with 49.74 % amino acid identity to an extracellular arabinanase in *Geobacillus stearothermophilus* [235] is located between 159 and 539 of AbnA; the same domain with 51.79 % amino acid identity to the extracellular arabinanase in *G. stearothermophilus* T-6 is located between positions 51 and 431 of AbnB (Table 5.3). The sequences of AbnA and AbnB in positions 548 to 662 and 440 and 549, respectively, are homologous to the C-terminal domain of GH43 enzymes. AbnA additionally includes a C-terminal surface layer protein (SLAP) domain at positions 764 to 817 (Figure 5.2A). The presence of a SLAP domain connected to a glycosyl hydrolase domain was unprecedented in other lactic acid bacteria but may indicate that the enzyme is attached to surface layer proteins of *L. crispatus* [203,236].

Table 5. 3 Sequence identity scores of ORFs in pentosan utilization operon and proposed biological function for encoded proteins

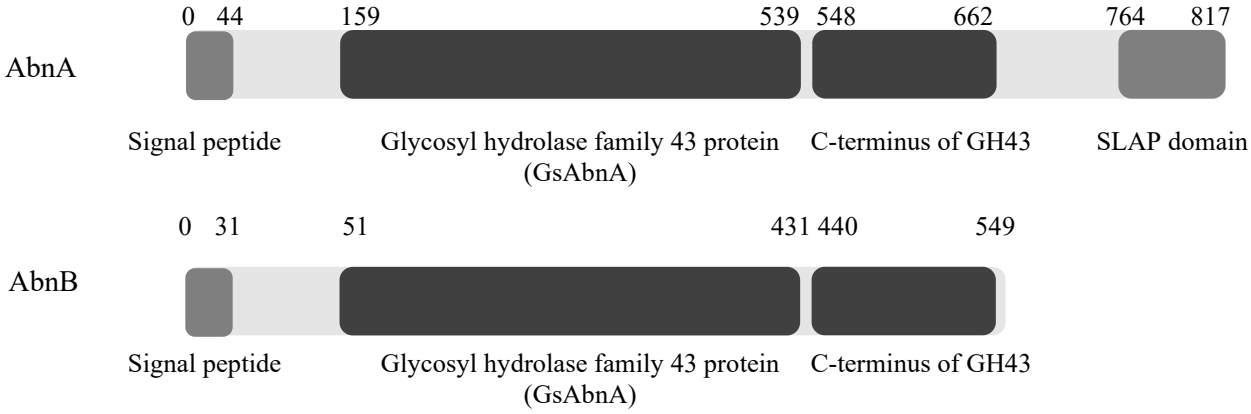
ORFs endoding proteins	Homologous protein (highest % identity) identified by BLASTp	Accession number (coverage%, identity %); putative function
Trn	IS4 family transposase of *L. crispatus*	WP 150398175.1 (100. 99.5); lateral gene transfer
Iep	group II intron reverse transcriptase/maturase of *L. crispatus*	WP 101887600.1 (100, 86.4) intron recognition, reverse transcriptase that enables group II introns to behave as mobile genetic elements, splicing and retrotransposition [237]
HypA	Hypothetical protein of *L. crispatus*	WP 060462779.1 (69, 80)
AbnA	Extracellular endo α-1,5-arabinanse of *Thermotoga petrophila*	UniProtKB: A5IKD4.1 (61, 34.5); internal cleavage of α-(1→5)-linked L-arabinofuranose residues (linear, debranched and branched) along the polysaccharide chain [231].
XylB	Xylulose kinase of *Klebsiella pneumoniae*/ ribulokinase in *Lactobacillus reuteri*	UniProtKB: P29444.1 (86, 21.9)/ CUR38122.1(100, 71.8); phosphorylation of xylulose or ribulose, second step of the pathway of L-arabinose degradation via L-ribulose [238]
AraD	L-ribulose-5-phosphate 4-epimerase of *Geobacillus stearothermophilus*	UniProtKB: Q9S469.1 (95, 63.04); involved in the degradation of L-arabinose; catalyzes the interconversion of L-ribulose 5-phosphate and D-xylulose 5-phosphate [239]
AraR	Arabinose metabolism transcriptional repressor of *Geobacillus stearothermophilus*	UniProtKB: Q9S470.1(92, 40.6); regulator of the arabinose metabolism [235]
LacM	β-galactosidase small subunit of *Lactobacillus helveticus*	UniProtKB: Q7WTB3.1 (99, 62.9); hydrolysis of terminal, non-reducing β-D-galactose residues in β-D-galactosides [240]
LacL	β-galactosidase large subunit of *Lactobacillus acidophilus*	UniProtKB: O07684.2 (100, 67.36); hydrolysis of terminal, non-reducing β-D-galactose residues in β-D-galactosides [241]
AraA	L-arabinose isomerase of *Latilactobacilus sakei* subsp. *sakei*	UniProtKB: Q38UH2.1 (99, 65.6); conversion of L-arabinose to L-ribulose [242]
AbnB	Extracellular endo-α-1,5-arabinanase of *Thermotoga petrophila*	UniProtKB/Swiss-Prot: A5IKD4.1 (90, 36.2); internal cleavage of α-(1→5)-linked L-arabinofuranose residues (linear, debranched and branched) along the polysaccharide chain [231].
AbfH	HypBA1 Intracellular β-L-arabinofuranosidase of *Bifidobacterium longum*	UniProtKB/Swiss-Prot: E8MGH8.1 (97, 45.7); liberate L-arabinose from the L-arabinofuranose (Ara*f*)-β-1,2-Ara*f* disaccharide and hydroxyproline-linked β -L-arabino-mono-, di- and tri-saccharides [233]
AbfA	Abf2 Intracellular exo-α-L-arabinofuranosidase of *Bacillus subtilis*	UniProtKB/Swiss-Prot: P94552.2 (96, 49.1); cleavage of terminal α-L-arabinofuranosyl residues in linear and branched arabinans and arabinoxylans, Abf2 is most active on α-(1→2) and α-(1→3) linkages of branched arabinan and arabinoxylan [243]
HypB	Hypothetical protein of *L. crispatus*	WP 150398101.1(100, 99.5)
AraN	Probable arabinose-binding protein on cell membrane of *Bacillus subtilis*	UniProtKB/Swiss-Prot: P94528.2 (93, 39.4); part of the binding-protein-dependent transport system for L-arabinose [232]
AraP	L-arabinose transport system permease protein AraP on cell membrane of *Bacillus subtilis*	UniProtKB/Swiss-Prot: P94529.1 (99, 44.6); part of the binding-protein-dependent transport system for L-arabinose. Probably responsible for the translocation of the substrate across the membrane [232]
AraQ	L-arabinose transport system permease protein AraQ on cell membrane of *Bacillus subtilis*	UniProtKB/Swiss-Prot: P94530.2 (94, 48.9); the same as AraP [232]

HypC	hypothetical protein of *L. crispatus*	WP 100732653.1 (100, 99.1)
AbfF	Araf43A Extracellular exo-α-1,5-L-arabinofuranosidase of *Streptomyces avermitilis*	UniProtKB/Swiss-Prot: Q82P90.1 (97, 52); hydrolysis of terminal non-reducing α-L-arabinofuranoside residues in α-L-arabinosides [234]
AbfB	AbfA Intracellular exo-α-1,5-L-arabinofuranosidase of *Geobacillus stearothermophilus*	UniProtKB/Swiss-Prot: Q9XBQ3.4 (99, 61.4); catalyzes the cleavage of terminal α-L-arabinofuranosyl residues in different hemicellulosic homopolysaccharides (branched and debranched arabinans) and heteropolysaccharides [244]
GalM	Aldose 1-epimerase of *E. coli*	UniProtKB/Swiss-Prot: P0A9C3.1 (94, 31.3); convert α-aldose to the β-anomer; active on D-glucose, L-arabinose, D-xylose, D-galactose, maltose and lactose [245]
Aga	α-galactosidase of *Cyamopsis tetragonoloba*	UniProtKB/Swiss-Prot: P14749.1(93, 24.8); hydrolysis of terminal, non-reducing α-D-galactose residues in α-D-galactosides, including galactose oligosaccharides, galactomannans and galactolipids [246]
MsmK	Multiple sugar-binding transport ATP-binding protein on cell membrane of *Streptococcus mutans*	UniProtKB/Swiss-Prot: Q00752.1 (99, 64); probably responsible for energy coupling to the transport system; belongs to the ABC transporter superfamily for the uptake of melibiose, raffinose and isomaltotriose [247]
YxkF	Uncharacterized protein of *Bacillus subtilis*	UniProtKB/Swiss-Prot: P94359.1(97, 27.2) [248]
XpkA	Xylulose-5-phosphate phosphoketolase of *Lactiplantibacillus pentosus*	UniProtKB/Swiss-Prot: Q937F6.3 (100, 66.1); D-xylulose 5-phosphate + phosphate is converted to acetyl phosphate + D-glyceraldehyde-3-phosphate + H_2O [249]

5.3.3 Phylogenetic analysis determined the distribution and frequency of arabinanases in bacteria

The amino acid sequences of AbnA and AbnB are 47.28 % identical. Homologous sequences in *Lactobacillaceae* species were exclusively present in strains of the vertebrate-host adapted genus *Lactobacillus; Lactobacillus gallinarum*, *Lactobacillus xujianguonis*, and *L. crispatus* [2,11,250,251] (Figure 5.2B). Homologous sequences were also identified in other lactic acid bacteria *(Lactobacillales*), predominantly *Enterococcus* species that are considered to be of intestinal origin [252] and species of *Streptococcus* that are also associated with animal or human hosts.

A



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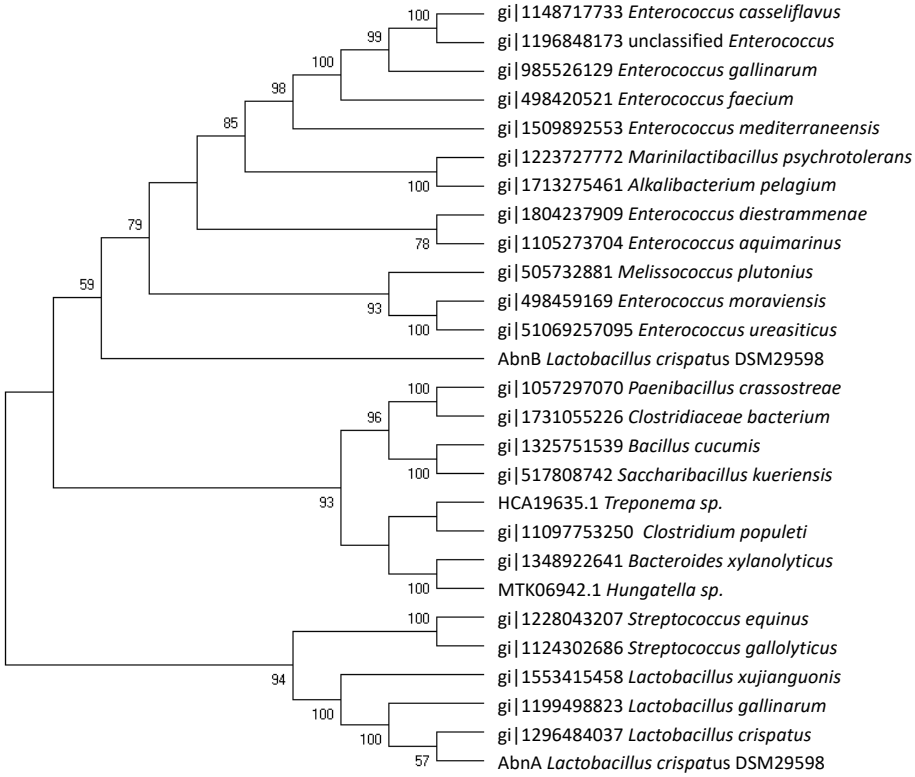

Figure 5. 2 Domain prediction of putative arabinanases in *L. crispatus* DSM29598

Panel A. Prediction of domains in AbnA and AbnB based on amino acid sequences. Amino acid sequences of both putative arabinanases were compared with conserved domains.
Panel B. Phylogenetic analysis of AbnA and AbnB. Amino acid sequences of AbnA and AbnB were used as query sequence to identify homologues by BLASTp on NCBI and results were cut by 45 % protein identity and 80 % coverage. The phylogenetic tree showed representative homologues from every lactic acid bacteria species and homologues from representative species of every other genus.

5.3.4 Growth of *L. crispatus* with different carbon source

The presence of extracellular arabinanases was confirmed by incubation of *L. crispatus* in media with linear arabinan, starch or pectin as sole carbon sources, as well as mMRS containing glucose, maltose and fructose (Figure 5.3). Extracellular enzymes are required for the utilization of polysaccharides to degrade these to oligosaccharides that can be transported to the cytoplasm [30]. *L. crispatus* grown in media with linear arabinan had a comparable OD_{600} and total production of lactic acid and acetic acid with that in regular mMRS, which indicates that linear arabinan was degraded extracellularly by AbnA or AbnB and utilized via AraA, XylB, AraD, and XpkA. *L. crispatus* did not harbor extracellular enzymes to utilize starch and pectin as carbon sources, which was consistent with higher pH and lower OD_{600} of culture, as well as the lower production of metabolites.

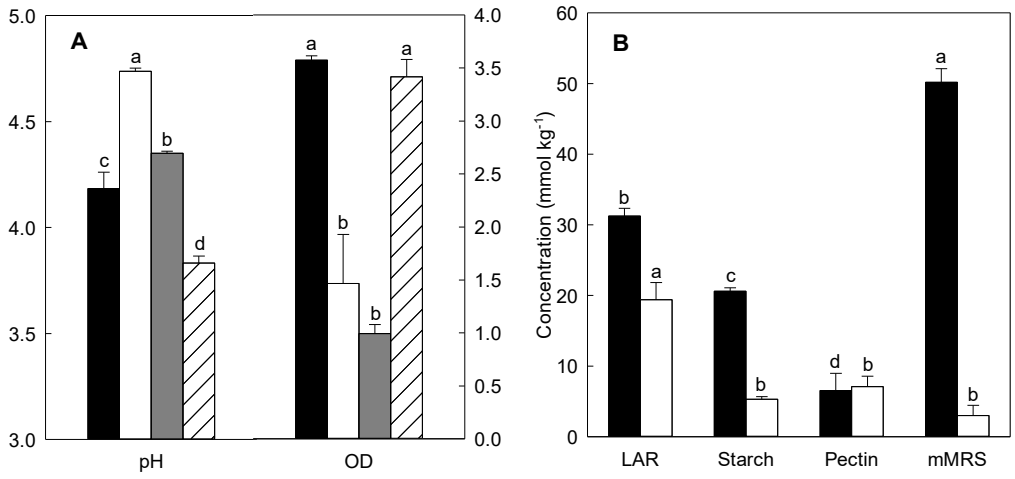

Figure 5. 3 Growth of *L. crispatus* DSM29598 with different carbon sources

Panel A. OD_{600nm} and pH of overnight culture grown in mMRS with linear arabinan (black bar), starch (white bar), Pection (gray bar), mMRS (coarse bar).
Panel B. Production of lactic acid (gray bar) and acetic acid (white bar) of *L. crispatus* after overnight growth in mMRS with linear-arabinan, starch, pection, mMRS. Values for the same analyte differ significantly ($P < 0.05$) unless they share a common superscript. Values ± standard deviation were calculated from three independent experiments.

5.3.5 Location of AbnA and AbnB in *L. crispatus*

The cellular location of AbnA and AbnB was assessed by determination of the arabinanase activity of the supernatant, the cell wall fraction, and the cytoplasmic fraction of *L. crispatus* grown with linear arabinan (Figure 5.4). The degradation of linear arabinan by the cell wall fraction and cytoplasmic fraction was higher than the degradation by enzymes present in the supernatant, which indicates the presence of cell wall-associated arabinanase in *L. crispatus*. However, the degradation of branch arabinan was comparable by different cell fractions.

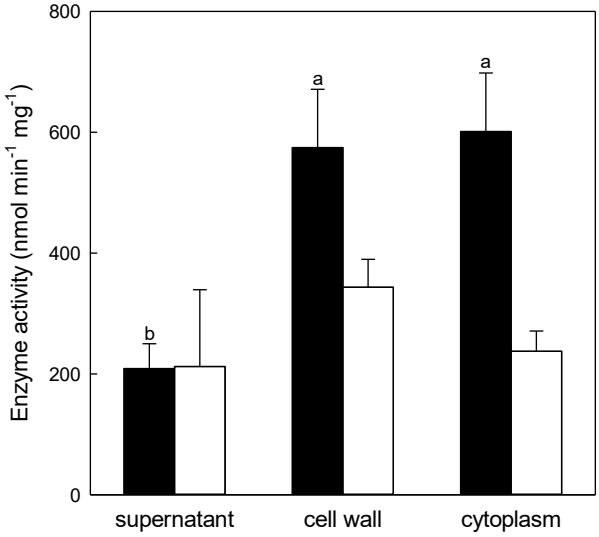

Figure 5. 4 Degradation of linear arabinan and branched arabinan by cell lysis of *L. crispatus* DSM29598

L. crispatus was grown in mMRS with 1 % linear arabinan. The enzyme activities were quantified with different cellular fractions with linear arabinan (black bar) or branched arabinan (white bar) as substrate and are expressed as the amount of reducing sugar released from linear-arabinan or arabinan per minute per milligram of protein. Values for the same substrate differ significantly ($P < 0.05$) unless they share a common superscript. Values indicate mean ± standard deviation from three biological replicates.

5.3.6 Cloning, expression and purification of AbnA and AbnB in *E. coli*

The recombined plasmids, pET-abnA and pET-abnB with catalytic domains of AbnA and AbnB respective, were transformed and expressed in *E. coli* BL21 (Table 5.1). Overexpressed AbnA and AbnB in soluble fraction of the cell lysate were purified by His pure columns. The predicted molecular weights of AbnA and AbnB with His-tag were 72.11

kDa and 58.56 kDa, in good accordance with the molecular weight of purified AbnA and AbnB (Figure 5.8).

5.3.7 Pentosan hydrolysis and enzymatic properties of AbnA and AbnB

The degradation of different substrates by purified AbnA and AbnB was assessed using HPAEC-PAD in comparison to the reaction of substrates with PBS buffer or cell lysate of *E. coli* BL21 with pET28a^{+} empty plasmid as control (Figure 5.5). The peaks between 20 and 90 min of retention time were present after reaction with AbnA and AbnB with linear arabinan but not in the control, and thus represent oligosaccharides produced from linear arabinan. AbnA but not AbnB also produced oligosaccharides with branched arabinan as substrate. Both AbnA and AbnB were inactive on any other substrate, including rye arabinoxylan, corn arabinoxylan, xylan, and arabinogalactan.

Purified AbnA and AbnB were incubated with linear arabianan in buffers with pH range of 2 to 10 at 37 °C. The activities of AbnA and AbnB were optimal at pH 6 and pH 7.5, respectively. The optimum temperature of both enzymes was 40 °C (Figure 5.6). Both enzymes maintained about 80 % of their activity after incubation at 40 °C for 30 min but were inactivated after 5 min at 50 °C or 60 °C (Figure 5.9).

Multiple additives were added into the reaction of AbnA or AbnB with linear arabinan to assess the effects of additives on the activities of two enzymes (Figure 5.7). The activity of AbnA was improved by Mn^{2+}, Mg^{2+}, Fe^{2+}, and Fe^{3+} and reduced by the addition of EDTA. The activity of AbnB was not affected by any of the additives.

The activities of AbnA and AbnB on linear and branched arabinan, rye arabinoxylan, corn arabinoxylan, xylan, and arabinogalactan were quantified at optimum pH and temperature (Table 5.3). AbnA degraded linear and branched arabinan with higher activity with the linear substrate while AbnB did not hydrolyze the branched arabinan.

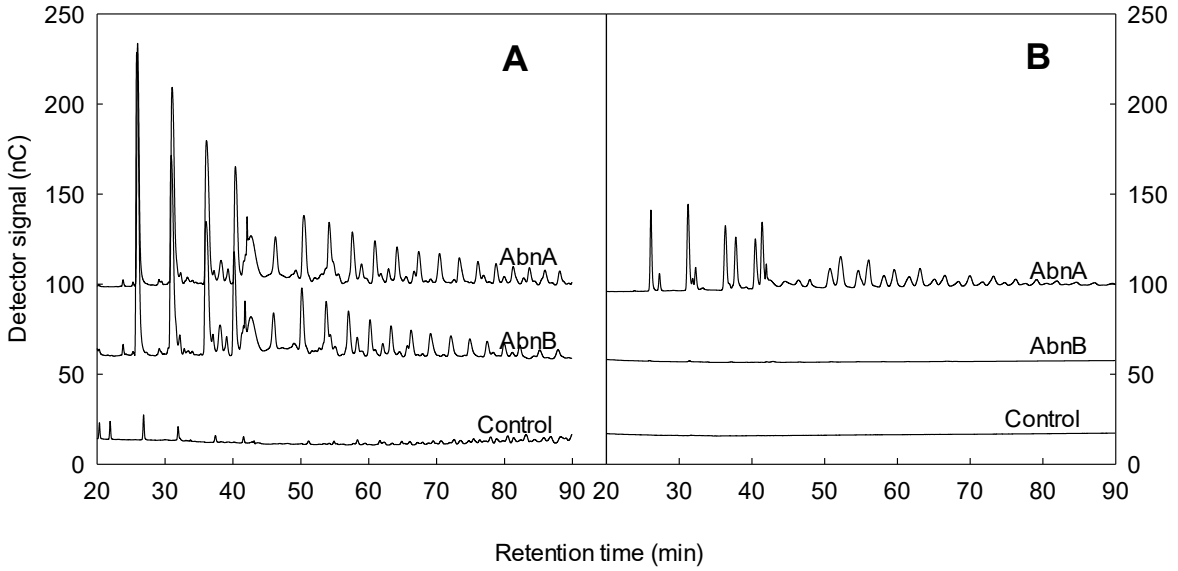

Figure 5. 5 Separation of oligosaccharides that were released by hydrolysis of different substrates by purified AbnA or AbnB

Panel A. Degradation of linear-arabinan
Panel B. Degradation of branched arabinan
Control shows the reaction of polysaccharides with cell lysis of *E. coli* BL21 with pET28a^{+} empty plasmid. The parallel reactions were also conducted with rye arabinoxylan, corn arabinoxylan, xylan, and arabinogalactan (data not shown), in which the differences between control and proteins were not detected.

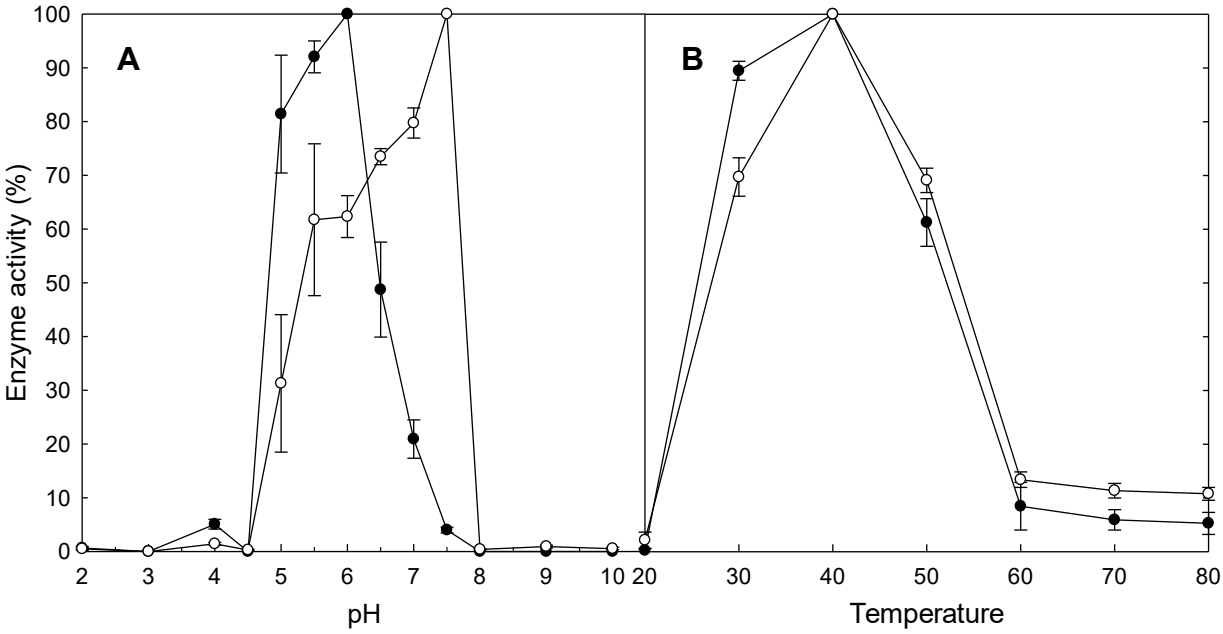

Figure 5. 6 Effects of pH and temperature on the relative activity of AbnA and AbnB

Relative activity of AbnA (black symbol) and AbnB (white symbol) under different pH (Panel A) and temperatures (Panel B) with linear arabinan as substrate were quantified. Enzyme activities were calculated relative to the maximum activity. Values represent mean ± standard deviation from three independent replicates.

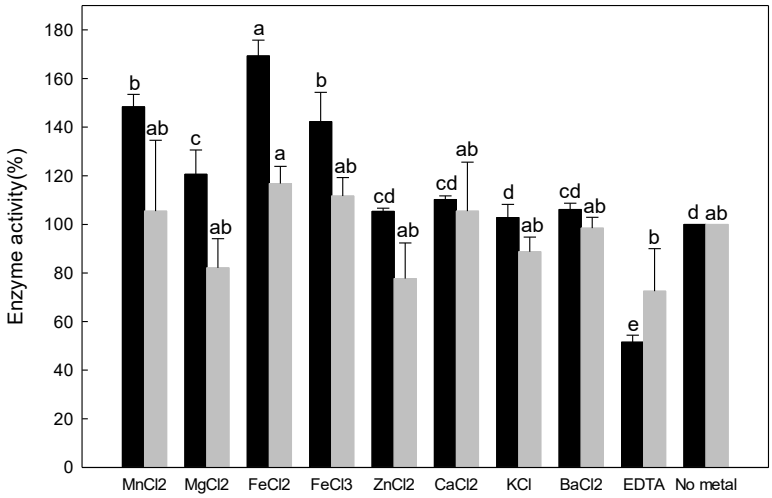

Figure 5. 7 Effect of different additives on the relative activities of AbnA and AbnB with linear arabinan as the substrate

Enzyme activities of AbnA (black bar) and AbnB (gray bar) were calculated relative to the reaction without additive. Values obtained for the same protein are significantly different ($P < 0.05$) unless they share the same superscript. Values represent mean ± standard deviation from three independent replicates.

Table 5. 4 Substrate specificity of AbnA and AbnB

Substrates	Enzyme activity (U mg^{-1})	
	AbnA	AbnB
Linear arabinan	22.41 ± 4.56^{Aa}	3.93 ± 0.70^{b}
Branched arabinan	6.55 ± 0.12^{B}	n.d.
Rye arabinoxylan	n.d.	n.d.
Corn arabinoxylan	n.d.	n.d.
Xylan	n.d.	n.d.
Arabinogalactan	n.d.	n.d.

***** Significant differences ($P < 0.05$) between different substrates with the same enzyme are indicated by uppercase letters; significant differences ($P < 0.05$) between different enzymes with the same substrates are indicated by lowercase letters.

5.4 Discussion

This study analysed an arabinan utilization operon of *L. crispatus* DSM29598 and the genetic and biochemical properties of AbnA and AbnB, two extracellular endo-arabinanases encoded by the operon. The operon encodes extracellular arabinan utilization enzymes as well as all the enzymes for metabolism of arabinose; which are exceptional in *Lactobacillus* species. Characterization of AbnA and AbnB demonstrated that these enzymes are extracellular endo-arabinanases. The homologous sequences in *Lactobacillaceae* species were exclusively present in vertebrate-host adapted genus *Lactobacillus*.

Until 2015, the fermentation of pentoses by lactobacilli has been used as a tool for their taxonomic identification [96,253,254], however, fermentation of pentoses in many homofermentative *Lactobacillaceae* is variable at a species- or strain- level [11]. *Lactobacillus* species do not encode pyruvate formate lyase or enzymes for homofermentative metabolism of pentoses [11,13,96]. Phosphoketolase is present in all *Lactobacillaceae* but and only few *Lactobacillus* species including *L. hamsteri*, *L. pasteurii*, and *L. xujianguonis* ferment pentoses [11,251]. To date, utilization of pentoses by *L. crispatus* has not been described. The genomic island encoding arabinan utilization in *L. crispatus* DSM29598 also encodes for AraA, an L-arabinose isomerase, AraD, a L-ribulose-5-phosphate 4-epimerase, XylB, a xylulose or ribulose kinase, and XpkA, a pentose phosphoketolase, the regulatory proteins AraR. The operon thus provides the full complement of enzymes to allow arabinose utilization via the phosphoketolase pathway [206]. The presence of an IS4 transposase implies that the arabinan genomic island was obtained by lateral gene transfer. The GC content of arabinan utilization operon is 34.67%, while GC content of genome sequence of *L. crispatus* DSM29598 is 37.6%. Arabinan metabolism via the phosphoketolase pathway was verified by metabolite analysis (this study), demonstrating lateral gene transfer bestows pentose utilization to homofermentative

lactobacilli, or, when using obsolete terminology, converts an “obligate homoferementative” to a “facultative heterofermentative” organism [11,254].

The arabinan utilization operon of *L. crispatus* also encodes for all enzymes that catalyse arabinan hydrolysis, including endo-active extracellular AbnA and AbnB, exo-acitve AbfA, AbfH, AbfB, and AbfF. AraNPQ transport arabinose or arabino-oligosaccharides into the cells and XylB, AraD and AraA utilize arabinose as carbon source. These functional enzymes are also encoded by an arabinan operon in *B. subtilis* [243]. The transcriptional regulater AraR was not identified in *B. subtilis*, but found in the arabinan utilization system of *G. stearothermophilus* [235].

The N-terminal export signal of AbnA and AbnB indicates their extracellular location, which was verified biochemically. AbnA additionally includes a C-terminal SLAP domain, which is also found in an extracellular fructosidase (FruA) of *L. crispatus* DSM29598 (Chapter 4)[236]. FruA has two SLAP domains, with identity of 48-55% with SLAP domain of AbnA. SLAP domain is found in multiple surface layer proteins or surface associated proteins of lactobacilli [211–214], and likely mediates association of the enzyme with cell wall [236]. T combination of a GH32 / 43 catalytic domain with SLAP domain are exclusive to vertebrate-host adapted *Lactobacillus* species (this study)[236]. In addition to degrading extracellular polysaccharides, the cell surface-associated enzymes of *L. crispatus* may contribute to the adhesion of cells to biofilm matrix and host epithelium [47,210].

AbnA was characterized as endo-arabinanase with activity on linear and branched arabinan, while AbnB only hydrolysed linear arabinan. Consistent with the activity of extracellular endo-arabinanase in *Thermotoga petrophila*, AbnA showed much lower activity on branched arabinan compared to linear arabinan [231]. AbnB is selective for linear arabinan, as was observed for an endo-arabinanase from *Bacillus licheniformis* [230]. AbnA activity is partially dependent on metal ions and its activity was reduced to 60% after addition of EDTA,

while AbnB activity is metal-independent, consistent with Abn2 in *Bacillus subtilis* [255]. Mn^{2+} stimulated the activity of AbnA in accordance with that of *Thermotoga thermarum* [256]. Fe^{2+} also improved arabinanase activity in *Caldicellulorsiruptor saccharolyticus* [257]. Arabinoxylans in food are recognized as dietary fiber and improve human health through multiple mechanisms, which include its influence on gut transit, prebiotic effects, and its fermentation in the large intestine to yield short-chain fatty acids [258]. Arabinoxylan is utilized by human intestinal microbiota including *B. thetaiotaomicron*, *B. ovatus* and *R. intestubalis* via multiple extracellular and intracellular glycoside hydrolases and transport system [38,226,227]. *B. ovatus* possesses 21 enzymes for hydrolysis of branched arabinoxylans. These enzymes are predicted to target glycosidic linkages, or chemical substituents present in arabinoxylans such as galactose and ferulic acid [38]. *Lactobacillus* species are stable components of animal intestinal microbiota but *L. crispatus* DSM29598 is the first strain equipped with arabinan utilization operon (this study). The lack of enzymes targeting xylans and other chemical substituents indicates that *L. crispatus* is unable to degrade most plant arabinoxylans, however, cooperation with other gut microorganisms may allow to degradation of arabinoxylans into linear arabinans, which are then metabolized by *L. crispatus*.

In conclusion, the arabinan utilization operon of *L. crispatus* DSM29598 encodes enzymes for hydrolysis of extracellular arabnan, arabino-oligosaccharides, and arabinose, which indicates pentosan and pentose metabolism enzymes of Lactobacillus species are acquired by lateral gene transfer. Two extracellular arabinanases, AbnA and AbnB, have been characterized as endo-1,5-arabinanase which, within the *Lactobacillaceae*, are exclusive to vertebrate-host adapted genus *Lactobacillus* species. AbnA acts on linear and branched arabinan, while AbnB only degrade linear arabinan. The arabinan operon of *L. crispatus* could be used together with other arabinoxylan-degrading enzymes in synergistic reaction

for the hydrolysis of branched arabinoxylans. Intestinal microbial metabolism of arabinoxylan generates health-beneficial short chain fatty acids. Short chain fatty acids improve the glucose and lipid metabolism to reduce the risk of developing cardiovascular disease, type 2 diabetes, and obesity, and also enhance gastrointestinal barrier function and exert immunoregulatory effects [259]. The application of arabinan degrading *L. crispatus* as probiotics or as synbiotics with arabinoxylan or arabinan may improve the utilization of arabinoxylan in intestinal tracts to yield energy for microbial growth and produce short chain fatty acids to increase host health [38].

5.5 Supplementary materials

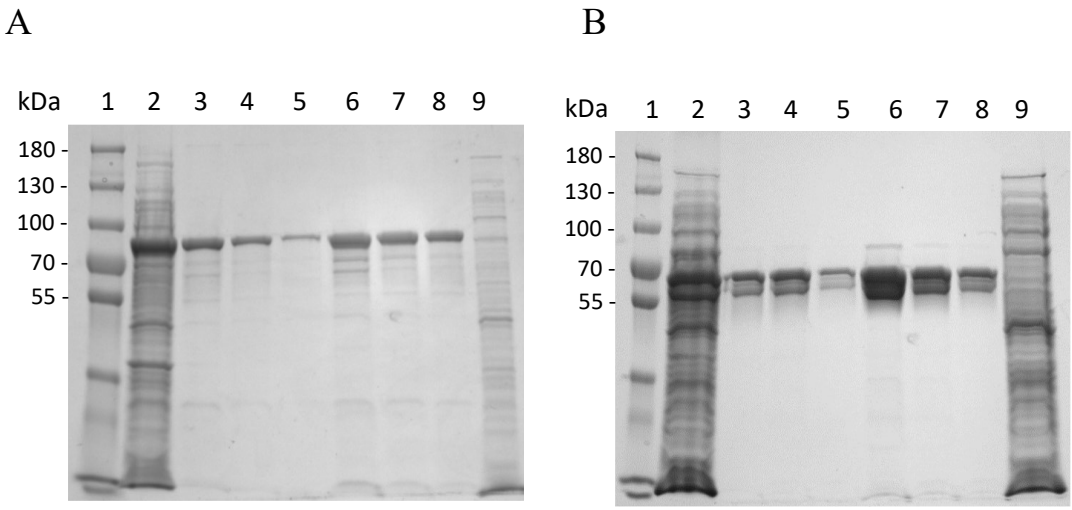

Figure 5. 8 SDS-PAGE analysis of the expression and purification of AbnA and AbnB

AbnA (Panel A) and AbnB (Panel B) were purified using His-pure Ni-NTA column and His-pure Cobalt column. For both images, from left to right: lane 1. Protein ladder, lane 2. Cell lysis supernatant, lane 3-5. Purified protein eluted by gradient imidazole using His-pur Cobalt column from Thermo Fisher, lane 6-8. The purified protein eluted by gradient imidazole using His-pur Ni-NTA column from Thermo Fisher, lane 9. Cell lysis supernatant of *E.coli* BL21 with pET 28a^{+}. The predicted molecular weight of AbnA with His-tag is 72.11 kDa, and AbnB with His-tag is 58.56 kDa.

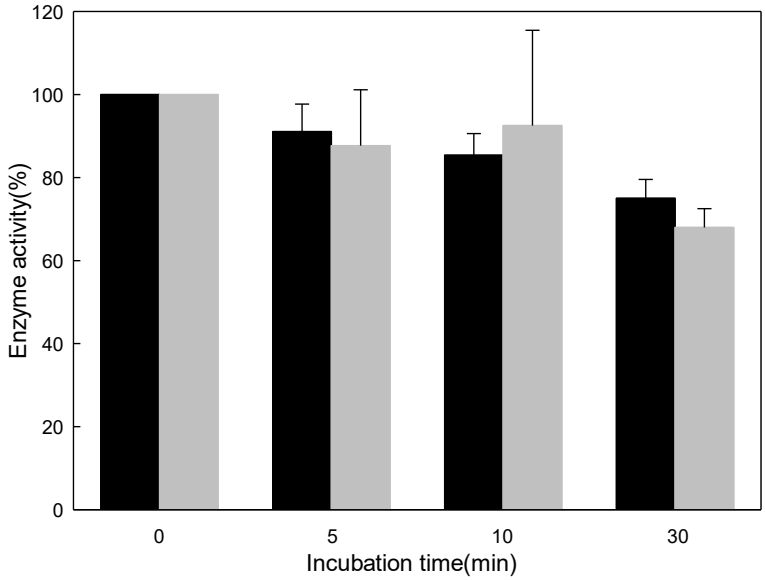

Figure 5. 9 Thermal stability of AbnA and AbnB

Thermal stability of AbnA (black bar) and AbnB (gray bar) were quantified after preincubation at 40 ℃ for 5, 10, 30 min, respectively. At indicated times, aliquots were withdrawn and analyzed at optimized temperature and pH for 20 min. Enzyme activities were calculated relative to the activity without heat treatment. The parallel experiments were also done at 50 and 60 ℃. For both of 50 and 60 ℃, both enzymes lost their catalytic activity after incubating for 5 min.

Chapter 6 General discussion and future directions

Fermented foods are populated with free-living, host-adapted, and nomadic lactobacilli. Numerous studies have focused on the metabolism and function of fermentation and spoilage lactobacilli in food environments [8,10,42]. However, the association of lactobacilli with their natural ecosystems instead of food habitats has shaped the metabolism and physiological properties of lactobacilli from an ecological and evolutionary perspective [2]. The exclusive presence of glutaminase, extracellular FruA, and extracellular arabinanases in host-adapted lactobacilli indicates the lifestyle association of these metabolic enzymes (Chapter 2, 3, 4, and 5).

6.1 Lifestyle-associated metabolism of host-adapted lactobacilli in fermented foods

Lifestyle-associated metabolic traits facilitate the ecological fitness of host-adapted lactobacilli in food environments that share selective factors with host habitats [10]. Moreover, these metabolic traits can impact food quality with a wide range of end products (Chapter 2). Type I sourdough selects for lactobacilli with a rapid growth rate, where *Fructilactobacillus sanfranciscensis* predominates relying on efficient utilization of maltose and the use of fructose as an electron acceptor, combined with its small genome size [116,260,261]. The carbohydrate metabolism and small genome size of *F. sanfranciscensis* has developed through its adaptation to suggested original habitats, including fruit flies and grain beetles [2,66]. During sourdough fermentation, the rapid growth of *F. sanfranciscensis* supports the leavening process via the correlated production of CO_2 [262], and acetate formation impacts the sensory quality and mold-free shelf life of bread [263].

In contrast, type II sourdough with long-term fermentation time accumulated lactic and acetic acids, and is populated mainly by vertebrate-adapted *Limosilactobacillus* and *Lactobacillus* species. These genera are mainly associated with the digestive tract of vertebrates [2,66]. *L. reuteri* has been specialized to several host lineages and the strains in sourdough microbiota belong to rodent and human lineages [51]. Urease and *gls3-gadB* operon are exclusively present in rodent-lineage *L. reuteri* strains, corresponding to their protective effects on the survival of cells under extreme acidic stress [16,47,51,79]. Glutamine metabolism protected *L. reuteri* at pH 2.5, therefore the disruption of *gadB* and *gls* genes did not affect the growth of *L. reuteri* in type II sourdough without propagation [24] (Chapter 3). However, glutamate decarboxylase contributed to the competitiveness of *L. reuteri* in type II sourdough fermentations after being back-slopped twice [116]. Acid resistance metabolism also improves the quality of fermented foods. Glutamine metabolism by lactobacilli produces glutamate or γ-aminobutyrate (GABA) during sourdough fermentation; glutamate accumulation exerts the final products an umami flavor and ensures the comparable bread quality with reduction of NaCl [29]. Dietary GABA benefits to human immunoregulation and helps lower the blood pressure of mild hypertensives [119,264]. However, glutaminase made a limited contribution to the accumulation of glutamate (Chapter 3). Arginine deamidation protected *L. reuteri* at pH 3.5 and produced crust odor compound, 2-acetyl-1-pyrroline, for bread flavor [21,98].

In addition, metabolic traits of host-adapted lactobacilli benefit food fermentation by degrading or converting intolerable components. FODMAPs include oligosaccharides (fructans and galactooligosaccharides), lactose, excess fructose, and sugar polyols (mannitol and sorbitol) [221]. During sourdough fermentation, FODMAPs from cereals or produced by microorganisms include fructans, fructose, raffinose, and mannitols. FODMAPs may trigger symptoms of IBS patients and low-FODMAP sourdough bread was shown to improve

symptoms of IBS patients including flatulence, abdominal pain, cramps, and stomach rumbling [36,130]. A low-FODMAP diet was defined with a total FODMAP cutoff value of ≦ 0.5 g per serving excluding lactose that with a low-FODMAP cutoff value of < 1g per serving [221]. The reduction of FODMAP by conventional sourdough fermentation may improve tolerance in healthy individuals. Fermentation with FruA-expressing *L. crispatus* DSM29598 produced a low-FODMAP bread that is more acceptable for IBS patients (Chapter 4). Moreover, arabinan utilization operon of *L. crispatus* DSM29598 encodes putative α-galactosidase by *aga* and β-galactosidase by *lacLM* genes, that mediate the hydrolysis of raffinose and lactose, other FODMAPs in cereals (Chapter 5). In addition, glutathione reductase activity of *L. sanfranciscensis* converts oxidized glutathione to reduced glutathione with the accumulation of thiols during sourdough fermentation, that supports the proteolytic degradation of ovotransferrin, a high disulfide-bonded allergen [265,266]. Conventional sourdough application in bread making likely improves tolerance in many individuals by partial detoxification of offending proteins including the amylase trypsin inhibitor [222].

6.2 Lifestyle-associated metabolisms for ecological fitness of host-adapted lactobacilli

Acid resistance systems of host-adapted lactobacilli are essential for the ecological success of organisms exposed to acidic habitats, such as forestomach of rodents [2]. The acid tolerance test of lactobacilli *in vitro* may provide clues about the ecological fitness of specific strains in animal forestomach. *In vitro*, glutamine metabolism mediated by glutaminase and glutamate decarboxylase provided the protection for survival of *L. reuteri* at pH 2.5 [21] (Chapter 3), arginine metabolism at pH 3.5, and urease pathway at pH ≦ 4 [16]. The expression of a cyclopropane-fatty-acyl-phospholipid synthase is increased when *Levilactobacillus brevis* was grown under pH 4.0 [267].

In vivo, the expression of multiple genes related to acid resistance system increased in murine forestomach biofilm microbiota compared to that in hindgut microbiota, including genes encoding ureases, glutaminase, glutamate decarboxylase, arginine deiminase, arginine/ornithine antiporter, and cyclopropane-fatty-acyl-phospholipid synthase [80]. The expression of *gls* genes, *gadB*, *gadC*, and genes encoding urease were also upregulated when *L. reuteri* 100-23 colonized the mouse forestomach compared with the expression *in vitro* at neutral pH [268]. Glutamate decarboxylase also contributed to both survival in the *in vitro* gastric model and ecological performance in mice cecum but did not contribute to the tolerance of host gastric acid secretion during forestomach colonization [16]. Urease pathway contributes the most to survival and persistence of *L. reuteri* in the mouse forestomach, which can be explained by the availability of urea as substrates in the habitat, while during cereal fermentation, free amino acids are released by cereal protein hydrolysis as the substrates for acid resistance system [16,268].

As shown in Figure 6.1, extracellular fructansucrase and dextransucrase hydrolyzes sucrose to glucose and fructose, and transfer one of the moieties to a fructan or glucan chain, respectively [30], contributing to sucrose metabolism and the production of extracellular polysaccharides (EPS). EPS produced by fructansucrases or glucansucrases of *S. mutans* form the biofilm matrix of dental plaque, that enhances adherence and coherence of microorganisms, act as the energy source, and protect microorganisms from environmental stressors [91]. In lactobacilli, glucansucrase (GtfA) and fructansucrase (FtfA) activity are frequently present in the host-adapted genera *Lactobacillus* and *Limosilactobacillus*, and in *Liquorilactobacillus*. Most of *Liquorilactobacillus* species are associated with liquid, including water, water kefir, and alcoholic beverages. Many *Liquorilactobacillus* strains encode dextransucrase to produce dextran from sucrose; the specific size and structure of the dextran produced by *Liquorilactobacillus hordei* induced *Saccharomyces cerevisiae*

aggregation and network formation on hydrophilic surfaces, contributing to water kefir granule growth [11,269,270]. *Limosilactobacillus* species produce EPS to form biofilm and support the colonization of host epithelia. Extracellular GtfA and inulosucrase of *L. reuteri* TMW1.106 contributed to cell aggregation and *in vitro* biofilm formation; GtfA affected the colonization of *L. reuteri* in mouse gut [90]. Moreover, the genes encoding fructansucrase and glucansucrase were overexpressed in mouse stomach [80], demonstrating their contribution in biofilm formation.

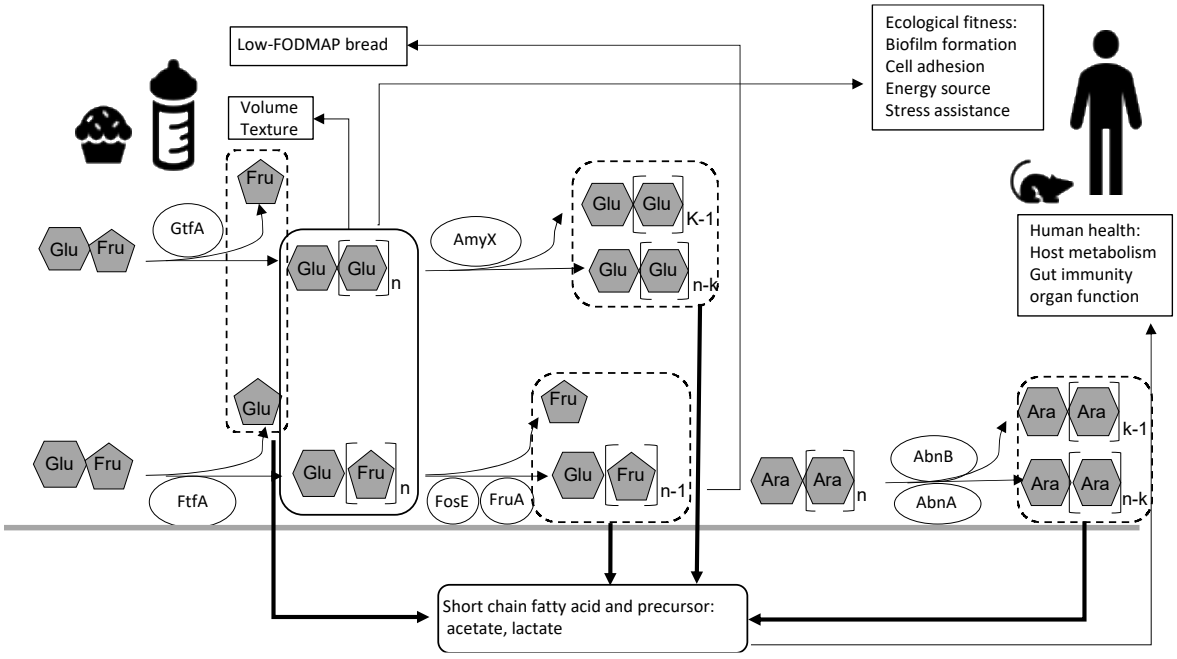

Figure 6. 1 Extracellular polysaccharide synthesis and hydrolysis by lactobacilli

Extracellular GtfA and FtfA mediate glucans and fructans synthesis with sucrose as the substrate [30]; Extracellular polysaccharides facilitate ecological fitness of lactobacilli by forming biofilm, improving cell adhesion, acting as energy source, and protecting microorganisms from environmental stressors. The synthesis of polysaccharides improve the volume and texture in bread and yogurt production[10]. Glucans and fructans in the habitat of lactobacilli can be hydrolyzed extracellularly by AmyX and FosE or FruA, respecively (Chapter 4) [30]. Degradation of fructans in cereals by FruA enable the production of low-FODMAP products by fermentation with lactobacilli (Chapter 4). Arabinanases, AbnA and AbnB, possess endo-activity to degrade Arabinan or Arabinose-containing polymers (Chapter 5). FtfA, FruA, and one of arabinanases are associated with cell wall. The degradation of polymers extracellularly enable their utilization of lactobacilli to produce acetate, and lactate as the precusors of propionate and butyrate production by other gut bacteria [224]. The production of short chain fatty acids in the intestine benefit human health in many ways.

To date, extracellular glycosyl hydrolases in lactobacilli includes endo-active amylopullulanases for starch hydrolysis [30], exo-active fructosidase FosE in *L. paracasei* [34] and FruA in *L. crispatus* for fructan hydrolysis (Chapter 4) [36], as well as endo-active

arabinanases in *L. crispatus* (Chapter 5, Figure 6.1). Extracellular amylopullulanase (AmyX) presents in several lactobacilli; most of these are homofermentative species of genera *Amylolactobacillus*, and *Lactobacillus* [13]. FruA is mostly present in oral organism *Streptococcus* and exclusively present in host-adapted genera *Lactobacillus* and *Ligilactobacillus* among *Lactobacillaceae* species (Chapter 4). Extracellular arabinanases are present in host-adapted *Streptococcus* and *Lactobacillus* species (Chapter 5). *Streptococcus mutans* produces FruA to release fructose from fructans generated by fructosyltransferase when exogenous carbon sources are limited [207,209], which contribute to the virulence of organisms in dental caries [35]. Similarly, extracellular glycosyl hydrolases in host-adapted *Lactobacillus* may contribute to the ecological fitness of lactobacilli through the degradation of polysaccharides that are formed by *Limosilactobacillus* species as part of the biofilm matrix [10,37] or plant fiber that is not digested by human enzyme and available in gastrointestinal tracts [271]. In addition, the surface layer associated protein (SLAP) domain identified in FruA and AbnA is mostly identified in surface layer protein of lactobacilli. Genetic analysis demonstrated that the SLAP domain and the combination of a GH32 / 43 catalytic domain with SLAP domain are exclusive to vertebrate-adapted *Lactobacillus* species, and may contribute to ecological fitness by attaching to the biofilm matrix or adhering to forestomach epithelium (Chapter 4 and 5)[80,272].

6.3 Lifestyle-associated metabolism for probiotic selection

Food fermentations with live probiotic organisms have been recognized as a tool to deliver beneficial microbes to the human or animal intestinal tract, and are also considered giving similar health benefits as intake of probiotic lactobacilli of the same specie [10]. Koumiss contains high cell counts of probiotic *Lactobacillus helveticus*, contributing to anti-

inflammatory attributes [133]; *Lactobacillus kefiranofaciens* in kefir decreased inflammation in a mouse model of obesity [74]. Moreover, food fermentation and gut environments habour closely related strains of lactic acid bacteria [5,7], and the consumption of fermented food can enrich conjugated linoleic acid in human gut, which is a putative beneficial molecule [6]. Host-adapted strains of lactobacilli show obvious competitive advantage in their respective hosts via host-specific features and therefore have a higher ecological fitness as a probiotic when compared to strains without a symbiosis history with the host [2,47,50,273]. The vertebrate host-adapted *L. reuteri* survives better during intestinal transit of piglets compared to *Lacticaseibacillus casei* and *Limosilactobacillus fermentum*, and reduced the abundance of total *E. coli* and the release of enterotoxins in swine intestine [138,274]. Ecology and lifestyle of lactobacilli thus are important criteria for the selection of probiotic strains. Vertebrate-adapted *L. reuteri* is autochthonous resident of human gastrointestinal tract and *L. crispatus* is a member of human vagina microbiota and found in various vertebrate hosts and body sites [2,89], which indicates their competitive advantage in human habitats. The study of their lifestyle associated metabolic traits may guide the application of these strains as probiotics selected for specific requirements.

The contribution of urease to acid tolerance of in *L. reuteri* 100-23 *in vitro* was consistent with its predominant protective effect against gastric acidity during the colonization of mouse intestinal tracts [16]. However, other acid resistant genes that also showed protective effects on acidic stress of *L. reuteri* 100-23 *in vitro* did not contribute to the survival *in vivo* [16]. Commercial probiotic strains that exhibited remarkable reduction *in vitro* acid tolerance test displayed high fecal recovery in the human feeding trails, such as *Lactobacillus acidophilus* LA5, *Lacticaseibacillus rhamnosus* GG, and *L. paracasei* IMPC 2.1 [275]. Taken together, acid tolerance *in vitro* may not be a suitable criteria for the probiotic selection to predict the persistent of them in host habitats.

Fructans and arabinopolysaccharides that are not digested by human enzymes or absorbed in the small intestine will feed symbiotic microorganisms residing in the human intestine as dietary fiber. Galactooligosaccharides administrated with specific probiotics were shown to increase the abundance of *Bifidobacterium adolescentis* and metabolic activity of *L. reuteri* [276,277]. Novel extracellular arabinanases and FruA facilitate *L. crispatus* DSM29598 to utilize dietary fiber as a probiotic or as a synbiotic served with arabinose-containing polysaccharides or inulin. The conversion of dietary fiber by lactobacilli in the gut can produce acetate, lactate and succinate as precursors of butyrate and propionate synthesis [96,224]. Acetate, butyrate, and propionate, as the major end products of dietary fiber fermentation in gut, exert anti-inflammatory and anti-tumor effects, and improve gut motility and secretory activity. Small amounts of acetate reach the circulation and directly induce beneficial effects in multiple organs, including the lung, brain, pancreas, bone marrow, and liver. Microbial succinate may have beneficial effects as a signaling molecule in human intestine [224]. Most of the vaginal *L. crispatus* carry a putative extracellular pullulanase that may mediate the degradation of glycogen as the major carbon source in the vagina. The degradation of glycogen by *L. crispatus* produces lactate that may affect physiological functions in the vagina [224,278].

6.4 Conclusion

Phylogenetic analysis of glutaminase, FruA, AbnA, and AbnB demonstrated their exclusive presence in host-adapted lactobacilli, indicating the lifestyle association of these metabolic enzymes. Lifestyle-associated metabolic traits of host-adapted lactobacilli contribute to their ecological fitness in host and food environments and improve food quality (Chapter 2). The contribution of glutaminase to acid resistance of *L. reuteri* 100-23 was demonstrated *in vitro*, while the contribution of glutaminase to metabolite accumulation by *L. reuteri* was limited

(Chapter 3).

The degradation activity of extracellular FruA in *L. crispatus* DSM29598 was observed with levan and inulin as substrates. The reduction of FODMAP by sourdough fermentation may improve tolerance in many individuals. Fermentation with FruA-expressing *L. crispatus* DSM29598 produced a low-FODMAP bread (Chapter 4). The identification of arabinan utilization operon in *L. crispatus* indicates its ability to utilize arabinan and arabinose as carbon sources. Extracellular AbnA and AbnB hydrolyzed linear arabinan and explained the growth of *L. crispatus* in mMRS with linear arabinan (Chapter 5). This study provides information on lifestyle associated metabolic traits of host-adapted lactobacilli, acid resistance and extracellular polysaccharide hydrolysis, which are essential to understand the ecological role and modulate application of lactobacilli in food fermentations. The investigation of those metabolic traits also guide the selection of strains as probiotics and exploration of their impact on host health.

6.5 Limitations and future directions

This study has demonstrated the contribution of glutaminase to acid resistance of *L. reuteri in vitro*, however, because of dynamic changes in host gastrointestinal tract and different acids used to mimic the gut environment *in vitro*, the role of glutaminase on survival and persistence of *L. reuteri* need to be tested in animal trials prior to the confirmation of its contribution to ecological success. The contribution of glutaminase to glutamate and GABA accumulation was not established in this study due to the interference of glutamine-amidotransferases. To analyze the function of glutaminase, exogenous and purified glutaminase needs to be tested on the production of glutamate and GABA, which is important for bread quality improvement. The supplementation of GABA in food may affect the brain and cognition through blood-brain barrier passage or the enteric nervous system [279].

Glutamate and GABA produced by probiotics in the gut act as excitatory and inhibitory neurotransmitters in the central nervous system of humans respectively, modulating excitation-inhibition balance and thereby play an essential role in neural disease prevention [280].

The contribution of SLAP domains to the attachment of FruA and AbnA to *L. crispatus* cells can be further confirmed by the comparison of enzyme location in wild type and *L. crispatus* derivative with deletion of SLAP domain. The effects of the SLAP domain on the competitiveness and ecological fitness of *L. crispatus* in hosts need to be studied by animal experiments.

Besides, the regulation of the expression of extracellular arabinanases in *L. crispatus* need further investigation. Further work could explore its function on arabinose metabolism and the regulation of gene expression on the operon. The contribution of extracellular FruA and arabinanases to ecological fitness and probiotic activities of *L. crispatus* should be investigated in animal or human trials.

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