Lehrstuhl für Technische Mikrobiologie

Regulation and ecological relevance of fructosyltransferases in *Lactobacillus reuteri*.

Clarissa Schwab

Regulation and ecological relevance of fructosyltransferases in *Lactobacillus reuteri*.

Clarissa Schwab

Doctoral thesis

Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung

und Umwelt

Lehrstuhl für

Technische Mikrobiologie

TECHNISCHE UNIVERSITÄT MÜNCHEN

ABBREVIATIONS

4dipyPC	1-pyrenebutanoyl-*sn*-glycerol-3-phosphatidylcholine
BW	bandwidth
CCCP	carbonyl cyanide m-chloro phenylhydrazone
Ftf	fructosyltransferase
FOS	fructooligosaccharides
GOS	glucooligosaccharides
GP	generalized polarization value
GPC	gel permeation chromatography
Gtf	glucosyltransferase
HoPS	homopolysaccharide
HPAEC-IPAD	high performance anion exchange chromatography with integrated pulsed amperometric detection
HPLC	high pressure liquid chromatography
I	fluorescence intensity
IMO	isomaltooligosaccharides
Laurdan	6-dodecanoyl-2-di-methyl-amino-naphtalene
mMRS	modified de Man, Rogosa, Sharp broth
PAS	Periodic acid/Schiff's reagent staining
PB	phosphate buffer
PE	phenylethanol
REST	relative expression software tool
RFU	relative fluorescence units
RLF-mice	reconstituted lactobacilli free mice
RTC	reutericyclin
SEM	scanning electron microscopy
TIH	trans-isohumulone
TLC	thin layer chromatography

TABLE OF CONTENTS

1 INTRODUCTION

Lactobacilli are members of the group of lactic acid bacteria. Lactic acid bacteria obtained their name due to the formation of lactic acid as the only or main final product of carbohydrate metabolism. The habitats of lactobacilli are characterized by the presence of fermentable carbohydrates and include: the bodies of humans and animals, plants and fermented or spoiled foods (45). Among the genus *Lactobacillus,* the species *Lactobacillus reuteri* stands out because strains are higly competitive in certain food fermentations and the gastrointestinal tracts of animals, form structurally different antimicrobial compounds and harbour several glycosyltransferases.

1.1 Habitats of *Lactobacillus reuteri*

1.1.1 The gastrointestinal tract

Microorganisms present within the gut flora can be divided into two groups:

- autochthonous organisms, which colonize the host at early age and persist and profilerate throughout the lifetime of the host

- allochthonous organisms, which are usually introduced via the uptake of foods. These organisms survive the passage but are only transient in the gut system

In humans, lactobacilli have been frequently isolated from feces or in a recent study, from the stomach mucosa (99). Yet, they only present a minority within the entire bacterial community of the human gut and are thought to originate from the oral cavity or the uptake of fermented foods (29). They are therefore considered allochthonous. Long-term studies indicated that individual humans harbour putative autochthonous lactobacilli, which can be detected over a period of time. The most persistent belonged to the species *L. ruminis* and *L. salivarius* (120).

In contrast, lactobacilli dominate the microflora in the proximal digestive tracts of rodents, pigs and birds. The forestomach of rodents, the esophagus pigs and the crops of birds are lined with non-glandular squamous stratified epithelium, whereas glandular mucosa lines the human stomach (15, 80, 116). Autochthonous lactobacilli directly adhere to the non-glandular epithelium and form dense colonies (118). The ability to adhere is considered animal host specific. However, exceptions have been reported (104, 121), for e.g. the sourdough isolates *L. reuteri* TMW1.106 and LTH5448 colonized reconstituted lactobacilli-free mice (RLF-mice) at numbers comparable to a rodent isolate (37). RLF-mice harbour a complex microflora but lack the presence of lactobacilli and enterococci (115, 119). The application of this colony of mice allows the determination of the lactobacilli associated impacts on the host animals. However, lactobacilli lack the competition of other lactic acid bacteria.

During colonization of RLF-mice, lactobacilli directly adhere to the forestomach epithelium and form a dense biofilm layer as illustrated in Figure 1 and Figure 2. Lactobacilli shed from the forestomach epithelium permeate the digesta and can be detected from the jejunum, cecum and feces. The application of IVET technology and further investigations employing mutants pointed out a high molecular mass surface protein (Lsp) and methionine sulfoxide-reductase B (MsrB) which defined performance of *L. reuteri* 100-23 during adhesion (142, 143).
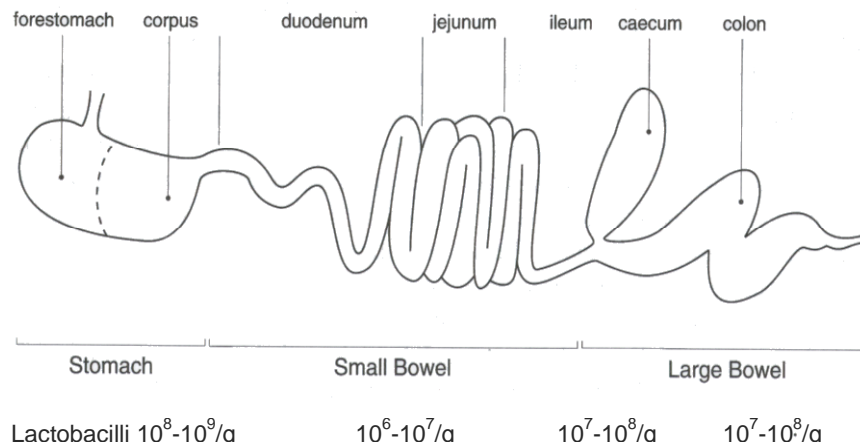
Figure 1 Distribution of lactobacilli in the murine intestinal tract (141).
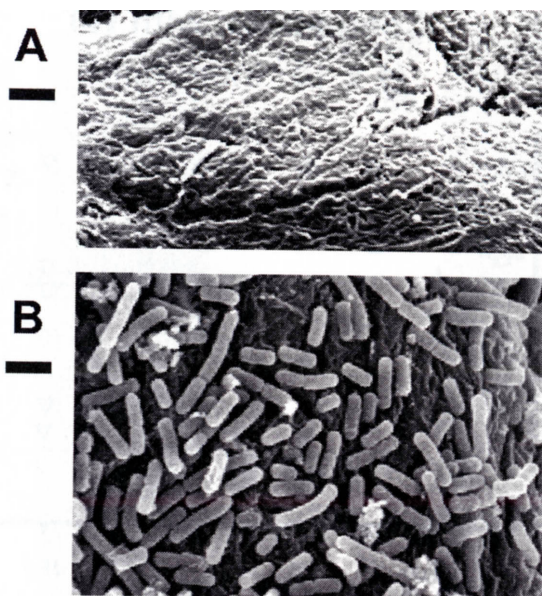
Figure 2. In vivo adherence of *L. reuteri* 100-23C to the forestomach epithelium of reconstituted lactobacilli-free mice (RLF-mice) determined using SEM. Bars represent one micrometer. Figure A shows the forestomach epithelium of a mouse colonized with *L. reuteri* 100-23C. Figures were taken from Walter et al. (2005) (142). *L. reuteri* 100-23C is a variant of *L. reuteri* 100-23 lacking the indigenious plasmid pGT232, which was generated for use as an electrotransformation host (50).

1.1.2   Presence of *L. reuteri* in food fermentations

Cereal fermentations. Sourdough is an intermediate product in bread production. The microflora of sourdough is composed of yeast and lactic acid bacteria. Lactobacilli represent the majority within the lactic acid bacteria (46). The prevalence of heterofermentative lactobacilli in cereal fermentations has been attributed to the combined use of maltose and electron acceptors like fructose thus producing lactate and acetate (111, 112, 113). Besides, lactobacilli form various structurally different antimicrobial compounds, for example bacteriocins, reuterin and reutericyclin (reviewed in 38). The formation of lactic acid bacteria furthermore leads to acidification of the dough and the formation of aroma precursors, which determine bread quality and bread aroma. Type II sourdoughs, which are used as dough acidifiers, are fermented for long periods at temperatures higher than 30 °C to obtain high amounts of lactic acid and acetic acid, demanding increased acid tolerance of the bacterial cultures. *L. reuteri* is a highly competitive and dominant strain in type II sourdough (78). Strains of *L. reuteri* persisted in continuously propagated type II sourdoughs over long periods of time (9, 39, 78). The enhanced competitiveness of *L. reuteri* strains has been partly attributed to the formation of reutericyclin, a tetramic acid with activity against mainly gram-positive bacteria, which is produced in significant amounts during growth in wheat sourdough (39, 41).

L. reuteri strains have been also associated with fermented maize and sorghum doughs in Benin and the Sudan, respectively. Due to higher environmental temperatures, these fermentations run at conditions similar to type II sourdough (1, 44, 56).

Cheese. *Lactobacillus reuteri* is a strain traditionally not applied in cheese manufacturing due to low milk acidification capacity (52). However, laboratory trails

showed that *L. reuteri* strains might act beneficial during cheese fermentation. Aminopeptidase activity of *L. reuteri* was lower compared to traditional starter cultures, however, the application of *L. reuteri* together with *L. johnsonii* resulted in improved texture of low fast cheeses (129).

1.2 Glycosyltransferases and homopolysaccharide formation in lactic acid bacteria with a focus on *L. reuteri*

Extracellular polysaccharides can be divided into two groups depending on the composition and the mechanism of formation: Heteropolysaccharides consist of more than one type of monosaccharide and are produced intracellularly through the combined action of several glycosyltransferases. The repeating monosaccharide units are derived from sugar nucleotides. In contrast, oligosaccharides and homopolysaccharides (HoPS) consisting of either fructosyl- (fructooligosaccharides (FOS) and fructans) or glucosyl- (gluco-oligosaccharides (GOS) and glucans) moieties are synthesized by the single action of extracellular glycosyltransferases termed fructosyltransferases (Ftfs) and glucosyltransferases (Gtfs), from sucrose. Energy gained through hydrolysis of the osidic bond of sucrose is used for the transfer of fructose or glucose to an acceptor molecule. Fructosyltransferases also use raffinose as a substrate. Glucosyltransferases trigger the cleavage of sucrose molecules, transfer of glucose units to a growing glucan chain (transglucosylation), to another substrate, e.g. maltose or isomaltose to synthesize gluco-oligosaccharides or to fructose (acceptor reaction) and hydrolysis (transfer of glucose units to water) (61, for a review 82). Fructosyltransferases cleave the substrates raffinose or sucrose, transfer the fructose units to a growing fructan (transfructosylation), to sucrose or

another acceptor, for example maltose, lactose, arabinose, raffinose and xylose, or to water (hydrolysis) (90,128, reviewed in 135).

Glucosyltransferases of lactic acid bacteria are composed of 4 domains: (i) a N-terminal signal peptide, (ii) a N-terminal variable region, (iii) a catalytic core, and (iv) the C-terminal. The catalytic core is highly preserved, the C-terminal contains the glucan-binding region. Fructosyltransferases are also divided into four structurally different regions: (i) signal peptide, (ii) a N-terminal variable region, (iii) the preserved catalytic core region, (iv) the C-terminal sometimes containing a cell wall binding domain (LPXTG). The function of the C-terminal domain is considered the anchoring to the producing organism. In contrast, the role of the N-terminus has not been assigned yet (for a review 135).

Homopolysaccharide formation has been frequently described in strains of *Streptococcus, Leuconostoc, Weissella* and *Lactobacillus* (22, reviewed in 83, 124, 135) and numerous glycosyltransferases have been isolated and described and are listed in Table 1. In lactobacilli, the formation of HoPS is often associated with strains isolated from animal intestines. Screenings by Tieking et al. (126, 127) showed that between 20 and 25% of isolates from sourdough, pig and duck origin synthesized HoPS. Glycosyltransferases are termed according to the main linkage type of the polymer synthesized. In *Lactobacillus* species, namely, *L. johnsonii, L. reuteri, L. sakei, L. fermentum, L. parabuchneri* and *L. sanfranciscensis* reuteransucrases, mutansucrases, dextransucrases, which all form α-glucan-type HoPS, as well as a levansucrases and inulosucrases, which synthesize fructan-like HoPS, have been characterized to date (63, 64, 66, 67, 126, 127, 132, 133, 134). The size of bacterial glucans is around 10^7 kDa (66). Bacterial levan sizes range between $10^4 – 10^7$ kDa.

Bacterial inulin, in contrast to plant inulin, also has a size of about 10^7 kDa (86, 132, 133, 134).

Various strains of *Streptococcus* and *Leuconostoc* harbour more than one glycosyltransferase (105, 109, 140). In the species *Lactobacillus,* the presence of various glycosyltransferases within one strain and the great variety of products formed by these glycosyltransferases is a characteristic trait of *L. reuteri* (64, 131, 133). *L. reuteri* 121 harbours two glucosyltransferase related genes, *gtfA* and the silent and inactive *gtfB*, as well as the fructosyltransferases encoding *lev* (Lev) and the silent *inu* (Inu) (64, 132, 133).

Table 1. Types of homopolysaccharides (HoPS) formed by lactic acid bacteria and examples of strains synthesizing these HoPS.

HoPS		strains	Main linkage types	gene	reference
glucan	dextran	*L. reuteri 180*	α-(1→6)	*gtf180*	64
		L. fermentum		*gtfKg3*	64
		S. mutans GS-5		*gtfD*	48
	mutan	*L. reuteri* ML1	α-(1→3)	*gtfML1*	64
		S. mutans GS-5		*gtfB*	36, 107
		S. mutans GS-5		*gtfC*	36, 130
	reuteran	*L. reuteri* 121	α-(1→4)	*gtfA*	66
fructan	levan	*L. reuteri* 121	β-(2→6)	*lev*	132
		L. sanfranciscensis		*levS*	125
	inulin	*L. reuteri* 121	β-(2→1)	*inu*	133
		L. citreum CW28		*islA*	89, 90
		S. mutans GS-5		*ftf*	51, 106

1.3   Role of glycosyltransferases and products formed

1.3.1   Sucrose metabolism in lactic acid bacteria

In *Streptococcus* and *Leuconostoc* species, the major proportion of sucrose is converted into HoPS by extracellular glycosyltransferases. A portion of the sucrose is transported into the cell, phosphorylated by sucrose phosphorylase and ultimately converted to lactate, acetate and ethanol via the heterofermentative pathway (33, 55). Regulation of glycosyltransferases in streptococci and *Leuconostoc* spp. is directed by sucrose. For example *Streptoccus mutans* harbours three Gtfs coded by *gtfB*, *gtfC* and *gtfD* and a Ftf, *ftf* (105, 106, Table 1). Ftf converts sucrose to a fructan carbohydrate reserve that is hydrolysed by the streptococcal fructanase upon carbohydrate depletion. Accordingly, Ftf is upregulated in the presence of sucrose or excess glucose and a protein termed RegM, which is similar to the carbon control protein A, is required for optimal ftfA expression (16, 60). A two-component regulatory system, covRS, mediates the up-regulation of ftfA in the presence of sucrose (69).

GtfB, GtfC and GtfD synthesize glucans, which are involved in cell-cell adhesive and cell-surface interactions. GtfB and GtfC are cell surface associated and form water-insoluble glucans rich in α-(1→3) linkages, GtfD is released in cell supernatant and forms water-soluble, mainly α-(1→6) linked glucan (see Table 1). Compared to FtfA, the Gtfs of *S. mutans* are much less susceptible to regulation by carbohydrate availability. The presence of sucrose increased *gtfD* transcription and decreased *gtfB* and *gtfC* transcription (35). In addition to carbohydrate levels, growth phase, environmental pH and growth conditions (planktonic versus biofilm culture), two quorum sensing signaling systems control the biofilm formation by *S. mutans* (73, 87, 97). The first quorum sensing systems consists of an autoinducer peptide, CSP, encoded by *comC*, a histidine kinase, ComD, that acts as a membrane-bound CSP-

receptor, and a response regulator, ComE, which is activated by ComD and in turn up-regulates the *comCDE* operon. The Com system is involved in initial adherence and biofilm architecture and *comC* deletion mutants exhibit an abnormal biofilm structure (for review, see 28). The second quorum sensing system employs the AI-2 molecule, a furanosyl borate diester produced by LuxS (102, 114). *LuxS* is present in many Gram-positive and Gram-negative bacteria and AI-2-mediated cell density signalling might enable inter-species communication (114). *S. mutans* harbours a *luxS* homologue, forms an AI-2 molecule, and *luxS*-dependent signaling is involved in the regulation of biofilm formation and stress tolerance in *S. mutans* (79, 147). The expression of *gtfB*, and *gtfC* promotor-gene fusions was not affected in a *luxS* deletion mutant of *S. mutans* UA159 (147) whereas *luxS* inactivation down-regulated *gtfG* expression in *S. gordonii* (77).

In *Lc. mesenteroides* the expression of dextransucrase in is relatively low in the presence of carbon sources other than sucrose and is increased by the addition of sucrose (84, 96). A sucrose concentration of 20 g L^{-1} stimulates dextransucrase DsrD expression in *L. mesenteroides* Lcc4 and increased sucrose levels do not result in a further increase in enzyme synthesis (85).

1.3.2 HoPS production and stress tolerance of lactobacilli

Exopolysaccharide production of LAB has repeatedly been reported to improve their survival at adverse environmental conditions. Dextran supported survival of *Lc. mesenteroides* during starvation under alkaline and acidic conditions. Exopolysaccharides produced by *Lactococcus lactis* protected the strain in the presence of nisin (58, 75). The application of the prebiotics FOS, inulin and polydextrose promoted survival of lactobacilli during spray-drying (4, 24). Desmond et al. (31) reported that the addition of polysaccharides inulin, dextran and

polydextrose to skim milk powder protected *L. paracasei* NFBC338 during freeze-drying, however, stabilizing effects became most apparent during storage.

1.3.3 Protective effects of sugars during freeze-drying

Viability loss of bacteria during freeze-drying is attributed to osmotic shock and membrane injury due to intracellular ice formation and recrystallization (49). Cryoprotectives are used to reduce viability loss during freeze-drying. Skim milk is widely applied as a cryoprotective in starter culture industry. Moreover, the application of a variety of chemical compounds either alone or in addition to skim milk has been tested (for a review, 18, 21). For example, carbohydrates offer cryoprotective properties. The mono- and disaccharides glucose, sucrose, maltose, lactose and trehalose as well as the oligosaccharide maltodextrin increased viability of lactic acid bacteria during freezing and freeze-drying (30, 88). The protective effect of sugars during freeze-drying is attributed to both direct interaction with proteins and membranes and the formation of a glassy matrix, which is characterized by high viscosity and low mobility (7, 26). In model mono- and bilayer membranes, fructans of the inulin and the levan type as well as fructooligosaccharides, but not glucans, have been shown to directly interact with phospholipids thus stabilizing these membranes during air-drying and freeze-drying (53, 54, 137, 138, 139).

1.3.4 Adherence to gastrointestinal or vaginal epithelium and biofilm formation

Lactobacillus species, mainly *L. iners, L. crispatus, L. jensenii, L. acidophilus* and *L. gasseri* constitute most of the vaginal flora of healthy women and reach up to 10^7 - 10^8 CFU g^{-1} vaginal fluid (110). The human vagina is lined by stratified, squamous, nonkeratinized, non-glandular epithelium, which is directly colonized by the *Lactobacillus* strains. Glycoproteins and carbohydrates of *L. acidophilus* and *L.*

gasseri, and *L. jensenii*, respectively, take part in adherence to vaginal epithelial cells (12, 136). In vitro experiments investigating the adherence mechanism of *Lactobacillus* species to rodent intestinal cell lines suggested that carbohydrates and proteins are involved in so far mainly unknown adherence mechanism (117). Sucrose-dependent adhesion of streptococci to tooth surfaces is triggered by combined actions of glycosytransferases, polysaccharides and extracellular glucan binding proteins (5, 28, 100, 101).

1.4   Motivation and objectives

Lactobacilli constitute the natural flora of cereal fermentations and have been identified as the dominating microflora of the gastrointestinal tract of rodents, porcine and the crop of birds. Competitiveness of lactobacilli has been attributed to the formation of organic acids and various structurally different antagonistic substances, however these traits alone do not fully explain the prevalence of specific *Lactobacillus* strains in certain intestinal environments and cereal fermentations (reviewed in 38). Numerous *Lactobacillus* strains isolated from cereal fermentations and intestinal regions form HoPS, and some corresponding glycosyltransferases have been isolated (124, 125); among those are strains of *L. reuteri*, which synthesize HoPS and appear competitive and adapted to these environments. However, the role of EPS formation in the prevalence and performance remains unclear.

It was therefore the objective of this work to determine the regulation and functionality of glycosyltransferases and their products formed in two strains of *L. reuteri,* which are both highly competitive in sourdough and in gastrointestinal tract of rodents. Investigations thereby focused on sucrose metabolism, stress tolerance and competitive performance in the habitats sourdough and gastrointestinal tract of mice.

2   MATERIALS AND METHODS

2.1   Glycosyltransferases of *L. reuteri*

2.1.1   Screening for the presence of glycosyltransferases

Bacterial cultures and growth conditions. *L. reuteri* LTH5448, TMW1106, TMW1.656 and LTH2584 were isolated from sourdough (9, 39, 78). *L. reuteri* TMW1.272, TMW1.279, TMW1.285, TMW1.146, TMW1.194, TMW1.138 and TMW1.967, TMW1.972, TMW1.973, TMW1.974, TMW1.975, TMW1.976, TMW1.980 were isolated from porcine and duck crop, respectively (68, 126). Strains were grown in mMRS (111) substituted with 10 g L^{-1} maltose, 5 g L^{-1} glucose and 5 g L^{-1} fructose anaerobically at 37 ºC unless otherwise stated.

General molecular techniques. Genomic DNA of *L. reuteri* strains was isolated according to Lewington et al. (72). DNA was amplified by PCR using dNTPs from Diagonal (Waldeck, Germany) and taq polymerase from Promega (Mannheim, Germany). Primers were purchased from MWG Biotech (Ebersberg, Germany) and are listed in Table 2. PCR products were visualized after electrophoretic separation on agarose gels. Amplicons were either eluted from the gels using the E.Z.N.A. gel extraction kit (PEQlab, Erlangen, Germany) or cleaned with the QIAquick PCR Purification KIT (Qiagen, Hilden, Germany) and sequenced by SequiServe (Vaterstetten, Germany). WuBlast2 (EMBL, Heidelberg, Germany) or Pubmed's BLAST were used for database comparisons. Reagents and enzymes were used according to the manufacturer's instructions unless otherwise indicated.

Initial screening of the genomes of *L. reuteri* LTH5448 and TMW1.106 for the presence of glycosyltransferase genes. Strategies used to investigate glycosyltransferases in both *L. reuteri* strains are summarized in Figure 3. Briefly,

degenerated primers DexWobV and DexWobR, Leu wob F and Leu wobR as well as Inu F and Inu R based on the *inu* sequence of *L. reuteri* 121 (133) were used to screen the genomes of *L. reuteri* TMW1.106 and LTH5448 for the presence of glycosyltransferases.

The complete sequence of *inu* of *L. reuteri* TMW1.106 was obtained using primers targeting C- and N-terminal regions of *inu* of *L. reuteri* 121.

In *L. reuteri* LTH5448, regions upstream of the fragment obtained using primer pair Leu Wob F and LeuWob R were amplified using a primer ISEFA F. ISEFA F was constructed on the sequence of an IS-element of *L. reuteri* (A. Giessler, master thesis) based on hypothesis, that a transposon or an IS element might be located upstream of *ftfA* in analogy to the gene assembly in *L. reuteri* 121 and *L. sanfranciscensis* TMW1.392 (125, 134). An amplicon was obtained, however, the region upstream had no homology to a mobile element. For inverse PCR, chromosomal DNA of *L. reuteri* LTH5448 was digested with various restriction enzymes (Fermentas, St Leon-Rot, Germany) at 37 °C and ligated overnight at 16°C with T4 Ligase (Fermentas). The primer pair Ftfinv F and Ftfinv R was used to obtain the C-terminal region of *ftfA*.

Occurence of glycosyltransferases in sourdough, duck and porcine isolates of *L. reuteri*. The genomes of 18 *L. reuteri* strains isolated from ducks, pigs, sourdough and rat, which had been tested for HoPS and oligosaccharide formation by Tieking et al. (2005) (126), were screened for the presence of *inu, ftfA, gtfA* and *lev* using primer pairs Inu F and Inu R, Leureu1001V and Leureu1001R, GlcV and GlcR, and LevV and LevR respectively (Table 2).
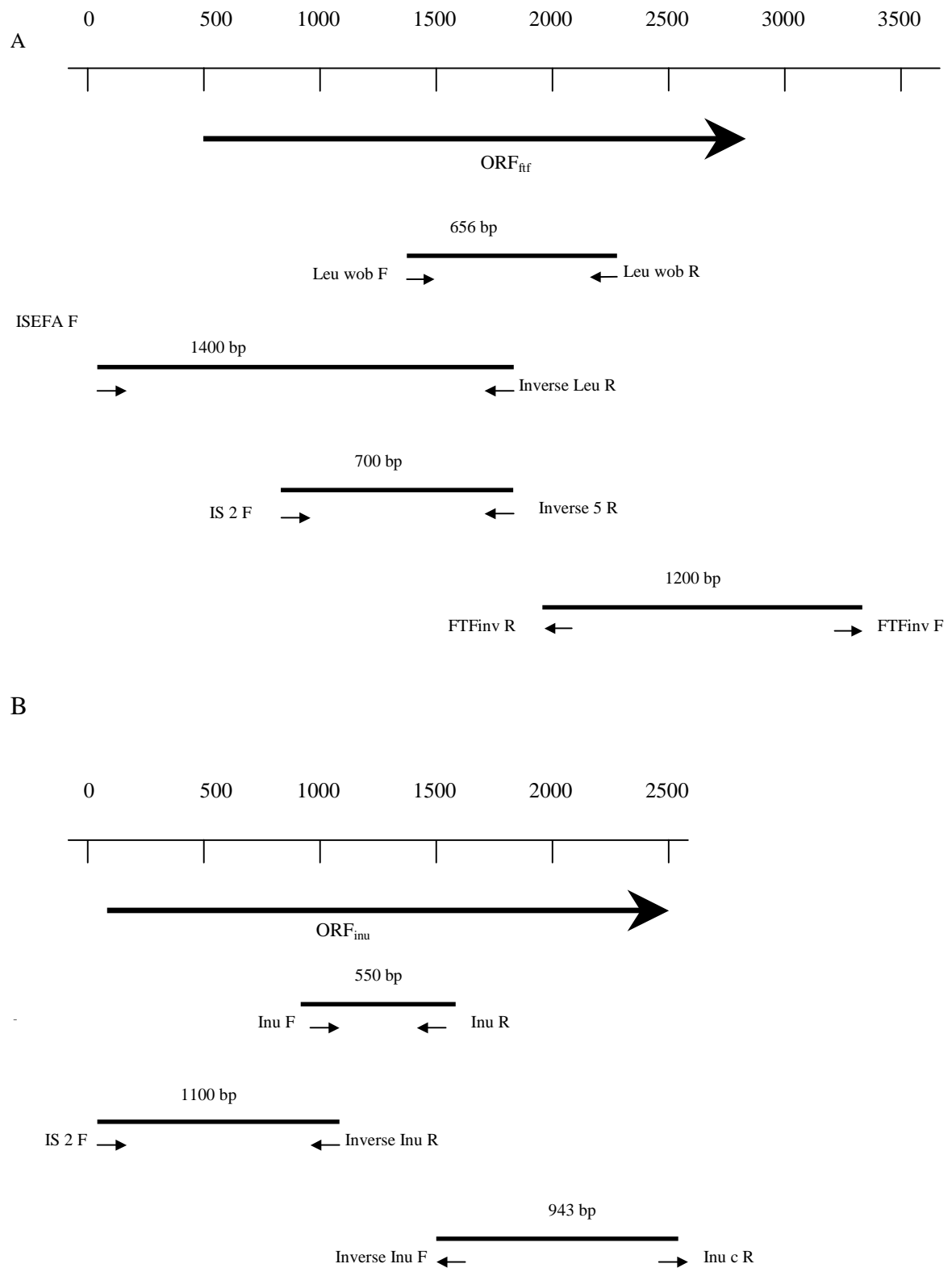
Figure 3. Strategy used for determination of *ftf* (A) and *inu* (B) genes in *L. reuteri* LTH5448 and TMW1.106, respectively.

2.1.2 Products formed by glycosyltransferases from sucrose under standard conditions

Growth conditions. *L. reuteri* strains were grown in mMRS with 100 g L^{-1} sucrose as carbohydrate source at 37 ºC (111). Sucrose was autoclaved separately. For polymer structure analysis of the polymer, *L. reuteri* LTH5448 was grown in mMRS medium containing 100 g L^{-1} sucrose at 37 ºC for one day (111).

Isolation of HoPS and determination of monosaccharide composition. Polysaccharides were precipitated from culture supernatants with three volumes of ethanol. The precipitate was harvested by centrifugation, dried, resuspended in deinonized water, and dialysed against ddH$_2$O overnight at 4 ºC using a dialysis membrane with a MW-cutoff of 1.2-1.4 x 10^4. For monosaccharide analysis, purified HoPS was hydrolysed with 2 M H$_2$SO$_4$ at 80 ºC for 2 h. Monosaccharides in the hydrolysate were quantified by HPLC as described (127).

Quantification of poly- and oligosaccharides from culture supernatants. HoPS in culture supernatants of *L. reuteri* LTH5448 and TMW1.106 was quantified by gel permeation chromatography (GPC) as described (127). Levan from *L. sanfranciscensis* was purified by ethanol-precipitation, dialysis, and GPC as described (126). To quantify glucan in culture supernatants of *L. reuteri* TMW1.106, glucan was purified, diluted to 5 different concentrations and analyzed by GPC. Corresponding volumes of these dilutions were hydrolysed, and the glucose concentrations were determined as described above. The glucan concentration in the samples was calculated based on the concentration of glucose using the conversion factor 162 g mol^{-1} polymeric glucose. A calibration curve previously established with levan from *L. sanfranciscensis* TMW1.392 was used to estimate fructan concentrations in culture supernatants of *L. reuteri* LTH5448. Oligosaccharides were quantified by HPLC as

described (62). Supernatants were furthermore analyzed after overnight incubation with 10 g L^{-1} inulinase (Fluka) at 37 °C. The error of HPLC quantification of HoPS and FOS was generally less than 10%.

Characterization of the fructan formed by *L. reuteri* LTH5448. HoPS were precipitated with three volumes of ethanol at –20 ºC for 24 h. The precipitate was collected, dialysed against ddH_2O and freeze-dried. HoPS was resuspended in ddH_2O, phenol (60 ºC) was added at the same volume, and the mixture was shaken for 1 h. HoPS was recovered from the phenolic phase and dried. The HoPS was characterized using NMR and identified through comparison with spectra obtained from pure inulin and levan (6). The structure of the fructan was determined by David Bundle, Carbohydrate Research Centre, University of Alberta, Canada.

2.1.3 Heterologous expression of FtfA and Inu

Strains and culture conditions. *E. coli* strains Top10 and LMG194, together with vector pBAD/Myc-HisB were purchased from Invitrogen (Burlington, Canada). *E. coli* TOP10 and LMG194 were grown in Luria-Bertrani broth (LB, Difco, Oakville, Canada) containing 60% sorbitol (Sigma, Mississauga, Canada) and minimal medium (RM medium), respectively, aerobically with shaking (220 pm) at 37 °C. Ampicillin (100 µg L^{-1}) and arabinose (1 mM) for gene induction were added when appropriate. The levansucrase (Lev) of *L. sanfranciscensis* TMW1.392 was expressed and purifed as described (127). Electrocompetent *E. coli* were prepared according to Tieking et al. (125).

Construction of plasmids for expression of *inu* and *ftfA* in *E.coli*. As *inu* of *L. reuteri* TMW1.106 and *L. reuteri* 121 are 99% identical (see section 3.1), primers were designed according to van Hijum et al. (2002) (133) with modifications. Instead of a BglII restriction site, a XbaI restriction site was introduced. Inuklon-for, Inuklon-

rev primers were used for expression of full length his-tagged *inu* (Inu). An *inu* variant C-terminally truncated from amino acid 699 upwards (InuklonC) was obtained using primers and Inuklon-C and Inuklon-for. As *ftfA* and *inu* are highly homologous within the C- and N-terminal, primers Inuklon-for and Inuklon-rev were also used to obtain a full-length his-tagged *ftfA*. A *ftfA* sequence truncated from aa 699 onwards was generated using primers Inuklon-for and FtfAklon-C. Primers are listed in Table 2. Genes were amplified using standard PCR techniques. Purified amplicons and pBAD/Myc-HisB were cut with NcoII and XbaI (Fermentas, Burlington, Canada). Restriction enzymes were heat inactivated at 70 ºC for 10 min. After purification, the vectors and PCR products were ligated overnight at 16 ºC using T4 Ligase (Invitrogen) and consequently heated at 70 ºC for 10 min. The vectors were transformed into electrocompetent *E. coli*. Clones growing on LB agar containing ampicillin were checked for the presence of plasmids of the correct size. Furthermore, primers InuF and InuR, and Leureu1001V and Leureu1001R targeting internal sequences of *inu* and *ftfA*, respectively, were used to verify presence of the genes by PCR.

FtfA and Inu purification. Overnight cultures of *E. coli* TOP10 and LMG194 harbouring the respective *inu* and *ftfA* constructs were subcultured in LB and RM media containing ampicillin (1%). At an optical density OD_{590nm} 0.6-0.7 1 mM arabinose was added and strains were grown for 4 more hours.

Cells were harvested by centrifugation, washed and resuspended in TE buffer. Cells were broken using zirconium silica beads and a Mini bead beater (both BioSpec Products, Bartlesville, USA). Cell debris was collected by centrifugation and the supernatant was used for further purification steps. Three volumes of buffer A (50 mM imidiazol, 300 mM NaCl, 50 mM NaH_2PO_4) were added to the supernatant and

the sample was loaded on a HisTrap™ (Amersham Biosciences, Oakville, Canada) HP column at a flow rate of 1 ml min^{-1}. After washing with 20 volumes of buffer A, proteins were eluted using buffer B (300 mM imidiazol, 300 mM NaCl, 50 mM NaH$_2$PO$_4$).

PAS staining. Crude extract, collected fractions and run-through were mixed with four volumes of SDS buffer containing 5% mercaptoethanol and loaded on 12% SDS gel. After the run, gels were washed for 2 x 10 min with ddH$_2$O and incubated in sucrose buffer (25 mM Na-acetat, 1 mM CaCl$_2$, 100 mM sucrose, pH 5.4, all Sigma) overnight. Gels were washed 2 x 10 min with water and fixed in 12.5% trifluoracetic acid (Sigma) for 30 min. After another washing step (2 x 10 min in ddH$_2$O) gels were incubated in 1% periodic acid, 3 % acetic acid buffer for 50 min, washed again 2 x 10 min with ddH$_2$O and resuspended in Schiff's reagent (Sigma). Purple bands occur where PAS stainable polymer is produced. Gels were consequently stained using EZ-Blue, a colloidal Coomassie Brilliant Blue stain (Sigma), to confirm spot identity.

Activity of FtfAC and crude extract of heterogously expressed Inu. FtfAC, crude extract of heterologously expressed Inu (InuCE) and Lev were incubated in 50 mM Na-acetate buffer, 1 mM CaCl$_2$, pH 5.4 containing 50 g L^{-1} sucrose and additionally 50 g L^{-1}maltose or 50 g L^{-1} arabinose (all Sigma). Enzymes were incubated at 37 ºC overnight. Oligosaccharides were separated using a Supelcosil™ LC-NH$_2$ (5 μm, Supelco, Oakville, Canada) column and detected with an evaporative light scattering detector (Alltech 500 ELSD, Alltech; Guelph, Canada). HPLC grade water (A) and acetonitril (B) were used as mobile phase (0 min 90% B, 1.5 ml min^{-1}; 25 min 75%, 1.5 ml min^{-1}; 26 min 50% B, 0.9 ml min^{-1}; 32 min 50% B, 0.9 ml min^{-1}, 33 min 30% B, 0.9 ml min^{-1}; 40 min 30% B, 0.9 ml min^{-1}; 45 min 90% B, 1.5 ml min^{-1}). Peaks

were assigned using glucose, maltose, maltotriose, fructose, sucrose (both Sigma) and 1-kestose (Fluka, Oakville, Canada) as external standards.

2.2 Characterization of *inu, gtfA* and *ftfA* deletion mutants

Organism and culture conditions. *Ftfa, inu* and *gtfA* mutants of *L. reuteri* LTH5448 and TMW1.106, respectively, were provided by Jens Walter, University of Otago, New Zealand. Gene inactivation was achieved by site-specific integration of plasmid pORI28, which carried internal fragments of the target genes characterized in this work, into the *L. reuteri* chromosomes as described previously (142). *L. reuteri* LTH5448 and TMW 1.106 as well as the *ftfA, inu* and *gtfA* mutants were grown at 37 ºC in mMRS (111) substituted with 50 g L^{-1} sucrose and additionally 50 g L^{-1} maltose (malsucMRS), 50 g L^{-1} arabinose (arasucMRS) and 10 g L^{-1} maltose and 5 g L^{-1} fructose and 5 g L^{-1} glucose (mMRS). Erythromycin was added when appropriate at 5 µg L^{-1} final concentration. *L. reuteri* TMW1.106 and the *gtfA* and *inu* mutants were also grown in mMRS containing 10 g L^{-1} or 50 g L^{-1} maltose, 10 g L^{-1} maltose and 5 g L^{-1} glucose or 5 g L^{-1} glucose. Sugars were filter sterilized and added to the autoclaved media.

For analysis of cell counts, carbohydrate utilization and organic acid formation, strains obtained from -80 C stock cultures were subcultured twice in mMRS, washed and inoculated in fresh sucMRS and mMRS at approximately 10^4 CFU ml^{-1}. CFUs were determined after 24 h of incubation (day 1). *L. reuteri* TMW1.106 and the *gtfA* and *inu* mutants adapted to sucMRS for 24 h were washed and reinoculated in the respective buffer medium. After 24 h, cell counts were determinded again on mMRS plates (with erythromycin) (day 2). Data was averaged from two independent experiments.

Determination of metabolites. Cells were harvested after 24 h incubation and the supernatant was used for analysis of metabolites. Organic acids were determined using an Aminex HPX-87H column with 5 mM H_2SO_4 as solvent at 0.4 ml min^{-1}. Mono- and disaccharides were separated using an Aminex HPX-87P column (both Biorad, Mississauga, Canada) as recommended by the manufacturer. Analysis of oligosaccharides is described in section 2.1.3.

Thin layer chromatography of products formed by *L. reuteri* TMW1.106 and the *gtfA* and *inu* mutants from maltose and sucrose. 5 μL of supernatant obtained from overnight cultures of *L. reuteri* TMW1.106 and its variants grown in malsucMRS were separated on TLC plates (Whatman, London, UK). Gels were run thrice in a butanol: ethanol: water (5:5:3, v/v/v %) (91) mixture and stained with an ethanol solution containing α-naphtole (w/v 0.3%) and H_2SO_4 (v/v 5%). The staining was developed at 110 ºC for 10 min (23).

SDS-PAGE of whole cells and PAS activity staining. 50 ml of overnight culture of *L. reuteri* and the *gtfA* or *inu* mutants grown in mMRS containing different carbohydrate combinations were harvested, the pellet was washed once with PB and the cells were disrupted using a MiniBead beater. Cell debris was removed by centrifugation. Total protein content was determined using Biorad protein assay. Proteins were separated on 9% SDS gels, stained using EZ-Blue (Sigma) or periodic acid and Schiff's reagent as described above (section 2.1.3).

LC-MS/MS. Total proteins of *inu* mutant were separated on an 8% SDS-Page and stained using the PAS method (section 2.1.3) and/or EZ-Blue. A band of approximately 200 kDa was excised from the gel and identified using LC-MSMS (Department of Chemistry, Mass Spectometry Facility, University of Alberta, Canada). The protein database NCBInr was applied for homology search.

2.3 Regulation of glycosyltransferases in *L. reuteri* in response to carbohydrates

Strains and culture conditions. Overnight cultures of *L. reuteri* TMW1.106 and LTH5448 were washed twice in PB and subcultured (1%) in mMRS (111) containing 5 g L^{-1} glucose, 5 g L-1 glucose and 10 g L^{-1} sucrose, 10 g L $^{-1}$ sucrose or 10 g L^{-1} maltose, 5 g L^{-1} glucose and 5 g L^{-1} fructose (mMRS) (111).

RNA extraction and preparation. RNA was isolated from 1 ml exponentially growing cells (OD_{590nm} of 0.4,). Two volumes of RNAprotect were added, cells were harvested, frozen with liquid nitrogen and RNA was isolated using peqGoldRNApure (PEQlab, Erlangen, Germany) according to the instructions of the supplier. mRNA was reverse transcribed as described in detail in section 2.8.1. Successful synthesis of cDNA (cDNA) was verified by PCR-amplification of the *ldh* of *L. reuteri* coding for the lactate dehydrogenase (Q8GMJ0) emplying the primer pair LdhF and LdhR.

Analysis of transcriptional levels of the *inu*, *ftfA*, *gtfA* genes by PCR and real-time PCR. Quantitative analysis of the expression of glycosyltransferases was carried out using cDNA as a template in Real-time PCR reactions. Total RNA preparations were used as negative control, and chromosomal DNA from *L. reuteri* TMW1.106 or LTH5448 was used as positive control. Primers were chosen to obtain amplicons not longer than 150 bp and are listed in Table 2. Real-time PCR was performed using a Light Cycler (Roche Molecular Biochemicals, Mannheim, Germany) essentially according to procotols established recently for mRNA quantification in lactobacilli (14). Primers were added to a concentration of 0.05 pmol to a master mix containing 10 µl QuantiTect SYBR Green PCR Master Mix (Qiagen), 2 µl cDNA and RNAse free water in a final volume of 20 µl. Melting curve analysis and determination of

amplicon size verified amplification of the appropriate transcripts. Transcription was quantified applying the REST software (93). The genes encoding a lactate dehydrogenase *ldh* and phosphoketolase (*pho*) were used as reference. The primer pair PhoketV and PhoketR targeting *pho* were constructed based on the sequence of a phosphoketolase of *L. reuteri* ATCC5730 (DQ466581) (Table 2). *Ldh* and *pho* were preferred as reference genes over ribosomal rRNAs to relate the transcription of the target genes to the central carbon metabolism, and to avoid the comparison of stable, high copy number rRNA with fast degradable, low copy number mRNA (32). The threshold cycle number C of the target- reference genes was determined and employed to calculate the normalized ratio

E_{target} and $E_{reference}$ represent the efficiencies of the respective PCR reactions and were determined as 2 in all PCR reactions. The ratio N determined for the individual genes using cDNA-libraries obtained from cultures grown at reference conditions (mMRS, 37°C) was designated N_0 and gene expression levels for cultures grown at different conditions were reported as $N/N_0 = E_{target}^{(C_T-C_R)} * E_{reference}^{(C_R-C_T)}$. Each sample was analysed in duplicate and samples from two independent fermentations were analysed for each condition.

2.4   Regulation of glycosyltransferases in response to environmental stress

2.4.1   Expression of glycosyltransferases and polymer formation

Chemicals. Stock solutions of the nigericin (Sigma, Deisenhofen, Germany), gramicidin D (Fluka, Buchs, Switzerland), carbonyl cyanide m-chloro phenylhydrazone (CCCP, Sigma) and nisin (Fluka) in sucMRS were stored at –20°C. Reutericyclin (RTC) was purified to homogeneity from cultures of *L. reuteri* as

described (41) Isomerised hop extract containing 11% (w/v) trans-isohumulone (TIH) was kindly provided by NateCO2, Wolnzach, Germany.

Organisms and culture conditions. The sourdough isolates *L. reuteri* TMW1.106 and LTH5448 were grown in modified MRS medium (111) containing 100 g L^{-1} sucrose (sucMRS) or 10 g L^{-1} maltose, 5 g L^{-1} fructose and 5 g L^{-1} glucose (mMRS) as carbon source. The temperature of incubation was 37°C unless otherwise stated.

Effect of antibiotics, phenylethanol and heat on polymer formation and expression of glycosyltransferases. Overnight cultures of *L. reuteri* TMW1.106 and LTH5448 were washed twice with 50 mM phosphate buffer pH 6.5, resuspended in fresh sucMRS and added to sucMRS in a ratio 1:100. For HoPS induction assays, the concentrations of antibiotics were adjusted by two-fold dilutions of antibiotic stock solutions. Maximum concentrations were: TIH: 320 mg L^{-1}, gramicidin: 0.5 and 5 mg L^{-1} for *L. reuteri* TMW1.106 and LTH5448, respectively, nisin: 500 AU L^{-1}, CCCP: 25 µM, and RTC: 50 mg L^{-1}. The MIC of the antibiotics in sucMRS was determined. Phenylethanol (PE, Sigma) was added directly to the growth medium to levels of 1 or 6 mM. Poly- and oligosaccharides were quantified after 24 h of incubation as described below, total RNA was isolated from exponentially growing cultures.

RNA extraction and preparation. RNA was isolated from 1 ml exponentially growing cells (OD 0.3-0.4). Two volumes of RNAprotect were added, cells were harvested, frozen with liquid nitrogen and total RNA was isolated using peqGoldRNApure (PEQlab, Erlangen, Germany) according to the instructions of the supplier. cDNA was obtained as described in section 2.8.1. Successful synthesis of cDNA (cDNA) was verified by PCR-amplification of the *ldh* of *L. reuteri* coding for the lactate dehydrogenase (Q8GMJ0).

Quantification of glycosyltransferase expression. cDNA libraries generated from *L. reuteri* LTH5448 and TMW1.106 grown during various stress conditions were quantified as described in section 2.3 using *ldh* as reference gene. Expression of target genes during growth of *L. reuteri* in sucMRS at 37 ºC was used as reference.

Quantification of poly- and oligosaccharides from culture supernatants. Oligosaccharides and HoPS in culture supernatants were quantified as described (62, 127). The levels of HoPS and FOS production after induction by antibiotics, PE and heat were determined in three independent experiments and the experimental error was less than 30% when HoPS and FOS levels were normalized to the reference during the same experiment.

2.4.2   Impact of fructosyltransferase-inducing conditions on membrane parameters

Determination of the membrane fluidity. Overnight cultures of *L. reuteri* TMW1.106 were labelled with Laurdan (6-dodecanoyl-2-di-methyl-amino-naphtalene) as described (81) and resuspended in 50 mM phosphate buffer, pH 6.5 (PB) or PB additionally containing 2.5 or 10 mg L^{-1} TIH, 25 mg L^{-1} RTC, 50 µM CCCP, 4 µg L^{-1} gramicidin, or 1 and 6 mM PE to an OD_{590nm} of 1. Spectra were recorded using a Spectrometer LS 50B (Perkin Elmer, Wiesbaden, Germany) as described (81) at 37°C or 45°C and the generalized polarization GP as measure for membrane fluidity was calculated as GP = $(I_{440nm} - I_{490nm})/(I_{440nm} + I_{490nm})$. Temperature was controlled using a circulating waterbath. Experiments were performed in triplicate with consistent results and representative data are shown.

Membrane vesicles and lateral pressure measurement. Membrane vesicles of *L. reuteri* TMW1.106 were prepared from cells harvested from 500 ml overnight culture in sucMRS, washed with PB, and resuspended in 50 ml PB. Cells were disrupted by

ultrasonificaction and cell debris was removed centrifugation (15000 x g, 20 min). DNAse I and Proteinase K (both Promega) were added and the supernatant was incubated at 30 ˚C for 20 min. Low molecular weight compounds were removed from membrane vesicles by GPC using a Superdex 200 column (Amersham Biosciences, Freiburg, Germany). The column was eluted at 1 mL min^{-1} with PB containing 10% glycerine and fractions corresponding to the column void volume were collected. A stock solution of 4dipyPC (1-pyrenebutanoyl-*sn*-glycerol-3-phosphatidylcholine, Molecular Probes, Eugene, USA, 1 mg ml^{-1} in chloroform) was added at 0.1% (v/v) level to achieve a 4dipyPC content of about 0.1 mol% in the membrane vesicles. To incorporate the dipy4PC into the membrane vesicles, the mixture was extruded three times through 0.2 µm membrane filters (Schleicher & Schuell, Dassel, Germany). The membrane vesicles labelled with 4dipyPC typically had an OD_{540nm} of 0.02. Prior to measurements, antibiotics and PE were added to the following final concentrations: 2.5 and 10 mg L^{-1} TIH, 25 mg L^{-1} RTC, 50 µM CCCP, 4 µg L^{-1} gramicidin, or 1 and 6 mM PE. Fluorescence spectra were recorded at 37 or 45°C using an excitation wavelength of 342 nm, emission wavelength ranging from 380 to 550 nm, and a monochromator bandpass of 2.5 nm. The emission bands of 4dipyPC monomers was expected at 377 and 397 nm, the emission bands of 4dipyPC excimers formed when ground-state and excited state moieties are in close proximity are present at 480 nm. Increased excimer formation in relation to monomers is a consequence of enhanced lateral packing, indicating an increased lateral pressure within the bilayer (122). The spectra of 4dipyPC labeled membrane vesicles prepared from *L. reuteri* cells are shown in Figure 4. The monomer bands M1 and M2 were present at 377 and 397 nm, respectively. The excimer emission was seen at approximately 475 nm. These spectra correspond well to those obtained from 4dipyPC and 10dipyPC incorporated in

chemically defined bilayers or proteoliposomes (10, 122). The lateral pressure was expressed as the ratio excimer to monomer fluorescence (E/M), i.e. fluorescence intensity at 475 nm over fluorescence intensity at 377 nm. Experiments were performed in duplicate with consistent results and representative data are shown.
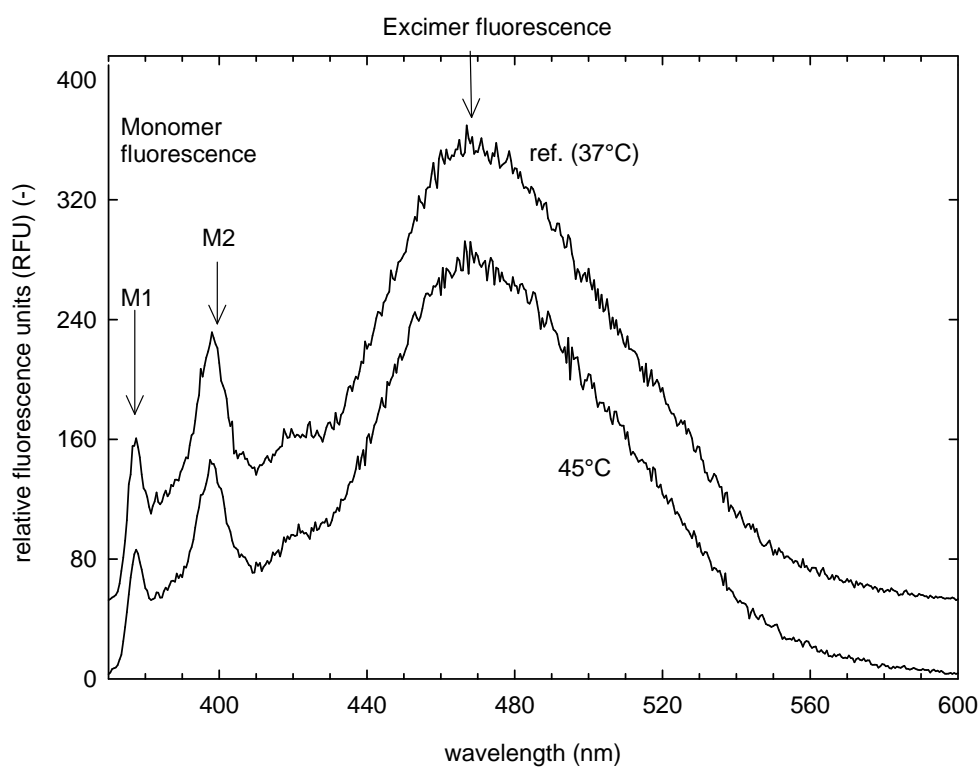
Figure 4. Fluorescence of 4dipyPC lipids incorporated in membrane vesicles of *L. reuteri* TMW1.106 at 37°C (upper trace) and 45°C (lower trace). The spectrum obtained at 37°C was offset by 50 RFU. Arrows indicate are fluorescence bands corresponding to the fluorescence of the pyrene monomers at 377 and 397 nm, and the pyrene excimers at 475 nm. Spectra are representative for duplicate independent experiments.

2.5   Effect of polysaccharides on the activity of membrane-active inhibitors.

Organisms and culture conditions. *L. reuteri* TMW1.106 and LTH5448 were grown in modified MRS medium (111) containing 10 g L^{-1} maltose, 5 g L^{-1} fructose and 5 g L^{-1} glucose (mMRS) as carbon source. The temperature of incubation was 37°C. Dextran from *Lc. mesenteroides* (Sigma, Deisenhofen, Germany), fructooligosaccharides (FOS) (Actilight, Gewürzmüller, Stuttgart, Germany) and levan from *L. sanfranciscensis* were added to mMRS at a level of 50 g L^{-1} as indicated.

Critical dilution assay. The minimum inhibitory concentration (MIC) of nisin, reutericyclin, and CCCP towards *L. reuteri* TMW1.106 and LTH5448 were determined in duplicate by a critical dilution assay as described (41) in mMRS, mMRS-dextran, mMRS-levan and mMRS-Actilight.

2.6   Impact of sucrose, FOS and inulin on membrane parameters

HPLC analysis of oligo- and polysaccharide preparations. FOS, IMO and inulin were resuspended in ddH$_2$0 and analysed using HPLC. Carbohydrates were analysed as detailed in section 2.1.3.

Determination of membrane fluidity. Membrane fluidity was determined using the fluorescence probe Laurdan (6-dodecanoyl-2-di-methyl-amino-naphtalene, Molecular Probes, Burlington, Canada) according to (81) with modifications. Briefly, stationary cells were washed twice in phosphate buffer (PB) and PB containing 7.5% of sucrose, FOS, inulin or IMO. OD$_{590nm}$ was adjusted 1.5. Laurdan was added at a final concentration of 40 μM and cells were incubated at 30 ºC for 30 min in the dark. Cells were washed twice in PB containing the additives and resuspended in the respective

buffer. Emission spectra (emission 360 nm, excitation 380-520 nm, 5 nm) were recorded using a Jasco FP6300 spectrafluorometer (Jasco, Victoria, Canada). Spectra were recorded over a temperature range from 20-50 ºC increasing in 10 ºC steps. Temperature was controlled using a circulating water bath. Membrane fluidity was calculated using the generalized polarization GP= ($I_{440\ nm}$ - I_{490nm}) / ($1_{440\ nm}$+ $I_{490\ nm}$).

Preparation of membrane vesicles and determination of membrane lateral pressure. Membrane lateral pressure was determined in membrane vesicles prepared from stationary phase cultures of *L. reuteri* TMW1.106. Briefly, cells were harvested by centrifugation and washed once in PB. Cells were disrupted using zirconium/silica beads and a Mini bead beater (both BioSpec products, Bartlesville, USA). DnaseI (Roche, Laval, Canada) and proteinase K (Oakville, Sigma, Canada) were added and the suspension was incubated for 30 min at 30 ºC. Cell debris was discarded after centrifugation and membrane vesicles were collected using a ultracentrifuge (Beckmann, Mississauga, Canada) at 63 000 x g (4 ºC, 1 h). Membrane vesicles were resuspended in PB containing 10% glycerol, and the fluorescence probe 4dipyPC (1-pyrenebutanoyl-*sn*-glycerol-3-phosphatidylcholine, Molecular Probes) was incorporated by passing the solution three times through a 22 μm filter. PB containing sucrose, FOS, inulin and IMO were added to obtain a final concentration of 7.5%, and emission spectra were recorded using a Jasco FP6300 spectrafluorometer (excitation 342 nm, emission 360-500 nm, BW 5 nm) over a temperature range from 10-50 ºC. Changes in membrane lateral pressure were calculated as the ratio E/M (emission of excimer at 475 nm / emission of monomer at 379 nm).

2.7   Freeze-drying of *L. reuteri* TMW1.106 in the presence of fructans

Buffer preparation. 7.5% inulin from chicory (SIGMA, Oakville, Canada), fructooligosaccharides (FOS) powder (Source natural, Scotts Valley, USA), sucrose (Difco, Oakville, Canada) and isomaltooligosaccharides IMO (BioNeutra, Edmonton, Canada) were added to 50 mM phosphate buffer (PB, pH 6.2) and sterilized by filtration. 5% skim milk was prepared using dehydrated skim milk powder (Difco) and sterilized.

Culture preparation. For preparation of exponentially growing cultures, overnight cultures of *L. reuteri* TMW1.106 were subcultured in modified MRS (111) substituted with 10 g L^{-1} maltose, 5 g L^{-1} fructose, 5 g L^{-1} glucose (all Sigma) and grown at 37 °C to an $OD_{595 nm}$ of 0.40 corresponding to approximately 10^6 CFU ml^{-1}. Cells were harvested, washed once in 60 mM phosphate buffer, pH 6.5 (PB), PB with additives or skim milk and resuspended in the corresponding buffer. Stationary cells were harvested after 18 h incubation, washed in PB containing the additives and the optical density was adjusted to $OD_{595 nm}$ 0.4. 3 ml of cell suspension were frozen at – 80 °C.

Freeze-drying. Frozen cells were freeze-dried using a Labconco FreeZone® 4.5 Liter Freeze Dry System (Labconco, Kansas City, USA) at a collector temperature of –50 °C. Upon dryness, cells were rehydrated using the appropriate volume of PB. Cell suspensions were freeze-dried and stored in duplicates, freeze-drying of stationary and exponential phase *L. reuteri* TMW1.106 was repeated three and four times, respectively.

Dry storage. Duplicates of each treatment were wrapped in parafilm and stored at room temperature in a closed container in the dark for 14 days.

Cell counts. CFU were determined before freezing, after freezing, after freeze-drying and after storage. Cell suspensions were serially diluted in phosphate buffered saline

(SIGMA) and plated on mMRS agar plates. Cell counts were determined after 24 h of anaerobic incubation at 37 ℃.

Membrane integrity. The dye exclusion assay Live/Dead BacLight Bacterial Viability kit (Molecular Probes, Burlington, Canada) was applied to investigate membrane integrity. Heat-treated (10 min, 80 ℃) and untreated cells from exponential and stationary growth phase ($OD_{595\ nm}$ adjusted to 0.4) were mixed in increments of 20 % to establish calibration curves. Frozen and rehydrated freeze-dried and stored cells were washed once and resuspended in PB. 10 μM and 1.67 μM of propidium iodide and Syto9 were added and the suspensions were incubated at room temperature for 15 min in the dark. Fluorescence emission spectra (excitation 470 nm, emission 490 - 670 nm, BW 5 nm) were recorded using a Jasco FP6300 spectrafluorometer (Victoria, Canada) at room temperature. Membrane integrity was calculated as the ratio of Syto9 fluorescence (integrated intensity between 510-540 nm) and propidium iodide fluorescence (intensity between 620 - 650 nm). Results are reported as percentage of intact membranes. Membrane integrity could not be determined in samples containing skim milk powder due to interaction of the powder with the fluorescence dyes.

Statistical analysis. The difference between two means was calculated using the t-test software provided by Sigmaplot8. Means were considered significantly different when $p < 0.05$.

2.8 Ecological impact of glycosyltransferases in *L. reuteri*

2.8.1 Expression of *ftfA, inu* and *gtfA* in vivo

Culture preparation. Cells grown overnight in 10 ml MRS broth (Difco, North Ryde, Australia, 20 g L^{-1} as only carbohydrate source) were harvested, washed once and resuspended in 0.9 % sodium chloride. Cultures of *L. reuteri* LTH5448 and TMW1.106 were spread on the fur of three weeks old mice and on commercially available rat chow. Mice were maintained in isolators.

RNA isolation from mice forestomach and buffer media. Five mice colonized by *L. reuteri* LTH5448 and TMW1.106 were killed 14 days after inoculation, and organs were removed immediately. Four uninoculated mice were treated in the same manner. Stomach and forestomach were separated, luminal contents were squeezed into 1.5 ml Eppendorf tubes filled with 0.75 ml RNAprotect (Qiagen, Auckland, New Zealand) and homogenize using plastic spatula. The forestomach was cut open, rinsed with phosphate buffered saline prepared using DEPC water, transferred into 1.5 ml Eppendorf tubes containing 0.75 ml RNA protect and homogenized. The homogenized contents were centrifuged twice at low speed (150 x g, 5 min, RT and 300 x g, 5 min, room temperature). Cells were pelleted at 9000 x g, 5 min, RT. Pellets were frozen at –70ºC until further use. RNA from forestomachs and stomach contents of five mice colonized by the respective strains were isolated. In vivo cell counts were determined according to (50) on MRS agar. RLF-mice were provided by Gerald Tannock, University of Otago, Dunedin, New Zealand. The Otago University Animal Ethics Committee approved animal experiments.

To compare in vivo and in vitro expression, overnight cultures of *L. reuteri* LTH5448 and TMW1.106 grown in MRS were harvested, washed twice in PB and resuspended in MRS. 1 % of the washed cell culture was inoculated in prewarmed, anaerobic

media. After approximately 3 h and 9 h, corresponding to cells in the exponential (OD$_{590nm}$ 0.35 - 0.45) and early stationary phase (OD$_{590nm}$ 2 - 3), respectively, one volume of culture was mixed with two volumes of RNAprotect (Qiagen). After incubation at RT for 10 min, cells were pelleted (9000 x g, 5 min, RT) and frozen at – 70º C until further processing. RNA was isolated from two independent experiments.

RNA procedures, **DNA digestion and Reverse Transcription**. 1 ml TRIzol (Invitrogen, Auckland, New Zealand) was added to cell pellets. Cells were disrupted using a MiniBeadBeater and 600 nm zirconium-silica beads. Supernatants were transferred to 1.5 ml Eppendorf tubes and RNA was isolated following the TRIzol manual. Additionally, redissolved RNA was incubated at 55 - 60ºC for 10 min to ensure complete resolution of the RNA pellet. DNA was removed by digestion with RQ1 RNAse-free DNAse at 37 ºC for 20 min. The reaction was stopped with the addition of DNAse stop buffer and incubation at 70 ºC for 10 min. Reverse transcription was prepared heating 1 μl of RNA, 1 μl random hexamer primers and 11 μl RNAse-free water for 10 min at 70 ˚C. After 5 min incubation on ice, 0.5 μl dNTPs (25 μM), 0.5 μl (100 U) Moloney murine leukemia virus reverse transcriptase (MMLV-RT), 5 μl MMLV-RT- buffer and 5 μl RNAse free water were added. The mixture was held at 25 ºC for 10 min, followed by incubation at 42 ºC for 1 h in a Primus Thermocycler. To inactivate the reverse transcriptase, temperature was finally increased to 72 ºC and held for 15 min. Reagents used for reverse transcription were purchased from Promega (Promega, Annandale, Australia).

Semi-quantitative real time PCR. Successful reverse transcription was checked using PCR and primers listed in Table 2. DNAse digested total RNA diluted in equal volume (negative control), chromosomal DNA (positive control) and cDNA was used as a template. An AbiPrism 7500 Fast Real-time PCR System and SYBR Green

(Applied Biosystems, Melbourne, Australia) were used for real-time PCR of cDNA libraries generated from RNA isolated from *L. reuteri* isolated from RLF-mice. All samples were run in duplicates. Specificity and identity of PCR products were confirmed by specific melting point analysis, molecular weight analysis on agarose gels and sequencing. Efficiency values for each primer pair were calculated as E=10^(-1/slope) of calibration curves of serially diluted chromosomal DNA. A r^2 value bigger than 0.92 was considered acceptable. The 16SrRNA gene was used as housekeeping gene. *Lactobacillus* specific primers were used to conform specificity (144). Employing 16SrRNA as reference gene brings about the disadvantage that expression of mRNA is compared to expression of the more stable and abundant rRNA. However, as no data existed which indicated genes constitutively expressed in mice by lactic acid bacteria, and as the lactate dehydrogenase *ldh* used as reference in buffer medium (section 2.3 and 2.4) was upregulated in *S. mutans* grown in biofilm (146), the use of the 16SrRNA gene as reference gene was considered best choice. cDNA libraries created from RNA isolated from uninoculated mice were included in PCR procedures to confirm that all primer pairs used were *L. reuteri* specific. The relative expression ratio of glycosyltransferase genes within individual mice were calculated according to Pfaffl (2000) (92) and further analysed applying the Relative Expression Software Tool (REST) established by Pfaffl et al. (93).

2.8.2   Impact of deletion of glycosyltransferase genes on metabolite formation of *L. reuteri* in RLF-mice

Stomach contents of 7 uninoculated RLF-mice as well as of 7 RLF-mice colonized by either *L. reuteri* TMW1.106 or its *gtfA* and *inu* mutants for two days were collected, pooled and freeze-dried. Carbohydrates in stomachs contents were extracted using ddH$_2$0 at 80 ºC for 2 h. The carbohydrate composition of the stomach contents were

analysed using HPLC as described in section 2.1.3. Organic acids were separated using Aminex HPX 87H as explained in section 2.2. Mice were fed with commercially available rat chow, and carbohydrates present within the feed were analysed in the same manner.

2.8.3 Performance of *gtfA, inu* and *ftfA* mutants in wheat sourdough

Performance of *L. reuteri* TMW1.106 and the *inu* and *gtfA* mutants, and of *L. reuteri* LTH5448 and the *ftfA* mutant in sourdough were compared when strains were inoculated alone or in competition. After subculturing the strains twice in mMRS containing 10 g L^{-1} maltose, 5 g L^{-1} fructose and 5 g L^{-1} glucose (111), overnight cultures were harvested, washed twice in sterile tap water and OD was adjusted to OD$_{590nm}$ 0.4. 5 ml of cell suspension (originating from the same preculture) was added to 50 g flour (Robin Hood all purpose flour, Smucker Foods, Markham, Canada) and 50 ml tap water in competitive and or 55 ml tap water in single inoculation. Initial cell counts were approximately 10^6 and 10^7 CFU g flour^{-1} for *L. reuteri* TMW1.106 and LTH5448, respectively. Doughs were inoculated at 37 °C and cell counts were determined after 0, 4, 10 and 24 h of incubation at 37 °C on mMRS plates and mMRS plates containing 5 µg L^{-1} erythromycin. Plates were incubated anaerobically at 37 °C for 48 h. Stability of the mutant strain was verified. For determination of organic acids and carbohydrates, samples were taken at the same time pointes, diluted with an equal volume of 7 % perchloric acid (Sigma, Oakville, Ontario) and incubated overnight at 4 °C (123). Organic acids and carbohydrates were analysed as described in section 2.2 and 2.1.2, respectively.

Table 2. List of primers used for PCR-amplification of DNA or cDNA. Primers are denoted according to International Union of Biochemistry group codes: *I* inosin, *Y* C or T, *S* G or C, *W* A or T, *R* A or G, *M* A or C

Primer	Sequence (5' to 3')	Use / Vector
Leu wob F	GAY GTI TGG GAY WSI TGG C	PCR[1]
Leu wob R	TCI TYY TCR TCI SWI RMC AT	PCR[1]
DexWobV	GAY CGI GTU GAY AAY GTI	PCR[2]
DexWobR	YTG RAA RTI ISW RAA ICC	PCR[2]
Lev F	GAA TGG CTA TCA ACT TGT G	PCR[1]
Lev R	CTT CTA CTT GCG GGT TC	PCR[1]
ISEFA F	CTT TGG GTC CAA AAA GCG	PCR[1]
Inverse 5 R	CAC AAG TTG ATA GCC ATT C	PCR[1]
IS 2 F	CTT AAT TGT TAA ACG TTT AGC	PCR[1,2]
Inverse inu R	GCA GCT TTC GAG AAG TTC	PCR[2]
InverseLeuR	CAT CAT AGC GAT CAC	PCR[1]
FTFinv F	GAT TAT AAA AAA GCT ATC TTT GC	Inverse PCR[1]
FTFinv R	GTG TAG TTG TCA GCA AAA C	Inverse PCR[1]
Ldh F	CAT GTT GAC CCT CGC GAT G	PCR, RT-PCR[3]
Ldh R	CGT ATG CCT TGT TAC GAA C	PCR, RT-PCR[3]
Ftf F	GGC ATT TGT ACA ACA TGT C	RT-PCR[3]
Ftf R	GTA GAG AAT AAT CAT GTG G	RT-PCR[3]
PhoketV	GTA ACC TTC AAG GAA TCC	RT-PCR[3]
PhoketR	CGT CTT TAC GCA TTC CTT G	RT-PCR[3]
Inu F	CTA TAA TTC TAC CGC GGT TTC	PCR[6], RT-PCR[3]
Inu3 R	GAT CAT TTG CTA CCT GAG CG	RT-PCR[3]
Inu R	GTA TAA CTC TGC CAC CTT AG	PCR[6]
Glc F	CAG AGC TAA TGC CTG TTA AGG	PCR[6], RT-PCR[3]
Glc R	CTT ACG TGG TTG ACC ACT TG	PCR, RT-PCR[3]
FTF-rt-forward	AGC CGA CAA TGA TGG TGT C	RT-PCR[4]
FTFrevTEST	GGC ATT TGT ACA ACA TGT CC	RT-PCR[4]
Dex-rt-reverse	GAG TTC ATA CCA TCT GCA GC	RT-PCR[4]

Lac2reverse	ATT CCA CCG CTA CAC ATG	RT-PCR[4]
InuforwTEST	GGGTAGACACGTCTGATAAC	RT-PCR[4]
Inu-rt-reverse	GTA GTA ATA GCC ATC ACC TTC	RT-PCR[4]
DexforTEST	AATTAAACTGGTTATACTATCTC	RT-PCR[4]
Leureu1001V	GAA TGG CTA TCA ACT TGT G	PCR[6]
Leureu1001R	CTT CTA CTT GCG GGT TC	PCR[6]
Lev V	CAG CYG CTA CTC AAG C	PCR[6]
Lev R	CTG AAG CAA AGT CCT VG	PCR[6]
Inuklon-for[1]	<u>TATACCATGGTT</u>ATGCTAGAACGCAAGGAAC	pBAD B
Inuklon-rev[1]	<u>TATATCTAGAGC</u>GTTAAATCGACGTTTGTTAA	pBAD B
Inuklon-C[1]	[5]<u>TATATCTAGAGCT</u>**TTA**ATCCATAACCAATTAAG	pBAD B
FtfAklon-C	<u>TATATCTAGAGCT</u>**TTA**ATCCATAACCACCAATTA	pBAD B

[1]Primers used to isolate *ftfA*

[2]Primers used to isolate *inu* and *gtfA,* primers were constructed based on known inulo-
and glucosyltransferase sequences of *L. reuteri* (67, 133)

[3]Real-time PCR using a LightCycler

[4]Real-time PCR using Taqman

[5]Restricton sites are underlined, introduced stop codons are marked bold

[6]Primers used in glycosyltransferase screening

3   RESULTS

3.1   Glycosyltransferases in sourdough, porcine and duck isolates of *L. reuteri*

3.1.1   Fructosyltransferase FtfA and Inulosucrase Inu

In *L. reuteri* TMW1.106, two glycosyltransferase genes were identified using PCR. A partial sequence of 856 bp encompassing the catalytic domain was 99 % identical to the gtfA of *L. reuteri* 121 (AAU08015), a second DNA fragment of 2412 bp encompassed an ORF that was 99 % identical to the inulosucrase of *L. reuteri* 121 (AF459437) (133) and which was therefore termed inulosucrase of *L. reuteri* TMW1.106 (AM293550). In *L. reuteri* LTH5448, a novel *ftf* gene was identified with degenerate primers targeting conserved regions of bacterial Ftfs (127) (FtfA, AJ812736). A DNA-fragment of 3090 bp encompassed an ORF termed *ftfA* corresponding to an enzyme of 804 aa. FtfA exhibited highest similarity to the *L. reuteri* 121 inulosucrase (Inu; AF459437, 79% identity and 88% similarity in 798 aa), *L. johnsonii* levansucrase (Q74K42; 55% identity, 72 % similarity in 797 aa) and *L. reuteri* 121 levansucrase (AA014618; 53% identity, 68% similarity in 753 aa). The putative protein shared the domain structure typical for Ftf enzymes of lactobacilli and is presented in Figure 5 (125, 133). Remarkably, the catalytic domain of FtfA (aa residues 231 to 682) shared only 72 % identity to the inulosucrase of *L. reuteri* 121, whereas the signal peptide (aa 1 – 44), the N-terminal domain (aa 45 – 230), and the C-terminal cell wall anchor (aa 663 – 804) were greater than 96% identical to the corresponding *inu* sequences (133). *FtfA* also shared high homology to a protein of *L. reuteri* 100-23 (77% identity, 84% similarity in 760 aa) which was listed as an anchor surface protein (Asp) in the database (ZP_01274373.1). Asp was highly homologous to other fructosyltransferases of lactic acid bacteria. *L. reuteri* 100-23 forms a fructan-

type HoPS from sucrose, therefore Asp is most likely the fructosyltransferase of this strain.
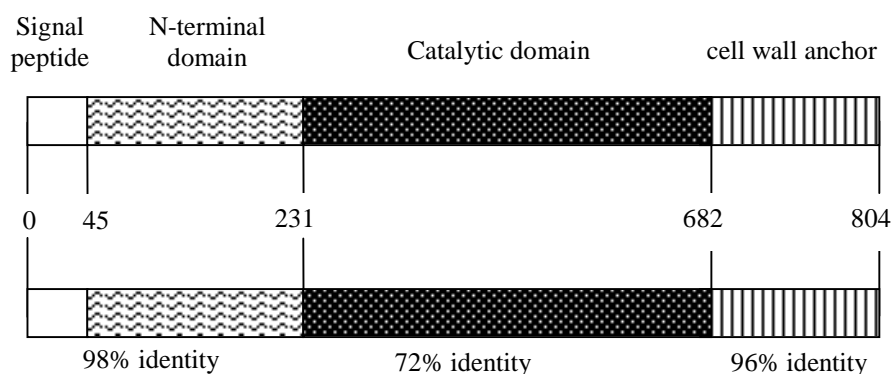
Figure 5. Domain structure of the FtfA (AJ812736) of *L. reuteri* LTH5448 and comparison with the inulosucrase (Inu) of *L. reuteri* LB121. Blast Search showed highest similarity of FtfA to Inu of *L. reuteri* LB121 (AF459437, 80% identity and 89% similarity in 798 aa) followed by a *L. johnsonii* levansucrase (Q74K42; 55% identity, 72 % similarity). Indicated in the graph is the similarity of individual domains (% aa identity) to Inu.

3.1.2   Screening for the presence of glycosyltransferase genes

Thirteen duck and porcine isolates, two other sourdough isolates and one rodent isolate of *L. reuteri* were screened for the presence of glycosyltransferase genes using primers based on sequence data collected to this point. Results are summarized in Table 3. All animal isolates harboured the genes for *inu,* and seven isolates additional harboured *gtfA. Lev* and *ftfA* each were present in one strain. The sourdough isolates LTH2584 and LTH5448 contained *ftfA* whereas TMW1.106 and TMW1.656 harboured *gtfA* and *inu.* Database analysis showed that *L. reuteri* 100-23 harboured a fructosyltransferase (ZP_01274373.1) with highest homology to FtfA of *L. reuteri*

LTH5448. However, there were two mismatches in each primer of the primer pair Leureu1001V and leureuL001R used, therefore no amplicon was obtained by PCR.

Table 3. Glycosyltransferase genes in *porcine, duck,* sourdough and rodent isolates of L. reuteri, and the production of poly- and oligosaccharides by these strains.

Origin[a]	Strain	Inulo-sucrase	Reuteran-sucrase	Fructosyl-transferase A	Levan-sucrase	Poly- and oligosacharides	
Porcine	TMW1.272	+[b]	+	-[C]	-	Glucan[d]	GOS[d]
	TMW1.279	+	-	-	-	-[d]	-[d]
	TMW1.285	+	-	-	-	-[d]	-[d]
	TMW1.146	+	-	-	-	-[d]	-[d]
	TMW1.194	+	+	-	-	-[d]	-[d]
	TMW1.138	+	+	-	-	Glucan[d]	GOS[d]
Duck	TMW1.967	+	-	-	-	-[d]	-[d]
	TMW1.972	+	-	-	-	-[d]	-[d]
	TMW1.973	+	-	-	-	-[d]	-[d]
	TMW1.974	+	+	-	-	Glucan[d]	GOS[d]
	TMW1.975	+	+	-	+	-[d]	-[d]
	TMW1.976	+	+	-	-	Glucan[d]	GOS[d]
	TMW1.980	+	-	+	-	-[d]	-[d]
Sourdough	TMW1.106	+	+	-	-	Glucan	GOS[d], FOS
	TMW1.656	+	+	-	-	Glucan	n.d.
	LTH2584	-	-	+	-	N.D.	FOS
	LTH5448	-	-	+	-	Levan	FOS
Rodent	100-23	-	-	-[e]	-	Fructan	n.d.

[a]Origin of strain: duck isolates (68), porcine isolates (126), sourdough isolates (9, 39, 78)

[b]positive PCR

[c]negative PCR

[d]HoPS and oligosaccharides analysis by Tieking et al. (126)

[e]*L. reuteri* 100-23 harbours a fructosyltransferases with highest homology to FtfA of *L. reuteri* LTH5448, which was not amplified with the primers used

N.D. not detected, n.d. not determined

3.1.3 Products formed by glycosyltransferases of *L. reuteri* TMW1.106 and LTH5448 from sucrose

At reference conditions (37 °C in sucMRS), *L. reuteri* TMW1.106 produced a HoPS composed of glucose to a concentration of 7 ± 1 g L^{-1}. *L. reuteri* LTH5448 produced a polymer composed of fructose and showed the structure of a levan. Levan levels were estimated as 0.7 ± 0.2 g L^{-1}. The presence of several oligosaccharides in supernatants of *L. reuteri* LTH5448 was shown by HPLC using HPAEC-IPAD as demonstrated in Figure 6. 1-kestose levels in culture supernatants of *L. reuteri* LTH5448 after incubation at reference conditions ranged from 0.2 to 2.4 mM L^{-1}. Treatment of the supernatants with inulinase degraded all oligosaccharides, indicating that unidentified peaks FOS1 and FOS2 were fructose-oligosaccharides. Low levels of 1-kestose and FOS1 were also detected in culture supernatants of *L. reuteri* TMW1.106 (data not shown).
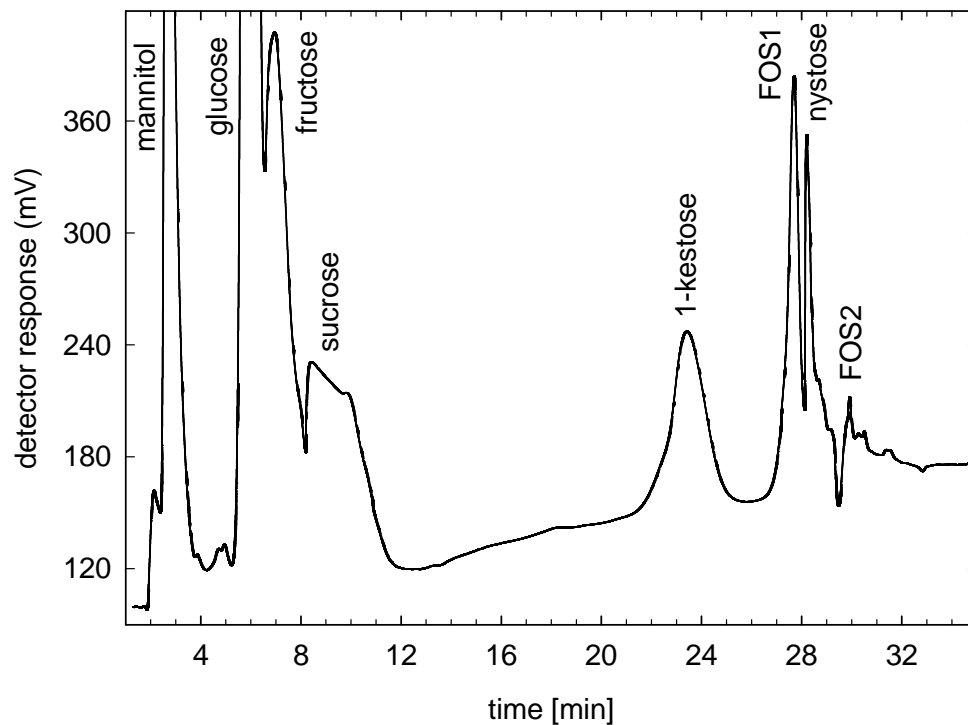
Figure 6. Separation of a culture supernatant of *L. reuteri* LTH5448 grown overnight at 37°C in sucMRS by HPAEC-IPAD. Mannitol, glucose, fructose, sucrose, 1-kestose, and nystose were identified by external standards, the peaks designated FOS1 and FOS2 could not be identified by external standards but were identified as FOS because they were hydrolysed by inulinase.

3.1.4 Heterologous expression of FtfA, FtfAC, Inu and InuC

E. coli Top10 overexpressed FtfA and a C-truncated form of FtfA (FtfAC), which is displayed in Figure 7. The purified protein FtfAC had a molecular weight of approx. 75 kDa on SDS-PAGE and was purified on HisTrap column. FtfA (approx. 80 kDa) did only bind weakly to the column. The better part was lost with the void volume. A clear overexpression of Inu or InuC was not obvious when heterologously expressed in neither *E. coli* TOP10, nor in *E. coli* LMG194, which has low basal expression of toxic proteins as clarified in Figure 8. Activity staining crude extracts of

heterologously expressed Inu (InuCE) showed that *E. coli* expressed a protein of the expected size, which formed polysaccharides from sucrose (data not shown). Active proteins were lost with the void volume, and binding to the HisTrap column could not be improved using increased concentrations of NaCl in combination with varying amounts of imidiazol. In consequence, oligosaccharides formed by purifed FtfAC and InuCE during incubation in buffer containing sucrose and additional acceptor sugars were analysed and products formed are described in section 3.2.2.
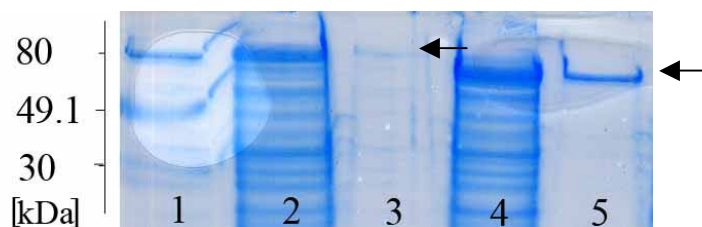
Figure 7. SDS-Page of the crude extract of *E. coli* Top10 heterologously expressing His-tagged FtfA and FtfAC (lane 2 and 4) and of heterologously expressed, purifed His-tagged proteins FtfA and FtfAC (lane 3 and 5), respectively, as indicated by the arrows. His-tagged FtfA had the expected size of approximately 80 kDa, FtfAC was approximately 75 kDa.
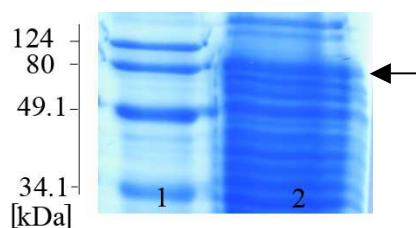
Figure 8. SDS-Page of crude extract of heterologous expression of Inu (InuCE) in *E. coli* LMG194. The arrow indicates the band of the expected size of Inu.

3.2   Characterisation of ftfA, inu and gtfA deletion mutants

3.2.1   Growth of mutants

L. reuteri LTH5448 and the *ftfA* mutant grew to 10^9 CFU ml^{-1} in mMRS and suc50MRS, formed comparable amounts of lactate, acetate and ethanol and reduced all fructose to mannitol. In sucMRS, *L. reuteri* LTH5448 metabolized almost all sucrose, whereas about 40 % remained after growth of the *ftfA* mutant. Data is summarized in Table 4.

In mMRS, *L. reuteri* TMW1.106 and the *gtfA* and *inu* mutants grew to 10^8 CFU ml^{-1} in 24 h as presented in Table 5. *L. reuteri* TMW1.106 and the *inu* mutant formed comparable amounts of lactate, acetate and ethanol; lactate and ethanol formation was lower in the *gtfA* mutant. The *inu* mutant reduced all fructose to mannitol. 21 and 10% fructose remained during growth of *L. reuteri* TMW1.106 and the *gtfA* mutant in mMRS, respectively (Table 5). Preculture conditions were strictly adjusted when *L. reuteri* TMW1.106 and the mutants were grown in suc50MRS. Strains were subcultured twice in mMRS and inoculated at 1 % in suc50MRS. *L. reuteri* TMW1.106 grew to 10^8 CFU ml^{-1} and utilized about 60 % sucrose. During the first inoculation in suc50MRS, the *inu* and *gtfA* mutant only reached about 10^6 CFU ml^{-1} and did not metabolize sucrose. Fermentation did not follow the classical heterofermentative pattern: mol lactate = mol acetate + mol ethanol as *gtfA* and *inu* mutants almost only formed ethanol. After reinoculating the mutants a second time in suc50MRS, overnight cell counts and sucrose utilization of the *gtfA* and *inu* mutants increased compared to day one. The amount of sucrose used by *L. reuteri* TMW1.106 and the mutant strains was alike.

During growth in mMRS containing maltose and sucrose, *L. reuteri* TMW1.106 preferably consumed sucrose (Table 5). *L. reuteri* TMW1.106 used less maltose than the mutants and consequently produced lower amounts of lactate and ethanol. The *gtfA* mutant did not metabolize any sucrose and therefore did not produce mannitol and acetate. Sucrose consumption increased in the order *gtfA* mutant < *inu* mutant < *L. reuteri* TMW1.106 and consequently correlated to mannitol and acetate formation

Table 4. Growth, carbohydrate utilization and formation of organic acids of *L. reuteri* LTH5448 and the *ftfA* mutant during growth in mMRS (23 mM maltose, 28 mM glucose, 56 mM fructose), or suc50MRS (178 mM)

	mMRS		sucMRS	
	L. reuteri LTH5448	*FtfA* mutant	*L. reuteri* LTH5448	*FtfA* mutant
CFU ml^{-1}	9.1 ± 0.1	9.0 ± 0.1	9.2 ± 0.2	9.1 ± 0.3
Maltose [mM]	2 ±0	2 ± 0	-	-
Sucrose [mM]	-	-	2 ± 0	40 ± 19
Glucose [mM]	0 ± 0	0 ± 0	7 ± 5	4 ± 0
Fructose [mM]	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Mannitol [mM]	53 ± 3	53 ± 9	92 ± 2	96 ± 5
Lactate [mM]	72 ± 1	72 ± 3	102 ± 10	102 ± 7
Acetate [mM]	28 ± 0	27 ± 0	61 ± 6	59 ± 4
Ethanol [mM]	50 ± 0	51 ± 1	28 ± 8	32 ± 0

Table 5. Growth, carbohydrate utilization and formation of organic acids of *L. reuteri* TMW1.106 and the *gtfA* and *inu* mutants during growth in mMRS (23 mM maltose, 28 mM glucose, 56 mM fructose), malsucMRS (136 mM maltose, 177 mM sucrose) or suc50MRS (182 mM).

	mMRS			malsucMRS		
	L. reuteri TMW1.106	*GtfA* mutant	*Inu* mutant	*L. reuteri* TMW1.106	*GtfA* mutant	*Inu* mutant
CFU ml^{-1}	8.4 ± 0.4	8.3 ± 0.7	7.8 ± 0.3	n.d.	n.d.	n.d.
Sucrose	-	-	-	54 ± 4	174 ± 1	83 ± 21
Maltose	11 ± 0	21 ± 7	10 ± 1	96 ± 3	44 ± 9	50 ± 8
Glucose	3 ± 1	3 ± 2	1 ± 0	4 ± 1	10 ± 4	7 ± 2
Fructose	12 ± 1	4 ± 3	0 ± 0	19 ± 1	1 ± 1	39 ± 6
Mannitol	48 ± 4	54 ± 6	54 ± 6	63 ± 3	0 ± 0	47 ± 24
Lactate	63 ± 13	41 ± 0	55 ± 2	68 ± 7	107 ± 15	82 ± 16
Acetate	25 ± 4	26 ± 2	27 ± 1	34 ± 4	0 ± 0	22 ± 5
Ethanol	43 ± 8	16 ± 4	34 ± 0	32 ± 4	107 ± 19	59 ± 10

	suc50MRS					
	L. reuteri TMW1.106		*GtfA mutant*		*Inu* mutant	
	Day 1	Day 2	Day 1	Day 2	Day 1	Day 2
CFU ml^{-1}	8.0 ± 0.3	8.2 ± 0.3	6.8 ± 0.1	7.9 ± 0.0	6.7 ± 0.6	7.8 ± 0.3
Sucrose	100 ± 13	100 ± 0	183 ± 10	127 ± 21	178 ± 19	109 ± 7
Glucose	4 ± 1	2 ± 0	0 ± 0	6 ± 2	2 ± 3	3 ± 0
Fructose	25 ± 2	26 ± 1	1 ± 1	37 ± 5	4 ± 1	28 ± 0
Mannitol	37 ± 0	41 ± 4	0 ± 0	31 ± 0	1 ± 2	33 ± 0
Lactate	33 ± 4	43 ± 3	2 ± 1	29 ± 6	3 ± 3	27 ± 1
Acetate	22 ± 4	28 ± 2	3 ± 1	20 ± 4	4 ± 2	23 ± 2
Ethanol	12 ± 2	18 ± 4	5 ± 5	11 ± 8	6 ± 5	8 ± 4

n.d. not determined

3.2.2 Heterooligosaccharide formation of *L. reuteri* LTH5448, TMW1.106, the mutant strains and purified Ftf enzymes

The fructosyltransferases of *L. reuteri* LTH5448 and TMW1.106 were purified to analyse products formed from sucrose with different acceptor molecules. Heterooligosaccharides were identified using the products synthesized by the purified Levansucrase (Lev) of *L. sanfranciscensis* TMW1.392 as standards (128) and through comparison of products formed by the parent and mutant strains. Carbohydrates were detected with an evaporated light scattering detector. Figure 9 shows that *L. reuteri* LTH5448 and Lev formed detectable amounts of 1-kestose in the presence of maltose. Formation of erlose could not be detected. *L. reuteri* TMW1.106 and the *inu* mutant synthesized glucooligosaccharides, which were identified as isomaltooligosaccharides by comparison with external standards isomaltotriose and an isomaltooligosaccharide preparation. The formation of arabsucrose from sucrose and arabinose by *L. reuteri* TMW1.106, the *gtfA* mutant, *L. reuteri* LTH5448, Lev and purified FtfAC is presented in Figure 10. 1-kestose formation from sucrose was only detected when *L. reuteri* LTH5448, the *gtfA* mutant of *L. reuteri* TMW1.106 and *L. reuteri* TMW1.106 were grown in the presence of sucrose as displayed in Figure 11. Neither 1-kestose nor FOS formation was observed when FtfAC, Inu CE or Lev were incubated in buffer containing sucrose (Figure 11).
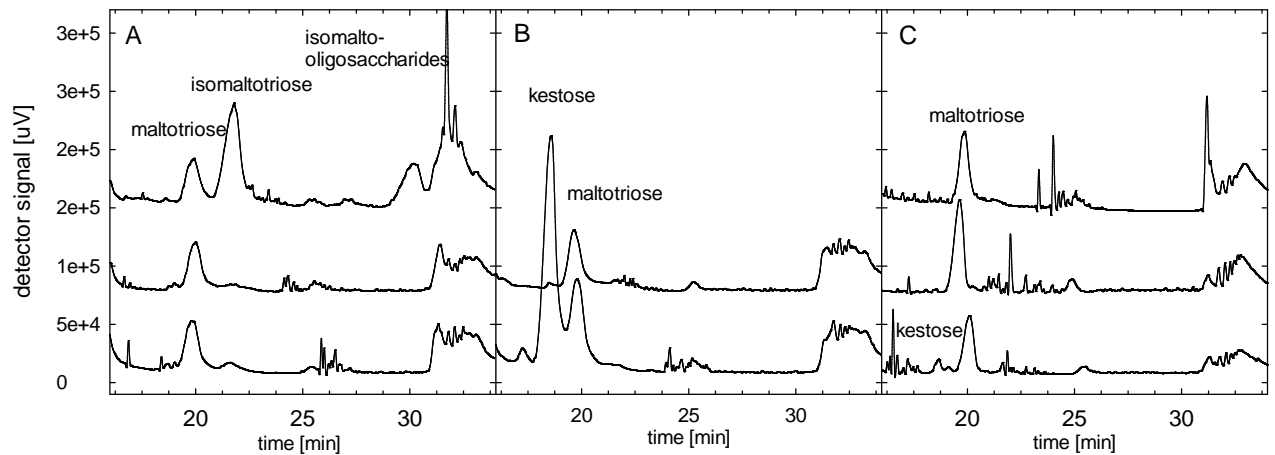
Figure 9. Oligosaccharides formed in the presence of maltose and sucrose by (A) *L. reuteri* TMW1.106 (lower trace), the *gtfA* mutant (middle trace) and the *inu* mutant (upper trace), (B) *L. reuteri* LTH5448 (lower trace) and the *ftfA* mutant (upper trace) and (C) the purified levansucrase of *L. sanfranciscensis* TMW1.392 (lower trace), FtfAC (middle trace) and of crude extract of heterologously expressed Inu (InuCE) (upper trace). Oligosaccharides were separated using a Supelcosil LC-NH$_2$ column and detected using an evaporative light scattering detector. Peaks were assigned using maltose, maltotriose, isomaltotriose and an isomaltoligosaccharide preparation as standards. Traces were offset 70000 µV. Maltotriose and maltooligosaccharides were present as part of maltose preparation.
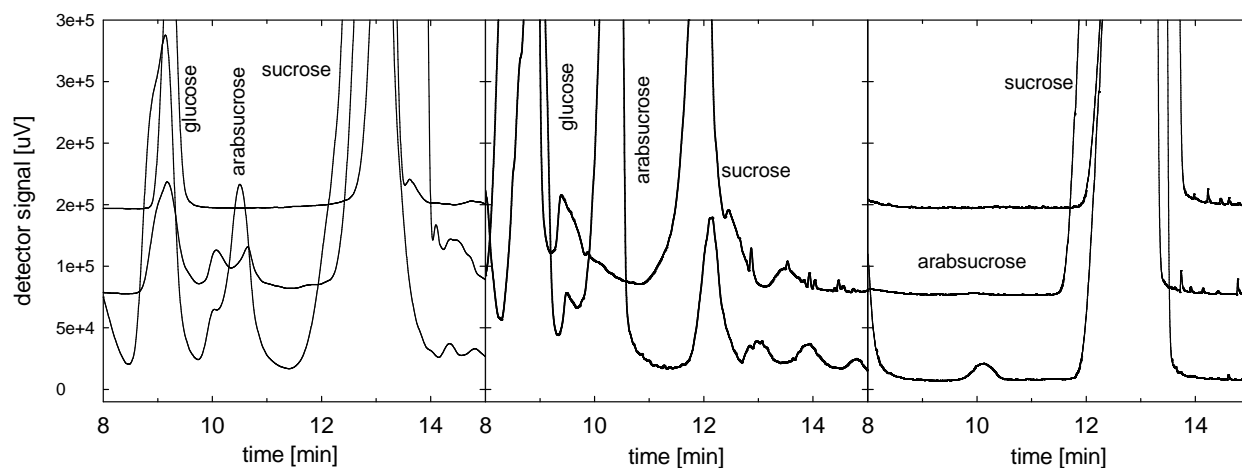
Figure 10. Oligosaccharides formed in the presence of arabinose and sucrose by (A) *L. reuteri* TMW1.106 (lower trace), the *gtfA* mutant (middle trace) and the *inu* mutant (upper trace), (B) *L. reuteri* LTH5448 (lower trace) and the *ftfA* mutant (upper trace) and (C) the purified levansucrase of *L. sanfranciscensis* TMW1.392 (lower trace), FtfAC (middle trace) and crude extract of heterologously expressed Inu (InuCE) (upper trace). Oligosaccharides were separated using a Supelcosil LC-NH$_2$ column and detected using an evaporative light scattering detector. Traces were offset 70000 µV. Peaks were assigned using the products formed by Lev as standards
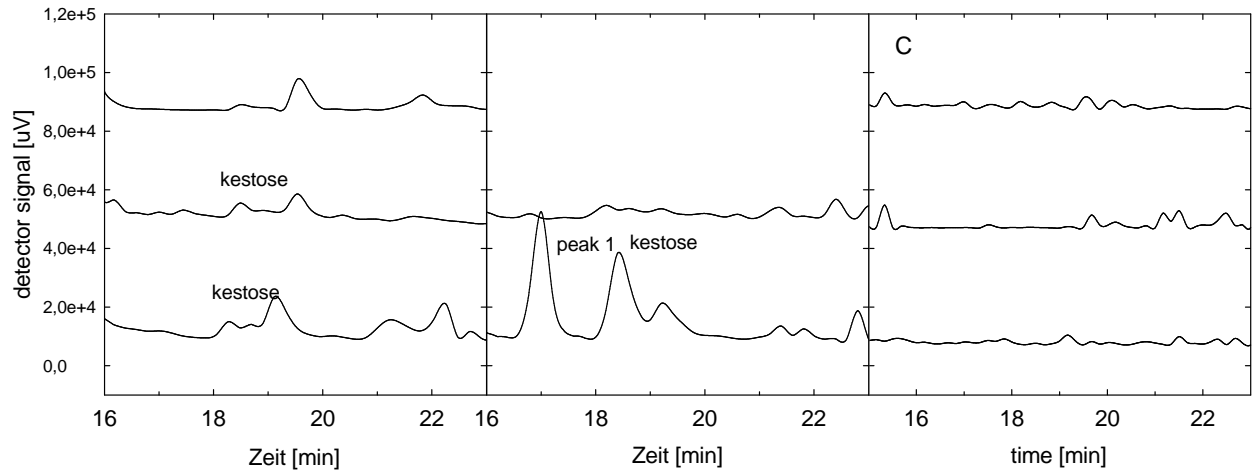
Figure 11. Oligosaccharides formed in the presence of sucrose by (A) *L. reuteri* TMW1.106 (lower trace), the *gtfA* mutant (middle trace) and the *inu* mutant (upper trace), (B) *L. reuteri* LTH5448 (lower trace) and the *ftfA* mutant (upper trace) and (C) the purified levansucrase of *L. sanfranciscensis* TMW1.392 (lower trace), FtfAC (middle trace) and crude extract of heterologously expressed Inu (InuCE) (upper trace). Oligosaccharides were separated using a Supelcosil LC-NH₂ column and detected using an evaporative light scattering detector. Traces were offset 40000 µV. Peaks were assigned using sucrose and 1-kestose as standards.

3.2.3 TLC of supernatants of *L. reuteri* TMW1.106 and the *inu* and *gtfA* mutants grown in MRS containing maltose and sucrose.

Thin layer chromatography (TLC) was applied as a second method to confirm the increased formation of isomaltooligosaccharides. As displayed in Figure 12, TLC verified the increased production of isomaltooligosacchrides by the *inu* mutant in the presence of 146 mM maltose and 177 mM sucrose. The chromatographical separation also revealed that the *inu* mutant formed more polymer than *L. reuteri* TMW1.106 when grown in the presence of different amounts of maltose and sucrose or sucrose. The application of HPLC methods verified that the polymer consisted of glucose (data not shown).
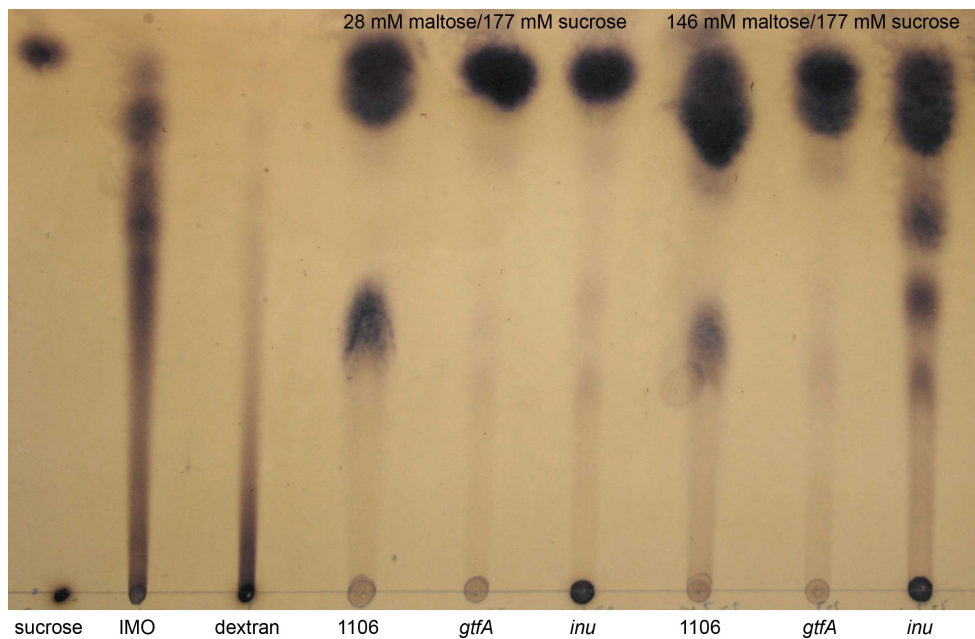
Figure 12. Separation of supernatants of *L. reuteri* TMW1.106 and the *gtfA* and *inu* mutants grown in mMRS containing maltose and sucrose on thin layer chromatography. Sucrose, isomaltooligosaccharides (IMO) and dextran of *Lc. mesenteroides* were used as standards.

3.2.4 Whole cell protein profile of *L. reuteri* TMW1.106 and the *inu* and *gtfA* mutants

In the presence of maltose and sucrose, the *inu* mutant synthesized higher amounts of isomaltooligosaccharides than *L. reuteri* TMW1.106. Whole cell proteins of both strains grown in malsucMRS were separated on SDS-PAGE to identify the protein responsible for increased oligosaccharide formation of the *inu* mutant. Protein profiles *L. reuteri* TMW1.106 and the *gtfA* and *inu* mutants grown in malsucMRS were highly similar. However, the *inu* mutant overexpressed a protein of approx. 200 kDa as pointed out in Figure 13, which was approximately the size calculated for GtfA based on amino acid sequence. The protein formed a polymer from sucrose, which was positively stained with PAS. LC-MS/MS identified the protein as a glucosyltransferase with highest homology to the reuteransucrase of *L. reuteri* 121 (66) and consequently as GtfA of *L. reuteri* TMW1.106 (section 3.1.1). Figure 14 shows that overexpression of GtfA also occurred when the *inu* mutant was grown in MRS containing maltose (10 g L^{-1} and 50 g L^{-1}) maltose and glucose or glucose as carbohydrate source (data not shown). *L. reuteri* TMW1.106 also constitutively expressed GtfA, however, EZ-Blue staining alone was not sensitive enough for visualization. Expression could only be detected using combined PAS and EZ-Blue staining as indicated in Figure 15. GtfA was never retrieved from whole cell protein of the *gtfA* mutant.
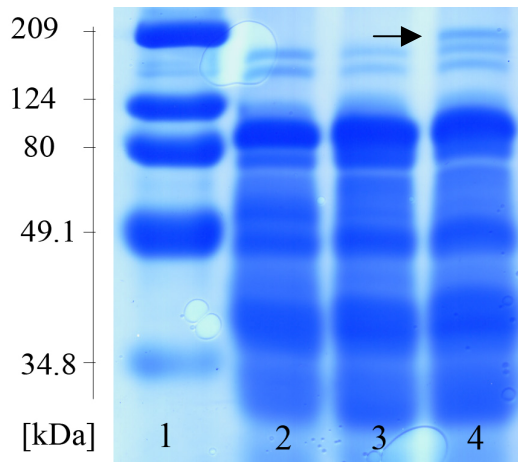
Figure 13. Whole cell protein profiles of *L. reuteri* TMW1.106 (Lane 2), the *gtfA* (Lane 3) and *inu* (Lane 4) mutant on 12 % SDS-Page. Strains were grown in mMRS containing 50 g L^{-1} maltose and sucrose. Proteins were separated on 8 % SDS-Page and stained using EZ-Blue stain. The arrow indicates the protein GtfA overexpressed by the *inu* mutant. Lane 1 molecular weight standard.
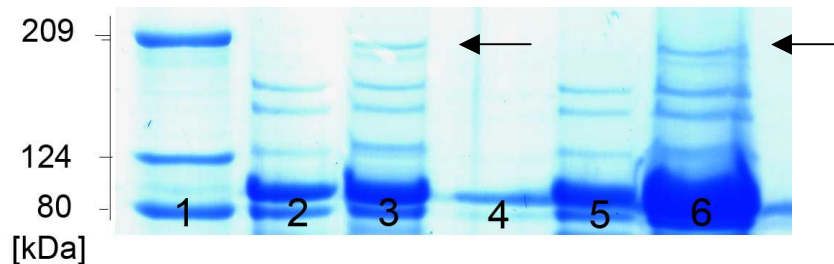
Figure 14. Whole cell protein profiles of *L. reuteri* TMW1.106 (Lanes 2 and 5) and the *inu* mutant (Lanes 3 and 6) grown in mMRS containing 10 g L^{-1} (Lanes 2 and 3) or 50 g L^{-1} (Lanes 5 and 6) maltose, separated on 8 % SDS-Page and stained using EZ-Blue stain. The arrows point out GtfA. Lane 1, molecular weight standards, Lane 4 empty.
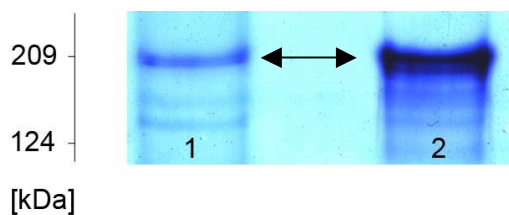
Figure 15. Whole cell protein profile of *L. reuteri* TMW1.106 (Lane 1) and the *inu* mutant (Lane 2) grown in MRS containing 50 g L-1 sucrose, separated on 8 % SDS-PAGE. The arrow indicates GtfA. Gels were incubated overnight in sucrose containing buffer, first stained using periodic/Schiff's reagent and consequently EZ-Blue stained.

3.3 Regulations of glycosyltransferases in *L. reuteri* in response to carbohydrates

Sucrose regulated glycosyltransferase expression has been shown for streptococci and strains of *Leuconostoc*. The impact of sucrose on expression of *L. reuteri* glycosyltransferases *ftfA, inu* and *gtfA* was investigated. Expression of glycosyltransferases in *L. reuteri* TMW1.106 and LTH5448 in response to glucose, sucrose, and maltose and fructose was set in relation to expression of the phosphoketolase (*pho*) and the lactatdehydrogenase (*ldh)* genes. Relative expression levels of *ftfA, inu* and *gtfA* were similar in relation to both reference genes as summarized in Table 6. *L. reuteri* LTH5448 and TMW1.106 expressed *ftfA, inu* and *gtfA,* respectively, when grown with and without sucrose. However, upon addition of sucrose, expression of *ftfA* was clearly induced in proportion to both housekeeping genes, whereas sucrose had no impact on transcription levels of *inu* and *gtfA*. An

increase of sucrose concentration up to 40% did not further enhance *ftfA* expression (data not shown).

Table 6. Expression of *ftfA, inu* and *gtfA* of *L. reuteri* LTH5448 and *L. reuteri* TMW1.106, respectively, during growth in the presence of glucose (5 g L^{-1}), glucose and sucrose (5 g L^{-1} and 10 g L^{-1}) and sucrose (10 g L^{-1}) in relation to expression of relevant genes during growth in mMRS (10 g L^{-1} maltose, 5 g L^{-1} glucose and 5 g L^{-1} fructose). Expression values of glycosyltransferase genes were calculated in relation to expression of the reference genes *ldh* and *pho* using the Pair-Waised Fixed Reallocation Randomization Test provided by the REST software (93).

Strain	Target Gene	Reference gene	Sugar composition of media		
			Glucose	Glucose Sucrose	Sucrose
L. reuteri LTH5448	*ftfA*	*pho*	1.01[#]	165*	101*
		ldh	0.67[#]	226*	113*
L. reuteri TMW1.106	*inu*	*pho*	1.91[#]	2.72[#]	2.41[#]
		ldh	2.30[#]	2.74[#]	1.44[#]
	gtfA	*pho*	1.31[#]	0.69[#]	0.61[#]
		ldh	1.59[#]	0.69[#]	0.34[#]

* significantly upregulated (p<0.001), [#] statistically not different

3.4 Regulation of glycosyltransferases in response to environmental stress

Sporadic reports link polysaccharide formation by lactobacilli to their tolerance towards inhibitors and adverse environmental conditions, suggesting a protective function (58, 75). The regulation of glycosyltransferases in *L. reuteri* upon exposure to stressors was monitored, to determine whether the expression of glycosyltransferases was affected by environmental stress conditions.

3.4.1 Glycosyltransferase expression and polymer formation

Effect of pore forming membrane stressors on HoPS and FOS formation. Nisin and gramicidin, which disrupt the transmembrane ΔpH through pore formation, were applied in concentrations encompassing the range of 0.1 to 2 x MIC towards *L. reuteri* TMW1.106 and LTH5448. Neither nisin nor gramicidin did increase HoPS- or FOS formation in the two strains at sub-inhibitory or inhibitory concentrations (data not shown).

Effect of proton ionophores. Data on polysaccharide formation of *L. reuteri* TMW1.106 and LTH5448 is collected in Table 7. The proton ionophores RTC, TIH and nigericin (40) were applied in concentrations encompassing the range of 0.1 to 2 x MIC towards *L. reuteri* TMW1.106 and LTH5448. Subinhibitory concentrations of RTC, TIH or nigericin stimulated levan and FOS production by *L. reuteri* LTH5448. Polysaccharide formation of *L. reuteri* TMW1.106 was unaffected upon addition of RTC, TIH or nigericin (Table 7). However, 1-kestose levels increased more than 15-fold in the presence of subinhibitory concentrations of TIH. Taken together, these results show that strains *L. reuteri* TMW1.106 and LTH5448 harbours inducible fructosyltransferases.

Effect of the uncoupler CCCP. CCCP was applied in concentrations spanning the range of 0.1 to 2 x MIC towards *L. reuteri* TMW1.106 and LTH5448. The application of CCCP did not increase HoPS or FOS formation in the two strains at sub-inhibitory or inhibitory concentrations (data not shown).

Table 7. Effect of antibiotics on the formation of HoPS and 1-kestose in L. *reuteri* LTH5448 and TMW1.106. Antibiotics were applied in eight different concentrations up to their minimal inhibitory concentration (MIC).

	L. reuteri LTH5448				*L. reuteri* TMW1.106			
	MIC	HoPS inducing concentration	Fold EPS induction	Fold 1-kestose Induction	MIC	HoPS inducing concentration	Fold EPS induction	Fold 1-kestose induction
nigericin [uM]	0.17	0.125-0.5 [a]	2.1-4 [b]	n.d. [c]	0.14	-	1 [d]	n.d.
reuteri-cyclin [mg L^{-1}]	25	6.25-25	2.5-7.4	6	25	-	1	n.d.
trans-isohumu-lone [mg L^{-1}]	33	1.25-10	2.2-4	8	85	5-20	1	17-19

[a] concentration range to achieve an twofold HoPS-production relative to reference conditions.

[b] Fold HoPS concentrations in culture supernatants relative to reference conditions (7 and 0.7 g L^{-1} in strains TMW1.106 and LTH5448, respectively).

[c] Fold 1-kestose concentrations in culture supernatants relative to reference conditions (less than 0.1 and 0.2 – 2.4 mM L^{-1} in strains TMW1.106 and LTH5448, respectively).

[d] Less than 1.5 fold increase of HoPS concentration relative to reference conditions.
n.d. not determined

Effect of increased temperature and phenylethanol on FOS formation. The effect of phenylethanol (PE) and elevated temperature on the FOS formation was determined to clarify whether the inducing effect of RTC, TIH and nigericin resulted from specific interactions with the bacterial membrane independent of their effects on the transmembrane ΔpH. Elevated growth temperature and phenylethanol are known to alter the membrane fluidity (57). The levels of 1-kestose produced by strains

LTH5448 and TMW1.106 were increased 1.5 fold and 10-fold, respectively, after incubation at either 45°C or with 6 mM PE compared to reference conditions.

Regulation of transcription. The expression of glycosyltransferases in *L. reuteri* TMW1.106 and LTH5448 at reference conditions (37°C in sucMRS) was determined by specific amplification of *gtfA, inu,* and *ftfA* fragments using cDNA-libraries as template in PCR reactions. At reference conditions, *L. reuteri* TMW1.106 transcribed *gtfA* whereas *inu* expression was below detection limit. *L. reuteri* LTH5448 transcribed *ftfA* (data not shown). To substantiate the results based on the biochemical analysis the expression of *gtfA, inu,* and *ftfA* was quantified relative to *ldh* transcription. Results are presented in Table 8. Two inducing antibiotics (RTC and TIH), one non-inducing antibiotic (CCCP), and incubation at elevated temperature were selected to evaluate whether increased fructan- and FOS production corresponds to increased transcription of *ftf*s. In *L. reuteri* TMW1.106, *gtfA* expression was independent of environmental conditions whereas *inu* expression was increased at 45°C and in the presence of TIH. In *L. reuteri* LTH5448, *ftfA* transcription was generally increased at conditions, which stimulated HoPS or FOS synthesis, although the inducing effect of 45°C incubation was not significant. CCCP slightly decreased transcription of *ftfA*.

Table 8. Analysis of transcription of *ftfA*, *inu* and *gtfA* using real-time PCR. Relative transcription levels N/N_0 were calculated relative to the transcription level at reference conditions (37 ℃, sucMRS) using *ldh* as reference gene according to Pfaffl (92).

L. reuteri	TMW 1.106		LTH 5448
	inu	*gtfA*	*ftfA*
trans-isohumulone (10 mg L^{-1})	7.7±2	1[a]	3.2±1.5
reutericyclin (25 mg L^{-1})	n.d.	1	12.4±2.4
cccp (50 μM)	n.d.	1	0.35 ± 0
45˚C	3.4±1	1	1.5 ± 0.9

[a] constitutive expression, difference to reference conditions less than 50%

n.d. not determined

3.4.2 Impact of fructosyltransferase inducing conditions on membrane parameters

Fructosyltransferases of *L. reuteri* were upregulated in response to the exposure to proton ionophores, PE and elevated temperature. The last mentioned are known to change membrane fluidity. To test the hypothesis that the increased fructosyltransferase is linked to alterations of membrane biophysical parameters, membrane fluidity and the membrane lateral pressure were determined in cells and membrane vesicles, respectively.

A temperature raise to 45°C or addition of inducing levels of PE decreased Laurdan GP-values, indicating increased membrane fluidity as presented in Figure 16. RTC or TIH at inducing concentrations decreased membrane fluidity. Gramicidin and CCCP had little or no effect on membrane fluidity. Comparable results were obtained with *L.*

reuteri LTH5448 (data not shown). Thus, a correlation of the effect of inducing agents to their effect on the fluidity of the membrane was not apparent.

The fluorescent dye 4dipyPC is a probe to determine the lateral pressure profile in liposomes (122). Figure 4 verified that the E/M ratio of 4dipyPC can be applied to probe lateral pressure in membrane vesicles with a complex composition. Increased temperature, inducing concentrations of PE, RTC or TIH resulted in increased E/M ratios (Figure 16). CCCP at concentrations close to its MIC did not affect lateral pressure, and gramicidin slightly decreased the lateral pressure in *L. reuteri* membranes. Taken together, an increased lateral pressure in *L. reuteri* membrane vesicles was determined in the presence of those chemical and physical agents, which increased the transcription of *ftf*s as well as fructan and FOS production in *L. reuteri*.
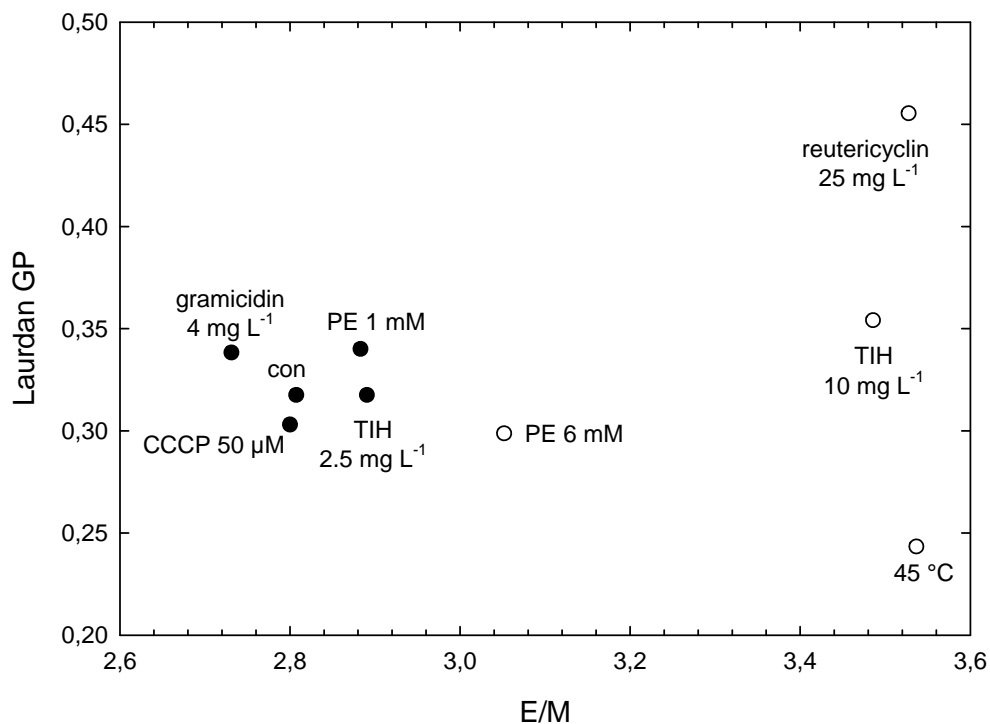
Figure 16. Plot of the fluidity of membranes of *L. reuteri* TMW1.106 against the membrane lateral pressure at various conditions. Closed circles, environmental conditions which do not induce *inu* expression and 1-kestose production; open circles, environmental conditions, which induce *inu* expression and 1-kestose formation. Membrane fluidity was determined in whole cells by using the Laurdan general polarisation (GP), membrane lateral pressure was determined in membrane vesicles by using the E/M ratio of 4dipyPC lipids incorporated into the membranes. con = reference conditions (37°C, no additives), PE = phenylethanol, TIH = trans-isohumulone. Laurdan GP was determined in three independent experiments, the E/M ratio was determined in two independent experiments and representative results are shown. The effects of the various conditions on the fluidity of the membrane of *L. reuteri* LTH5448 were essentially identical to those observed with strain TMW1.106 (not shown).

3.5 Impacts of fructan on membrane parameters

Fructans and fructooligosaccharides have been shown to directly interact with phospholipids of model mono- and bilayer membranes (53, 54, 137, 138, 139). Membrane fluidity and membrane lateral pressure were successfully applied to study changes within the bacterial membrane and were hence determined to detect interactions of fructose-based oligo- and polysaccharides with complex bacterial membranes. Chicory inulin preparation containing no low-molecular weight contaminations, FOS powder composed of sucrose (approx. 6%), 1-kestose, nystose and higher fructooligosaccharides up to DP 12 and an IMO preparation contained isomaltose (approx. 23 %), isomaltotriose, traces of maltose and maltotriose and higher isomaltooligosaccharides (data not shown) were used as test substances to assure standardized conditions.

Impact of fructans on membrane fluidity. Membrane parameters were determined at least four times, and results shown in Figure 17 are representative for trends observed in all replicates. The calculation of average values is not useful, as absolute values vary between independent experiments. Temperature increase from 10 to 50 ºC resulted in decreased GP values in all samples, indicating increased membrane fluidity. However, inulin clearly reduced membrane fluidity compared to control samples. In contrast, the application of FOS tended to result in increased membrane fluidity.

Impact of fructans on membrane lateral pressure. With increasing temperature, lateral pressure within membrane increased as outlined in Figure 18. Membrane pressure in vesicles in the presence of FOS and inulin were higher compared to controls. The presence of sucrose and IMO did not affect membrane lateral pressure.
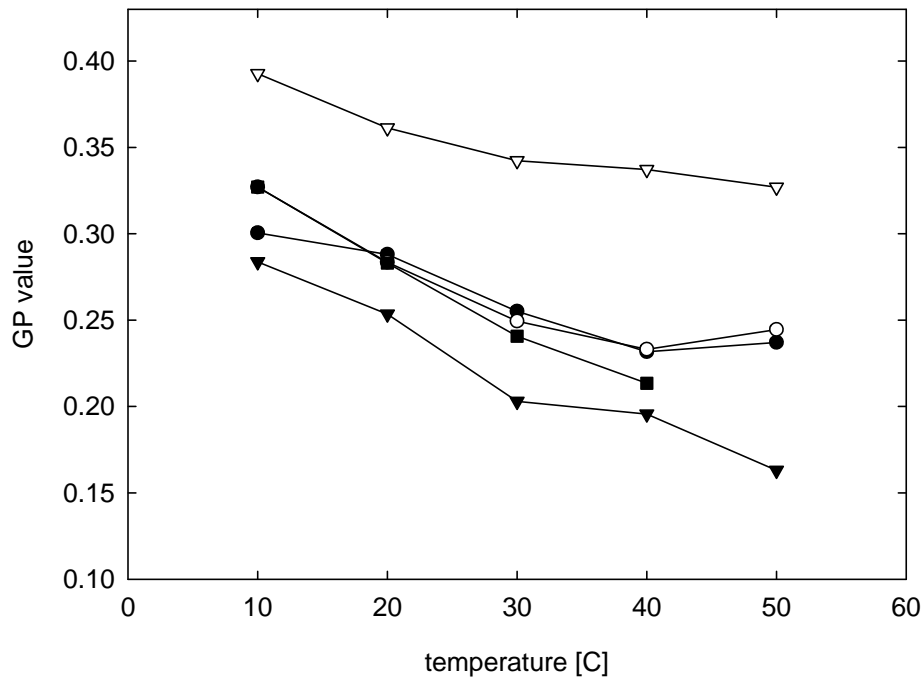
Figure 17. Shifts in membrane fluidity during temperature increase from 10 to 50 ℃ of *L. reuteri* TMW1.106 whole cells resuspended in phosphate buffer only ( ) or in phosphate buffer containing 7.5% fructooligosaccharides (q), inulin (s), sucrose ( ) or isomaltooligosaccharides (¢). Membrane fluidity was investigated using the fluorescence probe Laurdan. Emission spectra (excitation 360 nm, emission 400-520 nm) were recorded using a spectrafluorometer. The generalized polarization value GP was calculated as: GP= (I_{440nm} - $I_{490\ nm}$) / ($I_{440\ nm}$ + $I_{490\ nm}$). I stands for fluorescence intensity.
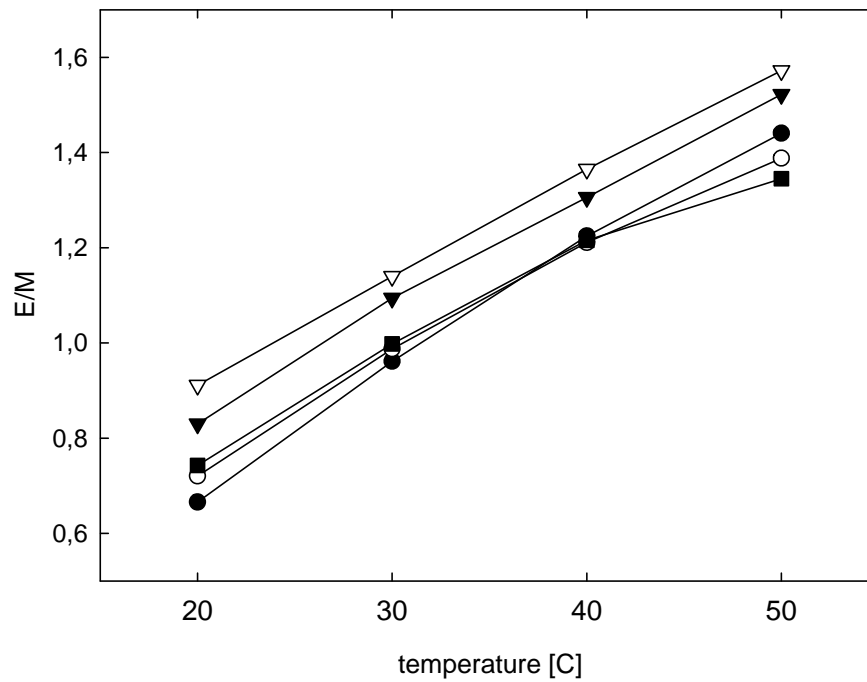
Figure 18. Membrane lateral pressure in membrane vesicles of *L. reuteri* TMW1.106 in phosphate buffer only ( ) or in phosphate buffer containing 7.5% fructooligosaccharides (q), inulin (s), sucrose (   ) or isomaltooligosaccharides (¢). Membrane lateral pressure was determined using the fluorescence probe dipy4-PC (excitation 342 nm, emission spectra 380-520 nm) and calculated as the excimer / monomer ratio (E/M).

3.6   Effects of HoPS and FOS survival of *L. reuteri* in the presence of antimicrobial substances and during freeze-drying.

3.6.1   Protective effect of of HoPS and FOS on the activity of inhibitors towards *L. reuteri.*

L. reuteri responded to environmental stress conditions causing increased membrane lateral pressure with the up-regulation of fructosyltransferases. This observation led to the question whether HoPS and oligosaccharides formed by glycosyltransferases offer

protection during exposure to stress. Therefore the effects of dextran, levan, and FOS, all produced by bacterial glycosyltransferases, on the activity of membrane-active inhibitors towards *L. reuteri* TMW1.106 were determined. The MIC of RTC was increased 2, 4 and 2 fold in the presence of 50 g L^{-1} FOS, dextran, and levan, respectively, compared to the MIC values in mMRS; the MIC of nisin was increased 4, 3, and 4 fold, and the MIC of CCCP was increased 2, 10, and 2 fold. Qualitatively comparable results were obtained with *L. reuteri* LTH5448. Thus, the products of glycosyltransferases provided significant protection towards membrane-active inhibitors.

3.6.2 Impact of FOS and fructans during freeze-drying of *L. reuteri* TMW1.106

Fructans and FOS interacted with model membranes thus protecting liposomes during air- and freeze-drying (53, 54, 138). Inulin and FOS also interacted with the bacterial membrane indicated by changes of biophysical membrane parameters. To investigate whether fructans offered protection of bacteria in analogy to observations made in liposomes, survival and membrane integrity of the *L. reuteri* TMW1.106 during freeze-drying was monitored. Results are summarized in Figure 19.

Survival and membrane integrity after freezing. Cells growing in the stationary phase resuspended in PB containing sucrose, FOS, inulin and skimmed milk showed slightly better viability after freezing than control samples. Approximately 75% membrane integrity remained. In exponential phase cells, membrane integrity during freezing dropped to less than 15 and 25% in cells resuspended in PB or PB containing sucrose, FOS and inulin, respectively. Cell counts of control cells dropped by two logs. About 10 % of cells frozen in the presence of sucrose, inulin and FOS survived the freezing.

Survival and membrane integrity after freezing and freeze-drying. The addition of FOS provided the best protection during freeze-drying of cells in the stationary phase. Viability and membrane integrity of cells freeze-dried without addition of protectants was the lowest. *L. reuteri* TMW1.106 in the stationary phase, which were freeze-dried in the presence of sucrose, inulin and fructan still retained up to 10 % membrane integrity, whereas cells freeze-dried in buffer containing FOS buffer had up to 20 % membrane integrity. In contrast, numbers of exponentially phase cells dropped between 3.5 and 2 logs in control and sugar containing samples, respectively. Membrane integrity decreased to less than 5 % in all treatments.

Survival and membrane integrity after freezing, freeze-drying and storage. FOS significantly (P<0.05) improved survival of stationary phase cells compared to control samples. Even after storage, cell counts of stationary phase *L. reuteri* TMW1.106 had only decreased by 0.5 log compared to initial cell counts. Concurrently, membranes of these cells retained up to 20 % membrane integrity, whereas membranes of cells freeze-dried and stored in the presence of sucrose and inulin where completely permeabilized. Cell counts of stationary *L. reuteri* TMW1.106 in PB, sucrose and skim milk decreased at similar amounts during storage. After storage, cell counts of exponential phase cells did not differ distinctly within treatments; membranes were completely disrupted. Viability in exponentially growing *L. reuteri* TMW1.106 decreased slightly less in cells treated in the presence of skim milk and inulin compared to control, sucrose and FOS. The total decline in cell viability during freezing, freeze-drying and storage in exponential and stationary phase *L. reuteri* TMW1.106 was comparable, whereas stationary growing cells in PB, sucrose or FOS survived better than exponential phase cells.
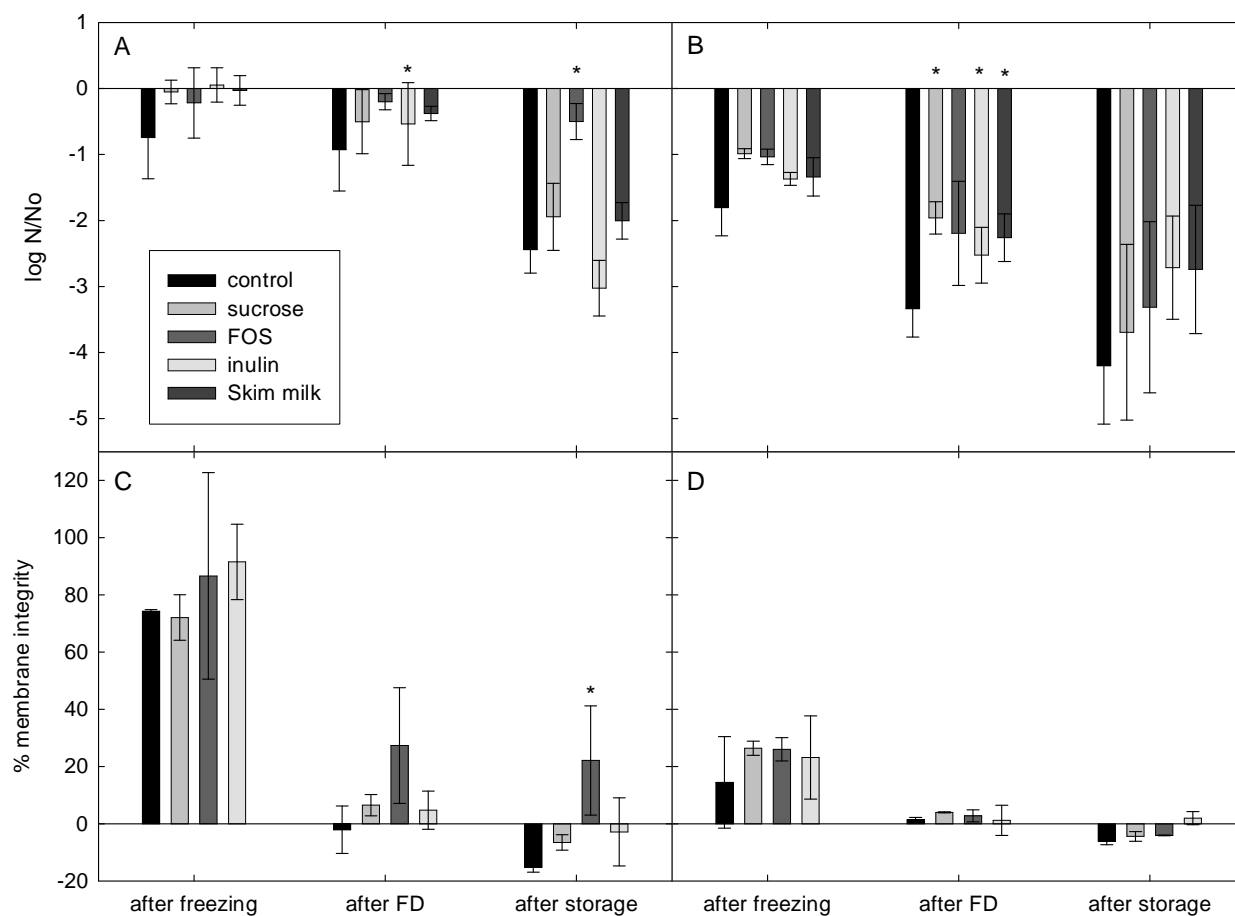
Figure 19. Survival of stationary (A) and exponentially growing (B) *L. reuteri* TMW1.106 resuspended in phosphate buffer or phosphate buffer with 7.5 % sucrose, fructooligosaccharides, inulin and skim milk powder after freezing, freeze-drying and storage. Membrane integrity of stationary (C) and exponentially (D) phase L. reuteri *TMW1.106 after* freezing, freeze-drying and storage was determined using BactoLive/Dead Kit. *means significantly different ($p < 0.05$) from control.

3.7 Performance of *ftfA*, *gtfA* and *inu* deletion mutants in natural habitats.

At the beginning of this work, the hypothesis was posted that the presence of glycosyltransferases may be considered a reason for the competitiveness of *L. reuteri* in cereal fermentations and the digestive tracts of pigs, birds and mice. In

consequence, the expression of glycosyltransferases in vivo was monitored, as a first indicator indicator whether glycosyltransferases play a role in biofilm formation in the forestomach of RLF-mice.

3.7.1 Expression of *inu, ftfA* and *gtfA* in vivo

Sourdough isolates of *L. reuteri* colonized RLF-mice after single application of cultures on fur and in drinking water at levels comparable to the rodent isolate *L. reuteri* 100-23 and reach CFUs of 10^9 per gram stomach content (37). *L. reuteri* TMW1.106 and LTH5448 were grown in MRS and total RNA was isolated from exponential and early stationary growth phase cells. Total RNA was furthermore isolated from the forestomach epithelium and luminal stomach contents of RLF-mice, which had been colonized with *L. reuteri* strains for two weeks. cDNA libraries were generated and analysed using real time PCR. Control PCRs showed that *L. reuteri* TMW1.106 and *L. reuteri* LTH5448 expressed *inu* and *gtfA* and *ftfA*, respectively, in MRS during both growth phases (data not shown). *GtfA* but not *inu* expression was detectable in *L. reuteri* TMW1.106 isolated from the forestomach epithelium and from luminal stomach contents of RLF-mice. In vivo grown *L. reuteri* LTH5448 expressed *ftfA*. Figure 20 shows that expression of *gtfA* varied from mouse to mouse. However, expression of *gtfA* was higher in cells isolated from luminal stomach contents than in cells isolated from the forestomach epithelium as displayed in Table 9. Expression of *ftfA* also varied within different mice (Figure 20). Expression of *ftfA* by cells isolated from luminal contents and the forestomach epithelium was not significantly different from expression of cells growing in the exponential or early stationary phase MRS (Table 9).
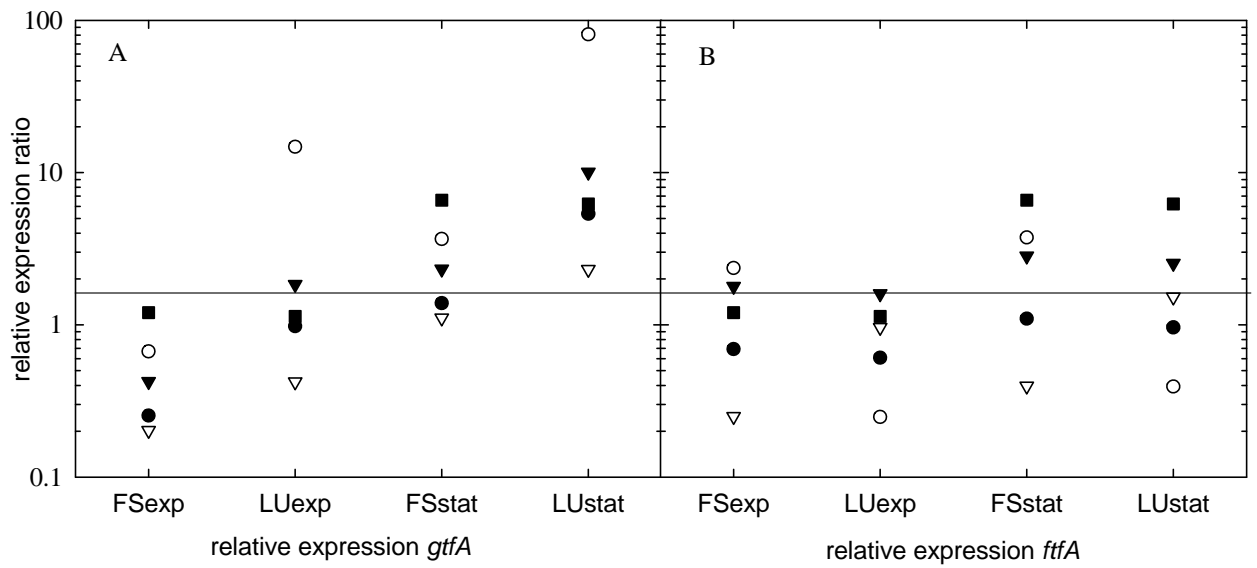
Figure 20. Relative expression ratios of *gtfA* (A) and *ftfA* (B) of *L. reuteri* TMW1.106 and LTH5448, respectively, isolated from forestomach epithelium (FS) and luminal (LU) stomach contents of five reconstituted lactobacilli-free mice in relation to expression of glycosyltransferase genes in *L. reuteri* strains grown to exponential (exp) or early stationary (stat) growth state in MRS. The 16SrRNA gene was used as reference gene. Identical symbols represent expression ratios within the same animal.

Table 9. Expression levels of *gtfA* and *ftfA* in *L. reuteri* TMW1.106 and LTH5448 isolated from the forestomach epithelium or within the stomach contents of RLF-mice compared to expression levels of strains grown in MRS. The 16SrRNA gene was used as reference. Expression levels were calculated using the Pair-Waised Fixed Reallocation Randomization Test provided by the REST software (93).

Expression ratios	Forestomach epithelium		Luminal stomach contents	
	exponential	stationary	exponential	stationary
gtfA	0.645*	2.274*	2.181*	8.481*,#
ftfA	1.070*	1.784*	0.993*	1.863*

*Expression ratios not significantly different (p > 0.05)

#90% probability that in vivo expression is different from expression during reference conditions (p < 0.1)

3.7.2   Carbohydrate utilization of *L. reuteri* in the forestomach of RLF-mice

RLF-mice were colonized with *L. reuteri* strains TMW1.106, LTH5448 and the *inu, gtfA* and *ftfA* mutants and cell counts were obtained after 7 and 14 of colonization. Data were collected by Jens Walter, University of Otago, New Zealand, and were provided for discussion (section 4.5.2). Stomach contents of RLF mice harbouring either *L. reuteri* TMW1.106 or the *gtfA* and *inu* mutants were analyzed in this work. RLF-mice were fed with commercially available rat chow containing mainly maltose, maltotriose, glucose and traces of fructose, raffinose and sucrose (data not shown). Maltose, maltotriose, glucose, fructose and lactate were detected in uninoculated mice. *L. reuteri* TMW1.106 and the *inu* and *gtfA* mutants metabolized maltose, glucose and fructose and formed lactate as shown in Table 10. Maltose utilization and lactate formation in stomach contents of RLF-mice colonized with the *inu* mutant was lower than in mice colonized with the *gtfA* mutant and *L. reuteri* TMW1.106. Malto-

or fructooligosaccharides were not detected within the stomach contents using various HPLC and TLC methods.

Table 10. Analysis of stomach contents of 7 uninoculated RLF-mice or 7 RLF-mice colonized with *L. reuteri* TMW1.106 and the *gtfA* and *inu* mutants after 2 days of colonization.

mM g^{-1} dried stomach contents	*L. reuteri* TMW1.106	*gtfA* mutant	*inu* mutant	uninoculated mice
Maltose	109 ± 6	73 ± 1	194 ± 18	759 ± 88
Glucose	75 ± 4	49 ± 2	101 ± 7	253 ± 2
Fructose	37 ± 1	25 ± 0	67 ± 5	105 ± 23
Lactate	168 ± 2	216 ± 2	83 ± 7	34 ± 10

3.7.3   Performance of *inu, gtfA* and *ftfA* mutants in wheat sourdough.

During 24 h of fermentation, *L. reuteri* TMW1.106, *L. reuteri* LTH5448 and the *gtfA, inu* and *ftfA* mutants acidified wheat sourdough reaching pH values between pH 3.2 and pH 3.4 (data not shown).

L. reuteri TMW1.106 and the *gtfA* mutant grew at a similar growth rate and reached similar cell counts when inoculated alone and in competition as shown in Figure 21 and Table 11. During competition experiments the *gtfA* mutant presented in average approximately 49% of the total lactobacilli population after 24 h as displayed in Figure 22. The *inu* mutant formed less lactate, acetate and ethanol than *L. reuteri* TMW1.106. The decreased formation of organic acid formation could be observed after 10 and 24 h of incubation (Figure 21, Table 11). The difference in lactate

formation between *L. reuteri* TMW1.106 and the *inu* mutant in three independent experiments ranged from 8-30 mM. A larger difference in lactate formation between *L. reuteri* TMW1.106 and *inu* mutant in single experiments correlated to a smaller proportion of mutant during growth in competition with *L. reuteri* TMW1.106. When *L. reuteri* TMW1.106 and *inu* mutant were inoculated together, the *inu* mutant constituted between 2.86 and 37.4% of total lactobacilli after 24 h.

When inoculated together, the *ftfA* mutant reached lower final cell counts than *L. reuteri* LTH5448. The *ftfA* mutant represented between 3.2 and 26.0% of total lactobacilli population when co-inoculated with *L. reuteri* LTH5448 (Figure 21). Doughs fermented with the *ftfA* mutant contained similar amounts of lactate, acetate and ethanol as doughs fermented with *L. reuteri* LTH5448.

Table 11. Cell counts and metabolite formation of *L. reuteri* TMW1.106, the *gtfA* and *inu* mutants, and of *L. reuteri* LTH5448 and the *ftfA* mutant in wheat sourdough after 24 h of incubation at 37 ℃.

L. reuteri strans	Log CFU/g flour	Metabolites formed [mM]					
		Maltose	Glucose	Fructose	Lactate	Acetate	Ethanol
TMW1.106*	9.3 ± 0.1	46 ± 5	2 ± 0	18 ± 2	58 ± 4	9 ± 1	41 ± 12
inu mutant[#]	9.0 ± 0.1	43 ± 5	1 ± 0	20 ± 4	41 ± 11	6 ± 1	31 ± 11
gtfA mutant[#]	8.6 ± 0.5	47 ± 6	1 ± 1	20 ± 4	49 ± 7	6 ± 0	47 ± 15
LTH5448*	9.8 ± 0.1	50 ± 9	2 ± 0	14 ± 2	52 ± 8	7 ± 1	31 ± 9
ftfA mutant[#]	9.6 ± 0.4	62 ± 11	2 ± 0	15 ± 1	53 ± 4	8 ± 0	42 ± 17

Cell counts determined on mMRS plates (*) or mMRS plates containing 5 µg L^{-1} erythromycin ([#])
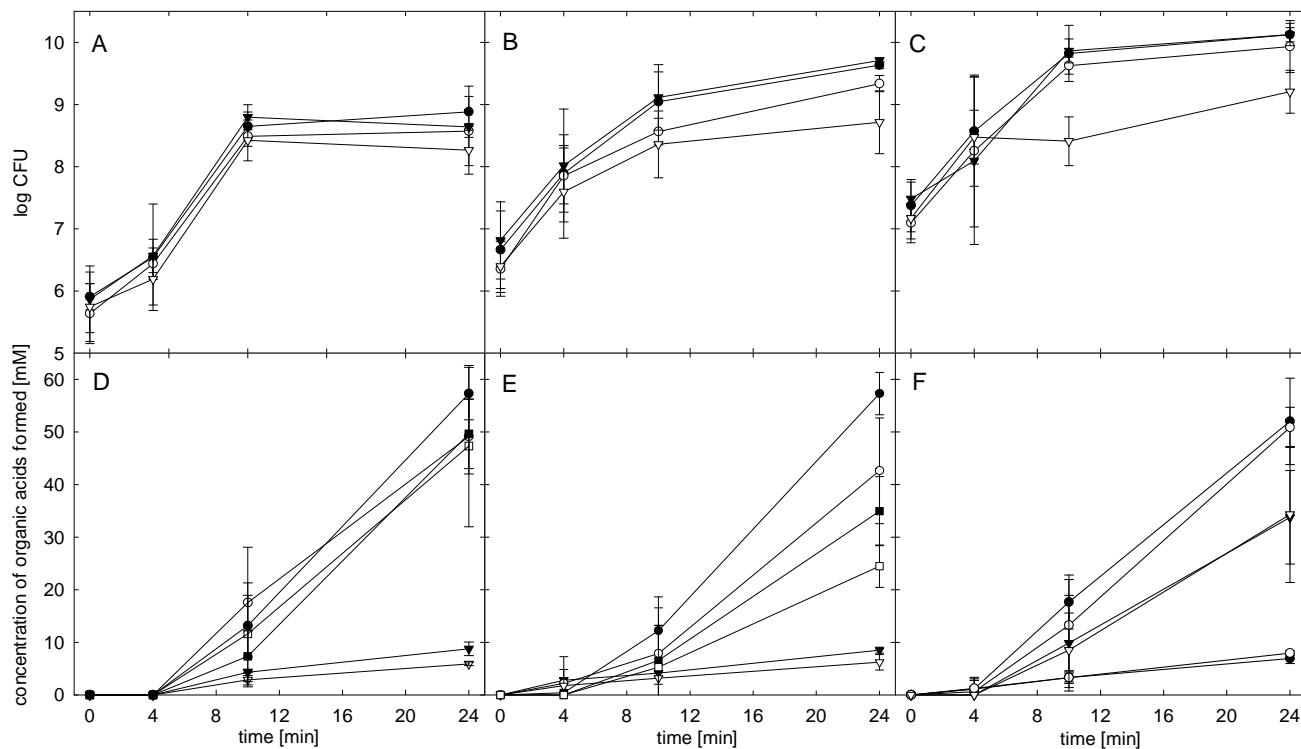
Figure 21. Illustrated is growth of *L. reuteri* TMW1.106 (,q) and the (A) *gtfA* (,s) or (B) *inu* mutants (,s), or (C) *L. reuteri* LTH5448 (,q) and the *ftfA* mutant (,s) inoculated in wheat sourdough alone (circles) or in competition (triangles). Kinetics of organic acid formation of *L. reuteri* TMW 1106 (closed symbols) and the (C) *gtfA* (open symbols) or (D) *inu* mutants (open symbols) or (E) *L. reuteri* LTH5448 (closed symbols) and the *ftfA* mutant (open symbols) are presented in figures C, D, and E. Concentrations of lactate (,), ethanol (■,□) and acetate (q,s) were determined after 0, 4, 10 and 24 h of incubation at 37 ℃.
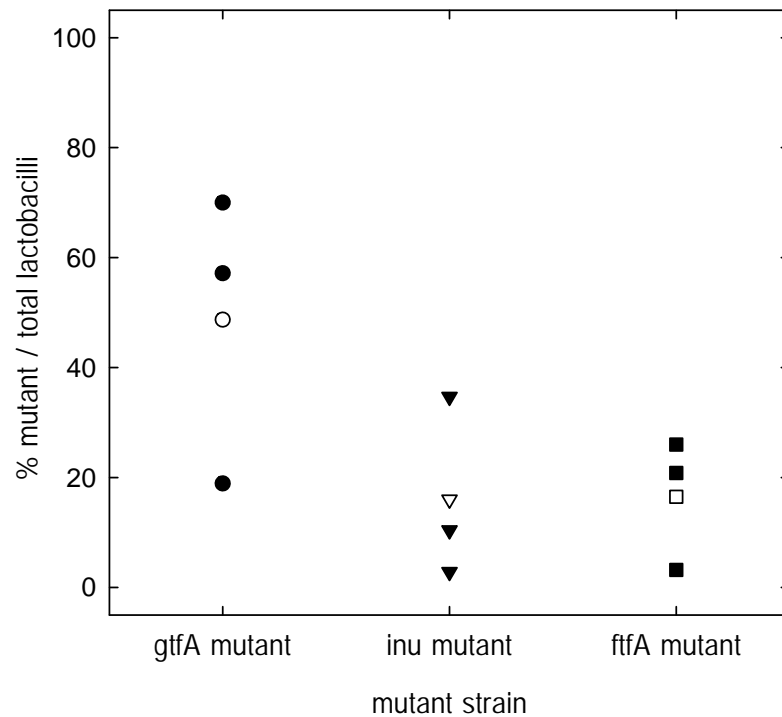
Figure 22. Distribution of mutant lactobacilli (circles *inu* mutant, triangles *inu* mutant, squares *ftfA* mutant) in total lactobacilli population in wheat sourdough after 24 h of incubation at 37 °C. Closed symbols represent to proportion of mutant strains within the entire lactobacilli population in three independent experiments. Open symbols show the average**.**

4 DISCUSSION

The results of the study indicated a role of fructosyltransferases in sucrose metabolism, stress tolerance and fitness of *L. reuteri* in cereal and intestinal environments. A correlation to stress tolerance was attributed due to the regulation of both fructosyltransferases by adverse environmental conditions and the protective effects of products formed by fructosyltransferases on the bacteria membrane. The activity of both fructosyltransferases determined competitiveness in wheat sourdough, whereas only the function of Inu affected colonization of the forestomach of RLF-mice. As maltose constituted the better part of total carbohydrate in wheat sourdough and in stomach contents of mice, protective effects offered by formation of FOS and HoPS or differences in substrate dependent regulation alone could not explain for the varying impacts of Inu and FtfA, which were rather caused by variations in functionality of structurally higly similar enzymes.

4.1 Characterization and occurrence of glycosyltransferases in *L. reuteri*

4.1.1 Glycosyltransferases of *L. reuteri* LTH5448 and TMW1.106

FtfA was a novel fructosyltransferase identified in the strain *L. reuteri* LTH5448 (Figure 5), which synthesized 1-kestose, nystose and levan from sucrose. Ftfs of lactobacilli share a common domain organisation, a signal peptide directing protein export, an N-terminal variable domain, a catalytic domain, and a C-terminal cell wall anchor comprising the LPXTG motif mediating covalent linkage to the cell wall (125, 132, 133). The signal peptide, N-terminal variable domain, and the C-terminal cell wall anchor of *ftfA* were essentially identical with the corresponding domains of the inulosucrase of *L. reuteri* 121. However, the similarity of the *ftfA* catalytic domain to Inu was lower (72 % identity, 83 % similarity to Inu, 67 % identity, 82 % similarity to

Lev of *L. reuteri* 121). The N-terminal variable domain of Ftfs of lactobacilli has no relevance for catalytic turnover (125) and the C-terminal cell wall anchors of functional Ftfs exhibit significant sequence variation. Therefore, it is unlikely that a high selective pressure accounts for the high sequence conservation in these domains. The unusual gene structure may rather result from genetic rearrangements, which replaced the catalytic domain of the *inu* gene.

The catalytic domains of Inu of *L. reuteri* 121 and TMW1.106, and of FtfA contained the eight higly preserved amino acid residues forming the active site of fructosyltransferases (91). Yet, FtfA of *L. reuteri* synthesized the ß-(2→6) linked levan, ß -(2→1) FOS and some FOS which might be ß-(2→6) linked, whereas Inu of *L. reuteri* 121 formed ß-(2→1) linked inulin. The study of three dimensional structures of lactobacilli Ftfs might help to understand which mechanism determined the linkage type of fructans.

Fructosyltransferases Inu and FtfA of *L. reuteri* and the Levansucrase Lev of *L. sanfranciscensis* were heterologously expressed to examine whether these fructosyltransferases form similar products from sucrose and various acceptor sugars. *E. coli* heterologously expressed His-tagged Inu, InuC and FtfA, however, purification of the proteins using Histrap columns failed. Van Hijum et al. (2004) (134) also reported difficulties while trying to purify Inu and a C-truncated version of Inu of *L. reuteri* 121. Crude extract of *E. coli,* which heterologously expressed Inu, contained a protein of the expected size which showed activity. Therefore oligosaccharides formed by this crude extract (InuCE) were analysed and compared to products of FtfAC.

Tieking et al. (128) observed that the Levansucrase of *L. sanfranciscensis* TMW1.392 synthesized FOS and heteroligosaccharides in the presence of sucrose and the

acceptor sugars maltotriose, raffinose, xylose, arabinose and maltose, respectively. Inu of *L. reuteri* 121 synthesized a broad range of FOS of the inulin type β-(2→1) glycosidic bond), whereas Lev formed 1-kestose, nystose and the polymer levan from sucrose (91). In contrast to these studies, formation of 1-kestose by the purified enzymes Lev, FtfAC and InuCE was not detected. Sucrose concentrations between studies varied greatly. In this study, FtfA and InuCE were incubated in buffer containing 50 g L^{-1} sucrose. Tieking et al. (128) incubated Lev of *L. sanfranciscensis* in 136 g L^{-1} sucrose, Ozimek et al. (91) used 286 g L^{-1} sucrose to investigate the product range of Inu and Lev of *L. reuteri* 121. The formation of 1-kestose and nystose and further FOS by *L. reuteri* LTH5448 in the presence of 100 g L^{-1} has been shown (section 3.1.3).

In the presence of the acceptor arabinose, InuCE and FtfAC formed the same heterooligosaccharide detected in the supernatants of *L. reuteri* TMW1.106 and LTH5448, confirming that the fructosyltransferases were responsible for oligosaccharide formation in the respective strains. It further indicated that truncation of the C-terminal of FtfA had no impact on product formation.

L. reuteri TMW1.106 was previously identified as a glucan producer (127) and the PCR-screening revealed the presence of two glycosyltransferase genes, *gtfA* and *inu*, that are essentially identical to the corresponding genes of *L. reuteri* 121 (67, 133). The GtfA of *L. reuteri* TMW1.106 shows high homology to the reuteransucrase of *L. reuteri* 121 (67, this work, PhD thesis of Susanne Kaditzky, Technische Universität München, Germany). GtfA forms a glucan of undefined structure from sucrose. The reuteransucrase of *L. reuteri* 121 synthesized panose and maltotriose, or isomaltotriose and isomaltotetraose in the presence of maltose and maltotriose (67). Two amino acid mutations within the catalytic core transformed the reuteransucrase

of *L. reuteri* 121 into a dextransucrase. This mutation also shifted the product spectrum in oligosaccharide synthesis from reuteran-type α-(1→4) linkages to dextran-like α-(1→6) (65). In the presence of maltose and sucrose, *L. reuteri* TMW1.106 and the *inu* mutant produced a glucooligosaccharides identified as isomaltotriose and isomaltooligosaccharides of higher polymerization degree by comparison with external standards. *L. reuteri* TMW1.106 also formed isomaltotriose and an isomaltooligosaccharide of higher polymerization grade from maltose and sucrose, implying that GtfA produced a dextran-like HoPS.

4.1.2   Occurrence of glycosyltransferases in *L. reuteri* intestinal isolates

Because additional sequence data on glycosyltransferases of lactobacilli became available in this work, the 13 strains of *L. reuteri* out of a pool of 43 *Lactobacillus* strains isolated from ducks and the feces of a pig were subjected to a second screening (126). An updated set of PCR-primers was used to detect genes coding for glycosyltransferases that are currently described in the genus lactobacillus. All strains carried at least one gene coding for a glycosyltransferase, independent on their capability to produce HoPS during growth in sucMRS (Table 3). In the species *L. reuteri*, the presence of several glycosyltransferases in a single strain was the rule rather than the exception and emphasized the metabolic diversity of this species (Table 3). From all *L. reuteri* strains, a DNA fragment of the appropriate size was amplified with primers targeting *inu*, about 50% of the strains harboured a gene related to *gtfA* coding for the reuteransucrase of *L. reuteri* 121, and one strain each harboured genes related to the *ftfA* and *lev*-genes of *L. reuteri* strains (Table 3). These data indicated that virtually all lactobacilli from intestinal habitats have the potential to produce HoPS but a majority of strains does not exhibit this metabolic activity during growth at reference conditions in laboratory media. A transposase and an IS-

element, were found adjacent to the levansucrase genes of *L. reuteri* 121 and *L. sanfranciscensis* TMW1.392, respectively (125, 131) and these mobile genetic elements may account for the rapid loss of glycosyltransferase expression that was observed in *L. reuteri* 121 during growth in chemostat culture and after a pH down shift (131).

4.1.3 Expression of *gtfA* in *inu* mutant of *L. reuteri* TMW1.106

Upon deletion of a second active glycosyltransferase Inu in *L. reuteri* TMW1.106, GtfA was overexpressed in the mutant strain grown in the presence of sucrose, glucose and various maltose concentrations. Increased glucan formation upon deletion of a fructosyltransferase had been reported before. Inactivation of Ftf in *Streptococcus mutans* V403 led to increased glucan production. The authors reasoned that the inactivation of Ftf might have abolished impaired functionality of Gtf in the parent strain caused by the formation of complexes between Gtfs and Ftfs. On the other hand, increased formation of glucan might have resulted from alterations in the regulation of *gtf* due to effects of the inserting plasmid on flanking regions of *ftf*, which might include a regulator for the glucosyltransferase (103).

Impaired function of GtfA in the parent strain due to the formation of complexes with Inu can be excluded, as increased glucan formation resulted from increased protein expression. *Inu* contains a putative rho-independent terminator as displayed in Figure 23, which minimized the chances of polar effects on regulatory elements of *gtfA*. However, the *inu* mutant of *L. reuteri* TMW1.106 was obtained through insertional inactivation of the *inu* gene without any intended changes of *gtfA,* notwithstanding it cannot be foreclosed that insertion of the plasmid affected regulatory segments of *gtfA*.

Overnight cell counts, maltose utilization and organic acid formation of the *inu* mutant during growth in mMRS containing maltose, fructose and glucose as carbohydrate sources were similar to *L. reuteri* TMW1.106, implying that overexpression of *gtfA* did not negatively affect performance of the mutant in vitro.

Figure 23. The putative rho independent terminator downstream of *inu.* The inverted repeats only contain one mismatch, but lack the stretch of adenines. The stop codon of *inu* is marked bold. CG rich inverted repeats are marked cursive. n stands for nucleotides, which were counted starting at the stop codon.

4.2   Sucrose metabolism and impact of sucrose on glycosyltransferase expression in *L. reuteri*

Heterofermentative lactic acid bacteria form lactate and ethanol from glucose and gain one mole of ATP via the pentose-phosphate pathway. The regeneration of reduced co-factors determines the energy yield of heterofermentative glucose metabolism (47). Glucosyltransferases and the hydrolysis activity of fructosyltransferases liberate fructose from sucrose. Fructose is further reduced to mannitol. Concurrently the oxidation of NADH to NAD^+ enables the formation of acetate from acetyl-phosphate and the gain of an additional mole of ATP via the pentose-phosphate pathway (125). In *L. sanfranciscensis* TMW1.392, sucrose metabolism and polymer formation of is directed by one levansucrase enzyme only (125). During growth in wheat sourdough

containing 9% sucrose, a *lev* mutant formed less mannitol and acetate, and more ethanol than *L. sanfranciscensis* TMW1.392 and did not synthesize 1-kestose (125).

L. reuteri LTH5448 produced levan and fructooligosaccharides during growth in mMRS containing solely sucrose as carbohydrate source. Metabolite analysis of *L. reuteri* LTH5448 and the *ftfA* mutant verified that FtfA was the only enzyme responsible for formation of fructooligosaccharides and the fructan polymer. However, the observed decrease of sucrose and the formation of similar amounts of mannitol, lactate, acetate and ethanol produced by the *ftfA* mutant in mMRS containing sucrose suggested that *L. reuteri* LTH5448 harbours at least one more sucrose-hydrolysing enzyme. Since an invertase has been characterized in *L. reuteri* CRL1100 (27), it can be assumed that the so far uncharacterized enzyme has a similar functionality. Conclusively, in *L. reuteri* LTH5448, at least two enzymes were responsible for sucrose metabolism and oligo- and polysaccharide formation.

L. reuteri TMW1.106 formed 1-kestose and glucans during growth at standard conditions, which showed that Inu and GtfA are expressed and active. Inactivation of either *inu* or *gtfA* resulted in reduced sucrose utilization and consequently decreased growth ability in mMRS with sucrose as carbohydrate source, although constitutive overexpression of GtfA in the *inu* mutant was observed. Improved growth after adaption to sucMRS implied either a change in the mechanism of sucrose utilization, or the induced expression of a further sucrose-utilizing enzyme.

Sucrose induced glucosyltransferase expression has been reported for *S. mutans* and *Lc. mesenteroides* (35, 60, 85, 96). The *Lactobacillus* strains *L. reuteri* 121 and *L. sanfranciscensis* TMW1.392 expressed a reuteransucrase and a levansucrase, respectively, even in the absence of sucrose (127, 131). *L. reuteri* LTH5448 expressed *ftfA* in the presence and absence of sucrose. However, expression was induced when

L. reuteri LTH5448 was grown in the presence of sucrose. Regulation of expression occurred on the transcriptional level. *L. reuteri* LTH5448 did not depend on its sucrose-inducible fructosyltransferase in sucrose utilization, possibly due to the presence of a second sucrose-metabolizing enzyme. In contrast, sucrose and had no impact on *inu* and *gtfA* expression of *L. reuteri* TMW1106, the only two enzymes found to be responsible for sucrose metabolism in *L. reuteri* TMW1.106. Constitutive expression of *gtfA* and *inu* and decreased growth in sucMRS upon inactivation of one of the genes implies that in *L. reuteri* TMW1.106 depended on GtfA and Inu and that GtfA and Inu act together in sucrose hydrolysis and/or product formation. Yet, the presence of an unknown, sucrose metabolizing enzyme in *L. reuteri* TMW1.106 cannot be ruled out.

4.3   Regulation of glycosyltransferases in *L. reuteri* by environmental factors

The abundance of glycosyltransferases in *Lactobacillus* strains suggested functions beneficial for the bacterial cell. Products formed by glycosyltransferases are likely to improve survival of bacterial strains in scenarios bacteria might encounter in nature, for e.g. pH fluctuations, drought, cold, heat and the exposure to antimicrobial substances. To identify whether regulation of glycosyltransferases and bacterial stress response correlated, the expression of glycosyltransferases of *L. reuteri* in response to the exposure to various stressors was investigated.

4.3.1   Fructosyltransferases regulation in response to changes in membrane
lateral pressure

The inhibitors used in this work have a comparable mode of action (2, 17, 40, 76, 108) and were employed at concentrations close to their MIC. Nevertheless, according

to results obtained in this study, these inhibitors can be classified as agents, which do not influence fructosyltransferase expression, and as agents, which induce fructosyltransferase expression. Gramicidin, nisin, and CCCP exerted no effect on the expression of Ftfs, excluding the possibility that a decreased proton motive force or the reduction of the growth rate affected gene expression. In contrast, RTC, TIH and nigericin induced Ftf expression. Furthermore, incubation at elevated temperature or in the presence of phenylethanol, agents known to alter the biophysical membrane properties, increased Ftf expression. Thus, the common denominator of inducing agents is their interaction with, or their effect on bacterial membranes. The biophysical properties of the cytoplasmic membrane are defined by several parameters including membrane fluidity and membrane lateral pressure (8, 11, 148). Bacterial cells may sense changes in environmental conditions through their effect on biophysical membrane properties (3, 94, 145). Accordingly, fructosyltransferase expression in *L. reuteri* may be mediated through alterations of the membrane. In *L. reuteri* the effect of the various agents on fructosyltransferase expression did not correlate to their effect on membrane fluidity but lateral pressure clearly distinguished between inducing agents and inhibitors with no effect on enzyme expression.

Lipid monolayers have the intrinsic propensity for interfacial curvature but in biological bilayers, both monolayers are held flat despite the desire for monolayer curvature. Therefore, the phospholipids in biological membranes exert a lateral pressure due to this curvature frustration (11, 148). A temperature increase is known to enhance interfacial curvature and hence the lateral pressure in the bilayer. The effects of RTC, TIH and nigericin on lateral pressure may be explained by their preferential interaction with the hydrophobic interior of the membrane, which increases the propensity of the monolayer for interfacial curvature. For the direct

measurement of lateral pressure in chemically defined liposomes, pyrene-labelled phosphatidylcholine have been proposed (122). Using pyrene-labelled phosphatidylcholine, it was shown that membrane lateral pressure modulates membrane protein folding and function *in vitro* (10, 11). So far, membrane lateral pressure had been commonly determined in membrane vesicles prepared from defined fatty acids and not from bacterial cells. The direct measurement of lateral pressure is not without controversy (11), regardless, membrane lateral pressure a likely candidate as mediator of the effect of environmental stressors on the expression of fructosyltransferases in *L. reuteri*.

4.4 Protective of effects of HoPS and fructooligosaccharides

4.4.1 Impact of HoPS in the presence of membrane active inhibitors.

Expression of both fructosyltransferases Inu and FtfA was induced by environmental stress conditions and resulted in increased formation of levan and FOS. Results obtained in this study further indicated that fructan and FOS increased the tolerance of *L. reuteri* towards membrane-active inhibitors two to tenfold. Taking into account that *L. reuteri* produces $10 - 20$ g L^{-1} (fructan + FOS) at inducing conditions, a protective effect of fructan and FOS at 50 g L^{-1} may be relevant in natural habitats. Dextran also increased resistance of *L reuteri* strains towards membrane active compounds. Protective effects could either be related to the presence of HoPS and FOS, which hamper diffusion of the antimicrobial substances, or by membrane stabilizing effects caused by interactions of FOS and HoPS with the bacterial membrane.

4.4.2 Beneficial effects of fructan and FOS during freeze-drying of *L. reuteri*

To further investigate whether products formed by fructosyltransferases protect *L. reuteri* during adverse environmental conditions, their impact during freeze-drying

was investigated. Freeze-drying was chosen as process relevant for starter culture industry comprising the two factors freezing and drying. The biophysical membrane parameters membrane lateral pressure and membrane fluidity were useful tools to characterize changes within the bacterial membrane (81, section 4.3), hence alterations in membrane lateral pressure and membrane fluidity caused by oligosaccharides and fructans were monitored and related to survival and membrane integrity of *L. reuteri* during freeze-drying.

The impact of sucrose, FOS and inulin during, freezing freeze-drying and atmospheric storage on the viability of *L. reuteri* TMW1.106 was investigated and compared to isotonic solution and reconstituted skim milk. In general, the addition of sugars protected *L. reuteri* TMW1.106 during freezing and freeze-drying compared to isotonic solution. Protective effects of sucrose, FOS and inulin were similar or slightly better than skim milk powder. It had been shown before, that the application of sucrose, trehalose, and maltodextrin resulted in improved cell viability during drying (71, 88). However, results on the effects of sugars on protein secondary structure and membrane phase behaviour were not consistent. In early stationary *E. coli* and *Bacillus thuringensis*, protective effects of sucrose and trehalose had been attributed to stabilization of proteins and decreased membrane phase transition temperature through replacement of water between the lipid headgroups which in consequence prevented phase transition and leakage during rehydration (71), whereas sucrose, skim milk and maltodextrin only had minor effects on protein structure and membrane phase of dried late exponential *L. bulgaricus.*

Membranes are primary targets of freezing and desiccation injury. During freeze-drying of *L. reuteri* TMW1.106, a correlation between survival rates and intact membrane was observed. In the presence of FOS, the highest rate of membrane

integrity corresponded to the highest rate of survival of stationary *L. reuteri* TMW1.106. This observation is in accordance with earlier studies (4, 13), which stated that membrane damage is a catalyst of cell death during drying. Membranes were disrupted during freezing and freeze-drying in stationary and exponential state *L. reuteri* TMW1.106, however, membrane integrity in stationary phase cells remained higher compared to exponentially state cells. In many bacterial species, entry into the stationary phase is accompanied by profound structural and physiological alterations, including changes in expression levels of stress related proteins, membrane composition and cell wall structure that result in increased resistance to heat shock, oxidative, osmotic, acid stresses and high pressure (25, 59, 74, 95). The acquisition of a more resilient cell envelope appeared to be partly accountable for the increased freezing and drying resistance in stationary phase *L. reuteri* TMW1.106.

FOS clearly supported viability of stationary-phase *L. reuteri* TMW1.106. Fructans, but not glucans or gluco-oligosaccharides, directly interact with model mono- and bilayer membranes. Interaction increases with increasing DP of the sugar molecule (54, 137, 139) Using membrane vesicles prepared from *L. reuteri* TMW1.106, it could be shown, that fructans also interacted with complex bacterial membranes. Interaction indeed increased with increasing chain length. Insertion of fructans into the aqueous phase of the membrane (137) led to increased curvature stress as indicated by increased membrane lateral pressure. In contrast, the presence of the disaccharide sucrose or IMO did not affect membrane lateral pressure. The insertion of inulin into the membrane resulted into a shift to a more gel-like state, whereas the membrane became more liquid-crystalline in the presence of FOS compared to control membrane state or in the presence of sucrose suggesting that interaction between fructans and the membrane is optimal at specific polymerization grades of the fructans. This

observation is in contrast to earlier studies reporting that fructans do not influence the liquid-crystalline-gel phase shift under fully hydrated conditions. However, these studies worked with single component model membranes and not with complex bacterial systems (137).

The beneficial effects of fructans during drying have been attributed to their ability to lower the liquid-crystalline-gel phase transition temperature. The membrane is thereby kept in a liquid-crystalline state during dry storage and consequently stabilized during rehydration. Increased membrane fluidity in the presence of FOS may therefore count for conserved viability of stationary *L. reuteri* TMW1.106.

In contrast, inulin and skim milk powder increased stability of exponential phase cells during storage. The effectiveness of a combination of skim milk and inulin in protecting exponential-phase lactobacilli had been reported before (31). Cell loss during storage depended on the degree of sublethal injury after freezing and freeze-drying. Furthermore, decline in viability during storage of freeze-dried lactobacilli has been attributed to further membrane damage caused by oxidation and lipolysis (19, 20). It is possible, that membrane-impairing reactions caused by enzymatical or chemical reactions superimpose beneficial effects achieved by the addition of fructans. Differences in membrane composition (ratio of saturated-unsaturated fatty acids) and consequently differences in susceptibility towards oxidation reactions in exponential and stationary phase cells may thus count for variations in protective effects of additives observed during storage.

Increased expression of fructosyltransferases in *L. reuteri* upon exposure to environmental stressors suggested a role of FOS and fructans in stress tolerance. Accordingly, fructans protected *L. reuteri* TMW1.106 during freezing and drying. Even though concentrations applied succeeded amounts produced by the strain, it is

imaginable that protective effects observed in this study are conferrable to natural drought conditions. Sucrose is ubiquitous, fructosyltransferases are attached to the cell envelope, so fructans formed are in close proximity to the bacterial cell membrane, which may favour interaction.

4.5   Ecological significance of glycosyltransferases

Sourdough is an ecological habitat offering lactobacilli conditions, which they also encounter in the murine forestomach: varying pH values, anaerobic atmosphere, a competitive microflora and the availability of carbohydrates derived from the degradation of starch through amylases. Carbohydrates detected within sourdough mainly comprised mainly maltose, glucose, fructose. The stomach contents of RLF mice fed with commercially available rat chow offered a similar carbohydrate spectrum: glucose, maltose, maltotriose and traces of raffinose and sucrose (data not completely shown and Table 10). The two habitats differ in a way that sourdough is a static ecosystem, whereas lactobacilli colonizing the forestomach of mice encounter mechanical movements and therefore need instruments to adhere and colonize. *L. reuteri* LTH5448 and TMW1.106 originating from sourdough colonized RLF-mice the forestomach epithelium at levels comparable to a rodent isolate and produced lactic acid from the available maltose and glucose (37). Glycosyltransferase expression in the forestomach of RLF-mice was monitored as an indicator to foretell whether glycosyltransferases are ecologically significant in this niche.

4.5.1   Expression of *ftfA, gtfA* and *inu* in vivo

Genes, which are expressed in a niche, are most likely ecologically significant genes as they positively contribute to a specific phenotype (70, 98). In the forestomach of

RLF-mice, *L. reuteri* TMW1.106 and LTH5448 expressed *gtfA* and *ftfA* at detectable levels*,* respectively, implying an ecological function. *Inu* was either not expressed or expression was below detection level.

The murine forestomach tract constitutes heterogeneous environments in which colonizing bacteria grow at different rates presumably due to nutrient availability and depth related conditions created within the colony. To show that glycosyltransferases were regulated by in vivo conditions and a not a consequence of stationary-like conditions prevailing within the biofilm, expression was related to exponentially and stationary phase cells grown with glucose as carbohydrate source. Relative expression ratios of *ftfA* were independent of in vitro growth state and in vivo location and rather dependent on the host animal. In contrast, expression of *gtfA* in *L. reuteri* TMW1.106 isolated from the luminal contents of five mice was upregulated (yet not significantly ($p > 0.05$), indiating a function of the enzyme in colonization of the forestomach of RLF-mice in accordance with regulation of GtfC in *S. mutans*, which is induced during growth in biofilm cultures compared to planktonic cultures (97).

Expression of *gtfA* was constitutive in laboratory media, upregulation of *inu* and *ftfA* in vitro has been correlated to the exposure of *L. reuteri* LTH5448 to environmental stresses. The observation that expression of *ftfA* was not increased in vivo and expression of *inu* was below detection level suggested that *L. reuteri* LTH5448 and TMW1.106 did not encounter stress conditions in the forestomach or lumen of RLF-mice.

Sucrose levels in the feed were low, and neither FOS and fructan nor GOS and glucans could be observed in stomach contents from RLF-mice colonized by *L. reuteri* TMW1.106 and its mutants. Nevertheless, when sucrose becomes available, especially the in vivo formation of FOS and levan through the sucrose inducible *ftfA*

seems possible in *L. reuteri* LTH5448. In *Streptococcus mutans,* induction of Ftf by sucrose could also be observed in vivo in rats. (43).

4.5.2 Performance of insertional mutants in mice

The in vivo expression of *gtfA* and *ftfA* in the forestomach of RLF-mice suggested that both glycosyltransferases contribute to the ecological fitness of *L. reuteri* TMW1.106 and LTH5448, respectively (70, 98). Jens Walter, University of Otago, New Zealand, tested performance of insertional *inu* and *gtfA,* and *ftfA* mutants in RLF-mice alone and in competition with the parent strains. Glucose grown parent and mutant strains were applied at similar inoculum levels. Cell counts in feces were determined after seven days of inoculation. Mice were sacrificed after 14 days of colonization, and lactobacilli counts in the forestomach and cecum were determined. When inoculated alone, *L. reuteri* TMW1.106, *L. reuteri* LTH5448 and the respective mutants colonized the forestomach in equal measure indicated by similar cell counts recovered from feces, forestomach and cecum after 7 days of colonization (data not shown). Population data of competitive colonization is summarized in Figure 24. Deletion of *gtfA* or *ftfA* did not affect the competitiveness of *L. reuteri* TMW1.106 and LTH5448, respectively. In average, the *ftfA* mutant constituted 39% of the total lactobacilli population detected in the feces, and 69% in forestomach and cecum. The *gtfA* mutant represented in average 35% of the total lactobacilli population in feces, and 47 and 41% of total lactobacilli population in forestomach and cecum, respectively. In contrast, the *inu* mutant constituted less than 12% of the total lactobacilli population after 7 and 14 days of colonization, indicating that, although expression was not proven using real-time PCR, the Inulosucrase was ecological significant.

GtfA and *ftfA* were expressed in vivo, yet, competitive performance of insertional mutants was not affected. Insertional mutagenesis is not in every case considered the

appropriate tool for characterization of ecological performance. In many environments ecological fitness of organisms is not determined by one factor alone and rather results from complex interactions of various gene products. Inactivation of genes with great impact on performance might not result in impaired phenotypes (98). Nevertheless, when tested in competition with the parent strains, the effects of inactivation of the genes of interest on ecological performance can be disclosed. In this study, the inactivation of *inu* resulted in reduced competitive fitness of *L. reuteri* TMW1.106.

In vitro, gowth of the *inu* mutant was impaired when grown in laboratory media containing only sucrose as carbohydrate source. The rat chow feeded to RLF-mice contained mainly maltose and only traces of sucrose, therefore carbohydrate limitation was not considered the reason for hampered competitive fitness of *inu* mutant in vivo. The frequent observation of HoPS formation in lactobacilli isolated from the gastrointestinal regions, and the isolation of related genes together with the observation, that carbohydrates and proteins are involved in in vitro adherence to ephitelial cells led to the hypothesis that glycosyltransferases may take part in biofilm formation to the epithelium of the forestomach (118, 124). The in vivo expression of *ftfA* and *gtfA* implied the potential formation of HoPS from sucrose available in rat chow in close proximity to the cell wall. In *S. mutans,* biofilm formation on oral tooth faces has been attributed to the combined action of glycosyltransferases, glucans formed by glucosyltransferases, and glucan-binding proteins, whereas the inulin-like fructans formed by Ftf are considered extracellular carbohydrate reserve within the dental plaque (5, 42). The fructosyltransferase of *S. mutans* showed great affinity for glucans and was involved in sucrose dependent adherence and biofilm formation (101). *L. reuteri* TMW1.106 harboured the glucosyltransferase GtfA and the

inulosucrase Inu. An adherence scenario in analogy to the sucrose-glycosyltransferase triggered adherence of *S. mutans* seemed plausible: The *gtfA* mutant lost the ability to synthesize glucans, but could attach itself to the glucans formed by the parent strain via the membrane anchored fructosyltransferase Inu. Colonization of *inu* mutant was hindered, because the strain lacked the ability to adhere through its inulosucrase. It is unlikely that the of overexpression of GtfA in the *inu* mutant observed in vitro affected performance of the strain in vivo, as the mutant was not hindered while colonizing without competition. Further studies testing the affinity of the inulosucrase for glucans need to be conducted to manifest this hypothesis.

The results obtained in this study also indicated, that glycosyltransferases were not the only factor determining in vivo performance of *L. reuteri* strains. When grown alone, the mutants lacked the competition of other lactobacilli and therefore colonized the forestomach of RLF-mice comparably to the parent strain. Even though the *inu* mutant consituted only a small proportion of the entire lactobacilli population, the strain was still detectable even after two weeks of colonization. *L. reuteri* LTH5448 apparently harboured only one fructosyltransferase FtfA, which was structurally highly similar to Inu. Inactivation of *ftfA* had no impact on competitive performance of *L. reuteri* LTH5448.
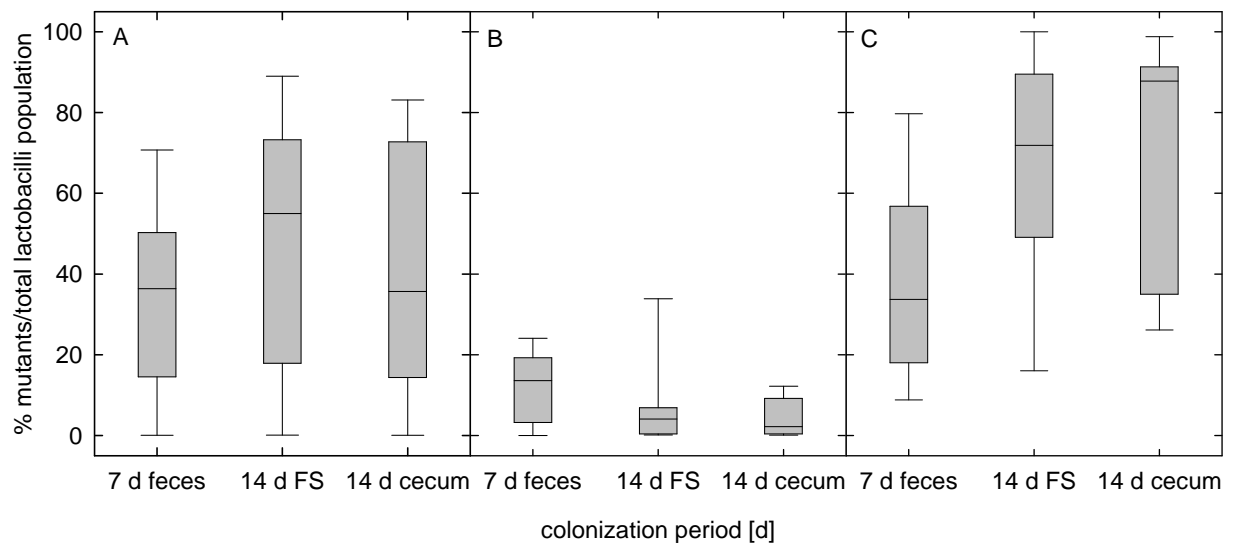
Figure 24. Proportion of of *gtfA* (A), *inu* (B), and *ftfA* (C) mutants of *L. reuteri* TMW1.106 (9 mice) and LTH5448 (11 mice) in total lactobacilli population in the gastro-intestinal tract of RLF-mice. RLF-mice were inoculated with equal amounts of parent and mutant *L. reuteri*. Lactobacilli were recovered from the feces 7 days after inoculation. Mice were killed after 14 days of colonization, and lactobacilli were isolated from the forestomach and the cecum. Cell counts were obtained from MRS plates prepared with our without 5 μM erythromycin. Shown are the median, the 25 and 75% percentile (box) and the 5 and 95% percentile (whiskers). Data was collected and provided by Jens Walter, University of Otago, Dunedin, New Zealand.

4.5.3 Performance of glycosyltransferase mutants in sourdough

L. reuteri TMW1.106 and LTH5448 both originate from commercially available sourdough, thus performance of glycosyltransferase mutants was also tested in the strains' original habitate. When inoculated alone, growth rate and final cell counts of mutants and parent strains were similar. Inactivation of the fructosyltransferases *ftfA*

and *inu* in *L. reuteri* LTH5448 and TMW1.106 resulted in cell counts lower than the parent strains when grown in competition, whereas the *gtfA* mutant was not affected.

During sourdough fermentation, the activity of α- and β-amylases of the flour releases maltose from starch. Maltose-phosphorylase is the key enzyme determining carbohydrate metabolism of heterofermentative lactobacilli during fermentation (reviewed in 38). As sucrose constitutes only about 1% of total carbohdrate in wheat and rye flour, it is unlikely that the impaired sucrose utilization observed during studies in broth culture was responsible for reduced competitiveness of the *inu* mutant.

The *inu* mutant still harboured *gtfA* and was yet impaired when grown in competition with *L. reuteri* TMW1.106 in sourdough and RLF-mice. In sourdough as well as in the forestomach of RLF-mice, the amount of lactate formed by the mutant was lower than in the wild-type strain when inoculated alone even though the *inu* mutant and *L. reuteri* TMW1.106 grew to similar cell counts. The decreased ability to produce lactic acid, maybe even decreased tolerance towards lactic acid/low pH might partly account for hindered performance of the *inu* mutant in vivo and in sourdough in competition.

HoPS formation has been frequently detected in sourdough isolates of *Lactobacillus* (127). The predominant lactobacilli of typeI and typeII sourdough, for e.g. *L. sanfranciscensis* and *L. panis, L. pontis, L. frumenti* as well as *L. johnsonii* were all identified as fructan producers, (34, 78, 127, for a review 124). Strains of *L. reuteri* synthesized glucan or fructan (this study, 127). Glucan, fructan and 1-kestose formation during sourdough fermentations in the presence of 9 and 12% sucrose has been shown (125, 127). In *L. reuteri* LTH5448, FtfA was the only enzyme responsible for the formation of FOS and levan. Inactivation of *ftfA* decreased competitive fitness,

implying a function of either the enzyme or the products formed for *L. reuteri* LTH5448 during sourdough fermentation.

Taking into account the the in vitro observations of interactions between FOS or fructan with the bacterial membrane, which resulted in increased membrane stability during the membrane disrupting processes, a beneficial role of the products synthesized by fructosyltransferases during might be proposed. The sucrose content of wheat sourdough might be sufficient for the formation of FOS and fructans in close proximity to the cell membrane, thus stabilizing latter during acidification during fermentation. Glucosyltransferase activity and the hydrolysis activity of fructosyltransferases liberate fructose and enables the formation of mannitol and acetate and consequently the gain of an additional mol ATP (111). This energy gain might result in competitive advantage for strains possessing functional glycoslyltransferases, especially in doughs with sucrose content higher than wheat sourdough, where the activity of wheat invertases is enough to hydrolyse the available sucrose.

5 SUMMARY

The frequent occurrence of homopolysaccharides (HoPS) formation from sucrose and the presence of one or more glycosyltransferase genes within the genomes of *L. reuteri* gastrointestinal and cereal raised the question on the regulation and function of these enzymes and their HoPS. Results obtained in this work with glycosyltransferases in two *L. reuteri* strains indicate a role of fructosyltransferases in sucrose metabolism, stress tolerance and competitiveness of *L. reuteri* in cereal and intestinal environments.

L. reuteri TMW1.106 harboured the glucosyltransferase GtfA and the inulosucrase Inu. *L. reuteri* possessed the levansucrase FtfA Regulation of glycosyltransferase genes in response to sucrose was strain dependent. Deletion of the sucrose inducible *ftfA* of *L. reuteri* LTH5448 had no impact on growth in sucrose containing MRS (sucMRS). In contrast, deletion of either *inu* or *gtfA* of *L. reuteri* TMW1.106, whose expression was sucrose independent, impaired growth of the respective mutants in sucMRS, indicating the combined activity of both enzymes in sucrose utilization. This observation was supported by the observation that the *inu* mutant constitutively overexpressed GtfA, yet failed to utilize sucrose comparably to *L. reuteri* TMW1.106. Expression of glycosyltransferase genes was further regulated by environmental stresses. Overexpression of the fructosyltransferases FtfA and Inu was observed in each strain of *L. reuteri* upon exposure to elevated temperatures, proton ionophores and phenylethanol. As fructosyltransferase-inducing conditions also increased membrane lateral pressure, it was hypothesized, that the membrane acts as environmental sensor and signal transmitter fructosyltransferase gene expression. The induction of fructosyltransferases in response to environmental stress strongly supported a role of fructooligosaccharides (FOS) and fructans in stress resistance of *L.*

reuteri. Accordingly, the presence of FOS and HoPS increased survival of *L. reuteri* in the presence of antimicrobial substances and during freeze-drying. Protective effects of FOS during freeze-drying were related to interactions between FOS with the bacterial membrane, which resulted in increased membrane integrity.

L. reuteri LTH5448 and TMW1.106 both originated from industrial sourdough and colonized the forestomach epithelium of reconstituted lactobacilli-free mice (RLF-mice). Deletion of fructosyltransferases *inu* and *ftfA* reduced competitiveness of *L. reuteri* TMW1.106 of LTH5448, respectively, during growth in sourdough indicating a role of fructosyltransferases during sourdough fermentation which can only be rudimentary explained with the gain of additional ATP through the hydrolysis activity of glucosyltransferases and the consequent formation of mannitol and acetate may account or beneficial effects caused by the formation of HoPS. In the forestomach of RLF-mice*, L. reuteri* LTH5448 and TMW1.106 expressed *ftfA* and *gtfA*, yet, inactivation of the respective genes had no affect on competitiveness of the strains. Only inactivation of *inu* impaired competitive growth of *L. reuteri* TMW1.106. Yet, despite the high structural similarity of fructosyltransferases Inu and FtfA, the varying impact they had on the performance of *L. reuteri* TMW1.106 and LTH5448, respectively, in different environments showed that factors exceeding structure-function relationship determine the functionality in vivo. The glucosyltransferase *gtfA* of *L. reuteri* TMW1.106 was not regulated by sucrose, environmental stress, and *gtfA* mutants were as competitive as *L. reuteri* TMW1.106

6 ZUSAMMENFASSUNG

Zahlreiche aus Getreidefermentationen oder dem Vertrauungstrakt von Schweinen und Enten isolierte *Lactobacillus reuteri* Stämme bilden Homopolysaccharide aus Saccharose und besitzen eines oder mehrere Glykosyltransferase-Gene. Diese Beobachtungen begründeten die Untersuchungen zur Regulation und Funktion von Glykosyltransferasen in zwei *L. reuteri*-Stämmen. Die Ergebnisse dieser Arbeit deuten an, das Fruktosyltransferasen am Saccharosestoffwechsel, an der Stresstoleranz und der Wettbewerbsfähigkeit von *L. reuteri* im Teig und im Gastrointestinaltrakt beteiligt sind.

L. reuteri TMW1.106 besaß die Glukosyltransferase GtfA und die Inulosucrase Inu. *L. reuteri* LTH5448 wies die Levansucrase FtfA auf. Die Regulation der Glykosyltransferasen in Abhängigkeit von Saccharose war stammabhängig. Die Deletion der Saccharose induzierbaren *ftfA* beeinflusste das Wachstum von *L. reuteri* LTH5448 in mMRS mit Saccharose als einzige Kohlenstoffquelle nicht. Im Gegensatz dazu verschlechterte die Inaktivierung der konstitutiv exprimierten *inu* und *gtfA* das Wachstum von *L. reuteri* TMW1.106, was den Schluss zulässt, dass Inu und GtfA in der Saccharoseverwertung zusammen wirken. Diese Folgerung wurde unterstützt durch die Tatsache, dass auch die Überexpression von GtfA die Inaktivierung von *inu* nicht ausgleichen konnte.

Die Expression der Glykosyltransferasen wurde außerdem von Umwelteinflüssen bestimmt. Temperaturerhöhung und die Gegenwart von Protonenionophoren oder Phenylethanol führten zur erhöhten Expression von *inu* und *gtfA*. Bedingungen, welche die Überexpression der Fruktosyltransferasen in den beiden *L. reuteri* Stämmen auslösten, bewirkten gleichzeitig eine Erhöhung des Lateraldruckes in der bakteriellen Membran. Es wurde daher die Hypothese aufgestellt, dass die Membran

als Umweltsensor und Signalleiter bei der Regulierung der Fruktosyltransferasen in *L. reuteri* dient. Die Induktion der Fruktosyltransferasen als Antwort auf Umweltstressbedingungen unterstützte die Annahme, dass Fruktooligosaccharide (FOS) und Fruktane die Stressresistenz von *L. reuteri* beeinflussen. Im Einklang dazu verbesserten FOS und HoPS das Überleben von *L. reuteri* in Gegenwart von antimikrobiellen Substanzen und während der Gefriertrocknung. Die Schutzwirkung der FOS während der Gefriertrocknung begründete sich in Wechselwirkungen zwischen FOS und der bakteriellen Membran, welche die Erhaltung der Membranintegrität verbesserten.

L. reuteri LTH5448 und TMW1.106 wurden beide aus industriellem TypII-Sauerteig isoliert und kolonisierten die Vormägen von rekonstituierten *Lactobacillus*-freien Mäusen (RLF-Mäuse). Die Inaktivierung von *inu* und *ftfA* reduzierte die Wettbewerbsfähigkeit der beiden Stämme im Sauerteig, was auf eine Funktion der Glykosyltransferasen während der Sauerteigfermentation hinweist. Die Rolle der Fruktsyltransferasen im Teig konnte nur ansatzweise mit dem Gewinn von zusätzlich ATP aufgrund der Aktivität der Glykosyltransferase und der daraus resultierenden Bildung von Mannitol und Acetat, oder durch die Bildung von HoPS und daraus entstehende Schutzfunktionen erklärt werden. In den Vormägen von RLF-Mäusen exprimierten *L. reuteri* LTH5448 und TMW1.106 *ftfA* und *gtfA*. Die Inaktivierung der Gene beeinflusste nicht die Wettbewerbskraft der beiden Stämme. Die Expression von *inu* konnte nicht nachgewiesen werden. Dennoch verminderte die Inaktivierung des Genes die Wettbewerbskraft von *L. reuteri* TMW1.106 in vivo. Trotz struktureller Homologien von Inu und FtfA variierte die Bedeutung der Fruktosyltransferasen für die jeweiligen Stämme in verschiedenen Habitaten. Diese Beobachtung zeigt, dass die Struktur alleine nicht die in vivo Funktionalität bestimmt. Die Expression der

Glucosyltransferase GtfA von *L. reuteri* TMW1.106 wurde nicht durch Saccharose oder Umwelteinflüsse reguliert. Die Inaktivierung von GtfA beeinflusste das Wachstumsvermögen von *L. reuteri* TMW1.106 weder in Sauerteig noch in Mausvormägen.

7 REFERENCES

1. Abriouel, H., Ben Omar, N., Lucas López, R., Martínez-Cañamero, Keleke, S., and A. Gálvez (2006). Culture-independent analysis of the microbial composition of the African traditional fermented foods poto poto and dégué by using three different DNA extraction methods. Int. J. Food Microbiol. E-paper ahead of publishing

2. Ahmed, S., and I.R. Booth (1983). The use of valinomycin, nigericin and trichlorocarbanilide in control of the protonmotive force in *Escherichia coli* cells. Biochem. J. 212: 105-112.

3. Albanesi, D., Mansilla, M.C., and D. de Mendoza (2004). The membrane fluidity sensor DesK of *Bacillus subtilis* controls the signal decay of its cognate response regulator. J. Bacteriol. 186: 2655-2663.

4. Ananta, E., Volkert, M., and D. Knorr (2005). Cellular injuries and storage stability of spray-dried *Lactobacillus rhamnosus* GG. Int. Dairy J. 15: 399-409

5. Banas, J.A., and M.M. Vickerman (2003). Glucan-binding proteins of the oral strepcocci. Crit. Rev. Oral Biol. Med. 14: 89-99.

6. Barrow, K.D., Collins, J.G. Rogers, P.L., and G.M. Smith (1984). The structure of a novel polysaccharide isolated from *Zymomonas mobilis* determined by nuclear magnetic resonance spectroscopy. Eur. J. Biochem. 145: 173-179

7. Bell, L.N., and M.J. Hagemann (1996). Glass transition explanation for the effect of polyhydroxy compounds on protein denaturation in dehydrated solids. J. Food Sci. 61 (2) 372-374, 378

8. Beney, L., and P. Gervais (2001). Influence of the fluidity of the membrane on the response of microorganisms to environmental stresses. Appl. Microbiol. Biotechnol. 57: 34-42.

9. Böcker, G., Stolz, P. and W.P. Hammes (1995). Neue Erkenntnisse zum Okosystem Sauerteig und zur Physiologie der sauerteigtypischen Staemme *Lactobacillus sanfrancisco* und *Lactobacillus pontis.* Getreide Mehl Brot 49: 370-374

10. Booth, P.J., Riley, M.L., Flitsch, S.L., Templer, R.H., Farooq, A., Curran, A.R., Chadborn, N., and P. Wright (1997). Evidence that bilayer bending rigidity affects membrane protein folding. Biochem. 36: 197-203.

11. Booth, P.J., Templer, R.H., Meijberg, W., Allen, S.J., Curran, A.R., Lorch, M. (2001). In vitro studies of membrane protein folding. Crit. Rev. Biochem. Mol. Biol. 36: 501-603.

12. Boris, S., Suarez, J.E., Vazquez, F., and C. Barbes (1998). Adherence of human vaginal lactobacilli to vaginal epithelial cells and interaction with uropathogens. Infect. Immun. 66 (5): 1985-1989

13. Brennan, M., Wanismail, B., Johnson, M.C., and B. Ray (1986). Cellular damage in dried *Lactobacillus acidophilus.* J. Food Prot. 49: 47-53

14. Bron, P.M., Marco, M., Hoffer, S.M., Van Mullekom, E., De Vos, M.W., and M. Kleerebezem (2004). Genetic characterization of the bile salt response in *Lactobacilus plantarum* and analysis of responsive promoters in vitro and in situ in the gastriontestinal tract. J. Bacteriol. 186: 7829-7835.

15. Brooker, B.E., and R. Fuller (1975). Adhesion of lactobacilli to the chicken crop epithelium. J. Ultrastruct. Res. 52 (1) 21-31

16. Browngardt, C.M., Wen, Z.R., and R.A. Burne (2004). RegM is required for optimal fructosyltransferase and glucosyltransferase expression in *Streptococcus mutans*. FEMS Microbiol. Lett. 240: 75-79.

17. Burkhart, B.M., Gassman, R.M., Langs, D.A., Pangborn, W.A., Duax, W.L., and V. Pletnev (1999). Gramicidin D conformation, dynamics and membrane ion transport. Biopolymers 51: 129-144.

18. Carvalho, A.S., Silva, J., Ho, P., Teixeira, P.M., Malcata, X.F., and P. Gibbs (2004). Relevant factors for the preparation of freeze-dried lactic acid bacteria. Int. Dairy J. 14: 835-847

19. Castro, H.P., Teixeira, P.M., and R. Kirby (1996). Changes in the cell membrane of *Lactobacillus bulgaricus* during storage following freeze-drying. Biotechnol. Letters 18 (1) 99-104

20. Castro, H.P., Teixeira, P.M., and R. Kirby (1997). Evidence of membrane damage in *Lactobacillus bulgaricus* during storage following freeze-drying. J. Appl. Microbiol. 82: 87-94

21. Champagne, C.P., Gardner, N., Brochu, E., and Y. Beaulieu (1991). The freeze-drying of lactic acid bacteria. A review. Can. Inst. Sci. Technol. J. 24: 118-128

22. Choi, H.J., Cheigh, C.I., Kim, S.B., Lee, J.C., Lee, D.W., Choi, S.W., Park, J.M., and Y.R. Pyun (2002). *Weissella kimchi* sp. *nov.*, a novel lactic acid bacterium from kimchi. Int. J. Syst. Evol. Microbiol. 52: 507-511

23. Chung, C.H., and D.F. Day (2002). Glucooligosaccharides from *Leuconostoc mesenteroides* B-742 (ATCC 13146): A potential prebiotic. J. Ind. Microbiol. Biotechnol. 29: 196-199

24. Corcoran B.M., Ross, R.P., Fitzgerald, G.F., and C. Stanton (2004). Comparative survival of probiotic lactobacilli spray-dried in the presence of prebiotic substances. J. Appl. Microbiol. 96: 1024-1039

25. Cronan, J.E. jun. (1968). Phospholipid alterations during growth in *Escherichia coli.* J. Bacteriol. 95 (6) 2054-2061

26. Crow, J.H., Carpenter, J.F., and L.M. Crowe (1973). The role of vitrification in anhydrobiosis. Ann. Rev. Physiol. 60: 73-103

27. Cuezzo de Gines, S., Maldonado, M.C., and G. Font de Valdez (2000). Purification and characterization of invertase from *Lactobacillus reuteri* CRL1100. Curr. Microbiol. 40 (3) 181-184

28. Cvitkovitch, D.G., Li, Y.H., and Ellen, R.P. (2003). Quorum sensing and biofilm formation in streptococcal infections. J. Clin. Invest. 112: 1626-1632

29. Dal Bello, F., and C. Hertel (2006). Oral cavity as natural reservoir for intestinal lactobacilli. Syst. Appl. Microbiol. 29: 69-79

30. De Guilio, B., Orlando, P., Barba, G., Coppola, R., De Rosa, M., Sada, A., De Prisco, P.P., and F. Nazarro (2005). Use of alginate and cryoprotective sugars to improve the viability of lactic acid bacteria after freezing and freeze-drying. World J. Mirobiol. Biotechnol. 21: 739-746

31. Desmond, C., Corcoran, B.M., Coakley, M., Fitzgerald, G.F., Ross, R.P., and C. Stanton (2005). Development of dairy-based functional foods containing probiotics and prebiotics. Austral. J. Dairy Technol. 60 (2) 121-126

32. Desroche, N., Beltramo, C., and J. Guzzo (2005). Determination of an internal control to apply reverse transcription quantitative PCR to study stress response in the lactic acid bacterium *Oenococcus oeni.* J. Microbiol. Meth. 60: 325–333.

33. Dols, M., Chraibi, W., Remaud-Simeon, Lindley, N.D., and P.F. Monsan (1997). Growth and energetics of *Leuconostoc mesenteroides* NRRL B-1299 during metabolism of various sugars and their consequences for dextransucrase production. Appl. Environm. Microbiol. 63: 2159-2165

34. Dubock, P., and R.D. Pridmore (2003). Levansucrase of *Lactobacillus johnsonii.* Europ. Patent EP 1 357 180 A

35. Fujiwara, T., Hashino, T., Oshima, T., and S. Hamada (2002). Differential and quantitative analysis of mRNA expression of glucosyltransferases from *Streptococcus mutans* MT8148. J. Dent. Res. 81 (2) 109-113

36. Fukuskima, K., Ikeda, T., and H.K. Kuramitsu (1992). Expression of *Streptococcus mutans gtf* genes in *Streptococcus milleri.* Infect. Immun. 60: 2815-2822

37. Gänzle, M.G. (2004). Reutericyclin: biological activity, mode of action, and potential applications. Appl. Microbiol. Biotechnol. 64 (3): 326-332

38. Gänzle, M.G. (2005). Physiologie und Biochemie der Mikroflora des Sauerteiges. In Handbuch Sauerteig (Hrsg. M.J. Brandt, and M.G. Gänzle). Behr's Verlag, Hamburg, pp. 109-184

39. Gänzle, M.G., and R.F. Vogel (2002). Contribution of reutericyclin production to the stable persistence of *Lactobacillus reuteri* in an industrial sourdough fermentation. Int. J. Food Microbiol. 80: 31-45

40. Gänzle, M.G., and R.F. Vogel (2003). Studies on the mode of action of reutericyclin. Appl. Environ. Microbiol. 69: 1305-1308.

41. Gänzle, M.G., Holtzel, A., Walter, J., Jung, G., and W.R. Hammes (2000). Characterization of reutericyclin produced by *Lactobacillus reuteri* LTH2584. Appl. Environm. Microbiol. 66: 4325-4333

42. Gibbons, R.J. (1968). Formation and significance of bacterial polysaccharides in caries etiology. Caries Res. 2: 164-171

43. Grey, W.T., Curtiss III, R., and M.C. Hudson (1997). Expression of the *Streptococcus mutans* fructosyltransferase gene within a mammalian host. Infect. Imm. 65 (6) 2488-2490

44. Hamad, S., Böcker, G., Vogel, R.F., and W.P. Hammes (1992). Microbiological and chemical analysis of fermented sorghum dough for Kisra production. Appl. Microbiol. Biotechnol. 37: 728-731

45. Hammes, W.P., and R.F. Vogel (1995). The genus *Lactobacillus. In* The lactic acid bacteria, vol. 2, The genera of lactic acid bacteria (eds. Wood, B.J.B., and W.H. Holzapfel). Blackie Acad. Prof., London, U.K., pp. 19-54

46. Hammes, W.P., Brandt, M.J., Francis, K.L., Rosenheim, J., Seitter, M.F.H., and S.A. Vogelmann (2005). Microbial ecology of cereal fermentations. Trends Food Sci. Techn. 16: 4-11

47. Hammes, W.P., Stolz, P., and Gänzle, M.G. (1996). Metabolism of lactobacilli in traditional sourdoughs. Adv. Food Sci. 18: 176-184

48. Hanada, N., and H.K. Kuramitsu (1989). Isolation and characterization of the *Streptococcus mutans gtfD* gene, coding for primer dependent soluble glucan synthesis. Infect. Immun. 57: 2079-2085

49. Heckl, R.J. (1985). Principles of preserving bacteria by freeze-drying. Dev. Ind. Microbiol. 26:379-395

50. Heng, N.C.K., Bateup, J. M., Loach, D.M. Wu, X., Jenkinson, H.F. Morrison, M., and G.W. Tannock (1999). Influence of different functional elements of plasmid pGT232 on maintenance of recombinant plasmids in *Lactobacillus*

reuteri populations in vitro and in vivo. Appl. Environ. Microbiol. 65**:** 5378-5385

51. Heyer, A.G., Schroeer, B., Wolff, D., Czapla, S., and J. Springer (1998). Structure of the enzymatically synthesized fructan inulin. Carbohydr. Res. 313: 165-174

52. Hidalgo-Morales, M., Robles-Olvera, V., and H.S. Garcia (2005). *Lactobacillus reuteri* beta-galactosidase activity and low milk acidification ability. Can. Microbiol. 51 (3) 261-267

53. Hincha, D.K., Hellwege, E.M., Heyer, A.G., and J.H. Crowe (2000). Plant fructans stabilize phophatidylcholine liposomes during freeze-drying. Eur. J. Biochem. 267: 535-540

54. Hincha, D.K., Zuther, E., Hellwege, E.M., and A.G. Heyer (2002). Specific effects of fructo- and gluco-oligosaccharides in the preservation of liposomes during drying. Glycobiol. 12 (2) 103-110

55. Hiratsuka, K., Wang, B., Sato, Y., and H. Kuramitsu (1998). Regulation of sucrose-6 phosphate hydrolase activity in *Streptococcus mutans*: Characterization of the *scrR* gene. Infect. Immun. 66 (8) 3736-3743

56. Hounhouigan, D.J., Nout, M.J., Nago, C.M., Houben, J.H., and F.M. Rombouts (1993). Characterization and frequenzy distribution of species of lactic acid bacteria involved in the processing of mawe, a fermented maize dough from Benin. Int. J. Food Microbiol. 18 (4) 279-287

57. Killian, J.A., Fabrie, C.H., Baart, W., Morein, S., and B. de Kruijff (1992). Effects of temperature variation and phenylethyl alcohol addition on acyl chain order and lipid organization. Biochim. Biophys. Acta. 603: 63-69.

58. Kim, D.-S., Thomas, S., and H.S. Fogler (2000). Effects of pH and trace minerals on long-term starvation of *Leuconostoc mesenteroides.* Appl. Environm. Microbiol. 66: 976-981

59. Kim, W.S., Perl, L., Park, J.H., Tandianus, J.E., and N.W. Dunn (2001). Assessment of the stress response of the probiotic *Lactobacillus acidophilus.* Curr. Microbiol. 43 (5) 346-350

60. Kiska, D.L., and F.L. Macrina (1994). Genetic regulation of fructosyltransferase in *Streptococcus mutans.* Infect. Immun. 62 (4) 1241-1251

61. Koepsell, H.J., Tsuchiya, H.M., Hellmann, N.N., Kazenka, A., Hoffman, C.A., Sharpe, E.S., and R.W. Jackson (1953). Enzymatic synthesis of dextran acceptor specificity and chain iniation. J. Biol. Chem. 200: 793-810

62. Korakli, M., Pavlovic, M., Gänzle, M.G., and R.F. Vogel (2003). Exopolysaccharide and 1-kestose production by *Lactobacillus sanfranciscensis* LTH2590. Appl. Environ. Microbiol. 69 : 2073-2079.

63. Kralj, S., Stripling, E., Van Geel-Schutten, G.H., Dondorff, M.M.G., Kirsanovs, S., Van der Maarel, M.J.E.C., and L. Dijkhuizen (2004). Highly hydrolytic reuteransucrase from the probiotic *Lactobacillus reuteri* ATCC55730. Appl. Environm. Microbiol. 71: 3942-3950

64. Kralj, S., Van Geel-Schutten, G.H., Dondorff, M.M.G., Kirsanovs, S., Van der Maarel, M.J.E.C., and L. Dijkhuizen (2004a). Glucan synthesis in the genus *Lactobacillus:* isolation and characterization of glucansucrase genes, enzymes and glucan products from six different strains. Microbiol. 150: 3681-3690

65. Kralj, S., Van Geel-Schutten, G.H., Faber, E.J., Van der Maarel, M.J.E.C., and L. Dijkhuizen (2005). Rational transformation of *Lactobacillus reuteri* 121 reuteransucrase into a dextransucrase. Biochem. 44: 9206-9216

66. Kralj, S., Van Geel-Schutten, G.H., Rahaoui, H., Leer, R.J., Faber, E.J., Van der Maarel, M.J.E.C., and L. Dijkhuizen (2002). Molecular characterization of a novel glucosyltransferase from *Lactobacillus reuteri* strain 121 synthesizing a unique, highly branched glucans with α-(1→4) and α-(1→6) glucosidic bonds. Appl. Environm. Microbiol. 62: 4283-4291

67. Kralj, S., Van Geel-Schutten, G.H., Van der Maarel, M.H.E.C., and L. Dijkhuizen (2004b). Biochemical and molecular characterization of *Lactobacillus reuteri* 121 reuteransucrase. Microbiol. 150: 2099-2112.

68. Kurzak, P., Ehrmann, M.A., and R.F. Vogel (1998). Diversity of lactic acid bacteria associated with ducks. System. Appl. Microbiol. 21: 588-592

69. Lee, S.F., Delaney, G.D., and M. Elkhateeb (2004). A two-component *covRS* regulatory system regulates expression of fructosyltransferase and a novel extracellular carbohydrate in *Streptococcus mutans*. Infect. Immun. 72: 3968-3973.

70. Lenski, R.E. (1995). Molecules are more than markers: new directions in molecular microbial ecology. Mol. Ecol. 4: 643-651

71. Leslie, S.B., Israrail, E., Lighthart, B., Crowe, J.H., and L.M. Crowe (1995). Trehalose and sucrose protect both membranes and proteins in intact bacteria during drying. Appl. Environ. Microbiol. 61 (10) 3592-3597

72. Lewington, J., Greenaway, S.D., and B.J. Spillane (1987). Rapid small scale preparation of bacterial genomic DNA, suitable for cloning and hybridization analysis. Lett. Appl. Microbiol. 5: 51-53.

73. Li Y., and R.A. Burne (2001). Regulation of *gtfBC* and *ftf* genes of *Streptococcus mutans* in biofilm in response to pH and carbohydrates. Microbiol. 147: 2841-2848

74. Loghardt, I.M., and H.Y. Neujahr (1975). Lysis of modified walls from *Lactobacillus fermentum*. J. Bacteriol. 124 (1) 73-77

75. Looijestijn, P.F., Trapet, L., Vries, E.E., Abee, T., and J. Hugenholtz (2001). Physiological function of exopolysaccharides produced by *Lactococcus lactis*. Int. J. Food Microbiol. 64: 71-80

76. McAuliffe, O., Ross, R.P., and C. Hill (2001). Lantibiotics: structure, biosynthesis and mode of action. FEMS Microbiol. Rev. 25: 285-308.

77. McNab, R., Ford, S.K., El-Sabaeny, A., Barbieri, B., Cook, G.S., and R.J. Lamont (2003). LuxS-based signaling in *Streptococcus gordonii*: Autoinducer 2 controls carbohydrate metabolism and biofilm formation with *Porphyromonas gingivalis*. J. Bacteriol. 185: 274-284.

78. Meroth, C.B., Walter, J., Hertel, C., Brandt, M.J., and W.P. Hammes (2003). Monitoring the bacterial population dynamics in sourdough fermentation processes by using PCR-denaturing gradient gel electrophoresis. Appl. Environ. Microbiol. 69: 475-82.

79. Merritt, J., Qi, F., Goodman, S.D., Anderson, M.H., and W. Shi (2003) Mutation of *luxS* affects biofilm formation in *Strepcococcus mutans*. Infect. Immun. 71: 1972-1979.

80. Mitsuoka, T. (1992). The human gastrointestinal tract. In: Wood, B.J.B., ed. The Lactic Acid Bacteria, vol. 1. The Lactic Acid Bacteria in Health and Disease. Elsevier Appl. Sci., London, pp. 69-114

81. Molina-Höppner, A., Doster, W., Vogel, R.F. and M.G. Gänzle (2004). Protective effect of sucrose and sodium chloride for *Lactococcus lactis* during sublethal and lethal high pressure treatments. Appl. Environm. Microbiol. 70: 2013-2020

82. Monchois, V., Villemot, R.M., and P. Monsan (1998). Glucansucrases: mechanism of action and structure-function relationships. FEMS Microbiol. Rev. 23: 131-151

83. Monsan, P, Bozonet, S., Albenne, C., Joucla, G., Willemot, R.-M., and M. Remaud-Simeon (2001). Homopolysaccharides from lactic acid bacteria. Int. Dairy J. 11: 675-585

84. Neely, W., and J. Nott (1962). Dextransucrase, an induced enzyme from *Leuconostoc mesenteroides*. Biochem. 1: 1136-1140.

85. Neubauer, H., Bauche, A., and B. Mollet (2003). Molecular characterization and expression analysis of the dextransucrase DsrD of *Leuconostoc mesenteroides* Lcc4 in homologous and heterologous *Lactococcus lactis* cultures. Microbiol. 149: 973-982.

86. Newbrun, E., and S., Baker (1968). Physico-chemical characteristics of the levan produced by *Streptococcus salivarius.* Carbohydr. Res. 6: 165-170

87. Newman, H.N., Donoghue, H.D., and A.B. Britton (1976). Effect of glucose and sucrose on the survival in batch cultures of *Streptococcus mutans* C67-1 and a non-cariogenic mutant C67-25. Morphological studies, Microbios. 15: 113-125

88. Oldenhof, H., Wolkers, W.F., Fonseca, F., Passot, S., and M. Marin (2005). Effect of sucrose and maltodextrin on the physical properties and survival of air-dried *Lactobacillus bulgaricus*: An in situ Fourier Transform Infrared Spectroscopy study. Biotechnol. Prog. 21: 885-892

89. Olivares-Illana, V., Lopez-Munguia, A., and C. Olvera (2003). Molecular characterization of inulosucrase from *Leuconostoc citreum*: a fructosyltransferase within a glucosyltransferase. J. Bacteriol. 185: 3606-3612.

90. Olivares-Illana, V., Rodarte, W.-C., LeBorgne, S., and L.-A. Mungia (2002). Characterization of a cell-associated inulosucrase from a novel source: A *Leuconostoc citreum* strain isolated from Pozol, a fermented corn beverage of Mayan origin. J. Ind. Microbiol. Biotechnol. 28: 112-117

91. Ozimek, L.K., Kralj, S., Van der Maarel, M.J.E.C., and L. Dijkhuizen (2005). The levansucrase and inulosucrase enzymes of *L. reuteri* 121 catalyze processive and non-processive transglycosylation reactions. Microbiol. 152: 1187-1196

92. Pfaffl, M.W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. Nuc. Acids Res. 29: 2002-2007

93. Pfaffl, M.W., Horgan, G.W., and L. Dempfle (2002). Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nuc. Acids. Res. 30 (9) e36

94. Poolman, B., Blount, P., Folgering, J.H.A, and T. Van der Heide (2002). How do membrane proteins sense water stress? Mol. Microbiol. 44: 889-902.

95. Prasad, J., McJarrow, P., and P. Gopal (2003). Heat and Osmotic stress responses of probiotic *Lactobacillus rhamnosus* HN001 (DR20) in relation to viability after drying. Appl. Environm. Microbiol. 69 (2) 917-925

96. Quirasco, M., Lopez-Munguia, A., Remaud-Simeon, M., Monsan, P., and A. Farres (1999). Induction and transcription studies of the dextransucrase gene in *Leuconostoc mesenteroides* NRRL B512-F. Appl. Environm. Microbiol. 65(12) 5504-5509

97. Rathsam, C., Eaton, R.E., Simpson, C.L., Browne, G.V., Valova, V.A., Harty, D.W.S., and N.A. Jaques (2005). Two-dimensional fluorescence difference gel

electrophoretic analysis of *Streptococcus mutans* biofilms. J. Prot. Res. 4: 2161-2173

98. Rediers, H., Rainey P.B., Vanderleyden, J., and R. De Mot (2005). Unraveling the secret lives of bacteria: Use of in vivo expression technology and differential fluorescence induction promoter traps as tool for exploring niche-specific gene expression. Microbiol. Mol. Biol. Rev. 69 (2) 217-261

99. Roos, S., Engstrand, L., and H. Jonsson (2005). *Lactobacillus gastricus* sp. *nov., Lactobacillus antri* sp. *nov.*, *Lactobacillus kalixensis* sp. *nov.* and *Lactobacillus ultunensis* sp. *nov.* isolated from human stomach mucosa. Int. Syst. Evol. Microbiol. 55: 77-82

100.   Rozen, R., Bachrach, M., Bronshteyn, M, Gedalia, I., and D. Steinberg (2001). The role of fructans on dental biofilm formation by *Streptococcus sorbinus, Streptococcus gordonii, Streptococcus mutans,* and *Actinomyces viscosus.* FEMS Microbiol. Lett. 195: 205-210

101.   Rozen, R., Steinberg, D., and G. Bachrach (2004). *Streptococcus mutans* fructosyltransferase interactions with glucans. FEMS Microbiol. Lett. 232 (1) 39-43

102.   Schauder, S., Shokat, K., Surette, M.G., and M.L. Bassler (2001). The LuxS family of bacterial autoinducers: biosynthesis of a novel quorum-sensing signal molecule. Mol. Microbiol. 41: 462-476.

103.   Schroeder, V.A., Michalek, S.M., and F.L. Macrina (1989). Biochemical characterization and evaluation of virulence of a fructosyltransferase-deficient mutant of *Streptococcus mutans* V403. Infect. Immun. 57 (11) 3560-3569

104.   Sherman, L.A., and D.C. Savage (1986). Lipoteichoic acids in *Lactobacillus* strains that colonise the mouse gastric epithelium. Appl. Environm. Microbiol. 52: 302-304

105.   Shimamura, A., Tsumori, H., and H. Mukasa (1983). Three kinds of extracellular glucosyltransferases from *Streptococcus mutans* 6715 (serotype g). FEBS Lett. 157: 79-84

106.   Shiroza, T., and H.K. Kuramitsu (1988). Sequence analysis *Streptococcus mutans* fructosyltransferase gene and flanking regions. J. Bacteriol. 170: 810-816

107.   Shiroza, T., Ueda, S., and H.K. Kuramitsu (1987). Sequence analysis of the *gtfB* gene from *Streptococcus mutans.* J. Bacteriol. 169: 4263-4270

108.   Simpson, W.J., and A. R. W. Smith (1992). Factors affecting antibacterial activity of hop compounds and their derivatives. J. Appl. Bacteriol. 72: 327-334

109.   Smith, M.R., Zahnley, J.C., Wong, R.Y., Lundin, R.E., and J.A. Ahlgren (1998). A mutant strain of *Leuconostoc mesenteroides* B-1355 producing a glucosyltransferase sythesizing α(1,2) glucosidic linkages. J. Ind. Microbiol. Biotechnol. 21: 37-45

110.   Sobel, J.D. (1996). Vaginitis and vaginal flora: controversies abound. Curr. Opin. Infect. Dis. 9: 42-47

111.   Stolz, P., Böcker, G., Hammes W. P. and R.F. Vogel (1995). Utilization of electron acceptors by lactobacilli isolated from sourdough. I. *Lactobacillus sanfrancisco*. Z. Lebensm. Unters. Forsch. 201: 91-96.

112. Stolz, P., Böcker, G., Vogel R.F., and W. P. Hammes (1993). Utilisation of maltose and glucose by lactobacilli isolated from sourdough. FEMS Microbiol. Lett. 109: 237-242

113. Stolz, P., Vogel R.F., and W. P. Hammes (1993). Utilization of electron acceptors by lactobacilli isolated from sourdough. II. *Lactobacillus pontis, L. reuteri, L. amylovorus,* and *L. fermentum.* Z. Lebensm. Unters. Forsch. 201: 401-411

114. Surette, M.G., Miller, M.B., and M.L. Bassler (1999). Quorum sensing in *Escherichia coli, Salmonella typhimurium,* and *Vibrio harveyi*: A new family of genes responsible for autoinducer production. Proc. Nat. Acad. Sci. USA 96: 1639-1644

115. Tannock, G. W., Crichton, C., Welling, G.W., Koopman, J.P., and T. Midtvedt (1988). Reconstitution of the gastrointestinal microflora of lactobacillus-free mice. Appl. Environ. Microbiol. 54: 2971-2975

116. Tannock, G.W. (1992). Lactic microbiota of pigs, mice and rats. *In* The Lactic Acid Bacteri, vol. 1. The Lactic Acid Bacteria in Health and Disease (ed. B.J.B. Wood). Elsevier Appl. Sci., London, pp.21-48

117. Tannock, G.W. (1997) Normal microbiota of the gastrointestinal tract of rodents. In: Mackie, R.I., White, B.A., and R.E. Isaacson., eds. Gastrointestinal Microbiology, vol. II. Chapman and Hall, London, pp. 187-215

118. Tannock, G.W. (2004). A special fondness for lactobacilli. Appl. Environ. Microbiol. 79: 3189-3194.

119.   Tannock, G.W., and R.D. Archibald (1984), The derivation and use of mice, which do not harbour lactobacilli in the gastrointestinal tract. Can. J. Microbiol. 30: 849-853

120.   Tannock, G.W., Munro, K., Harmsen, H.J.M., Welling, G.W., Smart, J., and P.K. Gopal. (2000). Analysis of the faecal microbiota of human subjects consuming a probiotic product containing *Lactobacillus rhamnosus* DR20. Appl. Environm. Microbiol. 66: 2578-2588

121.   Tannock, G.W., Szylit, O., Ducal, Y., and P. Ribaud (1982). Colonisation of tissue surfaces in the gastrointestinal tract of gnotobiotic animals by *Lactobacillus* strains. Can. J. Mirobiol. 28:1196-1198

122.   Templer, R.H., Castle, S.J., Curran, A.R., Rumbles, G., and D. R. Klug (1998). Sensing isothermal changes in the lateral pressure in model membranes using di-pyrenyl phosphatidylcholine. Faraday Discuss. 111: 41-53.

123.   Thiele, C., Gänzle, M.G., and R. F. Vogel (2002). Sample preparation for amino acid determination by integrated pulsed amperometric detection in foods. Anal. Biochem. 310: 171-178.

124.   Tieking, M., and M.G. Gänzle (2004). Exopolysaccharides from cereal-associated lactobacilli. Trends Food Sci. Tech. 16: 79-84

125.   Tieking, M., Ehrmann, M.A., Vogel, R.F., and M.G. Gänzle (2005). Molecular and functional characterization of a levansucrase from the sourdough isolate *Lactobacillus sanfranciscensis* TMW1.392. Appl. Microbiol. Biotechnol. 66: 655-663.

126.   Tieking, M., Kaditzky, S., Valcheva, R., Korakli, M., Vogel, R.F., and M.G. Gänzle (2005). Extracellular homopolysaccharides and oligosaccharides from intestinal lactobacilli. J. Appl. Microbiol. 99: 692-702

127.   Tieking, M., Korakli, M., Ehrmann, M.A., Gänzle, M.G., and R.F. Vogel (2003). In situ production of exopolysaccharides during sourdough fermentation by cereal and intestinal isolates of lactic acid bacteria. Appl. Environ. Microbiol. 69: 945-952.

128.   Tieking, M., Kühnl, W., and M.G. Gänzle (2006). Evidence for formation of heterooligosaccharides by *Lactobacillus sanfranciscensis* during growth in wheat sourdough. J. Agric. Food Chem. 53: 2456-2461

129.   Tungjaroenchai, M., Drake, M.A., and C.H. White (2001). Influence of adjunct cultures on ripening of reduced fat edam cheeses. J. Dairy Sci. 84: 2117-2124

130.   Ueda, S., Shiroza, T., and H.K. Kuramitsu (1988). Sequence analysis of the *gtfC* gene from *Streptococcus mutans* GS-5. Gene 69: 101-109

131.   Van Geel-Schutten, G.H., Faber, E.J., Smit, E., Bonting, K., Smith, M.R., Ten Brink, B., Kamerling, J.P., Vliegenthart, J.F., and L. Dijkhuizen (1999). Biochemical and structural characterization of the glucan and fructan exopolysaccharides synthesized by the *Lactobacillus reuteri* wild-type strain and by mutant strains. Appl. Environ. Microbiol. 65: 3008-3014.

132.   Van Hijum, S.A., Bonting, K., Van der Maarel, M.J.E.C., and L. Dijkhuizen (2001). Purification of a novel fructosyltransferase from *Lactobacillus reuteri* strain 121 and characterization of the levan produced. FEMS Microbiol. Letters, 205: 323-328

133.   Van Hijum, S.A., Szalowska, E., Rabaoui, M.J., Van der Maarel, M.J.E.C., and L. Dijkhuizen (2002). Characterization of a novel fructosyltransferase from *Lactobacillus reuteri* that synthesizes high molecular weight inulin and inulin oligosaccharides. Appl. Environm. 68: 4390-4398

134.   Van Hijum, S.A., Van Geel-Schutten, G.H., Van der Maarel, M.J., and L. Dijkkhuizen (2004). Biochemical and molecular characterization of a levansucrase from *Lactobacillus reuteri*. Microbiol. 150:621-630

135.   Van Hijum, S.A.F.T., Kralj, S., Ozimek, L.K., Dijkhuizen, L., and G.H. van Geel-Schutten (2006). Structure-function relationships of glucansucrase and fructansucrase enzymes from lactic acid bacteria. Microbiol. Mol. Biol. Rev. 70 (1) 157-176

136.   Vasquez, A., Jakobsson, T., Ahrne, S., Forsum, U., and G. Molin (2002). Vaginal *Lactobacillus* flora of healthy Swedish Woman. J. Clin. Microbiol. 40 (8) 2746-2749

137.   Vereyken, I.J., Chupin, V., Demel, R.A., Smeekens, S.C.M., and B. de Kruijff (2001). Fructans insert between the headgroups of phospholipids. Biochim. Biophys. Acta, 1510: 307-320

138.   Vereyken, I.J., Chupin, V., Islamov, A., Kuklin, A., Hincha, D.K., and B. de Kruijff (2003). The effect of fructan on membrane lipid organization and dynamics in the dry state. Biophys. J. 85: 3058-3065.

139.   Vereyken, I.J., van Kuik, J.A., Evers, T.H., Rijken, P.J., and B. de Kruijff (2003). Structural requirements of the fructan-lipid interaction. Biophys. J. 84:3147-3154

140.   Walker, G.J., Cheetham, N.W.H., Taylor, C., Pearce, B.J., and M.E. Slodki (1990). Productivity of four α-D-glucosyltransferases released by

Streptococcus sobrinus under defined conditions in continuous culture. Carb. Polymers 13: 399-421

141.   Walter, J. (2006). The microecology of lactobacilli in the gastrointestinal tract. In: Probiotics and Prebiotics-Scientific Aspects. G.W. Tannock, ed., Caister Academic Press, Norfolk, UK, pp 51-82

142.   Walter, J., Chagnaud, P., Tannock, G.W., Loach, D.M., Dal Bello, F., Jenkinson, H.F., Hammes, W.P., and C. Hertel (2005). A High-Molecular-Mass Surface Protein (Lsp) and Methione Sulfoxide Reductase B (MsrB) contribute to the ecological performance of *Lactobacillus reuteri* in the murine gut. Appl. Environm. Microbiol. 71 (2) 979-986

143.   Walter, J., Heng, N.C.K., Hammes, W.P., Loach, D.M., Tannock, G.W., and C. Hertel (2003). Identification of *Lactobacillus reuteri* genes specifically induced in the mouse gastrointestinal tract. Appl. Environ. Microbiol. 69: 2044-2051.

144.   Walter, J., Hertel, C., Tannock, G.W., Lis, C.M., Munro, K., and W.P. Hammes (2001). Detection of *Lactobacillus*, *Pediococcus*, *Leuconostoc*, and *Weissella* species in human feces by using group-speific PCR primers and denaturing gradient gel electrophoresis. Appl. Environ. Microbiol. 67 (6) 2578-2585

145.   Welch, T.J., and D.H. Bartlett (1998). Identification of a regulatory protein required for pressure-responsive gene expression in the deep-sea bacterium *Photobacterium* species strain SS9. Mol. Microbiol. 27: 977-985.

146.   Welin, J., Wilkens, J.C., Beighton, D., and G. Svensaeter (2004). Protein expression by *Streptococcus mutans* during initial stage of biofilm formation. Appl. Environm. Microbiol. 20 (7) 3736-3741

147.   Wen, Z.R., and R.A. Burne (2004). LuxS-mediated signalling in *Streptococcus mutans* is involved in regulation of acid and oxidative stress tolerance and biofilm formation. J. Bacteriol. 186: 2682-2691.

148.   Winter, R. (2002). Synchrotron X-ray and neutron small-angle scattering of lyotropic lipid mesophases, model biomembranes and protein in solution at high pressure. Biochim. Biophys. Acta 1595: 160-184.

Clarissa Schwab

Geburtsdatum: 15.12.1977
Geburtsort: Hanau

Ausbildung und Abschlüsse

1984 - 1988	Grundschule Kahl
1988 - 1997	Karl Rehbein Schule Hanau
1997	Allgemeine Hochschulreife
1997-2003	Studium der Technologie und Biotechnologie der Lebensmittel, Technische Universität München
2003	Diplom Ingenieur der Technologie und Biotechnologie der Lebensmittel
Seit Mai 2003	Promotion am Lehrstuhl für Technische Mikrobiolgie, Technische Universität München
Seit September 2005	Beschäftigt an University of Alberta, Edmonton, Canada

Studienbezogene Praktika

Döhler Darmstadt, Eurocitrus

New Zealand Dairy Board (jetzt Fonterra), Rehlingen

Flensburger Brauerei, Flensburg

Teilnahme an Austauschprogrammen

Food Atlantic	University of Alberta, Edmonton Canada
Sokrates	Agricultural University of Norway, Aas, Norwegen
DAAD Kurzstipendium	University of Otago, Dunedin, Neuseeland

Beschäftigung als studentische Hilfkraft

Lehrstuhl für Lebensmittelverfahrenstechnik und Milchwissenschaften, TU München

Lehrstuhl für Technische Mikrobiologie, TU München

Original papers resulting from this thesis

Schwab, C., and M.G. Gänzle (2006). Effect of membrane lateral pressure on the expression of fructosyltransferases in *Lactobacillus reuteri.* Syst. Appl. Microbiol. 29: 89-99

Gänzle, M.G., and **C. Schwab** (2005). Exopolysaccharide production by intestinal lactobacilli. *In* Probiotics and Prebiotics: Scientific Aspects (ed. G. W. Tannock), Caister Academic Press, New Zealand.