Microbial ecology of food fermentations and intestinal ecosystems

by

Xiaoxi B Lin

A thesis submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

in

Food Science and Technology

Department of Agricultural, Food and Nutritional Science
University of Alberta

Abstract

Microbiota are ubiquitous in nature. Similarities as well as differences are present between microbiota in animals and fermentation systems. The aim of the PhD project was to investigate factors affects microbial ecology in rodent and sourdough models.

To determine how compromised health of the host by tumor and CPT-11 therapies affects intestinal microbiota in tumor bearing rats, qualitative and quantitative taxonomic analyses were combined to characterize intestinal microbiota during CPT-11-based chemotherapy. Both tumor and CPT-11 chemotherapy increased cecal *Clostridium* cluster XI and Enterobacteriaceae. The effect of dietary fibre was evaluated in the same model. Cecal butyrate concentrations and feed intake were highly correlated. Moreover, a positive correlation of the host expression of MCT1 with body weight as well as a positive correlation of the abundance of bacterial butyryl-CoA gene with cecal butyrate concentrations were obeserved. These correlations support the interpretation that the influence of dietary fibre on CPT-11 toxicity is partially mediated by an increased cecal production of butyrate.

Similar to intestinal microbiota, in sourdough, cereal substrates contain various compounds that may have selective effect on sourdough microbiota. The effect of growth rate and acid resistance on microbial competitiveness in sourdoughs was evaluated by assessing competitiveness of glycerol-dehydratase (*gupCDE*) positive and glutamate-decarboxylase (*gadB*) positive strains of *L. reuteri* relative to the corresponding null mutants. Both glycerol and glutamate metabolism determine the competitiveness of *L. reuteri* in sourdough fermentations. Besides competition over shared energy source, microbes also compete with each other by producing antimicrobial compounds. Reutericyclin is an antibiotic produced by sourdough-

originated *L. reuteri* which is bactericidal against most gram-positive bacteria. A combination comparative genomics, bioinformatics analysis, and the characterization of null-mutants was used to determine the genetic determinants of reutericyclin biosynthesis. A gene cluster unique to reutericyclin producers was identified on a genomic island acquired through lateral gene transfer. It includes genes coding for a nonribosomal peptide synthetase (NRPS), a polyketide synthase (PKS), homologues of *phlABC,* and putative transport and regulatory proteins. The combination of PhlABC homologues with both a NRPS and PKS is exclusive to the lactic acid bacteria *Streptococcus mutans*, *L. plantarum* and *L. reuteri*, indicating that the genes in these organisms share a common evolutionary origin.

Preface

This thesis is an original work by Xiaoxi Lin.

Part of Chapters 1 and 7 has been included in the discussion of the submitted manuscript Zheng J, Gänzle MG, Lin XB, RuanL, Sun M. Diversity and dynamics of bacteriocins from human microbiome.

Chapter 2 has been published as Lin XB, Dieleman LA, Ketabi A, Bibova I, Sawyer MB, Xue H, Field CJ, Baracos VE, Gänzle MG (2012) Irinotecan (CPT-11) chemotherapy alters intestinal microbiota in tumour bearing rats. PLoS One 7: e39764. The study was designed by Drs Dieleman, Field, Sawyer, Baracos, and Gänzle. Animal experiments were designed and executed before I was recruited to the study. DGGE analysis was performed by Dr. Ketabi and Ms Bibova. I compiled and analysed the experimental data and wrote the manuscript together with Drs. Gänzle and Baracos.

Chapter 3 has been published as Lin XB, Farhangfar A, Valcheva R, Sawyer MB, Dieleman L, Schieber A, Gänzle MG, Baracos VE (2014) The role of intestinal microbiota in development of irinotecan toxicity and in toxicity reduction through dietary fibres in rats. PLoS One 9: e83644. The study was designed by Drs Sawyer, Gänzle, and Baracos. Dr Valcheva measured the expression of mucin genes and provided procotols for RNA extraction and reverse transcription. Mr Farhangfar measured the level of blood haptoglobin and glycoprotein. I compiled and analysed the experimental data and wrote the manuscript together with Drs. Gänzle and Baracos.

Chapter 4 has been published as Lin XB, Gänzle MG (2014) Quantitative high-resolution melting PCR analysis for monitoring of fermentation microbiota in sourdough. Int J Food Microbiol 186C: 42–48. I wrote the manuscript with input from the co-author and acted as corresponding author.

Chapter 5 has been published as Lin XB, Gänzle MG (2014) Effect of lineage-specific metabolic traits of *Lactobacillus reuteri* on sourdough microbial ecology. Appl Environ Microbiol 80:5782-9.

The study in Chapter 6 was designed by Drs Gänzle and Walter. Ms Duar coordinated genomic sequencing and assembly. Dr Jens performed comparative genomic analysis. Mr Lohans performed mass spectrometry analysis and participated in gene analysis. Mr Lohans and Dr Vederas proposed biosynthetic pathway of reutericyclin. Dr Zheng was responsible for constructing phylogenetic trees of reutericyclin biosythetic genes. I wrote the manuscript together with Drs. Walter and Gänzle. The manuscript has been submitted as Lin XB, Lohans CT, Duar R, Zheng J, Vederas J, Walter J, Gänzle MG. Genetic determinants of reutericyclin biosynthesis in *Lactobacillus reuteri*: a pathway to antibiotic synthesis that is unique to lactic acid bacteria?

Acknowledgement

I would like to express my sincere gratitude to my supervisor Dr Michael Gaenzle, for sparkling my interest in Food Microbiology, taking me as a student, and guiding me with patience and encouragement every step along the way. I am also extremely grateful to Dr Lynn McMullen for her support and advice throughout my undergraduate and graduate studies, to Dr Vickie Baracos for the opportunity to work on her animal projects and for introducing me to the fields of Nutrition and Medicine, to Drs Jens Walter and John Vederas for collaboration on my last project, which would have never been possible without their expertise, thank you.

I would like to thank my my past and present colleagues: Drs Marica Su, Rosica Valcheva, and Ryan Mercer for teaching me in and outside the lab. Drs Petya Koleva, Sandra Galle, Christine Liu, Yvonne Wang, Fernanda Sanchez Maldonado, Brenna Black, Januana Texiera, Bonno Sekwati-Monang, Clarissa Schwab, Simon Hofstetter, Petr Miller, Christina Engels, Ali Ketab; Mandi Hoke, Aaron Pleitner, Rebbeca Duar, Peter Kaluscha, Yalu Yan, Xiaoyan Chen, Yuanyao Chen, and Xiaohong Sun for their help and support, and Henan Wang for sharing my long journey towards PhD.

Thank you to Drs Wendy Wismer, Dirk Lutchman, Christopher Lohans, Ms Susan Gibson, and Mr Arazm Farhangfar. It's been great pleasure working with all of you. Thank you to Ms Heather Vandertol-Vanier, Ms Abha Dunichand-Hoedl, Ms Lisa Nikolai, and Ms Arlene Oatway for technical assistance.

Thank you mom and dad for your love and support. This thesis is dedicated to you.

Table of Contents

List of Figures

List of Tables

List of abbreviations

GUD	β-glucuronidase
CSR	competitor-stress tolerator-ruderal
RPS	rock-paper-scissors
DGGE	Denaturing Gradient Gel Electrophoresis
IBD	Inflammatory Bowel Disease
SCFA	short-chain fatty acids
IMO	isomaltooligosaccaride
FOS	fructooligoscaaride
SYN	Synergy, 50:50 mixture of FOS and inulin
RS	resistant starch
CEL	cellulose
PKS	polyketide synthase
NRPS	non-ribosomal peptide synthase
ORF	open reading frame
AT	acyltransferase
C	condensation
A	adenylation
T	thiolation
KS	ketosynthase
ACP	acyl carrier protein
TE	thioesterase
PP	5'-phosphopantetheinyl transferase

1. Introduction

Microbiota are the collectivity of microbes of a particular site, habitat, or geological period. Animals and humans can be viewed as "holobionts", ecosystems where hosts are environment to their microbiota. The understanding of human microbiota has progressed substantially since the availability of high throughput sequencing technology [1]. However, the majority of research efforts remain observational and descriptive rather than explanatory. To better understand microbial ecology in different habitat requires combined efforts from many disciplines, especially microbiology and ecology. Ecologists have a long history of using microbial model systems. The small size and short generation times of microbes allows the design of replicated experiments across a wide range of spatial and temporal scales. Abundant genetic and physiological information is available for commonly studied microorganisms. Microbes are also amenable to genetic manipulation. These advantages enable the ecologist to deconstruct the complexity of nature into its component parts and to explore the role of each part in creating patterns. Many ecological questions, including competition, community building, species diversity and ecological function, phenotypic diversity and niche specificity, multitrophic interactions, and coevolution, have been addressed with microbial models [2].

In comparison, the history of using ecological theories to study microbial communities by microbiologists is very recent [3,4]. Ecological theories such as community assembly theory, metacommunity theory, and competition theory are especially enlightening in explaining observations that cannot be readily explained on a micro scale, including the acquisition and development of gut microbiota, the dysbiosis following antibiotic treatment, the mutualism between commensals and host, the cooperation and competition between different microbes, and the infection, transmission, virulence of pathogens [3–6].

Great advances in microbiota research have been made since the beginning of this PhD research project. In retrospect, this review and subsequent work aim to investigate microbial ecology, especially microbial competition in different ecosystems and explore factors that might affect microbial ecology, in order to better understand the elements contributing to a healthy and stable microbiota.

1.1 Gut microbiota

Microbes that inhabit mucosal surfaces of the gastrointestinal tract (GI) are collectively known as gut microbiota. Bacteria predominate the gut microbiota but archaea and eukarya are also present. The structure and composition of the gut microbiota of an individual differ longitudinally, transversally, and chronologically. This is due to selection at both the microbial and host levels, which promotes mutual cooperation within and functional stability of this complex ecosystem [3,5]. Acid, bile, peristalsis, and pancreatic secretions hinder the colonization of the stomach and proximal small intestine by most bacteria. However, bacterial density increases in the distal small intestine, and reaches an estimated 10^{11}–10^{12} CFU per gram of colonic content. Mucosal and luminal microbial populations also differ, and the ratio of anaerobes to aerobes is lower at the mucosal surfaces than in the lumen [7,8]. The infant gut microbiota have low microbial density, phylogenetic diversity, and great interindividual variability, which are strongly dependent on mode of delivery. The adult-like microbiota are not formed until 3 year after birth. The bacterial diversity increases with age at an exponential scale within the first 3 year and continued at a lower rate thereafter [9,10]. The adult gut microbiota can be divided into enterotypes based on the abundance of specific bacterial groups, dominated by *Bacteroides*, *Prevotella* or *Ruminococcus* [11]. Aging (>65 yr) is associated with a number of changes in the microbiota, including an increase in Bacteroidetes, a greater variability than that of younger adults, and a distinct abundance patterns of *Clostridium* groups [12].

A number of factors influence interindividual variability of gut microbiota, including environment, diet, and genetics, disease, or medical treatment of disease. Lower abundance of the genera *Bacteroides*, *Bifidobacterium* and *Enterobacteriaceae* were observed in people on a vegan diet compared to those on an omnivorous diet [13,14]. Monozygotic twins were no more similar in their microbial composition than dizygotic twins, underscoring the importance of environment rather than genetics in shaping the microbiota [10]. Disease and medical treatment also impact gut microbiota and are in turn impacted by the composition and activity of gut microbiota (see below).

1.1.1 Gut microbiota and diseases

Gut microbiota is implicated in many diseases, especially the ones associated with Western life style, such as inflammatory bowel disease (IBD), Type I diabetes, colorectal cancer, obesity, and rheumatoid arthritis. However, whether microbiota is the cause or consequence of these diseases is still not clear [15]. There are different theories about the underlining reasons, but almost all converge at the event of industrialization and urbanization, where changes such as reduced family size, improved hygiene, altered diet pattern, increased antibiotic use all potentially contribute to altered gut microbiota which fail to provide optimal metabolic support to benefit human health [3,16,17].

1.1.2 Marker organisms in diseases

In healthy individuals, the normal physiological functions of the host as well as competition and cooperation among microbes maintain temporal stability of gut microbiota [18]. However, in event of diseases mentioned above, such homeostasis is often compromised and leads to dysbiosis, or alterations in the microbiota [19]. Dysbiosis is often characterized by reduced microbial diversity and by significant change in the abundance of marker organisms which can be either the cause or consequence of the disease. For example, decreased abundance of the butyrate-producing bacteria *Roseburia* and *Faecalibacterium prausnitzii* and acetate-producing *Ruminococcaceae* have been observed in IBD patients relative to controls [20,21]. Mucin-degrading bacterium *Akkermansia muciniphila* was reported to decrease in obesity and type 2 diabetes [19,22]. *Escherichia coli* has been linked with IBD and colorectal cancer [20,23]. Many factors might contribute to dysbiosis. For example, damaged epithelium can cause increased oxygen and heme at the musoca, and directly stimulate facultative aerobic organisms such as *Enterobacteriacae* [24]. Incomplete digestion of food by a dysfunctional digestive system could result in increased proportion of lipid, protein and starch reaching the colon, and thereby limit the fermentation substrates of fibrolytic organisms such as *Roseburia* [25].

1.1.3 The role of microbiota in colorectal cancer

Microbiota play multiple roles in colorectal cancer. They can both trigger and protect against development of colorectal cancer, both enhance and impair the efficacy of chemotherapy.

Cancer susceptibility and progression results from an interplay between gene regulation and the environment. Microbiota appears to have a role in carcinogenesis. A bacterial driver–

passenger model for microbial involvement in the development of colorectal cancer was proposed. According to this model, distinct indigenous intestinal bacteria, the 'driver bacteria' (e.g. *Enterococcus faecalis,* [26]) would create DNA damage and drive genome instability to initiate the first steps of tumorigenesis. Bacterial drivers may progressively disappear in favor of opportunistic bacteria, that is, 'passenger bacteria' (e.g. *Streptococcus gallolyticus* , [27]), which then overwhelm the intestinal niche alterations and corrupt the local innate immunity [28]. On the other hand, gut microbiota can also prime the innate and adaptive immune system of the host to defend against cancer [3,29]. For example, commensal bacteria *Bacteriodes fragilis* induces Treg differentiation and production of anti-inflammatory IL-10 in the colon through polysaccharide A [30]. *Clostridium* cluster IV and XIVa were found to induce Foxp3+ and IL-10 Treg in the colon [31]. Gut bacteria-triggered IL-10-dependent Treg may function to inhibit cancer even in extraintestinal sites such as breast [32].

1.1.4 Microbial interaction with chemotherapy

Microbiota can play an adjuvant role to certain cancer therapies. For example, the alkylating agent cyclophosphamide mobilizes Gram-positive bacteria such as *Lactobacillus johnsonii* or *Enterococcus hirae* from the gut to secondary lymphoid organs, where they prime pathogenic Th17 cells contributing to the activity of cytotoxic compounds [33]. In contrast, microbiota can aggravate chemotherapy-induced side effects such as mucositis. For example, CPT-11 is a drug commonly used to treat colorectal cancer. It is converted *in vivo* to the pharmacologically active SN-38, which has both antitumor activity and dose-limiting toxicity. SN-38 undergoes hepatic glucuronidation and is secreted into the bile as an inactive glucuronide SN-38G. Deconjugation of SN-38G in the colon by bacterial β-glucuronidases exposes intestinal epithelia to SN-38, mediating gut toxicity, causing diarrhea and systemic infection [34].

1.1.5 Prebiotics to modulate microbiota

Due to their critical role in many diseases, microbiota has been proposed as target of treatments. However, to this day, the knowledge of modulation of microbiota is still limited. Possible strategies include probiotics and prebiotics, antibiotics, drugs targeting microbial enzymes, and fecal transplantation [35]. Diet is one of the most important form of environmental selection imposed on gut microbiota by the host [5]. Prebiotics are currently defines as " a selectively fermented ingredient that results in specific changes, in the composition and/or

activity of the gastrointestinal microbiota, thus conferring benefit(s) upon host health" [36]. Prebiotics have most frequently been used to stimulate the growth of lactobacilli or bifidobacteria, organisms whose abundance often correlate positively with host health. The commonly used prebiotics include inulin, fructooligosaccharides, resistant starch [36,37]. Fibres provide substrate for microbial fermenation and thereby increase the amount of microbial metabolites. Among bacterial metabolites, short-chain fatty acids (SCFAs) have been proposed to regulate development and function of colonic Treg cells via epigenetic regulation of the *Foxp3* gene [38]. SCFAs, particularly butyrate, can act directly on T cells, increasing acetylation of the *Foxp3* locus [29,39]. SCFAs also can indirectly induce Treg cells by promoting TGFβ production [40], and by activating dendric cells and macrophages expressing glycoprotein receptor 109a [41]. SCFA can also promote homing of Treg cells to the large intestine by inducing GPR15 expression on $Foxp3^+$ Treg cells [42]. Other effect of prebiotics fermentation included decreased pH and increase abundance of fermenters such as *Clostridium* cluster XIVa [43], both of which may play a part in suppressing pathogens.

1.2 Sourdough as a model for forestomach microbiota

The complexity and instability of gut microbiota are due to the nutrient supply, microbial interactions, and particularly the interaction with the host. In comparison, the microbial composition and metabolite formation in fermented foods produced with defined technological parameters are usually much more predictable [44,45], making them an easier entry point to studying microbiota. Sourdough, which relies on continuous propagation for maintenance, provides an arena for long-term adaptation and competition and selects for the most adapted organisms, typically 2 – 5 species or bacterial strains in a given sourdough. As a result, different from other food fermentations, it is virtually impossible to control the microbiota of propagated sourdoughs through starter cultures [46,47]. Therefore, sourdough fermentation process represents an ideal tool to study microbial ecology, especially the relative competitiveness of the organisms.

Sourdough is used as acidification and/or and leavening agent in traditional bread baking [48]. Sourdough is a mixture of mainly flour and water that is fermented by lactic acid bacteria and may additionally contain yeasts. Sourdough microbiota are affected by the substrate, the

fermentation time and temperature, contamination from the environment, and the backslopping procedure [46,49]. The term backslopping (or refreshment, propagation) refers to the use of a previous batch as inoculum to start the fermentation of a new batch of sourdough [44]. Consecutive backslopping is essential to obtain and achieve the acidifying and leavening capability [50,51]; consecutive backslopping also selects for the most competitive microbiota.

Type I sourdoughs are traditional sourdoughs whose microorganisms are kept metabolically active through daily refreshments at ambient temperature. Type I sourdoughs are generally suitable for achieving dough leavening without addition of baker's yeast. In wheat and/or rye sourdoughs, dominating strains are often *L. sanfranciscensis* (see 1.3), which coexist with other species of lactic acid bacteria such as*, L. pontis*, *L. brevis*, *L. fermentum*, and *L. fructivorans*.

Type II sourdoughs are characterized by long fermentation time, high dough yield, and high temperature of fermentation. They are generally not suitable for achieving dough leavening but are used for dough acidification, and as dough improvers to enrich it with aroma and flavour compounds. These sourdoughs are produced at the industrial level using bioreactors or tanks at a controlled temperature that exceeds 30°C. Such a protocol aims at shortening the fermentation process [52]. Lactobacilli such as *L. panis* , *L. reuteri* , *L. johnsonii* , and *L. pontis* , which are resistant to low pH, dominate these sourdoughs [53]

As an ecosystem, sourdough resembles rodent forestomach in many aspects (Table 1-1); both environments are characterized by dominance of lactobacilli and a low pH as a result of their fermentation. Moreover, the metabolic functions that contribute to adaption in sourdough coincide with those that facilitate colonization in mice [54,55]. The similar carbon sources in both rodent forestomach and cereal sourdough result in glucose and maltose utilization being the major catabolic pathways for both rodent and sourdough microbiota. Lactobacilli from the two habitats also shared mechanisms to survive acidic and oxidative stress. The similarity between sourdough and rodent forestomach makes sourdough a useful *in vitro* model to study *in vivo* microbiota. However, its potential is limited by the lack of host-microbe interaction in the ecosystem. For example, the formation of biofilms is crucial for colonization in rodent forestomach [56] but is less important in sourdough.

1.3 Microbial competition

As briefly discussed above, microbial competition is a key element driving the genetic composition and metabolic activities of microbiota. Competition can be divided two broad categories: exploitative competition which indirectly limits available resources, and interference competition which directly harms other strains and species [64]. The following sections will discussion each type in host and sourdough ecosystems.

1.3.1 Exploitative competition in human and sourdough microbiota

The competitor–stress tolerator–ruderal (CSR) model states that organisms respond to conflicting selection pressures by allocating limited resources to growth, maintenance or regeneration [65,66]. Selection pressure may include competition (sharing of resources among neighboring organisms), stress (suboptimal conditions for growth or survival), and disturbance (destruction of biomass by external factors). Competitors are adapted for rapid resource utilization and long-term site occupation, stress tolerators are adapted to persist in low-resource environments and ruderals are adapted to highly disturbed environments by growing and reproducing quickly [65,66]. The model was originally developed for plant ecology, but seems to apply to competitive strategies of microbes in other microbial ecosystems as well [4].

The human body represents environments where resources, stress, and disturbance coexist. Successful commensal organisms are usually both competitive (i.e. versatile in utilizing available nutrients) and stress tolerant to adapt to the fluctuations caused by host activity and physiology (Table 1-2). For example, in the gut where sugars are not readily available, *Bacteroides* and *Roseburia* spp. have evolved ability to adhere and degrade various fibres [67]. The close interdependent syntrophic relationship among microbes normally maintains homeostasis in the gut. However, in time of disturbance (e.g. antibiotic treatment), the disrupted community gives ruderals opportunity to invade. Ruderals in the gut are often opportunistic pathogens which are equipped with various mechanisms to exploit the disturbant situation. For example, members of *Enterobacteriaceae* possess various virulent factors regulated by environmental conditions such as pH and host metabolites to ensure successful invasion during a narrow window of opportunity [68]. *C. difficile* uses a different approach by being able to switch

between heterotrophic growth on various substrates and autotrophy to increase its chance of survival under unfavorable conditions [69].

Table 1-1. Similarities between rodent forestomach and sourdough environments and metabolic properties that contribute to adaptation to such environments.

	Forestomach	Sourdough	Reference
Main species	*L. reuteri* *L. johnsonii*	*L. sanfranciscensis* *L. reuteri, L. plantarum, L. fermentum*	[57,58]
Energy metabolism	sugar transport		[57]
	maltose utilization		[57]
	pentose phosphate pathway		[54]
Protection against oxidative stress	glutathione reductase		[59]
	cystathionine-γ-lyase		[59]
Protection against acidic stress	glutaminase		[55]
	glutamate decarboxylase		[55]
	arginine deiminase		[60]
	urease		[55,61]
Extracelluar compound	levansucrase glucansucrase		[62]
	mucin-binding protein		[55]
Antimicrobial		reutericyclin	[63]
Structure	D-alanyl-lipoteichoic acid cyclopropane-fatty acyl-phsopholipid synthase		[55]

In controlled sourdough fermentation systems disturbance is rare, hence most organisms have adopted competitive or stress tolerant strategies depending on the fermentation conditions. In general, short fermentation time select for competitors such as *L. sanfranciscensis*, whose

efficient use of carbohydrate and electron acceptors, as well as minimal genome size enables it to outgrow other lactobacilli in short-fermented doughs [70–73]. Long fermentation times impose stress due to acid accumulation, and selects for stress tolerators with one or several mechanisms to maintain intracellular pH (see section 1.2). The presence of antimicrobial compounds such as phenolic compounds in cereals also cause stress, which selects for organisms with detoxification mechanisms [74].

Table 1-2. CSR stratiegies adopted by microbes in human and sourdough

	Competitor	**Stress tolerator**	**Ruderal**
Human	Gut: Fibrolytic bacteria (e.g. *Bacteroides* spp.) Tooth: *Streptococcus mutans* Stomach: *Helicobacter pylori* Vagina: *Lactobacillus* spp.		Opportunistic pathogens (e.g. *Clostridium difficile*, *Enterobacteriaceae*)
Sourdough	Fast-growing bacteria (short fermentation) Sugar utilization Electro acceptor utilization *L. sanfranciscensis*	Acid-resistant bacteria (long-fermenetation) with the following properties Antimicrobial resistant bacteria	

1.3.2 Interference competition in human and sourdough

A classic example of interference competition is the production of antimicrobial compound that harms other related or non-related organisms [64]. In natural ecosystems toxin (antimicrobial)-producing microbes which are immune to their own toxins usually coexist with immune microbes which lack the production but carry immunity genes, and susceptible organisms which lack both producing and immunity genes [75,76]. The dynamics among these three types organisms have been extensively studied. The rock-paper-scissors (RPS) model is the most studied model and is fundamental to understanding other models [77]. The susceptible strains are outcompeted by producer strains by direct inhibition. However, both the production of toxins and the immunity to toxins are costly, which results in producer strains being outcompeted

by immune strains, and immune strains outcompeted by susceptible strains. This can lead to cycling among the three strains, analogous to the dynamics of the classic rock-paper-scissors (RPS) game [78].

1.3.3 Factors influencing RPS dynamics

A number of factors can affect the maintenance of biodiversity in RPS model, including cost of toxin production and immunity, spatial dispersion, mobility of the organisms, etc. For example, a RPS relationship exists only if both the toxin production and immunity are costly, and the cost of immunity is less than the cost of toxin production [77]. If the costs of toxin production and immunity are minimal, then the growth of the susceptible strain will be inhibited by its weak advantage over the immune strain [79]. The localization of interaction, namely the competition for resources and the inhibition by toxins, also directly determine the community dynamics. Toxin production is beneficial only when the toxin concentration is high enough to inhibit susceptible strains and when the producer competes for resources with the organisms that it harms [77,79]. In a non-localized environment such as a shaken flask, the toxin's concentration is rapidly diluted once it's produced. The producers pay a cost to produce their toxin but gained no benefit and therefore will be outcompeted by others. In contrast, in a localized environment such as agar plate, toxin produced remains in close proximity to the producer cell, and thereby kill the neighboring susceptible cells to gain more access to resources [80].

One important family of natural antimicrobials is bacteriocin. Bacteriocins are ribosomally synthesized, heat-stable, antimicrobial peptides produced by bacteria [81]. The RPS dynamics were first and most well-studied in colicin-producing *Escherichia coli* [80,82,83] and has been extrapolated to other bacteriocins and antimicrobials in general. Another important category is antibiotics synthesized by polyketide synthases (PKS), nonribosomal peptide synthetases (NRPS), or their hybrids, which are molecular assembly lines that direct product formation on a protein template [84,85]. Antibiotics are different from bacteriocins in several aspects: bacteriocins are ribosomally synthesized primary metabolites that are usually produced in the growth phase, while antibiotics are secondary metabolites produced by special enzymatic systems in the stationary phase. In general, the target spectrum of bacteriocin is narrower than antibiotics. In bacteriocin producers, the immunity gene is usually linked to the synthesis gene, while in antibiotic producers, the immunity gene can be expressed separately [86]. In addition,

antibiotic producers are mainly found in highly structured environmental habitats such as soil [87]. The competition theory implies that antimicrobials need to be produced during the growth phase to inhibit competitors; however, the production of antibiotics and many Gram-positive bacteriocins are produced at stationary phase. It has therefore been proposed that the production of these antimicrobials are behaviors of predation (the ability to lyse live cells and exhibit growth when no other nutrients are available) rather than competition [87,88].

A variety of factors can have different effect on microbial competition. In the host-microbe ecosystem, diet and host physiology have a dominant effect on the microbiota. A fermentation system such as sourdough simplified the interaction by replacing living host to more predictable environment factors such as substrate, temperature, pH. The aim of this work was to study factors affecting microbial ecology using rodent and sourdough as models.

To determine the effect of host health and disease on gut microbial ecology, Chapter 1 investigated the effect of tumor and chemotherapy on gut microbiota of rats. Following Chapter 1, Chapter 2 explored the possibility of modulating gut microbiota of chemotherapy-treated rats with different dietary carbohydrates.

To study a more simple ecosystem which did not involve microbial interaction with host, Chapter 3 to 5 used sourdough as a model system. Chapter 3 developed melting-curve-based methods to differentiate closely-related organisms in order to study the coexistence of multiple strains and/or species. The methods were applied in Chapter 4 to study the effect of substrate and growth rates on exploitative competition of organisms with different metabolic traits. Chapter 5 looked at interfering competition by identifying the genetic determinants of biosynthesis of reutericyclin, an unsual antibiotic compound produced by *L. reuteri* from sourdough.

1.4 References

1. Ley RE, Lozupone CA, Hamady M, Knight R, Gordon JI (2008) Worlds within worlds: evolution of the vertebrate gut microbiota. Nat Rev Microbiol 6: 776–788.

2. Jessup CM, Kassen R, Forde SE, Kerr B, Buckling A, et al. (2004) Big questions, small worlds: microbial model systems in ecology. Trends Ecol Evol 19: 189–197.

3. Dethlefsen L, McFall-Ngai M, Relman DA (2007) An ecological and evolutionary perspective on human–microbe mutualism and disease. Nature 449: 811–818.

4. Prosser JI, Bohannan BJM, Curtis TP, Ellis RJ, Firestone MK, et al. (2007) The role of ecological theory in microbial ecology. Nat Rev Microbiol 5: 384–392.

5. Costello EK, Stagaman K, Dethlefsen L, Bohannan BJM, Relman DA (2012) The application of ecological theory toward an understanding of the human microbiome. Science 336: 1255–1262.

6. Lemon KP, Armitage GC, Relman D a, Fischbach M a (2012) Microbiota-targeted therapies: an ecological perspective. Sci Transl Med 4: 137rv5.

7. O'Hara AM, Shanahan F (2006) The gut flora as a forgotten organ. EMBO Rep 7: 688–693.

8. Eckburg PB, Bik EM, Bernstein CN, Purdom E, Dethlefsen L, et al. (2005) Diversity of the human intestinal microbial flora. Science 308: 1635–1638.

9. Palmer C, Bik EM, DiGiulio DB, Relman DA, Brown PO (2007) Development of the human infant intestinal microbiota. PLoS Biol 5: e177.

10. Yatsunenko T, Rey FE, Manary MJ, Trehan I, Dominguez-Bello MG, et al. (2012) Human gut microbiome viewed across age and geography. Nature 486: 222–227.

11. Arumugam M, Raes J, Pelletier E, Le Paslier D, Yamada T, et al. (2011) Enterotypes of the human gut microbiome. Nature 473: 174–180.

12. Claesson MJ, Cusack S, O'Sullivan O, Greene-Diniz R, de Weerd H, et al. (2011) Composition, variability, and temporal stability of the intestinal microbiota of the elderly. Proc Natl Acad Sci U S A 108 Suppl: 4586–4591.

13. De Filippo C, Cavalieri D, Di Paola M, Ramazzotti M, Poullet JB, et al. (2010) Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa. Proc Natl Acad Sci U S A 107: 14691–14696.

14. Zimmer J, Lange B, Frick J-S, Sauer H, Zimmermann K, et al. (2012) A vegan or vegetarian diet substantially alters the human colonic faecal microbiota. Eur J Clin Nutr 66: 53–60.

15. Cénit MC, Matzaraki V, Tigchelaar EF, Zhernakova a (2014) Rapidly expanding knowledge on the role of the gut microbiome in health and disease. Biochim Biophys Acta.

16. Blaser MJ, Falkow S (2009) What are the consequences of the disappearing human microbiota? Nat Rev Microbiol 7: 887–894.

17. Strachan DP (1989) Hay fever, hygiene, and household size. BMJ 299: 1259–1260.

18. Lahti L, Salojärvi J, Salonen A, Scheffer M, de Vos WM (2014) Tipping elements in the human intestinal ecosystem. Nat Commun 5: 4344.

19. Underwood MA (2014) Intestinal dysbiosis: Novel mechanisms by which gut microbes trigger and prevent disease. Prev Med (Baltim) 65C: 133–137.

20. Morgan XC, Tickle TL, Sokol H, Gevers D, Devaney KL, et al. (2012) Dysfunction of the intestinal microbiome in inflammatory bowel disease and treatment. Genome Biol 13: R79.

21. Machiels K, Joossens M, Sabino J, De Preter V, Arijs I, et al. (2013) A decrease of the butyrate-producing species *Roseburia hominis* and *Faecalibacterium prausnitzii* defines dysbiosis in patients with ulcerative colitis. Gut: gutjnl–2013–304833–.

22. Everard A, Belzer C, Geurts L, Ouwerkerk JP, Druart C, et al. (2013) Cross-talk between *Akkermansia muciniphila* and intestinal epithelium controls diet-induced obesity. Proc Natl Acad Sci U S A 110: 9066–9071.

23. Martinez-Medina M, Denizot J, Dreux N, Robin F, Billard E, et al. (2014) Western diet induces dysbiosis with increased *E coli* in CEABAC10 mice, alters host barrier function favouring AIEC colonisation. Gut 63: 116–124.

24. Schüller S, Phillips AD (2010) Microaerobic conditions enhance type III secretion and adherence of enterohaemorrhagic *Escherichia coli* to polarized human intestinal epithelial cells. Environ Microbiol 12: 2426–2435.

25. Metzler-Zebeli BU, Hooda S, Pieper R, Zijlstra RT, van Kessel AG, et al. (2010) Nonstarch polysaccharides modulate bacterial microbiota, pathways for butyrate production, and abundance of pathogenic *Escherichia coli* in the pig gastrointestinal tract. Appl Environ Microbiol 76: 3692–3701.

26. Huycke MM, Abrams V, Moore DR (2002) *Enterococcus faecalis* produces extracellular superoxide and hydrogen peroxide that damages colonic epithelial cell DNA. Carcinogenesis 23: 529–536.

27. Boleij A, van Gelder MMHJ, Swinkels DW, Tjalsma H (2011) Clinical Importance of *Streptococcus gallolyticus* infection among colorectal cancer patients: systematic review and meta-analysis. Clin Infect Dis 53: 870–878.

28. Tjalsma H, Boleij A, Marchesi JR, Dutilh BE (2012) A bacterial driver-passenger model for colorectal cancer: beyond the usual suspects. Nat Rev Microbiol 10: 575–582.

29. Kamada N, Núñez G (2014) Regulation of the immune system by the resident intestinal bacteria. Gastroenterology 146: 1477–1488.

30. Round JL, Mazmanian SK (2010) Inducible Foxp3+ regulatory T-cell development by a commensal bacterium of the intestinal microbiota. Proc Natl Acad Sci U S A 107: 12204–12209.

31. Atarashi K, Tanoue T, Oshima K, Suda W, Nagano Y, et al. (2013) Treg induction by a rationally selected mixture of Clostridia strains from the human microbiota. Nature 500: 232–236.

32. Erdman SE, Rao VP, Olipitz W, Taylor CL, Jackson EA, et al. (2010) Unifying roles for regulatory T cells and inflammation in cancer. Int J Cancer 126: 1651–1665.

33. Viaud S, Daillère R, Boneca IG, Lepage P, Langella P, et al. (2014) Gut microbiome and anticancer immune response: really hot Sh*t! Cell Death Differ.

34. Gupta E, Lestingi TM, Mick R, Ramirez J, Vokes EE, et al. (1994) Metabolic fate of irinotecan in humans: correlation of glucuronidation with diarrhea. Cancer Res 54: 3723–3725.

35. Holmes E, Kinross J, Gibson GR, Burcelin R, Jia W, et al. (2012) Therapeutic modulation of microbiota-host metabolic interactions. Sci Transl Med 4: 137rv6.

36. Roberfroid M, Gibson GR, Hoyles L, McCartney AL, Rastall R, et al. (2010) Prebiotic effects: metabolic and health benefits. Br J Nutr 104 Suppl: S1–63.

37. Kolida S, Tuohy K, Gibson GR (2007) Prebiotic effects of inulin and oligofructose. Br J Nutr 87: S193.

38. Smith PM, Howitt MR, Panikov N, Michaud M, Gallini CA, et al. (2013) The microbial metabolites, short-chain fatty acids, regulate colonic Treg cell homeostasis. Science 341: 569–573.

39. Furusawa Y, Obata Y, Fukuda S, Endo TA, Nakato G, et al. (2013) Commensal microbe-derived butyrate induces the differentiation of colonic regulatory T cells. Nature 504: 446–450.

40. Atarashi K, Tanoue T, Shima T, Imaoka A, Kuwahara T, et al. (2011) Induction of colonic regulatory T cells by indigenous Clostridium species. Science 331: 337–341.

41. Singh N, Gurav A, Sivaprakasam S, Brady E, Padia R, et al. (2014) Activation of Gpr109a, receptor for niacin and the commensal metabolite butyrate, suppresses colonic inflammation and carcinogenesis. Immunity 40: 128–139.

42. Kim S V, Xiang W V, Kwak C, Yang Y, Lin XW, et al. (2013) GPR15-mediated homing controls immune homeostasis in the large intestine mucosa. Science 340: 1456–1459.

43. Neyrinck AM, Possemiers S, Verstraete W, De Backer F, Cani PD, et al. (2012) Dietary modulation of clostridial cluster XIVa gut bacteria (*Roseburia* spp.) by chitin-glucan fiber improves host metabolic alterations induced by high-fat diet in mice. J Nutr Biochem 23: 51–59.

44. Corsetti A (2013) Technology of Sourdough Fermentation and Sourdough Applications. In: Gobbetti M, Gänzle M, editors. Handbook on Sourdough Biotechnology. Boston, MA: Springer US. pp. 85–103.

45. Vogelmann SA, Hertel C (2011) Impact of ecological factors on the stability of microbial associations in sourdough fermentation. Food Microbiol 28: 583–589.

46. Minervini F, De Angelis M, Di Cagno R, Gobbetti M (2014) Ecological parameters influencing microbial diversity and stability of traditional sourdough. Int J Food Microbiol 171: 136–146.

47. De Vuyst L, Vrancken G, Ravyts F, Rimaux T, Weckx S (2009) Biodiversity, ecological determinants, and metabolic exploitation of sourdough microbiota. Food Microbiol 26: 666–675.

48. Cappelle S, Guylaine L, Gänzle M, Gobbetti M (2013) History and Social Aspects of Sourdough. In: Gobbetti M, Gänzle M, editors. Handbook on Sourdough Biotechnology. Boston, MA. pp. 1–10.

49. Hammes WP, Brandt MJ, Francis KL, Rosenheim J, Seitter MFH, et al. (2005) Microbial ecology of cereal fermentations. Trends Food Sci Technol 16: 4–11.

50. Hammes W, Gänzle M (1997) Sourdough breads and related products. In: Woods B, editor. Microbiology of fermented foods. London: Blackie Academic and Professional. pp. 199–216.

51. De Vuyst L, Vancanneyt M (2007) Biodiversity and identification of sourdough lactic acid bacteria. Food Microbiol 24: 120–127.

52. Decock P, Cappelle S (2005) Bread technology and sourdough technology. Trends Food Sci Technol 16: 113–120.

53. Böcker G, Stolz P, Hammes W (1995) Neue Erkenntnisse zum Ökosystem Sauerteig und zur Physiologie der sauerteigtypischen Stämme *Lactobacillus sanfrancisco* und *Lactobacillus pontis*. Getreide Mehl Brot 49: 370–374.

54. Gänzle MG, Vermeulen N, Vogel RF (2007) Carbohydrate, peptide and lipid metabolism of lactic acid bacteria in sourdough. Food Microbiol 24: 128–138.

55. Schwab C, Tveit AT, Schleper C, Urich T (2014) Gene expression of lactobacilli in murine forestomach biofilms. Microb Biotechnol 7: 347–359.

56. Walter J, Chagnaud P, Tannock GW, Loach DM, Dal Bello F, et al. (2005) A high-molecular-mass surface protein (Lsp) and methionine sulfoxide reductase B (MsrB) contribute to the ecological performance of *Lactobacillus reuteri* in the murine gut. Appl Environ Microbiol 71: 979–986.

57. Tannock GW, Wilson CM, Loach D, Cook GM, Eason J, et al. (2012) Resource partitioning in relation to cohabitation of *Lactobacillus* species in the mouse forestomach. ISME J 6: 927–938.

58. De Vuyst L, Vrancken G, Ravyts F, Rimaux T, Weckx S (2009) Biodiversity, ecological determinants, and metabolic exploitation of sourdough microbiota. Food Microbiol 26: 666–675.

59. Jänsch A, Korakli M, Vogel RF, Gänzle MG (2007) Glutathione reductase from *Lactobacillus sanfranciscensis* DSM20451T: contribution to oxygen tolerance and thiol exchange reactions in wheat sourdoughs. Appl Environ Microbiol 73: 4469–4476.

60. Rollan G, Lorca GL, Font de Valdez G (2003) Arginine catabolism and acid tolerance response in *Lactobacillus reuteri* isolated from sourdough. Food Microbiol 20: 313–319.

61. Frese SA, Benson AK, Tannock GW, Loach DM, Kim J, et al. (2011) The evolution of host specialization in the vertebrate gut symbiont *Lactobacillus reuteri*. PLoS Genet 7: e1001314.

62. Tieking M, Korakli M, Ehrmann MA, Gänzle MG, Vogel RF (2003) In situ production of exopolysaccharides during Sourdough fermentation by cereal and intestinal isolates of lactic acid bacteria. Appl Environ Microbiol 69: 945–952.

63. Gänzle MG, Vogel RF (2003) Contribution of reutericyclin production to the stable persistence of *Lactobacillus reuteri* in an industrial sourdough fermentation. Int J Food Microbiol 80: 31–45.

64. Mitri S, Foster KR (2013) The genotypic view of social interactions in microbial communities. Annu Rev Genet 47: 247–273.

65. Grime J, Pierce S (2012) The Evolutionary Strategies that Shape Ecosystems. Chichester, UK: Wiley-Blackwell.

66. Grime J (1979) Plant Strategies and Vegetation Processes. New York, USA: John Wiley & Sons.

67. Mirande C, Kadlecikova E, Matulova M, Capek P, Bernalier-Donadille A, et al. (2010) Dietary fibre degradation and fermentation by two xylanolytic bacteria *Bacteroides xylanisolvens* XB1A and *Roseburia intestinalis* XB6B4 from the human intestine. J Appl Microbiol 109: 451–460.

68. Hernandez-Doria JD, Sperandio V (2013) Nutrient and chemical sensing by intestinal pathogens. Microbes Infect 15: 759–764.

69. Köpke M, Straub M, Dürre P (2013) *Clostridium difficile* is an autotrophic bacterial pathogen. PLoS One 8: e62157.

70. Gänzle M, Ehmann M, Hammes W (1998) Modeling of growth of *Lactobacillus sanfranciscensis* and *Candida milleri* in response to process parameters of sourdough fermentation. Appl Environ Microbiol 64: 2616–2623.

71. Stolz P, Böcker G, Hammes WP, Vogel RF (1995) Utilization of electron acceptors by lactobacilli isolated from sourdough. Z Lebensm Unters Forsch 201: 91–96.

72. Vogel RF, Pavlovic M, Ehrmann MA, Wiezer A, Liesegang H, et al. (2011) Genomic analysis reveals *Lactobacillus sanfranciscensis* as stable element in traditional sourdoughs. Microb Cell Fact 10 Suppl 1: S6.

73. Neubauer H, Glaasker E, Hammes WP, Poolman B, Konings WN (1994) Mechanism of maltose uptake and glucose excretion in *Lactobacillus sanfrancisco*. J Bacteriol 176: 3007–3012.

74. Sekwati-Monang B, Valcheva R, Gänzle MG (2012) Microbial ecology of sorghum sourdoughs: effect of substrate supply and phenolic compounds on composition of fermentation microbiota. Int J Food Microbiol 159: 240–246.

75. Gordon DM, O'Brien CL (2006) Bacteriocin diversity and the frequency of multiple bacteriocin production in *Escherichia coli*. Microbiology 152: 3239–3244.

76. Riley MA, Wertz JE (2002) Bacteriocins: evolution, ecology, and application. Annu Rev Microbiol 56: 117–137.

77. Kerr B, Riley MA, Feldman MW, Bohannan BJM (2002) Local dispersal promotes biodiversity in a real-life game of rock-paper-scissors. Nature 418: 171–174.

78. Abrudan MI, Brown S, Rozen DE (2012) Killing as means of promoting biodiversity. Biochem Soc Trans 40: 1512–1516.

79. Biernaskie JM, Gardner A, West S a (2013) Multicoloured greenbeards, bacteriocin diversity and the rock-paper-scissors game. J Evol Biol 26: 2081–2094.

80. Chao L, Levin BR (1981) Structured habitats and the evolution of anticompetitor toxins in bacteria. Proc Natl Acad Sci U S A 78: 6324–6328.

81. Cotter PD, Hill C, Ross RP (2005) Bacteriocins: developing innate immunity for food. Nat Rev Microbiol 3: 777–788.

82. Durrett R, Levin S (1997) Allelopathy in Spatially Distributed Populations. J Theor Biol 185: 165–171.

83. Iwasa YOH, Nakamaru ÃM, Levin SA (1998) Allelopathy of bacteria in a lattice population : Competition between colicin-sensitive and colicin-producing strains.

84. Fischbach MA, Walsh CT (2006) Assembly-line enzymology for polyketide and nonribosomal Peptide antibiotics: logic, machinery, and mechanisms. Chem Rev 106: 3468–3496.

85. Donadio S, Monciardini P, Sosio M (2007) Polyketide synthases and nonribosomal peptide synthetases: the emerging view from bacterial genomics. Nat Prod Rep 24: 1073–1109.

86. Dutton C, Haxwell M, McArthur H, Wax RG, editors (2002) Peptide Antibiotics: Discovery Modes Of Action And Applications. New York, USA: Marcel Dekker Inc.

87. Kumbhar C, Watve M (2013) Why antibiotics: A comparative evaluation of different hypotheses for the natural role of antibiotics and an evolutionary synthesis. Nat Sci 05: 26–40.

88. Leisner JJ, Haaber J (2012) Intraguild predation provides a selection mechanism for bacterial antagonistic compounds. Proc Biol Sci 279: 4513–4521.

2. Irinotecan (CPT-11) chemotherapy alters intestinal microbiota in tumour bearing rats

2.1 Introduction

Chemotherapy disrupts intestinal microbiota homeostasis, inducing mucositis and dysbiosis. This disruption may contribute to development of diarrhea, allow overgrowth of pathogenic bacteria, and exacerbate or perpetuate intestinal injury induced by chemotherapy [1]. Both the number and relative proportion of individual bacterial groups are important for maintaining the homeostasis of the intestine and host health. Microbiota changes during chemotherapy and their specific involvement in gut pathology and infection remain to be fully characterized [2, 3]. Irinotecan (CPT-11, 7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxy-camptothecin) is used to treat colorectal and other cancers. CPT-11 is noted for gastrointestinal side effects, especially severe diarrhea. The involvement of microbiota in this toxicity is linked to CPT-11 metabolism. *In vivo*, CPT-11 is converted to the pharmacologically active SN-38, which is responsible for both anti-tumor activity and dose-limiting toxicity. SN-38 undergoes hepatic glucuronidation and is secreted into the bile as inactive the glucuronide SN-38G [4]. Deconjugation of SN-38G in the colon by bacterial β-glucuronidases exposes intestinal epithelia to SN-38, mediating gut toxicity [5, 6]. Moreover, specific bacterial organisms translocate from the intestine of CPT-11 treated animals and cause systemic infection and sepsis [7]. Prophylaxis with antibiotics reduced SN-38 concentration and/or diarrhea both in animal models and patients [8, 9].

CPT-11 with 5 FU is the primary regimen to treat colon cancer around the world, in either 1st or 2nd line [10, 11, 12]. Diarrhea is one of the most clinically significant toxicities of CPT-11, and is experienced to varying degrees by more than 80 % of the patients [10]. Patients with diarrhea undergo changes in their chemotherapy, including dose reductions (45 % of patients), delays in therapy (71 %), reduction in dose intensity (64 %), and discontinuation of therapy (3 %) [12]. Therefore, diarrhea induced by CPT-11 limits CPT-11's utility and efficacy in colorectal cancer treatment. Glutamine, a key 'pharmaconutrient', protects the gut during a variety of stress conditions [13, 14], including cancer chemotherapy [15]. Oral glutamine reduced the incidence and severity of late-onset diarrhea following CPT-11 treatment in rats

[16]. Glutamine mediated several potentially protective responses, including heat shock protein induction, increase in the ratio of reduced to oxidized glutathione, and increased proportions of CD3+CD8+ and memory CD8+ cells in mesenteric lymph nodes. Glutamine also prevented the CPT-11-induced increase of β-glucuronidase activity in the cecum [16], suggesting that glutamine affected intestinal microbiota.

The contribution of intestinal β-glucuronidase activity in CPT-11 toxicity is well established; in addition, tumour growth may influence intestinal microbiota even in the absence of chemotherapy [17]. However, information on the interaction between CPT-11 chemotherapy, tumour, and intestinal microbiota as a basis for therapeutic intervention to mitigate adverse effects of chemotherapy is lacking. Past studies documenting CPT-11-induced changes of the intestinal microbiota [2, 18] remained restricted to the analysis of fecal microbiota, did not use tumor bearing animals, and a CPT-11 dose (200 mg/kg) that was too low to cause clinically comparable levels of diarrhea or weight loss [2, 18, 19]. This study aimed to employ a tumor-bearing rat model for CPT-11 chemotherapy [20] to investigate responses of intestinal microbiota to tumor implantation and CPT-11-based chemotherapies. A dose-intensive CPT-11 monotherapy regimen as well as a cyclic regimen with a 5-fluorouracil/CPT-11 combination was employed to match the incidence of moderate and severe diarrhea, mortality, and constitutional signs like weight loss that are observed in clinical practice [19]. Cecal and fecal microbiota were evaluated with qualitative and quantitative molecular methods using primers targeting 16S rRNA genes of major bacterial species, genes encoding virulence factors and toxins.

2.2 Materials and methods

2.2.1 Animals and treatments. Experimental conditions were described elsewhere [16, 20]. Experiments were approved by the University of Alberta Animal Policy and Welfare Committee (UAPWC) in accordance with the Canadian Council on Animal Care (CCAC) guidelines. Briefly, female Fisher 344 rats (body weight, 150–180 g), 11-12 weeks of age, were obtained from Charles River (QC, Canada). Rats were housed 2 per cage in a temperature (22°C) and light controlled (12 h light) room; water and food were available *ad libitum*. One week before chemotherapy rats were separated into individual housing in wire-bottom cages. Tumour pieces

(0.05 g) were transplanted subcutaneously on the flank via a trocar using light isoflurane anesthesia. Tumour volume was estimated as previously described [16].

2.2.2 Diet. Diets used in this study are described elsewhere [20]. Briefly, semi-purified diet was based on AIN-76 basal diet, with a modified fat component similar to a North American dietary pattern with respect to energy % as fat and levels of n-3, n-6, saturated and polyunsaturated fatty acids. Rats were initially fed Rodent Laboratory Chow (Harlan Teklad, Madison, WI). During the adaptation period, this non-purified diet was mixed with study diet (50/50, w/w) for one week, followed by transition to a 100% semi-purified diet starting 2 weeks prior to tumour implantation.

2.2.3 Chemotherapy regimens and glutamine administration. Two regimens were used (Figure 2-1) to deliver chemotherapy at the maximum tolerated dose, i.e. significant toxicity but without mortality, in keeping with clinical practice [10, 16, 19]. In both regimens intravenous chemotherapy was started when tumour volume reached ~2 cm^3 Atropine (1 mg/kg s.c.) was administered immediately before each CPT-11 injection to alleviate early-onset cholinergic symptoms.

During a dose-intensive regimen, tumour-bearing rats (n=6/group) received CPT-11 (125 mg/kg × 3 days) (Figure 2-1A), with or without bolus glutamine gavage [16]. Glutamine was prepared as a 3% (wt/v) solution immediately before use and filtered through a 0.45-nm filter. The solution was administered by oral gavage (0.75 g kg^{-1}) 30 min before each daily CPT-11 injection. The sham treatment group received an equal volume of sterile water. The day before CPT-11 administration was designated day 0. Rats were killed on day 0, on day 3 (6 hours after the 3rd injection of CPT-11) to capture early microbiota responses, and on day 7. In the dose-intensive regimen, diarrhea occurred in both sham- and glutamine-treated groups. Glutamine gavage decreased the incidence of severe diarrhea [16]. Relative food intake and relative body weight of both groups dropped immediately after CPT-11 treatment, but showed a trend towards recovery by day 7 [16].

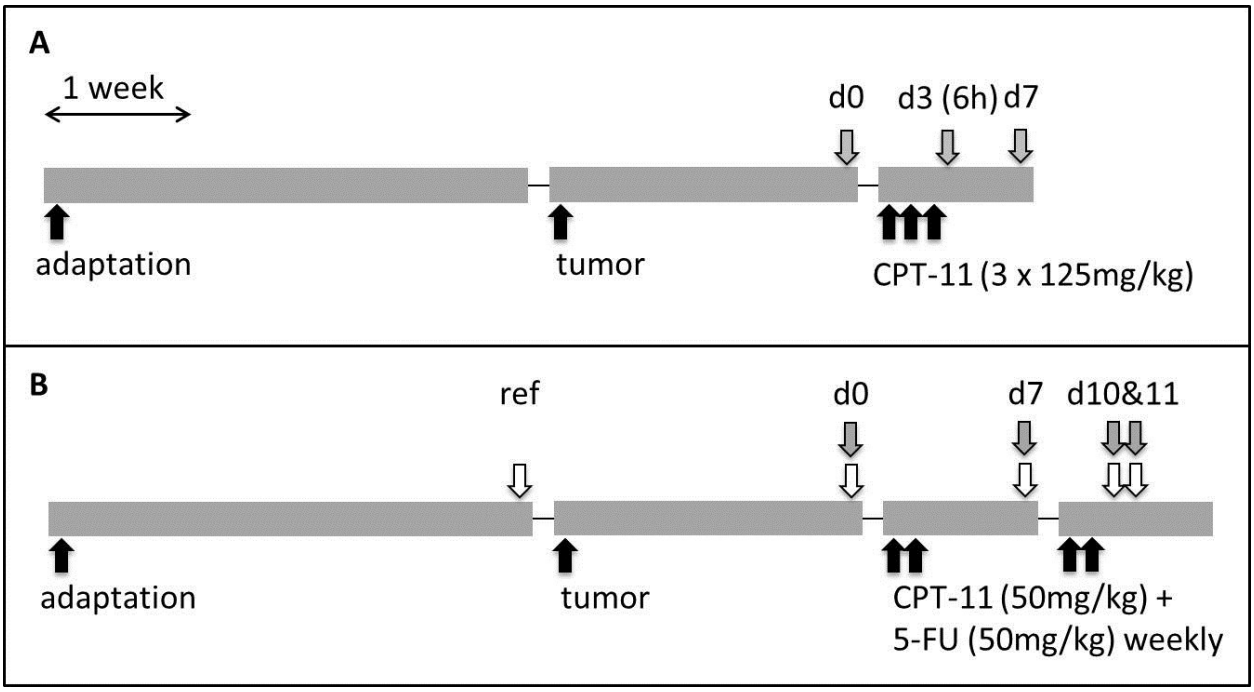


Figure 2-1. Experimental design of chemotherapy treatment of cancer-bearing rats.

The dose-intensive CPT-11 regimen is shown in panel A; the CPT-11/5-FU regimen is shown in panel B, Black arrows represent chemotherapy treatment at different time points. Grey and white arrows represent the time points at which cecal and fecal samples were taken, respectively (n=6/time point). For glutamine-treated rats in dose-intensive regimen, glutamine bolus was administered 30min before each CPT-11 dose.

In a second regimen designed to imitate clinical therapy of colorectal cancer, rats (n=6/group) received two cycles of CPT-11/5-fluorouracil (5-FU) treatment (Figure 1-1B). The day before the first CPT-11 injection was designated day 0. Animals received weekly CPT-11 (50 mg kg^{-1}) and 5-FU (50 mg kg^{-1}) injections on day 1 and 8 and on day 2 and 9, respectively. Animals were killed on day 0, day 7 (prior to the second treatment cycle), and on day 10 and 11 (one and two days after the 2nd treatment cycle) in order to assess intestinal microbiota changes after each cycle. Diarrhea, the relative body weight, and the relative food intake of animals was assessed as previously described [16]. During the clinical CPT-11/5-FU regimen, diarrhea was absent in animals at all time points (data not shown). The relative body weight and the relative

food intake showed little change after the 1st cycle of treatment, but were significantly reduced after the 2nd cycle (data not shown). Sampling in this regimen aimed to characterize microbiota after the 2nd cycle of chemotherapy, as it was at this time that the most prominent toxicity of CPT-11/5-FU chemotherapy was observed.

To assess the sequential effect of tumour implantation and chemotherapy on intestinal microbiota, fecal samples were obtained from animals after diet adaptation (healthy rats prior to any treatment), 2 weeks after tumour implantation, and throughout chemotherapy treatment (Figure 2-1B).

2.2.4 Sample collection and DNA isolation. Sampling schedules are shown in Figure 2-1. Rats were killed by CO_2 asphyxiation. Cecal contents were collected under aseptic conditions. In the second regimen, fecal samples from the same animals were collected over time. DNA was extracted from cecal or fecal samples using the QIAamp DNA Stool Mini Kit (Qiagen, Mississauga, Canada).

2.2.5 PCR-denaturing gradient gel electrophoresis (DGGE). PCR-DGGE was performed on cecal samples from the dose-intensive regimen as described previously [21]. Briefly, The V2-V3 region of the 16S rDNA gene of bacteria in the fecal samples was amplified by using primers HDA1-GC (5′-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAC TCC TAC GGG AGG CAG CAG T-3′) and HDA2 (5′-GTA TTA CCG CGG CTG CTG GCA C-3′). DGGE was performed by using a DCode universal mutation detection system (Bio-Rad, Richmond). Polyacrylamide gels (6%) were prepared and electrophoresed using 1× TAE buffer. The gels contained a 22 to 55% gradient of urea and formamide that increased in the direction of electrophoresis. A 100% denaturing solution contained 40% (v/v) formamide and 7.0 M urea. Electrophoresis was performed at 130 V and 60°C for about 4.5 h. The gels were stained with ethidium bromide (5 mg L^{-1}) for 20 min, washed with deionized water, and viewed by UV transillumination. Patterns were normalized by including PCR products from one sample on all gels. Cluster analysis was performed by an unweighted pair group method with arithmetic mean (UPGMA) algorithm based on the dice correlation coefficient using an optimization coefficient of 1% (Bionumerics software, version 3, Applied Maths, Sint-Martens-Latem, Belgium).

2.2.6 Quantification of major bacterial groups, virulence factors, and translocated species by quantitative PCR (qPCR). Quantitative PCR was performed as described [22]. Major bacterial groups in cecal and fecal microbiota were quantified using group-specific primers (Appendix A) targeting total bacteria, *Bacteroides-Prevotella-Porphyromonas* (*Bacteroides* group), *Lactobacillus-Pediococcus-Leuconostoc-Weissella (Lactobacillus* group), *Bifidobacterium* spp., *Clostridium* clusters I, IV, XI, and XIVa, and *Enterobacteriacaea*. Diarrhea- and enteric infection-associated virulence factors in cecal microbiota were quantified using primers (Appendix A) targeting virulence factor/toxin genes of enteropathogenic *Clostridium difficile* (tcdB) and *E. coli* (STa, STb, LT, EAST1). Samples from six animals per time point were analysed independently and data are reported as mean of six animals ± pooled standard error of the mean.

2.2.7 Antimicrobial activity of CPT-11 and SN-38 *in vitro*. The minimal inhibitory concentration (MIC) of CPT-11 and SN-38 was determined using a critical dilution assay. Four organisms of intestinal origin, *Lactobacillus reuteri* FUA3041, *Lactobacillus johnsonii* FUA3040 (both isolated from rodents), *E. coli* FUA1170 (isolated from cow rectum) and *Bifidobacterium animalis* DSM 10140 were used to represent Gram-negative and Gram-positive intestinal bacteria. CPT-11 and SN-38 concentrations ranging from 0.016 to 8 g L^{-1} and from 0.004 to 2 g L^{-1}, respectively, were tested to match or exceed concentrations found in the lumen of the colon *in vivo* [9]. Positive and negative controls (with and without inoculation of indicator strains) were used to compare the growth of bacteria in the wells.

2.2.8 Statistics. Data analysis was performed with PROC MIXED procedure (SAS v.9.2; SAS Institute, 2010) using one-way analysis of variance (ANOVA). Data were expressed as mean ± SEM. A p-value of ≤ 0.05 was considered statistically significant.

2.3 Results

2.3.1 Changes in cecal microbiota in CPT-11 chemotherapy. The effect of CPT-11 chemotherapy was initially assessed with dose-intensive CPT-11 monotherapy. The abundance of bacterial taxa in cecal samples in the dose-intensive treatment with CPT-11 is shown in Table 2-1. Data are reported as 16S rRNA gene copy numbers on a log scale. In sham-treated animals,

the total bacteria number decreased by ~1 log on the third day of treatment, and all bacterial groups except the *Clostridium* cluster XI were significantly lower compared to day 0. Particularly, the *Bacteroides* group and *Clostridium* clusters IV and XIVa were decreased by 1-3 logs. By day 7, the numbers of total bacteria and the *Bacteroides* group were restored. However, the abundance of *Clostridium* cluster XIVa, the *Lactobacillus* group, and *Bifidobacterium* spp. remained significantly lower than those at day 0. The amount of *Clostridium* cluster XI and *Enterobacteriaceae* remained ~0.5 and ~1.5 log higher than day 0, respectively. The number of *Clostridium* cluster I remained below detection limit at all time points.

Analysis of samples from CPT-11 treated animals receiving bolus glutamine gavage was carried out to allow differentiation between the effect of CPT-11 gavage and diarrhea on intestinal microbiota [16]. Bolus glutamine gavage immediately before CPT-11 injection reduced the CPT-11 induced decrease in several bacterial groups at day 3 (Table 2-1). The reductions in cecal abundance of the *Bacteroides* group, *Lactobacillus* group, *Clostridium* cluster IV, and *Enterobacteriaceae* were not as pronounced as in the sham-treated control group. The effect of glutamine was no longer observed 4 days after administration (day 7). The protective effect of glutamine gavage on cecal microbiota was confirmed by PCR-DGGE analysis (Figure 2-2). All samples from glutamine-treated animals clustered separately from samples obtained from sham-treated animals killed 6 hours after the 3rd CPT-11 dose, and most of these samples clustered together. This result further indicates that glutamine mitigated changes in intestinal microbiota. However, DGGE patterns from samples obtained four days after the last glutamine gavage (day 7) clustered together with control samples, indicating that the effect of glutamine was lost 4 days after administration.

Effects of CPT-11/5-FU therapy on cecal microbiota of tumor-bearing rats were additionally evaluated in a low-dose regimen corresponding to clinically relevant doses of chemotherapy in colorectal cancer (Table 2-2). The most pronounced changes were observed after the second cycle. At day 11, numbers of *Clostridium* cluster XI increased by ~2 logs, and *Clostridium* cluster XIVa and *Enterobacteriaceae* increased by ~0.5 log. *Clostridium* cluster IV decreased by ~0.5 log. No significant changes were detected for other bacterial groups.

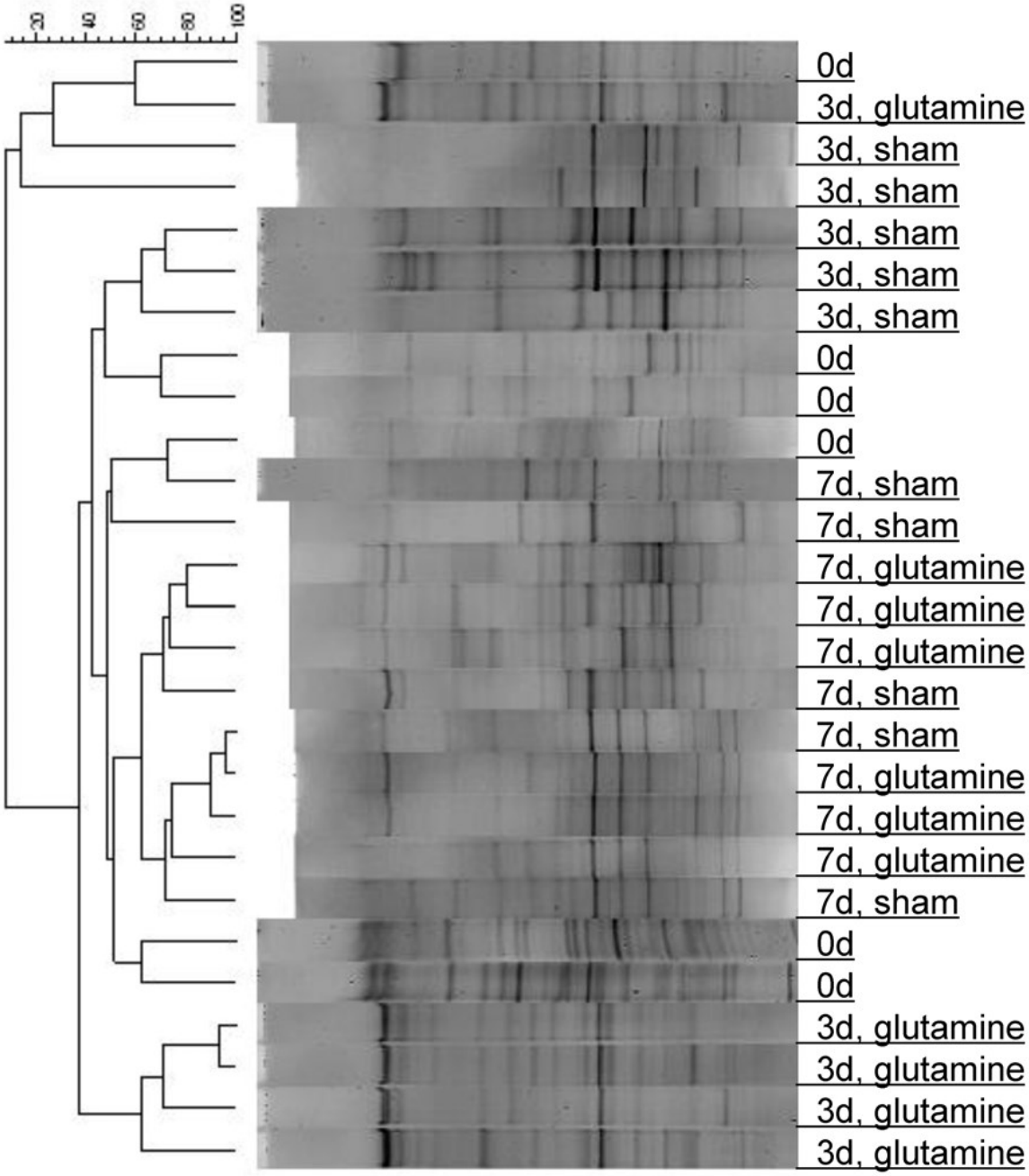


Figure 2-2. DGGE profiles of the cecal microbiota of sham- and glutamine-treated rats at day 0, day 3 (6h after the third DPT-11/glutamine administration), and day 7 in the dose-intensive regimen. Cluster analysis was performed by UPGMA algorithm based on the dice correlation coefficient.

Table 2-1. Gene copy numbers for major bacterial groups per gram of cecal contents of sham-treated rats (Cont.) and glutamine-treated rats (Gln) prior to the first cycle of chemotherapy (day 0), and 6h after the third cycle of chemotherapy (day 3), and at day 7.

	Total bacteria	***Bacteroid.***	***Lactobact.***	***Bifidobact.***	**Cluster IV**	**Cluster XI**	**Cluster XIV**	**Enterobact.**
Cont. 0 d	10.5^{a} (0.2)	8.0^{a} (0.4)	9.9^{a} (0.2)	9.4^{a} (0.2)	9.0^{a} (0.6)	7.0^{b} (0.0)	9.2^{a} (0.3)	7.7^{b} (0.6)
Cont. 3 d	9.4^{b} (0.2)	6.7^{b} (0.6)	7.3^{b} (0.7)	8.2^{c} (0.2)	7.1^{c} (0.4)	7.1^{b} (0.0)	8.7^{b} (0.1)	6.2^{c} (0.3)
Cont. 7 d	10.3^{a} (0.2)	6.9^{a} (0.2)	9.8^{a} (0.2)	8.3^{c} (0.2)	8.2^{bc} (0.5)	7.4^{a} (0.2)	8.8^{b} (0.3)	9.1^{a} (0.1)
Gln 3 d	10.6^{a} (0.1)	7.3^{a} (0.5)	10.2^{a} (0.0)	8.9^{b} (0.3)	8.4^{ab} (0.2)	7.0^{b} (0.0)	8.7^{b} (0.1)	8.1^{b} (0.7)
Gln 7 d	10.5^{a} (0.2)	6.9^{a} (0.3)	10.2^{a} (0.2)	8.4^{c} (0.2)	8.6^{ab} (0.4)	7.3^{a} (0.1)	9.1^{ab} (0.4)	9.2^{a} (0.1)

CPT-11 injections were carried out on day 1, 2, and 3 of the experiment (see Figure 1A). Glutamine bolus was administered 30 min before each CPT-11 dose. Shown are gene copy numbers of total bacteria, *Bacteroides* group (*Bacteroid.*), *Lactobacillus* group (*Lactobact.*), *Bifidobacterium* species (*Bifidobact.*) the *Clostridium* clusters IV, XI, and XIV, and Enterobacteriaceae. Data are shown as mean of six animals (pooled standard error of the mean). Values in the same column that do not share a common superscript differ significantly ($P \leq 0.05$).

Table 2-2. Gene copy numbers for major bacterial groups per gram of cecal digesta in CPT-11/5-FU regimen. Samples were taken at 0 d (prior to chemotherapy), 7 d (prior to the second cycle of thermotherapy), and 10 and 11 d (one and two days, respectively, after the second cycle of chemotherapy).

	Total bacteria	***Bacteroid.***	***Lactobact.***	***Bifidobact.***	**Cluster I**	**Cluster IV**	**Cluster XI**	**Cluster XIV**	**Enterobact.**
0 d	10.7 (0.1)	10.9^{a} (0.2)	8.1^{a} (0.4)	5.3^{b} (0.1)	5.5 (0.3)	8.7^{b} (0.2)	5.6^{c} (0.2)	8.8 (0.2)	4.4^{c} (0.1)
7 d	10.8 (0.1)	10.4^{b} (0.5)	8.1^{a} (0.4)	5.7^{a} (0.0)	5.5 (0.6)	9.2^{a} (0.2)	5.7^{bc} (0.3)	9.2 (0.1)	4.7^{b} (0.2)
10 d	10.9 (0.2)	11.1^{a} (0.3)	8.3^{a} (0.4)	5.6^{a} (0.2)	5.7 (0.3)	9.1^{a} (0.1)	6.0^{b} (0.3)	9.3 (0.1)	4.6^{bc} (0.2)
11 d	10.9 (0.1)	10.9^{a} (0.1)	7.6^{b} (0.5)	5.2^{a} (0.2)	5.7 (0.2)	8.4^{c} (0.1)	7.4^{a} (0.2)	9.1 (0.1)	5.0^{a} (0.1)

CPT-11 and 5-FU were administered as shown in Figure 1B. Shown are gene copy numbers of total bacteria, *Bacteroides* group (*Bacteroid.*), *Lactobacillus* group (*Lactobact.*), *Bifidobacterium* species (*Bifidobact.*) the *Clostridium* clusters I, IV, XI, and XIV, and Enterobacteriaceae. Data are shown as mean of six animals (pooled standard error of the mean). Values in the same column that do not share a common superscript differ significantly ($P \leq 0.05$). Superscripts are omitted for those bacterial groups that did not exhibit significant changes during the experiment.

2.3.2 Virulence factors of *E. coli* and *C. difficile* did not mediate chemotherapy-induced diarrhea. To determine whether the increased abundance of *Enterobacteriaceae* and *Clostridium* cluster XI after chemotherapy was associated with increased abundance of pathogenic or toxinogenic organisms in these groups, virulence factor/toxin genes of pathogenic *C. difficile* and *E. coli* in cecum were quantified by qPCR in cecal samples in the CPT-11/5-FU regimen. Gene copy numbers of all virulence factors or toxin gene quantified, TcdB from *C. difficile* as well as STa, STb, LT, and EAST1 from *E. coli*, were below detection limits at all time points.

2.3.3 CPT-11 and SN-38 had no antimicrobial activity *in vitro*. Dose-intensive CPT-11 therapy altered the abundance of total bacteria, and of several specific bacterial taxa, including the *Lactobacillus* group and *Enterobacteriaceae.* Prior observations indicated that antimicrobial activity of CPT-11 is not responsible for this effect [18] but did not include its active metabolite SN-38. The MICs of CPT-11 and SN-38 were determined using intestinal isolates of *Lactobacillus* spp., *E. coli*, and *Bifidobacterium animalis* as indicator strains. The MICs of CPT-11 and SN-38 were higher than 8 g L^{-1} and 2 g L^{-1}, respectively, confirming and extending previous findings that CPT-11 and its metabolites have no inhibitory effect on intestinal organisms [18].

2.3.4 Tumour induced changes in fecal microbiota. To characterize a potential effect of tumour-bearing state on host intestinal microbiota, fecal microbiota composition was continuously assessed on the same rats at the following time points: before tumour implantation (baseline); d0, before animals received a 2 cm^3 tumour burden; and after CPT-11/5-FU chemotherapy (Table 2-3). Tumour bearing state alone induced greater changes than chemotherapy. Particularly *Enterobacteriacea* and *Clostridium* cluster I and XI increased by about 1 log versus baseline. Chemotherapy-induced changes in fecal microbiota showed similar trends as in cecal samples (i.e. *Bacteroides* group, *Clostridium* cluster XI, and *Enterobacteriaceae* showed trends of increase over time) but with a smaller magnitude (Tables 2 and 3).

Table 2-3. Gene copy numbers for major bacterial groups per gram of feces from the same animals over time in CPT-11/5-FU regimen. Samples were taken from the same animal prior to tumor implantation (ref.), at 0 d (prior to chemotherapy), 7 d (prior to the second cycle of thermotherapy), and 10 and 11 d (one and two days, respectively, after the second cycle of chemotherapy).

	Total bacteria	***Bacteroid.***	***Lactobact.***	***Bifidobact.***	**Cluster I**	**Cluster IV**	**Cluster XI**	**Cluster XIV**	**Enterobact**
Ref.	10.1 (0.3)	10.7^{a} (0.4)	7.5 (0.5)	5.2 (0.2)	4.8^{b} (0.5)	8.4 (0.3)	5.6^{b} (0.2)	8.6 (0.4)	4.3^{b} (0.1)
0 d	10.4 (0.4)	10.0^{ab} (0.4)	7.7 (0.3)	4.8 (0.3)	5.7^{a} (0.5)	8.2 (0.4)	6.8^{a} (0.8)	8.8 (0.4)	5.4^{a} (0.7)
7 d	10.5 (0.2)	10.0^{ab} (0.3)	7.8 (0.4)	5.0 (0.1)	5.5^{a} (0.4)	8.4 (0.2)	7.2^{a} (0.2)	8.7 (0.4)	4.8^{a} (0.2)
10 d	10.2 (0.3)	9.8^{b} (0.4)	7.4 (0.6)	4.8 (0.2)	5.5^{a} (0.3)	8.1 (0.2)	7.0^{a} (0.3)	8.8 (0.2)	4.8^{a} (0.1)
11 d	10.5 (0.6)	10.6^{a} (0.5)	7.4 (0.2)	5.2 (0.5)	5.6^{a} (0.4)	8.3 (0.4)	7.3^{a} (0.3)	8.8 (0.5)	4.8^{ab} (0.4)

CPT-11 and 5-FU were administered as shown in Figure 1B. Shown are gene copy numbers of total bacteria, *Bacteroides* group (*Bacteroid.*), *Lactobacillus* group (*Lactobact.*), *Bifidobacterium* species (*Bifidobact.*) the *Clostridium* clusters I, IV, XI, and XIV, and Enterobacteriaceae (Enterobact). Data are shown as mean of six animals (pooled standard error of the mean). Values in the same column that do not share a common superscript differ significantly ($P \leq 0.05$). Superscripts are omitted for those bacterial groups that did not exhibit significant changes during the experiment.

2.4 Discussion

Mucositis is one of the most common side effects of radiotherapy and chemotherapy, including CPT-11 chemotherapy. Tissue damage induced by CPT-11 chemotherapy is well documented and includes apostosis of intestinal epithelial cells, resulting in malabsorption of water and electrolytes in the ileum, and hypersecretion of mucin [16, 23]. Mucositis is associated with abdominal pain, diarrhea, bacteremia, and weight loss [1]. Intestinal microbiota disruption or dysbiosis is associated with various mucositis-related diseases, including inflammatory bowel disease [24], irritable bowel syndrome [25], and colorectal cancer [26]. Especially CPT-11-induced late-onset diarrhea is linked with the function of intestinal microbiota because bacterial β-glucuronidase leads to the release of the toxic SN-38 from SN-38G in the intestine. To our knowledge, this study is the first in depth characterization of the changes of intestinal microbiota during CPT-11-based chemotherapy. Dysbiosis induced by CPT-11-based chemotherapy increased the abundance of intestinal *Enterobacteriaceae* and *Clostridium* cluster XI. These changes in intestinal microbiota are comparable to changes observed in other diseases associated with mucosal injury/and or inflammation and thus likely result from chemotherapy induced tissue damage.

CPT-11 chemotherapy consistently increased the abundance of the *Clostridium* cluster XI and *Enterobacteriaceae*, which was seen in cecal samples after both chemotherapy regimens. The diluting effect of diarrhea observed on day 3 in the dose-intensive regimen [16] reduced the abundance of most bacterial groups. In contrast, the abundance of *Clostridium* cluster XI did not decrease but increased about tenfold, indicating a dramatic increase in the proportion of this group within the total bacteria. *Enterobacteriaceae* and *Clostridium* cluster XI harbour several pathogens which induce diarrhea [27], and opportunistic pathogens which may translocate and cause systemic infections in oncology patients [28]. The absence of virulence factors of enterotoxigenic *E. coli,* enteroaggregative *E. coli* or toxin-producing *C. difficile* in cecal samples indicates that the increase of *Clostridium* cluster XI and *Enterobacteriaceae* virulence factors did not contribute to CPT-11-induced diarrhea.

The proportion of *Clostridium* cluster XI, especially *C. difficile* is low in healthy individuals for both human and rodents [29]. Although our approach quantified the *Clostridium*

cluster XI rather than *C. difficile,* the increase of *Clostridium* cluster XI in this study is consistent with the increase of the *Clostridium* cluster XI and / or *C. difficile* when human or rodent normal microbiota are severely altered in chemotherapies using various drugs [2, 3], radiotherapy [30, 31], inflammatory bowel disease [32, 33], chronic idiopathic diarrhea [34], or antibiotic treatment [35]. Infections of susceptible individuals with *C. difficile* are typically hospital-acquired, lead to damage of the colonic mucosa, and have significant mortality [35, 36]. Overgrowth of *Enterobacteriaceae* was also observed in chemotherapies [2], colitis [37, 38] and colorectal cancer [17]. Although these diseases are very distinct from each other, they are all characterized by disturbed host physiology and/or intestinal inflammation. The relative increase of *Clostridium* cluster XI and/or *Enterobacteriaceae* may thus reflect intestinal dysbiosis [24] as a result of altered function of the intestinal mucosa and the gut-associated immune system.

Changes in fecal microbiota were less pronounced compared to changes in cecal microbiota, which agreed with the observation that gut injury induced by CPT-11 chemotherapy was observed mostly in the cecum [6, 39]. In addition, the magnitude of changes of the composition of intestinal microbiota was greater in the dose-intensive regimen than in the CPT-11/5-FU regimen. This dose-dependent effect of CPT-11 chemotherapy on intestinal microbiota was in accordance with the dose-dependence of diarrhea severity [20]. In keeping with this, glutamine prevented CPT-11 induced diarrhea [16] and rats receiving glutamine experienced lesser intestinal dysbiosis than sham-treated rats despite receiving the same tumour and chemotherapy treatments. Because chemotherapy was administered exclusively to tumor-bearing animals, the dose-dependent effect of CPT-11 is independent of the effect of tumor implantation on intestinal microbiota. These findings thus support the hypothesis that mucosal injury and altered host physiology rather than chemotherapy caused dysbiosis. Glutamine can indirectly affect intestinal microbiota through prevention of damage to the intestinal mucosa [16], increased mucin production [40] and by modulating lymphocyte functions [41]. In clinical practice, chemotherapy is delivered at a dose causing significant toxicity without mortality and human CPT-11 chemotherapy results in mucosal injury and diarreal symptoms in a majority of patients [10, 12]. The dose of both CPT-11 regimens used in this study thus falls within the range used in the therapy of human colon cancer.

By following microbiota changes in the same animals over time in low-dose regimen, this study also showed that implantation of tumour per se significantly altered fecal microbiota. Microbial changes during colorectal cancer included an increase in bacterial diversity, and increase in species belonging to *Enterobacteriaceae*, but a decreased abundance of *Bacteroides* and of butyrate-producing bacteria [17, 26]. Interestingly, although tumours in this study were small and ectopic, similar microbiota changes were observed. This result indicates that tumour-bearing state can produce profound systemic effects affecting intestinal microbiota.

Studies on the interaction between host immune system and intestinal microbiota focused mainly on the modulating effect of microbes. Development of mucositis can be influenced by intestinal microbiota in several ways, including the inflammatory process, intestinal permeability, mucus secretion and composition, resistance to harmful stimuli, and the release of immune effector molecules [1]. This study indicates that changes to host physiology that are induced by tumour growth and chemotherapy-induced mucositis have a pronounced effect on intestinal microbiota. Commensal intestinal microbiota are affected by diet, the host genetic background [42] and its immune system [43]. The composition and function of mucosa-associated microbiota are regulated by mucosa-associated lymphoid tissue (MALT). The main components of MALT include mucus, secretory IgA, antimicrobial peptides secreted by Paneth cells, intraepithelial lymphocytes, dendritic cells, and macrophages [43]. Rodent quantitative trait loci that show genome-wide linkage with specific microbial taxa include loci related to host mucosal immune response such as Toll-like receptor 2 (TLR 2) pathway, lysozyme secretion, and interferon signaling by MALT [44]. CPT-11-based chemotherapy results in impaired immune functions exhibited by cytotoxic T cell depletion [16]. Cancer can induce impaired immune function [45], possibly through a modified serum cytokine profile [46, 47] and impaired interferon signaling [48], and thus may influence the MALT-mediated regulation of intestinal microbiota.

In conclusion, intestinal microbiota in rats were altered by tumor implantation, and by CPT-11-based chemotherapy. Bacterial dysbiosis in the gut induced by CPT-11-based chemotherapies must be taken into account for the etiology of mucositis and sepsis. The use of antibiotics alleviated the toxicity of CPT-11 chemotherapy [8, 9] but with apparent disadvantages. This study demonstrates that CPT-11 chemotherapy – induced changes in

intestinal microbiota favour potentially pathogenic bacteria, i.e. Enterobacteriaceae and the *Clostridium* cluster XI. Moreover the comparison of the effects of tumor, CPT-11 chemotherapy, and CPT-11 chemotherapy administered with glutamine strongly suggests that these changes are an indirect result of chemotherapy-induced by damage of the intestinal mucosa and likely involve an altered function of the mucosa-associated lymphoid tissue. In consequence, dietary intervention with glutamine, probiotics, or non-digestible carbohydrates [1] to maintain mucosal integrity during chemotherapy treatment may attenuate or mitigate the toxicity of CPT-11 chemotherapy in clinical practice.

Acknowledgements

Abha Dunichand Hoedl is acknowledged for expert technical assistance.

2.5 References

1. van Vliet MJ, Harmsen HJ, de Bont ES, Tissing WJ (2010) The role of intestinal microbiota in the development and severity of chemotherapy-induced mucositis. PLoS Pathogens 6:e1000879.
2. Stringer AM, Gibson RJ, Bowen JM, Keefe DM (2009) Chemotherapy-induced modifications to gastrointestinal microbiota: evidence and implications of change. Curr Drug Metab 10:79–83.
3. Zwielehner JC (2010) Molecular analysis of intestinal microbiota composition and function in individuals with different diets, age and disease status. Dissertation, Universität Wien, Fakultät für Lebenswissenschaften.
4. Gupta E, Lestingi TM, Mick R, Ramirez J, Vokes EE, et al. (1994) Metabolic fate of irinotecan in humans: correlation of glucuronidation with diarrhea. Cancer Res 54:3723–3725.
5. Sparreboom A, de Jonge MJA, de Bruijn P, Brouwer E, Nooter K, et al. (1998) Irinotecan (CPT-11) metabolism and disposition in cancer patients. Clin Cancer Res 4:2747–2754.
6. Takasuna K, Hagiwara T, Hirohashi M, Nomura M, Nagai E, et al. (1996) Involvement of β-glucuronidase in intestinal microbiota in the intestinal toxicity of the antitumor

camptothecin derivative irinotecan hydrochloride (CPT-11) in rats. Cancer Res 56:3752–3757.

7. Nakao T, Kurita N, Komatsu M, Yoshikawa K, Iwata T, et al. (2012) Irinotecan injures tight junction and causes bacterial translocation in rat. J Surg Res. 173:341-347.
8. Kurita A, Kado S, Matsumoto T, Asakawa N, Kaneda N, et al. (2011) Streptomycin alleviates irinotecan-induced delayed-onset diarrhea in rats by a mechanism other than inhibition of β-glucuronidase activity in intestinal lumen. Cancer Chemother Pharmacol 67:201–213.
9. Takasuna K, Hagiwara T, Hirohashi M, Kato M, Nomura M, et al. (1998) Inhibition of intestinal microbiota β-glucuronidase modifies the distribution of the active metabolite of the antitumor agent, irinotecan hydrochloride (CPT-11) in rats. Cancer Chemother Pharmacol 42:280–286.
10. Rothenberg ML (2001) Irinotecan (CPT-11): recent developments and future directions–colorectal cancer and beyond. Oncologist 6:66–80.
11. Tournigand C, André T, Achille E, Lledo G, Flesh M, et al. Mery-Mignard D, Quinaux E, Couteau C, Buyse M, Ganem G, Landi B, Colin P, Louvet C, de Gramont A (2004) FOLFIRI followed by FOLFOX6 or the reverse sequence in advanced colorectal cancer: A randomized GERCOR study. J Clin Oncol 22: 229 – 237.
12. Arnold RJ, Gabrail N, Raut M, Kim R, Sung JC, et al. (2005) Clinical implications of chemotherapy-induced diarrhea in patients with cancer. J Support Oncol 3:227-232.
13. Manhart N, Vierlinger K, Spittler A Bergmeister H, Sautner T, et al. (2001) Oral feeding with glutamine prevents lymphocyte and glutathione depletion of Peyer's patches in endotoxemic mice. Ann Surg 234:92–97.
14. Schloerb PR, Skikne BS (1999) Oral and parenteral glutamine in bone marrow transplantation: a randomized, double-blind study. JPEN J Parenter Enteral Nutr 23:117–122.
15. Decker-Baumann C, Buhl K, Frohmuller S, von Herbay A, Dueck M, et al. (1999) Reduction of chemotherapy-induced side-effects by parenteral glutamine supplementation in patients with metastatic colorectal cancer. Eur J Cancer 35:202–207.

16. Xue H, Sawyer MB, Field CJ, Dieleman LA, Murray D, et al. (2008) Bolus oral glutamine protects rats against CPT-11-induced diarrhea and differentially activates cytoprotective mechanisms in host intestine but not tumor. J Nutr 138:740–746.
17. Wang T, Cai G, Qiu Y, Fei N, Zhang M, et al. (2012) Structural segregation of gut microbiota between colorectal cancer patients and healthy volunteers. ISME J. 6:320-329.
18. Stringer AM, Gibson RJ, Logan RM, Bowen JM, Yeoh AS, et al. (2008) Faecal microbiota and beta-glucuronidase expression are altered in an irinotecan–induced diarrhea model in rats. Cancer Biol Ther 7:1919–1925.
19. Trifan OC, Durham FW, Salazar VS, Horton J, Levine BD et al. (2002) Cyclooxygenase-2 inhibition with celecoxib enhances antitumor efficacy and reduces diarrhea side dffect of CPT-11. Cancer Res 62:5778-5784.
20. Xue H, Sawyer MB, Field CJ, Dieleman LA, Baracos VE (2007) Nutritional modulation of antitumor efficacy and diarrhea toxicity related to irinotecan chemotherapy in rats bearing the ward colon tumor. Clin Cancer Res 3:7146–71-54.
21. Tannock GW, Munro K, Harmsen HJM, Welling GW, Smart J, et al. (2000) Analysis of the fecal microbiota of human subjects consuming a probiotic product containing *Lactobacillus rhamnosus* DR20. Appl Environ Microbiol 66:2578–2588.
22. Metzler-Zebeli BU, Hooda S, Pieper R, Zijlstra RT, van Kessel AG, et al. (2010) Nonstarch polysaccharides modulate bacterial microbiota, pathways for butyrate production, and abundance of pathogenic *Escherichia coli* in the pig gastrointestinal tract. Appl Environ Microbiol 76:3692–3701.
23. Ikuno N, Soda H, Watanabe M, Oka M (1995) Irinotecan (CPT-11) and characteristic mucosal changes in the mouse ileum and cecum. J Natl Cancer Inst. 87:1876-1883.
24. Marteau P, Chaput U (2011) Bacteria as trigger for chronic gastrointestinal disorders. Dig Dis 29:166-171.
25. Malinen E, Rinttilä T, Kajander K, Mättö J, Kassinen A, et al. (2005) Analysis of the fecal microbiota of irritable bowel syndrome patients and healthy controls with real-time PCR. Am J Gastroenterol 100:373-382.
26. Azcárate-Peril MA, Sikes M, Bruno-Bárcena JM (2011) The intestinal microbiota, gastrointestinal environment and colorectal cancer: a putative role for probiotics in prevention of colorectal cancer? Am J Physiol Gastrointest Liver Physiol 301:G401-424.

27. Janda JM, Abbott SL (2008) The family *Enterobacteriaceae*. In: Emanuel Goldman, Lorrence H. Green, editors. Practical Handbook of Microbiology, 2nd ed. Boca Raton: CRC Press LLC. pp. 217–229.
28. Picazo JJ (2005) Management of the febrile neutropenic patient. Int J Antimicrob Agents 26S:S120–S122
29. Franks AH, Harmsen HJM, Raangs GC, Jansen GJ, Schut F, et al. (2008) Variations of bacterial populations in human feces measured by fluorescent *in situ* hybridization with group-specific 16S rRNA-targeted oligonucleotide probes. Appl Environ Microbiol 64:3336–3345.
30. Hautmann MG, Hipp M, Kölbl O (2011) *Clostridium difficile*-associated diarrhea in radiooncology: an underestimated problem for the feasibility of the radiooncological treatment? Radiat Oncol 6:89.
31. Goodhand JR, Alazawi W, Rampton DS (2011) Systematic review: *Clostridium difficile* and inflammatory bowel disease. Aliment Pharmacol Ther 33:428-441.
32. Sinh P, Barrett TA, Yun L (2011) *Clostridium difficile* infection and inflammatory bowel disease: A review. Gastroenterol Res Pract. In press.
33. Koleva P, Valcheva R, Sun X, Gänzle M, Dieleman L. (2012) Inulin and fructo-oligosaccharides have divergent effects on colitis and commensal microbiota in HLA-B27 transgenic rats. Brit J Nutr 108:1633-1643.
34. Swidsinski A, Loening-Baucke V, Verstraelen H, Osowska S, Doerffel Y (2008) Biostructure of fecal microbiota in healthy subjects and patients with chronic idiopathic diarrhea. Gastroenterol 135:568–579.
35. Dawson LF, Valiente E, Wren BW (2009) *Clostridium difficile* - a continually evolving and problematic pathogen. Infect Genet Evol 9:1410-1417.
36. Wiegand PN, Nathwani D, Wilcox MH, Stephens J, Shelbaya A, Haider S (2012). Clinical and economic burden of *Clostridium difficile* infection in Europe: a systematic review of healthcare-facility-acquired infection. J Hosp Infect 81:1-14 .
37. Lupp C, Robertson ML, Wickham ME, Sekirov I, Champion OL, et al. (2007) Host-mediated inflammation disrupts the intestinal microbiota and promotes the overgrowth of *Enterobacteriaceae*. Cell Host Microbe 16:119-129.

38. Dorofeyev AE, Vasilenko IV, Rassokhina OA (2009) Joint extraintestinal manifestations in ulcerative colitis. Dig Dis 27:502–510.
39. Yang XX, Hu ZP, Xu AL, Duan W, Zhu YZ, et al. (2006) A mechanistic study on reduced toxicity of irinotecan by coadministered thalidomide, a tumor necrosis factor-alpha inhibitor. J Pharmacol Exp Ther 319:82–104.
40. Reeds PJ and Burrin DG (2001) Glutamine and the bowel. J Nutr 131: 2505S–2508S.
41. Xue H, Sawyer MB, Wischmeyer PE, Baracos VE (2011) Nutrition modulation of gastrointestinal toxicity related to cancer chemotherapy: from preclinical findings to clinical strategy. JPEN J Parenter Enteral Nutr 35:74-90.
42. Jermy A (2010) Symbiosis: Host genes shape the gut microbiota. Nat Rev Microbiol 8:838-839.
43. Duerkop BA, Vaishnava S, Hooper LV (2009) Immune responses to the microbiota at the intestinal mucosal surface. Immunity 31:368-376.
44. Benson A, Kelly SA, Legge R, Ma R, Low, SJ, et al. (2010). Individuality in gut microbiota composition is a complex polygenic trait shaped by multiple environmental and host genetic factors. Proc Nat Acad Sci U.S.A. 107:18933-18938.
45. Faber J, Vos AP, Kegler D, Argilés J, Laviano A, et al. (2009) Impaired immune function: an early marker for cancer cachexia. Oncol Rep 22:1403-1406.
46. Lv M, Xiaoping X, Cai H, Li D, Wang J, et al. (2011) Cytokines as prognstic tool in breast carcinoma. Front Biosci 17:2515-2526.
47. Hawinkels LJ, Ten Dijke P (2011) Exploring anti-TGF-β therapies in cancer and fibrosis. Growth Factors 29:140-152.
48. Critchley-Thorne RJ, Simons DL, Yan N, Miyahira AK, Dirbas FM, et al. (2009) Impaired interferon signaling is a common immune defect in human cancer. Proc Natl Acad Sci U S A 106:9010-9015.

3. The role of intestinal microbiota in development of Irinotecan toxicity and in toxicity reduction through dietary fibres in rats

3.1 Introduction

CPT-11 (irinotecan,7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxy-camptothecin) is a drug commonly used as a first-line chemotherapy for colorectal cancer. Therapeutic doses of CPT-11 cause prevalent toxic side-effects in patients. Late onset diarrhea is one of the most common symptoms that limit the application and efficacy of CPT-11, and has been attributed to enzymatic activities of intestinal microbiota. Gastrointestinal symptoms were substantially reduced when antibiotics or inhibitors of bacterial β-glucuronidase were used in combination with CPT-11 [1,2]. However, the use of broad spectrum of antibiotics often leads to severe disruption of the microbial homeostasis in the intestine and can result in other negative consequences [3]. A promising alternative of modulating microbiota is administration of dietary fibres, i.e. non-digestible polysaccharides that resist digestion in the small intestine and are fermented by intestinal microbiota in the large intestine. Dietary fibres not only stimulate beneficial bacteria but also to provide short chain fatty acids as an essential substrate for the colonic mucosa, and modulate activities of bacterial enzymes [4]. Therefore, they may ameliorate or mitigate CPT-11 toxicity without causing pronounced side-effects.

Figure 3-1 illustrates the potential roles of intestinal microbiota in CPT-11 toxicity. A major player in the pharmacokinetics of CPT-11 is microbial β-glucuronidase (GUD), which deconjugates the CPT-11 metabolite SN-38G to regenerate the toxic metabolite SN-38 in the large intestine. Microbial β-glucuronidase therefore is considered to be responsible for CPT-11-associated gut damage (Figure 3-1); intestinal injury and shifts in intestinal microbiota further facilitate bacterial translocation (Figure 3-1). However, microbiota can also positively affect host health through SCFA (especially butyrate) production. Bacterial groups differ in their contribution to these potential mechanisms. *Bifidobacterium* spp., *Enterobactericeae, Bacteroides* spp., *Lactobacillus* spp., *Staphylococcus* spp., and species from *Clostridium* Cluster XIVa and IV exhibit GUD activity [5]. Intestinal dysbiosis can be induced by both tumor and chemotherapy. Dysbiosis associated with cancer was characterized by an increase in *Enterobacteriaceae* and decrease in butyrl-CoA producing bacteria [6]. In dysbiosis caused by

various chemotherapies, increase in *Escherichia coli*, *Clostridium* spp., and *Staphylococcus* spp. and decrease in *Bifidobacterium* spp. and *Bacteroides* spp. were reported [7-9]. Bacterial species implicated in bacterial translocation were mostly facultative anaerobes and opportunistic pathogens, including *Enterococcus* spp., *Streptococcus* spp., *Staphylococcus* spp., and *Enterobacteriaceae* [10, 11]. Butyrate, the primary energy source for colonocytes, is mainly produced by *Clostridium* cluster IV and XIVa [12].

The multitude of potential involvements of microbiota in CPT-11 toxicity make CPT-11 treated animals a unique model for investigating the interaction between microbiota and host. This study aimed to explore the potential mechanisms through which microbiota and dietary fibres could modify host health: changes in intestinal microbial ecology, translocation, GUD activity, and SCFA production.

3.2 Methods

3.2.1 Animals and treatments. Animal use was approved by the Animal Care and Use Committee of the University of Alberta and conducted in accordance with Guidelines of the Canadian Council on Animal Care (AC08153). Female Fisher 344 rats (150–180 g of body weight and 11-12 weeks of age) were obtained from Charles River (QC, Canada). The use of female animals avoided potential confounding effects of sex, and allowed direct comparison with prior work in the same tumor model [13, 14]. Rats were housed 2 per cage in a temperature (22°C) and light controlled (12 h light) room; water and food were available *ad libitum*. One week before chemotherapy, rats were separated into individual cages. The Ward colon carcinoma was provided by Dr. Y Rustum, Roswell Park Institute. Tumor pieces (0.05g) were transplanted subcutaneously on the flank of the rats via trocar under slight isoflurane anesthesia. CPT-11 was provided by Pfizer as a ready-to-use clinical formulation. Atropine (0.6 mg/ml) was a clinical injectable formulation. Rats were killed by CO_2 asphyxiation.

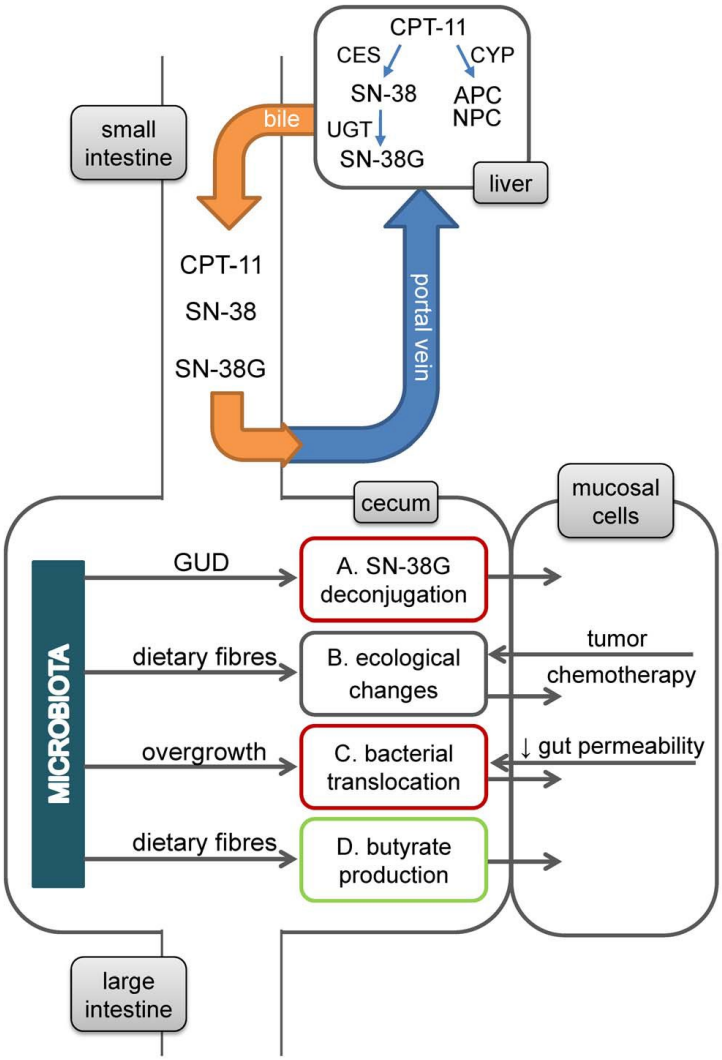


Figure 3-1. Potential roles of intestinal microbiota in development and mitigation of CPT-11 toxicity. *In vivo*, CPT-11 is converted to the pharmacologically active SN-38 (7-ethyl-10-hydroxy-camptothecin), which is responsible for both anti-tumor activity and dose-limiting toxicity. SN-38 undergoes hepatic glucuronidation and is secreted into the bile as inactive the glucuronide SN-38G. Deconjugation of SN-38G in large intestine by bacterial β-glucuronidases intensifies the epithelial exposure to SN-38 and mediates gut toxicity [2, 43, 44]. APC (7-ethyl-10-[4-N-(5-aminopentanoic acid)-1-piperidino]carbonyloxycamptothecin) and NPC (7-ethyl-10-(4-amino-1-piperidino)carbonyloxycamptothecine) are both important CPT-11 metabolites produced by alternative hepatic detoxification mechanism, but they play little role in gastrointestinal toxicity [45].

The composition of intestinal microbiota was reported to be altered in colorectal cancer patients [6, 8] and by various chemotherapeutic agents [7, 9]. On the other hand, dietary fibres are known to promote the growth of beneficial bacteria in animals and human [46]. Bacterial translocation to extraintestinal sites, was reported to be increased by CPT-11 treatment [7, 47] increased the risk of systemic infection. Bacterial translocation from intestine can be promoted by overgrowth of selective species in intestinal microbiota and/or a compromised host defense system, including immunodeficiency and increased intestinal permeability [11], both of which have been reported in CPT-11-treated animals [7, 48]. Dietary fibres have been shown to promote intestinal health through bacterial fermentation products short-chain fatty acids (SCFA), especially butyrate. The majority of butyrate is consumed by mucosal cells. Its main functions in intestine include (1) promoting proliferation and growth of normal colonocytes, (2) enhancing epithelial barrier function, and (3) suppressing inflammation and oxidative stress [36, 42] Therefore, the protective effect of butyrate may counteract injuries caused by SN-38.

3.2.2 Diet. Diets used in this study are described elsewhere [13]. Briefly, semi-purified diet was based on AIN-76 basal diet, with a modified fat component similar to a North American dietary pattern with respect to energy % as fat and levels of n-3, n-6, saturated and polyunsaturated fatty acids. Rats were initially fed Rodent Laboratory Chow (Harlan Teklad, Madison, WI). During the adaptation period, this non-purified diet was mixed with study diet (50/50, w/w) for one week, followed by transition to 100% semi-purified diet starting 2 weeks prior to tumor implantation.

3.2.3 Experimental design. All experiments used a two-cycle CPT-11/5-fluorouracil (5-FU) therapy which recapitulates clinical therapy of colorectal cancer (Figure 3-2). Rats received two cycles of CPT-11/5-FU treatment. The day before first CPT-11 injection was designated day 0. Animals received CPT-11 (50 mg/kg) and 5-FU (50 mg/kg) injections on days 1 and 8 and on days 2 and 9, respectively. **Exp 1** was designed to investigate the influence of chemotherapy on intestinal microbiota and bacterial translocation [9]. Animals (n=6/group) were killed on day 0, day 7 (prior to the second treatment cycle), and days 10 and 11 (one and two days after the 2nd treatment cycle). **Exp 2** was designed to evaluate and compare the effect of dietary fibres on CPT-11 toxicity. Animals (n=6/group) received diets containing the following dietary fibres:

commercial isomalto-oligosaccharides (IMO); fructooligosaccharides (FOS); inulin; a 1:1 mixture of FOS and inulin (synergy); and Type IV resistant starch (RS). These fibre sources differing in their structure and / or degree of polymerization were incorporated into the modified AIN-76 basal diet at 8% w/w. The dietary composition is shown in Appendix B1. A seventh diet where the fibres were substituted with equal amount of starch was used as control. All diets contained 2% w/w cellulose. **Exp 3** was designed to compare two different dietary fibres with larger sample size. IMO and synergy were selected as the treatment diet. Cellulose, which has the lowest fermentability in rat intestine, was used as control. Healthy rats without tumor or chemotherapy were used as reference. Sample size was 14 animals per treatment for synergy and IMO diets and 6 per treatment for healthy control and cellulose diet. In experiments 2 and 3, animals were killed at day 9 as chemotherapy-induced morbidity and mortality become apparent at this time [14].

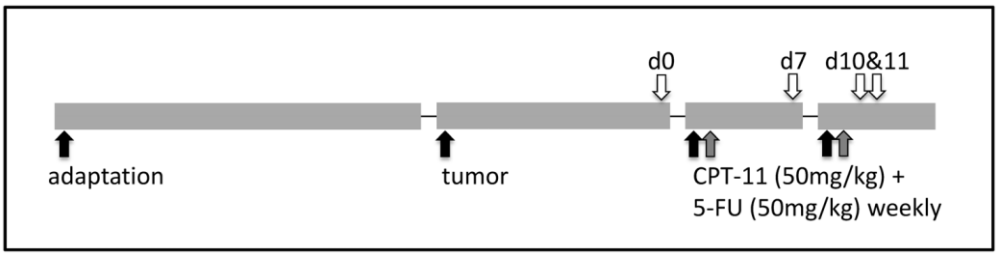


Figure 3-2. Experimental design. Black arrows represent diet, tumor, and CPT-11 treatments; grey arrows represent treatment with 5FU. Sampling at day 10 and 11 after the first CPT-11 treatment is indicated by white arrows.

3.2.4 Cecal gene expression of monocaboxylate transporter 1 (MCT1), glutathione S-transferase (GSTP1), and mucin genes. Gene expression was quantified using cecal tissue collected in Exp 3. Tissues were stored in Trizol® (Invitrogen) at -80°C. For RNA extraction, tissues were thawed in Trizol and homogenized using Kontes Pellet Pestle (Fisher Scientific). The homogenized solution was mixed with 20% v/v of chloroform and centrifuged for 15 min at 4°C. The supernatant mixed with an equal volume of 70% ethanol and applied to the column from Qiagen Rneasy Mini Kit (Qiagen, Mississauga, Canada). RNA was purified following the manufacturer's instructions and then reverse-transcribed to cDNA using QuantiTect Reverse Transcription Kit (Qiagen). Relative gene expression was calculated as

$(E_{target})^{\Delta CP(control-sample)} / (E_{reference})^{\Delta CP(control-sample)}$ [15]. Expression of MCT1, GSTP1, and the mucin genes MUC1 and MUC2 were quantified using the ubiquitin c gene *ubc* as reference gene due to its stable expression in normal and CPT-11-treated rats [16, 17]. Healthy rats fed a 10% cellulose diet were used as control. Primers used were: *gstp1,* forward: 5'-GAT GGG GTG GAG GAC CTT CGA TGC-3', reverse: 5'-CTG AGG CGA GCC ACA TAG GCA GAG-3' [18]; *mct1*, forward: 5'-CAG TGC AAC GAC CAG TGA AGT G-3', reverse: 5'-ATC AAG CCA CAG CCA GAC AGG-3' [19]; *ubc*, forward: 5'-ATC TAG AAA GAG CCC TTC TTG TGC-3', reverse: 5'-ACA CCT CCC CAT CAA ACC C-3' [17]; *muc1*, forward: 5'-CGC CGA AAG AGC TAT G-3', reverse: 5'-TAA GAG AGA CCG CTA CTG CC-3' [20]; *muc2*, forward: 5'-GCC AGA TCC CGA AAC CA-3' and reverse: 5'- TAT AGG AGT CTC GGC AGT CA-3' [21]; IL-1β, forward: 5'-GCA CCT TCT TTT CCT TCA TC-3' and reverse: 5'-CTG ATG TAC CAG TTG GGG AA-3'; TNF-α, forward: 5'-GGC AGG TCT ACT TTG GAG TCA TTG C-3', reverse: 5'-ACA TTC GGG GAT CCA GTG AGT TCC G-3' [22]. Quantitative PCR was performed on 7500 Fast Real-Time PCR (Applied Biosystem) using SYBR Green reagents (Qiagen). DNase-treated RNA samples were also amplified as negative controls to ensure the quality of reverse transcription.

3.2.5 DNA extraction and quantification of major bacterial groups by quantitative PCR (qPCR). DNA was extracted from digesta samples using the QIAamp DNA Stool Mini Kit (Qiagen). Quantitative PCR was performed. Major bacterial groups in cecal and fecal microbiota were quantified using group-specific primers targeting total bacteria, *Bacteroides-Prevotella-Porphyromonas* (*Bacteroides* group), *Lactobacillus-Pediococcus-Leuconostoc-Weissella (Lactobacillus* group), *Bifidobacterium* spp., *Clostridium* clusters I, IV, XI, and XIV, *Enterobacteriacaea* [23], *Ruminococcus gnavus* [24], and *Akkermansia muciniphila* [25]. Diarrhea- and enteric infection-associated virulence factors in cecal microbiota were quantified using primers described previously targeting virulence factor/toxin genes of enteropathogenic *Clostridium difficile* (tcdB) and *E. coli* (STa, STb, LT, EAST1) [22].

3.2.6 Identification of bacterial species carrying GUD genes in cecal microbiota. Cecal DNA in Exp 2 were amplified with primers targeting the bacterial GUD *gus* gene (forward: 5'-TAT TTA AAA GGI TTY GGI MRI CAY GAR-3', reverse: 5'-CCT TCT GTT GTI KBR AAR TCI GCR AAR-3') [26]. PCR amplification was performed using the following conditions: initial

denaturation (3 min at 94 °C), then 35 cycles of denaturation (30 s at 94 °C), ramped annealing (20 s at 55°C, 5 s at 50 °C, and 5 s at 45 °C), and elongation (1 min at 72 °C) and a final extension (7 min at 72 °C). PCR products were cloned with TOPO TA Cloning kit (Invitrogen) according to the manufacturer's instructions. Sequencing was done at Macrogen Inc. with vector primers M13F (5'-GTA AAA CGA CGG CCA G-3') and M13R (5'-GGAAACAGCTATGAC-3') and sequences were identified by comparing to the known *gus* genes in the GenBank database using BLAST.

3.2.7 Quantification of cecal lactate and SCFA concentrations. Cecal samples from Exp 2 and 3 were incubated with 7.5% perchloric acid at 4 °C overnight to remove proteins. Lactate and SCFA were separated using an Aminex 87H column (Bio-Rad, Mississauga, Ontario) at a temperature of 70 °C, and the solvent was 5 mM H_2SO_4, at a flow rate of 0.4 mL/min. Metabolites were visualized using a UV detector at 210 nm, and quantified using external standards.

3.2.8 Quantification of haptoglobin and acute phase proteins. For quantification of haptoglobin and acute phase proteins in blood, commercial ELISA kits for rat α-1-acid glycoprotein (AGP) and rat haptoglobin were purchased from Life Diagnostics (West Chester, Pennsylvania, USA) and used according to the manufacturer's instructions [27].

3.2.9 Quantification of CPT-11 metabolites in cecum and jejunum digesta. Cecal digesta from Exp 2 and cecal and jejunum digesta from Exp 3 were analyzed for CPT-11 and its metabolites. The digesta were extracted 3 times with 67% methanol overnight and solids were removed by centrifugation. The supernatant was then diluted 6 times with water. Metabolite concentration was quantified using a 4000 QTRAP® LC/MS/MS System (AB Sciex, Canada) following a protocol by Corona et al. [28] with slight modifications. A Kinetex™ 2.6 µm C18 100 x 3 mm column (Phenomenex, Canada) was used. Assays were performed using LC-MS/MS under ESI-MRM mode. The flow rate was 0.4 mL/min. The acetonitrile gradient increased from 10% to 80 % from 0 to 18 min, maintained at 100% from 18.1 to 19.0 min, and returned to 10% from 19.4 to 25 min. MRM transitions used were: *m/z* 587→167 for CPT-11, *m/z* 393→349 for SN-38 (7-ethyl-10-hydroxy-camptothecin), *m/z* 619→393 for APC (7-ethyl-10-[4-N-(5-aminopentanoic acid)-1-piperidino]carbonyloxycamptothecin), *m/z* 519→393 for NPC (7-ethyl-

10-(4-amino-1-piperidino)carbonyloxycamptothecine), and *m/z* 569→393 for SN-38-glucuronide (SN-38G). Quantification was done with standards prepared from pure CPT-11, APC, NPC, SN-38, SN-38G purchased from Tocris Bioscience, UK.

3.2.10 Isolation and identification of bacteria from mesenteric lymph nodes. Bacterial translocation to mesenteric lymph nodes was determined with samples obtained in Exp 1. Isolates from mesenteric lymph nodes were obtained by serial dilution streaking on sheep blood agar plates and incubation at 37°C for 48 h under aerobic conditions. Isolates were subsequently cultured in Brain Heart Infusion medium at 37°C. DNA was extracted from overnight cultures using DNeasy Blood, Tissue Kit (Qiagen, USA). Bacterial 16S rRNA genes were amplified using 616V (5' –AGA GTT TGA TYM TGG CTC-3') and 630R (5'-CAK AAA GGA GGT GAT CC-3') universal primers, and sequenced at Macrogen Corp. (MD, USA). For identification of isolates on genus or species level, sequences were matched to sequences of type strains deposited in the Ribosomal Database Project (rdp.cme.msu.edu).

3.2.11 Statistical analysis. Statistical analysis was performed with PROC MIXED procedure (SAS v.9.2; SAS Institute, 2010) using one-way analysis of variance (ANOVA). Associations between variables were analyzed using Spearman correlation. Variables with non-parametric distribution were log-transformed prior to analysis. A p-value of ≤ 0.05 was considered statistically significant.

3.3 Results

3.3.1 The diet effect on animal health was not dependent on the type of fibre. In all three experiments, animals experienced weight loss and a significant reduction in feed intake following each chemotherapy cycle, with the effect of the second cycle being more pronounced [14, 28]. Addition of non-digestible carbohydrates significantly influenced feed intake, body weight and other indicators of animal health [22]. However, all treatment groups displayed large intra-group variation indicating that factors other than the diet also had a major influence on animal health. The six different fibres used in Exp 2 resulted in a range of outcomes in rats: animals fed on RS and Synergy had a significantly lower weight loss and reduction in feed intake compared to those fed on starch and IMO [22]. In Exp 3, healthy rats without tumor and not

receiving chemotherapy had much higher body weight and food intake than chemotherapy-treated rats.

3.3.2 Overgrowth of the intestine by opportunistic pathogens is not a major contributor to CPT-11 toxicity. No bacterium was cultured from mesenteric lymph nodes of healthy animals. Bacteria in mesenteric lymph nodes from chemotherapy treated animals (Exp 1 and 2) were isolated and identified by partial sequencing of 16S rRNA genes. Isolates from infected mesenteric lymph nodes belonged to the family *Enterobacteriaceae* and the genera *Enterococcus* and *Staphylococcus* (Appendix B2). To determine whether translocation was favoured by a higher abundance of these opportunistic pathogens in the gut lumen, bacterial taxa representing translocated bacteria were quantified in cecal digesta by qPCR (Figure 3-3). *Morganella morganii* was below the detection limit in all samples. An increased abundance of *Citrobacter freundii, Klebsiella oxytoca,* and *Enterococcus* spp. following chemotherapy was observed, but the abundance of other translocated organisms remained unchanged (Figure 3-3). Despite unfavourable changes in intestinal microbiota during chemotherapy, compromised barrier properties of the gut mucosa as a result of CTP-11 toxicity rather than overgrowth of the intestine by opportunistic pathogens appears to be the primary cause of bacterial translocation.

3.3.3 Effect of fibres on intestinal microbiota and host health: Experimental design and data analysis. The effect of dietary fibre on intestinal microbiota and host health was assessed in two experiments. An initial, explorative experiment compared six different purified fibre types with minimal statistical power (N=6). A second experiment compared to purified fibre types, IMO and synergy, with a higher number of animals per group (n=14). Intestinal and host parameters for experiments 2 and 3 are reported by diet in tables 3-1 and 3-2, respectively. Because differences between diets were not greater than the differences between animals in the same group, results relating to host health and intestinal microbiota were also correlated to each other after pooling all animals used in experiment 2 and experiment 3. The results are depicted in heat maps (Figures 3-4 and 3-5, respectively).

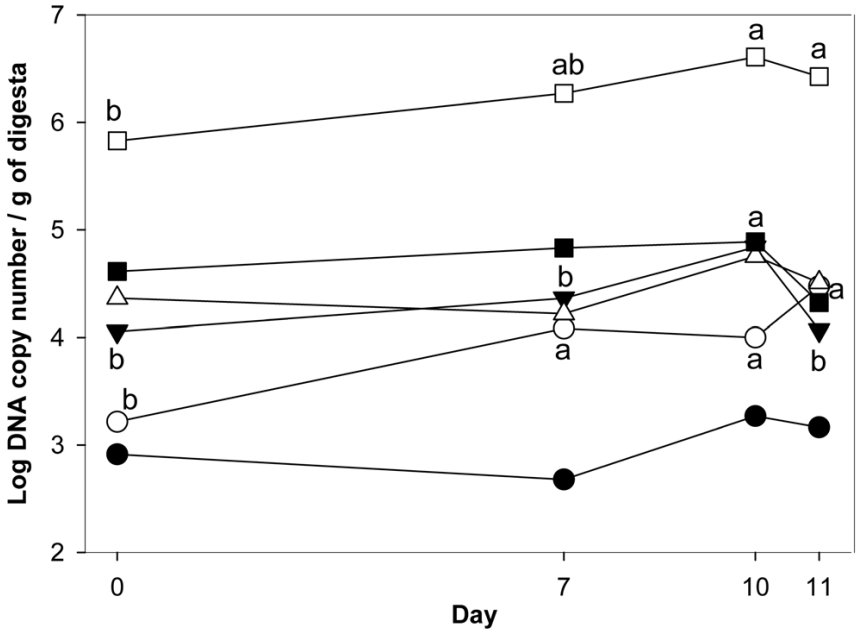


Figure 3-3. Gene copy numbers for major bacterial groups per gram of cecal digesta at day 0, 7, 10, and 11 in Exp 1. Symbols indicate *Proteus mirabilis* (●), *Citrobacter freundii* (○), *Klebsiella oxytoca* (▼), *Escherichia coli* (△), *Staphylococcus* spp. (■), and *Enterococcus* spp. (□). Data are shown as mean±SEM. Values obtained with the same primers that do not share a common letter differ significantly; i.e. vary over time.

3.3.4 Cecal GUD activity. Chemotherapy treated animals with a high body weight and feed intake surprisingly also had high intestinal GUD activity [22]. Correlation analysis confirmed that fecal GUD activity was positively correlated to body weight and feed intake but negatively correlated to the inflammation marker IL1-β (Figure 3-4). Protective effects of dietary fibre on CPT-11 toxicity were thus not mediated by reduced activity of intestinal GUD. To further elucidate roles of bacterial GUD, concentrations of CPT-11 and its metabolites were quantified in the cecum and the jejunum (Tables 3-1 and 3-2). Concentrations of CPT-11 and its metabolites varied over a wide range. Of the CPT-11 metabolites, SN-38G was present in the highest concentrations in the jejunum. Conversely, SN-38 was present in the highest concentrations in the cecum, where SN-38G was essentially absent (Tables 3-1 and 3-2). These data conform to the established role of bacterial GUD in CPT-11 toxicity, and additionally demonstrate that modulation of intestinal GUD activity by dietary intervention does not significantly reduce conversion of SN-38G to the toxic SN-38 in the cecum. Intestinal

concentrations of CPT-11 metabolites were positively correlated to each other and to CPT-11 but were not significantly related to body weight and feed intake (Figures 3-4 and 3-5). Interestingly, GUD activity was negatively correlated to cecal SN-38 concentrations (Figure 3-4), suggesting that deconjugation of SN-38G by GUD was not the main factor determining levels of SN-38 exposure.

To identify bacterial species responsible for GUD activity, cecal DNA in Exp 2 was amplified with degenerate primers specific for bacterial GUD and amplicons were sequenced (Table 3-3). In total, 23 out of 98 sequences were positively identified as GUD. two groups that were identified most frequently were *Ruminococcus gnavus* (8 out of 23) from *Clostridium* cluster XIVa, and *Enterobacteriaceae* (6 out of 23) which included the species *Edwardsiella, Escherichia,* and *Edwardsiella*.

3.3.5 Reduction in CPT-11 toxicity by dietary fibres was not due to stimulation of beneficial bacterial groups. Beneficial effects of inulin and fructo-oligosaccharides are often attributed to stimulation of specific bacterial groups according to the concept of "prebiotics" [4]. To determine whether the influence of dietary fibre on CPT-11 toxicity can be attributed to their prebiotic effects, abundance of major bacterial groups was determined in samples obtained from experiments 2 and 3. Tables 1 and 2 depict abundance bacterial groups in animals fed different diets in Exp. 2 and 3, respectively. Additionally, the abundance of bacterial groups was correlated to all other parameters that were quantified in Exp. 2 and 3. Heat maps depicting significant correlations in Exp. 2 and 3 are depicted in Figures 3-4 and 3-5, respectively. In Exp 2, only the abundance of the *Bacteroides* group (highest levels with FOS) and of the *Lactobacillus* group (highest levels with starch and resistant starch) differed among the seven diets (Table 3-1). However, none of the bacterial groups correlated to markers of animal health (Figure 3-4). Owing to the larger number of animals per group, Exp. 3 revealed more significant differences in intestinal microbiota of animals fed different dietary fibres (Table 3-2). Fructans increased abundance of lactobacilli and bifidobacteria but decreased abundance of the *Clostridium* cluster XI. However, none of the indicators of CPT-11 toxicity correlated to the abundance of specific bacterial groups. These results indicate that microbial ecology was altered by different fibre types; however, these changes did not mediate changes in CPT-11 toxicity.

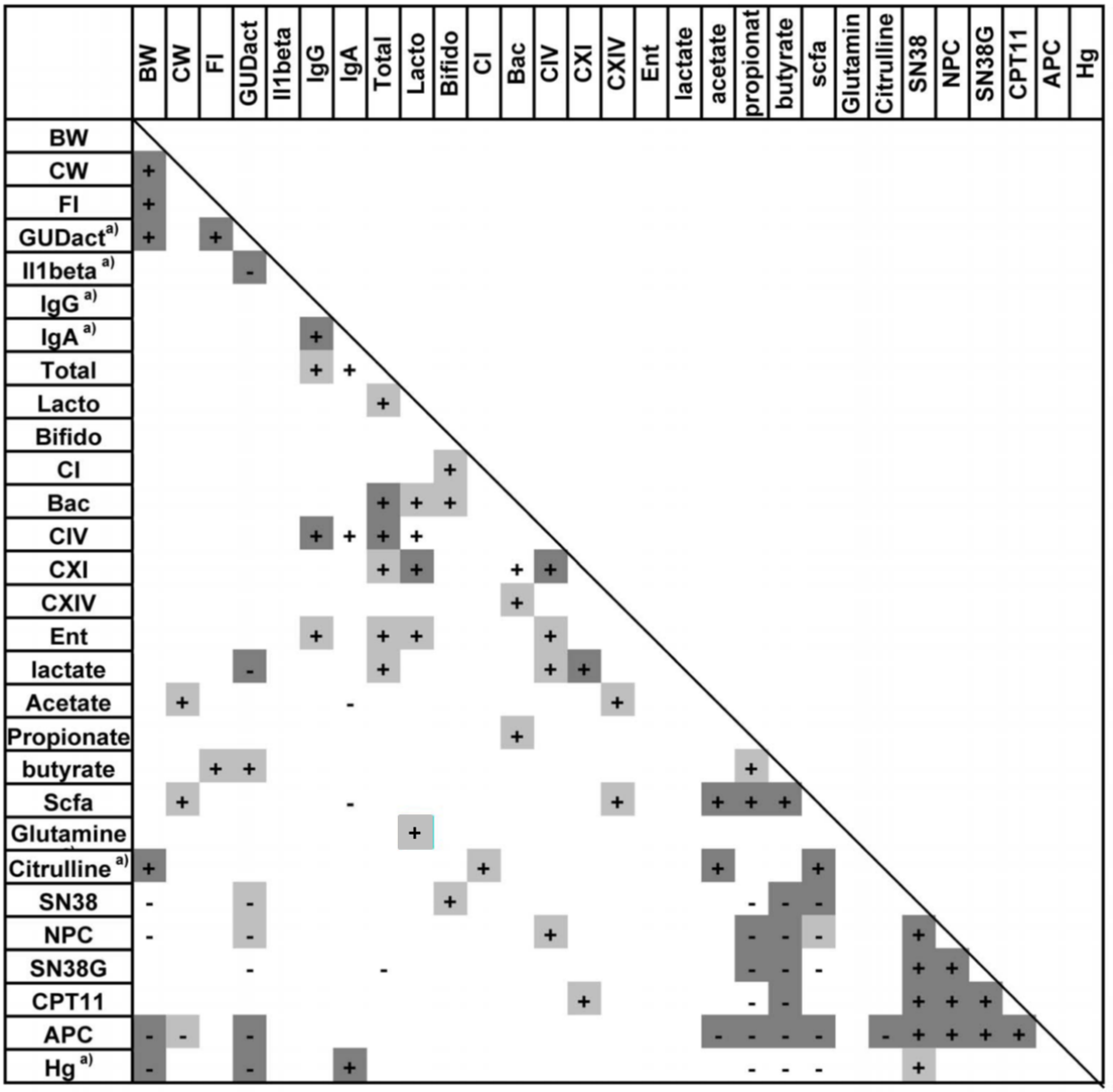


Figure 3-4. Pixel mapping highlights of major microbiota-host correlations of Exp 2. + indicates a positive correlation; - indicates negative correlation. Spearman correlation p-values are displayed as a greyscale, dark grey, $p<0.005$; light grey, $p<0.05$; no shading, trend ($p<0.1$). Key: BW, relative body weight; CW, colon weight; FI, cumulative food intake; Total, total bacteria; Lacto, *Lactobacillus* group; Bifido, *Bifidobacterium* spp.; CI, *Clostridium* cluster I, Bac, *Bacteroides* group; CIV, CXI, CXIV, *Clostridium* clusters IV, XI, and XIV; Ent, *Enterobacteriacaea*; scfa, short chain fatty acids; hg, haptoglobin

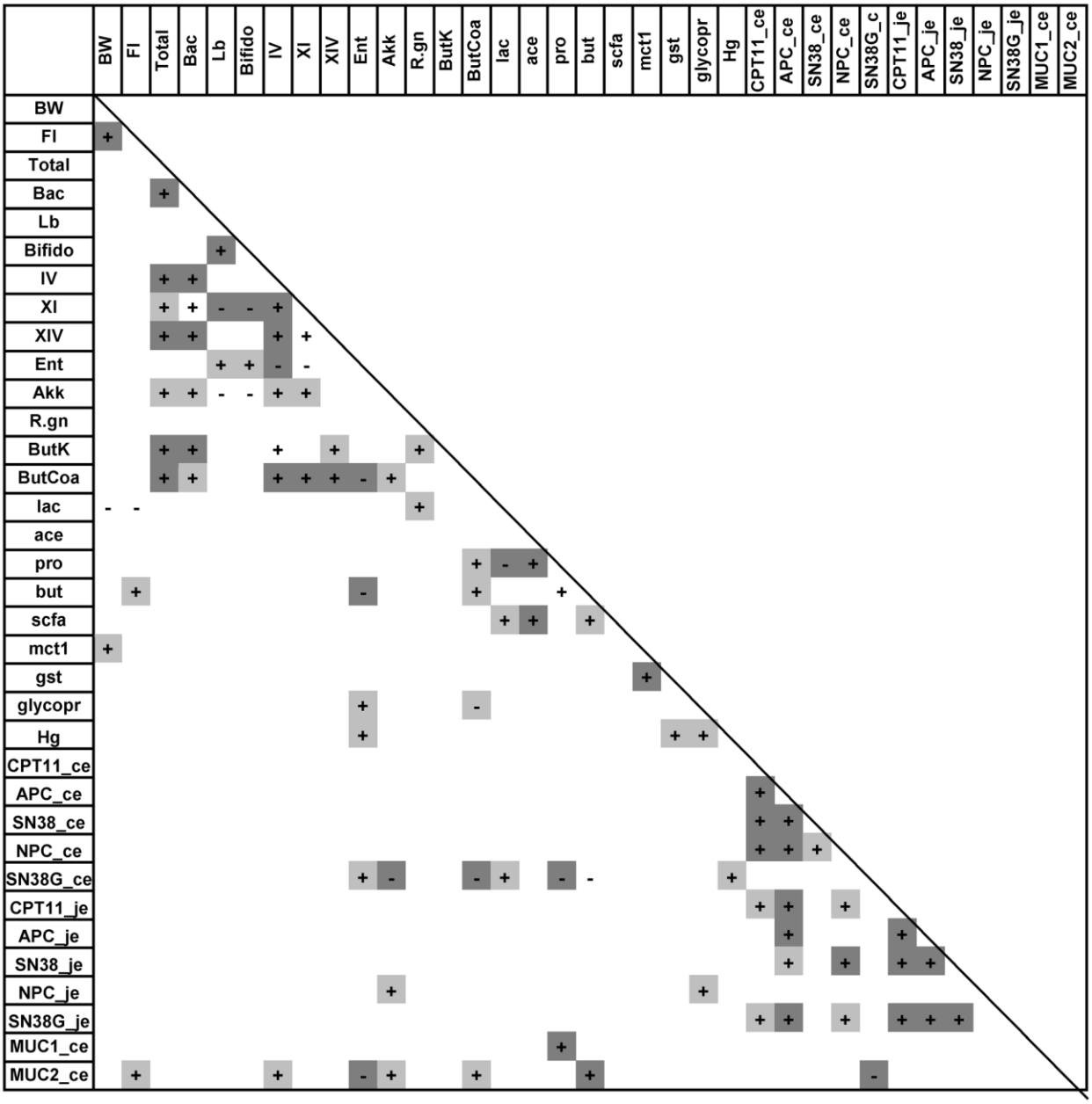


Figure 3-5. Pixel mapping highlights of major microbiota-host correlations of Exp 3. Spearman correlation p-values are displayed as a greyscale, dark grey, $p<0.005$; light grey, $p<0.05$; no shading, trend ($p<0.1$) . Key: BW, relative body weight; FI, cumulative food intake; Total, total bacteria; Bac, *Bacteroides* group; Lb, *Lactobacillus* group; Bifido, *Bifidobacterium* spp.; CIV, CXI, CXIV, *Clostridium* clusters IV, XI, and XIV; Ent, *Enterobacteriacaea*; Akk, *Akkermansia;* R. gn. *Ruminococcus gnavus*; lac, lactate; ace, acetate; pro, propionate; but, butyrate; scfa, short chain fatty acids; mct1, relative expression of monocarboxylate transporter 1; gst, relative expression of glutathione-S-transferase; glycopr, blood glycoprotein; hg, blood haptoglobin;

CPT11_ce and CPT11_je, cecal and jejunal concentration of CPT-11, respectively; APC_ce and APC_je, cecal and jejunal concentration of APC, respectively; SN38_ce and SN38_je, cecal and jejunal concentration of SN38, respectively; NPC_ce and NPC_je, cecal and jejunal concentration of NPC, respectively; SN38G_ce and SN38G_je, cecal and jejunal concentration of SN38-G, respectively; MUC1_ce and MUC2_ce, relative expression of *muc1* and *muc2*, respectively, in cecal tissue.

3.3.6 Reduction of toxicity by dietary fibres was associated with enhanced butyrate production. Whereas specific changes in composition of intestinal microbiota were unrelated to CPT-11 toxicity, activity of intestinal microbiota appeared to be a critical factor in CPT-11 toxicity. Cecal butyrate concentrations and feed intake were highly correlated in both experiments (Figure 3-4 and 3-5). Luminal concentrations of SCFA are a poor indicator of metabolic activity of intestinal microbiota because they represent a balance of microbial SCFA production and host absorption [29], therefore, abundance of genes coding for bacterial butyrate kinases and butyryl-CoA:acetate CoA-transferases as well as host expression MCT1 were additionally quantified. MCT1 expression had positive correlation with body weight and abundance of butyryl-CoA gene was positively correlated with butyrate concentration (Figure 3-5). Taken together, these correlations support the interpretation that the influence of dietary fibre on CPT-11 toxicity is partially mediated by an increased cecal production of butyrate.

Table 3-1. Abundance of major bacterial groups and SCFA concentration per gram of cecal digesta in Exp 2. Values within brackets are 95% confidence intervals for variables with parametric distribution and 5% and 95% percentiles for variables with non-parametric distribution. Values within a row without superscripts are not significantly different. Values without superscripts are not significantly different from any other value in the same row. Values within a row that do now share a common superscript are significantly different ($P<0.05$).

		Starch	IMO	Cellulose	FOS	Inulin	Synergy	RS
Total bacteria	copy number/ g of cecal digesta	9.0 (.4)	8.8 (.3)	8.7 (.3)	9.1 (.3)	8.8 (.6)	9.2 (.3)	9.3 (.3)
***Bacteroides* group**		9.6^{ab} (.4)	9.6^{ab} (.4)	9.5^{b} (.1)	1.0^{a} (.3)	9.7^{ab} (.4)	9.8^{ab} (.2)	1.0^{ab} (.4)
***Lactobacillus* group**		8.1^{a} (.4)	7.9^{ab} (.5)	7.4^{b} (.1)	7.8^{ab} (.7)	7.6^{ab} (.5)	7.8^{ab} (.1)	8.3^{a} (.4)
***Bifidobacterium* spp.**		6.0 (5.7-6.1)	5.7 (4.7-6.0)	5.7 (5.6-6.0)	5.8 (5.4-7.8)	5.9 (4.5-6.4)	5.8 (5.6-6.0)	5.8 (4.9-6.3)
Cl. cluster I		6.3 (.5)	6.0 (.5)	5.8 (.3)	6.6 (.6)	6.7 (1.0)	6.2 (.8)	6.1 (.4)
Cl. cluster IV		8.6 (.6)	8.2 (.5)	8.1 (.4)	8.2 (.6)	8.0 (.6)	8.5 (.5)	8.7 (.4)
Cl. cluster XI		4.9 (4.6-5.8)	4.8 (4.0-6.1)	4.4 (4.2-5.0)	4.7 (3.6-5.4)	3.8 (3.5-5.0)	4.7 (3.7-5.3)	4.8 (4.3-5.3)
Cl. cluster XIVa		8.1 (7.8-8.9)	8.7 (7.5-8.9)	8.1 (7.8-9.8)	8.7 (8.0-9.3)	8.6 (7.5-9.0)	9.0 (7.8-9.7)	8.8 (8.4-8.9)
Enterobacteriaceae		5.4 (.8)	4.1 (.2)	3.9 (.2)	4.5 (.8)	4.6 (1.0)	4.3 (.5)	4.2 (.3)
Lactate	µmol/g of cecal digesta	11.5 (6.16-23.1)	1.9 (7.54-14.9)	8.47 (6.34-1.7)	8.28 (5.22-11.1)	6.32 (4.37-8.03)	11.3 (7.27-19.9)	7.46 (4.05-19.0)
Acetate		32.7 (7.35)	24.1 (5.81)	28.4 (4.03)	37.0 (9.73)	3.6 (3.40)	3.5 (7.70)	28.7 (8.77)
Propionate		6.42 (1.30)	5.77 (.86)	5.52 (1.17)	7.70 (2.44)	6.54 (.49)	6.70 (.66)	7.34 (1.65)

Butyrate		4.02 (1.64-6.87)	3.35 (1.04-6.93)	3.21 (2.21-4.00)	8.51 (2.02-18.0)	1.7 (2.92-24.7)	4.19 (2.58-5.82)	7.33 (3.01-1.6)
CPT-11	µg/g cecal digesta	86.3 (35.2-123.0)	55.0 (15.1-93.2)	43.6 (27.1-68.1)	25.5 (1.4-8.5)	32.9 (9.55-84.2)	25.5 (12.6-62.3)	29.7 (21.9-88.1)
SN-38		13.9 (5.2-23.0)	7.83 (3.09-13.8)	9.29 (2.35-11.8)	3.70 (.93-5.55)	7.10 (3.65-18.5)	4.70 (1.68-8.48)	6.01 (2.60-2.0)
APC		9.02 (2.64-21.9)	18.8 (2.40-113.4)	8.47 (6.41-14.3)	3.68 (1.94-6.25)	5.55 (2.52-17.9)	4.02 (2.14-7.79)	3.74 (2.29-113.4)
NPC		.23 (.063-.25)	.062 (.023-.141)	.093 (.048-.164)	.038 (.015-.079)	.046 (.013-.119)	.039 (.021-.081)	.049 (.026-.386)
SN-38G		.041(.007-.138)	0	.047 (.009-.101)	0	0	0	0

Table 3-2. Abundance of major bacterial groups, stress response, MCT1 expression, SCFA concentration, and CPT-11 metabolite concentrations per gram of cecal digesta in Exp 3. Values within brackets are 95% confidence intervals for variables with parametric distribution and 5% and 95% percentiles for variables with non-parametric distribution. Values without superscripts are not significantly different from any other value in the same row. Values within a row that do now share a common superscript are significantly different (P<0.05).

		IMO	SYN	CEL	Ref
Relative body weight		.91 (.01)^{b}	.92 (.01)^{b}	.93 (.02)^{ab}	1.00 (.02)^{a}
Relative food intake		.73 (.07)^{b}	.76 (.07)^{b}	.78 (.014)^{b}	.99 (.07)^{a}
Total bacteria	Gene copy number/g of cecal digesta	1.7 (.1)	1.7 (.1)	1.7 (.2)	1.6 (.3)
Bacteroides group		9.9 (.1)	9.8 (.2)	9.8 (.3)	9.4 (.2)
Lactobacillus group		8.5^{b} (.2)	9.1^{a} (.3)	8.9^{ab} (.5)	9.0^{ab} (.3)
Bifidobacterium spp.		5.7^{c} (.3)	7.2^{a} (.3)	6.4^{b} (.5)	6.9^{ab} (.4)
Clostridium cluster IV		8.4 (7.9-8.7)	8.0 (6.3-8.8)	8.5 (7.3-8.8)	8.8 (8.2-8.8)
Clostridium cluster XI		5.2^{b} (.0-6.0)	.0^{c} (.0-1.9)	4.8^{b} (1.1-5.5)	5.6^{a} (5.4-5.8)
Clostridium cluster XIVa		10.0 (.1)	10.1 (.2)	10.0 (.2)	10.2 (.4)
Enterobacteriacea		8.2 (.3)	8.8 (.6)	7.8 (.7)	7.4 (1.0)
Akkermansia muciniphila		8.5 (.3)	8.0 (.2)	7.7 (.6)	8.6 (.5)
Ruminococcus gnavus		6.8 (.4)	7.0 (.3)	6.4 (.8)	7.1 (.3)
Butyrate kinase		6.3 (.4)	6.3 (.3)	6.1 (.6)	5.4 (.1)
Butyryl-CoA transferase		5.8 (.2)	5.3^{b} (.5)	5.6 (.8)	6.4^{a} (.3)
Lactate	µmol/g of cecal digesta	5.6 (.3-13.6)	6.1 (.6-33.2)	4.6 (3.8-6.3)	5.2 (.9-6.4)
Acetate		19.4 (1.4)	21.7 (4.1)	19.6 (2.0)	22.1 (1.2)
Propionate		3.6 (.6)	3.5 (1.8)	3.6 (.3)	3.4 (.8)
Butyrate		5.5^{b} (2.9-1.1)	5.3^{b} (2.1-22.4)	7.4^{ab} (4.6-11.0)	16.0^{a} (1.2-19.9)
MCT1		.33^{b} (.10)	.43^{ab} (.15)	.72^{ab} (.15)	1.16^{a} (.49)
Haptoglobin	g/L	1.611^{a} (.417)	1.847^{a} (.366)	1.428^{ab} (.471)	.477^{b}(.113)
GSTP1	c h	.70 (.28)	.63 (.24)	.77 (.25)	1.09 (.37)

TNF-α		.82 (.09-1.94)	1.12 (.24-9.83)	.58 (.14-1.07)	1.15 (.59-1.71)
IL1-β		1.38 (.42-2.67)	1.98 (.59-7.07)	3.14 (1.05-9.97)	1.16 (.76-1.22)
MUC1		2.33 (1.16-13.63)	5.77 (2.6-18.26)	2.08 (1.05-3.56)	
MUC2		2.20 (1.59)	1.85 (.94)	2.01 (.58)	
CPT-11	μg/g of cecal digesta	22.0 (8.77-8.4)	24.7(4.89-75.9)	22.7 (9.99-56.9)	
SN-38		3.52^{a} (.04-9.50)	$.80^{b}$ (.12-11.2)	2.44^{ab} (.56-6.54)	
SN-38G		$.02^{ab}$ (.02)	$.07^{a}$ (.06)	$.0^{b}$ (.0)	
APC		1.90 (1.06-5.93)	1.76 (.89-9.63)	2.35 (.75-7.64)	
NPC		.07 (.02-.24)	.04 (.02-.13)	.04 (.03-.12)	
CPT-11	μg/g of jejunum digesta	4.88 (1.43-1.5)	3.34 (.25-9.25)	5.14 (3.79-9.31)	
SN-38		.19 (.09-1.27)	.15 (.02-.55)	.43 (.31-.61)	
SN-38G		17.4 (3.23-39.6)	1.8 (.77-34.6)	16.77 (11.6-34.8)	
APC		.48 (1.44-1.49)	.37 (.04-2.84)	1.24 (.64-2.38)	
NPC		.03 (0-.05)	.00 (.00-.04)	.03 (0-.09)	

Table 3-3. Genera of bacterial GUD producers

Bacterial genus	Family, group or phylum	E-value	Max ident[a)]
Several unrelated genera	*Firmicutes*	9.1	89%
Frankia	*Actinobacteria*	1.1	92%
Sulfolobus	Archae	4.21	100%
Bacteroides	*Bacteroides*	0.009	51%
Bifidobacterium	*Bifidobacterium*	8.0E-13	76%
Clostridium	*Clostridum* cluster I	0.003	77%
Faecalibacterium	*Clostridum* cluster IV	4.0E-125	74%
Ruminococcus[b)]	*Clostridum* cluster XIV	0.12	84%
Ruminococcus	*Clostridum* cluster XIV	2.6	87%
Ruminococcus	*Clostridum* cluster XIV	0.16	88%
Ruminococcus	*Clostridum* cluster XIV	2.1	87%
Ruminococcus	*Clostridum* cluster XIV	0.029	91%
Ruminococcus	*Clostridum* cluster XIV	2.6	87%
Ruminococcus	*Clostridum* cluster XIV	0.35	88%
Ruminococcus	*Clostridum* cluster XIV	0.16	88%
Edwardsiella	*Enterobacteriaceae*	1.3	100%
Edwardsiella	*Enterobacteriaceae*	0.16	100%
Edwardsiella	*Enterobacteriaceae*	0.082	100%
Edwardsiella	*Enterobacteriaceae*	0.62	90%
Escherichia[b]	*Enterobacteriaceae*	1.1	87%
Escherichia[b)]	*Enterobacteriaceae*	3.3	87%
Lactobacillus	*Lactobacillaceae*	7.5	85%
Staphylococcus	*Staphylococcaceae*	1.3	90%

[a)] the minimum length of sequence data considered in the analysis was 200 bp.

[b)] Sequences attributed to *Ruminococcus* spp. all matched to *R. gnavus;* sequences attributed to *Escherichia* spp. all matched to *E. coli*.

3.4 Discussion

Intestinal microbiota interact with the host digestive and immune systems. They can play multiple roles in various health conditions and either promote or prevent gut injury. The role of intestinal microbiota in CPT-11 chemotherapy has long been clearly, and yet restrictedly defined as toxicity activation through bacterial GUD, while other aspects have been overlooked. The present study provided a comprehensive view of four mechanisms in which gut microbiota may affect toxicity development during CPT-11/5-FU treatment, and explored the potential of using dietary fibres to reduce CPT-11 toxicity.

Bacterial translocation from the intestine can be promoted by microbial and/or host factors. This study identified translocating bacteria, and quantified abundance of translocating bacterial taxa in the gut lumen. Only three of 7 bacterial taxa increased moderately during chemotherapy and abundance of opportunistic pathogens remained low. CPT-11-chemotherapy impairs immune functions, i.e. depletion of cytotoxic T cells, and damages intestinal mucosa [22]. Although a localized increase in opportunistic pathogens at the mucosal surface cannot be ruled out, bacterial translocation during CPT-11 chemotherapy is likely caused mainly by host factors.

Inclusion of dietary fibre induced specific changes in gut microbiota. Overgrowth of *Clostridium* cluster XI is an indicator of intestinal dysbiosis. Previously, *Clostridium* cluster XI was increased by both tumor and CPT-11 treatment [9] and reduced by inulin [30]. The reduced abundance of *Clostridium* cluster XI (~5 logs) after inclusion of synergy in the diet was not accompanied by a noticeable difference in animal health. Bifidogenic effects of dietary fibres are considered a main mechanism by which fibres promote health [4], however, protective effects of commensal bifidobacteria were attributed primarily to the production of acetate [31]. The abundance of bifidobacteria after inclusion of synergy in the diet was higher than after inclusion of other dietary fibres. However, animal health was not correlated to the abundance of bifidobacteria.

GUD activity of intestinal microbiota in healthy animals allows the use of glucuronides as a carbon source for bacterial metabolism. Cell wall of plants and microbes contain glucuronides [32, 33], and xenobiotic compounds are conjugated to water-soluble glucuronides

by liver UDP-glucuronosyltransferases and excreted with bile [34]. The release of xenobiotic compounds from glucuronides contributes to carcinogenesis in the lower gastrointestinal tract [35]. Effects of dietary fibres on GUD activity are specific for individual fibres [36-38]. Dietary effects on GUD activity are likely associated with group-specific changes in intestinal microbiota [32], the availability of energy source [39], as well as the availability of glucuronide substrate [40]. In this study, the primary determinant factor of GUD activity was the feed intake. Most rats showed substantial reduction in feed intake due to chemotherapy, reducing the availability of fermentable carbohydrates in the cecum and the overall activity of cecal microbiota. Quantification of SN-38G and SN-38 in jejunal and cecal digesta demonstrated that cecal GUD level was sufficient for complete deglucuronidation of SN-38 even in animals with low intestinal GUD activity. Strategies that target microbiota to reduce toxicity therefore require either drastic measures to suppress the entire flora (e.g. with antibiotics), or agents that specifically inhibit GUD activity [2]. In the present study, variation in SN-38 levels derived mainly from the upper gastrointestinal tract, most likely from hepatic metabolism, where dietary fibres have little impact. This also explains the large variations observed within treatments. The presence of GUD in intestinal bacteria is not as ubiquitous as other bacterial carbohydrate-active enzymes [20]. Major bacteria carriers of GUD were reported to be *Clostridium* cluster IV and XIVa [20]. In this study the most important producer was *Ruminococcus gnavus*. *R. gnavus* is a mucin-degrading species that increased disproportionately to total mucosa-associated bacteria in both Crohn's disease and ulcerative colitis [41]. *Enterbacteriaceae* accounted for less than 1% of total bacteria but also were significant contributors to intestinal GUD activity. Cecal *Enterobacteriaceae* were consistently increased by CPT-11-based regimens [9]. The role of *Enterobacteriaceae* in CPT-11 toxicity is thus three-fold: (i) their increased proportion in total bacteria reflects intestinal dysbiosis; (ii) they constituted the majority of bacteria that translocate across the intestinal barrier, and their lipopolysaccharide (LPS) can induce inflammatory responses and acute phase responses; (iii) they are important producers of GUD and may elevate SN-38 concentrations in the cecum.

Intestinal epithelial cells undergo considerable stress due to exposure to cytotoxic SN-38. This increases the requirement of energy for cellular repair and regeneration. Butyrate production is known as one of the key benefits of dietary fibres as it is an essential energy source

of colonic epithelial cells [42]. However, the amount of substrate that is entering the large bowel and is available for microbial fermentation can be quantified in surgically modified swine models [43] but not in a rodent model as used in this study. Moreover, SCFA are rapidly absorbed by the host and the luminal concentrations represent only a fraction of the SCFA produced in microbial metabolism [37]. Therefore, three different indirect measurements were used. Quantification of luminal butyrate levels was complemented by quantification of the expression of monocarboxylate cotransporter 1 (MCT1), a H+-dependent symporter of butyrate. It is expressed in the gastrointestinal tract, with the highest expression in the cecum [44]. Its expression reflects the health of mucosal cells but it is also induced by luminal butyrate [19, 45, 46]. Butyrate production was also assessed by quantification of bacterial genes related to bacterial butyrate forming pathways. Butyrate formation is catalyzed by two alternative enzymes: butyrate kinase and butyryl-CoA transferase; the butyryl-CoA transferase pathway is predominant in adult human microbiota [12]. A reduction in butyryl-CoA transferase gene was reported for colorectal cancer [6] and Type I diabetes [47] and was considered a reflection of dysbiosis. However, primers used in the current study were designed for human microbiota, and may not amplify the corresponding genes of all butyrate producers in rodent microbiota [12]. In our study, abundance of the butyryl-CoA transferase gene outnumbered abundance of the butyrate kinase gene by one log in healthy rats, however, the combination of tumor and chemotherapy reversed this difference by reducing the butyryl-CoA transferase gene and increasing the butyrate kinase gene. Abundance of cecal butyryl-CoA transferase but not butyrate kinase was positively correlated with butyrate concentrations (Figure 3-5), consistent with the role of butyryl-CoA transferase as the major enzyme for butyrate production. Butyrate kinases have minor contribution to total butyrate production compared to butyryl-CoA transferases. The butyrate kinase pathway is employed by clostridia including *Cl. acetobutylicum*, *Cl. perfringens*, *Cl. tetani*, *Cl. botulinum* and *Cl. difficile* [12], of which many species are also gastrointestinal pathogens. Therefore, in contrast to butyryl-CoA transferase, whose abundance indicates the level of butyrate production, butyrate kinase contributes only a minor proportion to total butyrate production and is more likely associated with potentially harmful bacteria. Therefore, similar to *Clostridium cluster* XI and *Enterobacteriaceae* [9], its increase can be considered a sign of dysbiosis.

MCT1 expression was positively correlated with body weight, and cecal butyrate concentrations were correlated to feed intake in both experiments. Remarkably, other SCFAs were not associated with animal health and feed intake, which reflects the importance of colonic butyrate formation for the energy supply and function of intestinal mucosa [23, 48]. The specific association of butyrate and host health also indicates that this association is not merely a consequence of higher feed intake of healthy animals but indicates that stimulation of butyrate production by dietary fibre improves the condition of cecal and colonic mucosa and thus improves barrier properties and immune function. Despite the strong correlation between butyrate production and host well-being, a clear diet effect on host health was not observed for any type of fibre. The variations in the hosts' metabolism of CPT-11 were large when compared to the modest effect of dietary fibre. A second possible reason could be the low dosage of fibre used. The quantitative assessment of starch digestion in swine revealed that inclusion of 40% of RS in diet is required to substantially increased colonic butyrate formation [37]. Our experimental diet contained 8% w/w fibre, which was marginal to produce any discernible difference.

In conclusion, this study demonstrates for the first time that dietary modulation by optimizing butyrate production reduces irinotecan-induced toxicity. This finding may be explored to reduce morbidity, and to improve tolerance of tolerability irinotecan chemotherapy.

3.5 References

1. Anthony L (2007) Irinotecan toxicity. Curr Opin Support Palliat Care 1: 35–39.
2. Wallace BD, Wang H, Lane KT, Scott JE, Orans J, et al. (2010) Alleviating cancer drug toxicity by inhibiting a bacterial enzyme. Science 330: 831–835.
3. Willing BP, Russell SL, Finlay BB (2011) Shifting the balance: antibiotic effects on host-microbiota mutualism. Nat Rev Microbiol 9: 233–243.
4. Roberfroid M (2007) Prebiotics: the concept revisited. J Nutr 137: 830S–7S.
5. Beaud D, Tailliez P, Anba-Mondoloni J (2005) Genetic characterization of the β-glucuronidase enzyme from a human intestinal bacterium, *Ruminococcus gnavus*. Microbiology 151: 2323–2330.
6. Wang T, Cai G, Qiu Y, Fei N, Zhang M, et al. (2012) Structural segregation of gut microbiota between colorectal cancer patients and healthy volunteers. The ISME J 6: 320–329.
7. Stringer AM, Gibson RJ, Bowen JM, Keefe DMK (2009) Chemotherapy-induced modifications to gastrointestinal microflora: evidence and implications of change. Curr Drug Metabol 10: 79–83.
8. Zwielehner J, Lassl C, Hippe B, Pointner A, Switzeny OJ, et al. (2011) Changes in human fecal microbiota due to chemotherapy analyzed by TaqMan-PCR, 454 sequencing and PCR-DGGE fingerprinting. PloSone 6: e28654.
9. Lin XB, Dieleman LA, Ketabi A, Bibova I, Sawyer MB, et al. (2012) Irinotecan (CPT-11) Chemotherapy alters intestinal microbiota in tumour bearing rats. PloSone 7: e39764.
10. Takesue Y, Kakehashi M, Ohge H, Uemura K, Imamura Y, et al. (2005) Bacterial translocation: not a clinically relevant phenomenon in colorectal cancer. World J Surg 29: 198–202.
11. Berg RD (1999) Bacterial translocation from the gastrointestinal tract. Adv Exp Med Biol 473: 11–30.
12. Louis P, Scott KP, Duncan SH, Flint HJ (2007) Understanding the effects of diet on bacterial metabolism in the large intestine. J Appl Microbiol 102: 1197–1208.

13. Xue H, Sawyer MB, Field CJ, Dieleman LA, Baracos VE (2007) Nutritional modulation of antitumor efficacy and diarrhea toxicity related to irinotecan chemotherapy in rats bearing the ward colon tumor. Clin Cancer Res 13: 7146–7154.
14. Xue H, Sawyer MB, Field CJ, Dieleman LA, Murray D, et al. (2008) Bolus oral glutamine protects rats against CPT-11-induced diarrhea and differentially activates cytoprotective mechanisms in host intestine but not tumor. J Nutr 138: 740–746.
15. Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. Nucleic acids Res 29: e45.
16. Al-Dasooqi N, Bowen JM, Gibson RJ, Logan RM, Stringer AM, et al. (2011) Selection of housekeeping genes for gene expression studies in a rat model of irinotecan-induced mucositis. Chemother 57: 43–53.
17. Martínez-Beamonte R, Navarro MA, Larraga A, Strunk M, Barranquero C, et al. (2011) Selection of reference genes for gene expression studies in rats. J Biotechnol 151: 325–334.
18. Ranganna K, Mathew OP, Yatsu FM, Yousefipour Z, Hayes BE, et al. (2007) Involvement of glutathione / glutathione S-transferase antioxidant system in butyrate-inhibited vascular smooth muscle cell proliferation. FEBS J 274: 5962–5978.
19. Thibault R, De Coppet P, Daly K, Bourreille A, Cuff M, et al. (2007) Down-regulation of the monocarboxylate transporter 1 is involved in butyrate deficiency during intestinal inflammation. Gastroenterol 133: 1916–1927.
20. Caballero-Franco C, Keller K, De Simone C, Chadee K (2007) The VSL#3 probiotic formula induces mucin gene expression and secretion in colonic epithelial cells. Am J Physiol Gastrointest Liver Physiol 292: G315–22.
21. Caballero-Franco C, Keller K, De Simone C, Chadee K (2007) The VSL#3 probiotic formula induces mucin gene expression and secretion in colonic epithelial cells. Am J Physiol Gastrointest Liver Physiol 292: G315–22.
22. Zhao L, Brinton RD (2004) Suppression of proinflammatory cytokines interleukin-1beta and tumor necrosis factor-alpha in astrocytes by a V1 vasopressin receptor agonist: a cAMP response element-binding protein-dependent mechanism. J Neurosci 24: 2226–2235.

23. Metzler-Zebeli BU, Hooda S, Pieper R, Zijlstra RT, van Kessel AG, et al. (2010) Nonstarch polysaccharides modulate bacterial microbiota, pathways for butyrate production, and abundance of pathogenic *Escherichia coli* in the pig gastrointestinal tract. Appl Environ Microbiol 76: 3692–3701.
24. Png CW, Lindén SK, Gilshenan KS, Zoetendal EG, McSweeney CS, et al. (2010) Mucolytic bacteria with increased prevalence in IBD mucosa augment in vitro utilization of mucin by other bacteria. Am J Gastroenterol 105: 2420–2428.
25. Collado MC, Derrien M, Isolauri E, de Vos WM, Salminen S (2007) Intestinal integrity and *Akkermansia muciniphila*, a mucin-degrading member of the intestinal microbiota present in infants, adults, and the elderly. Appl Environ Microbiol 73: 7767–7770.
26. Dabek M, McCrae SI, Stevens VJ, Duncan SH, Louis P (2008) Distribution of β-glucosidase and β-glucuronidase activity and of β-glucuronidase gene gus in human colonic bacteria. FEMS Microbiol Ecol 66: 487–495.
27. Corona G, Elia C, Casetta B, Toffoli G (2010) Fast liquid chromatography-tandem mass spectrometry method for routine assessment of irinotecan metabolic phenotype. Ther Drug Monit 32: 638–646.
28. Farhangfar A (2012) Non digestible carbohydrates in the diet determine toxicity of irinotecan (CPT-11)/5-fluorouracil in rats independently of β-glucuronidase activity in intestinal lumen. M.Sc. thesis, University of Alberta, http://hdl.handle.net/10402/era.28569. Accessed 2013 Nov 18.
29. Topping DL, Clifton PM (2001). Short-chain fatty acids and human colonic function: Roles of resistant starch and nonstarch polysaccharides. Physiol Rev 81: 1032-1064.
30. Van den Abbeele P, Gérard P, Rabot S, Bruneau A, El Aidy S, et al. (2011) Arabinoxylans and inulin differentially modulate the mucosal and luminal gut microbiota and mucin-degradation in humanized rats. Environ Microbiol 13: 2667–2680.
31. Fukuda S, Toh H, Hase K, Oshima K, Nakanishi Y, et al. (2011). Bifidobacteria can protect from enteropathogenic infection through production of acetate. Nature. 469: 543-547.
32. Reiter W-D (2008) Biochemical genetics of nucleotide sugar interconversion reactions. Curr Opin Plant Biol 11: 236–243.

33. Horecker BL (1966) The biosynthesis of bacterial polysaccharides. Ann Rev Microbiol 20: 253–290.
34. van der Logt EMJ, Roelofs HMJ, Nagengast FM, Peters WHM (2003) Induction of rat hepatic and intestinal UDP-glucuronosyltransferases by naturally occurring dietary anticarcinogens. Carcinogenesis 24: 1651–1656.
35. Gill CIR, Rowland IR (2002) Diet and cancer: assessing the risk. Brit J Nutr 88 S1: S73–87.
36. Juśkiewicz J, Głazka I, Król B, Zduńczyk Z (2006) Effect of chicory products with different inulin content on rat caecum physiology. J Anim Physiol Anim Nutr 90: 200–207.
37. Shiau SY, Chang GW (1983) Effects of dietary fiber on fecal mucinase and beta-glucuronidase activity in rats. J Nutr 113: 138–144.
38. Wu W-T, Cheng H-C, Chen H-L (2011) Ameliorative effects of konjac glucomannan on human faecal β-glucuronidase activity, secondary bile acid levels and faecal water toxicity towards Caco-2 cells. Brit J Nutr 105: 593–600.
39. Cenci G, Caldini G, Trotta F (2000) Bacterial amylolytic activity enhances β-glucuronidase expression of amylase-negative *Escherichia coli* strain in starch-medium. J Basic Microbiol 40: 311–318.
40. Rowland IR, Mallett AK, Wise A (1985) The effect of diet on the mammalian gut flora and its metabolic activities. Crit Rev Toxicol 16: 31–103.
41. Png CW, Lindén SK, Gilshenan KS, Zoetendal EG, McSweeney CS, et al. (2010) Mucolytic bacteria with increased prevalence in IBD mucosa augment in vitro utilization of mucin by other bacteria. Am J Gastroenterol 105: 2420–2428.
42. Hamer HM, Jonkers D, Venema K, Vanhoutvin S, Troost FJ, et al. (2008) Review article: the role of butyrate on colonic function. Aliment Pharmacol Ther 27: 104–119.
43. Regmi PR, Metzler-Zebeli BU, Gänzle MG, Kempen TATGV et al. (2011) Starch with high amylose content and low in vitro digestibility increases intestinal nutrient flow and microbial fermentation and selectively promotes bifidobacteria in pigs. J Nutr 141: 1273–1280.

44. Iwanaga T, Takebe K, Kato I, Karaki S-I, Kuwahara A (2006) Cellular expression of monocarboxylate transporters (MCT) in the digestive tract of the mouse, rat, and humans, with special reference to slc5a8. Biomed Res 27: 243–254.
45. Cuff MA, Lambert DW, Shirazi-Beechey SP (2002) Substrate-induced regulation of the human colonic monocarboxylate transporter, MCT1. J Physiol 539: 361–371.
46. Chapman MA, Grahn MF, Boyle MA, Hutton M, Rogers J, et al. (1994) Butyrate oxidation is impaired in the colonic mucosa of sufferers of quiescent ulcerative colitis. Gut 35: 73–76.
47. Brown CT, Davis-Richardson AG, Giongo A, Gano KA, Crabb DB, et al. (2011) Gut microbiome metagenomics analysis suggests a functional model for the development of autoimmunity for type 1 diabetes. PloSone 6: e25792.
48. Canani RB, Costanzo MD, Leone L, Pedata M, Meli R, et al. (2011) Potential beneficial effects of butyrate in intestinal and extraintestinal diseases. World J Gastroenterol 17: 1519–1528.

4. Quantitative high-resolution melting PCR analysis for monitoring of fermentation microbiota in sourdough

4.1 Introduction

The demand for gluten-free bread has been growing in recent years due to the increasing concern for coeliac disease, wheat allergy, gluten sensitivity, other autoimmune diseases, as well as the perceived healthiness by consumers [1,2]. However, gluten's unique ability to form thin gas-retaining films and to aggregate into a stretchable, extensible, coagulable, protein-starch matrix makes its presence essential to the overall appearance and textural properties of wheat bread. The replacement of the gluten in gluten-free bread often results in products with poor mouth-feel and off-flavour [3]. Sourdough fermentation has been used to improve the quality of gluten-free bread. Beneficial effects of sourdough include improved volume, texture, and shelf life through production of exopolysaccharides, and the formation of aroma and bioactive compounds [4]. The fermentation substrate strongly influences sourdough microbiota [5]; therefore, novel applications of sourdough in gluten-free products calls for a deeper understanding of microbial ecology in sourdoughs made of cereals and/or pseudocereals other than wheat and rye.

Sorghum is a major crop used for cereal fermentation in Africa and Asia [6], and is used as an important ingredient for gluten-free food products [3]. Sorghum selects for fermentation microbiota that differ from fermentation microbiota in wheat and rye sourdoughs [5,7]. The antimicrobial activity of sorghum phenolic compounds, low levels of maltose and the presence of glucose as major carbon source contribute to these differences [8]. *Lactobacillus reuteri* is a heterofermentative bacterium which can readily adapt to wheat, rye as well as gluten-free substrates [9,10]. However, *L. reuteri* strains from sorghum sourdough were phylogenetically different from strains that were isolated from wheat and rye sourdoughs [11]. Because the genetics, metabolism, and phylogeny of *L. reuteri* are well characterized [12–14], this species is suitable model for studying microbial ecology and metabolism of lactobacilli in sourdough.

The composition of sourdough microbiota is governed by the dynamics between environmental microbiota, properties of the fermentation substrate, and specific technological process parameters [15,16]. Given its complexity as well as importance to food quality, monitoring

sourdough microbiota throughout the fermentation progress has been challenging. Culture-dependent methods remain the reference method to characterize microbial composition of sourdough; however, these need to be complemented with molecular methods for identification of isolates on the species level. Methods for the identification of isolates include PCR-based methods such as randomly amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP) [17,18], and chemical methods such as Matrix Assisted Laser Desorption Ionization-Time-Of-Flight Mass Spectrometry (MALDI-TOF-MS) analysis [19]. Alternatively, culture-independent methods can also be used to characterize microbiota directly, these methods include denaturing gradient gel electrophoresis (DGGE), [18,20], quantitative PCR [17] and high-throughput sequencing of 16S rDNA [21]. Results of culture-independent characterization of sourdough microbiota, however, are not fully in agreement with culture-dependent method due to errors introduced by incomplete recovery of bacterial DNA from sourdoughs [8,17,20,21]. Moreover, the labour-intensive nature of most of the techniques mentioned above does not allow for rapid quality control during fermentation processes. High-Resolution melting (HRM)-qPCR methods, which can detect single nucleotide differences in target sequences, are more rapid and have been used for identification of clinical and foodborne pathogens [22–24], detection of food adulteration [25–27], and genotyping of diseases [28–31]. However, there has been very limited, if any application of HRM in food fermentation processes. The aim of the study was to develop HRM methods to monitor the microbial composition during sourdough fermentation, and to investigate factors affecting microbial ecology in sourdoughs made of different substrates.

4.2 Material and methods

4.2.1 Sourdough fermentation. *L. fermentum* FUA3165*, L. plantarum* FUA3309, *L. paracasei* FUA3166*,* and *L. reuteri* FUA3168 isolated from sorghum sourdough [7] were used for fermentation. Strains were inoculated in modified MRS (mMRS) broth. Each litre of mMRS contained 10 g tryptone, 10 g maltose, 5 g glucose, 5 g fructose, 5 g beef extract, 5 g yeast extract, 4.0 g potassium phosphate dibasic, 2.6 g potassium phosphate monobasic,2 g tri-ammonium citrate, 0.5 g L-cysteine, 0.2 g magnesium sulphate,0.05 g manganese sulphate, 1 mL vitamin mix (B12, folic acid, B1, B2,B6, panthothenic acid), and 1 g Tween80. Strains were incubated at 37°C overnight. The culture cocktails were prepared by mixing equal volume of the four cultures. Three pure sorghum cultivars, Segaolane (white), Town (red), and PAN8609 (red),

were obtained from the Food Technology Research Centre, Kanye, Botswana. Grains were ground in an UltraCentrifugalMillZM200 (Retsch, Burlington, Canada) to a size of 0.5mm. Commercial white sorghum flour was purchased from a local supermarket. Sourdoughs were prepared with by mixing 10 g of flour, 9 mL of autoclaved tap water, and 1 ml of the culture cocktail. The doughs were incubated at 37 °C and propagated every 24 h for 6 day. At each fermentation cycle, 2 g of the previous sourdough were mixed with 9 g of flour and 1 mL of tap water. Samples were taken for DNA extraction and plate count at the end of each fermentation cycle.

In a second experiment, five *L. reuteri* strains of three different sourdough origins were used. *L. reuteri* FUA5448 is a rodent-lineage strain isolated from rye sourdough; *L. reuteri* FUA3400 and 3401 are human lineage strains isolated from wheat sourdough; *L. reuteri* FUA3168 and 3324 are sorghum isolates that cannot be assigned to a known host lineage [11]. Strains were inoculated in mMRS broth and incubated at 37°C overnight. Two culture cocktails were prepared as biological repeats by mixing equal volume of one strain from each origin. Cocktail 1 contained *L. reuteri* FUA5448, FUA3400, and FUA3168. Cocktail 2 contained *L. reuteri* FUA5448, FUA3401, and FUA3324. Wheat, rye, and sorghum flours were purchased from local stores. Sourdoughs were prepared in the same way as described above. Samples were taken for DNA extraction at every second fermentation cycle.

4.2.2 Culture-dependent quantification of sourdough microbiota. Sourdough (0.5 g) was homogenized with 4.5 mL of 0.8% wt/vol NaCl solution by vortexing. Appropriate dilutions were plated on modified MRS (mMRS) agar to enumerate *Lactobacillus* spp. Plates were incubated anaerobically at 37 °C for 24 h. Differential cell counts are reported when the colony morphologies of strains in the same sourdough allowed their differentiation.

4.2.3 Identification and quantification of *Lactobacillus* spp using High-Resolution Melting (HRM) curves. Two grams of sourdough was homogenized with 80 mL of 0.8% wt/vol NaCl solution. The homogenate was centrifuged at 500g for 5 min to remove solids. The cells were harvested by centrifuging at 5000g for 15 min. DNA was extracted with DNeasy Blood & Tissue Kit (Qiagen, USA) following instructions of the manufacturer. PCR was performed on Rotor-Gene Q (Qiagen, USA) using Type-it HRM PCR Kit (Qiagen, USA). Universal primers

(forward, 5'-TCC TAC GGG AGG CAG CAG T-3'; reverse, 5'-GGA CTA CCA GGG TAT CTA ATC CTG TT-3') targeted at bacterial 16S rRNA gene were used. The PCR conditions were: denaturation 5 min at 95°C, followed by 45 cycles of denaturation at 95°C for 10 sec, annealing at 60°C for 30 sec, and extension at 72°C for 10 sec. At the final HRM stage, the temperature increased from 65°C to 90°C at 0.1°C/step with 2 sec holding time at each step. The raw melting curve data were reprocessed with PeakFit Software (Systat Software Inc., USA) using AutoFit Baseline followed by AutoFit Peaks I Residuals method. The areas under reprocessed peaks were calculated as % area of total peak area. This proportion was calibrated against % DNA, which was calculated by mixing known concentrations of DNA extracted from pure cultures. The ranges of calibrations were selected based on the results of sourdough samples. For *L. fermentum* and *L. plantarum*, varying concentrations of DNA of these two species were mixed. For *L. paracasei* and *L. reuteri*, varying concentrations of DNA of the species of interest was mixed with a DNA mixture which contained equal amount of DNA of each of the other three species.

4.2.4 Differentiation of *L. reuteri* strains using molecular beacon. DNA was extracted as described above. Oligonucleotide sequences of seven genes (*ddl, dlt, rpoA, recA, gyrB, leuS,* and *pkt*) previously used for multilocus sequence analysis (MLSA) of *L. reuteri* [12] from all strains were compared and selected to design molecular beacon in order to distinguish strains from different lineages based on temperature differences in melting temperatures. The primers sequences for amplification of *ddl* were: forward (limiting), 5'- AAT ATG CAG AAG CCT TAG-3'; reverse (excess), 5'-TAT CAC CCA TAT CAC CAT-3'. The sequence of molecular beacon was 5'-CGC GAT CAT GAT TAC GAA AAC AAG TTT GTG GAT GGA TCG CG-3'. Each PCR contained 1× TaqMan® Fast Universal PCR Master Mix (2X), No AmpErase® UNG (Applied Biosystem), 2 μM excess primer, 0.2 μM limiting primer, 0.2 μM of molecular beacon, and bacterial DNA in a final volume of 16 μl. PCR was done on Applied Biosystems 7500 Fast Real-time PCR System (Life Technologies, USA). The PCR conditions were: denaturation 5 min at 95°C, followed by 60 cycles of denaturation at 95°C for 10 sec, annealing at 50°C for 15 sec, and extension at 72°C for 20 sec. At the melting curve stage the temperature was held at 95°C for 2 min, at 25°C for 60 min, and then increased from 25°C to 90°C at 1°C/step with 25 sec holding time at each step.

4.2.5 Sensitivity of *Lactobacillus* spp. to antimicrobial phenolic compounds in sorghum. Phenolic compounds from sorghum flours were extracted with aqueous methanol followed by liquid–liquid extraction with ethyl acetate as described [32]. Extracts were evaporated to dryness and re-dissolved in methanol. Their antimicrobial activity was determined by a critical dilution assay performed as described [33]. Briefly, serial two-fold dilutions of 100 µL of phenolic extract with mMRS were prepared on 96 well microtiter plates. The plates were incubated at room temperature for 2 h in laminar flow hood to evaporate methanol. Overnight cultures of the different indicator strains were diluted 10 times which mMRS, and the microtitre plates were inoculated with 50 µL of the diluted cultures. The plates were incubated at 37 °C overnight and examined with pH strips. A pH below 5.0 indicated bacterial growth and organic acid production, and the MIC was defined as the lowest concentration of the substances that inhibited the acidification of the medium. Antimicrobial concentration was expressed as gram of flour needed for extraction.

4.2.6 Statistics. Experiments were performed in triplicate and means ± standard deviations are reported. Data analysis was performed with PROC MIXED procedure (SAS v.9.2; SAS Institute, USA) using two-way analysis of variance (ANOVA). A p-value of ≤ 0.05 was considered statistically significant.

4.3 Results

4.3.1 Resistance to sorghum phenolic compounds of *Lactobacillus* spp. The resistance to sorghum phenolic compounds were determined to investigate its effect on the competition among *Lactobacillus* spp. The antimicrobial activity of phenolic extracts from different sorghum cultivars was not different, but the resistance of different species varied greatly (Figure 4-1). *L. fermentum* had highest resistance to all sorghum phenolic extracts, followed by *L. plantarum*, while *L. paracasei* and *L. reuteri* showed little resistance to all extracts. Interestingly, *L. plantarum* was more sensitive to extracts from red sorghum cultivars (Town and PAN8609) than to extracts from white cultivars (Commercial and Segaloane).

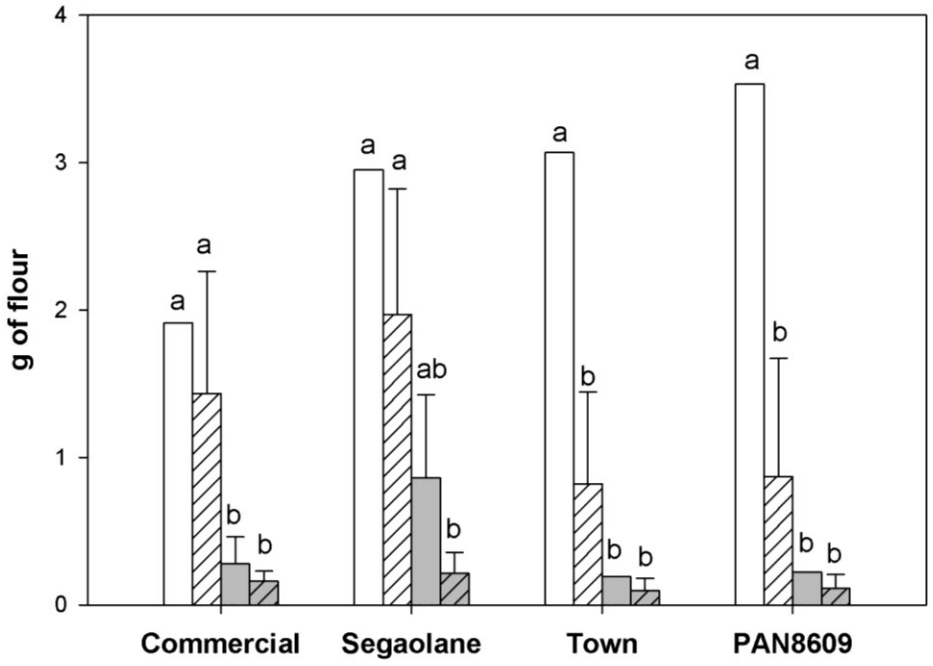


Figure 4-1. Minimal inhibitory concentration (MIC) of sorghum phenolic extracts on *Lactobacillus* spp. Results are shown as mean ± standard deviations of triplicate independent experiments. Bars indicate *L. fermentum* (white), *L. plantarum* (white hatched) , *L. casei* (grey), and *L. reuteri* (grey hatched). MIC obtained for different strains with extracts from the same flour are significantly different if they do not share a common superscript. There was no significant difference in the inhibitory activity of extracts from flours.

4.3.2 Identification and quantification of *Lactobacillus* spp using HRM curves. An HRM assay was developed in order to identify and quantify four species of *Lactobacillus* simultaneously. HRM curves were able to distinguish between *L. fermentum, L. plantarum, L. paracasei, L. reuteri*, separating melting peaks of 16S rDNA amplicons of each species by 0.5 to 1°C (Figure 4-2). Relative quantification was based on the relative areas of each melting peak. Quantification allowed detection of species if the corresponding rDNA amplicon comprised more 0.2% of the total rDNA. For *L. fermentum* and *L. plantarum*, a single linear regression was used for calibration to cover 20%-100% of total DNA (Table 4-1, Appendix C1). This range covered the range detected in all sourdough samples (Figure 4-3). For *L. paracasei* and *L. reuteri*, two linear regressions were calculated for higher and lower DNA % range (Table 4-1,

Appendix C2) because the slope decreased as melting peak area approached the detection limit. The R^2 value for all regressions were >98%.

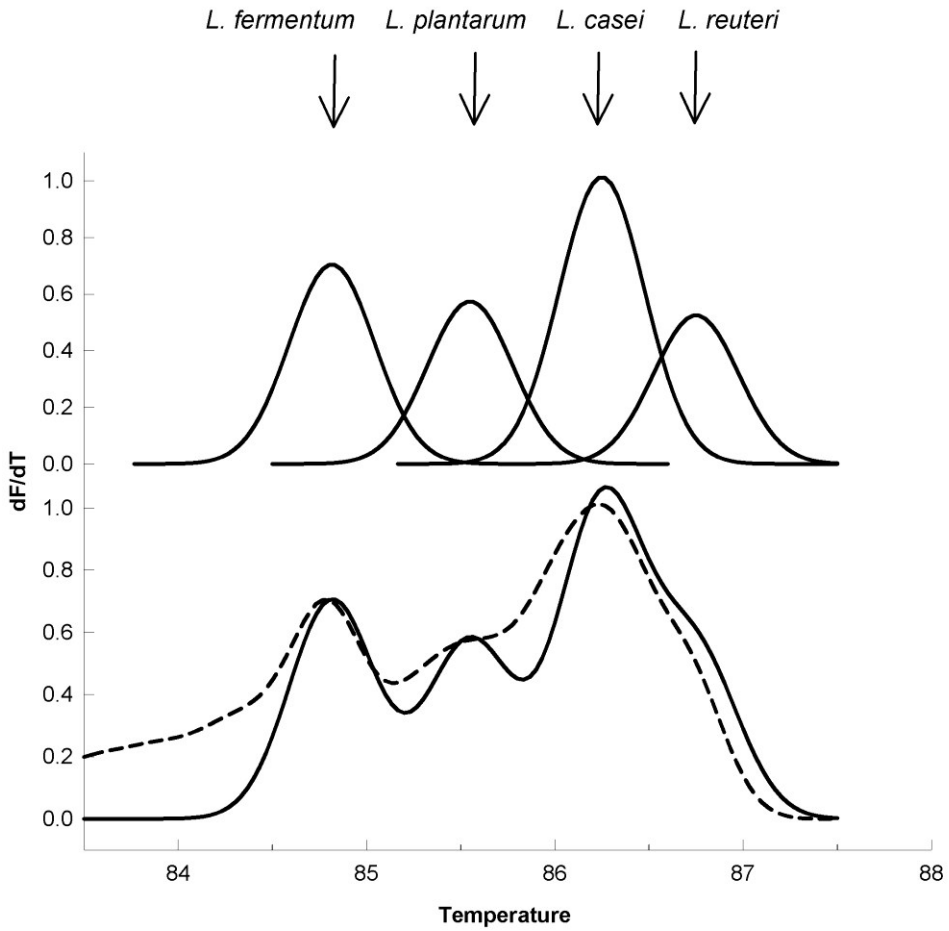


Figure 4-2. Melting curves of PCR products amplified from pure *Lactobacillus*. spp (top), the raw melting curve of a sourdough sample containing four different species (bottom, dotted), and the same melting curve after deconvolution by PeakFit software (bottom, solid).

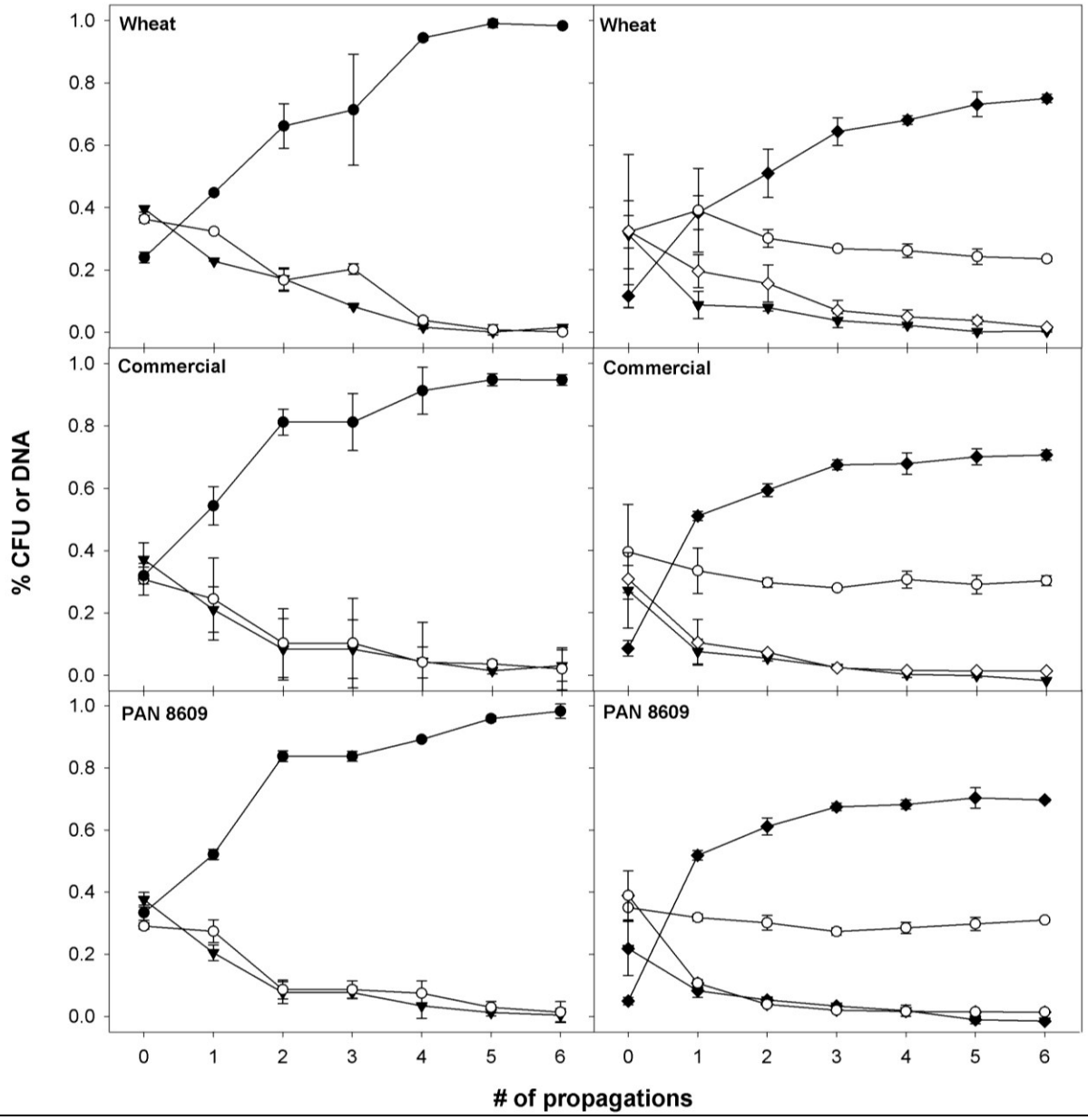


Figure 4-3. Microbial composition expressed as % CFU or % DNA in wheat and sorghum sourdoughs. Results are shown as mean ± standard deviations of triplicate independent experiments. *L. fermentum* and *L. reuteri* could not be distinguished by colony morhphology on mMRS agar but could be distinguished by different melting peaks. Symbols indicate *L. fermentum* + *L. reuteri* (●), *L. plantarum* (○), *L. casei* (▼), *L. fermentum* (◆), and *L. reuteri* (◇).There was no difference between the two white sorghum cultivars and two red sorghum cultivars. The figure shows the results of sourdough prepared with white commercial flour and flour from one of the two red cultivars.

Table 4-1. Calibration parameters of HRM-qPCR assays for simultaneous detection of *L. fermentum, L. plantarum, L.paracasei* and *L. reuteri*.

	L. fermentum	***L. platarum***	***L. paracasei***		***L. reuteri***	
% DNA range	20-100%	20-100%	0.5-30%	30-80%	0.2-0.8%	2.5-25%
Slope	1.15	3.79	0.79	0.97	0.84	1.92
R^2	0.996	0.998	0.999	0.980	0.994	0.989

4.3.3 Microbial composition of *Lactobacillus* spp. in different sorghum sourdough. Four species isolated from sorghum sourdough were co-inoculated in sourdoughs maintained by continuous propagation to study their competiveness. Sourdoughs were prepared with white commercial flour and flours obtained from three pure cultivar sorghum grains. Both culture-dependent and HRM-qPCR analysis showed that microbial composition of the four different sorghum sourdoughs did not differ (Figure 4-3 and Appendix C3). *L. fermentum* and *L. reuteri* shared the same colony morphology and could not be distinguished using culture-dependent method; however, they could be clearly distinguished by melting temperatures with HRM (Figure 4-2). After 3 propagations, *L. fermentum* prevailed in all sourdoughs. The second most abundant strain was *L. plantarum;* however, the proportion of *L. plantarum* in fermentation microbiota was over-estimated by qPCR-HRM analysis (Figure 4-3). After few fermentation cycles, *L. paracasei* and *L. reuteri* were only a minor component of the microbiota. The wheat sourdough had similar final composition as sorghum sourdoughs; however, *L. fermentum* reached 80% CFU by the 2nd propagation in sorghum sourdoughs but outcompeted the other strains only after 4 fermentation cycles in wheat sourdough.

4.3.4 Differentiation of *L. reuteri* strains using molecular beacon. A 27-bp segment of the *ddl* gene (Appendix C4) was selected to design molecular beacon because it contained the highest number of nucleotide differences among sorghum isolates, rodent lineage strains, and human lineage strains. A 1:10 ratio of limiting primer to excess primer was used for asymmetric PCR to produce an excess of template strands for molecular beacon in order to maximize fluorescence. The three lineages were separated by >7°C in melting temperature, which readily allowed the

strain-specific identification in sourdough samples. Quantification was not done due to the irregular shape of baseline (Figure 4-4).

4.3.5 Microbial ecology *L. reuteri* strains in wheat, rye, and sorghum sourdoughs. Three *L. reuteri* strains from of different lineages were inoculated into wheat, rye, and sorghum sourdoughs to investigate the effect of cereal substrate on competitiveness of *L. reuteri* strains of different origins. Two different cocktails were used to prevent strain-specific traits from affecting the final result. However, the rodent lineage strain, *L. reuteri* LTH5448, was used in both strain cocktails because other rye sourdough isolates that were assigned to rodent lineages produce reutericyclin [11]. Culture-dependent method was used for quantification of total bacterial counts while culture-independent method was used to distinguish strains. Cell counts in all sourdoughs were more than 10^9 cfu / g and fermentation microbiota consisted exclusively of *L. reuteri* (data not shown). The different strains of *L. reuteri* could not be differentiated on the basis of their colony morphology but strains of different host-adapted lineages were readily differentiated by HRM-qPCR analysis. Stable microbiota were observed after 8 – 10 fermentation cycles (Table 4-2). There was no preference of *L. reuteri* strains for a certain cereal substrate. Human lineage strains were detected in all sourdoughs after 12 fermentation cycles (Table 4-2). The microbial composition of the two strain cocktails were different for wheat and sorghum sourdoughs, indicating that strain-specific differences rather than lineage-specific differences accounted for the persistence of *L. reuteri* in wheat, rye, and sorghum sourdoughs.

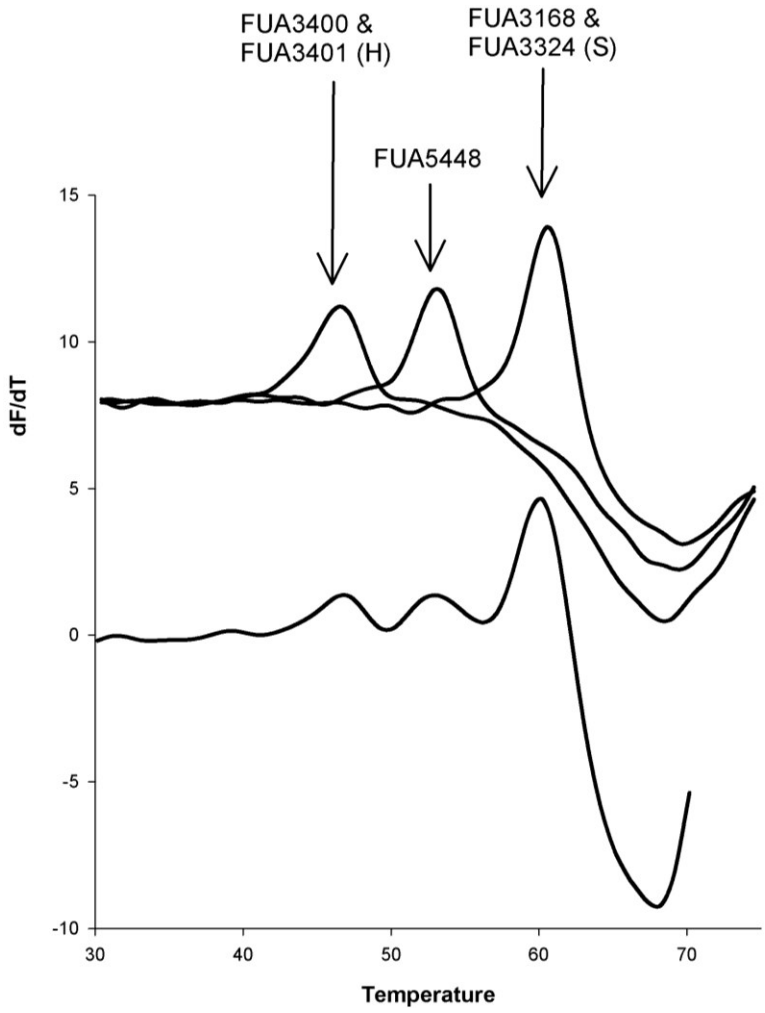


Figure 4-4. Melting peaks of pure *L. reuteri* strains (top) and a representative sourdough sample (bottom) in Exp 2.

Table 4-2. Qualitative detection of different lineages of L. reuteri in wheat, rye and sorghum sourdough by HRM-qPCR**.** Letters denote strains of the specific host lineage which were detectable by melting peaks: *L. reuteri* FUA3400 and FUA3401 (H), *L. reuteri* FUA5448 (R), *L. reuteri* FUA3168 and FUA3324 (S).

	Number of fermentation cycles				
	0	**4**	**8**	**10**	**12**
W1	H, R, S	H, R, S	H, S	H, R, S	H, R, S
W2	H, R, S	H, R	H	H, R	H, R
R1	H, R, S	H	H, R, S	H, R, S	H, R, S
R2	H, R, S	H, R, S	H, R, S	R, S	H, R, S
S1	H, R, S	H, R, S	H, S	H, S	H, S
S2	H, R, S	H, R	H	H, R	H, R

4.4 Discussion

Two HRM-qPCFR assays were developed for monitoring of sourdough microbiota during fermentation. Compared to other DNA-based methods, advantages of HRM-qPCR include the detection and quantification of several genotypes in qPCR reactions with a single primer pair, and the ability to detect single nucleotide differences in the amplicons (Table 4-3). However, the resolution of HRM-qPCR may not be suitable for resolution of multiple peaks in complex microbiota. For this reason, HRM are mostly used in relative pure samples that contain one or a few genotypes. In past studies, quantification of two genotypes in one sample was based on the initial melting curves [26,34,35]. To our knowledge, this study was the first that performed relative quantification of four genotypes simultaneously. Thus study analysed first derivative melting curves, which provides the advantage that data are readily processed by standard chromatography software, and account for the variation in melting temperature caused by DNA quality and/or concentration. HRM-qPCR can be readily adapted to other food fermentation processes. Most industrial fermentations are dominated by a very limited number of

species, which reduce the concern for loss of resolution due to complex or unknown samples [36]. The two common mechanisms used in real-time PCR, fluorescent probes and double-stranded DNA-binding dyes, can both be used. In this study, only the former was used for quantification because of the higher fluorescence generated by saturating double-stranded DNA-binding dyes; quantification can also be applied with fluorescent probes, however, probably with a lower accuracy and higher detection limit.

Table 4-3. Comparison of common PCR-based methods for characterizing microbiota in food fermentation.

	Specificity	**Quantitative**[a]	**Fast**	**Coverage/ detection limit**[b]	**Reference**
DGGE	genus/species	-	+	Universal or group specific / 1%	[20,37]
Pyrosequencing	genus/species	++	+	Universal / < 0.1%	[21]
qPCR	species	++	+++	Target group or species / < 0.01%	[8,17]
HRM	species/strain	++	+++	Universal / 0.1 - 1%	This study.

[a)] Systematic error due to differences in DNA recover from different strains is common to all DNA-based methods.

[b)] Universal, detection of all Eubacteria with universal 16S rRNA primers. The typical detection limit of the methods is indicated in % of total bacterial 16S rDNA.

Phenolic compounds present in sorghum have antimicrobial activities [32,38]. Sorghum sourdough was suggested to select for lactic acid bacteria that are able to metabolize phenolic acids [8]. Metabolism of phenolic compounds by lactobacilli generates decarboxylated or reduced metabolites with lower antimicrobial activity. Accordingly, metabolism of phenolic acids hence corresponded to higher resistance towards phenolic acids [38]. Organisms lacking such metabolic activity, i.e. *L. sanfranciscensis,* were outcompeted by others [8]. Lactic acid bacteria exhibit a strong strain-to-strain variation with respect to their tolerance to phenolic acids [39]. Of the strains used in this study, *L. fermentum* FUA3165 produced both decarboxylases and

reductases. *L. paracasei* FUA3166 exhibited glucosidase activity, *L. reuteri* FUA3168 had high esterase activity [32,38], and *L. plantarum* FUA3309 exhibited esterase and phenolic acid decarboxylase activities (unpublished data). The prevalence of different lactobacilli in sorghum sourdoughs thus corresponded to their resistance to phenolic extracts, and their ability to metabolise phenolic acids. *L. fermentum* FUA3165, the strain with highest resistance, was also the prevalent strain in sourdough microbiota. Red sorghum varieties, which differ from white varieties because they contain deoxyanthocanidins [40], did not select for different fermentation microbiota. It is noteworthy, however, that *L. plantarum* was less resistant to phenolic extracts from red sorghum when compared to white sorghum. In wheat sourdoughs lacking selective pressure from antimicrobial phenolic compounds, the final microbial composition was similar to sorghum sourdoughs. The increase of *L. fermentum* proportion was delayed, indicating that phenolic compounds are not only factor affecting microbial composition in sorghum sourdoughs. Because the genes for ferulic acid decarboxylases and phenolic acid reductases in lactobacilli remain to be identified, isogenic mutants of *L. fermentum* cannot be created to further confirm the role of phenolic acid metabolism in the ecology of sorghum sourdoughs.

It is well known that cereal type strongly affects microbial composition in sourdough [16]. However, the reasons have not been fully understood. Interestingly, *L. reuteri* from different sourdough origins were assigned to differently phylogenetic lineages [11]. This study demonstrated that the fermentation substrate exerts no lineage-specific selective pressure. Because the vertebrate hosts for human and rodent lineage strains of *L. reuteri* are typically present in bakery environments, different *L. reuteri* strains may have been introduced to the sourdough microbiota by accidental contamination by their host.

In conclusion, HRM-qPCR assays were established as rapid and highly specific tool for monitoring of sourdough microbiota. The ability to distinguish highly similar microbes in samples containing only few genotypes makes HRM-qPCR suitable for quality control in other food fermentation systems. The presence of phenolic compounds in sorghum sourdough favored organisms with higher resistance. Cereal type did not have selective effect on the lineage of *L. reuteri*.

Acknowledgements. Peter Kaluscha is acknowledged for technical assistance. The Alberta Livestock and Meat Agency and NSERC are acknowledge for financial support. Michael Gänzle acknowledges support from the Canada Research Chairs Program.

4.5 References

1. Gaesser GA, Angadi SS (2012) Gluten-Free Diet: Imprudent Dietary Advice for the General Population? J Acad Nutr Diet 112: 1330–1333.

2. Sapone A, Bai JC, Ciacci C, Dolinsek J, Green PHR, et al. (2012) Spectrum of gluten-related disorders: consensus on new nomenclature and classification. BMC Med 10: 13.

3. Zannini E, Jones JM, Renzetti S, Arendt EK (2012) Functional replacements for gluten. Annu Rev Food Sci Technol 3: 227–245.

4. Arendt EK, Moroni A, Zannini E (2011) Medical nutrition therapy: use of sourdough in gluten free bread. Microb Cell Fact 10 Suppl 1: S15.

5. Vogelmann SA, Seitter M, Singer U, Brandt MJ, Hertel C (2009) Adaptability of lactic acid bacteria and yeasts to sourdoughs prepared from cereals, pseudocereals and cassava and use of competitive strains as starters. Int J Food Microbiol 130: 205–212.

6. Nout MJR (2009) Rich nutrition from the poorest - cereal fermentations in Africa and Asia. Food Microbiol 26: 685–692.

7. Sekwati-Monang B, Gänzle MG (2011) Microbiological and chemical characterisation of ting, a sorghum-based sourdough product from Botswana. Int J Food Microbiol 150: 115–121.

8. Sekwati-Monang B, Valcheva R, Gänzle MG (2012) Microbial ecology of sorghum sourdoughs: effect of substrate supply and phenolic compounds on composition of fermentation microbiota. Int J Food Microbiol 159: 240–246.

9. Hamad SH, Dieng MC, Ehrmann MA, Vogel RF (1997) Characterization of the bacterial flora of Sudanese sorghum flour and sorghum sourdough. J Appl Microbiol 83: 764–770.

10. Olsen A, Halm M, Jakobsen M (1995) The antimicrobial activity of lactic acid bacteria from fermented maize (kenkey) and their interactions during fermentation. J Appl Bacteriol 79: 506–512.

11. Su MS-W, Oh PL, Walter J, Gänzle MG (2012) Intestinal origin of sourdough *Lactobacillus reuteri* isolates as revealed by phylogenetic, genetic, and physiological analysis. Appl Environ Microbiol 78: 6777–6780.

12. Oh PL, Benson AK, Peterson D, Patil PB, Moriyama EN, et al. (2010) Diversification of the gut symbiont *Lactobacillus reuteri* as a result of host-driven evolution. ISME J 4: 377–387.

13. Frese SA, Benson AK, Tannock GW, Loach DM, Kim J, et al. (2011) The evolution of host specialization in the vertebrate gut symbiont *Lactobacillus reuteri*. PLoS Genet 7: e1001314.

14. Gänzle MG, Vermeulen N, Vogel RF (2007) Carbohydrate, peptide and lipid metabolism of lactic acid bacteria in sourdough. Food Microbiol 24: 128–138.

15. De Vuyst L, Vrancken G, Ravyts F, Rimaux T, Weckx S (2009) Biodiversity, ecological determinants, and metabolic exploitation of sourdough microbiota. Food Microbiol 26: 666–675.

16. Vogel RF, Knorr R, Müller MRA, Steudel U, Gänzle MG, et al. (1999) Non-dairy lactic fermentations: the cereal world. Antonie Van Leeuwenhoek 76: 403–411.

17. Scheirlinck I, Van der Meulen R, Van Schoor A, Vancanneyt M, De Vuyst L, et al. (2008) Taxonomic structure and stability of the bacterial community in belgian sourdough ecosystems as assessed by culture and population fingerprinting. Appl Environ Microbiol 74: 2414–2423.

18. De Vuyst L, Vancanneyt M (2007) Biodiversity and identification of sourdough lactic acid bacteria. Food Microbiol 24: 120–127.

19. Albesharat R, Ehrmann MA, Korakli M, Yazaji S, Vogel RF (2011) Phenotypic and genotypic analyses of lactic acid bacteria in local fermented food, breast milk and faeces of mothers and their babies. Syst Appl Microbiol 34: 148–155.

20. Meroth CB, Walter J, Hertel C, Brandt MJ, Hammes WP (2003) Monitoring the bacterial population dynamics in sourdough fermentation processes by using PCR-denaturing gradient gel electrophoresis. Appl Environ Microbiol 69: 475–482.

21. Lattanzi A, Minervini F, Di Cagno R, Diviccaro A, Antonielli L, et al. (2013) The lactic acid bacteria and yeast microbiota of eighteen sourdoughs used for the manufacture of traditional Italian sweet leavened baked goods. Int J Food Microbiol 163: 71–79.

22. Safeukui I, Millet P, Boucher S, Melinard L, Fregeville F, et al. (2008) Evaluation of FRET real-time PCR assay for rapid detection and differentiation of *Plasmodium* species in returning travellers and migrants. Malar J 7: 70.

23. Chakravorty S, Helb D, Burday M, Connell N, Alland D (2007) A detailed analysis of 16S ribosomal RNA gene segments for the diagnosis of pathogenic bacteria. J Microbiol Methods 69: 330–339.

24. El-Hajj HH, Marras SAE, Tyagi S, Shashkina E, Kamboj M, et al. (2009) Use of sloppy molecular beacon probes for identification of mycobacterial species. J Clin Microbiol 47: 1190–1198.

25. Rensen GJ, Smith WL, Jaravata C V, Osburn B, Cullor JS (2006) Development and evaluation of a real-time FRET probe based multiplex PCR assay for the detection of prohibited meat and bone meal in cattle feed and feed ingredients. Foodborne Pathog Dis 3: 337–346.

26. Mader E, Ruzicka J, Schmiderer C, Novak J (2011) Quantitative high-resolution melting analysis for detecting adulterations. Anal Biochem 409: 153–155.

27. Ganopoulos I, Sakaridis I, Argiriou A, Madesis P, Tsaftaris A (2013) A novel closed-tube method based on high resolution melting (HRM) analysis for authenticity testing and quantitative detection in Greek PDO Feta cheese. Food Chem 141: 835–840.

28. Li C, Qiu X, Yang N, Gao J, Rong Y, et al. (2013) Common rs7138803 variant of FAIM2 and obesity in Han Chinese. BMC Cardiovasc Disord 13: 56.

29. Vaclavikova R, Ehrlichova M, Hlavata I, Pecha V, Kozevnikovova R, et al. (2012) Detection of frequent ABCB1 polymorphisms by high-resolution melting curve analysis and their effect on breast carcinoma prognosis. Clin Chem Lab Med 50: 1999–2007.

30. Vinci S, Gelmini S, Mancini I, Malentacchi F, Pazzagli M, et al. (2013) Genetic and epigenetic factors in regulation of microRNA in colorectal cancers. Methods 59: 138–146.

31. Gundry CN (2003) Amplicon melting analysis with labeled primers: a closed-tube method for differentiating homozygotes and heterozygotes. Clin Chem 49: 396–406.

32. Svensson L, Sekwati-Monang B, Lutz DL, Schieber A, Gänzle MG (2010) Phenolic acids and flavonoids in nonfermented and fermented red sorghum (*Sorghum bicolor* (L.) Moench). J Agric Food Chem 58: 9214–9220.

33. Engels C, Schieber A, Gänzle MG (2011) Inhibitory spectra and modes of antimicrobial action of gallotannins from mango kernels (*Mangifera indica* L.). Appl Environ Microbiol 77: 2215–2223.

34. Stuopelytė K, Daniūnaitė K, Laurinavičienė A, Ostapenko V, Jarmalaitė S (2013) High-resolution melting-based quantitative analysis of RASSF1 methylation in breast cancer. Medicina (Kaunas) 49: 78–83.

35. Vossen RHAM, Aten E, Roos A, den Dunnen JT (2009) High-resolution melting analysis (HRMA): more than just sequence variant screening. Hum Mutat 30: 860–866.

36. McKay LL, Baldwin KA (1990) Applications for biotechnology: present and future improvements in lactic acid bacteria. FEMS Microbiol Rev 7: 3–14.

37. Scheirlinck I, Van der Meulen R, Van Schoor A, Vancanneyt M, De Vuyst L, et al. (2007) Influence of geographical origin and flour type on diversity of lactic acid bacteria in traditional Belgian sourdoughs. Appl Environ Microbiol 73: 6262–6269.

38. Sánchez-Maldonado AF, Schieber A, Gänzle MG (2011) Structure-function relationships of the antibacterial activity of phenolic acids and their metabolism by lactic acid bacteria. J Appl Microbiol 111: 1176–1184.

39. Campos FM, Couto JA, Hogg TA (2003) Influence of phenolic acids on growth and inactivation of *Oenococcus oeni* and *Lactobacillus hilgardii*. J Appl Microbiol 94: 167–174.

40. Awika JM, Rooney LW, Waniska RD (2005) Anthocyanins from black sorghum and their antioxidant properties. Food Chem 90: 293–301.

5. Effect of lineage-specific metabolic traits of *Lactobacillus reuteri* on sourdough microbial ecology

5.1 Introduction

Sourdough fermentation with lactic acid bacteria has been used as leavening agent in artisanal baking, and sourdough or sourdough products increasingly find application as a baking improver in industrial baking [1]. In artisanal as well as industrial practice, sourdoughs are generally maintained by continuous back-slopping. Formation of a stable sourdough microbiota consisting of lactic acid bacteria alone or in association with yeasts depends on complex interactions between the inoculum, the cereal substrate, processing parameters, and environment [2]. Type I doughs are propagated by frequent inoculation at ambient temperature. The use of Type I sourdoughs as leavening agents requires fermentation processes that maintain continuous metabolic activity and CO_2 formation by fermentation microbiota [1]. Type I doughs propagated in temperate climates are generally dominated by *Lactobacillus sanfranciscensis* [3,4] but Type I doughs propagated in tropical climates harbour thermophilic lactobacilli including *Lactobacillus reuteri* [5,6]. The frequent occurrence of *L. sanfranciscensis* was attributed to the rapid growth of this organism, the efficient use of maltose as carbon source, the use of fructose as electron acceptor, and its small genome size [7–10]. In contrast, Type II doughs are used as baking improvers to modify flavour, texture, or shelf life in industrial processes in the form of active or dough that was stabilized by drying or pasteurization [1]. The elevated temperatures, long fermentation times, and high water content select for thermophilic and acid resistant organisms [9,11,12]. *L. reuteri* prevails in Type I and Type II sourdoughs fermented at elevated temperature [2,4,5].

L. reuteri not only occurs in food fermentations but is known predominantly for its lifestyle as vertebrate gut symbiont. *L. reuteri* is a vertebrate gut symbiont associated with humans, pigs, rodents, and different species of birds. Strains of *L. reuteri* have evolved into phylogenetically distinct and host-adapted lineages [13], with lineage-specific metabolic and genetic traits reflecting adaptation to different hosts [14]. Some rodent lineage strains are acid resistant due to the presence of glutamate decarboxylase GadB [12,15]. Human lineage strains convert glycerol or 1,2 propanediol to regenerate NAD^+ [14,16,17]. Sourdough isolates of *L.*

reuteri do not represent extra-intestinal lineages but can be assigned to host-adapted lineages, reflecting their long-term association with intestinal ecosystems prior to adopting an alternative lifestyle as fermentation organism in sourdough [18]. Genetics, metabolism, and phylogeny of *L. reuteri* have been studied extensively [14,19,20], making it an excellent model organism to identify strain-specific metabolic traits that affect the competitiveness in sourdough.

Past studies suggested that specific metabolic traits of lactic acid bacteria influence their growth rate in cereal substrates and determine their competitiveness in artisanal and industrial sourdough fermentations [8,10,12]. However, experimental validation for the effects of specific metabolic traits of lactobacilli on their competitiveness in sourdough is generally lacking. It was therefore the aim of this study to determine whether lineage specific metabolic traits contribute to competitiveness of *L. reuteri* in type I and type II sourdoughs. Experiments focused on glycerol metabolism, which may influence the growth rate in cereal substrates, and glutamate metabolism, which influences acid resistance in sourdough. Experiments evaluated the competitiveness of sourdough isolates of *L. reuteri* representing different host-adapted lineages in wheat and sorghum sourdoughs. Furthermore, the competitiveness of isogenic mutants with disruptions in glycerol- and glutamate metabolism was compared to that of the cognate wild type strains.

5.2 Material and methods

5.2.1 Sourdough fermentation. Previously, seven strains of *L. reuteri* isolated from rye, wheat, and sorghum sourdoughs were assigned to three host lineages [18]. To investigate the competitiveness of *L. reuteri*, this study used five strains – *L. reuteri* LTH5448 (rye / rodent-adapted lineage I), *L. reuteri* FUA3400 and FUA3401 (wheat / human-adapted lineage II), and *L. reuteri* FUA3168 and 3324 (sorghum / undefined lineage). Sourdough isolates of the rodent-adapted lineage III were not suitable for use in competition experiments because they produce reutericyclin, an antimicrobial compound that inhibits other strains of *L. reuteri* [21]. Strains were inoculated in modified MRS broth [8] and incubated at 37°C overnight. Two culture cocktails were prepared as biological repeats by mixing equal volume of one strain from each origin to obtain approximately equal cell counts at the time of inoculation. Cocktail 1 contained *L. reuteri* FUA5448, FUA3400, and FUA3168; cocktail 2 contained *L. reuteri* FUA5448,

FUA3401, and FUA3324. Whole wheat and white sorghum sourdoughs were prepared with commercial flours and a dough yield 200; sourdoughs were propagated at 37 °C by back-slopping every 12 or 72 h with 10% of the ripe sourdoughs. Sourdough samples were taken after inoculation and at the end of each fermentation cycle for analysis as outlined below. Two independent experiments with each cocktail were performed.

To determine the roles of glycerol and glutamate metabolism in sourdough ecology, sourdough was inoculated with equal cell counts of *L. reuteri* FUA3400 and *L. reuteri* FUA3400Δ*gupCDE*, or *L. reuteri* 100-23 and *L. reuteri* 100-23Δ*gadB*. *L. reuteri* FUA3400Δ*gupCDE* lacking the ability to use glycerol as electron acceptor was generated in this study (see below); generation of *L. reuteri* 100-23Δ*gadB* with a disruption in the gene coding for glutamate decarboxylase were described previously [12]. Competition experiments in sourdough were performed as described above in triplicate independent experiments.

5.2.2 Generation of *L. reuteri* FUA3400ΔgupCDE mutant. The gene *gupCDE* [17] in *L. reuteri* FUA3400 was truncated using pJRS233 [22] according to a deletion strategy described earlier [12]. The 5' flanking fragment of *gupCDE* was amplified from genomic DNA of *L. reuteri* FUA3400 using primers 5'-GGA GGT CGA CAG GCT TCA GTT GAT GCC GGA G-3' and 5'- ACC ATG CAT TGG GGT ACC TTA AAC AAA TGT ATC TTG ATG AAT TGG-3'. The 3' flanking fragment was amplified using primers 5'-CTG GTA CCT ATG AAA GTC GTA AGA AGC TAA AGG GCG ATA ACT AA-3' and 5'-CAA ATG CAT CGG ATC CCT TTC CTG TAA GAT CTG CCA TTG TTT -3'. The 5' flanking fragment was ligated into pGEMTeasy vector (Promega) to generate pGUP-A. The plasmid pGUP-A and 3' flanking fragment was digested with restrictive enzymes KpnI and NsiI, purified, and ligated to create pGUP-AB. The DNA fragment in pGUP-AB was digested with SalI and BamHI and ligated into pJRS233. The resulting plasmid pGUP-KO was electrotransformed in competent *L. reuteri* FUA3400 cells. Transformants were grown in mMRS erythromycin broth (5 mg/L) at 42 – 44°C for 80 generations to select for single crossover mutants. *L. reuteri* with pGUP-KO AB integrated into chromosome were cured by culturing in mMRS broth at 37°C for 100 generations. The culture was plated on mMRS agar and erythromycin-sensitive double crossover mutants were identified by replica plating mMRS and mMRS-erythromycin agar. The truncation of *gupCDE* in *L. reuteri* FUA3400Δ*gupCDE* was confirmed by PCR and sequencing. The

phenotype was confirmed by absence of 1,3 propanediol production using HPLC [6] and absence of 3-hydroxypropionaldhyde (reuterin) production using a colorimetric assay [23].

5.2.3 Culture-dependent quantification of sourdough microbiota. Sourdough samples were homogenized and diluted with 0.8% wt/vol NaCl solution. *L. reuteri* were enumerated after surface plating on modified MRS (mMRS) agar. Plates were incubated anaerobically at 37 °C for 24 h. When the individual strains used in the respective strain cocktails could be differentiated on the basis of their colony morphology, strains were differentially enumerated. The pH of sourdough was measured with a glass electrode; the sorghum sourdough pH was 3.44 ± 0.03 and 3.38 ± 0.09 after 12 and 72 h of fermentation, respectively, the wheat sourdough pH was 3.40 ± 0.06 and 3.46 ± 0.09 after 12 and 72 h of fermentation, respectively.

5.2.4 Differentiation of *L. reuteri* strains using molecular beacon. Two grams of sourdough was homogenized with 80 mL of 0.8% wt/vol saline. The homogenate was centrifuged at 1500 rpm for 5 min to remove solids. The cells were harvested by centrifuging at 5000 rpm for 15 min. DNA was extracted with DNeasy Blood & Tissue Kit (Qiagen, USA) following instructions of the manufacturer.

Molercular beacon was design to distinguish strains from different lineages based on temperature differences in melting temperatures [24]. The primers sequences were: forward (limiting), 5'-AAT ATG CAG AAG CCT TAG-3'; reverse (excess), 5'-TAT CAC CCA TAT CAC CAT-3'. The sequence of molecular beacon was 5'-CGC GAT CAT GAT TAC GAA AAC AAG TTT GTG GAT GGA TCG CG-3'. Each PCR contained 1× TaqMan® Fast Universal PCR Master Mix (2X), No AmpErase® UNG (Applied Biosystem, Canada), 2 μM excess primer, 0.2 μM limiting primer, 0.2 μM of molecular beacon, and bacterial DNA in a final volume of 16 μl. PCR was done on Applied Biosystems 7500 Fast Real-time PCR System (Life Technologies, USA). The PCR conditions were: denaturation 5 min at 95°C, followed by 60 cycles of denaturation at 95°C for 10 sec, annealing at 50°C for 15 sec, and extension at 72°C for 20 sec. At the melting curve stage the temperature was held at 95°C for 2 min, at 25°C for 60 min, and then increased from 25°C to 90°C at 1°C/step with 25 sec holding time at each step. The melting temperature was 47°C for human lineage, 53°C for rodent lineage, and 61°C for sorghum strains.

5.2.5 Relative quantification of *L. reuteri* wild type and mutant strains using quantitative PCR. DNA was extracted from sourdough as described above and *L. reuteri* wild type and mutant strains were quantified with strain-specific primers. The primers for wild types were: gad-WT-F, 5'-ATC TAG ATT ATC CTG CCA TAG ATA AAA-3'; gad-WT-R, 3'-TAA AGC ACG AGC ATC ATT CG-3'; gup-WT-F, 5'-GCA TTC GCA ACT GTT CTT GA-3'; gup-WT-R, 5'-ACT GTC GTC CCC TTT GAT TG-3'. The primers for mutants were: gad-M-F, 5'-AAA TTA ACC TAG GAG GTT TTA TCT ATG-3'; gad-M-R, 5'-CAG GAC GCA GCA AAG AAG TA-3'; gup-M-F, 5'-CTG TTA TGG CTG GAC GTG AA-3'; gup-M-R, 5'-TCG CCC TTT AGC TTC TTA CG-3'. Each PCR contained 1× QuantiFast SYBR Green PCR Master Mix (Qiagen, Canada), 1 μM of each primer, and 1 μL bacterial DNA in a final volume of 20 μl. PCR was done on Applied Biosystems® 7500 Real-Time PCR (Life Technologies, Canada). The PCR conditions were: denaturation 5 min at 95°C, followed by 60 cycles of denaturation at 95°C for 10 sec, annealing at 56°C for 30 sec. Standard curves for absolute quantification of the strains in the sourdoughs were generated with purified PCR products obtained from the respective strains as described [25].

5.2.6 *In vitro* growth rate of *L. reuteri* strains. The main sugars and electrons acceptor for wheat and rye sourdoughs are maltose and sucrose; in contrast, glucose and glycerol are most abundant substrates for use as carbon source and as electron acceptor, respectively, in sorghum sourdoughs [25]. Modified MRS that mimic sugar compositions of wheat and sorghum with and without electron acceptor was prepared with the following sugars as sole carbon source (W1) 10 g of maltose, 2 g of glucose; (W2) 10 g of maltose, 2 g of glucose, 10 g of sucrose; (S1) 2 g of maltose, 10 g of glucose; (W2) 2 g of maltose, 10 g of glucose, 5 g of glycerol. mMRS Glucose containing 10 g of glucose and lacking both maltose and electron acceptors was used to prepare the pre-cultures. Strains of *L. reuteri* were subcultured twice overnight in mMRS glucose, and grown to an optical density (600nm) of 0.5 corresponding to the exponential phase of growth. mMRS W1, W2, S1, S2 were inoculated with 10% inoculum of exponentially growing cultures and incubated in 96 well microtiter plates. The cultures were overlayered with 50 μL paraffin oil to maintain anaerobic environment. The plate was cultured at 37°C for 24 h and optical density was measured every 30 min with 10 s of shaking before each measurement. Growth rates were calculated by fitting the optical density data to the logistic growth curve [26].

5.2.7 Quantification of 1,3-propanediol concentration in bacterial culture and sourdoughs. Overnight bacterial cultures or sourdough samples were homogenized in equal volume of 7% $HClO_4$ and incubated at 4°C overnight to precipitate proteins. The sample was centrifuged at 14000 rpm for 10 min. The supernatant was diluted three times with water and then used for HPLC. 1,3-Propanediol concentration was quantified using Aminex HPX-87 column, 300 mm×7.8 mm (BioRad, USA) based on refractive index detection [6]. Samples were eluted with 5 mmol/L H_2SO_4 at 70 °C with a flow rate of 0.4 mL/min.

5.2.8 Statistics. Data analysis was performed with PROC MIXED procedure (SAS v.9.2; SAS Institute, USA) using two-way analysis of variance (ANOVA). A p-value of ≤ 0.05 was considered statistically significant.

5.3 Results

5.3.1 Microbial ecology *L. reuteri* strains in wheat and sorghum sourdoughs. To determine the effect of substrate and fermentation time on sourdough microbial ecology, wheat and sorghum sourdoughs were propagated every 12 or 72 h over 12 fermentation cycles. Culture dependent analysis and the characterization of sourdough microbiota by HRM-qPCR demonstrated that the *L. reuteri* strains used as inoculum accounted for more than 99% of bacteria in any of the sourdoughs (Figure 5-1 and Table 5-1). The rodent lineage strains and sorghum isolates could not be distinguished from each other by colony morphology; they were enumerated together by culture-dependent quantification (Figure 5-1) but were readily differentiated by HRM-qPCR (Table 5-1). The total cell count after 12h of fermentation was about 1 log higher when compared to cell counts after 72 h of fermentation (data not shown). After 12 fermentation cycles with 72 h fermentation time in wheat or sorghum sourdoughs, rodent lineage strains were identified in high cell counts in all sourdoughs; sorghum isolates were additionally identified in two of the four doughs. In wheat or sorghum sourdoughs fermented with 12h fermentation times, human lineage strains were identified in high cell counts 12 fermentation cycles; sorghum isolates were additionally present in the two of the four doughs (Figure 5-1 and Table 5-1). These results imply that different lineage-specific metabolic traits account for the competitiveness of *L. reuteri* in sourdoughs with short or long fermentation times.

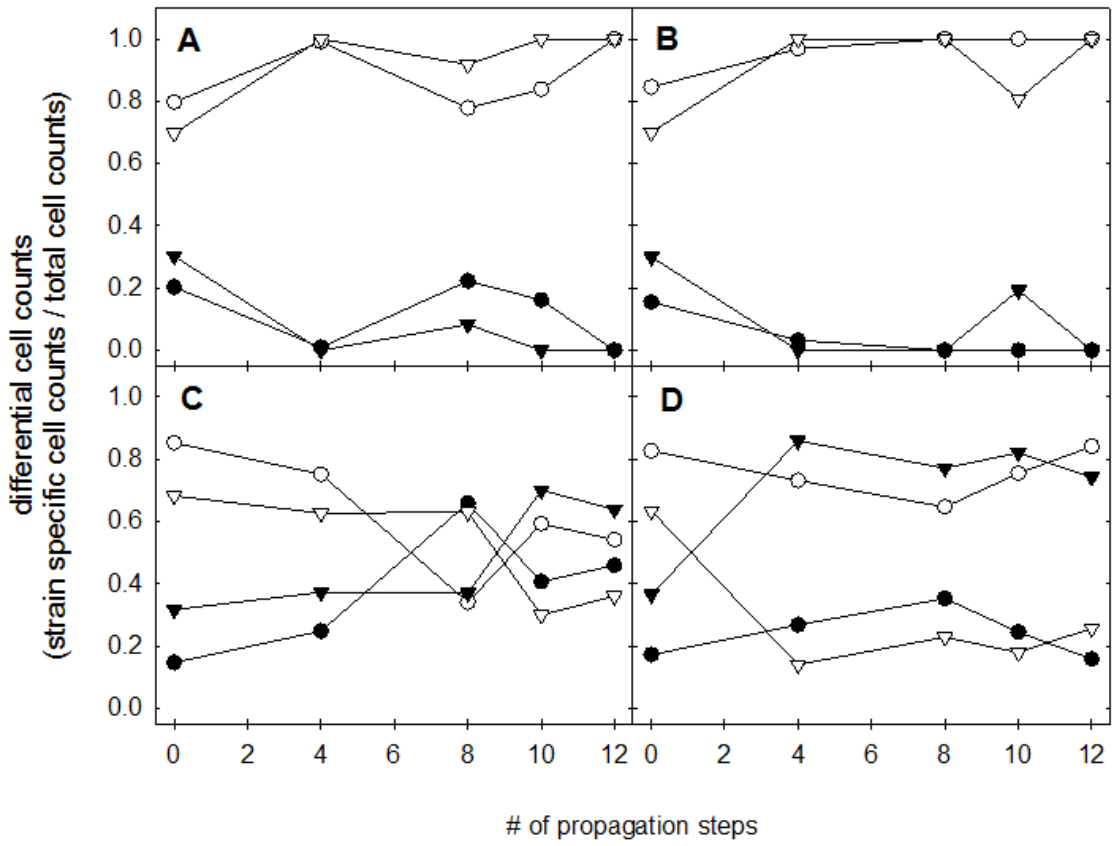


Figure 5-1. Microbial composition of wheat sourdoughs (Panels A and C) and sorghum sourdoughs (Panels B and D) that were inoculated with three *L. reuteri* strains. Sourdoughs were propagated with 72 h fermentation cycles (Panels A and B) or 12 h fermentation cycles (Panels C and D). Symbols indicate *L. reuteri* FUA3400 (●), *L. reuteri* FUA5448 or FUA 3168 (○) for cocktail 1, *L. reuteri* FUA3401 (▼), and *L. reuteri* FUA5448 or FUA 3324(△) for cocktail 2. Representative data of two independent experiments are shown.

Table 5-1. Lineage-specific detection of *L. reuteri* in wheat and sorghum sourdoughs propagated with 72 h and 12 h fermentation cycles. Duplicate experiments were conducted with two strain cocktails. Lineage specific detection of *L. reuteri* strains was achieved with HRM-qPCR.

		# of fermentation cycles				
		0	**4**	**8**	**10**	**12**
72 h fermentation time	**Wheat, cocktail #1** [a)]	H, R, S[c)]	R, S	H, R, S	R, S	R, S
	Wheat, cocktail #2 [b)]	H, R, S	H, R, S	H, R, S	R, S	R, S
	Sorghum, cocktail #1	H, R, S	R, S	R, S	R	R
	Sorghum, cocktail #2	H, R, S	R	R	R	R
12 h fermentation time	**Wheat, cocktail #1**	H, R, S	H, R, S	H, R, S	H, R, S	H, S
	Wheat, cocktail #2	H, R, S	H, R, S	H	H	H
	Sorghum, cocktail #1	H, R, S	H, R, S	H, S	H, S	H, S
	Sorghum, cocktail #2	H, R, S	H	H	H	H

[a)] Cocktail #1 was composed of *L. reuteri* FUA3400 (human lineage II; H), *L. reuteri* FUA5448 (rodent lineage I; R) and *L. reuteri* FUA3168 (undefined lineage; S)

[b)] Cocktail #2 was composed of *L. reuteri* FUA3401 (human lineage II; H), *L. reuteri* FUA5448 (rodent lineage I; R) and *L. reuteri* FUA3324 (sorghum isolate; undefined lineage; S)

[c)] Strains detected by observation of the lineage-specific melting peaks if they accounted for 1% or more of total fermentation microbiota. Letters denote strains of the same host lineage.

5.3.2 *In vitro* growth rate of *L. reuteri* strains. In order to determine the effect of electron acceptors on growth of *L. reuteri*, media were prepared to represent the main carbon sources in wheat and sorghum with sucrose and glycerol as electron acceptors (W2, S2) and without electron acceptors (W1, S1). For all strains, the addition of sucrose as the electron acceptor increased in growth rate (Figure 5-2). In contrast, glycerol as the electron acceptor increased growth rate of human lineage strains only. For human lineage strains, the increase in growth rate was less pronounced irrespective of whether sugar or glycerol was the electron acceptor.

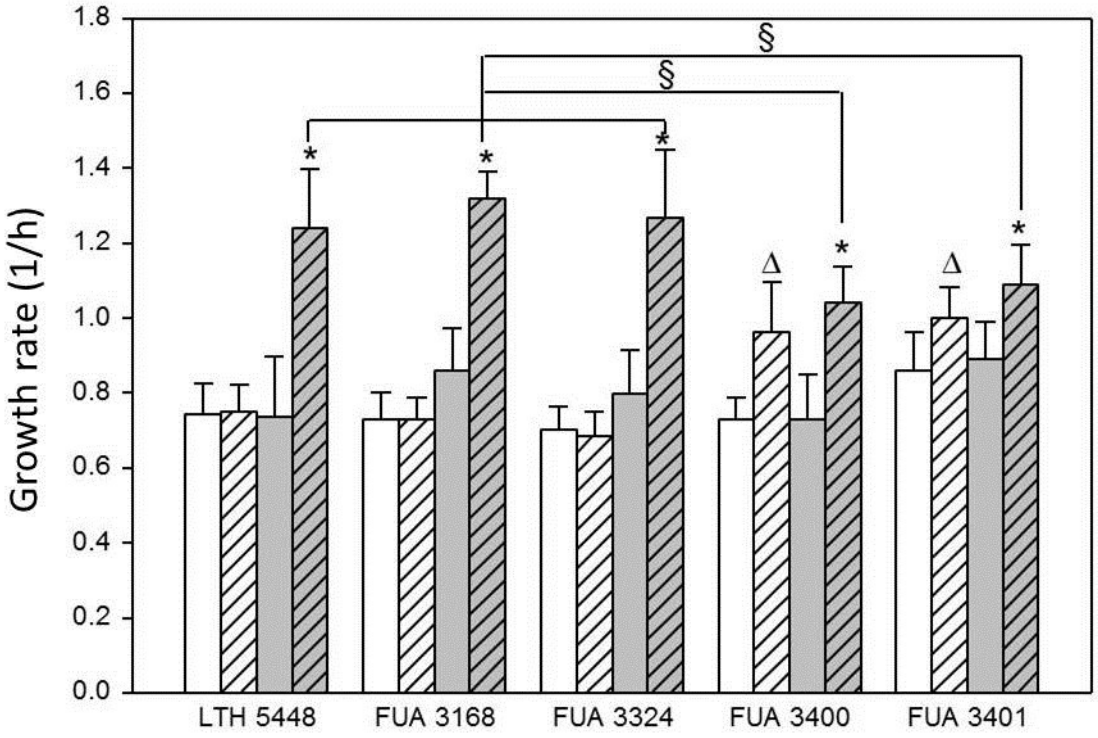


Figure 5-2. Growth rate of *L. reuteri* strains in mMRS broth. White bars indicate glucose as carbon source (S1 and S2), grey bars indicate maltose as carbon source (W1 and W2); hatched bars indicate the addition of electron acceptors (10 mmol / L glycerol in medium S2, 10 mmol / L sucrose in medium W2). Symbols indicate significant differences ($P<0.05$) between human lineage strains and other strains (§), between W1 and W2 media (*), and between S1 and S2 media (Δ). Data are shown as means ± standard deviation of triplicate independent experiments.

5.3.3 Generation of FUA3400ΔgupCDE mutant. In human lineage strains of *L. reuteri*, glycerol is converted to 1,3-propanediol by glycerol dehydratase and 1,3-propanediol dehydrogenase to regenerate NAD^+ [16,27]. In this study, the glycerol dehydratase gene was truncated to disable glycerol metabolism in *L. reuteri* FUA3400 because of the possible presence of more than one 1,3-propanediol dehydrogenase in the genome [28]. Truncation of the glycerol dehydratase avoids accumulation of the antimicrobially active intermediate reuterin; besides, the lack of propanediol dehydrogenase may be compensated by other dehydrogenase enzymes with

broad substrate specificity [29]. The candidate genes in *L. reuteri* FUA3400 show high sequence similarity to their counterparts in *L. reuteri* JCM1112 and were thus also designated as *gupCDE* [17]. The sequence of *gupCDE* and the truncated gene were deposited in GenBank (accession numbers: KJ435307-KJ435310). *L. reuteri* FUA3400 quantitatively converted glycerol to 1,3-propanediol (Figure 5-3 and data not shown). *L. reuteri* FUA3400Δ*gupCDE* did not convert glycerol to 1,3-propanediol and glycerol supplementation of mMRS-glucose did not support acetate formation (Figure 5-3). The colorimetric assay detected reuterin in cultures of *L. reuteri* FUA3400 but not in cultures of *L. reuteri* FUA3400Δ*gupCDE*. The growth rate of *L. reuteri* FUA3400 and FUA3400Δ*gupCDE* were compared in media containing maltose, maltose and fructose, glucose, or glucose and glycerol (designated W1, W2, S1, and S2 in Figure 5-4). The addition of sucrose in W2 enhanced the growth rate of both strains, while the addition of glycerol in S2 increased the growth rate of *L. reuteri* FUA3400 but not the growth rate of *L. reuteri* FUA3400Δ*gupCDE*, indicating glycerol metabolism had a positive effect on growth without affecting other key aspects of microbial metabolism.

5.3.4 Role of glycerol and glutamate metabolism in short-term and long-term fermentation. To investigate the role of glycerol and glutamate metabolisms during sourdough fermentation, wheat and sorghum sourdoughs were fermented using two pairs of wild type strains and their mutants. As observed above, the total cell counts of all doughs fermented with 72 h fermentation cycles were about 10-fold lower when compared to 12 h fermented doughs (data not shown), indicating cell death during extended incubation at acid conditions [30]. Disruption of *gupCDE* gene impaired growth of the mutant in both 12h and 72h cycles, but the strain disappeared faster in doughs fermented with 72h cycles (Figure 5-5). Disruption of *gadB* in *L. reuteri* 100-23 resulted in reduced competitiveness in doughs propagated with 72 h fermentation cycles. The wildtype outcompeted the GadB mutant faster in wheat than in sorghum; however, the competitiveness of the mutant was not affected in doughs propagated with 12h fermentation cycles.

5.3.5 Quantification of 1,3-propanediol in wheat and sorghum. Sorghum contains glycerol in the form of phenolic esters [31]. Lacking any indication that glycerol is present as electron acceptor in wheat sourdoughs, we expected that *L. reuteri* FUA3400 outcompetes the FUA3400 Δ*gupCDE* in sorghum but not in wheat sourdoughs. Surprisingly, no difference was observed

between wheat and sorghum doughs (Figure 5-5). To determine the level of glycerol conversion in wheat, rye, and sorghum, glycerol and 1,3 propanediol were quantified in unfermented sourdoughs and in sourdoughs fermented with *L. reuteri* FUA3400. The concentration of 1,3 propanediol in all sourdoughs was equivalent to the glycerol concentration in unfermented sourdoughs, indicating quantitative conversion by bacterial metabolism (Figure 5-6). Remarkably, glycerol and 1,3 propanediol concentrations in wheat demonstrate glycerol metabolism by *L. reuteri* in wheat and rye sourdoughs as well.

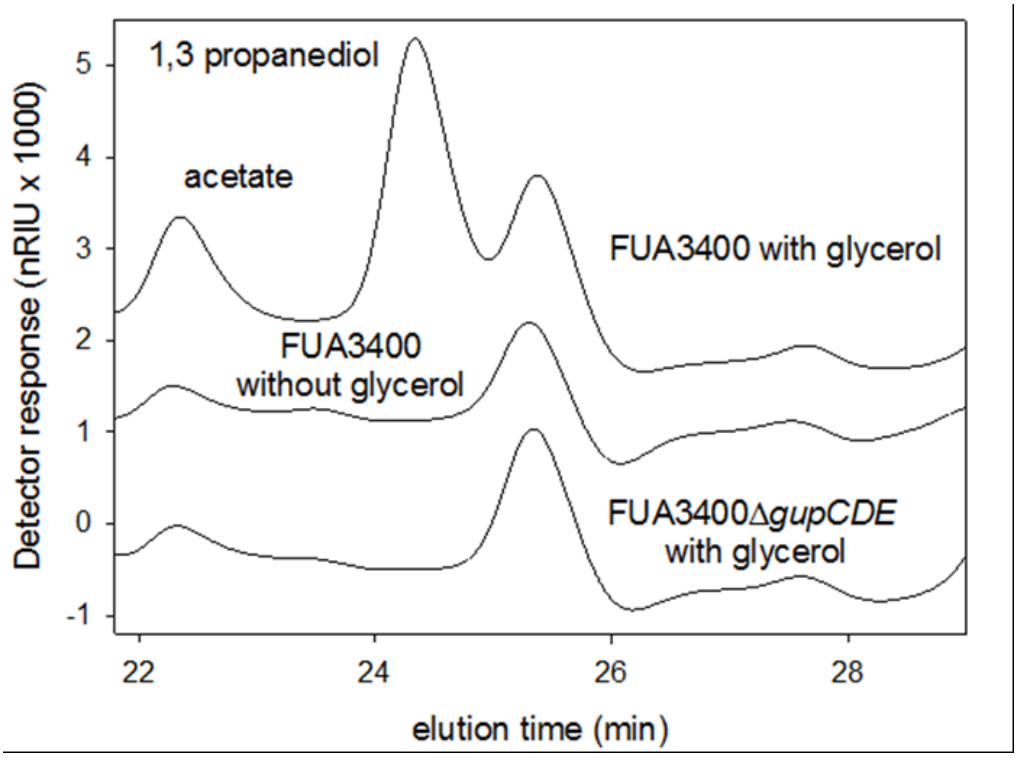


Figure 5-3. Separation of acetate and 1,3 propanediol in media fermented with *L. reuteri* FUA3400 or *L. reuteri* FUA3400Δ*gupCDE*. Where indicated, media were supplemented with 10 mmol / L glycerol as electron acceptor. Chromatograms were offset by 1500 nRIU.

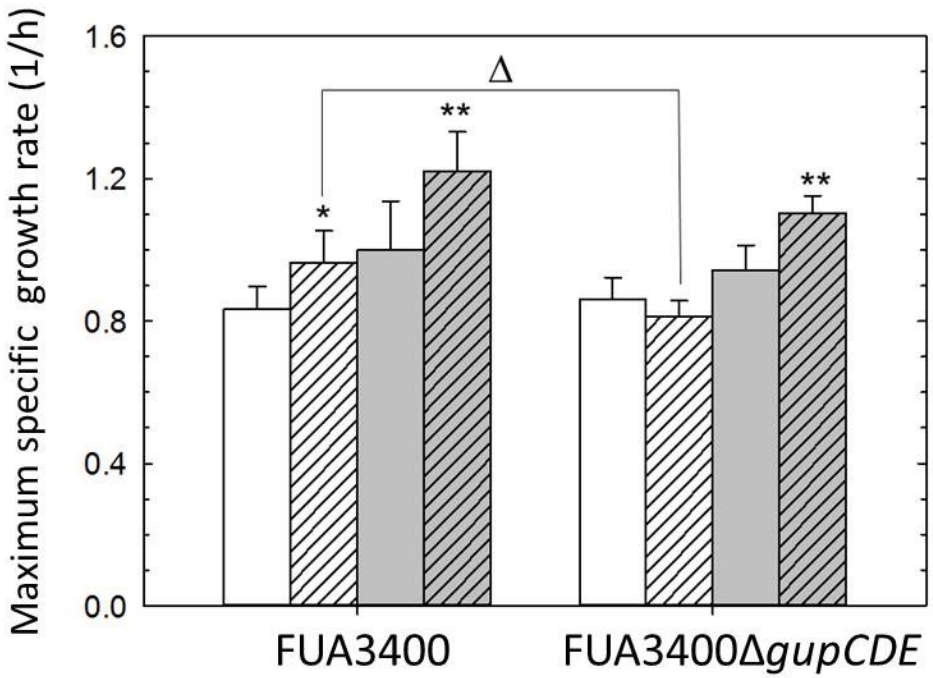


Figure 5-4. Growth rate of *L. reuteri* FUA3400 wildtype and *L. reuteri* FUA3400Δ*gupCDE* in mMRS broth. White bars indicate glucose as carbon source (S1 and S2), grey bars indicate maltose as carbon source (W1 and W2); hatched bars indicate the addition of electron acceptors (10 mmol / L glycerol in medium S2, 10 mmol / L sucrose in medium W2). Symbols indicate significant differences ($P<0.05$) between W1 and W2 (*), and between S1 and S2 (Δ). Data are shown as means ± standard deviation of triplicate independent experiments.

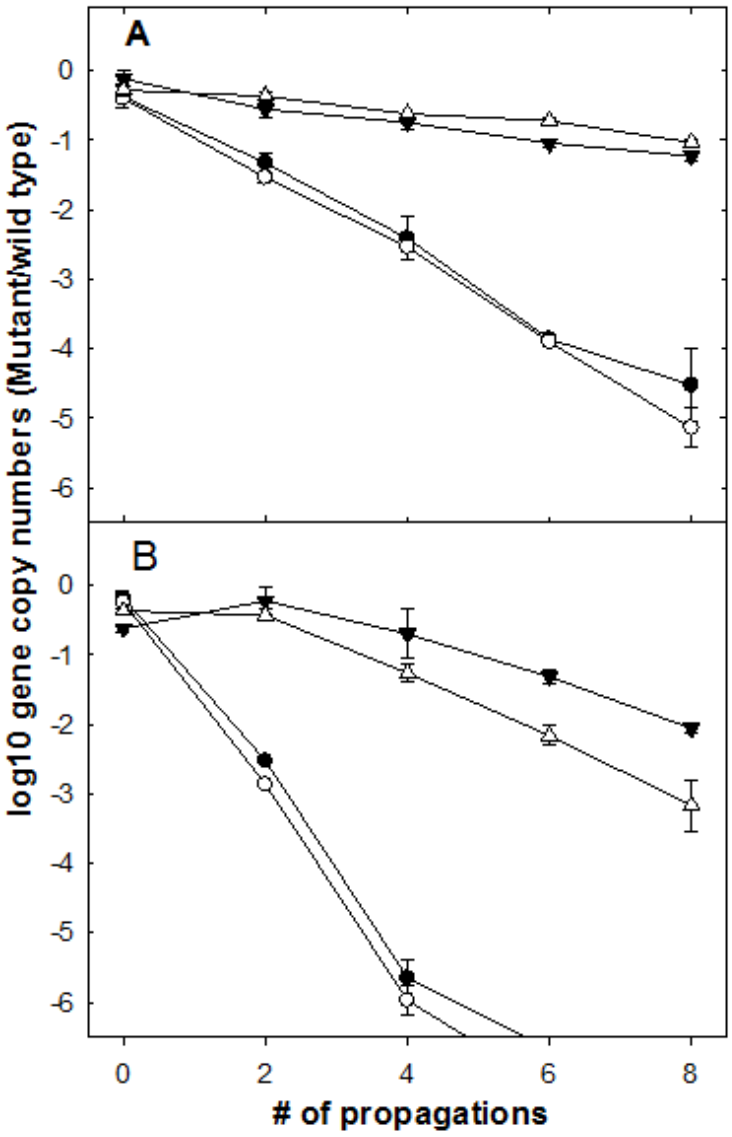


Figure 5-5. Microbial composition in sorghum and wheat sourdoughs fermentation by 12h and 72h propagations. Symbols indicate log ratio of *L. reuteri* FUA3400 / *L. reuteri* FUA3400□*gupCDE* in sorghum (●) and wheat (○) sourdoughs and the ratio of *L. reuteri* 100-23 / *L. reuteri* 100-23□*gadB* in sorghum (▼) and wheat (△) sourdoughs. The abundance of *L. reuteri* FUA3400□*gupCDE* was below detection after 6 propagations. Data are shown as means ± standard deviation of triplicate independent experiments.

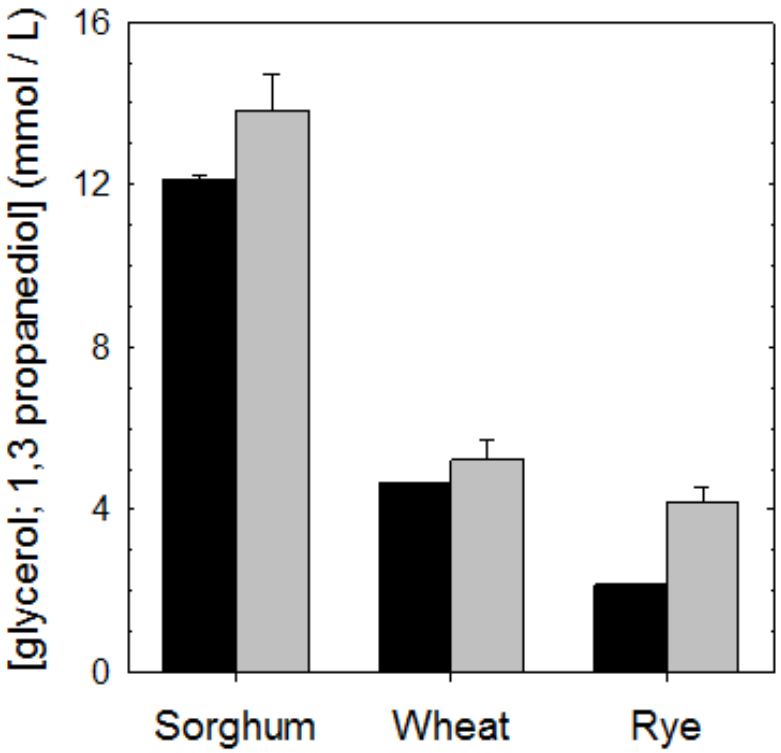


Figure 5-6. Concentration of glycerol (black bars) in unfermented wheat, rye, and sorghum sourdoughs, and concentration of 1,3 propanediol in wheat, rye, and sorghum sourdoughs after 24 h of fermentation with *L. reuteri* FUA3400.

5.4 Discussion

This study used microbial competition in back-slopped sourdough as a tool to study the impact of metabolic properties on ecological competitiveness of closely related organisms. Sourdoughs that are propagated in the bakery are continuously contaminated by organisms from ingredients, pests, bakers, and the bakery environment [3,4,18]. Continuous cycles of contamination and selection result in the establishment of highly competitive microbiota. The congruent evolution of microbiota in Type I sourdoughs worldwide demonstrates that this process is highly selective and reproducible [3,4,7,9,32,33].

Fermentations at the laboratory scale fail to reproduce this congruent selection process [34]. Sourdough fermentations in bakeries are controlled to achieve a consistent technological function (e.g. fermentation kinetics, level of acidity, leavening power) while sourdough fermentations in the laboratory are controlled to achieve consistent fermentation conditions with respect to time and temperature [6,11,12,34]. Moreover, flour is the only source of bacterial contamination in lab-scale fermentations, excluding other sources that are more relevant for sourdough ecology [18]. Third*, L. sanfranciscensis*, the key organism in Type I sourdoughs, was isolated only in sourdoughs with a long—month or years—history of continuous back-slopping [3,4,33] but lab-scale experiments are typically not conducted on that time scale. Competition experiments in back-slopped sourdoughs are nevertheless a sensitive tool to determine the competitiveness of different strains or species that are inoculated into the same sourdough [11,12,25,34]. This study additionally demonstrates that competition experiments with knockout mutant strains provide a quantitative assessment of the contribution of individual metabolic traits to overall competitiveness. In sourdoughs inoculated with pairs of wild type strains and knock down mutants, the ratio of wild type to mutant strains changed with a constant increment per fermentation cycle. For example, the log ratio of FUA3400Δ*gupCDE* to FUA3400 changed by 0.30±0.04 and 0.44±0.05 per fermentation cycle in sorghum and wheat sourdoughs, respectively. Despite the use of different flour and slightly different fermentation conditions, the rate of strain displacement and hence the impact of GadB on the competitiveness in wheat sourdough determined in this study was in excellent agreement with previous reports [12].

Short- and long-fermented sourdoughs select for different metabolic traits. Frequent propagation selects for fast growth. Long fermentation times select for acid resistance. The conversion of arginine to ornithine, which is species-specific in lactobacilli, and glutamine metabolism, which is lineage-specific in *L. reuteri* [14], contribute to acid resistance in lactobacilli [12,15,35]. Arginine and glutamine conversions partially compensate for the lack of glutamate decarboxylation [15,36,37]; glutamate decarboxylase positive rodent lineage strains of *L. reuteri* thus have only a modest advantage over other strains [12, this study]. The difference in competitiveness between *L. reuteri* 100-23 and 100-23Δ*gadB* was larger in wheat sourdoughs than in sorghum sourdoughs, possibly reflecting the higher content of (glutamine plus glutamate) in wheat proteins (31%, 37) when compared to sorghum proteins (19%, 38). *L. reuteri* 100-23

expressed *gadB* at the stationary stage but not at the exponential stage of growth [15], accordingly, *L. reuteri* 100-23 and 100-23Δ*gadB* were equally competitive in sourdoughs maintained by 12 h fermentation cycles.

In *L. reuteri*, enzymes for glycerol utilization are coded by the *pdu-cbi-cob-hem* cluster [16], which is conserved in human isolates but only in a small proportion of swine or rodent isolates [14]. Studies on glycerol metabolism by *L. reuteri* focused on the production of reuterin, a metabolic intermediate with antimicrobial activity at millimolar concentrations. 1,3-propanediol has no appreciable antimicrobial activity [40]. The use of glycerol as electron acceptor enhanced growth of obligate heterofermentative lactobacilli including *L. reuteri* [27,41]. This study demonstrates that glycerol conversion to 1,3-propanediol provides an ecological advantage during growth in cereal substrates. *L. reuteri* FUA3400Δ*gupCDE* failed to compete with the wild type in both short- and long-fermented doughs. The presence of glycerol as electron acceptor increased the growth rate by approximately 20% (Figure 5-4). A 10% inoculum in sourdoughs propagated with 12 h fermentation cycles corresponds to about 3 h of exponential growth [42]. Because cell counts decreased by about 90% during stationary phase of sourdoughs propagated with 72 h fermentation cycles, the exponential phase of growth upon back-slopping of 72 h fermented doughs is extended, enhancing the competitive advantage for the faster-growing wild type strain. Because *L. reuteri* FUA3400 and FUA3400Δ*gupCDE* did not differ in the stationary phase survival, this extended phase of exponential growth resulted in more rapidly decrease of the proportion of *L. reuteri* FUA3400Δ*gupCDE* in long-term fermented sourdoughs. However, in competition of glycerol metabolising human-lineage wild type strains with glutamate decarboxylating rodent lineage strains, the faster growth of the former was offset by the improved stationary phase survival of the latter (Figure 5-1, Table 5-1, [12]).

The conversion of glycerol to 1,3-propanediol was induced by 1,2-propanediol [43]. Interestingly, both 1,2-propanediol and 1,3-propanediol producing lactobacilli were isolated in traditional sorghum sourdoughs [6], suggesting possible symbiotic relationship that might benefit glycerol-metabolizing organisms. Glycerol is present in sorghum flour in the form of glycerol esters of phenolic acids [31], whereas the presence of 1,3-propropanediol from glycerol in wheat sourdough has not been described. However, results from this study showed that the amount of glycerol in wheat sourdough was sufficient to provide ecological advantage for glycerol-

metabolizing strains over non-metabolizing strains. This study did not confirm the source of glycerol in wheat; wheat lipids which are concentrated in the outer layers of the grain are a potential source for release of glycerol by esterases during fermentation [44].

In summary, glycerol metabolism enabled rapid growth and increased competitiveness of *L. reuteri* in Type I sourdoughs, while glutamate metabolism provided acid resistance and increased competitiveness in Type II sourdoughs. The use of glycerol as electron acceptor affects bread quality through increased levels of acetate; glutamate accumulation may alter the taste of bread [19]. The observation that the competitive advantage of glycerol-metabolising *L. reuteri* in sourdough is dependent on the use of reuterin as electron acceptor may also relate to intestinal ecosystems. Glutamate-mediated acid resistance may support the survival of probiotic lactobacilli during gastric transit [12,15]. In rodents, *L. reuteri* colonize the forestomach, where sucrose is available to provide fructose as electron acceptor [45]. The conserved *pdu-cbi-cob-hem* gene cluster in human-associated *L. reuteri* indicates that glycerol metabolism contributes to the competitiveness of the organism in humans. In humans, *L. reuteri* colonize the colon where sucrose is not available but glycerol or 1,2-propanediol may be available through lipid hydrolysis or as fermentation product of other bacteria [46,47].

Acknowledgements

Marcia Shu-Wei Su is acknowledged for support with generation of the deletion mutant. The National Science and Engineering Council of Canada and the Canada Research Chairs Program are acknowledged for financial support.

5.5 References

1. Brandt MJ (2007) Sourdough products for convenient use in baking. Food Microbiol 24: 161–164.

2. Hammes WP, Brandt MJ, Francis KL, Rosenheim J, Seitter MFH, et al. (2005) Microbial ecology of cereal fermentations. Trends Food Sci Technol 16: 4–11.

3. Vogel RF, Knorr R, Müller MRA, Steudel U, Gänzle MG, et al. (1999) Non-dairy lactic fermentations: the cereal world. Antonie Van Leeuwenhoek 76: 403–411.

4. De Vuyst L, Vrancken G, Ravyts F, Rimaux T, Weckx S (2009) Biodiversity, ecological determinants, and metabolic exploitation of sourdough microbiota. Food Microbiol 26: 666–675.

5. Hamad SH, Dieng MC, Ehrmann MA, Vogel RF (1997) Characterization of the bacterial flora of Sudanese sorghum flour and sorghum sourdough. J Appl Microbiol 83: 764–770.

6. Sekwati-Monang B, Gänzle MG (2011) Microbiological and chemical characterisation of ting, a sorghum-based sourdough product from Botswana. Int J Food Microbiol 150: 115–121.

7. Gänzle M, Ehmann M, Hammes W (1998) Modeling of growth of *Lactobacillus sanfranciscensis* and *Candida milleri* in response to process parameters of sourdough fermentation. Appl Environ Microbiol 64: 2616–2623.

8. Stolz P, Böcker G, Hammes WP, Vogel RF (1995) Utilization of electron acceptors by lactobacilli isolated from sourdough. Z Lebensm Unters Forsch 201: 91–96.

9. Vogel RF, Pavlovic M, Ehrmann MA, Wiezer A, Liesegang H, et al. (2011) Genomic analysis reveals *Lactobacillus sanfranciscensis* as stable element in traditional sourdoughs. Microb Cell Fact 10 Suppl 1: S6.

10. Neubauer H, Glaasker E, Hammes WP, Poolman B, Konings WN (1994) Mechanism of maltose uptake and glucose excretion in *Lactobacillus sanfrancisco*. J Bacteriol 176: 3007–3012.

11. Meroth CB, Walter J, Hertel C, Brandt MJ, Hammes WP (2003) Monitoring the bacterial population dynamics in sourdough fermentation processes by using PCR-denaturing gradient gel electrophoresis. Appl Environ Microbiol 69: 475–482.

12. Su MS, Schlicht S, Gänzle MG (2011) Contribution of glutamate decarboxylase in *Lactobacillus reuteri* to acid resistance and persistence in sourdough fermentation. Microb Cell Fact 10 Suppl 1: S8.

13. Oh PL, Benson AK, Peterson D, Patil PB, Moriyama EN, et al. (2010) Diversification of the gut symbiont *Lactobacillus reuteri* as a result of host-driven evolution. ISME J 4: 377–387.

14. Frese SA, Benson AK, Tannock GW, Loach DM, Kim J, et al. (2011) The evolution of host specialization in the vertebrate gut symbiont *Lactobacillus reuteri*. PLoS Genet 7: e1001314.

15. Teixeira JS, Seeras A, Sanchez-Maldonado AF, Zhang C, Su MS-W, et al. (2014) Glutamine, glutamate, and arginine-based acid resistance in *Lactobacillus reuteri.* Food Microbiol 42: 172–180.

16. Sriramulu DD, Liang M, Raux-deery E, Parsons JB, Warren MJ, et al. (2008) *Lactobacillus reuteri* DSM 20016 produces cobalamin-dependent diol dehydratase in metabolosomes and metabolizes 1 , 2-propanediol by disproportion. J Bact 190: 4559–4567.

17. Morita H, Toh H, Fukuda S, Horikawa H, Oshima K, et al. (2008) Comparative genome analysis of *Lactobacillus reuteri* and *Lactobacillus fermentum* reveal a genomic island for reuterin and cobalamin production. DNA Res 15: 151–161.

18. Su MS-W, Oh PL, Walter J, Gänzle MG (2012) Intestinal origin of sourdough *Lactobacillus reuteri* isolates as revealed by phylogenetic, genetic, and physiological analysis. Appl Environ Microbiol 78: 6777–6780.

19. Gänzle MG, Vermeulen N, Vogel RF (2007) Carbohydrate, peptide and lipid metabolism of lactic acid bacteria in sourdough. Food Microbiol 24: 128–138.

20. Gänzle MG, Höltzel A, Walter J, Jung G, Hammes WP (2000) Characterization of reutericyclin produced by *Lactobacillus reuteri* LTH2584. Appl Environ Microbiol 66: 4325–4333.

21. Gänzle MG, Vogel RF (2003) Contribution of reutericyclin production to the stable persistence of *Lactobacillus reuteri* in an industrial sourdough fermentation. Int J Food Microbiol 80: 31–45.

22. Perez-Casal J, Price JA, Maguin E, Scott JR (1993) An M protein with a single C repeat prevents phagocytosis of *Streptococcus pyogenes*: use of a temperature-sensitive shuttle vector to deliver homologous sequences to the chromosome of *S. pyogenes*. Mol Microbiol 8: 809–819.

23. Cadieux P, Wind A, Sommer P, Schaefer L, Crowley K, et al. (2008) Evaluation of reuterin production in urogenital probiotic *Lactobacillus reuteri* RC-14. Appl Environ Microbiol 74: 4645–4649.

24. Lin XB, Gänzle MG (2014) Quantitative high-resolution melting PCR analysis for monitoring of fermentation microbiota in sourdough. Int J Food Microbiol 186C: 42–48.

25. Sekwati-Monang B, Valcheva R, Gänzle MG (2012) Microbial ecology of sorghum sourdoughs: effect of substrate supply and phenolic compounds on composition of fermentation microbiota. Int J Food Microbiol 159: 240–246.

26. Zwietering MH, Jongenburger I, Rombouts FM, van 't Riet K (1990) Modeling of the bacterial growth curve. Appl Environ Microbiol 56: 1875–1881.

27. Talarico TL, Axelsson LT, Novotny J, Fiuzat M, Dobrogosz WJ (1990) Utilization of Glycerol as a Hydrogen Acceptor by *Lactobacillus reuteri*: Purification of 1,3-Propanediol:NAD Oxidoreductase. Appl Environ Microbiol 56: 943–948.

28. Stevens MJA, Vollenweider S, Meile L, Lacroix C (2011) 1,3-Propanediol dehydrogenases in *Lactobacillus reuteri*: impact on central metabolism and 3-hydroxypropionaldehyde production. Microb Cell Fact 10: 61.

29. Elleuche S, Fodor K, Klippel B, von der Heyde A, Wilmanns M, et al. (2013) Structural and biochemical characterisation of a NAD^+-dependent alcohol dehydrogenase from *Oenococcus oeni* as a new model molecule for industrial biotechnology applications. Appl Microbiol Biotechnol 97: 8963–8975.

30. Stromeck A, Hu Y, Chen L, Gänzle MG (2011) Proteolysis and bioconversion of cereal proteins to glutamate and γ-Aminobutyrate (GABA) in Rye malt sourdoughs. J Agric Food Chem 59: 1392–1399.

31. Svensson L, Sekwati-Monang B, Lutz DL, Schieber A, Gänzle MG (2010) Phenolic acids and flavonoids in nonfermented and fermented red sorghum (*Sorghum bicolor* (L.) Moench). J Agric Food Chem 58: 9214–9220.

32. Siragusa S, Di Cagno R, Ercolini D, Minervini F, Gobbetti M, et al. (2009) Taxonomic structure and monitoring of the dominant population of lactic acid bacteria during wheat flour sourdough type I propagation using *Lactobacillus sanfranciscensis* starters. Appl Environ Microbiol 75: 1099–1109.

33. Minervini F, Di Cagno R, Lattanzi A, De Angelis M, Antonielli L, et al. (2012) Lactic acid bacterium and yeast microbiotas of 19 sourdoughs used for traditional/typical italian breads: interactions between ingredients and microbial species diversity. Appl Environ Microbiol 78: 1251–1264.

34. Minervini F, Lattanzi A, De Angelis M, Di Cagno R, Gobbetti M (2012) Influence of artisan bakery- or laboratory-propagated sourdoughs on the diversity of lactic acid bacterium and yeast microbiotas. Appl Environ Microbiol 78: 5328–5340.

35. Rollan G, Lorca GL, Font de Valdez G (2003) Arginine catabolism and acid tolerance response in *Lactobacillus reuteri* isolated from sourdough. Food Microbiol 20: 313–319.

36. Arena ME, Manca de Nadra MC (2001) Biogenic amine production by *Lactobacillus*. J Appl Microbiol 90: 158–162.

37. Vermeulen N, Gänzle MG, Vogel RF (2007) Glutamine deamidation by cereal-associated lactic acid bacteria. J Appl Microbiol 103: 1197–1205.

38. Wieser H, Koehler P (2008) The biochemical basis of celiac disease. Cereal Chem 85: 1–13.

39. Mathieson M, Haikerwal AR (1971) The protein content and amino acid composition of sorghum grain. Cereal Chem 48: 690–699.

40. Vollenweider S, Evers S, Zurbriggen K, Lacroix C (2010) Unraveling the hydroxypropionaldehyde (HPA) system: an active antimicrobial agent against human pathogens. J Agric Food Chem 58: 10315–10322.

41. Schütz H, Radler F (1984) Anaerobic reduction of glycerol to propanediol-1.3 by *Lactobacillus brevis* and *Lactobacillus buchneri*. Syst Appl Microbiol 5: 169–178.

42. Brandt MJ, Hammes WP, Gänzle MG (2004) Effects of process parameters on growth and metabolism of *Lactobacillus sanfranciscensis* and *Candida humilis* during rye sourdough fermentation. Eur Food Res Technol 218: 333–338.

43. Amin HM, Hashem AM, Ashour MS, Hatti-Kaul R (2013) 1,2 Propanediol utilization by *Lactobacillus reuteri* DSM 20016, role in bioconversion of glycerol to 1,3 propanediol, 3-hydroxypropionaldehyde and 3-hydroxypropionic acid. J Genet Eng Biotechnol 11:53-39.

44. Morrison WR (1994) Wheat lipids: structure and functionality. In: Bushuk W, Rasper VF, editors. Wheat. Boston, MA: Springer US. pp. 128–142.

45. Sims IM, Frese SA, Walter J, Loach D, Wilson M, et al. (2011) Structure and functions of exopolysaccharide produced by gut commensal *Lactobacillus reuteri* 100-23. ISME J 5: 1115–1124.

46. Badía J, Ros J, Aguilar J (1985) Fermentation mechanism of fucose and rhamnose in *Salmonella* Typhimurium and *Klebsiella pneumoniae*. J Bacteriol 161: 435–437.

47. Scott KP, Martin JC, Campbell G, Mayer C-D, Flint HJ (2006) Whole-genome transcription profiling reveals genes up-regulated by growth on fucose in the human gut bacterium "*Roseburia inulinivorans*". J Bacteriol 188: 4340–4349.

6. Genetic determinants of reutericyclin biosynthesis in *Lactobacillus reuteri*: a pathway to antibiotic synthesis that is unique to lactic acid bacteria?

6.1 Introduction

Reutericyclin, produced by *Lactobacillus reuteri*, is the only chemically characterized low-molecular weight antibiotic produced within the genus *Lactobacillus* [1]. It is an N-acylated tetramic acid [2] with bacteriostatic and bactericidal activity against gram-positive bacteria, including *Staphylococcus aureus*, *Listeria innocua*, *Enterococcus faecium*, *Clostridium difficile,* and bacilli that cause ropy spoilage of bread [3,4]. Tetramic acids are mainly produced by fungi [5]; known bacterial producers of tetramic acids include *Streptomyces* spp., *Alteromonas* spp., *Stenotrophomonas* spp., *Lysobacter enzymogenes*, all of which are phylogenetically unrelated to lactobacilli [5–7]. Tetramic acids are produced by polyketide synthases (PKS) and nonribosomal peptide synthetases (NRPS). PKS and NRPS are enzymes responsible for the biosynthesis of many secondary metabolites with many function e.g. as pigments, virulence factors, infochemicals, or for defense [5,8]. However, genes coding for these enzymes are rarely found in genomes smaller than 3 Mbp [9]. *Lactobacillus reuteri* and other *Lactobacillus* spp. have evolved by reduction of genome size to succeed in very narrow ecological niches [10,11], and functional PKS/NRPS systems have not been described in lactobacilli. Moreover, despite the extensive body of literature related to antimicrobial activity of food-fermenting lactic acid bacteria, only few strains of *L. reuteri* were shown to produce reutericyclin, indicating that reutericyclin production is an exceptional trait of lactobacilli [1,12–14]. Reutericyclin production has only been described for four strains within the species *L. reuteri*; strains LTH2584, TMW1.106, TMW1.112, and *L. reuteri* TMW1.656 [15]. These strains were isolated in 1988, 1994, and 1998 from the same industrial sourdough (SER) that is maintained by continuous propagation [15]. Another strain, *L. reuteri* LTH5448, which was isolated from a sourdough in the same facility as SER lactobacilli [16,17], does not produce reutericyclin but its resistance to reutericyclin is comparable to the resistance of producing strains [18].

Although the chemical structure of reutericyclin had been elucidated 15 years ago [1,2], the genes involved in reutericyclin biosynthesis were unknown so far. The aim of this study was therefore to determine the genetic basis of reutericyclin biosynthesis and immunity by combining a comparative genomic gene finding approach with functional characterization of null mutants.

6.2 Materials and methods.

6.2.1 Strains and culture conditions. *L. reuteri* LTH2584, TMW1.106, TMW1.112, and LTH5448 were grown anaerobically on modified MRS (mMRS) broth 37°C overnight. Each litre of mMRS contained 10 g tryptone, 10 g maltose, 5 g glucose, 5 g fructose, 5 g beef extract, 5 g yeast extract, 4.0 g potassium phosphate dibasic, 2.6 g potassium phosphate monobasic,2 g tri-ammonium citrate, 0.5 g L-cysteine, 0.2 g magnesium sulphate, 0.05 g manganese sulphate, 1 g Tween 80, and 1 mL vitamin mix containing 0.5 µg of vitamins B1, B2, B6, B12, folic and panthothenic acids.

6.2.2 DNA extraction, genomic sequencing and assembly. Cells harvested from 10 mL overnight culture were flash frozen at -80 °C and shipped to the SMRT Sequencing Laboratory, Institute for Computational Biomedicine Weill Medical College of Cornell University (Ithaca, NY, United States) for genomic sequencing. Assemblies were produced by scaffolding PacBio long reads and medium insert (~550bp) Illumina paired end reads with the AHA scaffolding pipeline in the SMRT analysis version 2.0. Consensus sequences for the final drafts were produced with the Quiver algorithm. Final assemblies resulted in 48, 37, 25, and 24 scaffolds for *L. reuteri* TMW1.112, LTH5448, LTH2584, and TMW1.656, respectively, and the respective genome sizes are 2033533, 1980298, 1944170, and 2066054 base pairs. Genomes were annotated using the JGI annotation pipeline, and the genome sequences have been deposited in GenBank under the Bioproject PRJNA248653).

6.2.3 Determination of resistance to reutericyclin. To determine the resistance of wild type and mutant strains to reutericyclin, the compound was isolated from culture supernatant of *L. reuteri* TMW1.656 as previously described [4]. In brief, overnight *L. reuteri* cultures were subcultured with 10% inoculum and incubated overnight at 37°C. Cells were collected by centrifugation, washed with phosphate buffer, and extracted with 30% (v/v) isopropanol. NaCl

was added to the cell extract to saturation, and the organic phase was collected. The antimicrobial activity of reutericyclin was determined by using a critical-dilution assay on microtiter plates [4]. Twofold serial dilutions of the reutericyclin stock solution were prepared with mMRS and solvents were evaporated under a flow of sterile air for 2 h. The microtiter plates were then inoculated with the strains to a cell count of about 10^7 CFU/ml and incubated overnight at 37°C. *Lactobacillus sanfranciscensis* DSM20451 and *L. reuteri* LTH5448 were used as reutericyclin sensitive and reutericyclin resistant indicator strains, respectively. Growth of indicator strains was judged by measuring the optical density at 595 nm.

6.2.4 Identification of genes involved in reutericyclin production. Genomic analysis was performed using the Integrated Microbial Genomes (IMG/ER) system of the Joint Genome Institute [19]. Genome sequences of *L. reuteri* TMW1.656, TMW1.112, LTH2584, and LTH5448 were compared with the genomes of 11 strains of *L. reuteri,* i.e. strains ATCC53608, ATCC55730, mlc3, I5007, 100-23, JCM1112/F275, TD1, Ipuph, CF48-3A, MM2-3, and MM4-1A. None of the latter strains were reported to produce reutericyclin. The IMG Phylogenetic Profiler was used to identify genes present in reutericyclin producing strains and *L. reuteri* LTH5448 but not in other *L. reuteri* strains. Gene annotation was performed using Geneious 6.1.6 (Biomatters Limited). Database searches were performed using the BLASTP and CD-search programs. The NRPS-PKS program (http://www.nii.res.in/nrps-pks.html) [20] was used to predict the modules and domains of NRPSs and PKSs and to predict the substrate specificity of the NRPS adenylation (A) domains. The sequence of the gene cluster containing genes involved in reutericyclin biosynthesis was manually annotated and deposited in Genbank under accession number KJ659887 (identical for *L. reuteri* TMW1.656, TMW1.112, and LTH2584) and KJ659888 (*L. reuteri* LTH5448).

6.2.5 Generation of mutants of L. reuteri TMW1.656. Genes *rtcPKS, rtcPhlA,* in *L. reuteri* TMW1.656 were truncated using the double cross-over method described previously [21]. The genes *rtcT,* and *rtcR1/rtcR2* were deleted in *L. reuteri* TMW1.656Δ*rtcNRPS* using the same method. The plasmids and primers used are listed in Appendix D1 and Appendix D2 of the online supplemental material. Gene deletions in single and double knock out mutant strains were verified by Sanger sequencing, the sequences of the mutant strains are deposited with accession number KJ659887 as note to the sequence of the wild type strain.

6.2.6 Bioinformatic and phylogenetic analysis of genes coding for NRPS-PKS in the order Lactobacillales and related organisms. The sequences of all putative proteins encoded by the reutericyclin gene cluster were searched against nr database in NCBI using BLASTP. NRPS and PKS sequences from those strains which have both of the proteins sharing similar organization as in *L. reuteri* were collected. Likewise, sequences of homologous to RtcPhlA, RtcPhlB or RtcPhlC of *L. reuteri* were collected from bacterial strains which have all three proteins. Protein sequences were aligned by Muscle [22]. The phylogenetic trees were constructed using the best model estimated by MEGA 6 [23].

6.3 Results

6.3.1 Identification of candidate genes involved in reutericyclin biosynthesis. We applied a comparative genomic approach to identify the genes responsible for reutericyclin biosynthesis. We identified genes which are present in the three reutericyclin producing strains *L. reuteri* LTH2584, TMW1.656, and TMW1.112 but were absent in other strains of *L. reuteri. L. reuteri* LTH5448 was initially excluded from this analysis. The analysis revealed 16 ORFs that were unique to reutericyclin producing strains, 12 of which were organized together (Appendix D3). Interestingly, if *L. reuteri* LTH5448 was included in the analysis together with the non-reutericyclin producers, only 1 gene was detected (HQ33_02760 in *L. reuteri* TMW1.656), while when LTH5448 was included with the reutercyclin producing strains, 14 of the 16 genes were detected. This analysis suggests that *L. reuteri* LTH5448, a strain that does not produce reutericyclin but is reutericyclin resistant, contains most of the genes required for reutericyclin synthesis.

Three of the genes present in the three reutericyclin producing strains *L. reuteri* LTH2584, TMW1.656, and TMW1.112 but were absent in other strains of *L. reuteri* code for components of ABC transporters and are also present in *L. reuteri* LTH5448 (Appendix D3). The 12 genes co-localized in the three reutericyclin producers were located in a putative genomic island containing 14 ORFs which were absent in reutericyclin sensitive strains of *L. reuteri*. The cluster spans about 14 kb and contains two putative transcriptional terminators located 8 bp downstream of *rtcT* and 950 bp upstream of *rtcPP*. The sequence of the genomic island was identical in all reutericyclin producing strains, while it differed in the non-producing strain *L.*

reuteri LTH5448 at two sites (Figure 6-1). A single base deletion at bp 1812 in *rtcNRPS* introduces a stop codon and likely renders the gene non-functional. A transposon is inserted in *rtcPhlA* of *L. reuteri* LTH5448 at bp 850, which might also lead to loss of gene function. Either of these two mutations may render the open reading frames dysfunctional and thus account for the loss of reutericyclin production in *L. reuteri* LTH5448 [17, Figure 6-1].

The cluster contained nine genes whose annotated functions relate to the synthesis of reutericyclin when considering its chemical structure, or resistance against its action (Figure 6-2). RtcPP encodes a 24.9 kDa protein predicted to be 4'-phosphopantetheinyl transferase, which activates polyketide synthases (PKS) and non-ribosomal peptide synthases (NRPS) by transferring 4'-phosphopantetheinyl arm to a conserved serine residue [24]. RtcPP is 27% identical to 4'-phosphopantetheinyl transferase for iturin A and surfactin biosynthesis in Bacillus subtilis (Genbank accession P39144.1) (Appendix D4) [25].

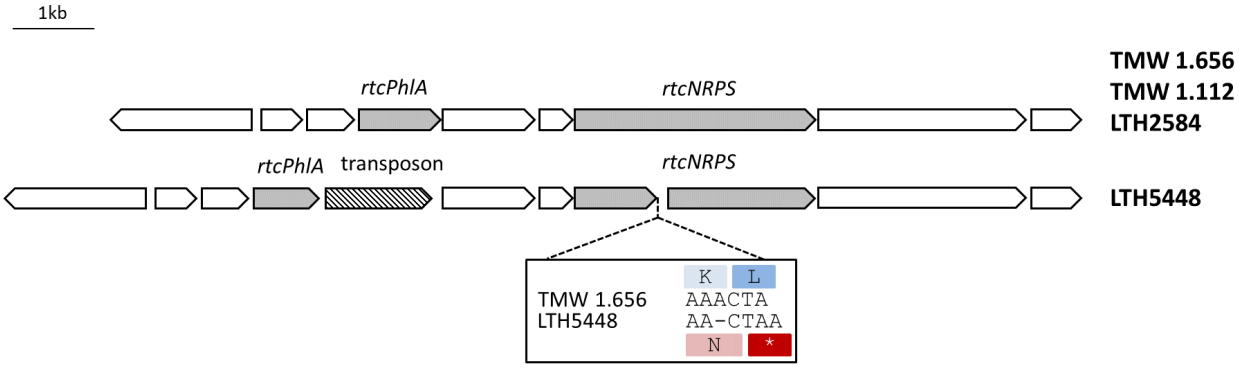


Figure 6-1. A comparison of the reutericyclin gene cluster of the reutericyclin producing strains of *L. reuteri* and the reutericyclin resistant non-producing strain *L. reuteri* LTH5448. Two mutations (indicated by grey arrow) resulted in loss of reutericyclin production in LTH5448, a single base pair deletion resulted in truncated *rtcNRPS*, and a transposon insertion in *rtcPhlA*. ORFs (indicated by arrows) with a deduced function in the synthesis and secretion of reutericyclin are denoted by the prefix *rtc*.

Table 6-1. Alignment of active site residues of RtcNRPS A domain with similar domains

compound	Adenylation domain active site residues										Specificity	Identity / Similarity	ref
	235	236	239	278	299	301	322	330	331	517			
reutericyclin	**D**	**Y**	**F**	**T**	**F**	**G**	**L**	**I**	**I**	**K**	**Leu**		
syringomycin	D	F	W	S	V	G	I	A	T	K	Thr	34% / 53%	[53]
bacillibactin	D	F	W	N	I	G	M	V	H	K	Thr	34% / 53%	[54]
fengycin	D	F	W	N	I	G	M	V	H	K	D-Thr	38% / 56%	[55]
actinomycin	D	F	W	N	V	G	M	V	H	K	Thr	34% / 52%	[56]
pristinamycin	D	F	W	N	V	G	M	V	H	K	Thr	36% / 52%	[57]
CDA a)	D	F	W	N	V	G	M	V	H	K	Thr	34% / 50%	[58]
lichenysin	D	L	T	K	V	G	H	I	G	K	Asp	33% / 53%	[59]
lichenysin	D	A	F	W	I	G	G	T	F	K	Val	35% / 50%	[59]
bactitracin	D	G	F	F	L	G	V	V	Y	K	Ile	32% / 52%	[60]
bactitracin	D	A	K	D	I	G	V	V	D	K	D-Glu	33% / 50%	[60]
bactitracin	**D**	**A**	**W**	**F**	**L**	**G**	**N**	**V**	**V**	**K**	**Leu**	33% / 51%	[60]

a) CDA, calcium-dependent antibiotic

RtcPKS encodes a protein of 99.5 kDa. RtcPKS is composed of a ketosynthase domain (KS), an acyl-carrier protein domain (ACP), and a thioesterase domain (TE). It is 32% identical to mycosubtilin synthase of *B. subtilis* (Genbank accession number Q9R9J1.1) (Appendix D4), a hybrid enzyme that combines functional domains of peptide synthetase, amino transferase, and fatty acid synthase.

RtcNRPS encodes a protein of 115.8 kDa. The protein is composed of a condensation domain (C), an adenylation domain (A), and a thiolation domain (T). The protein is 28% identical to the plipatatin synthase subunit B from *B. subtilis* (Appendix D4); the active sites are 33 – 38% identical to NRPS from *Bacillus* spp, *Streptomyces* spp. and *Pseudomonas syringae* (Table 6-1). Analysis of the adenylation domain active site residues with the NRPS-PKS program predicted possible specificity for leucine.

RtcPhlA, RtcPhlB, and *RtcPhlC* encode proteins with estimated molecular masses of 38.2, 16.7, 43.5 kDa, respectively. They share similarities with PhlA (33% identical, Genbank accession BAD00178.1), PhlC (32% identical, Genbank accession AAY86549.1), and PhlB (47% identical, Genbank accession BAD00180.1), proteins involved in 2,4-diacetylphloroglucinol biosynthesis in *Pseudomonas fluorescens* [26].

RtcR1 and *rtcR2* encode two distinct TetR family transcriptional regulators which are located adjacent to each other with molecular masses of 24.9 kDa and 21.8 kDa, respectively. RtcR1 is 29% identical to HTH-type transcriptional repressor Bm2R1 in *Bacillus megaterium* (Genbank accession P43506.1), and RtcR2 was 35% identical to HTH-type transcriptional repressor YerO in *Bacillus subtilis* (Genbank accession O31500.1).

RtcT encodes for a 63.9 kDa protein belonging to the Major Facilitator Superfamily (MFS). MFS proteins are often involved in the transport of synthesized compounds and provide immunity to various antibiotics [27]. RtcT is 32% identical to YusP in *Bacillus subtilis* (Genbank accession O32182.1).

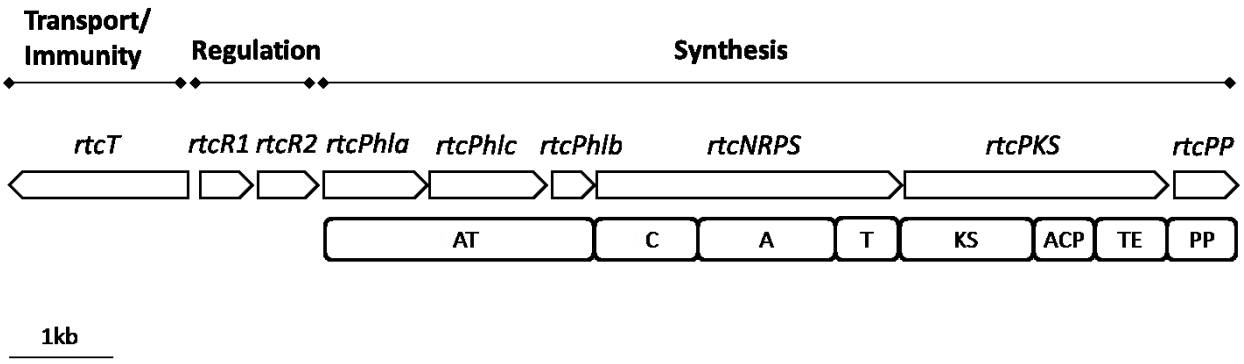


Figure 6-2. Graphic illustration of the rtc locus in reutericyclin producing strains of *L. reuteri*. ORFs (indicated by arrows) with a deduced function in the synthesis and secretion of reutericyclin are denoted by the prefix *rtc.* Sections assumed to be dedicated to transport/immunity, regulation and synthesis are indicated. The PKS and NRPS domains involved in reutericyclin synthesis were indicated below. Letters denote domain functions: AT, acyltransferase; C, condensation; A, adenylation; T, thiolation, also known as PCP (peptidyl carrier protein) domain, KS, ketosynthase; ACP, acyl carrier protein; TE, thioesterase; PP, phosphopantetheinyl transferase).

6.3.2 Gene function analysis. To elucidate the function of the genes on the genomic island, *rtcNRPS*, *rtcPhlA*, *rtcT*, and *rtcR1*/*rtcR2* were deleted in *L. reuteri* TMW1.656 by double-crossover mutagenesis and the phenotypes of the mutant strains were compared to the wild type. Mutants with a deletion in *rtcNRPS* or *rtcPhlA* lost the ability to synthesize reutericyclin but maintained their resistance to reutericyclin, providing direct evidence for the role of these genes in reutericyclin synthesis (Table 6-2). Attempts to delete *rtcT* or *rtcR1*/*rtcR2* in *L. reuteri* TWM1.656 were not successful. However, the same genes could be deleted in the reutericyclin-negative *L. reuteri* TMW1.656Δ*rtcNRPS* (Table 6-2). The disruption of *rtcT* or *rtcR1*/*rtcR2* in the reutericyclin-producing wild type thus likely generated the lethal phenotype of reutericyclin sensitivity coupled to reutericyclin production. Disruption of *rtcT* eliminated reutericyclin resistance; the sensitivity of *L. reuteri* TMW1.656Δ*rtcNRPS*Δ*rtcT* was comparable to the sensitivity of reutericyclin-susceptible *L. sanfranciscensis*. This finding suggests that RtcT is responsible for resistance against reutericyclin, probably through export of the substance. *L. reuteri* TMW1.656Δ*rtcNRPS*Δ*rtcR1R2* was also reutericyclin sensitive, suggesting that one or both regulators regulate expression of reutericyclin resistance.

Maintenance of reutericyclin resistance through active transport of the compound implies expenditure of metabolic energy at the expense of growth. To determine energetic consequences of reutericyclin production and resistance, growth of the *L. reuteri* TMW1.656 was compared to isogenic non-producing mutants. The growth rates *L. reuteri* TMW1.656Δ*rtcNRPS*, TMW1.656Δ*rtcNRPS*Δ*rtcT* and TMW1.656Δ*rtcNRPS*Δ*rtcR1R2* were increased by 20% when compared to *L. reuteri* TMW1.656 while the duration of the lag phase in the same strains was reduced by 25% (Figure S1 of the online supplementary material).

The second gene cluster which is shared by all reutericyclin resistant strains of *L. reuteri* but absent in other genomes of *L. reuteri* (Appendix D3 and D6) contains components of ABC transporters with a possible contribution to reutericyclin transport and/or immunity (*L. reuteri* TMW1.656 genome, nucleotide 50140-52314). However, the deletion of one of the three ORFs (*L. reuteri* TMW1.656 genome, locus tag HQ33_09475) in *L. reuteri* TMW1.656 or *L. reuteri* TMW1.656ΔrtcNRPS to generate *L. reuteri* TMW1.656ΔABC and *L. reuteri* TMW1.656Δ*rtcNRPS*Δ*ABC* did not affect reutericyclin biosynthesis or immunity (Table 2), indicating that these genes were not relevant to reutericyclin metabolism.

Table 6-2. Mutants of *L. reuteri* TMW 1.656 created to identify the genes involved in reutericyclin biosynthesis and immunity

***L. reuteri* TMW1.656 mutant**	**Reutericyclin synthesis**	**Resistance to reutericyclin**
Δ*rtcNRPS*	-[a)]	+[a)]
Δ*rtcPhlA*	-	+
Δ*rtcT*	Not obtained	
Δ*rtcR1R2*	Not obtained	
Δ*rtcNRPS*Δ*rtcT*	-	-
Δ*rtcNRPS*Δ*rtcR1R2*	-	-
Δ*ABC*	+	+
Δ*rtcNRPS*Δ*ABC*	-	+

[a)] + and – indicate presence and absence of reutericyclin biosynthesis or resistance

6.3.3 Homologues of reutericyclin biosynthesis genes in genomes of other lactic acid bacteria. The GC content of the genomic island harbouring genes coding for reutericyclin production, resistance to reutericyclin, and regulatory genes was 5 – 10 % lower when compared to the GC content of the overall genome sequence of *L. reuteri* LTH2584, TMW1.112, TMW 1.656, and LTH5448 (Figure 6-3), suggesting that the island may have been acquired by lateral gene transfer. The reutericyclin genomic island was inserted at two ORFs that encode for transposase and tRNA, respectively, which are common locations for horizontally acquired genes [28]. Moreover, *L. reuteri* LTH2584, TMW1.112, and TMW1.656 as well as the reutericyclin producing *L. reuteri* TMW1.106 all belong to the rodent-adapted lineage III of *L. reuteri* [10,15,29]. However, *L. reuteri* LTH5448 belongs to the rodent-adapted lineage I [29]. The presence of the reutericyclin genomic island in few phylogenetically unrelated strains of the species *L. reuteri* that were isolated from the same site further supports the hypothesis that the genomic island was transferred horizontally among these strains.

To gain insight into the evolution and potentially origin of the gene cluster, homologues of RtcPKS, RtcNRPS, RtcPhlA, RtcPhlB, and RtcPhlC were retrieved from the nr database in NCBI, and phylogenetic trees were inferred (Figure 6-4 and Appendix D7). Homologues of RtcPKS and RtcNRPS were present in *L. plantarum* and *Streptococcus mutans* and in members of the *Bacillales* (Figure 6-4A and Appendix D7A). Homologues of RtcPhlA, RtcPhlB, and RtcPhlC were also identified in *L. plantarum*, *Streptococcus mutans*; more distant relatives were found in *Clostridium* species, and *Pseudomonas* spp. (Figure 6-4B, Appendix D7B and D7C). RtcPhlA, RtcPhlB, and RtcPhlC thus have a different phylogenetic origin than RtcPKS and RtcNRPS. The combination of RtcPKS, RtcNRPS, RtcPhlA, RtcPhlB, and RtcPhlC was unique to members of the *Lactobacillales* and most frequently found in strains of *S. mutans* (Figure 6-4 and Appendix D7). Moreover, the organization of genes coding for RtcPKS RtcNRPS, RtcPhlA, RtcPhlB and RtcPhlC homologues in *S. mutans* and *L. plantarum* was similar to their organization in *L. reuteri* (Appendix D8).

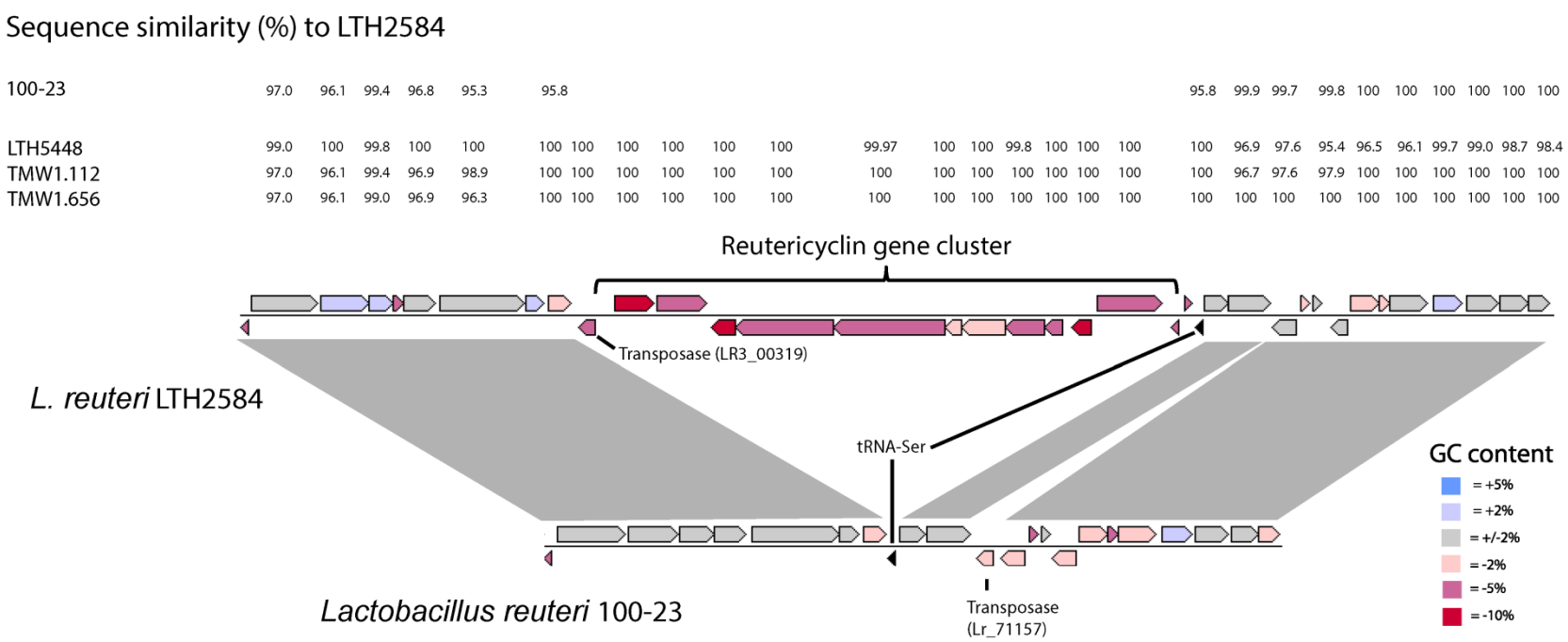
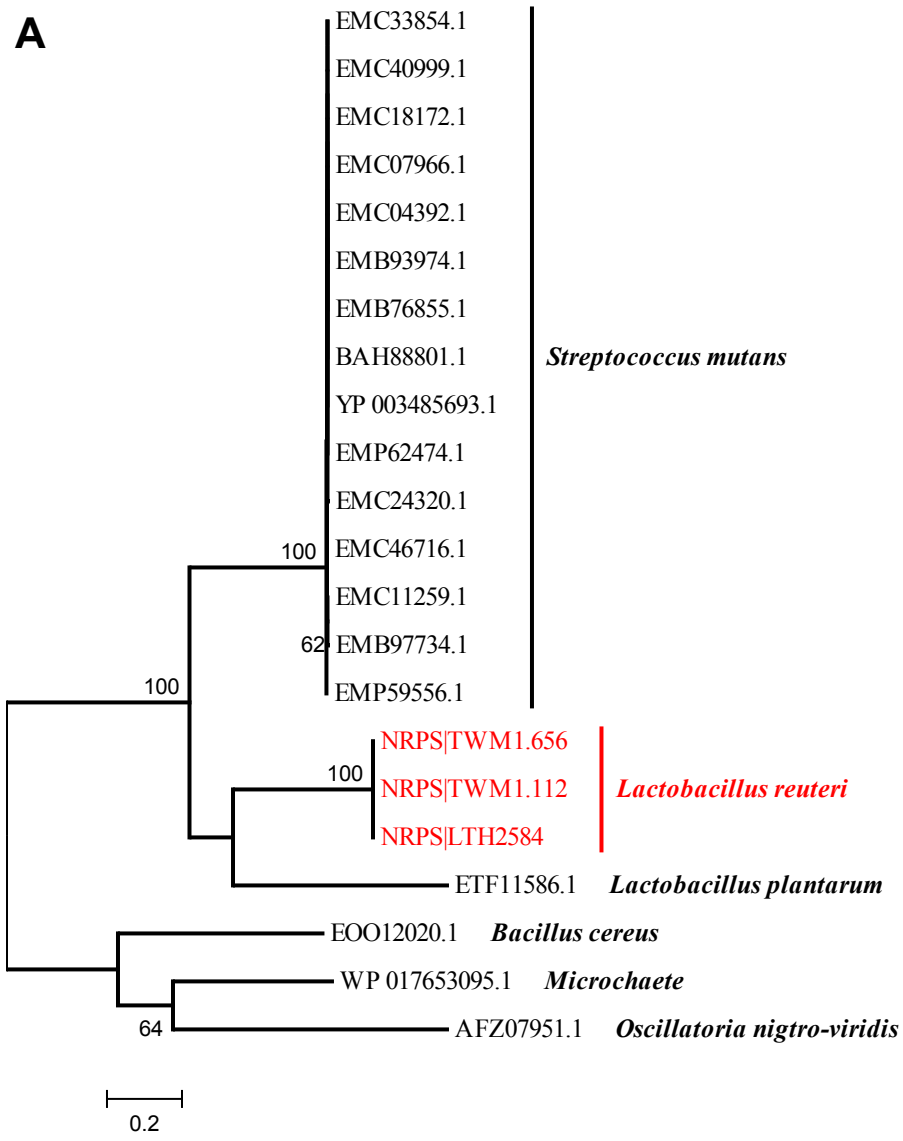


Figure 6-3. Genomic location and GC content of the genomic island of reutericyclin biosynthesis. Shown is the sequence identity of the reutericyclin producing *L. reuteri* TMW1.112, TMW1.656, and LTH5448 to *L. reuteri* LTH2584. The closely related rodent lineage III strain *L. reuteri* 100-23 was included as reference.


A
EMC33854.1
EMC40999.1
EMC18172.1
EMC07966.1
EMC04392.1
EMB93974.1
EMB76855.1
BAH88801.1
YP 003485693.1
EMP62474.1
EMC24320.1
EMC46716.1
EMC11259.1
EMB97734.1
EMP59556.1
Streptococcus mutans
100
62
100
NRPS|TWM1.656
NRPS|TWM1.112
NRPS|LTH2584
100
Lactobacillus reuteri
ETF11586.1
Lactobacillus plantarum
EOO12020.1
Bacillus cereus
WP 017653095.1
Microchaete
64
AFZ07951.1
Oscillatoria nigtro-viridis
0.2

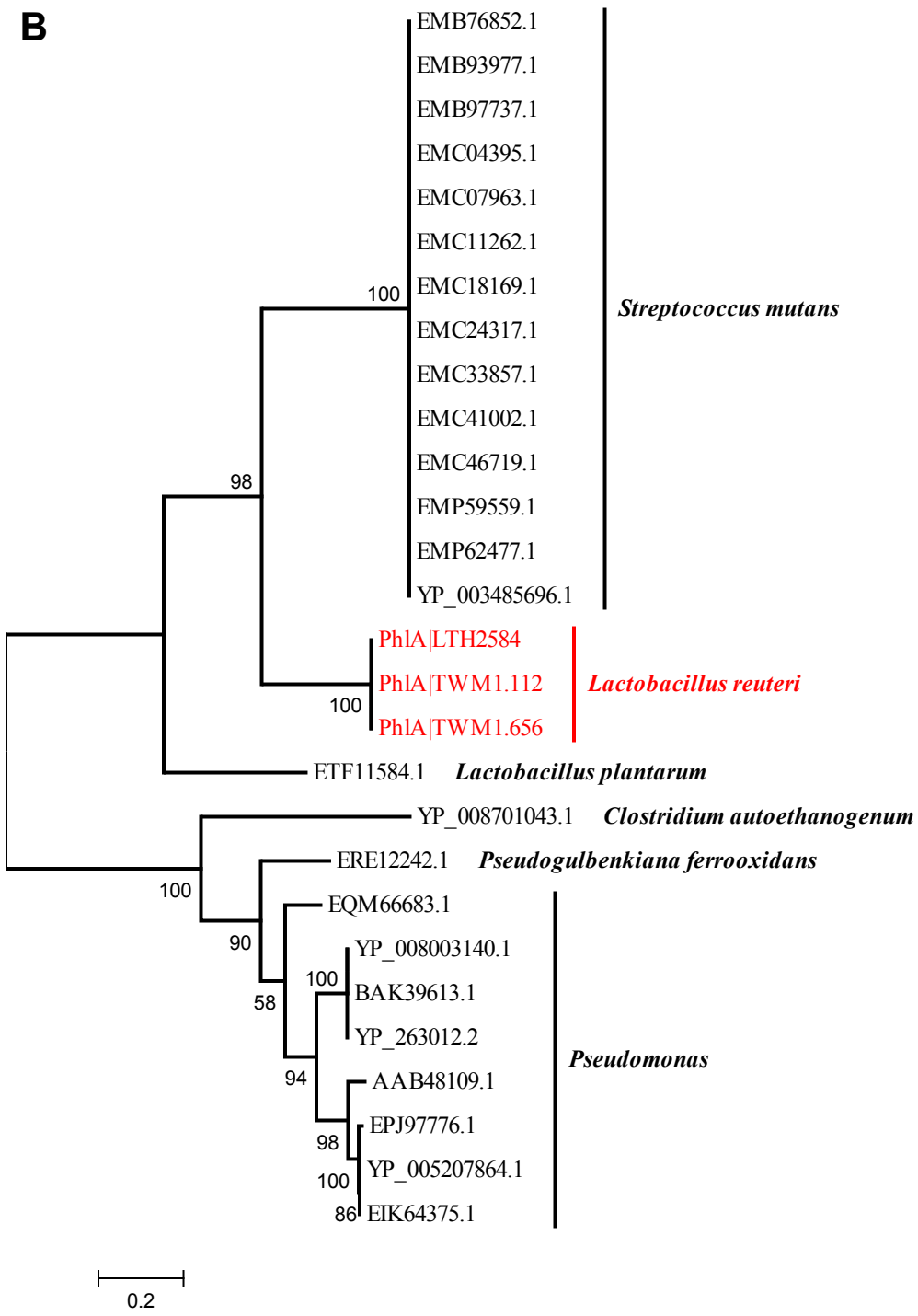


Figure 6-4. Unrooted phylogenetic tree of RtcNRPS (Panel A) and PhlA (Panel B) of *L. reuteri* and related proteins. The number at each branch point represents the percentage of bootstrap support calculated from 1,000 replicates. Only bootstrap values above 50 are shown.

6.4 Discussion

This study identified genetic determinants for reutericyclin biosynthesis, regulation, and immunity through an approach combining comparative genomics, bioinformatics analysis, and the characterization of null-mutants. Moreover, data mining of bacterial genome sequence data demonstrated that putative reutericyclin biosynthetic operons are present only in four strains of *L. reuteri*, one strain of *L. plantarum*, and several strains of *Streptococcu*s *mutant*s. The reutericyclin producing strains of *L. reuteri* persisted in an industrial sourdough over 10 years of continuous propagation [15]; reutericyclin thus provides an interesting model of the ecological effect of antibiotic production on the bacterial competitiveness.

6.4.1 Proposed pathway for reutericyclin biosynthesis. A genomic island was identified in reutericyclin resistant strains of *L. reuteri*; two open reading frames of this genomic island were disrupted in the reutericyclin resistant but non-producing strain *L. reuteri* LTH5448. Mutagenesis of four genes identified genetic determinants of reutericyclin biosynthesis and reutericyclin resistance. The annotations of the genes involved in reutericyclin biosynthesis and the characterization of reutericyclin production by isogenic mutants of *L. reuteri* TMW1.656 in combination with the chemical structure of reutericylin [2] allow suggestion of a putative biosynthetic pathway for reutericyclin (Figure 6-5). The assembly of the tetramic acid core structure by RtcNRPS and RtcPKS can be inferred from the well-characterized activity of bacterial NRPS and PKS. Because N-acylation and PhlABC-mediated acetylation of reutericyclin is exceptional among tetramic acids, the prediction of the function of the corresponding biosynthetic enzymes is plausible but more speculative (Figure 6-5).

Reutericyclin is likely exported by transporter protein RtcT. Both MFS and ABC transporters have been reported as export or immunity mechanisms to polyketides and/or non-ribosomal peptides [5,9]; this study demonstrated that deletion of RtcT eliminated resistance to reutericyclin while deletion of a putative ABC transporter that is also unique to reutericyclin resistant strains did not affect reutericyclin production or reduce reutericyclin resistance.

Reutericyclin resistance is regulated by the TetR family regulators RtcR1 and or RtcR2. TetR regulators are regulatory proteins with helix-turn-helix motifs with can act as either

transcriptional repressors or activators. RtcR1 and RtcR2 do not have identical sequences and thus cannot form homodimers as reported for other TetR regulators [30]. Whether they function alone or individually remains to be determined.

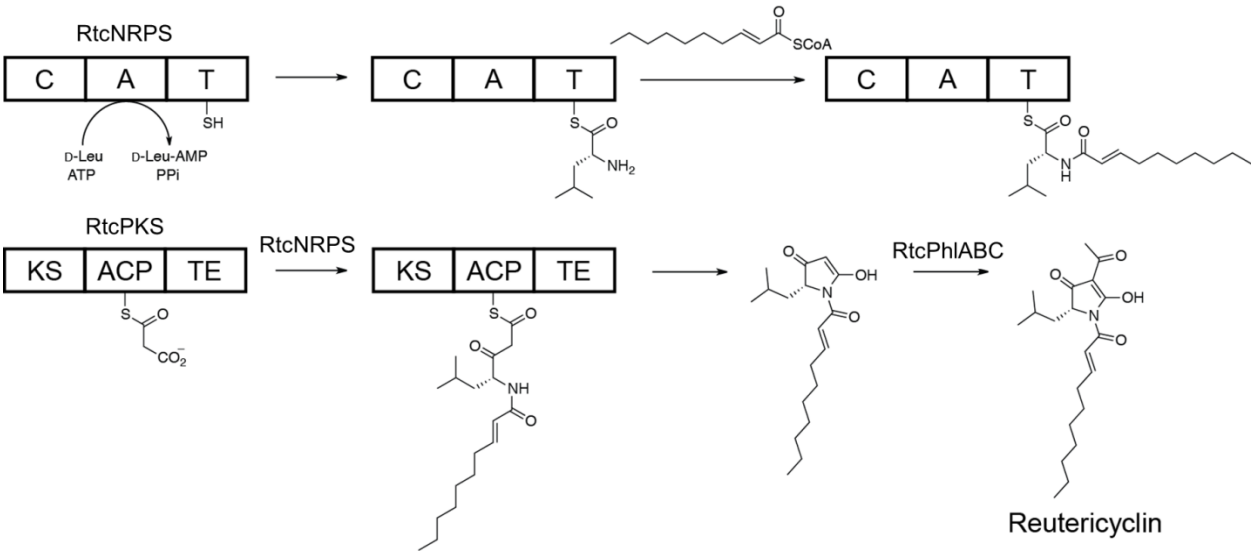


Figure 6-5. Proposed pathway for reutericyclin biosynthesis**.** The A-domain of RtcNRPS selects for D-leucine [2] but RtcNRPS was not found to have an epimerase domain. Most Gram-positive bacteria are capable of synthesizing D-alanine and D-glutamate as building blocks for peptidoglycan [43], whereas the synthesis of other D-amino acids is less common. An isoleucine 2-epimerase with leucine epimerase activity has been characterized in *L. otakiensis* JCM 15040 and *L. buchneri* JCM 1115 L [44]. An isoleucine 2-epimerase is conserved in all four strains of *L. reuteri* used in this study (WP_011953381.1). The enzyme is 98% identical to the isoleucine 2-epimerase in *L. reuteri* JCM1112 (YP_001841185) and strains of *L. reuteri* were reported to produce D-leucine [45]. It is therefore highly likely that the isoleucine 2-epimerase homologues, which were annotated as putative γ-aminobutyrate aminotransferases by automatic annotation, are responsible for D-leucine synthesis in *L. reuteri* [46,47]. D-amino acids can be directly activated by bacterial NRPS [48]. Once activated, the leucine aminoacyl adenylate is likely loaded onto the phosphopantetheinyl arm of the RtcNRPS T domain. Then, the C-domain of RtcNRPS may acylate the amino group of the NRPS-bound leucine with ACP- or CoA-activated 2-decenoic acid. The reutericyclin genomic island does not code for enzymes related to lipid metabolism; the C10 fatty acid in reutericyclin thus likely originates from general metabolism. Crosstalk between polyketide synthases and fatty acid synthases has been reported in *Escherichia coli* where a malonyl coenzyme A (CoA) acyltransferase component of the fatty

acid synthase complex was recruited for synthesis of the anticancer depsipeptide FK228 [49]. In bacteria, the 2-decenoic acid component can be synthesized by β-hydroxydecanoyl-ACP dehydratase (FabA) or a more common hydroxyacyl-ACP dehydratase (FabZ). FabA and FabZ are present in *Enterococcus faecalis* and *Streptococcus pneumonia* [46,47]. Two FabZ homologues were found in the genome of reutericyclin producers (*L. reuteri* TMW1.656 genome locus tag HQ33_05470, HQ33_05510).

Sequence analysis of RtcPKS suggests the presence of KS, ACP and TE domains. However, no acyltransferase (AT) domain is found, nor is one encoded elsewhere in the gene cluster. The amino acid sequence between the KS and ACP domains shows homology to the region N-terminal to AT domains found in other bacterial PKSs. This is similar to what is found in the AT-less type I PKSs, where the remnant AT sequence acts as a docking domain for a distinct AT enzyme [50]. Some AT enzymes are capable of operating on distinct biosynthetic pathways. For example, the malonyl-CoA:ACP transacylase involved in fatty acid biosynthesis acted in place of an artificially inactivated AT domain in the erythronolide biosynthetic pathway [51].

We propose that the KS domain of RtcPKS catalyzes a decarboxylative Claisen condensation. Once the enzyme-bound malonate decarboxylates, the resulting enolate attacks the leucine thioester loaded on the T domain of RtcNRPS. The TE domain of RtcPKS may then catalyze the cyclization and off-loading of the reutericyclin precursor via the nucleophilic attack of the amide nitrogen onto the thioester carbonyl. Finally, we propose that the resulting tetramic acid is acetylated by RtcPhlABC, thus forming reutericyclin. RtcPhlABC show sequence homology to PhlABC, proteins involved in the biosynthesis of 2,4-diacetylphloroglucinol [26]. Biosynthetic studies suggest that PhlABC are responsible for acetylating phloroglucinol, yielding 2-acetylphloroglucinol and 2,4-diacetylphloroglucinol [52]. This acetylation is highly analogous to the predicted role in reutericyclin biosynthesis.

6.4.2 NRPS/PKS mediated antibiotic production in lactic acid bacteria and lateral gene transfer. In bacteria, the number of genes is generally proportional to genome size. This relation holds also for specific gene classes but PKS/NRPS genes are an exception. Below a genome size of 3 Mbp, PKS/NRPS genes are rare, and a linear correlation between genome size and content

of PKS/NRPS genes could be established only for genomes above 5 Mb [9]. In the order *Lactobacillales*, PKS/NRPS genes have been annotated in *Lactobacillus plantarum* [31], *Streptococcus thermophiles* [32], and *S. mutans* [33,34], which have genome sizes of about 3.3, 1.8, and 2.2 Mbp, respectively. Among those organisms with functional NRPS/PKS systems, *L. reuteri* thus has an exceptionally small genome size. To our knowledge, the only PKS/NRPS of any lactic acid bacterium that was functionally characterized is a NRPS/PKS in *S. mutans* [33]. The characterized NRPS/PKS system in *S. mutans* synthesizes a pigment involved in oxidative stress tolerance [33] and the NRPS and PKS are 30 and 67% identical to the enzyme in *L. reuteri*. A PKS/NRPS with higher homology to RtcNRPS and RtcPKS (Figure 6-4) was annotated as putative bacitracin synthesis cluster [34]. To our knowledge, NRPS/PKS mediated production of antibiotics has not been described in *S. mutans*. However, the contribution of ribosomally synthesized peptide antibiotics, such as lantibiotics and class II bacteriocins, to competitiveness and persistence of *S. mutans* in its ecological niche, the dental biofilms, has been documented [35–37].

The low GC content of the reutericyclin genomic island and its presence in phylogenetically unrelated strains of *L. reuteri* originating from the same site suggest that the genomic island was acquired by lateral gene transfer. Homologous gene clusters that share a comparable organization and a common evolutionary origin were found in *S. mutans* and *L. plantarum*. These related gene clusters in streptococci are located on putative conjugative transposons [34], providing a potential vehicle for gene transfer between lactic acid bacteria. The low protein identities between PKS/NRPS systems in *S. mutans*, *L. plantarum* and *L. reuteri*, however, argue against a direct transfer between these three species. *S. mutans* was suggested to exchange genetic information with foodborne bacteria as they pass through the oral cavity [38]; *L. plantarum* adapts to a variety of ecological niches by acquisition of “life-style cassettes”, genomic islands appropriate to niche requirements [39].

The gene cluster of reutericyclin synthesis has two phylogenetically distinct components, the PKS/NRPS component which is related to bacitracin synthesis in the Gram positive *Bacillus* spp., and the PhlABC component related to 2,4-DAPG synthesis in the Gram negative *Pseudomonas fluorescens*. Analyses of currently available genome- and protein sequences indicates that combined activity of a PKS/NRPS system and PhlABC is restricted to the order

Lactobacillales and thus provides an unique solution to antibiotic synthesis within the lactic acid bacteria.

6.4.3 Ecological role of reutericyclin production for sourdough lactobacilli. Lactobacilli in sourdoughs that are maintained by continuous propagation are characterized by rapid growth rates, acid resistance, small genome size, and generally lack the ability to produce bacteriocins or other antimicrobial compounds [1,12,40,41]. The production of antimicrobial compounds and the maintenance of producer immunity diverts metabolic energy from metabolic functions that support rapid growth and thus may reduce the competitiveness of bacteriocin producing organisms [41,42]. This was confirmed by the observation that isogenic but reutericyclin-negative mutants of *L. reuteri* TMW1.656 grow faster than *L. reuteri* TMW1.656 (Appendix D5). However, reutericyclin producing strains are known to have persisted for at least 10 years of continuous propagation [15]; suggesting that reutericyclin production at least temporarily provided an advantage over reutericyclin non-producing strains.

Sourdough *L. reuteri* are of intestinal origin [29] and may have acquired the gene cluster before or after transfer to sourdough. Because the reutericyclin gene cluster is virtually absent in other lactobacilli (Figure 6-4), reutericyclin production is not essential in intestinal or sourdough ecosystems. It is tempting to speculate that the reutericyclin cluster was acquired by a rodent *L. reuteri* strain and that this novel trait, although energetically unfavorable, was selected for in the SER sourdough [15]. *L. reuteri* LTH5448, which harbored an inactivated version of the cluster, was isolated from a different dough in the same facility 15 years after the first reutericyclin producing strain was isolated, suggesting that the cluster became obsolete under different ecological conditions.

Acknowledgements. The Alberta Livestock and Meat Agency and the Natural Sciences and Engineering Research Council of Canada are acknowledged for funding. Michael Gänzle and John Vederas acknowledge funding from the Canada Research Chairs Program.

6.5 References

1. Gänzle MG (2004) Reutericyclin: biological activity, mode of action, and potential applications. Appl Microbiol Biotechnol 64: 326–332.

2. Höltzel A, Gänzle M, Nicholson G, Hammes W, Jung G (2000) The first low molecular weight antibiotic from lactic acid bacteria: reutericyclin, a new tetramic acid. Angew Chem Int Ed Engl 39: 2766–2768.

3. Hurdle JG, Heathcott AE, Yang L, Yan B, Lee RE (2011) Reutericyclin and related analogues kill stationary phase *Clostridium difficile* at achievable colonic concentrations. J Antimicrob Chemother 66: 1773–1776.

4. Gänzle MG, Höltzel A, Walter J, Jung G, Hammes WP (2000) Characterization of reutericyclin produced by *Lactobacillus reuteri* LTH2584. Appl Environ Microbiol 66: 4325–4333.

5. Fisch KM (2013) Biosynthesis of natural products by microbial iterative hybrid PKS–NRPS. RSC Adv 3: 18228.

6. Lou L, Qian G, Xie Y, Hang J, Chen H, et al. (2011) Biosynthesis of HSAF, a tetramic acid-containing macrolactam from *Lysobacter enzymogenes*. J Am Chem Soc 133: 643–645.

7. Wu Q, Wu Z, Qu X, Liu W (2012) Insights into pyrroindomycin biosynthesis reveal a uniform paradigm for tetramate/tetronate formation. J Am Chem Soc 134: 17342–17345.

8. Hertweck C (2009) The biosynthetic logic of polyketide diversity. Angew Chem Int Ed Engl 48: 4688–4716.

9. Donadio S, Monciardini P, Sosio M (2007) Polyketide synthases and nonribosomal peptide synthetases: the emerging view from bacterial genomics. Nat Prod Rep 24: 1073–1109.

10. Frese SA, Benson AK, Tannock GW, Loach DM, Kim J, et al. (2011) The evolution of host specialization in the vertebrate gut symbiont *Lactobacillus reuteri*. PLoS Genet 7: e1001314.

11. Makarova K, Slesarev A, Wolf Y, Sorokin A, Mirkin B, et al. (2006) Comparative genomics of the lactic acid bacteria. Proc Natl Acad Sci U S A 103: 15611–15616.

12. Messens W, De VL (2002) Inhibitory substances produced by lactobacilli isolated from sourdoughs--a review. Int J Food Microbiol 72: 31–43.

13. Cotter PD, Ross RP, Hill C (2013) Bacteriocins - a viable alternative to antibiotics? Nat Rev Microbiol 11: 95–105.

14. Rouse S, van Sinderen D (2008) Bioprotective potential of lactic acid bacteria in malting and brewing. J Food Prot 71: 1724–1733.

15. Gänzle MG, Vogel RF (2003) Contribution of reutericyclin production to the stable persistence of *Lactobacillus reuteri* in an industrial sourdough fermentation. Int J Food Microbiol 80: 31–45.

16. Meroth CB, Walter J, Hertel C, Brandt MJ, Hammes WP (2003) Monitoring the bacterial population dynamics in sourdough fermentation processes by using PCR-denaturing gradient gel electrophoresis. Appl Environ Microbiol 69: 475–482.

17. Brandt MJ (n.d.) Personal communication.

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

19. Markowitz VM, Szeto E, Palaniappan K, Grechkin Y, Chu K, et al. (2008) The integrated microbial genomes (IMG) system in 2007: data content and analysis tool extensions. Nucleic Acids Res 36: D528–33.

20. Ansari MZ, Yadav G, Gokhale RS, Mohanty D (2004) NRPS-PKS: a knowledge-based resource for analysis of NRPS/PKS megasynthases. Nucleic Acids Res 32: W405–13.

21. Su MS, Schlicht S, Gänzle MG (2011) Contribution of glutamate decarboxylase in *Lactobacillus reuteri* to acid resistance and persistence in sourdough fermentation. Microb Cell Fact 10 Suppl 1: S8.

22. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013) MEGA6: Molecular evolutionary genetics analysis version 6.0. Mol Biol Evol 30: 2725–2729.

23. Edgar RC (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res 32: 1792–1797.

24. Keating T a, Walsh CT (1999) Initiation, elongation, and termination strategies in polyketide and polypeptide antibiotic biosynthesis. Curr Opin Chem Biol 3: 598–606.

25. Shaligram NS, Singhal RS (2010) Surfactin -- A review on biosynthesis, fermentation, purification and applications. Food Technol Biotechnol 48: 119–134.

26. Bangera MG, Thomashow LS (1999) Identification and characterization of a gene cluster for synthesis of the polyketide antibiotic 2,4-diacetylphloroglucinol from *Pseudomonas fluorescens* Q2-87. J Bacteriol 181: 3155–3163.

27. Pao SS, Paulsen IT, Saier MH (1998) Major facilitator superfamily. Microbiol Mol Biol Rev 62: 1–34.

28. Tuller T (2011) Codon bias, tRNA pools and horizontal gene transfer. Mob Genet Elements 1: 75–77.

29. Su MS-W, Oh PL, Walter J, Gänzle MG (2012) Intestinal origin of sourdough *Lactobacillus reuteri* isolates as revealed by phylogenetic, genetic, and physiological analysis. Appl Environ Microbiol 78: 6777–6780.

30. Ramos JL, Martínez-Bueno M, Molina-Henares AJ, Terán W, Watanabe K, et al. (2005) The TetR family of transcriptional repressors. Microbiol Mol Biol Rev 69: 326–356.

31. Kleerebezem M, Boekhorst J, van Kranenburg R, Molenaar D, Kuipers OP, et al. (2003) Complete genome sequence of *Lactobacillus plantarum* WCFS1. Proc Natl Acad Sci U S A 100: 1990–1995.

32. Bolotin A, Quinquis B, Renault P, Sorokin A, Ehrlich SD, et al. (2004) Complete sequence and comparative genome analysis of the dairy bacterium *Streptococcus thermophilus*. Nat Biotechnol 22: 1554–1558.

33. Wu C, Cichewicz R, Li Y, Liu J, Roe B, et al. (2010) Genomic island TnSmu2 of *Streptococcus mutans* harbors a nonribosomal peptide synthetase-polyketide synthase gene cluster responsible for the biosynthesis of pigments involved in oxygen and H_2O_2 tolerance. Appl Environ Microbiol 76: 5815–5826.

34. Maruyama F, Kobata M, Kurokawa K, Nishida K, Sakurai A, et al. (2009) Comparative genomic analyses of *Streptococcus mutans* provide insights into chromosomal shuffling and species-specific content. BMC Genomics 10: 358.

35. Merritt J, Qi F (2012) The mutacins of *Streptococcus mutans*: regulation and ecology. Mol Oral Microbiol 27: 57–69.

36. Kreth J, Merritt J, Shi W, Qi F (2005) Competition and coexistence between *Streptococcus mutans* and *Streptococcus sanguinis* in the dental biofilm. J Bacteriol 187: 7193–7203.

37. Grönroos L, Saarela M, Mättö J, Tanner-Salo U, Vuorela A, et al. (1998) Mutacin production by *Streptococcus mutans* may promote transmission of bacteria from mother to child. Infect Immun 66: 2595–2600.

38. Cvitkovitch DG (2001) Genetic competence and transformation in oral streptococci. Crit Rev Oral Biol Med 12: 217–243.

39. Siezen RJ, van Hylckama Vlieg JET (2011) Genomic diversity and versatility of *Lactobacillus plantarum*, a natural metabolic engineer. Microb Cell Fact 10 Suppl 1: S3.

40. Vogel RF, Pavlovic M, Ehrmann MA, Wiezer A, Liesegang H, et al. (2011) Genomic analysis reveals *Lactobacillus sanfranciscensis* as stable element in traditional sourdoughs. Microb Cell Fact 10 Suppl 1: S6.

41. Lin XB, Gänzle MG (2014) Effect of lineage-specific metabolic traits of *Lactobacillus reuteri* on sourdough microbial ecology. Appl Environ Microbiol 80:5782-9.

42. Biernaskie JM, Gardner A, West S a (2013) Multicoloured greenbeards, bacteriocin diversity and the rock-paper-scissors game. J Evol Biol 26: 2081–2094.

43. Park JT, Strominger JL (1957) Mode of action of penicillin: Biochemical basis for the mechanism of action of penicillin and for its selective toxicity. Science (80-) 125: 99–101.

44. Mutaguchi Y, Ohmori T, Wakamatsu T, Doi K, Ohshima T (2013) Identification, purification, and characterization of a novel amino acid racemase, isoleucine 2-epimerase, from *Lactobacillus* species. J Bacteriol 195: 5207–5215.

45. Mutaguchi Y, Ohmori T, Akano H, Doi K, Ohshima T (2013) Distribution of D-amino acids in vinegars and involvement of lactic acid bacteria in the production of D-amino acids. Springerplus 2: 691.

46. Lu Y-J, White SW, Rock CO (2005) Domain swapping between *Enterococcus faecalis* FabN and FabZ proteins localizes the structural determinants for isomerase activity. J Biol Chem 280: 30342–30348.

47. Marrakchi H, Choi K-H, Rock CO (2002) A new mechanism for anaerobic unsaturated fatty acid formation in *Streptococcus pneumoniae*. J Biol Chem 277: 44809–44816.

48. Li J, Jensen SE (2008) Nonribosomal biosynthesis of fusaricidins by *Paenibacillus polymyxa* PKB1 involves direct activation of a D-amino acid. Chem Biol 15: 118–127.

49. Wesener SR, Potharla VY, Cheng Y-Q (2011) Reconstitution of the FK228 biosynthetic pathway reveals cross talk between modular polyketide synthases and fatty acid synthase. Appl Environ Microbiol 77: 1501–1507.

50. Cheng Y-Q, Coughlin JM, Lim S-K, Shen B (2009) Type I polyketide synthases that require discrete acyltransferases. Methods Enzymol 459: 165–186.

51. Kumar P, Koppisch AT, Cane DE, Khosla C (2003) Enhancing the modularity of the modular polyketide synthases: transacylation in modular polyketide synthases catalyzed by malonyl-CoA:ACP transacylase. J Am Chem Soc 125: 14307–14312.

52. Achkar J, Xian M, Zhao H, Frost JW (2005) Biosynthesis of phloroglucinol. J Am Chem Soc 127: 5332–5333.

53. Guenzi E, Galli G, Grgurina I, Gross DC, Grandi G (1998) Characterization of the syringomycin synthetase gene cluster. A link between prokaryotic and eukaryotic peptide synthetases. J Biol Chem 273: 32857–32863.

54. May JJ, Wendrich TM, Marahiel MA (2001) The dhb operon of *Bacillus subtilis* encodes the biosynthetic template for the catecholic siderophore 2,3-dihydroxybenzoate-glycine-threonine trimeric ester bacillibactin. J Biol Chem 276: 7209–7217.

55. Lin TP, Chen CL, Chang LK, Tschen JS, Liu ST (1999) Functional and transcriptional analyses of a fengycin synthetase gene, fenC, from *Bacillus subtilis*. J Bacteriol 181: 5060–5067.

56. Schauwecker F, Pfennig F, Schröder W, Keller U (1998) Molecular cloning of the actinomycin synthetase gene cluster from *Streptomyces chrysomallus* and functional heterologous expression of the gene encoding actinomycin synthetase II. J Bacteriol 180: 2468–2474.

57. De Crécy-Lagard V, Blanc V, Gil P, Naudin L, Lorenzon S, et al. (1997) Pristinamycin I biosynthesis in *Streptomyces pristinaespiralis*: molecular characterization of the first two structural peptide synthetase genes. J Bacteriol 179: 705–713.

58. Hojati Z, Milne C, Harvey B, Gordon L, Borg M, et al. (2002) Structure, biosynthetic origin, and engineered biosynthesis of calcium-dependent antibiotics from *Streptomyces coelicolor*. Chem Biol 9: 1175–1187.

59. Konz D, Doekel S, Marahiel MA (1999) Molecular and biochemical characterization of the protein template controlling biosynthesis of the lipopeptide lichenysin. J Bacteriol 181: 133–140.

60. Konz D, Klens A, Schörgendorfer K, Marahiel MA (1997) The bacitracin biosynthesis operon of *Bacillus licheniformis* ATCC 10716: molecular characterization of three multi-modular peptide synthetases. Chem Biol 4: 927–937.

7. Conclusions and Future Directions

7.1 General discussion

This thesis studied factors affecting microbial ecology using rodents and sourdough as systems. Experimentation on rodents focused on a model for CPT-11 chemotherapy. Physiological changes (such as reduced food intake) in the host caused by disease or medical treatment may disturb gut microbiota, giving opportunity for opportunistic pathogens (ruderals) to overgrow. This was observed as increased abundance of marker organisms *Clostridium* cluster XI and *Enterobacteriaceae* in response to both cancer and CPT-11 treatment. However, the converse does not hold; a higher abundance of marker organisms did not nececesarily indicate the health state of the host as other factors such as diet can strongly affect intestinal microbial composition. Therefore, marker organisms can be used as indicators for clinical diagnosis and/or prognosis, but should not be confused with the causing agent of diseases. On the other hand, microbial metabolism can either positively or negatively affect host health. Microbial GUD was blamed by the conventional perspective as the cause for CPT-11 toxicity; however, the negative effect of GUD can be compensated by the production of SCFA when fibres were used as dietary supplement for CPT-11-based therapies. In the hindgut, fibres as complex carbohydrate provide substrate for primary fermenters and stimulate secondary fermenters by crossfeeding [1]. The use of fibre on CPT-11-treated rats had two potential benefits: it could mitigate gut injury by enhancing SCFA production, the increase in fibre-fermenting organisms could also inhibit β-glucuronidase producers such as *Enteroacteriaceae* and potential pathogens by exploitative competition. Both effects could reduce the likelihood of microbial invasion of the mucosa.

Sourdough fermentation (without yeast) provides a nutrient-rich habitat where the pH and the buffering capacity limit bacterial growth. Acidification of sourdough reduces the pH to 3.5 –

4.0, depending on the pH tolerance of the fermentation organism. Successful organisms use various mechanisms to enhance their growth rate to maximize nutrient utilization and the number of generations within the limited window of growth (e.g. glycerol-metabolising bacteria, competititors). Alternatively, in case of prolonged acidification, organisms have evolved multiple acid-resistant mechanism to outlive others (e.g. glutamate-metabolising bacteria, ruderals). In comparison, interfering competition by producing antimicrobial compounds is a less preferable strategy in sourdough. If antimicrobial compounds are produced at growth phase, antimicrobial production reduces the lower growth rate. The cost therefore likely outweighs the benefit, which will result in either competitive dominance by faster-growing organism, or a priority effect with the outcome determined by the initial abundances of the producer and the target organism. In fact, the majority of antibiotics as well as many bacteriocins of lactic acid bacteria are produced at stationary phase. It has been proposed that their function is predation (to lyse target cells for resources) rather than competition (to outgrow others in population) when nutrients have been depleted [2,3]. However, as nutrients are not limiting in sourdough, and the lysis of others can not result in significant change in pH, the production of antimicrobials will bring little, if any benefit to the producer. Reutericyclin production in sourdough lactic acid bacteria therefore should be considered as an isolated incidence rather than the result of natural selection. The persistence of reutericyclin producing organisms in an industrial sourdough over ten years might be due to cooperation with others through other metabolic functions.

It appears that antimicrobial production is an important means to maintaining the niche only in relatively accomodating environments that lacks other forms of selection on the metabolism. A study on PKS and NRPS gene distribution in anaerobes reported that those genes are most likely to be present in organisms from soil [4] where the spatial heterogeneity of

microenvironment allows for one of the most biodiverse microbial habitats on earth [5]. On the other end of the scale, the organisms in extreme environments where there are strong physical and/or chemical selections contain next to no PKS or NRPS. The appearance rate of the enzymes is also low in organisms from animal and human microbiota. In the human body, it appears that importance of antimicrobials in colonization varies by body sites. In the gut, where the stability of ecosystem is driven by various syntrophic links within a community [1], microbial competition is mainly exploitative, which explains why PKS and NRPS is rare in the major phylums Firmutes and Bacteroidetes [4]. At other body sites, it has been reported that NRPS is conserved in *Propionibacterium acnes* [4,6], *Staphylococcus aureus* [7], and *Streptococcus mutans* [8], commensal bacteria on skin and dental biofilm, where the selection pressure through energy metabolism is less strict and interference competition might play a bigger role. The distribution of bacteriocin genes partially agrees with the pattern of PKS and NRPS. A study on the distribution of bacteriocin-producing microbes on different body sites reported that samples from the gut showed a significantly smaller proportion of bacteriocin genes than samples from the airway, oral cavity, skin, and vagina [9]. It has been demonstrated that bacteriocin production partially contribute to the colonization of *Streptococcus pneumoniae* in the nasal cavity and *Streptococcus mutans* in the dental biofilm [10,11].

A second factor that might also limit the benefit of antimicrobial production in the gut is the spatial structure of the microbial habitat. The habitats where antimicrobial producers are abundant (e.g. soil) generally have highly structured microbial communities. For example, specific temporal and spatial distribution is crucial for the development of dental biofilms [12]. The highly structured spatial distribution allows for localized interaction between bacterocin producer and its target organisms even when the producer species is a minority in the total

microbiota. For example, *S. mutans*, a minor species on health tooth, has been known to produce a diversity of bacteriocins with both wide and narrow spectrums. In contrast, the gut environment is less localized. The semi-liquid state of the digesta and frequent mixing by intestinal movement provide abundant opportunity for diffusion of bacteriocin, thus renders them ineffective if the producer a minority in the microbiota (e.g. streptococci and lactobacilli); the antimicrobial producer is favored only when present at a higher frequency, when the product can accumulate to a sufficient concentration to be effective, and thus allows the benefits of toxin production to exceed its costs [13]. However, if the minority bacteriocin-producer is ingested in large quantities such as in the case of probiotics, the population may reach sufficient number for temporary observation of bacteriocin-induced effect [14].

In summary, energy metabolism played a crucial role in determining the microbial ecology in both rodent intestine and sourdough. Other metabolic functions, such as stress resistance mechanisms and antimicrobial production serve a complementary role in fine-tuning the dynamics among individual microbes. *L. reuteri* is an excellent model system in studying microbial ecology because it is relevant in both food and intestinal ecosystems and thus can be used to differentiate between ecological factors relating to the substrate (diet) and ecological factors relating to the host. The HRM-qPCR methods that were developed for the lineage-specific differentiation of *L. reuteri* and the knockout mutants of reutericyclin biosynthesis and glycerol metabolism created in this project provide highly suitable tools to determine the role of energy metabolism and antimicrobial production in microbial ecology.

7.2 Future directions

This thesis proposed that significant changes in the abundance of ruderal organisms in

gut microbiota might reflect dysbiosis in patient experiencing physiological stress. Analysis of samples from humans subjects with various health states is needed to test this hypothesis.

This thesis proposed a link between *in vitro* and *in vivo* microbial habitats through competition strategies and demonstrated the ecological benefit of exploitative competition in both sourdough and gastrointestinal systems. Due to time constraint, the role of intereference competition through reutericyclin in these habitats has not been thoroughly studied. Competition experiments with *L. reuteri* TMW1.656 and its resistant and susceptible mutants are necessary to test the hypothesis that reutericylin production does not provide long-term benefit in sourdough or rodent. The degree of localization of microbial competition in sourdough and rodent gut can also be determined by comparing the two systems with liquid and static media.

7.3 References

1. Fischbach M a, Sonnenburg JL (2011) Eating for two: how metabolism establishes interspecies interactions in the gut. Cell Host Microbe 10: 336–347.

2. Leisner JJ, Haaber J (2012) Intraguild predation provides a selection mechanism for bacterial antagonistic compounds. Proc Biol Sci 279: 4513–4521.

3. Kumbhar C, Watve M (2013) Why antibiotics: A comparative evaluation of different hypotheses for the natural role of antibiotics and an evolutionary synthesis. Nat Sci 05: 26–40.

4. Letzel A-C, Pidot SJ, Hertweck C (2013) A genomic approach to the cryptic secondary metabolome of the anaerobic world. Nat Prod Rep 30: 392–428.

5. Vos M, Wolf AB, Jennings SJ, Kowalchuk GA (2013) Micro-scale determinants of bacterial diversity in soil. FEMS Microbiol Rev 37: 936–954.

6. Brüggemann H (2005) Insights in the pathogenic potential of *Propionibacterium acnes* from its complete genome. Semin Cutan Med Surg 24: 67–72.

7. Zimmermann M, Fischbach M a (2010) A family of pyrazinone natural products from a conserved nonribosomal peptide synthetase in *Staphylococcus aureus.* Chem Biol 17: 925–930.

8. Wu C, Cichewicz R, Li Y, Liu J, Roe B, et al. (2010) Genomic island TnSmu2 of *Streptococcus mutans* harbors a nonribosomal peptide synthetase-polyketide synthase gene cluster responsible for the biosynthesis of pigments involved in oxygen and H2O2 tolerance. Appl Environ Microbiol 76: 5815–5826.

9. Zheng J, Gänzle MG, Lin XB, Ruan L,Sun M (n.d.) Diversity and dynamics of bacteriocins from human microbiome. unpublished.

10. Lux T, Nuhn M, Hakenbeck R, Reichmann P (2007) Diversity of bacteriocins and activity spectrum in *Streptococcus pneumoniae*. J Bacteriol 189: 7741–7751.

11. Hillman JD, Dzuback AL, Andrews SW (1987) Colonization of the human oral cavity by a *Streptococcus mutans* mutant producing increased bacteriocin. J Dent Res 66: 1092–1094.

12. Rickard AH, Gilbert P, High NJ, Kolenbrander PE, Handley PS (2003) Bacterial coaggregation: an integral process in the development of multi-species biofilms. Trends Microbiol 11: 94–100.

13. Abrudan MI, Brown S, Rozen DE (2012) Killing as means of promoting biodiversity. Biochem Soc Trans 40: 1512–1516.

14. Riboulet-Bisson E, Sturme MHJ, Jeffery IB, O'Donnell MM, Neville BA, et al. (2012) Effect of *Lactobacillus salivarius* bacteriocin Abp118 on the mouse and pig intestinal microbiota. PLoS One 7: e31113.

Appendix A

Oligonucleotide primers used to quantify major bacterial groups and virulence factors in cecal and/or fecal samples.

Bacterial group	Amplicon size (bp)	Oligonucleotide sequence (5`->3`)	Reference
Lactobacillus group	341	F: AGCAGTAGGGAATCTTCCA R: CACCGCTACACATGGAG	1,2
Bifidobacterium spp.	243	F: TCGCGTCYGGTGTGAAAG R: CCACATCCAGCRTCCAC	3
Clostridium cluster XIVa	438-441	F: AAATGACGGTACCTGACTAA R: CTTTGAGTTTCATTCTTGCGAA	4
Clostridium cluster IV	239	F: GCACAAGCAGTGGAGT R: CTTCCTCCGTTTTGTCAA	5
Clostridium cluster I	120	F: ATGCAAGTCGAGCGAKG R: TATGCGGTATTAATCTYCCTTT	5
Bacteroides group	140	F: GGTGTCGGCTTAAGTGCCAT R: CGGAYGTAAGGGCCGTGC	5
Enterobacteriaceae spp.	195	F: CATTGACGTTACCCGCAGAAGAAGC R: CTCTACGAGACTCAAGCTTGC	6
Clostridium cluster XI	180	F: ACGCTACTTGAGGAGGA R: GAGCCGTAGCCTTTCACT	7
Total bacteria	200	F: CGGYCCAGACTCCTACGGG R: TTACCGCGGCTGCTGGCAC	8

tcdB	177	F:	GAAAGTCCAAGTTTACGCTCAAT	9
		R:	GCTGCACCTAAACTTACACCA	
		P:	FAM-ACAGATGCAGCCAAAGTTGTTGAATT-TAMRA	
STa	193	F:	ATGAAAAAGCTAATGTTGGC	10
		R:	TACAACAAAGTTCACAGCAG	
STb	204	F:	AATATCGCATTTCTTCTTGC	
		R:	GCATCCTTTTGCTGCAAC	
LT	291	F:	CTATTACAGAACTATGTTCGG	
		R:	TACTGATTGCCGCAATTG	
EAST1	109	F:	TGCCATCAACACAGTATATCC	
		R:	GCGAGTGACGGCTTTGT	

References

1. Walter J, Hertel C, Tannock GW *et al*. (2001) Detection of *Lactobacillus, Pediococcus, Leuconostoc*, and *Weissella* species in human feces by using group-specific PCR primers and denaturing gradient gel electrophoresis. Appl Environ Microbiol 67, 2578–2585.
2. Heilig HGHJ, Zoetendal EG, Vaughan EE et al. (2002) Molecular diversity of *Lactobacillus* spp. and other lactic acid bacteria in the human intestine as determined by specific amplification of 16S ribosomal DNA. Appl Environ Microbiol 68,114–123.
3. Rinttila T, Kassinen A, Malinen E et al. (2004) Development of an extensive set of 16S rDNA-targeted primers for quantification of pathogenic and indigenous bacteria in faecal samples by real-time PCR. J Appl Microbiol 97, 1166–1177.

4. Matsuki T, Watanabe K, Fujimoto J et al. (2002) Development of 16S rRNA-gene targeted group-specific primers for the detection and identification of predominant bacteria in human feces. Appl Environ Microbiol 68, 5445-5451.
5. Matsuki T, Watanabe K, Fujimoto J et al. (2004) Use of 16S rRNA gene-targeted group-specific primers for real-time PCR analysis of predominant bacteria in human feces. Appl Environ Microbiol 70, 7220-7228.
6. Bartosch S, Fite A, Macfarlane GT et al. (2004) Characterization of bacterial communities in feces from healthy elderly volunteers and hospitalized elderly patients by using real-time PCR and effects of antibiotic treatment on the fecal microbiota. Appl Environ Microbiol 70, 3575–3581.
7. Song Y, Liu C & Finegold SM (2004) Real-time PCR quantitation of clostridia in feces of autistic children. Appl Environ Microbiol 70, 6459–6465.
8. Lee DH, Zo YG & Kim SJ (1996) Nonradioactive method to study genetic profiles of natural bacterial communities by PCR-single-strand-conformation polymorphism. Appl Environ Microbiol 62, 3112-3120.
9. van den Berg RJ, Kuijper EJ, van Coppenraet LE et al. (2006) Rapid diagnosis of toxinogenic *Clostridium difficile* in faecal samples with internally controlled real-time PCR. Clin Microbiol Infect 12, 184-186.
10. Han W, Liu B, Cao B et al. (2007) DNA microarray-based identification of serogroups and virulence gene patterns of *Escherichia coli* isolates associated with porcine postweaning diarrhea and edema disease. Appl Environ Microbiol 73, 4082-4088.

Appendix B

B1. Dietary composition for Experiments 2 and 3..

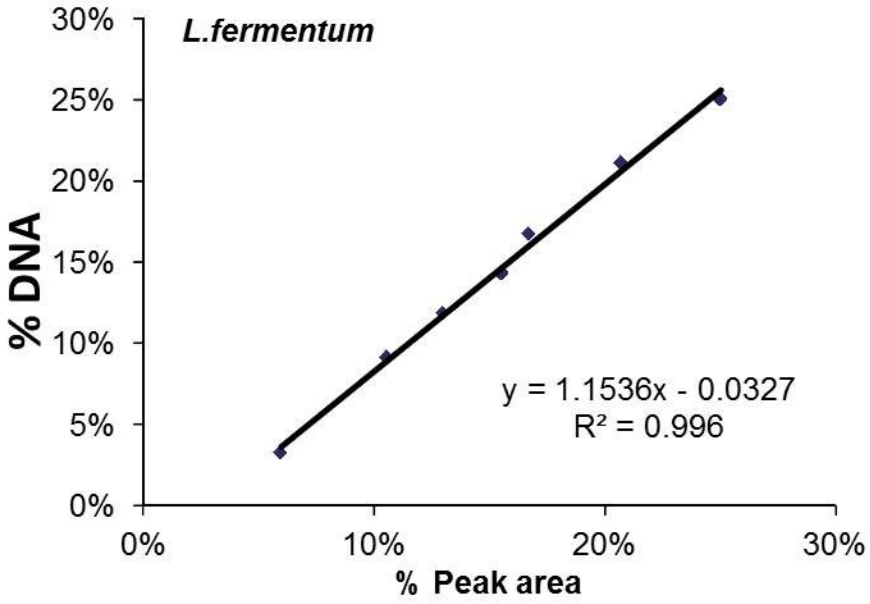
Ingredients [g]								
Variable portion (8%)								
		Cell.	Starch	IMO	FOS	In.	Syn	RS
Non digestible carbohydrates	Cellulose	8	0	0	0	0	0	0
	Cornstarch	0	8	0	0	0	0	0
	Isomalto-oligosacchrides	0	0	8	0	0	0	0
	Fructo-oligosaccharides	0	0	0	8	0	0	0
	Inulin	0	0	0	0	8	0	0
	Synergy	0	0	0	0	0	8	0
	Resistant starch	0	0	0	0	0	0	8
Constant portion (92%)								
Modified AIN-76 basal mix	Casein (25.2g), methionine (0.25g), glucose (13.95g), vitamins AIN 76 (1g), minerals AIN 76 (5g), inositol (0.6g), cornstarch (23.7g), cellulose 2 g	72	72	72	72	72	72	72
Lipids	canola stearine (11.4g), linseed oil (0.8g), sunflower oil (7.8g)	20	20	20	20	20	20	20
Total		100	100	100	100	100	100	100

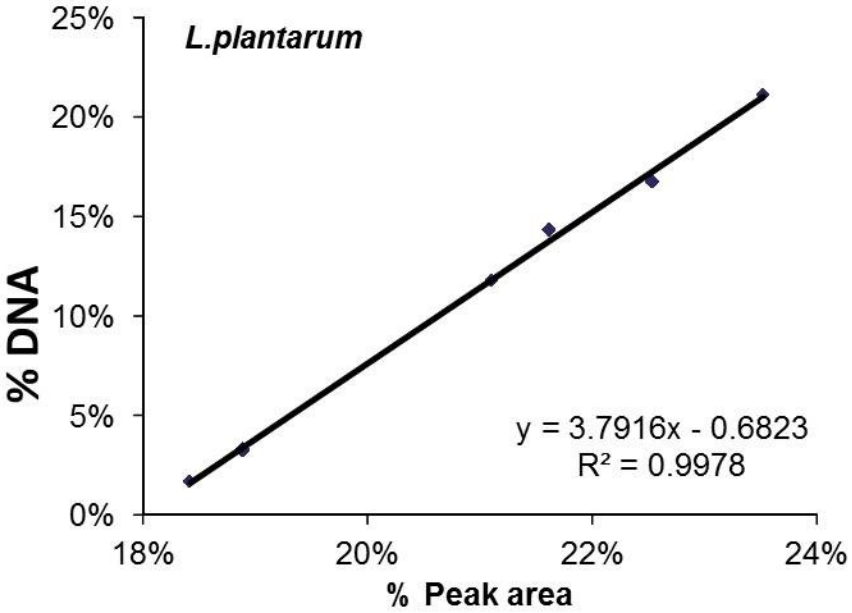
B2. Taxonomic identification of translocated bacteria isolated from mesenteric lymphnodes in two CPT-11-based regimens.

	Sequence Accession number	# of Base Pairs Sequenced	Homology to type strain
Morganella morganii FUA1235	HQ169114	1455	0.971
Escherichia coli FUA1236	HQ169115	612	1.000
Proteus mirabilis FUA1237	HQ169116	1454	0.982
Proteus mirabilis FUA1239	HQ169117	1455	0.983
Proteus mirabilis FUA1240	HQ169118	1454	0.979
Staphylococcus epidermidis FUA2058	HQ169119	1464	0.999
Enterococcus avium FUA3332	HQ169120	1471	1.000
Staphylococcus cohnii FUA2059	HQ169121	1464	0.981
Escherichia coli FUA1241	HQ169122	1455	1.000
Enterococcus faecalis FUA3333	HQ169123	653	1.000
Escherichia coli FUA1242	HQ169124	1454	0.998
Morganella morganii FUA1243	HQ169125	1454	0.989
Enterococcus faecalis FUA 3334	HQ184922	1475	0.976
Morganella morganii FUA1245	HQ169126	1455	0.983
Citrobacter freundii	HQ694731	1408	0.976
Klebsiella oxytoca	HQ694732	1403	0.983
Proteus mirabilis	HQ694733	1267	0.992
Staphylococcus warneri	HQ694734	1301	1.000

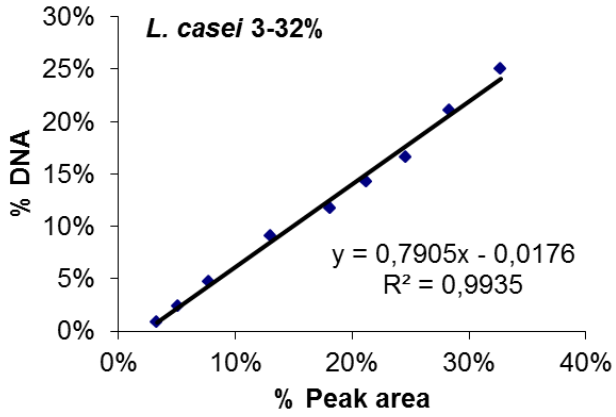
Appedix C

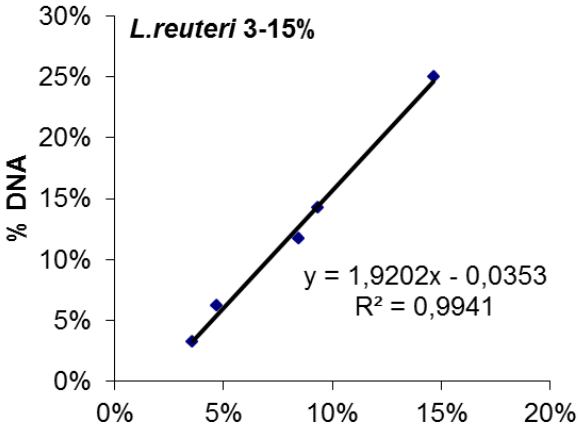
C1. HRM calibration curves for *L. fermentum* and *L. plantarum*

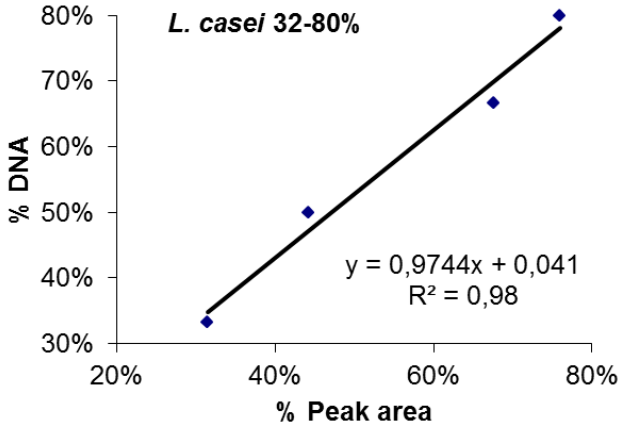


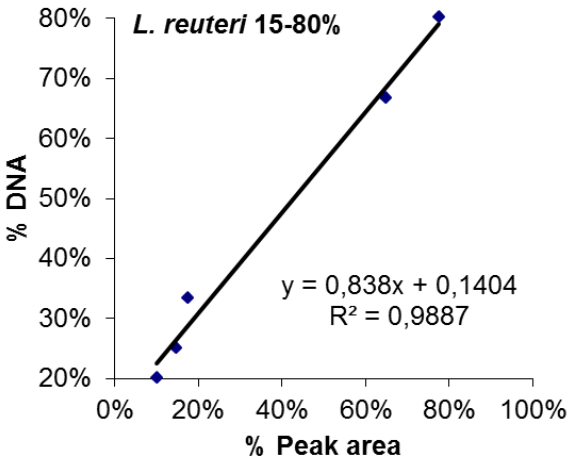


C2. HRM calibration curves for *L. casei* and *L. reuteri*

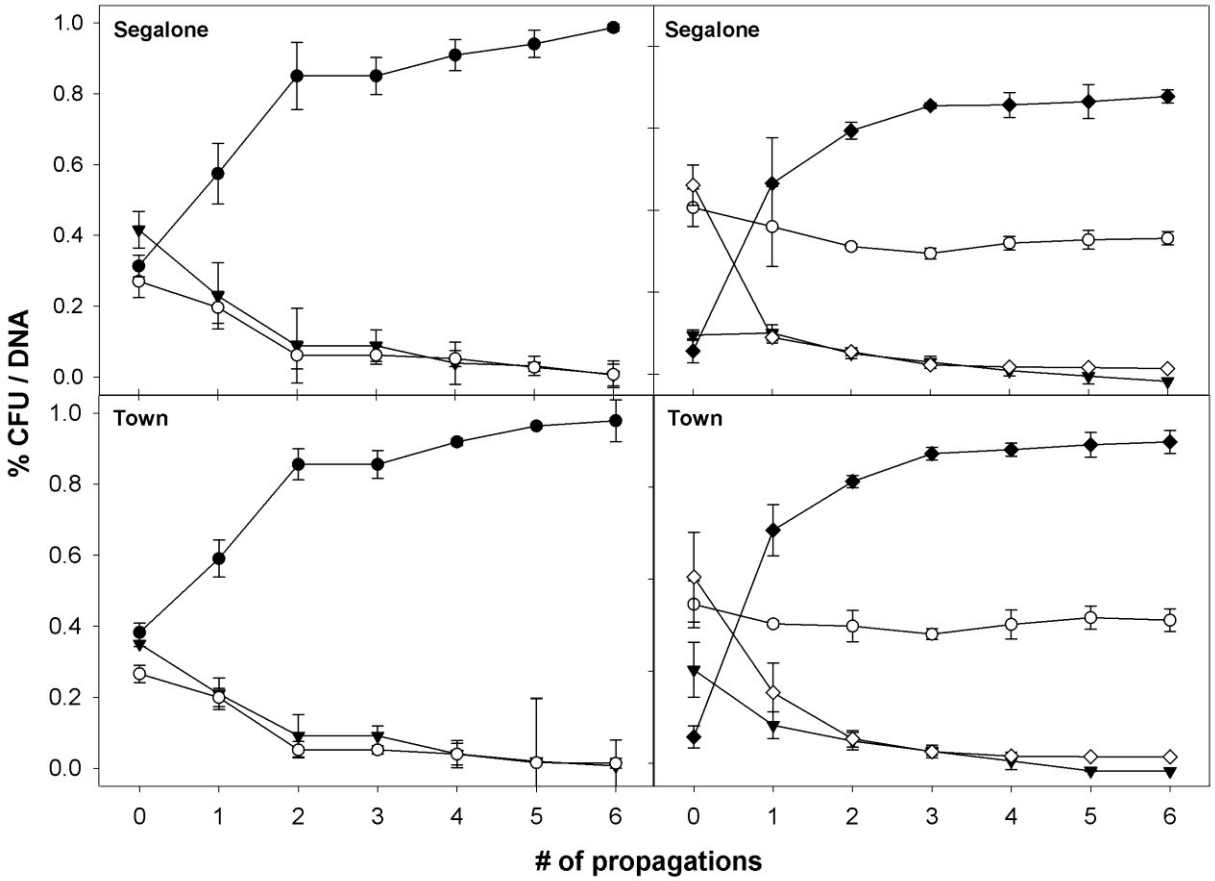








C3. Microbial composition expressed as % CFU or % DNA in wheat and sorghum sourdoughs in Exp 1. *L. fermentum* and *L. reuteri* could not be distinguished by colony morhphology on mMRS agar but could be distinguished by different melting peaks. Symbols indicate *L. fermentum* + *L. reuteri* (●), *L. plantarum* (○), *L. casei* (▼), *L. fermentum* (◆), and *L. reuteri* (◇).



C4. Nucleotide sequence of ddl gene selected for designing molecular beacon. Nucleotides which were different among the three lineages were bolded and underlined.

Sorghum	ATGATTA**C**GAAAA**C**AA**G**TTTGTGGATG
Rodent	ATGATTA**C**GAAAA**T**AA**G**TTTGTGGATG
Human	ATGATTA**T**GAAAA**C**AA**A**TTTGTGGATG

Appendix D

D1. Primers used to generate derivatives of *L. reuteri* TMW1.656 by double-crossover mutagenesis.

Primer name	sequence
N1	AAGGATCCGGTTGTTGGAAGAGGATTTGAA
N2	TATAGAGTCGACTtAACTAGTAAGATTCATTGATAACATCCTT
N3	ATAACAGTCGACAAGGAGCATTAATTTCCATGAAA
N4	AACTGCAGTGTTCAGGAATGATTTTTGAGG
P1	GCAGGATCCCGACTTGGAGGAATATGGAA
P2	AACGGCGTCGACGTtAGACATTATCCCAATATCAGTCATTAT
P3	GCATTAGTCGACTTTATCTAAAGGAAGTTATAGTTATGACTGAAAA
P4	TGCTGCAGCACGTGCAATATTTCCACCA
T1	TTAGGATCCGGAAGAATGGCAGATTCCAA
T2	ATTCGAGTCGACTTaTCCTTTCATCGGTACCTTTAATC
T3	ACGGATGTCGACTCTAAATCGTAATATTTATGCGTAATTACA
T4	GCCTGCAGGGGTTCAAATCCCCTTGTCT
R1	ATTAAGCTTGGTCCAATAATTGCAAAACCA
R2	ATTGGATCCTaACATTCTATTGGTCATTTATTGATTCTC
R3	GCAGGATCCAATGACTGATATTGGGATAATGTCTTA
R4	ACTTGAGTCGACATTGCTGTTGACCCAGAACC
A1	TGAGGATCCTGGACCATTTTCTGCAGTTG
A2	TTCAATGTCGACCTaATTCATTGGCTCATCAAG
A3	TCCGCTGTCGACGTAATTTAGACTAATAACGGGCTTGATG
A4	tatAAGCTTCTTTGCGCATAAGGATGACA

D2. Bacterial strains and plamids used to generate derivatives of *L. reuteri* TMW1.656 by double-crossover mutagenesis.

Strain or plamid	Description
pUC19	
pN-A	pUC19 containing 1 kb of the DNA sequence upstream of *rtcNRPS*; 4.0 kb; Ampr
pP-A	pUC19 containing 1 kb of the DNA sequence upstream of *rtcPKS*; 4.0 kb; Ampr
pT-A	pUC19 containing 1 kb of the DNA sequence upstream of *rtcT*; 4.0 kb; Ampr
pR-A	pUC19 containing 1 kb of the DNA sequence upstream of *rtcR1*; 4.0 kb; Ampr
pA-A	pUC19 containing 1 kb of the DNA sequence upstream of *abc*; 4.0 kb; Ampr
pN-AB	pUC19 containing 1 kb of the DNA sequence upstream and downstream of rtcNRPS; 5.0 kb; Ampr
pP-AB	pUC19 containing 1 kb of the DNA sequence upstream and downstream of rtcPKS; 5.0 kb; Ampr
pT-AB	pUC19 containing 1 kb of the DNA sequence upstream and downstream of rtcT; 5.0 kb; Ampr
pR-AB	pUC19 containing 1 kb of the DNA sequence upstream and downstream of rtcR1; 5.0 kb; Ampr
pA-AB	pUC19 containing 1 kb of the DNA sequence upstream and downstream of abc; 5.0 kb; Ampr
pJRS233	Shuttle vector used in *E. coli* and *L. reuteri*; 6.0 kb, Ermr
pKO-N	pJRS233 containing 2.0 kb of the flanking sequences of *rtcNRPS* ; 8.0 kb; Ermr
pKO-P	pJRS233 containing 2.0 kb of the flanking sequences of *rtcPKS*; 8.0 kb; Ermr
pKO-T	pJRS233 containing 2.0 kb of the flanking sequences of *rtcT*; 8.0 kb; Ermr
pKO-R	pJRS233 containing 2.0 kb of the flanking sequences of *rtcR1* and *rtcR2*

	; 8.0 kb; Ermr
pKO-A	pJRS233 containing 2.0 kb of the flanking sequences of *abc* ; 8.0 kb; Ermr
E. coli DH5α	Cloning host for pUC19- and pJRS233-derivative plasmids
Δ*rtcNRPS*	*L. reuteri* TMW1.656 derivative with a deletion in *rtcNRPS*
Δ*rtcPKS*	*L. reuteri* TMW1.656 derivative with a deletion in *rtcPKS*
Δ*rtcT*	*L. reuteri* TMW1.656 derivative with a deletion in *rtcT*
Δ*rtcR1R2*	*L. reuteri* TMW1.656 derivative with a deletion in *rtcR1R2*
Δ*rtcNRPS*Δ*rtcT*	*L. reuteri* TMW1.656 derivative with deletions in *rtcNRPS* and *rtcT*
Δ*rtcNRPS*Δ*rtcR1R2*	*L. reuteri* TMW1.656 derivative with deletions in *rtcNRPS* and *rtcR1R2*
Δ*ABC*	*L. reuteri* TMW1.656 derivative with a deletion in *abc*
Δ*rtcNRPS*Δ*ABC*	*L. reuteri* TMW1.656 derivative with deletions in *rtcNRPS* and *abc*

D3. Genes which are present in the three reutericyclin producing strains *L. reuteri* LTH2584, TMW1.656, and TMW1.112 but are absent in other strains of *L. reuteri*. *L. reuteri* LTH5448 was excluded from this analysis.

	Locus Tag	Gene Name	Prediction	Length (amino acids)
1	HQ33_01735	-	Hypothetical protein	353
2	HQ33_01740	-	Hypothetical protein	448
3	HQ33_01745	*rtcPP*	Phosphopantetheinyl transferase	212
4	HQ33_01750	*rtcPKS*	Polyketide synthase	891
5	HQ33_01755	*rtcNRPS*	Non-ribosomal peptide synthetase	1012
6	HQ33_01760	*rtcPhlB*	Acetyl transferase	145
7	HQ33_01765	*rtcPhlC*	Acetyl transferase	398
8	HQ33_01770	*rtcPhlA*	Acetyl transferase	356
9	HQ33_01775	*rtcR1*	TetR family regulator	157
10	HQ33_01780	*rtcR2*	TetR family regulator	181
11	HQ33_01785	*rtcT*	Major Facilitator Superfamily transport protein	585
12			Hypothetical protein	34
13	HQ33_09400		Hypothetical protein	188
14	HQ33_09460	-	ABC transporter component	72
15	HQ33_09465	-	ABC transporter component	91
16	HQ33_09475	-	ABC transporter component	81

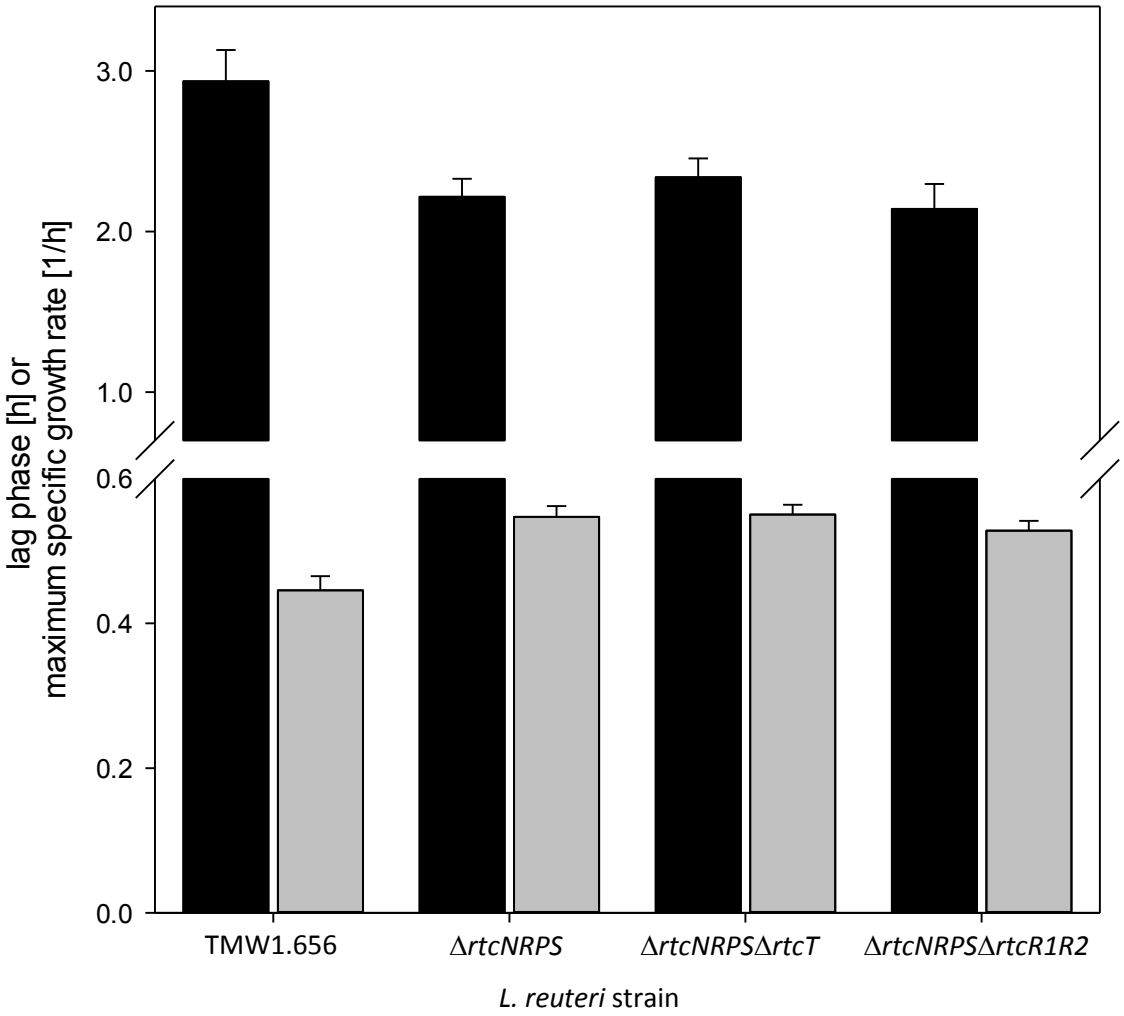
[a)] This hypothetical protein was not detected by automatic annotation.

D4. Proteins which are homologue to proteins encoded by the reutericyclin genetic island, and for which functional characterization has been published. Protein sequences were retrieved by Blastp.

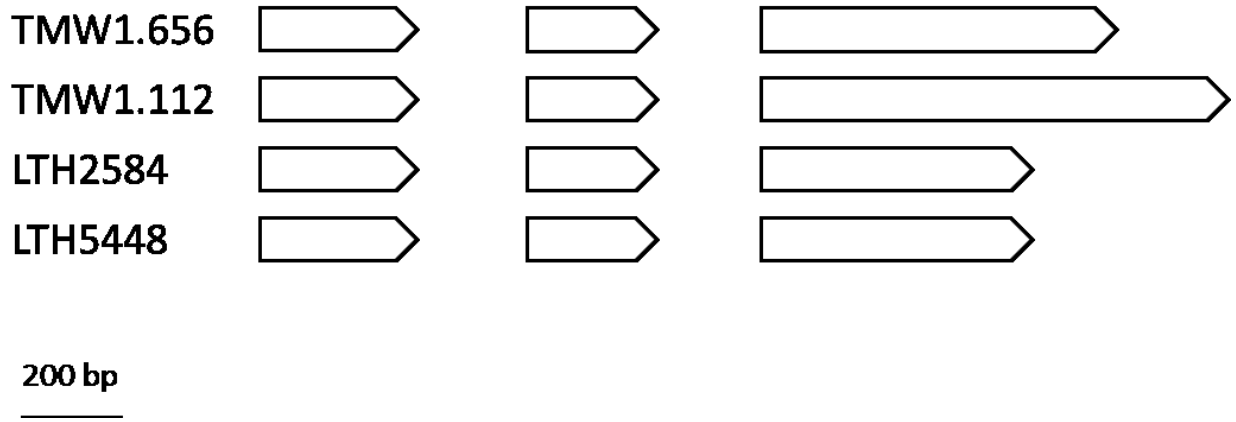
Name	Similar protein in NCBI database	Organism	Identity	Accession
RtcPP	4'-phosphopantetheinyl transferase	*B. subtilis*	27%	P39144.1
RtcPKS	Mycosubtilin synthase subunit A	*B. subtilis*	32%	Q9R9J1.1
RtcNRPS	Plipastatin synthase subunit B	*B. subtilis*	28%	P39846.1
RtcPhlA	PhlA	*P. fluorescens*	33%	BAD00178.1
RtcPhlC	PhlC	*P. fluorescens*	32%	AAY86549.1
RtcPhlB	PhlB	*P. fluorescens*	48%	AAM27407.1
RtcMFS	MFS-type transporter YusP	*B. subtilis*	27%	P39144.1
RtcR1	HTH-type transcriptional repressor BscR	*B. subtilis*	32%	O32182.1
RtcR2	HTH-type transcriptional regulator YerO	*B. subtilis*	41%	O08335.1

D5. *In vitro* growth of *L. reuteri* TMW1.656 and its reutericyclin-negative mutant strains.

The lag phase (black bars) and the maximum specific growth rate μ_{max} (gray bars) were determined by indubating *L. reuteri* strains in mMRS at 37°C, periodic measurement of the optical densitz at 600 nm, and determination of the parameters by non-linear curve fit to the logistic growth curve as described [Zwietering MH, Jongenburger I, Rombouts FM, van 't Riet K (1990) Modeling of the bacterial growth curve. Appl Environ Microbiol. 56:1875-1881.] Data are shown as means ± standard deviation of triplicate independent experiments.

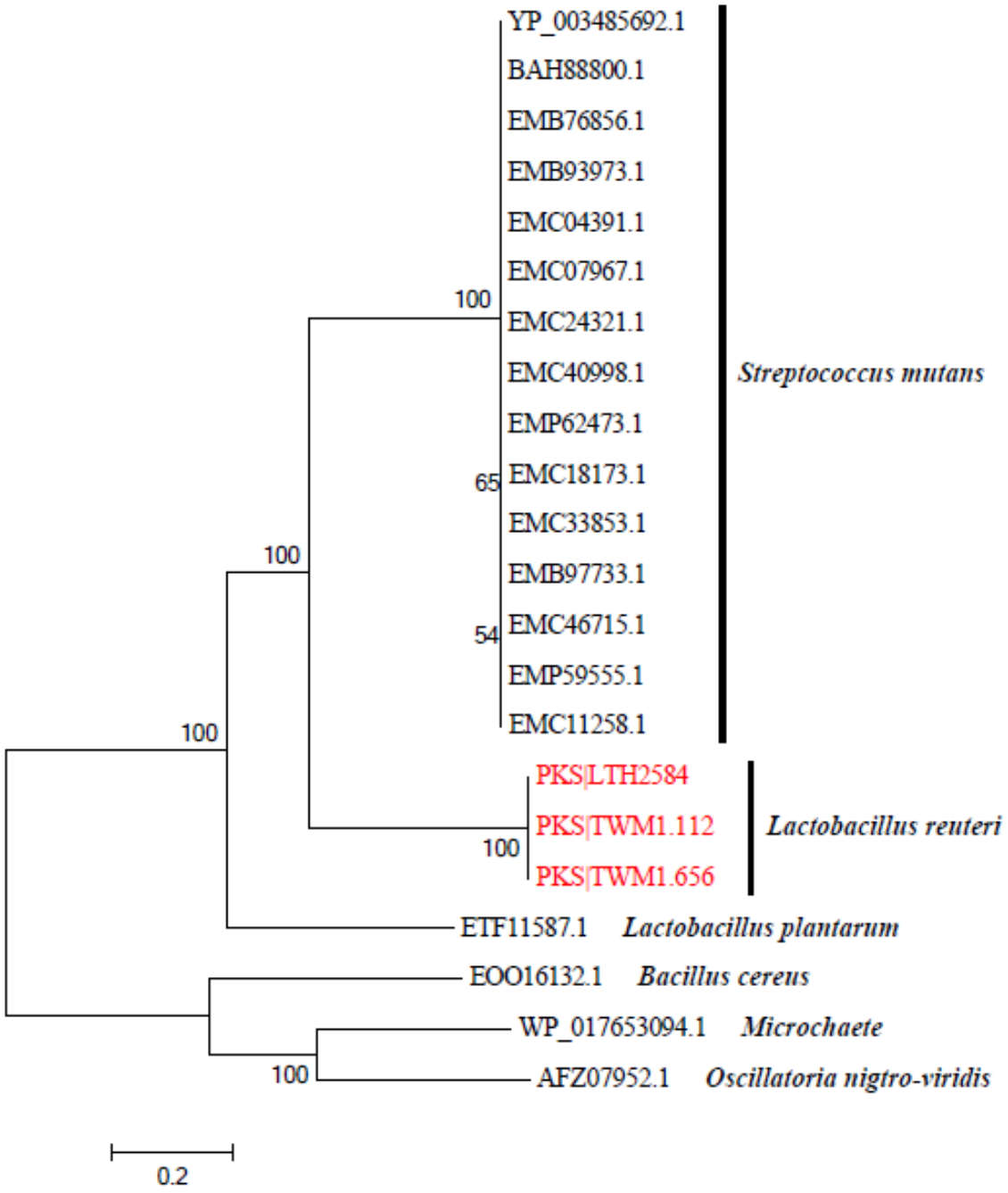


D6. Gene cluster containing ABC transporter unique to reutericyclin producing strains and LTH5448.

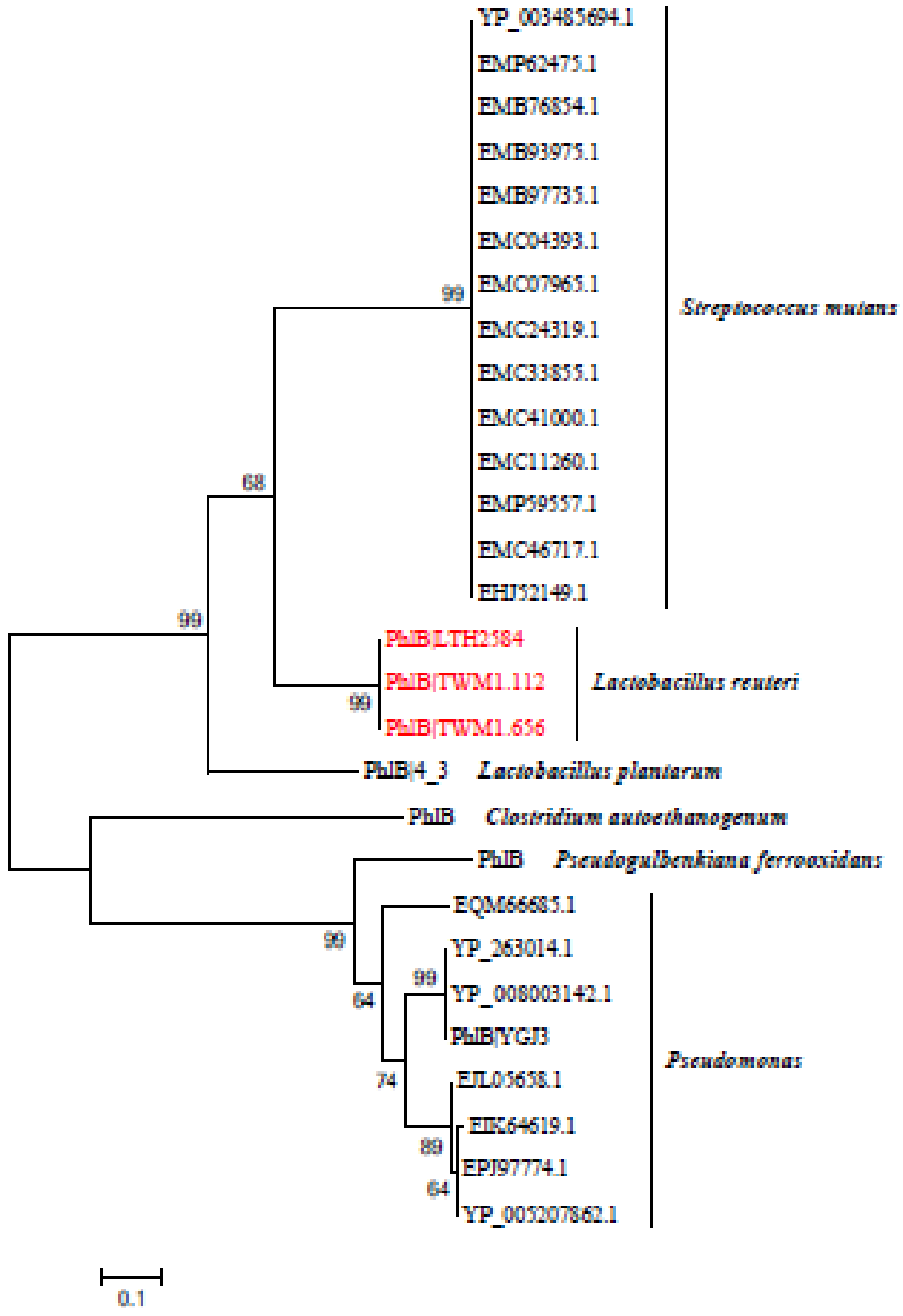


D7. Unrooted phylogenetic tree of RtcPKS (Panel A), PhlB (Panel B) and PhlC (Panel C) of *L. reuteri* and related proteins. The number at each branch point represents the percentage of bootstrap support calculated from 1,000 replicates. Only bootstrap values above 50 are shown.

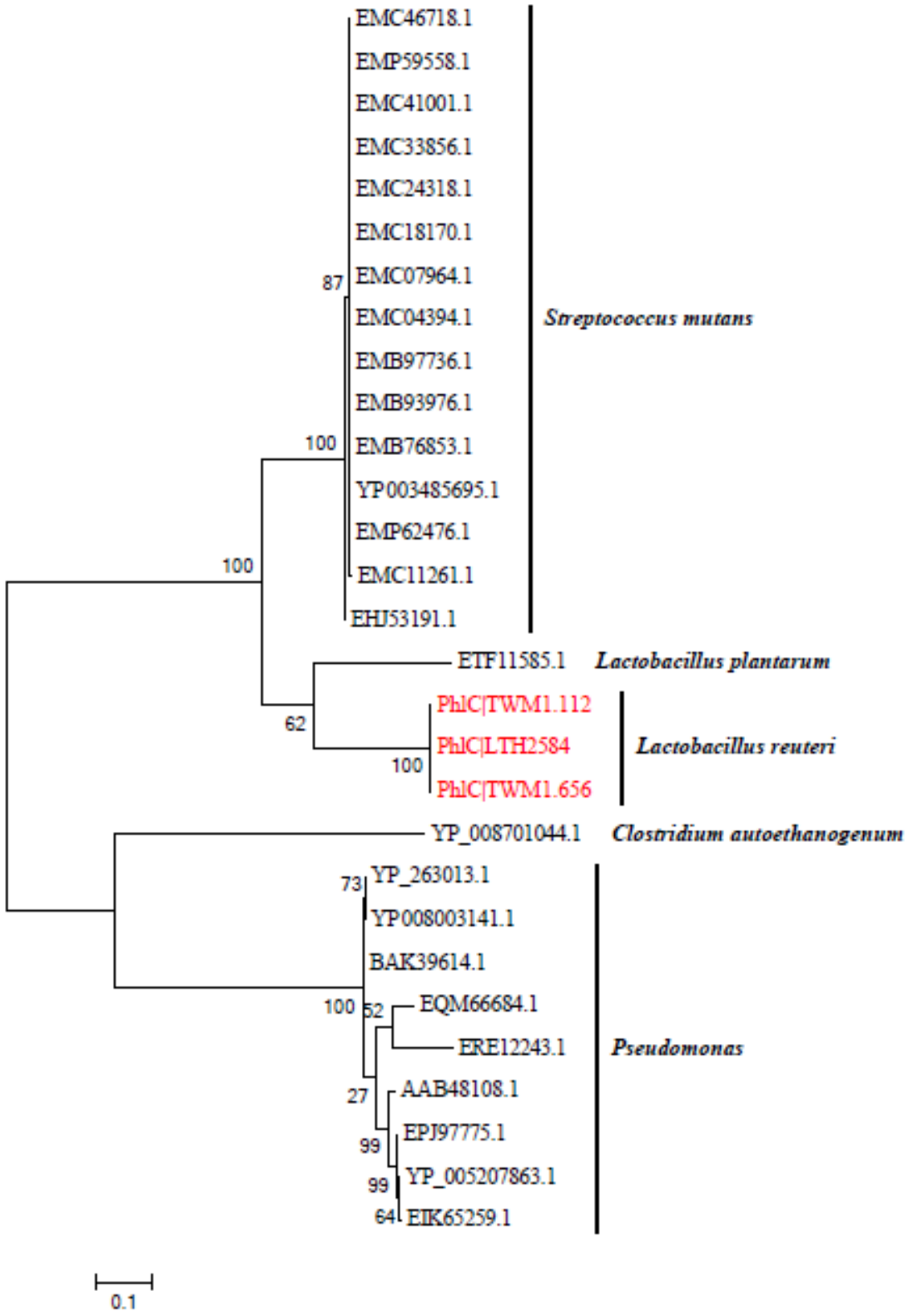
A.



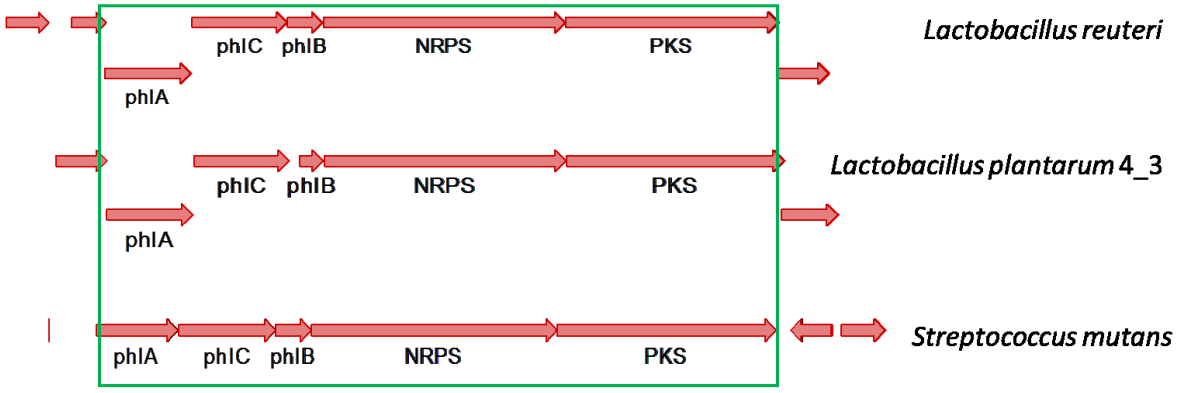
B


YP_003485694.1
EMP62475.1
EMB76854.1
EMB93975.1
EMB97735.1
EMC04393.1
EMC07965.1
EMC24319.1
EMC33855.1
EMC41000.1
EMC11260.1
EMP59557.1
EMC46717.1
EHJ52149.1
Streptococcus mutans
99
68
99
PhlB|LTH2584
PhlB|TWM1.112
PhlB|TWM1.656
Lactobacillus reuteri
99
PhlB|4_3
Lactobacillus plantarum
PhlB
Clostridium autoethanogenum
PhlB
Pseudogulbenkiana ferrooxidans
EQM66685.1
YP_263014.1
YP_008003142.1
PhlB|YGJ3
EJL05638.1
EIK64619.1
EPJ97774.1
YP_003207862.1
Pseudomonas
99
64
99
74
99
64
0.1

C.


EMC46718.1
EMP59558.1
EMC41001.1
EMC33856.1
EMC24318.1
EMC18170.1
87
EMC07964.1
EMC04394.1
Streptococcus mutans
EMB97736.1
EMB93976.1
100
EMB76853.1
YP003485695.1
EMP62476.1
100
EMC11261.1
EHJ53191.1
ETF11585.1
Lactobacillus plantarum
PhlC|TWM1.112
62
PhlC|LTH2584
Lactobacillus reuteri
100
PhlC|TWM1.656
YP_008701044.1
Clostridium autoethanogenum
73
YP_263013.1
YP008003141.1
BAK39614.1
100
52
EQM66684.1
ERE12243.1
Pseudomonas
27
AAB48108.1
99
EPJ97775.1
99
YP_005207863.1
64
EIK65259.1
0.1

D8. Comparison of the genetic locus containing homologues of *rtcphlA*, *rtcphlB*, *rtcphlC*, *rtcNRPS* and *rtcPKS* in *L. reuteri* TMW1.656, *L. plantarum* 4_3 and *S. mutans*. Genes coding for homologues of genes that are essential for reutericyclin biosynthesis are designated as phlA, phlC, phlB, NRPS, and PKS.



References

Abrudan MI, Brown S, Rozen DE (2012) Killing as means of promoting biodiversity. Biochem Soc Trans 40: 1512–1516.

Achkar J, Xian M, Zhao H, Frost JW (2005) Biosynthesis of phloroglucinol. J Am Chem Soc 127: 5332–5333.

Albesharat R, Ehrmann MA, Korakli M, Yazaji S, Vogel RF (2011) Phenotypic and genotypic analyses of lactic acid bacteria in local fermented food, breast milk and faeces of mothers and their babies. Syst Appl Microbiol 34: 148–155.

Al-Dasooqi N, Bowen JM, Gibson RJ, Logan RM, Stringer AM, et al. (2011) Selection of housekeeping genes for gene expression studies in a rat model of irinotecan-induced mucositis. Chemother 57: 43–53.

Amin HM, Hashem AM, Ashour MS, Hatti-Kaul R (2013) 1,2 Propanediol utilization by *Lactobacillus reuteri* DSM 20016, role in bioconversion of glycerol to 1,3 propanediol, 3-hydroxypropionaldehyde and 3-hydroxypropionic acid. J Genet Eng Biotechnol 11:53-39.

Ansari MZ, Yadav G, Gokhale RS, Mohanty D (2004) NRPS-PKS: a knowledge-based resource for analysis of NRPS/PKS megasynthases. Nucleic Acids Res 32: W405–13.

Anthony L (2007) Irinotecan toxicity. Curr Opin Support Palliat Care 1: 35–39.

Arena ME, Manca de Nadra MC (2001) Biogenic amine production by *Lactobacillus*. J Appl Microbiol 90: 158–162.

Arendt EK, Moroni A, Zannini E (2011) Medical nutrition therapy: use of sourdough in gluten free bread. Microb Cell Fact 10 Suppl 1: S15.

Arnold RJ, Gabrail N, Raut M, Kim R, Sung JC, et al. (2005) Clinical implications of chemotherapy-induced diarrhea in patients with cancer. J Support Oncol 3:227-232.

Arumugam M, Raes J, Pelletier E, Le Paslier D, Yamada T, et al. (2011) Enterotypes of the human gut microbiome. Nature 473: 174–180.

Atarashi K, Tanoue T, Oshima K, Suda W, Nagano Y, et al. (2013) Treg induction by a rationally selected mixture of Clostridia strains from the human microbiota. Nature 500: 232–236.

Atarashi K, Tanoue T, Shima T, Imaoka A, Kuwahara T, et al. (2011) Induction of colonic regulatory T cells by indigenous Clostridium species. Science 331: 337–341.

Awika JM, Rooney LW, Waniska RD (2005) Anthocyanins from black sorghum and their antioxidant properties. Food Chem 90: 293–301.

Azcárate-Peril MA, Sikes M, Bruno-Bárcena JM (2011) The intestinal microbiota, gastrointestinal environment and colorectal cancer: a putative role for probiotics in prevention of colorectal cancer? Am J Physiol Gastrointest Liver Physiol 301:G401-424.

Badía J, Ros J, Aguilar J (1985) Fermentation mechanism of fucose and rhamnose in *Salmonella* Typhimurium and *Klebsiella pneumoniae*. J Bacteriol 161: 435–437.

Bangera MG, Thomashow LS (1999) Identification and characterization of a gene cluster for synthesis of the polyketide antibiotic 2,4-diacetylphloroglucinol from *Pseudomonas fluorescens* Q2-87. J Bacteriol 181: 3155–3163.

Beaud D, Tailliez P, Anba-Mondoloni J (2005) Genetic characterization of the β-glucuronidase enzyme from a human intestinal bacterium, *Ruminococcus gnavus*. Microbiology 151: 2323–2330.

Benson A, Kelly SA, Legge R, Ma R, Low, SJ, et al. (2010). Individuality in gut microbiota composition is a complex polygenic trait shaped by multiple environmental and host genetic factors. Proc Nat Acad Sci U.S.A. 107:18933-18938.

Berg RD (1999) Bacterial translocation from the gastrointestinal tract. Adv Exp Med Biol 473: 11–30.

Biernaskie JM, Gardner A, West S a (2013) Multicoloured greenbeards, bacteriocin diversity and the rock-paper-scissors game. J Evol Biol 26: 2081–2094.

Blaser MJ, Falkow S (2009) What are the consequences of the disappearing human microbiota? Nat Rev Microbiol 7: 887–894.

Böcker G, Stolz P, Hammes W (1995) Neue Erkenntnisse zum Ökosystem Sauerteig und zur Physiologie der sauerteigtypischen Stämme *Lactobacillus sanfrancisco* und *Lactobacillus pontis*. Getreide Mehl Brot 49: 370–374.

Boleij A, van Gelder MMHJ, Swinkels DW, Tjalsma H (2011) Clinical Importance of *Streptococcus gallolyticus* infection among colorectal cancer patients: systematic review and meta-analysis. Clin Infect Dis 53: 870–878.

Bolotin A, Quinquis B, Renault P, Sorokin A, Ehrlich SD, et al. (2004) Complete sequence and comparative genome analysis of the dairy bacterium *Streptococcus thermophilus*. Nat Biotechnol 22: 1554–1558.

Brandt MJ (2007) Sourdough products for convenient use in baking. Food Microbiol 24: 161–164.

Brandt MJ (n.d.) Personal communication.

Brandt MJ, Hammes WP, Gänzle MG (2004) Effects of process parameters on growth and metabolism of *Lactobacillus sanfranciscensis* and *Candida humilis* during rye sourdough fermentation. Eur Food Res Technol 218: 333–338.

Brown CT, Davis-Richardson AG, Giongo A, Gano KA, Crabb DB, et al. (2011) Gut microbiome metagenomics analysis suggests a functional model for the development of autoimmunity for type 1 diabetes. PloSone 6: e25792.

Brüggemann H (2005) Insights in the pathogenic potential of *Propionibacterium acnes* from its complete genome. Semin Cutan Med Surg 24: 67–72.

Caballero-Franco C, Keller K, De Simone C, Chadee K (2007) The VSL#3 probiotic formula induces mucin gene expression and secretion in colonic epithelial cells. Am J Physiol Gastrointest Liver Physiol 292: G315–22.

Cadieux P, Wind A, Sommer P, Schaefer L, Crowley K, et al. (2008) Evaluation of reuterin production in urogenital probiotic *Lactobacillus reuteri* RC-14. Appl Environ Microbiol 74: 4645–4649.

Campos FM, Couto JA, Hogg TA (2003) Influence of phenolic acids on growth and inactivation of *Oenococcus oeni* and *Lactobacillus hilgardii*. J Appl Microbiol 94: 167–174.

Canani RB, Costanzo MD, Leone L, Pedata M, Meli R, et al. (2011) Potential beneficial effects of butyrate in intestinal and extraintestinal diseases. World J Gastroenterol 17: 1519–1528.

Cappelle S, Guylaine L, Gänzle M, Gobbetti M (2013) History and Social Aspects of Sourdough. In: Gobbetti M, Gänzle M, editors. Handbook on Sourdough Biotechnology. Boston, MA. pp. 1–10.

Cenci G, Caldini G, Trotta F (2000) Bacterial amylolytic activity enhances β-glucuronidase expression of amylase-negative *Escherichia coli* strain in starch-medium. J Basic Microbiol 40: 311–318.

Cénit MC, Matzaraki V, Tigchelaar EF, Zhernakova a (2014) Rapidly expanding knowledge on the role of the gut microbiome in health and disease. Biochim Biophys Acta.

Chakravorty S, Helb D, Burday M, Connell N, Alland D (2007) A detailed analysis of 16S ribosomal RNA gene segments for the diagnosis of pathogenic bacteria. J Microbiol Methods 69: 330–339.

Chao L, Levin BR (1981) Structured habitats and the evolution of anticompetitor toxins in bacteria. Proc Natl Acad Sci U S A 78: 6324–6328.

Chapman MA, Grahn MF, Boyle MA, Hutton M, Rogers J, et al. (1994) Butyrate oxidation is impaired in the colonic mucosa of sufferers of quiescent ulcerative colitis. Gut 35: 73–76.

Cheng Y-Q, Coughlin JM, Lim S-K, Shen B (2009) Type I polyketide synthases that require discrete acyltransferases. Methods Enzymol 459: 165–186.

Claesson MJ, Cusack S, O'Sullivan O, Greene-Diniz R, de Weerd H, et al. (2011) Composition, variability, and temporal stability of the intestinal microbiota of the elderly. Proc Natl Acad Sci U S A 108 Suppl: 4586–4591.

Collado MC, Derrien M, Isolauri E, de Vos WM, Salminen S (2007) Intestinal integrity and *Akkermansia muciniphila*, a mucin-degrading member of the intestinal microbiota present in infants, adults, and the elderly. Appl Environ Microbiol 73: 7767–7770.

Corona G, Elia C, Casetta B, Toffoli G (2010) Fast liquid chromatography-tandem mass spectrometry method for routine assessment of irinotecan metabolic phenotype. Ther Drug Monit 32: 638–646.

Corsetti A (2013) Technology of Sourdough Fermentation and Sourdough Applications. In: Gobbetti M, Gänzle M, editors. Handbook on Sourdough Biotechnology. Boston, MA: Springer US. pp. 85–103.

Costello EK, Stagaman K, Dethlefsen L, Bohannan BJM, Relman DA (2012) The application of ecological theory toward an understanding of the human microbiome. Science 336: 1255–1262.

Cotter PD, Hill C, Ross RP (2005) Bacteriocins: developing innate immunity for food. Nat Rev Microbiol 3: 777–788.

Cotter PD, Ross RP, Hill C (2013) Bacteriocins - a viable alternative to antibiotics? Nat Rev Microbiol 11: 95–105.

Critchley-Thorne RJ, Simons DL, Yan N, Miyahira AK, Dirbas FM, et al. (2009) Impaired interferon signaling is a common immune defect in human cancer. Proc Natl Acad Sci U S A 106:9010-9015.

Cuff MA, Lambert DW, Shirazi-Beechey SP (2002) Substrate-induced regulation of the human colonic monocarboxylate transporter, MCT1. J Physiol 539: 361–371.

Cvitkovitch DG (2001) Genetic competence and transformation in oral streptococci. Crit Rev Oral Biol Med 12: 217–243.

Dabek M, McCrae SI, Stevens VJ, Duncan SH, Louis P (2008) Distribution of β-glucosidase and β-glucuronidase activity and of β-glucuronidase gene gus in human colonic bacteria. FEMS Microbiol Ecol 66: 487–495.

Dawson LF, Valiente E, Wren BW (2009) *Clostridium difficile* - a continually evolving and problematic pathogen. Infect Genet Evol 9:1410-1417.

De Crécy-Lagard V, Blanc V, Gil P, Naudin L, Lorenzon S, et al. (1997) Pristinamycin I biosynthesis in *Streptomyces pristinaespiralis*: molecular characterization of the first two structural peptide synthetase genes. J Bacteriol 179: 705–713.

De Filippo C, Cavalieri D, Di Paola M, Ramazzotti M, Poullet JB, et al. (2010) Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa. Proc Natl Acad Sci U S A 107: 14691–14696.

De Vuyst L, Vancanneyt M (2007) Biodiversity and identification of sourdough lactic acid bacteria. Food Microbiol 24: 120–127.

De Vuyst L, Vrancken G, Ravyts F, Rimaux T, Weckx S (2009) Biodiversity, ecological determinants, and metabolic exploitation of sourdough microbiota. Food Microbiol 26: 666–675.

Decker-Baumann C, Buhl K, Frohmuller S, von Herbay A, Dueck M, et al. (1999) Reduction of chemotherapy-induced side-effects by parenteral glutamine supplementation in patients with metastatic colorectal cancer. Eur J Cancer 35:202–207.

Decock P, Cappelle S (2005) Bread technology and sourdough technology. Trends Food Sci Technol 16: 113–120.

Dethlefsen L, McFall-Ngai M, Relman DA (2007) An ecological and evolutionary perspective on human–microbe mutualism and disease. Nature 449: 811–818.

Donadio S, Monciardini P, Sosio M (2007) Polyketide synthases and nonribosomal peptide synthetases: the emerging view from bacterial genomics. Nat Prod Rep 24: 1073–1109.

Dorofeyev AE, Vasilenko IV, Rassokhina OA (2009) Joint extraintestinal manifestations in ulcerative colitis. Dig Dis 27:502–510.

Duerkop BA, Vaishnava S, Hooper LV (2009) Immune responses to the microbiota at the intestinal mucosal surface. Immunity 31:368-376.

Durrett R, Levin S (1997) Allelopathy in Spatially Distributed Populations. J Theor Biol 185: 165–171.

Dutton C, Haxwell M, McArthur H, Wax RG, editors (2002) Peptide Antibiotics: Discovery Modes Of Action And Applications. New York, USA: Marcel Dekker Inc.

Eckburg PB, Bik EM, Bernstein CN, Purdom E, Dethlefsen L, et al. (2005) Diversity of the human intestinal microbial flora. Science 308: 1635–1638.

Edgar RC (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res 32: 1792–1797.

El-Hajj HH, Marras SAE, Tyagi S, Shashkina E, Kamboj M, et al. (2009) Use of sloppy molecular beacon probes for identification of mycobacterial species. J Clin Microbiol 47: 1190–1198.

Elleuche S, Fodor K, Klippel B, von der Heyde A, Wilmanns M, et al. (2013) Structural and biochemical characterisation of a NAD^+-dependent alcohol dehydrogenase from *Oenococcus oeni* as a new model molecule for industrial biotechnology applications. Appl Microbiol Biotechnol 97: 8963–8975.

Engels C, Schieber A, Gänzle MG (2011) Inhibitory spectra and modes of antimicrobial action of gallotannins from mango kernels (*Mangifera indica* L.). Appl Environ Microbiol 77: 2215–2223.

Erdman SE, Rao VP, Olipitz W, Taylor CL, Jackson EA, et al. (2010) Unifying roles for regulatory T cells and inflammation in cancer. Int J Cancer 126: 1651–1665.

Everard A, Belzer C, Geurts L, Ouwerkerk JP, Druart C, et al. (2013) Cross-talk between *Akkermansia muciniphila* and intestinal epithelium controls diet-induced obesity. Proc Natl Acad Sci U S A 110: 9066–9071.

Faber J, Vos AP, Kegler D, Argilés J, Laviano A, et al. (2009) Impaired immune function: an early marker for cancer cachexia. Oncol Rep 22:1403-1406.

Farhangfar A (2012) Non digestible carbohydrates in the diet determine toxicity of irinotecan (CPT-11)/5-fluorouracil in rats independently of β-glucuronidase activity in intestinal lumen. M.Sc. thesis, University of Alberta, http://hdl.handle.net/10402/era.28569. Accessed 2013 Nov 18.

Fisch KM (2013) Biosynthesis of natural products by microbial iterative hybrid PKS–NRPS. RSC Adv 3: 18228.

Fischbach M a, Sonnenburg JL (2011) Eating for two: how metabolism establishes interspecies interactions in the gut. Cell Host Microbe 10: 336–347.

Fischbach MA, Walsh CT (2006) Assembly-line enzymology for polyketide and nonribosomal Peptide antibiotics: logic, machinery, and mechanisms. Chem Rev 106: 3468–3496.

Franks AH, Harmsen HJM, Raangs GC, Jansen GJ, Schut F, et al. (2008) Variations of bacterial populations in human feces measured by fluorescent *in situ* hybridization with group-specific 16S rRNA-targeted oligonucleotide probes. Appl Environ Microbiol 64:3336–3345.

Frese SA, Benson AK, Tannock GW, Loach DM, Kim J, et al. (2011) The evolution of host specialization in the vertebrate gut symbiont *Lactobacillus reuteri*. PLoS Genet 7: e1001314.

Fukuda S, Toh H, Hase K, Oshima K, Nakanishi Y, et al. (2011). Bifidobacteria can protect from enteropathogenic infection through production of acetate. Nature. 469: 543- 547.

Furusawa Y, Obata Y, Fukuda S, Endo TA, Nakato G, et al. (2013) Commensal microbe-derived butyrate induces the differentiation of colonic regulatory T cells. Nature 504: 446–450.

Ganopoulos I, Sakaridis I, Argiriou A, Madesis P, Tsaftaris A (2013) A novel closed-tube method based on high resolution melting (HRM) analysis for authenticity testing and quantitative detection in Greek PDO Feta cheese. Food Chem 141: 835–840.

Gänzle M, Ehmann M, Hammes W (1998) Modeling of growth of *Lactobacillus sanfranciscensis* and *Candida milleri* in response to process parameters of sourdough fermentation. Appl Environ Microbiol 64: 2616–2623.

Gänzle MG (2004) Reutericyclin: biological activity, mode of action, and potential applications. Appl Microbiol Biotechnol 64: 326–332.

Gänzle MG, Höltzel A, Walter J, Jung G, Hammes WP (2000) Characterization of reutericyclin produced by *Lactobacillus reuteri* LTH2584. Appl Environ Microbiol 66: 4325–4333.

Gänzle MG, Vermeulen N, Vogel RF (2007) Carbohydrate, peptide and lipid metabolism of lactic acid bacteria in sourdough. Food Microbiol 24: 128–138.

Gänzle MG, Vogel RF (2003) Contribution of reutericyclin production to the stable persistence of *Lactobacillus reuteri* in an industrial sourdough fermentation. Int J Food Microbiol 80: 31–45.

Gill CIR, Rowland IR (2002) Diet and cancer: assessing the risk. Brit J Nutr 88 S1: S73–87.

Goodhand JR, Alazawi W, Rampton DS (2011) Systematic review: *Clostridium difficile* and inflammatory bowel disease. Aliment Pharmacol Ther 33:428-441.

Gordon DM, O'Brien CL (2006) Bacteriocin diversity and the frequency of multiple bacteriocin production in *Escherichia coli*. Microbiology 152: 3239–3244.

Grime J (1979) Plant Strategies and Vegetation Processes. New York, USA: John Wiley & Sons.

Grime J, Pierce S (2012) The Evolutionary Strategies that Shape Ecosystems. Chichester, UK: Wiley-Blackwell.

Grönroos L, Saarela M, Mättö J, Tanner-Salo U, Vuorela A, et al. (1998) Mutacin production by *Streptococcus mutans* may promote transmission of bacteria from mother to child. Infect Immun 66: 2595–2600.

Guenzi E, Galli G, Grgurina I, Gross DC, Grandi G (1998) Characterization of the syringomycin synthetase gene cluster. A link between prokaryotic and eukaryotic peptide synthetases. J Biol Chem 273: 32857–32863.

Gundry CN (2003) Amplicon melting analysis with labeled primers: a closed-tube method for differentiating homozygotes and heterozygotes. Clin Chem 49: 396–406.

Gupta E, Lestingi TM, Mick R, Ramirez J, Vokes EE, et al. (1994) Metabolic fate of irinotecan in humans: correlation of glucuronidation with diarrhea. Cancer Res 54: 3723–3725.

Gupta E, Lestingi TM, Mick R, Ramirez J, Vokes EE, et al. (1994) Metabolic fate of irinotecan in humans: correlation of glucuronidation with diarrhea. Cancer Res 54:3723–3725.

Hamad SH, Dieng MC, Ehrmann MA, Vogel RF (1997) Characterization of the bacterial flora of Sudanese sorghum flour and sorghum sourdough. J Appl Microbiol 83: 764–770.

Hamer HM, Jonkers D, Venema K, Vanhoutvin S, Troost FJ, et al. (2008) Review article: the role of butyrate on colonic function. Aliment Pharmacol Ther 27: 104–119.

Hammes W, Gänzle M (1997) Sourdough breads and related products. In: Woods B, editor. Microbiology of fermented foods. London: Blackie Academic and Professional. pp. 199–216.

Hammes WP, Brandt MJ, Francis KL, Rosenheim J, Seitter MFH, et al. (2005) Microbial ecology of cereal fermentations. Trends Food Sci Technol 16: 4–11.

Hautmann MG, Hipp M, Kölbl O (2011) *Clostridium difficile*-associated diarrhea in radiooncology: an underestimated problem for the feasibility of the radiooncological treatment? Radiat Oncol 6:89.

Hawinkels LJ, Ten Dijke P (2011) Exploring anti-TGF-β therapies in cancer and fibrosis. Growth Factors 29:140-152.

Hernandez-Doria JD, Sperandio V (2013) Nutrient and chemical sensing by intestinal pathogens. Microbes Infect 15: 759–764.

Hertweck C (2009) The biosynthetic logic of polyketide diversity. Angew Chem Int Ed Engl 48: 4688–4716.

Hillman JD, Dzuback AL, Andrews SW (1987) Colonization of the human oral cavity by a *Streptococcus mutans* mutant producing increased bacteriocin. J Dent Res 66: 1092–1094.

Hojati Z, Milne C, Harvey B, Gordon L, Borg M, et al. (2002) Structure, biosynthetic origin, and engineered biosynthesis of calcium-dependent antibiotics from *Streptomyces coelicolor*. Chem Biol 9: 1175–1187.

Holmes E, Kinross J, Gibson GR, Burcelin R, Jia W, et al. (2012) Therapeutic modulation of microbiota-host metabolic interactions. Sci Transl Med 4: 137rv6.

Höltzel A, Gänzle M, Nicholson G, Hammes W, Jung G (2000) The first low molecular weight antibiotic from lactic acid bacteria: reutericyclin, a new tetramic acid. Angew Chem Int Ed Engl 39: 2766–2768.

Horecker BL (1966) The biosynthesis of bacterial polysaccharides. Ann Rev Microbiol 20: 253–290.

Hurdle JG, Heathcott AE, Yang L, Yan B, Lee RE (2011) Reutericyclin and related analogues kill stationary phase *Clostridium difficile* at achievable colonic concentrations. J Antimicrob Chemother 66: 1773–1776.

Huycke MM, Abrams V, Moore DR (2002) *Enterococcus faecalis* produces extracellular superoxide and hydrogen peroxide that damages colonic epithelial cell DNA. Carcinogenesis 23: 529–536.

Ikuno N, Soda H, Watanabe M, Oka M (1995) Irinotecan (CPT-11) and characteristic mucosal changes in the mouse ileum and cecum. J Natl Cancer Inst. 87:1876-1883.

Iwanaga T, Takebe K, Kato I, Karaki S-I, Kuwahara A (2006) Cellular expression of monocarboxylate transporters (MCT) in the digestive tract of the mouse, rat, and humans, with special reference to slc5a8. Biomed Res 27: 243–254.

Iwasa YOH, Nakamaru ÃM, Levin SA (1998) Allelopathy of bacteria in a lattice population : Competition between colicin-sensitive and colicin-producing strains.

Janda JM, Abbott SL (2008) The family *Enterobacteriaceae*. In: Emanuel Goldman, Lorrence H. Green, editors. Practical Handbook of Microbiology, 2nd ed. Boca Raton: CRC Press LLC. pp. 217–229.

Jänsch A, Korakli M, Vogel RF, Gänzle MG (2007) Glutathione reductase from *Lactobacillus sanfranciscensis* DSM20451T: contribution to oxygen tolerance and thiol exchange reactions in wheat sourdoughs. Appl Environ Microbiol 73: 4469–4476.

Jermy A (2010) Symbiosis: Host genes shape the gut microbiota. Nat Rev Microbiol 8:838-839.

Jessup CM, Kassen R, Forde SE, Kerr B, Buckling A, et al. (2004) Big questions, small worlds: microbial model systems in ecology. Trends Ecol Evol 19: 189–197.

Juśkiewicz J, Głazka I, Król B, Zduńczyk Z (2006) Effect of chicory products with different inulin content on rat caecum physiology. J Anim Physiol Anim Nutr 90: 200–207.

Kamada N, Núñez G (2014) Regulation of the immune system by the resident intestinal bacteria. Gastroenterology 146: 1477–1488.

Keating T a, Walsh CT (1999) Initiation, elongation, and termination strategies in polyketide and polypeptide antibiotic biosynthesis. Curr Opin Chem Biol 3: 598–606.

Kerr B, Riley MA, Feldman MW, Bohannan BJM (2002) Local dispersal promotes biodiversity in a real-life game of rock-paper-scissors. Nature 418: 171–174.

Kim S V, Xiang W V, Kwak C, Yang Y, Lin XW, et al. (2013) GPR15-mediated homing controls immune homeostasis in the large intestine mucosa. Science 340: 1456–1459.

Kleerebezem M, Boekhorst J, van Kranenburg R, Molenaar D, Kuipers OP, et al. (2003) Complete genome sequence of *Lactobacillus plantarum* WCFS1. Proc Natl Acad Sci U S A 100: 1990–1995.

Koleva P, Valcheva R, Sun X, Gänzle M, Dieleman L. (2012) Inulin and fructo-oligosaccharides have divergent effects on colitis and commensal microbiota in HLA-B27 transgenic rats. Brit J Nutr 108:1633-1643.

Kolida S, Tuohy K, Gibson GR (2007) Prebiotic effects of inulin and oligofructose. Br J Nutr 87: S193.

Konz D, Doekel S, Marahiel MA (1999) Molecular and biochemical characterization of the protein template controlling biosynthesis of the lipopeptide lichenysin. J Bacteriol 181: 133–140.

Konz D, Klens A, Schörgendorfer K, Marahiel MA (1997) The bacitracin biosynthesis operon of *Bacillus licheniformis* ATCC 10716: molecular characterization of three multi-modular peptide synthetases. Chem Biol 4: 927–937.

Köpke M, Straub M, Dürre P (2013) *Clostridium difficile* is an autotrophic bacterial pathogen. PLoS One 8: e62157.

Kreth J, Merritt J, Shi W, Qi F (2005) Competition and coexistence between *Streptococcus mutans* and *Streptococcus sanguinis* in the dental biofilm. J Bacteriol 187: 7193–7203.

Kumar P, Koppisch AT, Cane DE, Khosla C (2003) Enhancing the modularity of the modular polyketide synthases: transacylation in modular polyketide synthases catalyzed by malonyl-CoA:ACP transacylase. J Am Chem Soc 125: 14307–14312.

Kumbhar C, Watve M (2013) Why antibiotics: A comparative evaluation of different hypotheses for the natural role of antibiotics and an evolutionary synthesis. Nat Sci 05: 26–40.

Kurita A, Kado S, Matsumoto T, Asakawa N, Kaneda N, et al. (2011) Streptomycin alleviates irinotecan-induced delayed-onset diarrhea in rats by a mechanism other than inhibition of β-glucuronidase activity in intestinal lumen. Cancer Chemother Pharmacol 67:201–213.

Lahti L, Salojärvi J, Salonen A, Scheffer M, de Vos WM (2014) Tipping elements in the human intestinal ecosystem. Nat Commun 5: 4344.

Lattanzi A, Minervini F, Di Cagno R, Diviccaro A, Antonielli L, et al. (2013) The lactic acid bacteria and yeast microbiota of eighteen sourdoughs used for the manufacture of traditional Italian sweet leavened baked goods. Int J Food Microbiol 163: 71–79.

Leisner JJ, Haaber J (2012) Intraguild predation provides a selection mechanism for bacterial antagonistic compounds. Proc Biol Sci 279: 4513–4521.

Lemon KP, Armitage GC, Relman D a, Fischbach M a (2012) Microbiota-targeted therapies: an ecological perspective. Sci Transl Med 4: 137rv5.

Letzel A-C, Pidot SJ, Hertweck C (2013) A genomic approach to the cryptic secondary metabolome of the anaerobic world. Nat Prod Rep 30: 392–428.

Ley RE, Lozupone CA, Hamady M, Knight R, Gordon JI (2008) Worlds within worlds: evolution of the vertebrate gut microbiota. Nat Rev Microbiol 6: 776–788.

Li C, Qiu X, Yang N, Gao J, Rong Y, et al. (2013) Common rs7138803 variant of FAIM2 and obesity in Han Chinese. BMC Cardiovasc Disord 13: 56.

Li J, Jensen SE (2008) Nonribosomal biosynthesis of fusaricidins by *Paenibacillus polymyxa* PKB1 involves direct activation of a D-amino acid. Chem Biol 15: 118–127.

Lin TP, Chen CL, Chang LK, Tschen JS, Liu ST (1999) Functional and transcriptional analyses of a fengycin synthetase gene, fenC, from *Bacillus subtilis*. J Bacteriol 181: 5060–5067.

Lin XB, Dieleman LA, Ketabi A, Bibova I, Sawyer MB, et al. (2012) Irinotecan (CPT-11) Chemotherapy alters intestinal microbiota in tumour bearing rats. PloSone 7: e39764.

Lin XB, Gänzle MG (2014) Quantitative high-resolution melting PCR analysis for monitoring of fermentation microbiota in sourdough. Int J Food Microbiol 186C: 42–48.

Lin XB, Gänzle MG (2014) Effect of lineage-specific metabolic traits of *Lactobacillus reuteri* on sourdough microbial ecology. Appl Environ Microbiol 80:5782-9.

Lou L, Qian G, Xie Y, Hang J, Chen H, et al. (2011) Biosynthesis of HSAF, a tetramic acid-containing macrolactam from *Lysobacter enzymogenes*. J Am Chem Soc 133: 643–645.

Louis P, Scott KP, Duncan SH, Flint HJ (2007) Understanding the effects of diet on bacterial metabolism in the large intestine. J Appl Microbiol 102: 1197–1208.

Lu Y-J, White SW, Rock CO (2005) Domain swapping between *Enterococcus faecalis* FabN and FabZ proteins localizes the structural determinants for isomerase activity. J Biol Chem 280: 30342–30348.

Lupp C, Robertson ML, Wickham ME, Sekirov I, Champion OL, et al. (2007) Host-mediated inflammation disrupts the intestinal microbiota and promotes the overgrowth of *Enterobacteriaceae*. Cell Host Microbe 16:119-129.

Lux T, Nuhn M, Hakenbeck R, Reichmann P (2007) Diversity of bacteriocins and activity spectrum in *Streptococcus pneumoniae*. J Bacteriol 189: 7741–7751.

Lv M, Xiaoping X, Cai H, Li D, Wang J, et al. (2011) Cytokines as prognstic tool in breast carcinoma. Front Biosci 17:2515-2526.

Machiels K, Joossens M, Sabino J, De Preter V, Arijs I, et al. (2013) A decrease of the butyrate-producing species *Roseburia hominis* and *Faecalibacterium prausnitzii* defines dysbiosis in patients with ulcerative colitis. Gut: gutjnl–2013–304833–.

Mader E, Ruzicka J, Schmiderer C, Novak J (2011) Quantitative high-resolution melting analysis for detecting adulterations. Anal Biochem 409: 153–155.

Makarova K, Slesarev A, Wolf Y, Sorokin A, Mirkin B, et al. (2006) Comparative genomics of the lactic acid bacteria. Proc Natl Acad Sci U S A 103: 15611–15616.

Malinen E, Rinttilä T, Kajander K, Mättö J, Kassinen A, et al. (2005) Analysis of the fecal microbiota of irritable bowel syndrome patients and healthy controls with real-time PCR. Am J Gastroenterol 100:373-382.

Manhart N, Vierlinger K, Spittler A Bergmeister H, Sautner T, et al. (2001) Oral feeding with glutamine prevents lymphocyte and glutathione depletion of Peyer's patches in endotoxemic mice. Ann Surg 234:92–97.

Markowitz VM, Szeto E, Palaniappan K, Grechkin Y, Chu K, et al. (2008) The integrated microbial genomes (IMG) system in 2007: data content and analysis tool extensions. Nucleic Acids Res 36: D528–33.

Marrakchi H, Choi K-H, Rock CO (2002) A new mechanism for anaerobic unsaturated fatty acid formation in *Streptococcus pneumoniae*. J Biol Chem 277: 44809–44816.

Marteau P, Chaput U (2011) Bacteria as trigger for chronic gastrointestinal disorders. Dig Dis 29:166-171.

Martínez-Beamonte R, Navarro MA, Larraga A, Strunk M, Barranquero C, et al. (2011) Selection of reference genes for gene expression studies in rats. J Biotechnol 151: 325–334.

Martinez-Medina M, Denizot J, Dreux N, Robin F, Billard E, et al. (2014) Western diet induces dysbiosis with increased *E coli* in CEABAC10 mice, alters host barrier function favouring AIEC colonisation. Gut 63: 116–124.

Maruyama F, Kobata M, Kurokawa K, Nishida K, Sakurai A, et al. (2009) Comparative genomic analyses of *Streptococcus mutans* provide insights into chromosomal shuffling and species-specific content. BMC Genomics 10: 358.

Mathieson M, Haikerwal AR (1971) The protein content and amino acid composition of sorghum grain. Cereal Chem 48: 690–699.

May JJ, Wendrich TM, Marahiel MA (2001) The dhb operon of *Bacillus subtilis* encodes the biosynthetic template for the catecholic siderophore 2,3-dihydroxybenzoate-glycine-threonine trimeric ester bacillibactin. J Biol Chem 276: 7209–7217.

McKay LL, Baldwin KA (1990) Applications for biotechnology: present and future improvements in lactic acid bacteria. FEMS Microbiol Rev 7: 3–14.

Meroth CB, Walter J, Hertel C, Brandt MJ, Hammes WP (2003) Monitoring the bacterial population dynamics in sourdough fermentation processes by using PCR-denaturing gradient gel electrophoresis. Appl Environ Microbiol 69: 475–482.

Merritt J, Qi F (2012) The mutacins of *Streptococcus mutans*: regulation and ecology. Mol Oral Microbiol 27: 57–69.

Messens W, De VL (2002) Inhibitory substances produced by lactobacilli isolated from sourdoughs--a review. Int J Food Microbiol 72: 31–43.

Metzler-Zebeli BU, Hooda S, Pieper R, Zijlstra RT, van Kessel AG, et al. (2010) Nonstarch polysaccharides modulate bacterial microbiota, pathways for butyrate production, and abundance of pathogenic *Escherichia coli* in the pig gastrointestinal tract. Appl Environ Microbiol 76: 3692–3701.

Minervini F, De Angelis M, Di Cagno R, Gobbetti M (2014) Ecological parameters influencing microbial diversity and stability of traditional sourdough. Int J Food Microbiol 171: 136–146.

Minervini F, Di Cagno R, Lattanzi A, De Angelis M, Antonielli L, et al. (2012) Lactic acid bacterium and yeast microbiotas of 19 sourdoughs used for traditional/typical italian breads: interactions between ingredients and microbial species diversity. Appl Environ Microbiol 78: 1251–1264.

Minervini F, Lattanzi A, De Angelis M, Di Cagno R, Gobbetti M (2012) Influence of artisan bakery- or laboratory-propagated sourdoughs on the diversity of lactic acid bacterium and yeast microbiotas. Appl Environ Microbiol 78: 5328–5340.

Mirande C, Kadlecikova E, Matulova M, Capek P, Bernalier-Donadille A, et al. (2010) Dietary fibre degradation and fermentation by two xylanolytic bacteria *Bacteroides xylanisolvens* XB1A and *Roseburia intestinalis* XB6B4 from the human intestine. J Appl Microbiol 109: 451–460.

Mitri S, Foster KR (2013) The genotypic view of social interactions in microbial communities. Annu Rev Genet 47: 247–273.

Morgan XC, Tickle TL, Sokol H, Gevers D, Devaney KL, et al. (2012) Dysfunction of the intestinal microbiome in inflammatory bowel disease and treatment. Genome Biol 13: R79.

Morita H, Toh H, Fukuda S, Horikawa H, Oshima K, et al. (2008) Comparative genome analysis of *Lactobacillus reuteri* and *Lactobacillus fermentum* reveal a genomic island for reuterin and cobalamin production. DNA Res 15: 151–161.

Morrison WR (1994) Wheat lipids: structure and functionality. In: Bushuk W, Rasper VF, editors. Wheat. Boston, MA: Springer US. pp. 128–142.

Mutaguchi Y, Ohmori T, Akano H, Doi K, Ohshima T (2013) Distribution of D-amino acids in vinegars and involvement of lactic acid bacteria in the production of D-amino acids. Springerplus 2: 691.

Mutaguchi Y, Ohmori T, Wakamatsu T, Doi K, Ohshima T (2013) Identification, purification, and characterization of a novel amino acid racemase, isoleucine 2-epimerase, from *Lactobacillus* species. J Bacteriol 195: 5207–5215.

Nakao T, Kurita N, Komatsu M, Yoshikawa K, Iwata T, et al. (2012) Irinotecan injures tight junction and causes bacterial translocation in rat. J Surg Res. 173:341-347.

Neubauer H, Glaasker E, Hammes WP, Poolman B, Konings WN (1994) Mechanism of maltose uptake and glucose excretion in *Lactobacillus sanfrancisco*. J Bacteriol 176: 3007–3012.

Neyrinck AM, Possemiers S, Verstraete W, De Backer F, Cani PD, et al. (2012) Dietary modulation of clostridial cluster XIVa gut bacteria (*Roseburia* spp.) by chitin-glucan fiber improves host metabolic alterations induced by high-fat diet in mice. J Nutr Biochem 23: 51–59.

Nout MJR (2009) Rich nutrition from the poorest - cereal fermentations in Africa and Asia. Food Microbiol 26: 685–692.

O'Hara AM, Shanahan F (2006) The gut flora as a forgotten organ. EMBO Rep 7: 688–693.

Oh PL, Benson AK, Peterson D, Patil PB, Moriyama EN, et al. (2010) Diversification of the gut symbiont *Lactobacillus reuteri* as a result of host-driven evolution. ISME J 4: 377–387.

Olsen A, Halm M, Jakobsen M (1995) The antimicrobial activity of lactic acid bacteria from fermented maize (kenkey) and their interactions during fermentation. J Appl Bacteriol 79: 506–512.

Palmer C, Bik EM, DiGiulio DB, Relman DA, Brown PO (2007) Development of the human infant intestinal microbiota. PLoS Biol 5: e177.

Pao SS, Paulsen IT, Saier MH (1998) Major facilitator superfamily. Microbiol Mol Biol Rev 62: 1–34.

Park JT, Strominger JL (1957) Mode of action of penicillin: Biochemical basis for the mechanism of action of penicillin and for its selective toxicity. Science (80-) 125: 99–101.

Perez-Casal J, Price JA, Maguin E, Scott JR (1993) An M protein with a single C repeat prevents phagocytosis of *Streptococcus pyogenes*: use of a temperature-sensitive shuttle vector to deliver homologous sequences to the chromosome of *S. pyogenes*. Mol Microbiol 8: 809–819.

Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. Nucleic acids Res 29: e45.

Picazo JJ (2005) Management of the febrile neutropenic patient. Int J Antimicrob Agents 26S:S120–S122

Png CW, Lindén SK, Gilshenan KS, Zoetendal EG, McSweeney CS, et al. (2010) Mucolytic bacteria with increased prevalence in IBD mucosa augment in vitro utilization of mucin by other bacteria. Am J Gastroenterol 105: 2420–2428.

Prosser JI, Bohannan BJM, Curtis TP, Ellis RJ, Firestone MK, et al. (2007) The role of ecological theory in microbial ecology. Nat Rev Microbiol 5: 384–392.

Ramos JL, Martínez-Bueno M, Molina-Henares AJ, Terán W, Watanabe K, et al. (2005) The TetR family of transcriptional repressors. Microbiol Mol Biol Rev 69: 326–356.

Ranganna K, Mathew OP, Yatsu FM, Yousefipour Z, Hayes BE, et al. (2007) Involvement of glutathione / glutathione S-transferase antioxidant system in butyrate-inhibited vascular smooth muscle cell proliferation. FEBS J 274: 5962–5978.

Reeds PJ and Burrin DG (2001) Glutamine and the bowel. J Nutr 131: 2505S–2508S.

Regmi PR, Metzler-Zebeli BU, Gänzle MG, Kempen TATGV et al. (2011) Starch with high amylose content and low in vitro digestibility increases intestinal nutrient flow and microbial fermentation and selectively promotes bifidobacteria in pigs. J Nutr 141: 1273–1280.

Reiter W-D (2008) Biochemical genetics of nucleotide sugar interconversion reactions. Curr Opin Plant Biol 11: 236–243.

Rensen GJ, Smith WL, Jaravata C V, Osburn B, Cullor JS (2006) Development and evaluation of a real-time FRET probe based multiplex PCR assay for the detection of prohibited meat and bone meal in cattle feed and feed ingredients. Foodborne Pathog Dis 3: 337–346.

Riboulet-Bisson E, Sturme MHJ, Jeffery IB, O'Donnell MM, Neville BA, et al. (2012) Effect of *Lactobacillus salivarius* bacteriocin Abp118 on the mouse and pig intestinal microbiota. PLoS One 7: e31113.

Rickard AH, Gilbert P, High NJ, Kolenbrander PE, Handley PS (2003) Bacterial coaggregation: an integral process in the development of multi-species biofilms. Trends Microbiol 11: 94–100.

Riley MA, Wertz JE (2002) Bacteriocins: evolution, ecology, and application. Annu Rev Microbiol 56: 117–137.

Roberfroid M (2007) Prebiotics: the concept revisited. J Nutr 137: 830S–7S.

Roberfroid M, Gibson GR, Hoyles L, McCartney AL, Rastall R, et al. (2010) Prebiotic effects: metabolic and health benefits. Br J Nutr 104 Suppl: S1–63.

Rollan G, Lorca GL, Font de Valdez G (2003) Arginine catabolism and acid tolerance response in *Lactobacillus reuteri* isolated from sourdough. Food Microbiol 20: 313–319.

Rothenberg ML (2001) Irinotecan (CPT-11): recent developments and future directions–colorectal cancer and beyond. Oncologist 6:66–80.

Round JL, Mazmanian SK (2010) Inducible Foxp3+ regulatory T-cell development by a commensal bacterium of the intestinal microbiota. Proc Natl Acad Sci U S A 107: 12204–12209.

Rouse S, van Sinderen D (2008) Bioprotective potential of lactic acid bacteria in malting and brewing. J Food Prot 71: 1724–1733.

Rowland IR, Mallett AK, Wise A (1985) The effect of diet on the mammalian gut flora and its metabolic activities. Crit Rev Toxicol 16: 31–103.

Safeukui I, Millet P, Boucher S, Melinard L, Fregeville F, et al. (2008) Evaluation of FRET real-time PCR assay for rapid detection and differentiation of *Plasmodium* species in returning travellers and migrants. Malar J 7: 70.

Sánchez-Maldonado AF, Schieber A, Gänzle MG (2011) Structure-function relationships of the antibacterial activity of phenolic acids and their metabolism by lactic acid bacteria. J Appl Microbiol 111: 1176–1184.

Sapone A, Bai JC, Ciacci C, Dolinsek J, Green PHR, et al. (2012) Spectrum of gluten-related disorders: consensus on new nomenclature and classification. BMC Med 10: 13.

Schauwecker F, Pfennig F, Schröder W, Keller U (1998) Molecular cloning of the actinomycin synthetase gene cluster from *Streptomyces chrysomallus* and functional heterologous expression of the gene encoding actinomycin synthetase II. J Bacteriol 180: 2468–2474.

Scheirlinck I, Van der Meulen R, Van Schoor A, Vancanneyt M, De Vuyst L, et al. (2007) Influence of geographical origin and flour type on diversity of lactic acid bacteria in traditional Belgian sourdoughs. Appl Environ Microbiol 73: 6262–6269.

Scheirlinck I, Van der Meulen R, Van Schoor A, Vancanneyt M, De Vuyst L, et al. (2008) Taxonomic structure and stability of the bacterial community in belgian sourdough ecosystems as assessed by culture and population fingerprinting. Appl Environ Microbiol 74: 2414–2423.

Schloerb PR, Skikne BS (1999) Oral and parenteral glutamine in bone marrow transplantation: a randomized, double-blind study. JPEN J Parenter Enteral Nutr 23:117–122.

Schüller S, Phillips AD (2010) Microaerobic conditions enhance type III secretion and adherence of enterohaemorrhagic *Escherichia coli* to polarized human intestinal epithelial cells. Environ Microbiol 12: 2426–2435.

Schütz H, Radler F (1984) Anaerobic reduction of glycerol to propanediol-1.3 by *Lactobacillus brevis* and *Lactobacillus buchneri*. Syst Appl Microbiol 5: 169–178.

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

Schwab C, Tveit AT, Schleper C, Urich T (2014) Gene expression of lactobacilli in murine forestomach biofilms. Microb Biotechnol 7: 347–359.

Scott KP, Martin JC, Campbell G, Mayer C-D, Flint HJ (2006) Whole-genome transcription profiling reveals genes up-regulated by growth on fucose in the human gut bacterium "*Roseburia inulinivorans*". J Bacteriol 188: 4340–4349.

Sekwati-Monang B, Gänzle MG (2011) Microbiological and chemical characterisation of ting, a sorghum-based sourdough product from Botswana. Int J Food Microbiol 150: 115–121.

Sekwati-Monang B, Valcheva R, Gänzle MG (2012) Microbial ecology of sorghum sourdoughs: effect of substrate supply and phenolic compounds on composition of fermentation microbiota. Int J Food Microbiol 159: 240–246.

Shaligram NS, Singhal RS (2010) Surfactin -- A review on biosynthesis, fermentation, purification and applications. Food Technol Biotechnol 48: 119–134.

Shiau SY, Chang GW (1983) Effects of dietary fiber on fecal mucinase and beta-glucuronidase activity in rats. J Nutr 113: 138–144.

Siezen RJ, van Hylckama Vlieg JET (2011) Genomic diversity and versatility of *Lactobacillus plantarum*, a natural metabolic engineer. Microb Cell Fact 10 Suppl 1: S3.

Sims IM, Frese SA, Walter J, Loach D, Wilson M, et al. (2011) Structure and functions of exopolysaccharide produced by gut commensal *Lactobacillus reuteri* 100-23. ISME J 5: 1115–1124.

Singh N, Gurav A, Sivaprakasam S, Brady E, Padia R, et al. (2014) Activation of Gpr109a, receptor for niacin and the commensal metabolite butyrate, suppresses colonic inflammation and carcinogenesis. Immunity 40: 128–139.

Sinh P, Barrett TA, Yun L (2011) *Clostridium difficile* infection and inflammatory bowel disease: A review. Gastroenterol Res Pract. In press.

Siragusa S, Di Cagno R, Ercolini D, Minervini F, Gobbetti M, et al. (2009) Taxonomic structure and monitoring of the dominant population of lactic acid bacteria during wheat flour sourdough type I propagation using *Lactobacillus sanfranciscensis* starters. Appl Environ Microbiol 75: 1099–1109.

Smith PM, Howitt MR, Panikov N, Michaud M, Gallini CA, et al. (2013) The microbial metabolites, short-chain fatty acids, regulate colonic Treg cell homeostasis. Science 341: 569–573.

Sparreboom A, de Jonge MJA, de Bruijn P, Brouwer E, Nooter K, et al. (1998) Irinotecan (CPT-11) metabolism and disposition in cancer patients. Clin Cancer Res 4:2747–2754.

Sriramulu DD, Liang M, Raux-deery E, Parsons JB, Warren MJ, et al. (2008) *Lactobacillus reuteri* DSM 20016 produces cobalamin-dependent diol dehydratase in metabolosomes and metabolizes 1 , 2-propanediol by disproportion. J Bact 190: 4559–4567.

Stevens MJA, Vollenweider S, Meile L, Lacroix C (2011) 1,3-Propanediol dehydrogenases in *Lactobacillus reuteri*: impact on central metabolism and 3-hydroxypropionaldehyde production. Microb Cell Fact 10: 61.

Stolz P, Böcker G, Hammes WP, Vogel RF (1995) Utilization of electron acceptors by lactobacilli isolated from sourdough. Z Lebensm Unters Forsch 201: 91–96.

Strachan DP (1989) Hay fever, hygiene, and household size. BMJ 299: 1259–1260.

Stringer AM, Gibson RJ, Bowen JM, Keefe DMK (2009) Chemotherapy-induced modifications to gastrointestinal microflora: evidence and implications of change. Curr Drug Metabol 10: 79–83.

Stringer AM, Gibson RJ, Logan RM, Bowen JM, Yeoh AS, et al. (2008) Faecal microbiota and beta-glucuronidase expression are altered in an irinotecan–induced diarrhea model in rats. Cancer Biol Ther 7:1919–1925.

Stromeck A, Hu Y, Chen L, Gänzle MG (2011) Proteolysis and bioconversion of cereal proteins to glutamate and γ-Aminobutyrate (GABA) in Rye malt sourdoughs. J Agric Food Chem 59: 1392–1399.

Stuopelytė K, Daniūnaitė K, Laurinavičienė A, Ostapenko V, Jarmalaitė S (2013) High-resolution melting-based quantitative analysis of RASSF1 methylation in breast cancer. Medicina (Kaunas) 49: 78–83.

Su MS, Schlicht S, Gänzle MG (2011) Contribution of glutamate decarboxylase in *Lactobacillus reuteri* to acid resistance and persistence in sourdough fermentation. Microb Cell Fact 10 Suppl 1: S8.

Su MS-W, Oh PL, Walter J, Gänzle MG (2012) Intestinal origin of sourdough *Lactobacillus reuteri* isolates as revealed by phylogenetic, genetic, and physiological analysis. Appl Environ Microbiol 78: 6777–6780.

Svensson L, Sekwati-Monang B, Lutz DL, Schieber A, Gänzle MG (2010) Phenolic acids and flavonoids in nonfermented and fermented red sorghum (*Sorghum bicolor* (L.) Moench). J Agric Food Chem 58: 9214–9220.

Swidsinski A, Loening-Baucke V, Verstraelen H, Osowska S, Doerffel Y (2008) Biostructure of fecal microbiota in healthy subjects and patients with chronic idiopathic diarrhea. Gastroenterol 135:568–579.

Takasuna K, Hagiwara T, Hirohashi M, Kato M, Nomura M, et al. (1998) Inhibition of intestinal microbiota β-glucuronidase modifies the distribution of the active metabolite of the antitumor agent, irinotecan hydrochloride (CPT-11) in rats. Cancer Chemother Pharmacol 42:280–286.

Takasuna K, Hagiwara T, Hirohashi M, Nomura M, Nagai E, et al. (1996) Involvement of β-glucuronidase in intestinal microbiota in the intestinal toxicity of the antitumor

camptothecin derivative irinotecan hydrochloride (CPT-11) in rats. Cancer Res 56:3752–3757.

Takesue Y, Kakehashi M, Ohge H, Uemura K, Imamura Y, et al. (2005) Bacterial translocation: not a clinically relevant phenomenon in colorectal cancer. World J Surg 29: 198–202.

Talarico TL, Axelsson LT, Novotny J, Fiuzat M, Dobrogosz WJ (1990) Utilization of Glycerol as a Hydrogen Acceptor by *Lactobacillus reuteri*: Purification of 1,3-Propanediol:NAD Oxidoreductase. Appl Environ Microbiol 56: 943–948.

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013) MEGA6: Molecular evolutionary genetics analysis version 6.0. Mol Biol Evol 30: 2725–2729.

Tannock GW, Munro K, Harmsen HJM, Welling GW, Smart J, et al. (2000) Analysis of the fecal microbiota of human subjects consuming a probiotic product containing *Lactobacillus rhamnosus* DR20. Appl Environ Microbiol 66:2578–2588.

Tannock GW, Wilson CM, Loach D, Cook GM, Eason J, et al. (2012) Resource partitioning in relation to cohabitation of *Lactobacillus* species in the mouse forestomach. ISME J 6: 927–938.

Teixeira JS, Seeras A, Sanchez-Maldonado AF, Zhang C, Su MS-W, et al. (2014) Glutamine, glutamate, and arginine-based acid resistance in *Lactobacillus reuteri.* Food Microbiol 42: 172–180.

Thibault R, De Coppet P, Daly K, Bourreille A, Cuff M, et al. (2007) Down-regulation of the monocarboxylate transporter 1 is involved in butyrate deficiency during intestinal inflammation. Gastroenterol 133: 1916–1927.

Tieking M, Korakli M, Ehrmann MA, Gänzle MG, Vogel RF (2003) In situ production of exopolysaccharides during Sourdough fermentation by cereal and intestinal isolates of lactic acid bacteria. Appl Environ Microbiol 69: 945–952.

Tjalsma H, Boleij A, Marchesi JR, Dutilh BE (2012) A bacterial driver-passenger model for colorectal cancer: beyond the usual suspects. Nat Rev Microbiol 10: 575–582.

Topping DL, Clifton PM (2001). Short-chain fatty acids and human colonic function: Roles of resistant starch and nonstarch polysaccharides. Physiol Rev 81: 1032-1064.

Tournigand C, André T, Achille E, Lledo G, Flesh M, et al. Mery-Mignard D, Quinaux E, Couteau C, Buyse M, Ganem G, Landi B, Colin P, Louvet C, de Gramont A (2004)

FOLFIRI followed by FOLFOX6 or the reverse sequence in advanced colorectal cancer: A randomized GERCOR study. J Clin Oncol 22: 229 – 237.

Trifan OC, Durham FW, Salazar VS, Horton J, Levine BD et al. (2002) Cyclooxygenase-2 inhibition with celecoxib enhances antitumor efficacy and reduces diarrhea side dffect of CPT-11. Cancer Res 62:5778-5784.

Tuller T (2011) Codon bias, tRNA pools and horizontal gene transfer. Mob Genet Elements 1: 75–77.

Underwood MA (2014) Intestinal dysbiosis: Novel mechanisms by which gut microbes trigger and prevent disease. Prev Med (Baltim) 65C: 133–137.

Vaclavikova R, Ehrlichova M, Hlavata I, Pecha V, Kozevnikovova R, et al. (2012) Detection of frequent ABCB1 polymorphisms by high-resolution melting curve analysis and their effect on breast carcinoma prognosis. Clin Chem Lab Med 50: 1999–2007.

Van den Abbeele P, Gérard P, Rabot S, Bruneau A, El Aidy S, et al. (2011) Arabinoxylans and inulin differentially modulate the mucosal and luminal gut microbiota and mucin-degradation in humanized rats. Environ Microbiol 13: 2667–2680.

van der Logt EMJ, Roelofs HMJ, Nagengast FM, Peters WHM (2003) Induction of rat hepatic and intestinal UDP-glucuronosyltransferases by naturally occurring dietary anticarcinogens. Carcinogenesis 24: 1651–1656.

van Vliet MJ, Harmsen HJ, de Bont ES, Tissing WJ (2010) The role of intestinal microbiota in the development and severity of chemotherapy-induced mucositis. PLoS Pathogens 6:e1000879.

Vermeulen N, Gänzle MG, Vogel RF (2007) Glutamine deamidation by cereal-associated lactic acid bacteria. J Appl Microbiol 103: 1197–1205.

Viaud S, Daillère R, Boneca IG, Lepage P, Langella P, et al. (2014) Gut microbiome and anticancer immune response: really hot Sh*t! Cell Death Differ.

Vinci S, Gelmini S, Mancini I, Malentacchi F, Pazzagli M, et al. (2013) Genetic and epigenetic factors in regulation of microRNA in colorectal cancers. Methods 59: 138–146.

Vogel RF, Knorr R, Müller MRA, Steudel U, Gänzle MG, et al. (1999) Non-dairy lactic fermentations: the cereal world. Antonie Van Leeuwenhoek 76: 403–411.

Vogel RF, Pavlovic M, Ehrmann MA, Wiezer A, Liesegang H, et al. (2011) Genomic analysis reveals *Lactobacillus sanfranciscensis* as stable element in traditional sourdoughs. Microb Cell Fact 10 Suppl 1: S6.

Vogelmann SA, Hertel C (2011) Impact of ecological factors on the stability of microbial associations in sourdough fermentation. Food Microbiol 28: 583–589.

Vogelmann SA, Seitter M, Singer U, Brandt MJ, Hertel C (2009) Adaptability of lactic acid bacteria and yeasts to sourdoughs prepared from cereals, pseudocereals and cassava and use of competitive strains as starters. Int J Food Microbiol 130: 205–212.

Vollenweider S, Evers S, Zurbriggen K, Lacroix C (2010) Unraveling the hydroxypropionaldehyde (HPA) system: an active antimicrobial agent against human pathogens. J Agric Food Chem 58: 10315–10322.

Vos M, Wolf AB, Jennings SJ, Kowalchuk GA (2013) Micro-scale determinants of bacterial diversity in soil. FEMS Microbiol Rev 37: 936–954.

Vossen RHAM, Aten E, Roos A, den Dunnen JT (2009) High-resolution melting analysis (HRMA): more than just sequence variant screening. Hum Mutat 30: 860–866.

Wallace BD, Wang H, Lane KT, Scott JE, Orans J, et al. (2010) Alleviating cancer drug toxicity by inhibiting a bacterial enzyme. Science 330: 831–835.

Walter J, Chagnaud P, Tannock GW, Loach DM, Dal Bello F, et al. (2005) A high-molecular-mass surface protein (Lsp) and methionine sulfoxide reductase B (MsrB) contribute to the ecological performance of *Lactobacillus reuteri* in the murine gut. Appl Environ Microbiol 71: 979–986.

Wang T, Cai G, Qiu Y, Fei N, Zhang M, et al. (2012) Structural segregation of gut microbiota between colorectal cancer patients and healthy volunteers. ISME J. 6:320-329.

Wang T, Cai G, Qiu Y, Fei N, Zhang M, et al. (2012) Structural segregation of gut microbiota between colorectal cancer patients and healthy volunteers. The ISME J 6: 320–329.

Wesener SR, Potharla VY, Cheng Y-Q (2011) Reconstitution of the FK228 biosynthetic pathway reveals cross talk between modular polyketide synthases and fatty acid synthase. Appl Environ Microbiol 77: 1501–1507.

Wiegand PN, Nathwani D, Wilcox MH, Stephens J, Shelbaya A, Haider S (2012). Clinical and economic burden of *Clostridium difficile* infection in Europe: a systematic review of healthcare-facility-acquired infection. J Hosp Infect 81:1-14 .

Wieser H, Koehler P (2008) The biochemical basis of celiac disease. Cereal Chem 85: 1–13.

Willing BP, Russell SL, Finlay BB (2011) Shifting the balance: antibiotic effects on host-microbiota mutualism. Nat Rev Microbiol 9: 233–243.

Wu C, Cichewicz R, Li Y, Liu J, Roe B, et al. (2010) Genomic island TnSmu2 of *Streptococcus mutans* harbors a nonribosomal peptide synthetase-polyketide synthase gene cluster responsible for the biosynthesis of pigments involved in oxygen and H2O2 tolerance. Appl Environ Microbiol 76: 5815–5826.

Wu Q, Wu Z, Qu X, Liu W (2012) Insights into pyrroindomycin biosynthesis reveal a uniform paradigm for tetramate/tetronate formation. J Am Chem Soc 134: 17342–17345.

Wu W-T, Cheng H-C, Chen H-L (2011) Ameliorative effects of konjac glucomannan on human faecal β-glucuronidase activity, secondary bile acid levels and faecal water toxicity towards Caco-2 cells. Brit J Nutr 105: 593–600.

Xue H, Sawyer MB, Field CJ, Dieleman LA, Baracos VE (2007) Nutritional modulation of antitumor efficacy and diarrhea toxicity related to irinotecan chemotherapy in rats bearing the ward colon tumor. Clin Cancer Res 13: 7146–7154.

Xue H, Sawyer MB, Field CJ, Dieleman LA, Murray D, et al. (2008) Bolus oral glutamine protects rats against CPT-11-induced diarrhea and differentially activates cytoprotective mechanisms in host intestine but not tumor. J Nutr 138: 740–746.

Xue H, Sawyer MB, Wischmeyer PE, Baracos VE (2011) Nutrition modulation of gastrointestinal toxicity related to cancer chemotherapy: from preclinical findings to clinical strategy. JPEN J Parenter Enteral Nutr 35:74-90.

Yang XX, Hu ZP, Xu AL, Duan W, Zhu YZ, et al. (2006) A mechanistic study on reduced toxicity of irinotecan by coadministered thalidomide, a tumor necrosis factor-alpha inhibitor. J Pharmacol Exp Ther 319:82–104.

Yatsunenko T, Rey FE, Manary MJ, Trehan I, Dominguez-Bello MG, et al. (2012) Human gut microbiome viewed across age and geography. Nature 486: 222–227.

Zannini E, Jones JM, Renzetti S, Arendt EK (2012) Functional replacements for gluten. Annu Rev Food Sci Technol 3: 227–245.

Zhao L, Brinton RD (2004) Suppression of proinflammatory cytokines interleukin-1beta and tumor necrosis factor-alpha in astrocytes by a V1 vasopressin receptor agonist: a cAMP response element-binding protein-dependent mechanism. J Neurosci 24: 2226–2235.

Zheng J, Gänzle MG, Lin XB, Ruan L,Sun M (n.d.) Diversity and dynamics of bacteriocins from human microbiome. unpublished.

Zimmer J, Lange B, Frick J-S, Sauer H, Zimmermann K, et al. (2012) A vegan or vegetarian diet substantially alters the human colonic faecal microbiota. Eur J Clin Nutr 66: 53–60.

Zimmermann M, Fischbach M a (2010) A family of pyrazinone natural products from a conserved nonribosomal peptide synthetase in *Staphylococcus aureus*. Chem Biol 17: 925–930.

Zwielehner J, Lassl C, Hippe B, Pointner A, Switzeny OJ, et al. (2011) Changes in human fecal microbiota due to chemotherapy analyzed by TaqMan-PCR, 454 sequencing and PCR-DGGE fingerprinting. PloSone 6: e28654.

Zwielehner JC (2010) Molecular analysis of intestinal microbiota composition and function in individuals with different diets, age and disease status. Dissertation, Universität Wien, Fakultät für Lebenswissenschaften.

Zwietering MH, Jongenburger I, Rombouts FM, van 't Riet K (1990) Modeling of the bacterial growth curve. Appl Environ Microbiol 56: 1875–1881.