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Clinical and Mechanistic Insights into Novel Probiotic Functions and Formulations

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Graduate Program in Microbiology and Immunology
A thesis submitted in partial fulfillment of the requirements for the degree in Doctor of Philosophy
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CLINICAL AND MECHANISTIC INSIGHTS INTO NOVEL PROBIOTIC FUNCTIONS
AND FORMULATIONS

(Thesis format: Integrated Article)

by

Jordan Eduard Bisanz

Graduate Program in Microbiology and Immunology

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

The School of Graduate and Postdoctoral Studies
The University of Western Ontario
London, Ontario, Canada

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Abstract

Using a combination of hypothesis and discovery based approaches, the goal of this thesis was to better describe novel probiotic functions and their mechanisms while striving to better understand the effect of formulation on *Bifidobacterium animalis subsp. lactis*, *Lactobacillus paracasei* and *L. rhamnosus*.

Using RNA-Seq, a bacterial metatranscriptome analysis of a commonly consumed probiotic yogurt showed that the organisms adapted to storage time and flavor additions. This led to the discovery that in addition to the probiotic health benefits, members of the *L. casei* group (*L. rhamnosus* and *L. paracasei*) produce volatile sulfur compounds mediated by a novel sulfur/taurine metabolism gene cluster that affect taste and texture. The benefits of selected probiotic strains were tested in a further series of human studies.

A systems biology approach was developed and a double-blind, placebo-controlled clinical trial of post-menopausal women showed that vaginally administered probiotics could influence the microbiota and host responses. Changes in the vaginal microbiota were noted in late pregnancy in a rural Tanzanian population, and maternal intake of Moringa supplemented *L. rhamnosus* GR-1 yogurt appeared to improve the gut microbiota profile of the newborn babies. Having discovered that *L. rhamnosus* GR-1, and selected other lactobacilli, could sequester heavy metals *in vitro*, a randomized open-label pilot study was performed and showed a reduction in toxic metal uptake in Tanzanian pregnant women and school children. The latter series of findings led to the discovery, development and characterization of a new strain, *L. rhamnosus* Lr60, with high potential to reduce toxic metal accumulation in the host. Using a mouse model, strains of *L. rhamnosus* were tested to better understand mechanisms of protection against mercury as well as to examine potential modulation of host xenobiotic metabolism by probiotics. Data suggest it is possible to sequester mercury and prevent it from entering the bloodstream.

Collectively, these studies have increased our knowledge of probiotic mechanisms as well as lead to the development of novel applications of relevance to human health.

Keywords

Probiotics, Lactobacillus, microbiome, maternal and children's health, environmental toxicology, mercury, toxic metals, 16S rRNA sequencing, RNA sequencing

Co-Authorship Statement

The experiments and data analyses within this thesis were predominantly carried out by Jordan Bisanz with supervision from Gregor Reid. The manuscripts presented within were primarily written by Jordan Bisanz. Exceptions are listed below.

Chapter 2: Bacterial metatranscriptome analysis of a probiotic yogurt using an RNA-Seq approach

Jordan Bisanz and Gregor Reid conceived the experiment. Jordan Bisanz extracted and enriched RNA. Jean Macklaim and Gregory Gloor contributed code and had input into sample analysis. Jordan Bisanz performed analysis of data.

Chapter 3: Comparative genomics demonstrates production of volatile sulfur compounds in *Lactobacillus paracasei* and *Lactobacillus rhamnosus* is mediated by a novel sulfur/taurine metabolism gene cluster.

Jordan Bisanz and Gregor Reid conceived the experiments. Michiel Wels carried out clustering of orthologous proteins. Jordan Bisanz carried out assays and performed computational analysis. Jordan Bisanz, Johan van Hylckama Vlieg, Tamara Smokvina and Gregor Reid interpreted results.

Chapter 4: A systems biology approach investigating the effect of probiotics on the vaginal microbiome and host responses in a double-blind, placebo-controlled clinical trial of post-menopausal women.

Gregor Reid, Jeremy Burton, Barbara Dvoracek, David Koenig and Rebecca Vongsa conceived and designed study with input from Jordan Bisanz, Shannon Seney, Amy McMillan and Gregory Gloor. Shannon Seney, Brenda Ford and Dorli Herman were responsible for sample collection. Jordan Bisanz and Amy McMillan were responsible for DNA extraction, amplification, sequencing, and microbiota analysis with input from Gregory Gloor. Shannon Seney performed cytokine multiplex assays and Jordan Bisanz analyzed the results. Jordan Bisanz extracted RNA and analyzed microarray results. LungFai Wong performed statistical analysis of the primary study outcome. Amy McMillan and Mark Sumarah carried out untargeted metabolome studies. Jordan Bisanz primarily prepared the

manuscript with assistance from Amy McMillan who prepared the portions pertaining to metabolome analysis and associated methods.

Chapter 5: The microbiota at multiple body sites during pregnancy in a rural Tanzanian population and the effects of Moringa supplemented probiotic yogurt.

Gregor Reid, Gregory Gloor and Jeremy Burton conceived the study. Jordan Bisanz, Megan Enos, George PrayGod and Shannon Seney were responsible for sample collection. Megan Enos and Jordan Bisanz carried out DNA extraction. Dana Willner and Rob Knight were responsible for initial amplification and sequencing of samples. Megan Enos and Dana Willner carried out sequence de-multiplexing and OTU clustering. Jordan Bisanz performed all further microbiota analysis. Megan Enos and Stephanie Chilton carried out nutritional analysis. Megan Enos, Christoph Fusch and Gerhard Fusch analyzed breast milk composition. Jordan Bisanz primarily prepared the manuscript. Jordan Bisanz and Megan Enos are equal contributors to this manuscript.

Chapter 6: Randomized open-label pilot study of the influence of probiotics and the gut microbiome on toxic metal levels in Tanzanian pregnant women and school children

Jordan Bisanz, Jeremy Burton and Gregor Reid conceived and designed study. Jordan Bisanz, Megan Enos, Joseph Mwanga and John Chagalucha were responsible for sample collection. Jordan Bisanz carried out DNA extractions, amplifications and sequence analysis. Gregory Gloor assisted in data analysis. Metal quantifications were carried out at the trace elements laboratory as part of the London Health Sciences Laboratory Services group.

Chapter 7: Mercury-resistant lactobacilli to remediate toxic metals *in vitro* and *in vivo*.

Jordan Bisanz, Gregor Reid, Johan van Hylckama Vlieg, Tamara Smokvina and Benoit Foligne conceived the experiments. Jordan Bisanz carried out the *in vitro* experiments and all experiments with *Lactobacillus rhamnosus* Lr60. Jordan Bisanz, Mark Trinder, Colleen Ple and Benoit Foligne carried out the animal experiments. Jordan Bisanz and Mark Trinder carried out RNA extraction and qPCR from mouse tissues. Jordan Bisanz prepared samples for, and analyzed, microarrays. Jordan Bisanz and Benoit Foligne carried out the histology.

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Chapter 1

1 General Introduction

Microbes inhabited the earth long before humans and will remain long after we are gone. They were our earliest ancestors and according to the endosymbiont theory, their descendants live within our own cells in the form of mitochondria (Thrash *et al.*, 2011). Microbes have shaped our evolution both genetically and culturally (Diamond, 2000). We are born coated in microbes, we live immersed in microbes and eventually we die only to be recycled by microbes. In short, microbes are an intrinsic part of what it means to be human.

Traditionally, science has focused on the detrimental effects of microbes to human populations such as the fall of the Incan Empire due to smallpox (Diamond, 2000), or the emergence of new diseases like sickle cell anemia (Aidoo *et al.*, 2002) and cystic fibrosis (Gabriel *et al.*, 1994) as a result of microbial selective pressure. Beginning in the early 1900s with the fringe hypotheses of Nobel Laureate Élie Metchnikoff, the evidence for the beneficial effects of microbes has been slowly building (Mackowiak, 2013). The study of the body's microbial communities is now one of the fastest growing areas of science, with numerous implications for both human and animal health and disease.

1.1 The human microbiome

The human microbiome is the sum of all the microbial communities (the microbiota), their genes, transcripts and metabolic byproducts both on, and within the human host. It has been estimated that, by absolute count, microbes outnumber their human host's cells by a 10-to-1 ratio (Savage, 1977). Despite being ubiquitous in the literature, I would suggest this figure is misleading for many reasons, including the size difference between a prokaryotic and eukaryotic cell, and the varied sizes of humans. What is far more tangible is that on the genomic level, our microbes encode ~150-fold more unique genes than our own DNA (Qin *et al.*, 2010). Many of these genes include novel metabolic pathways which can have profound impacts on human physiology such as: recycling sex hormones including estrogen (DeRossi and Hersh, 1994; Flores *et al.*, 2012; Plottel and

Blaser, 2011), synthesizing nutrients for the host including folate and vitamin B₁₂ (LeBlanc *et al.*, 2013), and fermenting compounds with immunomodulatory activity important in disease such as short chain fatty acids in inflammatory bowel disease (Veiga *et al.*, 2010).

While much focus is on the gastrointestinal tract where cell densities can be up to 10¹¹ CFU/mL, the microbiotas of other body sites are also of great interest including the skin, oral, respiratory tract and urogenital tract communities (Costello *et al.*, 2009).

1.2 Microbiota dysbiosis

As opposed to traditional microbiology which has focused on single organisms fulfilling Koch's postulates of disease (Ferreira *et al.*, 2011), microbial dysbiosis involves altered states of whole communities which may be associated with aberrant conditions including those that cause disease.

Microbial dysbiosis is perhaps best typified in the human vagina. In the majority of healthy Caucasian women, the vaginal microbiota is dominated by *Lactobacillus iners* and/or *L. crispatus* with other species such as *L. gasseri* and *L. jensenii* occasionally found (Ravel *et al.*, 2011). In the dysbiotic state of bacterial vaginosis (BV), the relative proportions of *Lactobacillus spp.* are decreased coinciding with an increase in anaerobes invariably including *Gardnerella vaginalis*, with *Prevotella spp.*, *Atopobium vaginae* and others (Fredricks *et al.*, 2005; Hummelen *et al.*, 2010). BV is the most common vaginal "infection", with some studies showing one third of reproductive age females presenting with BV at any given time (Koumans *et al.*, 2007). The symptoms and signs of BV include vaginal odor, a milk-like discharge, and irritation with the latter commonly leading to improper self-misdiagnosis as a yeast infection.

While it is generally accepted that a *Lactobacillus*-dominated microbiota is favorable in the vagina, what defines dysbiosis in the gastrointestinal tract is far more varied. Examples of the influence of the gut microbiota on health include: obesity (Le Chatelier *et al.*, 2013; Turnbaugh *et al.*, 2006), diabetes (Giongo *et al.*, 2010), Crohn's Disease (Gevers *et al.*, 2014) and colon cancer (Devkota and Turnbaugh, 2013; Feng *et al.*, 2015).

In obesity, many researchers have focused on the crude metric of the ratio of *Firmicutes:Bacteroidetes* (Ley *et al.*, 2005; Mariat *et al.*, 2009) suggesting that a higher ratio is associated with adiposity, insulin insensitivity, dyslipidemia and chronic inflammation. More in-depth studies of individual taxa and gene-level differences have examined bacterial richness and gene content (Le Chatelier *et al.*, 2013). What is more relevant is do certain microbiota put a person at higher risk of obesity, and once obese, can alteration of the microbiota help a person lose weight?

Since there is no definition of a ‘healthy’ gut microbiome, it is difficult to define normality or use the microbiota as a biomarker for an unhealthy state. Gut enterotypes were proposed as a way of defining groups (Arumugam *et al.*, 2011) with three predominant microbiota types named by their dominant taxa: *Bacteroides* (enterotype 1), *Prevotella* (enterotype 2), and *Ruminococcus* (enterotype 3). Studies have suggested that diet may be a large driver of the enterotype (De Filippo *et al.*, 2010; Wu *et al.*, 2011). While it is appealing to have such a ternary classification system, it is a contentious notion (Jeffery *et al.*, 2012) and it is more likely there is a gradient of normality. It is also not unreasonable to think that there is a balance in microbiota composition wherein one configuration may be beneficial for some conditions but deleterious for others. Furthermore, the gut microbiota is not static and changes over life (Claesson *et al.*, 2011; Yatsunenکو *et al.*, 2012).

1.3 Probiotics

As our understanding of the role of the microbiota in health builds, the opportunities increase to translate this knowledge by modulating the microbiota for health. While antibiotics are capable of producing an immediate shift in microbiota composition (Dethlefsen *et al.*, 2008), other less drastic approaches include probiotics, prebiotics and synbiotics (a combination pro/prebiotic). The recently refined FAO/WHO definition of probiotics is “live microorganisms that, when administered in adequate amounts, confer a health benefit on the host” (Hill *et al.*, 2014). A prebiotic is a “non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria” (Manning and Gibson, 2004). The remainder of this work will focus on studies of probiotics.

Traditionally, the genera of *Lactobacillus* and *Bifidobacterium* have been the focus of probiotic study due to their historical associations with fermented foods and health (Farnworth, 2008; Naidu *et al.*, 1999; Yang *et al.*, 2014). Nonetheless, a number of other species being applied as human probiotics include *Bacillus coagulans* (Fitzpatrick *et al.*, 2011), *Saccharomyces boulardii* (Czerucka *et al.*, 2007), *Escherichia coli* (Schultz, 2008), and potentially *Faecalibacterium prausnitzii* (Sokol *et al.*, 2008). Additional species contained within a community structure analogous to an artificial stool transplant are also being tested, such as against antibiotic-associated *Clostridium difficile* diarrhea (Petrof *et al.*, 2013).

Much of the current thesis focuses on a group of 80 prototypical probiotic strains in the *L. casei* group. This group of phylogenetically similar, facultatively heterofermentative lactobacilli consists of *L. paracasei*, *L. rhamnosus*, *L. casei*, and *L. zaeae*. The differentiation and nomenclature of the *L. casei* group is a matter of debate and is far from fully established (Dellaglio *et al.*, 2002; Dicks *et al.*, 1996; Felis & Dellaglio, 2007; Sato *et al.*, 2012). This work focuses exclusively on members of the *L. rhamnosus* and *L. paracasei* lineages. Though *L. casei* and *L. paracasei* are used interchangeably in the literature often referring to the same strain, *L. paracasei* is the currently accepted nomenclature and will be used herein to refer to a set of the 40 strains of *L. paracasei* studied in this study (Klein, 2009). Further confusions relating to the delineation of this group led to the initial recategorization of *L. rhamnosus* GR-1 used in many of the early studies as *L. casei* GR-1 (Reid *et al.*, 1987). Despite a high degree of phylogenetic similarity among *L. casei* and *L. rhamnosus* strains, they can vary significantly in gene content and probiotic traits (Douillard *et al.*, 2013).

The intent of using this collection of strains was to uncover novel strain variable traits, and using the available genome sequences, to search for the genetic mediators of probiotic function.

1.4 The host-microbiota-probiotic interactome

How probiotic strains function is a million dollar question with no simple answer. There are probably a multitude of mechanisms working synergistically in a context specific

manor. For probiotic efficacy, the mechanisms likely involve the interactions of: (i) probiotic strain, (ii) host and, (iii) indigenous microbes which we refer to as the host-microbiota-probiotic interactome (Bisanz & Reid, 2011). Traditional understanding of probiotics has focused on only host and probiotic, or probiotic and indigenous microbes. Classical examples of probiotic action include production of hydrogen peroxide and/or bacteriocins to combat indigenous pathogens (Reid *et al.*, 2011) or direct interactions of probiotics and immune cells influencing inflammatory responses (Martins *et al.*, 2011). While science often takes a reductionist approach isolating individual phenomena, I suspect this results in many missed opportunities to better understand how probiotics work. Indeed it is becoming increasingly obvious that beneficial microbes often act through the microbiota as an intermediate. Recent studies have indicated that probiotic consumption does not significantly alter the microbiota composition at the community level; however, the effects can be seen at the transcriptional and metabolomics levels through interactions with the activity of indigenous microbes (Eloe-Fadrosh *et al.*, 2015; McNulty *et al.*, 2011).

The concept of probiotic-mediated detoxication/detoxification of environmental toxins presented later in this chapter is an example where a reductionist approach is fundamentally flawed, as it is highly likely there are multiple mechanisms acting in concert. We and others have known that strains of lactic acid bacteria are capable of interactions with a variety of environmental toxins (Halttunen *et al.*, 2008; Halttunen *et al.*, 2007; Peltonen *et al.*, 2001) and some of these have shown efficacy *in vivo* (Hernandez-Mendoza *et al.*, 2011; Zhai *et al.*, 2013). Despite predominantly surface binding mechanisms shown *in vitro*, it is also clear that intestinal microbes have impacts on host function at distal sites including xenobiotic metabolism (including pathways relevant to metals) in the kidney and liver (Björkholm *et al.*, 2009; Rawls *et al.*, 2004). Furthermore, experiments contrasting conventional and germ free animals have shown the importance of the microbiota in protection against mercury (Nakamura *et al.*, 1977) lead and cadmium (Breton *et al.*, 2013), raising the potential that modification of microbiota structure or activity could affect metal uptake. In this case, by understanding the probiotic-microbiota-host interactome, we will be able to capture the full spectra of

potential mechanisms involved in probiotic-mediated detoxification. To aid in such studies, cutting edge tools have become available for sequencing and data analysis.

1.5 Tools for studying the microbial communities

In the modern era of high-throughput sequencing technologies such as Illumina, SOLiD, and Ion Torrent sequencing platforms, there are numerous approaches available for studies of the human microbiota and microbiome depending on the researcher's question.

Currently, most studies of the microbiota rely on targeted amplicon sequencing of a particular gene or marker. The 16S rRNA gene is by far the most commonly applied target, and it was applied in three of the chapters in this thesis. While alternative markers such as Cpn60/GroEL can also be used, they are less common and lack the same level of pre-existing tools (Hill *et al.*, 2004; Schellenberg *et al.*, 2011). The 16S rRNA gene has been used since the 1980s (Stahl *et al.* 1984) and thus has large databases available for comparison. Though many early studies of the microbiota relied on the creation of 16S rRNA gene clone libraries followed by Sanger sequencing of individual inserts, the era of massively parallel sequencing platforms ushered a new era in microbiota analysis.

Formerly it was necessary to ligate amplicons into plasmids, transform into *E. coli* and then pick individual colonies for sequencing (Fredricks *et al.*, 2005). Alternatively, denaturing gradient gel electrophoresis (DGGE) was used (Burton and Reid, 2002) to separate amplicons of individual organisms followed by subsequent reamplification and sequencing. This approach gives limited sensitivity (Gloor *et al.*, 2010) and is tedious and time consuming requiring significant operator skill. By comparison, the modern approach requires nothing more than a simple PCR carried out with carefully designed primers (Gloor *et al.*, 2010). By introducing multiplexing DNA barcodes during PCR, hundreds of samples can be analyzed in a single run giving magnitudes more information at a fraction of the price.

One major limitation of the high-throughput sequencing for 16S rRNA gene analysis is the read length. While a typical Sanger sequencer, such as the Applied Biosystems 3730XL, can produce read lengths up to 1000 bp allowing the entire 16S rRNA gene to be determined in paired-end sequencing, high-throughput sequencers are significantly

limited. Due to this, it is necessary to carefully pick the region of the 16S rRNA gene to be sequenced and there is not necessarily a single best region (Soergel *et al.*, 2012). Much discussion on these regions focuses on the concept of variable regions, which as the term implies are found to be highly variable among even closely related species. The choice of which region to target depends highly on the community being sampled. Primers targeting the 6th variable region (V6), for example, are capable of differentiating many species of *Lactobacillus*, which is beneficial if studying the vaginal microbiome or fermented food products, but this has limited ability to differentiate the Gammaproteobacteria in the gut. Primers targeting the V4 region, which are applied in the Earth Microbiome Project (Earth Microbiome Project, 2012), are capable of improved differentiation of the Gammaproteobacteria, but lack the ability to discriminate many lactobacilli such as *L. crispatus*, *L. rhamnosus*, and *L. helveticus*. Other factors may influence the choice of primer including the availability of databases and classifiers (Wang *et al.*, 2007).

Depending on the sequencing modality, it is also possible to cover multiple variable regions in a single read if the given read is long enough to span the conjoining conserved regions. For this reason, the Roche 454 pyrosequencer was the favored instrument for many years for microbiota analysis due to its ability to provide read lengths of >500 bp at the cost of lower read output (Caporaso *et al.*, 2010; Finegold *et al.*, 2010; Turnbaugh *et al.*, 2009; Yatsunenko *et al.*, 2012). The first study to use Illumina sequencing to capture the entire V6 region with paired end sequencing covering 76 bases was performed on vaginal samples (Hummelen *et al.*, 2010). The newest incarnations of the Illumina HiSeq and MiSeq are capable of generating >600 bp reads and have now supplanted the 454 sequencer as the method of choice for most microbiota studies (Di Bella *et al.*, 2014).

The computational analysis of microbiota datasets is time-consuming, complex and with no one correct method. QIIME, Quantitative Insights into Microbial Ecology (Caporaso *et al.*, 2010), is one of the most commonly applied packages of tools despite having originally being designed for 454 pyrosequencing datasets. This tool allows relatively simple analysis of alpha and beta diversity. In microbial ecology alpha diversity refers to the within-sample diversity (such as number of species, or more complex metrics such as

Shannon's Diversity Index (Haegeman *et al.*, 2013)); while beta diversity refers to between-sample diversity. Weighted UniFrac (Lozupone and Knight, 2005) is a commonly applied technique which considers both the phylogenetic relationship of the organisms, as based on a user-generated tree, and also their abundances to provide a measure of similarity.

A common issue that arises in the analysis is that the microbiota data are compositional in nature. The resulting reads, despite being discrete count data, are but random samplings of the sequencing library and are heavily affected by technical non-biological factors. Thus, without extra lines of evidence such as qPCR, it is difficult to conclude that the absolute abundance of organisms between two samples is different. Rather, it is determined that their proportional abundance between communities is different. Furthermore, compositional data creates a number of challenges statistically. A great example of this lies in the attempts to carry out correlation analysis using compositional data. Karl Pearson, creator of the Pearson correlation coefficient, coined the term spurious correlation in 1896 to describe the false conclusions drawn as a result of the interdependence of observations inherent to compositional data (Pearson, 1896). Various approaches to overcome this problem include the log-ratio approach (Aitchison and Egozcue, 2005), utilized in the tool ALDEx (Fernandes *et al.*, 2013; Fernandes *et al.*, 2014) used for comparisons of individual community constituents in the following chapters. The literature is ripe with examples of ultra-low abundance organisms associated with disease in cross-sectional studies that are more likely to be due to sampling variation rather than biological reality, even in high impact papers (Hsiao *et al.*, 2013). The ALDEx tool models technical error based on a user-defined number of samplings from the Dirichlet distribution, as an additional tool to increase the confidence in results.

RNA-Seq, gene expression based on digital read counts from high-throughput sequencing, is quickly becoming the tool of choice for transcriptome studies. When the mixed pool of RNA belonging to a bacterial community is studied, it is referred to as the metatranscriptome. While approaches such as 16S rRNA gene sequencing determine the constituents of a community, metatranscriptome studies answer the question of what the

microbes are actually doing. It is more powerful than looking at just the community composition or their metagenomes, however it is limited in its broad range use due to sample preparation, sequencing cost and computationally intensive analysis. Given that >95% of total RNA is not coding but rather only the rRNA (Rosenow *et al.*, 2001), the bulk of the RNA must be removed prior to sequencing otherwise the vast majority of resultant data will be uninformative for gene expression. This can be accomplished through the use of magnetic beads carrying oligonucleotide capture probes for conserved rRNA regions such as that of MICROBExpress (Life Technologies). Additionally, in bacterial communities associated with a eukaryotic host, a significant portion of transcripts may be of host origin, therefore requiring further sample enrichment by depleting 3' poly-A tail mRNAs and host rRNAs.

While 16S rRNA gene sequencing and RNA-Seq answer the questions of what taxa are present, and how they transcriptionally/functionally react to stimuli, metabolomics identify a set of small molecules in a sampled environment. These molecules are the combination of microbial and host metabolism and represent the end by-products of the community. A limitation is that unlike sequence-based methods, it is often impossible to tell the origin of the molecule (microbe or host). Furthermore, as the methods have limited ability to identify all molecules given the dependence on spectra databases for identification. Despite these limitations, unlike RNA-Seq and shotgun metagenomics that infer functions, metabolomics is truly identifying end effector molecules.

These tools require biological questions and studies described herein sought to apply them to understanding probiotic function.

1.6 The microbiota in maternal and child health

Successful societies share a common trait: empowerment and care of women (Kar *et al.*, 1999). The developmental origins of health and disease would indicate that the key to a long healthy life begins before birth and even before conception (Fowden *et al.*, 2006; Myatt, 2006). The health of mothers, neonates and children is clearly crucial. Despite this, in many parts of the world, and even within Canada, many women do not receive the education and medical care to maximize health. Recognizing this, Canada launched the

Muskoka Initiative focusing on improving health of women and children, resulting in an investment of \$ 2.8 billion dollars into health programs and research between 2010 and 2015.

David Barker's seminal work in the United Kingdom showed that nutrition during fetal development impacted health of the baby into late life through increased risk of cardiovascular disease and metabolic syndrome (Barker *et al.*, 1993). But, the influence of diet on the composition and function of the gut microbiota was not considered until recently. Excess food intake and poor nutrient content has been the focus of microbiota studies in relation to obesity (Le Chatelier *et al.*, 2013; Turnbaugh *et al.*, 2006), due to its growing prevalence in developed countries. Logic dictates that if the gut microbiota can facilitate increased nutrient absorption resulting in obesity, it must also be able to decrease it. Furthermore, dysbiosis in the vagina (BV) certainly affects the fetus, increasing the risk of premature delivery (Mysorekar and Cao, 2014). Early neonatal sepsis, infection by group B streptococci and risk of necrotizing enterocolitis show the impact of an aberrant microbiota on infant health (Eickhoff *et al.*, 1964; Wang *et al.*, 2009). The ability of probiotics, particularly *L. rhamnosus* GR-1 and *L. reuteri* RC-14 to counter BV and restore vaginal health (Reid, 2014) as well as improve infant immune status (Isolauri *et al.*, 2001) and resolution of rotavirus diarrhea (Grandy *et al.*, 2010), illustrates benefits of microbial species in maternal and child health. Of additional interest to maternal and children's health is the issue of exposure to environmental pollutants and toxins.

1.7 Environmental toxin exposure and relevance to human health

As opposed to the definition of toxin in a microbiological context, environmental toxins are a broad group of synthetic and naturally occurring compounds which pose threats to human health in both chronic and acute doses (Grigg, 2004). The focus of much of this thesis lies on toxic metals, namely mercury, arsenic, cadmium, and lead. Toxic metals occur naturally in the environment, although anthropomorphic activity has served to increase their levels greatly since the industrial revolution (Lamborg *et al.*, 2014; Schwikowski *et al.*, 2004). While we may falsely assume the matters of toxic metal

exposure are limited to the developing world (Hossain, 2006) the reality is that it is a clear and present problem all over the world. In southwestern Ontario, a recent study conducted at the University of Western Ontario identified that 15% of women of reproductive age in Toronto have levels of mercury surpassing the minimum limit for adverse effects on cognitive development of their offspring (Schoeman *et al.*, 2010). The massive pollution from industry around Sarnia and Windsor would suggest that levels could be even higher west of Toronto. The situation is worrisome in all parts of the developed world, with an estimated 2 million children in the European Union born with unacceptably high levels of mercury estimated to result in a loss of 9 billion euros (12.5 billion CAD) to the EU economy (Bellanger *et al.*, 2013).

Mercury is one of the most toxic substances known to man. It is all around us and can be found in our food (Xue, 2007), drinking water (Mohapatra *et al.*, 2012) and air (Tian *et al.*, 2011). The diet is the most significant source of mercury in the body (Hrubá *et al.*, 2012; Koren and Bend, 2010). Mercury levels are low in natural water reservoirs but the metal is readily absorbed by algae and bioaccumulates in larger fish (Morel and Kraepiel, 1998) resulting in tissue concentrations of up to 1 part per million (ppm) (FDA, 2010). It is for this reason that public health agencies such as the FDA and EPA advise limiting consumption of fish for children and pregnant women as it can affect development (EPA, 2004; FDA, 2010). While high-level mercury poisoning results in acute neurological damage, chronic low-level exposure is associated with delayed neurological development in children, induction of preterm labor, as well as cardiovascular and autoimmune diseases (Karagas *et al.*, 2012). Limiting fish intake is not always easy, as it is one of the most plentiful and nutritious food sources on earth and is the primary source of dietary protein for many societies (Tidwell, 2001).

1.8 Toxicology and human metabolism of mercury

Mercury exists in three forms in nature: elemental (Hg^0), inorganic (Hg^{2+} , Hg^{1+}) and the organic form, predominantly as methylmercury (MeHg) (Morel & Kraepiel, 1998). The World Health Organization reports that people in North America and Europe consume daily 4.3 μg and 2.4 μg of inorganic and organic mercury respectively (ICPS, 1991). A significant source of MeHg occurs through conversion of inorganic mercury by microbial

processes including those performed by sulfate-reducing bacteria *Desulfovibrio* through a mechanism that has only recently been understood (Parks *et al.*, 2013). Inorganic mercury is not easily absorbed in the gastrointestinal tract, however MeHg and salts of Hg^{2+} are, and may enter systemic circulation. Once in the body, mercury has a half life of 40 and/or 70 days (Hg^{2+} and MeHg respectively) (Clarkson *et al.*, 2003). The metal is highly mobile forming complexes with thiols such as cysteine and being co-transported through the body (Clarkson *et al.*, 2007). In the case of MeHg, it is easily able to cross the blood-brain barrier which is why it is associated with cognitive development issues in children.

1.9 Interactions of microbes and metals

Because these toxic metals are elements, they can not truly be broken down or destroyed, rather they can only be transferred to less toxic forms, sequestered away from cellular processes to, or effluxed from, the cell (Bruins *et al.*, 2000). Given that many of the metals have unique properties, the mechanisms of resistance often differ. Resistance to some metals (lead and cadmium) is predominantly mediated by efflux transporters (Naik and Dubey, 2013). Cadmium resistance is often mediated by the *cadCA* operon wherein *cadA* is an ATP dependent cadmium efflux protein and *cadC* is a transcriptional repressor (Bruins *et al.*, 2000). Resistance to arsenic similarly involves the *ars* operon frequently containing *arsB* (encoding an efflux transporter), *arsC* (encoding arsenate reductase) and *arsR* (a transcriptional regulator)(Bruins *et al.*, 2000). The ArsC enzyme is particularly interesting as it converts arsenate to arsenite, a more toxic form of the metal, but one that is more easily transferred out of the cell (Rahman & Hassler, 2013).

In lactobacilli, toxic metals are often imported by nutrients acquisition systems, for example cadmium is imported through manganese transporters (Archibald and Duong, 1985). It is thought that cadmium sensitive organisms accumulate 3-15 more cadmium than resistant counterparts (Trevors *et al.*, 1986).

Mercury is unique among the toxic heavy metals in that mechanisms of resistance involve a true detoxification. Classical bacterial resistance to mercury involves the subsequent demethylation of organic mercury to inorganic mercury (organomercurial

lyase MerB), followed by the reduction of inorganic mercury to elemental mercury (mercuric reductase MerA) (Figure 1-1). While organic mercury can diffuse into the cell due to its lipophilic nature, inorganic mercury (Hg^{2+}) may be actively imported by the MerT transporter though this is variable among species (Bruins *et al.*, 2000). The operon is regulated by MerR which is a *trans*-acting repressor/activator protein which acts as a repressor in the absence of mercury, but an activator its presence (Condee and Summers, 1992).

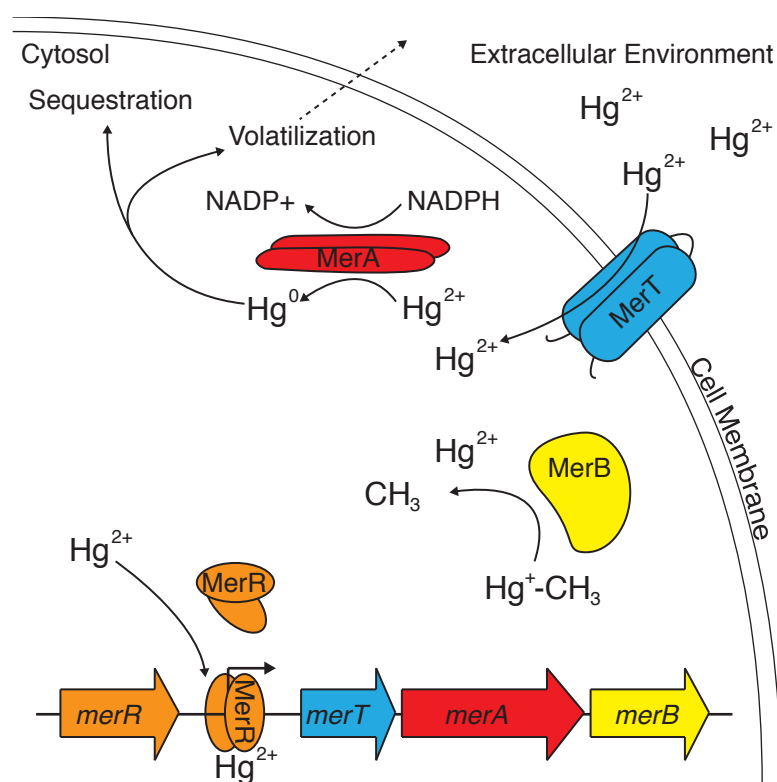


Figure 1-1. The Gram-positive *mer* operon of pI258.

In the mercury resistance operon of pI258 originally identified in *Staphylococcus aureus*, the gene product of *merT* facilitates the active import of inorganic mercury while the products of *merA* and *merB* enzymatically detoxify mercury from organic mercury to elemental mercury respectively. The operon is regulated by a *trans*-acting repressor/activator MerR.

Much of the early study of metals focused on environmental bacteria. Much of the field of bacterial/metal interactions was pioneered by Simon Silver and his trainees (Silver, 2011). It wasn't until 1972 that the nature of mercury resistance by volatilization of elemental mercury was discovered (Summers and Silver, 1972) and 1987 when the genetic basis was uncovered (Laddaga *et al.*, 1987). Since these early discoveries,

mercury resistance has been observed across a wide range of organisms including *Escherichia*, *Pseudomonas*, *Bacillus*, *Staphylococcus*, and *Streptococcus*. Despite being present in a wide range of phylogenies, there has never been a published report of mercury resistance elements present in species of *Lactobacillus*.

Not all biotransformations of mercury are favorable. It has long been known that certain organisms such as *Desulfovibrio* and *Geobacter* are capable of converting inorganic mercury to the more toxic methylmercury species (Schaefer *et al.*, 2011). The mechanism was not well understood until recently (Parks *et al.*, 2013) where it was determined through comparative genomics that a two-gene cluster (*hgcA* and *hgcB*) is sufficient and capable of methylating mercury by acting as a corrinoid protein and a methyl carrier/electron donor respectively. Furthermore, not all interactions of microbes and metals are enzymatically mediated, and passive electrostatic interactions with the cellular surface represent a major mechanism of interaction.

Early studies on interactions of bacterial cell wall used heavy metals as contrast agents in electronic microscopy (which was taken advantage of in Chapter 7 for imaging purposes). Much of this work was carried out at Western University and the University of Guelph in the 1980s and greatly contributed to the understanding of mechanisms in this thesis (Beveridge and Murray, 1980; Beveridge and Koval, 1981; Beveridge and Murray, 1985; Beveridge, 1985; Mullen *et al.*, 1989; Beveridge, 1989). Metals are normally found in association with the membrane, peptidoglycan and teichoic acid of bacterial cells where they have biological functions. For example, Mg^{2+} is essential for outer membrane integrity in *E. coli*. (Beveridge and Koval, 1981). While divalent calcium and magnesium are usually the preferred metals on the cell surface, it is perhaps not surprising that heavy metals can also be found in association with the cell surface. Some years ago, it was noted that different cells have varying metal binding capacities reflecting variations in the cell wall's chemistry, structure and constituent polymers. Lipopolysaccharide, for example, contains phosphoryl groups that are thought to be a main site for metal interactions in Gram-negative bacteria (Couglin *et al.*, 1983). In Gram-positive cells, the negatively charged phosphates of teichoic acid are the most likely repository of metals on the cell surface as extraction of teichoic acid from cells walls depletes most of the metal

content (Beveridge, 1989). Furthermore, additional cell surface features, such as the presence of capsule, may alter interactions. Capsule can block metals from interacting with the cell surface (Bitton and Frichofer, 1978) but may positively affect binding with the cell depending on the chemical nature of the capsule (McLean *et al.*, 1990). While many studies have examined metal/microbe interactions for environmental bioremediation, few have considered toxic metal interactions with members of the gut-microbiota.

Two studies have provided strong proof for a role of the gut microbiota in modulating toxic metal uptake from the gut. Nakamura *et al.* (1977) showed that germ-free mice lacking a gut microbiota absorbed considerably more mercury than their conventional counterparts, and Breton *et al.* (2013) showed the same effect with both lead and cadmium. While the germ-free mouse is a relatively extreme model for the role of a gut microbiota, more subtle microbiota modulating interventions have been tested using antibiotics (Seko *et al.*, 1981) and altered dietary fiber consumption (Rowland *et al.*, 1986). These have further implicated the gut microbiota in modulating host-mercury uptake. Little is known about mercury metabolism by the gut microbiota, but reference genomes available from the Human Microbiome Project, shotgun metagenomic sequencing of fecal samples by the MetaHit consortium show evidence of both mercury detoxifying and methylating enzymes at the genetic level (unpublished observations, JE Bisanz).

Motivated by the results of studies on mercury uptake at different stages of early life (Rowland *et al.*, 1983), it was hypothesized that a gut microbiota enriched in *Bifidobacterium* and *Lactobacillus* (prototypical probiotics) has a decreased ability to favorably metabolize mercury due to the lack of mercury detoxifying enzymes normally found in these two genera. This puts an even greater emphasis on the need to develop probiotic strains with these traits.

While germ-free mouse models provided proof that the gut microbiota affects metal uptake from the gut, these early studies did not address an important potential mechanism independent of direct metal sequestration: modulation of host xenobiotic metabolism.

1.10 Effect of microbes on xenobiotic metabolism

Along with the boom of microbiome studies in the mid 2000s came studies of how the presence of the gut microbiota affects host gene expression. One of the earliest findings involved differential expression cytochrome p450 oxidases (CYP enzymes) including CYP1A1, CYP2B6 and CYP2C19 (Rawls *et al.*, 2004; Toda *et al.*, 2009, Claus *et al.*, 2011; Bjorkholm *et al.*, 2009). A simple explanation for this phenomenon is via the TLR2 receptor (Do *et al.*, 2011) that is known to affect xenobiotic metabolism pathways. However, relatively little is known about how the gut microbiota and/or probiotics affect xenobiotic metabolism in the host and so this represents a novel field that will surely have profound impact on the field of pharmacology and toxicology. A recent example is of digoxin metabolism in the GI tract by strains of *Eggerthella lenta*, which metabolizes the drug contributing greatly to its inter-individual variability in effective dosing regimes (Haiser *et al.*, 2013).

1.11 The rationale for probiotic interventions to counter toxic metals

Given that consumption of mercury and other environmental toxins is more or less unavoidable, how does the body counter the potentially harmful effects? We hypothesized that bacteria can prevent heavy metal uptake into the host. The term detoxication is used in this thesis to describe the microbial role of preventing uptake while detoxification is used to describe the formation of less toxic products from the parent substance. There are three foreseeable mechanisms by which microbes may function to protect humans and other species from environmental toxins ingested in food and drink: (i) enzymatic conversion of toxic compounds to less toxic metabolites, (ii) manipulation of host processes to decrease uptake or increase xenobiotic metabolism, and (iii) microbial binding or sequestering of the toxin. These potential mechanisms are addressed in the context of mercury in Figure 1-2 and in the following paragraph.

We propose a model wherein lactobacilli, known to exist in the mouth, intestine and urogenital tract and commonly used as probiotics, can reduce mercury uptake into the host. This action, mediated primarily in the small intestine and colon, could be directed at

both organic and inorganic forms acting to make them less toxic, by sequestering or volatilizing them. Volatilized mercury is poorly absorbed in the GI tract and is of low toxicity. Lactobacilli acting on mercury may also prevent conversion of dietary inorganic mercury to methylmercury by resident microbes. Few probiotic strains appear able to colonize the gastrointestinal tract to any significant degree for a prolonged period (McNulty *et al.*, 2011; Smith *et al.*, 2011), but their administration and passage through the gut could sequester the toxins from the intestinal contents and carry them out in the feces. Such toxin-binding mediated detoxication has been demonstrated in murine models (Hathout *et al.*, 2011; Hernandez-Mendoza *et al.*, 2011; Kumar *et al.*, 2011). In addition, lactobacilli could act on the host to reduce intestinal permeability and modulate host detoxication pathways such as those involved in xenobiotic metabolism and efflux.

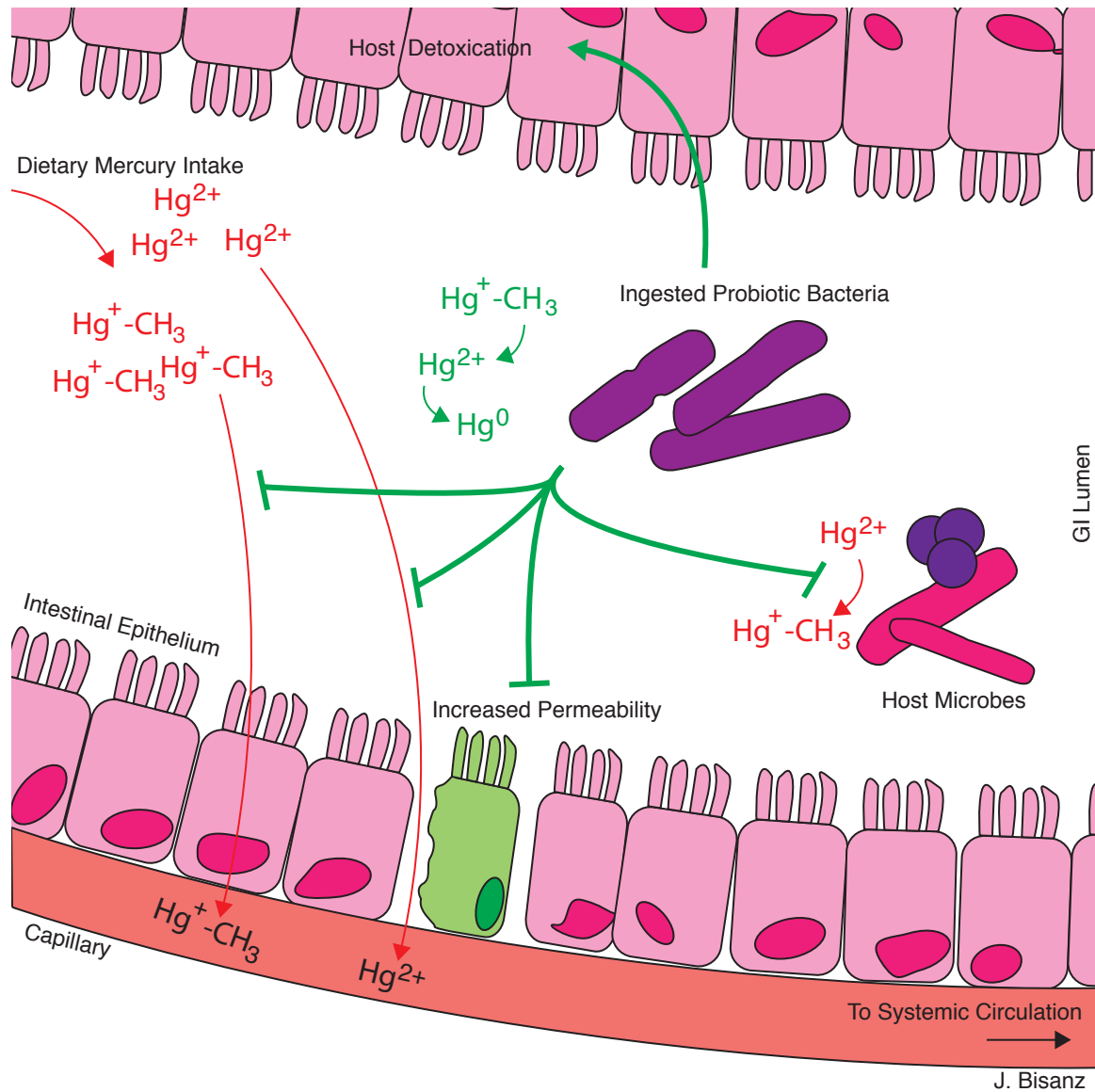


Figure 1-2. Potential mechanisms of probiotic mediated protection against dietary mercury exposure.

Orally administered probiotic bacteria may have protective effects against dietary mercury by: (i) directly binding mercury and sequestering it, (ii) enzymatically detoxifying mercury through mechanisms related to the *mer* operon, (iii) altering community composition or function to prevent indigenous microbes from methylating mercury increasing its absorption, (iv) affecting host barrier function or xenobiotic metabolism of mercury either locally or distally.

1.12 Scope and purpose

Probiotic function and mechanisms of action remain widely unknown. The purpose of the studies presented in this thesis was to increase our understanding of probiotic function and formulation as well as to develop the necessary tools to examine probiotic strain activity. Chapter 2 presents metatranscriptome studies of a commercially available probiotic yogurt, emphasising how formulation and storage can affect probiotic function. While being a pilot study performed early in my PhD studies (2010-2011), its primary purpose was to develop protocols and tools required for RNA sequencing. These were then used in other projects in our laboratory and are now being used to study the microbiota in the context of metal exposure. Chapter 3 describes a fortuitous discovery of a novel pathway involved in fermented food flavor and aroma. This work was initially focused on production of hydrogen sulfide as a potential mechanism of mercury precipitation; however, it is likely to be irrelevant *in vivo*. The discovery is important as production of hydrogen sulfide and volatile sulfur compounds is of great interest to food microbiology for their role in flavor and aroma of fermented foods. This work also increases knowledge of how *L. casei* group members adapt to their environment in fermented foods, and it may have yet unforeseen implications for probiotic function.

Chapter 4 describes a clinical study of vaginally administered probiotics to treat vaginal dysbiosis in post-menopausal women. It was the follow up to a previous study in which I was a co-first author (not included in this thesis: Hummelen *et al.*, 2011). The design of the study was constructed around the probiotic-microbiota-host interactome concept. Despite not showing a major impact on clinical parameters, novel observations were made in terms of how probiotics affect both host and microbiota, thereby adding to our understanding of how lactobacilli function in the vagina.

The work carried out in Chapter 5 describes a study in which I am co-first author (Bisanz *et al.*, 2015) on probiotic yogurt in pregnant Tanzanian women. This was the first study of its kind in pregnant women. It provided insight both into how the microbiota changes over pregnancy and how probiotic yogurt can modulate the microbiota of the newborn.

The human study in Chapter 6 was the first to test the probiotic-detoxication concept in vulnerable human populations and is the main translational component to the work presented in Chapter 7. We were the first to show a positive effect of yogurt containing probiotic lactobacilli on uptake of mercury and arsenic in pregnant women and school children, albeit the latter did not reach statistical significance. The study has many immediate real world applications. The work in chapter 7 builds on the lessons learned from the human studies with the intent of developing novel metal-detoxication oriented strains. The objective was to understand the mechanisms involved *in vivo*. It culminated in animal studies using the interactome approach of the microbiome, host and probiotic. We identified a new *L. rhamnosus* candidate probiotic strain for mercury remediation activity and genetically characterized a novel mercuric resistance operon in *L. rhamnosus*.

The unifying theme of this work is the desire to understand probiotic function. Through a combination of discovery and hypothesis driven research, we have increased our knowledge of how probiotics function in the human vagina and gut, and set the path for further investigations. As a result of these studies, I helped establish many platforms at Western University and Lawson Health Research including high throughput DNA extraction/amplification/sequencing and tools for genetic manipulations of *L. rhamnosus* strains which was previously not possible in our laboratory. It is my hope that the results contained within this thesis and the other publications resulting from my PhD studies will have translational impacts on medical and agricultural practice while increasing our understanding of how microbes function in host health.

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Chapter 2

2 Bacterial metatranscriptome analysis of a probiotic yogurt using an RNA-Seq approach

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2.1 Introduction

Yogurt is a fermented milk product created through the synergistic actions of a defined mixture of microbes, typically *Streptococcus thermophilus* and *Lactobacillus delbrueckii subsp. bulgaricus* (*L. bulgaricus*). It is one of the most common delivery vessels for probiotics, and many strains have been granted the “Generally Recognized as Safe” status by the American Food and Drug Administration allowing their use as food additives. Microbial fermentation of milk can have many effects on its contents including reduction of lactose allowing consumption for lactose intolerant individuals (Savaiano *et al.*, 1984). Bacteria may also produce metabolites beneficial to health during fermentation, the classical example being bacteriocins, which are present in the food matrix and when ingested may exert effects in addition to the actions of the live bacteria (Simova *et al.*, 2010). The addition of certain bacterial strains with known health benefits transforms the yogurt from a basic fermented food to a probiotic. Thus, the probiotic strain selected and its culture conditions are of importance, as strikingly indicated by differential anti-inflammatory signalling of *L. plantarum* WCFS1 depending on the growth phase in which it was administered (van Baarlen *et al.*, 2009). This suggests that modification of culture conditions is possible to optimize probiotics for maximum beneficial health effects. In addition, the interactions between the organisms in the mixture can influence the nutritional outcome of the yogurt. In point, microarray-based transcriptome studies on one or both organisms indicate *L. bulgaricus* provides free amino acids for both strains while formic acid, folic acid and fatty acids are supplied by *S. thermophilus* leading to

improved growth and acid production causing fermentation (Herve-Jiminez *et al.*, 2008; Herve-Jiminez *et al.*, 2009; Sieuwerts *et al.*, 2010). Besides being of industrial importance, better understanding of the interactions of the yogurt microbes may prove to be important to the emerging field of probiotics research.

Given that yogurt may be stored for weeks or months after fermentation before being consumed, we designed an experiment to analyse which genes are being expressed and how the expression varies with flavors and time to product expiry. In the past, most transcriptome studies on lactic acid bacteria have relied on cDNA microarrays, but technical advances and reduced costs have made RNA-Seq, a method of obtaining digital expression from high throughput sequencing, available to researchers. RNA-Seq has several advantages over microarray-based assays including greater sensitivity, greater specificity and nucleotide level resolution (Wang *et al.*, 2009). Given the interest in the health benefits of the commercially available probiotic yogurt in question, (Yang *et al.*, 2008; Guyonnet *et al.*, 2009; Veiga *et al.*, 2010), we sought to apply this technology to the study of probiotic bacteria and their associated bacterial community. A deeper understanding of how the yogurt milieu affects probiotic organisms may allow for maximization of probiotic effects and more guided utilization of these products.

2.2 Materials and Methods

2.2.1 Yogurt source

A mixed pack of 100 g cups of strawberry and vanilla flavored yogurt containing the probiotic bacterium *Bifidobacterium animalis* subsp. *lactis* (*B. lactis*) strain CNCM I-2494 was obtained from a local grocery store (London, Ontario, Canada) 23 days before expiry, and stored at 4 °C until further analysis. RNA isolation from pots of both strawberry and vanilla flavors took place 15 days before expiry (sample names: strawberry and vanilla) and vanilla 1 day before expiry (sample name: vanilla 2). This roughly represents the product at the recently purchased time point and close to expiry, though still in the time frame by which a consumer would be able to purchase and consume the product.

2.2.2 RNA isolation

The extraction protocol was based on previously published methods (Macklaim *et al.*, 2013; Smeianov *et al.*, 2007). Each product was thoroughly mixed and an aliquot of 10 mL of was resuspended in 20 mL of Bacterial RNAprotect (Qiagen) containing 50 µg/mL rifampicin (Sigma). The samples were mixed and incubated for 10 minutes at room temperature to stabilize RNA. The mixture was then centrifuged at 5,000 x g for 10 minutes, the supernatant was discarded, and the pellet was immediately frozen in liquid nitrogen and stored at -80°C until lysis and extraction. The pellet was next resuspended in 10 mL of DEPC-treated water containing 20 mg mL⁻¹ lysozyme (Sigma), 50 U mL⁻¹ mutanolysin (Sigma) and 100 µg mL⁻¹ rifampicin (Sigma) and incubated at 37 °C for 30 minutes. The mixture was then centrifuged as before and the supernatant was discarded. The pellet was then resuspended in 10 mL of TRIzol reagent (Invitrogen). The sample was fully homogenized and the sample was centrifuged for 10 minutes at 12,000 x g to clear excess un-dissolved milk components. The supernatant was then collected and the manufacturers protocol was followed to isolate total RNA (TRIzol Reagent Manual, Invitrogen). Total RNA in RNase-free water was then transferred to a RNeasy column (Qiagen) and treated with DNaseI (Qiagen) following the manufacturers protocol. mRNA was enriched with the MICROBExpress kit (Ambion) according to the manufacturer's protocol. mRNA purity was accessed using an BioPhotometer 6131 (Eppendorf) to ensure the sample was free of contaminants. Both the total RNA and mRNA were analysed with a Bioanalyzer 2100 (Agilent) to analyse RNA quality and confirm mRNA enrichment. All RNA samples used for sequencing had a RIN (RNA integrity number) >8.0 as total RNA.

2.2.3 RNA-Seq

Samples were sent to the Center for Applied Genomics (Toronto), and directional library preparation was carried out and the samples barcoded for 50 base pair sequencing on the ABI SOLiD 4 (Life Technologies).

2.2.4 Read mapping

The colorspace reads were mapped to the reference genomes of the strains present in the yogurt (*B. animalis* CNCM I-2494, *Lactobacillus bulgaricus* strains CNCM I-1632 and CNCM I-1519, *S. thermophilus* CNCM I-1630 and *Lactococcus lactis* CNCM-1631). At the time of publishing, these genomes are available under the following GenBank accessions: CP002915.1, NZ_AGFO00000000, NZ_AGHW00000000, NZ_AGFN00000000, NZ_AGHX00000000. Mapping was carried out with Bowtie 0.12.7 (bowtie-bio.sourceforge.net) using the '--best' and '-M 1' flags to report only best hits, and in the case of reads with multiple best hit mappings, randomly distributing them amongst matches. The first 10 nt at the 3' end of each read were trimmed prior to mapping. Using custom Perl scripts (JEB, JMM and GBG), the reads were assigned to open reading frames and the number of reads per ORF was enumerated. Proportional mRNA abundances per taxa were calculated by three methods: (i) finding the number of reads mapped to the *cpn60* gene of each strain and calculating the fraction of total mapped *cpn60* reads, (ii) the fraction of total mapped *rpoB* reads, and (iii) calculating each strains' total mapped reads as a fraction of reads mapping to all strains.

2.2.5 Expression analysis

For each organism's protein coding sequence, total reads per open reading frame were calculated. The reads per kilobase of exon model per million mapped reads (RPKM) method was used to normalize expression of each gene (Mortazavi *et al.*, 2008). DESeq (bioconductor.org/packages/2.6/bioc/html/DESeq.html), an open source R package, was used to calculate differential gene expression in each organism in each of the three conditions (Anders and Huber, 2010). The 'blind' method was used to allow statistical analysis without replicates. Genes were deemed to be significantly differentially regulated if they had a $p < 0.05$. Genes were functionally annotated using the Clusters of Orthologous Proteins (COGs) database with an e-value cut off of $1E-3$ to assign annotations and functions. Genes were also compared to the SEED subsystems database to assign function (<ftp.theseed.org/subsystems/>) and high quality matches were extracted ($\geq 80\%$ identity and $e\text{-value} < 1E-3$). Amino acid translations of predicted coding

sequences were submitted to the Kyoto Encyclopaedia of Genes and Genome-Automated Annotation Server (genome.ad.jp/tools/kaas/) for pathways analysis.

2.3 Results

2.3.1 Read mapping

A total of 48,658,804 reads (50 nt long) were successfully mapped to the reference database of whole genomes belonging to strains in the product. The average coding sequence coverage obtained for a given organism in a sample was 37.2-fold. Individual coverages are shown in Table 2-1 by number of reads and fold coverage of the strains coding sequences. Only *L. lactis* had poor sequencing depth having a coverage of ≤ 0.390 .

Table 2-1. Reads mapping and coding sequence coverage of strains present in each sample. All values are mapped reads in millions (fold-coverage of coding sequences).

Sample	<i>B. lactis</i>	<i>S. thermophilus</i>	<i>L. lactis</i>	<i>L. bulgaricus</i> CNCM I-1632	<i>L. bulgaricus</i> CNCM I-1519
Strawberry	1.13 (32.2)	1.15 (48.6)	4.76×10^{-4} (0.108)	0.48 (15.3)	0.78 (25.1)
Vanilla	0.85 (24.2)	5.11 (215.4)	1.72×10^{-3} (0.390)	0.86 (27.9)	1.12 (37.3)
Vanilla 2	0.82 (23.3)	1.98 (83.3)	1.03×10^{-3} (0.233)	0.26 (8.34)	0.46 (24.4)

2.3.2 Microbial mRNA composition

Figure 2-1 is a bar plot mapping microbial abundance by fraction of total *cpn60* reads, *rpoB* and total reads mapped to protein coding sequence in each sample. The dominant organism by *cpn60* mapped reads was *S. thermophilus*, while *B. lactis* and *L. bulgaricus* strains were also in high abundance. *B. lactis* and *L. bulgaricus* contributed a greater proportion of both *rpoB* mapped reads and total reads. *L. lactis* appeared to be present in relatively low abundance as compared to the other organisms both by *cpn60* ($\leq 0.01\%$), *rpoB* ($\leq 0.29\%$), which was consistent with its low transcriptome coverage. *L. lactis* was

excluded from further expression analysis, as it did not have sufficient coverage for meaningful analysis.

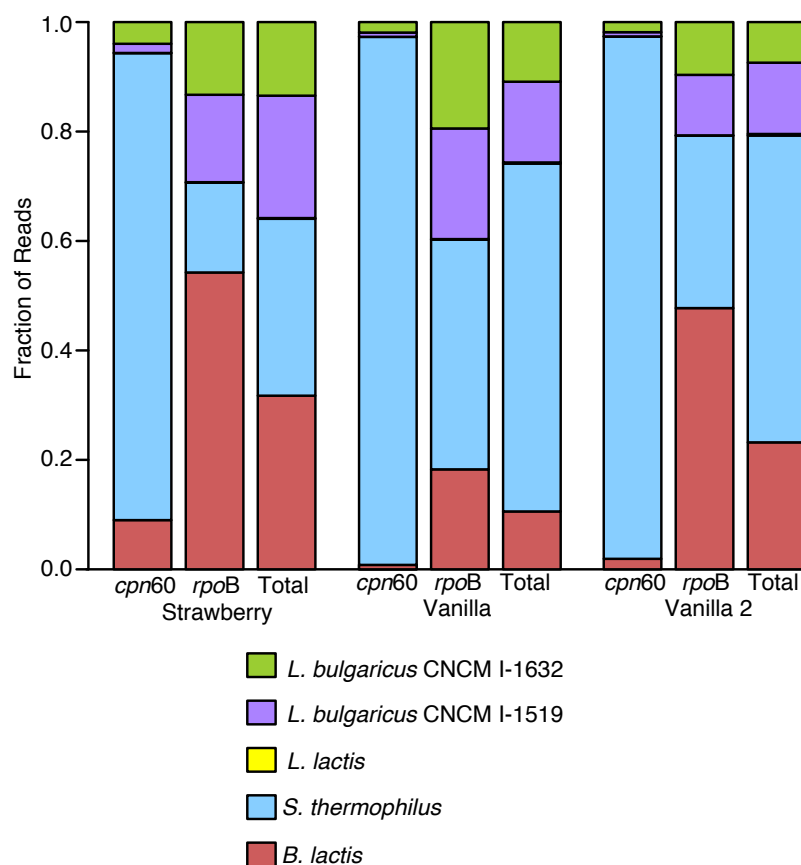


Figure 2-1. Microbial abundance as measured by the fraction of *cpn60* reads, *rpoB* reads and reads mapping to all coding sequences in the organisms.

By *cpn60*, but not *rpoB* and total reads, *S. thermophilus* was the most abundant organism in all conditions, however all strains, with the exception of *L. lactis*, appeared to be present and highly transcriptionally active.

2.3.3 Gene functions expressed in yogurt

In each organism an enrichment analysis was performed by analysing all COG functions present in the genome, genes expressed with a RPKM ≥ 200 in all samples, and genes expressed with a RPKM ≥ 1000 in all samples. This corresponds to all protein coding genes, the most highly expressed 22.4-37.8% and 5.26-9.94% of genes respectively in a

strain dependent manner. Of total genes with an assignable COG hit, the fraction of genes in each top-level function was calculated and is displayed in Figure 2-2. Highly represented functions in all organisms include translational machinery, carbohydrate transport and metabolism, amino acid transport and metabolism. In most strains such as *B. lactis*, there was an enrichment of genes involved in translation in the highly expressed genes (from 10% in the genome to 23.6% in genes with an RPKM ≥ 1000) with the exception of *S. thermophilus*. In this organism, at the highest RPKM cut-off, genes involved in this category only comprise 7.4% of genes while they comprise 8.6% in the genome. *S. thermophilus* showed higher enrichment in carbohydrate transport and metabolism from 6.1% in the genome to 17.2% at an RPKM cut-off of 1000. This included two genes annotated as *LacZ* beta-galactosidase, responsible for breaking down lactose, which was also highly expressed in *L. bulgaricus* strains but not *B. lactis*. Further KEGG pathway analysis showed the backbone genes of glycolysis were highly expressed (RPKM ≥ 1000) in *S. thermophilus* leading to its higher fraction of genes involved in carbohydrate transport and metabolism. Both *L. bulgaricus* strains displayed similar fractions of functions at all levels of enrichment. *B. lactis* had a higher fraction of genes involved in energy production and conversion at an RPKM ≥ 1000 with 8.9% of genes involved in this category. KEGG annotation showed the presence of 6 subunits of F₀F₁ type ATP synthase among these genes, supported by KEGG pathway analysis.

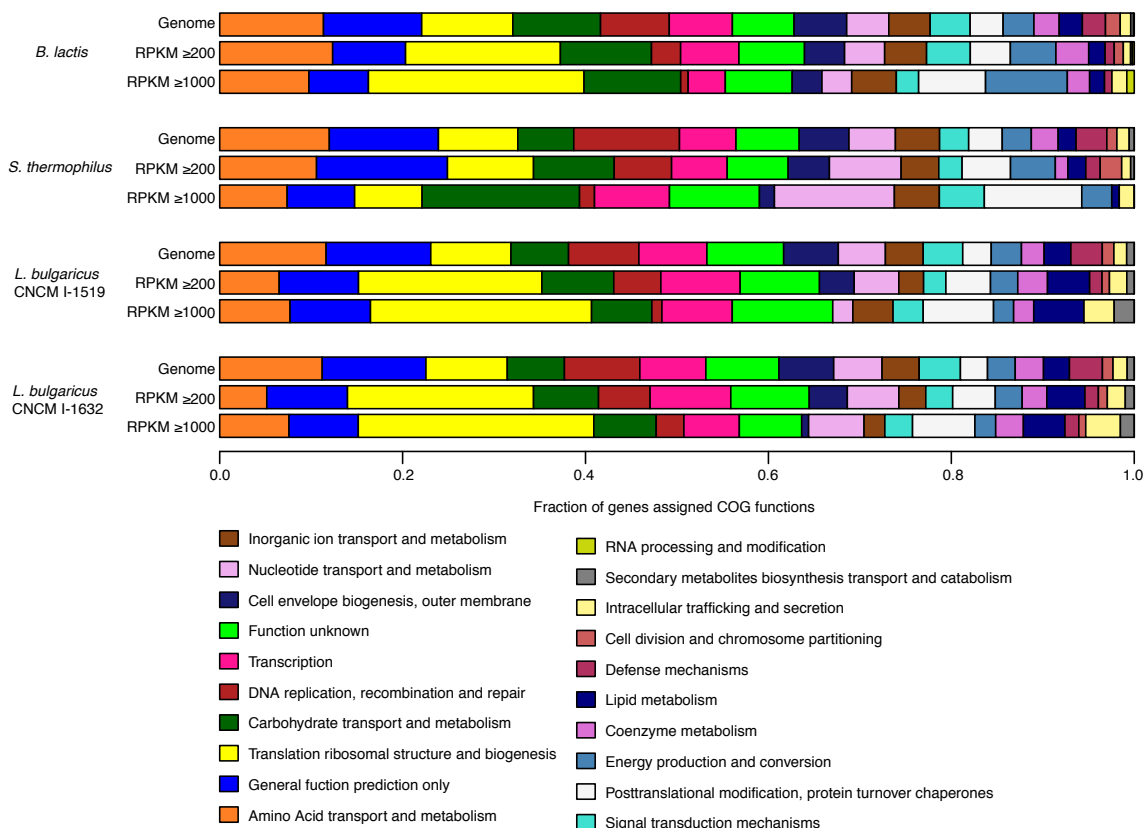


Figure 2-2. Enrichment of COG functions in all conditions by increasing RPKM cutoff demonstrating gene functions at different levels of expression.

Functions highly represented at the highest enrichment levels include those involved in translation, and carbohydrate and amino acid transport/metabolism. There was an enrichment of carbohydrate transport and metabolism genes in *S. thermophilus* at higher expression levels (RPKM \geq 1000) while other strains displayed enrichment of translation, ribosomal structure and biogenesis genes. *B. lactis* displayed an enrichment of genes involved in energy production and conversion. Both *L. bulgaricus* strains displayed similar profiles at the genome, and highly expressed gene levels.

2.3.4 Differential mRNA expression due to flavor and storage time

Differential expression analysis by DESeq was performed for flavor (strawberry vs. vanilla) and storage time (vanilla vs. vanilla 2). Statistical analysis without replicates was based on the assumption that no difference exists for most genes between samples compared, thus samples are treated as if they are replicates with differentially abundant genes as outliers. This approach is generally considered to be conservative, since it usually leads to an overestimation of variance making reported p-values conservative (Anders and Huber, 2010). Differential expression was plotted in the context of SEED

subsystems for all analyzed strains. Figure 2-3 shows a flavor comparison and storage time comparison at the most general SEED subsystem level. Genes identified as significantly differentially expressed are indicated by the strain of origin. Genes that had differential expression values of infinity due to read count values of zero in one sample were plotted as a 6-fold change for simplicity. Only 9 of these genes had a $p < 0.05$ including LDBUL1519_00397, a O-acetylhomoserine sulfhydrylase. In both comparisons, there were many up-regulated genes involved in protein metabolism in strawberry compared to vanilla and vanilla compared to vanilla 2. Inspection of SEED subsystem level 3 revealed most of these differentially expressed genes encode ribosomal proteins. In particular 13 genes, 11 up-regulated, involved in protein metabolism, were significantly differentially regulated by *B. lactis* in strawberry as opposed to vanilla flavors ranging from -1.4 to 1.4 fold (\log_2).

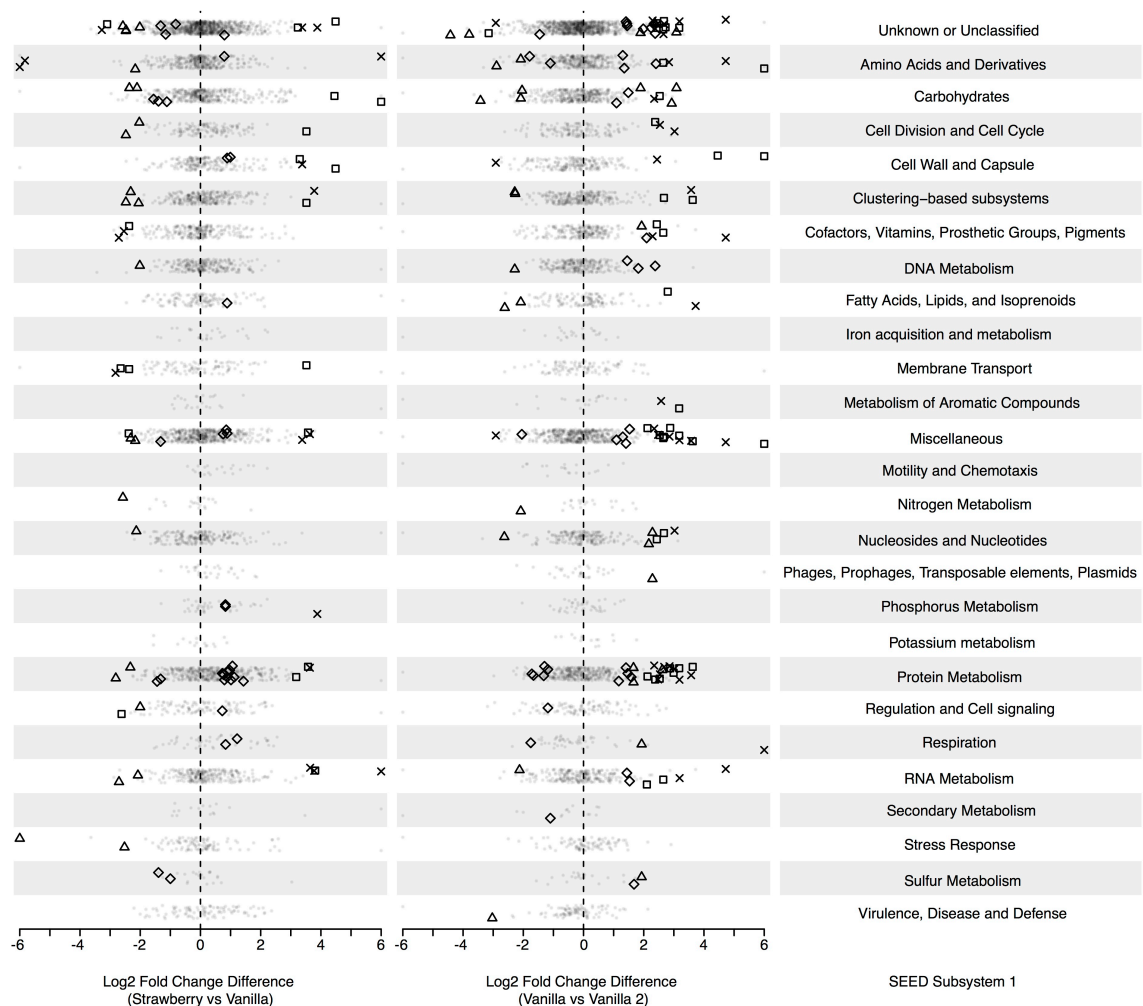


Figure 2-3. Differential expression measured by $\text{Log}_2(\text{fold change})$ in the highest level SEED subsystems in a comparison of flavors and storage time.

Genes with a $p < 0.05$ are displayed according to the strain of origin: *B. lactis* (\diamond), *S. thermophilus* (\triangle), *L. bulgaricus* CNCM I-1519 (\square), *L. bulgaricus* CNCM I-1632 (\times). Genes infinitely differentially regulated were assigned a fold-change value of ± 6 -fold.

2.3.5 *B. lactis* expression

The filtered, COG-annotated gene-by-gene results of a flavor (strawberry versus vanilla) and time comparison (vanilla versus vanilla 2) are displayed in Tables 2-2 and 2-3 respectively. In the flavor comparison, 90 genes were identified as being significantly differentially expressed with 59 being assigned COG annotations. In the storage comparison, 89 genes were significantly differentially expressed with 56 being assigned COG annotations. The most up-regulated gene in strawberry as compared to vanilla was *upgE*, a sugar transport permease (5.3-fold), while the most down-regulated was *salX*, an antimicrobial peptide transport system (-3.0-fold). In the time point comparison of vanilla to vanilla 2, *Ssb* single-stranded DNA-binding protein was most up-regulated in vanilla (12.6-fold) while COG1073, a hydrolase, was most down-regulated (infinitely down-regulated). Gene functions differentially regulated in both conditions include genes that are predominately involved in protein translation and DNA replication, or can't be assigned a function, and to a lesser extent those involved in DNA replication, recombination and repair, cell envelope biogenesis and transcription as demonstrated in Figure 2-4 which shows differential expression of COG functions in *B. lactis*.

Table 2-2. *B. lactis* genes with assigned COGs differentially regulated with $p < 0.05$ in a comparison of the strawberry and vanilla samples (flavor comparison).

Locus ID	Fold Change	P-value	Best COG Hit
BALAC2494_02066	5.28	8.79E-03	COG0395, UgpE, ABC-type sugar transport system, permease component
BALAC2494_01335	3.98	1.15E-07	COG5017, Uncharacterized conserved protein
BALAC2494_01292	3.66	6.58E-07	COG1403, McrA, Restriction endonuclease
BALAC2494_00517	3.64	4.95E-06	COG3039, Transposase and inactivated derivatives, IS5 family
BALAC2494_01290	3.47	1.02E-04	COG4974, XerD, Site-specific recombinase XerD
BALAC2494_01337	2.76	3.01E-04	COG0451, WcaG, Nucleoside-diphosphate-sugar epimerases
BALAC2494_01333	2.72	1.38E-04	COG1216, Predicted glycosyltransferases
BALAC2494_00723	2.69	8.28E-04	COG0203, RplQ, Ribosomal protein L17
BALAC2494_01334	2.55	3.48E-04	COG1216, Predicted glycosyltransferases
BALAC2494_00235	2.48	3.05E-03	COG1507, Uncharacterized conserved protein
BALAC2494_01304	2.33	4.28E-03	COG0224, AtpG, F0F1-type ATP synthase, gamma subunit
BALAC2494_00734	2.18	2.01E-03	COG0256, RplR, Ribosomal protein L18
BALAC2494_00989	2.15	2.59E-02	COG4177, LivM, ABC-type branched-chain amino acid transport system, permease component
BALAC2494_00987	2.13	7.96E-03	COG0410, LivF, ABC-type branched-chain amino acid transport systems, ATPase component
BALAC2494_01352	2.12	2.76E-03	COG1216, Predicted glycosyltransferases
BALAC2494_01336	2.11	3.50E-03	COG0707, MurG, UDP-N-acetylglucosamine:LPS N-acetylglucosamine transferase
BALAC2494_00329	2.11	2.63E-02	COG0239, CrcB, Integral membrane protein possibly involved in chromosome condensation
BALAC2494_00130	2.09	4.69E-03	COG1546, CinA, Uncharacterized protein (competence- and mitomycin-induced)
BALAC2494_00727	2.09	3.37E-03	COG0257, RpmJ, Ribosomal protein L36
BALAC2494_00651	2.07	3.59E-02	COG4487, Uncharacterized protein conserved in bacteria
BALAC2494_01817	2.02	1.46E-02	COG1203, Predicted helicases
BALAC2494_01248	2.02	7.01E-03	COG0360, RpsF, Ribosomal protein S6
BALAC2494_01776	2.00	8.92E-03	COG2244, RfbX, Membrane protein involved in the export of O-antigen and teichoic acid
BALAC2494_01354	1.99	6.60E-03	COG0463, WcaA, Glycosyltransferases involved in cell wall biogenesis
BALAC2494_01967	1.96	1.48E-02	COG0048, RpsL, Ribosomal protein S12
BALAC2494_00758	1.85	3.54E-02	COG0102, RplM, Ribosomal protein L13
BALAC2494_00797	1.85	1.41E-02	COG1211, IspD, 4-diphosphocytidyl-2-methyl-D-erythritol synthase
BALAC2494_00510	1.85	1.43E-02	COG0049, RpsG, Ribosomal protein S7
BALAC2494_00381	1.81	4.79E-02	COG0799, Uncharacterized homolog of plant Iojap protein
BALAC2494_01353	1.79	2.00E-02	COG2244, RfbX, Membrane protein involved in the export of O-antigen and teichoic acid
BALAC2494_01302	1.78	3.12E-02	COG0712, AtpH, F0F1-type ATP synthase, delta subunit
BALAC2494_00554	1.78	2.09E-02	COG1061, SSL2, DNA or RNA helicases of superfamily II
BALAC2494_01347	1.78	2.12E-02	COG1215, Glycosyltransferases, probably involved in cell wall biogenesis
BALAC2494_00785	1.78	2.66E-02	COG0581, PstA, ABC-type phosphate transport system, permease component
BALAC2494_00784	1.78	4.80E-02	COG1117, PstB, ABC-type phosphate transport system, ATPase component
BALAC2494_00780	1.74	3.29E-02	COG0222, RplL, Ribosomal protein L7/L12
BALAC2494_01492	1.73	3.33E-02	COG0118, HisH, Glutamine amidotransferase
BALAC2494_00746	1.71	3.00E-02	COG0185, RpsS, Ribosomal protein S19
BALAC2494_00185	1.69	4.80E-02	COG0268, RpsT, Ribosomal protein S20
BALAC2494_01079	1.68	4.42E-02	COG1196, Smc, Chromosome segregation ATPases
BALAC2494_00553	1.67	4.51E-02	COG1479, Uncharacterized conserved protein
BALAC2494_00168	1.65	4.25E-02	COG0740, ClpP, Protease subunit of ATP-dependent Clp proteases
BALAC2494_00446	-1.91	1.36E-02	COG3304, Predicted membrane protein
BALAC2494_00316	-1.93	3.55E-02	COG0861, TerC, Membrane protein TerC, possibly involved in tellurium resistance
BALAC2494_00765	-2.00	4.53E-02	COG4231, Indolepyruvate ferredoxin oxidoreductase, alpha and beta subunits
BALAC2494_00398	-2.17	1.91E-02	COG1028, FabG, Dehydrogenases with different specificities
BALAC2494_00859	-2.23	3.67E-03	COG1837, Predicted RNA-binding protein (contains KH domain)
BALAC2494_01319	-2.30	7.10E-03	COG0251, TdcF, Putative translation initiation inhibitor, yjgF family
BALAC2494_00825	-2.50	7.85E-04	COG0211, RpmA, Ribosomal protein L27
BALAC2494_00591	-2.56	2.05E-03	COG0629, Ssb, Single-stranded DNA-binding protein
BALAC2494_00637	-2.63	3.66E-02	COG1874, LacA, Beta-galactosidase
BALAC2494_00865	-2.71	1.54E-04	COG0227, RpmB, Ribosomal protein L28
BALAC2494_00612	-2.94	3.46E-02	COG3507, XynB, Beta-xylosidase
BALAC2494_00415	-2.98	1.78E-02	COG1136, SalX, ABC-type antimicrobial peptide transport system, ATPase component

Table 2-3. *B. lactis* genes with assigned COGs differentially regulated with $p < 0.05$ in a comparison of the vanilla and vanilla 2 samples (storage comparison).

Locus ID	Fold Change	p-value	Best COG Hit
BALAC2494_00591	12.61	4.83E-08	COG0629, Ssb, Single-stranded DNA-binding protein
BALAC2494_01069	10.79	7.53E-07	COG3464, Transposase and inactivated derivatives
BALAC2494_01866	10.16	1.19E-06	COG3464, Transposase and inactivated derivatives
BALAC2494_01860	9.40	2.02E-06	COG3464, Transposase and inactivated derivatives
BALAC2494_01696	7.67	1.39E-05	COG3464, Transposase and inactivated derivatives
BALAC2494_00118	5.70	1.65E-04	COG3464, Transposase and inactivated derivatives
BALAC2494_00693	5.30	1.09E-02	COG1605, PheA, Chorismate mutase
BALAC2494_01466	5.20	2.43E-04	COG1381, RecO, Recombinational DNA repair protein (RecF pathway)
BALAC2494_00859	5.13	1.10E-03	COG1837, Predicted RNA-binding protein (contains KH domain)
BALAC2494_01771	4.27	3.18E-02	COG0388, Predicted amidohydrolase
BALAC2494_00728	3.95	1.34E-02	COG0361, InfA, Translation initiation factor 1 (IF-1)
BALAC2494_01326	3.54	1.30E-03	COG1196, Smc, Chromosome segregation ATPases
BALAC2494_00488	3.53	5.57E-03	COG0776, HimA, Bacterial nucleoid DNA-binding protein
BALAC2494_01696	3.22	7.15E-03	COG3464, Transposase and inactivated derivatives
BALAC2494_00232	3.20	1.31E-02	COG0600, TauC, ABC-type nitrate/sulfonate/bicarbonate transport system, permease component
BALAC2494_00635	3.11	2.45E-02	COG1175, UgpA, ABC-type sugar transport systems, permease components
BALAC2494_00865	3.00	1.31E-02	COG0227, RpmB, Ribosomal protein L28
BALAC2494_00128	2.89	2.04E-02	COG0324, MiaA, tRNA delta(2)-isopentenylpyrophosphate transferase
BALAC2494_00398	2.80	1.49E-02	COG1028, FabG, Dehydrogenases with different specificities
BALAC2494_00770	2.77	4.57E-02	COG0234, GroS, Co-chaperonin GroES (HSP10)
BALAC2494_00031	2.73	2.55E-02	COG1974, LexA, SOS-response transcriptional repressors (RecA-mediated autopeptidases)
BALAC2494_00499	2.71	2.92E-02	COG1758, RpoZ, DNA-directed RNA polymerase, subunit K/omega
BALAC2494_00364	2.66	8.73E-03	COG0739, NlpD, Membrane proteins related to metalloendopeptidases
BALAC2494_00825	2.66	9.15E-03	COG0211, RpmA, Ribosomal protein L27
BALAC2494_02024	2.55	1.38E-02	COG0040, HisG, ATP phosphoribosyltransferase
BALAC2494_00909	2.47	1.74E-02	COG0527, LysC, Aspartokinases
BALAC2494_00389	2.35	2.17E-02	COG0637, Predicted phosphatase/phosphohexomutase
BALAC2494_00954	2.35	2.15E-02	COG4591, LolE, ABC-type transport system, involved in lipoprotein release, permease component
BALAC2494_01319	2.30	3.55E-02	COG0251, TdcF, Putative translation initiation inhibitor, yjgF family
BALAC2494_00446	2.30	2.57E-02	COG3304, Predicted membrane protein
BALAC2494_01603	2.27	2.95E-02	COG1278, CspC, Cold shock proteins
BALAC2494_01864	2.27	4.29E-02	COG2135, Uncharacterized conserved protein
BALAC2494_00085	2.25	3.28E-02	COG0292, RplT, Ribosomal protein L20
BALAC2494_01157	2.14	4.11E-02	COG2211, MelB, Na ⁺ /melibiose symporter and related transporters
BALAC2494_02003	2.10	4.97E-02	COG1765, Predicted redox protein, regulator of disulfide bond formation
BALAC2494_00341	-2.15	4.94E-02	COG0159, TrpA, Tryptophan synthase alpha chain
BALAC2494_00168	-2.27	3.35E-02	COG0740, ClpP, Protease subunit of ATP-dependent Clp proteases
BALAC2494_01335	-2.35	4.83E-02	COG5017, Uncharacterized conserved protein
BALAC2494_00735	-2.46	4.24E-02	COG0097, RplF, Ribosomal protein L6P/L9E
BALAC2494_00993	-2.46	3.50E-02	COG0239, CrcB, Integral membrane protein possibly involved in chromosome condensation
BALAC2494_00727	-2.50	2.18E-02	COG0257, RpmJ, Ribosomal protein L36
BALAC2494_01352	-2.55	1.49E-02	COG1216, Predicted glycosyltransferases
BALAC2494_01292	-2.57	3.86E-02	COG1403, McrA, Restriction endonuclease
BALAC2494_01353	-2.66	2.18E-02	COG2244, RfbX, Membrane protein involved in the export of O-antigen and teichoic acid
BALAC2494_00397	-2.74	4.08E-02	COG0162, TyrS, Tyrosyl-tRNA synthetase
BALAC2494_01360	-2.77	2.97E-02	COG4713, Predicted membrane protein
BALAC2494_01248	-3.16	1.41E-02	COG0360, RpsF, Ribosomal protein S6
BALAC2494_00732	-3.29	1.91E-02	COG1841, RpmD, Ribosomal protein L30/L7E
BALAC2494_01304	-3.37	2.14E-02	COG0224, AtpG, F0F1-type ATP synthase, gamma subunit
BALAC2494_00403	-3.46	1.52E-03	COG1438, ArgR, Arginine repressor
BALAC2494_00381	-4.14	2.97E-03	COG0799, Uncharacterized homolog of plant Iojap protein
BALAC2494_01007	-Inf	5.38E-03	COG1073, Hydrolases of the alpha/beta superfamily



Figure 2-4. Differential regulation of COG functions in *B. lactis* in both flavor (A) and storage time (B) comparisons.

Differential expression is represented by number of genes passing the filter and the bars are stacked showing the total number of genes separated by up and down-regulated. COG Functions: (J) translation, ribosomal structure and biogenesis; (R) general function prediction only; (S) function unknown; (L) DNA replication, recombination and repair; (M) cell envelope biogenesis, outer membrane; (G) carbohydrate transport and metabolism; (P) inorganic ion transport and metabolism; (C) energy production and conversion; (E) amino acid transport and metabolism; (D) cell division and chromosome partitioning; (I) lipid metabolism; (V) defense mechanisms; (K) transcription; (O) posttranslational modification, protein turnover, chaperones; (Q) secondary metabolites biosynthesis, transport and catabolism; (U) intracellular trafficking and secretion; and (T) signal transduction mechanisms.

2.4 Discussion

In this report, we describe one of the first metatranscriptome analyses of a probiotic yogurt. High coverage of all coding sequences of the organisms present in the yogurt was obtained with the high read output of the SOLiD 4 sequencer. We recognize the inherent limitations of the single replicate experimental design, and have been conservative in our analysis. However, several factors suggest that the majority of the findings are robust. First, the high read coverage partially compensated for the single replicate experimental design as the abundance values can be estimated with greater precision (Fernandes *et al.*, 2013). Second, the analysis tool used assumes that the majority of the expression values between samples do not change, and only identifies the expression outliers. This approach is expected to be conservative (Anders and Huber, 2010). Recent work has determined that genes with high intrinsic variation are also identified by this tool (Li and Tibshirani, 2013; Fernandes *et al.*, 2014), indicating that there may be contamination with genes of this type in the lists of differentially abundant genes. Validation thus will require additional samples. Third, many of the differentially abundant genes that are identified may be connected in pathways. This argues strongly for accepting as valid those genes that are identified as differentially abundant where multiple genes in the same pathway are also significantly different and share the same directionality of abundance difference.

Given that non-protein coding RNAs make up $\geq 90\%$ of total RNA, the enrichment of mRNA is necessary to optimize coverage of coding sequences. This makes expressed 16S rRNA an unsuitable marker of microbial abundances as it has been largely removed and the removal may not be consistent across all strains. We used instead the *cpn60* gene and *rpoB*, both are single copy, highly-transcribed genes with high levels of sequence variation allowing for unique identification of bacteria and quantification (Hill *et al.*, 2004; Rodrigues & Tiedje, 2007). The *cpn60*-mapped reads indicated that the most abundant organism was *S. thermophilus* while *L. bulgaricus* and the probiotic *B. lactis* also composed a large fraction of the population. Even though *B. lactis* may be rare according to *cpn60*, it still appears to be highly transcriptionally active as dictated by the proportion of total reads in the product. The proportional abundances of *rpoB* mapped

reads more closely resemble the proportional abundances determined by total reads. Given that two strains of *L. bulgaricus* were present, it was likely our quantitation may be skewed by an inability to distinguish between the strains given a high level of similarity in their 1614 bp *cpn60* genes (99% nucleotide sequence identity). In all three samples we observe *L. bulgaricus* CNCM I-1519 as being in higher quantities, thus we may be underestimating its abundance, and overestimating the abundance of *L. bulgaricus* CNCM I-1632 as reads with common mapping in these two organisms will be equally assigned to both. In addition, it is possible that levels of *cpn60* may fluctuate in response to cell stress which has been identified in *L. bulgaricus* (Lim *et al.*, 2000). This may also skew quantification making fraction of *cpn60* reads a good tool for identification of bacterial strains but as with any technique, it may have its own inherent biases. Indeed, recent studies have shown transcriptional activity is not always related to true microbial abundances which may suggest the need for culture or DNA-based quantification (McNulty *et al.*, 2011).

The *L. lactis* had extremely low coverage of ≤ 0.390 -fold, so we were unable to study its role further. While we can demonstrate its presence by the detection of uniquely mapping *cpn60* reads, there are less than 100 reads in any of the samples. This suggests *L. lactis* composes a small fraction of the total community and plays a minor role in the product as delivered to the consumer. Alternatively it may be present but in an extremely dormant state at the time of sampling with very little functional contribution in the finished product. Presumably during the production of the yogurt it contributes to the taste or texture of the product but could also function in improving growth and viability of the probiotic bifidobacteria strain as suggested by recent research (Yonezawa *et al.*, 2010; Odamaki *et al.*, 2011).

When comparing flavors, two genes of *L. bulgaricus* CNCM I-1519 involved in methionine pathways were differentially regulated infinitely (no transcripts detected in one condition), up-regulated in LDBUL1519_01373 (adenosylhomocysteinase) and down-regulated in LDBUL1519_00397 (O-acetylhomoserine sulfhydrylase or O-succinylhomoserine sulfhydrylase). The product of adenosylhomocysteinase reactions are L-cysteine and adenosine while O-succinylhomoserine sulfhydrylase consumes L-

cysteine to form L-cystathionine and succinate (KEGG Database). This may suggest a cellular response in *L. bulgaricus* to produce maximum quantities of L-cysteine in the strawberry flavor compared to vanilla. Cysteine can act as an antioxidant in cells (Qian *et al.*, 2010). It is also used in growth media to lower the redox potential and protect against reactive oxygen species for anaerobes such as bifidobacteria (Qian *et al.*, 2010). The importance of cysteine was underlined by the detection of a number of highly expressed components in the cysteine and methionine pathway during analysis of *B. lactis*. This suggests a mechanism to help protect viability of probiotics in yogurt, though as a double-edged sword, bifidobacteria grown in the absence of cysteine form anti-oxidant granules exhibiting higher acid tolerance and higher surface hydrophobicity which are beneficial characteristics for a probiotic to allow passage through the digestive system (Qian *et al.*, 2010). These antioxidant granules contain large quantities of polyphosphate and there was corresponding up-regulation of two *B. lactis* phosphate specific transporters in strawberry flavoring (*PstA* and *PstB*) that would supply phosphate for the production of polyphosphate.

O-acetylhomoserine sulfhydrylase, the other potential function of the *L. bulgaricus* gene LDBUL1519_00397 as reported by the KEGG Database, consumes methanethiol to produce L-methionine. Methanethiol is a key volatile sulfur compound important in giving flavor and aroma to cheese which has the potential to give rise to subtle flavor and scent differences between yogurts (Hanniffy *et al.*, 2009).

Components of the F₀F₁-ATP synthase machinery of *B. lactis* appeared to be highly expressed and differentially regulated between conditions being up-regulated (BALAC2494_1302 and BALAC2494_1304) in the strawberry flavor and in the fresher vanilla sample. This machinery has been implicated as being key for bile and acid resistance in both *B. animalis* and *L. plantarum* by increasing intracellular ATP and controlling intracellular pH (Sanchez *et al.*, 2006; Hammon *et al.*, 2011). Improved bile and acid resistance may aid a probiotic strain by improving resistance to stomach acid and bile in the stomach and early small intestine allowing more viable organisms to reach their site of action.

The SEED subsystem analyses indicated that ribosomal proteins were differentially regulated in all organisms in both flavor and storage comparisons. In the flavor comparison many of these changes are relatively small, less than 4-fold, while in the storage time comparison the changes were more drastic with up-regulation of the genes by 2-16 fold in the fresher sample. A probable explanation of why we were able to assign significance to relatively small 2-fold changes in the ribosomal subunits is a function of their extremely high abundance that allows higher confidence in their change. Higher expression of these transcripts may be indicative of higher translational activity, especially when coupled with up-regulation of RNA polymerase subunits such as *RpoZ* (BALAC2494_00499), or of significantly greater mRNA stability of these transcripts upon prolonged shelf storage. Recent work in *E. coli* suggests that bacterial cells have an upper limit on the amount of transcription supported. Scott *et al.* (2010) found that the transcription of house keeping genes such as ribosomal proteins change to compensate for increases in the transcription of genes expressed in response to environmental cues. This implies that there may be a number of smaller changes that occurred in the cell, however these most likely don't pass our filters. We suggest that a larger sample set would help improve our ability to detect smaller changes.

B. lactis adapts to its environment as indicated by the most up-regulated gene in strawberry in comparison to vanilla: *upgE*. It is most likely a broad specificity sugar uptake permease suggesting that this may be a response to differing sugar composition between flavors. This was further supported by the down-regulation in strawberry flavor of *lacA* β -galactosidase, a component of lactose metabolism, and *xynB*, β -xylosidase, an enzyme responsible for the break down of xylose and xylooligosaccharides, compounds with potential as a bifidogenic prebiotic (Lagaert *et al.*, 2011). Up-regulation of *xynB* has recently been identified as a key response of this *B. lactis* strain to its environment in the human body, raising the question whether modulating its expression in yogurt may have a positive effect on the strain once it enters the body (McNulty *et al.*, 2011). Alternately, the gene most up-regulated in vanilla as compared to strawberry shares significant similarity with SalX, an antimicrobial peptide transport component. This is of interest since the vanilla flavoring agent is from natural sources and vanillin, the primary component in vanilla bean extract, possesses anti-microbial properties (Negishi *et al.*,

2009). Influence of flavoring agents has been studied in both starter culture bacteria and probiotic strains (Vinderola *et al.*, 2002). It was found that probiotic strains were often resistant to the effects of flavoring agents and indeed our work may highlight a potential mechanism through which this occurs.

In culture, the half-life of total bacterial RNA has been estimated at 6.8 minutes (Selinger *et al.*, 2003), thus the gene expression of probiotic bacteria in the small intestine or distal colon is surely different from in the yogurt. This being said, genes expressed in the yogurt will likely influence the ability of the bacteria to exert a probiotic effect. For example, if the organisms are already expressing factors for improved acid and bile tolerance they will have an increased chance of passing through the acidic stomach and bile filled early duodenum allowing viable bacteria to reach multiple parts of the intestinal tract to exert their effects.

2.5 Conclusions

Given that to our knowledge, no publications exist on the transcriptomes of yogurt bacteria post-fermentation, the intent of this research was to perform a pilot study to rigorously, and scientifically, apply RNA-Seq to study the yogurt metatranscriptome post-fermentation and see the possible impacts on the organisms. This study offers a method for others to examine how bacterial strains in fermented food adapt to flavor and storage. The findings do not necessarily imply that flavor and/or storage alter clinical effects, but they illustrate the potential to further refine yogurt as a valuable method of delivering health through probiotics.

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Chapter 3

3 Comparative genomics demonstrates production of volatile sulfur compounds in *Lactobacillus paracasei* and *Lactobacillus rhamnosus* is mediated by a novel sulfur/taurine metabolism gene cluster.

This material in this chapter is currently under review at the International Journal of Food Microbiology as a short communication.

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3.1 Introduction

Flavor of many fermented milk products is determined by a complicated interaction of molecules acting in combination. In many fermented milk products including cheddar cheese, the actions of non-starter microorganisms, including lactobacilli, impart much of the characteristic flavor as a result of the amino acid metabolism (Weimer *et al.*, 1999). One of the most important groups of compounds in cheese flavor formation is volatile sulfur compounds (VSCs), including hydrogen sulfide (H₂S). These impart the often-desired scent and flavor colloquially referred to as “rotting egg”, but in lower levels they are required for basic cheese flavor. The lactic acid bacteria-mediated production of H₂S and other VSCs is of great interest for their role in this process. Some work has been undertaken to understand this phenomenon at the molecular level (Del Carmen Martínez-Cuesta *et al.*, 2013).

In the carbohydrate-limited environment of fermented milk, proteolysis is a key metabolic pathway for lactobacilli. The resultant methionine and cysteine produced are the intermediates from which VSCs are formed (Del Carmen Martínez-Cuesta *et al.*, 2013). An operon known as *cysK-ctl-cysE*, has been reported to putatively encode a cysteine synthase, cystathionine lyase and serine acetyltransferase (Bogicevic *et al.*, 2012). Further studies have demonstrated O-acetylserine sulfhydrylase and cysteine desulfurization activity by CysK (Bogicevic *et al.*, 2011), as well as cystathionine lyase activity of *ctl* leading to the production of VSCs (Bogicevic *et al.*, 2013). From

heterologous expression of these enzymes (Alting *et al.*, 1995; Aibel *et al.*, 2002; Bogicevic *et al.*, 2011; Bruinenberg *et al.*, 1997) it is clear that they function in VSC production, but the knockout of cystathionine lyases does not lead to a complete loss of activity (Lee *et al.*, 2007). Thus, we sought to identify novel pathways implicated in VSC production.

Our studies were primarily focused on screening a collection of sequenced *L. rhamnosus* and *L. paracasei* strains for tolerance to mercury and the results initially suggested that precipitation of mercury by hydrogen sulfide could be a potential mechanism of metal tolerance and remediation. This led us to further study the impact of the *cysK-ctl-cysE* operon in VSC production as it was found to be transcriptionally up-regulated by exposure to sublethal concentrations of mercury (data not shown). Given the sheer number of strains under study, a high-throughput, rapid, and economical method of screening for VSC production was required for which we adapted the method of Lee and Simard (1984). Briefly, when H₂S/VSCs are produced by lactobacilli grown in agar stabs, a black/brown PbS precipitate is formed.

The primary goal was to determine if under the assayed conditions, the presence of the *cysK-ctl-cysE* operon was sufficient for H₂S/VSC production.

3.2 Materials and Methods

3.2.1 Strains and genomes

A collection of 75 strains of *L. paracasei* and *L. rhamnosus* was obtained from the Danone strain collection (Danone Nutricia Research, Palaiseau, France). All strains were maintained on MRS agar medium (Becton Dickson and Company, Mississauga, Canada) and incubated at 37°C under anaerobic conditions. *Lactobacillus plantarum* ATCC 14917 was obtained from the ATCC as positive control as it had previously been shown to produce H₂S/VSCs under the assayed conditions. These strains have already been sequenced by 454 GS FLX (Smokvina *et al.*, 2013). Briefly, the predicted protein coding sequences were determined by consensus of Genemark (Borodovsky *et al.*, 2002), Glimmer, ZCurve (Guo, 2003), and Prodigal (Hyatt *et al.*, 2010). Using OrthoMCL (Li, 2003) a presence/absence matrix was created for all potential strains.

3.2.2 Determination of H₂S/VSC production

The modified lead acetate agar media was adapted from that reported by Lee and Simard (Lee and Simard, 1984). The media contained: 5.0 g/L proteose peptone, 15.0 g/L peptone #3, 1.0 g/L dextrose, 80 mg/L sodium thiosulfate, 200 mg/L lead acetate, 0.1% v/v tween 80, 0.005% w/v MnSO₄·4H₂O, and 15 g/L agar. For phenotyping the strains, the medium was placed in 5 mL tubes, stab inoculated and incubated for 72h at 37°C in anaerobic conditions to observe blackening of the media caused by the precipitation of PbS.

3.2.3 Genomic analysis

To identify the presence or absence of the *cysK-ctl-cysE* pathway, a BlastP (version 2.2.28+) was carried out to identify the corresponding gene orthologs in the presence/absence matrix using the predicted protein sequences previously identified in *L. casei* FAM18149 (Genbank accessions JF272516, EU340836 and JF272515 respectively). Statistical testing of the association of gene orthologs groups with production of VSCs/ H₂S was carried out by Fisher's exact test.

To further study genes implicated in the production of VSCs/ H₂S, matrices of gene presence/absence and phenotype were inputted into the random forest algorithm approach implemented in Phenolink (Bayjanov *et al.*, 2012) with default settings. GC Skew was visualized with Artemis 14.0.0 and IVOM analysis was carried out with Alien hunter 1.7 (Vernikos and Parkhill, 2006). To create a pan-genome tree, the strains were clustered based on gene presence/absence using the dist (binary method) and hclust (UPGMA) functions of R (r-project.org).

3.3 Results and Discussion

It was determined, with positive control *L. plantarum* ATCC 14917, that 24h incubation was insufficient to observe H₂S/VSC production, but after 72h production could be observed. Incubation for 72h gave rise to three distinct phenotypes (Figure 3-1): strong H₂S/VSC producers (positive reaction after 24h, complete blackening of media after 72h), weak H₂S/VSC producers including *L. plantarum* ATCC 14917 (positive reaction

after 72h) and non H₂S/VSC producers (no positive reaction after 72h). H₂S/VSC production was robustly assayed in all 75 strains in three independent assays revealing non-producers (n=45), weak producers (n=12) and strong producers (n=12) with few strains providing ambiguous results (n=6).

Little correlation was observed between H₂S/VSC production and the presence of *cysK-ctl-cysE* (Table 3-1, p=0.49, Fischer's exact) so gene-trait matching was carried out using a random forest algorithm approach which revealed 7 genes with the highest importance score (Figure 3-2). Upon closer inspection the cluster was revealed at the same chromosomal loci in each strain including *L. casei* Zhang (GenBank CP001084; 159190..166381). This cluster was robustly associated with strong H₂S/VSC production (p=1.8e⁻¹¹, Table 3-1). The gene cluster's organization and manually curated annotations are shown in Fig 3-3A while the heatmap in Fig 3-3B demonstrates its association with VSC production. The predicted functions of these genes involve the import and metabolism of sulfur and taurine. Many of the weak producers lack the downstream hypothetical protein of unknown function. There is a difference of 5.7% in GC content between in the gene cluster as compared to the remainder of the genome suggesting horizontal gene transfer (40.8% and 46.5% respectively). Additionally, application of interpolated variable order motif analysis provides evidence of horizontal gene acquisition.

To study the distribution of the gene cluster across *L. rhamnosus* and *L. paracasei*, a pan-genomic tree was created showing two major branches separating the *L. rhamnosus* strains (Lr) and *L. paracasei* strains (Lpp and Lpt). Strains containing at least 6/7 of the genes were indicated demonstrating that the gene cluster is uniformly distributed over both lineages independent of strain or internal node. Frequently, even in two strains with similar gene composition will be divergent in their possession of the cluster (ex. *L. rhamnosus* Lr52 and *L. rhamnosus* Lr35). Of note, the cluster was present in a mix of strains isolated from dairy, human/animal and plant environments.

The strength of this study compared to other gene-trait matching studies, is in the large number of strains and genomes. This allowed us to confidently examine the relevance of

the *cysK-ctl-cysE* operon and discover potential new pathways. Clear evidence of a novel gene cluster involved in VSC production has been provided but the intention is not to claim that *cysK-ctl-cysE* is irrelevant to VSC production, rather to provide evidence of an additional pathway. It is conceded that there are superior methods for determining VSC production, such as GC-MS, but our assay provided clear phenotypic traits allowing us to demonstrate that *cysK-ctl-cysE* is not the only pathway for H₂S production present in *L. paracasei* and *L. rhamnosus*.

The functions of the genes are putative and remain to be validated but the presence of domains relating to sulfur transport and metabolism offers interesting insights into potential mechanisms of action. The putative *tauB* and *tauC* genes are notable considering that taurine is a sulfur containing amino acid and high levels of taurine are found in many dairy products (Manzi and Pizzoferrato, 2013). In addition, taurine metabolism is related to methionine and cysteine metabolism. The putative pyridine nucleotide-disulfide oxidoreductase may be part of the H₂S-forming pathway, perhaps together with the hypothetical protein.

In summary, we identified a novel gene cluster involved in the formation of VSCs which has been previously unreported in *Lactobacillus* spp. Future studies are warranted to determine the function of these proteins *in situ*, in particular how they affect flavors in cheese.

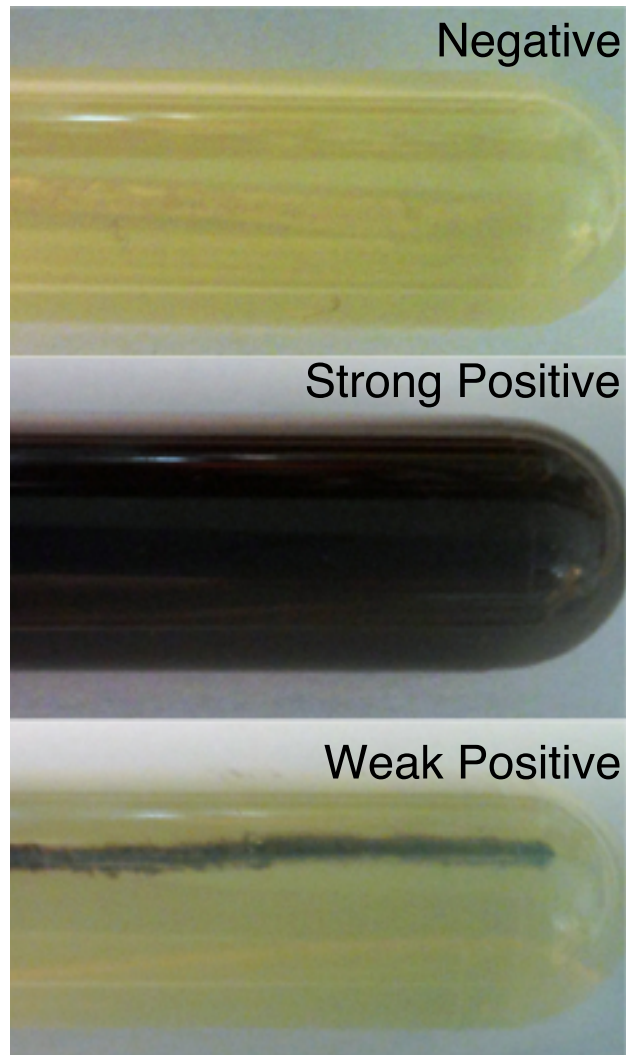


Figure 3-1. VSC production phenotypes observed after 72 h incubation at 37°C.

The dark precipitate formed is the result of lead acetate precipitated with volatile sulfur compounds to form PbS.

Table 3-1. Co-occurrence of the *cysK-ctl-cysE* operon and newly identified sulfur transport/utilization cluster with VSC production.

H ₂ S/VSC Production	<i>cysK-ctl-cysE</i>		Sulfur Transport Cluster ¹	
	Present	Absent	Present	Absent
Strong	2	10	12	0
Weak	4	8	2	10
Negative	13	32	1	44

¹Presence/absence of all 7 identified gene orthologs

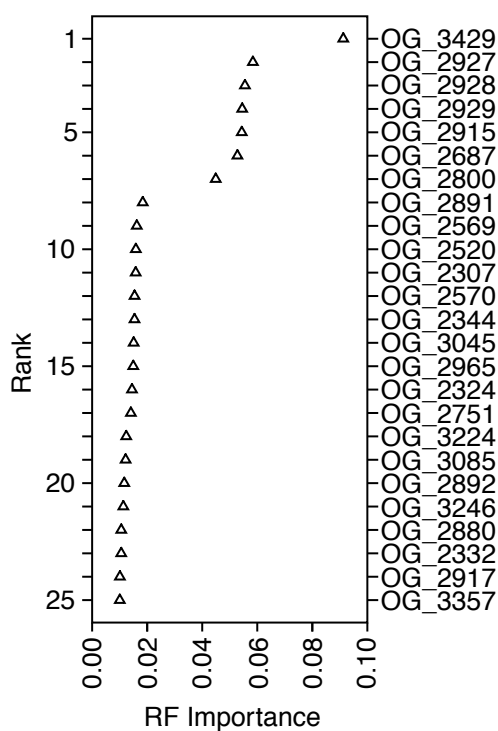


Figure 3-2. Results of gene trait matching demonstrate 7 gene orthologs with increased importance as identified by a random forest algorithm based approach.

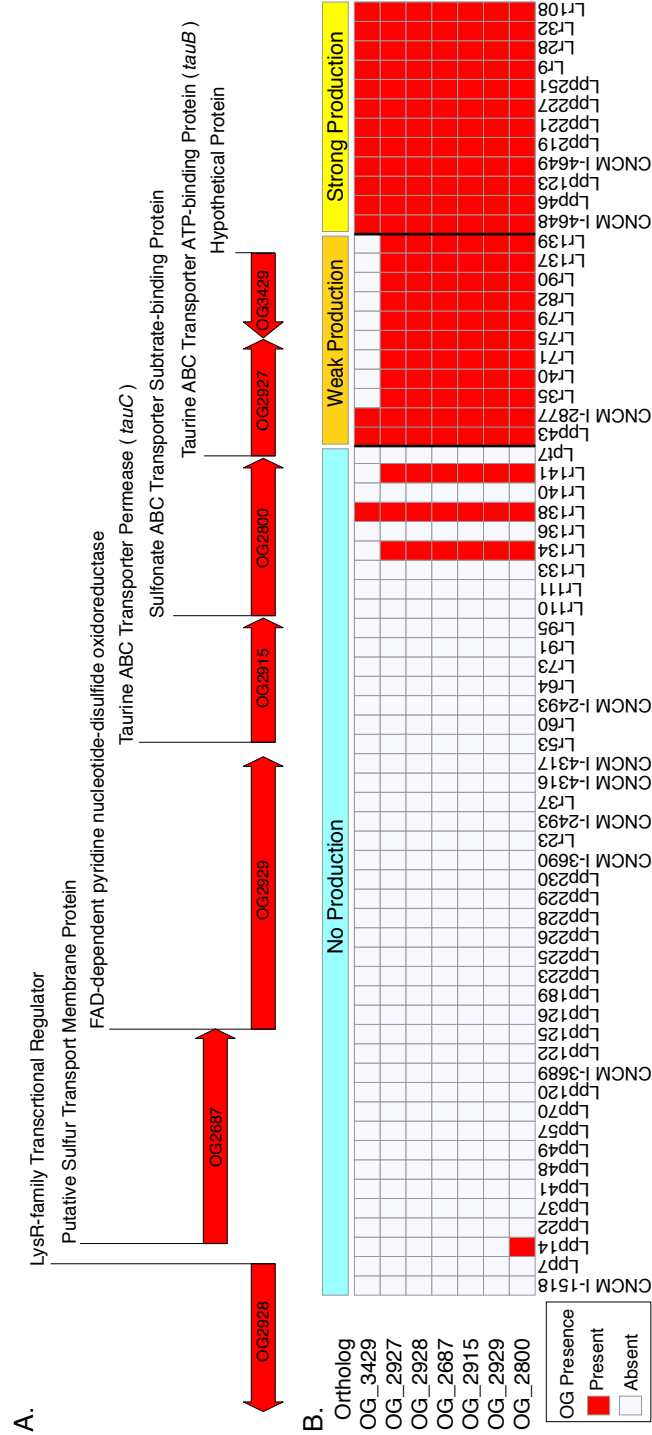


Figure 3-3. Identification of 7-gene locus in *L. rhamnosus* Lr9 and *L. casei* Zhang (A) and its co-occurrence with VSC production represented in heat map form (B).

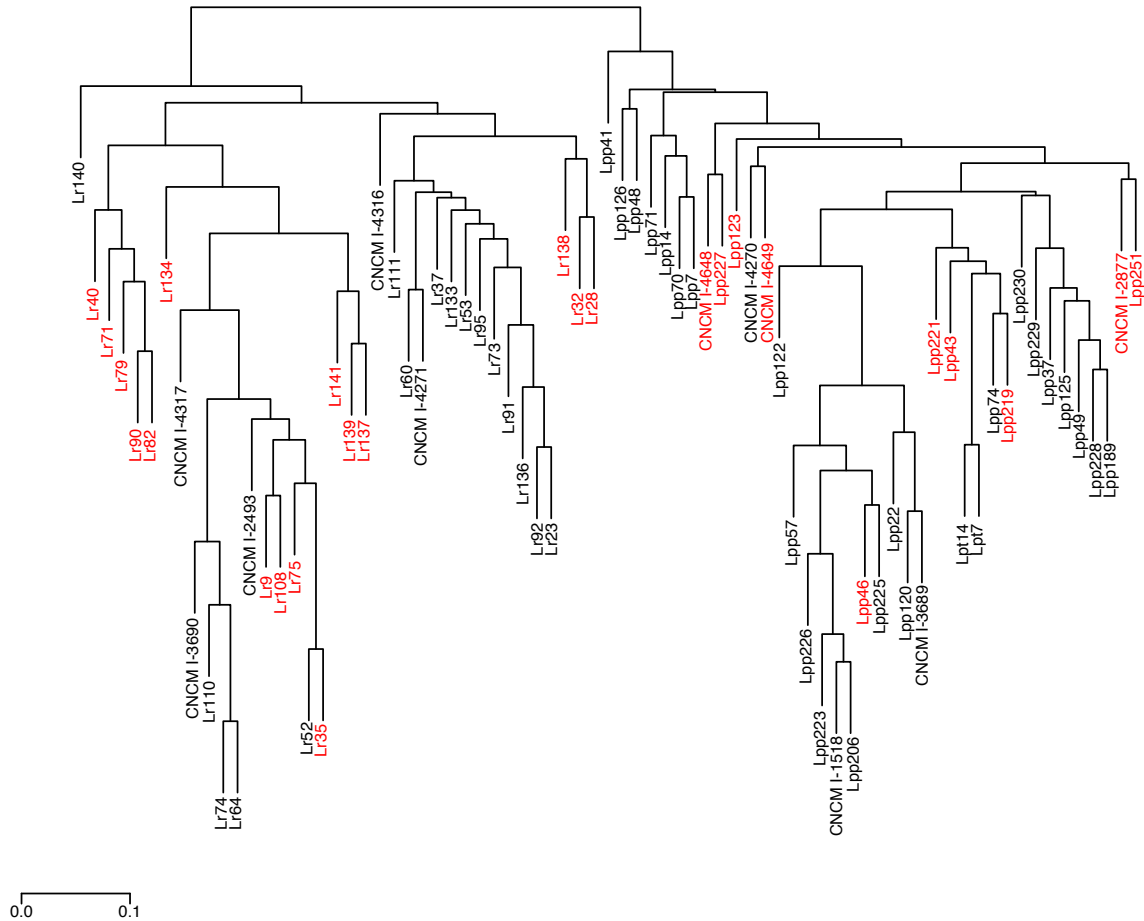


Figure 3-4. Pan-genome tree of strains studied with strains possessing at least 6/7 of the identified gene orthologs indicated in red.

3.4 Acknowledgements/funding

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Chapter 4

4 A systems biology approach investigating the effect of probiotics on the vaginal microbiome and host responses in a double-blind, placebo-controlled clinical trial of post-menopausal women

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Bisanz JE, Seney S, McMillan A, Vongsa R, Koenig D, Wong L, Dvoracek B, Gloor GB, Sumarah M, Ford B, Herman D, Burton JP and G Reid. 2014. A systems biology approach investigation the effect of probiotics on the vaginal microbiome and host responses in a double-blind, placebo-controlled clinical trial of post-menopausal women. PLoS One 9:e104511.

Supporting figures and tables (identified as S#) are available online at:

<http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0104511#s5>

Ethics approval documentation is provided in Appendix E

4.1 Introduction

The vaginal microbiota is a dynamic ecosystem that is usually mono dominated by the *Lactobacillus* genus in times of health but it can transform quickly to a dysbiotic state where a range of microorganisms rise in prominence and cause the polymicrobial bacterial vaginosis (BV) (Ma *et al.*, 2012). The pre-menopausal vaginal microbiota and its constituents have been extensively studied using genome sequencing, 16S rRNA community profiling and RNA-seq based meta-transcriptomics (Macklaim *et al.*, 2011; Ravel *et al.*, 2011; Hummelen *et al.*, 2010b; Macklaim *et al.*, 2013). However, until recently, few studies have used similar in-depth methods to decipher the vaginal microbiome of post-menopausal women, despite its impact on quality of life (Santoro and Komi, 2009; Nappi and Lachowsky, 2009). In previous studies, we have shown using high-throughput sequencing methods that contrary to conventional knowledge (Cauci *et al.*, 2002), the vaginal microbiome of post-menopausal women is not significantly

different from that of pre-menopausal women (Hummelen *et al.*, 2011). Our study showed that the post-menopausal vagina appeared to have greater stability than the rapidly fluctuating pre-menopausal microbiome (Hummelen *et al.*, 2011; Gajer *et al.*, 2012) presumably due to a lack of hormone cycling. We found that similar to premenopausal women, the healthy post-menopausal vaginal microbiome is dominated by a combination of *Lactobacillus iners* and *L. crispatus* while BV-associated organisms such as *Gardnerella vaginalis* and *Atopobium vaginae* are increased in states of dysbiosis (Hummelen *et al.*, 2011; Burton and Reid, 2002; Burton *et al.*, 2003). Despite some deficiencies, Nugent scoring is still the standard technique in most research and clinical settings to rapidly diagnose bacterial vaginosis (Nugent *et al.*, 1991). The score is calculated by microscopic examination of a Gram-stained vaginal smear and numeration of cell morphotypes to assign a score from 0 to 10 where 0-3 is considered normal, 4-6 is intermediate and 7-10 is BV (Nugent *et al.*, 1991). The intermediate scores are particularly interesting as they indicate a risk of transition to BV, or they could be a state that reverts to a healthy lactobacilli dominated microbiota.

Given vaginal contact with sanitary products by menstruating women and the large proportion of postmenopausal women whom suffer urinary incontinence requiring products such as incontinence pads, also in proximity to the vagina, these products may offer a vehicle to deliver a prophylactic probiotic to women that routinely suffer BV or urinary tract infections. We were interested to determine whether the microbiota and Nugent scores were altered by the administration of probiotics with the potential view of perhaps instilling these organisms in such products. *Lactobacillus rhamnosus* GR-1 and *Lactobacillus reuteri* RC-14 used in this prospective study are well-characterized probiotic strains used in combination to prevent and treat BV (Anukam *et al.*, 2006; Reid *et al.*, 2003; Martinez *et al.*, 2009; Hummelen *et al.*, 2010a). In addition to clinical and Nugent scoring, 16S rRNA sequencing was used to examine changes in the microbiota, GC-MS profiled metabolome changes, the host transcriptional responses were tested using an Affymetrix microarray and inflammatory mediators by multiplex cytokine analysis. This approach was designed to provide a holistic understanding of the probiotic-microbiota-host interactome (Bisanz and Reid, 2011).

4.2 Results

4.2.1 Participant recruitment and demographics

A total of 22 subjects were screened and 14 participants were enrolled into a 129-day prospective double-blind, cross-over placebo controlled study (design in Figure S1). A CONSORT flow diagram is displayed in Figure 4-1.

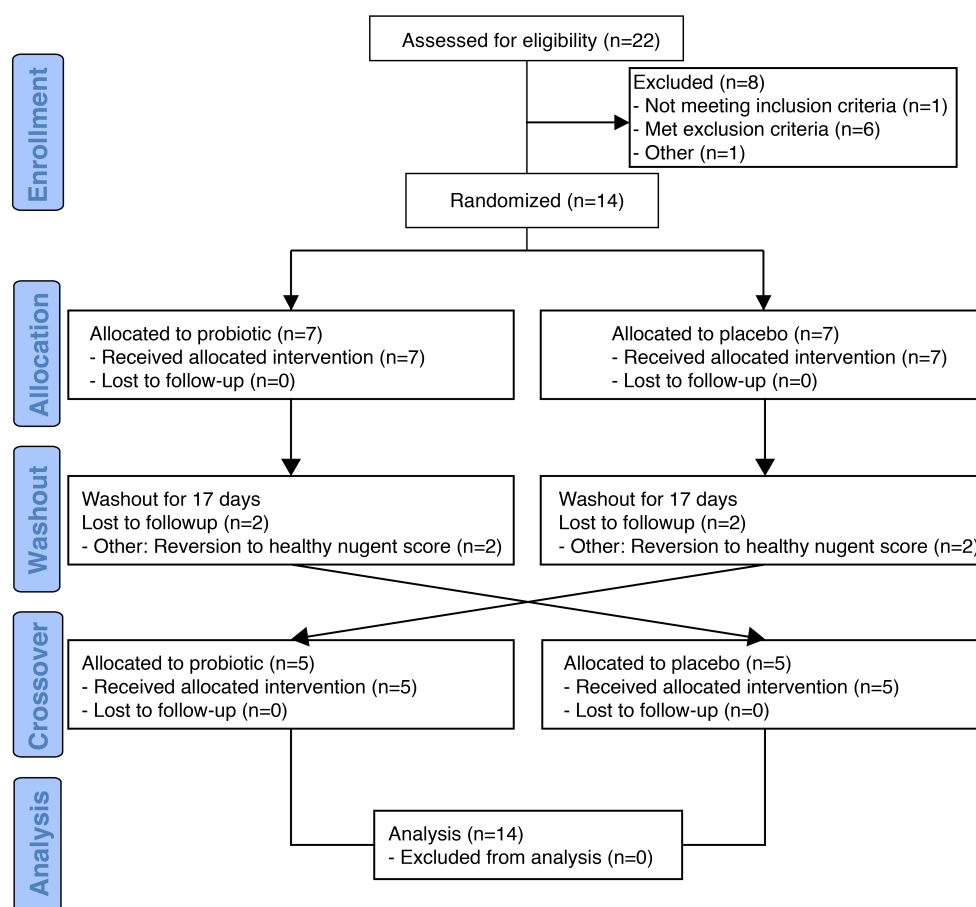


Figure 4-1. CONSORT flow diagram.

4.2.2 Clinical outcomes

Nugent score and vaginal pH measurements (data not shown) were taken before and after treatment with the probiotic and placebo. Table 4-1 shows there were no statistically significant improvement in Nugent scores between the treatment groups. Furthermore no statistical differences were observed in vaginal pH measurement.

Table 4-1. Summary of Nugent score change - Proportion of improvement from baseline

			Placebo		<i>p</i> -value ²
			Improved ¹	Not Improved	
<u>One-Day after last Test Article Use</u>	<u>Probiotics:</u>	Improved	0	2(20%)	0.1573
		Not Improved	0	8(80%)	
<u>Eight-Days after last Test Article Use</u>	<u>Probiotics:</u>	Improved	0	2(20%)	0.5637
		Not Improved	1(10%)	7(70%)	

¹Improvement in Nugent Score: indicated by shift of intermediate score (4-6) at baseline to normal score (0-3) at follow-up visits. Baseline Nugent score for study Phase-I was collected at visit 3, and baseline Nugent score for study Phase-II was collected at visit 6.

²*p*-value is based on McNemars test to determine results of cross-over treatment regimen.

4.2.3 Microbiome profiling

Sequences were successfully obtained for 104 of 106 samples with the mean number of reads being 33,396 (range 1,475 to 223,629) across 3 separate 316 Ion Torrent Sequencing Chips. A total of 221 Operational Taxonomic Units (OTUs) were defined with clustering at 97% identity (Table S1) at a minimum abundance of 1% in any one sample. A no template control was also sequenced and high-abundance contaminating sequences were filtered out *in silico* before OTU assignment.

Figure 4-2 shows a heat map of the 50 most abundant OTUs across all samples and time points. We were able to identify 2 OTUs which were assigned presumptive strain designations to *L. rhamnosus* GR-1 and *L. reuteri* RC-14 (OTU_12 and OTU_23 respectively) based upon 100% ID matches to the 16S rRNA gene of private genome assemblies of these strains. A separate heat map was created showing only the abundance of these two OTUs compared to all other OTUs detected (Figure S2). Furthermore with one notable exception (participant 19), these strains only appeared in combination following the probiotic treatment period. In the case of participant 19 however, the proportion of reads is still quite low (2.1% and 0.1%) for both OTUs. The *L. rhamnosus* GR-1 is far more abundant than the *L. reuteri* which may suggest it was a *L. casei* group member other than the GR-1 used in the study as this group has high sequence homology, especially in the V6 16S rRNA region (Vasquez *et al.*, 2005). In one individual (participant 09) both probiotic strains were detected even though only placebo should have been received. The probiotic strains could be unambiguously detected after probiotic treatment in 7 of 12 cases (04, 10, 15, 16, 17, 18, 21), which we termed responders. While in the non-responders (12, 14, 19, 05 and 07) the probiotic strains were not detected after the probiotic treatment. The abundance of *L. rhamnosus* GR-1 did appear to increase over the study period in participant 12, which may suggest temporary persistence or colonization. In all other cases, the probiotics were only detected in high abundance immediately after the treatment suggesting that the washout period chosen for the study was appropriate.

Samples were clustered based upon unweighted pair group method with arithmetic mean (UPGMA) using weighted UniFrac distance metrics (Lozupone and Knight, 2005) showing that participants were generally most similar to themselves over the study period and as we had previously shown, the post menopausal vaginal microbiome is more stable than that of pre menopausal women (Figure 4-3).

All samples were plotted on a PCoA using weighted Unifrac distances and overlaid with the contributions of the 20 most abundant genera (Figure S3). This shows 3 weak groupings, one dominated by *Lactobacillus*, another defined by *Atopobium* and *Gardnerella*, and a third defined by a large number of diverse genera.

Data were summarized to genus level and the relative abundance of *Lactobacillus* was considered. Increase in total *Lactobacillus* is significant (Figure 4-4; FDR<0.05) following probiotic administration though there is no significant difference in Shannon's diversity index ($p=0.25$, Wilcoxon signed-rank test). After probiotic administration, the proportion of total lactobacilli increased in all participants except participant 19 who appears to have had an indigenous *L. casei/rhamnosus* present. Even those without significant detection of the probiotic lactobacilli have an increase in indigenous lactobacilli (fold increases for the ambiguous participants are 1.27, 1.21, 2.35, 14.43 and 52.43). Additionally, in some cases, such as participant 17 (Figure 4-5), it is not only the probiotics that increased in proportional abundance, but also indigenous lactobacillus such as *L. gasseri/johnsonii*, ($p=0.011$ across all samples) which is not significant in the placebo group if the case of participant 09 is excluded (where probiotic appears to have been received rather than placebo). In the 7 individuals where the probiotics could be easily detected, the average fold increase was 120 fold (range 3.2 to 389). Significant decreases in the proportion of *Atopobium* were also observed after probiotic treatment with a trend of reduction of *Gardnerella* and *Prevotella*. Interestingly the placebo appears to have increased the proportion of *Streptococcus* (FDR<0.01) while both applications appear to have increased levels of *Staphylococcus*.

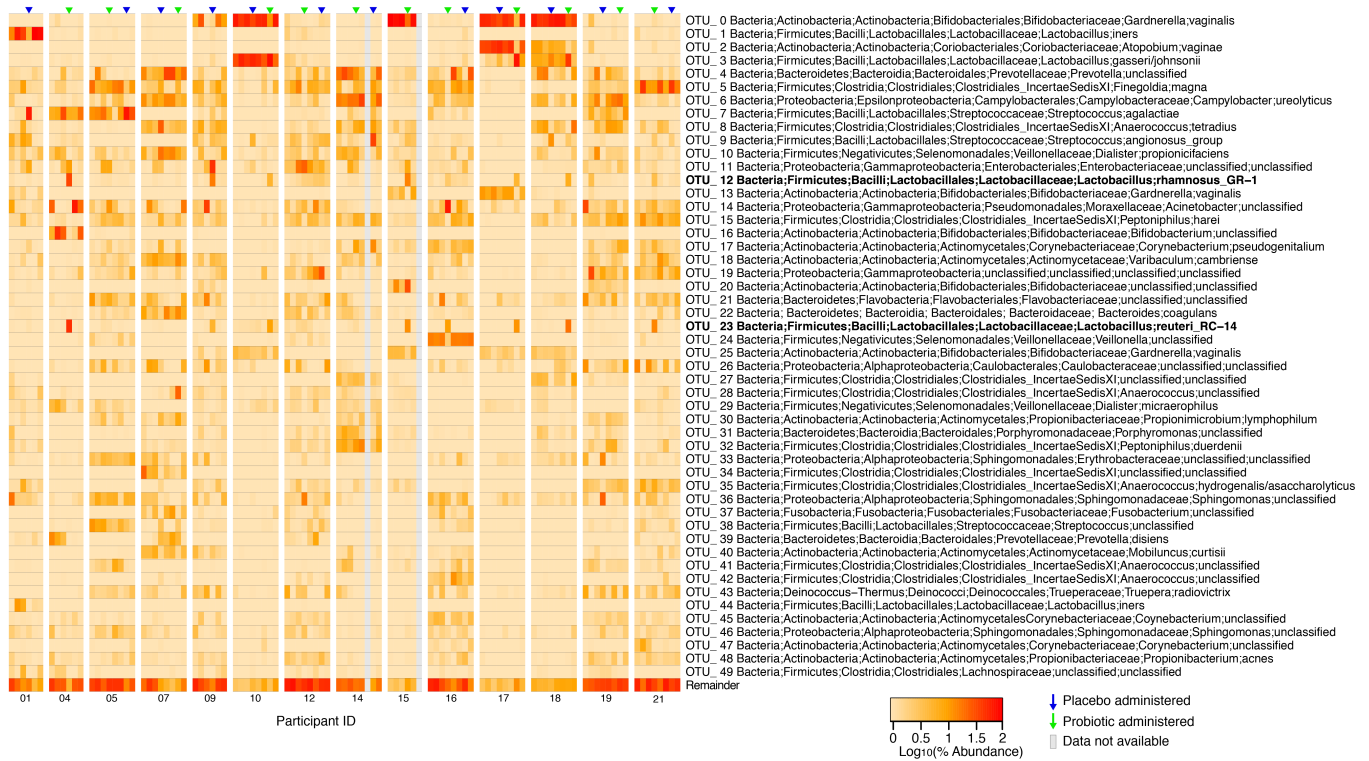


Figure 4-2. Microbiota heat map.

Each column represents the microbiota of a single sample with the 50 most abundant OTUs displayed with their taxonomies and the remainder pooled. Samples are clustered by the participant of origin and organized from first to last visit from left to right. The time points immediately following administration of the placebo or probiotic are indicated with a blue or green arrow (respectively). OTUs representing the putative OTUs for *L. rhamnosus* GR-1 and *L. reuteri* RC-14 have been bolded.

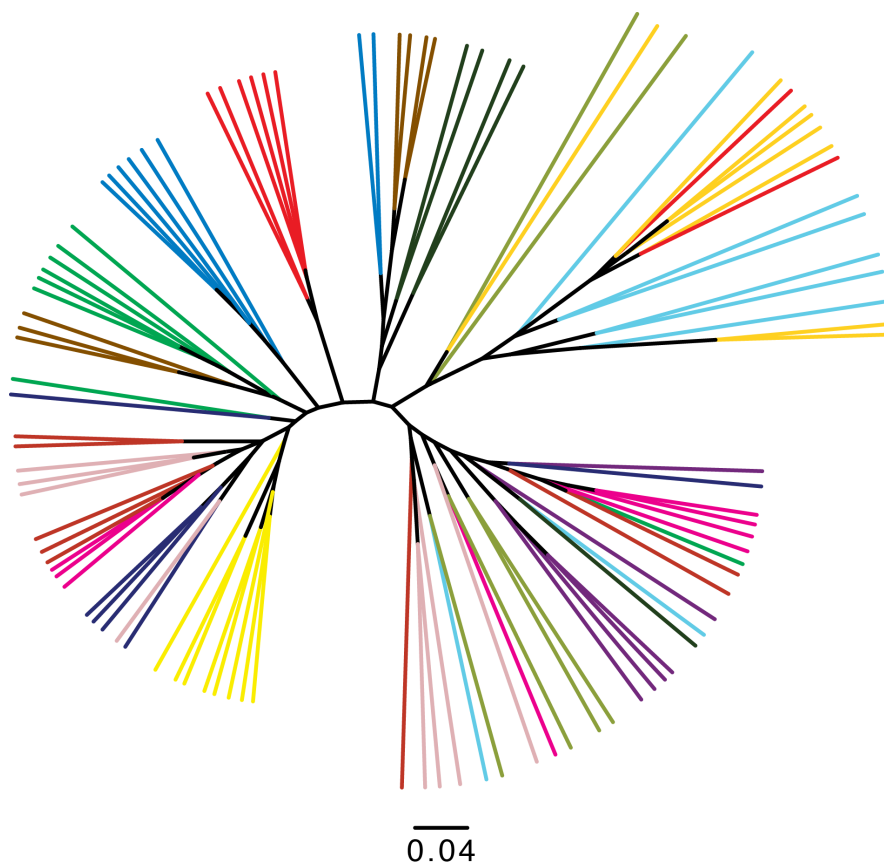


Figure 4-3. UPGMA clustering of all participants microbiota based upon weighted UniFrac distances.

In general, participants cluster most closely with themselves. Tree tips (samples) are colored by the participant of origin.

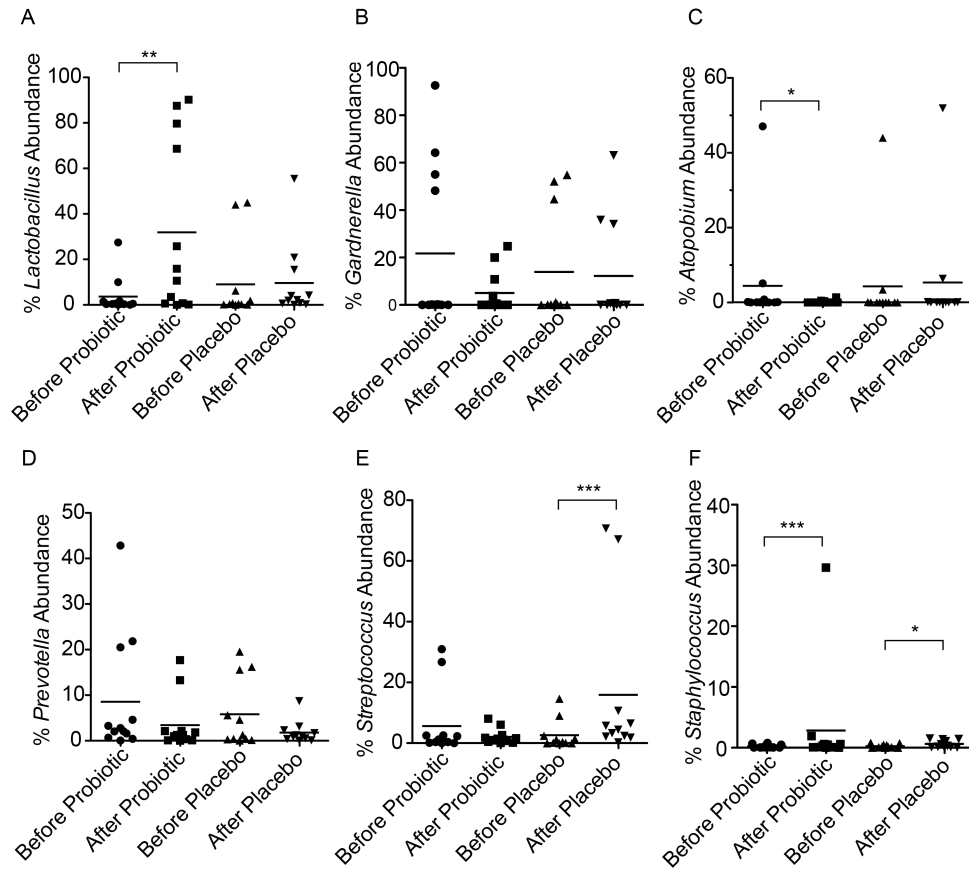


Figure 4-4. Selected genus relative abundances following probiotic and placebo interventions.

(A) *Lactobacillus*, (B) *Gardnerella*, (C) *Atopobium*, (D) *Prevotella*, (E) *Streptococcus*, (F) *Staphylococcus*. Following probiotic administration for 3 days, the proportion of *Lactobacillus* is significantly increased while that of *Atopobium* is decreased and *Staphylococcus* is increased. Placebo interventions increased *Streptococcus* and *Staphylococcus* abundance.

*FDR<0.1, **FDR<0.05, ***FDR<0.01

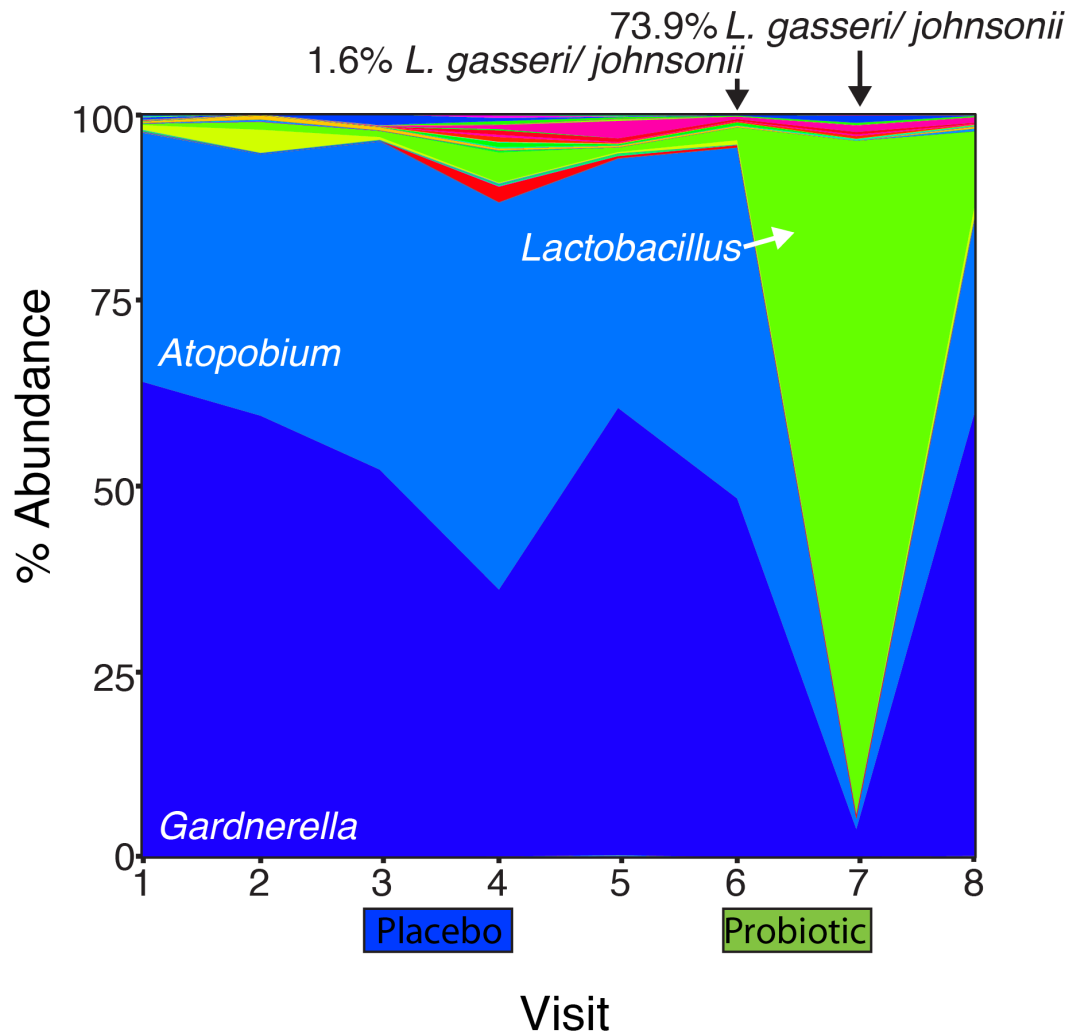


Figure 4-5. Time series of participant 17.

During the administration of probiotic between visits 6 and 7, the abundance of lactobacilli significantly increases, decreasing the proportional abundance of both *Atopobium* and *Gardnerella*. This increase is not due solely to the probiotic strains as a significant increase in indigenous *L. gasseri/johnsonii* takes place.

4.2.4 Metabolome

Using GC-MS, 68 metabolites were detected and a heat map is displayed in Figure S4. The Pearson's correlation coefficient between lactate and lactate producing bacteria was 0.47 ($p < 0.001$), indicating a weak positive correlation (Figure S5). Among those who responded to probiotic intervention and had sufficient material for metabolomics analysis ($n=4$), there was an increase in lactate levels ($p = 0.13$), whereas there tended to be a decrease with placebo ($p = 0.23$). There was no difference in the overall metabolome of all subjects after probiotic or placebo intervention (Figures S6A and S6B respectfully), nor was there a significant difference in any one metabolite. It is worth noting that low yields of metabolites were obtained from some of the collected swabs so lower abundance analytes may not be detectable.

4.2.5 Cytokine/chemokine analysis

Interleukin 5 (IL-5) was found to be significantly up-regulated following probiotic treatment but not following placebo treatment. In other cytokines such as IL-1B, IL-6, TNFa and GM-CSF there were weak positive trends in increase of cytokine levels, however this was also observed in the placebo in some cases (Figure S7).

4.2.6 Microarray

Due to limited yield from RNA samples, only 2 complete sample sets (before and after intervention from the same individual, 4 arrays) were obtained from the probiotic intervention and 3 sets from the placebo intervention (6 arrays). Each sample was analyzed on its own Affymetrix Human Gene 2.0 ST array. Using a paired t-test design to account for paired-samples before and after probiotic administration, 90 probe sets were detected as being differentially expressed (≥ 2 fold change, $p < 0.05$) of which 57 were coding genes or long non-coding RNA (a new feature of the Affymetrix 2.0 ST array) (Table S2A). Two of these were differentially regulated in both conditions (gene list differentially regulated by placebo is displayed in Table S2B), ANKRD20A5P pseudogene (ID cluster 16851230) and a long non-coding RNA (ID cluster 16851230) with no known biological significance.

Many genes of interest were differentially expressed by probiotic treatment including Interleukin 18 (IL18; -2.5 fold after probiotic), CR1-complement receptor (CR1; 10.4 fold), Caspase 14 (CASP14; 5.3 fold, previously observed to function in epithelial development and barrier function (Denecker *et al.*, 2008)) and Toll like receptor 2 (TLR2; 3.84 fold) (Table S2A). To look at functional consequences, GO enrichment was applied showing the most highly affected functions to be pattern recognition receptor activity, complement receptor activity, inflammatory response and gram-positive bacterial cell surface binding (Table 4-2). This was mirrored in the Ingenuity Pathway Analysis (Figure S8). In comparison, the placebo appears to have acted on functions related to epithelial cell differentiation, similar to our previous study (Hummelen *et al.*, 2011).

Table 4-2. Gene Ontology Enrichment in genes differentially expressed by probiotic intervention.

Genes differentially expressed (≥ 2 -fold change, $p < 0.05$) were subjected to GO enrichment analysis. Functions heavily relate to innate immune system responses including responses to both Gram-positive and Gram-negative bacteria.

Function	Enrichment Score	Enrichment p-value	# genes in list	% genes in group	GO ID
Pattern Recognition Receptor Activity	12.82	2.71E-06	3	18.75	8329
Complement Receptor Activity	10.9319	1.79E-05	2	50	4875
Inflammatory Response	10.4433	2.91E-05	6	1.70455	6954
Gram-positive Bacterial Cell Surface Binding	10.4222	2.98E-05	2	40	51637
LPS Receptor Activity	10.4222	2.98E-05	2	40	1875
Defense Response	9.64889	6.45E-05	9	0.840336	6952
Regulation of GM-CSF Production	8.92362	1.33E-04	2	20	32645
Positive Regulation of Response to External Stimulus	8.83166	1.46E-04	4	2.5974	32103
Response to Wounding	8.61585	1.81E-04	6	1.22449	9611
Positive Regulation of Inflammatory Response	8.25985	2.59E-04	3	4.22535	50729

4.3 Discussion

In this study, we aimed to evaluate the effect of 3 days of vaginal administration of *L. rhamnosus* GR-1 and *L. reuteri* RC-14 on the Nugent score of post-menopausal women as well as effects on the microbiota, metabolome and host. As the subjects under study had no symptoms of disease, not unexpectedly, the probiotic prophylaxis did not cause any clinical changes. There were no adverse effects of the probiotic therapy. The probiotic administration resulted in a temporary increase in the relative proportion of *Lactobacillus* in the vagina, which for women whose intermediate score tends to be a predecessor for BV, may help prevent or delay this transition. The near absence of *L. crispatus* (OTU_198), detected in only 1 of 15 women above a 1% threshold and throughout the study at maximum 1.4%, may indicate that the subjects were at a higher risk of BV, since *L. crispatus* is thought to play a protective role in maintaining the vaginal microbiota (Verstraelen *et al.*, 2009).

The increase in lactobacilli and potential decreases in BV-associated organisms such as *Atopobium* following probiotics could have been due to displacement of the host's microbes with the total bacterial load remaining the same; or the total bacterial load increased as a result of adding in exogenous lactobacilli. The observation that Shannon's diversity is not affected by probiotic instillation favors the second option. It is interesting to note the increased relative abundance of *Staphylococcus* as a result of placebo treatment, which may be the result of the antimicrobial actions of titanium dioxide used in the preparation. It is important to note that the probiotics may create an environment conducive to indigenous lactobacilli such as *L. gasseri* and *L. johnsonii*, however this effect may be short lived. A longer treatment time and follow-up might have determined if there was an effect on diversity, but we were interested in whether a short term probiotic application that women might find easy to administer monthly, could increase the lactobacilli count.

In some individuals it was extremely clear when the probiotic strains had been applied. The reason for this not being universal is unclear, but could be due to a particularly resilient indigenous microbiota or women who were non-responders for unexplained reasons (Reid *et al.*, 2010).

We identified a positive correlation between lactate abundance and percentage of lactate producers present $r=0.43$ ($p=9.6 \times 10^{-6}$). Although this correlation seems intuitive, to our knowledge it has never been directly shown. Only the relative abundance of bacterial taxa are measured, and therefore absolute changes affected lactate levels cannot be directly observed. Certain species of *Lactobacillus* have been associated with lower pH and may produce more lactate compared to others (Ravel *et al.*, 2011). There was a trend towards an increase in lactate after probiotic and decrease after placebo intervention, but as there were only four women whom responded to treatment and from whose samples enough material for metabolite analysis could be extracted, the sample size is likely too small to reach significance. Still, the observation that probiotics could potentially increase lactate levels is promising as lactate has been shown to have many beneficial properties in the vaginal tract such as HIV inactivation (Aldunate 2013).

Overall, the administration of the two lactobacilli strains did not induce any more changes than placebo in the metabolome, but lack of material makes this result inconclusive. In our experience and that of others, the metabolomic patterns differ between health and BV, with the latter showing odorous compounds such as cadaverine and putrescine, but these trends were not clearly observed in this study (Yeoman *et al.*, 2013).

In addition to looking at the bacterial communities, we sought to examine host responses to probiotic treatment. It has been well established that probiotic strains can affect host transcription in the gut in a strain specific manner (van Baarlen *et al.*, 2011) but this is the first time similar studies have been carried out in the vagina. Our findings show that *L. rhamnosus* GR-1 and *L. reuteri* RC-14 had an immunomodulatory effect working on important central inflammatory mediators complement receptor 1, toll like receptor 2 and IL-18. Though limited in the number of subjects available for analysis, the strength of this analysis is the use a paired study design. Interleukin 18 is a proinflammatory cytokine inducing cell-mediated immunity via interferon gamma though it also has effects on B cells and IgE production (Biet *et al.*, 2002). In the female reproductive tract it may be of relevance in preterm birth (Jacobsson *et al.*, 2003) indicating microbial invasion of the amnion, but it also has protective roles against genital herpes simplex 2

(Harandi *et al.*, 2001). The effect of probiotic treatment on IL-18 expression may not be a direct interaction, but rather indicative of anti-viral actions of the probiotics resulting which has previously been hypothesized and shown *in vitro* (Aldunate *et al.*, 2013; Bolton *et al.*, 2008). Alternatively it may reflect a general up-regulation of the innate immune system by the probiotic strains. Unfortunately, our cytokine/chemokine panel did not cover IL-18 to help support this finding. Alternatively the up-regulation, of complement receptors 1 and 3a, as well as TLR2 both are indicative of increased anti-bacterial innate immune system activity. TLR2 specifically is involved in detection of Gram-positive surface markers (including lactobacilli) (Schwandner *et al.*, 1999) while the complement receptors are important for phagocytosis and inflammatory cascades in response to bacterial infections (Zipfel and Skerka, 2009). *L. plantarum* has previously been shown to modulate TLR2 expression which may play a role in modifying infection response (Rizzo *et al.*, 2013). In addition, our cytokine/chemokine multiplex results may indicate the up-regulation of interleukin 5 which is most commonly associated with eosinophil activation (Molfino *et al.*, 2012). Though commonly associated with parasites, eosinophils have also been associated with *Actinomyces*-like organisms in the urogenital tract and may display some activity against bacterial infection (Kaya *et al.*, 2012). These seemingly pro-inflammatory responses may be beneficial in aiding the host immune system to help clear BV-associated pathogens to restore a more normal microbiota of symbionts. Microarray analysis failed to corroborate the finding that interleukin 5 was up-regulated, however the two individuals surveyed by microarray did not show up-regulation by cytokine analysis either. If all participants could have been surveyed this result may have been different.

Interestingly, caspase 14 plays a role in development and barrier function of the epidermis (Denecker *et al.*, 2008) and we had previously observed its differential regulation associated with vaginal dryness and dysbiosis in a subset of post-menopausal women. Its up-regulation following probiotic treatment may be evidence of improvement in strength and integrity of the vaginal epithelium, though further research is needed to verify this claim. Indeed probiotics are thought to positively act on barrier function (Sultana *et al.*, 2013).

In conclusion, this study demonstrated the use of a systems-wide interactomics approach to examine how probiotic application might modulate the vagina in post-menopausal women. The short duration therapy increased the lactobacilli, as well as modulating inflammatory markers. As the transition from healthy to intermediate to BV is such a common occurrence, women may feel that a three day intravaginal probiotic may be beneficial. A larger study is needed to support this, but the present study showed no harm and the potential for such a benefit.

4.4 Methods

4.4.1 Participant recruiting and study design

The protocol for this trial and supporting CONSORT checklist are available as supporting information; see Checklist S1 and Protocol S1. A total of fourteen post-menopausal women were enrolled in the study from a clinical site in Ontario, Canada. Inclusion criteria were individuals with an intermediate Nugent Score (4-6) who were post-menopausal (40 to 80 years old, not having had a menstrual period for the last 12 months, currently in a mutually monogamous sexual relationship or not sexually active, agreeing to be abstinent 72 hours prior to each study visit and agreeing to refrain from intercourse for 48 hours after treatment, agreeing to abstain from use of other intravaginal products throughout the study and in good general health. Exclusion criteria were use of intravaginal products within three months prior to visit 1, a history of immunosuppressive drug therapy, chemotherapy or radiation therapy, a medical condition which might compromise immune system function (ex. cancer, leucopenia, HIV, organ transplant), antibiotic and/or antifungal medication within the last 4 weeks, oral probiotic use within 3 months prior to visit 1, significant changes in diet during the course of the study (based on self report), induced menopause due to surgical/medical intervention (ex. hysterectomy), currently taking estrogen therapy, a history of drug or alcohol abuse, known allergy to either of the probiotic strains or product excipients and participation in a clinical trial involving an investigational product/device within the past three months or who are scheduled to participate in another clinical study concurrently. No formal sample

size calculation was carried out and the number of subjects to be randomized was based on feasibility and practical considerations for an exploratory study. The study was approved by Health Canada (Clinical Trial Application File No. 180061) and is registered with clinicaltrials.gov under accession number (NCT02139839).

The study design is diagramed in Figure S1. Briefly, after obtaining written informed consent, participants were enrolled on day one and subsequently returned for baseline appointments on days 5 and 15 (visits 2 and 3 respectively). On visit 3, the participants were randomized to either receive 3 days of *L. rhamnosus* GR-1/*L. reuteri* RC-14 probiotic (Chr. Hansen, minimum 2.5×10^9 CFU of each strain) or visually identical placebo (49mg Gelatin and 1mg titanium dioxide) self-administered by having the participant insert the capsule as far as comfortable into their vagina and then lying in a horizontal position for 15 minutes. Randomization was carried out using a random number generator. Both study staff and study participants were blinded as to the study treatment being given. The capsules were to be administered twice a day for 3 days with the participant returning on the fourth day for sampling. A feminine hygiene pad was applied for several hours after capsule implantation. There was then a 17 day washout period with sampling again at day 26. At day 36 participants were again sampled, individuals who received probiotic in the first treatment period then received placebo and vice versa. On day 40, participants returned for sampling after the treatment period and were followed up with on day 47 and again on day 129, only if they had positive culture results for the presence of the probiotic. At all visits, 5 swabs were collected: (i) Dacron swab for bacterial DNA extraction, (ii) Dacron swab for multiplex cytokine/chemokine analysis, (iii) Dacron swab for metabolomics analysis, (iv) a cytobrush (Cytobrush plus GT, Cooper Surgical Inc. USA) brushed against the vaginal wall and stored in 700 μ L RNeasy lysis buffer for total RNA extraction (Qiagen), and (v) Dacron swab for Nugent Score. McNemar's test was applied to examine changes in Nugent score and other symptoms.

4.4.2 Microbiome profiling

Vaginal swabs for microbiome analysis were extracted using the QIAamp DNA stool mini kit (Qiagen). Swabs were vortexed in 1 mL buffer ASL before removal of the swab

and addition of 0.1mm zirconia/silica beads (Biospec Products) with 2, 30 second rounds of bead beating at full speed with cooling on ice in between (Mini-BeadBeater; Biospec Products). Sample amplification for sequencing was carried out using the forward primer (CCATCTCATCCCTGCGTGTCTCCGACTCAGxxxxxCWACGCGARGAACCTTAC C) and the reverse primer (CCTCTCTATGGGCAGTCGGTGATACRACACGAGCTGACGAC) where xxxxx was a sample specific nucleotide barcode, the 5' end is the adapter sequence for the Ion Torrent sequencer and the sequences following the barcode are complementary to the V6 rRNA region. Amplification was carried out in 42 μ L with each primer present at a 10 μ L (3.2 pMol/ μ L stock), 20 μ L GoTaq hot start colorless master mix (Promega) and 2 μ L extracted DNA. The PCR protocol was as follows: initial activation step at 95°C for 2 minutes and 25 cycles of 1 minute 95°C, 1 minute 55°C and 1 minute 72°C. PCR products were quantified with a Qubit 2.0 Fluorometer and the high sensitivity dsDNA specific fluorescent probes (Life Technologies). Samples were mixed at equimolar concentrations and purified with the QIAquick PCR Purification kit (QIAGEN).

All subsequent work was carried out at the London Regional Genomics Centre (LRGC, lrgc.ca, London, Ontario, Canada). Briefly, samples were prepared with an Ion OneTouch System (Life Technologies) and sequenced on an Ion Torrent Personal Genome Machine sequencer on a 316 chip (Life Technologies).

Resulting Reads were extracted and de-multiplexed using modifications of in-house perl and unix-shell scripts (Hummelen *et al.*, 2011) with OTUs clustered at 97% identity. Table S3 displays the nucleotide barcodes and it's corresponding sample. Reads were deposited to the Short Read Archive (BioProject ID: PRJNA244441). To control for background contaminating sequences, a no-template control was also sequenced, and any individual sequence unit belonging to an OTU present at $\geq 1\%$ abundance in the NTC was removed before re-clustering with uclust 3.0.612.

Automated taxonomic assignments carried out by examining best hits from comparison the Ribosomal Database Project (rdp.cme.msu.edu) and manually curated as before (Hummelen *et al.*, 2011).

Alpha and beta-diversity analysis were made using the OSX distribution of QIIME (MacQIIME 1.70, wernerlab.org/software/macqiime). OTU tables were rarified to 1060 reads for all analysis to control for uneven sampling depth. To avoid inappropriate statistical inferences made from compositional data, log-ratios, a method previously described by Aitchison and adapted to microbiome data was used (Aitchison, 1981; Aitchison and Egozcue, 2005; Faust *et al.*, 2012) with paired t-tests for comparisons of genus and species level data. The False Discovery Rate (FDR) method was used to control for multiple testing with a significance threshold of FDR=0.1. All statistical analysis, unless otherwise indicated, was carried out using R (r-project.org).

4.4.3 Microarray analysis

Vaginal cytobrushes were extracted as previously (Hummelen *et al.*, 2011). Briefly a vaginal cytobrush was centrifuged at 5000xg for 10 min at 4°C and the supernatant discarded. The pellet was then extracted with TRIzol (Life Technologies) following the manufacturer's protocol before being DNase treated with Turbo DNA-free kit (Life Technologies). Sample quantity and quality were determined using a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, USA) and Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, USA). Samples were prepared for analysis on the GeneChip Human 2.0 ST array (Affymetrix, Santa Clara, USA) at the LRGC following standard protocols.

Probe level data was imported into Partek Genomics Suite (Partek, St. Louis, USA) using the RMA algorithm. Gene expression data was deposited in the Gene Expression Omnibus Database with the accession number GSE54363.

To study the effects of the probiotic and placebo treatments on host gene expression, and considering the study design, a paired t-test was used to evaluate gene expression changes in each individual immediately before and after probiotic/placebo administration. This yielded two individuals (14 and 16) with arrays immediately preceding and following the probiotic period treatment and three individuals (01, 14, 05) with arrays surrounding the placebo treatment period. For other participants, insufficient yield and/or quality resulted in a lack of paired samples, and they were thus excluded from downstream analysis.

4.4.4 Sample preparation GC-MS

Samples were collected from the mid-vaginal wall using a Dacron Swab. Vaginal swabs were pre-cut into 1.5 mL tubes and weighed prior to and after sample collection to determine the mass of vaginal fluid collected. Samples were stored at -80°C until analysis. After thawing, brushes were trimmed and eluted in methanol-water (1:1) in 1.5 mL microcentrifuge tubes. The weight of each sample was divided by the weight of the lightest sample and this fraction was multiplied by 200 μL to determine the volume of methanol-water to add to each sample. This corresponded to a volume of 200-382 μL , depending on the mass of vaginal fluid collected. A blank swab eluted in 200 μL methanol-water was included as a negative control. All samples were vortexed for 10 s to extract metabolites, centrifuged for 5 min at $10\,000 \times g$, vortexed again for 10 s after which time the brushes were removed from tubes. Samples were centrifuged a final time to pellet cells and 150 μL of the supernatant was transferred to a GC-MS vial. Next, 2.5 μL of 2 mg/mL ribitol was added to each vial as an internal standard. Samples were then dried to completeness using a SpeedVac. After drying, 100 μL of 2% methoxyamine•HCl in pyridine (MOX) was added to each for derivatization and incubated at 50°C for 90 min. 100 μL N-Methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) was then added to each vial and incubated at 50°C for 30 min, then transferred to micro inserts before running on GC-MS (Agilent 7890A GC, 5975 inert MSD with triple axis detector, 30m DB5-MS column with 10m duraguard column). Samples were analyzed once for untargeted whole metabolome analysis, and a second time on selective ion monitoring (SIM) mode specific for lactate using the reference ions 117, 147, 191 and 219. Solvent delay was 10 min and sample injection volume was 1 μL . All samples were run in random order and one was run multiple times throughout to ensure machine consistency.

4.4.5 Lactate relative abundance quantification

Chromatograms were deconvoluted in Chemstation (Agilent) and lactate relative abundance determined by peak area using the auto integration function. Lactate relative abundance was plotted against the percentage of lactate producers (*Lactobacillus*, *Bifidobacteria*, *Atopobium*, *Streptococcus*, *Staphylococcus*, *Weisella*) present in each sample to determine the correlation between lactate levels and lactate producing bacteria.

The correlation value was determined in R using a Pearson's correlation. Paired Welch's t-tests were used to determine if probiotic or placebo intervention had a significant effect on lactate levels using a significance threshold of $p=0.05$.

4.4.6 Whole metabolome analysis

Chromatogram files were converted to ELU format using the AMDIS Mass Spectrometry software (Stein, 1999). Chromatograms were then aligned and abundance of metabolites calculated using the Spectconnect software (Styczynski *et al.*, 2007), with the support threshold set to low. In order to determine changes in the metabolome due to probiotic or placebo, Principle component analysis (PCA) was conducted in SIMCA (Umetrics) using the relative abundance matrix (RA) output from Spectconnect. Data were mean centered and pareto scaled prior to PCA. A Mann-Whitney U test was then used to determine metabolites that were significantly altered by intervention ($p < 0.05$).

4.4.7 Cytokine/chemokine measurement

Swabs were resuspended in 200 μ L extraction buffer (20 mM Tris-HCl pH7.5, 150 mM NaCl, 1mM PMSF, 0.05% Tween 20 and 1 uL/mL protease inhibitor cocktail (Roche), vortexed, incubated overnight at 4°C, swab removed and then 50 μ L more extraction buffer was added before being stored at -80 °C. The resulting samples were thawed on ice and loaded onto a Milliplex Human High Sensitivity Cytokine/Chemokine Panel (EMD Millipore, Billerica, Mass, USA) and analyzed for IL-1B, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12p70, IL-13, IFN γ , GM-CSF, and TNF α . Results for IL-2, IL-7, IL-8, IL-10, IL-13, IFN γ were not displayed as they were consistently outside the assay range and there was limited material for reanalysis. The plate was analyzed using a Bio-Plex 200 System (Bio-Rad Laboratories, CA, USA) with cytokine/chemokine levels being generated automatically from standard curves using the Bio-Plex Manager software (v.4.1.1 Bio-Rad). In cases where the analyte was detected, but below the limit of detection, $\frac{1}{2}$ the LOD was used for analysis. Results were normalized to total protein as determined with the Qubit Protein Assay Kit and Qubit 2.0 Fluorometer (Life Technologies, CA, USA). Paired t-tests were used to make comparisons.

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Chapter 5

5 The microbiota at multiple body sites during pregnancy in a rural Tanzanian population and the effects of Moringa supplemented probiotic yogurt.

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Ethics approval documentation is provided in Appendix E

5.1 Introduction

The United Nations estimates that 805 million people are chronically undernourished throughout the world (FAO, 2014). Malnutrition is an additional challenge for pregnant women in many developing countries, along with access to medical care, exposure to malaria and HIV, and financial burden. Limited food accessibility, access to food with poor nutritional value, and cultural traditions result in deficiencies in essential nutrients. Micronutrients are dietary components, often referred to as vitamins and minerals. Iron, iodine, vitamin A, folate and zinc are recommended during pregnancy (CDC, 2014), although unfortunately not all women are recipients. In many developing countries, including Tanzania, maternal and newborn death and morbidity are major issues, often propagated by malnourishment (UNICEF, 2013; Tanzania National Bureau of Statistics, 2011). Efforts to reduce infant mortality by supplementation with vitamin A have failed (Masanja *et al.*, 2014). Fermented foods, including dairy products, have long been part of tradition in many parts of the African continent. In Tanzania (Franz *et al.*, 2014), while milk consumption is high (per capita milk consumption of 39 kg/annum), commercial yogurt is not affordable to many, and no health-promoting probiotic yogurt is sold in traditional retail outlets. The establishment of community kitchens in Mwanza in 2004

(Reid *et al.*, 2013) has provided affordable probiotic yogurt to around 4,000 people each day, with some measurable benefits to immunity and gut health (Irvine *et al.*, 2010; Hummelen *et al.*, 2011).

There is a clear link between the gut microbiota and nutrient uptake (Hullar and Lampe, 2012), and while the gut microbiota has been reported to be relatively stable in non-pregnant healthy individuals (Roager *et al.*, 2014), changes have been reported in pregnant American and European women, where Firmicutes, along with Bacteroides dominate. However, during the first and third trimesters, Proteobacteria and Actinobacteria increase while richness decreases (Collado *et al.*, 2008; Koren *et al.*, 2012; Jost *et al.*, 2014). Given the role of diet on shaping the microbiota (De Filippo *et al.*, 2010; Cotillard *et al.*, 2013) and the potential additional nutritional stresses within impoverished African rural settings, we sought to assess the gut microbiotas of Tanzanian pregnant women. Also, since vaginal and oral microbiomes are suspected to influence pregnancy outcomes (Mysorekar and Cao, 2014), and longitudinal studies seldom have included sampling after birth, we aimed to better characterize these microbiotas longitudinally.

Few studies have been undertaken on the human milk microbiota of African women, even though the transfer of these organisms to infants is critical to development, immunity, risk of disease later in life and cognitive function (Gruszfeld and Socha, 2013). In a Mozambique study, human milk was found to contain mostly lactic acid bacteria, but proportions were altered by HIV infection (Gonzalez *et al.*, 2013).

The probiotic *Lactobacillus rhamnosus* GR-1 was selected for use as an intervention in this study as it has a safe history of use (Irvine *et al.*, 2010; Hummelen *et al.*, 2011; Martinez *et al.*, 2009) and was already produced in Mwanza, Tanzania as part of the Western Heads East network of microenterprise community-run kitchens (Wenner, 2009). For the purposes of the present study, the yogurt was further supplemented with dried ground *Moringa oleifera* (Moringa), a readily available plant, rich in vitamin A, proteins, carbohydrate, fibre, minerals, calcium, magnesium, phosphorus, potassium, copper, iron, zinc, and manganese (van Tienen *et al.*, 2011) and used commonly in

management of malnutrition (Thurber and Fahey, 2009). An open label study was conducted to test a Moringa-supplemented probiotic yogurt, and to determine if it altered the oral, vaginal, gut or breast milk microbiotas, and had an impact on gestation and health parameters in newborns.

5.2 Materials and Methods

5.2.1 Study design and participants

The study protocol was approved and received ethical clearance from both the Medical Research Coordinating Committee of the National Institute for Medical Research (Mwanza, Tanzania) as well as from the Health Sciences Research Ethics Board at Western University (London, Canada). The study was registered with clinicaltrials.gov (NCT02021799). Participants provided written consent, and in the case where they could not write, a thumbprint was obtained before sample collection and subsequent analysis.

Women who were attending the antenatal clinic at Nyerere Dispensary in Buswelu, district of Ilemela, Mwanza Region, Tanzania, were recruited into the study if they were between the ages of 18 and 40 as well as between the gestational ages of 12 and 24 weeks. Gestational age was determined by last menstrual period as identified in the participant's clinic records. To confirm, or if last menstrual period was unavailable, approximation of gestational age was based upon measurement of fundal height.

Originally, participants were further grouped into states of nutritional status (nourished or under-nourished) based upon having a mid-upper arm circumference (MUAC) less than 235 mm if undernourished, and deviations from expected weight for gestational age (Gueri *et al.*, 1982). As this was a pilot study, sample size was based on participant availability.

5.2.2 Probiotic yogurt

Subjects were randomly assigned (using a random number generator) to the intervention group (produced fresh daily yogurt containing $\sim 1 \times 10^{10}$ CFU *Lactobacillus rhamnosus* GR-1 per 250 g unit confirmed by regular quality control tests, and 4.3 g of dried ground *Moringa oleifera* leaves), or control group (no intervention). Administration of the yogurt

amounted to 13% required daily intake of protein (9.95 g), 39% calcium (385 mg), 35% vitamin A (269 RAE), 81% vitamin B2 (1.1 mg) and 4% iron (1.2 mg). The probiotic group received the 250 g yogurt six days a week from the time of recruitment until exiting the study, which occurred one week to one month postpartum. Due to a lack of cold storage available to most participants, yogurt was delivered daily by a study employee who observed and recorded compliance. Participants in the probiotic group consumed yogurt for an average of 88 days \pm 31 SD, while the control group had no form of intervention.

5.2.3 Assessment and sample collection

At the initial and monthly follow up visits from recruitment until birth, which occurred between August 2012 and April 2013, weight was measured to the nearest 0.1 kg using an analogue scale and MUAC was measured to 1mm using a tape measure. A physical examination was also performed at these visits, which included measurement of blood pressure, heart rate and general medical history. Two vaginal swabs were collected at the mid-point of the vagina using CultureSwab polyester-tipped swabs (BD Biosciences, Mississauga, Ontario); one was used for evaluation of bacterial vaginosis (BV) by Nugent Scoring (Nugent *et al.*, 1991), while the second was used for microbial DNA extraction. Fecal samples and saliva samples were also collected in sterile collection containers and frozen at -80°C until analysis. A mid-stream urine sample was analyzed for urinary tract infection using a multi-test dipstick system (Rapid Response Urine Dipstick, BTNX Urinalysis, Laja Pharmacy, Mwanza, Tanzania). Hemoglobin levels were determined using a HemoCue® Hb 201+ analyser (HemoCue, Ängelholm, Sweden)

Blood samples were collected from all participants at the first, mid and final visits in Vacutainer tubes containing potassium EDTA (BD Biosciences, Mississauga, Ontario). Plasma was separated from the samples before being frozen at -80°C until analysis. Vitamin A levels were determined by HPLC, while the total plasma protein levels were determined by a colorimetric Biuret assay by the London Health Sciences Laboratory Services Group (London, Canada).

The first post-partum visits occurred at 3 days after birth and the final visit occurred one week to one month after the first post-partum visit. The same information and samples were collected as during the follow-up visits, however, a human milk sample was collected in sterile collection containers from the mother and stored at -80°C until analysis. Anthropometric data were collected from the infant including, weight as measured on an analogue baby scale to the nearest 0.1 kg, while head circumference, length and MUAC were measured using a tape measure to the nearest 0.1 cm. In addition, the sex of the infant, place of birth, and whether the baby was born vaginally or by caesarean section were recorded. Fecal samples were collected from the infant's diaper, and infant saliva samples were collected using a BBL™ CultureSwab™ (BD diagnostics, Mississauga, Ontario) and stored at -80°C until analysis.

5.2.4 Dietary recall analysis

Forty-eight hour dietary recall interviews were conducted by a trained study translator at each visit. Information from these interviews was analyzed for nutritional content and caloric intake using the ESHA Food Processor SQL (version 9.8) which provides access to an extensive database of comprehensive nutrition information to allow evaluation of a subject's diet and fitness. Although this program utilizes North American food composition tables, nutritional data for local Tanzanian foods and recipes were imputed into the software from Tanzanian food composition tables prepared by Muhimbili University of Health and Allied Sciences, Tanzania Food and Nutrition Center and Harvard School of Public Health (Lukmanji and Hertzmark, 2008). In addition, when serving sizes per se, or sizes of specific food items were not clearly stated in the interview, the average portion quantity, as outlined by the Tanzanian Food Composition Tables, was used. Nutritional data for Moringa were obtained (M. Broin, unpublished data) and also inputted into ESHA. The average intake of calories, protein, fat, carbohydrates, saturated fatty acids, monounsaturated fatty acids, polyunsaturated fatty acids, calcium, vitamin A, folate and zinc were calculated over the 48-hour period to determine daily consumption. The same was done for the 48-hour dietary recalls collected at the first post-partum and final visits, to obtain a value representing the daily average intake of the above listed nutrients postpartum.

5.2.5 Microbial DNA extraction and sequencing

DNA was extracted from all samples using the PowerSoil®-htp 96 Well Soil DNA Isolation Kit from MoBio (Carlsbad, California) as per the manufactures protocol with modifications as outlined by the Earth Microbiome Project (version 4_13).

Approximately 250 mg of fecal sample, 500 µL of saliva and 500 µL of breast milk were used for the extractions. Samples were sequenced by amplifying the V4 hypervariable region of the 16S rRNA gene using bacterial/archaeal primers 515F and 806R according to previously described methods (Caporaso *et al.*, 2012) modified for the Illumina MiSeq platform.

5.2.6 Microbial community analysis

Obtained reads were quality filtered and de-multiplexed using the open source software QIIME (split_libraries_fast.py with default parameters for Illumina sequencing) (Caporaso *et al.*, 2010). A total of 974 samples were sequenced, yielding 15,596,127 sequences total with an average of 16,012 sequences \pm 11,840 SD per sample. Samples that generated less than 1000 sequences after quality filtering were discarded. The remaining sequences were then binned into operational taxonomic units (OTUs) using closed-reference OTU picking based on 97% identity using the May 2013 build of the Greengenes reference database (McDonald *et al.*, 2012). Demultiplexed reads and associated metadata were deposited in the Qiita and EBI databases available through study ID 2024 (qiita.ucsd.edu).

5.2.7 Statistical analysis

All statistical analyses were carried out in R using unpaired Welch's t-tests with Benjamini Hochberg's false discovery rate (FDR) method with a $q < 0.05$ cutoff unless otherwise indicated. Analysis of alpha (Shannon diversity index) and beta diversity (weighted UniFrac and Bray Curtis) were performed in QIIME. All plotting was carried out in R with OTUs summarized to genus level and filtered to only include organisms which composed $>1\%$ of the total dataset (vaginal heat map Figure 5-6C) or $>0.01\%$ (entire dataset: Figure 5-3). For association of individual genera with yogurt/control treatment in infant fecal samples, the dataset was filtered to include only infant fecal

samples, summarized to the genus level and genera were only kept if they represented a minimum 0.1% of the total reads. Significantly differentially abundant genera were determined by comparing groups using ALDEx2 (version 0.99.2) (Fernandes *et al.*, 2013; Fernandes *et al.*, 2014) by Wilcoxon rank-sum test with FDR correction of centered log-ratio transformed data using 1000 Monte-Carlo instances drawn from the Dirichlet distribution with a minimally informative uniform prior of 0.5. Taxa that had 0 counts in all samples were removed. This approach accurately estimates the range of technical variation inherent in microbiome datasets for all taxa, including those where some samples contain a 0 and some where they contain a positive count (Fernandes *et al.*, 2013; Fernandes *et al.*, 2014).

5.2.8 Human milk analysis

The breast milk samples collected at the final visit for all women in the study were analyzed for fat, protein and lactose content. From the calculated lactose amount, total carbohydrates were estimated. A total of 12 milk samples were excluded from this analysis due to the provider being HIV positive. Thus, data was obtained for 9 participants from the control group and 6 participants from the probiotic yogurt group. Samples were homogenized for 10 seconds prior to a 500 μ L aliquot being removed for DNA extraction using a sonicator (VCX 130; Chemical Instruments AB, Sollentuna, Sweden). The remaining sample (approximately 500 μ L – 1 mL) was used for nutrient analysis. Lactose content was measured as described by Fusch *et al.* (Fusch *et al.*, 2011). Fat was extracted from the milk using Mojonnier ether extraction and then gravimetrically analyzed to get total fat content (Choi *et al.*, 2013). Protein content of the breast milk was determined as per the methods of Choi *et al.* (2013). The true protein content was determined by subtracting the non-protein nitrogen from the total nitrogen and multiplying this by 6.25 (Lonnerdal *et al.*, 1984).

5.3 Results

5.3.1 Clinical outcomes

The women were enrolled from a 20 km area around the Nyerere Dispensary on the outskirts of Mwanza. No verbal or written complaints or side effects were reported from consumption of the yogurt. Figure 5-1 shows a CONSORT-compliant study flow chart. Baseline demographic data for the nourished, undernourished, probiotic and control groups are presented in Table 5-1 and showed no differences between groups, except for weight related metrics between the nourished and undernourished groups. For interpretative purposes, a blood level for vitamin A of 0.9 mmol/L equals 25.8 mg/dL just below the normal range of 30-65 µg/dL.

Using 48-hour dietary recalls, women in both groups who were initially categorized as nourished or undernourished were found to have consumed similar calories and macronutrients. In comparison to reference dietary intake (RDI) values for Canadian pregnant women, the Tanzanian women only met requirements for carbohydrates and iron throughout pregnancy – all other micro- and macronutrients were below 90% of the Canadian RDI, including folate. Yet, only three babies (5%) were delivered preterm, while all other births occurred without adverse events, and overall, babies examined by the local physician were deemed to be healthy. It was noted at the study midpoint whereby there was increased calorie, carbohydrate, thiamine, folate and zinc intake in the probiotic group while blood protein levels were also higher, however these were not statistically supported after multiple testing corrections (Table 5-2). We noted that calcium levels in the probiotic group were significantly higher than all others (Tables 5-2 and 5-3).

Fat, carbohydrate and protein content of the breast milk and hemoglobin, blood retinol, and blood protein concentrations were not altered by yogurt intake, nor was gestational weight gain or newborn weight at the final visit (Table 5-3).

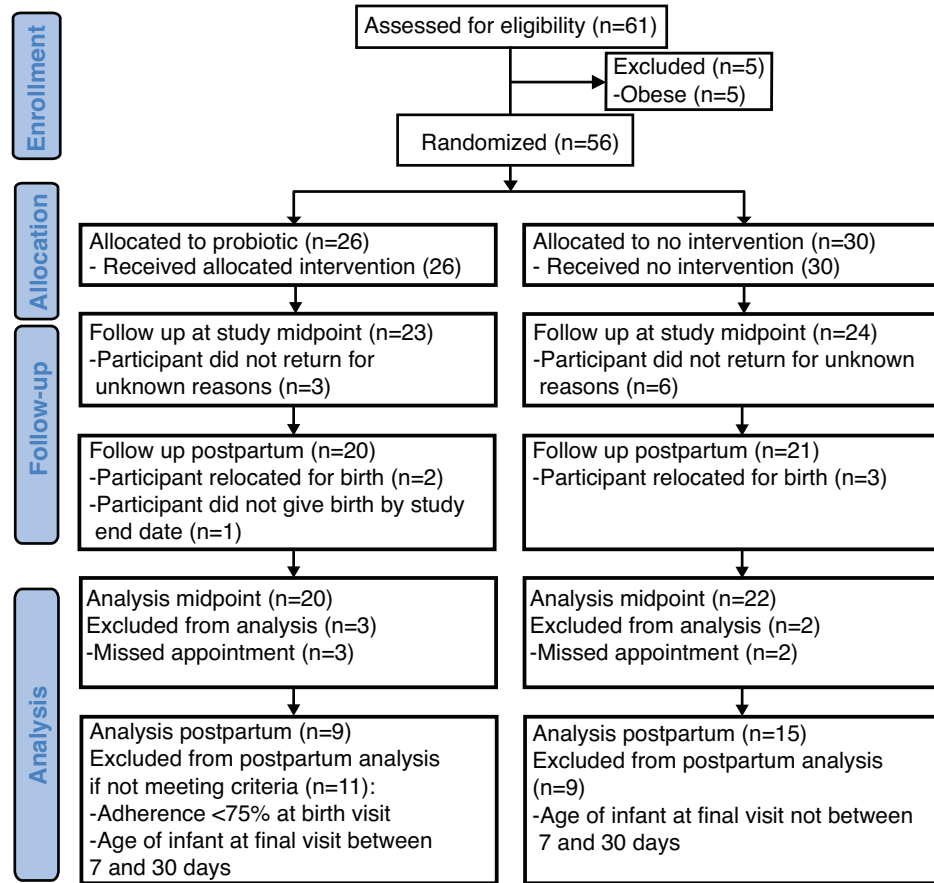


Figure 5-1. CONSORT flow diagram.

Table 5-1. Enrolment demographics for probiotic, control, undernourished and nourished groups (values \pm SD unless otherwise indicated).

	Probiotic (n=23)	Control (n=24)	q-value	Undernourished (n=29)	Nourished (n=18)	q-value
Gestational age (weeks)	21 \pm 3	20 \pm 5	0.915	21 \pm 4	21 \pm 4	0.976
Age (years)	23.30 \pm 4.42	24.38 \pm 5.01	0.915	24.31 \pm 5.47	23.11 \pm 3.12	0.958
Height (m)	1.60 \pm 0.07	1.62 \pm 0.06	0.915	1.61 \pm 0.06	1.62 \pm 0.07	0.976
Weight (kg)	54.8 \pm 5.8	57.5 \pm 7.4	0.915	52.6 \pm 4.3	62.0 \pm 5.9	3.11E-05
% Deviation ^a				72.71 \pm 10.14	96.75 \pm 6.58	2.19E-11
MUAC ^b (mm)	261 \pm 22	262 \pm 22	0.965	256 \pm 22	271 \pm 19	0.109
Hemoglobin (g/L)	110.48 \pm 13.68	103.38 \pm 17.21	0.915	107.34 \pm 16.70	106.06 \pm 14.75	0.976
HIV (n)	2	3		3	2	
Malaria (n)	3	4		4	3	
Syphilis(n)	2	2		4	0	
Previous pregnancies (n)	2.09 \pm 2.07	2.00 \pm 1.48	0.965	2.29 \pm 1.94	1.67 \pm 1.46	0.935
Age of first pregnancy	18.06 \pm 9.54	18.88 \pm 9.20	0.965	18.19 \pm 1.86	19.00 \pm 1.97	0.976
Calories (kcal/day)	1695 \pm 752	1759 \pm 781	0.965	1719 \pm 754	1742 \pm 789	0.976
Protein (g/day)	57 \pm 23	58 \pm 21	0.965	57 \pm 21	58 \pm 25	0.976
Carbohydrates (g/day)	278 \pm 118	276 \pm 121	0.965	277 \pm 122	277 \pm 116	0.997
Fat (g/day)	46 \pm 33	52 \pm 33	0.915	48 \pm 32	51 \pm 35	0.976
Vitamin B12 (μ g/day)	0.69 \pm 0.62	0.88 \pm 0.81	0.915	0.80 \pm 0.76	0.77 \pm 0.69	0.976
Thiamine (mg/day)	0.78 \pm 0.39	0.90 \pm 0.44	0.915	0.86 \pm 0.39	0.82 \pm 0.46	0.976
Calcium (mg/day)	187 \pm 119	236 \pm 175	0.915	199 \pm 168	233 \pm 120	0.976
Vitamin A (RAE ^c /day, median [IQR])	316 (70-869)	546 (123-1019)	0.915	261 (98-846)	636 (170-1018)	0.958
Folate (μ g/day)	249 \pm 212	294 \pm 199	0.915	269 \pm 206	276 \pm 208	0.976
Iron (mg/day)	49 \pm 51	40 \pm 41	0.915	41 \pm 45	50 \pm 48	0.976
Zinc (mg/day)	5.4 \pm 2.7	5.9 \pm 2.4	0.915	5.8 \pm 2.5	5.5 \pm 2.6	0.976
Blood vitamin A (mmol/L)	0.90 \pm 0.27	1.02 \pm 0.43	0.915	1.00 \pm 0.31	0.88 \pm 0.44	0.958
Blood protein (g/L)	64.4 \pm 8.9	63.5 \pm 6.9	0.965	65.8 \pm 4.7	60.9 \pm 10.7	0.398

^a% Deviation was calculated based on the participants gestational age (calculated from participant recall of the last menstrual period) and height as measured at baseline.

These were used to determine the ideal weight of the participants as outlined by Guerri *et al.* (25) The weight of participants measured at baseline was then used to calculate the % deviation from the ideal weight.

^bMUAC: Mid-upper arm circumference

^cRAE: retinol activity equivalents

Table 5-2. Comparison of probiotic and control group at the study midpoint. Values given are averages and standard deviation unless otherwise stated.

	Probiotic (n=20)	Control (n=22)	q-value
Gestational age (weeks)	32 ± 2	30 ± 4	0.582
Age (years)	23.5 ± 4.7	24.6 ± 5.1	0.582
Height (m)	1.59 ± 0.06	1.62 ± 0.06	0.304
Weight (kg)	58.5 ± 5.9	60.1 ± 7.8	0.582
MUAC^a (mm)	260 ± 23	263 ± 24	0.682
Hemoglobin (g/L)	102 ± 16	106 ± 24	0.619
Malaria (n)	2	1	-
Calories (kcal/day)	2065 ± 656	1701 ± 486	0.160*
Protein (g/day)	67 ± 12	63 ± 19	0.582
Carbohydrates (g/day)	326 ± 96	264 ± 96	0.160*
Fat (g/day)	60 ± 35	49 ± 19	0.386
Vitamin B12 (µg/day)	1.47 ± 0.83	1.41 ± 1.48	0.851
Thiamine (mg/day)	1.00 ± 0.28	0.82 ± 0.19	0.141*
Calcium (mg/day)	488 ± 148	190 ± 92	1.77E-7*
Vitamin A (RAE^b/day, median [IQR])	353 (129-985)	155 (102-268)	0.336
Folate (µg/day)	301 ± 160	224 ± 106	0.211*
Iron (mg/day)	44 ± 34	30 ± 22	0.304
Zinc (mg/day)	6.4 ± 2.3	5.1 ± 1.0	0.142*
Blood vitamin A (mmol/L)	0.82 ± 0.25	0.88 ± 0.31	0.607
Blood protein (g/L)	62.3 ± 8.6	54.8 ± 12.45	0.142*

^aMUAC: Mid-upper arm circumference

^bRAE stands for retinol activity equivalents

*Uncorrected p-value <0.05

Table 5-3. Demographics and birth data for participants in the probiotic and control groups at the final visit. Values represent a mean and standard deviation unless otherwise indicated.

	Probiotic (n=9)	Control (n=15)	q-value
Gestational age at birth (weeks)	40 ± 1	40 ± 2	0.976
Age (years)	24.9 ± 3.4	26.0 ± 5.6	0.787
Height (m)	1.58 ± 0.05	1.62 ± 0.05	0.598
Weight (kg)	56.2 ± 2.9	55.5 ± 6.7	0.973
MUAC^a (mm)	267 ± 19	260 ± 23	0.787
Hemoglobin (g/L)	107 ± 12	111 ± 18	0.787
No. of twin sets	2	0	
Infant			
Male	5	6	
Female	6	9	
Age (days)	13 ± 3	12 ± 2	0.787
Weight (kg)	3.5 ± 0.5	3.4 ± 0.4	0.787
Length (cm)	50.7 ± 2.9	48.7 ± 4.0	0.598
Head circumference (cm)	35.9 ± 1.3	35.3 ± 1.2	0.598
MUAC^a (mm)	11 ± 1	11 ± 1	0.787
Diet			
Calories (kcal/day)	1775 ± 565	2068 ± 383	0.646
Protein (g/day)	83 ± 38	61 ± 18	0.598
Carbohydrates (g/day)	283 ± 131	363 ± 53	0.598
Fat (g/day)	44 ± 14	49 ± 24	0.787
Vitamin B12 (mcg/day)	1.36 ± 1.14	0.51 ± 0.66	0.598
Thiamine (mg/day)	0.85 ± 0.25	1.00 ± 0.29	0.598
Calcium (mg/day)	394 ± 139	109 ± 11=2	0.003
Vitamin A (RAE^b/day, median [IQR])	190 (107-252)	85 (31-302)	0.899
Folate (mcg/day)	137 ± 66	157 ± 65	0.787
Iron (mg/day)	67 ± 38	56 ± 46	0.787
Zinc (mg/day)	5.1 ± 2.1	6.4 ± 1.6	0.141
Blood			
Blood vitamin A (mmol/L)	1.29 ± 0.34	1.58 ± 0.59	0.598
Blood protein (g/L)	62.13 ± 9.09	62.73 ± 12.22	0.598
Breast milk			
Fat (g/dL)	2.54 ± 0.26	2.80 ± 0.46	0.598
Carbohydrates (g/dL)	5.75 ± 0.18	5.78 ± 0.16	0.787
Protein (g/dL)	1.09 ± 0.06	1.14 ± 0.15	0.787

^aMUAC: Mid-upper arm circumference

^bRAE: retinol activity equivalents

5.3.2 Microbiome findings

Nutritional status did not correlate with a difference in microbiota profiles between groups at any body site both by diversity indices (Figure 5-2) or individual taxa (data not shown; determined by analysis with ALDEx2, which determines differential abundances for taxa that are robust to technical replication and that pass a stringent false discovery rate cutoff (Fernandes *et al.*, 2013; Fernandes *et al.*, 2014)). It can be noted there was a non-significant trend of increased microbial diversity in undernourished mother's breast milk and their infants oral cavities; this was not significant after multiple testing correction. After initial analysis of dietary data, blood protein and Vitamin A (Table 5-1), and bacterial communities, the classification based on nutritional status was rejected and participants were grouped as those receiving Moringa enriched probiotic supplementation ($n=23$, 14 undernourished) or those who did not ($n=24$, 7 undernourished).

The mothers' gut microbiotas were generally dominated by *Prevotella* (Figure 5-3) but the *L. rhamnosus* probiotic is not visible as it comprises a near-undetectable fraction of the fecal microbiota. For the oral microbiota, *Streptococcus*, *Prevotella*, *Fusobacterium*, *Porphyromonas* and other known oral commensals and pathogens were detected while the vaginal profiles were predominantly dominated by *Lactobacillus* (Figure 5-3). Clear clustering by body site was observed in PCoA using the Bray-Curtis dissimilarity measure, with the observation that the infants' oral and fecal microbiota seemed most closely related to breast milk (Figure 5-4A). Both 0-10 days and 10-25 days after birth, there was more similarity between the infant's oral microbiota and their mother's breast milk microbiota, than between the infant's feces and their mother's milk (Figure 5-4B, $p<0.0001$, FDR corrected Welch's T-test).

To determine the effect of probiotic administration, differences in the microbiota of each group at each body site were examined chronologically relative to enrollment using weighted UniFrac distances (Figure 5-5A), and no clear trend was detected comparing probiotic to control groups. When the infants of mothers in the probiotic and control groups are compared by PCoA of weighted UniFrac distances, weak clustering by

treatment group was observed (Figure 5B). The OTUs were summarized to genus level which revealed a 3-fold increase in relative abundance of *Bifidobacterium* and a 16.8-fold decrease in unclassified *Enterobacteriaceae* in the feces of infants whose mothers consumed the yogurt ($p < 0.05$ FDR-corrected Wilcoxon Rank Sum, Figure 5-5C). This trend was not observed in the breast milk nor was the primary macronutrient composition of the milk altered by yogurt intake (Table 5-3).

The vaginal microbiota showed a distinct change in beta diversity over gestational time irrespective of treatment group while the oral and fecal microbiotas did not (Figure 5-5A). To analyze this finding further, Shannon diversity index was considered over time (Figure 5-6A) showing a significant increase in alpha diversity in the 10 days following birth to a predominantly bacterial vaginosis (BV)-type communities as indicated by Nugent Score (Figure 5-6B, $p < 0.001$ Welch's t-test). In the postnatal period, there was a decrease in *Lactobacillus* with an increase in BV associated organisms such as *Prevotella*, *Veillonella*, *Poryphromonas* and *Megasphaera* (Figure 5-6C, $n=32$ participants, $p < 0.05$ FDR-corrected paired Wilcoxon test).

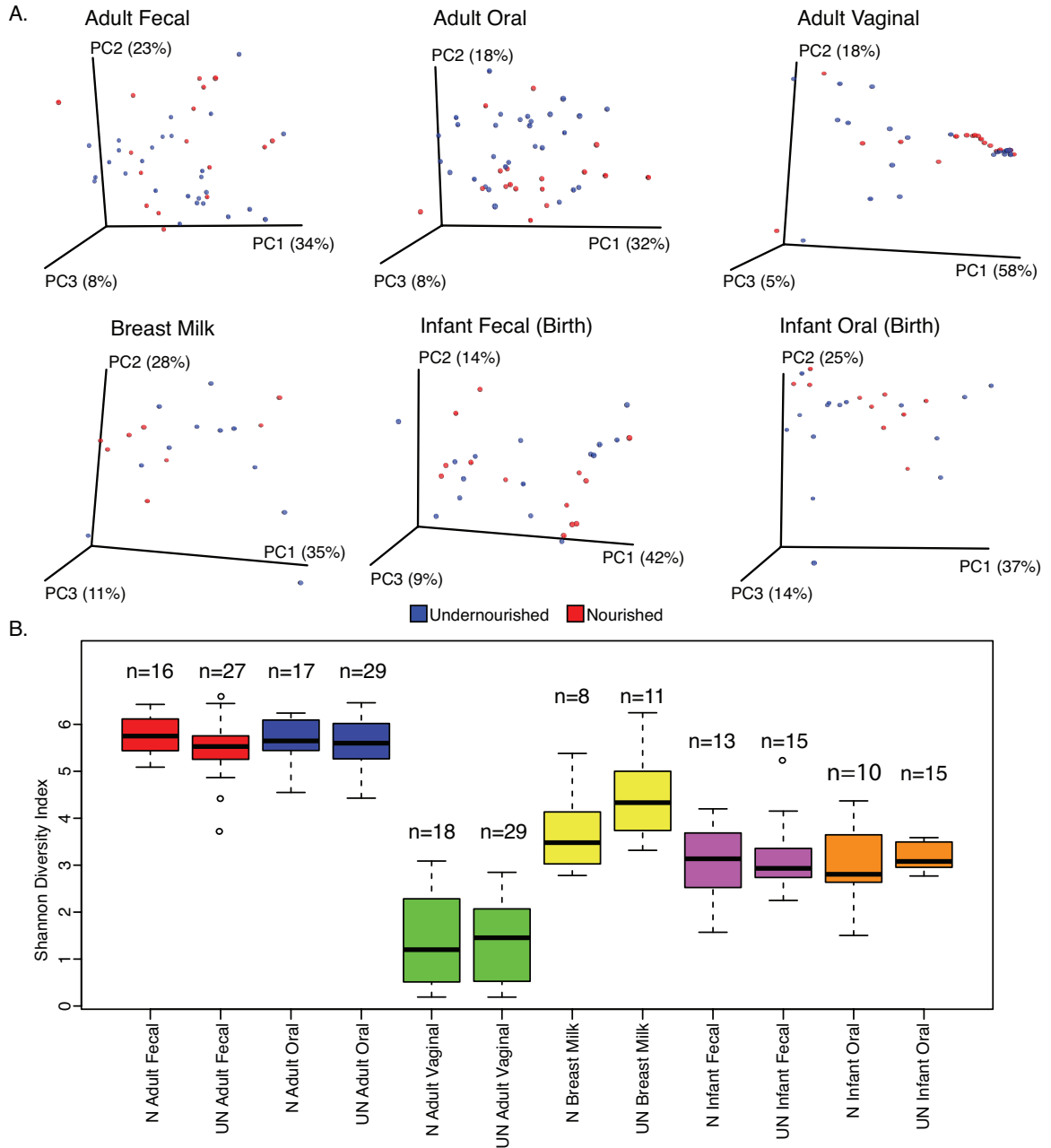


Figure 5-2. (A) PCoAs of weighted UniFrac distances and (B) Shannon's diversity index of samples taken at enrollment and birth show no effect of nourishment-status (N nourished, UN undernourished) on the microbiota at studied body sites.

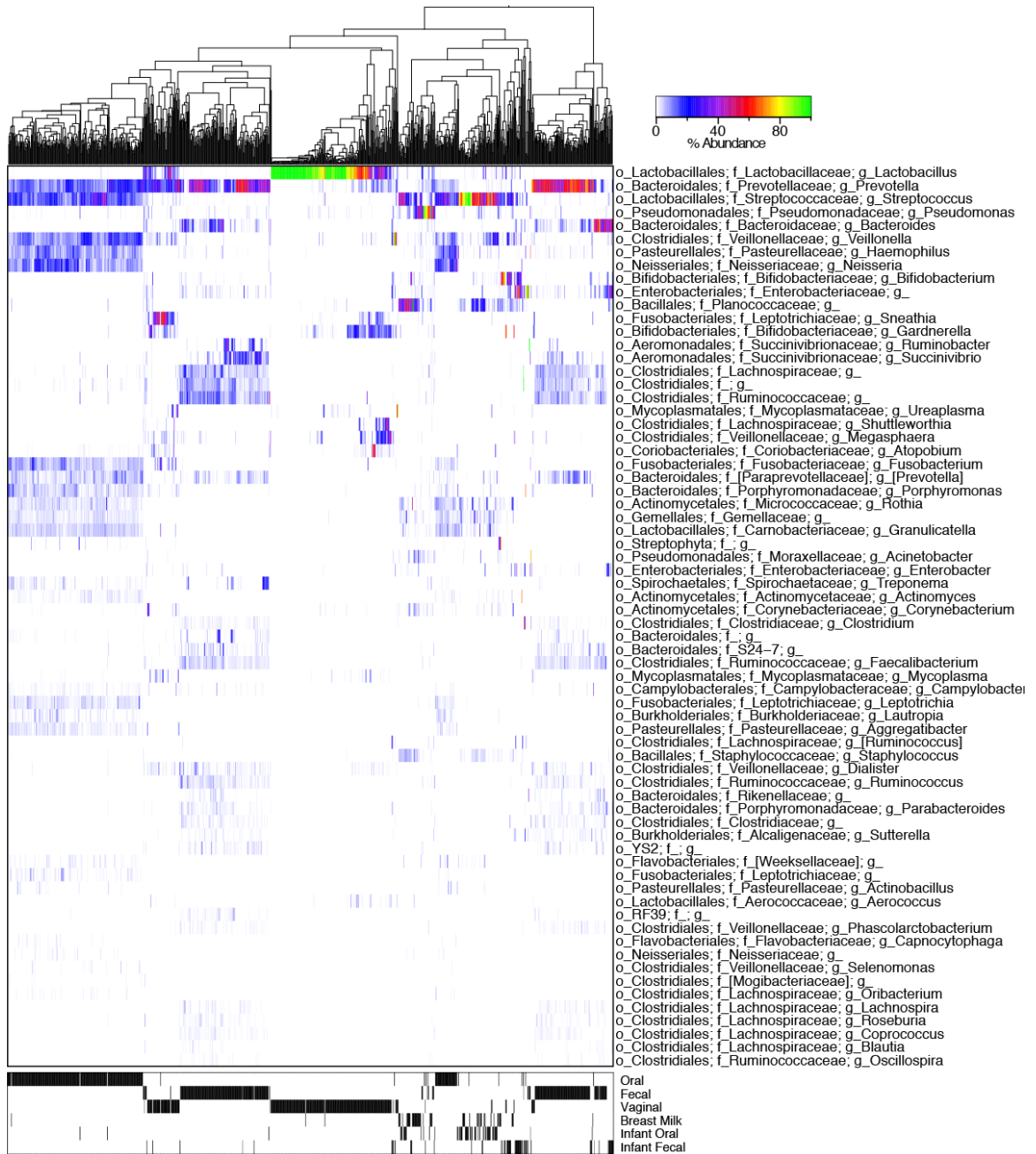


Figure 5-3. Heat map of all samples (n=974) at the genus level.

Samples were UPGMA clustered by weighted UniFrac distances showing strong clustering of samples by body site (as indicated in lower heat map).

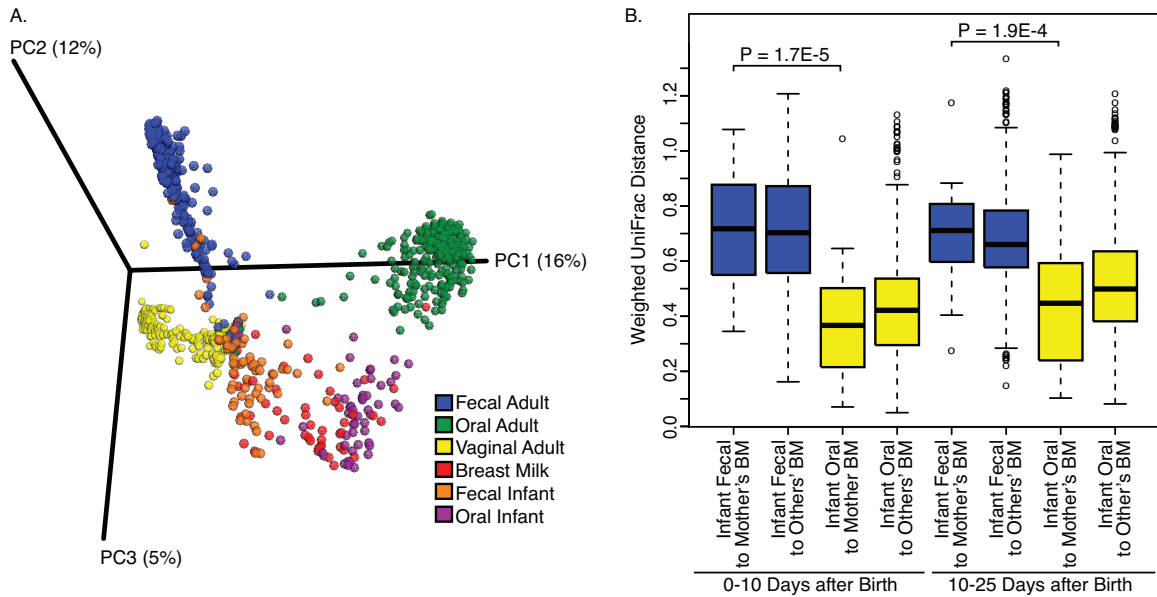


Figure 5-4. Clustering of samples and comparison of infant and mother microbiotas.

(A) PCoA of Bray-Curtis dissimilarities shows distinct clustering of samples based on body site. The infant's oral and fecal microbiomes cluster most closely with the breast milk. Analysis of weighted UniFrac distances shows that the oral sample of a baby is more similar to their own mother than their fecal sample (B). At both 0-10 days and 10-25 days after birth, there was more similarity between the infant's oral sample and their mother's breast milk ($n=22$ 0-10 days, $n=27$ 10-25 days), than between the infant's fecal sample ($n=25$ 0-10 days, $n=29$ 10-25 days) to their mother's breast milk ($p<0.0001$, FDR corrected Welch's T-test). There is also a non-significant trend that an infant's oral microbiota is more similar to their own mother's breast milk microbiota than that of other mothers.

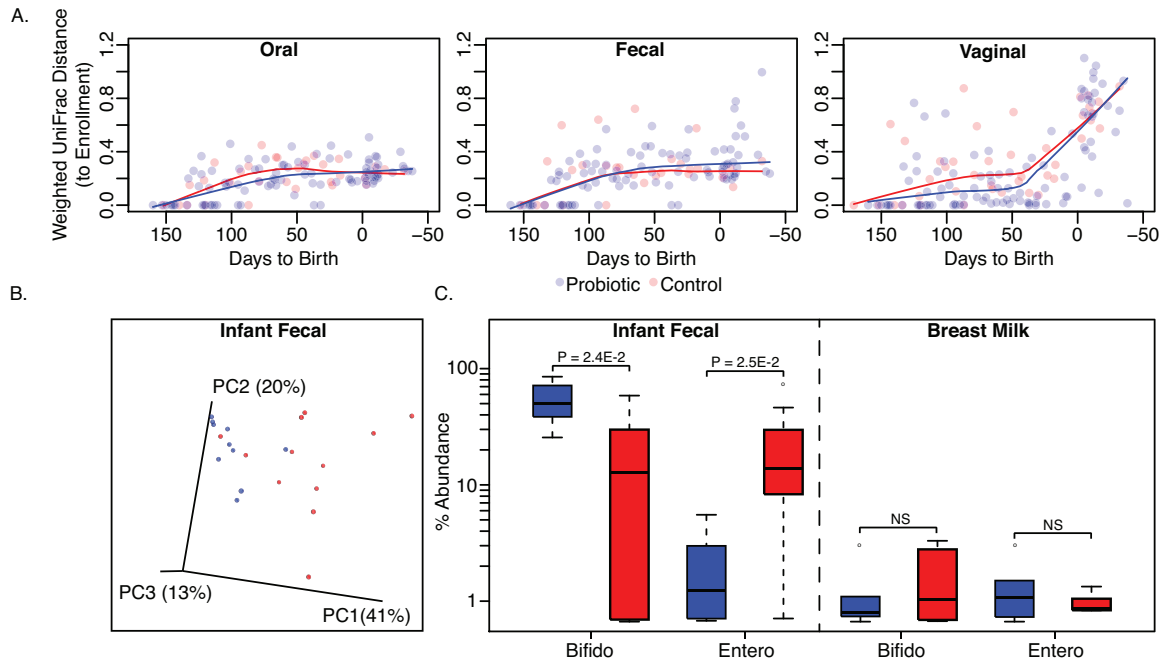


Figure 5-5. Change in the microbiota over time and the effect of yogurt on mother and infant microbiotas.

(A) Microbiota analysis by weighted UniFrac distances comparing samples to enrollment show that Moringa-supplemented probiotic yogurt does not affect the microbiota structure and that the oral and fecal microbiotas remain stable over pregnancy. The vaginal microbiota appears to be significantly altered at birth regardless of consumption of yogurt. (B) The fecal microbiota of infants aged 10-25 days who consumed the yogurt weekly clusters separately from controls by PCoA of weighted UniFrac distances (Red= Control, Blue=Probiotic). (C) There is an increase in the relative abundance of *Bifidobacterium* (Bifido) and decrease in *Enterobacteraceae* (Entero) (FDR corrected Wilcoxon Rank Sum Test < 0.05) in the feces of the infants whose mother's received probiotic yogurt intervention ($n=10$) as compared to controls ($n=12$). This difference was not present in the breast milk ($P > 0.05$).

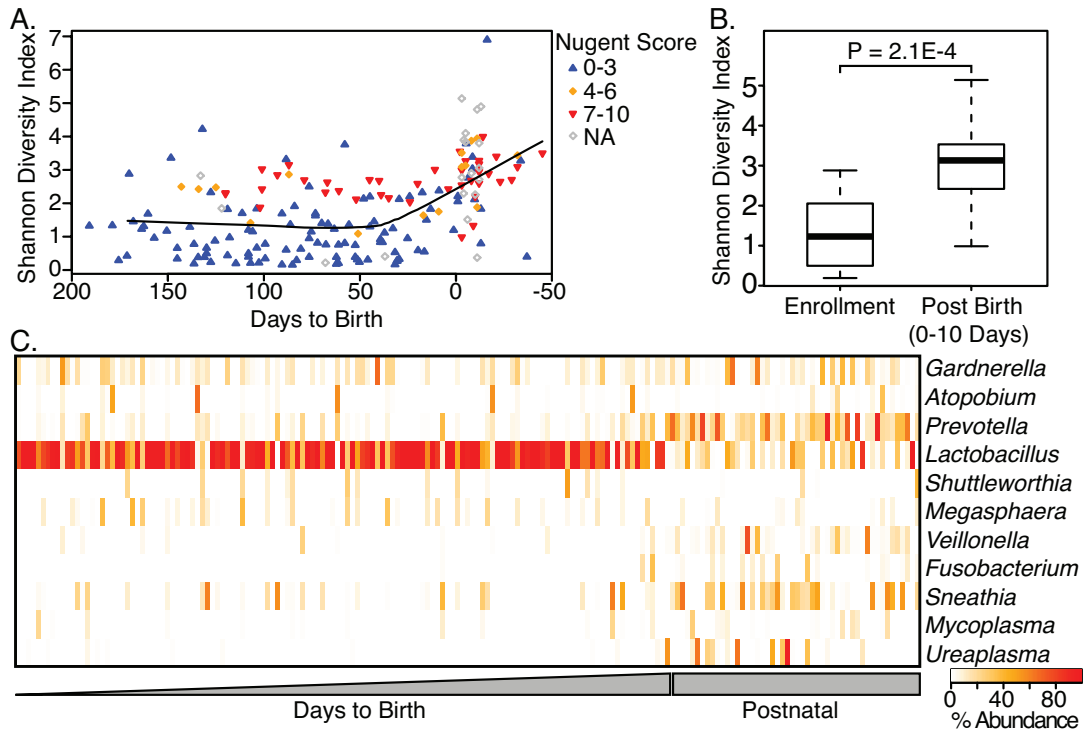


Figure 5-6. Vaginal microbiota over pregnancy reveals dysbiosis at birth.

(A) Time series of vaginal samples shows increasing diversity and leading up to and after birth. Nugent score based on microscopy (wherein 0-3 is considered normal, 4-6 intermediate, and 7-10 is a state of microbial dysbiosis referred to as bacterial vaginosis) is also displayed. (B) Comparison of Shannon's diversity shows an increase within 10 days after birth. (C) Heat map of genera composing >1% of vaginal organisms shows a loss of lactobacilli and an increase in bacterial vaginosis-associated organisms such as *Prevotella*.

5.4 Discussion

This study reports the oral, gut, vaginal and breast milk microbiotas of pregnant Tanzanian women, and assesses the effects of consumption of micronutrient-supplemented probiotic yogurt. None of the mothers or infants died during labor. This finding, plus the low incidence of preterm labor, no underweight births, and no serious malnutrition, suggested that this particular rural population did not typify the Tanzanian situation as a whole, where maternal mortality rate are 38.3 times higher than Canada and infant mortality under 1 year of age is 7.6 times higher (UNICEF, 2013). Sample size aside, according to the UNICEF statistics we would expect 2 infant deaths in a cohort of this size. There is an association between an aberrant urogenital microbiota and preterm labor (Giraldo *et al.*, 2012), but the incidence of the latter was low in this patient group (32%) and similar to that found in London, Canada (unpublished data, Mottola, Enos and Reid). Likewise, periodontal disease with poor oral hygiene has been linked with preterm labor (Macedo *et al.*, 2014), but the Tanzanian women studied here did not present with acute periodontal disease as identified during their examinations, and their oral microbiotas showed no obvious abnormality. Thus, with two risk factors for preterm delivery being absent, it was not surprising to find such a low rate of premature births. Nevertheless, this is all the more laudable since many subjects had poor caloric intake. In Tanzania, normally only around 50% women have a skilled attendant at birthing according to UNICEF, and many women have poor access to obstetrical care. The provision of the latter in our study, may have reduced the preterm labor rate and improved maternal outcome, albeit the majority of the women delivered at home.

In these settings, access to Moringa and cow's milk is good, making it feasible to have the yogurt produced locally. Whilst the yogurt was provided here free of charge, the community kitchen continues to operate and sell the product at a price (around \$0.15) affordable to members of the community. This is important for sustainability.

We had expected that the daily Moringa yogurt consumption would have improved nutritional parameters in women presenting with mid-upper arm circumference (MUAC) less than 235 mm (undernourished group), since this food provided a good portion of the required daily intake of calcium, vitamins, and proteins. However, the baseline levels of

hemoglobin, blood retinol and protein concentrations suggested they were not actually overtly malnourished. It is highly probable our study suffered from a lack of power, and with an increased sample size, we may have been able to conclusively demonstrate clinical outcomes as a result of the Moringa-supplemented yogurt consumption. A previous study in Mwanza, Tanzania using a different combination of micronutrients supplemented in yogurt showed benefits to HIV positive subjects (Hummelen *et al.*, 2011).

The mothers' gut microbiotas were generally dominated by *Prevotella*, in agreement with previous reports on children from Burkina Faso, Malawi, and Tanzania (De Filippo *et al.*, 2010; Bisanz *et al.*, 2014; Smith *et al.*, 2013) and adults (Yatsunenکو *et al.*, 2012). Potentially, this reflects a high carbohydrate diet which was noted in the dietary recalls.

The vaginal profiles were dominated by *Lactobacillus* species, similar to previous findings for American and Brazilian non-pregnant women (Romero *et al.*, 2014; Aagaard *et al.*, 2012; Gajer *et al.*, 2012; Reid, 2008; Martinez *et al.*, 2008) and for pregnant Canadian women (unpublished data, Mottola, Enos and Reid). It has long been believed that the vaginal tract is seeded from the rectum (Reid, 2008), and thus pathogens ascend and colonize. One would therefore expect that significantly different fecal microbiota patterns would result in differences amongst vaginal bacterial profiles. But this is not the case here or in other studies, suggesting that the reproductive tract has evolved receptivity to certain constituents of the gut, mainly lactobacilli, and an ability to repel others. This niche adaptation warrants further investigation.

Rapid development of an aberrant vaginal microbiota immediately after birth is also an interesting finding. A similar discovery was recently reported by Huang *et al.* (2014), however, that study was limited in the following ways: it was a cross-sectional design as opposed to longitudinal in the present study; postpartum samples were an average of 7 weeks after birth as opposed to 0-10 days in the present study; and sample size of postpartum individuals was only 5. Since our study showed stability in the fecal microbiota from the second trimester until after birth, this adds to previous findings of the microbiome across pregnancy in Finnish women (Collado *et al.*, 2008) where first and

third trimesters showed dramatic community shifts. A strength of this study is that unlike previous reports, we examined all three communities (fecal, oral, vaginal) simultaneously.

The significant increase in the relative abundance of *Bifidobacterium* spp. and a decrease in *Enterobacteriaceae* in the newborn gut of babies born to mothers who had received the yogurt is noteworthy. All the mothers breast-fed, so, since glycans in the milk promote bifidobacterial growth (Sela and Mills, 2014), it is not clear why only the milk from probiotic treated mothers further increased relative abundances of these organisms. We found no significant difference in milk microbiotas, fat, carbohydrate or protein concentrations between the two groups of women. Given the complex cocktail of bifidogenic glycans and bioactive compounds in breast milk, it will be necessary to apply further glycomic, proteomic and metabonomic approaches to study the mechanisms through which micronutrient and probiotic supplementation may affect breast milk composition and the infant's microbiota. Perhaps specific milk oligosaccharides increase following micronutrient/probiotic supplemented yogurt consumption.

The milk clearly supported a diverse range of organisms, which then colonized the newborn's intestine and oral cavity. Unlike North American society where caesarian delivery and use of infant formula is widespread, the women in this Mwanza community almost all deliver vaginally and breast-feed their babies. With inflammatory bowel disease and allergies increasing in Canadian children, potentially linked to environmental factors including caesarian section and formula feeding (Benchimol *et al.*, 2014; El-matary *et al.*, 2014), other lessons may be learned from studying pregnant women in eastern Africa.

In summary, daily ingestion of yogurt supplemented with probiotic *L. rhamnosus* GR-1 and locally sourced micronutrient-rich Moringa supplemented probiotic yogurt provided a safe, inexpensive food for pregnant women in rural Tanzania, without adversely changing gut or oral microbial diversity. The baseline nutritional deficiencies illustrated the problems faced by pregnant women in this region, however no effects of yogurt supplementation were observed in clinical parameters. The resultant improvement in the

gut microbial profile of infants, suggests consumption of this yogurt could be beneficial in this and other developing world settings but requires further work to understand the mechanism of action.

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Chapter 6

6 Randomized open-label pilot study of the influence of probiotics and the gut microbiome on toxic metal levels in Tanzanian pregnant women and school children

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Bisanz JE, Enos MK, Mwanga JR, Changalucha J, Burton JP, Goor GB and G Reid. 2014. Randomized open-label pilot study of the influence of probiotics and the gut microbiome on toxic metal levels in Tanzanian pregnant women and school children. *mBio*. doi:10.1128/mBio.01580-14.

Supplemental tables (Tables S1 and S2) are available online at <http://mbio.asm.org/content/5/5/e01580-14.full>.

Ethics approval documentation is provided in Appendix E

6.1 Introduction

Toxins in the environment are ubiquitous and exposure is often unavoidable. Their effects on human and animal life are usually seen over time, and can be serious. Acute exposure to high toxin levels is particularly detrimental. Anthropomorphic activity has only served to increase levels of toxins, such as heavy metals and pesticides, in the environment (Muir *et al.*, 2009). Due to lax regulations and exploitation, many environmental toxins disproportionately affect the developing world. Aflatoxin for example, is ubiquitous in East Africa due to *Aspergillus* contaminated cereal and grain crops (Wagacha and Muthomi, 2008). Metals such as mercury are released due to human activity such as mining, as seen along the shores of Lake Victoria, Africa, where the metal reaches the food web (Campbell *et al.*, 2003). Fish, while not as popular in the west, are one of the most important sources of dietary protein for many cultures (Tidwell and Allan, 2001). The effects of low-level mercury exposure include delayed neurological and cognitive development in children and more controversially, immune and cardiovascular diseases (Karagas *et al.*, 2012).

Metal chelating drugs such as dimercaptosuccinic acid (DMSA) and ethylenediaminetetraacetic acid (EDTA) are indicated for the treatment of acute exposure, however they are not intended for long term use and there is a lack of regulatory approved consumer products for chelation. Thus, alternative approaches are needed. Species of lactic acid bacteria including the *L. rhamnosus* GR-1 strain used here in the probiotic yogurt are known to have an affinity for many toxic metals including lead and cadmium *in vitro* (Ibrahim *et al.*, 2006), and we have also shown activity against mercury, arsenic and various organic pesticides (manuscripts in preparation). The mechanism is thought to be passive sequestration, however we have also discovered putative probiotic strains that have active enzymatic pathways for detoxification such as mercury demethylation and reduction. The concept of probiotic-mediated detoxification has recently been demonstrated in murine models (Tian *et al.*, 2012), but we explored whether such food-grade microbes could prevent up-take in the gastrointestinal tract (Monachese *et al.*, 2012).

Logic dictates that if probiotic organisms have these protective capacities, endogenous microbes of the GI tract, termed the microbiota, could also be of importance. Experiments contrasting conventional and germ free animals have shown the importance of the microbiota in protection against accumulation of mercury (Nakamura *et al.*, 1977), lead and cadmium (Breton *et al.*, 2013a). Furthermore, levels of indigenous lactobacilli appear to increase in response to metal exposure in murine models (Breton *et al.*, 2013b), perhaps conveying a natural protective effect. We sought to better understand the composition of the human gut microbiota exposed to toxic metals.

Two of the most vulnerable populations at risk from environmental toxin exposure are pregnant mothers and children. We suspected that in Mwanza, Tanzania, due to its proximity to Lake Victoria, and the fish-rich diet, women and children would have elevated toxic metal exposure and be ideal candidates for intervention. Furthermore, Mwanza is site to a network of community run probiotic yogurt kitchens that service people at the bottom-of-the-pyramid (Reid *et al.*, 2013). The aims of this study were: (i) determine the blood metal levels in the local population and from potential fish sources, (ii) measure if consumption of a probiotic yogurt has an effect on blood metal levels, and

(iii) characterize the gut microbiome of children to determine if there are bacterial genera associated with metal levels.

6.2 Results

6.2.1 Participant recruitment

Between November 2012 and December 2012, a total of 44 individuals were recruited into the 25-day study of school-aged children (SAC), with 22 in the control group receiving milk and 22 in the group receiving probiotic yogurt. Eight individuals withdrew during the course of the study, one due to suspected lactose intolerance which was not known to the child's guardian at the time of enrollment, and 7 for unknown reasons including not being present for final sample collection. A total of 24 individuals were selected for inclusion into blood metal analysis in the pregnant women (PW) group based on adherence of over 75% and matching of nutritional status and fish intake. A summary of recruitment is provided in Figure 6-1. Relevant participant demographics are represented in Table 6-1. *Z* scores based on weight-for-age indicate that the children enrolled were not malnourished at the point of enrollment. Based on the SAC enrollment questionnaire, 37% of children consumed local fish on a daily basis, 55% consumed multiple courses of fish a week and 8% multiple times a month. No guardians reported their children did not consume fish regularly as part of their diet.

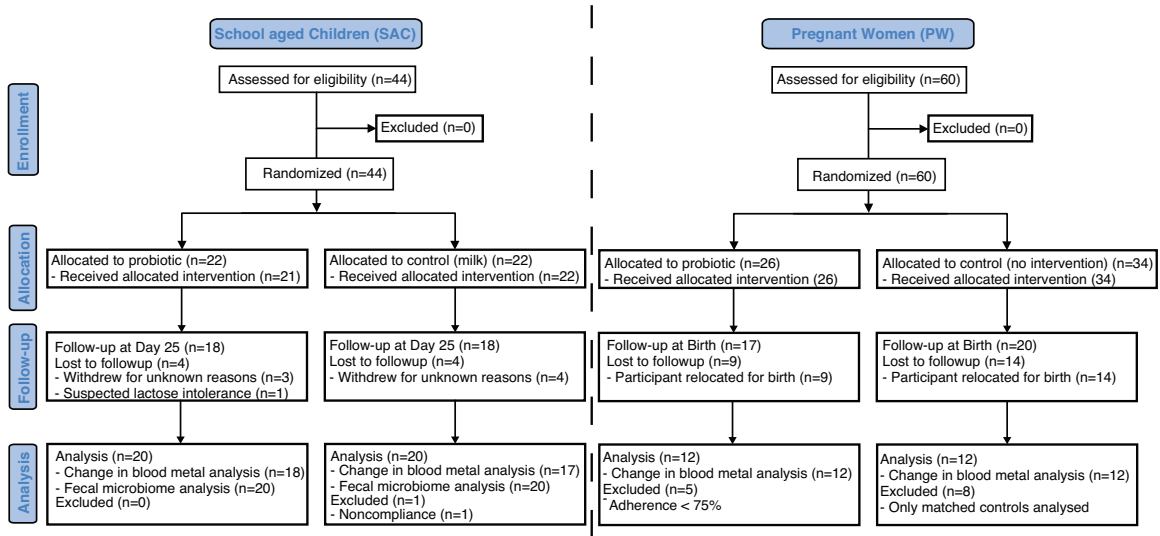


Figure 6-1. CONSORT flow diagram detailing participant enrollment, allocation, follow-up and analysis.

Table 6-1. Participant demographics for pregnant women (PW) and school-aged children (SAC) control and yogurt groups (mean \pm SD).

All relevant metrics are not statistically significant between groups. Z scores were calculated from WHO 5-19 year old children BMI-for-age tables (who.int/growthref/who2007_bmi_for_age/en/index.html).

	PW		SAC	
	Control	Yogurt	Control	Yogurt
Participants	n=12	n=12	n=22	n=21
Age (years)	24.5 \pm 3.9	23.5 \pm 3.6	8.3 \pm 1.1	8.4 \pm 1.2
Weight (kg)	58.0 \pm 6.8	55.0 \pm 4.9	23.1 \pm 3.6	23.6 \pm 3.2
Height (cm)	162.0 \pm 3.9	157.0 \pm 5.0	119.3 \pm 7.5	119.6 \pm 5.8
BMI (kg/m²)	22.0 \pm 1.9	22.3 \pm 2.0	16.2 \pm 1.9	16.5 \pm 2.0
Z score			-0.4 \pm 1.1	-0.3 \pm 0.8
Gender (M/F)			6/16	6/15
Fish Intake (g/day)	150.4 \pm 60.1	120.7 \pm 52.9		
Adherence (%)		90.6 \pm 5.4	89.8 \pm 10.0	86.1 \pm 11.4

6.2.2 Dietary and blood metal levels

Levels of metals across fish types for cadmium, lead, total mercury and total arsenic are shown in Table 6-2. Surprisingly the smaller fish type, Silver cyprinid, contained significantly higher levels of mercury and arsenic compared to the other piscine species tested.

Measures of blood lead, total mercury, total arsenic and cadmium are presented in Table 6-3 for both the SAC and PW groups. When comparisons were made to levels present in a developed country (Canada), lead and mercury were found to be elevated in both SAC and PW by up to 6.8 times. Levels of arsenic and cadmium appeared on par or lower than

the Canadian population values. The PW group tended to display lower levels of metals than the SAC group.

Table 6-2. Toxic metal levels in commonly consumed fish in the Mwanza Region. Mercury and arsenic are reported as total levels.

Species	Mercury (ng/g \pm SD)	Lead (ng/g \pm SD)	Arsenic (ng/g \pm SD)	Cadmium (ng/g \pm SD)
Tilapia (<i>Oreochromis niloticus</i>)	18.3 \pm 17.1	58.0 \pm 13.0	22.3 \pm 2.5	158 \pm 254
Nile Perch (<i>Lates niloticus</i>)	56.0 \pm 15.1	86.7 \pm 18.2	30.3 \pm 14.3	33.7 \pm 37.5
Silver cyprinid (<i>Rastrineobola argentea</i>)	77.3 \pm 40.5	78.0 \pm 18.3	664.3 \pm 159.9	113.0 \pm 54.7

Table 6-3. Blood metal levels at the time of recruitment and comparisons to levels found in a developed country.

	Average (\pm SD)	Range	Canadian Average ¹	Reference Range ²	Fold Difference
SAC					
Pb (μg/L)	47.1 \pm 16.2	22.5 - 91.3	9.0	0.0 - 17.7	5.2
Hg (nmol/L)	9.5 \pm 5.3	3.0 - 37.4	1.4	0.0 - 5.5	6.8
As (nmol/L)	6.5 \pm 2.1	2.7 - 10.8	7.8	0.0 - 21.4	-1.2
Cd (nmol/L)	1.2 \pm 0.7	0.9 - 4.4	0.89	0.0 - 4.6	1.3
PW					
Pb (μg/L)	22.6 \pm 9.6	7.3 - 40.5	8.9	0.0 - 45.0	2.5
Hg (nmol/L)	8.8 \pm 3.1	4.0 - 16.0	3.5	0.0 - 18.0	2.5
As (nmol/L)	3.0 \pm 1.6	1.3 - 6.7	11.7	0.0 - 21.4	- 3.9
Cd (nmol/L)	1.1 \pm 0.6	0.0 - 2.7	3.2	0.0 - 8.9	- 2.9

¹ Canadian averages are geometric means of males and females aged 6-11 (SAC) and females aged 20-39 (PW) based on the Canadian Health Measures Survey (2007-2009) (Health Canada, 2010). ² Reference ranges provided by Trace Elements Laboratory-London Laboratory Services Group.

6.2.3 Effect of probiotic yogurt on blood metal levels

Before and after intervention, samples were successfully collected from 36 individuals in the SAC group, 18 in each group. One individual was excluded from the control group after gut microbiome analysis showed a high number of reads presumptively mapping to the probiotic strain indicating non-compliance. After quantification by HR-SF-ICP-MS, no statistically significant differences were detected in blood metal levels of SAC receiving probiotic or milk control though it is noted that there was a weak trend of reduced lead and arsenic (Table 6-4). In the PW cohort, this effect was noted to a statistically significant level ($P<0.05$). It is noteworthy that levels of mercury and arsenic increased in the control groups ($P<0.05$) but remained stable in the probiotic group, indicating a protective effect of probiotic consumption.

Table 6-4. Blood metal levels between control and probiotic groups before and after intervention in school aged children (SAC) and pregnant women (PW)

Metal Analyzed	Control					Probiotic					Between-groups Comparison			
	Enrollment	Follow-up	Difference	P-value ^b	95% CI	Enrollment	Follow-up	Difference	P-value ^b	95% CI	Responder Rate (%) ^c	Difference (Prob-Con)	P-value ^d	95% CI
SAC														
	n=17					n=18								
Lead (µg/L)	48.6 ± 16.1	49.7 ± 21.8	1.1	0.79	-7.7 to 10.01	46.3 ± 16.7	47.3 ± 15.8	1.0	0.41	-1.6 to 3.6	53%	-0.1	0.98	-9.0 to 8.8
Mercury (nmol/L)	8.9 ± 2.8	9.4 ± 3.5	0.5	0.52	-1.1 to 2.1	10.3 ± 7.5	9.7 ± 4.9	-0.6	0.51	-2.6 to 1.3	29%	-1.1	0.36	-3.6 to 1.4
Cadmium (nmol/L)	1.4 ± 1.1	1.3 ± 1.2	-0.1	0.58	0.12 to -0.27	1.2 ± 0.4	1.1 ± 0.6	-0.1	0.43	0.17 to -1.6	13%	0	0.79	0.37 to -
Arsenic (nmol/L)	6.1 ± 2.3	6.3 ± 2.9	0.2	0.78	-1.5 to 1.9	6.7 ± 2.2	6.3 ± 2.3	-0.4	0.41	0.67 to -1.6	35%	-0.6	0.49	-2.6 to 1.3
PW														
	n=12					n=12								
Lead (µg/L)	25 ± 9.0	34 ± 13	9	0.011	2.4 to 15	20 ± 9.7	33 ± 19	13	0.0013	6.1 to 19	8%	4	0.35	-4.6 to 12
Mercury (nmol/L)	8.2 ± 3.5	11 ± 2.5	2.8	0.042	0.12 to 5.6	9.4 ± 2.7	9.0 ± 2.5	-0.4	0.60	-2.1 to 1.2	25%	-3.2	0.035	0.25 to -
Cadmium (nmol/L)	1.2 ± 0.59	1.3 ± 0.46	0.1	0.57	0.37 to -0.21	1.1 ± 0.65	1.4 ± 0.90	0.3	0.017	0.70 to 0.080	8%	0.2	0.13	0.69 to -
Arsenic (nmol/L)	2.4 ± 1.5	4.9 ± 2.5	2.5	0.0032	1.0 to 3.9	3.5 ± 1.7	3.7 ± 1.1	0.2	0.68	-0.85 to 1.3	0%	-2.3	0.011	-4.0 to 0.57

^a Values are mean ± SD, ^b Paired t-test used for within-group comparisons, ^c Responder defined as showing a decrease in blood metal levels over study period, ^d t-test used for between-group comparison

6.2.4 Gut microbiome and association with metal levels

A total of 74 fecal samples from the children were collected and processed for sequencing. For all but one of these samples, 16S rRNA gene sequences were successfully obtained with a total number of reads being 1,150,628 averaging 15,762 reads per sample. With clustering at 97% nucleotide identity, 177 OTUs were obtained at $\geq 1\%$ abundance. These data were summarized to the family level and presented in Figure 6-2. Nearly all samples contained *Prevotella* as the dominant genera and the *Prevotellaceae* as the dominant family. Administration of the probiotic was not observed to have an effect on the gut bacterial community composition based on analysis of weighted and unweighted UniFrac metrics (Lozupone and Knight, 2005; data not shown), as previously reported (McNulty *et al.*, 2011). The use of 16S rRNA sequencing also gave the unique opportunity to presumptively validate probiotic administration, as one OTU could be mapped back to the genome of *L. rhamnosus* GR-1 (OTU_140). This OTU was not observed in any sample prior to the start of the study and with only one exception, was only observed in the group that received probiotic yogurt. The exception (PDTX25) was excluded from analysis of probiotic efficacy in the SAC group.

To analyze the association between blood lead levels and the microbiotas, the initial analysis focused on only the first visit and the most extreme cases ($n=3$ per group) based upon visual inspection of the distribution of blood levels. Two OTUs were found to be elevated in conditions of high blood lead and to have a raw P -value < 0.05 : OTU_1 ($P=0.0205$) and OTU_215 ($P=0.0498$) representing *Succinivibrionaceae* and Gammaproteobacteria respectively. Due to the relatively small sample numbers used in this analysis, significance was not obtained with multiple testing corrections. In order to leverage the full power of the dataset, all samples regardless of visit or participant were considered. Quartile values for Q1 and Q3 based on blood lead concentration were used as cut offs to separate the microbiota samples ($n=16$ low, $n=18$ high). These two conditions were then compared using an FDR cutoff of 0.05, and again the increased proportional abundance of OTU_1 (2.9-fold, FDR=0.022) and OTU_215 (3.7-fold, FDR=0.023) with elevated lead levels was found (Figure 6-3). Significant associations were not found in the cases of mercury and arsenic.

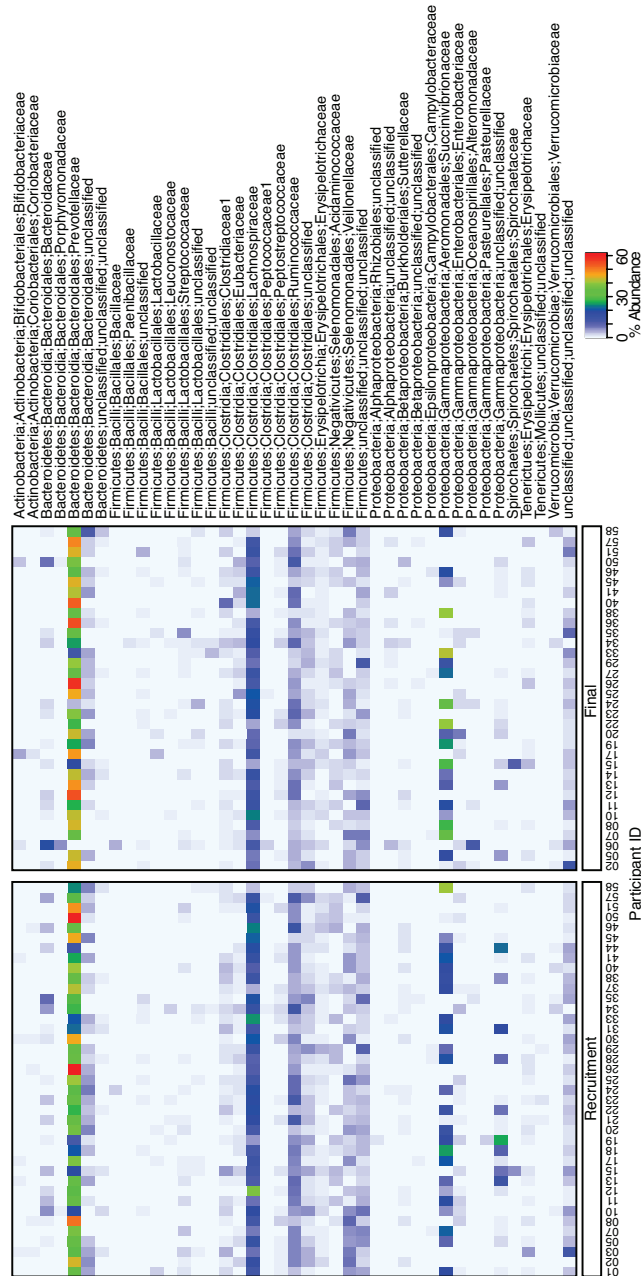


Figure 6-2. Heat map representation of the gut microbiomes of SAC at beginning and end point of study.

Data were summarized to family level and plotted as percent abundance. Across nearly all participants, the *Prevotellaceae* were the most dominant family observed while an unclassified *Succinivibrionaceae* is also of variably high abundance across many participants.

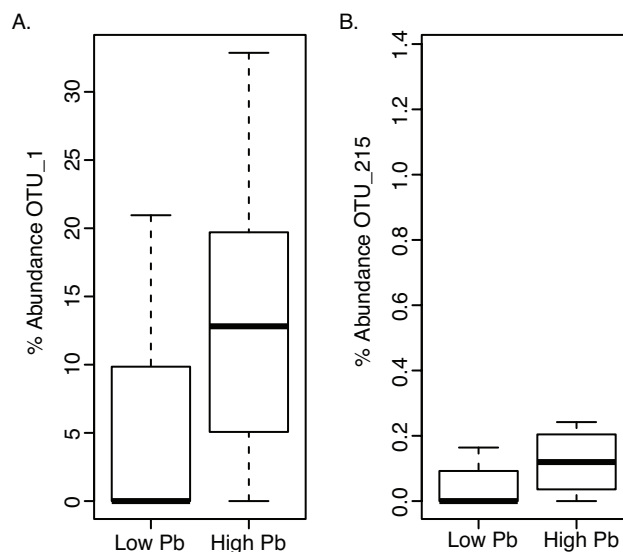


Figure 6-3. Association of (A) OTU_1 (*Succinivibrionaceae*) and (B) OTU_215 (*Gammaproteobacteria*) with elevated blood lead levels in a comparison of upper and lower quartiles of all samples (FDR<0.05).

6.3 Discussion

This is the first study to simultaneously evaluate toxic metal levels, associated microbiota and the potential for probiotics to convey a detoxification effect. It is also the first to assess the impact of a probiotic food on toxic metals in people living in the developing world.

Levels of metals in the fish tested were consistent with previous reports in Lake Victoria (Campbell *et al.*, 2003; Oyoo-Okoth *et al.*, 2010). Mercury limits in fish are well described but they are less well defined in the case of lead, cadmium and arsenic. It is greatly concerning to observe such high levels of metals in Silver cyprinid, as its daily consumption is common, especially in the economically disadvantaged due to its affordable price. This creates a disproportional burden on these individuals. Furthermore, this goes against the typical dogma that larger fish species are a greater concern for toxic metal exposure due to biomagnification (Bryan *et al.*, 1979).

Metal exposure from dietary fish intake likely explains why we see elevated levels of mercury in both the SAC and PW groups when compared to reported levels in Canadians, but the case of Cd and As is interesting as these were not dissimilar between the two countries. It is difficult to speculate why this was the case, and further studies of lake metal levels, and concentrations in other foods are needed. Unfortunately metal levels, particularly lead, were highly elevated in the SAC group where their effects may be particularly deleterious. The difference between the adults and children could be due to longer term exposure in adults as only 5-15% of ingested lead is absorbed in the gut while in children absorption can be up to 40% (International Programme on Chemical Safety, 1996).

The studies provided the first positive evidence for the use of yogurt containing probiotic lactobacilli to combat toxic heavy metal exposure in vulnerable human populations. The results comparing the short term and long term interventions (SAC vs PW) suggest that probiotic consumption does not have a fast acting effect like DMSA or EDTA, but rather acts over longer-term. This is likely because the mechanism of action is preventing uptake into the body from the GI tract, rather than scavenging what is already in the body as in chelation therapy. Alternatively it may be reflective of differing metabolic or hormonal differences and/or different indigenous microbes in the PW as compared to the SAC. Further studies involving time course interventions will be necessary to resolve this discrepancy and to rule out potential effects of nutrition affecting metal uptake.

Interestingly, in both study groups the mercury and arsenic increased in the control groups. A delay between sample collection and analysis was unavoidable due to the lack of instrumentation locally. But, sample storage of mercury, should not interfere with the analysis (Varian-Ramos *et al.*, 2011), and is more likely explained by seasonal changes in diet/exposure. Thus probiotic administration may be especially advocated at peak exposure times.

A high degree of homogeneity in a *Prevotella*-dominated microbiota is noteworthy. This profile, referred to as enterotype two (Arumugam *et al.*, 2011) has been previously observed to be predominant in African populations (De Filippo *et al.*, 2010) and is

presumed to be due to a carbohydrate rich diet (Wu *et al.*, 2011). Interestingly, enterotype two is often co-associated with *Desulfovibrio* spp. (Wu *et al.*, 2011). While we cannot definitively show the presence of these organisms due to their low abundance and lack of sequence diversity in the V6 rRNA region, they are associated with mercury methylation through a mechanism that was only recently understood (Parks *et al.*, 2013), and which could facilitate increased mercury uptake in the gut. In addition, mucin-degradation by this microbiome configuration could facilitate increased metal uptake by affecting gut barrier function (Wright *et al.*, 2000), putting these individuals at greater risk from metal exposure.

There are a number of mechanisms through which the *Succinivibrionaceae* and Gammaproteobacteria may function to facilitate greater lead uptake, including host-interactions and influencing other members of the microbiota. In fact, the mechanism may be relatively simple, since the cell wall structure of Gram-negative bacteria has lower metal binding activity than Gram-positive organisms (Beveridge and Fyfe, 1985). Given that probiotic treatment was not found to affect relative abundance of either of these two groups, or any bacterial population, this suggests the mechanism of action is independent of altering the microbiota, at least at the community structure level.

In summary, this work has demonstrated the potential value of long-term probiotic-based interventions to counter mercury and arsenic exposure in vulnerable populations, particularly in pregnant women. This approach can be disseminated at an affordable cost (equivalent of pennies) in developing countries where individuals are at high risk, however it could also be applied to developed world citizens and wildlife, for example those living near mining facilities. We hope that these studies help provide a framework for further human trials. Though it is reasonable to presume health benefits due to reduced toxin levels, long-term multi-year studies would help determine if reduction in toxin levels by probiotic foods results in improvements in physical and cognitive development in children.

6.4 Materials and Methods

6.4.1 Study design and participants

Two populations were recruited in the Mwanza region, Tanzania for this study: (i) 44 school aged children aged 6-10 years which will be further referred to as SAC, and (ii) a subset of 60 pregnant women in their first trimester who were being recruited for a separate study on nutrition and the microbiome which will be referred to as PW.

In the SAC group, consent was obtained from the child's guardian, as identified by school records, and assent was obtained from the child. If a signature could not be provided, a thumbprint was used in its place. Inclusion criteria was that the child was aged 6-10 years and in good health, the only exclusion criteria was known milk allergies and/or lactose intolerance. The guardians were surveyed for basic dietary information about their child, including the frequency with which they consumed fish and the species consumed. Blood was collected for determination of metal levels and feces was collected, stored on ice for less than 4 hours and stored at -80°C until processing and DNA extraction. Participants were then randomly assigned (using a random number generator) to receive either a locally produced yogurt containing 1×10^{10} CFU *Lactobacillus rhamnosus* GR-1 per 250 gram unit or an equivalent portion of ultra-heat treated milk as a control devoid of lactic acid bacteria. For 19 of the next 24 days, the children were supervised during administration of either yogurt or milk. Five days were missed due to logistical issues in administration/yogurt production. Upon completion of the study, blood and fecal samples were again collected.

As part of a separate study on maternal nutrition and the microbiome (PW), 60 pregnant women were recruited, of which 26 received a probiotic yogurt containing 1×10^{10} CFU *L. rhamnosus* GR-1 per 250 gram unit and supplemented with 4.3 g of Moringa, a micronutrient rich plant to enhance maternal nutrition. All women recruited were between 12 and 24 weeks pregnant and aged 18 to 40 years. Until their final visit after birth, individuals in the yogurt group received the product for six days a week with an average number of days consuming yogurt of 102 days \pm 19 SD. The control group had

no form of intervention. For blood trace metal analysis, individuals with $\geq 75\%$ compliance in the probiotic group were selected along with controls of appropriate age, nutritional status, and matched fish intake resulting in 12 PW per group. Given that this was a pilot study, sample size was based upon participant availability.

Both studies were registered with clinicaltrials.gov (NCT01904513, NCT02021799) and approved in Canada by the Health Sciences Research Ethics Board at Western University (#102881, #18850) and in Tanzania by the Lake Zone Institutional Review Board.

6.4.2 Dietary exposure

To assess potential exposure to dietary toxic metals via fish consumption, three of the most commonly consumed fish were collected. All samples were obtained from the main fish market in downtown Mwanza in early December 2012. Three specimens of each fish species were collected: Nile Perch (*Lates niloticus*), Tilapia (*Oreochromis niloticus*) and Dagaa/Silver Cyprinid (*Rastrineobola argentea*). Each was caught from a different area along the Mwanza coastline. Muscle tissue was removed from the Nile Perch (*Lates niloticus*) and Tilapia (*Oreochromis niloticus*) and frozen at -80°C until analysis. Dried *R. argentea* was frozen whole. Samples were digested in aqua regia and analyzed for lead, mercury, arsenic and cadmium by ICP-MS (Agilent 7700) at the UWO, Analytical Services Laboratory, London Canada.

6.4.3 Blood metal quantification

Samples were collected in Vacutainer Trace Elements Blood Tubes (Becton Dickinson) and frozen at -80°C until analysis. Whole blood samples were digested in ultrapure nitric acid before being analyzed on an ELEMENT 2 HR-SF-ICP-MS (Thermo Scientific) according to the standard operating procedures of the Trace Elements Laboratory of the London Health Sciences Centre for a panel of toxic metals (mercury, arsenic, cadmium, lead).

6.4.4 Microbiome analysis

DNA was extracted from frozen fecal samples of the SAC group using the E.Z.N.A Stool Kit (Omega Biotek) according to the manufacturers' instructions. Amplification of the V6 region of the 16S rRNA gene was carried out using the primers CCATCTCATCCCTGCGTGTCTCCGACTCAGnnnnnCWACGCGARGAACCTTACC and CCTCTCTATGGGCAGTCGGTGATACRACACGAGCTGACGAC where nnnnn is a sample specific nucleotide barcode. Amplification was carried out in 42 μ L reactions with 10 μ L of 3.2 pMol/ μ L of each primer, 20 μ L GoTaq hot start colorless master mix (Promega) and 2 μ L purified DNA. The PCR protocol was: 2 minutes 95 °C, and 25 cycles of: 1 minute each 95°C, 55°C, 72°C. PCR yield was assessed with a Qubit Fluorometer (Life Technologies) and samples were pooled at equimolar concentrations before a final cleanup with the QIAquick PCR Purification kit (Qiagen). Library preparation and sequencing was carried out at the London Regional Genomics Centre (London, Canada) on an Ion Torrent Personal Genome Machine (Life Technologies) with 316 chip following the manufacturers' instructions.

Resulting reads were extracted, de-multiplexed and grouped into operational taxonomic units (OTUs) at 97% identity in the manor previously reported (Gloor *et al.*, 2010). Reads were deposited into the Short Read Archive (BioProject ID: PRJNA244107) and barcodes and their corresponding sample IDs are available in Supplemental Table 1. Taxonomic assignments were made by extracting best hits from the Ribosomal Database Project (rdp.cme.msu.edu) Seqmatch tool. These were manually curated by comparison to the NCBI non-redundant database and the Green Genes database (greengenes.lbl.gov). OTU IDs, sequences and taxonomies have been supplied in Supplemental Table S2. Further analysis was carried out using Quantitative Insights Into Microbial Ecology (QIIME) (Caporaso *et al.*, 2010) and R (R-project.org). To better handle comparisons of compositional data, the centered log ratio transformation of Aitchison (1982) adapted to microbiome data (Fernandes *et al.*, 2013; Fernandes *et al.*, 2014) was used and then tested using ANOVA with FDR multiple testing corrections. Cadmium was excluded from analysis due to the limited range of concentrations observed.

6.5 Acknowledgements

We wish to thank the Lab Scientists at NIMR Mwanza for providing access to their facility, and to the women, children and their guardians who participated in the study. We also thank the African Probiotic Yogurt Network for logistical support and Mabatini/Tukwamuane, Buswelu and VSI yogurt kitchens for producing the probiotic yogurts. We appreciate the Trace Elements Laboratory at Victoria Hospital for their valuable guidance and blood sample analysis and the Geoanalysis and SWAP testing lab at the Biotron Climate Change Research Centre for their assistance with food testing. We also wish to express thanks to Jean Macklaim for assistance with analysis of the microbiome data. JEB and ME are recipients of NSERC Canada Graduate Scholarships. Funding was received from Bill and Melinda Gates Foundation. The funding sources for this work were not involved in the collection, analysis or decision to publish this work.

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Chapter 7

7 Mercury-resistant lactobacilli to remediate toxic metals *in vitro* and *in vivo*.

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7.1 Introduction

Many heavy metals including copper and chromium serve biological functions within multicellular organisms through specialized functions including as acting as enzyme co-factors (Mertz, 1981). Some metals such as mercury, lead, cadmium and arsenic have virtually no biological function and are considered detrimental at any concentration. Nonetheless, these compounds exist in our environment and have increased in concentration due to anthropogenic activity (Lamborg *et al.*, 2014; McConnell and Edwards, 2008). The detrimental effects of these elements often include delayed neurological development in children (Dovydaitis, 2008), cancer (Hughes *et al.*, 2011) and more controversially cardiovascular and autoimmune disease (Gallagher and Meliker, 2012; Karagas *et al.*, 2012). New concepts are required to counter these problems for human and animal health. Most interventions within the field of bioremediation focus on environmental applications; however given that much exposure to toxic metals is through the diet, we ask the question: if we cannot remove metals from food supply, can we prevent their absorption from the gut? The use of food-grade organisms, including probiotic strains, offers one such possibility.

We have recently shown that probiotic yogurt containing lactobacilli can counter toxic metal uptake in children (aged 6-10) and pregnant women (Bisanz *et al.*, 2014). We selected the *Lactobacillus rhamnosus* GR-1 strain based on unpublished *in vitro* metal binding data and the strain's safe history of use in vulnerable populations (Anukam *et al.*, 2006; Hummelen *et al.*, 2010; Hummelen *et al.*, 2011a). When a 25 day intervention was applied in children, toxin adsorption lowered but the levels failed to reach statistical significance; however, a significant effect was observed in protection against mercury and arsenic in pregnant women given the intervention for an average of 88 days.

It had previously been established that lactobacilli have strong affinity for lead and cadmium (Halttunen *et al.*, 2007b) so it is puzzling that effects were seen in the case of mercury and arsenic, but not cadmium or lead. This suggested there may be additional mechanisms of action that are not directly related to a surface binding mediated sequestration as the literature would suggest. The results of our human studies indicated that we need to develop new and improved strains specifically tailored for remediation of toxic metals to improve efficacy. This required further mechanistic studies.

We hypothesize there may be at least four distinct mechanisms involved in probiotic mediated protection against toxic metals: (i) passive sequestration as previously reported against cadmium, lead and arsenic (Halttunen *et al.*, 2007a; Halttunen *et al.*, 2007b), (ii) direct enzymatic detoxification to metal species of reduced toxicity or uptake in the GI tract, (iii) modulation of host xenobiotic metabolic pathways to favorably alter host metabolism and elimination of toxic substances, and (iv) modulation of microbiota composition or function.

As toxic metals are elements, they cannot be truly destroyed, but rather converted to less toxic forms. In the case of mercury, this is possible through the organomercurial lyase and mercuric reductase enzymes frequently found in bacterial mercury resistance (Nascimento and Chartone-Souza, 2003). The end product of this pathway, volatile elemental mercury (Hg^0), is negligibly absorbed in the GI tract while the starting product, methylmercury (MeHg) is nearly fully absorbed with the intermediate inorganic mercury (InHg) being absorbed at variable rates (Gochfeld, 2003). Not all interactions of bacteria and mercury are favorable as it is known many strains also have the capacity to methylate mercury and at least one human gut isolate (*Methanomassiliicoccus luminyensis*) has been shown to possess the necessary determinants for mercury methylation (Parks *et al.*, 2013). This suggests modulating of the microbiota's capacity for mercury species interconversion may impact host mercury uptake. Our previous work would suggest there is no shift in microbiota composition after *L. rhamnosus* GR-1 consumption which partially discounts this theory with the caveat of 16S rRNA sequencing being a somewhat coarse method to study composition which tells little about function.

Modulation of host xenobiotic metabolism represents a novel and unreported potential mechanism relevant to both toxic metals and a range of environmental pollutants as well as having pharmacological implications. Zhai *et al.*, (2014) demonstrated that orally administered *Lactobacillus* strains could protect against liver damage from intraperitoneally administered cadmium despite not having an impact on cadmium uptake and tissue distribution. Furthermore, studies in germ-free mice have indicated microbes play a role in expression of metallothioneins (Breton *et al.*, 2013) and cytochrome p450 enzymes at distal sites such as the liver (Toda *et al.*, 2009). Mechanistic studies of how microbes can impact function at distal sites are an exciting avenue in probiotic and microbiome research requiring further studies.

Given previous reports of strain specific toxin sequestering activity (Halttunen *et al.*, 2007b; Hernandez-Mendoza *et al.*, 2009; Ibrahim *et al.*, 2006) we sought to screen a collection of 80 *L. rhamnosus* and *L. paracasei* strains with draft genomes (Smokvina *et al.*, 2013) for activity against toxic metals to allow the opportunity to apply comparative genomics to identify the genetic basis for strain variability and potential effectors.

The goals of this study were to identify novel strains specifically tailored for activity against toxic metals. Binding and mercury resistance assays were used as a proxy to uncover lactobacilli harboring mercury-detoxifying enzymes. The strains were further characterized and the genomic basis of resistance was determined. Finally these strains were applied in murine models to determine the effect of mercury detoxifying enzymes *in vivo*.

7.2 Results

7.2.1 Binding of lead and mercury by strains of *L. rhamnosus*.

Consistent with previous reports of passive sequestration of lead by lactobacilli, all studied *L. rhamnosus* strains were capable of binding a significant portion of the 50 µg/mL lead inoculum in a strain variable manor (Figure 7-1A). Binding efficiencies varied from 42% (Lr64) to 99% (Lr60 and Lr63). Binding to InHg (as Hg²⁺) was assayed in strains Lr64 and Lr60 at 1 µg/mL (Figure 7-1B) and 10 ng/mL (Figure 7-1C) concentrations revealing up to 95% InHg removal by strain Lr60 with greatest removal at

higher concentrations. At the 1 $\mu\text{g/mL}$ concentration, Lr60 demonstrated significantly higher InHg removal than strain Lr64 which was not observed at 10 ng/mL . Neither strain resulted in production of MeHg from InHg. Removal of MeHg (as CH_3Hg^+) was also assayed again at 1 $\mu\text{g/mL}$ and 10 ng/mL (Figures 7-1D and 7-1E) showing significant removal with a maximum of 45% MeHg removed from solution by strain Lr60. The effect of viability was also examined in strain Lr60 where it was found that there was a statistically significant loss of mercury removal ability with a heat-killed strain, while other strains including Lr136 show no difference. The loss of mercury removal by Lr60 was coupled with a 16% difference in recovery of mercury by nitric acid digestion suggesting a loss of mercury from the system.

To examine passive binding, electron microscopy was used. TEM micrographs show the presence of metal precipitates in association with the cell in the case of both lead and mercury (Figure 7-2A-C). These precipitates do not appear to be in the surrounding capsule but rather in close association with the cell. To confirm the composition of the precipitates, SEM/EDX (representative image of Lr60 with InHg Figure 7-2E) was applied confirming the composition of the mercury precipitates (Figure 7-2F) and lack of metals in control cells (Figure 7-2D).

To further characterize the Lr60 strain as well as to screen the collection for the presence of mercury detoxifying enzymes, mercury resistance was screened in all strains. No strain showed increased resistance to MeHg and no *L. paracasei* strain displayed clear increased resistance to InHg (data not shown). Two strains showed elevated resistance to mercury in liquid media (strain Lr60 and Lr63), the organisms previously identified as displaying the highest lead sequestering activity (Figure 7-3A). Lr60 and Lr63 additionally displayed elevated resistance in disk diffusion assays with a difference of 6 mm in the zone of clearing (Figure 7-3B). To determine if resistance to mercury was an adaptive response, strains Lr60 and Lr63 were precultured in $\frac{1}{2}$ of their minimum inhibitory concentration (MIC) (6.25 $\mu\text{g/mL}$) and then were tested again in a MIC assay. Both strains showed a 2-fold increase in MIC as well as a reduced lag-phase during growth at 6.25 $\mu\text{g/mL}$ (Figure 7-3C; Lr60 shown as representative of both strains).

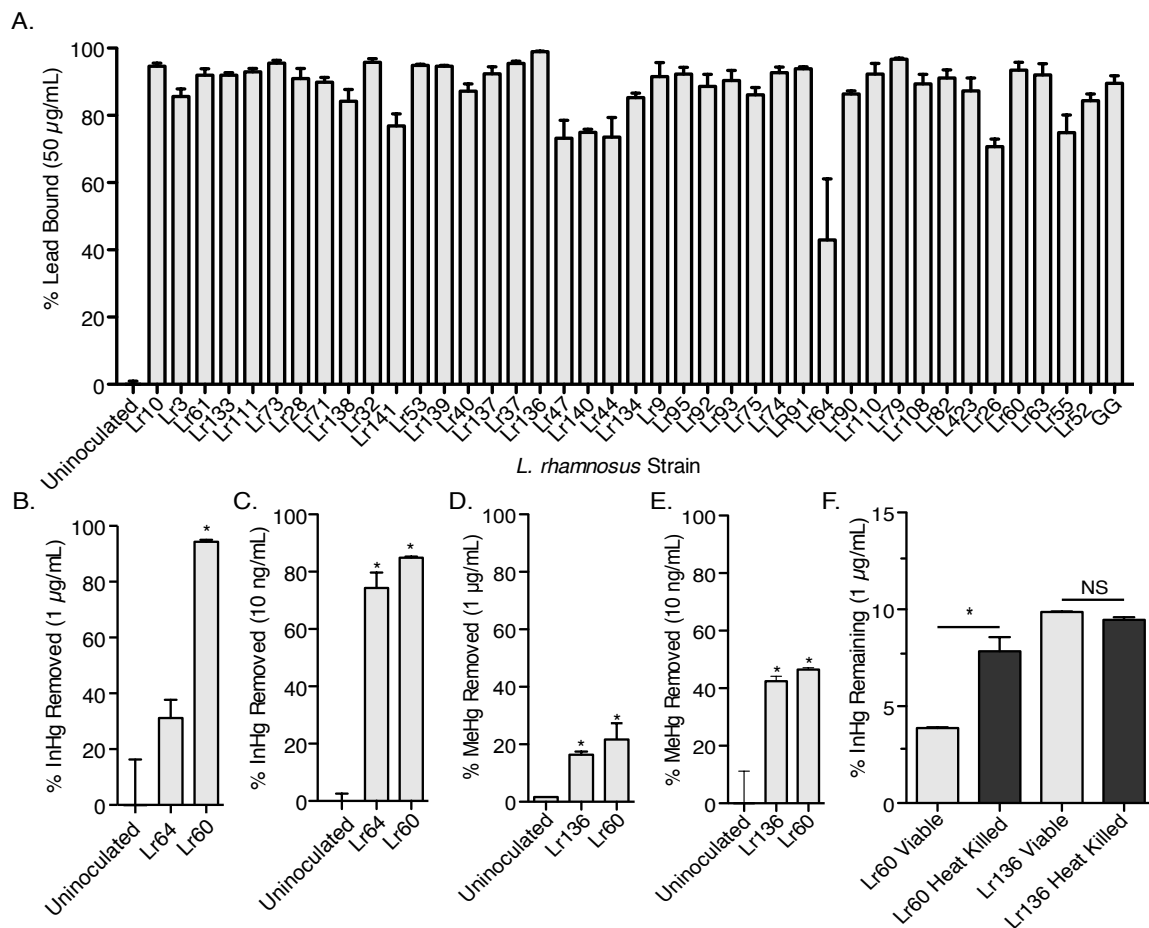


Figure 7-1. Removal capacities of lead and mercury by *L. rhamnosus* strains.

(A) Lead binding by 40 strains of *L. rhamnosus*. Mercury removal by select *L. rhamnosus* strains at 1 $\mu\text{g/mL}$ InHg (B), 10 ng/ μL InHg (C), 1 $\mu\text{g/mL}$ MeHg (D) and 10 ng/mL MeHg (E). The effect of viability on mercury removal by strains Lr60 and Lr136. Error bars \pm SEM, * $p < 0.05$ vs. control unless otherwise noted.

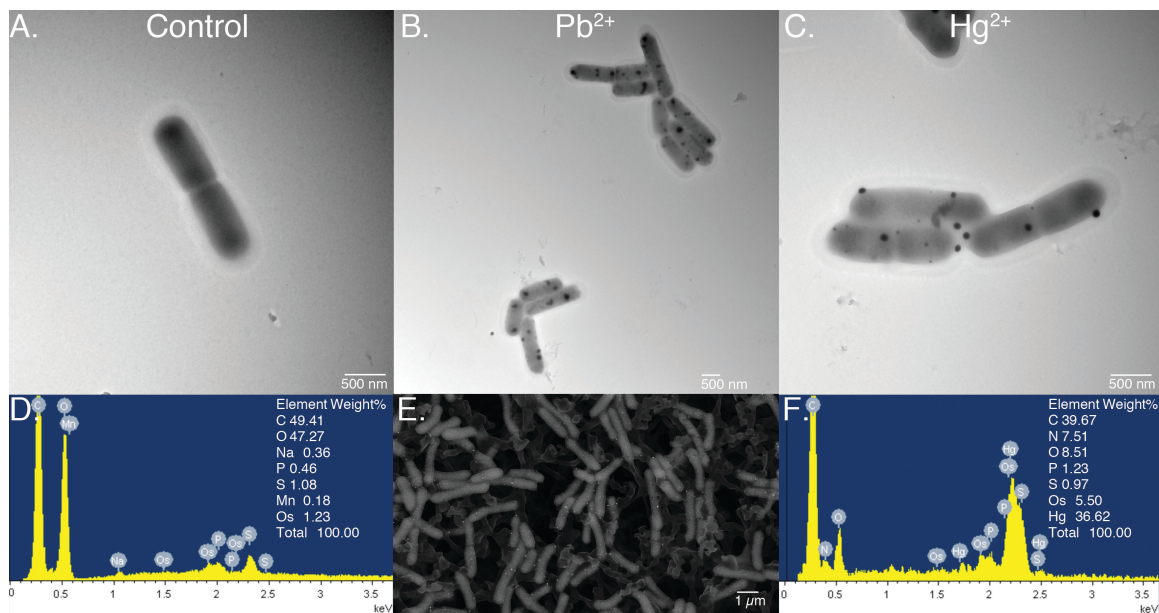


Figure 7-2. TEM and SEM/EDX analysis of metal precipitates in association with *L. rhamnosus* Lr60.

TEM micrographs of *L. rhamnosus* Lr60 incubated with Pb^{2+} (B), Hg^{2+} (C), or HEPES control (A). EDX compositional analysis of individual metal precipitates for mercury (F) and control (D). Representative SEM image of Lr60 incubated with Hg^{2+} for selecting individual precipitates for EDX analysis (E).

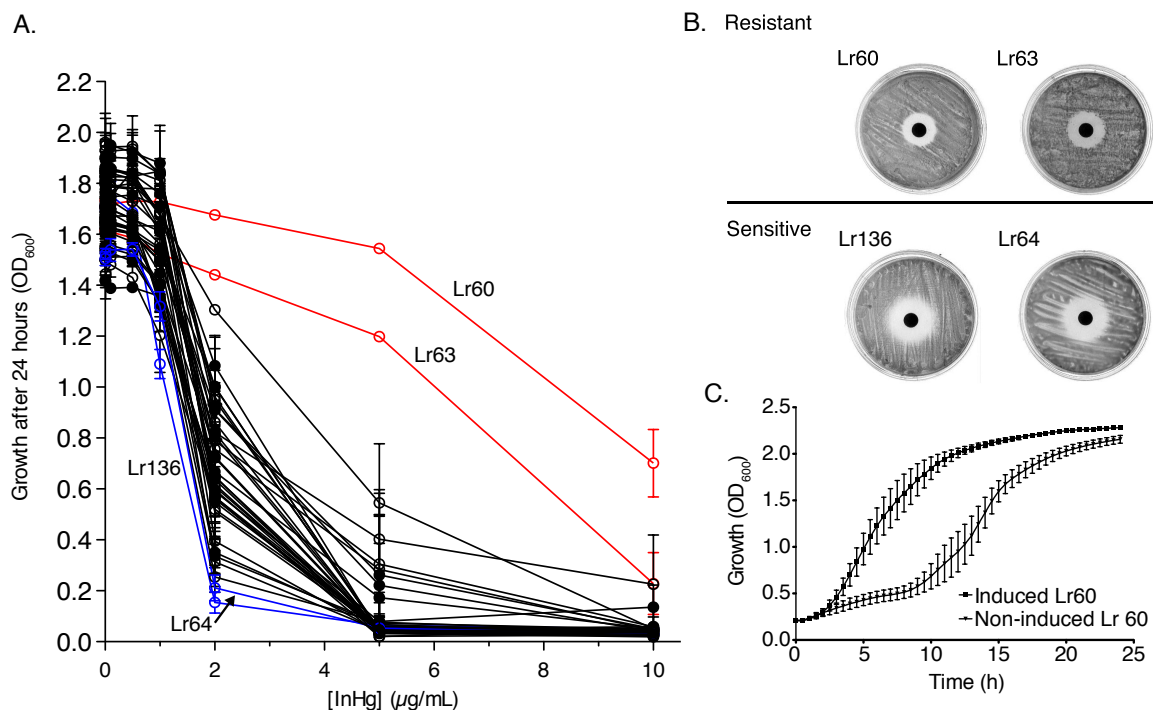


Figure 7-3. Mercury resistance screening in *L. rhamnosus* strains.

Growth across a gradient of InHg by *L. rhamnosus* strains demonstrates two strains of increased resistance (A). Disk diffusion assays confirm increased resistance (6mm) in Lr60 and Lr63 as compared to Lr136 and Lr64 (B). Induction in ½ MIC reduces growth inhibition upon re-culturing at ½ MIC as compared to Non-induced control (C).

7.2.2 Genetic determinants of mercury resistance in *L. rhamnosus*

At the time of this genomics work (2012-2013), no publically available genome of *Lactobacillus* were found to contain a full-length mercuric reductase (*merA*) or organomercurial lyase (*merB*). There has been a recently sequenced genome (Tanizawa *et al.*, 2015) of *Lactobacillus hokkaidonesis* JCM 18461 isolated from silage that likely contains a *merA* gene. It was determined that many *Lactobacillus* genomes contain a miss-annotated dihydrolipoamide dehydrogenase gene which is identified as *merA* due to homology in the pyridine nucleotide-disulphide oxidoreductase and dimerization domains. The dihydrolipoamide dehydrogenase critically lacks the N-terminal heavy metal associated domain containing two cysteine residues to bind the mercury ions. A

clustal alignment of the MerA of Lr60 and two miss-annotated dihydrolipoamide dehydrogenases is included in Figure 7-4.

To determine the genetic basis for increased mercury resistance in *L. rhamnosus* Lr60 and Lr63, a comparative genomics approach based on the machine learning approach used in chapter 3 was attempted which failed to yield meaningful results. Direct searches for *merB* and *merA* by BLASTP to validated Gram-positive genes discovered the presence of a full-length *merA* exclusively in the genomes of strains Lr60 and Lr63. It was determined that during clustering of orthologous protein groups, the true *merA* of Lr60 and Lr63 was inappropriately clustered with the dihydrolipoamide dehydrogenase present in other strains.

Due to low coverage in the initial genome sequencing, the genomes of strain Lr60 and Lr63 were resequenced via Illumina MiSeq. This resulted in draft genome assemblies for Lr60 (28-fold coverage, n50=125430bp) and Lr63 (27-fold, n50=100067bp) that were sufficient to resolve the genomic context of the *merA* gene. The mercuric reductase was found in a presumed operon structure with a *merR* transcriptional regulator (Figure 7-5A). Co-transcription of the *merR* and *merA* genes was verified by nested PCR from cDNA (Figure 7-5B) while their up-regulation in response to mercury was verified by qPCR (Figure 7-5C). Maximal up-regulation (10.6-fold *merA*; 11.3-fold *merR*) after 1 hour was observed at $\frac{1}{2}$ MIC (6.25 $\mu\text{g/mL}$).

The *merRA* operon appears to be found in an identical 10 kb horizontally acquired region in both Lr60 and Lr63 as determined by a significant GC-skew and interpolated variable order motif analysis (Vernikos and Parkhill, 2006) (Figure 7-5A). Additionally the insertion appears to have occurred in the vicinity of tRNA genes and it has remnants of transposon machinery, which is further indicative of horizontal gene transfer. The transfer also appears to have carried a *cadD* cadmium efflux protein as well as copper resistance determinants. Comparison to the Insertion Sequence database (<https://www.is.biotoul.fr>) reports no hits. The *merA* has high nucleotide identity to that of oral streptococci (99% nucleotide ID, 96% coverage; *S. oralis* Genbank: AJ582645.1, *S. mitis* Genbank: AJ582646) though the putative horizontally acquired region appears to be a

mosaic of other organisms, including regions homologous to *Bacillus cereus* RC607, thus it is not possible to identify a single possible source of the gene transfer. Additionally it was determined that Lr60 and Lr63, both isolates from fermented cereal, are extremely similar sharing 99.97% average nucleotide identity (ANI). This level of similarity may indicate they are in fact isolates of the same strain. For reference, Lr60 and *L. rhamnosus* GR-1 have a 99.20% ANI while the earlier 454 assembled draft genome of Lr60 and the newer MiSeq assembly have an ANI of 99.95%. The 16S rRNA, *cpn60*, *gyrB*, *dnaJ* and *ldhL* genes are also identical in nucleotide composition. Finally predictions of unique genes, based on reciprocal blasts of all predicted protein coding sequences suggest few genes with assignable functions unique to each strain. Further studies focused solely on strain Lr60 given the extreme similarity between the Lr60 and Lr63 strains.

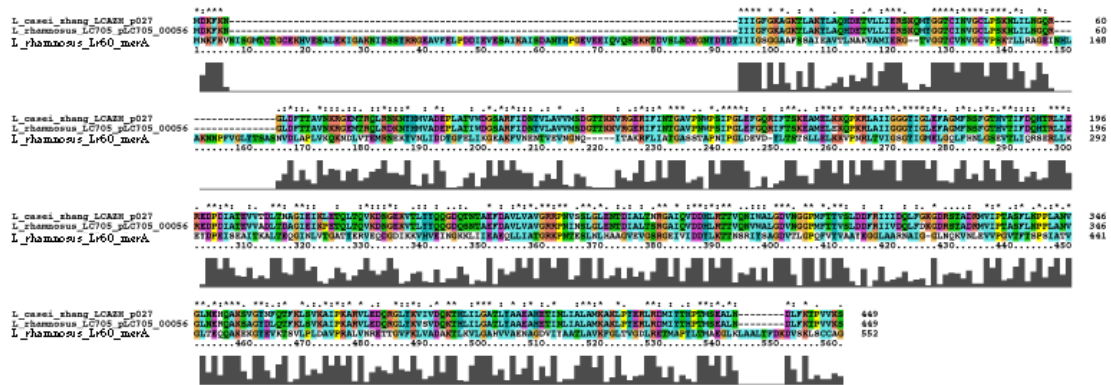


Figure 7-4. Clustal alignment of the MerA of Lr60 with the dihydroliipoamide dehydrogenase frequently misannotated as a mercuric reductase.

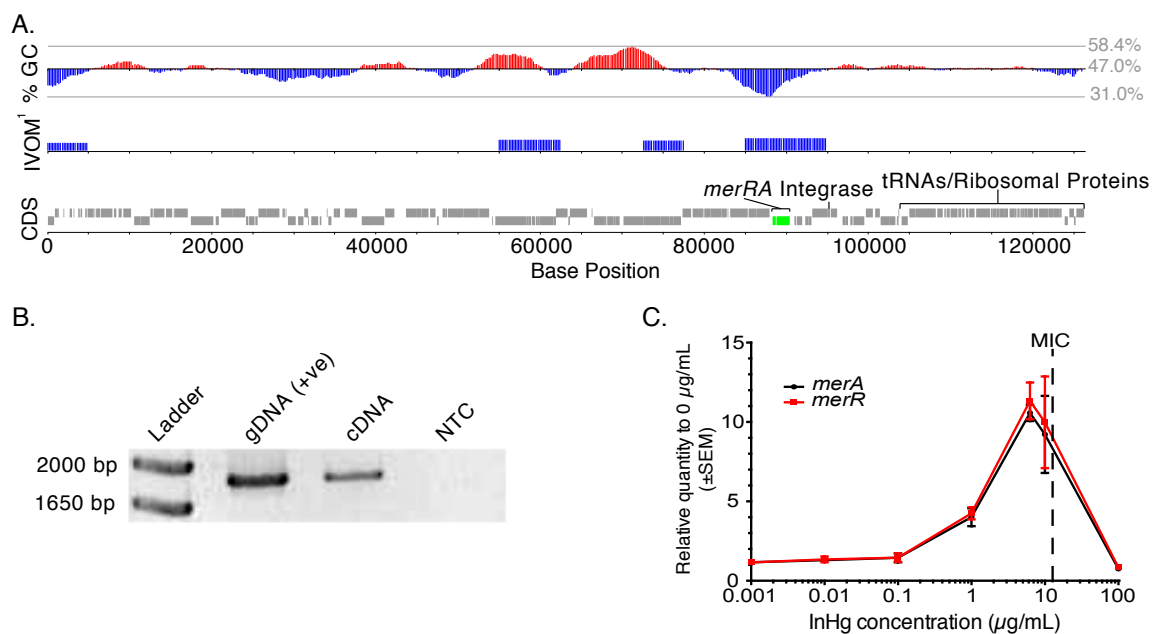


Figure 7-5. Genomic and transcriptional characterization of mercury resistance in *L. rhamnosus* Lr60.

(A) A minimal mercury resistance operon (*merRA*) was located in a horizontally acquired region as indicated by skewed GC composition and interpolated variable order motif analysis prediction of horizontally acquired regions (in blue). (B) It was determined that *merR* and *merA* are co-transcribed as indicated by a 1889 bp product bridging the genes amplified from cDNA prepared from DNA-free RNA from Lr60. (C) *merRA* is up-regulated by exposure to lnHg in a dose-dependent manner.

7.2.3 Validation of *merA* function

Given that a mercuric resistance operon (*merRA*) has never been described in a *Lactobacillus* species, we sought to validate the function of the genes through mutant analysis. The *merRA* operon from Lr60 was cloned into pLAB1301, a *Lactobacillus-E. coli* shuttle vector and electroporated into the mercury sensitive *L. rhamnosus* GR-1. MIC analysis of the resultant GR-1 pLAB1301-*merRA* showed a 4-fold increase in MIC as opposed to the WT and vector controls (Figure 7-6A). The *merRA* of Lr60 was knocked out through allelic exchange with chloramphenicol acetyltransferase (*cat*) (Lr60 Δ *merRA*::*cat*) revealing a 4-fold loss in MIC which was complemented by pLAB1301-*merRA* (Figure 7-6B). To demonstrate that the *merRA* operon results in volatilization of mercury as expected from the *merA*, the X-ray film method was used which demonstrated volatilization from the Lr60 WT and complemented strains, but not in the Lr60

$\Delta merRA::cat$ strain (Figure 7-6C). Mercury volatilization is determined by darkening of the film due to reaction of Hg^0 with the silver halides in the film.

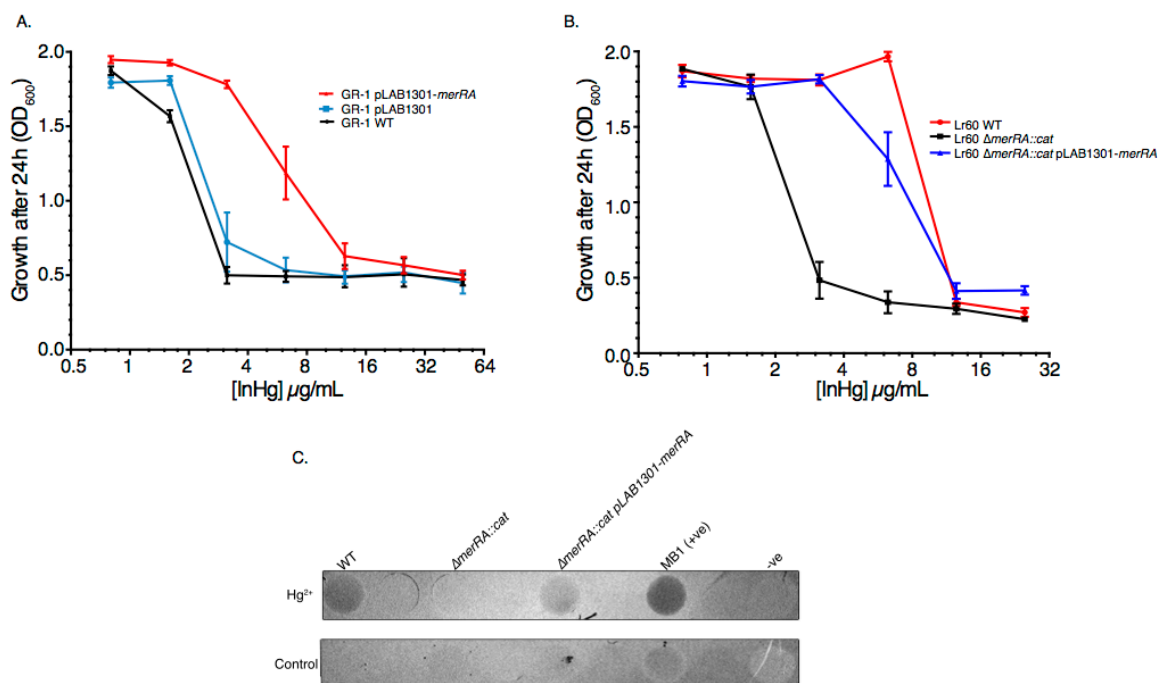


Figure 7-6. Validation of *merA* function by mutant analysis and volatilization assay.

(A) Expression of *merRA* amplified from Lr60 in the mercury sensitive *L. rhamnosus* GR-1 strain results in a 4-fold increase in resistance to mercury as indicated by MIC assay. (B) Knock-out of *merRA* in Lr60 results in a 4-fold loss in mercury resistance which is reverted by complementation. (C) X-ray film demonstrates production of Hg^0 , as indicated by a dark spot over the film, in WT and complemented Lr60, but not the knock-out strain.

7.2.4 Protective effects of lactobacilli in murine models of acute and sub-chronic metal exposure

To test the effect of lactobacilli on both acute and sub-chronic dosing schemes of both mercury and a combination of toxic metals (Hg, Pb, Cd; Figure S1), as well as to determine the effect of the Lr60 *merRA* operon *in vivo*, Balb/C mice were used. The ultimate endpoint was quantification of blood and tissue metals that will differentiate the effect of mercury resistance determinants. This analysis has been pending for 5 months due to logistical issues at the University Hospital of Lille and thus is unavailable for the purposes of this thesis.

As a marker of heavy metal toxicity, kidney histology was examined due to the extreme toxicity that these metals have for the kidney. Figure 7-7A shows representative images of the H&E stained transverse kidney sections from the saline control while 7-7B and 7-7C show the sections from the Hg and heavy metal cocktail (HMC) groups. It can be noted in both the mercury and HMC groups, that there is a loss of regularity and noticeable hypertrophy at the renal corpuscle. In the groups treated with *L. rhamnosus* Lr60, this damage seems to have been negated demonstrating a protective effect of Lr60 *in vivo*. Similar effects were observed in the sub-chronic dosing scheme but no differences were observed between the mutants of Lr60.

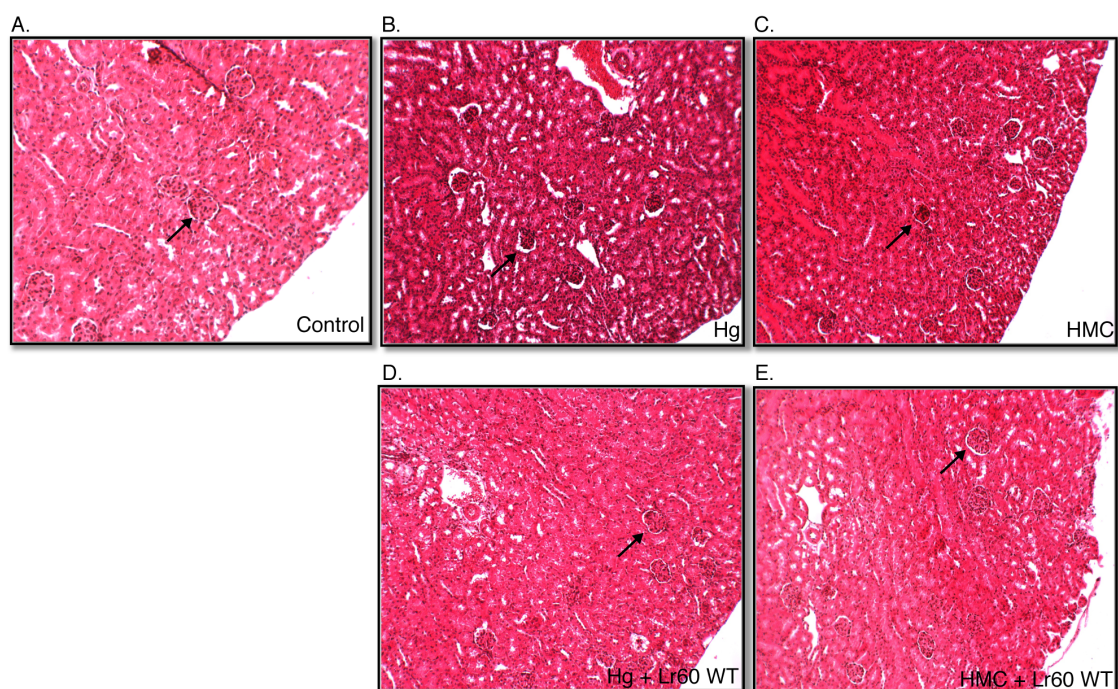


Figure 7-7. Histological analysis of effect of strain Lr60 on protection against mercury and a mixture of toxic metals.

H&E stained kidney sections of mice in acute-dosing study from control (A), mercury (B), heavy metal cocktail (C), mercury + Lr60 (D) and heavy metal cocktail + Lr60 (E)(representative images). Kidneys treated with metals alone (B and C) show damage at the level of the renal corpuscle (arrows). This damage appears to be negated by treatment with Lr60 (D and E).

7.2.5 Modulation of xenobiotic metabolism by *L. rhamnosus* GR-1

Given the previous effects of *L. rhamnosus* GR-1 in human populations and its previously observed abilities to act at distal sites from the gut (Gan *et al.*, 2014), it was selected for potential studies on modulation of xenobiotic metabolism. Balb/C mice were orally gavaged with either *L. rhamnosus* GR-1 or saline vehicle control daily for 3 days before sacrifice. The liver, kidneys and duodenum were harvested and RNA extracted and transcriptionally profiled by microarray and qPCR. Genes determined to be significantly differentially expressed ($p < 0.05$, ± 2 -fold) were subjected to GO enrichment analysis demonstrating that xenobiotic metabolic processes were significantly enriched in transcripts in the liver but not the kidney ($p < 0.05$). Table 7-1 contains a list of genes of interest related to xenobiotic metabolism and damage and which were found to be differentially regulated by *L. rhamnosus* GR-1. A subset of these genes was confirmed by qPCR (Figure 7-8) demonstrating significant increased expression of *Por* (8.3-fold), *Fmo2* (7.1-fold) and *Fmo3* (5.0-fold) in the liver after administration of *L. rhamnosus* GR-1.

Table 7-1. Differentially regulated liver transcript by *L. rhamnosus* GR-1 with functions related to xenobiotic metabolism.

Gene	Annotation	Fold-Change (GR-1 vs. Control)
<i>Por</i>	P450 Oxidase	2.58324
<i>Fmo2</i>	Flavin containing monooxygenase 2	2.45478
<i>Fmo3</i>	Flavin containing monooxygenase 3	2.07543
<i>Il1b</i>	Interleukin 1 beta	-2.68131
<i>Cyp2b10</i>	Cytochrome P450 2b10	-2.82371
<i>Gstm3</i>	Glutathione S-transferase (mu-3)	-2.13694

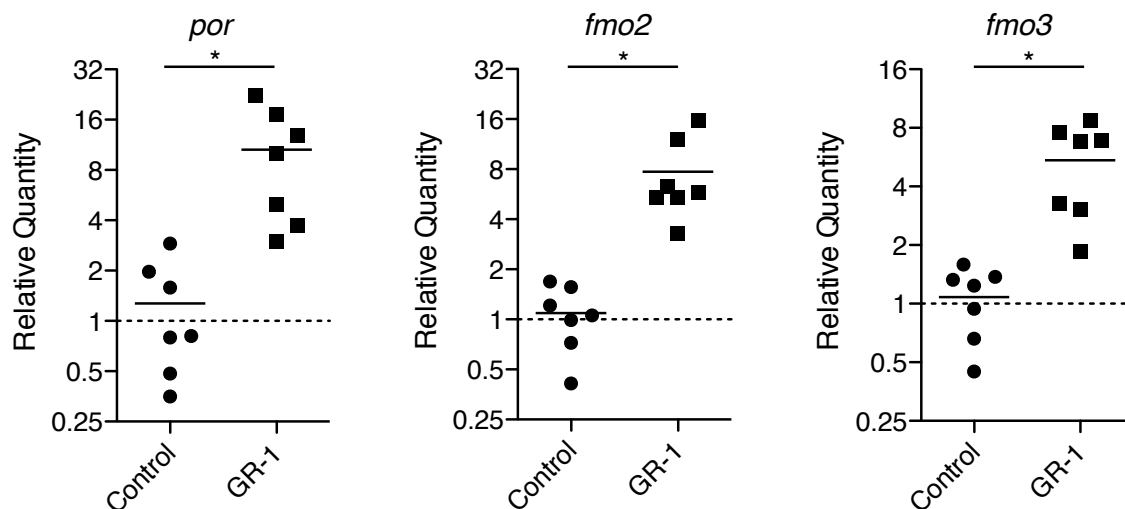


Figure 7-8. qPCR validation of *por*, *fmo2*, and *fmo3* genes show up-regulation of transcripts by *L. rhamnosus* GR-1. * $p < 0.05$

7.3 Discussion

The work described in this manuscript represents our intent to mechanistically understand how strains of lactobacilli may have protective effects against toxic metals *in vitro* and builds upon previous knowledge of binding-only mediated mechanisms.

Our results agree with others (Halttunen *et al.*, 2007b; Peltonen *et al.*, 2001; Stidl *et al.*, 2008) indicating lactobacilli have the capacity to interact with a variety of environmental pollutants via a passive binding. Given the surface of most Gram-positive bacteria is negatively charged, it makes sense that they should be able to interact with metals through electrostatic interactions and the nature of strain-variable binding of metals suggests altered composition of the cell surface (Beveridge and Fyfe, 1985). By far, the worst metal binding strain observed in these studies was strain Lr64, which is interesting as it has a unique mucoid phenotype when grown on solid media suggesting the presence of a large amount of extracellular polysaccharide (EPS). Though not the focus of these particular studies, the machine learning approach previously applied (chapter 3) would suggest that there is a negative association between a number of EPS-related glycosyltransferases and lead binding capacity. Furthermore, our TEM figures show little evidence of metal precipitates in the extracellular polysaccharides surrounding Lr60, but

rather in close association with the cell. Further localization would require ultra thin-sectioning but recent work would suggest that localization within the cell may depend on the particular metal under study (Monachese, 2012). The capsule has been suggested as a potential binding site for metals (McLean and Beauchemin, 1990) however the present data suggest the EPS of lactobacilli lacks this capacity. The observation of distinct precipitates on the cell begs the question of what may be the nucleation site and if there may be a more specific site of binding than negatively charged groups in the peptidoglycan and teichoic acid (Beveridge, 1989).

While binding has been shown to be the predominant mechanism of interaction with lead and cadmium, the case of mercury is more interesting given its known bacterial pathways of detoxification (Nascimento & Chartone-Souza, 2003). Highest mercury removal was obtained with InHg, but significant levels of removal were still observed with MeHg. The reduced capacity may be due to the lower positive charge of MeHg, however significant binding still occurred at very low levels (10 ng/mL). Our initial hypothesis that mercury-resistant lactobacilli would be superior remediators of metals resulted in the discovery of the Lr60 and Lr63 strains. While *in vitro*, the predominant mechanism is most likely binding, as indicated by elevated rates of removal by non-viable Lr60, there is a highly reproducible loss of removal efficiency coupled with increased recovery of mercury from the system indicating a volatilization mechanism as well. The sensitive Lr136 strain, also a strong binder of metals, shows no difference in mercury removal when killed. We would hypothesize that improved metal removal would be expected from non-viable strains due to a dissipation of the proton motive force increasing the net negative charge of the cell surface (Mera *et al.*, 1992).

The identification of mercury resistance in *L. rhamnosus* Lr60 and Lr63 is a novel finding in lactobacilli. Unlike common antibiotics in typical clinical isolates, agencies such as the Clinical and Laboratory Standards Institute have not established thresholds for mercury resistance in lactobacilli. The term resistance is used in this work due as the observed phenomenon fits two key criteria of resistance: (i) the resistant strains can grow in concentrations that kill the majority of the strains of the same species, (ii) the presence

of genetically encoded resistance elements with an observable phenotype when transferred to sensitive strains.

The discovery of a novel horizontally acquired minimal mercury resistance operon (*merRA*) present in the Lr60 and Lr63 strains is a unique observation. While there appear to be remnants of transposon machinery, the region is unlikely to still be mobile as it lacks the typical inverted repeats of a bacterial transposon (Foster *et al.*, 1981) and may represent a composite of multiple gene rearrangements in this region. Given that the acquired region also contains cadmium and copper resistance elements, it can be suggested that this acquisition was due to the exposure of the strain to toxic metal levels in the past, which could have occurred for any number of reasons. Despite being speculative, it is possible that transfer from streptococci could have occurred in the oral cavity due to mercury exposure from dental amalgam fillings.

To test the hypothesis that mercury-detoxifying enzymes confer increased mercury remediation capacity *in vivo* required the use of animal models, however due to logistical issues outside the control of the investigators, not all of this data are available for this thesis. Nonetheless, preliminary investigation by histology has demonstrated a protective effect against kidney damage by both the Lr60 and GR-1 strains in agreement with the observations of our previous human studies (Bisanz *et al.*, 2014).

Finally, the exploratory analysis of how oral probiotics may affect distal function in the liver is an exciting area for future research. The finding of the cytochrome P450 reductase (*por*) expression up-regulated by *L. rhamnosus* GR-1 is significant given that *Por* is an obligate electron donor for many of the cytochrome P450 enzymes which are key components in xenobiotic metabolism. Thus the altered regulation of cytochrome P450 reductase likely represents a significant alteration in the broad functions of many CYP enzymes. There is likely cross-over between the mechanisms by which the gut microbiota, and orally administered probiotics can modulate xenobiotic metabolism. Differential regulation of a number of the other CYP enzymes including Cyp2B10 is also noteworthy given links between gut microbiota modulation of xenobiotic metabolism through the CAR receptor (Björkholm *et al.*, 2009). Altered expression of the

inflammatory mediator interleukin 1 beta (IL1B) could represent one mechanism by which decreased liver damage could be observed without direct interaction of the metals and lactobacilli as demonstrated by Zhai *et al.* (2014) in models of intraperitoneally cadmium and oral lactobacilli administration. While modulation of cytochrome P450 function is of less significance to toxic metals, up-regulation of glutathione S-transferase (*gstm3*) is directly relevant as the glutathione S-transferases are important in countering metal-mediated oxidative damage as well as direct conjugation of metals to glutathione (Casalino, 2004; Gregus and Varga, 1985). In this case however, expression of *gstm3* was down-regulated by *L. rhamnosus* GR-1 which demonstrates on our limited understanding of how microbes may affect xenobiotic metabolism. This set of experiments sheds light on modulation of host xenobiotic metabolism as a potential mechanism through which lactobacilli may exert beneficial effects. This is not limited to toxic metals, but a broad range of dietary toxins as well as having implications for pharmacology.

We hypothesized multiple mechanisms involved in probiotic mediated protection against toxic metals and have provided evidence of three in the current manuscript: (i) passive sequestration, (ii) direct enzymatic detoxification, and (iii) modulation of host xenobiotic metabolic pathways to alter host metabolism and elimination of toxic substances. In addition we have characterized new strains specifically tailored to counter toxic metals. The strength of this study is that it provided multiple lines of evidence and attempted to describe probiotic function at the gene level. Future analysis and studies branching from this work will help to increase our understanding of probiotic function and create strain-level microbial therapies to counter environmental toxins.

7.4 Materials and Methods

7.4.1 Culture conditions

All *Lactobacillus* strains except *L. rhamnosus* GR-1 were obtained from the Danone Research collection (Palaiseau, France) and were maintained on de Man, Rogosa and Sharpe (MRS; BD Biosciences) agar at 4°C after culture at 37°C for 48 hours under anaerobic conditions (BD GasPack, BD Biosciences). Master stocks were maintained at -

80°C in MRS supplemented with 20% glycerol. *L. rhamnosus* GR-1 was obtained from an in-house strain collection and was originally isolated from the urogenital tract of a premenopausal woman (Reid *et al.*, 1987). When required, MRS was supplemented with 5 µg/mL erythromycin or 15 µg/mL chloramphenicol. *Lactobacilli* were heat-killed as needed by incubation at 60°C for 30 min followed by confirmation by plating on MRS agar and 72 hour incubation in anaerobic conditions at 37°C. Strains were subcultured twice in MRS broth (1 % v/v inoculum) before use in assays. Electrocompetent *Escherichia coli* TOP10 was obtained commercially (Thermo Fischer/Life Technologies) and used as the host for all cloning experiments. *E. coli* TOP 10 was maintained on LB (lysogeny broth; BD Biosciences) or BHI (Brain Heart Infusion; BD Biosciences) supplemented with 100 µg/mL ampicillin or 200 µg/mL erythromycin where appropriate. *Bacillus megaterium* MB1, a mercury resistant strain used as a positive control was obtained from CC Huang (National Chung Hsing University, Taiwan).

7.4.2 Metal binding assays

Lead binding assays were carried out in duplicate experiments with 3 technical replicates each using modified methods of Halttunen *et al.* (2007b). Strains were normalized to an $OD_{600nm}=2.0$ corresponding to a count of $\sim 2 \times 10^9$ CFU/mL and 200 µL of normalized culture was centrifuged at 5,000 g for 20 minutes before being washed twice in Milli-Q H₂O (pH=5) and recentrifuged as before with the supernatants discarded. Cells were then resuspended in 200 µL Milli-Q H₂O (pH=5) containing 50 µg/mL Pb²⁺ (prepared from 10 mg/mL analytical standard; Sigma Aldrich) before incubation for 4 hours at 37°C. After incubation, cells were centrifuged at 5,000 g for 20 minutes and the supernatant removed. The supernatant was acidified with 40 µL of 20% Nitric Acid (VWR) and frozen for analysis by inductively coupled plasma- atomic emission spectroscopy (ICP-AES) at the Biotron Facility (University of Western Ontario). Results were normalized to an uninoculated control and 50 µg/mL standard and converted to a percentage bound by the following formula $100 \times (1 - ([\text{sample}]/[\text{uninoculated control}]))$.

Mercury removal assays were performed by using 10 mL of culture normalized as before which was centrifuged for 20 minutes at 5,000 g. The supernatant was removed and replaced with 10 mL of MRS containing either 1 µg/mL or 10 ng/mL InHg (HgCl₂;

Sigma Aldrich) or MeHg (CH₃HgCl; Sigma Aldrich) from 10 mg/mL stocks prepared in Milli-Q H₂O or 100% ethanol respectively. After 24h of incubation at 37°C, the cultures were centrifuged as before and the supernatant immediately frozen for analysis by cold vapor atomic absorption spectroscopy at the Biotron Facility (University of Western Ontario). Samples were normalized as before. When cultures were digested in 20% nitric acid to recover bound mercury, they were resuspended in 12 mL nitric acid and heated at 60°C overnight. Wet mass (average = 300 mg) and cell concentration (average = 2x10⁹ CFU/mL) after experiments were recorded to ensure consistent normalization of cultures.

7.4.3 Electron microscopy

For both transmission electron microscopy (TEM) and scanning electron microscopy (SEM), 1 mL cultures were centrifuged at 5,000 g for 10 minutes and washed 3 times in 50 mM HEPES buffer before being resuspended in 10 mL 50 mM HEPES buffer containing 1 mM Hg²⁺ or Pb²⁺. Cultures were incubated for 4 hours at 37°C. For TEM, 10 µL was spotted directly on to electron microscopy grids (Formvar carbon film on 400 mesh copper, FCF400-CU; EMS Diasum) and allowed to dry. The whole mounts were then imaged using a Philips 420 Transmission Microscope at an accelerating voltage of 80 kV and approximate magnification of 19,000X. For SEM, the cultures were filtered through a 0.22 µm nitrocellulose filter that was then dried and coated with osmium tetroxide (5 nm; OPC80T Filgen Biosciences and Nanosciences). The filters were then imaged with a LEO 1540XB scanning electron microscope (Zeiss) fitting with EDX module (Oxford Instruments) for determining elemental composition at 10 kV and a working distance of 8.6 mm using backscatter electron detector mode.

7.4.4 Mercury resistance phenotyping.

Cultures normalized to an OD_{600nm}=2.0 were subcultured at 2.5% v/v into MRS media containing various concentrations of mercury prepared from mercury stocks as previously described in 96-well microplates (Corning Life Sciences). Plates were incubated for 24 hours at 37°C. Plates were either scanned after 24 hours or continuously read every 30 min (Figure 7-3C) at OD_{600nm} in a Multiskan Ascent (Thermo Fisher) plate reader. Minimum inhibitory concentration was defined as the lowest mercury concentration in

which no growth could be observed. Disk diffusion assays were prepared by swabbing (BD Culture Swab, BD Biosciences) cultures normalized to 1×10^8 CFU/mL (equivalent cell density to 0.5 McFarland *E. coli* standard) on 55 x 16 mm petri dishes (VWR) containing 10 mL MRS agar. Disks were prepared by placing 100 μ g HgCl₂ in Milli-Q H₂O on ¼ inch diameter paper disks (BD Biosciences) and air drying. Mercury volatilization assays were carried out using the X-ray film method of Nakamura and Nakahara (1988) with Amersham Hyperfilm ECL film (GE Life Sciences) using *Bacillus megaterium* MB1 as positive control (Oyetibo *et al.*, 2015). Mercuric reductase expression was induced by 24 hours growth in 1 μ g/mL HgCl₂ before assaying.

7.4.5 Genome sequencing

Original draft genomes were prepared by 454 pyrosequencing as previously described (Smokvina *et al.*, 2013). Resequencing of *L. rhamnosus* Lr60 and Lr64 was carried out by extracting gDNA from late-exponential cultures grown in MRS using the Purelink Genomic DNA kit with RNase kit (Life Technologies/ Thermo Fisher). The sequencing library was prepared with the Illumina Nextera XT kit and sequencing by Illumina MiSeq (London Regional Genomics Centre) using paired 2x150 bp reads. Reads were assembled using the Velvet short read assembler (Zerbino, 2010) using a 31 bp k-mer size and automatic coverage estimation with a minimum contig length of 100 bp. Gene predictions and annotations were carried out using the Rapid Annotation using Subsystem Technology pipeline (RAST.nmpdr.org). Comparison of genomes was carried out using command-line BLASTN and BLASTP (BLAST+ v2.2.30+) and average nucleotide identity was calculated using online tools (LM Rodriguez and K Konstantinidis; enve-omics.ce.gatech.edu/ani/). Prediction of horizontally acquired regions was carried out using GC-skew (Artemis Genome Browser) and interpolated variable order motif analysis (Vernikos & Parkhill, 2006).

7.4.6 Gene expression

RNA was extracted for all samples using TRIzol reagent (Invitrogen) and the Purelink mRNA Minikit with on-column DNase treatment following the manufacturers protocols (Life Technologies/ Thermo Fisher). RNA concentration and purity was accessed using

Qubit 2.0 Fluorometer (Life Technologies/ Thermo Fisher) and Nanodrop ND-1000 (Thermo Fisher) were required. RNA integrity was confirmed by Bioanalyzer analysis (Agilent Technologies) when required.

Bacterial samples were pretreated before RNA extraction by preservation in 2 volumes of Bacterial RNA Protect (Qiagen) following the manufacturers recommendation followed by 30 minute enzymatic treatment with 20 mg/mL lysozyme and 50 U/mL mutanolysin (Sigma Aldrich) at 37°C in pH=7 TE buffer. Cultures for *merRA* relative quantification were harvested at mid exponential growth (~6 hours) and repeated in triplicate experiments.

cDNA was prepared using the high-capacity reverse transcription kit (Life Technologies/ Thermo Fisher) from between 200ng-2µg total RNA following the manufacturers protocols. qPCR was performed in 10 or 20 µL reactions using Power SYBR Green master mix (Life Technologies/ Thermo Fisher) using *gyrB* and B-actin as endogenous reference genes for Lr60 and *Mus musculus* respectively. qPCR results were analyzed using SDS RQ 6.3 manager software (Applied Biosystems). Primers used for qPCR analysis and their amplification efficiencies are listed in Table S7-1. All target probe sets had an amplification efficiency within 5% of the endogenous reference gene. Nested PCR of cDNA to confirm co-transcription of *merR* and *merA* was accomplished using the *merA* forward and *merR* reverse qPCR primers to confirm an 1889 bp amplicon.

Microarrays were prepared and analyzed as before (Hummelen *et al.*, 2011b) with the Affymetrix Mouse Gene 2.0 ST array platform.

7.4.7 Mutant construction

The erythromycin resistance carrying pLAB1301 plasmid was derived from the CMPG10208 plasmid provided by S. Lebeer (U Antwerpen, Belgium) by the excision of the *dlt* promoter in the *EcoRI* site and re-ligation. The *merRA* fragment was amplified from Lr60 gDNA using the primer pair merRA_f_EcoRI and merRA_r_EcoRI and ligated into the *EcoRI* site of pLAB1301. Primers used for cloning are provided in Table S7-2. Orientation was confirmed by double-digest, PCR and sequencing. The construct

was then electroporated into *L. rhamnosus* GR-1 following the protocol of De Keersmaecker *et al.* (2006) followed by recovery on MRS supplemented with erythromycin. Potential transformants were screened by colony PCR using the primer pair *merRA_f_ECOR1* and *merA* reverse qPCR primer to screen for successful transformants. Colony PCR of *L. rhamnosus* was accomplished by microwaving for 3 minutes on high (Proctor Silex model 87007 microwave oven) prior to 35 cycles of PCR with AmpliTaq Gold hot start enzyme (Life Technologies/ Thermo Fisher). The parent strain was verified by ERIC-PCR to ensure the correct genetic background (Ventura & Zink, 2006).

For knockout of *merRA*, the method of Li *et al.* (2011) using the temperature sensitive plasmid pG+host5 carrying erythromycin resistance (Biswas *et al.*, 1993) was applied. 1000 bp of upstream (HR1) and downstream regions (HR2) of the *merRA* operon was amplified using the primer pairs KO_ *merRA*_HR1_f_SalI/KO_ *merRA*_HR1_r_EcoRV and KO_ HR2_f_BamHI/KO_ HR2_r_XbaI. These were sequentially cloned into pG+host5 flanking the chloramphenicol resistance gene (*cat*) of pLI50 amplified using the primer pair pLI50_ *cat*_f_EcoRV/pLI50_ *cat*_r_BamHI. The new plasmid, pG+host5-HR1-*cat*-HR2 was electroporated into Lr60 as before and transformants were selected on 5 µg/mL erythromycin with 48 hours incubation anaerobically at 30°C. To facilitate integration, successful transformants were then incubated at 40°C under chloramphenicol selection. Recovered clones carrying chloramphenicol and erythromycin resistance were then reverted to 30°C without selection for sequential subculturing without antibiotic selection to facilitate excision and curing of the plasmid. Final clones were screened for chloramphenicol resistance (due to *merRA/cat* exchange) with the absence of erythromycin resistance. Allelic exchange was verified by colony PCR and sequencing of the locus.

7.4.8 Mouse experiments

For the metal experiments, a total of 88 female Balb/C aged 6 weeks were randomly assorted into 11 groups of 8 as outlined in Figure S1. The mice were housed with *ad libitum* food and water at a temperature of 22°C on a 12h light/ 12h dark light cycle. These experiments were performed according to the policies of the Institut Pasteur de

Lille Animal Care and Use Committee. The study was covered under the protocol approved (number: 04/2011) by Nord-Pas-de-Calais Ethics and Welfare Committee for Experiments on Animals. All animals received daily oral gavage with 1×10^9 CFU of *L. rhamnosus* GR-1, Lr60 WT, Lr60 $\Delta merRA::cat$, Lr60 $\Delta merRA::cat$ pLAB1301-*merRA* or saline vehicle control. After 3 days of wash-in, metal dosing began. In the acute dosage, a single oral gavage was given of either mercury alone (5 mg/kg b.w. in a 2:1 ratio of MeHg:InHg mimicking human daily exposure) or a heavy metal cocktail (1 mg/kg Hg, 10mg/kg Pb^{2+} and Cd^{2+}). After 24 hours, sacrifice occurred. Similarly, in the sub-chronic dosing scheme, after 3 days wash-in, mercury (0.5 mg/kg/day) or a cocktail (0.3 mg/kg/day Hg, 1 mg/kg/day Cd and Pb) were administered through the drinking water for 28 days along with continual daily oral gavage of lactobacilli as before. Every 4 days, food and water consumption were measured to ensure consistent metal dosing between groups. On day 28 sacrifice occurred.

Prior to sacrifice by cervical dislocation, blood was collected by retro-orbital bleeding. Immediately after sacrifice, a portion of liver, kidney and a 1 cm segment of the duodenum were collected in RNA later while SI and caecal contents were collected for microbial DNA and RNA extraction (analysis pending). A portion of kidney, liver and spleen were additionally collected for metal quantification as well as histology by preservation in 4% formalin/PBS.

For the xenobiotic metabolism experiments, mice were orally gavaged with either *L. rhamnosus* GR-1 (n=7) or saline vehicle control (n=7) daily for 3 days before sacrifice. The liver, kidneys and duodenum were harvested and RNA extracted and transcriptionally profiled by microarray and qPCR as described in section 7.4.6.

7.4.9 Histology

After ≥ 48 hours of fixation in 4% formalin/PBS, tissue samples were sequentially passaged through 50% and 70% ethanol for 30 minutes each, 95% ethanol for 2 hours, and 100% ethanol over night. The ethanol was then replaced with toluene for 30 minutes

before being transferred to paraffin wax overnight. Samples were then embedded in paraffin blocks before sectioning into 5 μ M sections using a Leica RM2235 microtome (Leica Biosystems). After drying overnight, samples were deparaffinized and rehydrated through subsequent xylene and ethanol baths before staining with haematoxylin and eosin (Sigma Aldrich). Slides were then rehydrated and mounted using Permount medium (Sigma Aldrich). Images were obtained with a Zeiss Axioscope microscope with a total magnification of 100X using Northern Eclipse software (EMPix Imaging).

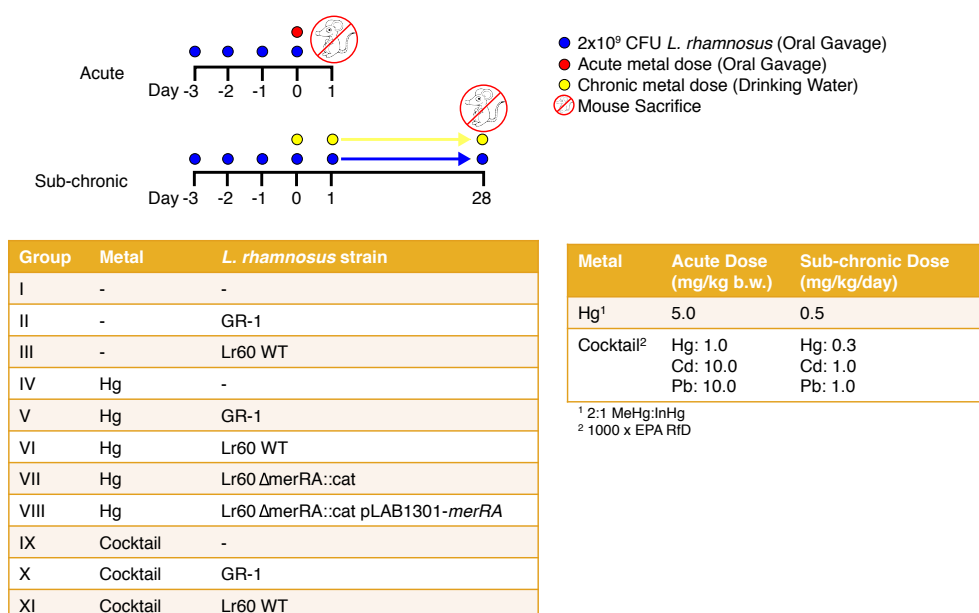


Figure S1. Murine study design.

Table S1. Primers used for qPCR.

Gene	Forward Primer Sequence	Reverse Primer Sequence	Efficiency	R ²
<i>gyrB</i>	CCGAGTTGTTTCGGTCAAG	CGTCAGCCGGATGTTCCATA	94.2	0.998
<i>merA</i>	GCGCTCGTTAATCGGGAAAC	ATGCGCCCCTAACACTTTCA	97.6	0.999
<i>merR</i>	CAAGAACCTCACCGAACGGA	CCCAACACGCTTAACGTCATC	93.8	0.999
<i>B-actin</i>	GATGTATGAAGGCTTTGGTC	TGTGCACTTTTATTGGTCTC	96.1	0.997
<i>fmo2</i>	AAAGAGTTAACCTGTTTGGG	GTACTGGTAGGAATTACAGGG	92.0	0.991
<i>por</i>	CTCCAAGATCAGGTATGAATC	GGAACGGATGCTTCTTATTC	92.4	0.979
<i>fmo3</i>	CCTTTAAACAGAACACTCAGG	GTGAACTCCTTTACATTAGGC	93.1	0.969

Table S2. Primer sequences used for cloning.

Primer	5' Extension	RE site	Primer
merRA_f_EcoRI	GGTGGT	GAATTC	TCCGTCGATGTGGAGAAATTGT
merRA_r_EcoRI	ATCATC	GAATTC	TCCCAGAAGGTAGATCCGCA
KO_merRA_HR1_f_Sall	GGTGGT	GTCGAC	ACTGTAGGTGCCGTCAGTTG
KO_merRA_HR1_r_ECORV	ATCATC	GATATC	ACATCCCACCAGAAAATCCGA
pLI50_cat_f_EcoRV	GGTGGT	GATATC	ACTTCAACGCACCTTTCAGC
pLI50_cat_r_BamHI	ATCATC	GGATCC	TTATAAAAGCCAGTCATTAGGCC
KO_merRA_HR2_f_BAMHI	GGTGGT	GGATCC	CTCCCTATTCAAGTGAACCAGC
KO_merRA_HR2_r_XbaI	ATCATC	TCTAGA	AGCCCGTGCCTAGATATTGC

7.5 References

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Chapter 8

8 General Discussion

Harnessing the power of the microbiome is increasingly finding its way into medical practice and indeed everyday life. This can be seen through the recent fascination with the effects of fecal transplants (Kelly *et al.*, 2015). Despite a recent boom in the probiotics sales, consumer interest and demand invariably requires constant documentation of benefits, an understanding of probiotic mechanisms, and support from caregivers who use them in clinical practice. Education programs on how microbe-mediated therapies can be best manipulated for health have not yet reached mainstream healthcare providers.

A relative of mine was recently prescribed *Lactobacillus* after visiting their general practitioner. No mention of what strain, formulation, or dosing regime was given: simply a written note saying “Lactobacillis”. More personally, on a recent visit to the dentist where I was prescribed antibiotics, my dentist, unaware of my background, advised that I should probably take probiotics to avoid antibiotic associated diarrhea (AAD). This is commendable. The problem lies in that they then recommended I take Activia, or that if I wanted a “real probiotic”, I should go to the health food store. There is no literature that suggests Activia would be effective in preventing AAD, despite a number of formulations providing protective effects (Goldenberg *et al.*, 2013). Additionally the current regulatory environment seems inappropriate where the word probiotic is all but banned in the European Union (Reid, 2011) yet it can be used virtually without restriction in the natural health supplements in Canada and the USA. Recently, when making a protein-shake I discovered that for the past 2 years I have been unknowingly consuming *L. fermentum* probiotics in the protein powder I purchase. There is no mention of the dose nor do I suspect that *L. fermentum* can survive particularly well in protein powder. Furthermore, no information on the strain is supplied despite many probiotic effects being strain specific.

The difficulty lies in that any competent microbiologist could go to the farmer’s market, bring home an artisan cheese, and isolate a handful of “probiotic” lactobacilli. Without

adequate testing in human populations we can never be sure of function. My intent is not to solely place the blame on clinicians or regulatory agencies, but perhaps we in the probiotics research field must also do more to force better control over products and generate understanding on how strains work. For probiotics to move into routine medical practice, convincing and reproducible clinical data is required with some understanding of mechanisms of function. Certainly in the case of AAD or necrotizing enterocolitis, convincing data is available (AlFaleh and Anabrees, 2014; Goldenberg *et al.*, 2013). It is also hard to convince clinicians when probiotic studies are frequently underpowered and apply different probiotic strains in different formulations that may affect efficacy. One cause of this is that probiotic research lacks the levels of the funding frequently found in the pharmaceutical industry. Unlike a drug with a defined chemical structure, it is more difficult to obtain a proprietary strain that cannot be easily copied. In the case of general probiotic function, there is nothing to suggest that company X's probiotic could be any better than company Y's. Thus it is difficult to justify the considerable financial investment in developing new probiotic technologies, as it would be difficult to get adequate intellectual property protection. I believe the answer to these problems is the development of new strains and formulations with defined mechanisms of action.

8.1 Effect of formulation on probiotic function

The results of chapter 2, while not conclusive, are valuable for the questions they raise. In probiotic formulation, there is a general assumption that if the product can maintain a consistent viable bacterial count over the shelf life, other aspects are fairly irrelevant. While the transcriptional responses of a probiotic strain will differ between its time in the product and those in varying portions of the gastrointestinal system, how the strain has adapted to initial entry into the body is of importance. Bacteria can transcriptionally respond to stimuli in a matter of minutes (Selinger *et al.*, 2003) but when we consider that an orally consumed probiotic will transit from the mouth to the stomach in a matter of seconds (Cordova-Fraga *et al.*, 2008), surely any effect of formulation that affects acid resistance will be of importance in aiding survival through the gut as was observed in chapter 2.

While fermented foods were primarily the focus of this thesis, chapter 4 used a probiotic administered as a suppository in capsule form, which presents a range of additional considerations in function. Because the bacteria in these capsules have been lyophilized and preserved, we must consider the time it takes for them to return to an active metabolic state from their dormancy. Enteric-coated tablets seem like an attractive approach for delivering probiotics as they will protect the cells through the harsh acidic conditions of the stomach and begin to release the cells in the small intestine. In this case however, the site of action must be carefully considered. If the site of action is suspected to be in the small intestine, as we would suspect protection against toxic metals or altered nutrient uptake would be, perhaps an enteric coated capsule is the wrong formulation to be delivered as we want metabolically active cells through their entire passage in the SI. The capsules applied in chapter 4 were indeed formulated for oral consumption and proved difficult to dissolve in water for metabolite analysis (observations of A. McMillan). This could partially explain why probiotic administration could not be observed in all participants as if the capsules did not fully dissolve in the vagina, dosing would have been highly inconsistent. Furthermore, when considering the appropriate control, many placebos contain nothing more than a sugar. In the case of studies on microbial populations, perhaps a placebo containing a fermentable sugar is the incorrect control as the sugars could be readily metabolized by the indigenous microbiota and thus inadvertently be biologically active.

Formulations offer potential ability to potentiate probiotic function. In the case of mercuric reductase, there is a basal level of expression, but it is greatly up-regulated upon exposure to mercury. Ideally we would want to administer a probiotic that has already up-regulated its mercury detoxifying machinery before coming into contact with mercury in the body. It would seem foolish to attempt to induce the machinery by including mercury in the formulation, but perhaps there are other non-toxic metals that could drive increased expression. Alternatively, in the case of genetically engineered strains, the genes could be expressed from strong constitutive promoters active both in culture outside the body and during passage through the GI tract such as *ldh1P* (Oozeer *et al.*, 2005). This would ensure the strain was always prepared to detoxify mercury upon exposure. Additionally, perhaps probiotic function in the gut can be altered by diet. In the

case of *E. lenta*, its digoxin metabolizing pathways are repressed by arginine allowing dietary protein to modulate its function (Haiser *et al.*, 2013). Though these examples of formulation affects function solely at the level of individual organisms, they are also relevant when considering the manipulations of the microbiome.

8.2 What is a healthy microbiome?

As identified in the Chapter 1, what constitutes health in the pre-menopausal vagina is well established. Until the work carried out in chapter 4 and its predecessor study (Hummelen *et al.*, 2011), what constituted health in post-menopause was less well established. Since production of glycogen is under the control of estrogen, estrogen is depleted after menopause, and vaginal lactobacilli use glycogen as a nutrient source, it had been suspected that the vaginal microbiota of post-menopausal women was also depleted in lactobacilli (Cauci *et al.*, 2002). Work presented in this thesis showed this to be incorrect where in fact, the majority of healthy post-menopausal women are in fact colonized predominantly by *Lactobacillus* species. The work in chapter 4 was unique in that it attempted to enroll only women in the intermediate stage between health and bacterial vaginosis. Despite an abundance of *L. iners*, there was virtually no *L. crispatus* identified in these women which fits with the hypothesis that *L. crispatus* is protective against transitioning to dysbiotic states (Verstraelen *et al.*, 2009). The one detail which does differ significantly between pre- and post-menopausal women lies in the stability of the microbiome. We found that the microbiota was extremely stable in post-menopausal women and that over time, the majority of women bore the greatest similarity with themselves. In pre-menopausal women the community shifts over the estrus cycle (Keane *et al.*, 1997).

The case of what defines healthy in the gut remains a complex issue. The children studied in chapter 6 had remarkably similar microbiotas that were nearly all dominated by *Prevotella* species (enterotype 2). Due to this homogeneous background we were able to determine that there was an association between *Succinovibrionaceae* and increased blood metal levels. Of the three predominant enterotypes (Arumugam *et al.*, 2011), there is not one that is healthier per se. Rather I suspect that the effects are contextual and that there is no one size fits all microbiome configuration that is beneficial in all settings. We

hypothesized in chapter 6 that a *Prevotella* enriched microbiota might predispose one to higher uptake of toxic metals and that perhaps a Gram-positive dominated microbiota such as the *Ruminococcus*-dominated enterotype 3 may be beneficial. However given that *Ruminococcus*, as a member of the Firmicutes, may have the effect of increased nutrient uptake (Ley *et al.*, 2005), this could predispose an individual to obesity. In the context of a resource-poor developing country such as Tanzania, this could be favorable to aid in nutrient absorption while in the context of the North American diet, be a dangerous predisposition to obesity and metabolic disease. Perhaps given the extreme control diet appears to have over microbiome composition, it is possible to modulate the microbiota to whatever configuration may best suit the current concerns of an individual at a given time (Carmody *et al.*, 2015; David *et al.*, 2014; Halmos *et al.*, 2015).

8.3 How can probiotics modulate the microbiota?

As identified in the previous section, using probiotics to modulate the composition of the gut may be putting the cart before the horse as we do not necessarily know exactly how we want to modulate the microbiome. There are certain cases such as inflammatory bowel disease where we may clearly want to increase *Faecalibacterium* (Sokol *et al.*, 2008) but for the most part, the desired modulation is unknown. Both the work presented in chapters 6 and 7 demonstrated that *L. rhamnosus* GR-1 is unable to modulate the gut microbiota composition, yet it can still confer a benefit. It has also been observed that the *B. animalis subsp. lactis* strain studied in chapter 2 is unable to modulate the microbiota (McNulty *et al.*, 2011) yet its inclusion in a yogurt modulated the function of the microbiota. It is not necessarily the bacteria present in the ecosystem that are important, but their functional roles. A recent study (Zhang *et al.*, 2014) has identified that *L. casei* Zhang may be capable of modulating microbiota composition, but it should be noted that this study lacked a control group and included samplings across large time frames in a population with a seasonal diet.

Again the vagina (chapter 4) appears to be unique. We found that the vaginally applied *L. rhamnosus* strain is capable of modulating the composition of the microbiota. While there was an immediate influx of lactobacilli driven by administration of 10^9 CFU of probiotic added, they were unable to colonize in the majority of individuals. Nonetheless they were

capable of restoring levels of the indigenous lactobacilli such as *L. gasseri/johnsonii*. The mechanism for this was not fully determined, however it could be speculated that the large influx of exogenous lactobacilli was able to re-acidify and condition the vaginal ecosystem to one more conducive to the growth of lactobacilli and deleterious for vaginal dysbiosis.

Given the inability of probiotics to modulate gut microbiota composition, perhaps alternative methods such as prebiotics or dietary interventions should be considered. While prebiotics are conventionally defined as selectively or specifically enriching certain microbes (Manning and Gibson, 2004), a new controversial definition supported by recent metagenomic studies (Bindels *et al.*, 2015) includes a broader definition of altering ecological and functional features of the microbiota. Ultimately without a greater understanding of mechanisms of both how the microbiota and probiotics affect health, we will never know exactly how it is we wish to modulate the microbiota of the gut.

8.4 Novel probiotic strains

Traditional selection of probiotic strains focused on traits such as acid/bile resistance, adhesion and colonization abilities, bacteriocin production, and immunomodulatory capability (Papadimitriou *et al.*, 2015). While these are justified desirable traits as for a general-purpose strain, I firmly believe the future of probiotics in clinical practice lies in strain-specific niche applications.

The final chapter of this work attempted to identify novel strains specifically selected to counter toxic metal exposure and resulted in the identification of *L. rhamnosus* Lr60. This strain delivered superior removal of both lead and mercury and its' ability to actively detoxify mercury was further investigated leading to the first description of a mercury resistant *Lactobacillus* with gene function validated through cloning and characterization of mutants. Given the apparent rarity of mercury resistance elements in *Lactobacillus* species, this discovery is all the more exciting in that we found a proverbial needle in a haystack.

While we were able to find a strain of *L. rhamnosus* carrying mercury-resistance elements, it would also be possible to engineer one for this purpose given the ubiquity of known genetic sequences for *merA* and *merB*. With the apparent absence of organomercurial lyase activity (*merB*) in the *L. casei* group, it is possible to engineer strains to carry it. In fact, work on this has already begun through a synthetic construct of a *ldhl* promoter fused to a codon optimized version of *merB* from *Bacillus megaterium* (Huang *et al.*, 1999). If a more broad-range metal sequestering strain was required, it would also be possible to express metallothioneins on the bacterial cell surface to potentiate sequestering activity (Deng and Wilson, 2001). Given the difficulties in genetically manipulating strains of *Lactobacillus*, this approach could be applied in *Lactococcus* species that are far more amenable to genetic manipulation (Pontes *et al.*, 2011).

While we tend to constrain our thoughts of new probiotics to those granted generally recognized as safe status (GRAS) by the FDA, the use of new species such as *Bacillus coagulans* (Fitzpatrick *et al.*, 2011) expands the landscape of available strains to apply. Indeed, it may be worth further studies on *Bacillus megaterium* MB1 to determine its safety profile to see if it could be applied directly to human and animal hosts forgoing the need for heterologous expression in food-grade vector organisms.

While the focus of these studies was primarily in the context of human health, these concepts should not be limited to humans or even mammals, as they are equally relevant to agricultural applications. An exciting and yet unexplored application of the mercury sequestration/detoxification probiotic technology lies in aquaculture. Probiotics are an emerging concept for commercial fish farming (Irianto and Austin, 2002; Mohapatra *et al.*, 2012) and it has been noted that levels of environmental pollutants are just as high or higher in commercially farmed fish (Bustnes *et al.*, 2011). Strains such as Lr60 could be incorporated into the feed as an additive. These approaches should be tested in aquaculture to determine if they could be used to potentially lower mercury in farmed fish, which may ultimately improve both production yields, and lower mercury in the human food supply.

8.5 How can we improve study design?

As identified in the general discussion, probiotic studies frequently suffer from problems of inconsistent strain selection, formulations and a lack of statistical power and the studies presented in this work possess some of the same issues.

The issue of sample size and power estimations is difficult in microbiota and probiotic studies as they present a number of unique traits not found in typical drug studies. If the primary end point of the study is an easily definable clinical parameter then traditional power estimations are valid. Where pre-existing literature on the effect is limited, as is the case with metal-detoxication, a pilot study is warranted to give a rough estimate of the effect size and variance of the response. While options to repeat the studies found in chapter 6 are being investigated, we can now predict based on the data obtained in the pregnant women that we would need 86 participants to show a 2 nmol/L difference (~23%) in mercury levels with power of 0.9, i.e. a 90% probability that the study will detect a treatment difference in a two-treatment parallel-design study. Alternative study designs such as a cross-over study should be also considered as should formulation of the probiotic yogurt. Milk, or no intervention depending on the cohort, was applied in the control group in the studies in chapter 6 due to logistical challenges of operating in Tanzania. It would be more ideal to carry out the study in a double-blinded method using a non-fermented control dairy product such as acidified, gelatinized, or heat compressed milk heat to rule out potential effects of nutrients affecting uptake (Zhai *et al.*, 2015). Furthermore, while the study in chapter 5 was not designed to differentiate the effects of micronutrients and probiotics, future studies should isolate the two effects and exclude the micronutrients from the formulation to allow study of probiotic function. Alternatively, a capsule formulation could be applied given careful design and testing to ensure timely release to be active in the small intestine.

How to estimate power in studies of microbiota modulation is far more complicated (La Rosa *et al.*, 2012). One issue lies in the sheer number of potential measurable outcomes such as alpha and beta diversity, or modulation of individual taxa. Number of reads per sample, which is often difficult to precisely control and estimate, heavily affects power as does the diversity of the environment. The vaginal sample of an average healthy women

may only have 1-3 major constituents, but the gut will have hundreds or thousands. Furthermore, the number of taxa (as measured by operational taxonomic units) is heavily influenced by upstream data processing, clustering and minimum abundance thresholds which vary from study to study. To control for multiple testing, approaches such as False Discovery Rate corrections are frequently applied (Fernandes *et al.*, 2013). This issue lies in that with the sheer number of OTUs frequently encountered in complex microbiome datasets, statistical significance can be near impossible to obtain even in the case of biological truth. The 16S rRNA dataset presented in chapter 6 demonstrates this phenomenon. At enrollment, no participant has detectable levels of any *L. casei* group member (the limit of resolution of the V6 hypervariable region is to the *L. casei* group so this group includes the probiotic *L. rhamnosus* GR-1). After intervention, the *L. casei* group is only detected in the group who received probiotic with one exception. Nonetheless, after multiple testing corrections for false discovery rate, this finding is not significant. One solution to this problem is to use these datasets as discovery tools and confirm all changes by a complimentary approach such as qPCR or culture. Alternatively the sample size could be greatly increased. Alternatively animal models informed by observations in human populations are becoming a tool to confirm effects of the microbiota as at the end of the day “phenotype trumps all” (personal communication, GB Gloor, 23 April 2015).

8.6 Future directions

There were many observations of the microbiota and probiotic function in this work that cannot be immediately pursued by me. Excellent examples lie in our observations that the infants of women who received the *L. rhamnosus* GR-1 probiotic yogurt have elevated levels of *Bifidobacterium* (chapter 5) or our hypotheses of microbiota structure effecting toxic metal uptake. Human-animal crossover models represent opportunities to address these questions in a controlled and mechanistic fashion. Traditionally this has involved the adoptive transfer of human communities to germ-free mice in an attempt to reproduce human phenotypes (Smith *et al.*, 2013). Alternatively gnotobiotic mice, with a defined microbiota controlled by the researcher can be applied or even built with a defined humanized gut microbiota (McNulty *et al.*, 2011). By feeding a humanized-microbiota

mouse breast milk from either women who received *L. rhamnosus* GR-1 or controls and selectively culturing or qPCR enumerating bifidobacteria, this finding could be confirmed. Individual components of the breast milk could be identified and tested in this model to identify the effectors. To study how microbiota affects metal uptake, gnotobiotic mice could be seeded with monocultures of *Succinovibrionaceae*, *Desulfovibrio* or *Lactobacillus*. Additionally, whole fecal samples from individuals with communities representative of the major enterotypes could be seeded to test our hypotheses.

Prior to initiating a new collaboration with Benoit Foligne at the Institut Pasteur de Lille, we did not have the capacity to carry out animal models with environmental toxins and this past winter we started our first experiments armed with the tools we need to establish the mechanisms behind probiotic mediated detoxication *in vivo*. Our studies have branched out from mercury to include a range of other environmental toxins such as aflatoxin, pesticides, nitrosamines and heterocyclic aromatic amines and it is my hope that these studies will lead to the development of new technologies which can be disseminated into both industrial and medical practice. Indeed through our partnerships with WORLDdiscoveries, we are currently investigating opportunities worldwide.

A unique opportunity in this thesis was to have a great amount of interaction with industry sponsors (Danone and Kimberly Clark), which gave me a unique view on translation of basic research. At the end of the day, the work carried out needed to have relevance to practical applications. Although this created unique obstacles and frustrations not experienced by many of my peers, it also provided uncommon opportunities for which I am truly grateful. As I go forward in my research career I will constantly draw from the diverse and varied skill set that my graduate studies fostered. It is my sincere wish that both the work included in this thesis and the other publications resulting from my graduate studies have improved our understanding of probiotic function and formulation while creating new areas of studies into novel probiotic applications.

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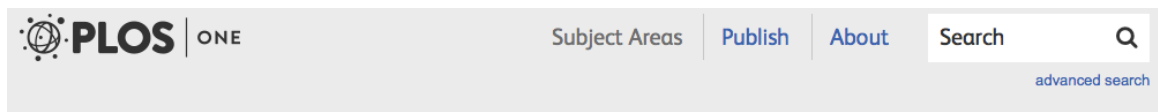
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Notice of Authorization

Company Code. 27645
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April 30, 2012

Dr. Shu-Ping Yang
c/o Ms. Maha Tawashi
Kimberly-Clark Corporation



Dear Dr. Yang

Re: CLINICAL TRIAL APPLICATION for UREX-CAP-5
Natural Health Products Regulations Section: 67

The Natural Health Products Directorate, Bureau of Clinical Trials and Health Sciences, is pleased to inform you that the information and material provided to support the above Clinical Trial Application have been assessed and we have no objection to your proposed study. Please consider this as your notice of authorization to sell or import this natural health product for the purposes of this clinical trial in Canada.

I would remind you of the necessity of complying with the *Natural Health Products Regulations, Part 4*, in the sale of this product for clinical testing. In addition, the Regulations (Part 4) impose responsibilities, including commencement notice, record keeping and reaction reporting, on those conducting clinical trials. Please ensure that all systems are compliant in order to meet these responsibilities.

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Should you have any questions concerning this letter, please contact the submission coordinator nhp-dta.dec-dpsn@hc-sc.gc.ca.



Senior Executive Director, Bureau of Product Review and Assessment
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Canada

UWO Ethical approval for study in chapter 5:



Use of Human Participants - Ethics Approval Notice

Principal Investigator: Dr. Gregor Reid
 Review Number: 18850
 Review Level: Full Board
 Approved Local Adult Participants: 60
 Approved Local Minor Participants: 0
 Protocol Title: The effect of under-nutrition on the human microbiota
 Department & Institution: Schulich School of Medicine and Dentistry/Microbiology & Immunology, University of Western Ontario
 Sponsor: Lawson Internal Research Fund

Ethics Approval Date: April 11, 2012
 Ethics Expiry Date: December 31, 2012

Documents Reviewed & Approved & Documents Received for Information:

Document Name	Comments	Version Date
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This is to notify you that the University of Western Ontario Health Sciences Research Ethics Board (HSREB) which is organized and operates according to the Tri-Council Policy Statement: Ethical Conduct of Research Involving Humans and the Health Canada/ICH Good Clinical Practice Practices: Consolidated Guidelines; and the applicable laws and regulations of Ontario has reviewed and granted approval to the above referenced study on the approval date noted above. The membership of this HSREB also complies with the membership requirements for REB's as defined in Division 5 of the Food and Drug Regulations.

The ethics approval for this study shall remain valid until the expiry date noted above assuming timely and acceptable responses to the HSREB's periodic requests for surveillance and monitoring information. If you require an updated approval notice prior to that time you must request it using the University of Western Ontario Updated Approval Request form.

Member of the HSREB that are named as investigators in research studies, or declare a conflict of interest, do not participate in discussions related to, nor vote on, such studies when they are presented to the HSREB.

The Chair of the HSREB is Dr. Joseph Gilbert. The HSREB is registered with the U.S. Department of Health & Human Services under the IRB registration number IRB 0000940.



Ethics Officer to Contact for Further Information





This is an official document. Please retain the original in your files.

The University of Western Ontario



Ethical approval from the National Institute of Medical Research (Tanzania) for chapter 5:

	<p>THE UNITED REPUBLIC OF TANZANIA</p>	
<p>National Institute for Medical Research</p> <div style="background-color: black; width: 150px; height: 40px; margin: 5px auto;"></div>	<p>Ministry of Health and Social Welfare</p> <div style="background-color: black; width: 150px; height: 40px; margin: 5px auto;"></div>	
	<p>23rd July 2012</p>	
<p>Dr Gregor Reid University of Western Ontario, Canada C/O John Chungalucha NIMR Mwanza</p> <div style="background-color: black; width: 150px; height: 20px; margin-top: 5px;"></div>		
<p>CLEARANCE CERTIFICATE FOR CONDUCTING MEDICAL RESEARCH IN TANZANIA</p>		
<p>This is to certify that the research entitled: The influence of nutrition on reproductive health at Nyamagana District Hospital in Mwanza, Tanzania (<i>Reid G et al</i>), whose local investigator is Mr John Chungalucha, NIMR Mwanza, has been granted ethics clearance to be conducted in Tanzania.</p>		
<p>The Principal Investigator of the study must ensure that the following conditions are fulfilled:</p>		
<ol style="list-style-type: none"> 1. Progress report is submitted to the Ministry of Health and the National Institute for Medical Research, Regional and District Medical Officers after every six months. 2. Permission to publish the results is obtained from National Institute for Medical Research. 3. Copies of final publications are made available to the Ministry of Health & Social Welfare and the National Institute for Medical Research. 4. Any researcher, who contravenes or fails to comply with these conditions, shall be guilty of an offence and shall be liable on conviction to a fine. NIMR Act No. 23 of 1979, PART III Section 10(2). 5. Approval is for one year: 23rd July 2012 to 22nd July 2013. 		
<div style="background-color: black; width: 400px; height: 40px; margin: 10px auto;"></div>		
<p>CHAIRPERSON MEDICAL RESEARCH COORDINATING COMMITTEE</p>	<p>ACTING CHIEF MEDICAL OFFICER MINISTRY OF HEALTH, SOCIAL WELFARE</p>	
<p>CC: RMO DMO</p>		

IWO Ethics approval for study in chapter 7:



Research Ethics

Use of Human Participants - Ethics Approval Notice

Principal Investigator:Dr. Gregor Reid
File Number:102881
Review Level:Full Board
Approved Local Adult Participants:60
Approved Local Minor Participants:0
Protocol Title:Investigating the effects of probiotic yogurt on reducing the levels of environmental toxins among school children in Mwanza, Tanzania
Department & Institution:Schulich School of Medicine and Dentistry/Microbiology & Immunology,Western University
Sponsor:
Ethics Approval Date:November 27, 2012
Ethics Expiry Date:December 31, 2013

Documents Reviewed & Approved & Documents Received for Information:

Table with 3 columns: Document Name, Comments, Version Date. Rows include Western University Protocol and Letter of Information & Consent.

This is to notify you that the University of Western Ontario Health Sciences Research Ethics Board (HSREB) which is organized and operates according to the Tri-Council Policy Statement: Ethical Conduct of Research Involving Humans and the Health Canada/CH Good Clinical Practice Practices: Consolidated Guidelines; and the applicable laws and regulations of Ontario has reviewed and granted approval to the above referenced study on the approval date noted above.

The ethics approval for this study shall remain valid until the expiry date noted above assuming timely and acceptable responses to the HSREB's periodic requests for surveillance and monitoring information.

Member of the HSREB that are named as investigators in research studies, or declare a conflict of interest, do not participate in discussions related to, nor vote on, such studies when they are presented to the HSREB.

The Chair of the HSREB is Dr. Joseph Gilbert. The HSREB is registered with the U.S. Department of Health & Human Services under the IRB registration number IRB 0000940.



Ethics Officer to Contact for Further Information



This is an official document. Please retain the original in your files.



Ethical approval from National Institute for Medical Research (Tanzania) for chapter 6:

LAKE ZONE INSTITUTIONAL REVIEW BOARD (LZIRB)



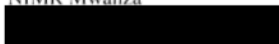
National Institute for Medical Research
Mwanza Medical Research Centre



MR/53/100/37

18th October 2012

Dr Gregor Reid
University of Western Ontario
c/o Dr Joseph Mwanga
NIMR Mwanza



CLEARANCE CERTIFICATE FOR CONDUCTING MEDICAL RESEARCH IN TANZANIA

This is to certify that the research entitled: Investigating the effects of probiotic yogurt on reducing the levels of environmental toxins among school children in Mwanza, Tanzania (Reid G *et al*) whose local Investigator is Dr Joseph Mwanga, NIMR Mwanza, has been granted ethics clearance by LZIRB .

The Principal Investigator (PI) of the study must ensure that the following conditions are fulfilled:

1. Progress report is submitted to the Ministry of Health and Mwanza Medical Research Centre, Regional and District Medical Officers after every six months.
2. Permission to publish the results is obtained from NIMR Headquarters.
3. Copies of final publications are made available to the Ministry of Health & Social Welfare Mwanza Medical Research Centre and the National Institute for Medical Research Headquarters.
4. Any researcher, who contravenes or fails to comply with these conditions, shall be guilty of an offence and shall be liable on conviction to a fine - NIMR Act No. 23 of 1979, PART III Section 10(2).
5. Approval is for this study, any other changes should be communicated to the committee for approval.
6. Approval is for one year: 18th October 2012 to 17th October 2013.
7. Since the study involves foreign collaborators, you are also directed to apply for National Ethics Clearance from NIMR Headquarters.

Na



Vice Chairperson LZIRB

Secretary

cc: Regional Medical Officer
District Medical Officer

Curriculum Vitae

Name: Jordan Eduard Bisanz

Post-secondary Education and Degrees: University of Western Ontario
London, Ontario, Canada
2006-2010 B.M.S.c.

The University of Western Ontario
London, Ontario, Canada
2010-2015 Ph.D.

Honours and Awards: Province of Ontario Graduate Scholarship
2011-2012

Province of Ontario Graduate Scholarship
2012-2013 (Declined)

Canadian International Development Agency
Students for Development Grant
2012

Natural Science and Engineer Research Council (NSERC)
Doctoral Fellowship
2012-2015

Natural Science and Engineer Research Council (NSERC)
Postdoctoral Fellowship
2015-2017

Related Work Experience Teaching Assistant
The University of Western Ontario
2011-2014

Summer Laboratory Assistant
London Public Health Laboratory
2008-2009

Publications:

Bisanz JE, Enos ME, PrayGod G, Seney S, Macklaim JM, Chilton S, Willner D, Knight R, Fusch C, Fusch G, Gloor GB, Burton JP and G Reid. The microbiota across pregnancy in a rural Tanzanian population and the effects of micronutrient supplemented probiotic yogurt. *Applied and Environmental Microbiology* doi: 10.1128/AEM.00780-15

Bisanz JE, Enos MK, Mwanga J, Chagalucha J, Burton JP, Gloor GB and G Reid. 2014. The influence of probiotics and the gut microbiome on toxic metal levels in Tanzanian pregnant women and school children: a randomized open-label pilot study. *mBio* 5:e01580-14.

Bisanz JE, Seney S, McMillan A, Vongsa R, Koenig D, Gloor GB, Sumarah M, Ford B, Herman D, Burton JP and G Reid. 2014. A systems biology approach investigating the effect of *Lactobacillus rhamnosus* GR-1 and *L. reuteri* RC-14 on the vaginal microbiome and host responses in a double blind, placebo-controlled clinical trial of post-menopausal women with intermediate Nugent scores. *PLoS ONE* 9:e104511.

Bisanz JE, Macklaim JM, Gloor GB and G Reid. 2014. Bacterial metatranscriptome analysis of a probiotic yogurt using a RNA-Seq approach. *International Dairy Journal* 39:284-292.

Bisanz JE and G Reid. 2011. Unraveling how probiotic yogurt works. *Science Translational Medicine* 3:106ps41.

Bisanz JE*, Hummelen R*, Macklaim JM*, Hammond JA, McMillan A, Vongsa R, Koenig D, Gloor GB, and G Reid. 2011. Vaginal Microbiome and epithelial gene array in post-menopausal women with moderate to severe dryness. *PLoS ONE*. 6: e26602. *Equal contributions

Trinder M, Bisanz JE, Burton JP and G Reid. 2015. Probiotic lactobacilli: a potential prophylactic treatment for pesticide bioaccumulation and associated toxicity. *Beneficial Microbes*, *In press*

Reid JNS, Bisanz JE, Monachese M, Burton JP and G Reid. 2013. The rationale for probiotics improving reproductive health and pregnancy outcome. *American Journal of Reproductive Immunology* 69:558-566.

Macphee RA, Hummelen R, Bisanz JE, Miller WL and G Reid. 2010. Probiotic strategies for the treatment and prevention of bacterial vaginosis. *Expert Opinion on Pharmacotherapy* 11:2985-2995.

PUBLICATIONS IN REVIEW AND SUBMISSION

McMillan A, Rulisa S, Sumarah M, Macklaim JM, Renaud J, Bisanz JE, GB Gloor and G Reid. A multi-platform metabolomics approach identifies novel biomarkers associated with bacterial diversity in the human vagina. *Under review: Scientific Reports, Manuscript ID: SREP-15-08796*

Bisanz JE, van Hylckama Vlieg JET, Wels M, Smokvina T and G Reid. Comparative genomics demonstrates production of volatile sulfur compounds in *Lactobacillus paracasei* and *Lactobacillus rhamnosus* is mediated by a novel sulfur/taurine metabolism gene cluster. *Under Review: International Journal of Food Microbiology, Manuscript ID: FOOD-D-15-00216*