Culture and Characterization of Kefir Organisms to Reproduce the Health Benefits of Traditional Kefir

by

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ABSTRACT

Fermented dairy products such as kefir have long been associated with a host of health benefits; however, the role that microbial composition has in these benefits has not been properly examined. This research aimed to examine how kefir impacts host metabolic health, as well as how alterations to the microbial populations of the kefir impacts these health benefits and the fermentation dynamics.

In order to examine how kefir impacts metabolic health, four traditional and one commercial kefir were administered to mice using a high fat diet-induced obesity model. Two traditional kefirs decreased weight gain and plasma cholesterol levels in mice and one also lowered liver triglycerides. Conversely, commercial kefir had no beneficial effect. Improvements to liver triglycerides corresponded with decreases in the expression of fatty acid synthase, a gene involved in liver lipogenesis. This study shows that traditional kefir has the potential for improving metabolic dysfunction associated with obesity and indicates that differences in kefir microbial populations may influence the ability of traditional kefir to positively impact host metabolic health.

As the metabolic health benefits associated with kefir were only associated with traditional examples, we set out to create a kefir product that utilized microbes from traditional kefir while maintaining a production method that is viable on an industrial scale. This was accomplished through the isolation of a large collection of organisms from a variety of traditional kefirs representing the most abundant species. Characterization of the isolates was carried out, and individual isolates were selected for use in the pitched kefir based on the characteristics of both the isolate and the kefir from which it was isolated. This proof of concept

study showed that it is possible for traditional kefir organisms to ferment milk outside of a kefir grain, and that the resulting product resembles kefir in microbial density and pH.

Following the development of the novel pitched kefir, we investigated the ability of this pitched culture kefir to recapitulate health benefits observed with a specific traditional kefir. This was accomplished using a mouse model of diet induced obesity. Additionally, we examined how differences in the microbial composition of kefir impacted these benefits. Both the traditional kefir and its pitched culture equivalent decreased plasma cholesterol and liver triglyceride levels by similar amounts when compared to commercial kefir. Furthermore, a pitched kefir produced without yeast and pitched kefir produced without lactobacilli did not show cholesterol-lowering effects. The traditional and pitched kefir with the full complement of microbes were able to impart corresponding decreases in the expression of multiple cholesterol and lipid metabolism genes in the liver. These results show that traditional kefir organisms can be utilized to create a more health promoting commercial kefir, while also highlighting the importance of microbial interactions during fermentation in the ability of fermented functional foods to beneficially impact host health.

To better understand how alterations to the microbial composition of kefir fermentations impact the dynamics of the fermentation, we used two-dimensional gas chromatography and time-of-flight mass spectrometry (GCxGC-TOFMS) to examine the metabolite profiles of traditional and pitched culture kefir, including pitched culture kefirs that had the yeast or lactobacilli removed. Interestingly, despite similarities in their ability to improve host metabolic health, the Pitch and traditional kefir differed significantly in the profile of metabolites present, especially in yeast associated metabolites such as ethanol and esters. In addition, the removal of lactobacilli from the Pitch fermentation resulted in lower levels of organic acid formation during

fermentation, while the removal of yeast appeared to result in little alteration in the metabolite profile.

This thesis provides insight into how microbial composition impacts kefir fermentation, and presents a new strategy for the development of fermented functional foods.

PREFACE

This thesis is an original work by Benjamin Bourrie.

Chapter 1 has been published as Bourrie BCT, Willing BP, Cotter PD. The microbiota and health promoting characteristics of the fermented beverage kefir. Frontiers in Microbiology. 2016;7:1–17. BB wrote the review and compiled, figures, tables, and references. PC supervised, edited, and approved the review. BW supervised, edited, and approved the review.

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The study in Chapter 3 was designed by BB, PC, and BW.
BB designed, conducted the experiment, collected and analyzed the data, and wrote the chapter. PC designed the experiment, supervised data analyses, and edited the chapter. BW designed the experiment, supervised data analyses, and edited the chapter.

The study in Chapter 4 was designed by BB, PC, and BW. BB conducted the experiment, collected and analyzed data, and wrote the manuscript. AF assisted with data collection. JF

assisted with data analysis. TJ assisted with data collection and analysis. CS assisted with histological analysis. The manuscript has been submitted as Benjamin C.T. Bourrie, Tingting Ju, Janelle Fouhse, Andrew Forgie, Consolato Sergi, Paul D. Cotter, Benjamin P. Willing. Microbial Composition is a Deciding Factor in the Health Benefits Conferred by Kefir.

The study in Chapter 5 was designed by BB, PC, and BW. BB conducted the experiment, collected and analyzed data, and wrote the chapter. ND assisting with data collection analysis and writing the chapter. PM assisted with data collection and analysis and writing the chapter

DEDICATION

This thesis is dedicated to my father, Sean Bourrie, who always believed in me and saw what I was capable of, even when I couldn't. And to my daughter Aoife Bourrie, my next great project.

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I would like to express my sincere gratitude to my co-supervisors, Dr. Ben Willing and Dr. Paul Cotter, for accepting me as a PhD student and allowing me to work on such an interesting and exciting project. Because of this PhD I was able to experience living in Ireland and made lifelong friends there. You both were extremely encouraging throughout this project and I am extremely thankful for you allowing me to pursue so many of my own ideas. I feel very lucky to have had two supervisors who were both so supportive throughout my PhD through hard times both academically, and in my non-academic life. I would also like to thank Dr. Colin Hill for your suggestions and help throughout my PhD, as well as to my examining committee members Dr. Lynn McMullen and Dr. Gisèle LaPointe for your time and support.

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Chapter 1: Literature Review

1.1 Introduction

Fermented dairy products have long been associated with the ability to confer health benefits in those who regularly consume them, with Ellie Metchnikoff first theorizing that their impact on the bacterial microbiota in the gut contributed to health and long life (1). Indeed many reportedly probiotic-containing foods come in the form of fermented milk products, such as yogurt, koumis, and kefir, many of which have been consumed for hundreds of years (2,3). Probiotics are live microorganisms which, when administered in adequate amounts, confer a health benefit on the host (4). As is the case with the fermented dairy products referred to above, probiotics are consumed in foods containing these organisms in sufficiently large quantities to pass safely to the gastrointestinal tract but can also come in the form of supplements consisting of live organisms such as pills.

Although not as widely popular as other fermented dairy products, such as yogurt and cheese, kefir has been consumed and associated with health benefits for hundreds of years; originally by communities in the Caucasian mountains. The beverage itself typically has a slightly viscous texture with tart and acidic flavour, low levels of alcohol, and in some cases slight carbonation. Kefir is traditionally made with cow's milk but it can be made with milk from other sources such as goat, sheep, buffalo, or soy milk (5–8). One of the features that distinguish kefir from many other fermented dairy products is the requirement for the presence of a kefir grain in fermentation and the presence and importance of a large population of yeasts (2,9). The aforementioned kefir grains are microbially derived protein and polysaccharide matrices that contain a community of bacterial and fungal species that are essential to kefir fermentation (10,11).

Traditionally, fermentation was initiated through the addition of kefir grains to milk, in a sheep or goat skin bag (6). Commercial, industrial-scale production rarely utilizes kefir grains for fermentation, but rather uses starter cultures of microbes that have been isolated from kefir or kefir grains in order to provide more consistent products (12). While this industrially produced kefir may have health benefits of its own, research examining such benefits has either not been performed or is not published. Thus, any kefir referred to in this review has been produced in a traditional manner using kefir grains or grain fermented milk as the inoculum. In addition to the microbial population present in kefir, these beverages typically also contain an abundance of fermentation products such as organic acids and multiple volatile flavour compounds including ethanol, acetaldehyde, and diacetyl (13). As part of the fermentation process, an exopolysaccharide unique to kefir, kefiran, is produced. Kefiran makes up a large proportion of the kefir grain itself and is also found dissolved in the liquid phase, where it contributes to the rheology and texture of the finished product (14–16).

In this review we will discuss the many health promoting effects that have been attributed to kefir, including tumour suppression and prevention, gastrointestinal immunity and allergy, wound healing, cholesterol assimilation and ACE inhibition, its antimicrobial properties, and the ability of kefir to modify the composition and activity of the gut microbiota (Figure 1.1).

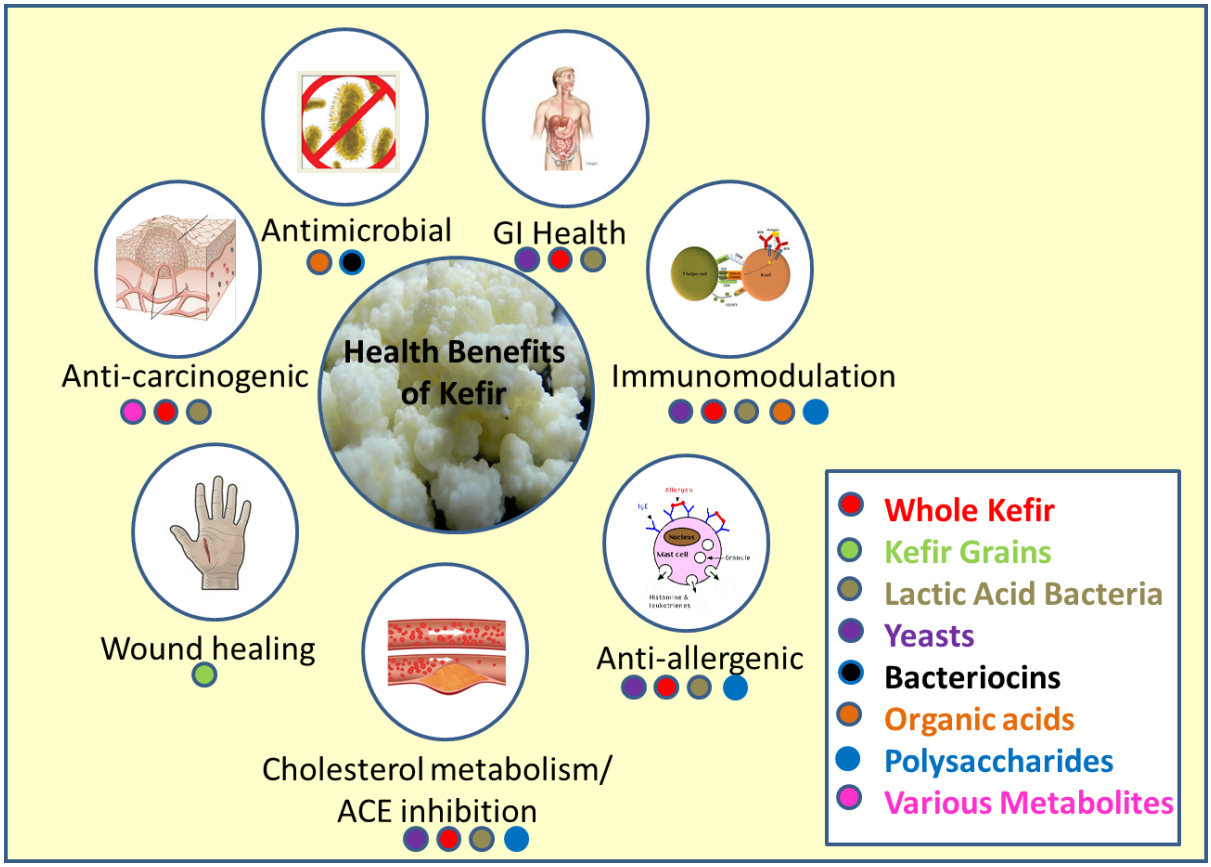

Figure 1.1. Major health benefits associated with kefir and the fractions or parts of kefir responsible for these benefits.

1.2 Bacterial and Fungal Populations of Kefir

Bacterial Populations

Since the first established use, hundreds of years ago, the propagation of kefir has been performed by transferring kefir grains from one batch to fresh milk and incubating at ambient temperature. Over this period there has been substantial opportunity for the microbial component of kefir grains to evolve and diverge, resulting in the addition or loss of bacteria and yeasts as well as the addition and loss of genes. The bacterial genera most commonly found in kefir using culture dependent techniques are *Lactobacillus*, *Lactococcus*, *Streptococcus*, and *Leuconostoc* (17–19). These genera tend to dominate the population present in both the kefir grain and milk, with

Lactococcus lactis subsp. *lactis*, *Streptococcus thermophilus*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lactobacillus helveticus*, *Lactobacillus casei* subsp. *pseudoplantarum*, *Lactobacillus kefiri*, *Lactobacillus kefir*, and *Lactobacillus brevis* accounting for between 37% and 90% of the total microbial community present (17,18,20). While these species commonly make up the majority of the microbial population present in kefir grains, some grains are dominated by yeast species or other bacterial species such as *Leuconostoc mesenteroides* (18). The proportions of species can also differ between the grain and milk (Figure 1.2). For example, *L. lactis* subsp. *lactis*, and *S. thermophilus* levels are generally much greater in the fermented kefir than in the kefir grains. The levels of these species increase further in kefir made from kefir as an inoculum. Indeed, the total increase observed has been as much as 30% in some cases (17). The reason for this increase during fermentation in the milk may be due to an increase in temperature created by the active fermentation or simply due to where these bacteria reside in the kefir grain, as organisms such as *Lactobacillus* may tend to reside deeper within the kefir grain, thus making it harder for them to escape in to the milk.

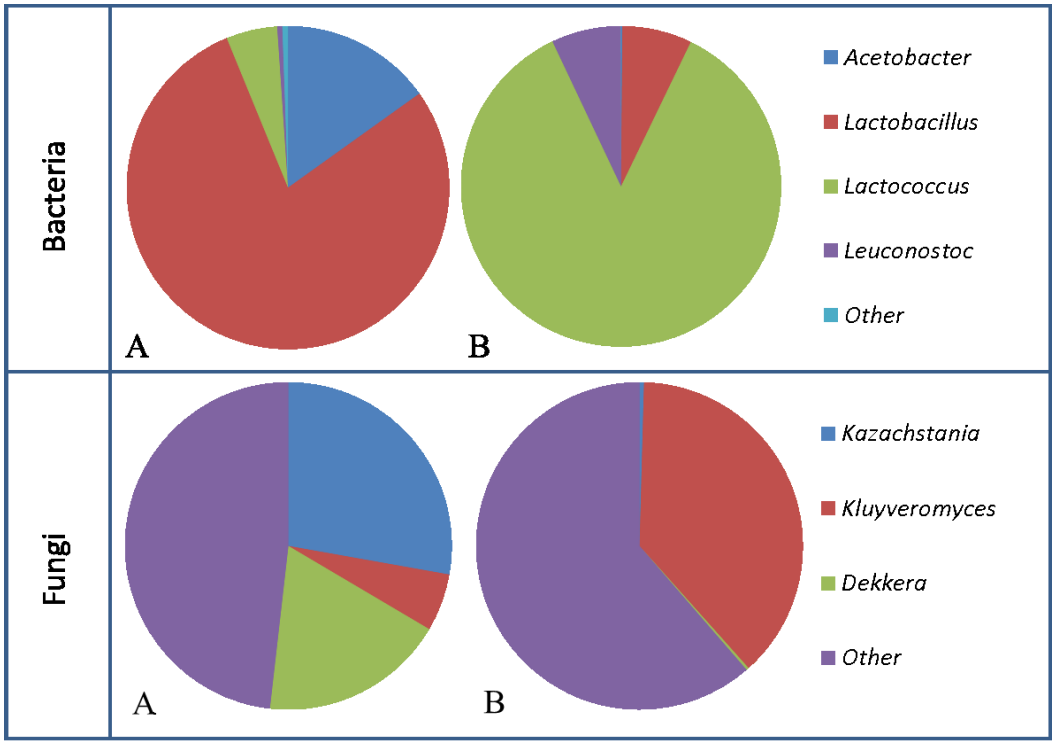

Figure 1.2. Representation of bacterial population changes from kefir grain (A) to fermented milk (B) and fungal population changes from kefir grain (A) to fermented milk (B). Figure generated using data from Marsh et al. 2013.

In agreement with the majority of culture base studies, investigation of the microbial composition of diverse kefir grains using culture independent techniques found that the overall bacterial populations were for the most part dominated by Firmicutes and Proteobacteria, and kefir milk contained a much higher level of representatives of the *Streptococcaceae* than any other family (11,21). Based on high-throughput sequencing of 16S genes present in kefir grains and milk, it was established that kefir grains typically have 1 (*Lactobacillus*) or 2 (*Lactobacillus* and *Acetobacter*) dominant bacterial genera (11,22–24). The most common species of *Lactobacillus* have been *L. kefiranofaciens*, *L. kefiri*, and *L. parakefiri* (21–27). There are many other genera present in these grains; however, they typically represent less than 10% of the community (11,22,23,27). When milk fermented by these same grains was examined, the relative abundance of the genera present vary much more than in the grain, with *Leuconostoc*, *Lactococcus*,

Lactobacillus, and *Acetobacter* being the most abundant (11,24). As has previously been stated, bacteria found at lower abundance in the kefir grain can become dominant, as species such as *Lactococcus* are minimally represented in kefir grain, but regularly become the most abundant genus present in the kefir milk (11,21). This observation is consistent with past culture based work, where *Lactococcus* was found to increase through the fermentation process (17). At the species level, high throughput 16S analysis showed the number of OTUs vary from 24 to 56 in the kefir grain, and 22 to 61 in kefir milk, i.e. much higher than what has been observed utilizing culture dependent techniques (11). These findings highlight the need for future studies to examine both the kefir grain and fermented milk rather than the previous tendency to focus solely on the population of the grain.

With respect to the non-lactic acid bacteria (LAB) that have been associated with kefir, it is notable that culture independent methods have revealed *Acetobacter* as one of the dominant genera present in grains. This is of interest as *Acetobacter* is not commonly isolated from kefir *via* culture dependent techniques and, indeed, has been described as a non-essential contaminant of kefir (18,28–30). While there are some studies that have found acetic acid bacteria in large quantities in kefir grains (30), many rely on isolation media that is not optimal for growth of acetic acid bacteria (31). *Bifidobacterium* species have also been identified through culture independent studies; however, *Bifidobacterium* has not been found in any culture based studies of the kefir microbiota (11,21,32). Table 1.1 contains a complete list of bacterial species found in both culture dependent and culture independent studies, while Figure 1.3 provides a breakdown of the distribution of species found in these studies.

Table 1.1. List of bacterial and fungal species found in kefir grains and milk using both culture dependent and culture independent techniques

Microbial Species	Authors
Lactobacillus	
Lactobacillus kefir	Miguel et al. 2010, Angulo et al. 1993, Pintado et al. 1996, Santos et al. 2003, Garrote et al. 2001, Mainville et al. 2006
Lactobacillus kefiranofaciens	Chen et al. 2008, Santos et al. 2003, Dobson et al. 2011, Garofalo et al. 2015, Vardjan et al. 2013, Nalbantoglu et al. 2014, Hamet et al. 2013, Zanirati et al. 2015, Korsak et al. 2015, Mainville et al. 2006
Lactobacillus delbrueckii	Simova et al. 2002, Santos et al. 2003, Witthuhn et al. 2004, Nalbantoglu et al. 2014
Lactobacillus helveticus	Simova et al. 2002, Dobson et al. 2011, Nalbantoglu et al. 2014, Chen et al. 2008
Lactobacillus casei	Angulo et al. 1993, Simova et al. 2002, Nalbantoglu et al. 2014, Zanirati et al. 2015
Lactobacillus kefiri	Miguel et al. 2010, Chen et al. 2008, Dobson et al. 2011, Garofalo et al. 2015, Vardjan et al. 2013, Nalbantoglu et al. 2014, Hamet et al. 2013, Zanirati et al. 2015, Korsak et al. 2015
Lactobacillus brevis	Angulo et al. 1993, Witthuhn et al. 2005 Simova et al. 2002, Santos et al. 2003, Nalbantoglu et al. 2014
Lactobacillus paracasei	Miguel et al. 2010, Santos et al. 2003, Nalbantoglu et al. 2014, Hamet et al. 2013
Lactobacillus parakefir	Miguel et al. 2010, Takizawa et al. 1994, Garrote et al. 2001
Lactobacillus plantarum	Miguel et al. 2010, Santos et al. 2003, Garrote et al. 2001, Nalbantoglu et al. 2014
Lactobacillus satsumensis	Miguel et al. 2010, Zanirati et al. 2015
Lactobacillus curvatis	Witthuhn et al. 2004
Lactobacillus fermentum	Witthuhn et al. 2004, Angulo et al. 1993, Witthuhn et al. 2005
Lactobacillus viridescens	Angulo et al. 1993
Lactobacillus acidophilus	Angulo et al. 1993, Santos et al. 2003, Dobson et al 2011, Nalbantoglu et al. 2014
Lactobacillus gasseri	Angulo et al. 1993, Nalbantoglu et al. 2014
Lactobacillus kefirgranum	Takizawa et al. 1994, Vardjan et al. 2013
Lactobacillus parakefiri	Dobson et al. 2011, Vardjan et al. 2013, Nalbantoglu et al. 2014, Hamet et al. 2013, Korsak et al. 2015

Lactobacillus parabuchneri	Dobson et al. 2011, Nalbantoglu et al. 2014
Lactobacillus garvieae	Dobson et al. 2011
Lactobacillus buchneri	Nalbantoglu et al. 2014, Garofalo et al. 2015
Lactobacillus sunkii	Nalbantoglu et al. 2014, Garofalo et al. 2015
Lactobacillus crispatus	Nalbantoglu et al. 2014, Garofalo et al. 2015
Lactobacillus otakiensis	Nalbantoglu et al. 2014, Garofalo et al. 2015
Lactobacillus instestinalis	Garofalo et al. 2015
Lactobacillus amylovorus, *L. pentosus*, *L. salivarius*, *L. johnsonii*, *L. rhamnosus*, *L. rossiae*, *L. sakei*, *L. reuteri*, *L. kalixensis*, *L. rapi*, *L. diolivorans*, *L. parafarraginis*, *L. gallinarum*, *Pediococcus claussenii*, *P. damnosus*, *P. halophilus*, *P. pentosaceus*, *P. lolii*	Nalbantoglu et al. 2014

Lactococcus/Streptococcus

Lactococcus lactis subsp. *lactis*	Witthuhn et al. 2004, Angulo et al. 1993, Pintado et al. 1996, Garrote 2001 Witthuhn et al. 2005, Simova et al 2002, Chen et al. 2008, Yuksekdag et al. 2004 , Garofalo et al. 2015, Zanirati et al. 2015, Mainville et al. 2006
Lactococcus lactis subsp. *cremoris*	Mainville et al. 2006, Yuksekdag et al. 2004, Korsak et al. 2015
Lactococcus lactis subsp. *lactis* biovar *diacetylactis*	Garrote et al. 2001
Lactococcus garvieae	Nalbantoglu et al. 2014
Streptococcus salivarius subsp. *thermophilus*	Angulo et al. 1993
Streptococcous thermophilus	Simova et al. 2002, Mainville et al. 2006, Yuksekdag et al. 2004, Garofalo et al. 2015
Streptococcus durans	Yuksekdag et al. 2004

Leuconostoc/Oenococcus

Leuconostoc spp.	Angulo et al. 1993
Leuconostoc mesenteroides subsp. *mesenteroides*	Witthuhn et al. 2004, Mainville et al. 2006
Leuconostoc mesenteroides subsp. *cremoris*	Witthuhn et al. 2005, Mainville et al. 2006
Leuconostoc mesenteroides	Simova et al. 2002, Chen et al. 2008, Nalbantoglu et al. 2014, Zanirati et al. 2015, Korsak et al. 2015
Leuconostoc pseudomesenteroides	Mainville et al. 2006
Oenococcus oeni	Nalbantoglu et al. 2014

Acetobacter

Acetobacter spp.	Angulo et al. 1993, Garrote et al. 2001, Marsh et al. 2013, Garofalo et al. 2015
Acetobacter sicerae	Li et al. 2014
Acetobacter orientalis, *Acetobacter lovaniensis*	Korsak et al. 2015
Bifidobacterium	
Bifidobacterium spp.	Marsh et al. 2013
Bifidobacterium breve, *B. choerinum, B. longum*, *B. pseudolongum*	Dobson et al. 2011
Yeast and Fungal Species	
Zygosaccharomyces spp.	Witthuhn et al. 2004, Witthuhn et al. 2005
Candida kefyr	Witthuhn et al. 2004, Angulo et al. 1993, Marquina et al. 2002
Candida lipolytica	Witthuhn et al. 2004
Saccharomyces cerevisiae	Witthuhn et al. 2004, Angulo et al. 1993, Simova et al. 2002, Marsh et al. 2013 Latorre-Garcia et al. 2007, Marquina et al. 2002, Garofalo et al. 2015, Vardjan et al. 2013, Diosma et al. 2014
Candida holmii	Witthuhn et al. 2004, Angulo et al. 1993, Latorre-Garcia et al. 2007
Torulaspora delbrueckii	Angulo et al. 1993, Vardjan et al. 2013
Saccharomyces unisporus	Angulo et al. 1993, Pintado et al. 1996, Latorre-Garcia et al. 2007, Marsh et al. 2013, Wang et al. 2008, Marquina et al. 2002, Garofalo et al. 2015, Vardjan et al. 2013, Diosma et al. 2014
Candida friedrichii	Angulo et al. 1993
Kluyveromyces lactis	Angulo et al. 1993, Latorre-Garcia et al. 2007, Marquina et al. 2002
Pichia fermentans	Angulo et al. 1993, Wang et al. 2008, Marsh et al. 2013
Issatchenkia orientalis	Latorre-Garcia et al. 2007, Marsh et al. 2013, Diosma et al. 2014
Kluyveromyces marxianus	Wang et al. 2008, Marsh et al. 2013, Marquina et al. 2002, Vardjan et al. 2013, Korsak et al. 2015, Diosma et al. 2014
Saccharomyces turicensis	Wang et al. 2008, Garofalo et al. 2015
Dekkera anomala	Garofalo et al. 2015, Marsh et al. 2013
Kazachstania exigua	Garofalo et al. 2015, Vardjan et al. 2013, Korsak et al. 2015
Naumovozyma spp.	Korsak et al. 2015
Cryptococcus humicolus,	Witthuhn et al. 2005

Geotricum candidum	
Kazachstania servazzii, *Ka. solicola*, *Ka. aerobia*, *Saccharomyces cariocanus*	Garofalo et al. 2015
Kluyveromyces marxianus var. *lactis*, *Candida inconspicua, C. maris*	Simova et al. 2002
Saccharomyces humaticus, *Candida sake, Yarrowia lipolytica, Dipodascus capitatus, Trichosporon coremiiforme*	Latorre-Garcia et al. 2007
Ganoderma lucidum, *Dioszegia hungarica*, *Heterbasidion annosum*, *Peziza campestris*, *Cyberlindnera jadinii*, *Malassezia pachydermatis*, *Teratosphaeria knoxdaviesii*, *Cryptococcus* sp. *Vega* 039, *Microdochium nivale*, *Wallemia sebi*, *Zygosaccharomyces lentus*, *Eurotium amsteldami*, *Dekkera bruxellensis*, *Kazachstania barnettii*, *Naumovozyma castelli*, *Davidiella tassiana*, *Penicillium* sp. *Vega* 347	Marsh et al. 2013

Yeast Populations

In addition to the large and variable bacterial population in kefir grains, there is an abundant yeast population that exists in a symbiotic relationship with the bacteria (11,17,18). Three genera of yeasts are commonly isolated from kefir grains or milk, and typically make up the majority of the total yeast population; *Saccharomyces*, *Kluyveromyces*, and *Candida* (17,28,33,34).

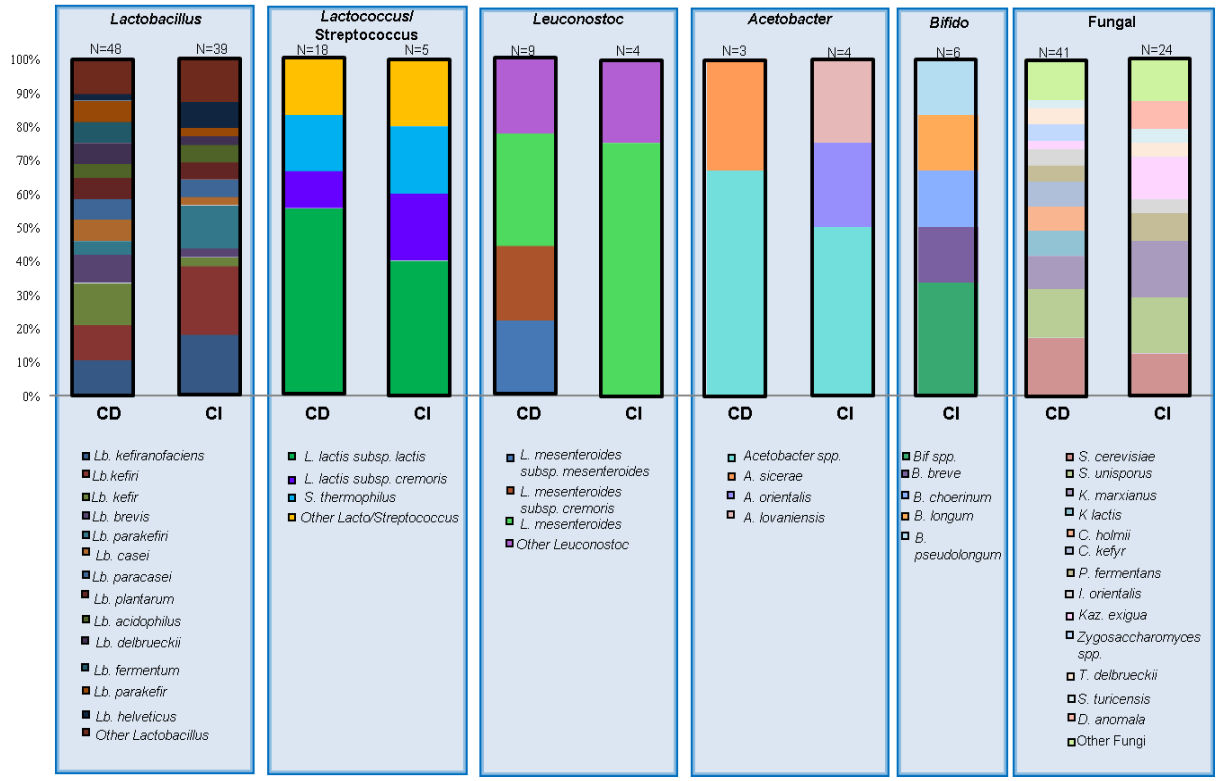

Figure 1.3. The number of times an individual species has been identified in kefir expressed as a percentage of the total number of species in the same genera. CD = Culture Dependent identification; CI = Culture Independent identification; N values represent the total number of times a species within the genus has been identified.

Many different species of *Saccharomyces* have been isolated from kefir; however, *S. cerevisiae* and *S. unisporus* are the most common and present in many varieties (28,33–35). *Kluyveromyces* make up the majority or entirety of the lactose utilizing yeast population, with *K. marxianus* and *K. lactis* being the two most common species (17,34,35). The *Candida* population is made up of a wide range of species with *C. holmii* and *C. kefyr* being the most prevalent (28,33). Outside of these three genera, only *Pichia* has been identified with any regularity and in each case the species was identified as *Pichia fermentans* (28,36). As fermentation progresses the proportions of some yeast species change with non-lactose fermenting yeasts, such as *Saccharomyces*, decreasing, whereas lactose utilising *K. marxianus* and *K. lactis* show a similar distribution between grain and kefir (17).

Unlike the bacterial population in kefir grain, the yeast component of the grain fluctuates considerably between grains when analyzed using culture independent techniques. Despite this, a small number of yeasts such as *Kazachstania*, *Kluyveromyces*, and *Naumovozyma* tend to be the dominant genera present in both the grain and fermented milk (11,22,24,26,37). Of these main genera, only *Naumovozyma* has not been isolated in culture based studies. *Kazachstania unispora*, the species of *Kazachstania* present is also known as *Saccharomyces unisporus* (11). Sequencing based approaches have also identified over a dozen yeast species that had not previously been associated with kefir, such as *Dekkera anomala*, *Issatchenkia orientalis*, and *Pichia fermentans*, and have even shown that, in some grains, the yeast population is dominated by a mix of these other species (11,22). Table 1.1 contains a complete list of yeast species found in culture dependent and culture independent studies.

Culture Dependent vs. Culture Independent Methods

As expected, sequencing based methods often identify organisms that are not readily isolated by traditional culture based methods. This may be due to the presence of these organisms in extremely low numbers, or some of these organisms may be unable to grow on traditional media due to the complex symbiotic relationships present in kefir. Indeed, this may account for why certain *Lactobacillus* species have only been identified in sequencing based studies (21). For example *L. kefiranofaciens* has not consistently been isolated in culture based methods but is regularly identified as a major part of the *Lactobacillus* population present in kefir when culture independent methods are used which may be due to the more strict anaerobic nature of this species when compared to other *Lactobacillus* species (38). While sequencing based methods have proven to be very valuable for identifying difficult to culture organisms, high throughput sequencing of 16S amplicons are limited with respect to their ability to consistently identify organisms at the

species level (11). Additionally, with metagenomic analyses there is the possibility that population dynamics may be skewed if there are dead cells present. While large numbers of dead cells from one species may indicate the importance of that species to kefir, the detection of these dead cells can still be problematic at later times during fermentation as they would not be actively involved in the community at these time points. Culture based methods remain essential as they allow organisms to be phenotypically tested. Regardless, the advent of sequence based technologies has increased the knowledge of which organisms are present in kefir grains and fermented milk and will allow for the development of new strategies to facilitate the isolation of organisms previously overlooked.

1.3 Cholesterol Metabolism and ACE Inhibition:

Due to the highly complex microbiota of kefir, there is a multitude of metabolic products and organisms present in the fermented milk. This combination of live microbial organisms and metabolites contributes to a wide range of effects attributed to kefir many of which are health benefits. Cardiovascular disease (CVD) is one of the leading causes of death in the western world, with high levels of serum cholesterol being a major risk factor for the disease. Diet can play a major role in the management of serum cholesterol levels and thus, ones risk of contracting CVD (39). It has been shown that milk and especially fermented milks are able to reduce serum cholesterol levels in animal trials (40,41). Kefir grains are capable of reducing the cholesterol levels of milk through the fermentation process and have been shown to reduce the levels of cholesterol present by between 41 and 84% after 24 hour fermentation and a further 48 hours of storage (42). While cholesterol reduction varied from one grain to another, these differences did not reflect the country of origin of the grain; Yugoslavian grains had both the highest and lowest levels of cholesterol assimilation. Single kefir isolates have also been shown to assimilate

cholesterol, with *K. marxianus* being one of the more effective. When *K. marxianus* strains K1 and M3 were inoculated in broth supplemented with cholesterol for 20 hours, cholesterol levels decreased 70 to 99% (43). These same strains of *K. marxianus* showed significant levels of bile salt hydrolase (BSH) activity which were proportional to the rate of cholesterol lowering (43). BSH deconjugates bile acids and, as deconjugated bile salts are less soluble and less efficiently reabsorbed from the intestinal lumen, this leads to increased bile salt excretion in the faeces (44). BSH deconjugation contributes to cholesterol lowering abilities of kefir as cholesterol is utilized in bile acid synthesis.

Cholesterol lowering properties of kefir have been validated in animal models. In a study using male golden Syrian hamsters fed a cholesterol free or cholesterol enriched diet, both milk kefir and soyamilk kefir reduced serum triacylglycerol and total cholesterol while improving the atherogenic index (i.e. ratio of non-HDL-cholesterol to HDL-cholesterol). The cholesterol lowering effect was independent of whether the hamsters were fed the cholesterol free or cholesterol enriched diet (7) indicating that kefir feeding altered endogenous cholesterol metabolism. Concentrations of cholesterol in the liver were also observed to decrease in both milk kefir and soyamilk kefir fed hamsters, and the levels of fecal bile acid and cholesterol secretions significantly increased for both groups. The increase in fecal bile acid is likely a result of the deconjugation of bile acid by microbes present in the kefir, while the higher levels of cholesterol secretion were likely due to the inhibition of cholesterol absorption in the small intestine due to the binding and assimilation of cholesterol by these same microbes (45).

Lactobacillus plantarum MA2 isolated from kefir has also shown hypocholesterolemic activity in male Sprague-Dawley (SD) rats fed a high cholesterol diet. Rats fed a diet supplemented with this organism had significantly lower total serum cholesterol, LDL-cholesterol,

triglycerides, liver cholesterol and triglycerides in conjunction with increased fecal cholesterol secretion (46). A similar study that used a high cholesterol diet supplemented with *L. plantarum* strains Lp09 and Lp45 in SD rats found that these strains had the same effect (47). Huang et al. (48) also found that *L. plantarum* Lp27 was able to decrease serum total cholesterol, LDL-cholesterol, and triglycerides in hypercholesterolemic SD rats that consumed a diet supplemented with Lp27. A proposed mechanism for decreased serum cholesterol is the inhibition of cholesterol absorption. The Niemann-Pick C1-like 1 (NPC1L1) gene, which plays a critical role in the absoption of cholesterol (49), is down-regulated in rats fed Lp27 and in *in vitro* tests with Caco-2 cells (48). Zheng *et al.* (50) found that *L. acidophilus* LA15, *L. plantarum* B23, and *L. kefiri* D17 were all able to lower serum total cholesterol, LDL, and triglyceride levels in SD rats fed a high cholesterol diet. The 3 strains also increased fecal cholesterol and bile acid secretion (50). *K. marxianus* YIT 8292 was also shown to decrease plasma and liver cholesterol levels in addition to increasing fecal sterol and bile acid excretion and the concentration of short chain fatty acids in the cecum (51), indicating that both bacteria and yeast can contribute to this characteristic. This effect was shown to be specific to α-mannan and β-glucan present in the cell wall of *K. marxianus* (51). In addition to individual microbes in kefir having an ability to reduce cholesterol, kefiran has also been shown to improve cholesterol and blood pressure levels. In a study using spontaneously hypertensive and stroke prone (SHRSP/Hos) rats fed a high fat diet, kefiran supplementation reduced serum total cholesterol, serum LDL-cholesterol, serum triglycerides, liver cholesterol, and liver triglycerides (52); however, the concentrations used for kefiran supplementation were not discussed. Decreases in the blood pressure and angiotensin converting enzyme (ACE) activity were also observed. ACE inhibitory action has been attributed to

commercial kefir made from caprine milk when tested *in vitro*, with the mode of action being attributed to two small peptides released from casein during the fermentation process (53).

In contrast to these studies, St-Onge et al. (54) found that when mildly hypercholesterolemic men consumed kefir as part of their diet for 4 weeks there was no significant change to total serum cholesterol, LDL-cholesterol, HDL-cholesterol, or triglyceride concentrations. They did note an increase in fecal bacterial counts and short chain fatty acid levels, including propionic acid. Additionally, a study examining Wistar rats fed a standard diet supplemented with kefir for 22 days found no significant differences in serum cholesterol when compared to rats on a control diet (55). While these two studies seem to conflict with other findings, this may be in large part due to the fact that different kefir grains were used for each of these studies. Additionally, the aforementioned Liu et al. (7) study had a timeline of 8 weeks, while St-Onge et al. (54) and Urdaneta et al. (55) had timelines of 4 weeks and 22 days, respectively. It may be significant that, in the study of hypercholesterolemic men, an increase in propionic acid was noted. Propionic acid has been shown to inhibit acetate incorporation into triacylglycerol and plasma cholesterol (56). Thus, a hypocholesterolemic effect may have been observed had the study continued for a longer time period.

1.4 Effects on the Host Gut and Gut Microbiome:

Pathogen Exclusion

One of the main ways through which probiotic-containing food products can exert beneficial effects is altering the gut microbiota. This can be done either through the introduction of new species or strains in to the gastrointestinal tract, or by promoting the growth of beneficial microbes which are already present. Some examples are presented here. In one such study, consumption of kefir in a rat model has been associated with an increase in microbes thought of

as beneficial, such as *Lactobacillus* and *Bifidobacterium*, while simultaneously decreasing harmful microbial species such as *Clostridium perfringens* (57,58). Kefir consumption was also able to reduce the severity of *Giardia intestinalis* infection in C75BL/6 mice, with the reported mechanism being through modulation of the immune system (59). Furthermore, specific strains of *Lactobacillus* isolated from kefir have been shown to adhere to Caco-2 cells and inhibit the adherence of *Salmonella enterica* serovar typhimurium and *E. coli* O157:H7 (48,60,61). The ability of these *Lactobacillus* species to bind to Caco-2 cells illustrates a likely mechanism for the increase in *Lactobacillus* species observed in the fecal microbiota of rats fed kefir (58,62). In an *in vivo* study where BALB/c mice were intragastrically challenged with *E. coli* O157:H7, mice receiving *L. kefiranofaciens* M1 prior to *E. coli* challenge showed reduced symptoms of infection, including intestinal and renal damage, bacterial translocation, and Shiga toxin penetration as well as increased EHEC-specific mucosal IgA responses (63).

Other *in vitro* work has also shown that lactobacilli isolated from kefir have the ability to protect Vero cells from type II Shiga toxin produced by *E. coli* O157:H7, leading to lower levels of cell death (64). Similar effects were apparent in another study where they observed that kefir fermented milk inhibited the ability of *Bacillus cereus* extracellular factors to cause damage to Caco-2 cells (65).

As well as regulating microbial composition, kefir can alter the activity of the microbiota. Certain *Bifidobacterium* strains have been shown to exhibit increases in growth rate when cultured in kefir and changes in gene expression have also been observed (66). These changes in gene expression resulted in increased expression levels of multiple genes associated with *pil3*, a sortase dependent pilus that has been shown to be extremely important for interaction with the host endothelial cells and is especially important for adherence and modulation of the host

inflammatory response (66,67). While this specific example shows the potential positive effects kefir can have on existing organisms within the gut microbiota, it is still unclear as to how this translates to the complex population of the whole microbiome.

Antibacterial and Antifungal Properties

Kefir, and kefir associated strains, have shown a multitude of antibacterial and antifungal activities (Table 1.2). Kefir fermented milk has been tested in disc diffusion experiments against a wide range of pathogenic bacterial and fungal species and found to have antimicrobial activity equal to ampicillin, azithromycin, ceftriaxone, amoxicillin, and ketoconazole against many of these species (68–71).

In addition to the antimicrobial effects of kefir fermented milk as a whole, there are also specific microbes which exert antimicrobial properties on their own. For instance, *L. plantarum* ST8KF produces the bacteriocin ST8KF which exhibits antimicrobial action against *Enterococcus mundtii* and *Listeria innocua* (72). Other kefir-derived *Lactobacillus* species such as *L. acidophilus* and *L. kefiranofaciens*, as well as some *S. thermophilus* strains have shown antimicrobial activity against a whole range of pathogenic organisms including *E. coli, L. monocytogenes, S. aureus, S.* typhimurium*, S. enteritidis, S. flexneri*, *P. aeruginosa*, and *Y. enterocolitica* when tested using an agar spot test (60,69,73). Other kefir lacotbacilli have also shown antimicrobial activity in *in vitro* tests against *S. typhimurium*, and *E. coli* that have already adhered to Caco-2 cells (73). Lacticin 3147 is produced by a strain of *L. lactis* isolated from kefir and has an extremely broad range of antimicrobial activity, affecting *B. cereus*, *B. subtilis*, *C. sporogenes*, *C. tyrobutyricum*, *Enterococcus faecium*, *E. faecalis*, *L. innocua*, *L. monocytogenes*, *S. aureus*, and *C. difficile* (74,75). Another bacteriocin of kefir origin is F1, which is produced by the *Lactobacillus paracasei* subsp. *tolerans* strain FX-6 source from a Tibetan kefir grain. F1 has been shown to

inhibit a wide range of bacterial and fungal species including *S. aureus*, *Shigella dysenteriae*, and *Aspergillus niger* (76). *L. kefiri* B6 isolated from kefir was also capable of inhibiting and inactivating *L. monocytogenes* when in the presence of galactooligosaccharide *in vitro*; however, this effect was not observed with *E. coli* and, in this case, further investigation of the mechanism of this inactivation is needed (77). Similarly, Leite et al. (78) isolated multiple strains of *L. lactis* and *Lb. paracasei* from kefir capable of producing bacteriocin-like substances that were inhibitory to *E. coli*, *S.* enterica, *S. aureus*, and *L. monocytogenes*; however, more work is needed in order to better characterize these substances and determine the range of their antimicrobial activity as well as their novelty. In a study examining lactic acid bacteria isolated from Brazilian kefir grains, *L. kefiranofaciens* 8U showed the ability to inhibit multiple pathogens including *P. aeruginosa*, *L. monocytogenes*, and *E. faecalis in vitro*, but again more work is needed in order to determine the mechanism behind this inhibition (79).

Table 1.2. List of pathogenic organisms that kefir or kefir-associated organisms have demonstrated antimicrobial effects against.

Microbial Species	References
Bacteria	
Staphylococcus aureus	Rodrigues et al. 2005, Cevikbas et al. 1994, Yuksekdag et al. 2004, Ryan et al. 1996, Miao et al. 2014, Leite et al. 2015, Zanirati et al. 2015
Pseudomonas aeruginosa	Rodrigues et al. 2005, Huseini et al. 2012, Cevikbas et al. 1994, Yuksekdag et al. 2004, Ryan et al. 1996, Zanirati et al. 2015
Salmonella typhimurium	Rodrigues et al. 2005, Santos et al. 2003, Golowczyc et al. 2008, Zanirati et al. 2015
Escherichia coli	Rodrigues et al. 2005, Santos et al. 2003, Yuksekdag et al. 2004, Golowczyc et al. 2008, Ryan et al. 1996, Leite et al. 2015, Zanirati et al. 2015
Salmonella enteritidis	Santos et al. 2003, Miao et al. 2014

Listeria monocytogenes	Rodrigues et al. 2005, Santos et al. 2003, Ryan et al. 1996, Likotrafiti et al. 2015, Leite et al. 2015, Zanirati et al. 2015
Bacillus subtilis	Cevikbas et al. 1994, Ryan et al. 1996
Salmonella enterica	Golowczyc et al. 2008, Miao et al. 2014, Leite et al. 2015
Enterococcus faecalis	Ryan et al. 1996, Zanirati et al. 2015
Shigella flexneri	Santos et al. 2003
Clostridium difficile	Rea et al. 2007
Klebsiella pneumonia, *Proteus vulgaris*	Cevikbas et al. 1994
Streptococcus pyogenes, *Staphylococcus salivarius*	Rodrigues et al. 2005
Bacillus cereus, *Clostridium sporogenes*, *C. tyrobutyricum*, *Enterococcus faecium*, *Listeria innocua*, *Salmonella typhi*	Ryan et al. 1996
Salmonella gallinarum, *Shigella sonnei*	Golowczyc et al. 2008
Bacillus thuringiensis, *Shigella dysenteriae*	Miao et al. 2014
Fungus	
Candida albicans	Rodrigues et al. 2005
Yersinia entocolitica	Santos et al. 2003
Aspergillus flavus, *A. niger*, *Rhizopus nigricans*, *Penicillium glaucum*	Miao et al. 2014
Staphylococcus epidermidis, *Candida stellatoidea*, *C. tropicalis*, *C. krusei*, *Saccharomyces cerevisiae*, *Rhodotorula glutinis*, *Torulopsis glabrata*	Cevikbas et al. 1994

1.5 Antitumor Effects:

Kefir also has significant antitumor activity against multiple cancer cell types. *L. kefiri* was shown to increase apoptosis of multiple drug resistant human myeloid leukemia cells *in vitro* through the activation of caspase 3 in a dose dependent manner (80). The cell free fraction of kefir has shown antitumor activity *in vitro* when it was observed to have a dose dependent anti-proliferative effect on the gastric cancer cell line SGC7901 (81). This study further demonstrated that cell free kefir was able to induce apoptosis in SGC7901 cells through up regulation of the *bax* gene, and apoptosis promoter and anti-oncogene, and down regulation of the *bcl-2* gene, which is an apoptosis inhibitor and known oncogene (82). In addition to the promotion of cell death in

cancerous cells, antimutagenic effects have been demonstrated in studies with known carcinogens such as methylmethanosulphate, methy-lazoxymethanol, sodium azide, aflatoxin B1, and 2-aminoanthracene as indicated by the Ames test (83).

In mouse models of fusiform cell sarcomas, mice receiving intraperitoneal kefir had reduced tumor size, with some tumors completely disappearing over a 20 day treatment period (68). While this is impressive, it has yet to be determined if these findings can be replicated in the case of oral consumption. A separate study utilizing a murine breast cancer model showed that kefir feeding prior to challenge with the tumor resulted in decreased size and increased apoptosis of the tumor, and that the levels of IgA+ cells and CD4+ T cells were also increased (84). Mice with breast cancer tumours fed kefir also showed increased serum levels of Il-10 and IL-4 (85). These studies both showed increases in immune cell populations and recruitment, pointing to a possible mechanism for the reduction of tumor size. These findings are consistent with other studies that have shown that kefir is able to modulate the immune system in the gut and show that the immunomodulatory abilities of kefir may not be limited to the gastrointestinal tract (59,86,87).

1.6 Wound Healing:

The antimicrobial properties of kefir may lead to its use for non-traditional applications. Indeed, when rats bearing open wounds inoculated with *S. aureus* were treated with a gel made from kefir grains, it was found that the wounds healed at a much faster rate than was observed in control rats that received no treatment or rats that received a traditional treatment of 5mg/kg neomycin-clostebol emulsion (70). Gels made from kefir and kefir grains were found to be more effective at reducing wound size in *P. aeruginosa* contaminated 3rd degree burns than a traditional silver sulfadiazine treatment in a rat model of burn wounds (71,88). Furthermore, a

study using a rabbit model for contaminated open wound also found that gel made from kefir grain resulted in quicker healing times and quicker clearing of infection (89).

These decreased healing times are likely due to multiple factors. One such factor is the ability of kefir to inhibit the growth of bacterial and fungal cells, thus leading to a cleaner wound, as shown to be the case in some studies (71,89). Another possible factor is the ability to modulate the immune system and recruit immune cells to help with the healing process. Although none of the studies examined the recruitment or population dynamics of immune cells at the site of injury, kefir has been shown to modulate the immune system and may be an area of interest for future studies.

1.7 Immunomodulatory Effects:

One of the major ways probiotic products such as kefir are able to produce health benefits is through the modulation of the gastrointestinal immune system. When young rats inoculated intra-duodenally with cholera toxin (CT) were fed kefir, the levels of anti-CT IgA in the serum increased as did the secretion levels of anti-CT IgA in the Peyer's Patches, the mesenteric lymph nodes, the spleen, and the intestinal lamina propria compared to CT alone (86). This same effect, however, was not observed in older mice that underwent the same treatment, suggesting that whatever mechanism is responsible for the observed change in the young rats is either no longer present in the senescent mice or requires a much larger dosage of kefir in order to activate it. Additional studies in to the mechanism as well as investigations with middle aged mice are needed to provide further insight in to this phenomenon. In an infection of C75BL/6 mice with *G. intestinalis*, kefir consumption reduced intensity of infection by mitigating the ability of *G. intestinalis* to suppress the mounting of an inflammatory response. This impact was mediated through increases in the levels of TNF-α and IFN-γ expression and through higher levels of IgA

positive and RcFcε positive cells (59). There have also been studies showing increases in IgA and IgG+ cells in the small intestine of rats that were fed both regular and pasteurized kefir, as well as increases in the levels of IL-4, IL-10, IL-6, and IL-2 positive cells in the lamina propria of these same rats. Increases were also seen in anti-inflammatory cytokines such as IL-10, IL-4, and IL-6, all of which promote a Th2 response (87). Interestingly, increases in IFN-γ, TNFα, and IL-12 (all of which are pro-inflammatory and promote a Th1 response) were observed only in rats fed pasteurized kefir. The increase in pro-inflammatory cytokines in the pasteurized kefir groups was likely due to the reduced cell wall integrity of heat killed cells exposing more inflammatory microbial products. The fact that pasteurized kefir was able to elicit an effect shows that the mechanisms behind this immune modulation are not entirely dependent on live cells, and may be due to metabolites present in the kefir (90). However, it should be noted that in this study live cells had a generally more substantial impact as live kefir was able to confer a similar effect at 1/10 the concentration and without eliciting a pro-inflammatory immune response (87).

When fed to mice over 2-7 days, solid fractions of kefir that contained live bacteria have been shown to increase the levels of IFN-γ, TNF-α, and IL-6 in peritoneal macrophages as well as to increase the levels of IL-1α, IL-10, and IL-6 in adherent cells isolated from the Peyer's patch of mice (91). IFN-γ and TNF-α increased early in feeding; however they quickly decreased back to control levels by day 7 along with IL-1α while IL-6 and IL-10 levels remained high through the 7 day feeding period (91). *In vitro* experiments with lactobacilli isolated from kefir have shown that they induce higher secretion levels of IL-1β, IL-6, TNF-α, IL-10, IL-8 and IL-12 in peripheral blood mononuclear cells and are able to decrease the ccl20 response in Caco-2 cells to TLR agonists such as bacterial flagella, with largely different effects being observed for different strains

of lactobacilli tested (62). In general, strains of *L. kefiri* that induced lower TNF-α/IL-10 and higher IL-10/IL-12 ratios showed a much greater decrease in the pro-inflammatory response of ccl20 to stimulation with bacterial flagella, indicating the importance of IL-10 in promoting a Th2 response while simultaneously inhibiting the pro-inflammatory Th1 response. Mice that were fed *L. kefiri* for a period of 21 days showed altered gene expression profiles in the ileum, colon, Peyer's Patches, and mesenteric lymph nodes, with proinflammatory cytokines such as IFN-γ and IL-23 being down regulated and IL-10 being up regulated (62). This further indicates that lactobacilli isolated from kefir have the ability to supress the production of pro-inflammatory cytokines while promoting anti-inflammatory cytokine production. *L. kefiranofaciens* co-incubation with mouse macrophage cells decreased the levels of pro-inflammatory cytokines IL-1β, and IL-12 while simultaneously increasing the level of the anti-inflammatory cytokine IL-10, which acts to specifically inhibit the production of IL-12 and IL-1β (92). Additionally, *L. kefiranofaciens* was able to ameliorate colitis in a DSS induced mouse model and enhance Th1 responses to TLR agonists in germ free mice by increasing the production of IFN-γ and IL-12 upon stimulation (63). Further investigation into the mechanisms of protection against colitis showed that *L. kefiranofaciens* M1 decreased the production of pro-inflammatory cytokines IL-1β and TNF-α, while increasing the production of IL-10 *in vivo* (93). This effect was also TLR-2 dependent as *L. kefiranofaciens* M1 was unable to improve DSS colitis in TLR-2 knockout mice (93).

The cell free fraction of kefir is also capable of modulating the immune system, and has been shown to modulate innate immune responses *in vitro* by lowering the activation of Caco-2-ccl20:luc cells that had been stimulated by *Salmonella* flagellar protein FliC, IL-1β, or TNF-α (90). One of the likely mechanisms was revealed when it was found that a 100mM lactic acid solution at pH 7 was able to elicit a comparable level of immune modulation in FliC stimulated cells when

preincubated with the solution (90). The lactic acid solution was also found to lower the level of NFκ-B activation in Caco-2 cells stimulated with FliC and was even able to down regulate the expression of pro-inflammatory cytokines ccl20, IL-8, CXCL 2, and CXCL 10 without affecting genes involved in the normal function of enterocytes (90). These results indicate just how important the metabolites produced during fermentation are to the ability of kefir to elicit beneficial responses or effects in the host.

In general studies using whole kefir, kefir fractions, or organisms isolated from kefir found that whether tested *in vitro* or *in vivo*, the result was a shift from a Th1 immune response to a Th2 response as well as increases in the levels of IgA present (62,86,87,91,92). The only study which seems to show a consistently increased Th1 response was conducted with germ free mice, while all other studies used conventional mice or rats (63). This may account for the difference in findings as it is quite possible that the observations from the germ free mice had more to do with the introduction of a bacterial population to the gut than it did with the specific bacterial species that comprised that population. The fact that most studies also observed increases in some pro-inflammatory cytokines such as TNF-α, IFN-γ, or IL-12 may be explained by an initial reaction of the immune system to common TLR agonists present, which was ultimately supressed following further interaction with the immune cells of the GI tract.

1.8 Anti-Allergenic Effects:

Allergic diseases have been on the rise in the developed world for decades, leading to higher incidences of conditions such as asthma and food allergy (94). Many allergies, especially those related to food, are developed early in life, with the majority of food allergies developing within the first 2 years of life (95). Although most food allergies developed early in life do not persist, some can become lifelong conditions (95). Recent work has shown that an increasingly

important factor in determining if a child develops allergic disease, be it food allergy or asthma, is the level of complexity and the specific organisms present in the gut microbiota (96–99). Higher levels of *Bifidobacterium* and group 1 lactobacilli (obligate heterofermentative lactobacilli such as *L. acidophilus, L. delbrueckii, and L. helveticus*) in the gut of infants have been associated with a lower incidence of allergic disease later in life (97), and both kefir and kefiran have been observed to exert these effects on the gut microbiota in animal trials (57,58). Supplementation with *Bifidobacterium* has been shown to influence the intestinal microbiota of weaning infants by reducing levels of *Bacteroides* and has been associated with lower incidence of food allergy (96). Studies with antibiotics in the early life period have also highlighted the importance of appropriate microbial stimulation of the immune system for protection against asthma development (100).

One of the main mechanisms behind food allergy is an imbalance in the Th1/Th2 cell ratio, leading to a heightened IgE response (101). Studies of *in vitro* reactions of human monocytes with a probiotic made up of multiple LAB showed that exposure to these LAB resulted in a much higher IFN-γ/IL-4 ratio, similar to what would be seen during a Th1 response (102). In addition to the *in vitro* studies carried out, Tsai *et al* found that both total IgE and OVA-specific IgE were significantly lower in mice that had been sensitized to OVA (ovalbumin) and then fed a LAB mixture than in control mice which had also been sensitized to OVA but did not receive any LAB mixture. Studies such as this indicate that kefir may help relieve some allergy symptoms.

In a study utilizing an ovalbumin sensitization mouse asthma model, it was found that mice receiving intra-gastric kefir showed lower levels of airway hyper-responsiveness (AHR) than control mice, and, impressively, had lower levels of AHR than the positive control group receiving an anti-asthma drug (103). This same study found that mice receiving kefir exhibited significantly lower levels of eosinophil infiltration in the lung tissue as well as in the brochoalveolar lavage

fluid (BALF). These mice also showed lower levels of IgE, IL-4, and IL-13 in the BALF, all of which are associated with the Th2 response which is responsible for allergic reaction (103). It has also been found that oral feeding of kefir in OVA sensitized mice resulted in significantly lower levels of anti-OVA serum IgE and IgG1 antibodies than those found in mice given water or unfermented milk (58). Studies examining the *in vitro* effect of heat-killed lactobacilli isolated from kefir on mouse peritoneal macrophages showed that even after being heat-inactivated, the lactobacilli were able to induce the expression of Th1 cytokines such as IFN-γ, TNF-α, IL-12, and IL-1β (104). These same heat-inactivated lactobacilli also reduced the levels of anti-OVA IgE in the serum when fed orally to OVA sensitized mice, while increasing the expression of IL-12 and decreasing the expression of IL-5 in splenocytes. An increase in the levels of regulatory T-cells was also detected in these mice (104). In a study of OVA sensitized mice fed with heat-inactivated strain M1 of *L. kefiranofaciens*, the inactivated M1 was able to decrease levels of pro-inflammatory and Th2 cytokines such as IL-4, IL-6, IL13, and ccl20 in both the splenocytes and BALF of the mice while decreasing OVA-specific IgE and the Th17 associated cytokine IL-17, both of which are strongly associated with an asthmatic response. The M1 treatment was also able to increase the levels of regulatory T cells present (105).

While all of these studies reveal a consistent pattern, it is interesting to note that many of the cytokine profiles are in stark contrast to those found in studies without antigen sensitization or challenge. This highlights both the complexity of the immune system and the need for a balance between the different possible reactions such as the Th1 and Th2 responses. The fact that kefir can induce shifts in the immune system in both directions is promising as it may mean that the organisms in kefir are capable of regulating this balance in the immune system. This may be in part due to the increased number of regulatory T-cells observed in some of these studies, as

regulatory T-cells play an important role in maintaining tolerance and supressing unnecessary inflammatory immune responses (106).

1.9 Health Benefits of Yeast in Kefir:

As noted above, one unique characteristic of kefir relative to other fermented dairy products is the presence of a large population of yeast in both the kefir grain and in the fermented milk (11). Although the majority of commercialised probiotic microbes are bacteria such as lactobacilli and bifidobacteria, there are some yeast species and strains that have been recognized to have probiotic properties, such as *Saccharomyces boulardii* (107,108). *S. boulardii* has been shown to improve the symptoms of *Clostridium difficile* associated diarrhoea as well as reduce inflammation and alter the immune state and reactions in the gut, leading to its adoption as a treatment for *C. difficile* diarrhoea (109–112).

Some yeasts from kefir have also shown immunomodulatory activities. For example *K. marxianus* B0399 has been shown to have the ability to adhere to Caco-2 cells (113). When co-incubated with LPS stimulated Caco-2 cells, a significant decrease in the secretion of IL-10, IL-12, IL-8, and IFN- γ was observed (113). Additionally, *K. marxianus* B0399 elicited a decrease in the secretion of pro-inflammatory cytokines TNF-α, IL-6, and MIP-1α when co-incubated with PBMCs that had been stimulated with LPS (113). This same study showed that in an *in vitro* colonic model system, *K. marxianus* was able to stably form a population in the model while simultaneously enhancing the levels of *Bifidobacterium*. Increases in the levels of the short chain fatty acids acetate and propionate were also observed. Utilizing a Caco-2 cell line with a ccl20 reporter gene, Romanin et al. (114) were able to show that multiple yeast strains of *S. cerevisiae* (CIDCA 81109, 81106, 8112, 9127, 9123, 9136, 9133, 9124, 81103, 9132, 81108, 81102, 8175, and 8111), *K. marxianus* (CIDCA 81111, 8116, 8118, 81105, 8153, 8154, 8113, 81104, and 9121),

and *Issatchenkia* spp. (CIDCA 9131) were able to inhibit the expression of the ccl20 reporter when incubated with the cells prior to stimulation with *Salmonella* flagellar protein FliC. From these yeasts, *K. marxianus* CIDCA 8154 was selected for further testing and showed the ability to inhibit the levels of ccl20 expression in Caco-2 cells regardless of whether the stimulation came from FliC, IL-1β, or TNF-α. The strain also inhibited the expression of IL-8 and MIP-2α in HT-29 cells and inhibited ccl20 expression in a mouse ligated intestinal loop model when administered prior to stimulation with FliC (114). Yeasts isolated from kefir have also shown the ability to improve the probiotic properties of bacterial species by improving the viability of these bacterial strains over time in simulated gastric and intestinal juice, and through improving the adhesion of lactic acid bacteria to Caco-2 cells in an *in vitro* model. This effect is likely due to the co-aggregation of the two microbial species (115).

1.10 Kefiran and the Cell Free Fraction of Kefir:

In addition to the microbial populations present in kefir and other fermented probiotics, there are also fermentation products and other by-products of the metabolism of these microbes that possess bioactivity. Some of these by-products may have a profound effect on the host without the presence of the microbial population. Such a by-product is kefiran, the exopolysaccharide produced by *L. kefiranofaciens* during fermentation (52,116). Mice fed kefiran dissolved in drinking water showed increases in the levels of IgA+ B cells, as well as increases in IL-6, IL-10 and IL-12 in the lamina propria of the small intestine after 7 days of feeding (116). In a murine model of asthma using OVA sensitization, kefiran introduced intra-gastrically one hour prior to challenge reduced levels of the Th2 cytokines IL-4 and IL-5 and lowered AHR when compared to OVA challenged mice that did not receive kefiran (117). After the same period the study showed increases in serum levels of IL-4, IL-6, IL-10, and IFN-γ (117). Addition of kefiran to a co-

incubation of *B. cereus* culture supernatant and Caco-2 cell monolayer resulted in reduced cell detachment and greater mitochondrial activity, as well as negated the haemolytic effect of the *B. cereus* culture supernatant on human red blood cells (118). Genetically diabetic (KKAy) mice fed kefiran were found to have decreasing levels of blood glucose throughout a 30 day examination while a control group was found to have constantly increasing and generally higher levels of blood glucose throughout the same timeline (119). Using SD rats as a model for constipation, it was also found that kefiran significantly improved the symptoms of constipation over the control group (119).

The polysaccharide KGF-C was shown to improve humoral immune response in mice against Sheep Red Blood Cells (SRBC). The levels of anti-SRBC cells isolated from the spleen of mice immunized with SRBC while being intubated with KGF-C was significantly higher than in control mice 4 days post immunization (120). However, this effect was not seen in nu/nu mice (no thymus or T cell population) immunized with SRBC, or in conventional mice immunized with thymus-independent antigens, indicating that the mechanism of action is likely through the T cell population (120). Sphingomyelin isolated from kefir has been shown to increase IFN-β secretion in human MG-63 cells when compared to commercial sphingomyelin and sphingosine (121).

Kefir cell-free supernatant (KCFS) has been shown to increase the levels of IFN-β, IL-6, IL-12, and TNF-α secreted by RAW 264.7 cells through a TLR2 dependent mechanism (92). Cell-free fractions of kefir have also been shown to increase the levels of these cytokines in peritoneal macrophages and adherent cells from the Peyer's patches of mice (91). In addition, KCFSs were found to have a significant impact on tumour size, apoptosis, and immune recruitment in a murine breast cancer model, resulting in increased apoptosis of tumour cells and increases in the CD4+ T cell population (85). In *in vitro* studies utilizing human T-lymphotropic virus 1 (HTLV-1) positive

HuT-102 Malignant T lymphocytes as a model for T cell leukemia, the KCFS was found to inhibit proliferation by up to 98% while simultaneously decreasing the transcriptional levels of TGF-α. These effects have also been observed in HTLV-1 negative malignant T cells with the same decrease in TGF-α transcription being observed (122,123). In addition to anti-proliferative effects, KCFS was found to induce apoptosis in both HTLV-1 positive and negative malignant T cells through the up regulation of *bax* and down regulation of *bcl-2* in a dose dependent manner (124).

1.11 Conclusion:

The purpose of this review has been to collate and summarise that which is known about the microbial composition of kefir and how this composition plays a role in the health benefits associated with kefir consumption. Kefir is a dynamic fermented dairy product with many different factors affecting the benefits associated with its consumption. These factors include the variable yeast and bacterial species present, as well as metabolites such as kefiran and other exopolysaccharides. While kefir has been associated with health benefits for hundreds of years, the exact form of these benefits has, until recently, not been studied. The use of animal models and other *in-vitro* analyses has allowed for the elucidation of how kefir positively impacts host health. Whole kefir, as well as specific fractions and individual organisms isolated from kefir, provide a multitude of positive effects when consumed. These range from improved cholesterol metabolism and wound healing, to the modulation of the immune system and microbiome, and even the potential alleviation of allergies and cancers. Further studies into the mechanisms behind these effects will allow scientists to better understand exactly how kefir and other fermented dairy products confer these benefits as well as how to harness these traits outside of kefir itself.

The wide range of potential health promoting effects of kefir could lead to a further expansion on the popularity of both traditional fermented kefir and products that are manufactured with kefir fractions or organisms. In order to fully exploit the beneficial characteristics of kefir, a more in-depth understanding of the composition of kefir is critical. With advances in metagenomic analysis through the development of high-throughput sequencing technology, this is a very realistic prospect. Armed with this knowledge, it should be possible to more readily isolate and examine the phenotypic characteristics of individual organisms present in a kefir blend while also providing a greater insight into the evolution of these organisms and how they became specialized to the kefir ecosystem. The additional knowledge gained can also provide crucial information relating to the mechanisms and exact agents responsible for beneficial effects that have been attributed to kefir (70,71,88,89).

The need for further research does not only apply to the mechanisms by which kefir consumption exerts these effects but also which organisms or parts of kefir are responsible for each benefit. By determining which organisms and metabolites are essential for each process, the possibility arises for the commercial manufacturing of kefir that is specifically designed to create the most profound effect in those that consume it. The ability to combine the best possible strains of the best organisms from multiple sources of kefir would create the potential for greater benefits than have been previously observed, with a measure of control over these effects that has not been possible in traditional kefir.

1.13 Hypotheses and Objectives

This thesis set out to determine how different variations of kefir impacted host cholesterol and lipid metabolism as well as how variations to the microbial composition of kefir impacted fermentation metabolites with the following objectives and hypotheses.

Hypotheses

1. Different examples of traditional kefir vary in their ability to impact host health.
2. Traditional kefir is more beneficial than commercial kefir in relation to cholesterol metabolism and obesity.
3. Pitched culture kefir made from organisms present in traditional kefir is able to recapitulate health benefits associated with traditional kefir.
4. Pitched culture kefir closely resembles traditional kefir in metabolite profile, while alterations to the microbial composition of pitched kefir significantly alter said profile.

Objectives

1. To determine the impact of different examples of kefir on cholesterol and lipid metabolism in a mouse model of obesity (Chapter 2 and 4).
2. To generate a pitched culture kefir that is able to elicit the same health benefits as traditional kefir while employing a production method that is viable on an industrial scale (Chapter 3 and 4).
3. To investigate how the removal of certain groups of microbes from kefir impacts the ability of kefir to improve host health (Chapter 4).
4. To understand how kefir fermentation dynamics are affected by differences in the microbial composition at the onset of fermentation (Chapter 5).

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Chapter 2: Traditional Kefir Reduces Weight Gain and Improves Plasma and Liver Lipid Profiles More Successfully than a Commercial Equivalent in a Mouse Model of Obesity

2.1 Introduction

Obesity and metabolic disease are a growing problem in the developed world, and have been shown to be a contributing factor in a variety of chronic diseases, such as type 2 diabetes (T2D), cardiovascular disease (CVD), and atherosclerosis. While the link between obesity and diet is well established, recent research has shown that multiple factors, including the gut microbiota, play a significant role in the mechanisms underlying diet induced obesity and the associated disease states (1–5). Specifically, the gut microbiota has been found to have an impact on energy metabolism through processes such as bile acid breakdown (6,7), fatty acid metabolism (8), immunomodulation (9,10), and regulating host physiology (11). Additionally, probiotic and prebiotic interventions that influence the gut microbiota and metabolic health have shown promising results in preventing and improving some of the complications of metabolic syndrome (12), with fermented milk products (13) and associated microorganisms (14) being particularly effective.

Although consumed for thousands of years, kefir has recently gained popularity as a health promoting beverage and source of organisms. While kefir has been associated with diverse health benefits, recent studies have begun to examine the mechanisms behind them (15). Kefir has demonstrated ACE inhibitory activity (16), the ability to improve levels of serum cholesterol (17,18), and immunomodulatory characteristics (19). These attributes, and others such as bile salt hydrolase activity (17), have been associated with individual microorganisms isolated from kefir. Kefir and kefir-derived peptides have also been shown to be effective at alleviating non-alcoholic fatty livery disease (NAFLD) and obesity (20–24). These

characteristics all point to kefir having the potential to positively impact metabolic syndrome, either through effects on diet, direct interactions with the host, or through altering the microbiota and its associated metabolic profile. However, individual examples of traditional kefir differ in their microbial populations, with the major differences being in the ratios of key microorganisms (25,26). Given that these differences impact the fermentation by-products and development of flavour (27), it is likely that they also affect the impact that individual kefirs have on consumer health. Additionally, some commercially produced beverages that are labelled as 'kefir' differ significantly from traditional kefir from a microbiological perspective. While such commercial products and traditional kefir contain *Lactobacillus*, *Lactococcus*, and *Leuconostoc*, most commercial kefir lack acetic acid bacteria, which is present in the vast majority of traditional examples (25–27). Additionally, kefir contains *Lactobacillus kefiri* and *L. kefiranofaciens,* both of which have exhibited health benefits *in vivo* (28,29). *L. kefiranofaciens* also produces an exopolysaccharide unique to kefir called kefiran, which has shown beneficial effects *in vivo* (30,31). Another important aspect of traditional kefir that is not present in most commercial examples is the presence of a complex fungal community. While commercial kefir can contain yeast, the complexity of the yeast population is often significantly lower than what is found in traditional kefir, and sometimes only contain *Saccharomyces cerevisiae*, while traditional kefir contains *S. cerevisiae*, *Pichia fermentans*, *Kazachastania unispora*, and *Kluyveromyces marxianus* and *lactis* along with many other smaller populations of yeast.

To date no studies have compared the health benefits of different traditional kefirs, or of how mass-produced commercial products compare to traditional kefir made with grains. We therefore set out to determine how examples of traditional kefir with differing microbial compositions (25–27) and *in vitro* characteristics compare to both each other and a widely

available commercial product in their ability to affect weight gain and lipid profiles using a mouse model of diet induced obesity.

2.2 Materials and Methods

Kefir Grain Sourcing and Kefir Production

Kefir grains were obtained in a previous study (25) from Ireland, Canada, Germany, United Kingdom, United States of America, Greece and Italy, and were labelled according to their country of origin. The grain ICK has an unknown country of origin and thus stands for Indeterminate Country Kefir. Grains selected for animal experiments were inoculated at 1% weight/volume in fresh 2% milk daily for the course of the study. Fermentation was carried out in glass jars at room temperature (22℃) for 18 hours each day. Commercial kefir contained a microbial composition of *Streptococcus thermophilus*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lb. casei*, *Lb. acidophilus*, *Lb. delbrueckii* subsp. *lactis*, *Lb. rhamnosus*, *Bifidobacterium lactis*, *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis*, *L. lactis* subsp. *cremoris*, and *Leuconostoc mesenteroides* subsp. *cremoris* and had a CFU/ml of 8.0×10^6. The grains used in this study were previously sequenced by our group (25,27), and have varying microbial composition (Table 2.1).

Table 2.1. Relative abundance of bacterial and fungal genera in the four traditional kefir used in this study

Bacterial Genus	ICK	IR9	IR10	GER2
Acetobacter	0.046997	0.199694	0.531256	0.102909
Lactobacillus	0.519055	0.422822	0.157705	0.25657
Lactococcus	0	0.363729	0.263348	0.559077
Leuconostoc	0.344207	0.00866	0.044461	0.079238
Propionibacterium	0	0.002208	0	0
Gluconobacter	0.010318	0	0	0
Other	0.079423	0.002887	0.00323	0.002207

Fungal Genus	ICK	IR9	IR10	GER2
Kazachstania	0.157029	0.068585	0.885288	0.548772
Kluyveromyces	0.114724	0.001679	0.001193	0
Naumovozyma	0	0.818705	0	0
Saccharomyces	0	0.009353	0	0
Davidella	0	0.008393	0	0
Dekkera	0.003695	0	0	0
Wallerria	0	0	0.005765	0
Eurotium	0	0	0.00159	0
Cryptococcus	0	0.006235	0	0
Teratoshpaeria	0	0.001199	0	0
Debaromyces	0	0.002878	0	0
Cyberlinchera	0	0.002878	0	0
Malassezia	0	0.002158	0	0
Other	0.724552	0.077938	0.106163	0.451728

Animals and Treatments

Fifty six 8-week old wild type C57BL/6 female mice were obtained from Jackson Labs. Mice were allocated into 7 groups (n=8) consisting of low fat diet (LFD) control, high fat diet (HFD) control, HFD + commercial kefir (COM), and four groups of HFD + traditional kefir (HFD + ICK, HFD + IR9, HFD + IR10, HFD + Ger2). The LFD group received standard rodent chow, while the HFD groups received a diet consisting of 40% calories from fat supplemented with 1.25% cholesterol by weight (Research Diets D12108C). Mice were housed in a temperature-controlled room (22°C–23°C) under a 12 hr light/12 hr dark cycle and fed chow and water *ad libitum*. Animals received an oral gavage of 100ul of either kefir (treatment groups) or

milk (control groups) daily for 12 weeks. Body weights were taken weekly for the duration of the study and fecal samples were collected on days 0, 28 and 84. After 12 weeks, the animals were sacrificed and tissues were collected, snap-frozen, and stored at -80°C until further analysis. All experiments were carried out with approval from the Animal Care and Use Committee at the University of Alberta (AUP 00000671).

Physiochemical Analysis of Traditional Kefir

Viscosity was tested using a Discovery HR-3 hybrid rheometer (TA Instruments, New Castle, USA) with a cone-plate method and was determined at a shear rate of 3.5 Pa/s as this is similar to shear forces encountered in the human stomach (32). Analysis of pH was conducted using an Orion 2 star benchtop pH meter (Thermo Scientific, Burlington, ON).

In vitro Cholesterol Assimilation

The ability of kefir grains to lower the level of cholesterol in whole milk was determined by inoculating whole milk with kefir grains at 1% weight/volume and fermenting for 24 hours at 22°C. Total cholesterol was determined in mg/dl using a commercial fluorometric kit (Cholesterol Quantitation Kit, Sigma Aldrich, Oakville, ON).

Plasma Cholesterol Measurements

At termination, following a 6 hr fast, blood was collected via heart puncture in an EDTA lined blood collection tube (Fisher Scientific, Ottawa, ON). Blood samples were centrifuged and plasma was collected and stored at -80°C until further analysis. Plasma total cholesterol and high-density lipoprotein (HDL) were determined using commercial colorimetric kits (Wako Diagnostics, Richmond, VA). Non-HDL cholesterol was determined by subtracting HDL cholesterol from total cholesterol.

Liver Triglyceride Analysis

Liver triglycerides were quantified using a chloroform methanol extraction method. Approximately 30mg of frozen liver tissue was homogenized using a bead beater (MP Biomedicals, Solon, OH) in homogenization buffer (10mM Tris-HCl pH 7.4, 150mM NaCl, 1mM EDTA and 1mM DTT containing phosphatase and protein inhibitor cocktails). Protein content was analyzed using a bicinchoninic acid assay (Fisher Scientific, Ottawa, ON) and samples were normalized by protein content. Total lipids were extracted from liver homogenate in methanol-chloroform (2:1). The organic extract was dried under N_2 gas and reconstituted in isopropanol. Triglycerides were then quantified according to manufacturer's instructions using a commercial colorimetric kit (Wako Diagnostics, Richmond, VA).

Gene Expression

Total RNA was isolated from ileum and liver tissue using the GeneJET RNA Purification Kit (Thermo Scientific, Burlington, ON) according to manufacturer's instructions. Following isolation, 1µg aliquots of RNA were used to synthesize cDNA using the qScript Flex cDNA Synthesis Kit (Quantabio, Beverly, MA) according to manufacturer's instructions. Real-time PCR was performed using PerfeCTa SYBR Green Supermix (Quantabio, Gaithersburg, MD). Primers for host genes are listed in Table 2.2. Real-time PCR was performed on an ABI StepOne^{TM} real-time System (Applied Biosystems, Foster City, CA) using the conditions as follows: 95^{o}C for 3 minutes, followed by 40 cycles of 95^{o}C for 10 seconds and 60-62^{o}C for 30 seconds. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene and fold changes of gene expression compared to HFD group were calculated using the $2^{-\Delta\Delta Ct}$ method.

Table 2.2. Specific primer sequences used for quantitative real-time PCR. GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; FGF-15: Fibroblast growth factor 15; Cyp7a1: Cytochrome P450 family 7 subfamily A member 1; PPARγ: Peroxisome proliferator-activated receptor gamma; FASN: Fatty acid synthase; IL-1β: Interleukin 1β; IL-18: Interleukin 18.

Target Gene	Forward (5'-3')	Reverse (5'-3')
GAPDH	ATTGTCAGCAATGCATCCTG	ATGGACTGTGGTCATGAGCC
FGF-15	ATGGACTGTGGTCATGAGCC	GAGGACCAAAACGAACGAAATT
Cyp7a1	GGGATTGCTGTGGTAGTGAGC	GGTATGGAATCAACCCGTTGTC
PPARγ	TTGCTGAACGTGAAGCCCATCGAGG	GTCCTTGTAGATCTCCTGGAGCAG
FASN	AGGGGTCGACCTGGTCCTCA	GCCATGCCCAGAGGGTGGTT
IL-1β	GGAGAACCAAGCAACGACAAAATA	TGGGGAACTCTGCAGACTCAAAC
IL-18	CAGGCCTGACATCTTCTGCAA	TCTGACATGGCAGCCATTGT

Microbiota Analyses

Total DNA was extracted from either faecal pellets or caecal content using the QIAmp DNA stool mini kit (Qiagen, Montreal, QC) according to manufacturer's instructions, with the addition of a bead-beating step (33). Following DNA isolation, amplicon libraries were constructed of the V3/V4 region of the 16S rRNA gene according to the protocol from Illumina (16S Metagenomic Sequencing Library Preparation). Primers targeting the region were: (Forward: 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3' Reverse: 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3'). Raw data was filtered through a quality control pipeline, with bases of quality score <33 being filtered using the FASTX-Toolkit. Paired-end reads were merged using PANDAseq. QIIME 1.9.1 (Quantitative Insights Into Microbial Ecology) software package (34) was applied for obtaining an operational taxonomic units (OTUs) table. This was performed by first dereplicating merged sequences and filtering out chimeras using the ChimeraSlayer database. Next, high-quality reads were mapped against the database of usearch_global and the OTU table was obtained using the 'uc2otutab.py' script. The classification of sequences for each OTU was

carried out using QIIME with the Ribosomal Database Project classifier (confidence threshold, 80%). Greengenes v.13_8 clustered at 97% identity was used for taxonomy assignment.

Statistical Analyses

Cholesterol assimilation *in vitro* was analyzed using a 2 tailed student's T-test comparing kefir grains to unfermented milk. Percent weight gain (calculated as $\frac{weight\ gain\ in\ grams}{starting\ weight\ in\ grams} \times 100$), plasma cholesterol, liver triglyceride, and gene expression data was analyzed using Analysis of Variance with Tukey post-hoc for multiple comparisons utilizing the R packages multcompView, ggplot2, plyr, and lmPerm. Effect of treatment on microbiota was determined using analysis of similarities (ANOSIM) while relative abundance from phylum to genus taxonomic levels were determined using the Kruskal-Wallis test. Principal-coordinate analysis (PCoA) of data derived from Bray Curtis distance matrices was performed to evaluate the overall differences between groups using the R packages ecodist, ape, and vegan.

2.3 Results

Kefir Grains Vary in their Ability to Decrease Cholesterol in Milk

As different examples of traditional kefir have previously been shown to differ in their ability to decrease cholesterol levels in milk (35), our library of 14 different kefir grains was analyzed *in vitro* prior to *in vivo* work. Of the 14 grains tested, 5 (IR10, Ger2, UK4, IR9, and ICK) significantly lowered cholesterol levels following a 24 hour fermentation (figure 2.1). On the basis of the cholesterol lowering phenotype, 4 of the best performing grains were selected for *in vivo* studies to assess impacts on host metabolic health.

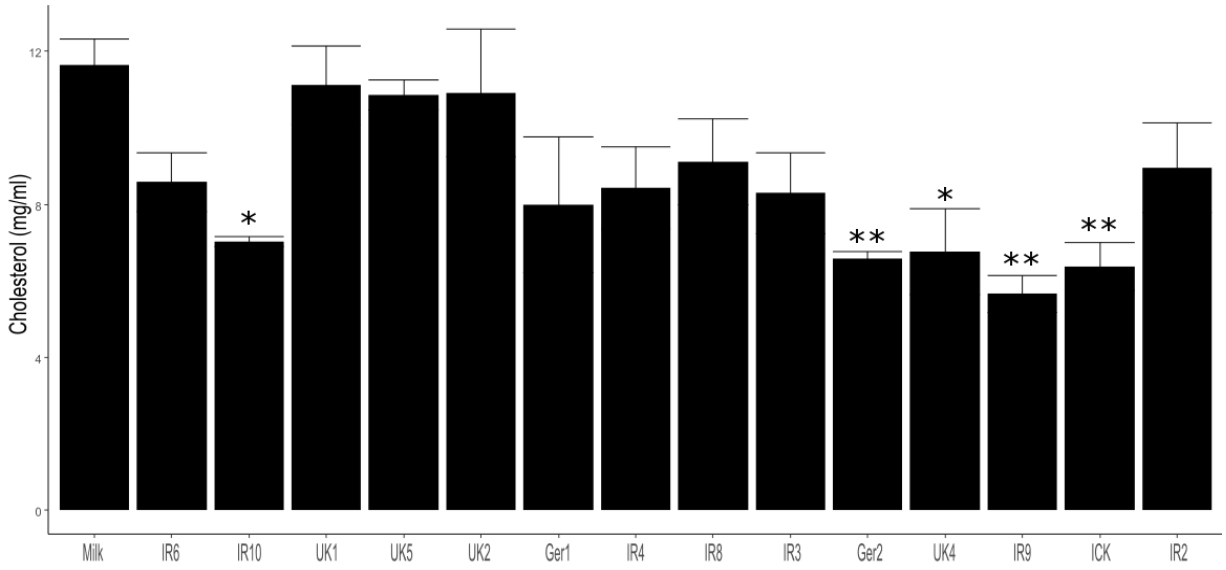

Figure 2.1. Cholesterol levels in whole milk following a 24hr fermentation with different kefir grains. Data expressed as means ± SEs (n=3). * = P ≤0.05 **=P≤0.01 when compared to Milk. Each group corresponds to a different example of traditional kefir.

Physiochemical and Microbial Characteristics of Traditional Kefir

ICK kefir had the highest viscosity (0.43715 ± 0.15605) while IR10 had the lowest (0.00188 ± 0.00039), with IR9 (0.00242 ± 0.00079) and GER2 (0.00309 ± 0.00041) had viscosities closer to that of IR10 (Table 2.3). While there was no significant difference in viscosity between groups, ICK exhibited a trend (P<0.10) when compared to all three of the other kefirs using an ANOVA. The pH of the kefirs had greater differences than viscosity with ICK (4.56 ± 0.08) having a significantly lower pH (P<0.05) than both IR10 (5.72 ± 0.10) and IR9 (5.56 ± 0.12), while Ger2 (5.08 ± 0.06) had a significantly lower pH than IR10. Ger2 and ICK did not differ significantly in pH; however, there was a trend (P<0.10) for ICK to be lower than Ger2. Different traditional kefirs had highly variable microbial compositions, with differences in the abundance of both bacterial and yeast genera observed (Table 2.1). Yeast populations were much more variable with a total of 13 high abundance genera identified for yeast when compared to 6 high abundance bacterial genera. The dominant bacterial genera were

Acetobacter, *Lactobacillus*, *Lactococcus*, and *Leuconostoc*, while *Propionibacterium* and *Gluconobacter* were detected in only IR9 and ICK, respectively. *Acetobacter* was the most abundant bacterial genus in IR10 kefir (53.1% relative abundance), while *Lactobacillus* was most abundant in ICK and IR9 (51.9% and 42.2% relative abundance, respectively), and *Lactococcus* was highest in GER2 (55.9% relative abundance). The dominant yeast genera were *Kazachstania* in ICK, IR10, and GER2 (15.7%, 88.5%, and 54.8% relative abundance, respectively) and *Naumovozyma* in IR9 (81.8% relative abundance).

Table 2.3. Viscosity and pH of traditional kefirs used in this study following an 18 hour fermentation. Viscosity was measured at a shear rate of 3.5 Pascal/second.

Kefir	Viscosity (Pa·s)	pH
IR9	0.00242 ± 0.00079	5.56 ± 0.12^{bc}
IR10	0.00188 ± 0.00039	5.72 ± 0.10^{b}
ICK	0.43715 ± 0.15605	4.56 ± 0.08^{a}
GER2	0.00309 ± 0.00041	5.08 ± 0.06^{ac}

Effects of Kefir on Weight Gain

The ICK and IR10 kefir fed groups both had lower (P<0.05) weight gain over the 12 weeks than the HFD control group, while the LFD fed group had the lowest weight gain (figure 2.2). The Com mice gained more weight (P<0.05) than LFD control, whereas none of the mice receiving high fat diet with traditional kefir gained significantly more weight than LFD control. No differences between groups in terms of feed intake were detected; for instance, daily feed intake for the HFD control, Commercial kefir, and ICK mice averaged 2.63, 2.65, and 2.75 grams per mouse; while the IR9, IR10, and GER2 fed mice averaged 2.33, 2.11, and 2.04 grams per mouse respectively.

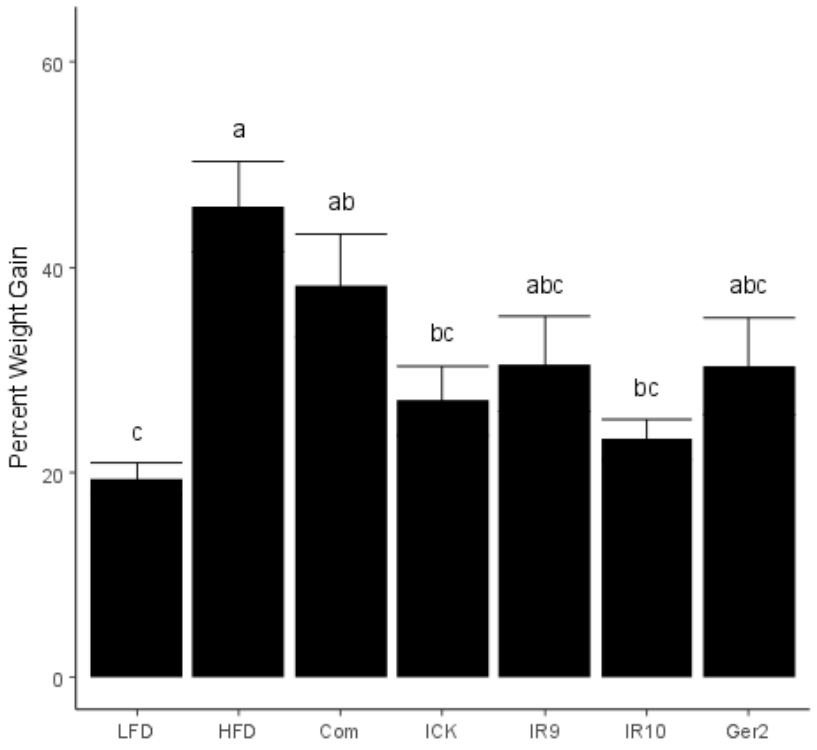

Figure 2.2. Weight gain of mice fed either milk or different examples of kefir for 12 weeks. Data are expressed as means ± SEs (n=7-8) in percentage of starting body weight. Means that do not share a letter are significantly different (P<.05). LFD, mice fed a low fat diet with daily gavage of milk for 12 weeks; HFD, mice fed a high fat diet with daily gavage of milk for 12 weeks; Com, mice fed a high fat diet with daily gavage of commercial kefir for 12 weeks; ICK/IR9/IR10/Ger2, mice fed a high fat diet with daily gavage of a traditional kefir made with the grain corresponding to the group name for 12 weeks.

Traditional Kefir Improved Plasma Cholesterol Profiles and Liver Triglyceride Levels

To examine how kefir impacted cholesterol metabolism, total plasma cholesterol and non-HDL cholesterol levels were determined. Groups treated with the ICK and IR10 kefir had total plasma cholesterol levels similar to the LFD control group (104.372 and 106.174 mg/dl respectively for ICK and IR10 vs. 81.1551 for LFD; figure 2.3A), while the levels of cholesterol in the HFD control and commercial kefir fed groups were higher (P<0.05; 196.039 and 190.811 mg/dl respectively). The same pattern between treatments was observed for plasma non-HDL cholesterol.

We analyzed triglyceride levels in the liver to determine if kefir might have a protective effect against the development of NAFLD. Liver triglycerides were significantly reduced in the ICK kefir group when compared to the HFD control group (figure 2.3C). However, all high fat diet fed groups had significantly higher levels of liver triglycerides as compared to LFD control.

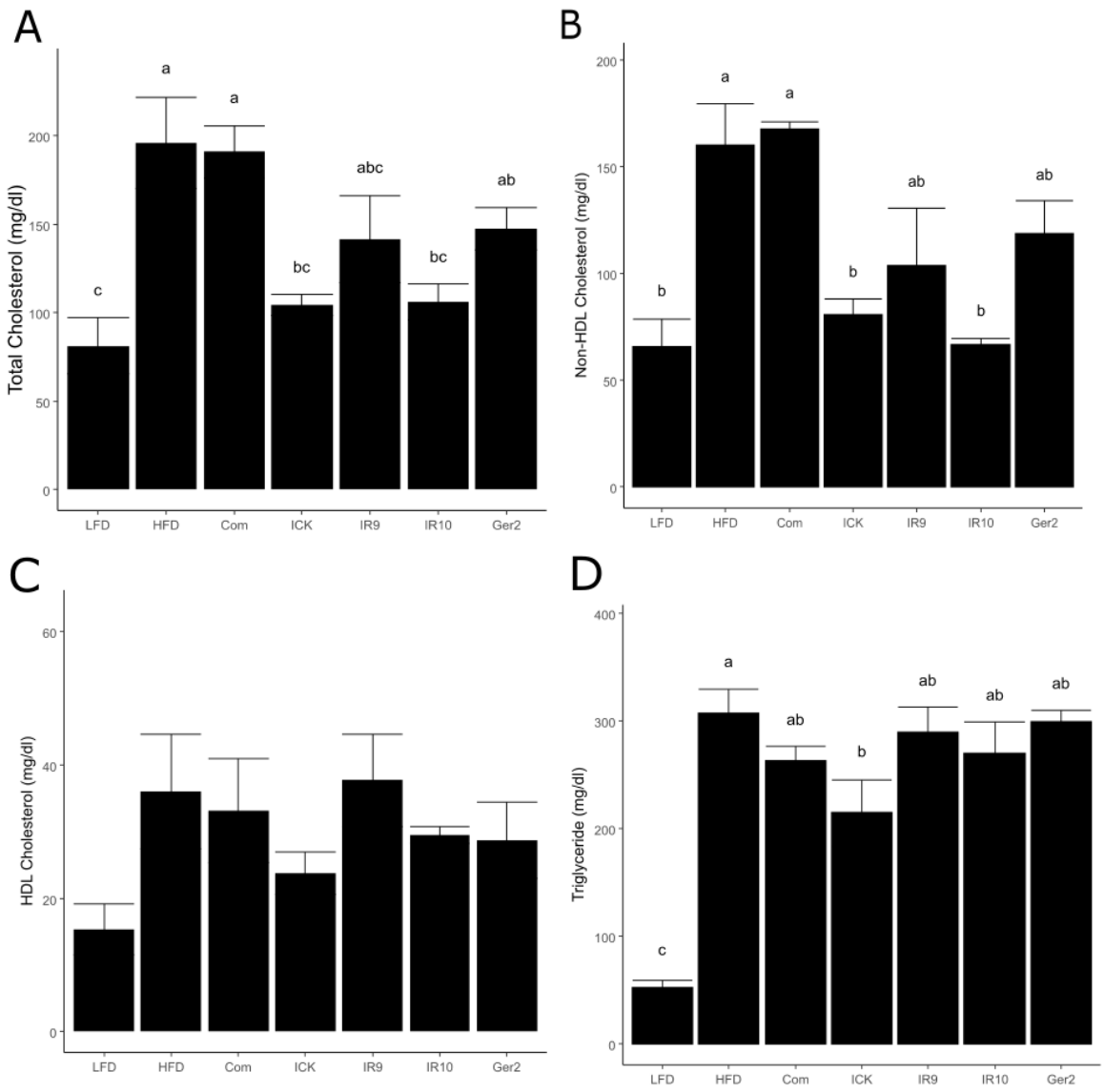

Figure 2.3. Concentrations of plasma total cholesterol (A), non-HDL cholesterol (B), HDL cholesterol (C) and liver triglyceride levels (D) in mice fed either milk or different examples of kefir for 12 weeks. Data are expressed as means ± SEs (n=7-8). Means that do not share a letter are significantly different (P<.05). LFD, mice fed a low fat diet with daily gavage of milk for 12 weeks; HFD, mice fed a high fat diet with daily gavage of milk for 12 weeks; Com, mice fed a high fat diet with daily gavage of commercial kefir for 12 weeks; ICK/IR9/IR10/Ger2, mice fed a high fat diet with daily gavage of a traditional kefir made with the grain corresponding to the group name for 12 weeks.

The Effect of Kefir Feeding on Cholesterol and Fatty Acid Metabolism

Expression levels of FGF-15 and Cyp7a1 were examined in the ileum and liver, respectively, in order to determine whether the differences in plasma cholesterol levels/profiles could be due to a change in bile acid synthesis. Although both the ICK and IR10 groups had decreased FGF-15 expression the ileum as well as increased Cyp7a1 expression in the liver, these changes were not statistically significant (figure 2.4).

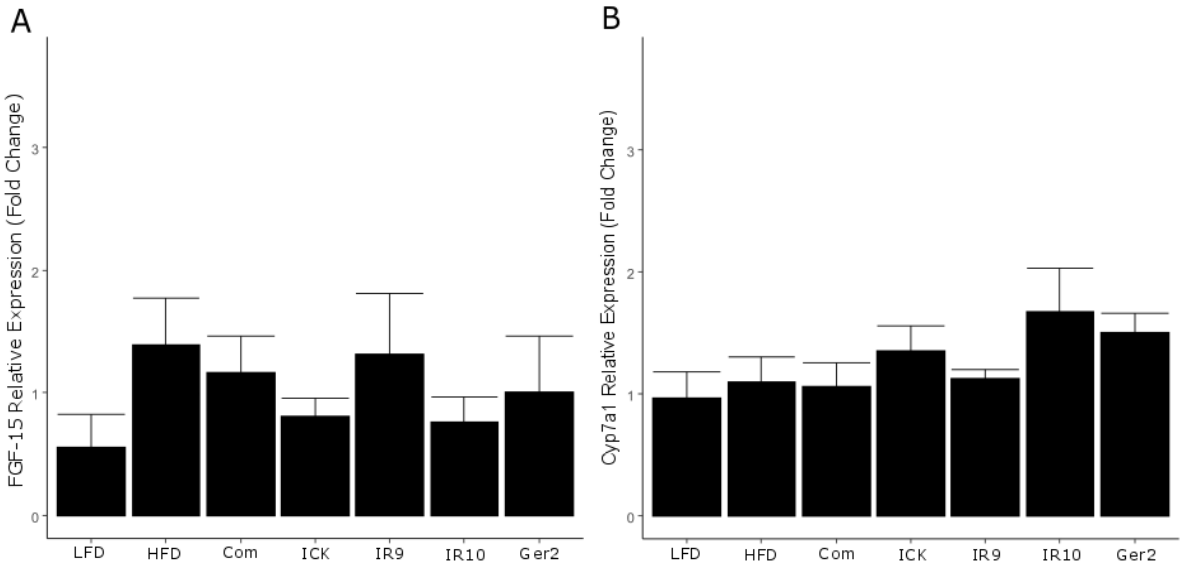

Figure 2.4. Relative expression levels of (A) FGF-15 in the ileum and (B) Cyp7a1 in the liver of mice fed either milk or different examples of kefir for 12 weeks. Data are expressed as means ± SEs (n=7-8). Means that do not share a letter are significantly different ($P<.05$). LFD, mice fed a low fat diet with daily gavage of milk for 12 weeks; HFD, mice fed a high fat diet with daily gavage of milk for 12 weeks; Com, mice fed a high fat diet with daily gavage of commercial kefir for 12 weeks; ICK/IR9/IR10/Ger2, mice fed a high fat diet with daily gavage of a traditional kefir made with the grain corresponding to the group name for 12 weeks.

To examine the effect of kefir feeding on fatty acid metabolism, fatty acid synthase (FASN) and peroxisome proliferator-activated receptor gamma (PPARγ) expression were measured in the liver. As with previous results, the ICK and IR10 groups showed a significant decrease in expression of FASN; however, the commercial kefir also exhibited a significant decrease (figure 2.5A). PPARγ, however, only showed a significant reduction in expression in

the ICK fed group. The LFD, IR9 and Ger2 groups did not show a significant reduction in the expression levels of FASN or PPARγ relative to HFD.

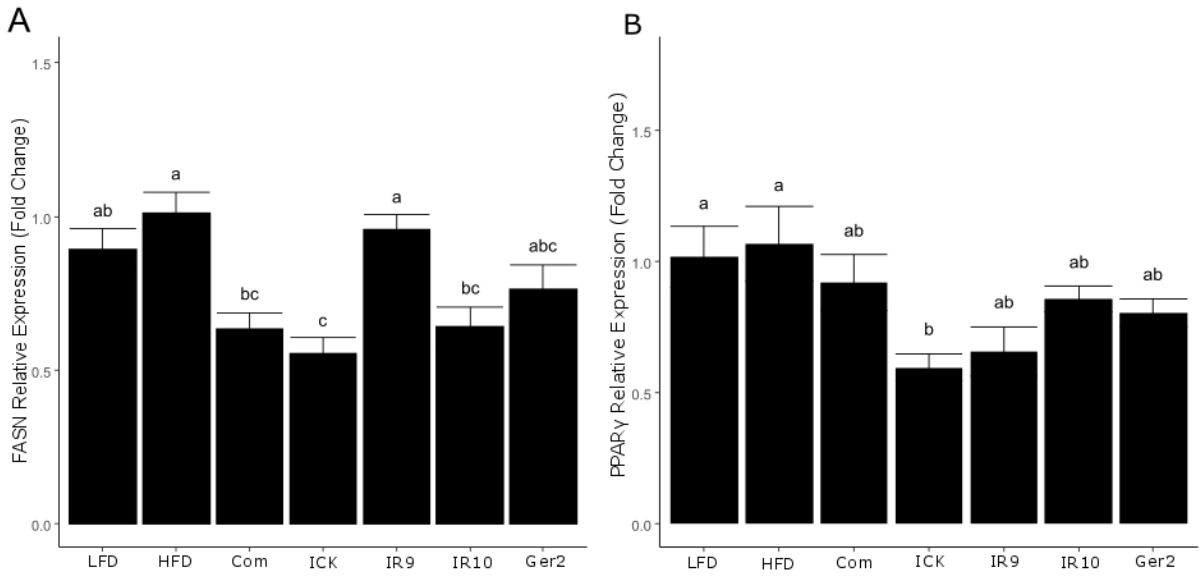

Figure 2.5. Relative Expression levels of fatty acid synthase (A) and PPARγ (B) in the liver of mice fed either milk or different examples of kefir for 12 weeks. Data are expressed as means ± SEs (n=7-8). Means that do not share a letter are significantly different (P<.05). LFD, mice fed a low fat diet with daily gavage of milk for 12 weeks; HFD, mice fed a high fat diet with daily gavage of milk for 12 weeks; Com, mice fed a high fat diet with daily gavage of commercial kefir for 12 weeks; ICK/IR9/IR10/Ger2, mice fed a high fat diet with daily gavage of a traditional kefir made with the grain corresponding to the group name for 12 weeks.

Kefir had a Varied Effect on IL-18 and IL-1β Expression

To determine whether kefir affected inflammasome activation, IL-18 and IL-1β expression were measured in the ileum. None of the kefir fed groups showed significant reductions compared to the HFD group; however, ICK mice had significantly higher levels of IL-18 than the LFD group while IR10 fed mice had levels similar to the LFD group. Similar but not significant (P = 0.20) changes were observed for the expression of IL-1β, with ICK increasing expression levels compared to the LFD group, while IR10 mice had comparable levels to LFD (Figure 2.6).

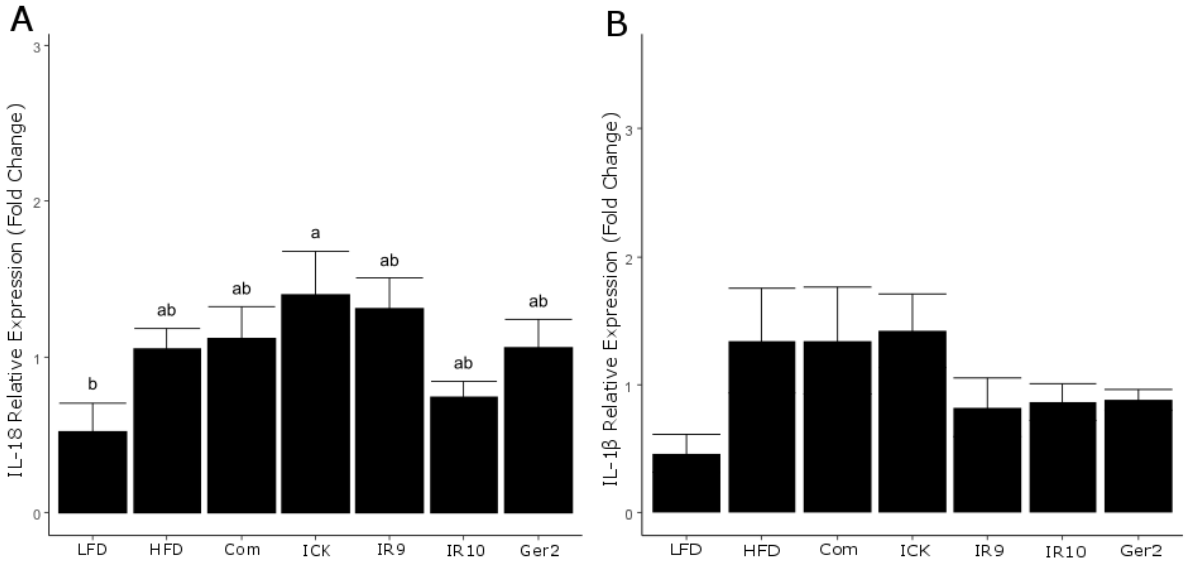

Figure 2.6. Relative expression of IL-18 (A) and IL-1β (B) in the ileum of mice fed either milk or different examples of kefir for 12 weeks. Data are expressed as means ± SEs (n=7-8). Means that do not share a letter are significantly different (P<.05). LFD, mice fed a low fat diet with daily gavage of milk for 12 weeks; HFD, mice fed a high fat diet with daily gavage of milk for 12 weeks; Com, mice fed a high fat diet with daily gavage of commercial kefir for 12 weeks; ICK/IR9/IR10/Ger2, mice fed a high fat diet with daily gavage of a traditional kefir made with the grain corresponding to the group name for 12 weeks.

Microbiota Composition Analysis

Fecal microbiota was analyzed at 28 days and beta-diversity was compared using a Bray Curtis distance matrix and visualized utilizing PCoA (Figure 2.7). ANOSIM of day 28 microbiota showed a significant effect of treatment (P<0.01). The LFD group separated from the HFD fed mice, largely due to a significant increase in Erysipelotrichaceae (P<0.01), while the Ger2 and IR10-fed groups showed significant separation from the other HFD mice, which coincided with a significant increase in the bacterial genus *Akkermansia* (18% relative abundance in IR10 and 42% relative abundance in Ger2 vs <1% in all other groups; P<0.01). Caecal microbiota was analyzed at day 84 using the same method, and once again the LFD fed mice separated from the HFD fed mice. ANOSIM of the day 84 caecal microbiota showed a significant effect of treatment again (P<0.01) despite less obvious clustering in the PCoA plots.

However, removing the LFD group from the analysis eliminated any significance in the ANOSIM, indicating that no kefir treatment had an appreciable effect on overall microbial community composition. Comparisons of individual bacterial families showed only 5 families with significant differences ($P<0.05$) between HFD fed mice were at extremely low relative abundances (<0.001%) and showed no discernible pattern among HFD, Commercial, and traditional kefir groups (Tables 2.4 and 2.5).

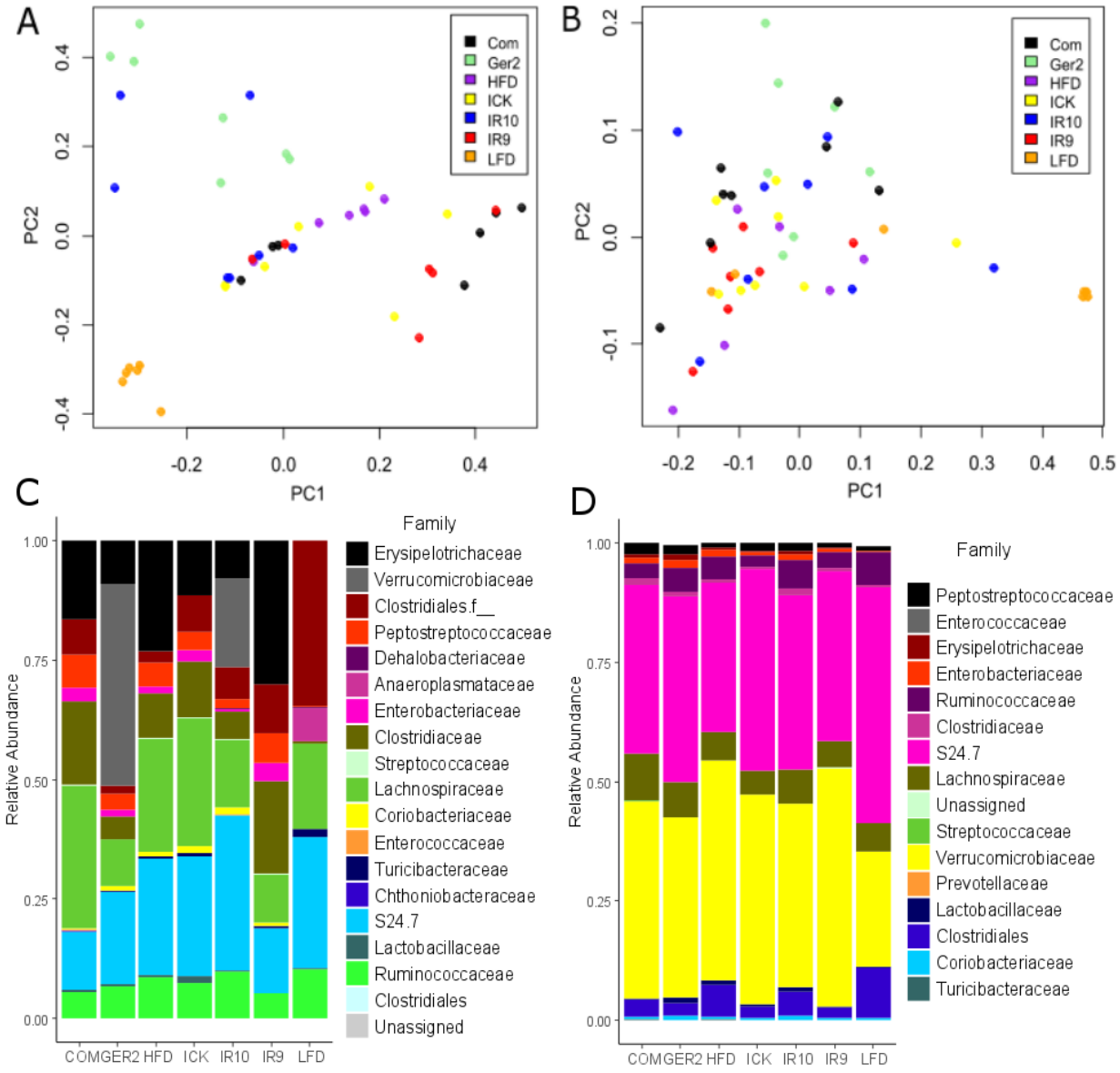

Figure 2.7 Principle coordinate analysis (PCoA) of Bray Curtis dissimilarity matrix for (A) day 28 faecal and (B) day 84 caecal microbiota as well as stacked bar charts representing the relative abundance at Family level for (C) day 28 faecal and (D) day 84 caecal microbiota. LFD, mice fed a low fat diet with daily gavage of milk for 12 weeks; HFD, mice fed a high fat diet with daily gavage of milk for 12 weeks; Com, mice fed a high fat diet with daily gavage of commercial kefir for 12 weeks; ICK/IR9/IR10/Ger2, mice fed a high fat diet with daily gavage of a traditional kefir made with the grain corresponding to the group name for 12 weeks.

Table 2.4. Relative abundances and P values (calculated by ANOSIM) of bacterial genera/families in the faecal microbiota at day 28.

Genus/Family	P Value	ICK	LFD	IR9	IR10	Ger2	Com	HFD
Blautia	2.67E-05	1.18E-01	0.00E+00	1.54E-05	1.51E-05	4.47E-06	2.30E-06	7.16E-06
Erysipelotrichaceae genus unassigned	4.78E-05	1.11E-01	4.00E-04	2.98E-01	7.73E-02	8.52E-02	1.02E-01	2.22E-01
Akkermansia	6.90E-05	0.00E+00	5.07E-05	2.94E-05	1.85E-01	4.22E-01	2.30E-06	1.58E-05
Epulopiscium	7.34E-05	1.05E-01	6.73E-06	4.23E-02	6.81E-02	3.70E-02	2.33E-01	1.62E-01
Clostridiales family unassigned genus unassigned	7.93E-05	7.58E-02	3.46E-01	1.05E-01	6.75E-02	1.75E-02	7.28E-02	2.49E-02
Peptostreptococcaceae genus unassigned	1.20E-04	3.86E-02	3.90E-04	6.02E-02	1.92E-02	3.24E-02	6.97E-02	4.99E-02
Dehalobacterium	1.50E-04	5.30E-05	2.57E-03	0.00E+00	1.49E-03	0.00E+00	1.80E-05	1.39E-05
Anaeroplasma	1.50E-04	2.51E-05	6.93E-02	2.80E-04	5.34E-05	0.00E+00	2.40E-04	0.00E+00
Citrobacter	1.80E-04	2.24E-02	3.90E-06	3.87E-02	6.46E-03	1.39E-02	2.74E-02	1.28E-02
Lactococcus	4.20E-04	2.38E-03	0.00E+00	3.07E-03	2.06E-03	9.80E-04	2.70E-03	2.01E-03
Clostridium	1.25E-03	9.08E-02	1.90E-05	9.98E-02	5.24E-02	4.04E-02	9.18E-02	7.65E-02
Coprobacillus	2.07E-03	3.87E-03	8.44E-05	1.92E-03	1.84E-03	2.94E-03	3.36E-03	9.42E-03
Coriobacteriaceae genus unassigned	2.33E-03	1.48E-02	9.21E-05	4.87E-03	1.49E-02	1.03E-02	2.15E-03	7.76E-03
Coprococcus	2.36E-03	5.56E-03	3.41E-03	2.78E-03	2.90E-04	3.69E-03	4.39E-03	1.21E-02
Eubacterium	2.70E-03	0.00E+00	0.00E+00	0.00E+00	0.00E+00	1.41E-03	5.92E-02	0.00E+00
Enterococcaceaegenus unassigned	1.51E-02	3.89E-04	0.00E+00	1.69E-03	2.86E-04	2.93E-04	1.20E-03	3.33E-04
Ruminococcus	1.54E-02	4.49E-03	6.09E-03	1.83E-03	8.33E-03	4.24E-03	6.12E-03	5.57E-03
Clostridiaceae genus unassigned	1.62E-02	2.70E-02	4.35E-03	9.33E-02	3.00E-03	8.22E-03	8.34E-02	1.70E-02
Ruminococcaceae genus unassigned	1.93E-02	1.25E-02	1.59E-02	1.36E-03	1.29E-02	6.04E-03	6.12E-03	8.03E-03
Lachnospiraceae genus unassigned	2.91E-02	2.75E-02	1.58E-01	4.15E-02	5.81E-02	4.06E-02	4.75E-02	3.94E-02
Turicibacter	4.67E-02	4.86E-03	1.66E-02	3.50E-03	8.21E-04	2.66E-03	1.63E-03	5.04E-03
Oscillospira	5.29E-02	2.47E-02	3.84E-02	1.31E-02	4.53E-02	2.32E-02	1.55E-02	3.41E-02
Dorea	6.21E-02	5.20E-03	2.76E-03	3.02E-03	1.03E-02	7.97E-03	6.51E-03	1.20E-02
Ruminococcus	1.10E-01	5.57E-03	1.56E-02	1.05E-02	5.18E-03	6.72E-03	6.91E-03	1.17E-02

Delftia	1.31E-01	0.00E+00	7.02E-06	0.00E+00	0.00E+00	8.18E-06	0.00E+00	0.00E+00
S24-7 genus unassigned	1.64E-01	2.52E-01	2.74E-01	1.36E-01	3.24E-01	1.92E-01	1.23E-01	2.43E-01
Lactobacillus	2.44E-01	1.44E-02	1.67E-03	7.72E-04	2.09E-03	4.25E-03	3.87E-03	4.65E-03
Ruminococcaceae genus unassigned	5.08E-01	3.93E-04	5.72E-04	3.68E-04	6.38E-04	2.92E-04	2.84E-04	3.09E-04

Table 2.5. Relative abundances and P values (calculated by ANOSIM) of bacterial genera/families in the caecal microbiota at day 84

Genus/Family	P value	ICK	LFD	IR9	IR10	Ger2	Com	HFD
Ruminococcaceae genus unassigned	2.98E-05	3.63E-03	1.52E-02	4.19E-03	8.39E-03	5.28E-03	7.78E-04	1.39E-03
Eubacterium	2.69E-04	1.81E-04	4.05E-05	3.96E-04	2.03E-04	2.72E-03	1.81E-03	2.55E-04
Coprococcus	4.17E-04	1.05E-03	1.60E-03	3.03E-03	3.75E-03	7.52E-04	2.39E-03	4.17E-03
Epulopiscium	2.25E-03	3.27E-02	9.88E-03	3.13E-02	2.37E-02	4.35E-02	6.20E-02	1.63E-02
Dorea	4.68E-03	1.23E-03	1.66E-03	2.67E-03	7.92E-03	2.67E-03	4.60E-03	1.51E-03
Peptostreptococcaceae genus unassigned	1.14E-02	1.61E-02	9.42E-03	8.83E-03	1.69E-02	1.92E-02	2.47E-02	9.85E-03
Ruminococcaceae genus unassigned	1.59E-02	1.02E-02	2.30E-02	1.70E-02	2.83E-02	3.15E-02	1.14E-02	3.29E-02
Lachnospiraceae genus unassigned	3.05E-02	8.58E-03	4.00E-02	9.90E-03	2.01E-02	1.46E-02	1.14E-02	2.80E-02
Citrobacter	3.07E-02	7.38E-03	2.58E-03	8.73E-03	1.22E-02	1.69E-02	1.07E-02	1.52E-02
Clostridium	3.49E-02	3.84E-03	3.04E-03	7.17E-03	1.29E-02	8.21E-03	1.34E-02	4.82E-03
Erysipelotrichaceae genus unassigned	4.73E-02	2.30E-03	7.86E-04	1.71E-03	6.70E-03	6.92E-03	4.89E-03	3.97E-03
Coprobacillus	9.50E-02	3.44E-04	9.26E-05	1.97E-04	5.03E-04	3.28E-03	6.40E-04	3.44E-04
S24-7 genus unassigned	1.08E-01	4.21E-01	4.93E-01	3.54E-01	3.66E-01	3.88E-01	3.53E-01	3.13E-01
Blautia	1.33E-01	3.97E-03	2.50E-03	4.77E-03	1.06E-02	7.36E-03	1.36E-02	2.37E-03
Unassigned	1.49E-01	3.46E-04	2.22E-04	4.78E-04	5.46E-04	3.59E-04	5.32E-04	5.83E-04
Clostridiaceae genus unassigned	1.81E-01	6.92E-04	4.79E-04	4.06E-04	7.65E-04	1.01E-03	3.74E-04	1.13E-03
Akkermansia	2.43E-01	4.38E-01	2.41E-01	4.99E-01	3.84E-01	3.77E-01	4.14E-01	4.59E-01
Oscillospira	2.67E-01	8.85E-03	2.68E-02	1.04E-02	2.02E-02	1.15E-02	1.84E-02	1.20E-02
Lactobacillus	3.19E-01	4.73E-03	2.05E-03	1.30E-03	7.42E-03	1.23E-02	2.47E-03	9.77E-03

Ruminococcus	3.38E-01	1.05E-03	4.14E-03	1.08E-03	2.50E-03	1.45E-03	1.54E-03	1.26E-03
Coriobacteriaceae genus unassigned	3.43E-01	3.72E-03	2.33E-03	2.48E-03	8.82E-03	8.66E-03	4.84E-03	4.63E-03
Clostridiales family unassigned genus unassigned	3.45E-01	2.43E-02	1.05E-01	2.33E-02	5.05E-02	2.54E-02	3.50E-02	6.69E-02
Turicibacter	4.42E-01	1.10E-03	1.94E-03	1.89E-03	1.98E-03	1.46E-03	2.96E-03	3.26E-03
Ruminococcus	7.57E-01	3.32E-03	5.19E-03	4.40E-03	4.20E-03	4.48E-03	3.95E-03	7.20E-03

2.4 Discussion

Because each traditional kefir has a different population of microbes, and the commercial kefir used in this study is microbially very distinct from traditional kefir, we expected that they would differ in their ability to improve metabolic health outcomes in a high fat/high cholesterol diet challenge model. Indeed our study showed that certain traditional kefirs are able to alleviate weight gain, plasma cholesterol levels, and triglyceride deposition in the liver associated with high fat diet feeding. Specifically, the IR10 and ICK kefirs resulted in weight gain and plasma cholesterol levels similar to those seen in the LFD mice. These results indicate that traditional kefir could potentially be used to alleviate excess weight gain and cholesterol deposition in the blood. This is especially important as both obesity and circulating cholesterol levels have been associated with metabolic syndrome and increased risk of cardiovascular disease and diabetes (36).

In addition to cardiovascular disease and diabetes, hyperlipidemia and obesity have been linked with NAFLD, with elevated triglyceride levels in the liver being a common marker of NAFLD and hepatic steatosis (37). While not all traditional kefir had an impact on triglycerides, ICK was able to reduce liver triglyceride levels. Triglyceride levels in the liver have been strongly correlated to the expression of specific genes. For example, fatty acid synthase is an

important modulator of *de novo* lipogenesis and has been shown to be elevated in both human and murine subjects with NAFLD (38). PPARγ expression has also been shown to increase in high fat diet induced liver steatosis in mice (39). In our study mice fed ICK, IR10 and commercial kefir had significant reductions in the expression of FASN. ICK also resulted in reductions in PPARγ expression when compared to the HFD group, which may help to explain the corresponding reduction in liver triglyceride levels that were observed.

While there was a strong plasma cholesterol reduction associated with IR10 and ICK kefir feeding, the analysis of the FGF-15/Cyp7a1 signalling axis showed no significant differences. FGF-15 and Cyp7a1 were examined as they play an important role in bile acid signalling and controlling the size of the bile acid pool (40). FGF-15 expression is controlled by the bile acid receptor FXR and directly inhibits Cyp7a1 expression, with Cyp7a1 expression being the rate limiting factor in bile acid synthesis (7). This means that as FGF-15 expression decreases, Cyp7a1 expression increases leading to greater synthesis of bile acids, and thus increased utilization of cholesterol in the liver. Additionally, the kefir grains tested in this trial were shown to assimilate cholesterol *in vitro,* which may explain the observed reduction *in vivo*.

One of the major contributors to increased metabolic dysfunction in obesity is the induction of chronic low-grade inflammation by the inflammasome (41–43). As IL-18 and IL-1β are the main cytokines involved in activation of the inflammasome (44), we used expression levels of IL-18 and IL-1β in the ileum as markers of inflammasome activation. The role of the inflammasome in the development of metabolic dysfunction is complex and the exact mechanisms behind how IL-1β and IL-18 interact and, in turn, impact metabolic health are still being elucidated (43,45). We found that traditional kefir elicited a varied response in regards to both IL-18 and IL-1β expression, with ICK increasing expression compared to the LFD fed

group, while IR10 fed mice exhibited expression levels similar to the LFD group; however, none of the traditional or commercial kefir fed groups showed significantly different expression levels than the HFD control group. The common ability of ICK and IR10 to improve plasma cholesterol profiles did not consistently correlate with markers of inflammasome activation.

Additionally, as recent work has begun to highlight the role of the gut microbiota in the development of metabolic dysfunction associated with obesity (1–3), we examined the composition of the fecal and cecal microbiota at day 28 and 84 of the study. At week 4, the microbiomes of the IR10 and Ger2 kefir fed groups showed strong separation from the rest of the mice fed high fat diets based largely on an increased incidence of the genus *Akkermansia*. Analysis of the cecal microbiota at week 12 failed to show any consistent differences between treatment groups fed HFD. The early increase in *Akkermansia* is interesting as it has previously been associated with improved metabolic health outcomes (46) and may contribute to the metabolic phenotypes observed. Although the changes to the microbiome were not consistent, this is likely due to differences in collection point as fecal and caecal microbial communities commonly differ (47). The longer timeline of this trial along with the increased stress associated with a daily gavage in the mice may have played a role in overcoming the influence of kefir administration (48,49). Additionally, the lack of difference in the caecal microbiota may point to a mechanism of action that is not tied to alterations to the microbiome and instead may involve fermentation and metabolic products present in the kefir itself.

This study is the first of our knowledge to compare traditional examples of kefir from multiple origins in an *in vivo* model examining metabolic health. However, different grains have previously been compared for a small number of health relevant characteristics *in vitro* (35). Our analysis agrees with past results in showing that kefir can vary in its ability to lower cholesterol

levels in milk. Additionally, different components of kefir have been examined for their potential health benefits, such as kefiran (30,50), lactic acid (51), and filtered cell free kefir (52,53). While the traditional kefirs examined collectively exhibited decreases in weight gain and plasma cholesterol, only IR10 and ICK showed statistically significant decreases, and only ICK decreased liver triglyceride levels. While viscosity and pH varied among the traditional kefirs, ICK and IR10 were the highest and lowest kefirs in both viscosity and pH, indicating that these physiochemical characteristics are not indicative of the ability of traditional kefir to improve weight gain and lipid profiles. These results show that, while traditional kefirs have largely the same microbes present regardless of origin (25), the differences in the relative abundances of these organisms or their behaviours may be important. The variation in effect between kefirs is consistent with studies examining in vitro characteristics of different kefirs. For example, differences in the quantities of certain microbes have been shown to impact the flavour profile and fermentation by-products (27,54). These findings point to the potential importance of microbial interactions during fermentation on the efficacy of functional fermented foods.

While traditional kefir showed promise in reducing adverse health outcomes associated with an unhealthy diet, commercial kefir did not. Indeed, commercial kefir fed mice showed near identical weight gain, plasma cholesterol levels, and liver triglyceride levels as the HFD control group. This indicates that traditional kefir may better prevent weight gain and metabolic dysfunction compared to commercial examples. The results from the current study may explain why commercial kefir was ineffective in improving host metabolic health in a human trial (55). While commercial kefir lowered fatty acid synthase levels in the liver and may be beneficial, the beneficial effects of the commercially available kefir used in this study differ from those imparted by traditional kefir.

The results of this study agree with recent work showing kefir or kefir organisms to be protective against NAFLD (23,56) and obesity (24,57). It should be noted that we did not see as marked changes in the expression of genes related to lipogenesis and fatty acid metabolism. This may be explained by differences in diet or tissue examined in the other studies. For instance, many of these studies have been carried out with knockout strains, such as ob/ob mice, or used diets consisting of significantly higher levels of fat (ie 60% kcal from fat) or sugar (high fructose corn syrup) in order to induce obesity/NAFLD. This may have led to the development of a more significant phenotype and thus resulted in greater alterations to basal gene expression levels. Many other studies have utilized freeze dried kefir as a delivery method through either rehydration in water or mixing with food, which may lead to increased dosages (>10 times) of microorganisms or other kefir components beyond what would be consumed under normal circumstances. Additionally, no previous studies have analyzed gene expression related to bile acid metabolism and production. While our findings were not significant the patterns observed may indicate a valuable area of further study.

It should be noted that this study only examined one commercially available product. The majority of commercial kefirs available in Canada, including from international kefir producers contain *S. thermophilus*, *Lactobacillus* species such as *Lb*. *acidophilus*, *Lb. casei*, *Lb. delbrueckii*, *Bifidobacterium* species, *Lactococcus lactis* strains, and *Leuconostoc mesenteroides* strains. In contrast, traditional kefir contains the *Lactobacillus* species *Lb*. *kefiri* and *Lb*. *kefiranofaciens*, as well as a variety of yeast and fungal species in addition to examples of *Lactococcus lactis* and *Leuconostoc mesenteroides*. Since performing this study we have become are aware of at least one commercially available kefir that indicates inclusion of kefir specific isolates and will merit further investigation.

2.5 Conclusion

These findings show that traditional kefir has promise in reducing adverse metabolic outcomes associated with a high fat western diet. It was also observed that traditional kefir exhibited varying levels of effectiveness alleviating metabolic dysfunction and weight gain, suggesting that differences in microbial population of the kefir play an important role in how fermented foods impact host health. Most importantly traditional kefir outperformed commercial kefir indicating that substantial consideration is needed in future selection of commercial kefir organisms.

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Chapter 3: Isolation and Characterisation of Traditional Kefir Microorganisms

3.1 Introduction

As individual microorganisms present in kefir have been shown to provide health benefits when administered on their own, outside of a kefir matrix (1–5), we set out to generate a large library of kefir microorganisms in order to identify microorganisms of interest and potential future use from both fermented kefir milk and kefir grains. Kefir microorganisms, especially lactobacilli, have been shown in the past to exhibit pH and bile tolerance, both of which are thought of as important characteristics for the gastrointestinal tract survival of microorganisms which are administered orally, as this can improve survivability through the gastrointestinal tract (6–9). Additionally, kefir microorganisms have exhibited bile salt hydrolase activity, as well as bacteriocin production (10–13) all of which are thought of as beneficial and probiotic properties (14–16).

Given the greater ability of traditional kefir to improve cholesterol metabolism in a mouse model of obesity when compared to a commercial example or milk (17; Chapter 2, this thesis), we set out to recapitulate these health benefits while utilizing a method or manufacturing kefir that is more easily controllable and applicable to commercial scale production. As previous work had shown that kefir made with the grain ICK (indeterminate country kefir) had the most significant impact on plasma cholesterol and liver triglyceride levels, isolates from this grain were screened using a variety of methods to identify specific microorganisms of interest which were deemed important for recapitulating the health benefits observed in the ICK kefir. In order to accomplish this, we set out to isolate and identify microbes with potentially health promoting characteristics, while also attempting to obtain representatives of each of the major bacterial and yeast species present in most traditional kefir. These included *Lactobacillus kefiri*, *Lactobacillus*

kefiranofaciens, *Lactococcus lactis*, *Leuconostoc mesenteroides*, and *Acetobacter pasteurianus* for bacteria and *Saccharomyces cerevisiae*, *Pichia fermentans*, *Kazachstania unispora*, and *Kluyveromyces marxianus* for yeast (18–20). Additionally, many of these microorganisms were targeted as they have been associated with health benefits when ingested on their own, including hypocholesterolaemic affects being attributed to *K. marxianus* (10,21), and the exopolysaccharide kefiran which is uniquely produced by *L. kefiranofaciens* (22–24).

3.2 Methods

Bacterial and Fungal Isolation

Bacterial and yeast isolates were obtained from plating either serial dilutions of kefir following 18 hours of fermentation, or kefir grains which had been homogenized in PBS. Bacteria were isolated using De Man, Rogosa and Sharpe (MRS) agar, MRS agar pH adjusted to 5.4 with hydrochloric acid, and MRS supplemented with vancomycin (0.5g/L) and erythromycin (0.5g/L). Yeasts were isolated using yeast extract, glucose, and chloramphenicol (YEGC), yeast extract, lactose, and chloramphenicol (YELC), Sabouraud, and malt extract media. Incubations with each media were performed at both 30 and 37ºC for 24-72h. Following growth on plates, isolates were re-streaked and then individual colonies were picked and placed into 24 well plates with 1mL of liquid media corresponding to the agar used for isolation supplemented with 20% glycerol and grown in the corresponding conditions prior to being frozen at -80ºC for storage.

pH Tolerance Testing

Bacterial and fungal cultures at a concentration of 10^6 cfu/mL were incubated for 4h at 30ºCin MRS or YEGC adjusted to a pH of 2.5 with hydrochloric acid. Following the 4h incubation, isolates were plated on their respective media and compared to control counts (from

isolates incubated in non-pH adjusted media) following 24-48h of growth at 30°C. Isolates with greater than 75% survival relative to the paired control counts were deemed to be pH tolerant.

Bile Tolerance Testing

Isolates exhibiting pH tolerance were inoculated into either MRS or YGC containing 3% oxgall at 10^6 cfu/mL and incubated at 30°C for 6h before being plated as in the pH tolerance assay. Isolates with >75% survival were determined to be bile tolerant and carried through to bile salt hydrolase testing.

Bile Salt Hydrolase Activity

Isolates were tested using a plate assay where overnight cultures were spot plated (10µL) onto MRS supplemented with 0.5% taurodeoxycholic acid and 0.037% calcium chloride. BSH activity was indicated by the formation of a halo of precipitated deconjugated bile acids around spots.

Bacteriocin Production

Bacteriocin production was assayed using a spot plate overlay method where isolates were spot plated (2µL) on either MRS or YGC and incubated for 24h at their required growth conditions. Spots were then overlaid with 10mL soft agar (0.7%) that had been inoculated at 1% with an indicator strain (*Lactobacillus delbruickii* subsp. *bulgaricus* DPC5383) and incubated anaerobically for 24h. Inhibition zones were then read and any isolates showing a clear zone of >1mm were identified for further testing.

Gram Staining and Microscopy

Bacterial isolates of interest were stained using a Gram stain followed by visualization on an EVOS FL Auto microscope (Life technologies, Carlsbad, USA) in order to determine cell morphology and identify isolates for downstream Sanger sequencing.

Identification of Isolates Using DNA Sequencing

Selected isolates were identified via Sanger sequencing of the 16S rRNA or ITS genes for bacteria and yeast, respectively. The 16S rRNA primers used were 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and 926R (5'- CCGTCAATTCNTTTRAGT-3'). ITS primers used were: ITS1F (5'-CTTGGTCATTTAGAGGAAGTAA-3') and ITS 2 (5'-GCTGCGTTCTTCATCGATGC-3'). Prior to ITS sequencing, likely *Kluyveromyces* isolates were identified using the *Kluyveromyces* specific primers KLOC1 (5'-ATCGGGTACCTTCAATGG-3') and KLOC2 (5'-TTGCGCACGGATCTGTAAC-3') under previously described PCR conditions (25).

Kefir Production

The ICK kefir grain was obtained in a previous study (18) and fermentation was carried out as previously described (17; Chapter 2, this thesis). Pitched kefir was prepared by inoculating 2% milk with *Acetobacter pasteurianus*, *Lactococcus lactis*, *Leuconostoc mesenteroides*, *Lactobacillus kefiri*, *Lactobacillus kefiranofaciens*, *Pichia fermentans*, *Saccharomyces cerevisiae*, *Kazachstania unispora*, and *Kluyveromyces marxianus*. Both bacteria and yeast were cultured at 30ºC and 5% CO_2 overnight prior to inoculation. Overnight cultures were inoculated at a starting concentration of 10^4 colony forming units (CFU)/mL of bacteria and 10^3 CFU/mL of yeast and fermentation occurred under the same conditions as grain fermentation.

Microbial Density and pH of Kefir

Following 18h of fermentation, the pH of ICK and Pitch kefir were measured using an Orion 2 star benchtop pH meter (Thermo Scientific, Ottawa, Canada), then serially diluted in PBS and plated on MRS or YEGC media to obtain the density in CFU/mL of bacteria and yeast, respectively.

3.3 Results

Composition of Isolate Collection and Selection of Acetobacter and Kluyveromyces

A total of 1,296 bacterial and 768 yeast isolates were generated and carried forward to pH and bile tolerance testing. A subset of 200 bacteria and 200 yeast were selected to undergo 16S and ITS sequencing, respectively in order to determine the general proportion of species present in the culture collection. Initial results indicated that bacterial isolates included 44% *Lactococcus*, 31% *Leuconostoc*, 24% *Lactobacillus*, and 2 *Bifidobacteria* isolates. All lactobacilli were isolated from MRS media with the pH adjusted to 5.4, while *Leuconostoc* and *Lactococcus* dominated the isolates from non-pH adjusted media. Given the lack of *Acetobacter* in the initial isolation of organisms, we set out to isolate *Acetobacter* in a more targeted matter using the previously described vancomycin and erythromycin supplemented MRS. Following growth of organisms, 3 isolates were sequenced with 100% returning an identification of *Acetobacter pasteurianus*. Initial yeast isolates included 62% *Saccharomyces*, 28% *Kazachstania*, 6% *Pichia*, and 4% *Vanderwaltozyma*. *Pichia* was only isolated from YELC media, while *Saccharomyces* and *Kazachstania* were isolated from all media and *Vanderwaltozyma* was only isolated from YEGC. As there were no *Kluyveromyces* in the initial sequencing, the *Kluyveromyces* specific primers KLOC 1 and 2 were used to first screen pools of each 24 well plate in order to identify plates that were likely to contain *Kluyveromyces*. Individual isolates from each KLOC positive plate were then tested using the KLOC primers and positive isolates underwent ITS sequencing for final identification with 100% of KLOC positive isolates being identified as *Kluyveromyces*.

Probiotic Properties of Kefir Isolates

Testing resulted in 42% of bacterial isolates (540 of 1,296) showing pH tolerance while 18% exhibited bile tolerance. A total of 80% of fungal isolates (620 of 768) exhibited both pH and bile tolerance. Following bile salt hydrolase and antimicrobial testing, no bacterial or fungal isolates exhibited bile salt hydrolase activity or antimicrobial production.

Selection of Microorganisms for Use in Pitched Culture Kefir

Isolates were selected based on BLAST results of 16S or ITS sequences for bacteria and yeast respectively. One isolate corresponding to each of *Acetobacter pasteurianus*, *Leuconostoc mesenteroides*, *Lactococcus lactis*, *Lactobacillus kefiri*, *Lactobacillus kefiranofaciens*, *Saccharomyces cerevisiae*, *Pichia fermentans*, *Kazachstania unispora*, and *Kluyveromyces marxianus* was selected for use in pitched culture kefir. All isolates used for pitched kefir were confirmed to be pH tolerant, while each of the *Lactobacillus* and yeast isolates were confirmed to be bile tolerant.

Cell Densities and pH of Pitched Kefir are Similar to a Grain Fermented Kefir

Bacterial and fungal cell densities of pitched and grain fermented kefir were compared by plating on MRS and YEGC media, respectively. In each case, there was no significant difference between the cell counts of pitched or grain fermented kefir (Figure 3.2).

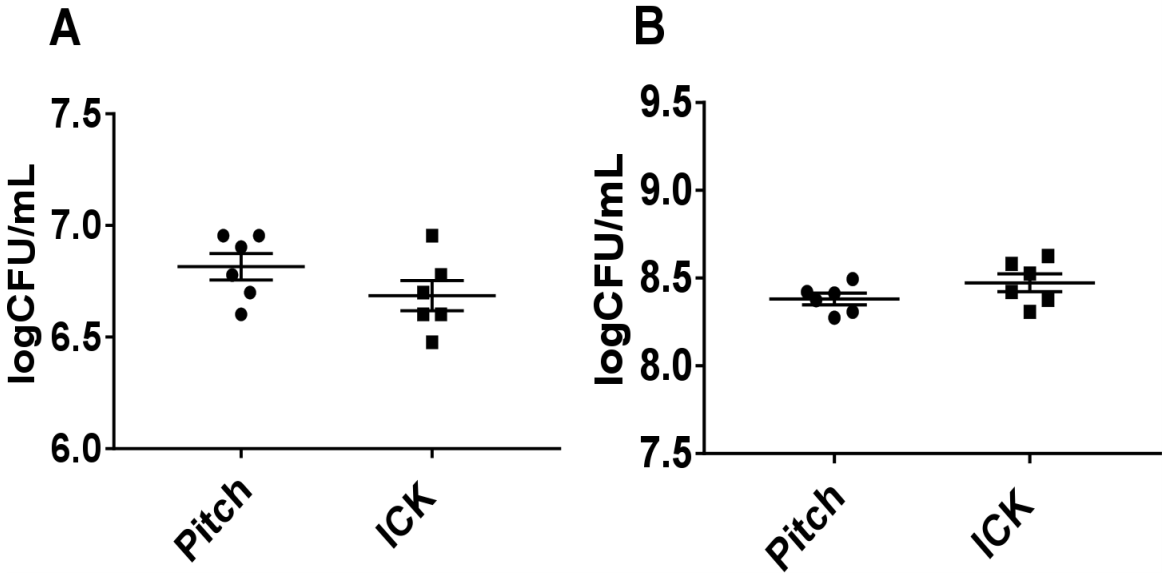

Figure 3.1. Yeast (A) and Bacterial (B) counts in both pitched kefir and grain fermented (ICK) kefir after 18h of fermentation. Data are expressed as means ± SEs (n=6). Pitch, kefir made with a defined mixture of microorganisms; ICK, kefir made with the traditional kefir grain ICK.

The pH of pitched kefir also did not differ from grain fermented kefir when measured after 18h of fermentation (Figure 3.3).

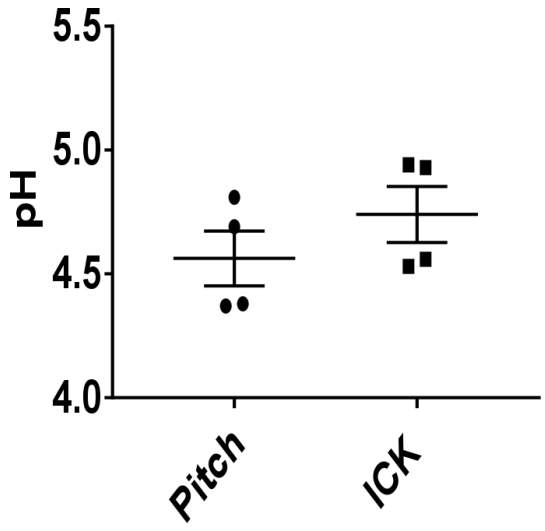

Figure 3.2. pH values of Pitch and ICK kefirs following 18h of fermentation. Data are expressed as means ± SEs (n=4). Pitch, kefir made with a defined mixture of microorganisms; ICK, kefir made with the traditional kefir grain ICK.

3.4 Discussion

This study led to the generation of a large library of kefir bacteria and yeast totaling greater than 2,000 microorganisms representing the most prevalent microbes present in traditional kefir, with over 200 bacteria and 600 yeast exhibiting both pH and bile tolerance. Further testing revealed that none of the isolates showed BSH activity, which is in contrast to other studies that have identified bile salt hydrolase activity in kefir isolates (10,11). While these results failed to identify any isolates with BSH activity, this could be due the low percentage of bacterial isolates which displayed bile tolerance. The lack of bile tolerant microorganisms may be due to the culture conditions utilized in the initial isolation, as pre-selection of microorganisms on bile containing media may have resulted in greater selection for BSH containing microorganisms. Additionally, while the vast majority of yeast isolates were bile tolerant, BSH activity has not been commonly described in yeast, although *Kluyveromyces marxianus* isolated from kefir has shown BSH activity in a previous study (10). As such, the lack of BSH activity present in the yeast component of the isolates could simply be due to rarity of BSH activity in yeast despite the previously described study.

In addition to a lack of bile salt hydrolase activity in the isolates tested, there was no bacteriocin production observed following spot plating and overlay testing which, similarly to BSH activity, is in contrast to previous studies performed (12,13). While there was no bacteriocin activity detected against the indicator strain used in this experiment, it is possible that one of the isolates produces a bacteriocin that has activity against a different strain, especially as many bacteriocins have relatively narrow spectrums of action (26). Further testing using multiple indicator strains may have revealed bacteriocin production that went undetected with the gram positive *Lactobacillus* strain used.

We were also able to generate a fermented dairy product that resembles traditional kefir in microbial density and pH is possible utilizing microorganisms isolated from traditional kefir and kefir grains. This is an important finding given that traditional kefir has been shown to better alleviate markers of metabolic dysfunction in animal models of obesity than current commercial examples (17; Chapter 2, this thesis), and that current commercial examples have failed to exhibit such health benefits in human trials (27). Future work examining the ability of this Pitch kefir to recapitulate health benefits associated with traditional kefir has the potential to significantly change how future fermented food products are developed. Another important implication of the development of the Pitch kefir is the ability to use this microbial community to study how bacteria and yeast interact in as a community during fermentation. Recent work has indicated that there are symbiotic relationships between yeast and lactic acid bacteria during fermentation (28–30), and the ability to study these microorganisms in a defined community made up of members that are traditionally found together could have major implications for our understanding of microbial interactions and how these interactions affect fermentation from both a sensory and health benefit perspective. Kefir fermentation has been shown to vary in the final composition of volatile flavour compounds and sensory characteristics (19,31,32), with Walsh *et al.* (19) establishing that increases in certain microorganisms at the beginning of fermentation can alter the levels of volatile compounds produced during fermentation. While these studies are interesting, they largely use either starters which do not use kefir microorganisms such as *L. kefiranofaciens* or *K. marxianus*, or they use cultures added to kefir grains. By allowing the removal of certain microorganisms from the fermentation matrix, there is potential for us to gain an even greater understanding of how specific constituents of the kefir microbiota interact to create health benefits.

3.5 Conclusion

This study identified over 200 bacterial and 600 yeast isolates with both pH and bile tolerance from multiple kefir sources originating from a wide variety of countries, while there was a lack of other probiotic characteristics such as BSH activity. The large number of bile and pH tolerant isolates is encouraging in regards to the ability of these isolates to survive passage through the gastrointestinal tract following feeding; whether in a kefir product or in pure culture form. Additionally, using both phenotypic and DNA based identification methods, we were able to select a group of isolates and develop a scalable method for the generation of a kefir product using traditional kefir microorganisms. The development of this kefir product is especially promising as it allows the further study of how kefir microorganisms behave and interact outside of a kefir grain and has the potential to significantly increase our understanding of how these interactions relate to the health benefits associated with kefir.

3.6 References

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Chapter 4: Microbial Composition is a Deciding Factor in the Health Benefits Conferred by Kefir

4.1 Introduction

Fermentation is one of the oldest forms of food preservation (1), with dairy being one of the most common substrates for food fermentation. Fermented dairy products, such as yogurt and kefir, have long been associated with health benefits and longevity in those who consume them (2). Of these fermented dairy products, kefir in particular has been associated with a large amount of health benefits that have been ascribed to whole kefir, kefir microorganisms, lactic acid and/or exopolysaccharides (3,4). These health benefits include serum and plasma cholesterol lowering abilities (5–7), ACE inhibitory activity (8), improved cardiac function (9), immunomodulatory characteristics (10), and an ability to improve NAFLD and obesity (5; Chapter 2, this thesis,11–15). These characteristics have led to a surge in the popularity of kefir as a functional probiotic food with the ability to improve one's health.

One overlooked factor in the health benefits associated with kefir is that significant differences exist between the microbial composition of individual examples of kefir (16,17) and that these differences impact the final flavour development and fermentation by-products present (18). We have recently shown that the impact of these microbial differences extends to the ability of kefir to improve circulating cholesterol levels and markers of NAFLD in obese mice, and that traditional kefir was better able to improve these phenotypes than a commercial example (5; Chapter 2, this thesis). This is especially important given the number of commercially available products labelled as kefir that do not contain the microorganisms described as core members of traditional kefir microbial communities (3,5; Chapter 2, this thesis). For example, commercial examples do not typically contain acetic acid bacteria, which are ubiquitous among traditional

kefirs (16–18). Additionally, while such products may contain *Leuconostoc* and *Lactococcus* similar to those found in traditional kefir, the *Lactobacillus* species contained in many commercial examples are different than those found in kefir grains and grain fermented milk. This is especially important as *Lactobacillus kefiranofaciens* and *L. kefiri*, both species unique to kefir, have been shown to have beneficial effects on host health (19–22). Kefiran, an exopolysaccharide produced by *L. kefiranofaciens*, has also proven beneficial *in vivo* (23–25). Another major difference between traditional kefir and commercial varieties is the lack of a complex yeast community in many commercial products. While some commercial kefir may contain a single species of *Saccharomyces*, traditional kefir generally contains *Saccharomyces cerevisiae*, *Pichia fermentans*, *Kazachstania unispora*, *Kluyveromyces marxianus* and *K. lactis* as well as a multitude of other yeast species at lower levels (17).

Although this thesis has shown kefir grain fermented milk to be more beneficial in improving cholesterol and lipid metabolism in mice than a commercial kefir product, the manufacturing of industrial scales of such milks using kefir grains is challenging. This is due to multiple factors such as the need to store and maintain the necessary volume of grains as well as natural variability in microbial composition, and indeed fermentation by-products, over time. It was also notable that fermented milk produced from different kefir grains differed in their abilities to improve these metabolic markers, with some performing significantly better than others (5; Chapter 2, this thesis). Due to these factors, we set out to make a kefir product better suited to commercial scale-up using bacteria and yeast isolated from a kefir grain previously shown to improve plasma cholesterol and liver triglyceride levels (5; Chapter 2, this thesis) and determine if these health benefits could be recapitulated using this pitched culture method. In order to examine how the microbial composition of kefir impacts its ability to impart health benefits, we

also made pitched culture kefir that lacked either the lactobacilli or yeast population (referred to as PNL or PNY, respectively) while containing all the other organisms present in the pitched kefir. The ability of these three pitched kefir examples to reduce weight gain, plasma cholesterol profiles, and markers of NAFLD in a mouse model of obesity was then compared to both a commercial kefir and traditional kefir made with the grain from which the pitch organisms were isolated

4.2 Methods

Kefir Grain Sourcing and Kefir Production

Kefir grains were acquired for a previous study (20) and fermentation was carried out as previously described (5; Chapter 2, this thesis). Pitched kefir was prepared by inoculating 2% milk with a mixture of microbes consisting of *Acetobacter pasteurianus*, *Lactococcus lactis*, *Leuconostoc mesenteroides*, *Lactobacillus kefiri*, *Lactobacillus kefiranofaciens*, *Pichia fermentans*, *Saccharomyces cerevisiae*, *Kazachstania unispora*, and *Kluyveromyces marxianus*. Overnight cultures were inoculated at a starting concentration of 10^4 colony forming units (CFU)/ml of bacteria and 10^3 CFU/mL of yeast. Fermentation occurred under the same conditions as grain fermentation and microbial density of the pitched kefir was 2.4±0.7 x 10^8 for bacteria, and 6.8±2.8 x 10^6 for yeast. The microbial density of ICK kefir was 3.0±1.0 x 10^8 for bacteria, and 5.2±2.2 x 10^6 for yeast. The microbial density of PNL was 1.9±1.0 x 10^8 and 7.0±2.0 x 10^6 for bacteria and yeast respectively, while the PNY kefir had a bacterial density of 2.5±0.6 x 10^8 while having zero yeast present. The commercial kefir used a microbial composition of *Lactobacillus lactis*, *Lb. rhamnosus*, *Streptococcus diacetylactis*, *Lb. plantarum*, *Lb. casei*, *Saccharomyces florentinus*, *Leuconostoc mesenteroides* subsp. *cremoris*, *Bifidobacterium longum*, *Bif. breve*, *Lb. acidophilus*, *Bif. lactis*, and *Lb. reuteri*, totaling 8.0 x 10^6 CFU/mL. The ICK kefir grain used in this study was previously sequenced by our group (17,18), and contains the major bacterial and

fungal genera *Lactobacillus*, *Acetobacter*, *Leuconostoc*, *Gluconobacter*, *Kluyveromyces*, *Kazachstania*, and *Dekkera*; along with a multitude of other low abundance genera.

Animals and Treatments

Forty 8 week old wild type C57BL/6 female mice were obtained from Jackson Labs. Mice were allocated into 5 groups (n=8) consisting of HFD + commercial kefir (COM), HFD + traditional kefir (ICK), HFD + pitched kefir, HFD + pitched kefir without the inclusion of lactobacilli, and HFD + pitched kefir without the inclusion of yeast species. Mice received a diet consisting of 40% calories from fat supplemented with 1.25% cholesterol by weight (Research Diets D12108C). Mice were housed under the same conditions as previously described (5; Chapter 2, this thesis). Kefir was mixed into the food daily at a ratio of 2mL kefir to 20g of food, which equates to approximately ¼ cup of kefir for a human on a 2000kcal per day diet. Body weights were taken weekly for the duration of the study and fecal samples were collected at day 0 and day 28. After 8 weeks, the animals were sacrificed and tissues collected, snap-frozen, and stored at -80^{o}C until further analysis. All experiments were carried out with approval from the Animal Care and Use Committee at the University of Alberta (AUP 00000671).

Quantification of Fecal Fungal Population

Fecal samples were collected after 8 weeks of HFD + kefir feeding and weighed prior to being homogenized in phosphate buffered saline (PBS). Homogenized samples were then serially diluted and plated on yeast extract glucose chloramphenicol media. Fungal colonies were counted and quantified as CFU/g feces. To determine survival of all kefir yeasts through the tract, DNA was extracted from representative colonies and ITS sequences determined to identify isolates using NCBI BLAST.

Plasma Cholesterol Measurements

Plasma was prepared and total cholesterol and high-density lipoprotein were determined as previously described (5; Chapter 2, this thesis). Non-HDL cholesterol was determined by subtracting HDL cholesterol from total cholesterol.

Liver Triglyceride Analysis

Liver lipids were extracted using a chloroform methanol extraction method and triglycerides were quantified as previously described (5; Chapter 2, this thesis).

Liver Histopathology

Liver tissue was cut and fixed in 10% neutralized formalin buffer for downstream histological analysis. All histological assessments were performed by a single investigator (CS) who was blinded to treatment. As manual measurement and counting of vacuoles can be error-prone, hepatocyte of zone 2 according to Rappaport were assessed using an operator-interactive, semi-automated method for quantification of data as previously reported (26). The parameters measured from Haemotoxylin and Eosin (H&E) stained sections were the variation of area, perimeter, and width of the vacuoles as well as the variation of their angle, circularity, and Feret, skewness and kurtosis. The Feret diameter is the longest distance between any two points along the selection boundary.

Gene Expression

Total RNA was isolated from liver tissue and gene expression analysis was carried out as previously described (5; Chapter 2, this thesis). Primers for host genes are listed in table 4.1.

Table 4.1. Specific primer sequences used for quantitative real-time PCR. GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; CD36: Cluster of differentiation 36; HMG-CoA Reductase: 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase; TNFα: Tumor necrosis factor alpha.

Target Gene	Forward (5'-3')	Reverse (5'-3')
GAPDH	ATTGTCAGCAATGCATCCTG	ATGGACTGTGGTCATGAGCC
CD36	GATCGGAACTGTGGGCTCAT	GGTTCCTTCTTCAAGGACAACTTC
PPARγ	TTGCTGAACGTGAAGCCCATCGAGG	GTCCTTGTAGATCTCCTGGAGCAG
HMG-CoA Reductase	CAGGATGCAGCACAGAATGT	CTTTGCATGCTCCTTGAACA
TNFα	CCACCACGCTCTTCTGTCTAC	AGGGTCTGGGCCATAGAACT

Microbiota Analyses

Total DNA was extracted from caecal content as previously described (27). 16S rRNA gene amplicon sequencing and data analysis was performed as previously described (5; Chapter 2, this thesis).

Statistical Analyses

Plasma cholesterol, liver triglyceride, gene expression, and histology data was analyzed using Analysis of Variance with Tukey post-hoc for multiple comparisons utilizing the R packages multcompView, ggplot2, plyr, and lmPerm. Permutational multivariate analysis of variance using distance matrices (ADONIS) was used to determine effect of treatment on microbiota while relative abundance from phylum to genus taxonomic levels were determined using the Kruskal-Wallis test. Principal-coordinate analysis (PCoA) of data derived from Bray Curtis distance matrices was performed to evaluate the overall differences between groups using the R package phyloseq.

4.3 Results

Kefir Composition Did Not Impact Weight Gain

As our group had previously observed a reduction in weight gain in HFD mice fed a traditional kefir (ICK), here we investigated the relative ability of a Pitch culture containing key ICK strains, and PNL and PNY variants thereof, to reduce weight gain in mice fed a high fat diet over an 8 week feeding period, relative to ICK and Commercial kefir controls. After 8 weeks, there was no significant difference in weight gain between any of the groups.

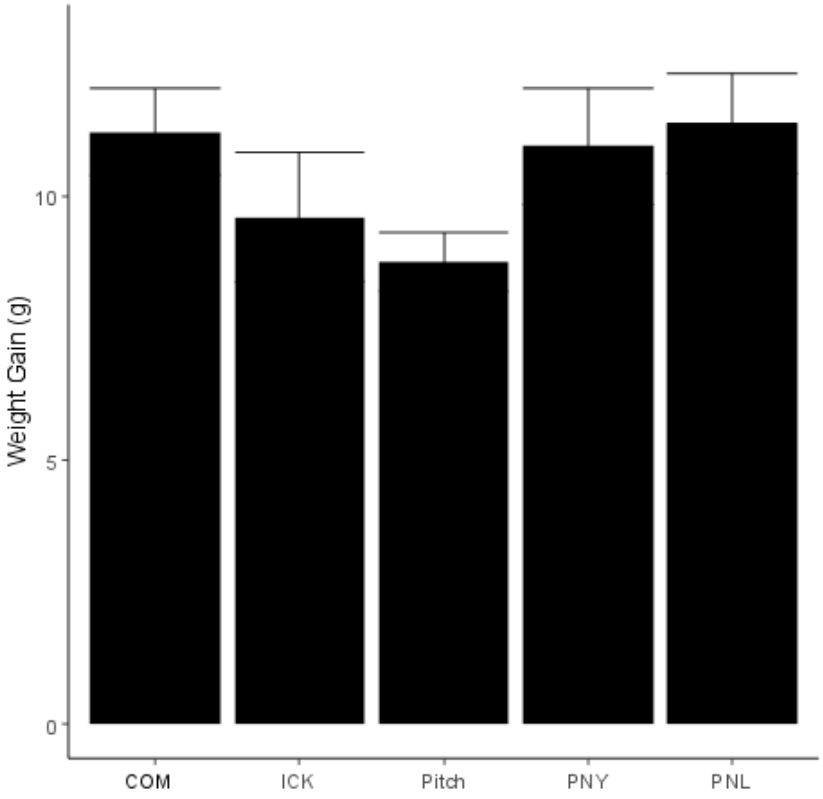

Figure 4.1. Weight gain of mice fed a high fat diet supplemented with different examples of kefir for 8 weeks. Data are expressed as means ± SEs (n=8). COM, mice fed a high fat diet supplemented with commercial kefir for 8 weeks; ICK, mice fed a high fat supplemented with traditional kefir ICK for 8 weeks; Pitch, mice fed a high fat diet supplemented with pitched culture kefir for 8 weeks; PNY, mice fed a high fat diet supplemented with pitched kefir containing no yeast population; PNL, mice fed a high fat diet supplemented with pitched kefir containing no lactobacilli.

Kefir Yeast Survived Passage through the Gastrointestinal Tract

As individual yeasts present in kefir have been associated with decreases in cholesterol (6,28), we determined whether kefir yeast were able to survive transit through the mouse gut. Following kefir feeding, the ICK, Pitch, and PNL mice had significantly higher levels of fecal fungal colonies than both the commercial and PNY fed mice, with approximately a 2 log difference being observed (Figure 4.2). Additionally, the fecal-derived colonies from each of the ICK, Pitch, and PNL-treatment groups were made up of representatives of each of the major species of yeast present in the kefir (*Pichia fermentans*, *Saccharomyces cerevisiae*, *Kazachstania unispora*, and *Kluyveromyces marxianus*), while all colonies isolated from the COM and PNY groups belonged to the genus *Rhizopus*.

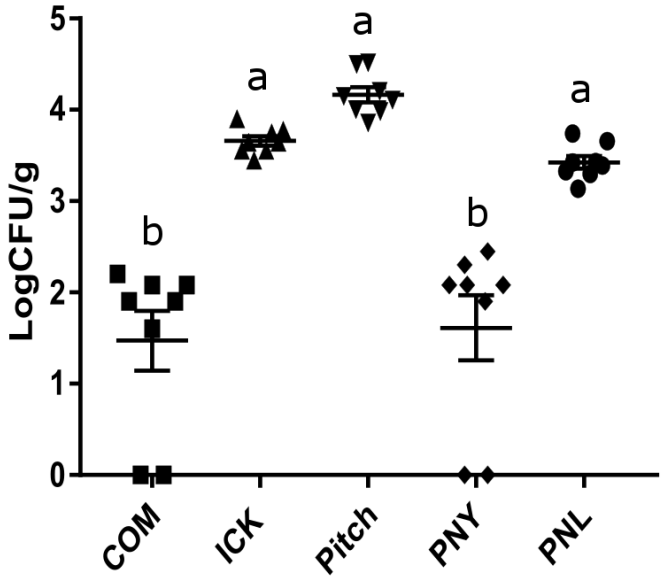

Figure 4.2. Enumeration of fungi from feces of mice fed a high fat diet supplemented with different examples of kefir for 8 weeks. Data are expressed as means ± SEs (n=8). Means that do not share a letter are significantly different (P<.05). COM, mice fed a high fat diet supplemented with commercial kefir for 8 weeks; ICK, mice fed a high fat supplemented with traditional kefir ICK for 8 weeks; Pitch, mice fed a high fat diet supplemented with pitched culture kefir for 8 weeks; PNY, mice fed a high fat diet supplemented with pitched kefir containing no yeast population; PNL, mice fed a high fat diet supplemented with pitched kefir containing no lactobacilli.

Certain Kefir Improved Plasma Cholesterol Levels and Profiles

To determine if kefir composition impacted cholesterol metabolism, total plasma cholesterol, HDL, and non-HDL cholesterol levels were analyzed, and the HDL/total cholesterol ratio was calculated. Both the ICK and Pitch groups had similar total cholesterol levels, which were lower than those observed among the COM, PNL, and PNY animals ($P<0.05$ Figure 4.3A). The same pattern between treatments was observed for plasma non-HDL cholesterol; however plasma HDL cholesterol levels were not significantly different between groups (Figure 4.3B and C). Additionally, the Pitch treated group showed improved HDL:total cholesterol ratios when compared to the COM, PNL, and PNY treated groups ($P<0.05$; Figure 4.3D).

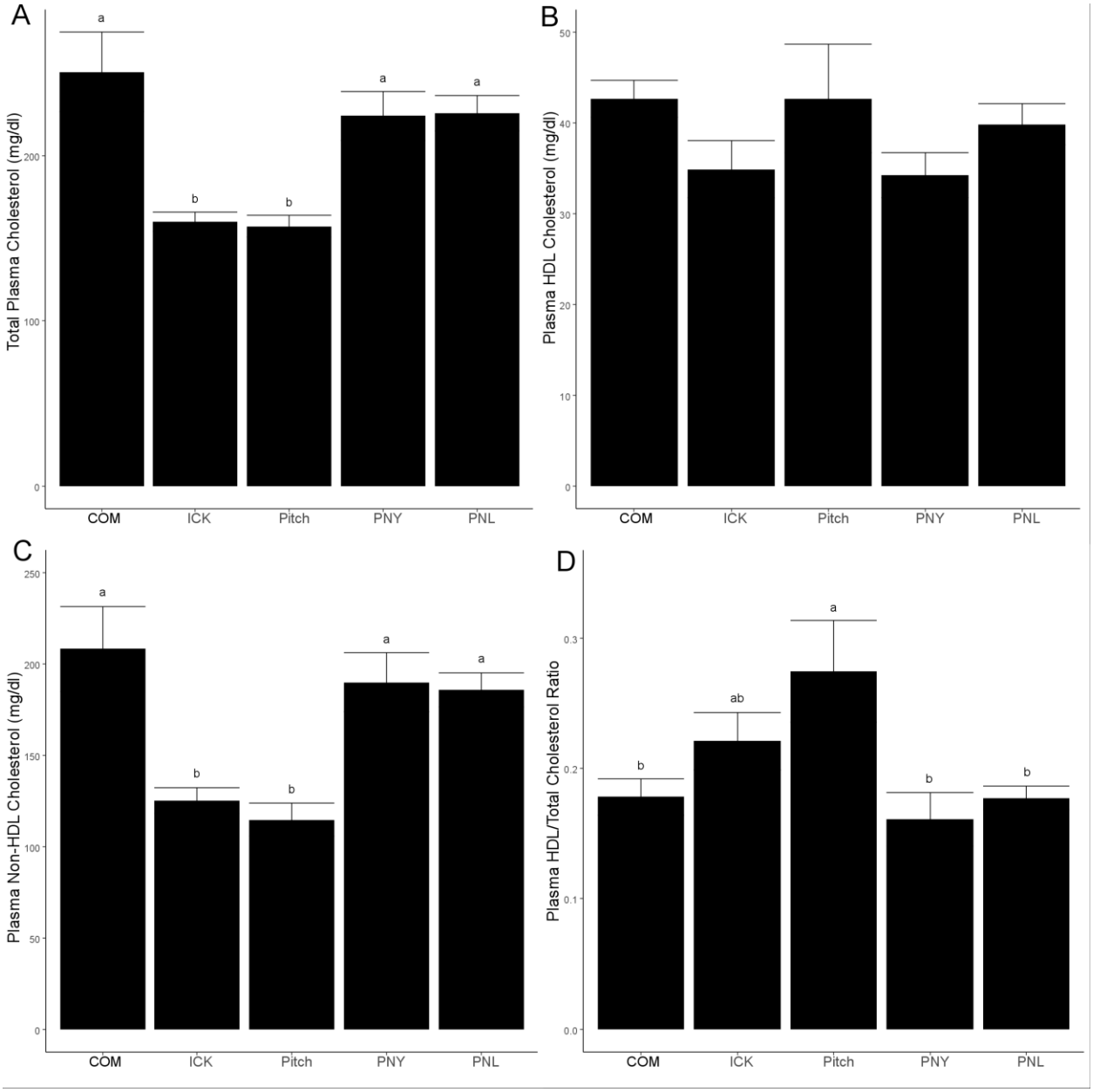

Figure 4.3. Concentration of plasma total cholesterol (A), non-HDL cholesterol (B), HDL cholesterol (C), and HDL/total cholesterol ratio (D) of mice fed a high fat diet supplemented with different examples of kefir for 8 weeks. Data are expressed as means ± SEs (n=8). Means that do not share a letter are significantly different (P<.05). COM, mice fed a high fat diet supplemented with commercial kefir for 8 weeks; ICK, mice fed a high fat supplemented with traditional kefir ICK for 8 weeks; Pitch, mice fed a high fat diet supplemented with pitched culture kefir for 8 weeks; PNY, mice fed a high fat diet supplemented with pitched kefir containing no yeast population; PNL, mice fed a high fat diet supplemented with pitched kefir containing no lactobacilli.

Traditional (ICK) and Pitched Kefir Improved Liver Triglyceride Levels

Triglycerides in the liver were measured in order to determine if kefir composition plays a role in protection against NAFLD. Both the ICK and pitched kefir groups showed significantly lower levels of triglycerides when compared to commercial kefir, PNL, and PNY fed mice (Figure 4.4).

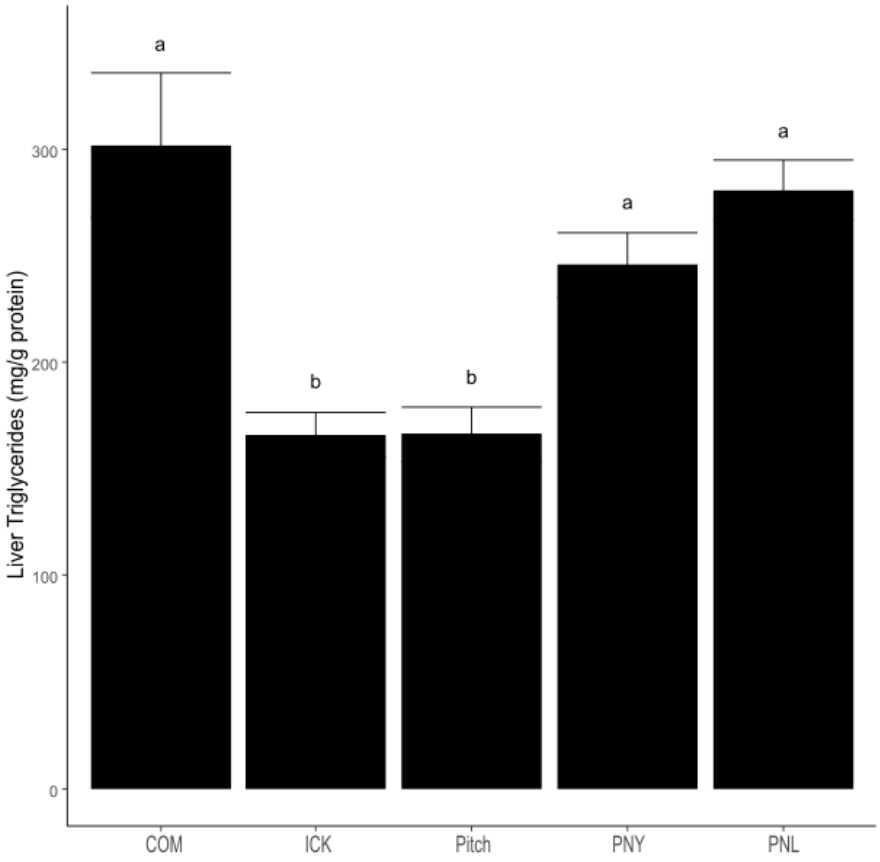

Figure 4.4. Total triglycerides in the liver of mice fed a high fat diet supplemented with different examples of kefir for 8 weeks. Data are expressed as means ± SEs (n=8). Means that do not share a letter are significantly different (P<.05). COM, mice fed a high fat diet supplemented with commercial kefir for 8 weeks; ICK, mice fed a high fat supplemented with traditional kefir ICK for 8 weeks; Pitch, mice fed a high fat diet supplemented with pitched culture kefir for 8 weeks; PNY, mice fed a high fat diet supplemented with pitched kefir containing no yeast population; PNL, mice fed a high fat diet supplemented with pitched kefir containing no lactobacilli.

Kefir Microbiota did not Impact Liver Histopathology

As increased liver triglycerides have been associated with the development of NAFLD and steatohepatitis, we examined the average size of lipid droplets in the liver and assigned histological

scores for the degree of steatohepatitis present. Average size of lipid droplets in the liver showed no significant differences between the groups (Figure 4.5A). Histopathological scoring showed no significant differences between groups (Figure 4.5B).

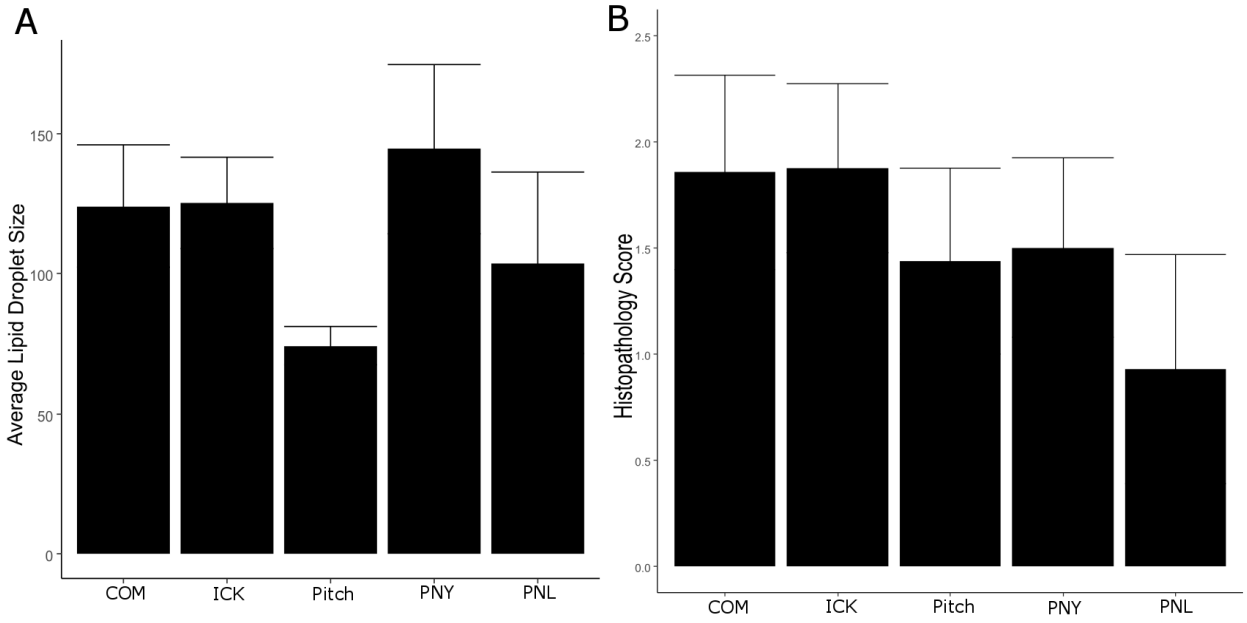

Figure 4.5. Average size of lipid droplets (A) and histopathology scores (B) of livers from mice fed a high fat diet supplemented with different examples of kefir for 8 weeks. Data are expressed as means ± SEs (n=6-8). COM, mice fed a high fat diet supplemented with commercial kefir for 8 weeks; ICK, mice fed a high fat supplemented with traditional kefir ICK for 8 weeks; Pitch, mice fed a high fat diet supplemented with pitched culture kefir for 8 weeks; PNY, mice fed a high fat diet supplemented with pitched kefir containing no yeast population; PNL, mice fed a high fat diet supplemented with pitched kefir containing no lactobacilli.

Kefir Composition is a Factor in Improving Host Lipid Metabolism but not Inflammatory markers

To determine how different kefir was able to alter circulating cholesterol levels in mice, the expression of PPARγ, CD36, and 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase were measured in the liver. PPARγ expression was significantly lower in the Pitch group when compared to the PNL and PNY (P<0.05, Figure 4.6A), while there was a trend for Pitch to be lower than the COM group. ICK did not have significantly lower expression than any of the other groups; however, there was a trend for ICK to be lower than PNL. There was also a trend for

mice fed PNY and PNL kefir to have higher expression levels of CD36 than both the ICK and Pitch kefir fed groups (Figure 4.6B). HMG-CoA reductase expression was significantly reduced in ICK mice compared to PNY and PNL, while there was a trend for expression levels in Pitch mice to be lower than both PNY and PNL (Figure 4.6C). In contrast to the alterations to expression levels of cholesterol related genes in the liver, TNFα expression was not significantly changed by any of the kefir treatments (Figure 4.6D)

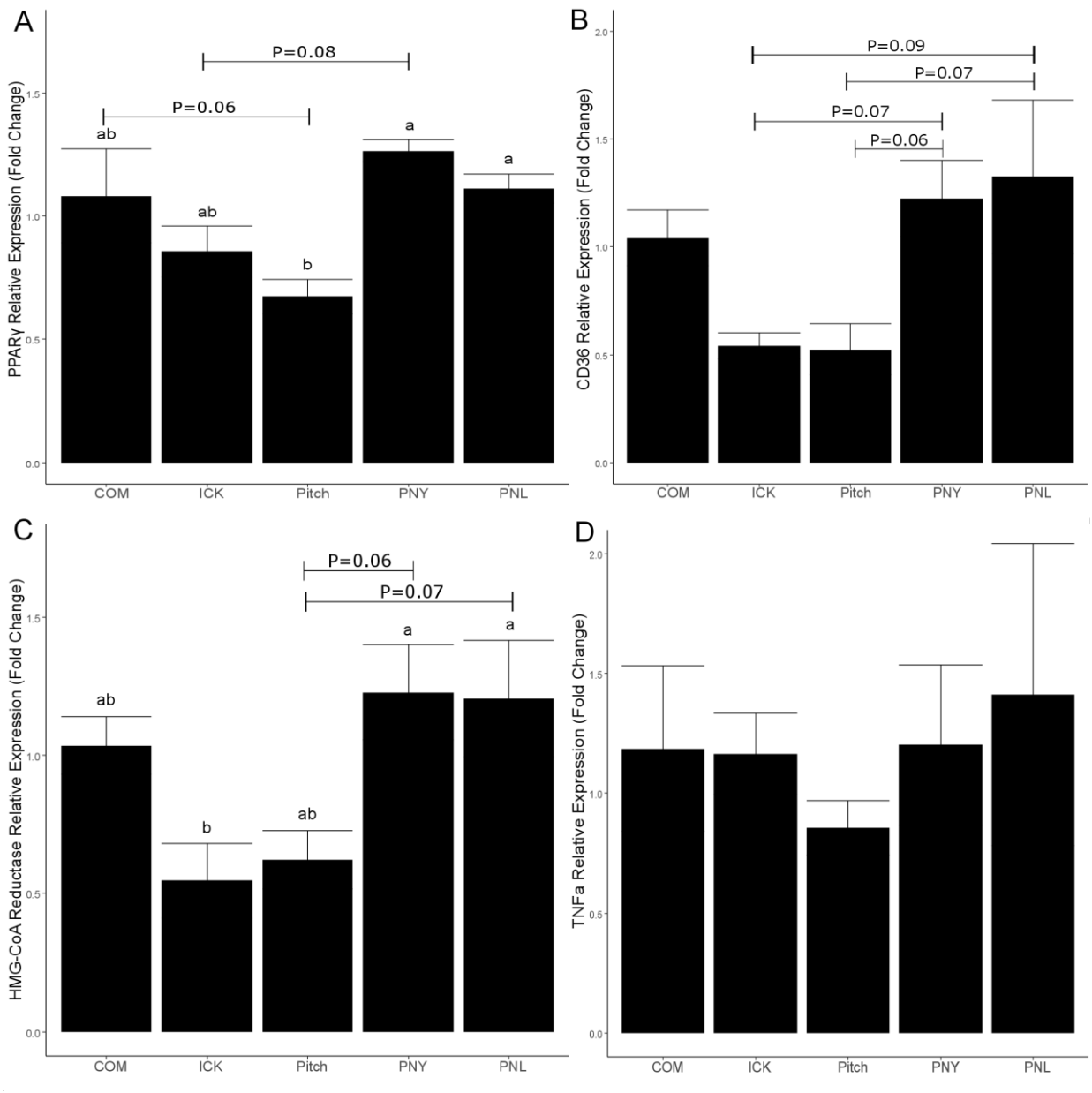

Figure 4.6. Relative expression of PPARγ (A), CD36 (B), HMG-CoA Reductase (C), and TNFα (D) in the liver of mice fed a high fat diet supplemented with different examples of kefir for 8 weeks. Data are expressed as means ± SEs (n=8). Means that do not share a letter are significantly different (P<.05). COM, mice fed a high fat diet supplemented with commercial kefir for 8 weeks; ICK, mice fed a high fat supplemented with traditional kefir ICK for 8 weeks; Pitch, mice fed a high fat diet supplemented with pitched culture kefir for 8 weeks; PNY, mice fed a high fat diet supplemented with pitched kefir containing no yeast population; PNL, mice fed a high fat diet supplemented with pitched kefir containing no lactobacilli. PPARγ, peroxisome proliferator-activated receptor gamma; CD36, cluster of differentiation 36; HMG-CoA Reductase, 3-hydroxy-3-methylglutaryl-coenzyme A reductase; TNFa, tumor necrosis factor alpha.

Analysis of the Composition of the Microbiota

As the gastrointestinal microbiota has been linked to metabolic health, and kefir is often touted as a probiotic product with beneficial effects on the gut microbiota, we set out to determine how feeding kefir to mice impacted microbiome composition. Beta-diversity of the caecal microbiota of mice fed different kefirs for 56 days was compared using a Bray Curtis distance matrix and visualized with PCoA (Figure 4.7A). ADONIS analysis showed a significant effect of treatment ($P<0.05$) and the COM group separated slightly from the rest of the kefir groups on the PCoA. This may have been due to a complete lack of the bacterial genera *Leuconostoc* in the caeca of commercial kefir fed mice while all other groups contained reads assigned to this genus, although at relatively low abundance (0.0209% to 0.00039% relative abundance). Outside of this genus, there were no other significantly different genera between groups. Alpha diversity was measured using both the Shannon and Simpson indices and was not significantly different among groups (Figure 4.7B).

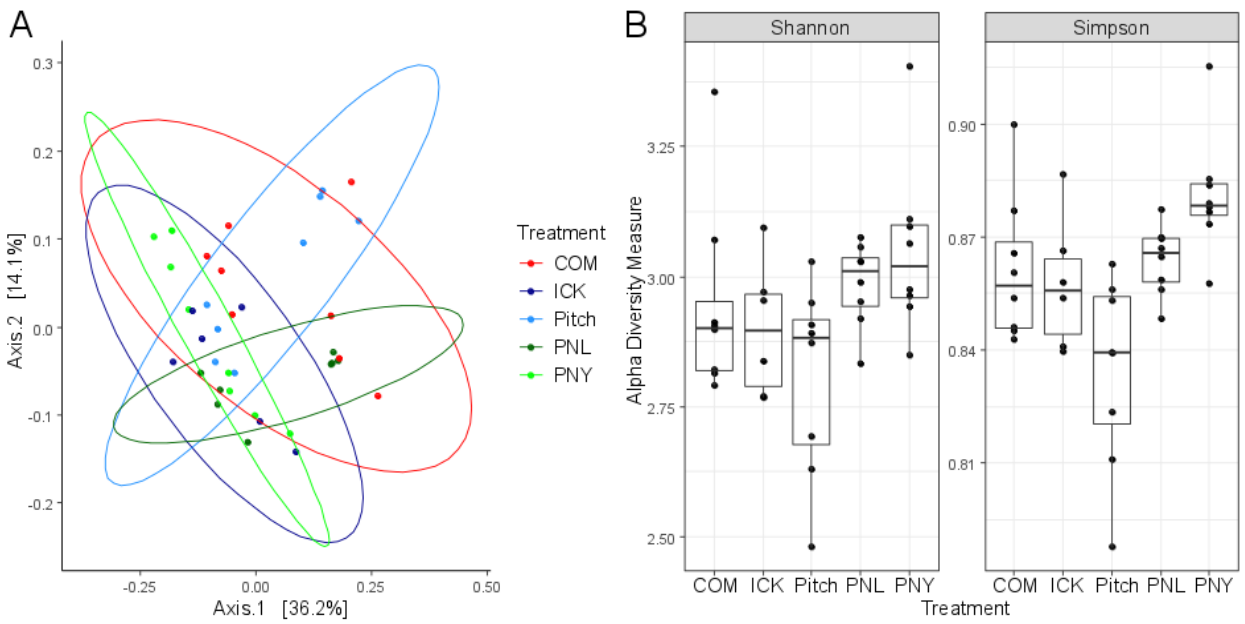

Figure 4.7. PCoA of cecal microbiota separated by Bray Curtis distance matrix (A) and Alpha diversity measures of cecal microbiota (B) of mice fed a high fat diet supplemented with different examples of kefir for 8 weeks. Data for alpha diversity is expressed as means ± SEs (n=8). COM, mice fed a high fat diet supplemented with commercial kefir for 8 weeks; ICK, mice fed a high fat supplemented with traditional kefir ICK for 8 weeks; Pitch, mice fed a high fat diet supplemented with pitched culture kefir for 8 weeks; PNY, mice fed a high fat diet supplemented with pitched kefir containing no yeast population; PNL, mice fed a high fat diet supplemented with pitched kefir containing no lactobacilli.

4.4 Discussion

This is the first study to examine how specific changes to the microbial composition of kefir fermentations impact the ability of said kefir to improve cholesterol levels and metabolism. Given the significant differences in microbial communities present in the different kefirs, we expected that there would be some variability in their ability to impact cholesterol metabolism phenotypes in the presence of a high fat/high cholesterol diet. Indeed, we found that both the traditional (ICK) and Pitch kefir fed groups had significantly lower levels of both total and non-HDL cholesterol than the commercial, PNY, and PNL groups while having unaltered levels of HDL cholesterol. This is in line with previous results from our group showing that traditional kefir can outperform a commercial example in improving cholesterol profiles in a mouse model of obesity (5; Chapter 2, this thesis) while also highlighting the importance of the microbial

composition of kefir in the health benefits associated with kefir. This is especially important as elevated circulating cholesterol levels are indicative of an increased risk of metabolic syndrome and cardiovascular disease (29). Additionally, functional food products are becoming more popular among the public as a means to improve metabolic health, and these results indicate that the microbial composition of these products needs to be taken into consideration when evaluating their health benefits.

HMG-CoA reductase is an especially important component of cholesterol homeostasis as it is the rate limiting enzyme in the biosynthesis of cholesterol. HMG-CoA reductase inhibitors have been utilized to treat hypercholesterolemia for decades (30,31). In our trial we found that the ICK group had significantly lower levels of HMG-CoA reductase expression when compared to PNY and PNL groups, while Pitch fed mice showed a trend to have lower expression levels than PNY and PNL fed mice. This pattern of HMG-CoA reductase expression was consistent with differences in plasma cholesterol. These differences in gene expression may explain the reduction in plasma cholesterol levels observed in the ICK and Pitch groups as expression levels of HMG-CoA reductase in the liver have been found to contribute to increased circulating cholesterol (32,33).

Another potential disease state associated with obesity and hyperlipidemia is NAFLD, a significant risk factor for the development of steatosis and liver cancer, which is increasing in prevalence world-wide and is threatening to reach epidemic levels (34,35). One common indicator of NAFLD and non-alcoholic steatohepatitis (NASH) is the level of triglycerides in the liver (36). We found that, concurrent with the changes observed in plasma cholesterol, mice fed ICK and Pitch kefir exhibited significantly decreased liver triglycerides when compared to mice fed Com, PNY, and PNL kefir. We examined how hepatic expression of PPARγ and CD36 were

altered by kefir feeding as both of these genes have been shown to have increased expression levels when liver triglycerides are increased (37–39). Hepatic PPARγ expression was significantly reduced in Pitch mice when compared to PNY and PNL mice and showed a trend to be lower when compared to COM mice, while ICK mice trended towards decreased expression when compared to PNY mice. CD36 expression was also altered, with both ICK and Pitch showing a trend to be lower than PNY and PNL. Additionally, HMG-CoA reductase expression has been shown to correlate with NAFLD and NASH (40), which may help to further explain the differences observed in liver triglycerides. These changes together may point to an ability of specific kefirs to alter host lipid metabolism in the liver, leading to a decrease in the hyperlipidemia commonly associated with obesity.

Interestingly, we found that when histopathological scoring or liver lipid droplet size was measured there was no difference between treatment groups. This is in contrast to our findings related to liver triglyceride levels and lipid metabolism in the liver, which pointed towards certain kefirs being protective against NASH. The lack of a correlation between liver triglyceride levels and lipid droplet size in the liver may simply be due to an increased deposition of triglycerides which are not present in large lipid droplets. Histopathological scoring showed a distinct lack of a trend and also exhibited extremely high variation. This may be due to a lack of differences observed in the expression levels of the inflammatory cytokine TNFα in the liver as TNFα has been shown to be important in the development of NASH (41,42). Additionally, recent work has shown the importance of IL-1β and the activation of the NLRP3 inflammasome in the development of steatohepatitis in mice (43,44), while previous work from our group failed to find any differences in intestinal expression of the NLRP3 inflammasome markers IL-1β or

IL-18 in mice fed kefir on a high fat diet (5). This lack of an anti-inflammatory effect of kefir may explain the similarities in histopathology scoring between the treatment groups.

Recently, it has been shown that the gastrointestinal microbiota plays an important part in the development of obesity associated metabolic disorders (45–47). Given this, and the fact that kefir is generally regarded as a health promoting beverage with beneficial effects on the gut, we examined the bacterial composition of the cecal microbiota following 8 weeks of HFD feeding supplemented with kefir. Although there was a significant effect of treatment in the ADONIS, there was minimal separation of groups on the PCoA, with the commercial group clustering somewhat separately from the grain fermented and pitched culture kefir fed groups. This was likely due to the lack of detection of *Leuconostoc* in the gut of commercial mice while each of the other groups contained this genus. Data from the present study suggests that the *Leuconostoc* strain present in this commercial product is less adept at surviving passage through the gastrointestinal tract than those contained in the freshly fermented kefir. It was also notable that, despite containing *Saccharomyces florentinus*, the mice fed commercial kefir presented significantly lower levels of fecal fungal colonies when compared to mice fed ICK, Pitch, and PNL kefir. Furthermore, there were no *S. florentinus* identified among the yeast isolated from mice fed commercial kefir. The lack of dramatic differences in the gut microbiota of the different groups, as assessed via 16S taxonomic sequencing, suggested that the mechanism of action of kefir is not tied to large scale microbial changes in the gut and is instead dependent on more subtle changes in composition, changes to the microbiome on a functional metabolic level or fermentation products present in the kefir acting directly on the host.

As previously stated, one possible reason for the differential effects of the different kefirs used in this study is a difference in the fermentation products generated during the fermentation

of each individual kefir. Interestingly, the removal of both yeast and lactobacilli from the pitched kefir fermentation resulted in the loss of a beneficial impact on cholesterol metabolism. This may point to a relationship between the yeast component and *Lactobacilli* present in the kefir fermentation which results in the production of a metabolite that is unable to be produced when one group is missing. This hypothesis is supported by recent work identifying interactions between lactobacilli and *Saccharomyces* species in various fermentations (48–50) with some of these interactions being shown to be strain dependent. In fact, metabolic by-products of kefir fermentation, such as small peptides and the exopolysaccharide kefiran, have been identified to have potentially positive effects on cholesterol metabolism (11,51–54). These studies indicate that there is a possibility that the absence or lowering of a specific metabolic by-product or products could result in a drastically different impact on the host, once again highlighting the importance of microbial composition in the ability of kefir to benefit host health. Furthermore, the fact that similar fecal yeast counts were obtained for mice fed Pitch and PNL kefir suggests that the yeast survival in the tract may not be important to the mechanism. This is in contrast to previous studies which have shown that kefir yeast can lower plasma cholesterol in animal models (6,28); however, these studies utilized pure cultures or cell components of yeast which may explain these differences.

This study expands on previous work showing that kefir is able to improve cholesterol metabolism, and that traditional kefir is more capable of improving metabolic health than certain commercial examples (5,12,55); however, this is the first study to our knowledge to examine how specific alterations to the microbial composition of kefir impacts host health and lipid metabolism in an *in vivo* model of diet induced obesity. Both the grain fermented ICK kefir and a lab produced commercial process kefir (Pitch) utilizing organisms isolated from ICK lowered

plasma total cholesterol, non-HDL cholesterol, and liver triglyceride levels when compared to a widely available commercial kefir as well as lab produced kefir that lacked either the lactobacilli or yeast population. These greater impacts were likely due to an alteration of the host cholesterol and lipid metabolism in the liver based on observed changes to gene expression profiles. Our results show that, although many commercial kefirs have microbes of the same genera as those present in traditional kefir, the exact species and perhaps even strain of these species may be essential to the health benefits observed in previous studies utilizing traditionally fermented kefir. This is not surprising given the importance of species and strain level differences in other fermented foods (56). We also showed that the health benefits of traditional kefir can be recapitulated utilizing traditional kefir organisms in a process of producing kefir using pitched cultures, indicating a potential important consideration in the future development of large scale kefir production. Additionally, this study highlights the importance of microbial composition and interactions in functional fermented foods and indicates that a failure to accurately replicate or retain key microbes present in such foods can have detrimental effects on the ability of said functional food to exert a positive influence on the host. Future work should focus on identifying the specific species necessary to achieve the health benefits observed while also examining how microbial interactions during fermentation may play a role in these benefits.

4.5 Conclusion

This study has shown that traditional and pitched culture kefir are capable of improving hyperlipidemia associated with diet induced obesity. Importantly, we identified that the health benefits of traditional grain fermented kefir can be recapitulated in a commercial process pitched culture kefir by using microbes that make up the majority of the traditional kefir microbiota. Additionally, the microbial composition of the kefir fermentation is an essential component of

the ability of kefir to exert positive influence over the host's metabolism, with both lactobacilli and yeast populations identified as being necessary to produce these benefits. These results should be strongly considered in the development of future commercial kefir products and any other functional products that wish to mimic a traditional fermented food product.

4.6 References

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Chapter 5: Metabolomics of Kefir Fermentation

5.1 Introduction

While traditional kefir has been shown to improve markers of metabolic dysfunction such as plasma cholesterol and liver triglycerides in mouse models of obesity (1–3), the inability of commercial kefir to improve these markers may prove problematic for individuals who wish to take advantage of these benefits (1,4). As we have previously shown in chapter 4, a pitched kefir product-utilizing organisms isolated from traditional kefir was able to recapitulate the health benefits of traditional kefir while also maintaining a scalable manufacturing process. Additionally, we were able to show that upon removal of the lactobacilli or yeast populations from the fermentation, the associated benefits were no longer present. These findings allow us a unique opportunity to examine the differences present in these fermentations in order to determine how alterations to the kefir microbiota impact the health benefits of kefir.

Kefir metabolites have been shown to improve cholesterol and lipid metabolism when administered separately from the organisms that produce them. For instance, kefir peptides have been shown to have ACE inhibitory activity, as well as improve cholesterol and liver lipid profiles in animal models of obesity (5–7), while the exopolysaccharide kefiran has improved cholesterol metabolism and atherosclerosis, as well as demonstrating bifidogenic effects in mice, and antimicrobial activity *in vitro* (8–11). Additionally, lactic acid has recently been shown to exert immunomodulatory benefits on the host, including lowering proinflammatory cytokine levels and reinforcing intestinal barrier function (12,13). Given these impacts on the host, it is possible that other metabolites, such as organic acids, may play a greater role in the health benefits associated with fermented foods than previously thought.

In addition to potential health benefits of metabolites present in kefir fermentations, these compounds are also major components of the aroma and taste of the finished product. Due to the complex microbial make-up of kefir, the composition of the final product is extremely complex and can vary greatly from one fermentation to another due to differences in the microbial composition of different kefir grains (14,15). There have been multiple studies analyzing organic acids and flavour compounds present in kefir fermentations; however, few have examined how differences in the microbial composition of the fermentation impact metabolites, and there are none to our knowledge that have utilized kefir with demonstrated differences in their ability to benefit the host.

This study sought to examine how metabolite profile was impacted by varying the starting composition of bacteria and yeast present in kefir fermentation. Of particular interest was how the removal of the lactobacilli (PNL) or yeast (PNY) population from the Pitch kefir impacted these profiles, and whether there are any specific metabolic differences that might help explain the results presented in Chapter 4. Additionally, we hoped to determine how the composition of metabolites present in our Pitch kefir when compared to a traditional, grain fermented example (ICK). The similarity of this Pitch kefir to the ICK is especially important with regards to potential commercialization of this product, as palatability is especially important to consumers and has been shown to be varied between different kefir in the past (14). In order to obtain a view of how kefir fermentation progresses, we sampled fermentations after both 12 and 18 hours and utilized two-dimensional gas chromatography-time-of-flight mass spectrometry (GC×GC-TOFMS) in order to characterize metabolite profiles over the course of fermentation.

5.2 Materials and Methods

Kefir Production

Kefir production was performed as previously described in Chapter 4 for each of the ICK, Pitch, PNL, and PNY kefirs. Fermentations were performed in triplicate, and 5mL samples were taken after 12 and 18 hours of fermentation and stored at -20ºC until GCxGC-TOFMS analysis.

Metabolite Analysis

Kefir samples were profiled using headspace solid-phase microextraction (SPME) for sample preparation. Samples were analyzed by comprehensive two-dimensional gas chromatography-time-of-flight mass spectrometry (GC×GC-TOFMS). Approximately 0.5 g kefir samples were provided in headspace vials for analysis. The tubes were kept on ice until 2 minutes before incubation. The tube was incubated at 75 °C for 5 min, followed by 20 minutes of extraction using a Divinylbenzene/Carboxen/Polydimethylsiloxane (DVB/CAR/PDMS) fibre. Samples were desorbed for 3 minutes into the injector of the GC. Chromatograms were processed using a data processing method to find all peaks with S/N>100. Statistical Compare tools in ChromaTOF® were used to align the peaks from all samples based on retention times and mass spectra. Compounds were manually identified following alignment using a similarity threshold of 600 and verified through comparison of retention indices. Comparisons of fermentation types at 18 hours, and comparisons within fermentation at 12 and 18 hours were performed using Metaboanalyst. Briefly, normalized data was range scaled, and a t-test performed. Volcano plots were generated for all compounds with a false discovery rate (FDR)-adjusted p-value < 0.10. Intensity data was analyzed using Analysis of Variance with Tukey post-hoc for multiple comparisons utilizing the R packages multcompView, ggplot2, plyr, and lmPerm. Effect of fermentation type on metabolite composition was determined using ADONIS.

5.3 Results

Kefir Fermentations Cluster by Fermentation Time and Microbial Composition

Principal component analysis (PCA) of the samples from both 12 and 18 hours did not show a significant level of clustering among the ICK, Pitch, PNY, and PNL kefir after 12 hours of fermentation; however, there did appear to be some separation of the samples by 18 hours (ADONIS = 0.001, Figure 5.1A). There was also clear separation of the 12 and 18 hour samples of all groups along PC2. Among the groups, the 18 hour Pitch kefir samples clustered significantly more tightly than every other group, potentially indicating lower levels of variation between samples. Interestingly, the Pitch kefir was no more similar to ICK kefir than the PNY and PNL kefirs. PCA analysis of only the 18 hour samples revealed a significant effect of kefir composition (ADONIS=0.003, Figure 5.1B), continuing the trend of the Pitch kefir being no more similar to ICK than PNY and PNL fermentations. Among the pitched culture kefirs, PNL seemed to cluster distinctly, while PNY and Pitch less distinctly separated.

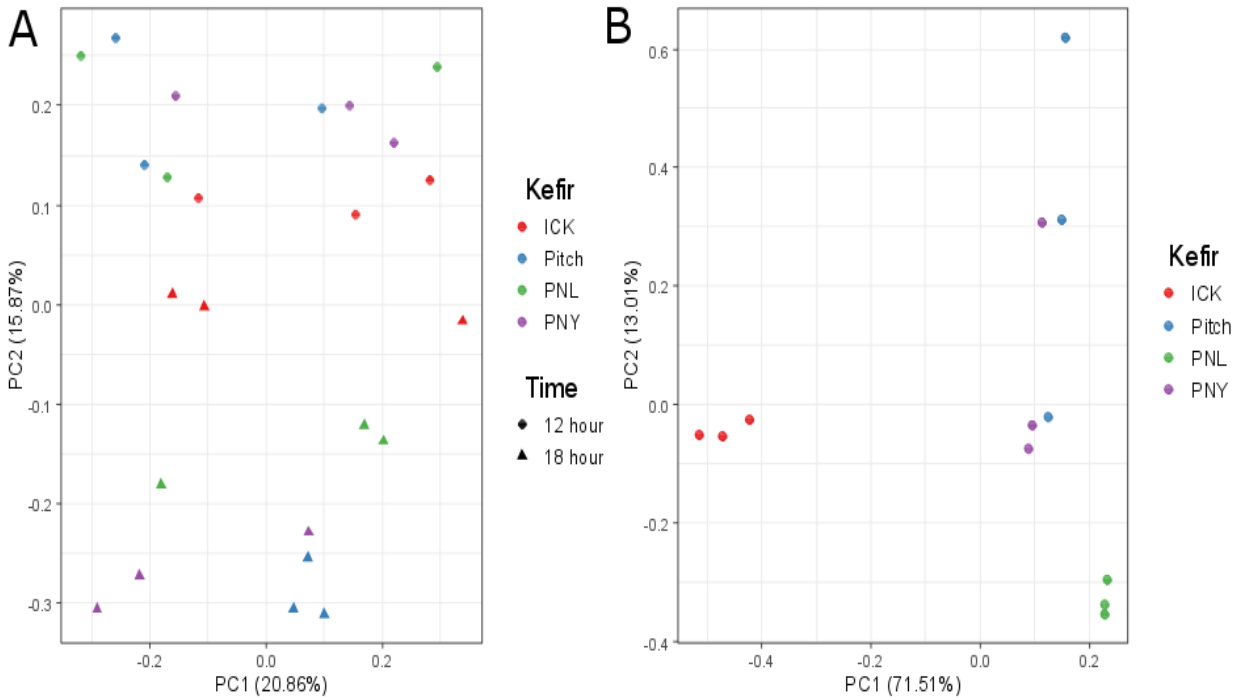
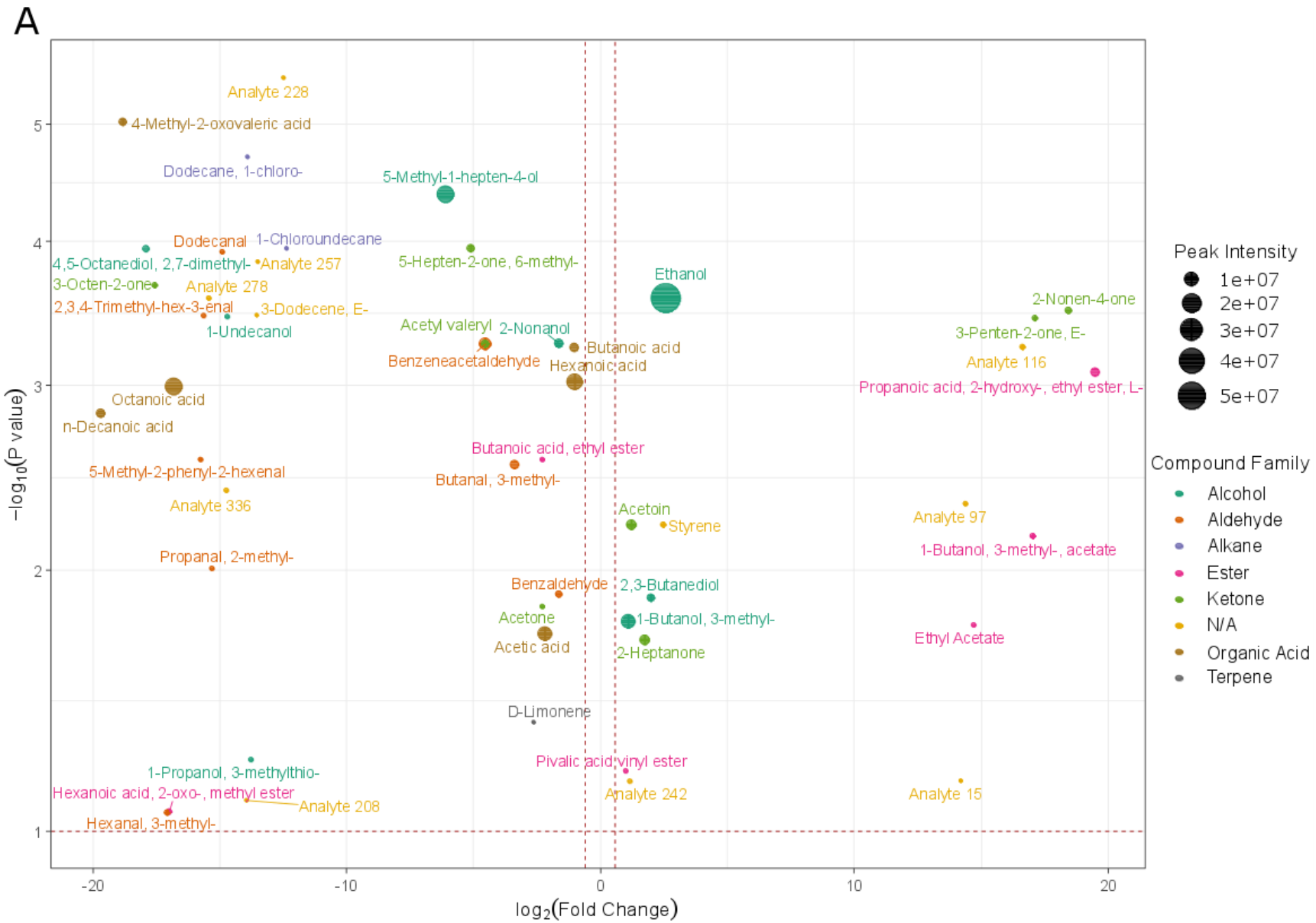


Figure 5.1 PCA of volatile compound profiles in different kefir fermentations after 12 and 18 hours of fermentation (A) and after 18 hours of fermentation only (B). Each symbol represents one kefir fermentation sample. ICK, kefir fermented with the kefir grain ICK; Pitch, kefir fermented with a defined mixture of microorganisms; PNL, kefir fermented with a defined mixture of microorganisms that lacks lactobacilli; PNY, kefir fermented with a defined mixture of microorganisms that lacks yeast.

Lab Generated Pitched Culture Kefirs Differ Significantly from Traditional ICK Kefir

A total of 51 compounds were present with significantly different intensities between the ICK and Pitch kefir following 18 hours of fermentation (Figure 5.2). ICK had significant increases in the concentration of many esters, such as ethyl acetate, as well as ethanol, which were a major component of the fermentation as measured by peak intensity. Conversely, the Pitch kefir had significantly higher concentrations of organic acids and aldehydes; including butanoic acid, acetic acid, and benzaldehyde.


A
Analyte 228
4-Methyl-2-oxovaleric acid
Dodecane, 1-chloro-
5-Methyl-1-hepten-4-ol
Dodecanal
1-Chloroundecane
4,5-Octanediol, 2,7-dimethyl-
Analyte 257
5-Hepten-2-one, 6-methyl-
3-Octen-2-one
Analyte 278
Ethanol
2,3,4-Trimethyl-hex-3-enal
3-Dodecene, E-
1-Undecanol
Acetyl valeryl
2-Nonanol
Butanoic acid
Benzeneacetaldehyde
Hexanoic acid
Octanoic acid
n-Decanoic acid
Butanoic acid, ethyl ester
5-Methyl-2-phenyl-2-hexenal
Butanal, 3-methyl-
Analyte 336
Acetoin
Styrene
Propanal, 2-methyl-
Benzaldehyde
2,3-Butanediol
Acetone
1-Butanol, 3-methyl-
Acetic acid
2-Heptanone
D-Limonene
1-Propanol, 3-methylthio-
Pivalic acid, vinyl ester
Hexanoic acid, 2-oxo-, methyl ester
Analyte 208
Analyte 242
Hexanal, 3-methyl-
2-Nonen-4-one
3-Penten-2-one, E-
Analyte 116
Propanoic acid, 2-hydroxy-, ethyl ester, L-
Analyte 97
1-Butanol, 3-methyl-, acetate
Ethyl Acetate
Analyte 15
−log10(P value)
log2(Fold Change)
Peak Intensity
1e+07
2e+07
3e+07
4e+07
5e+07
Compound Family
Alcohol
Aldehyde
Alkane
Ester
Ketone
N/A
Organic Acid
Terpene

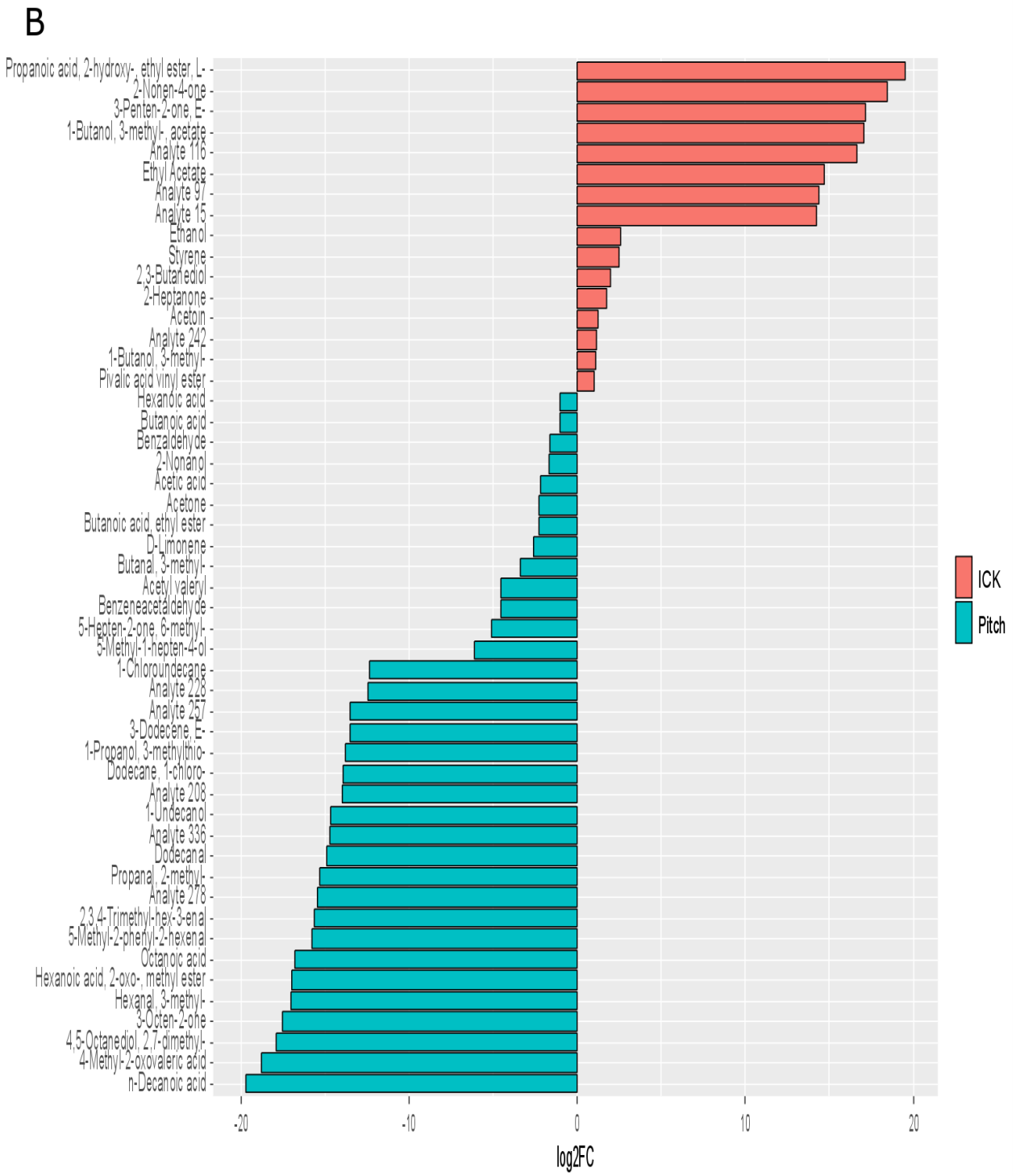
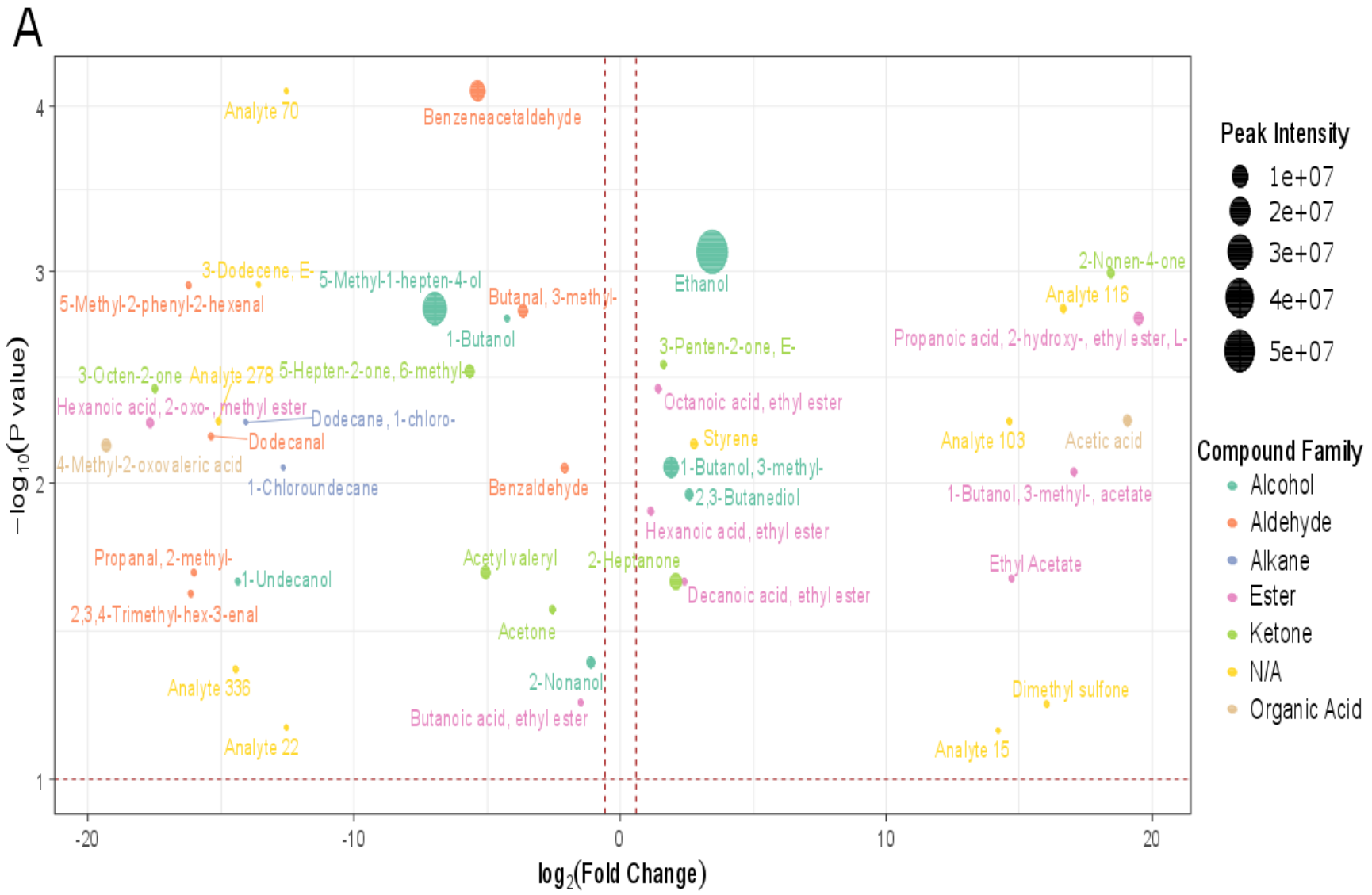


Figure 5.2. Volcano plot (A) and LEfSe plot (B) of compounds with significant differences in peak intensity between ICK and Pitch kefirs following 18 hours of fermentation (n=3). ICK, kefir fermented with the kefir grain ICK; Pitch, kefir fermented with a defined mixture of microorganisms. Compounds with a negative fold change had increased peak intensities in Pitch kefir while those with positive fold changes had increased peak intensities in ICK kefir.

Comparison of ICK and PNL kefirs showed a total of 43 compounds with significantly different intensities between the two fermentations (Figure 5.3). Similarly to the ICK and Pitch comparison, there was a general increase in esters as well as ethanol in the ICK kefir, while PNL had significantly higher levels of esters. However, in contrast to Pitch kefir, PNL did not have higher levels of organic acids than ICK; in fact, ICK had increased levels of acetic acid when compared to PNL. Similarly to both Pitch and PNL, the comparison of ICK and PNY kefir revealed a total of 40 compounds that were significantly different between the two (figure 5.4). PNY and PNL showed very similar profiles in the compounds that were different to those found in ICK; however, there were slightly higher levels of significance in the compounds that were different.


A
Analyte 70
Benzeneacetaldehyde
3-Dodecene, E-
5-Methyl-1-hepten-4-ol
5-Methyl-2-phenyl-2-hexenal
Butanal, 3-methyl-
1-Butanol
Ethanol
2-Nonen-4-one
Analyte 116
Propanoic acid, 2-hydroxy-, ethyl ester, L-
3-Penten-2-one, E-
3-Octen-2-one
Analyte 278
5-Hepten-2-one, 6-methyl-
Octanoic acid, ethyl ester
Hexanoic acid, 2-oxo-, methyl ester
Dodecane, 1-chloro-
Dodecanal
Styrene
Analyte 103
Acetic acid
4-Methyl-2-oxovaleric acid
1-Chloroundecane
Benzaldehyde
1-Butanol, 3-methyl-
2,3-Butanediol
1-Butanol, 3-methyl-, acetate
Hexanoic acid, ethyl ester
Propanal, 2-methyl-
Acetyl valeryl
2-Heptanone
Ethyl Acetate
1-Undecanol
Decanoic acid, ethyl ester
2,3,4-Trimethyl-hex-3-enal
Acetone
Analyte 336
2-Nonanol
Dimethyl sulfone
Butanoic acid, ethyl ester
Analyte 22
Analyte 15
4
3
2
1
-20
-10
0
10
20
−log10(P value)
log2(Fold Change)
Peak Intensity
1e+07
2e+07
3e+07
4e+07
5e+07
Compound Family
Alcohol
Aldehyde
Alkane
Ester
Ketone
N/A
Organic Acid

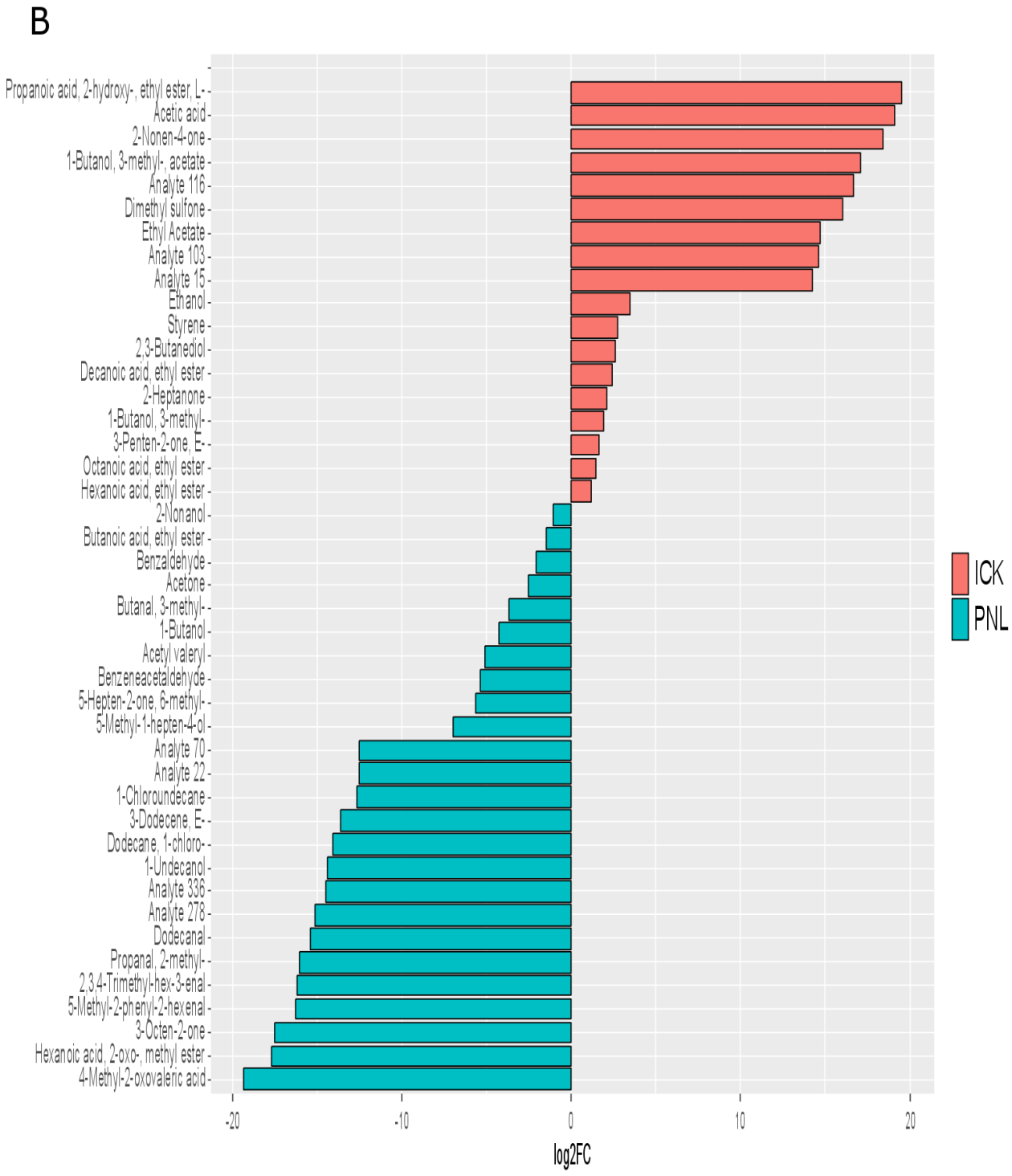
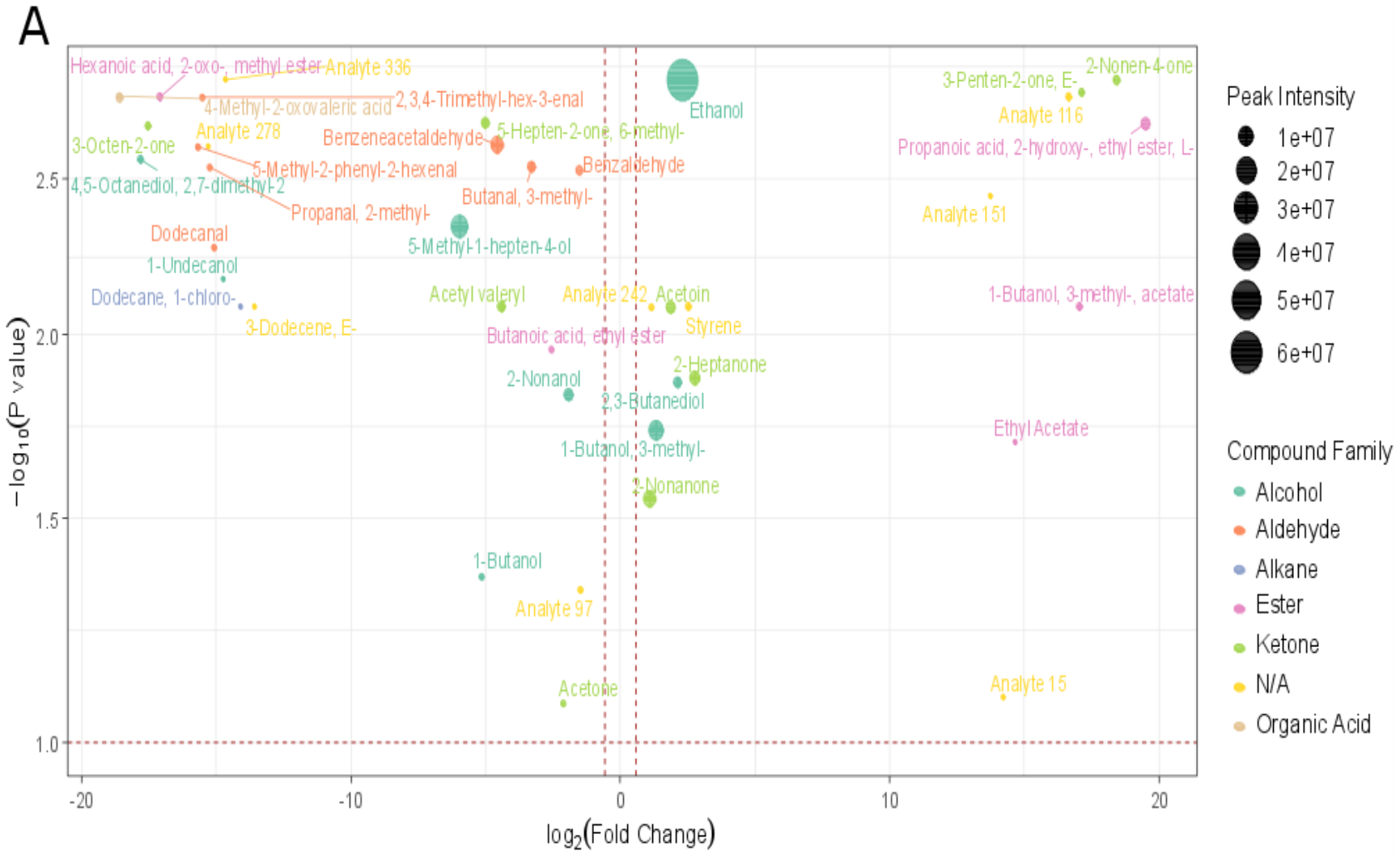


Figure 5.3. Volcano plot (A) and LEfSe plot (B) of compounds with significant differences in peak intensity between ICK and PNL kefirs following 18 hours of fermentation (n=3). ICK, kefir fermented with the kefir grain ICK; PNL, kefir fermented with a defined mixture of microorganisms that lacks lactobacilli. Compounds with a negative fold change had increased peak intensities in PNL kefir while those with positive fold changes had increased peak intensities in ICK kefir.


A
Hexanoic acid, 2-oxo-, methyl ester
Analyte 336
4-Methyl-2-oxovaleric acid
2,3,4-Trimethyl-hex-3-enal
Ethanol
2-Nonen-4-one
3-Penten-2-one, E-
Analyte 116
Analyte 278
Benzeneacetaldehyde
5-Hepten-2-one, 6-methyl-
Propanoic acid, 2-hydroxy-, ethyl ester, L-
3-Octen-2-one
5-Methyl-2-phenyl-2-hexenal
Benzaldehyde
4,5-Octanediol, 2,7-dimethyl-2
Butanal, 3-methyl-
Analyte 151
Propanal, 2-methyl-
Dodecanal
5-Methyl-1-hepten-4-ol
1-Undecanol
Dodecane, 1-chloro-
Acetyl valeryl
Analyte 242
Acetoin
1-Butanol, 3-methyl-, acetate
3-Dodecene, E-
Styrene
Butanoic acid, ethyl ester
2-Heptanone
2-Nonanol
2,3-Butanediol
Ethyl Acetate
1-Butanol, 3-methyl-
2-Nonanone
1-Butanol
Analyte 97
Acetone
Analyte 15
2.5
2.0
1.5
1.0
−log10(P value)
-20
-10
0
10
20
log2(Fold Change)
Peak Intensity
1e+07
2e+07
3e+07
4e+07
5e+07
6e+07
Compound Family
Alcohol
Aldehyde
Alkane
Ester
Ketone
N/A
Organic Acid

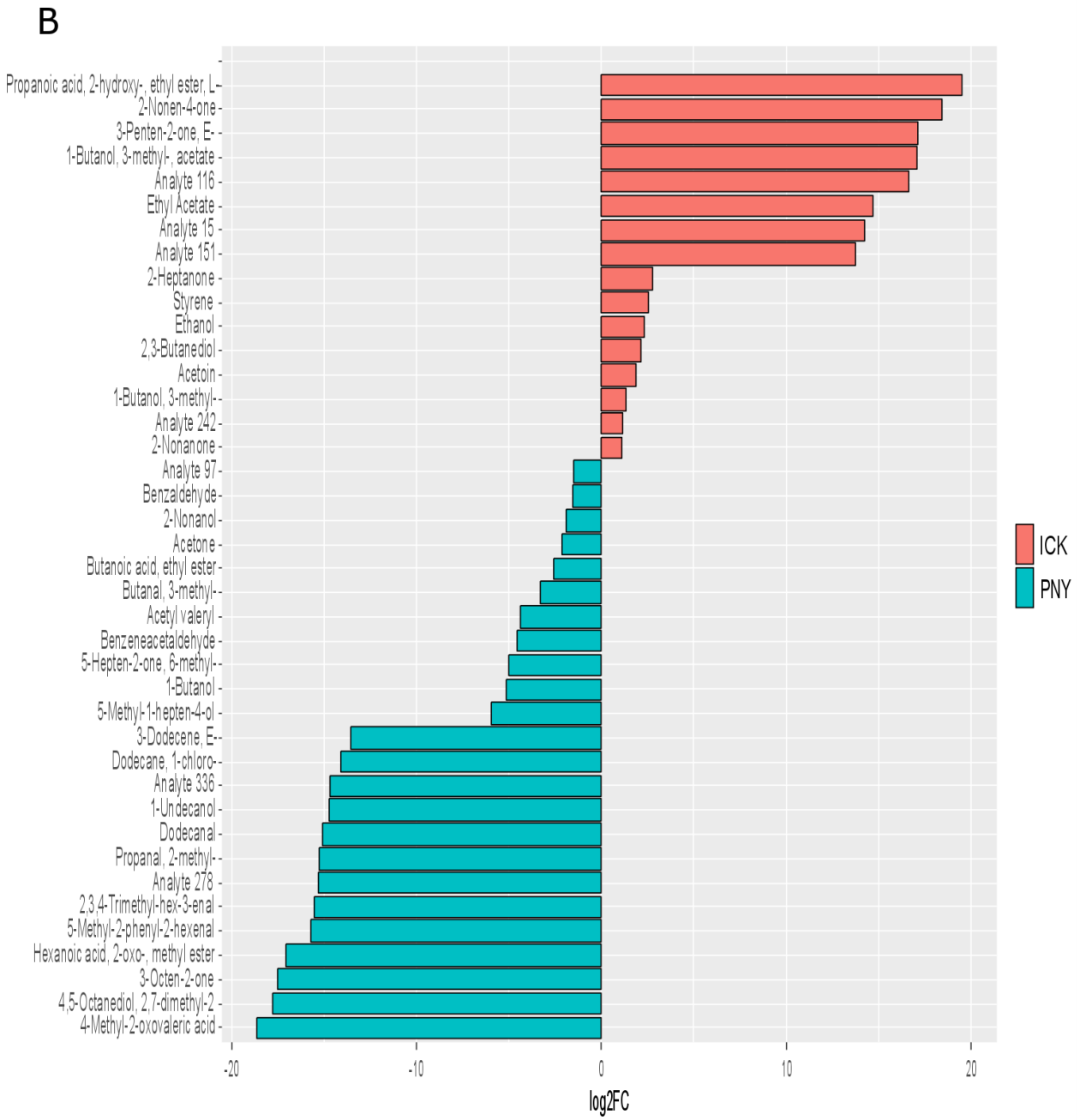
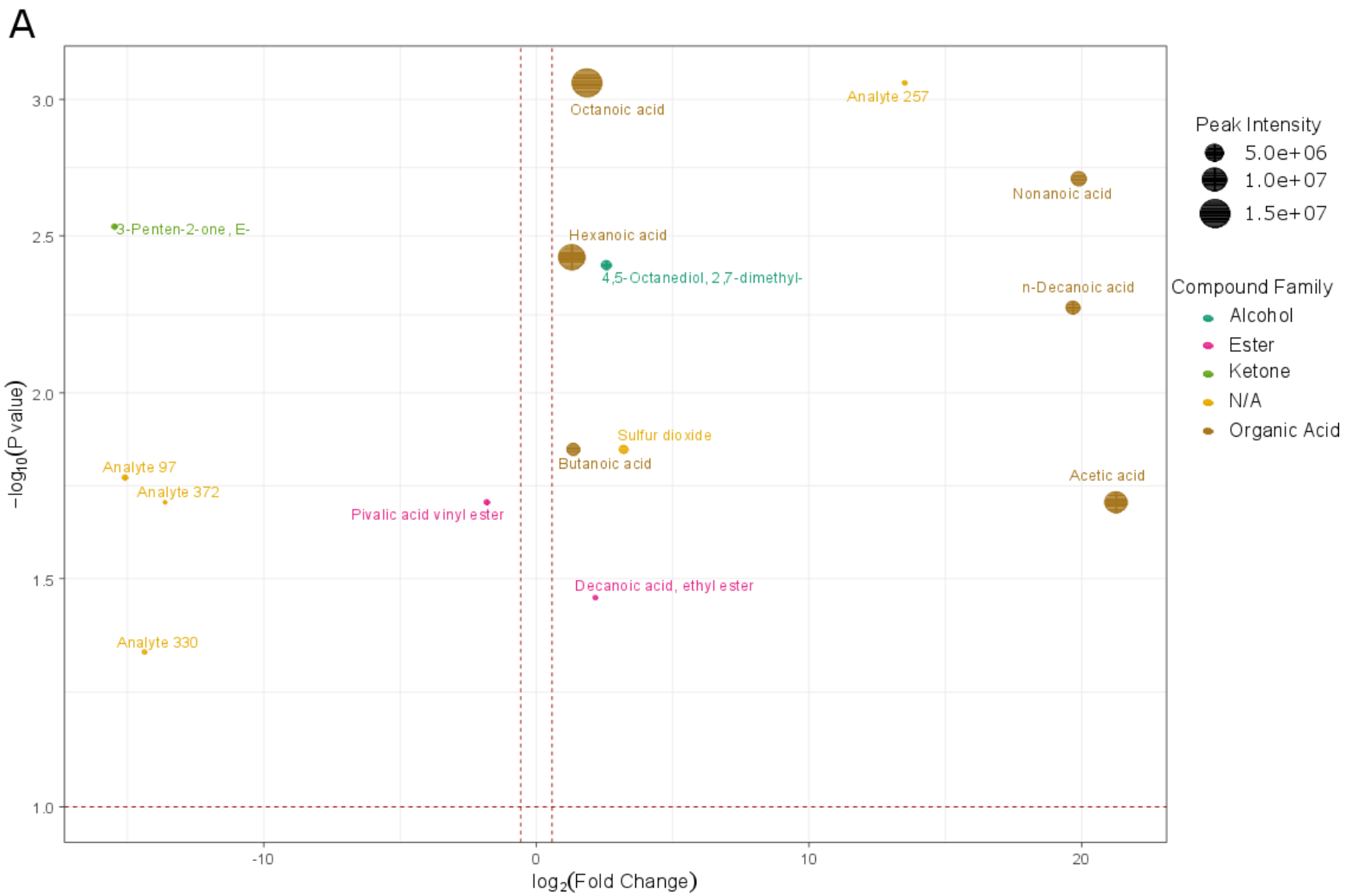


Figure 5.4. Volcano plot (A) and LEfSe plot (B) of compounds with significant differences in peak intensity between ICK and PNY kefirs following 18 hours of fermentation (n=3). ICK, kefir fermented with the kefir grain ICK; PNY, kefir fermented with a defined mixture of microorganisms that lacks yeast. Compounds with a negative fold change had increased peak intensities in PNY kefir while those with positive fold changes had increased peak intensities in ICK kefir.

Pitched Culture Kefirs Have Distinct Compound Profiles

A total of 16 compounds were identified as having significantly different intensities between the Pitch kefir and PNL kefir groups (Figure 5.5). Of these 16 compounds 11 were present at significantly higher levels in the Pitch kefir, with organic acids especially being increased in the Pitch. Meanwhile, PNL had increased levels of the ketone 3-Penten-2-one (E), as well as three compounds that we were unable to identify. In contrast to the other 18 hour comparisons, only 5 compounds were identified as having significantly different intensities between PNY and pitch groups, with D-limonene, 2-heptanone, and nonanoic acid being the only compounds that were successfully identified (Figure 5.6). Of these compounds, all but one were significantly increased in the Pitch kefir.


A
3.0
2.5
2.0
1.5
1.0
−log10(P value)
-10
0
10
20
log2(Fold Change)
Octanoic acid
Analyte 257
Nonanoic acid
3-Penten-2-one, E-
Hexanoic acid
4,5-Octanediol, 2,7-dimethyl-
n-Decanoic acid
Sulfur dioxide
Butanoic acid
Analyte 97
Analyte 372
Acetic acid
Pivalic acid vinyl ester
Decanoic acid, ethyl ester
Analyte 330
Peak Intensity
5.0e+06
1.0e+07
1.5e+07
Compound Family
Alcohol
Ester
Ketone
N/A
Organic Acid

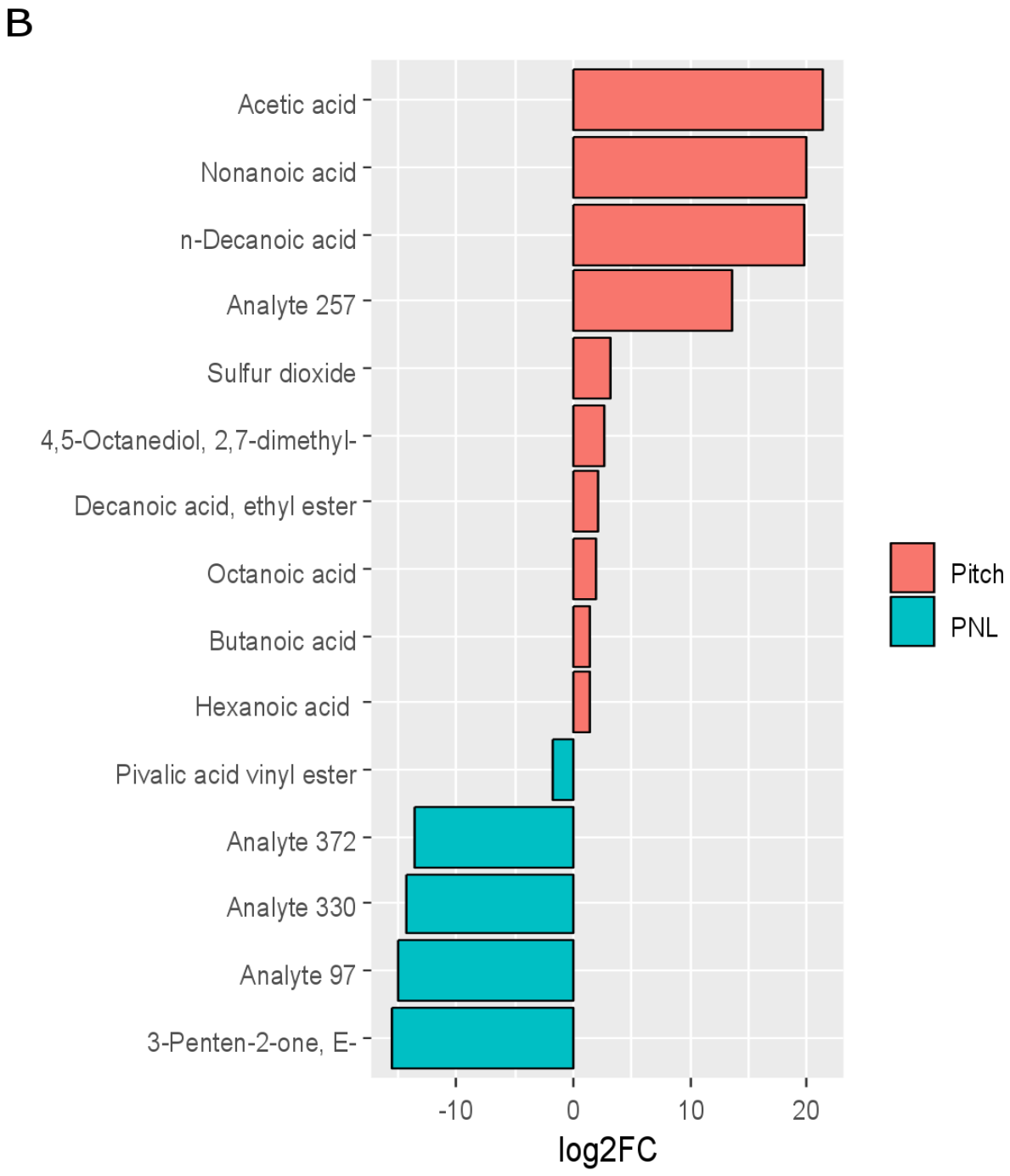
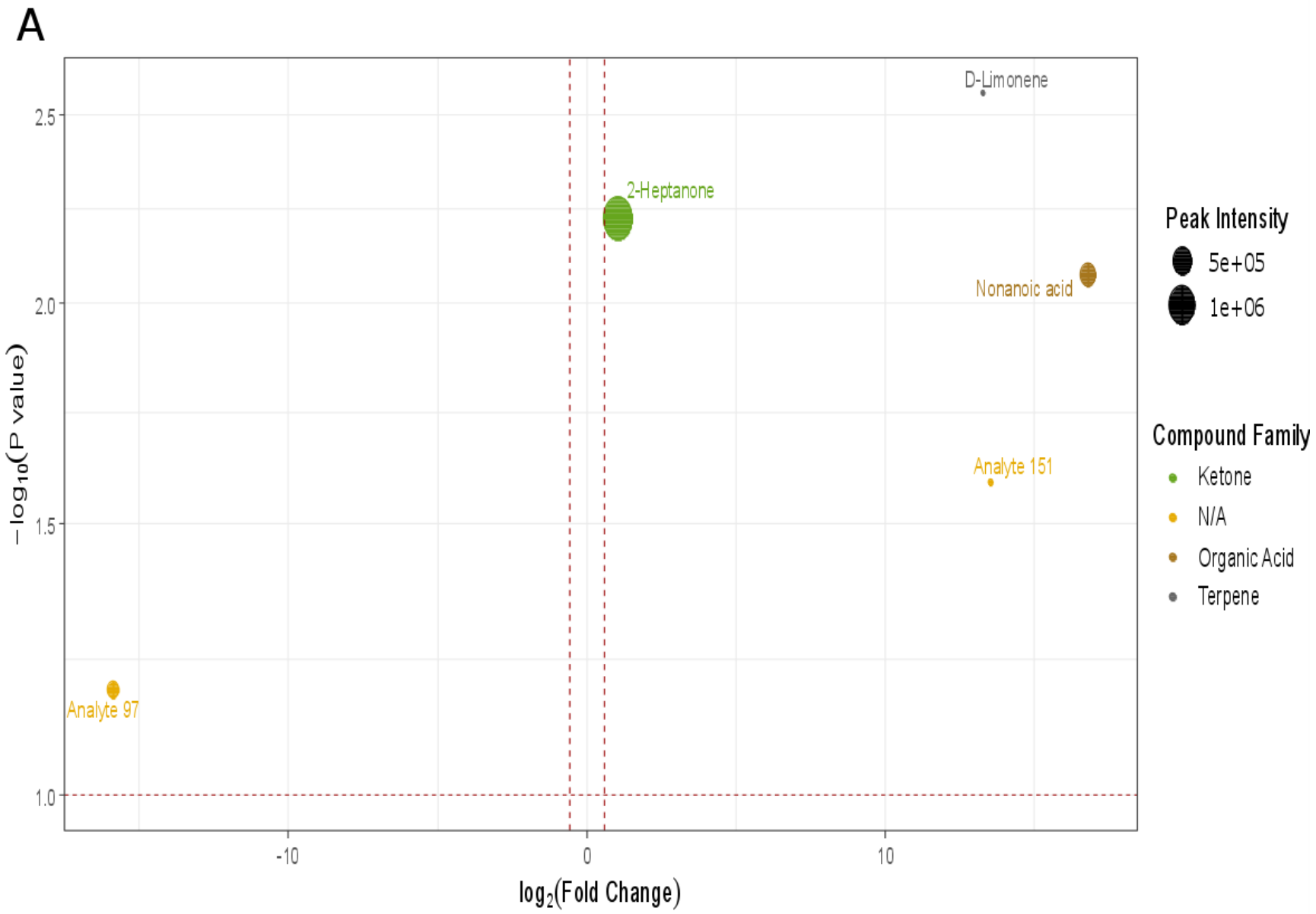


Figure 5.5. Volcano plot (A) and LEfSe plot (B) of compounds with significant differences in peak intensity between Pitch and PNL kefirs following 18 hours of fermentation (n=3). Pitch, kefir fermented with a defined mixture of microorganisms; PNL, kefir fermented with a defined mixture of microorganisms that lacks lactobacilli. Compounds with a negative fold change had increased peak intensities in PNL kefir while those with positive fold changes had increased peak intensities in Pitch kefir.


A
D-Limonene
2-Heptanone
Nonanoic acid
Analyte 151
Analyte 97
2.5
2.0
1.5
1.0
−log10(P value)
-10
0
10
log2(Fold Change)
Peak Intensity
5e+05
1e+06
Compound Family
Ketone
N/A
Organic Acid
Terpene

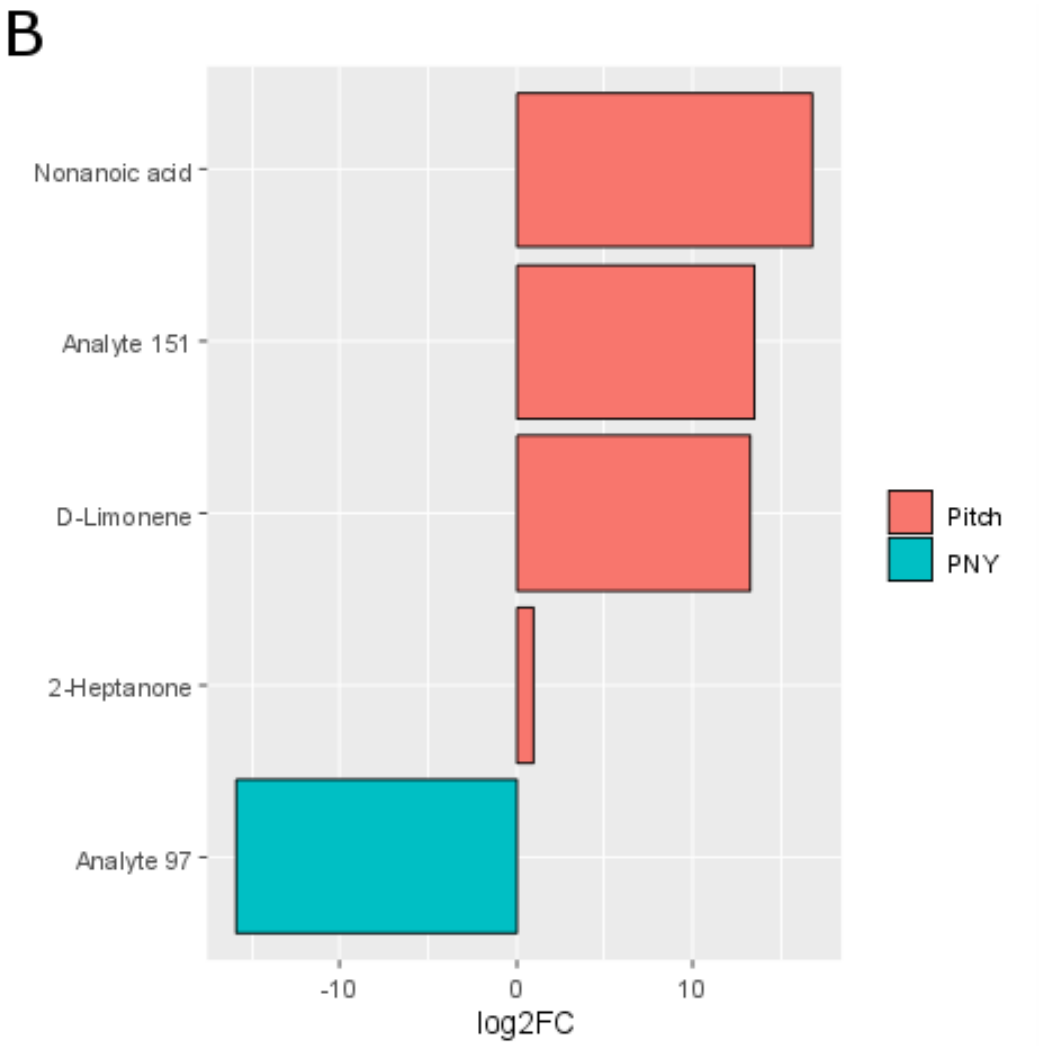


Figure 5.6. Volcano plot (A) and LEfSe plot (B) of compounds with significant differences in peak intensity between Pitch and PNY kefirs following 18 hours of fermentation (n=3). Pitch, kefir fermented with a defined mixture of microorganisms; PNY, kefir fermented with a defined mixture of microorganisms that lacks yeast. Compounds with a negative fold change had increased peak intensities in PNY kefir while those with positive fold changes had increased peak intensities in Pitch kefir.

The differences in fermentations were further analyzed by comparing the intensity levels of the major organic acids, esters, and ethanol across all four fermentation types. For each of butanoic, acetic, hexanoic, and octanoic acid, the Pitch kefir had the highest intensities followed by PNY, ICK, and PNL (Figure 5.7). Pitch had significantly higher peak intensities than both

ICK and PNL for all four acids, while also having a significantly higher intensity of acetic acid than the PNY fermentation. Additionally, the PNY fermentation had significantly higher intensities of both butanoic and hexanoic acid when compared to the ICK and PNL kefirs. Analysis of the esters showed even more marked differences than the organic acids. Of the 4 esters analyzed, only butanoic acid ethyl ester was identified in the Pitch, PNY, or PNL kefirs, with PNY and Pitch kefir having significantly higher intensity levels present than PNL (Figure 5.8). ICK, however, showed significantly higher intensities of 1-butanol-3-methyl-acetate, ethyl acetate, and propanoic acid 2-hydroxy-ethyl ester while displaying significantly lower intensities of butanoic acid ethyl ester than all three pitched kefir variations. In addition, the intensity of the ethanol peak was significantly higher in ICK kefir than all three pitched kefirs, while also being increased in Pitch and PNY when compared to PNL (Figure 5.9).

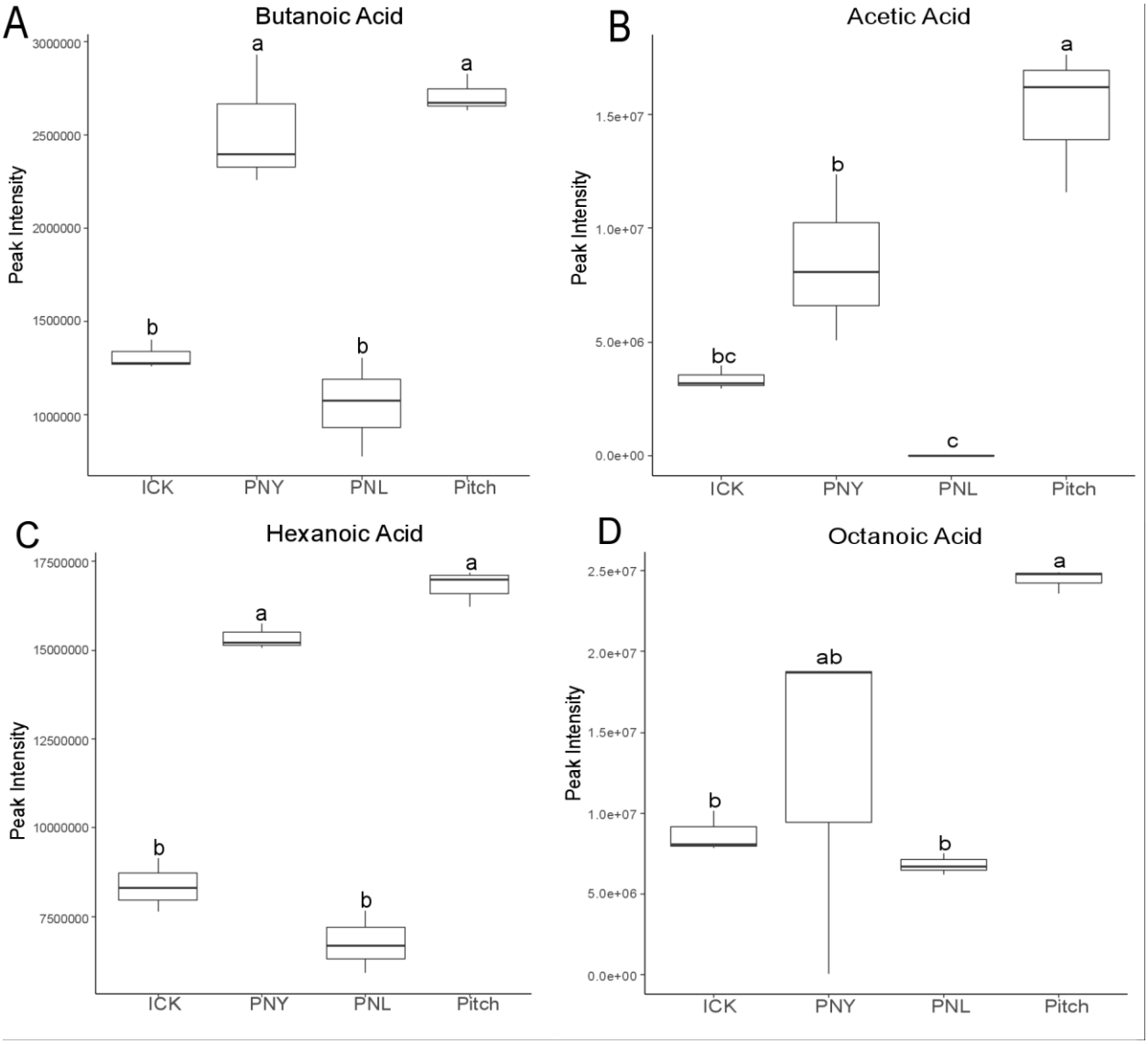


Figure 5.7. Boxplots showing peak intensities of butanoic acid (A), acetic acid (B), hexanoic acid (C), and octanoic acid (D) between ICK, PNY, PNL, and Pitch kefirs following 18 hours of fermentation. Data are expressed as means ± SEs (n=3). Means that do not share a letter are significantly different ($P<.05$). ICK, kefir fermented with the kefir grain ICK; Pitch, kefir fermented with a defined mixture of microorganisms; PNL, kefir fermented with a defined mixture of microorganisms that lacks lactobacilli; PNY, kefir fermented with a defined mixture of microorganisms that lacks yeast.

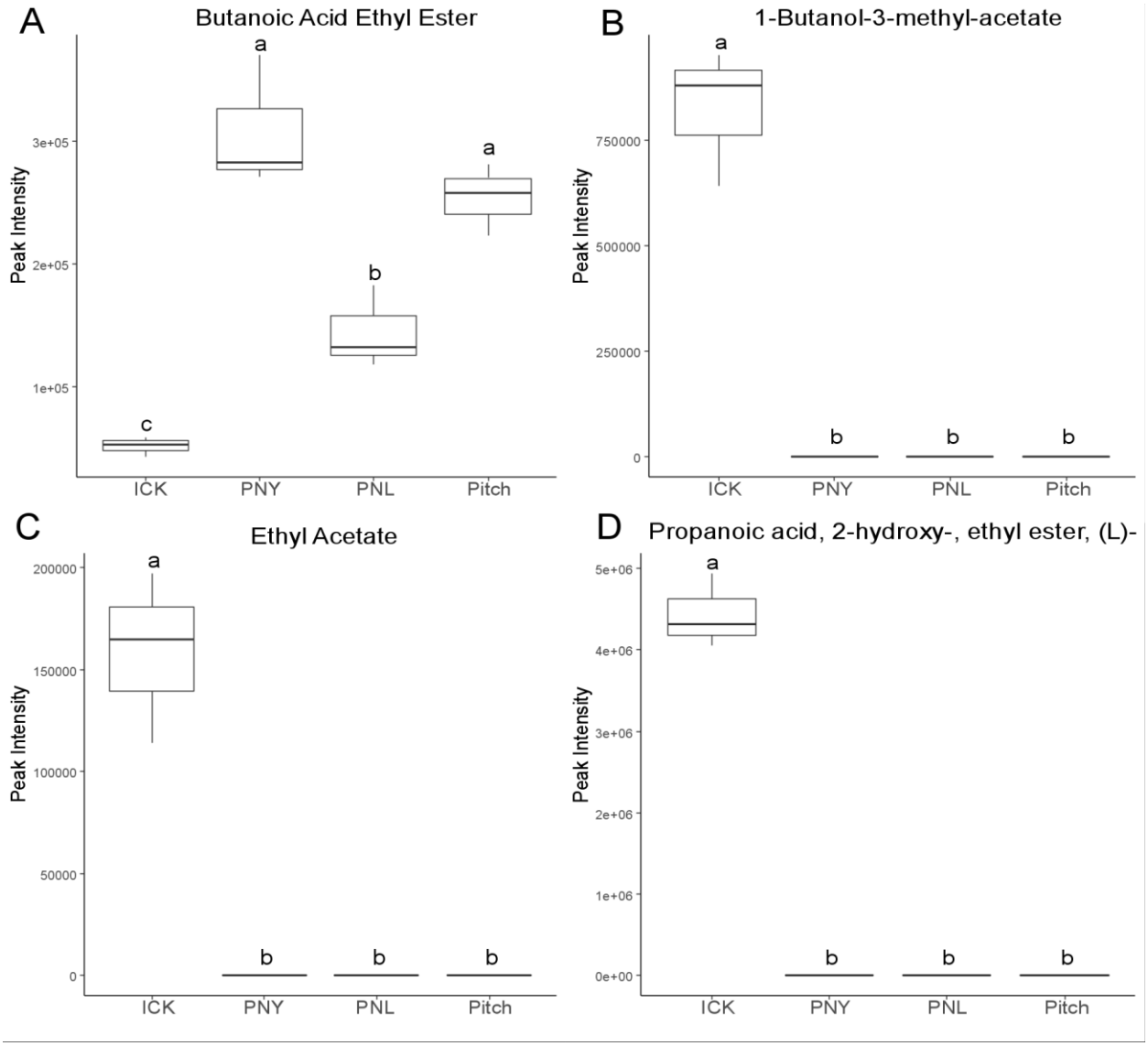


Figure 5.8. Boxplots showing intensity levels of butanoic acid ethyl ester (A), 1-butanol-3-methyl-acetate (B), ethyl acetate (C), and propanoic acid 2-hydroxy-ethyl ester (D) between ICK, PNY, PNL, and Pitch kefirs following 18 hours of fermentation. Data are expressed as means ± SEs (n=3). Means that do not share a letter are significantly different ($P<.05$). ICK, kefir fermented with the kefir grain ICK; Pitch, kefir fermented with a defined mixture of microorganisms; PNL, kefir fermented with a defined mixture of microorganisms that lacks lactobacilli; PNY, kefir fermented with a defined mixture of microorganisms that lacks yeast.

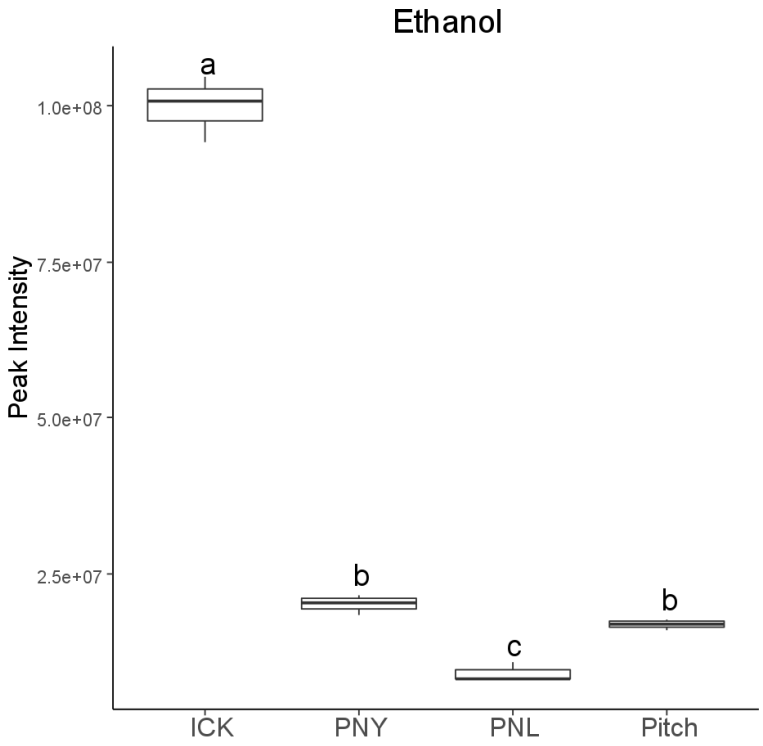
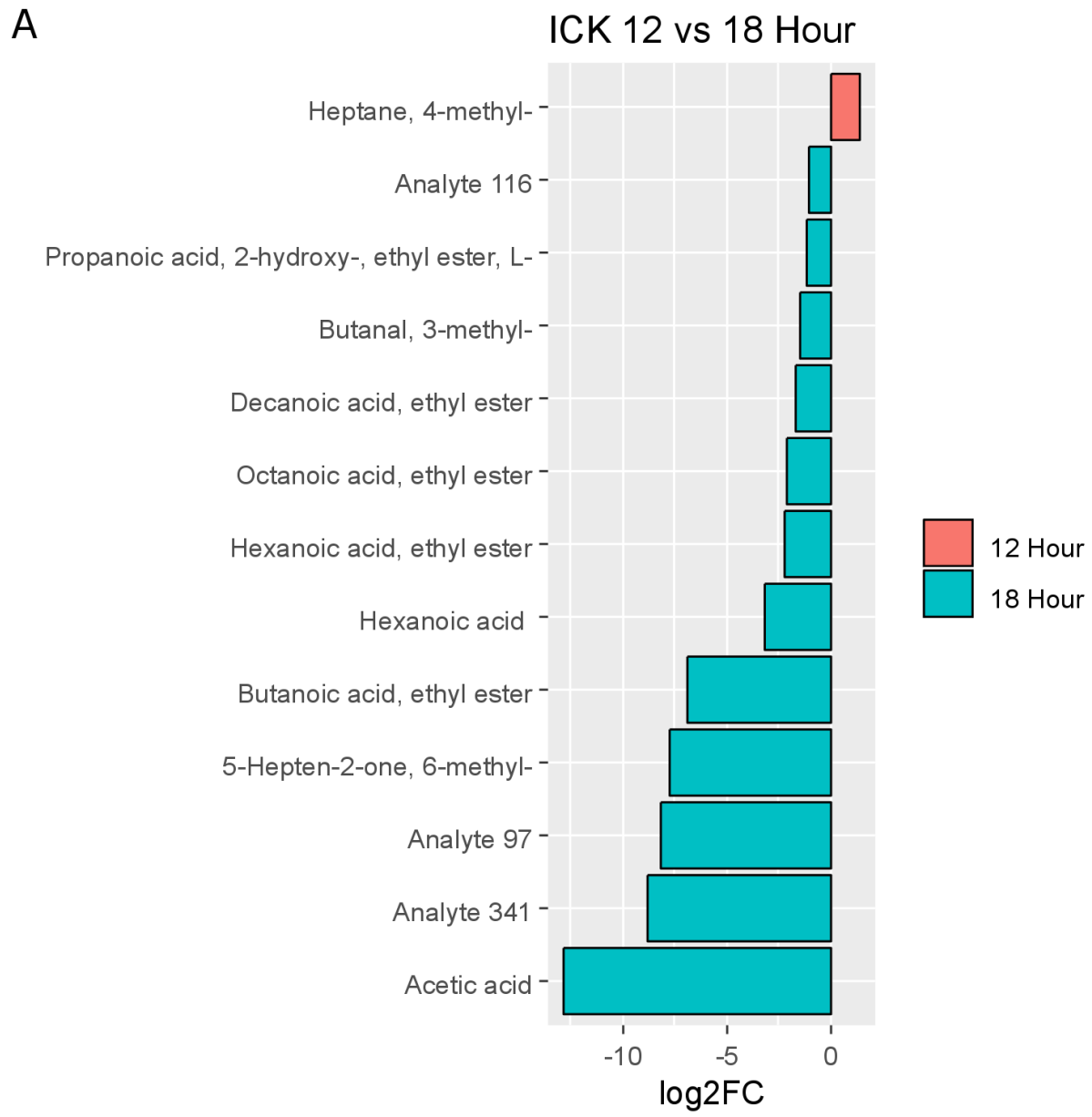


Figure 5.9. Boxplot showing intensity levels of ethanol between ICK, PNY, PNL, and Pitch kefirs following 18 hours of fermentation. Data are expressed as means ± SEs (n=3). Means that do not share a letter are significantly different (P<.05). ICK, kefir fermented with the kefir grain ICK; Pitch, kefir fermented with a defined mixture of microorganisms; PNL, kefir fermented with a defined mixture of microorganisms that lacks lactobacilli; PNY, kefir fermented with a defined mixture of microorganisms that lacks yeast.

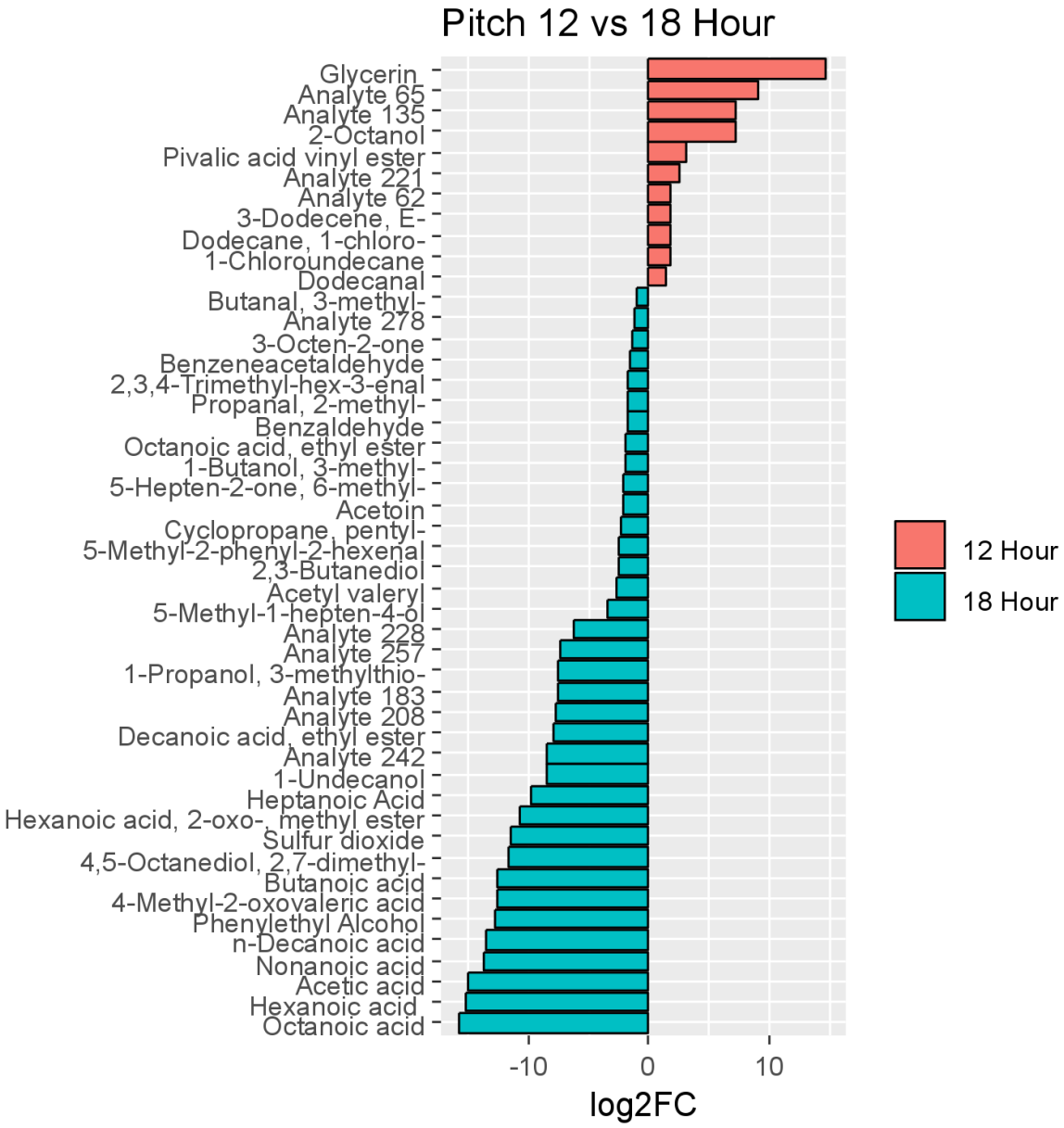
Metabolite Levels Change over the Course of Fermentation

In order to determine how the metabolite profile of the different kefirs progressed over time, we compared fermentations after both 12 and 18 hours. Kefir made with the ICK grain showed significant increases in multiple ethyl esters, as well as the organic acids hexanoic and acetic acid after 18 hours of fermentation. The only compound with significantly higher levels in the 12 hour ICK samples was heptane, 4-methyl- (figure 5.10A). There was a much larger number of compounds that were significantly different between 12 and 18 hours of fermentation in the Pitch kefir than in ICK kefir. Pitch kefir had a significant increase in the level of a number of organic acids, including heptanoic, butanoic, n-decanoic, nonanoic, acetic, hecanoic, and octanoic acid; as well as various esters after 18 hours(figure 5.10B). Both the PNL and PNY

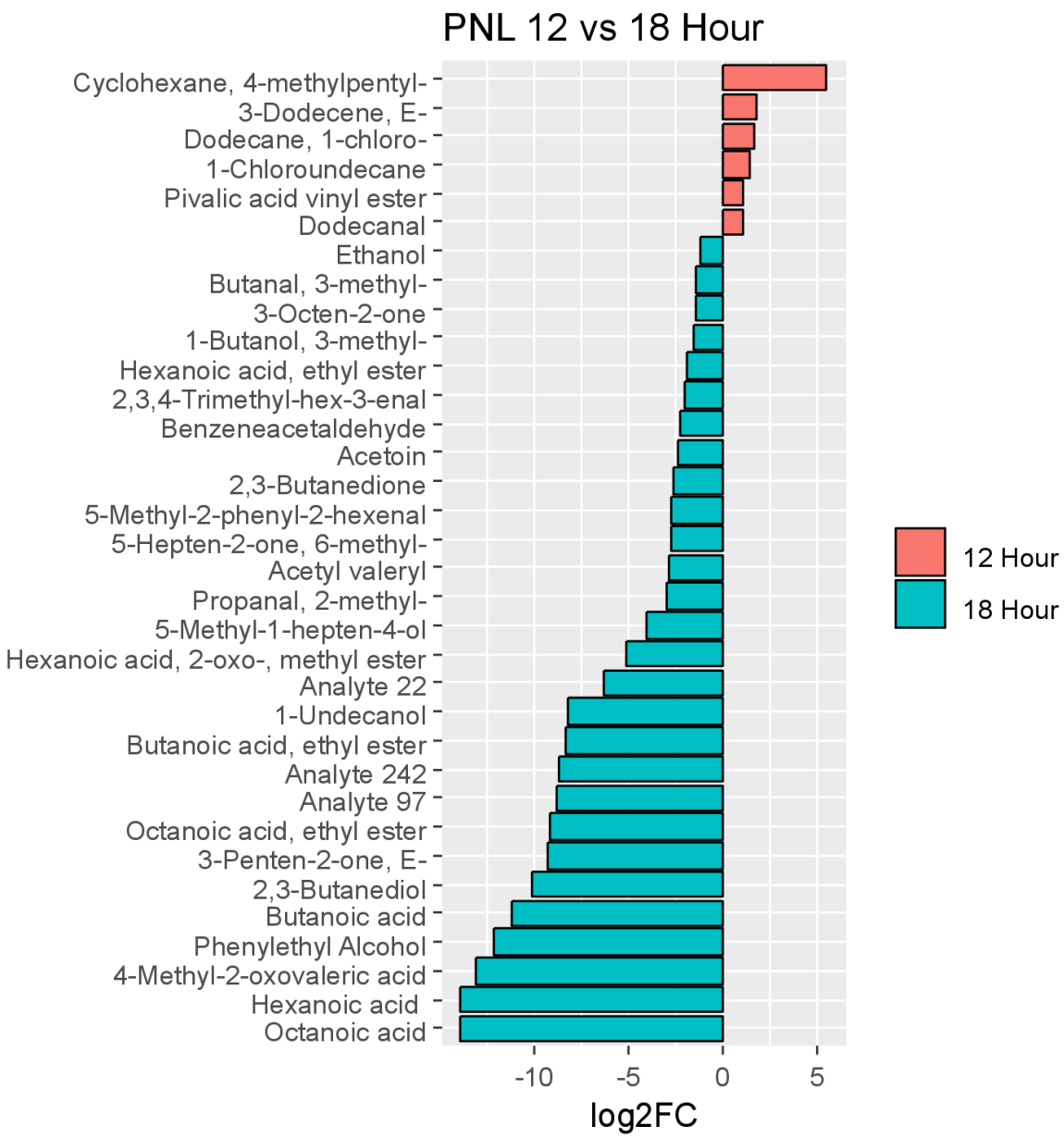
closely resembled the Pitch kefir in the changes observed between the 12 and 18 hour time points with increases in a number of organic acids in the 18 hour samples along with increases in a small number of esters (Figure 5.10C and D).



B


Pitch 12 vs 18 Hour
Glycerin
Analyte 65
Analyte 135
2-Octanol
Pivalic acid vinyl ester
Analyte 221
Analyte 62
3-Dodecene, E-
Dodecane, 1-chloro-
1-Chloroundecane
Dodecanal
Butanal, 3-methyl-
Analyte 278
3-Octen-2-one
Benzeneacetaldehyde
2,3,4-Trimethyl-hex-3-enal
Propanal, 2-methyl-
Benzaldehyde
Octanoic acid, ethyl ester
1-Butanol, 3-methyl-
5-Hepten-2-one, 6-methyl-
Acetoin
Cyclopropane, pentyl-
5-Methyl-2-phenyl-2-hexenal
2,3-Butanediol
Acetyl valeryl
5-Methyl-1-hepten-4-ol
Analyte 228
Analyte 257
1-Propanol, 3-methylthio-
Analyte 183
Analyte 208
Decanoic acid, ethyl ester
Analyte 242
1-Undecanol
Heptanoic Acid
Hexanoic acid, 2-oxo-, methyl ester
Sulfur dioxide
4,5-Octanediol, 2,7-dimethyl-
Butanoic acid
4-Methyl-2-oxovaleric acid
Phenylethyl Alcohol
n-Decanoic acid
Nonanoic acid
Acetic acid
Hexanoic acid
Octanoic acid
-10
0
10
log2FC
12 Hour
18 Hour

C


PNL 12 vs 18 Hour
Cyclohexane, 4-methylpentyl-
3-Dodecene, E-
Dodecane, 1-chloro-
1-Chloroundecane
Pivalic acid vinyl ester
Dodecanal
Ethanol
Butanal, 3-methyl-
3-Octen-2-one
1-Butanol, 3-methyl-
Hexanoic acid, ethyl ester
2,3,4-Trimethyl-hex-3-enal
Benzeneacetaldehyde
Acetoin
2,3-Butanedione
5-Methyl-2-phenyl-2-hexenal
5-Hepten-2-one, 6-methyl-
Acetyl valeryl
Propanal, 2-methyl-
5-Methyl-1-hepten-4-ol
Hexanoic acid, 2-oxo-, methyl ester
Analyte 22
1-Undecanol
Butanoic acid, ethyl ester
Analyte 242
Analyte 97
Octanoic acid, ethyl ester
3-Penten-2-one, E-
2,3-Butanediol
Butanoic acid
Phenylethyl Alcohol
4-Methyl-2-oxovaleric acid
Hexanoic acid
Octanoic acid
-10
-5
0
5
log2FC
12 Hour
18 Hour

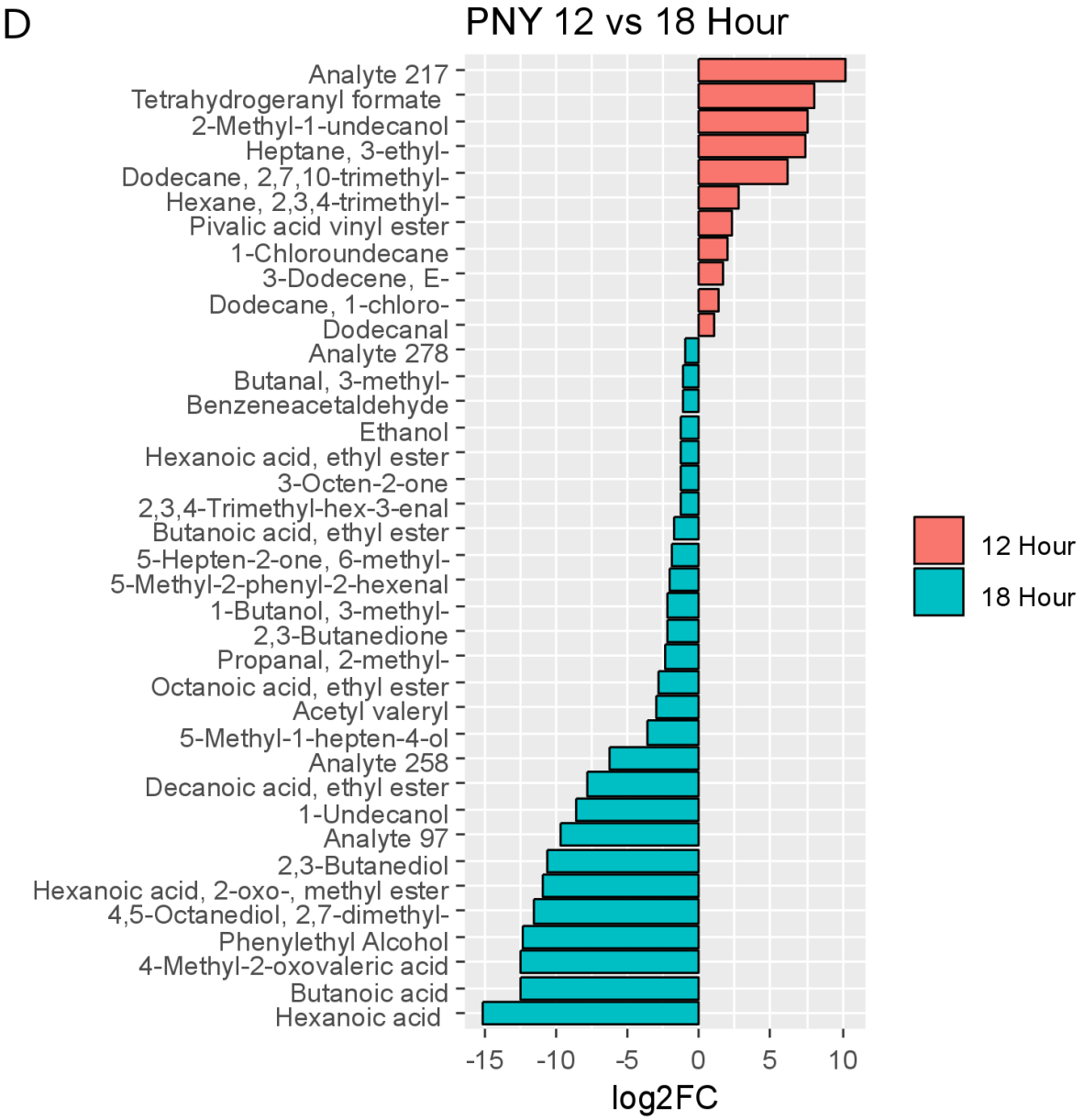


Figure 5.10. LEfSe plot of compounds with significantly different peak intensities between 12 and 18 hours of fermentation in ICK (A), Pitch (B), PNL (C), and PNY (D) kefir. ICK, kefir fermented with the kefir grain ICK; Pitch, kefir fermented with a defined mixture of microorganisms; PNL, kefir fermented with a defined mixture of microorganisms that lacks lactobacilli; PNY, kefir fermented with a defined mixture of microorganisms that lacks yeast.

5.4 Discussion

This study examined similarities and differences between a pitched culture kefir utilizing traditional kefir organisms and a traditional grain fermented kefir, as well as how variations in the starting microbial composition of pitched kefir impacted fermentation dynamics, and thus, the final product by analyzing metabolites utilizing GCxGC-TOFMS. In the process, we identified potential compounds of importance to the sensory characteristics of traditional kefir, as well as possible contributions made by specific groups present in the fermentation. For instance, it was observed that ICK kefir had increased levels of multiple esters and ethanol following 18 hours of fermentation when compared to Pitch kefir, while the Pitch kefir contained significantly more organic acids and aldehydes. Conversely, while the PNL and PNY kefir maintained an increase in aldehydes in comparison to ICK, there was not a significant difference in organic acid levels. The increased levels of esters in the ICK kefir, along with higher levels of ethanol, may indicate an increased role of yeast metabolism in the grain fermented kefir given that esterification and ethanol production are metabolic processes that are typically associated with yeast, although there is evidence for a role of lactic acid bacteria in ester production (14,16–20). Indeed, Walsh *et al.* found that *Saccharomyces cerevisiae* was strongly correlated with levels of both esters and ethanol in kefir fermentations (14). Another factor that may influence increased ester formation in grain fermented kefir is the presence of smaller populations of yeast present in grain fermented kefir that are not present in the pitched culture version. For instance the yeast genus *Dekkera* has been shown to produce esters, including ethyl acetate, in multiple fermentations, and this genus has also been identified as a minor member of the kefir yeast microbiota (21,22). Additionally, yeast from the genus *Dekkera* have been found to utilize many different nitrogen sources during fermentation, which may allow it to thrive in a specific niche in

kefir fermentation (23). A decreased role of yeast metabolism and subsequent increase in the role of lactobacilli in the Pitch kefir in comparison to ICK would help explain the increase in levels of organic acids present in the Pitch kefir, as these compounds have been associated with lactic acid bacteria in multiple different fermentations and specifically with *Lactobacillus kefiranofaciens* in kefir (14,24–27). Additionally, lactic acid bacteria in dairy have been associated with the formation of multiple aldehydes, such as benzaldehyde, benzeneacetaldehyde, and butanal 3-methyl (28–30). This apparent shift towards a more lactic acid bacteria, and seemingly specifically lactobacilli, dominant fermentation may be due to differences in the starting ratios of bacteria and yeast between pitched kefir and traditional kefir, as traditional kefir grains do not contain an equal mix of all organisms present (21,31). There is also the possibility that the arrangement of microorganisms within the kefir grain plays a role in the fermentation dynamics, as different microbes are found in different areas of the grain (32–34) These differences between Pitch and ICK kefir are interesting as, although they do not seem to alter the impact of the kefir on host cholesterol and lipid metabolism, they may prove to be important to the sensory characteristics of the finished product, as esters such as ethyl acetate and 1-butanol-3-methyl-acetate are generally associated with frutiy aromas such as banana, pineapple, or apple, while organic fatty acids such as octanoic or acetic acid produce aromas described as cheesy or vinegar-like, and aldehydes can range in aroma from cheesy (butanal 3-methyl) to almond or cherry (benzaldehyde) (14,29). These possible sensory differences should be further examined using a professional tasting panel, as the perceived flavours and aromas of a product are the result of complex interactions between compounds present in the product, and sensory analysis is important in understanding how these differences impact the palatability of the product (35–39).

Comparison of the Pitch kefir to PNL revealed significantly higher levels of a number of organic acids in the Pitch kefir, while there were very few compounds that were different between the Pitch and PNY kefir. This increase in a significant amount of organic acids in the Pitch kefir when compared to the PNL kefir at 18 hours, and the relatively minor differences observed between Pitch and PNY kefir point to the lactobacilli population being a major contributor to organic acid production during in the fermentation. Lactobacilli are capable of producing large organic acids such as octanoic and nonanoic acid, as well as butanoic and acetic acid in addition to lactic acid and are major producers of organic acids in multiple food fermentations (24,27,40). The observed differences between the Pitch and PNL/PNY kefirs showed only nonanoic acid was similarly increased in Pitch when compared to both the PNL and PNY kefirs. While nonanoic acid has not been associated with any cholesterol lowering effects, it has been shown to have mild antiinflammatory potential due to cyclooxygenase (COX)-1 inhibitory activity (41). Other fatty acids, such as butyrate and acetate, have shown potential to benefit host health (42–45); however, the likelihood of nonanoic acid being solely responsible for the previously observed improvements in host cholesterol and lipid metabolism is low.

Characterization of changes to the metabolite profile of ICK over the course of fermentation revealed a trend towards an increase in ester levels from 12 to 18 hours potentially indicating that alcohol and organic acid production is relatively constant throughout fermentation while esterification takes place later. As organic acids and alcohols are utilized by yeast in the formation of esters, this would explain why there was not significantly more organic acids or alcohols present in the 12 hour fermentation samples (46–48). Contrary to the ICK kefir, the Pitch, PNL, and PNY in a number of organic acids, including hexanoic and butanoic acid, as well as phenylethyl alcohol, and a small number of large fatty acid esters. This may further

support the possibility of the pitched kefir fermentations being more bacterially, and specifically lactobacilli driven as the increase in organic acids could indicate a lack of ester production by the yeast present in the fermentation.

This study is the first of our knowledge to compare the volatile compound profiles of kefir made with a traditional kefir grain and multiple pitched culture examples using defined communities of microorganisms isolated from traditional kefir. While there were differences in volatile compound profiles among different kefirs, there were no distinct compounds which could be linked to the previously observed physiological changes present in mice fed both ICK and Pitch kefir (1; Chapter 2, this thesis). It should be noted, however, that kefir contains a complex mixture of microbial metabolites which may be responsible for these observed effects. In addition to aromatic compounds, kefir also contains microbially produced peptides and exopolysaccharides which have been associated with health benefits (5–7,9,49–53). As such, future work to identify potential compounds of interest in regards to these benefits should focus on the utilization of peptidomic approaches to ascertain how the proteolytic activity of the community is impacted by changes to said community. The recent advent of databases to better identify bioactive peptides present in food fermentations may also allow more streamlined and accurate ID of compounds of interest for future study (54). Additionally, recent work utilizing a multi-omic approach to analyzing food fermentations has been successful in determining how community structure and behaviour impacts the development of these products (14,55,56). By taking a metagenomic or metatranscriptomic approach and pairing it with metabolomic or peptidomic data, we can gain valuable insight into how microbes interact and influence eachother during fermentation, and in turn how these interactions shape the final product from both a flavour and health perspective.

5.5 Conclusion

This study examined metabolite profiles of kefir products generated using grain fermentation or pitched culture fermentation with varying microbial compositions. We were able to identify a distinct difference in metabolite profiles between traditional grain fermented kefir and a pitched culture example, with the traditional kefir exihibting a trend towards increased ester production and the Pitch kefir having significantly higher levels of organic acids. Comparisons of the Pitch and PNL/PNY kefir showed lower levels of organic acid production in the PNL kefir, yet relatively unchanged metabolite profiles between PNY and Pitch; potentially indicating an important role for the lactobacilli population in the fermentation profile of Pitch kefir. This study presents an important look into understanding how alterations to the community of microorganisms present in a food fermentation can impact the final composition of the product. This is especially important given the potential for future studies utilizing multi-omic approaches to better understand these microbial interactions during fermentation.

5.6 References

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Chapter 6: General Discussion

The impact of fermented and functional foods on host health has been a growing topic of interest; however, the variability, especially in microbial composition, of these traditional fermented foods is rarely considered when discussing these purported benefits. Among these fermented foods, kefir has generated significant interest due to a multitude of health claims associated with the fermented milk beverage. Kefir has been associated with a variety of health claims including cholesterol lowering effects and antimicrobial activity (1). This research set out to examine how variations in the microbial population of kefir affect different qualities of kefir, such as metabolite profiles and associated benefits with host metabolism, and in turn how we can use this knowledge to construct a more health promoting kefir using an industrially viable production technique.

6.1 The Importance of Microbial Composition in Kefir

Traditional kefir can be extremely varied, both in the level of microbes present, as well as the presence or absence of specific microorganisms (2,3), which may lead to variation in the ability of different kefirs to affect the host in a specific manner. Indeed, variation in the qualities of kefir grains from different sources has been seen *in vitro* (4), suggesting that slight differences in the microbial populations of kefir may alter said kefir's capabilities in certain conditions. The study in chapter 2 found that, similar to Vujicic *et al.* (4), kefir grains from different sources had varying abilities to lower cholesterol levels, both in whole milk over the course of fermentation, and in a mouse model of obesity. Interestingly, however, these levels of cholesterol lowering *in vitro* did not necessarily correlate to the ability of a corresponding kefir drink to lower plasma cholesterol levels *in vivo*, which may be due to a difference in the mechanism of action behind each of these findings.

As more individuals reach for probiotic or functional fermented foods as a way to improve their health, particular attention needs to be paid to the inherent differences in commercially available products and traditional examples of fermented foods. This is especially important given that research on the health benefits of functional fermented foods are often carried out using traditional versions of the food in question. Indeed, kefir has been proclaimed to help improve circulating lipid levels and profiles and fatty liver disease (5–7), yet these studies generally examine traditional, grain fermented kefir, which does not resemble the majority of commercial examples on a microbial level. While consumers may read these health claims and attempt to utilize these products to improve their metabolic health, most commercial examples of kefir do not contain the same microorganisms present in traditional kefir. For instance, while many traditional kefirs contain the specific lactobacilli species *Lb. kefiri* and *Lb. kefiranofaciens*, commercial examples generally contain one or more of *Lb. bulgaricus*, *Lb. acidophilus*, *Lb. delbrueckii*, *Lb. reuteri*, and *Lb. plantarum*. Additionally, commercial examples often lack *Acetobacter* while also omitting the yeast population or simply including a single *Saccharomyces* strain. By consuming a product which shares no microbial similarities to traditional kefir, greatly reduces the chance that the individual will receive the same health benefits as those associated with said traditional kefir.

6.2 Towards Evidence Based Commercial Product Development

As we have shown in chapter 2 and 4, common commercial kefirs are not able to replicate the improvement to plasma cholesterol and liver lipid levels seen with traditional kefir. Therefore, it is paramount to the design of a kefir product with the goal of improving host health to generate a product that recreates the traditional example as faithfully as possible; however, the production of an industrial scale kefir product using kefir grains is not feasible. In order to accurately represent traditional kefir, while maintaining industrially scalable production, the isolation of individual

microorganisms from traditional kefir for use in pure culture kefir fermentation is required. One important consideration is the potential for desirable characteristics of microorganisms to be species or even strain specific (8). Additionally, yeast and lactic acid bacteria have been shown to have symbiotic relationships with one another during the fermentation process, potentially impacting the growth, survivability, and metabolisms of the microbes (9,10). Specifically, lactic acid bacteria can benefit yeast through the breakdown of lactose to galactose and glucose, while yeast are capable of producing excess amino acids that allow for improved survival of lactic acid bacteria (9). Indeed, there is evidence that co-cultivation of yeast and lactic acid bacteria can induce the release of specific amino acids by yeast which are essential to lactic acid bacterial growth (10). Therefore, selection of the specific organisms is key, with the symbiotic nature of kefir proving extremely important in producing a successful fermentation while maintaining the health benefits observed in traditional kefir. Our work in Chapter 4 showed that a kefir produced with an industrially viable method and, using organisms isolated from traditional kefir was able to recapitulate health benefits observed in said traditional kefir. This is an especially important finding as it shows that through careful consideration, and by utilizing existing knowledge of a traditional fermented food product, there is potential to create a functional fermented food product that mirrors the health benefits of a traditional fermentation, while maintaining the possibility of commercial scale production. Additionally, the findings that upon removal of either the yeast population or the lactobacilli population any health benefits were lost, is another indication of the importance of maintaining a healthy and representative population is paramount when developing these products. The determination of which organisms from traditional kefir are required to recapitulate health benefits in the host is of the utmost importance in the development of such as product, as including fewer organisms in a fermentation can significantly lower the cost of

production. Additionally, the kefir produced in our lab was highly reproducible in both pH and microbial density, indicating potential for future commercialization.

The manner by which microbes of interest for inclusion are selected for a fermentation can have a large impact on the outcomes of the fermentation. Although we attempted to select for isolates with desirable characteristics for inclusion in our pitched culture kefir, we were unable to identify any microbes with BSH activity or bacteriocin production (chapter 3). The lack of microbes displaying these characteristics was in contrast to much of the research carried out on kefir microbes (11–15). We did, however, succeed in isolating and identifying a wide range of microorganisms which encompassed the most ubiquitous and plentiful members of traditional kefir by utilizing multiple culture methods along with phenotypic and genotypic analyses in order to select for the organisms of interest. The methods described in chapter 3 may prove useful for future projects aiming to isolate a wide range of microbes from fermented foods.

6.3 How Does Microbial Composition Impact Volatile Compound Production

The composition of volatile compounds present in fermented products is the outcome of a complex interaction between the entire community of microorganisms present. As has been previously discussed, yeast and lactic acid bacteria are able to co-exist in a symbiotic relationship during fermentation in order to create combinations of compounds which would not be possible without the inclusion of both communities (9,10,16,17). The volatile compounds produced during fermentation help to drive the aroma and flavour profile of the finished product and play a vital role in the palatability of the fermented food. In addition, these compound profiles can help inform us about the behaviour of the microbes present during fermentation, thus increasing our understanding of the roles of these microorganisms. The results presented in chapter 5 show that, although a lab generated pitched culture kefir was able to lower cholesterol and liver lipid levels

similarly to its traditional counterpart (chapter 4), there are still distinct and vast differences in the fermentation profile between the two. Specifically, there appeared to be significantly more yeast metabolites present in the traditional kefir, potentially indicating a large role for other species of yeast which were not included in the lab generated kefir. Additionally, there were differences observed between the Pitch and PNL kefir, though the PNY kefir did not appear to differ as much from the Pitch. Regardless, these results are supported by other work which has shown variation in kefir volatile compounds, as well as changes to the levels of these compounds when the starting microbial composition is altered (18). These results show that the ability of kefir to benefit host health may not be correlated with levels of volatile compounds present during fermentation, especially given how similar the pitched kefir variants were to each other.

6.4 Limitations and Future Directions

While these studies were successful in showing that traditional kefir was better able to improve host cholesterol and liver lipid levels, it is important to note that we were unable to establish a clearly defined mechanism for these phenotypic changes in the host. Although evidence suggests that these effects are rooted in alterations to host cholesterol and fatty acid metabolism, we were unable to identify how the kefir is acting on the host to exact these changes. It may be beneficial to consider the use of RNA-Seq as a method of measuring differential expression of each gene in the liver as a way to gather a more complete picture of how these changes come about. In addition, although traditional kefir and Pitch kefir were able to lower triglyceride levels in the liver, these changes did not impact the histological profiles of liver tissue. The inclusion of other types of histological preparations and stains in order to better visualize immune cell infiltration may help to elucidate the impact that kefir has on the development of NAFLD and NASH. It may also be prudent to examine the livers of mice at multiple time points in order to determine whether

kefir has an impact on the rate of onset of these histological changes. Another important consideration is that these trials were conducted while utilizing kefir in a preventative manner and not in the treatment of already obese, dyslipidemic mice. While kefir proved to be capable of preventing the onset of hypercholesterolaemia and liver lipid accumulation, many human subjects interested in these potential benefits are already suffering from high cholesterol and/or NAFLD. The inclusion of future studies analyzing the ability of kefir to effectively treat already dyslipidemic subjects is a must in the development of kefir for real-world clinical use, especially when considering the mixed results generated by previous human trials involving kefir (19–22). Additionally, the inherent differences in mouse and human cholesterol should be considered and human trials are necessary to determine how these findings transfer to a human population.

The findings of both the yeast and lactobacilli populations being necessary in kefir in order for health benefits to be observed is intriguing and suggests a potentially symbiotic relationship which allows for the production of some form of bioactive compound or compounds. However, we still do not know whether the entirety of these populations is required for these benefits, or whether simply a single representative from one or both communities is sufficient. This can be examined through experimentation removing a single member of each community in order to better understand the role that each individual microbe present plays. In addition, the possibility still has to be considered that these benefits are not due to a bioactive component of kefir, and, in fact, there is simply a single organism in each of these populations that is able to exert a cholesterol lowering effect on the host. There are studies that show that both *Kluyveromyces marxianus* and multiple lactobacilli species including *Lb. kefiri* are capable of lowering cholesterol levels when administered as single organisms (11,23,24). Future studies utilizing the administration of either single organisms or a cell free fraction of kefir should be performed in order to better understand

the potential components of kefir which are causing these phenotypes. It is also important to note that, although we were able to generate a promising pitched culture kefir after one attempt, there was a significant possibility of failure given that none of our isolates showed strong health promoting characteristics in the screening performed. While the strategy of utilizing only organisms from the best performing traditional kefir in chapter 2 was successful, it is unrealistic to expect that a similar approach would be successful on a consistent basis; especially considering the potential for strain level differences within the same species of microbe. Indeed, there is ample opportunity for improvement of the screening protocols used in chapter 3, such as the inclusion of multiple bile salts in the BSH assay and the testing of isolates against a broader range of indicator organisms for antimicrobial activity.

Although the development of a kefir product that is able to be produced using an industrially viable method while maintaining the health promoting characteristics of traditional kefir is promising, the differences observed in volatile compound profiles are potentially troubling as it pertains to the palatability of said product. The inclusion of a trained tasting panel in evaluating each of the kefirs tested would help to identify how these changes in volatile compounds impact consumer perception of the beverages, as well as determining the feasibility of continuing development of the pitched kefir as a commercial product. GCxGC-TOFMS also failed to identify a likely candidate compound that existed commonly between the Pitch and ICK kefir, while being absent or lowered in the PNL and PNY kefir, indicating that, if it is in fact a bioactive compound responsible for cholesterol lowering, it is unlikely to be a volatile compound. Future studies in this regard should focus on the analysis of peptides produced during fermentation, as lactobacilli are adept at digesting dairy proteins into smaller peptides and have been documented to produce a variety of bioactive peptides (25). In fact kefir fermentation has been shown to produce a wide

range of peptides and kefir peptides have also been shown to have a variety of benefits (6,26–30). Attention should also be paid to the potential production of exopolysaccharides in the various fermentations, as *Lactobacillus kefiranofaciens* produces an exopolysaccharide unique to kefir which has also been shown to lower cholesterol levels when administered on its own (31,32) In addition, the inclusion of metagenomics and metatranscriptomic analyses of the kefir fermentations would allow for a better understanding of how microorganisms interact during the fermentation in order to create complex fermented foods. The potential for further alterations of fermentation parameters utilizing kefir isolates from the library generated in chapter 4 allows for improvements in our knowledge of how complex microbial communities interact; especially as it pertains to bacterial and yeast interactions. The findings of these studies would also allow for a better potential understanding of how other communities such as the gastrointestinal microbiome operate.

These studies set out to examine how different varieties of kefir, including traditional, commercial, and lab generated, impacted host metabolic health in the context of obesity. Additionally, we attempted to identify organisms and components of kefir that are essential to the observed health benefits reported in this study. In order to examine how kefir impacted host metabolic health a mouse model of obesity was used with supplementation of high fat diet with differing kefir with the goal of identifying improvements in host metabolic parameters. The role of kefir organisms in the volatile compound profile of kefir was examined using GCxGC-TOFMS with the intent of describing how alterations to the kefir microbiota change fermentation by-products as well as potentially determining if any volatile compounds present in kefir play a role in improving host metabolic health.

In summary, the microbial composition of kefir proved to be an important determinant in any ability to improve plasma cholesterol and liver triglyceride levels; however, the exact mechanism of action is still undetermined. In addition, through extensive isolation and library screening of microbes from traditional kefirs, we were able to generate a pitched culture kefir which recapitulated the health benefits observed in traditional examples. Volatile profiling revealed that although the pitched culture kefir resembled traditional kefir in health promoting characteristics, cell density, and pH, there were distinct differences in the volatile compound profiles of the two beverages. Overall, the studies outlined in this thesis represent a first step to understanding how the microbes in fermented foods interact in order to create a product that is able to benefit those that consume it, as well as a potential blueprint for future development of fermented foods with an aim towards human health.

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