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

Benjamin C.T. Bourrie[a,b], Paul D. Cotter[b,c], Benjamin P. Willing[a*]

[a]Agricultural Food and Nutritional Science, Agriculture/Forestry Center,

University of Alberta, Edmonton, Alberta, Canada

[b]Teagasc Food Research Centre, Moorepark, Fermoy, Co. Cork, Ireland

[c]APC Microbiome Ireland, Cork, Ireland

[*]Corresponding Author

Author emails:

Ben Bourrie: bourrie@ualberta.ca

Paul Cotter: Paul.Cotter@teagasc.ie

Ben Willing (Corresponding Author): willing@ualberta.ca

Abstract

Kefir, a fermented milk beverage, has shown promise in alleviating obesity and associated metabolic dysfunction. However, microbial characteristics are variable among traditional kefirs, and commercial kefirs drastically differ from traditional kefir. This study investigated the ability of four traditional and one commercial kefir to control weight gain, plasma cholesterol, and liver triglycerides in a high fat diet-induced obesity mouse model. Two traditional kefirs decreased weight gain and plasma cholesterol levels. Conversely, commercial kefir had no beneficial effect. Additionally, one of the four traditional kefirs lowered liver triglycerides, which corresponded with decreases in the expression of fatty acid synthase, a gene involved in liver lipogenesis. Together with evidence of gut microbiome modulation, this study shows that traditional kefir has the potential for improving metabolic dysfunction associated with obesity. Notably, differences in kefir microbial populations may influence the ability of traditional kefir to positively impact host metabolic health.

Keywords

Kefir; Metabolic Health; Cholesterol; Gut Microbiome

Abbreviations

Type 2 Diabetes (T2D), Cardiovascular Disease (CVD), Angiotensin Converting Enzyme (ACE), non-alcoholic fatty livery disease (NAFLD), low fat diet (LFD), high fat diet (HFD), high-density lipoprotein (HDL), Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), operational taxonomic units (OTUs), analysis of similarities (ANOSIM)

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 (Bäckhed et al., 2004; Everard & Cani, 2013; Gérard, 2016; Pedersen et al., 2016; Rosenbaum, Knight, & Leibel, 2015). Specifically, the gut microbiota has been found to have an impact on energy metabolism through processes such as bile acid breakdown (Joyce & Gahan, 2016; Ridlon, Kang, & Hylemon, 2006), fatty acid metabolism (Ley, Turnbaugh, Klein, & Gordon, 2006), immunomodulation (Cani et al., 2008; Cani, Osto, Geurts, & Everard, 2012), and regulating host physiology (Zhang, Osaka, & Tsuneda, 2015). 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 (Li et al., 2013), with fermented milk products (Kullisaar et al., 2003) and associated microorganisms (Naruszewicz, Johansson, Zapolska-downar, & Bukowska, 2002) 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 (Bourrie, Willing, & Cotter, 2016). Kefir has demonstrated ACE inhibitory activity (Quiro, 2005), the ability to improve levels of serum cholesterol (H. Liu et al., 2012; J.-R. Liu et al., 2006), and immunomodulatory characteristics (C. G. Vinderola et al., 2005). These attributes, and others

such as bile salt hydrolase activity (H. Liu et al., 2012), 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 (H.-L. Chen et al., 2013; H. L. Chen et al., 2016; Choi et al., 2017; Fathi, Ghodrati, Zibaeenezhad, & Faghih, 2016; Ostadrahimi et al., 2015). 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 (Dobson, O'Sullivan, Cotter, Ross, & Hill, 2011; Marsh, O'Sullivan, Hill, Ross, & Cotter, 2013). Given that these differences impact the fermentation by-products and development of flavour (Walsh et al., 2016), 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 (Dobson et al., 2011; Marsh et al., 2013; Walsh et al., 2016). Additionally, kefir contains *Lactobacillus kefiri* and *L. kefiranofaciens*, both of which have exhibited health benefits *in vivo* (Carasi et al., 2015; Zhou et al., 2013). *L. kefiranofaciens* also produces an exopolysaccharide unique to kefir called kefiran, which has shown beneficial effects *in vivo* (Maeda, Zhu, & Mitsuoka, 2004; G. Vinderola, Perdigón, Duarte, Farnworth, & Matar, 2006). 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 (Dobson et al., 2011; Marsh et al., 2013; Walsh et al., 2016) 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. Materials and Methods

2.1 Kefir Grain Sourcing and Kefir Production

Kefir grains were obtained in a previous study (Marsh et al., 2013) 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°C) 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 (Marsh et al., 2013; Walsh et al., 2016), and have varying microbial composition (table S1).

2.2 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^{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).

2.3 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 (Pal, Abrahamsson, Schwizer, Hebbard, & Brasseur, 2003). Analysis of pH was conducted using an Orion 2 star benchtop pH meter (Thermo Scientific, Burlington, ON).

2.4 *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).

2.5 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.

2.6 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).

2.7 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 S2. 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.

2.8 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 (Willing, Vacharaksa, Croxen, Thanachayanont, & Finlay, 2011). 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 (Caporaso et al., 2010) 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.

2.9 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.

3. Results

3.1 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 (Vujicic, Vulic, & Konyves, 1992), 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 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 1. Cholesterol levels in whole milk following a 24hr fermentation with different kefir grains, expressed in mg/dl. * = P ≤0.05 **=P≤0.01 when compared to Milk

3.2 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 S3). 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 S1). 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).

3.3 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). 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. Weight gain of each groups expressed as a percentage of starting body weight. Means that do not share a letter are significantly different ($P<.05$). N=7-8.

3.4 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 3), 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 3C). However, all high fat diet fed groups had significantly higher levels of liver triglycerides as compared to LFD control.

Figure 3. Plasma total cholesterol (A), non-HDL cholesterol (B), HDL cholesterol (C) and liver triglyceride levels (D) in mice fed different kefir. Levels are expressed in mg/dl for both cholesterol and triglycerides. Means that do not share a letter are significantly different ($P<0.05$). N=7-8

3.5 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 4).

Figure 4. Expression levels of (A) FGF-15 in the ileum and (B) Cyp7a1 in the liver. Expression levels are expressed as fold change relative to HFD using the ΔΔCT method. N=7-8

To examine the effect of kefir feeding on fatty acid metabolism, FASN and 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 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 5. Fatty acid synthase (A) and PPARγ (B) expression levels in the liver, expressed as fold change relative to HFD. Means that do not share a letter are significantly different (P<.05). N=7-8.

3.7 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 6).

Figure 6. IL-18 and IL-1β expression in the ileum expressed as fold change relative to HFD using the ΔΔCT method. Means that do not share a letter are significantly different (P<.05). N=7-8.

3.8 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 S1). 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.

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 (Després & Lemieux, 2006).

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 (Angulo, 2002). 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 (Dorn et al., 2010). PPARγ expression has also been shown to increase in high fat diet induced liver steatosis in mice (Inoue et al., 2005). In our study ICK, IR10, and commercial kefir fed mice showed 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 (Tinting Ju, Li, & Willing, 2016). 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 (Joyce & Gahan, 2016). 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 (Henao-mejia et al., 2012; Stienstra, Tack, Kanneganti, Joosten, & Netea, 2012; Vandanmagsar et al., 2011). As IL-18 and IL-1β are the main cytokines involved in activation of the inflammasome (Guo, Callaway, & Ting, 2015), 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 (Murphy et al., 2016; Vandanmagsar et al., 2011). 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 (Everard & Cani, 2013; Gérard,

2016; Rosenbaum et al., 2015), 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 high fat diet fed groups 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 (Dao et al., 2016) and may contribute 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 (Gu et al., 2013). 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 (Bailey et al., 2011; Konturek, Brzozowski, & Konturek, 2011). 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* (Vujicic et al., 1992). 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 (Hamet, Medrano, Perez, & Abraham, 2016; G. Vinderola et al., 2006), lactic acid (Iraporda, Romanin, Rumbo, Garrote, & Abraham, 2014), and filtered cell free kefir (de Moreno de LeBlanc, Matar, Farnworth, &

Perdigon, 2006; Rizk, Maalouf, & Baydoun, 2009). 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 (Marsh et al., 2013), 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 (Dertli & Çon, 2017; Walsh et al., 2016). 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 and plasma cholesterol levels as the HFD control group, while the reduction observed in liver triglycerides was not significant. 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 (St-Onge et al., 2002). 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 (H.-L. Chen et al., 2013; D. H. Kim et al., 2017) and obesity (Fathi et al., 2016; D.-H. Kim et al., 2017). 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 *Streptococcus 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.

5. Conclusion

Our findings show that traditional kefir has promise in reducing adverse metabolic outcomes associated with a high fat western diet. We 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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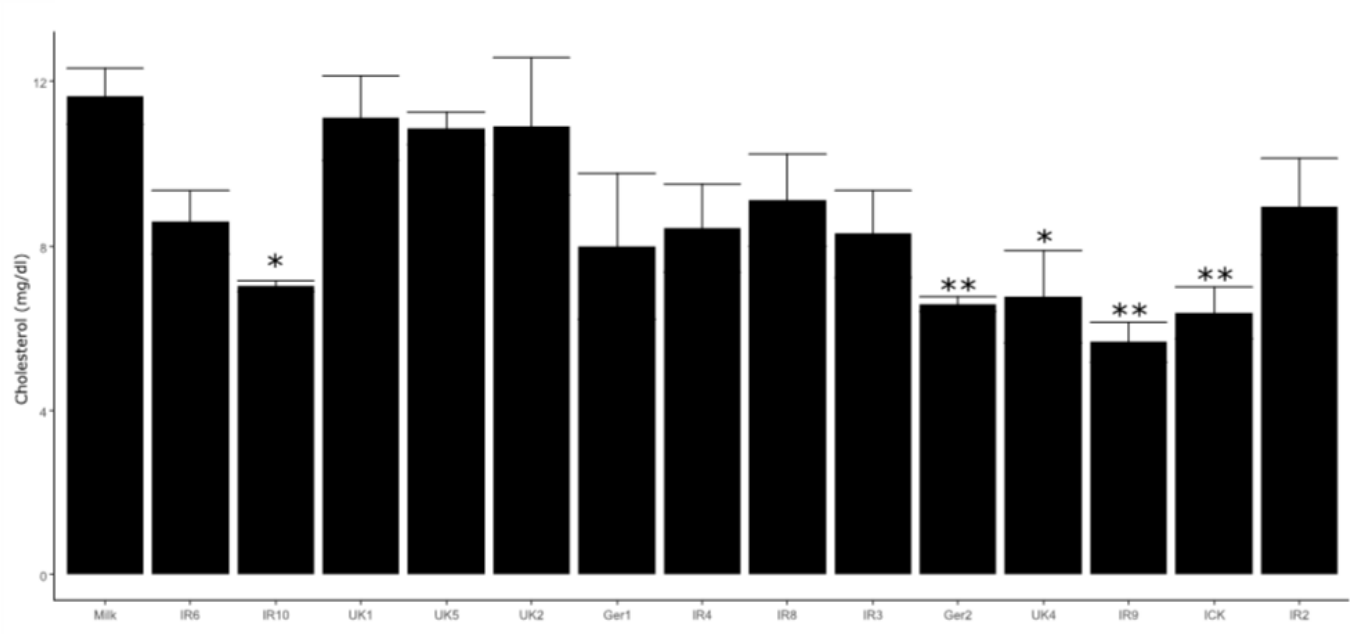
Figures

Figure 1



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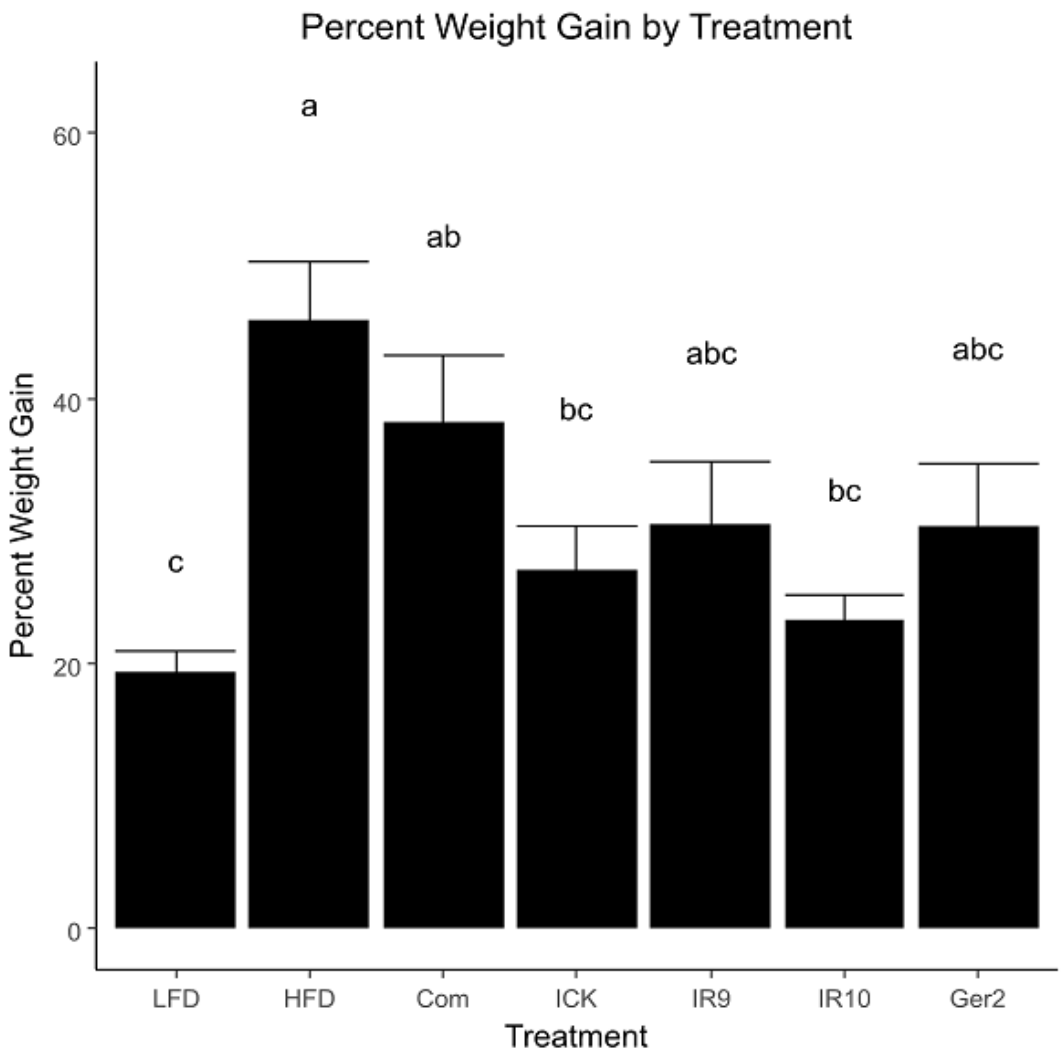

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Figure 3

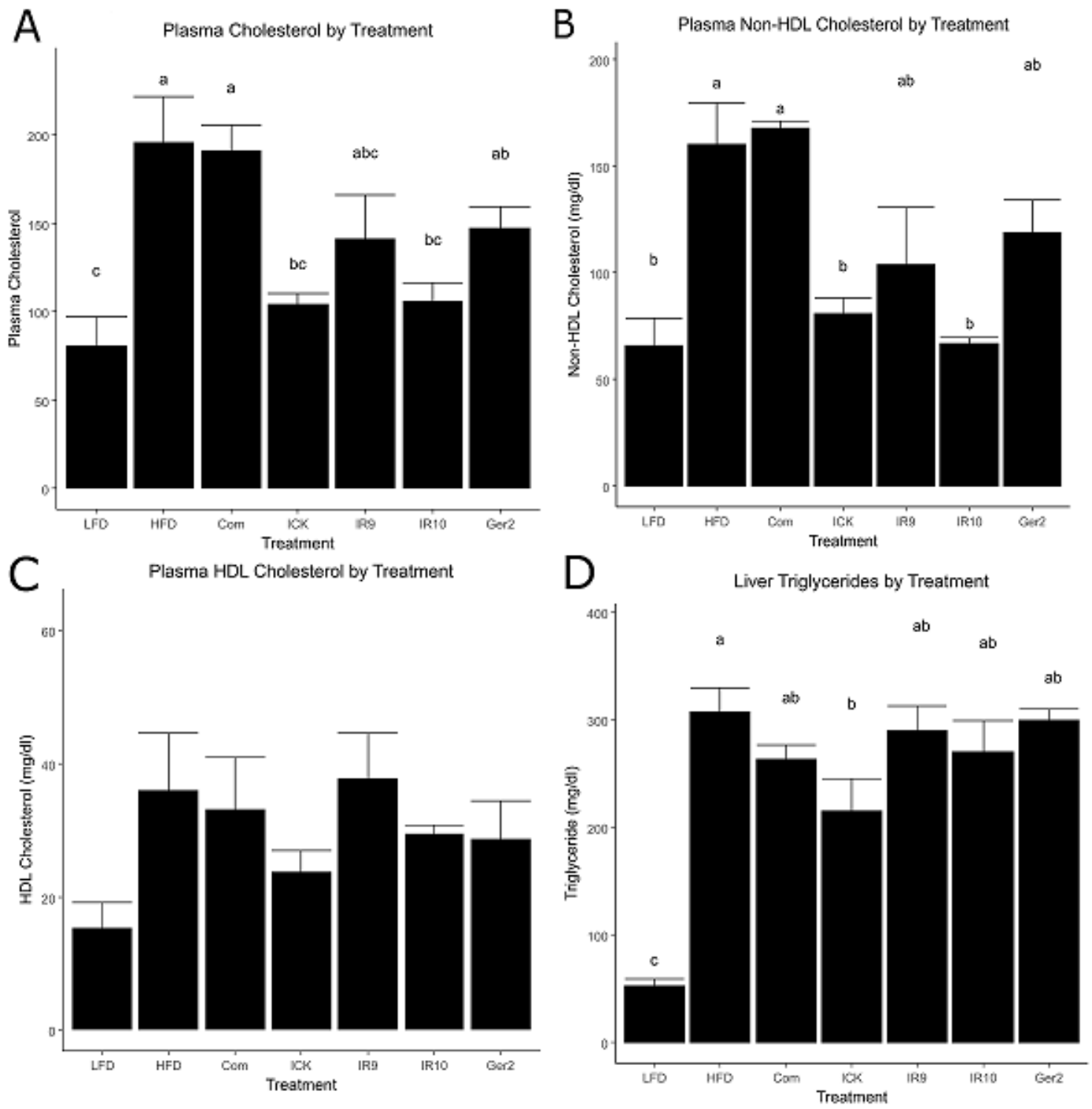
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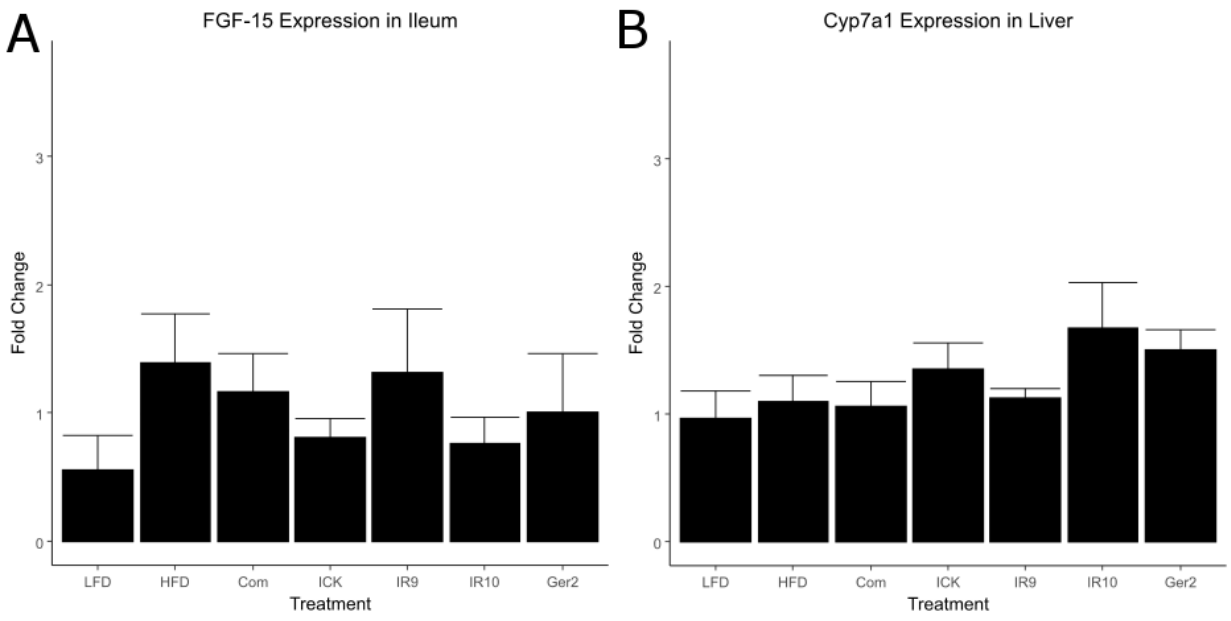
Figure 4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673 Figure 5

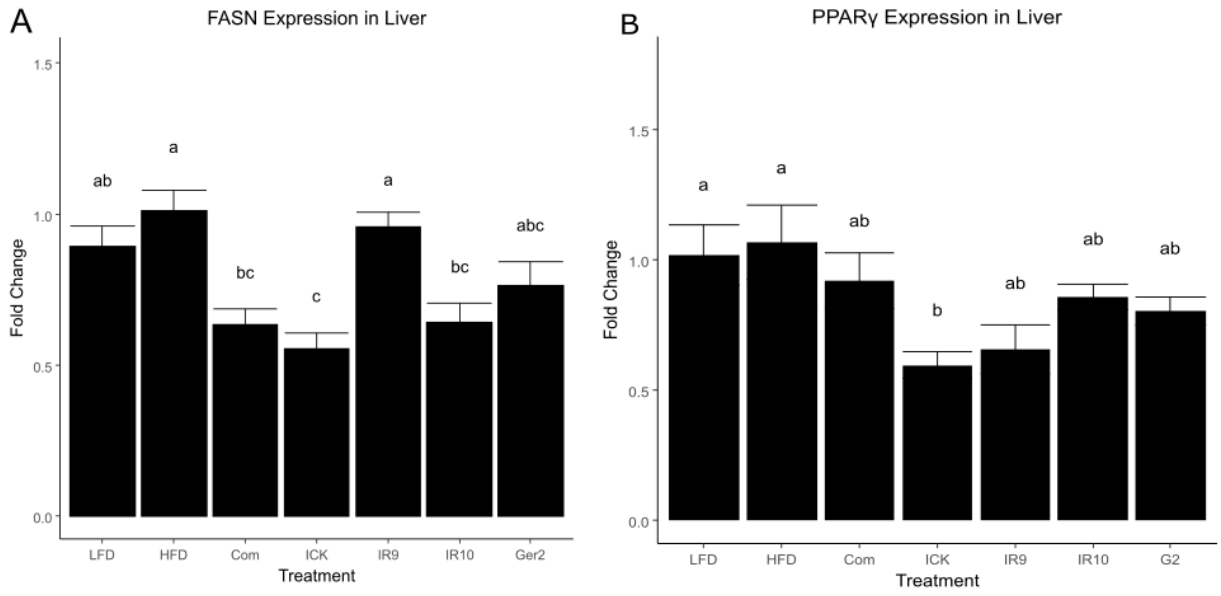

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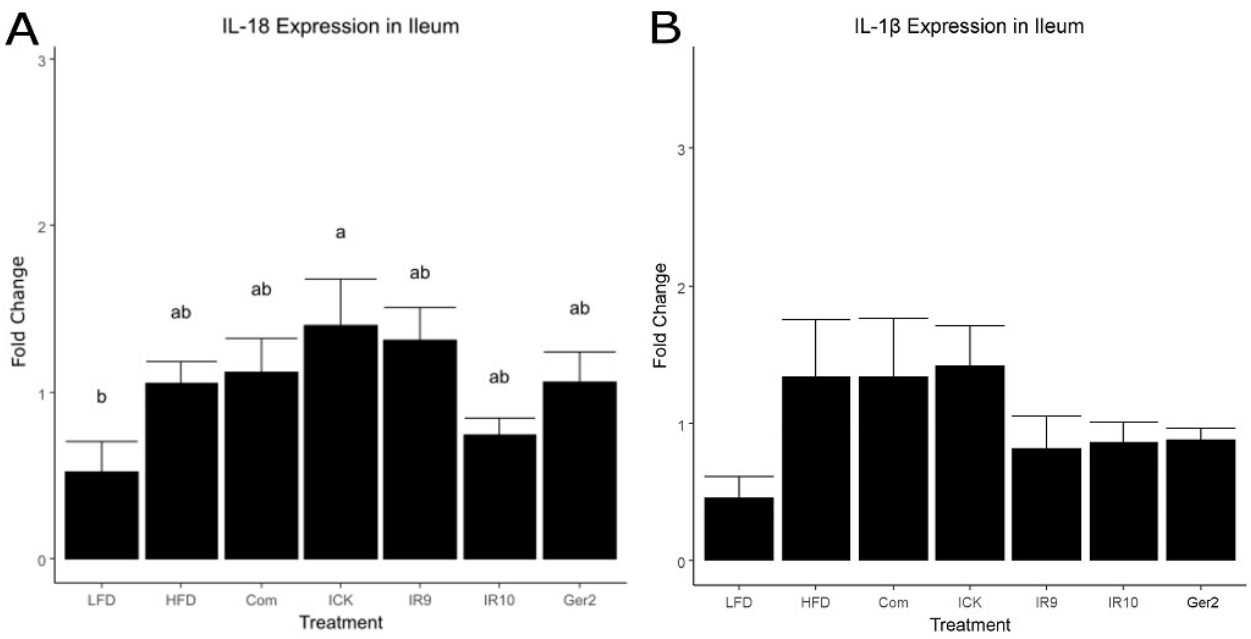
Figure 6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697 Figure S1

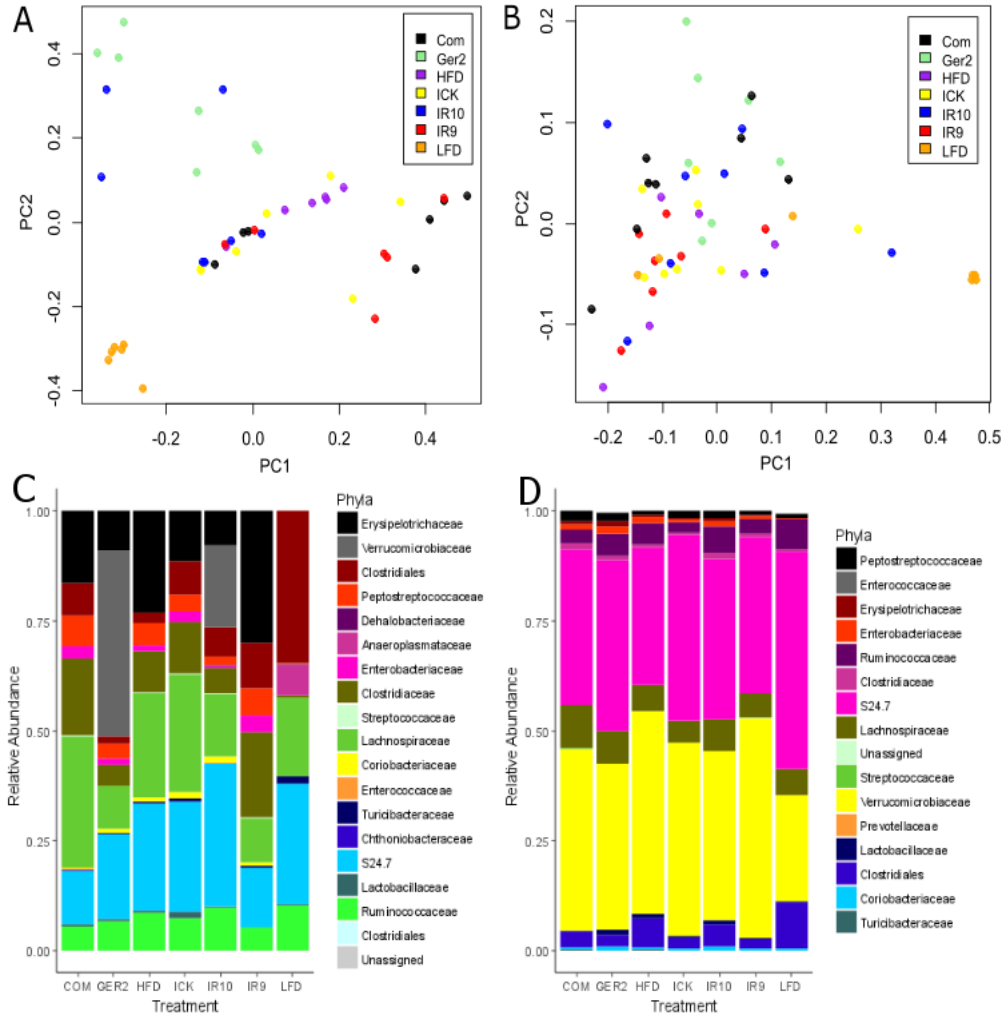

Figure S1. Principle coordinate analysis (PCoA) ofBray Curtis dissimilarity matrix for (A) day 28 faecal and (B) day 84 caecal microbiota as well as stacked bar charts representing the relative abundance at Family level for (C) day 28 faecal and (D) day 84 caecal microbiota.

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Table S1

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

Supplementary Table 1. Relative abundance of bacterial and fungal genera in the four traditional kefir used in this study

713 Table S2

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

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715 Table S2. Specific primer sequences used for quantitative real-time PCR. GAPDH: Glyceraldehyde 3-
716 phosphate dehydrogenase; FGF-15: Fibroblast growth factor 15; Cyp7a1: Cytochrome P450 family 7
717 subfamily A member 1; PPARγ: Peroxisome proliferator-activated receptor gamma; FASN: Fatty acid
718 synthase; IL-18: Interleukin 18.

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731 Table S3

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}

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733 Supplementary Table 3. Viscosity and pH of traditional kefirs used in this study following an 18 hour
734 fermentation. Viscosity was measured at a shear rate of 3.5 Pascal/second.

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749 Table S4

Genus/Family	P	ICK	LFD	IR9	IR10	Ger2	Com	HFD
Blautia	2.67E-05	0.118272	0	1.54E-05	1.51E-05	4.47E-06	2.30E-06	7.16E-06
Erysipelotrichaceae genus unassigned	4.78E-05	0.111113	0.000404	0.297744	0.077329	0.0852	0.102288	0.221883
Akkermansia	6.90E-05	0	5.07E-05	2.94E-05	0.184574	0.422434	2.30E-06	1.58E-05
Epulopiscium	7.34E-05	0.10493	6.73E-06	0.042293	0.068053	0.037031	0.2329	0.162398
Clostridiales family unassigned genus unassigned	7.93E-05	0.075759	0.345705	0.104624	0.067517	0.017508	0.07277	0.024948
Peptostreptococcaceae genus unassigned	0.000128	0.038614	0.000392	0.060215	0.019229	0.032435	0.069698	0.049866
Dehalobacterium	0.000153	5.30E-05	0.002575	0	0.001491	0	1.80E-05	1.39E-05
Anaeroplasma	0.000159	2.51E-05	0.069311	0.00028	5.34E-05	0	0.000241	0
Citrobacter	0.000185	0.022364	3.90E-06	0.038694	0.006466	0.013921	0.027365	0.012821
Lactococcus	0.000424	0.002381	0	0.003071	0.00206	0.000982	0.002706	0.002016
Clostridium	0.001256	0.090775	1.90E-05	0.099827	0.052446	0.040386	0.091779	0.076546
Coprobacillus	0.002071	0.00387	8.44E-05	0.00192	0.00184	0.002949	0.003367	0.009426
Coriobacteriaceae genus unassigned	0.00233	0.014812	9.21E-05	0.004878	0.014871	0.010318	0.002152	0.007769
Coprococcus	0.002362	0.005565	0.003416	0.002781	0.000296	0.003692	0.004389	0.012067
Eubacterium	0.002699	0	0	0	0	0.001409	0.059179	0
Enterococcaceaegenus unassigned	0.015149	0.000389	0	0.001694	0.000286	0.000293	0.001201	0.000333
Ruminococcus	0.015396	0.004485	0.006089	0.001828	0.008329	0.004244	0.006122	0.005566
Clostridiaceae genus unassigned	0.016222	0.026995	0.004346	0.093302	0.003	0.008224	0.083403	0.017013
Ruminococcaceae genus unassigned	0.019342	0.012499	0.015909	0.00136	0.012918	0.006044	0.00612	0.008032
Lachnospiraceae genus unassigned	0.029061	0.027489	0.158359	0.041454	0.058071	0.040633	0.047461	0.039407
Turicibacter	0.046729	0.004858	0.016611	0.003497	0.000821	0.002662	0.001634	0.005038
Oscillospira	0.052924	0.024691	0.038367	0.013129	0.045261	0.023167	0.015457	0.034146
Dorea	0.062129	0.005197	0.002764	0.003022	0.010273	0.007966	0.006508	0.012024
Ruminococcus	0.11049	0.005568	0.015593	0.01053	0.005178	0.006721	0.006914	0.011716
Delftia	0.130953	0	7.02E-06	0	0	8.18E-06	0	0
S24-7 genus unassigned	0.163779	0.2517	0.27422	0.135991	0.324165	0.191931	0.122652	0.243108
Lactobacillus	0.243882	0.014417	0.001669	0.000772	0.002089	0.004246	0.003867	0.004652
Unassigned	0.508077	0.000393	0.000572	0.000368	0.000638	0.000292	0.000284	0.000309
Ruminococcaceae genus unassigned	0.776417	0.031822	0.041871	0.03602	0.031364	0.034436	0.0281	0.038469

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751 Table S4. Relative abundances and P values (calculated by ANOSIM) of bacterial genera/families in the
752 faecal microbiota at day 28.

Genus/Family	P value	ICK	LFD	IR9	IR10	Ger2	Com	HFD
Ruminococcaceae genus unassigned	2.98E-05	0.003626	0.015243	0.004191	0.008386	0.005276	0.000778	0.001387
Eubacterium	0.000269	0.000181	4.05E-05	0.000396	0.000203	0.002717	0.001814	0.000255
Coprococcus	0.000417	0.001049	0.001603	0.003029	0.003746	0.000752	0.002393	0.00417
Epulopiscium	0.002252	0.032719	0.00988	0.031267	0.023693	0.043536	0.061952	0.016324
Dorea	0.004681	0.001225	0.001657	0.002674	0.007922	0.002674	0.004595	0.00151
Peptostreptococcaceae genus unassigned	0.01137	0.016139	0.009422	0.008827	0.016878	0.01923	0.024697	0.009847
Ruminococcaceae genus unassigned	0.015865	0.010217	0.022981	0.016956	0.028286	0.031496	0.011438	0.032871
Lachnospiraceae genus unassigned	0.030471	0.008576	0.039975	0.009903	0.020062	0.014635	0.011361	0.027957
Citrobacter	0.030671	0.007384	0.002579	0.008725	0.012192	0.016885	0.010719	0.015153
Clostridium	0.034922	0.003838	0.003041	0.007168	0.012883	0.008205	0.013383	0.004823
Erysipelotrichaceae genus unassigned	0.047276	0.002303	0.000786	0.001709	0.006695	0.006924	0.004886	0.003965
Coprobacillus	0.09503	0.000344	9.26E-05	0.000197	0.000503	0.003281	0.00064	0.000344
S24-7 genus unassigned	0.108435	0.420998	0.493339	0.354305	0.36574	0.387891	0.352851	0.312552
Blautia	0.132584	0.00397	0.002503	0.004769	0.010553	0.007361	0.01364	0.002369
Unassigned	0.148857	0.000346	0.000222	0.000478	0.000546	0.000359	0.000532	0.000583
Clostridiaceae genus unassigned	0.180998	0.000692	0.000479	0.000406	0.000765	0.001008	0.000374	0.001125
Akkermansia	0.242513	0.438458	0.241041	0.498901	0.384428	0.376875	0.41381	0.459001
Oscillospira	0.267119	0.008845	0.026817	0.010423	0.020202	0.011543	0.018403	0.01196
Lactobacillus	0.319268	0.00473	0.002053	0.001302	0.007424	0.012255	0.002469	0.009768
Ruminococcus	0.337874	0.001045	0.00414	0.001075	0.002496	0.001449	0.001539	0.001258
Coriobacteriaceae genus unassigned	0.34322	0.003721	0.002329	0.002477	0.008817	0.008655	0.004839	0.004631
Clostridiales family unassigned genus unassigned	0.345019	0.024256	0.105405	0.023342	0.050533	0.025376	0.034996	0.066912
Turicibacter	0.441948	0.001104	0.001935	0.001893	0.001982	0.00146	0.002959	0.003259
Ruminococcus	0.756579	0.003321	0.005191	0.004402	0.0042	0.004478	0.003947	0.007197

753 Table S5

754 Table S5. Relative abundances and P values (calculated by ANOSIM) of bacterial genera/families in the
755 caecal microbiota at day 84

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