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EVALUATING TRADITIONAL AND COMMERCIAL KEFIR PRODUCTS FOR
HUMANS AND COMPANION ANIMALS

BY

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DISSERTATION

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Abstract

Many traditional fermented foods, such as the dairy beverage kefir, are produced by small-scale artisans using milk and kefir grains. Increasing interest in fermented foods has demanded higher production, thus food and beverage companies have commercialized the fermentation process of kefir. To do this, companies have removed the use of kefir grains, which host novel microorganisms such as *Lactobacillus kefiranofaciens* and *Kluyveromyces marxianus* and contribute flavor compounds, carbonation, exopolysaccharides, and peptides. The resulting products sold in stores contain microbes starter cultures, but none are garnered from kefir grains.

Given the lack of research in the field, the first aim was to evaluate five commercial kefir products for humans for label accuracy, microbial consistency, and viable live microorganisms. Our objectives were to measure viable microbial counts, characterize bacterial taxa of commercial kefir products, and compare enumeration and taxonomy data to label claims. To enumerate viable lactic acid bacteria (LAB), products were plated upon opening and following 14 d on deMan Rogosa and Sharpe (MRS) agar and incubated under anaerobic and aerobic conditions. High-throughput full-length 16S rRNA sequencing was used to assess microbial composition. Overall, each product was lacking in one or all categories. Our findings revealed that 66% of products with a guaranteed count of colony-forming units (CFU)/g overstated microorganism density by at least 1 log and that a moderate level of labeling accuracy is still needed for commercial kefir products intended for human consumption.

The second aim was to evaluate six companion animal commercial kefir products, with the objectives of measuring viable microbial counts and characterizing bacterial taxa of commercial kefir products, and comparing them to label claims. High-throughput full-length 16S rRNA sequencing revealed significant microbial labeling inaccuracies and culturing techniques demonstrated there were fewer live microorganisms present at the time of opening than what the

labels claimed. Microbial plating methods from Aim 1 were repeated for the companion animal products. Five products (83%) listed specific microorganisms, and four products (66%) guaranteed CFU/g on their label. Results from point of opening revealed that all commercial kefir products with a guaranteed CFU/g overstated the number of microorganisms present by at least 1 log, with only one product exceeding 1×10^9 CFU/g. Sequencing results demonstrated that none of the labels claiming specific bacterial genera and species on their labels were correct, and all products contained at least three additional bacterial species above the minimum detectable threshold (0.001% relative abundance) not disclosed by the manufacturer. The results from this aim demonstrated a low level of accuracy in the labeling of commercial kefir products intended for use in dogs and cats.

The objective of the third aim was to test the effects of commercial or traditional kefir supplementation on apparent total tract macronutrient digestibility (ATTD) and fecal characteristics, microbiota populations, and metabolite and immunoglobulin (Ig) A concentrations of healthy adult dogs. Using a 3x3 replicated Latin square design, 12 healthy dogs were provided 60 mL/day of either non-fermented milk (control), commercial kefir, or traditional kefir for 28 d. Fresh fecal and blood samples were collected and analyzed at the end of each experimental period. Our results found that kefir supplementation had minor effects on the fecal microbiota populations, with a significantly higher relative abundance of *Lactococcus* in feces of dogs fed the traditional kefir (0.47%) than the controls (0.00%) or those fed the commercial kefir (0.00%). No significant differences were observed in ATTD, fecal characteristics, and fecal metabolite and IgA concentrations.

The fourth aim was to use in vitro fermentation assays to determine the fermentation characteristics of dietary fibers using 1) fecal inoculum from dogs supplemented with starter- or

grain-based kefir; and 2) a starter bacterial culture or a grain kefir culture as inoculum. Canine feces or kefir inoculum were added to tubes containing a semi-defined medium and one of the fiber sources: pectin, cellulose, beet pulp, or chicory pulp. Over 18 h, increases in butyrate were greater ($P<0.05$) in tubes inoculated with feces from dogs fed grain kefir than those fed starter kefir or control tubes, and greater ($P<0.05$) in tubes inoculated with feces from dogs fed starter kefir than controls. In vitro fermentations inoculated with kefir demonstrated that the microbiota present in both kefir were able to ferment the fibers, but at a slower rate and incomplete metabolite profile.

In conclusion, our data suggest that the current labeling of kefir products is often inaccurate and requires improvement as fermented beverages increase in consumption by both humans and companion animals. It is clear that the inclusion of kefir grains significantly impacts the microbial community of kefir beverages. Modifying the fermentation process by excluding kefir grains significantly impacts alpha diversity, minimally impacts the gut microbiota in vivo, and modifies the fermentation of dietary fibers and subsequent SCFA production in vitro. These studies have explored the different effects that grain and commercial kefir can have in dogs and how they impact microbial functionality in vitro. These data provide justification for further research into kefir products and other fermented foods and beverages.

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“There is a principle which is a bar against all information, which is proof against all arguments, and which cannot fail to keep a man in everlasting ignorance – that principle is contempt prior to investigation.”

Herbert Spence

1931

This dissertation is dedicated in loving memory to my grandma, Lorna T. Glendinning.

April 13, 1942 – September 3, 2023

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CHAPTER 1: INTRODUCTION

With increasing consumer interest in fermented food, better scrutiny of microbial and fungal communities is needed especially when attempting to determine physiological outcomes, mechanisms of action, and conservation of authentic microbial profiles traditionally found in fermented foods (Dobson et al., 2011; Markowiak-Kopeć and Śliżewska, 2020; Walsh et al., 2023). The fermented food market is currently valued at \$1.83 trillion in 2024, with an average growth rate of 6.7% estimated in the next five years (Mordor Intelligence, 2024). As new products continue to enter the market, labeling integrity is crucial for accuracy and to better attribute conserved effects from specific fermented foods (Mukherjee et al., 2022). The fermented dairy beverage kefir can be produced through traditional methods by inoculating bacterial kefir grains with milk, or through the combination of preselected starter bacteria and milk for large-scale commercial production (Rosa et al., 2017).

Due to a lack of reporting or labeling standards, many commercial kefir products do not contain clear ingredient labeling, leading to unintentionally vague disclosures of microbial contents (Nejati et al., 2020a). Specific deviations in the production of fermented foods and defining their composition will become more important in the context of dietary research interventions, as fermented food consumption increases, and as companies adjust large-scale fermentation methods that may alter the microbial contents (Hansen, 2002; Bourrie et al., 2016; Nejati et al., 2022).

Kefir is a Slavic word meaning 'well-being' or 'living well'. The origins of kefir can be traced back to the Caucasus, Tibetan, and Mongolian Mountains in Asia, where kefir grains have been recovered and dated back to 2,000 years ago (Guzel-Seydim et al., 2011). Historically, kefir grains were passed down through generations as a form of family wealth. The inclusion of kefir

grains differentiates the beverage from other fermented dairy due to the specific and complex symbiotic composition of the grains (Rosa et al., 2017). The beverage can be produced from various types of milk, including cow, goat, camel, ewe, sheep, or buffalo milk, with higher fat content resulting in a creamier kefir (Verruck et al., 2019). Kefir's popularity has expanded beyond Middle Eastern regions due to increased industrial systems transporting and producing milk (Kazou et al., 2021). Today, it is consumed worldwide and is one of several popular fermented foods that contribute to a person's daily intake of live dietary microbes (Iyer et al., 2024).

Recently, there has been a growing number of publications exploring the health-affecting properties of fermented foods with novel microbial profiles including kefir (Rezac et al., 2018). According to the Codex Standard for Fermented Milks, kefir is defined as fermented milk prepared from kefir grains containing specific microorganisms, including *Lentilactobacillus kefiri*, *Leuconostoc*, *Lactococcus*, *Lactobacillus kefirianofaciens*, and lactose-fermenting yeasts (Wang et al., 2020). Kefir fermentation involves adding kefir grains to milk, incubating for 22-24 hours at 25°C, and results in a final pH of 4.2-4.6. The fermentation process includes the digestion of lactose in milk, producing lactic acid, flavor compounds, ethanol, and CO₂ (Rosa et al., 2017). The unique feature of kefir fermentation is the recycling of grains through a practice known as backslopping, which facilitates the activity of endogenous enzymes, making the product and nutrients more bioavailable (Bourrie et al., 2016; Heilbronner et al., 2021). Variations at different points in fermentation will impact the resulting overall flavor, food matrix, and microbial profile, which makes mass production of grain kefir difficult (Nejati et al., 2022).

Kefir grains are a symbiotic culture of yeasts and bacteria, forming semi-soft granular particles. The grains consist of a protein-polysaccharide matrix called kefiran and a water-soluble galactoglucan portion (de Sainz et al., 2020). The microbial composition of kefir grains includes

both heterofermentative and homofermentative bacteria, along with low-abundance fungi (Rosa et al., 2017; Georgalaki et al., 2021; González-Orozco et al., 2023). Differences in grain composition affect the production of volatile compounds, which are correlated with specific bacterial and yeast abundances (Diez-Ozaeta and Astiazaran, 2022). For example, *L. kefiranofaciens* has been shown to correlate with flavor compounds 2-nonanone and *n*-decanoic acid whereas *Leuconostoc mesenteroide* correlates with the production of 2,3-butanedione and acetic acid compounds (Walsh et al., 2016). Due to the stark differences in production and profile, commercially-produced kefir more closely resembles dairy yogurt in flavors, food matrix, and microbe profiles than it does with traditionally-produced kefir (Nejati et al., 2022). Commercial kefir uses manufactured starter bacterial cultures, resulting in a more consistent product for large-scale production (Mukherjee et al., 2022; Walsh et al., 2023). However, this approach removes the microbial diversity present in kefir grains, reducing alpha diversity, bioactive peptides, exopolysaccharides, and palatable product characteristics (Bourrie et al., 2018). Recent sequencing analysis of commercial kefir products revealed dominance by specific yeasts, as well as differences in microbial diversity, nutrient composition, and volatile organic compound profile compared with grain kefir products (Dobson et al., 2011; Leite et al., 2013; Nejati et al., 2020a; de Sainz et al., 2020).

A myriad of health benefits are associated with kefir consumption, such as improvement of blood lipids (Liu et al., 2006; Liu et al., 2011; Bourrie et al., 2018; Bourrie et al., 2021), immunomodulatory effects (Vinderola et al., 2006), and alleviation of inflammatory gastrointestinal symptoms (Yilmaz et al., 2020). Studies using humans and animal experimental models have explored the impact kefir consumption has on immune health, gut microbiome modulation, and the reduction of metabolic syndrome symptoms (Bellikci-Koyu et al., 2019; Kim et al., 2019; Gaspardo et al., 2020; Walsh et al., 2023). The impact that regular kefir consumption

has on type 2 diabetes is the most well-studied; with HbA1c shown to significantly reduce delta level group effects ($p < 0.01$) and decrease fasting blood glucose levels ($p < 0.015$) (Jiang et al., 2022). Symptoms from inflammatory bowel disease and effects on mucosal barrier integrity have also been shown to improve with regulator kefir consumption, but more research is needed to understand the specific mechanisms and potential variations based on kefir composition (Yilmaz et al., 2020; Bourrie et al., 2021). Traditional kefir consumption has been associated with weight loss, decreased serum lipid levels, and improved immune responses (Bourrie et al., 2016; Kim et al., 2019; Bourrie et al., 2021). However, the variability of microbial composition between kefir products lacks conservative and clear discrimination in clinical trials; resulting in inconclusive findings (St-Onge et al., 2002; Fathi et al., 2016; Bellikci-Koyu et al., 2019).

The research aims of this dissertation were to characterize and compare microbial communities of commercial and grain kefir products, investigate the physiological effects of kefir supplementation in healthy dogs, and understand the in vitro fermentation dynamics of fibers inoculated with starter bacterial culture, grain kefir culture, or canine feces. In the assessment of kefir products, the objectives were to measure viable microbial counts, characterize bacterial taxa of commercial kefir products intended for human and companion animal consumption, and compare enumeration and taxonomy data to label claims. During a canine in vivo kefir intervention study, key objectives were to determine the effects of commercial or traditional kefir supplementation on apparent total tract macronutrient digestibility and fecal characteristics, microbiota populations, metabolite concentrations, and immunoglobulin A concentrations of healthy adult dogs. In the first in vitro fermentation experiment, feces from dogs consuming starter or grain kefir were used to determine the in vitro fermentation characteristics of dietary fibers common in pet foods. In the second in vitro fermentation experiment, starter kefir or grain kefir

were used to inoculate tubes and test the fermentation dynamics of the same dietary fibers. The research is significant in contributing to the understanding of microbial dynamics in kefir, particularly in the context of canine health. By elucidating the microbial differences and physiological effects, these studies provide valuable insights into the potential benefits or drawbacks associated with different kefir products, informing both the scientific community and consumers.

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CHAPTER 2: LITERATURE REVIEW

Gastrointestinal (GI) Microbiome

Gut Physiology and Gut Microbiome

Microbiome ecosystems thrive throughout the human host, including the oral cavity, within pores on our skin, and the gastrointestinal tract (GIT). Residential microorganisms are thought to encode over 45,666,334 non-redundant genes in the human oral and gut microbiome, which dwarfs the 20,000 to 25,000 genes present in the human genome (Tierney et al., 2019). The GIT is the pivotal point for communicating the host's health status throughout the body; it reflects changes to the host's diet, environment, and lifestyle. Each physiological region of the GIT is unique in terms of substrate availability, pH, oxygen tension, host secretions, and transit time. Microbiota niches are considered self-discrete communities cohabitating within their host and are made up of bacteria, archaea, and eukaryota (Flint et al., 2012). While a select few bacteria can survive the acidic conditions of the stomach (from 0.9 to 1.5-3.0 pH), the environment of the small intestine is slightly more favorable for colonization (Castro-Lopez et al., 2022). However, the slightly acidic conditions (pH ~6.2), high levels of enzymatic activity, and short residence time of digesta in the small intestine (e.g., 3-5 h) provide a challenging environment for microbiota unable to adhere to the intestinal mucus (Castro-Lopez et al., 2022; de Vos et al., 2022).

Oxygen tension decreases as bacterial colonization increases and oxygenation occurs (He et al., 1999). Using electron paramagnetic resonance oximetry to measure low-oxygen tension environments, the oxygen tension in the mouse duodenum has been reported to be 32 PO₂ mmHg (Zheng et al., 2015). The amount of diffusive oxygen movement and resulting partial pressure depends on several factors such as the partial pressure gradient of oxygen, the available surface area to diffusion, the thickness and permeability of diffusion barriers, and local metabolic demand

(Ortiz-Prado et al., 2019). Many bacteria survive in hypoxic conditions (<12% O₂, 90 mmHg), which are common through the GIT. As bacteria proliferate, the greater oxygen tension increases the reduction reaction of oxygen to superoxide anion (O₂⁻). These reactive oxygen species can interact with other compounds to generate more destructive molecules that can be bactericidal (Park et al., 1999).

The jejunum and ileum host gram-positive facultative anaerobic bacteria such as *Streptococcus*, *Lactobacillus*, *Enterococcus*, and gram-negative bacteria such as Proteobacteria and Bacteroides. The ileum is an interface for immune system interaction with resident microbes, who synthesize short-chain fatty acids (SCFA) and other bioactive components that initiate the production of B cells and memory T cells (Shi et al., 2017). Compared with the small intestine, the large intestine provides a more suitable environment for microbial colonization. Digesta residence time is longer (20–50 h) and a neutral to mildly acidic pH exists (5.5–7.5 pH), with variations of oxygen tension at each location in the large intestine (Castro-Lopez et al., 2022). In humans, the terminal ileum has a low-oxygen tension of 34 PO₂ mmHg, the cecum is around 30 PO₂ mmHg, and the sigmoid colon or end of the large intestine is around 39 PO₂ mmHg (Sheridan et al., 1990; Zheng et al., 2015). The sigmoid colon is dominated by obligate anaerobes such as Firmicutes and Bacteroides (Hassen., 2012).

Commensal microbes provide the host with protection against pathogenic infection through many mechanisms: competition for nutrients, competition for binding sites, bacteriocin and metabolite production, and communication with the host's immune system. In some cases, commensals suppress the expression of lipopolysaccharide (LPS) (Everarda et al., 2013). Commensals are also known to produce antimicrobial peptides such as defensins and lysozymes, which attack pathogenic bacteria (Martino et al., 2022). These residential microbes may also

interact directly with the host immune system. Naive B cells receive activating antigen signals which initiate the migration of B cells to the borders of lymphoid follicles where they can interact with T cells, cytokines, and Toll-like receptors that secretory immunoglobulin A (IgA). IgA antibodies interact with and neutralize pathogenic microbiota by performing agglutination or bacterial clustering. Intestinal IgA also interacts with commensals to maintain a balanced microbiome and generate a feedback loop producing more FOXP3⁺ regulatory T cells and sustaining IgA concentrations (Zheng et al., 2020). Without the presence of B cells or IgA, immune mechanisms mediated by interferon-gamma (IFN- γ)-inducible pathways may be upregulated to induce a multifaceted host immune response. This response may include effects such as the stimulation of bactericidal activity in phagocytes, orchestration of leukocyte-endothelium interactions, changes to cell proliferation and apoptosis, and the stimulation or repression of a variety of genes with obscure functional significance (Zheng et al., 2020). There is a delicate balance between maintaining homeostasis and managing elements of inflammation.

The makeup of the gut microbiota determines which genes are expressed to produce metabolites and contribute to metabolic pathways. Residential microbes may express bile salt hydrolases, gain energy from the fermentation of non-digestible substrates, and digest amino acids escaping host digestion. This allows bacteria to produce vitamins and organic acids, which affect local pH and available oxygen. Changes in the environment such as a pH change or decreased oxygen send signals into the local environment to facilitate immune system crosstalk. Microbial metabolites may contribute to cross-feeding between bacteria and ultimately signal the GIT's immune status to the brain via the vagus nerve (Flint et al., 2012; Leeming et al., 2021).

The mucus layer of the small and large intestinal epithelia provides a protective layer from toxins and pathogens. Three layers of mucin cover the epithelium of the colon, whereas the small

intestine has only one layer. Mucins consist of large, glycosylated proteins made up of thousands of amino acids with *O*-glycan attachment sites, which not only provide protection but energy for certain commensals, notably *Akkermansia Muciniphila* (Dicks et al., 2018). However, the cells that make up the epithelial layer are connected by tight junctions, adherent junctions, and gap junctions that allow amino acids and vitamins to enter the bloodstream across the gut-vascular barrier. The gut-vascular barrier is selective and vulnerable to pathogens and environmental disturbances. Thus, the epithelial lining is the first line of defense for the gut vascular barrier and can become inflamed due to damage. Inflammation of epithelial cells can affect intestinal permeability and encourage a dysbiotic state whereby abnormal bacterial growth is associated with the dysregulation of the host's immunoinflammatory response (Dicks et al., 2018). A state of dysbiosis may occur when mucosal immune responses are modulated during gut inflammation. In response to inflammation and dysbiosis, epithelial cells may secrete antimicrobial compounds such as bactericidal defensin peptides that disrupt the membrane integrity of pathogenic microorganisms and prevent their translocation into tissue (Bevins and Salzman, 2011). Epithelial tight junctions are weakened by inflammatory compounds such as procalcitonin, adrenomedullin, and histamine, with GI mucus production being reduced (Hellenthal et al., 2022). Collectively, the weakened barrier exposes the mucosal immune system to luminal contents, which triggers the release of proinflammatory cytokines such as IL-6, IL-17, and IL-22 (Bevins and Salzman., 2011; Fakhoury et al., 2014). Bacteria produce a wide range of compounds to protect the intestinal lining and communicate the fitness of local sites and species to the host immune system. Endogenously-produced compounds may include branched-chain fatty acids (BCFA), SCFA, phenols, indoles, ammonia, bacteriocins, and immunoregulatory peptides, which also send important signals about the environment to the immune system.

Diet and the Human GI Microbiome

Diet is an important modulator of the gut microbiota, evidenced by whole community shifts when eating patterns are changed (Willis and Slavin, 2020). Dietary nutrient consumption and digestibility dictate which substrates are available for the GI microbiota. Most GI microbes obtain energy through the fermentation of dietary fibers and resistant starches, leading to the production of SCFA such as butyric acid, propionic acid, and acetic acid; but some are derived from non-digestible dietary proteins as well. SCFA are organic acids produced by the gut microbiota in the large intestine as products of fermentation from food components unabsorbed or undigested in the small intestine. These acids are characterized by containing less than six carbons and have a branched or straight chain configuration. Foods leading to high production of SCFA may include wheat bran, whole grain bread, or inulin from chicory root (Rios-Covian et al., 2016). A review paper that summarized nutritional-microbiome study results concluded that eating patterns high in fiber and antioxidants (e.g., olive oil, coffee, tea, nuts, fruits, vegetables, herbs, and spices) improve gut microbiome stability (Flint et al., 2012). High-fiber diets fuel microbes and lead to the production of mucins by intestinal goblet cells, which protects the gut mucosal layer from pathogen transport, as well as SCFA. The production of SCFA also reduces local luminal pH, which can inhibit pathogenic microorganism growth and increase mucin production to alter the rate of bacterial adhesion (Bevins and Salzman., 2011). Meanwhile, diets following a Western diet pattern low in dietary fiber, yet high in animal proteins, added sugars, ultra-processed foods, and saturated fats, increase the chance of gut dysbiosis (Filippo et al., 2010; Flint et al., 2012; Ghosh et al., 2020; Bolte et al., 2021; Leeming et al., 2021; Wastyk et al., 2021; Cotillard et al., 2022).

Geographical location can affect which microbes are dominant in the host in addition to diet and genetics (Nishijima et al., 2016). People living in Western countries such as the United States

have colonic microbiota profiles dominated by *Firmicutes*, *Bacteroides*, *Actinobacteria*, *Proteobacteria*, and *Verrucomicrobiota* phyla, with 92% being comprised of *Firmicutes* and *Bacteroides* (Flint et al., 2012). In contrast, research conducted on people living in Japan has shown a higher abundance of *Actinobacteria* and the genus *Bifidobacterium* than in other nations (Nishijima et al., 2016). Environmental factors, including diet, were shown to affect the abundance of common genera such as *Bifidobacterium*, *Bilophila*, *Oscillibacter*, and the *Prevotella copri* species in Dutch adults (Gacesa et al., 2022). In older adults, the gut microbiota undergoes further changes in which it declines in *Bifidobacterium* relative abundance and increases in Enterobacteriaceae (Flint et al., 2012; Ghosh et al., 2020; Martino et al., 2022). The various factors that can impact a person's gut microbiome demonstrate that variation among people is common and dynamic.

When the gut microbiome became an emerging field in the 2000s, researchers used germ-free (GF) mice models to better understand the complex interplay between the host and its gut microbiota. Preliminary knowledge of how microbial inhabitants impact gut maturation and nutrient absorption was reinforced by a study, whereby GF mice were inoculated with the microbiota of conventionally raised mice (GF-conv); after 14 d, the GF mice had a 60% increase in body fat despite reduced food intake (Backed et al., 2004). Measurements of gene expression from real-time polymerase chain reaction (qRT-PCR) showed that GF-conv mice had upregulated expression of key enzymes in the de novo fatty acid biosynthesis pathway, namely acetyl-CoA carboxylase and fatty acid synthase; and suppressed expression of fasting-induced adipocyte factor (Backed et al., 2004). Other researchers reported that 3-phenylpropanoid acid, indole-3-propionic acid, phenyl sulfate, and *p*-cresol sulfate were among the metabolites only observed in conventional mice and not in GF mice (Wikoff et al., 2009). The production of indole-3-propionic

acid was determined to come from *Clostridium sporogenes*, a bacterial species only present in conventional mice with diverse microbiomes. Although researchers have been studying the gut microbiome as an important internal ecosystem through selective microbial culturing for over a century, advancements in technology such as DNA sequencing and large information processing systems catapulted our understanding of inhabiting microorganisms and their relationship to health (Rosenbaum et al., 2015).

Connecting host diet with the dynamic activity of the intestinal microbiome began after the Human Microbiome Project (HMP), a massive effort that surveyed 4,788 healthy adults who submitted microbiome samples from up to 18 body sites for 16S rRNA profiling and Illumina sequencing (Turnbaugh et al., 2007). Before this study, the relationship between types of fiber fermented by the gut microbiome and dietary intake in humans was unclear. Diet analysis paired with HMP microbiota reported that prebiotics such as inulin, galactooligosaccharides, and xylooligosaccharides were fermented by genera within the Firmicutes and Bacteroides phyla. In addition, methods of dietary recall such as food frequency questionnaires, motivational interviews, and food diaries were employed as methods to capture dietary intake and correlate nutrient digestion (fiber, carbohydrates, lipids, protein, vitamin, mineral) with fecal metabolites and microbial populations (Candela et al., 2010).

The ability to sequence the human genome allowed researchers to perform a whole genome analysis scan (GWAS) to detect mutated genes called single nucleotide polymorphisms (SNP) which may be related to chronic diseases. By extracting DNA from a person, a GWAS can detect SNP that relates to the gene expression of chronic diseases in the gut, such as inflammatory bowel disease (IBD). The presence of 3 SNP of the caspase-activated recruitment domain (CARD15) in patients with IBD was discovered (Hampe et al., 2002). These SNP send a defective bacterial

signal, which results in the host immune system mounting an excessive immune response. The resulting chronic gut inflammation begins the cyclic cycle of symptoms for patients with IBD. That paper suggested that SNP may affect bacterial recognition pathways, immune responses, and mucosal transport or polarity. However, a deeper knowledge of the mechanisms that govern gut microbes and their connection to chronic disease states and host nutrition was needed at that time.

Recent human observational trials studying diet and the microbiome have analyzed the eating patterns of subjects to better understand their microbial community shifts rather than focusing on isolated nutrients or ingredients. An eating pattern-based approach affords more correlations between biomarkers, bacteria, and dietary habits due to the high inter-individuality of each person's gut microbiota and diet (Cotillard et al., 2022). Food choices that are the most consequential to the microbiome's composition include the amount of fiber consumed per day, the ratio of animal-derived protein compared with plant-derived protein, the amount of sugar or ultra-processed foods consumed, and the number of drinks such as coffee, tea, wine, or spirit-based alcohol consumed (Bolte et al., 2021). In a recent study, five a posteriori dietary patterns (DP5) were developed in an attempt to account for the primary global dietary intake and gut microbiota variations (Cotillard et al., 2022). These diets were characterized as plant-based, flexitarian, health-conscious Western, standard Western, and an elimination eating pattern. While many core functions of the gut microbiota stem from diet, researchers are still learning how to better capture dietary intake as playing a critical role in microbial metabolism and host interaction (Leeming et al., 2021).

Canine Gut Microbiome

The canine gut microbiome shows comparable abundance and richness in residential microorganisms to the human gut microbiome, with reported averages between colony-forming units (CFU) per gram depending on the location in the GIT (Pilla and Schodolski., 2019). In a study using 22 healthy adult beagles, it was reported that their jejunum had microbial densities ranging from 10^2 to 10^6 CFU/g, and feces ranging from 10^8 to 10^{11} CFU/g (Mentula et al., 2005). In healthy adult dogs, the predominant bacterial phyla in the GIT include Bacteroidetes, Fusobacteria, Proteobacteria, Firmicutes, and Actinobacteria, but relative abundances differ depending on GI region, animal characteristics (e.g., breed, sex), and diet composition (Deng and Swanson, 2015). The canine microbiome has been demonstrated to have similar predominant phyla when compared with the human microbiome. In a study performed by Swanson et al. (2011), healthy adult dogs were fed either a high-fiber diet containing 7.5% beet pulp or a low-fiber control diet in a crossover design. After the interventions, dog feces were pooled by treatment, and the DNA was extracted and subjected to pyrosequencing with a 454 Genome Sequencer using FLX titanium reagents, with the data being organized into diet-assembled canine metagenomic datasets (Swanson et al., 2011). It was reported that the dominant bacterial phyla in dogs were the Bacteroides/Chlorobi group and Firmicutes. Results from that study were compared with data sets within metagenomics Rapid Annotation using Subsystem Technology (MG-RAST) that had similar characteristics such as sequence length and average sequence length at a phylogenetic and metabolic level. Using a double hierarchical dendrogram, two profiles from the canine groups clustered together with a healthy human and a mouse metagenome than with an obese mouse metagenome. The results of that study demonstrated that the GI metagenomes and phylogenetic diversity between dogs, humans, and mice are comparable. A more recent study fed two groups of

dogs, either a low-protein/high-carbohydrate diet or a high-protein/low-carbohydrate diet, and extracted the DNA from the stool from each treatment after 4 weeks (Coelho et al., 2018). The results of 16S rRNA sequencing showed the canine microbiome is directly affected by diet shifts and is stable in the absence of interventions. The researchers hypothesized that dog studies may be predictive of results in humans, as human studies have been predictive of results in dogs (Coelho et al., 2018).

Despite similarities in terms of taxonomy and function, there are differences between the human and canine microbiomes. From a taxonomy perspective, one of the largest differences is the abundance of *Fusobacterium* and its relationship with disease. In humans, the presence of *Fusobacterium* is a sign of proinflammatory diseases such as colon cancer and IBD (Bashir et al., 2016); yet, in dogs, it is part of a healthy digestive tract. Despite differences between dogs and humans, the dog microbiome is well suited to help researchers better understand the functions of the human microbiome (Coelho et al., 2018).

Fermented foods

The intentional application of fermentation to food and beverages has been practiced for centuries to improve food preservation, nutritional value, and flavor (Selhub et al., 2014). While typically produced at home or by artisans, recent interest in improving GI health via fermented food consumption has fueled consumer demand for kimchi, kefir, kombucha, lactic-acid fermented pickles, and sauerkraut. Many traditional fermented foods are still being produced by small artisans, but there are exceptions such as markets for cheese, yogurt, and in recent years fermented beverages such as kombucha and kefir (Terefe and Augustin., 2020). The revenues coming from the international digestive health food and drink market were estimated to be \$25.9 billion in 2015

and \$37.6 billion in 2020, with a 7.77% average growth rate (Technavio, 2016). The market for fermented foods is surging due to evidence-based research demonstrating increased bioavailability of vitamins and minerals from these foods, activation of functional phytochemicals through the fermentation process, and health benefits such as enhanced glucose responses and improved lactic acid tolerance (Taylor et al., 2020; Leeuwendaal et al., 2022).

For centuries, the fermentation of food has been used as a form of food preservation that requires no heat or additional energy (Hutkins, 2018). During the fermentation process, microbes thrive on digestible cell components within the food, while the salt draws out moisture. Lactic acid-producing bacteria (LAB) and acetic acid-producing bacteria, as well as yeasts (e.g., *Saccharomyces cerevisiae*), are mainly responsible for the fermentation process in foods. Bacteria largely produce lactic acid, CO₂, and alcohol, thus reducing the final pH of fermented foods to around 4.0 (Hutkins, 2018). The microbial metabolism during fermentation generates novel compounds such as antimicrobials, bacteriocins, peptides, vitamin K, and exopolysaccharides (EPS). The transformation of texture and flavor by these microbes and compounds accounts for unique physical, sensorial, and nutritional attributes in the final product (Terefe and Augustin, 2020). Dairy-based fermented products are higher in fat compared with vegetable- or legume-based fermented products, providing an environment optimal for microbial growth and that easily buffers changes in pH to extend the milk's shelf life (Vardjan et al., 2013; Marco et al., 2021).

Several potential health benefits from kimchi consumption have been reported, including reductions in fasting blood glucose and/or total cholesterol concentrations, and blood pressure in obese and overweight subjects (Kim et al., 2011). Even so, the consumption of kimchi and miso has also been investigated in relation to gastric cancer development, but conclusive evidence is lacking (Dimidi et al., 2019). Many people consuming Western diets high in processed foods and

added sugar have increased GIT inflammation, which induces a dysbiotic state and increases their susceptibility to developing non-communicable chronic diseases such as heart disease and type 2 diabetes (Malesza et al., 2021). Due to potential anti-inflammatory effects and microbial metabolites, fermented food consumption may help non-communicable chronic disease symptoms (Gille et al., 2018). Consuming fermented foods may also provide compensatory exposure to safe and foodborne microbes lost in today's modern industrialized environment (Rezac et al., 2018; Marco et al., 2021). The antimicrobial compounds naturally produced by LAB may strengthen the host's natural immunity when exposed to pathogens. This natural form of protection has weakened as society becomes increasingly sterilized and consumes less LAB fermented foods (Marco et al., 2021). More evidence-based research is needed for fermented foods so that we have a better understanding of their specific effects: such research may also aid in the development of appropriate regulations.

Elucidating the health effects of fermented foods in randomized human clinical trials poses several challenges, including providing an ideal placebo, supplying consistent microbial profiles among products, obtaining a microbial baseline for participants and products, and participant tolerance of the fermented foods (Marco et al., 2021). Wastyk et al. (2021) conducted a 17-wk randomized prospective study consisting of 36 healthy adults ($52 \text{ yr} \pm 11$) consuming either an average of $45.1 \pm 10.7 \text{ g}$ of self-selected fiber per day or an average of 6.3 ± 2.9 servings of self-selected fermented foods, with exclusion criteria of a $\text{BMI} \geq 40$ or a history of IBD, chronic gastric diseases, diabetes, renal disease, significant liver enzyme abnormality, pregnancy or lactation, smoking, a history of CVD, inflammatory disease, malignant neoplasm, or consuming $20+ \text{ g}$ of fiber per day already. Participants were randomly placed into two groups for a 10-wk intervention phase, with one group ($n=18$) consuming an average of 6.3 ± 2.9 servings of fermented foods per

day and a second group (n=21) consuming an average of 45.1 ± 10.7 g fiber per day. In the fermented foods group, some participants largely consumed fermented dairy products while others consumed fermented vegetables. Fecal samples were frozen and collected every 2 wk for the duration of the study, with blood being collected 7 times throughout the experiment. Baseline measurements were based on participant samples before the dietary intervention, and baseline gut microbiota measurements of alpha and beta diversity in both arms showed no significant differences. The fermented food intervention group showed increased bacterial alpha diversity ($p = 0.016$) measures when compared with baseline, as well as decreases ($p < 0.05$) in 19 of 93 circulating cytokines in serum. There was an incremental dietary effect in this group, which demonstrated increased alpha diversity and decreased inflammatory markers as more servings of fermented food were consumed per day (Wastyk et al., 2021). Those results suggest that the increase in diversity came from taxa already in the GIT, potentially with increased abundance or strain mutations. The decrease in inflammatory markers, including IL-6 ($p < 0.05$), IL-10 ($p < 0.05$), and IL-12 ($p < 0.05$), supports research showing the inflammatory state is modulated by the gut microbiota. The high fiber arm had a significant increase ($p < 0.05$) in the relative abundance of 11 microbiome-encoded glycan-degrading carbohydrate-active enzymes (CAZymes) compared with the CAZymes present at baseline (Wastyk et al., 2021). Despite the variation in fermented food sources, the participants in this group had consistent trends suggesting fermented foods can be beneficial.

Kefir

The origins of kefir are traced to the Caucasus, Tibetan, and Mongolian Mountains in Asia where recovered kefir grains have been dated back 2,000 yr (Seyim, 2001). Kefir grains were

passed down generations as a form of family wealth. The word kefir is Slavic, meaning ‘well-being’ or ‘living well’ due to people feeling better after drinking it. The grains differentiate kefir from other fermented foods due to their specific and complex symbiotic composition (Rose et al., 2017). The beverage can be made with cow, goat, camel, ewe, sheep, or buffalo milk (Irigoyen et al., 2015). Higher fat content in milk will produce a creamier kefir, with the microbes thriving on the lactose present in milk (Rosa et al., 2017). Kefir is commonly consumed in Middle Eastern parts of the world, but its popularity has spread as industrial systems transporting and producing milk have increased (Irigoyen et al., 2015).

Over the last decade, there have been an increasing number of publications investigating fermented milk products such as yogurt and kefir for their health-affecting properties (Savaiano and Hutkins., 2020). According to the Codex Standard for Fermented Milks (Codex Stan 243-2003), kefir is defined as fermented milk prepared from a unique starter culture known as kefir grains, which contain LAB such as *Lentilactobacillus kefiri*; members of the genera *Leuconostoc* and *Lactococcus*; acetic acid bacteria, and lactose-fermenting (*Kluyveromyces marxianus*) yeasts and non-fermenting yeasts (*Saccharomyces unisporus*, *Saccharomyces cerevisiae*, and *Saccharomyces exiguus*). Due to variations in geographic and environmental conditions, kefir grains vary and these recommendations are minimum inclusion goals (Wang et al., 2021). Kefir is also recommended by the Codex Standard for Fermented Milks to contain at least 2.7% protein, 0.6% lactic acid, and less than 10% fat. The percentage of alcohol is not established, but the total number of microorganisms in the fermented milk is recommended by the Codex Standard for Fermented Milks to be at least 10^7 CFU/g and the yeast not less than 10^4 CFU/g. These regulations are not enforced but are a means of the international standard reference for countries and local governances to form regulations around.

Kefir Fermentation Process

Kefir fermentation begins with the addition of kefir grains to milk that are incubated for 22-24 h at 25°C (Rosa et al., 2017). Typically, 5-10% w/v of grains is added to the milk for a palatable consistency, becoming more viscous and acidic with increasing grain (w/v%) inclusion. During kefir production, the microorganisms present in the grains digest the lactose in milk to proliferate and produce lactic acid, flavor compounds (e.g., acetaldehyde), ethanol, and CO₂. The final pH of kefir (4.2-4.6) depends on the grain inclusion level, fermentation temperature, and fermentation length (Guzel-Seydim et al., 2013; Rosa et al., 2017). The bacteria present can also affect the pH of the final product due to their metabolism and fermentative products. *L. kefir* and *Leuconostoc mesenteroides* are heterofermentative bacteria, which produce lactic acid, ethanol, CO₂, and acetic acid, and die at pH < 4. These compounds are generated via the phosphogluconate and phosphoketolase pathways (for hexoses) and the phosphoketolase pathway for pentoses (Abdel-Rahman et al., 2013). *L. kefir* and *L. plantarum* are homofermentative LAB that survive pH < 4, eventually becoming the dominant species, while heterofermentative microbes die off. Homofermentative LAB are common in commercial use due to the utilization of the enzyme aldolase which produces lactic acid as its major end product without many byproducts (Abdel-Rahman et al., 2013). The production of lactic acid by homofermentative bacteria decreases the pH, generating a sour and tart flavor.

A unique feature of kefir fermentation is the recycling of the grains from batch to batch. The practice of using a portion of the current product to inoculate new milk to restart the process is referred to as backslopping. Over time, the biomass of kefir grains increases in size and number. In vitro research has shown that the traditional method of backslopping facilitates the activity of endogenous enzymes to increase tannin breakdown and protein digestibility, making the product

and nutrients more bioavailable (Hassan and El Tinay, 1995). A diet containing bacteria and their resulting metabolites may be a promising direction for the development of fermented beverages (Heilbronner et al., 2021). The recent surge in personalized health has fueled consumer interest in fermented food products, yet mass production and commercial scale are relatively new applications to this market.

Composition of Kefir Grains

Kefir grains are a symbiotic culture of yeasts and bacteria that form semi-soft particles resembling a granular shape. These grains are comprised of a protein-polysaccharide matrix called kefiran and a water-soluble galactoglucan portion containing galactose and glucose units (Nejati et al., 2020; Diez-Ozaeta and Astiazaran, 2022; Leeuwendaal et al., 2022). In a review paper by Sarkar (2008), it was stated that common genera in kefir grains including *Lentilactobacillus kefir*, *Lentilactobacillus parakefir*, and *Lactobacillus brevis* that are heterofermentative in nature and *Lactobacillus plantarum*, *Lactobacillus acidophilus*, *Lactobacillus kefirgranum*, *Lactobacillus kefiranofaciens* and *Lactobacillus casei* that are homofermentative (Sarkar., 2008). It has been hypothesized that the self-aggregation of *L. kefiranofaciens*, *Kazachstania turicensis*, and *Acetobacter orientalis* initiate the formation of a biofilm on the milk that coagulates to form small granules over time (Wang et al., 2012). In addition to bacteria and yeasts, a low abundance of molds, including *Aspergillus*, *Alternaria*, and *Malassezia* have also been detected in kefir grains (Dertli & Con, 2017). Differences in grain composition also affect the production of volatile compounds, which are highly correlated with *L. kefiranofaciens* and *Saccharomyces* spp. abundance (Wang et al., 2021).

Composition of Traditional Kefir

Traditional kefir is a slightly tart beverage that can be refrigerated for months. The dominating bacteria in traditional kefir include *L. kefirifaciens* and *L. kefir*, with predominant yeasts being *Kluyveromyces marxianus*, *Kazachstania turicensis*, *Kz. unispora*, and *Dekkera anomala* (Netaji et al., 2020). *S. cerevisiae* has been recovered from traditional kefir, demonstrating choice of grains largely affects the resulting microbial composition of the final product (Kim et al., 2015a).

The nutrient composition of traditional kefir made from cow milk in Korea was reported to be $90.93 \pm 1.01\%$ water, 3.73% carbohydrates, $1.34 \pm 0.34\%$ fat, $3.25 \pm 0.16\%$ protein, and $0.75 \pm 0.03\%$ ash; the number of samples was not disclosed, but the milk was changed 4 times a wk for 40 d before nutrient analysis (Gamba et al., 2019). Kefir has also been reported to contain formic, propionic, and succinic acids, aldehydes, traces of acetone, and isoamyl alcohol (Rosa et al., 2017). The final product contains a variety of vitamins, including thiamin (B1), riboflavin (B2), pantothenic acid (B5), folic acid (B9), vitamin C, vitamin A, and vitamin K, with the concentrations being dependent on the milk used for production. Traditional kefir is also a good source of calcium, magnesium, phosphorus, zinc, copper, manganese, cobalt, and molybdenum (Otle and Cagindi., 2003; Rosa et al., 2017). The ethanol content of kefir ranges from 0.5-2% (v/v), depending on the yeasts present during production (Rosa et al., 2017). Due to differences in local geographical regions, microbial environments, and milk used, the nutritional composition of kefir is expected to vary.

Traditional Kefir Byproducts

The fermentation process of milk with kefir grains also produces beneficial byproducts such as EPS, water-soluble polysaccharides (KGF-C), kefir-fermented peptides (KP), and kefir cell-free supernatant (KCFS) that are not retained in commercial kefir products (Kim et al., 2019). Water soluble polysaccharides isolated from kefir (KGF-C) have been shown to improve the immune response in mice through T-cell proliferation and antibody responses to thymus-independent antigens, such as dinitrophenyl-ficoll and trinitrophenyl-lipopolysaccharide (Murofushi et al., 1986). KP are bioactive peptides shown to increase enrichment levels in the gut microbiota (Tu et al., 2020). The sphingolipid sphingomyelin, which is essential to animals, was also isolated from traditional kefir as a naturally-occurring product. Kefir-sphingomyelin has been applied to human MG-63 cells in vitro, with significant increases in IFN- β ($p < 0.05$) being observed when compared with commercially produced sphingomyelin (Osada et al., 2013). The byproduct KCFS has been demonstrated to have effects on the immune response, including changes to murine Peyer's patches (Vinderola et al., 2006), reduced tumor size and apoptosis in mice injected with breast tumor cells (de Moreno de LeBlanc et al., 2007), and inhibited T-lymphocytes in vitro.

Small compounds called bacteriocins can be produced by ingested microbes and cross the gut-blood barrier, providing indirect protection against infectious microbial agents by exerting antimicrobial effects against pathogens. Bacteriocins act synergistically with other bacteria or host-derived antimicrobial molecules to facilitate commensal community invasion, prevent a pathogenic community invasion, or facilitate community differentiation (Dicks et al., 2018).

The bacteriocin ST8KF has been isolated from grain kefir *L. plantarum* and displayed strong antimicrobial properties such as proliferating after growth suppression by glycerol and triammonium citrate and producing more bacST8KF (Powell et al., 2007). Many LAB from the

Firmicutes phylum produce bacteriocins, justifying further research into other bioactive compounds produced in kefir (Dicks et al., 2018). Intentionally consuming foods with live probiotics may provide therapeutic benefits to the microbiome due to bacteriocins, which affect the microbial environment.

Microbes in fermented foods may be encapsulated by EPS and may produce bacteriocins to aid in their colonization fitness. Ingested bacteria from fermented foods also produce EPS, which enables agglutination and protects them from environmental changes throughout the GIT. EPS are large hydrophobic proteins, which enhance their ability to bind to intestinal mucus, especially in the presence of bile acids in the large intestine (Flint et al., 2012; Dicks et al., 2018; Lu et al., 2022). The literature suggests that in conditions of a neutral pH and high bile salt concentrations, EPS increase mucoadhesive forces with mucin, allowing bacterial pili to be partially released for improved adherence to the mucus layer. Kefiran is an EPS produced by *L. kefirano**faciens* during fermentation. An in vivo murine study supplemented kefiran and observed improvements in asthma pathogenesis via suppression of eosinophils and other inflammatory cells in the bronchoalveolar lavage (Kwon et al., 2008). This study also observed a reduction in IL-4 and IL-5 concentrations in the bronchoalveolar lavage (BAL) fluid from Th2 cytokines produced by activated T cells compared with normal ranges (Kwon et al., 2008). Another study administered kefiran to mice consuming a high-fat diet and observed decreased fasting glucose concentrations, reduced serum cholesterol concentrations, and suppressed increases in blood pressure, potentially due to increased angiotensin I-converting enzyme (ACE) protein expression in blood serum and thoracic aorta (Maeda et al., 2004). Cell line in vitro research showed that kefiran improved bacterial adhesion properties and increased mitochondrial activity (Medrano et al., 2008).

Composition of Commercial Kefir Products

Commercially-produced kefirs more closely resemble yogurt due to the use of manufactured starter bacterial cultures typically containing *S. thermophilus*, *L. acidophilus*, *L. casei*, *L. bulgaricus*, and *L. delbrueckii* (Nejati et al., 2022). Instead of fermenting kefir grains, commercial kefir manufacturers use preset bacterial cultures of select taxa that provide more consistent products for large-scale producers (Hansen, 2002; Leeuwendaal et al., 2022; Nejati et al., 2022). These cultures contain microbes such as *Streptococcus thermophilus*, *Lactobacillus delbrueckii* ssp. *bulgaricus*, *Bifidobacterium lactis*, *Bifidobacterium bifidum*, *Lactobacillus casei*, *Lactobacillus acidophilus*, and *Lactobacillus rhamnosus* along with yeast in the environment. The exclusion of grains also removes novel yeasts present in traditional kefir, so the final product contains minimal ethanol concentrations, and it is more palatable for consumers (Nejati et al., 2022). While this practice increases consistency from batch to batch, the absence of kefir grains and the yeasts they contain (e.g., *Saccharomyces*, *Kluyveromyces*, *Naumovozyma*, and *Candida*) results in a different final product. Recent sequencing analysis of commercial kefir products demonstrated that they were dominated by the yeasts *Kazachstania* spp. *unispora* and *turicensis*, and *Debaryomyces hasenii* (Nejati et al., 2022). Although they are not intentionally added, naturally-occurring yeast inoculate the milk vats used to brew kefir. These results are consistent with research reported by Kazou et al. (2021), who also detected a high abundance of this yeast in commercial Greek kefir, but it is hypothesized that *D. hasenii* does not make up a large part of the kefir composition.

Few studies have characterized the microbial composition of kefir at a species- and strain-specific level due to limitations of traditional analytical methods (e.g., culturing, polymerase chain reaction) and lack of affordable high-throughput sequencing methods. Traditional culturing

methods are unable to study unknown bacteria and growth media is selective to the nutrient requirements of certain species (Kim et al., 2018). Despite these limitations, media culturing allows for species-specific identification of purified colonies through RTq-PCR and MALDI-TOF MS for information beyond bacterial phylum and genera. Modern advancements in technology have allowed untargeted metagenomic approaches such as next-generation high-throughput sequencing to discover that *L. kefiranofaciens* and *L. kefiri* dominate traditional grain-based kefir cultures and that these taxa are not detected in commercially-produced kefirs (Kim et al., 2018; Yegin and Sudagidan, 2022). With recent food technology developments and increased consumer interest, a new approach to commercially-produced kefir with more similarities to grain kefir is needed.

LAB are commonly chosen in commercial starter cultures due to their in-depth characterization, safety, and potential for genetic manipulation (Hansen, 2002). The number of bacterial cultures used in industry is not large and is limited to a small number of well-characterized microbes. Companies are most concerned with food safety and providing a pleasing, consistent flavor for consumers. Research by Netaji et al. (2022) supported previous findings that the microbial diversity of commercial kefirs is dominated by a small selection of bacterial species (Korsak et al., 2015), but also that these products had more galactose ($p < 0.05$) and lactate ($p < 0.05$) compared with grain kefir as well as less glycerol ($p < 0.05$). In addition, that research demonstrated that the volatile organic compound profile of commercial kefirs clustered closely with the milk and yogurt tested rather than with grain kefirs. While many commercial kefir producers strive to suggest their product as an equal alternative to grain kefir, current research demonstrates that commercial kefir has large variations in dominating bacteria, yeast, nutrient composition, and volatile organic compound profile from grain kefir.

In Vitro and In Vivo Research on Traditional Kefir

Many animal studies have been conducted in an attempt to understand the mechanisms of action attributed to the health effects observed from kefir consumption. Due to differences in bacteria, yeast, fungi, and contributing metabolites, commercial and grain kefirs are hypothesized to impart different effects on the host. To investigate this question, two murine studies were conducted by Bourrie et al. (2018; 2021) to compare grain kefirs and commercial brand kefirs. Those researchers reported a significant increase in weight loss ($p < 0.05$), decreases in serum lipid-lowering effects ($p < 0.05$), and decreased gene expression of liver HMG-CoA reductase ($p < 0.05$) in the groups consuming grain kefir. Those results suggest that consistent grain kefir consumption may reduce endogenous cholesterol synthesis (Bourrie et al., 2018; Bourrie et al., 2021). The decreased expression of HMG-CoA reductase observed only in grain kefir-fed mice suggests that it interacts with the host differently from commercial kefir. Kefir consumption in vivo demonstrated an increase in ACE activity in mice (Maeda et al., 2004), suggesting a potential mechanism by which kefir reduces blood pressure. Product consistency, participant compliance, and overall diet can also affect blood lipid concentrations, so more research is needed to make an evidence-based conclusion on kefir's effects on endogenous lipid and cholesterol concentrations. What exactly is mediating these effects is unclear due to the many variables between kefirs, but these studies demonstrate the importance of consistent kefir fermentation and the need for clear labeling due to the significant effects that kefir grains can impart.

Differences in the relationship with *Fusobacterium* in dogs and humans were highlighted previously concerning a study that provided grain kefir to 6 healthy dogs for 2 wk; qRT-PCR results demonstrated that the relative abundance of fecal *Fusobacterium* decreased ($p < 0.05$) after kefir intervention (Kim et al., 2019). A similar reduction in fecal *Fusobacterium* was observed by

another lab, which fed the supplement Kefibios® with $\geq 10^9$ active fluorescent units (AFU) of *L. kefir* to 10 healthy dogs for 30 d (Gaspardo et al., 2021).

An in vitro study designed to assess the immunomodulatory properties of *L. kefir* CIDCA 8348 applied to 19 mucosal biopsy samples from IBD patients compared with 5 healthy control mucosal biopsy samples, was conducted by Curciarello et al. (2021). Cytokine activity was quantified by ELISA, while cell proliferation and viability were assessed via flow cytometry. Increased immune cell numbers (i.e., CD4⁺ T cells) and IL-10 concentrations, and reduced IL-6 and IL-8 concentrations were observed (Curciarello et al., 2021). IL-10 is activated by B cells and increases their function as an antigen-presenting cell (APC) to employ a more adaptive immune response in the host (Ghosh et al., 2021), while CD4⁺ T cells employ effective immune protection against cancer cells (Saito et al., 2016). The increase of both IL-10 and CD4⁺ T cells suggests that kefir impacts several immunoregulatory mechanisms in the body to exert such effects on the immune system (Curciarello et al., 2021).

A handful of randomized clinical trials assessing a traditional kefir intervention have been conducted in healthy adult humans (Pražnikar et al., 2020; Wang et al., 2020), in patients who are overweight (Fathi et al., 2017a), diabetic (Judiono et al., 2014; Ostadrahimi et al. 2015; Bellikci-Koyu et al., 2019; El-Bashiti et al., 2019), or hypercholesteremic (St-Onge et al., 2002). Fathi et al. (2017a) conducted a human kefir intervention through a single-center, multi-arm parallel group randomized control trial offering 58 asymptomatic overweight or obese premenopausal women either kefir, milk, or a control (low-fat milk) in 300 mL servings/d for 8 wk. Dietary records, anthropometrics, and fasting blood samples were drawn at baseline and the end of the intervention (Fathi et al., 2017a). St-Onge et al. (2002) performed a similar randomized crossover placebo-controlled trial in 13 overweight or obese asymptomatic non-smoking men. The participants were

randomized into two groups and consumed 500 mL/d of kefir or milk for 4 wk, then consumed the alternative beverage for another 4 wk after a 4-wk washout period in between experimental periods. Researchers collected fasting blood samples throughout the experiment as well as fecal samples at the beginning and end of intervention periods (St-Onge et al., 2002).

The lack of agreement on kefir's impact on lipid metabolism and the gut microbiome can be attributed to human clinical studies reporting conflicting outcomes. After 8 wk, Fathi et al. (2017a) did not detect any significant differences in serum lipid panels between the kefir and milk group; yet when kefir was compared to the low-fat milk control group a significant decrease in total cholesterol ($p < 0.024$) was observed in the kefir group, as well as decreases in low-density lipoprotein cholesterol (LDL-C) ($p < 0.03$), and non-high-density lipoprotein cholesterol (HDL-C) ($p < 0.014$). St-Onge et al. (2002) reported no significant effect on serum lipids in hypercholesteremic men between treatments, yet both groups saw significant increases in fecal isobutyric acid ($p < 0.05$), isovaleric acid ($p < 0.05$), propionic acid ($p < 0.05$), and SCFA ($p < 0.05$) in each treatment group. The only difference detected between kefir and milk treatments was increased fecal propionic acid ($p < 0.05$) and increased fecal bacterial concentration in the kefir-consuming group (St-Onge et al., 2002). The lack of information on the nutrition composition or microbial environment in the kefir products used in these studies adds limitations for comparing their results with studies using better-profiled products.

Overall effects on the gut microbiome varied in each study from increased *Lactobacillus* detected in feces (Fathi et al., 2017b), increased Actinobacteria in men (Bellikci-Koyu et al., 2019), increased *Bifidobacterium* in men, and increased total abundance of total anaerobes and bacteria in females (Wang et al., 2020). These results suggest novel bacteria from the kefir are not colonizing in the gut, but rather influencing commensals as they pass through the GIT.

Knowing the microbial composition of kefir used in an RCT is crucial for researchers to understand the potential interactions of host microbes with those from the kefir product. A randomized controlled trial was conducted that included a parallel group of 22 participants diagnosed with metabolic syndrome (Bellikci-Koyu et al., 2019). Participants were instructed to ingest 180 mL/d of milk or a kefir product from Danisco that claimed to contain *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris*, *Lactococcus lactis* subsp. *diacetylactis*, *Leuconostoc mesenteroides* subsp. *cremoris*, *Lactobacillus kefir*, *Kluyveromyces marxianus*, and *Saccharomyces unisporus*. That study reported significant decreases in fasting insulin levels ($p < 0.05$), tumor necrosis factor (TNF- α) ($p < 0.05$), IFN- γ ($p < 0.05$), and in systolic ($p < 0.05$) and diastolic ($p < 0.05$) blood pressure in groups consuming kefir from their baseline, but no health parameters had significant decreases when compared to the control group (Bellikci-Koyu et al., 2019). Another randomized controlled trial had 29 asymptomatic overweight adults ingest 300 mL/d of kefir containing *Lentilactobacillus parakefiri*, *Lentilactobacillus kefiri*, and *Lactobacillus kefiranofaciens* spp. *kefirgranum*, *Kluyveromyces marxianus*, *Kazachstania exigua*, and *Rhodospiridium kratochvilova* or milk for 21 d before a washout period where the intervention groups switched. Serum was collected to analyze serum zonulin concentrations and lipid panels (Praznikar et al., 2020). After the intervention, the kefir-consuming group had lower serum C-reactive protein ($p < 0.018$), HDL/C ($p < 0.013$), and fasting blood sugar ($p < 0.001$) concentrations than milk-consuming groups, yet both groups saw significant decreases in other health parameters; low-density lipoprotein cholesterol ($P < 0.001$), total cholesterol ($P < 0.001$), and fasting blood sugar ($P < 0.006$). In a single-blind randomized placebo-controlled study, 54 healthy adults were instructed to consume 2 g of AB-kefir microbes (*Bifidobacterium longum*, *Lactobacillus acidophilus* LA1063, *Lactobacillus fermentum* LF26, *Lactobacillus helveticus* LH43,

Lactobacillus paracasei LPC12, *Lactobacillus rhamnosus* LRH10, and *Streptococcus thermophilus* ST30) or a placebo sachet for 3 wk (Wang et al., 2019). Consumption of the AB-kefir reduced symptoms of abdominal pain, bloating ($P=0.014$), and appetite ($P=0.041$), while also increasing the abundance of total anaerobes ($P=0.038$) and total bacteria ($P=0.049$) in the feces of females. These studies demonstrate the potential variety of microbes in different kefir products used in human research, which may play a role in outcomes and reinforce the need for species-specificity when conducting microbiome research with fermented foods.

Several studies have investigated kefir's effects on patients with diabetes (Judiono et al., 2014; El-Bashiti et al., 2019; Ostadrahimi et al. 2015), and a meta-analysis determined that kefir has a significant impact on reducing insulin ($P<0.05$) and fasting blood sugar concentrations ($P<0.05$) when at least 200 mL were consumed for at least 4 wk consecutively (Salari et al., 2021). Potential mechanisms of action stem from bacteria inhibiting endotoxins from entering the bloodstream and reducing LPS, which is produced by gram-negative bacteria. The reduction in circulating LPS may reduce the release of proinflammatory cytokines (Negkelo et al., 2012). LPS has auto-amplification loops after its activation by the immune system, which can activate the inflammasome, a molecular platform that releases mature proinflammatory cytokines such as IL- 1β and IL-18 (Cavaillon., 2018). Reducing LPS and its capacity to induce inflammation allows the intestinal microbiota to modulate the immune system and produce anti-inflammatory metabolites. Reduced inflammation improves insulin resistance and prevents the breakdown of β -pancreatic cells (Salari et al., 2021). The bacteria present in kefir, as well as organic compounds and byproducts, may also play a role in its fasting blood sugar lowering effects.

In a recent study, kefir was provided to patients with active IBD consisting of ulcerative colitis (UC) or Crohn's disease (CD). One group ($n=25$) was assigned to consume 800 mL/d of kefir,

while the control group (n=20) did not consume any placebo beverages due to possible microbial content. MALDI-TOF mass spectrometry was employed to detect the bacterial species of kefir, with *Lactobacillus pentosus*, *Lactobacillus brevis*, *Lactobacillus plantarum*, *Lactobacillus fermentum*, *Lactobacillus kefir*, and *Lactobacillus lindneri* being measured. Patients diagnosed with CD experienced significant improvements in abdominal pain ($p < 0.012$), increased blood hemoglobin concentrations ($P < 0.029$), and decreased serum C-reactive protein concentrations ($P < 0.015$) (Yilmez et al., 2019). C-reactive protein is an acute inflammatory protein that is increased at places of inflammation in the body and can be detected in the blood (Sproston and Ashworth, 2018). A reduction in this protein implies that inflammatory measures declined in the disease state of patients diagnosed with IBD. Significant differences were not observed in the UC group receiving kefir, so the mechanisms by which kefir has on these pathologies may differ (Flint et al., 2012; Leeuwendaal et al., 2022).

In Vitro and In Vivo Research on Commercial Kefir

Recent advances in technology have allowed researchers to differentiate between core microbial populations in kefir and observe molecular variations in fermentation to better attribute resulting host effects (Dobson et al., 2011; Marsh et al., 2013; Goldstein et al., 2013; Walsh et al., 2016; Sindi et al., 2020; Wastyk et al., 2021; Nejati et al., 2022). Recent developments in microbiome clinical trials demonstrate that small changes in the fermentation or strain specificity can have differing effects on the host and microbial metabolism (Johnson et al., 2020). Yet, there is a lack of conclusion to health effects attributed to kefir due to the compositional discrepancies between commercial and grain-produced kefir products. Many human clinical trials (St-Onge et al., 2002; Ostadrahimi et al., 2015; Fathi et al., 2017; Gölünük et al., 2017; Bellikci-Koyu et al.,

2019; Yilmez et al., 2019; Pražnikar et al., 2020) and animal trials (de Moreno et al., 2007; Senol et al., 2015; Murray et al., 2019) applied commercial kefir interventions without discriminating between potential compositional differences and limitations.

From the human research conducted so far, health benefits from commercial kefir products are inconsistent (St-Onge et al., 2002; Fathi et al., 2017; Bellikci-Koyu et al., 2019), and product accuracy relating to bacterial enumeration and microbial composition have also proved to be unreliable (Weese and Martin, 2011; Prados-Bo et al., 2022). Weese and Martin (2011) assessed 25 live probiotic-containing products for bacterial enumeration counts and accurate descriptions of microbes. Only 2 of the 25 products had accurate CFU/g counts and appropriate labeling. In addition, 8 products (32%) had less than 100 million CFU/g, even though all had label claims containing higher amounts. Prados-Bo et al. (2022) investigated the health claim efficacy and level of evidence provided by 133 probiotic and fermented beverages sold in Spain. Only 52.6% (70/133 products) had scientific evidence linked to the product online, with 18.4% reporting study conclusions and 7.9% quantifying the product's effects. Until sequencing methods become more widely adopted by companies producing probiotic-containing foods, transparency of probiotic contents and quality is needed.

Conclusions, Research Aims, and Hypotheses

Traditional fermented foods, such as the dairy beverage kefir, are normally produced by small-scale artisans or in households using milk and kefir grains. Increasing interest in fermented foods has demanded higher production, thus food and beverage companies have commercialized the fermentation process of kefir to meet demand. To scale up, companies have removed the use of kefir grains, which host novel microorganisms such as *Lactobacillus kefirianofaciens*,

Lentilactobacillus kefir, and *Kluyveromyces marxianus* and contribute flavor compounds, carbonation, exopolysaccharides, and peptides (Rosa et al., 2017). For more consistent batches, large-scale kefir producers may use vats of milk with preset starter cultures such as *Lactococcus lactis*, *Lactococcus cremoris*, *Lactobacillus diacetylactis*, *Lactobacillus acidophilus*, and *Saccharomyces cerevisiae*. The sequencing of commercial kefirs sold in Germany confirmed these results and demonstrated that commercial kefirs contained more galactose ($p < 0.05$) and lactate ($p < 0.05$), and less glycerol ($p < 0.05$) than grain kefirs. In addition, that research trial reported that the volatile organic compound profile of commercial kefirs clustered closely with the milk and yogurt tested rather than with grain kefirs (Nejati et al., 2022). Grain kefirs consist of a similar nutrient composition and microbial environment with variations of subdominant species (Bourrie et al., 2016; Nejati et al., 2022). Lack of differentiation between commercial kefir and grain kefir has resulted in randomized controlled trials being conducted with both styles of kefir; and some not specifying at all (St-Onge et al., 2002; Fathi et al., 2017a). More questions regarding the regulation of fermented foods arise when papers such as by Weese and Martin (2011) demonstrated that only 2/25 probiotic-containing supplements had matching CFU/g counts and accurate labeling, and by Prado-Bo et al. (2022) who showed that 52.6% (70/133) of probiotic products with websites in Spain did not have systematic reviews to back their health claims.

Given the need for more research in this area, the primary objective of Chapter 3 of this dissertation was to measure the microbiota of commercial kefir products intended for human consumption, including characterization using high-resolution, long-read 16S rRNA gene amplicon sequencing and quantitative measures using standard plating techniques. The secondary objective of this experiment was to evaluate label claims of bacterial taxonomy present and viable bacterial counts in the products tested. Recent studies have demonstrated the discrepancies and

inaccuracies that exist regarding bacterial counts and identity listed on companion animal supplements and product labels containing probiotics (Weese and Arroyo, 2003). Due to the growing market for fermented foods and probiotics in pets, the objective of Chapter 4 of this dissertation was to investigate the accuracy of label claims made by commercial kefir products designed for companion animals.

A few studies have investigated kefir's effect on canine health, including one study demonstrating that grain kefir modulated the gut microbiota of canines, including increases in LAB (Kim et al., 2019). Another study reported that dogs supplemented with *Lentilactobacillus kefir* did not have changes to fecal IgA concentrations or microbiota populations (Gaspardo et al., 2020). Neither study reported health benefits and it is clear that more clinical research is needed to test what benefits may be provided by kefir products in companion animals. Therefore, Chapter 5 of this dissertation was designed to determine the effects of a commercial kefir or traditional kefir on apparent total tract macronutrient digestibility of a diet when fed to healthy adult dogs and the fecal characteristics, metabolites, microbiota, and IgA concentrations of healthy adult dogs consuming them.

In addition to kefir consumption altering the gastrointestinal microbiota populations of hosts, its consumption may also alter the fermentation characteristics of dietary fibers present in pet foods. Due to the lack of previous research on this topic, Chapter 6 of this dissertation was conducted to assess the ability of fecal microbiota collected from kefir-fed dogs to ferment common dietary fibers using an in vitro fermentation assay. Many bacteria in fermented foods do not survive the journey through the host's GIT, yet fit species may survive and be candidates for future probiotic research. It is important to understand how different microbes from different fermented foods ferment dietary fibers found in food. Previous research has demonstrated robust

survival of bacteria from traditional grain-based kefirs (Nehir et al., 2015), LAB in kefir (de Moura et al., 2021), *Lentilactobacillus kefir* (Likotrafiti et al., 2015), and *Saccharomyces cerevisiae* (de Lima et al., 2017) using in vitro assays. Because the properties of commercial and grain kefir products need to be distinguished, another objective of Chapter 6 was to determine the in vitro fermentation characteristics of fibers using starter kefir (S-Kefir) or grain kefir (G-Kefir) as inoculum. These studies have explored the potentially different effects that grain-based and commercial kefirs can have physiology in canine models, and microbial functionality in vitro, and establish preliminary rationale for a better regulation of fermented dairy foods such as kefir.

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CHAPTER 3: COMMERCIAL KEFIR PRODUCTS ASSESSED FOR LABEL ACCURACY OF MICROBIAL COMPOSITION AND DENSITY¹

Abstract

Kefirs are fermented beverages containing yeast and bacteria produced by the fermentation of water or milk with kefir grains. Because microorganism density may influence a product's health benefits, label accuracy regarding viable bacterial density and taxonomy of fermented foods is important. In this study, the microbiota of 5 commercial kefir products were measured quantitatively using standard plating techniques and characterized using high-resolution, long-read 16S rRNA gene amplicon sequencing. To enumerate viable lactic acid bacteria, 2 lots of each product were plated on de Man, Rogosa and Sharpe agar upon opening and following 14 d and incubated under anaerobic and aerobic conditions. Results revealed that 66% of products with a guaranteed count of colony-forming units per gram overstated microorganism density by at least 1 log, with only product E exceeding 1×10^9 cfu/g. Sequencing results demonstrated moderate product label accuracy in regard to taxonomy, yet several products contained bacterial species above the minimum detectable threshold (0.001% relative abundance) that were not included on the labels (e.g., *Streptococcus salivarius*, *Lactobacillus paracasei*). Our results demonstrate a moderate level of labeling accuracy for commercial kefir products intended for human consumption. Regulatory agencies and consumers must continue to scrutinize these products and demand a higher level of accuracy and quality.

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Introduction

The importance of the gut microbiome to human health has become increasingly relevant in fields pertaining to nutrition and nutrient quality. When considering the effect of microorganisms on human health, the International Scientific Association for Probiotics and Prebiotics states that several probiotic strains have documented health benefits, and probiotic fermented foods are defined as having documented health benefits due to the actions of the probiotic (Hill et al., 2014). Probiotic mechanisms can include modulation of immune function, production of antimicrobial compounds, interactions with resident microbiota, and improvement of barrier integrity and enzyme formation (Verruck et al., 2019; Marco et al., 2021), but these effects cannot be expected from consumption of fermented foods. Recently, many fermented foods such as kombucha, kefir, and kimchi have been marketed as beneficial to human health, yet no documented health benefit is necessary for sale (Hill et al., 2014; Marco, 2020). It is important for consumers to understand this difference and know the accurate contents of the fermented foods they consume.

Kefir is a common fermented dairy beverage that contains both yeast and bacteria produced by the fermentation of water or milk with kefir grains (Rosa et al., 2017). Kefir has been consumed for centuries, and due to its affordability and potential to improve health status, many consumers are incorporating it into their lifestyle as a fermented beverage that may contain live microorganisms (Stanton et al., 2001; de Sainz et al., 2020; Mitra and Ghosh, 2020). In addition to testing sensory acceptance (Larosa et al., 2021), several randomized human clinical trials have been conducted in recent years bolstering kefir's health effects (Rosa et al., 2017). These include lower fasting insulin levels, lower levels of serum zonulin, lower erythrocyte sedimentation rate, lower C-reactive protein levels in patients with Crohn's disease, and lower serum lipid profiles in

premenopausal women (Fathi et al., 2017; Bellikci-Koyu et al., 2019; Yılmaz et al., 2019; Pražnikar et al., 2020). Each human trial used a different kefir product, which must be considered given the vast bacterial variability present between homemade and commercially produced kefir products (Bourrie et al., 2021). Because the benefits associated with added microorganisms are largely strain specific, verifying the bacterial taxa of kefir products used in clinical trials is of extreme importance when it comes to applying the results to human health (Kechagia et al., 2013).

The US Food and Drug Administration (FDA) considers microorganisms added to products as dietary supplements, and according to the FDA Code of Federal Regulations Title 21 (Code of Federal Regulations, 1975), kefir is categorized as a cultured milk that contains aroma- and flavor-producing microbial cultures. Under this title, kefir companies can make claims pertaining to colony-forming units per gram (cfu/g) and viable bacteria content without being closely monitored or regulated. Research by Merenstein et al. (2019) showed that 65% of the 93 commercial probiotic-containing products assessed lacked supporting clinical research. Correspondingly, research by Weese and Martin (2011) showed that many commercial pet products containing probiotics do not contain what their labeling suggests. Our recent assessment of pet kefir products showed similar inaccuracies in that segment of the market (Metras et al., 2020). Despite a surge in the market for fermented foods such as kefir, little research has been done to confirm the product claims of colony-forming units per gram and bacterial taxa. Regulatory agencies will need to identify core microbial components to impose novel regulations and labeling for fermented foods (Marco et al., 2021).

To address this potential gap in label accuracy, the objectives of this study were to measure viable microbial counts and characterize bacterial taxa of commercial kefir products intended for human consumption as well as compare enumeration and taxonomy data to label claims. Based on

the results of previous studies (Weese and Martin, 2011; Merenstein et al., 2019; Metras et al., 2020), we hypothesized that commercially available kefir products would not closely match label claims of viable colony counts or bacterial taxonomy.

Kefir products were purchased from online vendors and local stores in the Urbana, Illinois, area. Products included the following: product A, Maple Hill Plain Kefir; product B, Siggi's Plain Filmjölk; product C, Redwood Hill Farm Plain Goat Milk Kefir; product D, CoYo Kefir; and product E, Lifeway Original Kefir. Chobani Plain Yogurt 0% Milk Fat (YOG) was included as a validation of techniques. For each product, 2 lots were obtained for analysis, and each lot was plated on d 1 and d 14 for thorough enumeration results. Lots were chosen at random, and products were outwardly labeled by their chosen product letter. Labels were evaluated for colony-forming units per gram claims and a clear description of microbial taxa; lot numbers and expiration dates were recorded as stated on the labels. All liquid samples were refrigerated at 4°C until analysis.

To begin, 2 lots of each product were thoroughly vortexed until homogenized, 1 g of product was added to 9 mL of PBS, and the product–PBS mixture was vortexed again. Serial 10-fold dilutions were prepared with PBS. A 50-μL volume of 10⁵ dilutions and a 50-μL volume of 10⁷ dilutions were both plated in duplicate via spiral plater (Eddy Jet Spiral Plater, Neutec Group Inc.) onto de Man, Rogosa and Sharpe (MRS) medium (BD Difco). The MRS plates were incubated anaerobically at 37°C for 48 h and aerobically at 30°C for 48 h to culture lactic acid bacteria. Colony counts were measured via enumeration software (Colony Counter, IUL Flash and Go, Neutec Group Inc.). No attempt to distinguish organisms was made, only enumeration. Fourteen days after product opening, enumeration was repeated for all product lots in identical conditions. Results at time of opening and at d 14 are averages from the duplicate plates of each product lot.

For DNA extraction, 250 mg of kefir product samples from the day of opening was used (DNeasy PowerLyzer PowerSoil Kit, Qiagen) following the manufacturer's instructions. The DNA quantity was determined using a Qubit 3.0 fluorometer (Life Technologies), and DNA quality was assessed via gel electrophoresis (E-Gel Power Snap and E-Gel EX 1% Agarose, Invitrogen). The DNA extracts were sequenced at the W. M. Keck Center for Biotechnology at the University of Illinois at Urbana–Champaign using the Loop Sequencing 16S Long Read Kit (Loop Genomics) at a 1 ng/μL concentration. All samples were processed following the standard Loop Genomics protocol for full-length 16S sequencing. Sequence results were processed through the Silva132 Large Subunit rRNA Database (<https://www.arb-silva.de/documentation/release-132/>). All taxa with a relative abundance greater than 0.001% were retained for analysis. For plating procedures and DNA extraction, researchers were not blinded to products. For sequencing methods, researchers were blinded to product identification.

Statistical analyses were conducted using R x64 version 4.0.2 (R Foundation for Statistical Computing). Data obtained from enumeration samples on d 1 and 14 and relative abundance lot 1 and relative abundance lot 2 were tested for significance via paired Student's t-test. A P-value <0.05 was considered statistically significant.

All 5 kefir products tested claimed the presence of specific microorganisms in their products, and 3 of 5 products guaranteed minimum counts in colony-forming units per gram (**Table 3.1, 3.2**). Analysis of product A specifically identified 5 out of the 10 species claimed on the label, with 7 species being detected in total. In addition, although the label claimed 3 different *Lactococcus lactis* subspecies, Loop sequencing was unable to distinguish and grouped them together as *Lactococcus sp.* *Streptococcus thermophilus* was the majority species detected at 84.50%, with *Streptococcus salivarius* second highest at 9.30%. These 2 species have very similar

16S rRNA gene sequence, and it is possible that small errors in sequence assembly resulted in misidentifying *Strep. thermophilus* sequence reads as *Strep. salivarius*. This product did not make colony-forming units per gram claims, but at opening we detected similar results between lots, with 2.03×10^8 and 1.66×10^8 cfu/g in lots 1 and 2, respectively, under anerobic conditions, and 1.02×10^9 and 1.36×10^9 cfu/g in lots 1 and 2, respectively, under anaerobic conditions. Product B fared well when comparing its label claims and Loop sequencing; 4 out of the 4 genera claimed were detected. As with product A, Loop sequencing was unable to distinguish the *Lc. lactis* subspecies and identified both *Strep. thermophilus* and *Strep. salivarius*. The top 3 genera were *Streptococcus* (53.60%), *Lactococcus* (24.15%), and *Lactobacillus* (3.75%). Although the label did not specify a *Leuconostoc* species, Loop sequencing identified *Leuconostoc mesenteroides* in both lots. This product did not make colony-forming units per gram claims, and enumeration values did not change from d 1 to d 14. When considering product C, sequencing specifically detected 4 of 11 species claimed on the label (*Strep. thermophilus*, *Lactobacillus rhamnosus*, *Lactobacillus casei*, and *Lactobacillus acidophilus*). In addition, Loop sequencing was unable to distinguish the 2 *Lactobacillus delbrueckii* subspecies and 3 *Lc. lactis* subspecies that were indicated on the label. No *Bifidobacteria* or *Leuconostoc* were detected by sequencing. *Lactococcus sp.* was the second most abundant genus (1.65%) after *Streptococcus sp.* (79.35%). This product claimed 1×10^{11} cfu/g in its product, but our culturing methods never observed counts as high; lot 1 contained the most viable bacteria, 1.71×10^8 cfu/g at d 1 under anaerobic conditions. Product D contained *Strep. thermophilus* (88.05%) and *Strep. salivarius* (7.75%) as the majority taxa. The label claimed 2 different *Bifidobacteria*, but Loop sequencing was able to identify only *Bifidobacterium sp.* In addition, *L. acidophilus* and *L. rhamnosus* were not specifically identified with sequencing but *Lactobacillus sp.* might include these 2 species. Interestingly, *Pseudomonas*

was detected in both lots of product D. With claims of 1×10^9 cfu/g on its label, we detected this quantity on d 14 in anaerobic conditions in lot 2. No other culturing test achieved counts as high for product D. The last commercial kefir product we tested was product E, and sequencing confirmed 3 of 11 species claimed on its label (*L. rhamnosus*, *L. acidophilus*, and *L. casei*). Although the label claimed *Streptococcus diacetylactis* as the only *streptococcus* added, sequencing identified *Strep. thermophilus*, *Strep. salivarius*, and *Streptococcus sp.* In addition, sequencing did not identify any *Bifidobacteria* or *Leuconostoc* despite the label claim for each. For product E, culturing results were consistent between lots, with counts exceeding 1×10^9 cfu/g, but none reaching 1×10^{10} cfu/g as its label claims. The YOG sequenced to validate our techniques specifically identified 4 of 5 species on its label, and counts matched the label claims of 1×10^{10} cfu/g. These results give confidence in our methods applied to the kefir products. The data from **Table 3.1** and **Table 3.2** justify an expansion of testing to other kefirs and commercialized fermented foods. Out of the 3 products that guaranteed viable bacteria counts, none of those products met the colony-forming units per gram listed at the time of opening. Product D (lot 2) did meet claims when tested at d 14 in anaerobic conditions.

Based on the results in **Table 3.1**, there should be more regulatory oversight on label accuracy for commercial kefir products to reduce the number of claims that can be misleading to consumers. Classification as a “cultured milk product” by the FDA requires disclosure of added microorganisms, yet regulation of ingredient quality and viability need to be better scrutinized. All 5 kefir products guaranteed specific bacterial species used in fermentation, yet no product matched its labeling completely. Incomplete labeling status can be defined by whether species are detected by sequencing and not listed on the label or when the label did not have enough DNA present of the species listed to be detected by 16S sequencing. Although our results demonstrated close

matches across product labels and their stated contents, several products contained bacterial species above the minimum detectable threshold that were not included on the labels. For example, all products contained *Strep. salivarius*, and 4 out of 5 products contained *Lactobacillus paracasei*. Although there is regulatory oversight preventing companies from making unproven health claims, they should be held to higher standards of ingredient accuracy in their products (Marco et al., 2021). Previous research enumerating commercial pet probiotics and kefir showed that label claims of colony-forming units per gram and microbial taxa are likely to be incorrect (Weese and Martin, 2011; Metras et al., 2020), and no other recent literature has investigated the efficacy of labels in this lucrative market.

Limitations of DNA-based sequencing methods could explain why taxa stated on labels were not detected and why unclaimed viable species were identified. Perhaps species were present but not in high enough abundance for Silva132 species-level classification; thus, they were classified under the bacterial genera (sp.) by the Loop Genomics pipeline [B. J. Callahan, D. Grinevich, and S. Thakur (North Carolina State University), M. A. Balamotis and T. B. Yehezkel (Loop Genomics, San Jose, CA); unpublished data]. For example, *L. casei* and *L. paracasei* both may exist in a sample, yet appear grouped as *Lactobacillus sp.* Databases also have limitations, as they are constantly updated with new species and nomenclature adjustments; running the data against updated databases may provide better categorization (Zheng et al., 2020). A limitation to our bacterial plating was that it was not specific to any taxa. The goal was to culture as much lactic acid bacteria as possible. Thus, the use of specific plating assays may yield different colony-forming units per gram results. It should also be noted that nonviable bacteria may exist in these products. If so, they might not have been cultured on MRS medium (Adams, 2010) and thus would not be included in final counts (cfu/g). Using methods such as sonication instead of vortexing the

kefir products before plating might have broken up cell clumps better and resulted in more accurate counts. The YOG product was used to verify method accuracy. The results of the YOG enumeration and Loop sequencing in **Tables 3.1** and **Table 3.2** closely matched YOG label claims, giving confidence to our methods. The small sample size of 5 products can also be seen as a limitation in the ever-growing market of commercial kefir products. More fermented food research is needed so health professionals can identify which commercial products are truthful on their labels; then consumers might have more protection when purchasing from the vast market of commercial fermented foods.

In conclusion, our analysis of commercial kefir products via culture enumeration and sequencing revealed many deficiencies in label accuracy. Better quality control of these fermented foods is required to demonstrate and understand their potential health benefits for humans. Given the absence of regulatory scrutiny, consumers should be wary of this segment of the market and demand better quality commercial fermented foods.

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Tables and Figures

Table 3.1. Comparison of bacterial cell counts (colony forming units/gram) label claims and enumeration results on deMan Rogosa and Sharpe (MRS) media in aerobic (AER) and anaerobic (ANA) conditions

Product ¹	Claimed	Lot	Day 1		Day 14	
			MRS AER	MRS ANA	MRS AER	MRS ANA
A	N/A	1	2.03X 10 ⁸ ±	1.02 X 10 ⁹ ±	7.53 X 10 ⁸ ±	1.37 X 10 ⁹ ±
			6.77 X 10 ⁷	6.62 X 10 ⁷	4.74 X 10 ⁶	2.97 X 10 ⁸
			1.66 X 10 ⁸ ±	1.36 X 10 ⁹ ±	8.71 X 10 ⁹ ±	1.80 X 10 ¹⁰ ±
		2	5.62 X 10 ⁷	2.22 X 10 ⁸	7.72 X 10 ⁹	3.84 X 10 ⁸
B	N/A	1	4.74 X 10 ⁸ ±	< 2.0 X 10 ⁶	8.49 X 10 ⁸ ±	9.92 X 10 ⁸ ±
			2.81 X 10 ⁸		1.66 X 10 ⁷ *	6.24 X 10 ⁸
			5.46 X 10 ⁸ ±	5.84 X 10 ⁸ ±	2.56 X 10 ⁸ ±	8.81 X 10 ⁸ ±
		2	3.08 X 10 ⁸	1.66 X 10 ⁷	5.62 X 10 ⁷ *	8.63 X 10 ⁷
C	1.00 X 10 ¹¹	1	2.24 X 10 ⁷ ±	1.71 X 10 ⁸ ±	1.43 X 10 ⁷ ±	3.46 X 10 ⁷ ±
			1.00 X 10 ⁻³	1.73 X 10 ⁸	1.00 X 10 ⁻³	1.15 X 10 ⁷
			2.04 X 10 ⁷ ±	7.13 X 10 ⁷ ±	2.55 X 10 ⁷ ±	6.83 X 10 ⁷ ±
		2	1.44 X 10 ⁷	8.65 X 10 ⁶	1.44 X 10 ⁶	1.44 X 10 ⁶
D	1.00 X 10 ⁹	1	1.89 X 10 ⁸ ±	5.67 X 10 ⁸ ±	2.69 X 10 ⁸ ±	3.69 X 10 ⁸ ±
			7.64 X 10 ⁷	1.18 X 10 ⁷	1.00 X 10 ⁻³	8.99 X 10 ⁶
			8.97 X 10 ⁷ ±	3.59 X 10 ⁸ ±	1.86 X 10 ⁸ ±	1.20 X 10 ⁹ ±
		2	1.00 X 10 ⁻³	2.31 X 10 ⁷	5.62 X 10 ⁷	6.24 X 10 ⁷
E	3.00 X 10 ¹⁰	1	1.45 X 10 ⁹ ±	1.10 X 10 ⁹ ±	1.69 X 10 ⁹ ±	1.67 X 10 ⁹ ±
			3.95 X 10 ⁸	9.74 X 10 ⁷	4.07 X 10 ⁷	1.00 X 10 ⁻³
			1.55 X 10 ⁹ ±	1.28 X 10 ⁹ ±	1.00 X 10 ⁹ ±	9.36 X 10 ⁸ ±
		2	1.44 X 10 ⁸	2.57 X 10 ⁸	7.10 X 10 ⁶	2.60 X 10 ⁷
YOG	1.00 X 10 ¹⁰	1	5.12 X 10 ⁹ ± 1	5.71 X 10 ⁹ ±	3.14 X 10 ⁹ ±	4.08 X 10 ⁹ ±
			X 10 ⁻³	2.51 X 10 ⁸	2.12 X 10 ⁸	7.94 X 10 ⁷
			9.00 X 10 ⁹ ±	1.19 X 10 ¹⁰ ±	4.64 X 10 ⁹ ±	1.09 X 10 ¹⁰ ±
		2	1.08 X 10 ⁹	6.96 X 10 ⁹	1.01 X 10 ⁹	1.30 X 10 ⁹

¹Product A: Maple Hill Plain Kefir (Kinderhook, NY); Product B: Siggi's Plain Filmjölk (Richland County, WI); Product C: Redwood Hill Farm Plain Goat Milk Kefir (Sebastopol, CA); Product D: CoYo Kefir (Omaha, NE); Product E: Lifeway Original Kefir (Waukesha, WI); YOG: Chobani Plain Yogurt 0% Milk Fat (South Edmeston, NY).

* Indicates a significant difference from 1 d to 14 d (P < 0.05 by paired Student's t-test).

Table 3.2. Comparison of label claims and 16S rRNA sequencing results (relative abundance) of commercial kefir products

Product ¹	Claimed	Species	Lot 1	Lot 2
A	<i>Streptococcus thermophilus</i>	<i>Streptococcus thermophilus</i>	0.843	0.847
	<i>Lactobacillus acidophilus</i>	<i>Streptococcus salivarius</i>	0.090	0.096
	<i>Lactobacillus delbrueckii</i> subsp. <i>bulgarius</i>	<i>Lactobacillus acidophilus</i>	0.024	0.021
	<i>Lactobacillus rhamnosus</i>	<i>Lactobacillus</i> sp.	0.016	0.011
	<i>Lactococcus lactis</i> subsp. <i>lactic</i> biovar <i>diacetylactis</i>	<i>Streptococcus</i> sp.	0.013	0.013
	<i>Lactococcus lactis</i> subsp. <i>cremoris</i>	<i>Lactobacillus paracasei</i>	0.006	0.005
	<i>Lactococcus lactis</i> subsp. <i>lactis</i>	<i>Lactobacillus delbrueckii</i>	0.003	0.003
	<i>Lactobacillus casei</i>	<i>Lactobacillus rhamnosus</i>	0.002	0.001
	<i>Bifidobacterium lactis</i>	<i>Lactococcus</i> sp.	0.002	0.001
	<i>Leuconostoc cremoris</i>	<i>Lactobacillus casei</i>	0.001	0.001
B	<i>Streptococcus thermophilus</i>	<i>Streptococcus thermophilus</i>	0.489	0.583
	<i>Lactococcus lactis</i> subsp. <i>lactic</i> biovar <i>diacetylactis</i>	<i>Lactococcus</i> sp.	0.329	0.154
	<i>Lactococcus lactis</i> subsp. <i>lactis</i>	<i>Lactobacillus</i> sp.	0.049	0.026
	<i>Lactococcus lactis</i> subsp. <i>cremoris</i>	<i>Streptococcus salivarius</i>	0.045	0.107
	<i>Lactobacillus delbrueckii</i> subsp. <i>bulgaricus</i>	<i>Lactococcus lactis</i>	0.043	0.051
	<i>Leuconostoc</i> sp.	<i>Leuconostoc mesenteroides</i>	0.010	0.004
		<i>Streptococcus</i> sp.	0.010	0.023
		<i>Lactococcus raffinolactis</i>	0.007	0.005
		<i>Lactobacillus rhamnosus</i>	0.006	0.016
		<i>Lactobacillus delbrueckii</i>	0.005	0.011
		<i>Lactobacillus paracasei</i>	0.005	0.012
		<i>Lactobacillus casei</i>	0.002	0.006
		<i>Leuconostoc pseudomesenteroides</i>	0.001	0.000
C	<i>Streptococcus thermophilus</i>	<i>Streptococcus thermophilus</i>	0.823	0.620
	<i>Lactococcus lactis</i> subsp. <i>lactic</i> biovar <i>diacetylactis</i>	<i>Streptococcus salivarius</i>	0.081	0.063
	<i>Lactococcus lactis</i> subsp. <i>cremoris</i>	<i>Lactococcus</i> sp.	0.032	0.001
	<i>Lactobacillus delbrueckii</i> subsp. <i>bulgaricus</i>	<i>Lactobacillus</i> sp.	0.029	0.023

Table 3.2 (cont.)

	<i>Lactobacillus delbrueckii</i> subsp. <i>lactis</i>	<i>Streptococcus</i> sp.	0.014	0.007
	<i>Lactobacillus rhamnosus</i>	<i>Lactobacillus delbrueckii</i>	0.007	0.282
	<i>Lactobacillus casei</i>	<i>Lactobacillus rhamnosus</i>	0.004	0.001
	<i>Lactococcus lactis</i> subsp. <i>lactis</i>	<i>Lactobacillus casei</i>	0.003	0.001
	<i>Lactobacillus acidophilus</i>	<i>Lactobacillus paracasei</i>	0.003	0.001
	<i>Leuconostoc mesenteroides</i> subsp. <i>cremoris</i>	<i>Lactococcus lactis</i>	0.003	0.000
	<i>Bifidobacterium lactis</i>	<i>Lactobacillus acidophilus</i>	0.001	0.001
D	<i>Streptococcus thermophilus</i>	<i>Streptococcus thermophilus</i>	0.884	0.877
	<i>Lactobacillus delbrueckii</i> subsp. <i>bulargicus</i>	<i>Streptococcus salivarius</i>	0.069	0.086
	<i>Bifidobacterium lactis</i>	<i>Streptococcus</i> sp.	0.011	0.012
	<i>Bifidobacterium bifidum</i>	<i>Lactobacillus delbrueckii</i>	0.011	0.009
	<i>Lactobacillus casei</i>	<i>Bifidobacterium</i> sp.	0.007	0.005
	<i>Lactobacillus acidophilus</i>	<i>Leuconostoc mesenteroides</i>	0.005	0.000
	<i>Lactobacillus rhamnosus</i>	<i>Pseudomonas</i> sp.	0.005	0.000
		<i>Pseudomonas lundensis</i>	0.003	0.003
		<i>Lactobacillus</i> sp.	0.002	0.002
		<i>Lactococcus</i> sp.	0.000	0.001
		<i>Lactobacillus casei</i>	0.000	0.001
E	<i>Streptococcus diacetylactis</i>	<i>Streptococcus thermophilus</i>	0.648	0.680
	<i>Lactobacillus rhamnosus</i>	<i>Lactobacillus delbrueckii</i>	0.119	0.108
	<i>Lactococcus lactis</i>	<i>Lactobacillus paracasei</i>	0.103	0.103
	<i>Lactobacillus acidophilus</i>	<i>Streptococcus salivarius</i>	0.065	0.055
	<i>Lactobacillus reuteri</i>	<i>Lactococcus</i> sp.	0.030	0.024
	<i>Lactobacillus plantarum</i>	<i>Lactobacillus casei</i>	0.014	0.010
	<i>Lactobacillus casei</i>	<i>Streptococcus</i> sp.	0.009	0.011
	<i>Bifidobacterium longum</i>	<i>Lactobacillus</i> sp.	0.006	0.001
	<i>Bifidobacterium lactis</i>	<i>Lactobacillus rhamnosus</i>	0.004	0.001
	<i>Bifidobacterium breve</i>	<i>Lactococcus lactis</i>	0.002	0.001
	<i>Leuconostoc cremoris</i>	<i>Lactobacillus acidophilus</i>	0.000	0.001
YOG	<i>Streptococcus thermophilus</i>	<i>Streptococcus thermophilus</i>	0.775	
	<i>Lactobacillus acidophilus</i>	<i>Streptococcus salivarius</i>	0.087	
	<i>Lactobacillus casei</i>	<i>Lactobacillus paracasei</i>	0.046	
	<i>Lactobacillus bulgaricus</i>	<i>Lactobacillus</i> sp.	0.044	
	<i>Lactobacillus bifidus</i>	<i>Lactobacillus acidophilus</i>	0.014	
		<i>Streptococcus</i> sp.	0.014	
		<i>Lactobacillus casei</i>	0.009	

Table 3.2 (cont.)

<i>Lactobacillus rhamnosus</i>	0.008
<i>Lactobacillus delbrueckii</i>	0.002

¹ Product A Maple Hill Plain Kefir (Kinderhook, NY); Product B: Siggi's Plain Filmjölk (Richland County, WI); Product C: Redwood Hill Farm Plain Goat Milk Kefir (Sebastopol, CA); Product D: CoYo Kefir (Omaha, NE); Product E: Lifeway Original Kefir (Waukesha, WI); YOG: Chobani Plain Yogurt 0% Milk Fat (South Edmeston, NY).

* Indicates a significant difference from Lot 1 abundance to Lot 2 abundance ($P < 0.05$ by paired Student's t-test).

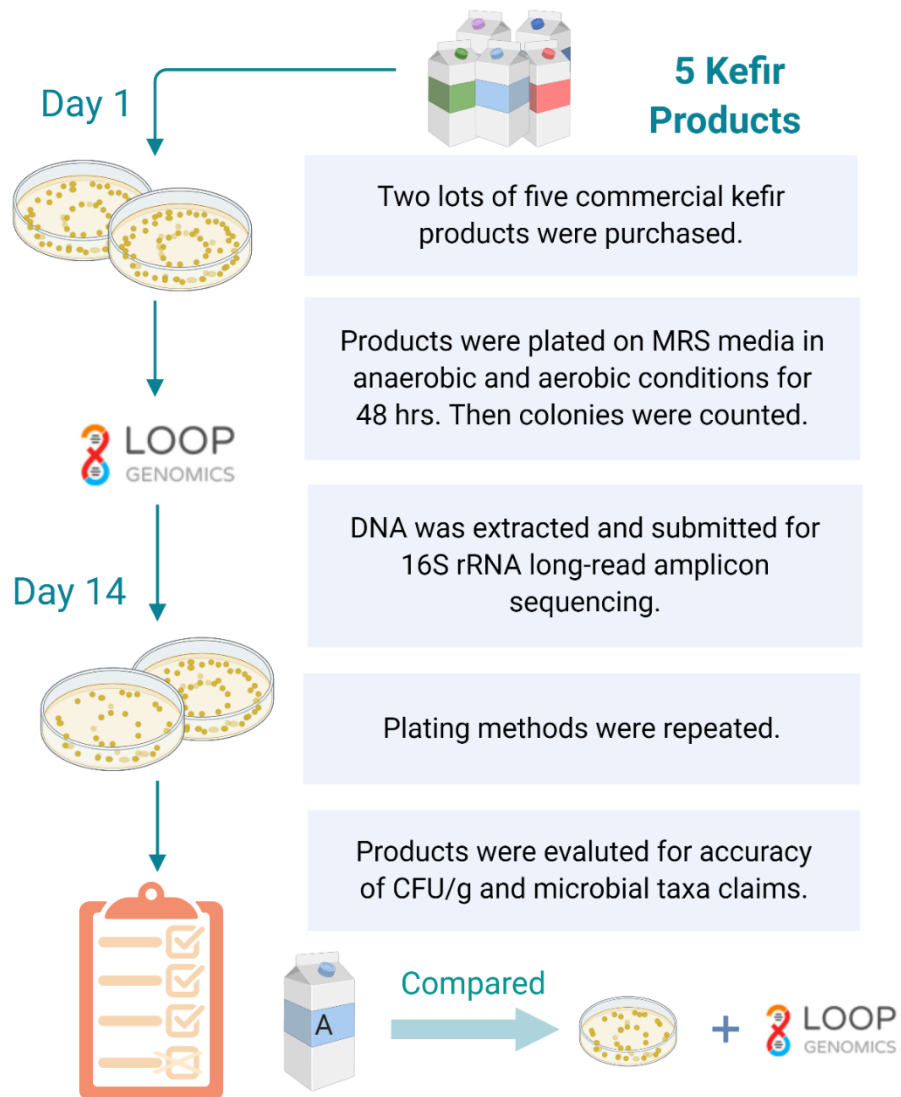


Figure 3.1: This graphical abstract describes the overall methods of bacterial culturing for enumeration and DNA extraction for microbial profiling.

CHAPTER 4: ASSESSMENT OF COMMERCIAL COMPANION ANIMAL KEFIR PRODUCTS FOR LABEL ACCURACY OF MICROBIAL COMPOSITION AND QUANTITY²

Abstract

Kefir is a fermented beverage containing yeast and bacteria produced by the fermentation of water or milk with kefir grains. Lack of regulation for probiotic-containing fermented food sold for companion dogs and cats creates the potential for misreporting on viable microbial counts, taxonomy, and label claims. In this study, the microbiota of six companion animal kefir products were measured quantitatively using standard plating techniques. Microbial composition of these products was also characterized by using high-resolution, long-read amplicon sequencing of the 16S rRNA gene. Five products (83%) listed specific microorganisms, and four products (66%) guaranteed colony forming units (CFU)/g on their label. To enumerate viable lactic acid bacteria (LAB), two lots of each homogenized product were plated upon opening and following 14 d on deMan Rogosa and Sharpe (MRS) agar and incubated under anaerobic and aerobic conditions. Results from point of opening revealed that all commercial kefir products with a guaranteed CFU/g overstated the number of microorganisms present by at least 1 log, with only one product exceeding 1×10^9 CFU/g. Sequencing results demonstrated that none of the labels claiming specific bacterial genera and species on their labels were correct, and all products contained at least three additional bacterial species above the minimum detectable threshold (0.001% relative abundance) that were not disclosed by the manufacturer. In addition to the incorrect viable CFU

² Previously published as Assessment of commercial companion animal kefir products for label accuracy of microbial composition and quantity, by B. N. Metras, 2020, Journal of Animal Sciences (doi: 10.1093/jas/skaa301). Copyright 2020 by Journal of Animal Sciences. Reprinted with permission.

and bacterial taxonomies, several of the product labels and websites contained a wide range of health claims, none of which are supported by the companion animal literature. Our results demonstrate a low level of accuracy in the labeling of commercial kefir products intended for use in dogs and cats. Regulatory agencies, veterinarians, pet food professionals, and pet owners must scrutinize these products and demand a higher level of accuracy and quality in the future.

Introduction

Probiotics are living microorganisms that, when administered in adequate amounts, confer a health benefit on the host (Hill et al., 2014). Probiotics are becoming increasingly popular, as more information is learned of their potential health benefits, with annual worldwide revenue predicted to reach 64 billion dollars by 2023 (Freedman et al., 2020). Probiotics may come in many forms, including capsules, fermented foods, and dairy supplements. In addition to their potential benefits, their ease of intake, low cost, and low incidence of associated adverse events make them appealing to a broad range of consumers (Reid et al., 2019). For companion animals, veterinarians often prescribe probiotics for those with gastrointestinal (GI) disorders, under stress, or during antibiotic treatment. There is mixed support in regard to research demonstrating the impact of introduced bacterial species in animals with GI disease (Suchodolski and Jergens, 2016) or after a round of antibiotics (Reid et al., 2019), but one study did show reduced antibiotic-associated GI signs following probiotic and yeast treatment after antibiotics in cats (Stokes et al., 2017).

Due to the differences that exist between companion animal and human gut microbiomes (Deng and Swanson, 2015), niche markets for probiotic supplements, probiotic foods (e.g., yogurt), and kefirs made specifically for companion animals have emerged. Kefir contains both

yeast and bacteria produced by the fermentation of water or milk with kefir grains (Rosa et al., 2017); it may serve as an alternative to probiotic supplements in companion animals.

The US Food and Drug Administration (FDA) considers probiotic products to be dietary supplements, and according to the FDA Code of Federal Regulations Title 21, kefir is categorized as a cultured milk that contains aroma- and flavor-producing microbial cultures. Where there are nomenclature and manufacturing regulations in place mandating the fermented beverage to be identified as “Kefir cultured milk,” there is little existing regulation regarding the added probiotic quality, quantity, or viability. This lack of regulation has resulted in widespread use of structure-function claims for included microbial cultures that come without or with little evidence of clinical efficacy. Several companion animal kefir product labels contain claims of supporting healthy skin and coat or cognitive function, providing benefit for candidiasis and heart problems, providing anti-fungal properties, preventing allergies, alleviating gas, bloating, and heartburn, and numerous other claims. The popularity of these fermented products in the human consumer market has incentivized misleading label claims resulting in higher sales (Freedman et al., 2020). Randomized clinical trials testing the effects of kefir consumption on humans have been published (Bellikci-Koyu et al., 2019; Wang et al., 2019). Results have suggested that the beverages improve elements of metabolic syndrome, but more clinical trials are needed to understand their effects.

Currently, there are only two published clinical trials that report on testing the effects of kefir in dogs. One study demonstrated that kefir modulated the gut microbiota, including increases in LAB (Kim et al., 2019). The other study reported that dogs supplemented with *Lactobacillus kefir* did not have changes to fecal immunoglobulin A concentrations or microbiota populations (Gaspardo et al., 2020). Because neither study reported health benefits, it is clear that more clinical

research is needed to test what benefits may be provided by kefir products in companion animals. Recent studies have demonstrated the discrepancies and inaccuracies that exist in regard to bacterial counts and identity listed on probiotic labels compared to those measured in a laboratory (Weese and Arroyo, 2003; Weese et al., 2005; Weese and Martin, 2011). Genomic sequencing of probiotic supplements and foods has been limited in the past due to assay and database limitations that made it difficult to identify bacteria at the species level. Recent advances, however, have resulted in the development of assays able to sequence the majority of the bacterial 16S rRNA gene, which is commonly used for identification purposes.

The objective of this study was to investigate the accuracy of label claims made by commercial kefir products designed for companion animals. Given the lack of regulation and results from previous studies testing companion animal probiotic supplements, we hypothesized that the products tested would not match label claims of viable colony counts or bacterial taxonomy.

Materials and Methods

Sample preparation and culture conditions

Companion animal kefir products were purchased from online vendors and local stores in Urbana, IL. This is a niche market and at the time of this study, six products were available for purchase. For each product, two separate lots were ordered to address batch variability, and experiments on each lot were performed in duplicate. Products included the following: Product A: The Honest Kitchen Daily Boosters Instant Goat's Milk w/ Probiotics for Dogs & Cats (Chicago, IL); Product B: Open Farm Raw Organic Grass-Fed Kefir Topper for Dogs & Cats (Toronto, Canada); Product C: Answers Pet Food Raw Cow's Milk Kefir (Fleetwood, PA); Product D: Coco

Love Coconut Kefir for Dogs Nuggets (Chenango County, NY); Product E: Champions Choice Natural Kefir (Millersburg, IN); and Product F: Only Natural Raw Goat Milk Kefir (Boulder, CO). A Chobani Plain Yogurt 0% Milk Fat (YOG; South Edmeston, NY) was included as a standard for assessing technique validity. Claims for CFU/g, bacterial taxonomy, lot numbers, and expiration dates were recorded as stated on the labels. Labels and product websites were evaluated for a clear description of microbial taxa (genus and species) and health claims. Products B, C, D, and F were shipped frozen and were kept frozen until 24 h before analysis. These products were thawed, then refrigerated at 4 °C before analysis as per individual manufacturer instructions. Product E and YOG arrived in refrigerated liquid form and were stored at 4 °C until analysis. Product A was refrigerated at 4 °C and rehydrated prior to analysis as recommended on the label. This was done with DNA-free water to avoid foreign bacterial contamination.

To begin, all products were vortexed and thoroughly homogenized, 1-g product was added to 9 mL of phosphate-buffered saline (PBS), and the product-PBS mixture was vortexed again. Serial 10-fold dilutions were prepared with PBS. A 50-μL volume of 10^5 and 10^7 dilutions were enumerated in duplicate via spiral plater (Eddy Jet Spiral Plater, Neutec Group Inc., Farmingdale, NY) onto deMan Rogosa Sharpe (MRS) Abbreviations CFU/g colony forming units/gram GI gastrointestinal FDA Food and Drug Administration LAB lactic acid bacteria MRS deMan Rogosa and Sharpe PBS phosphate-buffered saline media (BD Difco, Franklin Lakes, NJ). MRS plates were incubated anaerobically at 37 °C for 48 h and aerobically at 30 °C for 48 h to culture lactic acid bacteria (LAB). Colony counts were measured via spiral plating software (Colony counter, IUL Flash and Go, Neutec Group Inc., Farmingdale, NY). No attempt to distinguish organisms was made, only enumeration. Fourteen days after product opening, enumeration was repeated for

all product lots in identical conditions. Results at time of opening and results at 14 d are reported as the average of each duplicate from both product lots.

DNA extraction and sequencing

Samples were vortexed until homogenized, then 250 mg of each kefir product sample on the day of opening was used for DNA extraction (DNeasy PowerLyzer PowerSoil Kit, Qiagen, Germantown, MD) following manufacturer's instructions. DNA quantity was determined using a Qubit 3.0 Fluorometer (Life Technologies, Carlsbad, CA) and DNA quality was assessed via gel electrophoresis (E-Gel Power Snap and E-Gel EX 1% Agarose, Invitrogen by Thermo Fisher Scientific, Carlsbad, CA). DNA extracts were sequenced at the W. M. Keck Center for Biotechnology at the University of Illinois at Urbana Champaign using the LoopSeq 16S Long Read Kit (Loop Genomics, San Jose, CA) at a 1 ng/ μ L concentration. All samples were processed following the standard Loop Genomics protocol for full length 16S sequencing. Sequence results were processed through the SILVA132 Large Subunit rRNA Database. All taxa with a relative abundance greater than 0.001% were retained for analysis.

Statistical analysis

Statistical analyses were conducted using R x64 version 4.0.2 (R Foundation for Statistical Computing, Vienna, Austria). Data obtained from enumeration samples on 1 and 14 d, and relative abundance of Lot 1 and relative abundance of Lot 2 were tested for significance via paired Student's t-test. A P value less than 0.05 was considered statistically significant.

Results

Bacterial enumeration

Bacterial plating on MRS media results is presented in Table 1. Anaerobic conditions at 37 °C were maintained to culture LAB, and aerobic conditions were held at 30 °C to culture aerobes such as *Streptococcus thermophilus* and *Lactobacillus lactis*. The label for Product A claimed that each sachet contained 5×10^9 CFU/g at point of rehydration. Using our culture media and conditions, Lot 2 failed to reach the 2.0×10^6 threshold of the assay on 1 and 14 d, yet Lot 1 reached $2.04 \times 10^8 \pm 1 \times 10^3$ and $1.53 \times 10^9 \pm 1.87 \times 10^9$ in anaerobic conditions. Product B is sold as a kefir topper for dogs and cats, claiming 1.5×10^{10} CFU/g. Using our culture media and conditions, Lot 1 and Lot 2 contained colonies much lower than the counts claimed on the label. Although Product C claimed 1×10^{10} CFU/g, our culture methods showed that neither lot surpassed 1.4×10^8 CFU/g at opening. Colony counts decreased when tested 14 d later. Product D's label had no CFU/g claims. Upon enumeration, both Lot 1 and Lot 2 contained less than our detection limit (2.0×10^6 CFU/g). Product E did not make claims for amount of viable CFU on their label, but had the highest cell counts of the kefir products tested. Our enumeration results at time of opening showed that Lot 1 contained $9.68 \times 10^8 \pm 3.69 \times 10^7$ and Lot 2 contained $9.14 \times 10^8 \pm 7.11 \times 10^7$ when tested under aerobic conditions. Retesting 14 d after opening showed that viable colony counts significantly decreased. Product F claimed to contain 1.00×10^6 CFU/g at opening, yet neither lot contained enough bacteria to exceed the detectable colony threshold. After 14 d, viable colonies were still below the threshold. The standard product used to validate techniques (YOG) claimed to contain 1×10^{10} CFU/g. Our enumeration tests estimated that Lot 1 and Lot 2 met these claims. When products were tested again 14 d after opening, the values were largely unchanged.

Sequencing bacterial DNA

Loop Genomics sequencing was used to assess the 16S (bacteria) rRNA components of the test products (Table 2). The most common and abundant genera were *Lactococcus*, *Streptococcus*, and *Bacillus*. For some products, the bacterial genus and species listed on the labels appeared to differ significantly from our results. Product A claimed to contain four bacterial species (*B. coagulans*, *L. casei*, *L. acidophilus*, and *L. rhamnosus*), which were all detected in the sequencing results. In addition, nearly 10 unclaimed species were detected. For Product B, sequencing results only detected the *Lactobacillus* and *Propionibacterium* species claimed on its label. However, the *Bifidobacterium*, *Enterococcus* and *Leuconostoc spp.* listed were not detected. Product C claimed nine bacterial species on their label, yet sequencing detected 15 bacterial species among seven bacterial genera. For Product D, sequencing results confirmed seven bacterial species, all LAB, yet none were claimed on the label. Product E claimed to contain four bacterial genera and one bacterial species, yet only DNA from the *Lactococcus*, *Streptococcus*, and *Lactobacillus* genera were detected. No *Acetobacter* were detected (less than 0.1% abundance). Product F only claimed one bacterial species, yet our analyses detected nine bacterial genera, including 10 bacterial species but not the species on the label. The yogurt sample claimed to contain five bacterial species. Our sequence data were successful for Lot 1 but failed to generate sufficient data for Lot 2 due to extremely low DNA concentrations. Four of the five species claimed were clearly identified via sequencing. Sequencing also identified a significant abundance of unidentified lactobacilli, which may explain the lack of *L. bifidus* in the sequencing results. An additional three bacterial species were detected via sequencing but each is closely related to another identified species, suggesting a limitation for using Loop for species discrimination of very similar species.

Discussion

The market for companion animal probiotic products has increased in demand in recent years but has little regulatory oversight. Previous studies have demonstrated deficiencies in companion animal probiotic quality, quantity, and label accuracy (Weese and Martin, 2011). After analyzing six pet kefir products, all three brands that made CFU/g claims failed to provide the guarantees stated on their labels. These results suggest a lack of quality control, accurate testing, and/or deficiencies in bacteria stability during storage. Even if label claims were met at the point of manufacture, viability should be maintained until the expiration date is reached. Literature supports that bacteria do not need to be alive to provide an anti-inflammatory response, but must be viable to modulate the microbiota (Adams, 2010). Certain products had counts up to 10^7 – 10^8 CFU/g, so it is reasonable to assume that these products with higher counts would be more likely to be effective than those with lower counts. It should be noted that cultivation of LAB was attempted in both anaerobic and aerobic conditions to enumerate as many species as possible in each product. Previous in vivo probiotic studies in pets have tested doses of 10^7 – 10^9 CFU/g and observed effective responses as measured by microbial culture (Strompfová et al., 2006; Huys et al., 2013). Of the three brands that did not make claims, two of them did not have enough viable colonies to meet our minimum detection threshold of 2.0×10^6 CFU/g.

Despite there being only two published clinical trials in dogs whereby kefir response was minimal (Kim et al., 2019; Gaspardo et al., 2020), the six kefir products tested in this study all made health claims on their websites or on their product labels. Product A claimed to support digestion and the immune system. Several claims were made by product B, including support for strong teeth, soft skin and coat, improved gut health and digestion, as well as a strong immune system. Product C claimed to assist in the ease of digestion, and that consuming live enzymes will

maximize nutrient absorption. A range of health claims were made by product D, including support for healthy skin and coat, cognitive function, delayed osteoarthritis, and improved blood circulation. Product E made the most health claims for their product, stating anti-fungal properties, restoration of the digestive tract after a course of antibiotics, prevention of allergies, promotion of healthy skin, alleviation of gas, bloating, and heartburn, and several more diseases. Product F claimed to settle a sensitive stomach, support a healthy immune system, and entice picky eaters. Clinical trials have been performed to understand the effects of kefir on humans, but these conclusions cannot be directly translated to dogs. Like claims for probiotics (Hill et al., 2014) and prebiotics (Gibson et al., 2017), kefir products should be tested in the target host animal. Until these studies are conducted, these broad claims are not supported by the published literature.

Product A contained high amounts of *Bacillus coagulans*. *B. coagulans* are spore-forming bacteria, which when sporulated can withstand processing and acidic environments such as the stomach thus able to reach the small intestine for germination (Ara et al., 2002). If present as spores in the products, it is possible that the spores did not germinate and form colonies on our plates and thus underrepresent the counts in product A. This species has been demonstrated to assist companion animals in carbohydrate utilization and to create an environment inhospitable to pathogens (Jäger et al., 2018). However, further studies are needed to fully understand the safety and dose of *B. coagulans* probiotics for pets. In Products B and D, the main bacteria detected were of the *Streptococcus* genera (**Table 4.2**). Streptococci are Gram-positive bacteria and are normal inhabitants of animal and human GI microbiota. *Streptococcus thermophilus* is often essential in industrial dairy production as yogurt starters, so it is unsurprising that these bacteria appear in high amounts in kefir products. Other pathogenic members of streptococci such as *Streptococcus mutans* and *Streptococcus pneumoniae* are pathogenic to mammals, but *Streptococcus*

thermophilus has adapted to the dairy environment and lost functionality of genes not involved in basic cellular functions (Price et al., 2012).

The genera *Lactococcus* made up the majority of sequences measured in Products C, E, and F, and is a LAB common in dairy products. The species *L. lactis* is commonly used as a starter culture for dairy products, but no companion animal studies have researched effective dose or observed its impacts on the animal's GI tract and health indices. Many of the tested kefir products contained numerous members of the *Lactobacillus* genera, including *L. acidophilus*, *L. casei*, *L. delbrueckii*, *L. paracasei*, and *L. rhamnosus*, several of which have been investigated individually and as “cocktails” for use in cats and dogs (Baillon et al., 2004; Garcia-Mazcorro et al., 2011; Ferandez et al., 2019; Fusi et al., 2019). It should be noted that in Product D and YOG, their labels claim to contain *Lactobacillus bulgaricus* whose proper name is *Lactobacillus delbreuckii* subsp. *bulgaricus*. Because the names of many *Lactobacillus* spp. have now been changed (Zheng et al., 2020), labels for many commercial probiotic products will require revision in the near future.

Considering our results, it would be very difficult to detect and enumerate all claimed bacteria present in equal amounts. Identification of *Enterococcus* and *Leuconostoc* genera by LoopSeq was extremely low; this suggests poor survival in the final fermented product, potential bias in the DNA extraction procedure, or limitations in the primers used for sequencing. Products were thoroughly homogenized before samples were taken, but sonification might have broken up cell clusters better to provide a more accurate enumeration of bacteria. Further tests using real-time polymerase chain reaction for individual genera could confirm these hypotheses. Many bacterial species not claimed by the kefir labels were detected in sequencing, suggesting those species might originate from the manufacturing process and were not unintentionally listed, or

were misidentified by SILVA132 data base due to high sequence similarity with other species. For example, *Lactobacillus casei* and *L. paracasei* have very similar 16S rRNA sequences and may lead to misidentification when only one of these is present in the sample such as with products A, B, and YOG. Some labels had the issue of inconsistency in labeling bacterial genera and species properly, while others had issues with correct spellings and proper capitalization.

In conclusion, our analysis of companion animal kefir products via culture enumeration and sequencing revealed many deficiencies in label accuracy. Better quality control of these products and clinical trials to demonstrate and understand their potential health benefits for animals are required. Given the various health claims, inconsistent product quality, and absence of regulatory scrutiny, consumers should be wary of this segment of the market and demand better quality commercial probiotics for their pets.

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Tables

Table 4.1. Comparison of bacterial cell counts (colony forming units/gram) label claims and enumeration results on deMan Rogosa and Sharpe (MRS) media in aerobic (AER) and anaerobic (ANA) conditions.

Product	Claimed	Day 1	Day 14
		Highest Amount Detected	Highest Amount Detected
A	5.00×10^9	$2.04 \times 10^8 \pm 1 \times 10^{-3}$	$1.53 \times 10^9 \pm 1.87 \times 10^9$
B	1.50×10^{10}	$4.79 \times 10^7 \pm 4.32 \times 10^6$	$7.22 \times 10^6 \pm 7.81 \times 10^5$
C	1.00×10^{10}	$1.4 \times 10^8 \pm 5.33 \times 10^7$	
D	N/A	$< 20 \times 10^5$	
E	N/A	$9.68 \times 10^8 \pm 3.69 \times 10^{7*}$	$< 20 \times 10^5$ $4.48 \times 10^7 \pm 1 \times 10^{-3*}$
F	1.00×10^6	$< 20 \times 10^5$	$< 20 \times 10^5$
YOG	1.00×10^{10}	$1.19 \times 10^{10} \pm 6.96 \times 10^9$	$1.09 \times 10^{10} \pm 1.30 \times 10^9$

¹Product A: The Honest Kitchen Daily Boosters Instant Goat's Milk w/ Probiotics for Dogs & Cats (Chicago, IL); Product B: Open Farm Raw Organic Grass-Fed Kefir Topper for Dogs & Cats (Toronto, Canada); Product C: Answers Pet Food Raw Cow's Milk Kefir (Fleetwood, PA); Product D: Coco Love Coconut Kefir for Dogs Nuggets (Chenango County, NY); Product E: Champions Choice Natural Kefir (Millersburg, IN); Product F: Only Natural Raw Goat Milk Kefir (Boulder, CO); YOG: Chobani Plain Yogurt 0% Milk Fat (South Edmeston, NY).

*Indicates a significant difference from 1 d to 14 d ($P < 0.05$ by paired Student's t-test).

Table 4.2. Comparison of label claims and 16S rRNA sequencing results (relative abundance) of commercial kefir products.

Product¹	Claimed	Species	Abundance Lot 1	Abundance Lot 2
A	<i>Bacillus coagulans</i>	<i>Bacillus coagulans</i>	0.793	0.759
	<i>Lactobacillus casei</i>	<i>Lactobacillus sp.</i>	0.098	0.144
	<i>Lactobacillus acidophilus</i>	<i>Lactobacillus casei</i>	0.044	0.060
	<i>Lactobacillus rhamnosus</i>	<i>Lactobacillus acidophilus</i>	0.023	0.008
		<i>Lactobacillus paracasei</i>	0.012	0.005
		<i>Lactobacillus plantarum</i>	0.011	0.006
		<i>Lactobacillus rhamnosus</i>	0.010	0.004
		<i>Pseudomonas stutzeri</i>	0.003	0.003
		<i>Bacillus sp.</i>	0.002	0.002
		<i>Lactobacillus pentosus</i>	0.001	0.000
		<i>Staphylococcus sp.</i>	0.000	0.001
		<i>Lactococcus sp.</i>	0.000	0.001
		<i>Streptococcus salivarius</i>	0.000	0.001
		<i>Streptococcus thermophilus</i>	0.000	0.001
B	<i>Bifidobacterium animalis</i>	<i>Streptococcus thermophilus</i>	0.420	0.352
	<i>Enterococcus cremoris</i>	<i>Lactobacillus paracasei</i>	0.285	0.174
	<i>Enterococcus diacetyenterococcus thermophilus</i>	<i>Lactococcus sp.</i>	0.137	0.125
	<i>Enterococcus diacetylacti</i>	<i>Lactobacillus delbrueckii</i>	0.055	0.041
	<i>Enterococcus thermophilus</i>	<i>Streptococcus salivarius</i>	0.037	0.130
	<i>Lactobacillus acidophilus</i>	<i>Lactobacillus casei</i>	0.025	0.068
	<i>Lactobacillus casei</i>	<i>Lactococcus lactis</i>	0.014	0.046
	<i>Lactobacillus delbrueckii</i>	<i>Lactobacillus sp.</i>	0.010	0.014
	<i>Leuconostoc mesenteroides</i>	<i>Lactobacillus rhamnosus</i>	0.007	0.015
	<i>Propionibacterium freudenreichii</i>	<i>Streptococcus sp.</i>	0.007	0.031
	<i>Propionibacterium freudenreichii</i>	<i>Lactobacillus acidophilus</i>	0.003	0.002
		<i>Propionibacterium sp.</i>	0.001	0.000
C	<i>Lactobacillus acidophilus</i>	<i>Lactococcus sp.</i>	0.753	0.724
	<i>Lactobacillus bulgaricus</i>	<i>Lactococcus lactis</i>	0.079	0.079
	<i>Lactobacillus caucasicus</i>	<i>Streptococcus thermophilus</i>	0.070	0.065

Table 4.2 (cont.)

		<i>Lactobacillus</i>		
	<i>Lactobacillus kefir</i>	<i>kefiranofaciens</i>	0.053	0.089
	<i>Lactococcus cremoris</i>	<i>Pseudomonas sp.</i>	0.012	0.010
	<i>Lactococcus lactis</i>	<i>Leuconostoc mesenteroides</i>	0.009	0.010
	<i>Leuconostoc cremoris</i>	<i>Streptococcus salivarius</i>	0.005	0.006
	<i>Leuconostoc</i>			
	<i>mesenteroides</i>	<i>Streptococcus uberis</i>	0.004	0.003
	<i>Saccharomyces kefir</i>	<i>Citrobacter freundii</i>	0.004	0.003
		<i>Lactobacillus kefir</i>	0.003	0.004
		<i>Streptococcus sp.</i>	0.003	0.002
		<i>Lactobacillus sp.</i>	0.001	0.001
		<i>Lactococcus raffinolactis</i>	0.001	0.002
		<i>Raoultella ornithinolytica</i>	0.001	0.001
		<i>Pseudomonas fluorescens</i>	0.001	0.001
D	N/A	<i>Streptococcus thermophilus</i>	0.567	0.669
		<i>Lactobacillus sp.</i>	0.122	0.097
		<i>Lactobacillus acidophilus</i>	0.113	0.094
		<i>Streptococcus salivarius</i>	0.091	0.067
		<i>Lactobacillus paracasei</i>	0.035	0.027
		<i>Lactobacillus rhamnosus</i>	0.026	0.014
		<i>Lactobacillus casei</i>	0.016	0.009
		<i>Streptococcus sp.</i>	0.015	0.010
		<i>Lactobacillus delbrueckii</i>	0.012	0.012
		<i>Lactococcus sp.</i>	0.001	0.000
E	<i>Acetobacter</i>	<i>Lactococcus sp.</i>	0.823	0.881
	<i>Lactobacillus caucasicus</i>	<i>Lactococcus lactis</i>	0.169	0.112
	<i>Lactobacillus</i>	<i>Lactobacillus sp.</i>	0.004	0.002
	<i>Leuconostoc</i>	<i>Streptococcus uberis</i>	0.002	0.002
	<i>Streptococcus</i>	<i>Lactobacillus casei</i>	0.001	0.000
	<i>Saccharomyces kefir</i>	<i>Lactobacillus plantarum</i>	0.001	0.000
		<i>Streptococcus sp.</i>	0.000	0.001
		<i>Streptococcus thermophilus</i>	0.000	0.001
F	<i>Lactobacillus acidophilus</i>	<i>Lactococcus sp.</i>	0.891	0.878
		<i>Lactococcus lactis</i>	0.075	0.090
		<i>Lactobacillus</i>		
		<i>kefiranofaciens</i>	0.003	0.004
		<i>Streptococcus sp.</i>	0.002	0.001
		<i>Enterococcus faecalis</i>	0.001	0.001

Table 4.2 (cont.)

		<i>Lactobacillus paracasei</i>	0.001	0.000
		<i>Streptococcus thermophilus</i>	0.001	0.001
YOG	<i>Lactobacillus bulgaricus</i>	<i>Streptococcus thermophilus</i>	0.775	
	<i>Lactobacillus acidophilus</i>	<i>Streptococcus salivarius</i>	0.087	
	<i>Lactobacillus bifidus</i>	<i>Lactobacillus paracasei</i>	0.046	
	<i>Lactobacillus casei</i>	<i>Lactobacillus sp.</i>	0.044	
	<i>Streptococcus thermophilus</i>			
		<i>Lactobacillus acidophilus</i>	0.014	
		<i>Streptococcus sp.</i>	0.014	
		<i>Lactobacillus casei</i>	0.009	
		<i>Lactobacillus rhamnosus</i>	0.008	
		<i>Lactobacillus delbrueckii</i>	0.002	

¹Product A: The Honest Kitchen Daily Boosters Instant Goat's Milk w/ Probiotics for Dogs & Cats (Chicago, IL); Product B: Open Farm Raw Organic Grass-Fed Kefir Topper for Dogs & Cats (Toronto, Canada); Product C: Answers Pet Food Raw Cow's Milk Kefir (Fleetwood, PA); Product D: Coco Love Coconut Kefir for Dogs Nuggets (Chenango County, NY); Product E: Champions Choice Natural Kefir (Millersburg, IN); Product F: Only Natural Raw Goat Milk Kefir (Boulder, CO); YOG: Chobani Plain Yogurt 0% Milk Fat (South Edmeston, NY).

*Indicates a significant difference from Relative Abundance Lot 1 to Relative Abundance Lot 2 ($P < 0.05$ by paired Student's t-test).

CHAPTER 5: EFFECTS OF COMMERCIAL AND TRADITIONAL KEFIR SUPPLEMENTATION ON APPARENT TOTAL TRACT MACRONUTRIENT DIGESTIBILITY AND THE FECAL CHARACTERISTICS, METABOLITES, AND MICROBIOTA OF HEALTHY ADULT DOGS³

Abstract

Kefir is a fermented dairy beverage that has been consumed by humans for centuries, but poorly studied in pets. The objective of this study was to determine the effects of commercial or traditional kefir supplementation on apparent total tract macronutrient digestibility (ATTD) and fecal characteristics, microbiota populations, and metabolite and immunoglobulin (Ig) A concentrations of healthy adult dogs. Twelve healthy adult dogs (5.67 ± 1.72 y, 7.27 ± 1.15 kg) were used in a replicated 3x3 Latin square design (n=12/group). All dogs were fed a commercial diet and allotted to 1 of 3 treatments (60 mL/d): 2% reduced-fat milk treated with lactase [CNTL; 4.57×10^3 lactic acid bacteria (LAB) colony-forming units (CFU)/mL], commercial kefir (C-Kefir; 6.95×10^4 LAB CFU/mL), or traditional kefir brewed daily from 2% reduced-fat milk and kefir grains (T-Kefir; 1.79×10^9 LAB CFU/mL). The experiment was composed of three 28-d periods, with each consisting of a 22-d transition phase, a 5-d fecal collection phase, and 1 d for blood collection. Fecal samples were collected for determination of ATTD and fecal pH, dry matter, microbiota, and metabolite and IgA concentrations. Data were analyzed using the Mixed Models procedure of SAS 9.4. The main effects of treatment were tested, with significance set at $p \leq 0.05$ and trends set at $p \leq 0.10$. Kefir products differed in microbial density and profile, but fecal

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microbiota populations were weakly impacted. Bacterial alpha diversity tended to be greater ($p=0.10$) in dogs fed T-Kefir than those fed CNTL. Bacterial beta diversity analysis identified a difference ($p<0.0004$) between dogs fed CNTL and those fed C-Kefir. Dogs fed C-Kefir tended to have a greater ($p=0.06$) relative abundance of Fusobacteriota than those fed CNTL or T-Kefir. Dogs fed T-Kefir had a greater ($p<0.0001$) relative abundance of *Lactococcus* than those fed CNTL or C-Kefir. Dogs fed T-Kefir also tended to have a lower ($p=0.09$) relative abundance of *Escherichia-Shigella* and greater ($p=0.09$) relative abundance of *Candidatus stoquefichus* than dogs fed CNTL or C-Kefir. Dogs fed C-Kefir tended to have lower ($p=0.08$) fecal valerate concentrations than those fed CNTL or T-Kefir. All other measures were unaffected by kefir treatments. Our results suggest that kefir supplementation had minor effects on the fecal microbiota populations and fecal metabolite concentrations of healthy adult dogs without impacting ATTD, fecal characteristics, or fecal IgA concentrations.

Introduction

Kefir is a fermented milk beverage traditionally produced from the fermentation of kefir grains with milk. Kefir has been consumed for centuries, originating from the Caucasus Mountains and Tibet (Rosa et al., 2017). Kefir products falls into the category of fermented foods, which have been defined by the International Scientific Association of Probiotics and Prebiotics to be “foods made through desired microbial growth and enzymatic conversions of food components” (Marco et al., 2021). DNA sequencing has shown that traditionally-produced kefir contains bacteria such as *Lactobacillus kefiranofaciens*, *Lentilactobacillus kefir*, and *Lentilactobacillus parakefiri* and yeasts such as *Saccharomyces*, *Kluyveromyces*, *Naumovozyma*, and *Candida* (Guzel-Seydim et al., 2011; Nejati et al., 2022). In contrast to traditional kefir, commercial kefir sold in stores are

based on bacterial cultures (typically no yeast) that ferment milk. Because the commercial kefir products are not brewed from kefir grains, but from defined cultures, they do not possess the traditional kefir microbial profiles (Nejati et al., 2022). Human kefir products sold in the United States have a microbial profiles containing *Lactobacillus*, *Lactococcus*, *Streptococcus*, and/or *Leuconostoc* (Metras et al., 2021a). Similarly, commercial companion animal kefir products primarily consist of *Bacillus*, *Streptococcus*, *Lactobacillus*, and *Lactococcus* (Metras et al., 2020a). Although fermented foods and beverages are believed to provide health benefits, further work is needed to better understand the potential physiological benefits they provide to companion animals.

In addition to microbiota, kefirs contain other fermentation by-products, such as exopolysaccharides, water-soluble polysaccharides, kefir-fermented peptides, and kefir cell-free supernatants that may provide host benefits (Simova et al., 2002a; Medrano, 2008). Traditional grain-based kefirs have been shown to modulate the gut microbiome and improve cholesterol metabolism in mice (Bourrie et al., 2016a). A handful of randomized clinical trials assessing a traditional kefir intervention have been conducted in healthy adult humans (Pražnikar et al., 2020; Wang et al., 2020a) as well as in patients who are overweight (Fathi et al., 2017), diabetic (Judiono et al., 2014; Bellikci-Koyu et al., 2019; El-Bashiti et al., 2019; Ostadrahimi et al., 2015), or hypercholesteremic (St-Onge et al., 2002a). A recent meta-analysis determined that kefir has a significant impact on reducing insulin ($p<0.05$) and fasting blood sugar concentrations ($p<0.05$) when at least 200 mL were consumed for at least 4 wk consecutively (Salari et al., 2021). In one study, Praznikar et. al. (2020) reported that kefir consuming people had lower serum C-reactive protein ($p<0.018$), high density lipoprotein ($p<0.013$), and fasting blood sugar ($p<0.001$) concentrations than those consuming milk. Other human clinical trials conducted, however, have

not observed consistent physiological outcomes, which could be attributed to the large microbial variations among traditional and commercial kefir products used.

Research on the health effects of various kefir products has been lacking, especially in companion animals. This is especially important as there are commercial kefir products that are advertised for dogs. A couple studies have investigated kefir in dogs, but neither specified if traditional or commercial kefir was used, providing uncertainty to kefir's overall attributes. In one study, healthy adult dogs receiving 200 mL/day of traditional grain kefir [9.32 ± 0.23 log CFU/mL of lactic acid bacteria (LAB); 7.12 ± 0.36 log CFU/mL of yeast] for 2 weeks had some shifts in the fecal microbiota (Kim et al., 2019). While alpha diversity indices were unchanged, kefir consumption led to shifts in beta diversity and changes to several individual bacterial taxa. Based on sequencing data, the relative abundances of several bacterial families (Prevotellaceae, Selenomonadaceae, and Sutterellaceae) were increased ($p < 0.05$), while the relative abundances of other bacterial families (Clostridiaceae, Fusobacteriaceae, and Ruminococcaceae) were decreased ($p < 0.05$) after kefir consumption. Based on qPCR data, kefir consumption was shown to increase ($p = 0.012$) LAB abundance, increase ($p = 0.04$) the LAB:Enterobacteriaceae ratio, and decrease ($p = 0.03$) the Firmicutes:Bacteroidetes ratio. In another study, healthy adult dogs were fed *Lentilactobacillus kefiri* (from Kefibios®; 1×10^7 CFU/day) for 30 days, but no significant differences in fecal IgA or microbiota were observed (Gaspardo et al., 2020a).

Given the lack of research on kefir products in dogs, especially in comparison of grain-based and commercial kefirs, further in vivo research in this area is justified. The objective of this study was to determine the effects of commercial or traditional kefir supplementation on apparent total tract digestibility (ATTD) and the fecal characteristics, microbiota, and metabolite and immunoglobulin A (IgA) concentrations of healthy adult dogs. Without negatively affecting

ATTD or fecal characteristics, we hypothesized that kefir supplementation would beneficially shift the fecal microbiota and metabolites and increase fecal IgA concentrations of adult dogs compared with controls. We also hypothesized that the traditional kefir would have greater effects than commercial kefir product.

Materials and Methods

All procedures were approved by the University of Illinois Institutional Animal Care and Use Committee prior to experimentation. All methods were performed in accordance with the United States Public Health Service Policy on Humane Care and Use of Laboratory Animals.

Animals, treatments, and experimental design

Twelve healthy adult spayed female dogs (age: 5.67 ± 1.72 y; body weight: 7.27 ± 1.15 kg) were used in a replicated 3 x 3 Latin square design (n=12/group). The experiment consisted of three 28-d periods, each consisting of a 22-d transition phase, a 5-d fecal collection phase, and 1 d for blood collection. All dogs were housed individually in pens (1.0 m wide by 1.8 m long) in an environmentally controlled facility at the University of Illinois Urbana-Champaign. Dogs had free access to fresh water at all times. All dogs were fed a commercial diet without probiotics or prebiotics, having low levels of fermentable fiber, and formulated to meet all essential nutrients recommended by the Association of American Feed Control Officials (AAFCO, 2022) for adult dogs at maintenance (Best Dog 21/12, Coker Feed Mill, Inc., Goldsboro, NC). On the basis of the maintenance energy requirement for adult dogs and information from previous feeding records, an amount of food to maintain body weight was offered and intake was measured twice daily (8 am;

4 pm). Dogs were weighed and body condition scores were assessed (9-point scale) once a week prior to feeding.

The following treatments were tested: diet + 2% milk treated with lactase (CNTL; 2% Reduced Fat Milk, Great Value Farm, Fort Wayne, IN; 60 mL/d); diet + commercial kefir (C-Kefir; Champions Choice Kefir; Champions Choice, Millersburg, IN, 60 mL/d); and diet + traditional kefir (T-Kefir; Traditional Kefir; Kefir Garden Grains, Hamilton Ontario, Canada; 60 mL/d). Whole milk and kefir products were stored at 4°C. Before feeding, milk and kefir product containers were inverted 10 times to homogenize the products. In addition, 1 gallon of 2% reduced fat milk was treated with 14 lactase drops 24 h before being fed to dogs (Lactase Drops Seeking Health, Bellingham, WA). Servings of 30 mL were aliquoted and given to dogs at each meal (60 mL/dog/d).

The commercial kefir product was ordered from a local vendor, shipped in liquid form, and refrigerated immediately upon arrival. The product was refrigerated at 4°C between feedings and was thoroughly homogenized before feedings. The kefir brewing process to prepare the traditional kefir began with the addition of 5 % w/v kefir grains to 2% reduced-fat dairy milk (Great Value Farm, Fort Wayne, IN). Kefir grains and kefir mixtures were incubated at 25°C in a sterilized lightly sealed glass jar for 24 h. The pH of the kefir was measured after 24 h of fermentation using a pH meter equipped with a pH and temperature probe (Orion™ Triode™ 3-in-1 pH/Automatic Temperature Compensation Probe, ThermoFisher Scientific, Waltham, MA), with an ideal pH range maintained between pH 4-5. After 24 h, grains were removed using a stainless-steel strainer, with the kefir refrigerated at 4°C until it was fed. Kefir grains were rinsed with fresh 2% reduced-fat milk and stored in 2% reduced-fat milk at 25°C for 24 h, when the process was repeated. Each

batch of grain kefir was viable for use up to 72 h after the initial product was refrigerated. Fresh grain kefir batches were prepared daily to maintain freshness.

Milk and kefir bacterial quantification

To begin, all milk and kefir products were inverted 10 times for homogenization, with 1 mL of product added to 9 mL of phosphate-buffered saline (PBS), and the product-PBS mixture inverted 10 times again. Serial 10-fold dilutions were prepared with PBS. A 50- μ L volume of 10^5 and 10^7 dilutions were enumerated in duplicate via spiral plater (Eddy Jet Spiral Plater, Neutec Group Inc., Farmingdale, NY) onto deMan Rogosa Sharpe media (BD Difco, Franklin Lakes, NJ). deMan Rogosa Sharpe plates were incubated anaerobically at 37°C for 48 h and aerobically at 30°C for 48 h to culture LAB. Colony counts were measured via spiral plating software (Colony counter, IUL Flash and Go, Neutec Group Inc., Farmingdale, NY) once each experimental period, using composite treatment samples in triplicate. Average anaerobic and average aerobic counts were combined to calculate a total LAB count (CFU/mL). No attempt to distinguish organisms were made, only enumeration.

Fecal collection, scoring, and handling

Total feces excreted during the collection phase were collected from each dog, weighed, and frozen at -20°C until analyses. During the collection phase, all fecal samples were scored using the following scale: 1 = hard, dry pellets, small hard mass; 2 = hard, formed, dry stool; remains firm and soft; 3 = soft, formed, and moist stool, retains shape; 4 = soft, unformed stool, assumes shape of container; and 5 = watery, liquid that can be poured. During the collection phase, one fresh fecal sample (within 15 min of defecation) was collected for the measurement of pH and dry

matter (DM) content, microbiota populations, and metabolite and IgA concentrations. Fecal metabolites of interest included short-chain fatty acids (SCFA), which serve as an important energy source for colonocytes, and protein fermentative products [ammonia; branched-chain fatty acids (BCFA); phenols; indoles] that are responsible for fecal odor and are associated with gastrointestinal disease. Fecal aliquots for analysis of phenols and indoles were frozen at -20°C immediately after collection. One aliquot was collected and placed in 2 N hydrochloric acid for ammonia, SCFA, and BCFA analyses. An aliquot of fresh feces was immediately transferred to sterile cryogenic vials (Nalgene, Rochester, NY), snap-frozen in liquid nitrogen, and stored at -80°C for microbiota and IgA analysis. An additional aliquot was used for fecal DM determination.

Blood collection and analysis

Blood samples were immediately transferred to appropriate vacutainer tubes for hematology (#367841 BD Vacutainer Plus plastic whole blood tube – with K₂EDTA additive) and serum collection (#367974 BD Vacutainer Plus plastic serum tube – red/grey with clot activator and gel for serum separation; BD, Franklin Lakes, NJ). The blood tube for serum isolation was centrifuged at $1,300 \times g$ at 4°C for 10 min (Beckman CS-6R centrifuge; Beckman Coulter Inc., Brea, CA). Once serum was collected, it was transported to the University of Illinois Veterinary Medicine Diagnostics Laboratory for serum chemistry analysis. K₂EDTA tubes were cooled (but not frozen) and then transported to the University of Illinois Veterinary Medicine Diagnostics Laboratory for hematology analyses.

Chemical analyses and ATTD calculations

Before analyses, milk and kefir samples were lyophilized (Dura-Dry MP microprocessor-controlled freeze-dryer, FTS Systems, Stone Ridge, NY) and fecal samples were dried at 55°C in a forced-air oven. All samples were then ground in a Wiley mill (model 4, Thomas Scientific, Swedesboro, NJ) through a 2-mm screen. Milk and kefir sugar (lactose; glucose; tagatose; galactose), lactate, glycerol, acetate, and ethanol concentrations were measured using high-performance liquid chromatography. Composite samples were run on a 1260 Infinity high-performance liquid chromatography (Agilent Technologies, Santa Clara, CA) equipped with a refractive index detector using a Rezex ROA-Organic Acid H+ (8%) column (Phenomenex, Torrance, CA). The column was eluted with 0.005 N H₂SO₄ at a flow rate of 0.6 mL/min at 5°C, with fucose, salicylic acid, 2'-fucosyllactose, 3'-sialyllactose, lacto-N-neotetraose, and lacto-N-triose II (Carbosynth US, San Diego, CA) used as detection standards.

Dried and ground diet, milk, kefir, kefir grain, and fecal samples were analyzed for DM and ash according to AOAC (2006; methods 934.01 and 942.05), with organic matter calculated. Crude protein was calculated from Leco (FP2000 and TruMac) total nitrogen values according to AOAC (2006; method 992.15). Total lipid content (acid-hydrolyzed fat) was determined according to the methods of the American Association of Cereal Chemists (1983) and Budde (1952). Total dietary fiber of the diet was determined according to Prosky et al. (1992). Gross energy was measured using an oxygen bomb calorimeter (model 6200, Parr Instruments, Moline, IL). ATTD of nutrients and energy were calculated using the following equation:

$$\% \text{ Digestibility} = \frac{\text{Nutrient Intake} \left(\frac{g}{d} \right) - \text{Fecal output} \left(\frac{g}{d} \right)}{\text{Nutrient Intake} \left(\frac{g}{d} \right)} * 100\%$$

Fecal IgA concentrations

Fecal proteins were extracted according to Vilson et al. (2016). Fecal samples (250 mg) were vortexed with 750 μ L extraction buffer containing 50 mM ethylenediaminetetraacetic acid (ThermoFisher, Waltham, MA) and 100 μ g/L soybean trypsin inhibitor (Sigma, St. Louis, MO) in PBS/1% bovine serum albumin (Tocris Bioscience, Bristol, UK). Phenylmethanesulphonyl fluoride (12.5 μ L, 350 mg/L; Sigma, St. Louis, MO) was added into each tube, followed by centrifugation for 10 min at $2,500 \times g$. Supernatants were collected for the measurement of IgA using a commercial enzyme-linked immunosorbent assay kit (MBS018650; MyBioSource, Inc., San Diego, CA).

Milk, kefir, and fecal DNA extraction and MiSeq Illumina sequencing of 16S rRNA gene amplicons

Total DNA from fecal, commercial kefir product, traditional kefir product, and whole milk samples were extracted using Mo-Bio PowerSoil kits (MO BIO Laboratories, Inc., Carlsbad, CA). The concentration of extracted DNA was quantified using a Qubit 3.0 Fluorometer (Life Technologies, Grand Island, NY). 16S rRNA gene amplicons were generated using a Fluidigm Access Array (Fluidigm Corporation, South San Francisco, CA) in combination with a Roche High Fidelity Fast Start Kit (Roche, Indianapolis, IN). The primers 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') that target a 252 bp-fragment of the V4 region of the 16S rRNA gene were used for amplification (primers synthesized by IDT Corp., Coralville, IA) (Caporaso et al., 2012). The CS1 forward and CS2 reverse tags were added according to the Fluidigm protocol. The quality of the amplicons was assessed using a Fragment Analyzer (Advanced Analytics, Ames, IA) to confirm amplicon regions and sizes. A DNA pool was generated by combining equimolar amounts of the amplicons from

each sample. The pooled samples were then size selected on a 1-2% agarose E-gel (Life Technologies, Grand Island, NY) and extracted using a Qiagen gel purification kit (Qiagen, Valencia, CA). Cleaned size-selected pooled products were run on an Agilent Bioanalyzer to confirm the appropriate profile and average size. Illumina sequencing was performed on a MiSeq using v3 reagents (Illumina Inc., San Diego, CA) at the Roy J. Carver Biotechnology Center at the University of Illinois.

Microbial data analysis

Forward reads were trimmed using the FASTX-Toolkit (version 0.0.13) and QIIME 2.0 (Caporaso et al., 2010) was used to process the resulting sequence data. Briefly, high-quality (quality value ≥ 20) sequence data derived from the sequencing process was demultiplexed. Data was then denoised and assembled into amplicon sequence variants using DADA2 (Callahan et al., 2016). The SILVA 132 database (Quast et al., 2013) was used to assign taxonomy. An even sampling depth (sequences per sample) was used for assessing alpha- and beta-diversity measures. Beta-diversity was assessed using weighted and unweighted UniFrac distance measures (Lozupone and Knight, 2005) and presented using principal coordinates analysis (PCoA) plots.

Statistical analysis

Data were analyzed using the Mixed Models procedure of SAS (SAS Institute, Inc., Cary, NC), testing for differences due to treatment. Dog and period were considered random effects. Differences among dietary treatments were determined using a Fisher-protected least significant difference with a Tukey adjustment to control for experiment-wise error. A probability of $p \leq 0.05$ was accepted as being statistically significant, with $p \leq 0.10$ accepted as statistical trends.

Results

Milk and kefir samples were similar in moisture, protein, calcium, and phosphorus concentrations, but C-Kefir contained higher fat concentrations than CNTL and T-Kefir (**Table 5.1**). Sugar content differed among CNTL and kefir treatments. Both kefirs contained much higher lactose and ethanol concentrations than the CNTL, while the CNTL contained higher glucose and galactose concentrations. These differences were most likely the result of the lactase treatment to the CNTL. Lactate, acetate, glycerol, and tagatose concentrations were similar among CNTL and kefir samples.

As predicted, the T-Kefir contained a much higher bacterial density [$1.79\text{E}+09$ colony-forming units (CFU)/mL] than the CNTL ($4.57\text{E}+03$ CFU/mL) or C-Kefir ($6.95\text{E}+04$ CFU/mL) treatments. In regard to bacterial profile, the CNTL was primarily composed of *Lactococcus* (61.80%), *Leuconostoc mesenteroides* (20.72%), Enterobacteriaceae (11.86%), and Yersiniaceae (4.73%) (**Table 5.2**). C-Kefir was primarily composed of two genera, namely *Lactococcus* (91.94%) and *Lactobacillus* (7.97%). T-Kefir also contained high amounts of *Lactococcus* (72.69%) and *Lactobacillus* (25.18%) including species such as *L. kefiranoferiens* (4.99%) and *Gluconobacter* (1.85%). T-Kefir grains, which were used to produce T-Kefir primarily contained *Lactococcus* (68.38%), *Lactobacillus* (31.38%) and *L. kefiranoferiens* (30.25%).

Fecal characteristics, including pH, scores, and DM % were normal for all dogs throughout the study and were not affected by treatment (**Table 5.3**). The ATTD of DM, organic matter, crude protein, fat, and energy were not affected by treatment. Fecal valerate concentrations tended to be higher ($p=0.08$) in CNTL dogs than those supplemented with C-Kefir or T-Kefir. All other fecal metabolites and fecal IgA were unaffected by treatment.

Fecal bacterial alpha diversity, as measured by Faith's phylogenetic diversity, was higher ($p=0.03$) in dogs fed C-Kefir than those fed CNTL (**Figure 5.1a**). All other measures of alpha diversity, however, were unchanged by treatment (data not shown). Fecal bacterial beta diversity, as assessed using unweighted and weighted PCoA plots, showed bacterial shifts among treatments (**Figure 5.1b, 5.1c**). The unweighted PCoA plot showed that CNTL was different ($p=0.01$) from C-Kefir, while CNTL tended to be different ($p=0.10$) from T-Kefir. The weighted PCoA plot, however, did not identify treatment differences. At the phyla level, the relative abundance of fecal Fusobacteriota tended to be higher ($p=0.06$) in dogs fed C-Kefir than those fed CNTL or T-Kefir (**Table 5.4**). At the genus level, dogs fed T-Kefir had a higher ($p<0.0001$) relative abundance of fecal *Lactococcus* than those fed CNTL or C-Kefir. The relative abundance of fecal *Escherichia-Shigella* tended to be higher ($p=0.10$) in CNTL dogs than those fed C-Kefir or T-Kefir. Finally, the relative abundance of fecal *Candidatus stoquefichus* tended to be greater ($p=0.09$) in dogs fed T-Kefir than those fed CNTL or C-Kefir.

All serum metabolites were within reference ranges throughout the study with the exception of the albumin:globulin ratio exceeding the reference range (0.6-1.1) in CNTL (1.13) and C-Kefir (1.13) groups (**Table 5.5**). Serum metabolites were not affected by treatment.

Discussion

Because companion animals are considered to be members of the family in many households worldwide, pet owners seek to feed them high quality ingredients and provide them with supplements that are believed to promote health. Dogs are metabolically omnivorous and can tolerate a range of diets varying in ingredient profile, nutrient composition, and forms (Lee et al., 2022). In addition to their metabolic flexibility, the canine gastrointestinal system contains

complex microbial communities that provide many benefits to the host. In their search for health promotion, many owners look for products that may target the gastrointestinal tract and/or microbiota. Key outcomes include fecal scores, fecal metabolites (SCFA; phenols and indoles), and microbiota populations. Traditionally, dietary fibers, probiotics (*Bifidobacterium*, *Lactobacillus*, *Streptococcus*), prebiotics (galactooligosaccharides, fructooligosaccharides, inulin), synbiotics (prebiotic + probiotic), and postbiotics (bacterial and yeast fermentation products) have been used in this fashion (Swanson et al., 2002; Lin et al., 2019; Lee et al., 2022; Palade et al., 2022; Koziol et al., 2023). Several studies have shown benefits on satiety, reduced blood lipid and glucose concentrations, improved stool quality, greater fecal SCFA concentrations, reduced fecal protein catabolites, and altered microbiota populations (de Godoy et al., 2013; Panasevich et al., 2013; Palmqvist et al., 2023). Many of the same potential benefits may be derived from probiotics and prebiotics (Lin et al., 2023).

A recent addition to this area is that of fermented foods and beverages (Vinderola et al., 2023). Fermented foods have been consumed for centuries by humans, as this process typically extends the duration by which a food can be stored and consumed. Lactic acid bacteria are often responsible for the fermentation process within foods, as is the yeast *Saccharomyces*. These organisms work, often in concert, to transform dairy, vegetables, meats, and cereals into new products full of live microorganisms (Marco et al., 2021). Such foods may not only be more stable and delicious but may provide health benefits as well. The regular consumption of yogurt, for example, has been shown to reduce symptoms of type 2 diabetes in humans (Ostadrahimi et al.). Consistent physiological outcomes from other fermented foods have yet to be observed, but consumers have become more health-conscious so interest in fermented foods has increased. Given the success of the yogurt industry, companies have expanded to produce commercialized versions

of the fermented dairy beverage kefir. Traditional kefir is made through the fermentation of kefir grains with milk to produce a fermented milk beverage. In the US, commercial kefir production does not use kefir grains in order to avoid yeast related ethanol issues and to provide product consistency possible with the use of defined starter cultures. Consequently, commercial kefir products will have a different microbial communities and thus might have different potential impacts on host health (Metras et al., 2021; Nejati et al., 2022).

Similar to humans, several commercial kefir products for companion animals exist (Metras et al., 2020a). To our knowledge, only a couple studies on kefir have been conducted in dogs. Kim et al. (2019) reported mild changes to the fecal microbiota, showing changes in beta diversity and a few bacterial taxonomic groups. The study conducted by Gaspardo et al. (2020), however, did not identify treatment differences. Treatment duration, dosage, animals and housing conditions, or other factors may explain the differences between studies. In both studies, the microbial profiles and nutrient compositions of test products were not presented, making direct comparisons challenging.

Because kefir products are being sold commercially, but are not well studied, we aimed to conduct the current study in hopes of gaining a better understanding of what effects these products may have on the stool quality, fecal metabolites and microbiota, and serum metabolites of dogs. Our initial focus was on microbial and nutrient differences among the milk, commercial kefir, and traditional kefir treatments. The kefir and milk treatments were not dramatically different in regard to nutrient composition, but sugar, lactate, and ethanol concentrations were slightly different. Many commercial kefir products claim to be lactose-free, but low amounts were detected in both kefir products. The lactase treatment of the milk, which was done in an attempt to avoid a lactose effect, converted most of the lactose into glucose and galactose. Because the kefirs were not treated

with lactase, they ended up containing much higher lactose concentrations than the milk. Both kefirs contained about twice the ethanol that milk contained.

Variation in the microbial profiles of treatments was also of great interest given that commercial and traditional kefirs have been reported to differ (Nejati et al., 2022). Most importantly, the traditional kefir brewed in our laboratory contained much higher CFU/mL than the commercial kefir and milk. Based on the results of our previous work in this area (Metras et al., 2020), this was expected. *Lactococcus* was the primary genera present in kefir grains, kefir products, and milk. Not surprisingly, however, the microbial profile of the traditional kefir was more similar to that of the kefir grains. Most notable was that the traditional kefir contained higher amounts of *Lactobacillus* in general and was the only treatment to contain *L. kefirianofaciens* that is present in the kefir grains. The kefirs tested in this study did not contain any measurable *Bacillus*, *Streptococcus*, and *Leuconostoc*, which were measured in a few commercial kefir products in our previous study (Metras et al., 2020). Despite the large differences in bacterial counts and different microbial profiles of kefir products, minor changes were noted in fecal microbiota. It is interesting to note that although the C-Kefir product had the highest relative abundance of *Lactococcus*, it was only the T-Kefir product that increased fecal *Lactococcus* in the dogs studied. Overall, minor changes to the fecal microbiota were observed, while serum metabolites, fecal metabolites, and fecal IgA were unchanged. Further study is required to determine whether kefirs derived from different kefir grains or containing higher microbial densities or different microbial profiles may provide benefits to dogs.

In conclusion, we observed that the traditional kefir brewed in our laboratory contained significantly more live microorganisms and a different microbial profile than the commercial kefir tested. Of note, the traditional kefir was the only one to contain *L. kefirianofaciens* that is derived

from kefir grains. The supplementation of commercial and traditional kefir products had minor effects on fecal microbiota populations and metabolite concentrations of healthy adult dogs. In addition to slight changes in the bacterial beta diversity for both kefirs, the traditional kefir led to an increase in the relative abundance of fecal *Lactococcus*. The commercial kefir tended to reduce fecal valerate concentrations. Stool quality and all other physiological measures were unaffected by treatments. Further research is needed to test whether kefirs derived from different kefir grains or those containing higher microbial densities or different microbial profiles may provide benefits to dogs.

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Tables and Figures

Table 5.1: Analyzed nutrient concentrations (as-is values) of milk and kefir treatments tested.

Item	CNTL ¹	C-Kefir ²	T-Kefir ³
Moisture, %	93.30	93.03	93.43
		--- % ---	
Crude protein	4.86	4.46	4.90
Acid-hydrolyzed fat	3.47	5.27	3.93
Calcium	0.14	0.09	0.12
Phosphorus	0.11	0.08	0.09
		--- g/L ---	
Lactose	2.12	29.81	31.75
Glucose	13.33	0.03	0.02
Tagatose	0.00	0.01	0.01
Galactose	22.14	0.01	0.01
Lactate	8.28	7.71	9.25
Glycerol	0.52	0.37	0.39
Acetate	0.99	0.87	1.26
Ethanol	3.43	8.46	7.02
Gross energy, kcal/g	0.83	0.89	0.86

¹2% milk treated with lactase.

²C-Kefir: commercial kefir.

³T-Kefir: traditional kefir.

Table 5.2: Microbial composition (% of sequences) of kefir grains and milk and kefir treatments tested.

Phyla	Genus	CNTL ¹	C-Kefir ²	T-Kefir ³	Kefir Grains
Bacteroidota		0.05	0.02	0.04	0.05
	<i>Bacteroides</i>	0.02	0.00	0.00	0.01
	Muribaculaceae uncultured	0.03	0.01	0.01	0.04
Firmicutes		82.91	99.96	98.01	99.81
	Total <i>Lactobacillus</i>	0.09	7.97	25.18	31.38
	<i>Lactobacillus</i> spp. (unclassified)	0.09	7.86	20.19	1.13
	<i>Lactobacillus kefiranofaciens</i>	0.00	0.00	4.99	30.25
	<i>Lactococcus</i>	61.80	91.94	72.69	68.38
	<i>Leuconostoc mesenteroides</i>	20.72	0.00	0.00	0.00
Fusobacteriota		0.03	0.01	0.04	0.02
	<i>Fusobacterium</i>	0.00	0.00	0.02	0.00
Proteobacteria		17.01	0.01	1.91	0.11
	Enterobacteriaceae	11.86	0.00	0.00	0.00
	<i>Gluconobacter</i>	0.00	0.00	1.85	0.11
	<i>Pseudomonas</i>	0.38	0.00	0.00	0.00
	Yersiniaceae	4.73	0.00	0.00	0.00
Verrucomicrobiota		0.03	0.01	0.02	0.02
	<i>Akkermansia</i>	0.03	0.01	0.02	0.02

¹2% milk treated with lactase.

²C-Kefir: commercial kefir.

³T-Kefir: traditional kefir.

Table 5.3. Fecal characteristics and metabolite concentrations of healthy adult dogs consuming commercial or traditional kefir.

Characteristics	Treatment			SEM	p-values
	CNTL ¹	C-Kefir ²	T-Kefir ³		
pH	6.71	6.60	6.50	0.19	0.747
Fecal score ⁴	3.13	3.33	3.17	0.11	0.385
Fecal DM (%)	29.64	30.46	30.29	0.71	0.687
Digestibility, %					
Dry matter	69.47	69.71	69.25	1.73	0.982
Organic matter	74.37	74.65	74.47	1.45	0.990
Crude protein	72.59	72.26	71.94	1.66	0.976
Acid-hydrolyzed fat	87.75	87.67	86.80	0.67	0.536
Energy	74.59	74.81	74.62	1.66	0.976
Metabolites, μmole/g (DMB)					
Total SCFA ⁵	651.50	612.85	622.38	33.44	0.699
Acetate	379.62	368.21	355.54	23.65	0.773
Propionate	190.36	166.06	176.27	13.21	0.436
Butyrate	81.53	78.59	90.57	9.51	0.654
Total BCFA ⁵	24.96	22.73	26.15	2.19	0.542
Isobutyrate	9.79	10.34	11.40	0.92	0.467
Isovalerate	12.65	11.84	13.60	1.18	0.577
Valerate	2.53	0.56	1.15	0.57	0.081
Total phenols and indoles	2.98	2.91	3.18	0.19	0.589
4-methylphenol	0.29	0.28	0.27	0.03	0.955
Indole	1.61	1.66	1.76	0.18	0.847
Ammonia	106.23	93.27	100.36	6.16	0.342
Fecal IgA, mg/g	29.26	29.76	30.27	0.03	0.761

¹2% milk treated with lactase.

²C-Kefir: commercial kefir.

³T-Kefir: traditional kefir.

⁴Fecal score: 1 = hard, dry pellets, small hard mass; 2 = hard, formed, dry stool; remains firm and soft; 3 = soft, formed, and moist stool, retains shape; 4 = soft, unformed stool, assumes shape of container; and 5 = watery, liquid that can be poured.

⁵Total SCFA: total short-chain fatty acids = acetate + propionate + butyrate; total BCFA: total branched-chain fatty acids = isobutyrate + isovalerate + valerate; valerate is not technically a SCFA, but is derived from amino acid fermentation so it was included in the BCFA calculation.

Table 5.4. Fecal microbiota relative abundances (% of sequences) of healthy adult dogs consuming commercial or traditional kefir.

Phyla	Genus	Treatment			SEM	P-values
		CNTL ¹	C-Kefir ²	T-Kefir ³		
Actinobacteriota		2.98	3.04	3.23	0.83	0.853
	<i>Bifidobacterium</i>	1.91	1.79	1.95	0.52	0.783
	<i>Coriobacteriaceae</i> UCG-002	0.51	0.89	0.88	0.37	0.179
Bacteroidota		26.56	29.51	26.91	1.43	0.997
	<i>Alloprevotella</i>	3.71	2.87	2.64	0.51	0.322
	<i>Bacteroides</i>	10.15	13.26	10.49	2.03	0.501
	<i>Muribaculaceae</i>	3.51	3.28	4.31	1.41	0.723
	<i>Prevotella</i>	6.80	6.93	6.64	1.62	0.986
	<i>Prevotellaceae</i> Ga6A1	1.31	1.41	1.51	0.21	0.751
Firmicutes		46.83	37.65	44.86	4.78	0.214
	<i>Allobaculum</i>	4.53	4.48	6.68	2.27	0.784
	<i>Blautia</i>	2.75	2.13	2.49	0.63	0.741
	<i>Candidatus Stoquefichus</i>	0.08	0.04	0.24	0.08	0.086
	<i>Faecalibacterium</i>	1.19	1.23	1.09	0.27	0.889
	<i>Holdemanella</i>	1.02	0.31	0.35	0.31	0.586
	<i>Lactobacillus</i>	3.07	2.03	2.63	0.38	0.174
	<i>Lactococcus</i>	0.04 ^a	0.03 ^a	0.39 ^b	0.07	<0.001
	<i>Peptoclostridium</i>	3.93	3.67	4.26	0.84	0.850
	<i>Phascolarctobacterium</i>	1.10	0.95	0.75	0.17	0.379
	<i>Romboutsia</i>	0.77	1.07	0.98	0.27	0.548
	<i>Ruminoccus</i>	3.31	2.88	3.59	0.73	0.880
	<i>Ruminococcus torques</i>	1.34	1.06	1.60	0.22	0.279
	<i>Streptococcus</i>	1.09	0.31	0.22	0.27	0.173
	<i>Turicibacter</i>	1.42	1.79	1.38	0.56	0.869
Fusobacteriota		14.01	18.37	15.61	1.25	0.056
	<i>Cetobacterium</i>	0.88	1.44	1.25	0.32	0.445
	<i>Fusobacterium</i>	12.84	14.45	13.04	1.44	0.692
Proteobacteria		9.55	11.26	9.30	1.43	0.576
	<i>Anaerobiospirillum</i>	2.99	4.63	3.36	0.83	0.352
	<i>Escherichia-Shigella</i>	0.24	0.02	0.00	0.07	0.095
	<i>Parasutterella</i>	4.10	3.70	4.37	1.02	0.747
	<i>Sutterella</i>	1.310	1.47	1.11	0.22	0.530

¹2% milk treated with lactase.

²C-Kefir: commercial kefir.

³T-Kefir: traditional kefir.

Table 5.5. Serum chemistry of healthy adult dogs consuming commercial or traditional kefirs

Item	Reference Range ¹	Treatment			SEM	P-value
		CNTL ²	C-Kefir ³	T-Kefir ⁴		
Creatinine, mg/dL	0.5 - 1.5	0.65	0.66	0.61	0.066	0.724
Blood urea nitrogen, mg/dL	6 - 30	16.67	16.92	15.50	0.317	0.744
Total protein, g/dL	5.1 - 7.0	5.76	5.73	5.76	0.084	0.949
Albumin, g/dL	2.5 - 3.8	3.03	3.01	3.00	0.044	0.858
Globulin, g/dL	2.7 - 4.4	2.73	2.72	2.76	0.082	0.931
Albumin:globulin ratio	0.6 - 1.1	1.13	1.13	1.10	0.041	0.838
Ca, mg/dL	7.6 - 11.4	8.64	8.42	8.30	0.587	0.723
P, mg/dL	2.7 - 5.2	4.10	4.13	3.95	0.213	0.821
Na, mmol/L	141 - 152	146.75	146.08	146.42	0.402	0.510
K, mmol/L	3.9 - 5.5	4.76	4.70	4.86	0.156	0.709
Na: K ratio	28 - 36	31.17	31.67	30.33	1.021	0.651
Cl, mmol/L	107 - 118	112.00	112.42	111.92	0.696	0.863
Glucose, mg/dL	68 - 126	82.17	84.25	82.58	2.901	0.866
Alkaline phosphatase (ALP), U/L	7 - 92	82.17	84.25	82.58	2.901	0.710
Corticosteroid-induced alp, U/L	0 - 40	13.42	9.33	7.33	7.576	0.564
Alanine transaminase, U/L	8 - 65	25.25	24.33	24.75	2.735	0.857
Gamma-glutamyl transferase, U/L	0 - 7	2.75	2.75	3.08	0.374	0.769
Total bilirubin, mg/dL	0.1 - 0.3	0.25	0.26	0.24	0.028	0.986
Creatine phosphokinase, U/L	26 - 310	111.83	105.33	109.83	0.908	0.961
Cholesterol, mg/dL	129 - 297	212.67	206.42	211.33	0.511	0.898
Triglycerides, mg/dL	32 - 154	42.75	42.92	43.75	0.199	0.723
Bicarbonate, mmol/L	16 - 24	21.58	21.00	21.58	0.471	0.604
Anion gap	---	17.92	17.42	17.67	0.854	0.723
Hemolytic indicator	---	0.35	0.621	0.45	0.235	0.715

¹University of Illinois Veterinary Diagnostic Laboratory Reference Ranges.²2% milk treated with lactase.³C-Kefir: commercial kefir.⁴T-Kefir: traditional kefir.

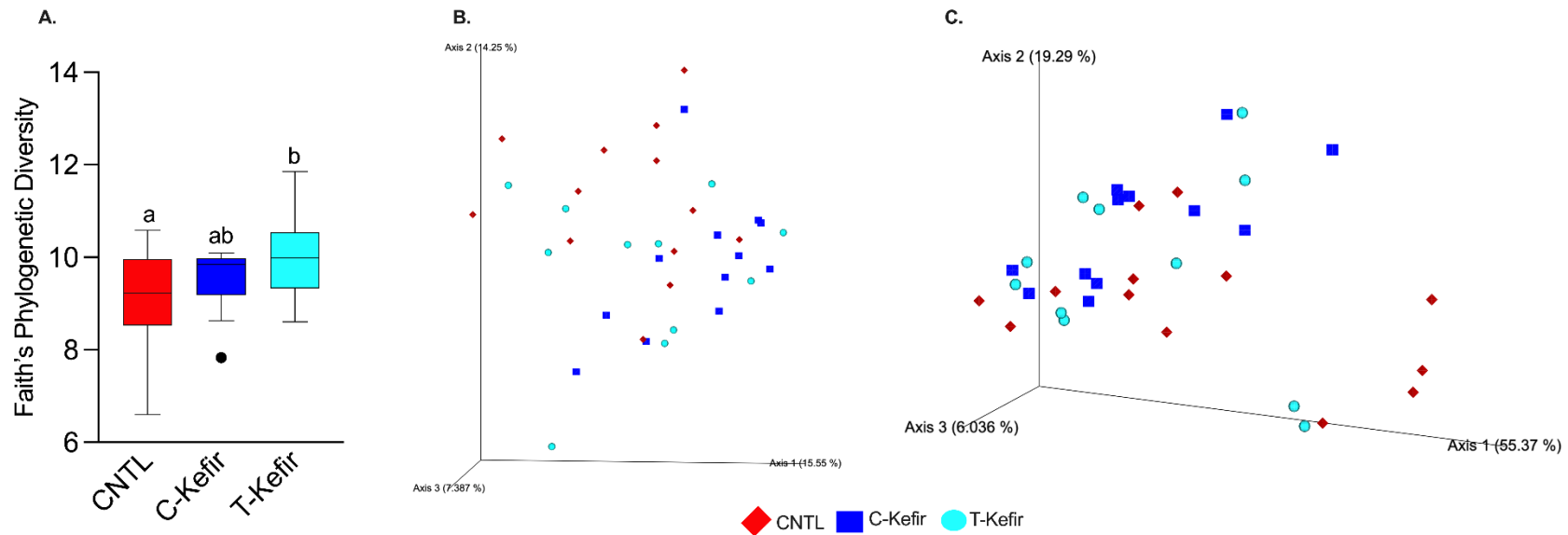


Figure 5.1: (a) Bacterial alpha diversity indices of canine treatments as assessed by Faith's phylogenetic diversity. Superscripts indicate a significant difference ($P < 0.05$). PCoA plots of (b) unweighted and (c) weighted Unifrac distances of canine fecal microbial communities performed on the 97% OTU abundance matrix using QIIME. The unweighted PCoA plot (b) showed that CNTL was different ($P = 0.01$) from C-Kefir, while CNTL tended to be different ($P = 0.10$) from T-Kefir. The weighted PCoA plot (c) did not identify treatment differences.

CHAPTER 6: IN VITRO FERMENTATION CHARACTERISTICS OF DIETARY FIBERS USING STARTER BACTERIAL CULTURE, GRAIN KEFIR CULTURE, OR CANINE FECES AS INOCULUM

Abstract

Traditionally, grain kefir is produced from the fermentation of milk with yeast- and bacteria-containing cultures. To maintain consistency and adhere to food safety guidelines, industrial producers sell kefir products based on starter bacterial cultures (typically no yeast). Bacterial profiles of starter vs. grain kefirs differ, but their influence on health effects is unknown. The objectives of this experiment were to determine the in vitro fermentation characteristics of common dietary fibers using 1) fecal inoculum from dogs supplemented with starter- or grain-based kefirs; and 2) a starter bacterial culture or a grain kefir culture as inoculum. Healthy adult dogs were allotted to one of 3 treatments (60 mL/d) and supplemented for 14 d (n=4/treatment): 1) 2% reduced-fat milk treated with lactase (control), 2) starter kefir (S-Kefir), or 3) grain kefir (G-Kefir). After 14 d, fresh fecal samples were collected and frozen in a 20% glycerol solution for use in vitro at a later date. For the in vitro experiment, fecal samples were thawed, pooled by treatment, and diluted in an anaerobic diluting solution under CO₂. Blended, diluted feces inoculated tubes containing semi-defined medium and one of the fiber sources: cellulose (CEL, negative control), pectin (PC, positive control), beet pulp (BP), or chicory pulp (CP). Tubes were incubated for 0, 6, 12, or 18 h with periodic mixing. At each time point, the incubation was stopped and samples were processed immediately. Short-chain fatty acid (SCFA) concentrations, pH, and microbiota populations were measured. Blank tubes (inocula and media, but no fiber) and baseline (0 h) tubes were used to calculate change from baseline values. A second in vitro experiment was conducted using similar methods and measurements, but by using S-Kefir and G-Kefir as inoculum sources. Effects of treatment (control, S-Kefir, G-Kefir), time, and treatment*time interactions

within the fiber source were analyzed statistically, with significance considered $P < 0.05$. Using fecal inoculum, BP and PC were rapidly fermented, leading to large pH reductions, SCFA increases, and microbiota shifts. pH change was of greater ($P < 0.05$) magnitude (PC) and higher ($P < 0.05$) kinetic rate (CP) when using feces from dogs fed S-Kefir or G-Kefir than controls. Butyrate increases were greater ($P < 0.05$) in tubes inoculated with G-Kefir feces than in S-Kefir or control feces. When PC and BP were fermented, tubes with S-Kefir feces had greater ($P < 0.05$) acetate, propionate, and total SCFA increases than G-Kefir or control feces. Fermentations were slower using starter and kefir cultures, but some differences were noted. Butyrate was higher ($P < 0.05$) in CEL and CP tubes fermented by S-Kefir inoculum, but higher ($P < 0.05$) in PC and BP tubes fermented by G-Kefir inoculum. Bacterial beta diversity and relative abundances shifted over time within each substrate and were unique to the inoculum source. These data suggest that the activity of kefir bacterial populations differs and that kefir consumption changes the abundance and activity of the fecal microbiota of dogs, justifying in vivo investigation.

Introduction

The market size for fermented dairy beverages, including kefir products, is expected to increase by \$456 million from 2021 to 2025, with a compound annual growth rate of 4.37% in 2021 (González-Orozco et al., 2023). Given their success in the human market, the companion animal supplement market has recently included fermented food products such as kefir too. Traditionally, kefir is generated from the fermentation of kefir grains with milk (Rosa et al., 2017b). A range of purported benefits has been attributed to grain-fermented kefir, which includes the presence of kefiran, the exopolysaccharide produced by lactic acid bacteria (e.g., *Lactobacillus kefirifaciens*) in kefir (Georgalaki et al., 2021). Kefiran acts as a biofilm that may serve as a

protective barrier for probiotic strains being delivered to the host gastrointestinal tract as well as antibacterial properties that target the cytoplasmic membrane of sensitive pathogens and other bacteria (Simova et al., 2002; Guzel-Seydim et al., 2011).

While grain-fermented kefir may provide health benefits, it is unknown if similar benefits may hold for commercialized starter kefir products. Starter kefir production is quite different than grain kefir due to the exclusion of kefir grains. Rather, its production involves the inoculation of dairy milk with preset microbial cultures commonly derived from yogurt suppliers (Nejati et al., 2022b). The rational use of preformed microbial starters to ferment milk instead of kefir grains is due to concerns of product consistency, microbial consistency, presence or absence of yeast, and consumer palatability (Hansen, 2002). Due to stark differences in processing and omission of grain fermentation, starter kefir is more similar to yogurt than to grain kefir. Not surprisingly, the differences in production materials and procedures lead to commercial products having different food matrix properties and microbial profiles (Nejati et al., 2022; Metras et al., 2023).

The microbial profiles of grain kefir derived from fermentation with microbial grains have been shown to consist of bacterial species such as *L. kefiranofaciens*, *Lentilactobacillus kefir*, *Lentilactobacillus parakefir* and fungal species such as *Kluyveromyces lactis*, *Kluyveromyces marxinus*, *Saccharomyces cerevisiae*, *Saccharomyces boulardii*, and *Saccharomyces unisporus* (Gut et al., 2019; Slattery et al., 2019). A high variability in the kefir microbial profile exists, however, research using 16S rRNA sequencing has been used to characterize kefir grains and consistently conserved microbes from around the world (Sindi et al., 2020). While the taxa listed above are often predominant, many other bacterial species have been detected (e.g., *Lactobacillus plantarum*, *Lactobacillus delbrueckii*, *Lactobacillus casei*, *Lactobacillus reuteri*, *Lactobacillus buchneri* groups, *Leuconostoc mesenteroides*, *Lactococcus lactis*, *Gluconobacter frateurii*,

Acetobacter orientalis, *Acetobacter lovaniensis*) (Korsak et al., 2015; Wang et al., 2020b; Georgalaki et al., 2021; Youn et al., 2022).

Little research has been conducted on companion animal kefir products and how they may impact the health of dogs. In one study, our laboratory characterized six starter kefir products, demonstrating that the predominant microbial species included *Bacillus coagulans*, *Lactobacillus paracasei*, *Lactobacillus acidophilus*, *L. delbrueckii*, *Streptococcus thermophilus*, *Streptococcus salivarius*, and *L. lactis* and that label inaccuracies regarding microbial abundance and identity were frequent (Metras et al., 2020). Researchers reported that traditionally made grain kefir brewed daily with 200 mL given to dogs once per day for 14 d modulated the gut microbiota and increased lactic acid bacteria (Kim et al., 2019). In another study, however, dogs supplemented with a daily dose of $>10^9$ AFU (Active fluorescent units) *L. kefir* for 30 days were reported to have no changes to fecal IgA concentrations or microbiota populations (Gaspardo et al., 2020b). Most recently, our laboratory investigated the effects of supplementing a grain kefir or starter kefir vs. a lactase-treated milk control in healthy adult dogs (Metras et al., 2023). In that study, no significant physiological changes were detected, but fecal *Lactococcus* relative abundance was greater ($P<0.05$) in dogs supplemented with grain kefir.

Currently, the field relies on the use of fecal microbiomics to assess the effects of gut-modifying substances such as dietary fibers, biotics, and fermented foods. Because many of the fermentation end-products [e.g., short-chain fatty acids (SCFA)] are rapidly absorbed by the host, fecal samples are unable to accurately assess microbial activity. Because in vitro fermentation assays allow for the measurement of microbial abundances and metabolite concentrations over time, however, they are a useful way to assess the activity of fecal microbiota (Payne et al., 2012; de Godoy et al., 2015; Traughber et al., 2020). Therefore, the objectives of the current study was

to determine the in vitro fermentation characteristics of common dietary fibers using 1) fecal inoculum from dogs supplemented with starter- or grain-based kefir; and 2) a starter bacterial culture or a grain kefir culture as inoculum. We hypothesized that differences in fecal microbial populations would impact dietary fiber fermentation, as evidenced by greater decreases in pH and greater production of SCFA such as butyrate in tubes inoculated by feces of dogs fed kefir. We also hypothesized that fiber fermentation by kefir beverages would occur at a slower rate than fecal sample fermentations, with changes in pH and SCFA production differing between tubes inoculated with starter- and grain-based kefir.

Materials and Methods (Study #1: In Vitro Experiment using Fecal Inoculum)

All procedures were approved by the University of Illinois Institutional Animal Care and Use Committee prior to experimentation. All methods were performed in accordance with the United States Public Health Service Policy on Humane Care and Use of Laboratory Animals.

Substrates and analysis:

Test fibers included beet pulp (BP; Archer Daniels Midland Co., Decatur, IL), chicory pulp (CP; BioMatrix International, Princeton, MN), pectin (PC; Modernist Pantry LLC; Eliot, ME) and cellulose (CEL; Solka Floc[®] International Fiber Corporation; North Tonawanda, NY). Samples were analyzed for dry matter (DM) and ash according to AOAC (2006; methods 934.01 and 942.05), with organic matter (OM) calculated.

Animals, experimental design, and fecal sample collection:

Twelve healthy female adult beagle dogs (age: 5.67 ± 1.72 yr; body weight: 7.27 ± 1.15 kg) were used in a completely randomized design. All dogs were housed individually in pens (1.0 m wide by 1.8 m long) in an environmentally controlled facility at the University of Illinois Urbana-Champaign. Dogs had free access to fresh water at all times. All dogs were fed a commercial diet that was formulated to meet all essential nutrients recommended by the Association of American Feed Control Officials (AAFCO, 2023) for adult dogs (Best Dog 21/12, Coker Feed Mill, Inc., Goldsboro, NC). Dogs were then randomly assigned to one of the following treatments and fed for 2 wk ($n=4/\text{treatment}$): 2% milk treated with lactase (CNTL; 2% Reduced Fat Milk, Great Value Farm, Fort Wayne, IN; 60 mL/d); starter kefir (S-Kefir; Champions Choice Kefir; Champions Choice, Millersburg, IN, 60 mL/d); and grain kefir (G-Kefir; Kefir Garden Grains, Hamilton Ontario, Canada; 60 mL/d). On the basis of the maintenance energy requirement for adult dogs and information from previous feeding records, an amount of food to maintain body weight was offered and intake was measured twice daily (8 am; 4 pm).

After 14 d of treatment, fresh fecal samples were collected within 15 min of defecation. For each dog, a 10 g aliquot of feces was collected into a 50 mL conical tube and mixed with 10 mL of 20% glycerol solution according to Cammarota et al. (2017). Samples were immediately placed on dry ice and then stored at -80°C until the in vitro fermentation assay.

In vitro fermentation procedures:

On the day of the in vitro experiment, fecal samples were carefully thawed and heated to 39°C using a water bath, pooled by treatment, and then diluted 1:4 (wt/vol) in anaerobic diluting solution (**Table 6.5**) and blended for 15 s in a Waring blender (Waring Products, New Hartford,

CT). Blended, diluted feces were filtered through 4 layers of cheesecloth and sealed in 125 mL serum bottles under a stream of CO₂. Sample and blank tubes were then aseptically inoculated with diluted feces and added to the medium as described by Bourquin et al. (1993). Four mL of diluted feces was used to inoculate tubes containing 26 mL of semi-defined medium and one of the following fiber sources (300 mg/tube); cellulose (CEL, negative control), pectin (PC, positive control), beet pulp (BP), or chicory pulp (CP). Triplicate tubes of each fibrous substrate were incubated at 39°C for 0, 6, 12, or 18 h, with periodic mixing. At each time point, the incubation was stopped, and samples were processed immediately. For each time point, the pH of tube contents was measured using a pH meter. Samples to be analyzed (2 mL) for SCFA were mixed with 0.5 mL of 25% metaphosphoric acid and processed according to Erwin et al. (1961) using a Hewlett-Packard (Avondale, PA) Model 5890A gas chromatograph equipped with a flame ionization detector on a column (1.8 m × 4 mm i.d.) packed with GP 10% SP-1200/1% H₃P₀₄ on 80/100 chromosorb WAW (Supelco, Bellefonte, PA). The carrier gas was nitrogen, with a flow rate of 75 mL/min. The oven, injection port, and detector port temperatures were 125, 175, and 180°C, respectively. Aliquots for microbial analyses were collected into sterile cryogenic vials and placed on dry ice until being transferred to a -80°C freezer where they were stored until analysis. Data were corrected by blank tube (inocula and media, but no fiber source) and baseline (0 h) sample production. Negative values were expressed as 0.0 and subsequent acetate, propionate, and butyrate production values from each set of triplicate tubes were averaged.

Microbial DNA extraction:

Genomic DNA was extracted from fermentation media using the Mo-Bio PowerSoil Kits (MO BIO Laboratories, Inc., Carlsbad, CA) according to the manufacturer's specifications. The

quality of DNA was assessed via gel electrophoresis (E-Gel EX Gel 1%; Invitrogen, Carlsbad, CA) and the concentration of extracted DNA was quantified using a Qubit 3.0 Fluorometer (Life Technologies, Grand Island, NY) with a Qubit dsDNA BR assay kit (Invitrogen, Carlsbad, CA). 16S rRNA gene amplicons were generated using a Fluidigm Access Array (Fluidigm Corporation, South San Francisco, CA) in combination with Roche High Fidelity Fast Start Kit (Roche, Indianapolis, IN). The primers 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') target a 252 bp-fragment of the V4 region of the 16S rRNA gene was used for amplification (primers synthesized by IDT Corp., Coralville, IA) (Caporaso et al., 2012). The CS1 forward tags and CS2 reverse tags were added according to the Fluidigm protocol. The quality of the amplicons was assessed using a Fragment Analyzer (Advanced Analytics, Ames, IA) to confirm amplicon regions and sizes. A DNA pool was generated by combining equimolar amounts of the amplicons from each sample. The pooled samples were then size selected on a 1-2% agarose E-gel (Life Technologies, Grand Island, NY) and extracted using a Qiagen gel purification kit (Qiagen, Valencia, CA). Cleaned size-selected pooled products were run on an Agilent Bioanalyzer to confirm the appropriate profile and average size. Illumina sequencing was performed on a MiSeq using v3 reagents (Illumina Inc., San Diego, CA) at the Roy J. Carver Biotechnology Center at the University of Illinois.

Microbial data analysis:

Forward reads were trimmed using the FASTX-Toolkit (version 0.0.13) and QIIME 2.0 (Caporaso et al., 2010) was used to process the resulting sequence data. Briefly, high-quality (quality value ≥ 20) sequence data derived from the sequencing process was demultiplexed. Data was then denoised and assembled into amplicon sequence variants using DADA2 (Callahan et al.,

2016). The SILVA 132 database (Quast et al., 2013) was used to assign taxonomy. An even sampling depth (sequences per sample) was used for assessing alpha and beta diversity measures. Beta diversity was assessed using weighted and unweighted UniFrac distance (Lozupone and Knight, 2005) measures and presented using principal coordinates analysis (PCoA) plots.

Statistical analysis:

Blank-corrected data were analyzed using the PROC MIXED procedure in SAS (SAS Institute Inc., version 9.4, Cary, NC). Data normality was analyzed using PROC UNIVARIATE. Differences among substrates, time, and substrate*time interactions were determined using a Fisher-protected least significant difference test with a Tukey adjustment to control for type-1 experiment-wise error. Data were presented as least squares means. Statistical significance was set at $P < 0.05$.

Materials and Methods (Study #2: In Vitro Experiment using Starter or Grain Cultures as Inoculum)

Substrates, kefir preparation, and analysis:

Test fibers included beet pulp (BP; Archer Daniels Midland Co., Decatur, IL), chicory pulp (CP; BioMatrix International, Princeton, MN), pectin (PC; Modernist Pantry LLC; Eliot, ME) and cellulose (CEL; Solka Floc[®] International Fiber Corporation; North Tonawanda, NY). Samples were analyzed for dry matter (DM) and ash according to AOAC (2006; methods 934.01 and 942.05), with organic matter (OM) calculated.

The starter kefir product (S-Kefir) was purchased from a local vendor, shipped in liquid form, and refrigerated (4°C) immediately upon arrival. The kefir brewing process to prepare the

grain kefir (G-Kefir) began with the addition of 5 % w/v kefir grains to 2% reduced-fat dairy milk (Great Value Farm, Fort Wayne, IN). Kefir grains and kefir mixtures were incubated at 25°C in a sterilized lightly sealed glass jar for 24 h. The pH of the kefir was measured after 24 h of fermentation using a pH meter equipped with a pH and temperature probe (Orion™ Triode™ 3-in-1 pH/Automatic Temperature Compensation Probe, ThermoFisher Scientific, Waltham, MA), with an ideal pH range maintained between pH 4-5. After 24 h, grains were removed using a stainless-steel strainer, with the kefir refrigerated at 4°C until it was used for the in vitro assay.

Kefir bacterial quantification:

To begin, kefir products were inverted 10 times for homogenization, with 1 mL of product added to 9 mL of phosphate-buffered saline (PBS), and the product-PBS mixture inverted 10 times again. Serial 10-fold dilutions were prepared with PBS. A 50-μL volume of 10^5 and 10^7 dilutions were enumerated in duplicate via spiral plater (Eddy Jet Spiral Plater, Neutec Group Inc., Farmingdale, NY) onto deMan Rogosa Sharpe media (BD Difco, Franklin Lakes, NJ). deMan Rogosa Sharpe plates were incubated anaerobically at 37°C for 48 h and aerobically at 30°C for 48 h to culture lactic acid bacteria. Colony counts were measured via spiral plating software (Colony counter, IUL Flash and Go, Neutec Group Inc., Farmingdale, NY) once each experimental period, using composite treatment samples in triplicate. No attempt to distinguish organisms were made, only enumeration. For microbial dilution purposes, completed grain kefir underwent sterilization of live microorganisms through high temperature and pressure autoclave cycles.

In vitro fermentation procedures:

On the day of the in vitro experiment, kefir samples were prepared so that both contained an equal microbial density (10^6 CFU/mL). A total of 100 mL of S-Kefir was combined with an anaerobic dilution solution (**Table 6.5**) by blending it for 15 s in a Waring blender (Waring Products, New Hartford, CT) under a stream of CO₂. To achieve the same microbial density, G-Kefir (20 mL of fresh G-Kefir + 80 mL of autoclaved G-Kefir) was diluted 1:40 in an anaerobic dilution solution by blending it for 15 s in a Waring blender (Waring Products) under a stream of CO₂. Blended, diluted kefir was filtered through 4 layers of cheesecloth and sealed in 125 mL serum bottles under CO₂. Appropriate samples and blank tubes were then aseptically inoculated with diluted kefir (4 mL) and added to a semi-defined medium (26 mL) as described by Bourquin et al. (1993).

Triplicate tubes of each fibrous substrate (300 mg/tube) were incubated at 39 °C for 0, 6, 12, and 18 h, with periodic mixing. At each time point, the incubation was stopped and samples were processed immediately. For each time point, the pH of tube contents was measured using a pH meter. Samples to be analyzed (2 mL) for SCFA were mixed with 0.5 mL of 25% metaphosphoric acid and processed according to Erwin et al. (1961) using a Hewlett-Packard (Avondale, PA) Model 5890A gas chromatograph equipped with a flame ionization detector on a column (1.8 m × 4 mm i.d.) packed with GP 10% SP-1200/1% H₃P0₄ on 80/100 chromosorb WAW (Supelco, Bellefonte, PA). The carrier gas was nitrogen, with a flow rate of 75 mL/min. The oven, injection port, and detector port temperatures were 125, 175, and 180°C, respectively. Aliquots for microbial analyses were collected into sterile cryogenic vials and placed on dry ice until being transferred to a -80°C freezer where they were stored until analysis. Data were corrected by blank tube (inocula and media, but no fiber source) and baseline (0 h) sample production. Negative

values were expressed as 0.0 and subsequent acetate, propionate, and butyrate production values from each set of triplicate tubes were averaged.

Microbial DNA extraction:

Genomic DNA was extracted from fermentation media using the Mo-Bio PowerSoil Kits (MO BIO Laboratories, Inc., Carlsbad, CA) according to the manufacturer's specifications. The quality of DNA was assessed via gel electrophoresis (E-Gel EX Gel 1%; Invitrogen, Carlsbad, CA) and the concentration of extracted DNA was quantified using a Qubit 3.0 Fluorometer (Life Technologies, Grand Island, NY) with a Qubit dsDNA BR assay kit (Invitrogen, Carlsbad, CA). 16S rRNA gene amplicons were generated using a Fluidigm Access Array (Fluidigm Corporation, South San Francisco, CA) in combination with Roche High Fidelity Fast Start Kit (Roche, Indianapolis, IN). The primers 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') target a 252 bp-fragment of the V4 region of the 16S rRNA gene was used for amplification (primers synthesized by IDT Corp., Coralville, IA) (Caporaso et al., 2012). The CS1 forward tags and CS2 reverse tags were added according to the Fluidigm protocol. The quality of the amplicons was assessed using a Fragment Analyzer (Advanced Analytics, Ames, IA) to confirm amplicon regions and sizes. A DNA pool was generated by combining equimolar amounts of the amplicons from each sample. The pooled samples were then size selected on a 1-2% agarose E-gel (Life Technologies, Grand Island, NY) and extracted using a Qiagen gel purification kit (Qiagen, Valencia, CA). Cleaned size-selected pooled products were run on an Agilent Bioanalyzer to confirm the appropriate profile and average size. Illumina sequencing was performed on a MiSeq using v3 reagents (Illumina Inc., San Diego, CA) at the Roy J. Carver Biotechnology Center at the University of Illinois.

Microbial data analysis:

Forward reads were trimmed using the FASTX-Toolkit (version 0.0.13) and QIIME 2.0 (Caporaso et al., 2010) was used to process the resulting sequence data. Briefly, high-quality (quality value ≥ 20) sequence data derived from the sequencing process was demultiplexed. Data was then denoised and assembled into amplicon sequence variants using DADA2 (Callahan et al., 2016). The SILVA 132 database (Quast et al., 2013) was used to assign taxonomy. An even sampling depth (sequences per sample) was used for assessing alpha and beta diversity measures. Beta diversity was assessed using weighted and unweighted UniFrac distance (Lozupone and Knight, 2005) measures and presented using principal coordinates analysis (PCoA) plots.

Statistical analysis:

Blank-corrected data were analyzed using PROC MIXED in SAS (SAS Institute Inc., version 9.4, Cary, NC). Data normality was analyzed using PROC UNIVARIATE. Differences among treatments, time, and treatment*time interactions were determined using a Fisher-protected least significant difference test with a Tukey adjustment to control for type-1 experiment-wise error. Data were presented as least squares means. Statistical significance was set at $P < 0.05$.

Results (Study #1)

The microbial profiles of the baseline microbial communities (CNTL; S-Kefir; G-Kefir) of the feces (**Table 6.6**) and fecal inocula (**Table 6.1**) were different from one another. In total, the relative abundances of 48 bacterial genera were different ($P < 0.05$) in the fecal inoculum sources among the three treatments. Significant treatment*time interactions were observed for pH change in tubes containing CEL ($P = 0.0495$), PC ($P = 0.0007$), BP ($P = 0.0044$), and CP ($P = 0.0302$) (**Table**

6.2). Despite the significant interaction, the pH change for CEL fermentations, the negative control, was quite small for all treatments (0.03 to -0.08). The pH change for CP fermentations was moderate for all treatments (-0.18 to -0.34), but the reduction was slightly faster in tubes containing G-Kefir inoculum than in tubes containing CNTL inoculum. The pH reductions were much larger for BP (-0.30 to -0.85) and PC (-0.4 to -1.49) fermentations. While there were small differences among treatments for BP fermentations, tubes containing S-Kefir and G-Kefir inoculum had greater pH reductions over time than tubes containing CNTL inoculum for PC fermentations.

As expected, SCFA production was highest for PC fermentations (**Figure 6.1**). Significant time and treatment differences were observed for acetate and propionate production. Acetate production was higher ($P<0.0001$) in tubes containing S-Kefir inoculum than in tubes containing CNTL or G-Kefir inoculum. Propionate production was higher ($P<0.0001$) in tubes containing S-Kefir inoculum than tubes containing CNTL or G-Kefir inoculum, and higher ($P<0.0001$) in tubes containing CNTL inoculum than G-Kefir inoculum. Significant treatment*time interactions were observed for butyrate and total SCFA production. While butyrate production increased in all tubes, its production increased at a greater ($P<0.0001$) rate in tubes containing G-Kefir inoculum than in tubes containing CNTL or S-Kefir inoculum and increased at a greater ($P<0.0001$) rate in tubes containing S-Kefir inoculum than tubes containing CNTL inoculum. Total SCFA production increased in all tubes but increased at a greater ($P<0.0001$) rate in tubes containing S-Kefir inoculum than in tubes containing CNTL or G-Kefir inoculum.

Compared with PC, SCFA production was slightly lower in BP fermentations but led to great increases over time (**Figure 6.2**). Significant time and treatment differences were observed for acetate, propionate, and butyrate production. Acetate production was higher ($P<0.0001$) in

tubes containing S-Kefir inoculum than in tubes containing CNTL or G-Kefir inoculum. Propionate production was higher ($P<0.0001$) in tubes containing S-Kefir inoculum than in tubes containing G-Kefir inoculum. Butyrate production was higher ($P<0.0001$) in tubes containing S-Kefir or G-Kefir inoculum than in tubes containing CNTL inoculum. A significant treatment*time interaction was observed for total SCFA production. While total SCFA production increased in all tubes, its production increased at a greater ($P<0.0001$) rate in tubes containing G-Kefir inoculum than in tubes containing CNTL or C-Kefir inoculum.

Short-chain fatty acid production was moderate in all CP fermentations (**Figure 6.3**). Significant time and treatment differences were observed for propionate production. Propionate production was higher ($P<0.0001$) in tubes containing CNTL or S-Kefir inoculum than in tubes containing G-Kefir inoculum. Significant treatment*time interactions were observed for acetate, butyrate, and total SCFA production. While acetate and total SCFA production increased in all tubes, their production increased at a greater ($P<0.0001$) rate in tubes containing CNTL or S-Kefir inoculum than in tubes containing G-Kefir inoculum. Butyrate production also increased in all tubes, but its production increased at a greater ($P<0.0001$) rate in tubes containing S-Kefir or G-Kefir inoculum than in tubes containing CNTL inoculum. The production of SCFA for CEL fermentations was low and variable for all treatments (**Figure 6.10**).

Bacterial alpha diversity indices (i.e., Shannon Index, Observed features) of fermentation media fluctuated over time, with significant treatment, time, and treatment*time interactions being observed depending on the fiber source (**Figure 6.4**). Alpha diversity fluctuated over time but was higher ($P<0.01$) in BP fermentations than in the PC and CP fermentations. For PC, BP, and CP fermentations, alpha diversity was typically higher ($P<0.05$) in tubes containing S-Kefir inoculum than in tubes containing CNTL or G-Kefir inoculum. Bacterial beta diversity is represented by

principal coordinates analysis plots of weighted (**Figures 6.5-6.6**) and unweighted (**Figures 6.12-6.13**) UniFrac distances of fermentation media. When all fibers and time points were considered, both weighted and unweighted plots showed the separation of bacterial populations over time (**Figures 6.5, 6.12**). Within each fiber source, 0 h samples clearly clustered together and separately from other time points (**Figures 6.6; 6.13**). Over time, treatment affected clustering patterns, with greatest differences noted in PC and BP fermentations.

Significant treatment, time, and treatment*time interactions were observed for several bacterial taxa within all fiber fermentations (**Table 6.3; Figures 6.7-6.9; Tables 6.7, 6.8, 6.9, 6.10; Figure 6.14**). While many bacterial genera were altered, *Bacteroides*, *Prevotella*, *Faecalibacterium*, and *Ruminococcus torques* group were predominant taxa altered in all fiber fermentations. In CEL fermentations, *Bacteroides* relative abundance increased slightly by 6 h and then decreased over time in all tubes, but was higher ($P=0.01$) in tubes containing S-Kefir inoculum (**Figure 6.14**). *Prevotella* relative abundance increased ($P<0.01$) over time in all tubes, but was higher ($P<0.01$) in tubes containing CNTL inoculum than those containing S-Kefir or G-Kefir inoculum. *Faecalibacterium* relative abundance stayed relatively low over time but was higher ($P<0.01$) in tubes containing G-Kefir than those containing CNTL or S-Kefir inoculum. *Ruminococcus torques* group relative abundance increased consistently over time in tubes containing S-Kefir and was greater ($P<0.01$) than in tubes containing CNTL or G-Kefir inoculum that had small changes over time.

In PC fermentations, *Bacteroides* relative abundance increased over time in all tubes but was higher ($P=0.01$) in tubes containing CNTL and S-kefir at 12 h (**Figure 6.7**). *Prevotella* relative abundance increased ($P<0.0001$) over time in all tubes, but was higher ($P<0.0001$) in tubes containing S-Kefir or G-Kefir inoculum than those containing CNTL inoculum. *Faecalibacterium*

relative abundance increased ($P<0.0001$) over time in all tubes, but was higher ($P<0.0001$) in tubes containing CNTL inoculum than tubes containing S-Kefir or G-Kefir inoculum, and higher ($P<0.0001$) in tubes containing G-Kefir inoculum than tubes containing S-Kefir inoculum. *Ruminococcus torques* group relative abundance increased in all tubes but was greater ($P<0.01$) in tubes containing S-Kefir inoculum than in tubes containing CNTL or G-Kefir inoculum.

In BP fermentations, *Bacteroides* relative abundance increased over time in all tubes but increased at a greater ($P<0.0001$) in tubes containing CNTL inoculum than in tubes containing S-Kefir or G-Kefir inoculum (**Figure 6.8**). *Prevotella* relative abundance increased ($P<0.0001$) over time in all tubes, but increased at a greater ($P<0.0001$) rate in tubes containing S-Kefir inoculum than tubes containing CNTL or G-Kefir inoculum, and at a greater rate in tubes containing G-Kefir inoculum than those containing CNTL inoculum. *Faecalibacterium* relative abundance increased ($P<0.0001$) over time in all tubes, but increased at a greater ($P<0.01$) rate in tubes containing CNTL or G-Kefir inoculum than tubes containing S-Kefir inoculum. *Ruminococcus torques* group relative abundance increased over time in all tubes but increased at a greater ($P<0.05$) rate in tubes containing S-Kefir inoculum than in tubes containing CNTL or G-Kefir inoculum.

In CP fermentations, *Bacteroides* relative abundance increased over time in all tubes but was higher ($P<0.0001$) in tubes containing CNTL inoculum than in tubes containing S-Kefir or G-Kefir inoculum (**Figure 6.9**). *Prevotella* relative abundance increased over time in all tubes. While *Prevotella* increased consistently over time in tubes containing G-Kefir inoculum, it increased from baseline to 6 h and then stayed stable in tubes containing CNTL or G-Kefir inoculum, leading to greater ($P<0.01$) relative abundances in G-Kefir tubes vs. CNTL or S-Kefir tubes. *Faecalibacterium* relative abundance increased over time in all tubes but increased at a greater ($P<0.001$) rate in tubes containing G-Kefir inoculum than in tubes containing CNTL or S-Kefir

inoculum. *Ruminococcus torques* group relative abundance increased over time in tubes containing S-Kefir but stayed relatively low over time in tubes containing CNTL or G-Kefir inoculum. This resulted in a treatment*time interaction ($P < 0.0001$).

Results (Study #2)

Based on culture on MRS media for 48 h, S-Kefir contained 2.21×10^6 CFU/mL and G-Kefir contained 4.31×10^9 CFU/mL. The microbial profile of S-Kefir demonstrated that *Lactococcus* was the dominant genus present (98.26% relative abundance) (**Table 6.6**). Low levels of *Streptococcus* (1.20%), *Lactobacillus* (0.19%), *Enhydrobacter* (0.13%), *Enterococcus* (0.10%), *Clostridium sensu stricto 1* (0.03%), *Allobaculum* (0.02%), *Turicibacter* (0.02%), *Limosilactobacillus fermentum* (0.01%), *Leuconostoc* (0.01%), Planococcaceae (0.01%), and *Hafnia-Obesumbacterium* (0.01%) were detected. The microbial profile of G-Kefir was much more diverse. *Lactococcus* (72.59%), *Lactobacillus Kefiranofaciens* (17.42%), *Lactobacillus* (2.41%), *Bacillus* (2.07%), and *Ammoniphilus* (1.90%) were the predominant genera present in G-Kefir, with 15 other genera detected at lower levels ($< 1.0\%$). Not surprisingly, similar microbial relative abundances were observed in the baseline fermentation media samples (**Table 6.7**).

In the fermentations of PC (**Table 6.8**), BP (**Table 6.9**), CP (**Table 6.10**), and CEL (**Table 6.11**), similar genera were observed due to the similar nature of both treatments, yet overall trends of increase in *Lactobacillus* by G-Kefir were seen in all fibers, while increases in Proteobacteria were observed in S-Kefir throughout all fibers.

Significant treatment*time interactions were observed for pH change in tubes containing CEL ($P = 0.0201$), PC ($P = 0.0473$), BP ($P = 0.0061$), and CP ($P = 0.0073$) (**Table 6.8**). While the reduction in pH was a bit more rapid in tubes inoculated with S-Kefir for the BP and CP

fermentations (at 12 h), the final pH values were not different between kefir sources for all fibers. Acetate production increased over time in all fermentations, but treatment differences were not observed for CEL, PC, or BP fermentations (**Table 6.8**). For CP fermentations, a treatment*time interaction ($P=0.0054$) was observed whereby the increase in acetate production was greater in tubes inoculated with G-Kefir than those inoculated with S-Kefir. Propionate concentrations remained low in all fermentations, slightly fluctuating with time. For CP fermentations, propionate concentrations were higher ($P=0.0436$) in tubes inoculated with S-Kefir than those inoculated with G-Kefir. Butyrate production fluctuated over time depending on the fiber source and inoculum source. While no significant differences were noted due to treatment or time in PEC fermentations, significant treatment*time interactions were noted in CEL ($P=0.0003$), BP ($P<0.0001$), and CP ($P=0.0204$) fermentations. For CEL fermentations, butyrate concentrations slightly increased over time in tubes inoculated with S-Kefir but decreased in tubes inoculated with G-Kefir. For BP fermentations, butyrate concentrations slightly increased over time in tubes inoculated with S-Kefir but fluctuated in tubes inoculated with G-Kefir (decreased at 12 h, but increased at 18 h). Finally, butyrate production was increased in all tubes containing CP, with fermentation patterns differing between inoculum sources. Butyrate increased consistently over time in tubes inoculated with S-Kefir, which ended with higher concentrations than in tubes inoculated with G-Kefir.

Bacterial alpha diversity measures such as Observed features and the Evenness Index demonstrate that the diversity of fermentation media fluctuated over time, with significant treatment, time, and treatment*time interactions being observed depending on the fiber source (**Figure 6.10**). In general, alpha diversity was higher in tubes inoculated with G-Kefir than those inoculated with S-Kefir ($P<0.05$; Evenness Index). In regard to the fiber source, alpha diversity was also higher in tubes containing CP than in tubes containing the other fibers ($P<0.05$; Observed

features). Bacterial beta diversity is represented by principal coordinate analysis (PCoA) plots of unweighted and weighted UniFrac distances (**Figures 6.12, 6.13, 6.14, 6.15**). No significant differences were observed between fiber beta diversity in the unweighted PCoA (**Figure 6.12**) or weighted (**Figure 6.13**). The PCoA plots displayed significant differences between 0 h and 6 h, 12 h, 18 h occurred ($q=0.002$), and a clustering effect by treatment was present in both weighted PCoA plots (**Figures 6.14, 6.15**).

Several bacterial genera/families were consistently abundant above baseline values in the fermentation of all fibers. *Hafnia-Obesumbacterium*, Yersiniaceae, and *Lactobacillus* increased in abundance in S-Kefir samples throughout the fermentation (**Table 6.9**). The species *Clostridium sensu stricto 1* bloomed to 41% abundance in both treatments when combined with CP (**Table 6.6**). G-Kefir did have persistent *L. kefiranoferiens* and *L. fermentum* abundance throughout the fermentation, although not exceeding baseline abundance.

Discussion

The increasing popularity of foods containing live microorganisms such as fermented foods ushers in questions regarding their effects on host health (Vinderola et al., 2023). Variation in microbial composition is expected among fermented foods because environmental factors largely impact which species proliferate. Kefir grains are defined by the Codex Alimentarius Standard (CODEX STAN 243-2003) as containing *L. kefir*, as well as species of *Leuconostoc*, *Lactococcus*, and *Acetobacter*. Both lactose-fermenting yeasts (*K. marxianus*) and non-lactose-fermenting yeasts (*Saccharomyces cerevisiae*) are also commonly identified in kefir grains (González-Orozco et al., 2023). Despite many attempts to replicate grains or their effects on large-scale production, the fermentation of kefir grains is the only way to achieve an authentic kefir product (Rosa et al.,

2017). A regulatory standard of identity currently exists only for yogurt, which establishes a minimal inclusion of specific microbes such as *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* to be labeled as such (21 CFR 131.200). However, kefir does not have an established standard of identity, allowing companies to sell a variety of products without the use of kefir grains, the inclusion of authentic kefir microbes, or a minimal microbe inclusion level. This discrepancy highlights the need for label differentiation between starter and grain kefir, as well as investigations into the impacts of associated microbial communities (Bourrie et al., 2021; Nejati et al., 2022). The success of the companion animal probiotic supplement industry has welcomed an influx of fermented foods and beverages in the market. Recent testing, however, demonstrates a lack of ingredient label integrity and accuracy within this niche market (Metras et al., 2020). Moreover, while kefir products for companion animals have been tested recently (Kim et al., 2019; Gaspardo et al., 2020; Metras et al., 2023), more testing of such products is necessary to establish agreeable label regulations.

Because fecal samples are unable to accurately assess microbial activity, we chose to use an in vitro fermentation assay to measure changes to microbiota and metabolite concentrations from fecal inoculum of dogs fed grain or starter kefir. In vitro fermentation assays are often used to test the fermentability of individual fiber sources (Payne et al., 2012; de Godoy et al., 2015; Carlson et al., 2017; Traughber et al., 2020), but may also be used to assess the activity of fecal microbiota collected from animals given different treatments. Our results demonstrate that fecal inoculum from dogs supplemented with S-Kefir and G-Kefir produced higher butyrate concentrations in all fiber fermentations when compared with fecal inoculum from control dogs, with those supplemented with G-Kefir producing the greatest amounts. While the fecal microbiota populations of our previous study testing these treatments did not identify many changes (Metras

et al., 2023), these data suggest that the activity of the microbial communities differed. The primary SCFA, namely acetate, propionate, and butyrate, have been estimated to provide 60–70% of the energy requirements of colonic epithelial cells (Brahe et al., 2013). Of the SCFA, butyrate is the preferred energy source and is known to enhance intestinal barrier function and support mucosal immunity (Fu et al., 2019). Therefore, the changes to the gastrointestinal microbiota with kefir supplementation would be deemed to be beneficial.

This research is the first to observe microbial syntrophy by microbes within a system supported by fermented food-derived inoculums. Increases of acetate and butyrate occurred during fecal in vitro fermentations, with much lower conversion in tubes inoculated with kefir inocula. The presence of lactate could also have been used by butyrate and propionate-producing bacteria such as *Lactobacillus*, *Faecalibacterium*, *Prevotella*, and *Ruminococcus* (Boger et al., 2018). Unfortunately, lactate concentrations were not measured in the current study.

The chicory pulp used is comprised of several types of fibers, including pectin (27%), cellulose (18.5%), hemicellulose (8%), and inulin (7-8%). Inulin is a type of highly fermentable fructooligosaccharide (FOS). FOS are linear polymers consisting of β -2,1-linked fructose monomers with a glucose molecule on the terminal end. The degradation of FOS such as inulin by bacteria is largely dependent on enzymes associated with certain phylum and the degree of polymerization. Actinobacteria such as *Bifidobacteria* can digest shorter FOS segments with <7 degrees of polymerization to produce acetate and lactate while releasing fructose residues into the microbial environment. By use of FOS, this study reinforced that members of Bacteroides and Firmicutes such as *Lactobacillus* and *Clostridium* can break down longer FOS chains >6-7 degrees of polymerization. These gram-negative bacteria were shown to “cross-feed” on lactate and fructose while utilizing environmental acetate to release butyrate into the local environment

(Rivière et al., 2016). This functionality is owed to fructan polysaccharide utilization loci (PUL) which encodes the system necessary for uptake and degradation of fructose-based dietary polysaccharides (Joglekar et al., 2018). PUL are responsible for the transcription of carbohydrate active enzymes (CAZymes) such as glycoside hydrolases (GH) specific for degrading glycosidic linkages in shorter FOS, polysaccharide lyases, carbohydrate esterases, and β -fructofuranosidases (Fernandez-Julia et al., 2022).

SCFA represents the main carbon flow from the diet to the host microbiome, with commensal microbes such as *Faecalibacterium*, Lachnospiraceae, lactic acid bacteria, and *Streptococcus* known to convert bacterial metabolites such as lactate and acetate into butyrate via the acetyl-CoA pathway (Singh et al., 2023). Although it is not possible to know the exact source of SCFA producers in the current study, many commensal microbes such as *Ruminococcus torques* and *Clostridium* detected in fecal kefir tubes are known to produce SCFA by converting pyruvate via the acetyl-CoA pathway (Markowiak-Kopeć and Śliżewska, 2020; Deleu et al., 2021; Singh et al., 2023). Total SCFA concentrations differed significantly by treatment*time interaction effect in PC, BP, and CP fermentations during the fecal in vitro fermentation.

Of the SCFA, butyrate is the preferred energy source and is known to enhance intestinal barrier function and support mucosal immunity (Fu et al., 2019). Our results demonstrate that butyrate concentrations were highest in both fermented milk-treated fecal samples when fermenting selected dietary fibers with G-Kefir supplementation produced the highest butyrate output overall. While the fecal microbiota populations from our previous study did not detect any significant microbiota changes in abundance (Metras et al., 2023), these data suggest that the functional activity of the microbial communities differed. Butyrate producers such as *Lactobacillus* bloomed alongside increases in butyrate concentrations during fecal G-Kefir pectin

fermentation (Rivière et al., 2016). The significant abundance of *Faecalibacterium* in all fecal treatments fermenting BP and CP is also supported by literature demonstrating this genus can break β -(2,1) glycosidic bonds while being a prolific butyrate producer (Moens et al., 2017). Therefore, changes to the gastrointestinal microbiota by kefir supplementation could be deemed to be beneficial in vitro.

While bacteria can utilize dairy proteins as energy sources, plant-derived polymers (hemicellulose and xylans) can be fermented as well (Petrova and Petrov, 2020). *Lactococcus lactis* spp. *cremoris* is enriched in genes encoding CAZymes such as glycosyltransferase to support fiber fermentation (Apostolakos et al., 2023). Despite both treatments having a majority *Lactococcus* abundance, the phylum did not increase over time with fiber supplementation.

Over time, PC fermentation supported an increase in the abundance of *Ruminococcus torques* in fecal S-Kefir, yet was 1.22% more abundant in fecal G-Kefir at 18 h. While *Ruminococcus torques* increased in fecal G-kefir, *Fusobacterium* also increased over time with PC in CNTL and S-Kefir fecal tubes. In the fermentation of BP, *Ruminococcus* increased in both CNTL and S-Kefir fecal tubes over time, and yet another butyrate producer *Peptosclostridium* peaked in fecal G-Kefir. The interplay of butyrate producers could explain high levels of this SCFA in fecal tubes, yet microbes known to be secondary butyrate producers should also be considered.

The secondary butyrate producer Lachnospiraceae increased in both fecal kefir samples in BP fermentation and remained above baseline in fecal G-Kefir at 12h and 18h. The potential production of lactic acid and acetic acid by Lachnospiraceae may have supported butyrate production in fecal kefir samples (Singh et al., 2023). Other probiotic bacteria such as *Bifidobacterium* can undergo cross-feeding interactions with butyrate-producing commensal bacteria, where exogenous acetate produced by *Bifidobacterium* is a co-substrate to butyrate

production (Rivière et al., 2016; Singh et al., 2023). While levels of *Bifidobacterium* did not exceed baseline levels throughout the experiment, its population maintained a presence over 18 h in all fibers and treatments by potentially utilizing local acetate and lactic acid (Drey et al., 2022).

Our previous dog study testing kefir demonstrated an increase ($P < 0.0001$) in fecal *Lactococcus* in dogs supplemented with G-Kefir (Metras et al., 2023). Lactic acid bacteria, including *Lactococcus* and *Lactobacillus*, are generally viewed as beneficial to gastrointestinal and host health due to their protection from pathogenic bacteria, modulation of the immune system, reduction of radical oxidative species, and inulin degrading enzymes. (Slattery et al., 2019; Marco, 2020). More research is needed on *Lactococcus* SCFA production, yet *Lactococcus* has been shown to produce several B vitamins, including thiamin and riboflavin, while LAB produce folate; all of which function as cofactors to enzymes in metabolic pathways producing SCFA (LeBlanc et al., 2017). While the relative abundance of *Lactobacillus* declined over time, the fermentation of PC supported *Lactobacillus* better in fecal G-Kefir tubes. This finding is consistent with other in vitro studies fermenting pectin which also observed *Lactobacillus* stability (Beukema et al., 2020; Pascale et al., 2022).

During the drink in vitro fermentation, several changes in phyla were observed. While both kefir drinks were dominated by Firmicutes, their abundance increased during PC fermentation in G-Kefir likely due to *Bacillus* and *Clostridium sensu stricto 1* thriving on the fiber source and outcompeting other genera. The opposite effect was observed in Proteobacteria, which increased during PC fermentation in S-Kefir drinks due to the growth of *Hafnia-Obesumbacterium undf* and *Yersiniaceae undf*. Over time, the fermentation of CP by S-Kefir displayed a significant treatment effect of *Hafnia-Obesumbacterium undf* which reached an average of 40% abundance. CP was heavily fermented by *Turicibacter*, *Streptococcus*, and *Clostridium sensu stricto 3* in G-Kefir

drinks. Both Yersiniaceae and *Hafnia* have been observed to demonstrate a negative correlation with glucose, thus as lactose was consumed throughout the 18 h fermentation and glucose decreased, these bacterial families thrived on the remaining bacterial protein and contributed to the increased acetate production from S-Kefir (Shang et al., 2023).

During the fermentation of CP in our drink in vitro experiment, a significant increase in alpha diversity was observed. The relative abundance of *Clostridium sensu stricto 1* increased markedly both in G-kefir and S-Kefir drinks. Previous studies fermenting CP have not seen an increase in *Clostridium sensu stricto 1* specifically which provides novelty to our findings (Yasuda et al., 2006). During the fermentation of PC by microbes in the S-kefir drink, significant effects were observed in the production of *Lactobacillus spp.* and *L. fermentum*. A decline in *Lactobacillus* was expected over time due lack of preferred substrates by kefir microbes, yet the community within S-Kefir surprisingly increased using PC and BP, leading to areas of future investigation of conserved fiber degrading genes conserved in starter kefirs. Given the low SCFA production in tubes inoculated with kefirs, it appears that the strains present were not as effective as the fecal communities at breaking down these fibers. This was expected due to the presence of fiber-fermenting commensal bacteria derived from the gut microbiota in fecal samples and absent from kefir beverages.

These in vitro fermentation assays using fecal inoculum of dogs fed grain vs. starter kefir as well as direct kefir beverages were done to measure changes in microbiota, their within-sample interactions, and resulting metabolite concentrations. In vitro fermentation assays are often used to test the fermentability of individual fiber sources (Payne et al., 2012; de Godoy et al., 2015; Carlson et al., 2017; Traughber et al., 2020) as well as assess the functionality of fecal microbiota derived from different treatment (Barry et al., 2011; Christensen et al., 2022). The fecal inoculum

and kefir inoculum contain dynamic microbial communities that utilize the complex array of microbes in these products better than a monoculture or a selected closed community. In addition, this in vitro assay enables the measurement of SCFA production, which is not possible from fecal samples that only resemble terminal gut conditions.

There are several limitations to this study and to using in vitro fermentations as a model for the host gastrointestinal system. While the assay includes a microbiological medium and is conducted in a way to control temperature and pH within an anaerobic system, it does not correct for gastrointestinal secretions (e.g., mucins, host enzymes, bacteriocins), and SCFA are not removed from the system as they are in vivo. In terms of microbial density, the consumption of kefir imparts a higher volume of live microorganisms than tested in this study and results should be interpreted with that in mind. In addition, the journey live microbes undergo throughout the digestive tract involves passage through extreme acidity in gastric digestion and competition with native commensal microbes. These differences may impact how microbiota respond in the system, cross-feed, and consequently how fibers are fermented. While three-time points were sampled for outcome measures, more frequent sampling may provide a more complete picture of the fermentation kinetics. The microbial inoculum was only tested in four fiber sources commonly used in pet foods. Dietary fibers and other potential substrates containing other monosaccharide components, macronutrient profiles, and chemical linkages may generate different results. In addition, the preferred substrate of kefir bacteria is lactose, thus using fiber as the sole energy source may explain reduced microbial abundances and SCFA production beyond baseline. Efforts were made to correct for the higher microbial density in G-kefir by diluting it 1:4 with sterilized G-Kefir product so that upon inoculation, both kefirs contained about 10^6 CFU/mL in the second fermentation experiment. The focus of these experiments was on changes in microbiota, yet it

should be noted many species of yeast are present in G-Kefir and may have contributed to SCFA generation and fiber digestion. Fermented foods are expected to vary in microbial contents even among similar batches, so truly encapsulating an all-encompassing comparison of starter and grain kefir will need more research.

This experimental design allows for a snapshot into a controlled environment. Using animal models and in vitro, simulations can be effective ways to better understand the differences between microbial communities among fermented foods and their SCFA production with different fibers, yet we demonstrated significant functional differences among S-Kefir and G-Kefir. In conclusion, our in vitro fermentation system confirmed the fermentability of common fiber sources, with PC having the highest fermentability, followed by BP, CP, and CEL. Primary and secondary butyrate producers *Ruminococcus torques* and *Faecalibacterium prausnitzii* proliferated in G-Kefir treatment fermentations which observed significant butyrate production. Our data also demonstrated that the activity of microbiota-derived from kefir-supplemented dogs promoted the growth of SCFA-producing bacteria and the production of butyrate, with greater promotion in dogs fed traditional vs. commercial kefir.

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Tables and Figures

Table 6.1. Relative abundances (% of sequences) of bacterial phyla and genera of fecal inoculum from dogs fed the control (CNTL), starter kefir (S-Kefir), or grain kefir (G-kefir) treatments at baseline (0 h) among all fiber tubes

Phyla	Genera	CNTL	S-Kefir	G-Kefir	SEM	p-value
Actinobacteriota		4.98 ^c	7.53 ^b	10.00 ^a	0.274	<0.0001
	<i>Adlercreutzia</i>	0.12 ^b	0.07 ^c	0.17 ^a	0.009	<0.0001
	<i>Atopobiaceae undf</i>	0.11 ^b	0.13 ^b	0.24 ^a	0.022	0.0002
	<i>Bifidobacterium</i>	4.02 ^c	5.39 ^b	8.71 ^a	0.223	<0.0001
	<i>Collinsella</i>	0.09 ^b	0.24 ^a	0.09 ^b	0.033	0.0013
	<i>Coriobacteriaceae UCG-002 undf</i>	0.59 ^b	1.61 ^a	0.76 ^b	0.091	<0.0001
Bacteroidota		7.09 ^b	8.12 ^{ab}	8.96 ^a	0.508	0.0431
	<i>Alloprevotella</i>	0.12 ^b	0.25 ^a	0.06 ^b	0.021	<0.0001
	<i>Bacteroides</i>	2.49 ^a	2.28 ^a	1.22 ^b	0.177	<0.0001
	<i>Bacteroides caecigallinarum</i>	1.43 ^a	0.31 ^b	0.26 ^b	0.088	<0.0001
	<i>Bacteroides plebeius</i>	0.17 ^b	0.60 ^a	0.12 ^b	0.025	<0.0001
	<i>Muribaculaceae undf</i>	3.23 ^b	3.30 ^b	6.64 ^a	0.256	<0.0001
	<i>Parabacteroides</i>	0.08 ^b	0.16 ^a	0.09 ^b	0.01	<0.0001
	<i>Parabacteroides merdae</i>	0.04 ^b	0.05 ^b	0.09 ^a	0.007	<0.0001
	<i>Prevotella</i>	0.34 ^b	0.89 ^a	0.48 ^b	0.041	<0.0001
	<i>Prevotellaceae Ga6A1 group</i>	0.57 ^b	0.94 ^a	0.44 ^b	0.056	<0.0001
	<i>Rikenellaceae undefined</i>	0.05 ^b	0.30 ^a	0.04 ^b	0.012	<0.0001
	<i>Rikenellaceae R9 gut group</i>	0.21 ^a	0.00 ^b	0.00 ^b	0.017	<0.0001
Campilobacterota		0.01 ^b	0.06 ^a	0.00 ^b	0.004	<0.0001
	<i>Helicobacter</i>	0.01 ^b	0.06 ^a	0.00 ^b	0.004	<0.0001
Firmicutes		72.21 ^a	69.21 ^{ab}	67.10 ^b	1.095	0.0077
	<i>Allobaculum</i>	8.10 ^b	6.26 ^c	18.47 ^a	0.454	<0.0001
	<i>Allobaculum stercoricanis</i>	0.15 ^b	0.22 ^a	0.05 ^c	0.008	<0.0001
	<i>Anaerovoracaceae undf</i>	0.44 ^a	0.41 ^a	0.19 ^b	0.027	<0.0001
	<i>Bacilli undf.</i>	2.27 ^a	2.34 ^a	1.61 ^b	0.077	<0.0001
	<i>Blautia</i>	1.79 ^b	3.81 ^a	1.87 ^b	0.078	<0.0001
	<i>Butyricicoccus</i>	0.02 ^b	0.06 ^a	0.06 ^a	0.004	<0.0001
	<i>Butyricicoccus pullicaecorum</i>	0.01 ^b	0.04 ^a	0.05 ^a	0.006	<0.0001
	<i>Candidatus Stoquefichus</i>	0.08 ^a	0.04 ^b	0.03 ^b	0.005	<0.0001
	<i>Cellulosilyticum</i>	0.04 ^b	0.02 ^b	0.08 ^a	0.007	<0.0001
	<i>Clostridium sensu stricto 1</i>	0.55 ^a	0.26 ^b	0.72 ^a	0.074	<0.0001
	<i>Dubosiella</i>	2.96 ^b	6.68 ^a	6.45 ^a	0.183	<0.0001
	<i>Dubosiella newyorkensis</i>	0.19 ^c	1.08 ^a	0.59 ^b	0.035	<0.0001
	<i>Enterococcus</i>	2.42 ^a	1.64 ^b	0.72 ^c	0.084	<0.0001
	<i>Enterococcus cecorum</i>	2.42 ^a	1.63 ^b	0.72 ^c	0.084	<0.0001
	<i>Erysipelatoclostridium</i>	0.48 ^a	0.27 ^c	0.36 ^b	0.013	<0.0001
	<i>Erysipelotrichaceae undf</i>	10.87 ^a	8.48 ^b	8.52 ^b	0.432	0.0003
	<i>Faecalibacterium</i>	0.30 ^a	0.11 ^{ab}	0.09 ^b	0.013	<0.0001
	<i>Faecalibaculum</i>	2.27	2.05	2.3	0.097	0.1566
	<i>Fournierella</i>	0.24 ^b	0.31 ^a	0.21 ^b	0.024	0.0003
	<i>Holdemanella</i>	0.14 ^b	0.26 ^a	0.09 ^c	0.013	<0.0001
	<i>Lachnospiraceae undf</i>	7.24 ^a	6.30 ^b	4.42 ^c	0.183	<0.0001
	<i>Lactobacillus</i>	14.90 ^b	18.71 ^a	8.77 ^c	0.538	<0.0001
	<i>Lactococcus</i>	0.00 ^b	0.00 ^b	0.47 ^a	0.035	<0.0001

Table 6.1 (cont.)

	<i>Peptoclostridium</i>	2.84 ^a	1.99 ^b	2.59 ^a	0.159	0.0016
	<i>Peptococcus</i>	0.47 ^b	0.59 ^a	0.34 ^c	0.03	<0.0001
	<i>Peptostreptococcus</i>	1.34 ^c	2.60 ^b	3.94 ^a	0.12	<0.0001
	<i>Phascolarctobacterium</i>	0.04 ^b	0.24 ^a	0.20 ^a	0.018	<0.0001
	<i>Romboutsia</i>	1.49 ^a	0.71 ^b	0.89 ^b	0.104	<0.0001
	<i>Ruminococcus gausvreauii group</i>	0.36 ^b	0.51 ^a	0.28 ^b	0.016	<0.0001
	<i>Ruminococcus gnavus group</i>	0.12 ^b	0.44 ^a	0.41 ^a	0.016	<0.0001
	<i>Ruminococcus torques group</i>	2.18 ^a	1.86 ^b	1.21 ^c	0.052	<0.0001
	<i>Streptococcus</i>	5.57 ^a	0.70 ^b	0.26 ^b	0.273	<0.0001
	<i>Terrisporobacter</i>	0.09 ^b	0.00 ^c	0.17 ^a	0.009	<0.0001
	<i>Turicibacter</i>	2.11 ^a	1.16 ^b	1.15 ^b	0.152	<0.0001
Fusobacteriota		12.36	13.05	11.15	0.573	0.0722
	<i>Cetobacterium</i>	2.16 ^b	2.76 ^b	4.26 ^a	0.156	<0.0001
	<i>Fusobacterium</i>	10.20 ^a	10.28 ^a	6.89 ^b	0.447	<0.0001
Proteobacteria		3.35 ^a	2.02 ^b	2.78 ^a	0.193	<0.0001
	<i>Anaerobiospirillum</i>	0.04	0.05	0.05	0.006	0.2125
	<i>Anaerobiospirillum succiniciproducens</i>	0.03 ^b	0.04 ^{ab}	0.05 ^a	0.006	0.0392
	<i>Parasutterella</i>	3.21 ^a	1.88 ^b	2.70 ^a	0.181	<0.0001

undf.; Undefined

Table 6.2. Change in pH during cellulose, pectin, beet pulp, and chicory pulp in vitro fermentation assays using fecal inocula from dogs fed the control (CNTL), starter kefir (S-Kefir), or grain kefir (G-Kefir) treatments

Fiber source	CNTL			S-Kefir			G-Kefir			SEM	p-values		
	6 h	12 h	18 h	6 h	12 h	18 h	6 h	12 h	18 h		Trt	Time	Trt*Time
Cellulose	0.00	0.02	-0.08	0.03	0.01	-0.01	-0.03	-0.03	0.03	0.027	0.4066	0.5788	0.0495
Pectin	-0.74 ^a	-0.86 ^{ab}	-1.15 ^c	-0.72 ^a	-1.09 ^c	-1.49 ^d	-0.89 ^b	-1.09 ^c	-1.4 ^d	0.033	<0.0001	<0.0001	0.0007
Beet pulp	-0.30 ^a	-0.67 ^b	-0.85 ^c	-0.40 ^a	-0.79 ^{bc}	-0.82 ^c	-0.40 ^a	-0.77 ^{bc}	-0.75 ^{bc}	0.025	0.0191	<0.0001	0.0044
Chicory pulp	-0.18 ^a	-0.22 ^{ab}	-0.30 ^{ab}	-0.20 ^{ab}	-0.33 ^{ab}	-0.30 ^{ab}	-0.34 ^b	-0.33 ^{ab}	-0.27 ^{ab}	0.032	0.0195	0.1157	0.0302

^{a-d}Means in the same row with different superscript letters are different (P<0.05)

Table 6.3. Change in bacterial phyla and genera relative abundances (% of sequences) from 0h during cellulose, pectin, beet pulp, and chicory pulp in vitro fermentation assays using fecal inocula from dogs fed the control (CNTL), starter kefir (S-Kefir), or grain kefir (G-Kefir) treatments.

Fiber	Phyla	Genus	CNTL			S-Kefir			G-Kefir			SEM	p-values			
			6 h	12 h	18 h	6 h	12 h	18 h	6 h	12 h	18 h		Trt	Time	Trt*Time	
Cellulose	Bacteroidota	<i>Parabacteroides</i>	0.22 ^a	0.95 ^a	0.93 ^a	0.40 ^a	0.82 ^a	0.52 ^a	0.06 ^{ac}	0.46 ^{ab}	0.73 ^a	0.074	<0.0008	<0.0001	0.0016	
		<i>Rikenellaceae undf</i>	0.13 ^{ab}	0.36 ^a	0.33 ^{ab}	0.21 ^a	0.16 ^a	0.02 ^{ab}	0.15 ^a	0.21 ^a	0.38 ^a	0.047	0.0037	0.0839	0.0009	
	Firmicutes	<i>Negativibacillus</i>	-0.01 ^{ab}	-0.03 ^a	0.06 ^a	0.07 ^a	0.21 ^a	0.38 ^a	-0.04 ^{ac}	-0.03 ^{ab}	0.03 ^a	0.037	<0.0001	0.0004	0.0318	
		<i>Oscillospiraceae UCG-005 undf</i>	0.10 ^b	0.19 ^a	0.22 ^a	0.10 ^b	0.10 ^b	0.11 ^b	0.03 ^{bc}	0.15 ^{ab}	0.14 ^{ab}	0.021	0.0014	0.0003	0.0279	
		<i>Peptoclostridium</i>	4.11 ^{ab}	1.16 ^{bc}	3.50 ^{ab}	1.80 ^{bc}	0.69 ^c	3.52 ^{ab}	6.82 ^{ab}	5.01 ^{ab}	3.66 ^b	0.654	<0.0001	0.0058	0.0091	
		<i>Peptostreptococcus</i>	-0.10 ^{ab}	-0.27 ^b	-0.29 ^b	-1.63 ^{bc}	-0.33 ^b	1.42 ^{ab}	-1.07 ^b	2.06 ^{ab}	2.05 ^{ab}	0.251	<0.0001	<0.0001	<0.0001	
	Proteobacteria	<i>Sutterella</i>	1.61 ^a	4.48 ^a	5.87 ^a	2.67 ^{ab}	4.80 ^a	5.85 ^a	0.21 ^{abc}	0.63 ^{abc}	1.94 ^{ab}	0.226	<0.0001	<0.0001	<0.0001	
		<i>Parasutterella</i>	-0.23 ^{bc}	-0.89 ^c	-1.28 ^c	2.31 ^a	0.27 ^b	-0.66 ^{bc}	0.01 ^{bc}	-1.01 ^c	-0.88 ^{bc}	0.327	<0.0001	<0.0001	0.0344	
	Pectin	Bacteroidota	<i>Parabacteroides</i>	0.10 ^{bc}	0.14 ^b	0.25 ^a	0.13 ^{ab}	0.22 ^{ab}	0.08 ^{bc}	0.03 ^c	0.18 ^{ab}	0.11 ^{bc}	0.020	0.0096	<0.0001	<0.0001
			<i>Rikenellaceae undf</i>	0.01 ^a	0.06 ^a	0.07 ^a	0.02 ^a	0.07 ^a	-0.12 ^b	0.02 ^a	0.08 ^a	0.08 ^a	0.021	0.0015	0.0041	0.0003
Firmicutes		<i>Enterococcus</i>	4.77 ^b	2.71 ^c	6.99 ^a	1.88 ^c	0.72 ^{cd}	0.53 ^{cd}	1.37 ^c	0.89 ^{cd}	0.62 ^{cd}	0.402	<0.0001	0.0013	<0.0001	
		<i>Lactobacillus</i>	-6.67 ^c	-6.14 ^c	-11.22 ^{cd}	-3.37 ^c	-10.35 ^{cd}	-8.02 ^c	16.78 ^a	8.92 ^b	5.11 ^b	1.394	<0.0001	<0.0001	0.0098	
		<i>Phascolarctobacterium</i>	-0.01 ^{bc}	0.07 ^b	0.58 ^a	-0.11 ^c	0.01 ^{bc}	0.56 ^a	-0.13 ^c	-0.11 ^c	-0.01 ^{bc}	0.036	<0.0001	<0.0001	<0.0001	
		<i>Ruminococcus gnavus group</i>	0.14 ^c	0.70 ^b	0.37 ^{bc}	-0.06 ^c	0.75 ^b	0.31 ^{bc}	-0.11 ^c	0.37 ^{bc}	1.22 ^a	0.079	0.0676	<0.0001	<0.0001	
Fusobacteriota		<i>Fusobacterium</i>	14.81 ^a	9.09 ^b	5.87 ^b	14.90 ^a	14.50 ^a	5.21 ^b	13.07 ^{ab}	9.76 ^{ab}	6.37 ^b	1.046	0.0949	<0.0001	0.0355	
Proteobacteria		<i>Sutterella</i>	0.34 ^c	1.36 ^{bc}	1.88 ^b	0.74 ^c	2.85 ^a	2.25 ^{ab}	0.05 ^c	0.60 ^c	1.15 ^{bc}	0.159	<0.0001	<0.0001	0.0011	
Beet Pulp		Actinobacteriota	<i>Collinsella</i>	0.06 ^b	0.01 ^b	0.19 ^a	0.05 ^b	0.19 ^a	0.08 ^b	0.06 ^b	0.11 ^{ab}	0.06 ^b	0.042	0.7690	0.2952	0.0300
			Firmicutes	<i>Anaeroplasma</i>	0.95 ^b	1.31 ^a	-0.02 ^{bcde}	0.24 ^b	0.28 ^b	0.02 ^{bcde}	0.67 ^{ab}	0.17 ^{bc}	0.05 ^{bcd}	0.171	0.0116	<0.0001
	<i>Blautia</i>	0.05 ^{ab}		0.00 ^{ab}	-0.05 ^{ab}	-0.30 ^b	0.45 ^a	-0.67 ^b	-0.27 ^b	-0.14 ^{ab}	0.05 ^{ab}	0.167	0.4394	0.0586	0.0095	
	<i>Lachnospiraceae undf</i>	-0.65 ^{abc}		-0.50 ^{abc}	1.34 ^{ab}	-0.99 ^{bc}	2.31 ^a	-0.27 ^b	-0.22 ^{ab}	1.05 ^{ab}	0.73 ^b	0.408	0.4000	0.0004	0.0008	
	<i>Peptoclostridium</i>	1.80 ^{ab}		-0.64 ^b	0.79 ^b	0.75 ^b	0.30 ^b	0.00 ^b	1.99 ^{ab}	0.31 ^b	-0.28 ^b	0.336	0.4349	<0.0001	0.0142	
	<i>Streptococcus</i>	5.91 ^a		0.31 ^b	5.62 ^a	1.29 ^b	1.77 ^b	1.50 ^b	0.68 ^b	0.71 ^b	0.66 ^b	0.348	<0.0001	<0.0001	<0.0001	
	<i>Rikenellaceae undf</i>	0.03 ^{bc}		0.23 ^{ab}	0.07 ^b	0.19 ^{ab}	-0.14 ^c	-0.13 ^c	0.09 ^b	0.15 ^{ab}	0.18 ^{ab}	0.034	<0.0001	0.0934	<0.0001	
	Fusobacteriota	<i>Fusobacterium</i>	14.67 ^{ab}	11.13 ^{ab}	4.14 ^{bc}	16.67 ^a	8.97 ^{bc}	9.70 ^b	23.92 ^a	14.51 ^{ab}	12.49 ^{ab}	1.196	<0.0001	<0.0001	0.0281	
		<i>Parabacteroides</i>	0.09 ^b	0.65 ^a	0.39 ^{ab}	0.19 ^b	0.19 ^b	0.35 ^b	0.03 ^{bc}	0.17 ^b	0.24 ^b	0.047	<0.0001	<0.0001	<0.0001	
	Proteobacteria	<i>Sutterella</i>	0.72 ^{bc}	1.40 ^b	1.81 ^{ab}	0.87 ^{bc}	0.94 ^b	1.95 ^a	0.13 ^d	0.31 ^{bcd}	0.70 ^{bcd}	0.097	<0.0001	<0.0001	0.0041	

Table 6.3 (cont.)

Chicory															
Pulp	Bacteroidota	<i>Rikenellaceae undf</i>	0.10 ^b	0.18 ^{ab}	0.17 ^{ab}	0.01 ^{bc}	0.04 ^b	-0.02 ^{bc}	0.12 ^b	0.26 ^{ab}	0.31 ^a	0.036	<0.0001	<0.0001	0.0003
	Firmicutes	<i>Anaeroplasma</i>	0.28 ^a	0.11 ^b	0.01 ^b	0.10 ^b	0.03 ^b	0.01 ^b	0.39 ^a	0.09 ^b	0.05 ^b	0.029	<0.0001	<0.0001	0.0034
		<i>Negativibacillus</i>	-0.05 ^b	-0.03 ^b	-0.02 ^b	-0.06 ^b	0.09 ^a	0.07 ^{ab}	-0.06 ^b	-0.06 ^b	-0.04 ^b	0.020	<0.0001	0.0031	0.0145
		<i>Ruminococcaceae undf</i>	0.13 ^{ab}	0.25 ^a	0.24 ^a	0.02 ^b	0.26 ^a	0.28 ^a	0.06 ^b	0.33 ^a	0.28 ^a	0.021	0.1568	<0.0001	0.0054

CNTL Control; 2% Reduced Fat Milk, Great Value Farm, Fort Wayne Indiana, USA

S-Kefir Starter kefir; Champions Choice Kefir; Champions Choice, Millersburg Indiana, USA

G-Kefir Grain Kefir; Kefir Garden Grains, Hamilton Ontario, Canada

undf.; Undefined

Table 6.4. Change in pH and SCFA from baseline (0h) levels

Fiber		S-Kefir			G-Kefir			p-values			
Cellulose	µmole/g	6 h	12 h	18 h	6 h	12 h	18 h	SEM	Trt	Time	Trt*Time
	pH	7.30 ^z	6.80 ^{zy}	6.78 ^{zy}	7.25 ^{zy}	6.92 ^{zy}	6.65 ^{zy}	0.078	0.4261*	0.0022*	0.0201*
	Acetate	5.23	59.07	96.84	4.53	163.21	56.34	59.307	0.6725	0.2291	0.4746
	Propionate	0.00	0.00	-0.42	0.00	0.00	1.53	1.644	0.6350*	0.0272*	0.1626*
	Butyrate	0.00 ^{ab}	3.17 ^a	1.09 ^{ab}	-0.26 ^{ab}	-15.63 ^b	-61.29 ^c	5.463	<0.0001	0.0003	0.0003
	Total SCFA	5.23	62.24	97.50	4.27	147.58	-1.96	60.251	0.9203	0.2862	0.3412
Pectin	pH	7.15 ^a	5.99 ^b	5.85 ^{bc}	7.10 ^a	6.53 ^b	5.85 ^{bc}	0.116	0.1105	<0.0001	0.0473
	Acetate	28.72	337.79	347.61	28.38	327.28	517.33	82.751	0.4484	0.0010	0.4939
	Propionate	0.00	0.00	-0.43	0.00	0.00	-3.13	0.322	0.7590*	0.0221*	0.1615*
	Butyrate	0.00	7.16	56.7	-0.61	-5.36	78.89	43.957	0.6041*	0.1112*	0.3013*
	Total SCFA	28.72	344.96	403.89	27.77	321.92	593.70	119.27	0.5808	0.0062	0.6293
Beet Pulp	pH	7.12 ^a	6.44 ^b	6.42 ^b	7.14 ^a	6.87 ^a	6.43 ^b	0.060	0.0080	<0.0001	0.0061
	Acetate	158.25	182.24	171.02	148.27	217.76	312.16	56.632	0.6911*	0.1443*	0.4046*
	Propionate	0.00	0.00	0.78	0.00	0.00	-3.53	0.672	0.2694*	0.0278*	0.0549*
	Butyrate	2.73 ^b	1.76 ^b	3.58 ^b	0.77 ^b	-17.56 ^c	17.99 ^a	39.701	<0.0001	<0.0001	<0.0001
	Total SCFA	160.98	183.99	176.58	149.03	200.20	322.59	87.467	0.6911*	0.1426*	0.4451*
Chicory Pulp	pH	7.16 ^a	6.52 ^c	6.51 ^c	7.23 ^a	6.71 ^b	6.52 ^c	0.024	0.0007	<0.0001	0.0073
	Acetate	37.66 ^b	94.15 ^b	276.80 ^a	26.46 ^b	301.36 ^a	252.10 ^a	31.903	0.0488	<0.0001	0.0054
	Propionate	0.00	3.71	1.99	0.00	2.48	0.34	1.694	0.0436	0.0334	0.6072
	Butyrate	2.89 ^b	8.60 ^b	66.38 ^a	0.55 ^b	25.28 ^{ab}	16.09 ^b	10.427	0.1845	0.0084	0.0204
	Total SCFA	40.56 ^b	106.46 ^b	345.17 ^a	27.01 ^b	329.12 ^a	264.67 ^{ab}	40.783	0.2223	<0.0001	0.0073

* Indicates non-parametric tests were used; ^{z-y} denotes significance ranking; ^{a-c}Means with different superscript letters are different (P<0.05).

S-Kefir Starter Kefir

G-Kefir Grain Kefir

Table 6.5. Composition of microbiological medium used in the in vitro experiment.

Component	Amount
Liquid solutions	
Solution A (mL/L) ^a	330.0
Solution B (mL/L) ^b	330.0
Distilled water (mL/L)	296.0
Water-soluble vitamin mix (mL/L) ^c	20.0
Trace mineral solution (mL/L) ^d	10.0
Folate/biotin solution (mL/L) ^e	5.0
Riboflavin solution (mL/L) ^f	5.0
Hemin solution (mL/L) ^g	5.0
Resazurin (mL/L) ^h	1.0
Short-chain fatty acid mix (mL/L) ⁱ	0.4
Solid chemicals, g in medium	
Yeast (g/L)	0.5
Trypticase (g/L)	0.5
Na ₂ CO ₃ (g/L)	4.0
Cysteine hydrochloride H ₂ O (g/L)	0.5

^aComposition (g/L): NaCl, 5.4; KH₂PO₄, 2.7; CaCl₂·H₂O, 0.18; MgCl₂·6H₂O, 0.12; MnCl₂·4H₂O, 0.06; CoCl₂·6H₂O, 0.06; (NH₄)₂SO₄, 5.4.

^bComposition: K₂HPO₄, 2.7 g/L.

^cComposition (mg/L): thiamin hydrochloride, 100; *D*-pantothenic acid, 100; niacin, 100; pyridoxine, 100; *p*-aminobenzoic acid, 5; vitamin B₁₂, 0.25.

^dComposition (mg/L): EDTA (EDTA, disodium salt), 500; FeSO₄·7H₂O, 200; ZnSO₄·7H₂O, 10; MnCl₂·4H₂O, 3; H₃PO₄, 30; CoCl₂·6H₂O, 20; CuCl₂·2H₂O, 1; NiCl₂·6H₂O, 2; and Na₂MoO₄·2H₂O, 3.

^eComposition (mg/L): folic acid, 10; *D*-biotin, 2; NH₄HCO₃, 100.

^fHemin, 500 mg/L, in 10 mmol/L NaOH.

^gComposition: riboflavin, 10 mg/L, in 5 mmol/L of 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid, *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid) (HEPES).

^hResazurin, 1 g/L, in distilled H₂O.

ⁱContained 250 µL/L each of *n*-valerate, isovalerate, isobutyrate, and *DL*-α-methylbutyrate.

Table 6.6. Baseline microbiota from fecal samples of dogs fed the control (CNTL), starter kefir (S-Kefir), or grain kefir (G-kefir) treatments.

Phylum	Genus	CNTL	S-Kefir	G-Kefir
Fusobacteriota	<i>Cetobacterium</i>	3.06	2.13	2.84
	<i>Fusobacterium</i>	13.53	12.07	4.44
Proteobacteria	<i>Sutterella</i>	0.18	0.15	0.00
	<i>Parasutterella</i>	4.38	2.21	1.72
Actinobacteriota	<i>Adlercreutzia</i>	0.13	0.05	0.24
	<i>Atopobiaceae undf</i>	0.09	0.12	0.44
	<i>Bifidobacterium</i>	3.52	5.03	10.96
	<i>Collinsella</i>	0.19	0.25	0.32
	<i>Coriobacteriaceae UCG-002 undf</i>	0.98	2.33	1.08
Bacteroidota	<i>Alloprevotella</i>	0.13	0.17	0.03
	<i>Bacteroides</i>	3.69	2.32	0.55
	<i>Muribaculaceae undf</i>	5.26	4.89	4.34
	<i>Parabacteroides</i>	0.13	0.11	0.06
	<i>Prevotella</i>	0.43	0.94	0.17
	<i>Prevotellaceae Ga6A1 group</i>	1.00	1.04	0.24
	<i>Rikenellaceae undf</i>	0.58	0.44	0.00
Firmicutes	<i>Allobaculum</i>	6.15	5.45	20.90
	<i>Anaeroplasm</i>	0.19	0.06	0.00
	<i>Anaerovoracaceae undf</i>	0.56	0.48	0.22
	<i>Bacilli</i>	1.67	1.81	1.58
	<i>Blautia</i>	1.85	3.95	2.12
	<i>Clostridium sensu stricto 1</i>	0.54	0.33	2.24
	<i>Dubosiella</i>	2.50	6.31	4.68
	<i>Enterococcus</i>	1.88	1.03	0.52
	<i>Erysipelatoclostridium</i>	0.39	0.28	0.30
	<i>Erysipelotrichaceae undf</i>	9.94	8.05	8.09
	<i>Faecalibacterium</i>	0.34	0.11	0.06
	<i>Faecalibaculum</i>	1.80	1.71	2.73
	<i>Fournierella</i>	0.24	0.25	0.18
	<i>Holdemanella</i>	0.15	0.24	0.07
	<i>Lachnospiraceae undf</i>	8.20	6.97	5.46
	<i>Lactobacillus</i>	10.86	14.41	7.62

Table 6.6 (cont.)

<i>Lactococcus</i>	0.00	0.00	0.49
<i>Negativibacillus</i>	0.07	0.22	0.14
<i>Peptoclostridium</i>	2.91	3.55	4.56
<i>Peptococcus</i>	0.36	0.64	0.38
<i>Peptostreptococcus</i>	1.75	3.33	3.85
<i>Phascolarctobacterium</i>	0.08	0.22	0.06
<i>Romboutsia</i>	1.64	0.82	1.36
<i>Ruminococcus gauvreauii</i> group	0.51	0.47	0.38
<i>Ruminococcus gnavus</i> group	0.14	0.41	0.35
<i>Ruminococcus torques</i> group	2.69	1.88	1.20
<i>Streptococcus</i>	2.92	0.47	0.23
<i>Terrisporobacter</i>	0.11	0.00	0.31
<i>Turicibacter</i>	1.57	1.75	2.00

CNTL Control; 2% Reduced Fat Milk, Great Value Farm, Fort Wayne Indiana, USA

S-Kefir Starter kefir; Champions Choice Kefir; Champions Choice, Millersburg Indiana, USA

G-Kefir Grain Kefir; Kefir Garden Grains, Hamilton Ontario, Canada

undf; Undefined

Table 6.7. Bacterial phyla and genera relative abundances (% of sequences) during cellulose in vitro fermentation assays at 0h, 6h, 12h, and 18h using fecal inocula from dogs fed the control (CNTL), starter kefir (S-Kefir), or grain kefir (G-kefir) treatments.

Phyla	Genus	CNTL				S-Kefir				G-Kefir				SEM	p-values		
		0 h	6 h	12 h	18 h	0 h	6 h	12 h	18 h	0 h	6 h	12 h	18 h		Trt	Time	Trt*Time
Proteobacteria		2.93 ^b	4.27 ^b	6.48 ^a	7.48 ^a	2.09 ^b	7.01 ^a	7.12 ^a	7.24 ^a	2.87 ^b	3.01 ^b	2.41 ^b	3.84 ^b	0.314	<0.0001	<0.0001	<0.0001
	<i>Parasutterella</i>	2.83 ^b	2.60 ^b	1.94 ^b	1.55 ^{bc}	1.94 ^{bc}	4.25 ^a	2.21 ^b	1.28 ^{bc}	2.77 ^b	2.79 ^b	1.76 ^{bc}	1.88 ^b	0.194	0.7153	<0.0001	<0.0001
	<i>Sutterella</i>	0.07 ^d	1.67 ^c	4.54 ^b	5.94 ^a	0.10 ^d	2.76 ^c	4.91 ^b	5.95 ^a	0.02 ^d	0.23 ^d	0.65 ^d	1.9 ^{6c}	0.197	<0.0001	<0.0001	<0.0001
Fusobacteriota		9.74 ^c	40.91 ^{ab}	41.39 ^{ab}	38.60 ^b	12.90 ^c	46.64 ^a	46.38 ^a	37.37 ^b	11.32 ^c	36.78 ^b	37.44 ^b	35.35 ^b	1.332	<0.0001	<0.0001	0.0138
	<i>Cetobacterium</i>	1.50 ^c	0.41 ^d	0.41 ^d	0.51 ^d	3.04 ^b	0.63 ^d	0.65 ^d	0.87 ^{cd}	4.54 ^a	0.67 ^d	0.44 ^d	0.39 ^d	0.129	<0.0001	<0.0001	<0.0001
	<i>Fusobacterium</i>	8.24 ^c	40.50 ^{ab}	41.01 ^a	38.10 ^{ab}	9.868 ^c	46.01 ^a	45.72 ^a	36.50 ^b	6.78 ^c	36.11 ^{ab}	36.40 ^b	34.93 ^{ab}	1.312	<0.0001	<0.0001	0.0323
Firmicutes		76.45 ^a	44.56 ^c	38.80 ^{cd}	41.96 ^{cd}	68.01 ^b	35.06 ^d	33.34 ^d	43.42 ^c	65.96 ^b	48.26 ^c	48.13 ^c	47.57 ^c	1.553	<0.0001	<0.0001	<0.0001
	<i>Allobaculum</i>	8.25 ^c	3.75 ^d	3.76 ^d	3.27 ^d	5.92 ^{dc}	3.15 ^d	3.06 ^d	3.19 ^d	16.36 ^a	10.95 ^b	9.48 ^{bc}	10.36 ^b	0.301	<0.0001	<0.0001	<0.0001
	<i>Bacilli undf</i>	2.44 ^a	1.05 ^{bc}	1.82 ^{ab}	1.92 ^{ab}	2.18 ^a	0.78 ^c	0.86 ^{bc}	0.85 ^{bc}	1.77 ^{ab}	1.13 ^{bc}	0.89 ^{bc}	0.65 ^c	0.142	<0.0001	<0.0001	0.0017
	<i>Blautia</i>	1.99 ^b	1.25 ^{bc}	0.78 ^c	0.89 ^c	4.21 ^a	2.14 ^b	1.92 ^b	2.39 ^b	1.89 ^b	1.12 ^{bc}	1.50 ^{bc}	1.69 ^{bc}	0.109	<0.0001	<0.0001	<0.0001
	<i>Dubosiella</i>	2.83 ^b	1.47 ^c	1.23 ^c	1.25 ^c	7.15 ^a	2.70 ^b	2.40 ^b	2.27 ^b	6.43 ^a	3.36 ^b	2.96 ^b	2.46 ^b	0.149	<0.0001	<0.0001	0.0025
	<i>Enterococcus</i>	1.75 ^a	1.39 ^a	1.83 ^a	1.84 ^a	1.42 ^a	0.76 ^b	0.62 ^b	0.66 ^b	0.63 ^b	0.62 ^b	0.74 ^b	0.59 ^b	0.099	<0.0001	0.0028	0.0004
	<i>Faecalibaculum</i>	2.47 ^a	1.49 ^{ab}	1.36 ^{ab}	1.55 ^{ab}	2.11 ^{ab}	1.15 ^b	1.37 ^{ab}	1.86 ^a	2.17 ^a	1.96 ^a	2.60 ^a	2.21 ^a	0.138	<0.0001	<0.0001	0.0003
	<i>Lachnospiraceae undf</i>	7.07 ^a	4.20 ^{bc}	4.65 ^b	6.39 ^a	6.29 ^a	4.03 ^{bc}	3.48 ^{bc}	5.14 ^b	4.35 ^b	3.01 ^c	3.76 ^{bc}	4.78 ^b	0.189	<0.0001	<0.0001	0.0006
	<i>Lactobacillus</i>	15.87 ^a	7.24 ^{bc}	6.21 ^b	5.43 ^b	16.90 ^a	7.17 ^{bc}	5.48 ^b	6.08 ^b	9.97 ^c	4.99 ^b	4.59 ^b	3.50 ^b	0.678	<0.0001	<0.0001	0.0052
	<i>Peptoclostridium</i>	3.45 ^c	7.57 ^{ab}	4.61 ^{bc}	6.95 ^{ab}	2.25 ^c	4.06 ^{bc}	2.94 ^c	5.77 ^{bc}	2.55 ^c	9.37 ^a	7.56 ^{ab}	6.21 ^b	0.514	<0.0001	<0.0001	0.0001
	<i>Peptostreptococcus</i>	1.24 ^d	1.15 ^d	0.97 ^d	0.95 ^d	2.76 ^c	1.13 ^d	2.43 ^c	4.18 ^b	3.83 ^b	2.77 ^c	5.90 ^a	5.89 ^a	0.181	<0.0001	<0.0001	<0.0001
	<i>Ruminococcus torques</i>	2.30 ^b	1.27 ^{bc}	1.36 ^{bc}	1.73 ^{bc}	1.87 ^b	1.77 ^b	2.84 ^{ab}	3.65 ^a	1.18 ^{bc}	0.80 ^{cd}	0.86 ^{cd}	0.87 ^{cd}	0.178	<0.0001	<0.0001	<0.0001
	<i>Streptococcus uncultured</i>	6.47 ^a	3.67 ^b	2.92 ^b	3.04 ^b	0.72 ^c	0.81 ^c	0.85 ^c	1.42 ^{bc}	0.24 ^c	0.46 ^c	0.92 ^c	0.70 ^c	0.334	<0.0001	0.0023	<0.0001
		10.97 ^a	3.85 ^{cd}	2.68 ^{cd}	1.67 ^{cd}	8.40 ^b	2.57 ^{cd}	1.96 ^{cd}	1.33 ^d	8.33 ^b	3.49 ^c	2.16 ^{cd}	2.05 ^{cd}	0.330	<0.0001	<0.0001	0.0182
		5.45 ^c	7.66 ^{bc}	11.64 ^a	10.38 ^{ab}	8.31 ^{bc}	8.90 ^{ab}	10.05 ^{ab}	7.91 ^{bc}	9.37 ^{ab}	6.72 ^{bc}	6.45 ^{bc}	7.75 ^{bc}	0.624	0.0151	0.0081	<0.0001
	<i>Bacteroides</i>	1.95 ^{cd}	4.47 ^b	6.99 ^a	6.31 ^a	2.32 ^c	4.02 ^{bc}	5.15 ^{ab}	4.36 ^{bc}	1.33 ^{cd}	2.45 ^c	2.88 ^c	3.49 ^{bc}	0.292	<0.0001	<0.0001	0.0001
	<i>Muribaculaceae undf</i>	2.56 ^b	1.91 ^{bc}	2.46 ^b	2.08 ^b	3.38 ^b	2.13 ^b	2.03 ^b	1.48 ^{bc}	6.82 ^a	2.97 ^b	2.08 ^b	2.33 ^b	0.287	<0.0001	<0.0001	<0.0001
Actinobacteria		5.40 ^b	2.53 ^{bc}	1.51 ^b	1.42 ^c	8.27 ^a	2.31 ^{bc}	2.96 ^{bc}	3.92 ^{bc}	10.21 ^a	5.19 ^b	5.46 ^b	5.37 ^b	0.490	<0.0001	<0.0001	0.0859
	<i>Bifidobacteria</i>	4.42	1.98	1.27	1.16	6.09	1.68	2.08	3.03	9.13	4.61	4.87	4.65	0.408	<0.0001	<0.0001	0.085
	<i>Coriobacteriaceae undf</i>	0.41 ^c	0.21 ^c	0.13 ^c	0.11 ^c	1.30 ^a	0.52 ^{bc}	0.75 ^b	0.71 ^b	0.52 ^{bc}	0.32 ^c	0.25 ^c	0.28 ^c	0.069	<0.0001	0.035	0.0006

^{a-d}Means in the same row with different superscript letters are different (P<0.05).

undf; Undefine

Table 6.8. Bacterial phyla and genera relative abundances (% of sequences) during pectin in vitro fermentation assays at 0h, 6h, 12h, and 18h using fecal inocula from dogs fed the control (CNTL), starter kefir (S-Kefir), or grain kefir (G-kefir) treatments.

Phyla	Genus	CNTL				S-Kefir				G-Kefir				SEM	p-values		
		0 h	6 h	12 h	18 h	0 h	6 h	12 h	18 h	0 h	6 h	12 h	18 h		Trt	Time	Trt*Time
Proteobacteria		2.11 ^b	1.37 ^b	2.51 ^b	2.61 ^{ab}	1.28 ^b	2.06 ^b	3.92 ^a	2.79 ^{ab}	2.19 ^b	0.92 ^b	1.60 ^b	1.92 ^b	0.276	0.0009	<0.0001	0.0005
	<i>Parasutterella</i>	2.04 ^a	1.00 ^b	1.12 ^b	0.69 ^b	1.21 ^b	1.26 ^b	1.02 ^b	0.49 ^b	2.14 ^a	0.87 ^b	0.99 ^b	0.74 ^b	0.201	0.1449	<0.0001	0.0170
	<i>Sutterella</i>	0.03 ^c	0.36 ^{bc}	1.38 ^b	1.91 ^{ab}	0.04 ^c	0.79 ^b	2.90 ^a	2.30 ^a	0.01 ^c	0.06 ^c	0.61 ^{bc}	1.17 ^b	0.139	<0.0001	<0.0001	0.0002
Fusobacteriota		9.88 ^c	23.35 ^a	17.53 ^b	14.34 ^{bc}	11.37 ^c	24.28 ^a	23.75 ^a	14.34 ^{bc}	10.75 ^c	19.88 ^{ab}	16.45 ^{bc}	13.02 ^c	0.862	<0.0001	<0.0001	0.0033
	<i>Fusobacterium</i>	8.26 ^c	23.07 ^a	17.33 ^b	14.13 ^b	8.87 ^{bc}	23.77 ^a	23.37 ^a	14.08 ^b	6.35 ^c	19.42 ^{ab}	16.11 ^b	12.7 ^b	0.796	<0.0001	<0.0001	0.0054
Firmicutes		77.98 ^a	63.35 ^b	62.64 ^b	64.04 ^b	71.99 ^{ab}	55.60 ^{bc}	47.38 ^c	51.62 ^c	68.75 ^b	67.36 ^{bc}	59.41 ^{bc}	52.85 ^c	1.577	<0.0001	<0.0001	<0.0001
	<i>Allobaculum</i>	8.54 ^b	3.00 ^c	2.91 ^c	2.78 ^c	7.22 ^b	2.61 ^c	2.74 ^c	3.96 ^c	16.94 ^a	7.19 ^b	7.81 ^b	7.59 ^b	0.465	0.0005	0.0046	0.0006
	<i>Bacilli undf</i>	2.54 ^a	1.73 ^b	1.42 ^{bc}	1.74 ^b	2.30 ^{ab}	1.16 ^{bc}	0.80 ^c	0.85 ^c	1.94 ^b	1.46 ^{bc}	0.89 ^c	0.88 ^c	0.113	<0.0001	<0.0001	0.0365
	<i>Blautia</i>	1.62 ^{bc}	1.05 ^c	1.01 ^c	0.74 ^c	3.28 ^a	2.01 ^{bc}	1.48 ^c	2.09 ^b	1.83 ^{bc}	1.12 ^c	1.36 ^c	1.36 ^c	0.108	<0.0001	<0.0001	<0.0001
	<i>Dubosiella</i>	3.69 ^d	2.02 ^e	2.15 ^e	2.28 ^e	7.55 ^a	5.60 ^b	3.86 ^d	3.75 ^d	7.08 ^a	4.93 ^c	3.85 ^d	3.46 ^d	0.200	<0.0001	<0.0001	0.0002
	<i>Enterococcus</i>	2.77 ^d	7.54 ^b	5.48 ^c	9.76 ^a	1.75 ^d	3.63 ^d	2.47 ^d	2.28 ^d	0.79 ^d	2.16 ^d	1.68 ^d	1.41 ^d	0.311	<0.0001	<0.0001	<0.0001
	<i>Faecalibacterium</i>	0.29 ^d	1.13 ^c	2.96 ^b	5.34 ^a	0.09 ^d	0.39 ^d	1.00 ^{cd}	1.64 ^c	0.12 ^d	1.09 ^c	2.62 ^b	3.05 ^b	0.130	<0.0001	<0.0001	<0.0001
	<i>Faecalibaculum</i>	2.91 ^{ab}	2.23 ^{bc}	1.99 ^{bc}	1.83 ^{bc}	1.84 ^{bc}	1.56 ^c	1.47 ^c	2.13 ^{bc}	2.38 ^b	2.84 ^{ab}	3.15 ^{ab}	3.16 ^a	0.152	<0.0001	0.3093	<0.0001
	<i>Lachnospiraceae undf</i>	8.21 ^a	4.91 ^b	4.38 ^{bc}	7.23 ^a	6.92 ^a	4.01 ^{bc}	3.03 ^c	4.43 ^{bc}	5.18 ^b	4.80 ^b	3.92 ^{bc}	3.09 ^c	0.330	<0.0001	<0.0001	<0.0001
	<i>Lactobacillus</i>	17.38 ^c	10.71 ^{de}	11.24 ^d	6.16 ^e	22.25 ^b	18.88 ^{bc}	11.91 ^d	14.28 ^{cd}	10.18 ^{de}	26.96 ^a	19.10 ^{bc}	15.29 ^{cd}	0.903	<0.0001	<0.0001	<0.0001
	<i>Peptoclostridium</i>	3.08 ^b	2.98 ^b	1.79 ^b	2.05 ^b	2.26 ^b	1.81 ^b	1.49 ^b	2.34 ^b	3.08 ^b	4.27 ^a	2.99 ^b	2.23 ^b	0.294	<0.0001	0.0007	0.0039
	<i>Peptostreptococcus</i>	4.19 ^{bc}	2.74 ^c	2.42 ^c	2.39 ^c	5.26 ^b	3.14 ^c	2.08 ^c	2.68 ^c	8.28 ^a	4.57 ^{bc}	3.81 ^c	3.67 ^c	0.605	<0.0001	0.0026	0.1052
	<i>Ruminococcus torques group</i>	2.01 ^{cd}	1.69 ^{cd}	2.08 ^{cd}	2.63 ^c	1.64 ^d	3.06 ^{bc}	4.42 ^a	2.46 ^{cd}	1.19 ^d	0.99 ^d	1.16 ^d	1.23 ^d	0.198	<0.0001	<0.0001	<0.0001
	<i>Streptococcus</i>	8.00 ^b	16.84 ^a	16.56 ^a	15.25 ^a	0.82 ^c	3.48 ^{bc}	2.08 ^{bc}	3.01 ^{bc}	0.32 ^c	1.52 ^c	1.24 ^c	1.14 ^c	0.899	<0.0001	<0.0001	0.0021
	<i>uncultured</i>	8.33 ^a	3.07 ^c	2.98 ^c	2.22 ^c	5.79 ^b	3.04 ^c	2.39 ^{bc}	1.73 ^c	6.56 ^b	2.33 ^c	2.48 ^c	1.82 ^c	0.217	<0.0001	<0.0001	0.0002
Bacteroidota		4.51 ^d	8.49 ^d	14.43 ^c	16.31 ^c	7.05 ^d	14.00 ^c	22.39 ^b	27.92 ^a	7.75 ^d	6.39 ^d	18.14 ^{bc}	28.08 ^a	1.052	<0.0001	<0.0001	<0.0001
	<i>Bacteroides</i>	1.51 ^c	4.76 ^{bc}	8.27 ^{ab}	8.71 ^a	1.72 ^c	4.92 ^b	8.24 ^{ab}	5.65 ^b	0.91 ^c	2.21 ^{bc}	4.94 ^b	4.99 ^b	0.597	<0.0001	<0.0001	0.0278
	<i>Muribaculaceae undf</i>	2.21 ^c	2.24 ^c	1.94 ^c	1.61 ^c	3.14 ^{bc}	3.68 ^b	2.26 ^c	1.23 ^c	5.74 ^a	2.17 ^c	1.98 ^c	1.19 ^c	0.250	0.0012	<0.0001	<0.0001
	<i>Prevotella</i>	0.25 ^d	1.10 ^{cd}	3.73 ^{cd}	5.44 ^c	0.75 ^{cd}	4.75 ^c	11.13 ^b	20.65 ^a	0.50 ^{cd}	1.81 ^{cd}	10.85 ^b	21.61 ^a	0.726	<0.0001	<0.0001	<0.0001
Actinobacteriota		5.52 ^c	3.43 ^d	2.88 ^d	2.69 ^d	8.27 ^b	4.02 ^{cd}	2.56 ^d	3.32 ^d	10.56 ^a	5.45 ^c	4.39 ^{cd}	4.13 ^{cd}	0.292	<0.0001	<0.0001	<0.0001
	<i>Bifidobacterium</i>	4.19 ^{bc}	2.74 ^c	2.42 ^c	2.39 ^c	5.26 ^b	3.14 ^c	2.08 ^c	2.68 ^c	8.28 ^a	4.57 ^{bc}	3.81 ^c	3.67 ^c	0.259	<0.0001	<0.0001	0.0002

^{a-e}Means in the same row with different superscript letters are different (P<0.05).

undf; Undefined

Table 6.9. Bacterial phyla and genera relative abundances (% of sequences) during beet pulp in vitro fermentation assays at 0h, 6h, 12h, and 18h using fecal inocula from dogs fed the control (CNTL), starter kefir (S-Kefir), or grain kefir (G-kefir) treatments.

Phyla	Genus	CNTL				S-Kefir				G-Kefir				SEM	p-values		
		0 h	6 h	12 h	18 h	0 h	6 h	12 h	18 h	0 h	6 h	12 h	18 h		Trt	Time	Trt*Time
Proteobacteria		4.53 ^a	2.52 ^b	3.00 ^b	2.73 ^b	2.30 ^b	2.89 ^b	2.20 ^{bc}	2.81 ^b	3.09 ^b	1.98 ^{bc}	1.33 ^c	1.50 ^c	0.155	<0.0001	<0.0001	<0.0001
	<i>Parasutterella</i>	4.29 ^a	1.61 ^{cd}	1.43 ^{cd}	0.74 ^d	2.12 ^c	1.89 ^c	1.12 ^d	0.73 ^d	2.98 ^b	1.81 ^{cd}	0.98 ^d	0.75 ^d	0.138	0.9009	<0.0001	0.0006
	<i>Sutterella</i>	0.16 ^d	0.88 ^c	1.56 ^b	1.97 ^{ab}	0.13 ^d	1.00 ^c	1.07 ^c	2.08 ^a	0.04 ^d	0.17 ^d	0.35 ^d	0.74 ^{cd}	0.083	<0.0001	<0.0001	<0.0001
Fusobacteriota		15.82 ^{bc}	27.49 ^a	23.83 ^{ab}	16.93 ^{bc}	14.36 ^c	28.56 ^a	20.76 ^{bc}	21.46 ^b	11.31 ^c	31.67 ^a	21.96 ^b	19.86 ^{bc}	1.046	0.9336	<0.0001	0.0012
	<i>Cetobacterium</i>	3.21 ^b	0.24 ^c	0.11 ^c	0.19 ^c	2.83 ^b	0.38 ^c	0.28 ^c	0.25 ^c	4.11 ^a	0.54 ^c	0.25 ^c	0.17 ^c	0.083	<0.0001	<0.0001	<0.0001
	<i>Fusobacterium</i>	12.59 ^{bc}	27.26 ^{ab}	23.72 ^{ab}	16.73 ^b	11.51 ^{bc}	28.18 ^a	20.48 ^b	21.21 ^b	7.20 ^c	31.12 ^a	21.71 ^b	19.69 ^b	1.034	0.8468	<0.0001	0.0005
Firmicutes		64.42 ^a	54.49 ^b	40.90 ^c	49.93 ^{bc}	67.51 ^a	51.54 ^b	52.57 ^b	42.09 ^c	66.41 ^a	51.37 ^b	43.67 ^{bc}	40.10 ^c	1.746	0.0611	<0.0001	0.0003
	<i>Allobaculum</i>	7.86 ^{bc}	3.33 ^d	2.28 ^d	1.80 ^d	5.86 ^c	2.92 ^d	2.43 ^d	2.12 ^d	18.70 ^a	6.79 ^c	4.75 ^c	3.72 ^{cd}	0.248	<0.0001	<0.0001	<0.0001
	<i>Bacilli</i> undf	2.01 ^b	1.36 ^c	0.62 ^d	1.21 ^c	2.62 ^a	0.81 ^d	0.65 ^d	0.48 ^d	1.52 ^c	0.74 ^d	0.40 ^d	0.32 ^d	0.076	<0.0001	<0.0001	<0.0001
	<i>Blautia</i>	1.61 ^c	1.65 ^c	1.61 ^c	1.56 ^c	3.61 ^{ab}	3.31 ^b	4.05 ^a	2.94 ^b	1.79 ^c	1.52 ^c	1.66 ^c	1.84 ^c	0.126	<0.0001	0.0137	0.001
	<i>Dubosiella</i>	2.74 ^c	1.49 ^d	1.14 ^d	1.17 ^d	5.61 ^a	3.22 ^c	4.18 ^b	2.16 ^c	6.30 ^a	4.15 ^b	2.38 ^c	2.19 ^c	0.135	<0.0001	<0.0001	<0.0001
	<i>Enterococcus</i>	2.62 ^a	1.14 ^c	0.71 ^c	1.84 ^b	1.61 ^b	0.95 ^c	0.71 ^c	0.55 ^c	0.65 ^c	0.51 ^c	0.57 ^c	0.42 ^c	0.068	<0.0001	<0.0001	<0.0001
	<i>Faecalibacterium</i>	0.30 ^c	3.33 ^{ab}	4.25 ^{ab}	5.86 ^a	0.12 ^c	1.06 ^{bc}	1.74 ^{bc}	3.10 ^{ab}	0.10 ^c	1.80 ^{bc}	4.68 ^a	5.45 ^a	0.453	0.0670	0.0002	0.0006
	<i>Faecalibaculum</i>	1.77 ^{ab}	2.12 ^{ab}	1.21 ^b	1.83 ^{ab}	2.04 ^{ab}	1.76 ^{ab}	2.02 ^{ab}	1.64 ^b	2.11 ^{ab}	2.48 ^{ab}	2.65 ^a	2.07 ^{ab}	0.186	0.0004	0.3847	0.0181
	<i>Lachnospiraceae</i> undf	6.44 ^{bc}	5.79 ^c	5.94 ^c	7.77 ^{ab}	6.12 ^{bc}	5.13 ^c	8.42 ^a	5.84 ^c	4.31 ^c	4.10 ^c	5.36 ^{7c}	5.04 ^c	0.362	<0.0001	<0.0001	0.0003
	<i>Lactobacillus</i>	13.91 ^{ab}	8.97 ^{bc}	8.50 ^{bc}	4.73 ^c	17.36 ^a	16.40 ^{ab}	11.27 ^b	6.74 ^c	8.45 ^{bc}	13.60 ^{ab}	8.40 ^{bc}	6.93 ^c	0.847	<0.0001	<0.0001	<0.0001
	<i>Peptoclostridium</i>	1.66 ^{bc}	3.46 ^{ab}	1.02 ^c	2.45 ^{bc}	1.70 ^{bc}	2.45 ^{bc}	2.00 ^{bc}	1.70 ^{bc}	2.36 ^b	4.34 ^a	2.66 ^b	2.07 ^b	0.297	0.0006	<0.0001	0.0098
	<i>Peptostreptococcus</i>	1.14 ^b	0.64 ^b	0.61 ^b	0.89 ^b	2.25 ^b	1.38 ^b	1.74 ^b	2.51 ^{ab}	3.50 ^a	2.07 ^b	3.31 ^a	3.31 ^a	0.196	<0.0001	<0.0001	0.041
	<i>Ruminococcus torques</i> group	2.19 ^c	3.28 ^c	3.45 ^{bc}	6.41 ^a	2.11 ^c	4.52 ^{bc}	5.77 ^{ab}	7.05 ^a	1.31 ^c	1.74 ^c	1.88 ^c	2.48 ^c	0.357	<0.0001	<0.0001	0.0014
	<i>Streptococcus uncultured</i>	3.54 ^{bc}	9.46 ^a	3.85 ^b	9.17 ^a	0.63 ^c	1.92 ^c	2.41 ^{bc}	2.13 ^{bc}	0.22 ^c	0.90 ^c	0.93 ^c	0.88 ^c	0.357	<0.0001	<0.0001	<0.0001
		11.44 ^a	4.11 ^b	1.66 ^c	0.91 ^c	10.27 ^a	2.69 ^{bc}	1.96 ^{bc}	0.91 ^c	9.95 ^a	3.20 ^{bc}	1.34 ^c	0.94 ^c	0.302	0.8651	<0.0001	0.0005
Bacteroidota		10.64 ^d	12.90 ^d	30.89 ^a	28.01 ^{ab}	9.02 ^d	14.06 ^d	21.13 ^c	30.60 ^{ab}	10.42 ^d	10.99 ^d	29.00 ^{ab}	35.31 ^a	1.088	0.0051	<0.0001	<0.0001
	<i>Bacteroides</i>	3.83 ^{bc}	9.57 ^b	21.90 ^a	17.32 ^a	2.79 ^{bc}	7.52 ^b	7.95 ^b	15.24 ^a	1.80 ^c	5.75 ^{bc}	8.66 ^b	15.00 ^a	0.796	<0.0001	<0.0001	<0.0001
	<i>Muribaculaceae</i> undf	4.67 ^b	1.95 ^d	4.35 ^{bc}	2.53 ^{cd}	3.66 ^{bc}	2.28 ^{cd}	1.22 ^d	1.69 ^d	7.24 ^a	3.00 ^{cd}	4.40 ^{bc}	3.58 ^{bc}	0.278	<0.0001	<0.0001	<0.0001
	<i>Prevotella</i>	0.44 ^e	0.63 ^e	2.83 ^{de}	7.24 ^c	0.88 ^e	3.19 ^{de}	11.39 ^b	12.91 ^{ab}	0.61 ^e	1.88 ^{de}	15.46 ^a	16.14 ^a	0.649	<0.0001	<0.0001	<0.0001
Actinobacteriota		4.57 ^{ab}	2.58 ^{bc}	1.38 ^c	2.37 ^{bc}	6.71 ^a	2.94 ^{bc}	3.32 ^{bc}	3.04 ^{bc}	8.75 ^a	3.98 ^b	4.00 ^b	3.22 ^{bc}	0.422	<0.0001	<0.0001	0.0147
	<i>Bifidobacterium</i>	3.77 ^b	2.23 ^b	1.16 ^{bc}	1.96 ^b	5.14 ^b	2.32 ^b	2.53 ^b	2.41 ^b	7.93 ^a	3.54 ^b	3.60 ^b	2.89 ^b	0.373	<0.0001	<0.0001	0.0052

^{a-e}Means in the same row with different superscript letters are different (P<0.05).

undf; Undefined

Table 6.10. Bacterial phyla and genera relative abundances (% of sequences) during chicory pulp in vitro fermentation assays at 0h, 6h, 12h, and 18h using fecal inocula from dogs fed the control (CNTL), starter kefir (S-Kefir), or grain kefir (G-kefir) treatments.

Phyla	Genera	CNTL				S-Kefir				G-Kefir				SEM	p-values		
		0 h	6 h	12 h	18 h	0 h	6 h	12 h	18 h	0 h	6 h	12 h	18 h		Trt	Time	Trt*Time
Proteobacteria		3.78 ^{abc}	3.78 ^{abc}	4.72 ^b	6.40 ^a	2.07 ^c	4.57 ^a	4.95 ^{ab}	6.02 ^{ab}	3.38 ^{bc}	2.85 ^{bc}	2.77 ^{bc}	4.17 ^b	0.377	<0.0001	<0.0001	0.0013
	<i>Parasutterella</i>	3.60 ^a	2.18 ^{ab}	1.62 ^b	1.38 ^b	1.94 ^b	2.75 ^{ab}	1.63 ^b	1.14 ^b	3.28 ^a	2.60 ^{ab}	1.59 ^b	1.43 ^b	0.217	0.0490	<0.0001	0.0016
	<i>Sutterella</i>	0.10 ^c	1.57 ^c	3.08 ^b	4.98 ^a	0.09 ^c	1.82 ^{bc}	3.32 ^b	4.89 ^a	0.02 ^c	0.24 ^c	1.17 ^c	2.74 ^b	0.288	<0.0001	<0.0001	0.0049
Fusobacteriota		15.28	38.70	36.22	37.01	13.93	35.65	33.37	39.26	12.46	38.38	35.62	34.39	1.270	0.1963	<0.0001	0.0902
	<i>Fusobacterium</i>	12.59 ^b	38.46 ^a	36.06 ^a	36.83 ^a	11.47 ^b	35.27 ^a	33.0 ^a	38.97 ^a	8.07 ^b	37.95 ^a	35.40 ^a	34.18 ^a	1.233	0.0730	<0.0001	0.0384
Firmicutes		68.42	42.00	42.39	39.26	68.86	43.29	45.24	37.22	64.55	42.96	43.83	42.31	2.279	0.9231	<0.0001	0.5012
	<i>Blautia</i>	1.60 ^c	1.11 ^c	1.29 ^c	1.00 ^c	4.17 ^a	2.32 ^{bc}	2.57 ^b	2.25 ^{bc}	2.02 ^{bc}	1.53 ^c	1.49 ^c	1.43 ^c	0.120	<0.0001	<0.0001	<0.0001
	<i>Dubosiella</i>	2.88 ^{bc}	1.60 ^b	1.80 ^b	1.53 ^{bc}	5.94 ^a	3.94 ^b	3.36 ^b	2.51 ^{bc}	6.76 ^a	3.77 ^b	2.92 ^{bc}	2.40 ^{bc}	0.284	<0.0001	<0.0001	0.0021
	<i>Faecalibacterium</i>	0.25 ^c	3.24 ^b	4.66 ^b	5.08 ^b	0.12 ^c	0.88 ^{bc}	2.26 ^{bc}	3.14 ^b	0.09 ^c	1.89 ^{bc}	6.27 ^b	9.36 ^a	0.480	<0.0001	<0.0001	<0.0001
	<i>Lachnospiraceae undf</i>	7.61 ^a	3.82 ^b	4.78 ^b	5.04 ^b	6.55 ^{ab}	5.36 ^b	5.27 ^b	4.84 ^b	4.20 ^b	4.10 ^b	3.76 ^b	3.29 ^b	0.322	<0.0001	<0.0001	0.0005
	<i>Lactobacillus</i>	12.98 ^b	6.12 ^c	5.12 ^c	3.39 ^c	17.46 ^a	10.68 ^{bc}	8.07 ^c	4.96 ^c	7.94 ^c	7.32 ^c	4.74 ^c	4.48 ^c	0.648	<0.0001	<0.0001	<0.0001
	<i>Peptostreptococcus</i>	1.47 ^c	0.43 ^d	0.70 ^d	0.56 ^d	2.71 ^b	1.00 ^{cd}	1.34 ^{cd}	1.67 ^c	3.85 ^a	1.41 ^c	2.78 ^b	1.97 ^c	0.097	<0.0001	<0.0001	<0.0001
	<i>Ruminococcus torques group</i>	2.13 ^{bc}	1.23 ^{cd}	1.29 ^{dc}	2.10 ^c	1.95 ^c	1.50 ^c	3.83 ^a	2.72 ^b	1.33 ^{cd}	0.84 ^d	0.83 ^d	0.79 ^d	0.120	<0.0001	<0.0001	<0.0001
Bacteroidota		7.70	14.30	15.37	16.48	6.92	14.15	13.89	15.08	9.83	12.43	14.83	16.63	1.068	0.3762	<0.0001	0.4398
	<i>Prevotella</i>	0.31 ^c	1.04 ^c	1.94 ^{bc}	1.41 ^c	0.72 ^c	4.11 ^b	3.70 ^b	3.50 ^b	0.46 ^c	3.31 ^b	4.39 ^{ab}	5.56 ^a	0.277	<0.0001	<0.0001	<0.0001
	<i>Muribaculaceae undf</i>	3.51 ^b	2.10 ^b	2.41 ^b	2.92 ^b	3.04 ^b	1.94 ^b	1.82 ^b	1.94 ^b	7.51 ^a	3.27 ^b	2.97 ^b	2.64 ^b	0.450	<0.0001	<0.0001	0.0167
Actinobacteriota		4.83 ^c	1.22 ^e	1.30 ^e	0.85 ^e	8.16 ^b	2.31 ^{de}	2.53 ^{de}	2.41 ^{de}	9.76 ^a	3.39 ^d	2.95 ^d	2.49 ^{de}	0.279	<0.0001	<0.0001	<0.0001
	<i>Bifidobacterium</i>	3.94 ^c	0.89 ^e	0.82 ^e	0.52 ^e	5.90 ^b	1.50 ^{de}	1.67 ^{de}	1.60 ^{de}	8.59 ^a	2.93 ^{cd}	2.44 ^d	2.03 ^d	0.212	<0.0001	<0.0001	0.0653

^{a-e} Means in the same row with different superscript letters are different (P<0.05)

undf; Undefined

Table 6.11. Change in bacterial phyla and genera relative abundances (% of sequences) from baseline (0h) % abundance

Fiber	Phylum/Family/Species	S-Kefir			G-Kefir			SEM	p-values		
		6	12	18	6	12	18		Trt	Time	Trt*Time
Cellulose	Firmicutes	-0.09 ^{abc}	-5.80 ^{bc}	-17.53 ^c	-8.82 ^c	1.56 ^a	1.52 ^{abc}	3.312	0.0562	0.2359	0.0045
	<i>Lactobacillus kefiranofaciens</i>	0.00	0.00	0.00	20.76	-18.8	-18.83	11.778	0.0045*	0.3528*	0.0318*
	<i>Lactobacillus spp.</i>	4.89	1.34	1.02	15.31	-27.7	-27.73	11.504	<0.0001	0.0089	0.0219*
	<i>Limosilactobacillus fermentum</i>	0.97	1.41	1.23	0.59	0.02	0.08	0.328	0.0034	0.9324	0.3069
	<i>Romboutsia</i>	-0.01	-0.02	-0.02	1.13	-0.02	-0.02	0.309	0.0373	0.0400	0.0344
	Proteobacteria	0.16 ^y	5.87 ^{zy}	17.61 ^z	6.41 ^{zy}	-1.14 ^y	-1.14 ^y	3.052	0.0092*	0.8844*	0.0423*
	<i>Hafnia-Obesumbacterium undf</i>	0.05	3.38	10.33	0.13	0.01	0.00	0.695	<0.0001	<0.0001	<0.0001
	<i>Yersiniaceae undf</i>	0.02	2.6	7.37	0.01	0.01	0.02	0.414	<0.0001	<0.0001	<0.0001
Pectin	Firmicutes	-0.05 ^{ab}	-0.37 ^b	-2.93 ^b	-0.80 ^{ab}	1.46 ^a	1.46 ^a	0.872	0.0249	0.3400	0.0383
	<i>Enterococcus</i>	1.71	9.08	17.48	-0.16	-3.86	-3.03	2.264	<0.0001	0.0389	0.0049
	<i>Lactobacillus kefiranofaciens</i>	0.00 ^{zy}	0.00 ^{zy}	0.00 ^{zy}	7.74 ^z	-10.39 ^y	-10.41 ^y	3.742	0.0407*	0.2213*	0.0276*
	<i>Lactobacillus spp.</i>	9.21	17.72	14.49	4.99	-15.48	-15.05	3.346	<0.0001	0.1041	0.0019
	<i>Leuconostoc</i>	0.18	0.14	0.14	0.06	0.00	0.00	0.054	0.0014*	0.5557*	0.0434*
	<i>Limosilactobacillus fermentum</i>	2.72 ^b	11.11 ^a	8.29 ^a	0.20 ^b	-0.55 ^b	-0.28 ^b	0.97	<0.0001	0.0061	0.0016
	Proteobacteria	0.08 ^{ab}	0.34 ^{ab}	2.91 ^a	-0.09 ^{ab}	-0.62 ^{ab}	-0.62 ^b	0.599	0.0079	0.1016	0.0395
	<i>Hafnia-Obesumbacterium undf</i>	0.01	0.58	1.85	-0.03	-0.12	-0.13	0.304	0.0054	0.1840	0.0352
Chicory Pulp	<i>Yersiniaceae undf</i>	-0.01	0.38	1.31	-0.01	-0.01	0.00	0.198	0.0235*	0.0431*	0.0313*
	Firmicutes	0.02 ^{ab}	-2.77 ^{ab}	-12.18 ^b	5.33 ^a	4.54 ^a	9.87 ^a	1.993	<0.0001	0.1997	0.0023
	<i>Clostridium sensu stricto 1</i>	0.03	7.76	41.04	0.93	41.25	22.4	8.801	0.2272	<0.0001	0.0178
	<i>Lactobacillus kefiranofaciens</i>	0.00 ^z	0.00 ^z	0.00 ^z	-4.86 ^{zy}	-16.06 ^y	-16.05 ^y	2.662	0.0048*	0.4132*	0.0336*
	<i>Lactobacillus spp.</i>	4.88	3.23	1.98	1.03	-18.34	-17.78	2.907	<0.0001	0.0041	0.0189
	<i>Limosilactobacillus fermentum</i>	1.51	1.28	1.00	0.31	0.02	0.51	0.202	<0.0001	0.4676	0.1501
	<i>Paenibacillus</i>	0.00	0.02	0.53	2.74	0.14	3.23	0.617	<0.0001	<0.0001	0.0022
	<i>P. Barengoltzii</i>	0.01 ^{ab}	0.16 ^{ab}	0.00 ^b	0.09 ^{ab}	0.65 ^a	0.00 ^c	0.057	0.0038	<0.0001	0.0342
Beet Pulp	<i>Romboutsia</i>	0.00	0.00	0.06	0.00	0.00	0.34	0.096	0.8669	0.0018	0.0261
	<i>Streptococcus</i>	-0.83	-0.58	-0.31	0.00	2.69	0.00	1.089	0.0035	0.2844	0.0283
	Proteobacteria	0.07 ^b	2.81 ^b	12.28 ^a	-1.99 ^{bc}	-1.29 ^{bc}	-3.29 ^{bc}	0.823	<0.0001	<0.0001	<0.0001
	<i>Hafnia-Obesumbacterium undf</i>	0.01	1.43	6.13	0.00	0.00	0.01	0.319	0.0022	0.1946	0.0177
	<i>Yersiniaceae undf</i>	0.02	1.52	6.23	0.00	0.01	0.00	0.271	<0.0001	<0.0001	<0.0001
	Firmicutes	0.25 ^{ab}	-3.03 ^b	-11.87 ^c	-7.68 ^{bc}	2.35 ^a	2.42 ^a	1.116	0.0011	0.0051	<0.0001
	<i>Bacillus</i>	-0.02	-0.01	0.24	1.10	5.60	0.15	2.712	0.0055	0.0542	0.0376
	<i>Lactobacillus kefiranofaciens</i>	0.00 ^z	0.00 ^z	0.00 ^z	-6.64 ^{zy}	-27.87 ^x	-27.94 ^x	3.467	0.0046	0.4603	0.0275
	<i>Lactobacillus spp.</i>	6.05	4.51	5.68	-5.01	-29.78	-28.73	3.611	0.0003*	0.3722*	0.0103*
	<i>Limosilactobacillus fermentum</i>	2.05	2.32	2.73	-0.05	-0.31	0.69	0.214	<0.0001	0.0084	0.3429
	<i>Streptococcus</i>	-1.19	-1.11	-0.21	1.80	1.26	9.80	4.059	0.0013*	0.6300*	0.0219*
	Proteobacteria	-0.26 ^x	3.08 ^y	11.72 ^z	3.40 ^y	-0.79 ^x	-0.85 ^x	0.674	0.0192	0.6726	0.0079
	<i>Hafnia-Obesumbacterium undf</i>	0.03	1.97	7.19	0.01	0.04	0.00	0.256	<0.0001	<0.0001	<0.0001
	<i>Yersiniaceae undf</i>	0.02	1.48	4.92	-0.04	-0.02	-0.04	0.165	<0.0001	<0.0001	<0.0001

S-kefir Starter Kefir

G-Kefir Grain Kefir

* Indicates non-parametric tests were used; ^{a-c}Means with different superscript letters are different (P<0.05).

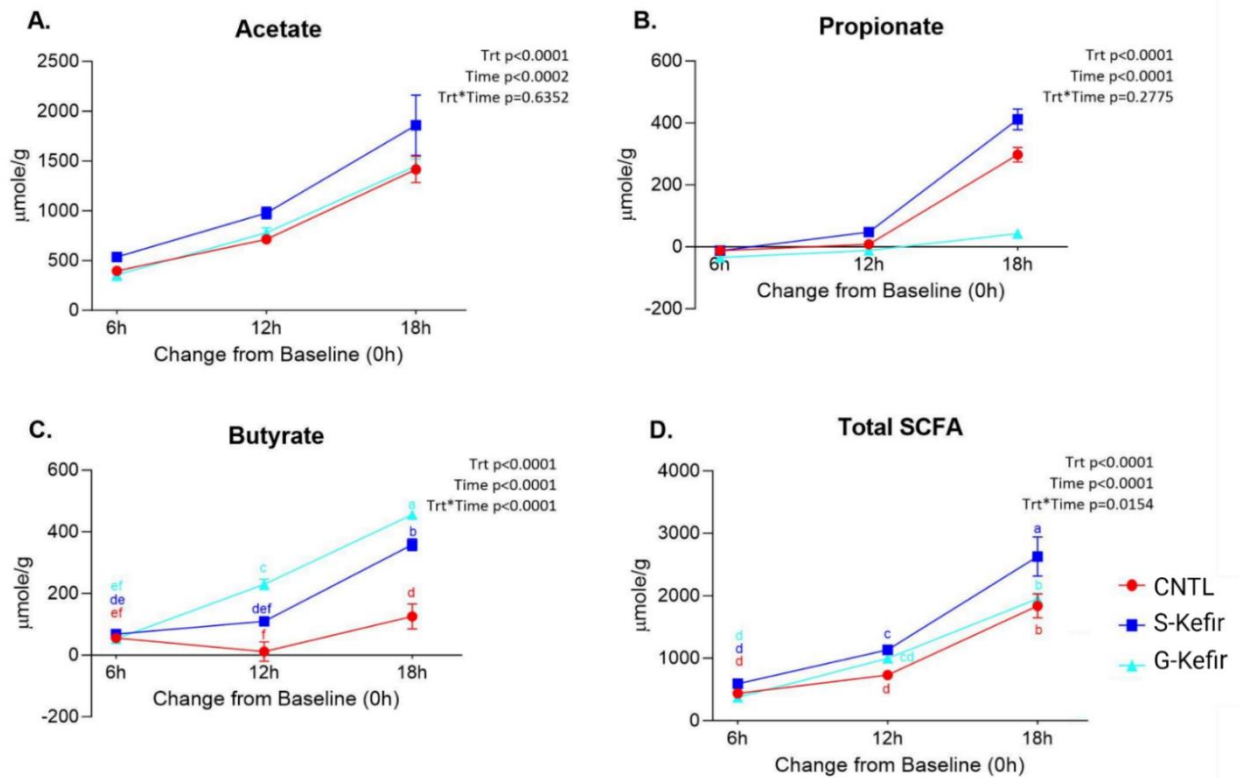


Figure 6.1. Change in acetate (A), propionate (B), butyrate (C), and total short-chain fatty acid (SCFA) concentrations ($\mu\text{mol/g}$; D) during pectin in vitro fermentation assays using fecal inocula from dogs fed the control (CNTL), starter kefir (S-Kefir), or grain kefir (G-Kefir) treatments.

^{a-f}Means in the same graph with different superscript letters are different ($P < 0.05$).

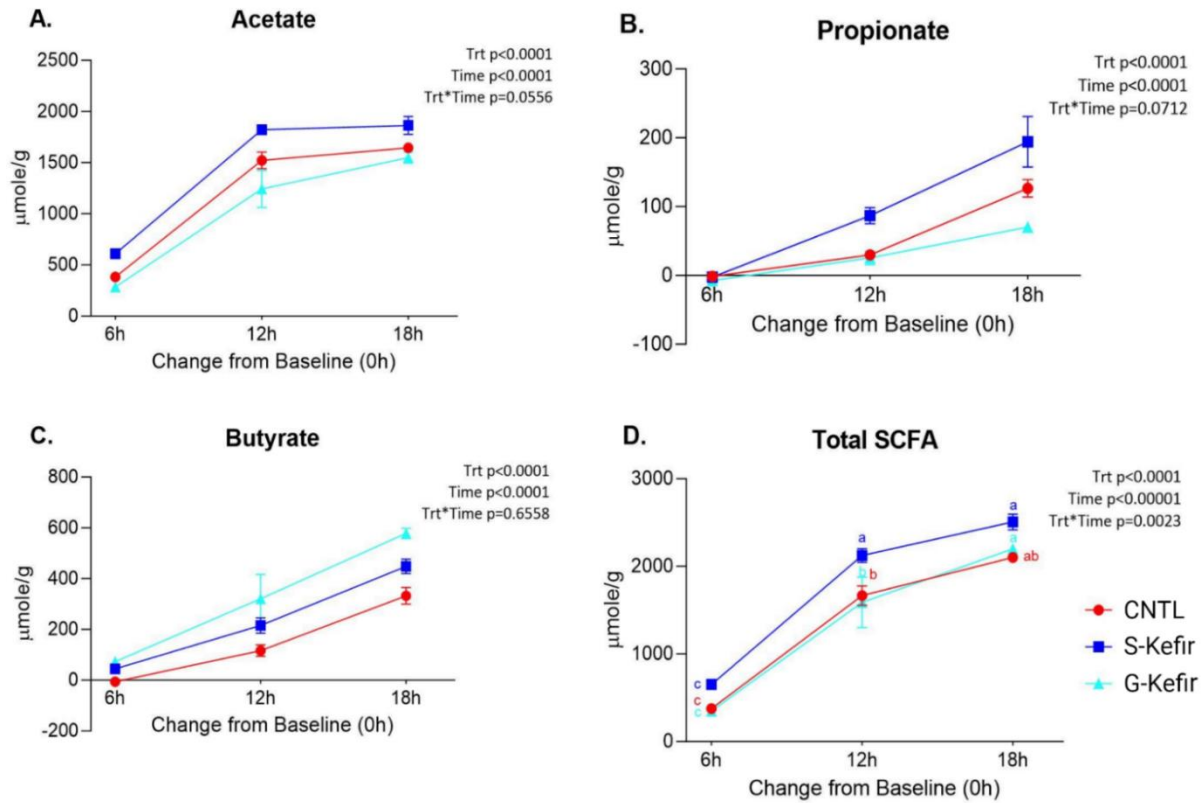


Figure 6.2. Change in acetate (A), propionate (B), butyrate (C), and total short-chain fatty acid (SCFA) concentrations ($\mu\text{mol/g}$; D) during beet pulp in vitro fermentation assays using fecal inocula from dogs fed the control (CNTL), starter kefir (S-Kefir), or grain kefir (G-Kefir) treatments. ^{a-b}Means in the same graph with different superscript letters are different ($P < 0.05$).

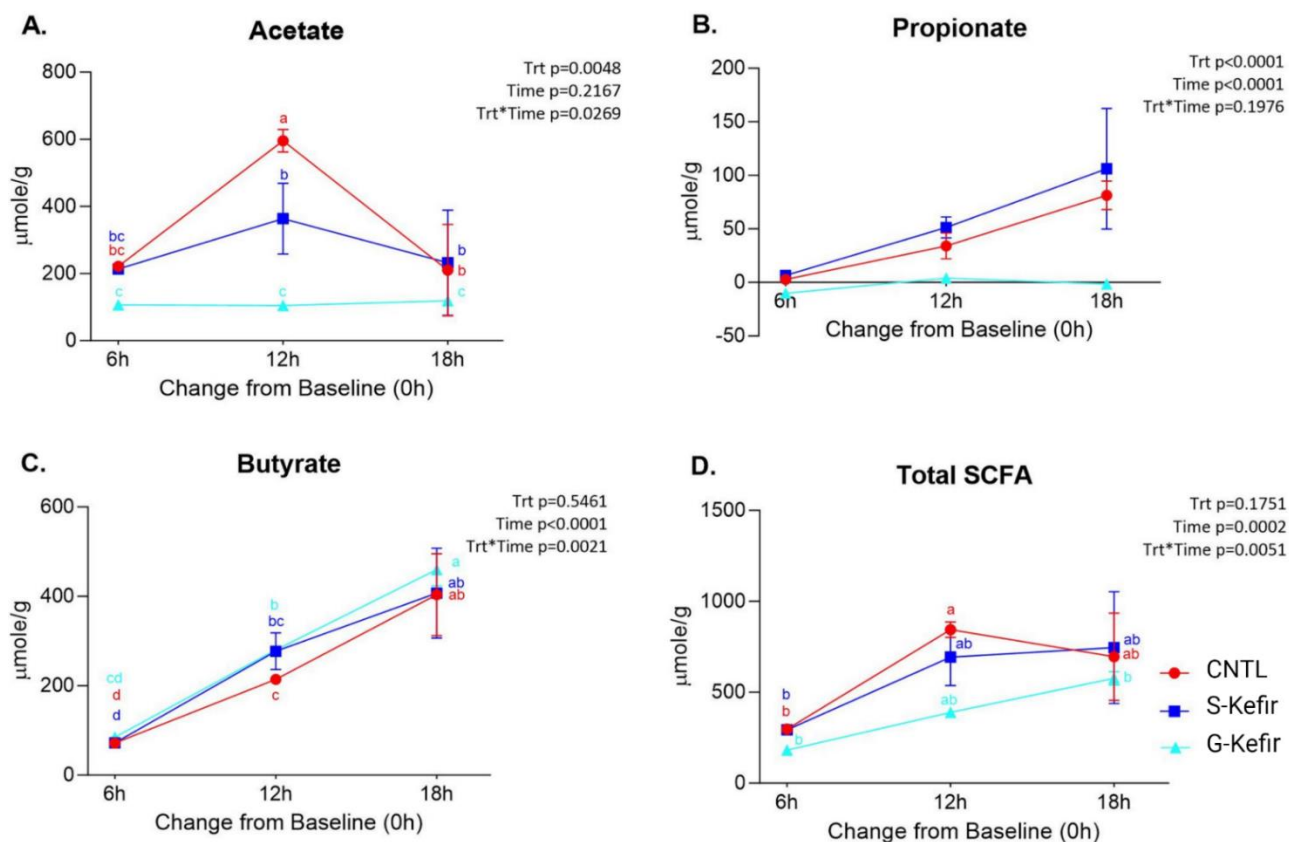


Figure 6.3. Change in acetate (A), propionate (B), butyrate (C), and total short-chain fatty acid (SCFA) concentrations ($\mu\text{mol/g}$; D) during chicory pulp in vitro fermentation assays using fecal inocula from dogs fed the control (CNTL), starter kefir (S-Kefir), or grain kefir (G-Kefir) treatments. ^{a-d}Means in the same graph with different superscript letters are different ($P<0.05$).

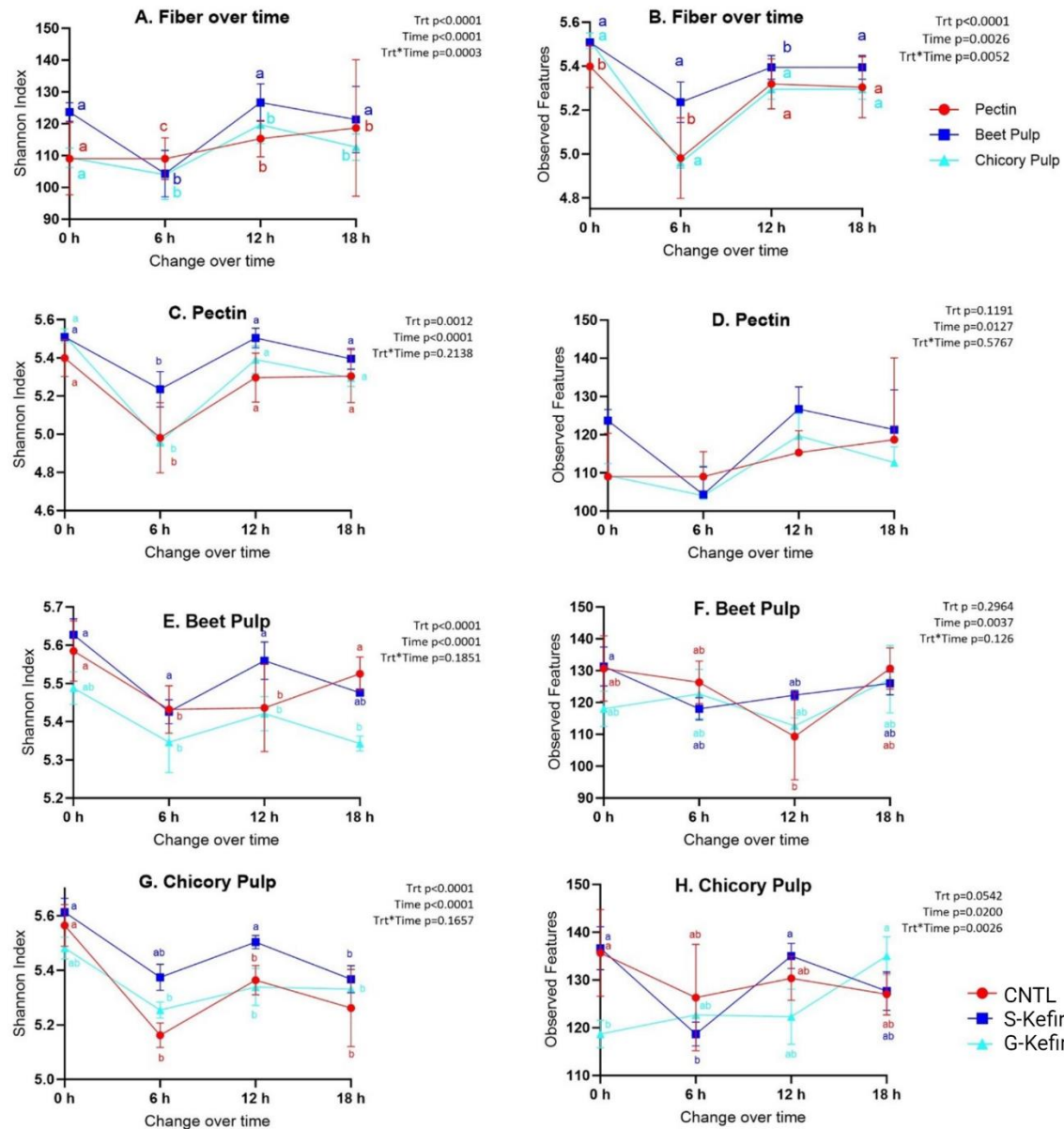


Figure 6.4. Change in bacterial alpha diversity indices during in vitro fermentation assays of all fiber sources (A, B), pectin (C, D), beet pulp (E, F), and chicory pulp (G, H) using fecal inocula from dogs. ^{a-c}Means in the same graph with different superscript letters are different ($P < 0.05$).

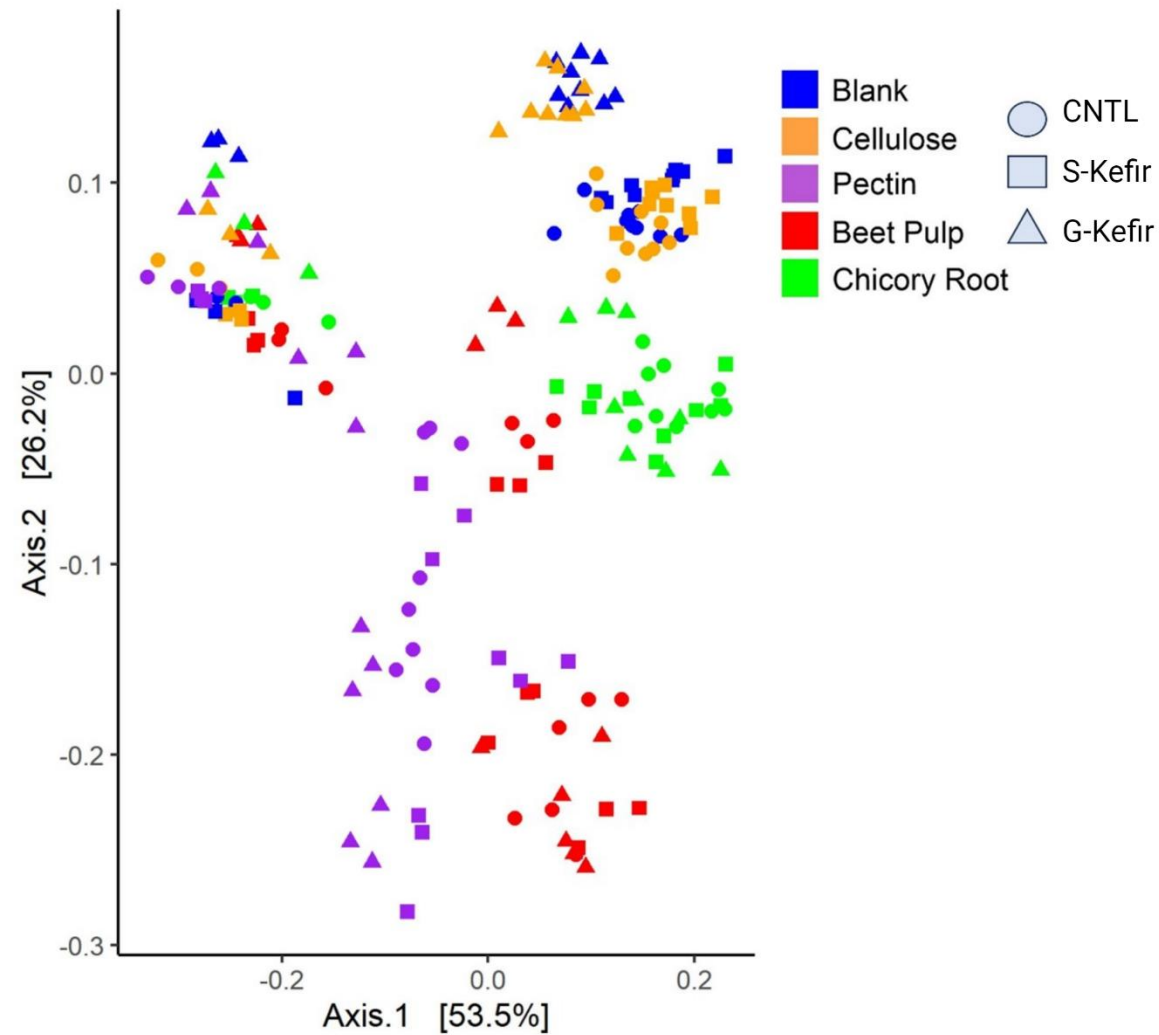


Figure 6.5. Bacterial beta diversity of all fiber in vitro fermentation assays using fecal inocula from dogs fed the control (CNTL), starter kefir (S-Kefir), or grain kefir (G-Kefir) treatments. The principal coordinate analysis plot is based on the weighted Unifrac distances of microbial communities performed on the 97% operational taxonomic unit abundance matrix using QIIME2. All time points were different ($q < 0.005$) from one another.

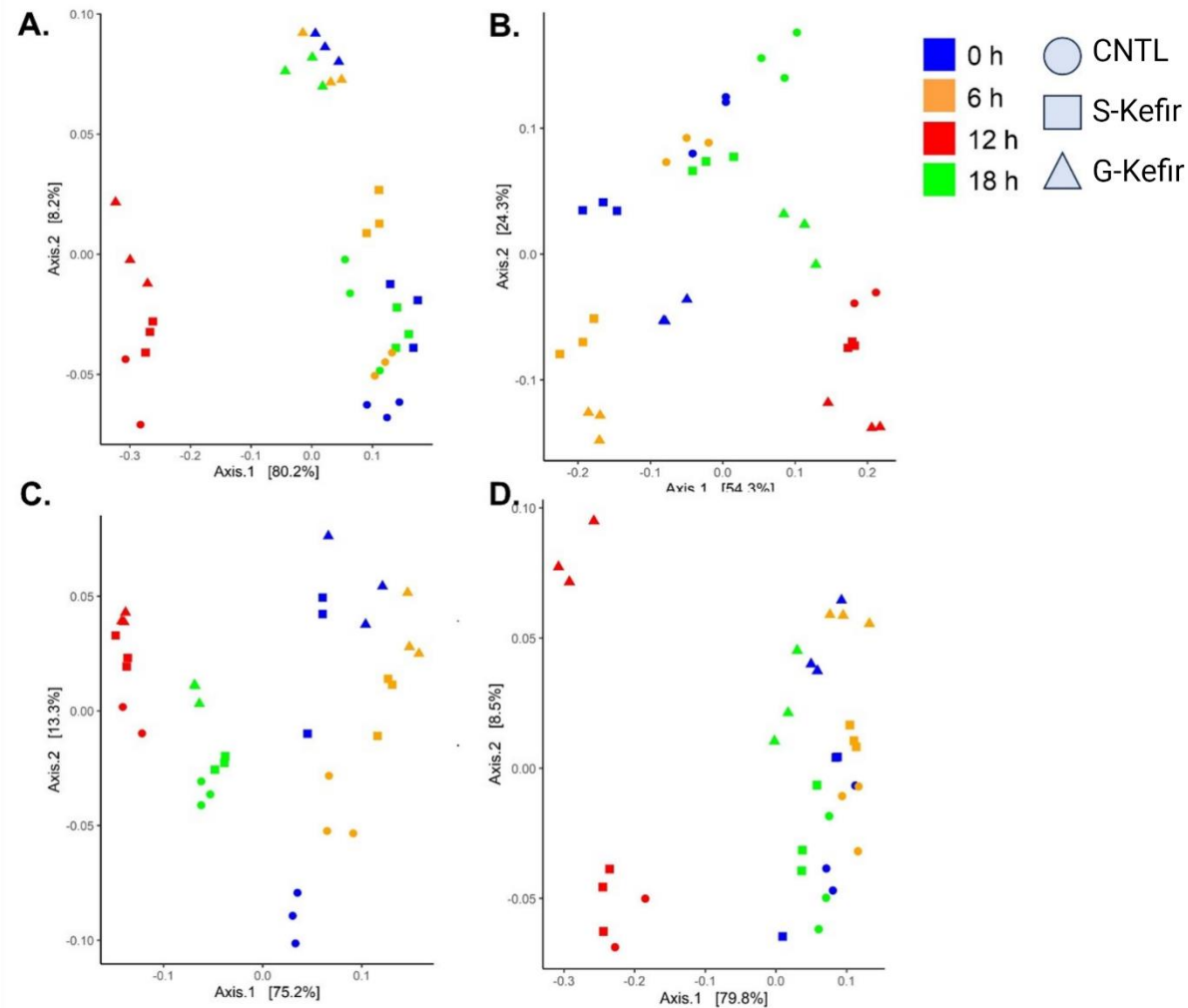


Figure 6.6. Principal coordinate analysis plots based on weighted UniFrac distances for cellulose (A), pectin (B), beet pulp (C), and chicory pulp (D) in vitro fermentation assays using fecal inocula from dogs fed the control (CNTL), starter kefir (S-Kefir), or grain kefir (G-Kefir) treatments.

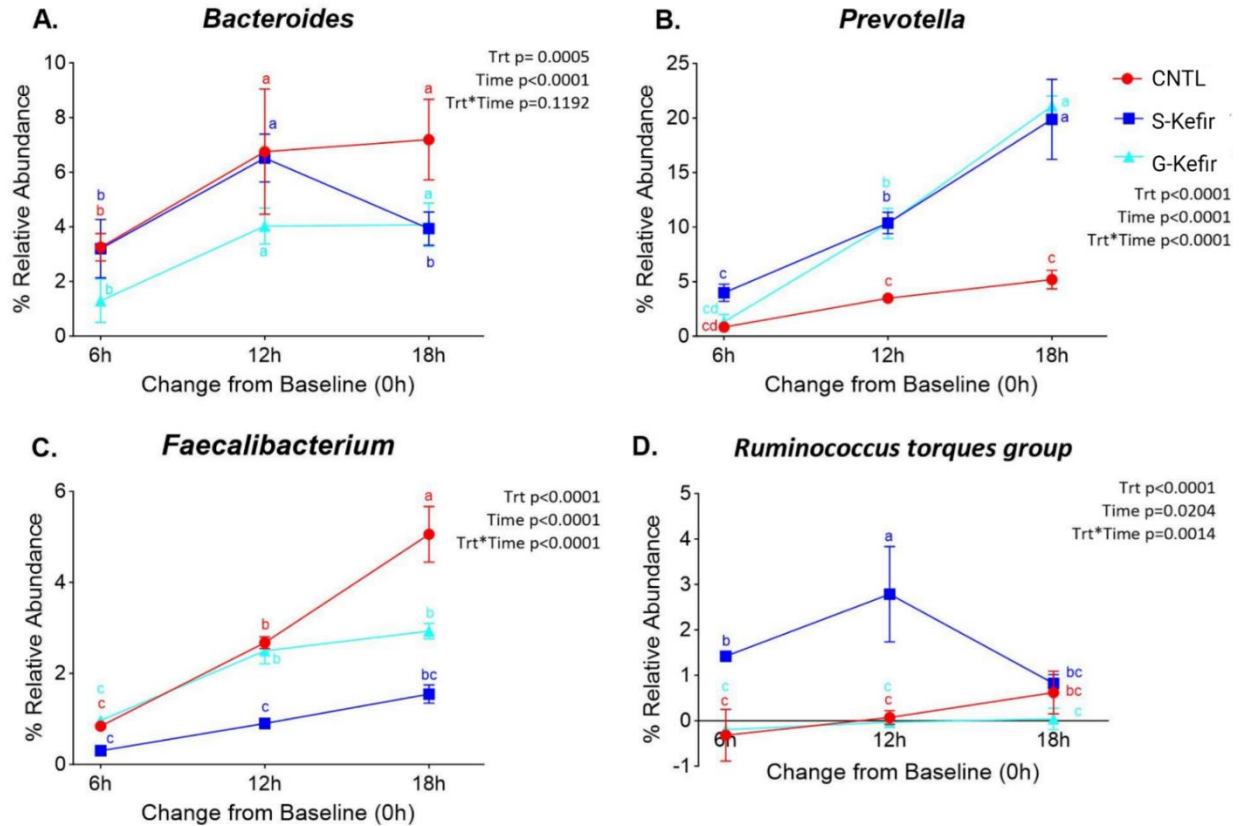


Figure 6.7. Change in *Bacteroides* (A), *Prevotella* (B), *Faecalibacterium* (C), and *Ruminococcus torques* group (D) relative abundances (% sequences) during pectin in vitro fermentation assays using fecal inocula from dogs fed the control (CNTL), starter kefir (S-Kefir), or grain kefir (G-Kefir) treatments. ^{a-d}Means in the same graph with different superscript letters are different (P<0.05).

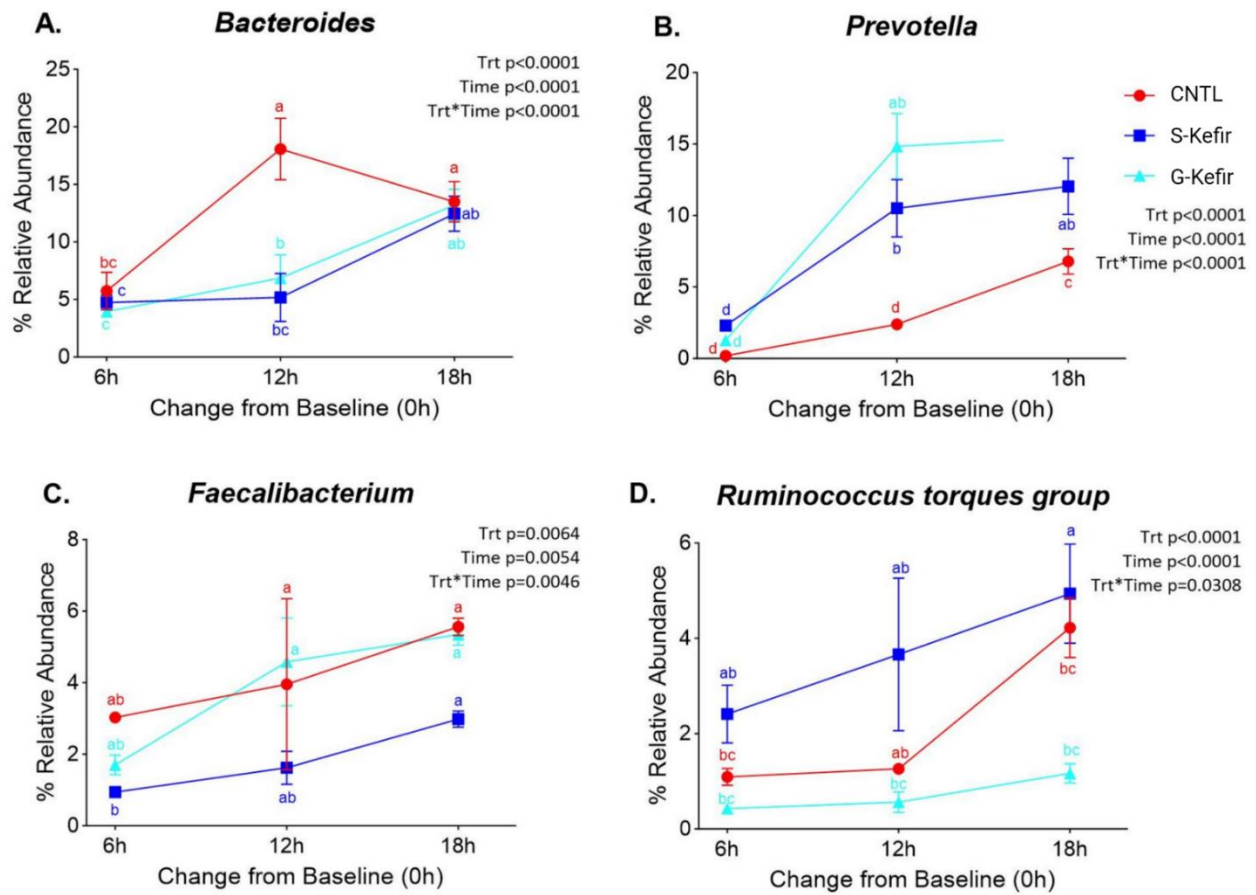


Figure 6.8. Change in *Bacteroides* (A), *Prevotella* (B), *Faecalibacterium* (C), and *Ruminococcus torques* group (D) relative abundances (% sequences) during beet pulp in vitro fermentation assays using fecal inocula from dogs fed the control (CNTL), starter kefir (S-Kefir), or grain kefir (G-Kefir) treatments. ^{a-d}Means in the same graph with different superscript letters are different ($P < 0.05$).

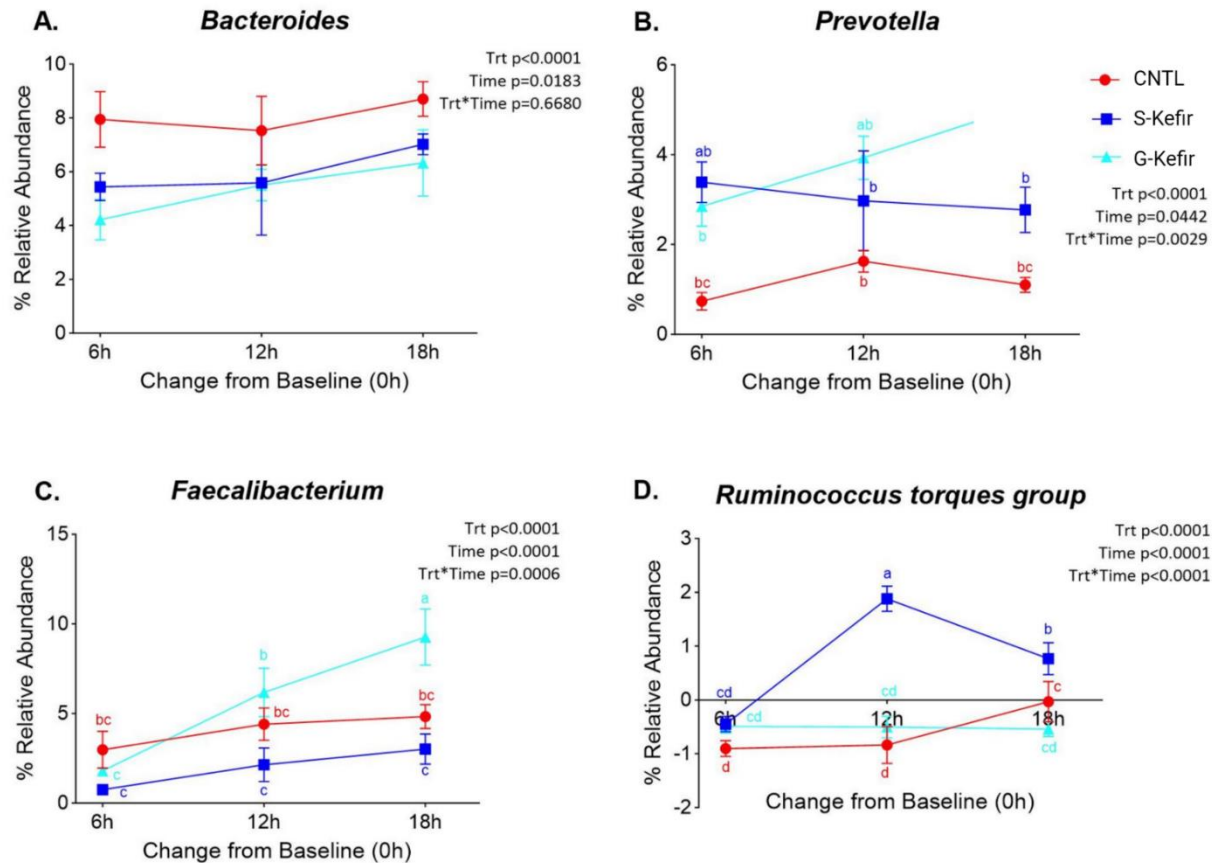


Figure 6.9. Change in *Bacteroides* (A), *Prevotella* (B), *Faecalibacterium* (C), and *Ruminococcus torques* group (D) relative abundances (% sequences) during chicory pulp in vitro fermentation assays using fecal inocula from dogs fed the control (CNTL), starter kefir (S-Kefir), or grain kefir (G-Kefir) treatments. ^{a-d}Means in the same graph with different superscript letters are different ($P < 0.05$).

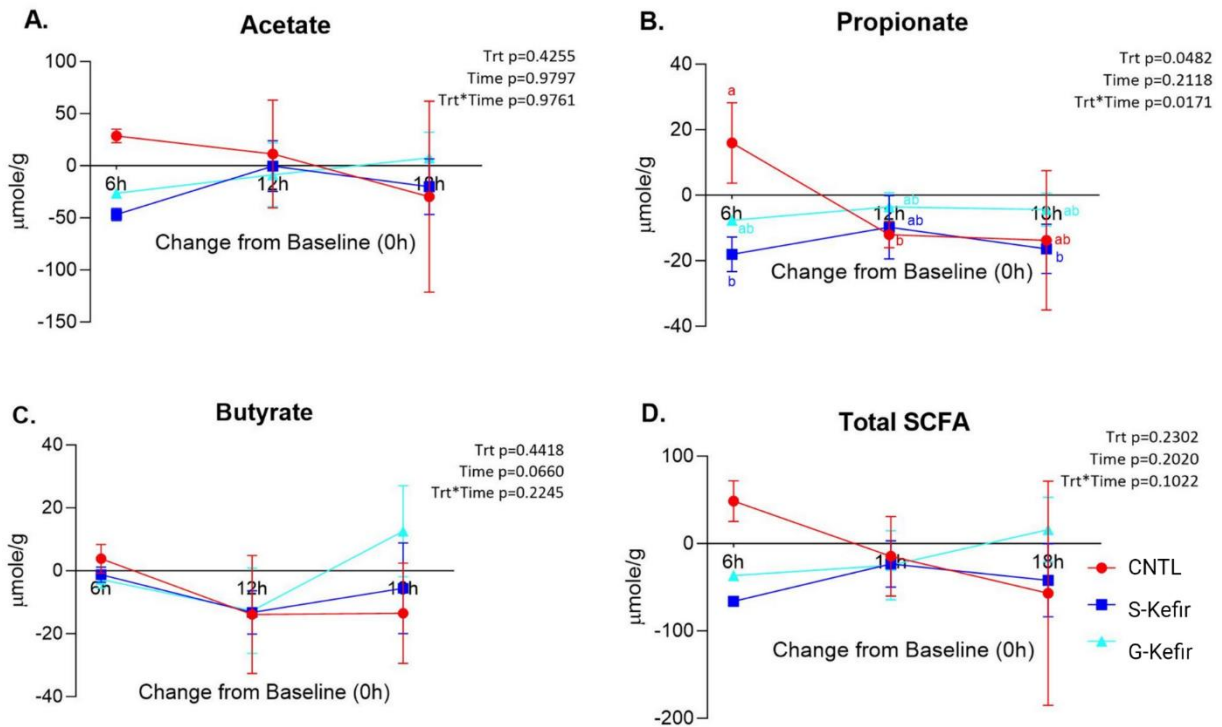


Figure 6.10. Change in acetate (A), propionate (B), butyrate (C), and total short-chain fatty acid (SCFA) concentrations ($\mu\text{mol/g}$; D) during cellulose in vitro fermentation assays using fecal inocula from dogs fed the control (CNTL), starter kefir (S-Kefir), or grain kefir (G-Kefir) treatments. ^{a-b}Means in the same graph with different superscript letters are different ($P < 0.05$).

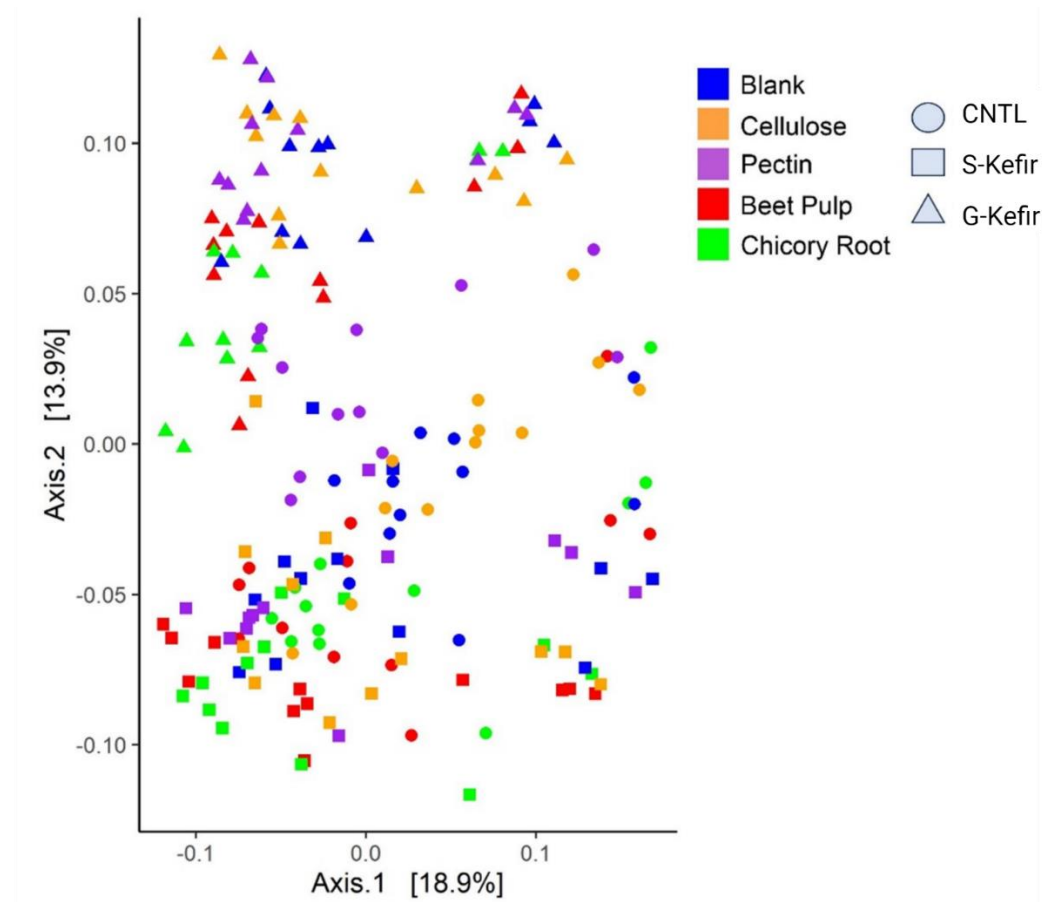


Figure 6.11. Principal coordinate analysis plots of unweighted Unifrac distances of microbial communities from fibers fermented in vitro over time with canine fecal inoculum performed on the 97% OTU abundance matrix using QIIME2. All time points were different ($q < 0.005$) from one another, except for 12 h vs. 18 h ($q = 0.1$).

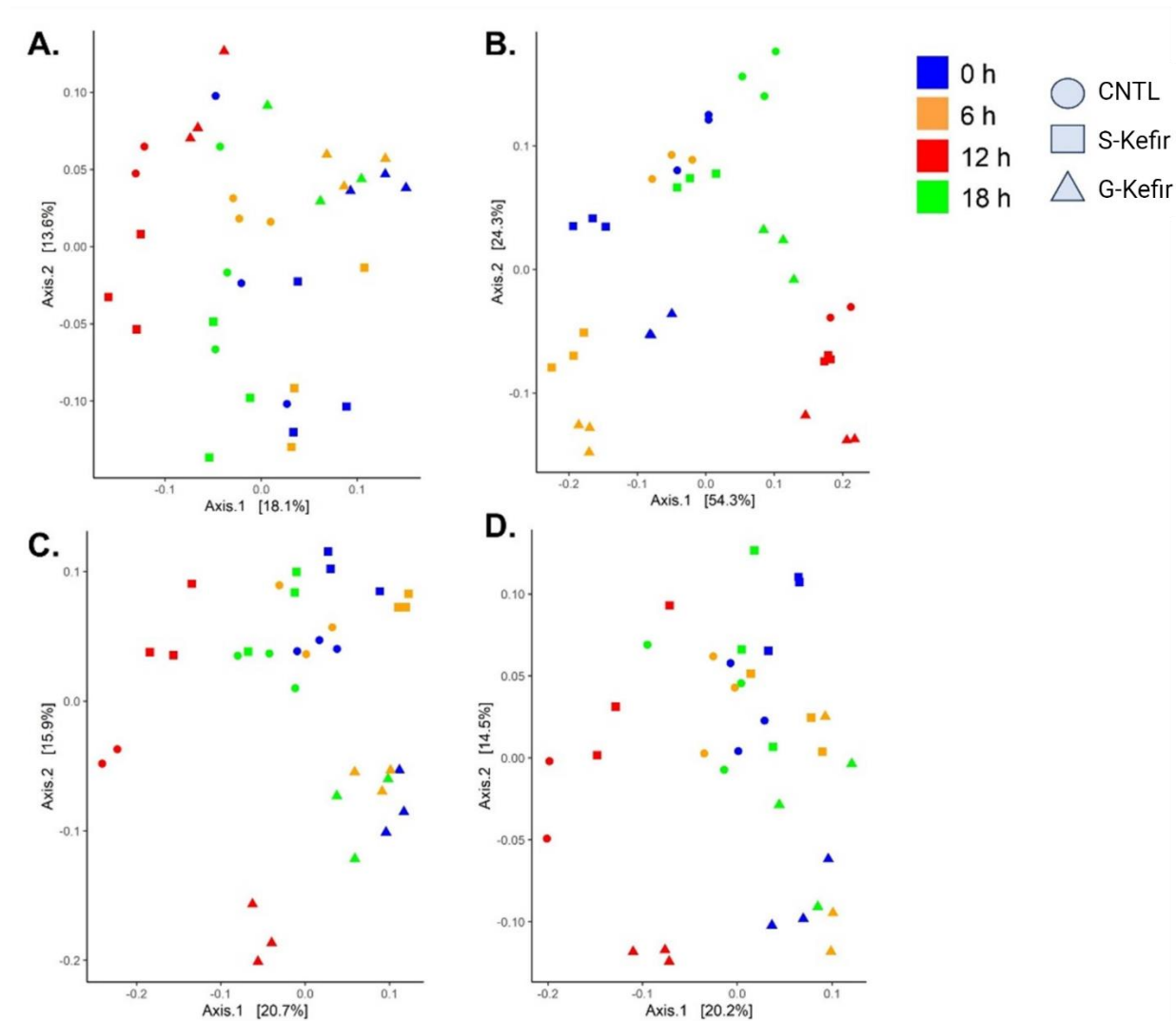


Figure 6.12. Principal coordinate analysis plots based on unweighted UniFrac distances for cellulose (A), pectin (B), beet pulp (C), and chicory pulp (D) in vitro fermentation assays using fecal inocula from dogs fed the control, starter kefir (S-Kefir), or grain kefir (G-Kefir) treatments.

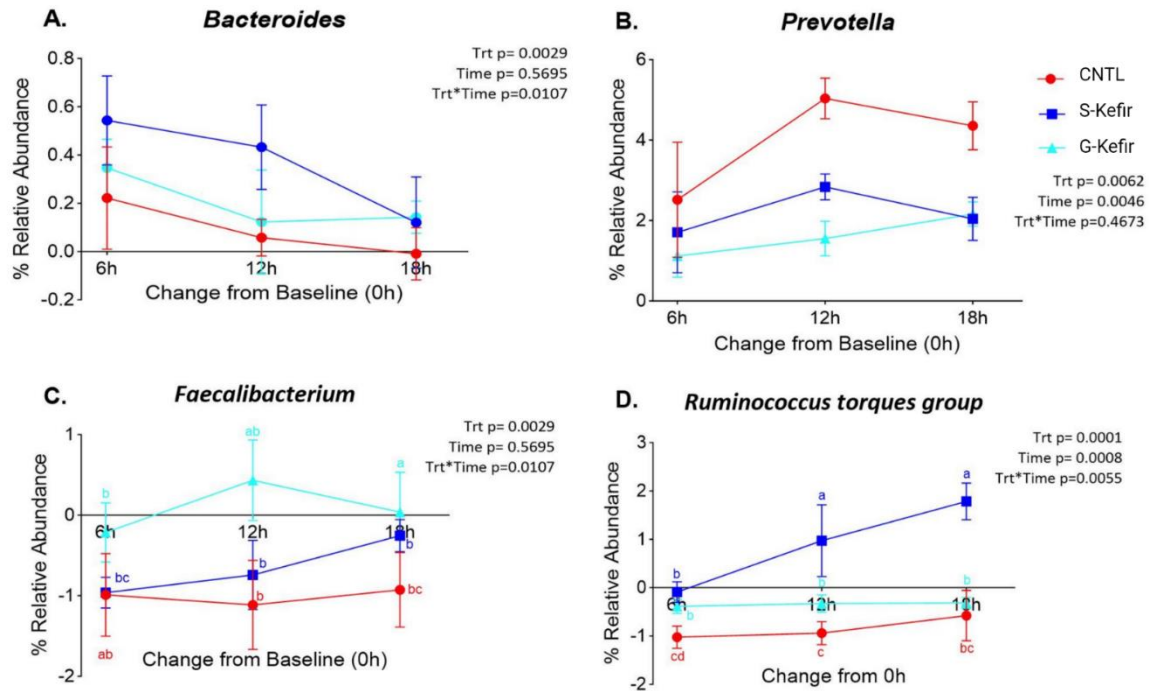


Figure 6.13. Change in *Bacteroides* (A), *Prevotella* (B), *Faecalibacterium* (C), and *Ruminococcus torques* group (D) relative abundances (% sequences) during cellulose in vitro fermentation assays using fecal inocula from dogs fed the control (CNTL), starter kefir (S-Kefir), or grain kefir (G-Kefir) treatments. ^{a-d}Means in the same graph with different superscript letters are different (P<0.05).

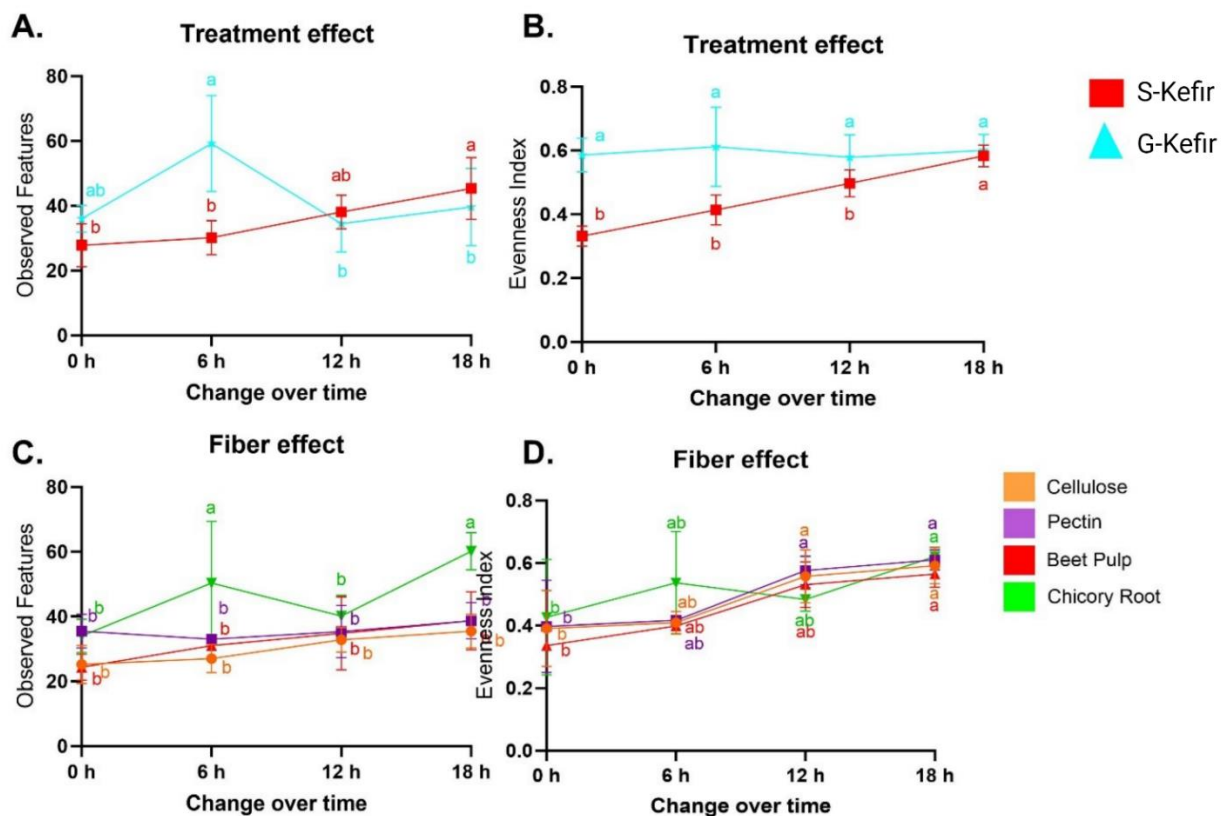


Figure 6.14. Treatment bacterial alpha diversity indices over time assessed by Observed Features (a) and Evenness Index (b). Superscripts indicate a significant difference ($P < 0.05$). Change in fiber bacterial alpha diversity indices over time was also assessed by Observed Features (c) and Evenness Index (d). Superscripts indicate a significant difference ($P < 0.05$).

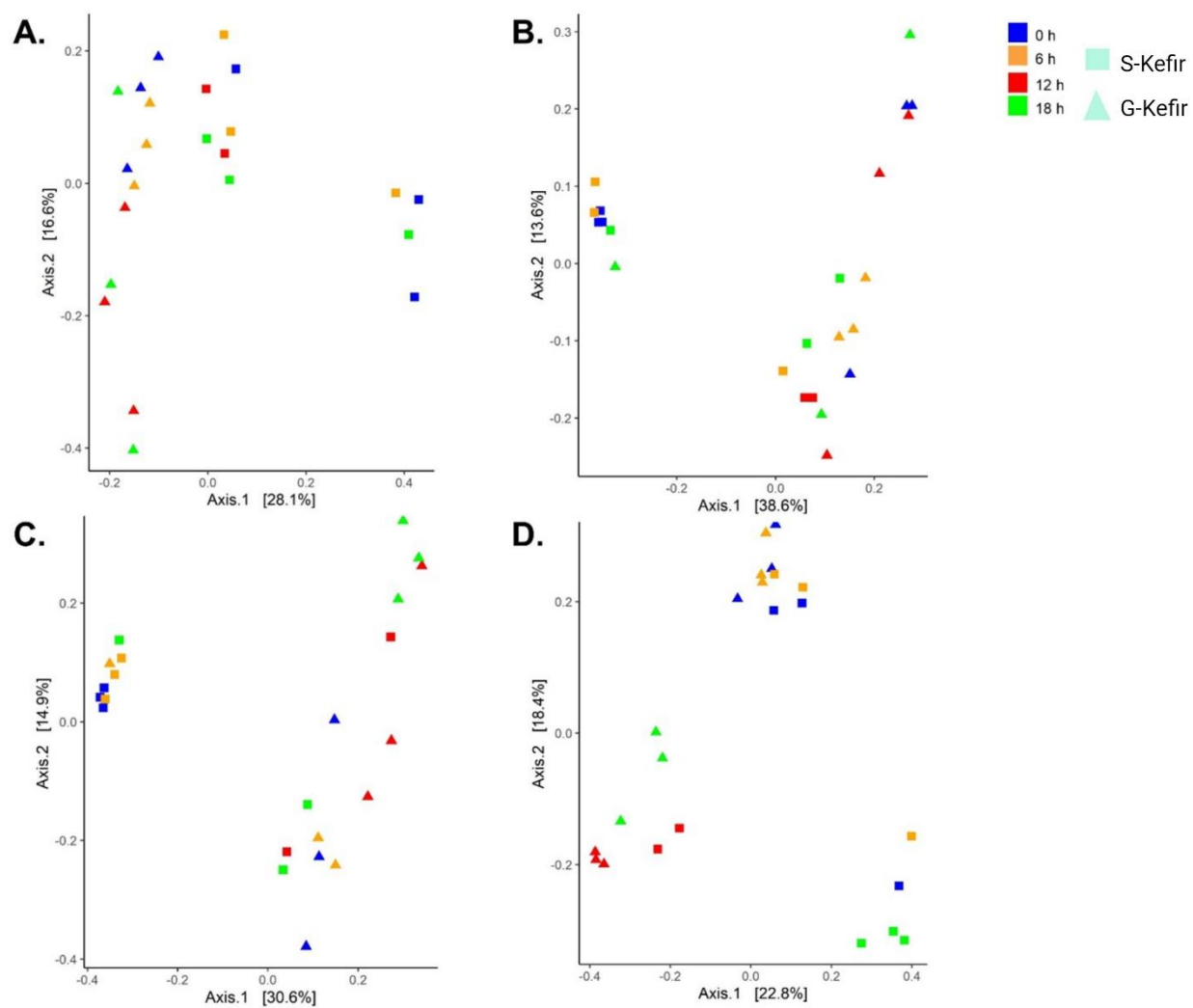


Figure 6.15. Principal coordinate analysis plots based on unweighted UniFrac distances for cellulose (A), pectin (B), beet pulp (C), and chicory pulp (D) in vitro fermentation assays using microbial inocula from starter kefir (S-Kefir), or grain kefir (G-Kefir) treatments.

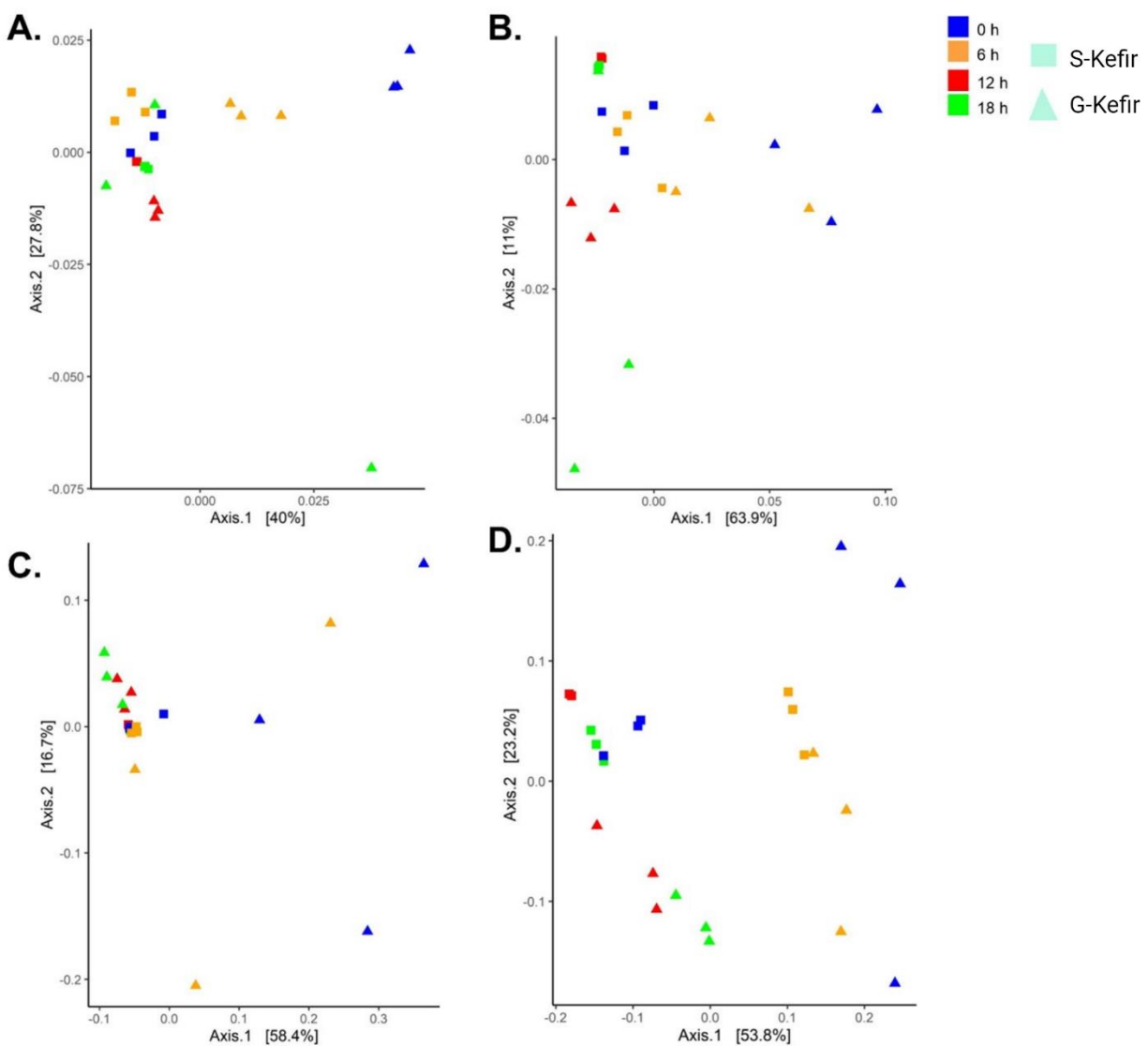


Figure 6.16. Principal coordinate analysis plots based on weighted UniFrac distances for cellulose (A), pectin (B), beet pulp (C), and chicory pulp (D) in vitro fermentation assays using microbial inocula from starter kefir (S-Kefir), or grain kefir (G-Kefir) treatments.

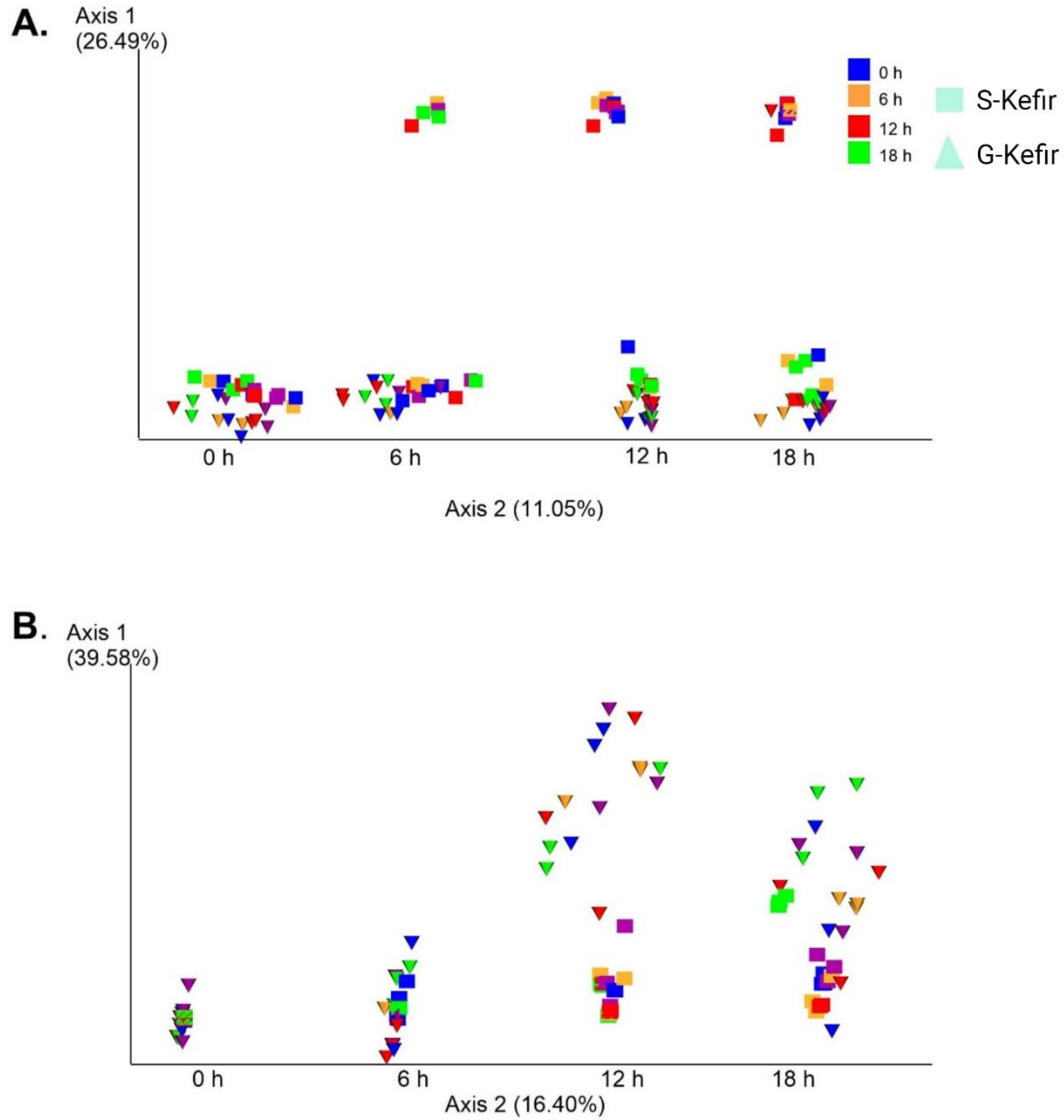


Figure 6.17. PCoA plots of (a) unweighted and (b) weighted Unifrac distances of treatment microbial communities over time performed on the 97% OTU abundance matrix using QIIME. The unweighted PCoA plot (a) showed that treatments were different ($P = 0.01$) over time, while fibers did not have significant differences.

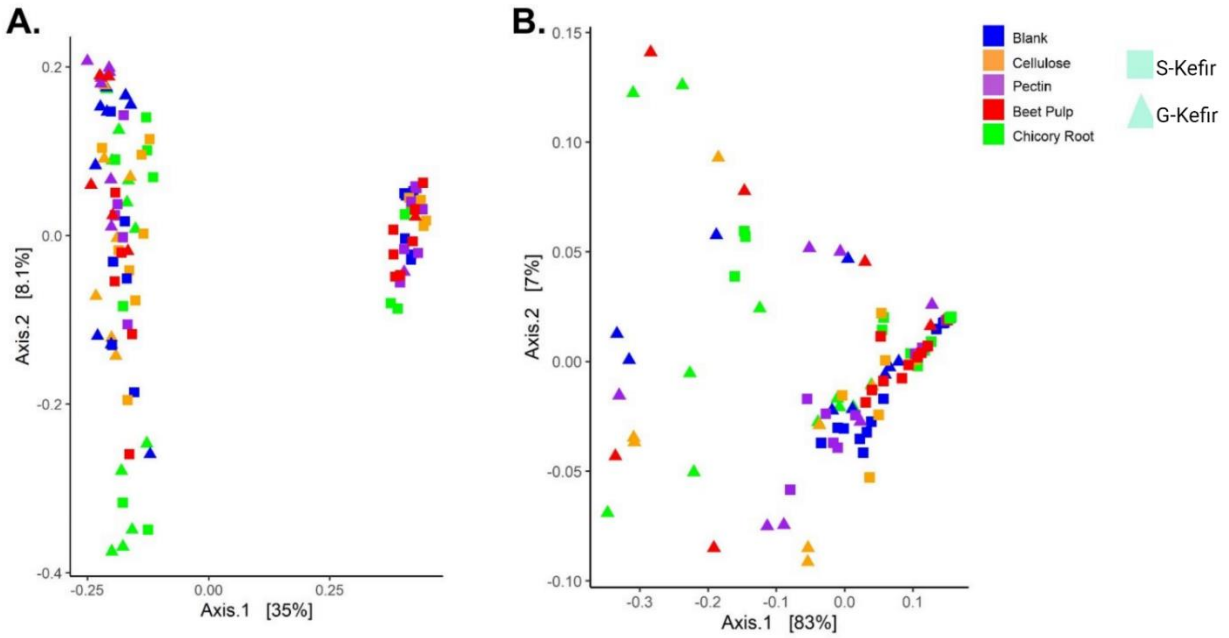


Figure 6.18. PCoA plots of (a) unweighted and (b) weighted Unifrac distances of treatment microbial communities by fiber on the 97% OTU abundance matrix using QIIME. The unweighted PCoA plot (a) showed that treatments were different ($P = 0.01$) over time, while fibers such as CP clustered.

CHAPTER 7: GENERAL DISCUSSION AND CONCLUSIONS

Fermented foods have been a part of the human diet for centuries and the modern era will continue to usher in new forms of these foods (Marco, 2020). The live microorganisms that produce fermented foods require more research to better understand how their presence impacts food properties, the health of those consuming the foods, and the gut microbiome of the host (Wastyk et al., 2021). Specific to kefir products, understanding the impact of various microbial communities derived from kefir grains or starter communities *in vivo* and *in vitro* is the first step in accurately accounting for potential mechanistic differences (Bourrie et al., 2021; Nejati et al., 2022). Current regulations for fermented food contents are lacking, which has generated a need for research endeavors comparing product label claims with actual live microbial counts and components (Metras et al., 2020; Metras et al., 2021). This dissertation has reviewed the literature on the connection between diet, gut microbiota, fermented foods, and kefir as well as how variations in kefir fermentation impact research outcomes. Our analyses of commercial kefir products sold for human and companion animal consumption revealed conflicting results in regard to product integrity, requiring scrutiny as the fermented food market increases in popularity (Chapters 3 and 4)(Metras et al., 2020; Metras et al., 2021). The impact of kefir consumption on nutrient digestibility and the fecal characteristics of dogs was studied in Chapter 5 (Metras et al., 2023). Follow-up *in vitro* fermentation experiments assessing the functionality of associated fecal microbiomes as well as a direct comparison of microbial population functionality sourced directly from both kefir fermentations (Chapter 6).

There are a handful of studies that have evaluated the ingredient accuracy of foods containing live microorganisms or probiotics (Weese and Arroyo, 2003; Weese et al., 2005); yet to our knowledge, an assessment of such products in the United States had not been done before

(Chapters 3 and 4). Although researchers have begun to explore how the inclusion or exclusion of kefir grains impacts host health, none have directly compared whole products through a canine in vivo intervention to observe community-wide changes (Kim et al., 2019; Gaspardo et al., 2020) (Chapter 5). Finally, Chapter 6 focused on in vitro fermentation kinetics of dietary fibers using fecal inoculum of dogs consuming kefir or the kefirs directly.

We demonstrated that many inaccuracies currently exist in commercial kefir products, with none of the products tested meeting 100% of the label claims microbial identification or viable microbial counts. As the market for commercial foods containing live microorganisms is still in its early years, our research demonstrates that more attention is needed for these products in the absence of federal regulations or standards of identity. While kefir is an extremely popular beverage, other fermented foods with label claims would benefit from a non-biased third-party assessment. There are also no stipulations on what can be labeled as “kefir”, and we have demonstrated that there is no market consensus on this product definition either.

Performing an intervention directly comparing kefir produced from grains or starter kefir microbes revealed a significant increase in fecal *Lactococcus* from regular consumption of grain kefir, while no other significant differences were observed between treatment groups. While colonization is atypical in probiotic or fermented food interventions, the significant increase of *Lactococcus* abundance was retained in our in vitro fermentation using feces from a separate grain kefir intervention period (Adams, 2010). The significant increase of butyrate and low propionate production detected during our in vitro fermentation experiments derived from grain kefir feces and beverages provide encouraging insight into further functional differences of these microbial communities.

Strengths and Limitations

There are important strengths and limitations to consider in the current effort that can inform future research pursuits. The products chosen for label analysis are all actively sold on the market in the United States, directly relaying relevant information to consumers. Additionally, the inclusion of a yogurt control confirmed that our findings were accurate by the methods used. The *in vivo* intervention utilized a rigorous replicated 3x3 Latin square crossover design. Similar experiments have utilized privately owned canines without randomization or crossover interventions (Kim et al., 2019; Gaspardo et al., 2020). To capture the complexity of fermented foods, our pursuits to compare the microbial differences of kefir products utilized whole foods rather than a mono-culture or preselected group of microbes and yeast (Chen and Chen, 2013; Carasi et al., 2015; Bourrie et al., 2018; Bourrie et al., 2021; Georgalaki et al., 2021; González-Orozco et al., 2023). This approach allowed us to assess the realistic impacts of these foods on the host through dynamic microbial communities as well as including other elements of their food matrix such as exopolysaccharides, bacteriocins, and unique flavor compounds generated through fermentation (Maeda et al., 2004; Powell et al., 2007; Walsh et al., 2016; Nejati et al., 2020; Lu et al., 2022). The lack of significant physiological outcomes from the intervention may be explained by our canine population being of optimal health before the investigation, reducing the possibility for an alteration in inflammatory markers or improvement in blood panels. Finally, the choice to perform *in vitro* fermentations with fecal samples and dietary fiber provided insight into the functional fitness of resulting microbial communities post-kefir intervention (Sunvold et al., 1995; Traughber et al., 2020; Pérez-Burillo et al., 2021). The measurements of SCFA production and population shifts by fiber fermentation allow for insight into these communities as they might

behave in the gut microbiome rather than observing the terminal populations from simple fecal analysis (Boisen and Eggum, 1991; Barry et al., 2011; Carlson et al., 2017).

In performing research involving fermented foods, understanding the microbial communities and microbial density of the products is of utmost importance. Over a decade of previous research on kefir did not consider these factors by omitting sequencing and attempts to culture their kefir intervention products (St-Onge et al., 2002; Judiono et al., 2014; Bellikci-Koyu et al., 2019; El-Bashiti et al., 2019; Yilmaz et al., 2020; Salari et al., 2021). For researchers to study potential mechanisms occurring, a thorough knowledge of the initial product is crucial. Methods disclosing product fermentation, sourcing, microbial density, and microbial contents are imperative for moving forward in fermented food research (Sindi et al., 2020; Bourrie et al., 2021; Nejati et al., 2022).

Experimental design is a crucial component of the successful analysis and comparison of fermented foods. In our product analysis, two different lots of each kefir were used in an attempt to capture slight variations among products as a result of fermentation. All culturing measures were done in triplicate under sterile conditions with the same intention. For our in vivo study, a replicated 3x3 Latin square crossover design allowed each subject to experience each treatment, which strengthened our findings due to their repetition. The in vitro fermentations pooled fecal samples by treatment to strengthen treatment group effects. In addition, samples were collected at 4 time points during fermentation, up to 18 h. The persistence of alpha diversity through to 18 h demonstrated that the microbial communities were still utilizing metabolites within their environment to grow and replicate. Ending the fermentation prematurely would not have allowed us to observe this persistence.

The dose of kefir used in our dog study aimed to capture what a household pet may receive at home. This decision allows our results to be directly translated into consumer knowledge. Due to grain kefir being more microbially dense than starter kefir, a dilution factor of 1:4 was applied in the last in vitro fermentation so the microbial density would be equal between products. Had this not been considered, the SCFA output could have been interpreted incorrectly due to higher concentrations generated by grain kefir in vitro.

Standardization is needed when fermenting kefir for results to be reproduced and mechanistic effects to be appropriately quantified. Sterilization of jars and tools was practiced in all fermentations to prevent contamination. The same kefir grains were recycled in all grain kefir experiments, allowing for consistency of the microbial communities and food matrix throughout each aim. Kefir was brewed in a temperature-controlled setting with daily pH testing to ensure consistent products. All beverages were used before expiration to prevent spoilage of products. Additionally, DNA extraction and microbial quantification techniques were replicated at each aim to allow for the most consistent results and for direct comparison over time.

Conclusions

In summary, interest in the impact of fermented foods on human health is on the rise. While traditionally-fermented foods have been produced within individual households, growing consumer interest has forced food companies to mass produce these foods while taking fermentation shortcuts along the way. As a result, many products labeled and sold as kefir bare little to no resemblance to the authentic brewing style of grain inclusion or through backslopping. Once mechanistic differences between microbial communities derived from kefir grains or starter

cultures are better understood, standards of identity will be necessary so dietary recommendations of fermented foods can be supported by quality assurance to provide confidence to consumers.

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