



Manipulating the Gut Microbiome to Improve Health

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HARVARD UNIVERSITY
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Manipulating the Gut Microbiome to Improve Health

A dissertation presented

by

Theodore Andrew Chavkin

to

The Division of Medical Sciences

in partial fulfillment of the requirements
for the degree of

Doctor of Philosophy

in the subject of

Microbiology

Harvard University

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Manipulating the Gut Microbiome to Improve Health

Abstract

The human gut microbiome is rapidly gaining attention for its connections to a long list of health and disease states, presenting new opportunities to modulate human health using microbiome therapeutics. In this dissertation, I will explore the concept of the microbiome as a metabolic “organ,” where the metabolic reactions encoded in microbial genomes can have profound effects on the health of the host. In the context of athletic performance, distinct exercise-associated microbiome states are characterized by increases in distinct microbial taxa, some of which are relatively unique in their preference for lactate as a carbon source. We show that isolates of lactate-utilizing members of the genus *Veillonella* can improve athletic performance in mice, and further show that the principal metabolic byproduct of *Veillonella*, propionate, mimics this performance enhancing effect. In a similar vein, we investigate the metabolic potential for probiotic *E. coli* Nissle to absorb glucose in the context of diabetic postprandial hyperglycemia. We show that *E. coli* Nissle can reduce the glycemic impact of a large glucose bolus, however this effect is not dependent on *E. coli* glucose metabolism. Thus, while we observe improvements in glycemic profiles, these improvements are not due to a metabolic interaction, but likely a physiological one. Overall, the work I describe here represents a step towards manipulating the gut microbiome to improve health and manage disease. By focusing on the molecular effectors at the host-microbiome interface, we can target and modify those signals to the benefit of host wellbeing.

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Introduction

Microbial Metabolites and Their Impact on Human Health

Overview

The human microbiome has been dubbed the “other you” and described as a metabolic organ, similar in importance to human health as the liver or pancreas. This understanding of the intestinal microbiome as a mediator of health and development is not a new concept, and was suggested over a century ago.¹ However, the rapid development of sequencing technology has enabled an explosion in microbiome science and deeper understanding. Indeed, microbes have been shown to play a role in mediating all manner of diseases from gastrointestinal to metabolic to neurological. The broad health effects of the microbiome present a unique opportunity for a new class of microbial therapeutics: modifying the microbiome to promote health.

In this introduction I review the state of the microbiome field, within the context of the metabolic interactions between the microbiome and disease. These microbe/host interfaces represent just a few of the possible avenues for microbiome manipulation, and focusing on molecular effectors highlights the mechanisms by which these manipulations can act. The work described in this dissertation will discuss further the links between lactate, propionate, and exercise, as well as glucose and diabetes, but the other interactions discussed highlight the potential for therapeutic development across a wide range of diseases.

Microbial Metabolites

Microbial interactions have been implicated in cancer, immune function, metabolic disease, and neurochemistry, to name only a few. These interactions are often studied in terms of metagenomic associations, where samples are sequenced in bulk and species are identified. Correlations are then formed between presence of a given species and disease, and subsequent experimentation often focuses on bacterial isolates and probiotic supplementation. However,

while taxonomic units are helpful in describing the microbiome, effects on health and disease are mainly propagated by the metabolites exchanged between diet, host, and microbe.

With this mindset, it has been proposed to think of the microbiome not as a list of species, but as a metabolic organ, with inputs and outputs that mediate the microbiome's many health effects. In this framework, the metagenome of a microbiome represents metabolic potential, and the various reactions encoded therein will alter dietary molecules, produce beneficial and harmful compounds, and react to host factors. This simplifies the complex interactions present in the microbiome to a series of inputs and outputs: the metabolites that are consumed, produced, and biotransformed. Thinking of the microbiome in this way allows one to focus on the molecular mediators of health and disease and works as a supplement to the traditional taxonomic way of thinking.

This conception of the microbiome is crucial to the work described in this dissertation. In this review, I will describe the well-studied molecular effectors exchanged and influenced by the microbiome, focusing on each metabolite in turn and the state of the literature supporting its effects.

Dietary Fiber

One of the earliest proposed metabolic interchanges between host and microbiome is the extraction of energy from dietary fiber.¹ This class of molecules include resistant starch, defined loosely as starch variants that are poorly degraded and absorbed by the host, as well as nonstarch polysaccharides, such as the widely studied fructo-oligosaccharides. These molecules escape host absorption in the proximal small intestine, instead reaching the colon where they feed resident gut microbes.² The primary fermentation products are the short chain fatty acids (SCFAs) acetate, propionate, and butyrate, discussed further in the next section. SCFAs serve

many functions, but in the context of dietary fiber they are most well known for their role in feeding colonic epithelial cells. Butyrate in particular serves as the primary carbon source for intestinal epithelial cells, and this energy extraction from indigestible fiber comprises a major function of the gut microbiome.³

Short Chain Fatty Acids

Short chain fatty acids (SCFAs) refer to the grouping of formate (C1), acetate (C2), propionate (C3), and butyrate (C4) produced and secreted in abundance by the microbiome. Though acetate is the most abundant SCFA in the gut, studies primarily focus on propionate and butyrate, or discuss the class of molecules as a whole. SCFAs are a broad topic in the microbiome with many disease associations, well reviewed in the context of colorectal cancer by Wang et. al⁴, inflammation by Vinolo et. al⁵, and host metabolism by den Besten et. al.⁶

SCFAs selectively bind and activate the G-protein coupled receptors FFAR2 and FFAR3 (also called GPR43 and 41, respectively). FFAR2 is primarily activated by propionate, butyrate, and to a lesser extent acetate.⁷ FFAR3 is primarily activated by propionate and butyrate, with strong activity from pentanoate (C5 saturated fatty acid), which is not discussed much in the context of the microbiome.⁷ The functions of FFAR signaling have been elucidated in knockout mice and different effects have been observed for different tissue types. Within adipose tissue, FFAR2 activation reduces insulin-mediated fat accumulation.^{8,9} Within immune cells, FFAR2 activation reduces inflammatory response and plays an important role in resolving inflammatory disorders in mouse models such as colitis, arthritis, and asthma.¹⁰ In colonic endocrine L cells, SCFAs stimulate glucagon-like peptide (GLP-1) secretion *in vitro*, in a mechanism requiring either FFAR2 or FFAR3.¹¹ GLP-1 is an incretin hormone associated with improved glucose regulation and reduced weight gain.¹² Strangely, in mice lacking FFAR3, propionate and

butyrate still protect against diet induced obesity and insulin resistance, suggesting multiple independent mechanisms of regulating weight gain and glucose homeostasis.¹³ Taken together, FFAR2 and 3 appear to facilitate communication between the microbiome and host, with SCFAs inducing anti-inflammatory effects as well as improved metabolic effects in glucose regulation and adiposity.

Butyrate

Butyrate is well studied for its immunomodulatory effects. In vivo, butyrate induces differentiation of regulatory T cells (Tregs) and reduction in inflammatory cytokine response, ameliorating colitis symptoms in rodent models.^{14–16} Tregs in the intestine act to suppress inflammation and the balance between Tregs and inflammatory effector T cells has been highlighted as a key factor in inflammatory bowel disease.¹⁷ Metagenomic human studies implicate butyrate in inflammatory bowel disease as well, with ulcerative colitis patients showing increased levels of butyrate-producing bacteria and butyrate treatment improving symptoms in clinical trial.¹⁸ However, these clinical trial results are not consistent across studies, with several confounding null results.^{19,20} It has been suggested that these mixed results may be a result of variable butyrate uptake rates in patients with inflammatory bowel disease, and more work is needed to establish the efficacy of butyrate treatments.²¹

Propionate

Propionate has an interesting property among SCFAs in that microbial propionate has been shown to reach systemic circulation at high concentrations.²² In contrast, microbially produced butyrate and acetate are primarily metabolized by the liver or colonocytes and subsequently incorporated into gluconeogenesis or lipid biosynthesis.²² Overall SCFA concentrations have been observed to be elevated in obese subjects, with greater increases in

propionate over other SCFAs.²³ This is in contrast to mechanistic mouse studies discussed earlier where propionate administration reduces diet induced obesity and improves glucose homeostasis in mice.^{11,13}

Lactate

Lactate is a short chain carboxylic acid that often comes up in microbiome metabolic analyses, and is therefore often grouped with the SCFAs. Lactate utilizing bacteria (LUB) are readily isolated from intestinal microbiota, notable examples being *Eubacterium hallii*, *Anaerostipes* spp., as well as the genus *Veillonella*.²⁴ In vitro, LUB co-cultured with starch-degrading lactic acid bacteria result in rapid conversion of starch to lactate, with subsequent conversion to butyrate, providing evidence that lactate acts as a metabolic niche in the gut.²⁴ This two-stage production of butyrate from starch likely represents one metabolic pathway explaining the SCFA production from resistant starch and dietary fiber, discussed above.

Lactate has been implicated in inflammatory bowel disease, but the directionality of this relationship is still not clear. Severe ulcerative colitis is characterized by high lactate concentrations and low fecal pH.²⁵ In vitro fecal culture studies have shown that while LUB effectively maintain low lactate concentrations except in the case of low pH, where lactate accumulates.^{26,27} These results support a connection between inflammatory bowel disease, reduced intestinal pH, and inactivation of bacterial lactate metabolism, though the casual direction of this relationship is unknown.

Succinate

Succinate is another microbially produced organic acid that is sometimes discussed alongside the SCFAs. Microbially derived succinate has received attention recently in the contexts of metabolic disorders and inflammatory bowel disease, where its role has not been

fully elucidated. The difficulty in elucidating function comes from host-derived succinate, which is connected to hypoxic stress response and inflammation.²⁸ In the intestinal lumen, succinate concentrations are generally low, likely due to scavenging by microbial conversion to propionate.²⁹ Studies have implicated succinate accumulation as a component of intestinal inflammation and inflammatory bowel disease, corroborated by a reduction in succinate consuming species in inflammatory bowel disease patients.^{29,30} Succinate also appears to be relevant in metabolic disease, where it can serve as a precursor for intestinal gluconeogenesis, which improves glucose homeostasis in a similar mechanism to propionate and butyrate.^{31,32} However, in metabolic disease succinate may play a more complicated role, as circulating succinate concentrations are elevated in cases of obesity, type 2 diabetes, and cardiovascular disease.³³ Ultimately, it still remains to be shown whether microbially derived succinate has a causal relationship with inflammatory bowel disease or metabolic disease, and which direction that causality may go.

Bile Acids

Bile acids represent a class of metabolites with well-studied host/microbe interactions but without consensus on function or health outcomes. Bile acids are hydrophobic sterol compounds produced in the liver and secreted into the duodenal lumen and primarily function to improve uptake of hydrophobic nutrients.³⁴ The liver produces conjugated bile acids which are metabolized to form secondary bile acids by select members of the microbiome, primarily Clostridia clusters XVIa and XI.³⁴ Secondary bile acids are reabsorbed and incorporated into the circulating bile acid pool, meaning that the microbiome has a direct impact on the distribution of bile acids in their host. The size of the bile acid pool is significantly larger in germ free mice than

conventionalized mice, suggesting the microbiome regulates the quantity of bile acids as well as structure.³⁵

Microbially transformed bile acids act as signaling molecules in the host. Secondary bile acids can interact with hepatic macrophages to reduce pro-inflammatory cytokine response, potentially mediating a microbial tolerance effect.³⁵ Specific bile acids may have different regulatory effects, such as tauro-conjugated bile acids inhibiting the farnesoid X receptor (FXR) in mice, which regulates synthesis of bile acids in the liver.³⁶ One study also linked bile acid metabolism to post-antibiotic *Clostridium difficile* infection, stating that alterations in the bile acid pool promote germination of *C. difficile* preceding infection.³⁷

Secondary bile acid metabolism represents an important area of study in microbe/host metabolic interactions. It is clear that the microbiome alters the bile acid pool, both in size and structure. These altered bile acid pools likely have downstream effects, as the secondary bile acids are potent signaling molecules, but specific effects on health and promotion or attenuation of disease are not fully understood.

Carcinogens

Due to proximity, much work has been done investigating the interaction between the gut microbiome and colorectal cancer. Of particular interest are the various mutagens, carcinogens, and toxins produced in the microbiome and their potential effects on carcinogenesis. These molecules are produced in all manner of host/microbe interactions, including activation of dietary molecules, biotransformation of host metabolites, and direct production of cytotoxins.

The class of molecules called heterocyclic amines (HAs) are studied for their carcinogenic activity in the context of cooked red meat. Many bacterial species bind HAs, and certain microbes have been associated with increased host HA uptake.^{38,39} In germ free animals,

liver and colon DNA damage is reduced, suggesting that the microbiome plays a role in inducing DNA damage.⁴⁰ Specific work has highlighted *B. fragilis* as increasing the mutagenic potential of fried meat extract, an activity mediated primarily by HAs.³⁸ In addition, lactic acid bacteria, found in dairy fermentation, reduce the uptake of a HA carcinogen in rats, though the specific mechanism has not been elucidated.³⁹

Another potentially toxic activity of the microbiome is the widespread production of B-glucuronidases. In the liver, toxins are often marked for excretion via glucuronidation, where attachment of a glucuronate moiety facilitates secretion into the intestinal lumen. Many species within the microbiome express B-glucuronidases, which harvest glucuronate for bacterial consumption, reactivating toxins and leading to their reuptake and recirculation. This activity has been implicated in colon cancer, as increased activity has been shown in patients with colon cancer and treatment with an enzyme inhibitor reduces tumor prevalence in a carcinogen induced colon cancer model in rodents.^{41,42}

B-glucuronidase activity is also connected to breast cancer and endometrial cancer, as it can impact the levels of circulating estrogen. This emerging conception of the “estrobolome” suggests that the microbiome influences estrogen pools and estrogen exposure, perturbations of which are associated with breast cancer and post-menopausal endometrial cancer.⁴³ Conjugated estrogens are subject to biliary excretion into the intestinal lumen, where microbes can deconjugate and reactivate them.^{44,45} Human fecal extracts metabolize estrogens, converting them between different functional forms, and antibiotic treatments increase the presence of conjugated estrogens in feces.^{46,47} Human colonic microbiota can also metabolize dietary compounds into estrogenic compounds, further supporting their role in modulating estrogen exposure and circulating estrogen pools.⁴⁸

Hydrogen Sulfide

Hydrogen sulfide (H_2S) is rapidly gaining focus as a regulator of various diseases, And its production in the microbiome represents a potential link. H_2S is a gasotransmitter produced in the body from cysteine and is produced in a variety of tissues, affecting a wide range of functions.^{49,50} H_2S exhibits antioxidant cytoprotective effects, acting both as a reducing agent and as a stimulator of expression of oxidative stress response genes. H_2S has also been highlighted for therapeutic potential in cardiovascular disease, where it acts as a vascular relaxant and potential treatment for hypertension. Similarly, it has been shown to increase vascular angiogenesis, which combined with alterations in H_2S levels in atherosclerosis rodent models suggests therapeutic potential as an anti-atherosclerotic.^{51,52}

In addition to endogenous production, H_2S is also produced and consumed by microbiota, where its interactions in the gut have been associated with improving gastrointestinal health. H_2S promotes gastrointestinal ulcer healing and reduces inflammation in the colon, making it a promising target for ulcerative colitis therapies.⁵³ H_2S addition via H_2S chemical donors also induces changes in microbiome composition and structure, increasing the stability of mucosal biofilms.⁵⁴ This has been linked to colitis in mice, where addition of exogenous H_2S both restored mucosal biofilm structure and reduced inflammation.⁵⁵ There is also data suggesting that intestinal epithelial cells can utilize H_2S as an electron donor, making it a possible mechanism for colonocyte feeding via the microbiome.⁵⁶

While H_2S has clear physiological effects, it is still unclear the extent to which the microbiome influences systemic H_2S . Concentrations are highest in the colon,⁵⁴ and the presence of sulfate reducing bacteria in the microbiota suggests microbial production, but the specific effects of microbial H_2S have not been fully elucidated. However, the effects of H_2S on

microbiome composition and biofilm structure, as well as the localized effects of H₂S on colonic epithelial cells and colitis suggest a link that requires further study.

Neurotransmitters

A growing body of evidence supports the presence of a microbial gut-brain axis, with alterations in microbiota resulting in changes in central and enteric nervous system phenotypes. Intestinal microbes have been shown to produce neurotransmitters *in vitro*, including serotonin, dopamine, norepinephrine, and gamma-aminobutyric acid (GABA).⁵⁷

Serotonin, or 5-HT, has been identified as a potential mediator of gut-brain signaling, as 90% of total serotonin synthesis occurs in the gut. Intestinal serotonin is not thought to act in the central nervous system, as it cannot cross the blood-brain barrier, but has broad signaling activity and regulates enteric nervous system development, intestinal motility, and inflammation.⁵⁸ Experiments in germ-free mice show increased production and release of 5-HT in conventionalized mice. This change in 5-HT signaling causes an increase in intestinal motility and enteric neuronal proliferation, implicating the microbiome in mediating enteric nervous system maturation and health.⁵⁹ These effects have notably been confirmed for spore forming intestinal bacteria, and follow-up metabolomics further implicated several candidate metabolites as potential 5-HT inducers, including SCFAs butyrate and propionate, and bile acids cholate and deoxycholate. Indeed, supplementation of deoxycholate in germ free mice restored 5-HT levels in the colon and serum.⁶⁰

5-HT is also implicated as a mediator of a gut/brain interaction with glucose regulation. Antibiotic treatment reduces the total intestinal 5-HT, which induces impaired glucose tolerance.⁶¹ This mechanism requires host 5-HT synthesis to function, suggesting that the microbiota can modulate glucose regulation via induction of endogenous 5-HT synthesis.⁶¹

GABA is a neurotransmitter with wide ranging functions in the central and enteric nervous systems. In the microbiota, GABA is primarily thought to be produced as a regulator of internal pH, though it can also be consumed via the GABA shunt, where it is converted to succinate and incorporated into the TCA cycle.⁶² GABA is a required nutrient for certain intestinal microbes, and production of GABA is widespread among certain genera of intestinal microbiota, including *Bacteroides*, *Parabacteroides*, and *Escherichia*.⁶³

Early work connecting the microbiome to neuronal GABA signaling demonstrated that treatment with *L. rhamnosus* induces transcriptional increases in GABA receptor mRNA, as well as reductions in stress and anxiety metrics.⁶⁴ More recent work has connected GABA signaling with autism in a knockout mouse model, where KO mice show reduced GABA receptor expression.⁶⁵ Treatment with *L. reuteri*, a GABA producer, restored expression of GABA receptors and increased social behaviors, suggesting a link between the microbiome and autism, mediated by GABA signaling.⁶⁵ GABA has also been implicated as a potential microbial mediator in schizophrenia, with fecal transplant into germ free mice causing altered behavior and increases in GABA levels in the brain.⁶⁶ Notably, these studies highlight a link between GABA, neurological function, and the microbiome, but molecular mechanisms are still unclear and more work must be done to solidify these connections.

Discussion

The far-reaching biological effects of the microbiome are both a blessing and a curse. The numerous health impacts of the microbiome give the field near unlimited potential for therapeutics, diagnostics, and quality of life improvements. But these many impacts mean that careless treatment can have massive ripple effects, potentially causing more harm than good. Rational microbiome engineering, the goal of the work described in this dissertation, requires

tremendous respect for the impacts of microbiome perturbations on overall health. It is then crucial to understand microbiome interactions at a fine resolution. By focusing on the metabolites exchanged between microbiome and host, we can achieve that resolution, and identify the specific metabolic reactions involved in health and disease. While this level of understanding is difficult to achieve, it enables fine-tuned manipulations of the microbiome, which will be crucial for effective treatments.

Thesis Summary

This dissertation reports on efforts to manipulate the microbiome to improve host health. In Chapter 1, we demonstrate an exercise-associated microbial genus, *Veillonella*, and proceed to isolate a strain of *Veillonella atypica* from elite athletes which displays performance-enhancing effects in mice. We further demonstrate that *Veillonella* carbon metabolism, which converts lactate to acetate and propionate, increases in abundance post-exercise, and that propionate supplementation mirrors the performance-enhancing effects of *Veillonella atypica*. We propose a model where lactate produced during exercise can feed the microbiome, causing a bloom in lactate utilizers such as *Veillonella*, and the subsequent production of propionate improves exercise performance. In Chapter 2, we utilize a probiotic *E. coli* Nissle strain to compete with the host for absorption of glucose in an oral glucose bolus, to reduce the glycemic impact of a meal. However, while *E. coli* Nissle gavage reduced the glycemic impact of a glucose bolus, there was no difference when *E. coli* Nissle was compared to a glucose-null mutant, suggesting this effect did not depend on glucose metabolism.

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Chapter 1

Performance Enhancing Microbes in Elite Athletes

This Chapter is a reproduction of a published manuscript:

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Author Contributions:

T.A.C., J.S., and J.M.L contributed equally to the project. J.S., G.M.C. and A.D.K. conceived the project. J.S. collected the athlete samples and metadata. J.M.L. performed the computational analysis and modeling, with assistance from T.A.C., M.C.W., R.C.W., B.T.T., Z.Y. and M.W.H. T.A.C. designed and performed the model organism experiments, with assistance from J.M.L., T.M., A.T., L.-D.P., M.C.W., S.P. and S.L. J.A.-P. and C.B.C. processed the metabolomic samples. J.M.L., T.A.C. and A.D.K. wrote the manuscript. G.M.C. and A.D.K. supervised the project.

Abstract

The human gut microbiome encodes a vast metabolic repertoire, yet it is unknown whether it can impact exercise performance. To begin to address this question, we performed longitudinal metagenomic analysis on runners in the Boston Marathon to identify microbiome features associated with intense exercise. The strongest microbiome feature enriched post-marathon was an increase in the abundance of the bacterial genus *Veillonella*. We isolated *Veillonella atypica* directly from the marathon runners and found that inoculation in laboratory mice increased exhaustive treadmill runtime by 13% in a *V. atypica*-dependent manner. *Veillonella* utilize lactate as their sole carbon source, which prompted us to perform shotgun metagenomic analysis in a cohort of elite athletes, finding that every gene in a major pathway metabolizing lactate to propionate is at higher relative abundance post-exercise. Using $^{13}\text{C}_3$ -labeled lactate in mice we demonstrate that serum lactate crosses the epithelial barrier into the lumen of the gut. We also show that intrarectal instillation of propionate is sufficient to reproduce the increased treadmill runtime performance observed with *V. atypica* gavage. Taken together, these studies reveal that *V. atypica* improves runtime via its metabolic conversion of exercise-induced lactate into propionate, thereby identifying a natural, microbiome-encoded enzymatic process that enhances athletic performance.

Introduction

Human microbiome studies have generally examined individuals who are “healthy” or diseased and identified features of the microbiome associated with these states.¹⁻⁴ Athlete microbiomes have been found to contain distinct microbial compositions defined by elevated abundances of *Veillonella*, *Bacteroides*, *Prevotella*, *Methanobrevibacter* or the

Akkermansiaceae.^{5,6} These studies show exercise is associated with microbiome composition. However, the effect of these specific microbiome compositions on phenotype remains unknown.

Results

To identify gut bacteria associated with athletic performance and recovery states, we recruited athletes (n=15) who ran in the 2015 Boston Marathon along with a set of sedentary controls (n=10) and conducted 16S rDNA sequencing on approximately daily samples collected up to one week before and one week after marathon day (n=209 samples, **Supplementary Tables 1 and 2**). Phylum-level relative abundance partitioned by individual, time (-5 to +5 days in relation to running the Marathon), and whether the participant was an athlete (**Figure 1-1A**) shows that, at this high-level taxonomic view, any orthogonal differences are likely due to variation at the level of the individual. The bacterial genus *Veillonella* is the most differentially abundant microbiome feature between pre- and post-exercise states (**Supplementary Table 2**). *Veillonella* has a significant difference in relative abundance ($P = 0.02$; Wilcoxon rank sum test with continuity correction) between samples collected before and after exercise (**Figure 1-1B**). To validate the significance of the association between *Veillonella* and post-marathon state, we constructed a series of generalized linear mixed effect models (GLMMs) to predict *Veillonella* relative abundance in the marathon participants (**Figure 1-1C**, methods). Subsequently, significance was calculated for all coefficients included in the GLMM (**Figure 1-1D**, Wald Z-tests); revealing no coefficients were significant except time in relation to the marathon in days ($P = 0.0014$, Wald Z-test, n=15). Both leave-one-out cross-validation (LOOCV) and iterative permutation of labels were conducted as part of the GLMM analysis

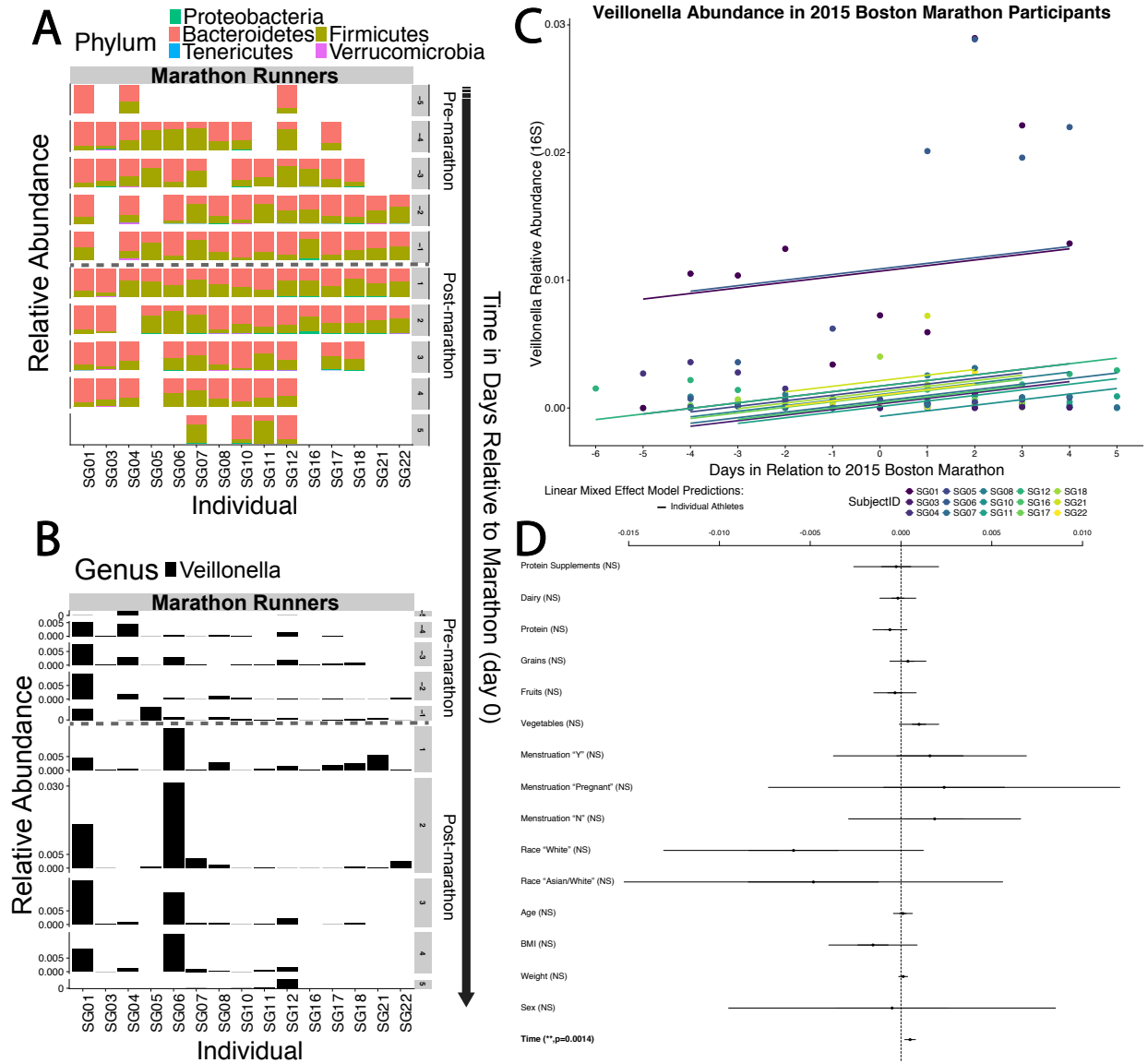


Figure 1-1: Gut Veillonella abundance is significantly associated with marathon running. **A:** Phylum level relative abundance partitioned by individual and time (-5 to +5 days in relation to running the Marathon) shows few global differences in composition. **B:** Veillonella relative abundance at the Genus level partitioned by individual and time (-5 to +5 days in relation to running the Marathon) shows that Veillonella has a significant difference in relative abundance ($P = 0.02$; two-sided Wilcoxon rank sum test with continuity correction; $n = 15$ individuals) between samples collected before and after exercise. **C:** Generalized linear mixed effect models (GLMMs) predicting longitudinal Veillonella relative abundance in the marathon participants. Differences in intercept between fits for different marathoners represent random effects. **D:** 95% confidence intervals for all fixed effects (coefficients) included in the GLMMs. The Y-axis represents Veillonella relative abundance and the X-axis represents (time days in relation to running the Marathon). All coefficients except time ($P = 0.0014$, Wald Z-test, post-marathon time points correspond with increased Veillonella relative abundance) are not significant, suggesting that Veillonella blooms in runners correspond with exercise state and not other fixed effects ($n = 15$ individuals).

(**Supplementary Figure 1**, methods). Additionally, it appears that *Veillonella* is more prevalent among runners than non-runners (**Supplementary Figure 2**), though this is not statistically significant. These correlations raise the question whether there is a causal link between *Veillonella* and marathon runner's performance, but no conclusions can be made without proper validation.

To assess whether there are any potential benefits of *Veillonella* on performance in an animal exercise model, we designed an AB/BA crossover mouse experiment spanning two weeks consisting of a control group (*Lactobacillus bulgaricus* gavage, n=16) and a treatment group (*Veillonella* gavage, n=16) with a treatment/control crossover happening between weeks (n=32 total mice). *Lactobacillus bulgaricus* was chosen as a control due its inability to catabolize lactate, mimicking bacterial load without impacting lactate metabolism.⁷ The *Veillonella* strain used, *Veillonella atypica*, was directly isolated from one of the marathon runners. Mice were administered either *Veillonella atypica* or *Lactobacillus bulgaricus* and run to exhaustion five hours later (methods). In aggregate, on both sides of the crossover, mice gavaged with *Veillonella atypica* have statistically significant longer maximum runtimes than mice gavaged with *Lactobacillus bulgaricus* ($P = 0.02$ paired t-test, **Figure 1-2A**, **Supplementary Table 3**, **Supplementary Figure 3**). Both leave-one-out cross-validation (LOOCV) and iterative permutation of labels were conducted as part of the GLMM analysis (**Supplementary Figure 3**, methods). Per-mouse run times overlaid on the GLMM fits (**Supplementary Figure 4**) and the difference between max run time in *Lb. Bulgaricus* versus *V. atypica* gavage show a distinction between “responders” and “non-responders” to *V. atypica* gavage (**Supplementary Figure 5**). Mice treated with *Veillonella atypica* run on average 13% longer than the control group (**Figure 1-2A**). Testing the significance of coefficients in the GLMM for their contribution to

treadmill runtime (Wald-Z test) shows that sequence effect is not significant ($P = 0.758$) while treatment day ($P = 0.031$, negative effect on runtime) and *Veillonella* treatment ($P = 0.016$, positive effect on runtime) are significant (**Figure 1-2B; Supplementary Figure 3**). In a separate experiment, levels of inflammatory cytokines were quantified post exercise and were significantly reduced in *Veillonella*-treated animals compared to *Lb. bulgaricus* or PBS (**Supplementary Figure 6, Supplementary Table 4**). To assess changes in muscle physiology, the glucose transporter GLUT4 was quantified via western blot, but we observed no changes regardless of treatment (**Supplementary Figure 7**).

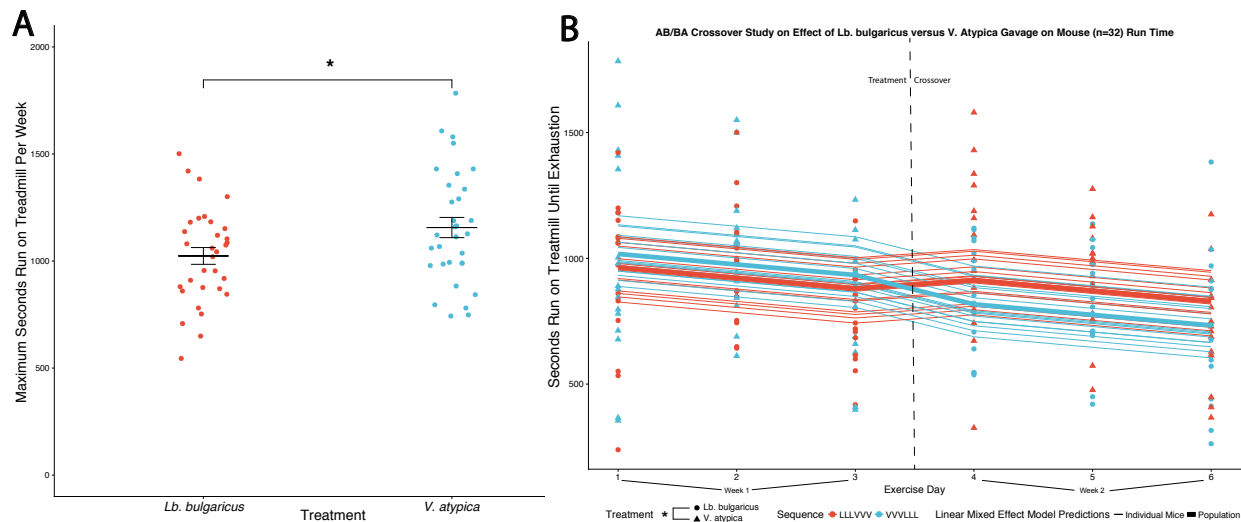


Figure 1-2: *Veillonella atypica* gavage improves treadmill runtime in mice. **A:** Mice gavaged with *Veillonella atypica* have greater maximum run time per week than mice gavaged with *Lactobacillus bulgaricus* in an AB/BA crossover trial. Data shown are the maximum run time out of 3 days of consecutive treadmill running for a given treatment (all mice switched treatments second week). The jitter plot shows each mouse as an individual point, with the central bar representing the mean and error bars representing s.e.m. ($n = 32$). ($*P = 0.02$, using two-sided paired t-test). **B:** Generalized linear mixed effect models (GLMMs) predicting runtime in the 2 week AB/BA crossover trial. The Y-axis shows seconds run on treadmill until exhaustion and the X-axis shows days which the mice were run in the 2 week crossover. Color of lines (GLMM fits) and points (runs by an arbitrary mouse) represents treatment sequence; shape of points represents treatment at a given time point. These models incorporate both random effects (individual variation per mouse that manifests longitudinally) and fixed effects (treatment day, treatment sequence, and treatment given). Visualization of all longitudinal data points with the GLMM predictions overlaid show both the effect of *Veillonella atypica* increasing performance on both sides of the crossover when aggregated by treatment group (thick lines) as well as the trends for each of the 32 individual mice (thin lines). ($*P=0.016$, Wald-Z test on model coefficients).

To test whether our results would be replicated in an independent cohort of human athletes, we performed shotgun metagenomic sequencing of stool samples (n=87) from ultra-marathoners and Olympic trial rowers both before and after exercise (**Supplementary Table 6**). Putative taxonomic abundances reproduced the previous 16S sequencing-based association with *Veillonella* (**Supplementary Figure 8**).⁸ By utilizing novel algorithms that allow for cheap construction of metagenomic gene catalogs at massive scale through efficient use of cloud computing, we investigated phenotypic modulating effects of millions of microbial genes on athletes by building a sample (n=87) by gene (n=2,288,155) relative abundance matrix (**Supplementary Figure 8 and 9**; methods).^{9–13} The inability of *Veillonella* to ferment carbohydrates, coupled with high observed abundance of the lactate import permease in previously sequenced isolates, suggests that metabolic enzymes facilitating lactate breakdown are likely conserved.¹⁴ Across the entire ultramarathon and rower cohorts, there exist a group of gene families with differential relative abundance pre and post exercise (**Supplementary Figure 9**) representing every step of the enriched methylmalonyl-CoA pathway ($P = 0.00147$; methods) degrading lactate into propionate as assigned by EC IDs (**Figure 1-3A**). Given the limited prevalence of the methylmalonyl-CoA pathway across lactate-utilizing microbes, this enrichment post-exercise may implicate *Veillonella* in causing functional change in the metabolic repertoire of the gut microbiome. We verified strong production of acetate and propionate by performing mass spectrometry on spent media collected after growing three *Veillonella* strains isolated from the human athletes (*V. parvula*, *V. dispar*, and *V. atypica*) in lactate-supplemented BHI media and semi-synthetic lactate media (**Figure 1-4A and Supplementary Tables 7 and 8**; methods).

Veillonella species metabolize lactate into the SCFAs acetate and propionate via the methylmalonyl-CoA pathway.¹⁵ Lactate dehydrogenase (LDH), the enzyme responsible for the

first step of lactate metabolism, is present in a phylogenetically diverse group of bacteria (**Figure 1-3B**). Querying microbial isolate strain genome annotations from NCBI show that unlike *Veillonella atypica*, many other microbes are theoretically capable of utilizing lactate through LDH, but do not possess the full pathway to convert lactate into propionate (**Figure 1-3C**). Other obligate anaerobes such as *Anaerostipes caccae* and *Eubacterium hallii* commonly ferment

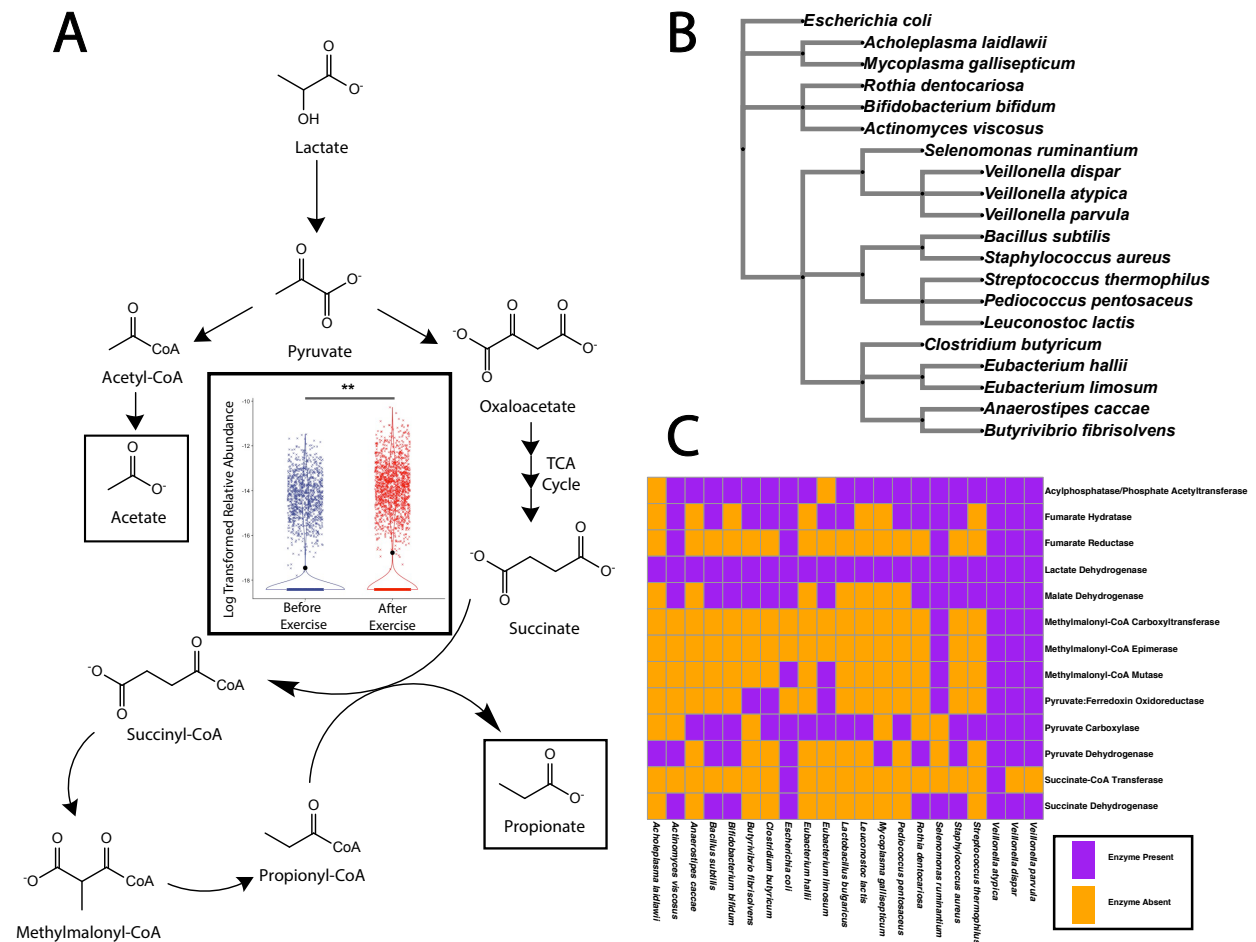


Figure 1-3: The athlete gut microbiome is functionally enriched for the metabolism of lactate to propionate post-exercise. **A:** methylmalonyl-CoA pathway and inset showing significant differentially expressed gene families pathway wide in a pair of non-redundant gene catalogs created from metagenomic sequencing of athlete stool samples. Log transformed relative abundance increases after exercise for every enzyme in the methylmalonyl-CoA pathway. (**P = 0.00147; two-sided Fisher's exact test; n = 8, contingency table constructed for enzymes in pathway). Data represented as a violin plot, which displays the distribution of data as a rotated kernel density distribution. **B:** Bacterial phylogenetic tree showing diversity of microbes that have the ability to utilize Lactate as a carbon source. **C:** Prevalence of enzymes in the methylmalonyl-CoA pathway that breaks down lactate into acetate and propionate in reference genomes from this represent subset of lactate processing microbes.

lactate into butyrate via different pathways (**Figure 1-3C**). *Eubacterium hallii* can also produce propionate, however this has been demonstrated as a biotransformation of 1,2-propanediol, rather than a complete pathway from lactate to propionate. Of note, both the reference genomes on NCBI for *Veillonella dispar* and *Veillonella parvula* are not annotated to have the succinate-CoA transferase needed for propionate production to occur; this is likely an annotation error as we validated production of propionate via mass spectrometry on isolates of these species (**Supplementary Table 7**).

Taken together, these results show that not only is the genus *Veillonella* enriched in athletes after exercise but the metabolic pathway that *Veillonella* species utilize for lactate metabolism is also enriched. This result raised the possibility that systemic lactate resulting from muscle activity during exercise may enter the gastrointestinal lumen and become metabolized by *Veillonella*.

We next sought to determine whether systemic lactate is capable of crossing the epithelial barrier into the gut lumen, as this has not been demonstrated before to our knowledge. To investigate this, we performed tail-vein injection of $^{13}\text{C}_3$ sodium lactate into mice colonized with either *Veillonella atypica* or *Lactobacillus bulgaricus* and sacrificed 12 minutes after injection. This time-point was chosen because it was the earliest time at which we observed serum lactate levels return to baseline levels after tail-vein injection in pilot experiments. At sacrifice, we immediately collected serum and plasma following cardiac puncture and collected intestinal luminal contents by removing the colon and cecum from the mice and gently sampling the inner surface of the tissue. By performing liquid-chromatography and mass spectrometry (LC-MS) on these tissues, we were able to identify $^{13}\text{C}_3$ -labelled lactate present in both the serum and plasma as well as in the lumen of the colons and ceca (**Figure 1-4B-D, Supplementary Table 9**). We

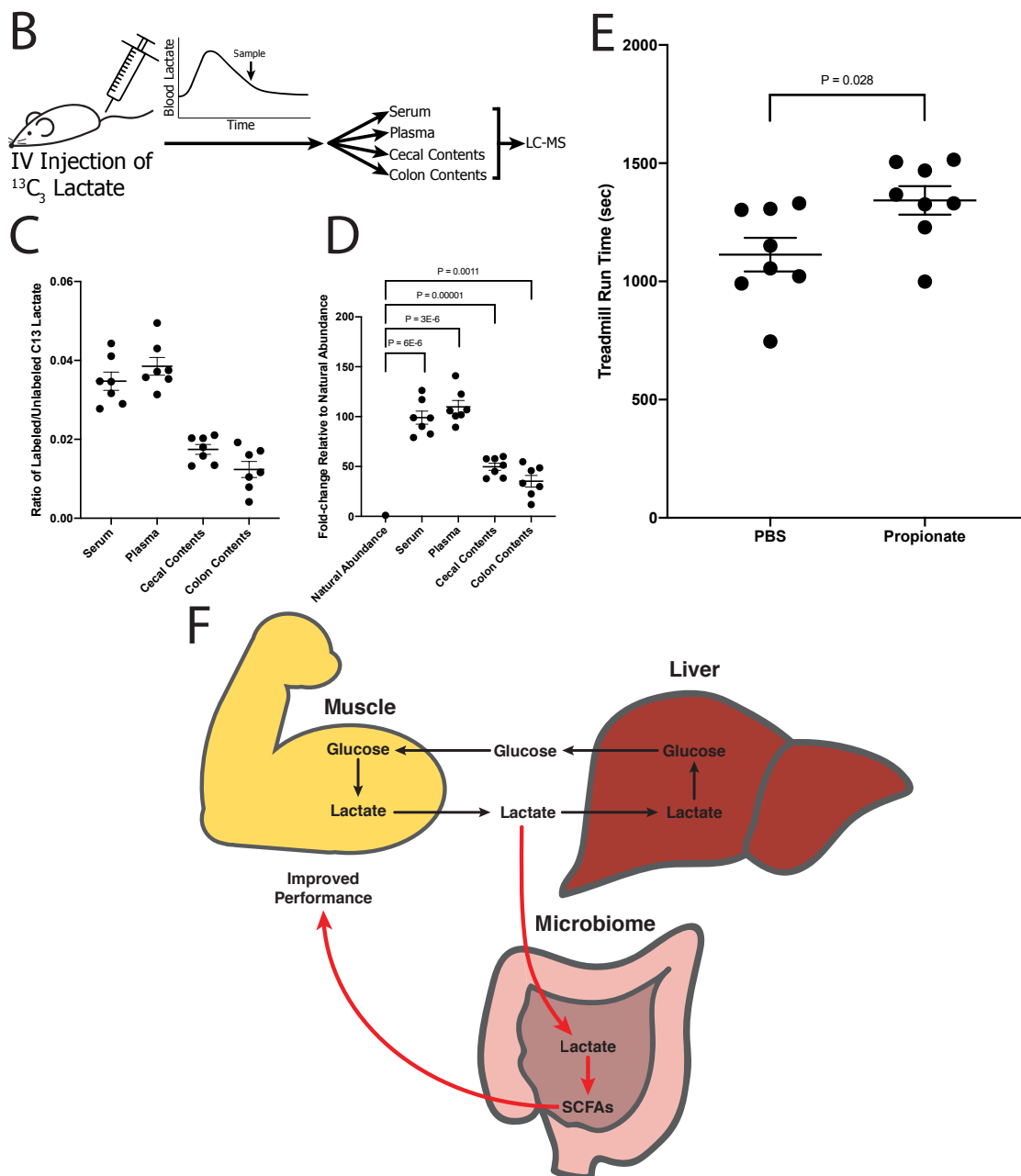
were unable to detect any $^{13}\text{C}_3$ -labelled propionate in these tissues, however the 12-minute time-point from tail-vein injection to sacrifice is likely insufficient time for labelled lactate crossing the gut barrier to be metabolized into propionate by gut *Veillonella*.

As we have shown that serum lactate is capable of entering the intestinal lumen, we sought to determine whether *Veillonella* colonization may actively limit blood lactate levels by serving as a metabolic “sink.” To test the capability of *Veillonella* to accelerate blood lactate clearance *in vivo*, we performed intraperitoneal injections of sodium lactate in mice colonized with either *V. atypica* or *L. bulgaricus* and monitored blood lactate over time. Neither the basal nor the peak lactate levels between the treatment groups were significantly different (**Supplementary Figure 10, Supplementary Table 10**). The vast majority of lactate processing occurs in the liver¹⁶, and although systemic lactate infiltrates the intestinal lumen, we did not observe a change in overall lactate clearance upon inoculation with *Veillonella*.

Propionate has been shown to increase heart rate, VO_2 max, and affect blood pressure in mice^{17–19} as well as raise the resting energy expenditure and lipid oxidation in fasted humans.²⁰ To test whether the exercise-enhancing effects of *Veillonella* may be attributable at least in part to propionate, we performed intrarectal instillation of propionate in our mouse treadmill model. Propionate was introduced intrarectally rather than orally because colonic absorption provides a more direct route for propionate to reach systemic circulation, mirroring the location of *Veillonella*-sourced propionate. Intrarectal propionate instillation (n=8) compared with saline vehicle (n=8) resulted in increased treadmill runtime similar to that of *Veillonella atypica* gavage ($P = 0.03$, **Figure 4E**). As in the *Veillonella* gavage experiments, we ran the same panel of inflammatory cytokines on serum taken 40 minutes after treadmill running but found no significant differences in cytokine levels (**Supplementary Figure 6, Supplementary**

A

	Butyrate (μM)	Propionate (μM)	Pyruvate (μM)	Lactate (μM)	Acetate (μM)
<i>V. atypica</i> LM	19 $\pm$ 0.3***	10611 $\pm$ 584**	4 $\pm$ 0.4***	431 $\pm$ 5.6***	92432 $\pm$ 3129**
<i>L. bulgaricus</i> LM	9 $\pm$ 0.5*	3 $\pm$ 1.5	32 $\pm$ 2.7	737 $\pm$ 45	4985 $\pm$ 247**
LM Alone	11 $\pm$ 0.1	4 $\pm$ 0.5	41 $\pm$ 0.4	851 $\pm$ 6.6	1741 $\pm$ 12.2
<i>V. atypica</i> BHIL	69 $\pm$ 0.5*	2286 $\pm$ 68*	n/a	n/a	4149 $\pm$ 118*
BHIL Alone	147 $\pm$ 4.1	160 $\pm$ 1.4	n/a	n/a	7557 $\pm$ 30



(Legend on next page)

Figure 1-4: Serum lactate crosses the epithelial barrier into the gut lumen and colorectal propionate instillation is sufficient to enhance treadmill runtime. **A:** SCFAs detected in spent media after 48 hours of growth with the indicated strain. LM = semi-synthetic lactate media; BHIL = brain-heart infusion media supplemented with sodium lactate; n/a = not quantified. Each table entry shows the mean $\pm$ s.e.m. (BHIL, n = 2; LM, n = 3). (p values from left to right, top to bottom: .0008, .003, 4.4E-7, 1.4E-6, .001, .023, .006, .03, .02, .015; compared with media control using two-sided Welch's t-test). **B:** Schematic of the $^{13}\text{C}_3$ flux tracing experimental design. Mice were injected with $^{13}\text{C}_3$ sodium lactate, then sacrificed after 12 minutes. Serum and plasma were collected via cardiac puncture. Cecum and colon contents were collected by dissection. **C:** Abundance of $^{13}\text{C}_3$ lactate quantified relative to abundance of unlabeled lactate. Each mouse sample is represented as an individual point, with the central bar representing the mean and error bars representing s.e.m. (n = 7). **D:** $^{13}\text{C}_3$ lactate abundance normalized to the expected natural abundance of $^{13}\text{C}_3$ lactate. Ratio of labeled/unlabeled lactate was quantified for experimental samples as well as for unlabeled lactate standard. Experimental samples are represented as fold-change relative to unlabeled standard. Each mouse sample is represented as an individual point, with the central bar representing the mean and error bars representing s.e.m. (n = 7). (p values are from two-sided one sample t-test vs natural abundance). **E:** Intracolonic infusion of propionate improves maximum run time in mice. Data shown are the maximum run time out of 3 days of consecutive treadmill running. The jitter plot shows each mouse as an individual point, with the central bar representing the mean and error bars representing s.e.m. (n = 8). (p value from two-sided unpaired t-test). **F:** Proposed model of the microbiome-exercise interaction. Black arrows represent the well-known steps of the Cori cycle, where glucose is converted to lactate in the muscle, enters the liver via blood circulation, then is converted back to glucose in the liver via gluconeogenesis. Red arrows represent the steps proposed in this work. First, lactate produced in the muscle enters the intestinal lumen via blood circulation. In the intestine it acts as a carbon source for specific microbes, including *Veillonella* species. This causes the observed bloom in intestinal *Veillonella*, as well as production of SCFA byproducts (predominantly propionate), which are taken up by the host via the intestinal epithelium. Presence of microbiome-sourced SCFAs in the blood improves athletic performance via an unknown mechanism. Together, this creates an addendum to the Cori cycle by converting an exercise byproduct into a performance-enhancing molecule, mediated by naturally occurring members of the athlete gut microbiome.

Table 5). Therefore, introduction of propionate into the colon is sufficient to result in an enhanced exercise phenotype via a mechanism that does not impact the inflammatory cytokines measured.

Discussion

Coupling computational approaches, multi-omic data collection approaches, and experimental validation looks promising as a method to approach unvalidated metagenomic associations that have been proposed in the past decade. Acting on this principle, we observe: 1) *Veillonella* abundance increases in the gut microbiome post-exercise in two independent cohorts of athletes; 2) the *Veillonella* methylmalonyl-CoA pathway is overrepresented in athlete metagenomic samples post exercise; 3) systemic lactate can cross the gut barrier into the lumen

of the gut; 4) in a longitudinal AB/BA crossover study in mice, *Veillonella* inoculation improves treadmill performance; and 5) treadmill performance is improved in mice administered propionate via intracolonic infusion.

These data illustrate a model in which systemic lactate produced during exercise crosses to the gut lumen and is metabolized by *Veillonella* into propionate in the colon, which in-turn serves to promote performance. Gut colonization of *Veillonella* may be augmenting the Cori cycle by providing an alternate lactate processing method where systemic lactate is converted into SCFAs that re-enter circulation (**Figure 1-4F**). SCFAs are absorbed in the sigmoid and rectal region of the colon as it enters the pelvic plexus, bypassing the liver and draining via the vena cava to reach systemic circulation directly.²¹ Microbiome-derived SCFAs then augment performance directly and acutely, suggesting that lactate generated during sustained bouts of exercise could be accessible to the microbiome and converted to these SCFAs that improve athletic performance.

In conclusion, we demonstrate for the first time that the microbiome may be a critical component of physical performance and the benefit derived from it. An important question is how this performance-facilitating organism has come to be more prevalent amongst athletes in the first place. We propose that the high-lactate environment of the athlete provides a selective advantage for colonization by lactate metabolizing organisms such as *Veillonella*. Future studies are needed to help explain why there is an apparent preference for *Veillonella* and not one of the many other lactate-metabolizing organisms. *Veillonella* in the physically active host therefore serves as a potential example of a symbiotic relationship in the human microbiome.

Methods

Participant recruitment

All study participants were recruited following an IRB approved Sports Genomics protocol (#IRB15-0869), conducted at the Wyss Institute for Biologically Inspired Engineering. Each participant read and signed a consent form prior to study enrollment. We have complied with all relevant ethical regulations.

Sample collection, extraction, and library preparation

As collection materials, study participants were provided a 15ml falcon tube with a 1ml pipette tip inserted inside. Participants were instructed to dip pipette tips into soiled toilet tissue then place back into tubes and label with date and time of collection. Samples were kept at 4°C for short term storage until sample pickup, at which time they were immediately placed onto dry ice, then transferred to a -80°C freezer for long term storage.

Fecal samples were thawed on ice and resuspended into 2-5ml of Phosphate Buffered Saline, of which 250µl was used for DNA extraction using the MOBIO Power Soil high throughput DNA extraction kit, following manufacturer's protocol. For 16s rDNA library construction, 1-5µl of purified DNA was used for PCR amplification of the V4 variable region using the Q5 hotstart polymerase (NEB). Primers were adapted from the Earth Microbiome Project (<http://www.earthmicrobiome.org/>), attaching illumina PE adaptors (Forward: CTT TCC CTA CAC GAC GCT CTT CCG ATC TGT GCC AGC MGC CGC GGT AA, Reverse: GGA GTT CAG ACG TGT GCT CTT CCG ATC TGG ACT ACH VGG GTW TCT AAT). illumina barcodes were added to libraries during a second PCR step (Forward: AAT GAT ACG GCG ACC ACC GAG ATC TAC ACT CTT TCC CTA CAC GAC GCT C, Reverse: CAA GCA GAA GAC GGC ATA CGA GAT GTG ACT GGA GTT CAG ACG TGT GCT C) and end

products were purified via column (Zymo Research). Individual libraries were quantified and normalized for sequencing using the Quant-It Picogreen reagent (Thermo Fisher). For whole genome shotgun library construction, 1ng of purified DNA was used for illumina's Nextera XT Tagmentation kit, following manufacturer's protocol. Libraries were submitted to the Harvard Biopolymers core sequencing facility for bioanalyzer QC and 150 b.p. paired-end sequencing reads using either illumina miseq or Hiseq 2500 (high output mode) for 16s rDNA and shotgun analysis, respectively.

Metadata collection

Each study participant was provided a questionnaire to collect health, dietary, and athletic background information (adapted from The American Gut, <http://americangut.org/>). Additionally, for each sample collection, study participants filled out a daily annotation sheet to collect dietary, exercise, and sleep information.

16S Analysis

Each subject in our study provided us with fecal samples on a daily basis, up to one week before and one week after the marathon (controls did not run in the marathon but provided fecal samples). We next extracted genomic DNA from these samples and performed 16S rDNA amplicon sequencing followed by bioinformatic analysis to obtain genus level resolution of bacteria in each individual's microbiome (Supplementary Tables 1 and 2).

16S reads were processed with the dada2 pipeline and phyloseq.^{27,28} Default settings were used for filtering and trimming. Built in training models were utilized to learn error rates for the amplicon dataset. Identical sequencing reads were combined through dada2's dereplication functionality and the dada2 sequence-variant inference algorithm was applied to each dataset. Subsequently, paired end reads were merged, a sequence table was constructed, taxonomy was

assigned, and abundance was calculated at all possible taxonomic levels.

16S Mixed Effect Modeling in Human Cohort

We constructed a series of generalized linear mixed effect models (GLMMs) to predict *Veillonella* relative abundance in the marathon participants from both random effects (individual variation per athlete that manifests longitudinally) and fixed effects (USDA MyPlate consumption categories, protein powder supplementation, menstruation status, race, time, BMI, weight, gender, and age).

The longitudinal nature of the microbiome sampling coupled with the unique lifestyles of athletes means that diet, physical characteristics, age, gender, ethnicity, and the menstrual cycle could potentially confound the association between post-marathon state and *Veillonella* relative abundance.^{29,30} As some food compounds can selectively increase relative abundance of *Veillonella*, 1,267 meal records logging every instance of food consumption over the course of the study (Supplementary Table 1) were quantified according to USDA MyPlate and associated with daily microbiome samples. Leave-one-out cross-validation (LOOCV) was performed for the GLMM analysis where the p-value for the time coefficient was calculated for all permutations of eliminating one athlete, which revealed a general trend of no individual athlete driving significance, with one minor outlier (Supplementary Figure 1, Wald Z-tests). To ensure that an arbitrary shuffling of participant labeling would not yield significant results, the GLMM was trained 1000 times on input data with permuted labels, which generated uniformly distributed p-values and shows the significance of the original labeling (Supplementary Figure 1, Wald Z-tests). Thus, the observed significance of the association between *Veillonella* relative abundance and pre- and post-marathon state is likely not confounded by any fixed effects. To test

whether *Veillonella* has any phenotypic impact on running ability, we next introduced *Veillonella* to mice in a treadmill experiment.

16S *Veillonella* relative abundance modeling for athletes participating in the marathon was done with the R nlme package.³¹ 1,267 meal records logging every instance of food consumption over the course of the study were quantified according to USDA MyPlate and associated with daily 16S samples by a nutritionist. Relative abundance was first modeled as:

$$\text{Abundance} = \beta_0 + \beta_{\text{Time}} + \beta_{\text{Sex}} + \beta_{\text{Weight}} + \beta_{\text{BMI}} + \beta_{\text{Age}} + \beta_{\text{Race}} + \beta_{\text{Menstruation}} + \beta_{\text{Vegetables}} + \beta_{\text{Fruits}} + \beta_{\text{Grains}} + \beta_{\text{Protein}} + \beta_{\text{Dairy}} + \beta_{\text{Dietary Protein Supplementation}}$$

Subsequently, a second model was generated that included interaction terms of Time:Vegetables and Time:Menstruation. Significance was calculated for all coefficients included in the GLMM with Wald Z-tests (default calculation in the library utilized). Coefficients were created with the coefplot2 package.³² Model results were validated with both leave-one-out cross-validation (LOOCV) and permutation testing on shuffled labels.

Preparation of bacteria for gavage

Veillonella atypica and *Lactobacillus bulgaricus* were grown in 250mL BHI broth supplemented with lactate (10mL 60% sodium lactate/L) and MRS broth, respectively. OD₆₀₀ was monitored and at OD 0.4-0.6, cells were pelleted by refrigerated centrifugation at 5,000g for 10 min. The pellet was washed in PBS and resuspended in 2mL residual PBS. 100μL aliquots were frozen at -80°C and CFU/mL measured by serial dilution onto BHI lactate agar plates. *Veillonella atypica* was gavaged in wild-type C57BL/6 mice to determine viability and transit time through the GI tract, observing peak viable bacterial CFU counts in fecal pellets 5h after gavage.

Treadmill crossover experiment

Animal research was approved by the Joslin Diabetes Center IACUC. We have complied with all relevant ethical regulations. For treadmill experiments, 8-12 week old CL57BL/6 mice (n=32) were acclimated to treadmilling with 2 bouts of 30 minutes of 5 m/min walking, split over 2 consecutive days. For exhaustion measurements, mice were fasted for 7 hours prior to exercise. 6 hours prior to exercise, mice were gavaged with 200 μ L of 2.5% sodium bicarbonate to neutralize stomach contents, then 20 minutes after the first gavage, mice were gavaged 200 μ L of either *V. atypica* or *Lb. bulgaricus*, prepared as above and normalized to 5×10^9 CFU/mL. 5 hours post gavage, mice were run on the treadmill, starting at 5 m/min and increasing speed by 1 m/min every minute until exhaustion. Time of exhaustion was recorded for every animal, defined as a mouse failing to return the treadmill from the rest platform after three consecutive attempts to continue running. This protocol was repeated for 2 more days, followed by 4 days of rest and 3 days of crossover treatment. On the first day of treatment, serum was collected 40 minutes post-exhaustion via tail-vein bleed and measured using the Ciraplex multiplex mouse cytokine assay (Aushon BioSystems).

Treadmill Runtime Mixed Effect Modeling

Despite the high number of mice utilized in the AB/BA crossover experiment, comparisons of raw runtime in this context could be confounded both by carryover effect (modeled as a sequence effect) inherent in the longitudinal study design as well as unavoidably high inter-mouse variation. To account for this, we constructed a series of GLMMs predicting runtime (methods). These models incorporate both random effects (individual variation per mouse that manifests longitudinally) and fixed effects (treatment day, treatment sequence, and treatment given). Modeling was conducted with the R nlme package.³¹ Visualization of coefficients was conducted using the coef2plot R package.³² Visualization of predictions

overlayed on data was conducted using the R ggplot2 package.²³

Visualization of all longitudinal data points with the GLMM predictions overlayed show both the effect of *Veillonella atypica* increasing performance on both sides of the crossover when aggregated by treatment group (thick lines) as well as the trends for each of the 32 individual mice (thin lines) (**Figure 1-2B**). LOOCV was performed for the GLMM analysis where the p-value for the *V. atypica* treatment coefficient was calculated for all permutations of eliminating one mouse, which revealed that no individual mice are driving significance (**Supplementary Figure 3**, Wald Z-tests). To ensure that an arbitrary shuffling of mouse labeling would not yield significant results, the GLMM was trained 1000 times on input data with permuted labels, which generated uniformly distributed p-values and shows the significance of the original labeling (**Supplementary Figure 3**, Wald Z-tests). This longitudinal modeling approach allows us to interpret that as the treadmill runs are conducted back-to-back each week on subsequent days, the mice in aggregate have decreasing runtimes as time to exhaustion decreases (visible as slope of predictions in **Figure 1-2B**), while *Veillonella atypica* treatment independently increases runtime (visible as crossover of predictions showing *Veillonella* treatment group having longer time to exhaustion on both sides of the crossover in **Figure 1-2B**). To identify possible biological mechanisms of *Veillonella* effect, we quantified levels of various inflammatory cytokines in the blood immediately following treadmill run. We observed several pro-inflammatory cytokines, including TNFa and IFNg, were significantly reduced in *V. atypica*-treated mice compared to both baseline and control treatment (**Supplementary Figure 6**, **Supplementary Tables 4 and 13**). In a separate experiment, we quantified levels of the muscle glucose transporter GLUT4 to assess effects on muscle physiology but found no difference between *V. atypica*-treatment and control (**Supplementary Figure 7**). Altogether, taking into

account inter-mouse variation, longitudinal study design, and possible carryover effect in an AB/BA crossover trial, *Veillonella atypica* treatment causes substantial increases in treadmill runtime in mice.

Models were constructed to predict treadmill runtime in the AB/BA crossover experiment to include treatment effect of *Veillonella*, period effects (time of treatment), carry-over effects due to the treatment crossover, and effects for naturally occurring mouse variation. In general, we can model expected runtime as:

Sequence: *V. atypica* → *L. bulgaricus* Week 1: $\mu + \pi_1 + \alpha_A$ Week 2: $\mu + \pi_2 + \alpha_B + \lambda_A$

Sequence: *L. bulgaricus* → *V. atypica* Week 1: $\mu + \pi_1 + \alpha_B$ Week 2: $\mu + \pi_2 + \alpha_A + \lambda_B$

Where α_A and α_B are treatment effects, λ_A and λ_B are carry-over effects, and π_1 and π_2 are period effects.

Metagenomic Analysis

All steps in the processing of raw metagenomic data were done utilizing the Aether package.³³ Raw reads were *de novo* assembled using megahit.³⁴ Open reading frames and annotations were generated using prokka³⁵. A gene family catalog was generated from the called open reading frames at 95% identity utilizing the CD-HIT software package.³⁶ A raw abundance count matrix was generated utilizing the gene family catalog, bowtie2, and samtools.^{37,38} The raw abundance count matrix was normalized both by sample and by gene length.³⁹ Metabolic pathways were queried using MetaCyc and EC IDs were pulled from prokka annotations.^{35,40} R was utilized to perform the majority of statistical tests with the exception of pairwise ANOVA tests, for which the SciPy library in python was used.⁴¹ Root mean square error calculations were performed using the plotrix package.⁴²

Metaphlan2 Taxonomy in Metagenomics Data

Putative taxonomic abundances were calculated with Metaphlan2⁴³ and found to have the same association between *Veillonella* and exercise status as the previous marathon runner results (p=0.03; **Supplementary Figure 8**)

Annotations

To compare trends in the aggregate microbiome with the metabolic processes of microbes that had elevated 16S abundance in the prior experiment, a pairwise ANOVA was performed on all ~2.3M genes in the catalog to look for significant differences before and after exercise. 396 gene families with unique annotations showed statistically different relative abundance (p < 0.005). While FDR correction did not yield significant individual genes, of these 396 gene families, 391 share functional annotations with the reference assemblies of the *V. atypica* type strain on NCBI. Of the significant genus level results from the 16S data, *Veillonella* has extremely high-quality assemblies of cultured isolates.

Significant alleles are present in each of the 87 samples (Supplementary Figure 8). Interestingly, when all 396 significant alleles are segregated by exercise state and sample, discordant shifts of relative abundance are observed (Supplementary Figure 8). This suggests changes in global microbiome function associated with *Veillonella* abundance, and that conserved *Veillonella* genes may generally play metabolic roles.

Comparative Genomics

Genome annotations were retrieved from NCBI reference genomes. Phylogenetic trees were generated from NCBI taxonomy and visualized with phylo.io.⁴⁴ Heatmaps were generated with the pheatmap package in R.⁴⁵

Gene Catalog Creation

Raw reads were processed and *de novo* assembled into 4,802,186 contigs.^{33,34} 4,792,638

total Open Reading Frames were called, which were subsequently clustered into 2,288,155 gene families with a threshold of 95% identity to create a gene catalog alongside putative annotations assigned by homology.^{35,36} Of these gene families, 801,307 were assigned annotations and 1,486,948 were putatively classified as hypothetical proteins. Comparing annotation state versus gene family size yields the expected result that larger families, which are likely to be present in more microbes, tend to have many more annotations (Supplementary Figure 8). Raw reads were then aligned back to the gene catalog to create a raw count abundance matrix.^{37,38} This matrix was normalized both per sample and by gene length to create a relative abundance matrix.³⁹

Pathway Elucidation

Reactions involved with the breakdown of lactate to both propionate and acetate were manually associated with EC IDs using MetaCyc.⁴⁰

In vitro growth and SCFA analysis

Veillonella species (*V. dispar*, *V. parvula*, and *V. atypica*) were isolated and purified from several study participants and grown in three different media compositions: 1) Brain Heart Infusion Broth (BHI) supplemented with lactate (10mL 60% sodium lactate/L) 2) MRS broth (BD) supplemented with lactate (10ml 60% sodium lactate/L) 3) Semi-synthetic lactate medium (per liter: 5g bacto yeast extract, 0.75g sodium thioglycolate, 25ml basic fuchsin, 21ml 60% sodium lactate, pH 7.5). *Veillonella* species were inoculated into each medium, under anaerobic conditions, and allowed to grow for 48h to reach stationary phase. After 48h, bacteria were pelleted and supernatants were collected for lactate and SCFA measurements. Approximately 10µl of supernatant was used to measure lactate via the Lactate Scout (Lactate.com). The remaining supernatants were frozen at -80°C and then submitted to the Harvard Small Molecule Mass Spectrometry core facility for butyrate, propionate, and acetate quantitative analysis.

SCFAs identified from the mass spectrometry in all three media conditions corresponded with the propionate end product suggested by the metagenomic results. Acetate was not observed in MRS or BHI, likely due to high existing concentrations in the media making the forward reaction thermodynamically unfavorable. However, acetate production was observed in semi-synthetic lactate media (**Supplementary Table 8**).

$^{13}\text{C}_3$ -lactate flux tracing

10 week old C57BL/6 mice were treated with sodium bicarbonate followed by 10^9 CFU of either *Veillonella atypica* (n=4) or *Lactobacillus bulgaricus* (n=4), prepared as above. 20% w/w $^{13}\text{C}_3$ sodium lactate (Cambridge Isotope Laboratories) was diluted with PBS to a concentration of 400mM in PBS. Mice were injected with 100 μL intravenously via the tail vein and after 9 minutes anaesthetized with isoflurane. One mouse treated with *Veillonella atypica* was unable to be injected due to vein clamping and had to be removed. 10 minutes post-injection, anesthetic was confirmed via foot pinch and mice were sacrificed via cardiac puncture. Whole blood was divided into two samples to obtain both serum and plasma, which was flash frozen in liquid nitrogen at 12 minutes post-injection and stored at -80°C .

Immediately following cardiac puncture, mice were dissected to remove colon and cecum and the contents were removed by squeezing with sterilized forceps into pre-weighed tubes. Contents were immediately flash frozen in liquid nitrogen. Timing varied slightly, between 17 and 19 minutes post-injection.

Samples were analyzed for lactate and propionate by the Broad Institute Metabolomics Platform. LC-MS metabolomics were performed as previously described.⁴⁶ LC-MS traces were identified and integrated to quantify presence of $^{13}\text{C}_0$ - and $^{13}\text{C}_3$ - lactate isotopes.

Colorectal propionate instillation

Treadmilling followed the same protocol as above. Mice were fasted 7 hours prior to exercise, to normalize metabolic profiles. 30 minutes prior to exercise, mice were treated with 200 μ L of either PBS vehicle alone (n=8) or 150mM sodium propionate in PBS (n=8) using a flexible gavage needle to introduce 200 μ L of solution into the colon. Mice were run to exhaustion as above. This protocol was repeated for 3 consecutive days. On the first day of treatment, serum was collected 40 minutes post-exhaustion via tail-vein bleed and measured using the Ciraplex multiplex mouse cytokine assay (Aushon BioSystems).

Lactate clearance

To measure lactate clearance rate, mice were first fasted for 7 hours prior to measurement to stabilize basal lactate levels. 5 hours prior to measurement, mice were treated with sodium bicarbonate followed by 10⁹ CFU of either *Veillonella atypica* or *Lactobacillus bulgaricus*, prepared as above (n=8). 30 minutes prior to measurement, mice were weighed, individually caged, and a baseline blood lactate reading was taken using a Lactate Scout meter. Mice were administered sodium lactate via IP injection with a dosage of 750mg/kg, prepared as a 75mg/mL solution of sodium lactate in pH7.0 PBS. Blood lactate was monitored with a Lactate Scout meter at 5, 15, 25, 35, and 45 minutes post-injection.

Statistics

Figures 1A and 1B:

Wilcoxon rank sum test with continuity correction were used to look at differences in taxonomic composition before and after exercise. Mean *Veillonella* abundance is 0.9 orders of magnitude greater 1 day post exercise compared to one hour prior to exercise.

Figure 1-1C and 1D:

Longitudinal data was modeled with a GLMM approach. In our model, the random effect is

individual variation per marathon runner. Fixed effects are shown in Figure 1-1D. An advantage of this type of statistical analysis is that it can account for the large variation between marathon participants in this type of study.

To determine statistical significance, a Wald Z-test was used to assign p-values to coefficients in the GLMM. No outliers were removed in this analysis.

Figure 1-2A:

Each animal was treated with both *Veillonella atypica* and *Lactobacillus bulgaricus* as part of the AB/BA crossover. Because all 32 animals were treated twice and compared between treatments, p-value was generated using a paired t-test ($p=0.022$). The normality assumption was assessed via Shapiro-Wilk's normality test ($p = 0.67$), validating the use of the t-test.

Figure 1-2B:

Longitudinal data was modeled with a GLMM approach. In our model, the random effect is individual variation per mouse. Fixed effects are treatment effect, period effect (at what time point measurements are made), and carryover/sequence effect (if the order of treatments in the crossover affects later results). An advantage of this type of statistical analysis is that it can account for the large variation between mice in this type of study.

The figure shows seconds run until exhaustion at 6 timepoints, with each of the 32 mice having one measurement per time point. For each treatment order (LLL VVV and VVV LLL) the GLMM is fitted both to each individual mouse (skinny blue and red lines; note that these are all parallel for mice in the same treatment order—the space between these lines represents the “random effect” of natural variation between mice) and all mice (Population) with the same treatment order (thick blue and red lines).

To determine statistical significance, a Wald Z-test was used to assign p-values to coefficients in

the GLMM. No outliers were removed in this analysis.

Figure 1-3A and Supplementary Figure 9:

p-values for individual genes were generated utilizing pairwise ANOVA comparing before and after exercise relative abundance. Non-significant families are associated with homologs common in other microbes that do not change in abundance. To determine the significance of potential overrepresentation, 1,000 global EC IDs were randomly selected and mean difference in relative abundance between samples taken before and after exercise. These EC IDs were used to construct an odds table to determine the probability of having a set of 8 selected EC IDs with increases in mean gene-level relative abundance after exercise. This calculation determined that the relative abundances changes in Figures 3B-3I are significant ($p=0.00147$ using a Fisher's Exact Test for count data).

Table 1:

p-values were generated using Welch's t-test (unequal variances t-test).

Figure 1-4D:

p-value was generated using a one-sample t-test. Ratios of labeled/unlabeled lactate from samples were compared to the expected ratio determined mathematically. Each sample was independently compared to the expected ratio, then multiple hypothesis correction was performed using the FDR correction method of Benjamini & Hochberg (1995). (Serum $p=0.00001$; plasma $p=0.00001$; cecum content $p=0.00001$; colon content $p=0.001$).

Figure 1-4E:

p-value was generated using Welch's t-test (unequal variances t-test). ($p=0.028$).

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Chapter 2

E. coli Nissle 1917 Modulates Host Glucose Metabolism Without Directly Acting on Glucose

This Chapter is part of a submitted manuscript:

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Author Contributions:

T.A.C. and A.D.K. conceived the project. T.A.C. generated bacterial samples and mutants. T.A.C. and L.D.P. performed glucose tolerance tests. T.A.C. wrote the manuscript and generated all images and model figures using PowerPoint. A.D.K. supervised the project. All authors reviewed the manuscript.

Abstract

Managing postprandial glycemic response, or the increase in blood sugar following a meal, is a crucial component to maintaining healthy blood sugar in patients with diabetes. To test whether oral probiotics can impact postprandial glycemic response, *E. coli* Nissle 1917 (EcN) was evaluated in an oral glucose tolerance test. Oral gavage of EcN concurrent with a glucose bolus reduced the post-gavage glycemic response in mice. However, there was no difference in glycemic response when comparing EcN to a mutant deficient in glucose metabolism. This suggests that while EcN can alter glycemic response to a glucose bolus, this effect is not mediated by direct uptake of glucose. Of the possible indirect effects EcN could have, gastric emptying rate was highlighted as a likely cause, but EcN had no effect on gastric emptying rate in mice. This leaves many more possible indirect explanations for the interaction between EcN and host glucose metabolism to be explored in future work.

Introduction

One of the key aspects of maintaining healthy blood sugar in patients with diabetes is managing the postprandial glycemic response.¹⁻³ Shortly after a carbohydrate rich meal, free sugars reach the small intestine where they are rapidly absorbed into the intestinal lumen and into the bloodstream.^{4,5} Rising blood sugar levels cause a glucose stimulated insulin secretion (GSIS), stimulating glucose uptake in peripheral tissue.⁶ However, in patients with diabetes, GSIS is impaired, either by reduced function of the insulin producing pancreatic beta islets (as in type I diabetes and MODY genetic diabetes), or by reduced sensitivity of peripheral tissue to insulin signals (as in type II diabetes).^{7,8} This leads to impaired postprandial glycemic response,

causing long periods of elevated blood sugar after meals, and often requiring pharmaceutical intervention to manage.⁹

The study of the human gut microbiome has uncovered many promising targets for "functional probiotics" with experimentally verified effects. For example, *Akkermansia muciniphila* has been targeted as an anti-obesogenic microbe, with supporting data in mice and in clinical trials.^{10–12} *Bacteroides thetaiotaomicron* has received similar attention as a potential probiotic for reducing weight gain in a high fat diet context.^{13–15} And recently our group has identified *Veillonella atypica* as a candidate for an endurance-enhancing microbe in the context of exercise.¹⁶ Microbes are a potentially powerful tool for impacting health, as they exist naturally in the gut at high abundance, have biological connections to a massive number of diseases and biological processes, and can be isolated, cultured, and formulated for diet supplementation.¹⁷

In the context of altering postprandial glycemic response, we hypothesized a clear mechanism by which the microbiome could impact glucose uptake and improve glycemic profiles. Many bacteria have high affinity for glucose, and in the gut microbiome, bacteria could feasibly compete with the host for glucose absorption and degradation. To combat this, the sites of carbohydrate degradation and glucose uptake, the stomach and upper small intestine, are harsh environments with low colonization rates due to high acidity, fast lumen flow rate, antimicrobial compounds, and a loose mucus layer that impairs adhesion.^{18,19} We hypothesized that by boosting the local concentration of glucose-degrading bacteria in the stomach and small intestine, the microbiome could compete with the host for glucose uptake, reducing the amount of glucose that makes it to the bloodstream. To this end, we chose *Escherichia coli* Nissle 1917, a probiotic strain that is used commercially as a probiotic and is closely related to model strains

of *E. coli* that are well characterized for their fast growth rates, high affinity for glucose, and relative ease of engineering.²⁰

In this study, we propose that oral supplementation of probiotic bacteria with high affinity for glucose could impact glycemic response to an oral glucose bolus. We show that concurrent administration of probiotic *E. coli* Nissle 1917 with a glucose bolus causes a reduction in the glycemic response. We then tested whether *E. coli* glucose consumption is necessary to see this change in glycemic response but found that a glucose-null mutant had no effect when compared to wild type. We then hypothesized that bacterially-induced changes in gastric emptying rate could explain the change in glycemic response, however we observed no change in gastric emptying in *E. coli* Nissle supplemented mice versus PBS control. This suggests that while *E. coli* Nissle appears able to modify the glycemic response, the mechanism is complex. Importantly, we demonstrate that the intuitive explanation of simple bacterial absorption of dietary glucose is likely not responsible for this effect.

Results

We first set out to test the hypothesis that the glucose metabolic capacity of live probiotics can impact glucose uptake rate in the gastrointestinal tract (**Figure 2-1A**). 8-week-old C57-BL/6J mice were gavaged with 10^{10} CFU of *E. coli* Nissle 1917, concurrently with 2mg/kg glucose, and blood glucose was monitored over 2 hours (**Figure 2-1B**). At each time point measured, Nissle-treated mice showed a trend of lower average blood glucose than vehicle control, though this difference was not significant. However, the area under the blood glucose curve (AUC) was significantly lower in Nissle-treated mice compared to vehicle control (**Figure 2-1C**). We then explored whether other probiotic bacteria could elicit a similar response.

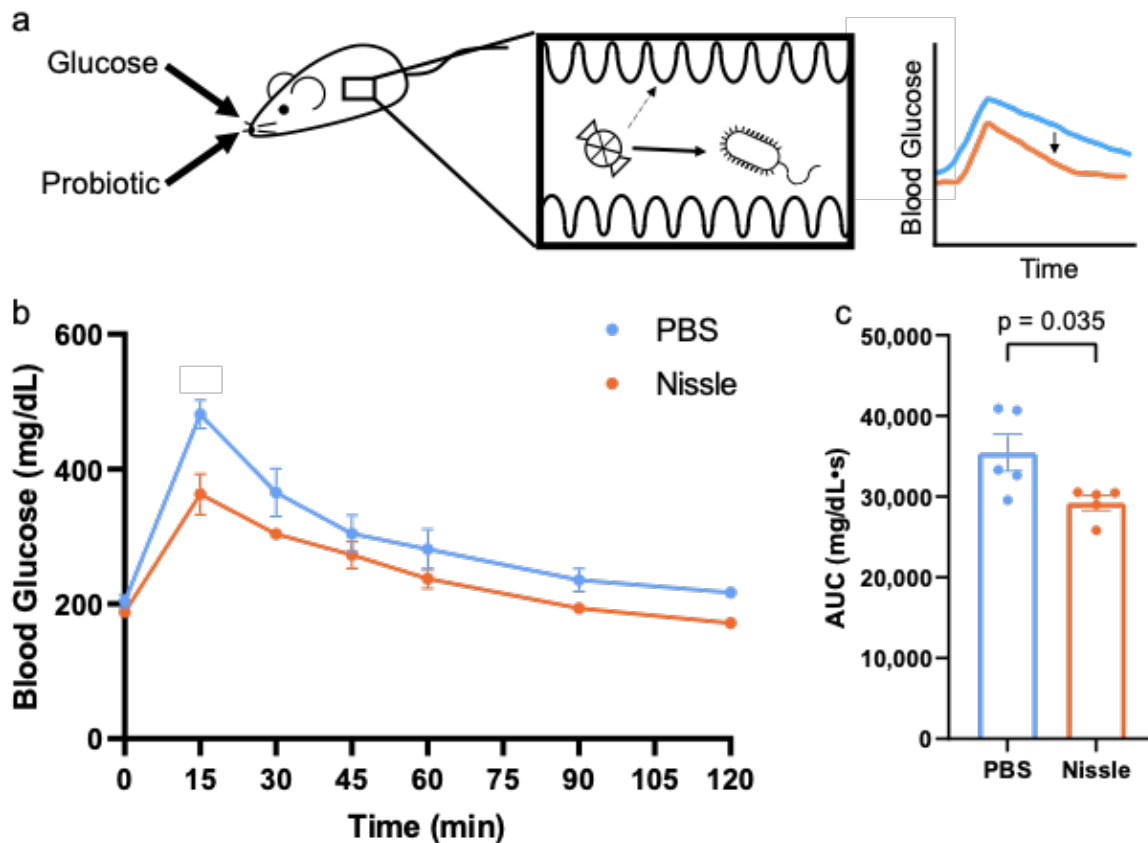


Figure 2-1. *E. coli* Nissle 1917 improves glycemic response in healthy mice. **A** Experimental design. **B** Oral glucose tolerance test (OGTT) showing blood glucose over time following oral glucose gavage. Points represent mean and bars represent SEM. **C** Area under the curve (AUC, in mg/dL*s) of **B**. Points represent individual mice and bars represent mean and SEM. p values calculated in **B** using repeated measures ANOVA with Tukey's post-hoc test, and in **c** using student's T-test.

However, administration of a cocktail of probiotic *Lactobacillus* strains showed no impact on average blood glucose at any time point measured nor the average AUC (**Supplementary**

Figure 11). These data suggest that while probiotic bacteria can impact the glycemic response to a bolus of glucose, this response is species specific, prompting further investigation into what causes *E. coli* Nissle specifically to have this effect.

E. coli strains are well characterized for their high rate of glucose uptake. To test whether glucose uptake was necessary for the improvement of glycemic response, we first generated a glucose-null mutant of *E. coli* Nissle. *E. coli* metabolizes glucose via central carbon metabolism,

primarily through glycolysis and the pentose phosphate pathway (**Figure 2-2A**). Hexose transporters take up glucose and phosphorylate to glucose-6-phosphate, which is incorporated into glycolysis via phosphoglucose isomerase (*pgi*), or into the pentose phosphate pathway via glucose-6-phosphate dehydrogenase (G6PDH, encoded by gene *zwf*).²¹ In *E. coli* strains lacking both *pgi* and *zwf*, glucose-6-phosphate accumulates in the cell, inhibiting hexokinase transporters via feedback inhibition. Using *E. coli* Nissle 1917 as a base strain, we generated the double knockout EcN $\Delta pgi\Delta zwf$ and confirmed that it was unable to grow in minimal media with glucose as the sole carbon source, while growth on glycerol was unaffected (**Supplementary Figure 12**). In an OGTT, mice supplemented with EcN $\Delta pgi\Delta zwf$ showed no significant change in glycemic profile compared to wild type EcN, and the AUC was unchanged as well (**Figure 2-2B-C**). Thus, while EcN appears to affect the glycemic profile in OGTT, this effect is not dependent on EcN possessing functional glucose metabolism, suggesting that this effect is dependent on some other property of the bacteria.

For *E. coli* Nissle to impact glycemic response without directly interacting with glucose, it would have to have some effect on host physiology, impacting glycemic response indirectly. A prime candidate for this indirect effect is gastric emptying, as microbiota and their small molecule effectors have been shown to impact gastric emptying rate, and gastric emptying rate has a direct effect on glucose absorption rate.²² To test whether Nissle impacts gastric emptying rate, we used acetaminophen as a tracer, co-administering a bolus of acetaminophen with either Nissle or PBS as an oral gavage. By monitoring concentration of acetaminophen in the blood, gastric emptying rate can be estimated as the rate of influx of acetaminophen into the bloodstream from the stomach (**Figure 2-3B**). However, we observed no clear effect of Nissle on

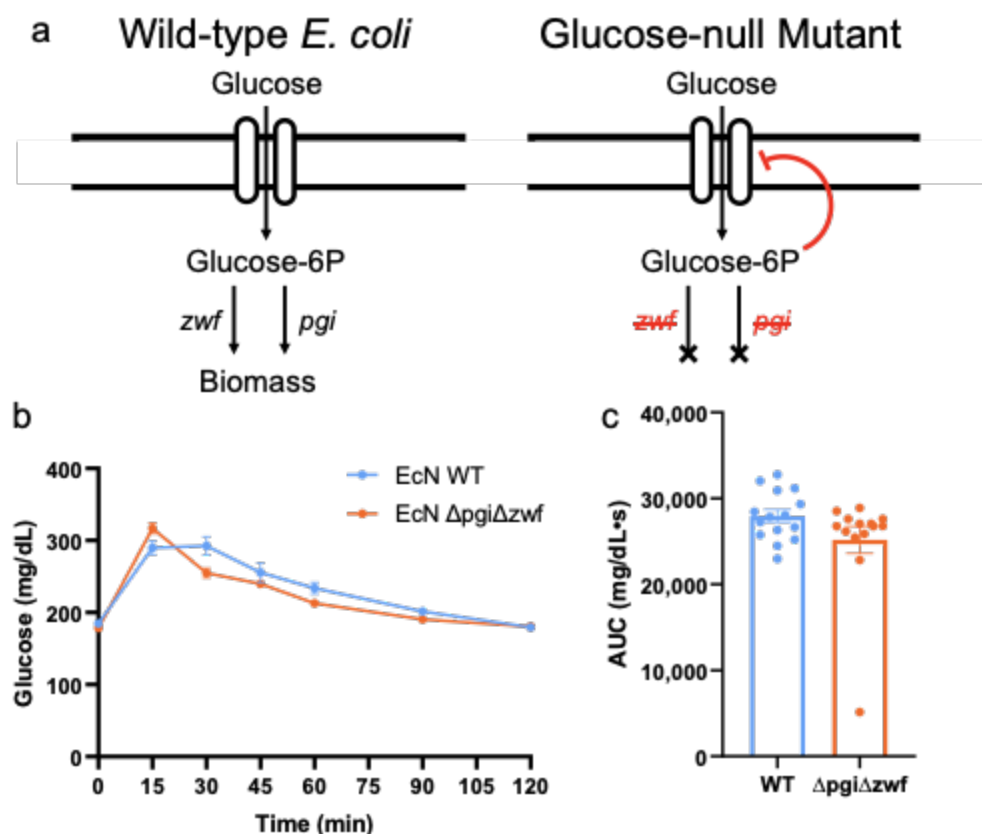


Figure 2-2. A glucose-null mutant of *E. coli* Nissle 1917 has no impact on blood glucose AUC compared to wild type. **A** Simplified view of glucose metabolism in *E. coli*. Hexokinase transports glucose from extracellular space into the cytoplasm and phosphorylates, generating glucose-6-phosphate. Glucose-6P is processed via *zwf* into the pentose phosphate pathway or via *pgi* into glycolysis. Knocking out both genes causes buildup of glucose-6P, eliminating further glucose uptake via feedback inhibition. **B** OGTT showing blood glucose over time following oral glucose gavage. Points represent mean and bars represent SEM **C** AUC of the OGTT. Points represent individual mice and bars represent mean and SEM. p values calculated in **B** using repeated measures ANOVA with Tukey's post-hoc test, and in **c** using student's T-test.

gastric emptying rate. Inter-mouse variability was high, as was the batch effect between the two trials conducted (**Figure 2-3B**). Aggregated, we observed no difference in average blood concentration of acetaminophen at any time point measured (**Figure 2-3C**). These data suggest that Nissle has no observable impact on gastric emptying rate in mice, and thus gastric emptying cannot explain our initial observation.

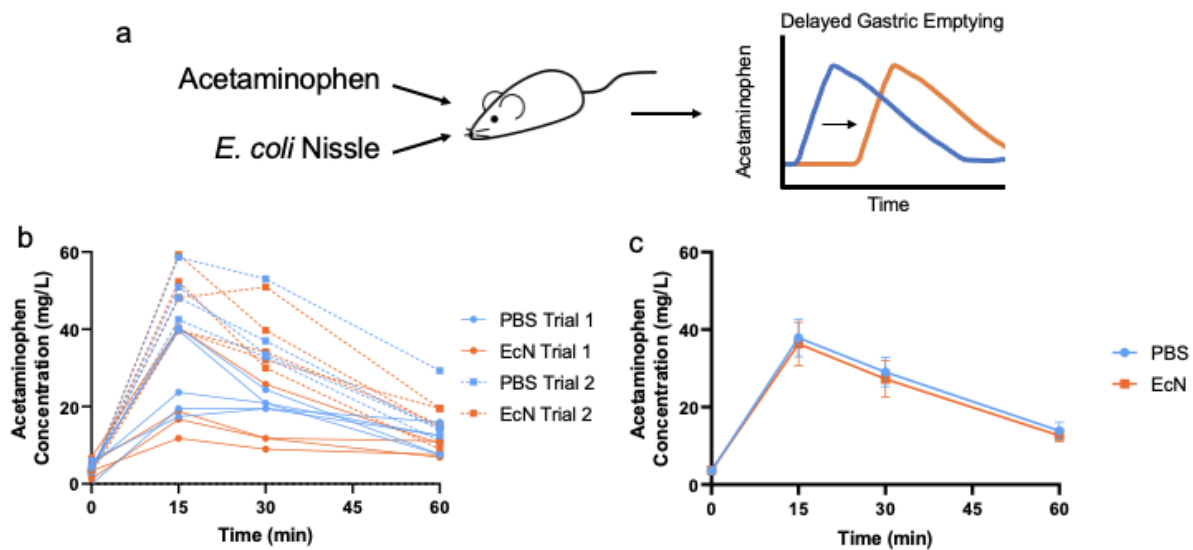


Figure 2-3. Impact of *E. coli* Nissle on gastric emptying rate. **A** Experimental design. Acetaminophen is co-administered with bacteria or PBS as an oral gavage. Acetaminophen acts as a tracer to monitor rate of gastric emptying and further clearance from the blood. **B, C** Concentration of acetaminophen in the blood over time following oral gavage. In **B**, points represent individual mice, while in **c** points represent mean and bars represent SEM. p values calculated in **B** and **C** using repeated measures ANOVA with Tukey's post-hoc test.

Discussion

In this study, we proposed the hypothesis that *E. coli* Nissle could act as a glucose “sponge” by competing with the host for glucose uptake. Promisingly, we observed that Nissle reduced the glycemic response in mice compared to PBS vehicle control. However, we acknowledged that PBS was not an adequate control to conclude that this effect was caused by direct competition. To test this further, we generated a glucose-null mutant of Nissle (EcN Δ pgi Δ zwf), which is unable to grow on or metabolize glucose. However, in a mouse OGTT, EcN Δ pgi Δ zwf showed no change in glycemic response compared to wild type, meaning that functional glucose metabolism was not necessary for the glycemic impact we initially observed. Furthermore, this observation meant that in absence of a direct effect, the next best explanation for our initial findings would be an indirect effect: bacterial gavage impacting an aspect of host physiology which alters glycemic response in a separate downstream response. We then tested

one likely candidate for this indirect effect via gastric emptying rate, however we found that *E. coli* Nissle had no effect on gastric emptying.

Eliminating this possibility does little to whittle down the long list of potential bioprocesses a probiotic microbe could be interacting with that alter glycemic response. Microbiome signals have been shown to affect the secretion of several endocrine hormones, including GLP-1 and GIP²² which are both involved in postprandial insulin secretion.²³ Other affected signals include PYY, which has a more complicated role in glucose homeostasis,²⁴ and amylin, which affects blood glucose via delaying gastric emptying.²⁵ Microbiome signals have also been shown to interact with the neurological system, via the gut-brain axis.²⁶ Neurological connections to glucose sensing and homeostasis represent another possible explanation.²⁷ The microbiome has also been shown to interact with metabolism directly via small molecule secretion. Microbiome derived succinate absorbed from the intestinal lumen can induce intestinal gluconeogenesis, which improves glucose homeostasis and reduces glycemic response.²⁸ There is even emerging research suggesting that there may be a resident pancreatic microbiome, and oral supplementation of *E. coli* specifically has been observed entering the pancreatic ducts at high concentrations.²⁹ Direct pancreatic stimulation could affect glucose homeostasis via a unique mechanism, making this avenue particularly hazy and unknown. We are left without a clear explanation for our initial finding and a wide expanse of possible avenues towards an explanation which lies outside the scope of this study.

The key finding of this work is the observation that probiotic bacteria given just prior to a glucose bolus can impact the body's response to the glucose. Our initial assumption was that this effect was caused by direct competition for glucose, as this was the most likely and most exciting possibility. However, our further experimentation showed that competition could not explain the

initial observation, demonstrating that the microbiome and microbe-host interactions are much more complicated than they appear.

Materials and Methods

Bacterial Strains and Growth

To generate material for gavage, bacterial strains were grown in LB at 37°C overnight, then sub-inoculated into LB at 1:100 dilution and incubated at 37°C until OD₆₀₀ 0.4-0.8. Cultures were then pelleted at 4000g, 4°C, and washed once in 1x PBS. Pellets were resuspended in PBS to a final concentration of $\sim 5 \times 10^{10}$ CFU/mL and flash frozen in liquid nitrogen. Aliquots were stored at -80°C. CFU/mL was determined by plating a dilution series of the aliquots onto LB agar plates and counting colonies after 24 hour incubation at 37°C.

To generate the glucose-null mutant, we utilized the lambda red recombineering system encoded on plasmid pREDTKI.³⁰ Deletion cassettes were designed for Δ pgi and Δ zwf by PCR amplifying FRT-CatR-FRT (chloramphenicol resistance gene) from pKD3 with 60bp homology to the upstream and downstream regions of each gene. EcN cultures were made electrocompetent by growing cultures to OD₆₀₀ 0.4-0.6 and washing with 10% glycerol twice and resuspending in 1/100th the original volume. Electrocompetent cultures were transformed with 10ng plasmid DNA or 100ng linear DNA at 3kV 2mm gap with 1 hour recovery in SOC media before plating on LB agar plates. EcN pREDTKI cells were grown in the presence of 0.1% arabinose and electrotransformed with the Δ pgi deletion cassette, then plated on LB Chlor. Activation of the FRT recombinase at 42°C removed the CatR gene, then the process was repeated for the Δ zwf deletion. Deletions were confirmed by sequencing across the gene and glucose-null growth

phenotype confirmed by streaking overnight LB cultures on M9 minimal media with glucose or glycerol as the sole carbon source.

Oral Glucose Tolerance Test

Protocols for mouse experiments were reviewed and approved by the Joslin Diabetes Center Animal Care and Use Committee (Protocol #2016-05). The study was carried out in accordance with the ARRIVE guidelines. All experiments were performed according the relevant guidelines and recommendations. All mouse experiments were performed using 8-12 week old C57/B6J mice ordered from Jackson Labs. To reduce confounding effects, mice were distributed between groups from different litters and cages. For OGTT, standard best practice guidelines were followed.³¹ Mice were individually caged and fasted for 5 hours prior to gavage. Roughly 1 hour prior to gavage, bacterial aliquots were thawed on ice. Immediately before gavage, 670 μ L of 50% w/v glucose was added to 1mL bacteria maintained at $\sim 10^{10}$ CFU/mL and gavaged at 10 μ L per gram body weight. This gives final dosages of 2g/kg BW glucose and 10^9 CFU/g BW bacteria. Gavages were timed exactly 1 minute apart, then blood glucose was measured via tail vein bleed and a handheld glucose meter at 15, 30, 45, 60, 90, and 120 minutes post-gavage. Area under the curve was calculated using the trapezoid rule with 0mg/dL as baseline. For probiotic blend gavage, a commercial probiotic blend was obtained (CVS) and suspended in PBS to a concentration of 10^{10} CFU/mL, as measured by CFU plating. Probiotic blend was administered identically to the frozen bacterial aliquots. For all mouse experiments, the administering researcher was blinded to the aliquot contents.

Gastric Emptying

Gastric emptying rate was measured as previously described.^{32,33} Briefly, mice were fasted overnight for 20 hours. Gavage mixture was prepared by adding equal volumes 2% w/v

acetaminophen and either PBS or bacteria maintained at 10^{10} CFU/mL, thawed on ice as above. Baseline blood samples were collected via tail vein bleed, and mice were gavaged 250 μ L gavage mixture, giving final dosage of 2.5mg acetaminophen and $\sim 10^9$ CFU bacteria per animal. Blood samples were collected at 15, 30, and 60 minutes into heparinized tubes and stored on ice. Blood samples were centrifuged at 4°C for 20 minutes at 2000g. Plasma was collected and stored in separate tubes at -20°C. Acetaminophen was quantified in the plasma using an enzymatic colorimetric assay specific to acetaminophen (Cambridge Life Sciences).

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Conclusion

Impacts on the Field of Microbiome Engineering

The work discussed in this dissertation represent a small step towards the goal of engineering the human microbiome to treat disease and improve health. I hope to effectively convey the difficult work required to achieve this goal, as the field is still very much in its infancy. Truly improving health using the microbiome requires one to understand and manipulate a complicated web of interacting components.

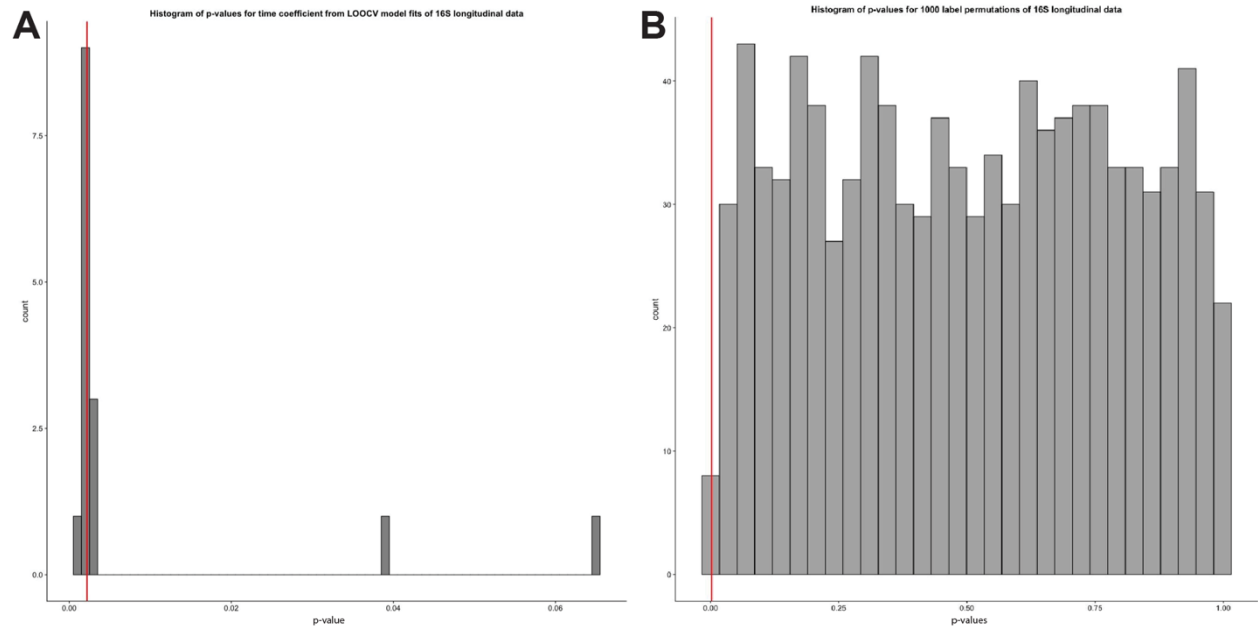
In Chapter 1, I have tried to communicate the complexity of these interactions, demonstrating the various molecular signals exchanged across the host/microbiome interface. These interactions involve a long list of molecular intermediates whose impacts are still poorly understood. In Chapters 2 and 3, I hope to have demonstrated two possible paths towards microbiome engineering. In Chapter 2, we started from a simple observation of 16S abundance in runners. We then dissected this using shotgun metagenomics to identify a relevant metabolic pathway, strain isolation to culture a functional strain, then demonstration of that effect in a sufficiency mouse model. Ultimately, we were able to implicate a specific metabolite in improvement of athletic performance, providing a potential model for performance enhancement and a hypothetical probiotic treatment. To contrast this, I have presented Chapter 3, where I instead started from a functional hypothesis that *E. coli* glucose metabolism may be able to alter host glycemic response. While I did observe a glycemic effect, this was not dependent on *E. coli* glucose metabolism, contradicting my initial hypothesis and making this observation much harder to explain. Without a simple explanation, this effect instead was likely mediated by one of the many complex interactions occurring across the host/microbiome interface, as discussed briefly in Chapter 1. Together, I hope this work represents a framework for moving towards microbiome manipulation, as well as a blunt demonstration of the pitfalls of this goal.

Much of the work in the microbiome has relied heavily on 16S rRNA gene sequencing and simple correlations between microbial taxonomy and health or disease. This style of research has become so common, my colleagues have jokingly mused on the “prototypical microbiome paper.” This is a template for microbiome research wherein a group identifies their disease of interest, recruits a metagenomic cohort (figure 1), sequences stool samples via 16S sequencing (figure 2), and generates huge numbers of correlations between microbes and host factors, highlighting the correlations that support their hypothesis (figure 3). In more recent publications, we will often see researchers test their hypothesis in a single mouse or *in vitro* cell model. This style of paper is ubiquitous, and despite being well designed with reasonable experiments, in many areas of microbiome research this is as far as our understanding goes.

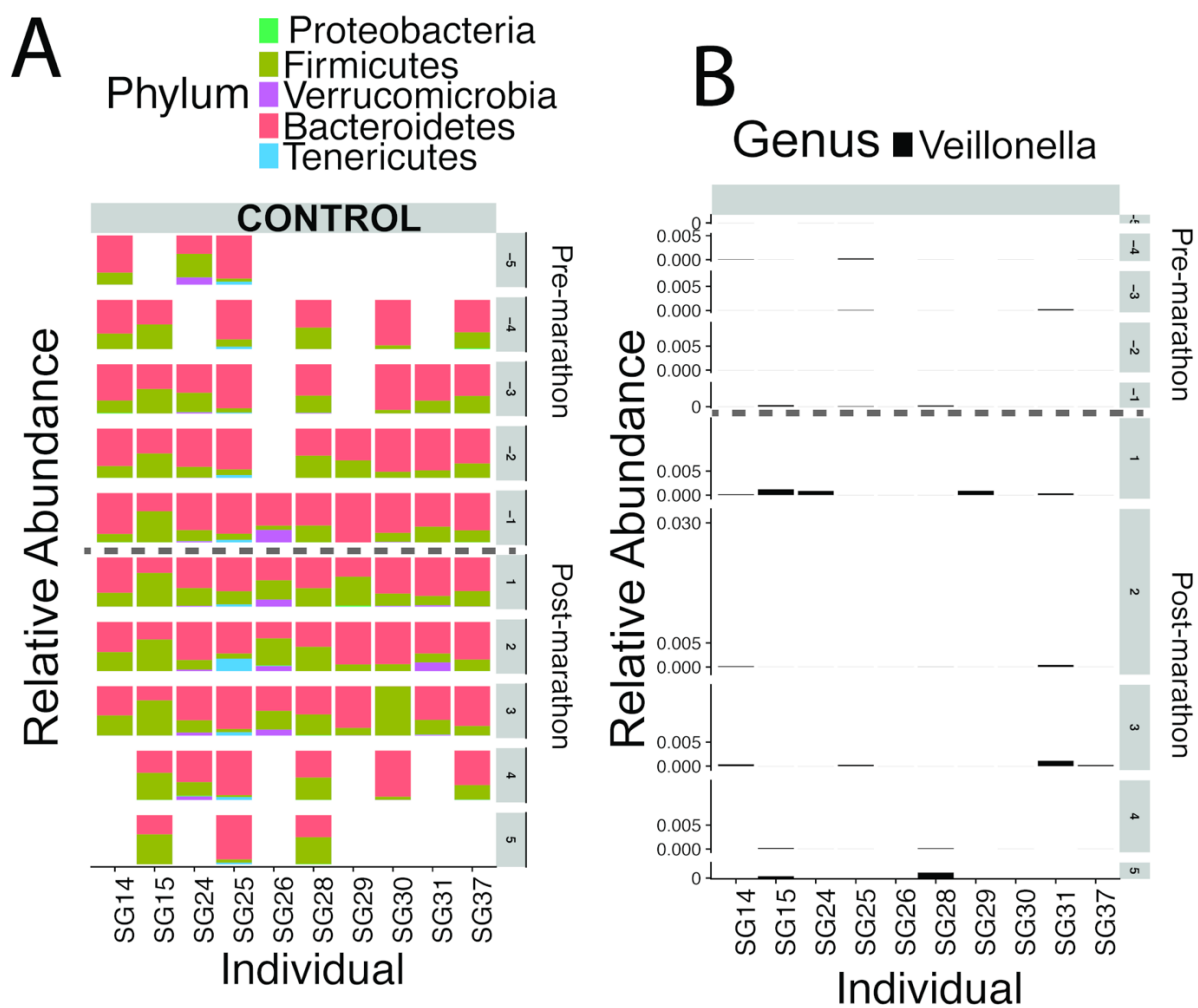
In order to move towards rational microbiome engineering, I believe the field needs to shift away from simple correlations and low resolution 16S sequencing data. 16S sequencing generates lists of taxa, which ideally represent species but often can only be characterized on the level of genus, if not phylum. This low-resolution representation of microbes seems reasonable, but consider the case of antibiotic resistance: single gene mutations imparting antibiotic resistance can cause two organisms to display different phenotypes while maintaining over 99.9% genetic identity. In this way, our understanding of genus or species imparting certain functional phenotypes will always come with a grain of salt. This is demonstrated in Chapter 2, where 16S sequencing simply identified a genus. Further work via shotgun metagenomics was required to identify genes and potential metabolites responsible. And more work still was required to test those strains, genes, and metabolites using supplementation studies. These studies are the next area I believe the microbiome field needs to embrace as required to make any substantive claims about function.

Appendix

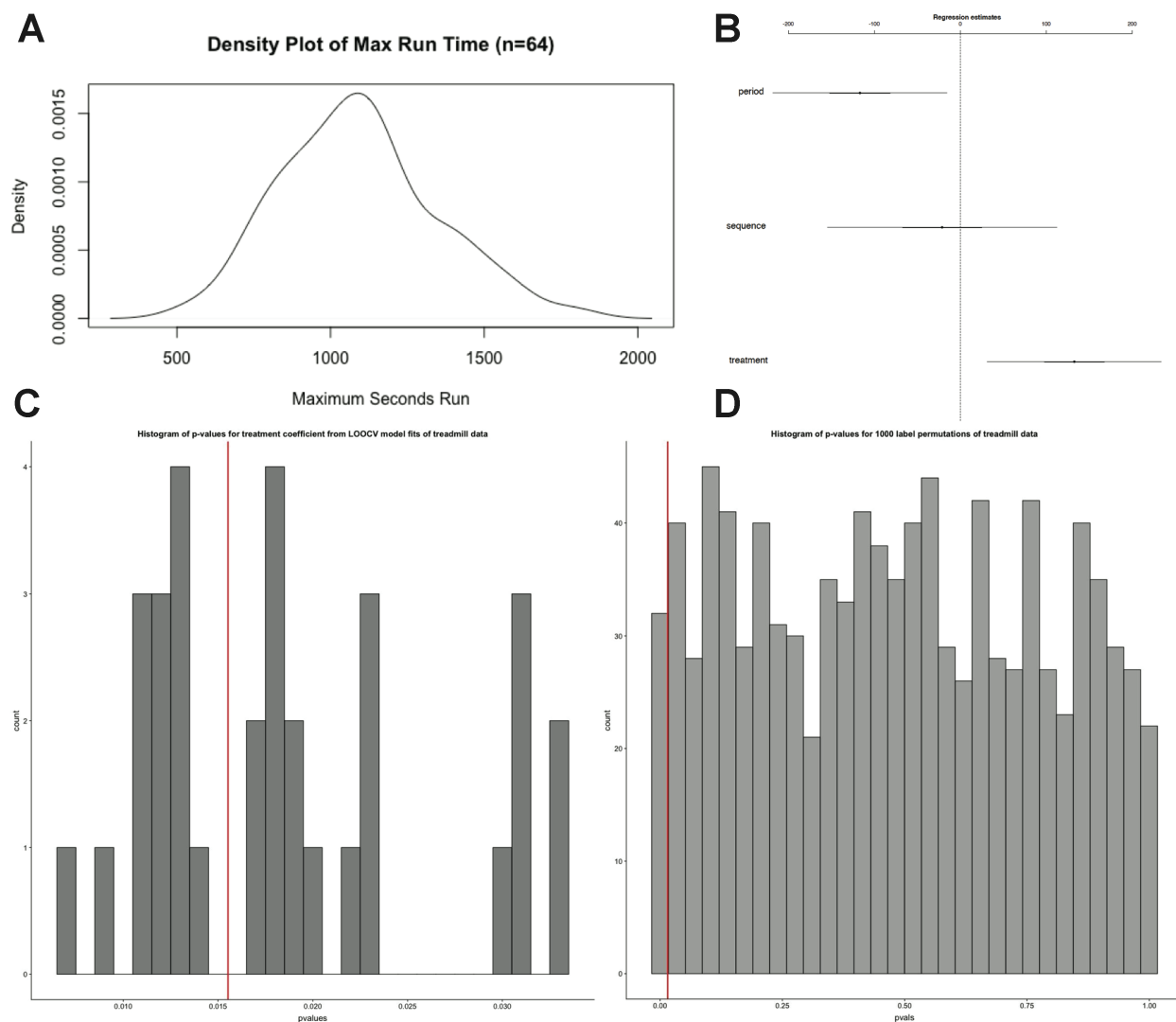
Supplementary Figures



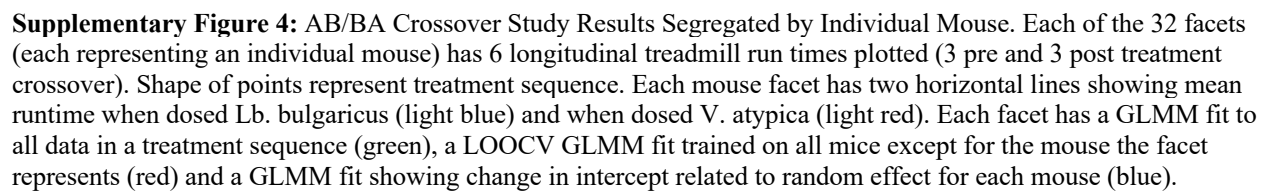
Supplementary Figure 1: A: Histogram of two-sided p-values (Wald-Z tests) for time coefficient from LOOCV models predicting 16S *Veillonella* abundance. Red line represents p value for model trained without any hold outs. **B:** Histogram of two-sided p-values for time coefficient from 1000 label permutations in GLMM models predicting *Veillonella* relative abundance. Red line represents p value for model trained without any label permutation.

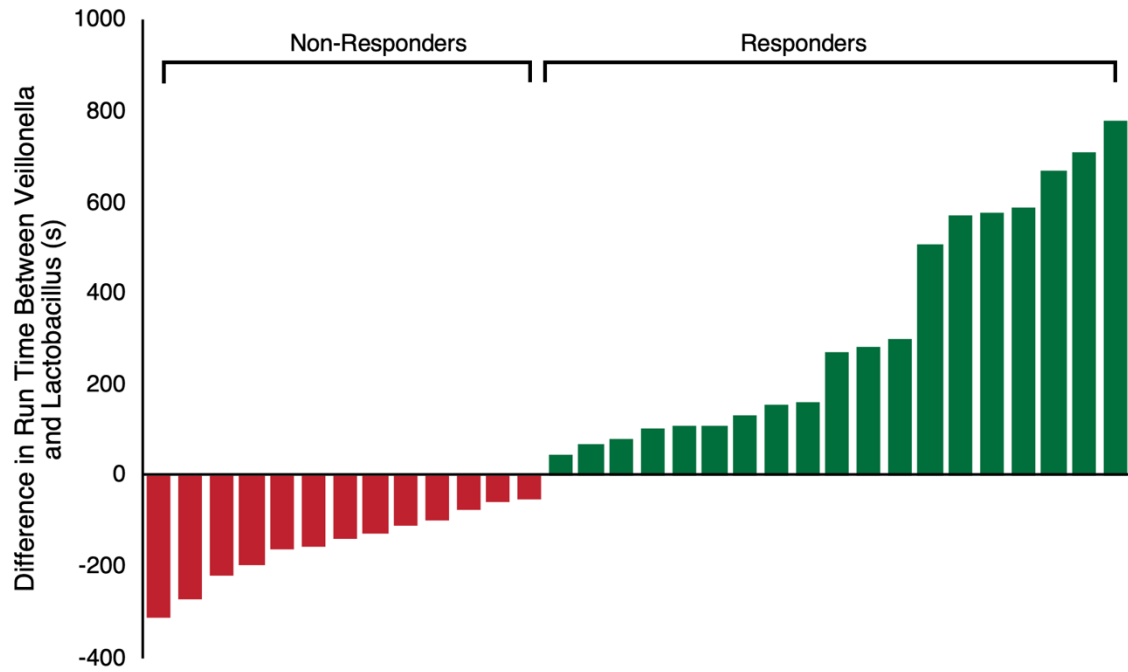


Supplementary Figure 2: A:16S composition in control subjects **B:***Veillonella* relative abundance in control subjects.

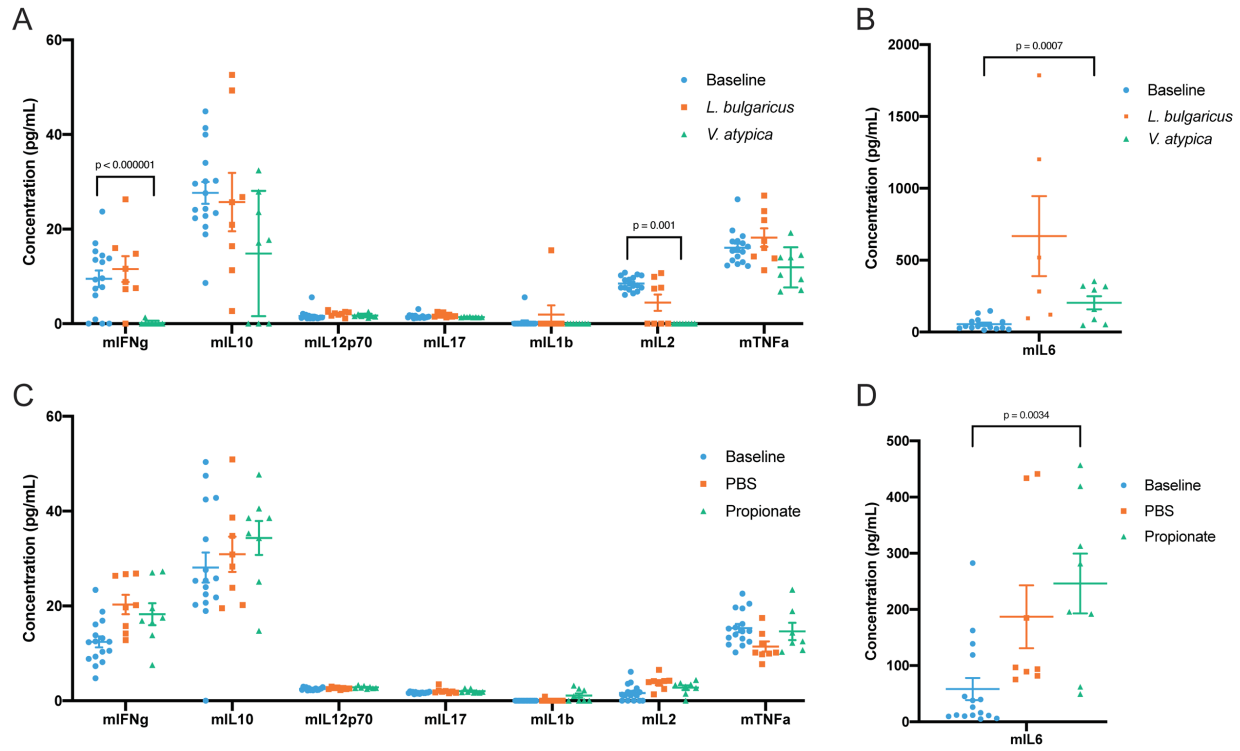


Supplementary Figure 3: **A:** Density plot of maximal run times in AB/BA crossover study. Two-sided Shapiro-Wilk normality test on the max run times for each mouse in each treatment group results in $p = 0.67$ with a null hypothesis that the distribution of the data is normal ($n = 64$). **B:** 95% confidence intervals for coefficient effect on treadmill runtime in AB/BA crossover (Wald-Z tests, $n=64$). Center values are the regression estimate for each coefficient. Error bars represent the 95% confidence interval. **C:** Histogram of p-values for treatment coefficient from LOOCV models predicting treadmill runtime. Red line represents p value for model trained without any hold outs (Wald-Z tests, $n=64$). **D:** Histogram of p-values for treatment coefficient from 1000 label permutations in GLMM models predicting treadmill runtime. Red line represents p value for model trained without any label permutation (Wald-Z tests, $n=64$ per permutation).

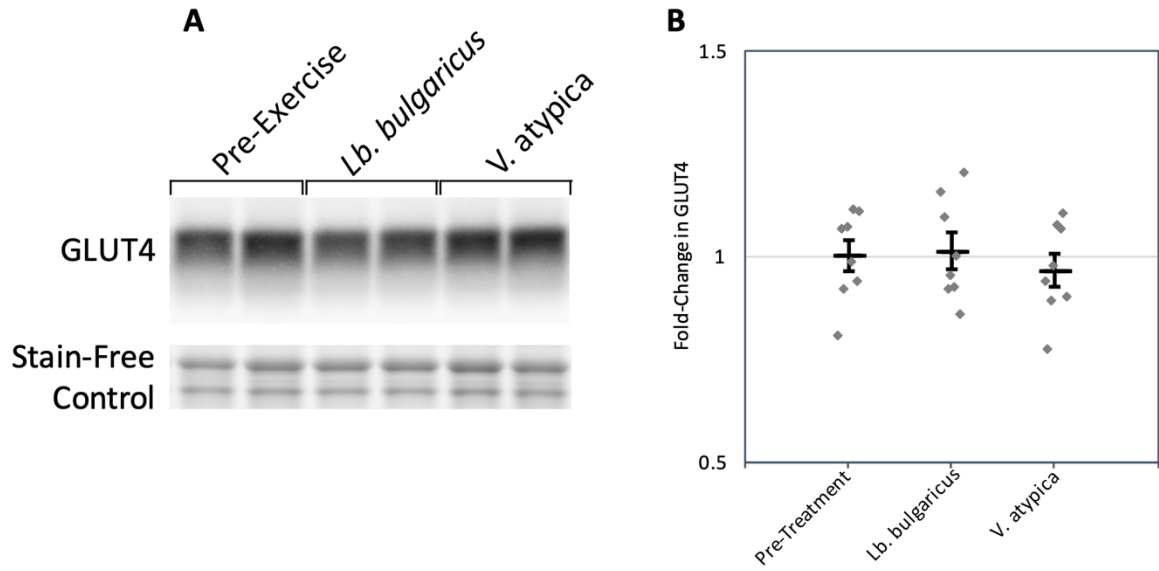




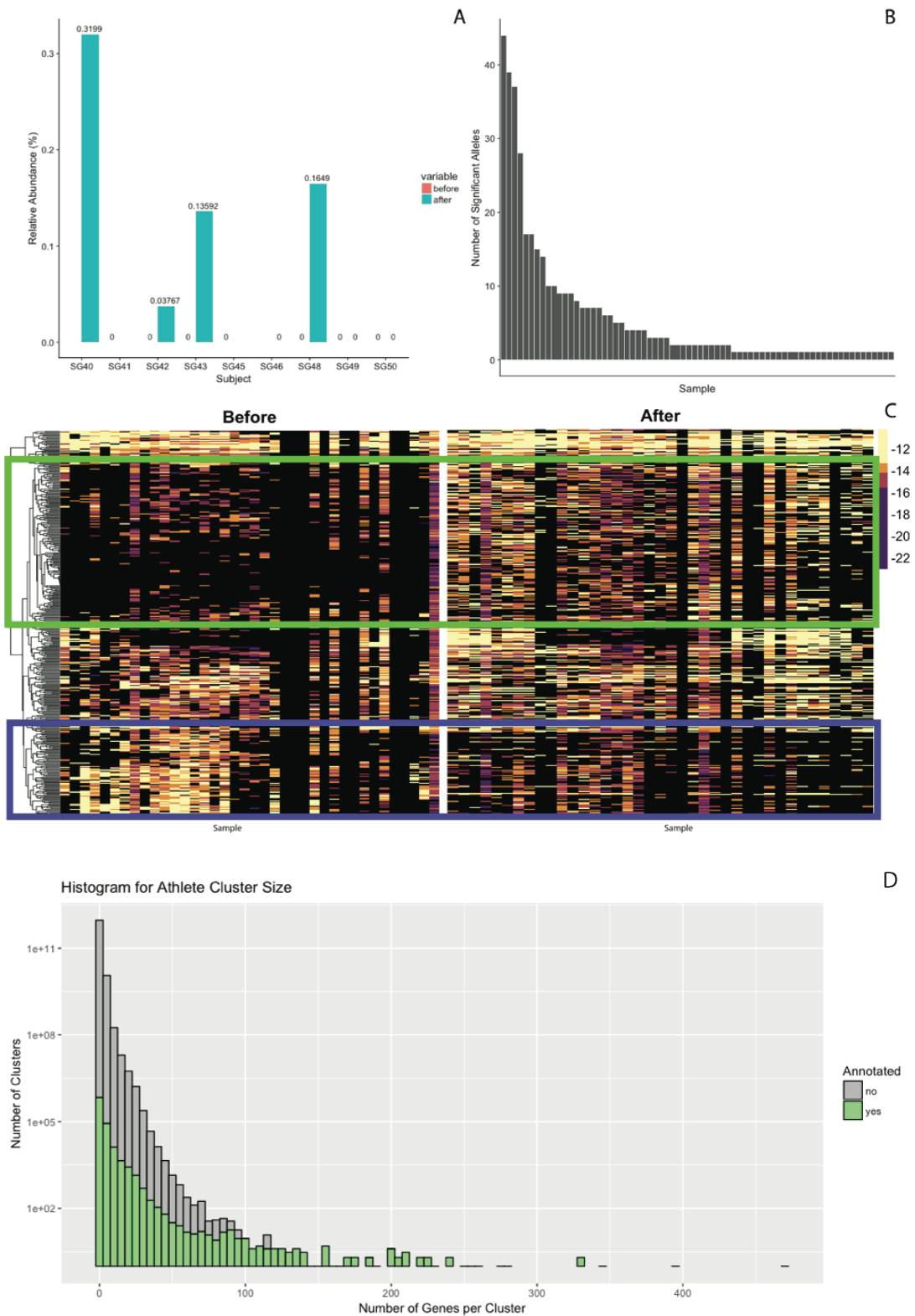
Supplementary Figure 5: Difference in maximal run time between *V. atypica* gavage periods and *L. bulgaricus* gavage treatment periods segregated into “responders” and “non-responders” to *V. atypica* treatment (n = 32).



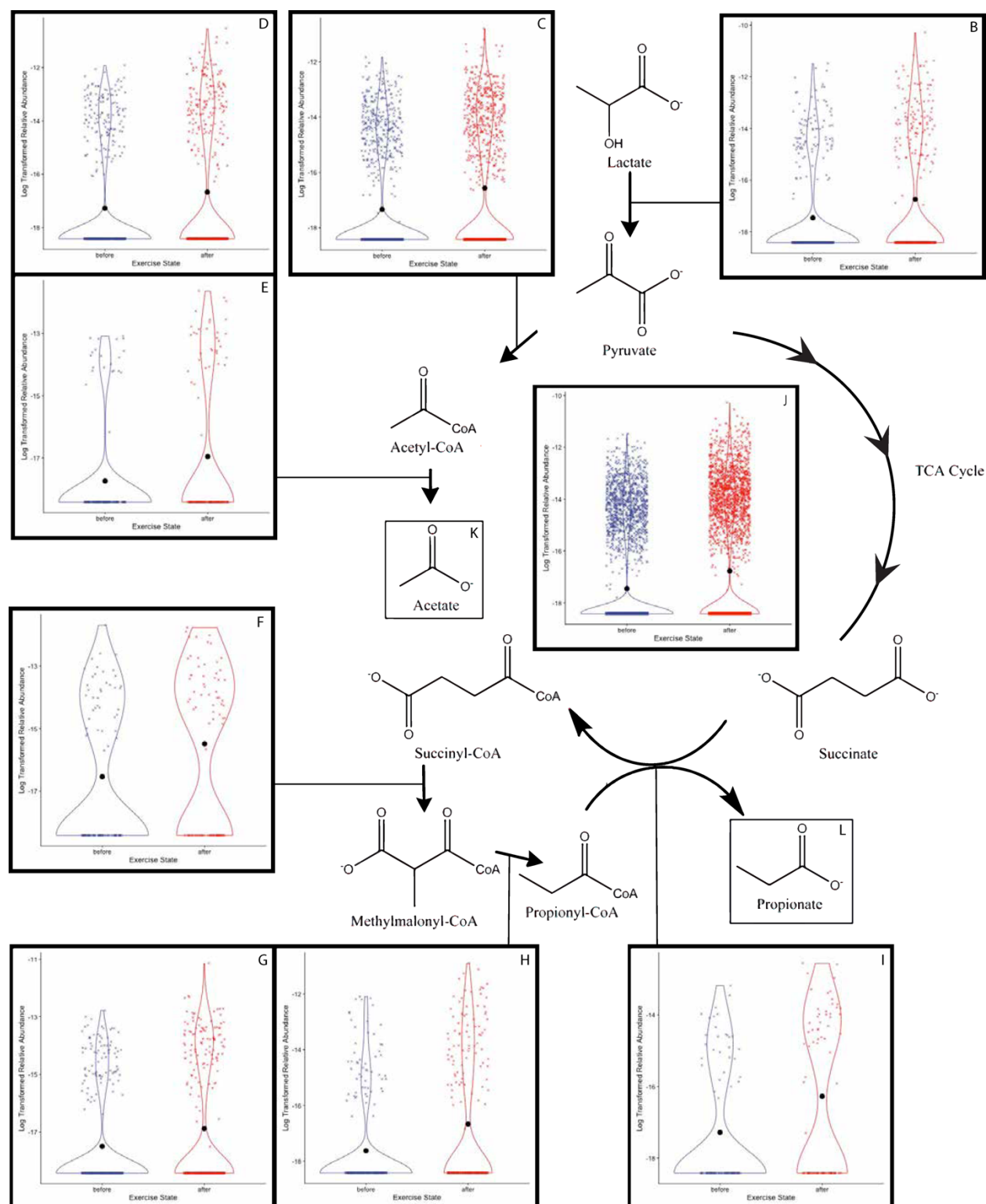
Supplementary Figure 6: A, B: Cytokines after *V. atypica* and *L. bulgaricus* gavage. Each mouse sample is represented as an individual point, with the central bar representing the mean and error bars representing s.e.m. ($n = 64, 32$, and 32 , for baseline, *L. bulgaricus*, and *V. atypica*, respectively). **C, D:** Cytokines after intra-rectal propionate instillation. Each mouse sample is represented as an individual point, with the central bar representing the mean and error bars representing s.e.m. ($n = 32, 16$, and 16 , for baseline, *L. bulgaricus*, and *V. atypica*, respectively). P values determined using one-way ANOVA followed by Tukey's post-hoc test.



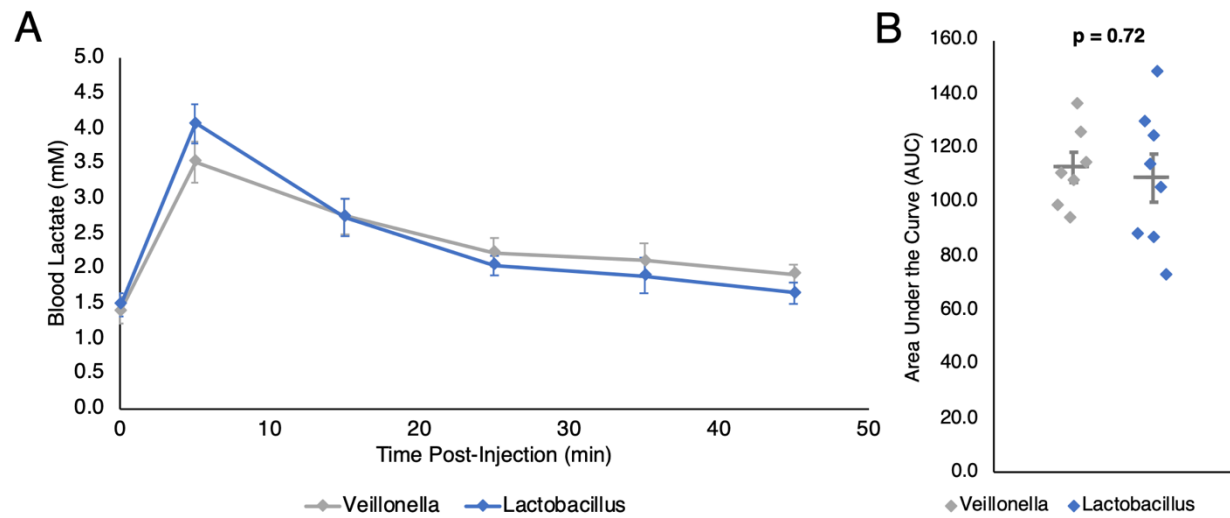
Supplementary Figure 7: A: Representative section of western blot showing GLUT4 abundance in pre-exercise states as well as following *Lb. bulgaricus* and *V. atypica* gavage. Stain-free control used to normalize densitometry analysis shown. Experiment was performed once (n = 8). **B:** Fold-change in GLUT4 abundance. Each point represents an individual mouse sample, the center bar represents the mean, and error bars represent s.e.m. (n = 8).



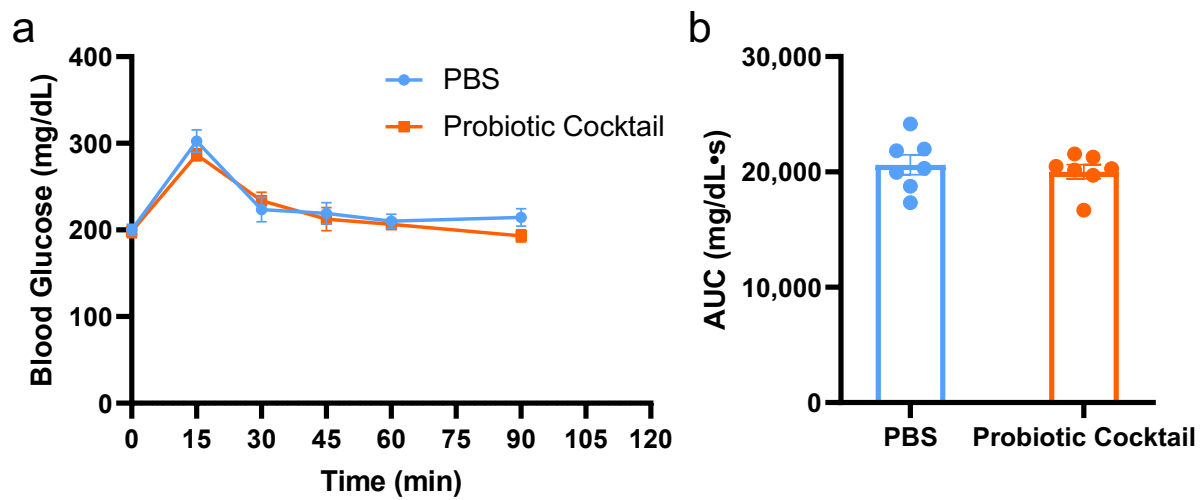
Supplementary Figure 8: **A:** Fraction of putative *Veillonella* relative abundance from metagenomics (calculated utilizing metaphlan2) before and after exercise in rowers and runners. **B:** Significant alleles (calculated from pairwise ANOVA) that are present in each of the 87 samples. **C:** Aforementioned 396 significant alleles are segregated by exercise state and sample. **D:** Histogram comparing non-redundant gene family size and annotation fraction.



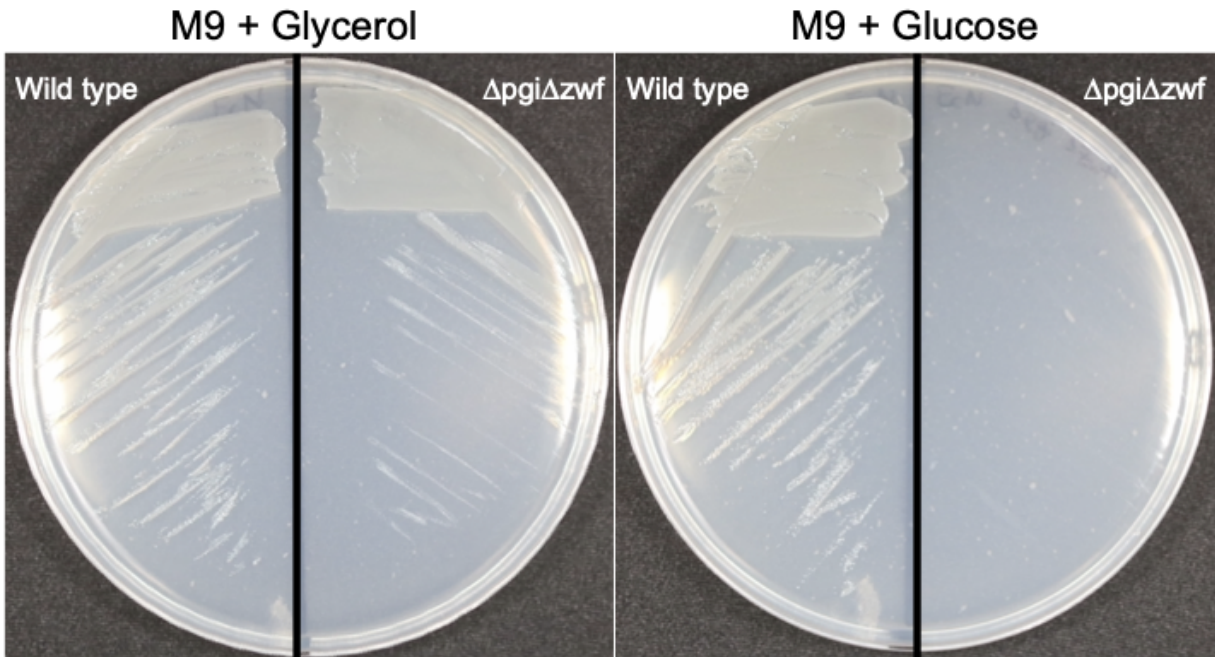
Supplementary Figure 9: Enzyme-resolution log-transformed relative abundances of differentially abundant non-redundant gene families mapped by EC ID to Methylmalonyl-CoA pathway components. Panel A represents the pathway in aggregate and panels B-I represent individual reactions in the pathway (n=8). This data is represented as a violin plot, which displays the distribution of data as a rotated kernel density distribution.



Supplementary Figure 10: Lactate clearance following IP injection in mice. **A:** Mice were gavaged either *Veillonella atypica* or *Lactobacillus bulgaricus* and 5 hours later injected with sodium lactate (750 mg/kg). Blood lactate was measured 5 minutes post-injection and every subsequent 10 minutes ($n = 8$). Points are means $\pm$ s.e.m. **B:** Area under the curve (AUC) was determined for each mouse and compared between treatments. Each mouse is represented as an individual point, with the central bar representing the mean and error bars representing s.e.m. ($p = 0.72$ using two-sided unpaired t-test, $n = 8$).



Supplementary Figure 11: A mixture of probiotic species does not impact OGTT in mice. **a** Blood glucose over time following a co-administered bolus of 1g/kg glucose and 10^9 CFU of freeze-dried probiotic mixture. **b** Area under the curve of **a**.



Supplementary Figure 12: EcN $\Delta pgi\Delta zwf$ is incapable of growth in minimal media with glucose as the sole carbon source.

Supplementary Table 1: Metadata for 2015 Marathon 16S rDNA Sequencing – Too large for print, access via publisher:

Chavkin TA*, Scheiman J*, Luber JM*, MacDonald T, Tung A, Pham LD, Wibowo MC, Wurth RC, Punthambaker S, Tierney BT, Yang Z, Hattab M, Avila-Pacheco J, Clish CB, Lessard S, Church GM, Kostic AD. Meta-omics analysis of elite athletes identifies a performance-enhancing microbe that functions via lactate metabolism. *Nat. Med.* 25, 1104–1109 (2019)

Supplementary Table 2: 16S rDNA taxonomic results from 2015 Boston Marathon – Too large for print, access via publisher:

Chavkin TA*, Scheiman J*, Luber JM*, MacDonald T, Tung A, Pham LD, Wibowo MC, Wurth RC, Punthambaker S, Tierney BT, Yang Z, Hattab M, Avila-Pacheco J, Clish CB, Lessard S, Church GM, Kostic AD. Meta-omics analysis of elite athletes identifies a performance-enhancing microbe that functions via lactate metabolism. *Nat. Med.* 25, 1104–1109 (2019)

Supplementary Table 3: Data from treadmill performance experiments in which mice were either gavaged with either *Lactobacillus bulgaricus* or *Veillonella atypica*

Week 1				Week 2			
	Day 1	Day 2	Day 3		Day 1	Day 2	Day 3
1	1151	1050	708	1	994	980	688
2	1060	744	802	2	326	748	710
3	753	643	743	3	796	573	629
4	1086	869	917	4	1188	1080	805
13	1080	1501	920	13	1336	1020	749
14	1063	1301	418	14	672	1164	850
15	1200	1098	1149	15	1093	1127	614
16	1181	1000	880	16	813	986	408
5	1608	1056	1233	5	1020	900	616
6	1003	1550	832	6	853	840	880
7	890	990	660	7	707	708	571
8	780	689	398	8	546	876	596
9	1354	612	858	9	780	701	676
10	1784	1521	1075	10	990	1075	408
11	1430	1092	626	11	919	450	412
12	1408	1066	926	12	1070	1137	876

Week 3				Week 4			
	Day 1	Day 2	Day 3		Day 1	Day 2	Day 3
17	551	1104	600	17	883	780	367
18	860	848	830	18	1160	929	915
19	239	753	954	19	912	1019	1037
20	973	1208	855	20	950	1276	1175
25	831	871	553	25	743	477	448
26	1420	1041	720	26	1580	1041	914
27	1183	991	684	27	1290	757	843
28	534	649	615	28	1430	893	729
21	979	909	807	21	537	807	845
22	846	1189	688	22	1080	975	970
23	877	944	990	23	640	1043	1035
24	678	812	1113	24	1114	941	1383
29	713	843	409	29	956	693	735
30	355	1068	893	30	822	711	910
31	797	1121	940	31	545	420	316
32	366	1060	946	32	1120	780	263

<i>Lactobacillus bulgaricus</i>
<i>Veillonella atypica</i>

Supplementary Table 4: Serum cytokines quantified before and after treatment with *V. atypica* and exercise

Mouse #	Treatment	mIFNg	mIL10	mIL12p70	mIL17	mIL1b	mIL2	mTNFa	mIL6	<i>V. atypica</i>
		pg/ml	pg/ml	pg/ml	pg/ml	pg/ml	pg/ml	pg/ml	pg/ml	
17	Pre-Exercise	13.8	22.3	1.4	1.3	0.0	6.8	12.6	19.0	Lactobacillus
18	Pre-Exercise	15.3	8.6	1.2	3.1	0.0	7.6	17.5	23.1	
19	Pre-Exercise	17.0	30.2	1.3	1.3	0.0	10.8	15.2	31.4	Below Calibration
20	Pre-Exercise	0.0	30.1	1.1	1.2	0.0	7.2	17.0	13.7	
25	Pre-Exercise	13.5	44.9	1.5	1.7	5.6	10.4	26.3		None Detected
26	Pre-Exercise	13.0	20.5	1.9	1.7	0.0	9.3	12.2	40.9	
27	Pre-Exercise	0.9	23.4	1.1	1.2	0.0	10.2	13.0	71.8	None Detected
28	Pre-Exercise	9.6	27.6	1.2	1.3	0.0	6.1	19.7		
21	Pre-Exercise	0.0	40.0	1.1	1.2	0.0	7.6	18.5	84.6	None Detected
22	Pre-Exercise	14.4	22.8	1.3	1.1	0.0	9.2	14.2	39.2	
23	Pre-Exercise	23.7	34.0	5.6	1.6	0.0	7.6	17.4	131.8	None Detected
24	Pre-Exercise	6.0	41.4	1.7	1.7	0.0	8.0	15.4	147.6	
29	Pre-Exercise	0.0	18.9	1.1	1.4	0.0	10.2	16.3	32.9	None Detected
30	Pre-Exercise	9.3	29.6	1.3	1.5	0.0	8.1	13.4	29.5	
31	Pre-Exercise	7.4	24.3	1.2	1.4	0.0	6.4	15.8	73.9	None Detected
32	Pre-Exercise	7.7	24.1	2.1	1.8	0.0	9.7	12.3	26.4	
17	Lactobacillus	14.8	49.3	1.1	1.7	0.0	7.6	16.0	1201.9	None Detected
18	Lactobacillus	7.3	11.3	1.7	2.5	0.0	7.4	17.5	1786.1	
19	Lactobacillus	26.3	52.6	2.4	2.4	0.0	10.7	27.1	518.1	None Detected
20	Lactobacillus	8.8	16.4	1.9	1.9	0.0	9.9	13.8	96.6	
25	Lactobacillus	0.0	2.7	2.1	1.5	15.5	0.0	23.8		None Detected
26	Lactobacillus	7.5	25.7	2.5	1.3	0.0	0.0	11.3	121.5	
27	Lactobacillus	16.0	26.8	2.9	1.5	0.0	0.0	14.2	282.1	None Detected
28	Lactobacillus	11.6	20.9	2.2	1.6	0.0	0.0	21.9		
21	Veillonella	1.3	17.1	2.0	1.3	0.0	0.0	14.0	352.6	None Detected
22	Veillonella	0.0	27.9	1.6	1.4	0.0	0.0	19.2	320.4	
23	Veillonella	0.0	32.4	1.6	1.4	0.0	0.0	14.5	317.0	None Detected
24	Veillonella	0.0	23.6	1.5	1.5	0.0	0.0	14.0	293.8	
29	Veillonella	0.0	0.0	2.5	1.3	0.0	0.0	10.3	159.2	None Detected
30	Veillonella	0.0	17.7	1.8	1.4	0.0	0.0	7.1	88.7	
31	Veillonella	0.0	0.0	1.6	1.3	0.0	0.0	9.4	52.6	None Detected
32	Veillonella	0.0	0.0	1.2	1.5	0.0	0.0	6.8	47.7	

Supplementary Table 5: Serum cytokines quantified before and after treatment with propionate and exercise

Mouse #	Treatment	mIFN γ pg/ml	mIL10 pg/ml	mIL12p70 pg/ml	mIL17 pg/ml	mIL1b pg/ml	mIL2 pg/ml	mTNFa pg/ml	mIL6 pg/ml
33	Pre-Exercise	8.8	20.2	2.4	1.6	0.0	1.8	16.2	10.1
34	Pre-Exercise	13.8	25.8	2.9	1.6	0.0	6.1	15.2	16.3
35	Pre-Exercise	13.2	27.6	2.2	1.4	0.0	1.1	14.0	26.3
36	Pre-Exercise	8.2	47.4	2.6	1.5	0.0	1.8	19.7	38.3
41	Pre-Exercise	18.8	20.7	2.3	1.7	0.0	1.3	15.4	162.5
42	Pre-Exercise	16.1	18.9	2.6	1.9	0.0	1.8	13.1	39.8
43	Pre-Exercise	12.5	22.5	2.2	1.8	0.0	0.0	14.7	11.3
44	Pre-Exercise	12.4	25.3	2.3	1.8	0.0	0.0	12.4	139.0
37	Pre-Exercise	10.3	34.1	2.2	1.7	0.0	1.1	14.6	6.2
38	Pre-Exercise	9.3	42.5	2.4	1.4	0.0	2.6	20.5	282.4
39	Pre-Exercise	7.3	42.8	2.6	1.7	0.0	0.0	19.5	45.2
40	Pre-Exercise	10.5	50.3	2.6	1.6	0.0	3.8	22.6	119.0
45	Pre-Exercise	4.7	0.0	2.4	1.7	0.0	0.0	10.2	4.9
46	Pre-Exercise	12.5	21.8	2.3	1.7	0.0	0.0	11.6	11.8
47	Pre-Exercise	16.9	24.0	3.0	2.0	0.0	3.6	13.3	12.0
48	Pre-Exercise	23.4	25.4	2.8	1.9	0.0	1.1	11.8	9.6
33	PBS	26.7	28.3	2.6	1.9	0.9	6.5	12.1	96.8
34	PBS	15.8	19.5	2.8	1.7	0.0	2.5	10.2	185.0
35	PBS	12.8	50.9	2.4	1.8	0.0	3.9	10.0	93.7
36	PBS	26.3	38.6	2.6	1.6	0.0	4.1	14.1	441.0
41	PBS	19.7	23.8	2.3	1.9	0.0	4.2	10.2	89.0
42	PBS	26.8	20.2	2.7	2.0	0.0	4.2	7.7	81.8
43	PBS	20.2	30.9	2.9	2.1	0.0	1.3	9.8	75.1
44	PBS	14.2	34.8	2.4	3.5	0.0	3.6	17.5	433.5
37	Propionate	16.5	38.5	2.5	1.8	0.0	3.7	23.4	49.4
38	Propionate	27.3	34.3	2.7	2.6	2.5	3.0	18.9	195.7
39	Propionate	27.0	35.3	2.7	1.7	0.0	4.4	10.3	62.1
40	Propionate	19.5	25.1	2.6	1.8	1.0	3.4	14.4	192.0
45	Propionate	13.8	14.7	2.9	1.9	3.1	0.0	12.2	456.7
46	Propionate	7.5	38.5	2.6	1.9	0.0	3.3	10.7	419.2
47	Propionate	17.5	40.6	3.3	2.6	2.2	2.7		312.6
48	Propionate	16.9	47.7	3.1	1.7	0.0	1.5	12.5	281.5

Propionate
PBS
Below Calibration
None Detected

Supplementary Table 6: Metadata for metagenomic sequencing of runners and rowers before and after exercise – Too large for print, access via publisher:

Chavkin TA*, Scheiman J*, Luber JM*, MacDonald T, Tung A, Pham LD, Wibowo MC, Wurth RC, Punthambaker S, Tierney BT, Yang Z, Hattab M, Avila-Pacheco J, Clish CB, Lessard S, Church GM, Kostic AD. Meta-omics analysis of elite athletes identifies a performance-enhancing microbe that functions via lactate metabolism. *Nat. Med.* 25, 1104–1109 (2019)

Supplementary Table 7: In vitro metabolomics quantification of select SCFAs from spent media of 48-hour BHI cultures

Sample	Internal Std	Valerate and Isovalerate		Butyrate and Isobutyrate		Propionate	
	Area	Area	uM	Area	uM	Area	uM
V. dispar 1	5E+10	9E+09	103.47	8E+09	84.83	7E+10	2329.98
V. dispar 2	6E+10	9E+09	97.46	7E+09	78.79	7E+10	2212.05
V. atypica 1	6E+10	9E+09	94.80	7E+09	69.73	8E+10	2354.02
V. atypica 2	6E+10	9E+09	87.22	7E+09	68.68	8E+10	2218.25
V. parvula 1	6E+10	1E+10	97.13	7E+09	74.38	7E+10	2119.28
V. parvula 2	6E+10	1E+10	97.32	7E+09	73.47	8E+10	2090.90
Media Ctrl 1	5E+10	1E+10	121.48	1E+10	143.37	5E+09	161.62
Media Ctrl 2	5E+10	1E+10	124.00	1E+10	151.49	5E+09	158.78
		uM average	% vs media control	uM average	% vs media control	uM average	% vs media control
Media Ctrl		123	100%	147	100%	160	100%
V. dispar		100	82%	82	55%	2271	1418%
V. atypica		91	74%	69	47%	2286	1427%
V. parvula		97	79%	74	50%	2105	1314%

Sample	Internal Std	Acetate		GABA	
	Area	Area	uM	Area	uM
V. dispar 1	5E+10	8E+10	4914.49	3E+09	5.63
V. dispar 2	6E+10	8E+10	4589.87	3E+09	5.22
V. atypica 1	6E+10	8E+10	4266.98	3E+09	5.50
V. atypica 2	6E+10	8E+10	4031.71	3E+09	5.09
V. parvula 1	6E+10	8E+10	4395.88	3E+09	4.90
V. parvula 2	6E+10	8E+10	4059.10	3E+09	4.87
Media Ctrl 1	5E+10	1E+11	7587.36	3E+09	6.48
Media Ctrl 2	5E+10	1E+11	7527.05	3E+09	6.51
		uM average	% vs media control	uM average	% vs media control
Media Ctrl		7557	100%	6	100%
V. dispar		4752	63%	5	84%
V. atypica		4149	55%	5	82%
V. parvula		4227	56%	5	75%

Supplementary Table 8: In vitro metabolomics data from MRS media and semi-synthetic lactate media experiments

Sample Label	Internal Std Area	Butyrate Area	Butyrate uM	Propionate Area	Propionate uM	Pyruvate Area	Pyruvate uM	Lactate Area	Lactate uM	Acetate Area	Acetate uM
V. dispar MRS	8E+08	2E+09	1.92	1E+10	1137	2E+09	0.31	2E+11	25.4	7E+09	26794
V. dispar MRS	8E+08	2E+09	1.99	1E+10	1094	1E+09	0.25	2E+11	22.0	7E+09	28025
V. dispar MRS	7E+08	2E+09	2.02	1E+10	1092	1E+09	0.27	1E+11	24.0	7E+09	28655
			1.98		1108		0.28		23.8		27824
V. dispar lactate	1E+08	3E+09	17.74	2E+10	9371	3E+09	9.54	4E+11	420.1	3E+09	77048
V. dispar lactate	1E+08	3E+09	16.21	2E+10	8431	3E+09	4.60	4E+11	371.8	3E+09	65318
V. dispar lactate	1E+08	3E+09	18.94	1E+10	9358	3E+09	11.14	3E+11	429.3	3E+09	76230
			17.63		9053		8.43		407.0		72866
V. parvula MRS	8E+08	2E+09	1.74	1E+10	885	8E+08	0.16	1E+11	23.7	7E+09	28178
V. parvula MRS	8E+08	2E+09	1.84	1E+10	966	8E+08	0.15	2E+11	24.7	7E+09	29361
V. parvula MRS	7E+08	2E+09	1.86	1E+10	1113	8E+08	0.18	2E+11	26.4	7E+09	31456
			1.81		988		0.16		24.9		29665
V. parvula Lactate	3E+08	2E+09	5.09	1E+10	2883	3E+09	2.03	4E+11	153.6	3E+09	27470
V. parvula Lactate	3E+08	2E+09	5.02	1E+10	2924	4E+09	2.44	4E+11	150.5	3E+09	27317
V. parvula Lactate	3E+08	2E+09	4.98	1E+10	2943	3E+09	2.27	4E+11	159.9	3E+09	28501
			5.03		2917		2.25		154.6		27763
V. atypica MRS	8E+08	2E+09	1.97	1E+10	1153	7E+08	0.11	1E+11	16.5	7E+09	28402
V. atypica MRS	8E+08	2E+09	1.97	1E+10	1144	7E+08	0.12	1E+11	16.3	7E+09	28686
V. atypica MRS	7E+08	2E+09	2.07	1E+10	1224	7E+08	0.14	1E+11	17.4	7E+09	30338
			2.00		1174		0.12		16.7		29142
V. atypica Lactate	8E+07	2E+09	18.90	1E+10	11773	2E+09	3.93	3E+11	441.8	3E+09	98661
V. atypica Lactate	9E+07	2E+09	18.01	1E+10	9929	2E+09	5.13	3E+11	422.8	3E+09	88786
V. atypica Lactate	9E+07	2E+09	18.93	1E+10	10131	2E+09	4.23	3E+11	429.2	3E+09	89850
			18.61		10611		4.43		431.3		92432
L. plantarum MRS	8E+08	7E+08	0.62	4E+07	6	2E+09	0.52	7E+11	114.5	5E+09	19800
L. plantarum MRS	8E+08	7E+08	0.59	4E+07	6	3E+09	0.58	7E+11	114.7	5E+09	19423
L. plantarum MRS	9E+08	7E+08	0.59	4E+07	5	3E+09	0.55	7E+11	108.0	5E+09	17566
			0.60		6		0.55		112.4		18930
L. plantarum Lactate	3E+08	2E+09	7.06	1E+07	6	8E+09	13.21	1E+12	597.7	5E+08	6011
L. plantarum Lactate	3E+08	2E+09	6.68	1E+07	5	8E+09	13.79	1E+12	584.5	4E+08	5141
L. plantarum Lactate	3E+08	2E+09	6.47	1E+07	5	8E+09	13.14	1E+12	565.1	5E+08	5554
			6.74		5		13.38		582.4		5569
E. coli Nissle MRS	5E+08	1E+09	2.10	1E+07	2	5E+09	1.71	4E+11	108.4	7E+09	39949
E. coli Nissle MRS	5E+08	1E+09	2.12	5E+06	1	5E+09	1.98	4E+11	118.1	7E+09	44334
E. coli Nissle MRS	5E+08	1E+09	2.19	4E+06	1	5E+09	2.04	4E+11	118.5	7E+09	45727
			2.14		1		1.91		115.0		43337
E. coli Nissle Lactate	2E+08	2E+09	7.78	8E+06	4	2E+10	60.93	1E+12	613.7	7E+08	9056
E. coli Nissle Lactate	2E+08	3E+09	8.48	9E+06	4	3E+10	90.38	1E+12	622.1	7E+08	9302
E. coli Nissle Lactate	2E+08	3E+09	7.78	9E+06	4	3E+10	86.64	1E+12	613.9	6E+08	8181
			8.01		4		79.32		616.6		8846
L. bulgaricus MRS	5E+08	2E+09	2.38	4E+07	8	3E+09	1.13	8E+11	195.6	6E+09	36775
L. bulgaricus MRS	6E+08	2E+09	2.20	4E+07	7	3E+09	1.00	8E+11	176.8	5E+09	30058
L. bulgaricus MRS	5E+08	2E+09	2.18	4E+07	7	3E+09	1.12	8E+11	189.2	5E+09	31157
			2.25		7		1.08		187.2		32663
L. bulgaricus Lactate	2E+08	2E+09	8.39	9E+06	4	1E+10	31.04	1E+12	693.5	4E+08	5160
L. bulgaricus Lactate	2E+08	2E+09	8.10	4E+05	0	1E+10	30.84	1E+12	690.9	3E+08	4497
L. bulgaricus Lactate	2E+08	2E+09	9.56	8E+06	5	1E+10	38.99	1E+12	826.2	3E+08	5297
			8.68		3		33.62		736.9		4985
B. longum MRS	5E+08	1E+09	2.21	6E+07	11	2E+09	0.97	5E+11	128.8	9E+09	63506
B. longum MRS	5E+08	1E+09	2.32	6E+07	11	2E+09	0.98	5E+11	128.2	9E+09	62189
B. longum MRS	6E+08	2E+09	2.16	6E+07	10	2E+09	0.76	4E+11	101.3	9E+09	52160
			2.23		11		0.90		119.5		59285
B. longum Lactate	2E+08	2E+09	9.64	2E+07	8	8E+09	25.91	1E+12	767.3	1E+09	20760
B. longum Lactate	2E+08	2E+09	8.96	1E+07	7	8E+09	22.86	1E+12	772.0	1E+09	18769
B. longum Lactate	2E+08	2E+09	9.06	1E+07	7	8E+09	21.92	1E+12	732.5	1E+09	20094
			9.22		7		23.56		757.2		19874
MRS Media Control	6E+08	2E+09	2.00	6E+06	1	3E+09	0.97	5E+11	108.2	6E+09	32467
MRS Media Control	5E+08	1E+09	2.30	3E+06	1	3E+09	1.25	5E+11	142.5	2E+09	29034
MRS Media Control	6E+08	2E+09	2.02	6E+06	1	3E+09	0.85	5E+11	98.5	6E+09	31823
			2.10		1		1.02		116.4		31108
Lactate Media Control	2E+08	3E+09	11.37	6E+06	4	1E+10	41.99	1E+12	853.6	1E+08	1761
Lactate Media Control	2E+08	3E+09	11.20	6E+06	3	1E+10	41.06	1E+12	861.7	1E+08	1719
Lactate Media Control	2E+08	3E+09	11.52	8E+06	5	1E+10	40.53	1E+12	839.1	1E+08	1742
			11.36		4		41.19		851.5		1741

Supplementary Table 9: $^{13}\text{C}_3$ -labeled lactate experiment raw data

Method	m/z	Retention Time (min)	Metabolite	M1-Serum	M2-Serum	M3-Serum	M5-Serum	M6-Serum	M7-Serum	M8-Serum
HILIC-neg	89.0244	3.62	lactate	376300502	358227850	339057538	339705180	240526799	233256360	295133344
HILIC-neg	90.0278	3.62	lactate-13C1	15545778	14683365	14647973	13518428	10624305	11451584	12439107
HILIC-neg	91.0311	3.62	lactate-13C2	3677504	3322930	4153291	2897782	2836431	3780091	3288951
HILIC-neg	92.0345	3.62	lactate-13C3	11914741	12439502	11732833	9847273	9879545	10328704	8190177
HILIC-neg	73.0295	3.53	propionate	1657276	1735182	1325080	1194357	1837389	2128644	1242876
HILIC-neg	74.0329	3.53	propionate-13C1	34134	36972	0	0	47971	38956	35547
HILIC-neg	75.0362	3.53	propionate-13C2	0	0	0	0	0	0	0
HILIC-neg	76.0396	3.53	propionate-13C3	0	0	0	0	0	0	0

Method	m/z	Retention Time (min)	Metabolite	M1-Plasma	M2-Plasma	M3-Plasma	M5-Plasma	M6-Plasma	M7-Plasma	M8-Plasma
HILIC-neg	89.0244	3.62	lactate	294485889	297700107	233249383	392924912	305906911	299700261	191954734
HILIC-neg	90.0278	3.62	lactate-13C1	12914708	12204663	10266267	15571857	12557107	12904095	9552152
HILIC-neg	91.0311	3.62	lactate-13C2	3822765	3283453	2832395	3760721	2510479	2999187	3348467
HILIC-neg	92.0345	3.62	lactate-13C3	11046154	11062748	10035327	14043859	9592413	10584360	9496671
HILIC-neg	73.0295	3.53	propionate	1266839	1589489	1815344	1829473	1316050	1492924	2145134
HILIC-neg	74.0329	3.53	propionate-13C1	14217	46157	40467	33302	25967	36696	53048
HILIC-neg	75.0362	3.53	propionate-13C2	0	0	0	0	0	0	0
HILIC-neg	76.0396	3.53	propionate-13C3	0	0	0	0	0	0	0

Method	m/z	Retention Time (min)	Metabolite	M1-Cecum	M2-Cecum	M3-Cecum	M5-Cecum	M6-Cecum	M7-Cecum	M8-Cecum
HILIC-neg	89.0244	3.62	lactate	10061949	8591729	7225127	5513199	7852259	7989688	11611597
HILIC-neg	90.0278	3.62	lactate-13C1	407633	391560	336458	262746	325021	359817	534867
HILIC-neg	91.0311	3.62	lactate-13C2	34235	44279	43926	18731	37678	31635	84775
HILIC-neg	92.0345	3.62	lactate-13C3	135097	174076	146578	87142	141420	105893	244573
HILIC-neg	73.0295	3.53	propionate	2799701	3916689	3463899	3187542	3888359	3470150	2766376
HILIC-neg	74.0329	3.53	propionate-13C1	104931	137030	102293	125091	148330	136999	97572
HILIC-neg	75.0362	3.53	propionate-13C2	0	0	0	0	0	0	0
HILIC-neg	76.0396	3.53	propionate-13C3	0	0	0	0	0	0	0

Method	m/z	Retention Time (min)	Metabolite	M1-Colon	M2-Colon	M3-Colon	M5-Colon	M6-Colon	M7-Colon	M8-Colon
HILIC-neg	89.0244	3.62	lactate	16653281	4792503	7229712	11169351	13031645	3978496	4615575
HILIC-neg	90.0278	3.62	lactate-13C1	651963	216703	353960	473421	585478	202225	219230
HILIC-neg	91.0311	3.62	lactate-13C2	68332	0	42597	13424	94204	17450	13158
HILIC-neg	92.0345	3.62	lactate-13C3	174267	37842	116220	46476	250430	67910	53765
HILIC-neg	73.0295	3.53	propionate	1903582	2458468	2261953	2553629	2472535	2222644	2107514
HILIC-neg	74.0329	3.53	propionate-13C1	51073	80521	54803	73507	71262	69404	72045
HILIC-neg	75.0362	3.53	propionate-13C2	0	0	0	0	0	0	0
HILIC-neg	76.0396	3.53	propionate-13C3	0	0	0	0	0	0	0

Supplementary Table 10: Lactate clearance data following IP injection in mice either gavaged with *Veillonella atypica* or *Lactobacillus bulgaricus*

	Mouse	Weight (g)	0 min	5 min	15 min	25 min	35 min	45 min
Veillonella-Treated	A1	22.0	1.6	2.3	1.9	2.2	2.4	2.5
	A2	20.5	2.3	4.6	2.3	1.9	1.8	2.1
	A3	21.0	0.8	3.7	2.2	2.1	0.9	
	A4	19.5	1.2	3.4	2.7	1.8	1.4	1.4
	B1	29.0	1.4	2.8	2.4	2.2	2.8	2.0
	B2	29.5	0.9	4.5	4.3	2.6	2.2	1.9
	B3	28.0	1.6	3.8	3.0	3.0	2.3	2.1
	B4	28.5	1.4	3.0	3.1	2.0	3.1	1.4
Lactobacillus-Treated	C1	28.0	1.4	3.6	2.1	2.2	0.8	1.4
	C2	27.0	1.7	3.5	2.1	1.5	1.3	1.5
	C3	28.5	2.4	4.7	3.2	2.6	2.4	1.4
	D1	25.5	1.7	4.6	2.6	1.9	2.3	1.5
	D2	26.0	1.0	4.6	3.4	2.4	2.0	1.9
	D3	24.0	1.1	5.0	3.9	2.8	2.9	2.4
	D4	21.5	1.8	4.1	2.6	1.7	1.9	1.7
	D5	26.5	0.8	2.4	1.9	1.2	1.5	1.4