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Understanding Veillonella atypica as a potential metabolic mediator in diabetic exercise

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Understanding *Veillonella atypica* as a potential metabolic mediator in diabetic exercise resistance

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ABSTRACT

Diabetes is one of the leading health threats to Western society. While several treatment options and preventative measures exist for diabetes, physicians routinely recommend exercise to improve glucose metabolism in diabetics. However, many patients still struggle with both excess glucose production and inefficient glucose uptake, despite adherence to an exercise regimen. This phenomenon, known as exercise resistance, means that exercise is either ineffective or harmful for diabetics. While some diabetics may not respond well to exercise due to mitochondrial limitations, other factors, such as gut microbiome dysbiosis and lactic acidosis, could be contributors. Lactate-metabolizing gut bacteria, like *Veillonella atypica*, could ameliorate exercise's negative effects in diabetics by improving dysbiosis and lactic acidosis. *V. atypica* is traditionally classified as a strict anaerobe that could metabolize lactate into usable short-chain fatty acids (SCFA) to improve diabetic exercise resistance symptoms. However, many characteristics of *V. atypica* have only been supported by genomic studies, so this project's goal was to begin to characterize the metabolic features of *V. atypica* in the laboratory. In this study, fluid thioglycollate tests supported literature that *V. atypica* was an obligate anaerobe. Growth on Brain heart infusion (BHI) versus BHI plus lactate (BHIL) media showed that *V. atypica* could grow with or without lactate at 25°C and 37°C; however, *V. atypica* grew better in the presence of lactate at 37°C. GC-MS analysis detected high quantities of potentially beneficial SCFAs, acetic and propionic acids, from metabolism of *V. atypica,* particularly in BHIL media. These data could contribute to the potential development of a well-tested, clinically-safe, *V. atypica*-based probiotic to amend exercise resistance in diabetics.

KEYWORDS: *Veillonella atypica*, diabetes, exercise resistance, lactate, lactic acidosis, gut microbiome, dysbiosis, SCFA

INTRODUCTION

Despite its prevalence in many Western societies, diabetes can be an especially difficult metabolic disorder to manage. Type I and type II diabetes (T1D and T2D) patients struggle with both excess glucose production in the liver and ineffective glucose uptake by skeletal muscles and other tissues. Particularly, T2D patients cannot manage the metabolic load of excess glucose, so they are often categorized as insulin resistant (Kim et al. 2008). Eventually, excess glucose in the bloodstream can lead to other systemic complications, resulting in symptoms like excessive urination and thirst, fatigue, blurry vision, immune system complications, and weight loss (Keays 2007). Such problems could occur during states of hyperglycemia because the body often resorts to pathways other than the traditional glycolysis pathway to process glucose. Pathways include glycation, hexosamine, protein kinase C, alpha-ketoaldehyde, and sorbitol pathways. Such pathways are problematic because they all produce reactive oxygen species (ROS) (Srikanth and Orrick 2022). ROS are unstable molecules that have a "free," unpaired electron in their outer shell, predisposing them to "steal" electrons from other species. This "stealing" damages cellular structures that normally build DNA, proteins, and lipids, suggesting that ROS are largely responsible for diabetic systemic complications associated with hyperglycemia (Pham-Huy et al. 2008; Shields et al. 2021).

Considering the pathologic damages associated with diabetes, its increasing prevalence is concerning. For example, although only about 11 percent of America's population currently has diabetes, nearly 40 percent of Americans over the age of 18 and 50 percent of Americans over the age of 65 have prediabetes (National Diabetes Statistics…2022). A prediabetic individual has a higher glycemic reading than a normal, healthy individual; however, the hyperglycemia level is still below the classification for that of a diabetic (Bansal 2015). With high percentages

of prediabetics, the United States is at risk of a heavy increase in the number of diabetes cases, especially if preventative measures and treatments against hyperglycemia and insulin impairments are insufficient. The World Health Organization (WHO) anticipates that type II diabetes (T2D) will move from the eighth leading cause of death in the United States to the seventh position by 2030. Therefore, it is imperative to find new preventative measures and treatments to either delay or prohibit the disease's growing prevalence (Böhm et al. 2016).

While treatments and preventative measures against diabetes have varied between insulin injections, glucose-lowering medications, and dietary alterations, exercise is also highly recommended to help manage glucose homeostasis. With this idea, various exercise studies used the maximum oxygen uptake (VO_2max) to assess the training's effectiveness in terms of cardiorespiratory fitness. The VO_2max is the maximum oxygen uptake by mitochondria during exercise (Parikh 2009). Mitochondria aerobically maintain energy homeostasis by metabolizing nutrients into ATP; however, with a lower VO_2max, mitochondria cannot appropriately balance energy production versus respiration (Kim et al. 2008). Therefore, with an elevated VO_2max during exercise, glucose homeostasis should improve, as mitochondria operate more efficiently with more oxygen; however, improvements are not universal to all diabetics (Böhm et al. 2016). According to a phenomenon known as exercise resistance, exercise can sometimes become harmful or unproductive for diabetics, suggesting that glycemic control and cellular responses to insulin do not improve with exercise (Liu et al. 2020). One explanation could be that there are fewer and smaller mitochondria in diabetic skeletal muscle. With this perspective, Kim et al. (2008) showed that endurance training improved the VO_2max to increase the number, oxidative activity, and size of mitochondria. Despite these findings, another study showed that an enhanced VO_2max did not consistently help all individuals manage glucose levels (Böhm et al.

2016). Considering the additional evidence, there are likely other factors influencing some diabetics' limited responses to exercise and increased glucose levels during or after exercise.

One possible explanation for increased glucose, in the context of exercise resistance, could be the imbalance between mitochondrial impairments and excess lactate production during exercise. In general, lactate is a metabolic product of glycolysis and is often produced from active skeletal muscle under lower oxygen conditions (Kreisberg 1980). Typically, glucose is metabolized into pyruvate, and under exercise's more anaerobic conditions, pyruvate, NADH, and H^+ are converted into lactate and NAD^+ (Kreisberg 1980). Lactate is then used as a substrate in mitochondrial respiration, which contributes to ATP production (Brooks 2018). Under aerobic conditions, mitochondria reoxidize NAD^+ and lactate to NADH and pyruvate, respectively, and these products can then be used in the citric acid cycle to produce ATP (Kreisberg 1980). The citric acid cycle creates a connected metabolic pathway for all aerobic processes to eventually lead to more energy (Akram 2014). This emphasizes that when used appropriately, lactate can be an important energy resource; however, it can also sometimes become problematic in excess quantities, especially in diabetics. Kreisberg (1980) explained that lactate oxidation in diabetes patients is inhibited because their skeletal muscles cannot utilize lactate as well as people without diabetes. This can lead to higher arterial lactate concentrations after exercise, potentially leading to higher amounts of lactate reaching the liver, rather than being used for energy. This could relate to impaired mitochondrial number and size, as the higher lactate levels found in diabetics were serving as precursors for gluconeogenesis once they reached the liver, producing more glucose than what would occur in healthy individuals (Wahren et al. 1975). Furthermore, lactate can be particularly problematic for diabetics because diabetics often suffer from lactic acidosis, a condition of hyperlactatemia and a low pH in the blood. This condition can not only contribute

to health issues, such as neurotoxicity and cardiac arrhythmias, but it is also associated with inadequate tissue oxygenation, which could contribute to impaired mitochondrial functioning (Duncan et al. 2004; Kreisberg 1980). This implies that not only do diabetics struggle to keep pace with skeletal muscle's lactate production, but the effects of lactate are intensified under the conditions of lactic acidosis, potentially contributing to inefficient mitochondria and excess glucose production.

Based on diabetic lactic acidosis and related mitochondrial impairments during exercise, lactic acidosis could be connected to exercise resistance. While some researchers suggest that lactic acid is supposed to be metabolized by muscle cells as a source for ATP recovery, excess lactate often converts into glucose in the liver, resulting in higher blood glucose levels, rather than helping maintain glucose homeostasis in diabetics (Roosterman and Cottrell 2021; Kreisberg 1980). This means that diabetics may be unable to process sufficient amounts of lactic acid due to mitochondrial inefficiencies and the overabundance of lactate from lactic acidosis; therefore, excess lactate could be converting into the resulting excess glucose found in diabetics experiencing exercise resistance. This could also mean that diabetics do not have sufficient energy harvests to help them perform, enhancing lactic acidosis' negative effects and potentially contributing to exercise resistance. Therefore, decreasing lactate levels or finding a way to appropriately use lactate could help diabetics respond better to exercise, rather than their bodies being overwhelmed with too much lactate.

Considering that many diabetics struggle with mitochondrial limitations and excess lactate production, some studies suggested that the problem could be related to the gut microbiome. The human gut microbiome is a collection of all bacteria that live within the oral cavity, stomach, small intestine, and large intestine. It is composed of five different phyla,

including Firmicutes (79.4 percent), Bacteroides (16.9 percent), Actinobacteria (1 percent), Proteobacteria (0.1 percent), and Verrucomicrobia (0.1 percent) (Pokusaeva et al. 2011). The gut microbiome mediates between diet and host physiology, as bacteria can metabolize typically indigestible plant fibers, ensure that various sugar polymers are broken down from dietary glycans, and ferment sugars to produce short-chain fatty acids (SCFAs) (Peterson et al. 2022). As a potential way to describe exercise resistance, one study divided a sample population of diabetics into "responders" and "non-responders." Responders' glucose homeostasis and insulin responses improved with exercise, but non-responders either did not improve or became worse. Based on the experiment, researchers identified a compositional maladaptation in non-responders, showing that their gut microbiomes unexpectedly resembled sedentary individuals, rather than active people (Liu et al. 2020). The findings could be explained by gut dysbiosis, a common condition in diabetics. Dysbiosis occurs when a person's gut composition changes to have more pathogenic than beneficial bacteria (Qin et al. 2012). Based on a metagenome-wide analysis of diabetic gut microbiomes, Qin and colleagues suggested that patients with T2D experienced functional dysbiosis, a condition that associates diabetes with a loss of several bacterial species, rather than just a few particular species. This is typically associated with having fewer metabolically advantageous bacteria and more opportunistic pathogens (2012). However, unlike diabetics, non-diabetic athletes who frequently exercise are often associated with having higher gut microbial diversity and more beneficial bacteria than non-athletes (Wosinska et al. 2019). Considering both studies, exercise should balance diabetics' gut microbiomes, as athletes, thought to exercise more frequently than non-athletes, had more beneficial bacteria than pathogenic bacteria. However, unlike non-diabetic athletes, even with

augmented exercise, diabetics could still be negatively impacted by increased exercise, potentially due to functional dysbiosis.

As gut microbiome diversity seems vital for effective exercise, gut microbes' metabolic products are possibly important for managing diabetes-related symptoms. Beneficial gut bacteria metabolically produce SCFAs. Unlike athletes, who have diverse microbiomes, dysbiosis in diabetics reduces metabolic efficiency by decreasing the number of SCFA pathways (Qin et al. 2012). This means that in addition to mitochondrial deficits, diabetics have fewer ways to break down nutrients in the gut microbiome. SCFA are signaling molecules that can provide energy for the host, help with hormone regulation, improve insulin sensitivity, and maintain glucose homeostasis (De Vadder et al. 2014). Some researchers assume that SCFAs are only metabolic end-products; however, SCFAs can also be substrates for other purposes (Peterson et al. 2022). For example, glucose tolerance and insulin resistance are worse when more propionate, a type of SCFA, converts to glucose in the liver. However, with an efficient gut microbiome, propionate could act as a substrate for certain bacteria that can metabolize it into energy before it reaches the liver, helping insulin-resistant patients suppress glucose production in the liver (De Vadder et al. 2014). In connection to exercise, Liu et al. showed that in responders, exercise often improved SCFA biosynthesis; however, many test subjects were non-responders (2020). This lack of universal improvement could be related to dysbiosis. For example, in children with T1D, Murri et al. found a significant prevalence of Bacteroidetes, which are often pathogenic, and a significant decrease of Firmicutes and Actinobacteria, which are often beneficial for SCFA production (2013). Such patients may have lacked essential SCFA production, which could contribute to exercise resistance.

One possibility for improving dysbiosis and lactic acidosis-induced exercise resistance could be to enhance diabetic gut microbiomes with beneficial lactate-metabolizing bacteria, which could help manage lactate levels during exercise. Due to the prevalence of dysbiosis in diabetics, these beneficial bacteria are less likely to be as commonly found in a diabetic's gut microbiome. The prevalence of dysbiosis could be due to heavier antibiotic-use in diabetes, as diabetes can make certain bacterial infections more common, particularly those due to skin ulceration (Lipsky and Berendt 2000). Such infections would require antibiotic treatment, which could limit certain species within the gut microbiome, as high antibiotic-use is associated with increasing dysbiosis (Deshmukh et al. 2014). While they may be diminished in cases of dysbiosis, lactate-metabolizing bacteria convert lactate into SCFAs, such as acetate, propionate, and butyrate, which can be used as energy sources for other processes when metabolized by gut microbes (Duncan et al. 2004). One lactate-metabolizing gut microbe genus that could be used is *Veillonella.* These bacteria are anaerobic, non-motile, gram-negative cocci bacteria that often appear in short chains or pairs (Vesth et al. 2013). They are found in the oral cavity, esophagus, stomach, small intestine, and large intestine (van den Bogert et al. 2013). In order to survive these areas, *Veillonella* have an outer lipopolysaccharide envelope and autotransporter proteins that can help them aggregate with other organisms in order to form biofilms (Béchon et al. 2020). A biofilm forms when communities of two or more bacterial species adhere themselves within the extracellular matrix, serving as a protective measure against environmental stressors or as a way to exchange nutrients between the different species (Baumgartner et al. 2021). This adaptation likely helps *Veillonella* survive harsh environments within the human body. Some research suggests that *Veillonella* forms biofilms with *Streptococcus* species, especially in the oral microbiome. In one oral biofilm study, *Streptococci* catabolized carbohydrates into lactic

acid, and *Veillonella* consumed this product (Palmer et al. 2006). This indicates a potential symbiotic relationship between *Streptococci* and *Veillonella* for survival and metabolic purposes, and it shows that the *Veillonella* species could help reduce lactic acidosis by metabolizing lactate.

One specific species that could diminish lactate levels in exercising diabetics is *Veillonella atypica*. An exercise study found that *V. atypica* could metabolize exercise-induced lactate into SCFA that could then be used for host energy and to decrease overall systemic lactate levels in the blood (Scheiman et al. 2019). As a gut microbe, *V. atypica* could be useful in the context of both exercise and elevated lactate levels to potentially help diabetics experiencing lactic acidosis and exercise resistance. Furthermore, *Veillonella* bacteria metabolize lactate into butyrate and propionate, which help regulate hormones like glucose-dependent insulinotropic polypeptide, glucagon-like peptide I, and ghrelin (Murri et al. 2013). With this understanding, mitigating dysbiosis in diabetics with more *V. atypica* could improve glycemic levels and help regulate energy balance during exercise. While not much information explains how *V. atypica* bacteria survive lower pH conditions in the gut, especially in the additional context of lactic acidosis, one study demonstrated that other lactate-utilizing bacteria could survive low pH conditions in a starch-enriched environment (Biddle et al. 2013). Based on the study, *V. atypica* does not metabolize starch itself, but its survival under low pH conditions could depend upon the presence of starch. Starch could fuel *Streptococci* bacteria, which ferment sugars into lactic acid and enhance *V. atypica*'s ability to form a protective biofilm to survive the gut (van den Bogert et al. 2013). Furthermore, Zoetendal et al. emphasized that the small intestine, an area in which *V. atypica* could live, was an especially harsh environment for gut bacteria due to its bile excretion and digestive enzymes. Microbes in the small intestine would require stronger survival strategies (Zoetendal et al. 2012). This implies that *V. atypica* could have adapted an outer

lipopolysaccharide envelope and biofilm communities in order to survive harsher areas in the body (Béchon et al. 2020). With an outer lipopolysaccharide envelope fueled by products from starch-metabolizing bacteria, *V. atypica* could survive lower pH ranges in diabetics suffering from lactic acidosis.

In addition to *V. atypica*'s potential ability to tolerate low pH environments, studies also made predictions about *V. atypica*'s aerotolerance based upon where it could survive in the body. Most studies classified *V. atypica* as an obligate anaerobe; however, some other studies implied that it could be a facultative anaerobe. Facultative anaerobes generally prefer aerobic conditions; however, they can adapt their metabolisms to survive anaerobic conditions (Stieglmeier et al. 2009). As opposed to studies that indicated that *V. atypica* was an obligate anaerobe, Zoetendal et al. studied bacteria, including *Veillonella* species, from ileostomy subjects. Researchers emphasized that most past research focused on the large intestine, so they wanted to address how bacteria behaved in the small intestine. They indicated that there were higher amounts of facultative anaerobes due to oxygen exposure to these subjects' small intestines (Zoetendal et al. 2012). Although Zoetendal et al. did not directly study *V. atypica*, since *V. atypica* was found in subjects' small intestines and survived, Zoetendal et al.'s results imply that *V. atypica* could be a facultative anaerobe that adapted its metabolism to the large intestine's anaerobic environment.

Despite its potential benefits for exercising diabetics, *V. atypica* is not especially well-characterized with *in vivo* or *in vitro* studies. Most studies focus on genomic sequencing predictions, thus much of the data collected about *V. atypica* has yet to be tested under *in vitro* or *in vivo* conditions, including information about its metabolism, aerotolerance, and pH tolerance. The focus of this study was to better understand *V. atypica* as a potential gut microbe that could amend the effects of lactic acidosis-induced exercise resistance in diabetics. Our experiments

involved learning more about *V. atypica*'s lactate metabolism, addressed predictions about its aerotolerance, and identified what kinds of SCFA products could be produced by *V. atypica*. By studying *V. atypica in vitro*, this microbe could be examined with subsequent *in vivo* and clinical studies to identify its potential as a future probiotic product that could be used to amend exercise resistance's negative effects in diabetic or prediabetic individuals.

METHODS

BHIL Media

Brain heart infusion broth with lactate (BHIL) was used for broth culture and agar culture, based on successful *V. atypica* growth with such media in a study by Scheiman et al. (2019). Brain heart infusion (BHI) broth or agar powder and SIGMA L4263-500mL Sodium DL-lactate solution syrup, BioReagent, 60 percent (w/w), synthetic were used for the culture's broth media. Media was prepared with reverse osmosis filtered deionized water containing 0.6 percent sodium lactate broth media by following the manufacturer's (BBL) instructions. Media was autoclaved per BBL's instructions, and broth was stored at room temperature.

V. atypica Growth and Maintenance

A freezer stock culture of a human mouth sample of *V. atypica* strain KON (ATCC 17744) that had been isolated and stored in glycerol was inoculated onto a fresh BHIL plate with a simple streak. Since multiple sources cited *V. atypica* as an obligate anaerobe, anaerobic conditions were maintained during growth. The plate was stored in a quart-sized Ziploc bag with a BD GasPack™EZ, Catalase #260001 Anaerobe Container System with Indicator to maintain anaerobic conditions. A second Ziploc bag was used to further ensure anaerobic conditions. Excessive oxygen exposure could be indicated if the normally white indicator turned blue. The

freezer stock culture plate was stored at 25°C for six days to slowly acclimate *V. atypica* to its new growth environment. The plate was then examined for growth characteristics.

An additional series of simple streaks on fresh BHIL plates were performed using the same technique and storage procedures to maintain the *V. atypica* sample for further experimentation across the next few months. All plates were inoculated with a *V. atypica* sample from the plate's direct predecessor plate. The 25°C incubation temperature was selected for plates when a slow growth period was desired; however, 37°C was preferred when expedited growth or mimicking internal human body temperature was required. Plates incubating at 25°C typically grew for between five and seven days, while plates incubating at 37°C grew between two and three days. After growing, each plate was observed to ensure that consistent growth and pure culture were maintained. Plates were also monitored for disposal if the anaerobe pack indicator turned blue from excessive oxygen exposure.

Aerotolerance Test with Thioglycollate Broth

SIGMA-ALDRICH 90404-500G Thioglycollate Broth with Resazurin was prepared according to the manufacturer's instructions. Tubes were autoclaved and cooled according to the manufacturer's instructions. *V. atypica* was inoculated into the bottom of three individual tubes containing thioglycollate broth, per standard thioglycollate inoculation technique (Leboffe and Pierce 2019). Tubes were incubated at 37°C in a Scientific Industries Inc: ENVIRO-GENIE incubator for 24 hours and then observed for aerotolerance conditions. After 24 hours, tubes were analyzed for growth and compared to a control that was prepared in the same manner as the other three tubes but lacked *V. atypica* inoculation. The control tube had been stored at 4℃, while the other tubes incubated for 24 hours, and then the control was warmed at 37℃ for one hour, prior to comparison with the *V. atypica*-inoculated tubes.

V. atypica Growth Comparison: BHI Versus BHIL

V. atypica growth on BHIL plates was compared to growth on BHI plates to determine if *V. atypica* could successfully grow without lactate. The BHI media prep procedure was repeated in the same manner as previously referenced with media prep but omitting the 0.6 percent sodium lactate. A sample of *V. atypica* was inoculated with a simple streak pattern onto one BHIL and one BHI plate. For the broths, 5 mL of BHI and BHIL were heavily inoculated with *V. atypica.* The broth tubes were placed into a tube holder, and all broths and plates were stored in the same two Ziploc bags with an anaerobe pack at 37°C for 48 hours.

In order to assess colony morphology comparisons between BHI and BHIL plates, two quadrant streaks were created from *V. atypica*, and both plates were placed in the same set of Ziploc bags with an anaerobe pack at 37°C for 48 hours. Once colony morphology was determined for each plate, colony sizes were measured using a calibrated ocular micrometer on a NIKON Eclipse E200 microscope's scale. The ocular micrometer was calibrated with the microscope's stage micrometer according to a laboratory manual (LeBoffe and Pierce 2006). Five different colonies were measured on each plate, and only isolated colonies were selected from quadrant III or IV on each plate.

SCFA Analysis

An overnight culture of *V. atypica* was heavily inoculated into a 4 mL tube of BHIL broth using the same inoculation and incubation procedure as previous broth cultures. Following the overnight growth period, the culture was centrifuged at 4,000 rpm for five minutes. After discarding the supernatant, the bacterial cell pellet was resuspended in 400 µL of BHI broth. *V. atypica* was added into two 3 mL BHI tubes and two 3 mL BHIL tubes, each receiving 100 µL of bacteria. One 3 mL tube of BHI and BHIL was reserved as uninoculated controls. All tubes

incubated for 24 hours at 37°C, and then each was vortexed before transferring 1 mL of each culture sample into a sterile microcentrifuge tube. Each microcentrifuge tube was centrifuged at 14,000xg for five minutes before collecting 500 µL of supernatant from each. The cell pellet was not disturbed in the process. Samples were snap frozen on dry ice and then shipped to Creative Proteomics (Shirley, NY, USA) for GC-MS analysis of seven different SCFAs, including acetic, propionic, butyric, isobutyric, valeric, isovaleric, and hexanoic acid. The SCFA measurements for the uninoculated sterile media were considered background levels and subtracted from the values for the *V. atypica* inoculated experimental samples. Measurements were then averaged, standard deviation determined, and compared using a student's t-test. Statistical significance was assumed with a $p<0.05$.

RESULTS

V. atypica Plate Growth on BHIL Agar

After six days at 25℃, the freezer stock culture plate showed a translucent mass of growth that included small, buff colonies. The later simple streak plates were observed for similar growth to ensure that pure culture was maintained throughout experimentation (Figure 1). In general, plates that grew at 25℃ tended to have colonies that appeared smaller than those that incubated at 37℃. Plates that incubated at 37℃ showed larger accumulations of growth in fewer days than plates that incubated at 25℃. According to the anaerobe pack indicator for each plate's incubation period, anaerobic conditions were maintained for all plates at 25℃ or 37℃; however, the maximum growth period at 25℃ was determined when the indicator on the anaerobe pack began turning blue after seven days of incubation.

Figure 1. *V. atypica* freezer stock culture (left) and streak plate 3 (right) growth. The freezer stock culture was grown at 25℃, and streak 3 was grown at 37℃ for six days and 48 hours, respectively. Visually, all colonies were small and buff; however, colonies on the plate that incubated at 25℃ appeared slightly smaller than those on the plate that incubated at 37℃. The plate at 37°C had growth in fewer days than the plate incubating at 25°C. Plates were stored at 4°C after growth observations.

V. atypica Growth Comparison: BHI Versus BHIL Plates

After 24 hours, the BHI simple streak plate produced some growth; however, overall growth seemed smaller and less extensive than growth on the BHIL plate. After 48 hours, growth on both plates was about the same as the previous day (Figure 2). Following simple streaks, quadrant streaks were used to assess colony morphology. The BHIL quadrant streak plate's colony morphology was round, smooth, buff or no pigmentation, flat-slightly convex, and shiny. The BHI plate's colony morphology was the same; however, colonies appeared slightly smaller (Figure 3). According to average colony measurements, the BHI plate had smaller colonies than the BHIL plate (Figure 4).

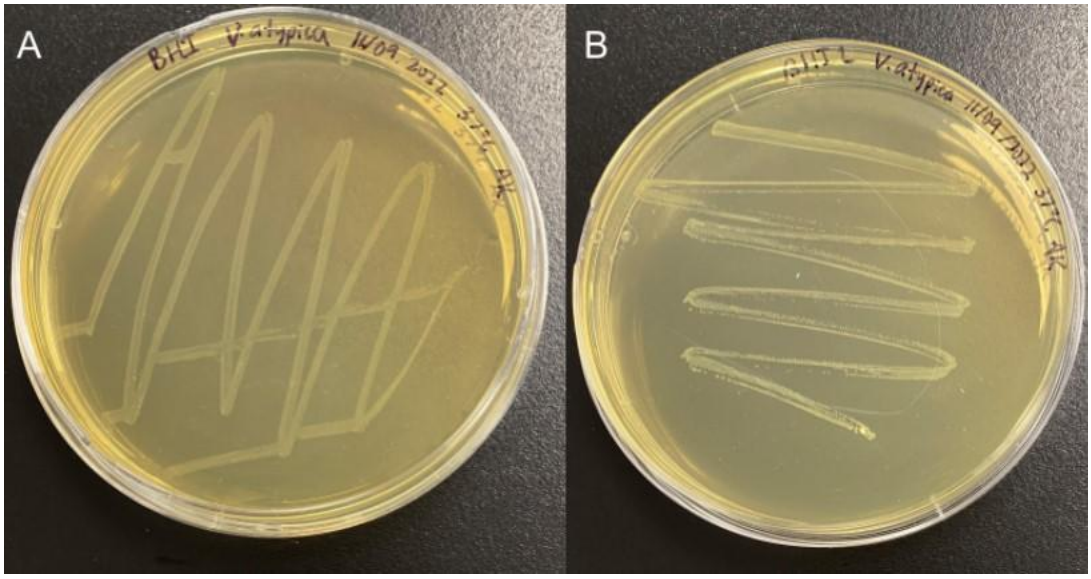

Figure 2. BHI (A) and BHIL (B) *V. atypica* simple streaks after 48 hours at 37°C. Plates were simple streaked with a sample of *V. atypica* from the most recent simple streak plate, and growth was compared between BHI versus BHIL plates. Growth was similar between the two plates.

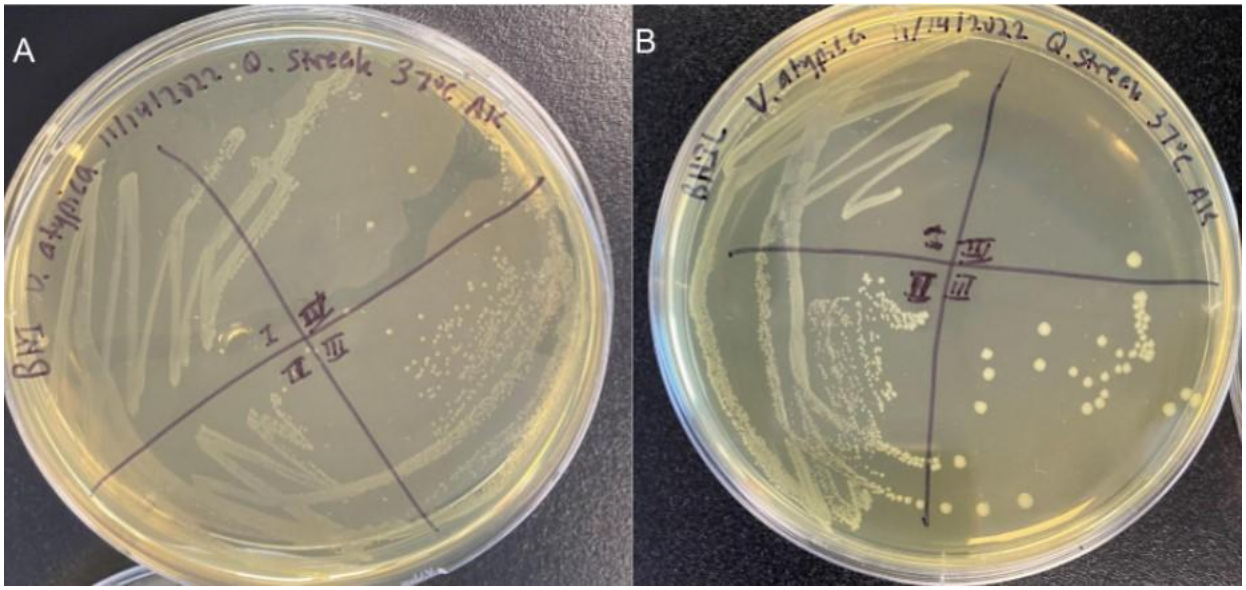

Figure 3. *V. atypica* BHI (A) and BHIL (B) quadrant streaks after 48 hours of incubation at 37°C. A quadrant streak procedure was performed by transferring a sample of *V atypica* from simple streak 9 to individual BHI and BHIL plates. The plates were incubated in a double-Ziploc bag with an anaerobe pack. Colony isolation was observed on each plate, and the plates were compared for growth differences.

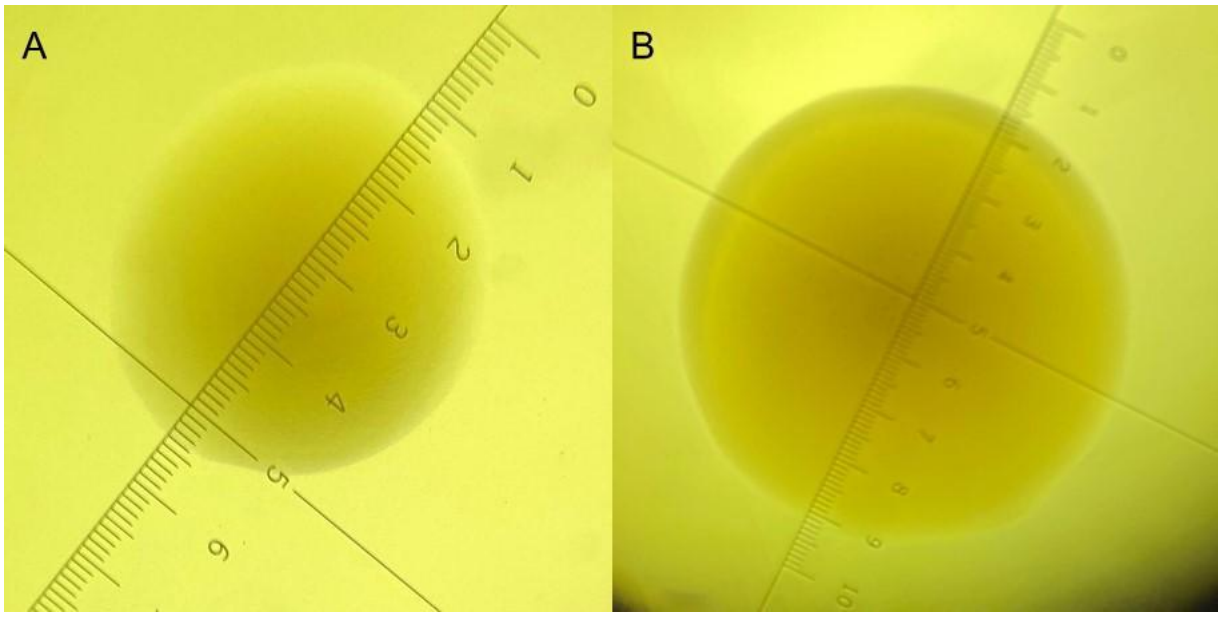

Figure 4. *V. atypica* quadrant streak BHI (A) colony 4 and BHIL (B) colony 2 at 40x magnification. The quadrant streaks plates were incubated at 37°C for 48 hours with an anaerobe pack in two Ziploc bags. A NIKON Eclipse E200's stage micrometer was calibrated at 40x magnification, meaning that one ocular unit was equivalent to 25µm. The BHI colony was about 4.5 ocular units (112.5 µm), and the BHIL colony was larger at about 7.5 ocular units (187.5 µm) at 40x magnification. Five total colonies were measured for both BHI and BHIL. The average colony sizes were 100 µm±17.7 and 165 µm±26.7.

V. atypica Growth Comparison: BHI Versus BHIL Broths

In addition to comparing BHI versus BHIL growth conditions on plates, growth comparisons of *V. atypica* in the two conditions were also compared in broth. Both showed uniform fine turbidity for *V. atypica* growth in broth. Prior to using a vortex on each broth, some sediment was present at the bottom of both BHI and BHIL tubes. Based on visual observations, the BHIL broth seemed to have denser *V. atypica* growth than the BHI broth (Figure 5).

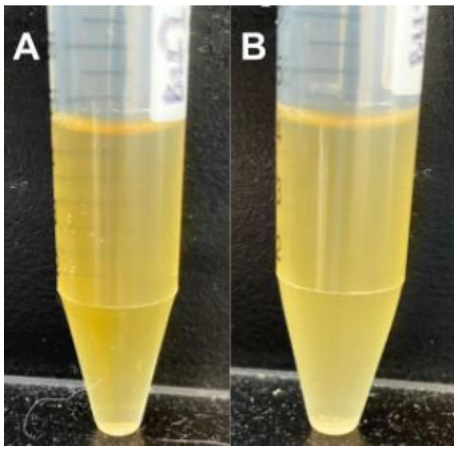

Figure 5. BHI (A) and BHIL (B) *V. atypica*-inoculated 15-mL tube broths after 48 hours at 37°C. Both the BHI and BHIL broths did not have visible growth after 24 hours at 37°C; however, after being heavily inoculated with additional *V. atypica* and incubating for an additional 24 hours, both broths had uniform fine turbidity growth. Based on observations, BHIL broth (B) appeared to have denser growth than BHI broth (A).

V. atypica Thioglycollate Aerotolerance

After 24 hours at 37℃, *V. atypica*'s growth in fluid thioglycollate was interpreted based on where it grew along the concentration of oxygen gradient created by the thioglycollate. In all three tubes of thioglycollate, *V. atypica* only grew in the regions without oxygen (Figure 6). The colorless regions were anaerobic, while the pink regions were aerobic, so based on images and descriptions from Leboffe and Pierce (2019) and based on growth solely below the pink region, *V. atypica* grew in thioglycollate as an obligate anaerobe.

Figure 6. *V. atypica* growth in thioglycollate broth at 37°C (left) and control thioglycollate broth (right). Three tubes of thioglycollate broth were inoculated with *V. atypica* from plate 3 (Figure 1) and incubated for 24 hours at 37°C. With thioglycollate, the oxygen concentration gradient created had high [O_2] near the tops of tubes (pink region) and [O_2] decreased when closer to the bottoms of each tube. *V. atypica*'s growth in thioglycollate tubes suggested that it only grew in oxygen-free environments in the pattern of an obligate anaerobe.

SCFA Analysis

Following the GC-MS analysis for comparison of non-lactate versus lactate metabolic products of *V. atypica*, most SCFA detection in BHI media was limited; however, detection increased in BHIL media (Figure 7). The most prominent SCFA product of non-lactate metabolism in the unsupplemented media was acetic acid at 30.7 ±4.5 µg/mL, while the other SCFA products showed minimal increases in the presence of *V. atypica* (Figure 7). The most prominent SCFA products of lactate metabolism detected were acetic acid (821.6 ±8.6 µg/mL) and propionic acid (1090.1 ± 65.2 µg/mL). Most other SCFA products showed minimal increases or decreases in the presence of *V. atypica* (Figure 7). Between BHI and BHIL results, in the presence of lactate, *V. atypica* produced significantly higher amounts of both acetic and propionic acids ($p=0.0008$ and $p=0.0009$; t-test, $n=2$); however, lower amounts were detected for all other SCFA products (Figure 7).

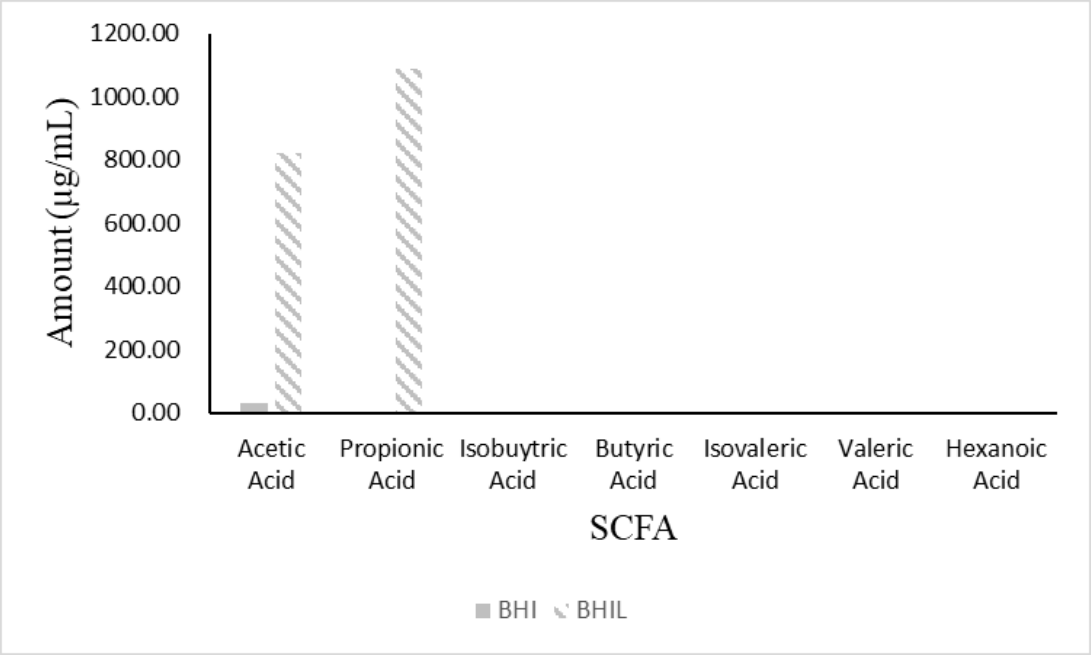

Figure 7. SCFA comparison of *V. atypica* metabolism in BHI versus BHIL media. Samples of BHI (A), BHI plus *V. atypica* (B), BHIL (C), and BHIL plus *V. atypica* (D) were processed using GC-MS to compare SCFA products of non-lactate versus lactate metabolism. Control values were subtracted from values of samples containing bacteria, and all samples were averaged for each SCFA. Compared to *V. atypica* in BHI (B), *V. atypica* in BHIL had higher quantities of both acetic acid and propionic acid ($p=0.0008$ and $p=0.0009$; t-test, $n=2$); however, minimal to no amount of any other SCFA product was detected in either BHI or BHIL.

DISCUSSION

Considering exercise resistance and its potential links to gut microbiome dysbiosis and lactic acidosis, lactate-metabolizing bacteria, like *V. atypica*, could help diabetics respond better to exercise by mediating glucose intolerance and improving energy supply. While potentially beneficial, *V. atypica* has been poorly characterized by scientific literature in terms of its growth preferences, aerotolerance, and general metabolic profile, especially in regard to SCFA products. This study used *in vitro* experiments to address previous predictions about growth and metabolic behaviors of *V. atypica* to better understand how this microbe could potentially help diabetics who are negatively impacted by exercise resistance.

Initially, *V. atypica* was characterized for its growth preferences on plates by culturing samples at either 25℃ and 37℃ on both lactate-supplemented and non-lactate-supplemented growth media. In comparison to BHIL plates grown at 37℃, *V. atypica* grew slower and produced visibly smaller colonies on BHIL plates at 25℃. Considering that human body temperature is normally about 37℃, this portion of the experiment supported that *V. atypica* prefers growing at temperatures closer to human body temperature, rather than at room temperature. This supports that *V. atypica* may survive well in the human body; however, if temperature varies between healthy individuals and diabetics, *V. atypica* may not survive as well. For example, Stapleton et al. (2013) demonstrated that at 40 percent maximal aerobic capacity, type I diabetics showed no differences in heat loss with skin or core body temperature in comparison to non-diabetic controls; however, Carter et al. (2014) showed that with more intense exercise (above 40 percent maximal aerobic capacity), type I diabetics' heat loss mechanisms were impaired compared to healthy controls. With type II diabetics, Kenny et al. (2016) explained that patients often have compromised sweat responses to exercise, suggesting that exercise affects heat loss mechanisms in type II diabetics. Researchers expanded on this idea by stating that even seemingly minor internal temperature fluctuations of up to 3°C could be harmful. Considering such implications, more prevalent changes in core body temperature during exercise could impact gut microbes' preferences, including how *V. atypica* would survive within an exercising diabetic. Although homeostasis tends to maintain core body temperature at or near 37℃, periods of intense exercise could seemingly disrupt the microbiome's balance if temperature changes can harm other systemic functions within a human's body. Since *V. atypica* appears to prefer 37℃, further experimentation may be necessary to determine if temporary temperature fluctuations during intense exercise affect microbial growth and survival.

While temperature was important for determining the growth preferences of *V. atypica*, as findings supported its potential to grow well at normal human body temperature, another important consideration for specifically growing within the human gut was to address predictions regarding aerotolerance. Although most studies used genomic predictions to explicitly list *V. atypica* as an obligate anaerobe, previous research with *in vitro* experiments suggested that *V. atypica* was more aerotolerant than predicted. For example, a previous study found that *V. atypica* was still alive after 24 hours of continuous oxygen exposure (E. Druebbisch, *unpublished data*). Since *V. atypica* did not die upon immediate oxygen exposure, it seemed more likely that *V. atypica* could be able to tolerate some oxygen, rather than being strictly anaerobic. A fluid thioglycollate experiment was used to officially assess the aerotolerance of *V. atypica* under *in vitro* conditions. Fluid thioglycollate was originally developed by Brewer (1940) as a way to culture anaerobes in open tubes. Thioglycollate can convert oxygen into water to create an anaerobic environment in broth conditions. With this test, *V. atypica* only grew in colorless regions, indicating that it was strictly anaerobic. Such results not only support genomic studies' predictions, but they also seem typical for microbes residing in the large intestine. Most gastrointestinal bacteria are obligate anaerobes, which has made culturing them especially difficult (Maier et al. 2015). Considering the difficulties with maintaining anaerobic conditions, genomic studies' predictions may have been the most efficient way to understand *V. atypica* in the past. With this experiment's findings and findings from the previous unpublished data, *V. atypica* may be easier to culture as an obligate anaerobe, since it can survive for a short period of time in the presence of oxygen. This could make *V. atypica* more accessible for being developed into a future probiotic product.

Considering that *V. atypica* was identified as an obligate anaerobe, such results also support that *V. atypica* could be used to assist diabetics struggling with exercise resistance. Since Böhm et al. (2016) implied that diabetic mitochondria had a limited aerobic capacity to produce ATP during exercise, impairing glucose homeostasis, diabetics may not be getting as much oxygen or be able to use as much oxygen as healthy individuals during exercise. With potentially more anaerobic conditions, anaerobic bacteria, like *V. atypica*, could help mediate such oxygen limitations. Limited oxygen might be a problem for mitochondria, but *V. atypica* does well under more anaerobic conditions; therefore, if *V. atypica* produced or effectively used SCFAs to provide more energy during exercise, a diabetic might be able to better tolerate oxygen-limited conditions during exercise.

After supporting genomic studies' predictions regarding aerotolerance, the next portion of the experiment focused on addressing metabolic capabilities and products of *V. atypica* in the presence and absence of lactate. As a way to assess this factor, *V. atypica* growth in lactate supplemented (BHIL) and non-lactate supplemented (BHI) media was compared for both plate and broth cultures. With plate cultures, the overall growth of *V. atypica* on BHI media appeared smaller and less extensive than *V. atypica* grown with lactate. For more specific comparisons, quadrant streaks were used to compare colony morphology and colony sizes. While both BHI and BHIL cultures had round, smooth, generally flat, somewhat convex, and shiny colonies without pigmentation, the average colony size for BHI plates was only about 100 μm (0.1 mm), compared to an average size of 165 μm (0.165 mm) for colonies on BHIL plates. Broth cultures suggested similar results. For instance, while both BHI and BHIL broths produced uniform fine turbidity growth, BHIL broth seemed denser than BHI growth. This supports the larger colony sizes found in quadrant streak assessments, suggesting that *V. atypica* grows more effectively

with lactate than without lactate. Considering that BHIL media produced consistently larger colonies than BHI media, *V. atypica* seems to prefer environments with lactate, suggesting that it could be successful in helping to metabolize some of the lactate present in diabetics' guts.

Although results from BHIL versus BHI culture conditions supported that *V. atypica* seems to prefer lactate-supplemented media, since the growth in BHI media was still successful, metabolic products of *V. atypica* could be compared between lactate-supplemented and non-lactate-supplemented plates in the form of SCFA analysis. During or after exercise, systemic lactate can enter the large intestines by crossing the gut lumen. Once inside the large intestine, lactate-metabolizing bacteria, like *V. atypica*, could break down the lactate into potentially beneficial SCFA products (Scheiman et al. 2019). While breaking down lactate would help decrease the amount of lactate converting into glucose, the SCFA products could also have some benefits. Following a GC-MS analysis of seven different SCFA products from non-lactate and lactate metabolism of *V. atypica*, higher levels of acetic acid were detected with *V. atypica* in BHIL than in BHI, suggesting that lactate could be metabolized by *V. atypica* to enhance acetic acid production. Acetic acid could be particularly beneficial for people with T2D, as it can decrease glucose production in the liver, improve skeletal muscle-use of glucose, and help improve beta cell function to boost insulin levels (Valdes et al. 2021). With such benefits, acetic acid could help exercising diabetics by regulating glucose homeostasis and improving insulin sensitivity, which were recognized as problems for exercising diabetics with imbalanced gut microbiomes.

Like acetic acid, propionic acid levels were also notable results from the SCFA analysis. Between BHI and BHIL media inoculated with *V. atypica*, BHIL media had significantly higher levels of propionic acid than BHI media, suggesting that propionic acid was being produced at

elevated levels from lactate metabolism (Figure 7). Like acetic acid, propionic acid could also benefit exercising diabetics. For example, Scheiman et al. (2019) indicated that exercising mice with enhanced propionate levels had a higher VO_2max, suggesting that mitochondria could potentially benefit from propionate due to a higher oxygen uptake. While some diabetics with exercise resistance still may not improve with an enhanced VO_2max, it seems possible that this could have been linked to the already present gut microbiome dysbiosis. Based on genomic analysis, lactate-metabolizing microbes are able to use lactate; however, unlike *V. atypica*, they do not have the complete methylmalonyl-CoA pathway to fully convert lactate to propionate. In this sense, *V. atypica* is uniquely specific for deriving propionate more specifically from lactate metabolism (Scheiman et al. 2019). With this in mind, if *V. atypica* is more commonly limited in diabetics, the VO_2max may not have been improving sufficiently enough to boost mitochondria. Additionally, propionic acid is linked to improving gut hormones related to improved glucose homeostasis, such as glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide 1 (GLP-1) (Murri et al. 2013). According to Ding et al. (2006), GIP is a hormone released in the small intestine that stimulates insulin release. It is typically released after a meal; however, when stimulated by propionic acid, it could potentially be released to help stimulate insulin release during exercise to remove some of the excess glucose. Similarly, GLP-1 is a hormone that is linked to improving beta cells, which produce insulin, and suppressing glucagon, a hormone that enhances glucose production (Gupta 2013). Therefore, the negative effects of increased glucose or no effects from exercise could be mediated by a combination of enhanced GIP and GLP-1.

Although SCFA products have numerous benefits for diabetics, it is unclear whether or not these products would be officially safe for clinical-use. For instance, some studies suggest

that SCFAs could disrupt normal immune functions. Ciarlo et al. (2016) explained that propionic acid could potentially damage immune cells and immune responses, due to its potential to disrupt immune cell communication. However, the study found that propionic acid's impact on a host's risk of infection or ability to respond to infections was not significantly problematic. Considering that there is potential for propionic acid to impair immune responses, further testing on SCFA products would be necessary before introducing something like *V. atypica* to a diabetic to treat exercise resistance; however, since propionic acid did not have significant negative effects on host immunity, it seems promising that propionic acid, as a metabolic product of *V. atypica*, could likely be a safe probiotic product. Another concern with SCFA products is that despite the suggestion that they could benefit people with hyperglycemia or insulin resistance, some studies suggest the opposite. For instance, Perry et al. (2016) found that when rats were fed a high fat diet and had a continuous infusion of acetate, the rats experienced higher rates of insulin resistance, hunger, and weight gain. Although this study suggests that acetate could become harmful, it seems more likely that future studies with *V. atypica* and acetic acid would be necessary to determine the specific level of acetate that would be beneficial for diabetics and how often it would be safe to use the product for exercise. Furthermore, the highest levels of acetate were found in BHIL media, so the lower levels detected in BHI could indicate that *V. atypica* would only produce higher amounts of acetate under certain conditions. If such conditions were monitored to determine how much acetate exposure is safe, *V. atypica* could potentially be appropriately used for exercise alone, rather than as a probiotic product under any conditions.

CONCLUSION

Identifying optimal ways to treat and prevent diabetes is essential to diminish its prevalence, especially in the United States. While physicians commonly recommend exercise to help manage hyperglycemia in diabetics, not all exercise is beneficial. Since this phenomenon, exercise resistance, could potentially be derived from a combination of hyperglycemia, excess lactate, ineffective mitochondria, and a lack of metabolically beneficial bacteria, using a lactate-metabolizing gut microbe, like *V. atypica*, could be promising to make exercise more effective for all diabetics. This study's results supported bioinformatic genomic predictions that *V. atypica* is an obligate anaerobe; however, considering past data from our lab that showed *V. atypica* could survive for a short period of time in oxygen's presence, this particular microbe could be easier to culture than other obligate anaerobes. This study also demonstrated what is believed to be the first *in vitro* culture of *V. atypica* without lactate, which lead into successful GC-MS analysis of the SCFA products of *V. atypica* in both the presence and absence of lactate to see which products were solely due to lactate metabolism. Results showed that diabetics could potentially benefit, in terms of glucose homeostasis and insulin sensitivity, from the high levels of both acetic and propionic acids found in lactate-supplemented media, which seemed to outweigh the potential harm that could come from overuse of SCFA products. Overall, this study demonstrates that *V. atypica* has the potential to be developed into a safe, well-tested probiotic that could be specifically used to mitigate the negative effects of lactic acidosis and gut microbiome dysbiosis-induced exercise resistance in diabetics.

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