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Effect of 8 Weeks Prebiotics/Probiotics Supplementation on Alcohol Metabolism and Blood Biomarkers of Healthy Adults: A Pilot Study

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ABSTRACT

Purpose: Modulating gut bacteria via regular prebiotics/probiotics consumption may improve the metabolism of acute alcohol ingestion. This study investigated the impact of 8 weeks prebiotics/probiotics supplementation on microbiome changes and responses to acute alcohol consumption.

Methods: 38 participants (21 females, $23.6 \pm 3.4 \text{ kg} \cdot \text{m}^{-2}$, mean \pm SD) attended the laboratory on two occasions separated by an 8 week intervention period. On each of these visits, a dose of alcohol ($0.40 \pm 0.04 \text{ g} \cdot \text{kg}^{-1}$, Vodka+Soda-Water) was consumed over 10 min. Breath alcohol concentration was sampled over 5 h and alcohol pharmacokinetics was analysed using WinNonlin non-compartmental modelling (C_{max} , t_{max} , AUC_{last}). For the intervention, participants were randomised to receive Placebo+Placebo (PLA), Placebo+Prebiotics (PRE), Probiotics+Placebo (PRO), or Probiotics+Prebiotics (SYN) in a double blinded manner. Probiotics were a commercially available source of *Lactobacillus acidophilus* (NCFM[®]) and *Bifidobacterium lactis* (Bi-07). Prebiotics were a commercially available source of Larch Gum (from *Larix occidentalis*). Placebo was microcrystalline cellulose. Each visit, participants provided a stool sample, which was analysed to determine presence of *L. acidophilus* and *B. lactis*. Differences between trials were analysed using paired samples *t*-tests.

Results: Increased counts for at least one bacterial strain (*L. acidophilus* or *B. lactis*) were observed for all participants on SYN ($n=10$) and PRO ($n=10$) trials. No difference in C_{max} or t_{max} was observed between trials when analysed by treatment condition or microbiome outcome. A significant decrease in AUC_{last} was observed between trials for PLA ($p=0.039$) and PRE ($p=0.030$) treatments, and when increases in at least one bacterial strain ($p=0.003$) and no microbiome changes ($p=0.016$) were observed.

Conclusion: Consumption of probiotics appears to alter faecal counts of supplemental bacterial strains in otherwise healthy individuals. However, translation to any possible beneficial impact on alcohol metabolism remains to be elucidated.

Keywords: Alcohol · Pharmacokinetics · Prebiotics · Probiotics · Synbiotics · Lipids

Introduction

Alcohol is one of the most widely used recreational drugs in the world [1]. Consequently, alcohol-related harm is a major public health concern, with over 5% of the global burden of disease attributed to alcohol consumption [1]. In Australia, the annual cost of alcohol-related social problems (e.g. lost workplace productivity, road accidents) is estimated to be over \$15 billion [2]. Recent data from the U.S. indicates that excessive drinking costs the nation almost \$210 billion [3]. Accordingly, there is significant interest in strategies designed to attenuate the negative side effects associated with acute alcohol consumption.

Following ingestion, alcohol is rapidly absorbed [4-6] and alcohol concentrations in the gastrointestinal tract quickly equilibrate with levels observed in blood soon after drinking [7,8]. The vast majority of alcohol is removed metabolically in a process involving the alcohol dehydrogenase (ADH) and acetaldehyde dehydrogenase (ALDH) enzymes, where ADH converts alcohol to acetaldehyde, and ALDH converts acetaldehyde to acetate [4,9]. Acetaldehyde is a highly reactive and toxic compound, and is generally considered responsible for a range of alcohol-related health consequences (e.g. alcoholic liver injury, rectal carcinogenesis, tissue damage) [10]. Thus, efficient removal of acetaldehyde (via conversion to acetate) from the body is particularly important. The alcohol elimination process is variable, but for most individuals, it takes over an hour for the body to eliminate the alcohol contained in a single standard drink (i.e. 10 g of ethanol) [11,12].

While the liver is often reported as the primary site of ethanol metabolism [6], alcohol is also readily metabolised by enteric microorganisms [7,13] as a means of eliminating alcohol produced by fermentation in the gut [14]. Reports from cell culture studies [15] and animal models [10,16-18] indicate that lactic acid and other genus (i.e. *Bacillus spp.*) bacteria can convert ethanol into acetaldehyde and further to acetic acid. Increasing numbers of these gram-positive bacteria of the intestine may increase alcohol metabolism, thereby enhancing alcohol elimination and attenuating alcohol related toxicity in the liver and other organs [15,19,18].

Probiotics are live microorganisms (e.g. *Lactobacillus* and *Bifidobacterium*) that may aid in improving gastrointestinal health, lowering cholesterol, reducing blood pressure, improving immune function, and decreasing inflammation [20,19,11]. Prebiotics are selectively fermented ingredients that support the composition and/or activity of the gastrointestinal microbes [21]. Acute consumption of probiotic products has been implicated in the attenuation of alcohol intoxication levels [18]. After consuming 100 mL of a *Bacillus* natto-fermented product, lower breath alcohol (44% reduction) and aldehyde (45% reduction) concentrations were observed in a group of participants 1 h after drinking whisky, compared to a control group [18]. However, in this study the probiotic product was consumed acutely prior to the ingestion of alcohol. This may have provided some mechanistic effect by slowing gastric emptying [22-25], exposing alcohol to greater first pass metabolism via gastric ADH enzymes [26,27], as opposed to modulating the gut microbiome. Nevertheless, a decrease in alcohol intoxication levels of this magnitude is likely to have positive effects on reducing acute alcohol-related harm and the potential to reduce subsequent negative side-effects of alcohol (i.e. hangover).

The gut acts as a dynamic environment, with bacterial numbers being influenced by many factors (e.g. a high fat/low fibre diet and illness). The regular consumption of alcohol has been identified as a cause of gut dysbiosis (defined by the disturbed balance between pathogenic and healthy bacteria) [28,29]. Conversely, prebiotics/probiotics consumption may improve this balance [30,31], with proliferation of gut bacteria providing health benefits when duration of probiotics supplementation is ≥ 8 weeks [20]. Understanding the effect of prebiotics/probiotics consumption in healthy individuals, and its influence on alcohol metabolism is of interest.

Therefore, the purpose of this pilot investigation was to determine if an 8 week intervention involving the consumption of commercially available prebiotics/probiotics could improve the gut microbiota of healthy individuals, and if these improvements would assist alcohol metabolism and reduce intoxication effects following acute alcohol ingestion.

Methods

Participants

Forty healthy individuals participated in this study. Two individuals withdrew from the study prior to completion (one was lost to follow up, one contracted a medical illness unrelated to the investigation), resulting in complete data for 38 participants ($n=21$ female). Participant characteristics are displayed in Table 1. Eligibility for inclusion required participants to be healthy, aged between 18-70 years, consume alcohol, not be taking prebiotics or probiotics supplements and have a systolic blood pressure of <140 mmHg. All participants initially completed a self-administered health assessment questionnaire. Participants scoring ≥ 3 on the Short Michigan Alcoholism Screening Test (SMAST) [32], indicating potential for alcohol addiction, were excluded. Alcohol consumption was assessed using a modified personal

drinking history questionnaire (PDHQ) [33]. Participants were excluded from the study if they were breastfeeding, pregnant, had a medical condition or were taking medications that would interact with alcohol. Ethical approval for the study was obtained by the University's Human Research Ethics Committee (Protocol No: AHS/74/14/HREC). All participants provided written informed consent prior to commencement.

INSERT TABLE 1 HERE

Experimental Design

This investigation employed a double blind, placebo controlled, parallel-group study design. Participants completed two experimental trials, each separated by eight weeks (intervention period). Participants were randomised (using a block randomisation process in groups of 2) into one of four intervention groups (PLA, Placebo; PRE, Prebiotics; PRO, Probiotics; SYN, Synbiotics) and received supplements to consume daily for 8 weeks (Fig. 1). Each of the experimental trials involved consumption of a set volume of alcohol (40.0% v/v, GREY GOOSE[®] Vodka, Bacardi Limited, France) mixed with soda water and lime juice (Woolworths Select, Woolworths Ltd, Sydney, Australia). Breath alcohol concentration (BrAC) and subjective ratings of alcohol intoxication and impairment were measured throughout a subsequent 300 min observation period in each trial. All BrAC measures were analysed using a calibrated police grade portable breathalyser (Alcolizer LE5, Alcolizer Technology, Brisbane, QLD, Australia). All breathalyser measurements were taken in duplicate, with a triplicate measure recorded if readings differed by ≥ 0.005 g·dL⁻¹. The measures were averaged to provide the final assessment of BrAC.

INSERT FIG 1 HERE

Pre-experimental Trial Procedures

Participants were asked to refrain from alcohol consumption for 24 h and caffeine consumption for 12 h prior to each trial. In addition, participants were required to fast from all food and beverages (except water, which was encouraged) from 21:00 h the night before each trial. During the 24 h period immediately preceding the first trial, participants provided a stool sample for faecal bacterial analysis and recorded all food and beverages consumed as well as any exercise completed. A copy of the food and exercise record with this information was supplied to each participant at the end of the first trial and they were asked to repeat this on the day prior to the second trial, making note of any changes to dietary intakes. Collection of the faecal sample and compliance with the pre-experimental procedures was verbally confirmed by participants on arrival to the laboratory. Food records were analysed by a trained dietitian to examine total energy, water, and macronutrient (carbohydrate, protein, fat) consumption for both trials.

Experimental Trial Procedures

On arrival at the laboratory (~06:00 h), an initial BrAC sample was obtained to confirm abstinence from alcohol. Participants were also asked to confirm that they had fasted from foods and beverages overnight. A urine sample was then collected from participants which was subsequently analysed to determine urine specific gravity (U_{sg}) as a measure of hydration status (U_{sg} refractometer UG- α [®], Atago Co., Ltd., Tokyo, Japan). Body mass and height were then measured, before participants rested in a supine position for 5 min. A resting blood pressure recording was then taken using an automatic blood pressure monitor (Omron HEM-7203, Omron HealthCare Co. Ltd., Kyoto, Japan) and immediately after, a 10 mL blood sample was collected from the antecubital forearm vein, using standard phlebotomy procedures. Participants were then asked to complete a food frequency questionnaire (FFQ)

[34,35], which indicated dietary consumption patterns over the previous 4 weeks. The FFQ was modified to also contain a detailed section for evaluating consumption of prebiotic and probiotic foods and fluids. The FFQ was analysed by a trained dietitian to examine differences in diet patterns between trials.

Following completion of the FFQ, participants were given an alcoholic beverage to consume. Each participant received an individualised dose of alcohol, determined in the first trial and replicated in the second trial. The dose of alcohol was calculated using the modified Widmark equation and designed to illicit a peak BrAC equivalent to $0.050 \text{ g}\cdot\text{dL}^{-1}$ [36]. The mean dose of alcohol provided was $28.1\pm 5.8 \text{ g}$. The total beverage volume was prepared by adding two parts soda water to one part vodka with the addition of 10 mL lime juice (total drink volume = $328\pm 100 \text{ mL}$). Three equal (weighed) amounts were then partitioned into different glasses and were stored in a refrigerator at 4°C until required for consumption.

Participants were required to consume the entire content of their beverage over a period of 10 mins (on an empty stomach). Following consumption of the final beverage aliquot, participants were provided with 200 mL of water in order to rinse their mouths, expelling the water without swallowing. This was completed to assist with the reduction of residual mouth alcohol in preparation for the first BrAC measure (15 min post ingestion). Following this, participants had BrAC and subjective ratings of alcohol intoxication and impairment measured over a 300 min observation period. The first BrAC measure was collected 15 min following alcohol ingestion, with the next measure taken 5 min later. BrAC measures were then collected every 10 min for the next 80 min period. Following this, measures were collected at 100 min and then every 30 min until trial completion. Subjective ratings were collected prior to alcohol ingestion, immediately after alcohol ingestion (0 min), at 10, 20, 40

and 60 min of observation, then at 30 min intervals until 210 min of observation was completed. At the end of the observation period participants provided another urine sample for hydration analysis.

Intervention

The probiotics were a commercially available source of *Lactobacillus acidophilus* (NCFM[®]) (12.5×10^9 colony-forming units/dose) and *Bifidobacterium lactis* (Bi-07) (12.5×10^9 colony-forming units/dose) (Inner Health Plus Dairy Free; Health World Limited, Brisbane, Australia). The probiotics were provided in capsule form. The prebiotics were a commercially available source of Larch Gum (from *Larix occidentalis*) (Arabino Guard; Health World Limited, Brisbane, Australia), provided in powder form. Placebo was microcrystalline cellulose, presented in both capsules and powder form to simulate the active treatment supplements.

All products were blinded from participants and the research team (blinding performed by Health World Limited, Brisbane, Australia). At the end of the first trial, participants were provided with one bottle of capsules (probiotics or placebo) and one container of powder (prebiotics or placebo) based on their allocated intervention group. Participants were instructed to keep the bottle of capsules refrigerated at 4°C for the length of the study. Participants were instructed to consume one capsule and one half metric teaspoon of powder (dissolved into a liquid of their choosing) daily. Participants were free to choose the timing of supplement ingestion. Participants were required to keep a record of their intake of study products using a calendar to mark off daily consumption, and these were collected at the second experimental trial. A compliance score was calculated as the percentage of the prescribed test products a participant reported to have consumed during the 8 weeks intervention. To assist with monitoring of compliance, participants received a weekly email

containing a link to a short survey (Google Forms, Google Inc., California, USA). The survey asked questions relating to “supplement days missed”, “alcohol consumption”, “side effects experienced from taking the supplements” and “any major changes to dietary intake” in the past week. Participants were also required to return any unused supplements at the second experimental trial and these were weighed/counted as a measure of consumption compliance. The participants were otherwise instructed to maintain usual dietary habits.

Stool Samples and Microbiota Analyses

Collected faecal samples were returned to the laboratory within 24 h of collection and stored at -80°C until analysis. For DNA extraction, samples were brought to room temperature and total DNA was extracted from 2.5 g of faecal material using the QIAGEN DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. Following extraction, DNA concentrations were determined using a Nanodrop Spectrophotometer (Thermo Scientific, Scoresby, VIC, Australia). Isolated faecal DNA was used for the detection of the bacterial strains (*B. lactis*, *L. acidophilus*) contained within the supplement.

Separate real-time polymerase chain reactions (PCR) were used for microbial identification. Sequence specific primers and probes were used as described previously [37,38]. Reactions included Universal Fast PCR Master Mix (Life Technologies, Carlsbad, CA, USA), 100 nM of both forward and reverse primers, 50 nM probe and total of 30 ng of isolated DNA to a final reaction volume of 10 µL. Cycling conditions were, for *B. lactis*: 50°C for 2 min, 95°C for 20 s, and 40 cycles of 95°C for 5 s, 52°C for 15 s and 60°C for 20 s; for *L. acidophilus*: 50°C for 2 min, 95°C for 20 s, and 40 cycles of 95°C for 5 s, 48°C for 15 s and 60°C for 20 s. All samples were assessed in duplicate and all reactions were performed using a QuantStudio6 Flex Real-Time PCR system (Life Technologies, Carlsbad, CA, USA).

The mean cycle threshold (C_T) value was determined for duplicate samples and the change in C_T values (ΔC_T) between pre- and post-intervention visits determined. A decrease in the C_T value post-intervention was interpreted as an increase in the bacterial count.

Blood Sampling and Biochemical Analyses

Samples of venous blood were separated into two 5 mL aliquots and placed into vacutainer tubes, one containing lithium heparin for the collection of blood plasma, and one free from anti-coagulant substances for collection of serum. Vacutainers were centrifuged at $1350 \times g$ for 10 min. Plasma and serum were stored at -80°C until analysis. All blood biomarkers were determined on a COBAS Integra 400 auto-analyser (Roche Diagnostics[®], Mannheim, Germany). Plasma and serum samples were prepared with standard reagent cassettes and results compared to commercially available calibrators (Calibrator For Automated Systems; CFAS; Roche Diagnostics; Dee Why, Australia) and quality controls (PeciControl ClinChem Multi 1 and 2; PCCC1 and 2) in accordance with the manufacturers specifications. Blood biomarkers included: albumin concentration (ALB), alanine aminotransferase (ALT), aspartate aminotransferase (AST), direct bilirubin (BIL-D), total bilirubin (BIL-T), total cholesterol (CHOL), creatinine (CRE), high sensitivity C-reactive protein (CRP), γ -glutamyltransferase (GGT), glucose (GLUC), high-density lipoprotein cholesterol concentration (HDL-C), lactate dehydrogenase (LDH), lipase (LIP), total protein (TR2), triglycerides (TRIGL), and urea concentration (UA2). All analyses were made in duplicate, with the average of duplicates calculated as individuals' final results.

WinNonlin Parameter Analysis

Analysis of breath alcohol data was performed using Phoenix WinNonlin 6.4 (Certara, St. Louis, USA). All BrAC data was computed via non-compartmental analysis to determine parameters including peak breath alcohol concentration (C_{\max}), time to peak alcohol concentration (t_{\max}) and area under the breath alcohol curve at last measurement (AUC_{last}).

Subjective Ratings

Participants were required to estimate their BrAC level (BrAC_{est}) at several times throughout the observation period (15 min, 30 min, and every 30 min thereafter). As a guide, participants were informed that the legal driving limit in Queensland for an individual holding an open class license was $0.050 \text{ g}\cdot\text{dL}^{-1}$. Subjective ratings to a set of pre-defined questions were collected at specified time points throughout the trial using an adaptive visual analogue scale (AVAS) [39]. Participants answered six questions presented on a laptop computer by making a mark on a 100 mm line between two anchor points; ‘not at all’ and ‘very much so’. Questions included: How much do you feel the effects of alcohol right now? How able are you to concentrate right now? How dizzy do you feel right now? How much do you feel the effects of headache right now? How nauseas do you feel right now? How able are you to drive a car at this time? These questions have been used in previous research investigating intoxication effects of alcohol [40,41].

Statistical Analysis

All statistical procedures were performed using SPSS for Windows, Version 23.0 (IBM SPSS Inc., Chicago, USA). Differences between trials (Trial 1, T1 vs. Trial 2, T2) were analysed using paired samples (*a-priori*) *t*-tests for both treatment intention (PLA, PRE, PRO, SYN) and microbiome outcome (no change (NC) or decrease (DEC) in faecal count for both bacterial strains, increase (INC) in faecal count for at least one bacterial strain using a 1.5

fold change as a threshold for an increase). Repeated measures intervention effect sizes (ES) were calculated as Hedges' g . The mean difference between trials in each intervention was standardised against the standard deviation (SD) of the change and corrected for bias due to small sample size [42,43] using the supplementary spreadsheet by Lakens [44]. FFQ data were first converted to daily intakes prior to analysis. The relationship between BrAC and BrAC_{est} for each trial (T1, T2) was investigated using Pearson product-moment correlation coefficient. Subjective AVAS questionnaire data was non-normally distributed, thus statistical analyses comparing T1 and T2 data for corresponding intervention groups were performed using Wilcoxon matched pairs signed rank tests. Statistical significance was accepted at $p < 0.05$. All data are reported as mean \pm SD.

Results

Participant Characteristics and Intervention Compliance

Baseline characteristics for $n=38$ participants by intervention group are presented in Table 2. There was a significant increase in mean body weight ($t(8)=5.69$, $p < 0.001$, ES=0.13) and BMI ($t(8)=7.25$, $p < 0.001$, ES=0.12) for participants in the PLA intervention group at T2 compared to T1. No other differences in anthropometric measures or BP were found between trials ($p > 0.05$). The mean compliance scores calculated from the remaining supplements returned were above 90% in all groups (PLA=93 \pm 7%, PRE=92 \pm 6%, PRO=93 \pm 8%, SYN=94 \pm 7%). Based on calendar entries and completion of compliance forms, all participants reported to have consumed at least 78% of the study products during the 8-week intervention.

Adverse Events

Nine participants reported experiencing gastrointestinal (GI) symptoms throughout the intervention period (PLA, $n=4$; PRE, $n=2$; PRO, $n=1$; SYN, $n=2$). These most commonly occurred within the first 3 weeks of the intervention ($n=8$). The most frequently reported GI symptoms were bloating ($n=6$), diarrhoea ($n=1$), gas/flatulence ($n=6$), stomach pain/cramps ($n=2$), constipation ($n=2$) and nausea ($n=1$). No serious adverse events were registered during the study.

FFQ and 24 h Record Dietary Assessment

Analysis of FFQ data indicated that participant's dietary habits were similar between trials for most items. There was a significant increase in consumption of tomatoes (T1 = 0.40 ± 0.32 , T2 = 0.58 ± 0.41 serves \cdot day $^{-1}$; $t(8)=2.38$, $p=0.044$, ES=0.48), beef (T1 = 0.21 ± 0.14 , T2 = 0.38 ± 0.23 serves \cdot day $^{-1}$; $t(8)=3.81$, $p=0.005$, ES=0.88), and rice (T1 = 0.17 ± 0.11 , T2 = 0.32 ± 0.21 serves \cdot day $^{-1}$; $t(8)=3.20$, $p=0.009$, ES=0.89) reported by the PLA group. The PRE group reported a significant increase in added salt consumption between trials (T1 = 0.44 ± 0.44 , T2 = 0.81 ± 0.34 serves \cdot day $^{-1}$; $t(8)=2.36$, $p=0.047$, ES=0.90). The PRO group reported a significant decrease in consumption of bread (T1 = 1.13 ± 0.64 , T2 = 0.88 ± 0.79 serves \cdot day $^{-1}$; $t(9)=2.52$, $p=0.035$, ES=0.34) between trials and the SYN group reported a significant decrease in consumption of cereal (T1 = 1.07 ± 1.01 , T2 = 0.50 ± 0.46 serves \cdot day $^{-1}$; $t(9)=2.35$, $p=0.045$, ES=0.74) and added sugar (T1 = 0.68 ± 0.80 , T2 = 0.27 ± 0.62 serves \cdot day $^{-1}$; $t(9)=2.24$, $p=0.049$, ES=0.55) between trials. There were no differences in consumption of prebiotics and probiotics products between trials (other than the intervention treatments) for any of the intervention groups ($p>0.05$). Analysis of participant's 24 h dietary intakes prior to each experimental trial revealed no differences in energy, water or macronutrient consumption ($p>0.05$) between T1 and T2 for any of the intervention groups (see Online Resource Table 1 for data).

INSERT TABLE 2 HERE

*Intervention Effects on Faecal Detection of *L. acidophilus* and *B. lactis**

Based on the PCR detection of microbial DNA, changes in the detection of the supplemental strains were most evident for the PRO and SYN groups post-intervention; all participants had an increased detection of either *B. lactis* or *L. acidophilus* post-intervention with 50% of the PRO group and 60% of the SYN group having increased detection of both strains. In contrast, increased detection of either strain was noted in only $n=3$ individuals (33%) from the PRE group (two of these three participants had increased detection of both strains). For the PLA group increased detection of either strain was observed for $n=4$ participants (44%; only one of these individuals had increased detection of both strains).

Intervention Effects on Biochemical Markers

Blood was unable to be collected from one participant from the PLA group. Data from the remaining 37 participants was analysed and mean results for each of the biochemical markers are shown in Table 3. There were few significant differences in biomarkers between trials for all intervention groups. A significant increase in GLUC was observed for the PLA group, ($t(7)=-2.451$, $p=0.044$, $ES=0.38$). A significant decrease in CRE ($t(7)=2.457$, $p=0.044$, $ES=0.52$) and increase in LIP ($t(7)=-2.391$, $p=0.048$, $ES=0.31$) was observed for the PRE group. A significant increase in LDH ($t(9)=-2.573$, $p=0.032$, $ES=0.63$) and UA2 ($t(9)=-2.329$, $p=0.045$, $ES=0.21$) was observed for the PRO group. No other differences were observed between trials ($p>0.05$). Analysis of chemical biomarkers by microbiome outcome (see Online Resource Table 2 for data) also revealed few significant differences between trials. A significant increase in LIP ($t(9)=-2.465$, $p=0.036$, $ES=0.19$) was observed for the NC / DEC

group and a significant increase in UA2 ($t(25)=-2.557$, $p=0.017$, $ES=0.33$) was observed for the INC group. No other differences were observed between trials ($p>0.05$).

INSERT TABLE 3 HERE

Experimental Trials

Pre- and Post- Trial Physiological Measures

All participants reported to the laboratory and verbally confirmed compliance to pre-experimental standardisation procedures. All participants produced a $0.000 \text{ g}\cdot\text{dL}^{-1}$ BrAC reading at the initial testing time. Participants pre-trial urine samples indicated a mean $U_{\text{sg}} < 1.020$ with no significant differences observed across the two trials for each intervention group ($p>0.05$) (Table 2).

WinNonlin Parameters

Mean BrAC responses for each intervention group are shown in Fig. 2 and summary data from WinNonlin analysis by treatment intervention are presented in Table 4. No significant differences in C_{max} or t_{max} were observed between trials ($p>0.05$). A significant decrease in AUC_{last} was observed for the PLA ($t(8)=2.49$, $p=0.039$, $ES=0.67$) and PRE ($t(8)=2.66$, $p=0.030$, $ES=0.55$) groups. There was a trend for a decrease in AUC_{last} for the SYN group ($t(9)=1.85$, $p=0.091$, $ES=0.43$). No difference in AUC_{last} was observed between trials for the PRO group ($t(9)=1.50$, $p=0.112$, $ES=0.28$). Analysis by microbiome outcome 4 (see Online Resource Table 3 for data) revealed no significant differences in C_{max} between trials ($p>0.05$). A significant increase in t_{max} was observed in the NC/DEC outcome group ($t(10)=2.34$, $p=0.041$, $ES=0.44$), but not for the INC group ($t(26)=1.31$, $p=0.203$, $ES=0.32$).

A significant decrease in AUC_{last} was observed for both the NC/DEC ($t(10)=2.81$, $p=0.016$, $ES=0.68$) and INC ($t(26)=3.45$, $p=0.003$, $ES=0.45$) groups.

BrAC Estimations ($BrAC_{est}$) and Subjective Ratings

There were no significant differences in mean peak $BrAC_{est}$ between trials (T1 vs T2) for any of the intervention groups ($p>0.05$); (PLA: 0.072 ± 0.014 vs 0.085 ± 0.018 $g\cdot dL^{-1}$; PRE: 0.088 ± 0.030 vs 0.087 ± 0.016 $g\cdot dL^{-1}$; PRO: 0.088 ± 0.026 vs 0.087 ± 0.014 $g\cdot dL^{-1}$; SYN: 0.077 ± 0.011 vs 0.080 ± 0.005 $g\cdot dL^{-1}$). In general, when asked to guess their BrAC, participant's tended to overestimate peak BrAC in all trials except for T1 in the PLA group and in both trials for the SYN group. There was no difference between peak BrAC and peak $BrAC_{est}$ in trials for any of the groups ($p>0.05$). There was a strong, positive and significant correlation between the two variables in each trial, for all intervention groups ($p<0.001$; PLA: 0.78 and 0.82; PRE: 0.81 and 0.84; PRO: 0.77 and 0.89; SYN: 0.73 and 0.68, for T1 and T2 respectively).

Ratings for feeling the effects of alcohol, ability to concentrate, experiencing dizziness, headache and ability to drive a car were influenced by ingestion of alcohol and also varied over time throughout trials ($p<0.05$). However, no differences were observed for scores on any of the subjective ratings scales between trials (T1 vs T2) for any of the intervention groups ($p>0.05$).

INSERT FIG 2 HERE

INSERT TABLE 4 HERE

Discussion

This pilot investigation demonstrates that 8 weeks supplementation of probiotics (with or without concurrent prebiotics supplementation) in healthy adults, increases the number of supplement specific bacteria (*L. acidophilus* and/or *B. lactis*) of the intestine (determined by faecal abundance). However, this increase in bacterial colonisation did not translate to improved metabolism of an acute dose of alcohol.

Supplementation with probiotics or a combination of prebiotics and probiotics resulted in increased detection of *B. lactis* and/or *L. acidophilus* in all participants. This finding is in contrast to a recent review indicating a lack of evidence for the impact of probiotics on faecal microbiota composition and richness in healthy adults [45]. Whilst the review included interventions involving probiotics belonging to the genus *Lactobacillus* and *Bifidobacterium*, the specific strains employed in the current study (*L. acidophilus* NCFM[®] and *B. lactis* Bi-07) were not included. The ability of *L. acidophilus* NCFM[®] and *B. lactis* Bi-07 to adhere to human epithelial cells, modulate the population or activity of human intestinal microbiota, and increase the number of faecal bacteria in healthy human subjects has been confirmed in a number of studies [46-50,30,51]. Thus, results from the present study suggest that the prescribed prebiotics/probiotics contributed to increased counts of the supplemental bacterial strains and represented a viable approach to exploring whether supplementation of the gut microbiome influences alcohol metabolism.

Despite detected increases in gram positive bacteria, current results suggest probiotics supplementation has no effect on C_{\max} and t_{\max} parameters. Furthermore, no differences in subjective ratings of intoxication or impairment were observed in the present study, suggesting that probiotics supplementation do not impact the cognitive effects associated

with acute alcohol consumption. While no study had previously investigated the effects of probiotics consumption on influencing alcohol responses in humans (via changes to the gut microbiome as opposed to acute consumption of a probiotic product [18]), these results were expected, given that changes in C_{\max} and t_{\max} are measures associated with alcohol absorption rather than alcohol metabolism or elimination [11].

Probiotic *Lactobacillus* and *Bifidobacterium* strains possess ADH and ALDH activity *in vitro*, and demonstrate a positive correlation between rising bacterial concentrations and increased acetaldehyde-metabolising capacity [15]. Thus, increasing intestinal gram-positive bacteria may assist with alcohol metabolism. However, in the present study, probiotics supplementation had no influence on alcohol AUC values. While lower values were observed in the second trial for both the PRO and SYN groups, these changes were not significantly different from the first trial. In contrast, significantly lower AUC values were observed in the second trial for groups that received PLA and PRE treatments. One possible explanation for this in the PLA group could be the significant increase in body weight and BMI found between trials. Participants received identical doses of alcohol at each trial (based on Widmark calculations in trial 1 that considered anthropometric values (body weight, height) to determine total body water content). A greater body weight is therefore reflected in the equation by a larger total body water volume [36]. AUC is significantly correlated with total body water; with lower AUC values observed as total body water content increases [52]. However, this does not help explain the significant AUC differences observed in the PRE group, where anthropometric values were not significantly different between trials.

When considered relative to microbiome outcome, AUC was significantly lower in the group that demonstrated an increase (INC) in faecal count of at least one bacterial strain

(irrespective of intervention). However, similar results were observed in the group where no change or decreases (NC/DEC) in faecal counts of both bacterial strains were observed. Subsequent analysis of body weight between trials for these groups indicated no difference in the INC group (T1 = 69.38±12.90kg, T2 = 69.73±12.89kg; $p=0.092$), but a significant increase for the NC/DEC group (T1 = 70.68±12.93kg, T2 = 71.52±13.26kg; $p=0.047$). Again, this may provide partial explanation for the AUC results observed, particularly where changes were not expected. However, a more likely explanation is that alcohol metabolism is highly variable [11], which may have precluded an ability to detect subtle changes as a result of treatment effects. In addition, the low dose of alcohol, use of breath alcohol measures opposed to blood concentrations, and the short time period of measurement may also be factors that influenced the ability to detect changes (should they exist). Furthermore, results may have been different if an alcohol beverage with a more nutritional matrix, such as beer or wine was used instead of vodka. There is clearly scope for further research to be conducted in this area with different doses and types of alcohol provided.

While few differences in blood biomarkers were detected between trials, there was no obvious or consistent effect of probiotics consumption on these variables. Previous reports have identified positive effects of probiotics consumption (over similar time frames of supplementation) at reducing blood pressure [20], cholesterol [53,54], blood lipids [55], and improving liver function and enzyme activity [56,57]. However, these changes have typically been observed in high risk groups (i.e. the elderly) or individuals that have demonstrated some degree of baseline abnormality, illness or injury. Participants in the present study were healthy adults, and on average no abnormalities were detected from blood measures collected in the first trial. Results may have been different in a population with some degree of dysbiosis or underlying biochemical irregularity. Given that regular alcohol consumption can

cause gut dysbiosis [28,29], probiotics/prebiotics supplementation may benefit those who are chronic alcohol users. In fact, one study has demonstrated that probiotics have the capacity to restore gut microbiota and improve liver enzymes in alcoholic adult male patients with alcohol-induced liver injury [56]. However, promotion of probiotics for recovery of alcohol-induced gut dysbiosis in these individuals may endorse a message supporting alcohol consumption and requires important ethical consideration.

The present study demonstrated a high degree of participant compliance with probiotics/prebiotics supplementation. This was likely a result of incorporating several regular monitoring measures into the study. Nonetheless, this demonstrates excellent feasibility for the study protocol, particularly given that supplementation resulted in increased faecal detection of the bacterial strains. The compliance procedures employed here may prove useful for future investigations where probiotics supplementation is employed over longer treatment durations. This study used a commercially available source of probiotics and prebiotics with a specific strain and dose of bacteria, consumed as per manufacturer's recommendations. Results may vary with the provision of different doses and bacterial strains of probiotics and prebiotics. For example, the probiotic *Lactobacillus GG ATCC 53103* has been demonstrated to have high acetaldehyde metabolising capacity in vitro, even in the presence of ethanol [15], and rodents fed a medium containing a culture broth of *Lactobacillus fermentum MG590* and alcohol have demonstrated enhanced alcohol metabolism [17]. Furthermore, other species such as *Lactobacillus rhamnosus GG*, *Lactobacillus casei* and *Saccharomyces boulardii* are considered to be some of the most beneficial probiotic strains to human health [58-60]. Future research could target these specific probiotic strains in individuals with existing gut dysbiosis and investigate their impact on supporting alcohol metabolism.

Overall, this pilot study demonstrates that consumption of probiotics alters faecal bacterial content in otherwise healthy individuals. However, translation to any possible beneficial impact on alcohol metabolism remains to be elucidated. Nonetheless, probiotics supplementation in this study supported proliferation of *L. acidophilus* and *B. lactis* bacterial strains, with negligible incidence of adverse gastrointestinal symptoms. Supporting the gut microbiome with probiotics may have important implications for consumers of alcohol that extend beyond the acute ingestion period examined in this study, into longitudinal and broader elements of human health (i.e. reducing incidence of alcohol related gastrointestinal complications such as dysbiosis, acute bacterial gastroenteritis, small bowel bacterial overgrowth, and antibiotic-associated diarrhoea).

Compliance with Ethical Standards

Conflict of Interest

The authors declare that they have no conflict of interest.

Ethical Standards

This study was conducted according to the guidelines laid down in the 1964 Declaration of Helsinki and all procedures involving human subjects/patients were approved by the University's Human Research Ethics Committee (Protocol No: AHS/74/14/HREC). Written informed consent was obtained from all subjects/patients.

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Figure Captions

Fig. 1 Illustration of the 8 weeks parallel-group intervention, with participants randomised to one of the four different groups. Placebo = microcrystalline cellulose; Prebiotics = Larch Gum (from *Larix occidentalis*); Probiotics = Inner Health Plus Dairy Free (*L. acidophilus* NCFM[®] and *B. lactis* Bi-07).

Fig. 2a BrAC responses (mean±SD) for PLA, placebo+placebo intervention ($n=9$); Placebo = microcrystalline cellulose; T1, trial 1; T2, trial 2; SD, standard deviation. ^aSignificant difference between T1 and T2 ($p<0.05$).

Fig. 2b BrAC responses (mean±SD) for PRE, prebiotics+placebo intervention ($n=9$); Placebo = microcrystalline cellulose; Prebiotics = larch gum (from *Larix occidentalis*); T1, trial 1; T2, trial 2; SD, standard deviation. ^aSignificant difference between T1 and T2 ($p<0.05$).

Fig. 2c BrAC responses (mean±SD) for PRO, placebo+probiotics intervention ($n=10$); Placebo = microcrystalline cellulose; Probiotics = Inner Health Plus Dairy Free (*L. acidophilus* NCFM[®] and *B. lactis* Bi-07); T1, trial 1; T2, trial 2; SD, standard deviation. ^aSignificant difference between T1 and T2 ($p<0.05$).

Fig. 2d BrAC responses (mean±SD) for SYN, prebiotics+probiotics intervention ($n=10$); Prebiotics = larch gum (from *Larix occidentalis*); Probiotics = Inner Health Plus Dairy Free (*L. acidophilus* NCFM[®] and *B. lactis* Bi-07); T1, trial 1; T2, trial 2; SD, standard deviation. ^aSignificant difference between T1 and T2 ($p<0.05$).

Tables

Table 1. Participant characteristics and drinking related habits

Parameter	Total <i>n</i>=38	Males <i>n</i>=17	Females <i>n</i>=21
Age (yrs)	26.2±8.4	27.9±10.2	24.9±6.5
BW (kg)	69.8±12.7	77.3±10.8	63.6±10.9
BMI (kg·m ⁻²)	23.7±3.5	24.4±3.1	23.1±3.7
Drinking history (yrs)	8.2±6.5	11.3±6.2	5.7±5.8
PDHQ drinking frequency·wk ⁻¹	1.2±1.1	1.7±1.3	0.8±0.6
No. of standard drinks per drinking occasion	3.6±1.8	3.6±1.5	3.5±2.0

BW, body weight; BMI, body mass index; PDHQ, personal drinking history questionnaire; yrs, years, Values are mean±SD.

Table 2. Participant characteristics and experimental trial values for each intervention group ($n=38$)

Parameter	PLA ($n=9$, 5m)		PRE ($n=9$, 2m)		PRO ($n=10$, 5m)		SYN ($n=10$, 5m)	
	T1	T2	T1	T2	T1	T2	T1	T2
Age (yrs)	27.6±8.0	27.6±8.0	26.4±7.4	26.4±7.4	27.9±6.5	27.9±6.5	26.1±7.7	26.1±7.7
BW (kg)	74.5±11.0	76.0±10.6 ^a	62.5±10.0	62.9±10.5	69.4±14.2	69.7±14.3	72.4±13.7	72.3±13.6
BMI (kg·m ⁻²)	24.8±4.1	25.3±4.0 ^a	22.0±3.3	22.2±3.5	23.0±3.3	23.1±3.2	24.6±2.7	24.6±2.7
SBP (mmHg)	123±13	118±13	120±12	120±12	122±10	121±12	120±10	118±9
DBP (mmHg)	72±8	70±8	70±11	70±8	69±9	68±9	68±7	68±6
Pre-Trial Usg	1.015±0.009	1.015±0.007	1.012±0.005	1.007±0.004	1.015±0.008	1.013±0.005	1.016±0.008	1.014±0.008
Post-Trial Usg	1.015±0.002	1.015±0.004	1.013±0.005	1.012±0.004 ^b	1.015±0.004	1.016±0.004	1.012±0.004	1.015±0.002
Alcohol Dose Provided (g)	29.7±4.8	29.7±4.8	24.8±4.1	24.8±4.1	28.3±7.0	28.3±7.0	29.5±6.6	29.5±6.6

Values are mean±SD; m, male; BW, body weight; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; Usg, urine specific gravity; PLA, placebo+placebo intervention; PRE, prebiotics+placebo intervention; PRO, placebo+probiotics intervention; SYN, prebiotics+probiotics intervention; Placebo = microcrystalline cellulose; Prebiotics = larch gum (from *Larix occidentalis*); Probiotics = Inner Health Plus Dairy Free (*L. acidophilus* NCFM[®] and *B. lactis* Bi-07). ^aSignificant difference between T1 and T2 measures ($p<0.001$), ^bSignificant difference between pre- and post- trial measures ($p=0.01$).

Table 3. Biochemical markers for each intervention group ($n=37$)

Parameter	PLA ($n=8$, 5m)		PRE ($n=9$, 2m)		PRO ($n=10$, 5m)		SYN ($n=10$, 5m)	
	T1	T2	T1	T2	T1	T2	T1	T2
ALB ($\text{g}\cdot\text{L}^{-1}$)	46.50±3.18	47.19±2.88	47.70±1.92	43.22±9.74	47.71±1.71	48.01±2.56	48.23±1.77	48.30±2.05
ALT ($\text{U}\cdot\text{L}^{-1}$)	18.13±2.90	28.88±25.17	17.91±5.36	16.20±6.12	18.04±10.61	18.35±8.91	23.53±13.37	20.46±5.03
AST ($\text{U}\cdot\text{L}^{-1}$)	22.09±3.14	43.22±50.49	22.98±3.20	22.50±5.14	23.50±12.26	22.64±7.76	33.03±27.10	27.89±12.36
BIL-D ($\mu\text{M}\cdot\text{L}^{-1}$)	4.51±2.06	4.29±1.33	6.26±3.36	5.55±2.80	4.65±1.28	4.83±1.74	3.55±0.97	3.66±0.91
BIL-T ($\mu\text{M}\cdot\text{L}^{-1}$)	11.09±5.60	9.78±3.55	16.69±12.17	13.09±7.77	10.73±3.81	11.68±4.45	8.18±2.55	9.15±2.95
CHOL ($\mu\text{M}\cdot\text{L}^{-1}$)	4.48±1.22	4.86±1.31	4.07±0.50	3.68±1.05	4.78±0.82	4.71±0.63	4.94±1.04	4.86±0.75
CRE ($\mu\text{M}\cdot\text{L}^{-1}$)	73.38±17.30	78.13±21.64	74.44±8.69	69.00±11.08 ^a	76.05±12.98	76.05±12.98	79.00±10.47	76.20±12.07
CRP ($\text{mg}\cdot\text{L}^{-1}$)	1.34±1.98	1.41±1.82	1.02±0.76	0.99±0.59	0.82±0.70	0.75±0.69	1.22±1.36	0.47±0.22
GGT ($\text{U}\cdot\text{L}^{-1}$)	18.67±6.91	21.78±14.25	18.65±7.26	15.24±7.66	19.32±9.16	21.32±10.52	23.51±15.71	23.06±13.42
GLUC ($\text{mM}\cdot\text{L}^{-1}$)	5.23±0.44	5.43±0.55 ^a	5.04±0.34	5.16±0.24	5.13±0.42	5.16±0.42	5.20±0.42	5.30±0.36
HDL-C ($\text{mM}\cdot\text{L}^{-1}$)	1.55±0.41	1.51±0.46	1.54±0.50	1.53±0.38	1.63±0.47	1.72±0.48	1.52±0.38	1.42±0.37
LDH ($\text{U}\cdot\text{L}^{-1}$)	160.19±29.14	182.88±61.34	148.67±9.40	154.28±18.16	146.05±21.29	158.60±16.70 ^a	176.20±29.46	158.85±18.42
LIP ($\text{U}\cdot\text{L}^{-1}$)	33.36±9.87	34.29±10.50	42.17±12.49	46.44±13.61 ^a	33.02±10.15	34.95±8.96	43.15±12.11	43.97±14.87
TP2 ($\text{g}\cdot\text{L}^{-1}$)	69.49±4.54	70.61±4.39	70.50±2.53	68.52±3.84	69.83±5.26	69.78±4.75	72.48±5.06	71.47±3.49
TRIGL ($\text{mM}\cdot\text{L}^{-1}$)	1.25±0.53	2.48±3.71	0.97±0.50	0.76±0.33	0.95±0.62	0.88±0.63	1.25±0.51	1.32±0.96
UA2 ($\text{mM}\cdot\text{L}^{-1}$)	4.97±1.54	5.51±1.81	4.52±1.60	4.67±1.62	4.97±2.08	5.43±2.10 ^a	4.68±0.93	5.56±1.93

Values are mean±SD; m, male; PLA, placebo+placebo intervention; PRE, prebiotics+placebo intervention; PRO, placebo+probiotics intervention; SYN, prebiotics+probiotics intervention; Placebo = microcrystalline cellulose; Prebiotics = larch gum (from *Larix occidentalis*); Probiotics = Inner Health Plus Dairy Free (*L. acidophilus* NCFM[®] and *B. lactis* Bi-07). ^aSignificant difference between T1 and T2 measures ($p<0.05$).

Table 4: WinNonlin parameters for each intervention group ($n=38$)

Parameter	PLA ($n=9, 5m$)		PRE ($n=9, 2m$)		PRO ($n=10, 5m$)		SYN ($n=10, 5m$)	
	T1	T2	T1	T2	T1	T2	T1	T2
t_{max} (min)	28.9±11.4	27.8±12.8	25.0±7.9	25.0±9.4	25.5±10.1	22.0±8.6	31.5±21.4	31.0±9.9
C_{max} (g·dL ⁻¹)	0.084±0.018	0.083±0.021	0.085±0.013	0.080±0.016	0.082±0.015	0.082±0.013	0.083±0.011	0.080±0.009
AUC_{last} (min×g·dL ⁻¹)	12.6±2.4	11.0±2.4 ^a	11.9±2.3	10.4±2.9 ^a	11.8±1.3	11.4±1.4	12.0±2.7	10.9±2.2

Values are mean±SD; m, male; t_{max} , mean time to peak concentration; c_{max} , mean peak concentration; AUC_{last} , area under the curve to the last measured time. PLA, placebo+placebo intervention; PRE, prebiotics+placebo intervention; PRO, placebo+probiotics intervention; SYN, prebiotics+probiotics intervention; Placebo = microcrystalline cellulose; Prebiotics = larch gum (from *Larix occidentalis*); Probiotics = Inner Health Plus Dairy Free (*L. acidophilus* NCFM[®] and *B. lactis* Bi-07). ^aSignificant difference between T1 and T2 measures ($p<0.001$).