Tufts University
School of Dental Medicine

EFFECT OF ALCOHOL CONSUMPTION ON RODENT PERIODONTAL HEALTH AND IMPROVEMENT BY THE PROBIOTIC *LACTOBACILLUS REUTERI*

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Abstract

Background: The purpose of this study was to evaluate the effect of chronic alcohol consumption, in high and low doses, on periodontal health and to investigate the potential of the probiotic *Lactobacillus reuteri* (*L. reuteri*) to counteract the deleterious effect of alcohol on periodontal health. **Materials and methods:** Twenty-four adult male Sprague-Dawley rats (2 months old) were divided randomly into three groups: control group consumed liquid diet (CG; n=8); low alcohol group consumed 3.1% alcohol liquid diet (LAG; n=8); and high alcohol group consumed 6.2% alcohol liquid diet (HAG; n=8). After eight weeks of alcohol consumption, the three groups were divided randomly into six subgroups to either receive *L. reuteri* or not. *L. reuteri* (DSM17938, 2×10^8 CFU/day and ATCC PTA5289, 2×10^8 CFU/day) was added daily to the liquid diet for two weeks. The periodontal parameters: plaque index (PI), bleeding on probing (BOP), and gingival index (GI); and subgingival bacterial count were measured at baseline, after eight weeks of alcohol consumption, and after 2 weeks of *L. reuteri* intake. **Results:** BOP increased in both the LAG and HAG after 8 weeks of alcohol consumption and this increase was statistically significant in the HAG [median (IQ): 6 (6.5) to 15.63 (6.25), p=0.035]. The GI score was also significantly increased in the LAG [median (IQ): 0.12 (0.18) to 0.25 (0.15), p=0.020] and in the HAG [median (IQ): 0.12 (0.13) to 0.37 (0.25), p=0.021]. There was no difference in the amount of plaque between the different groups. After two weeks of *L. reuteri* intake, BOP and GI were decreased in the LAG and HAG and the decreases were statistically significant in the LAG [BOP, 12.50 (4.87) to 3 (6), p=0.048] and [GI, 0.25 (0.09) to 0.06 (0.12), p=0.048]. Subgingival bacterial count was diminished in the LAG and HAG and was not affected by *L. reuteri* intake. **Conclusions:** Chronic

alcohol consumption may be considered a risk factor for gingivitis in a dose-dependent manner and *L. reuteri* supplements could be a useful strategy to treat gingival inflammation caused by chronic alcohol consumption.

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I. INTRODUCTION:

1. Periodontal Disease Periodontal disease is an inflammatory disease of bacterial origin that results in the progressive destruction of the tissues that support the tooth, specifically the gingiva, periodontal ligament, and alveolar bone (Fig. 1).[1] Periodontal disease is classified into two types: gingivitis and periodontitis. Gingivitis is caused by inflammation of the gingiva characterized clinically by inflammation limited to the unattached gingiva, changes in color, gingival form, position, surface appearance, and presence of bleeding and/or exudate.[2] In contrast, periodontitis is a progressive, destructive disease that affects all supporting tissues of the tooth, including the alveolar bone crest which causes bone loss.[2, 3] The initiator of periodontal disease is microbial dental plaque. The main periodontal pathogens associated with the periodontal destruction are *Porphyromonas gingivalis, Tannerella forsythia, Treponema denticola,* and *Aggregatibacter actinomycetemcomitans*.[3, 4]

According to the American Academy of Periodontology (AAP), 75% of adults in the United States are affected by periodontal disease ranging from mild gingivitis to severe periodontitis.[5] There are several factors that influence the progression of periodontitis: subject characteristics; psychological and stress factors; social and behavioral factors; systemic factors; genetic factors; socioeconomic status; nutritional status; tooth factors; and excessive alcohol consumption.[4, 6]

Several methods have been used for the recognition of periodontal pathogens in subgingival plaque samples. The well known method used in etiological research and in clinical treatment of periodontitis is *in vitro* culture and subgingival microbiological analysis include curettes, scalers, paper points, broaches within cannula, and irrigation of

periodontal pockets.[7-9] They specified that the paper point technique be used for *in vitro* culture studies. This technique has the capability to remove loosely adherent tissue-associated microorganisms.[7]

The diagnosis of periodontal disease relies on clinical evaluation including bleeding on probing, clinical attachment level, probing depth, and radiographic examination.[1] In general, the healthy periodontal tissue has proper color, size, shape, contour, consistency, and texture.[2] In bleeding on probing test, healthy tissue does not bleed after gentle probing and probing depths are 1 to 3 mm.[2] Radiographic examination of bone tissue shows intact crestal lamina dura around dentition and intact inter-radicular bone.[2] Inflamed tissue becomes enlarged, rolled margin, and flattened shape interdental papilla.[2] The gingival texture loses its stippled character and becomes smooth and glossy.[2] Bleeding occur with gentle probing force, deep pocket depths are recorded, as well as attachment loss.[10] Radiographic evaluation of osseous tissues shows loss of interproximal and interradicular bone (Fig. 1).[2]

The purpose of treating periodontal disease is to eliminate the etiological factors and re-establish gingival health.[11] There are different treatment considerations for periodontal disease and choosing the proper treatment depends on understanding the etiology and pathogenesis of the disease. A treatment plan for periodontal disease includes the following: oral hygiene instruction; scaling and root planing; antimicrobial agents; correction of plaque retentive factors; surgical correction; and follow-up.[11] Additionally, the success of treatment requires patient cooperation.

2. Chronic Alcohol Intake and Oral Health Several studies reported that when alcohol is consumed in large amounts and chronically, it may cause harmful effects.[12] The World Health Organization (WHO) reported that alcohol consumption is the world's third largest risk factor for disease and disability in middle-income countries.[12] The 2010 National Health Interview Survey (NHIS) reported that 51% of adults aged 18 years and over were currently regular drinkers, 14% were currently infrequent drinkers, 6% were former regular drinkers, 8% were former infrequent drinkers, and 21% were lifetime abstainers.[13]

As discussed earlier, periodontal disease is caused by bacteria originating from dental plaque and there are several factors that influence disease expression. Excessive alcohol consumption is a risk factor for periodontal disease. Alcohol may affect periodontal tissues through different mechanisms (Fig. 2). First, alcohol has an adverse effect on host defense by impairing neutrophils, phagocytic monocytes and macrophages, and T-cell functions which lead to increases the frequency of infections.[14] The function of the phagocytic cells is to protect the body by locating, ingesting, and killing the harmful foreign particles through a complex process.[14] Some studies in human and animal models reported that acute and moderate alcohol intake can impair host defense resulting in bacteria and viral stimulation.[14] Second, ethanol alters bone metabolism: it increases bone resorption and decreases bone formation by changing the number and activity of the osteoblasts and osteoclasts as well as an increase in osteocyte apoptosis; altering cell differentiation that might cause low bone mass; and an increases of fat accumulation in the bone marrow.[15, 16] Third, alcohol has a toxic effect on the liver.[17] It may disrupt the production of prothrombin, vitamin K activity, and clotting mechanisms which might

lead to hemorrhage and increases production of inflammatory cytokines.[17,18] Additionally, alcohol may interfere with protein metabolism which affects tissue healing by impairing the digestion of proteins to amino acids, impairing the synthesis of proteins from amino acids, and impairing protein secretion by the liver.[16, 19]

It is reported that alcohol consumption may be harmful to the oral cavity resulting in dental caries, tooth loss, and periodontal disease.[20, 21] It is suggested that alcohol may affect soft and hard tissues of the periodontium differently, and concluded that drinking alcohol may be a risk indicator for periodontal disease.[18] Later, in another study, it is found that there is a significant relationship between alcohol consumption and clinical attachment loss.[22] In addition, other studies showed that periodontal disease can be a result of self-neglect due to chronic alcohol consumption.[23] In contrast, other studies did not support an association between alcohol intake and periodontal disease.[24, 25] These differences may be because of methodological issues and the etiology of dental and periodontal diseases. In our study, we hypothesize that alcohol consumption is a risk factor for periodontal disease.

One study showed that the adverse effect of alcohol on the periodontium is dose-dependent.[26] A cross-sectional study, involving 13,198 individuals, reported that there is a dose-dependent relationship between alcohol consumption and increased severity of periodontal disease.[22] In addition, it is reported that alcohol consumption increases alveolar bone loss in a dose dependent manner.[27] Therefore, there is a positive relationship between chronic alcohol intake and harm to the periodontium. However, others reported that there is no dose–response relationship between alcohol consumption and periodontal disease.[25] In our study, we will evaluate the effect of alcohol intake in

high and low doses on periodontal health.

Few studies focused on the effect of frequency and types of alcohol consumed on periodontal disease. A study evaluated the effect of alcohol beverage types (wine, beer, hard liquor) but reported no apparent differences in the association with periodontal variables.[18] In the Health Professionals prospective study done on 51,529 male, it is concluded that wine, beer, and hard liquor intake had no clear effect on periodontitis.[28] One study revealed a significant relationship between beer and hard liquor consumption and clinical attachment loss measures.[22] Also, in the same study, the effect of frequency of alcohol consumption has been mentioned. It showed a significant linear relationship between frequency of beer and hard liquor consumption (times/month) and clinical attachment loss. Another study reported that there is no significant relationship between frequency of consumption and severity of disease.[29] Hence, this question remains unanswered.

3. Health Benefits of Probiotic Supplements Probiotics are defined by The Food Agricultural Organization/World Health Organization (FAO/WHO) as "live microorganisms which when administered in adequate amounts confer a health benefit on the host".[30] In 1907, Ukrainian bacteriologist and Nobel Laureate Elie Metchnikoff showed that the lactic acid produced by *Lactobacilli* could inhibit growth of some pathogenic bacterial species in the human body. He presented the first scientific explanation of the beneficial effects of yogurts.[31, 32] There are numerous studies reporting the positive effect of probiotics on systemic health.[32, 33] Probiotics have been investigated in different scopes. They have multifactorial anti-carcinogenic activity and can attenuate

the adverse effects of *Helicobacter pylori* in the gastrointestinal system. Probiotics have been linked with cancer risk reduction, coaggregation with uropathogens preventing urogenital infections, and reduction of high blood pressure.[32]

Probiotics could prevent periodontal diseases either by inhibition of specific pathogens or by altering the host immune response.[34] Probiotics can act through several mechanisms to improve oral health (Fig. 3). First, they can adhere to the hard and soft surfaces in the oral cavity or attach to other bacteria.[35, 36] Second, they can interfere with the growth of pathogens by secreting antimicrobial substances such as organic acids, hydrogen peroxide, carbon peroxide, bacteriocins, or adhesion inhibitors.[33, 37] Third, they can stimulate nonspecific immunity and modulate the humoral and cellular immune responses.[3] Finally, probiotics can alter the environmental conditions of the oral cavity by modulating the pH and/or the oxidation-reduction potential.[3]

Past studies focused on using probiotics for medical conditions such as prevention or treatment of gastrointestinal infections.[38] During the last few years, an increasing number of studies have reported that probiotics could be beneficial for oral health including caries inhibiting effect, reducing gingival and periodontal inflammation, and eliminating halitosis.[39] The broad and most commonly used probiotic bacterial strains belong to the genera *Lactobacillus, Bifidobacterium, Propionibacterium* and *Streptococcus.*[3] The difference between these probiotics is that *Lactobacilli* have better adherence capability to saliva-coated surfaces.[3]

L. reuteri is one of the *Lactobacillus* species. *L. reuteri* is a Gram-positive, rod-shaped and anaerobic bacterium. This heterofermentatic lactic acid bacterium is found in the gastrointestinal tract of human and animals.[40] It is also found in human breast milk.[41]

The safe and efficient doses of *L. reuteri* are 10^{10} - 10^{11} CFU/day for 21 days.[42, 43]

Many of the benefits of *L. reuteri* have been studied against periodontal inflammation. *L. reuteri* have three properties which could help improve periodontal condition: first, it secretes bacteriocins, reuterin and reutericyclin, which inhibit the growth of a wide variety of pathogens; second, it has a strong capacity to adhere to host tissues and compete with pathogenic bacteria; and third, it inhibits secretion of proinflammatory cytokines.[3] In a recent study, gingivitis and plaque were reduced after chewing gum containing *L. reuteri* in patients with moderate to severe forms of gingivitis.[44] In another study with patients with moderate gingivitis, *L. reuteri* improved gum bleeding on probing, decreased gingival crevicular fluid volume, and decreased the levels of the proinflammatory cytokines tumor necrosis factor α (TNFα) and interleukin-8 (IL-8).[45] Therefore, *L. reuteri* is selected for the study.

The risk of infection from *Lactobacilli* and *Bifidobacteria* derived probiotic intake is rare since they are normally present in the body's commensal microflora.[46, 47] In addition, fermented foods such as cheese and yogurt; and non-fermented products such as fresh salad and vegetables are a natural source of probiotics.[47] The Food and Drug Administration reported that *Lactobacilli* and *Bifidobacteria* used for food production are "generally recognized as safe" (GRAS).[48] Moreover, no cases of infection have been reported in people who consume food containing probiotics or in those exposed to bacteria in probiotic industry.[47]

Because of the experimental difficulties and ethical concerns in studying the effect of alcohol in humans, we choose rodents as experimental animals to evaluate the effect of chronic alcohol intake on periodontal health. Rodent models have been

developed to study many aspects of human alcoholism.[49] Fortunately, rodent models can be used to study the causes of human diseases and efficiency of treatments with predictive validity.[49] In addition, there are more than 15 bacterial species from the oral cavity of rats, and the most two common genera are *Lactobacillus* and *Streptococcus* in all laboratory animals.[50] Any proportional changes in the gingival plaque flora may lead to gingival inflammation in rats.[51]

Significance

The effect of chronic alcohol consumption on periodontal disease is evaluated in different studies. Some of these studies reported a significant relationship between alcohol consumption and periodontal disease whereas others did not. In addition, the benefits of the probiotic *L. reuteri* on periodontal health were assessed in several studies. However, to the best of our knowledge, none of these studies tested if the probiotic can reduce the effect of alcohol on periodontal tissue and improve oral health. Therefore, the purpose of this study was to evaluate the effect of chronic alcohol intake on periodontal health and investigate the potential of the probiotic *L. reuteri* to counteract the deleterious effects of alcohol.

II. SPECIFIC AIMS AND HYPOTHESIS

Specific Aims

1- Evaluate the deleterious effect of alcohol intake, in high and low doses, on periodontal health in rats by using plaque index, bleeding on probing, gingival index, and presence of subgingival microorganisms as outcome variables.

2- Investigate the potential of the probiotic *L. reuteri* to counteract the deleterious effect of alcohol on periodontal health in rats.

Hypothesis

We hypothesize that alcohol consumption will be a greater risk for periodontal disease in rats and that the alcohol effects can be counteracted with the probiotic *L. reuteri* (DSM17938 & ATCC PTA5289) supplements.

III. MATERIALS AND METHODS

1. Sample Size Calculation

Based on published data, we defined the mean (SD) values of *Streptococcus* bacteria (CFU) 5.63 (4.33) for the control group and 8.17 (6.69) for the high alcohol group to run nQuery.[52] nQuery (Version 7.0) was used to determine the sample size needed to get significance level at 0.05 and the power = 95%. We ended up with a sample size of twenty-four (eight for each group) which was adequate to detect significant differences between groups.

Twenty-four adult male Sprague-Dawley rats (2 months old) were purchased from Taconic (Hudson, NY, USA). Animals were housed in individual cages and their room was under controlled temperature (72.5°F) and constant humidity (27%) with fixed light/dark intervals of 12 hours length and were fed ad libitum. The study was approved by the Tufts Medical Center Institutional Animal Care and Use Committee (IACUC).

2. Materials

The Materials that have been used in this experiment were: Lieber-DeCarli liquid rat diet, was from Dyets, Inc. (Bethlehem, PA, USA); blood agar plates, trypticase soy agar with 5% sheep blood, MRS broth and agar, and 100% alcohol, were from Fisher Scientific (Waltham, MA, USA); periodontal probes (PCPUNC 15), were from Hu Friedy (Chicago, IL, USA); absorbent paper point size fine, was from SybronEndo (Glendora, CA, USA); *L. reuteri* DSM17938 and ATCC PTA5289, were a generous gift from BioGaia Biologics Inc. (Raleigh, NC, USA); Ketamine, was from Putney (Portland, ME, USA); Xylazine, was from Llayd Laboratories (Shenandoah, IA, USA); and

Isoflurane, was from Abbott (North Chicago, IL, USA).

3. Experimental Design

Alcohol Consumptions (Phase I) Animals were randomly divided into three groups (Fig. 4):

- Control group (CG, n=8): liquid nutrition
- Low alcohol group (LAG, n=8): 3.1% alcoholic solution in liquid nutrition
- High alcohol group (HAG, n=8): 6.2% alcoholic solution in liquid nutrition

a. Diet Components The diet mix components for each group was (Appendix 1):

- Control diet (CD), 132.18 g of 710260 Lieber-DeCarli regular rat diet + 89.6 g maltose dextrin + 1 L of water
- Low alcohol diet (LAD), 132.18 g of 710260 Lieber-DeCarli regular rat diet + 44.8 g of maltose dextrin + 31.97 mL of 100% ethanol + 1 L of water
- High alcoholic diet (HAD), 132.18 g of 710260 Lieber-DeCarli regular rat diet + 63.94 mL of 100% ethanol + 1 L of water

All the components were mixed in a blender for 30 seconds. Ethanol amount that was added to the LAG and HAG represented 18% and 36% of the total energy of the diet, respectively. In the CG and LAG, ethanol was substituted by isocaloric maltodextrin. Therefore, CG, LAG, and HAG consumed equal amount of total energy. Because the liquid diet provides physiologic amounts of fluid, extra water was not given. The diets were prepared once per week and were stored at 4°C.

The alcohol intake in HAG (63.94 mL of 100% ethanol) was approximately equal

to the consumption of 100 g/day of alcohol in a 2000-kcal human diet (7.1 kcal/g alcohol; 1 kcal = 4.184 kJ).[53] In other words, the 63.94 mL of ethanol consumed by the HAG is equivalent to consuming 7 standard drinks of wine for human daily, with one standard drink being equal to 150 mL of wine.[54]

b. Adaptation Period Alcohol was mixed in the rats' diet. Initially, the 3.1% alcohol and the 6.2% alcohol fed animals went through an adaptation period in which the alcohol concentration was increased gradually until it reached the experimental concentrations. A diet containing 1.5% of alcohol was administered for 3 days to the LAG group. The HAG group received a diet containing 1.5%, 3.1%, and 4.6% alcohol for 3 days each. After this adaptation period, the animals received diet containing the experimental concentrations (3.1% for the LAG and 6.2% for the HAG group).

c. Pair-feeding Technique After the adaptation period, the pair-feeding technique was done. It is a technique in which the amount of food provided to a control group of animals is matched with that consumed by the experimental groups, so as to determine the extent to which the effect of a treatment on body weight or body composition occurred independently of changes of energy intake.[55] The weight of the diet consumed by the LAG and HAG was measured and it was 60 mL/day. Therefore, we added 60 mL of diet/day for the CG, LAG, and HAG. In this case, all the groups were consumed the same mount of liquid diet (Fig. 6).

d. Diet and Body Weights The amount of consumed diet was measured and recorded daily before addition of fresh diet (Fig. 6). The rats' weights were measured and

recorded every 3 days (Fig. 11). If animals in the alcohol groups lost more than 15% of their body weight, the percentage of alcohol was reduced to half of the experimental concentration for 7 days; afterward, it was increased back to the experimental concentration. If the rat did not recover, it was euthanized.

Probiotic Intake (Phase II) After eight weeks of consuming the alcoholic diet, the outcomes were measured to ensure that gingival disease was established. Animals were then divided into sub-groups randomly by using the following website *www.random.org*. The first sub-group of each group received 500 μL of *L. reuteri* (DSM17938 & ATCC PTA5289) in their diet and the second sub-group received nothing. There were six sub-groups as follow (Fig. 4):

- CG: liquid nutrition with *L. reuteri* (n=4); liquid nutrition without *L. reuteri* (n=4)
- LAG: 3.1% alcohol diet with *L. reuteri* (n=4); 3.1% alcohol diet without *L. reuteri* (n=4)
- HAG: 6.2% alcohol diet with *L. reuteri* (n=3); 6.2% alcohol diet without *L. reuteri* (n=3)

Probiotic Preparation Two vials of *L. reuteri* DSM17938 and two vials with *L. reuteri* ATCC PTA5289 were received from BioGaia Biologics Inc. and were stored at 4°C. Before addition to the diets, the probiotics were propagated as follow:

- A sample of each strain was inoculated in MRS broth (Mann-Rogosa-Sharp) and incubated at 37°C for 24 hrs then was cultured on MRS agar and incubated at 37°C for an additional 48 hrs. Agar plates were incubated in anaerobic bags

- One colony from each strain was inoculated in 4 mL of MRS broth, and incubated at 37°C for 18 hrs. Then, the 4 mL of MRS broth was transferred to 40 mL of MRS broth, and incubated for an additional 18 hrs at 37°C
- Four mL of the incubated samples were mixed with 40 mL of MRS broth and incubated for 18 hrs at 37°C to be used for the next day. The remaining of the samples was centrifuged at 3500 rpm for 10 minutes, and discarded the supernatant. The pellet was resuspended in 6 mL distilled water. To determine the dose of each strain, 2 μL of each strain were diluted in 998 μL of sterile water, and a 2 μL aliquot was plated in MRS plates and incubated for 24 hrs at 37°C. Therefore, the dose of each strain was DSM17938 2×10^8 CFU/day and ATCC PTA5289 2×10^8 CFU/day. Later, the 2 strains were mixed together before being added to the diet

The optical density (590 nm) of the probiotic samples was measured daily to ensure that the same amount was mixed in the diet (Fig. 5).

In our study, a blend of two different strains were used: *L. reuteri* DSM17938 & ATCC PTA5289. *L. reuteri* DSM17938 was used to support the immune system and ATCC PTA5289 to support the oral health.

The first strain, DSM17938, originally called *L. reuteri* SD2112, was isolated from the breast milk of a young mother and deposited in ATCC (American Type Culture Collection).[56] The second strain, ATCC PTA5289 was isolated from the oral cavity.[58]

Testing the Probiotic in the Diet Before being added to the diet, the probiotics were tested to ensure that they would withstand the 3 types of diets (control diet, low alcoholic diet, and high alcoholic diet). One mL of each strain (from the 6 mL stock in distilled water) was added to 50 mL of each type of diet. Tubes were kept at room temperature for

24 hrs. In addition, an aliquot was removed with a sterile loop, inoculated in MRS agar and incubated for 24 hrs at 37°C. As shown in (Table 1), neither the liquid diet nor the alcohol content inhibited the growth of both strains of *L. reuteri.*

4. Periodontal Parameters

Experimental Teeth The four experimental teeth that were examined are upper right central incisor, upper left first molar, lower left central incisor, and lower right first molar.

Time Period of Measuring the Outcomes The periodontal parameters and microbiological analysis were measured at different times during the study:

- Baseline: 2 days after starting the study and consuming regular liquid diet
- Phase I: 8 weeks of consuming alcohol in liquid diet
- Phase II: 10 weeks (i.e., after 2 weeks of probiotics intake)

Before measuring the outcomes, rats were anesthetized with Isoflurane for 2 minutes, and then injected with 0.6 mL of an anesthesia mix (5 mL of Ketamine + 2.2 mL of Xylazine) that was administered intraperitoneally (i.p.). A single blinded investigator evaluated the following parameters:

- Plaque Index (PI) Supragingival plaque on the tooth four surfaces (buccal, lingual, mesial, and distal) was measured by passing sterile probe across the tooth surfaces, then scored using published criteria as 0 = no plaque in the gingival area, 1 = a film of plaque adhering to the free gingival margin and adjacent area of the tooth, 2 = moderate accumulation of soft deposits within the gingival pocket, on the gingival margin and/or adjacent tooth surface, which can be seen by the naked eye, and 3 =

abundance of soft matter within the gingival pocket and/or on the gingival margin and adjacent tooth surface.[59] Each surface was given a score from 0 - 3. The scores from the four surfaces of the tooth were added and divided by four to get the PI for the tooth. Then we added the PI for the four experimental teeth and divided it by four to get the PI for the rat.

- Bleeding on Probing (BOP) It was measured from four surfaces (buccal, lingual, mesial, and distal) from each experimental tooth by placing a sterile probe (tip diameter 0.45 mm) into the gingival sulcus, until tissue resistance, for 10 seconds, then score as 1 for bleeding and 0 for no bleeding.[60] Then, the percentage was calculated by adding the numbers of bleeding sites divided by the numbers of sites evaluated * 100. The severity of gingivitis is reported as percentage.
- Gingival Index (GI) was assessed based on a combination of symptoms including redness and swelling as well as presence of bleeding on probing and registered as 0 = normal gingiva; 1 = mild inflammation: slight change in color, slight edema, no bleeding on probing; 2 = moderate inflammation: redness, edema, glazing, bleeding on probing; and 3 = severe inflammation: marked redness, edema, ulceration, tendency to spontaneous bleeding.[59] Each surface was given a score from 0 - 3. The scores from the four surfaces of the tooth were added and divided by four to get the GI for the tooth. The GI index for the rat was obtained by summing the indices for all experimental teeth and divided by four.

5. Microbiological Analysis

a. Subgingival Plaque Collection Microbiological samples were collected at

baseline, 8 weeks, and 10 weeks. Subgingival bacteria were collected from one site (buccal site) of each of the four experimental teeth. Subgingival bacteria sampling were performed after measuring the periodontal parameters. Prior to sampling, the sites were isolated, dried and any supragingival deposits were removed with sterile cotton pellets. After the sample site was isolated by retracting the cheek and tongue, subgingival bacteria was collected using the paper point technique.[7] Sterile fine absorbent paper points were inserted gently using sterile dental tweezers into gingival sulcus until tissue resistance was met. After placement for 20 seconds, the four paper points from each rat were removed carefully without touching the adjacent tissues and placed into a microfuge tube containing 100 µL of sterile water. All samples were taken by the same investigator in order to standardize the sampling procedure.

b. Subgingival Plaque Samples Processing Samples were processed immediately after collection. The vials were mixed by vigorous vortexing for 20 seconds. A 2 μL aliquot was plated onto blood agar plates, containing pancreatic digest of casein 14.5g/L, papaic digest of soybean meal 5 g/L, sodium chloride 5 g/L, agar 14 g/L, growth factors 1.5 g/L, and defibrinated sheep blood 5%. All plates were incubated in a standard incubator at 37°C for 48 hrs. After incubation, the numbers of colony-forming units per 100 μL (CFU/100μL) were determined.

6. Data Presentation and Statistical Analyses

Data is presented as means (SD) for the microbiological and body weight data or medians (IQ) for the periodontal parameters. Data were analyzed using SAS software package version 9.2 or GraphPad Prism software package version 5.0. Wilcoxon signed

rank test (non-parametric test) was used for phase I data (after alcohol consumption) and Mann-Whitney U test (non-parametric test) for phase II data (after probiotic intake) to analyze the PI, BOP and GI scores because the data were ordinal. The normal distribution for the bacterial colony counts data and body weight was assessed using the Q-Q plot. The data were normally distributed, so paired *t*-test was used to analyze the bacterial colony counts data in phase I and two samples *t*-test in phase II. Repeated measures analysis was performed for the body weight data. p values < 0.05 were considered to be significant.

IV. RESULTS

Effect of Alcohol Intake on Plaque Index (PI)

Intra-group Comparison Wilcoxon signed rank test showed no significant difference of PI score in control group and low alcohol group after consuming liquid diet and alcohol compared to baseline ($p > 0.05$). In contrast, there was a statistically significant difference of PI score in high alcohol group after 8 weeks of alcohol consumption [median (IQ): 0.06 (0.09)] compared to baseline [median (IQ): 0 (0)] ($p = 0.008$) (Table 2).

Inter-group Comparison Mann-Whitney U test showed that PI was statistically significantly different in high alcohol group compared to control group ($p = 0.004$). The PI scores were not statistically significantly different when comparing the control group to the low alcohol group and the low alcohol group to the high alcohol group ($p > 0.05$) (Table 6).

Effect of Alcohol Intake on Bleeding on Probing (BOP)

Intra-group Comparison Wilcoxon signed rank test showed no significant difference ($p > 0.05$) in BOP percentage in the control group after liquid diet consumption [median (IQ): 6 (0)] compared to baseline [median (IQ): 12.5 (20.56)]. In the low alcohol group, although there were more sites with positive BOP after alcohol consumption [median (IQ): 12.5 (9.56)] compared with the baseline [median (IQ): 9.25 (11)], the difference was not statistically significant ($p = 0.057$). Comparing the median percentage of BOP in the high alcohol group [median (IQ): 15.63 (6.25)] to the baseline

[median (IQ): 6 (6.5)], we found a statistically significantly increase in BOP (p = 0.035) (Fig. 7) (Table 3).

Inter-group Comparison Mann-Whitney U test showed that BOP was statistically significantly different between the low alcohol group and control group (p = 0.003), and between the high alcohol group and control group (p = 0.002). However, when comparing the high alcohol group to the low alcohol group the difference was not statistically significant (p = 0.162) (Table 6).

Effect of Alcohol Intake on Gingival Index (GI)

Intra-group Comparison Wilcoxon signed rank test showed that there was a significant difference (p = 0.028) in GI score in the control group after liquid diet consumption [median (IQ): 0.12 (0)] compared to baseline [median (IQ): 0.25 (0.5)]. In contrast, animals in the low and high alcohol groups had several sites with BOP and slight redness on the gingival margin after alcohol consumption [median (IQ): 0.25 (0.15) and 0.37 (0.25), respectively] compared to baseline [median (IQ): 0.12 (0.18) and 0.12 (0.13), respectively] (p = 0.020 and p = 0.021, respectively) (Fig. 8) (Table 4).

Inter-group Comparison Mann-Whitney U test showed that GI scores were statistically significantly different between the low alcohol group and control group (p = 0.001) and between the high alcohol group and control group (p = 0.001). However, when comparing the high alcohol group to the low alcohol group the GI scores were not statistically significantly different (p = 0.48) (Table 6).

Effect of Alcohol Intake on Subgingival Bacterial Count

Intra-group Comparison The paired *t*-test showed no statistically significant differences ($p = 0.962$) in the mean of subgingival bacterial count (CFU/100 μL) in the control group [mean (SD) at baseline: 1650 (1289) and after liquid diet consumption: 1669 (1801)]. In the low alcohol group, the mean subgingival bacterial count (CFU/100 μL) was almost the same at baseline and after alcohol consumption [mean (SD): 2050 (1187) and 1931 (2224), respectively] and it was not statistically significant ($p = 0.875$). In the high alcohol group, the mean bacteria colony count (CFU/100 μL) was less than half compared to baseline; at baseline [mean (SD): 1713 (1053)] and after alcohol consumption [mean (SD): 756.3 (813)] and it was not statistically significant ($p = 0.052$) (Fig. 10) (Table 5).

Inter-group Comparison Two Sample *t*-test showed that the subgingival bacterial count (CFU/100 μL) was not significantly different between the low alcohol group and the control group ($p = 0.800$), the high alcohol group and control group ($p = 0.256$), and the high alcohol group and low alcohol group ($p = 0.313$) (Table 6).

Effect of Alcohol Intake on Body Weight

Repeated measures analysis showed that after 8 weeks of chronic alcohol consumption, the body weight of the animals in the high alcohol group was significantly lower than the control group and the low alcohol group ($p = 0.0030$ and $p < 0.0001$, respectively) (Fig. 11).

Effect of *L. reuteri* Intake on plaque Index (PI)

Mann-Whitney U test showed no statistically significant difference in PI score in the *L. reuteri* groups compared to the non *L. reuteri* groups in all 3 groups ($p > 0.05$) (Table 2).

Effect of *L. reuteri* Intake on Bleeding on Probing (BOP)

In the control group, Mann-Whitney U test showed no significant differences in the BOP percentage ($p > 0.05$) between the non *L. reuteri* and *L. reuteri* groups. In the low alcohol group, the BOP was statistically less ($p = 0.048$) in the *L. reuteri* group [median (IQ): 3 (6)] than the non *L. reuteri* group [median (IQ): 12.5 (4.87)]. Although animals in the high alcohol group with *L. reuteri* had decreased BOP [median (IQ): 6 (12.5)] compared to those without *L. reuteri* [median (IQ): 12.5 (6.25)], it was not statistically significant ($p = 0.164$) (Fig. 7) (Table 3).

Effect of *L. reuteri* Intake on Gingival Index (GI)

Mann-Whitney U test showed no significant difference in the GI score in both groups of the control group ($p > 0.05$). In the low alcohol group, the GI score in animals with *L. reuteri* [median (IQ): 0.06 (0.12)] showed significant less redness and bleeding compared to low alcohol group without *L. reuteri* [median (IQ): 0.25 (0.09)] ($p = 0.048$). The GI score in the high alcohol group with *L. reuteri* [median (IQ): 0.12 (0.25)] was less than non *L. reuteri* group [median (IQ): 0.25 (0.12)] but it was not statistically significant ($p = 0.164$) (Fig. 8) (Table 4).

Effect of *L. reuteri* Intake on Subgingival Bacterial Count

In the control group, two samples *t*-test found no significant difference (p = 0.443) by comparing the mean bacterial colony count (CFU/100 μL) of the control group with *L. reuteri* [mean (SD): 1113 (787.8)] to the control group without *L. reuteri* [mean (SD): 2488 (3260)]. In the low alcohol group, the mean bacteria colony count (CFU/100 μL) was slightly higher in the *L. reuteri* group [mean (SD): 875 (499.2)] than the non *L. reuteri* group [mean (SD): 662.5 (585.1)] but was not statistically significant (p = 0.60). In the high alcohol group, the mean bacteria colony count was almost the same in the non *L. reuteri* and the *L. reuteri* group [mean (SD): 283.3 (208.2) and 433.3 (160.7), respectively] and it was not statistically significant (p = 0.379) (Fig. 10) (Table 5). The presence of *lactobacilli* in the saliva and subgingival plaque was not assessed in this study.

Effect of *L. reuteri* Intake on Body Weight

Repeated measures analysis showed that after 8 weeks of alcohol intake, animals in the high alcohol group lost weight when compared to the control group and the low alcohol group (p = 0.003 and p < 0.0001, respectively) (Fig. 11).

V. DISCUSSION

The data of the present study shows that there is a deleterious relationship between chronic alcohol consumption and periodontal disease. The GI score showed that low alcohol group and high alcohol group established gingivitis compared to control group. Although BOP showed a statistically significant trend when using Wilcoxon signed rank test, the relationship between BOP and low alcohol group was not statistically significant whereas it was statistically significant in the high alcoholic group. The results of this study were consistent with those reported by Tezal et al.[18] He suggested in a cross-sectional study of 1,371 subjects utilizing a self-reported questionnaire that the strongest effect of alcohol seems to be on the gingiva, followed by periodontal ligament and lastly on alveolar bone.

Although plaque is the cause of periodontal disease, it had no-significant effect on periodontal disease in our study. There was no difference in the amount of plaque between the different groups. This effect could partly be due to the fact that in our study the rats were fed a liquid diet; therefore, the amount of plaque accumulation was negligible.

In our study, we found a dose-dependent effect of alcohol consumption on gingivitis. In BOP scores, the median of the low alcoholic group after alcohol consumption [median (IQ): 12.5 (9.56)**]** was lower than the median of the high alcohol group after alcohol consumption [median (IQ): 15.63 (6.25)]. Also, in GI scores, the median of the low alcohol group after alcohol consumption [median: 0.25 (0.15)] was less than the median of the high alcohol group after alcohol consumption [median: 0.37 (0.25)]. Therefore, our data suggest that there was a dose-dependent relationship between

chronic alcohol consumption and periodontal disease. There are two studies that showed similar findings to ours. Tezal et al. reported after controlling for major confounders that self-reported alcohol consumption was related to CAL in a dose-dependent manner, with ORs of 1.36 [1.02–1.80] and 1.44 [1.04–2.00] for ≥ 5 and ≥10 drinks/week, respectively.[18] Another study utilized three groups of rats, which received a water diet, 10% alcohol diet, and 20% alcoholic diet, a dose-dependent effect of alcohol on alveolar bone loss in experimental periodontitis was observed.[27]

There are some studies which claimed that the effect of alcohol consumption on oral tissuet is simply due to poor oral hygiene and self neglect.[23, 61] However, alcohol may also affect periodontal tissues through different mechanisms explained by biological plausibility. First, alcohol has an adverse effect on the host defense. It is associated with impairing neutrophils, phagocytic monocytes and macrophages, T-cell functions which increases the frequency of infections.[14] Second, alcohol alters bone metabolism. It increases bone resorption and decreases bone formation.[16] Third, alcohol has a toxic effect on the liver.[17] It may disrupt the production of prothrombin, vitamin K activity, and clotting mechanisms which might lead to hemorrhage.[18] Finally, alcohol may interfere with protein metabolism which affects tissue healing.[16]

Due to the fact that alcohol is part of the daily caloric intake that is provided by the alcoholic drinks, its consumption reduces the human body weight.[62] In the present study, 3.1% of alcohol for low alcohol group and 6.2% of alcohol for high alcohol group provided18% and 36% of the total energy of the diet, respectively. Rats' body weights were measured every 3 - 4 days and it was found that chronic alcohol consumption in the high alcohol group led to a significant loss in body weight compared to control group and

low alcohol group. Souza et al. evaluated the relationship between eight weeks of alcohol consumption and rats' body weights after consumption of 5.80 g/kg/day for the 10% ethanol group and 10.74 g/kg/day for the 20% ethanol group and reported no statistically significant difference between baseline and final weight in the rats receiving 20% ethanol in a liquid diet.[27] However, the impact of alcohol consumption on weight is infrequently observed in rats.[63]

In the present study we also investigated the potential of the probiotic *L. reuteri* to counteract the deleterious effects of alcohol on periodontal health by measuring the periodontal and microbiological parameters. We found that the oral administration of *L. reuteri* DSM17938 & ATCC PTA5289 significantly decreased the BOP and GI scores in the low alcoholic group, suggesting that probiotic intervention could prevent or reverse the effect of chronic alcohol consumption on periodontal health. The beneficial effect of *L. reuteri* on periodontal inflammation could be either due to the secretion of bacteriocins, reuterin and reutericyclin, which inhibit the growth of a wide variety of pathogens; or, the strong capacity that probiotics have to adhere to host tissues and compete with pathogenic bacteria; and lastly, the ability to inhibits secretion of proinflammatory cytokines.[3]

Several studies reported the positive effect of probiotics on systemic health after Elie Metchnikoff presented the first scientific explanation of the beneficial effects of lactic acid produced by *lactobacilli* in the gastrointestinal tract.[31, 32] Nikawa et al. suggested that bovine milk fermented by *L. reuteri* may help decrease the risk of dental caries by reducing the oral carriage of *Streptococcus mutans*.[64] Moreover, probiotic bacteria are effective in controlling oral yeast infection.[65] Krasse et al. concluded that

administration of probiotic *L. reuteri* reduced plaque and gingivitis.[44] In addition, a randomized controlled trial confirmed the plaque inhibition, anti-inflammatory, and antimicrobial effects of *L. reuteri* prodentis and suggested that probiotic *L. reuteri* could be an alternative to periodontal treatment when scaling and root planing are contraindicated.[66] Mayangi et al. reported that administration of oral tablets containing *Lactobacillus salivarius* WB21 to healthy volunteers without severe periodontitis reduced the numerical sum of five selected periodontopathic bacteria in subgingival plaque.[67]

We used a combination of two different strains DSM17938 & ATCC PTA5289. DSM17938 derived from a mother's milk and ATCC PTA5289 isolated from the oral cavity.[56, 58] The aim of this mixture is to provide the benefit of oral and systemic immuno-modulating defense mechanisms. Twetman et al. used chewing gum containing *L. reuteri* in two different strains ATCC 55730 and ATCC PTA 5289 daily for 2 weeks to adults with moderate levels of gingivitis. He reported a decrease in gingival bleeding on probing, gingival crevicular fluid volume and the levels of the proinflammatory cytokines tumor necrosis α (TNFα) and interleukin-8 (IL-8).[45]

In the control group, low alcohol group, and high alcohol group, the BOP and GI scores were reduced in the probiotic subgroups but it did not reach statistical significance in the control group and high alcohol groups. It is possible that the 2-week probiotic supplement was not long enough to have significant effects and longer treatment durations are warranted.

Although the subgingival bacterial count (CFU) in the control group + *L. reuteri* showed a trend compared to the control group – *L. reuteri*, it was not statistically significant. The inhibitory activity displayed by *L. reuteri* against subgingival bacaeria

was principally related to the production of acid such as reuterin.[3] There were diminished counts in the subgingival bacteria in the low and high alcohol groups with or without *L. reuteri*. This could be because chronic alcohol consumption killed oral bacteria that may effects also the beneficial bacteria. In our study, the mechanisms of reduced subgingival bacterial count have not been fully clarified.

There are certain limitations of this study that should be kept in mind. First, the study was conducted on animals and as such extrapolation to the human condition warrants further investigation. Although the embryonic development in animals is closely related to that of humans and can be used to study the causes of human diseases and efficiency of treatments with predictive validity, animal studies do not reliably predict human outcomes.[49] Second, after chronic alcohol consumption, the role of systemic inflammation and inflammatory cytokines in alcohol-induced gingivitis remain to be determined.

VI. CONCLUSIONS

In the present study, we evaluated the effect of chronic alcohol consumption, in high and low doses, on periodontal health and investigated the potential effect of probiotic *L. reuteri* to counteract the effect of alcohol. The study results suggested that chronic alcohol consumption is a risk factor for gingivitis and the relationship found to be dose dependent. Furthermore, *L. reuteri* supplements could be a useful strategy to prevent or treat gingival inflammation caused by chronic alcohol consumption. That was apparent in the low alcohol group as the BOP and GI scores were significantly decreased after probiotic intervention.

Clinical Implications

Since chronic alcohol consumption is a risk factor for periodontal disease, dental practitioners should enhance knowledge of alcoholic patients and develop clinical strategies for earlier detection to prevent periodontal disease progression. Moreover, as our study results showed that probiotic *L. reuteri* counteracted the effect of alcohol, intake of probiotics dietary supplement could be recommended for chronic alcohol drinker.

Future Studies

In this study, we evaluated the effect of alcohol on periodontal health by measuring the periodontal parameters and microbiological analysis. As alcohol has an adverse effect on host defense, further studies are needed to elucidate the cellular mechanisms/mediators involved in alcohol-induced gingivitis with emphasis on inflammatory cytokines. In addition, clinical trials over longer periods of time are needed to build up strong evidence for alcohol and probiotics effect on periodontal health.

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Table 1. Effect of liquid diet and alcohol on *L. reuteri* growth.

	MRS Broth	Control Diet	Low Alcohol Diet	High Alcohol Diet
DSM17938	0.24 (0)	0.33 (0)	0.33 (0.05)	0.37 (0)
ATCC PTA5289	0.25 (0)	0.36 (0.02)	0.34 (0.01)	0.32 (0.01)

One mL of each strain was added to 50 mL of MRS broth, control diet, low alcoholic diet, or high alcoholic diet; and incubated at room temperature for 24 hrs. The O.D. (590 nm) was determined and is expressed as means (SD). The liquid diet and alcohol did not affect the growth of either strains of *L. reuteri*.

Table 2. Plaque index intra-group comparison.

	Plaque Index (PI)						
	Phase I				Phase II		
	Baseline	8 Weeks	Differences	*P	10 Weeks		**P
					- L. reuteri	+ L. reuteri	
Control Group	0 (0)	0 (0)	0 (0)	1	0 (0)	0 (0)	1
Low Alcohol Group	0 (0)	0 (0.06)	0.0 (0.06)	1	0 (0)	0 (0)	1
High Alcohol Group	0 (0)	0.06 (0.09)	0.06 (0.09)	0.008	0 (0.04)	0 (0)	1

Data represent medians (IQ) of PI score. * Wilcoxon signed rank test ** Mann-Whitney U test

Table 3. Bleeding on probing intra-group comparison.

	Bleeding on Probing (BOP)						
	Phase I				Phase II		
	Baseline	8 Weeks	Differences	*P	10 Weeks		**P
					- L. reuteri	+ L. reuteri	
Control Group	12.5 (20.56)	6 (0)	-6.5 (-20.56)	0.061	0 (4.5)	6 (2)	0.057
Low Alcohol Group	9.25 (11)	12.5 (9.56)	6.12 (6.43)	0.057	12.5 (4.87)	3 (6)	0.048
High Alcohol Group	6 (6.5)	15.63 (6.25)	9.5 (11.12)	0.035	12.5 (6.25)	6 (12.5)	0.164

Data represent medians (IQ) of BOP %. * Wilcoxon signed rank test ** Mann-Whitney U test

Table 4. Gingival index intra-group comparison

	Gingival Index (GI)						
	Phase I				Phase II		
					10 Weeks		
	Baseline	8 Weeks	Differences	*P	- L. reuteri	+ L. reuteri	**P
Control Group	0.25 (0.5)	0.12 (0)	-0.12 (0.55)	0.028	0.18 (0.3)	0 (0.09)	0.054
Low Alcohol Group	0.12 (0.18)	0.25 (0.15)	0.15 (0.06)	0.020	0.25 (0.09)	0.06 (0.12)	0.048
High Alcohol Group	0.12 (0.13)	0.37 (0.25)	0.18 (0.21)	0.021	0.25 (0.12)	0.12 (0.25)	0.164

Data represent medians (IQ) of GI score. * Wilcoxon signed rank test ** Mann-Whitney U test

Table 5. Bacterial colonies count intra-group comparison.

	Bacterial Colonies Count (CFU/100 µL)						
	Phase I				Phase II		
	Baseline	8 Weeks	Differences	*P	10 Weeks		**P
					- L. reuteri	+ L. reuteri	
Control Group	1650 (1289)	1669 (1801)	-293.8 (1082)	0.962	2488 (3260)	1113 (787.8)	0.443
Low Alcohol Group	2050 (1187)	1931 (2224)	-81.25 (2069)	0.875	662.5 (585.1)	875 (499.2)	0.600
High Alcohol Group	1713 (1053)	756.3 (813)	-956.3 (1157)	0.052	433.3 (160.7)	283.3 (208.2)	0.379

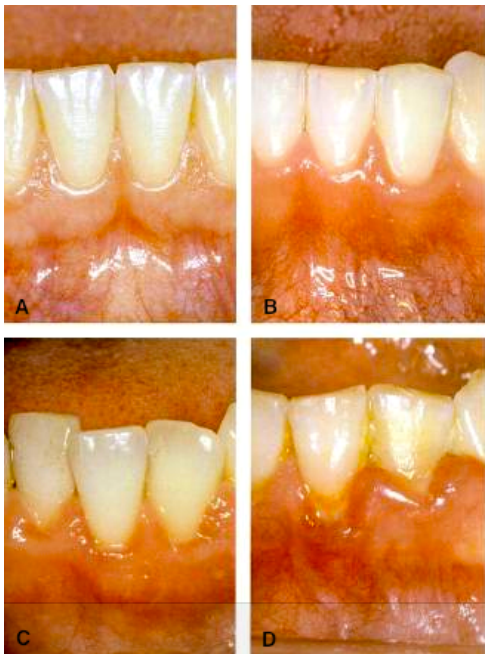
Data represent means (SD) of CFU/100 µL. * Paired *t*-test ** Two samples *t*-test

Table 6. Inter-group comparison after 8 weeks of alcohol consumption.

	*PI	*BOP	*GI	**Bacterial Count
CG vs LAG	0.053	0.003	0.001	0.800
CG vs HAG	0.004	0.002	0.001	0.256
LAG vs HAG	0.095	0.162	0.480	0.313

Data represent Inter-group *p* value. * Mann-Whitney U test ** Two samples *t*-test

I.



II.

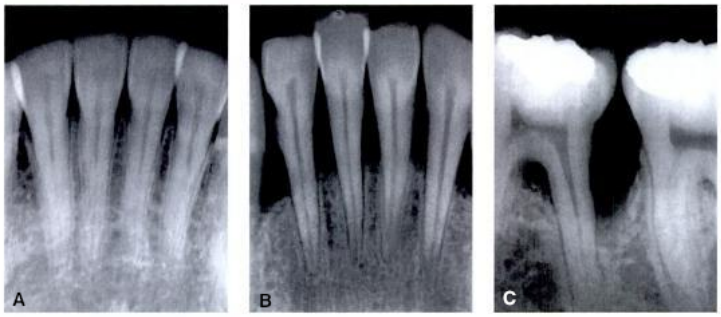


Figure 1. I: The stages of gingivitis. **A.** Healthy gingiva. **B.** Mild gingivitis. **C.** Moderate gingivitis. **D.** Severe gingivitis. **II:** Types of bone loss. **A.** No attachment loss. **B.** Horizontal bone loss. **C.** Vertical bone boss. (H. F. Wolf, T. M. Hassell. Color atlas of dental hygiene: Periodontology. 2006)

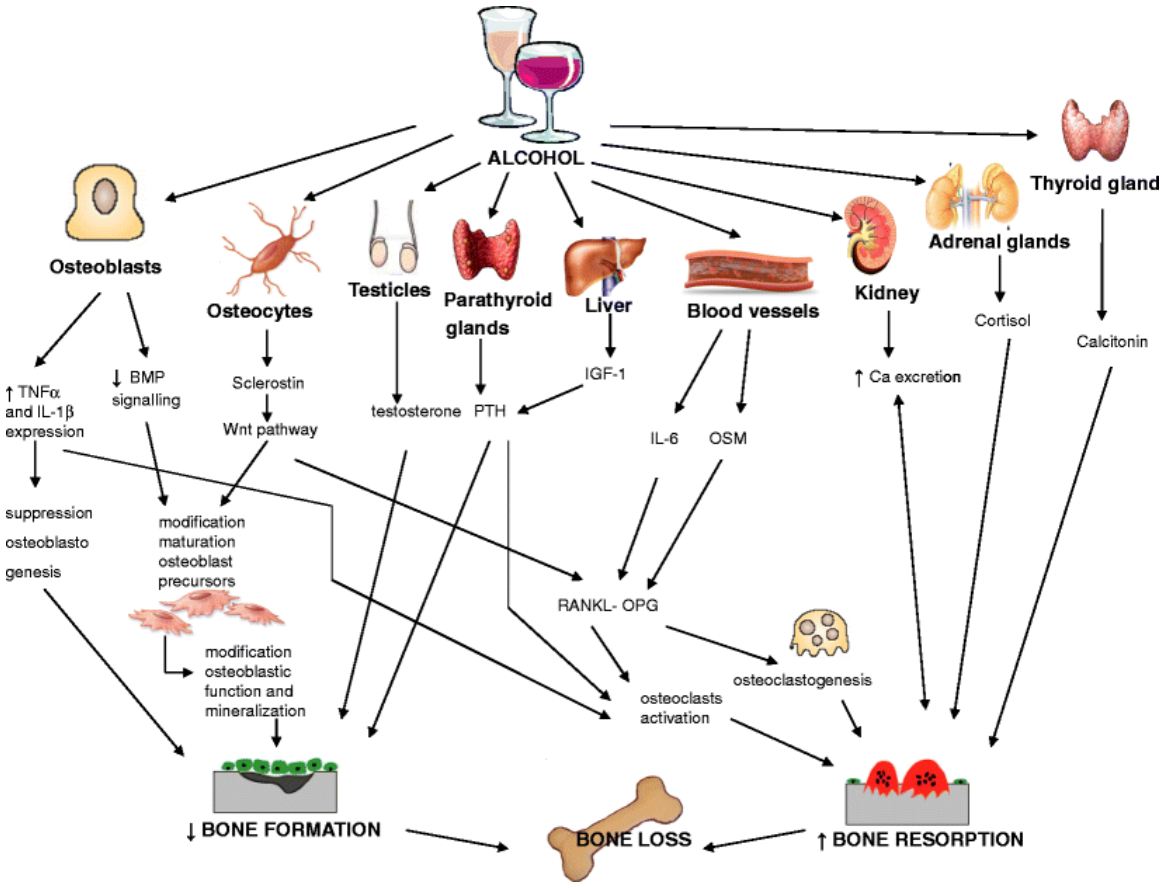


Figure 2. Mechanisms which may be implicated in bone loss due to heavy chronic alcohol consumption. (Maurel et al., Osteoporos. Int. 2012;23(1):1-16)

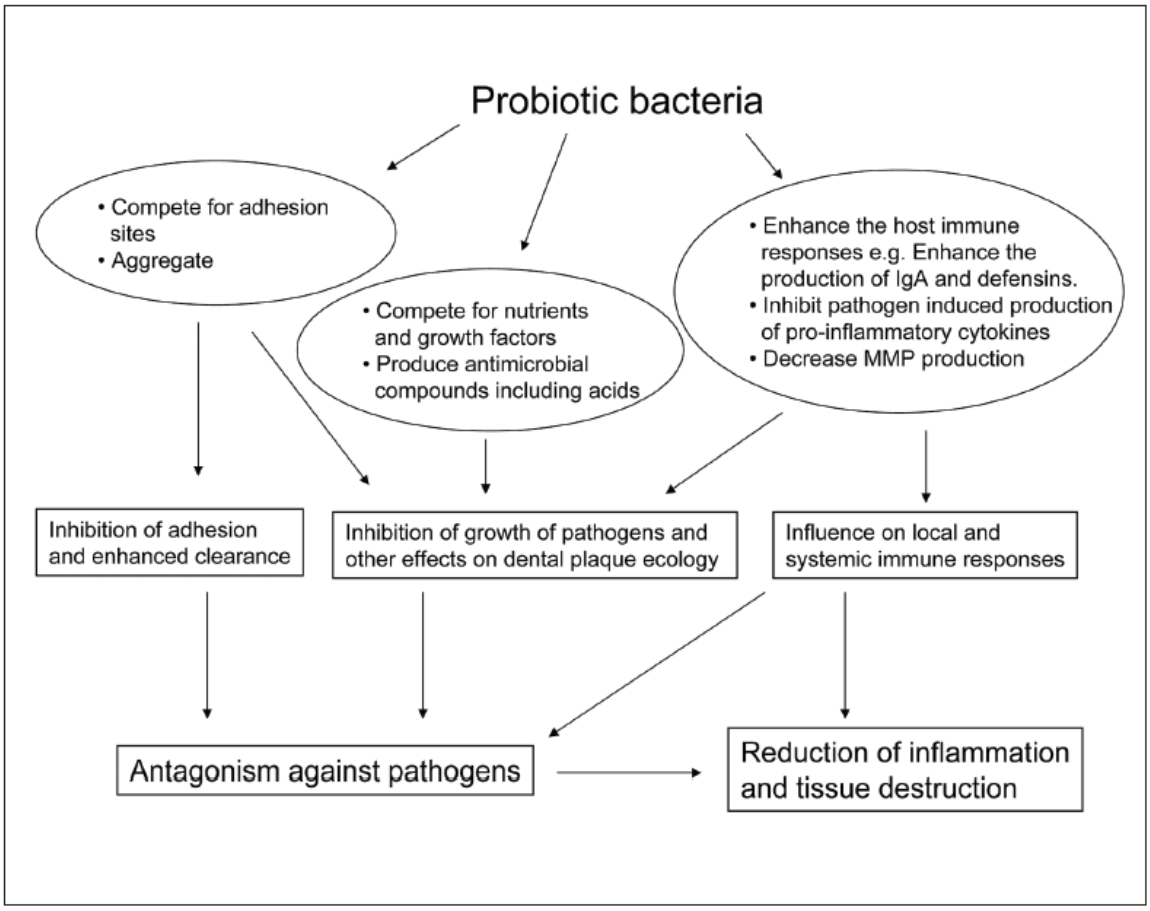


Figure 3. Potential mechanisms by which probiotic bacteria could affect oral health.
(Anna Haukioja, Eur J Dent 2010;4:348-355)

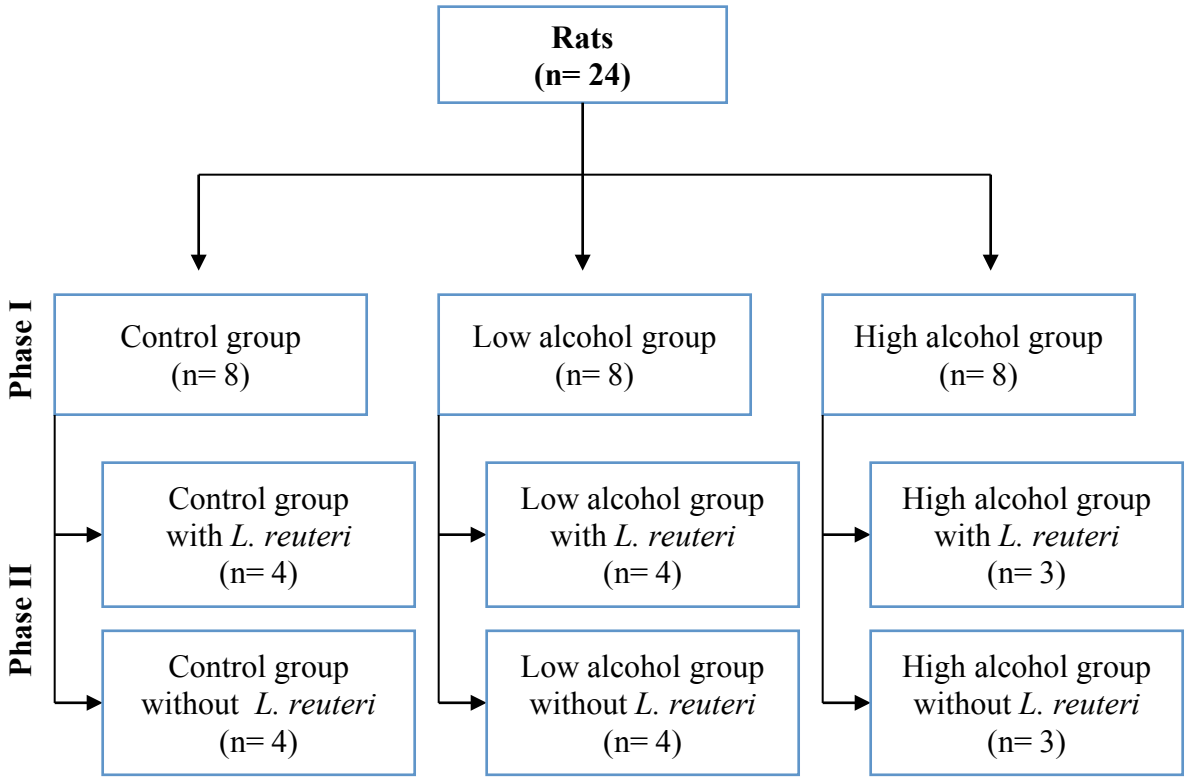


Figure 4. Study flowchart.

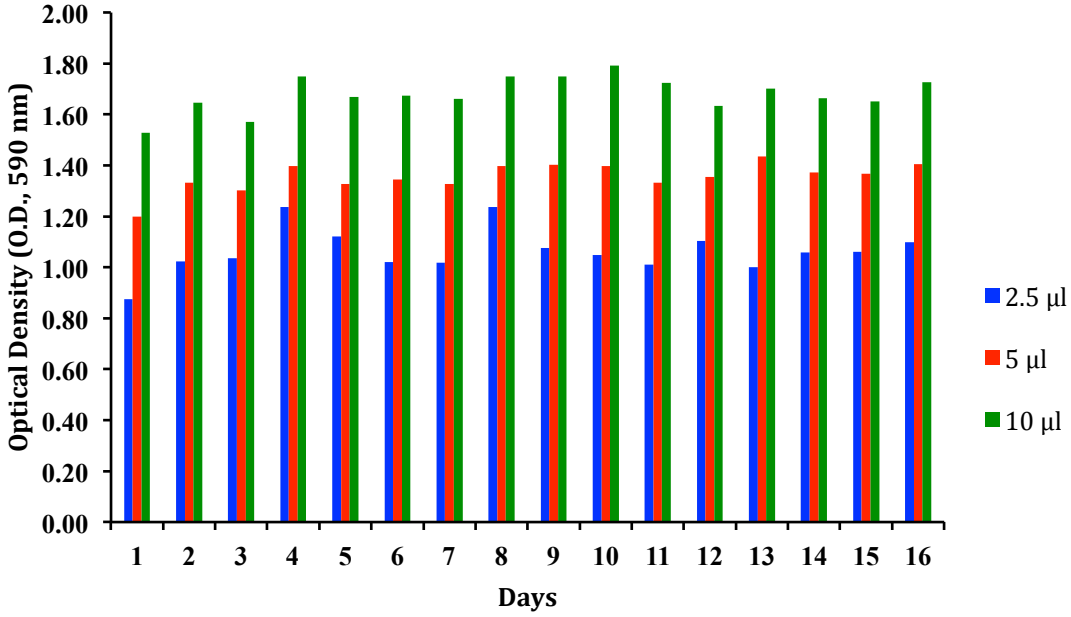


Figure 5. Daily optical density (O.D.) measurements of *L. reuteri*.
The O.D. of 2.5 *µL*, 5 *µL*, and 10 *µL* of *L. reuteri* (both strains combined) were measured daily showing that the amounts were constant.

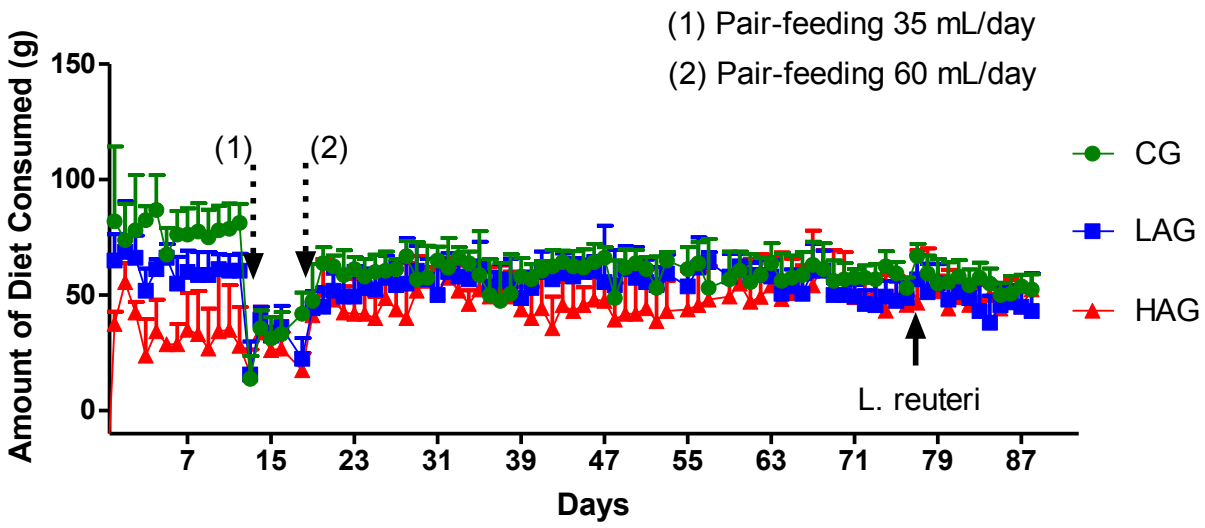


Figure 6. Daily diet weight during the study period. (1) Refers to 35 mL of diet consumed per day, (2) refers to 60 mL of daily consumption of diet. *L. reuteri* supplement was started on day 76.

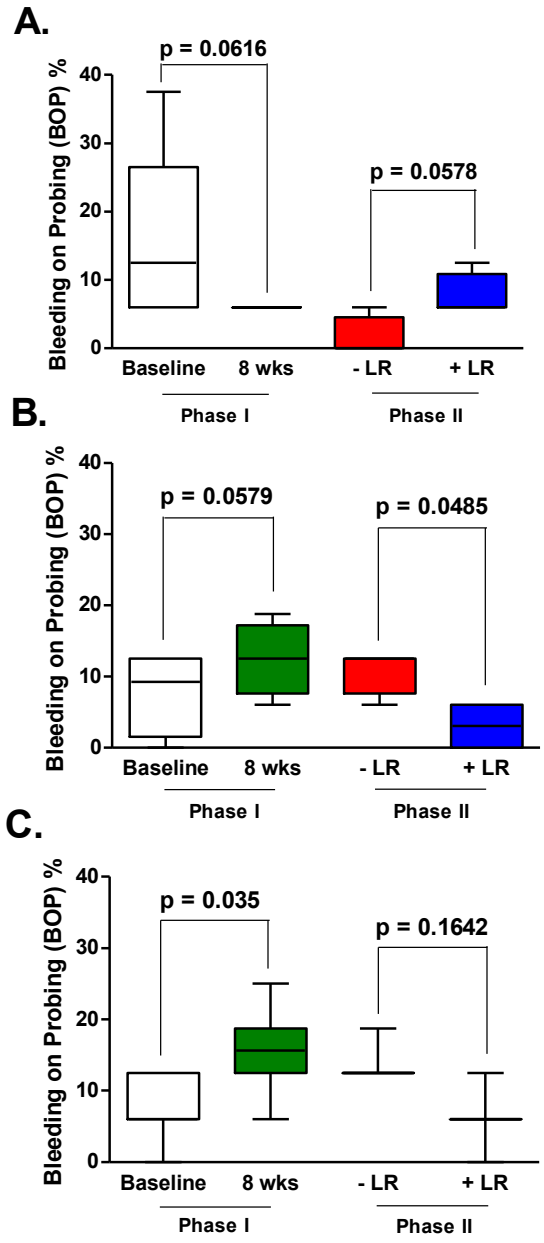


Figure 7. Effect of chronic alcohol consumption and probiotic intake on bleeding on probing. Data are expressed as medians **(**IQ). **A.** Control group (CG), **B.** Low alcohol group (LAG), and **C.** High alcohol group (HAG). BOP increased in both alcohol groups (statistically significantly in the HAG) and was decreased following probiotic intake (statistically significantly in the LAG).

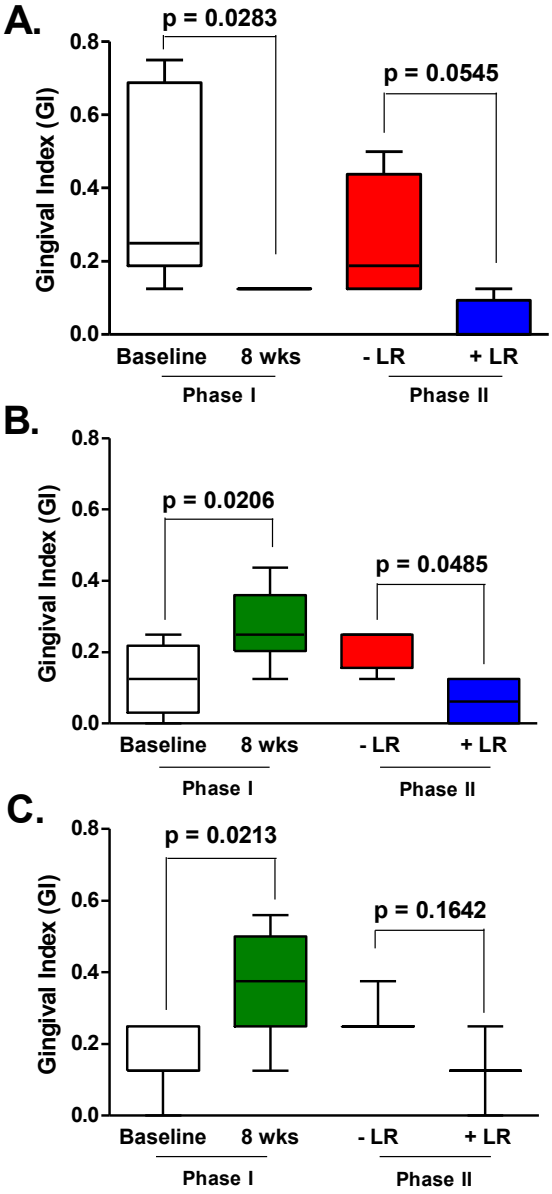


Figure 8. Effect of chronic alcohol consumption and probiotic intake on gingival index. Data are expressed as medians **(**IQ). **A.** Control group (CG), **B.** Low alcohol group (LAG), and **C.** High alcohol group (HAG). GI increased in both alcohol groups (statistically significantly in both) and was decreased following probiotic intake (statistically significantly in the LAG).

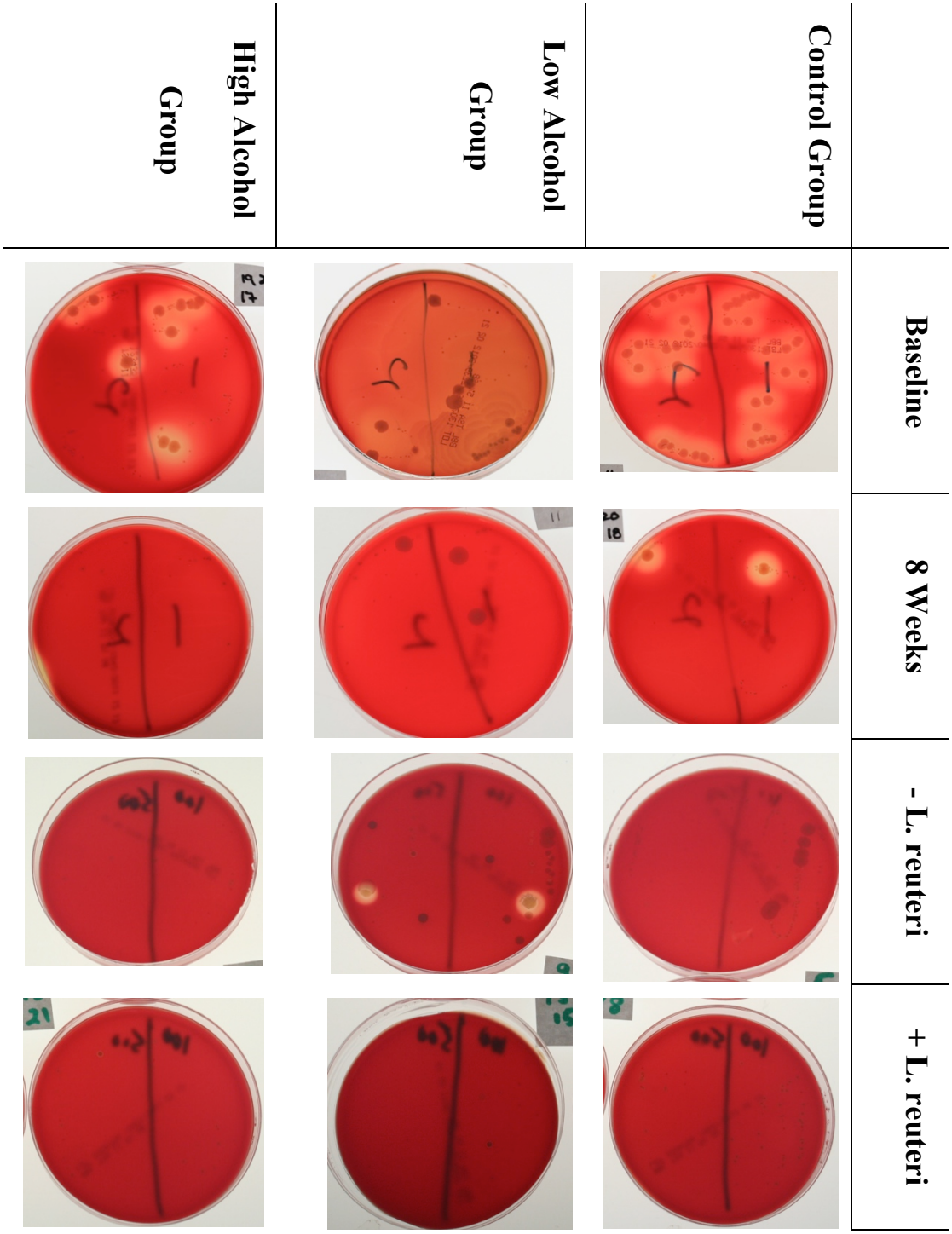


Figure 9. Examples of subgingival bacterial cultures. Samples were collected using the paper point technique, diluted and plated on blood agar plates. Plates were incubated for 24 hrs at 37°C. The number of colonies was counted and is reported in figure 10.

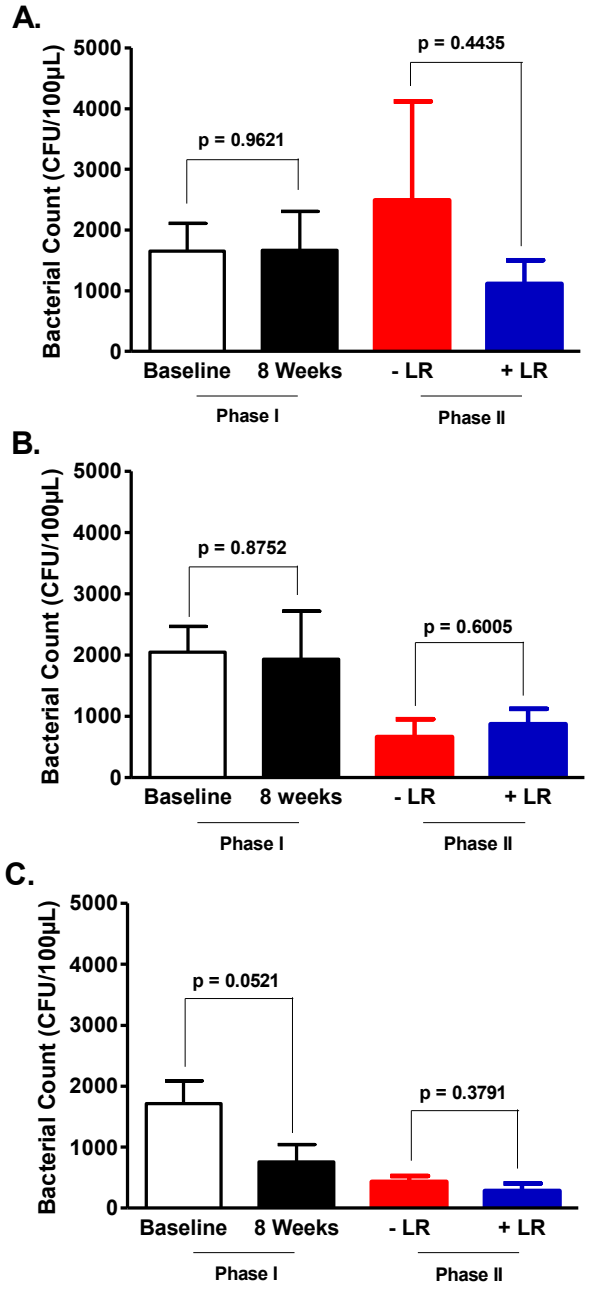


Figure 10. Effect of chronic alcohol consumption and probiotic intake on subgingival bacteria counts. Data are expressed as means **(**SD). **A.** Control group (CG), **B.** Low alcohol group (LAG), and **C.** High alcohol group (HAG).

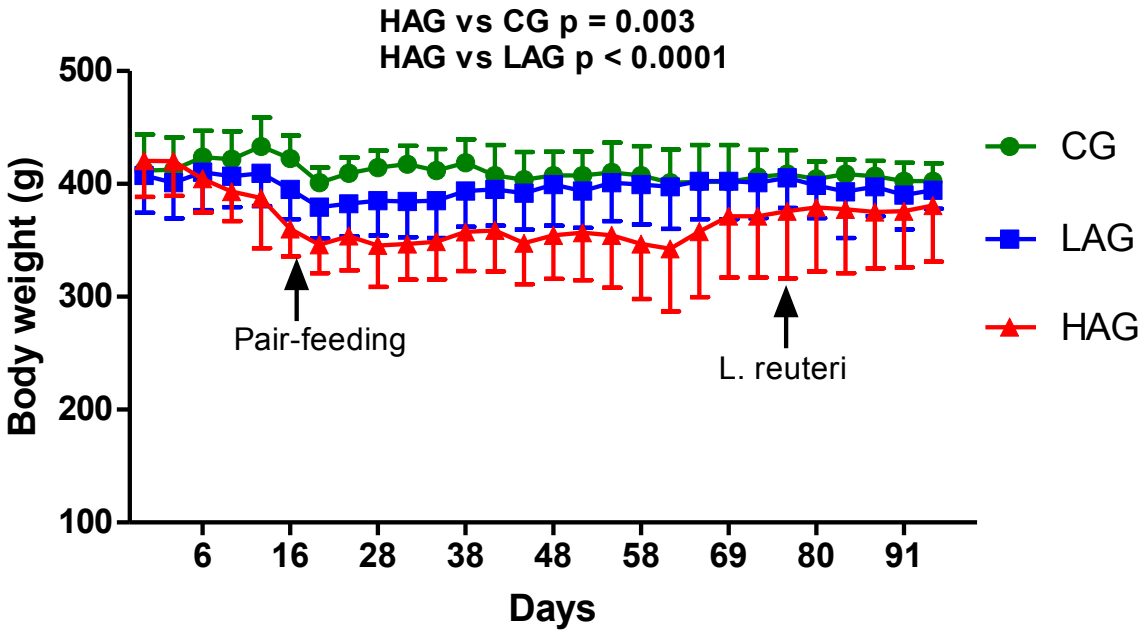


Figure 11. Effect of liquid diet and alcohol consumption on body weight. Data are expressed as means (SD). The body weight in the high alcohol group was significantly lower when compared to the control group ($p = 0.003$) and to the low alcohol group ($p < 0.0001$).

Appendices

Dyets Inc.

2508 Easton A Bethlehem, Pennsylvania 18017
Within 610 Area Code: 868-7701, FAX -868-5170
Outside 610 Area Code: 800-275-3938
FAX: 800-329-3938
E-Mail Address: dyets@enter.net

DYET# 710027

Lieber-DeCarli Regular Control Rat Diet

Ingredient	kcal/gm*	grams/L	kcal/Liter
Casein, (100 Mesh)	4.27	41.4	176.778
L-Cystine	4	0.5	2
DL-Methionine	4	0.3	1.2
Corn Oil	8.84	8.5	75.1
Olive Oil	8.84	28.4	251.056
Safflower Oil	8.84	2.7	23.868
Maltose Dextrin	3.96	115.2	456.192
Cellulose	0	10	0
Mineral Mix #210011	0.47	8.75	4.1125
Vitamin Mix # 310011	3.8	2.5	9.5
Choline Bitartrate	0	0.53	0
Xanthan Gum	0	3	0
		221.78	999.8465

DIRECTIONS: To 221.78 grams of Dyets #710027, Q.S. with cold water to one liter and mix for 30 seconds in a blender.

Lieber and DeCarli, Alcoholism-Clinical and Experimental Research 6, 523-531(1982)

*Based on original Lieber-DeCarli values

NOTE: This diet contains 1.0 Kcal/ml. Of which 35% are fat derived, 47% are derived from carbohydrate, and 18% are derived from protein.

Appendix A. Lieber-DeCarli regular control rat diet ingredient.

Dyets Inc.
2508 Easton A'Bethlehem, Pennsylvania 18017
Within 610 Area Code: 868-7701, FAX -868-5170
Outside 610 Area Code: 800-275-3938
FAX: 800-329-3938
E-Mail Address: dyets@enter.net

DYET# 710260

Lieber-DeCarli Ethanol Rat Diet (Regular)

Ingredient	kcal/gm*	grams/L	kcal/Liter
Casein, (80 Mesh)	4.27	41.4	176.778
L-Cystine	0	0.5	0
DL-Methionine	0	0.3	0
Corn Oil	8.84	8.5	75.14
Olive Oil	8.84	28.4	251.056
Safflower Oil	8.84	2.7	23.868
Maltose Dextrin	3.96	25.6	101.376
Cellulose	0	10	0
Mineral Mix #210011	0.47	8.75	4.1125
Vitamin Mix # 310011	3.8	2.5	9.5
Choline Bitartrate	0	0.53	0
Xanthan Gum	0	3	0
		132.18	641.8305
Ethanol	**5.35**	**67**	358.45
Grand total		**132.18**	1000.2805

DIRECTIONS: To 132.18 grams of Dyets #710260 add Ethanol (67 ml of 95% ethanol) and QS to one liter with cold water and mix for thirty seconds in a blender.

Lieber & DeCarli, Alcohol and Alcoholism 24: 197-211(1989)

*Based on original Lieber-DeCarli values

NOTE: This diet contains 1 Kcal/ml, of which 35% are fat derived, 11% derived from carbohydrate, 18% are derived from protein, & 36% are derived from ethanol.

Appendix B. Lieber-DeCarli ethanol rat diet ingredient.

Chart for Exchanging Ethanol and Maltose Dextrin to Keep the Lieber-DeCarli Diets Isocaloric*
Revised 11/15/94

ETOH Derived Kcal	ml of 95% ETOH	gm Maltose Dextrin
360 Kcal	67.29	0
350	65.42	2.49
340	63.55	4.98
330	61.68	7.47
320	59.81	9.96
310	57.94	12.44
300	56.07	14.93
290	54.21	17.42
280	52.34	19.91
270	50.47	22.40
260	48.60	24.89
250	46.73	27.38
240	44.86	29.87
230	42.99	32.36
220	41.12	34.84
210	39.25	37.33
200	37.38	39.82
190	35.51	42.31
180	33.64	44.80
170	31.78	47.29
160	29.91	49.78
150	28.04	52.27
140	26.17	54.76
130	24.30	57.24
120	22.43	59.73
110	20.56	62.22
100	18.69	64.71
0 (control)	0	89.60

* Based on the following information:

1. The original Lieber-DeCarli Diet formulations replace 360kcal/L of 95% EtOH with 89.6g/L of maltose dextrin Hence, this exchange chart reduces 89.6g/L of maltose dextrin(in the control diet - 0.0% EDC) proportionately until 67.3ml/L of 95% EtOH(36% EDC) is reached. Differences in caloric density values for maltose dextrin(360kcal/89.6 = 4.02kcal/gm vs. the original 3.96kcal/gm are due to variations incurred in rounding and reformulating.

2. 95% Ethanol at 25 C has a density of 0.80424 g/ml. 0.80424 x .95 x 7.0= 5.35 Kcal/ml.

Appendix C. Chart for exchanging ethanol and maltose dextrin.