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Porphyromonas gingivalis, ethanol, and chronic diseases.

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PORPHYROMONAS GINGIVALIS, ETHANOL, AND CHRONIC DISEASES

By

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A Dissertation Approved on

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DEDICATION

This dissertation is dedicated to my parents

Kathy Sue Short

and

Alan Eugene Short

who have supported me through everything. You've taught me so much.

This dissertation is also dedicated to my first mentor

Fabián Crespo

who taught me how to be a scientist. You've been a fantastic role model.

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ABSTRACT

PORPHYROMONAS GINGIVALIS, ETHANOL, AND
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This dissertation is an investigation into the relationships among *Porphyromonas gingivalis*, ethanol, and a series of chronic diseases, focusing primarily on atherosclerosis. It uses evolutionary theory to understand clinical parameters related to chronic disease biology. The initial research question was, "If people who drink a glass of wine each day have a lower risk for atherosclerosis, could one explanation involve antibacterial effects on pathogens associated with causing atherosclerosis, namely, *Porphyromonas gingivalis?".*

This dissertation is divided into four chapters. Chapter One provides the foundational information pertinent to the dissertation. Chapter Two describes an in-vitro experiment aimed at understanding how ethanol influences planktonic *Porphyromonas gingivalis*. Chapter Three details an in-vitro experiment aimed at learning how ethanol influences *Porphyromonas gingivalis* when it exists in a biofilm. Chapter Four explores how *Porphyromonas gingivalis* and ethanol influence rheumatoid arthritis, osteoporosis, Alzheimer's disease, chronic kidney disease, and type II diabetes.

Chapters Two and Three provide primary information resulting from experiments that I designed and performed, while Chapter Four is more theoretical in nature. The experiments detailed in Chapters Two and Three were designed to understand how ethanol may differentially impact *Porphyromonas gingivalis* in the bloodstream relative to the oral cavity.

The value of this dissertation lies in the synthesis of new ideas related to how the most widely used drug (ethanol) can influence the leading cause of death worldwide, heart disease. The use of ethanol as a systemic antimicrobial agent with regards to chronic infectious diseases has generally been overlooked. The hypothesis that ethanol consumption suppresses *P. gingivalis* growth in the blood, but not the oral cavity, is supported by experiments and a review of the literature presented in this dissertation.

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CHAPTER I

ATHEROSCLEROSIS, PERIODONTITIS, AND ETHANOL: PARADOXICAL RELATIONS

Introduction

Atherosclerosis is the leading cause of death worldwide, causing approximately one-third of all human deaths globally [1]. It is a chronic disease characterized by lipid depositions within arterial walls that progressively decrease luminal diameter [2]. Atherosclerotic progression often results in ischemic or hemorrhagic stroke or myocardial infarction [2]. According to the current paradigm, atherosclerosis often begins prior to adulthood [3] and progresses throughout a lifetime [4, 5].

Risk factors for atherosclerosis include smoking, elevated serum low-density lipoprotein (LDL), decreased serum high-density lipoprotein (HDL), elevated serum triglycerides, hypertension, obesity, and lack of exercise [6]. Evidence supports an etiological role for bacterial pathogens in atherosclerosis [7, 8], though the results are not consistent across studies or among patients within studies [9].
Moderate ethanol consumption has been implicated as protective against the development of atherosclerosis, but the reasons for a protective effect are unclear [10, 11] (Figure 1). This thesis will address the hypothesis that moderate alcohol consumption could protect against atherosclerosis by inhibiting bacteria that may contribute to atherosclerosis.

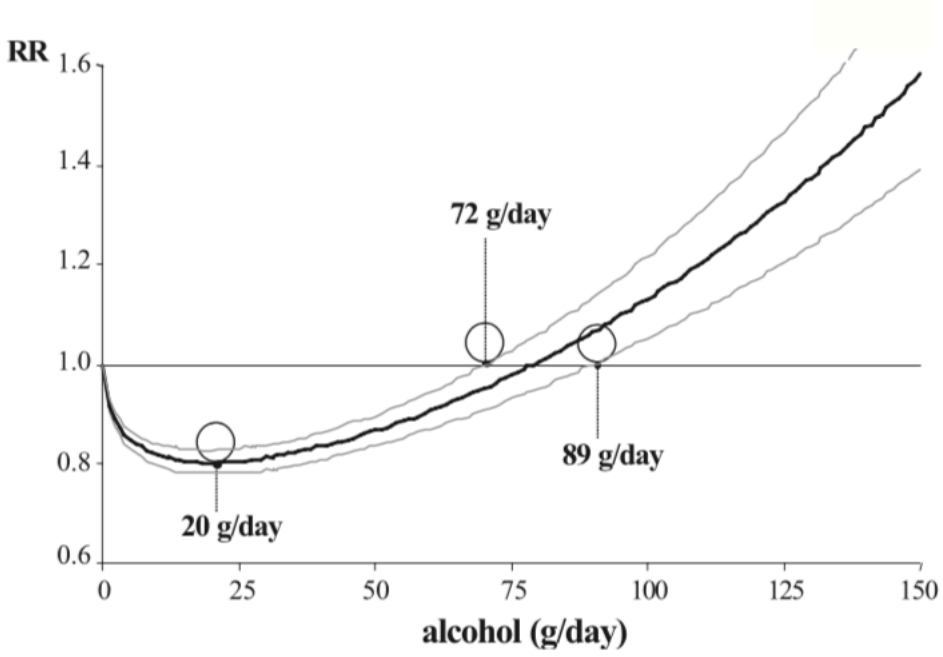


Fig. 2. Relative risk function and corresponding 95% confidence intervals describing the dose-response relationship between alcohol consumption and the risk of coronary heart disease.

Figure 1. Effect of ethanol on relative risk of coronary heart disease displays a J-shaped curve.

The curve favors low ethanol consumption for maximal benefits, while heavy consumption increases risk of total mortality. The curve crosses RR=1.0 at approximately 6-7 standard drinks/day. Adapted from [11].

Cardiovascular System

The cardiovascular system includes the heart and blood vessels [12]. The heart is a muscular organ that pumps blood throughout the body within a series of blood vessels [12]. Blood travels away from the heart in arteries and returns to the heart through veins [12]. Capillaries lie between arteries and veins and serve as a site for exchange between blood and interstitial fluid of the tissues being served [12].

Atherosclerosis Categorization and Diagnosis

Atherosclerosis is a cardiovascular disease characterized by plaque accumulation within the walls of arteries [13]. Atherosclerosis diagnosis is categorized according to the location of the diseased vasculature [14]. Atherosclerosis of the blood vessels serving the heart is referred to as coronary artery disease [15]. Atherosclerosis of carotid arteries and other vessels supplying blood to the brain is referred to as cerebrovascular disease. Atherosclerosis of blood vessels in the upper and lower limbs is referred to as peripheral arterial disease [16, 17]. Each of these categories share an arterial pathology characterized by lipid accumulation, decreased arterial elasticity, and decreased luminal diameter. For all categories, major risks involve chronic ischemia or hemorrhage.

Physicians use a combination of laboratory tests and imaging techniques to diagnose atherosclerosis risk and severity [18]. They detect the earliest stages of atherosclerosis using non-invasive imaging techniques, measuring blood pressure,

and performing blood tests. Blood samples from high-risk individuals display high LDL, low HDL, high blood sugar, and elevated C-reactive protein [19].

Carotid intimal-medial thickness (c-IMT) is performed as a diagnostic measure of clinical and subclinical atherosclerosis [20]. It is defined as the distance from the luminal edge of the tunica intima to the outer edge of the tunica media [21]. c-IMT increases as atherosclerosis progresses [20] and is associated with increasing age [5]. The increased risk associated with age does not justify the conclusion that atherosclerosis is a normal part of human senescence, as some [22] claim. Rather, it may result from repeated or ongoing damage from the factors that may contribute to atherosclerosis, such as infection.

Atherosclerotic plaques cause localized blood pressure changes due to decreased luminal volume and decreased arterial elasticity. The ankle-brachial pressure index (ABI) non-invasively compares blood pressure in the upper limbs relative to lower limbs Peripheral arterial disease (PAD) diagnosis requires an ABI value of 0.9 or less [23]. Low ABI suggests that blood pressure in the lower limbs exceeds that from the upper limbs, and indicates lower limb atherosclerosis. The 0.9 value is used instead of 1.0 because the brachial artery is smaller than the femoral artery and, therefore, experiences greater pressure in healthy individuals. ABI values greater than one indicate that upper limb blood pressure exceeds lower-limb blood pressure, and thus atherosclerosis in the upper limbs.

Porphyromonas gingivalis **and Oral Biofilms**

Porphyromonas gingivalis (P. gingivalis) is a gram-negative bacterium that contributes to periodontal disease when it proliferates in subgingival biofilms and forms anaerobic pockets; damage to periodontal tissues results directly from pathogen activity [24] and indirectly through immune responses [25]. *P. gingivalis* is referred to as a keystone species because of its predominant impact on communities of periodontal pathogens in biofilms where it is present in relatively low abundance [26]. *P. gingivalis* uses two fimbriae, FimA and Mfa1, to adhere to other bacteria and gingival epithelial cells[27]; *P. gingivalis* can invade human epithelial cells [24], a process that involves FimA [28].

P. gingivalis relies on other organisms to colonize before it can become incorporated into biofilms *in vivo*. Streptococcal species adhere to the acquired pellicle on teeth, enabling additional organisms to attach to their surface proteins. *Streptococcus gordonii* expresses membrane-bound proteins that facilitate *P. gingivalis* adherence and biofilm integration [29]. Biofilms accumulate biomass as they recruit bacteria into the microbial community and as adherent cells replicate within the biofilm [30].

Bacteria that colonize biofilms are not necessarily permanently embedded [31, 32]. Rather, they are able to leave the biofilm to allow transmission or to colonize other sites [31, 32]. Although *P. gingivalis* is non-motile, it moves throughout the oral cavity in saliva. *P. gingivalis* is transmitted from person to person through when intimate relations and shared utensils allow spread via saliva [33]. The ability to exit from biofilms therefore seems essential for its transmission.

P. gingivalis can spread from oral biofilms by several routes. It can infect lungs via aspiration and cause pneumonia [34]. It can invade gingival epithelial cells [24] and spread intercellularly without entering the extracellular matrix [35]. This attribute helps protect bacteria from the host's immune system. Infected host cells are killed by cytotoxic T cells, whereas extracellular infections are cleared without the requirement of killing host tissue. Extracellular *P. gingivalis* can also gain entry into the bloodstream by invading capillaries or damaged gingiva [36].

Periodontitis and Atherosclerosis

Gingival tissue serves as a physical barrier and protects teeth, maxillae, and mandibulae from infection. Gingivitis and periodontitis exist on a spectrum, whereby untreated gingivitis can progress to periodontitis [37]. Subgingival bacteria cause periodontitis when they damage host tissue and promote chronic inflammation. Tissue damage can result from microbial proteases and stimulation of chronic inflammation [38]. Subgingival bacteria are embedded in a biofilm [39] that helps them adhere to teeth. Biofilms contain living microorganisms and their extracellular components within a matrix that attaches to a surface [29]. One of the most important of these bacteria is *P. gingivalis* [39] [40] [41].

Periodontitis is a risk factor for atherosclerosis [42]. *P. gingivalis* is an etiological agent of periodontitis and is thought to contribute to atherosclerosis [43-45]. *P. gingivalis* spreads systemically when it penetrates capillary beds of breached gingiva [7, 46]. It and several other periodontal pathogens (e.g., *Trepomena denticola*, *Tannerella forsythia*, and *Aggregatibacter actinomycetecomitans*) have been detected in atherosclerotic lesions excised from larger blood vessels, such as

coronary arteries [47, 48]. One group of researchers claims to have successfully demonstrated that resected tissues contain viable *P. gingivalis*; although, the report was a letter to the editor and not a peer-reviewed article [49].

It is thought that the oral pathogens travel from the oral cavity to the sites where atherosclerosis develops via the bloodstream. Bacteria may gain entry to the bloodstream by penetrating vascular tissue [24, 50, 51] or by travelling within macrophages [52]. Poor oral hygiene is associated with increased risk of bacteremia [53]. At the other extreme, people who brush and floss too roughly can damage gingiva and cause bacteremia [54, 55].

Within an individual, the microbial compositions of subgingival and atherosclerotic plaques are positively correlated [56]. Tissue from bypass surgeries revealed that atherosclerotic and healthy arteries contain *P. gingivalis*, indicating that infection may precede and potentially play a causal role in cholesterol accumulation associated with atherosclerotic plaque synthesis rather than simply being an opportunistic bystander that infects damaged arterial tissue [57].

When subgingival bacteria spread systemically they may infect arteries [57]. Oral pathogens are commonly detected in resected arterial tissue from individuals who required surgical intervention for treatment of atherosclerosis [50, 57]. These pathogens have not been viably cultured after resection [58], but their presence is often recognized using polymerase chain reaction (PCR) [59] or fluorescent in-situ hybridization (FISH) [45] to detect DNA or RNA. Pathogens may invade arteries directly [60] or by traveling intracellularly within macrophages [61].

Arterial walls increase in stiffness as atherosclerosis progresses, a process that can be quantified before life-threatening cardiovascular complications arise. Arterial stiffness is associated with decreased lipid concentration and increased calcification [62]. Flow-mediated dilation is a measure of endothelial function that quantifies how peripheral arteries dilate in response to increased blood flow following artificial vasoconstriction using a sphygmomanometer cuff [63]. Individuals with periodontitis have higher c-IMT values and impaired flow-mediated dilation when compared than those without periodontitis; both of these values improve with periodontal treatment [64, 65]. This association provides a correlation between oral pathogens and atherosclerosis development.

It is not known whether arterial tissue is a dead-end for colonizing pathogens or if they are able to exit arteries and re-enter circulation. Arterial anatomy is such that pathogens would have to invade multiple tissue layers to reach or exit the tunica media, the region that typically accumulates cholesterol that contributes to atherosclerosis. From the lumen, pathogens would have to pass through the tunica intima, which contains an endothelial layer, the subendothelium, and an internal elastic lamina. Alternatively, they could invade arteries via the vasa vasora, blood vessels that extend superficially into deeper layers of large arteries and veins. While all arteries and veins do not contain vasa vasora, they are present in the arteries which atherosclerosis typically develops. These include the coronary arteries, aorta, carotid arteries, brachial artery, iliac artery, and the femoral artery [66, 67].

As mentioned in the introduction, moderate ethanol consumption is associated with a decreased risk for atherosclerosis [11]. The following sections will

explore the effects of human ethanol consumption. Ethanol metabolism will be reviewed, followed by a discussion of ethanol as an antimicrobial agent. These contents will be applied to understanding how ethanol influences bacteria and human tissue throughout the body and how these interactions influence an individual's risk for developing chronic diseases.

Ethanol Metabolism and Pharmacokinetics

After ethanol is swallowed, it passes down the esophagus and enters the stomach. Gastric tissue is about 400 micrometers thick, lending to poor diffusion when compared to the small intestine, which is only about 25 micrometers thick [68]. Gastric emptying into the small intestine is dramatically influenced by the presence of food. Consumed ethanol reaches the small intestine much faster when someone has not recently eaten; moreover, some of the ethanol is oxidized by the bolus before emptying into the small intestine. Ethanol is primarily absorbed in the small intestine into the capillaries of the hepatic portal system [69].

Once ethanol enters the liver via the portal vein, the deoxygenated blood mixes with oxygenated blood from the hepatic artery within sinusoids. Ethanol will be diluted within sinusoids, as roughly 1/3 of the blood serving the liver comes from the hepatic artery. Hepatocytes catabolize ethanol, and what remains enters systemic circulation via the hepatic vein. Ethanol is further diluted as the superior and inferior vena cava mix blood from the upper and lower body.

Ethanol is typically metabolized at a rate of roughly ten grams per hour, but some experience a blood alcohol concentration plateau for the first two hours

following consumption [70]. Ethanol is primarily degraded in the liver, with only about 5% of ingested amounts collectively remaining in urine, sweat, and breath [71-75]. Blood alcohol concentration is usually highest about 30-60 minutes after consumption [70, 76].

Chronic ethanol consumption can lead to increased ethanol tolerance, suggesting that regular drinking patterns decrease ethanol's effects and require increased consumption to obtain effects that used to be reached with less consumption [77, 78]. The National Institute of Health (1995) considers multiple ways in which individuals can become tolerant as a result of chronic alcohol consumption, including functional, acute, environment-dependent, learned, environment-independent, and metabolic tolerance [78]. Tolerant individuals can have a blood ethanol concentration of 0.1% and show no signs of intoxication; whereas, those without tolerance would show visible signs of intoxication at this concentration [77]. Highly tolerant individuals may reach blood ethanol concentrations around 0.4-0.5%, even though such high levels could be lethal for social drinkers [77].

Metabolic tolerance arises from increased efficiency at metabolizing ethanol and can be considered a physiological adaptation to regular ethanol consumption [79]. Alternatively, functional tolerance arises when the brain adapts to chronic ethanol consumption to compensate ethanol's physiological and behavioral effects [79]. Individuals with functional tolerance can have elevated blood ethanol concentrations while displaying no clear signs of intoxication, while the same ethanol concentrations could be fatal for those without functional tolerance [80]. S.

C. Lapham quotes a DUI offender who said, “Usually I was the designated driver because my friends say I can drive better than anybody when I’m intoxicated… Five beers is nothing to me” [77].

Blood alcohol concentrations in the hepatic portal system may be elevated relative to concentrations in the rest of the bloodstream. Ethanol is degraded primarily in the liver after leaving the hepatic portal system. Of the ethanol that enters hepatocytes, roughly 75% of ethanol is catabolized to acetaldehyde by alcohol dehydrogenase (ADH); the remaining 25% is catabolized to acetaldehyde using cytochrome (CYP) P450 enzymes like CYP2E1. Catalase is a peroxisomal enzyme to catabolize ethanol to acetaldehyde. Acetaldehyde is converted to acetate by aldehyde dehydrogenase 2 (ALDH2), and this is the rate-limiting step that raises concern regarding aldehyde reactivity.

Blood flows three times faster in the portal vein than the liver (0.75 and 0.25 liters/minute/kg, respectively) [81]. Bacteria in the portal vein blood can, therefore, be exposed to ethanol at higher concentrations than elsewhere in the body for a brief period of time [82]). This exposure may be important during first-pass metabolism (FPM), which is defined as the amount of ethanol that is catabolized prior to entering systemic circulation, aka “peripherally available (PA)”[81]. Simply put, FPM= total oral dose – PA [83]. Although the contribution of first-pass metabolism remains under debate, recent research suggests that only about 10% of consumed ethanol is degraded in its first passage [84, 85].

Blood flows through the portal vein at a velocity of approximately 13 cm/second [86], and the portal vein is about 25 cm in length; therefore, ethanol

traveling from the gastrointestinal tract to the liver would remain within the portal vein for roughly two seconds before entering the liver [86-90]. This duration in noteworthy because it is time that systemic bacteria would be exposed to the highest possible blood alcohol concentration.

Ethanol as an Antimicrobial Agent

Ethanol is an aliphatic hydrocarbon with strong antimicrobial properties [91-95]. The CDC (2008) recommends using 60-90% ethanol solutions in water (volume/volume) to adequately kill microbes [95]. Ethanol damages microbes by increasing membrane permeability and denaturing intracellular and extracellular proteins, which explains why it is still commonly used in antimicrobial mouthwashes [91-95]. Not all alcohols denature proteins, though. Glycerol is commonly used to freeze microbial stock cultures at -80° C [94].

Bacterial species vary in their response to ethanol exposure. The CDC (2008) refers to Morton's 1950 publication, which claims that ethanol concentration ranging within 30-100% killed *Pseudomonas aeruginosa* in ten seconds, and any concentration within 40-100% ethanol killed *Escherichia coli* or *Salmonella typhosa* in ten seconds [95, 96]. *Staphylococcus aureus*, a gram-positive bacterium, requires 60-95% ethanol to kill it within ten seconds [97].

Ethanol and Biofilms

Ethanol (70%) is commonly used to disinfect catheters, as they have a tendency to accumulate bacterial biofilms [98]. Recent findings demonstrate that ethanol concentrations as low as 2.5% inhibit *Pseudomonas aeruginosa* biofilms [98]. Other studies have shown that 1% ethanol stimulates *P. aeruginosa* biofilms [99]. Polymicrobial biofilms display increased resistance to ethanol, as different organisms have different capacities to withstand ethanol exposure [100, 101]. Understanding how ethanol influences microbial fitness becomes more complicated when multiple species are involved, such as in oral biofilms.

Ethanol and Oral Microbiome

Ethanol consumption is positively associated with periodontitis risk [102]. Chronic ethanol consumption promotes a dysbiotic oral microbiome, increasing one's risk for periodontal disease [103-105]. Amaral et al (2011) demonstrated that the subgingival microbiota in alcoholics was contained higher counts of pathogenic bacteria than in non-alcoholics; although, the prevalence of bacterial species was similar between groups [105]. Ethanol also directly damages gingival tissue and decreases salivary secretions, both of which facilitate microbial pathogenesis [104, 106, 107]. Ethanol's effects extend beyond the oral cavity once someone swallows and subsequently absorbs it into their bloodstream.

Ethanol and Cardiovascular Disease

One "standard drink" contains fourteen grams of ethanol [108]. Therefore, for comparative purposes, the volume of a "standard drink" is inversely related to

the concentration of ethanol in the beverage (i.e., lower volume for spirits and higher volume for wine and beer). Because alcohol can damage human tissue, the U.S. Department of Agriculture and U.S. National Institute of Health and Human Services recommends that women consume no more that one drink per day and men consume no more that two standard drinks per day [109].

Adults over twenty-one years of age are legally allowed to purchase and consume ethanol in the United States. Underage drinking occurs frequently and often results in binge drinking [110, 111]. Adolescent ethanol consumption is a disease risk factor that tends to be more prevalent in boys than girls [112]. Results from a 2015 survey of adolescents in the U.S. indicate that about 30% of fifteen year-olds and 60% of eighteen year-olds have consumed at least one alcoholic drink [110].

Health surveys [113] and meta-analyses [10] suggest that moderate ethanol consumption reduces risk of cardiovascular disease-related morbidity and mortality but that these ameliorating effects are lost when ethanol consumption continues past a threshold amount. This dose/effect pattern is commonly referred to as a U- or J-shaped curve [114]. If the negative effects of high alcohol consumption on cardiovascular disease offset the beneficial effects associated with low alcohol consumption the curve is U-shaped. If the negative effects of high alcohol consumption are greater than the beneficial effects of low alcohol consumption, then the curve is J-shaped [115] (Figure 1). The type of alcoholic beverage seems to have little, if any, effect on this association, suggesting that major physiological

effects are due to ethanol rather than other constituents, though, some research supports a protective effect of resveratrol in wine (discussed below) [116, 117].

The protective effect of moderate ethanol consumption and the mechanisms that could generate such protection are still being debated [118]. One possible protective mechanism for ethanol-mediated protection from cardiovascular disease could be through improvement of lipid profiles by increasing serum high-density lipoprotein (HDL) and decreasing serum low-density lipoprotein (LDL) [119]. Low to moderate alcohol consumption is associated with reduced inflammatory biomarker concentration and improved glucose metabolism [120].

Meta-analyses suggest that moderate ethanol consumption decreases risk of dying from a cardiovascular disease [116, 121]. Correlates of atherosclerosis progression, however, do not necessarily show concordant associations. Carotid artery intimal-medial thickness, for example, correlates with progression of atherosclerosis. As discussed below, some analyses suggest that moderate ethanol consumption promotes thickening of arterial walls [65, 122], while others generate evidence of a J-shaped association between ethanol consumption and arterial wall thickness [123].

Another uncertainty in this area of research involves ethical issues pertaining to a researcher who has reported protective effects of moderate alcohol consumption. The National Institute of Health and Human Services terminated funding for a major prospective clinical trial that aimed to determine the health

outcomes of moderate ethanol consumption when the principal investigator was found to have a conflict of interest arising from his association with the alcoholic beverage industry [124].

In spite of these uncertainties, the current state of research suggests that moderate alcohol consumption provides protective effects. The lack of a clear protective mechanism for such protection, however, may contribute to legitimate reservations about the validity of the association. By testing a hypothetical mechanism for this protection, the work described in this dissertation might be useful to the evaluation of the validity of the hypothesized beneficial effects of moderate alcohol consumption.

Experimental Animal Models Investigating the Effects of Ethanol Consumption on Cardiovascular Disease Risk

Experimental investigations of animal models of cardiovascular disease have provided evidence that bears on the effects of moderate alcohol consumption. Removing the alcoholic fraction of a beverage allows researchers to compare the effects of secondary compounds in the presence and absence of ethanol. An experimental rabbit model was used to determine if ethanol or secondary compounds found in dealcoholized red wine, such as resveratrol, conferred cardiovascular protection [125]. The researchers found that red wine and dealcoholized red wine provided similar increases in cardiovascular protection when compared to controls that received neither beverage, suggesting that wine components aside from ethanol provide cardiovascular protection [126].

Apolipoprotein E-null mice (ApoE -/-) are commonly used as a model for cardiovascular disease. These mice are unable to express apolipoprotein E, which typically functions to bind to circulating serum lipoproteins and direct them to cell-specific receptors [127]. ApoE -/- mice are hypercholesterolemic and form arterial lesions that resemble those found in humans [128]. These mice have been used to experimentally investigate how daily moderate ethanol consumption or weekend binging influenced atherosclerotic plaque development [129]. Compared with mice that received no ethanol, daily ethanol consumers displayed less atherosclerotic plaque and larger luminal diameters [129]. Mice provided with a binging dose of ethanol had more plaques and smaller luminal diameters [129]. These results suggest that moderate ethanol consumption is protective in mice, but heavy consumption is harmful.

Following chapters

This dissertation aims to evaluate one aspect of a hypothetical mechanism by which moderate alcohol intake could ameliorate atherosclerosis: though inhibition of *P. gingivalis*. Chapter 2 addresses the extent to which different ethanol concentrations inhibit *P. gingivalis* cultures that are in suspended in media rather than in a biofilm. The suspended state is used as an indicator of effects of ethanol on *P. gingivalis* that have left their biofilm microenvironment to move systemically through the bloodstream. Chapter 3 addresses the extent to which different ethanol concentrations inhibit *P. gingivalis* that are in a biofilm model, which is used as an indicator of the effects of ethanol on *P. gingivalis* in the biofilm environment.

Chapter 4 discusses the implications of the results for the spectrum of diseases that are associated with *P. gingivalis* and for which correlations with alcohol intake have been reported: periodontal disease, atherosclerosis, Alzheimer's disease, type 2 diabetes, and rheumatoid arthritis, as well as overall conclusions and directions for future studies.

CHAPTER II

EFFECTS OF ETHANOL ON PLANKTONIC PORPHYROMONAS GINGIVALIS

Introduction

This chapter reports experiments that investigated whether *in vitro* growth of planktonic *Porphyromonas gingivalis* (i.e., suspended in media rather than in biofilms) was inhibited by ethanol across a range of concentrations that include the concentrations associated with low levels of alcohol ingestion. The goal is to evaluate whether concentrations of ethanol over a range that might occur in the bloodstream could inhibit *P. gingivalis* and thus reduce the risk for systemic diseases caused by this pathogen.

Materials and Methods

General approach

Planktonic *Porphyromonas gingivalis* were exposed to a series of ethanol concentrations *in vitro* over a twenty-four-hour period to allow most bacteria to reach their stationary phase. To assess bacterial density, absorbance was measured using spectrophotometry and colony-forming units were counted from growth on agar plates. Absorbances provide precise measurements of a presumed indicator of bacterial density, whereas the counts of colony-forming

units allowed assessment of whether the absorbance in fact reflected the density of viable bacteria.

Porphyromonas gingivalis

All experiments used *Porphyromonas gingivalis* wild-type (ATCC 33277) obtained from Dr. Richard J. Lamont. Cultures were grown from frozen stocks and maintained on blood agar plates. Trypticase Soy Broth (TSB, BD) was supplemented with yeast extract (1 mg/mL), hemin (5 μg/mL), and menadione (1 μg/mL) to create "TSB complete". Fresh blood agar plates containing TSB complete and sheep's blood were prepared weekly for each experiment. The primary subculture was created by inoculating TSB complete with isolated *P. gingivalis* colonies and pipetting to resuspend the cells. The primary subculture (5.0 mL) was incubated anaerobically at 37°C overnight. A secondary subculture was created the following morning by combining 500 μL of primary subculture and 5.0 mL sterile TSB complete and incubating anaerobically at 37°C overnight.

The secondary subculture was centrifuged at 3,000 RPM at 4°C for 10 minutes to create a pellet. The supernatant was aspirated using a Pasteur pipet, and the resulting pellet was resuspended in 950 μL sterile TSB complete. Cells were resuspended using gentle micropipetting. Optical density (O.D.) was measured using an Eppendorf spectrophotometer at 600 nm.

Growth curves

Broth containing 0.001% to 10% molecular biology-grade ethanol was prepared by performing ten-fold serial dilutions from a 10% ethanol broth created with 4.5 mL sterile TSB complete and 0.5 mL ethanol. These ethanol concentrations were chosen to encompass blood ethanol concentrations occurring after consumption of feasible ranges of ingested ethanol. A blood ethanol concentration of approximately 0.001% would be expected during the slow consumption of a standard drink. The drunk driving limit of 0.08% blood ethanol conforms roughly to the experimental concentration of 0.1% ethanol. The 1% and 10% ethanol concentrations was used as negative controls under in recognition that these levels of ethanol was far outside of the range of blood ethanol concentrations associated with moderate ethanol consumption [130]. Absorbances and colony counts for each ethanol concentration were compared statistically with positive controls (*P. gingivalis* in 0% ethanol).

All samples initially contained 5.0 mL sterile TSB complete in 15.0 mL centrifuge tubes and were inoculated with 5 x 10^8 cells (O.D.= 0.1). Each preparation was performed in duplicate, and each duplicate was replicated independently six times. Caps were loosened prior to anaerobic incubation. Data were collected in the form of optical density measurements and colony-forming units at baseline, 30 minutes, 4 hours, 6 hours, 12 hours, 18 hours, and 24 hours. Optical density measurements used 500 μL from each sample after homogenization using micropipetting.

Bacterial concentrations at each time point were determined using a modified spread plate method, in which each plate contained twelve samples (four

sets of triplicates) for quantifying colony forming units (CFUs). Each plate was divided into four quadrants to save materials, and triplicate 20 μL volumes from each experimental sample were diluted to either 1/10,000 or 1/1,000,000, plated, and spread into each quadrant using a pipette tip. Dilutions were chosen based on results from prior experiments, depending on how many colonies were expected to grow. Triplicate samples were used to verify CFU counts due to the variability associated with small volumes. Samples were incubated anaerobically on blood-agar plates containing TSB complete for seven days to allow colonies to grow and display black pigmentation, a visual indication of colony maturation. Averages were calculated from the triplicated counts, and colony-forming units (CFUs) per mL were calculated using the appropriate dilution factor from the day the sample was plated.

P. gingivalis growth rates were calculated using the following formula: $\mu = ((\log_{10} N - \log_{10} N_0) 2.303) / (t - t_0)$ [131]. Optical density values were multiplied by 10^9 before performing growth rate calculations. The initial time point was at 6 hours, and the final time point was at 12 hours, corresponding to the two points encompassing logarithmic growth. The data are presented in Table 1.

Statistical analysis

Two-way ANOVA tests were performed to compare bacterial density under experimental and control conditions. Absorbances were compared with the corresponding measurements for controls. Statistical tests were performed on the data at four and six hours because these time intervals corresponded to the time period in which *P. gingivlalis* cells replicates; statistical tests were run at 24 hours to

determine whether the ethanol concentrations inhibited growth at a time when the density in the positive control samples had reached stationary phase. Statistical analyses were performed using R. The threshold for ascribing statistical significance was p=0.05.

Results

Ethanol inhibits planktonic *Porphyromonas gingivalis* growth.

Measurements of absorbance as a function of time show that 10% ethanol was associated with a steady decline in absorbance (Figure 2), indicating that this level of ethanol killed *P. gingivalis*. Absorbance increased over the 24-hour experimental period for ethanol concentrations of 1.0%, 0.1%, 0.01%, 0.001%, and 0% ethanol (Figure 2). These increases indicate that *P. gingivalis* was able to survive and replicate under these ethanol concentrations. The absorbance curves appeared to rise more slowly, however, with increasing ethanol concentration (Figure 2). This hypothesis was tested for statistical significance as discussed below.

Under the conditions used in this study, *P. gingivalis* replicates roughly every four to five hours [24] [132]. The approximate doubling of absorbance at four hours relative to zero hours (Figure 2) is consistent with this replication rate. The absorbance curve for 0% ethanol rose and then flattened sigmoidally during the 24-hour experimental period with an inflection point between 6 and 12 hours (Figure 2). The leveling off of absorbance by 18 hours (Figure 2) indicates some inhibition of bacterial growth by this time, probably due to depletion of the resources needed for bacterial growth. This feedback inhibition associated with bacterial growth is also

apparent in the absorbance curves for each ethanol concentration ranging from 0.001% to 1.0%.

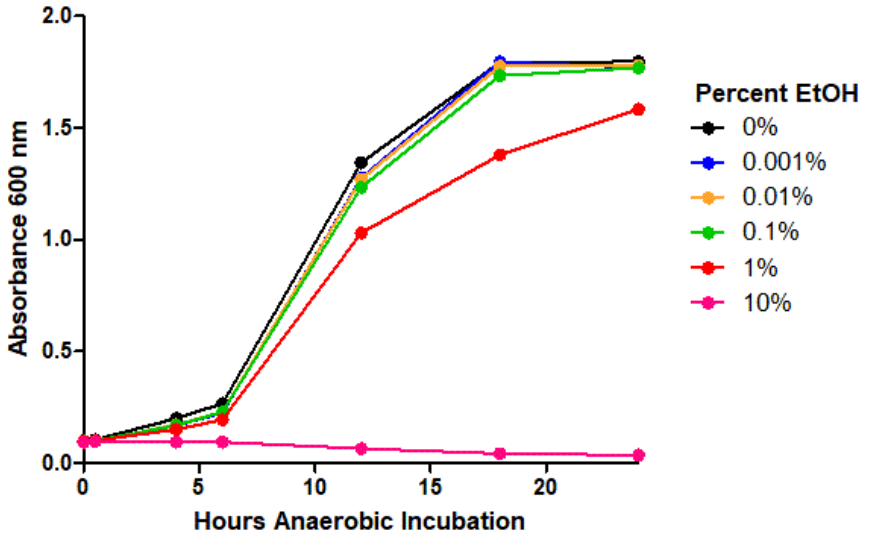
In accordance with these considerations, assessments of the ethanol inhibition at four and six hours were used as the best indicators of ethanol inhibition that is not confounded by the inhibitory effects of resource depletion. Statistical tests to evaluate ethanol therefore focused on differences in the absorbances at 4 and 6 hours (Figure 3). Each experimental ethanol concentrations ranging from 0.001% to 0.1% significantly inhibited planktonic bacteria after four hours and six hours of ethanol exposure relative to the 0% controls (Figure 3).

Overall, the results presented in Figures 1 and 2 suggest that ethanol concentrations from 0.001% to 1% ethanol inhibited but did not prevent replication of *P. gingivalis*; 10% ethanol, however, prevented growth.

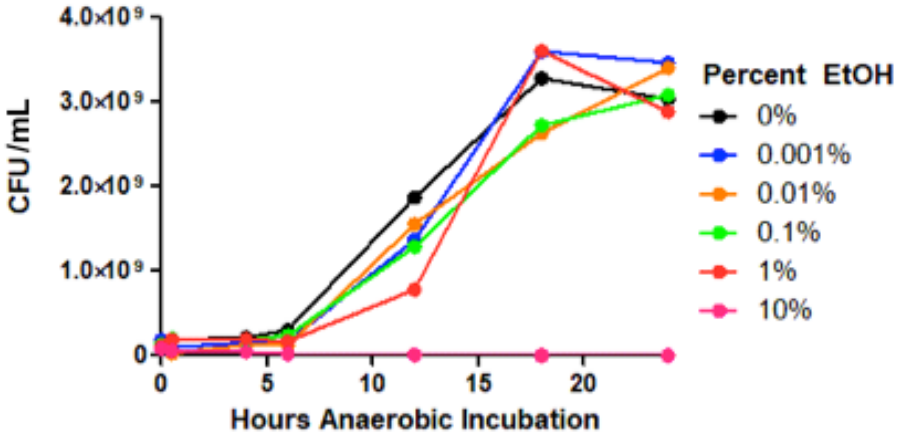
At 24 hours of exposure, absorbances for ethanol concentrations at 0.001%, 0.01% and 0.1% were not significantly lower than absorbances associated with 0% ethanol (Figure 4). This lack of difference suggests that these concentrations slowed growth to the equilibrium but still allowed the bacteria to grow to the equilibrium value. This finding accords with the hypothesis that the equilibrium at these concentrations is constrained by the resources in the media rather than by ethanol inhibition.

The CFUs recorded from inoculated agar plates confirmed that absorbance readings reflected viable *P. gingivalis* (Figure 2b). For ethanol concentrations from 0.001% to 0.1% CFUs increased and then leveled off over the 24-hour experimental period with a pattern that accorded with the changes in absorbance (Figures 2b and

2a, respectively). For *P. gingivalis* exposed to 10% ethanol, CFUs declined steadily over this period, indicating that ethanol at this level prevented bacterial proliferation and eventually killed the bacteria.



a.



b.

Figure 2. Planktonic *P. gingivalis* growth curves.

(a.) Absorbance of *P. gingivalis* in liquid growth media with different ethanol concentrations as functions time exposed to ethanol (hours after inoculation). Each data point is based on 12 measurements: six independent experimental replicates with 2 measurements per replicate for each time and each concentration. (b.) Samples were plated each time that an optical density measurement was obtained.

Ethanol Concentration (%)	Average Growth Rate (μ)	Standard Deviation
0	0.264236559	0.045858639
0.001	0.281291402	0.046835392
0.01	0.280184977	0.049862388
0.1	0.274182489	0.046838472
1	0.259925377	0.106589981
10	-0.114344069	0.129258963

Table 1: *P. gingivalis* mid-logarithmic growth rates.

P. gingivalis growth rates were calculated using the following formula: $\mu = ((\log_{10} N - \log_{10} N_0) 2.303) / (t - t_0)$ [131].

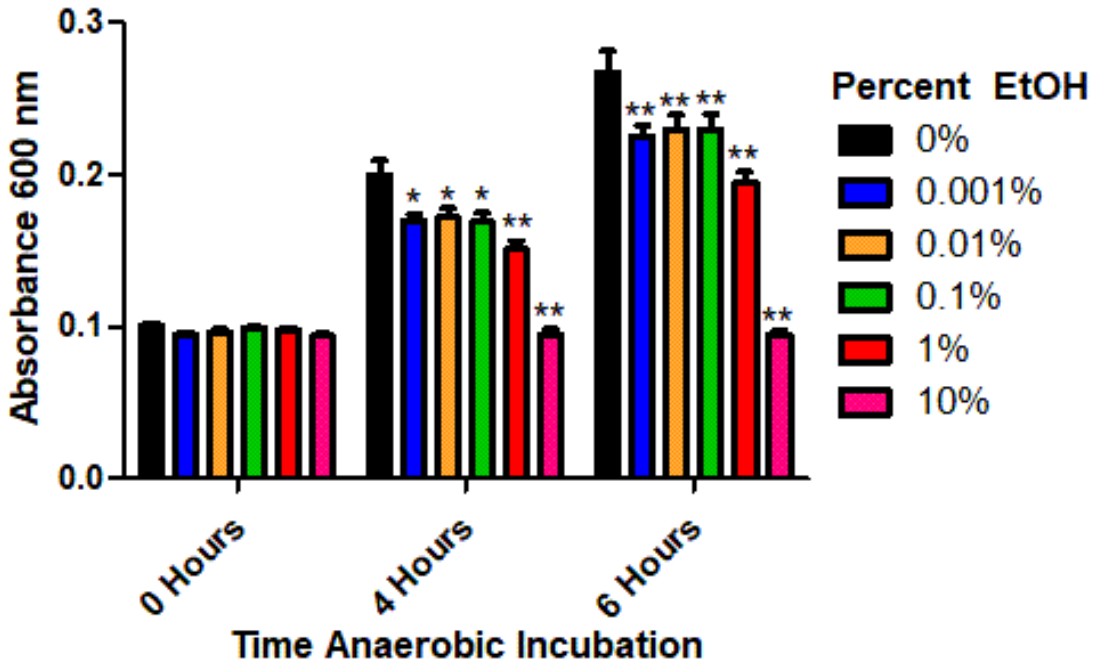


Figure 3. Absorbance of *P. gingivalis* in liquid growth media with different ethanol concentrations at four and six hours after inoculation.

Each riser in the histogram corresponds to a data point in Figure 2. Asterisks designate statistically significant differences from the absorbances associated with the 0% ethanol controls at each time period. * $p<0.05$, ** $p<0.01$

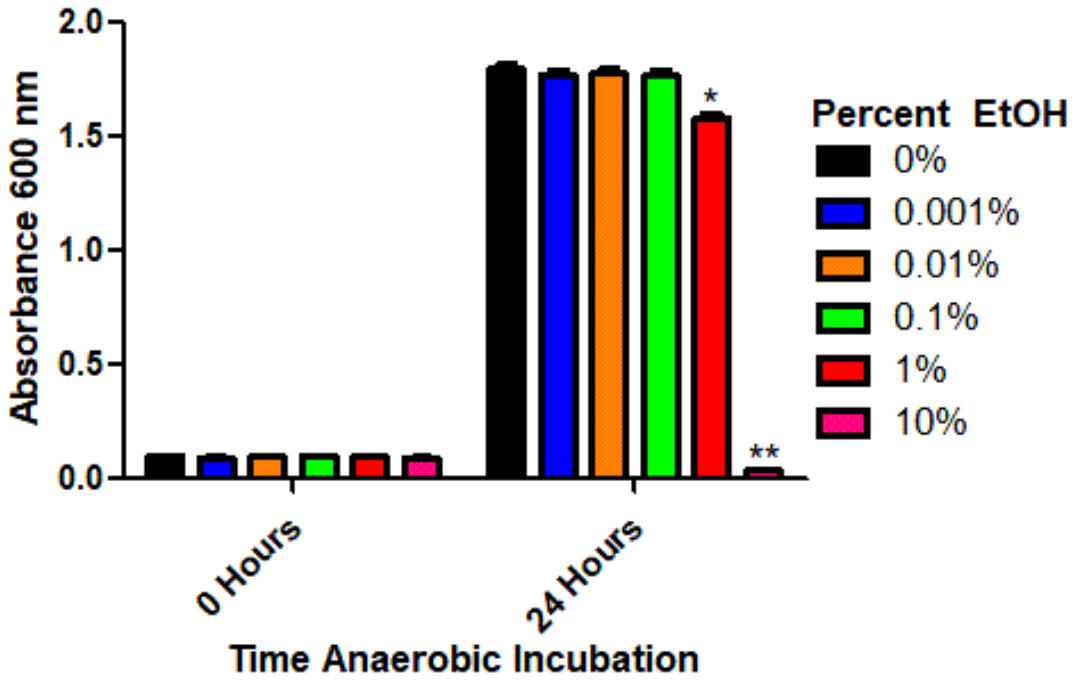


Figure 4. Absorbance of *P. gingivalis* in growth media with different ethanol concentrations at twenty-four hours after inoculation.

Each riser in the histogram corresponds to a data point in Figure 2. Asterisks designate statistically significant differences from the absorbances relative to the 0% ethanol controls at each time period. * $p<0.05$, ** $p<0.01$

Discussion

The results show that physiologically relevant ethanol concentrations (i.e., 0.01%-0.1%) inhibit planktonic growth of *P. gingivalis*. This finding is consistent with the hypothesis that low blood ethanol concentrations could inhibit *P. gingivalis* bacteremia and, thus, protect against atherosclerosis. Although the inhibitory effects of physiologically relevant ethanol concentrations were slight, they might contribute to protection against *P. gingivalis* by tipping the balance in favor of immunological control.

The lack of conspicuous systemic *P. gingivalis* infections in humans suggests that the immune system is broadly able to suppress systemic *P. gingivalis* infections [133-135]. This suppression apparently occurs even though periodontal bacteria frequently enter the bloodstream as a result of activities such as eating, brushing teeth, and flossing [55, 136]. Presumably, frequent pathogen exposure allows for relatively continuous mobilization of immunological defenses against them [134, 135]. Transient bacteremia typically contains about 10-100 bacteria per mL blood and is resolved in about an hour (reviewed by [134]).

Higher concentrations of ethanol increasingly inhibited *P. gingivalis*. Humans presumably have evolved immune systems that function well at levels of ethanol that were regularly present during our evolutionary history from fermented foods and symbiotic microbiota [137]. Accordingly, negative effects of ethanol on immune function may occur at higher concentrations but not at concentrations associated with low levels of ethanol consumption [138]. The net effect of ethanol on control of candidate causal pathogens may thus become negative at higher ethanol intake

because the negative effects on the immunological function outweighs the direct negative effect of the ethanol on the pathogens.

Physiologically relevant ethanol concentrations span a range because pathogens are exposed to different ethanol concentrations depending on the amount of ethanol ingested, the time since ingestion and their location in the body. The oral cavity and esophagus experience the highest ethanol concentrations. Absorption occurs slightly in the stomach but mostly in the small intestine. Plasma ethanol concentrations decline as ingested ethanol in the blood travels throughout the body as a result of degradation and dilution. The portal vein transports blood from the gastrointestinal tract to the liver where substantial degradation of ethanol occurs. The liver receives approximately 25% of cardiac output at rest, with two-thirds coming from the portal vein and one-third from the hepatic artery [90, 139]. Upon leaving the liver, blood that arrived from the portal vein is mixed with blood from the hepatic artery.

Calculations based on enzyme kinetics indicate that ethanol concentrations in the portal vein are roughly ten times higher than in the blood leaving the liver [140]. *P. gingivalis* in systemic circulation may therefore often be subjected to ethanol concentrations in the portal vein that are far higher than the blood concentrations that are measured in blood draws or breaths. Someone who drank a sip or two of a diluted beverage would have approximately 0.001% of their total blood volume as ethanol. A standard drink would result in 0.02-0.04% blood ethanol, similar to the 0.01% experimental condition. The drunk driving limit in

most states is 0.08% blood ethanol, similar to the experimental concentration of 0.1% ethanol.

One caveat for interpretation of these experimental results is that exposure to the ethanol concentrations used in the experiments were continuous, whereas, exposure *in vivo* would vary depending on the aforementioned factors. My results leave open the question of whether alcohol in the oral cavity inhibits *P. gingivalis* or other organisms that might contribute to cardiovascular disease. Although the levels of ethanol in the oral cavity could be high, *P. gingivalis* in the oral cavity is most often embedded with other organisms in biofilms [29, 141]. Biofilm formation might protect bacteria from the damaging effects of ethanol, a possibility that needs to be investigated experimentally. This hypothesis is addressed in the next chapter.

CHAPTER III

EFFECTS OF ETHANOL ON PORPHYROMONAS GINGIVALIS IN A BIOFILM MODEL

Introduction

Biofilms contain living microorganisms and their extracellular components within a matrix that attaches to a surface [29]. Biofilms may benefit microbes by allowing them to adhere to a substrate, stimulate nutrient secretion [39], facilitate metabolically mutualistic relationships with other microbes [142], and allow organisms to evade host defenses [143]. In the case of oral biofilms, microorganisms adhere to teeth [144]. Oral biofilms can contain approximately 700 microbial species, about half above and half below the gumline [145]. Anaerobic pathogens in oral biofilms persist in subgingival pockets where they can avoid oxygen exposure and access nutrients from gingival crevicular fluid [39], a serum-based exudate fluid that contains inflammatory cells and antimicrobial biochemicals [146].

This chapter reports an experimental investigation of whether the range of ethanol concentrations reported in Chapter 2 adversely affect *P. gingivalis* when it is present in an *in vitro* biofilm model. This work addresses a paradox arising from the association between ethanol consumption and risks of periodontitis relative to atherosclerosis. Specifically, in contrast with the J-shaped association of atherosclerosis with ethanol consumption, periodontal disease increases linearly with increases in ethanol consumption [10, 113, 147].

If atherosclerosis and periodontitis are both caused by the same periodontal pathogens [43], then why does ethanol consumption influence the disease risk profiles so differently? The findings reported in Chapter 2 suggest that moderate ethanol consumption may inhibit planktonic *P. gingivalis.* The inhibitory influence on pathogens may occur without compromising immune responses [148], generating a net protective effect against reducing the probability infections of vascular tissue [50]. If *P. gingivalis* biofilms protect it from exposure to ethanol, then moderate ethanol consumption may fail to inhibit *P. gingivalis* in the oral cavity sufficiently to generate protection from periodontal disease.

The experiment reported in Chapter 2 showed that moderate concentrations of ethanol inhibited planktonic *P. gingivalis* growth and viability. This chapter reports a complementary experiment that investigated whether the same ethanol concentrations had inhibitory effects on *P. gingivalis* in an *in vitro* biofilm model.

Materials and Methods

Culturing of *Porphyromonas gingivalis* from stock

All experiments used *Porphyromonas gingivalis* wild-type (ATCC 33277) obtained from Dr. Richard J. Lamont. Cultures were grown from frozen stocks and maintained on blood agar plates. Trypticase Soy Broth (TSB, BD) was supplemented with yeast extract (1 mg/mL), hemin (5 μg/mL), and menadione (1 μg/mL) to create "TSB complete". Fresh blood agar plates containing TSB complete and sheep's blood were prepared weekly for each experiment. The primary subculture was created by inoculating TSB complete with isolated *P. gingivalis* colonies and pipetting to

resuspend the cells. The primary *P. gingivalis* subculture (5.0 mL) was incubated anaerobically at 37°C overnight. A secondary subculture was created the following morning by combining 500 µL of primary subculture and 5.0 mL sterile TSB complete and incubating anaerobically at 37°C overnight.

The secondary subculture was centrifuged at 3,000 RPM at 4°C for 10 minutes to create a pellet. The supernatant was aspirated using a Pasteur pipet, and the resulting pellet was resuspended in 950 µL sterile 0.5x TSB complete (TSB complete diluted into sterile phosphate buffered saline, PBS). Cells were resuspended using gentle micropipetting. Optical density (O.D.) was measured using an Eppendorf spectrophotometer at 600 nm.

Experimental design optimization

Prior to conducting the experiments, *P. gingivalis* biofilm development was optimized by measuring the extent to which biofilms grow in response to either (1) buffer (1x PBS) or broth (0.5x TSB complete), (2) high (5 x 10^8 cells/mL) or low (2 x 10^7 cells/mL) inoculum concentration, (3) time exposed to ethanol (0, 0.5, 1, or 4 hours), and (4) concentration of ethanol exposure (0.001% - 50%). Data are presented in Figure 5. Experiments were performed using 96-well plates that contained either 1x PBS or 0.5 x TSB complete. Different 96-well plates were used to expose biofilms to ethanol for various amounts of time (0, 0.5, 1, or 4 hours). The lower inoculation concentration was used on the left side of the 96-well plates, and the higher inoculation concentration was used on the right side of the 96-well plates. The full spectrum of ethanol concentrations was included in each 96-well

plate. Taken together, one complete set of data required eight different 96-well plates. All experimental conditions were run in triplicate. Biofilm growth was measured via spectrophotometry at 595 nm using the crystal violet staining technique [149]. Results from these experiments (n=6) were used to design subsequent biofilm experiments (see below).

Biofilm experiment

Four 24-well plates were used for each replicated experiment. One "set" of plates includes two 24-well plates in order to accommodate all experimental conditions. One set of plates was used to determine the biofilm density at 24 hours, and the other set of plates was used to determine the biofilm density at 48 hours.

One mL of *P. gingivalis* subculture (2×10^7 cells/mL) in 0.5x TSB complete was inoculated into each experimental well of four 24-well plates. Control wells received only sterile medium (0.5x TSB complete). All four plates were incubated

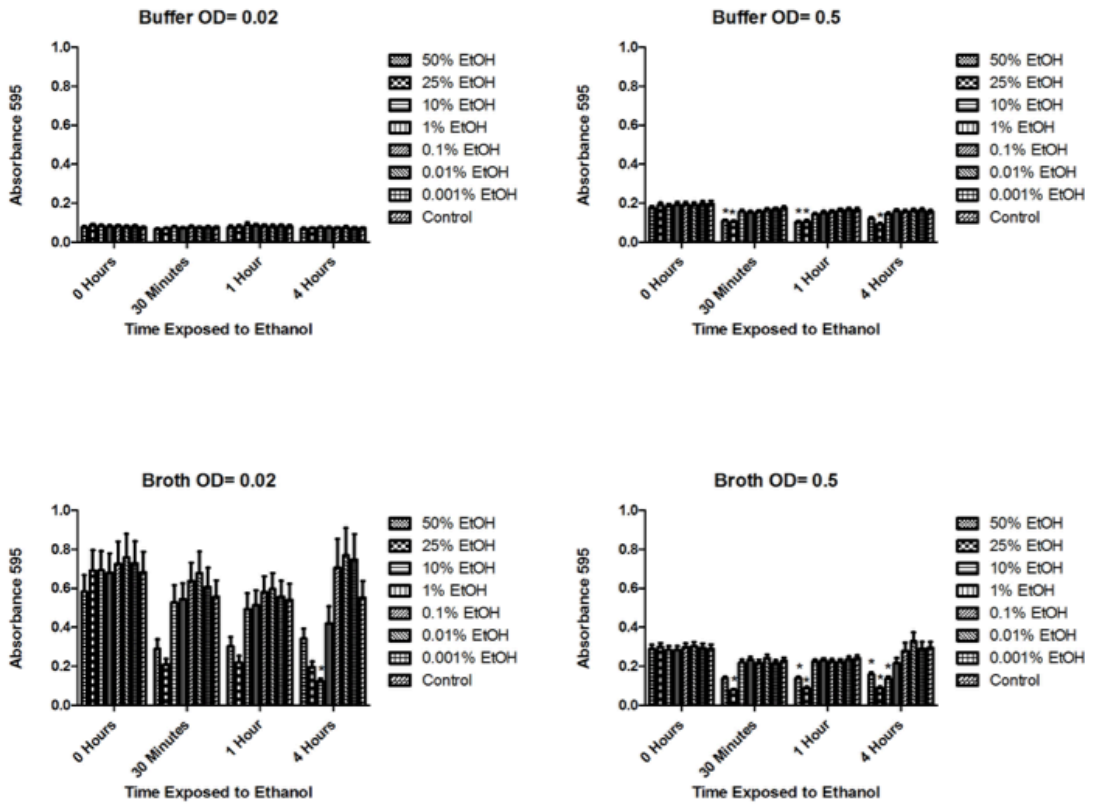


Figure 5: Optimization of *P. gingivalis* Biofilms (n=6).

Biofilms were grown overnight in either 1x PBS or 0.5x TSB complete, treated with ethanol, stained using crystal violet, and the absorbance was measured at 595 nm. Asterisks designate significant differences between absorbances for each ethanol concentration at relative to the broth control. Results from this set of experiments were used to optimize the conditions for subsequent experiments, which used 2 x 10^7 *P. gingivalis* cells/mL in 0.5x TSB complete. ANOVAs were performed the cutoff for statistical significance at $p<0.05$ (*).

anaerobically on a 3D rocker for 24 hours hours. Following overnight incubation, the supernatants were discarded, leaving *P. gingivalis* in a biofilm at the bottom of each experimental well [150]. The remaining biofilms were then treated with one of several concentrations of ethanol (0%, 0.01%, 0.1%, 1.0% or 10%) diluted into 0.5x TSB complete and incubated anaerobically for four hours with 3D rocking. The four-hour ethanol exposure time was chosen based on results from the experiments contained in Chapter 2 and preliminary data collected while optimizing the experiments contained within this chapter. Experimental wells were created using the same 10-fold serial dilutions used in the experiments described in Chapter 2, but this experiment used 0.5x TSB complete instead of 1x TSB complete.

Following the four-hour ethanol treatment, one set of plates was washed, stained with crystal violet, and the absorbance at 595 nm was measured. The other set was rinsed with 1x PBS and provided with sterile TSB complete for an additional 18-hour incubation period. Following overnight 3D rocking, the remaining biofilms were washed with 1x PBS and stained with crystal violet. Absorbances were measured at 595 nm to assess whether the *P. gingivalis* remaining in the biofilm after the various four-hour ethanol treatments were viable. Viable biofilms should increase in biomass between 24 hours and 48 hours when provided with fresh medium, while dead biofilms should not increase in biomass when provided fresh medium. This experiment was performed three times (n=3), and the data were averaged for further analysis.

Statistical analysis

The data were normalized by subtracting the broth control from the experimental values. Two-way ANOVA was performed in R across ethanol concentrations at 24 and 48 hours to determine if growth was significantly different. Statistical tests were performed on the resulting data. A p-value of 0.05 was used as the threshold for statistical significance.

Results

Physiological concentrations of ethanol do not inhibit *P. gingivalis* in biofilms

Absorbances of *P. gingivalis* after 24 hours of biofilm development were similar across ethanol concentrations from 0% to 1% (Figure 6). The absorbances associated with each ethanol concentration from 0.001% to 1.0% were not significantly different from the absorbance associated with 0% ethanol ($P>0.05$ for each comparison; Figure 6). These results indicate that *P. gingivalis* under the biofilm conditions were not adversely affected by four hours of exposure to ethanol concentrations ranging from 0.001% to 1.0%.

Absorbances after 48 hours of incubation (an additional 24 hours of after the four hours of ethanol exposure) were significantly greater than absorbances at 24 hours for ethanol concentrations from 0% to 1% (Figure 6). This difference indicates that *P. gingivalis* in the biofilm environment not only survived four hours of ethanol exposure at concentrations up 0.001 to 1%, but it also replicated at these concentrations over the 48-hour study period (Figure 7, red bars). The consistently lower absorbances associated with 10% ethanol indicate that this concentration killed *P. gingivalis* in biofilms (Figure 7).

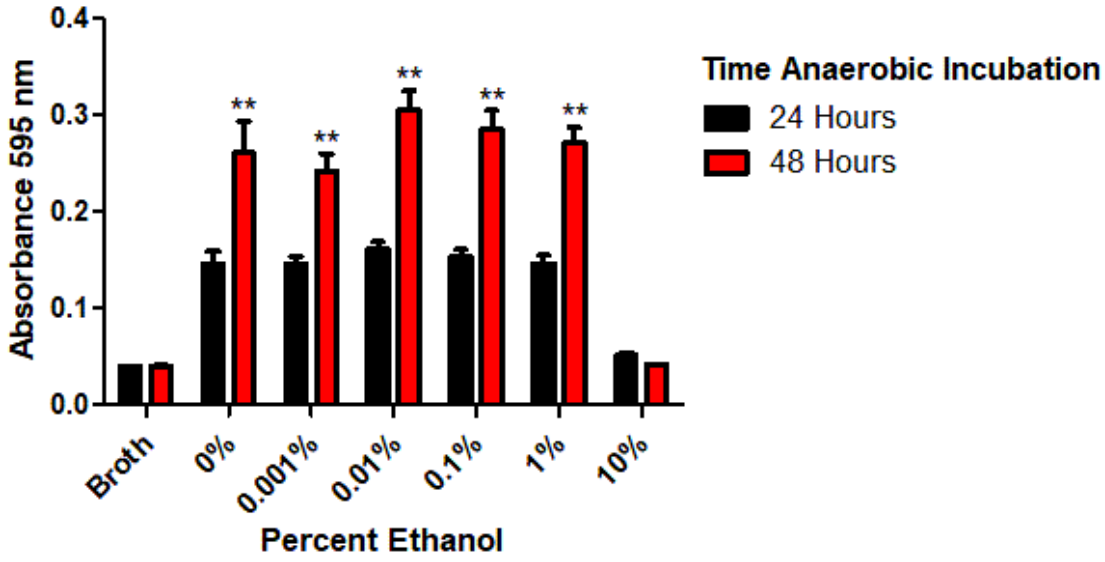


Figure 6: *P. gingivalis* live/dead biofilms (n=3).

Optical densities were measured after 4 hours ethanol treatment using a plate reader at 24- and 48-hours of biofilm development. Asterisks designate significant differences between absorbances for each ethanol concentration at 48 hours relative to 24 hours. ANOVA was performed the cutoff for statistical significance at $p<0.01$ (**).

Discussion

The results suggest that biofilms offer protection against ethanol exposure. In the planktonic experiments reported in Chapter 2, concentrations of ethanol ranging from 0.001% to 1.0% inhibited *P. gingivalis* after four hours of ethanol exposure. The experiment reported in this chapter indicates that, under biofilm conditions, these concentrations did not inhibit *P. gingivalis* after four hours of exposure.

Unlike larger molecules, ethanol can cross plasma membranes and penetrate biofilm [98, 151]. It therefore would be reasonable to hypothesize that ethanol could negatively affect *P. gingivalis* in biofilms. The results of this experiment indicate, however, that the *in vitro* biofilm does afford protection against the low-to-moderate ethanol concentrations that adversely affect planktonic *P. gingivalis*.

Several large-scale clinical trials and meta-analyses suggest that ethanol consumption increases risk for periodontal disease linearly with no protection associated with low levels of consumption [102, 147]. This association contrasts with the J-shaped curve that characterizes ethanol consumption and atherosclerosis risk. This difference would seem to negate the idea that moderate ethanol consumption reduces atherosclerosis risk by inhibiting *P. gingivalis*. The results of the experiments in Chapters 2 and 3, however, provide the basis for an alternative hypothesis: moderate ethanol consumption may protect against atherosclerosis by inhibiting planktonic *P. gingivalis* but may fail to protect against periodontal disease because the biofilm in which *P. gingivalis* is embedded protects against the antimicrobial effects of ethanol. In the absence of a protective antimicrobial effect,

even low levels of ethanol consumption may exacerbate periodontal disease. The increased damage from periodontal disease with increasing ethanol consumption presumably occurs because host defenses are increasingly compromised.

CHAPTER IV

CHRONIC DISEASES ASSOCIATED WITH PORPHYROMONAS GINGIVALIS

Introduction

Porphyromonas gingivalis is positively associated with several chronic diseases in addition to atherosclerosis: rheumatoid arthritis, osteoporosis, Alzheimer's disease, chronic kidney disease, and type 2 diabetes. If *P. gingivalis* contributes causally to any of these diseases as a result of systemic planktonic spread, the logic presented in this thesis suggests that moderate alcohol consumption might also be associated with protection. In this chapter, I discuss the evidence from the biomedical literature pertaining to associations of these chronic diseases with periodontal disease, *P. gingivalis*, and ethanol consumption. I use the J-shaped association between ethanol consumption and atherosclerosis presented in Chapter 1 as a benchmark for comparison. Specifically, this chapter assesses whether low-to-moderate ethanol consumption is associated with protection and high ethanol consumption is associated with exacerbation of each chronic disease, taking into account disease-specific pathologies (Figure 7).

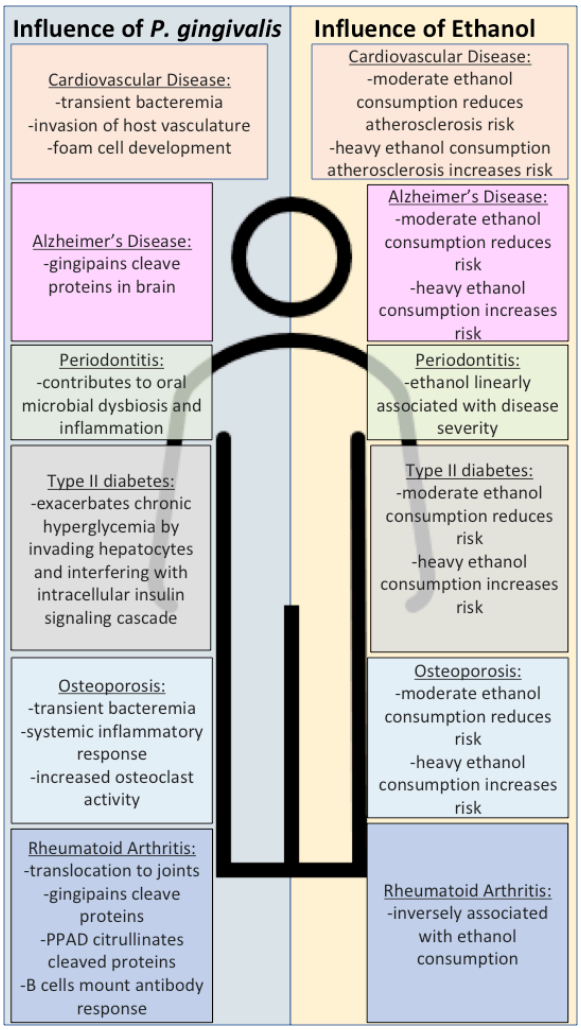


Figure 7. Contributions of *P. gingivalis* and ethanol to chronic diseases throughout the human body.

P. gingivalis invades host tissues throughout the body. The left panel provides mechanisms regarding *P. gingivalis*-mediated pathology, and the right panel describes how ethanol influences each chronic disease.

Rheumatoid Arthritis

P. gingivalis is thought to contribute to rheumatoid arthritis when it stimulates an autoimmune response in joints [152]. *P. gingivalis* produces gingipains, which cleave proteins at specific amino acid residues [153]. *P. gingivalis* then citrullinates cleaved proteins using peptidyl arginine deiminase (PPAD) [152].The immune system produces anti-citrullinated protein antibodies (ACPAs) in response to the citrullinated proteins [152]. Citrullinated proteins and the ACPAs are hallmarks of rheumatoid arthritis [152].

A meta-analysis found that people who consume ethanol were less likely to develop rheumatoid arthritis [154]. One hypothesis for this association is that ethanol suppresses immune function. Ethanol exposure may interfere with T cell function, dysregulating communication between T cells and B cells that would lead to antibody responses to extracellular infections [155]. Additionally, ethanol interferes with antigen presentation and cytokine production [156].

The general argument presented in this thesis provides an alternative hypothesis for the ameliorative effect of ethanol on rheumatoid arthritis by invoking inhibition of planktonic *P. gingivalis*. Rheumatoid arthritis is negative associated with amount of ethanol consumed rather than having a J-shaped association [154]. That is, even high levels of ethanol consumption are associated with amelioration of rheumatoid arthritis. Because rheumatoid arthritis is an autoimmune disease, ameliorative effects at all levels of ethanol consumption is consistent with both antimicrobial and immunosuppressive effects of ethanol occurring jointly: ethanol could be ameliorating rheumatoid arthritis in part by inhibiting *P. gingivalis* but the

inhibitory effects of ethanol on immune function could be ameliorating rather than exacerbating the immunopathology.

Osteoporosis

Osteoporosis is a disease characterized by decreased bone density, which typically occurs during the later decades of life, but it can occur earlier in association with calcium deficiency, use of certain pharmaceuticals, or chronic infection. Additional risk factors include cigarette smoking, sedentary lifestyle, and high alcohol consumption (more than seven standard drinks per week) [157]. Periodontitis is one infectious risk factor, as the causal pathogens are well known to stimulate bone resorption [158]. *P. gingivalis* may induce bone resorption when its LPS activates TLR-2 on osteoblasts, promoting periosteal osteoclast maturation that leads to pathogenic bone loss [159].

Experiments performed on rats demonstrated that periodontal disease contributes to increased severity of osteoporosis [158]. Researchers induced periodontitis using a ligature model over an eight-week period, while control rats did not receive ligation wires. The animals were sacrificed after eight weeks, and the fourth vertebra, the femur, the tibia, periodontal tissues, and blood were analyzed for evidence of osteoporosis, periodontal disease, or systemic pathogens, respectively. The researchers performed PCR on the periodontal silk and blood samples to look for nine periodontal pathogens. ELISA was used to quantify tumor necrosis factor alpha, interleukin 6, bone glutamic acid protein, and type 1 collagen C-terminal peptide (CTX-1). Bone resorption was measured using micro-computer

tomography. Bone histopathology was performed by staining tissues with hematoxylin-eosin (H & E) and tartate-resistant acid phosphatase.

Rats in the PD group displayed more bone resorption than controls. Staining revealed bone-resorbing osteoclasts in the PD group but not in the control group. Pro-inflammatory cytokines and CTX-1 were significantly more elevated in rats with PD than in control rats. Rats in the PD group also displayed more bone resorption and more osteoclasts than WT controls. *Porphyromonas gingivalis* was one of several pathogens detected in blood samples and from silk ligatures from sacrificed mice with experimentally induced PD [158]. Taken together, this study provides evidence for the association of *P. gingivalis* infection and subsequent bone loss *in vivo*.

Studies suggest that low ethanol consumption may improve bone mineral density, while heavy ethanol consumption decreases bone mineral density [160]. Different types of alcoholic beverages seem to affect bone physiology differently; distilled liquor is more damaging than beer or wine [160], perhaps because distilled liquor can be associated with higher ethanol levels. The effects of moderate ethanol consumption seem to depend largely on age, sex, and lifestyle. For example, moderate alcohol may increase bone density in men and postmenopausal women but not in premenopausal women [161]. Ethanol may not increase bone density in premenopausal women because they have elevated antibody responses relative to postmenopausal women [162 {Fish, 2008 #6599, 163]. A reliance on humoral immunity in premenopausal women is one hypothesis to explain this association if

planktonic bacteria are controlled sufficiently well by antibodies in the absence of ethanol.

Alzheimer's Disease

Olsen and Singhrao reviewed potential mechanisms by which *P. gingivalis* can contribute to Alzheimer's disease [164]. Gingipains damage tau proteins and contribute to tangle formation [165], a hallmark of Alzheimer's disease. If damaged tau proteins are a contributing cause of Alzheimer's disease rather than a side effect, then this damage may be a mechanism by which *P. gingivalis* contributes to Alzheimer's disease. Gingipains appear to enhance growth of *P. gingivalis* in the brain. Small-molecule inhibitors of gingipains, termed Kgps, reduce the bacterial load in the brain and prevent some neuronal damage [165].

Plaques of amyloid beta in the brain are another hallmark of Alzheimer's disease [166]. Amyloid beta has antimicrobial effects against *P. gingivalis* [165]. Higher levels of amyloid beta in the brains of people with Alzheimer's disease may be a response to *P. gingivalis* in neuronal tissue; increased beta amyloid might itself then cause neuronal damage and/or be a side effect of damage caused by *P. gingivalis*. Elevated levels of amyloid beta seem to be associated with a reduction in living bacteria, most likely due to the effects of amyloid beta on membrane integrity [165].

Light to moderate ethanol consumption appears to reduce risk of developing Alzheimer's disease, particularly in people who do not carry the ApoE4 allele [167]. Heavy ethanol consumption, together with positivity for the ApoE4 allele, increase

risk of developing Alzheimer's disease [167]. These results accord with the hypothesis that planktonic *P. gingivalis* plays a role in sporadic Alzheimer's disease and that moderate ethanol intake confers some protection.

Chronic Kidney Disease

Chronic kidney disease is diagnosed either by finding renal damage or by measuring a decline in filtration over three months. People with periodontal disease have an increased risk for developing chronic kidney disease [168, 169]. An association between subgingival periodontal pathogen detection and severity of kidney disease suggests that systemic infections play a role the development or exacerbation of renal disease [168, 170]. Some researchers report that elevated antibody production against *P. gingivalis* is associated with reduced kidney function [170, 171], although one study found an inverse relationship between antibodies against periodontal pathogens and chronic kidney disease [172].

Periodontal disease is associated with renal insufficiency, defined as a glomerular filtration rates less than 60 mL/min/1.73 m^2 [173].
One possible pathogenic mechanism could involve infection of the glomerulus or renal artery. A recent review (2019) suggests that *P. gingivalis* contributes to atherosclerotic clot formations in renal vasculature; by this mechanism periodontitis contributes to atherosclerosis, and atherosclerosis causes chronic kidney disease [174]. Pathological outcomes involve occluded vasculature and blood clot formation, both of which decrease renal perfusion.

Ethanol consumption has a J-shaped risk profile relative to chronic kidney disease; lower consumption reduces risk and high consumption increases risk relative to nondrinkers [175, 176]. If *P. gingivalis* plays a role in chronic kidney disease, then ethanol may protect the kidneys from infection until the point at which heavy concentrations of ethanol generate renal damage that outweighs any beneficial effect of inhibiting *P. gingivalis*.

Type 2 Diabetes

Periodontitis risk and severity is elevated in type 1 [177] and type 2 diabetics [178]. Diabetes diagnosis requires evidence of chronic hyperglycemia, typically involving glycated hemoglobin, fasting plasma glucose, random glucose tests, or an oral glucose tolerance test [179].

Experimental approaches demonstrate the multifactorial nature of insulin resistance, as evidenced by dietary, genetic, and infectious contributions. High-fat [180] and high-sugar diets [181] exacerbate insulin resistance [180] and alveolar bone loss [181, 182] in periodontitis-diabetes comorbid mice. Moreover, mice with experimentally induced periodontitis and diabetes develop atherosclerotic lesions that contain *Porphyromonas gingivalis* [181]. Systemic *P. gingivalis* infects the liver and is able to invade hepatic cells, where it may alter glucose uptake or metabolism [183].

Porphyromonas gingivalis contributes to insulin resistance by inhibiting insulin receptor substrate 1 (IRS1), Akt, and glycogen synthase kinase-3 beta (GSK-3B) phosphorylation, each of which are involved in the response to the binding of

insulin to its receptor [183]. Disrupted insulin signal transduction leaves diabetics unable to import and store blood glucose levels because cells fail to appropriately translocate GLUT 4 transporters to the cell's surface and the liver fails to synthesize glycogen [183].

Although *P. gingivalis* has been thought to be unable to use glucose, the emerging sense is that it uses glucose facultatively depending on microenvironmental conditions [184]; moreover, chronic hyperglycemia may favor species in the oral microbiome that may, in turn, favor *P. gingivalis*. Saccharolytic streptococcal species colonize sugar-protein dental pellicles and provide "landing zones" for incoming microbiota. *Streptococcus gordonii* binds with the major and minor fimbriae of *P. gingivalis* and thus fosters the ability of P. gingivalis to persist in biofilms [132, 185]. These findings emphasize the importance of considering relationships between diabetes and microbial species that interact with *P. gingivalis* in the oral microbiome when assessing the possibility that ethanol could ameliorate the negative effects of diabetes.

GLUT 2 transports glucose bidirectionally across the intestines, kidneys, liver, and pancreas. Hyperglycemia induces gut permeability and systemic microbial dissemination. Glucose enters intestinal cells via GLUT2 from the intestinal lumen and from systemic circulation; intracellular protein glycosylation then leads to chronic inflammation [186]. Enzymes glycosylate proteins and lipids, whereas glycation occurs non-enzymatically in blood by chance interactions.

Irreversible Advanced Glycation End products (AGEs) result when proteins or lipids are exposed to glucose, resulting in glycation [187]. AGEs are used as a

diagnostic marker for type 2 diabetics because chronic hyperglycemia results in protein or lipid glycation. Considerations of the interplay between periodontitis and the AGEs implicate contributions of periodontitis to diabetes and hence the potential for amelioration of diabetes through inhibition periodontal pathogens (e.g., through ethanol consumption). Diabetics with severe periodontitis accumulate more AGEs than diabetics with less advanced periodontitis, suggesting that periodontal pathogens may promote AGE formation [178].

Glycated proteins and lipids fail to function properly and may eventually become pro-inflammatory AGEs [187]. AGEs are recognized by the AGE receptor (RAGE), which stimulates the NF-kB inflammatory pathway when activated. AGEs may thus contribute to inflammation-associated pathology of diabetes. Oral AGEs may also enhance the success of *P. gingivalis* in biofilms by providing non-dental colonization sites for streptococci such as *S. gondonii*..

Treatment of periodontitis provides additional evidence that periodontal pathogens contribute to type 2 diabetes. Treating periodontitis with systemic antibiotics reduces probing depth, subgingival *Porphyromonas gingivalis* colonization, and serum glycated hemoglobin [188].

A 2016 compilation of fourteen meta-analyses [189] provides a large body of evidence that periodontal treatment reduces circulating levels of glycated hemoglobin. Only one study [190] found HbA1c reductions in response to treatment of periodontal disease that exceeded one percent. Such reductions are associated with improved vascular health and lower risk of mortality caused by diabetes or myocardial infarction [189]. This analysis suggests that periodontal pathogens

contribute to the chronic hyperglycemia conditions that promote HbA1c formation, and removing pathogens from the oral cavity reduces this process.

A meta-analysis including nearly two million subjects suggests that moderate ethanol consumption reduces risk of developing type 2 diabetes [191]. The peak risk reduction was reached at about one standard drink per day, and the risk of developing type 2 diabetes increased after about five standard drinks per day [191]. Consumption of one standard drink per day reduced diabetes risk by 18% relative to nondrinkers. This finding is consistent with the J-shaped relationship between ethanol consumption and atherosclerosis.

Overview of Ethanol and Chronic Diseases associated with *P. gingivalis*

This chapter assessed whether chronic diseases that are associated with *P. gingivalis* are ameliorated in people who consume ethanol. For each of the five chronic diseases characterized by pathology outside of the oral cavity, low-to-moderate ethanol consumption was associated with protection. These findings contrast with the progressively increasing exacerbation of periodontal disease that is associated with increased ethanol consumption. These findings accord with the idea that ethanol has inhibitory effects on *P. gingivalis* when it is planktonic (Chapter 2) but not when it is embedded in its periodontal biofilms (Chapter 3). For five of these chronic diseases-- atherosclerosis, chronic kidney disease, Alzheimer's disease, osteoporosis and type 2 diabetes—evidence implicates a J-shaped curve: amelioration associated with moderate ethanol consumption but exacerbation for high ethanol consumption. In contrast, rheumatoid arthritis appears to be

ameliorated across the spectrum of ethanol consumption. This difference could be related to the tendency for rheumatoid arthritis to be an autoimmune disease, with the inhibition of *P. gingivalis* by high levels of ethanol consumption being supplemented by suppression of an autoimmune pathology.

If ethanol ameliorates these chronic diseases outside the oral cavity by suppressing *P. gingivalis,* it could do so directly by reducing the ability of *P. gingivalis* to cause tissue damage that leads to the diseases; for example, *P. gingivalis* could contribute to chronic kidney disease by damaging the glomerulus. However, comorbidities of these diseases raise the possibility that effects could be indirect; for example, *P. gingivalis* could cause atherosclerosis of the renal artery that could, in turn, damage kidney function. Chronic infections may take decades to diagnose, and bacteremia can lead to infections throughout various organs. Therefore, it may useful to determine the sequence of chronic disease development; for example, periodontitis may cause atherosclerotic plaque of the renal artery, which in turn may chronic kidney disease as a result of hypoperfusion through the renal artery [174].

A related point is that ethanol may suppress the early development of particular chronic diseases. Future studies could consider, for example, how the combination of ethanol and *P. gingivalis* influences foam cell formation, an aspect of the early pathogenesis of atherosclerosis. *P. gingivalis* can induce foam cell formation, and moderate ethanol consumption is associated with reduced risk of atherosclerosis,; however, the influence of ethanol on foam cell formation has not been investigated.

The elevated blood glucose associated with type 2 diabetes may result in increased oral biofilm formation which in turn could generate more planktonic *P. gingivalis* which could cause an upward spiral in glucose levels and diabetes severity if unchecked by ethanol. The elevated glucose might also exacerbate other systemic infections that play a role in these chronic diseases (e.g., *C. pneumoniae* in atherosclerosis and Alzheimer's disease or Hepatitis C in type 2 diabetes).

Concluding Remarks

It is evident that the association between oral and systemic disease requires a great deal of attention moving forward. Elucidating disease etiology facilitates treatment and prevention strategies. *P. gingivalis* can infect a variety of human tissues, some of which may require specialized drugs depending on where the infection localizes. For example, a systemic antibiotic may be useful for bacteremia but do nothing to treat a brain infection. Small molecule inhibitors are one strategy that can be utilized without generating antibiotic resistance. Public health measures to maintain oral hygiene may also be a helpful strategy.

Regarding how ethanol consumption affects chronic disease biology, moderate amounts of ethanol may protect the consumer if the ethanol negatively impacts infectious agents more than it damages the host. It seems counter-intuitive that a poison would reduce risk for developing a chronic disease until one considers the etiological events that produce disease. Ethanol travels systemically, but it becomes diluted as it is absorbed into the bloodstream. These diluted volumes of

ethanol may be elevated enough to curb pathogenicity but low enough to avoid damaging host tissues.

Studies concerning the ability of *P. gingivalis* to evolve ethanol tolerance could also be valuable. Oral pathogens may adapt to increasingly elevated ethanol concentrations over decades of alcohol use, and such organisms could pose a greater risk to systemic dissemination if they evolve tolerance mechanisms.

The value of this dissertation lies in the synthesis of new ideas related to how the most widely used drug (ethanol) may influence the leading cause of death worldwide, heart disease, as well as other chronic diseases. The use of ethanol as a systemic antimicrobial agent with regards to chronic infectious diseases has generally been overlooked. The hypothesis that ethanol consumption suppresses *P. gingivalis* growth in the blood, but not the oral cavity, is supported by experiments and review of the literature presented in this dissertation.

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CURRICULUM VITA

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EDUCATION

PhD Candidate — **University of Louisville- Louisville, KY**
Department of Biology
Advisor: Paul W. Ewald, PhD Lab: Richard J. Lamont, PhD

Bachelor of Arts — **University of Louisville- Louisville, KY**
Graduated: 2012 — Department of Anthropology (natural sciences), minor Biology
Advisor: Fabián Crespo, PhD

ACADEMIC AWARDS AND HONORS

2017 **Faculty Favorites nominee**
University of Louisville Delphi Center

2016 **William Furnish Teaching Award** ($500)
University of Louisville Biology Department

2016 **Graduate Student Service Award** ($150)
University of Louisville Biology Department

2016 **Best 3-Minute Thesis presentation**
University of Louisville Graduate Student Council Regional Research Conference

2014 **Best overall presentation and best presentation in session**

University of Louisville Graduate Student Council Research Symposium

TEACHING EXPERIENCE

Adjunct Professor

Indiana University Southeast

Spring 2022	Physiology 215, Basic Human Physiology (Lecture and Laboratory)

Bellarmine University, Department of Biology

Spring 2022	Biology 317, Molecular Biology Laboratory
Spring 2022	Biology 130, Principles of Biology Laboratory
Fall 2021	Biology 314, Vertebrate Physiology Laboratory
Fall 2021	Biology 231, Cell Biology Laboratory
Fall 2021	Biology 108, Anatomy & Physiology Laboratory
Spring 2021	Biology 317, Molecular Biology Laboratory
Spring 2021	Biology 260, Genetics Laboratory
Fall 2020	Biology 130, Principles of Biology Laboratory

Spalding University, Department of Natural Sciences

Winter 2020	Biology 256, Microbiology

Graduate Teaching Assistant

University of Louisville, Department of Biology

Spring 2020	Biology 262, Anatomy and Physiology Laboratory
Fall 2019	Biology 240, Unity of Life Recitation
2018-2019	Biology 262, Anatomy and Physiology Laboratory
2017-2018	Biology 262, Anatomy and Physiology Laboratory

2016-2017	Biology 262, Anatomy and Physiology Laboratory
2014- 2016	Biology 258, Introduction to Microbiology Laboratory
Spring 2014	Biology 244, Introduction to Biology Laboratory

Academic Tutor

University of Louisville, REACH (resources for academic achievement)

2013	CRLA level III Master Tutor certification
2012- 2013	REACH tutor peer-supervisor
2013	Biology 242, Diversity of Life
2012	Anthropology 314, Underwater Archaeology
2012	Anthropology 202, Introduction to Biological Anthropology
2011	Anthropology 202, Introduction to Biological Anthropology

GRANTS FUNDED

2017	$350 travel grant University of Louisville Graduate Student Council
2017	$100 travel grant University of Louisville Biology Graduate Student Association
2016	$300 travel grant University of Louisville Graduate Student Council
2016	$150 travel grant University of Louisville Engage Lead Serve Board
2016	$150 travel grant University of Louisville Biology Graduate Student Association
2015	$300 research grant University of Louisville Graduate Student Council
2015	$250 travel grant University of Louisville Graduate Student Council

2015 $150 travel grant
University of Louisville Biology Graduate Student Association

2015 $100 research grant
University of Louisville Graduate Student Union

2015 $100 research grant
University of Louisville Biology Graduate Student Association

INVITED PRESENTATIONS

2019 Guest instructor for Biology 372, Chronic Disease Biology
University of Louisville, Fall 2019

2017 3-Minute Thesis information and demonstration for Graduate Seminar in Department of Biology at University of Louisville

2016 Guest instructor for Anthropology 353, Evolutionary Anthropology
University of Louisville, Fall 2016
"Ecological Immunology". 21 March 2016
"Evolutionary Medicine". 23 March 2016

2015 Radio Interview with Mark Hebert, University of Louisville UofL Today
https://soundcloud.com/uofl/dec-15-2015-uofl-today-nik-short-gems-lila-lutfi

2015 "Ethanol's influence on *Porphyromonas gingivalis*: proliferation and colonization." University of Louisville Oral Biology Student Research Group, Louisville, KY.

PRESENTATIONS

2017 **Short NA**, Lamont RJ, Ewald PE. "Effects of ethanol on *Porphyromonas gingivalis* planktonic and biofilm monocultures." Human Biology Association annual conference 2017; American Association of Physical Anthropologists annual meeting 2017, New Orleans, LA. (poster)

2017 **Short NA**. "Bacteria with a drinking problem." University of Louisville 3-Minute Thesis competition. Louisville, KY. (podium)

2017 **Short NA**. "Bacteria with a drinking problem." Conference of Southern Graduate Schools 3-Minute Thesis regional finals. Annapolis, MD. (podium)

2016 **Short NA**, Lamont RJ, Ewald PE. "Effects of ethanol on *Porphyromonas gingivalis* in planktonic and biofilm monocultures". Kentucky Academy of Sciences annual meeting. Louisville, KY. (podium)

2016 **Short NA**, Lamont RJ, Ewald PE. "Effects of ethanol on *Porphyromonas gingivalis* planktonic and biofilm monocultures". Research! Louisville. Louisville, KY. (poster)

2016 **Short NA**, Lamont RJ, Ewald PE. "Just one drink". University of Louisville Graduate Student Council regional research conference 3-Minute Thesis. Louisville, KY. (podium)

2016 **Short NA**, Lamont RJ, Ewald PE. "Moderate alcohol consumption influences chronic disease risk". University of Louisville Biology Department awards day. Louisville, KY. (podium)

2015 **Short NA**, Lamont RJ, Ewald PE. "Proliferation response to ethanol: *Porphyromonas gingivalis*." Human Biology Association annual conference 2015, St. Louis, MO. (podium, abstract published)
American Journal of Human Biology 27(2):287. February 2015

2015 **Short NA**, Lamont RJ, Ewald PE. "Proliferation response to ethanol: *Porphyromonas gingivalis*." American Association of Physical Anthropology annual conference 2015, St. Louis, MO. (poster, abstract published)
American Journal of Physical Anthropology 156 (S60): 287. March 2015

2015 **Short NA**, Lamont RJ, Ewald PE. "The consequences of drinking." University of Louisville Biology Department awards day, Louisville, KY. (podium)

2014 **Short NA**, Lamont RJ, Ewald PE. "Proliferation response to ethanol: *Porphyromonas gingivalis*." Ohio Valley Society of Toxicology annual meeting 2014, Louisville, KY. (podium)

2014 **Short NA**, Lamont RJ, Ewald PE. "Proliferation response to ethanol: *Porphyromonas gingivalis*." Graduate Seminar, Louisville, KY. (podium)

2014 **Short NA**, Lamont RJ, Ewald PE. "Proliferation response to ethanol: *Porphyromonas gingivalis*." University of Louisville Graduate Student Council's Graduate Student Research Symposium 2014, Louisville, KY. (podium)

2013 **Short NA**, Keeton K, Fernandez-Botrán R, Crespo F. "Phenotypic plasticity in humans: Lessons from the immune system." American Association of Physical Anthropology annual meeting, Knoxville, TN (podium, abstract published)
American Journal of Physical Anthropology 150 (S56): 254-255. February 2013

2012 **Short N**, Keeton K, Fernandez-Botrán R, Crespo F. "Differential immune responses in human populations: searching for evolutionary clues." American Association of Physical Anthropologists undergraduate symposium, Portland, OR (poster)

2012 **Short N**, Keeton K, Fernandez-Botrán R, Crespo F. "Differential immune responses in human populations: searching for evolutionary clues." University of Louisville undergraduate symposium, Louisville, KY (poster).

PROFESSIONAL DEVELOPMENT, SERVICE, AND ACTIVITIES

2021 Attended Gross Anatomy with Dr. David Porta at Bellarmine University

2018-2019 Femur extraction with Dr. David Porta for cadaver workshop at Bellarmine University (HAPS-AACA regional meeting 2019)

2016-2017 Created Anatomy and Physiology YouTube channel

Biology Graduate Student Association outreach chair

AAPA undergraduate symposium mentor

Louisville Regional Science and Engineering Fair mentor for two students

Youth Science Summit volunteer

Planted trees in Old Louisville with Second Street Neighborhood Association

Sustainability Council student representative

BioBlitz in Lincoln Boyhood Memorial National Park

Kentucky Science Center exBEERiment volunteer

2015-2016 Biology Graduate Student Association president and outreach chair

Graduate Student Council internal vice-president and Biology Department representative

Mentor and science advocate with Science Policy and Outreach Group

Louisville Regional Science and Engineering Fair mentor for two students

Youth Science Summit volunteer (speed-mentor; microbiology demo)
Gear-Up! Kentucky volunteer (human evolution presentation)
Healthworks at KY Science Center volunteer (brain demo for all ages)

Organized first 3-Minute Thesis competition at University of Louisville

Engage Lead Serve Board executive council, graduate representative

Invited Dr. Jan Potempa, PhD to present at Biology departmental seminar series

Bike MS volunteer (provided cyclists with water and food)

2014-2015 Biology Graduate Student Association vice-president and treasurer

Graduate Student Council Biology Department representative

Edited manuscript for international post-doctoral fellow

Educational outreach project with local Seneca high school students (microbiology demo)

Annual spring clean at Beckley Creek, Louisville, KY

2013 i2a (ideas to action) project for Anthropology department to evaluate critical thinking assessment within multiple-choice exams

MEMBERSHIPS

American Association of Physical Anthropologists
Human Biology Association
Human Anatomy and Physiology Society
Kentucky Academy of Sciences
Society for Neuroscience

REFERENCES

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