The Impact of Antibiotics and HAMLET on Microbial Ecology: From *Ex Vivo* to *In Vivo* Studies

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Abbreviations

ALA	Alpha-lactalbumin
AMR	Antimicrobial Resistance
ARG	Antimicrobial Resistance Gene
BMM	basal medium mucin
Bp	Base pair
CNS	Central nervous system
CARD	Comprehensive Antibiotic Resistance Database
DNA	Deoxyribonucleic acid
EPS	Extracellular polymeric substance
EDTA	Ethylenediamine tetra-acetic acid
eDNA	Extracellular DNA
FOTS	Norwegian Food and safety Authority
FMT	Fecal microbiome transplantation
HAMLET	human alpha-lactalbumin made lethal to tumor cells
HGT	Horizontal Gene Transfer
HMP	The Human Microbiome Project
HMO	human milk oligosaccharide
HOMD	Human Oral Microbiome Database
MIC	Minimum Inhibitory Concentration
MRSA	Methicillin-resistant *Staphylococcus aureus*
NAM	N-acetylmuramic acid
NGS	Next Generation Sequencing
OA	Oleic Acid
PCR	Polymerase Chain Reaction
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
WHO	World Health Organization
QS	Quorum sensing

Introduction

1. Background

1.1 Antimicrobial Resistance

Infectious diseases have played a significant role in human history, impacting both mortality and morbidity. The advent of antibiotics in the early 20th century marked a transformative milestone in healthcare, contributing considerably to increased life expectancies through a marked reduction in deaths by infectious diseases (1). Antibiotics have since become a cornerstone of medical practice, essential in the treatment and prevention of infections (2).

However, today's medical landscape faces the concerning issue of antimicrobial resistance (AMR). This global health crisis manifests when bacteria, formerly susceptible to antibiotics, evolve to resist their effects (1). The recent report, “No Time to Wait: Securing the Future from Drug–Resistant Infections”, jointly released by the World Health Organization (WHO) and the United Nations Interagency Coordination Group (IACG), highlights the imminent threat of entering a post-antibiotic era in the 21st century, leaving no room for delay in finding novel approaches to treat or prevent disease with resistant organisms (3).

The present situation exhibits the emergence of hard-to-treat infections caused by multidrug resistant organisms. Without effective infection control measures, critical medical procedures such as organ transplantation, cancer treatments, and major surgeries become considerably unsafe (1, 4). Additionally, there is an increased risk of future pandemics driven by antibiotic-resistant pathogens (1, 4). Finally, if the medical community does not act to counter antibiotic resistance, minor infections will again become life threatening, similar to the pre-antibiotic era.

1.2 History

The precise historical origins of antimicrobial agents remain obscured by time. However, evidence from the literature indicates that various forms of antimicrobial agents have been used worldwide for centuries. Over millions of years of evolution, microorganisms have naturally produced antibiotic molecules (5). Interestingly, these molecules not only play a crucial role in bacterial communication but also carry out other metabolic functions. In the environment, the concentrations of these antimicrobial molecules vary considerably, ranging from levels below the minimum inhibitory concentrations (MIC) for bacteria to concentrations sufficient to compete with and eliminate other microorganisms (6-8).

The modern European history of antimicrobials dates back to 1900 when German medical scientist Paul Ehrlich (1854-1915) had the groundbreaking idea of selectively targeting disease-causing microbes in the body without harming the body itself. In 1909, Ehrlich, and his colleagues Alfred Bertheim and Sahachiro Hata, developed the first 'magic bullet' known as 'Salvarsan.' This drug was employed to treat syphilis, albeit with several side effects, until the discovery of penicillin and other novel antibiotics in the mid-20th century (9, 10). In 1935, another pivotal moment occurred when German pathologist Gerhard Domagk (1895-1964) developed the first sulfonamide, named 'Prontosil,' which effectively combated streptococcal infections (2, 11).

The serendipitous discovery of penicillin by Sir Alexander Fleming (1881-1955) in 1928 marked a significant breakthrough in medical science (12). It wasn't until 1945 that this drug became available on the market, marking the commencement of the 'Antibiotic era' that revolutionized the treatment of infectious diseases worldwide. Before this time, diseases such as pneumonia and minor infections could often lead to death due to septicemia. In an interview from 1945 with The New York Times, Fleming cautioned that a substantial number of *Staphylococcus aureus* strains had already developed resistance to penicillin (13). The discovery of these first three antimicrobials; Salvarsan, Prontosil, and Penicillin served as exemplary milestones, setting the stage for future drug discovery research (2). The period between 1945 and 1962 is often referred to as the ‘Golden Era‘ in antibiotic history, witnessing significant developments across various antibiotic classes such as tetracycline, chloramphenicol, aminoglycoside, macrolide, glycopeptide, fluoroquinolone, cephalosporin, carbapenem and rifamycin (14, 15). Since that time, few new antibiotic classes have been developed.

1.3 Antibiotics

Antibiotics are drugs that possess the ability to inhibit bacterial growth or even eliminate bacteria, thus aiding the body's immune system in combating bacterial infections (16). These essential pharmaceuticals can be categorized based on various criteria, including their molecular structures, spectral coverage (broad or narrow), targeting characteristics based on cell wall structure (gram-positive or gram-negative), bactericidal or bacteriostatic action, and specific mechanisms of action or target sites (17).

The primary target sites within bacterial cells can be broadly categorized into four major classes **(Figure 1)** (18):

1. DNA/RNA Metabolism: Antibiotics such as fluoroquinolones, sulfonamides, and trimethoprim exert their effects by disrupting processes related to the synthesis of DNA and RNA.
2. Cell Wall Biosynthesis: This category includes well-known antibiotics, such as beta-lactams (e.g., penicillin, such as ampicillin and amoxicillin), cephalosporins, and carbapenems, which specifically target the construction of bacterial cell walls.
3. Components of Translational Machinery: Antibiotics, such as aminoglycosides, tetracyclines, macrolides, and chloramphenicol act by interfering with various components of the bacterial translational machinery, such as ribosomes, thereby affecting protein synthesis.
4. Other Cellular Components: Some antibiotics, such as polymyxin, impact various cellular components beyond the previous categories, disrupting vital bacterial functions in different ways.

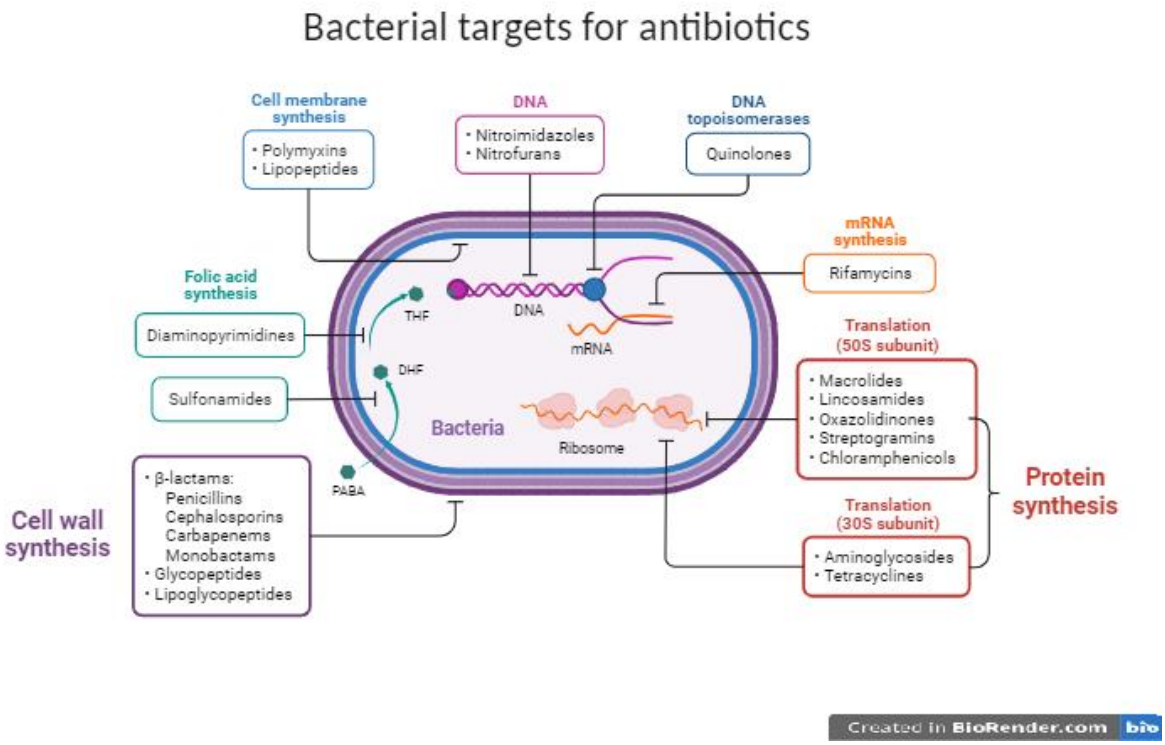

Figure 1: An overview of target sites for different antibiotics within bacterial cells. Created in BioRender.com

1.4 Drivers of AMR

AMR refers to the ability of microorganisms, including bacteria, viruses, fungi, and parasites, to develop mechanisms that render antimicrobial drugs ineffective against them (19). This resistance can occur through intrinsic resistance mechanisms, from de novo mutations or the acquisition of resistance mechanisms from the environment (20, 21). The consequences of AMR include prolonged illness, increased disability, and, in the worst cases, death.

AMR is a natural evolutionary response to the exposure of antimicrobials. However, a significant source of AMR selection arises from increased antimicrobial exposure in healthcare, agriculture, and the environment (22). Animals have been identified as major drivers of AMR, as antimicrobial resistance genes (ARGs) can transfer to human pathogens through various mechanisms (22). Antibiotics are extensively used in animal husbandry, with a larger proportion allocated to non-therapeutic purposes (22, 23). Additionally, high levels of antibiotic exposure can be found in various settings such as the food production chain, wastewater, and soil samples (22). The One Health approach serves as a crucial framework that establishes connections among various consumers of antimicrobials, including humans, animals, agriculture, plants, food, soil, water, and the environment (24). It adopts a multidisciplinary and holistic perspective to address complex health challenges more effectively. This approach encompasses the development of surveillance systems that reflect the interconnected nature of global challenges such as AMR, climate change, and biodiversity loss.

Educating both communities and governmental bodies plays a vital role in fostering awareness regarding how individuals can minimize the spread of AMR within their respective domains (25, 26). By recognizing the broader implications of AMR and adopting a collaborative One Health approach, we can better navigate this multifaceted health concern.

1.5 AMR Mechanisms

AMR mechanisms can be categorized into three main groups.

Table 1: Overview of Key Antimicrobial Resistance Mechanisms

Mechanism:	**Function**	**Reference:**
1. Intrinsic mutations	Spontaneous genetic mutations in microbial DNA that can impact the target site of antimicrobial drugs or alter metabolic pathways.	(27-29)
2. Extrinsic/acquired resistance	Bacteria acquire resistance to antibiotics by obtaining resistance genes. This category includes horizontal gene transfer (HGT) via three main mechanisms:	(27, 29)
	1. *Conjugation*: Direct transfer of plasmids containing resistance genes from one bacterium to another.	(27-29)
	2. *Transduction*: Transfer of resistance genes with the help of bacteriophages from one bacterium to another.	(27-29)
	3. *Transformation*: Bacteria take up resistance genes from their environment.	(27-29)
3. Adaptive resistance	Microorganisms produce enzymes responsible for the degradation or modification of antimicrobial drugs. Examples include beta-lactamases, which break down beta-lactam antibiotics like penicillin and cephalosporins. Adaptive resistance mechanisms impact antibiotic effectiveness through:	(27-29)

	a. *Decreased Antibiotic Penetration*: Reducing the drug's effectiveness and concentration.	(27-29)
	b. *Efflux Pumps*: Transport proteins specialized in pumping antimicrobial drugs out of bacterial cells before they can take effect.	(27-29)
	c. *Changes in Target Sites*: Protective measures or modifications to antibiotic target sites to reduce affinity.	(27-29)
	d. Biofilm Formation: The development of bacterial communities encased in a protective matrix that acts as a physical barrier, preventing antimicrobial drugs from reaching the bacteria and increasing the MIC for effective treatment.	(30, 31)

2. The Human Microbiome and Microbiota

The human body is home to a vast number of symbiotic microbial cells, estimated to range from 10 to 100 trillion, roughly equivalent to the number of human cells (32, 33). These microorganisms include various types such as bacteria, viruses, fungi, archaea, and other microorganisms. They form site-specific niches within the body, with distinct microbial communities found in different areas, such as the skin, oral cavity, respiratory tract, genital tract, and gastrointestinal tract (34). These niche-specific microbial communities, often referred to as microbiomes, perform crucial functions essential for our well-being. Key functions of the human microbiome includes: protective barrier against overgrowth of harmful pathogens (35), development and function of the immune system (36), endocrine functions within the body (37), digestion and absorption of nutrients (38-40), synthesize essential vitamins (38, 39), regulate inflammation (35) and contribute to maintaining a healthy metabolism (40).

2.1 Microbiome vs. Microbiota

In the scientific literature, two main terms are used to describe the microorganisms residing in the human body, which can sometimes lead to confusion. "Microbiome" has been defined as the catalog of human microbes and their genes (32). More recently, the definition was expanded to include their activities, rather than centering on the genes alone (41). On the other hand, "microbiota" refers to the specific microbial taxa associated with humans. Microbiota is often niche-specific, meaning it relates to particular sites or habitats in the human body. The two terms are often used interchangeably. In this thesis I will use the term microbiome as proposed by the Waterloo Centre for Microbial Research expert panel in 2020 (41).

2.2 Microbiome Development

The composition of the human microbiome is unique to each individual. Its initial development begins from birth and is influenced directly and indirectly by various factors. For instance, the mode of birth, whether vaginal or cesarean section, has a direct impact on shaping the gut microbiome. Vaginally delivered infants tend to harbor bacterial communities resembling their mother's vaginal microbiome, characterized by high levels of *Lactobacillus*, *Prevotella*, or *Sneathia* species (42). In contrast, infants born via cesarean section tend to have microbiomes resembling their mother's skin surface, dominated by *Staphylococcus*, *Corynebacterium*, and *Propionibacterium* species (42). At birth, the oral microbiome will begin to shape. The baby's mouth will be exposed to microorganisms through breathing, breastfeeding, and contact with people. Within 24 hours, the establishment of pioneer bacteria has begun, such as *Streptococcus* and *Staphylococcus* (43).

Beyond birth, the microbiome continues to evolve due to factors like diet, stress, or neuroendocrine exposure, which can mediate epigenetic programming (44, 45). After the age of three, the microbiome stabilizes and becomes more resilient to changes. However, throughout life, other factors like genetics (46), age (47), diet (48), hygiene practices (49), medication (especially antibiotic exposure) (50), and lifestyle choices (51) continue to play important roles in shaping the microbiome.

2.3 Human Microbiome Project (HMP)

The Human Microbiome Project (HMP) was initiated in 2007 by the National Institutes of Health (NIH) in the United States (52, 53). It was one of the pioneering large-scale initiatives dedicated to mapping the human microbiome. The primary goal of the HMP was to comprehensively characterize the human microbiome and gain insights into its role in health and disease. HMP aimed to identify and analyze microorganisms residing at various human body sites, including the skin, mouth, nose, gastrointestinal tract, and urogenital tract (34). The project sought to establish a reference for microbial communities in healthy individuals, which could then be compared with the microbiomes of individuals with specific diseases. It also aimed to investigate microbiome diversity, function, and its interactions with the host.

2.4 The Human Microbiome in Health and Disease

Advancements in culture-independent approaches, such as DNA and RNA-based techniques facilitated by next-generation sequencing (NGS) and bioinformatics, have been influential in uncovering the complexities of different niches within the human microbiome in both health and disease (54). The human microbiome, often referred to as "the hidden organ," serves multiple functions tied to fundamental biological processes within the human body, as mentioned earlier. These microbial communities, each adapted to its site-specific environment, contribute to maintain homeostasis and regulate the immune system while in symbiosis with the host.

To maintain homeostasis and to keep the external pathogens at bay, strategies like nutrition competition and antimicrobial production are employed by microorganisms within the human microbiome (55). Under stable conditions, potential pathogens are effectively restrained in the human microbiome, preventing their colonization and/or overgrowth. This phenomenon is referred to as colonization resistance and is associated with stable and diverse microbiome, as well as a lack of inflammation (56, 57). However, any disruption or imbalance in the microbiome, known as dysbiosis, creates an opportunity for pathogens to invade. This can lead to dysregulation of the body's functions and contribute to a wide range of diseases, including obesity (58), type 1 and 2 diabetes (59), colon cancer (60), cardiovascular diseases (61), chronic respiratory diseases (62), inflammatory bowel disease (63), chronic kidney diseases (64), chronic liver diseases (55), chronic inflammatory skin conditions (55), and Alzheimer's disease (65).

Recent evidence also suggests that the gut microbiome can influence the physiology and inflammation of the central nervous system (CNS) through a network of signaling pathways known as the gut-brain axis (66). The microbiome and the brain communicate via various routes, including the immune system, tryptophan metabolism, the vagus nerve, and CNS. Metabolites such as short-chain fatty acids, branched-chain amino acids, and peptidoglycan play key roles in this communication. The gut microbiome has been implicated in affecting cognitive conditions, including autism, anxiety, obesity, schizophrenia, Parkinson's disease, and Alzheimer's disease (67).

2.5 The Impact of Antibiotic Treatment on the Human Microbiome

Antibiotics have played a pivotal role in human medicine and have been considered essential medicines throughout history. In 2015, worldwide antibiotic consumption, measured as defined daily doses, was estimated to be 34.8 billion, which is expected to continue to rise in the future (68). The effects of antibiotic treatment depend on both pharmacodynamic and pharmacokinetic aspects of the antibiotic drug (69). Factors such as antibiotic class, spectrum, route of administration, dose, treatment duration, or the combination of different antibiotics are essential considerations in the treatment (70).

Antibiotic treatment not only impacts the target pathogenic bacteria but also has collateral effects on the off-target microbiome, which has been identified as a primary cause of antibiotic-driven dysbiosis. The loss of colonization resistance has been linked to various health consequences, as mentioned earlier. Moreover, off-target antibiotic exposure can promote antimicrobial resistance within the human microbiome (70).

Age is a factor that can significantly affect the outcomes of antibiotic treatment (71). Infants and children, especially underweight and preterm infants in the Western world, are among the groups for which most antibiotics are prescribed. Frequently prescribed antibiotics for this age group include broad-spectrum antibiotics like amoxicillin, azithromycin, and amoxicillin/clavulanate (71). The infant microbiome is more dynamic and less diverse in children up to 2 years of age, contributing to making them more susceptible to infections. Antibiotic treatment at an early age, whether prophylactic or therapeutic, can disturb the development of the human microbiome. Since the microbiome is immature and the immune system is not fully developed, the consequences of antibiotic treatment can be long-lasting (72). Some studies have linked early antibiotic exposure to the development of various diseases later in life, including asthma (73), allergies (74), obesity (75), and autoimmune diseases such as inflammatory bowel disease (76).

The most studied microbiome in humans under and after antibiotic exposure is the gut microbiome (77). Some studies indicate that the effect of antibiotic treatment on human gut microbial ecology reverses after the treatment ends (77). However, in other studies, dysbiosis may persist for weeks to months, and some reports suggest prolonged disruption of the microbiome for at least two years (77, 78). A major pattern observed under or after antibiotic exposure in the human gut microbiome include a decrease in alpha-diversity (richness).

Normal microbial homeostasis is disrupted, leading to a disturbance in microbial ecology composition. This often includes a reduction in the *Actinobacteria* phylum and an increase in the abundance of the *Bacteroidetes* phylum (77). It is important to note that some studies may yield contradictory results due to variations in factors such as antibiotic dose, class, and combinations, as well as demographics, lifestyle, age, and the location of the subjects. Different measurement techniques, analyses, and reporting measurements used by researchers can also impact the study outcomes (77, 79).

Understanding the effects of antibiotic treatment can aid in tailoring treatments to minimize collateral effects (80). Prolonged use of amoxicillin for three months in patients with chronic low back pain significantly reduced health-associated short-chain fatty acid-producing species immediately after treatment, but the gut microbiome recovered within 9 months post-treatment (81). In another study, the effects of a single dose of different antibiotics, including clindamycin, ciprofloxacin, amoxicillin, and minocycline, each with distinct modes of action, were tested on healthy individuals, impacting both the gut and oral microbiomes. The salivary microbiome quickly recovered and was surprisingly robust against antibiotic-induced disturbances. However, ciprofloxacin significantly affected the fecal microbiome for months, leading to a strong underrepresentation of health-associated butyrate-producing species in both the clindamycin and ciprofloxacin groups. Interestingly, short-term amoxicillin exposure did not significantly impact gut microbiome diversity (82).

2.6 The Human Resistome

The concept of the resistome or antibiotic resistome in humans has gained prominence over the past decade. Historical analysis of ancient DNA extracted from permafrost sediments dating back 30,000 years has unveiled a diverse array of ARGs. These ARGs encode resistance to antibiotics like beta-lactams, tetracyclines, and glycopeptides, underscoring the natural existence of antibiotic resistance as an existing feature before the discovery of antibiotics (83, 84). The resistome refers to the collection of ARGs in each environment and has persistently coexisted with the biosphere. However, in contemporary times, this has increased dramatically by the imprudent prescription and overuse of antibiotics, culminating in the selection of antibiotic-resistant bacterial strains, including critical human and animal pathogens (85).

A significant proportion of antibiotics employed in clinical settings today are either structurally identical or semi-synthetic derivatives of compounds initially isolated from natural sources. Consequently, it is evident that the microbial inhabitants of the human host carry ARGs for the majority of antibiotics in use (86, 87). The advent of high-throughput culture-independent methodologies, such as sequence-based shotgun metagenomic, have been a transformative tool for identifying new ARGs and interpreting mechanisms in microbial communities relevant for antimicrobial resistance. Such methodologies are also emerging as potential valuable tools for the functional genetic surveillance of ARGs (88, 89).

The term "human resistome" encompasses the totality of all ARGs and their precursors within pathogenic and non-pathogenic bacteria residing in the human microbiome.(90, 91). Much of our comprehension of antibiotic treatment in the human microbiome is based on the study of antibiotic resistance mechanisms within single species of antibiotic-resistant pathogens isolated from the broader microbial community (50). Less studied are dysbiosis in microbial communities, particularly due to prolonged exposure to sub-MIC of antibiotics. This is an important field, given the potential of antibiotics to reduce bacterial diversity, and consequently give pathogens opportunities to survive (92). Antibiotic treatments can also induce bottleneck events where smaller populations are susceptible to genetic drift (77). From a One Health perspective, sub-therapeutic or sub-MIC antibiotic concentrations are extensively employed in livestock as growth promoters (93).

Understanding the human resistome is of paramount importance in combatting multidrug-resistant pathogenic infections (85). This comprehension helps not only to foster awareness

but has the potential to be of future use for surveillance systems and strategies to mitigate the selection and dissemination of antibiotic-resistant strains. Moreover, it can help to explore novel interventions aimed to prevent dissemination of antibiotic resistance and developing new treatment modalities. Pathogens have been observed to acquire ARGs through HGT mechanisms, either from commensal or environmental bacteria or via mobile genetic elements and bacteriophages present in the human resistome **(Table 1)**. The transfer of ARGs between non-pathogenic and pathogenic bacteria has been identified as having the most substantial impact on the dissemination of ARGs within the human resistome (94, 95). Furthermore, the human resistome exhibits a degree of specificity to different bodily niches and can also vary by geographical region (96). On a population scale, human movement, migration, and travel serve as vectors for the transmission of ARGs (97). This ecological dynamic is not confined to human hosts alone but also extends to the environment, with ARG transmission routes including the transfer from soil to animals or humans (84).

3. The Oral Microbiome

The oral microbiome is recognized as one of the largest and most diverse microbiomes in the human body, second only to the gut microbiome (98). It is well-established that the oral cavity hosts a remarkable diversity of more than 700 different species of bacteria, of which only 54% have been successfully cultivated and identified. Another 14% are cultivable but not yet identified, while a substantial 32% remain uncultivable and unidentified (98, 99). The non-uniform nature of the oral cavity creates a complex environment where each surface offers a unique ecological niche, capable of supporting only specific bacterial populations. Consequently, subpopulations of bacteria preferentially colonize specific microenvironments based on factors such as oxygen availability, nutrient resources, adhesion properties, redox potential, and pH (100).

The integration of culture-independent techniques, such as targeted polymerase chain reaction (PCR), in conjunction with advanced next-generation DNA sequencing methods, has heralded a revolutionary era in oral microbiome research. These techniques enable more comprehensive analyses of the intricate interactions within the oral microbial ecology, although much remains to be uncovered. Some studies have utilized *in vitro* model systems to investigate the microbiome within specific microenvironments, such as saliva, tooth surfaces, subgingival/supragingival plaque, buccal mucosa, dorsal tongue, and the hard and soft palate (98, 101, 102). Due to their ease of collection, non-stimulated saliva samples are a predominant choice in oral microbiome research, providing insights into the microbial ecology in healthy and diseased states (101, 102).

3.1 Ecology

The study of oral microbial ecology was a significant component of the HMP. It aimed to identify the bacterial species associated with specific oral microenvironments and elucidate their genetic potential in the context of health and disease. To accomplish this, sequences of the 16S rRNA gene were compiled into the Human Oral Microbiome Database (HOMD), which is a phylogeny-based database (103-105). The HMP represents the most extensive exploration of the human oral microbiome to date, focusing not only on microbial ecology but also on the interactions between species and the host (106). This ambitious research initiative has sparked greater curiosity among researchers to understand the oral microbiome in various health and disease conditions, leading to more advanced research inquiries over the past decade.

HOMD encompassed 13 phyla that were identified within the oral microbiome, including *Actinobacteria, Bacteroides, Chlamydiae, Chloroflexi, Euryarchaeota, Firmicutes, Fusobacteria, Proteobacteria, Spirochaetes, SR1, Synergistetes, Tenericutes,* and *TM7*. However, the six major phyla predominant in the oral cavity were *Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, Spirochaetes,* and *Fusobacteria*, which accounted for 96% of the taxa (105). Additionally, the database included 169 genera (105). It is important to note that different studies have reported varying numbers of bacterial genera in the oral cavity, with some reporting as few as 15 genera (107) and others reporting over 200 genera (98). These discrepancies can be attributed to individual variations, sequencing depth, and the diverse microenvironments within the oral cavity. Among the most prevalent genera in the oral cavity are: *Streptococcus, Prevotella, Veillonella, Neisseria, Haemophilus, Campylobacter, Fusobacterium, Rothia, Mycoplasma, Actinomyces, Aggregatibacter, Granulicatella, Corynebacterium*, and *Actinomyces* (98, 101, 107).

Gram-positive *Streptococci* have been notably abundant in the oral cavity and the upper respiratory tract. This predominance is observed across various microenvironments in the oral cavity. Furthermore, *Streptococci* are particularly dominant in infants below the age of four, a period during which the oral microbiome exhibits high heterogeneity and dynamics (108, 109).

3.2 Oral Microbiome in Health and Disease

The oral microbiome serves as the primary gateway for bacteria to enter the human body, and it significantly impacts overall health. Within the oral cavity, commensal and pathogenic bacteria coexist in a harmonious balance. Dysbiosis, which refers to ecological changes in the oral microbiome due to various factors, can disrupt the interplay between microbial species in the oral cavity, potentially leading to an overabundance of pathogenic species (110). Factors such as changes in lifestyle or the presence of diseases can contribute to this dysbiosis. For instance, inadequate oral hygiene practices have been linked to an increased presence of potential respiratory pathogens in the oral cavity, including *Streptococcus pneumoniae*, *Klebsiella pneumoniae*, *Haemophilus influenza*, and *Staphylococcus aureus*, all of which are classified as WHO Global priority pathogens with heightened antibiotic resistance development (111-113).

Maintaining a healthy oral microbiome relies on metabolic and functional interactions between the host and bacteria, as well as within bacterial habitats. One critical mechanism for sustaining the oral microbiome's health is colonization resistance, which helps segregate microenvironments in the oral cavity and prevents the colonization of niches by pathogenic bacteria (114, 115).

Oral diseases, such as periodontal disease or dental caries, are often the result of shifts or dysbiosis in the bacterial ecology, marked by a decrease in microbial alpha-diversity in caries patients, while increased alpha-diversity is observed in patients with periodontal disease (116, 117). However, it is worth noting that some individuals appear to be more resilient to changes in the oral microbiome than others. NGS has revealed that dysbiosis in the oral microbiome, characterized by an increased presence of genera such as *Synergistes*, *Prevotella*, and *Fusobacterium*, is associated with periodontitis (110, 118). The excessive accumulation of plaque also increases the risk of periodontal disease. Pathogens, in conjunction with the host's inflammatory response, have been identified as the main risk factors for the progression of periodontal disease. On the other hand, genera like *Streptococcus*, *Actinomyces*, and *Granulicatella* have been associated with periodontal health (110). Specific species, such as the "red complex" (*Porphyrmonas gingivalis*, *Treponema denticola*, and *Tannerella forsythia*), are strongly associated with periodontal disease (121-123).

In the case of dental caries, bacterial species like *Streptococcus mutans* and some *lactobacilli* have shown strong associations. Dental caries is a result of dysbiosis in microbial

homeostasis, where acid-selected or adapted microbiome can thrive (119). It is a multifactorial, non-communicable disease influenced by extrinsic factors like carbohydrate consumption and intrinsic factors such as reduced saliva flow, which create an environment conducive to cariogenic dental plaque formation, ultimately contributing to caries progression (124-127).

The oral microbiome is not isolated; it is interconnected with other microbiomes in the human body. The mouth can also act as a reservoir for pathogenic bacteria originating from other microbiomes. For example, individuals with cystic fibrosis often experience lung infections caused by bacteria like *Pseudomonas aeruginosa, H. influenza*, and *S. aureus* (120). Interestingly, these pathogens are not rarely found in the oral cavity, suggesting that the oral microbiome may serve as a reservoir for these organisms (121). *Helicobacter pylori* is another pathogen occasionally detected in dental plaque, despite its origin in the gut microbiome. Its presence in the oral cavity may be associated with gastroesophageal reflux (122). Bacteria originating from the oral microbiome can also act as opportunistic pathogens at distant sites, such as aspiration into the lungs or entry into the bloodstream, leading to conditions like bacteremia (123) **(figure 2)**. **Table 2** provides an overview of bacteria originating from the oral cavity and their associations with different diseases.

Table 2: Oral bacterial species associated with oral- or systemic diseases

Disease	**Associated bacterial species**	**Reference**
Caries	*Streptococcus mutans, Lactobacillus spp. Streptococcus sobrinus, Veillonella dispar, Veillonella parvula*	(110, 119)
Periodontitis	*Porphyromonas gingivalis, Treponema denticola, Tannerella forsythia, Filifactor alocis, Parvimonas micra, Aggregatibacter actinomycetemcomitans*	(110, 124-126)
Sjøgren's syndrome	*Veillonella parvula, V. dispar, Prevotella melaninogenicus, Prevotella histicola*	(127, 128)
Oral cancer	*Fusobacterium nucleatum, Pseudomonas aeruginosa*	(110, 129)
Upper respiratory tract infection	*Staphylococcus aureus, Streptococcus pneumoniae, Hemophilus influenzae, Moraxella catarrhalis, Streptococcus pyogenes*	(130, 131)
Cystic fibrosis	*Streptococcus oralis, Streptococcus mitis, Streptococcous gordonii and Steptococcous sanguinis, Pseudomonas aeruginosa, H. influenzae, S. aureus*	(110, 120)
Cardiovascular disease	*Campylobacter rectus, Porphyromonas endodontalis, Prevotella intermedia, Prevotella nigrescens, P. gingivalis*	(110, 132)
Endocarditis	*S. mutans, S. gordonii, S. sanguinis, Streptococcous gallolyticus, S. mitis, S. oralis*	(133)
Diabetes	*Porphyromonas gingivalis, Treponema denticola, Tannerella forsythia*	(110, 134)
Chronic gastritis, Peptic ulcer	*Helicobacter pylori, Fusobacterium nucleatum, P.gingivalis*	(135)
Gastric cancer	*H. pylori, F. nucleatum, P. gingivalis, P. intermedia, A. actinomycetemcomitans*	(135)
Gastro-esophageal reflux	*H. pylori*	(122)

Colorectal cancer	*F. nucleatum, P. gingivalis, P. intermedia, A. actinomycetemcomitans*	(135, 136)
Pancreatic cancer	*P. gingivalis, A.actinomycetemcomitans*	(110, 136)
Rheumatoid arthritis	*Rothia mucilaginosa, Rothia dentocariosa, Lactobacillus salivarius, Cryptobacterium curtum, P. gingivalis, A. actinomycetemcomitans*	(110, 136)
Alzheimer's disease	*P. gingivalis, A. actinomycetemcomitans, , T. denticola, T. forsythia, Campylobacter rectus, P. Intermedia*	(110, 137)

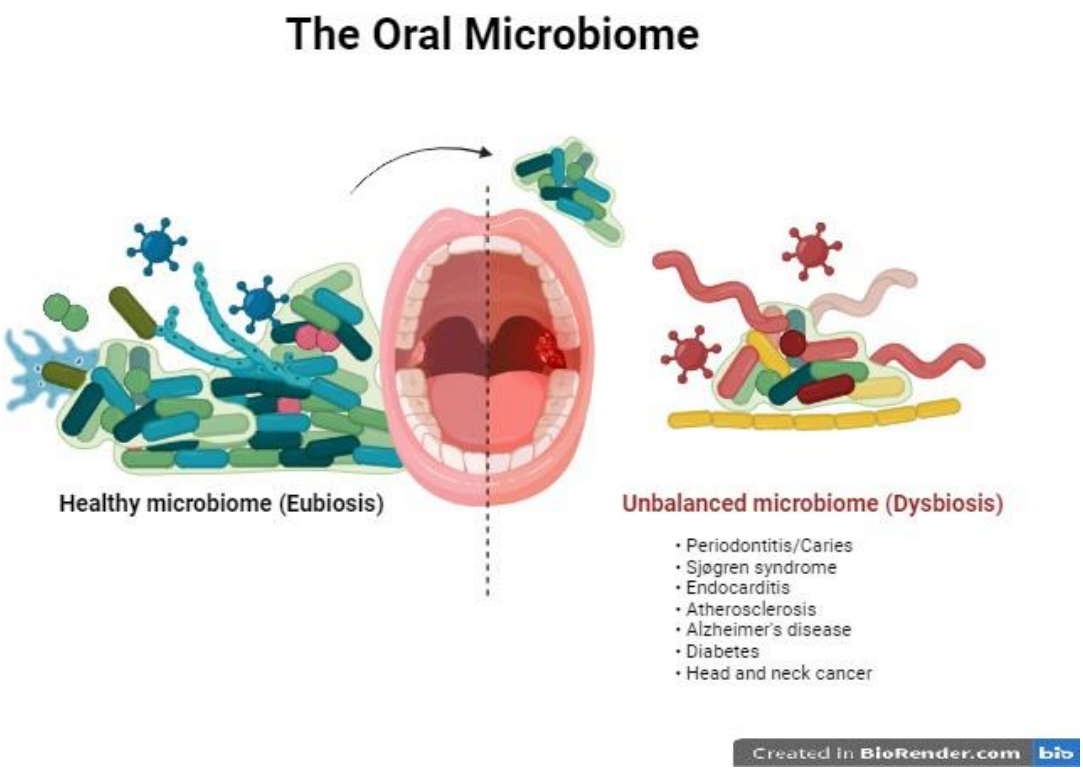

Figure 2: A comparison between a healthy vs. unbalanced oral microbiome with microbial dysbiosis leading to several unwanted conditions/diseases.

3.3 Oral Biofilm

Biofilms are a common microbial lifestyle in nature and represent a distinct mode of growth for bacteria. This concept was introduced by Bill Costerton in 1978 when he highlighted how biofilms could lead to chronic infections in patients with medical devices. He also emphasized that bacteria within biofilms possess mechanisms to resist antibiotic treatment and immune host defenses (138, 139).

A biofilm is characterized by its heterogeneous structure, consisting of various populations of microorganisms encased in an extracellular polymeric substances (EPS) matrix. This matrix allows the microorganisms to attach to different surfaces, which can be abiotic (e.g., rocks, glass, plastic, and medical devices like implants and prosthetics) or biotic (e.g., skin, cuticles, mucosa, and teeth) (140, 141). Biofilms form adaptable and diverse microbial communities in various environments.

The EPS matrix is primarily composed of polysaccharides, but it can also contain proteins, lipids and extracellular DNA (eDNA). This matrix is produced intracellularly by the bacteria and released into the surrounding environment. Once EPS components are released by producer species, extracellular enzymes have the ability to degrade the EPS components to low-molecular-mass products that can be used as carbon and energy sources by the bacteria (142). The EPS matrix functions to anchor the bacterial communities together and to create structured channels. Additionally, EPS plays a role in establishing gradients of nutrients and waste products, provides protection against host defense systems, and shields the bacteria from stressful conditions such as antibiotic treatment (140, 143). In a mature biofilm, bacterial cells typically account for only 5-25% of the total biomass, with the remaining 75-95% comprising of EPS (144).

Dental plaque, one of the most extensively studied biofilms, adheres to surfaces in the oral cavity (145). The study of oral biofilms dates back to 1683 when Anton van Leeuwenhoek used a self-constructed microscope to examine his own dental plaque, describing the small creatures (bacteria) as "animalcules" (146). Oral biofilms are easily accessible, making them a common subject for investigating bacterial adhesion, biofilm development, and antibiotic resistance in model systems (147, 148). Dental caries and periodontitis are two diseases directly linked to the metabolic activity within dental plaque (108). The composition of oral biofilm formation can vary based on the environmental conditions influenced by the

community's intrinsic metabolism, as the oral cavity presents a broad range of such conditions (145).

The initial step in oral biofilm formation involves the attachment of oral bacteria to a salivary pellicle or to bacteria already attached to a surface **(figure 3)**. The salivary pellicle is a protein-containing film produced by glycoproteins and other substances in saliva and is the first step in oral biofilm formation (144, 149, 150). The biofilm formation involves the initial adhesion of free-floating planktonic bacteria to a surface. *Streptococcus* genus, specifically *S. mitis*, *S. oralis*, *S. sanguinis*, and *S. gordonii*, are among the initial colonizers in oral biofilms (108, 145). These bacteria express adhesin molecules on their surfaces that bind to the pellicle and interact with new species. These pioneer bacteria multiply, contributing to biofilm growth. Coaggregation follows, involving the binding of genetically distinct bacterial cells. For instance, specific *Actinomyces spp*., *Haemophilus spp*., and *Neisseria spp*. will attach to the pioneer bacteria (145). *Veillonella spp*. or *Prevotella spp*. play a bridging role in creating multispecies biofilms (145, 151). These bacteria produce EPS, which are essential components of the extracellular matrix. Interactions among different species within the biofilm involve physical and nutritional competition, synergistic associations, antagonism, neutralization of virulence factors, gene transfer, and cell-to-cell communication through quorum sensing (QS) (152, 153).

As a biofilm matures, the metabolic activity of the multispecies biofilm alters environmental conditions by producing metabolites such as lactate, acetate, and butyrate, leading to increased acidity in the environment. Bacteria within mature biofilms experience shifts in nutritional resources, oxygen levels, and waste products. Some bacteria that do not thrive in this environment disperse and attach to other surfaces **(figure 3)** (154).

Many human infections are biofilm-mediated, where approximately 80% of chronic and recurrent microbial infections involves bacterial biofilms. These include chronic lung infections, chronic osteomyelitis, chronic otitis media, chronic wounds, endocarditis, periodontitis, and dental caries (31, 155, 156). An understanding of the nature and physiology of biofilms is crucial for implementing effective management strategies. Biofilms exhibit increased resilience to antibiotic treatment, being up to 10-1000 times more resistant to antibiotics compared to the same bacteria in planktonic form. This resilience in biofilm is due to factors such as decreased antibiotic penetration and occurrence of ARGs within the bacteria's chromosome or on mobile genetic elements (30, 157). In addition, differences in gene expression has been observed between planktonic cells and biofilm communities. For

instance, genes important in iron-sulfur metabolism, lipid metabolism, amino acids, carbohydrate transport, secondary metabolites and stress-response are up-regulated in biofilm, while DNA-repair genes are down regulated (141). Exposure to low antibiotic concentrations, which often occurs in biofilms, can have significant adverse effects on the oral microbiome (158).

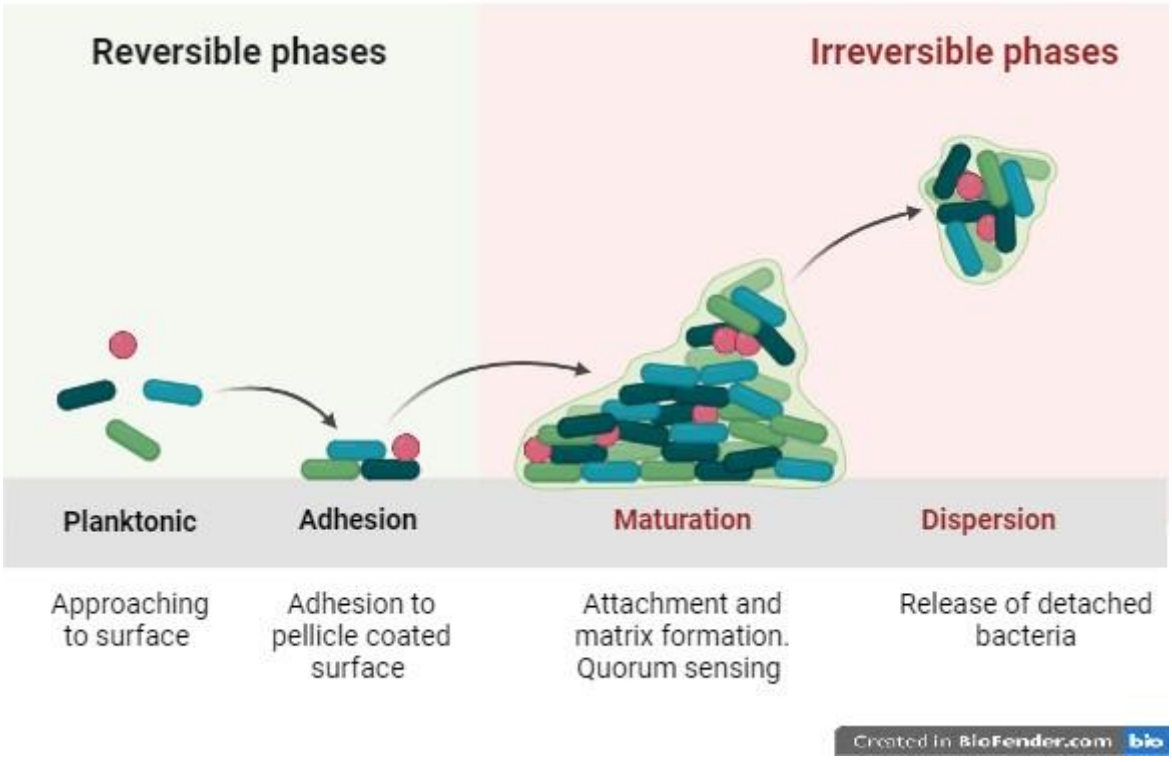

Figure 3: A schematic illustration of the four different stages of dental biofilm formation and the most important factors involved in these stages. The biofilm formation is divided into reversible and irreversible phase. Created in BioRender.com

3.4 Effects of Low Concentrations of Antibiotics on the Microbiome

The concentration of antibiotics detected in saliva is typically lower than that observed in the serum. For some bacteria, antibiotic concentrations in saliva have been detected at levels around the MIC or sub-inhibitory concentrations for many species in the oral microbiome (159). Variability in antibiotic concentration in saliva can be attributed to factors such as the type of antibiotic, dosage, treatment duration, as well as inter-individual differences, including age and various other factors (69, 160). Metagenomics studies correlating antibiotic levels in the oral cavity with their impact on the oral microbiome are relatively scarce. While some studies suggest that the oral microbiome is resilient to changes after antibiotic treatment (82, 161, 162), others indicate an increase in dysbiosis in the oral microbiome (163, 164).

The human oral microbiome and the pharynx are known to host five of the 12 priority pathogens listed by the WHO, including *S. pneumoniae*, *K. pneumoniae*, *S. aureus*, *Enterococcus faecium* and *H. influenzae* (113, 165-168). All of these pathogens display widespread antibiotic resistance patterns. Exposure to low antibiotic concentrations has played a significant role in the development of AMR. Low antibiotic concentrations can impact gene expression in bacteria, influencing horizontal gene transfer and disrupting QS functions. This exposure can also induce stress in bacteria and trigger behavioral changes, such as increased biofilm formation, which may lead to the selection of antibiotic-resistant bacteria (158, 169-173).

3.5 Oral Resistome

The oral resistome, encompassing all ARGs present in the oral cavity, represents a complex and relatively understudied domain. Notably, certain studies involving Native American populations have uncovered ARGs that appear to be inherited through evolution, firmly integrated into the oral microbiome (96, 174).

Investigations into the oral resistome abundance, diversity, and composition have been somewhat limited but have nonetheless indicated intriguing variations during the first decade of life (96, 98, 175, 176). Accounting for individual differences, it is apparent that the oral resistome exhibits a relatively high abundance of ARGs, although its diversity remains lower in comparison to the gut resistome (96). Notably, the most prevalent ARG drug classes identified within the oral resistome include aminoglycoside, macrolide-lincosamide-streptogramin B, beta-lactam, fluoroquinolone, tetracycline, and vancomycin (96, 98).

Despite the limited exploration of the oral resistome, some studies have delved into the oral cavity's role as a reservoir for ARGs (177-179). It has been established that commensal bacteria within the oral microbiome can serve as essential reservoirs of antibiotic resistance genes. For instance, organisms such as *S. mitis* and closely related streptococci possess the potential to transmit ARGs to pathogens such as *S. pneumoniae* through HGT, specifically via natural transformation. Such dynamics contribute to the challenge of eliminating infections associated with *S. pneumoniae* (178).

4. Host Response to Antibiotic Treatment

The human body responds to antibiotic treatment in various ways, including interaction with the human immune system and the microbiome (180-182). The primary goal of antibiotic treatment is to eliminate or inhibit the growth of the causative bacteria to allow the body's immune system to effectively clear the infection and resolve symptoms. There are, however, potential unwanted effects. Some studies have, for instance, highlighted that antibiotics can impair children's immunity to pathogens (72, 183). Perturbations in the microbiome, known as dysbiosis, have been linked to several diseases, such as diarrhea and immune- related diseases such as allergies (180). In some cases, pathogens can develop resistance to antibiotics, while many commensal bacteria act as a reservoir for ARGs, leading to antibiotic treatment failure. To mitigate the side-effects of antibiotic treatments, new strategies to modulate the microbiome have gained focus.

4.1 Modulation Strategies

Modulation of microbiome dysbiosis is an emerging field of research with potential implications for health and well-being. Different therapeutic strategies, such as prebiotics and probiotics, have been developed to modify the immune system and prevent pathogen colonization. For the gut microbiome, fecal microbiome transplantation (FMT) has also shown promising results (184).

4.2 Prebiotics/Probiotics

Probiotics are defined as “live microorganisms when administrated in adequate amounts, confer health benefits on the host”, while prebiotics does not contain microorganisms, only substances which can promote the growth of probiotic species (185). Breast milk has shown to have both pre- and probiotic properties. It contains components that increase the growth of beneficial bacteria. Additionally, it has a microbiome that can impact the ecological diversity in the infant microbiome (186-188). Some probiotics, such as *Limosilactobacillus reuteri*, have shown to prevent caries by inhibiting acid-tolerant biofilm development (189). Although, some studies indicate that both pre- and probiotics can help the microbiome to maintain the ecological diversity and homeostasis after antibiotic treatment, more investigation is needed to understand it fully (190).

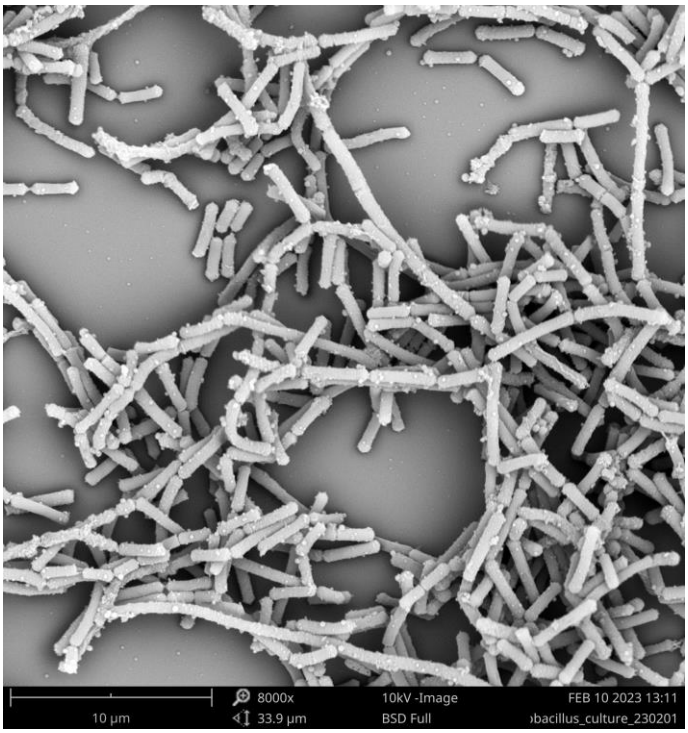

Figure 4: Scanning electron microscope image of probiotic bacterial species, *Lactobacillus crispatus* (CCUG 30722) biofilm. Sample prepared by Navdeep Kaur Brar. Photo by: Maria Baumgarten, at Infection Medicine in Lund University, Sweden.

4.3 HAMLET

Human milk contains crucial nutrients for the growth and development of infants during their first years of life such as amino acids, lactose as a carbohydrate source, and 100-200 human milk oligosaccharides (HMOs) that are specifically evolved to feed the human bacterial ecology. HMOs are directly involved in development of human microbiome (191). In addition, it also contains various components with antimicrobial and immunomodulatory effects that are part of the innate immune system. These components include alpha-lactalbumin (ALA), lactoferrin, lysozyme, and secretory IgA, which protect infants' initial mucosal surfaces against pathogenic bacteria (192, 193). Additionally, fatty acids such as oleic acid (OA), linoleic acid, eicosapentaenoic acid, arachidonic acid, and docosahexaenoic acid play essential roles in the development of the immune system and central nervous system (194).

HAMLET (Human alpha-lactalbumin made lethal to tumor cells) is a protein-lipid complex that consists of ALA and specific fatty acids, primarily oleic acid (OA, C18:1:9 cis), which is the most abundant fatty acid in human milk. The protein is partially unfolded in the HAMLET-complex and is stabilized by fatty acids, where each ALA molecule is bound to 5-8 oleic acids (195-198). HAMLET was discovered by researchers at Lund University in Sweden during their investigation of the antimicrobial properties of human milk against upper respiratory pathogens such as *S. pneumoniae* and *H. influenzae*. Serendipitously, it was first observed that the milk fraction killed cancer cells while leaving healthy cells unaffected (199-201).

In cancer cells, this protein-lipid complex induces apoptosis-like death in 50 different cancer cell lines. The first human study of muscle invasive bladder cancer with HAMLET began in 2018 with a Phase I/II trial (201-203). The morphological characterization of tumor cell death involves cell shrinkage, DNA condensation, and degradation into high molecular weight fragments of similar sizes, suggesting apoptotic cell death. HAMLET internalizes into the tumor cell and interacts with the inner mitochondrial membrane, increasing its permeability and causing rapid depolarization with influx of calcium ions, which leads to the release of apoptogenic factors into the cytosol and initiation of the caspase-9/APAF-1 cell death program. This apoptosis pathways is activated only in tumor cells without affecting healthy differentiated cells (199). In healthy cells, binding of HAMLET to the outside of the cells is observed, but no internalization of interaction with mitochondria is seen. Other studies have

also found that HAMLET interferes with glycolysis by binding and inhibiting hexokinase as well as activating protein kinases such as serine/threonine kinases (213-214).

HAMLET also has bactericidal activity against several pathogens, including *Streptococcus pyogenes, Streptococcus agalactiae, H. influenzae, M. catarrhalis,* and *Mycobacterium tuberculosis*, with the highest activity observed against *S. pneumoniae* (204). However, HAMLET shows no bactericidal activity against other pathogens such as *S. aureus, Escherichia coli, K. pneumoniae, P. aeruginosa, Haemophilus parainfluenzae,* and *Enterobacter cloacae* (204-208). Nonetheless, HAMLET has demonstrated promising results in resensitizing previously resistant bacteria to antibiotics, such as *methicillin-resistant Staphylococcus aureus* (MRSA), which became susceptible to antibiotics when treated with a combination therapy of HAMLET and methicillin (207, 208) . HAMLET induces a sodium-dependent influx of calcium in *S. pneumoniae,* leading to chromatin condensation and DNA fragmentation, as well as activation of eukaryotic-type serine kinases. Additionally, similar to tumor cells, HAMLET interferes with glycolysis in *S. pneumoniae* (201, 209).

5. Oral-Lung-Gut Microbiome Crosstalk

The interrelationship between the oral, gut, and lung microbiomes and their association with the immune system plays a critical role in human health and disease (210). Some studies have shed light on the resilience of the oral microbiome compared to the gut microbiome in response to changes in the environment, such as exposure to antibiotics (82, 161). However, low concentrations of antibiotics have been shown to stimulate the biofilms of respiratory pathogens in the oral cavity, including *S. pneumoniae, S. aureus, H. influenzae, Streptococcus intermedius,* and *Enterococcus fecalis* (165, 166, 170-172). Dental plaque has been identified as a reservoir for respiratory pathogens such as *S. pneumoniae* (165, 166). Furthermore, oral anaerobic bacteria such as *Veillonella spp.* and *Prevotella spp.* often colonize both the upper and sometimes lower respiratory tracts, resulting in activating mucosal immunity (210).

Research has shown that gut and oral dysbiosis can alter the microbial and inflammatory environment in the lungs (210). The mechanisms of communication between these different microbial niches are not well understood; however, dysbiosis in one compartment can impact another, potentially leading to various diseases, including periodontitis, dental caries, inflammatory bowel disease, asthma, chronic obstructive pulmonary disease, and Alzheimer's (210-212). Dysbiosis and dysregulation of immune-inflammatory responses in interconnected niches are relevant mechanisms involved in the manifestation of such diseases.

Aims of the Research

General aim

- To investigate the microbial ecology under antibiotic stress conditions, and interventions with HAMLET.

Specific aims

- To study the effect of different concentrations of a broad-spectrum antibiotic, ampicillin, on oral microbiome ecology. (Paper I)
- To investigate HAMLET's potential as an antimicrobial to modulate oral microbiome ecology in polymicrobial biofilms untreated or treated with amoxicillin. (Paper II)
- To explore the impact of short-term treatment of infant mice with amoxicillin and/or HAMLET on lung T cell responses to *Streptococcus pneumoniae.* (Paper III)

Metodological Considerations

In this section, I will discuss the methodological considerations and common challenges encountered in all three papers (Paper I-III). Most of the methods employed in these studies are described in detail in the respective "Materials and Methods" sections of each paper. This section is divided into two main parts: the first will discuss the choice of laboratory techniques and metagenomic sequencing used in Paper I and II, while the second part will focus on the methodological considerations for the *in vivo* study in Paper III.

Paper I and Paper II

Ethics

In both studies, saliva-derived oral microbiomes were obtained from human donors and used in *ex vivo* experiments. Prior to the commencement of these studies, proper informed consent was obtained from all donors, and all experiments were carried out within strict principles of confidentiality and privacy. The studies were conducted in accordance with the Declaration of Helsinki, which outlines the basic ethical principles for conducting research involving human subjects.

To ensure adherence to ethical standards and guidelines, these studies were subjected to review by the Norwegian Regional Ethics Committee (REK20152491) before their commencement. The approval of the ethics committee indicates that the studies were conducted in accordance with established ethical standards, ensuring that the rights and welfare of human subjects were respected throughout the course of the experiments.

Ex vivo Oral Microbiome Model

The *ex vivo* oral microbiome model utilized in Paper I and II offered a controlled experimental setup for investigating the impact of specific treatments on oral biofilms. However, it is important to acknowledge that *ex vivo* models only partially emulate the intricate dynamics of the *in vivo* oral microbiome and may not fully translate to clinical or *in vivo* conditions.

In these studies, the biological saliva samples were utilized in the *ex vivo* oral microbiome model outside in the human body. This approach avoided the limitations associated with *in vivo* studies, which are susceptible to various confounding factors (102, 213). By using individual saliva samples in Paper I, a more accurate representation of the diverse oral microbiomes among individuals was achieved. Conversely, pooling saliva samples from multiple donors in Paper II ensured a representative microbial diversity. Nevertheless, the use of individual saliva samples consistently yielded more reliable biological replicates compared to pooled samples. The biofilm harvesting time also played a crucial role in the reproducibility of results as previously reported by Edlund et al. (102)(231).

To mimic the oral cavity environment, the 24-well plates employed in these studies were coated with pellicle. Oral biofilms were cultured anaerobically in these plates, with a gas composition of 5% carbon dioxide balanced with nitrogen. SHI media was utilized in both papers to culture the biofilms (214). It is important to note, however, that SHI media does not perfectly replicate the diversity or complexity of the *in vivo* oral microbiome. Additionally, the choice of growth media can influence the composition and behavior of oral biofilms.

SHI Growth Medium

In the *ex vivo* oral microbiome model, one of the main challenges is to find a culture medium that can support bacterial growth and maintain the diversity of the oral microbiome. In Paper I and II, SHI medium was used as the bacterial culture medium. This medium has been previously shown to support microbial diversity, reproducibility, and stability, closely resembling the original saliva sample (102, 213, 215-217).

The ingredients in SHI medium includes: proteose peptone (10g/L), trypticase peptone (5 g/L), yeast extract (5.0 g/L), KCl (2.5 g/L), sucrose (5 g/L), haemin (5 mg/L), Vitamin K (1 mg/L), urea (0.06 g/L), arginine (0.174 g/L), porcine mucin (2.5 g/L), sheep blood (5%), and N-acetylmuramic acid (NAM) (10 mg/L) (214). The SHI medium is a modified version of the previous BMM (basal medium mucin) growth medium, which includes proteose peptone, trypticase peptone and yeast extract as the basic components. However, it is supplemented with mucin, urea, vitamin K, haemin, and arginine, which are the active ingredients of the BMM medium. Mucin is the glycoprotein found in saliva, while haemin has been shown to stimulate the growth of important species in the oral cavity such as cocci, rods, and filaments (214). The SHI medium also includes some additional components. NAM has been found to

facilitate the growth of subgingival anaerobic bacteria, such as *T. forsythia* and *P. gingivalis*. Sheep blood is important for the growth of fastidious and slow-growing, obligate anaerobic bacteria. Glucose, a substitute for sucrose, is important for the recovery of streptococci from saliva samples (214).

Choice of Antibiotic

The antibiotics used in Paper I, II, and III belong to the amino-penicillin class, recognized as a broad-spectrum bactericidal beta-lactam antibiotic group. Amino-penicillins are obtained by the addition of an amino group to benzyl penicillin, which combat antibiotic resistance (218). While ampicillin was employed in Paper I, amoxicillin was used in Paper II and III. Both antibiotics are typically prescribed for respiratory tract and odontogenic infections, with amoxicillin having the additional feature of possessing a hydroxyl group on its phenyl side chain. Amino-penicillin is able to display bactericidal activity against both gram-positive and gram-negative bacteria (218-220). While the concentration of antibiotics in saliva may differ depending on varying factors such as dose, treatment duration, and patient age, both ampicillin and amoxicillin have been detected in saliva (159, 160, 164, 221, 222).

Preparation of HAMLET

The process of producing HAMLET from human milk involves three steps: 1) purification of ALA, 2) conversion and 3) dialysis and lyophilization. In the first step, the ALA and other proteins are precipitated from defatted milk. ALA concentration in milk is stable over time, approximately 2 g/L (223). Ethylenediamine tetra-acetic acid (EDTA) is added to make the protein hydrophobic, followed by the use of hydrophobic interaction chromatography (HIC) to specifically capture the ALA apo-protein (treated with EDTA to remove calcium ions) (198). The second step entails the production of the complex between the apo-protein of ALA and OA. This process involves using DEAE Trisacryl M, an exchange matrix in which oleic acids binds to ALA molecule. The final steps include the removal of salt and buffer followed by dialysis of the HAMLET complex with de-ionized water. Lastly, the HAMLET complex is lyophilized and can be stored at -20 ^{o}C for several years (198).

Laboratory Technique Employed Before Shotgun Metagenomic Sequencing

Before performing shotgun metagenomic sequencing, a preliminary screening of biofilm samples from the different treatment groups was conducted by different methods, such as the biofilm viability assay. This experiment provided a broader understanding of the effects of antibiotics, while giving preliminary insights into the changes occurring in the oral microbiome and guided the selection of samples for subsequent shotgun metagenomic sequencing. The biofilm viability assay was employed in Paper I and II to cultivate, dilute and count bacteria from the different treatment groups on selective SHI agar plates under anaerobic conditions. Although this assay does not capture the diversity of bacterial species within biofilms, it provided a general insight into the alterations occurring in live bacteria in the different treatment groups.

Metagenomics Analysis

Shotgun metagenomics analysis was employed to investigate changes in the oral microbiome composition and resistome. NGS offers the advantage of sequencing the entire microbial genome without the need for culturing bacteria, allowing for a deeper exploration of microbial abundance and diversity. However, NGS also poses challenges, such as handling and interpreting the large amount of data generated, as well as the inability to provide information on bacterial viability. Metagenomics analysis encompasses several steps, including sample collection, storage, DNA extraction, library preparation, sequencing, upstream bioinformatics analysis, and downstream bioinformatics analysis. However, it is important to note that there is currently no standardized protocol for conducting metagenomics studies (224).

DNA extraction

During DNA extraction, the choice of method can impact DNA yield, purity, molecular weight, and degradation, thus affecting the sequencing results (225). DNA extraction methods can rely on mechanical, chemical, or a combination of both approaches. Mechanical methods involve bead-beating, while enzymatic processes are common in chemical methods (226). In both, Paper I and II, the MasterPure Gram Positive DNA Purification Kit (Epicentre, Madison, WI, USA) was used for DNA extraction. This kit contains Ready-Lyse ™ Lysozyme, which has a highly specific activity to facilitate the lysis of Gram-positive

bacteria. This is of high relevance since the oral microflora consists mainly of gram-positive bacteria (227). This kit has been used successfully in previous oral metagenomic studies, and its effectiveness was validated in our studies as well.

Library preparation and sequencing

Library preparation and sequencing are critical steps in metagenomic analysis and can significantly impact the quality and accuracy of results obtained from sequencing (228). The choice of DNA library preparation kit and sequencing platform depends on various factors, including the research question, the type of sample, and available resources.

During library preparation, the bacterial DNA is fragmented and attached to adapters for sequencing. Different DNA library preparation kits use different fragmentation methods, such as sonication, enzymatic fragmentation, or tagmentation by transposomes. The choice of kit can affect the size distribution and quality of the library, thus influencing the downstream sequencing data (226).

In both, Paper I and II, we tested different DNA library preparation kits before selecting the Illumina DNA Prep kit (m) (Illumina, Inc., San Diego, CA, USA). This kit consistently generated high-quality DNA libraries that were suitable for downstream sequencing. We collaborated with the Norwegian Sequencing Center for the sequencing in both papers.

For Paper I, the sequencing platform used was Illumina 3000/4000 High Seq (Illumina, Inc., San Diegi, CA, USA), while for Paper II, we used the Illumina NovaSeq 6000 SP platform (Illumina, Inc., San Diego, CA, USA), which allowed for high-throughput sequencing with paired-end reads of 150 bp.

Bioinformatics analysis

During the upstream bioinformatics analysis, the raw sequencing reads underwent rigorous preprocessing steps, including the removal of adapter sequences, low-quality reads, and potential contamination from host DNA. To accomplish this, FastQC and Trimmomatic tools were utilized in both Paper I and II as they are widely recognized for their efficiency and suitability for this specific application (228, 229). It is worth noting that no notable instances of human DNA contamination were observed.

Subsequently, the remaining high-quality reads were aligned against microbiome and resistome reference databases to profile the microbial ecology. Considering the aim of the thesis, a read-based analysis approach was chosen due to its overall higher accuracy, faster processing times, and diminished computational requirements (224). MetaPhlAn2 and MetaPhlAn3 were employed as the analytical tool in Paper I and II, respectively, which facilitated the characterization of the microbial community structure by adopting a marker gene approach (230). This tool has proven to be efficient, particularly due to its faster performance and has previously been utilized in the Human Microbiome Project.

To evaluate the presence of ARGs, the Comprehensive Antibiotic Resistance Database (CARD) was employed. CARD has been widely utilized in similar experiments owing to its comprehensive ARG information (231, 232). In Paper I, the alignment tool Bowtie 2 (233) was employed, while in Paper II, KMA (k-mer alignment) (234) was employed to address the challenge of ARG sequences mapping to multiple alleles, which can noticeably increase the false positive rate. In both studies, ARGs were filtered to include only those presenting an identity threshold of 80%. Moreover, to assess the actual abundance of ARGs in Paper I and II, read counts were normalized by gene length and bacterial abundance using the reads per kilobase of reference gene per million bacterial reads (RPKM) metric.

For downstream bioinformatic analysis, the microbiome and resistome data were analyzed using user-friendly software tools. MicrobiomeAnalyst (235, 236) and ResistoXplorer (237) were chosen for their capability to efficiently handle data exploration, visualization, and analysis in both Paper I and II.

Paper III

Study Design

Paper III used a combination of *in vivo* and *in vitro* methods to investigate T cell responses, notably Th17 and Th1 responses. In this study, 16/17 days old infant mice were subjected to HAMLET and/or amoxicillin treatment via intranasal route. Furthermore, lung and splenic cells from these infants were stimulated *in vitro* with killed *S. pneumoniae* to measure key T cell cytokine production.

The first step was to get an approval of the application to the Norwegian Food and safety Authority (FOTS) to conduct mouse experiments. In this application, we described our project in detail, including how the three R's: replacement, reduction and refinement, would be taken into consideration. There were some challenges related to carrying out mouse experiments under COVID-19 pandemic restrictions. Hence we decided to go with an experimental design that involves antimicrobial treatment, but not the *in vivo* infection with *S. pneumoniae*. Splenic and lung cells were isolated from antibiotic and/or HAMLET exposed mice and stimulated *in vitro* with UV-killed *S. pneumoniae*. Both HAMLET and amoxicillin were administrated intranasally. Amoxicillin intranasal delivery was primarily used in our study to reduce stress and disturbance in young pups that would occur by using an additional route for drug administration. While it showed an effect on immune responses, it is noteworthy that amoxicillin has not yet been developed for the prevention or treatment of human infections using the intranasal route. Of note, HAMLET has previously been administrated intranasally in *in vivo* studies. This is a route for which HAMLET has been shown to have a bactericidal and immunomodulatory effect (218,219). The HAMLET and amoxicillin dosage were calculated from previous studies (263, 264).

The mouse equivalent dose was calculated by multiplying the human dose (mg/kg) by the ratio of Km, namely the correction factor estimated by dividing the average body weight (kg) of species to its body surface area (m2), which has been provided by the Food and Drug Administration (FDA) guideline (263, 264).

Summary of Results

Paper I

Ecological Consequences of Low Ampicillin Concentrations on an *Ex vivo* Biofilm Model of the Human Oral Microbiome

In this study, we aimed to evaluate the ecological effects of ampicillin, a broad-spectrum beta-lactam antibiotic, on the oral microbiome using an *ex vivo* biofilm model. To achieve this objective, we utilized a unique *ex vivo* oral biofilm model in which experimental conditions could be controlled and tested different interventions.

The results of this study revealed that a single exposure to low concentrations of ampicillin led to an increase in the viability of multi-species oral biofilms in all individuals. We observed donor-specific clustering for both the oral microbiome and resistome, while the biological replicates were highly reproducible within the same donor. However, we did note an increase in dissimilarity in biological samples for both microbiome and resistome composition in antibiotic-treated samples. Furthermore, an antibiotic dose-dependent shift in both oral microbiome composition and oral resistome was detected, accompanied by a noteworthy reduction in microbial alpha-diversity and antimicrobial resistance gene load. The reduction in antimicrobial resistance gene load observed in our study may also occur *in vivo* during shorter courses of antibiotic treatment. This study provides important information for designing future studies aiming at increasing the understanding of the responses also under prolonged exposure to the antibiotic.

Paper II

HAMLET, a Human Milk Protein-lipid Complex, Modulates Amoxicillin Induced Changes in an *Ex vivo* Biofilm Model of the Oral Microbiome

Polymicrobial infections present significant challenges in treatment and prevention, as biofilm formation reduces the efficacy of antibiotics. In this study, we utilized a human *ex vivo* biofilm model to investigate the ecological impact of HAMLET, a protein-lipid complex derived from human milk, in combination with amoxicillin. Whole metagenomics sequencing data provided an ecological perspective for analysis. HAMLET has been previously shown to display synergistic effects with antibiotics in single bacterial models while limiting microbial

resistance. Results demonstrated that the combination of HAMLET and amoxicillin significantly reduced biofilm formation, whereas individual treatments had minimal impact. Additionally, the combination treatment promoted a shift in the overall microbial composition, favoring lactobacilli-enriched communities, particularly *Lactobacillus crispatus*. Resistome analysis revealed no significant shifts in alpha-diversity, while principal component analysis showcased distinct clusters for each treatment. *TEM* beta-lactamase genes were detected in low proportions in all treated samples but were absent in untreated samples. These findings underscore the potential of HAMLET as a synergistic antimicrobial agent when combined with amoxicillin and its ability to modulate the proportion of probiotic bacteria within polymicrobial biofilms. The implications of this discovery are significant for the design of future experiments exploring the potential of HAMLET in combination with antibiotics to promote beneficial shifts in microbiome composition. Specifically, by using a larger variety of samples, and examine individual samples for ecological responses.

Paper III

Treatment of Mouse Infants with Amoxicillin, but Not the Human Milk-Derived Antimicrobial HAMLET, Impairs Lung Th17 responses

In this study, we aimed to investigate the impact of amoxicillin, a therapeutic antibiotic, and HAMLET derived from human milk, on T-cell response of infant mice when stimulated with *S. pneumoniae*. In an *in vitro* stimulation setup, lung cells from mice treated with amoxicillin, either alone or in combination with HAMLET, exhibited reduced levels of Th17 cytokine (IL-17A) upon stimulation with *S. pneumoniae*. However, no significant differences in the production of Th1 cytokine (IFN-γ) were observed among the treatment groups. Notably, the stimulated splenocytes showed no significant differences in cytokine production between the treatment groups. Furthermore, flow cytometry analysis of T-cell cytokine profiles revealed that lung CD4+ T cells, but not CD8+ T cells, from mice treated with amoxicillin or HAMLET and amoxicillin combination displayed decreased levels of IL-17A compared to those from mice exposed to HAMLET alone or the control group. These results indicate that exposure of infant mice to amoxicillin but not HAMLET, may modulate the immune response to *S. pneumoniae* by suppressing Th17 cytokine production in lung CD4+ T cells.

Discussion

Antibiotic resistance has emerged as a significant global health issue in the 21st century, posing challenges in treating life-threatening bacterial infections. NGS technology has revolutionized microbiome studies, allowing for comprehensive investigations of microbial communities as well as dynamic changes in ARGs within these communities. In this thesis, we employed a metagenomic approach in both Paper I and II to explore the oral microbiome's microbial composition and resistome under different stress conditions, including low antibiotic treatment and interventions with HAMLET. This approach provides a holistic view of the oral microbiome and its response to stressors. Furthermore, the thesis investigated the immunological response, focusing on particular subsets of T-cells, in infant mice subjected to antibiotic/HAMLET treatment and then challenged with *S. pneumoniae* to shed light on the immune system's response to treatments and their potential impact on combating bacterial infections.

Studying the oral polymicrobial biofilm in humans poses considerable challenges due to difficulties in controlling and quantifying confounding factors *in vivo*. The oral microbiome is one of the most diverse microbial niches in the human body, consisting of over 700 species of microorganisms (110). Each surface of the oral cavity has its ecological niche, and the environment is highly unstable, with rapid fluctuations in pH, organic carbon levels, and oxygen levels that can vary by several orders of magnitude within minutes (238). To overcome these challenges and effectively examine the complexity of oral polymicrobial biofilms, an *ex vivo* oral biofilm model was employed in Paper I and II. This approach provided a more stable and controlled environment, to test different interventions, to generate biological replicates and perform metagenomic analyses of polymicrobial oral biofilms. The human oral microbiome model has been used previously to study the reproducibility in oral microbiome taxonomic composition across replicates and to look at the complex microbiome activities in oral biofilms (102). In Paper I, individual saliva was used as an inoculum, resulting in more similar biological replicates. However, Paper II, when using pooled saliva from eight individuals, a higher variation in biological replicates was observed. In polymicrobial community, inter-species interaction plays a vital role. The microbial dynamics in biofilm formation depend on which pioneer bacteria attach to the surface, and which bacteria benefit from the metabolic environment in this polymicrobial biofilm (239). In both papers, treating the biofilm with low antibiotic concentration resulted in more divergent

biological replicates, with increased variation in beta diversity observed in both the microbial composition and resistome.

Research on the impact of antibiotics on the oral microbiome and resistome is still limited, despite the oral cavity being a significant source of pathogen transmission and containing a high abundance of ARGs compared to the gut microbiome (96, 177). Polymicrobial biofilms commonly found in the oral cavity often include priority pathogens listed by the WHO, such as *S. pneumoniae*, *K. pneumoniae*, *H. influenzae*, and *S. aureus* (113, 165, 166). A review by Morley et al. emphasizes the challenges posed by the off-target effects of antibiotics on the oral microbiome (70). A prediction model suggests that *S. pneumoniae* and other pathogens commonly present in the oral cavity and adjacent anatomical sites are exposed to antibiotics in 90% of cases when they are not the primary targets of the antibiotics (70). In Paper I, *S. pneumoniae* was detected in low abundance in two out of three donors, while in Paper II, where pooled saliva from eight donors was used as an inoculum, *S. pneumoniae* was also detected in both untreated and antibiotic/HAMLET treated samples. However, no significant changes in *S. pneumoniae* abundance occurred upon treatment with low concentrations of ampicillin and amoxicillin. However, previous studies have shown that HAMLET alone has bactericidal activity against *S. pneumoniae* biofilm (204, 208). Possible reasons for why this effect was not observed in a polymicrobial biofilm may relate to several factors, such as strain dependency on the response to HAMLET, complex interactions with species that offer *S. pneumoniae* protection against HAMLET, or even differences on the environmental conditions used in the experiments. Future studies with possible modifications in the model, such as using donor samples from patients with higher carriage of *S. pneumoniae*, or adding *S. pneumoniae* to the original samples may bring more light to our understanding of how antibiotics impact *S. pneumoniae* in polymicrobial biofilms.

The concentration of antibiotics detected in saliva varies depending on the specific antibiotic drug's pharmacodynamics and pharmacokinetics. Ampicillin and amoxicillin are second-generation semi-synthetic broad-spectrum beta-lactam antibiotics widely used to treat oral infections and surrounding tissues, such as oro-pharyngeal polymicrobial biofilm infections (219, 220). However, the precise quantity of antibiotics reaching the oral cavity varies. Past studies have monitored saliva concentration levels of ampicillin ranging from 0.1-0.28 μg/mL, while concentrations of amoxicillin ranged from 0.0 to 0.43 μg/mL (159, 160, 162-164, 221, 222). In both Paper I and II, the selected antibiotic concentrations favor increased biofilm viability within the polymicrobial community. However, extended exposure to low

concentrations of antibiotics has been associated with amplified bacterial virulence, increased biofilm formation, and increase of antibiotic resistance transmission (158). Bacteria perceive antibiotics as stressors and respond by promoting the survival of polymicrobial biofilms (158, 171, 177, 240). Our studies in both Paper I and Paper II demonstrates that low concentrations of ampicillin and amoxicillin, at sub-MIC concentrations for a variety of oral bacteria, enhance biofilm viability in polymicrobial biofilms.

There is limited knowledge regarding the effects of antibiotics in saliva on the oral microbiome and resistome, with current understanding based on prediction models and 16S rRNA gene data (82, 161, 162). *In vivo* studies have suggested that the oral microbiome is more resilient to changes than the gut microbiome, although only a few have investigated how antibiotic exposure affects the oral cavity's ecological environment. A study conducted with rats has shown that ampicillin administration significantly perturbed gut microbiome composition, while no substantial changes were observed in oral microbiome composition (161). A similar finding was observed in a study conducted in humans using different antibiotics such as amoxicillin (82). In this study, they concluded that the oral microbiome was more resilient to exposure to antibiotics than the fecal microbiome in the same population. A third study investigating the effect of amoxicillin on the oral microbiome indicated that the salivary microbiome is resilient to an antibiotic challenge by a low dose regiment (162). However, other studies found that the use of amoxicillin has been correlated with an increase in oral microbiome dysbiosis (163, 164). The reason for such difference in results might be due to different antibiotic treatment length, patients age or use of different methods.

In Paper I, the human oral microbiome model was used to investigate low ampicillin concentration-dependent shifts in the oral microbiome for individual donors using a sequence-based metagenomic approach. After 24 hours of ampicillin treatment, a reduction in alpha-diversity with respect to Shannon index for both donors was found, but surprisingly also a reduction in the ARG load was observed. Additionally, a significant increase in dissimilarity in biological replicates was observed in both donors after ampicillin treatment. These findings suggest that even short-term, single exposure to ampicillin treatment can have a significant impact on the oral microbiome and resistome. A positive correlation was also observed between reduced microbial taxonomy alpha diversity and reduced ARG abundance on both donor A and B. In contrast, Paper II where HAMLET’s antimicrobial synergistic effect alone and in combination with low amoxicillin concentration was investigated on polymicrobial oral

biofilms for 24 hours, no major difference in alpha-diversity in the oral microbiome and resistome was observed compared to the untreated negative control. Future studies should design long-term treatment experiments with different antibiotic classes to investigate the potential impact on oral microbiome.

In Paper I, each donor had a unique and specific microbiome, with the untreated negative control samples containing the highest relative abundance of *Streptococcus* and *Veillonella* genus. Although the composition varied among individuals, *V. atypica*, *S. salivarius*, and *S. mitis* were among the most abundant species. These bacterial species play a critical role in biofilm formation and establishment (151). During low concentrations of ampicillin treatment, there was minimal change in the relative abundance and bacterial composition profile. In Paper II, the negative untreated control also contained bacterial species including *V. atipica*, *S. salivarius*, and *S. mitis*, as observed in individual donors in Paper I. However, the use of pooled saliva samples from eight donors, might obscure individual differences characteristics of human microbiome studies. In these samples, the polymicrobial biofilm had a high abundance of other *Lactobacillus* species such as *Lactobacillus fermentum*. The most significant shift in microbial composition was observed in the combination treatment of HAMLET and amoxicillin. *Lactobacillus crispatus* was particularly enriched in the combination treatment of HAMLET and amoxicillin, with an increase of up to 90%. This species is known for its probiotic and antimicrobial properties and has been shown to alleviate symptoms of periodontal diseases in mice (241-243). It has also been demonstrated to reduce the development of other dental diseases, such as caries, by maintaining the pH above 6 in the oral cavity (244). Notably, unlike polymicrobial biofilms treated with amoxicillin alone, the combination treatment of HAMLET and amoxicillin enriched in probiotic bacteria. HAMLET has demonstrated synergistic antibiotic bactericidal activity against certain single-species biofilms, such as *S. aureus* and *S. pneumoniae* (204, 207, 208), but its effects on polymicrobial biofilms is yet to be unrevealed. The bactericidal mechanism of HAMLET's antimicrobial activity differs from that of amoxicillin. Previous studies have demonstrated that HAMLET interacts with bacterial cell membranes, leading to a rapid depolarization of calcium ions, increasing cell membrane permeability and depolarization. Consequently, this results in cell shrinkage, DNA condensation, and fragmentation, leading to apoptosis execution (201). Additionally, studies on *S. pneumoniae* have shown another bactericidal pathway by inhibiting glycolytic enzymes, such as fructose-bisphosphate aldolase and glyceraldehyde-3 phosphate (GAPDH) (209).

In both Paper I and II, the oral resistome composition included efflux pump ARGs *patA*, *patB*, and *pmrA*, as well as the antibiotic target alteration ARG *RlmA(II)*, all showing a strong correlation with *Streptococcus* genus, including species like *S. mitis*. The *Streptococcus* genus has been discovered to be a well-established reservoir for ARGs (178, 245, 246). Notably, *S. mitis* is closely related to *S. pneumoniae*, and prior studies have demonstrated the potential for ARGs to be readily transmitted from *S. mitis* to *S. pneumoniae* via horizontal gene transfer, a phenomenon known as natural transformation (178). Furthermore, despite being in low abundance, an increase in beta-lactam genes was also observed in samples treated with antibiotics and HAMLET in both Paper I and II. The rise of beta-lactam genes in antibiotic-treated samples has previously been documented as a survival mechanism adopted by bacterial species in polymicrobial biofilms (247). However, the increase in beta-lactam genes in HAMLET-treated samples in Paper II presents a complex phenomenon that requires further investigation. Moreover, several tetracycline related ARGs were detected in oral resistome samples from both Paper I and II. The escalation of these ARGs in samples treated with low concentrations of antibiotics has been frequently reported in metagenomics studies (248-250), with suggestions that they may be co-carried in genetic mobile elements with other ARGs. This underscores the complex ecological dynamics within polymicrobial biofilms.

In Paper III, the same therapeutic agents used in Paper II, including amoxicillin alone, HAMLET alone, and a combination of HAMLET and amoxicillin, were utilized. However, unlike Paper II, Paper III employed an *in vivo* model using infant mice to measure the immunological response of T-cell subsets Th17/Th1 following *in vitro* stimulation with *S. pneumoniae*. The Th17 response has been recognized as crucial in protecting infants against bacterial pathogens, including *S. pneumoniae*. HAMLET, in addition to its selective bactericidal properties, has also demonstrated immunomodulatory features in previous studies (208, 251). Specifically, intranasal administration of a combination treatment of HAMLET and gentamicin, but not HAMLET alone, was found to significantly enhance pneumococcal death in the nasal passages in an *in vivo* study. In Paper III, a significant amoxicillin-induced IL-17A/Th17 responses was observed exclusively in lung Th17 cells, but not in the spleen. Previous research has shown that intraperitoneal administration of piperacillin and the beta-lactamase inhibitor, tazobactam reduced both splenic and lung CD4+IL-17A+T cell numbers in response to *in vitro* stimulation with *S. pneumoniae* (183). A major difference between these two studies lies in the administration route of antibiotics and HAMLET. In Paper III, intra-nasal administration was chosen to minimize stress in infant mice and because it had

previously been demonstrated to impact immune responses in an *in vivo* HAMLET study (208). This suggests that the local lung microbiome and T-cell responses may have a stronger impact compared to systemic exposure. In addition, no significant difference was observed between the untreated negative control and the HAMLET-treated group in terms of immune responses, specifically Th17/Th1. However, although not significantly, the combination of HAMLET and amoxicillin, tended to suppress Th17 responses in the lungs more effectively than amoxicillin alone, indicating a potential synergistic antimicrobial effect. A similar effect was also observed in Paper II, where the combination of HAMLET and amoxicillin increased the efficacy of amoxicillin and reduced cell viability in the polymicrobial biofilm. Further investigation is needed to understand the mechanism of impairment of Th17 cell responses and whether Th17 suppression can lead to increased susceptibility to pneumococcal infection.

Conclusions and Future Perspectives

This thesis provides valuable insights into the effects of antibiotics and HAMLET on the oral microbiome, biofilm formation, antimicrobial resistance, and adaptive immune responses. The research fills a gap in our understanding of how antibiotics affect the oral microbiome and resistome, emphasizing the significance of this area of study given the oral cavity's role in pathogen transmission and the high abundance of antibiotic resistance genes. It highlights the potential risks associated with prolonged exposure to low antibiotic concentrations, including the development of bacterial virulence, biofilm formation, and antibiotic resistance.

The results of the research demonstrate that low concentrations of ampicillin and amoxicillin can enhance the viability of polymicrobial biofilms in the oral cavity. Also, the combination of HAMLET and amoxicillin has a synergistic effect that reduces biofilm viability and promotes the growth of bacteria with probiotic and antimicrobial potential. Additionally, the study uncovers the acquired immune responses triggered by amoxicillin, but not HAMLET, resulting in the suppression of IL-17A production in lung CD4+ T cells after exposure to *S. pneumoniae*.

The methodologies employed shed light on the complex interplay between the oral microbiome, antibiotic resistance, and immune responses. The study highlights in particular the possibilities of combining *in vivo* and *ex vivo* models to better understand the dynamics and complexity of the response of microbiomes to antibiotics and other possible interventions. The individuality of microbiomes and its variability in response to external factors such as genetics, diet and several other environmental influences, are a recognized challenge in microbiome studies. However, the continuing reduction in costs to run and analyze shotgun metagenomics data will likely help in extending the use of such models to a larger set of samples and donor source, as well as to different experimental conditions. Combining such methods with clinical studies on metagenomics are promising approaches to move the field of microbiome research from association with conditions and diseases to a better understanding of the mechanisms involved.

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Original papers

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Ecological consequences of low ampicillin concentrations on an *ex-vivo* biofilm model of the human oral microbiome

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Abstract

Antibiotics at low concentration can promote processes such as biofilm formation, virulence and antibiotic resistance. This can be of high relevance in microbial communities like the oral microbiome, where commensals and pathogens share a common habitat and where the abundance of antibiotic resistance genes surpasses the abundance in the gut. Here, we used an *ex vivo* model of human oral biofilms to investigate the impact of ampicillin on biofilm viability. Further, the ecological impact on the microbiome and resistomes was investigated using shotgun metagenomics. The results showed that low concentrations promoted significant shifts in microbial taxonomic profile and could enhance biofilm viability by up to 1 to 2-log. For the resistome, low concentrations had no significant impact on antibiotic resistance gene (ARG) diversity, while ARG abundance decreased by up to 84%. A positive correlation was observed between reduced microbial diversity and reduced ARG abundance. The WHO priority pathogens *Streptococcus pneumoniae* and *Staphylococcus aureus* were identified in some of the samples, but their abundance was not significantly altered by ampicillin. Yet, most of the antibiotic resistance genes that increased in abundance in the ampicillin group were associated with streptococci, including *Streptococcus mitis*, a well-known potential donor of ARGs to *S. pneumoniae*. To our knowledge, this is the first report on antibiotic effects on oral microbial communities using an *ex-vivo* human microbiome model combining biofilms and shotgun metagenomics. Overall, the results highlight the potential of using the model to further our understanding of ecological and evolutionary forces driving antimicrobial resistance in oral microbiomes.

INTRODUCTION

Since their widespread availability and use beginning in the late 1940s, antibiotics have saved millions of lives. However, the current rise of antibiotic-resistant infections represents a serious and growing threat to global health (1–3). Multiple factors, including poor sanitation, limited access to clean water, raise in global travel and migration, further contribute to transmission of drug-resistant microorganisms among populations (4–6). Additionally, overuse and misuse of antibiotics creates a selective pressure where antibiotic susceptible bacteria are killed or inhibited, while antibiotic resistant bacteria survive (7–9).

Historically, antibiotic resistance studies have focused on specific pathogens and antibiotic concentrations used to eliminate bacteria at infection sites. An issue that recently has gained attention is the collateral impact of antibiotics on the human microbiome at different anatomical sites. The biological response to an antibiotic drug depends on the different pharmacokinetic aspects (7, 10). When antibiotics reach the different sites within the human body, the concentrations of the drug differ in time and space, often resulting in prolonged exposure to low antibiotic concentrations. In addition, the impact of antibiotics on the microbiome may be affected by factors related to how microbes are organized, with microbial composition and biofilm mode of growth playing an important role. Microbes organized in biofilms are generally less susceptible to the effect of antibiotics. Two major concerns regarding the off-target effects of antibiotics on biofilm communities is the loss of colonization resistance, and the

enrichment for antimicrobial resistance genes (ARGs) and drug resistant bacteria in the human microbiome (11, 12).

Most of the research reporting on the impact of antibiotics on the human microbiome and the associated ARGs (resistome) has focused on the gut. Results of these studies vary with some reporting an increase in ARG load, while others demonstrates no effect. Results for changes in ARG diversity also differs, varying from slightly increased diversity, to no effect, and some studies showing decreased richness (13–15). Multiple factors have been proposed to explain these differences, such as variations in the populations studied, the type of antibiotics used, age differences, and a range of other factors (15–18). Although less well-studied in this context, the oral microbiome is thought to be less prone to changes in microbial ecology due to antibiotic treatment than the gut (19, 20). The oral cavity and adjacent anatomical regions are, however, important ecological niches that serve as reservoirs for the emergence and dissemination of antibiotic resistance genes and antibiotic-resistant bacteria, often thriving in the form of biofilms (21–24). Four of the 12 WHO Global priority pathogens are often found in the oral cavity including; *Streptococcus pneumoniae*, *Haemophilus influenzae, Klebsiella pneumoniae*, and *Staphylococcus aureus* (25).

One of the most worldwide prescribed antibiotics by clinicians and other healthcare professionals is ampicillin (26, 27). This is a broad-spectrum antibiotic that interferes with the cross-linkage of peptidoglycans in the bacterial cell wall to inactivate and kill bacteria (28). Ampicillin is widely used to treat infections in the respiratory tract and other body sites (29). When administered orally, ampicillin concentration in saliva can reach peak concentrations close to or higher than minimum inhibitory concentrations (MIC) for several of the most studied bacteria in the oral cavity (30, 31). At concentrations below the MIC, ampicillin and several other antibiotics have shown to stimulate biofilm formation by *S. aureus* and *H. influenzae*, as well as by other pathogens found in the oral cavity and at close anatomical sites, such as *Streptococcus intermedius* and *Enterococcus faecalis* (12, 32–35).

To address the inherent limitations of human studies related to multiple cofounding factors, various *ex-vivo* models have been proposed (36–39). Such models are particularly relevant to generate hypotheses for further *in vivo* investigations. So far, they have been applied mostly to get a better understanding of ecological changes associated with specific disease states (39–41). Here, we investigated the ecological impact of ampicillin on the oral microbiome using an *ex vivo* highly reproducible biofilm model of the human oral microbiome (42). Our focus was on the effect of ampicillin on viability and microbiome composition, alongside assessing changes in diversity, composition, and abundance of ARGs. Our results showed an increase in biofilm viability by low ampicillin concentrations and an unexpected decrease in ARG abundance by both low and high concentrations.

METHODS

Sample collection

The study was conducted in accordance with the Declaration of Helsinki and approved by the National Regional Ethics Committee (REK20152491) for studies involving human samples. The participants were asked to brush their teeth after breakfast and refrain from any food or drink two hours before donating saliva. They rinsed the mouth three times with water 10 minutes prior to saliva collection. Non-stimulated saliva was collected from each person. Saliva samples were centrifuged at 6000 x g for 5 minutes at 4^{o}C to spin down large debris and eukaryotic cells. The supernatant was used as saliva derived inoculum. Cell-free saliva was obtained by centrifuging saliva samples at 10 000 x g for 7 minutes at 4^{o}C. The upper fraction was used as pellicle to coat the bottom of the wells prior to growing the biofilms as previously described (42). Saliva from three donors was used in the study. All experiments were conducted in triplicates, including controls and ampicillin treated biofilms.

The human oral microbiome biofilm model

A previously described *ex vivo* biofilm model that maintains a highly reproducible species and metabolic diversity of the human oral microbiome was utilized (42, 43). Briefly, SHI media was pre-reduced for 4 hours in anaerobic conditions (carbon dioxide, 5%; balanced with nitrogen) (44). Saliva samples were used to inoculate the SHI media (2 µl saliva/ mL), which was then distributed to the wells of a 24-well plate (1 mL per well) followed by incubation in an anaerobic chamber at 37^{o}C for 24 h. The liquid phase was then removed and fresh SHI medium was added to the pre-formed oral biofilms. Samples were either not treated (control) or treated with ampicillin ranging from 0.025-200 µg mL^{-1} (Sigma-Aldrich). The ampicillin stock (50 mg mL^{-1} in distilled water) was diluted in SHI medium before adding to the biofilms. After 48 h, the oral biofilms were washed and resuspended in 1 mL PBS, and 20% glycerol was added before the samples were stored at -80^{o}C.

Oral biofilm viability assay

For biofilm viability assessment, the samples obtained as described above from controls and treated biofilms were diluted in 10-fold dilution series, and 20 µL of each dilution was then plated on SHI agar plates. The plates were incubated for 48 hours at 37^{o}C in the anaerobic chamber to calculate colony forming units per milliliter (CFUs mL^{-1} $\log_{10}$).

DNA extraction

Bacterial DNA was extracted using the MasterPure™ Gram Positive DNA Purification Kit (Epicentre, Madison, WI, USA) using the manufacturer's protocol. Precipitated DNA was resuspended in 35 µl milliQwater. A NanoDrop™ 2000c spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and a Qubit ™ 4 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA) were used to measure the quality and the amount of extracted DNA.

DNA library preparation and sequencing

DNA library preparation was conducted using an Illumina DNA Prep kit, (m) (Illumina, Inc., San Diego, CA, USA) following the manufacturer's protocol. The final DNA library was retrieved by resuspending it in the

provided buffer, and each sample adjusted to 500 ng in 30 µL using nuclease-free water.The quality and the concentration of the DNA library was measured using a NanoDrop™ 2000c spectrophotometer and Qubit ™ 4 Fluorometer, and further analysed using a Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) and a High Sensitivity DNA kit (Agilent Technologies, Santa Clara, CA, USA) using the manufacturer's protocol. Metagenomic shotgun sequencing was conducted at the Norwegian Sequencing Centre (Oslo, Norway) using Illumina 3000/4000 High Seq (Illumina, Inc., San Diego, CA, USA).

Quality control and pre-processing of metagenomic data

The quality of raw and preprocessed sequencing reads were evaluated by FastQC tool (v.0.11.8) (45). Low quality reads and adapter sequences were trimmed using Trimmomatic (v.0.35) (46) with the following parameters: ILLUMINACLIP: Nextera PE:2:30:10 LEADING:3 TRAILING:3 SLIDING WINDOW:4:15 MINLEN:36. Reads which mapped to the human genome (GRch38) using Bowtie 2 (v.2.3.4) (47) were removed. The remaining high-quality reads were then subjected to microbiome and resistome profiling.

Taxonomic and resistome profiling

MetaPhlAn (v.2.0) (48) was used to profile the bacterial composition in the oral biofilm samples and to determine their abundance at the species-level. For ARG prediction, reads were mapped against the Comprehensive Antibiotic Resistance Database (CARD) using the Bowtie 2 alignment tool (49, 50). ARGs with > 80% gene fraction (proportion of nucleotides that align with at least one read to the reference ARG) were considered to be positively detected in a sample. Read counts were normalized for differences in both gene lengths and bacterial abundances for each sample by calculating reads per kilobase of reference gene per million bacterial reads (RPKM).

Downstream analysis

MicrobiomeAnalyst (51, 52) and ResistoXplorer (53) were used to carry out comprehensive exploration, analysis and visualization of the microbiome and resistome count data. GraphPad (Prism 9 software) and R (v3.6.0) were used for graphical representation and statistical analysis. Alpha diversity using the Shannon and Chao1 diversity indexes were calculated at the genus, species and ARG level. Beta diversity was estimated using Aitchison distance on centered log-ratio (CLR) transformed counts, using the *ordinate* function from the phyloseq (v.1.34.0) R package (54, 55), and visualized in a compositional principal component analysis (PCA) ordination plot. Differences in beta diversity were tested using permutational multivariate analysis of variance (PERMANOVA) using the *adonis* function (vegan (v.2.5.7) package) with 999 permutations. The dissimilarity in oral microbiome and resistome composition within replicates were calculated using the Aitchison distance. Pairwise comparisons of log-fold change in abundance between groups were performed using DESeq2 (v.1.36.0) (55), adjusted for multiple comparisons using the Benjamini-Hochberg (BH) procedure. We conducted the association analysis based on the pairwise Spearman's correlations among microbial taxa and ARG using the integration module in ResistoXplorer. One-way analysis of variance (ANOVA) adjusted for multiple comparisons using the Benjamini-Hochberg (BH) procedure and two –tailed unpaired t-test were used to compare group differences, as appropriate. P values of < 0.05 were considered statistically significant.

RESULTS

The impact of different ampicillin concentrations on oral biofilm viability

Ampicillin ranging from 0 to 200 µg mL^{-1} was used to investigate the impact on biofilm viability using saliva inoculum from two different donors (Fig. 1). Despite some variation in the curve response shape for each of the donor samples, low concentrations, including 0.05 µg mL^{-1} and 0.1 µg mL^{-1}, resulted in increased biofilm viability by approximately 1.2 log_{10} compared to the negative control. The viability decreased in biofilm samples at high concentrations until viable cells could no longer be detected.

The impact of low ampicillin concentrations on oral microbiome ecology

In total, 24 samples from donor A and B were analyzed by shotgun metagenomic sequencing to investigate the impact of 0.025, 0.05 and 0.1 µg mL^{-1} ampicillin on oral microbiome ecology. These concentrations were chosen based on clinical data reporting levels up to 0.12–0.28 µg mL^{-1} ampicillin in saliva followed by oral administration (30, 31, 56). They were also within the range in which biofilm viability was enhanced. (Fig. 1). Compared to the untreated samples, there was a significant increase in DNA concentration for donor A in response to antibiotic treatment. Although not statistically significant, a similar trend was observed for donor B (Fig. 2a).

A total of 234 million (M) paired reads across all samples were obtained after quality filtering, with an average of 9.8 M-reads per sample (minimum of 3.9 M and maximum of 19.9 Mreads per sample). In total, 48 bacterial species were detected across all samples.

Alpha diversity (intra-sample diversity) using the Shannon index, which takes into account both taxonomic abundance and evenness, revealed a significant reduction in alpha diversity at genus level for donor A, but not donor B, compared to the untreated samples (Fig. 2b). At species level, a tendency in alpha-diversity reduction was also observed for both donors. (Fig. 2c). No significant differences were observed for either donor A and B using Chao1 index for alpha diversity at genus and species level, which takes into account only microbial taxonomic richness (Fig. 2d,e). Beta diversity (inter sample diversity) showed a significant difference between the two donors ($P = 0.001$, $R^2 = 0.548$, $F = 26.706$, permutational multivariate analysis of variance [PERMANOVA]) (Fig. 2f). Compared to the control, a significant increase in microbiome dissimilarity was observed for the highest ampicillin concentrations for both donors (Fig. 2g).

The relative taxonomic composition varied with respect to ampicillin treatment compared to the non-treated controls. The untreated samples in both Donor A and B showed *Veillonella atypica* being the most abundant species (Fig. 2h) **(Supplementary table 1)**. Differential abundance analysis using DESeq2

revealed a statistically significant increase in gram-negative bacteria within the genus *Veillonella* and *Prevotella* for the antibiotic-treated biofilm samples from both donors (Fig. 3). For donor A, *V. atypica*, *V. infantium, P. jejuni, P. histicola, P.* sp. Oral taxon 306, *P. salivae* and *P. melaninogenica* increased significantly. In contrast, *Streptococci* such as *S. oralis*, *S. mitis, S. parasanguinis, S.* sp. HMSC034E03, *S* sp. HMSC067H01 were reduced. In donor B, only *V. atypica*, *V. infantium, V. dispar, V.* sp T11011 6 and *P. jejuni* increased significantly. On the other hand, *V. parvula*, *S.* sp.12 and *S. sanguinis* were decreased. The only *Streptococcous* species which showed a significant increase under ampicillin treatment in both donors was *S. salivarius*. Among the pathogen priority list by the WHO, we found *S. pneumoniae* in all samples from donor A, and *S. aureus* in donor B. Their relative abundance was not significantly changed in ampicillin treated samples at all concentrations **(Supplementary table 1).**

The impact of low ampicillin concentrations on oral resistome

A total of 129 258 paired reads were annotated as ARGs across all samples, with an average of 5 385 reads (min-max:1 620 – 13 067) per sample. In total, 28 ARGs were detected. The ARGs were found to belong to 10 different antibiotic drug classes associated with three different mechanisms of resistance; antibiotic efflux, antibiotic inactivation and antibiotic target protection **(Supplementary table 2)**.

For both donors, treatment with low concentration of ampicillin decreased ARG abundance compared to the untreated samples, visualized by bar plots for each ARG (Fig. 4a). The reduction in ARG load by ampicillin was statistically significant for both donors (Fig. 4b). Compared to the untreated samples, both donor A and B failed to show major changes in ARG alpha diversity (Fig. 4c). For beta diversity, the samples clustered according to individuals ($P = 0.001$, $R^2 = 0.3357$, $F = 11.117$), permutational multivariate analysis of variance [PERMANOVA]) (Fig. 4d). A significant increase in oral resistome dissimilarity was observed within the samples exposed to ampicillin compared to the control in donor B, but not donor A **(**Fig. 4e**).**

The most abundant ARG classes detected in both donors were fluoroquinolone, as well as macrolide, lincosamide, streptogramin (MLS) followed by tetracycline in both control and ampicillin treated samples **(**Fig. 4f**)**. Donor A showed an increased abundance of the *mel* gene in ampicillin treated samples, which is associated with macrolide resistance. In addition, some beta-lactam genes such as *PC1* and *CfxA3* were detected only in samples treated with ampicillin in donor A. Oral biofilms treated with ampicillin in donor B harbored more beta-lactam genes in *CfxA* family, compared to the untreated control. (Fig. 4g**)** **(Supplementary table 3)**

Association between the oral microbiome and resistome

A Spearman correlation matrix revealed a strong positive correlation between ARG abundance and microbial alpha-diversity (Shannon index, species level) in donor A (R = 0.8322) and donor B (R = 0.8252). **(**Fig. 5a**)**. To predict the origin of ARGs, Spearman's pairwise correlation analysis was also conducted between ARGs and species abundance (Fig. 5b**).** Efflux pump ARGs such as *patA*, p*atB* and *pmrA* were

strongly correlated with *Streptococcus* species, including *S.* sp. HMSC067H01, *S. mitis*, *S. vestibularis*, *S. infantis*, *S.* sp. HMSC034E03, *S. parasanguinis*, *S. australis* and *S. salivarius*. A positive correlation between increased *Veillonella* sp. and tetracycline resistant genes *tet(O)* and *tet(M)* was also observed indicating *Veillonella* sp. as the potential host for *tet(O)* and *tet(M)*.

The impact of ampicillin on the microbiome and resistome by high ampicillin concentration

Although the primary focus of the study was on low ampicillin concentrations, an additional experiment to investigate the impact of a high concentration was performed. Saliva samples from a third donor (donor C) were first investigated for the impact of ampicillin at different concentrations on biofilm viability. This was chosen as no more samples from donor A and B were available. Here, similar to donors A and B, we also found that low concentrations favored biofilm viability **(Fig. S1)**. Results for a high concentration (approximately 10 μg mL^{-1}), showed a significant reduction in DNA concentration compared to the control (**Fig. S2 a**). No significant difference was observed in alpha diversity **(Fig. S2 b,c).** Compared to the untreated samples *V. atypica* reduced significantly. In contrast, there was an increase in the relative abundance of *Streptococcus* species, including *S. salivarius*, *S. parasanguinis* and *S. infantis*. Among the pathogen priority list by the WHO, we found *S. pneumoniae* in all samples from donor C **(Fig. S2 d,e) (Supplementary table 4).**

Results from oral resistome revealed a tendency for a decrease in ARG load and an increase in ARG alpha diversity in the treated samples compared to the control, although these findings were not statistically significant **(Fig. S3 a,b) (Supplementary table 5).** A relative increase was observed for ARGs such as *mel*, *tetA(46)*, *tetB(46)* and others, while *patA*, *patB* and *pmrA* reduced. **(Fig. S3 c,d) (Supplementary table 6).**

DISCUSSION

Sub-inhibitory concentration of antibiotics can impact bacterial gene expression, and trigger behaviors involved in virulence, such as biofilm formation, quorum sensing, and horizontal gene transfer (12, 33, 57, 58). Most research on sub-inhibitory concentrations of antibiotics has been conducted on single bacteria and rarely on a consortium of defined microbial species (59, 60). Here we demonstrate that ampicillin, at low concentrations, favored biofilm viability within a diverse oral microbial community. To the best of our knowledge, this is the first study to report antibiotic effects in an *ex-vivo* human microbiome model combining biofilms and shotgun metagenomics. The metagenomic changes induced by ampicillin were highly reproducible between replicates from the same donor and showed donor-specific clustering features in line with inter-individual variations characteristic of human microbiomes (61, 62). Additionally, low concentrations were associated with changes in both microbial and antimicrobial resistance gene composition. Defying expectations, we found an overall decrease in the abundance of ARGs in ampicillin treated samples.

The oral microbiome is best understood within the framework of the most prevalent oral diseases, namely dental caries and periodontal disease. However, the oral microbiome also represents an important reservoir of pathogens and antibiotic resistance genes (63–66). *S. pneumoniae*, *K. pneumoniae*, *H. influenzae* and *S. aureus*, for instance, are mostly often thought of as residents of the nasopharynx, but their presence in the oral cavity is of relevance, particularly due to saliva being a main route for dissemination of microorganisms between humans, both via direct contact and droplets. In saliva samples collected from healthy donors for this study, *S. pneumoniae* was identified in the samples from two of the three donors. Previous studies have also demonstrated *S. pneumoniae* in biofilms using a similar *ex-vivo* model as the one used in our study (42, 60). We found that either of the concentrations used resulted in changes in *S. pneumoniae* abundance. Yet, among the 12 most prevalent antibiotic resistance genes, four were correlated with *S. mitis* and other oral streptococci closely related to *S. pneumoniae*. Of note, clinical and laboratory data indicate that oral streptococci are an important reservoir of antibiotic resistance genes that can be readily transmitted to *S. pneumoniae* by horizontal gene transfer via natural transformation, thus comprising treatment of invasive pneumococcal diseases (67–69). In line with *in vivo* studies, oral streptococci were also among the most prevalent bacteria in our model, independent of donor, indicating the possibility of using variations of the current model to investigate this important phenomenon in complex communities that approximate *in vivo* conditions. Another WHO priority pathogen, *S. aureus*, was also found in all samples from one of the donors exposed to low ampicillin concentration, but its abundance remained unchanged.

The reduction in antimicrobial resistance gene load by both low and high ampicillin concentration was somewhat surprising. However, most available studies using metagenomics have focused on the gut resistome. In these studies, the reported outcomes on the resistome vary from no significant effects to increased antibiotic resistance gene load and diversity following antibiotic therapy (20, 70–72). For the oral microbiome, our knowledge is mostly restricted to a few studies indicating that the oral microbiome is more resilient to changes than the gut microbiome (19, 20). Reports on the resistome are mostly based on functional predictions using 16S rRNA gene data (20). Resilience of the oral microbiome can be a result of evolutionary processes by having evolved in the presence of mechanical disrupting forces from salivary flow and mastication, as well as fluctuations in diet, temperature, and chemical agents (73), or that oral biofilms may be more impermeable to antimicrobials. Alternatively, pharmacodynamics and pharmacokinetics of antibiotics can also be relevant as for at least some antibiotics, the concentrations that reach saliva following absorption are lower than in the gastro-intestinal tract (15, 74). Also, in our model the ecological effects were studied after 24 hours of antibiotic exposure, while in clinical studies on the oral microbiome and resistome the reported effects are after seven days antibiotic course (74–76). The possibility that the observed reduction in antibiotic resistance gene load in our study may occur also *in vivo* during shorter-courses are warranted, since such a finding would provide relevant information for antibiotic-stewardship programs aiming at reducing the length of antibiotic therapies (77). Another interesting finding was the increased dissimilarity in microbiome composition within the samples exposed to low antibiotic concentrations compared to the control. Such findings have also been observed in human clinical studies (70). Increased dissimilarity in resitome compostion was also observed, but

only for one of the donors. This is an interesting phenomenon that is not yet understood or universally proven, but that may relate to stochastic mechanisms involved in the response to antibiotics (9).

Overall, the results indicate that the model can be useful for future studies investigating the impact of different antibiotics or examining other interventions with the potential to alter the ecology and evolution of antimicrobial resistance in oral biofilms, and that include main pathogens listed by the WHO as priority microorganisms for controlling antimicrobial resistance (25). The current model has previously been adapted to investigate conditions such as caries by changing the carbohydrate substrate used in the growth medium (40), or from patients with periodontal disease to obtain a specific disease-state sub gingival community (41). For *S. pneumoniae*, for instance, samples could be from children, as young age is associated with prevalent carriage of pneumococci, and these are one of the most vulnerable age groups to pneumococcal infections (78). Of notice, recent studies using prediction models estimate that exposure of *S. pneumoniae* and other pathogens found in the oral cavity and adjacent anatomic regions to antibiotics are in more than 90% of the cases when they are not the target of the antibiotic therapy (7). This highlights the importance of advancing our understanding of the impact of antibiotics on antimicrobial resistance from an ecological and evolutionary perspective. *Ex-vivo* models are particularly relevant as they are useful for mechanistic studies and are not subjected to main ethical issues. Since the environment is stable and controlled, it has the potential to generate highly reproducible results, thus avoiding the shortcomings of high variability observed in clinical studies of human microbiomes (42). In addition, since shotgun approaches are still costly and will require large clinical data before finding its way from bench to bed side, modelling can be a cost-effective way to help develop new hypothesis and predictive models to test against available and future human data sets.

Declarations

ACKNOWLEDGMENTS:

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DATA AVAILABITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) is submitted to NCBI.

AUTHOR CONTRIBUTION:

Conceptualization of the project was by FCP and AE. All authors contributed to the design of experiments. Laboratory work was by NKB, FCP, JLB, HAÅ. Downstream metagenomics analysis was by NKB and AD. First drafting of manuscript by NKB and FCP. All authors contributed to critical review of data and writing the final manuscript.

COMPETING OF INTERESTS

No potential conflict of interest was reported by the author(s).

ADDITIONAL INFORMATION

Ethics statement:

The study was conducted in accordance with the Declaration of Helsinki and approved by the National Regional Ethical Committee (REK20152491)

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Figures

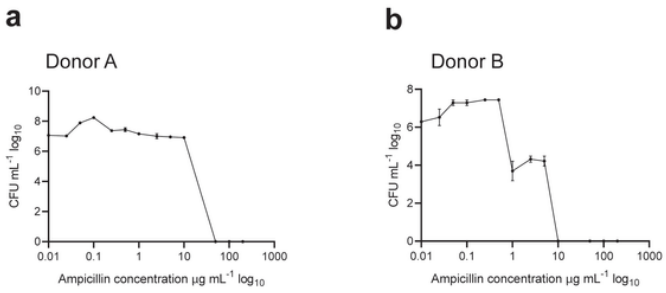

Figure 1

Oral microbiome biofilm viability treated with different ampicillin concentrations. Numbers of viable cells in the community, as determined by colony-forming units counted on SHI agar plates for (a) donor A and (b) donor B. The data are shown for triplicate experiments. Error bars represent mean SEM.

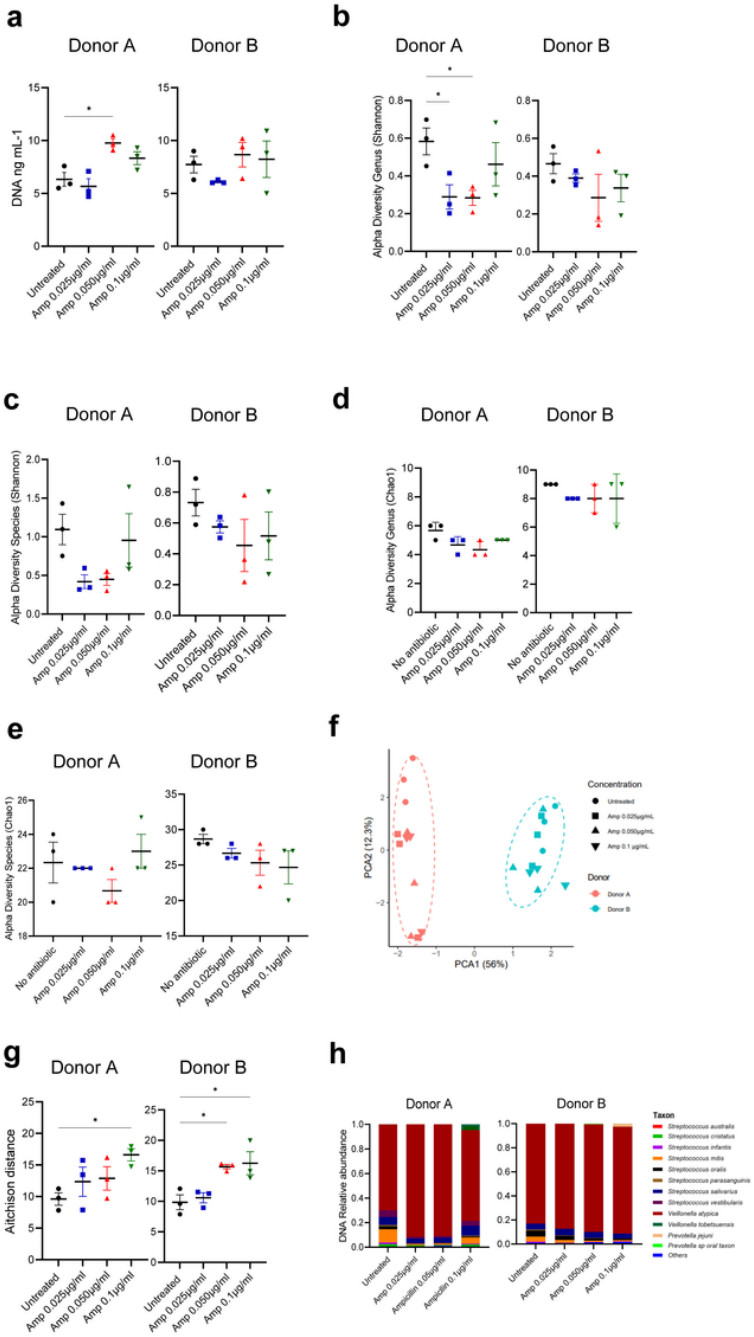

Figure 2

The effect of low ampicillin concentrations on the microbiome of *ex vivo* oral biofilm communities. (a) DNA concentration measured by Qubit4 in ng mL^{-1}. (b and c) Alpha-diversity measured by Shannon index which indicates richness and evenness on (b) genus and (c) species level. (d and e) Alpha diversity measured by Chao1 indicates the total richness at (d) genus level and (e) species level. (f) Principal component analysis (PCA) ordination plot with Aitchison distance illustrating beta-diversity. (g)

Dissimilarity in oral microbiome within each treatment group, each point shown as Aitchison distance. (a-e, g) Error bars represent mean SEM. One-way ANOVA followed by Benjamini-Hochberg (BH) posthoc test, * *P*<0.05 (h) Stacked bar plots displays the relative abundance of the 12 most abundant species in the microbiomes.

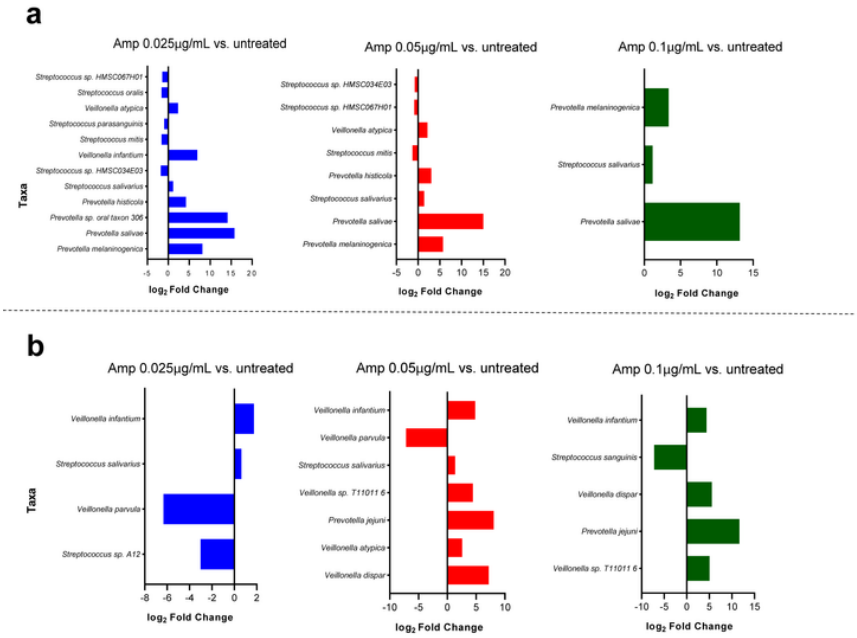

Figure 3

Taxa with significantly different abundances upon treatment with low concentration of ampicillin. Bar charts illustrate the $\log_2$ fold change of taxa, adjusted for false discovery rate (FDR), *p*-values <0.05. (a) Donor A and (b) donor B (based on DESeq2).

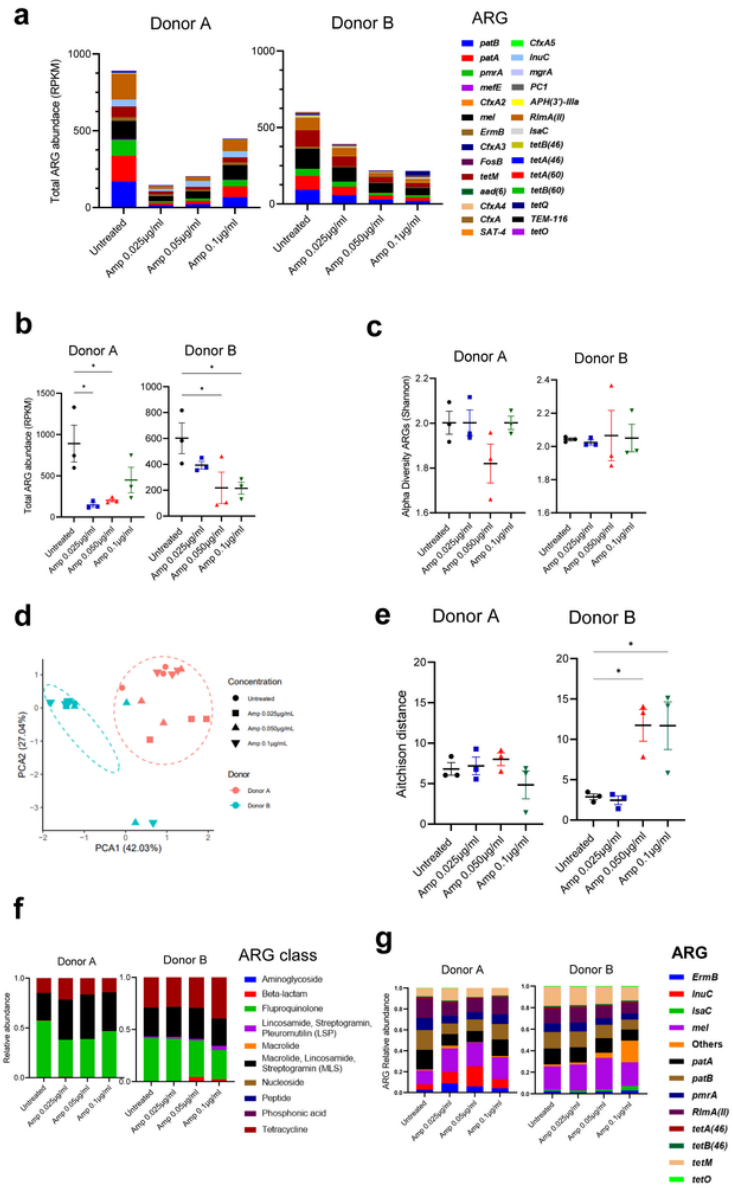

Figure 4

The effect of low ampicillin concentrations on the resistome of *ex vivo* oral biofilm communities. (a) The total ARG abundance visualized as reads per kilobase million values (RPKM) in the oral biofilm community. (b) Total mean RPKM display error bars with mean SEM. One-way ANOVA followed by Benjamini-Hochberg (BH) posthoc test. * $P<0.05$. (c) Alpha diversity measured at ARG level by Shannon index which indicates richness and evenness. (d) Principal component analysis (PCA) ordination plot with Aitchison distance illustrating beta-diversity. (e) Dissimilarity in oral resistome within each treatment group, each point shown as Aitchison distance. Error bars represent mean SEM. One-way ANOVA followed by BH posthoc test.* $P<0.05$. (f and g) Stacked bar plots displays the relative abundance of (f) all antimicrobial classes and (g) the 12 most abundant antimicrobial resistance genes (ARGs) found in the oral biofilm community.

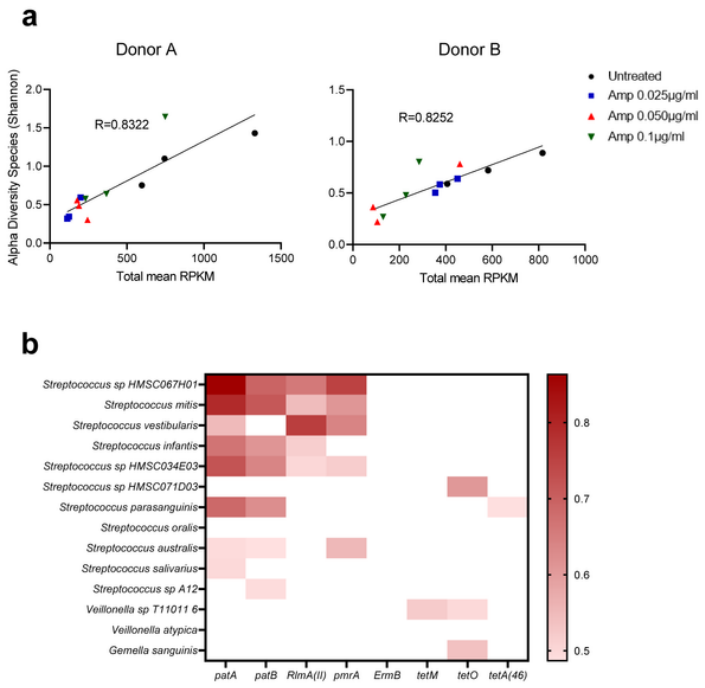

Figure 5

Correlations between the oral microbiome and resistome. (a) Spearman correlation matrix between microbiome alpha-diversity and ARG in RPKM. Correlation coefficients “R” were between 0.8-1 showing positive correlation between the two variables, (P<0.001). (b) Heatmap represents pairwise Spearman correlation between ARG and bacterial species abundance from oral biofilms in Donor A and B.

Correlation cut off was 0.1. Benjamini-Hochberg method was used to adjust for multiple comparisons. Adjusted for false discovery rate (FDR), *p*-values <0.05.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementarytable1.pdf
- Supplementarytable2.pdf
- Supplementarytable3.pdf
- Supplementarytable4.pdf
- Supplementarytable5.pdf
- Supplementarytable6.pdf
- FinalfiguresS1.tif
- FinalfiguresS2.tif
- FinalfiguresS3.tif

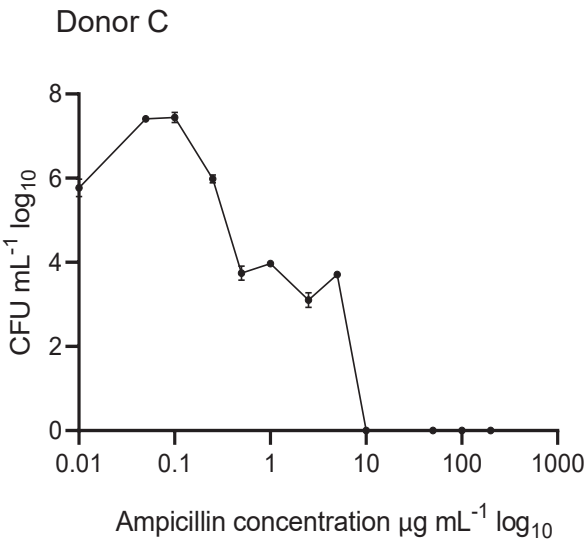

Figure S1: Oral microbiome biofilm viability treated with different ampicillin concentrations. Numbers of viable cells in the community, as determined by colony-forming units counted on SHI agar plates for donor C. The data are for triplicate experiments. Error bars represent mean SEM.

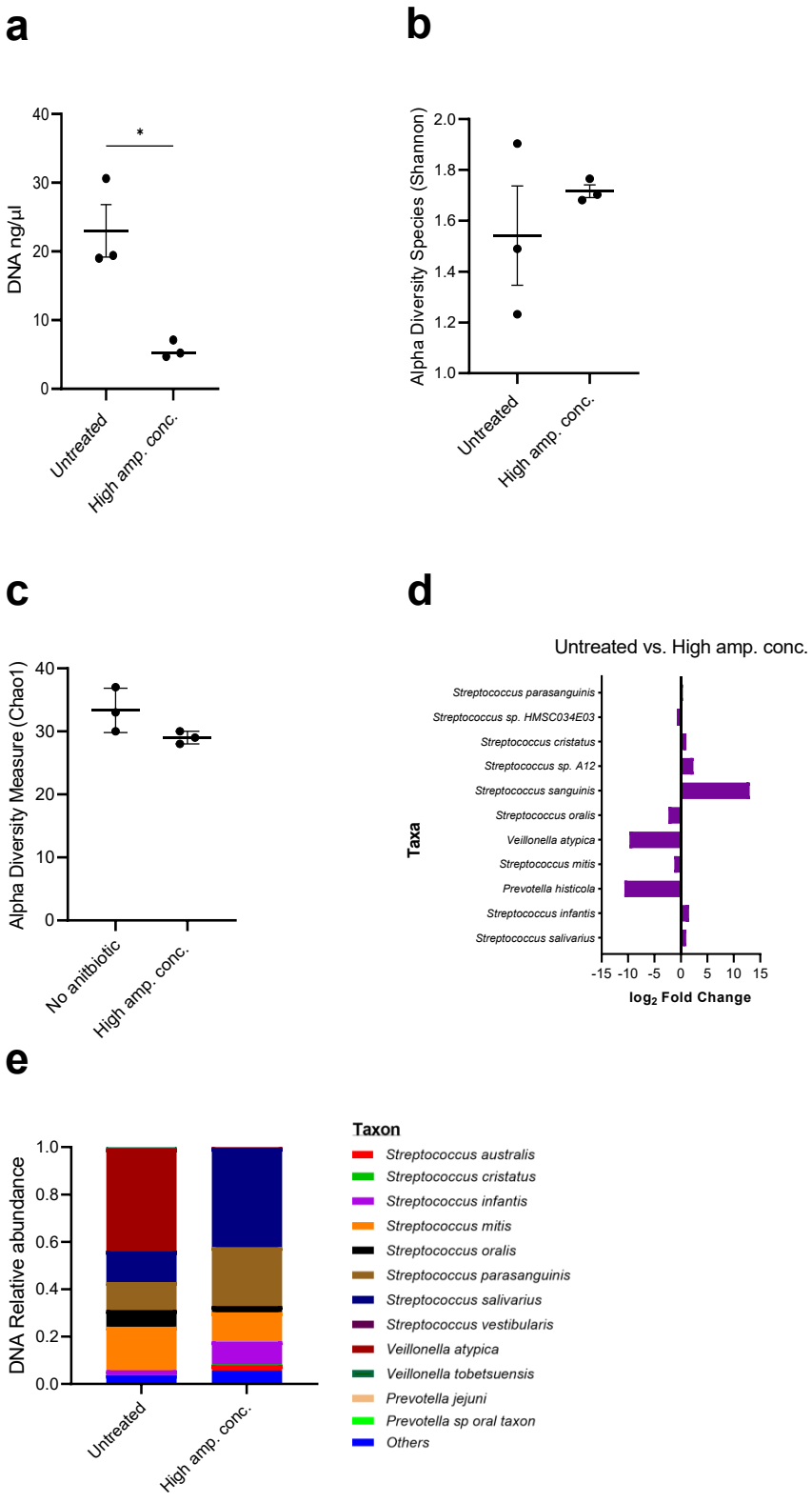

Figure S2: The effect of high ampicillin concentration on the microbiome of *ex vivo* oral biofilm communities. (a) DNA concentration measured by Qubit4 in ng mL^{-1}. (b) Alpha-diversity measured by Shannon index indicates richness and evenness on species level. (c) Alpha diversity measured by Chao1 indicates the total richness at species level. (a-c) Two –tailed unpaired t-test, **P*<0.05. Error bars represent mean SEM. (d) Bar charts illustrates the $\log_2$ fold change of taxa adjusted for false discovery rate (FDR), *p*-values <0.05 (based on DESeq2). (e) Stacked bar plots display the relative abundance of the 12 most abundant species in the microbiomes.

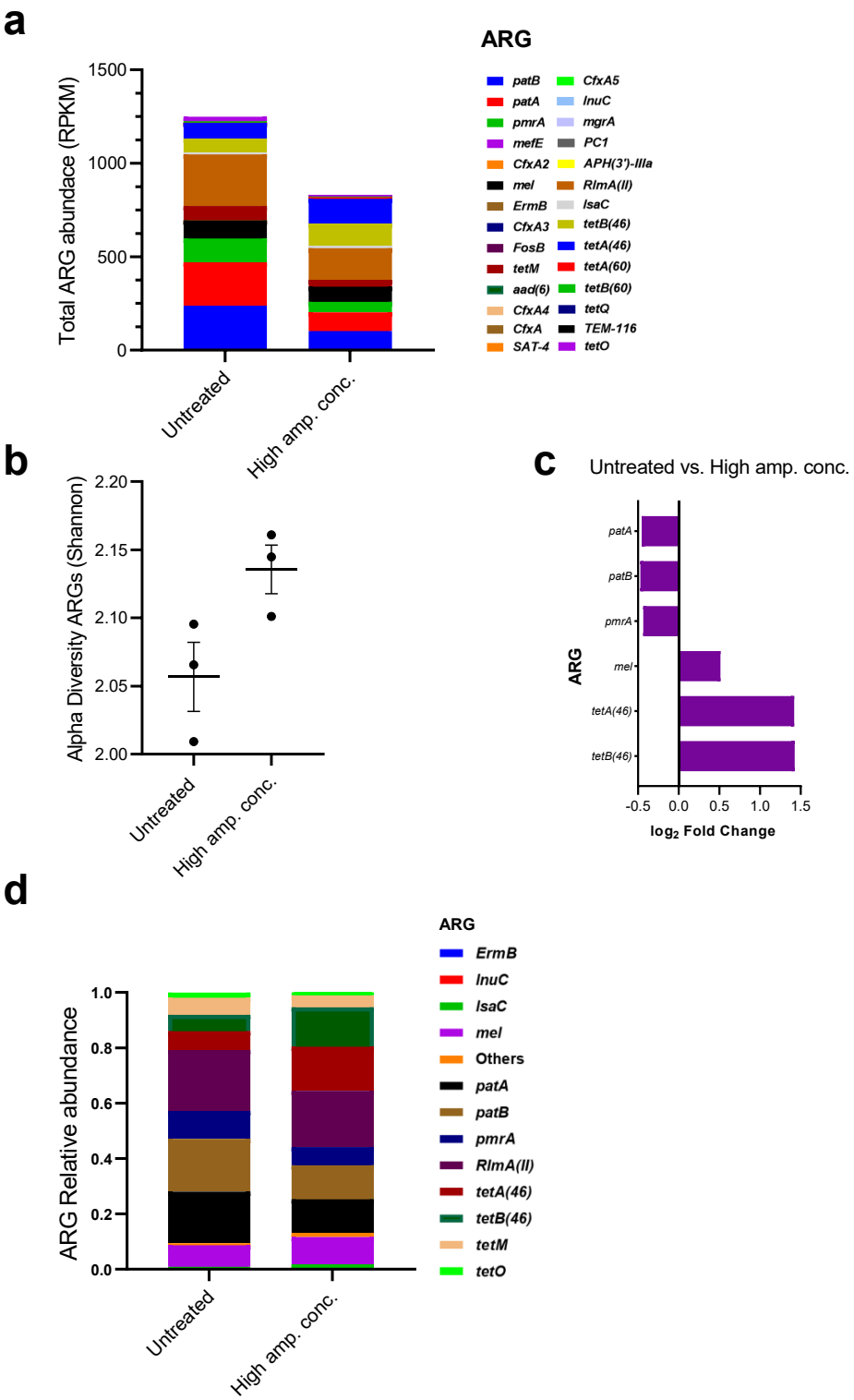

Figure S3: The effect of high ampicillin concentration on the resistome of *ex vivo* oral biofilm communities. (a) ARG abundance visualized as reads per kilobase million values (RPKM) in the oral biofilm community. (b) Alpha diversity measured at gene level by Shannon index which indicates richness and evenness**.** (c) Stacked bar plots displays the relative abundance of the 12 most abundant antimicrobial resistance genes (ARGs) in the resistome. (d) Bar charts illustrates the $\log_2$ fold change of taxa adjusted for false discovery rate (FDR), *p*-values <0.05 (based on DESeq2).

Supplementary Table S1: Reative abundance table of shotgun metagenomic sequencing of *ex-vivo* biofilm community exposed to low ampicillin concentrations. Untreated [Control], Ampicillin 0.025µg/mL [0.025],Ampicillin 0.050µg/mL [0.050] and Ampicillin 0.1µg/mL [0.1]

Feature.ID	DonorA-Control-1	DonorA-Control-2	DonorA-Control-3	DonorA-0.025-1	DonorA-0.025-2	DonorA-0.025-3	DonorA-0.05-1	DonorA-0.05-2	DonorA-0.05-3	DonorA-0.1-1	DonorA-0.1-2	DonorA-0.1-3	DonorB-Control-1	DonorB-Control-2	DonorB-Control-3	DonorB-0.025-1	DonorB-0.025-2	DonorB-0.025-3	DonorB-0.05-1	DonorB-0.05-2	DonorB-0.05-3	DonorB-0.1-1	DonorB-0.1-2	DonorB-0.1-3
Actinomyces odontolyticus	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	7.309999e-05	7.620000e-05	2.090000e-05	1.007009e-04	6.010001e-05	4.890001e-05	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00
Alloscardovia omnicolens	0.000221800	0.001883000 6	0.004418455	0.000000000	0.000000000 0	0.000000000	0.000000000 0	0.000000000	0.000000000	0.000508900 2	0.000000000 0	0.000000000	0.000000000	0.000000000 0	0.000000000	0.000000000 0	0.000000000	0.000000000	0.000000000 0	0.000000000	0.000000000	0.000000000 0	0.000000000 0	0.000000000
Campylobacter concisus	0.000000e+00	2.860001e-05	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	6.400000e-06	4.009999e-05	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00
Gemella haemolysans	1.240000e-03	2.332401e-03	1.891932e-03	5.990399e-03	5.000099e-03	5.878300e-03	3.551200e-03	5.747154e-03	3.641500e-03	4.097701e-03	4.640221e-03	2.396000e-03	2.999001e-04	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00
Gemella morbillorum	3.120000e-05	1.097000e-04	0.000000e+00	2.382000e-04	0.000000e+00	8.470000e-05	1.417000e-04	3.700035e-05	6.110001e-05	3.017001e-04	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00
Gemella sanguinis	9.200001e-04	2.050501e-03	7.783956e-04	3.216699e-03	2.728099e-03	1.378700e-03	9.399001e-04	3.539233e-03	2.266300e-03	1.678601e-03	1.807164e-03	1.713500e-03	2.720001e-04	0.000000e+00	0.000000e+00	0.000000e+00	3.540001e-05	0.000000e+00	0.000000e+00	0.000000e+00	2.370000e-05	9.800000e-06	2.920000e-05	1.416000e-04
Granulicatella adiacens	2.660000e-05	8.270002e-05	2.760339e-05	8.549998e-05	1.500000e-04	2.790000e-05	1.750000e-04	3.787036e-04	1.334000e-04	1.613000e-04	9.780888e-05	8.340002e-05	0.000000e+00	2.150000e-05	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00
Granulicatella elegans	4.055000e-04	1.670401e-03	7.323899e-04	4.254999e-04	3.260999e-04	4.664000e-04	5.759001e-04	4.134039e-04	6.699001e-04	2.926001e-04	4.827438e-04	3.281001e-04	1.017000e-04	0.000000e+00	3.100000e-05	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00
Haemophilus parainfluenzae	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	2.287021e-04	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00
Haemophilus sputorum	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00
Neisseria flavescens	0.002517400 3	0.006033501 8	0.003420120 0	0.010499297 9	0.009659098 1	0.007599600 0	0.007956300 8	0.009759098	0.009997401 0	0.005797001 7	0.005153868 0	0.004564000 9	0.000000000 0	0.000000000 0	0.000000000 0	0.000000000 0	0.000000000 0	0.000000000 0	0.000000000 0	0.000000000 0	0.000000000 0	0.000000000 0	0.000000000 0	0.000000000 0
Neisseria perflava	0.000000e+00	2.270001e-05	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	5.430001e-05	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00
Neisseria subflava	1.050900e-03	2.737901e-03	1.277957e-03	7.380299e-03	3.328499e-03	2.455300e-03	3.115700e-03	2.025019e-03	3.135700e-03	1.988501e-03	2.144195e-03	1.190300e-03	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00
Porphyromonas somerae	0.000000e+00	2.350001e-05	0.000000e+00	2.104000e-04	1.856000e-04	7.430000e-05	1.761000e-04	2.018019e-04	1.608000e-04	3.360001e-04	4.000363e-06	3.810001e-05	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00
Prevotella histicola	6.370001e-05	1.093000e-04	3.090379e-05	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	7.680002e-05	8.410001e-05	1.484000e-04	4.914999e-04	6.271002e-04	3.548000e-04	2.973999e-04	1.792000e-04	2.446000e-04	1.802000e-04	9.839999e-05	1.392000e-04
Prevotella jejuni	3.320000e-05	5.900002e-05	4.510554e-05	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00
Prevotella melaninogenica	0.000000e+00	3.070001e-05	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	3.800001e-05	1.790000e-05	2.570000e-05	1.451300e-03	2.610301e-03	1.777200e-03	5.048998e-04	1.614000e-04	5.181999e-04	1.747000e-04	1.103000e-04	5.300001e-06
Prevotella salivae	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	2.534999e-04	5.987002e-04	4.871000e-04	2.387999e-04	1.088000e-04	4.005999e-04	2.144000e-04	7.229999e-05	4.100000e-06
Prevotella sp oral taxon 306	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	1.114000e-04	7.540002e-05	1.499000e-04	4.529999e-05	0.000000e+00	4.499999e-06	0.000000e+00	0.000000e+00	0.000000e+00
Rothia mucilaginosa	3.755000e-04	9.773003e-04	4.895601e-04	2.826699e-03	2.447500e-03	3.732600e-03	2.275800e-03	1.984519e-03	1.966600e-03	1.863901e-03	2.388217e-03	2.242200e-03	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00
Staphylococcus aureus	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	3.860001e-04	7.900001e-05	3.457000e-04	2.999999e-05	1.372000e-04	7.790001e-05	4.179999e-05	8.470000e-05	1.660000e-05	3.000000e-05	2.390000e-05	6.060001e-04
Streptococcus anginosus group	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	8.790000e-05	0.000000e+00	0.000000e+00	1.440000e-05	0.000000e+00	6.980634e-05	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	1.309000e-04	0.000000e+00	0.000000e+00
Streptococcus australis	1.207000e-03	2.464101e-03	1.670905e-03	1.935460e-02	2.218040e-02	2.369860e-02	1.456780e-02	1.676636e-02	1.782700e-02	2.763601e-02	1.694474e-02	1.956860e-02	9.070002e-05	3.000000e-05	3.810000e-05	0.000000e+00	5.060002e-05	1.260000e-05	2.159999e-05	0.000000e+00	4.739999e-05	3.400000e-06	0.000000e+00	4.530000e-05
Streptococcus cristatus	0.000612900 1	0.002088000 6	0.000800298 3	0.003726599 3	0.002729699 5	0.004931400 0	0.003314100 3	0.003043728 6	0.002547000 3	0.003258901 0	0.002674242 8	0.003814300 8	0.016247603 2	0.022727502 3	0.023536400 0	0.003686099 3	0.024406007 3	0.002318900 2	0.010877196 7	0.005760400 0	0.005649398 9	0.011641200 0	0.010151099 0	0.026491402 6
Streptococcus gordonii	0.000000e+00	1.270000e-05	0.000000e+00	3.084999e-04	0.000000e+00	0.000000e+00	2.478000e-04	1.307012e-04	0.000000e+00	4.111001e-04	1.151105e-04	0.000000e+00	1.790000e-04	2.127000e-04	2.878000e-04	3.509999e-05	1.216000e-04	7.040001e-05	8.989997e-05	4.770000e-05	0.000000e+00	7.110000e-05	1.494000e-04	0.000000e+00
Streptococcus infantis	0.012873 1013	0.030898 3093	0.014634 8972	0.095047 6810	0.106836 0786	0.087749 6000	0.078919 8079	0.080871 4602	0.090836 8091	0.084253 3253	0.062153 6436	0.077330 7155	0.018768 4038	0.005941 9006	0.013469 7000	0.001718 2997	0.003049 3009	0.001606 3002	0.002712 5992	0.002327 9000	0.003260 9993	0.004323 1000	0.003177 6997	0.010845 5011
Streptococcus mitis	0.100287 210	0.319939 896	0.130458 320	0.140161 072	0.109865 778	0.120021 500	0.202681 520	0.198577 767	0.118751 512	0.105485 132	0.146477 400	0.126932 525	0.192413 338	0.044563 204	0.085596 800	0.005129 999	0.010425 503	0.009309 801	0.009892 297	0.007004 000	0.012895 197	0.031542 700	0.010512 399	0.108671 011
Streptococcus oralis	0.077363 408	0.094184 328	0.039422 441	0.031708 994	0.026876 195	0.019996 200	0.030773 903	0.039411 170	0.025226 003	0.037390 511	0.035148 591	0.031092 306	0.036825 107	0.012128 201	0.026117 300	0.001172 300	0.001404 300	0.002935 500	0.001126 300	0.003953 600	0.001862 900	0.010548 700	0.001241 400	0.013504 201
Streptococcus parasanguinis	0.065796 1066	0.202590 7608	0.087443 3380	0.273954 0452	0.233375 0533	0.240005 5000	0.243824 6244	0.209471 1690	0.236046 7236	0.255265 0766	0.232976 7562	0.227358 0455	0.013279 3027	0.007365 4007	0.008486 3000	0.000730 2999	0.002025 2006	0.001391 8001	0.001338 2996	0.001187 8000	0.002149 9996	0.003781 6000	0.002122 1998	0.012476 7012
Streptococcus peroris	6.510001e-05	1.412000e-04	6.350780e-05	0.000000e+00	0.000000e+00	1.945000e-04	1.379000e-04	0.000000e+00	8.730001e-05	0.000000e+00	0.000000e+00	1.316000e-04	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00
Streptococcus pneumoniae	0.000509 0001	0.002942 7009	0.001157 1421	0.000827 4998	0.000729 1999	0.000422 2000	0.001071 3001	0.001184 1111	0.000278 8000	0.000539 0002	0.000694 4631	0.000420 5001	0.000000 0000	0.000000 0000	0.000000 0000	0.000000 0000	0.000000 0000	0.000000 0000	0.000000 0000	0.000000 0000	0.000000 0000	0.000000 0000	0.000000 0000	0.000000 0000
Streptococcus pseudopneumoniae	2.731200e-03	7.910802e-03	4.566261e-03	8.715598e-03	6.376299e-03	6.274900e-03	7.267601e-03	7.781973e-03	2.885000e-03	4.338201e-03	9.348149e-03	7.322901e-03	5.107001e-04	2.740000e-05	8.880000e-05	1.750000e-05	0.000000e+00	6.790001e-05	0.000000e+00	0.000000e+00	6.549999e-05	3.010000e-05	0.000000e+00	2.765000e-04
Streptococcus salivarius	0.06079901	0.22023287	0.10921371	0.37158653	0.43790691	0.44296710	0.36795494	0.39023717	0.45596955	0.43505723	0.44693278	0.46049869	0.10096482	0.03296760	0.06163130	0.01911020	0.07097882	0.03068300	0.04546829	0.02276230	0.05785039	0.05060480	0.04400560	0.14949581
Streptococcus sanguinis	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	6.025999e-04	5.072000e-04	2.190000e-04	8.155077e-04	2.770000e-04	1.260300e-03	2.555232e-04	7.204001e-04	1.732000e-04	0.000000e+00	9.500000e-05	0.000000e+00	6.710002e-05	5.010001e-05	1.320000e-05	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	9.020001e-05
Streptococcus sp A12	2.786000e-04	9.205003e-04	8.930097e-04	2.508099e-03	5.374299e-03	1.013720e-02	2.191800e-03	2.371922e-03	5.197201e-03	3.993801e-03	3.449713e-03	3.632801e-03	3.456001e-04	4.440000e-05	1.578000e-04	0.000000e+00	6.540002e-05	2.020000e-05	0.000000e+00	2.800000e-06	2.710999e-04	1.450000e-05	0.000000e+00	1.189000e-04
Streptococcus sp F0442	1.617000e-04	4.507001e-04	4.077501e-04	3.390899e-03	3.819699e-03	4.062300e-03	3.954200e-03	3.253931e-03	3.875400e-03	5.935602e-03	4.531711e-03	5.724401e-03	0.000000e+00	0.000000e+00	6.920000e-05	0.000000e+00	0.000000e+00	1.000000e-05	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	8.480001e-05
Streptococcus sp HMSC034E03	2.800600e-03	9.719603e-03	3.234897e-03	6.053899e-03	4.028499e-03	4.256500e-03	9.659901e-03	6.211558e-03	5.058901e-03	5.341102e-03	4.885544e-03	5.008001e-03	2.573601e-03	7.516001e-04	1.439200e-03	5.099999e-05	1.748001e-04	1.346000e-04	1.894999e-04	2.011000e-04	1.564000e-04	4.266000e-04	1.995000e-04	1.854600e-03
Streptococcus sp HMSC067H01	2.869200e-03	9.103003e-03	3.977788e-03	7.286499e-03	8.690798e-03	9.759700e-03	9.189101e-03	1.026770e-02	8.739901e-03	1.015180e-02	7.126047e-03	1.154030e-02	2.134200e-03	5.530001e-04	1.138200e-03	6.309999e-05	1.031000e-04	1.576000e-04	1.626000e-04	1.152000e-04	2.028000e-04	4.758000e-04	4.076000e-04	1.449500e-03
Streptococcus sp HMSC071D03	1.755000e-04	1.004900e-03	3.021371e-04	3.956999e-04	3.013999e-04	0.000000e+00	3.048000e-04	7.117067e-04	1.583000e-04	1.207000e-04	4.057368e-04	0.000000e+00	4.547001e-04	1.230000e-04	1.718000e-04	0.000000e+00	3.310001e-05	0.000000e+00	0.000000e+00	6.370000e-05	0.000000e+00	3.250000e-05	3.450000e-05	4.758000e-04
Streptococcus sp HPH0090	2.190000e-05	0.000000e+00	2.130262e-05	0.000000e+00	1.559000e-04	7.210000e-05	2.745000e-04	1.445014e-04	0.000000e+00	1.023000e-04	2.636239e-04	1.437000e-04	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00
Streptococcus sp oral taxon 058	0.000000e+00	0.000000e+00	1.400172e-05	1.746000e-04	0.000000e+00	0.000000e+00	0.000000e+00	5.350050e-05	0.000000e+00	0.000000e+00	4.850044e-05	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00
Streptococcus vestibularis	0.001016100	0.003165401	0.001932037	0.003342399	0.002950599	0.002645200	0.003650800	0.003972737	0.003963900	0.002195301	0.008568178	0.006098201	0.066295713	0.039162504	0.058833600	0.013659697	0.017606805	0.012591501	0.013905796	0.008005200	0.019351096	0.024578100	0.013935199	0.078318508
Veillonella atypica	6.635093e-01	5.451462e-02	5.734878e-01	1.375000e-04	3.292099e-03	2.234000e-04	8.479001e-04	9.300087e-06	4.810000e-05	1.622000e-04	1.026093e-04	0.000000e+00	5.475326e-01	8.331947e-01	7.182699e-01	9.445142e-01	8.653989e-01	9.357340e-01	8.860221e-01	9.466408e-01	8.949579e-01	8.611425e-01	8.854949e-01	4.677416e-01
Veillonella dispar	0.000000e+00	1.866001e-04	9.621482e-03	4.039999e-04	0.000000e+00	2.130000e-04	0.000000e+00	2.397023e-04	6.410001e-05	0.000000e+00	8.800799e-05	1.205000e-04	0.000000e+00	0.000000e+00	0.000000e+00	6.906199e-03	0.000000e+00	0.000000e+00	3.241999e-03	3.690000e-05	0.000000e+00	4.330000e-05	5.766399e-03	2.782000e-04
Veillonella infantium	3.730000e-05	1.620000e-05	2.764740e-03	4.229999e-05	3.039999e-05	0.000000e+00	1.820000e-05	5.020047e-05	0.000000e+00	2.730001e-05	0.000000e+00	1.290000e-05	1.650000e-05	4.400000e-06	1.560000e-05	5.412999e-04	5.400002e-06	5.890001e-05	3.912999e-04	1.110000e-05	2.569999e-05	0.000000e+00	1.259100e-03	1.578000e-04
Veillonella parvula	0.000000e+00	1.472000e-04	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	1.852000e-04	0.000000e+00	0.000000e+00	2.339179e-02	1.345400e-03	0.000000e+00	0.000000e+00	2.742000e-03	5.929001e-04
Veillonella sp T11011 6	0.000000e+00	0.000000e+00	2.546313e-04	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	1.017000e-04	0.000000e+00	0.000000e+00	2.699999e-05	0.000000e+00	0.000000e+00	0.000000e+00	9.049999e-05	0.000000e+00
Veillonella tobetsuensis	0.000000e+00	1.921441e-02	5.217641e-04	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	2.050000e-05	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	1.837640e-02	1.261345e-01

Supplementary Table S2: Total ARG abundance, RPKM (reads per kilobase per million). Shotgun metagenomic sequencing of *ex-vivo* biofilm community exposed to low ampicillin concentrations. Untreated [Control], Ampicillin 0.025µg/mL [0.025],Ampicillin 0.050µg/mL [0.050] and Ampicillin 0.1µg/mL [0.1]

Feature.ID	DonorA-Control-1	DonorA-Control-2	DonorA-Control-3	DonorA- 0.025-1	DonorA-0.025-2	DonorA-0.025-3	DonorA-0.05-1	DonorA-0.05-2	DonorA-0.05-3	DonorA-0.1-1	DonorA-0.1-2	DonorA-0.1-3	DonorB-Control-1	DonorB-Control-2	DonorB-Control-3	DonorB-0.025-1	DonorB-0.025-2	DonorB-0.025-3	DonorB-0.05-1	DonorB-0.05-2	DonorB-0.05-3	DonorB-0.1-1	DonorB-0.1-2	DonorB-0.1-3
patB	185.506091830803	329.12143501333 9	198.756382941826	92.18462327328 32	104.937773636337	108.619130236 48	161.337391191856	170.831280741016	116.704349815948	89.62269672256 11	136.330406572 138	127.428784315637	267.950466165674	87.4260118219767	149.837877840089	10.526860280794 8	15.445932336056 5	17.945174803003 6	23.259730754542 8	16.330700621387 2	28.013421427812 3	55.662645742205 4	18.420994893273 1	127.294024048512
patA	175.759296902 29	318.340479452363	203.989669315599	96.728537667030 2	108.399411728267	98.459666980691 1	154.399798286885	158.615812781967	117.208401967705	92.006313531307 8	131.914670387693	128.019353163302	270.365980717 66	85.991167367 4018	145.073729595421	10.491162113895 7	19.361565557259 7	18.913931685353 7	21.879926999421 9	17.212508951033 7	26.125381699850 1	58.924402640318 6	25.481540281058 9	127.386614645015
pmrA	83.67318337797 88	188.983023991182	106.800768965537	50.06352364062 96	58.43226239981 3	55.854629816550 59	75.738839187319	82.856478826559 7	62.902873245992 7	45.919889913216 1	75.689521729974	67.986965831935 8	174.468402531637	50.20966752566 2	87.356245641451 1	6.6343431547653 4	11.878516040748 1	12.832978758130 2	15.918396204647 9	9.1670718778148 9	17.814807693604 6	31.095334510088 9	12.91041626468 33	80.612095789143 9
metE	0	0	0	0	0	0	0	0	0	0	0	0	11.516066173705 4	0	4.446855132044 65	0	0	0	0	0	0	0	0	6.975145327438 77
CfxA2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
mel	60.904109887939 2	162.710156619149	68.506240371367 5	69.92722638586 41	91.363708952088 6	83.097773059899	95.421757613705	97.100302912524 3	97.866128736581 4	86.520959314713 5	78.982803314410 9	93.825773476239 1	195.318029179735	59.986193808418 4	100.667599799562	17.898462725898 8	43.999543894014 7	36.665653594657 8	43.170370170345 3	38.482460656494 3	57.878275054286 8	64.102076388864	50.23608259432 9	170.447474400522
ErmB	1.704327742170 28	2.483330774105 76	0	0	2.955074998651 86	0	0	0	3.368293078768 02	3.746479759961 251	0	0	14.947761394946 2	30.573024204832 5	27.958038992834 9	3.984141818723 32	31.063645198556 6	3.654517499577 07	20.198939044605 8	8.323515586631 3	7.154541242411 51	13.302526549627 7	12.35997825023 43	28.385521296967 9
CfxA3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1.232361879331 81	2.058965657792 53	1.231850021954 91	0.420732026024 79	0	0	0	0	0
tetM	57.114153049980 38	112.196593358773	59.375427503078	38.419001528014 8	36.182014333217 6	33.971985416918 5	62.491353376227 7	59.162734708452 9	47.450170874025	39.104284536429 6	45.462862067871 8	69.802307783497 4	93.453676726437 2	55.895970396441 9	59.439861872034 9	8.292928934456 7	34.875875584754 5	8.093526375866 23	20.877018958555 9	13.119541274409	19.286119936156 8	29.574367061225 9	17.442102781832 2	50.310379673740 4
aad(6)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CfxA4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CfxA5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
lnuC	0	0	0	0	0	0	0	0	0	0	0	0	0	122.095507528434	13.882273203128 5	38.629876879630 5	8.705889134862 1	0	0	117.396493613123	0	44.656013986820 8	67.021934450268 7	5.319544514000 99
mgrA	0	0	0	0	0	0	0	0	0	0	0	0	1.397144366177 20	0	0	0	0	0	0	0	0	0	0	0
PC1	0	0	0	0	0	0	0	0	0	0	0	0	1.466506145845 5	1.426582474349 136	0	0	0	0	0.600512909485 737	0	0	0	0	2.621052168152 78
APH(3')-III	0	0	0	0	0	0	0	0	0	0	0	0	0	0.817437968845 261	0	0	0	0	0	0	0	0	0	2.091896353072 88
RlmA(II)	218.536973062821	372.721185381045	233.122997684641	141.343186124996	182.615158501182	182.972139318741	205.823573458179	236.005043082753	180.163510141153	150.269119244699	195.747099530552	189.464501627069	268.335645406596	87.698178454666 18	139.500831013354	12.356822888980 2	24.077853206655 1	18.880514849202	26.030006574775 7	21.594986755747 7	30.575641675040 8	62.821270365289 6	23.040325989012 5	130.752761891936
lsaC	9.1245489070961 94	12.019975780344 6	9.0872865237976 5	10.797586427229	16.944522039161 5	15.004669784759 1	15.252323621127 5	10.228075682988 2	19.280574175016 9	15.072655808219 3	12.229105305613 2	15.641353044383 7	0	0	0	0	0	0	0	0	0	0	0	0
tetB(46)	52.283712187268 6	100.566318352064	72.078814879959 9	36.928918336157	125.139905657631	131.126616810241	105.118330254638	109.652713834 24	131.032563631789	141.561638671512	121.556869590 04	135.759585584403	11.963802693911 3	6.573992512981 9	6.593480364180 19	1.113701819275 25	2.290110334401 36	1.450737118205 96	2.018096751774 35	1.606205179936 06	0	3.385765190330 33	2.072117193264	5.106298524484 18
tetA(46)	56.977306647288 5	114.887515928239	79.351295967012 1	105.538426501413	135.118725935799	156.446676462929	130.677334049813	128.353051570881	148.718190414492	158.378689827841	141.321297834168	150.200512991077	11.237900324099	7.749912121518894	9.060757334383 219	0	3.266892177030 81	1.298514846672 47	1.767074509304 12	1.247692202651 55	2.065484949983 15	3.232974287438 36	2.267969479961 92	8.757163211378 29
tetA(60)	0	3.686996918710 04	3.402323347302 81	6.643086506657 09	11.641676799962 7	7.238739342100 676	9.958219987417 28	11.460093890275 72	10.363308619071 6	13.255454664244 14	7.833716732001 52	8.686164723981 11	1.693435324700 44	0	0	0	0	0	0	0	0	0	0	0
tetB(60)	0	16.125058813153 6	3.310368662240 57	4.545269715081 17	7.238734933310 17	0	0	7.982548158041 24	5.603416869614 29	7.154617789485 93	4.670100359462 45	0	0	0	0	0	0	0	0	0	0	0	0	0
tetO	15.383688282776 45	34.943180134702 3	2.233494134911 3	6.337154891218 93	7.912702199974 68	11.792574248171 7	11.663917455780 9	13.966898428751	9.555558011778 4	7.438861325696 12	10.853746349537	11.486455711208 4	5.088661740506 09	0.870352480221 41	0	0	0	1.166634432557 3	0	0	0	0	0	0
SAT-4	0	0	0	0	0	0	0	0	0	0	0	0	1.428020483172 8	0	0.992557720806 84	0	0	0	0	0	0	0	0	2.552267649927 78
TEM-116	0	0	0	0	0	0	0	2.657969439807 65	2.875006132747 46	2.435264159736 46	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CfxA	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
tetQ	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1.733828228357 78	2.630900562734 9	2.304900854596 48	0.849296829129 828	0	0	0	0	0

Supplementary Table S3: Relative abundance table of antimicrobial resistance genes (ARGs). Shotgun metagenomic sequencing of *ex-vivo* biofilm exposed to low ampicillin concentrations. Untreated [Control], Ampicillin 0.025µg/mL [0.025],Ampicillin 0.050µg/mL [0.050] and Ampicillin 0.1µg/mL [0.1]

Gene.ID	DonorA-Control-1	DonorA-Control-2	DonorA-Control-3	DonorA- 0.025-1	DonorA-0.025-2	DonorA-0.025-3	DonorA-0.05-1	DonorA-0.05-2	DonorA-0.05-3	DonorA-0.1-1	DonorA-0.1-2	DonorA-0.1-3	DonorB-Control-1	DonorB-Control-2	DonorB-Control-3	DonorB- 0.025-1	DonorB-0.025-2	DonorB-0.025-3	DonorB-0.05-1	DonorB-0.05-2	DonorB-0.05-3	DonorB-0.1-1	DonorB-0.1-2	DonorB-0.1-3
aad(6)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0,003499
APH(3)-IIIa	0	0,000893997	0	0	0	0	0	0	0	0	0	0,001639568	0,000841815	0,001530724	0	0	0,001567574	0,003642384	0,002375767	0	0,006935414	0	0,006849315	0	0
CfxA	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0,027741656	0	0	0,012408635	0
CfxA2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0,039011704	0	0	0,017678055	0
CfxA3	0	0	0	0,009821977	0,007659574	0,006964359	0,001718951	0	0	0	0	0	0	0	0	0	0	0	0	0	0,018205462	0	0	0,009688934	0
CfxA4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0,015604681	0	0	0,006459289	0
CfxA5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0,022973559	0	0	0,006969233	0
ErmB	0,00598106	0,031417625	0,020248204	0,024554942	0,089361702	0,015977059	0,063816072	0,027187173	0,020750988	0,021267361	0,036151961	0,020904495	0,005280478	0,022195495	0,013302752	0,00481469	0,004470199	0,012274797	0,012311358	0,007368877	0,016049383	0,021481943	0,004929458	0,018894
lnuC	0	0,083141762	0,006662312	0,1577655	0,016595745	0	0	0,254095504	0	0,047309028	0,129901961	0,002595983	0	0	0	0	0	0	0	0	0	0	0	0	0
lsaC	0	0	0	0	0	0	0	0	0	0	0	0	0,010025254	0,014104527	0,019877676	0,014556041	0,010430464	0,027123342	0,021577972	0,022540095	0	0,077833126	0,010538841	0,031491
mefE	0,007476325	0	0,00522534 3	0	0	0	0	0	0	0	0	0,008334472	0	0	0	0	0	0	0	0	0	0	0	0	0
mel	0,127429806	0,100510856	0,118876551	0,179864948	0,206382979	0,261368292	0,222389343	0,20494946	0,273715415	0,167100694	0,239583333	0,20467277	0,175556746	0,176361251	0,198929664	0,191020043	0,22102649	0,193823005	0,268599418	0,189423494	0,209876543	0,210772105	0,110317865	0,249825
mgrA	0,000332281	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
patA	0,24547267	0,200510856	0,23840627	0,146715777	0,126382979	0,187628021	0,156854319	0,127570582	0,171936759	0,213758681	0,169117647	0,212870611	0,182903497	0,166192871	0,159480122	0,175680215	0,163245033	0,150069293	0,171035213	0,11053316	0,154320988	0,146637609	0,070882203	0,145906
patB	0,253613557	0,212515964	0,256694971	0,153468386	0,105106383	0,18557968	0,173828964	0,126176368	0,192193676	0,210503472	0,12745098	0,221751605	0,198285758	0,177782637	0,174923547	0,192587616	0,177980132	0,165907741	0,157797194	0,110099697	0,193209877	0,137297634	0,064252932	0,145906
PC1 beta-lactamase (blaZ)	0,000664562	0,001660281	0	0	0	0	0,002148689	0	0	0	0	0,002186091	0	0	0	0	0	0	0	0	0	0	0	0	0
pmrA	0,112144875	0,082886335	0,10163292	0,065684469	0,054893617	0,090126997	0,080790718	0,048100383	0,083003953	0,079861111	0,060661765	0,09536822	0,070942068	0,055762082	0,064525994	0,080170194	0,07384106	0,048703227	0,051098756	0,045513654	0,058641975	0,063200498	0,023967364	0,048286
RlmA(II)	0,122030238	0,102426564	0,114826911	0,086556169	0,078723404	0,093814011	0,093467985	0,080167306	0,100790514	0,114149306	0,076593137	0,10944118	0,075074615	0,080581675	0,085779817	0,09103124	0,068708609	0,077410414	0,066851999	0,044646727	0,077777778	0,082191781	0,026857046	0,060182
SAT-4	0,000415351	0	0,00052253 4	0	0	0	0	0	0	0	0	0,001366307	0,001683631	0	0,001376147	0,001343635	0,002483444	0	0	0	0	0,002801993	0	0
Staphylococcus aureus FosB	0	0	0	0	0	0	0,000644607	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
tetA(46)	0,010383785	0,018390805	0,015153494	0	0,021702128	0,013109381	0,012892136	0,009410945	0,013833992	0,011935764	0,015318627	0,014892745	0,016071019	0,020883446	0,012691131	0,009629381	0,014900662	0,019006137	0,014826582	0,009969658	0	0,014009963	0,007139215	0,016445
tetA(60)	0,001578335	0	0	0	0	0	0	0	0	0	0	0	0,007346751	0,005669582	0,005504587	0,00727802	0,009437086	0,007325282	0,007678051	0,015604681	0	0,01992528	0	0,012946
tetB(46)	0,011131417	0,015708812	0,011103854	0,015960712	0,015319149	0,014748054	0,014825956	0,012199373	0	0,012586806	0,014093137	0,008744364	0,014999617	0,015416576	0,015749235	0,015563767	0,01705298	0,016036428	0,015753243	0,011270048	0,014197531	0,012764633	0,011048785	0,017495
tetB(60)	0	0	0	0	0	0	0	0	0	0	0	0	0,00742328	0,005669582	0,009480122	0,009181503	0,004635762	0,005741437	0,003839026	0	0	0,019613948	0	0,006998
tetM	0,096112311	0,147637292	0,110646636	0,13136893 8	0,25787234	0,090946333	0,169531586	0,110142907	0,143774704	0,121527778	0,131127451	0,095231589	0,22560649	0,251585393	0,227217125	0,198298063	0,217384106	0,260740447	0,200423617	0,238838318	0,259876543	0,174968867	0,123746388	0,222883
tetO	0,005233427	0,002298851	0	0	0	0,013109381	0	0	0	0	0	0	0,007958981	0,004264159	0,01116208	0,00727802	0,010761589	0,013462681	0,008207572	0	0,016049383	0,009651308	0,003059663	0,019244
tetQ	0	0	0	0,028238183	0,02	0,026628431	0,007090675	0	0	0	0	0	0	0	0	0	0	0	0	0,063719116	0	0	0,490056094	0

Supplementary Table S4: Reative abundance table of shotgun metagenomic sequencing of *ex-vivo* biofilm community exposed to high ampicillin concentrations. Untreated [Control], Ampicillin 10 µg/mL [10.0]

Feature.ID	DonorC-Control-1	DonorC-Control-2	DonorC-Control-3	DonorC-0.10-1	DonorC-0.10-2	DonorC-0.10-3
Actinomyces odontolyticus	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00
Alloscardovia omnicolens	0.0000000000	0.0000000000	0.0000000000	0.0000000000	0.0000000000	0.0000000000
Campylobacter concisus	9.230000e-05	1.940000e-05	3.639999e-05	0.000000e+00	0.000000e+00	0.000000e+00
Gemella haemolysans	1.641000e-04	2.303000e-04	1.300000e-05	5.409999e-05	1.171000e-04	3.096000e-04
Gemella morbillorum	1.650000e-05	2.340000e-05	1.350000e-05	0.000000e+00	0.000000e+00	1.090000e-05
Gemella sanguinis	4.884000e-04	3.348000e-04	2.269999e-04	2.439000e-04	2.066000e-04	1.675000e-04
Granulicatella adiacens	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00
Granulicatella elegans	2.611000e-04	1.239000e-04	3.409999e-05	1.110000e-04	2.320000e-05	3.760000e-05
Haemophilus parainfluenzae	7.480000e-05	1.690000e-05	3.739999e-05	5.899999e-06	0.000000e+00	0.000000e+00
Haemophilus sputorum	2.784000e-04	1.378000e-04	1.268000e-04	3.250000e-05	1.059000e-04	4.640000e-05
Neisseria flavescens	0.0037186000	0.0014754000	0.0014628996	0.0013601999	0.0012808003	0.0013984999
Neisseria perflava	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00
Neisseria subflava	0.000000e+00	1.800000e-05	7.869998e-05	0.000000e+00	0.000000e+00	4.200000e-05
Porphyromonas somerae	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00
Prevotella histicola	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00
Prevotella jejuni	6.190000e-05	1.870000e-05	3.419999e-05	7.539999e-05	1.381000e-04	3.110000e-05
Prevotella melaninogenica	1.110000e-05	4.250000e-05	3.939999e-05	2.040000e-05	2.420000e-05	2.291000e-04
Prevotella salivae	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00
Prevotella sp oral taxon 306	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00
Rothia mucilaginosa	3.086000e-04	4.678000e-04	1.832999e-04	1.381000e-04	1.102000e-04	1.277000e-04
Staphylococcus aureus	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00
Streptococcus anginosus grou	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00
Streptococcus australis	4.940000e-05	3.600000e-06	3.869999e-05	5.749999e-05	0.000000e+00	0.000000e+00
Streptococcus cristatus	0.0000000000	0.0000000000	0.0000000000	0.0000000000	0.0000000000	0.0000000000
Streptococcus gordonii	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00
Streptococcus infantis	0.0078342000	0.0153985000	0.0038168989	0.0031003997	0.0029855006	0.0032928997
Streptococcus mitis	0.067872700	0.036007800	0.024458193	0.032634297	0.020367704	0.019988798
Streptococcus oralis	0.068925400	0.047951800	0.031521291	0.037331296	0.021755604	0.040139996
Streptococcus parasanguinis	0.0122011000	0.0096592000	0.0057256983	0.0048244995	0.0039719008	0.0056831994
Streptococcus peroris	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00
Streptococcus pneumoniae	0.0016724000	0.0005060000	0.0005777998	0.0008539999	0.0003944001	0.0005571999
Streptococcus pseudopneum	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	2.860001e-05	0.000000e+00
Streptococcus salivarius	0.05054790	0.05085230	0.04550859	0.06223939	0.05344511	0.04796000
Streptococcus sanguinis	4.790000e-05	0.000000e+00	8.289998e-05	5.269999e-05	5.830001e-05	0.000000e+00
Streptococcus sp A12	2.552000e-04	1.259000e-04	1.005000e-04	1.320000e-05	1.450000e-05	9.899999e-06
Streptococcus sp F0442	1.010000e-05	0.000000e+00	0.000000e+00	8.019999e-05	6.700001e-06	0.000000e+00
Streptococcus sp HMSC034E0	1.364800e-03	6.610000e-04	3.376999e-04	4.813000e-04	2.885001e-04	3.247000e-04
Streptococcus sp HMSC067H0	3.247000e-04	1.004000e-04	2.783999e-04	1.380000e-04	1.513000e-04	1.265000e-04
Streptococcus sp HMSC071D0	2.294700e-03	1.236100e-03	1.412600e-03	2.493300e-03	1.870200e-03	1.957700e-03
Streptococcus sp HPH0090	0.000000e+00	0.000000e+00	5.199998e-05	0.000000e+00	2.020000e-05	0.000000e+00
Streptococcus sp oral taxon 0	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00
Streptococcus vestibularis	0.000000000	0.000000000	0.000000000	0.000000000	0.000000000	0.000135000
Veillonella atypica	7.798714e-01	8.343972e-01	8.754070e-01	8.525568e-01	8.916748e-01	8.735733e-01
Veillonella dispar	8.062000e-04	1.148000e-04	3.467999e-04	7.393999e-04	6.671001e-04	2.695000e-03
Veillonella infantium	3.414000e-04	3.800000e-05	2.157999e-04	3.517000e-04	1.115000e-04	8.919999e-04
Veillonella parvula	0.000000e+00	2.060000e-05	7.825198e-03	0.000000e+00	8.900002e-06	8.259999e-05
Veillonella sp T11011 6	1.047000e-04	1.790000e-05	7.199998e-06	1.050000e-05	2.660001e-05	1.808000e-04
Veillonella tobetsuensis	0.000000e+00	0.000000e+00	0.000000e+00	0.000000e+00	1.465000e-04	0.000000e+00

Supplementary Table S5: Total ARG abundance, RPKM (reads per kilobase per million). Shotgun metagenomic sequencing of *ex-vivo* biofilm community exposed to high ampicillin concentration. Untreated [Control], Ampicillin 10 µg/mL [10.0]

Feature.ID	DonorC-Control-1	DonorC-Control-2	DonorC-Control-3	DonorC- 10.0-1	DonorC-10.0-2	DonorC-10.0-3
patB	135.19171949134	85.2199651679682	57.9484459692142	70.6566432002398	51.9296888196123	51.5585227906257
patA	130.001219637175	83.0483954495842	55.0765749225346	67.1915012386426	49.6536316126694	48.6174862233053
pmrA	71.2225824174355	39.3592025473646	31.4762889711887	43.310468309561	31.7246922669284	22.2867559702414
mefE	0	0	0	0	0	0
CfxA2	0	0	0	0	0	0
mel	173.646227903558	122.643472827005	95.6053789169951	101.670004315314	93.5573221770957	87.3832916611252
ErmB	8.51623657400354	25.1670780915506	10.4243976493402	4.17838984386961	3.08522589445519	9.02327273422123
CfxA3	0	0	0	0	0	0
tetM	141.561605290318	110.987163065547	69.273941663487	66.9542941482476	58.3723667971932	74.5723008455487
aad(6)	0	0	0	0	0	0
CfxA4	0	0	0	0	0	0
CfxA5	0	0	0	0	0	0
lnuC	0	0	0	0	0	0
mgrA	0	0	0	0	0	0
PC1	0	0	0	0	0	0
APH(3')-IIIa	1.2756889240468	1.63086595830738	0	1.27826827518468	2.36210586300846	1.64099445708174
RlmA(II)	106.532114082822	80.3928006025296	59.1435637560895	69.5094670739939	41.7238342891673	50.0682098558671
lsaC	8.16624396059093	8.07753590042562	7.86733235198856	6.38022922053206	3.6359355875831	10.0703691285248
tetB(46)	10.4034044143493	7.5175557034487	5.30749660557496	5.80866172174041	5.06152296520772	5.06965256494813
tetA(46)	11.2240456321272	10.2541963328854	4.30666719078287	3.61884894303837	4.45345650851793	6.05027521567529
tetA(60)	5.08675959682611	3.24666849951216	1.85184899977577	2.71159741872612	2.79620760664129	2.31177451892047
tetB(60)	5.13974667595971	3.24666849951216	3.18929549961382	3.42078443593142	1.37357566642028	1.81193137969442
tetO	4.9940322083423	1.88113835704316	3.40309403865044	2.45738516072055	2.88972112857392	3.85035418185065
SAT-4	3.73544270577239	0	1.48352544180932	1.60414092939514	2.35795349207902	0
TEM-116	0	0	0	0	0	0
CfxA	0	0	0	0	0	0
tetQ	0	0	0	0	0	0

Supplementary Table S6: Relative abundance table of antimicrobial resistance genes (ARGs). Shotgun metagenomic sequencing of *ex-vivo* biofilm exposed to high ampicillin concentration. Untreated [Control], Ampicillin 10µg/mL [10.0]

Gene.ID	DonorC-Control-1	DonorC-Control-2	DonorC-Control-3	DonorC- 10.0-1	DonorC-10.0-2	DonorC-10.0-3
APH(3)-IIIa	0.0015620099197918	0.0027989400201988	0	0.00283586541272528	0.00665424594127732	0.00438399107015834
CfxA	0	0	0	0	0	0
CfxA2	0	0	0	0	0	0
CfxA3	0	0	0	0	0	0
CfxA4	0	0	0	0	0	0
CfxA5	0	0	0	0	0	0
ErmB	0.0104276565839332	0.0431924780225457	0.0256532452827541	0.00926984692426983	0.0086913343756552	0.0241060820892576
lnuC	0	0	0	0	0	0
lsaC	0.00999911015407932	0.0138629041713265	0.0193606012870473	0.014154674510053	0.0102427287469689	0.0269034475662777
mefE	0	0	0	0	0	0
mel	0.212620118750701	0.21048432739864	0.235273856409684	0.225557071505802	0.263558649560612	0.233448424319896
patA	0.159179240981518	0.142530093569668	0.135537124863151	0.149065777576483	0.139878352533898	0.129883818032848
patB	0.165534718487088	0.14625700525145	0.14260446964979	0.156753268872203	0.146290192353865	0.13774093052491
PC1 beta-lactamase (blaZ)	0	0	0	0	0	0
pmrA	0.0872080788287394	0.0675494185231904	0.0774595318339432	0.0960851970375885	0.0893710599772234	0.059540078721588
RlmA(II)	0.13044263051676	0.137972483756922	0.145545517253671	0.154208234185916	0.117539463121101	0.133759491971277
SAT-4	0.00457384120160028	0	0.00365078571655882	0.00355882084154159	0.00664254837181753	0
TEM-116	0	0	0	0	0	0
tetA(46)	0.013743217713267	0.0175985526860076	0.0105982132985243	0.00802849351006325	0.0125457522292067	0.0161635844673664
tetA(60)	0.00622845334788453	0.0055720277618859	0.00455718768756653	0.00601573666677253	0.00787714615540706	0.00617601040846301
tetB(46)	0.0127383883237535	0.0129018497230111	0.0130611395344091	0.0128866398319437	0.014258725307518	0.0135438066092327
tetB(60)	0.00629333307025832	0.0055720277618859	0.00784848990636456	0.00758908317962074	0.00386947530441048	0.00484065680663317
tetM	0.173334288286896	0.19047942649738	0.170475213722215	0.148539528590755	0.164439744604953	0.199223281697997
tetO	0.00611491383373038	0.00322846485588778	0.00837462355432147	0.00545176135426261	0.00814058141608679	0.0102863957140955
tetQ	0	0	0	0	0	0

II

HAMLET, a human milk protein-lipid complex, modulates amoxicillin-induced changes in an *ex vivo* biofilm model of the oral microbiome

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ABSTRACT (250 words):

Challenges from infections caused by biofilms and antimicrobial resistance highlight the need for novel antimicrobials that work synergistically with antibiotics and minimize resistance risk. In this study we investigated the potential synergistic effect of HAMLET (human alpha-lactalbumin made lethal to tumor cells), a human milk protein-lipid complex and amoxicillin on microbial ecology using an *ex-vivo* oral biofilm model. HAMLET was chosen due to its multi-targeted antimicrobial mechanism, together with its synergistic effect with antibiotics on single species pathogens, and low risk of resistance development. The combination of HAMLET and amoxicillin significantly reduced biofilm viability, while each of them alone had little or no impact. Using a whole metagenomics approach, we found that the combination group promoted a most remarkable shift in overall microbial composition compared to the untreated samples. Up to 90% of the bacterial species in the combined treatment were *Lactobacillus crispatus*, a species with probiotic effects, whereas it was detected in minor fraction in untreated samples. Resistome analysis indicated no major shifts on alpha-diversity, while beta-diversity revealed distinct clustering patterns for each treatment group, signifying that each treatment group harbors a unique resistome. TEM beta-lactamase genes were detected in low proportions in all treated samples but absent in untreated samples. Our study highlights the potential of HAMLET to synergize with amoxicillin in an *ex-vivo* model of the oral microbiome and modulate the proportion of probiotic bacteria. The findings extend the knowledge on the synergistic effects of HAMLET and antibiotics from single-species studies to polymicrobial biofilms of human origin.

Importance (150 words):

Polymicrobial infections are challenging to treat and prevent, requiring the use of antibiotics that exhibit reduced efficacy due to biofilm formation. HAMLET has recently emerged as an antimicrobial agent that can synergize with antibiotics while limiting microbial resistance. We investigated the effects of HAMLET, alone and combined with low concentrations of amoxicillin, on *ex vivo* oral biofilms to simulate complex microbial interactions observed in the oral cavity. The combination of HAMLET and amoxicillin effectively targeted polymicrobial biofilms and led to an increase in *Lactobacillus crispatus*. The potency of this combination appears to be due to the synergistic effect of HAMLET and amoxicillin. These findings underscore the potential of combining antimicrobials with different modes of action for the development of more effective strategies for preventing and treating polymicrobial infections.

Key words: Antibiotic resistance, HAMLET, oral microbiome, oral resistome, amoxicillin, probiotics

INTRODUCTION

The human oral microbiome is considered the second-largest microbial community, following the gut microbiota, in terms of both diversity and complexity (1). A broad range of odontogenic inflammatory infections, from periodontitis and peri-implantitis to posttraumatic osteomyelitis and facial cellulitis, have been associated with oral biofilms featuring polymicrobial communities (2-7). These biofilms are difficult to treat because of their intrinsic antibiotic tolerance and resistance to the host's immune system. Additionally, the oral cavity harbors the highest abundance of antimicrobial resistance genes (ARGs) in the entire human body, surpassing the abundance of ARGs in the gut (8). As such, the oral cavity serves as a significant potential source for the dissemination of antibiotic resistance (9, 10). Combining different therapies with potential synergistic antimicrobial activities has emerged as a novel strategy to overcome the challenges posed by polymicrobial infections (11). One particular combination that has demonstrated promising results is the utilization of HAMLET (Human Alpha-lactalbumin Made Lethal to Tumor Cells), a protein-lipid complex, in conjunction with antibiotics.

HAMLET is a complex comprised of alpha-lactalbumin and oleic acid that has demonstrated potent cancer cell killing capabilities, while sparing healthy, differentiated cells, rendering it a promising potential therapeutic. (12-14). Additionally, HAMLET exhibits antimicrobial properties against key human pathogens. Although mostly active against gram-positive bacteria, such as *Streptococcus pneumoniae* and *Streptococcus pyogenes*, HAMLET has also shown bactericidal effects on selected gram-negative species, such as *Haemophilus influenzae* and *Moraxella catarrhalis* (15). Notably, HAMLET's bactericidal activity has not been detected in other gram-negative pathogens, including *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Haemophilus parainfluenzae*, and *Enterobacter*

cloacae (15-21). When used in combination with antibiotics, HAMLET has demonstrated the ability to lower the minimum inhibitory concentration (MIC) of methicillin for methicillin resistant *Staphylococcus aureus* (MRSA) strains, bringing them within the sensitive range. HAMLET augment also the efficacy of selected antibiotics against antibiotic-resistant bacterial strains such as *S. pneumoniae* and *M. tuberculosis* (15, 18, 19).

Among the most commonly prescribed antibiotics in primary healthcare settings and for odontogenic infections is amoxicillin (22). This broad-spectrum β-lactam antibiotic is a modified form of penicillin with an extra amino group. Its mechanism of action involves disrupting peptidoglycan cross-linking in the bacterial cell-wall. Amoxicillin inactivates and kills pathogens by binding to penicillin-binding-proteins (PBPs) located on the bacterial membrane (22-24). However, its efficacy against polymicrobial biofilms such as in the oral cavity can be limited due to a number of factors, including the formation of a protective barrier that can prevent antibiotics from effectively reaching and killing the bacteria within the biofilms. Further, the production of beta-lactamases by members of microbial communities can reduce the concentration of active amoxicillin available. In combination with HAMLET, other beta-lactam antibiotics has shown synergistic effects against both *S. pneumoniae* and MRSA biofilms (15, 18). This suggests that the inclusion of HAMLET in combination with amoxicillin may have potential as an effective strategy for treating polymicrobial biofilms.

In this study, we used an *ex vivo* oral microbiome model to provide a relevant testbed for investigating the effects of HAMLET and amoxicillin on microbial ecology. Our findings indicate that the combination of amoxicillin and HAMLET act synergistically to inhibit bacterial viability in polymicrobial biofilms. Furthermore, the combination at low concentrations influenced the microbial ecology of the oral microbiome, leading to a proportional increase in bacterial species exhibiting probiotic characteristics.

METHODS

Sample collection

The research followed the ethical principles directed in the Declaration of Helsinki and received approval from the National Regional Ethics Committee (REK20152491) for studies involving human samples. Eight participants were instructed to brush their teeth following breakfast and to abstain from food or drink for a minimum of two hours before providing saliva samples. Additionally, they rinsed their mouths three times with water, 10 minutes before saliva collection. Non-stimulated saliva was collected from eight participants, and these samples were centrifuged at 6000 x g for 5 minutes at 4^{o}C. This centrifugation step effectively precipitated larger debris and eukaryotic cells. The resulting supernatant was pooled and utilized as the inoculum in the human oral microbiome biofilm model, as described below.

A second centrifugation was conducted to obtain cell-free saliva by spinning down the samples at 10 000 x g for 7 minutes at 4^{o}C. The upper fraction was used to coat the bottom of the wells prior to biofilm growth in a process termed as 'pellicle formation' to mimic the establishment of an oral biofilm (25).

HAMLET production

HAMLET was produced in three steps; : 1) purification of alpha-lactalbumin from human milk, 2) converting native alpha-lactalbumin to partially unfolded protein in the presence of oleic acid (C18:1) and 3) dialysis and lyophilization as previously described (14, 21).

The human oral microbiome biofilm model

We utilized a previously established *ex vivo* biofilm model designed to preserve a highly reproducible diversity of species and metabolic activity within the human oral microbiome (25, 26). In summary, SHI media was pre-reduced for four hours under anaerobic conditions, characterized by a carbon dioxide level of 5%, balanced with nitrogen. SHI media was prepared as previously described (27). The pooled saliva samples were added at a ratio of 2 µl of saliva per mL SHI medium. These were allotted into the wells of a 24-well plate, with each well containing 1 mL of the mixture. The plate was then incubated within an anaerobic chamber at 37^{o}C for 24 hours.

After this incubation period, the supernatant was removed and replaced with fresh SHI medium to support the pre-formed oral biofilms. In the first set of experiments, the samples were either left untreated (control), or treated with amoxicillin ranging from 0-200 µg mL^{-1} (Sigma-Aldrich). In the second set of experiments, the preformed biofilms were not treated (control), treated with amoxicillin 0.1 µg mL^{-}1, HAMLET ranging between 125-250 µg mL^{-1}, or with a combination treatment composed of HAMLET ranging between 125-250µg mL^{-1}in conjunction with amoxicillin at 0.1 µg mL^{-}1. The stock solution of amoxicillin (2 mg/mL in distilled water) and HAMLET (5 mg/mL in phosphate-buffered saline (PBS)) were appropriately diluted in SHI medium before adding to the biofilms.

Following an incubation period of another 24 hours, the oral biofilms were washed with PBS, followed by suspension in 1 mL of PBS. Glycerol (20%) was added to the samples before they were archived and stored at -80^{o}C.

169 **Oral biofilm viability assay**

170 To evaluate the viability of the biofilms, samples obtained from both the control and the
171 treatment groups, were subjected to a ten-fold dilution series. Subsequently, 20 µL of each
172 dilution was plated onto SHI agar plates. These plates were then incubated for 48 hours at 37^{o}C
173 within an anaerobic chamber. Then the number of colony forming units per milliliter (CFUs
174 $mL^{-1)}$ was calculated, and represented as log 10-transformed values.

175

176 **DNA extraction**

177 Bacterial DNA was extracted using the MasterPure^{TM} Gram Positive DNA Purification Kit
178 (Epicentre, Madison, WI, USA), following the manufacturer's established protocol.
179 Subsequently, the precipitated DNA was resuspended in 35 µl milliQ water. To assess the
180 quality and quantity of the extracted DNA, NanoDropTM 2000c spectrophotometer (Thermo
181 Fisher Scientific, Waltham, MA, USA) was used for initial evaluation. This was followed by
182 quantification using Qubit TM 4 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA)
183 to yield precise measurements of the DNA's concentrations.

184

185 **DNA library preparation and sequencing**

186 The preparation of the DNA libraries was executed with the Illumina DNA Prep (M) kit,
187 (Illumina, Inc., San Diego, CA, USA), in strict adherence to the manufacturer's protocol. To
188 assess the quality and concentration of the DNA library, initial measurements were conducted
189 using the NanoDrop^{TM} 2000c spectrophotometer and Qubit ^{TM} 4 Fluorometer. Finally, analysis
190 involved the utilization of a Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) using
191 a High Sensitivity DNA kit (Agilent Technologies, Santa Clara, CA, USA).
192 The DNA library was obtained by resuspending it in the provided buffer. Each sample was
193 adjusted to 500 ng DNA in a 30 µL volume using nuclease-free water.

For the metagenomic shotgun sequencing approach, services at the Norwegian Sequencing Centre (Oslo, Norway) were utilized, using the Illumina NovaSeq 6000 SP platform (Illumina, Inc., San Diego, CA, USA). The paired-end sequencing reads were generated with a corresponding read length of 150 base pairs.

Assessment of sequencing read quality

The evaluation of sequencing read quality, both in raw and preprocessing state, was conducted utilizing FastQC tool (v.0.11.9) (28). The identification and removal of low-quality reads, as well as the elimination of adapter sequences, was achieved using Trimmomatic (v.0.39). The following parameters were used during this process: ILLUMINACLIP: Nextera PE:2:30:10 LEADING:3 TRAILING:3 SLIDING WINDOW:4:15 MINLEN:36.. The remaining high-quality reads were subjected to microbiome and resistome profiling.

Taxonomic and resistome profiling

MetaPhlAn3 software (v.3.7.0) (29) was used to profile the bacterial composition in the oral biofilm samples and to determine their abundance at species-level using default settings. The '*merge metaphlan tables.py*' script was used to merge the profiled metagenomes into an abundance table. To detect the hits to known Antibiotic Resistance Genes (ARGs), "high quality" paired-end reads were mapped against the Comprehensive Antibiotic Resistance Database (CARD) (v.3.2.2) (30, 31) by using the KMA alignment tool (1.4.12) (32) with parameters: *-ipe*, *-tmp*, *-1t1*, *-and*, *-apm f*, *-ef*. The list of detected ARGs was filtered to include only those with a minimum threshold of 80% identity between the query and reference gene over at least 80% of the reference gene length.

Downstream analysis

Two key software tools were used to conduct comprehensive exploration, analysis, and visualization of the microbiome and resistome count data: MicrobiomeAnalyst (33, 34) and ResistoXplorer (34).

For graphical representation and statistical analysis, GraphPad Prism (Prism 9 and 10 software) as well as the R programming (version 4.2.1) were utilized. Alpha-diversity was calculated using the Shannon and Chao1 diversity indexes at species level, as well as ARG level. The top 10 most abundant features of the microbiome (species) and resistome (ARGs) data were plotted using *aggregate top taxa* and plotting functions of the microbiome R package (35-37). For Beta-diversity, Aitchison distance metric on centered log-ratio (CLR) transformed counts were performed using the *transform* and *ordinate* (RDA) function of the microbiome and phyloseq R packages. The resulting data was visualized as compositional principal component analysis (PCA) ordination plot using the *plot_ordination* function of the phyloseq package.

Pairwise comparisons of log-fold changes in the abundance of microbial species and ARGs between different groups were performed using DESeq2 (38). In order to account for multiple testing, Benjamini-Hochberg (BH) procedure was employed to adjust the results (adjusted p-values).

In case where “one-way analysis of variance” (ANOVA) was conducted, the results were adjusted for multiple comparisons using the Dunnett’s multiple comparison test. Adjusted p-values lower than 0.05 were considered statistically significant.

RESULTS

Dose-dependent effects of amoxicillin on oral biofilms

Pre-formed oral biofilms in new fresh SHI media were initially subjected to varying concentrations of amoxicillin, ranging from 0-200 µg/ml **(Figure 1)**. The inoculum was prepared using pooled saliva obtained from eight donors. Notably, when exposed to low amoxicillin concentration within the range of 0.025-0.1 µg/ml, a slight increase in biofilm viability was observed in comparison to the negative control. The peak of this increase was remarkably evident under treatment with 0.1 µg/ml amoxicillin. However, as the amoxicillin concentration exceeded 0.1 µg/ml, a contrasting effect was observed where biofilm viability was gradually inhibited. The reduction in viability continued until the highest amoxicillin concentration was reached, at which point viable cells were almost undetectable.

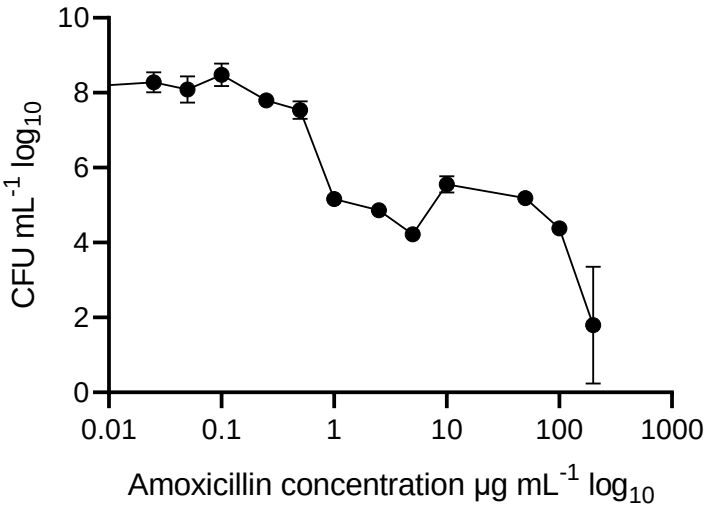

Figure 1: Oral biofilm treated with different amoxicillin concentrations. Number of viable cells in the polymicrobial biofilm community, as determined by colony-forming units, counted on SHI agar plates. The data are shown for triplicate experiments as mean± SE.

The Efficacy of HAMLET and low concentrations of amoxicillin on oral biofilms

To evaluate the impact of HAMLET, both as a standalone treatment and in combination with amoxicillin at low concentrations, pre-formed oral biofilms were subjected to two different HAMLET concentrations, alone or in combination with 0.1 µg/ml of amoxicillin for 24 hours. Bactericidal activity with HAMLET alone was observed with a concentration of 250 µg/ml **(Figure 2A)** In context, neither HAMLET at 125 µg/ml alone nor amoxicillin alone, when assessed in comparison to the negative control, displayed any significant reduction in bacterial cell viability. However, oral biofilm viability showed to be affected by the combination treatment of HAMLET at 125 µg/ml and 0.1 µg/ml amoxicillin, leading to significant decrease in bacterial viability compared to untreated samples **(Figure 2B)**.

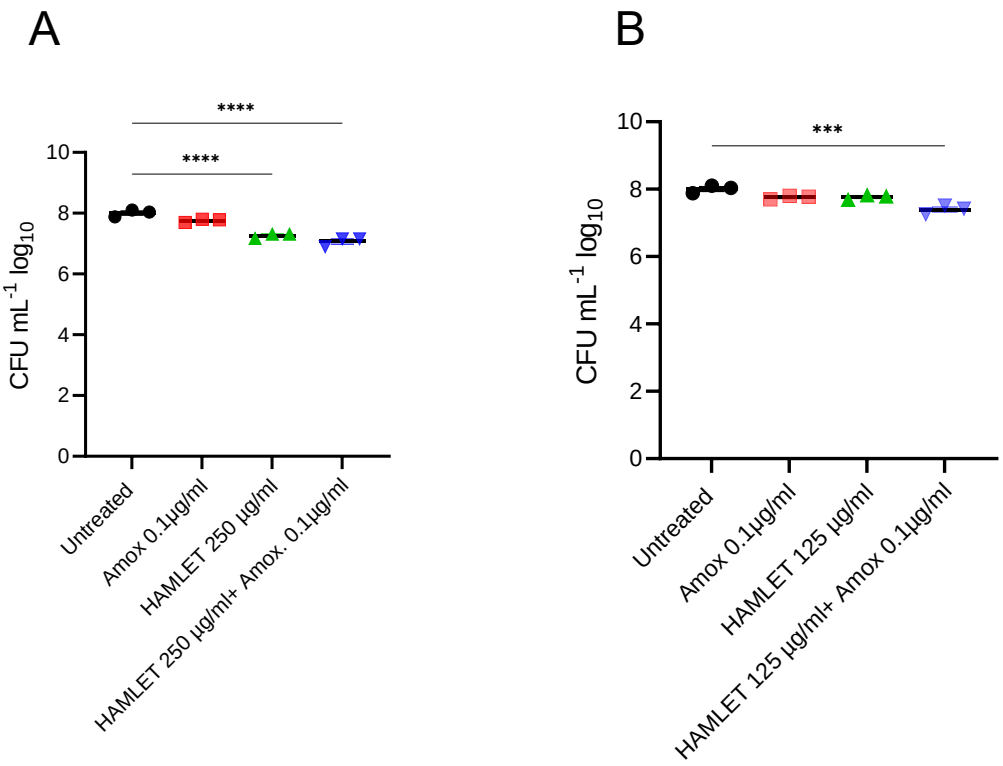

Figure 2: The effect of HAMLET alone or in combination with low amoxicillin concentration on oral biofilm community. (A) HAMLET concentration 250 µg/mL and (B) HAMLET concentration 125 µg/mL. Numbers of viable cells in the community are determined by colony-forming units. All results are based on three independent experiments with triplicate samples. Data are shown as mean± SE. *** P<0.001. **** P< 0.0001. One-way ANOVA followed by Dunnett's multiple comparison test.

Impact of HAMLET alone or in combination with amoxicillin: Oral microbiome ecology

A total of eight samples, representing two samples from each treatment group, underwent shotgun metagenomic sequencing. This analysis resulted in the generation of approximately 90.2 million paired reads per sample after quality filtering, yielding an average of 11.3 million reads per sample (with a minimum of 6.8 million and a maximum of 18.5 million reads per sample).

The metagenomic analysis provided insights into the effect of HAMLET, administrated either as a standalone treatment or in combination with amoxicillin, on the ecology of the oral microbiome. To assess changes in alpha diversity, metrics such as Chao1 index, which quantifies only microbial richness and Shannon index, which accounts for both richness and evenness (abundance) were employed. At species level, no significant changes in either alpha diversity index were observed in treatment samples when compared to the untreated control **(Figure 3A, B)**. Beta diversity analysis revealed increased variability in the biological replicates subjected to antibiotic treatment, contrasting with the biological replicates from untreated or HAMLET solely treated samples **(Figure 3C)**.

In total, 44 bacterial species spanning eight bacterial genera across all the samples were identified **(Supplementary Table 1)**. Despite biological sample variation, alterations in the relative abundance of taxonomic composition were evident in the treatment groups compared to the negative control **(Supplementary Table 2)**.

Analyzing the taxonomic composition at the species level revealed the emergence of new species in the amoxicillin, HAMLET and HAMLET combined with amoxicillin treated biofilm groups **(Figure 3D)**. In comparison to the untreated samples, *Streptococcus salivarius* emerged as the dominant species in both biological replicates, while *Lactobacillus fermentum* decreased significantly in amoxicillin treated samples **(Figure S1)**. Both *L. fermentum* and *S. salivarius* reduced in proportion when subjected to the combination of HAMLET and

amoxicillin. In contrast, *Lactobacillus crispatus* increased significantly in proportion and dominated in the combination treatment group **(Figure S1)**. *Lactobacillus paragasseri* was detected in at least one replicate of samples treated with amoxicillin, either alone or in combination with HAMLET. Predominant colonizers were gram-positives, with the exception of two samples that had more than 20% *Veillonella atypica*. However, these were in replicates that belonged to different treatment groups.

Furthermore, the presence of the pathogen *Streptococcus pneumoniae* was noted in all samples, with an increase in amoxicillin-treated samples compared to the untreated control **(Supplementary Table 1)**.

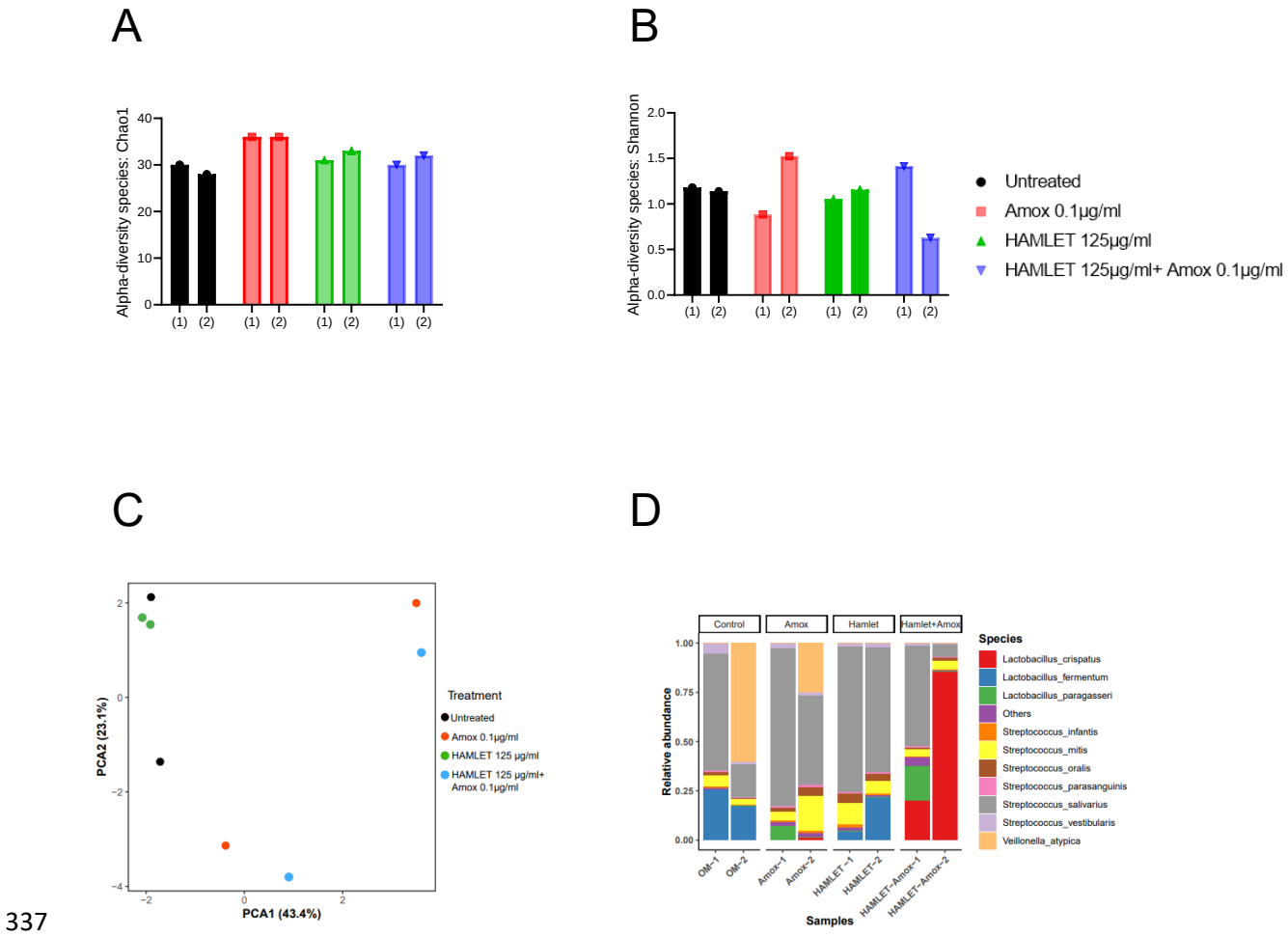

Figure 3: Ecological impact of HAMLET alone or in combination with amoxicillin on oral biofilm community. (A and B) Alpha-diversity on species level measured by (A) Chao1 index indicate the total richness and (B) Shannon index which indicate richness and evenness. (C) A principal component analysis plot (PCA) with Aitchison distance illustrating beta-diversity. (D) Stacked bar plots illustrate the relative abundance of all replicates for 10 most abundant species. All results are based on two biological replicates from the same day.

Impact of HAMLET alone or in combination with amoxicillin on Oral resistome

Across all the eight samples, a total of 123,350 paired reads were annotated as antimicrobial resistant genes (ARGs). On average, there were 15,418 reads per sample, with a minimum count of 3,625 and a maximum count of 24,976. In total, 22 distinct ARGs associated with seven antibiotic drug classes and four antibiotic resistance mechanisms: antibiotic efflux, antibiotic inactivation, antibiotic target alteration, and antibiotic target protection were identified **(Figure S2, Supplementary Table 3).**

Alpha-diversity, as measured by Chao 1 and Shannon indexes exhibited no major changes in the treatment groups when compared to the untreated control **(Figure 4A, B)**. However, beta-diversity analysis revealed distinct clustering patterns of biological samples within each treatment group, signifying that each treatment group harbors a unique resistome (**Figure 4C)**.

ARGs associated with all four antibiotic resistance mechanisms were detected in all treatment groups, with the highest relative abundance observed in ARGs related to antibiotic target protection and antibiotic efflux **(Figure S2)**. The three most prevalent classes of ARGs in all treatment groups included fluoroquinolone, tetracycline, and macrolide-lincosamide-streptogramin (MLS). Although there was a modest increase in the beta-lactam ARG class in HAMLET and the combination treatment with HAMLET and amoxicillin. **(Figure S2)**.

Regarding specific ARGs, despite some variations between the replicates, *mel*, *patA*, *patB*, *pmrA*, *RImA(II)*, and *tetM* genes were detected in high abundance across all treatment groups **(Figure 4D and Supplementary Table 3)**. Furthermore, the relative abundance of the *tet(C)* gene showed an increase in the HAMLET and HAMLET combined with amoxicillin-treated samples. For the beta-lactam antibiotic resistance genes, TEM genes were detected in all treated samples, although they comprised a low proportion of all ARGs.

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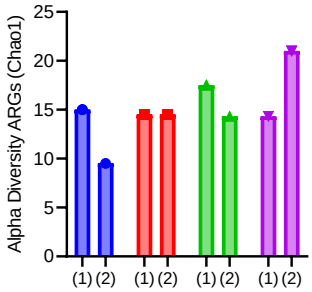

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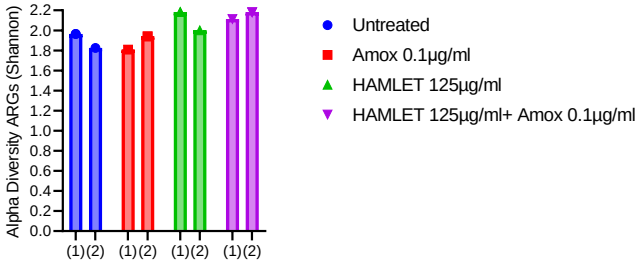

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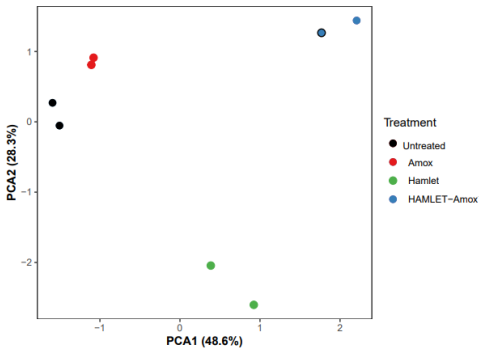

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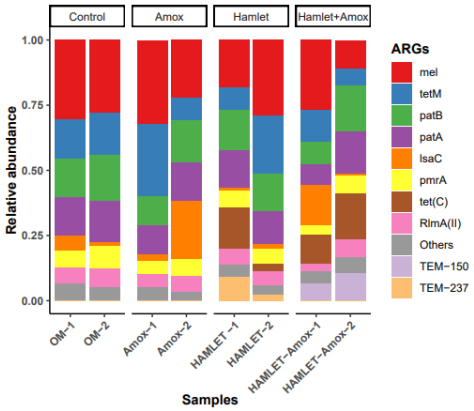

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Figure 4: Impact of HAMLET alone or in combination with amoxicillin on oral resistome.

(A and B) Alpha-diversity on ARG level measured by (A) Chao1 index indicates the total richness and (B) Shannon index which indicates richness and evenness. (C) Principal component analysis (PCA) ordination plot with Aitchison distance illustrating beta-diversity. (D) Stacked bar plots illustrate the relative abundance of all replicates for the 10 most abundant ARG's. All results are based on two biological replicates from the same day.

DISCUSSION

With the treatment challenges of infections caused by biofilms and the growing global issue of antimicrobial resistance, there is an increased interest in identifying novel antimicrobials that can work synergistically with antibiotics while lowering the likelihood of microbial resistance (39, 40). Here, we investigated the combined usage of HAMLET and amoxicillin. HAMLET was specifically chosen due to its unique multi-targeted antimicrobial mechanism including inhibition of glycolytic pathways (20). Other promising properties of HAMLET such as, lack of resistance development in studies with *S. pneumoniae* and *S. aureus (15, 18)*, and established low- or non-toxic profile in prior animal and human studies investigating its potential as an anticancer drug (41-44).

Our focus was on polymicrobial biofilm communities, an area that has received less attention compared to single bacterium in biofilms (45, 46). Our results revealed that neither HAMLET nor amoxicillin individually in our comparative analysis had a significant effect on the overall cell viability of the polymicrobial community compared with the untreated control at the chosen concentrations. However, their combination resulted in a significant reduction in biofilm viability, indicating a synergistic effect. Furthermore, through metagenomic analysis, our data suggested that this combination may skew the polymicrobial community towards populations with potential probiotic effects, thereby representing a potential new approach on managing polymicrobial biofilms.

One of the most studied probiotic bacteria are lactobacilli. Several species in this genus have been shown to have beneficial effects, including improving gut and oral health, boosting the immune system, aiding in the digestion of lactose, and reducing the risk of certain infections (47-51). *Lactobacillus fermentum* was the dominant species of lactobacilli in the non-treated

control samples, comprising approximately 25% of the microbiome. These were practically absent in amoxicillin treated samples. It was therefore interesting that the combination of HAMLET and low-amoxicillin concentration in our study resulted in a microbiome dominated by lactobacilli. *Lactobacillus crispatus*, in particular, was among those that increased in abundance from very low detected levels in the non-treated control and single treatments with HAMLET or amoxicillin to up to 90% with the combination of the agents. This species is known for its probiotic and antimicrobial properties (52), and has been associated with oral health, particularly in the context of dental and periodontal diseases (53-55) These results highlight the potential of the combination of HAMLET and amoxicillin to modulate the composition of the microbiome towards a community enriched in probiotic bacteria, compared to samples treated with amoxicillin alone.

Although our study primarily aimed to investigate the combined effects of HAMLET and amoxicillin, the observations on HAMLET alone are also of relevance. We initially tested two concentrations of HAMLET for its potential effect on cell viability. From these, we chose the lowest concentration of HAMLET for the metagenomics studies to underscore its potential synergistic effect with amoxicillin. Although at this low dosage HAMLET alone had no discernible effects on the overall number of viable bacteria, the metagenomics analysis indicated potential changes in the microbiome composition with increased relative abundance of *S. salivarius*. It is possible that the changes by HAMLET, alone or in combination with amoxicillin, are a result of its influence on glycolytic pathways. Previous studies have shown that HAMLET binds to and inactivates two key glycolytic enzymes, fructose-bisphosphate aldolase and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (20). However, the mechanism of HAMLET's antimicrobial effect is not yet fully understood.

In the case of antibiotics, it is known that at low concentrations bacteria can sense antibiotics as a stress, and rather than eliminating them, these low concentrations may promote stress

435 responses that can favor overall survival (10, 56-58). This adds a level of complexity that
436 needs to be considered in interpreting results using polymicrobial communities. Small
437 changes in one microbial species caused by low concentrations of antimicrobials can trigger
438 major ecological shifts in the community due to interdependent and nonlinear interactions
439 between microbial species.

440 In our study, we observed that *TEM* genes encoding beta-lactamases (59) were present in
441 samples exposed to HAMLET alone, amoxicillin alone or in combination with amoxicillin.
442 While the increase in abundance of this gene in samples exposed to amoxicillin could
443 potentially be linked to survival mechanisms in the presence of this beta-lactam antibiotic, it
444 is difficult to explain the presence of this beta-lactamase in the HAMLET group alone. We
445 also observed an increase in the abundance of *(tet)C* in both the groups treated with HAMLET
446 alone and in combination with amoxicillin, despite tetracycline not being used in the study.
447 Such increases in antibiotic resistance genes in response to the presence of low concentrations
448 of antimicrobials are frequently reported in metagenomic studies and are often not solely
449 attributed to the co-carriage of different antibiotic resistance genes in mobile genetic elements
450 (60-62). Instead, this phenomenon is reflective of the complex and intricate ecological
451 dynamics within microbial communities, as discussed above.

452 In conclusion, our results highlight the potential of HAMLET as a synergistic antimicrobial
453 agent when combined with amoxicillin. The significant shift in the oral microbiome towards
454 an increase in *Lactobacillus crispatus*, a potential probiotic, by the combined agents presents
455 a promising strategy for combatting polymicrobial infections and reducing the burden of
456 antibiotic resistance. The findings of our study suggest that this approach may contribute to
457 the development of more effective strategies for combating drug-resistant polymicrobial
458 infections and underscore the importance of continued research in this area.

459

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AUTHOR CONTRIBUTION:

Conceptualization of the project was by FCP and APH. All authors contributed to the design of experiments. Laboratory work was by NKB, APH, FCP. Downstream metagenomics analysis was by NKB and AD. First drafting of manuscript by NKB, FCP, AD. All authors contributed to critical review of data and writing the final manuscript.

COMPETING OF INTERESTS

No potential conflict of interest was reported by the author(s).

ADDITIONAL INFORMATION

Ethics statement:

The study was conducted in accordance with the Declaration of Helsinki and approved by the National Regional Ethical Committee (REK20152491)

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SUPPLEMENTARY FIGURES

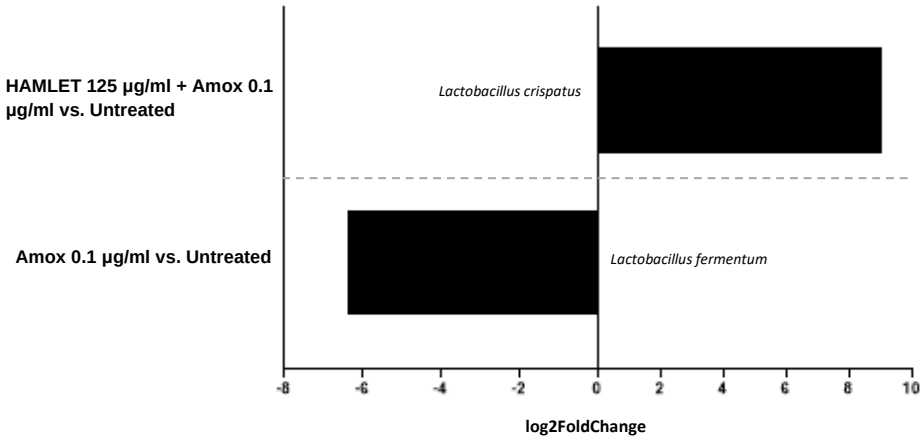


Figure S1: Taxa with significantly different abundance upon treatment. Bar charts illustrate the $\log_2$ fold change of taxa, adjusted for false discovery rate (FDR), *p*-values <0.05. (based on DESeq2).

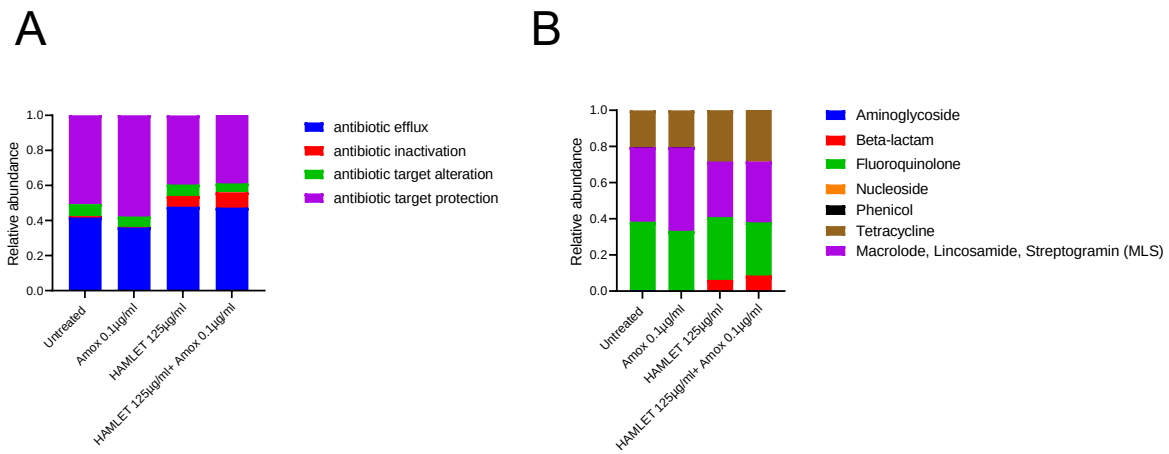
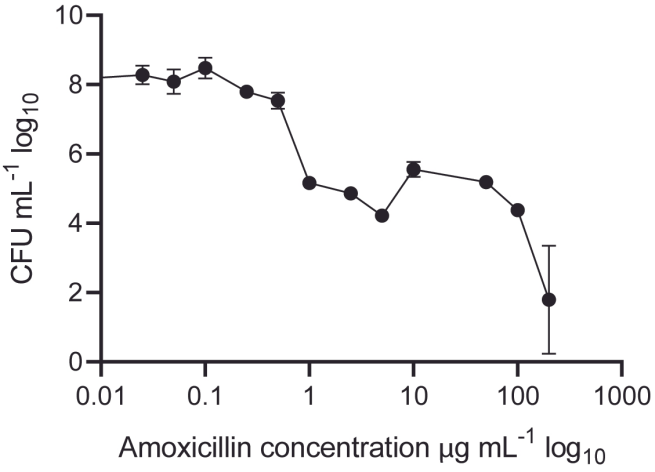
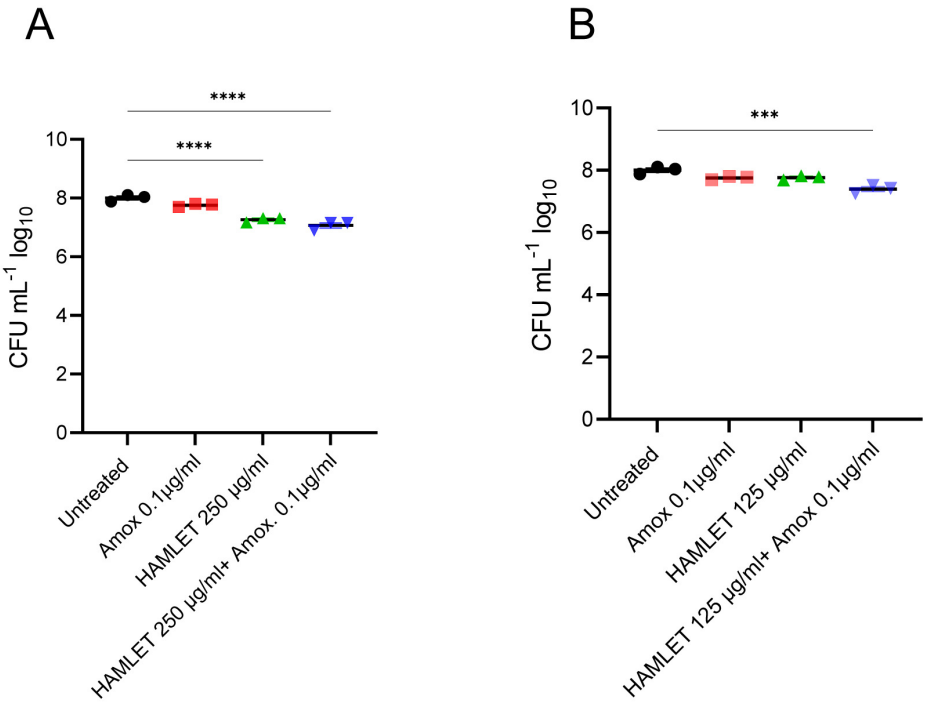
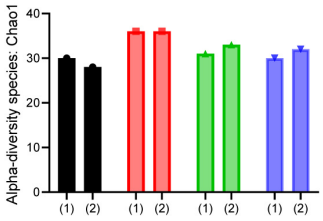


Figure S2: Impact of HAMLET alone or in combination with amoxicillin on oral resistome. (A and B) Stacked bar plots display the relative abundance of (A) all antibiotic mechanisms (B) all ARG classes.





A



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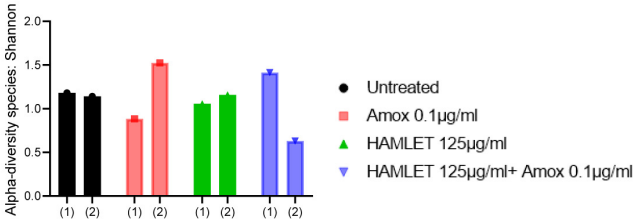

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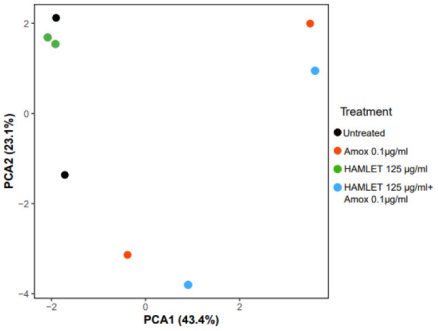

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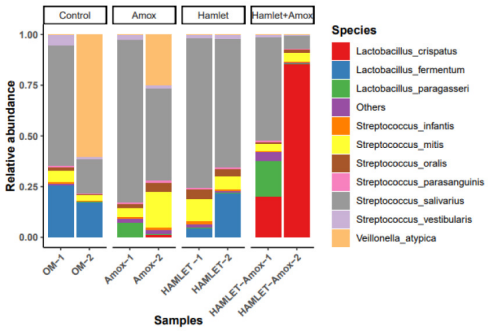
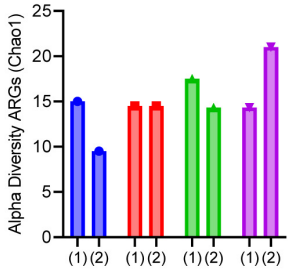


A



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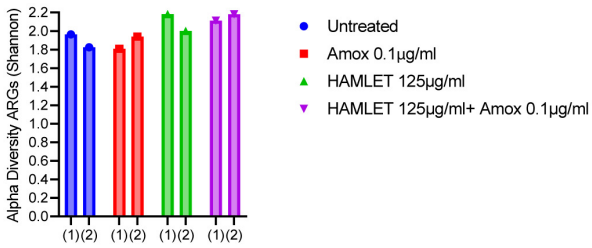

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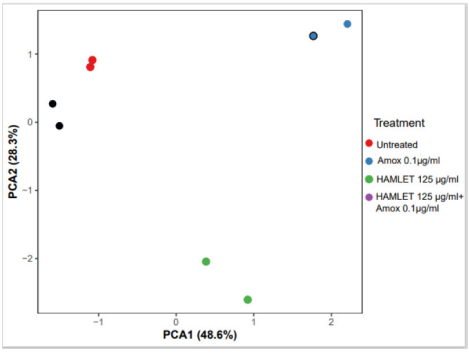
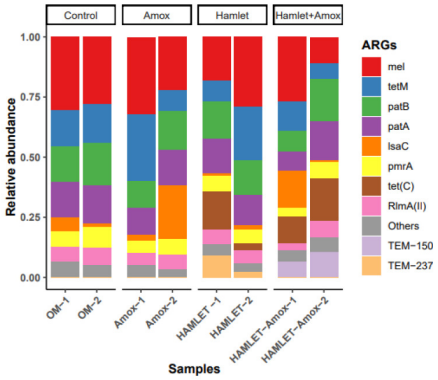


D



Supplementary Table S1: Count table of shotgun metagenomic sequencing of biofilm community exposed to amoxicillin and HAMLET. Untreated [Control], Amoxicllin 0.1 µg/mL [Amox], HAMLET 125 µg/mL [HAMLET], HAMLET 125 µg/mL+ amoxicillin 0.1 µg/mL [HAMLET +Amox]

Feature.ID	Control-1	Control-2	HAMLET-1	HAMLET-2	HAMLET-Amox-1	HAMLET-Amox-2	Amox-1	Amox-2
Alloscardovia omnicolens	0	0	0	0	0	76	0	28499
Rothia mucilaginosa	1961	162	2236	2505	3103	3262	2854	1944
Gemella haemolysans	864	207	1859	1054	812	2303	1141	1384
Gemella morbillorum	72	245	220	0	0	323	0	0
Gemella sanguinis	3784	3336	6816	8158	5543	5165	7359	3383
Granulicatella adiacens	738	0	948	989	2324	4237	1185	1623
Granulicatella elegans	1974	852	2493	2041	1483	2309	1364	4051
Lactobacillus crispatus	0	6313	0	0	3041170	15822483	34739	111099
Lactobacillus fermentum	2109792	1496844	323095	2450459	0	0	0	52
Lactobacillus gasseri	0	0	0	0	565389	0	166561	0
Lactobacillus oris	0	0	0	0	0	0	455333	0
Lactobacillus paragasseri	0	0	0	0	2696443	0	989946	0
Lactobacillus pontis	0	0	0	0	0	0	1254423	0
Lactobacillus salivarius	0	0	0	0	0	73595	0	0
Streptococcus anginosus group	1041	1946	1239	360	3012	530	12438	435
Streptococcus australis	1796	340	929	1261	1424	666	2660	387
Streptococcus cristatus	4087	1980	14706	9690	13151	5537	13180	7877
Streptococcus gordonii	1916	0	0	2972	1499	293	1542	153
Streptococcus infantis	65845	21871	84989	80807	66429	65020	118229	62417
Streptococcus mitis	434727	236026	754729	704640	519234	831197	643245	1325673
Streptococcus oralis	150473	47337	305725	403706	159091	263555	250032	320118
Streptococcus parasanguinis	57479	17852	80618	91116	68530	75351	102708	99100
Streptococcus peroris	0	0	0	0	0	0	877	1304
Streptococcus pneumoniae	12647	7401	19247	17360	13095	28647	20291	50367
Streptococcus pseudopneumoniae	3764	554	3723	7863	5318	4886	5847	7494
Streptococcus salivarius	4878480	1467533	4981891	7042957	7750616	1210911	11557253	3338069
Streptococcus sanguinis	727	0	1322	1270	805	0	1927	601
Streptococcus sinensis	0	0	310	296	147	399	148	439
Streptococcus sp A12	3239	288	1282	2236	2749	859	3807	872
Streptococcus sp F0442	1164	87	1251	2075	1553	1020	2806	1003
Streptococcus sp HMSC034E03	8497	4040	21168	16858	18411	20618	20116	16534
Streptococcus sp HMSC067H01	10253	2230	16385	16878	11651	12312	22095	17377
Streptococcus sp HMSC070B10	1488	548	3964	3086	3339	0	3366	4447
Streptococcus sp HMSC071D03	9576	2249	17330	12750	10580	25313	26559	9651
Streptococcus sp HPH0090	270	0	1976	1762	537	211	2936	1660
Streptococcus sp M334	1137	1033	1297	286	0	838	715	3254
Streptococcus sp NLAE zl C503	2429	0	4290	4251	0	5721	5667	7738
Streptococcus vestibularis	429118	82052	121102	258666	200707	57107	364019	126149
Streptococcus viridans	0	0	0	489	0	0	0	0
Veillonella atypica	179	5205749	557	2617	0	19030	575	1853020
Veillonella dispar	0	426	0	1665	0	0	1027	219
Veillonella infantium	0	0	0	435	0	41	127	98
Veillonella parvula	0	245	0	260	0	0	93	1251
Escherichia coli	0	0	692	0	306	573	0	0

Supplementary Table S2: Relative abundance table of shotgun metagenomic sequencing of biofilm community exposed to amoxicillin and HAMLET. Untreated [Control], Amoxicllin 0.1 µg/mL [Amox], HAMLET 125 µg/mL [HAMLET], HAMLET 125 µg/mL+ amoxicillin 0.1 µg/mL [HAMLET +Amox]

Feature.ID	Control-1	Control-2	HAMLET-1	HAMLET-2	HAMLET-A	HAMLET-A	Amox-1	Amox-2
Alloscardovia omnicolens	0	0	0	0	0	0.00041	0	0.38461
Rothia mucilaginosa	0.02392	0.00188	0.03298	0.02246	0.02046	0.01759	0.01773	0.02623
Gemella haemolysans	0.01054	0.00241	0.02743	0.00945	0.00535	0.01242	0.00709	0.01868
Gemella morbillorum	0.00088	0.00285	0.00325	0	0	0.00174	0	0
Gemella sanguinis	0.04615	0.03875	0.10055	0.07314	0.03654	0.02785	0.04571	0.04565
Granulicatella adiacens	0.009	0	0.01398	0.00887	0.01532	0.02285	0.00736	0.02191
Granulicatella elegans	0.02407	0.0099	0.03678	0.0183	0.00978	0.01245	0.00847	0.05467
Lactobacillus crispatus	0	0.07332	0	0	20.04931	85.32222	0.21578	1.49937
Lactobacillus fermentum	25.73069	17.38546	4.76655	21.96969	0	0	0	0.0007
Lactobacillus gasseri	0	0	0	0	3.73	0	1.03459	0
Lactobacillus oris	0	0	0	0	0	0	2.8283	0
Lactobacillus paragasseri	0	0	0	0	17.77665	0	6.14904	0
Lactobacillus pontis	0	0	0	0	0	0	7.79184	0
Lactobacillus salivarius	0	0	0	0	0	0.39686	0	0
Streptococcus anginosus group	0.01269	0.0226	0.01828	0.00323	0.01986	0.00286	0.07726	0.00587
Streptococcus australis	0.0219	0.00395	0.01371	0.01131	0.00939	0.00359	0.01652	0.00522
Streptococcus cristatus	0.04985	0.023	0.21695	0.08688	0.0867	0.02986	0.08187	0.1063
Streptococcus gordonii	0.02337	0	0	0.02665	0.00988	0.00158	0.00958	0.00207
Streptococcus infantis	0.80304	0.25403	1.25383	0.72448	0.43794	0.35062	0.73438	0.84237
Streptococcus mitis	5.30186	2.74138	11.13434	6.31748	3.42312	4.48	3.99551	17.89095
Streptococcus oralis	1.83515	0.54981	4.51029	3.61944	1.04883	1.42121	1.55307	4.32023
Streptococcus parasanguinis	0.701	0.20735	1.18934	0.8169	0.45179	0.40633	0.63797	1.33743
Streptococcus peroris	0	0	0	0	0	0	0.00545	0.0176
Streptococcus pneumoniae	0.15424	0.08596	0.28394	0.15564	0.08633	0.15448	0.12604	0.67974
Streptococcus pseudopneumoniae	0.04591	0.00644	0.05493	0.0705	0.03506	0.02635	0.03632	0.10114
Streptococcus salivarius	59.49717	17.04502	73.49668	63.14391	51.09694	6.53	71.78778	45.04974
Streptococcus sanguinis	0.00887	0	0.0195	0.01139	0.00531	0	0.01197	0.00811
Streptococcus sinensis	0	0	0.00458	0.00265	0.00097	0.00215	0.00092	0.00592
Streptococcus sp A12	0.0395	0.00335	0.01891	0.02005	0.01812	0.00463	0.02365	0.01177
Streptococcus sp F0442	0.0142	0.00101	0.01845	0.0186	0.01024	0.0055	0.01743	0.01354
Streptococcus sp HMSC034E03	0.10363	0.04692	0.31229	0.15114	0.12138	0.11118	0.12495	0.22314
Streptococcus sp HMSC067H01	0.12504	0.0259	0.24173	0.15132	0.07681	0.06639	0.13724	0.23452
Streptococcus sp HMSC070B10	0.01815	0.00636	0.05848	0.02767	0.02201	0	0.02091	0.06001
Streptococcus sp HMSC071D03	0.11679	0.02612	0.25567	0.11431	0.06975	0.1365	0.16497	0.13025
Streptococcus sp HPH0090	0.00329	0	0.02915	0.0158	0.00354	0.00114	0.01824	0.0224
Streptococcus sp M334	0.01387	0.012	0.01914	0.00256	0	0.00452	0.00444	0.04392
Streptococcus sp NLAE zl C503	0.02962	0	0.06329	0.03811	0	0.03085	0.0352	0.10443
Streptococcus vestibularis	5.23346	0.95301	1.78659	2.31908	1.32319	0.30795	2.2611	1.70248
Streptococcus viridans	0	0	0	0.00438	0	0	0	0
Veillonella atypica	0.00218	60.46343	0.00821	0.02346	0	0.10262	0.00357	25.00789
Veillonella dispar	0	0.00495	0	0.01493	0	0	0.00638	0.00295
Veillonella infantium	0	0	0	0.0039	0	0.00022	0.00079	0.00132
Veillonella parvula	0	0.00284	0	0.00233	0	0	0.00058	0.01688
Escherichia coli	0	0	0.01021	0	0.00202	0.00309	0	0

Supplementary Table S3: count table of antimicrobial resistance genes (ARGs). Shotgun metagenomic sequencing of *ex-vivo* biofilm exposed to amoxicillin and HAMLET. Untreated [Control], Amoxiclin 0.1 µg/mL [Amox], HAMLET 125 µg/mL [HAMLET], HAMLET 125 µg/mL+ amoxicillin 0.1 µg/mL [HAMLET +Amox]

Feature.ID	Control-1	Control-2	HAMLET-1	HAMLET-2	HAMLET-Amox-1	HAMLET-Amox-2	Amox-1	Amox-2
gb|AB039845.1|+|25-1945|ARO:3000186|tetM [Erysipelothrix rhusiopathiae]	1436	581	1615	3252	2213	972	5314	2180
gb|AE005672.3|-|1980557-1982324|ARO:3000025|patB [Streptococcus pneumoniae TIGR4]	1391	640	2884	2037	1575	2586	2159	4051
gb|AE005672.3|-|1983115-1984810|ARO:3000024|patA [Streptococcus pneumoniae TIGR4]	1362	571	2629	1863	1511	2425	2130	3670
gb|AE007317.1|+|866210-867410|ARO:3000822|pmrA [Streptococcus pneumoniae R6]	637	318	1184	841	635	1019	1017	1670
gb|AF227521.1|+|3269-4487|ARO:3000616|mel [Streptococcus pyogenes]	2867	1018	3354	4177	4906	1655	6234	5544
gb|AF242872.1|+|2131-2878|ARO:3000375|ErmB [Enterococcus faecium]	48	17	105	99	87	69	124	108
gb|AM183304.1|+|208-1069|ARO:3001017|TEM-150 [Escherichia coli]	0	0	0	0	1212	1586	0	0
gb|AY043299.1|-|3984-5175|ARO:3000167|tet(C) [Aeromonas salmonicida]	0	0	2927	410	2071	2640	0	0
gb|AY712687.1|+|0-831|ARO:3002628|aad(6) [Streptococcus oralis] Partial	0	0	0	0	0	13	0	17
gb|CP004067.1|+|52914-53709|ARO:3002647|APH(3')-IIIa [Campylobacter coli CVM N29710]	0	0	0	0	21	22	0	0
gb|CP007593.1|+|2148923-2149772|ARO:3001301|RlmA(II) [Streptococcus pneumoniae]	569	260	1159	772	556	991	931	1464
gb|HM990671.1|+|5192-6671|ARO:3003112|lsaC [Streptococcus agalactiae]	526	51	210	229	2817	93	471	5559
gb|HQ652506.1|+|2099-3836|ARO:3004033|tetB(46) [Streptococcus australis]	180	54	226	152	236	139	291	190
gb|HQ652506.1|+|373-2098|ARO:3004032|tetA(46) [Streptococcus australis]	166	39	189	156	176	138	219	165
gb|KX000272.1|+|0-1740|ARO:3004035|tetA(60) [uncultured bacterium]	77	0	122	65	125	179	113	114
gb|KX000273.1|+|0-1740|ARO:3004036|tetB(60) [uncultured bacterium]	0	0	68	0	79	160	85	91
gb|M18896.2|+|206-2126|ARO:3000190|tetO [Campylobacter jejuni]	79	35	101	63	72	119	116	118
gb|M55620.1|+|0-660|ARO:3002687|catQ [Clostridium perfringens]	22	0	0	0	0	0	41	14
gb|NG_062250.1|+|100-961|ARO:3005268|TEM-237 [Escherichia coli]	0	0	1692	325	0	0	0	0
gb|U01945.1|+|373-916|ARO:3002897|SAT-4 [Campylobacter coli]	0	0	0	0	0	53	8	0
gb|U36911.1|+|1429-2290|ARO:3000979|TEM-116 [Staphylococcus aureus]	0	0	0	0	0	0	16	21
gb|V00618.1|+|150-945|ARO:3002644|APH(3')-IIa [Escherichia coli]	43	40	21	0	0	0	0	0

III

antibiotics

MDPI

Article

Treatment of Mouse Infants with Amoxicillin, but Not the Human Milk-Derived Antimicrobial HAMLET, Impairs Lung Th17 Responses

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Abstract: Emerging evidence suggests differential effects of therapeutic antibiotics on infant T cell responses to pathogens. In this study, we explored the impact of the treatment of mouse infants with amoxicillin and the human milk-derived antimicrobial HAMLET (human alpha-lactalbumin made lethal to tumor cells) on T cell responses to *Streptococcus pneumoniae*. Lung cells and splenocytes were isolated from the infant mice subjected to intranasal administration of amoxicillin, HAMLET, or a combination of HAMLET and amoxicillin, and cultured with *S. pneumoniae* to measure T cell responses. After *in-vitro* stimulation with *S. pneumoniae*, lung cells from amoxicillin- or amoxicillin plus HAMLET-treated mice produced lower levels of Th17 (IL-17A), but not Th1 (IFN-γ), cytokine than mice receiving HAMLET or PBS. IL-17A/IFN-γ cytokine levels produced by the stimulated splenocytes, on the other hand, revealed no significant difference among treatment groups. Further analysis of T cell cytokine profiles by flow cytometry showed that lung CD4+, but not CD8+, T cells from amoxicillin- or HAMLET plus amoxicillin-treated mice expressed decreased levels of IL-17A compared to those from HAMLET-exposed or control mice. Collectively, these results indicate that exposure of infant mice to amoxicillin, but not HAMLET, may suppress lung Th17 responses to *S. pneumoniae*.

Keywords: amoxicillin; HAMLET; infants; lungs; Th17 immunity

Citation: Shekhar, S.; Brar, N.K.; Håkansson, A.P.; Petersen, F.C. Treatment of Mouse Infants with Amoxicillin, but Not the Human Milk-Derived Antimicrobial HAMLET, Impairs Lung Th17 Responses. *Antibiotics* **2023**, *12*, 423. https://doi.org/10.3390/antibiotics12020423

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1. Introduction

Antibiotics are among the most commonly used drugs for neonates and infants that are suffering from or are prone to bacterial infections, particularly sepsis, which causes severe morbidity and mortality across the globe [1–4]. Among newborns, neonatal sepsis causes an estimated 600,000 deaths per annum worldwide [5]. Amoxicillin, a β-lactam penicillinase-susceptible semisynthetic amino-penicillin antibiotic with activity against a wide range of bacteria, is one of the most commonly prescribed antibiotics for the treatment or prevention of neonatal sepsis [6,7]. Although antibiotics are desired to specifically target pathogenic bacteria, many antibiotics, including amoxicillin, have reported adverse side effects on the neonatal and infant microbiota and immunity that can contribute to the development of dysbiosis, microbiota perturbance, and impaired immunity to pathogens [8,9]. These side effects are much more profound and long-lasting in neonates and infants due to their evolving and immature immunophysiological systems [8–10].

There are a limited number of studies that focus on the impact of antibiotic regimens on neonatal and infant T cell responses to pathogens [8–14]. Gonzalez-Perez et al. demonstrated that perinatal exposure of mice to a combination of ampicillin, streptomycin, and clindamycin after vaccinia virus infection reduced the number of virus-specific neonatal/infant CD8+ T cells expressing IFN-γ [11]. Not only that, but the infants showed altered peripheral CD8+ T cell receptor signaling due to the gastrointestinal microbiome

dysbiosis [12]. In accordance with these suppressive effects on T cell function, we recently showed that upon in vitro stimulation with *Streptococcus pneumoniae*, CD4+, but not CD8+, T cells from neonatal mice exposed to piperacillin in combination with the beta-lactamase inhibitor tazobactam expressed lower levels of IL-17A (Th17) and IFN-γ (Th1) cytokines compared to unexposed mice [13]. On the other hand, when newborn pigs exposed to therapeutic amoxicillin doses were challenged with *Salmonella enterica* serovar Typhimurium, their whole blood analysis exhibited an enhanced upregulation of effector T cell surrogate cytokine, IFN-γ, gene expression compared to those treated with placebo [15]. Similarly, CD4+ T cells from antibiotic-exposed (ampicillin plus neomycin) infant mice immunized with the antipneumococcal conjugate vaccine PCV13 showed increased IFN-γ recall responses [16]. Altogether, while these data have shed some light on the differential effects (stimulatory versus suppressive) of different antibiotic types on T cell immunity, the impact of amoxicillin on T cell responses to pathogens, despite being routinely administered to neonates and infants in clinical settings, is largely unknown.

HAMLET (human alpha-lactalbumin made lethal to tumor cells) is a human milk-derived lipid-protein complex that possesses bactericidal activities against certain Gram-positive and Gram-negative bacteria [17–19]. Recent studies have shown that HAMLET augments the activity of antibiotics against *S. pneumoniae*, *Streptococcus pyogenes*, *Streptococcus agalactiae*, and *M. tuberculosis* [19–22]. For instance, treatment of antibiotic-resistant *S. pneumoniae*, *S. pyogenes*, and *S. agalactiae* isolates with HAMLET in combination with antibiotics (e.g., penicillin and erythromycin) reduced their Minimum Inhibitory Concentrations (MICs) [22]. These findings highlight the vast potential for the use of a novel therapeutic strategy based on HAMLET-antibiotic combination against antibiotic-resistant pathogens, which pose a menace to global public health. Furthermore, Vansarla et al. have further pointed out that HAMLET holds the ability to modulate the function of antigen-presenting cells (APCs) like dendritic cells (DCs) [23]. In vitro stimulation of primary human monocyte-derived DCs (Mo-DCs) with HAMLET not only led to the enhanced production of proinflammatory cytokines like IL-6 and IL-12, but also upregulated the surface costimulatory molecule CD83 [23]. Furthermore, HAMLET-stimulated Mo-DCs were more effective in eliciting allogeneic T cell proliferation in a mixed lymphocyte reaction (MLR) assay compared to unstimulated Mo-DCs, underscoring the HAMLET's immunomodulatory properties [23]. However, it remains unknown whether treatment with HAMLET alone or in combination with antibiotics can alter T cell immunity against infections.

Here, we sought to determine whether amoxicillin and/or HAMLET alter T cell immunity using a combination of an infant mouse model and in vitro antigenic (killed *S. pneumoniae*) stimulation assays. We selected *S. pneumoniae* as a model organism in this study because it is a pathogen of public health significance and induces T cell responses, particularly Th17 and Th1 responses [24–27]. Our results furnish crucial information on how amoxicillin, HAMLET, or a combination of both modulates peripheral and lung Th17 and Th1 immunity to *S. pneumoniae*, which may be important for designing better therapeutic strategies.

2. Results

2.1. Treatment of Infant Mice with Amoxicillin, but Not HAMLET, Suppresses Lung IL-17A Responses to S. pneumoniae

To assess the impact of antimicrobial therapy on infant T cell responses, we treated infant mice intranasally with amoxicillin, HAMLET, or a combination of both and stimulated the lung cells and splenocytes isolated from them with UV-killed *S. pneumoniae* to measure the production pattern of T cell surrogate cytokines (IL-17A and IFN-γ). The lung cells isolated from amoxicillin- or amoxicillin plus HAMLET-treated infants produced reduced quantities of IL-17A but not IFN-γ, compared to mice receiving PBS (control) or HAMLET (Figure 1). No difference was observed with HAMLET alone compared to the control. In the case of splenocytes, none of the treatments had a significant effect on IL-17A

and IFN-γ production (Figure 1). Without antigenic stimulation, the levels of IL-17A and IFN-γ produced by lung cells and splenocytes from infant mice treated with amoxicillin, HAMLET, amoxicillin plus HAMLET, or PBS did not differ statistically (Supplementary Figure S2).

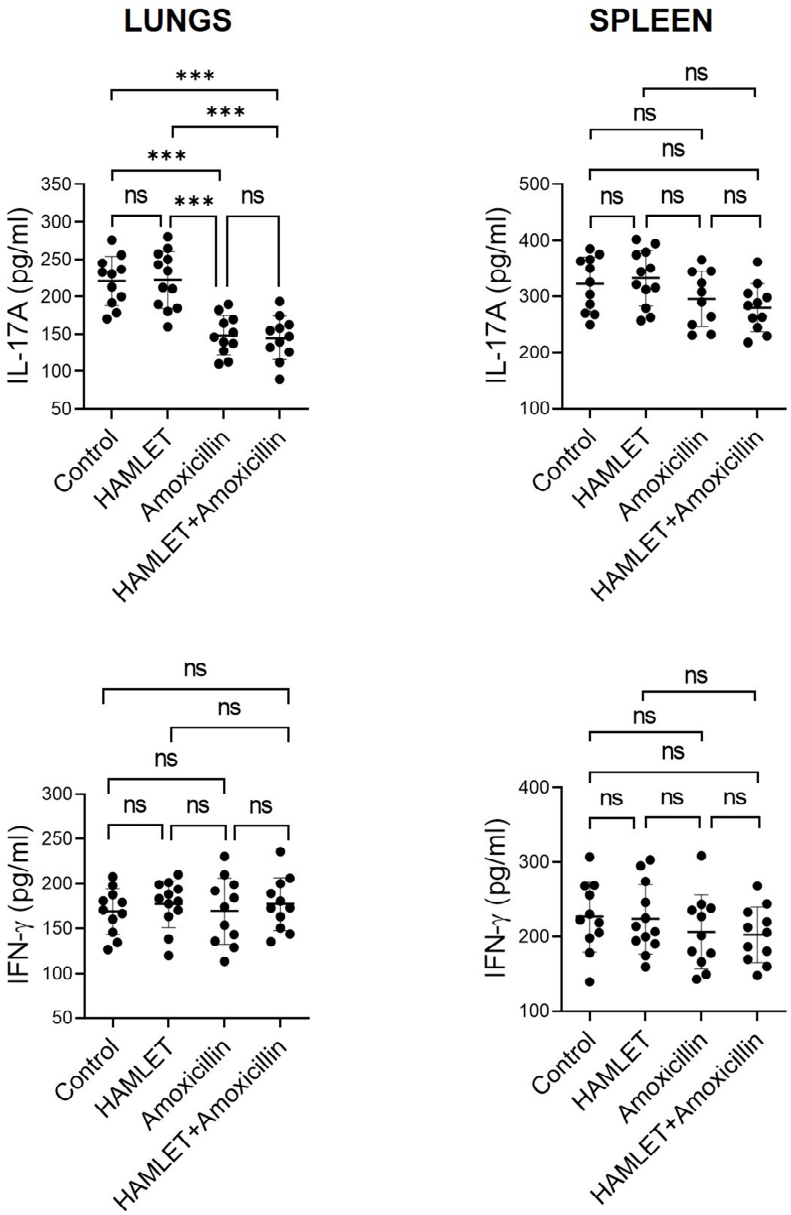

Figure 1. IL-17A and IFN-γ production by lung cells and splenocytes from the infant mice treated with antimicrobials. Lung cells and splenocytes from the infant mice treated with amoxicillin, HAMLET, amoxicillin plus HAMLET, or PBS (control) were stimulated with killed *S. pneumoniae* for 72 h, and Th17 (IL-17A) and Th1 (IFN-γ) cytokine levels in the culture supernatants were measured by ELISA. Each experimental group had 11–12 mice. The data are represented as the mean ± SD of two independent experiments. The dots represent the data for each mouse, and the horizontal bars are the mean values for the groups. *** $p < 0.001$. ns = non-significant. One-way ANOVA and Tukey post hoc test.

2.2. Exposure of Infants to Amoxicillin Diminishes Th17, but Not Th1, Responses

We assessed the immune responses induced by the lung and splenic CD4+ and CD8+ T cells of infant mice that received antimicrobial treatment via the intranasal route. Flow

cytometric intracellular cytokine analysis demonstrated that lung CD4+, but not CD8+, T cells from amoxicillin- or HAMLET plus amoxicillin-exposed mice expressed lower levels of IL-17A than those from HAMLET alone-exposed or control mice (Figure 2 and Supplementary Figure S3). In addition, there was no difference between mouse groups treated with amoxicillin and amoxicillin plus HAMLET (Figure 2). On the other hand, splenic CD4+IL-17A+ and CD8+IL-17A+ T cells did not show a significant difference between the exposed and control groups (Figure 2). Furthermore, we investigated the effect of amoxicillin and/or HAMLET on T cell responses characterized by IFN-γ production in response to *S. pneumoniae* in vitro stimulation (Figure 3). IFN-γ levels were similar in splenic and lung CD4+ and CD8+ T cells from amoxicillin- or HAMLET plus amoxicillin-exposed mice (Figure 3). Overall, these findings show that amoxicillin treatment regimens are mainly responsible for suppressing lung Th17 immunity to *S. pneumoniae*.

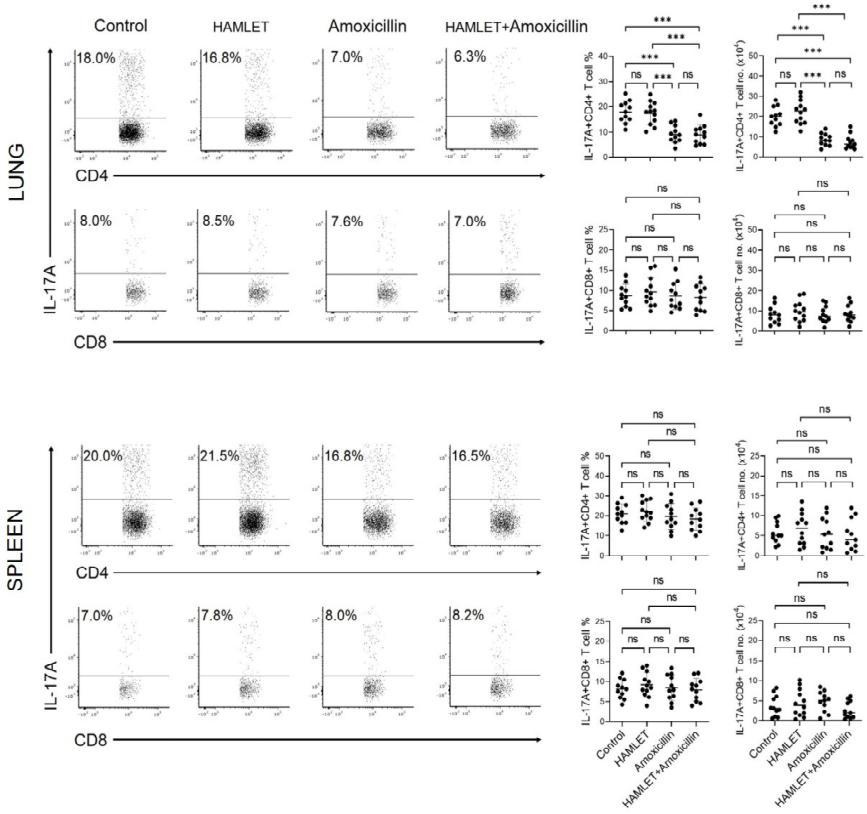

Figure 2. Production of IL-17A by infant CD4+ and CD8+ T cell subsets following stimulation with killed *S. pneumoniae*. The production of IL-17A by CD4+ and CD8+ T cells in the lungs and spleen was examined using flow cytometric intracellular cytokine analysis. Images of flow cytometric dot plots (**left**) and a summary of the percentages and numbers of IL-17A+ T cells (**right**). CD3+ cells were gated and presented as CD3+CD4+ and CD3+CD8+ T cells. In Supplementary Figure S1, the strategies for CD4+ and CD8+ T cell gating and intracellular cytokine expression analysis are shown. There were 11–12 infant mice in each experimental group. The information on the right graph is presented as the mean ± SD and represents two independent experiments. The horizontal bars show the mean values for the groups, and the dots show data from each individual mouse. *** $p < 0.001$. ns = non-significant. One-way ANOVA and Tukey post hoc test.

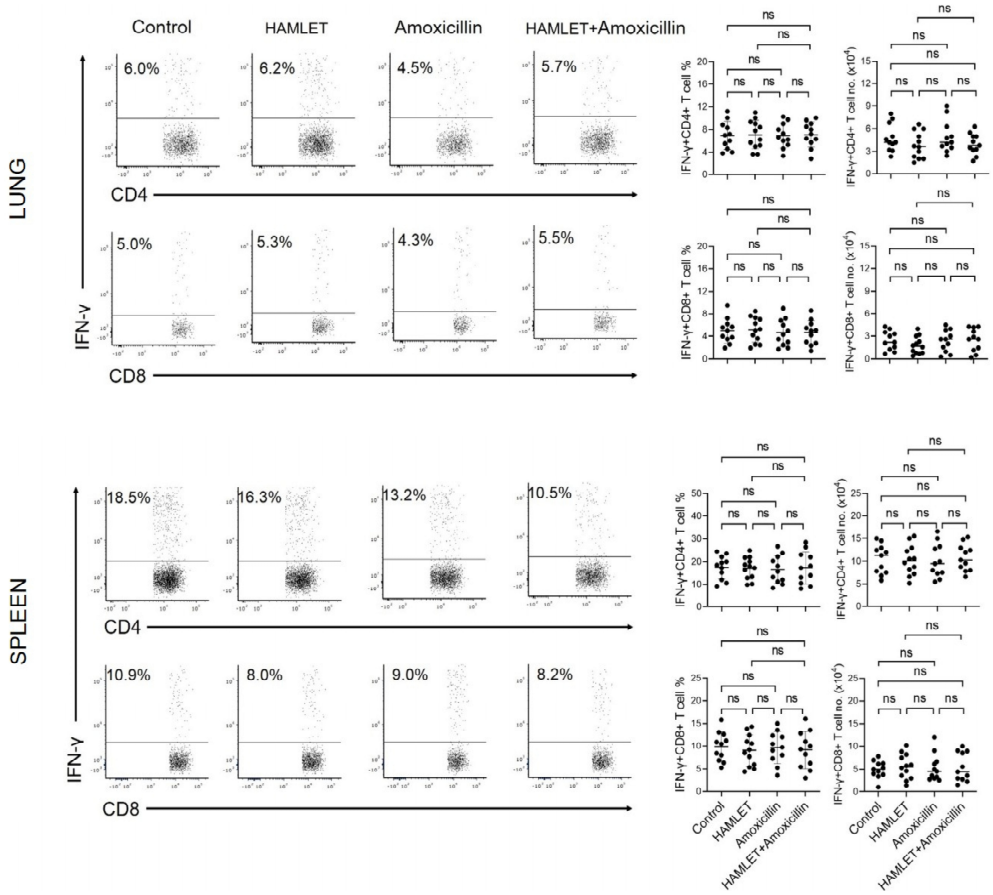

Figure 3. Th1 responses characterized by IFN-γ production after stimulation with killed *S. pneumoniae*. IFN-γ production by lung and splenic CD4+ and CD8+ T cells was analyzed by flow cytometric intracellular cytokine analysis. Images of flow cytometric dot plots (**left**) and a summary of the percentages and numbers of IFN-γ+ T cells (**right**). CD3+ cells were gated and presented as CD3+CD4+ and CD3+CD8+ T cells. In Supplementary Figure S1, specific strategies for CD4+ and CD8+ T cell gating and intracellular cytokine expression analysis are shown. There were 11–12 infant mice in each experimental group. The information on the right graph is presented as the mean ± SD and represents two independent experiments. The horizontal bars show the mean values for the groups, and the dots show data from each individual mouse. ns = non-significant. One-way ANOVA and Tukey post hoc test.

2.3. Amoxicillin Alone or with HAMLET Reduces the CD4+ T Cell Number

Neonatal exposure to antibiotics, including amoxicillin, has been shown to alter the number of T cells in the blood and the spleen [11,15]. We sought to assess whether the exposure to amoxicillin or HAMLET plus amoxicillin alters the number of T cell subsets in the spleen and lungs of infant mice. Following intranasal administration of mice with amoxicillin or HAMLET plus amoxicillin, we noticed a significant decline in the percentage and absolute number of CD4+, but not CD8+, T cells in the lungs compared to PBS-treated mice (Figure 4). Furthermore, there was no difference between groups treated with amoxicillin and amoxicillin plus HAMLET, suggesting that the suppressive effect on CD4+ T cell number was related mainly to amoxicillin (Figure 4). However, the number of CD4+ and CD8+ T cells in the spleen remained unaffected (Figure 4).

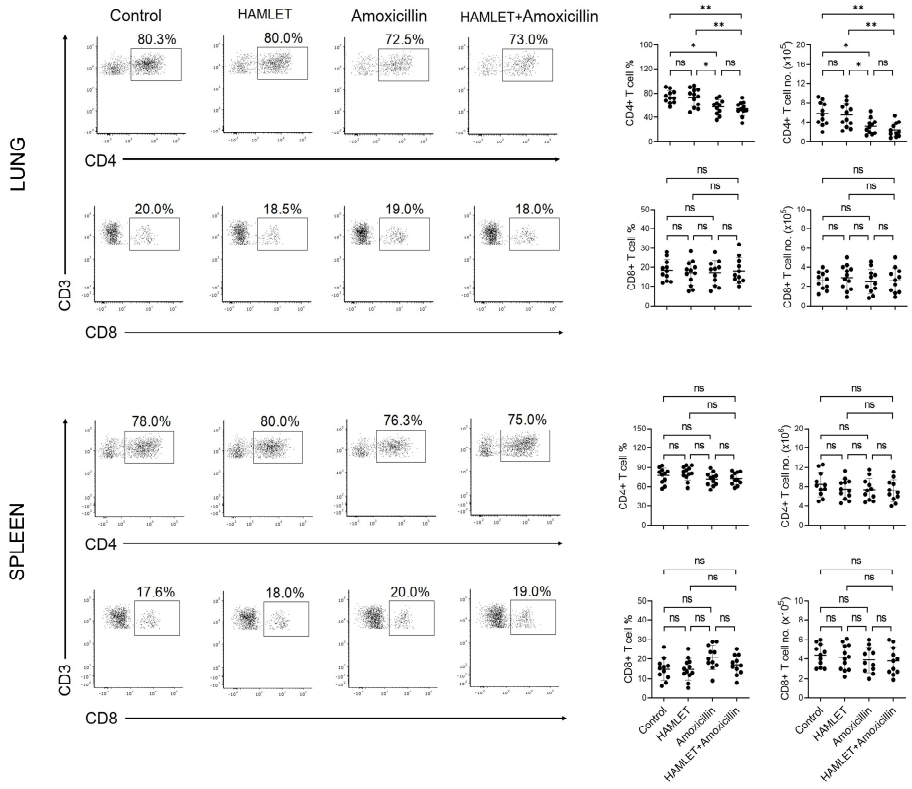

Figure 4. Effect of antimicrobial treatment on the percentage and number of T cells. Lung cells and splenocytes isolated from amoxicillin-, HAMLET-, or amoxicillin plus HAMLET-treated or control infant mice were stained with antibodies and analyzed by flow cytometry. The T cell subsets were gated and presented as described in Supplementary Figure S1. Representative flow cytometric dot plot images (**left**) and a summary of the percentages and numbers of CD4+ and CD8+ T cells (**right**). Each experimental group had 11–12 mice. The data, which are shown as mean ± SD, represent two independent experiments. The horizontal bars represent the mean values for the groups, and each dot symbol represents data from a single mouse. ns = non-significant. * $p < 0.05$; ** $p < 0.01$. One-way ANOVA and Tukey post hoc test.

3. Discussion

In this study, we focused on how amoxicillin treatment of infant mice alters peripheral (splenic) and local (lung) Th17/Th1 responses to *S. pneumoniae*. We also investigated whether HAMLET, alone or with amoxicillin, impacts Th17/Th1 antipneumococcal immunity. Our main findings were that: (1) amoxicillin treatment suppressed lung IL-17A/Th17 responses to *S. pneumoniae*, but not IFN-γ/Th1 responses; (2) HAMLET treatment had no significant effect on splenic and lung Th17/Th1 immunity; and (3) amoxicillin exposure resulted in decreased CD4+ T cell numbers in the lungs. Overall, these findings provide important information on the potential impact of amoxicillin and HAMLET on infant T cell immunity to *S. pneumoniae*.

Our finding that amoxicillin suppressed lung Th17 immunity to *S. pneumoniae* is consistent with our previous findings that therapeutic regimens containing piperacillin and the β-lactamase inhibitor tazobactam reduced the frequencies of neonatal splenic and lung CD4+IL-17A+ T cells in response to *S. pneumoniae* in vitro stimulation [12]. However, we did not find any impact of amoxicillin treatment on peripheral (splenic) CD4+IL-17A+ T cells, which could be due to different routes of antibiotic administration in these studies, as well as to responses specific to the antibiotic type used or the presence/absence of beta-lactamase inhibitor. While mice received amoxicillin intranasally in this study, piperacillin plus tazobactam was injected via the intraperitoneal route [13]. It is possible that the effect of intranasal amoxicillin treatment was mainly confined to the local respiratory

microbiota and T cell responses with a lower systemic exposure. Moreover, in line with the immunosuppressive role of amoxicillin, intramuscular injection of neonatal rats with meropenem and vancomycin resulted in diminished intestinal Th17 immunity to the fungus *Candida albicans* [28]. On the other hand, it remains unclear as to how antibiotic regimens alter infant T cell immunity to pathogens. Recent reports have shown that infant mice infected with vaccinia virus and exposed to antibiotics exhibited reduced frequencies of splenic DCs expressing $CD11c^{hi}MHC\text{-}II^{hi}$ [11]. The question of whether antibiotic exposure modulates DC function to generate neonatal and infant T cell immunity, including Th17, warrants further investigation. Overall, suppression of Th17 function by antibiotic regimens as shown in this study could have important implications because a Th17 response is critical to protection against neonatal and infant infections by extracellular bacterial and fungal pathogens, including *S. pneumoniae* [29].

In the present study, we chose the intranasal route of delivery for HAMLET and amoxicillin. This route was previously used to demonstrate the effect of HAMLET in combination with gentamicin in protecting mice from pneumococcal colonization [20]. The advantages of the intranasal route include ease of use and the potential to augment bioavailability and reduce adverse effects. Intranasal delivery of antibiotics faces, however, numerous challenges, particularly in relation to drug stability. Several lines of study are now being explored in an attempt to expand the range of antibiotics for intranasal delivery [30]. Amoxicillin intranasal delivery was primarily used in our study to reduce stress and disturbance in young pups by having an additional route of administration. While it showed an effect on immune responses, it is noteworthy that amoxicillin has not yet been developed for the prevention or treatment of human infections using the intranasal route.

HAMLET is not only bactericidal against certain pathogens, but also immunomodulatory [20,23]. In a mouse model of nasopharyngeal colonization with *S. pneumoniae*, intranasal administration of a combination of HAMLET and gentamicin, but not HAMLET alone, showed a significantly enhanced pneumococcal death in the nasal wash compared with the mice exposed to gentamicin alone. This indicates the ability of HAMLET to increase the efficacy of antibiotic activity in vivo [20]. In addition, using primary human immune cells in an in vitro setting, HAMLET was shown to possess immunomodulatory properties as exhibited by increased T cell proliferation by HAMLET-pulsed Mo-DCs [23]. In this study, we assessed, for the first time, the role of HAMLET in eliciting T cell immunity to *S. pneumoniae* using an infant mouse model. Our findings showed that antipneumococcal lung Th17 responses induced by intranasal HAMLET or PBS in infant mice were significantly higher than those induced by a combination of HAMLET and amoxicillin, and that there were no significant differences between Th17/Th1 responses in HAMLET- and PBS-treated mice. Thus, HAMLET exposure is neither suppressive nor stimulatory in generating Th17/Th1 responses to *S. pneumoniae* under the conditions used in this study. A positive inference drawn out of this finding is that HAMLET can potentially be used as a safe antimicrobial drug that does not suppress T cell immunity required for specific and long-lasting protection against pathogens. It is important to note that mouse infants were not weaned while receiving HAMLET or HAMLET plus amoxicillin, and it is possible that their dams' milk may have contained HAMLET-like antimicrobials. Future studies are required to explore whether HAMLET-like antimicrobials are present in murine milk.

Collectively, our study found that exposure of infant mice to amoxicillin impairs lung Th17 responses to *S. pneumoniae*. Considering an important role for Th17 immunity in contributing to pathogen defense and the protection mediated by vaccines [25,27,31–33], our findings that show amoxicillin-induced suppression of Th17 responses could have important implications for the development of better therapeutic and prophylactic strategies for neonates and infants. Although the use of amoxicillin in neonatal and infantile clinical settings is appreciated, it is worth keeping in mind that treatment with amoxicillin may have suppressive effects on immune function. Amoxicillin-induced Th17 suppression can: (1) raise susceptibility to bacterial, parasitic, and viral infections; (2) change

the clinical features of an infection; and (3) increase the likelihood that the live vaccine strain will develop virulence upon administration and reduce the efficacy of inactivated vaccines [34]. Additionally, unlike amoxicillin regimens, HAMLET alone did not suppress T cell immunity, accentuating its potential therapeutic role as a bactericidal without being immunosuppressive. However, caution should be taken while extrapolating our mouse data to human infants due to differences in their microbiota proportion and abundance. There are some limitations to this study. We did not evaluate the susceptibility to *S. pneumoniae* in vivo and did not explore the mechanisms of action by amoxicillin on impaired T cell responses. Future work is needed to investigate: (1) the long-term effects of amoxicillin and/or HAMLET treatment; (2) whether amoxicillin-induced Th17 immunosuppression can lead to increased susceptibility to pneumococcal infection; and (3) the underlying mechanisms by which amoxicillin exposure alters Th17 immunity.

4. Materials and Methods

4.1. Streptococcus pneumoniae

We used the *S. pneumoniae* TIGR4 strain throughout this study [35]. Pneumococcal cells were maintained in TSB (Beckton Dickinson, NJ, USA) and glycerol (15%), and kept at −80 °C. This stock culture was taken out, thawed, and grown at 37 °C to an optical density (OD) of 0.5 at 600 nm in a 5% CO_2 incubator. Harvesting of pneumococcal cells was done by centrifugating at 5000× *g* for 10 min at 4 °C and subsequent washing in endotoxin free Dulbecco's-PBS (Sigma-Aldrich, St. Louis, MO, USA). The pneumococcal suspension was UV-inactivated at the rate of 1200 J/m^2 UV radiation for 30 min, aliquoted, and frozen at −80 °C for further use. The pneumococcal colonies were confirmed to be dead by culture, with the probable limit of detection being less than one in one million pneumococci.

4.2. HAMLET Production

Human alpha-lactalbumin was enriched from human milk and was converted into HAMLET by complexing the apo-protein (treated with EDTA to remove its calcium ion) with oleic acid (C18:1; Sigma-Aldrich) on a DEAE-containing ion exchange matrix as described [17,36]. The HAMLET complex was eluted with salt and dialyzed with water to remove salt, and the desalted protein-lipid complex was lyophilized and saved at −20 °C until use.

4.3. Mice

Specific pathogen free (SPF) pregnant Swiss mice were purchased from the commercial animal supplier JANVIER LABS, France. The mice were kept at the animal facility at the Oslo University Hospital, Rikshospitalet, Norway. The pregnant mice delivered pups in IVC cages, and the newborn pups stayed with their dams. Each mouse litter of 11–12 infants was taken as an experimental group. To get rid of litter bias, we randomly mixed the newborn littermates across the four experimental groups in the first few days after birth. The 16–17-day-old mouse infants in different groups were intranasally administered with HAMLET (100 μg in 10 μL PBS per pup), amoxicillin (200 μg in 10 μL PBS per pup), HAMLET plus amoxicillin (100 μg HAMLET + 200 μg amoxicillin in 10 μL PBS per pup), or PBS (10 μL per pup) daily for 7 consecutive days. The HAMLET dosage was calculated as described previously [20]. The infant mice receiving HAMLET, amoxicillin, HAMLET plus amoxicillin, or PBS were euthanized using an intraperitoneal pentobarbital injection (dose rate of 0.05–0.5 mL per mouse) under isoflurane anesthesia (4–5%). Of note, mouse experimental protocols were approved by the Norwegian Food Safety Authority, Oslo, Norway (FOTS number 21062), and the experiments were conducted in line with the institutional guidelines.

4.4. Cell Isolation and Antigenic Stimulation

Spleens were mashed on a 70 μm cell strainer (ThermoFisher Scientific, Rockford, IL, USA) and washed with the washing buffer (PBS, 0.5% BSA and 5 mM EDTA). The splenic

cell suspension was lysed with red blood cell (RBC) lysis buffer and washed two times. On the other hand, lungs were digested in 10 mg/mL collagenase XI (Sigma-Aldrich, Israel) in RPMI 1640 supplemented with 10% heat-inactivated FBS and gentamicin (25 μg/mL) (Sigma-Aldrich, United Kingdom) for 1 h at 37 °C [37]. The lung cell suspension was treated with RBC lysis buffer (eBioscience, San Diego, CA, USA), and washed with a washing buffer containing PBS, 0.5% BSA, and 5 mM EDTA. Trypan blue was used to count live cells in the hemocytometer. In addition to evaluating cell viability by Trypan blue staining (90–96% viability), we used flow cytometric FSC versus SSC analysis to exclude debris and dead cells (Supplementary Figure S1), which have low forward scatter. 2.5×10^6 splenocytes in 500 μL or 5×10^5 lung cells in 200 μL of complete RPMI 1640 medium having 10% heat-inactivated FBS (Sigma-Aldrich, UK) were cultured at 37 °C. The cell culture was stimulated with UV-killed *S. pneumoniae* TIGR4 (10^5 CFU/mL) for 72 h. The culture supernatants were frozen at −80 °C, and the supernatant concentrations of IL-17A and IFN-γ were measured by Ready-SET-Go ELISA kits (eBioscience, San Diego, CA, USA) as per the manufacturer's instructions. The cytokine detection limit of the ELISA kit for IL-17 was 4 pg/mL, whereas the limit for IFN-γ was 15 pg/mL.

4.5. Flow Cytometric Analysis

To perform cell surface staining, lung and splenocytes were stained with anti-CD4-Phycoerythrine (PE), anti-CD8-Flurorescein isothiocyanate (FITC), and anti-CD3-PE-Cy7 (eBioscience, San Diego, CA, USA). The isotype controls of these fluorochrome-conjugated antibodies were also used. To perform intracellular cytokine staining by flow cytometry, 2.5×10^6 splenocytes in 500 μL or 5×10^5 lung cells in 200 μL of complete RPMI 1640 medium with 10% heat-inactivated FBS and gentamicin (25 μg/mL) (Sigma-Aldrich, UK) were cultured at 37 °C. The cell culture was stimulated with the UV-killed *S. pneumoniae* TIGR4 (10^5 CFU/mL) for 72 h. Following cell stimulation, splenic and lung cells were washed, cultured in the complete RPMI, and treated with a cell stimulation cocktail for 18 h (eBioscience, San Diego, CA, USA). Of note, the cell stimulation cocktail contains a mixture of phorbol 12-myristate 13-acetate (PMA), ionomycin, brefeldin A, and monensin. Brefeldin A and monensin result in the accumulation of secreted proteins in the endoplasmic reticulum and Golgi apparatus. The cells were then washed and incubated with FcR-blocking antibodies (anti 16/32; eBioscience) for 15 min. The cell surface markers (CD4, CD8, and CD3) were stained with anti-CD3-PECy7, anti-CD8-FITC, and anti-CD4-PE (eBioscience, San Diego, CA, USA). After washing, cells were treated with IC fixation buffer (Invitrogen, CA, USA), followed by permeabilization with permeabilization buffer (eBioscience, San Diego, CA, USA). Intracellular cytokine staining of cells was performed with anti-IL-17A–allophycocyanin (APC), anti-IFN-γ-APC, or isotype control antibodies (eBioscience, San Diego, CA, USA). At the end, cells were washed and resuspended in Dulbecco's PBS mixed with 0.5% BSA and 1mM EDTA. The samples were run on a BD LSR II flow cytometer (BD Biosciences, San Diego, CA, USA) to collect experimental data. The flow cytometry data analysis was performed by the FCS Express software (De Novo Software, Los Angeles, CA, USA).

4.6. Statistics

One-way ANOVA and Tukey post hoc test were used for comparing four experimental groups of infant mice using GraphPad Prism Software (version 9, San Diego, CA, USA). A p value of less than 0.05 was considered significant.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/antibiotics12020423/s1, Figure S1: Gating strategy for flow cytometric analysis. Figure S2. IL-17A and IFN-γ production by lung cells and splenocytes without pneumococcal stimulation in vitro. Figure S3. Production of IL-17A by CD4+ and CD8+ T cell subsets following stimulation with killed S. pneumoniae.

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Informed Consent Statement: Not applicable.

Data Availability Statement: We confirm that the data supporting the findings of this study are available within the manuscript and its Supplementary Figures S1–S3.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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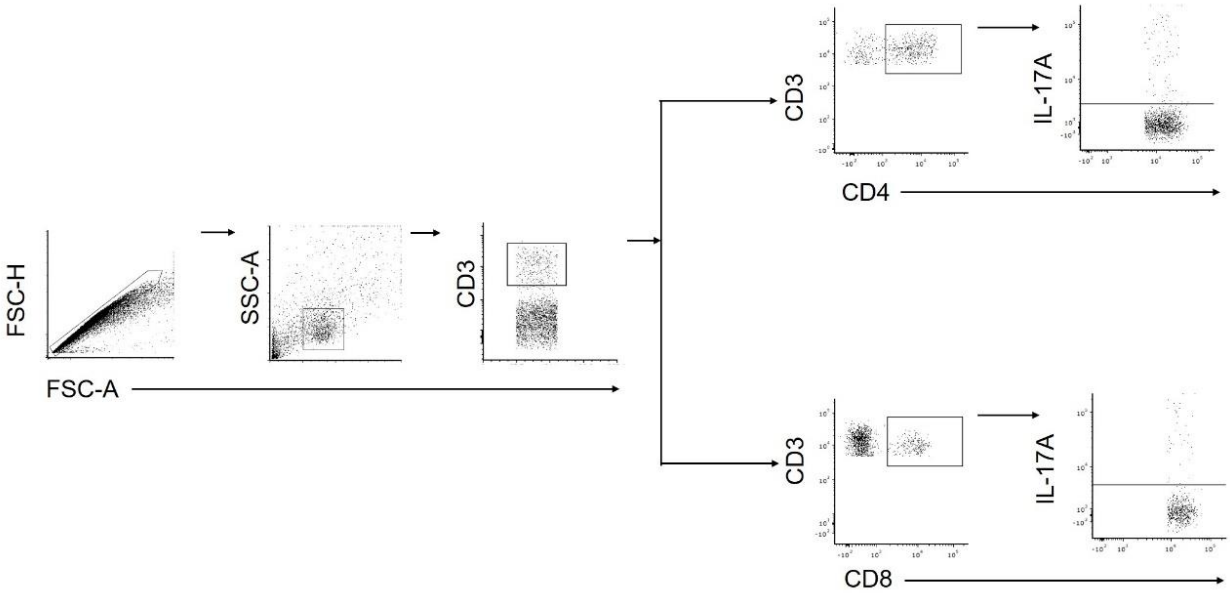
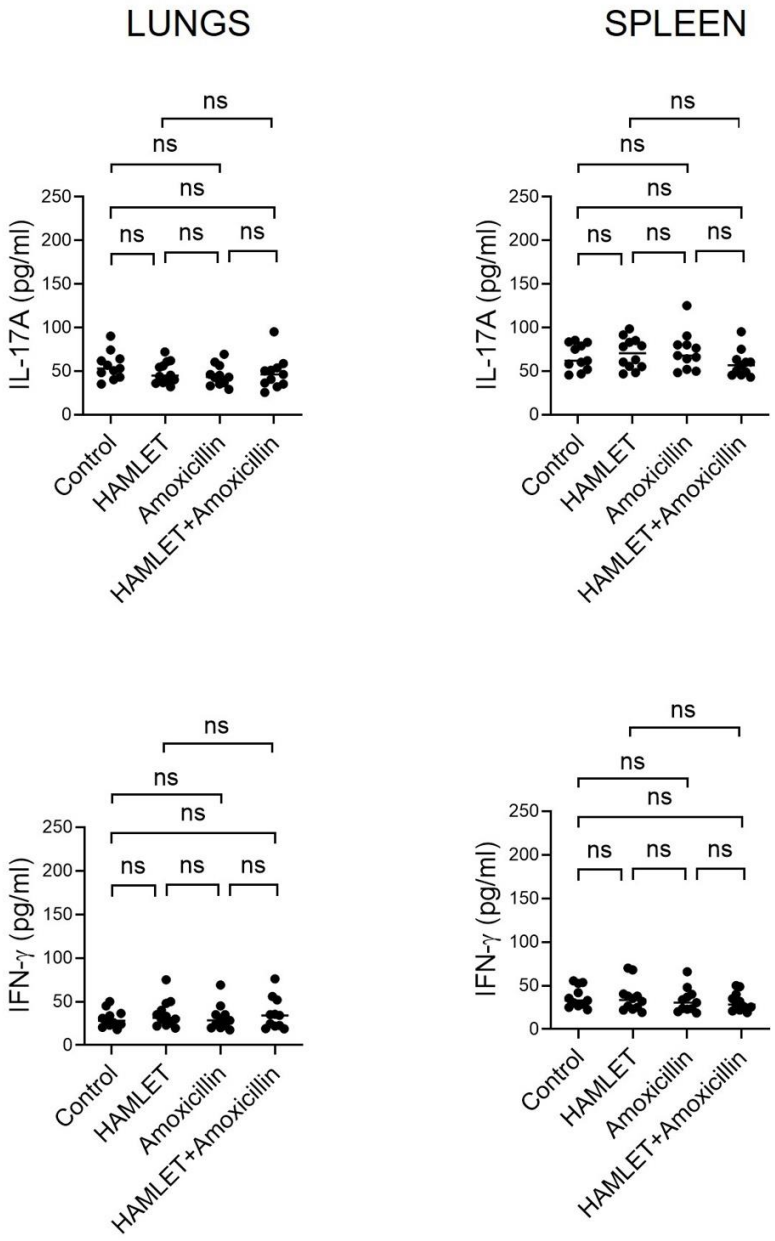
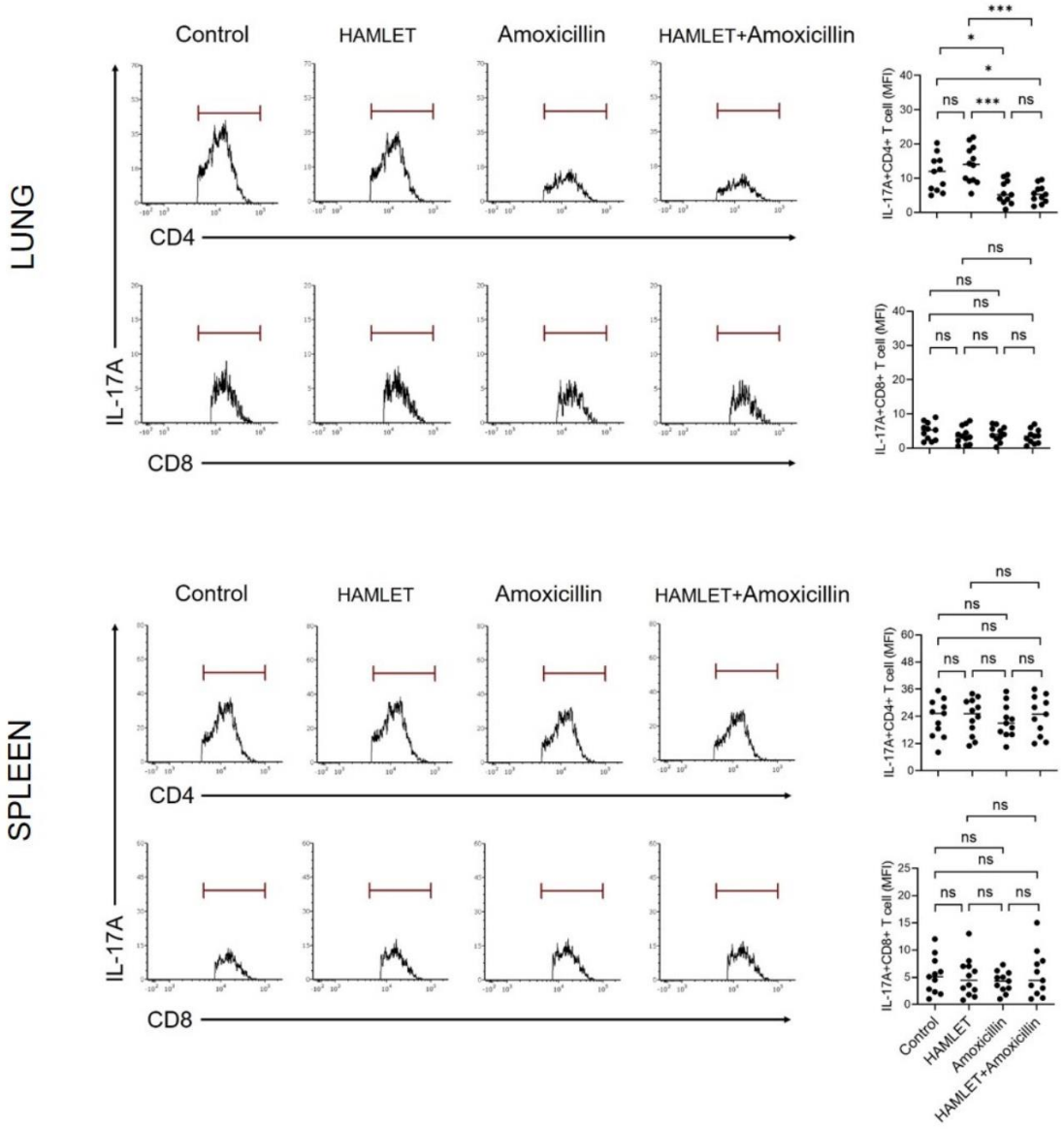


Supplementary Figure S1. Gating strategy for flow cytometric analysis. Lung cells isolated from the infant mice treated with amoxicillin were stimulated with UV-killed *S. pneumoniae* TIGR4, and stained with antibodies specific for CD3, CD4, CD8, IL-17A markers, and analyzed by flow cytometry. The cells were first plotted against FSC-H and FSCA to exclude the doublet cells and then plotted against SSC-A and FSC-A. Analysis was performed on gated CD3+ (T cells) lung cells based on a gate that mainly contains lymphocytes in the SSC-A versus FSC-A plot. CD3+ cells were displayed into CD4+ and CD8+ T cells, which were further shown to express IL-17A. Representative flow cytometric dot plot images are given.



Supplementary Figure S2. IL-17A and IFN-γ production by lung cells and splenocytes without pneumococcal stimulation *in vitro*. Lung cells and splenocytes from the infant mice treated with amoxicillin, HAMLET, amoxicillin plus HAMLET or PBS (control) were cultured for 72 hours without stimulation with the UV-killed *Streptococcus pneumoniae*, and Th17 (IL-17A) and Th1 (IFN-γ) cytokine levels in the culture supernatants were measured by ELISA. Each experimental group had 11-12 mice. The data represent the mean ± SD of two independent experiments. The dots represent the data for each mouse, and the horizontal bars are the mean values for the groups. ns = non-significant. One-way ANOVA and Tukey post hoc test.



Supplementary Figure S3. Production of IL-17A by CD4+ and CD8+ T cell subsets following stimulation with killed *S. pneumoniae*. The production of IL-17A by CD4+ and CD8+ T cells in the lungs and spleen was examined using flow cytometric intracellular cytokine analysis. Images of flow cytometric histograms (left) and a summary of the mean fluorescence intensity (MFI) of IL-17A expression in CD4+ and CD8+ T cells. CD3+ cells were gated and presented as CD3+CD4+ and CD3+CD8+ T cells as described in Supplementary Figure 1. There were 11–12 infant mice in each experimental group. The information on the right graph is presented as the mean ± SD and is a summary of the two separate experiments. The horizontal bars show the mean values for the groups, and the dots show data from each individual mouse. **P*<0.05; ****P*<0.001. ns = non-significant. One-way ANOVA and Tukey post hoc test.