ARCHAEA IN MAMMALIAN GUT MICROBIOMES

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Abstract: Archaea are the most enigmatic of the three domains of life. Most archaea are extremophiles found in highly acidic, high-salt, or high-temperature environments. However, members of the archaea also have been discovered in animal and human intestines, albeit their functional roles in host health or disease are poorly understood. Interestingly, archaea are now considered as indigenous microorganisms of the human gut microbiota and their biological importance has recently been reevaluated. Targeted sequencing of the 16S rRNA gene, metagenomic shotgun sequencing, or other metaomic approaches are used to define the composition, activity, and dynamics of the microbial communities. Archaea themselves are not known to be pathogenic; however, the presence of methanogens, a group of archaea in the human gut, has been linked to several digestive disorders such as IBD and IBS and metabolic diseases such as obesity and diabetes. Our study aimed to mine rat 16S rRNA gene amplicon and shotgun metagenomic sequencing datasets in our laboratory for the presence of archaeal sequence reads. Additionally, we investigated whether these sequences are present in 16S sequencing data from pig fecal DNA samples. Taxonomic profiling workflows in the Qiagen CLC Genomics Workbench were used to elucidate the relative abundances of archaeal and bacterial reads in the sequence datasets. Our results showed that archaeal sequences were either absent or present in extremely low abundance in the investigated rat metagenomes. In contrast, we found evidence that methanogens were abundant in sow and piglet fecal microbiotas. The correlation of sow and offspring archaea profiles was evaluated using longitudinal sequence data and quantitative PCR with group-specific primers. The genus *Methanobrevibacter* was dominant in sows and piglets, while *Methanosphaera* and *Methanomethylophilaceae* showed disparate abundances. Future studies will include investigation of archaeome inheritance and development as well as study of archaea-bacteria co-occurrence networks and correlations with host health status.

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CHAPTER I

INTRODUCTION

A diverse and healthy microbiome is essential to human and animal health. The existence of microbial life in a healthy human gut has been known for more than a century. Although the terms "microbiome" and "microbiota" are frequently used interchangeably, there are some distinctions between the two. The term “microbiota” is described as the collection of living microorganisms in a given population or habitat and includes bacteria, archaea, fungi, algae, and protists [1]. The majority of microbiome researchers concur with this definition [2]. As initially proposed by Whipps and colleagues the term "microbiome" [3], includes both the population of microorganisms and their "theatre of activity" [2]. This definition includes molecules produced by microorganisms, their structural components (nucleic acids, proteins, lipids, and polysaccharides), microbial metabolites (signaling molecules, toxins, organic, and inorganic molecules), and molecules created by coexisting hosts and shaped by the environment. Consequently, all mobile genetic components—including phages, viruses, plasmids, prions, virioids, and "relic DNA" (extracellular DNA obtained/procured from dead cells)—should be incorporated in the term “microbiome” rather than “microbiota.” The term “microbiome” is sometimes mixed up with “metagenome.” However, metagenome is explicitly defined as the collection of genomes and genes from members of a microbiota [1, 2]. In this study, the term "microbiota” is used when referring exclusively to microorganisms [4].

Mammals harbor communities of microbiota inside and on various parts of their bodies such as the oral cavity, respiratory tract, skin, gastrointestinal and urogenital tract [5]. Most members of the microbiota, primarily bacteria, reside in the intestine of animals including humans, where they make vitamins, protect against pathogens, boost the immune system, and ferment food [5]. The gut microbiota is crucial to human health, and dysbiosis of the microbiota may affect the onset of chronic illnesses such as colorectal cancer, gastrointestinal disorders, and metabolic diseases [6].

Bacteria are among the most studied microbes in the microbiota. The resident bacteria colonizing humans are essential for health, but the composition and characteristics of a healthy microbiota are still unclear. To maintain optimum health in populations that are typically healthy and improve the health status of individuals who have altered or aberrant microbiota (dysbiosis) it is important to better understand the characteristics of a healthy microbiota.

While bacteria are known to play important roles in human and animal health and disease, archaea are often overlooked. However, these microorganisms comprising the third domain of life as proposed by Woese and coauthors [7] have unique metabolic pathways and are being investigated for their potential use in biotechnology, medicine, and environmental remediation. Understanding the diversity and functions of both bacteria and archaea is crucial for advancing our knowledge of microbiology. Archaea are prokaryotic microorganisms found ubiquitously on earth, especially in extreme environments [8]. Initially thought to be ancient extremophiles, archaea also were discovered in moderate environments such as animal and human intestinal tracts [9]. Although archaea have been extensively studied in different environmental habitats, they have been largely neglected in microbiome studies, and therefore very little is known about the impact of the human archaeome on mammalian health [8].

Archaea are now considered as indigenous microorganisms of the human gut microbiota and their biological importance has recently been reevaluated [10]. The archaea found in human and

animal gastrointestinal tracts are comprised primarily of methanogenic archaea and halophilic (salt loving) methanogens. Two species of methanogens, *Methanobrevibacter smithii* and *Methanosphaera stadtmanae*, were identified in breath methane, isolated with culture-based approaches, and quantified by qPCR-based quantification method [10]. Haloarchaea were first found in fecal samples of a Korean cohort [9]. Recently other haloarchaeal strains such as *Haloferax massiliensis* and *Halorubrum lipolyticum* were also isolated from human feces [9].

Archaea themselves are not known to be pathogenic. Because archaeal pathogens have not been identified yet, it is assumed that they do not exist. The presence of methanogens in the human gut has been linked to several digestive disorders such as IBD and IBS and metabolic diseases such as obesity and diabetes. However, their involvement with IBD pathogenesis and other diseases is not fully understood.

Methane-producing archaea in the gut break down bacterial end products of fermentation such as hydrogen, carbon dioxide, formate, acetate, methanol, and perhaps ethanol as well as methyl compounds to generate gaseous methane, which contributes to global warming and digestive energy loss in animals [11,12]. Methanogenic archaea in the gut of monogastric (single chambered stomach) animals such as pigs and humans are thought to be linked to energy metabolism and adipose tissue deposition [12,13]. Pigs with a lean phenotype have higher methanogen abundances and more diversity, and higher abundance of *Methanosphaera spp*. and *Methanobrevibacter smithii* has been shown to reduce body fat accumulation in pigs [14]. Methane production from pigs is significant because it contributes to greenhouse gas emissions, which can have a negative impact on the environment. Reducing methane emissions from pig farming can help mitigate climate change.

Microbial communities are vital to human health. Targeted sequencing of the 16S rRNA gene, metagenomic shotgun sequencing, or other metaomic approaches are frequently used in

microbiome studies to define the composition, activity, and dynamics of the microbial communities [15].

The purpose of my research was to detect and identify archaea in mammalian gut microbiomes. The aim of the study was to analyze available 16S rRNA gene sequencing and metagenomic data and perform qPCR using archaea-specific primers, validate the results, and compare the results with next generation sequencing (NGS) data. For the present study, we analyzed the 16S rRNA sequencing and metagenomic data in rats, and 16S rRNA gene sequencing data in pig samples to detect the presence of methanogenic archaea. Then the NGS data was used to compare the qPCR data to confirm for presence of methanogens in rat small intestine and colon samples and pig fecal samples.

CHAPTER II

REVIEW OF LITERATURE

Brief History of Microbiome Research

The existence of microbial life in a healthy human gut has been known for more than a century. The term "microbiota," which first appeared in the early 1900s, refers to the microorganisms that inhabit different sites in or on the body, such as the skin, lung, oral cavity, and vagina in females [16, 17]. However, microbiota is predominantly found in the animal or human gut. These microorganisms include a wide variety of bacteria, archaea, viruses, fungi, and protozoans. The human microbiome contributes more genetic information than the human genome by a factor of over 150 [16, 18]. The term 'microbiome' was first used by American Nobel laureate Joshua Lederberg in 2001, but the term has been well documented since the 1960s. John M. Whipps and colleagues coined the term in the late 1980s by combining the two words 'micro' and 'biome'. He described the microbiome as a "characteristic microbial community" in a "reasonably well-defined habitat" that had distinct physio-chemical properties" as their "theatre of activity" [2, 19, 20]. It was previously suggested that the word "microbiota" refers to the taxonomic collection of microbial populations and that the term "microbiome" exclusively refers to the metagenome of the microorganisms [20, 21].

Today however, the term “microbiota” is described as the collection of living microorganisms in a given population or habitat [1]. The microbiota is made up of all the living organisms that make up the microbiome. Members of the microbiota should include bacteria, archaea, fungi, algae, and protists [1]. The majority of microbiome researchers concur with this definition. However, the most controversial aspect was whether to include phages, viruses, plasmids, prions, virioids, and free DNA in the definition of microbiome as they are considered non-living microorganisms [22] and do not belong to the microbiota [2].

As initially proposed by Whipps and colleagues the term "microbiome" [3], includes both the population of microorganisms and their "theatre of activity." This includes molecules produced by microorganisms such as nucleic acids, proteins, lipids, and polysaccharides, microbial metabolites (signaling molecules, toxins, organic, and inorganic molecules), and molecules created by coexisting hosts and shaped by the environment. Consequently, all mobile genetic components—including phages, viruses, "relic DNA" (extracellular DNA obtained/procured from dead cells)—should be incorporated in the term “microbiome” rather than “microbiota”. Sometimes, the terms "microbiota" and "microbiome" are used interchangeably, yet the microbiome is more comprehensive than the microbiota. The term “microbiome” is sometimes mixed up with “metagenome”. However, metagenome is explicitly defined as a collection of genomes and genes from members of a microbiota [1, 2] and a “genome” is defined as an individual organism’s genetic makeup. In this study, the term "microbiota” is used when referring exclusively to microorganisms [4].

Overview of the Microbiome in Health and Disease

Humans

Microbes are ubiquitous in nature, inhabiting all potential environments, and are crucial to human survival. Despite being mostly invisible, microbes are pivotal to the health of ecosystems as they

control vital processes like plant growth, soil nutrient cycling, and marine biogeochemical cycling [23-26]. The human microbiota is made up of many symbiotic, pathogenic, and commensal bacteria that have colonized the human body. Numerous elements of human health are known to be influenced by interactions between the human body and gut flora [27].

The role of the microbiome in human health and disease is still unclear and not fully understood. In humans, the composition of microbiota varies from one site to another due to differences in environmental conditions and host factors, such as diet and genetics. Understanding these variations can help in developing targeted microbiome-based therapies for various diseases. The majority of microbiota, mostly bacteria, reside in the animal or human gut, performing functions such as vitamin generation, pathogen defense, immune response stimulation, and food fermentation [28]. Human disease state and wellness are directly related to microbiome diversity and microbial balance in the gut. As more research indicates the involvement of microbes in disease development, studies are expanding beyond the focus of the microbiome's composition and focusing on establishing the cause of microbiota functions with the help of new high throughput sequencing techniques. While bacteria have been studied extensively, other components of the microbiome, such as archaea, fungi, and virus, need more comprehensive understanding of the role of microbiome in disease development. More research is needed to determine how the human microbiota functions and how the development of microbiome-based therapeutic treatments can be promoted [29].

Several studies have revealed the connection between the microbiome and illnesses like cancer, diabetes, and neurological conditions [16]. Hou et al. reported that the microbial populations generally work in harmony with the host to maintain homeostasis and control immune response, but microbial dysbiosis can lead to dysregulation of physiological functions [16, 30] and various disease conditions such as metabolic diseases [31-34], cardiovascular diseases (CVDs) [16, 30], neurological disorders [35, 36], and cancers [34, 37]. In one study, researchers studied microbiota

in healthy individuals. They looked at how disease developed, progressed, and caused pathogenicity due to microbial dysbiosis and concluded that disease development and progression was linked to the dysregulation of microbial composition, modulation of host immune response, and generation of chronic inflammation. To treat diseases, researchers are exploring the use of fecal microbial transplantation and microbiota manipulation such as use of probiotics and prebiotics to treat diseases. Since the study was conducted in mice additional research is needed to translate the results to humans [29].

Animals

Animals' microbiomes, or the microbial communities that live inside and on animals, play crucial roles in the health of their hosts, much like in humans. Microbes may positively impact animals by regulating processes such as digestion, reproduction, infection resistance, and other activities, allowing animal host to adapt and maintain their fitness. Microorganisms can also negatively impact animal health by causing infections and spreading diseases. Research suggests that microbial communities residing in animal's gut or skin may provide useful and insightful information and suggest conservation practices for animals. Although microbiome analyses have the potential to be useful in the field of conservation, there are problems scientists must overcome such as sample collection, data creation, and interpretation to make microbiome analysis more useful in conservation [38].

Protection of Animal Health using Microbiome in Captivity

Researchers are attempting to understand how microbiome analysis might be used in conservation. Some efforts have been made to protect animals in the wild by using strategies based on their microbiomes. However, most of the work done so far has been done on changing the microbiomes of animals that are born or brought into captivity. Many animals do not breed well under human care, and it is not clear why, but it is possible that microbiomes might have

something to do with it. In fact, the microbiomes of captive animals are different from those of wild animals because of factors such as diet, living conditions/habitat, and exposure to humans which are different from those in the wild. These factors can affect the composition and function of microbiomes in captive animals and the animals in the wild [38]. A multidisciplinary approach is important to understand how these microbes' function within communities. This will help to figure out if certain microbes are advantageous or detrimental for their hosts. This can be achieved with metabolomics. In the future, scientists will need to "figure out how to link the culturing and sequencing to move forward" [38].

Methods to Study Microbiomes

Culture-Based Methods

Before NGS became available, early studies on human microbiome were limited to light microscopy and culture-based techniques. One of the advantages of culture-based techniques is that they allow for the isolation and identification of specific microorganisms, that can be useful in understanding their characteristics and behaviors. One of the disadvantages of culture-dependent technique is that not all microbes could be cultured. Despite the fact that culture-independent genetic techniques have allowed us to get insight into their possible roles, culture-based approaches are still necessary to comprehend their distinctive characteristics and phenotypes [39].

Bioinformatics has allowed scientists to analyze and assess whole genome sequences from gut bacteria from metagenomic data. As a result, we have learned more about their habitat, the role they play in their environment, nutrient requirements, and potential roles in maintaining our health [40]. Thus, the importance of microbial culture techniques may seem to have decreased in recent years. However, there are limitations to what can be learned from molecular approaches alone. Pure intestinal microbiota cultures are still necessary to understand the functions of

particular bacteria in the intestine. Moreover, use of selective medium allows less abundant bacteria to grow which may go undetected in culture-independent studies due to low sequencing depth. [41]. Recently, several methods have been established to cultivate bacteria that were previously uncultivable [39]. The limitation of culturing technique may be overcome by the development of NGS technologies [27].

Non-Culture-Based Methods

NGS

Next-generation sequencing (NGS), sometimes referred to "massively parallel sequencing," is a DNA sequencing technique that offers high through put and speed to determine the order of nucleotides in entire genomes or targeted regions of DNA or RNA and is used in clinical and translational research. Millions to billions of DNA nucleotides can be sequenced at the same time, which gives a much higher throughput and reduces the need for the fragment-cloning methods that were used with Sanger sequencing [42]. Because the NGS platforms can sequence such a large amount of DNA/RNA fragments simultaneously, they need large data storage capability to analyze high data loads and they also need sophisticated bioinformatics systems and fast data processing infrastructure. With second-generation sequencing methods, amplified sequencing libraries must be prepared first before the amplified DNA clones [43] can be sequenced. However, third-generation single molecular sequencing can be done without the preparation of expensive amplification libraries and also saves time.

Improvements in NGS technology and bioinformatics tools have made it possible for research groups to build de novo draft genome sequences for any organism of interest. NGS produces millions of short reads of nucleotides in parallel in a short time than the Sanger sequencing method. The read types made by NGS are digital, so direct quantitative comparisons are possible. At fragment ends, you can get either a single end read, or a pair end read [44].

NGS technology can make specific sequencing biases and errors and these errors must be found and fixed. Most of the major sequencing mistakes are caused by high-frequency indel polymorphisms, homo polymeric regions, GC- and AT-rich regions, replicate bias, and substitution errors [45-47]. Obtaining adequate sequence depth and coverage for statistical certainty is a crucial factor in next-generation sequencing research that aims to provide high-quality, unbiased, and interpretable data. For appropriate sequence depth and coverage to be produced, a sequence library must be properly prepared. Depending on the NGS applications, a variety of alternative library approaches are available to reach this goal [44].

One of the biggest disadvantages of NGS is that it cannot distinguish between living and nonliving microorganisms. This limitation can lead to the detection of nonviable or dead organisms, which can lead to false positives and incorrect results, and in some applications, it can also be misleading. However, researchers are continually working on developing new techniques to improve the accuracy of NGS and address this limitation.

Characterization of Microbiome using 16S rRNA Gene Sequencing and Shotgun Metagenomic Sequencing.

16S rRNA gene amplicon based NGS sequencing is a preferred method of choice in microbiome research. Microorganisms can be reliably and accurately detected using metagenomic sequencing approach [48]. Although shotgun metagenomics and 16S both provide functional profiling of the microbiome, shotgun metagenomics provides a more accurate evaluation, given that there are sufficient sequences available. Ideally, shotgun metagenomics should also be combined with meta transcriptomics analysis [49]. One of the most important differences between the two techniques is that 16S sequencing examines only 16S rRNA genes and therefore is helpful in detecting bacteria and archaea while Shotgun sequencing examines all metagenomic DNA from all microorganisms in a given population.

16S rRNA Gene Amplicon Sequencing Approach

The 16S rRNA gene is a conserved region found in bacteria and archaea. The 16S rRNA gene sequencing is a targeted approach and still one of the preferred methods to explore microbial communities as it is relatively inexpensive and is computationally less challenging than shotgun metagenomics [48]. The 16S rRNA sequencing technique is applicable to samples contaminated by host DNA and without host-DNA interference. The16S sequencing can be used for analysis of large numbers of samples but gives limited taxonomical resolution, commonly not beyond genus level. The 16S rRNA gene marker only allows for detection of bacteria and archaea [48]. Functional profiling may be possible with the 16S technique using PICRUSt: Phylogenetic Investigation of Communities by Reconstruction of Unobserved States, but false positives can be found in low-biomass samples due to PCR biases and possible DNA contamination; therefore, it is important to carefully assess the quality of the samples, evaluate the results, and consider using alternative methods such as shotgun metagenomics for more accurate functional analysis. The 16S r RNA sequencing technique provides information on microbiota only and not the genomes. It does not explain the role bacteria and archaea within their environment [48].

Shotgun Metagenomic Sequencing Approach.

Shotgun metagenomics approach is expensive and time consuming but relatively more reliable than 16S in that it allows for the identification of all genetic material present in a sample, including non-bacterial organisms (archaea, viruses, fungi, protists, and DNA viruses), and provides more detailed information on the functional potential of the microbiome. However, it requires more computational power and expertise to analyze the large amount of data generated. Additionally, shotgun sequencing allows for the identification of unknown, uncultured microbial genomes within microbial communities. Metagenomic data can be used to analyze, evaluate, and assess the composition, diversity, and functional capacities of microbiomes [49-54]. Taxonomic

profiling up to species or even strain level is possible with shotgun sequencing. Shotgun sequencing makes it possible to generate metagenome-assembled genomes MAG's [55] from samples. MAGs are useful to study non-culturable and novel or unknown microbes in metagenomic data [55].

Mammalian Gut Microbiomes

Mammalian guts are home to a gastrointestinal microbiome composed of bacteria, archaea, fungi, protozoa, and viruses. Human and other mammalian studies have linked the microbiome to a variety of physiologic processes critical to host health, such as energy balance, metabolism, gut epithelium health, immunologic activity, and neurobehavioral development. Changes in the gut microbiome have been linked to diseases such as inflammatory bowel disease, asthma, obesity, metabolic syndrome, cardiovascular disease, immune-mediated ailments, and neurodevelopmental disorders. The field of Microbiomics aims to identify components of the microbiome, allows us to study the microbial genome, analyze interactions between the microbiome and host, and determine the disease mechanisms. Currently, there is limited information on the importance of intestinal microbiome in animals, and we need a thorough review of the human intestinal microbiota to be able to study the gut microbiota in animals [56].

Formation of the Mammalian Gut Microbiota

All mammals are thought to be sterile before birth and colonization of microbes occurs during birth. There is some evidence to support the theory that vertical transfer of microbes may occur before birth, but more research is needed to verify these claims. Human neonatal research reveals that infants get their intestinal microbes in the first few hours and days of life from their mother and environment. Infants born vaginally get colonized with *Lactobacillus spp*. and *Bifidobacterium sp*. [57]. Infants delivered through cesarean section get colonized by skin microbes such as *Staphylococcus* and *Ruminococcaceae* [57]. An experimental study done on

babies born via C section reports that when exposed to mother's vaginal secretions at birth, the infants partially restored the microbiome like seen during vaginal birth. These findings indicated that the gut, oral, and skin microbiomes in C section born babies showed increased bacterial populations similar to vaginally born neonates [56,58].

Maternal gut microbiome composition changes from first to third trimester, with increased abundance of *Proteobacteria* and *Actinobacteria* and decrease in *Fecalibacterium* in the third trimester. Surprisingly, newborns do not inherit these microbial markers from their mothers. Instead, the infant gut microbiome resembles that of its mothers in the first trimester. Research done in human newborns indicates that the mode of delivery, antibiotics, and nutrition all altered the rate of microbial colonization, but not the pattern of development. Infant gut microbes carry genes capable of metabolizing sugars found in milk and are responsible for de novo production of folate [59], which prepares the neonatal gut to receive plant derived nutrients. This finding indicates how the microbiota affects the host's developmental course [56].

Role of Gut Microbiota in Health and Disease

Human disease state and wellness are directly related to the microbiome diversity and microbial balance in the gut. The gut microbe helps to breakdown and ferment dietary fiber in colon. Microbes produce short chain fatty acids (SFCA). The three main types of short chain fatty acids produced are acetate, propionate, and butyrate. Butyrate is the main source of energy for human colon cells and can cause apoptosis in colon cancer cells. Acetate is the most abundant SCFA and an essential metabolite for the growth of other bacteria. Acetate may play a role in regulating the appetite [60]. Propionate is transported to the liver, where it interacts with the gut fatty acid receptors to control gluconeogenesis and appetite signaling [61]. The gut bacteria perform several functions such as synthesize vitamin generation (vitamin B12, folate, vitamin K, riboflavin, biotin), that the host cannot produce [62], pathogen defense, immune response stimulation, and

food fermentation [28]. Obesity and type 2 diabetes are correlated with changes in the co-metabolism of bile acids, branched fatty acids, choline, vitamins such as niacin, purines, and phenolic substances [63, 64]. The correlation between lower bacterial diversity and disease suggests that the species rich gastrointestinal ecosystem is more resistant to environmental changes. This is because functionally related microbes in a healthy ecosystem can take over for missing species. So, in general diversity is a good indicator of "healthy gut" [65, 66]. (Consequently, higher microbial diversity generally indicates a healthy gut [65, 66]. However, recent interventional studies suggest that increased dietary fiber can temporarily reduce diversity as the microbes that digest fiber become specifically enriched, leading to a change in composition and reduced diversity [61, 67].

Factors Influencing Gut Microbiota in Animals and Humans.

-Antibiotics

Medical interventions such as antibiotics can influence the stability of the gut microbiome in animals and humans. Ciprofloxacin, for example, has a significant impact on the human fecal microbiota, decreasing the taxonomic diversity and richness [56, 68, 69]. After the medicine is stopped, the microbiome partially returns to its pre-antibiotic state which means that the microbiome diversity and richness is partially recovered. However, this stable state is different from the pre-antibiotic state [56].

-Birth Mode

In mammals and animals, colonization of the microbes happens at birth. Mode of delivery, vaginal or cesarean section (CS) and diet provided during infancy significantly influence the gut flora. A study on pigs' microbial composition of umbilical cord found that vertical transfer is possible during gestation, but there is no evidence to support that concept. Furthermore, pigs

delivered vaginally had a higher bacterial abundance and a higher concentration of SCFAs such as acetate, propionate, and butyrate than pigs born through CS [70].

-Diet and Exercise

Lkhagva et al. compared the effects of dietary shift in carnivore diet versus vegetarian diet and effects of physical exercise on the composition of gut microbiome, after 3 months. The results indicated that both dietary shift and exercise affected the composition of the gut microbiome but in different ways [71]. Even though host factors play a major role in defining the composition of the gut microbiome, diet is still considered a primary determining factor of the gut microbiome. [71-73]. In summary, both the host and nutrition play a role in determining the composition of the gut microbiome, but we don't know which one plays a more important role.

-Age

The gut microbiome changes with aging, which is characterized by a gradual loss of homeostasis, reduced function, and vulnerability to death. The microbiome changes associated with aging are impacted by personal and environmental factors, such as diet and social interactions. Microbiome-based therapies supporting healthy aging in older adults are still being developed, but postbiotic interventions such as heat-killed Lactobacilli and butyrate have shown some promise. There are reasons to be hopeful about microbiome-targeted interventions, especially when it comes to unhealthy aging [74].

-Genetics

The influence of host genetics on the composition of the gut microbiome is unknown. According to several 16S rDNA sequencing data, the gut microbiome of related humans is more similar in composition than that of unrelated people, and there may be some host genes contributing to the inheritance of gut microbiome [31, 75, 76]. However, studies on several hundred humans,

including mono and dizygotic twins reported that the gut microbiome of related individuals was not significantly different than unrelated people living in the same cultural and geographic region [59]. This and other studies show that the composition and genetic traits of the gut microbiome may vary between individuals from different regions [28, 59].

-Household Cohabitation

The host habits and lifestyle (increased frailty, medication intake, surgery, decreased physical activity, and diet quality) can worsen the effects on the gut microbiome. Even though the spread of pathogens within social groups has been closely studied, strain tracking studies have shown that commensals and mutualistic microorganisms are also shared within social networks [77]. People living in the same house tend to have similar composition of microbiomes compared to people living in other households [78, 79]. House pets can also help share microbes by acting as vectors of transmission [74, 80].

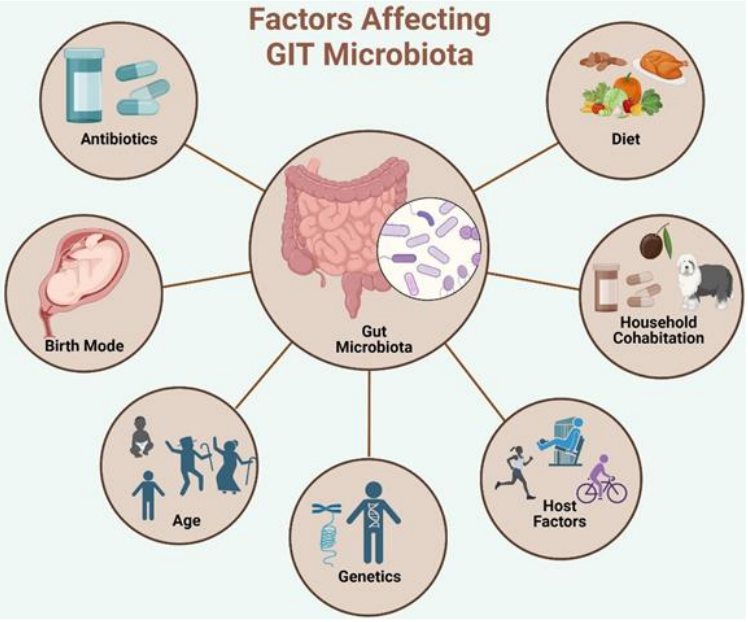


Fig.2.1 Factors Affecting GIT Microbiota. Factors influencing gut microbiota in animals and humans include diet, age, genetics, birth mode, host factors, environmental factors, and medication use. These factors can impact the composition and diversity of gut microbiota, significantly affecting overall health .and disease susceptibility.

Image created by BIORender.com.

Effects of Captivity on Mammalian Gut Microbiome

Mammalian gut microbiome performs a variety of functions such as digestion of food and signaling of the host immune system. Both host genetics and nutrition influence the composition and function of mammalian gut microbiome. Additionally, alteration in the host's living environment can significantly change the microbiome. Animals in man-made captive habitats experience different living conditions than the wild animals, and changes in gut microbiomes may be linked to distinct lifestyles, such as those of animals in captivity or in the wild [81]. Animals in captivity may experience changes that affect their microbiome, such as having limited access to a diverse diet, being exposed to antibiotics and other veterinary treatments (maintain animal health in captivity), having limited access to different habitats, fewer interactions with other animals or species, and having more access to human microbes. All these factors may impact the microbiome in animals [82].

Previous studies on rodents and primate gut bacterial communities [83, 84] suggested that bacterial diversity was greatly reduced in animals kept in the captive state as compared to the wild state. However, a decrease in gut bacterial diversity was not observed in all mammals. Therefore, we can conclude that not all mammals will show a similar pattern. Host genetics may be a deciding factor in determining whether an animal will show altered gut bacteria associated with captivity [82].

Gastrointestinal Microbe-Host Interaction Influencing the Gut Microbiome.

The microbiome is involved in several essential physiological and immune processes, such as energy homeostasis and metabolism, production of vitamins and other nutrients, endocrine signaling, prevention of enteropathogen colonization, regulation of immune function, and the metabolism of xenobiotic compounds [85]. Abnormal gut microbial communities have been linked to several digestive and systemic diseases, and research suggests that it plays a direct role

in the pathogenesis of diseases through intricate interactions with the host's metabolic and immunological systems. The host-cell-microbe interaction has an intricate structure, and the interacting cells vary greatly [56]. Recent metabolomic studies have shown that the microbiome is a key part of the host's systemic metabolism. Some gut bacteria, like Faecalibacterium prausnitzii, Roseburia spp., and Butyricicoccus pullicaecorum, produce short chain fatty acids, like butyrate, acetate, and propionate, by fermenting dietary fibers, resistant starches, and undigested proteins in the absence of oxygen [86]. These SCFA can help the host in many ways, especially butyrate, keeping people from becoming obese, while the health benefits of phytochemicals could be due to improved butyrogenic microbes in the intestine [87]. Short-chain fatty acids (SCFAs) such as acetate, propionate, and butyrate, produced by microbial fermentation of complex carbohydrates help regulate intestinal motility, create tight junctions, maintain the epithelial barrier, and synthesize anti-inflammatory chemicals [88]. Decreased SCFA synthesis, toxic microbial metabolite production, mucosal barrier damage, and microbe-mediated host immune dysregulation all contribute to a new long-term pro-inflammatory state [89, 90]. The diversity of gut microbiome is a major driver of host health, with clear links between the GI microbiome and overall health in humans and other mammals [56, 91, 92].

Archaea in Mammalian Gut Microbiome

Human Archaeome

The human microbiome is known to be made up of bacteria, archaea, viruses, and eukaryotes. Most microbiome studies focus on bacteria, but in recent years, there has been an increase in the number of human microbiome studies on eukaryotes (like fungi) and viruses [93-95]. However, the human Archaeome is still largely ignored in microbiome research despite the fact that methanogenic archaea are known to be commensals of the human gut and influence human health and wellbeing [96, 97, 107]. Most archaea are extremophiles and are known to survive in extreme

environments. Interestingly, archaea are now known to inhabit animal and human intestines where they constitute a significant part of the gut microbiota [98, 99]. Some of the reasons why archaea are under-represented in microbiome studies include primer mismatches of the "universal primers" [99] low abundance of archaeal DNA in samples [100] improper DNA extraction methods [101] and incompleteness of the 16S rRNA gene [100, 102]. Additionally, there is no clinical interest in archaea because no pathogens from archaea have been found [48].

Non-methanogens such as *Desulfurococcales*, *Sulfolobales*, *Thermoproteales*, *Nitrososphaerales*, and *Halobacteriales* have been found in the human intestine [10]. *Haloferax massiliensis* [103] a new species of halophilic archaea, was recently isolated from the human gut, raising the possibility that they may be permanent inhabitants [104]. Archaea are also found in the vaginal cavity (*M. smithii*) and skin (*Thaumarchaeota)* [105, 106]. Skin-associated *Thaumarchaeota* can represent about 10% of the microbiome, especially in elderly and children. Although archaea can be discovered in the oral cavity, skin, and lungs, the majority of archaea are known to reside in the human gastrointestinal tract [107].

Till date *Methanobrevibacter smithii* [108], *Methanosphaera stadtmanae* [109], and *Methanomassiliicoccus luminyensis* [110] have all been cultivated and isolated from human feces. *Methanobrevibacter oralis* was isolated from oral mucosa. Molecular studies have discovered other archaeal groups in humans. Two candidate species— "*Candidatus Methanomassiliicoccus intestinalis*" and "*Candidatus Methanomethylophilus alvus*"—and other unknown members of *Methanosarcinales, Methanobacteriales, Methanococcales, Methanomicrobiales,* and *Methanopyrales* have been identified in the human gut [10]. According to this article, up to 96% of individuals are known to carry *M. smithii* in their intestines [111, 112]. This archaeon makes up to 10% of the colon's [13] anaerobic microorganisms in humans. *M. stadtmanae* was abundant and found in 30% of patients examined [111]. *M. smithii, M. stadtmanae, and M. luminyensis*, were found to inhabit the gut in about 4% of the population [113].

In summary, archaea have been recognized as a distinct domain of life for more than 40 years, but little is known about the composition and function of human Archaeome. It has been reported that archaea may be found in the human microbiome in the first year of life, but it is unknown how humans get colonized with these microorganisms. Since amplicon-based NGS sequencing is the preferred method for microbiome research, we may need to focus on archaea-specific approaches, such as using methanogen-specific primers and more aggressive DNA extraction methods to break the archaeal cell wall (pseudopeptidoglycan) as Lysozyme, a common component of DNA extraction is not suitable for archaeal DNA extraction [48].

Archaea in Rats

Rats have been used in scientific research since the 1850s as a model for studying the human microbiome. They share four dominant bacteria that are commonly found in the GI tract [114], with Firmicutes (74%) and Bacteroidetes (23%) making up the most [115]. Rats have an advantage over other rodent models for studying the human microbiome because they can reproduce quickly, their genomes have been fully sequenced, and they are easy to care for because of their small size. However, their diet and environment can affect the gut microbiota. Rat diet high in fiber may change the diversity of intestinal microbiota [116]. *Methanobrevibacter* is the most commonly found archaea in the GI tract of rats [10, 117], followed by *Methanosphaera*, *Nitrososphaera*, *Thermogynomonas*, and *Thermoplasma*, and a novel candidate species, *Methanomethylophilus alvus* [118]. *Methanobrevibacter* species play a significant role in methanogenesis [10], but more research is needed to understand how they interact with other microbiota. To further comprehend archaea's fitness and role (apart from methanogenesis) in the microbiome, more research is required to understand how they interact with other microbiota [119].

Archaea in Pigs

Archaea are an important group of microorganisms in the intestines of both humans and animals, but they have been ignored for a long time. A few studies have investigated the composition of archaea using 16S rRNA sequencing technique, but most studies have only looked at the composition of methanogenic archaea in the gut. Pigs have been used to study the human microbiome because their genes, physiology, behavior, metabolism, and immune functions are similar to those of humans [120]. Because most studies use fewer pigs than humans for research, the pig core microbiome at the genus level may alter as more pigs are researched [119]. In one study when human feces were transplanted into humanized GF pigs, their microbiomes looked more like those of the humans than pigs [119, 121].

The GI tract in pigs is divided into 4 parts: stomach, small intestine, cecum, and colon. According to Hillman et al, two dominant genera of *Methanomicrobia* are found in the intestine of the pig. Archaeal 16S rRNA gene clone library analysis indicates that *Methanobrevibacter* is the predominant methanogen found in pig feces. In pigs and sows [12, 122] the genus *Methanobrevibacter* predominates, accounting for 57–100% of the archaeal communities. Moreover, Su et al and Federici et al. claim that the archaeal composition in the swine GIT is dynamic and diet-driven [123]. While the sample sizes of these exploratory investigations were limited, they still offer vital information on archaea in pigs [124].

Archaea Along the GIT of Rats, Pigs, and Humans

The structure of the GI tract shares similarities with humans and most animal models but differences in anatomy and pH at different locations along the GI tract may be a reason why the microbiota found in humans and animal models [125] are different. The mucosal layer of the human colon is thicker than those of mice and rats [126], which may affect the diversity of microbiota present in the colon. Different regions of the GI tract show different kinds of

microbial activity with fermentation happening in the ceca of most animal models but not in humans [126]. While performing translational research, it is important to consider the strengths and weaknesses of the main animal models being used [119].

Beneficial Characteristics of Archaea

Methanogenic archaea are among the most heritable groups of microbes in the human microbiota, which suggests a substantial dependence on the host gene. The presence of methanogens indicates lower BMI [127, 128]. They have a syntrophic relationship with *Christensenellaceae* and are significantly more abundant in people with BMIs in the normal range (18.5-24.9) than those who are obese [129] ($P< 0.05$). Additionally, an increase in levels of short-chain fatty acids (propionate, butyrate) is indicative of presence of methanogens. Several animal and cell culture studies have focused on methane's action as a gasotransmitter, with [130] demonstrating that inhaling 2.5% methane reduced the severity of ischemia-reperfusion injury in dogs, or that exogenous methane prevented leukocyte infiltration in vitro.

Methanomassiliicoccales species have been linked to lower levels of TMA in fecal samples, and experiments with rodents have shown that the presence of methanogenic archaea reduces the amount of TMA oxide in the blood. *Methanomassiliicoccales* archaea could be thought of as potential archaeal probiotics, or Archaebiotics, to lower the risk of atherosclerosis [131].

Potential Characteristics of Archaea Mimicking Pathogens

The genetic, metabolic, and structural characteristics of archaea are distinct from those of eukarya and bacteria. For instance, the cell walls of archaea lack murein [132] and they have distinctive flagellins and ether-linked lipids [133]. Despite new discoveries concerning archaeal genomes, structure, and function, much remains unclear. More than half of archaeal genes encode proteins with uncertain functions [134]. Furthermore, difficulties in isolating and cultivating archaea lead to a general lack of understanding. Yet, no clear virulence genes or factors have been identified in

archaea. However, archaea may have the ability and opportunity to cause disease [135]. Archaea share some of the characteristics with pathogens, such as easy access to a host and the ability to colonize the host for a long time. Anaerobic archaea can colonize humans since they have been found in the oral, vaginal, and colonic microbial flora of humans. It is unclear whether members of archaea show presence of virulence factors or have the ability to cause illness. Researchers would like to know if archaea share any essential characteristics that should prevent them from serving as pathogens [135].

Potential Role of Archaea in Human Gut Linked Diseases-

Cardiovascular Diseases (CVD)

Methanogenic archaea (hydrogenotrophic) in absence of oxygen convert carbon dioxide (CO_2) to methane (CH_4) in humans. *Methanobrevibacter smithii* represents 95.7% [111] of intestinal microbiota. Archaea are found in patients with inflammatory bowel disease, periodontal disease [136], obesity, cancer [137], and diverticulosis [138]. As a result, there is increased interest in learning about the role of methanogenic archaea in human disease processes [139]. It is established in literature that archaea are not capable of causing pathogenicity. However, their involvement with IBD pathogenesis and in chronic illnesses like cardiovascular disease (CVD) is poorly understood. Understanding the role of gut microbiota and its function may suggest some strategies to reverse the dysbiosis in CVD [140].

Archaebiotics have been proposed as a therapy to prevent trimethylaminuria and cardiovascular disease. One study reported that *Methanomassiliicoccus luminyensis B10* can decrease TMA by reducing it with hydrogen for methanogenesis and suggested that *M. luminyensis* could be used in the treatment of metabolic disorders [141].

Colorectal Cancer (CRC)

The American Cancer Society reports that colorectal cancer is the third most common cancer diagnosed in the United States. Studies have shown that some microorganisms in the gut microbiota, such as *Fusobacterium nucleatum* [142, 143], are involved in the process of colonic carcinogenesis [131]. The human microbiome includes microbes such as bacteria, archaea, and fungus, which live in synchrony and communicate with their host through a complex network [117, 144]. Methanogenic archaea show mutualistic interaction with bacteria in the human body [145], and methanogens interact syntrophically with bacteria to increase the synthesis of SCFA [96], which aids in the host's ability to fight cancer [146]. However, microbial dysbiosis can disrupt the mutualism between bacteria and methanogenic archaea, leading to diseases like colorectal cancer [147].

Human associated archaea are more likely to interact with *Helicobacter pylori*, a pathogenic bacteria known to cause cancer [137]. Evidence suggests that microbial metabolites made in the human body affect diseases like cancer [148, 149]. Some microbial metabolites, like butyrate, prevent inflammation and cancer [150, 151], while others, like secondary bile acids, causes cancer [144, 152]. Research on the metabolites of the human microbiome could help us figure out how microbes and humans work together. Recent evidence on how archaea, fungi, parasites, and viruses affect human health and disease suggests that archaea play an important role in human health [153-155].

Small Intestinal Bacterial Overgrowth and Intestinal Methanogen Overgrowth (SIBO/IMO)

Intestinal microbial imbalances may result in SIBO. Many reports indicate that colonic Gram-negative, anaerobic bacteria play a major role in SIBO condition [156]. Yet, several articles suggest that methanogenic archaea may be potentially involved in this disorder. The latter is

crucial. A study of 400 SIBO patients from 2021 showed that 49.8% produced only hydrogen, 38.8% produced only methane, and 11.4% produced both gases [157]. Interestingly, patients with SIBO who produced methane showed a range of symptoms, including a decrease in vitamin B12, suggesting that methanogens are not dependent on dietary vitamins to function [158] Guidelines from American College of Gastroenterology (ACG) recommend using the term "intestinal methanogen overgrowth" (IMO) when excess of methane is produced on the breath test [159]. Fasting single methane breath measurements of 10 ppm accurately diagnosed IMO but was also found to be associated with constipation and showed dominance of *M. smithii*. However, these measurements decreased after antibiotic treatment. A meta-analysis of more than 3,000 patients showed that SIBO is more common in people with IBS but its role in causing IBS pathogenesis is not well understood [131].

In short, IMO is not recommended for GI symptoms and disorders due to lack of evidence. Small clinical trials have shown that antibiotic treatment can reduce intestinal methane production and help with constipation [160, 161], but this observation needs to be confirmed by large, high-quality randomized controlled trials. Therefore, antibiotic treatment to treat IMO is not currently recommended [131].

Obesity (BMI)

Intestinal methane production in obese individuals is associated with a higher body mass index (BMI). A study looked at the correlation between methane-producing archaea and obesity in people, showing that an increased level of methane in a person's breath is a good predictor of obesity in overweight people. Previous animal research showed that composition and abundance of gut microbiota changes in obese mice, and that presence of *Methanobrevibacter smithii* in animal models showed increased weight gain. This study hypothesized that people with higher concentrations of methane in their breath may be more likely to be overweight than people with

lower concentrations of methane [162]. This study was the first to show a significant correlation between obesity and the methane on breath tests. Their studies reported that obesity may be associated with type 2 diabetes mellitus, coronary artery disease, hypertension, several malignancies, and other diseases leading to morbidity and mortality. According to the article two big factors, increased calorie consumption and decreased physical activity contributed to high incidence of obesity [163]. In summary, increased breath methane concentration is associated with higher BMI in obese subjects likely due to *M. smithii* colonization, supporting the role of gut flora (methanogenic archaea) in obesity. Further studies are needed to confirm these findings [162].

Inflammatory Bowel Disease (IBD)

Inflammatory Bowel Disease (IBD) includes two main types of chronic inflammatory conditions: ulcerative colitis (UC) and Crohn's disease (CD), the causes of which are still unknown [164, 165]. The role of archaea in the development of inflammatory bowel disease (IBD) is not yet fully understood [166]. Studies on *M. stadtmanae* indicate that it stimulates the production of TNF in vitro and is commonly found in the stools of IBD patients, suggesting that it may be involved in gut inflammation [167]. A recent study found that *M. smithii* levels were considerably lower in IBD patients as compared to healthy individuals and the count returned to normal after remission [101].

Recently, a meta transcriptomics meta-analysis analyzed a large sample size [168] to study the composition of archaea. Both samples showed differences in archaeal composition, with CD ileum showing more "halobacteria" and UC ileum showing an abundance of *Methanococcales*, *Methanobacteriales*, *Methanosarcinales*, and *Methanomicrobiales*. It is possible that the bacterial dysbiosis linked to IBD may contribute to the change in the composition of the archaeome, giving *M. stadtmanae* the credit for increasing the inflammatory response in the human gut [167]. The

two probable causes of dysbiosis; overgrowth of archaea and removal of SCFA from the biofilms can cause bacteria to become endoparasites, enter intestinal epithelial tissues, and causes inflammation in the human gut [98].

Weaning Time Affects the Archaeal Community Structure and Functional Potential in Pigs.

Although few studies have been performed on gut archaea of piglets, little is known about the impact of weaning on structure and function of archaea. Deng et al. looked at how weaning affects the changes in archaeal composition, diversity, and functional potential in pigs over time. They reanalyzed a published metagenomic dataset and found that the richness and diversity of archaeal species increased, and weaning significantly affected the richness of the species. Within two weeks of weaning, abundance of *Methanobrevibacter smithii* decreased and was replaced by *Methanobrevibacter sp900769095* [169]. Deng et al showed how archaeal diversity and functions change over time and how weaning affects the archaea in pigs' guts, suggesting that archaea may play important roles in pigs' nutrition, metabolism, and growth, especially during the weaning process [169]. *Methanobrevibacter* was reported to be the dominant methanogen in pigs' digestive tract especially in the hindgut [170, 171] and the gut colonized after birth and changed over time as the piglet grew [12].

Holman et al. (2021) studied the relationship between weaning age and the gut microbiome in piglets, using shotgun metagenomic sequencing and 16S rRNA-based sequencing techniques. Their research provides experimental data to identify the impacts of weaning on intestinal archaeal structure, diversity, and functional potential in piglets over time [169].

Development of Methanogenic Archaea in Piglets

The gut methanogenic archaea of swine and other animals are associated with energy metabolism and fat deposition. However, the development of methanogenic archaea in piglets is poorly understood. The GI microbiota of newborn animals plays an important role in determining how

the intestine functions and how the immune system develops [172, 173]. The infant gut changes from being sterile to being densely populated, and then acquiring adult like microbial community at adulthood [174, 175]. A study performed by Su et al. on Meishan (obese breed) and Yorkshire (lean breed) piglets revealed that from one to 14 days of age, while the total methanogen populations increased, the diversity of the methanogenic community in the feces of newborn piglets declined. The genus of *Methanobrevibacter* was dominated by the age of the piglets but not by the breed, suggesting that other methanogens may have colonized the gut later in the piglet's life. As the intestinal microbiota of newborn piglets (Yorkshire and Meishan) [176] developed, the bacterial diversity increased, the diversity of methanogenic community dropped from 1 day to 14 days, but the abundance of methanogens significantly increased to 109 copies of 16S rRNA gene per gram of feces in Meishan and Yorkshire piglets indicating that methanogens colonize in the piglet gut as a result of mutual selection between methanogens and the host.

Additionally, the abundance of *Methanobrevibacter smithii* increased from day 1 to day 14, while the abundance of *M. thaueri* and *M. millerae* decreased significantly. The latter two species were found in Meishan and Yorkshire piglets three days after birth. This suggests that replacing *M. smithii* with other *Methanobrevibacter* spp. may be advantageous to the gut microbiome. This substitution happened faster in Yorkshire piglets than in Meishan pigs. Further research is required to determine whether or not this variation is related to the phenotypical differences between the two breeds [12]. The results of the investigation on Yorkshire and Meishan piglets were consistent with the findings from Landrace and Erhualian piglets. The article reported that *M. smithii* concentration was higher in anorexic individuals than in lean people and was depleted in obese individuals [177]. These findings suggest that *M. smithii* may play an important role in host energy metabolism [12].

The objective of the present study was to analyze the metagenomic data set for presence of archaeal sequences in samples from colon and small intestine of rats. If archaea were present,

then we wanted to compare if there was any difference in relative abundance of archaea in male and female rats as well as in the small intestine versus colon. In pigs, we analyzed the 16S rRNA gene sequencing data from pig feces for the presence of archaeal sequences. In the pig data we looked for indications of transfer of archaea from mother to offspring during birth and lactation as well as a difference in the relative abundance of archaea in male and female piglets.

The research goal was to investigate the relative abundance of archaea in diverse sets of fecal/digesta samples from rats and pigs and seek correlations with metadata. To accomplish this objective, we used two different approaches: Bioinformatics and quantitative PCR (qPCR) approach.

Hypothesis 1- We hypothesize that a portion of NGS data indicates presence of archaea, and that most of the archaea, in fecal and digesta samples will be methanogens.

Aim - Investigate the presence of archaea by mining our NGS data for relative abundance of archaeal (methanogens) sequences.

Hypothesis 2: We hypothesize that we will find similar relative abundances of archaea with NGS data and qPCR data.

Aim: Confirm and compare NGS data with qPCR data for relative abundance of archaeal sequences.

- Use archaea-specific primers to perform qPCRs and then compare the data with the NGS data.

- Future validated qPCR assays could allow for quick quantitative testing of samples for presence of archaeal DNA

CHAPTER III

METHODOLOGY

This section summarizes different approaches used in the project. The research began with the use of bioinformatic approach, followed by qualitative PCR, gel electrophoresis, and quantitative PCR assays. DNA was extracted from small intestine and colon (digesta) samples in rats using three different DNA extraction protocols, namely the HostZERO DNA kit, the Qiagen DNA extraction kit, and the ZymoBIOMICS DNA extraction kit. DNA was isolated from fecal samples of sows and piglets using ZymoBIOMICS DNA extraction kit.

Sequencing Data

Rat NGS Data Set

Short sequence read data sets based on 16S rRNA gene amplicon sequencing were derived from digesta or fecal DNA samples from Sprague Dawley rats generated in the Intestinal Microbiome and Regeneration in Opioid Misuse project (G. Koehler, PI; D. Vazquez Sanroman, Co-PI). Paired-end sequencing (2 x250 cycles) for this data set was performed on an Illumina MiSeq System.

This project also included metagenomic sequencing of digesta DNA samples from rat small intestine and colon. For this purpose, DNA sample aliquots were processed by the OSU Stillwater Genomics Core Facility for paired-end sequencing on an Illumina NextSeq 500 system (double-indexed, 2 x 151 cycles).

Pig NGS Data Set

The 16S rRNA gene amplicon short read data set (Illumina MiSeq double-indexed paired end reads 2x 251 cycles) from sows and their offspring was generated through a collaboration with Dr. Janeen Salak-Johnson and Lilly Hernandez, Animal Sciences, OSU Stillwater.

The rat and pig-derived datasets were screened for archaeal sequence reads as described below.

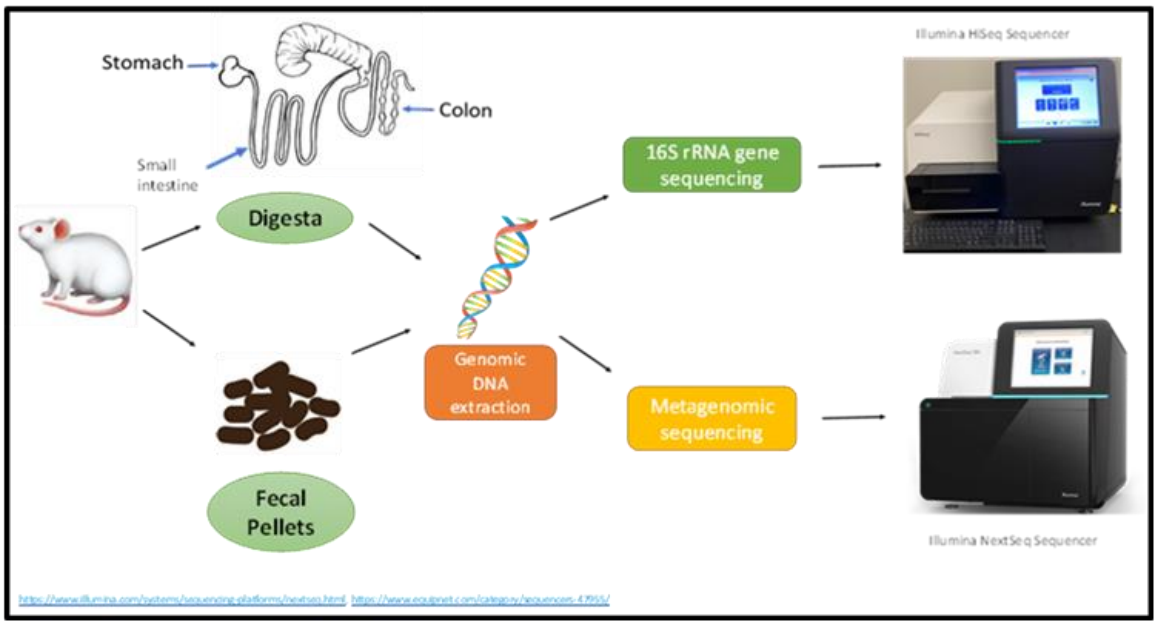


Fig: 3.1. Schematic Illustration Showing DNA Extraction, Sequence Library Preparation, and Sequencing in Rat Digesta Samples. Genomic DNA extraction from rat small intestine and colon digesta samples using ZymoBIOMICS, QIAamp, and HostZERO DNA extraction kit. DNA was sequenced with 16S rRNA gene sequencing technique using Illumina MiSeq sequencer and the metagenomics sequencing technique using NextSeq sequencer.

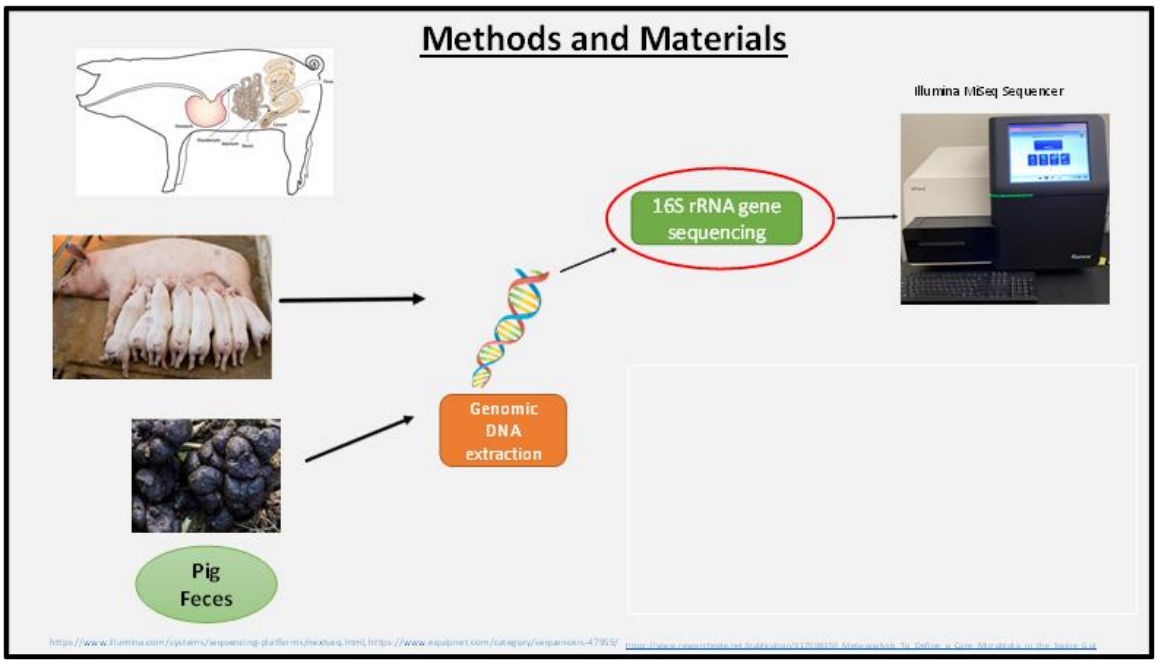


Fig: 3.2 Schematic Illustration Showing DNA Extraction, Sequence Library Preparation, and 16S rRNA Sequencing in Pig Fecal Samples. Genomic DNA extraction from sows and piglets' fecal samples using ZymoBIOMICS DNA extraction kit. DNA was sequenced using 16S rRNA **gene sequencing technique using Illumina MiSeq sequencer.**

Bioinformatic Analysis

CLC Genomics Workbench with CLC Microbial Genomics Module

16S rRNA Gene Amplicon Data Analysis

Taxonomic profiling of 16S rRNA gene amplicon sequence reads was conducted with adaptations following the OTU clustering procedure as detailed in the respective Qiagen tutorial (Tutorial – OTU Clustering Step by Step, July 9, 2021, Qiagen) [178]. The following outlines the procedure: Demultiplexed Illumina MiSeq fastq read files were imported as paired-end reads with default parameters (file names and quality scores retained). Following import, reads were trimmed using the Trim Reads tool with default quality score parameters and Illumina adapter trimming.

Samples with low numbers of reads (< 10,000 reads) were identified and discarded from further analysis using the tool Filter Samples Based on Number of Reads.

Taxonomic profiling was performed using the OTU Clustering tool in conjunction with the SILVA v123 16S rRNA database for reference-based clustering. Figure 3.3

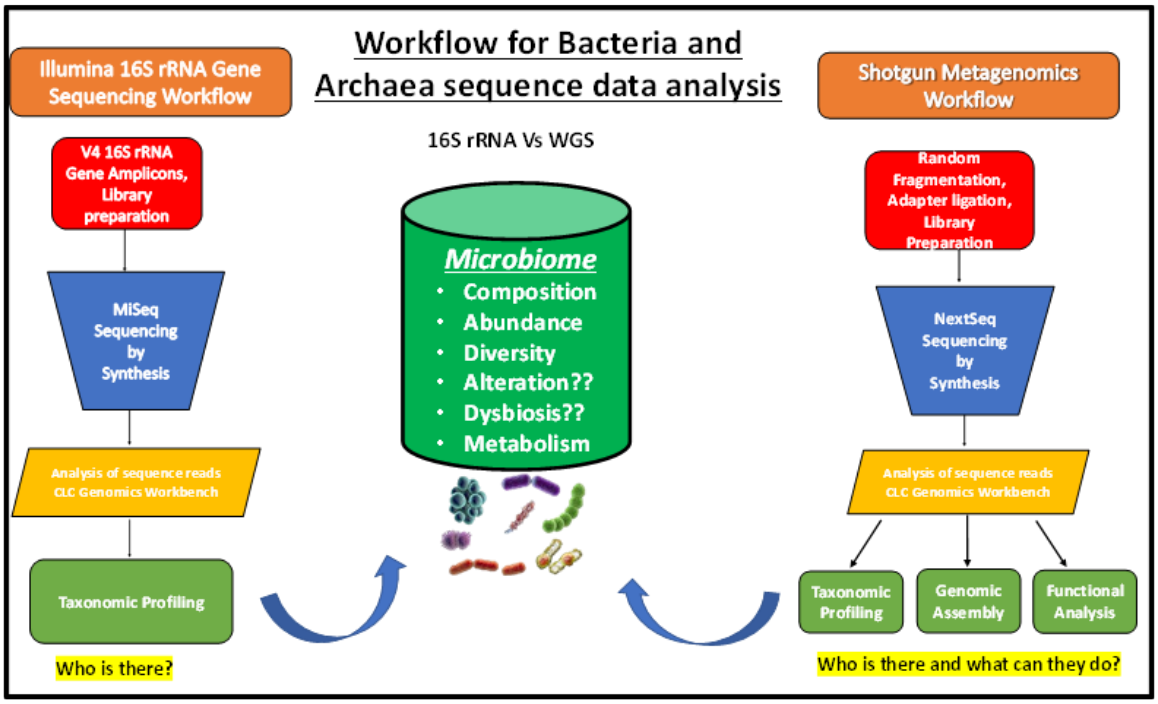


Fig: 3.3. Workflow for Bacteria and Archaea Sequence Data Analysis. Two main approaches to characterize microbiome are 16S rRNA gene sequencing and Metagenomic sequencing. For microbiota sequencing 16S rRNA sequencing is preferred and targeted approach. Workflow includes library preparation for MiSeq sequencer, sequence by synthesis, analysis of sequencing reads using bioinformatics and taxonomic profiling. Metagenomic sequencing uses a random approach. Workflow includes library preparation for NextSeq sequencer, sequence by synthesis, analysis of sequencing reads using bioinformatics and taxonomic profiling, genomic assembly, and functional analysis.

Sample metadata were joined with the OTU abundance table using the tool Add Metadata to Abundance Table.

Taxonomic Profiling of Shotgun Metagenomic Data (Citation: Tutorial – Taxonomic Profiling of Whole Shotgun Metagenomic Data, July 2021, QIAGEN) [178].

The CLC Genomics Workbench with CLC Microbial Genomics Module 21.04 was employed for taxonomic profiling of rat small intestinal and colon metagenomes using the following workflow.

Demultiplexed Illumina NextSeq 500 fastq sequence read files were imported using the paired-end, discard read names and discard quality scores options. The minimum distance was set to 50, the maximum distance to 500.

Metadata were imported and associated with sample reads using Import>Import Metadata.

The metagenomics workflow Data QC and Taxonomic Profiling was used in batch mode with the reference data base index for taxonomic profiling and the rat host genome index for removal of host reads.

The resulting individual sample abundance tables were merged with Merge Abundance Tables.

Archaeal abundance data were selected in the merged abundance table and a filtered archaea abundance sub-table was generated for further analysis such as generation of relative abundance bar charts.

Functional Profiling of Shotgun Metagenomic Data

The main goal is to demonstrate the assembly of metagenomes derived from two different groups of samples and the subsequent investigation of functional differences. It serves as a template for performing a comparative investigation into the functional composition and diversity of microbial communities. The tools provide a way of looking at different samples in aggregate views and to

drill down into differentiating functional categories that result from the comparative analysis. QIAGEN CLC Genomics Workbench version 12.0 or higher with CLC Microbial Genomics Module is required. Refer to Figure 3.4.

• First, import "raw" NGS sequencing data into the workbench and prepare the samples for analysis.

• Then assemble the reads using De Novo Assemble Metagenome into contigs.

• Map the reads to the assembled contigs using Map Reads to Contigs.

• The tool Bin Pangenomes by Sequence assigns the reads to the bin of the contig they belong to.

• With Find Prokaryotic Genes, identify genes and coding DNA sequences (CDS) on the contigs.

• Subsequently, functional annotation of the CDS with Gene Ontology (GO) terms and Pfam domains will be performed with Annotate CDS with Pfam Domains.

• Based on the annotations, construct a Gene Ontology profile using the Build Functional Profile tool for measuring functional diversity.

• Create a multi-sample abundance table using Merge Abundance Tables.

• Finally, set up the data for additional statistical analyses and visualizations

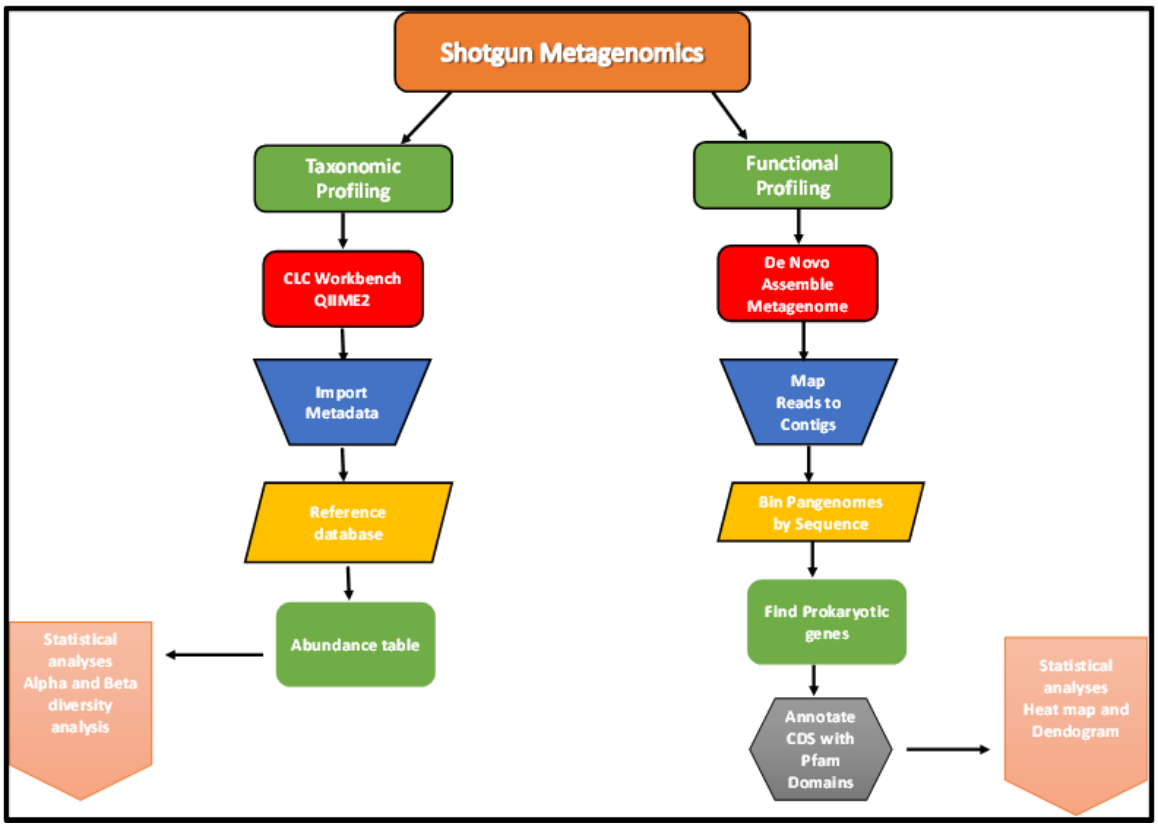


Fig: 3.4. Bioinformatic Analysis; Taxonomic Profiling and Functional Profiling of Shotgun Metagenomic Data. Shotgun metagenomics: two possibilities, taxonomic profiling, and functional profiling. **Taxonomic profiling** uses bioinformatics in combination with metadata, 16S rRNA reference database, abundance table, statistical analyses. **Functional profiling** workflow includes De Novo assembly of metagenome into contigs, map reads to assembled contigs, Bin Pangenomes by Sequence assigns reads the right bin of the contig, find prokaryotic genes in the sequence data, functional annotation of the CDS with Gene Ontology (GO), construct a Gene Ontology profile, set up data for statistical analyses.

16S rRNA Gene Primer Selection and Evaluation of Primers *In-Silico*

Several primer pairs targeting the archaeal 16S rRNA gene were chosen from different publications. Primers were chosen based on how specific they are for an Archaeal sequence in-silico, their melting temperature, the size of the amplicon (about 150-200bp), and the length of

the primer. Primer pairs were tested and selected using SILVA TestPrime 1.0 (Silva database). TestPrime explains how well the primer pair covers different taxa. TestPrime evaluates the performance of primer pairs by running an in-silico PCR on the SILVA databases. From the results of the PCR, TestPrime computes coverages for each taxonomic group in all the taxonomies offered by SILVA. A specific pair of primers' strengths and weaknesses are determined by their coverage in the taxonomy.

Fecal Sample Collection and DNA Extraction

Different DNA extraction techniques were used to eliminate host read contamination. Genomic DNA was extracted from rat fecal and digesta samples using three DNA extraction methods: ZymoBIOMICS, HostZERO, and QIAamp techniques. Figure 3.1 ZymoBIOMICS DNA Miniprep Kit (Zymo Research Irwing, California) was used to extract genomic DNA from pig fecal samples. Figure 3.2 Approximately 100mg of each fecal and digesta sample were used to extract DNA using the kit, and the manufacturer's instructions were followed. Following the extraction, the concentration of the extracted DNA samples (2 µL) was measured using BioTek Synergy 2 Multi mode Microplate reader (BioTek Instruments, Inc. Winooski, Vermont).

Qualitative Analysis- Amplification of the 16S rRNA Gene

Genomic DNA was amplified using Archaeal-specific primers, Arch 958F and Arch 1114aR followed by touch down (TD) amplification protocols. Standard PCR analysis was performed using 16S rRNA universal primer sets for bacteria and archaea and archaeal 16S rRNA primers sets specific to archaea. All PCR amplifications were performed in a final volume of 20µL that contained 100ng/µL of template, 1µL of 10 µM of forward primer and 1µL of 10 µM of Reverse primer, and GoTaq (2X master mix). The endpoint PCR reactions were performed in the PTC 200 DNA engine thermocycler (Bio-Rad, Hercules, CA, USA) in the following Touch Down (TD) PCR mode: 95°C - 2 mins to activate the enzyme, followed by 39 cycles of denaturation at 95°C

for 30 seconds, annealing at 65°C for 30 seconds (-0.5°C per cycle), extension at 72°C for 30 seconds, and final extension at 72°C for 5 minutes.

Table:**1 Oligonucleotide Primers Used in the Study.**

Primer Name	5'-Sequence-3'	Tm °C	Primer length (nt)	Amplicon size (bp)	Specificity
Arch 915F	AGGAATTGGCGGGGGAGCAC	64	20	141	Archaea
Arch 1017R	GGCCATGCACCWCCTCTC	59	18		Archaea
Arch 915F	AGGAATTGGCGGGGGAGCAC	64	20	195	Archaea
1114aR	GGGTCTCGCTCGTTRCC	60	17		Archaea
Arch 958F	AATTGGANTCAACGCCGG	58	18	97	Archaea
Arch 1017R	GGCCATGCACCWCCTCTC	59	18		Archaea
Arch 958F	**AATTGGANTCAACGCCGG**	**58**	**18**	**151**	**Archaea**
1114aR	**GGGTCTCGCTCGTTRCC**	**60**	**17**		**Archaea**
Arch 349F	GYGCASCAGKCGMGAAW	62	17	139	Archaea
Eury 514	GCGGCGGCTGGCACC	64	15		Archaea
Arch 344F	ACGGGGYGCAGCAGGCGCGA	73	20	144	Archaea
Eury 514	GCGGCGGCTGGCACC	64	15		Archaea
GK1053F	ATGGCTGTCGTCAGCTCGT	66.2	19	353	Bacteria
1391R	GACGGGCGGTGTGTRCA	60.3	17		Bacteria

Evaluation of Amplified DNA using Gel Electrophoresis

Amplified PCR reactions were then evaluated using gel electrophoresis. 1.5% agarose gel was prepared using 0.75g of agarose in 1X TAE (Tris-acetate-EDTA). About 3µL of SYBR safe was added to visualize DNA on the agarose gel. A final volume of 15 µL of amplified DNA was loaded into each well.

Real-Time PCR Analysis

Real-time PCR assays were performed using the QuantStudio™5 Design & Analysis instrument, Real Time PCR System (Applied Biosystems, Fischer Scientific) Waltham MA. In quantitative PCR, the amount of DNA was measured at each cycle. The 16S rRNA gene was quantified with SYBR Green real-time PCR analysis. Universal 16S rRNA primer sets GK 1053F and 1391R and Archaea-specific primers Arch 958F and Arch 1114aR were used to quantify the 16S rRNA gene under the following conditions: initial denaturation step at 95°C for 10 mins, denaturation at 95°C for 15 seconds, and annealing and extension at 60°C for 1 min. The reaction volumes of 10µL consisted of 5.5µL of SYBR Green enzyme, 0.22µL of 10µM forward primer, 0.22µL of 10µM reverse primer, 1µL of 1ng/µL of template DNA, and 3.96µL of molecular water. The qPCR reactions were carried out in triplicates and the mean values were calculated. ΔCrt and ΔΔCrt values were recorded. qPCR data was used to compare the relative abundance of archaea with the 16S rRNA sequencing data and metagenomic data.

Statistical Analyses

Statistical analyses were reported on the 16S rRNA gene sequencing data in pigs. One-way ANOVA analysis was performed to determine significant difference in the relative abundance of methanogens in all sows and their piglets. Tukey's multiple comparisons test was performed to

compare the multiple possible pairwise comparisons. Furthermore, a non-parametric test, Kruskal-Wallis test with Dunn’s multiple comparisons test was performed to confirm the difference in significance. Two-way ANOVA analysis followed by a post-hoc test, Šídák's multiple comparisons test for pair wise comparison was performed to determine significant difference in relative abundance of methanogens based on sex of the piglets.

CHAPTER IV

FINDINGS

The purpose of the current study was to identify archaeal sequences in NGS datasets derived from mammalian gut microbiomes such as rat intestinal and pig fecal samples. Data from rat small intestinal and colon digesta samples were generated by 16S rRNA gene amplicon sequencing and shotgun metagenomic sequencing. Profiling of pig fecal microbiota employed solely 16S rRNA gene amplicon sequencing. The following shows the results obtained from these various approaches.

Evidence for Archaea in Rat Gut Microbiota and Metagenomes

Rat Data Sets

The following datasets were generated in the project entitled “Intestinal Microbiome and Regeneration in Opioid Misuse” by Alejandro Torres, Senait Assefa, PhD, Dolores Vazquez Sanroman, PhD (Co-PI), Gerwald Koehler, PhD (PI) funded by an award from the Oklahoma Center for Adult Stem Cell Research (OCASCR), a program of the Tobacco Settlement Endowment Trust (TSET).

The experimental animal research associated with the datasets was approved by the OSU Center for Health Sciences Institutional Animal Care and Use Committee (PI: Dolores Vazquez Sanroman; protocol number 2021-1288).

- 16S rRNA gene amplicon sequencing reads of digesta or fecal DNA via paired-end sequencing (2 x 250 cycles) on an Illumina MiSeq System using the method described by [179]
- Shotgun metagenomics reads (2 x 150 cycles) on an Illumina NextSeq 500 using the Kapa HyperPrep Kit (Roche Diagnostics Corporation, Indianapolis, IN)

The current study was directed solely towards identification of archaeal sequences in NGS datasets and rat intestinal samples. Additional results associated with the aforementioned research project will be published elsewhere.

Bioinformatics and Databases

Next Generation sequencing (NGS) data analysis was performed using QIAGEN CLC Genomics Workbench (GWB; version 21.0.4) in conjunction with a Microbial Genomics Module for amplicon-based and metagenomic analyses. QIAGEN CLC Genomics Workbench has been developed to support a wide range of bioinformatics applications for genomic, metagenomic, and transcriptomic analyses.

The following databases were used:

- SILVA 16S rRNA database (v123; 99%) for amplicon-based analyses [180]
- QMI-PTDB taxpro index (January 2022) for taxonomic profiling of shotgun metagenomic sequence reads (Qiagen) [178]

- *Rattus norvegicus* (mRatBN7.2) (taxpro index) for removal of host DNA sequences from shotgun metagenomic sequence read datasets.

Archaeal Sequences in the Rat 16S rRNA Gene Amplicon Sequence Datasets

Scrutiny of the rat small intestine, colon, and fecal 16S rRNA gene amplicon sequencing datasets available to this study did not reveal reads reliably classified as archaeal. Taxonomic profiling using SILVA 16S rRNA databases was performed with the GWB and additionally, with QIIME 2 (data not shown) [181].

Archaeal Sequences in Shotgun Metagenomic Sequencing Data

Shotgun metagenomic sequencing of digesta DNA samples from rat small intestine and colon was performed at the OSU Stillwater Genomics Core Facility using Illumina NextSeq 500 system. Metagenomic paired-end and double-indexed sequence reads were paired, and quality controlled using the GWB. Taxonomic profiling was performed with host read removal as detailed in Materials and Methods. The resulting sample abundance tables were merged into a single abundance table containing bacterial and archaeal OTUs, which subsequently was parsed for archaeal reads to generate a sub table that only contained the archaeal OTUs. Metagenomic data for rats indicate the presence of methanogens and haloarchaea.

Metagenomic Profiles of Archaeal Taxa in Rat Small Intestine

Small intestinal digesta samples from male and female rats were processed for metagenomic sequencing. Rat feed was used as control for identification of potential allochthonous DNA in the small intestinal metagenome. The following figures show the taxonomic profiles at kingdom (Figure 4.1) level as well as archaeal phylum (Figure 4.2) and genus (Figure 4.3) levels obtained from metagenomic sequencing of the rat small intestinal digesta samples.

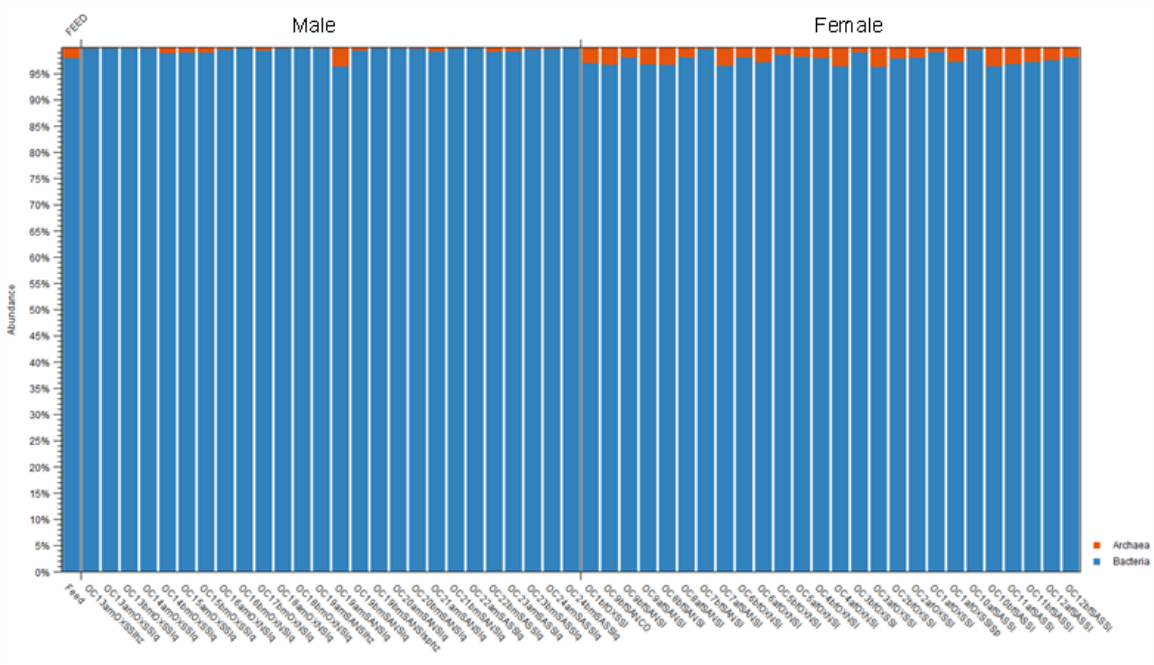
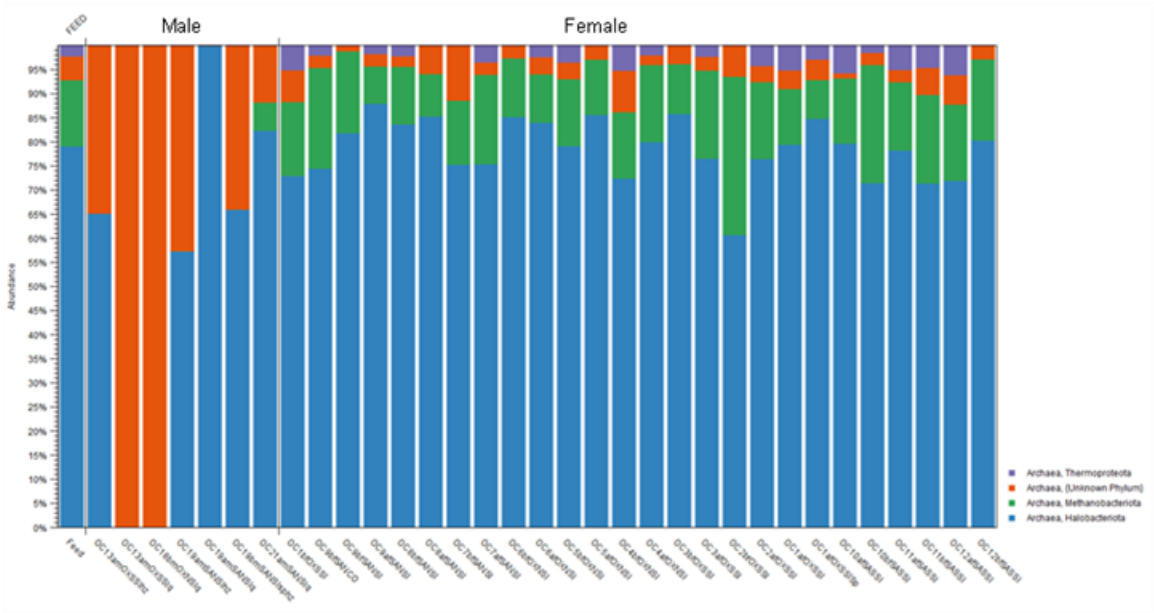


Fig. 4.1. Relative Abundance of Archaea and Bacteria in Male and Female Rat Small Intestine at Kingdom Level. Compared to bacterial sequences, the relative abundance of archaeal sequences was quite low or absent in male and female small intestinal samples. Sequencing data from rat feed (leftmost column) indicated presence of archaeal sequences in similar low abundance.



(Previous page) Fig. 4.2. Relative Abundance of Archaea in Male and Female Rat Small Intestine at the Phylum Level. Relative abundance analysis of archaeal sequences in samples from the male and female rat small intestine as well as feed revealed four phyla: *Halobacteriota*, *Methanobacteriota*, *Thermoproteota*, and an *Unknown* phylum. Samples with an archaeal sequence abundance of less than 10 were excluded from this bar graph.

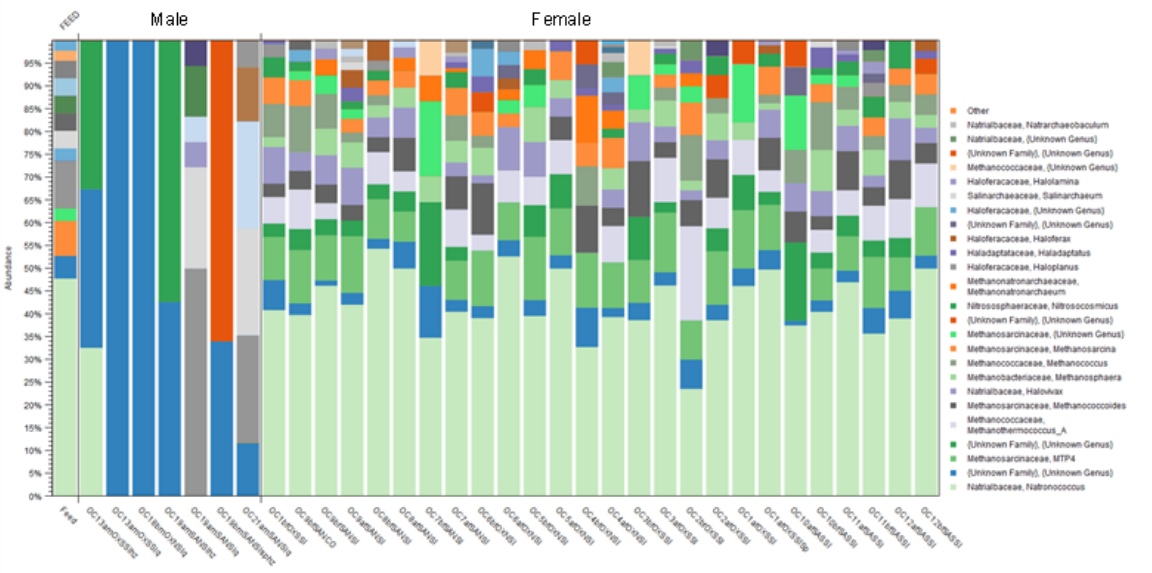


Fig. 4.3. Relative Abundance of Archaea in Male and Female Rat Small Intestine at the Genus Level. The relative abundance of archaeal sequences in female rat small intestine appears to be dominated by the genus *Natronococcus*. Results from male samples were quite different, with dominance of Haloplanus or an unknown archaeal taxon. Rat feed data also indicated abundant presence of *Natronococcus* sequences as well as sequences from additional genera that also appeared to be represented in the small intestinal samples. Only the top 25 genera are shown.

Metagenomic Profiles of Archaeal Taxa in Rat Colon

Similar to the results from small intestine, metagenomic sequencing of colon digesta DNA from male and female rats indicated very low archaeal abundance. It appears that bacterial sequences vastly outnumbered archaeal sequences. For example, bar graphs were not useful to depict

kingdom-level taxonomic profiles due to the low abundance of archaeal sequences (data not shown). Figure 4.4 shows bacterial and archaeal phylum-level profiles while Figure 4.5 highlights the relative abundances of Halobacteriota, Methanobacteriota, and unclassified archaea. Classification to genus level was not possible in the colonic samples.

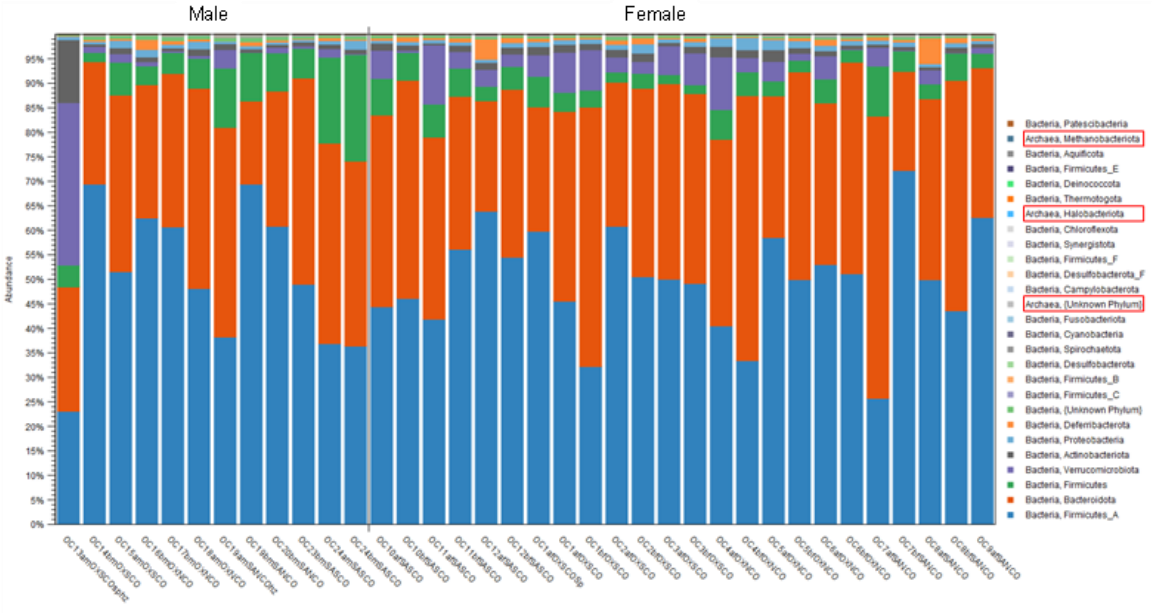


Fig. 4.4. Relative Abundances of Archaeal and Bacterial Phyla in Male and Female Rat Colon Samples. The bar graphs depict the relative abundances of archaeal phyla (Halobacteriota, Methanobacteriota, and an unknown phylum; all highlighted by red frames) with the dominant bacterial phyla in male and female rat colon shotgun metagenomic sequencing results.

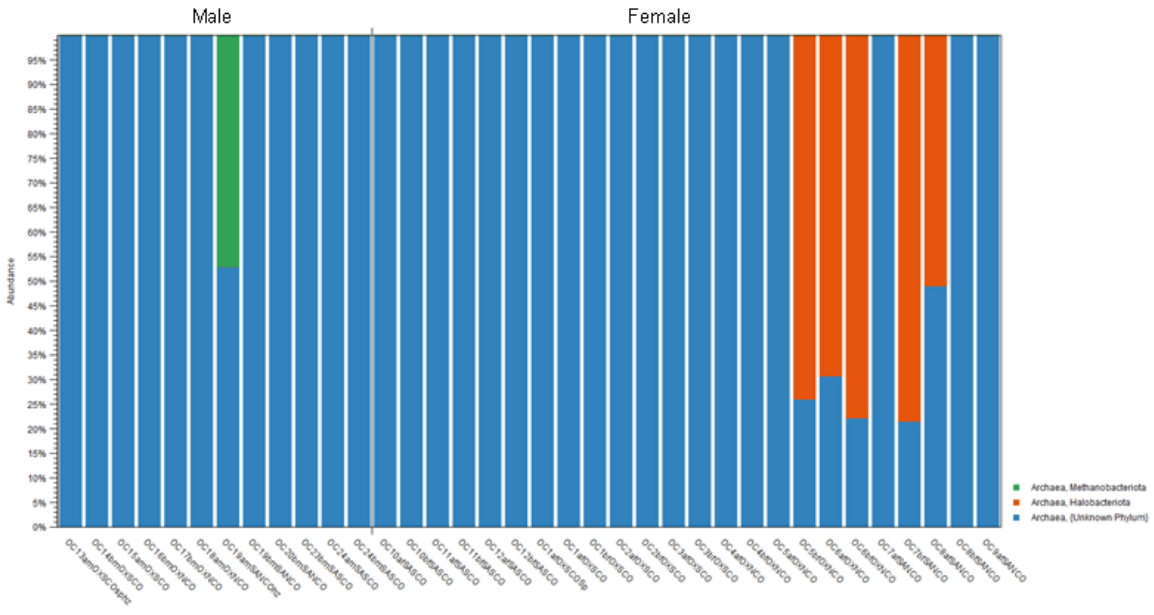


Fig. 4.5. Relative Abundances of the Three Archaeal Phyla in Male and Female Rat Colon Samples. The bar graphs depict the relative abundances of *Halobacteriota*, *Methanobacteriota*, and an unknown Archaea phylum in male and female colon digesta metagenomic sequencing results.

In summary, shotgun metagenomic sequencing of rat small intestinal digesta revealed low abundance of archaea in samples from males and females, with apparent higher diversity of phyla (*Halobacteriota, Methanobacteriota, Thermoproteota, and an unclassified* phylum) and genera in the female samples (see Figures 4.2 and 4.3). The genus *Natronococcus* dominated in the female rat small intestine and the feed samples, while the male rat digesta sample indicated disparate communities. The rat colon digesta metagenomes also revealed a very low abundance of archaea. Three phyla were represented in these samples, the *Halobacteriota, Methanobacteriota*, and an *unclassified* phylum; however, no classification to genus level was possible with the employed QMI-PTDB reference database.

Detection of Archaeal Sequences by PCR

Qualitative and quantitative PCR assays were attempted on male and female rat small intestine and colon digesta samples for development of rapid detection assays and confirmation of the metagenomic data.

Endpoint PCR Attempts

Several male and female rat intestinal digesta DNA samples were isolated and used as templates to test archaea-specific PCRs. Samples were amplified using a touch-down amplification protocol on a PTC 200 DNA Engine thermocycler (Bio-Rad, Hercules, CA, USA) with 16S rRNA gene universal primer sets and archaea-specific primer combinations (see Table 1). Amplified PCR reactions were then evaluated using agarose gel electrophoresis. The gels revealed very faint bands at the expected size ranges and in some cases strong bands that were too large. The qualitative assays were performed with different samples and PCR optimization steps to try obtaining more accurate results, but all the experiments indicated non-specific amplification. The following figures show representative PCR results using rat small intestine (Figure 4.6) and colon (Figure 4.7) DNA samples as templates.

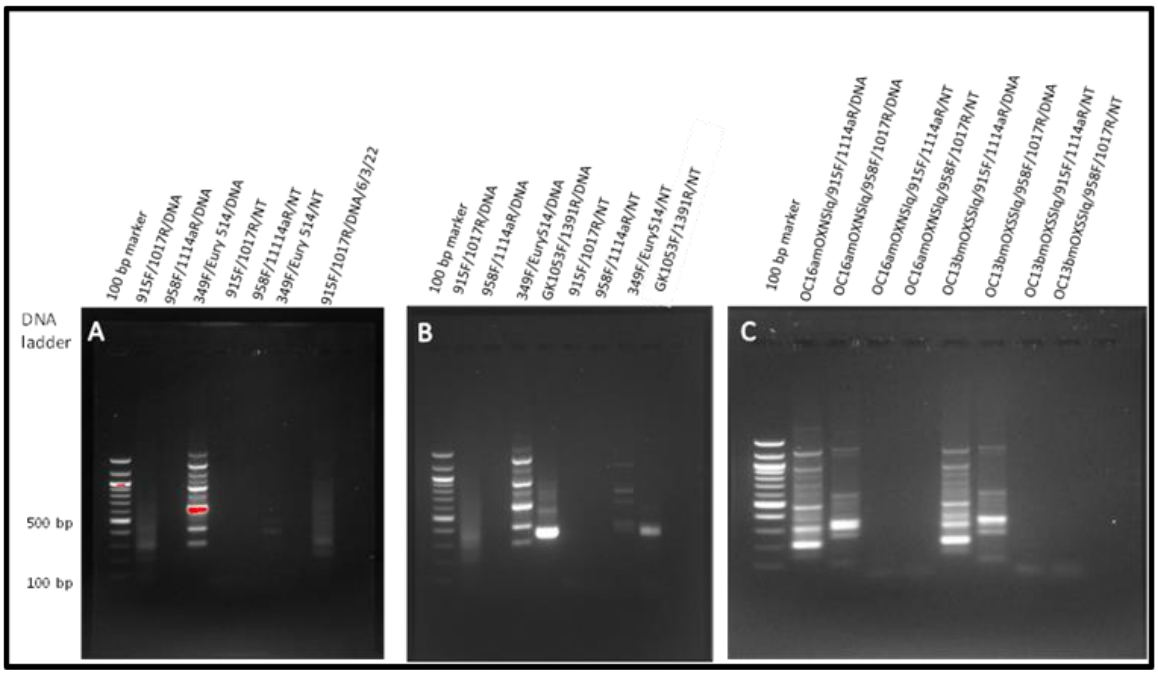


Fig. 4.6. Endpoint PCR with Small Intestinal DNA Samples. Agarose gel electrophoresis (1.5%) showing PCR amplifications using the indicated archaea-specific primer pairs with the small intestinal genomic DNA samples OC16amOXNSIq (A), OC13bmOXSSIq (B) as well as OC16amOXNSIq and OC13bmOXSSIq (C). Only the universal 16S rRNA gene control primer pair GK1053-1391R yielded the expected band size. NT: no template control. The GK1053-1391R amplicon in (B) indicates bacterial DNA contamination.

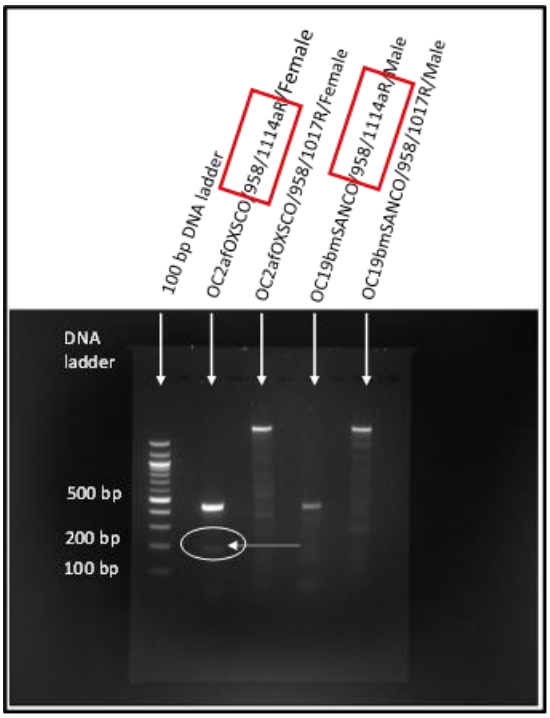


Fig. 4.7. Endpoint PCR with Colon DNA Samples. Agarose gel electrophoresis (1.5%) showing PCR amplicons from two rat colon DNA samples (OC2afOXSCO, OC19bmSANCO) using different the archaea-specific primer pairs 958-1114aR and 958-1017R. The ellipse and arrow show a faint band with the expected size; however, the additional bands appear to represent non-specific PCR products.

Endpoint PCR results were inconclusive and failed to confirm unequivocally the presence of archaeal sequences in rat small intestine and colon metagenomic DNA. Some of the chosen putative archaea-specific primer combinations (e.g., 958-1114aR and 958-1017R), however, yielded specific amplicons with pig fecal DNA as shown later in this chapter. I tested these primers also with quantitative PCR in conjunction with rat small intestinal and colon DNA as shown in the following section.

Quantitative PCR Attempts

Quantitative real-time PCR assays were performed with a QuantStudioTM 5 Real-Time PCR System using PowerUp SYBR master mix (Applied Biosystems, Thermo Fischer Scientific, Waltham MA) for SYBR Green-based qPCR. The universal primers GK1053F and 1391R were used to quantify 16S rRNA genes for normalization, while the archaea-specific primers Arch958F and 1114aR were employed for quantitation of archaeal 16S rRNA gene sequences. Small intestine and colon DNA samples from male and female rats were used as templates in the

qPCRs. Reactions were run in triplicates and the means of cycle relative threshold values (Crt), ΔCrt, and relative quantity (RQ) values were determined and analyzed further using QuantStudio5 Design & Analysis software (Applied Biosystems) as well as Microsoft Excel and GraphPad (version 9.5.1) software.

Figures 4.8 and 4.9 show plots with qPCR Crt values derived from male and female rat small intestine and colon DNA samples. The results indicated that archaeal sequence targets were not reliably detectable under the chosen reaction conditions. In contrast, a control sample with pig fecal DNA as template showed efficient quantitation (see Figure 4.8).

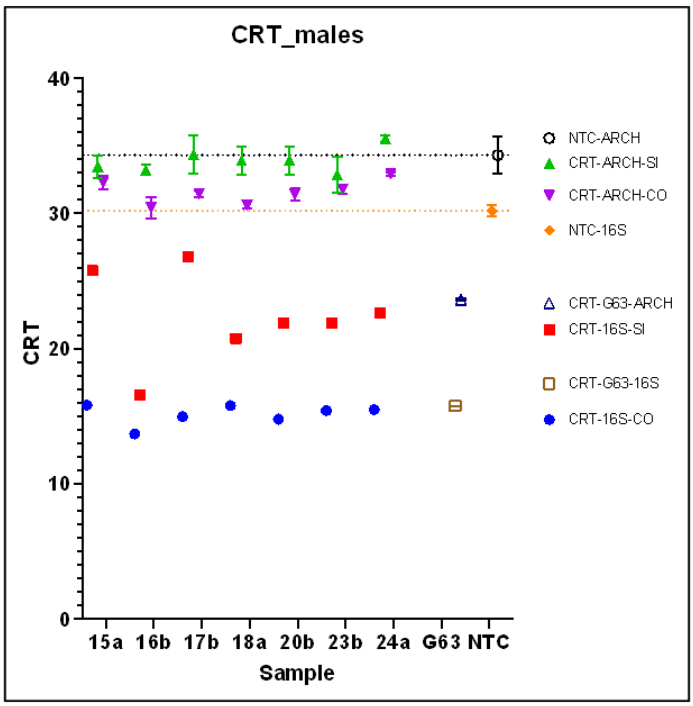


Fig. 4.8. Quantitative PCR of Digesta DNA Samples from Male Rats. Graph of Crt values for male rat small intestine (SI) and colon (CO) digesta DNA samples in comparison to a pig fecal DNA sample (G63) showing the Crt values for the universal 16S rRNA-specific qPCR (primer pair GK1053F - 1391R; 16S) and archaea-specific PCR (primer pair Arch958F - 1114aR; ARCH). The dotted lines across the graph show the non-template control (NTC) Crt values for the two primer pairs. The graph reveals that archaea-specific Crt values for both CO and SI samples are close to the NTC values indicating very low target concentration or non-specific amplification. The pig control values show robust amplification with an archaea-specific Crt around 23, far below the NTC Crt.

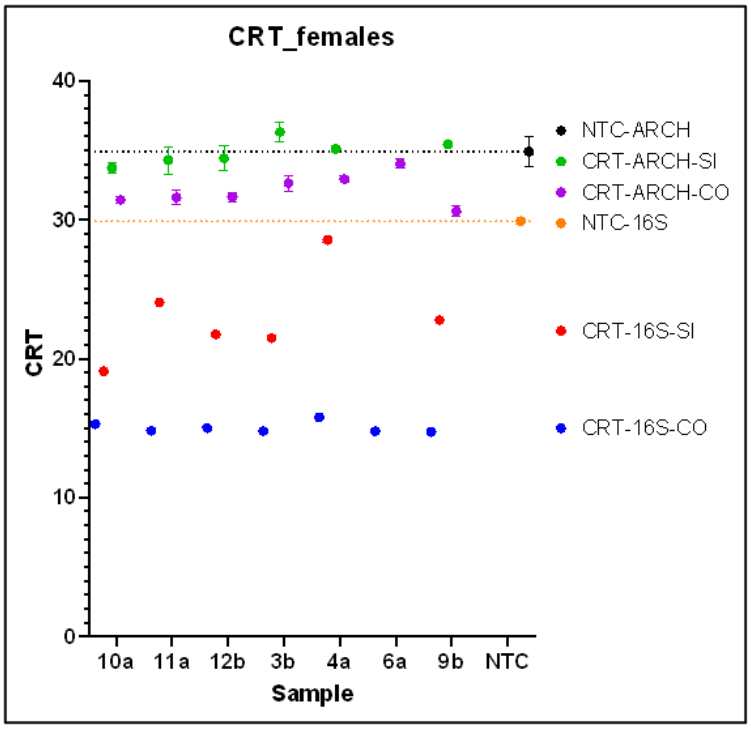


Fig. 4.9. Quantitative PCR of Digesta DNA Samples from Female Rats. Graph of Crt values for female rat small intestine (SI) and colon (CO) digesta DNA samples showing the Crt values for the universal 16S rRNA-specific qPCR (primer pair GK1053F - 1391R; 16S) and archaea-specific PCR (primer pair Arch958F - 1114aR; ARCH). The dotted lines across the graph show the non-template control (NTC) Crt values for the two primer pairs. The graph reveals that archaea-specific Crt values for both CO and SI samples are close to the NTC values indicating very low target concentration or non-specific amplification.

Summary of Archaeal Sequence Detection Results in Rat Small Intestine and Colon

Analysis of 16S rRNA gene amplicon sequencing reads from the rat small intestine and colon digesta DNA samples did not show archaeal reads, despite sufficient sequencing depth for reliable detection of many bacterial operational taxonomic units (OTUs; data not shown). Shotgun metagenomic sequencing followed by taxonomic classification of the rat gut archaeome showed archaeal sequences in low abundance. The phyla Halobacteriota and Methanobacteriota, in addition to unclassified archaeal sequences were dominant in most samples. Remarkably high taxonomic diversity was detected in female rat small intestinal samples, but also in feed (see Figure 4.3).

Several different male and female rat small intestine and colon samples, as well as different primer pair combinations, were used in qualitative PCR experiments to identify and detect archaea in these samples utilizing touch-down PCR conditions. Standard polymerase chain reaction (PCR) indicated ample non-specific amplification. Quantitative PCR was utilized to test different reaction conditions and potentially improve the assay specificity and sensitivity. However, the low target-specific amplification results suggest that further optimization is needed.

In conclusion, while metagenomic data indicated the presence of methanogens and haloarchaea in low abundance, data from qualitative and quantitative assays suggest that archaea may be absent or found in extremely low abundance, potentially below the limit of detection, in rat small intestine and colon samples. Further assay optimization is needed to fully gauge the contributions of archaeal taxa and their interactions with other members of the gut microbiota in this experimental animal model.

Evidence for Archaea in Pig Fecal Microbiota

Pig Dataset

The dataset analyzed for this study stems from a collaborative project on the analysis of pig fecal microbiota by Lily Hernandez, Cassidy Reddout, and Janeen Johnson, PhD, OSU Stillwater Department of Animal and Food Sciences, with Senait Assefa, PhD, and Gerwald Koehler, PhD, OSU Center for Health Sciences Department of Biochemistry and Microbiology. The animal work associated with the collaborative project was approved by the OSU Stillwater Institutional Animal Care and Use Committee (Protocol Title: Influence of Prenatal Stress on Immune Function, Behavior, and Welfare of the Progeny, Principal Investigator: Dr. Janeen Johnson; protocol number: IACUC_20-19).

The dataset encompassed16S rRNA gene amplicon sequencing reads from sow and piglet fecal DNA generated via paired-end sequencing on an Illumina MiSeq System (2 x 250 cycles) using the method described by [179].

The current study was directed solely towards identification of archaeal sequences in the NGS dataset and fecal DNA samples. Additional results associated with the collaborative research project will be published elsewhere.

Bioinformatics and Databases

Next Generation sequencing (NGS) data analysis was performed using QIAGEN CLC Genomics Workbench (GWB; version 21.0.4) in conjunction with a Microbial Genomics Module for amplicon-based analyses. QIAGEN CLC Genomics Workbench has been developed to support a wide range of bioinformatics applications for genomic, metagenomic, and transcriptomic analyses.

The SILVA 16S rRNA database (v123; 99%) for amplicon-based analyses (Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, Peplies J, Glöckner FO (2013) [178] was used for taxonomic profiling of the pig fecal microbiota.

Profiling of the Pig Fecal Archaeome

For sows and piglet samples, 16S rRNA gene sequencing data showed presence of substantial amounts of archaeal reads (see Figure 4.10). Taxonomic classification of the pig fecal archaeome showed that archaeal sequences belonged to the phylum Euryarchaeota. At genus level, the sequences were assigned to *Methanobrevibacter*, *Methanosphaera*, and yet *unclassified* or

candidate members of the family *Methanomethylophilaceae*. The following figures demonstrate individual archaeal profiles of sows and piglets.

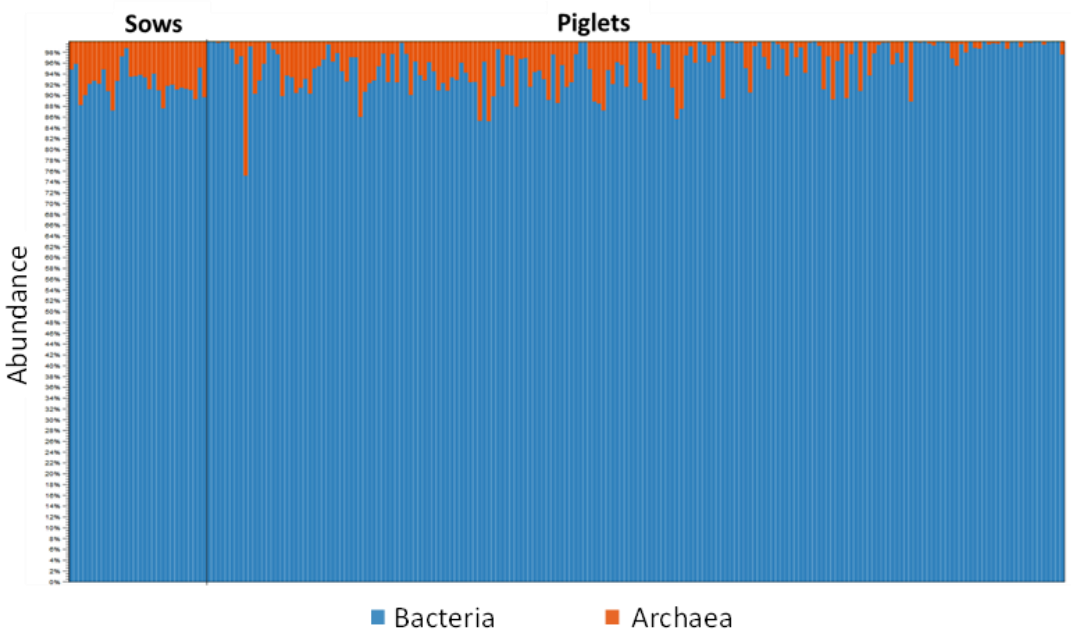


Fig. 4.10. Overview of Kingdom-Level Classification in Sow and Piglet Fecal Microbiota. All sow and most piglet fecal samples showed substantial presence of archaeal reads.

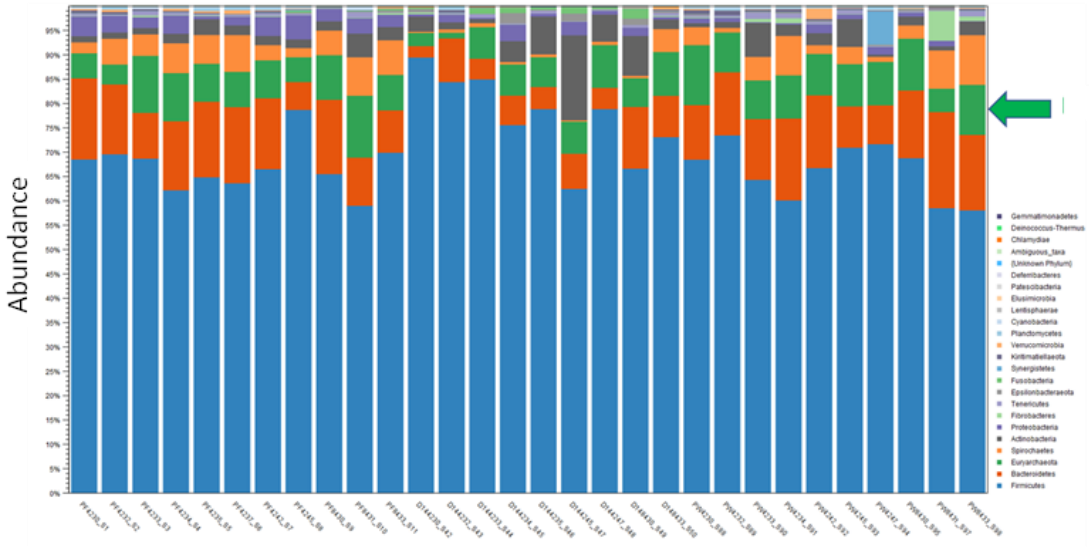


Fig. 4.11 Bacterial and Archaeal Phyla in Sow Fecal Microbiota. The single archaeal phylum Euryarcheota is indicated by the green arrow.

Archaeal Sequence Reads in Pig Feces

Fecal samples from sows and piglets were collected at specific timepoint:

Sow fecal samples were collected on day 114 of gestation (Sow_d114G) as well as day 14 (Sow_d14Lact) and day 20 of lactation (Sow_d20Lact).

Piglet fecal samples were collected on day 0 to 3 after birth (d0-3Lact), day 14 (d14Lact) and day 20 (d20Lact) of lactation as well as post weaning at 24 hours (24hPW), 7 days (7dPW), 14 days (14dPW), and 21 days (21dPW). Figure 4.12 illustrates the timeline of fecal sample collection.

Fig. 4.12 Timeline of Fecal Sample Collection in Sows and Piglets.

The following representative figures illustrate the relative abundances of archaeal 16S rRNA gene amplicon sequences classified as *Methanobrevibacter* (Figure 14.13) and *Methanosphaera* (Figure 14.14) in sow and piglet fecal samples. Comparisons of the *Methanobrevibacter* abundances of male and female piglets at the investigated time points are shown in Figure 14.15 – no significant differences were found in this comparison and between male-female abundance data with the other archaeal groups (data not shown).

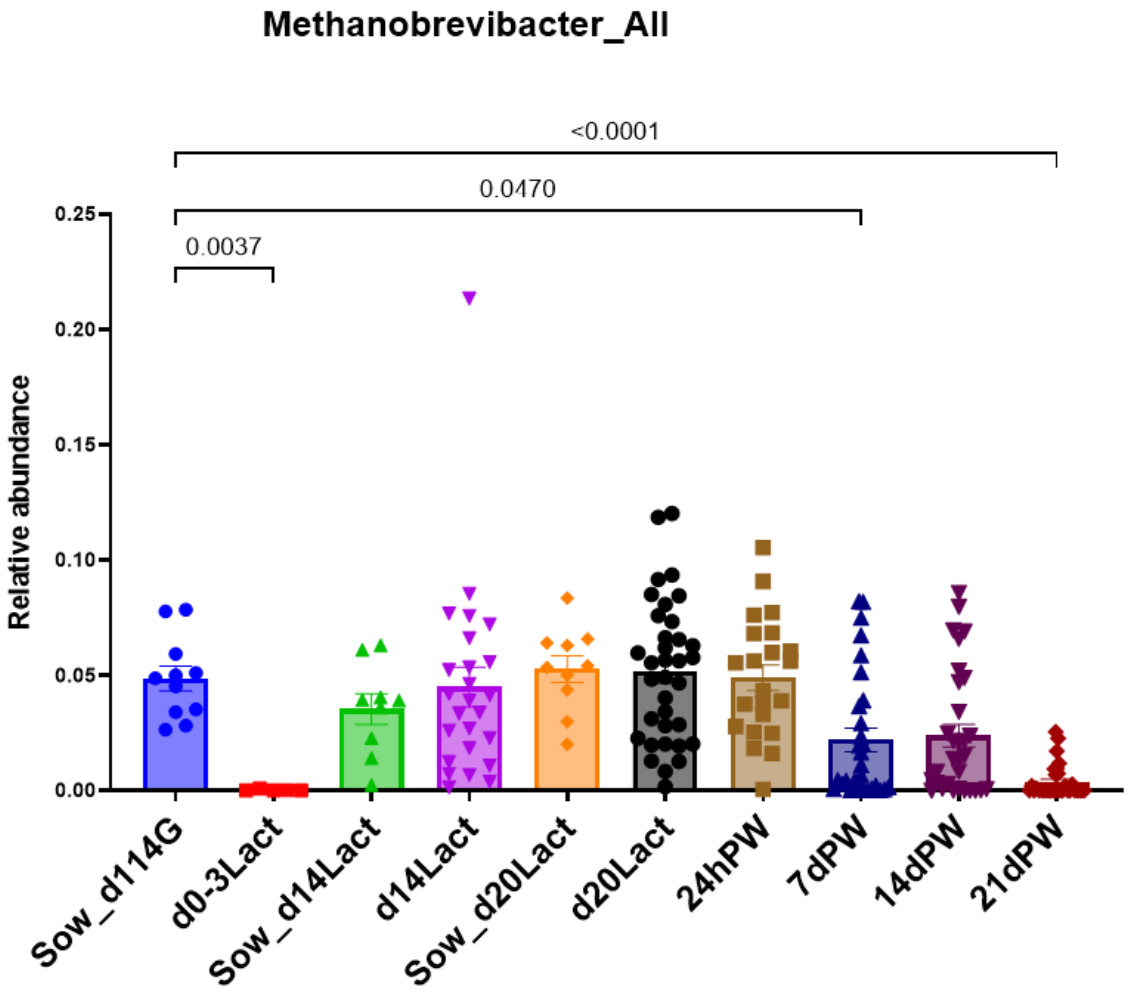


Fig. 4.13. Relative Abundance of *Methanobrevibacter* Sequences in Sow and Piglet Samples. Shortly after birth piglets appeared to have very low abundances in fecal *Methanobrevibacter;* however, a bloom of the taxon appeared later in lactation and was reduced again in the tested post-weaning period. Significant differences to the sow day 114 gestation abundances are indicated with p-values (Kruskal-Wallis test with Dunn's multiple comparisons test).

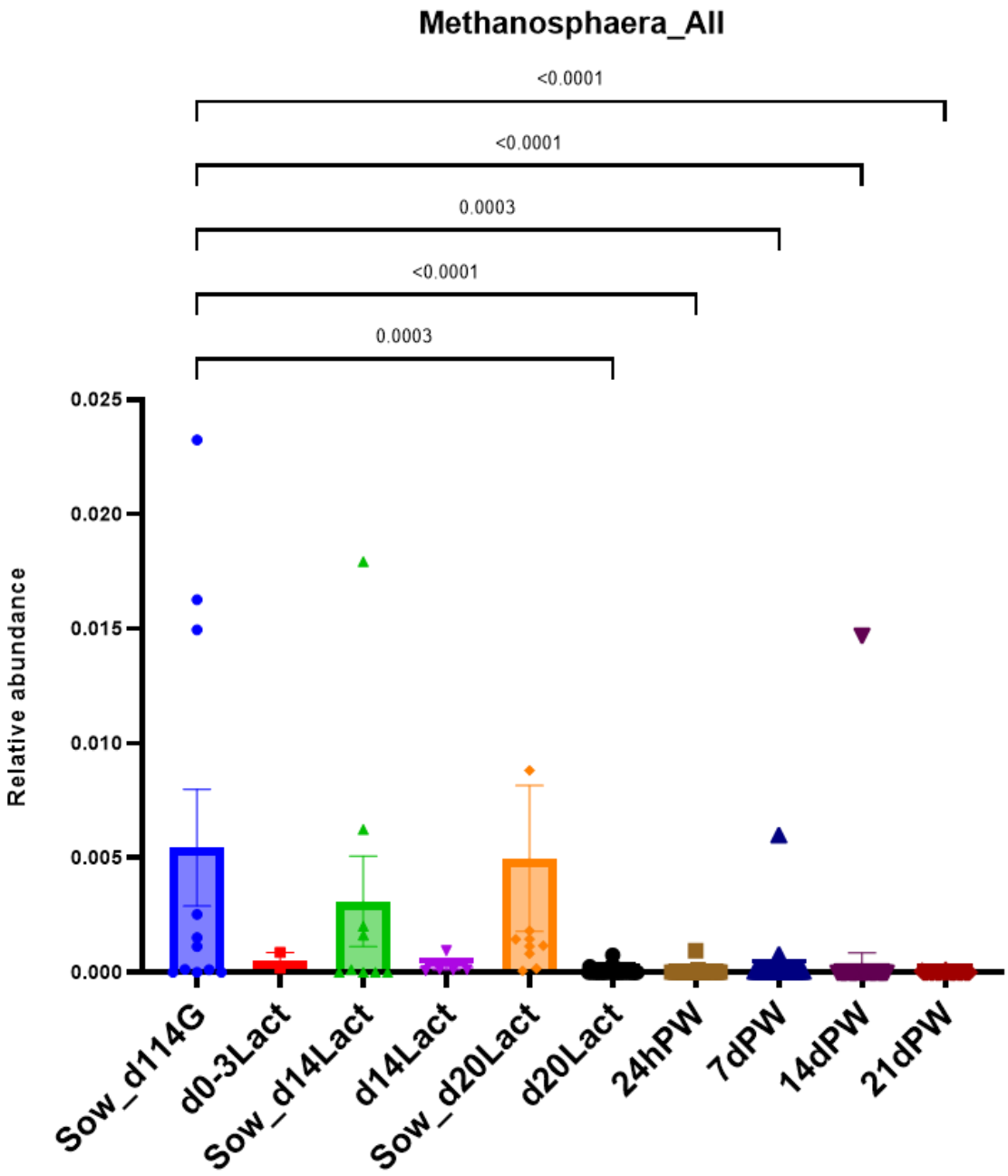


Fig. 4.14. Relative Abundance of *Methanosphaera* Sequences in Sow and Piglet Samples. Most piglets appeared to have very low abundances in fecal *Methanosphaera* while the genus was strongly represented in some sows. Significant differences to the sow day 114 gestation abundances are indicated with p-values (Kruskal-Wallis test with Dunn's multiple comparisons test)

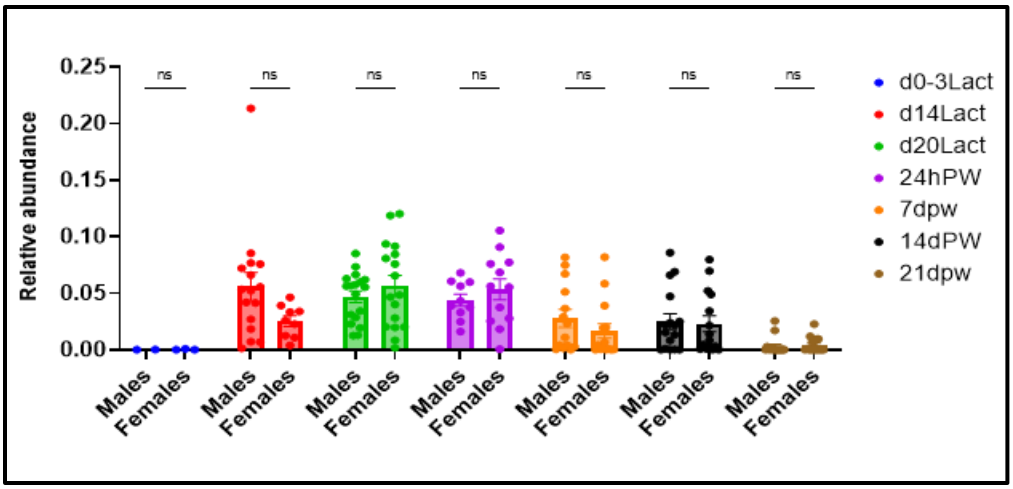


Fig. 4.15 Relative Abundances of *Methanobrevibacter* in Male and Female Piglets. The means of the relative abundances for male versus female piglets at the indicated time points were not significantly different ($p<0.05$; Two-way ANOVA).

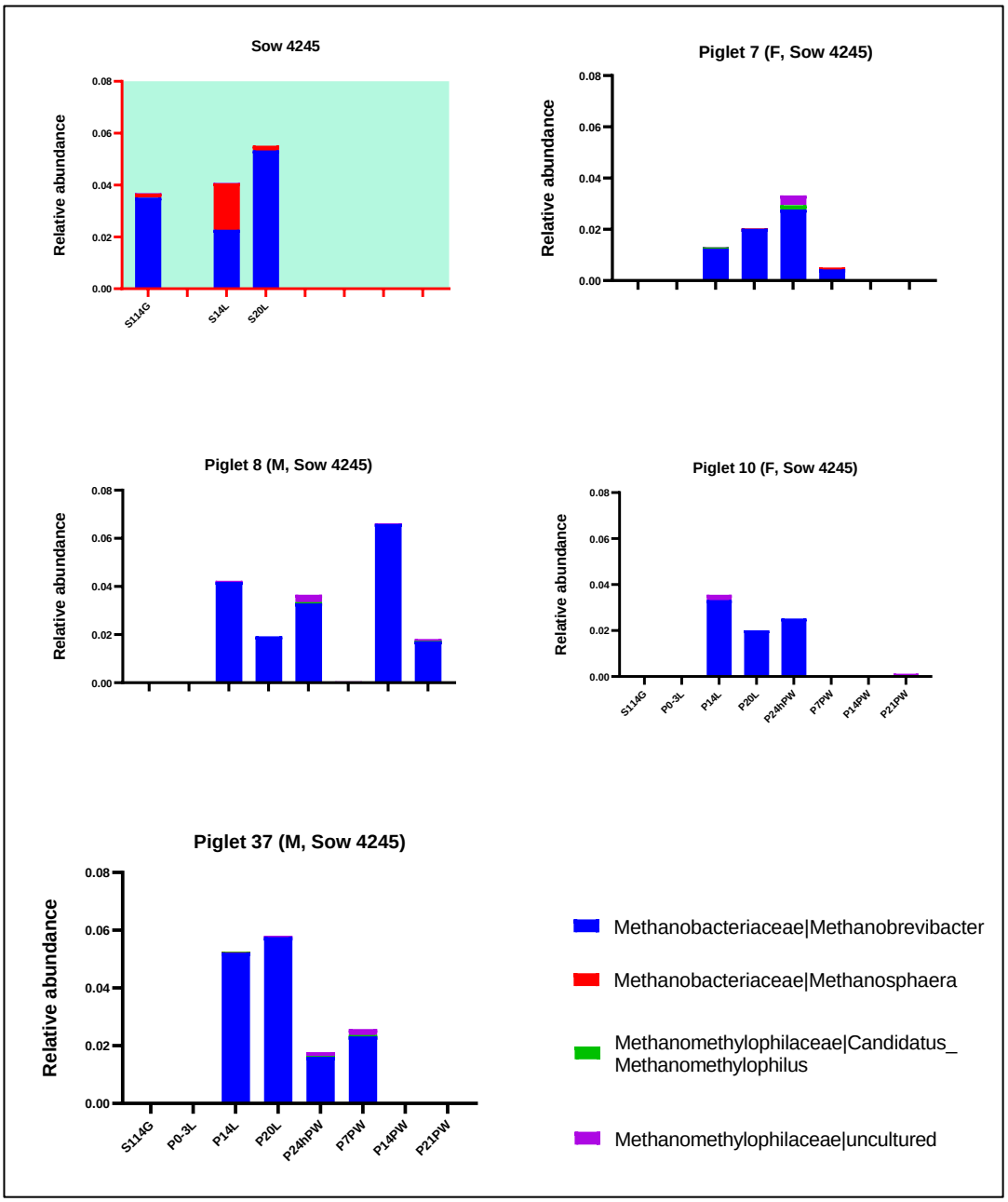


Fig 4.16. Relative Abundances for Archaeal Sequences in a Sow and her Piglets. The comparative analysis of fecal abundances of the archaeal taxa between a sow and her piglets indicates similar predominance of *Methanobrevibacter* in the offspring. However, *Methanosphaera* was detected in the sow, but not in the piglets. Conversely, the piglet samples indicated presence of *Methanomethylophilaceae,* which were absent in the sow feces and thus may have been acquired from elsewhere.

Qualitative PCR for Detection of Archaeal DNA in Pig Feces

To further identify archaea, qualitative PCR was performed using sow and and piglet samples at different time points). Standard polymerase chain reaction (PCR) showed strong bands in pig samples (Figure 14.17). This assay confirmed the presence of archaeal targets.

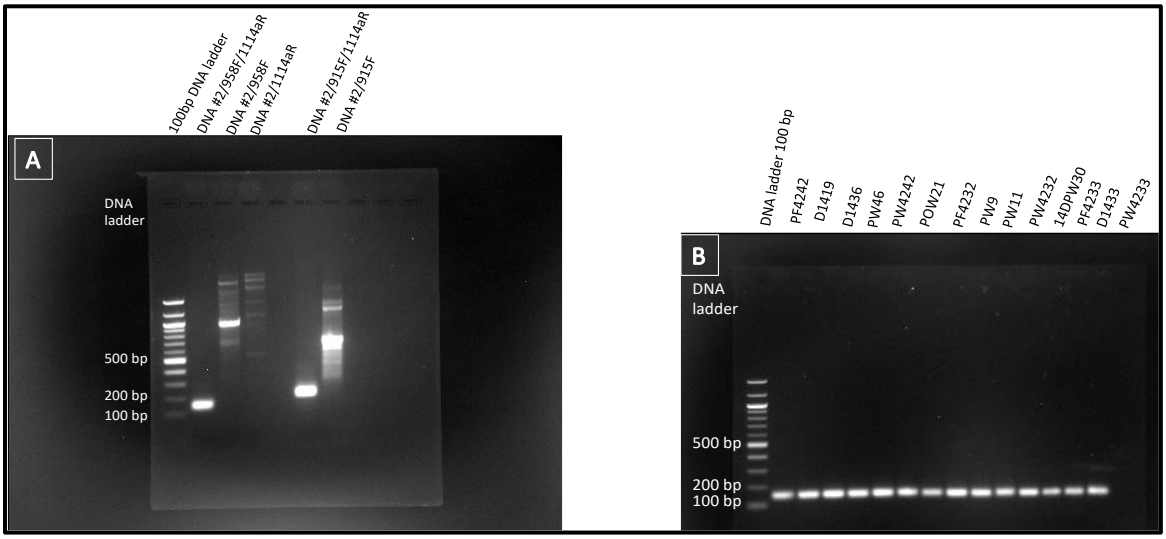


Fig. 4.17. Agarose Gel Electrophoresis Showing Endpoint PCR Results from Pig Fecal DNA. Correct size PCR amplicons are shown with primer pairs ARCH958F-1114aR and 915F-114aR (A). Multiple pig fecal DNA samples yielded the expected PCR bands (B).

Quantitative PCR (qPCR) for Archaeal Sequences in Pig Feces

Quantitative PCR (qPCR) assays targeting the archaeal 16S ribosomal RNA genes were performed as described above. The universal primers, GK1053F and 1391R were used to quantify 16S rRNA genes from bacteria and archaea and the archaea-specific primers Arch 958F and 1114aR were used to quantify 16S rRNA genes of archaea. The quantity of DNA in each sample was measured in triplicates and the mean values, ΔCrt, relative quantification (RQ) values, were determined. Figure 4.18 shows representative results for a sow and some of her piglets.

Correlation of qPCR results with relative abundances in 16S rRNA gene amplicon sequencing data was investigated and yielded mixed results (see Figure 4.18).

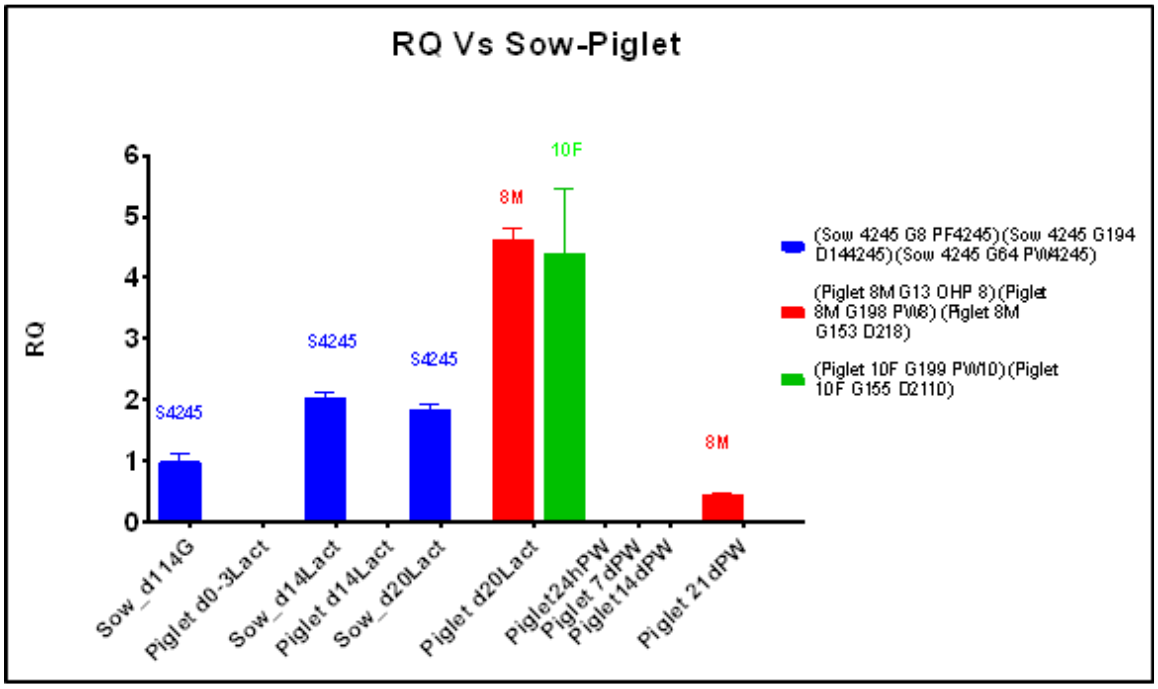


Fig. 4.18. Relative Quantities of Archaeal Targets in Sow 4245 and her Piglets. Sow timepoint day 114 gestation was used as calibrator. The sow qPCR data show relatively good correlation with the animal's sequence data (see Fig. 4.16). However, the relative quantities (RQs) of archaeal targets in piglet samples were higher than anticipated from sequencing results.

CHAPTER V

CONCLUSION

Several studies have reported that gut archaea may play a key role as an important member of the microbiota in animals and humans. According to Hillman et. Al, rat gut microbiota shows presence of *Methanobrevibacter* as the predominant archaea (methanogen) and pig gut microbiota should include *Methanomicrobia* and *Methanosphaera* [119]. It is established in the literature that *Methanobrevibacter smithii* is the predominant genus found in the mammalian gut microbiome [182]. Bioinformatic tools and NGS technologies may allow us to detect archaea in rat small intestine and colon [183]. For our rat project we received 16S rRNA sequencing data and metagenomic data. There was little evidence of archaea in the 16S rRNA sequencing data and therefore, metagenomic data was screened for archaeal sequences. The metagenomic data indicated presence of methanogens and haloarchaea in rat small intestine and colon digesta samples.

Archaeal sequences in rat samples resembled more with haloarchaea and less with methanogens. To further confirm the presence of haloarchaeal sequences and methanogenic archaeal sequences, we performed qualitative and quantitative PCR assays.

The qualitative PCR showed faint bands (expected band size was 150bp) and we did not get a strong band in the agarose gel. Most of the band sizes were greater than the band of interest. There were many non-specific bands in the gel. We optimized the experiment by using different primer combinations and used the touch down approach for amplification of DNA. We further confirmed the qualitative data using qPCR approach. The qPCR data did show lower abundance of archaea in digesta samples from rats and did not match with the NGS data. Further optimization of qPCR assays and sequencing data should be performed. When we compared the qPCR data with NGS data for the archaeal sequences, the data did not match, and we did not find any methanogens in rat samples. It is possible that the archaeal sequences in rat intestinal microbiota may be misclassified and potentially be derived from other microorganisms or extraneous DNA from plants. Both 16S rRNA and metagenomic studies indicate the presence of low quantities of archaea in samples; however, Shotgun metagenomics detected higher diversity of archaea. Therefore, we conclude that in 16S rRNA gene sequencing data in rats, there was no convincing evidence of presence of archaea in the rat small intestine and colon samples. In metagenomic data archaeal sequences may be found in lower abundance DNA samples from the small intestine and colon however, in much lower abundance than bacterial sequences.). Hence, we have reason to believe that metagenomic data needs further analysis.

Limitations

With 16S data only bacteria and archaea can be detected but with WGS species from all 3 domains can be detected and identified. Some of the limitations of 16S data includes taxonomic profiling up to genus level, possibility of PCR and primer biases, and false positive in low-biomass samples. False positives may be found in low-biomass samples due to PCR biases and possible DNA contamination. In our qPCR assay with rat samples non- specific bands were detected indicating that the primers used may not detect archaea. study with rat samples, we saw a lot of non-specific bindings. The reason for non-specific binding could be that the primers were

not specific. It's possible that primers did not pick up the archaea. The 16S rRNA sequencing data for rats indicated absence of archaea. The reason for that could be there might be low archaea in the lab rats, or the sequencing approach might have missed the archaeal sequences due to primer biases. Wild rats may show presence of diverse archaea in their small intestine and colon. Alternatively, studies could be performed with different strains of rats.

Furthermore, in our study the data was collected from adolescent rats (about 8-10 weeks old). To our knowledge there is no comprehensive study published with microbiota analysis at different ages.

Metagenomic data allows for taxonomic profiling to species or strain level. The limitation with metagenomic data is that it can generate false positives and possibly show overwhelmingly high levels of host read contamination, which could reduce the yield of microbial sequences.

The 16S rRNA gene sequencing data for pigs was analyzed and indicated presence of methanogens which was confirmed by qualitative and qPCR. This supports the hypothesis that methanogens are present in pigs. We found *Methanobrevibacter, Methanosphaera, and Methanomethylophilaceae* in pig fecal samples. The presence of *Methanobrevibacter* in sows and early in piglets suggests that this genus could be transferred from mother to offspring at birth or soon thereafter. On the other hand, *Methanosphaera* was detected in sows but absent in most piglets. Indicating that this genus may not be able to immediately establish in the piglet gut. Conversely, *Methanomethylophilacea*e were associated solely with piglets, thus an environment could be a source for colonization of piglets with this genus. Interestingly, *Methanomethylophilus* has been found to be a part of skin microbiome [169] and *Methanobrevibacter* is known to be found in mothers' milk and in feces [169].

In our study, we analyzed the 16S rRNA sequencing data from pigs to determine if there was a difference in the relative abundance of methanogens between sows and their piglets. Our experiment lasted 45 days. *Methanobrevibacter* was found in the majority of the sows and piglets as the leading methanogen. More studies are needed to confirm vertical transmission of *Methanobrevibacter* from sow to piglets. Several findings suggest that *Methanobrevibacter* may be found in mothers' milk and that could be a source of *Methanobrevibacter* heredity in piglets. According to one study, in mammals, the initial exposure to microbes occurs at parturition in the birth canal. The mode of delivery, vaginal or cesarean section (CS), and nutrition provided during early stages of life, have a significant impact on the intestinal flora. Furthermore, a recent study on the microbial composition of the umbilical cord found that maternal transfer is possible and that it may occur during gestation [70]. Methanogen abundance was decreased in both male and female pigs by 42-45 days after birth. Because there is no data available 45 days after birth, it is impossible to anticipate how the archaeome will develop until adulthood.

Few reports indicate that *Methanomethylophilus* may be a part of skin microbiome and therefore found on the mother's skin. Literature suggests that as piglets grow, they will acquire adult microbes just like humans. The structure and composition of the gut microbiota in animals are determined by many factors such as genetics, age, phylogeny, diet, and surrounding environmental conditions during birth [5, 184]. In animals and humans, microbial colonization, therefore, begins at birth and continues to diversify in the initial days of life based on exposure to environmental microbiota, which depends on the host habitat, diet, and physiology [185]. The intestinal microbiota diversity has been reported to change three times from birth to after weaning in young piglets [186]. The diet of the sow affects the piglet microbiota, and the post weaning diet affects the microbial diversity significantly [70].

Our results indicate that there was no statistically significant difference in the microbiome between males and female piglets. More archaeal diversity was observed during lactation (pre

wean) than immediately after birth (day 0-3), or post wean period. However, with separation of the sexes, archaea might show diversification.

We analyzed the 16S rRNA gene sequencing data to confirm studies performed by different researchers to see if archaea specifically, methanogens are present in animal gut of rats and pigs. We did not gather strong evidence for archaea in lab rats but did find relatively high abundance of methanogens in pig samples find any methanogens in lab rats used in our study, but we found methanogens in pig samples. Study performed by Su et al observed *M. thaueri* and *M. millerae* in Yorkshire and Meishan piglets [12].

Our study was similar to other studies in which *M. smithii* was found to be a dominant methanogen in piglets after birth and that methanogen diversity decreased after birth, but abundance of methanogens increased after birth in piglets at least up to 14 days after birth. Study performed by Su et al observed *M. thaueri* and *M. millerae* in Yorkshire and Meishan piglets. Interestingly, our study found other methanogens such as *Methanosphaera, Methanomethylophilaceae* in piglet feces. The difference in methanogens profiles could be because of differences in diet or breeds used in these studies.

Future studies should include metagenomic analysis of fecal samples from sows and piglets to compare and confirm findings from 16S rRNA gene sequencing data and take advantage of greater taxonomic resolution of this technology. Metagenomic analysis will allow for functional analysis and provide a comprehensive understanding of the role of methanogens in the pig intestine.

In summary, my research hypothesis that “The rat small intestine and colon digesta samples would show presence of methanogens” was not unequivocally supported with the qPCR and sequencing data. The reason could be that there might not be enough archaea present in rat small intestine and colon digesta samples and/or the primers for sequencing library preparation did not

pick up archaeal sequences. It is reasonable to conclude that very low abundances of archaea could still be present in rat small intestine and colon samples.

In pigs, however, my research hypothesis that "The pig fecal samples would show presence of archaea specifically methanogens" was supported by the qPCR and sequencing data. We found *Methanobrevibacter* to be the dominant genus in sow and piglet fecal samples. Sequencing data shows that piglets may inherit *Methanobrevibacter* (dominant) from their sow and possibly other methanogens (*Methanomethylophilus*) from the environment. Further studies are necessary to determine the colonization of methanogens in piglets from mother and the environment and the impact of archaea on pig health and disease.

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