DIETARY SODIUM AND THE HUMAN GUT MICROBIOTA

by

Katarina Smiljanec

A dissertation submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Applied Physiology

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by

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Every time I played the Game of Life as a kid, I wanted to be a scientist or a doctor. Little did I know that I could be both. Running had made me curious about the human body and how it responds to different foods and nutrients. I did not know that it would lead me to Dr. Brenda Davy's lab at Virginia Tech for a master's degree nor to Dr. Shannon Lennon's lab at the University of Delaware (UD) for a doctorate degree, but I knew that I would be a scientist.

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ABSTRACT

A disrupted gut microbial profile mediates pathogenesis of hypertension (HTN) and cardiovascular disease (CVD), and humans with HTN present with lower microbial diversity than their normotensive counterparts. High sodium intake is a modifiable risk factor for HTN and CVD but only 11% of the United States (U.S.) adults meet the recommended dietary sodium intake set by the Dietary Guidelines for Americans (2,300 vs 3,400 mg/d). High sodium diets (HSDs) disrupt the microbial profile through increased inflammation, in particular by activating proinflammatory T helper 17 (TH17) cells and suppressing anti-inflammatory T regulatory cells (Treg). **OBJECTIVES:** 1.a) Determine the effect of an HSD on gut microbiota composition in healthy adults. 1.b) Determine the effect of an HSD on gut microbiota composition in men and women. 2. Determine the effect of an HSD on the abundance of TH17 and Treg cells in the peripheral blood of healthy adults. **HYPOTHESES:** 1.a) Gut microbial composition will exhibit more deleterious changes following an HSD compared to a recommended sodium diet (RSD) as evidenced by a reduction in microbial diversity. 1.b) Gut microbial composition in men will exhibit more deleterious changes following an HSD compared to women, as evidenced by greater reductions in gut microbial diversity. 2) An HSD would increase the serum abundance

of TH17 cells and reduce abundance of Treg cells compared to an RSD. **METHODS:** Healthy, non-hypertensive and non-obese young adults were recruited. Subjects underwent a 10-day HSD (6,900mg sodium/d) and a 10-day RSD (2,300 mg sodium/d) intervention in random order. Microbial diversity (Shannon diversity index) was assessed from a fecal sample that subjects provided on Day 0 and Day 10 of each intervention. A blood sample was obtained on Day 10 of each intervention to quantify TH17 and Treg cells. Compliance was assessed from urinary sodium excretion obtained via 24-hour urine collection on Day 9, and blood pressure (BP) was measured using a 24-hour ambulatory BP monitor on the same day. **RESULTS:** Thirty-one subjects completed both interventions. Day 10 fecal samples were analyzed from 20 subjects (8M/12W, 25 ± 1 yrs, body mass index 23.1 ± 0.5 kg/m2), and day 0 and day 10 samples (complete sets) were analyzed from a subset of 16 subjects. Urinary sodium excretion and serum sodium levels were increased following an HSD ($p = <0.001$, $p = <0.002$, respectively; $n = 20$). Systolic BP remained stable ($p = 0.222$). Shannon diversity index was comparable between RSD and HSD in all subjects (RSD: 2.62 ± 0.18; HSD: 3.11 ± 0.21; $p = 0.088$; $n = 20$). There were also no significant differences in Shannon diversity index between sexes (men: RSD: 2.52 ± 0.29, HSD: 3.01 ± 0.27; women: RSD: 2.69 ± 0.23, HSD: 3.18 ± 0.32; diet * sex interaction $p = 0.937$). There were no differences in diversity within and between each intervention in samples from complete sets (RSD: Day 0: 2.91 ± 0.35, Day 10: 2.54 ± 0.27; HSD: Day 0: 3.36 ± 0.33, Day 10: 3.19 ± 0.33; diet * day interaction $p = 0.062$; $n = 16$). Inclusion of sex in the analysis revealed no significant differences in Shannon

diversity index (diet * day * sex interaction p = 0.925). TH17 and Treg cells were quantified from 22 subjects (13M/9W, 25 ± 1 yrs, BMI 22.9 ± 0.5 kg/m2). Urinary sodium excretion and serum sodium levels were increased following an HSD (p = <0.001, p = <0.010, respectively). Systolic BP remained stable (p = 0.129). Abundance of TH17 cells did not differ between HSD and RSD (RSD: 0.35 ± 0.06%, HSD: 0.28 ± 0.02%, p = 0.262). Abundance of Treg also remained unchanged (RSD: 0.99 ± 0.11%, HSD: 0.28 ± 0.02%, p = 0.552). **CONCLUSION:** An HSD did not affect the gut microbial diversity nor the abundance of TH17 and Treg cells in the peripheral blood. Further studies should investigate the influence of HSD in a larger sample and target individual characteristics, such as salt sensitivity and middle to advanced age, that may be mediating changes in the gut microbial composition on HSD.

Chapter 1

REVIEW OF LITERATURE

1.1 Introduction

Dietary sodium intake by Americans greatly exceeds the amount recommended by the Dietary Guidelines for Americans (DGA)[1] and American Heart Association (AHA)[2]. The DGA state sodium intake should be no more than 2,300 mg/d[1] whereas AHA further reduces this recommendation to 1,500 mg/d for optimal health, and in particular for older adults, African Americans, and individuals with hypertension (HTN)[2]. Both recommendations call for major diet modifications as sodium intake in Americans is 50% above the recommended intake encouraged by the DGA. According to the National Health and Nutrition Examination Survey, the median sodium consumption in the United States (US) has steadily increased from 1999 to 2016[3]. Adults in the age range of 18-50 years consume the greatest amount of sodium per day: 3,427 mg/d[3].

High dietary sodium intake is a risk factor for HTN and cardiovascular disease (CVD), the leading cause of death in the US[4]. Adherence to sodium intake guidelines has decreased from 34% in 1999-2004 to 23% in 2011-2016[3] while the DGA

recommendations remained unchanged (2,300-2,400 mg/d)[5; 6; 7; 8]. Alarmingly, less than 10% of individuals at high risk for sodium-related conditions (i.e. adults over 51 years of age, African Americans, those with HTN, chronic kidney disease, and diabetes mellitus) adhere to the guidelines[3]. In addition to those well-known consequences of chronic sodium intake, new findings suggest dietary sodium may be involved in pathogenesis of conditions such as inflammatory bowel disease[9] and multiple sclerosis[10] indicated by immune system activation and alterations of gut microbial composition and function.

Human gastrointestinal (GI) microbiota is a rich population of bacteria, viruses, archaea, and eukaryotic microbes that reside in the GI tract [11]. The gut microbiota communicates with an array of bodily systems, and especially with the immune system[12]. With the advancement of sequencing technologies[13], research on the so called "forgotten organ"[14], the microbiota, has bloomed in the past two decades and is focused on examining the role gut microbiota may have in various disease states. Disruption in the gut microbiota composition and function (i.e. gut dysbiosis) is observed in HTN[15] and recent evidence links the gut microbiota to CVD pathophysiology via modulation of gut-immune axes [16; 17]. This review of literature focuses on the effects of dietary sodium on the gut microbiota composition in men and women, as well as the effects of dietary sodium on the immune system.

1.2 Gut microbiota overview

1.2.1 Gut microbiota composition and function

Human gastrointestinal (GI) microbiota refers to a population of bacteria, viruses, archaea, and eukaryotic microbes that resides in the GI tract. However, in this review the term "gut microbiota" refers exclusively to bacterial communities as most research done thus far examines bacterial populations. Contrary to widespread belief that the ratio of bacterial to human cells is 10:1 [18; 19], which stems from an inaccurate estimate from more than five decades ago [20], the ratio of bacterial cells to human cells is 1:1 [21] in the human body. Nevertheless, the microbial genome should not be understated as it is ~150 times the size of the human genome [22]. The microbiota populates the GI tract in different abundances. In the upper GI tract, such as the stomach, there are 10^3 - 10^4 bacteria present per milliliter of content, a number that increases along the distal GI tract and reaches 10^{12} bacteria per milliliter of content in the colon [23].

Gut microbiota communicates with many organ systems and mediates multiple physiological functions. In the gut, bacteria utilize dietary fiber as an energy source that cannot be broken down by host enzymes thus, resulting in various metabolites, such as short-chain fatty-acids (SCFA). Bacteria also produce vitamins (biotin, vitamin K), stimulate gut tissue (villi, intestinal crypts) and immune system development, and metabolize primary bile acids into secondary bile acids. Gut

microbiota is predominantly composed of Bacteroidetes and Firmicutes phyla, with lower abundances of Actinobacteria, Proteobacteria, and Verrucomicrobia [24]. Even though all humans share these common phyla, the proportions of each slightly differ. These differences in microbial abundance and diversity depend on genetic factors [25], dietary patterns [26], and geographical region [27], among others. Over 2,900 bacterial species that reside in the human gut have been identified [28; 29], ranging from mutualistic (a term often used interchangeably with commensal) to opportunistic that compete for resources to establish their niche and ensure survival.

1.2.2 Gut microbiota assessment

The beginnings of mammalian GI microbiota research date to the early 1960's when it was evident that the microbiota influences growth rate, food utilization, and resistance to infection in mice [30]. At the time the only method of assessing microbial species present in the GI tract was culturing them. However, only aerobic species could be identified with culturing since anaerobes cannot grow in a Petri dish. This presented a challenge as the gut is dominated by anaerobes which can be cultured in now-available anaerobic chambers [31]. Recent advancements in DNA sequencing technology and computational methods have increased availability and feasibility of assessing the gut microbiota. The main three methods used today are 16S rRNA sequencing to create the compositional profile, metagenomics sequencing to assess the

function, and metatranscriptomics to assess the active gene expression in microbial communities [32].

Various indices are used to evaluate gut microbial diversity within (alpha diversity) and between (beta diversity) samples, and can be visualized using ordination techniques (Table 1.1) [33]. Diversity can be assessed using qualitative measures that evaluate for presence or absence of data for each taxon (measure of richness) or quantitative measures where the abundance of each taxon is considered (measure of evenness). Taxons are also referred to as operational taxonomic units (OTUs), which are gradually being replaced with amplicon sequence variants (ASV) that provide greater resolution and exact sequence match to the species. Alpha diversity is expressed as richness and evenness of the sample. Richness refers to how many different species there are in a sample (i.e. how many different OTUs or ASVs), whereas evenness refers to how even or uneven distribution of those OTUs/ASVs is. For example, a community with 200 OTUs/ASVs has greater richness than a community with 50 OTUs/ASVs. A community in which all 200 OTUs/ASVs are evenly abundant has high evenness, opposed to a community that has different abundances of each OUT/ASV (low evenness). Aside from OTUs/ASVs, another metric is phylogenetic diversity that calculates the distance of phylogenetic branches between species.

Table 1.1: Categories of diversity measurements.

	α diversity	**β diversity**
Qualitative	**Richness:** **Species-based:** Chao 1 Abundance-based Coverage Estimator Rarefaction **Divergence-based:** Phylogenetic Diversity (PD)	**Species-based:** Sörensen index Jaccard index **Divergence-based:** Unweighted UniFrac Taxonomic Similarity
Quantitative	Richness and/or Evenness: **Species-based:** Shannon's index Simpson's index **Divergence-based:** Theta	**Species-based:** Bray-Curtis dissimilarity Sörensen quantitative index Morisita-Horn measure **Divergence-based:** Weighted UniFrac
Compositional Statistical Approaches		Aitchison distance
Ordination Techniques		Principal coordinates analysis (PCoA) Principal components analysis (PCA) Non-metric multidimensional scaling (NMDS)

Adapted from: Lozupone and Knight. *FEMS Microbiol Rev* 2008.[33]

One can also use species-based measures where all taxa are treated as equally related to one another (phylogeny unknown) (Figure 1.1.A), or divergence-based measures where the phylogenetic distance between each pair of taxa is considered (phylogeny known) (Figure 1.1.B). Figure 1 illustrates three communities of three

different animal species represented by individual OTUs. In Figure 1A, the star phylogeny portrays unknown phylogeny between these animals and in the principal coordinates analysis (PCoA) plot at the bottom they all appear equally related (red, blue, and yellow circles are equally close to each other). In Figure 1B, phylogeny of the communities is known and represented by phylogenetic branches of different lengths (mammals are more related to each other than to birds). This is represented in the PCoA plot where birds (yellow circle) are further away from mammals (red and blue circles).

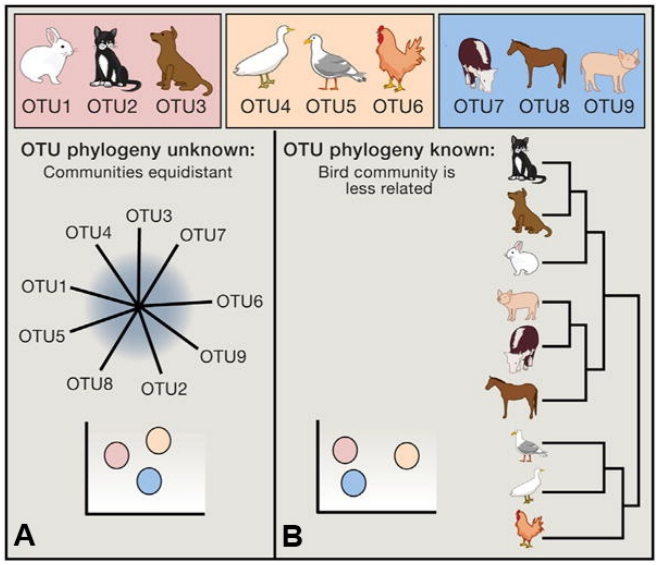


Figure 1.1: Phylogeny-based beta diversity metrics. OTU, operational taxonomic unit. Source: Goodrich et al. [32]

There is a rigorous debate that traditionally used metrics of alpha and beta diversity are misleading as they treat microbial counts as absolute microbial abundances when they are, in reality, relative abundances [34]. Sequencing platforms have a fixed number of slots for DNA reads so sequenced genetic material portrays relative abundance of material present in the ecosystem, not absolute [34]. For example, if lions and ants are a part of the same ecosystem, an increase in the number of lions may not impact the number of ants, but their proportion will change. The relation between absolute abundances in the ecosystem and relative abundances after sequencing are not predictable [34]. For this reason, microbial data should be analyzed as compositional data, or data described as proportions [34]. A metric proposed in calculation of beta diversity is Aitchison distance (Table 1.1). While the majority of current research uses traditional methods of microbiome data analysis, new research should turn to compositional approaches.

Visualization of beta diversity is achieved using ordination techniques that reduce dimensionality of data to two or three-dimensions visualized in a scatterplot. The most common traditional ordination technique is PCoA in which principal coordinates represent a fraction of variability (Figure 1.2.A). PCoA considers presence or absence of operational taxonomic units (OTUs). Points that are closer together or appear to cluster represent microbial communities that are more similar in their sequence composition. A compositional alternative to PCoA is a principal component analysis (PCA) biplot that also identifies which variables contribute most to clustering

or spread of data (ex. age). Heatmaps are also used to visualize under or over representation of certain OTUs between samples where a color spectrum is used to indicate higher and lower abundance (Figure 1.2.B).

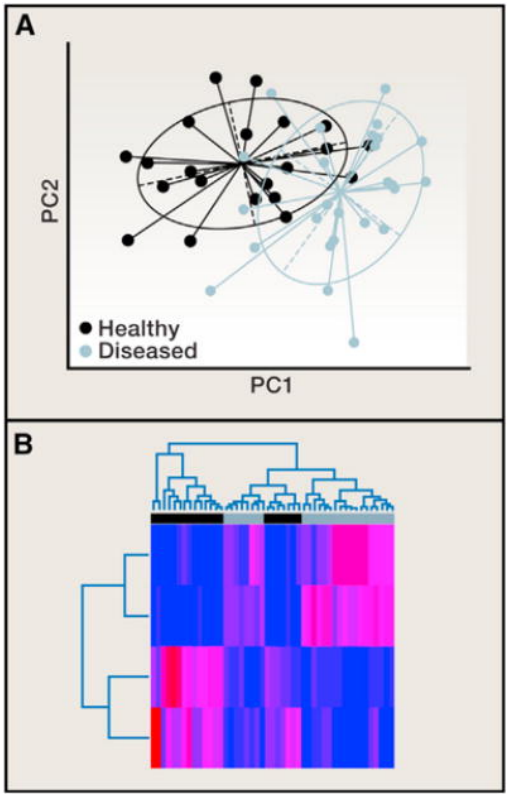


Figure 1.2: A) Principal Coordinates Analysis, B) Heatmaps. Blue indicated low abundance and red indicated high abundance. Source: Goodrich et al. [32]

1.3 Gut microbiota and the immune system

1.3.1 Brief immune system overview

The two main branches of the immune system are the innate and adaptive immune systems. They differ in speed and specificity of defense as innate responses are the quick, first line of defense to pathogens, whereas the adaptive response is slower, antigen-specific, and has memory [35]. Innate immune responses include actions from neutrophils, monocytes, macrophages, complement, cytokines, and acute phase proteins. Adaptive immune responses include specific actions of the T and B lymphocytes [35].

All immune cells are recognized by a specific receptor on the cell surface. T cells are recognized by the CD3 receptor and are identified as CD3+. Subpopulations of CD3+ T cells are T helper (T_H) cells, cytotoxic T cells, and natural killer cells, identified by CD4, CD8, and CD56 receptors, respectively. Naïve CD4+ T cells differentiate to T_H1, T_H2, T_H17, and T regulatory (T_{reg}) cells that further differ by extracellular and intracellular receptors and the cytokines they produce.

T_H1 and T_H2 cells are morphologically the same but produce different cytokines and elicit different responses [35]. T_H1 cells produce interleukin (IL)-2 and interferon γ (IFNγ) that induce cell-mediated inflammatory responses as they prompt

cytotoxic T cell development and macrophage activation to control the replication of intracellular pathogens [35]. T_H2 cells produce IL-4, IL-5, IL-6, and IL-10 that stimulate antibody production by B cells and their response is associated with allergic reactions [35].

In the presence of transforming growth factor β (TGFβ) and the proinflammatory cytokines IL-6 and/or IL-21, naïve CD4+ T cells differentiate into T_H17 cells. This causes inflammation and autoimmunity through production of IL-17, IL-22, and IL-23, and recruitment of neutrophils. On the other hand, in the presence of TGFβ but without the presence of proinflammatory cytokines, naïve CD4+ cells differentiate into T_{reg} that oppose the effects of T_H17 cells. T_{reg} cells release anti-inflammatory IL-10 and TGFβ and maintain immune homeostasis [36]. Thus, TGFβ is a critical factor for both T_{reg} and T_H17 homeostasis.

1.3.2 The immune system in the GI tract

The gut-associated lymphoid tissue (GALT) and draining lymph nodes are the main locations for priming adaptive immune cell responses in the gut. Effector immune cells are largely present in the lamina propria (B cells, T cells, dendritic cells, macrophages, eosinophils and mast cells) and the intestinal epithelial layer (T cells)

(see Figure 1.3). Altogether, lamina propria and the epithelium house the largest population of T cells, plasma cells, and macrophages in the body.

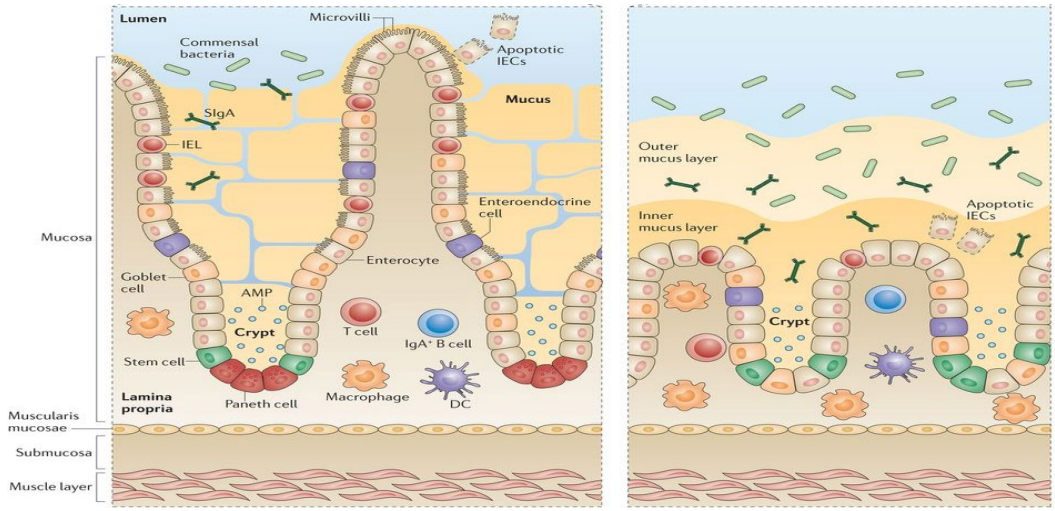


Figure 1.3: Mucosal surface in the small (left panel) and large intestine (right panel) and associated immune system framework. Source: Mowat AM & Agace WM. [12]

Figure 1.3 represents the mucosal surface in the small (left) and large (right) intestine and the associated immune system framework. The small intestine has deeper crypts due to long microvilli. The surface epithelium contains various cells including intestinal epithelial cells (IECs), enteroendocrine, intraepithelial lymphocytes (IELs),

Paneth cells, and goblet cells. Stem cells at the bottom of the crypts develop into intestinal epithelial cells (IEC) that migrate towards the tip of the villi and are replaced every few days. Similarly, stem cells give rise to mucous-secreting goblet cells and Paneth cells. Paneth cells contain antimicrobial peptides (AMP) and migrate towards the bottom of the crypts. The majority of immune cells are found in lamina propria, with exception of IELs (mainly T cells) which are found between IECs in the epithelium layer. Moving distally along the small intestine, villi become shorter and thicker, goblet and Paneth cells increase in number, and IELs decrease in number. The large intestine does not contain villi and has smaller crypts, but has a thicker mucous layer produced by goblet cells that house commensal bacteria. Paneth cells and IELs are very rare in this section of the intestine. The epithelium acts as a protective barrier from commensal bacteria and serves mainly for reabsorption of water from feces.

1.3.3 Gut microbiota and T cell interactions

Interactions between the host immune system and gut microbiota is at the forefront of immune system action as commensal bacteria must be tolerated by the immune system. There are several adaptations that allow bacterial tolerance. For instance, bacteria is coated with the highly abundant secreted Immunoglobulin A (sIgA) [37], a critical component of both host and bacterial homeostasis as it creates biofilms over bacterial communities to reduce adherence of pathogens [38] and bacterial inflammatory signaling [39; 40]. Another example is that the capsule of

Bacteroides fragilis contains immunomodulatory molecule polysaccharide A (PSA) that signals T_{reg} cells to produce anti-inflammatory IL-10 [18] which allows it to enter the colonic mucosa [41].

Induction of various lymphocyte subsets may be regulated by a distinct component of the microbiota [42; 43; 44; 45; 46]. This has been demonstrated in germ-free (GF) mice as they have significantly lower abundance of CD4+ T cells compared to mice colonized with specific-pathogen-free (SPF) microbiota [44] (i.e. more diverse microbiota, indicating microbial colonization induces immune system response). However, colonizing GF mice with the commensal bacteria *Bacteroides fragilis* restores CD4+ T cells to a level comparable with conventionally raised mice, as well as corrects splenic anatomical and developmental defects seen in GF mice [44]. The driver of these changes is PSA as CD4+ T cell abundance is lower in GF mice colonized with *Bacteroides fragilis* lacking PSA [44]. Furthermore, cytotoxic T cells (CD3+CD8+) and B cells (CD19+) are not affected by PSA presence or absence [44] indicating PSA is only necessary for development of CD4+ T cells.

Commensal microbiota also induce peripheral T_{reg} cell generation [42; 43]. T_{reg} cells are present in the highest abundance in the small intestine and colonic lamina propria than in any other tissue [42]. GF mice have comparable levels of small intestine T_{reg} cells as SPF mice, however they present with much lower levels of colonic T_{reg} cells [42]. Colonizing GF mice with microbiota from the SPF mice increases colonic

T_{reg} levels indicating colonic microbiota drives T_{reg} cell accumulation in the large intestine [42]. Specifically, colonizing GF mice with commensal *Clostridium* strains increases T_{reg} levels with a concomitant increase in TGFβ production necessary for their development [42].

The most prominent immune cells in the gut are T_H17 cells that accumulate in the presence of commensal gut microbiota [45]. Their maturation is dependent on gut microbiota-induced cytokines, particularly IL-1β [47]. T_H17 cell development is induced by certain mutualistic bacteria, such as those from the genus *Arthromitus* belonging to the group of segmented filamentous bacteria (SFB) which are known to enhance resistance to pathogens [48]. GF mice present with greater numbers of anti-inflammatory T_{reg} cells and lower amount of T_H17 cells [45]. Consecutively colonizing the gut with SFB induces T_H17 cell development and protects against pathogens [45]. The relation between T_H17 and T_{reg} cells is inverse as T_H17 cells are decreasing in number along the GI tract, whereas T_{reg} cells are increasing [49]. On the other hand, T_H1 and T_H2 cells are represented in similar number along the GI tract [50]. T_H1 cells are also increased by colonizing GF mice with SFB but to a lesser extent than T_H17 cells [48].

Certain gut microbial taxa are associated with specific bacterial-induced cytokine responses. In a study by Schirmer et al., three bacterial stimulations (*Escherichia coli*-derived lipopolysaccharide (LPS), *Staphylococcus aureus*, and

Bacteroides fragilis) were used to induce cytokine production in human peripheral blood mononuclear cells (PBMCs) or whole blood from 500 healthy subjects [51]. The results show that the gut microbiome highly impacts TNFα and IFNγ production capacity, but to a lesser degree IL-1β, IL-6, IL-17 and IL-22 production. However, the latter cytokines are associated with specific taxa. These cytokines are also associated with the microbiome in a stimulus-dependent manner. For example, associations with microbial taxa and IL-17 was related to a *S. aureus*-induced response. Genera *Clostridium IV* and *XIV* were positively associated with IL-17, whereas genera *Faecalibacterium* and *Atopobium* were negatively associated with IL-17 production[51], as they may inhibit it [52]. Some microbial taxa (*Alistipes spp*., *Clostridium spp*., and *Bilophila spp*.) and TNFα were related exclusively to an LPS-induced response [51]. These observations were also followed by certain functional changes and release of metabolic products that serve as communication between microbes and the host immune system, opposed to direct interaction with the immune cells. Altogether, the immune system recognizes gut bacteria and their products with high specificity.

1.4 Sex differences in gut microbiota composition

Gut microbial composition is dependent on sex [53; 54] and distinct differences exist in the gut microbiota between male and female rodents [54; 55; 56] and men and women [57; 58; 59]. Term *microgenderome* was coined as the evidence for sex-specific

microbe-host interactions was mounting. These sex differences become most evident at puberty and are most distinct in adulthood [56]. Male mice have reduced alpha diversity as evidenced by lower Chao1 and Shannon diversity indices, and a lower number of OTUs compared to female mice [55]. Differences in specific taxa were also observed as Ruminococcaceae, *Ruminococcus*, and *Anaerostipes* were higher in male mice and Peptostreptococcaceae were higher in female mice [55]. Metabolic profiling of SPF and GF mice reveals clustering based on sex in SPF mice but not in GF mice [56]. Gut microbiota influences the levels of sex hormones as fecal transfer from adult male mice to female SPF weanlings resulted in elevated testosterone during puberty in recipients, but not adulthood, compared to female to female transfer or unmanipulated females [56]. These changes also induced sex-specific metabolic outcomes and protection from type 1 diabetes [56]. Org and colleagues [54] took it a step further and examined the gut microbiota of males and females from three strains of gonadectomized mice while on regular chow and a high fat/high sucrose diet. Aside from strain-specific differences in the gut microbiota stemming likely from different genetic backgrounds, they observed clear differences between male and female sham and gonadectomized mice. The hormonal status affected the male gut microbiota while on the chow diet, whereas the female microbiota was affected on the high fat/high sucrose diet. These findings indicate the gut microbiota can regulate sex hormones and sex hormones mediate the gut microbiota composition.

In three large cohort studies from the U.S. and United Kingdom (US/UK), Colombia, and China, microbial alpha diversities depended on host age and sex [57].

Younger (20-45 years) women have greater alpha diversity than younger men confirming findings from a Dutch cohort [58], however those differences are less pronounced with age (46-69 years) [57]. These differences persisted in the Colombian cohort after adjusting for cardiometabolic parameters, and in the US/UK cohort after adjusting for antibiotic use. Interestingly, the association of age and sex with gut microbial diversity was not noted in the Chinese cohort [57]. In terms of bacterial taxa, men present with greater abundance of Bacteroidetes [53], and *Bacteroides* and *Prevotella* genera [59], however no differences were observed for *B. vulgatus*, the most dominant *Bacteroides* species, and *B. putredinis* [59]. Body mass index (BMI) and dietary factors may have different associations with gut microbiota in men and women. Haro et al. [60] reported a decrease of *Bacteroides* genus abundance in men as BMI increased, but not in women. Palmu and colleagues [61] reported a negative association between *L. paracasei* and 24-hour urinary sodium excretion as an indicator of dietary sodium intake in a cohort of Finnish adults that was stronger in women than men.

In conclusion, existing evidence points at gut microbial differences between men and women. The work in microgenderome is limited and the mechanisms are more established in rodent models. Future research in humans should expand current findings on compositional differences in the gut microbiota to functional implications.

1.5 Dietary sodium and the immune system

Dietary sodium induces inflammation on several fronts. Rodent models have shown that high sodium diets activate the innate [62; 63] and adaptive [64; 65] immune system, and promote tissue inflammation and autoimmune disease [65]. High sodium diets in rodents exacerbate inflammatory bowel disease [9; 66], experimental autoimmune encephalomyelitis [65; 67], and lung inflammation [63] via immune system activation. These effects are mainly achieved through modulation of innate immune cells such as macrophages [62; 63] and adaptive immune cells such as T_H17 and T_{reg} cells [65; 67; 68; 69].

1.5.1 Dietary sodium and the innate immune system

Sodium activates the innate immune system by affecting both monocytes and macrophages. Sodium promotes pro-inflammatory macrophage (M1) polarization [70] and dose-dependent expression of pro-inflammatory genes and suppresses expression of anti-inflammatory genes in human macrophages *in vitro* [63]. Alternatively activated macrophages (M2) which are not considered inflammatory, demonstrate reduced anti-inflammatory potential when stimulated in the presence of high sodium [71]. In the skin interstitium, macrophages act to buffer hypertonicity caused by a high sodium diet by augmenting nitric oxide production [62]. A dietary intervention study conducted in

healthy humans showed 7 days of a high sodium diet (6,000 mg/d) followed by 7 days of low sodium diet (2,000 mg/d) induced monocyte expansion [72], cells that actively participate in inflammation. These findings were also confirmed *in vitro* as incubation of isolated monocytes with additional sodium increased their expansion and induced oxidative stress, indicating sodium acts on monocytes via a reactive oxygen species-dependent pathway [72].

1.5.2 Dietary sodium and the adaptive immune system

High salt intake and specifically the sodium cation has been shown to activate T_H17 cells in murine and human cell cultures [65; 67; 68] and increased production of IL-17A, IL-23, IL-6, and TNF-α [70; 73]. Culturing naïve CD4+ T cells with increasing sodium concentrations reinforces differentiation of naïve T cells into the pathogenic T_H17 phenotype [65; 67]. In the mouse gut, a high sodium diet increases T_H17 cell activation and IL-17A secretion, while it reduces T_{reg} cell abundance and IL-10 production in the small and large intestine [64; 74]. In addition, a high sodium diet increases leukocytes, T helper and cytotoxic T cells in mesentery arteries and mesenteric lymph nodes [75]. In the mouse plasma, a high sodium intake increased levels of IL-17A [74], while fecal transplant from high sodium-fed mice to GF mice increased plasma levels of IL-6 and IL-17 to greater levels than fecal transplant from normal sodium-fed to GF mice [75]. Although levels of these cytokines in human plasma were not replicated, leukocytes of healthy men who consumed 12 g/d of salt (4,800 mg sodium) for 30-60 days produced more IL-6 and IL-23 compared to

consumption of 6 g/d (2,400 mg sodium) and 9 g/d (3,600 mg sodium) when stimulated to elicit an immune response [69]. Furthermore, 7 days of a high sodium diet (7,000 mg/d) increased plasma IL-17 levels compared to a low sodium diet (1,200 mg/d) in subjects with normal blood pressure (BP), elevated BP, and stage 1 HTN [76].

While homeostasis requires a balance between T_H17 and T_{reg} cells, exposure to high sodium impairs regulatory functions of T_{reg} cells and shifts the T_H17/T_{reg} ratio [64; 77]. Mechanisms involve expression of serum glucocorticoid regulated kinase (SGK)-1 which was previously shown to respond to increased hypertonicity in the kidney, whether from mineralocorticoid excess or a high sodium environment. SGK-1 promotes the IL-23 receptor—T_H17 cell pathway [67] crucial for expression of pro-inflammatory cytokines such as IL-6 and IL-23, while the same pathway inhibits T_{reg} cell differentiation [67; 78]. This leads to increased production of IFNγ and reduced production of IL-10 [64; 78; 79]. On the other hand, it has been shown that dendritic cells exposed to excess sodium produce IL-1β that stimulate T cells to produce IL-17A and IFNγ [75; 80]. Thus, sodium can stimulate T cells directly, as well as indirectly through activation of dendritic cells. Notably, sodium exposure has no effect on murine T_H1 and T_H2 CD4+ cell differentiation, indicating this pathway is specific for T_H17 cells [65; 74]. While both the immune and adaptive immune system are activated with high sodium diets, the most pronounced mechanism of action in response to sodium is through upregulation of T_H17 cells and inhibition of T_{reg} cells.

1.6 Dietary sodium and gut microbiota

Interactions of dietary sodium and the gut microbiota have recently sparked interest. This results from the discovery of gut dysbiosis in rodents [15; 81; 82; 83; 84; 85] and humans [15; 82; 85; 86] with HTN, as well as due to evidence that the gut microbiota takes part in the pathogenesis of HTN in rodents [15; 75; 81; 85]. The number of studies examining the relation of dietary sodium and the gut microbiota is mounting as highlighted in Table 1.2.

Table 1.2: Bacterial taxa altered by dietary sodium with their full classification.

Phylum	Class	Order	Family	Genus	Species	Reference
Human taxa negatively associated with dietary sodium intake						
Firmicutes	Bacili	Lactobacillales	*Lactobacillaceae*	*Lactobacillus*	*paracasei*	Palmu et al.[61]
Human taxa positively associated with dietary sodium intake						
Firmicutes	Bacili	Lactobacillales	*Lactobacillaceae*	*Lactobacillus*	*salivarius*	Palmu et al.[61]
	Clostridia	Clostridiales	*Ruminococcaceae*			Ferguson et al.[75]
Bacteroidetes	Bacteroidia	Bacteroidales	*Prevotellaceae*	*Prevotella*		Ferguson et al.[75]
			Bacteroidaceae	*Bacteroides*		Ferguson et al.[75]
Proteobacteria						Ferguson et al.[75]
Rodent taxa reduced in abundance following a high sodium diet (3.15-8% Na; 14 days - 8 weeks)						
Actinobacteria	Actinobacteria	Actinomycetales	*Micrococcaceae*	*Rothia*		Wilck et al.[68]
Bacteroidetes	Bacteroidia	Bacteroidales	*Bacteroidaceae*	*Bacteroides*	*fragilis*	Yan et al.[85]
					acidifaciens	Yan et al.[85]
					*finegoldii**	Yan et al.[85]
			Bacteroidales incertae sedis	*Phocaeicola*	*massiliensis*	Yan et al.[85]
			Odoribacteraceae	*Butyricimonas*		Yan et al.[85]
				Odoribacter		Yan et al.[85]
			Porphyromonadaceae	*Parabacteroides*	*distasonis*	Yan et al.[85]

			Rikenellaceae	*Alistipes*	*putredinis finegoldii**	Wang et al.[87], Yan et al.[85]
				Alistipes	*indistinctus*	Yan et al.[85]
			S24-7			Wang et al.[87]
			Tannerellaceae	*Parabacteroides*	*gordonii*	Yan et al.[85]
Firmicutes	Bacili	Lactobacillales	*Lactobacillaceae*	*Lactobacillus*	*vaginalis*	Wilck et al.[68], Wang et al.[87], Miranda et al.[66], Ferguson et al.[75], Yan et al.[85]
				Lactobacillus	*murinus*	Wilck et al.[68]
			Leuconostocaceae	*Leuconostoc*		Ferguson et al.[75]
				Weissella		Ferguson et al.[75]
			Streptococcaceae			Ferguson et al.[75]
		Bacillales				Ferguson et al.[75]
	Clostridia	Clostridiales	*Clostridiaceae*	*Unclassified*		Miranda et al.[66]
				Candidatus Arthromitus		Yan et al.[85]
				Clostridium	*cocleatum*	Yan et al.[85]
			Lachnospiraceae	*Anaerostipes*		Bier et al.[88]
				Clostridium XIVa		Wilck et al.[68]
				Johnsonella		Wilck et al.[68]

			Oscillospiraceae	*Oscillibacter*		Wilck et al.[68]
			Peptococcaceae	*Dehalobacterium*		Yan et al.[85]
			Ruminococcaceae	*Anaerotruncus*		Wang et al.[87], Yan et al.[85]
			Family unassigned	*Pseudoflavonifractor*		Wilck et al.[68]
	Erysipelotrichia	Erysipelotrichales	*Erysipelotrichaceae*	*Holdemania*		Yan et al.[85]
	Mollicutes	Anaeroplasmatales	*Anaeroplasmataceae*	*Anaeroplasma*		Yan et al.[85]
Proteobacteria	Gammaproteobacteria	Pasteurellales	*Pasteurellaceae*	*Aggregatibacter*	*pneumotropica*	Yan et al.[85]
Varrucomicrobia						Yan et al.[85]
Human taxa reduced in abundance following a high sodium diet (5,500 mg Na/d; 14 days)						
Firmicutes	Bacili	Lactobacillales	*Lactobacillaceae*	*Lactobacillus*		Wilck et al.[68]
Rodent taxa increased in abundance following a high sodium diet (3.15-8% Na; 14 days - 8 weeks)						
Actinobacteria	Actinobacteria	Corynebacteriales	*Corynebacteriaceae*	*Corynebacterium*		Bier et al.[88], Yan et al.[85]
		Coriobacteriales	*Coriobacteriaceae*	*Adlercreutzia*		Yan et al. [85]
Bacteroidetes	Bacteroidia	Bacteroidales	*Prevotellaceae*	*Prevotella*		Wilck et al.[68], Ferguson[75]
			Rikenellaceae	*Alistipes*		Wilck et al.[68]
Firmicutes	Bacilli	Lactobacillales	*Aerococcaceae*	*Aerococcus*		Yan et al.[85]
			Streptococcaceae	*Streptococcus*	*sanguinis*	Yan et al.[85]
	Clostridia	Clostridiales	*Christensenellaceae*			Bier et al.[88], Ferguson et al.[75]

				Roseburia		Wang et al.[87], Miranda et al.[66], Ferguson et al.[75]
				Lachnospiraceae UCG-006		Ferguson et al.[75]
				Incertae Sedis		Ferguson et al.[75]
				Lachnospiraceae FCS020		Ferguson et al.[75]
			Oscillospiraceae	*Oscillospira*		Miranda et al.[66]
				Oscillibacter		Wang et al.[87]
			Ruminococcaceae	*Ruminococcus*		Wang et al.[87]
	Erysipelotrichia	Erysipelotrichales	*Erysiopelotrichaceae*	*P-75-a5*		Yan et al.[85]
Proteobacteria	Betaproteobacteria	Burkholderiales	*Sutterellaceae*	*Parasutterella spp*		Wilck et al.[68]
	Gammaproteobacteria	Enterobacteriales	*Enterobacteriaceae*	*Erwinia*		Bier et al.[88]
		Pseudomonadales	*Moraxellaceae*	*Psychrobacter*		
	Deltaproteobacteria	Desulfovibrionales	*Desulfovibrionaceae*	*Desulfovibrio*		Yan et al.[85]
	Epsilonproteobacteria	Campylobacterales	*Helicobacteraceae*	*Flexispira*	*rappini*	Yan et al.[85]
Spirochaetes						Yan et al. [85]
Verrucomicrobia	Verrucomicrobiae	Verrucomicrobiales	*Verrucomicrobiaceae*	*Akkermansia*		Wilck et al.[68]

Bacterial taxa found to be altered in the original publication are bolded. *Genus unclear.

1.6.1 Effects of dietary sodium on gut microbiota in rodents

High sodium intake is associated with lower microbial diversity. Wang et al. [87] observed greater intra-group variation in bacterial species when mice were fed normal amounts of sodium for 8 weeks compared to high amounts of sodium. Ferguson and colleagues [75] reported a lower number of OTUs in mice fed a high sodium diet for 3 weeks compared to a low sodium diet with a clear separation of high and low sodium clusters when data were visualized [75]. They also observed an increase in the phylum Firmicutes but not in Bacteroidetes [75], thus increasing the Firmicutes:Bacteroidetes ratio, a ratio that has been considered an indicator of gut health. Yan et al.[85] also observed an increase in the Firmicutes:Bacteroidetes ratio but did not observe differences in diversity between rats fed a normal and high sodium diet for 4 weeks. Overall, high amounts of dietary sodium appear to reduce microbial diversity and unfavorably change abundances of the two biggest phyla.

Although microbial diversity and the Firmicutes:Bacteroides ratio has been considered a landmark of gut health, the field is moving towards delineating individual genera and species affected by dietary changes as there are beneficial taxa belonging to Firmicutes phylum, and vice versa. Lactic acid-producing bacteria have garnered a lot of attention as several research groups demonstrated a reduction in abundance when consuming a high sodium diet [61; 68; 75; 85]. Ferguson and colleagues [75] reported a reduction in abundance of the class Bacilli, orders Lactobacillales and Bacillales,

families *Leuconostocaceae* and *Streptococcaceae*, and genera *Leuconostoc* and *Weissella* in mice after 3 weeks of high sodium diets. Wilck and colleagues [68] demonstrated that a high sodium intake changed the gut microbiota profile and depleted *Lactobacillus murinus*, a species present in the mouse gut, but not found in humans. Feeding salt-sensitive mice a high sodium diet and supplementing it with *L. murinus* and *L. reuteri* reduced systolic and diastolic BP and T_H17 cells.

In addition to *Lactobacillus*, genera in order Clostridiales have shown to be greatly affected by high sodium as a reduction [66; 68; 87; 88] or an increase [75] in abundance of several genera have been noted in rodent models (Table 1.2). Family *Christensenellaceae* in the order Clostridiales was increased along with genus *Erwinia* and family *Corynebacteriaceae* following 8 weeks of high sodium [88]. Family *Lachnospiraceae* [66; 75; 87] and genera *Ruminococcae* [87], and *Oscillospira* increased upon a 3-, 4- and 8-week long high sodium diet which also exacerbated colitis in mice [66]. Mice on a normal sodium diet demonstrate higher abundance of *Lactobacillus* and lower abundance of Lachnospiraceae and Ruminococcae [87]. *Prevotellaceae* and *Prevotella*, family and genus associated with chronic inflammation and metabolic syndrome, are also shown to be increased upon high sodium feeding which was observed along with reduction of lactic acid-producing bacteria [68; 75]. Similarly, phyla Firmicutes and Proteobacteria were significantly associated when rats were fed a high sodium diet, implicating that an increase in one could directly or indirectly increase abundance of the other phylum [85]. These observations highlight that changes in

microbial populations should be looked at as a part of an ecosystem and not in isolation.

1.6.2 Effects of dietary sodium on gut microbiota in humans

The first evidence of dietary sodium's deleterious effect on the human gut microbiota was a controlled trial in a cohort of 12 healthy men in a study by Wilck et al. [68]. Men consumed high amounts of dietary sodium (5,500 mg/d) for 14 days. An increase in T_H17 cells and depletion of 90% of the *Lactobacillus* species present at baseline was noted, indicating low resilience of *Lactobacillus* spp. to high sodium intake. After Wilck et al. published their controlled trial in 2017, more research groups began investigating the relation between sodium and the gut microbiota resulting in publications of several observational studies [61; 75; 89]. Recently, Palmu and colleagues [61] reported a negative association of *L. paracasei*, and a positive association of *L. salivarius* with 24-hour urinary sodium excretion as an indicator of dietary sodium intake in a large sample of 829 Finnish adults. *L. paracasei* was negatively associated with systolic BP, diastolic BP, and mean arterial pressure, while *L. salivarius* was positively associated with pulse pressure. *Lactobacillus* genus was not associated with urinary sodium excretion nor BP [61]. These findings emphasize the importance of using sequencing methodologies with high resolution at the species level and suggest that not all species within the same genera perform the same functions. In general, metabolites from *Lactobacillus* spp. confer benefits for the host as they are rich in

bacteriocins that impede pathogen growth and activation [90; 91], as well as other mediators necessary for optimal gut microbial structure and composition [90]. *Lactobacillus* spp. are already lower in abundance in westernized societies compared to non-industrialized, agriculture-based societies [92]. *Lactobacillus* spp. were only found in 42% (five out of 12) of the individuals in the study by Wilck and colleagues [68] and in 15.5% of individuals (128 out of 829) in the study by Palmu and colleagues [61]. Restoring *Lactobacillus* spp. abundance in the gut could have health implications.

Ferguson et al. [75] sequenced stool samples from 135 adults with a low (<2,300 mg/d) and high (>2,300 mg/d) sodium intake. They reported no difference in alpha diversity and a significant difference in beta diversity but without distinct clustering of low- and high-sodium consumers. Distinct differences in clustering of high and low sodium diets appears to be more pertinent in rodents than in humans, perhaps due to an ability to achieve larger differences in sodium intake. Ferguson et al. [75] also reported that genera *Prevotella* and *Bacteroides*, family *Ruminococcaceae*, and phyla Firmicutes and Proteobacteria were associated with a high sodium intake, while Yan and colleagues [85] strengthened these findings by noting a significant relation between Firmicutes and Proteobacteria phyla in rats fed a high sodium diet. In a large multi-center study, Seck and colleagues [89] used stool salinity as a proxy for dietary sodium intake to observe compositional changes in the gut microbiota. They assessed salinity of stools from 1326 human subjects and reported a reduction in the Shannon diversity index with an increase in salinity above a 1.5% threshold for presence of halophiles. A major drawback to this study was not including dietary

intake data from these individuals, making it difficult to extrapolate stool salinity levels to dietary sodium intake. They identified 64 halophilic prokaryotes in feces, suggesting salinity of the intestinal environment can be high enough to harbor halophiles. In contrast, bacterial resistance to salt is an important survival strategy in stressful environments such as increased extracellular osmolarity due to greater concentration of sodium ions. Several genes in the human gut microbiome have been detected as the metabolic activity of salt tolerance genes increases in high sodium environments [93]. Identification of these genes has implications in the development of novel therapeutics (for example, functional foods, dietary supplements, and drugs) that target specific members of the gut microbiota in conditions induced or mediated by a high sodium intake [94]. Evidence from these observational studies is an indispensable starting point to advance the field of sodium and gut microbiota interaction, but randomized controlled trials in humans are needed to verify findings in rodents.

1.6.3 Intestinal sodium absorption

The GI tract is the first organ encountered by ingested dietary sodium. Even though the majority of sodium is absorbed in the upper GI tract, mainly the stomach, a significant amount is also absorbed in the large intestine [9]. For example, sodium concentration in the colon of mice fed a high sodium diet is greater compared to those fed a low sodium diet [9]. The sodium/hydrogen exchanger 3 (NHE3) in the proximal colon is a site of intestinal regulation of sodium absorption, water absorption, and pH

[95; 96]. Linz et al. [97; 98] demonstrated pharmacologic inhibition of NHE3 increased fecal sodium and water content, decreased urinary sodium excretion, and lowered BP [97; 98], as well as reduced left-ventricular end-diastolic pressure, mass, and fibrosis [98] in spontaneously-hypertensive rats. NHE3 knockout mice present with an increased number of bacteria in most luminal and all mucosal sites from ileum to distal colon along with regional shifts in bacterial phyla and/or genera [99]. *Firmicutes* were present in lower abundance in these NHE3 knockout mice and *Bacteriodetes* in greater abundance in luminal and mucosal sites in the terminal ileum, cecum, and distal colon. A prominent increase in abundance was noted with a commensal *Bacteriodetes* member *Bacteroides thetaiotaomicron* in the lumen of the terminal ileum. *Bacteroides thetaiotaomicron* ferments glutamate, a precursor for anti-inflammatory glutathione. It has also been identified as a species depleted in the gut of obese individuals [100]. Results of this study favor positive outcomes with NHE3 inhibition, whereas other studies report that NHE3 deficiency induces irritable bowel disease-like symptoms, gut dysbiosis, and inflammatory immune system response [101]. While these results confirm that intestinal ion concentration and pH alter bacterial composition driven by NHE3, further research is needed to elucidate specific changes in bacterial genera and species.

1.6.4 Short-chain fatty acids and high dietary sodium

Short-chain fatty acids (SCFAs) are products of anaerobic fermentation of complex carbohydrates by the gut microbiota that humans are unable to digest. The most abundant SCFAs are acetate, propionate, and butyrate accounting for 80% of total SCFA production [102]. Concentration of SCFAs are altered under high sodium conditions. In response to a high sodium diet, colonocytes may increase butyrate uptake as a protective mechanism. Mice fed high sodium diet demonstrate a higher abundance of butyrate-producing *Roseburia* [87] and lower intestinal levels of the SCFA butyrate [66]. A reduction in *Lactobacillus* spp. may in part be responsible for accompanying lower butyrate levels [66] due to bacterial cross-feeding between lactate-producing bacteria and lactate-utilizing, butyrate-producing bacteria [103]. In contrast to butyrate levels, fecal contents of acetate and propionate [88], as well as plasma acetate [84] are reported to be higher under high sodium conditions in rodents. Since acetate and propionate are strong ligands for the SCFA receptor in the vasculature, Olfr78, that stimulates renin secretion and increases BP [104; 105], this suggests different physiological roles of SCFAs as acetate and propionate may act to increase BP and butyrate may offer protection. These findings are in conflict with Ganesh et al. [106] who demonstrated a BP reduction with intracolonic acetate infusion in rats with obstructive sleep apnea-induced HTN.

In humans, modest reduction in dietary sodium intake from ~4,000 mg/d to ~2,200 mg/d in 145 untreated hypertensive adults increased circulating SCFAs [107].

Increased concentrations of butyrate, 2-methylbutyrate, isobutyrate, and hexanoate persisted even after adjusting for age, sex, race, and BMI. However, a sex interaction revealed increases in butyrate, hexanoate, isobutyrate, isovalerate, and valerate only in women and not in men. Certain SCFAs were further associated with reduced BP [107]. Altogether, SCFAs are emerging as regulators of BP with rodent evidence even suggesting BP-lowering effects. However, the response of SCFAs to dietary sodium levels in both rodents and humans warrants further investigation.

1.7 Concluding remarks

The gut microbiota interacts with numerous organ systems and influences various autoimmune and metabolic diseases. Research in rodents and humans demonstrates altered gut microbial composition is evident in HTN which can also be evoked with high sodium diets. While certain mechanistic pathways are being revealed, much remains to be elucidated regarding the interplay of the gut microbiota and high sodium diets. Targeted pathways include immune cell signaling and mechanisms of the NHE3 and SCFA action. Identification of specific salt tolerance genes may offer novel therapeutics to target the gut microbiota in conditions caused or exacerbated by high sodium consumption. Future experimental research should focus on this vastly unexplored area on the role of excess sodium consumption on the gut microbiota in men and women which has implications for the cardiovascular system and other organs associated with HTN development.

Chapter 2

HIGH DIETARY SODIUM INTAKE AND GUT MICROBIOTA COMPOSITION IN HEALTHY YOUNG ADULTS

2.1 Introduction

The gut microbiota is a dynamic organ with an immense influence on the host metabolism. The gut microbial composition and function appears to be disrupted (i.e. dysbiotic) in individuals with chronic autoimmune [108; 109] and cardiometabolic diseases [110], as well as in hypertension (HTN) [15; 82; 86]. As already confirmed in animal models [15; 81; 82; 83; 84], individuals with HTN present with lower microbial diversity, richness, and a clear taxonomic distinction compared to normotensive individuals [15; 82; 86]. Given that high dietary sodium intake is a risk factor for HTN and cardiovascular disease [4], and that the gastrointestinal (GI) tract is in direct contact with ingested sodium, it is plausible that the gut microbiota composition could change under high sodium conditions.

High dietary sodium intake is a modifiable risk factor for HTN [4]. While Dietary Guidelines for Americans recommend an intake of 2,300 mg sodium/d, the

American Heart Association recommends an intake of 1,500 mg sodium/d for optimal health, older adults, African Americans, and individuals with HTN [2]. It is alarming that the average intake of sodium among adults in the United States (U.S.) is ~3,400 mg sodium/d and that only 11% of Americans meet the recommendations set by the Dietary Guidelines. While high sodium diets (HSD) have been mostly associated with their adverse effects on blood pressure (BP), emerging findings suggest that HSD may lead to a disbalance of gut microbial composition and function [61; 66; 68; 75; 85; 87; 88]. Work in rodents demonstrates that gut microbial diversity decreases on an HSD with certain bacterial species either increasing or decreasing in abundance [66; 68; 75; 85; 87; 88], while there has been only one randomized controlled trial done in men in which HSD led to depletion of the beneficial *Lactobacillus* species [68].

Sex differences in the gut microbiota composition are evident [54; 111] as distinct differences exist in the gut microbiota between male and female rodents [54; 55; 56] and men and women [57; 58; 59]. These differences become apparent at puberty and are most pronounced in adulthood [56]. Epidemiological studies in humans report women 20 - 45 years of age have greater gut microbial diversity than men [57; 58] even after adjusting for cardiometabolic parameters and antibiotic use [57]. These findings suggest the gut microbiota may respond to dietary interventions differently in men than in women. Indeed, the magnitude and direction of diet-induced change among microbial taxa differs among male and female rodents [54], but the same is unexplored in humans.

Considering that the gut microbiota mediates HTN and that an HSD leads to gut dysbiosis in rodents, it is plausible that the effects of an HSD on gut microbiota may be a new approach to optimize cardiovascular health. Our primary objective was to assess the effect of an HSD on gut microbial diversity in healthy young adults. We hypothesized that gut microbial diversity would decrease following an HSD compared to a recommended sodium diet (RSD). Our secondary, exploratory objective was to assess the effect of HSD on gut microbial diversity in men and women. We hypothesized that men would demonstrate a larger decrease in gut microbial diversity following an HSD compared to women.

2.2 Methods

2.2.1 Study design

This was a single-blind randomized crossover study with two 10-day interventions of RSD or HSD to determine the effect of sodium on the gut microbiota composition. The study protocol and procedures were approved by the Institutional Review Board of the University of Delaware (ID# 1423170) and conform to the provisions of the Declaration of Helsinki. Subjects were recruited through flyers posted around the University of Delaware campus and the surrounding community, as well as through online advertisements. Informed consent was obtained from all

subjects prior to enrollment in the study. The study was registered on clinicaltrials.gov (NCT04229680).

2.2.2 Subject screening

Healthy adults between the ages of 18-45 were recruited to participate in the study. A prescreening questionnaire was administered via phone before the screening visit to ensure age, body mass index (BMI), and main GI health criteria were met. After subjects signed the informed consent, their height, weight, and resting BP were measured. Physical activity levels, medical history including GI health, dietary supplementation, and antibiotic and medication use were assessed via questionnaires. Inclusion criteria was apparently healthy men and women, age 18-45 years with a systolic BP <130mmHg, diastolic BP <80mmHg and BMI between 18.5-29.9 kg/m^2. Exclusion criteria were the following: history of cardiovascular disease, HTN, diabetes or kidney disease; history of intestinal disease or surgery, malabsorption disorder, or food allergies/sensitivities; antibiotic, prebiotic, or probiotic use in past 3 months; use of medications for mood disorders (fluoxetine, escitalopram, venlafaxine, aripiprazole, lithium, olanzapine, valproate); malignant cancer; pregnancy or lactation; smoking; and highly trained endurance athletes.

2.2.3 Dietary interventions

Subjects were instructed to provide a food record for three consecutive days (2 weekdays and 1 weekend day) to assess their habitual diet. Eligible participants were instructed to consume a recommended amount of dietary sodium (2,300 mg/d) [8] for 10 days for both interventions and then supplement with either sodium pills (4,600 mg/d for a total of 6,900 mg/d; HSD) or placebo pills (dextrose; RSD). At least a 2-week washout occurred between the interventions. Participants were asked again about GI health, dietary supplementation, and antibiotic and medication use prior to starting the second intervention period to ensure no changes occurred to their GI health. Research staff provided subjects with instructions and strategies to consume a diet with the recommended amount of sodium during both interventions. These instructions were based on subjects' habitual dietary intake and food choices following the nutrient analysis of the 3-day food record. Subjects recorded their dietary intake during the intervention and were instructed to consume similar foods during both trials. To ensure compliance to sodium intake, food intake was assessed via two 24-hour dietary recalls during each 10-day intervention using the multiple-pass approach [112]. The first recall was performed through an unannounced phone call between days 4 and day 7, i.e. the exact day was unknown to the participant. The second recall was performed during the in-lab testing visit on day 10 and recalled foods consumed on day 9. Diet records were analyzed using Nutrition Data System for Research software (NDS-R 2018 and NDS-R 2019, University of Minnesota, Minneapolis).

2.2.4 Sodium and placebo supplementation

Subjects supplemented their diet with 12 enteric, slow-release pills per day containing either table salt (4,600 mg sodium total) or dextrose (10 g total) as a placebo. They were randomly assigned to the sodium and placebo conditions and were blinded to the pill contents. They were instructed to space out their pill intake throughout the day and take them with food only and not on an empty stomach to minimize GI side-effects. For the first three days of the intervention, subjects gradually increased the number of pills they took (5 pills on day 1, 7 pills day 2 and 9 pills on day 3). For days 4-10, they were instructed to consume all pills daily. Subjects were asked to note the number of pills taken each day to ensure compliance. They were also provided with GI side-effects forms to note any side-effects they might experience.

2.2.5 Ambulatory BP, urine collection, and blood draw

Ambulatory BP monitoring (Oscar 2 with SphygmoCor, SunTech Medical) was conducted on the last day of each diet to collect 24 hours of BP data [113]. BP was automatically taken every 20 min during the day and every 30 min at night. Data was accepted if at least 20 daytime and 7 nighttime BPs were recorded [114]. Urine was also collected for 24 hours on the last day. Urine volume, electrolyte concentrations (Na^+,

K^+, Cl^-) (EasyElectrolyte Analyzer, Medica), and osmolality (Advanced 3D3 Osmometer, Advanced Instruments) were assessed as per standard lab protocol. Urine collections were considered incomplete if there were ≥2 missed collections or if urine volume was <500 mL [115]. Subjects were asked to fast, avoid caffeine and alcohol for at least 12 hours and not exercise for 24 hours prior to the testing visit on day 10. A venous blood sample was collected on day 10 of both interventions. Hematocrit and hemoglobin in the whole blood, serum electrolytes (EasyElectrolyte Analyzer, Medica), and plasma osmolality (Advanced 3D3 Osmometer, Advanced Instruments) were assessed.

2.2.6 Physical activity monitoring

Subjects were instructed to not alter their habitual physical activity levels as exercise may influence the gut microbial composition [116; 117]. They were issued an accelerometer (ActiGraph, Florida, USA; CE; FDA cleared Class II medical device) and were instructed to wear it on the hip for 10 days during both interventions. Subjects received a log to record times when the accelerometer was worn, and not worn, to improve compliance and data quality. Data was accepted if there were at least 4 days with 10 hours of wear time [118]. Subjects returned the device at the testing visit on day 10.

2.2.7 Fecal sample collection

Subjects collected a stool sample before and after RSD and HSD for a total of 4 stool samples. They collected them within 24 hours of starting each 10-day intervention and within 24 hours of each testing visit on day 10. They were instructed to place the sample in a sterile sample tube (Sarstedt Inc., Newton, NC) and bring it to research staff refrigerated or on ice. All samples were aliquoted, flash frozen in liquid nitrogen, and stored at 80°C within 4 hours of collection.

2.2.8 Fecal sample preparation

Approximately 1-5 mg of fecal material was used as input into the StrainID kit (Shoreline Biome, Farmington, CT, USA). Sample DNA was isolated as per manufacturer's instructions. Briefly, 50 µl of reconstituted lysis reagent was added to each sample. Subsequently, 50 µl of 0.4 M KOH solution was added and the samples were heated to 95°C for 5 minutes to lyse the cells. The plate was spun briefly to pellet the fecal debris, and 50 µl of the supernatant was transferred to a clean plate. 50 µl of DNA Purification Beads were added, the DNA was allowed to bind, and the pellets were washed with 70% ethanol. DNA was eluted in TE, and 10 µl eluted DNA was transferred to the corresponding well in the PCR plate. 2x PCR mix was added, and PCR was performed as per instructions to amplify and barcode each sample. Samples

were pooled, purified via spin column (Qiagen MinElute, Qiagen, Valencia, CA, USA).

2.2.9 Library construction and sequencing

Long reads were obtained using single-molecule real-time (SMRT) sequencing technology (PacBio Sequel IIe, Pacific Biosciences, Menlo Park, CA, USA). The resultant 16S/ITS/23S amplicon pool (~2500bp) was used as template to construct a SMRTbell library using Express Template Prep 2.0 (Pacific Biosciences, Menlo Park, CA, USA) as per the manufacturer's Template Preparation protocol. The quality of the amplicon library was assessed using the Qubit 3.0 Fluorometer (Thermo Fisher Scientific Inc., Waltham, MA, USA) and the Agilent Femto Pulse System (Agilent Technologies, Santa Clara, CA, USA). The amplicon library was run on a single Sequel IIe system 8M SMRT cell using sequencing chemistry 3.0 with a 4-hour pre-extension and a 30-hour movie time.

2.2.10 Bioinformatics processing

Sequence data was demultiplexed and filtered using SBPipe (ver. 3.1.R38b5a8f; Shoreline Biome). Denoising and clustering of data into amplicon sequence variants was performed in DADA2 (ver 1.18.0) [119] via Shoreline Biome DADA2_R_pipeline.R with OMEGAA 1e-10 and OMEGAC 1e-80. Taxonomy was assigned to amplicon sequence variants (ASVs) using SBPipe against a merged Athena (ver 2.2) and SILVA (ver 138) [120] database. Taxonomy and ASV feature table were imported into QIIME2 (ver 2021.4) [121] for data visualization and further processing. The feature table was filtered to remove samples with less than 300 total sequences and remove features that had a combined count ≤5 and/or were represented in ≤2 samples. Analysis of taxonomic composition was performed using ANCOM [122]. Sequences were aligned MAFFT [123] and an approximate maximum likelihood tree was constructed using FastTree [124] with posterior mid-point rooting. Alpha and beta diversity analyses were rarefied by subsampling 600 sequences, except compositional statistical tests that utilized complete data normalized using centered log ratio after zero replacement with a pseudocount of 1 [125]. Alpha diversity was assessed using Shannon Index, Chao1, Observed Features, Pielou Evenness, and Faith's Phylogenetic Diversity (PD) [126]. Beta Diversity analysis examined Aitchison distance [125], Jaccard distance, Bray-Curtis dissimilarity, and Weighted [127] and Unweighted Unifrac [128] distances and were visualized using principal coordinate analyses.

2.2.11 Statistical analysis

The primary outcome was the index of microbial diversity (Shannon diversity index) during the two interventions. Group significance of alpha diversity metrics and relevant metadata was performed using Kruskal Wallis test and beta diversity group significance utilized ANOSIM [(129)]. Additional alpha and beta statistical analyses were performed using q2-longitudinal including, analysis of pairwise differences/distances, and non-parametric microbial independence testing [(130)]. Benjamini-Hochberg false discovery rate (FDR) correction [(131)] was applied to all statistical tests as appropriate. *A priori* power analysis was performed using G*Power statistical software. Based on findings of Li et al. [(132)], a sample size of 24 participants was needed to detect a 9% difference in Shannon diversity index on day 10 between HSD and RSD. A sample size of 28 participants was needed to detect a 9% difference in Shannon diversity index within (before vs after RSD/HSD) and between (RSD vs HSD) conditions. Inclusion of sex as a between-subjects factor would require a sample size of 36 participants to detect the same difference in Shannon diversity index and explore sex differences. Significance was set *a priori* at $p<0.05$.

2.3 Results

Thirty-one subjects completed both interventions. Good quality DNA sequences from the final day of each intervention (i.e. day 10 sets) were obtained from 20 subjects, while good quality DNA sequences from all 4 samples (i.e. complete sets) were obtained from 16 subjects.

Subject characteristics for the 20 subjects are presented in Table 2.1. Overall, subjects were young, had a normal screening BP, and normal BMI. Urinary sodium excretion and serum sodium levels were greater on an HSD compared to RSD indicating study compliance. Urine osmolality and urine specific gravity were greater on HSD compared to RSD suggesting greater urine concentration during HSD. There were no differences in subjects' ambulatory systolic and diastolic overall, nighttime or daytime BP between the two interventions, nor mean arterial pressure and heart rate. Subject characteristics separated by sex are presented in Appendix A (Table A1, Table A2). Dietary intake data as an average of the two 24-hour recalls within each intervention demonstrated subjects' energy, macronutrient, and micronutrient intake was comparable between the diets (Table 2.2, Appendix A Table A3).

We further examined subjects' consistency in energy and nutrient intake within each intervention by correlating energy and nutrient intakes from the two dietary

recalls. Energy intake and intake of protein, carbohydrates, and fiber from the two 24-hour dietary recalls in RSD were significantly correlated, while energy intake and intake of protein, fat, and sodium from the two 24-hour dietary recalls in HSD were significantly correlated (Appendix A, Table A4).

These results indicate that subjects were consistent in energy and protein intake on the days that recalls were performed, however carbohydrate, fat, and micronutrient intake was more variable. Note that urinary sodium excretion in Table 2.1 reflects overall sodium intake as supplemented with the pills, while sodium intake presented in Table 2.2 reflects intake from the diet. Habitual dietary intake for all subjects and separated by sex is presented in Appendix A (Table A5). Subjects spent 35 min more being sedentary during HSD than RSD, with no differences in absolute time spent doing light, moderate, vigorous, and very vigorous physical activity (Table 2.3). As they wore the accelerometer for an average of 43 min longer during HSD than RSD (a non-significant but trending finding), this could account for the difference in the sedentary time. Relative time spent in each physical activity category was not different between RSD and HSD (data not shown).

Table 2.1: Subject characteristics.

	Screening	Recommended Sodium	High Sodium	p-value*
Demographic data				
n (men/women)	20 (8/12)			
Ethnicity (H/NH)[a]	0/17			
Race (W/B/A)	18/0/2			
Anthropometrics				
Age (yr)	25 ± 1			
Height (cm)	169 ± 2			
Mass (kg)	66.4 ± 2.4	65.7 ± 2.3	65.9 ± 2.3	0.623
Body mass index (kg/m^2)	23.1 ± 0.5			
Hemodynamics				
Laboratory[b]				
Systolic BP (mmHg)	112 ± 2	110 ± 2	111 ± 2	0.361
Diastolic BP (mmHg)	65 ± 1	62 ± 2	63 ± 1	0.813
MAP (mmHg)	81 ± 1	78 ± 1	79 ± 1	0.595
Heart rate (bpm)	66 ± 2	57 ± 2	55 ± 2	0.277
24h Ambulatory				
Systolic BP, overall (mmHg)[c]		114 ± 2	116 ± 3	0.222
Diastolic BP, overall (mmHg)[c]		66 ± 1	65 ± 1	0.871
MAP, overall (mmHg)[c]		82 ± 1	82 ± 2	0.491
Heart rate, overall (bpm)[c]		70 ± 2	70 ± 2	0.948
Systolic BP, daytime (mmHg)		119 ± 2	122 ± 2	0.068
Diastolic BP, daytime (mmHg)		69 ± 1	69 ± 1	0.802
MAP, daytime (mmHg)		86 ± 1	87 ± 1	0.439
Heart rate, daytime (bpm)		74 ± 2	72 ± 2	0.314
Systolic BP, nighttime (mmHg)[c]		104 ± 2	105 ± 4	0.773
Diastolic BP, nighttime (mmHg)[c]		55 ± 1	54 ± 2	0.392
MAP, nighttime (mmHg)[c]		71 ± 2	71 ± 2	0.859

Table 2.1 continued.

Heart rate, nighttime (bpm)[c]	60 ± 2	60 ± 2	0.869
Biochemical parameters			
Hemoglobin (g/dL)	14.4 ± 0.3	14.1 ± 0.3	**0.026**
Hematocrit (%)	42.9 ± 0.9	43.4 ± 0.9	0.424
Serum sodium (mmol/L)	137.8 ± 0.4	139.6 ± 0.4	**0.002**
Serum potassium (mmol/L)	3.8 ± 0.1	3.9 ± 0.0	0.435
Serum chloride (mmol/L)	104.4 ± 0.4	105.3 ± 0.4	**0.059**
Serum osmolality (mOsm/kg)	285 ± 1	286 ± 1	0.452
Urinary sodium excretion (mmol/24h)	115 ± 12	278 ± 17	**<0.001**
Urinary potassium excretion (mmol/24h)	51 ± 4	54 ± 5	0.444
Urine osmolality (mOsm/kg)	416 ± 52	576 ± 48	**<0.001**
Urine specific gravity	1.011 ± 0.001	1.014 ± 0.001	**0.028**

A, Asian; B, Black; BMI, body mass index; BP, blood pressure; H, Hispanic; MAP, mean arterial pressure; NH, non-Hispanic; W, White. Values are means ± SEM. [a]Three subjects (1 man, 2 women) did not report ethnicity. [b]Screening measured after 5min seated rest, Recommended/High Sodium measured after 20min supine rest. [c]n=15. *Recommended Sodium vs. High Sodium.

Table 2.2: Dietary intake during Recommended/High Sodium interventions.

	Recommended Sodium	High Sodium	p-value
Energy intake (kcal/d)	1,951 ± 102	2,040 ± 101	0.367
Carbohydrates (g/d)	214 ± 12	229 ± 13	0.247
Fiber (g/d)	22 ± 2	24 ± 2	0.520
Added sugar (g/d)	31 ± 3	37 ± 4	0.179
Protein (g/d)	87 ± 7	95 ± 7	0.262
Fat (g/d)	77 ± 5	81 ± 5	0.460
Saturated fat (g/d)	25 ± 2	24 ± 2	0.783
Sodium (mg/d)	2,542 ± 160	2,651 ± 197	0.573
Potassium (mg/d)	2,606 ± 153	2,622 ± 141	0.924
Phosphorus (mg/d)	1,276 ± 80	1,270 ± 67	0.941
Calcium (mg/d)	967 ± 56	865 ± 54	0.169
Magnesium (mg/d)	337 ± 30	334 ± 20	0.913
Alcohol (g/d)	17 ± 8	10 ± 4	0.411

Values are means ± SEM. n=20.

Table 2.3: Subjects' physical activity levels during Recommended/High Sodium interventions.

	Screening	Recommended Sodium	High Sodium	p-value
Habitual PA[a]				
Moderate (min/wk)	257 ± 53			
Vigorous (min/wk)	172 ± 30			
Accelerometry[b]				
Sedentary (min/d)		630 ± 12	665 ± 16	**0.046**
Light PA (min/d)		116 ± 6	117 ± 6	0.883
Moderate PA (min/d)		29 ± 3	32 ± 4	0.103
Vigorous PA (min/d)		3 ± 1	7 ± 3	0.171
Very vigorous PA (min/d)		2 ± 1	1 ± 1	0.742
Steps (/day)		5,390 ± 484	6,005 ± 533	0.073
Time accelerometer worn (hrs/d)		13.0 ± 0.3	13.7 ± 0.3	0.059

PA, physical activity. Values are means ± SEM. [a]Reported during screening. n=20. [b]n=14.

2.3.1 Results from subjects with samples on day 10 of RSD and HSD (day 10 sets)

Figure 2.1 represents rarefaction curves of sequenced samples. A plateau of observed features with an increase in sequencing depth indicates achievement of sufficient sequencing depth which was reached at ~500 sequences.

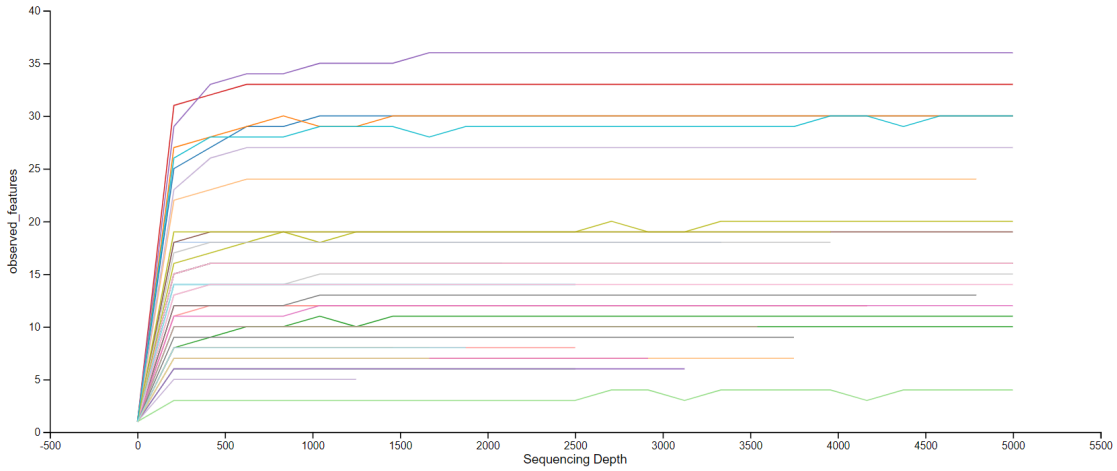


Figure 2.1: Rarefaction curves of sequenced samples. Each curve represents a sample, i.e. there are 40 curves for 40 samples (Day 10 of RSD and HSD for each subject). The X-axis represents sequencing depth, or the number of sequenced nucleotides. The y-axis represents observed ASVs (features), or sequences assigned to species. As the number of sequences from a sample increases, the number of observed ASVs converges on the true diversity. n=20. ASV, amplicon sequence variant; HSD, high sodium diet; RSD, recommended sodium diet.

Shannon diversity index at day 10 of each intervention is represented in Figure 2.2.a. The diversity indices were comparable between RSD and HSD (RSD: 2.62 ± 0.18; HSD: 3.11 ± 0.21; $p = 0.088$) suggesting sodium loading did not negatively affect microbial diversity. Faith's PD and and Pielou Evenness metrics showed similar results, while Chao1 richness and observed features metrics trended in the direction that the richness was lower following RSD compared to HSD ($p = 0.054$ and $p = 0.062$, respectively). There were also no significant differences in the Shannon

diversity index between sexes (men: RSD: 2.52 ± 0.29, HSD: 3.01 ± 0.27; women: RSD: 2.69 ± 0.23, HSD: 3.18 ± 0.32; diet * sex interaction p=0.937; Figure 2.2.b).

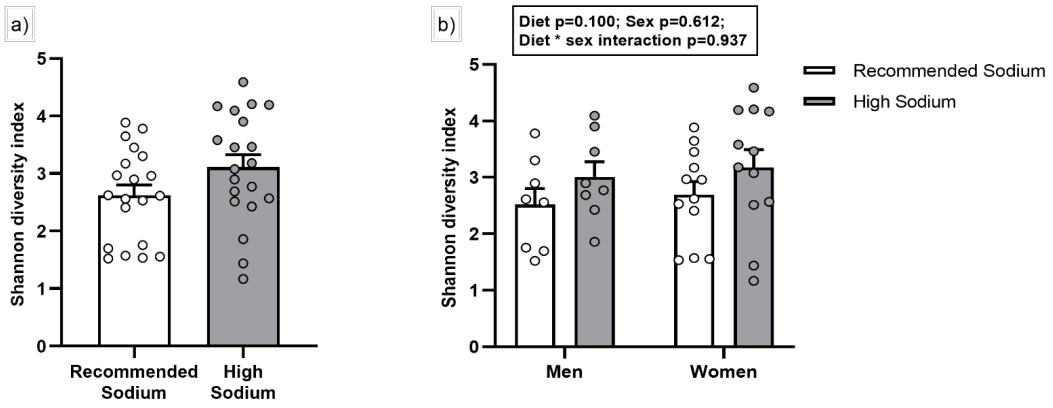
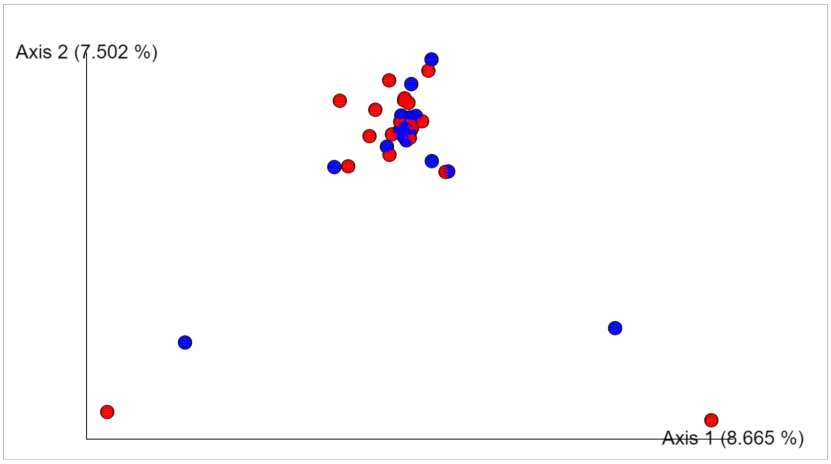


Figure 2.2: Shannon diversity index on Day 10 of high sodium (gray) and recommended sodium (white) interventions in a) all subjects (n=20), and b) men (n=8) and women (n=12) separately. Values are means ± SEM.

Figure 2.3 depicts principal coordinate analysis for beta diversity using Aitchison distance. There is no apparent separation between samples following HSD and RSD. Most samples from the same subject were clustered together suggesting a strong intraindividual microbial stability. Similar results were observed when Jaccard distance, Bray-Curtis dissimilarity, and weighted and unweighted Unifrac were used to visualize beta diversity (plots not shown).

a)



b)

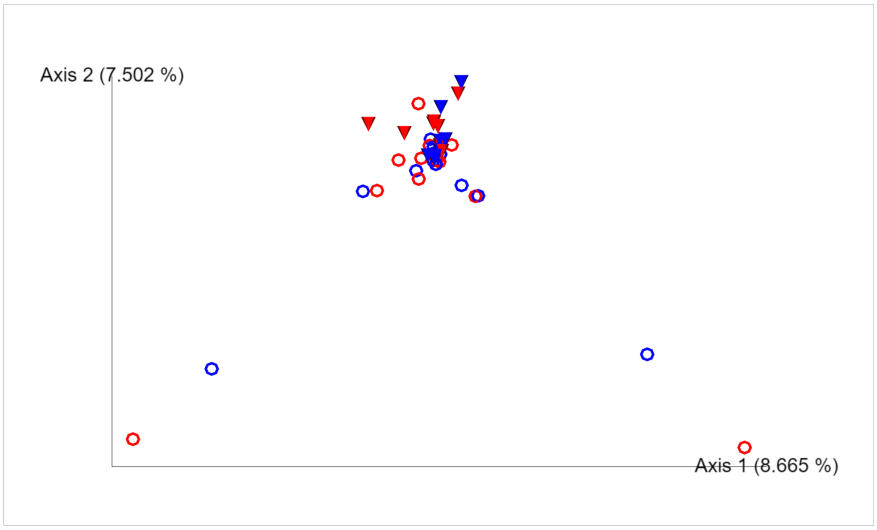


Figure 2.3: Beta diversity represented using Aitchison distance visualized in a principal coordinate analysis. a) Data points are samples collected from all subjects on day 10 of the recommended sodium diet (blue) and the high sodium diet (red). b) Data points are samples collected from men (cones) and women (rings) on day 10 of the recommended sodium diet (blue) and high sodium diet (red). n=20.

As fiber intake and BP are related to gut microbial composition, we examined their associations with the Shannon diversity index. There was no correlation between Shannon diversity index and fiber intake during interventions ($\rho = -0.029$, $p = 0.859$), nor 24h overall, nighttime, or daytime SBP, DBP, MAP, and PP (Spearman correlation coefficient ranges: -0.198 – 0.109, $p = 0.255 – 0.946$).

Analysis of taxonomic composition (ANCOM) was used to assess differences in microbial taxa between the two interventions. No specific taxa were statistically enriched in HSD nor RSD.

2.3.2 Results from subjects with samples on day 0 and day 10 of RSD and HSD (complete sets)

Figure 2.4 represents rarefaction curves of sequenced samples. A plateau of observed features with an increase in sequencing depth indicates achievement of sufficient sequencing depth which was reached at ~500 sequences.

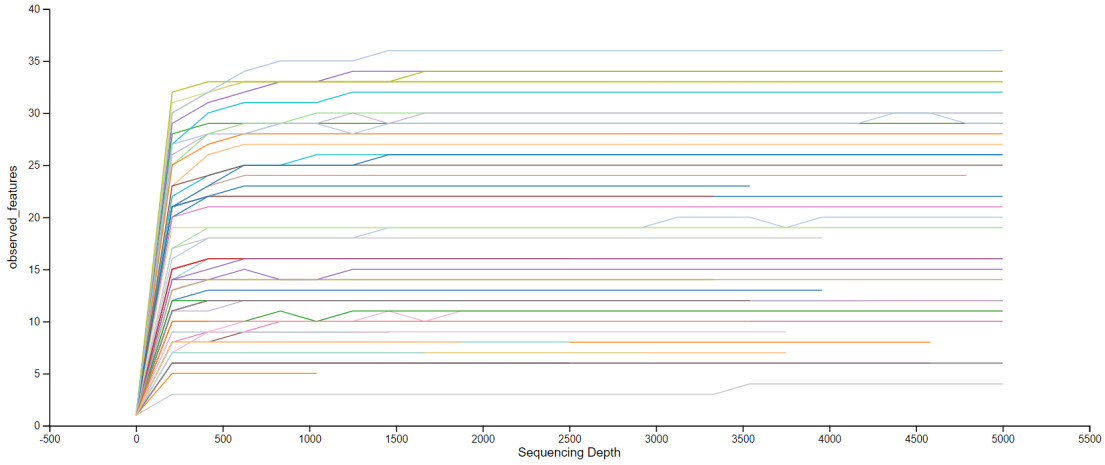


Figure 2.4: Rarefaction curves of sequenced samples. Each curve represents a sample, i.e. there are 64 curves for 64 samples (Day 0 and 10 of RSD and HSD for each subject). The X-axis represents sequencing depth, or the number of sequenced nucleotides. The y-axis represents observed ASVs (features), or sequences assigned to species. As the number of sequences from a sample increases, the number of observed ASVs converges on the true diversity. n=16. ASV, amplicon sequence variant; HSD, high sodium diet; RSD, recommended sodium diet.

Shannon diversity indices before and after each 10-day intervention are represented in Figure 2.5.a. There were no differences between samples before starting each intervention, indicating the samples had comparable microbial diversity (Day 0 RSD: 2.91 ± 0.35, Day 0 HSD: 3.36 ± 0.33; $p = 0.200$). There were also no differences between men and women before starting each intervention (Day 0 RSD, men: 2.90 ± 0.40, women: 2.91 ± 0.30, $p = 0.987$; Day 0 HSD, men: 3.18 ± 0.44, women: 3.54 ± 0.18, $p = 0.462$). Group means when all subjects were included trended to be significant ($p = 0.062$) suggesting there may be differences in Shannon diversity

between HSD and RSD. Since the p-value was trending, we further examined pairwise comparisons revealing a significant difference after 10 days of HSD and RSD with Shannon diversity being lower after RSD than HSD (Day 10 RSD: 2.54 ± 0.27, Day 10 HSD: 3.19 ± 0.33; p = 0.046). Since the main effects were non-significant, these results should be interpreted with caution. However, Faith's PD, Chao1 and observed features metrics reached significance and showed diversity and richness were lower after RSD than HSD (data not shown). Inclusion of sex in the analysis revealed no significant differences in Shannon diversity index (p = 0.925), suggesting there were no sex effects in response to sodium loading. However, there was a significant effect for diet with RSD overall having lower diversity than HSD (p = 0.021). Additionally, there were no differences in change in Shannon diversity index from day 0 to day 10 of each intervention ($\Delta_{day0\text{-}10}$ RSD: -0.37 ± 0.21, $\Delta_{day0\text{-}10}$ HSD: -0.17 ± 0.23; p = 0.534) (Figure 2.5.b). The change was also non-significant between men and women (men: $\Delta_{day0\text{-}10}$ RSD: -0.35 ± 0.13, $\Delta_{day0\text{-}10}$ HSD: -0.11 ± 0.30; women: $\Delta_{day0\text{-}10}$ RSD: -0.38 ± 0.21, $\Delta_{day0\text{-}10}$ HSD: -0.22 ± 0.29; diet * sex interaction p = 0.865). Faith's PD, Pielou Evenness, Chao1 and observed features metrics showed similar results (data not shown).

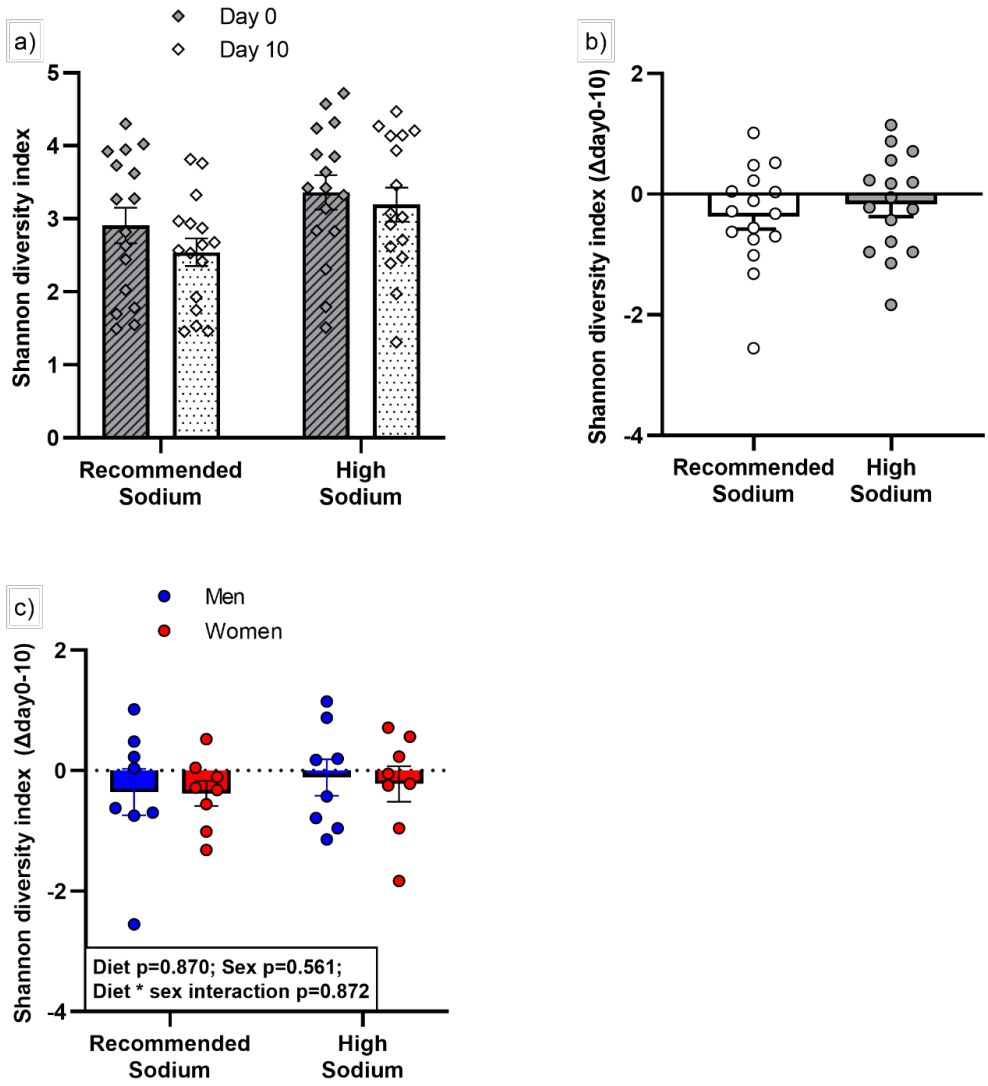


Figure 2.5: a) Shannon diversity indices at day 0 (hatched gray) and at day 10 (dotted white) of each intervention in all subjects (n=16). b) Change in Shannon diversity index from day 0 to day 10 during each intervention in all subjects (n=16). c) Change in Shannon diversity index from day 0 to day 10 during each intervention in men (blue, n=8) and women (red, n=8). Values are means ± SEM.

Figure 2.6. represents the principal coordinate analysis for beta diversity using Aitchison distance. There is no apparent separation within or between RSD and HSD diets, suggesting the gut microbiota diversity was not affected by sodium loading. Subjects' samples tended to cluster around each other regardless of the study day or the intervention. Similar results were observed when Jaccard distance, Bray-Curtis dissimilarity, and weighted and unweighted Unifrac were used to visualize beta diversity.

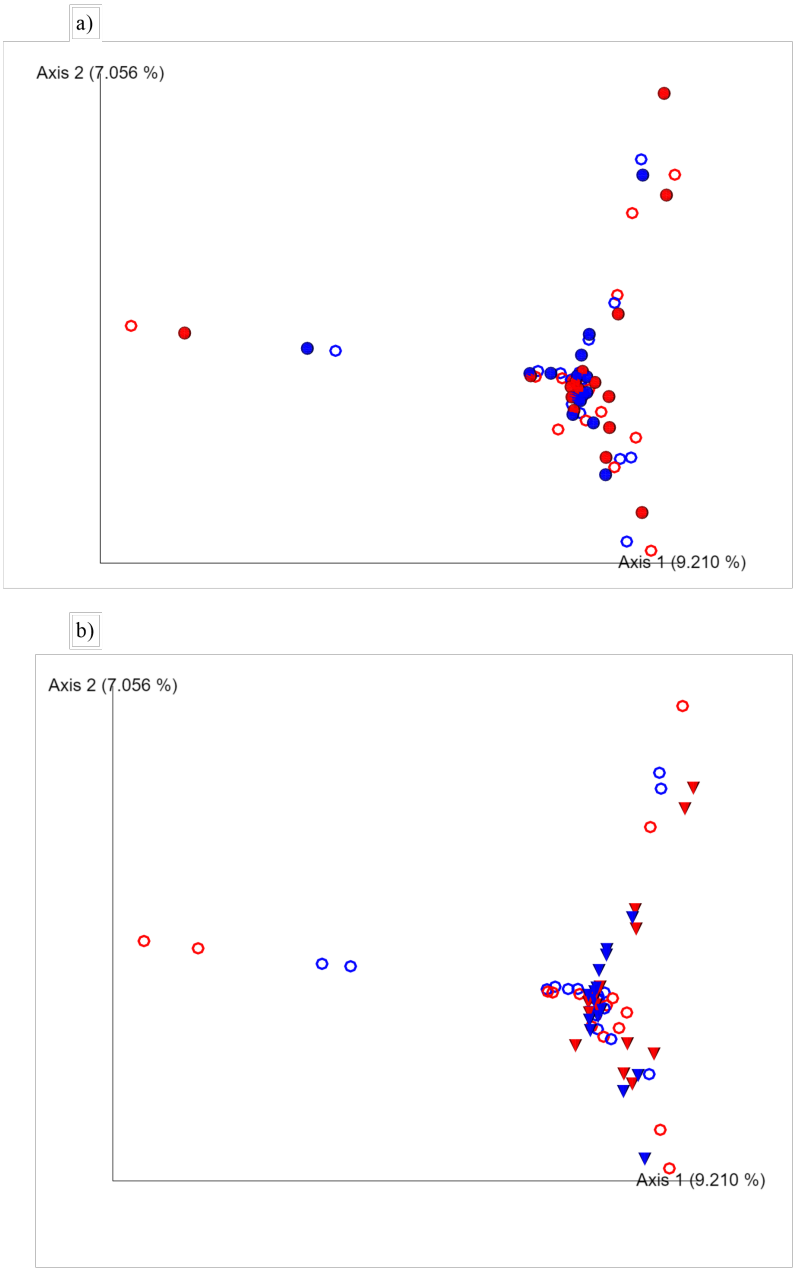


Figure 2.6: Beta diversity represented using Aitchison distance visualized in a principal coordinate analysis. a) Data points are samples collected from all subjects before (rings) and on day 10 (full circles) on recommended sodium diet (blue) and high sodium diet (red). b) Data points are samples collected from men (cones) and women (rings) during recommended sodium diet (blue) and high sodium diet (red). n=16.

As fiber intake and BP are related to gut microbial composition, we examined their associations with the Shannon diversity index. There was no correlation between Shannon diversity index and fiber intake during interventions ($\rho = -0.052$, $p = 0.777$), nor 24h overall, nighttime, and daytime SBP, DBP, MAP, and PP (Spearman correlation coefficient ranges: -0.252 – 0.110, $p = 0.155 – 0.956$).

ANCOM was used to assess differences in microbial taxa. No specific taxa were statistically enriched across HSD and RSD.

2.4 Discussion

This study was the first randomized controlled trial in humans to investigate the effects of a HSD on gut microbial composition. Our objective was to assess changes in gut microbial diversity following 10 days of a HSD and examine sex differences as exploratory analyses. Contrary to our hypotheses, microbial diversity did not change after 10 days of HSD compared to RSD, nor were there were sex differences in this sample of healthy young adults.

This study was designed to utilize salt pills to increase sodium intake over a 10 day period. Subject compliance was confirmed by an increase in 24-hour urinary sodium excretion and an increase in plasma sodium levels. Subjects reported a comparable sodium intake from foods during both the RSD and HSD interventions (~2,500 – 2,650 mg/d), although it was slightly above the target level instructed through the dietary counseling. Dietary macronutrient and micronutrient intake during the RSD and HSD interventions was similar, indicating subjects consumed foods of a similar nutritional profile during both interventions. We observed slight inconsistencies in carbohydrate, fat, and micronutrient intakes between the two days on which dietary recall was performed in each intervention. This may be due to some recalls reflecting an intake on a weekend-day that is known to be different than on a weekday [133].

We did not observe changes in alpha (Shannon diversity index) nor beta diversity (Aitchison distance) between RSD and HSD. Trending group differences and consequent exploratory pairwise differences in complete sets (stool samples collected on day 0 and day 10 of each intervention) suggested microbial diversity may be lower following RSD than HSD, which contrasts with our hypothesis. This should be interpreted cautiously since pairwise differences should not be examined if the group effect is not present. Reaching the number of samples set in the power calculation should be a priority ahead of making premature conclusions. Null findings from our study are in agreement with an observational study by Ferguson et al. [75] who examined microbial diversity in 135 individuals habitually consuming less or more

sodium than the Dietary Guidelines (~1,700 – 2,100 mg/d and ~3,100 – 3,400 mg/d). They reported no differences in alpha diversity between groups. The alpha diversity metric used in this study was not described, but operational taxonomic units were used to cluster and assign taxonomy to sequences in contrast to ASVs in our study. Beta diversity was different when sodium intake from a 3-day food record was included in analyses and the first two axes explained 23.8% of variance. This captured more variance than in our study, as the first two axes in our analyses explained 16.17% (day 10 sample sets) and 16.27% (complete sets), suggesting our subjects' samples were less dissimilar than in the study by Ferguson et al. However, beta diversity was not different when sodium intake over a 1-year period was analyzed [75]. This finding also points at inconsistencies in dietary assessments. We verified subject compliance and objectively assessed sodium intake by calculating urinary sodium excretion from a 24-hour urine collection. In addition, the sample in the mentioned study was slightly older (mean of ~30 yrs, range 18 - 50 yrs) and more racially and ethnically diverse, which could both have implications on the gut microbiota composition [57; 134].

ANCOM analyses revealed no taxa were enriched during each diet. This is in contrast with several groups who reported depletion or enrichment of certain taxa upon HSD [66; 75; 85; 87; 88]. The beneficial *Lactobacillus spp.* was depleted following a HSD in both rodents and humans [68], while *Prevotella* and *Bacteroides*, family *Ruminococcaceae*, and phyla Firmicutes and Proteobacteria was associated with high sodium intake [75]. However, these are results of single studies and need to be replicated by other investigators.

Laboratory and ambulatory BP remained consistent between RSD and HSD suggesting our subjects may have been salt resistant. However, we cannot conclusively state that since we did not have a low sodium intervention in our study design. Wilck and colleagues [68] were the first group who reported changes in microbial composition in humans upon sodium loading. They instructed 12 men ages 18 - 50 to supplement their diets with sodium tablets for 14 days and reach a total sodium intake of 5,500 mg/d, ~2,500 mg/d more than at baseline. Subjects' nighttime systolic and diastolic BP significantly rose by ~6 - 7 mmHg from baseline to HSD, suggesting they may have been salt sensitive. Changes in microbial composition during HSD are often accompanied by an increase in BP in both rodent and human experiments [68; 75; 85; 88]. Ferguson and colleagues reported a positive correlation between sodium intake and systolic BP [75]. It has also been reported in other studies that systolic BP [61; 135], diastolic BP [61], and mean arterial pressure were inversely associated with alpha [61; 135] and beta [61] diversity in a large sample of middle-aged adults. The samples in these studies were racially diverse with smokers and hypertensive individuals included, and age ranges from 25 – 74 yrs [61] and 48 – 60 years. In our study, there was no correlation between habitual sodium intake and screening systolic and diastolic BP, nor between microbial diversity and 24-hour systolic and diastolic BP at the end of the RSD and HSD interventions. Our subjects were young adults and therefore, age may influence the results that other groups have reported.

Since sex differences in microbial composition were reported in men and women [57; 58; 59], we investigated whether men and women would exhibit different responses to a HSD. In contrast to large observational studies [57; 58], women in our sample did not exhibit greater diversity than men at the beginning of each intervention. We also did not find sex differences when diversity was assessed at the end of the RSD and HSD in a sample of 20 subjects (day 10 sets) nor when the absolute diversity and change in diversity from day 0 to day 10 of RSD and HSD was assessed in a sample of 16 subjects (complete sets). We were likely underpowered to assess these differences as only 8 men were included in the analyses. Although we did not explore taxonomic differences between men and women in RSD and HSD, it was reported that men have a greater abundance of Bacteroidetes [53], and *Bacteroides* and *Prevotella* genera [59] than women. In terms of sodium intake, a negative association between *L. paracasei* and 24-hour urinary sodium excretion was reported in a cohort of middle-aged adults that was stronger in women than men [61]. Findings from rodent studies suggest male mice have greater abundances of Ruminococcaceae, *Ruminococcus*, and *Anaerostipes*, while female mice have greater abundance of Peptostreptococcaceae [55]. These results should be extrapolated to humans cautiously since rodent and human gut microbiota composition do not entirely overlap. Overall, studies investigating sex differences in microbial composition in humans are scarce.

It is important to note that some discrepancies between our study and other mentioned studies may lie in sequencing methodology and bioinformatics. The most widely sequenced genes are the V4-V6 variable regions of the 16S ribosomal RNA

(rRNA), while 16S/ITS/23S sequencing used in our study sequences all 9 variable regions of the 16S rRNA, the Internal Transcribed Spacer (ITS) rRNA, and the 23S rRNA gene. This methodology offers superior representation of the microbial community diversity which, when paired with the sparsity of microbial data, may result in a reduced ability to detect different communities between each intervention. Methodology used to assess the gut microbiome is certainly an area in the field that requires standardization.

This study is not without limitations. First, 10 days may have not been enough time for compositional changes to occur. Zhao et al. [136] emphasized that at least 28 days of fiber consumption is required to illicit changes in the gut microbial composition, which is in contrast to early published findings that the gut microbial composition shifts even after only 5 days of dietary intervention consisting of plant or animal foods [137]. It is plausible that 10 days may be too long to see transitional changes that may have occurred in the first several days of the interventions, and too short to observe permanent microbial changes. Second, dietary counseling accompanied by sodium supplementation allows for certain amount of leeway in food choices and sodium intake compared to a controlled feeding study in which researchers can strictly control nutrient intake. Third, although microbial diversity has been used as an indicator of gut microbial health, it may be too simplistic as it does not give information about the functional capacity of microbial communities. Assessing taxonomic and functional differences between the two interventions may be a better indicator of the effects of an HSD on the gut microbiota. Lastly, we have not

achieved the calculated statistical power due to lower quality of several samples after sequencing. However, both compositional analyses using ASVs and traditional analysis using operational taxonomic units (data not shown) revealed no changes in diversity indices following a HSD which strengthens our findings. Nevertheless, we have additional samples stored which could be sequenced and included in the analysis.

In conclusion, we carried out a dietary counseling protocol accompanied with sodium pill supplementation to increase sodium intake, as confirmed by increased urinary sodium excretion and serum sodium levels. Our findings revealed that the gut microbial diversity did not change after 10 days of an HSD compared to RSD in a cohort of young healthy adults, nor did the gut microbiota composition respond differently to sodium in men and women. While high sodium intake may be detrimental for several organ systems [138], it appears that the gut microbial composition of young healthy adults may not be affected by an HSD. Further research should focus on standardizing methods of gut microbial composition assessment and targeting individual characteristics, such as salt sensitivity, that may be mediating changes in the gut microbial composition upon high sodium consumption. In addition, comparing young to middle-aged adults in a similar intervention may identify potential differences in sodium-induced microbial changes that arise with aging.

Chapter 3

HIGH DIETARY SODIUM INTAKE AND CIRCULATING T-HELPER 17 AND T-REGULATORY CELLS IN HEALTHY YOUNG ADULTS

3.1 Introduction

Sodium intake among United States (U. S.) adults is 57% greater than recommended by the Dietary Guidelines for Americans [1; 139]. While the recommended intake is 2,300 mg sodium/d, Americans consume an average of 3,600 mg/d [139] with only 11% of adults meeting the recommendations [140]. High consumption of dietary sodium is widely known for its effects on blood pressure (BP) as it is a modifiable risk factor for hypertension (HTN) [4]. HTN remains a major public health problem with nearly 1 in 2 Americans diagnosed with this chronic disease [4]. In addition to BP, high sodium consumption has detrimental effects on the vasculature, heart, kidneys, skin, brain, bone, and most recently, the gut microbiota [138]. There is emerging evidence that the gut microbiota mediates the pathophysiology of HTN and that high intake of sodium alters gut microbial composition and function [15; 66; 68; 87; 88]. A plausible link between sodium's effects on the gut microbiota is immune system activation. The immune system constantly surveys the microbial population in the body, particularly in the gastrointestinal (GI) tract which harbors over 2900 identified bacterial species [28; 29]. Considering implications of the gut microbiota in the pathophysiology of HTN and the vital gut-immune communication,

investigating whether the immune system is activated upon high sodium intake is warranted.

The gut microbiota is necessary for proper maturation of immune cells [48]. Bacterial species and their metabolites impact the immune cell profile along the GI tract and thus regulate inflammation [12]. High sodium conditions have been shown to activate proinflammatory T-helper 17 (T_H17) cells and suppress anti-inflammatory T-regulatory (T_{reg}) cells that are most abundant in the gut. While homeostasis requires a balance between the two, exposure to high salt can impair the regulatory functions of T_{reg} cells and shift the T_H17/T_{reg} ratio [64; 77]. Addition of sodium activates T_H17 cells in murine and human cell cultures [65; 67; 68] which rapidly increased their proliferation and was followed by an increase in production of the inflammatory cytokines interleukin (IL)-17A, IL-23, IL-6, and TNF-α [73]. T_H17 cells have also been activated in the mesenteric lymph nodes [66; 74] , the spleen [74], and small intestinal lamina propria [74] in mice fed a high sodium diet (HSD). The same was not observed in the blood of HSD-fed mice although plasma levels of IL-17A and the mRNA levels of proteins required for T_H17 cell polarization (IL-23, IL-22, and serum amyloid proteins) were elevated [74]. Findings from studies in humans demonstrate an increase in circulating IL-6 and IL-23 following long-term consumption of HSD compared to lower amounts (4,800 mg/d vs. 3,600 and 2,400 mg/d for 30 - 60 days) [69]. Plasma IL-17 is increased in subjects with normal BP, elevated BP, or stage 1 HTN consuming a HSD compared to a low sodium diet (7,000 mg/d vs 1,200 mg/d) [76]. Only one pilot study has investigated the status of T cells in humans. This study only included men

who supplemented their diet with salt tablets resulting in an increase in T cells producing IL-17A and TNF-α [68].

Addition of sodium to culture media, and rodents fed a HSD suppresses anti-inflammatory T_{reg} cells and the release of anti-inflammatory cytokines in *in vitro* and *in vivo* experiments [77]. This is accompanied by a shift towards a pro-inflammatory T_{reg} phenotype [77]. There are no studies in humans investigating T_{reg} cell status following a HSD. The limited data in humans warrants a new focus on the effect of a HSD on T cell activation and suppression.

The objective of this study was to determine the effect of a HSD on the abundance of T_H17 and T_{reg} cells in the peripheral blood of healthy adults compared to a recommend sodium diet (RSD). We hypothesized that a HSD would increase the abundance of T_H17 cells and reduce abundance of T_{reg} cells compared to RSD.

3.2 Methods

3.2.1 Study design

This was a single-blind randomized crossover study with two 10-day interventions of RSD or HSD to determine the effect of sodium on T_H17 and T_{reg} cell populations. The study protocol and procedures were approved by the Institutional Review Board of the University of Delaware (ID# 1423170) and conform to the provisions of the Declaration of Helsinki. Subjects were recruited through flyers posted around the University of Delaware campus and the surrounding community, as well as through online advertisements. Informed consent was obtained from all subjects prior to enrollment in the study. The study was registered on clinicaltrials.gov (NCT04229680).

3.2.2 Subject screening

Healthy adults between the ages of 18-45 were recruited to participate in the study. A prescreening questionnaire was administered via phone before the screening visit to ensure age, body mass index (BMI), and main GI health criteria were met. After subjects signed the informed consent, their height, weight, and resting BP were measured. Medical history including GI health, dietary supplementation, and antibiotic and medication use were assessed via questionnaires. Inclusion criteria was apparently

healthy men and women, age 18-45 years with a systolic BP <130mmHg, diastolic BP <80mmHg and BMI between 18.5-29.9 kg/m^2. Exclusion criteria were the following: history of cardiovascular disease, HTN, diabetes or kidney disease; history of intestinal disease or surgery, malabsorption disorder, or food allergies/sensitivities; antibiotic, prebiotic, or probiotic use in past 3 months; use of medications for mood disorders (fluoxetine, escitalopram, venlafaxine, aripiprazole, lithium, olanzapine, valproate); malignant cancer; pregnancy or lactation; smoking; and highly trained endurance athletes.

3.2.3 Dietary interventions

Subjects were instructed to provide a food record for three consecutive days (2 weekdays and 1 weekend day) to assess their habitual diet. Eligible subjects were instructed to consume a recommended amount of dietary sodium (2,300 mg/d)[8] for 10 days for both interventions and then supplement with either sodium pills (4,600 mg/d for a total of 6,900 mg/d; HSD) or placebo pills (dextrose; RSD). At least a 2-week washout occurred between the interventions. Subjects were asked again about GI health, dietary supplementation, and antibiotic and medication use prior to starting the second intervention period to ensure no changes occurred to their GI health. Research staff provided subjects with instructions and strategies to consume a diet with the recommended amount of sodium during both interventions. These instructions were based on subjects' habitual dietary intake and food choices following the nutrient

analysis of a 3-day food record. Subjects recorded their dietary intake during the intervention and were instructed to consume similar foods during both trials. To ensure compliance to sodium intake, food intake was assessed via two 24-hour dietary recalls during each 10-day intervention using the multiple-pass approach[112]. The first recall was performed through an unannounced phone call between days 4 and day 7, i.e. the exact day was unknown to the participant. The second recall was performed during the in-lab testing visit on day 10 and recalled foods consumed on day 9. The average of two 24-hour recalls was reported. Diet records were analyzed using Nutrition Data System for Research software (NDS-R 2018 and NDS-R 2019, University of Minnesota, Minneapolis).

3.2.4 Sodium and placebo supplementation

Subjects supplemented their diet with 12 enteric, slow-release pills per day containing either table salt (4,600 mg sodium total) or dextrose (10 g total) as a placebo. They were randomly assigned to the sodium and placebo conditions and were blinded to the pill contents. They were instructed to space out their pill intake throughout the day and take them with food only and not on an empty stomach to minimize GI side-effects. For the first three days of the intervention, subjects gradually increased the number of pills they took (5 pills on day 1, 7 pills day 2 and 9 pills on day 3). For days 4-10, they were instructed to consume all pills daily. Subjects were asked to note the number of pills taken each day to ensure compliance. They

were also provided with GI side-effects forms to note any side-effects they might experience.

3.2.5 Ambulatory BP, urine collection, and blood draw

Ambulatory BP monitoring (Oscar 2 with SphygmoCor, SunTech Medical) was conducted on the last day of each diet to collect 24 hours of BP data [113]. BP was automatically taken every 20 min during the day and every 30 min at night. Data was accepted if at least 20 daytime and 7 nighttime BPs were recorded [114]. Urine was also collected for 24 hours on the last day. Urine volume, electrolyte concentrations (sodium, potassium, and chloride) (EasyElectrolyte Analyzer, Medica), and osmolality (Advanced 3D3 Osmometer, Advanced Instruments) were assessed as per standard lab protocol. Urine collections were considered incomplete if there were ≥2 missed collections or if urine volume was <500 mL [115]. Subjects were asked to fast, avoid caffeine and alcohol for at least 12 hours and not exercise for 24 hours prior to the testing visit on day 10. A venous blood sample was collected on day 10 of both interventions. Hematocrit and hemoglobin in the whole blood, serum electrolytes (EasyElectrolyte Analyzer, Medica), and plasma osmolality (Advanced 3D3 Osmometer, Advanced Instruments) were assessed.

3.2.6 Isolation, storage, and stimulation of peripheral blood mononuclear cells (PBMCs)

Whole blood was collected in K2EDTA tubes and processed within 24h of collection. A complete blood count with differential was completed for each sample (VetScan Hm5 Hematology Analyzer, Abaxis, Union City, CA). PBMCs were harvested by a gradient density separation technique (Fico/Lite LymphoH, R&D Systems, Minneapolis, MN), and assessed for viability and cell count. The cells were frozen at a density of 1 x 107 cells/mL in a freezing medium (RPMI-1640 (Gibco) with 20% fetal bovine serum (FBS, Gibco), 10% DMSO (Sigma-Aldrich), and 1% Penicillin-Streptomycin (Gibco) and stored at -196ºC in the vapor phase of liquid nitrogen until analysis. Prior to analysis, cells were thawed, washed in complete media (RPMI-1640 (Gibco) with 10% FBS (Gibco) and 1% Penicillin-Streptomycin (Gibco), and resuspended at a concentration of 0.5 - 1 x 106 cells/mL. PBMCs were stimulated with Phorbol 12-Myristate 13-Acetate (PMA; 10 ng/mL; Sigma-Aldrich), ionomycin (1 µg/mL; Calbiochem), Brefeldin A (5 µg/mL; Sigma-Aldrich), and monensin (5 µg/mL; BioLegend) and incubated for 4 hours at 37ºC and 5% CO2 in complete media to elicit cytokine production.

3.2.7 Immunophenotypic identification and analysis

Cell populations were characterized based on the expression of cell surface and cytoplasmic markers. Extensive flow cytometric analysis of lymphocytes was conducted using TNF-α (MAb11 clone, BV421, BioLegend), CD4 (OKT4 clone, BV605, BioLegend), CD8 (SK1 clone, BV650, BioLegend), IL-17A (BL168 clone, BV785, BioLegend), CD127 (A019D5 clone, FITC, BioLegend), CD45 (2D1 clone, PerCP, BioLegend), RORγt (Q21-559 clone, PE, BD Pharmingen), CD25 (BC96 clone, PE-Cy5, BioLegend), CD56 (5.1H11 clone, Pe-Cy7, BioLegend), CD3 (SK7 clone, APC, BioLegend), CD14 (M5E2 clone, AlexaFluor 700, BioLegend), and CD19 (SJ25C1 clone, APC-Cy7, BioLegend) antibodies. Briefly, 1 x 106 cells were stained with antibodies against cell surface markers, then fixed and permeabilized for intracellular staining (Fix and Perm kit, Caltag, Buckingham, UK). Compensation beads were used for positive and negative staining controls and to avoid spillover signals from fluorochromes (BD Biosciences, San Jose, CA). Fluorescence minus one (FMO) controls were employed in addition to compensation controls.

3.2.8 Flow cytometry

Samples were analyzed on a 15-color flow cytometer (BD FACSAria Fusion, BD Biosciences, San Jose, CA). Thirty thousand events were acquired for each

sample. Data were analyzed using Becton-Dickinson FACSDiva™ software (v9.0.1) and DeNovo FCS Express (v5.01.0050).

3.2.9 Statistical analysis

Differences in T_H17 and T_{reg} abundance on day 10 of RSD and HSD were evaluated using a paired t-test. We used relative and absolute counts in the analyses, as well as fold change from RSD to HSD. To account for potential differences in cell abundance resulting from a change in BP we used a linear mixed model with the 24h systolic BP (overall, nighttime, and daytime) as a time-varying covariate. In addition, we included sex as a between-subjects factor to explore sex differences. Other cells of interest included CD4+IL-17A+, CD4+TNF-α+, and CD4+IL-17A+TNF-α+ that were analyzed as described above. Subject characteristics and dietary intake during RSD and HSD was compared using a paired t-test. *A priori* power analysis was performed using G*Power statistical software. Based on findings of Luo et al.[132], a sample size of 19 participants was needed to detect a 0.43% absolute difference in T_H17 (CD4+RORγt+) cells between the two conditions. Based on findings of Hu et al.[141], we needed a sample size of 6 participants to detect a 1.73% absolute difference in T_{reg} (CD4+CD25+CD127-) cells. Significance was set *a priori* at $p<0.05$.

3.3 Results

Twenty-two healthy adults completed both study interventions and PBMCs were isolated from 20 subjects. Subject characteristics are presented in Table 3.1 and are further separated by sex in Appendix B, Table B1 and Table B2. Overall, subjects were young, had a normal screening BP, and normal BMI. Urinary sodium excretion and serum sodium levels were greater on HSD compared to RSD indicating study compliance, while serum osmolality was unchanged. There were no differences in subjects' ambulatory systolic and diastolic overall, nighttime or daytime BP between the two interventions. There was a slight reduction in HR on HSD compared to RSD but the values were within normal limits. Dietary intake data as an average of two 24-hour recalls demonstrated subjects' energy, macronutrient, and micronutrient intake was comparable between the diets (Table 3.2). We further examined subjects' consistency in energy and nutrient intake within each intervention by correlating energy and nutrient intakes from the two recalls. Energy intake and intake of protein, fat, and fiber from the two 24-hour dietary recalls in RSD were significantly correlated, while energy intake and intake of protein, fat, and sodium from the two 24-hour dietary recalls in HSD were significantly correlated (Appendix B, Table B3). These results indicate that subjects were consistent in energy and macronutrient intake on the days that recalls were performed, however micronutrient intake was more variable. Note that urinary sodium excretion in Table 3.1 reflects overall sodium intake as supplemented with the pills, while sodium intake presented in Table 3.2 reflects intake from the diet. Habitual dietary intake is presented in Appendix B (Table B4).

Table 3.1: Subject characteristics.

	Screening	Recommended Sodium	High Sodium	p-value*
Demographic data				
N (men/women)	22 (13/9)			
Ethnicity (H/NH)[a]	0/20			
Race (W/B/A)	19/1/2			
Anthropometrics				
Age (yr)	25 ± 1			
Height (cm)	171 ± 2			
Mass (kg)	67.0 ± 2.0	66.3 ± 2.0	66.5 ± 2.0	0.442
Body mass index (kg/m^2)	22.9 ± 0.5			
Hemodynamics				
Laboratory[b]				
Systolic BP (mmHg)	114 ± 2	112 ± 2	113 ± 2	0.412
Diastolic BP (mmHg)	65 ± 1	62 ± 1	64 ± 1	0.134
MAP (mmHg)	81 ± 1	79 ± 1	80 ± 1	0.171
Heart rate (bpm)	66 ± 2	58 ± 2	55 ± 2	**0.043**
24h ambulatory				
Systolic BP, overall (mmHg)[c]		116 ± 2	117 ± 3	0.405
Diastolic BP, overall (mmHg)[c]		66 ± 1	66 ± 1	0.625
MAP, overall (mmHg)[c]		83 ± 2	83 ± 2	0.806
Heart rate, overall (bpm)[c]		70 ± 2	69 ± 2	0.695
Systolic BP, daytime (mmHg)		120 ± 2	122 ± 2	0.129
Diastolic BP, daytime (mmHg)		70 ± 1	70 ± 1	0.843
MAP, daytime (mmHg)		87 ± 2	87 ± 1	0.855
Heart rate, daytime (bpm)		73 ± 2	71 ± 2	0.155
Systolic BP, nighttime (mmHg)[c]		107 ± 3	107 ± 4	0.958
Diastolic BP, nighttime (mmHg)[c]		56 ± 2	55± 2	0.611
MAP, nighttime (mmHg)[c]		73 ± 2	72 ± 2	0.838
Heart rate, nighttime (bpm)[c]		60 ± 2	61 ± 2	0.774
Biochemical parameters				
Hemoglobin (g/dL)		14.8 ± 0.3	14.4 ± 0.3	**0.023**
Hematocrit (%)		44.0 ± 0.8	44.2 ± 0.8	0.760

Table 3.1 continued.

Serum sodium (mmol/L)	138.4 ± 0.4	139.7 ± 0.4	**0.010**
Serum potassium (mmol/L)	3.8 ± 0.1	3.9 ± 0.0	0.205
Serum chloride (mmol/L)	103.9 ± 0.5	105.3 ± 0.4	**0.003**
Serum osmolality (mOsm/kg)	286 ± 1	288 ± 1	0.095
Urinary sodium excretion (mmol/24h)	114 ± 9	288 ± 15	**<0.001**
Urinary potassium excretion (mmol/24h)	51 ± 5	56 ± 6	0.209
Urine osmolality (mOsm/kg)	436 ± 50	579 ± 42	**0.001**
Urine specific gravity	1.012 ± 0.001	1.014 ± 0.001	0.076

A, Asian; B, Black; BMI, body mass index; BP, blood pressure; H, Hispanic; MAP, mean arterial pressure; NH, non-Hispanic; W, White. Values are means ± SEM. [a]Two subjects (1 man, 1 woman) did not report ethnicity. [b]Screening measured after 5min seated rest, Recommended/High Sodium measured after 20min supine rest. [c]n=17. *Recommended Sodium vs. High Sodium.

Table 3.2: Dietary intake during Recommended/High Sodium interventions.

	Recommended Sodium	High Sodium	p-value
Energy intake (kcal/d)	2,126 ± 115	2,020 ± 93	0.378
Carbohydrates (g/d)	234 ± 14	226 ± 11	0.579
Fiber (g/d)	22 ± 2	23 ± 2	0.836
Added sugar (g/d)	39 ± 5	40 ± 4	0.885
Protein (g/d)	94 ± 7	93 ± 7	0.972
Fat (g/d)	85 ± 5	82 ± 5	0.631
Saturated fat (g/d)	26 ± 2	24 ± 2	0.331
Sodium (mg/d)	2,761 ± 198	2,756 ± 179	0.980
Potassium (mg/d)	2,747 ± 160	2,631 ± 140	0.528
Phosphorus (mg/d)	1,348 ± 82	1,246 ± 64	0.198
Calcium (mg/d)	939 ± 52	847 ± 53	0.204
Magnesium (mg/d)	351 ± 28	327 ± 19	0.343
Alcohol (g/d)	12 ± 6	5 ± 2	0.218

Values are means ± SEM. n=22.

Flow cytometric analysis of lymphocytes and lymphocyte subpopulations demonstrated no change in relative nor absolute counts of lymphocytes, T cells, B cells, NK cells, and monocytes between RSD and HSD (Table 3.3). Representative plots of unstimulated stained and stimulated unstained controls for quantification of CD4+RORγt+ T_H17 and CD4+CD25+CD127- T_{reg} cells are presented in Figure 3.1. These plots demonstrate lower abundance of CD4+RORγt+ T_H17 and CD4+CD25+CD127- T_{reg} cells without stimulation (Figure 3.1.a) and a successful staining protocol as there were no cells detected in an unstained sample (Figure 3.1.b). Sorting of T cell subpopulations revealed no change in percentage of CD4+RORγt+ T_H17 nor CD4+CD25+CD127- T_{reg} cells between RSD and HSD (T_H17: RSD: 0.35 ±

0.06%, HSD: 0.28 ± 0.02%, p = 0.262; $T_{reg:}$ RSD: 0.99 ± 0.11%, HSD: 0.28 ± 0.02%, p = 0.552) (Figures 3.2.b and 3.3.b). There was also no change in absolute numbers of CD4+RORγt+ T_H17 nor CD4+CD25+CD127- T_{reg} cells between RSD and HSD (T_H17: RSD: 3.6 ± 0.7, HSD: 2.9 ± 0.4 cells/μL, p = 0.263; T_{reg}: RSD: 8.9 ± 1.1, HSD: 9.0 ± 0.9 cells/μL, p = 0.826) (Figures 3.2.c and 3.3.c). Fold change from RSD to HSD remained statistically comparable between the diets (T_H17: RSD: 1.03 ± 0.16, HSD: 1.28 ± 0.29, p = 0.562; T_{reg}: RSD: 1.05 ± 0.16, HSD: 1.13 ± 0.08, p = 0.719) (Figures 3.2.d and 3.3.d). There was also no change in abundance of these cells when values were covaried for 24h systolic BP (overall, nighttime or daytime) (T_H17, p = 0.126 - 0.958; T_{reg}, p = 0. 225 - 0.940, respectively), nor when sex was included as a between subjects factor (T_H17, p = 0.077 - 0.607; T_{reg}, p = 0.311 – 0.873, respectively). Relative count, absolute count, and fold change from RSD to HSD of CD4+IL-17A+ and CD4+TNF-α+ cells (i.e. IL-17A- and TNF-α-producing T helper cells, respectively) were also not different between RSD and HSD (CD4+IL-17A+ relative count: RSD: 0.73 ± 0.09%, HSD: 0.70 ± 0.07%, p = 0.688; absolute count: RSD: 7.6 ± 1.0 cells/μL, HSD: 7.4 ± 1.1 cells/μL, p = 0.811; fold change: RSD: 1.05 ± 0.12, HSD: 1.04 ± 0.09, p = 0.981; CD4+TNF-α+ relative count: RSD: 44.3 ± 2.0%, HSD: 40.9 ± 2.3%, p = 0.170; absolute count: RSD: 436 ± 47 cells/μL, HSD: 455 ± 73 cells/μL, p = 0.620; fold change: RSD: 0.99 ± 0.05, HSD: 0.94 ± 0.05, p = 0.573). In the interest of replicating an increase in CD4+IL-17A+TNF-α+ cells following a HSD as shown by Wilck and colleagues, we assessed differences in the mentioned cells but did not observe significant changes between the interventions (relative count: RSD: 0.80 ± 0.08%, HSD: 0.74 ± 0.07%, p = 0.401; absolute count: RSD: 8.4 ± 1.1 cells/μL,

HSD: 7.9 ± 1.2 cells/μL, p = 0.688; fold change: RSD: 1.03 ± 0.11, HSD: 1.03 ± 0.10, p = 0.976).

Table 3.3: Relative and absolute counts of lymphocytes and lymphocyte subpopulations from flow cytometric data.

	Recommended Sodium	**High Sodium**	**p-value***
Relative counts			
Lymphocytes (% PBMCs)	87.9 ± 1.2	84.3 ± 2.4	0.139
CD3+ T cells (% lymphocytes)	84.4 ± 1.1	85.9 ± 1.4	0.292
CD4+ T helper cells (%)	58.6 ± 2.5	59.1 ± 2.4	0.789
CD8+ cytotoxic T cells (%)	25.2 ± 2.1	24.4 ± 1.8	0.342
CD19+ B cells (%lymphocytes)	5.35 ± 0.39	5.19 ± 0.54	0.682
CD56+ natural killer cells (% lymphocytes)	10.20 ± 1.21	8.89 ± 1.22	0.297
CD14+ monocytes (% lymphocytes)	2.59 ± 0.35	3.20 ± 0.55	0.180
Absolute counts (cells/μL)			
Lymphocytes	2067 ± 161	1954 ± 127	0.319
CD3+ T cells	1732 ± 130	1681 ± 122	0.521
CD4+ T helper cells	1006 ± 89	1020 ± 102	0.741
CD8+ cytotoxic T cells	437 ± 60	396 ± 37	0.243
CD19+ B cells	101 ± 8	97 ± 10	0.666
CD56+ natural killer cells	224 ± 41	173 ± 25	0.232
CD14+ monocytes	51 ± 7	57 ± 9	0.470

Values are means ± SEM. PBMC, peripheral blood mononuclear cell. n=19.
*Recommended Sodium vs. High Sodium

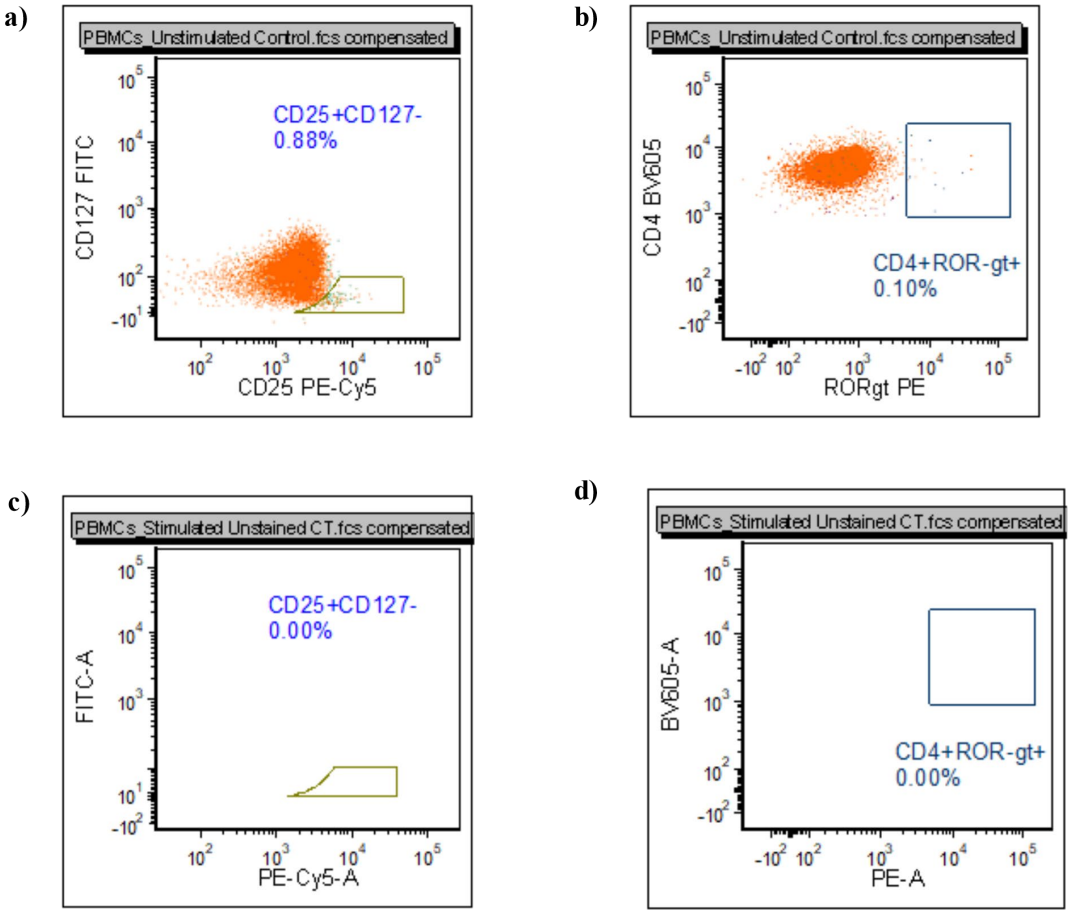
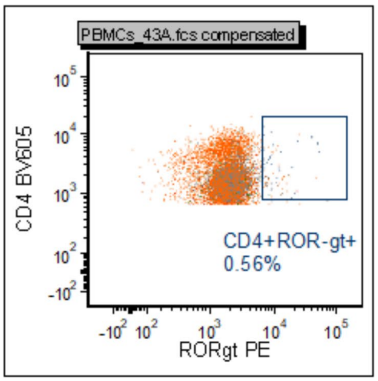
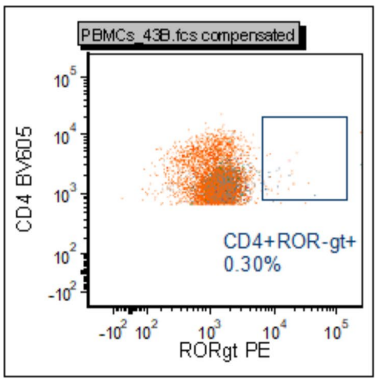


Figure 3.1: Representative plots of experiment controls: a) unstimulated stained CD4+CD25+CD127- T-regulatory cells, b) unstimulated stained CD4+RORgt+ T-helper 17 cells, c) stimulated unstained CD4+CD25+CD127- T-regulatory cells, d) stimulated unstained CD4+RORgt+ T-helper 17 cells.

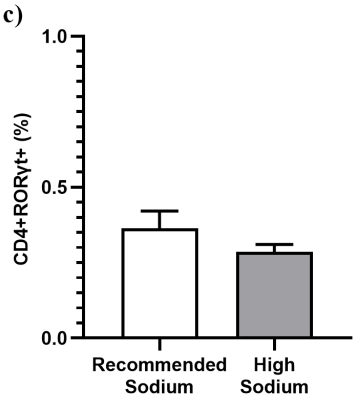
a) Recommended sodium



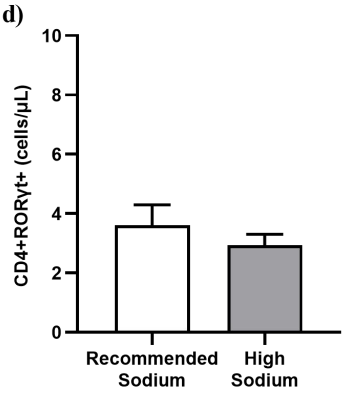
b) High sodium



c)



d)



e)

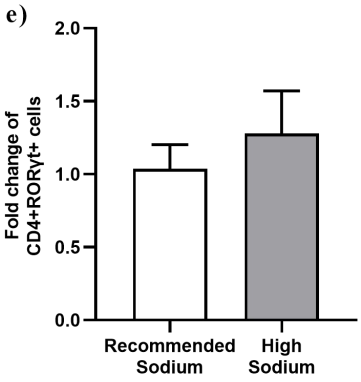


Figure 3.2: Representative plots of CD4+RORgt+ T-helper 17 cells following a a) recommended sodium, and b) high sodium diet. c) Relative count of CD4+RORgt+ cells. d) Absolute count of CD4+RORgt+ cells. e) Fold change of CD4+RORgt+ cells from recommended to high sodium diet. n=19.

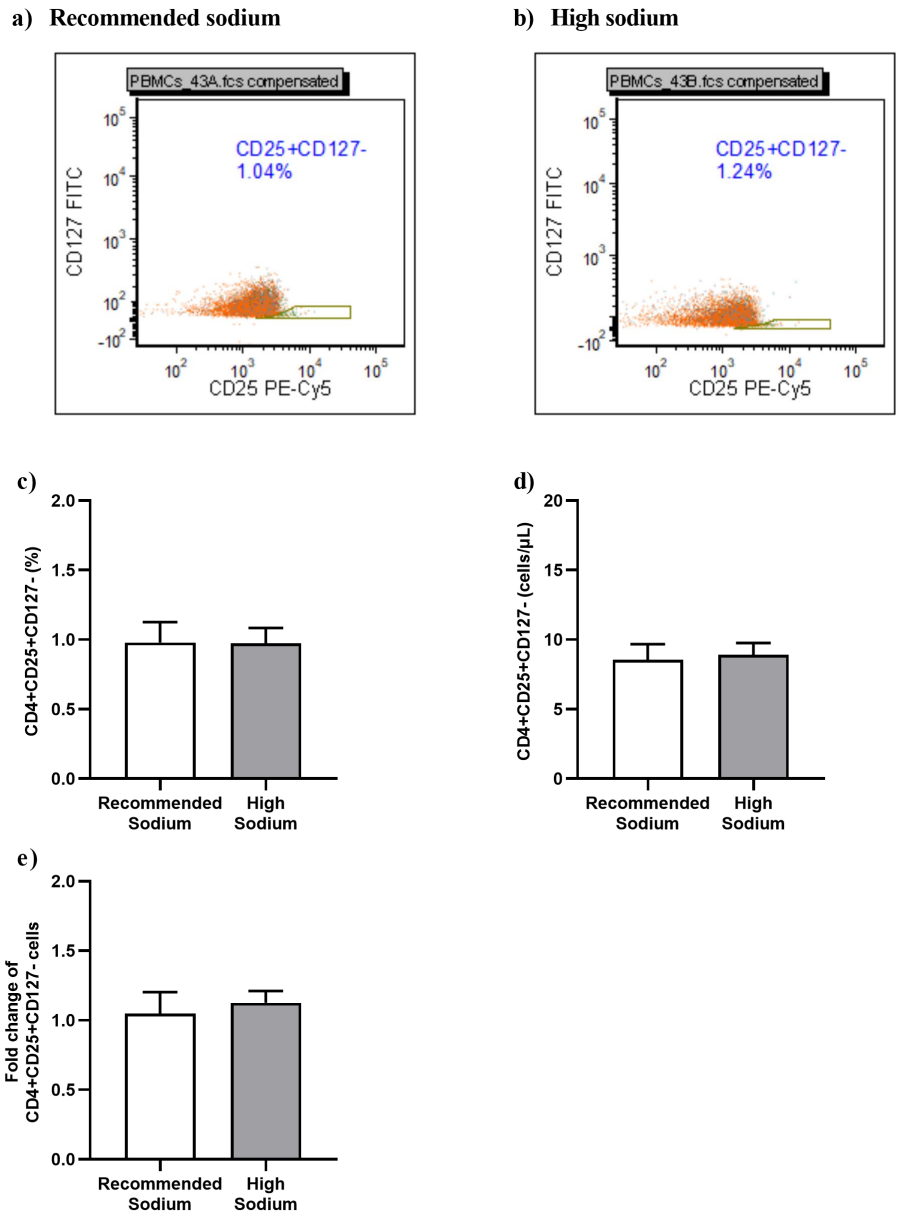


Figure 3.3: Representative plots of CD4+CD25+CD127- T-regulatory cells following a a) recommended sodium, and b) high sodium diet. c) Relative count of CD4+CD25+CD127- cells. d) Absolute count of CD4+CD25+CD127- cells. e) Fold change of CD4+CD25+CD127- cells from recommended to high sodium diet. n=19.

3.4 Discussion

The objective of this study was to assess the changes in T_H17 and T_{reg} cell abundances between recommended and high levels of sodium in the diet. We hypothesized that T_H17 cells would increase in abundance and T_{reg} cells decrease in abundance with a higher amount of sodium in the diet. Contrary to our hypothesis, the obtained results demonstrated no change in T_H17 nor T_{reg} cell abundances between RSD and HSD in a sample of healthy young adults.

Habitual dietary intake of our subjects revealed a macronutrient intake distribution common for Westernized societies with ~45%, ~15%, and ~35% of energy coming from carbohydrates, protein, and fat, respectively. Similar patterns for macronutrients and micronutrients were observed during both interventions, suggesting that subjects followed their dietary habits. We observed slight inconsistencies in micronutrient intakes between the two days on which dietary recall was performed in each intervention. This may be due to some recalls reflecting an intake on a weekend-day that is known to be different than a weekday [133]. Americans consume 150% of the dietary sodium recommended by the Dietary Guidelines for Americans [1; 139] and it is also not uncommon for a single meal at restaurants to exceed the recommended daily intake of 2,300 mg/d [142; 143]. Although our subjects habitually consumed ~600 mg sodium/d more than the recommendations, they consumed ~700 mg/d less than the U.S. average. During the two interventions, their reported sodium intake remained consistent, while the urinary sodium excretion

demonstrated a significant increase on HSD, indicating compliance to taking sodium pills. We also did not observe changes in overall, daytime nor nighttime systolic BP, diastolic BP, and mean arterial pressure, suggesting that the HSD did not have adverse effects on BP. Nevertheless, high sodium intake has negative implications on physiological systems independent of a rise in BP [138; 144] and recent evidence points at immune system activation upon high sodium consumption [68; 69; 74].

We did not observe any differences in the major lymphocyte subpopulations including: CD3+ T cells, CD3+CD4+ T-helper cells, CD3+CD8+ cytotoxic T cells, CD19+ B cells, and CD56+ natural killer cells. Results from a longitudinal study by Yi et al. [69] demonstrated no differences in abundance of peripheral T cells, T-helper cells, cytotoxic T cells, nor natural killer cells when 6 healthy male astronauts were placed on 2,200, 3,300 and 4,500 mg sodium/d diets for 30 - 60 days each. They did however report a reduction in B cells on the 2,200 mg sodium/d diet compared to the 4,500 mg sodium/d diet. BP measures were not provided to assess whether the observed differences were BP-dependent. These findings suggest that a longer duration of HSD may be required to observe changes in lymphocyte subpopulations.

In addition to lymphocytes, high sodium also activates monocytes. Even small transient changes in plasma sodium concentration (2-4 mmol/L) can rapidly inhibit mitochondrial respiration of circulating monocytes [145]. We observed a change of 1.3 mmol/L in serum sodium concentration between RSD and HSD, which may be the

reason why there were no differences in monocyte abundances in our study. Trials in humans report monocyte expansion during a HSD compared to lower sodium diets [69; 72; 146]. A randomized controlled trial in healthy young volunteers demonstrated expansion of circulating monocytes when subjects consumed a HSD (7,500 mg/d) compared to low sodium diet (2,000 mg/d) with no increase in BP [72]. In another trial, circulating monocytes of healthy young men who consumed a high (7,500 mg/d) or a very low sodium diet (750 mg/d) tended to increase on the high sodium diet [146]. The monocytes also exhibited a proinflammatory phenotype when cultured *in vitro* in a high sodium media. These observations were concomitant with an increase in systolic BP and body mass [146]. A smaller change in sodium intake between the two interventions in our study compared to the mentioned trials (4,000 mg/d in our trial vs. 5,500 mg/d [72] and 6,750 mg/d [146]) could be another reason for the lack of differences in monocyte abundances, as well as consistent BP measures on RSD and HSD.

It has been suggested that high sodium microenvironments drive inflammation in HTN [147] by acting directly on T cells [148]. We quantified peripheral T_H17 cells following a HSD and demonstrated no change in relative counts, absolute counts, nor fold change compared to RSD. Only two trials to date have investigated the effects of a HSD on T_H17 cells and IL-17 in humans, both having a very small sample size [68; 76]. Wilck et al. [68] recruited 8 healthy males, aged 18-50, and instructed them to consume slow-release sodium tablets for 14 days. Subjects' sodium intake increased from ~3,000 mg/d to 5,500 mg/d and was accompanied by an increase in nocturnal systolic and diastolic BP (a ~6-7 mmHg increase from baseline to HSD). Analysis of

T-cell-enriched PBMCs revealed a greater fold change in T-helper cells that produce IL-17A and TNF-α (CD4+IL-17A+TNF-α+ T_H17 cells) on HSD compared to baseline. To compare our findings with Wilck et al., we analyzed CD4+IL-17A+TNF-α+ cells in men separately, but we found no difference between RSD and HSD (data not shown). Wen et al. [76] assessed plasma IL-17A when subjects were placed on a controlled feeding HSD (7,000 mg/d) and a low sodium diet (1,200 mg/d) for 7 days each, and reported a large increase of IL-17A following a HSD. The sample size of 45 subjects was fairly heterogenous as recruited subjects had normal BP, elevated BP, or stage 1 HTN, and 45% of them were smokers. They were on average 50 years old and their systolic BP was 7 mmHg higher on HSD than on low sodium diet suggesting salt sensitivity. The heterogeneity of age, BP, and smoking status among recruited subjects makes it difficult to compare findings to a sample of healthy young adults in our study.

When human T_H17 cells are cultured in a high sodium media, they increase production of IL-17A [65]. Under these conditions, CD4+IL-17A+TNF-α+ and CD4+RORγt+IL-17A+ are also increased compared to no additional sodium present in the media [65]. We did not observe a change in peripheral CD4+RORγt+, CD4+IL-17A+, nor CD4+IL-17+TNF-α+ T cells between RSD and HSD which could be due to two main reasons: 1) BP of our subjects did not increase on HSD, and 2) our intervention was not long enough. It is not clear whether high sodium-induced changes in $T_H17$cells in humans are dependent on BP. Although we cannot determine with certainty that our subjects were salt-resistant and the subjects in the Wilck et al. [68]

study were salt-sensitive due to study design, it is plausible that is the case due to no observed change in BP in our study and a >5 mmHg increase in nocturnal BP in the Wilck study. Subjects in that study were also older than the subjects in our study. Second, it is possible that our intervention was not long enough and that 4 additional days of HSD could elicit greater immune activation. However, our subjects achieved a ~4,000 mg/d difference between the RSD and HSD interventions while the subjects in the Wilck et al. study achieved a ~2,500 mg/d difference from baseline to HSD. We confirmed subject compliance due to increased urinary sodium excretion and increased serum sodium levels. Faraco and colleagues [74] first observed an increase in circulating IL-17 after 8 weeks of feeding mice a HSD that were still elevated at 24 weeks, suggesting a longer exposure to a HSD may be needed to observe an increase in plasma IL-17. In addition, subjects in both Wilck et al. [68] and our study were generally healthy which does not exclude for potential onset of colds or infections that may have arisen during the interventions and subsequently impacted T cell populations more so than the HSD. Many of our subjects completed the study during the winter months. Lastly, there appears to be inconsistencies with assessing circulating IL-17A levels as there is a high variability between the studies using ELISAs. Most report undetectable serum and plasma levels of IL-17 protein [149] which we also observed in our lab with a small sample of healthy human subjects consuming a HSD (unpublished data). Hence, assays that evaluate functional IL-17 would be a better assessment of this marker [150], including flow cytometry utilized in our study.

Abundance of T_{reg} cells is inversely proportional to abundance of T_H17 cells and activation of T_H17 cells is accompanied with reduced suppressive potential of T_{reg} cells [64; 77]. Findings in rodents demonstrate reduced [74] or unaffected [64] intestinal T_{reg} cells on HSD, however their production of IL-10 [64], and their suppressive potential [64; 77] is reduced on HSD compared to a normal sodium diet. We did not find differences in relative and absolute counts of T_{reg} cells between RSD and HSD, and fold change from RSD to HSD remained unchanged. We did not assess IL-10 levels, however Yi et al. [69] reported reduced IL-10 levels in whole blood when 6 healthy male astronauts consumed 4,500 mg sodium/d for ~50 days compared to 2,200 and 3,300 mg/d. These findings suggest T_{reg} cell counts may remain unchanged on HSD while their IL-10-producing potential may be increased. More research is needed to investigate how sodium affects T_{reg} cells in humans.

This study is not without limitations. First, circulating T cells may not be a good indicator of T cells in the intestines. Small changes in serum sodium such as those observed in this study may not be sufficient to drive inflammation [147]. For instance, individuals with HTN tend to have a greater infiltration of CD3+ T cells in the colon compared to normotensive individuals [75]. Performing a biopsy and assessing the immune cells in the obtained colonic tissue and the associated mucosa would be a better indicator of T cells in the intestines. Furthermore, Wei at al. observed an increase in IL-17A-producing cells and T_H17 cells in small and large intestine lamina propria of mice fed HSD, but not in small and large intraepithelial lymphocytes, mesenteric lymph nodes and Peyer's patches [64]. Most studies

investigating the effects of sodium on T cells are conducted in rodents which allows for assessments of inflammation in the intestinal or lymphoid tissues. Second, T_H17 and T_{reg} cells are present in low numbers in the circulation as seen from absolute counts in our analyses. Although we collected 30,000 events per sample, more accurate counts of these cells could have been obtained with a greater number of events (up to 100,000) or by enriching PBMCs with T cells. Similarly, we used PMA to activate cells at a concentration that is at the lower end of those seen in the literature. PMA is a potent carcinogen so using a greater concentration may lead to greater cell death and lower cell counts. Third, 10 days of HSD might have not been long enough to elicit an immune response in healthy young adults. Wilck et al. [68] conducted a HSD intervention in middle-aged men for 14 days, while Yi et al. [69] in adult men for 30 - 60 days. Fourth, we used salt pills in addition to counseling as a mode of increasing sodium levels in the diet. As such, the subjects were consuming an *ad libitum* diet of their choice. Providing subjects with foods to reach the specific amount of sodium in the diet in a controlled feeding study would allow for a tighter control of this mineral in the diet.

Future studies should focus on interventions of longer duration that may elicit a greater immune response. They should also examine T cell responses to a HSD in relation to BP, as changes in T cells in rodents and humans were mainly observed along with an increase in BP. In a seminal paper from 2007, Guzik et al. [151] demonstrated that RAG1-deficient mice have a blunted increase in BP upon angiotensin II infusion or DOCA/salt administration. RAG-1 knockout mice lack T

cells and B cells, and adoptive transfer of T cells, but not B cells restores the BP response to angiotensin II [151]. Faraco and colleagues [74] treated mice on HSD with IL-17-neutralizing antibodies which prevented endothelial nitric oxide synthase (eNOS) inhibition and ameliorated endothelial dysfunction induced by HSD without a change in mean arterial pressure. Administration of exogenous IL-17 to mice fed a normal sodium diet increased circulating IL-17 levels and endothelial dysfunction to the levels of mice fed the HSD [74]. Interestingly, these effects were not due to inflammation as IL-17 did not upregulate inflammatory genes, but due to eNOS inhibition [74]. These results suggest that circulating IL-17 may have detrimental effects on the vasculature independent of BP and inflammation.

In conclusion, we carried out an intervention to increase dietary sodium intake as evidenced by an increase in urinary sodium excretion and serum sodium levels.. We did not observe changes in peripheral T_H17, T_{reg}, nor TNF- α-producing T cell abundances between an RSD and HSD. Similarly, we did not observe changes in the gut microbial diversity, which we hypothesized would occur on an HSD. There is limited knowledge about the role of excess sodium consumption on T cell populations closely related to the gut microbiota in humans. Future research is needed, as activation of immune cells upon high sodium consumption may be a factor in compromising cardiovascular and GI health.

Chapter 4

CONCLUSION

4.1 Summary

Gut microbiota is a highly dynamic organ composed of billions of bacteria mediating numerous host physiological functions. Disruption in the gut microbiota composition and function (i.e. gut dysbiosis) is observed in various disease states, including hypertension (HTN) and cardiovascular disease (CVD) [15; 82; 86; 88]. Nearly 50% of United States (U.S.) adults has HTN and 89% of adults exceeds daily sodium intake as recommended by the Dietary Guidelines for Americans [4; 140]. The gut microbiota composition of individuals with HTN is less diverse than of normotensive individuals [15; 86], hence determining the effects of sodium on the gut microbiota is of high importance considering that excessive sodium intake is a major risk factor for HTN [4]. Recent evidence demonstrates that the gut microbiota takes part in pathogenesis of HTN via modulation of gut-immune axes [16; 17]. Inflammatory T helper 17 (T_H17) and anti-inflammatory T regulatory (T_{reg}) in the gut are in constant interaction with the gut microbiota.

The evidence for the deleterious effects of sodium on the gut microbial composition and T_H17 and T_{reg} cells in rodent models is mounting [45; 68; 87]. Despite a few observational studies in humans and only one randomized controlled trial in men investigating the gut microbial composition after a high sodium diet (HSD) showing

similar findings [61; 68; 75; 135], we were not able to replicate them in our study. Similarly, only two studies in humans investigated the influence of an HSD on T_H17 and T_{reg} cells, and had small and heterogenous samples [68; 76]. The reasons for the lack of significant findings in our study may lie with the young age of our cohort and a normal to overweight body mass index (BMI). These are factors that are not tightly regulated in existing studies but that have an impact on the gut microbiota and the immune function. In addition, determining potential taxonomic differences accompanied by their functional capacity could offer more information on the effects of an HSD on the gut microbiota.

4.2 Perspectives

Research in the gut microbiota field is in its infancy. Assessment of the gut microbiota is not standardized which poses an obstacle when comparing results from studies in which data were obtained from different DNA sequencing methods and statistical approaches [34]. In addition to standardizing methodology, further studies should factor in differences in BP and salt sensitivity, as well as age and BMI on the gut microbiota composition. It is unknown, but plausible, that some of these factors may have a greater influence in the gut microbiota composition that high sodium diet itself.

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Appendix A

SUPPLEMENTARY TABLES - EFFECTS OF HIGH DIETARY SODIUM INTAKE ON MODULATING GUT MICROBIOTA COMPOSITION IN HEALTHY, YOUNG ADULTS

Table A1: Screening subject characteristics separated by sex.

	Men	Women	p-value
Demographic data			
N	8	12	
Ethnicity (H/NH)[a]	0/7	0/10	
Race (W/B/A)	6/0/2	12/0/0	
Anthropometrics			
Age (yr)	25 ± 1	25 ± 1	0.639
Height (cm)	177 ± 3	164 ± 2	0.001
Mass (kg)	73.7 ± 3.3	61.6 ± 2.5	0.008
Body mass index (kg/m^2)	23.5 ± 1.1	22.9 ± 0.5	0.542
Hemodynamics[b]			
Systolic BP, laboratory (mmHg)	117 ± 2	109 ± 2	0.004
Diastolic BP, laboratory (mmHg)	66 ± 2	64 ± 1	0.477
MAP, laboratory (mmHg)	83 ± 1	79 ± 1	0.059
Heart rate, laboratory (bpm)	66 ± 3	66 ± 3	0.937

A, Asian; B, Black; BMI, body mass index; BP, blood pressure; H, Hispanic; MAP, mean arterial pressure; NH, non-Hispanic; W, White. Values are means ± SEM. [a]Three subjects (1 man, 2 women) did not report ethnicity. [b]Measured after 5min seated rest.

Table A2: Hemodynamics and biochemical parameters following recommended and high sodium diets separated by sex.

	Recommended Sodium		High Sodium	
	Men	**Women**	**Men**	**Women**
Mass (kg)	72.7 ± 3.3	61.1 ± 2.4	73.2 ± 3.3	61.1 ± 2.4
Hemodynamics				
Laboratory[a]				
Systolic BP (mmHg)	117 ± 1	105 ± 1	119 ± 3	106 ± 1
Diastolic BP (mmHg)	62 ± 1	63 ± 1	63 ± 2	63 ± 1
MAP (mmHg)	80 ± 1	77 ± 1	81 ± 2	77 ± 1
Heart rate (bpm)	58 ± 2	57 ± 3	56 ± 3	55 ± 2
24h ambulatory				
Systolic BP, overall (mmHg)[b]	119 ± 1	111 ± 2	124 ± 5	112 ± 2
Diastolic BP, overall (mmHg)[b]	68 ± 1	64 ± 2	68 ± 2	64 ± 1
MAP, overall (mmHg)[b]	85 ± 1	80 ± 2	87 ± 3	80 ± 1
Heart rate, overall (bpm)[b]	69 ± 3	70 ± 3	71 ± 4	69 ± 3
Systolic BP, daytime (mmHg)	125 ± 2	115 ± 2	130 ± 3	116 ± 2
Diastolic BP, daytime (mmHg)	72 ± 2	67 ± 2	72 ± 2	68 ± 1
MAP, daytime (mmHg)	90 ± 1	83 ± 2	91 ± 2	84 ± 1
Heart rate, daytime (bpm)	74 ± 3	74 ± 3	73 ± 4	72 ± 2
Systolic BP, nighttime (mmHg)[b]	111 ± 3	100 ± 3	112 ± 8	101 ± 3
Diastolic BP, nighttime (mmHg)[b]	58 ± 2	53 ± 2	55 ± 3	53 ± 2
MAP, nighttime (mmHg)[b]	76 ± 2	69 ± 2	74 ± 5	69 ± 2
Heart rate, nighttime (bpm)[b]	59 ± 3	60 ± 2	60 ± 3	60 ± 2
Biochemical parameters				
Hemoglobin (g/dL)	15.9 ± 0.2	13.6 ± 0.3	15.6 ± 0.1	13.1 ± 0.4
Hematocrit (%)	46.3 ± 1.0	41.1 ± 1.0	46.8 ± 0.8	41.1 ± 1.1
Serum sodium (mmol/L)	138.0 ± 0.7	137.7 ± 0.5	141.0 ± 0.6*	138.6 ± 0.3
Serum potassium (mmol/L)	3.8 ± 0.1	3.9 ± 0.1	4.0 ± 0.1*	3.8 ± 0.0

Table A2 continued.

Serum chloride (mmol/L)	104.4 ± 0.9	104.3 ± 0.4	104.2 ± 0.3	106.6 ± 0.6[#]
Serum osmolality (mOsm/kg)	286 ± 1	285 ± 1	290 ± 1*	283 ± 2
Urinary sodium excretion (mmol/24h)	107 ± 11	137 ± 18	294 ± 15*	279 ± 23[#]
Urinary potassium excretion (mmol/24h)	52 ± 7	50 ± 6	57 ± 8	52 ± 6
Urine osmolality (mOsm/kg)	410 ± 84	420 ± 69	582 ± 74*	571 ± 65[#]
Urine specific gravity	1.011 ± 0.002	1.012 ± 0.002	1.014 ± 0.002	1.013 ± 0.002

BP, blood pressure; MAP, mean arterial pressure. Values are means ± SEM.
[a]Measured after 20min supine rest. [b]men: n = 6; women: n = 10. *$p < 0.05$ RSD vs. HSD for men; [#]$p < 0.05$ RSD vs. HSD for women (paired t-test)

Table A3: Dietary intake during Recommended/High Sodium interventions separated by sex.

	Recommended Sodium		High Sodium	
	Men (n=8)	**Women (n=12)**	**Men (n=8)**	**Women (n=12)**
Energy intake (kcal/d)	2,374 ± 160	1,669 ± 99	2,278 ± 160	1,881 ± 16
Carbohydrates (g/d)	255 ± 23	187 ± 10	242 ± 19	220 ± 17
Fiber (g/d)	26 ± 3	20 ± 2	24 ± 3	23 ± 2
Added sugar (g/d)	32 ± 4	31 ± 3	41 ± 7	35 ± 5
Protein (g/d)	109 ± 12	73 ± 7	121 ± 14	78 ± 5
Fat (g/d)	88 ± 7	69 ± 6	91 ± 9	74 ± 5
Saturated fat (g/d)	27 ± 3	23 ± 3	29 ± 3	21 ± 2
Sodium (mg/d)	2,966 ± 254	2,260 ± 189	3,191 ± 359	2,292 ± 200
Potassium (mg/d)	2,975 ± 258	2,360 ± 174	2,669 ± 202	2,591 ± 196
Phosphorus (mg/d)	1,514 ± 143	1,117 ± 79	1,466 ± 122	1,140 ± 71
Calcium (mg/d)	942 ± 57	983 ± 87	942 ± 58	813 ± 68
Magnesium (mg/d)	443 ± 60	266 ± 18	358 ± 35	317 ± 23
Alcohol (g/d)	27 ± 3	6 ± 6	29 ± 3	10 ± 5

Values are means ± SEM.

Table A4: Energy and nutrient intake correlations between the two 24-hour dietary recalls during Recommended/High Sodium interventions.

	r-value, Recommended Sodium	**r-value, High Sodium**
Energy intake	0.719*	0.563*
Carbohydrates	0.764*	0.391
Fiber	0.505*	0.234
Added sugar	0.197	0.282
Protein	0.794*	0.613*
Fat	0.406	0.589*
Saturated fat	0.395	0.327
Sodium	0.386	0.583*
Potassium	0.506*	0.413
Phosphorus	0.703*	0.704*
Calcium	0.484*	0.488*
Magnesium	0.849*	0.587*
Alcohol	0.563*	0.093

Values are means ± SEM. n=20. *$p<0.05$.

Table A5: Habitual dietary intake in all subjects and men and women separately.

	All subjects (n=20)	Men (n=8)	Women (n=12)
Energy intake (kcal/d)	2,053 ± 93	2,526 ± 163	1,738 ± 76
Carbohydrates (g/d)	229 ± 11	272 ± 22	201 ± 9
Fiber (g/d)	22 ± 2	25 ± 3	20 ± 2
Added sugar (g/d)	43 ± 4	48 ± 6	39 ± 5
Protein (g/d)	87 ± 6	115 ± 13	69 ± 3
Fat (g/d)	78 ± 4	93 ± 5	69 ± 4
Saturated fat (g/d)	25 ± 1	30 ± 2	21 ± 2
Sodium (mg/d)	2,882 ± 177	3,322 ± 380	2,589 ± 136
Potassium (mg/d)	2,505 ± 120	2,746 ± 230	2,343 ± 123
Phosphorus (mg/d)	1,263 ± 70	1,540 ± 140	1,078 ± 52
Calcium (mg/d)	923 ± 66	1,067 ± 128	827 ± 67
Magnesium (mg/d)	312 ± 17	364 ± 34	276 ± 15
Alcohol (g/d)	18 ± 5	25 ± 11	13 ± 4

Values are means ± SEM.

Appendix B

SUPPLEMENTARY TABLES - EFFECTS OF HIGH DIETARY SODIUM INTAKE ON MODULATING CIRCULATING T-HELPER 17 AND T-REGULATORY CELLS IN HEALTHY YOUNG ADULTS

Table B1: Screening subject characteristics separated by sex.

	Men	Women	p-value
Demographic data			
N	13	9	
Ethnicity (H/NH)[a]	0/12	0/8	
Race (W/B/A)	11/0/2	8/1/0	
Anthropometrics			
Age (yr)	25 ± 1	25 ± 1	0.758
Height (cm)	176 ± 2	164 ± 2	<0.001
Mass (kg)	71.6 ± 2.3	60.4 ± 2.4	0.003
Body mass index (kg/m^2)	23.1 ± 0.7	22.5 ± 0.4	0.463
Hemodynamics[b]			
Systolic BP, laboratory (mmHg)	118 ± 2	108 ± 2	0.004
Diastolic BP, laboratory (mmHg)	66 ± 2	62 ± 2	0.102
MAP, laboratory (mmHg)	83 ± 2	78 ± 2	0.016
Heart rate, laboratory (bpm)	66 ± 3	65 ± 3	0.908

A, Asian; B, Black; BMI, body mass index; BP, blood pressure; H, Hispanic; MAP, mean arterial pressure; NH, non-Hispanic; W, White. Values are means ± SEM. [a]Two subjects (1 man, 1 woman) did not report ethnicity. [b]Measured after 5min seated rest.

Table B2: Hemodynamics and biochemical parameters following recommended and high sodium diets separated by sex.

	Recommended Sodium		High Sodium	
	Men	**Women**	**Men**	**Women**
Mass (kg)	70.6 ± 2.5	60.0 ± 2.2	71.0 ± 2.4	60.1 ± 2.2
Hemodynamics				
Laboratory[a]				
Systolic BP (mmHg)	116 ± 2	105 ± 2	116 ± 2	107 ± 3
Diastolic BP (mmHg)	63 ± 1	61 ± 2	64 ± 1	64 ± 3
MAP (mmHg)	81 ± 1	76 ± 2	82 ± 1	78 ± 3
Heart rate (bpm)	57 ± 2	60 ± 4	54 ± 2	57 ± 2
24h ambulatory				
Systolic BP, overall (mmHg)[b]	120 ± 2	111 ± 3	117 ± 3	111 ± 3
Diastolic BP, overall (mmHg)[b]	67 ± 1	65 ± 3	66 ± 1	64 ± 2
MAP, overall (mmHg)[b]	85 ± 2	80 ± 3	83 ± 2	80 ± 2
Heart rate, overall (bpm)[b]	67 ± 3	74 ± 5	69 ± 2	72 ± 3
Systolic BP, daytime (mmHg)	124 ± 2	115 ± 2	126 ± 3	116 ± 2
Diastolic BP daytime (mmHg)	71 ± 1	68 ± 3	70 ± 1	68 ± 2
MAP, daytime (mmHg)	89 ± 1	83 ± 3	87 ± 1	84 ± 2
Heart rate, daytime (bpm)	70 ± 3	77 ± 3	71 ± 2	74 ± 3
Systolic BP, nighttime (mmHg)[b]	109 ± 3	101 ± 4	112 ± 5	102 ± 5
Diastolic BP nighttime (mmHg)[b]	56 ± 2	55 ± 3	55± 2	55 ± 3
MAP, nighttime (mmHg)[b]	74 ± 2	70 ± 3	72 ± 2	70 ± 4
Heart rate, nighttime (bpm)[b]	58 ± 2	64 ± 3	61 ± 2	65 ± 2
Biochemical parameters				
Hemoglobin (g/dL)	15.6 ± 0.3	13.6 ± 0.3	15.3 ± 0.2	13.1 ± 0.4[#]

Table B2 continued.

Hematocrit (%)	45.9 ± 0.8	41.1 ± 1.0	46.3 ± 0.6	41.1 ± 1.1
Serum sodium (mmol/L)	138.4 ± 0.6	138.4 ± 0.5	140.6 ± 0.6*	138.5 ± 0.3
Serum potassium (mmol/L)	3.8 ± 0.1	3.9 ± 0.1	3.98 ± 0.1	3.8 ± 0.7
Serum chloride (mmol/L)	103.4 ± 0.7	104.5 ± 0.6	104.4 ± 0.3	106.6 ± 0.6[#]
Serum osmolality (mOsm/kg)	286 ± 1	285 ± 1	291 ± 1*	283 ± 2
Urinary sodium excretion (mmol/24h)	119 ± 12	107 ± 14	312 ± 12*	252 ± 28[#]
Urinary potassium excretion (mmol/24h)	55 ± 6	44 ± 7	66 ± 6*	42 ± 8
Urine osmolality (mOsm/kg)	407 ± 55	478 ± 96	558 ± 50*	609 ± 75
Urine specific gravity	1.011 ± 0.002	1.013 ± 0.003	1.013 ± 0.001	1.014 ± 0.002

BP, blood pressure; MAP, mean arterial pressure. Values are means ± SEM.
[a]Measured after 20min supine rest. [b]n=17. *$p < 0.05$ RSD vs. HSD for men; [#]$p < 0.05$ RSD vs. HSD for women (paired t-test)

Table B3: Energy and nutrient intake correlations between the two 24-hour dietary recalls during Recommended/High Sodium interventions.

	r-value, Recommended Sodium	**r-value, High Sodium**
Energy intake	0.596*	0.544*
Carbohydrates	0.412	0.374
Fiber	0.491*	0.370
Added sugar	0.377	0.422
Protein	0.614*	0.596*
Fat	0.538*	0.599*
Saturated fat	0.503	0.388
Sodium	0.128	0.590*
Potassium	0.537*	0.370
Phosphorus	0.436*	0.685*
Calcium	0.025	0.421
Magnesium	0.731*	0.628*
Alcohol	0.567*	0.183

Values are means ± SEM. n=22. *$p<0.05$.

Table B4: Habitual dietary intake.

Energy intake (kcal/d)	2,148 ± 92
Carbohydrates (g/d)	240 ± 12
Fiber (g/d)	21 ± 1
Added sugar (g/d)	48 ± 4
Protein (g/d)	90 ± 6
Fat (g/d)	84 ± 4
Saturated fat (g/d)	28 ± 2
Sodium (mg/d)	2,929 ± 165
Potassium (mg/d)	2,506 ± 115
Phosphorus (mg/d)	1,314 ± 67
Calcium (mg/d)	975 ± 62
Magnesium (mg/d)	316 ± 17
Alcohol (g/d)	16 ± 5

Values are means ± SEM. n=22.

Appendix C

IRB APPROVAL LETTER

Institutional Review Board
210H Hullihen Hall
Newark, DE 19716
Phone: 302-831-2137
Fax: 302-831-2828

DATE:	May 1, 2019
TO:	Katarina Smiljanec, MS
FROM:	University of Delaware IRB
STUDY TITLE:	[1423170-1] The Salty Gut
SUBMISSION TYPE:	New Project
ACTION:	APPROVED
APPROVAL DATE:	May 1, 2019
EXPIRATION DATE:	April 16, 2020
REVIEW TYPE:	Full Committee Review

Thank you for your New Project submission to the University of Delaware Institutional Review Board (UD IRB). The UD IRB has reviewed and APPROVED the proposed research and submitted documents via Full Committee Review in compliance with the pertinent federal regulations.

As the Principal Investigator for this study, you are responsible for and agree that:

- All research must be conducted in accordance with the protocol and all other study forms as approved in this submission. Any revisions to the approved study procedures or documents must be reviewed and approved by the IRB prior to their implementation. Please use the UD amendment form to request the review of any changes to approved study procedures or documents.
- Informed consent is a process that must allow prospective participants sufficient opportunity to discuss and consider whether to participate. IRB-approved and stamped consent documents must be used when enrolling participants and a written copy shall be given to the person signing the informed consent form.
- Unanticipated problems, serious adverse events involving risk to participants, and all non-compliance issues must be reported to this office in a timely fashion according with the UD requirements for reportable events. All sponsor reporting requirements must also be followed.

Oversight of this study by the UD IRB REQUIRES the submission of a CONTINUING REVIEW seeking the renewal of this IRB approval, which will expire on April 16, 2020. A continuing review/progress report form and up-to-date copies of the protocol form and all other approved study materials must be submitted to the UD IRB at least 45 days prior to the expiration date to allow for the required IRB review of that report.

If you have any questions, please contact the UD IRB Office at (302) 831-2137 or via email at hsrb-research@udel.edu. Please include the study title and reference number in all correspondence with this office.