THE EFFECTS OF A HIGH-PROTEIN DIET WITH A PREBIOTIC AND MULTI-STRAIN PROBIOTIC ON MICROBIOTA, GASTROINTESTINAL FUNCTION AND WELLNESS IN OLDER WOMEN

By

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A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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To my family for instilling in me the importance of growing from both my successes and failures and showing me infinite grace along the way
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Abstract of Dissertation Presented to the Graduate School of the University of Florida in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

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By
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August 2017

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Major: Nutritional Sciences

Protein intake exceeding requirements may benefit physiological processes and reduce the risk of sarcopenia in older adults. Research has demonstrated the helpful effects of protein and potential benefits involving energy balance, wound/injury healing, recovery from trauma, cardiovascular function, in addition to other conditions that commonly affect older adults. However, high-protein, animal-based diets may produce undesirable perturbations in microbiota, resulting in inflammatory conditions. With the decline in diversity of colonic microbiota among older adults, prebiotics and probiotics may confer benefits in this cohort. The aim of this research was to examine the effects of a high-protein (animal and plant protein) diet on microbiota profile and digestive health in healthy older adults, and the potential mitigating role of a prebiotic, multi-strain probiotic or synbiotic. A randomized, crossover, placebo-controlled feeding trial was conducted. Healthy older women (n=26; 73.7±5.6 y) were randomized to an 18-week crossover study design consisting of four interventions; i) high-protein diet (1.5-2.2 g/kg/d), ii) high-protein diet with a multi-strain probiotic (2×10^{10} CFU/d), iii) high-protein diet with inulin and iv) high-protein diet with synbiotic (inulin + probiotic). Each 14-d
intervention period consisted of the same eucaloric, high-protein diet separated by 14-day washout periods. Gastrointestinal wellness, including stool frequency and weekly gastrointestinal symptoms measured by Gastrointestinal Symptom Rating Scale (GSRS), were evaluated. Stools were collected during baseline, intervention and washout periods and quantitative polymerase chain reaction (qPCR) and 16S rRNA gene sequencing was conducted. The results showed no phyla level changes, whereas high intra-individual variations in genera and significant changes in operational taxonomic units (OTUs) were observed with treatments. Stool frequency was not different among periods. A significant increase in GSRS scores of abdominal pain (p=0.011) and indigestion syndromes (p<0.0001), but not reflux, constipation and diarrhea syndrome, compared to the baseline and washout combined were observed during treatments. The results of this study provide evidence that contrary to the possible negative effects of a high-protein diet, a diet that maintains the Acceptable Macronutrient Distribution Range (AMDR) does not have profound changes on microbiota in older women, a group that may benefit from the physiological benefits of higher protein intake.
CHAPTER 1
INTRODUCTION

Significance and Rationale

Throughout the lifespan, nutrient recommendations and energy needs may change. With a continuous rise in the aging population, it is necessary to examine the physiological and biochemical alterations that result with age and the role of nutrition and its contribution to optimizing health and longevity. Aging is associated with a decline in lean mass and an increase in fat mass. Research examining the decline of skeletal muscle associated with age, known as sarcopenia, has demonstrated higher levels of mortality and impairment in these individuals.

Protein intake has been identified by researchers as a “modifiable risk factor” for sarcopenia. Protein requirements during injury, hospitalization, surgery, etc. are elevated -- conditions more prevalent as we age. Scientific evidence and research contemplate whether the current dietary recommendation for protein is optimal for older adults due to metabolic and physiological changes.

Most studies in the literature evaluating protein intake in an older adult cohort have focused on stimulating muscle protein synthesis and measures of bone health. Additionally, little research has evaluated the effect of dietary changes in older adults using controlled feeding methods and altering the distribution of macronutrients, particularly a mixed protein diet (animal and plant protein sources). Although evidence confirming the relationships among diet, gut microbiota and health and disease is mounting, few studies have examined the effects of a mixed protein diet on gastrointestinal health and protein fermentation. Dietary interventions in humans that
demonstrate diet-specific effects and short-term consequences of alterations in specific macronutrients are lacking.

It has been suggested that microbiota dysbiosis may be more common in older adults\(^1\), reduced meat protein digestion has recently been confirmed in older men\(^2\), and that many older adults may be at nutritional risk\(^3\), thus older adults were the target population in this research.

This research aims to fill the void in the breadth of current scientific knowledge by exploring the feasibility of the current dietary recommendation for protein in addition to the effects of protein fermentation on microbiota and measures of gastrointestinal health with and without prebiotic, probiotic and synbiotic supplementation. Because research indicates a declining diversity of colonic microbiota among older adults, prebiotics and probiotics were utilized to investigate potential affirmative benefits on colonic metabolism in this cohort.

A high-protein diet composed of commercially available protein products and supplemented with a prebiotic (inulin) or multi-strain probiotic may have considerable health advantages and provide scientific support for the incorporation of prebiotics and probiotics into protein products marketed to consumers, in particular the growing product market directed at older adults.

**Specific Aims**

The primary objective of this research was to determine the effects of a high-protein diet on fecal microbial communities (microbiota diversity and profile) in community-dwelling older women. Additionally, to investigate any perturbations in microbiota and the potential ability to mitigate them with a multi-strain probiotic product, prebiotic (inulin) and synbiotic were explored. Further investigation into compounds
produced during protein fermentation may allude to the potentially beneficial role of prebiotics, probiotics or synbiotics in the reduction of proteolytic fermentation\(^{(5)}\).

It is hypothesized that consuming a high-protein diet will produce undesirable changes in gut microbiota including suppression of *Bifidobacteria* spp. proportions and promotion of *Clostridia* spp., ammonia-producing bacteria, whereas a high-protein diet consumed with a prebiotic (inulin) or multi-strain probiotic will demonstrate opposing effects.

Secondary aims of this research explore the effects of high-protein diets on measures of gastrointestinal, digestive and general health in older adults. Hypothesizing that older adults on high-protein diets will have decreased intestinal motility and gastrointestinal wellness, whereas a high-protein diet, consumed with a prebiotic (inulin) or multi-strain probiotic, will maintain motility and measures of general health and wellness.

Additional research aims investigated the impact of a high-protein diet on urinary metabolites of protein fermentation, measures of QoL (includes the SF-36v2 Health Survey and GFI) and measures of nutritional status (handgrip strength and body composition).
CHAPTER 2
REVIEW OF THE LITERATURE

Introductory Notes

The foods and nutrients consumed throughout an individual’s life have both short and long-term implications, influencing biochemical reactions and processes in the body and impacting health and disease risk. While macronutrient and micronutrient requirements change throughout the lifespan, the ability of the body to absorb and utilize nutrients also changes.

Across the lifespan, protein plays a critical role in maintaining health and promoting growth, development and general health. Research has elucidated that the functions of protein are diverse in the body (vital functions include: repair and maintenance, energy production, body hormones, enzymes involved in our bodies chemical reactions, transportation and immune functions).

Research on the health benefits of protein have largely focused on its relation to bone, muscle and weight management (specifically satiety). Previous research in these areas support the recommendation of increasing dietary protein in older adults. While potential outcomes have been cited and it is known that high-protein diets, particularly those high in meat, result in significant protein reaching the colon, the effects of this malabsorbed protein on microbiota profile, putrefactive microbial activities and health had not been previously explored. Knowing the functionality of dietary protein and its role in the microbiota should be evaluated.

Topics covered in this literature review will include a general discussion of aging, nutritional implications of aging, dietary changes that occur with age and provide a more detailed discussion of protein requirements for older adults. Specifically, examining
protein requirements as they relate to older adults. The review will include a discussion of the adequacy of the current recommendations versus recommendations that are optimal for health in this population. Digestive health will also be reviewed and will include discussion of the microbiota and protein fermentation and potential applications of probiotics, prebiotics and synbiotics.

**Aging**

Globally, the population is readily changing as healthcare, access to preventative care, research and consumer knowledge advances. The World Health Organization (WHO) estimates that from 2015 to 2050, the proportion of individuals over age 60 will rise from approximately 900 million to 2 billion\(^6\). Worldwide, the major health burdens among this cohort are non-communicable diseases/conditions (greatest contributors to mortality include heart disease, stroke and lung disease). The Census reports that similarly to the changing global population, the demographic of the United States’ is also vastly changing and the cohort of aging adults will rapidly increase from the years 2012 to 2050\(^7\). The Administration on Aging reports that in the year 2014, individuals over the age of 65 represented approximately 14.5% of the U.S. population and that this statistic is likely to double by the year 2040, when individuals over age 65 will represent approximately 21.7%\(^8\). The population of individuals 85 years of age and older is estimated to triple from 5.7 million in 2011 to 14.1 million by 2040\(^9\).

While the population of older adults continues to rise (older adults are the fastest growing cohort which is primarily attributed to an increase in life expectancy and declining rates of fertility), overall health among these individuals must be considered\(^10; 11; 12\). While it is expected that an individual will live longer today than previous
generations, there is little evidence to support that the health and quality of life of these individuals is higher than those of previous generations\(^{(11)}\).

Current scientific theories that address the aging process include programmed theories and damage or error theories. Aging is a multidimensional process associated with biochemical and physiological changes that are often characterized as impairments in immune function, respiratory function, bone health and metabolic function\(^{(13)}\). As longevity increases and the population of aging adults grows, a focus on “healthy aging” has emerged. While currently little agreement exists regarding the definition of “healthy aging” or the quantitative outcome measures that can be used for assessment, there is a general consensus among the field that it is multidimensional and encompasses an individual’s quality of life in addition to physical and biochemical measures. Rowe and Kahn suggest that “healthy aging” or “successful aging” is measured by determining an individual’s probability of disease and disease-related disability, cognitive capacity, physical functional capacity and their active participation and engagement in activities of living\(^{(14)}\). Comparatively, the World Health Organization (WHO) defines “healthy aging” as a process which includes both the processes of developing and maintaining functional ability as an individual ages\(^{(15)}\).

**Aging and Quality of Life**

Instead of using lifespan, which refers to living an additional set number of years compared to life expectancy, most individuals prefer to focus on the quality of life (QoL) associated with longevity. QoL is a construct that consists of multiples components including psychological, clinical and geriatric outcomes\(^{(16)}\). QoL can be measured both subjectively and objectively, and some measurement tools are disease or condition specific.
The most widely used tool in clinical research in which patient reported outcomes were an aim was the SF-36\textsuperscript{(17)}. The Optum\textsuperscript{TM} SF-36v2\textsuperscript{®} Health Survey is a validated 36-item self-report questionnaire, that is available in multiple translations with two recall periods (standard 4-week or acute 1-week) and has been used by researchers, clinicians, patients and administrators. The SF-36v2\textsuperscript{®} Health Survey measures physical and mental health across eight domains (physical functioning, role-physical, bodily pain, general health, vitality, social functioning, role-emotional and mental health)\textsuperscript{(18)}.

**Aging and Immune Health**

It is known that functional capacity and immune function declines with normal aging\textsuperscript{(19; 20)}. The age associated changes of the immune system are collectively referred to as immunosenescence and impact both the innate and adaptive immune systems. These age-related associations are characterized by a chronic low-grade inflammation\textsuperscript{(21)} and have been termed “inflamm-aging”\textsuperscript{(22)}. This chronic state of low grade inflammation may elicit immunomodulation and mechanisms eliciting inflammatory cascades. Changes in inflammatory mediators are most notable in T and B cells, characteristically involve a reduction in function and an increase in the expression of pro-inflammatory cytokines (IL-6 and TNF-\textalpha)\textsuperscript{(23)}. These age-related changes in the immune system may be the result of change in body composition (increase in fat mass with age), viral infection, dietary and the microbiota\textsuperscript{(24; 25)}. Markers of pro-inflammation have been researched in regards to a role in age related impairments and physical decline, specifically CRP, IL-6 and TNF-\textalpha\textsuperscript{(26; 27)}.

Cytokines, cell signaling proteins, are diverse and include interleukins, interferons, tumor necrosis factors, chemokines and lymphokines. IL-6 is a tightly
regulated pro-inflammatory cytokine. IL-6 is secreted by both T cells and macrophages, and is a mediator that elicits fever and serves to mediate and regulate cellular processes and the acute phase response. IL-6 is tightly controlled and normally expressed at low levels, however IL-6 levels increase during and after injury, disease, infection and stress. An age-related increase in IL-6 occurs around midlife (testosterone and estrogen are known to down-regulate IL-6 gene expression). Levels of IL-6 increase with aging and elevated levels have also been reported in many age related conditions\(^{(28;29)}\). Tumor necrosis factor-alpha (TNF-\(\alpha\)), a cytokine, is also involved in the acute phase response and systemic inflammation and increased levels have been observed in aging. TNF-\(\alpha\) is produced by multiple cell types and can stimulate IL-6 and chemokines.

C-reactive protein (CRP) is a non-specific acute phase protein marker of systemic inflammation\(^{(30)}\). In the liver, CRP production is induced by IL-6 kinase activation. CRP is associated with functional impairment in older adults\(^{(31)}\).

**Physiological Changes- Musculoskeletal**

The age-related loss of skeletal muscle mass (sarcopenia), a physiological change that occurs with aging and decreasing strength, is associated with functional impairment, disability, increased risks of falls, hospitalization\(^{(32;33;34;35;36)}\) and increases the risk of frailty\(^{(37)}\). Yearly health care costs related to frailty in the U.S. are estimated at over 18.4 billion dollars, affecting approximately 5-13\% of adults between 60-70 years of age (prevalence increasing with age)\(^{(38;39)}\). Sarcopenia is thought to be the result of inactivity and influenced by age-related changes in muscle function, regeneration and increased prevalence of injury. The physiology and changes characteristic of sarcopenia are: decreased activity\(^{(40)}\), decreased growth hormone and insulin-like
growth factor (IGF-1) with age\textsuperscript{(41)}, altered antioxidant cellular defenses\textsuperscript{(42)}, anorexia and decreased dietary protein intake\textsuperscript{(43; 44)}, pro-inflammatory cytokines and increased levels of TNF and IL-6\textsuperscript{(45)}. Currently, the emphasis is on the management of sarcopenia and includes increasing resistance exercise and consuming dietary protein\textsuperscript{(39)}.

Research supporting the loss of muscle mass and strength among older adults indicate a progressive loss over time that is associated with disability and functional decline. Goodpaster et al. examined muscle mass and strength in older adults and reported strength loss and a 1\% loss of leg lean mass per year over a 3-year period. Strength loss occurred at much higher rates of decline (approximately 3.4\% loss per year in Caucasian men) than loss of muscle mass\textsuperscript{(46)}. In a cross-sectional study using data collected from the Third National Health and Nutrition Examination Survey (NHANES III), Janssen et al. demonstrated among older Americans, a reduced relative skeletal mass (measured using bioimpedance analysis) is independently associated with functional impairments and disability\textsuperscript{(47)}.

Voluntary and involuntary muscle function can be measured by multiple methods. To measure involuntary muscle contraction, electrical stimulation is generally used and considered the best objective measure\textsuperscript{(48)}. In contrast, voluntary muscle strength can be measured via handgrip, knee extension or hip flexion strength. Within clinical settings handgrip, a validated method, is generally the preferred method because of its quickness and ease of use.

Grip strength measured with a hand dynamometer is a general measure of muscle strength and function. Grip strength has been shown to be a strong predictor of mortality and morbidity\textsuperscript{(49; 50; 51)}. Age and gender are significantly associated with
handgrip strength\(^{(52)}\). Alley et al. suggested grip strength ranges associated with functional impairment and weakness. The intermediate cutoff proposed for men was 26-32 kg and 16-20 kg for women. A cutoff for weak was proposed at <26 kg for men and <16 kg for women\(^{(53; 54)}\).

In community-dwelling older adults, Springstroh et al.\(^{(55)}\) used handgrip to identify nutritional risk. Researchers reported that handgrip strength (using the strength ranges/cutoffs established by Alley et al.) was weakly associated with nutritional risk (assessed using the SCREEN 1, self-administered questionnaire) and suggested alternate cutoffs. Suggesting that a handgrip strength higher than that suggested previously, 33 kg for men and 22 kg of women provided the best comparison for nutritional risk in community-dwelling older adults\(^{(55)}\).

**Aging and Frailty**

In a population based study of community dwelling older adults, Fried et al. proposed a standardized phenotype for frailty\(^{(56)}\). Frailty was defined as a clinical syndrome and the operational criteria includes the presence of three or more of the following: un-intentional weight loss (10 lbs. in past year), self-reported exhaustion, weakness (grip strength), slow walking speed and low physical activity\(^{(57)}\). Frailty may include all or a subset of the following characteristics: low physical activity, muscle weakness, slowed performance, fatigue/poor endurance and unintentional weight loss\(^{(58)}\). In a prospective cohort study of 24,417 community-living women aged 65 to 79, researchers classified 13.5% as frail after a three-year period\(^{(59)}\).

To better detect and identify frailty among individuals, screening tools have been developed. The Groningen Frailty Indicator (GFI), a 15-item tool, measures frailty based on four domains: physical (mobility functions, multiple health problems, physical fatigue,
vision, hearing), cognitive (cognitive dysfunction), social (emotional isolation) and psychological (depressed mood and feelings of anxiety)\(^{60; 61}\). The GFI measures the loss or decline reported by an individual in the four identified functional domains. The GFI has been used in both home-dwelling and institutionalized older adults. Frailty should be used to identify individuals at risk or needing intervention instead of age alone\(^{62}\).

**Nutritional Status**

Aging is accompanied with physiological changes and sensory impairments that readily alter food and nutrition choices\(^{63}\). Additionally, psychological factors (isolation, depression) and socioeconomic factors (decreasing finances) may influence food choices and security within this population. While food choice is multifactorial, the contribution of nutrition (specifically the changes and requirements of macronutrients and micronutrients) are not well defined in the aging population. Factors influencing “healthy aging” and “active life expectancy” (the National Institute on Aging refers to active life expectancy as the time during late life that is free of disabilities) includes the treatment and prevention of diseases and chronic conditions, promoting optimal health and decreasing the risk of comorbidities\(^{64}\).

The incidence of chronic diseases/conditions increases significantly with age, and nutrition is a critical component in the development, progression, vulnerability and outcomes associated with these diseases. While community nutrition places a substantial effort on educating and combating overnutrition and the prevention of obesity, it is recognized that the aging population is at a higher risk for undernutrition\(^{65}\). Margetts et al. examined the prevalence of undernutrition in older adults (65 years and older) by using a cross-sectional nationally representative sample of both home-
dwelling and institutional adults in the United Kingdom. Results indicated that approximately 14% of home dwelling and 21% of institutionalized were at medium or high risk for undernutrition\(^{(66)}\). Additionally, significantly increased risk of undernutrition was associated with those individuals who reported lower energy intake including meat products, fruits, vegetables, and decreased blood values for zinc, vitamins A, D, E and C. With approximately 80% of older adults living in low and middle income countries by 2050 and the need for long term care increasing, this cohort is at greater nutritional risk\(^{(6)}\). Factors such as diet, food access, inability to obtain adequate nutrition, limited income, isolation, chronic illness and physiologic changes place aging adults at increased risk of malnutrition and weight loss\(^{(67)}\). A compromised nutritional status has been strongly associated with negative long term outcomes, including an impaired quality of life and higher morbidity\(^{(68)}\).

Malnutrition, defined as a nutritional imbalance, is a common concern among older adults and is strongly associated with reduced quality of life and morbidity, decreased recovery time, and is a key factor leading to hospitalization, increased care and mortality\(^{(69; 70)}\). In a retrospective study of 4,570 older adults (representing 12 countries including the United States) residing in the community, rehabilitation facility, nursing home and hospital, approximately two-thirds were classified as malnourished or at nutritional risk using the Mini Nutritional Assessment\(^{(4)}\). Higher levels of malnutrition have been documented in multiple countries in older patients who are hospitalized, living in nursing home or are in home-care programs\(^{(71; 72; 73; 74)}\).

It is recognized that among older adults, good nutrition is associated with overall health, recovery from illness/injury and ability to perform activities of daily living.
Research has demonstrated that protein is a vital nutrient throughout life, especially in older adults\(^{(75)}\).

**Aging: Protein Requirements**

The Food and Nutrition Board of the Institute of Medicine, National Academy of Sciences was responsible for establishing and rendering guidelines for the Dietary Reference Intakes (DRI). For adults 19 years of age and older, the current DRI for protein proposed to meet the needs of most (97-98%) healthy individuals is 0.8 g/kg/day (Recommended Dietary Allowance, RDA)\(^{(76)}\). The RDA is set to meet the needs and decrease risk of chronic disease in most healthy individuals based on sex and age groupings. The Estimated Average Requirement (EAR) is the average amount of a nutrient (protein) required by half of healthy individuals (based on sex and age). Americans typically meet their protein requirement with \(\frac{1}{3}\) of their requirements coming from plant sources and \(\frac{2}{3}\) from animal sources of protein. The RDA for protein, determined by the Institute of Medicine, does not increase and/or decrease in healthy adults over 19 years of age regardless of the many physiological and metabolic changes that occur with increasing age. The current RDA was originally proposed in the 1980s and was established based on studies conducted in young adults in which nitrogen balance (equilibrium) was assessed\(^{(77)}\). The Acceptable Macronutrient Distribution Range (AMDR) for protein is currently set at 10-35% of energy intake and represents a range of intakes that are associated with reduced risk of chronic disease and providing individuals with the essential nutrients\(^{(76)}\). Discussion among the research and nutrition education community has questioned whether the current requirements are optimal for specific populations, of particular interest are older adults\(^{(78; 79)}\).
Scientific evidence and research debate the adequacy of the current dietary recommendation for protein and whether it is adequate for older adults due to physiological changes and optimal to promote lean muscle anabolism. Researchers have demonstrated that the pattern of protein distribution does not affect anabolic response\(^{(80)}\).

Nitrogen balance studies, on which the RDA was set are in disagreement with more recent research and the literature. Studies that are in agreement with the current recommendation provide data the recommendation may be adequate with successful adaption but also requires metabolic accommodation\(^{(81; 82)}\) among older adults. Additionally, research indicates that most older individuals are not currently meeting the RDA (0.8 g/kg per day), and it has been estimated that between 22 to 38% of men 50 years of age and older consume less than the current RDA for protein\(^{(83)}\). In community-dwelling older adults (70-79 years old), researchers found by using a food frequency questionnaire and Dual-energy X-ray absorptiometry (DEXA) that protein intake over a three year period that individual consuming the highest intake of dietary protein had 40% less loss of total lean mass and appendicular lean mass\(^{(43)}\).

A large proponent of the scientific community suggests raising the RDA for older adults in an attempt to improve strength and muscle mass in this population, combating the age-related loss of lean body mass\(^{(79; 84; 85)}\). In the absence of adequate protein, lean body mass is catabolized. In a controlled study, Campbell et al., measured urinary nitrogen excretion and body composition to assess the adequacy of the protein RDA in a group of older men and women over 14 weeks. Researchers demonstrated that a
eucaloric diet providing 0.8 g protein/kg body weight/day resulted in a significant decrease in skeletal muscle, specifically mid-thigh muscle area.

Research has reported the beneficial effects of increasing protein intake in older adults on muscle strength. In a study of community dwelling older adults (n=960) aged 55 to 92, it was reported that for every 15 g/day increase in animal protein intake, investigators measured a significant increase in bone mineral density increased at the hip, femoral neck and total body\(^{86}\). Researchers reported that energy-adjusted protein intake was associated with measurable changes in lean mass, participants consuming the highest amount of protein lost the least amount of lean mass. In older adults, dietary protein has been referred to as a “modifiable risk factor” for sarcopenia due to its ability to preserve and promote lean mass repletion\(^{43}\). Symons et al. demonstrated with a stable isotope method that a protein rich food (4-oz serving of lean ground-beef patty) could increase skeletal muscle protein anabolism by 50% in both healthy young and older participants\(^{87}\). During a fasting period prior to meat ingestion and for 5 hours after, consumption plasma amino acid concentrations and a mixed-muscle fractional synthesis rate was calculated\(^{87}\). Additionally, Isanejad et al. reported that over 3 years older women consuming a higher protein intake were associated with less decline in handgrip strength and body mass and that higher protein intake along with less fat mass may be associated with physical functioning\(^{88}\).

Protein requirements during injury, hospitalization and surgery are elevated, conditions more prevalent with increasing age\(^{89}\). Diets high in protein are commonly recommended for individuals at nutritional risk, older adults, those seeking weight loss and for various athletic pursuits. The food industry has responded to consumer
interest/demand and diversified its product offerings to include a vast selection of high-protein products (bars, cereals, drinks, shakes, etc.). High-protein products are commonly marketed at older adults (eg. Ensure® High-Protein, BOOST® High-Protein, ProSource Gelatine® 20, Special K® Protein Cereal, etc.) for their potential effects on bone health and decreasing the risk of sarcopenia.

**Protein Digestion and Absorption**

Investigation into the digestion of proteins has been limited to few studies in humans\(^{(90;91)}\). Protein turnover and digestion rates were shown to differ when in young vs. elderly individuals\(^{(92)}\).

Unlike lipids and carbohydrates, the digestion of dietary protein is not initiated until the food/protein containing food reaches the stomach. The stomach functions in protein digestion to initiate and promote the production of gastric secretions. Dietary protein provides the body with a source of amino acids that are irreversibly oxidized during normal metabolism. Regardless of dietary intake, there are obligatory losses that will be excreted as nitrogen waste products (ammonia, uric acid, etc.)\(^{(93)}\). The primary nitrogen waste product, composing approximately 70-90% of total nitrogen waste, is urea.

Any distension of the stomach or sensory stimulus results in vagal stimulation beginning with gastric secretions and includes the hormone gastrin. G cells in the antrum of the stomach secrete gastrin, a polypeptide hormone into the bloodstream. When the blood supply comes in contact with these specialized cells in the fundus of the stomach, histamine is secreted (derivative of amino acid histidine). Histamine signals specialized cells of stomach to secrete acid, HCl (hydrochloric acid) into the lumen. The secretion of hydrochloric acid lowers the pH, and when a pH of
approximately three is established a feedback loop is initiated to raise the pH and prevent auto digestion\(^{(93)}\).

As acid is secreted, inactive proteolytic enzymes (pepsinogens) are secreted from specialized stomach cells, upon exposure to acid the enzymes are activated and pepsin functions to hydrolyze proteins. The primary function of the stomach is to act as a “holding tank” for protein digestion. Partially digested food, known as chyme, travels through the pyloric sphincter into the duodenum of the small intestine. Additionally, acid aids in the inhibition of pathogens (coming from foods that are consumed). Atrophic gastritis, loss of acid production, is common among older adults and causes an increased risk and infections of the GI tract\(^{(94)}\).

The duodenum is the main site of protein digestion and absorption. Once chyme enters the duodenum, the pancreas secretes the enzymes trypsinogen, chymotrypsinogen and procarboxypeptidase. In addition, when the acidic chyme comes in contact with specialized endocrine cells, they function to secrete two chief hormones, secretin and cholecystokinin (CCK), into the circulation, both playing vital roles in protein digestion. Secretin, a polypeptide, stimulates the exocrine cells of the pancreas to secrete bicarbonate into the pancreatic fluid. The bicarbonate increases the pH of the lumen contents to 6 to 6.5. CCK, a group of peptides, act on the gallbladder and cause it to contract and send bile down the gallbladder duct to the common bile duct and into the small intestine. Bile does assist proteolytic enzymes, but is not required. CCK also signals the pancreas to secrete fluids that are rich in pancreatic digestive enzymes (ex. lipase, amylase, proteolytic enzymes) to digest macronutrients.
The proteolytic enzymes: trypsinogen, chymotrypsinogen, procarboxypeptidase and proelastase, from the pancreas are also present as zymogens. Trypsinogen is activated first by enterokinase from the mucosal wall of duodenum. Enterokinase cleaves a peptide of trypsinogen activating trypsin. Trypsin in turn activates chymotrypsinogen to chymotrypsin (active form), and can activate procarboxypeptidase to carboxypeptidases (active form) and proelastase to elastase (active form). These are the four major proteolytic enzymes secreted by the pancreas that act within the small gut and hydrolyze/further digest the dietary proteins down to polypeptides.

Peptides are cleaved to approximately eight or 12 amino acids in length. Brush border or microvilli have attached digestive enzymes, made within the enterocyte and extruded by Golgi apparatus out onto brush border. Brush border peptidases hydrolyze the peptides into approximately $\frac{1}{3}$ free amino acids, $\frac{1}{3}$ di-peptides and $\frac{1}{3}$ tri-peptides. They are then taken up to the brush border membrane into the enterocyte. Within the lumen of small intestine, the breakdown of dietary proteins results in an approximate equal proportion of free amino acids, di-, and tri-peptides. Once inside the enterocyte, there are cytoplasmic peptidases. If a small protein meal is consumed, there is generally enough time for the cytoplasmic peptidases to complete the hydrolysis of the di- and tri- peptides into free amino acids that then are absorbed into the circulation. Generally, time is insufficient and high quantities of protein are consumed so di- and tri-peptides are absorbed into the circulation with the free amino acids. When nutrients are digested in the lumen of the small intestine, they are absorbed into the venuel. The venuel empties into a larger vessel, eventually emptying into the portal vein and liver. The liver is involved in the synthesis of new proteins from amino acids exclusively. In
the jejunum, protein digestion is mostly complete and the ileocecal valve empties the contents from the ileum into the large intestine where microorganisms are abundant and metabolize any undigested foods, secretions, etc.

Animal protein has a high true digestibility (on average 95%)\(^{(95)}\), whereas plant protein has a lower digestibility, highly attributed to the anti-nutritional factors they contain. True digestibility, the most accurate determination of digestibility, adds back metabolic nitrogen (refers to the bacteria, digestive secretions and sloughed mucosal surface enzymes that are found in part of the fecal nitrogen). Efficiency is no higher than the most limiting amino acid in a food, therefore to get true digestibility an individual must consume a highly digestible protein.

The protein digestibility corrected amino acid score (PDCAAS) is a method used to assess protein quality. PDCAAS assesses protein quality by evaluating a foods amino acid composition in relation to those required by the body and also its digestibility by assigning a numerical value between 0 and 1 (1 is the highest). The PDCAAS is used by the FDA and the FAO/WHO as the favored method for assessing protein value in human nutrition\(^{(96)}\). The PDCAAS is calculated as a percentage and measures the amount of the limiting amino acid (in g) in a specified food item compared to the 1 g of that amino acid in a reference and also considers the fecal true digestibility.

Although it is known that high-protein diets, particularly those high in meat, result in significant protein reaching the colon, the effects of this malabsorbed protein on microbiota profile and putrefactive microbial activities is not known\(^{(97)}\).

Protein fermentation, occurring in the distal colon, can result in the production of ammonia, phenols, amines and sulfides, potentially toxic metabolites\(^{(5)}\). The amount of
protein consumed determines the amount that reaches the colon and the digestibility. Proteins derived from animal sources have the higher digestibility (dairy proteins and meat proteins) compared to plant proteins. Evenepoel et al. compared the digestibility of a meal consisting of raw and cooked egg protein, finding a higher concentration of malabsorbed raw egg protein as metabolites of fermentation in the collected urine\(^{98}\). Research in ileostomy patients exploring the digestibility of different proteins on a normal mixed diet suggested that the amount and not the source of protein consumed in a normal mixed diet is indicative of what reaches the colon\(^{97}\).

Silvester and Cummings fed participants with ileostomies a base diet and research diets with cheese, small beef steak, large beef steak, and a large steak with resistant starch\(^{97}\). The study reported the true nitrogen digestibility measured by ileal output was 86.3% for the control diet, 89.4% for the diet containing cheese, 88.6% for small beef steak, 88.6% for the large beef steak and 88.7% for the large steak with resistant starch. Thus, suggesting that in males 53-62 years old that beef was as well digested as other foods and nitrogen output was related to dietary nitrogen consumed. It was Silvester and Cummings who provided support to previous studies that suggested consumption of meat as a risk factor for cancer (specifically large bowel) because of protein metabolites. Thus, these studies provide evidence and support the notion that it is the amount of protein entering the colon that corresponds to the amount of protein consumed.

**Products of Protein Fermentation**

Fermentation is defined as, “the extraction of energy from carbohydrates and other organic substrates without using \(O_2\) as an electron acceptor. Hence, fermentation is an energy-yielding catabolic pathway that proceeds with no net change in the
oxidation state of the products compared to that of the substrate\textsuperscript{(99)}. Recognized as deleterious to gut health, protein fermentation is the digestion of protein by microorganisms that occurs under anaerobic conditions in the colon\textsuperscript{(100)}. Protein degradation in the colon occurs when proteins are hydrolyzed by bacterial peptidases and proteases to small peptides and amino acids in the colon. These activities generally occur at an alkaline pH. The amount of dietary protein consumed and digestibility of the protein determined the amount of protein that will go to the large intestine for fermentation of the microflora, producing ammonia, phenols, \textit{N}-ntiroso compounds and amines.

The environment of the proximal colon is more acidic due to the production of short chain fatty acids (SCFA) from carbohydrate fermentation\textsuperscript{(101)}. Carbohydrates may be depleted as movement to the distal colon occurs, causing an increase in pH and increased efficiency of protein fermentation.

Short chain fatty acids (SCFA), generally recognized as beneficial to the host, are the primary product of carbohydrate (largely dietary fiber and resistance starch) fermentation\textsuperscript{(102)}. However, deamination of amino acids can also produce SCFA\textsuperscript{(103)}. Butyrate, a SCFA, is the major energy source for colonocytes and functions in colonic homeostasis, specifically in the reduction/inhibition of inflammation and differentiation\textsuperscript{(104; 105)}. Branched chain fatty acids (BCFA) may result from the fermentation process of branched chain amino acids (valine, leucine and isoleucine)\textsuperscript{(106)}.

Bacterial degradation of aromatic amino acids in the colonic lumen results in the production of phenolic and indolic compounds\textsuperscript{(107)}. Bacterial degradation products of the aromatic amino acid tyrosine results in p-cresol, phenol and 4-ethylphenol. The
degradation of the amino acids phenylalanine and tryptophan result in additional metabolites. Research exploring amino acid degradation has demonstrated that phenolic compounds are primarily absorbed by the large intestine and excreted in the urine\(^\text{(108)}\). The major excretory compound is \(p\)-cresol sulfate\(^\text{(107)}\). The excretion of metabolites, specifically those not produced by human enzymes (BCFA, indoles and phenols), has been used as markers to estimate colonic protein fermentation\(^\text{(109)}\).

Russell et al. examined the effects of a high-protein diet, intended for weight loss on measured of gastrointestinal/colonic health\(^\text{(110)}\). Phenolic metabolites, nitrogenous compounds and short-chain fatty acids were measured after a four-week period. On the high-protein diet, an increase in phenylacetic acid, \(N\)-nitroso compounds and branched chain fatty acids were reported. This research suggested that long-term adherence to a high-protein reduced carbohydrate diet may result in increased metabolites that may increase risk of certain diseases. Specifically, Poesen et al. suggest that serum levels of phenylacetyl glutamine is an independent risk factor for cardiovascular and disease and mortality\(^\text{(111)}\).

The large intestine plays a critical role in the nitrogen metabolism\(^\text{(112)}\). Free nitrogen waste products that end up in large intestine and the blood stream such as urea, uric acid, creatinine and other sources of nitrogen enter the large bowl as largely undigested dietary proteins, in particular plant proteins that are not as readily digestible as animal proteins\(^\text{(113)}\). Some plant proteins are only 70% digestible and the remaining end up in the large intestine. Undigested digestive secretions, such as mucins that come from mucosa, get sloughed off from the turnover of epithelial cells go to the large intestine. Ammonia, other byproducts, endogenous and exogenous losses will go to the
large intestine where metabolism will occur. The bacteria in the large intestine play a role in nitrogen economy. Bacteria in the large intestine produce the enzyme urease, resulting in urea production. Urea diffuses through the bloodstream to the mucosal surface of colonocytes where it can be freely absorbed back into circulation and into the portal vein. The enzyme uricase is also produced by bacteria and in the large intestine is responsible for converting urea back to ammonia and creatinine. Most creatinine produced is cleared by the kidney and excreted in the urine. Approximately 25% of total urea production is recycled through the gut\textsuperscript{(114)}.

The potential toxicity and genotoxicity of protein fermentation has been demonstrated in an animal model where rats received a high casein diet (25% of weight)\textsuperscript{(115)}. Urinary levels of \textit{p}-cresol significantly correlated with observed genetic damage\textsuperscript{(115)}. The presence of adequate carbohydrate substrate (fiber and resistant starch) in the colon supporting saccharolytic microbial activity may depress colonic protein fermentation, and recent research has shown in individuals with reduced kidney function, that the addition of fiber (including pulse fiber) not only improves blood urea nitrogen\textsuperscript{(116)}, gastrointestinal function and specific scores of quality of life\textsuperscript{(117)}, but also lowers \textit{p}-cresol, a gut-generated, protein fermentation product with known systematic inflammatory effects\textsuperscript{(118)}, suggesting suppression of proteolytic microbial activities.

A feeding study in rats confirmed, that in an animal model, a potato fiber diet elicited effects on the colonic environment, and the concentration of phenol and \textit{p}-cresol were significantly reduced in animals fed a potato fiber diet compared to controlled diets of cellulose and potato-resistant starch\textsuperscript{(119)}. Improved colonic structure was reported in
the potato fiber fed animals and colonic fermentation stimulated growth of bifidobacteria\(^{(119)}\).

In addition to \(p\)-cresol, another uremic solute is indoxyl sulfate. Also found in the urine and a product of protein degradation is indoxyl sulfate a metabolite of the breakdown of the amino acid tryptophan. Protein-bound uremic retention solutes \(p\)-cresol and indoxyl sulfate, have been shown to inhibit/decrease overall endothelial proliferation and wound repair\(^{(120)}\).

Probiotics (\textit{Lactobacillus casei} Shirota, and \textit{Bifidobacterium breve} Yakult) administered in healthy adults resulted in a significant decrease in \(p\)-cresol excretion\(^{(121)}\). Researchers proposed that the reduced level of \(p\)-cresol excretion observed is a result of an increased uptake of tyrosine or other possible metabolic products from protein putrefaction. Further explaining that the reduced level of \(p\)-cresol excretion was caused by a higher colonic availability of probiotic microorganisms or the inhibition of proteolytic bacteria by the probiotic microorganisms consumed. Patel et al. measured the uremic solutes, \(p\)-cresol and indoxyl sulfate in vegetarian participants in comparison to participants consuming an unrestricted diet. Researchers noted participants consuming the unrestricted diet had urinary excretion rates of \(p\)-cresol that were 62% higher and indoxyl sulfate rates 58% higher than the participants consuming the vegetarian diet\(^{(122)}\).

At a time when high-protein diets are common, their impact on microbiota and wellness needs examination.

\textbf{Digestive Health}

Aging is accompanied by changes and decreased functionality of the gastrointestinal tract. Changes commonly include: a reduction in gut motility, reduced
blood flow throughout the gastrointestinal tract, a reduction in gastric acid production and the rate of gastric emptying, and a reduction in absorption surface and uptake and transport\(^{(123)}\). Polypharmacy often exacerbates and also contributes to these changes and can result in malabsorption and diarrhea in older adults. In a sample of community-dwelling older adults (65-93 years of age) 24.3% reported suffering from frequent abdominal pain, and 24.1% reported symptoms of chronic diarrhea or constipation\(^{(124)}\). With the prevalence of gastrointestinal symptoms validated assessment methods are needed, specifically in healthy and older adult populations.

**Gastrointestinal Symptom Rating Scale**

The Gastrointestinal Symptom Rating Scale (GSRS), originally developed and validated for patients with peptic ulcer disease and irritable bowel syndrome (IBS), is a 15-item questionnaire that measures gastrointestinal symptoms and discomfort\(^{(125)}\). The GSRS was later adapted and evaluated for individuals with gastroesophageal reflux disease (GERD)\(^{(126)}\). The 15-item questionnaire is scored using a seven-point Likert scale where 1 indicates no discomfort and a score of 7 indicates very severe discomfort. The 15-item questionnaire can be further grouped into five scales, which include: abdominal pain, reflux syndrome, diarrhea syndrome, indigestion syndrome and constipation syndrome. In addition to its use as a disease-specific instrument, the GSRS has also been used in clinical studies in healthy adults. It has been used by researchers investigating potential changes in gastrointestinal symptoms when administered probiotics\(^{(127,128)}\).

**Microbiota**

The gut microbiome is highly diverse and its complexity has only recently been alluded to by researchers, along with its influential roles nutrition, immunology and gut-
brain regulation. The human microbiota contains approximately ten fold more cells than the human body\textsuperscript{(129)}. The human intestinal tract contains trillions of microbes and is comprised of a diverse composition of species. It has been estimated that the bacteria present in the colon contains approximately 35-50\% of the colonic volume\textsuperscript{(130)} and these bacteria can impact health. Over 90\% of the bacteria in the gastrointestinal tract belong to the phylum Firmicutes and Bacteriodetes\textsuperscript{(131; 132)}. The intestinal environment readily changes with stress, dietary intake, changes in daily living and illness\textsuperscript{(132; 133)}. The use of specific medications, including antibiotics and long-term dietary changes, disrupt the natural balance in gut microbiota\textsuperscript{(134; 135)}. The microbes found in the gastrointestinal tract aid in digestion, provide essential vitamins (includes, vitamin K, B vitamins, etc.), provide immune system stimulation, influence the gastrointestinal barrier, improve bioavailability of nutrients and stimulate bowel motility and function\textsuperscript{(136)}. The microbes can also help ferment oligosaccharides and fiber, functioning in energy salvage. Oligosaccharides and fiber assist in the catabolism of SCFA and the level of SCFA often decreases with disease.

Previous research supports the statement that shifts in microbiota can be made with long-term dietary changes, however inter-individual variation is noted and any changes noted after dietary intervention are relative to the initial composition\textsuperscript{(135)}. The time required for microbiota changes after dietary changes, specifically changes in macronutrients, was previously unknown. Research conducted by Ley et al. supported that notion that changes in microbial communities and diversity could result on a controlled diet (fat-restricted or carbohydrate restricted low-calorie diets) during a 1-year period\textsuperscript{(137)}. However, David et al. demonstrated that the gut microbiome of
humans can be shifted by dietary changes in a rapid manner\textsuperscript{(138)}. Participants received an \textit{ad libitum} diet consisting of entirely animal or plant products for five days and differences in microbial colonization and diversity were measured. Based on their findings researchers suggested that the gut microbiota can change functional profiles in a rapid and diet specific manner (herbivore vs carnivore diet).

Research demonstrating disturbances and alterations in the microbiota with dietary changes are limited and studies that have been conducted have not been well controlled or applicable to the population at large. The need exists for additional research in this area that utilizes a controlled diet and objective measures for health.

**Aging and the Microbiota**

Researchers have demonstrated that older adults generally have higher populations of fungi and enterobacteria and lower counts of bifidobacteria\textsuperscript{(139; 140)}. The microbiota of older adults is distinct compared to younger and middle age adults. The microbiota of older adults consists of a greater abundance of \textit{Bacteroides} spp. And \textit{Clostridium} groups and a lower proportion of the phylum \textit{Firmicutes}\textsuperscript{(2)}. Additionally, the microbiota of older adults is often characterized as unstable or in a state of flux\textsuperscript{(141)}. While the mechanisms are not clearly known, immune functions have been demonstrated to benefit from lactobacilli and bifidobacteria\textsuperscript{(142; 143)}.

Cleasson et al. used a food frequency questionnaire (147-item) and the healthy food diversity index to identify differences in food intake between community-dwelling and institutionalized older adults\textsuperscript{(144)}. Researchers showed significant differences between older adults living in the community vs institutionalized older adults. Additionally, using the healthy food diversity index researchers demonstrated that a high diversity in food intake supports higher diversity of the microbiota.
A declining diversity of colonic microbiota has been demonstrated to occur with aging, thus prebiotics and probiotics may confer affirmative benefits on colonic metabolism of older adults.

**Prebiotics**

Prebiotics are generally defined as “nondigestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacterial species already established in the colon, and thus in effect improve host health”\(^{(145)}\). To be considered a prebiotic, Collins and Gibson provide the following criteria that must be met: “1) neither be hydrolyzed nor absorbed in the upper part of the gastrointestinal tract; 2) be a selective substrate for one or a limited number of potentially beneficial commensal bacteria in the colon, thus stimulating the bacteria to grow, become metabolically activated, or both; and 3) be able as a consequence to alter the colonic microflora toward a more healthier composition”\(^{(146)}\). Research on prebiotics and their ability to selectively stimulate growth in the colon have focused on bifidobacteria and lactobacilli. Presently only inulin and galactooligosaccharides (GOS) meet the specified criteria to be classified as a prebiotic\(^{(147)}\).

**Inulin**

Inulin, a dietary fiber, is structurally a polymer of fructose units joined by \(\beta(2\rightarrow1)\) glycosidic bonds and a terminal glucose unit (fructan)\(^{(148)}\). Degree of polymerization for inulin varies from 2 to 60. If inulin has a degree of polymerization \(\leq 10\) it is further classified as oligofructose. The chemical structure makes inulin non-digestible and is fermented by colonic microflora, thus a lower caloric value\(^{(149)}\).

Natural sources of inulin include: garlic, agave, jicama, banana, wheat and onion among others. Commercially, inulin is used to improve organoleptic properties;
specifically taste and mouth feel\(^{(150)}\). The nutritional and functional properties of inulin make it a widely used product in the food industry\(^{(151)}\).

**Inulin- clinical application**

Because of the ease of incorporation into many food products, and their physiological properties prebiotics have the potential to have widespread impact and maintain and/or enhance the microbiota. The ability of inulin to influence gastrointestinal function has been demonstrated. Digestibility studies in ileostomy patients demonstrate that inulin reaches the large intestine, with a percent recovery of 86–89\%\(^{(149, 152)}\). Specifically, its ability to increase stool frequency, decrease pH of the stool\(^{(153)}\), increase stool weight\(^{(154)}\) and regulate blood cholesterol levels in individuals with hypercholesterolemia\(^{(155)}\).

Used for industrial purposes, inulin is largely extracted from chicory root \((Cichorium intybus)\) using a hot water extraction process.

Research has demonstrated the ability of inulin to stimulate specific species of the genus *Bifidobacterium*\(^{(154)}\), a genera considered beneficial to overall health. Bifidobacteria are saccharolytic microorganisms that are important in carbohydrate metabolism and are known for their protective role against pathogens (produce antimicrobial agents, inhibit adhesion pathogens and are involved in immune modulation).

In addition to the use and commercialization of inulin by the food industry, inulin has been researched for its clinical application, most notably in type 2 diabetes mellitus\(^{(156)}\) and renal disease.
Probiotics

Probiotics are defined by the World Health Organization as, “live organisms which when administered in adequate amounts confer a health benefit on the host”. In recent years, probiotics have become increasingly popular in the United States as well as in Europe and Asia\(^\text{157; 158}\). Probiotics have been suggested and used as a therapy due to the symbiotic relationship they may confer and to promote good health and strengthen the host system. Additionally, probiotics may aid in the recovery of certain diseases or conditions. Many probiotic strains have been investigated for clinical efficacy, including multiple bacterial strains: *Lactobacilli, Bifidobacteria, Streptococci, Clostridia* and the fungal strains: *Saccharomyces boulardi (S. boulardi) and S. cerevisiae*\(^\text{159; 160}\).

For probiotics to have optimal effect, it is critical that they are able to survive the gastrointestinal tract and transit to the target organ. In general, when probiotics survive the gastrointestinal tract they provide a microbial barrier against antigens and toxins, and allow for specific immune functions to modulate and regulate throughout the gastrointestinal tract\(^\text{136}\). The competitive and mutualistic relationship that is found in the gastrointestinal tract allows for certain bacteria to prevail over other bacterial species in a phenomenon termed “colonization resistance”\(^\text{161}\). When the concentration of beneficial bacteria is high through the microbial environment with *lactobacilli* and *bifidobacteria*, for example, other harmful bacteria such as *Escherichia coli, Campylobacter, Salmonella*, etc. are less likely to proliferate and adhere to the intestinal wall of the gastrointestinal tract\(^\text{133; 162}\). The mechanisms probiotics use to regulate the microbiota include their ability to produce bacteriocins/defensins, participate in
competitive inhibition with pathogens, inhibit pathogenic mucosal adhesion, reduce the luminal pH and increase mucus production\textsuperscript{(163)}.

As demonstrated to confer a benefit on the host, the probiotic must be able to survive the gastrointestinal tract. If the probiotic survives, the strain should be found in the individuals’ feces. By definition probiotics should only transiently colonize the hosts’ gastrointestinal tract. Limited studies have been conducted that study the survival of administered probiotic as it transits the gastrointestinal tract and completed fecal recovery. In general, oral doses that survive transit are approximately 100-1000 times lower that which is found in the oral dose\textsuperscript{(164)}. After oral administration of live yeast only 1 to 3\% is recovered in feces\textsuperscript{(165)}.

Few studies have shown the potential of probiotics and prebiotics to shift from proteolytic to saccharolytic fermentation. Ndagijimana et al. reported that healthy adults who consumed a synbiotic food product containing fructooligosaccharides (FOS), \textit{Bifidobacterium longum} and \textit{Lactobacillus acidophilus} for 30 days demonstrated a shift from proteolytic to saccharolytic fermentation, as indicated by an increase in SCFA concentrations, a decrease in amino acids and in associated protein metabolites when fecal samples were analyzed\textsuperscript{(166)}. Researchers suggested a potential health benefit and modulation of microbiota when the synbiotic food product was consumed.

\textbf{Probiotics and ageing}

Gastrointestinal conditions, particularly constipation and diarrhea are common in older adults. To modulate the microbiota, probiotics have been suggested. However, research exploring the use and efficacy of probiotics in an older adult population has been limited to largely disease/condition specific and their potential impact on immunity.
This review will focus on the use of probiotics in the elderly regarding gastrointestinal function, wellness and immune function.

The use of lactic acid bacteria (LAB) strains and clinical efficacy for reducing constipation on older adults had been well researched. In a parallel study older participants consuming a juice with *L. rhamnosus* and *Propionibacter freudenreichii* exhibited an increase in bowel movement frequency (24% increase)(167). Researchers supplementing frail elderly adults residing in a nursing home with a daily fermented milk beverage containing *Lactobacillus casei* Shirota for 6 weeks significantly decreased the percentage of diarrhea and constipation type stools and significantly increased the number of normal type stools per week(168). A randomized, double-blind controlled study in elderly individuals residing in nursing homes investigated the use of a probiotic product Duolac® Care and reported after two weeks a significant improvement in constipation (using the Rome III criteria)(169). The conclusions of a recent systematic review conducted by Martinez-Martinez et al. suggests that probiotic administration compared to placebo may significantly improve constipation in older adults by approximately 10-40%, in a strain specific manner (*Bifidobacterium longum* was the most widely used)(170).

In addition to the effects probiotics may elicit on the gastrointestinal tract, their impact on the immune system has also been explored. Some probiotic, particularly lactic acid bacteria (LAB) strains, may enhance properties of the immune system. Immunomodulatory effects of probiotics are under investigation and has been demonstrated in strain specific mechanisms. The effect has been demonstrated with increased levels of interferon-alpha and increases in phagocyte mediated bactericidal
activity in older adults consuming milk with *B. lactis*\(^ {171}\). Modulation of the immune system has been demonstrated and seem to suggest strain specificity, however the clinical significance of these changes needs to be further investigated.

**Synbiotics**

The combination of probiotic and prebiotic supplementation is referred to as a synbiotic and should have “synergistic” effect. A synbiotic refer to the use of a prebiotic (inulin or GOS) that selectively favors a probiotic product and benefits the host by enhancing survival\(^ {172}\). In clinical studies, synbiotics have been investigated primarily in adults with illness or diarrhea\(^ {173}\). In surgical patients a probiotic mix was provided with oligofructose and researchers examined the effects on the microbiota, inflammation and sepsis\(^ {174; 175}\). The use and investigation of synbiotics in healthy adults is limited in clinical research. Although because of the “synergistic” relationship between prebiotic and probiotics this area should be further investigated with clinical trials.

**Summary**

Globally, the cohort of aging adults continues to rise and is associated with increasing health care costs and age related physiological and metabolic changes. These age related changes include “inflamm-aging,” the chronic low-grade state of inflammation, characterized by increased level of IL-6, TNF-α and reduced levels of the anti-inflammatory cytokine IL-10. In addition to a suppressed immune system, changes to the musculoskeletal system include: sarcopenia, the loss of lean mass, and strength and are associated with increased risks including falls and disability. Dietary protein has been identified as a “modifiable risk factor” for sarcopenia and has been associated with a reduction in lean mass loss in older adults.
Research has demonstrated that the microbial community of the gastrointestinal tract, known as the microbiota, also changes with age. In addition to age, the microbiota is influenced by other factors including diet and disease. Research suggests that older adults exhibit decreased microbial diversity and a decline in bifidobacteria. Current research exploring the microbiota is rapidly increasing and further exploring perturbations in composition and their potential influence on human health and disease. The effect of diet and diet-induced changes to the microbiota are also under investigation. However, research on dietary protein intake and potential disruptions or dysbiosis in the microbiota is largely unknown. Research examining the effects of dietary protein on microbiota in the current literature is limited to a short-term exclusively animal based high-protein diet void of dietary fiber conducted in healthy young adults.

Because many older adults do not consume the recommendations for dietary protein – and protein exhibits potential physiological benefits to older adults to maintain muscle mass further suppressing some of the age related changes – further research examining the effect of high-protein diets should be examined. Research is needed to explore high-protein diets that are feasible (animal and plant based protein sources), contain practical macronutrient distributions, and contain a fiber intake consistent with the typical North American diet.

The purpose of this study is to fill the current void in the scientific literature and examine a high-protein diet on microbiota in older adults -- a cohort that may benefit from increased protein intake. Additionally, this research will further explore the potential effects of high-protein diet with a prebiotic (inulin), probiotic and synbiotic.
CHAPTER 3
METHODS

Experimental Design

An eighteen-week randomized, double-blind, placebo-controlled, crossover study (Figure 3-1) was conducted at the University of Florida, Food Science and Human Nutrition Department Clinical Laboratory and the Marion County Cooperative Extension Office in 2015. Healthy, community-dwelling, older adult women aged ≥ 65 years were recruited from Alachua and Marion counties and surrounding areas through posters, flyers, announcements, approved list-serves, word of mouth, presentations to clubs/organizations/church groups, television commercials (aired through the Public Broadcasting Service) and community/newspaper advertisements.

Interested individuals were initially screened (pre-screening) via a telephone contact script. The study was described to the potential study participant using the approved script and the inclusion/exclusion criteria was read. Potential study participants were directed to listen to the criteria, without directly answering (without stating “yes” or “no”) after each criterion was read, and then asked to decide whether they would like to continue with study procedures. If the potential participant was still interested in participating and still believed they may qualify, an appointment was scheduled for consenting.

Approval was obtained from the Institutional Review Board (IRB) at the University of Florida, IRB-01 (IRB# 2014-00955) and ClinicalTrials.gov (NCT# 02445560). All participants provided written informed consent.
Participants

Participants were excluded if they did not meet the specified criterion. Criterion included the following: participants could not i) be currently being treated for any known illnesses or conditions that may impact perceived health such as (human immunodeficiency virus (HIV) / acquired immune deficiency syndrome (AIDS), immune modulating diseases (autoimmune, hepatitis, cancer, etc.), diabetes or chronic kidney disease; ii) be vegetarian; iii) have any known food allergies or dietary restrictions; iv) have a physician-diagnosed gastrointestinal disease or condition (such as ulcerative colitis, Crohn’s disease, gastroparesis, peptic ulcer disease, cancer, celiac disease, short bowel disease, ileostomy); v) be taking medication for constipation or diarrhea, have received an antibiotic therapy during the previous 2 months; vi) be a current smoker; vii) typically consume no more than one alcoholic beverage per day (Dietary Guidelines for Americans, 2010); vix) have a BMI > 30 or x) be planning on losing/gaining weight during the next 6 months.

Additionally, potential participants had to: i) be a woman 65 years of age or older; ii) be willing to complete daily and weekly questionnaires; iii) be willing to wear a SenseWear Pro Armband (BodyMedia, Inc.) to monitor energy expenditure and establish dietary energy needs; iv) be willing to provide blood samples, stool samples and urine samples throughout the course of the study; v) be willing to discontinue prebiotics, probiotics and/or any fiber supplements for the duration of the study; vi) be willing to consume the provided diet for the designated eight weeks of the study; vii) be willing to report and maintain their usual alcohol intake throughout the study; vix) be willing to provide a social security number to receive study payment; x) be able to take foods, study fiber, probiotic and placebo without the aid of another person xi) be able to
attend all scheduled study appointments for the duration of the study; xii) consume <15% of daily energy intake from protein as assessed by dietary analysis and xiii) consume a usual diet <20 g/day fiber as assessed by dietary analysis.

Participants were excluded if they do not meet the inclusion/exclusion criteria. It was noted, that while participants would only be included in the study if they had originally reported that they could attend all study visits for the duration of the 18-week study, it was also stated that if any extenuating circumstances occurred study staff would attempt to make alternate arrangements to complete any study procedures that had been missed, if possible. In the event that a participant was unable to attend a scheduled study visit, researchers attempted to reschedule their study appointment and/or make arrangements with the participant on a case by case basis.

This 18-week study consisted of nine periods, each two weeks in length. The first period (two weeks) consisted of a baseline period during which screening was conducted and eligibility was confirmed. The baseline period was followed by alternating two-week periods of study treatments and washouts. Each treatment period included a “standard” high-protein diet (kept consistent for each participant through each of the four treatment periods) and in random order the administration of a probiotic and/or prebiotic. Participants attended a study appointment at either the Food Science and Human Nutrition department clinical lab or the Marion County Extension office every two weeks (at the end of each period) for monitoring, questionnaire completion, blood sample collection at specified time points, anthropometric measurements and handgrip measurements.
After consenting and prior to the baseline period, participants attended a study appointment to pick up study materials, receive additional instructions regarding the study procedures, and to collect the armband (SenseWear Pro Armband BodyMedia, Inc.) that would be used to estimate their energy requirements and set dietary requirements (for the "standard" high-protein diet) during the intervention periods. In addition to biweekly study appointments, participants were contacted regularly to encourage compliance and reiterate study protocol.

Using the National Institutes of Health categories, participants were asked to self-identify their race (American Indian/Alaska Native, Asian, Native Hawaiian or Other Pacific Islander, Black or African American, White, More Than One Race, Unknown or Not Reported, or Other) and ethnicity (Hispanic or Latino, Not Hispanic or Latino, Unknown or Not Reported)\(^{(176)}\). Participants were also asked to report their current age.

**Screening and Baseline**

Screening occurred during the baseline period and consisted of a two-week period. Participants were screened during the baseline period using a three-day, 24-hour recall to select individuals who typically consume an average protein intake of <15% of daily energy and an average fiber diet (<20 g/day). Participants were directed to consume their usual diet and not to make any dietary changes during this period. Dietary recalls were collected by randomly contacting the participants by telephone on three days (two weekdays and one weekend day were collected) and asking them to report their dietary intake from the previous day\(^{(177)}\). The 24-hour recall method was used, a retrospective assessment method during which an interviewer asks the respondent to recall and describe all foods and beverages that were consumed during the previous 24-hour period while using prompts to encourage the respondent to
remember all items consumed\textsuperscript{(178)}. Portion size estimating aids were provided to assist in the collection of 24-hour dietary recalls and participants were instructed and given directions on how to use them to assist with the recalls, if needed. Tools included a 12-inch ruler, USDA Food Model Booklet, plastic measuring cups (four-piece set including \(\frac{1}{4}\) cup, 1/3 cup, \(\frac{1}{2}\) cup, 1 cup) and plastic measuring spoons (4-piece set including \(\frac{1}{4}\) tsp., \(\frac{1}{2}\) tsp., 1 tsp., 1 Tbsp.)\textsuperscript{(179)}. The USDA Food Model Booklet is an estimating aid used in NHANES to assist with portion size estimations\textsuperscript{(180)}. Permission was granted by a research leader at the USDA to allow access and files to use the USDA Food Model Booklets. A multiple pass method, originally developed by the USDA Human Nutrition Information Service (HNIS), was used when interviewing participants\textsuperscript{(181)}. A three-pass method was used when interviewing participants. Prompting was used by the interviewer to assist participants in remembering commonly forgotten foods (i.e. butter, jam or spreads on toast). The three-pass method consists of first asking the participant to provide a quick list, followed by the next pass that is a detailed description and the final pass is a review\textsuperscript{(182)}. Dietary intake was analyzed using food analysis software (ESHA Food Processor\textsuperscript{®}, version 11.0.117) to estimate mean intake. If the individual did not meet the inclusion criteria for average protein or fiber intake, they were excluded prior to randomization.

During the baseline period, participants were also provided with and asked to wear SenseWear Pro 2 Armband (SWA; Body Media, Pittsburgh, PA), a multi-sensor device that is portable and is worn over the tricep muscle\textsuperscript{(183)}. The use of Sensewear Pro Armbands was validated in community-dwelling older adults by Mackey et al. who reported no difference in total energy expenditure (TEE) when the method was
compared to doubly labeled water\textsuperscript{(184)}. To determine usual energy requirement, participants were asked to monitor energy expenditure for a seven-day period, wearing the armband as close to 24 hours a day as possible and only removing the armband during periods when it may come in contact with water (i.e. swimming, showering, etc.). After the seven-day period, study staff picked up armbands from the participants for analysis. To calculate energy expenditure, data was downloaded and used in combination with participant characteristics (gender, age, height, weight, dominant hand and smoking status). SenseWear Professional software version 8.0; BodyMedia Inc.) was used for analysis.

Participants were also asked to record their daily weight in kilograms (kg) using a portable scale (Eat Smart Precision Digital Bathroom Scales were provided to all participants prior to the baseline period) in the morning while still fasting. Fasting weights were self-recorded daily in a daily questionnaire booklet provided to participants. Participants were asked to record their weight throughout the entire 18-week study.

**Randomization**

In July 2015, on the day of randomization participants met with study staff. Anthropometric data, bioelectrical impedance analysis (BIA) and handgrip strength were collected and measured at the appointment. Weight was measured using the Seca\textsuperscript{®} 874 flat scale for mobile use, height using Seca\textsuperscript{®} 217 portable stadiometer, and handgrip strength using a digital dynamometer (JAMAR\textsuperscript{®} Plus+; Patterson Medical, Warrenville, IL). Fasting venous blood was drawn on the day of randomization by a licensed phlebotomist to assess baseline measurements.
Participants were randomized via sealed envelopes to receive each of the two-week study interventions (high-protein diet + fiber sachet + probiotic capsule, high-protein diet + placebo sachet + probiotic capsule, high-protein diet + fiber sachet + placebo capsule, high-protein diet + placebo sachet + placebo capsule) in random order. Randomization followed a Latin square design. A total of four treatment sequences were used and participants were randomized to receive their treatment allocation in one of the four designated orders (ABCD, BDAC, CADB or DCBA). Randomization envelopes were prepared by an individual unaffiliated with the study. Participants and study staff were blinded to the sequence that sachets and capsules were given during the treatment periods. During each treatment period, participants received a high-protein diet that was kept consistent through each treatment period and customized to each participant (see “Treatment Periods with Controlled Diet Intervention”).

**Supplement Administration Protocol**

**Prebiotic Administration**

Prebiotic supplements were provided in coded silver opaque sachets. Sachets were labeled with a four-digit numerical code and directions for use. Sensus FRUTAFIT® IQ, inulin was provided by SENSUS, part of the Royal Consun group. The placebo, potato maltodextrin (PenNovo® MD10), was indistinguishable in color and similar in appearance and texture. FRUTAFIT IQ and placebo was weighed to 5.6 g ± 0.02 using electronic precision scales (Adam Equipment® PGW 4502e Precision Balance). The weighed FRUTAFIT® IQ and placebo were placed in individual silver opaque sachets, labeled and sealed. Codes were generated using a random number
generator and assigned by an individual unaffiliated with the study to ensure the study team remained blinded. Frutafit IQ is labeled as ≥90% inulin so to ensure the supplementation of 5 g of inulin 5.6 g ± 0.02 was provided to participants during assigned treatment periods. Placebo and FRUTAFIT® IQ were visually similar, a white powder and could not be distinguished from each other. Participants were instructed to consume one sachet a day and to consume the sachet in its entirety by thoroughly mixing the contents of the packet with water or a beverage.

**Probiotic Administration**

Probiotic capsules were provided in opaque white bottles with supplement codes (four-digit numeric code) and study information (study name, lot number, expiry date, contact information, sponsor information, storage information and directions for use) on the label. Participants were instructed to consume one capsule daily with a meal. Probiotic and placebo were provided by Lallemand Health Solutions Inc. The probiotic supplement contained the following: *Bifidobacterium bifidum* HA-132 (8%; 1.54 billion), *Bifidobacterium breve* HA-129 (23%; 4.62 billion), *Bifidobacterium longum* HA-135 (23%; 4.62 billion), *Lactobacillus acidophilus* HA-122 (23%; 4.62 billion) and *Lactobacillus plantarum* HA-119 (23%; 4.62 billion). The strains used in the product were based on existing probiotic formulations that are currently marketed and sold in the U.S. Placebo capsules were identical in appearance to the probiotics and consisted of encapsulated potato starch, magnesium stearate and ascorbic acid. Each bottle provided to participants contained 15 capsules to account for accidental loss. Participants were instructed that bottles contained enough capsules for the designated treatment period but were not informed of the exact number of capsules in each bottle.
A two-week supply was provided of the sachets and capsules during each treatment period and participants were asked to return any unconsumed supplements at the end of each period and record their intake compliance of the probiotic and prebiotic on their daily questionnaire.

Treatment Periods with Controlled Diet Intervention

A high-protein diet composed of commercially available protein products and further supplemented with an prebiotic (inulin) and/or multi-strain probiotic was provided during each of the four two-week treatment periods. During each treatment period, all food, snacks and calorie-containing beverages were provided to participants.

Participants were provided with a weight maintenance diet during each controlled feeding treatment period based on a 1600-calorie low fiber base menu providing 1.5-2.2 g/kg/day of protein with additional macronutrient sources to raise the energy content and meet each participant’s personal requirements, as defined by the acceptable macronutrient distribution range (AMDR). The AMDR is defined by the Institute of Medicine as, “the range of intake for a particular energy source that is associated with reduced risk of chronic disease while providing intakes of essential nutrients.”

A weight maintenance 1600-calorie low fiber base diet was provided to each participant which was then customized individually for each participant by a dietitian and was comprised of the base diet and adjusted according to their dietary and energy needs. Energy needs were determined by the SenseWear Pro 2 Armband (SWA; Body Media, Pittsburgh, PA).

Study Diet: The diet was comprised of commercially available fresh meats (chicken and red meat) packaged by the meat department at Publix® supermarkets.
Meats were cut, weighed and packaged individually by Publix®. Additionally, provided were fresh fruit and vegetables, frozen meals, pre-packaged foods such as fruits, salads, vegetables, protein drinks, breakfast foods, and an assortment of snacks. Shelf-stable foods with acceptable expirations were purchased in bulk prior to the study start, to limit the possibility of any changes in product formulation. Perishable products were ordered and delivered by Publix® approximately one-to-two days prior to when the food would be delivered to the participants. Food was then sorted, packaged and labeled individually for each participant according to their menu specifications. Food was provided to participants in freezer bags and shelf-stable food was packaged in grocery bags. Participants were given four-day rotations of this menu with low to moderate fiber levels for 14 days during each treatment period. Participants received a one-week supply of food during their study appointments and the second week of food in each of the two-week periods was delivered to the participant the day prior to when they would require it. During treatment periods, food was delivered to the participants' home and/or picked up weekly by participants at the research site.

Participants were instructed to consume all food that was given to them during the treatment periods and record any food that was not consumed via daily questionnaire and by taking a picture of the unconsumed foods. Participants were provided digital cameras (Vivitar ViviCam F128) to document any food that was not consumed during the controlled dietary interventions. Participants were asked to bring their cameras in at the end of each controlled diet intervention. Study staff downloaded any pictures that were taken by the participant during that period, and the camera was returned at the beginning of the next diet intervention. For food safety and proper
handling instructions, participants received the USDA Food Safety Information
documents entitled “Chicken from Farm to Table” and “Beef from Farm to Table”.

Washout Periods

Washout periods were two weeks in duration and occurred after each treatment
period (total of four washout periods during the 18-week study). During each washout
period, participants were asked to return to their usual diet (prior to the study start) and
asked not to make any dietary changes. Participants were responsible for providing
their own food during each washout period. Dietary recalls were collected during each
washout period to ensure participants were not changing their dietary habits. During
each washout period, three days of dietary recalls were collected (two weekdays and
one weekend day respectively). The same 24-hour recall method was used for
collecting dietary recalls (see “Screening and Baseline”). The three-pass method was
again used with portion size estimating aids and the USDA Food Model Booklet.

Participants were instructed to continue to complete daily questionnaires and the
weekly GSRS. Fasting weight was also collected daily during washout periods and self-
recorded daily using the provided scales (Eat Smart Precision Digital Bathroom Scale)
and daily questionnaire booklet provided.

Final Appointment

In addition to the standard data collected at each appointment, BIA was again
measured at the final study appointment.

Study Questionnaires

Daily Questionnaire

During each study appointment participants received a paper booklet of
questionnaires that contained a two-week supply of daily questionnaires (daily and
GSRS) for each study period (baseline, treatments and washouts). Participants were asked to bring in their completed questionnaire packet and were given a new packet for the current study period at each appointment.

Daily questionnaires asked participants to record their fasting morning weight in kg, hours of sleep (asked to record how many of hours they slept last night, not including the time it took to fall asleep or anytime they were awakened during the night; responses were reported as <5 hours, 5-6 hours, 6-7 hours, 7-8 hours, 8-9 hours, >9 hours). The daily questionnaire also contained a “Yes” or “No” response regarding the consumption of study supplements (probiotic and fiber), adherence to study diet during controlled feeding periods, antibiotic use, consultation with a doctor, changes in physical activity, medication usage and changes. Participants were asked to expand in an open answer format if they reported that they did not completely adhere to the study diet, there were any changes in their level of physical activity, and/or changes in their medications.

Bowel movement frequency and consistency, level of hunger and a series of questions pertaining to symptoms and psychological well-being were included. Symptoms included; bloating, flatulence, abdominal cramping, abdominal noises, headache, dizziness, nausea, vomiting, constipation, fatigue, feeling anxious, feeling depressed, and feeling stressed. Participants were asked to report any of the mentioned symptoms experienced in the previous 24-hours and asked to report using a scale of 0 to 6 (anchors were assessed at the following values 0=none, 3=moderate, 6=very severe).
In the daily questionnaire, participants were also asked to report their bowel movement frequency and consistency using the Bristol Stool Form Scale. Transit time was evaluated using the Bristol Stool Form Scale\(^{(185)}\). The Bristol Stool Form Scale provides standard descriptors and pictures and asks participants to rate the consistency of each stool on a scale of Type 1 to Type 7 (Type 1: Separate hard lumps, like nuts (hard to pass), Type 2: Sausage-shaped but lumpy, Type 3: Like a sausage but with cracks on the surface, Type 4: Like a sausage or snake, smooth and soft, Type 5: Soft blobs with clear-cut edges (passed easily), Type 6: Fluffy pieces with ragged edges, a mushy stool or Type 7: Watery, no solid pieces. Entirely Liquid). The Bristol Stool Scale is a validated scale to assess/predict transit time\(^{(186)}\).

**Gastrointestinal Symptom Rating Scale**

Gastrointestinal symptoms were also assessed using the Gastrointestinal Symptom Rating Scale (GSRS). The GSRS was included in the questionnaire booklet (new booklets were provided to participants every two weeks and included the daily questionnaire and GSRS questionnaire) and participants were asked to complete the GSRS every seventh day (a total of 18 questionnaires were completed) throughout the entire study.

The GSRS is a 15-item questionnaire, with a one week recall period that asks individuals to report gastrointestinal symptoms using a seven-point Likert scale and can be reported as pooled data or as five clinical syndromes (reflux, abdominal pain, indigestion, diarrhea and constipation)\(^{(126)}\). The five syndromes are composed of a series of symptom questions that include: abdominal pain (abdominal pain, hunger pains and nausea), reflux syndrome (heartburn and acid regurgitation), diarrhea syndrome (diarrhea, loose stools and urgent need for defecation), indigestion syndrome
(stomach rumbling, abdominal distension, burping and increased gas/flatus) and constipation syndrome (constipation, hard stools and feeling of incomplete evacuation)\(^{(187; 188)}\).

Syndromes were created and formed based on clinical experience\(^{(125; 189)}\).

General population norm values are available for the GSRS\(^{(189)}\).

**SF-36v2 Health Survey**

At study appointments (every two weeks at the end of each period), participants were asked to complete the Optum™ SF-36v2 Health Survey a Quality of Life (QoL) and self-administered questionnaire. Participants completed the SF-36v2 Health Survey nine times throughout the study. The Optum™ SF-36v2 Health Survey is a general tool used to measure well-being and functional health in healthy and disease/condition specific adults 18 years of age and older. The SF-36v2 acute Health Survey is a 36-item questionnaire with an acute one week recall and is scored on eight health domains (physical functioning, role-physical, bodily pain, general health, vitality, social functioning, role-emotional and mental health)\(^{(190)}\). Summary scores are also reported for physical component summary (PCS) and mental component summary (MCS). Scores (originally reported on a 0 to 100 scale) are normalized to the 2009 U.S. population using a linear transformation with a mean score of 50 and a standard deviation of 10 (QualityMetric Health Outcomes™ Scoring Software 4.5).

**Groningen Frailty Indicator**

At study appointments (every two weeks at the end of each period), participants were also asked to complete the Groningen Frailty Indicator (GFI) in addition to the SF-36v2 Health Survey.
The GFI is a 15-item self-reported screening tool used to identify frailty, in both institutionalized and community-dwelling older adults\(^\text{(60)}\). The GFI measures frailty as a loss of function and represents four domains (physical, cognitive, social and psychological). Questions asked participants to report their ability to complete activities of daily living (shopping, walking, dressing, using the restroom), their level of physical activity (scale from 0 to 10), whether they reported difficulty with vision, hearing, unintentional weight loss, medication usage and memory. Using a four-point Likert scale (reported as never, sometimes, often, or all the time) participants were also asked about their beliefs regarding their social network, attention from others, and amount of assistance they require from others. A six-point Likert scale (reported as never, seldom, sometimes, often, very often, all the time) asked participants about psychological well-being (feeling of downhearted or sad and feeling calm and relaxed). Questionnaire responses were then scored and given a value of 0 or 1 by researchers according to the protocol and scoring guidelines outlined by Peters et al.\(^\text{(61)}\).

**Biological Measurements**

Primary endpoints included digestive health and the microbiota. To measure digestive health, gastrointestinal symptoms were captured with the GSRS and daily questionnaire (gastrointestinal symptoms, bowel movement frequency and transit time using the Bristol Stool Scale).

**Fecal Collection**

To explore digestive health, stool samples were collected during the last two-to-three days of every period (a total of nine stool samples were collected). Coolers and collection kits (plastic frame, plastic collection container with a lid, plastic bag and disposable pen) were provided and participants were asked to record their study
number, date and time on the lid of the collection container (Fisher Scientific Commode Collection System, Cat# 02-544-208). After collecting the sample, participants were asked to place the collection kit in a provided plastic bag on ice and call between 7:30 a.m. and 5 p.m. to arrange a pick-up of their sample. A 28-quart carrier cooler was provided to each participant for sample transportation so samples could be kept on ice. Participants were instructed to call the study phone and a member of the study team would arrange a time to pick up the sample from their home or another location -- whatever was most convenient for the participant. Once samples were picked up, they were transported to the Food Science and Human Nutrition department for processing. Samples were recorded (time of defecation and time of processing) and then homogenized. Once homogenized, samples were aliquoted for storage until future analysis and an additional aliquot was sampled for measuring pH. Aliquots for future analysis were stored at -80 °C until needed for future procedures.

**Stool pH**

An aliquot of fresh stool was sampled in a 15 mL conical tube and pH was then measured. Stool pH was measured by first weighing 1 g of stool (if the weight of the stool was not precisely 1 g, the volume of the NaCl was adjusted to yield a 1 to 10 dilution) into a 15 mL conical tube, 9 mL of NaCl (0.9%, or 0.155M) pH 7.00 and 10 glass beads to the tubes, sample was vortexed (Fisher Mini Vortexer 120V) for 1 minute or until the sample was homogenized (as much as possible, as fibrous pieces of plant material were usually present). Tubes were centrifuged (Thermo Scientific IEC CL31R) for 10 minutes (3700 x g), a pH measurement was taken of the supernatant using the accumet® basic AB15 pH Meter.
Microbial quantification

To prepare for deoxyribonucleic acid (DNA) extraction procedures, 5 mL labeled tubes with aliquoted stool samples were left in ice at 4 °C to thaw. Genomic DNA was extracted from homogenized, clinical fecal samples with the QIAamp® Fast DNA Stool Mini Kit (Qiagen, Cat# 51604) as per manufacturer’s protocol, with the following modifications: two 0.05 M sodium phosphate buffer washes prior to the addition of InhibitEX, and a 0.1 mm zirconia/silica bead beating step (~300 mg/tube, 4 m/s for 1 minute x 3) following incubation with InhibitEx. The eluted samples' DNA concentrations were read by a Nanodrop, and samples were stored at -20°C. Prior to qPCR analysis, the samples were diluted 5-fold.

The generation of the template DNA for standard curve formation was performed by spiking $10^9$ bacteria into ~250 mg of a clinical feces matrix. 1 g of lyophilized bacteria ($L.\ plantarum$ HA119, $B.\ bifidum$ R0071, or $L.\ helveticus$ R0052; Lallemand Health Solutions) was first dissolved in 99 ml HyClone Phosphate-Buffered Saline (PBS). Total bacterial cell counts of the resulting bacterial suspensions were determined by flow cytometry using the BD Accuri cytometer and SYTO® 24 labelling. Feces were spiked with a volume equivalent to $10^9$ bacteria, and then subjected to DNA extraction as described above. Feces without the addition of bacterial suspension were used as a negative control.

Quantification of $L.\ plantarum$ HA119, $B.\ bifidum$ HA132 and $L.\ acidophilus$ HA122 in clinical fecal samples was performed by real-time PCR (qPCR) using the ViiA™ 7 Real-Time PCR System (Thermo Fisher Scientific). Standard curves for HA119, HA132 and HA122 were generated from feces spiked with $10^9$ HA119, R0071,
and R0052, respectively. DNA recovered from spiked feces was serially diluted (10-fold) to generate template for the standard curve ranging from $10^8$ to $10^8$ bacteria. DNA samples to be quantified were diluted 5-fold in molecular biology grade water prior to qPCR.

The qPCR reaction mixture (25 μl) consisted of 300 nM of the appropriate primers, 1X SYBR® Select Master Mix (Thermo Fisher Scientific, Cat# 4472908) and 2.5 μl diluted DNA. Standard curve samples were run in duplicate and unknown samples were tested in triplicate. Cycling conditions consisted of initial incubations at 50°C and 95°C for 2 minutes each, followed by 40 cycles of 95°C for 15 s, 60°C for 30 s and 72°C for 30 s. A dissociation curve analysis (60°C to 95°C) was also performed to ensure amplification specificity of the primers.

**Microbial diversity**

Microbial diversity measured by denaturing gradient gel electrophoresis (DGGE) profiling (detects large distortions), qPCR (polymerase chain reaction) to quantify changes, and 16S rRNA sequencing (454) to identify treatment effects on specific bacteria was undertaken.

DNA was amplified using gene-specific primers for the V4 hypervariable region of the 16S rRNA gene and tagged with unique identifiers. The tagged amplicons were pooled and sequenced together on an Illumina MiSeq apparatus. The resulting reads were processed using a data analysis pipeline from the National Research Council (NRC) Canada\(^\text{(191)}\). An average of at least 20,000 reads of the V4 region per sample was obtained, QCed, paired-end assembled and clustered at 97% similarity. Resulting operational taxonomic units (OTUs) were assigned a taxonomic lineage using the Ribosomal Database Project (RDP) classifier\(^\text{(192)}\) with Greengenes (v13.5) training.
set\(^{(193)}\). Taxonomic classification and alpha/beta-diversities were computed using Qiime\(^{(194; 195)}\). Downstream analysis was done with in-house Perl and R scripts.

**Biological and Clinical Measurements**

Secondary endpoints included blood sample analysis, urine analysis for p-cresol, indoxyl sulfate and phenylacetyl glutamine. Also, measures of QoL (includes the SF-36v2 Health Survey and GFI) and measures of well-being (hand-grip strength and compliance) were undertaken.

**Venous Blood**

Fasting blood samples were collected at five time points by a licensed phlebotomist. Blood was collected at the end of baseline and each treatment period (treatment 1, treatment 2, treatment 3 and treatment 4). Participants were asked to fast for 12 hours (nothing to eat or drink except water and black coffee) prior to their study appointment the following morning. Blood was collected and Vista Laboratories was contracted to perform the following profiles: lipid, comprehensive metabolic profile (CMP) (includes blood urea nitrogen (BUN) and creatinine) and for C-reactive protein (CRP). Breakfast was provided to all participants following each blood draw and fasting appointment.

**Serum Collection and Analysis**

An additional tube of blood was collected for serum. The serum tubes were centrifuged for 10 minutes at 4 °C in a Hettich Instruments ROTINA 420R centrifuge at 800 x g (1,500 rpm) to collect the serum.

Serum (250 μl) was aliquoted and stored at -80 °C until assayed. After collection and storage, serum was analyzed for inflammatory markers (tumor necrotic factor
(TNF)-α, interleukin (IL)-6, and interleukin (IL)-10) at the Food Science and Human Nutrition department.

**TNF-α, IL-6 and IL-10:** (TNF)-α, interleukin (IL)-6 and interleukin (IL)-10 were measured using a human high-sensitivity T cell magnetic bead panel, 96-well plate assays. The Milliplex® Map Human High-Sensitivity T-Cell Panel Immunology Multiplex Assay Kit (Cat# HSTCMAG-28SK) was used and methods followed. Following an overnight (16-18 hour) incubation at 4 °C plates were read on a Luminex® xMAP Technology 200.

**Urine Analysis**

Analysis of 24-hour complete urine collections at five time points throughout the 18-week study (last two days of baseline and each treatment period) was conducted. Urine collection containers (Fisherbrand™ Low Form 24-Hour Urine Collection Containers; 3.5 L) and apparatus (Dover™ Commode Specimen Collector 800 mL) were provided and participants were asked to begin the collection the morning before their study appointment. Participant instructions were provided and discussed at consenting. Participant instructions for collecting a 24-hour urine specimen were also included on the container. The first urination of the day was not collected, however the date and time was recorded directly onto the specimen container. The second urination of the day was collected with the specimen collector and transferred into the container. Participants were asked to collect all the urine that was passed for the next 24 hours. At the end of the 24-hour period, participants recorded the time of their last collection. All specimen containers were kept on ice or in the refrigerator until they were delivered to study staff at the end of the 24-hour period.
Total volume was recorded, with starting time and date, and ending time and date, and collection containers were mixed thoroughly before being sampled. A volume of fresh sample was aliquoted and transferred to a sterile urine specimen container. The time, date and total volume of the 24-hour collection was recorded on the container. Once labeled and recorded, the urine specimen containers were delivered to the nearest drop-off location for Vista Laboratories for analysis of urea nitrogen and 24-hour urea nitrogen. Additional aliquots of the 24-hour collection were taken and stored at −80 °C until analysis of p-cresol, phenylacetyl glutamine and indoxyl sulfate was conducted\(^{196;197}\).

**Urinary metabolites:** Metabolites measures included: cresol sulfate (ug/mL), phenylacetyl glutamine (ug/mL), and indoxyl sulfate (ug/mL). P-cresol is not formed by human enzymes and is a metabolite of tyrosine. It is a bacterial metabolite, absorbed from the colon and is excreted in urine. The use of p–cresol as a marker can be used to quantify proteolytic activity in the colon\(^ {121}\). A metabolite of tryptophan, indoxyl sulfate, is also a uremic solute normally excreted by the kidneys. The conjugation of phenylacetate and glutamine produces the uremic solute phenylacetyl glutamine.

The method of quantification of metabolites was adapted from Cuoghi et al.\(^ {198}\). All solvents used were of the highest available grade and purchased from Fisher Scientific (Waltham, MA, USA). P-cresol sulfate and indoxyl sulfate were purchased from Alsachim (Illkirch-Graffenstaden, France) and phenylacetyl glutamine was purchased from LGC (Middlesex, UK). P-Toluene sulfonic acid sodium salt was sourced from Sigma Aldrich (St. Louis, MO, USA). Formic acid was LC/MS grade and purchased from Fisher Scientific.
Extraction of p-cresol sulfate, indoxyl sulfate and phenylacetyl glutamine from urine was conducted using the following methods. Urine samples were stored at -80 °C until analysis. Twenty microliters of the urine sample were mixed with 5 µL p-toluene sulfonic acid (200 µg/mL, as internal standard) and followed by addition of 975 µL acetonitrile for protein precipitation. After shaking for two minutes, the mixture was centrifuged at 20,000 g at 4 °C for five minutes. After centrifugation, 500 µL supernatant is diluted with 500 µL 0.5% formic acid aqueous solution and then filtered by a 0.22 µm nylon filter prior to LC-MS analysis.

Preparation of the standard calibration curves was conducted using the methods described below. Before preparing the calibration curve, the stock solutions were prepared by dissolving p-cresol sulfate, indoxyl sulfate and phenylacetyl glutamine in water at a concentration of 10 mg/mL. The working solutions of p-cresol sulfate and phenylacetyl glutamine were prepared at concentrations of 5000, 2500, 1000, 500, 250, 100, 50, 25 and 10 µg/mL, respectively, by dilution of stock solutions with water. For indoxyl sulfate, the working solutions were prepared at concentrations of 1000, 500, 250, 100, 20, 25 and 10 µg/mL, respectively. The calibration curve was prepared in artificial urine (Aldon, Avon, NY, USA). Five microliters of internal standard and 5 µL working solutions of p-cresol sulfate, indoxyl sulfate and phenylacetyl glutamine were added into 20 µL artificial urine and then mixed with 960 µL acetonitrile. After centrifugation at 20,000 g at 4 °C for five minutes, 500 µL supernatant was diluted with 500 µL 0.5% formic acid aqueous solution prior to LC-MS analysis. Linear regression for each calibration curve was achieved by plotting the peak area ratio of the target compound/internal standard versus the concentration of target compound added. The
calibration curves of target compounds showed good linearity and the $r^2$ for $p$-cresol sulfate, phenylacetyl glutamine and indoxyl sulfate were 0.9980, 0.9943 and 0.9985, respectively.

$P$-cresol sulfate, indoxyl sulfate and phenylacetyl glutamine in urine was quantified using the following methods. A Thermo Ultimate 3000 HPLC was equipped with a Thermo Quantiva triple quadrupole electrospray ionization tandem mass spectrometer (Thermo, Waltham, MA, USA). Chromatographic separations were performed using a Acquity BEH C18 column (1.7 µm, 2.1 x 100 mm) (Waters, Milford, MA, USA) with a mobile phase consisting of 0.1% formic acid aqueous solution (A) and 0.1% formic acid in acetonitrile (B). The gradient program was set as follows: 0-3 min, 50% B, 3-3.5 min, ramped to 95% B; 3.5-6 min, 95% B; 6-6.1 min, 50% B; 6.1-9 min, 50% B. The flow rate was set at 0.2 ml/min. The column temperature was maintained at 25 °C. The injection volume was 10 µL. Multiple reaction monitoring (MRM) in negative mode was employed for quantification at m/z 171 → 80 for $p$-toluene sulfonic acid; m/z 187 → 107 for $p$-cresol sulfate and m/z 212 → 80 for indoxyl sulfate while m/z 265 → 130 for phenylacetyl glutamine in positive mode. The spray voltage in positive and negative modes were set at 3,500 and 2,500 V, respectively. Other MS parameters were as follows: sheath gas, 45 Arb; Aux gas, 15 Arb, Sweep gas, 1 Arb; CID gas, 2 mTorr, Ion transfer tube temp: 325 °C, Vaporizer temp: 300 °C.

**Measures of Well-being**

Measures of well-being included handgrip strength (screening tool for assessing risk of malnutrition and as an indicator of muscle strength) and compliance (recorded intake of the provided diet and supplements). Participants were also asked to report
their compliance and intake of study food, prebiotic study food/placebo and probiotic capsule/placebo on the daily questionnaire, acceptability of the diet, antibiotic use, doctors’ visits -- all assessed on the daily questionnaire.

**Handgrip Strength**

Handgrip strength was assessed at study appointments (at the end of each period; assessed a total of nine times throughout the study) as a screening tool for nutritional risk/status\(^{(199;200)}\) using the Jamar® Plus+ digital dynamometer (Patterson Medical, Warrenville, IL).

The method described by Flood et al. was adapted in this study to assess handgrip strength\(^{(54)}\). One at a time while being seated, study staff asked participants to bend their arms to a 90-degree angle. Participants were then prompted to squeeze the dynamometer as hard as they could for three seconds. The measurement was recorded in kg and the same process was conducted in duplicate steps, while alternating hands after each measurement. A total of four handgrip strength measurements were recorded, and the participants’ dominant hand as well as any injuries, surgeries or medical conditions (i.e. arthritis) that may affect the readings were documented.

**Bioelectrical Impedance Analysis**

To estimate body composition BIA was used, a widely used method in clinical settings\(^{(201;202)}\). BIA was measured two times throughout the study, on the day of randomization and at the final study appointment using the HYDRA ECF/ICF Bio-Impedance Spectrum Analyzer (XITRON® Technologies, Model 4200).

Before the BIA was performed, participants were asked to fast overnight and abstain from any exercise prior to testing. On the day of testing, the participant was
directed to remove any metal jewelry and lay in a supine position with arms and legs separated from the body, with no limbs touching. Four electrodes were placed on the right hand and foot (ankle, foot, wrist and hand). Extracellular fluid (ECF) in Liters, total body water (TBW) in Liters, intracellular fluid (I) in Liters and fat-free mass (FFM) in kg was recorded -- fat and percent fat then being calculated. Fat was calculated as the difference of weight (in kg) and FFM. Percent fat was calculated at fat divided by weight (in kg) multiplied by 100.

**Statistical Analysis**

All data was analyzed on an intent-to-treat and compliance basis. Unless noted otherwise, data representing the least square means ± SEM and significance is denoted at a P-value <0.05.

**Microbiota**

Statistical methods were adapted from Tremblay et al.\(^{191}\). Alpha-diversity was statistically analyzed for the gut microbiota using t/test and Wilcox test. Metrics corresponding to diversity and taxonomic classification were calculated using QIIME software suite.

Stool pH was analyzed with Tukey-Kramer groupings for treatment least squares means. Statistical analysis of stool pH used measured pH from stool samples collected at baseline and at the end of each treatment period.

**Body Weight and Bioelectrical Impedance Analysis (BIA)**

For body weight, equivalence testing was performed. A paired t-test of body weight at baseline vs. washout 4 (final study visit) was used with a tolerance level set at 1 kg. Only those participants with both measurements (baseline and washout 4) were included in the analysis.
BIA was analyzed with a paired t-test (baseline vs. washout 4). Only those participants with both measurements (baseline and washout 4) were included in the analysis. Analysis included: extracellular fluid (L), intracellular fluid (L), total body water, fat free mass (kg), fat (kg) and percent fat.

**Daily Questionnaire Analysis**

Daily questionnaires evaluated a variety of general wellness symptoms (Table 3-1). Symptoms were rated on a scale of 0 (no symptoms present) to 6 (very severe). The mean of each symptom was calculated by period (two weeks in length) for each participant. Period means for each corresponding symptom was grouped to determine the mean syndrome score for daily questionnaire syndromes (gastrointestinal distress, psychological health, cephalic and emetic) and individual syndromes (diarrhea, constipation, fatigue and hunger). Individual syndromes consist of symptoms collected on the daily questionnaire that could not be grouped with other symptoms and were consequently analyzed individually. Symptoms that could not be grouped into a syndrome were evaluated individually.

Syndromes were unable to be analyzed using parametric analysis procedures due to the large number or zeroes (indicating no symptoms present)/too few non-zeroes reported by participants. Individual symptoms that could not be subsequently analyzed included, diarrhea and constipation, and syndromes that could not be analyzed included, psychological health, cephalic and emetic.

Non-parametric analysis, including REML variance component, estimates and least squares means difference Tukey HSD was used for gastrointestinal distress, diarrhea and constipation.
Bowel movement frequency and Bristol Stool Form Scale category (slow transit - types 1 and 2, normal transit- types 3 to 5 and fast transit- types 6 and 7) were collected in the daily questionnaire and were analyzed with a random effect of subject included in the model. Each period (treatment or washout) was two weeks in length. The results are reported for the entire treatment period, not each individual week within those two-week periods. Means of daily bowel movement frequency for each two-week period of each treatment were analyzed. Treatment least squares means, the estimated mean value per day (multiple the estimate and the standard error by 7 for weekly totals and SE of total), were calculated. BSS category (constipation, regular or diarrhea) due to treatment – probability of individual BM for each two-week period of each treatment. These are predicted probability of an individual BM being in the listed category. Transit time, measured by the Bristol Stool Form Scale, was also analyzed as means of each two-week period of each treatment.

Sleep was compared using a quasi-binomial distribution of daily category modeling using mean daily proportion of people with sleep <7 hrs. Sleep categories were grouped to include <7 or ≥7.

**Gastrointestinal Symptom Rating Scale**

The 15-item GSRS evaluated five scales including: abdominal pain, reflux syndrome, diarrhea syndrome, indigestion syndrome and constipation syndrome (Table 3-2). Preliminary analysis determined log transformation was appropriate, that sequence did not have an effect on syndromes (except for constipation syndrome), week had no effect, and there was no washout/carryover effect. All washouts were combined because there were no statistical differences among the four washout means.
The model used for analysis was a general linear mixed model with a random subject effect and a fixed effect of treatment. Differences of treatment least square means were reported. For log constipation, an effect of sequence and its interaction with treatment was added. No interaction was noted and the main effect still present. A pairwise comparison of the sequences was then conducted. Analysis suggest that the DCBA sequence group started out higher even during baseline than the other three groups. As a result, DCBA tends to have higher means throughout the entire study for the GSRS.

**Inflammatory Markers - serum**

The Milliplex® Map Human High-Sensitivity T-Cell Panel Immunology Multiplex Assay Kit was used to measure pro-inflammatory (IL-6 and TNF-α) and anti-inflammatory (IL-10) cytokines. The assay kit was read on a Luminex® xMAP Technology 200. Pro-inflammatory cytokines, IL-6 and TNF-α were measured in the serum and for statistical analysis a log transformation was performed. Data was analyzed utilizing a fixed effect of treatment model. For IL-6 a measure of <0.04 was recorded as 0.04 and <0.14 was recorded as 0.14 for statistical analysis.

Data for the anti-inflammatory cytokine, IL-10 was analyzed using a fixed effect of treatment model. For IL-10 a measure of <0.43 was recorded as 0.43 and <1.33 was recorded as 1.33 for statistical analysis.

**Laboratory Analysis – serum**

Blood urea nitrogen, creatinine, liver enzymes (alanine transaminase, alanine aminotransferase, and alkaline phosphatase), total protein, albumin and c-reactive protein were analyzed using a general linear mixed model with a random subject effect.
and a fixed effect of sequence and time point. Differences of treatment least square means were reported.

C-reactive protein <0.3 was set to 0.15, however there were too many non-detectable values for analysis. An indicator variable was then used for above and below detection values and an analysis using only values above the detection limit but neither analysis would converge.

A time effect did occur with total protein and albumin, however a sequence effect was not seen. Thus, both were reanalyzed with treatment effect.

For total cholesterol and triglycerides, a generalized linear-mixed model was fitted with intervention as fixed effects and a random subject effect (to include any correlation among repeated observations).

**Laboratory Analysis – urine**

Twenty-four-hour nitrogen was analyzed with a generalized linear-mixed model was fitted with intervention as fixed effects and a random subject effect (to include any correlation among repeated observations).

The urinary metabolites (cresol sulfate, phenylacetyl glutamine and indoxyl sulfate) were analyzed using two models, both using log-transformed metabolite values. The first examined whether there was a sequence or time effect. The second model is the effect of treatment on the metabolite. A random effect of subject was included due to the multiple measurements on the same individual and heterogeneous variance (varies by treatment) was incorporated in the model. In all cases, different treatments had different variances. Pairwise comparisons of each treatment against baseline using Dunnett’s correction for multiple comparisons and all possible pairwise tests of treatments using Tukey’s method to control for multiple comparisons were performed.
Handgrip Strength

For analysis, the maximum handgrip strength measure recorded in either hand was used. A paired t-test of maximum handgrip at baseline vs. washout 4 was conducted to analyze handgrip strength. Only participants with a baseline and washout 4 measurement were included in analysis. No handgrip strength measurements were excluded based on surgeries or rheumatoid arthritis.

GFI and QoL

The GFI questionnaire was scored and distribution of total GFI scores over all days of the study was analyzed by treatment. A binomial model with a random effect of subject (accounting for repeated observations on each participant).

Data from the SF-36v2 Health Survey, a QoL tool, was entered and scored using the QualityMetric Health Outcomes™ Scoring Software 4.5. Scores were standardized to norm values (scoring software performs linear transformation of data; original data scale 0-100) for comparison purposes. Norm-based scores for each domain has a mean of 50 (standard deviation of 10) set to the 2009 U.S. population (most recent norm set available for comparison). The scored norm-based data was analyzed with a generalized linear-mixed model was fitted with intervention as fixed effects and a random subject effect (to include any correlation among repeated observations).

Compliance

Participant compliance was determined as adherence to the stool protocol, supplement protocol (probiotic and prebiotic) and daily questionnaires. To measure adherence to the stool protocol, compliance was determined by summing the number of stool samples received (out of nine total) and calculating the percentage. Questionnaire compliance was measured as adherence to the daily questionnaire and was included if
any questions were completed for the daily questionnaire on a given day (a total of 126 daily questionnaires were released during the study). Supplement compliance was measured with the questionnaire. Participants were asked to indicate whether they consumed the probiotic capsule and fiber sachet with a “Yes” or “No” response daily. Compliance was assessed by calculating non-compliance as the number of times “No” was indicated during the treatment periods. If a participant withdrew from the study, compliance was measured as their percent compliance up until the time of their withdrawal. If the question regarding probiotic or fiber compliance was unanswered, a “No” was assumed indicating the capsule or sachet was not consumed for the specified day.
Figure 3-1. Study design for the 18-week randomized, double-blinded, crossover study. A Latin squares randomization design was used and participants were randomized to one of four sequences: ABCD, BDAC, CADB, DCBA. Each treatment period consisted of a standard high-protein diet that was provided during each treatment period. Treatment periods were as follows: treatment A (inulin+placebo), treatment B (inulin +probiotic), treatment C (control+placebo) and treatment D (control+probiotic).
Table 3-1. Daily Questionnaire syndrome components

<table>
<thead>
<tr>
<th>Gastrointestinal Distress (GID)</th>
<th>Psychological Health (PSY)</th>
<th>Cephalic (CEP)</th>
<th>Emetic (EM)</th>
<th>Individual Syndromes</th>
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<tr>
<td>Bloating</td>
<td>Anxious</td>
<td>Headaches</td>
<td>Nausea</td>
<td>Diarrhea</td>
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<tr>
<td>Flatulence</td>
<td>Depressed</td>
<td>Dizziness</td>
<td>Vomiting</td>
<td>Constipation</td>
</tr>
<tr>
<td>Cramping</td>
<td>Stressed</td>
<td></td>
<td>Fatigue</td>
<td>Hunger</td>
</tr>
<tr>
<td>Abdominal noises</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Abdominal Pain</td>
<td>Reflux Syndrome</td>
<td>Diarrhea Syndrome</td>
<td>Indigestion Syndrome</td>
<td>Constipation Syndrome</td>
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<td>----------------</td>
<td>-----------------</td>
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<td>----------------------</td>
<td>----------------------</td>
</tr>
<tr>
<td>Hunger Pain</td>
<td>Heartburn</td>
<td>Burping</td>
<td>Constipation</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Acid Reflux</td>
<td>Abdominal Distention</td>
<td>Hard Stools</td>
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<tr>
<td>Nausea</td>
<td>Urgency of Defecation</td>
<td>Rumbling</td>
<td>Incomplete Evacuation</td>
<td></td>
</tr>
</tbody>
</table>
CHAPTER 4
RESULTS

Twenty-nine participants were consented and initially assessed for eligibility. The study flow diagram demonstrating the recruitment, allocation and analysis is depicted in Figure 4-1. Of those consented, one reported no longer meeting the inclusion criteria and two declined to participate. Twenty-six participants were randomized and five withdrew during the study intervention. Twenty-five participants completed at least one of the diet interventions and 21 completed the entire study. No serious adverse events (as defined by the IRB-01 evaluation and reporting guide) were reported during the study. All participants were female and their age ranged from 65 to 87 years old (73.7±5.6 y) at the time of the screening visit (Table 4-1). Participants’ race and ethnicity were self-reported and recorded (Table 4-1). Participant compliance measured as adherence to the stool, supplement and questionnaire is also reported in Table 4-1.

Diet Composition

The energy needs of participants was measured by SenseWear Pro Armband (BodyMedia, Inc.). Participants wore armbands during the baseline periods and the estimated mean energy requirement for weight maintenance was 1838.9±38.9 kcal/d. The high-protein controlled diet was adjusted for participants individually based on their energy requirements indicated by accelerometer data and was adjusted to increase protein intake and maintain the AMDR distribution. The mean intake provided to participants during the treatment periods, after adjusting the base diet (provided 1616±20 kcal/d) was 1792±36 kcal/d (Table 4-2). The provided intake was within 50 kcal/d of their estimated energy requirement. Complete reporting of the foods and the
diet composition provided during the high-protein treatment periods is reported in Supplementary Tables located in Appendix C.

The high-protein diet provided to participants during the controlled periods, as a percent of total energy intake was 29.8% protein, 46.3% carbohydrates and 26.8% fat. The high-protein controlled diet provided a mean of 121.3±1.2 g protein/d. All participants received 1.5-2.2 g protein per kilogram body weight (1.8±0.04 g protein/kg body weight). Percent estimates were within the AMDR ranges for macronutrients.

**Anthropometrics**

**Body Mass Index and Weight**

The body mass index (BMI) of all participants during screening were categorized as either normal weight (46.2%) or overweight (53.8%) according to the World Health Organization (WHO) classifications (Table 4-1).

Participant weight throughout the study was evaluated using equivalence (tolerance = 1 kg) paired t-test of body weight at baseline vs. washout 4 (end of study). Only participants with both measurements were included in equivalence analysis (n=22; PAM 06, 08, 13, 20 and 28 not included in analysis because washout 4, weight not taken) (Figure 4-2). Participant weight was demonstrated as statistically equivalent at a tolerance level of 1 kg (2.2 lbs).

**Bioelectrical Impedance Analysis**

Analyzing BIA using a 1-tailed paired t-test there was a significant increase in lean/fat-free mass at the study completion (end of 18-week intermittent high-protein intervention) compared to randomization/baseline (p=0.027). A 2.1 kg increase in lean mass was observed at the final visit compared to baseline. Additionally, a trend (defined as a p-value <0.10) was observed for both measured fat (p=0.050) and percent (%) fat
The trend demonstrated a decrease in fat and percent fat from baseline to study end. A 5% increase in lean mass (fat-free mass) in kg was measured at the final visit compared to participant measurements at randomization. Additionally, a 7% decrease in fat mass in kg was measured at the final visit compared to participant measurements at randomization (Figure 4-3). Intracellular fluid (p=0.025) and total body water (p=0.030) also significantly increased compared to baseline (Table 4-3).

**Microbiota Profile**

No phyla level changes were observed with treatments compared to baseline (Figure 4-4). However, high intra individual variations in genera and significant changes in operational taxonomic unites (OTUs) were observed with treatments. Taxonomy at the Genus level for all samples and their relative abundance was reported for the 20 most abundant taxons across all samples (baseline, treatments and washout) (Figure 4-5). The taxon in highest abundance was Blautia followed by the family Lanhcospiraceae and Coprococcus. Some OTUs were statistically differentially abundant (important to note that sample size for each OTU was low). Some DAOs (Differentially Abundant OTUs) are statistically significant.

Principle Coordinate (PCo) analysis describes the evolution of the microbiome of each individual across their visits (time points; includes baseline, all treatments and washout), measured by clustering of enterotypes (Figure 4-6). The different clustering demonstrates that some individuals have either an unstable microbiome or that their microbiome is sensitive to the given treatment.

To look at changes in global microbiota, alpha diversity was measured. Diversity was not statistically significant from treatment periods (A, B, C, and D) compared to
baseline and washouts (Figure 4-7). Diversity was also not significant when comparing all treatment periods (all protein interventions) to washout and baseline (Figure 4-8).

To examine any differences among the treatment sequences, the twenty most abundant OTUs reported as relative abundance were plotted for the 4 treatment sequences (ABCD, CADB, BDAC and DCBA) comparing baseline, treatments and washouts (Figure 4-9). Core microbiome seems to be consistent across approximately 60% of microbiota and the other 40% unique to individual. The twenty most abundant OTUs were reported as relative abundance plots for the 4 treatment sequences (ABCD, CADB, BDAC and DCBA) and compared baseline, treatment periods (at time points; treatment 1, 2, 3 and 4) and washout periods (Figure 4-10). Figure 4-11 shows the twenty most abundant OTUs reported as relative abundance plotted for the four treatment sequences and comparing relative OTUs at treatment periods (A, B, C and D). No statistical difference was reported. The relative abundance of specific taxon (Bifidobacterium, Lactobacillus and Clostridium) compared at baseline, treatment period (A, B, C and D) and washout is shown in Figure 4-12.

Survival of strains was shown using qPCR. Quantification of L. plantarum HA119, B. bifidum HA132 and L. acidophilus HA122 in stool samples collected at the end of each period (baseline, treatment and washouts) is shown in Figure 4-13. A carryover of strains (assessed by quantifying strains) is observed in some participants during the washout periods.

**Self-reported Gastrointestinal Wellness**

**Daily Questionnaire Analysis**

Paper questionnaire booklets were distributed to participants during each study period and collected at the end of that period. Participants’ questionnaire responses
were analyzed as syndromes (individual symptom scores grouped) or individual symptoms. Individual symptoms, not incorporated into a syndrome, were also analyzed. Syndromes included: gastrointestinal distress (syndrome included individual symptom questions referencing bloating, flatulence, cramping and abdominal noises), psychological health (anxiety, depression, stress), cephalic (headaches and dizziness) and emetic (nausea and vomiting). Symptoms reported individually include diarrhea, constipation, fatigue and hunger.

Of the symptoms that were reported individually, constipation and diarrhea could not be statistically analyzed. Descriptive statistics of individual and component syndromes for the daily questionnaire are reported in the Supplementary Tables located in Appendix D. The data set contained 97.5% zeroes (0=none on severity scale of 0 to 6) for diarrhea, 86% for constipation (individual syndromes), thus they could not be statistically analyzed (Table 4-4). Fatigue and hunger, both individual symptoms, are presented in Table 4-5. The mean proportion of non-zero values/responses reported for fatigue by participants during each period/treatment (14 day periods) were not significantly different (p=0.286). For the self-reported symptom of fatigue, the mean of the non-zero values reported by participants by period/treatment was also not significantly different (p=0.415) (Table 4-5). There were significant differences observed for hunger when comparing the mean hunger among participants by period/treatment (p<0.0001) (Figure 4-14). Hunger was significantly lower during every treatment and washout period compared to baseline.

Component syndrome scores for psychological health, cephalic and emetic were also unable to be analyzed statistically because of the large number of zeroes reported
by participants in the data set. Gastrointestinal distress (syndrome includes sum of
symptom scored for bloating, flatulence, abdominal cramping and abdominal noises)
was analyzed and significant differences were observed when looking at the mean
gastrointestinal distress among participants by period/treatment (p<0.0001) (Table 4-5).
Gastrointestinal distress was significantly higher during treatment A (inulin+placebo)
and treatment B (inulin+probiotic) compared to baseline.

Sleep was analyzed by grouping responses into two sleep categories (sleep
categories <7 or ≥7). The mean daily proportion of participants reporting sleep <7 hrs
was modeled using a quasi-binomial distribution. Treatment did not significantly affect
sleep category means (p=0.433) (Table 4-6).

**Bowel Movement Frequency and Transit**

Mean bowel movement frequency was self-reported daily and was analyzed as
the entire two-week treatment and washout periods. Mean bowel movement frequency
was 1.38±0.10 at baseline with no effect of treatment or washout period (p=0.999)
(Table 4-7).

Bristol Stool Scale was also reported daily for each bowel movement and
analyzed by grouping responses in categories based on transit time (slow transit - types
1 and 2, normal transit- types 3 to 5 and fast transit- types 6 and 7). Data is reported for
each period and the predicted probabilities of each Bristol Stool Form Scale category
did not significantly differ based on treatment (p=0.184) (Figure 4-15). Transit time,
measured by the Bristol Stool Scale, was also analyzed as means of daily Bristol Stool
Form Scale for each two-week period of each treatment and no significant treatment
effect was observed (p=0.892) (Table 4-7).
Stool pH was statistically higher compared to baseline when participants consumed the high-protein diet. This statistically significant increase was also observed during treatment A (inulin+placebo) and treatment D (control+probiotic). However, the pH was not statistically different from baseline when participants received treatment B (inulin+probiotic) (Figure 4-16).

**Gastrointestinal Symptom Rating Scale Analysis**

Gastrointestinal symptom domains measured by the Gastrointestinal Symptom Rating Scale (GSRS) were statistically analyzed. A significant treatment effect was observed for abdominal pain (p=0.011) and indigestion syndrome (p<.0001). Overall, a fixed effects model for treatment did not demonstrate any statistical differences between treatment groups for reflux (p=0.154), constipation (p=0.116) and diarrhea (p=0.107) syndromes (Table 4-8).

Gastrointestinal symptom scores were significantly higher for abdominal pain when participants received treatment A (inulin+placebo) (p=0.046), treatment B (inulin +probiotic) (p=0.009), and treatment C (control+placebo) (p=0.014) but not treatment D (control+probiotic) (p=0.697) compared to baseline+washout (Table 4-8). Compared to baseline, Treatments A (p=0.380), treatment B (p=0.138), treatment C (p=0.186), treatment D (p=0.9995) and washout periods (p=0.999) were not statistically different (Table 4-9).

Indigestion syndrome was significantly higher when participants received treatment A (inulin+placebo) (p=0.0001), treatment B (inulin+probiotic) (p<.0001) and treatment D (p=0.033) but not treatment C (control+placebo) (p=0.242) compared to baseline+washout (Table 4-8). Compared to baseline, Treatments A (inulin+placebo)
(p=0.0029) and B (inulin +probiotic) (p<.0001) were statistically higher for symptoms composing indigestion syndrome (Table 4-9).

Gastrointestinal symptom domains measured by the Gastrointestinal Symptom Rating Scale (GSRS) were also analyzed for treatment effects compared to the washout periods (Table 4-10). A significant treatment effect was observed for abdominal pain (p=0.01) and indigestion syndrome (p<.0001). Gastrointestinal symptom scores were significantly higher for abdominal pain when participants received treatment B (inulin +probiotic) (p=0.027) and treatment C (control+placebo) (p=0.043) but not treatment A (inulin+placebo) (p=0.148) or treatment D (control+probiotic) (p=0.990) compared to washout (Table 4-10). Symptom scores for indigestion were higher for treatments when inulin was received, treatment A (inulin+placebo) (p=0.001) and treatment B (inulin+probiotic) (p<.0001) compared to washout.

**Laboratory Analysis**

**Blood Analysis**

There was no significant sequence (ABCD, BDAC, CADB, DCBA), time or sequence*time effect for cholesterol or triglycerides. There was not an observed treatment effect for cholesterol (p=0.732) or triglycerides (p=0.854) (Table 4-11). Total protein and albumin had significant effects for time but not sequence thus they were reanalyzed for treatment effects. No treatment effect was found for total protein (p=0.871). A trend for albumin (p=0.051) was reported for treatment, however no treatment periods were statistically different from baseline (Table 4-11).

There was no significant statistical difference in sequence, time or sequence*time effect in liver function tests (alanine transaminase, alanine aminotransferase, and alkaline phosphatase) thus treatment effect was not evaluated.
There was a significant treatment effect for blood urea nitrogen (BUN) \((p<.0001)\). BUN was significantly elevated for all treatment periods (inulin+placebo \(p<0.0001\), inulin+probiotic \(p<0.0001\), control+placebo \(p<0.0001\) and control+probiotic \(p<0.0001\)) compared to baseline (Table 4-11). Baseline characteristics of participants are reported as arithmetic means in Table 4-12 and include lipid panel, comprehensive metabolic panel, liver enzymes and CRP measured at baseline and the end of each treatment period.

**Immune Function**

Laboratory results for C-reactive protein had too many non-detects in the data set thus, results could not be statistically analyzed (C-reactive <0.3 was set to 0.15 for analysis).

Analysis of pro-inflammatory cytokines, IL-6 \((p=0.461)\) and TNF- \(\alpha\) \((0.275)\) showed no statistical differences between baseline and treatment periods (Figure 4-17).

Additionally, there were no statistical differences \((p=0.862)\) between baseline and treatments in IL-10, an anti-inflammatory cytokine (Figure 4-18).

**Urinary Analysis**

A significant treatment effect was observed for 24-hour nitrogen \((p<.0001)\). Compared to baseline all treatments (inulin+placebo \(p<0.0001\), inulin+probiotic \(p<0.0001\), control+placebo \(p<0.0001\) and control+probiotic \(p<0.0001\)) were statistically higher compared to baseline (Table 4-13).

For the urinary metabolite, p-cresyl sulfate a sequence \((p=0.046)\), day \((p=0.233)\) and sequence*day \((p=0.626)\) are reported. A treatment effect for p-cresyl sulfate was not significant \((p=0.314)\). For indoxyl sulfate a sequence \((p=0.398)\), day \((p=0.023)\) and sequence*day \((p=0.375)\) are reported. A treatment effect for indoxyl sulfate was not
significant but a trend was reported (p=0.053). For phenylacetyl glutamine a sequence (p=0.176), day (p=0.005) and sequence*day (p=0.623) are reported. A treatment effect for phenylacetyl glutamine was significant (p=0.022). An effect of day was observed for both phenylacetyl glutamine and indoxyl sulfate, however no effect of sequence*day was observed.

Phenylacetyl glutamine was significantly increased during the high-protein treatment (treatment C control+placebo p=0.019) and all other treatment periods (treatment A inulin+placebo p=0.024, treatment B inulin+probiotic p=0.021, and treatment D control+probiotic p=0.013) compared to baseline (Table 4-11). Periods when probiotic, prebiotic or synbiotic were supplemented to the high protein diet did not mitigate the measured increased in phenylacetyl glutamine (Figure 4-19).

**Handgrip Strength**

For handgrip strength, no statistical difference was observed between baseline and washout 4 (end of study). Only those participants with both measurements were included in the analysis and results were not significant (p= 0.503) (PAM 06, 08, 13, 20 and 28 not included in data set both values were not recorded). Handgrip strength using the maximum single measurement (in kg), maximum single measurement from the dominant hand (in kg) and maximum measurement from the non-dominant hand (in kg) was also assessed at baseline, the end of all treatment and the final visit for any differences (Table 4-14). Handgrip strength was measured at the end of baseline, all treatment and washout periods, however washout is not reported in Table 4-14.

**Groningen Frailty Indicator and Quality of Life Measures**

The Groningen Frailty Indicator (GFI) was completed by participants at the end of all periods. When looking at risk of frailty, no significant differences were observed
based on treatment period (p=0.851). Twenty-two participants were included in the analysis and 4 participants (PAM 08, 18, 20 and 29) were removed from the analysis due to little/no variation in reporting.

Health related quality of life measures were collected using the SF-36v2 at the end of baseline and each treatment and washout period. There were no statistical differences among any SF-36v2 domains (eight domains measuring physical functioning, role-physical, bodily pain, general health, vitality, social functioning, role-emotional and mental health), MCS, or PCS between baseline, intervention/treatment and washout periods. Graphical depiction using a radar plot (Figure 4-20) demonstrates no statistical differences between baseline, washouts and treatment periods.
Figure 4-1. Flow diagram of participant recruitment, allocation and analysis.
Figure 4-2. Equivalence testing using paired profiles for participant body weight (kg). Data was analyzed using a paired t-test of body weight at baseline vs. washout 4 (end of study). A threshold of 1 kg (2.2 lbs.) was used for equivalence testing. Participants weight was concluded equivalent at the indicated threshold. Only participants with both measures were included in statistical analysis (n=22).
Figure 4-3. Effect of 18-week intermittent high protein diet on body composition. Mean Bioelectrical Impedance Analysis (BIA) measurements and weight (kg) taken at randomization (study start) and again at the end of the study. Measurements included lean mass, fat mass, and total body weight. A 1-tail, paired t-test was performed for total body weight (kg) (p=0.316), lean mass (kg) (p=0.027) and fat mass (kg) (p=0.050).
Figure 4-4. Heat map cluster analysis, 16S Microbiome. Analysis shows all participants and data from all stool samples collected (baseline, treatment periods and washout periods; nine stool samples in total).
Figure 4-5. Taxonomy at the Genus level for all samples - twenty most abundant taxon/operational taxonomic units (OTUs). Abundance is reported for taxon.
Figure 4-6. Investigation of enterotype clustering, 16S microbiota. Principal Coordinate (PCo) analysis of microbiota for each participant at baseline (left). Principal Coordinate (PCo) analysis of microbiota for each participant at baseline, treatment A (inulin + placebo), treatment B inulin + probiotic), treatment C (control + placebo) and treatment D (control + probiotic) (right).
Figure 4-7. Alpha diversity boxplot of treatment periods (A, B, C and D) compared to baseline and washout. Treatment periods were as follows: A (inulin+placebo), B (inulin +probiotic), C (control+placebo) and D (control+probiotic). No statistical difference was observed.
Figure 4-8. Alpha diversity boxplot of treatment period (all protein interventions; treatment A, B, C and D combined) compared to washout and baseline. Treatment periods were as follows: A (inulin+placebo), B (inulin+probiotic), C (control+placebo) and D (control+probiotic). No statistical significant difference was observed.
Figure 4-9. Twenty most abundant Operational taxonomic units (OTUs) reported as relative abundance for baseline, treatment and washout. Plots show the four treatment sequences (ABCD, CADB, BDAC and DCBA) and compare baseline, treatment and washout. The four treatment sequences compare relative OTUs at treatment period (all protein interventions) compared to washout and baseline. Plots show the four treatment sequences (ABCD, CADB, BDAC and DCBA). Treatment periods were as follows: A (inulin+placebo), B (inulin +probiotic), C (control+placebo) and D (control+probiotic). Taxon key shows least abundant (top) to most abundant (bottom). No statistical significant difference was observed.
Figure 4-10. Twenty most abundant Operational taxonomic units (OTUs) reported as relative abundance for baseline, Tx1, Tx2, Tx3, Tx 4 and washout. Plots show the four treatment sequences and compare relative OTUs at treatment period (A, B, C and D) compared to washout and baseline. Treatment sequences include ABCD, CADB, BDAC and DCBA. Treatment periods were as follows: A (inulin-placebo), B (inulin +probiotic), C (control-placebo) and D (control+probiotic). Taxon key shows least abundant (top) to most abundant (bottom). No statistical significant difference was observed.
Figure 4-11. Twenty most abundant Operational taxonomic units (OTUs) reported as relative abundance. Plots compare relative OTUs at treatment period (A,B,C and D) compared to washout and baseline. Treatment periods were as follows: A (inulin+placebo), B (inulin +probiotic), C (control+placebo) and D (control+probiotic). Taxon key shows least abundant (top) to most abundant (bottom). No statistical significant difference was observed.
Figure 4-12. Relative abundance of specific taxon (Bifidobacterium, Lactobacillus and Clostridium). Taxon key shows least abundant (top) to most abundant (bottom). Plots compare relative abundance of specified genus at baseline, treatment period (A, B, C and D) and washout. Treatment periods were as follows: A (inulin+placebo), B (inulin +probiotic), C (control+placebo) and D (control+probiotic). No statistical significant difference was observed.
Figure 4-13. Quantification of *L. plantarum* HA119, *B. bifidum* HA132, and *L. acidophilus* HA122 in stool samples using qPCR. Strains were quantified as LOG bacterial per gram of stool. Numerical entry indicates a specific sample was not received (a total of 9 stool samples were collected throughout the 18-week study).
Figure 4-13. Continued.
Figure 4-14. Effect of treatment (Tx) and washout (Wo) on self-reported hunger level compared to baseline. Hunger was reported on the daily questionnaire throughout the entire 18-week study. Participants scored the severity of symptoms from 0 (none) to 6 (very severe). An anchor of 3 was also provided and corresponded to moderate symptom intensity. Mean hunger per person per period (14 days/period) was calculated. Statistically a fixed effects test for treatment was performed and Least Squares means differences and Tukey honest significance difference (HSD) test was performed. Bars with different letters are significantly different (p<0.05).

**Main Effects**

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<tr>
<td>Treatment</td>
<td>&lt;0.0001</td>
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Figure 4-15. Predicted probabilities for Bristol Stool Form Scale categories (constipation, regular, diarrhea) during baseline, treatment A, B, C, D and washouts 1, 2, 3 and 4. Treatment periods were as follows: A (inulin+placebo), B (inulin +probiotic), C (control+placebo) and D (control+probiotic). Tx refers to Treatment, Base refers to the Baseline period, and Wo refers to Washout periods. Bristol Stool Scale category (slow transit; constipation- types 1 and 2, normal transit; regular types 3 to 5 and fast transit; diarrhea types 6 and 7) were collected in the daily questionnaire and were analyzed. All participants (n=26) were included in statistical analysis. The overall model (main effect) was not statistically significant (p=0.184).
Figure 4-16. Stool pH measured at the end of the baseline and treatment periods. Tukey-Kramer grouping for treatment least square means was used to analyze for any differences between treatment periods and baseline. Treatments A (inulin + placebo), C (control + placebo) and D (control + probiotic) were statistically higher than baseline. Treatment B (inulin + probiotic) was not statistically different from baseline. Data from all participants was analyzed for baseline (n=26), treatment A (n=23; PAM 08, 13, 20 excluded), treatment B (n=22; PAM 06, 08, 20, 28 excluded), treatment C (n=23; PAM 08, 13, 20 excluded) and treatment D (n=21; PAM 06, 13, 20, 23, 28 excluded). For IL-6 a measure of <0.04 was entered as 0.04 and <0.14 was entered as 0.14 for statistical analysis. Bars with different letters are significantly different (p<0.05).
Cytokines measured in the serum (pg/ml) were not significantly different at the end of any treatment period compared to baseline measures. For analysis both IL-6 and TNF-α values were log-transformed. Data was analyzed utilizing a fixed effect of treatment model. Effect of treatment was not statistically significant for IL-6 ($p=0.4610$) or TNF-α ($p=0.2759$). Data from all participants was analyzed for baseline ($n=26$), treatment A ($n=22$), treatment B ($n=22$), treatment C ($n=22$) and treatment D ($n=21$). For IL-6 a measure of $<0.04$ was entered as 0.04 and $<0.14$ was entered as 0.14 for statistical analysis. PAM 04 was not included in analysis at treatment A or C because Luminex software was unable to provide a measurement. Bars with different letters are significantly different ($p<0.05$).
Figure 4-18. Effect of treatments on anti-inflammatory cytokine, IL-10. IL-10 measured in the serum (pg/ml) was not significantly different at the end of any treatment period compared to baseline measures. Data was analyzed utilizing a fixed effect of treatment model. Effect of treatment was not statistically significant (p=0.8622). Data from all participants was analyzed for baseline (n=26), treatment A (n=22), treatment B (n=22), treatment C (n=22) and treatment D (n=21). For IL-10 a measure of <0.43 was entered at 0.43 and <1.33 was entered as 1.33 for statistical analysis. PAM 04 was not included in analysis at treatment A or C because Luminex software was unable to provide a measurement. Bars with different letters are significantly different (p<0.05).
Figure 4-19. Effect of treatment on the metabolite phenylacetyl glutamine in the urine. Data was analyzed utilizing two models to analyze metabolite values, both log-transformations. One model looked for a possible sequence effect and the other effect of treatment on metabolite. The model was a random effect of subject and heterogeneous variance. Effect of sequence (p=0.1762) was not statistically significant, effect of day (p=0.0045) was significant, however sequence*day (p=0.6229) was not statistically significant. The overall model (main effect of treatment) was statistically significant (p=0.0219). Data from all participants was analyzed (n=26). Bars with different letters are significantly different (p<0.05).
Figure 4-20. Radar chart of SF-36v2 health domains, Mental Component Summary (MCS) and Physical Component Summary (PCS). Scored were compared at end of baseline and treatment periods. No statistical difference observed for SF-36v2 health domains (eight domains include: physical functioning, role-physical, bodily pain, general health, vitality, social functioning, role-emotional and mental health), MCS or PCS scores from baseline and treatments.
Table 4-1. Participant demographics and compliance

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Total (n=26)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (M/F)</td>
<td>0/26</td>
</tr>
<tr>
<td>Age (y, mean±SD)</td>
<td>73.7±5.6</td>
</tr>
<tr>
<td>Race [n, (%)]</td>
<td></td>
</tr>
<tr>
<td>White</td>
<td>23 (88.5)</td>
</tr>
<tr>
<td>Black</td>
<td>2 (7.7)</td>
</tr>
<tr>
<td>American Indian or Alaska Native</td>
<td>1 (3.8)</td>
</tr>
<tr>
<td>Ethnicity [n, (%)]</td>
<td></td>
</tr>
<tr>
<td>Not Hispanic or Latino</td>
<td>26 (100)</td>
</tr>
<tr>
<td>Hispanic or Latino</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Unknown or Not Reported</td>
<td>0 (0)</td>
</tr>
<tr>
<td>BMI percentiles [n, (%)]</td>
<td></td>
</tr>
<tr>
<td>Normal weight (5th - 84th)</td>
<td>12 (46.2)</td>
</tr>
<tr>
<td>Overweight (85th - 95th)</td>
<td>14 (53.8)</td>
</tr>
<tr>
<td>Total initial GFI score</td>
<td>1.6±1.7</td>
</tr>
<tr>
<td>Compliance (%)</td>
<td></td>
</tr>
<tr>
<td>Stool protocol</td>
<td>99.5</td>
</tr>
<tr>
<td>Supplement protocol, probiotic</td>
<td>96.6</td>
</tr>
<tr>
<td>Supplement protocol, prebiotic (inulin)</td>
<td>95.0</td>
</tr>
<tr>
<td>Questionnaire protocol</td>
<td>99.8</td>
</tr>
</tbody>
</table>

1Abbreviation: BMI, body mass index (kg/m²); d, day; GFI, Groningen Frailty Indicator.
2Values are means ± SDs.
3No statistical test was performed on BMI, because adiposity categories are presented.
4Total GFI score measured at baseline (n=26); reported at means ± SDs. Individuals with a score 4 or higher out of a maximum score of 15 are considered to have “moderate to severe frailty”. A total of 4 participants scored a 4 or higher on the GFI at baseline.
5Questionnaire compliance reported as a percent of completion of all daily questionnaires.
Table 4-2. Energy and macronutrients provided during treatment periods¹

<table>
<thead>
<tr>
<th></th>
<th>(n=26)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estimated Energy Requirement², kcal/d</td>
<td>1839±39</td>
</tr>
<tr>
<td>Provided Energy³, kcal/d</td>
<td>1792±36</td>
</tr>
<tr>
<td>Provided Protein⁴, g/d</td>
<td>121.3±1.2</td>
</tr>
<tr>
<td>Provided Protein⁵, g/kg body weight</td>
<td>1.8±0.04</td>
</tr>
</tbody>
</table>

Controlled High-Protein Diet (base diet)

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Energy, kcal/d</td>
<td>1616±20</td>
</tr>
<tr>
<td>Protein, g/d (% of total energy)</td>
<td>120.5±1.7 (29.8)</td>
</tr>
<tr>
<td>Carbohydrate, g/d (% of total energy)</td>
<td>187.0±2.1 (46.3)</td>
</tr>
<tr>
<td>Fat, g/d (% of total energy)</td>
<td>48.1±4.0 (26.8)</td>
</tr>
<tr>
<td>Fiber, g/d</td>
<td>15.5±0.4</td>
</tr>
</tbody>
</table>

¹Abbreviations: kcal, kilocalorie; L, Liter; d, day; g, gram; %, percent.
²Estimated Energy Requirement was calculated as a mean from the SenseWear Pro Armband (BodyMedia, Inc.) to calculate requirements for weight-maintenance. Armband was worn by participants during the baseline periods.
³Provided Energy is the mean energy that was provided to participants during the controlled diet periods after adjusting the base diet to their specified requirements (measured with SenseWear Pro Armband).
⁴Provided Protein is the mean grams of protein that was provided to participants during the controlled diet periods after adjusting the base diet to their specified requirements (measured with SenseWear Pro Armband).
⁵Provided Protein is the mean protein reported as grams per kilogram body weight that was provided to participants during the controlled diet periods after adjusting the base diet to their specified requirements (measured with SenseWear Pro Armband).

kcal/d reported as means of 4-day menu rotation ± SE
Table 4-3. Body composition of participants using Bioelectrical Impedance Analysis (BIA) (baseline and final/end of study)¹

<table>
<thead>
<tr>
<th>Measurements</th>
<th>Baseline (randomization)</th>
<th>Final, end of study</th>
<th>P value²</th>
<th>P value³</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight, kg</td>
<td>67.2±1.90</td>
<td>67.3±1.84</td>
<td>0.632</td>
<td>0.316</td>
</tr>
<tr>
<td>Extracellular fluid, L</td>
<td>13.5±0.29</td>
<td>13.7±0.32</td>
<td>0.303</td>
<td>0.151</td>
</tr>
<tr>
<td>Intracellular fluid, L</td>
<td>17.2±0.56</td>
<td>18.4±0.57</td>
<td>0.049*</td>
<td>0.025*</td>
</tr>
<tr>
<td>Total body water, L</td>
<td>30.7±0.75</td>
<td>32.1±0.85</td>
<td>0.059</td>
<td>0.030*</td>
</tr>
<tr>
<td>Fat free mass, kg</td>
<td>41.1±1.05</td>
<td>43.2±1.18</td>
<td>0.054</td>
<td>0.027*</td>
</tr>
<tr>
<td>Fat, kg</td>
<td>26.1±1.69</td>
<td>24.2±1.05</td>
<td>0.099</td>
<td>0.050</td>
</tr>
<tr>
<td>Fat, %</td>
<td>38.2±1.63</td>
<td>35.7±0.95</td>
<td>0.101</td>
<td>0.050</td>
</tr>
</tbody>
</table>

¹Abbreviations: BIA, bioelectrical impedance analysis; L, Liter; kg, kilograms; %, percent.
²Differences were calculated using a 2 tail, paired t-test.
³Differences were calculated using a 1 tail, paired t-test.
Data are reported as means ± SEs for measurements taken at baseline/randomization and final study visits. Only participants with both measurements were included in analysis (n=21; excluded PAM 06, 08,13,20,28).
*p-value < 0.05; **p-value <0.01; ***p-value <0.001
### Table 4-4. Frequency table of self-reported individual symptoms as reported on the daily questionnaire

<table>
<thead>
<tr>
<th>Level</th>
<th>Diarrhea Count</th>
<th>Probability</th>
<th>Constipation Count</th>
<th>Probability</th>
<th>Fatigue Count</th>
<th>Probability</th>
<th>Hunger Count</th>
<th>Probability</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2770</td>
<td>0.97</td>
<td>2436</td>
<td>0.86</td>
<td>2109</td>
<td>0.74</td>
<td>1298</td>
<td>0.46</td>
</tr>
<tr>
<td>1</td>
<td>47</td>
<td>0.01</td>
<td>226</td>
<td>0.08</td>
<td>456</td>
<td>0.16</td>
<td>491</td>
<td>0.17</td>
</tr>
<tr>
<td>2</td>
<td>16</td>
<td>0.01</td>
<td>57</td>
<td>0.02</td>
<td>130</td>
<td>0.046</td>
<td>459</td>
<td>0.16</td>
</tr>
<tr>
<td>3</td>
<td>4</td>
<td>0.00</td>
<td>50</td>
<td>0.02</td>
<td>55</td>
<td>0.02</td>
<td>536</td>
<td>0.19</td>
</tr>
<tr>
<td>4</td>
<td>2</td>
<td>0.00</td>
<td>28</td>
<td>0.01</td>
<td>56</td>
<td>0.02</td>
<td>22</td>
<td>0.01</td>
</tr>
<tr>
<td>5</td>
<td>1</td>
<td>0.00</td>
<td>36</td>
<td>0.01</td>
<td>40</td>
<td>0.01</td>
<td>0</td>
<td>0.00</td>
</tr>
<tr>
<td>6</td>
<td>2</td>
<td>0.00</td>
<td>10</td>
<td>0.00</td>
<td>1</td>
<td>0.00</td>
<td>0</td>
<td>0.00</td>
</tr>
</tbody>
</table>

1. Frequency table of participant responses for individual symptoms diarrhea, constipation, fatigue and hunger (these are individual symptoms that were not further combined into a syndrome) from daily questionnaire responses. Individual daily symptoms were self-reported daily throughout the 18-week study and participants scored the severity of symptoms from 0 (none) to 6 (very severe). An anchor of 3 was also provided and corresponded to moderate symptom intensity.

2. For the individual symptom, diarrhea the data set contained 97.5% zeroes on the severity scale (scale from 0 to 6). Data could not be further statistically analyzed.

3. For the individual symptom, constipation the data set contained 86% zeroes on the severity scale (scale from 0 to 6). Data could not be further statistically analyzed.

4. For the individual symptom, fatigue response variation was sufficient among participant responses to statistically analyze results.

5. For the individual symptom, hunger response variation was sufficient among participant responses to statistically analyze results. Data are reported as a total count of all participant responses throughout the 18-week study. All participant responses were included in analysis (n=26).
Table 4-5. Effect of period on self-reported symptoms/syndromes of the daily questionnaire\(^1\)

<table>
<thead>
<tr>
<th>Period</th>
<th>Fatigue(^2)</th>
<th>Hunger(^3***)</th>
<th>Gastrointestinal distress(^4***)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>1.39±0.20</td>
<td>1.76±0.22</td>
<td>0.31±0.07</td>
</tr>
<tr>
<td>Tx A (inulin + placebo)</td>
<td>1.40±0.20</td>
<td>1.20±0.22(^*)</td>
<td>0.58±0.08(^*)</td>
</tr>
<tr>
<td>Tx B (inulin + probiotic)</td>
<td>1.73±0.21</td>
<td>1.23±0.23(^*)</td>
<td>0.62±0.08(^*)</td>
</tr>
<tr>
<td>Tx C (control + placebo)</td>
<td>1.56±0.20</td>
<td>1.20±0.22(^*)</td>
<td>0.36±0.08</td>
</tr>
<tr>
<td>Tx D (control + probiotic)</td>
<td>1.34±0.21</td>
<td>1.01±0.22(^*)</td>
<td>0.39±0.08</td>
</tr>
<tr>
<td>Washout 1</td>
<td>1.60±0.21</td>
<td>1.31±0.22(^*)</td>
<td>0.31±0.08</td>
</tr>
<tr>
<td>Washout 2</td>
<td>1.54±0.21</td>
<td>1.32±0.22(^*)</td>
<td>0.32±0.08</td>
</tr>
<tr>
<td>Washout 3</td>
<td>1.41±0.22</td>
<td>1.10±0.23(^*)</td>
<td>0.29±0.08</td>
</tr>
<tr>
<td>Washout 4</td>
<td>1.46±0.22</td>
<td>1.15±0.23(^*)</td>
<td>0.29±0.08</td>
</tr>
</tbody>
</table>

\(^1\)Abbreviations: Tx, treatment.
Effect of treatment group on self-reporting individual symptoms (fatigue, hunger) and syndrome (gastrointestinal distress) responses. Fatigue, hunger and gastrointestinal distress were the only individual symptoms and component syndrome that could be statistically analyzed due to variation in non-zero response reported by participants. Symptoms were self-reported daily throughout the 18-week study and participants scored the severity of symptoms from 0 (none) to 6 (very severe).

\(^2\)For the individual symptom fatigue, response means were analyzed as the mean of non-zeroes values of fatigue per person per period (14 days/period). The overall model (main effect) was not statistically significant (\(p=0.415\))

PAM 01,02,03,04,07,09,17,18 were removed from fatigue symptom analyses due to little to no variation in reported daily values (mostly zeroes); \(n=18\).
For the individual symptom hunger, response means were analyzed as the mean of non-zeroes values of hunger per person per period (14 days/period). The overall model (main effect) was statistically significant (p<0.0001). Data from all participants was analyzed (n=26).

For the component syndrome gastrointestinal distress (syndrome includes sum of symptom scored for bloating, flatulence, abdominal cramping and abdominal noises), response means were analyzed as the mean of values of bloating, flatulence, abdominal cramping and abdominal noises per person per period (14 days/period). The overall model (main effect) was statistically significant (p<0.0001). Data from all participants was analyzed (n=26).

Data was analyzed using a fixed effect model with treatment as main effect. Data are presented as Least Sq Means ± SE with *p<0.05; **p<0.01; ***p<0.001.
Table 4-6. Sleep scores reported as mean daily proportion of people with sleep <7 hrs as reported in the daily questionnaire.1

<table>
<thead>
<tr>
<th>Period</th>
<th>Sleep (Categorical &lt;7 or ≥7)2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>0.40±0.17</td>
</tr>
<tr>
<td>Tx A (inulin + placebo)</td>
<td>0.32±0.16</td>
</tr>
<tr>
<td>Tx B (inulin + probiotic)</td>
<td>0.36±0.16</td>
</tr>
<tr>
<td>Tx C (control + placebo)</td>
<td>0.33±0.16</td>
</tr>
<tr>
<td>Tx D (control + probiotic)</td>
<td>0.34±0.16</td>
</tr>
<tr>
<td>Washout 1</td>
<td>0.40±0.17</td>
</tr>
<tr>
<td>Washout 2</td>
<td>0.32±0.16</td>
</tr>
<tr>
<td>Washout 3</td>
<td>0.31±0.15</td>
</tr>
<tr>
<td>Washout 4</td>
<td>0.37±0.17</td>
</tr>
</tbody>
</table>

1Abbreviations: Tx, treatment; hrs, hours. Self-reported sleep; Participant asked to report how many hours they slept previous night asked not to include the time it took to fall asleep or anytime they were awakened during the night. The following response categories were provided, <5hrs 5-6 hrs 6-7 hrs 7-8 hrs 8-9 hrs >9 hrs.

2A quasi-binomial distribution of daily category (<7 or ≥7 hrs) modeling mean daily proportion of people with sleep <7hrs. The overall model (main effect) was not statistically significant (p=0.433). Data from all participants was analyzed (n=26). Data was analyzed using a fixed effect model with treatment as main effect. Data are presented as Least Sq. Means ± SE with *p<0.05; **p<0.01; ***p<0.001
Table 4-7. Period effects of bowel movement frequency and Bristol Stool Scale as reported in the daily questionnaire

<table>
<thead>
<tr>
<th>Period</th>
<th>Bowel Movement Frequency&lt;sup&gt;2&lt;/sup&gt;</th>
<th>Bristol Stool Scale&lt;sup&gt;3&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>1.38±0.10</td>
<td>3.84±0.21</td>
</tr>
<tr>
<td>Tx A (inulin + placebo)</td>
<td>1.38±0.10</td>
<td>3.71±0.21</td>
</tr>
<tr>
<td>Tx B (inulin + probiotic)</td>
<td>1.43±0.10</td>
<td>3.79±0.22</td>
</tr>
<tr>
<td>Tx C (control + placebo)</td>
<td>1.39±0.10</td>
<td>3.78±0.21</td>
</tr>
<tr>
<td>Tx D (control + probiotic)</td>
<td>1.39±0.10</td>
<td>3.58±0.21</td>
</tr>
<tr>
<td>Washout 1</td>
<td>1.42±0.10</td>
<td>3.75±0.21</td>
</tr>
<tr>
<td>Washout 2</td>
<td>1.38±0.10</td>
<td>3.74±0.21</td>
</tr>
<tr>
<td>Washout 3</td>
<td>1.36±0.11</td>
<td>3.77±0.22</td>
</tr>
<tr>
<td>Washout 4</td>
<td>1.38±0.11</td>
<td>3.78±0.22</td>
</tr>
</tbody>
</table>

<sup>1</sup>Abbreviations: Tx, treatment.
Self-reported bowel movement frequency and Bristol Stool Scale (categorically classifies stool based on stool appearance and consistency). Both were self-reported as part of the daily questionnaire and participants were asked to record the Bristol Stool Scale for each stool sample reported.

<sup>2</sup>Bowel movement frequency was estimated as mean values per day (multiple the estimate and the standard error by 7 for weekly totals and SE of total). The overall model (main effect) was not statistically significant (p=0.999). Data from all participants was analyzed (n=26).

<sup>3</sup>Bristol Stool Scale categories were analyzed statistically. The mean of daily Bristol Stool Scale responses for each 2-week period of each treatment was analyzed. Responses were grouped in categories based on transit time (slow transit - types 1 and 2, normal transit- types 3 to 5 and fast transit- types 6 and 7). The overall model (main effect) was not statistically significant (p=0.892). Data from all participants was analyzed (n=26).

Data was analyzed using a fixed effect model with treatment as main effect. Data are presented as Least Sq. Means ± SE with *p<0.05; **p<0.01; ***p<0.001
Table 4-8. Gastrointestinal Symptom Rating Scale scores reported weekly compared to scores reported during combined baseline and washout periods.

<table>
<thead>
<tr>
<th>Period</th>
<th>Abdominal Pain(^2)*</th>
<th>P value</th>
<th>Constipation(^3)</th>
<th>P value</th>
<th>Diarrhea(^4)</th>
<th>P value</th>
<th>Indigestion(^5)***</th>
<th>P value</th>
<th>Reflux(^6)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tx A (inulin + placebo)</td>
<td>0.20±0.04*</td>
<td>0.046</td>
<td>0.32±0.07</td>
<td>NS</td>
<td>0.14±0.04</td>
<td>NS</td>
<td>0.43±0.06***</td>
<td>0.0001</td>
<td>0.13±0.06</td>
<td>NS</td>
</tr>
<tr>
<td>Tx B (inulin + probiotic)</td>
<td>0.22±0.04**</td>
<td>0.009</td>
<td>0.33±0.07</td>
<td>NS</td>
<td>0.13±0.04</td>
<td>NS</td>
<td>0.50±0.06***</td>
<td>&lt;0.0001</td>
<td>0.15±0.06</td>
<td>NS</td>
</tr>
<tr>
<td>Tx C (control + placebo)</td>
<td>0.21±0.04*</td>
<td>0.014</td>
<td>0.38±0.07</td>
<td>NS</td>
<td>0.06±0.04</td>
<td>NS</td>
<td>0.33±0.06</td>
<td>NS</td>
<td>0.25±0.06</td>
<td>NS</td>
</tr>
<tr>
<td>Tx D (control + probiotic)</td>
<td>0.14±0.04</td>
<td>NS</td>
<td>0.36±0.07</td>
<td>NS</td>
<td>0.06±0.04</td>
<td>NS</td>
<td>0.36±0.06*</td>
<td>0.033</td>
<td>0.17±0.06</td>
<td>NS</td>
</tr>
</tbody>
</table>

\(^1\)Abbreviations: Tx, treatment.
The Gastrointestinal Symptom Rating Scale was a self-reported questionnaire completed weekly throughout the 18-week study. Individual symptoms were given a value from 1 (no discomfort at all) to 7 (very severe discomfort) and a sum and then mean was calculated for each scale (abdominal pain, constipation, diarrhea, indigestion and reflux).

\(^2\)Abdominal pain scale includes individual symptom questions regarding abdominal pain, hunger pains and nausea symptoms. The overall model (main effect) was statistically significant (p=0.011). Data from all participants was analyzed (n=26).

\(^3\)Constipation scale includes individual symptom questions regarding constipation, hard stools and feeling of incomplete evacuation symptoms. Data were transformed by the natural log and an effect of sequence and interaction with treatment was analyzed. Effect of sequence (p=0.006) was statistically significant, however, the overall model (main effect) was not statistically significant (p=0.116). Data from all participants was analyzed (n=26).

\(^4\)Diarrhea scale individual symptom questions regarding diarrhea, loose stools and urgent need for defecation symptoms. The overall model (main effect) was not statistically significant (p=0.107). Data from all participants was analyzed (n=26).

\(^5\)Indigestion scale includes stomach rumbling, bloating, burping and increased flatus symptoms. The overall model (main effect) was statistically significant (p<0.0001). Data from all participants was analyzed (n=26).
Reflux scale includes individual symptom questions regarding heartburn and acid regurgitation symptoms. The overall model (main effect) was not statistically significant (p=0.154). Data from all participants was analyzed (n=26). Data presented as Least Squares Mean ± SEM compared to baseline+washout periods. Data was analyzed using a general linear mixed model with a random subject effect and a fixed effect of treatment. Data are presented as Least Squ Mean ± SE with *p<0.05; **p<0.01; ***p<0.001
Table 4-9. Gastrointestinal Symptom Rating Scale scores reported weekly compared to scores reported during the baseline period

<table>
<thead>
<tr>
<th>Period</th>
<th>Abdominal Pain²</th>
<th>Constipation³</th>
<th>Diarrhea⁴</th>
<th>Indigestion⁵</th>
<th>Reflux⁶</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P value</td>
<td>P value</td>
<td>P value</td>
<td>P value</td>
<td>P value</td>
</tr>
<tr>
<td>Tx A (inulin + placebo)</td>
<td>0.20±0.04</td>
<td>NS</td>
<td>0.32±0.07</td>
<td>NS</td>
<td>0.43±0.06**</td>
</tr>
<tr>
<td>Tx B (inulin + probiotic)</td>
<td>0.22±0.04</td>
<td>NS</td>
<td>0.33±0.07</td>
<td>NS</td>
<td>0.50±0.06***</td>
</tr>
<tr>
<td>Tx C (control + placebo)</td>
<td>0.21±0.04</td>
<td>NS</td>
<td>0.38±0.07</td>
<td>NS</td>
<td>0.33±0.06</td>
</tr>
<tr>
<td>Tx D (control + probiotic)</td>
<td>0.14±0.04</td>
<td>NS</td>
<td>0.36±0.07</td>
<td>NS</td>
<td>0.36±0.06</td>
</tr>
<tr>
<td>Washout</td>
<td>0.13±0.04</td>
<td>NS</td>
<td>0.28±0.06</td>
<td>NS</td>
<td>0.08±0.02</td>
</tr>
</tbody>
</table>

¹Abbreviations: Tx, treatment.
The Gastrointestinal Symptom Rating Scale was a self-reported questionnaire completed weekly throughout the 18-week study. Individual symptoms were given a value from 1 (no discomfort at all) to 7 (very severe discomfort) and a sum and then mean was calculated for each scale (abdominal pain, constipation, diarrhea, indigestion and reflux).

²Abdominal pain scale includes individual symptom questions regarding abdominal pain, hunger pains and nausea symptoms. The overall model (main effect) was statistically significant (p=0.011). Data from all participants was analyzed (n=26).

³Constipation scale includes individual symptom questions regarding constipation, hard stools and feeling of incomplete evacuation symptoms. Data were transformed by the natural log and an effect of sequence and interaction with treatment was analyzed. Effect of sequence (p=0.006) was statistically significant, however, the overall model (main effect) was not statistically significant (p=0.116). Data from all participants was analyzed (n=26).

⁴Diarrhea scale individual symptom questions regarding diarrhea, loose stools and urgent need for defecation symptoms. The overall model (main effect) was not statistically significant (p=0.107). Data from all participants was analyzed (n=26).

⁵Indigestion scale includes stomach rumbling, bloating, burping and increased flatus symptoms. The overall model (main effect) was statistically significant (p<0.0001). Data from all participants was analyzed (n=26).

⁶Reflux scale includes individual symptom questions regarding heartburn and acid regurgitation symptoms. The overall model (main effect) was not statistically significant (p=0.154). Data from all participants was analyzed (n=26).
Data presented as Least Squares Mean ± SEM compared to the baseline period. Data was analyzed using a general linear mixed model with a random subject effect and a fixed of treatment. Data are presented as Least Sq. Means ± SE with *p<0.05; **p<0.01; ***p<0.001
Table 4-10. Gastrointestinal Symptom Rating Scale scores reported weekly compared to scores reported during the washout periods.$^1$

<table>
<thead>
<tr>
<th>Period</th>
<th>Abdominal Pain$^2*$</th>
<th>P value</th>
<th>Constipation$^3$</th>
<th>P value</th>
<th>Diarrhea$^4$</th>
<th>P value</th>
<th>Indigestion$^5***$</th>
<th>P value</th>
<th>Reflux$^6$</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>0.13±0.04</td>
<td>NS</td>
<td>0.26±0.07</td>
<td>NS</td>
<td>0.13±0.03</td>
<td>NS</td>
<td>0.28±0.05</td>
<td>NS</td>
<td>0.13±0.05</td>
<td>NS</td>
</tr>
<tr>
<td>Tx A (inulin + placebo)</td>
<td>0.20±0.04</td>
<td>NS</td>
<td>0.32±0.07</td>
<td>NS</td>
<td>0.14±0.04</td>
<td>NS</td>
<td>0.43±0.06**</td>
<td>0.001</td>
<td>0.13±0.06</td>
<td>NS</td>
</tr>
<tr>
<td>Tx B (inulin + probiotic)</td>
<td>0.22±0.04*</td>
<td>0.027</td>
<td>0.33±0.07</td>
<td>NS</td>
<td>0.13±0.04</td>
<td>NS</td>
<td>0.50±0.06***</td>
<td>&lt;.0001</td>
<td>0.15±0.06</td>
<td>NS</td>
</tr>
<tr>
<td>Tx C (control + placebo)</td>
<td>0.21±0.04*</td>
<td>0.043</td>
<td>0.38±0.07</td>
<td>NS</td>
<td>0.06±0.04</td>
<td>NS</td>
<td>0.33±0.06</td>
<td>NS</td>
<td>0.25±0.06</td>
<td>NS</td>
</tr>
<tr>
<td>Tx D (control + probiotic)</td>
<td>0.14±0.04</td>
<td>NS</td>
<td>0.36±0.07</td>
<td>NS</td>
<td>0.06±0.04</td>
<td>NS</td>
<td>0.36±0.06</td>
<td>NS</td>
<td>0.17±0.06</td>
<td>NS</td>
</tr>
</tbody>
</table>

$^1$ Abbreviations: Tx, treatment.
The Gastrointestinal Symptom Rating Scale was a self-reported questionnaire completed weekly throughout the 18-week study. Individual symptoms were given a value from 1 (no discomfort at all) to 7 (very severe discomfort) and a sum and then mean was calculated for each scale (abdominal pain, constipation, diarrhea, indigestion, and reflux).

$^2$ Abdominal pain scale includes individual symptom questions regarding abdominal pain, hunger pains and nausea symptoms. The overall model (main effect) was statistically significant (p=0.011). Data from all participants was analyzed (n=26).

$^3$ Constipation scale includes individual symptom questions regarding constipation, hard stools and feeling of incomplete evacuation symptoms. Data were transformed by the natural log and an effect of sequence and interaction with treatment was analyzed. Effect of sequence (p=0.006) was statistically significant, however, the overall model (main effect) was not statistically significant (p=0.116). Data from all participants was analyzed (n=26).

$^4$ Diarrhea scale individual symptom questions regarding diarrhea, loose stools and urgent need for defecation symptoms. The overall model (main effect) was not statistically significant (p=0.107). Data from all participants was analyzed (n=26).

$^5$ Indigestion scale includes stomach rumbling, bloating, burping and increased flatus symptoms. The overall model (main effect) was statistically significant (p<0.0001). Data from all participants was analyzed (n=26).

$^6$ Reflux scale includes individual symptom questions regarding heartburn and acid regurgitation symptoms. The overall model (main effect) was not statistically significant (p=0.154). Data from all participants was analyzed (n=26).

Data presented as Least Squares Mean ± SEM compared to washout.
Data was analyzed using a general linear mixed model with a random subject effect and a fixed of treatment. Data are presented as Least Sq. Means ± SE with *p<0.05; **p<0.01; ***p<0.001
### Table 4-11. Biochemical characteristics of participants from treatment periods compared to baseline values

<table>
<thead>
<tr>
<th>Measurements</th>
<th>Reference Range</th>
<th>Baseline</th>
<th>Tx A (inulin + placebo)</th>
<th>P value</th>
<th>Tx B (inulin + probiotic)</th>
<th>P value</th>
<th>Tx C (control + placebo)</th>
<th>P value</th>
<th>Tx D (control + probiotic)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol (mg/dL)²</td>
<td>120-200</td>
<td>197.4±7.6</td>
<td>198.0±7.8</td>
<td>NS</td>
<td>199.0±7.8</td>
<td>NS</td>
<td>202.4±7.8</td>
<td>NS</td>
<td>195.1±7.9</td>
<td>NS</td>
</tr>
<tr>
<td>Triglycerides (mg/dL)³</td>
<td>30-149</td>
<td>95.4±7.4</td>
<td>97.5±7.6</td>
<td>NS</td>
<td>99.3±7.6</td>
<td>NS</td>
<td>94.3±7.6</td>
<td>NS</td>
<td>94.7±7.7</td>
<td>NS</td>
</tr>
<tr>
<td>Blood urea nitrogen (mg/dL)⁴</td>
<td>6-20</td>
<td>16.7±1.2</td>
<td>22.0±1.3</td>
<td>&lt;.0001</td>
<td>23.8±1.3</td>
<td>&lt;.0001</td>
<td>22.6±1.3</td>
<td>&lt;.0001</td>
<td>21.9±1.3</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Albumin (g/dL)⁵</td>
<td>3.5-5.2</td>
<td>4.39±0.05</td>
<td>4.30±0.05</td>
<td>NS</td>
<td>4.30±0.05</td>
<td>NS</td>
<td>4.30±0.05</td>
<td>NS</td>
<td>4.33±0.05</td>
<td>NS</td>
</tr>
<tr>
<td>Total protein (g/dL)⁶</td>
<td>6.0-8.3</td>
<td>6.99±0.08</td>
<td>6.97±0.08</td>
<td>NS</td>
<td>6.93±0.08</td>
<td>NS</td>
<td>6.94±0.08</td>
<td>NS</td>
<td>6.98±0.08</td>
<td>NS</td>
</tr>
</tbody>
</table>

¹Abbreviations: Tx, treatment; mg, milligram; dL, deciliter; g, gram.
²Total cholesterol was analyzed using a general linear mixed model with a random subject effect and a fixed effect of treatment. Previous analysis reported no significant effects for sequence or time point. The overall model (main effect) was not statistically significant (p=0.732). Data from all participants was analyzed (n=26).
³Triglycerides were analyzed using a general linear mixed model with a random subject effect and a fixed effect of treatment. Previous analysis reported no significant effects for sequence or time point. The overall model (main effect) was not statistically significant (p=0.854). Data from all participants was analyzed (n=26).
⁴Blood urea nitrogen were analyzed using a general linear mixed model with a random subject effect for sequence and time point. The overall model (main effect) was statistically significant (p<0.0001). Data from all participants was analyzed (n=26).
⁵Albumin analyzed using a general linear mixed model with a random subject effect for sequence and time point. A significant time effect (p=0.0002) was reported but not sequence (p=0.260) or sequence*time (p=0.770) so data was reanalyzed for treatment effect. The overall model (main effect) was not statistically significant (p=0.051). Data from all participants was analyzed (n=26).
⁶Total protein were analyzed using a general linear mixed model with a random subject effect for sequence and time point. A significant time effect (p=0.004) was reported but not sequence (p=0.967) or sequence*time (p=0.958) so data was reanalyzed for
treatment effect. The overall model (main effect) was not statistically significant ($p=0.871$). Data from all participants was analyzed ($n=26$).

Data are presented as Least Sq. Means $\pm$ SE with $^*p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$
### Table 4-12. Characteristics of participants

<table>
<thead>
<tr>
<th>Measurements</th>
<th>Reference Range</th>
<th>Baseline $^2$</th>
<th>Tx A (inulin + placebo)$^3$</th>
<th>Tx B (inulin + probiotic)$^4$</th>
<th>Tx C (control + placebo)$^5$</th>
<th>Tx D (control + probiotic)$^6$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight (kg)</td>
<td>N/A</td>
<td>67.7±1.7</td>
<td>67.6±1.7</td>
<td>67.5±1.8</td>
<td>67.3±1.8</td>
<td>67.1±1.9</td>
</tr>
<tr>
<td>Lipid Panel</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cholesterol (mg/dL)</td>
<td>120-200</td>
<td>197.9±7.4</td>
<td>197.9±8.0</td>
<td>198.4±8.0</td>
<td>198.5±7.8</td>
<td>199.2±8.2</td>
</tr>
<tr>
<td>Triglycerides (mg/dL)</td>
<td>30-149</td>
<td>92.3±6.9</td>
<td>91.7±7.3</td>
<td>93.9±7.8</td>
<td>95.6±7.7</td>
<td>96.0±8.3</td>
</tr>
<tr>
<td>HDL (mg/dL)</td>
<td>35-85</td>
<td>68.9±3.3</td>
<td>68.7±3.6</td>
<td>68.5±3.6</td>
<td>68.1±3.5</td>
<td>68.4±3.6</td>
</tr>
<tr>
<td>LDL (calc)</td>
<td>0-99</td>
<td>110.6±6.6</td>
<td>110.9±7.1</td>
<td>111.1±7.1</td>
<td>111.2±6.9</td>
<td>111.7±7.2</td>
</tr>
<tr>
<td>VLDL (calc)</td>
<td>8-39</td>
<td>18.5±1.4</td>
<td>18.3±1.5</td>
<td>18.8±1.6</td>
<td>19.1±1.5</td>
<td>19.2±1.7</td>
</tr>
<tr>
<td>Complete Metabolic Profile</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fasting glucose (mg/dL)</td>
<td>70-99</td>
<td>92.1±1.8</td>
<td>92.1±1.9</td>
<td>92.0±1.9</td>
<td>92.0±1.9</td>
<td>91.9±2.0</td>
</tr>
<tr>
<td>Sodium (mmol/L)</td>
<td>136-145</td>
<td>141.1±0.5</td>
<td>141.1±0.6</td>
<td>141.0±0.6</td>
<td>141.0±0.6</td>
<td>141.0±0.6</td>
</tr>
<tr>
<td>Potassium (mmol/L)</td>
<td>3.5-5.3</td>
<td>4.58±0.07</td>
<td>4.58±0.07</td>
<td>4.57±0.07</td>
<td>4.57±0.07</td>
<td>4.56±0.08</td>
</tr>
<tr>
<td>Calcium (mg/dL)</td>
<td>8.6-10.2</td>
<td>9.67±0.07</td>
<td>9.66±0.08</td>
<td>9.66±0.08</td>
<td>9.66±0.08</td>
<td>9.65±0.08</td>
</tr>
<tr>
<td>Blood urea nitrogen (mg/dL)</td>
<td>6-20</td>
<td>20.7±1.2</td>
<td>20.8±1.3</td>
<td>20.7±1.3</td>
<td>20.6±1.3</td>
<td>20.6±1.4</td>
</tr>
<tr>
<td>Creatinine (mg/dL)</td>
<td>0.5-1.2</td>
<td>0.73±0.02</td>
<td>0.74±0.03</td>
<td>0.74±0.03</td>
<td>0.74±0.03</td>
<td>0.74±0.03</td>
</tr>
<tr>
<td>Total protein (g/dL)</td>
<td>6.0-8.3</td>
<td>6.97±0.08</td>
<td>6.97±0.09</td>
<td>6.97±0.09</td>
<td>6.97±0.09</td>
<td>6.98±0.09</td>
</tr>
<tr>
<td>Albumin (g/dL)</td>
<td>3.5-5.2</td>
<td>4.34±0.05</td>
<td>4.34±0.06</td>
<td>4.33±0.06</td>
<td>4.33±0.06</td>
<td>4.33±0.06</td>
</tr>
<tr>
<td>Measurements</td>
<td>Reference Range</td>
<td>Baseline&lt;sup&gt;2&lt;/sup&gt;</td>
<td>Tx A (inulin + placebo)&lt;sup&gt;3&lt;/sup&gt;</td>
<td>Tx B (inulin + probiotic)&lt;sup&gt;4&lt;/sup&gt;</td>
<td>Tx C (control + placebo)&lt;sup&gt;5&lt;/sup&gt;</td>
<td>Tx D (control + probiotic)&lt;sup&gt;6&lt;/sup&gt;</td>
</tr>
<tr>
<td>------------------------------------------</td>
<td>-----------------</td>
<td>-----------------------</td>
<td>-------------------------------------</td>
<td>-------------------------------------</td>
<td>-------------------------------------</td>
<td>-------------------------------------</td>
</tr>
<tr>
<td>Alanine transaminase (U/L)</td>
<td>0-33</td>
<td>16.6±1.3</td>
<td>16.6±1.4</td>
<td>16.6±1.4</td>
<td>16.7±1.4</td>
<td>16.8±1.4</td>
</tr>
<tr>
<td>Alanine aminotransferase (U/L)</td>
<td>0-32</td>
<td>21.7±1.0</td>
<td>21.7±1.0</td>
<td>21.7±1.0</td>
<td>21.7±1.0</td>
<td>21.7±1.1</td>
</tr>
<tr>
<td>Alkaline phosphatase (U/L)</td>
<td>40-129</td>
<td>72.1±3.5</td>
<td>72.0±3.7</td>
<td>72.0±3.8</td>
<td>72.1±3.7</td>
<td>72.1±3.9</td>
</tr>
<tr>
<td>C-reactive protein (mg/dL)&lt;sup&gt;7&lt;/sup&gt;</td>
<td>0.10-0.50</td>
<td>0.50±0.13</td>
<td>0.72±0.29</td>
<td>0.65±0.26</td>
<td>0.55±0.12</td>
<td>0.56±0.16</td>
</tr>
</tbody>
</table>

<sup>1</sup>Abbreviation: Tx, treatment; kg, kilogram; mg, milligram; dL, deciliter; calc, calculated; mmol, millimole; U, unit; L, liter; HDL, high-density lipoprotein; LDL, low-density lipoprotein; VLDL, very-low-density lipoprotein.

Blood was drawn and laboratory analysis conducted at the end of the two-week baseline period and at the end of each treatment period.

<sup>2</sup>For baseline laboratory testing, data from all participants was analyzed (n=26). Participant weight was recorded for all participants (n=26).

<sup>3</sup>For Tx A, laboratory testing data was analyzed from 23 participants (PAM 08,13,20 excluded from analysis; no sample collected). Additionally, for Tx A results for cholesterol, triglycerides, HDL, LDL and VLDL for PAM 29 were missing (lab did not perform). Participant weight was recorded for 23 participants (PAM 08,13,20 excluded).

<sup>4</sup>For Tx B, laboratory testing data was analyzed from 22 participants (PAM 06,08,20,28 excluded from analysis; no sample collected). Participant weight was recorded for 22 participants (PAM 06,08,20,28 excluded).

<sup>5</sup>For Tx C, laboratory testing data was analyzed from 23 participants (PAM 08,13,20 excluded from analysis; no sample collected). Participant weight was recorded for 23 participants (PAM 06,08,13,20 excluded).

<sup>6</sup>For Tx D, laboratory testing data was analyzed from 21 participants (PAM 06,13,20,23,28 excluded from analysis; no sample collected). Participant weight was recorded for 21 participants (PAM 06,13,20,23,28 excluded).

<sup>7</sup>For C-reactive protein, a laboratory value of <0.3 was adjusted to 0.30 to calculate arithmetic means and the standard error for baseline and treatment periods.
For baseline n=26, Tx A (n=22; PAM 08, 13, 20 excluded and a CRP value was not reported for PAM 24), Tx B (n=22; PAM 06, 08, 20, 28 excluded), Tx C (n=23; PAM 08, 13, 20 excluded), and Tx D (n=20; PAM 06, 13, 20, 23, 28 excluded and a CRP value was not reported for PAM 05).

All values are reported as arithmetic means ± SE
Table 4-13. Urinary metabolites and 24 hr nitrogen measured at the end of treatment periods compared to participant baseline values\(^1\)

<table>
<thead>
<tr>
<th>Measurements</th>
<th>Reference Range</th>
<th>Baseline (inulin + placebo)</th>
<th>P value</th>
<th>Tx A (inulin + placebo)</th>
<th>P value</th>
<th>Tx A (probiotic)</th>
<th>P value</th>
<th>Tx B (control + placebo)</th>
<th>P value</th>
<th>Tx C (control + probiotic)</th>
<th>P value</th>
<th>Tx D (control + probiotic)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>cresol sulfate (ug/mL)(^2)</td>
<td>-</td>
<td>4.39±0.20</td>
<td>NS</td>
<td>4.66±0.15</td>
<td>NS</td>
<td>4.76±0.17</td>
<td>NS</td>
<td>4.73±0.19</td>
<td>NS</td>
<td>4.78±0.20</td>
<td>NS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>phenylacetyl glutamine (ug/mL)(^3)*</td>
<td>-</td>
<td>5.40±0.15</td>
<td>0.024</td>
<td>5.75±0.12</td>
<td>0.021</td>
<td>5.86±0.14</td>
<td>0.019</td>
<td>5.82±0.13</td>
<td>0.013</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>indoxyl sulfate (ug/mL)(^4)</td>
<td>-</td>
<td>4.05±0.16</td>
<td>NS</td>
<td>4.32±0.12</td>
<td>NS</td>
<td>4.30±0.14</td>
<td>NS</td>
<td>4.43±0.15</td>
<td>NS</td>
<td>4.49±0.14</td>
<td>NS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>24 hr Nitrogen (g/24 h)(^5)***</td>
<td>12.0-20.0</td>
<td>8.31±0.73</td>
<td>&lt;.0001</td>
<td>13.53±0.77</td>
<td>&lt;.0001</td>
<td>13.07±0.77</td>
<td>&lt;.0001</td>
<td>13.75±0.77</td>
<td>&lt;.0001</td>
<td>13.12±0.77</td>
<td>&lt;.0001</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^1\)Abbreviations: Tx, treatment; ug, microgram; mL, milliliter; g, gram; hr, hour; log, logarithm.

Urine was collected from participants at the end of the two-week baseline period and at the end of each two-week treatment period for analysis.

\(^2\)Cresol sulfate was analyzed utilizing two models to analyze metabolite values, both log-transformations. One model looked for a possible sequence effect and the other effect of treatment on metabolite. The model was a random effect of subject and heterogeneous variance. Effect of sequence (p=0.046), effect of day (p=0.233) and sequence*day (p=0.626) are reported. The overall model (main effect of treatment) was not statistically significant (p=0.314). Data from all participants was analyzed (n=26).

\(^3\)Phenylacetyl glutamine was analyzed utilizing two models to analyze metabolite values, both log-transformations. One model looked for a possible sequence effect and the other effect of treatment on metabolite. The model was a random effect of subject and heterogeneous variance. Effect of sequence (p=0.176) was not statistically significant, effect of day (p=0.005) was significant, however sequence*day (p=0.623) was not statistically significant. The overall model (main effect of treatment) was statistically significant (p=0.022). Data from all participants was analyzed (n=26).

\(^4\)Indoxyl sulfate was analyzed utilizing two models to analyze metabolite values, both log-transformations. One model looked for a possible sequence effect and the other effect of treatment on metabolite. The model was a random effect of subject and heterogeneous variance. Effect of sequence (p=0.398) was not statistically significant, effect of day (p=0.023) was significant,
however sequence*day (p=0.375) was not statistically significant. The overall model (main effect of treatment) was not statistically significant (p=0.053). Data from all participants was analyzed (n=26).

Nitrogen was analyzed using a general linear mixed model with a random subject effect and a fixed of treatment. The overall model (main effect) was statistically significant (p<0.0001). Data from all participants was analyzed (n=26).

Data are presented as Least Sq. Means ± SE with *p<0.05; **p<0.01; ***p<0.001
Table 4-14. Handgrip strength measured as maximum grip strength, maximum of dominant and maximum of non-dominant hand

<table>
<thead>
<tr>
<th>Period</th>
<th>Dominant(^2)</th>
<th>Non-dominant(^3)</th>
<th>Maximum(^4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline(^5)</td>
<td>20.3±0.98</td>
<td>18.3±0.90</td>
<td>20.9±0.96</td>
</tr>
<tr>
<td>Tx A (inulin + placebo)(^6)</td>
<td>21.8±1.00</td>
<td>18.4±0.99</td>
<td>21.6±1.00</td>
</tr>
<tr>
<td>Tx B (inulin + probiotic)(^7)</td>
<td>20.6±1.10</td>
<td>18.5±1.04</td>
<td>21.6±1.05</td>
</tr>
<tr>
<td>Tx C (control + placebo)(^8)</td>
<td>20.7±1.06</td>
<td>18.6±0.99</td>
<td>21.6±1.00</td>
</tr>
<tr>
<td>Tx D (control + probiotic)(^9)</td>
<td>20.7±1.12</td>
<td>18.7±1.04</td>
<td>21.6±1.00</td>
</tr>
<tr>
<td>Final(^10)</td>
<td>20.4±1.09</td>
<td>18.4±1.00</td>
<td>20.9±1.06</td>
</tr>
</tbody>
</table>

\(^1\)Abbreviations: Tx, treatment; kg, kilogram.
Hand grip strength (recorded in kg) was measured at the end of baseline, treatment periods and at the end of 18-week study.
\(^2\)The maximum recorded measurement from the participants’ dominant hand was used for analysis.
\(^3\)The maximum recorded measurement from the participants’ non-dominant hand was used for analysis.
\(^4\)The maximum recorded measurement from the participant was used for analysis.
\(^5\)For Baseline, data from n=26 participants included in analysis.
\(^6\)For Tx A, data from n=23 participants included in analysis (PAM 08,13,20). Measurement for PAM 15 non-dominant hand not taken (injury on non-dominant hand); reported highest value for dominant measurement as maximum for analysis.
\(^7\)For Tx B, data from n=21 included in analysis (PAM 03,06,08,20,28 excluded). Measurement for PAM 03 not taken for Tx B.
\(^8\)For Tx C, data from n=23 included in analysis (PAM 08,13,20 excluded).
\(^9\)For Tx D, data from n=21 included in analysis (PAM 06,13,20,23,28 excluded).
\(^10\)For time point, Final data from n=21 included in analysis (PAM 06,08,13,20,28 excluded).
Data are presented as Means ± SE
CHAPTER 5
DISCUSSION AND CONCLUSIONS

The primary objective of this study was to determine the effects of a high-protein diet on fecal microbial communities (microbiota, diversity and profile) in older community dwelling adults, specifically women. Based on previous findings, it was hypothesized that consuming a high-protein diet in a cohort of older women (≥65 yrs) would result in undesirable changes in gut microbiota. Specifically, a reduction in overall diversity of microbiota, including suppression of Bifidobacteria spp., the proportions and promotion of Clostridia spp. and ammonia-producing bacteria. However, these outcomes were not observed.

Results demonstrated that there were no phyla level changes on microbiota from baseline to a high-protein diet. Our results indicate that there is a core microbiota (consisting of Blautia, family Lachnospiraceae, Coprococcus, family Ruminococcaceae, order Clostridiales, and Bacteroides) (Figure 4-9 and Figure 4-10) that is relatively consistent between all the participants, but for the less abundant microbiota there is a lot inter-individual variation. Large inter-individual variation among participants was observed and is consistent with previous research examining fecal microbiota composition in older adults\(^{203}\).

Claesson et al. reported that in community-dwelling older adults there are higher proportions of the phylum Firmicutes. Additionally, Claesson et al. reported in community-dwelling participants compared to individuals residing in residential care at a genus level, Coprococcus and Roseburia where observed at a higher abundance\(^{144}\). These observations are consistent with our findings that were observed in Figure 4-10 of the most abundant OTUs. At the genus level, we observed the relative abundance of
Coprococcus and Roseburia among the 20 most abundant OTUs at baseline and throughout all treatment and washout periods. The family Lachnospiraceae was the most dominant reported by Claesson et al. This was supported by our results where the family Lachnospiraceae was among the second most abundant among baseline and all treatment periods.

In addition to the age related decline in bifidobacteria that has been reported, additional perturbations have been previously demonstrated in frail older adults\(^{(204)}\). Relative abundance of Bifidobacterium is reported in Figure 4-12 and did not significantly change on the high-protein diet or alternate treatments.

Frailty and microbiota have been previously investigated using the GFI\(^{(205)}\). Researchers grouped older adults into two groups: high frailty (GFI score of 5 or more) or low frailty (GFI score 1 to 4). Researchers reported that individuals in the high frailty group had a significant decrease in lactobacilli. Additionally, Bacteroides, Prevotella and Faecalibacterium prausnitzii significantly decreased and Enterobacteriaceae increased. While in our study at baseline, there were only two participants with a GFI score of 5 or more, making it difficult to create groups to further analyze microbiota data based on frailty.

Examining the twenty most abundant OTUs, we are able to identify the family Enterobacteriaceae and Bacteroides. Bacteroides is one of the most predominant genera and is involved in carbohydrate digestion in the large intestine. Other Bacteroides have also been discovered as opportunistic pathogens\(^{(206)}\). Enterobacteriaceae, gram-negative bacteria shown to increase with age contain
symbionts but also contain potential pathogens and disease causing bacteria (i.e. *Salmonella, Escherichia coli*, etc.).

Additionally, our results demonstrated that there were no phyla level changes between baseline, a high-protein diet, a high-protein diet with probiotic, a high-protein diet with fiber, or high-protein diet with synbiotic. Significant changes in OTUs were observed and these changes/shifts may provide insight to potential health implications. However, these results should be viewed cautiously and additional research is needed with a larger number of participants and replicates to broaden these findings.

These study findings regarding gut microbiome are in some disagreement with previous work on the microbiota composition of a high-protein diet. David et al. examined the microbiomes response to altered diet<sup>138</sup>. In the study conducted by this group, a plant-based diet was compared with an animal-based diet and both were provided *ad libitum* to participants. Diets were consumed by healthy adult men and women for a 5-day period. The animal diet was composed of 30.1±0.5% kcal protein and 69.5±0.4% dietary fat and fiber intake approximately zero. This study did not control intake or provide a weight maintenance diet. Additionally, for many a high-protein animal-based diet is largely unrealistic for many, especially among older adults. Researchers did report that the microbiome responded to short term changes in macronutrients and in the high-protein animal based diet displayed functional properties of carnivores affecting the resident microbiota. Microbiota and 16S data from our current study suggests that when provided a controlled-diet with mixed protein sources and a fiber intake consistent with U.S. intake disruptions in the microbiota are not seen over a short term period. Thus, this diet was also feasible and acceptable as noted by study
participants. All foods were purchased from local food suppliers or commercially available and simulated a more typical and attainable diet for many older adults.

Results from qPCR data (Figure 4-13) indicate that probiotic strains were quantifiable with specified primers for *L. acidophilus* HA122, *L. plantarum* HA119 and *B. bifidum* HA132. These strains were detected at the specified treatment periods when the probiotic was administered, treatment B (inulin+probiotic) and treatment D (control+probiotic). Indicating that a 2-week washout was not adequate and strains may have persisted into the following treatment period.

**Effect of Protein Intake on Gastrointestinal Symptoms and Function**

A secondary aim of this research was to explore the effects of high-protein diet on measures of gastrointestinal and digestive health in older adults. The effects of a high protein diet on gastrointestinal symptoms (measured with the GSRS and daily questionnaire) was investigated. The potential effects of supplementation with a prebiotic (inulin), probiotics and synbiotic were also investigated while on a high-protein diet.

Gastrointestinal wellness measured by the GSRS and daily (individual and syndrome) questionnaires indicated that scores were significantly higher for abdominal pain when participants received the high protein diet (treatment C; control + placebo) compared to baseline and washouts combined. Reviewing the literature, it was found that in a randomized trial conducted by Skov et al. participants were provided an *ad libitum* reduced fat and high-protein diet (25% protein and 30% fat as percent of total energy) for weight loss. Researchers noted that there were no differences in gastrointestinal discomfort or symptoms (participants were asked about abdominal symptoms and discomforts (specifically rumbling, constipation, diarrhea and abdominal
pain after a meal). These results are largely in agreement from the findings of this study. In the study by Skov et al., protein was approximately 25% of total daily energy intake and in our study participants were provided approximately 29%. Our findings demonstrate there were no significant increases in gastrointestinal complaints relating to diarrhea, constipation, reflux or indigestion syndrome when participants were receiving only the high-protein diet. However, there was a statistically significant increase in abdominal pain (measured using the GSRS) and gastrointestinal distress (component score assessed using the daily questionnaire) compared to the baseline and washout periods combined. While statistically significant increases were observed for abdominal pain and gastrointestinal distress on the high-protein diet alone, the mean score reported was not clinically significant.

Gastrointestinal symptoms of abdominal pain and indigestion (assessed by the GSRS) were significantly higher for periods when inulin was consumed (treatment A–inulin+placebo and treatment B—inulin+probiotic) compared to baseline and washout combined. However, the mean score reported was still below clinical significance. Indigestion syndrome also increased significantly on treatment A (inulin + placebo) and treatment B (inulin + probiotic) compared to baseline and washout combined. Symptoms for indigestion were statistically higher when participants were receiving the inulin but the mean was again not clinically significant.

Fermentation products of prebiotics include SCFAs and gases (H₂ and CO₂). The production of gases via fermentation are the cause of gastrointestinal discomfort/symptoms in clinical settings. Inulin has previously demonstrated impaired gastrointestinal tolerance in some clinical studies. Pederson et al. supplemented a low-
fat diet with 14 g of inulin in healthy young women (n=64) and reported that self-reported gastrointestinal symptoms (assessed using a health questionnaire) were higher when the inulin was given compared to control. Gastrointestinal symptoms assessed by Pederson et al. included: stomach noises/rumbling, rumbling in the gut, nausea, burping/belching, cramping, bloated, reflux, flatulence, diarrhea and vomiting\(^\text{(208)}\). Discomfort was reported for stomach rumbling, rumbling in gut, cramping, bloated and flatulence. Flatulence was rated most severe by participants when consuming inulin. These findings are consistent with the increase in abdominal pain and indigestion reported by participants in this study. The discrepancy in the severity and lack of other symptoms is likely due to the dose amount of inulin provided in this study.

Pederson et al. reported no adaption to the inulin as indicated by the symptoms and this is consistent with the results of this study as indicated by symptoms being reported during both treatments when inulin was provided\(^\text{(208)}\).

Bowel movement frequency and transit (measured by the Bristol Stool Form Scale) did not change from baseline when given the high-protein or accompanying interventions. The supplementation of inulin did not have any effect on bowel movement frequency. The literature suggests that inulin does not affect stool weight (i.e. 2 g increase in weight for each g of inulin consumed)\(^\text{(209)}\). Studies that reported an increase in stool weight were not controlled for diet or intake. Thus, additional research is needed to confirm inulin’s effect on stool weight.

Stool pH increased on the high-protein diet, and this significant increase was observed on the high-protein diet with inulin and the high-protein diet with probiotic. However, this increase from the high-protein diet seemed to be mitigated when provided
the synbiotic. Research has demonstrated that prebiotics, including inulin, can decrease stool pH\textsuperscript{210}.

**Biochemical**

Biochemical measurements (triglyceride, total cholesterol, glucose) did not significantly change and based on previous literature this was expected. A longer duration may impact biochemical measurements, however, short term feeding has not been suggested to alter these parameters. Sacks et al. demonstrated in a controlled diet intervention (high carbohydrate, low glycemic index) a significant increase in LDL levels. Total cholesterol, triglycerides and LDL were unaffected\textsuperscript{211}. Rousell et al. demonstrated in a dietary intervention study a decrease in total cholesterol and LDL in hypercholesterolemic individuals after 5-weeks in individuals adhering to the Dietary Approaches to Stop Hypertension (DASH) diet with 28 g beef/d, DASH diet with 113 g beef/d, and DASH diet with 153 g beef/d compared to a healthy American diet\textsuperscript{212}.

**Protein Intake and Immune Function**

Mixed findings have been reported on the association between the consumption of red meat and coronary events/diseases. Research has proposed an association of red meat intake and systemic inflammation. It has been demonstrated that certain markers of inflammation are associated with increased risk for vascular episodes and/or the increase in certain inflammatory markers may actually contribute to the pathogenesis of certain diseases.

Most of the studies examining associations between red meat consumption, disease, cancer and inflammation are epidemiological studies. In a cross-sectional study, red meat intake in women 40-60 years of age was associated with greater risk of inflammation and metabolic syndrome\textsuperscript{213}. Another cross-sectional study evaluating
participants in the Nurses’ Health Study (N=2690) reported higher CRP levels with high total red meat intake. However, after adjusting for BMI, some was attenuated\(^{(214)}\). A case-control study, using medical history and a food frequency questionnaire suggested that increased consumption of red meat was associated with cardiac disease risk\(^{(215)}\). Additional research is needed to elucidate these relationships using randomized, double-blind placebo controlled clinical studies.

In an 8-week parallel study participants receiving lean red meat (replacement of dietary carbohydrates) did not increase markers of oxidative stress or inflammation\(^{(216)}\). Using a parallel study, the effects of lean red meat on changes in blood pressure and markers of cardiovascular risk were also examined in hypertensive adults\(^{(217)}\). Participants were randomized to a control group and a protein group where they replaced carbohydrates with protein from lean red meat. Systolic blood pressure decreased in the protein group.

Rousell et al. showed that a diet incorporating lean beef (the Dietary Approaches to Stop Hypertension diet was used incorporating lean beef) decreased LDL and total cholesterol in hypercholesterolemic participants after 5 weeks\(^{(212)}\). They also reported no differences in CRP but noted that participants with a baseline CRP value ≥1 mg/L had significant decreases in total cholesterol after adhering to the lean beef diet. Research in elderly women showed that a protein-enriched diet (lean red meat; 160 g) with resistance training for 4 months decreased IL-6 by 16\%\(^{(218)}\).

In our study, the high-protein diet did not increase levels of CRP, IL-6 or TNF- in the serum. There was no change in IL-10 and anti-inflammatory cytokine. Red meat was included in the high-protein diet provided to participants during each treatment
period. Our findings with the results of Rousell et al. provide supporting evidence that animal protein provided as lean red meat can be incorporated into the diet and without increasing inflammatory markers\(^{212}\). Thus, the inflammatory markers measured in this study (CRP, IL-6, and TNF-\(\alpha\)) and Rousell et al. did not increase after a short-term dietary intervention\(^{212}\).

**Protein Intake and Body Composition**

Aging is associated with a decline in lean mass and increase in fat mass. Research examining the decline of skeletal muscle associated with age, known as sarcopenia, has demonstrated higher levels of mortality and impairment in these individuals. The data from this study suggests that older women on an intermittent high-protein may alter body composition. Specifically, an increase in fat free mass. A trend was also demonstrated for decreasing fat and percent fat. This was a surprising result considering the study duration and that it was a weight-maintenance study. Previous research has shown that dietary protein intake was associated with a decreased loss of lean mass\(^{43}\). Protein intake, assessed using a food-frequency questionnaire in older men and women, used DEXA to measure changes in body composition after a three-year period. Researchers reported that individuals reporting protein intake at the highest quintile lost 40% less lean mass and appendicular lean mass than those reporting the lowest intake of dietary protein. While this research shows an association, the use of a food frequency has limitations\(^{43}\).

Additional research should be conducted over a longer period to determine if these trends become significant over time and with increasing statistical power.

Research has previously demonstrated the helpful effects resistance training and exercise to combat the loss of muscle strength that occurs with age. Research suggests
that dietary protein intake is required to maximize skeletal mass during resistance exercise. The current consensus for counteracting sarcopenia and frailty is to recommend optimal dietary protein intake and resistance exercise activities in individuals at risk. In a group of frail elderly participants consuming supplementation with dietary protein (twice a day with 15 g protein) and a resistance exercise training program for 24 weeks compared to a control group only conducting the exercise program the participants receiving the protein increased lean body mass. Strength and physical performance increased in both control and protein supplemented group\(^{(219)}\).

The results of the present study are among the first to show an increase in lean mass and decrease in fat mass in older adults on an intermittent high-protein diet without added resistance exercise. Participants were asked to maintain their usual physical activity level and stable weight suggests that participants were compliant. The potential implication of these findings are significant given the prevalence and health care related costs of sarcopenia.

Hunger was also assessed with the daily questionnaire. Participants were asked to self-report hunger daily throughout the 18-week study and results demonstrated a significant decrease during all intervention periods. This suppression in hunger was also observed and carried into all washout periods. Previous research on high-protein diets and their effect in hunger have been primarily limited to weight loss studies\(^{(220, 221)}\). In the current literature this study is among the first to indicate that in a controlled study a high-protein diet can reduce hunger and maintain weight in older women.

**Protein Intake and Metabolites of Fermentation**

P-cresyl-sulfate and indoxyl sulfate, both protein bound solutes are the major metabolites produced from microbial metabolism in the colon. Colonic microbial
metabolite from aromatic amino acid fermentation result in phenolic and indolic compounds. Most phenolic compounds are excreted in the urine as p-cresol\(^{(107)}\). P-cresol is the metabolite produced by the fermentation of tyrosine and indoxyl sulfate the metabolite produced from tryptophan metabolism.

Urinary excretion of p-cresyl-sulfate and indoxyl sulfate did not differ from baseline on the high-protein diet. However, phenylacetyl glutamine (ug/mL) did increase significantly on the high protein intervention and this increase was not mitigated/reduced on high-protein treatments with a probiotic, prebiotic or synbiotic.

Phenylacetyl glutamine is also produced by microbial metabolism of phenylalanine but has a lower protein binding affinity compared to P-cresol sulfate and indoxyl sulfate. While research on phenylacetyl glutamine still remains novel it has been identified as an independent risk factor for mortality and cardiovascular disease\(^{(111)}\).

Barrios et al. investigated microbiota profiles in 855 individuals with renal function decline (minimal decline) with metabolites for any associations. OTUs that were significantly associated with p-cresyl-sulfate, indoxyl sulfate and phenylacetyl glutamine were reported.

**Protein Intake and Measures of Quality of Life and Frailty**

Our study did not demonstrate any significant effects of a high-protein diet on measured of QoL or frailty. Literature is limited regarding the effect of dietary interventions and changes in quality of life. We did not observe any statistical differences in health related quality of life, specifically domains measuring physical functioning, role-physical, bodily pain, general health, vitality, social functioning, role-emotional and mental health), MCS, or PCS between suggesting that the diet was well tolerated among older women.
Frailty measured using the GFI did not statically change throughout the intervention. However, at baseline only four participants were classified with “moderate to severe frailty” (defined by a GFI score of four or higher out of a maximum score of 15) thus dividing our participants into groups based on frailty status was not statistically possible.

**Study Limitations**

A limitation of this study is the study length. While the complete study was 18-weeks with only two weeks for each intervention and washout periods it may not have been adequate to see statistical and clinical implications for some outcomes.

The qPCR data suggest that after probiotic intake strains were detected and measured into the intervention periods. While some participants harbored resonant strains that were quantified it was also shown that in some participants strains were quantified in the washout period following the treatment period. Thus, two weeks may not be long enough and additional time may be needed.

Additionally, future studies may choose to use the baseline stool sample to stratify participants based on microbiota composition. As noted previously there is a lot of inter-individual variation among older adults, and this study provided additional evidence that to examine changes after the administration of a probiotic, prebiotic and synbiotic stratifying may be needed to classify participants into responders and non-responders.

One of the significant findings measured in this study was the change in body composition noted during the 18-week study. A change in lean mass and decrease of fat mass has potential major implications among older adults and clinical
recommendation. However, future studies should use diagnostic measurements to quantify changes in body composition like DEXA scanning, etc.

**Conclusions**

A high-protein diet (1.5-2.2 g protein/kg body weight), composed of commercially available foods and that maintains the AMDR for each macronutrient, did not demonstrate any major disruptions in microbiota or clinically relevant gastrointestinal symptoms or complaints. Furthermore, the high protein diet had positive effects on body composition (changes in lean mass and fat mass). A high protein-diet further supplemented with a multi-strain probiotic or synbiotic may have benefits on gastrointestinal wellness.

Therefore, a high-protein diet may have health advantages and this research provides scientific support for a higher protein diet in older women, a group who may benefit from the physiological benefits of increasing dietary protein intake. However, a high-protein diet significantly elevates the urinary metabolite phenylacetyl glutamine but not indoxyl sulfate or cresol sulfate. This significant increase was not mitigated by probiotic, prebiotic or synbiotic. This is an area requiring further investigation and evaluation of physiological relevance. Additional studies are needed to further elucidate these finding but these results will further contribute to the body of literature in support of increasing dietary protein recommendations in older adults.
DATE: 4/10/2015
TO: Wendy Dahl 
359 FSHN Building Newell Drive 
Gainesville, Florida 32611
FROM: Peter Iafrate, Pharm.D 
Chair IRB-01
IRB#: IRB201400955
TITLE: The effects of a high protein diet on microbota, gastrointestinal function, wellness and the potential mitigating effects of a multi-strain probiotic, prebiotic, and symbiotic: a randomized, double-blinded crossover study in older women.

You have received IRB approval to conduct the above-listed research project. Approval of this project was granted on 4/6/2015 by IRB-01. This study is approved as expedited because it poses minimal risk and is approved under the following expedited categories:

2. Collection of blood samples by finger stick, heel stick, ear stick, or venipuncture as follows: a. Subjects are healthy, non-pregnant adults who weigh at least 110 pounds; amount drawn may not exceed 550 ml over 8 weeks; and collection may not occur more frequently than 2 times per week. OR b. Subjects are other adults and children*, considering the age, weight, and health of the subjects; the collection procedure; the amount of blood to be collected; and the frequency with which it will be collected. For these subjects, the amount collected may not exceed the lesser of 50 ml or 3 ml per kg over 8 weeks, and collection may not occur more frequently than 2 times per week. [* - Children are defined in the HHS regulations as "persons who have not attained the legal age for consent to treatments or procedures involved in the research, under the applicable law of the jurisdiction in which the research will be conducted." (45 CFR 46.402(a).)]

3. Prospective collection of biological specimens for research purposes by noninvasive means. [ Examples: (a) hair and nail clippings, if collected in a non-disfiguring manner; (b) deciduous teeth at time of exfoliation or if routine patient care indicates a need for extraction; (c) permanent teeth, if routine patient care indicates a need for extraction; (d) excreta and external secretions (including sweat); (e) uncannulated saliva collected either in an unstimulated fashion or stimulated by chewing gumbase or wax or by applying a dilute citric solution to the tongue; (f) placenta removed at delivery; (g) amniotic fluid obtained at the time of rupture of the membrane before or during labor; (h) supra- and subgingival dental plaque and calculus, provided the collection procedure is not more invasive than routine prophylactic scaling of the teeth and the process is accomplished in accordance with accepted prophylactic techniques; (i) mucosal and skin cells collected by buccal scraping or swab, skin swab, or mouth washings; (j) sputum collected after saline mist nebulization. ]
2. Collection of data through noninvasive procedures (not involving general anesthesia or sedation) routinely employed in clinical practice, excluding procedures involving x-rays or microwaves. Where medical devices are employed, they must be cleared/approved for marketing. (Studies intended to evaluate the safety and effectiveness of the medical device are not generally eligible for expedited review, including studies of cleared medical devices for new indications). [ Examples: (a) physical sensors that are applied either to the surface of the body or at a distance and do not involve input of significant amounts of energy into the subject or an invasion of the subject’s privacy; (b) weighing or testing sensory acuity; (c) magnetic resonance imaging; (d) electrocardiography, electroencephalography, thermography, detection of naturally occurring radioactivity, electroretinography, ultrasound, diagnostic infrared imaging, doppler blood flow, and echocardiography; (e) moderate exercise, muscular strength testing, body composition assessment, and flexibility testing, where appropriate to the age, weight, and health of the individual. ]

7. Research on individual or group characteristics or behavior (including, but not limited to, research on perception, cognition, motivation, identity, language, communication, cultural beliefs or practices, and social behavior) or research employing survey, interview, oral history, focus group, program evaluation, human factors evaluation, or quality assurance methodologies. [ Note: Some research in this category may be exempt from the HHS regulations for the protection of human subjects. (45 CFR 46.101(b)(2) and (b)(3).) This listing refers only to research that is not exempt. ]

**Approval Includes, but is not limited to:**

- Dated and watermarked IRB-approved Informed Consent Form

**Special notes to Investigator:** None

**Reviewer Notes:** 0 Reviewer Notes

**Principal Investigator Responsibilities:**

The PI is responsible for the conduct of the study. Please review these responsibilities described at: [http://irb.ufl.edu/irb01/researcher-information/researcherresponsibilities.html](http://irb.ufl.edu/irb01/researcher-information/researcherresponsibilities.html)

Important responsibilities described at the above link include:

- Using currently approved consent form to enroll subjects (if applicable)
- Renewing your study before expiration
- Obtaining approval for revisions before implementation
- Reporting Adverse Events
- Retention of Research Records
- Obtaining approval to conduct research at the VA
- Notifying others about this project’s approval status

**CC:** Amanda Ford
      Nancy Gal
INTRODUCTION

Name of person seeking your consent: __________________________

Place of employment & position: ________________________________________

Please read this form which describes the study in some detail. A member of the research team will describe this study to you and answer all of your questions. Your participation is entirely voluntary. If you choose to participate you can change your mind at any time and withdraw from the study. You will not be penalized in any way or lose any benefits to which you would otherwise be entitled if you choose not to participate in this study or to withdraw. If you have questions about your rights as a research subject, please call the University of Florida Institutional Review Board (IRB) office at (352) 273-9600.

GENERAL INFORMATION ABOUT THIS STUDY

1. Name of Participant ("Study Subject")

_____________________________________

2. What is the Title of this research study?

   The effects of a high protein diet on microbiota, gastrointestinal function, wellness and the potential mitigating effects of a multi-strain probiotic, prebiotic, and synbiotic: a randomized, double-blinded crossover study in older women.
3. Who do you call if you have questions about this research study?

Principal Investigator: Wendy Dahl, PhD, RD
Cell: 352-226-1773
Email: wdahl@ufl.edu

Co-Investigator: Amanda Ford, MS
Study phone: 352-263-9136
Cell: 352-317-6969
Email: ufnutrition@ifas.ufl.edu

4. Who is paying for this research study?

The sponsor of this study is Lallemand Health Solutions and the University of Florida.

5. Why is this research study being done?

The purpose of this study is to determine the effects of consuming a higher protein diet with and without a probiotic and fiber supplement on gastrointestinal function, survival through the gastrointestinal tract and general well-being in healthy older women.

You are being asked to be in this research study because you are a healthy woman 65 years of age or older and meet the inclusion and exclusion criteria.

A description of this clinical trial will be available on http://www.ClinicalTrials.gov, as required by U.S. Law. This Web site will not include information that can identify you. At most, the Web site will include a summary of the results. You can search this Web site at any time.

WHAT CAN YOU EXPECT IF YOU PARTICIPATE IN THIS STUDY?

6. What will be done as part of your normal clinical care (even if you did not participate in this research study)?

This study is not related to your normal clinical care.
7. What will be done only because you are in this research study?

This study is divided into 3 periods: baseline, study treatment periods (when you will receive a controlled high protein diet with a probiotic, fiber, or probiotic and fiber), and wash-out periods.

If you decide to take part in this study, following informed consent, you will be asked to review the inclusion/exclusion criteria. Additionally, you will be asked to complete the following as part of the 2 week baseline period:

- Complete 3 dietary recalls. Each recall is a 24-hour dietary recall, in which you will be asked to report all the food and beverages you consumed over the previous 24-hour period.
- Wear SenseWear Pro Armband (BodyMedia, Inc.) to monitor your energy expenditure for 7 days.
- Turn in the SenseWear Pro Armband after the 7 day period.
- Complete daily and weekly questionnaires that will ask you to report questions regarding your gastrointestinal health (bowel movement frequency and questions regarding gastrointestinal and other symptoms, i.e. constipation, diarrhea, abdominal cramping, etc.) and general wellness (if you consumed an antibiotic, visited a doctor, etc.).

Next, if you still meet the screening criteria, you will be scheduled to come in for a study appointment and randomization. On the day of randomization or prior to randomization your height, weight, body compositions via Bioelectrical Impedance Analysis (BIA) and demographic information will be obtained. To assess body composition study staff will use BIA, the procedure will take approximately 10-15 minutes and you should not experience any discomfort. You will be asked to take off one shoe and sock and lie on the exam table. Electrodes will be placed on your hand and foot and will analyze how much fat and lean body tissue you have.

You will also continue the daily questionnaire, weekly questionnaire, complete a quality of life questionnaire (will ask you to report and will contain questions regarding your physical health, mental health, etc.), and the Groningen Frailty Indicator questionnaire (asks you to report your ability to complete activities of daily living and questions regarding your general health, i.e. mood, memory, etc.). You will be asked to continue completing these questionnaires throughout the entire study. Hand grip will also be assessed on or before the randomization appointment and at the end of each treatment and washout period. To assess hand grip strength you will be asked to hold a device called a dynamometer that measures force and will be asked to squeeze the dynamometer with as much effort as possible, hand grip strength will be measured in both your left and right hand. Researchers will demonstrate and show you how hand grip strength will be assessed and the process will take approximately 5-10 minutes to complete.
You will then be randomly assigned (much like the flip of a coin) to receive all of the following in random order: a probiotic capsule and fiber sachet for 2-weeks, a probiotic capsule and placebo sachet for 2-weeks, a fiber sachet and placebo capsule for 2-weeks, and placebo capsule and placebo sachet for 2-weeks. Each treatment period is separated by a 2-week washout. The sachets are small disposable baggies that will contain the fiber or placebo. The fiber or placebo in the sachet should be mixed and dissolved into a beverage that you will be asked to consume.

A placebo is a substance that looks like and is given in the same way as an experimental treatment but contains no medicine, for example a sugar pill. A placebo is used in research studies to show what effect a treatment has compared with taking nothing at all. When you are assigned to receive the placebo, you will not receive the benefits of the probiotic or fiber. If there are any, nor will you be exposed to its risks, which are described below under "What are the possible discomforts and risks?"

Studies have shown, however, that about 1 in 3 persons who take a placebo do improve, if only for a short time. You and study staff and other persons doing the study will not know whether you are receiving placebo pill or probiotic pill and fiber sachet or placebo sachet but that information is available if it is needed. Also, you will have approximately a 100% chance of receiving a probiotic and a 100% chance of receiving placebo. In the remainder of the description of what will be done, both the probiotic, fiber and the placebo will be called "study treatment."

Treatment 1: Fiber sachet and Probiotic pill
Treatment 2: Probiotic capsule and Placebo sachet
Treatment 3: Fiber sachet and Placebo probiotic
Treatment 4: Placebo capsule and Placebo sachet

Treatment 2 and Treatment 4 contain a probiotic supplement consisting of strains that are all commercially available and sold in the United States. The supplements will contain *Bifidobacterium bifidum* HA-132, *Bifidobacterium breve* HA-129, *Bifidobacterium longum* HA-135, *Lactobacillus acidophilus* HA-122 and *Lactobacillus plantarum* HA-119

Treatment 2 and Treatment 4 contain 5 g of chicory root fiber as the fiber source and a high protein diet with the probiotic and fiber for 2-weeks. You will be asked to consume the controlled diet (high protein diet) during each treatment period.

During each treatment period, in addition to consuming a probiotic capsule or placebo and/or a fiber sachet or placebo sachet you will be on a controlled diet provided by study staff. The controlled diet is a weight maintenance diet, so study staff will set your individual diet during the controlled periods to your personal energy needs and try to minimize and weight gain or loss throughout the study. The controlled diet consists of foods that provide an average of approximately 2 g/kg/day of protein with additional macronutrient (carbohydrates and fat) sources to raise your energy content to meet your personal requirements. The foods provided to you will be comprised of commercially available frozen meals and pre-packaged foods and may include foods
such as fruits, salads, vegetables, protein drinks, breakfast, lunch and dinner foods, and an assortment of snacks. You will be given all of your food during each treatment period (4 periods total of the provided diet throughout the study and each period consist of 2-weeks) and asked not to consume any foods and beverages that are not provided or approved by study staff. You will be given 4-day rotations of this menu for each 2-week treatment period and all study foods. You will be asked to pick-up your study foods during designated study appointments and/or study foods will be delivered to you by study staff. You will also be asked to record any study foods and/or supplements that are not consumed throughout the study.

During the washout periods (2-week periods) you will not consume the study supplements (probiotics and/or fiber) or the controlled diet. Instead you will be asked to consume your regular diet (not provided by the study) and complete 3 days of 24-hour dietary recalls (you will be asked to report all the food and beverages you consumed over the previous 24-hour period).

You will also be asked to provide blood, fecal and urine samples throughout the study.

At the randomization appointment, a baseline blood sample will be collected and you will begin the study treatment (provided high protein diet with Treatment 1, 2, 3, or 4). Participants will consume 1 capsule (probiotic or placebo) and 1 sachet (fiber or placebo) daily during each 2 week treatment period. Blood samples will also be collected on or around weeks 2, 6, 10 and 14 of the study treatment phase. All blood samples will be drawn by a licensed phlebotomist and approximately 2-3 tablespoons of blood will be drawn at each blood draw appointment. On the day of all scheduled blood draws, you will be scheduled to come in during the morning and you will be asked to fast overnight. A light breakfast will be provided after each blood draw. There will be a total of 5 blood draws throughout the study.

Stool samples will also be collected and analyzed throughout the study. In the baseline period, a stool sample will be collected on or before the randomization appointment. Additional stool samples will be collected on or around weeks 2, 4, 6, 8, 10, 12, 14 and 16 of the study treatment phase. A total of 9 stool samples will be collected throughout the study. Stool sample collection kits will be provided to you. The stool collection kit may be taken home and you will be asked to drop off the stool within 4 hours of defecation or call the study staff who can pick up the stool sample from you (collection kits will be provided and you will be asked to keep the collection kit on ice or use the provided ice packs and cooler).

In addition to blood and stool samples, 24-hour complete urine collections will also be collected and analyzed throughout the study. In the baseline period, a urine sample will be collected on or before the randomization appointment. Additional urine samples will be collected on or around weeks 2, 6, 10 and 14 of the study treatment phase. A total of 5 urine samples will be collected throughout the study. Urine collection containers will be provided to you. The urine collection kit may be taken home and you will be asked to collect all urine during a 24-hour period. You can drop
off the urine collection or call the study staff who can pick up the collection from you (collection kits will be provided and you will be asked to keep the collection kit on ice or use the provided ice packs and cooler). Blood, stool, and urine samples will be analyzed at a UF lab and additional analysis will be sent to a contracted and outside lab for analysis. Blood, stool, and urine samples will not contain your name or contact information; instead samples will be labeled with participant number that is assigned to you after consenting.

You will also be asked to complete daily, weekly, a quality of life questionnaire, and a frailty questionnaire throughout the entire study. Paper questionnaires will be provided to you throughout the study. The daily questionnaire should take approximately 5-7 minutes, the weekly questionnaire will take approximately 5 minutes, and the quality of life questionnaire and frailty questionnaire will each take approximately 5-10 minutes to complete. You will also be provided with a scale and asked to report your fasting weight on the daily questionnaire to ensure that you are receiving a weight maintenance diet throughout the study.

In addition to randomization and blood draw days, you will be asked to come for a final visit. At the final appointment, you will be asked to bring any unconsumed capsules and/or sachets and the Bioelectrical Impedance Analysis (BIA) will be conducted again to assess body composition.

The study coordinator and/or study staff may contact you via phone or email to remind you to complete your stool or urine collections, questionnaires or of study appointments.

If you have any questions now or at any time during the study, please contact one of the research team members listed in question 3 of this form.

If you have any questions now or at any time during the study, please contact one of the research team members listed in question 3 of this form.

8. How long will you be in this research study?

The total amount of time you will spend participating in this study is 18 weeks (126 days).

9. How many people are expected to take part in this research study?

Up to 50 people may participate in this research study.
**WHAT ARE THE RISKS AND BENEFITS OF THIS STUDY AND WHAT ARE YOUR OPTIONS?**

10. What are the possible discomforts and risks from taking part in this research study?

There are no known discomforts or risks associated with the consumption of the commercially available and marketed strains used in this supplement. Additionally, potential discomforts and risks may include:

- Some people may feel uncomfortable when body weight is measured.
- Some people may feel uncomfortable answering questions about their stool habits.
- Some people may feel uncomfortable providing stool samples or 24-hour urine samples.
- Some people may feel uncomfortable answering questions about their physical and emotional health.
- Food allergy symptoms, such as tingling or itching in the mouth, hives, swelling of the lips, tongue, or other body parts, abdominal or diarrhea, or lightheadedness, may be experienced within a few minutes to two hours after consuming supplements if you are allergic to any of the ingredients.
- Some people may experience increased flatulence from the consumption of fiber.

If severe emotional problems are noted participants will be referred to their physician.

Other possible risks to you may include:

- Possible risks associated with drawing blood from a vein include discomfort at the site of puncture; possible bruising and swelling around the puncture site; rarely, an infection; and uncommonly faintness from the procedure. Although proper procedures and steps will be taken to protect all collected information, there is a slight risk that information could be revealed inappropriately or accidentally. Researchers will take appropriate steps to protect any information they collect about you.

- No serious side effects or adverse events have been reported due to the probiotics used in this study or other commonly used commercially available probiotics, although, some isolated case studies have reported side effects such as infection associated with the use of certain probiotics.
Researchers will take appropriate steps to protect any information they collect about you. However, there is a slight risk that information about you could be revealed inappropriately or accidentally. Depending on the nature of the information, such a release could upset or embarrass you, or possibly affect your insurability or employability. Questions 17-21 in this form discuss what information about you will be collected, used, protected, and shared. This study may include risks that are unknown at this time.

Participation in more than one research study or project may further increase the risks to you. If you are already enrolled in another research study, please inform one of the research team members listed in question 3 of this form or the person reviewing this consent with you before enrolling in this or any other research study or project.

Throughout the study, the researchers will notify you of new information that may become available and might affect your decision to remain in the study.

If you wish to discuss the information above or any discomforts you may experience, please ask questions now or call one of the research team members listed in question 3 in this form.

11a. What are the potential benefits to you for taking part in this research study?

You may or may not benefit from your participation in this study. Some individuals may experience improved bowel habits (i.e. less gas and bloating, softer stools).

11b. How could others possibly benefit from this study?

Research into the efficacy of a high protein diet may lead to a better understanding of digestive health and measures of well-being in healthy adults. Additionally, the study results may demonstrate the potential effects probiotics and fiber when consumed with a high protein diet.

11c. How could the researchers benefit from this study?

In general, presenting research results helps the career of a scientist. Therefore, the Principal Investigator listed in question 3 of this form may benefit if the results of this study are presented at scientific meetings or in scientific journals.

12. What other choices do you have if you do not want to be in this study?

If you do not wish to participate in this study, you have the option of not signing this consent form.
13a. Can you withdraw from this study?

You are free to withdraw your consent and to stop participating in this study at any time. If you do withdraw your consent, you will not be penalized in any way and you will not lose any benefits to which you are entitled.

If you decide to withdraw your consent to participate in this study for any reason, please contact one of the research team members listed in question 3 of this form. They will tell you how to stop your participation safely.

If you have any questions regarding your rights as a research subject, please call the Institutional Review Board (IRB) office at (352) 273-9600.

13b. If you withdraw, can information about you still be used and/or collected?

Yes, your information can be used for data analysis.

13c. Can the Principal Investigator withdraw you from this study?

Yes, you may be withdrawn from the study for non-compliance if you do not follow the study instructions given, (e.g. do not consume the study treatments, do not consume the study foods provided, miss required appointments, fail to complete questionnaires, do not provide stool or urine samples, etc.).

Additionally, you may be withdrawn if during the study you no longer meet the inclusion/exclusion criteria.

What are the Financial Issues if You Participate?

14. If you choose to take part in this research study, will it cost you anything?

No, all study materials will be provided to you at no cost while you are participating in this study.

The Sponsor will pay for all probiotic capsules, fiber sachets, lab work, and supplies required as part of your participation in this study as described above in the question “What Will Be Done Only Because You Are in This Research Study”.

If you receive a bill for these services, please contact Wendy Dahl at 352-392-1991 ext 224.

Any other medical services you receive would have been provided to you even if you were not in the study. These services will be billed to you or your insurance company.

You will be responsible for paying any deductible, co-insurance, and/or co-payments for these services, and any non-covered or out-of-network services.
Some insurance companies may not cover costs associated with studies. Please contact your insurance company for additional information.

15. Will you be paid for taking part in this study?

Yes. Participants will receive a maximum of $675 for attending study appointments, blood draw appointments, completing the questionnaires, consuming the high protein diet during study treatments, consuming the supplements provided (probiotic capsule and/or fiber sachet), and turning in stool and urine samples throughout the duration of the study.

Study payment will be pro-rated for early drop-out or withdrawal. Participants will receive $17.50/week ($2.50 per day) for completing study procedures: consuming a probiotic or placebo capsule and fiber or fiber sachet daily for 8 weeks (during treatment periods), consume all study foods and adhere to provided study diet during the treatment periods, complete all questionnaires during all study periods and attend study appointments. A total of $315 will be compensated for the 18 week study. Participants will be compensated $15 per stool and blood sample (9 stool samples and 5 blood samples collected throughout the study) for a total of $210. Participants will be compensated $30 per 24-hour complete urine collection (5 urine collections throughout the study) for a total of $150. Compensation will be provided after the study is completed and will be available for pick-up during the last study visit.

If you are paid more than $75 for taking part in this study, your name and social security number will be reported to the appropriate University employees for purposes of making and recording the payment as required by law. You are responsible for paying income taxes on any payments provided by the study. If the payments total $600 or more or you are a nonresident alien, payment will be processed through the University of Florida Accounts Payable department and the University must report the amount you received to the Internal Revenue Service (IRS). The IRS is not provided with the study name or its purpose. If you have questions about the collection and use of your Social Security Number, please visit: http://privacy.ufl.edu/SSNPrivacy.html.

Your payment for participation in this research study is handled through the University of Florida’s Human Subject Payment (HSP) Program. Your information which will include your name, address, date of birth, and SSN (depending on amount of money you are paid) is protected. Access to the (HSP) Program site is limited to certain staff with the assigned security role. You will be randomly assigned a specific identification (ID) number to protect your identity.

The study team will provide you with an informational form called the Prepaid Card Facts document. If you have any problems regarding your payment call the HSP Office (352) 392-9057.
16. What if you are injured because of the study?

If you are injured as a direct result of your participation in this study, only professional care that you receive from any University of Florida Health Science Center health care provider will be provided without charge. These healthcare providers include physicians, physician assistants, nurse practitioners, dentists or psychologists. Any other expenses, including Shands hospital expenses, will be billed to you or your insurance provider.

You will be responsible for any deductible, co-insurance, or co-payments. Some insurance companies may not cover costs associated with research studies or research-related injuries. Please contact your insurance company for additional information.

The Principal Investigator will determine whether your injury is related to your participation in this study.

No additional compensation is offered. The Principal Investigator and others involved in this study may be University of Florida employees. As employees of the University, they are protected under state law, which limits financial recovery for negligence.

Please contact Wendy Dahl, PhD at 352-226-1773 if you experience an injury or have questions about any discomforts that you experience while participating in this study.

17. How will your health information be collected, used and shared?

If you agree to participate in this study, the Principal Investigator will create, collect, and use private information about you and your health. This information is called protected health information or PHI. In order to do this, the Principal Investigator needs your authorization. The following section describes what PHI will be collected, used and shared, how it will be collected, used, and shared, who will collect, use or share it, who will have access to it, how it will be secured, and what your rights are to revoke this authorization.

Your protected health information may be collected, used, and shared with others to determine if you can participate in the study, and then as part of your participation in the study. This information can be gathered from you or your past, current or future health records, from procedures such as physical examinations, x-rays, blood or urine tests or from other procedures or tests. This information will be created by receiving study treatments or participating in study procedures, or from your study visits and telephone calls. More specifically, the following information may be collected, used, and shared with others:

- Name
- Address
- Your social security number for compensation purposes
- Telephone number
- Email address (if applicable)
- Height, Weight, Body composition, Age, Sex
- Dietary intake data
- Fecal microbial information
- Gastrointestinal symptoms and wellness
- Study questionnaire responses
- Laboratory results
- Blood chemistry information
- Urinary metabolites

This information will be stored in locked filing cabinets or on computer servers with secure passwords, or encrypted electronic storage devices.

Some of the information collected could be included in a "limited data set" to be used for other research purposes. If so, the limited data set will only include information that does not directly identify you. For example, the limited data set cannot include your name, address, telephone number, social security number, photographs, or other codes that link you to the information in the limited data set. If limited data sets are created and used, agreements between the parties creating and receiving the limited data set are required in order to protect your identity and confidentiality and privacy.

18. For what study-related purposes will your protected health information be collected, used, and shared with others?

Your PHI may be collected, used, and shared with others to make sure you can participate in the research, through your participation in the research, and to evaluate the results of the research study. More specifically, your PHI may be collected, used, and shared with others for the following study-related purpose(s):

To determine the effects of consuming a higher protein diet with and without a probiotic and fiber supplement on gastrointestinal function, survival through the gastrointestinal tract and general well-being in healthy older women.

Once this information is collected, it becomes part of the research record for this study.
19. Who will be allowed to collect, use, and share your protected health information?

Only certain people have the legal right to collect, use and share your research records, and they will protect the privacy and security of these records to the extent the law allows. These people include:

- the study Principal Investigator (listed in question 3 of this form) and research staff associated with this project.
- other professionals at the University of Florida or Shands Hospital that provide study-related treatment or procedures.
- the University of Florida Institutional Review Board (IRB; an IRB is a group of people who are responsible for looking after the rights and welfare of people taking part in research).

20. Once collected or used, who may your protected health information be shared with?

Your PHI may be shared with:

- the study sponsor (listed in Question 4 of this form).
- United States and foreign governmental agencies who are responsible for overseeing research, such as the Food and Drug Administration, the Department of Health and Human Services, and the Office of Human Research Protections.
- Government agencies who are responsible for overseeing public health concerns such as the Centers for Disease Control and federal, state and local health departments.

Otherwise, your research records will not be released without your permission unless required by law or a court order. It is possible that once this information is shared with authorized persons, it could be shared by the persons or agencies who receive it and it would no longer be protected by the federal medical privacy law.

21. If you agree to take part in this research study, how long will your protected health information be used and shared with others?

Your PHI will be used and shared with others for two years following study completion.

You are not required to sign this consent and authorization or allow researchers to collect, use and share your PHI. Your refusal to sign will not affect your treatment, payment, enrollment, or eligibility for any benefits outside this research study. However, you cannot participate in this research unless you allow the collection, use
and sharing of your protected health information by signing this consent and authorization.

You have the right to review and copy your protected health information. However, we can make this available only after the study is finished.

You can revoke your authorization at any time before, during, or after your participation in this study. If you revoke it, no new information will be collected about you. However, information that was already collected may still be used and shared with others if the researchers have relied on it to complete the research. You can revoke your authorization by giving a written request with your signature on it to the Principal Investigator.
SIGNATURES

As an investigator or the investigator’s representative, I have explained to the participant the purpose, the procedures, the possible benefits, and the risks of this research study; the alternative to being in the study; and how the participant’s protected health information will be collected, used, and shared with others:

Signature of Person Obtaining Consent and Authorization    Date

You have been informed about this study’s purpose, procedures, possible benefits, and risks; the alternatives to being in the study; and how your protected health information will be collected, used and shared with others. You have received a copy of this Form. You have been given the opportunity to ask questions before you sign, and you have been told that you can ask questions at any time.

You voluntarily agree to participate in this study. You hereby authorize the collection, use and sharing of your protected health information as described in sections 17-21 above. By signing this form, you are not waiving any of your legal rights.

Signature of Person Consenting and Authorizing    Date
APPENDIX C
DIET COMPOSITION OF CONTROLLED HIGH-PROTEIN DIET PROVIDED DURING TREATMENT PERIODS
Table C-1. Energy intake and diet composition, Day A (1-4 diet rotations provided during treatment periods)\(^1\)

<table>
<thead>
<tr>
<th>Item</th>
<th>Weight</th>
<th>Quantity</th>
<th>Energy (kcal)</th>
<th>Protein (g)</th>
<th>Carbs (g)</th>
<th>Fat (g)</th>
<th>Fiber (g)</th>
<th>Sugar (g)</th>
<th>Sodium (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breakfast</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Evol.® Ham, Egg and Cheddar on Multi-grain Flatbread</td>
<td>104 g</td>
<td>3.7 oz</td>
<td>210</td>
<td>14</td>
<td>17</td>
<td>10</td>
<td>2</td>
<td>1</td>
<td>510</td>
</tr>
<tr>
<td>Tropicana®, orange juice single</td>
<td>240 mL</td>
<td>8 fl oz</td>
<td>110</td>
<td>2</td>
<td>26</td>
<td>0</td>
<td>0</td>
<td>22</td>
<td>0</td>
</tr>
<tr>
<td>Lunch</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Healthy Choice®, Café Steamers Simply-Chicken Pasta Primavera</td>
<td>283 g</td>
<td>10 oz</td>
<td>220</td>
<td>20</td>
<td>29</td>
<td>2.5</td>
<td>5</td>
<td>8</td>
<td>390</td>
</tr>
<tr>
<td>Crunch Pak®, Sweet Apple Slices</td>
<td>57 g</td>
<td>2 oz</td>
<td>30</td>
<td>0</td>
<td>7</td>
<td>0</td>
<td>1</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Babybel®, mini cheese</td>
<td>21 g</td>
<td>1</td>
<td>70</td>
<td>5</td>
<td>0</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>160</td>
</tr>
<tr>
<td>Snack</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Organic Valley®, Organic Balance Protein Shake Vanilla Bean</td>
<td>325 mL</td>
<td>11 fl oz</td>
<td>190</td>
<td>16</td>
<td>24</td>
<td>3</td>
<td>0</td>
<td>23</td>
<td>220</td>
</tr>
<tr>
<td>Breakstone®, Cottage Doubles Pineapple</td>
<td>110 g</td>
<td>3.9 oz</td>
<td>100</td>
<td>7</td>
<td>13</td>
<td>1.5</td>
<td>0</td>
<td>11</td>
<td>290</td>
</tr>
<tr>
<td>Dinner</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Beef, top sirloin fillet</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Potato, flesh and skin, raw</td>
<td>170 g</td>
<td></td>
<td>131</td>
<td>3.5</td>
<td>29.7</td>
<td>0.2</td>
<td>3.6</td>
<td>1.4</td>
<td>10</td>
</tr>
<tr>
<td>Land O’Lakes®, sour cream</td>
<td>28 g</td>
<td>1 oz</td>
<td>50</td>
<td>1</td>
<td>2</td>
<td>5</td>
<td>0</td>
<td>1</td>
<td>30</td>
</tr>
<tr>
<td>Land O’Lakes®, butter</td>
<td>5 g</td>
<td>1</td>
<td>35</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>35</td>
</tr>
<tr>
<td>Publix®, Individual Carrot Pack</td>
<td>85 g</td>
<td>3 oz</td>
<td>35</td>
<td>1</td>
<td>8</td>
<td>0</td>
<td>2</td>
<td>5</td>
<td>65</td>
</tr>
<tr>
<td>Snack</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>thinkThin® High-Protein Bar - White Chocolate</td>
<td>60 g</td>
<td>2.1 oz</td>
<td>240</td>
<td>20</td>
<td>25</td>
<td>9</td>
<td>1</td>
<td>0</td>
<td>200</td>
</tr>
</tbody>
</table>

Controlled diet provided during treatment periods. Controlled diet had four-day rotation during each treatment period. Diet Day A (1 of 4 controlled diets that were rotated).
Table C-2. Energy intake and diet composition, Day B (2 of 4 diet rotations provided during treatment periods)\(^1\)

<table>
<thead>
<tr>
<th>Item</th>
<th>Weight</th>
<th>Quantity</th>
<th>Energy (kcal)</th>
<th>Protein (g)</th>
<th>Carbs (g)</th>
<th>Fat (g)</th>
<th>Fiber (g)</th>
<th>Sugar (g)</th>
<th>Sodium (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breakfast</td>
<td>Egg Beaters®, Smart Cup™</td>
<td>113.5 g</td>
<td>4 oz</td>
<td>60</td>
<td>11</td>
<td>2</td>
<td>0</td>
<td>&lt;1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Thomas®, double protein oatmeal english muffin</td>
<td>61 g</td>
<td>1 muffin</td>
<td>150</td>
<td>7</td>
<td>26</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Smucker’s Jelly, individual pack</td>
<td>14 g</td>
<td>2 oz</td>
<td>35</td>
<td>0</td>
<td>9</td>
<td>0</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td>Lunch</td>
<td>Artisan Bistro, wild alaskan salmon bake</td>
<td>227 g</td>
<td>8 oz</td>
<td>240</td>
<td>16</td>
<td>30</td>
<td>7</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Dannon® Light &amp; Fit®, greek, strawberry cheesecake</td>
<td>150 g</td>
<td>5.3 oz</td>
<td>80</td>
<td>12</td>
<td>9</td>
<td>0</td>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td>Snack</td>
<td>Breakstone®, cottage cheese, fat-free, snack size</td>
<td>113 g</td>
<td>4 oz each / 2</td>
<td>70 / 140</td>
<td>10 / 20</td>
<td>7 / 14</td>
<td>0</td>
<td>0</td>
<td>6 / 12</td>
</tr>
<tr>
<td></td>
<td>Kellogg’s Special K Protein Bar, honey almond</td>
<td>45 g</td>
<td>1.59 oz</td>
<td>180</td>
<td>10</td>
<td>22</td>
<td>6</td>
<td>5</td>
<td>12</td>
</tr>
<tr>
<td>Dinner</td>
<td>Beef, cubed steak</td>
<td>-</td>
<td>5 oz</td>
<td>203</td>
<td>28.8</td>
<td>0</td>
<td>9.8</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Potato, flesh and skin, raw</td>
<td>170 g</td>
<td>-</td>
<td>131</td>
<td>3.5</td>
<td>29.7</td>
<td>0.2</td>
<td>3.6</td>
<td>1.4</td>
</tr>
<tr>
<td></td>
<td>Libby’s® vegetable cup, cut green beans</td>
<td>113 g</td>
<td>-</td>
<td>10</td>
<td>1</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Snack</td>
<td>Justin’s, classic peanut butter, individual</td>
<td>32 g</td>
<td>1.15 oz</td>
<td>190</td>
<td>8</td>
<td>7</td>
<td>17</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Saltines, original fresh stack</td>
<td>-</td>
<td>1 pack</td>
<td>220</td>
<td>4</td>
<td>39</td>
<td>4.5</td>
<td>1</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

\(^1\)Controlled diet provided during treatment periods. Controlled diet had four-day rotation during each treatment period. Diet Day B (2 of 4 controlled diets that were rotated).
<table>
<thead>
<tr>
<th>Item</th>
<th>Weight</th>
<th>Quantity</th>
<th>Energy (kcal)</th>
<th>Protein (g)</th>
<th>Carbs (g)</th>
<th>Fat (g)</th>
<th>Fiber (g)</th>
<th>Sugar (g)</th>
<th>Sodium (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breakfast</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Quaker, instant grits, butter flavor</td>
<td>28 g</td>
<td>1 pack</td>
<td>100</td>
<td>2</td>
<td>21</td>
<td>1.5</td>
<td>1</td>
<td>0</td>
<td>340</td>
</tr>
<tr>
<td>Cream of Wheat®, original, instant</td>
<td>28 g</td>
<td>1 pack</td>
<td>100</td>
<td>3</td>
<td>20</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>170</td>
</tr>
<tr>
<td>Eggs, whole</td>
<td>100 g</td>
<td>2 eggs</td>
<td>140</td>
<td>12</td>
<td>2</td>
<td>9</td>
<td>0</td>
<td>0</td>
<td>130</td>
</tr>
<tr>
<td>Jimmy Dean®, fully cooked turkey sausage patties</td>
<td>68 g</td>
<td>2 patties</td>
<td>100</td>
<td>11</td>
<td>1</td>
<td>7</td>
<td>0</td>
<td>1</td>
<td>420</td>
</tr>
<tr>
<td>Horizon Organic, low-fat milk, vanilla</td>
<td>236 mL</td>
<td>8 fl oz</td>
<td>140</td>
<td>8</td>
<td>22</td>
<td>2.5</td>
<td>0</td>
<td>21</td>
<td>130</td>
</tr>
<tr>
<td>Lunch</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lean Cuisine®, creamy basil chicken with tortellini</td>
<td>241 g</td>
<td>8.5 oz</td>
<td>230</td>
<td>19</td>
<td>28</td>
<td>5</td>
<td>2</td>
<td>6</td>
<td>490</td>
</tr>
<tr>
<td>Quaker, protein baked bar, oatmeal raisin nut flavor</td>
<td>47 g</td>
<td>1.65 oz</td>
<td>190</td>
<td>10</td>
<td>25</td>
<td>5</td>
<td>1</td>
<td>15</td>
<td>170</td>
</tr>
<tr>
<td>Snack</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yoplait®, greek 100 calories, peach</td>
<td>150 g</td>
<td>1 container</td>
<td>100</td>
<td>10</td>
<td>14</td>
<td>0</td>
<td>0</td>
<td>9</td>
<td>45</td>
</tr>
<tr>
<td>Luna protein bar, lemon vanilla</td>
<td>45 g</td>
<td>1 bar</td>
<td>170</td>
<td>12</td>
<td>21</td>
<td>4.5</td>
<td>3</td>
<td>15</td>
<td>160</td>
</tr>
<tr>
<td>Dinner</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Perdue®, boneless skinless chicken breast, individually wrapped</td>
<td>120 g</td>
<td>1 piece</td>
<td>120</td>
<td>22</td>
<td>0</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>290</td>
</tr>
<tr>
<td>Birds Eye Steamfresh®, sweet peas, singles</td>
<td>-</td>
<td>3.25 oz</td>
<td>70</td>
<td>5</td>
<td>13</td>
<td>0</td>
<td>4</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Uncle Ben's®, natural, whole grain brown rice, individual</td>
<td>-</td>
<td>1 container</td>
<td>170</td>
<td>5</td>
<td>35</td>
<td>3</td>
<td>3</td>
<td>0</td>
<td>15</td>
</tr>
<tr>
<td>Snack</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crunch Pak®, sweet apple slices</td>
<td>57 g</td>
<td>0.2 oz</td>
<td>30</td>
<td>0</td>
<td>7</td>
<td>0</td>
<td>1</td>
<td>5</td>
<td>0</td>
</tr>
</tbody>
</table>

1Controlled diet provided during treatment periods. Controlled diet had 4-day rotation during each treatment period. Diet Day C (3 of 4 controlled diets that were rotated). For breakfast participants were given the option of the Quaker Instant Grits or Cream of Wheat®.
Table C-4. Energy intake and diet composition, Day D (4 of 4 diet rotations provided during treatment periods)\(^1\)

<table>
<thead>
<tr>
<th>Item</th>
<th>Weight</th>
<th>Quantity</th>
<th>Energy (kcal)</th>
<th>Protein (g)</th>
<th>Carbs (g)</th>
<th>Fat (g)</th>
<th>Fiber (g)</th>
<th>Sugar (g)</th>
<th>Sodium (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breakfast</td>
<td>Horizon Organic, 1% low-fat milk</td>
<td>236 mL</td>
<td>8 fl oz</td>
<td>1</td>
<td>110</td>
<td>8</td>
<td>13</td>
<td>2.5</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Kellogg’s® Special K® Original, cereal</td>
<td>36 g</td>
<td>1 container</td>
<td>140</td>
<td>6</td>
<td>26</td>
<td>0.5</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Banana, medium</td>
<td>118 g</td>
<td>1</td>
<td>105</td>
<td>1.3</td>
<td>29.7</td>
<td>0.4</td>
<td>3.1</td>
<td>14.4</td>
</tr>
<tr>
<td>Lunch</td>
<td>Ready Pac®, bistro bowl(^{TM}) chicken caesar salad</td>
<td>177 g</td>
<td>6.25 oz</td>
<td>230</td>
<td>16</td>
<td>8</td>
<td>16</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>DelMonte® fruit naturals®, yellow cling peach chunks</td>
<td>184 g</td>
<td>6.5 oz</td>
<td>50</td>
<td>1</td>
<td>12</td>
<td>0</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>Snack</td>
<td>Bear Naked® To-Go Pouch, original cinnamon protein</td>
<td>42 g</td>
<td>1.5 oz</td>
<td>180</td>
<td>13</td>
<td>19</td>
<td>7</td>
<td>4</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>Breakstone®, cottage cheese, fat-free, snack size</td>
<td>113 g</td>
<td>4 oz / 2</td>
<td>70 / 140</td>
<td>10 / 20</td>
<td>7 / 14</td>
<td>0</td>
<td>0</td>
<td>6 / 12</td>
</tr>
<tr>
<td>Dinner</td>
<td>Beef, new york strip</td>
<td>-</td>
<td>5 oz</td>
<td>220</td>
<td>32.3</td>
<td>0</td>
<td>8</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Potato, flesh and skin, raw</td>
<td>170 g</td>
<td>1</td>
<td>131</td>
<td>3.5</td>
<td>29.7</td>
<td>0.2</td>
<td>3.6</td>
<td>1.4</td>
</tr>
<tr>
<td></td>
<td>Land O’Lakes®, butter</td>
<td>5 g</td>
<td>1</td>
<td>35</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Birds Eye Steamfresh®, super sweet corn, singles</td>
<td>92 g</td>
<td>3.25 oz</td>
<td>80</td>
<td>3</td>
<td>14</td>
<td>1</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>Snack</td>
<td>thinkThin® High-Protein Bar, brownie crunch</td>
<td>60 g</td>
<td>2.1 oz</td>
<td>230</td>
<td>20</td>
<td>23</td>
<td>8</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

\(^1\)Controlled diet provided during treatment periods. Controlled diet had four-day rotation during each treatment period. Diet Day D (4 of 4 controlled diets that were rotated).
Table C-5. Energy intake and diet composition of additional foods added to individual diets

<table>
<thead>
<tr>
<th>Item</th>
<th>Weight</th>
<th>Quantity</th>
<th>Energy (kcal)</th>
<th>Protein (g)</th>
<th>Carbs (g)</th>
<th>Fat (g)</th>
<th>Fiber (g)</th>
<th>Sugar (g)</th>
<th>Sodium (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sargento®, reduced fat, Colby-jack cheese stick</td>
<td>21 g</td>
<td>1 piece / 2</td>
<td>60 / 120</td>
<td>5 / 10</td>
<td>0 / 0</td>
<td>4.5</td>
<td>0 / 0</td>
<td>0 / 0</td>
<td>135 / 270</td>
</tr>
<tr>
<td>Nestle® Juicy Juice, 100% juice, apple</td>
<td>125 mL</td>
<td>4.23 fl oz</td>
<td>60</td>
<td>0</td>
<td>15</td>
<td>0</td>
<td>0</td>
<td>14</td>
<td>10</td>
</tr>
<tr>
<td>Mott's®, 100% apple juice</td>
<td>-</td>
<td>1 bottle</td>
<td>120</td>
<td>0</td>
<td>29</td>
<td>0</td>
<td>0</td>
<td>28</td>
<td>10</td>
</tr>
<tr>
<td>Cheez-it®, snack mix, 100 calorie portions</td>
<td>0.74 oz</td>
<td>21 g</td>
<td>100</td>
<td>2</td>
<td>14</td>
<td>3</td>
<td>&lt;1</td>
<td>0</td>
<td>180</td>
</tr>
<tr>
<td>Emerald®, natural walnuts and almonds, 100 calorie pack</td>
<td>0.56 oz</td>
<td>15.8 g</td>
<td>100</td>
<td>3</td>
<td>3</td>
<td>9</td>
<td>2</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Bumble Bee®, snack on the run, chicken salad with crackers</td>
<td>99 g</td>
<td>3.5 oz</td>
<td>230</td>
<td>9</td>
<td>22</td>
<td>13</td>
<td>2</td>
<td>8</td>
<td>545</td>
</tr>
<tr>
<td>Bumble Bee®, snack on the run, tuna salad with crackers</td>
<td>99 g</td>
<td>3.5 oz</td>
<td>300</td>
<td>7</td>
<td>18</td>
<td>23</td>
<td>2</td>
<td>5</td>
<td>425</td>
</tr>
<tr>
<td>Bumble Bee®, lunch, tuna salad with crackers and cookie</td>
<td>118 g</td>
<td>4.2</td>
<td>390</td>
<td>7</td>
<td>30</td>
<td>27</td>
<td>2</td>
<td>12</td>
<td>450</td>
</tr>
<tr>
<td>Jelly Belly®, jelly beans, original</td>
<td>-</td>
<td>5 jelly beans</td>
<td>20</td>
<td>0</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Land O'Lakes®, butter</td>
<td>5 g</td>
<td>1</td>
<td>35</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>35</td>
</tr>
</tbody>
</table>

¹Controlled diet provided during treatment periods. Additional foods that’s were added to the base high-protein controlled diet. Additional foods added to individual based diet were provided daily to participants.
APPENDIX D
DESCRIPTIVE STATISTICS OF REPORTED DAILY QUESTIONNAIRE RESPONSES
Figure D-1. Reported daily response to daily questionnaire regarding fatigue.
Figure D-2. Reported daily response to daily questionnaire regarding hunger.
Figure D-3. Reported mean daily responses to gastrointestinal distress (component syndrome from the daily questionnaire).
Figure D-4. Reported mean daily responses to cephalic syndrome (component syndrome from the daily questionnaire).
Figure D-5. Reported mean daily responses to emetic syndrome (component syndrome from the daily questionnaire).
Figure D-6. Reported mean daily responses to psychological syndrome (component syndrome from the daily questionnaire).
LIST OF REFERENCES


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BIOGRAPHICAL SKETCH

Amanda L. Ford was born and raised in Alachua, Florida. She attended the University of Florida and earned her B.S. in nutritional sciences from the Food Science and Human Nutrition department in 2010. Upon completing her B.S. she entered the Hough Graduate School of Business at the University of Florida where she earned her M.S. in management in 2011. After completing an internship at Walt Disney World® Disney’s Animal Kingdom and EPCOT in an analytical chemistry lab, she returned to Gainesville, Florida, where she worked under Dr. Wendy Dahl as a research coordinator conducting clinical trials. It was during this time that Amanda discovered her interest in clinical and probiotics research. In the fall of 2012, Amanda began the Ph.D. program in the Food Science and Human Nutrition Department under the mentorship of Dr. Wendy Dahl. Amanda was the recipient of the 2014-2015 Dannon Yogurt and Probiotics Fellowship Recipient and the College of Agricultural and Life Sciences Jimmy G. Cheek Graduate Student Medal of Excellence in 2015.

Amanda graduated with her Doctor of Philosophy in Nutritional Science and a minor in Agricultural Education and Communication from the College of Agricultural and Life Sciences in the summer of 2017.