EFFECTS OF INULIN SUPPLEMENTATION ON MARKERS OF MINERAL AND BONE METABOLISM AND THE GUT MICROBIOTA IN HEMODIALYSIS PATIENTS

BY

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DISSERTATION

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ABSTRACT

Up to 80% of patients with chronic kidney disease (CKD) have some degree of mineral and bone disorder (MBD), which is associated with higher morbidity and mortality, specifically from cardiovascular causes. Despite the pharmacological treatment for MBD, its prevalence remains high, especially for patients with kidney failure undergoing chronic hemodialysis (HD) treatment. Restriction of dietary phosphorus along with phosphate binders represents an important part of the treatment of MBD. However, due to this restriction along with a potassium-restricted diet, HD patients limit a variety of foods that contain dietary fiber. The supplementation of dietary fiber, specifically fermentable fiber, has been explored in other clinical populations with MBD, such as female adolescents and postmenopausal women. While it is known that this supplementation leads to a higher production of short-chain fatty acids (SCFA) by the gut microbiota and an effect on the absorption of calcium and magnesium, this mechanism remains unexplored in HD patients. The supplementation of inulin, a fermentable fiber, represents a feasible and low-cost co-adjuvant therapy for the treatment of MBD in HD patients. Therefore, the objective of this dissertation is to examine the effects of the supplementation of inulin on bone and mineral metabolism and the gut microbiota in HD patients.

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CHAPTER 1

INTRODUCTION

According to the United States Renal Data System (USRDS), up to 2014 the overall prevalence of chronic kidney disease (CKD) was 14% of adults, which has remained stable since 2004.[1] Once patient's glomerular filtration rate (GFR) reaches less than 15ml/min/1.73m^2, it is considered end-stage renal disease (ESRD), which represents a non-reversible damage to the kidney where there is a need of a renal replacement therapy or a kidney transplant. In the United States, the most common form of renal replacement therapy is hemodialysis (HD), used in almost 64% of the prevalent ESRD patients.[1] One of the most prevalent problems in ESRD patients undergoing chronic HD treatment is mineral and bone disorder (MBD), which can be seen as early as CKD stage 2, where the kidney function has declined by 10-40%.[1,2] The diagnosis of MBD includes at least one of the following: a) laboratory abnormalities (e.g., hypocalcemia, hyperphosphatemia, secondary hyperparathyroidism, low concentrations vitamin D [calcidiol and/or calcitriol]) and high fibroblast growth factor-23 (FGF-23); b) bone abnormalities (e.g., altered turnover, volume and mineralization); and c) extra-skeletal calcifications (e.g., vascular calcification).[3] The disruption in bone and mineral homeostasis critically affects the bone structure and function by altering bone turnover and can lead to a negative bone balance (high bone resorption and normal-to-low bone formation) in this clinical population.[4] Up to 80% of CKD patients have some degree of MBD, which is associated with increased risk of fractures, cardiovascular disease, hospitalization, and mortality and lower quality of life.[5,6]

Despite the pharmacological treatment for MBD, which include phosphate binders, calcimimetics, and vitamin D analogs, the prevalence of MBD remains high, especially for

patients with kidney failure undergoing chronic HD treatment. Restriction of dietary phosphorus along with phosphate binders represents a main part of the treatment of MBD.[7] However, due to this dietary phosphorus restriction along with a potassium-restricted diet, HD patients limit intake of certain food groups, such as fruits, vegetables, legumes, whole grains, nuts, and legumes. These food groups, however, are also good sources of fiber.[8] The supplementation of dietary fiber, specifically fermentable fiber, has been explored in other clinical populations with MBD, such as female adolescents and postmenopausal women.[9–12] While it is known that this supplementation leads to a higher production of short-chain fatty acids (SCFA) by the gut microbiota and an effect on the absorption of minerals, especially calcium and magnesium, this mechanism remains unexplored in HD patients. The supplementation of inulin, a fermentable fiber, represents a feasible and low-cost co-adjuvant therapy for the treatment of MBD in HD patients. Therefore, the objective of this trial is to assess the effects of a four-week supplementation with inulin on markers of MBD. Additionally, as secondary outcomes, the effects of the supplementation of inulin on the gut microbiota will be assessed.

CHAPTER 2

LITERATURE REVIEW: MODIFIED NUTRITIONAL RECOMMENDATIONS TO IMPROVE DIETARY PATTERNS AND OUTCOMES IN HEMODIALYSIS PATIENTS

Introduction

The renal diet has traditionally been one of the most complex medical nutrition therapies to teach, understand, and implement. End stage renal disease (ESRD) medical nutrition therapy dictates that patients shift their nutrition goals from standard dietary recommendations to a pattern that manages levels of circulating waste products and minerals between dialysis treatments. This is especially true for patients undergoing chronic hemodialysis (HD) treatment due to the 48-72h span between treatments. Specifically, patients are instructed to limit fruits, vegetables, nuts, legumes, dairy, and whole grains because of both phosphorus and potassium (K^+) concerns.[13] These dietary restrictions are even more challenging due to elevated protein and energy needs. Further, HD patients are often encouraged to decrease fluid intake to control interdialytic weight gain (IDWG).[14,15] These restrictions can result in frustration, lack of autonomy, and the perception that there is nothing left to eat.[13,16] In some cases, this can result in a disregard for the nutrition recommendations altogether and a reliance on processed, convenience, and restaurant foods.[17,18] These choices can further exacerbate complications and comorbidities associated with ESRD including cardiovascular (CV) disease, poor glycemic control, large IDWG, continued struggles with phosphorus and K^+ regulation, with a potentially confounding low intake of other vitamins, minerals, antioxidants, and dietary fiber.[8,19,20] Indeed, it appears that standard dietary prescription for HD patients may be doing more harm than good.

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There has been a slowly emerging discussion of 'liberalizing' the diet prescription in an effort to decrease the total sodium and phosphorus additive load while inducing a corresponding increase in fiber, antioxidants, and phytochemicals, resulting in an overall improved dietary profile, particularly for CV health.

Considering the burden associated with ESRD and HD treatment, every effort must be made to support quality of life and patient dietary options. It is possible that the traditional ESRD medical nutrition therapy may be liberalized in order to afford patients greater choices and ultimately improved outcomes. The objective of this review is to assess the evidence in support of a renal diet focused on reducing the intake of sodium and inorganic phosphorus. In addition, the limited evidence for restricting dietary K^+ intake is summarized.

Sodium

Sodium is the main cation in the extracellular space and a key contributor to plasma osmolality.[21] In HD patients, increases in plasma osmolality may occur due to excessive dietary sodium intake or from treatment-related factors, such as the use of high-sodium dialysate solutions or hypertonic saline infusion used for the prevention of cramping and intradialytic hypotension. Each of these sodium sources may increase thirst and have been associated with higher IDWG, pre-dialysis systolic blood pressure (BP) and chronic fluid overload,[22] which are in turn associated with acute and chronic CV complications and mortality.[23,24] In order to prevent the aforementioned complications, dietary sodium restriction is advised to HD patients.

<u>Dietary sodium intake and clinical outcomes in HD patients</u>

Guidelines from the Kidney Disease Outcomes Quality Initiative (KDOQI) recommend to limit dietary sodium.[15] However, the expert panel did not recommend a specific dietary sodium

prescription and called for the assessment of an ideal dietary sodium intake for HD patients, although previous guidelines recommended a dietary sodium intake below 2000mg/day.[14] Surprisingly, there is little data assessing the relationship between dietary sodium intake and clinical outcomes in HD patients. Furthermore, most studies in the United States have been observational and based on self-reported intake, which may underestimate dietary intake due to underreport and variability of sodium content of foods.[25,26] In a post-hoc analysis of the Hemodialysis (HEMO) Study, McCausland et al.[23] reported a mean sodium intake of 2240±1050 mg/day, which was higher in younger, male, non-black participants with longer dialysis vintage. Moreover, there was a positive association between dietary sodium intake and mortality risk. Interestingly, they did not find an association between dietary sodium intake and BP. In a cohort of 122 HD patients, Clark-Cutaia et al.[27] reported a mean dietary sodium intake of 2,346±904 mg/day and found that younger participants had higher dietary sodium intakes, elevated IDWG, and lower adherence to dietary sodium restriction.

Evidence of potential benefits of dietary sodium restriction in HD patients

It has long been recognized that hypertension and related CV co-morbidities can be controlled in many HD patients through non-pharmacological means. Dietary sodium restriction in conjunction with persistent ultrafiltration can limit extracellular volume expansion and hypertension in HD patients, without the need of antihypertensive medications.[28] The contributions of a sodium-restricted diet as a component of a comprehensive volume control strategy has been highlighted by data from the HD populations in Tassin, France, and Izmir, Turkey. For over 40 years, HD clinics in Tassin have used a protocol to control hypertension that includes the use of sodium-restricted diets in combination with longer dialysis sessions (up to 8h).[29] Interestingly, they have reported controlled BP in 90% of their patients without the use of

antihypertensive medications, as well as lower mortality rates.[30] HD clinics in Izmir have used a similar protocol, though with shorter HD sessions (~4h), which may be more applicable to the United States.[31] In a retrospective cross-sectional study, Kayikcioglu et al.[24] compared CV parameters in patients at two HD centers that managed BP using different protocols. The first center controlled BP without the use of antihypertensive medications, primarily using dietary sodium-restriction (< 5g of salt, or 1,950mg of sodium/day) in conjunction with intensive ultrafiltration, while the second clinic controlled BP using antihypertensive medications. Patients relying on sodium-restriction and enhanced ultrafiltration had lower IDWG, lower left-ventricular mass index, better systolic and diastolic function and higher serum albumin. Furthermore, only 7% of patients were using antihypertensive medications vs. 42% in the clinic utilizing standard BP control practices.[24] In the United States, the application of Izmir's protocol was explored in a pilot study by Williams et al.[32] Dietary sodium restriction, as part of an intensive volume control strategy that involved slowly reducing post-dialysis weight by 200-300g/treatment, resulted in a decrease in systolic BP in patients whose pre-dialysis systolic BPs were above 160mmHg. Additionally, IDWG was reduced in those whose pre-dialysis systolic BP was above 140mmHg. This data suggests that dietary sodium restriction is a valuable component of a comprehensive volume reduction strategy aimed at improving CV function.

To date, only two randomized-controlled trials (RCTs) have assessed the lone effects of dietary sodium restriction in HD patients. First, Rodriguez-Telini et al.[33] examined the effect of sodium restriction (prescribed reduction of 5g of salt/1,950mg of sodium per day) on BP, IDWG, and inflammatory markers. Although there was no change in BP or IDWG after 16 weeks, inflammatory markers (C-reactive protein, tumor necrosis factor alpha, and interleukin 6) were reduced by 54%, 64%, 56%, respectively in the sodium-restricted group, but no change in the

control group. However, the small sample size in this study (n=21 in sodium restricted group) suggests that additional studies are needed before definitive conclusions can be drawn. Finally, in the BalanceWise study, which aimed to assess the efficacy of a technology-supported behavioral intervention for dietary sodium restriction, Sevick et al. reported baseline dietary sodium intakes between 2,298±957 and 2,555±2,090 mg of sodium/day. After 8 weeks, patients in the intervention group reduced dietary sodium an average of 371mg/day, but these changes were not sustained at 16 weeks. Furthermore, there were no changes in IDWG at 8 or 16 weeks.[34] Despite the aforementioned potential benefits of dietary sodium restriction, more adequately-powered RCTs are needed to guide a specific recommendation in HD patients.

Potential concerns with dietary sodium restriction

In a recent review by Kalantar-Zadeh et al.,[13] it was stated that sodium restriction may be beneficial as long as optimal nutritional status and food intake is not compromised. Indeed, malnutrition and the risk of protein-energy wasting are primary concerns with a sodium-restricted diet due to the wide use of sodium in foods. Data supporting this concern was reported by Dong et al.,[35] who found an association between low-dietary sodium intake and higher mortality[25]. However, these results should be interpreted with caution considering this was in a cohort of peritoneal dialysis patients. Furthermore, the average body-mass index was ~23 kg/m^2 and serum albumin concentrations were low across all sodium quartiles (~3.5g/dl), which suggests possible malnutrition among all participants in this study. By contrast, in the RCT by Rodrigues-Telini noted previously,[33] patient's sodium intake was reduced by 2g/d (baseline dietary sodium intake of 9.25g/d) for 16 weeks without a reduction in total caloric or protein intake. This demonstrates that a sodium-restricted diet with uncompromised energy and protein intakes may be achieved with adequate nutritional counseling.

Finally, an additional concern regarding dietary sodium restriction is the loss of residual renal function. Residual renal function has been associated with positive outcomes in HD including survival benefit.[36] Additionally, the preservation of residual renal function has an important role in nutrition, in part due to a better control of K^+ and phosphorus.[37] In a retrospective study by Ozkahya et al.,[38] loss of residual renal function was observed in nearly all patients after three years of implementation of Izmir's volume reduction protocol. However, little information was provided regarding the percentage of patients that were anuric/oliguric at baseline, their average residual urine output, or how long it was before their residual urine output was lost. Because few patients maintain significant urine output long after initiating HD, it is impossible to determine if this loss of residual function was related to the dietary sodium restriction, volume reduction, or dialysis vintage. Furthermore, some antihypertensive medications (e.g., loop-diuretics and angiotensin-converting enzyme inhibitors) have been proposed to preserve residual renal function in HD patients,[39] which were discontinued in the aforementioned study. Despite this, it should be noted that several factors impacting CV disease risk in HD patients, including BP, cardiothoracic index, and IDWG, improved in this study. This suggests that even if a loss of residual renal function is a manifestation of sodium restriction, the CV-related benefits may outweigh this potential concern. Studies that aim to assess the effect of dietary sodium restriction on residual renal function and clinical outcomes are needed.

Limitations and barriers with dietary sodium restriction

Dietary sodium is found ubiquitously in the western diet and is particularly excessive in processed foods. It has been estimated that 75% of dietary sodium in the United States diet comes from processed foods, 15% is added in food preparation as salt, and 10% is endogenous to foods.[40] Due to the high proportion of sodium coming from processed foods, the concept of

"foods consumed away from home", such as fast-food and full-service restaurants, being associated with unhealthy eating patterns has been reported in the general population.[41] This concept, however, assumes that foods prepared and consumed at home are a healthier option. To examine this, we recently conducted a pilot study examining food's point of purchase in 60 HD patients (56±14 years old, 62% male, 71% African American). On dialysis days, 75% of the dietary sodium came from grocery/convenience stores, while 15% and 5% of sodium came from fast-food restaurants and full-service restaurants, respectively. By contrast, on non-dialysis days, 49% of dietary sodium came from grocery stores, 31% from full-service restaurants, and 14% from fast-food restaurants. Surprisingly, in this study, the ratio of sodium to kcal was similar from foods purchased at each location. This challenges the concept that healthier foods, at least in terms of sodium content, are consumed at home. This information may be helpful for renal dietitians for implementing nutritional education and counseling techniques focused on improving shopping skills and reading labels to limit major sources of dietary sodium.

Salt-taste perception may represent an additional barrier to comply with sodium-restricted diets. Salt-taste perception has been reported to be altered in ESRD patients.[42,43] Changes in taste acuity may occur with increased age and nutrient deficiencies, such as zinc.[44] In addition, it may take up to three months to adjust to a low-sodium diet in healthy populations,[45] with no difference reported in HD patients.[46] Therefore, palate adjustment to low-sodium diets may represent a challenge to HD patients due to repeated exposure to foods high in dietary sodium, and an altered salt-taste acuity.

Phosphate

<u>Hyperphosphatemia and its clinical management</u>

Phosphate is the second most abundant mineral in the body, with 85% stored in bone and the other 15% in soft tissues, blood and extracellular fluid.[47,48] Factors that influence phosphate metabolism have been reviewed elsewhere.[49] Hyperphosphatemia, or serum phosphorus ≥5.5mg/dl, is one component of chronic kidney disease- mineral and bone disorder (CKD-MBD) and its prevalence in HD patients is as high as 50%.[50] Hyperphosphatemia has been associated with an increased risk of mortality,[51] specifically from CV causes, where it may increase the risk vascular calcification and left-ventricular hypertrophy.[52] CKD-MBD is also associated with decreased physical function, increased risk of fractures, and reduced quality of life.[53] The current clinical management of hyperphosphatemia is focused on dialysis treatment, phosphate-binder therapy, and dietary phosphorus restriction.[7,54] The efficiency with which phosphate is removed by dialysis and control of phosphate with phosphate binders have been reviewed elsewhere.[55–57] Dietary phosphorus restriction represents a cornerstone of the renal diet and the treatment of CKD-MBD. The recommended dietary phosphorus intake is less than 800 to 1000mg/day.[7] However, the efficacy of this recommendation has not been established. Furthermore, this restriction does not distinguish between types of dietary phosphorus, and in conjunction with dietary K^+ restriction may lead to a lower intake of other nutrients, such as dietary fiber.[8]

Types of dietary phosphate: focus on avoidance of phosphate additives

Dietary phosphorus can be divided in two kinds: organic and inorganic. Organic phosphate is found mostly as phosphoproteins and membrane phospholipids in animal sources (e.g., meat and dairy) and as phytate in vegetable sources (e.g., legumes, whole grains and nuts). Animal sources have an absorption rate of 40% to 80%, being higher when vitamin D is present.[58] Meanwhile, vegetable sources have an absorption rate of 20% to 40%[58] because most of the phosphorus is found as part of phytate, which must be hydrolyzed by phytase to be

released and absorbed in the small intestine. Phytase, however, is not expressed in the small intestine; therefore, absorption of phytate-containing foods is even more limited. Vegetable sources containing yeast may have increased phosphate availability due to yeast phytase activity.[59] Phosphate concentrations in both animal[60] and vegetable sources[61] also may be reduced with some cooking methods, such as boiling, slicing, and pressure cooking.

By contrast, inorganic phosphate is not normally found in fresh/unprocessed foods. Instead, it is used as an additive in processed foods to increase palatability and shelf-life, among other reasons.[58] Inorganic phosphate has an absorption rate of more than 90%, representing a major concern for HD patients.[62] Consumption of phosphate additives has been estimated to be as high as 1000mg/day.[40] However, it is difficult to precisely determine intake because the food industry is not required to report the quantity used in their products. Instead, they only need to list the additives in the ingredient list.[62] This generally leads to an underestimation of dietary phosphorus intake, and represents a challenge to nutrition professionals when assessing patients' intake. Recently, the Academy of Nutrition and Dietetics submitted a request to the Food and Drug Administration (FDA) to add dietary phosphorus to the nutrition facts panel and make a distinction between naturally-contained phosphate and phosphate additives.[63] Unless this is adopted and enforced, assessing patients' true phosphorus load and corresponding dietary prescriptions will remain a challenge.

Liberalized organic phosphate restriction in HD patients

Intensive nutritional counseling has been used by dietitians as a tool to reduce serum phosphate.[64] The focus of the counseling sessions has evolved, from a phosphate restriction without a differentiation between organic and inorganic sources of phosphate, to a more recent focus on phosphate additives. This shift occurred due to a concern of low-protein and energy

intakes, which may lead to malnutrition and protein-energy wasting, and associated with lower physical function, reduced quality of life, and higher mortality.[65] Furthermore, foods with only organic phosphorus typically are more nutrient-dense and have a higher nutritional value compared to processed foods containing phosphate additives, which tend to have a lower nutritional value, and are often paired with sodium and K^+ additives.[40] In a post- hoc analysis from the HEMO study, Lynch et al. found that phosphate restriction was not associated with improved survival, and was associated with a higher risk of mortality. Conversely, a more liberal phosphorus prescription tended to improve survival.[66] Furthermore, Sullivan et al.[67] found that by educating patients on how to avoid foods with phosphate additives by reading food labels and providing renal-friendly options at local fast-food restaurants, serum phosphorus decreased by 0.6mg/dl compared to controls with standard dietary recommendations. Recently, a meta-analysis by Karavetian et al.[68] suggested that nutritional counseling based on a structured behavioral change may be successful in HD patients. However, only half of the studies reviewed were RCTs and most were short-term with a follow-up ranging from one to six months. Although more RCTs are needed to assess the efficacy of dietary phosphate restriction on hyperphosphatemia, nutritional education focused on restricting processed foods and cooking methods to reduce the availability of phosphate may benefit HD patients.

Potassium

Potassium (K^+) is the main intracellular cation, which is important to maintain the cell's membrane potential, heart function, nerve-impulse transmission, and skeletal muscle contraction.[69,70] Regulation of serum K^+ is an important concern for HD patients due to its effect on acute CV complications and mortality.[70,71] In HD patients, the prevalence of hyperkalemia, defined as $K^+ \geq 5.5$ mmol/L, has been reported to be 4.5%-6.3%.[70] Serum K^+ levels are

influenced by many factors, including HD-related variables (blood and dialysate flow rate, dialysate K^+ and buffer concentrations, dialysis length and efficiency), residual renal function, acid-base balance, fecal excretion, glucose metabolism, and shifts from intracellular compartments.[72–74]

Current dietary guidelines for K^+ in HD patients

HD patients are recommended to restrict certain foods, such as fruits, vegetables, nuts, legumes, and dairy products that are high in K^+[70], primarily due to concerns with hyperkalemia. The KDOQI guidelines do not have a specific recommendation for dietary K^+ for HD patients. However, the Joint Standards Task Force of the Academy of Nutrition and Dietetics and the National Kidney Foundation Council on Renal Nutrition recommend an intake of 2-4g/day.[75] However, there is little evidence to support these recommendations. In a cohort of 224 HD patients with 5-year follow-up, Noori et al.[76] found that the association between dietary K^+ (estimated by food-frequency questionnaire) and serum K^+ was weak ($r=0.14$, $p<0.05$). Furthermore, St-Jules et al.[77] found no association between dietary K^+ intake and serum K^+ in the BalanceWise Study cohort of 140 HD patients. Dietary K^+ intake was positively associated with energy, protein and phosphorus intake, which have been associated with better outcomes in other large HD cohorts, such as the HEMO study.[65,78] This suggests that dietary K^+ restriction may be potentially deleterious if not implemented with caution in the face of increased risk of malnutrition and protein-energy wasting.[76] Indeed, low K^+ levels have also been associated with increased mortality in dialysis patients.[71,79] Further studies are needed to assess whether the current restrictions on dietary K^+ in HD patients are warranted.

Alternative approaches to restricting dietary potassium

When restricting foods that naturally contain K^+, other important nutrients are also restricted, such as dietary fiber. Indeed, dietary fiber intake in HD patients has been reported below the adequate intake of 25g/day for women and 38g/day for men.[8,80] Fortunately, dietary K^+ exposure can be limited using alternative cooking methods, such as boiling and leaching, particularly vegetables and legumes.[81–83] Depending on the cut and pre-preparation steps, the amount of K^+ loss can be reduced by as much as 75%.[81] Moreover, K^+ salts may be added to foods, such as meats, fish, dairy, and legumes, to enhance flavor or as a preservative.[84] Sherman et al.[85] compared the K^+ content in a variety of enhanced and non-enhanced meat and poultry products and found that K^+ levels averaged 8.7% higher in the enhanced products. The highest K^+ concentration found in a non-enhanced product was 387mg K^+ /100g, while the average K^+ concentration in the top-five enhanced products analyzed was 692 mg/100g (max of 930mg/100g). Moreover, some salt-substitutes are K^+-based, containing an average of 600mg of K^+ per ¼ teaspoon, potentially contributing to excessive dietary K^+ load.[75] Similar to phosphorus, dietary K^+ is not required to be reported on the nutrition facts label of packaged foods and a recent study found K^+ levels were included on less than 10% of the foods studied.[86] The addition of dietary K+ to nutrition fact labels has been recommended. If these changes are adopted, new labels should include total K^+.[84]

Because of these concerns, we propose an alternative approach to broad restrictions on high K^+ foods for HD patients: 1) focus on limiting foods with added K^+; and 2) liberalize the restriction on foods that naturally contain K^+, while utilizing food preparation and cooking methods such as boiling and leaching to limit K^+ intake, especially in vegetables and legumes. We recognize that some adjustments to this approach may be warranted for patients with overt hyperkalemia. However, since K^+ is an intracellular ion, other causes should be also suspected,

such as inflammation, hemolysis, acidosis, as well as nutrition-related causes including poor appetite and low food intake that potentially promote muscle catabolism.[77,87] A liberalized dietary approach for controlling K^+ intake may have important health consequences for the majority of HD patients. Prospective studies using this approach are needed in addition to studies that aim to explore the efficacy of current practices, as well as the safety of this approach.

Conclusion and Future Directions

It is well accepted that the HD dietary recommendations, namely to reduce the intake of sodium, phosphorus and K^+, while increasing protein and total energy, may be confusing, counterintuitive, and thus difficult to follow. As a result, patient compliance with the renal diet is comprehensibly low.[88] Based on the data presented herein, an alternative approach consisting of a simplified message focusing on dietary patterns to limit the intake of processed foods (from grocery/convenience stores, fast-food and full-service restaurants), enhancing patient's self-efficacy on how to shop and read nutrition fact labels, as well as promoting a whole-foods approach, may be warranted. We hypothesize that this approach will be associated with an overall decrease in IDWG and chronic volume overload, secondary to a decrease in overall dietary sodium, while supporting a dietary pattern consistent with lowered inorganic phosphorus and K^+ additives, and increased fiber. This dietary pattern is likely to reduce risk factors for CV disease, the primary cause of death in HD patients.[1] A similar dietary strategy, along with persistent volume reduction has consistently proven to significantly lower BP, nearly eliminate the need for antihypertensive medications, improve indices of cardiac structure and function, and reduce mortality rates in HD patients in Tassin[31] and Izmir.[38,89]

An added challenge to this approach is the need for patient and staff education. Intensive dietary counseling has been shown to improve nutritional markers such as serum albumin and

phosphorus,[67] but renal dietitians lack the time to provide this level of patient interaction.[90,91] However, other members of HD clinic staff (nurses, social workers, technicians, and physicians) represent an underutilized opportunity for consistently reinforcing basic nutrition messages. Enlisting the support from the dialysis team may represent the best approach to achieve these food intake patterns without compromising energy and protein intake. The World Health Organization's (WHO) Ottawa Charter states the need to create a supportive environment in order to empower patients and promote health.[92] Additionally, family and/or caregivers should be included, since they are often responsible for handling food purchasing and preparation. Evidence that this approach can work in HD patients has been demonstrated by the data from Tassin[31] and Izmir,[38] as well as the studies by Krautzig et al.[93] and Sullivan et al.,[67] where the dietary message was based on simple recommendations, such as encourage home cooking, read food labels, avoid salt in food preparation, as well as obvious high-salt foods. Furthermore, these protocols have been structured to involve the entire dialysis clinic staff supporting the dietitians in helping counsel patients on how to lower their dietary sodium intake while maintaining an adequate nutritional status.[31,89]

In contrast, in the United States the responsibility for nutritional counseling is usually guided by the renal dietitian with little support from the clinic staff.[94] Dialysis providers in the United States have taken preliminary steps to remedy this with development of "Tech Talks", which serve as talking points for dialysis technicians to utilize with patients. These "Tech Talks" consist of simple nutrition-focused messages aimed at decreasing phosphorus and sodium intake while increasing albumin; however, they are rarely utilized. Therefore, a simplified message focused on dietary patterns, led by renal dietitians, with support of the whole clinic staff, may

help improve patient's overall diet in a manner that can help reduce chronic volume overload, CKD-MBD and enhance overall nutritional status and patient's quality of life.

Recommendations for Implementation

Based on the concerns described above, we propose the following modifications to the dietary recommendations for HD patients (Table 2.1):

1) Dietary restrictions on K^+ and phosphorus from non-processed/whole foods should be largely eliminated. Specifically, few restrictions should be placed on fresh fruit, vegetables, nuts, legumes, and dairy products, as the health benefits associated with these foods likely outweigh the unsubstantiated risks attributed to them. This strategy will result in a shift from highly-processed food products with added sodium, K^+, and efficiently-absorbed inorganic phosphorus additives, to low-sodium foods with primarily poorly-absorbed organic sources of phosphorus that have the added benefits of additional antioxidants, vitamins, and dietary fiber. This recommendation should be taken within the context of clinical judgement and patient-specific considerations, particularly in terms of K^+ intake. However, it is critically important to remember that little evidence exists in support of dietary K^+ intake and hyperkalemia.[77] Patients should also be instructed in food preparation methods known to reduce phosphorus and K^+ quantities.

2) Instead, the primary focus of dietary restrictions should be on the reduction/elimination of processed, restaurant, and convenience foods that are almost universally high in sodium, inorganic phosphorus, and added K^+. Apart from these obvious benefits is the advantage of a far simplified message compared to current HD diet recommendations. Patients adhering to these recommendations may also realize cost-savings through increased food consumption within the home.

Implementing this approach will take a concerted effort focused on several factors. First, patient education must shift from providing lists of foods to *not* eat to instruction on how to identify and shop for unprocessed whole foods and label reading with an emphasis on sodium, K^+, and phosphorus additives. One method that we have implemented in our counseling experience is to encourage patients to look for packaged products that have a sodium (mg) to kcal ratio of 1:1 or less. Products that contain fewer mg of sodium than kilocalories are generally lower sodium options and this concept is easily understood by patients. In addition, we firmly believe that HD clinic staff and caregiver support is essential to overall patient success. The clinic staff have the unique opportunity of extended face time with patients, allowing for consistent reinforcement of general nutrition principles. Caregiver support also is essential to ensure consistent messages and to eliminate a reversion to habits at home. This can be accomplished through continued staff and caregiver education alongside, and independent of, patient education. Development of staff and caregiver specific tools and resources may be necessary. Overall, improved patient health and quality of life may be within reach through a simplification and corresponding liberalization of the HD diet prescription. Further work must be conducted to establish protocols that are appropriate for implementation in the United States, taking into consideration HD treatment standards, the Western diet, and clinic staff education.

Table 2.1 Current dietary recommendations for HD patients and summary of proposal for a more liberalized diet approach

Nutrient	Current Recommendations	Hypothesized rationale	Evidence/Comments	Proposed changes and potential benefits
Sodium	<2,000mg/day	↓ thirst stimulation: prevention of plasma expansion, chronic volume overload, and CV complications	Observational studies showing benefits of dietary Na^+ restriction along with intensive ultrafiltration on CV complications and reduced use of antihypertensive medications [30,31,38]	**1)** ↓ processed food intake (grocery store, fast-food restaurants, full-service restaurants, convenience stores), which are usually ↑ in Na^+ and P additives, as well as K^+ additives - If processed foods are consumed, educate on how to read food label to avoid ↑ Na^+ and P additives
Phosphorus	<800-1000mg/day	Prevention of hyperphosphatemia and consequences: vascular calcification, CV mortality and overall mortality	↓ inflammatory biomarkers[33] Overall P restriction has been associated with lower protein and energy intake[65,66] Recommendation does not differentiate between organic and inorganic P[57]	**2)** ↑ intake of whole-foods and meals prepared at home: - Ease restrictions on fruits, vegetables, whole-grains, nuts, legumes, and dairy. Despite containing P and K^+, they also contain fiber and other heart-healthy nutrients. - Educate on food-preparation methods that can ↓ P and K^+, such as boiling and leaching - Introduce use of spices without Na^+ to ↑ palatability of foods
Potassium	<2-4g/day	Prevention of hyperkalemia and consequences: cardiac arrhythmia, cardiac arrest and mortality	Very weak association between dietary K^+ intake and serum K^+[77]	- Use P-bioavailability as education tool on *good* food options (↑ organic P) vs. *questionable* options (↑ inorganic P) - Educate on portion control of known foods with ↑ or moderate K^+; monitor blood K^+

CV, cardiovascular; HD, hemodialysis; Na^+, sodium; P, phosphorus; K^+, potassium

CHAPTER 3

GUT MICROBIOTA AND CLINICAL RISK FACTORS IN HEMODIALYSIS PATIENTS

Abstract

The gut microbiota (GM) is important for human health and has been implicated in the pathogenesis of many chronic diseases. However, little is known about the composition and effects of the GM in patients with end-stage renal disease undergoing chronic hemodialysis (HD). The aim of this study was to examine the GM structure and its association with clinical risk factors in HD patients.

Methods: In this cross-sectional study, ten HD patients (7M, 50 ± 4years, 80% African American) were assessed on a non-dialysis day. Assessment included: bone and body composition by DEXA; arterial function by applanation tonometry; dietary intake through dietary recalls over the 48h prior to the fecal sample collection; and plasma lipopolysaccharide-binding protein (LBP). Participants were asked to collect a complete fecal sample; DNA was extracted and the V4 hypervariable region of the bacterial 16S rRNA gene was sequenced using Illumina MiSeq. Sequence data was analyzed using QIIME 1.9.1. Descriptive statistics were reported while Spearman correlations were used to compare GM operational taxonomic units (representative of ≥1% of total bacterial population) and clinical risk factors.

Results: Firmicutes-to-*Bacteroidetes* ratio (1.40±0.37) was positively associated with resting brachial and aortic systolic blood pressures (ρ= 0.648 and 0.636, respectively; $p<0.05$). Additionally, it was positively associated with dietary intake of total and saturated fat (ρ=0.667 and 0.636, respectively; $p<0.05$). At the genus level, *Bacteroides* was the most abundant genus in

all patients, ranging from 26.91% to 43.22% (mean 33.59±5.71%) of total sequences. Meanwhile, *Faecalibacterium* was variably represented between patients (median 7.54%; range 0.1 to 23.17% of the total sequences) and was positively associated with total carbohydrate intake (ρ=0.636; $p<0.05$) and negatively associated with carotid-femoral pulse wave velocity (ρ=-0.867, p=0.001), a surrogate of arterial stiffness. LBP (32.35 ± 4.05µg/ml) was inversely associated with butyrate producers *Ruminococcus* and *Roseburia* (ρ=-0.733, -0.697, p=0.016, 0.025, respectively) and positively associated with *Bilophila*, (ρ=0.644, p=0.044).

Conclusions: The *Firmicutes*-to-*Bacteroidetes* was associated with traditional risk factors for cardiovascular disease. At the genera level, *Faecalibacterium*, was strongly associated with lower arterial stiffness, which is an independent risk factor for cardiovascular mortality in this population. Finally, LBP was positively associated with *Bilophila*, and inversely associated with some genera that possess the enzymes necessary for butyrate production. Future studies are needed to further explore the relationship between the gut microbiome and cardiovascular health in HD patients and to assess if interventions that target the gut microbiome translate into a lower burden for clinical risk factors in this clinical population

Introduction

The gut microbiota is the community of microorganisms residing in the gut, including bacteria, archaea, viruses, and fungi.[95,96] These microorganisms are especially concentrated in the distal ileum and throughout the colon.[97] The gut microbiota is important for human health and has been implicated in the pathogenesis of many chronic diseases, including kidney disease.[98] A unique gut microbiota has been described in chronic kidney disease (CKD) and end-stage renal disease (ESRD), including bacterial overgrowth in the duodenum and jejunum, overgrowth of some aerobic species, such as *Enterobacteria* and *Enterococci*, and a decrease in commensal bacteria genera, such as *Bifidobacteria*.[99,100]

Besides the gut microbiota composition, metabolites produced by these bacteria have been associated with cardiovascular disease and the progression of CKD.[101,102] Specifically, p-cresyl sulfate and indoxyl sulfate, which are products of the fermentation of tyrosine and tryptophan, respectively, have been associated with higher cardiovascular mortality, endothelial dysfunction, and mineral and bone disorder.[103–106] Furthermore, trimethylamine-oxide (TMAO), which is derived from the bacterial metabolite trimethylamine through the metabolism of choline, betaine, and carnitine, has also been associated with the progression of CKD.[99,104]

In healthy individuals these metabolites are excreted in the urine, but due to the reduced or blunted ability to produce urine in ESRD patients, the concentrations of these bacterial metabolites are higher than in adults with preserved kidney function.[107–109] However, little is known about the structure of the gut microbiota and its relationship with clinical risk factors in ESRD patients undergoing maintenance hemodialysis (HD). Therefore, the objective of this study was to examine the gut microbiota composition and its association with clinical variables,

such as body composition, arterial stiffness, physical function, dietary intake, and blood biomarkers in HD patients.

Methods

HD patients were recruited from local dialysis clinics in Central Illinois. Patients were approached for recruitment if they received HD treatment 3 days/week, were ages >30 years, did not have gastrointestinal disease (e.g., inflammatory bowel disease), did not take antibiotic treatment the month before testing, and had been receiving dialysis treatment for at least three months. Consent was obtained from each participant and all protocols were approved by the University of Illinois Institutional Review Board and were in accordance with the Declaration of Helsinki.

Following the initial recruitment, all individuals who qualified for the study and agree to participate underwent a battery of tests on a single occasion at the beginning of the study:

Anthropometry, Bone, and Body Composition

Barefoot standing height was measured to the nearest 0.1 cm with a stadiometer (Seca 222, Hamburg, Germany). Body weight was measured on a digital scale (Tanita BWB-800, Arlington Heights, IL) with shoes and superfluous outer garments removed. All measurements were taken in duplicate and averaged. Whole-body fat, lean, bone mass, and bone mineral density was measured by dual emission x-ray absorptiometry (DXA) (Hologic QDR 4500A, Bedford, Massachusetts) following manufacturers protocol.

Wave Reflection and Arterial Stiffness

Blood pressure was measured in duplicate using an automated cuff following a 10-minute quiet rest in the supine position (Omron IntelliSense HEM-907XL, Lake Forest, IL). Radial wave forms were then collected via tonometry and were used to estimate central pressures using a validated transfer function (SphygmoCor, AtCor Medical, Sydney, Australia). Aortic pulse wave velocity (PWV) was determined by tonometry (SphygmoCor, AtCor Medical, Sydney, Australia).[110] In short, aortic PWV was calculated as the time delay (Δt) between the R-wave of the ECG and the foot of the forward pressure wave form (Intersecting Tangent) between the carotid and femoral arteries using the equation: PWV= D/Δt (m/sec); where D is distance in meters and Δt is the time interval in seconds. D was calculated by subtracting the distance between the sternal notch and the location the carotid pressure was measured from the distance between the sternal notch and the location the femoral pressure was measured.

Physical Function

Normal gait speed was determined as the average walking speed (meter per second) recorded over a 10-m course. The measurement was taken in triplicate and averaged. After the measurement of gait speed, participants underwent a validated incremental shuttle walk test to assess physical performance as previously described.[111] In short, this test involved walking back and forth over a 10-meter course to successively faster time constraints until the participant was no longer able to complete the course in the allotted time. Finally, participants completed the 8ft up-and-go test, in which starting in a seated position, participants are asked to walk as fast as possible to a mark (cone)placed 8ft apart and return back to their seat, to assess functional fitness.[112]

Dietary Intake

Dietary recalls covering the 48 hours prior to receiving the fecal sample for analysis were collected by a trained researcher using the modified version of the USDA 5-pass method.[113] In short, the first pass asks for the patients to recall everything they ate during the previous 24-hours, the second pass probes for foods that may have been forgotten including beverages, snacks, and condiments, the third pass includes prompts for portion sizes and food amounts, the fourth pass asks for details about the food including brand names, and the fifth pass consists of a final review of the record. The records were analyzed for macronutrient and micronutrient composition, as well for food groups and individual food-item consumption using Nutrition Data System for Research (NDSR 2014 version, University of Minnesota, Minneapolis, MN).

Fecal Collection and Consistency

Participants were asked to collect a complete fecal sample (Commode Specimen Collection System Sage Products, Crystal Lake, IL). Participants were asked to rate consistency and ease of passage for the bowel movement. Stool consistency was scored according to the Bristol Stool Scale. Ease of stool passage was ranked on a 5-point scale (1=very easy, 2=easy, 3=neither easy nor difficult, 4=difficult, 5=very difficult). Samples were weighed, homogenized and three-2ml aliquots were stored at -80°C within 30 minutes of collection.

DNA was extracted (Powerlyzer PowerSoil DNA Isolation Kit MO BIO, Carlsbald, CA) and its quantification was performed by Qubit Fluorometric Quantitation (ThermoFisher Scientific, Waltham, MA), while quality was assessed by electrophoresis with agarose 2% Agarose E-gels using the E-Gel iBase (Invitrogen, Grand Island, NY); primers targeting the V4 hypervariable region of the bacterial 16S rRNA gene amplicons of 250bp were generated as described previously.[114] Sequencing was performed through Illumina Mi-seq V3 platform. Relative changes in bacterial diversity (α-diversity) and taxonomical changes were analyzed

through the open software QIIME (version 1.9.1). In short, sequenced data was demultiplexed and went through quality filtering using split_libraries_fastq.py default parameters. Sequences were clustered into operational taxonomic units (OTU) using closed-reference OTU picking against the Greengenes 13_8 reference OTU database with a 97% similarity threshold.[115] Since it was a cross-sectional study without a control group, α-diversity, or within-sample diversity was assessed through phylogenetic metrics (e.g., phylogenetic distance) and non-phylogenetic metrics (e.g., OTU count and Chao1).[116] OTU count describes the number of OTUs in a given sample, whereas Chao1 takes into account the of rare species (i.e., singletons). Phylogenetic distance sums the branch length in a phylogenetic tree covered by the sample of interest.

Blood chemistry

Plasma samples were collected in lithium-heparin coated tubes at the start of the dialysis treatment immediately after the fecal sample collection. Whole blood samples were centrifuged and separated into 500 µl aliquots and were stored at -80˚C until analyzed. A renal function panel that included albumin (bromocresol purple method), blood urea nitrogen, total calcium, chloride, creatinine, glucose, potassium, and sodium was measured by a point-of-care autoanalyzer (Piccolo Xpress, Abaxis Inc., Union City, CA).

Lipopolysaccharide-binding protein (LBP)

Plasma LBP was determined by a commercially-available enzyme-linked immunosorbent assay (ELISA) kit (Cell Sciences, Canton, MA). Inter-assay Coefficient Variation (CV) was 9.8-17.8%, while the intra-assay CV was 6.1%. The effective range of the assay was 5-50 ng/ml. All standards, blank, and samples were performed in triplicate and averaged. If there was a percent

coefficient of variation (% CV) above 5%, the values were analyzed and assessed if one of the measurements should be deleted to decrease variation.

Statistical Analysis

All data were analyzed using SPSS 24 (IBM Corporation, Armonk, NY). Data are presented as the mean ± standard error of the mean (SEM), unless otherwise indicated. Spearman correlations were used to compare gut microbiota operational taxonomic units (representative of ≥1% of total bacterial population) and variables of interest. For all statistical tests, significance was considered as $p \leq 0.05$.

Results

In this cross-sectional study, ten HD patients (7 males/ 3 females, age 50 ± 4years, 80% African American, Table 3.1) were recruited and completed the assessments. For the fecal microbiota, a total of 308,536 bacterial 16S rRNA sequences were obtained, with a median of 31,199 (range 25,056-35,617) sequences per sample. For diversity and species richness, the data was rarified to 25,056 sequences. We observed a mean species richness (Chao1) of 339±66 species, which was inversely associated with age (ρ=-0.806; p=0.005) (Figure 3.2).

At the phyla level (Figure 3.1), *Firmicutes* and *Bacteroidetes* were the most abundant, followed by *Proteobacteria* and *Verrucomicrobia* (54.02±1.53, 40.07±2.18, 2.63±0.59, and 1.63±1.22% of total sequences, respectively). There was a *Firmicutes-to-Bacteroidetes* ratio of 1.40±0.11, which was positively associated with traditional risk factors for cardiovascular disease, such as resting brachial and aortic systolic blood pressures (ρ= 0.648 and 0.636, respectively; $p<0.05$). Additionally, it was positively associated with dietary intake of total fat, saturated fat, and meat (ρ=0.667, 0.636, 0.661, respectively; $p<0.05$). At the genera level,

Bacteroides was the most abundant genus in all patients, with a mean of 33.59±1.81% of total sequences. Meanwhile, *Faecalibacterium* was variably represented between subjects (mean 9.08±2.3% of total sequences; range 0.10-23.17% of total sequences) and was positively associated with total carbohydrate intake (ρ=0.636; p=0.048) and negatively associated with carotid-femoral pulse wave velocity (ρ=-0.867, p=0.001) (Figure 3.3). Concurrently, the genus *Akkermansia* was not present in 40% of the participants and its concentration ranged from 0 to 12.02% of sequences (median 0.008%, interquartile range 0.0024-1.1081% of total sequences). Furthermore, *Prevotella* was only present in one participant, but was not associated with any variable tested. Other bacterial genera that have been associated with negative physiological effects were expressed in various relative abundances: *Desulfovibrio* (present in two subjects, 1.41-1.51% of total sequences) and *Bilophila* (present in six subjects, 0.01-0.75% of total sequences), but no associations with any clinical risk factors were observed. Finally, LBP concentration was 32.35 ± 4.05 µg/ml, and was positively associated with *Bilophila* (ρ=0.644, p=0.044), and inversely correlated with *Ruminococcus* and *Oscillospira.* (ρ=-0.733, -0.697, p=0.016, 0.025, respectively).

Discussion

In this cross-sectional study, we assessed the relationship between the fecal microbiota and clinical variables of interest in ten HD patients. First, we observed that bacterial richness was inversely associated with age. At the phylum level, the two most abundant phyla were *Firmicutes* and *Bacteroidetes.* Furthermore, the *Firmicutes-to-Bacteroidetes* ratio was positively associated with traditional risk factors for cardiovascular disease, such as resting brachial and arterial systolic blood pressure, total fat and saturated fat intake, as well as meat intake. Moreover, plasma concentration of LBP, a marker of bacterial translocation, was positively associated with

Bilophila, and negatively associated with the known butyrate producing bacterial phyla *Roseburia* and *Oscillospira,* but not *Faecalibacterium.* Finally, and perhaps the most interesting finding, was that *Faecalibacterium* was associated with aortic pulse-wave velocity, a surrogate of arterial stiffness. *Faecalibacterium prausnitzii* is the most common species within the *Faecalibacterium* genus and it is a known butyrate producer.

Bacterial species richness has been suggested as an important factor in the gut microbiome of healthy individuals.[117] We observed a mean species richness of 339±66, which is below what has been reported in healthy adults and healthy elderly populations.[118] Additionally, we found that species richness was inversely associated with age (*Figure 3.2*). These data support previous findings suggesting reduced species richness with age.[9] Since CKD has been described as an accelerated aging process, we also assessed if there was a relationship between bacterial species richness and dialysis vintage. While no relation between these variables were found, this may have been due to the specifics of our study population, in which the younger patients tended to have spent more time on dialysis compared to older patients (<50y: 95±40 months vs. 49±19 months). Previous studies have reported that species richness is lower in obesity.[119] However, we did not find an association between species richness and body mass index or any other anthropometric measures, such as weight, whole-body fat, or waist-to-hip ratio.

The ratio of *Firmicutes*-to-*Bacteroidetes* is commonly assessed in both healthy and clinical populations since these two phyla are the most abundant in the gut.[120] In our study, *Firmicutes* were represented by 54.02±1.53% of the total sequences, while *Bacteroidetes* were represented by 40.07±2.19% of the total sequences, accounting for 94.09% of the total sequences. The *Firmicutes*-to-*Bacteroidetes* ratio has been reported to be greater in experimental

models of obesity, without a difference in food consumption, suggesting an increased capacity for energy harvest.[121] However, several studies have found a higher ratio in lean individuals or did not agree with this association.[122] In our study, the *Firmicutes*-to-*Bacteroidetes* ratio was 1.40±0.37. Furthermore, we found a positive association between this bacterial ratio and intakes of total fat, saturated fat, and meat. This was somewhat surprising because a higher abundance of *Bacteroides,* a genera within the *Bacteroidetes* phylum, has been associated with an animal-based diet, which is usually higher in total and saturated fat.[123] Additionally, we did not find an association between *Bacteroidetes* phylum or *Bacteroides* genus and meat, fat, and saturated fat intake. Finally, we found no associations with other bacteria genera that traditionally have been associated with a higher consumption of animal-based products, such as *Desulfovibrio* and *Bilophila*. Larger scale studies should aim to assess the relationship between dietary patterns across CKD and changes in the gut microbiota structure and function.

Interestingly, we found an inverse association between the genus *Faecalibacterium* and carotid-femoral pulse wave velocity, a surrogate of arterial stiffness. Within the genus *Faecalibacterium, Faecalibacterium prausnitzii* is a known butyrate producer.[124,125] A decrease in this bacteria species has been reported with age.[118] Concurrently, arterial stiffness increases with age.[126] However, we did not find an association between *Faecalibacterium* and age. Interestingly, there have not been any reports of a direct relationship between the relative abundance of *Faecalibacterium prausnitzii* and cardiovascular disease. One possible explanation that may be driving this relationship is the inverse correlation between butyrate production and systemic inflammation.[124] Unfortunately, we did not assess inflammation, which was a limitation of the study due to the link between the gut microbiota and immunity; the only indirect measurement of inflammation that we had available was albumin, which has been described as a

reverse acute-phase protein. Nevertheless, this relationship was not significant (ρ=0.526; p=0.118). Further studies should aim to explore at a larger scale the relationship between *Faecalibacterium prausnitzii* and cardiovascular disease in CKD and ESRD.

Endotoxemia, or high concentrations of lipopolysaccharide (LPS) or proteins associated with it, such as LBP, have been reported prevalent in CKD and ESRD undergoing HD treatment.[127] This may be in part due to the direct effect of uremia on the intestinal tight-junction proteins, increasing the risk of bacterial translocation from the intestinal lumen to the bloodstream.[128] Moreover, endotoxemia contributes to the increased systemic inflammation in HD patients.[129] In our study, we found that LBP was 32.35 ± 4.05µg/ml, which is higher than what has been reported in healthy subjects, but similar to other studies in CKD, ESRD, and kidney transplant subjects.[127,130] LBP was inversely associated with *Ruminococcus* and *Roseburia,* genera with known butyrate producers, but not *Faecalibacterium* or other butyrate producers. Furthermore, we observed a positive correlation between LBP and *Bilophila,* which is a genus that has been associated with a dietary pattern high in animal products, such as meat.[123] However, we did not find an association between LBP and any nutrient intake or food group consumption. Future studies should aim to assess if interventions that target the gut microbiota in order to increase butyrate production decreases endotoxemia and inflammation in HD patients.

There are several limitations in our study. First, this was a cross-sectional study with a small sample size. Second, we did not have a control group to compare our variables against. Lastly, our patients were recruited from only one clinic in Central Illinois and cannot be generalized to other HD patients. However, we believe this study offers clear rationale to further explore the gut microbiota and clinical risk factors in HD patients and to develop targeted interventions to assess if changes in the gut microbiota translate into better outcomes in HD

patients, especially due to the association between the gut microbiota and cardiovascular disease, which is the main cause of mortality in HD patients.

Conclusions

We observed an inverse association of α-diversity with age, similar to what is reported in the aging population. Like other metabolic diseases, the gut microbiome showed a high *Firmicutes-to-Bacteroidetes* ratio at the phylum level. However, at the genus level there was high variability across individuals with some bacteria associated with positive health outcomes. *Akkermansia,* a gram-negative bacterium that preferentially colonizes the mucus layer and is associated with improved metabolic health, was expressed in relatively low concentrations and was not present in some individuals, similar to *Prevotella,* which previously has been associated with higher dietary fiber intake. This association, however, was not found because it was present in only one individual. In addition, dietary fiber intake was less than 50% of the recommendation (6.26±2.48g/1000kcal). Furthermore, *Faecalibacterium,* a known butyrate producer, was inversely correlated with arterial stiffness, an independent risk factor for cardiovascular mortality in this population. Finally, LBP was positively associated with *Bilophila,* and inversely associated with genera that possess the enzymes necessary for butyrate production. Future studies are needed to further explore the relationship between the gut microbiome and cardiovascular health in HD patients and to assess if interventions that target the gut microbiome translate into a lower burden for clinical risk factors in this clinical population

Table 3.1 Patient characteristics

Variable	Mean ± SD /Median (IQR)
Demographics	
Age (years)	50 ± 4
Sex (M/F)	7/3
Ethnicity (% African American)	80%
Anthropometry, bone, and body composition	
BMI (kg/m^2)	31.04 ± 7.4
Waist-to-hip circumference ratio	0.94 ± 0.014
Whole-body fat (%)	30.21 ± 3.37
Whole-body lean mass (kg/m^2)	21.09 ± 1.22
Whole-body BMD (g/cm^2)	1.2 ± 0.03
Cardiovascular	
Brachial SBP (mmHg)	157.3 ± 8.39
Brachial DBP (mmHg)	82.3 ± 3.09
Aortic SBP (mmHg)	143.6 ± 8.38
Aortic DBP (mmHg)	84 ± 3.07
Augmentation Index @75bpm	28.3 ± 3.58
cfPWV (m/s)	9.21 ± 0.63
Physical function	
Gait speed (m/s)	9.06 (8.61-10.23)
ISWT (s)	291.4 ± 44.05
8ft up-and-go (s)	3.86 ± 0.82
Dietary intake	
Energy (kcal/kg/d)	24.37 ± 2.77
Protein (g/kg/d)	0.86 ± 0.09
Total fat (% total kcal)	37.91 ± 2.39
Carbohydrates (% total kcal)	48.23 ± 2.53
Fiber (g/1000kcal)	6.24 ± 0.79
Protein-to-fiber ratio	6.39 ± 0.57
Blood parameters	
Albumin (g/dl)	4.05 ± 0.09
Phosphorus (mg/dl)	5.98 ± 0.58
Potassium (mg/dl)	4.95 ± 0.18
LBP (µg/mL)	32.35 ± 4.05

SEM, standard error of the mean (for normally distributed data); IQR, interquartile range for (for non-normally distributed data); M, male; F, female; BMI, body mass index; BMD, bone mineral density; SBP, systolic blood pressure; DBP, diastolic blood pressure; bpm, beats per minute; cfPWV, carotid-femoral pulse-wave velocity; ISWT, incremental shuttle-walk test; LBP, Lipopolysaccharide-binding protein.

Table 3.2 Relative abundance (% of total sequences) of bacteria (phyla and genera)

Bacteria Genera	Mean ± SD /Median (IQR)
Bacteroidetes	
Bacteroides	33.59±1.81
Parabacteroides	4.66±1.14
Firmicutes	
Faecalibacterium	9.08±2.30
Lachnospiraceae	5.80±1.02
Clostridiales	5.12±0.78
Blautia	4.68(1.52, 7.56)
Ruminococcus	1.95(0.49, 4.34)
Dorea	1.53±0.45
Eubacterium	1.37±0.27
Streptococcus	0.94 ± 0.014
Oscillospira	0.59±0.12
Roseburia	0.32(0.10,1.14)
Actinobacteria	
Bifidobacterium	0.33(0.03,1.19)
Proteobacteria	
Bilophila	0.11(0.00,0.42)
Verrucomicrobia	
Akkermansia	0.0081(0.0024,1.11)

SEM, standard error of the mean (for normally-distributed data); IQR, interquartile range (for non-normally distributed data).

Figure 3.1 *Firmicutes*-to-*Bacteroidetes* ratio in HD patients

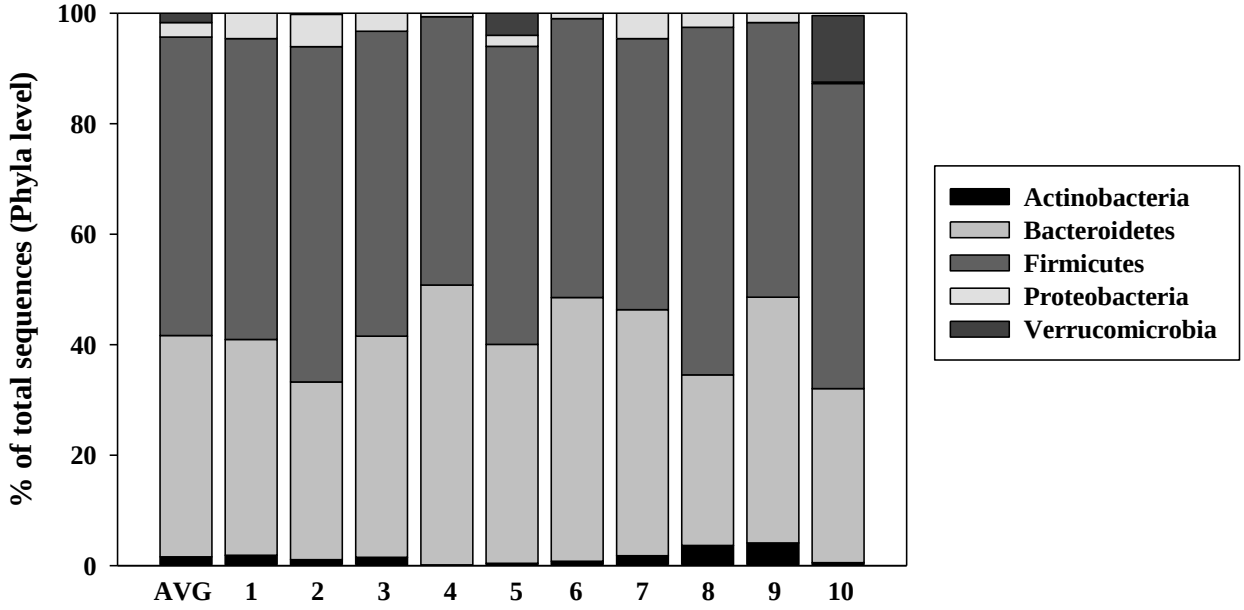


Figure 3.1 *Firmicutes* and *Bacteroidetes* were the two most abundant phyla in our subjects and accounted for ~94% of the total sequences. The *Firmicutes*-to-*Bacteroidetes* ratio was 1.4±0.37

Figure 3.2 Age is associated with a lower α- diversity

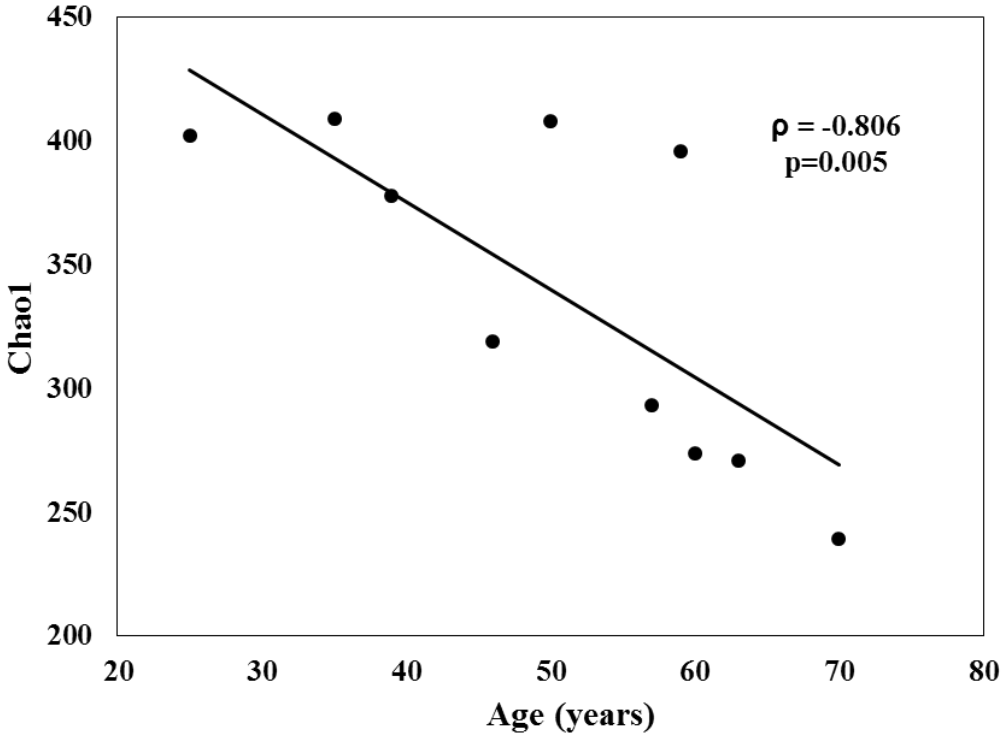


Figure 3.1 There was a negative association between age and Chao1, a metric of α-diversity (ρ=-0.806, p=0.005)

Figure 3.3 *Faecalibacterium* is inversely associated with arterial stiffness

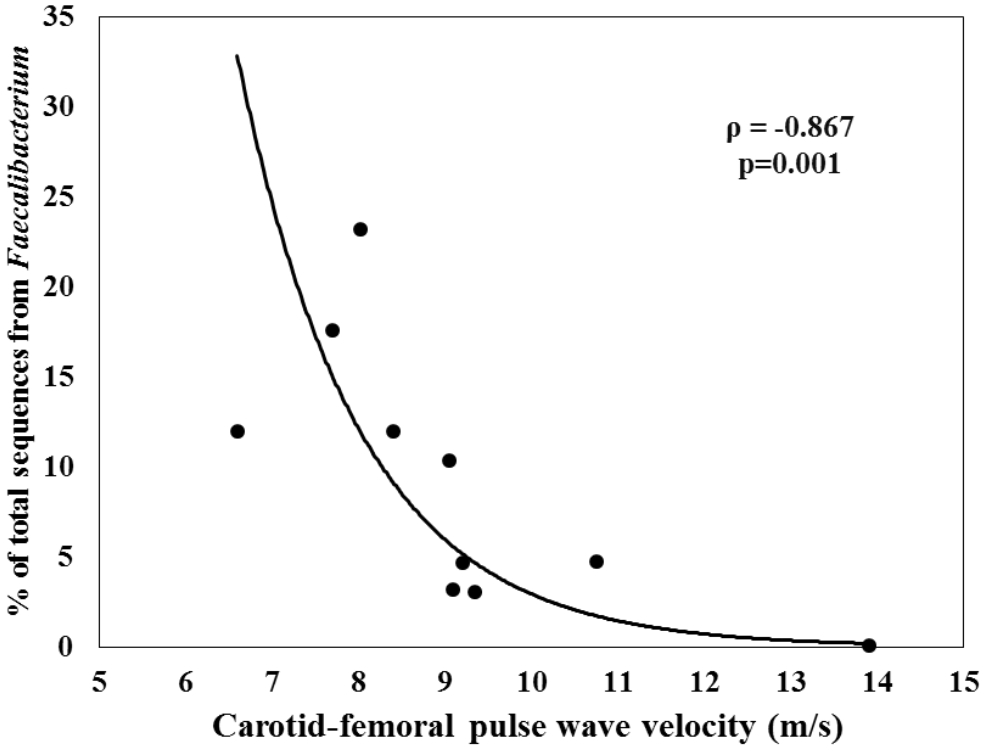


Figure 3.2 There was a negative association between carotid-femora pulse wave velocity and the relative abundance of *Faecalibacterium* (ρ=-0.867, p=0.001).

CHAPTER 4

EFFECT OF INULIN SUPPLEMENTATION ON MARKERS OF BONE AND MINERAL BIOMARKERS IN HEMODIALYSIS PATIENTS

Abstract

Mineral and bone disorder (MBD) is highly prevalent among hemodialysis patients (HD) and is associated with increased morbidity and mortality. Despite the pharmacological treatment for MBD, its prevalence remains high. The supplementation of dietary fiber may lead to an enhanced absorption of minerals mediated by the production of short-chain fatty acids (SCFA) by the gut microbiota, which has benefited other populations with MBD. However, this mechanism remains unexplored in HD patients. Our objective was to examine the effect of a 4-week supplementation of a fermentable dietary fiber (inulin) on markers of mineral and bone metabolism and fecal excretion of minerals in HD patients.

Methods: This was a randomized, double-blind, placebo-controlled, crossover design in which subjects consumed inulin (IN) [10g/d for females; 15g/d for males] or maltodextrin (CON) [6g/d for females; 9g/d for males] for 4 weeks, separated by a 4-week washout period. Plasma and fecal samples were obtained before and after each supplementation period. Blood chemistries were obtained using an autoanalyzer, while mineral and bone biomarkers were assessed using commercially available ELISA assays. Outcomes were assessed using a within-subjects repeated measures ANOVA with treatment (IN, CON) and time (Pre, Post) as the independent variables.

Results: Twelve HD patients completed the study (56±10 y, 50% male, 58% African American, 31.2±9.2kg/m2). IN supplementation corrected dietary fiber intake (IN 7.9±5.3 to 15.4±2.7g/1000kcal vs. CON 6.7±2.5 to 7.8±5.2g/1000kcal; p interaction=0.024). However, IN

did not produce any changes in blood calcium, phosphorus, and magnesium ($p>0.05$ for all), nor any of the biomarkers of bone and mineral metabolism of interest, including FGF-23, BALP, and CTX ($p>0.05$ for all). There was a time effect on iPTH, decreasing by 35% in both IN and CON groups (time $p=0.031$). Furthermore, there were no differences in the fecal excretion of calcium or magnesium ($p>0.05$). However, fecal excretion of phosphorus was maintained during IN supplementation (Pre 22.73±1.46 vs. Post 21.24±2.0, $p=0.44$), while it was decreased in the CON group (Pre 25.25±1.94 vs. Post 19.49±1.85; group-by-time interaction $p=0.025$), despite a similar phosphorus intake at both CON timepoints (Pre 914.96±134.72 vs. Post 983.54±152.06mg/d, $p=0.586$).

Conclusion: IN did not produce significant changes in plasma minerals or markers of bone metabolism, or fecal excretion of calcium and magnesium. Future studies should aim to explore whether isolated sources of fiber have an effect on the efficacy of phosphate binders and MBD markers in HD patients.

Introduction

One of the most prevalent problems in ESRD patients undergoing chronic hemodialysis (HD) treatment is mineral and bone disorder (MBD), which manifests as early as chronic kidney disease (CKD) stage 2, where kidney function has declined by 10-40%.[1,2] The diagnosis of MBD includes at least one of the following: a) laboratory abnormalities (e.g., hypocalcemia, hyperphosphatemia, secondary hyperparathyroidism, low concentrations vitamin D [calcidiol and/or calcitriol]) and high concentrations of fibroblast growth factor-23 (FGF-23); b) bone abnormalities (e.g., altered turnover, volume and mineralization); and c) extra-skeletal calcifications (e.g., vascular calcification).[3] The disruption in bone and mineral homeostasis may critically affect bone structure and function by altering bone turnover, with a negative bone balance phenotype, characterized by a high bone resorption and normal-to-low bone formation.[4] Up to 80% of CKD patients have some degree of MBD, which has been associated with increased risk of fractures, cardiovascular disease, hospitalization and mortality and lower quality of life.[5,6]

The Kidney Disease Improving Global Outcomes (KDIGO) guidelines recommend to monitor serum concentrations of calcium, phosphorus and parathyroid hormone (PTH). Recently, they recommended the addition of bone-specific alkaline phosphatase (BALP) as a confirmatory and complementary test to assess bone turnover.[4] Furthermore, the National Kidney Foundation- Kidney Disease Outcomes Quality Initiative (NKF-KDOQI) guidelines recommend pharmacological and nutritional therapies targeted to decrease MBD abnormalities in HD patients.[7] Standard pharmacological therapies for MBD target a reduction in absorption of dietary phosphate (e.g., phosphate binders), mimic the action of calcium in the parathyroid gland (e.g., calcimimetics), and supplement vitamin D to offset its reduced activation by the kidney

(e.g., vitamin D analogues).[3] Despite these therapies, it has been estimated that approximately 50% of HD patients still have some degree of MBD.[131] Furthermore, these therapies are expensive[132] and may increase pill-burden,[133] which is a major problem in HD patients. Therefore, low-cost therapies targeting improved bone and mineral metabolism are needed.

Dietary phosphate restriction constitutes a vital part of the treatment for MBD along standard pharmacological treatment.[7] For dialysis patients, the NKF-KDOQI guidelines recommend a dietary phosphate intake of 800-1000mg/day.[7] This is in addition to restrictions on other nutrients, including potassium.[94] To achieve this restriction, renal dietitians often advise HD patients to limit food groups that are high in these nutrients, such as fruits, vegetables, whole grains, nuts and legumes. However, these food groups are also good sources of dietary fiber, so their restriction may lead to a low fiber intake.[8,134] Additionally, we have observed in a representative sample from our local dialysis units that patients consume less than 60% of the adequate intake (AI) of fiber, which is 14g/1000kcal or 25g and 38g/day for adult females and males, respectively.[80] Recently, Chiavaroli et al.[135] conducted a systematic review and meta-analysis of the effect of supplementing dietary fiber on serum urea, creatinine, and phosphorus in CKD and ESRD patients. From the fourteen studies analyzed, they concluded that dietary fiber caused a significant decrease in urea (n=120; mean difference -4.9 (95% CI -8.4, 1.43) mg/dl), and creatinine (n=120; mean difference -0.25 (95% CI -0.47, -0.33) mg/dl), but not phosphorus (n=66; mean difference 0.12 (95% CI -0.22,0.49) mg/dl). The potential explanation for these effects include the effect of fermentable fiber on bacterial growth,[136] displacement of protein as source of energy and use of it for growth,[99] and the elevation of ammonium excretion due to a decreased pH through the production of SCFA. This causes a shift from readily diffusible ammonia to less-diffusible ammonium.[137] To date, the decrease on concentrations of creatinine

may be due to the expression of creatinase in some bacteria.[138] Besides this study, there are no reports of the effect of fiber on mineral metabolism in CKD or ESRD patients.

Dietary Fermentable Fibers: Inulin-type Fructans and Mineral Metabolism

Dietary fiber is defined by the Food and Nutrition Board of the Institute of Medicine as those non-digestible carbohydrates and lignin that are intrinsic and intact in foods.[139] Additionally, they defined functional fiber as non-digestible carbohydrates that provide a beneficial physiologic effect, with the total fiber being the sum of dietary fiber and functional fiber.[139] According to the dietary fiber's physicochemical properties, they can be classified by their viscosity and fermentability properties, which also provide physiological benefits.[140] In terms of fermentability, commensal bacterial in the distal ileum, but more importantly in the colon metabolize fibers partially or totally to hydrogen, methane, carbon dioxide and short-chain fatty acids (SCFA), such as acetate, propionate and butyrate.[141] These SCFA have been shown to have effects locally (e.g., butyrate as energy source for colonocytes) and peripherally (e.g., adipose tissue, skeletal muscle, liver, brain, and kidney) by binding free-fatty acid receptors (FFAR) 2 and 3.[142] Among dietary fermentable fibers, inulin-type fructans (ITFs) have been extensively studied in animal (Table 4.1), healthy populations, and clinical populations (Table 4.2) due to their potential effect on mineral metabolism.

Inulin is a plant-derived fermentable fiber (from chicory root and Jerusalem artichoke, principally) with β (2-1) fructosyl-fructose linkages. ITFs degree of polymerization ranges from 2-70 fructose units with a low branching degree.[143] However, those that have a degree of polymerization of 2-10 could also be named fructo-oligosaccharides and those with >10 subunits as inulin.[141,143] Due to the β-linkages, ITFs cannot be hydrolyzed in the small intestine and, therefore, follow the criteria to be classified as a dietary fiber. In a review by Coxam,[144] several

mechanisms were highlighted by which ITFs could improve mineral absorption. First, the bacteria in the distal gastrointestinal tract produces SCFA through the fermentation of ITFs. These SCFA in turn reduce the pH within the portion of the colon that are produced, improving the solubility of minerals (e.g., dissociating from other compounds, such as phosphates) and enhancing their absorption, especially through the paracellular route.[145] Furthermore, butyrate is generally recognized as the major source of energy for colonocytes, and by enhancing its production, it enhances colonocyte growth and functional surface area for absorption. Finally, there is some indication from experimental models that transcellular absorption of calcium may also be increased after the supplementation of ITFs, mainly through vitamin D-dependent transporters (e.g., calbindin) and the enhanced hydrogen-calcium exchanger.[144]

Supplementation of ITFs has been used in animal models (Table 4.1) of MBD showing an enhanced calcium and magnesium absorption, contributing to mineral and bone homeostasis. Overall, an effect on fractional calcium absorption and retention has been shown across animal studies. Effects in clinical populations (Table 4.2) also have been consistent, demonstrating up to a 12% increase in calcium absorption, as well as changes in bone turnover biomarkers. The effect of ITFs on mineral and bone metabolism in CKD and HD, however, has not been assessed.

In a representative sample of our two dialysis clinics, 60.1% of patient's had some alteration in markers of bone and mineral metabolism*:* 33.3% had hyperphosphatemia (serum phosphorus >5.5mg/dl), 68.6% had hyperparathyroidism (PTH >300ng/mL), and 17.5% had hypocalcemia (serum calcium concentrations corrected for serum albumin of <8.4mg/dl). Despite this high prevalence of MBD, the impact of supplementation of ITFs on mineral and bone homeostasis has not been documented in this clinical population. Therefore, our objective was to examine the changes in bone and mineral metabolism markers after a 4-week

supplementation of inulin. Our hypothesis was that four weeks of inulin supplementation would increase calcium and magnesium absorption, downregulating the secretion of parathyroid hormone (PTH) and decreasing the plasma concentration of phosphorus, compared to maltodextrin. Additionally, we tested the effect of the supplementation of inulin on bone turnover biomarkers: bone-specific alkaline phosphatase (BALP), as a marker of bone formation, and C-terminal telopeptide (CTX), as a marker of bone resorption.

Methods

HD patients were recruited from local dialysis clinics in Central Illinois. Inclusion criteria for participants included: 1) Received HD therapy 3 days per week and for at least 3 months. Exclusion criteria includes: 1) Sustained hypercalcemia (>3months). 2) Previous major gastrointestinal disease diagnosis (e.g., inflammatory bowel disease and celiac disease). 3) Antibiotic treatment less than two weeks prior the start of the study.

Intervention Protocol

As shown in Figure 4.1, in a randomized, placebo-controlled, cross-over design, patients were randomized to the intervention group (inulin [IN]) or placebo group (maltodextrin [CON]). For one month, patients consumed iso-caloric amounts of either: 1) IN (90% inulin with a degree of polymerization of 2-60 and 10% short-chain fructooligosaccharides, with a degree of polymerization of 2-8; ORAFTI SYNERGY, Beneo, Belgium) (IN; females: 10g/day; males: 15g/day), or 2) CON (100% Maltodextrin, Now Foods Carbogain, Bloomingdale, IL) (females: 6g/day; males: 9g/day). Between treatment periods, all subjects underwent a four-week washout period before continuing with the other treatment (IN or CON) for another month. The first week of each intervention period was an adaptation week, in which patients consumed half of the dose

(5g/day and 7.5g/day for females and males, respectively in the IN group; 3g/day and 4.5g/day for females and males, respectively in the CON group).

Supplementation Compliance

Supplement compliance was self-reported. Participants were asked at every HD treatment if they took the supplement that day and the previous day(s). Additionally, subjects were asked the type of fluid or food the supplement was mixed with, time of the day, and if the full or half dose was taken. Finally, bowel movement type was asked to be classified according to the Bristol Stool Chart at every HD treatment.

Blood Minerals

Blood samples (4ml) were obtained at the beginning and end of every period (Figure 4.1) during the HD treatment following the fecal sample collection (Chapter 5) in Lithium heparin-coated tubes. Whole-blood calcium, magnesium, and serum phosphorus were measured using an auto-analyzer (MetLac12 panel, Piccolo-Xpress, Abaxis, Union City, CA). Calcium was corrected according to Obi et al.[146] formula, which is specific for HD patients:

$$Corr.Ca\left({}^{mg}/_{dl}\right) = 1.35 \times total\ Ca\left({}^{mg}/_{dl}\right) - 0.65 \times Alb\left({}^{g}/_{dl}\right) - 0.5 \times P\left({}^{mg}/_{dl}\right) + 0.3$$

Where Ca is total calcium, Alb is albumin, and P is phosphorus.

Fecal minerals

Participants were asked to collect a complete fecal sample (Commode Specimen Collection System Sage Products, Crystal Lake, IL) at the beginning and end of Period 1 and 2 (Figure 4.1). Samples were weighed, homogenized, and stored at -80°C within 30 minutes of

collection until analysis. Fecal minerals (calcium, magnesium, and phosphorus) were assessed by atomic absorption spectroscopy and reported on a mg/g of dry matter basis.

Intact PTH (iPTH)

Plasma was collected in EDTA-coated tubes, then centrifuged at 2,400 rpm for fifteen minutes at 4°C, aliquoted and stored at -80°C until analysis. Intact PTH was measured by a commercially available enzyme-linked immunosorbent assay (ELISA) (ALPCO, Salem, NH). This assay had an intra-assay variability of 3.68-6.08% and inter-assay variability of 2.8-3.6%. The assay had a maximum detectable concentration of 1000 pg/ml. All standards, blank, and samples were performed in triplicate and averaged. Samples were diluted based on iPTH values from the patient's clinical record of the month the samples were collected. If there was a percent coefficient of variation (% CV) above 5%, the values were analyzed and assessed if one of the measurements should be deleted to decrease variation.

Fibroblast Growth Factor-23 (FGF-23)

Plasma FGF-23 was measured by a commercially available ELISA kit (RayBiotech, Norcross, GA). This assay had an intra-assay variability of <10% and inter-assay variability of <12%. The assay had a detectable range from 300 to 100,000pg/ml. All standards, blank, and samples were performed in triplicate and averaged. If there was a percent coefficient of variation (% CV) above 5%, the values were analyzed and assessed if one of the measurements should be deleted to decrease variation.

Bone-specific Alkaline Phosphatase (BALP)

Plasma BALP was measured by a commercially available ELISA kit *(*BioTrend, Destin, FL). This assay had an intra-assay variability of <8% and inter-assay variability of <10%. The

assay had a detectable range from 3.12-200ng/ml. All standards, blank, and samples were performed in triplicate and averaged. If there was a percent coefficient of variation (% CV) above 5%, the values were analyzed and assessed if one of the measurements should be deleted to decrease variation.

C-terminal Telopeptide (CTX)

Plasma CTX was measured by a commercially available ELISA kit (NEO Scientific, Cambridge, MA). This assay had an intra-assay and inter-assay variability of <10%. The assay had a detectable range from 0 to 10ng/ml. All standards, blank, and samples were performed in triplicate and averaged. If there was a percent coefficient of variation (% CV) above 5%, the values were analyzed to assess if one of the measurements should be deleted to decrease variation.

Bone and Mineral Metabolism Composite Score

We created a scoring system in which we classified our outcomes into positive, neutral, or negative, giving a value of +1, 0, or -1, respectively. Our scoring system was based on the expected concentration range of minerals and iPTH,[3] previously reported significant effect on the FGF-23,[147]and expected positive/negative effect on the rest of the parameters (Figure 4.20).

Body composition and bone mineral density

Whole-body, lumbar spine, and hip scans were measured by dual emission x-ray absorptiometry (DXA) (Hologic QDR 4500A, Bedford, Massachusetts) following the manufacturer's protocols. These measurements were used as descriptive variables and were performed only at baseline testing since we were not expecting changes in bone mineral density in a 4-week period.

Dietary intake

Dietary recalls covering the 48 hours prior to receiving the fecal sample (Chapter 5) for analysis were collected by a trained researcher using the modified version of the USDA 5-pass method.[113] In short, the first pass asks for the patients to recall everything they ate during the previous 24-hours, the second pass probes for foods that may have been forgotten including beverages, snacks, and condiments, the third pass includes prompts for portion sizes and food amounts, the fourth pass asks for details about the food including brand names, and the fifth pass consists of a final review of the record. The records were analyzed for macronutrient and micronutrient composition, as well for food groups and individual food-item consumption using Nutrition Data System for Research (NDSR 2014 version, University of Minnesota, Minneapolis, MN).

Wave Reflection and Arterial Stiffness

Blood pressure was measured in duplicate using an automated cuff following a 10-minute quiet rest in the supine position (Omron IntelliSense HEM-907XL, Lake Forest, IL). Radial wave forms were then collected via tonometry and were used to estimate central pressures using a validated transfer function (SphygmoCor, AtCor Medical, Sydney, Australia). Aortic pulse wave velocity (PWV) was determined by tonometry (SphygmoCor, AtCor Medical, Sydney, Australia).[110] In short, aortic PWV was calculated as the time delay (Δt) between the R-wave of the ECG and the foot of the forward pressure wave form (Intersecting Tangent) between the carotid and femoral arteries using the equation: $PWV = D/\Delta t$ (m/sec); where D is distance in meters and Δt is the time interval in seconds. D was calculated by subtracting the distance between the sternal notch and the location the carotid pressure was measured from the distance between the sternal notch and the location the femoral pressure was measured. These

measurements were performed in a subset of patients (n=5) during the same visit that the bone and body composition measurements were performed.

Statistical Analysis

Mean and standard error of the mean is reported unless otherwise noted. Baseline subject characteristics were analyzed using a one-way ANOVA, where the grouping factor was sex and medications against the variable of interest (e.g., baseline blood minerals, bone biomarkers, etc.). Repeated measures ANOVA was performed in a within-subjects analysis with two groups (IN, CON) and two time-points (Pre, Post), comparing the variables of interest with significance at $p<0.05$. Pearson and Spearman's correlations were performed between blood minerals, bone biomarkers, and other variables of interest. All statistical analysis was performed using SPSS version 24.

Results

From the fifteen HD patients recruited, twelve patients finished the intervention, two patients dropped before starting the intervention, and one patient died (Figure 4.2). Patient baseline characteristics can be found in Table 4.3. At baseline, 33% had hypocalcemia (corrected calcium <8.2mg/dl), 50% had hyperphosphatemia (phosphorus ≥5.5mg/dl), and 83% had secondary hyperparathyroidism (iPTH ≥300pg/ml). Female subjects tended to have lower femoral neck bone mineral density (BMD) (female 0.705±0.07g/cm^2 vs. male 0.922±0.037; p=0.019) and lower total hip BMD (female 0.827±0.49 vs. male 1.0417±0.089 g/cm^2; p=0.051). Additionally, African American (AA) subjects had higher total hip BMD (AA 1.04±0.049 vs. Hispanic and non-Hispanic White 0.825±0.087g/cm^2; p=0.045). Ninety-two percent of subjects were prescribed phosphate binders: 50% calcium-based phosphate binders (e.g., calcium

acetate), 50% non-calcium phosphate binders (e.g., sevelamer carbonate, sevelamer hydrochloride, and sucroferric oxyhydroxide), and one subject was prescribed one calcium and one non-calcium-based phosphate binder (calcium acetate and sucroferric oxyhydroxide). Additionally, phosphate binder prescriptions varied from one (sucroferric oxyhydroxide) to five (calcium acetate) pills per meal, with some patients having an additional recommendation for intake with snacks. Furthermore, fifty-eight percent of patients were prescribed a calcimimetic (e.g., cinacalcet), while 25% were prescribed vitamin D (e.g., cholecalciferol). Finally, patients that were prescribed a calcimimetic tended to have lower lumbar spine BMD (yes 0.95±0.059 vs no 1.16±0.043g/cm^2; p=0.017), higher iPTH (yes 640.23±98.93 vs. no 222.62±25.05pg/ml; p=0.006), and lower BALP (yes 35.21±6.18 vs. no 79.55±46.18ng/ml; p=0.039).

IN corrected dietary fiber intake (Pre 7.806±1.36 vs. Post 15.405±0.796 g/1000kcal, group-by-time interaction p=0.006) whereas in the CON group it was maintained (Pre 7.17±0.78 vs. Post 7.58±1.21g/1000kcal) (Table 4.6). However, we did not observe any main effects or group-by-time interactions for blood calcium, phosphorus, and magnesium (p>0.05 for all) (Table 4.4, Figure 4.3-4.8). Additionally, IN did not produce any effects on FGF-23, BALP, or CTX-1 (Table 4.4, Figures 4.11-4.16). However, there was an iPTH time effect, where the concentrations decreased in IN and CON similarly by 34.95% and 34.60%, respectively (Table 4.4, Figure 4.9-4.10). Furthermore, there was a numerical increase in phosphorus, FGF-23, and BALP after a 4-week consumption of inulin of 12%, 9.9%, and 9.96%, respectively, though these changes did not reach statistical significance (Table 4.4).

Fecal excretion of calcium and magnesium did not differ after the supplementation of IN or CON (p>0.05) (Table 4.5, Figure 4.17 and 4.19). Concurrently, we assessed the dietary intake of minerals through dietary recalls of the 48h prior to the fecal sample collection. Despite a

lower dietary calcium intake in the CON group (IN 775.35±88.29 vs. CON 600.76±69.50mg/d; group p=0.042), fecal calcium excretion was not different (Table 4.5). However, we observed a group-by-time interaction in the fecal excretion of phosphorus, where IN phosphorus excretion was maintained, while phosphorus excretion was lower after a 4-week supplementation with CON (Table 4.5, Figure 4.18). This may be explained by an overall lower phosphorus intake in the CON group (IN 1148.72±130.03 vs. CON 949.25±113.53mg/d; group p=0.043); however, the phosphorus intake at both CON time points were not different (Pre 914.96±134.72 vs. Post 983.54±152.06mg/d, p=0.586). Nevertheless, it is important to highlight that the exact amount of dietary phosphorus consumed cannot be accurately assessed since current nutrition databases only report phosphorus that is naturally contained in foods, and not the amount that is used as phosphate additives.

Finally, we designed a *MBD Composite Score*, in which we grouped the outcomes of interest (e.g., blood minerals, mineral and bone plasma biomarkers, and fecal excretion of calcium and phosphorus; Figure 4.20) to assess the effect of IN on MBD. In this composite score, a neutral (e.g., zero) or positive score is desirable. We observed that after a 4-week IN supplementation, subjects in the IN group were more likely to have a neutral or positive MBD Composite Score (IN 61.5% vs. CON 38.5%), though this difference did not reach statistical significance (p = 0.415).

Discussion

In this randomized, placebo-controlled, cross-over design, IN corrected the low dietary fiber intake in HD patients. However, IN did not produce any changes in blood calcium, phosphorus, and magnesium, nor any of the biomarkers of bone and mineral metabolism of interest, including FGF-23, BALP, and CTX. Contrary to what we expected, iPTH change after a

4-week supplementation with IN and CON was very similar. Furthermore, there were no differences in the fecal excretion of calcium or magnesium. However, fecal excretion of phosphorus was maintained in the IN group, while it was decreased in the CON group, which may be explained by a lower phosphorus intake in the CON group. Finally, intakes of energy, macronutrients, and minerals were maintained during the three months the study lasted.

While there were no significant changes in our primary outcomes, it is important to mention that there were numerical increases in several markers, including blood phosphorus, FGF-23, and BALP after a 4-week supplementation with IN. Higher concentrations of phosphorus and FGF-23 have been associated with poor outcomes, including higher vascular calcifications,[148] cardiovascular mortality,[2,149] and overall mortality.[6,150] Furthermore, although BALP is a marker of bone formation,[4,151,152] it has also been associated with higher vascular calcifications.[153,154] In the present study, we assessed the relationship between baseline concentrations of blood minerals, biomarkers of bone and mineral metabolism, and total hip, femoral neck, and lumbar spine bone mineral densities. However, we did not find any significant associations. Moreover, we assessed if there was relationship between carotid-femoral pulse wave velocity (cfPWV), a surrogate of arterial stiffness, and baseline blood minerals and bone and mineral metabolism biomarkers in a subset of our subjects (n=5). However, we did not find any significant associations, but our study was not powered to assess differences in markers of arterial stiffness.

Our hypothesis was based on several studies in other populations with MBD, in which the supplementation of fermentable dietary fibers led to an increase in the absorption of calcium and magnesium. Calcium and magnesium absorption may be increased due to the fermentation of the supplemented dietary fibers, which yields SCFA and decreases the intracolonic pH.[155]

This decrease in pH may affect the solubility of intracolonic minerals, causing a dissociation from other minerals, and thus enhancing their absorption.[155] However, most absorption of calcium and magnesium occurs within the proximal portions of the small intestine (duodenum and jejunum), with a small portion of these dietary minerals reaching the colon.[69,156] In most of the studies in postmenopausal women and female adolescents, calcium and magnesium stable isotopes were used to assess fractional absorption rates. [157] However, in the present study, we used fecal excretion and plasma concentrations of minerals as a proxy to assess changes in absorption. We observed that, at a similar calcium and magnesium dietary intake, fecal excretion and plasma concentration of calcium and magnesium were not different after a 4-week supplementation with IN or CON.

Dietary phosphorus is a mineral that theoretically would be more likely to reach the colon due to the use of binders to reduce phosphorus absorption in HD patients. Phosphorus binders are widely prescribed to reduce serum phosphorus and the negative outcomes associated with hyperphosphatemia, including increased vascular calcifications, all-cause mortality, and cardiovascular mortality.[57,132] In present study, 92% of the subjects were prescribed phosphate binders: 50% calcium-based phosphate binders (calcium acetate), 42% non-calcium phosphate binders (sevelamer hydrochloride, sevelamer carbonate, and sucroferric oxyhydroxide), and one subject taking both types of phosphate binders (calcium acetate and sucroferric oxyhydroxide). The phosphorus-binding capacity differs between phosphate binders, where calcium acetate binds ~26.5mg/g, sevelamer hydrochloride/carbonate ~33mg/g, and sucroferric oxyhydroxide ~240mg/g.[158,159] Thus, by assuming patient's compliance to their phosphate binder prescription and by knowing the number of meals and snacks that subjects consumed, we could calculate an approximate amount of dietary phosphorus that may have reached the colon. Although our

hypothesis was related to calcium and magnesium absorption, it may be possible that the pH reduction due to the fermentation of IN may have affected the solubility of phosphorus, ultimately reducing the efficacy of the binder, and increasing colonic phosphate absorption. However, we observed a lower fecal excretion of phosphorus only in the CON group. We would not expect to see an enhanced phosphorus absorption with CON because maltodextrin is a readily digestible carbohydrate. Future studies should aim to assess if isolated sources of fiber impact the solubility of phosphorus and thus decrease the efficacy of phosphate binders. If there is a reduction in the efficacy of phosphate binder therapy, the use of isolated sources of fiber may not be suitable for HD patients that are prescribed phosphate binders. Rather, an increase in dietary fiber through high-fiber foods and the effect on mineral metabolism should be explored.

Due to the lack of an effect of IN on the individual parameters measured, we developed a composite score to collectively the impact of IN on all the MBD related measures (e.g., blood minerals, mineral and bone metabolism biomarkers, and fecal excretion of minerals). Based on this MBD composite score, 23% more subjects had a neutral or positive value after IN, compared to CON, meaning that they had more MBD variables within a desirable concentration (e.g., blood minerals). This suggests that future studies should aim to assess if an overall improvement in markers of MBD translate into better outcomes in HD patients.

There are several limitations to our study. First, we measured plasma minerals and mineral fecal excretion as a proxy for absorption instead of using stable isotope methodologies. Furthermore, we did not control for dietary intake prior to the specimen collection. However, there were no overall differences in dietary intake at the four timepoints. Moreover, the supplements' compliance was assessed verbally every treatment, without any physiological measurement, such as breath hydrogen. Finally, our power analysis was based on data from other

clinical populations (e.g., postmenopausal women), in which the measurements were less variable than our population. With the effect size seen on blood calcium (η^2=0.016), we would have needed a sample size of 255 HD patients to observe an effect. Thus, it is likely that inulin may not influence blood minerals.

Conclusion

IN did not cause a change in blood minerals and biomarkers related to mineral and bone metabolism, such as iPTH, FGF-23, BALP, and CTX. However, there was a ~10% increase in blood concentrations of phosphorus, FGF-23, and BALP. Furthermore, we did not observe any effects of IN on the fecal excretion of calcium and magnesium. However, after four weeks of CON, fecal excretion of phosphorus was lower. Future studies should aim to explore the effect that isolated sources of fiber have on the efficacy of phosphate binders and MBD markers in HD patients.

Table 4.1 Summary of supplementation of ITFs for bone and/or mineral metabolism in animal models

Study	Fiber	Study Design	Animal model	Findings
Legette 2012[160]	ITFs or PDX	1) AIN-93M control diet, 2) AIN-93M+ 5% ITFs (50-50% scFOS and lcFOS) 3) AIN-93M diet+ ITFs (98% inulin+2% FOS) 4) AIN-93M diet+ 55% PDX for 4 weeks	5-month OVX Sprague-Dawley rats (n=16 to 18 per group)	ITFs ↑ Ca, SCFA and cecal wall weight. All fibers ↑Mg absorption.
Naughton 2011[161]	Oligofructose-enriched inulin	1) AIN-93M 2) AIN-93M + synbiotics (inulin 1.44g/24h and *Lactobacilus GG* and *Bifidobacterium lactis* $3.96x10^{10}$ CFU of each	21-week and 61-week old Sprague-Dawley rats (n=16, respectively)	↑ Ca concentration ↓ Osteocalcin in aged rats with synbiotics
Varley 2010[162]	Inulin	1) low P 2) low P + 20g/kg inulin 3) high P 4) high P + 20g/kg inulin	Finisher pigs (n= total 40, 10 per treatment)	No effect in Ca and P digestibility or bone mineralization
Coudray 2005[163]	Inulin	1) Control diet 2) Control diet + 3.75% inulin for 4 days and then 7.5% inulin for 3 weeks	2, 5, 10 and 20-month Wistar rats (n= 8 per group)	Inulin ↑ Ca and Mg absorption in all 4 age groups. ↑↑Ca absorption in aged rats Similar Mg absorption was in all groups
Raschka 2005[164]	1:1 inulin and oligofructose	1) Control diet (2% maltodextrin) 2) Control diet +10% inulin-oligofructose for 21 days.	Male Sprague-Dawley rats (n=24 per treatment)	↑ cecal contents, ↓ cecal pH ↑total, soluble, and ionized Ca ↑ surface area

Table 4.1 Cont.				
Coudray 2005[165]	Inulin	Ca (0.25%, 0.50% or 0.75%) with or without inulin 5% during the first 4 days and 10% for the rest: short-term: day 13-17 and long-term: day 36-40	10-week Wistar rats (n=10 per group)	↑Ca and Mg absorption in the short and long term Ca absorption depended on Ca consumed
Zafar 2004[166]	Inulin+FOS	1) AIN 93 2) AIN 93 + inulin and FOS 55g/kg for 21 days	6-month Sprague-Dawley OVX rats (n=13 per group)	↑ Ca absorption, femoral Ca content, BMD, and bone balance Bone resorption rate relative to formation was lower
Kruger 2003[167]	FOS and inulin	1) Semisynthetic diet with 0.5% Ca, 2) same diet +5% FOS (DP 2-8) 3) same diet + 5% inulin (DP>23) 4) same diet + 5% inulin and FOS	7-week old male Sprague-Dawley rats (n=10 per group)	↑ Femoral BMD femurs and spine BMC ↓ CTx-1
Coudray 2003[168]	FOS, inulin, branched-chain inulin	1) Purified diet, 2) same diet+ FOS, 3) same diet + inulin, 4) same diet + inulin/FOS, 5) same diet + branched-chain inulin. First week 2.5% fiber, second week 5% fiber, and third and fourth week 10%	Male Wistar rats (n=10 per group)	All fiber groups increased Mg absorption and balance. FOS/Inulin group increased Ca absorption and balance.
Younes 2001[169]	Inulin and resistant starch	1) fiber-free 2) 100g/kg inulin 3) 150g/kg potato resistant starch 4) 50g/kg inulin and 75g/kg resistant starch.	Adult male Wistar rats (n=8 per group)	Both ↑ absorption of Ca and Mg, without changing plasma levels

Table 4.1 Cont.				
Lopez 2000[170]	FOS	1) Fiber-free 2) 100g/kg FOS 3) 10g/kg phytic acid 4) FOS+ phytic acid for 21 days	Male Wistar rats (n=8 per group)	FOS ↑ absorption by 120% Ca, 150% Mg, 123% Fe, 145% Cu. Phytic acid ↓ absorption by 248% Fe, 262% Zn, 231% Cu, and affected plasma Mg FOS reduced this effects.
Ohta 1998[171]	FOS	1) Gastrectomy + control diet 2) Gastrectomy + 75g/kg FOS 3) Sham + control 4) Sham + FOS 7d post-operation for 25d.	4-week Sprague-Dawley rats (n=14 per group)	↑Ca absorption ↑Ca absorption with FOS and prevented decrease in Ca content and BMD in gastrectomized rats
Rémésy 1993[172]	Inulin	1) Fiber-free 2) Fiber-free, 8g Ca/kg 3) 15% inulin, 3g Ca/kg 4) 15% inulin, 8g Ca/kg for 21d	6-week male Wistar rats (n=12 per group)	↑ cecum and fermentation profile ↑ soluble Ca and P ↑ Ca absorption was higher.

ITFs, inulin-type fructans;OVX: ovariectomized, FOS: fructooligosaccharides, scFOS: short-chain fructooligosaccharides, lcFOS: long-chain FOS, PDX: polydextrose, RM: resistant maltodextrin, RMH: hydrogenated resistant maltodextrin, Ca: calcium, Mg: magnesium, P: phosphorus, Fe: iron, Zn: zinc, Cu: copper, SCFA: short-chain fatty acids, CFU: colony forming units, E: experiment, BMD: bone mineral density, DP: degree of polymerization,

Table 4.2 Summary of supplementation of ITFs for bone and/or mineral metabolism in humans

Study	Fiber	Study Design	Clinical Population	Findings
Kruger 2015[173]	Ca and Vit D fortified milk with FOS-inulin	RCT: 1) 2 glasses of regular (500mg of Ca) 2) Fortified milk (1000mg for premenopausal and 1200mg for postmenopausal women, 96mg Mg, 2.4mg zinc, 15mcg vitamin D, 4g FOS) for 12 weeks.	Premenopausal (41±5y, n=136) and postmenopausal (59±4y, n=121) females	↓CTx-1 and PINP ↓PTH
Slevin 2014[174]	scFOS	RCT: 1) 800 mg of Ca 2) 800 mg of Ca with 3.6 g of scFOS 3) 9 g of maltodextrin.	45-75-year-old postmenopausal women (n=300)	↓CTX-1 at 12 months ↓osteocalcin at 24 months in the Ca + scFOS group.
Van den Heuvel 2009 [175]	scFOS	Randomized, double-blind, crossover design: 1) 10g of scFOS 2)Placebo (maltodextrin) for 37d with washout period of 12 days	12-14-year-old females (n=14)	scFOS ↑ Mg absorption by 18%
Abrams 2007[157]	ITFs	8g/day for 8wk	18-27yo healthy adults (n=13)	In responders to the fiber (n=8), ↑ Ca absorption by ~9%
Holloway 2007[176]	Oligofructose-enriched inulin or maltodextrin (placebo)	Double-blind, placebo-controlled, cross-over design. 2 doses of 5g a day for 6 weeks	Postmenopausal women (72±6y, n=15)	↑Ca and Mg Responders tended to have ↓ lumbar spine BMD
Abrams 2005[177]	Short and long-chain ITFs	RCT, 8w and 1y follow up	Pubertal adolescents (11±0.2y, ITFs n=48, control group n=50)	↑ Ca absorption by 8.5% higher at 8 weeks and 5.9% at 1 year BMC was higher

Table 4.2 Cont.				
Coudray 1997[178]	Inulin and sugar beet fiber	3x3 Latin Square, 28d periods, 18g of total fiber in control diet, and 58g in the inulin and sugar beet fiber	Male students (21±2y, n=9)	Inulin ↑ Ca (12%), while sugar beet fiber increase Ca intake and balance, without a modification in Ca absorption.

ITFs, inulin-type fructans;RCT: randomized-controlled trial, FOS: fructooligosaccharides, SCF: soluble corn fiber, GOS: galactooligosaccharides, scFOS: short-chain fructooligosaccharides, Ca: calcium, Mg: magnesium, BMD: bone mineral density, BMC: bone mineral content, CTx-1: C-terminal telopeptide of type I collagen, PINP: serum procollagen type 1N propeptide, PTH: parathyroid hormone.

Figure 4.1 Study Design

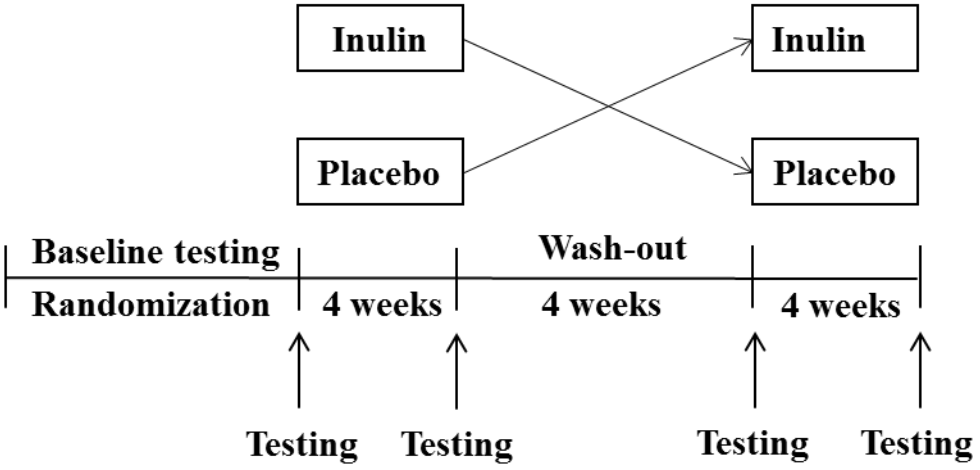


Figure 4.1 Hemodialysis Inulin Trial was randomized, placebo-controlled, crossover study with a wash-out period of 4 weeks. At the beginning and end of each period, patients provided a fecal sample and the immediate HD treatment a blood sample was collected for the measurement of blood minerals and mineral metabolism biomarkers.

Figure 4.2 Study Enrollment Tree

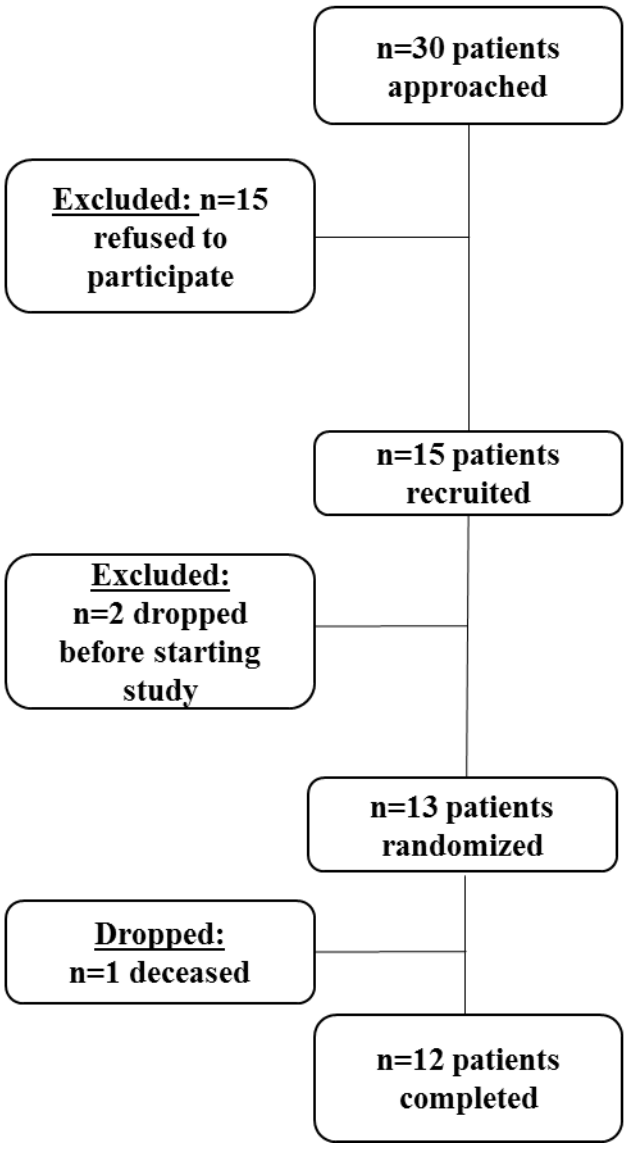


Table 4.3 Patient Characteristics

Variable	Mean ± SEM	Reference Value
Age (years)	56 ± 9	N.A.
Gender (M/F)	6/6	N.A.
African American (%)	58.3%	N.A.
BMI (kg/m2)	31.06 ± 2.58	18.5-24.9
Diabetes (%)	46%	N.A.
Albumin (g/dl)	3.27 ± 0.27	3.3-5.5
Corrected calcium (mg/dl)	8.85 ± 1.32	8.0-10.3
Phosphorus (mg/dl)	5.98 ± 1.54	2.2-4.4
Magnesium (mg/dl)	2.2 ± 0.3	1.6-2.3
Intact PTH (pg/ml)	502.73 ± 301.52	<300
FGF-23 (pg/ml)	4321.64 ± 674.93	N.A.
BALP (ng/ml)	53.69 ± 10.96	N.A.
CTX (ng/ml)	3.81 ± 0.52	N.A

SEM, standard error of the mean; N.A., not applicable; M, male; F, female; BMI, body mass index; PTH, parathyroid hormone; FGF-23, fibroblast-growth factor-23; BALP, bone-specific alkaline phosphatase; CTX, C-terminal Telopeptide

Table 4.4 Blood mineral and bone biomarkers after 4-weeks of IN or CON consumption

	IN		CON		Group *P*	Time *P*	G x T *P*/Effect size (n^2)
	Pre	**Post**	**Pre**	**Post**			
Calcium (mg/dl)	8.94 ± 0.40	8.85 ± 0.38	8.84 ± 0.41	8.56 ± 0.34	0.498	0.195	0.677/ 0.016
Phosphorus (mg/dl)	5.84 ± 0.42	6.55 ± 0.56	6.1 ± 0.52	6.24 ± 0.42	0.965	0.232	0.352/ 0.079
Magnesium (mg/dl)	2.24 ± 0.11	2.19 ± 0.096	2.17 ± 0.07	2.25 ± 0.08	0.622	0.918	0.162/ 0.170
iPTH (pg/ml)	379.52±54.49	246.86±27.98	379.94±74.04	248.47±24.14	0.970	**0.031**	0.990/ 0.000
FGF-23 (pg/ml)	4184.8±810.1	4599.1±841.79	3978.5±655.2	4004.7±656.9	0.152	0.156	0.602/ 0.025
BALP (ng/ml)	63.76 ±14.91	70.11 ±13.03	62.59 ±10.73	61.9 8± 11.3	0.622	0.918	0.162/ 0.030
CTX (ng/ml)	3.76 ± 0.48	3.74 ± 0.42	3.92 ± 0.59	3.83 ± 0.51	0.596	0.749	0.811/ 0.005

G, group; T, time; G x T, group-by-time; n^2, eta squared (effect size); IN, inulin, CON, maltodextrin; iPTH, parathyroid hormone; FGF-23, fibroblast-growth factor-23; BALP, bone-specific alkaline phosphatase; CTX, C-terminal telopeptide.

Figure 4.3 Plasma calcium concentration after 4 weeks with IN or CON

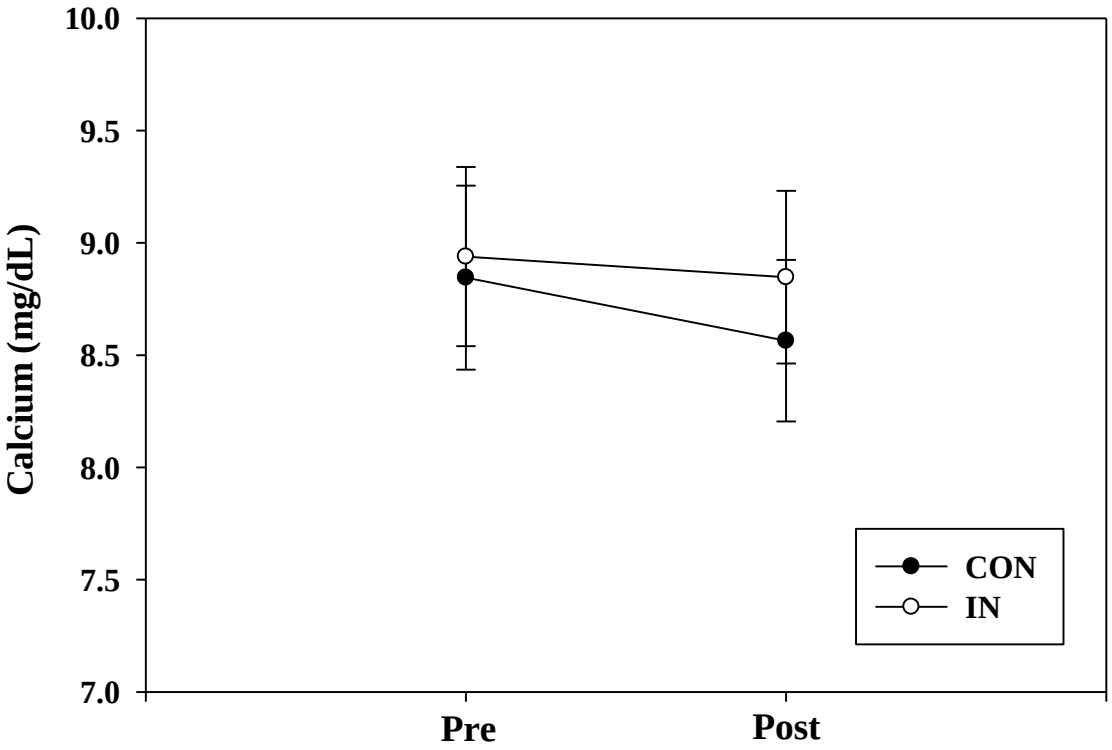
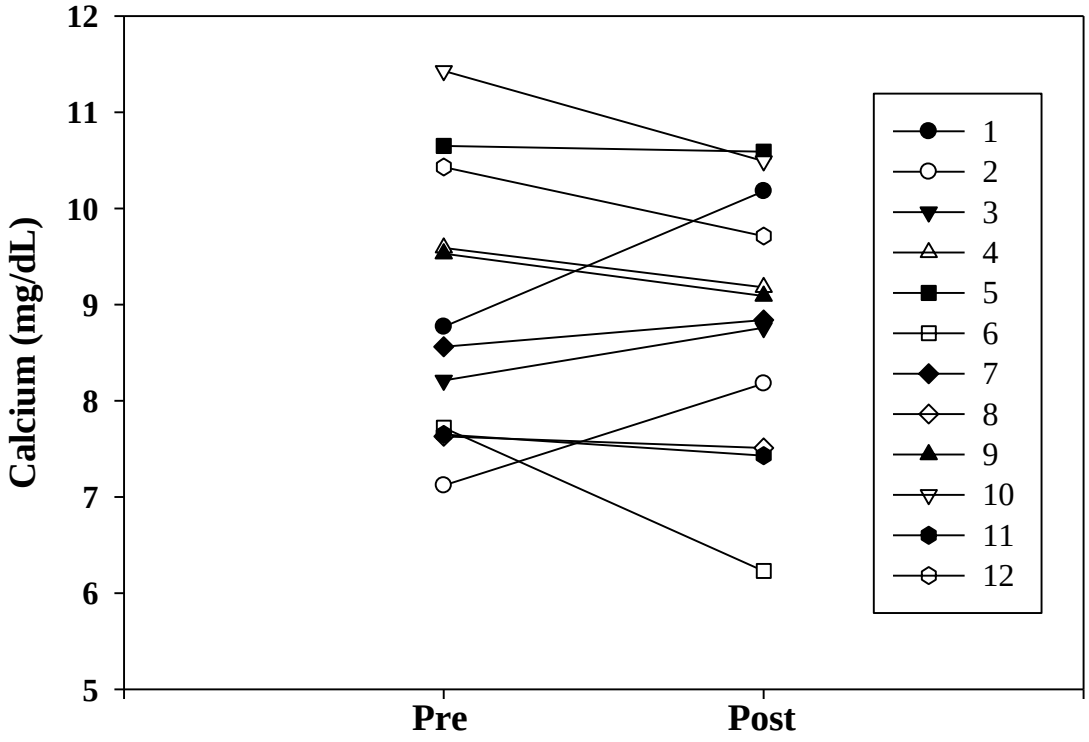


Figure 4.3 Plasma calcium concentrations did not change after a 4-week supplementation with inulin (IN) or maltodextrin (CON).

Figure 4.4 Individual changes in plasma calcium after 4-weeks

A. IN



B. CON

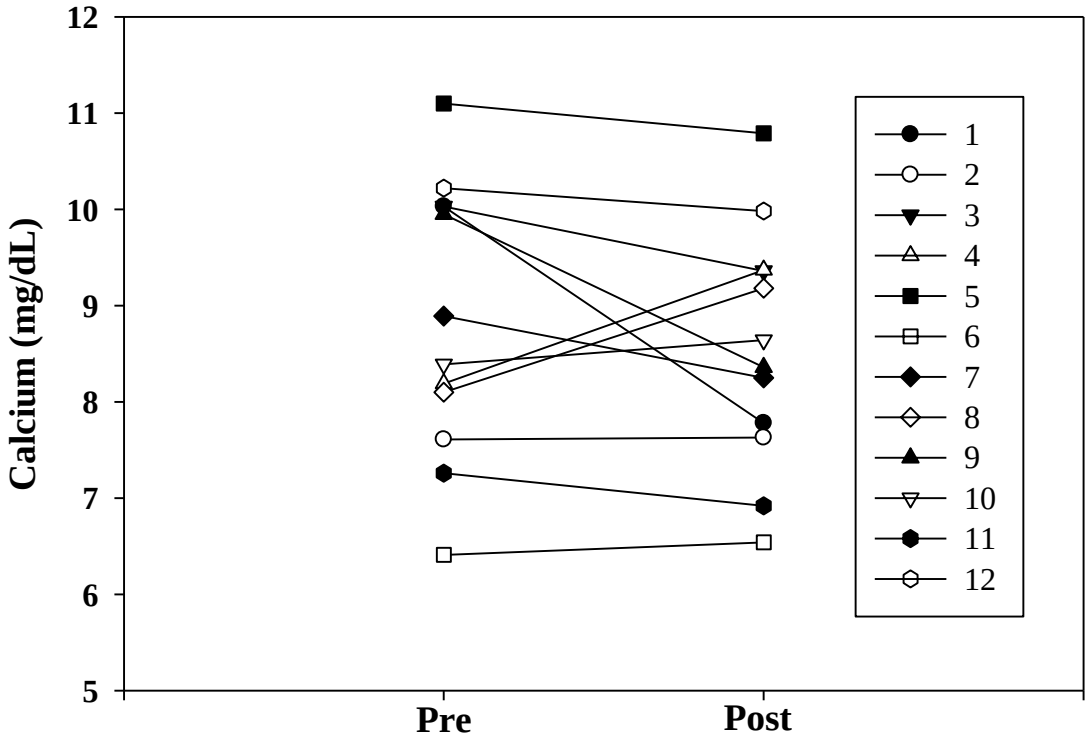


Figure 4.4 Hemodialysis patients show an inconsistent change in plasma calcium concentration after a 4-week supplementation with A. inulin (IN); and B. maltodextrin (CON).

Figure 4.5 There were no changes in plasma phosphorus concentrations after 4 weeks with IN or CON

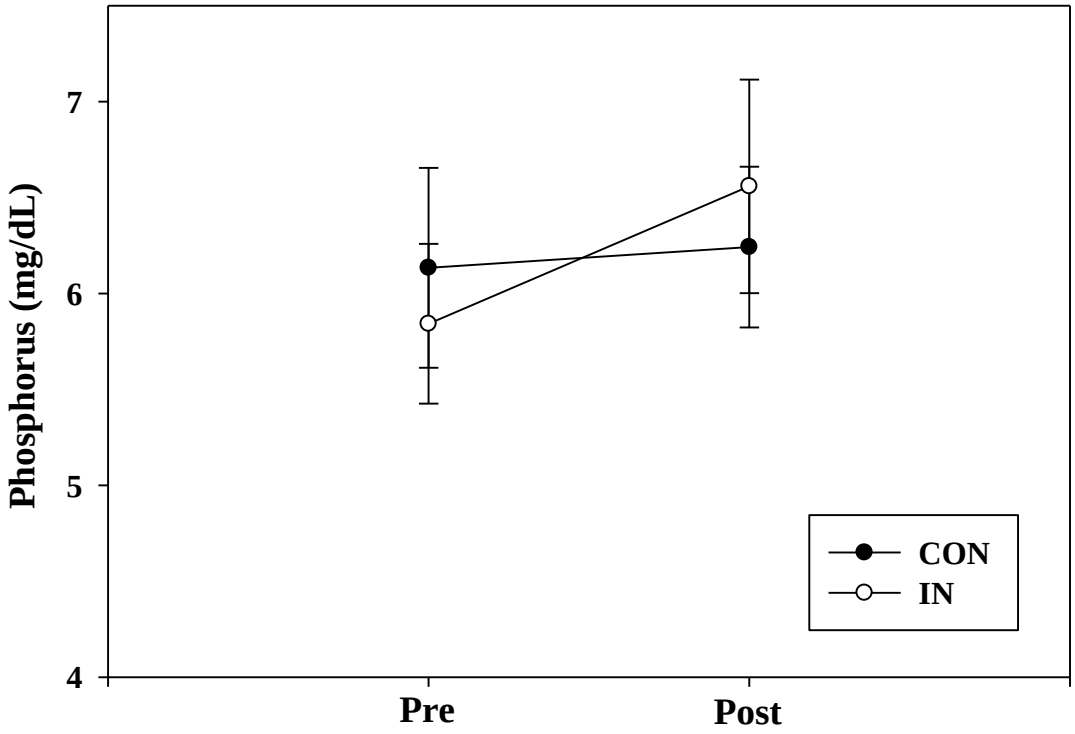
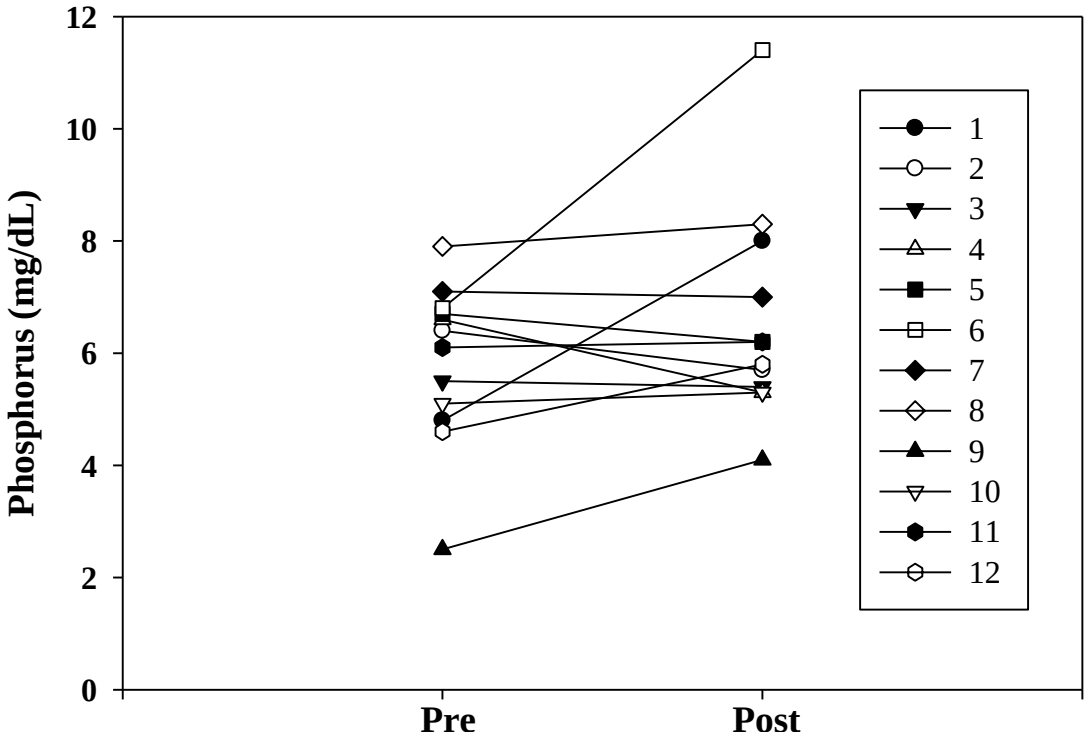


Figure 4.5 Plasma phosphorus concentrations did not change after a 4-week supplementation with inulin (IN) or maltodextrin (CON).

Figure 4.6 Individual changes in plasma phosphorus after 4-weeks
A. IN



B. CON

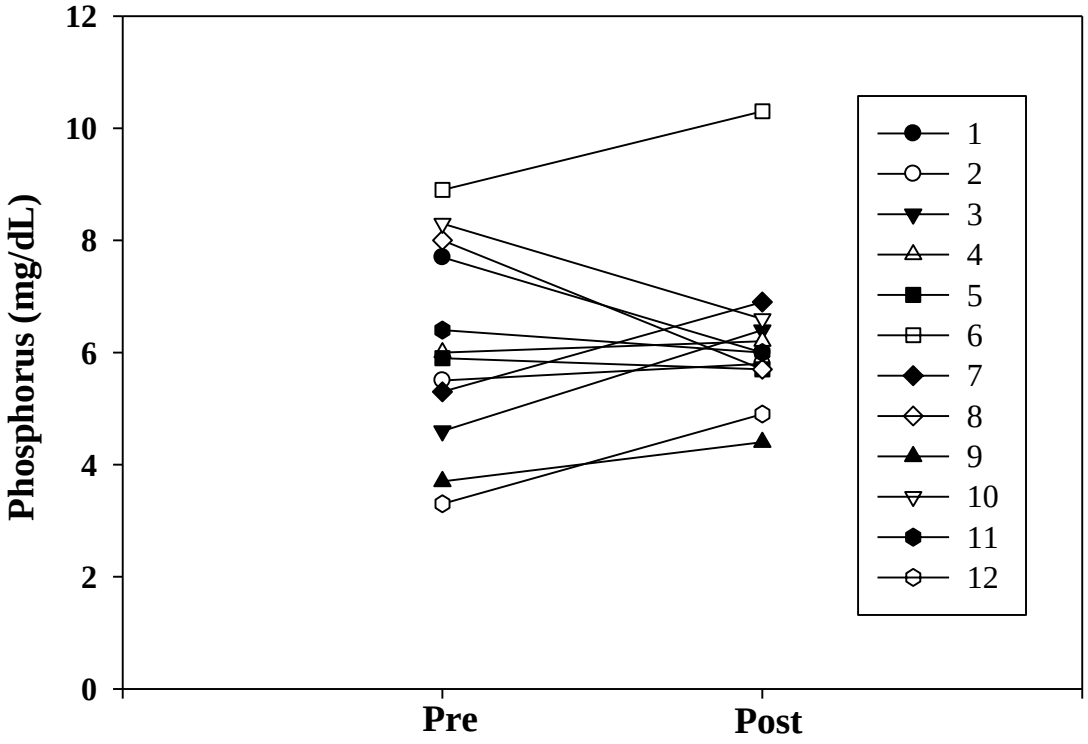


Figure 4.6 Hemodialysis patients show an inconsistent change in plasma phosphorus concentration after a 4-week supplementation with A. inulin (IN); and B. maltodextrin (CON).

Figure 4.7 There were no changes in plasma magnesium concentrations after 4 weeks with IN or CON

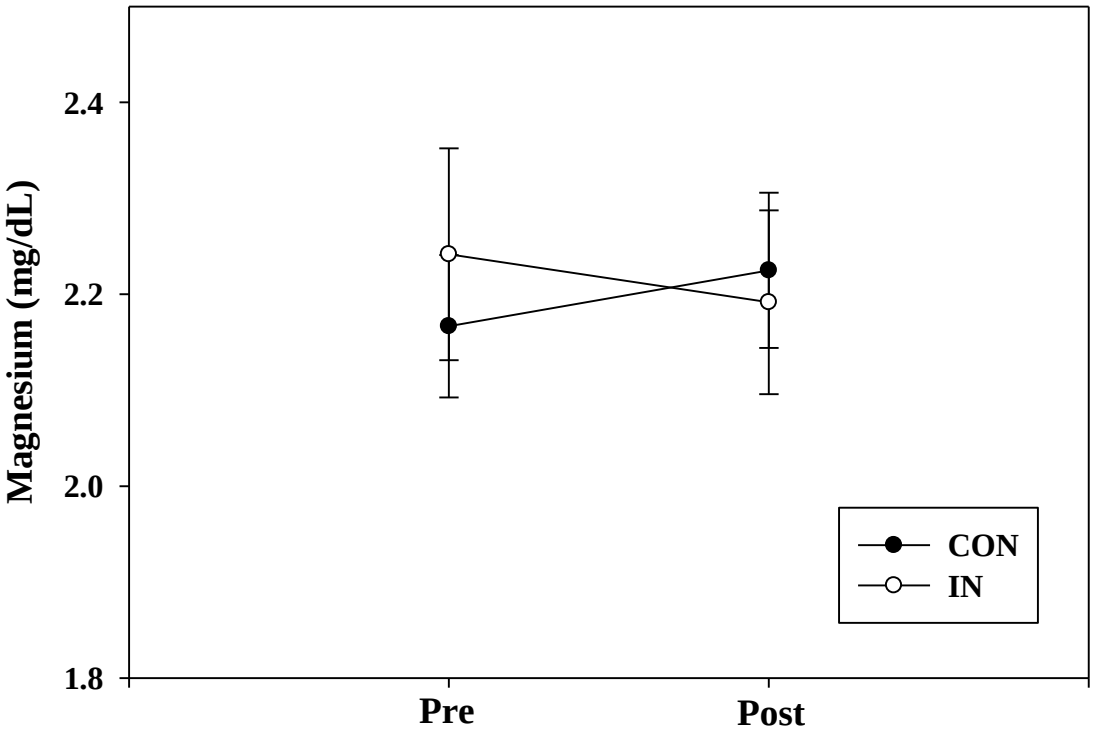
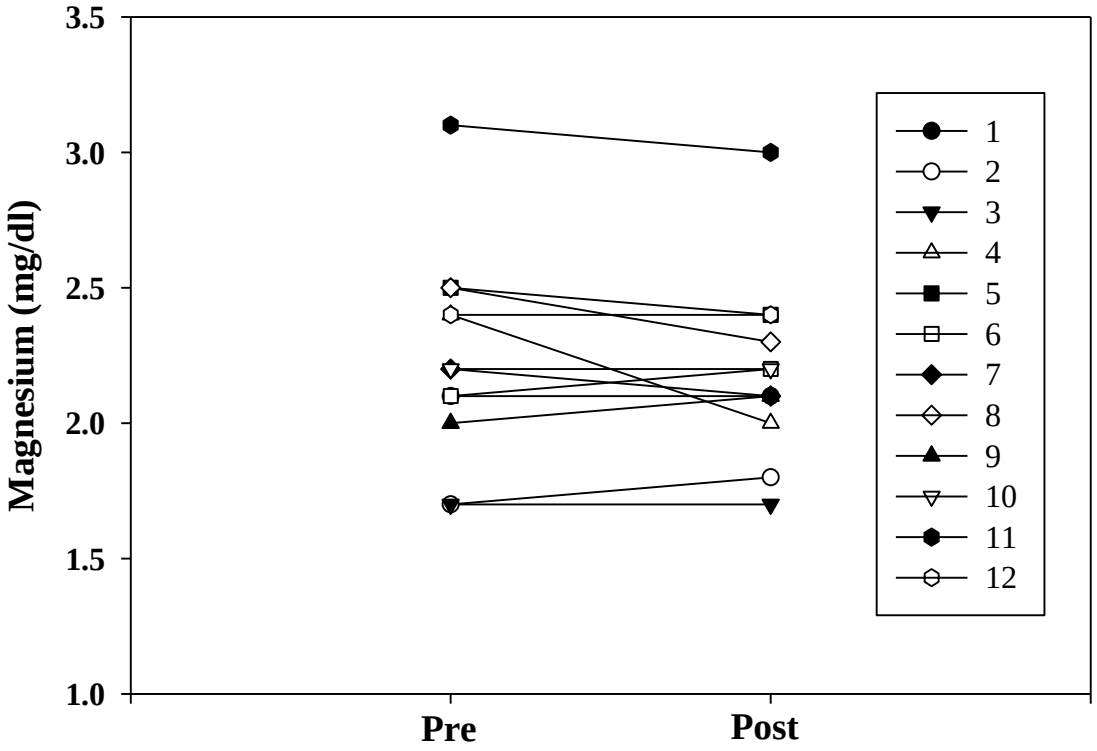


Figure 4.7 Plasma magnesium concentrations did not change after a 4-week supplementation with inulin (IN) or maltodextrin (CON).

Figure 4.8 Individual changes in magnesium after 4-weeks with IN or CON
A. IN



B. CON

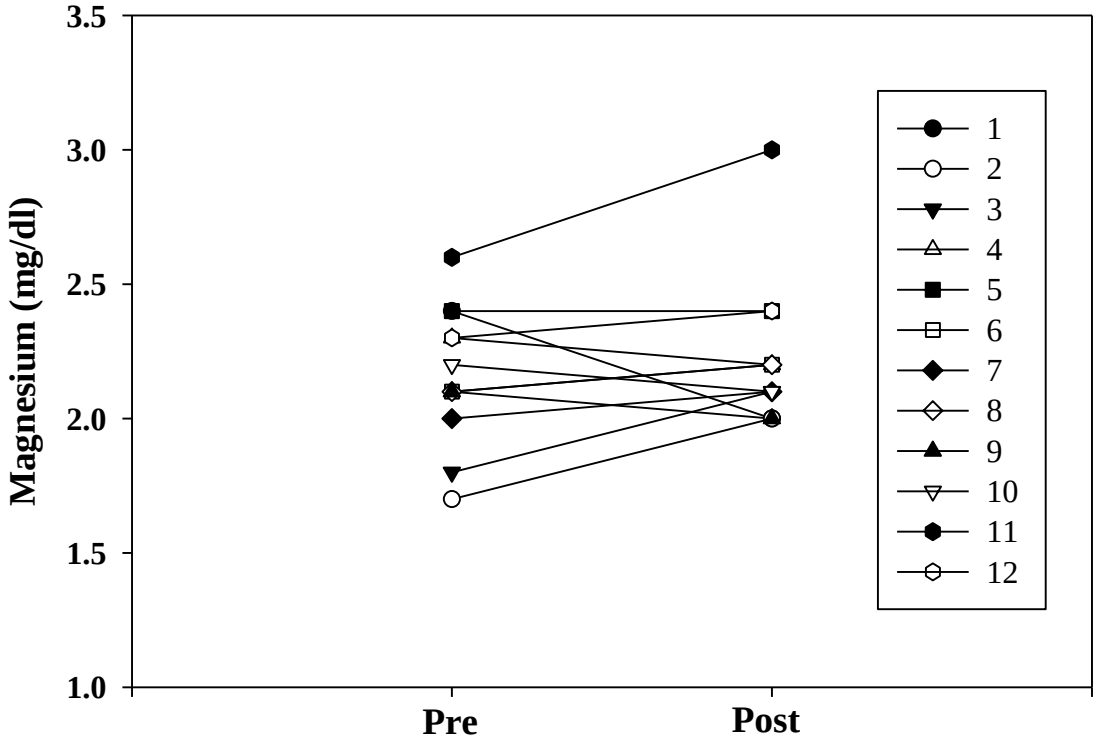


Figure 4.8 Hemodialysis patients show an inconsistent change in plasma magnesium concentration after a 4-week supplementation with A. inulin (IN); and B. maltodextrin (CON).

Figure 4.9 Plasma iPTH decrease is similar after 4 weeks of IN or CON

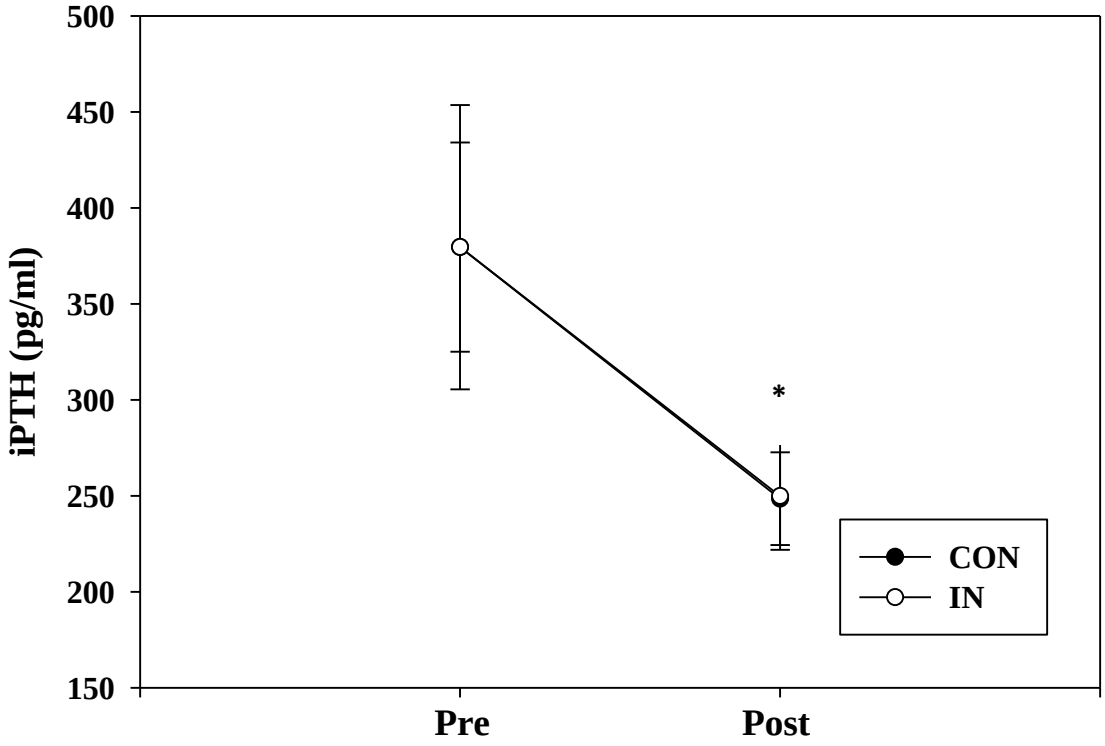
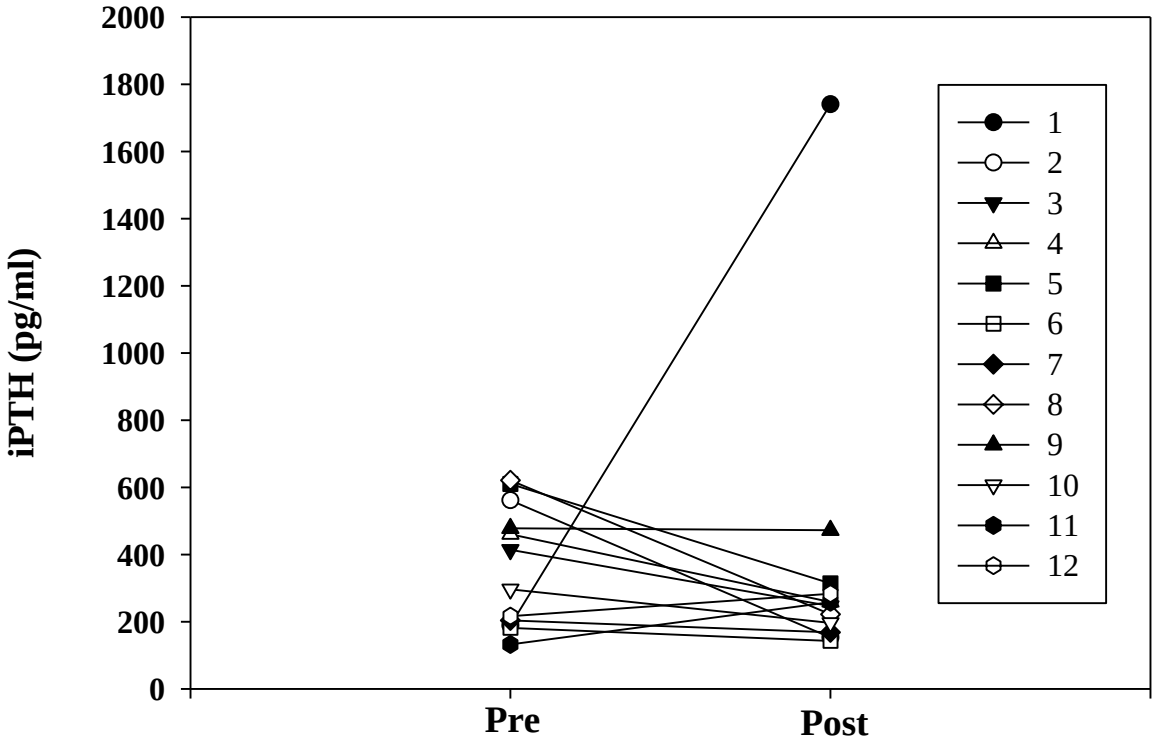


Figure 4.9 There was a time effect on intact parathyroid hormone (iPTH), decreasing to the same extent after a 4-week supplementation with inulin (IN) or maltodextrin (CON). * p time <0.05

Figure 4.10 Individual changes in iPTH after 4-weeks with IN or CON

A. IN



B. CON

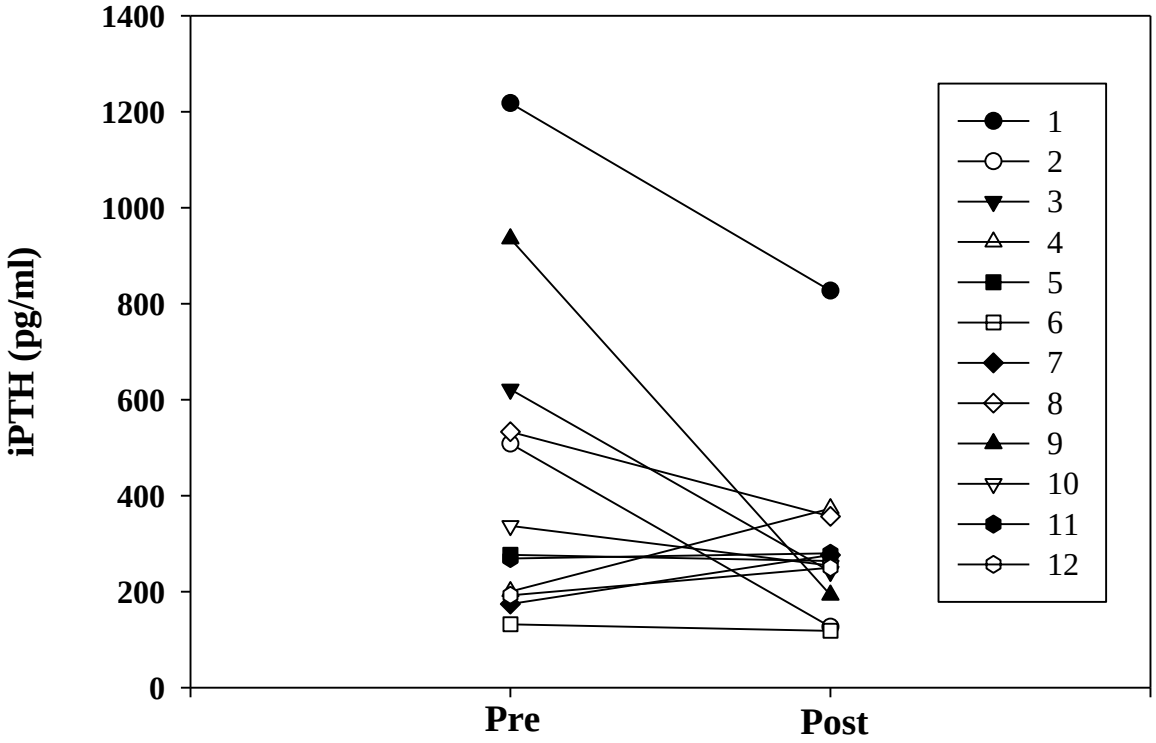


Figure 4.10 Individual changes in intact parathyroid hormone (iPTH) after a 4-week supplementation with A. inulin (IN); and B. maltodextrin (CON).

Figure 4.11 Plasma FGF-23 concentrations after 4-week supplementation with IN or CON

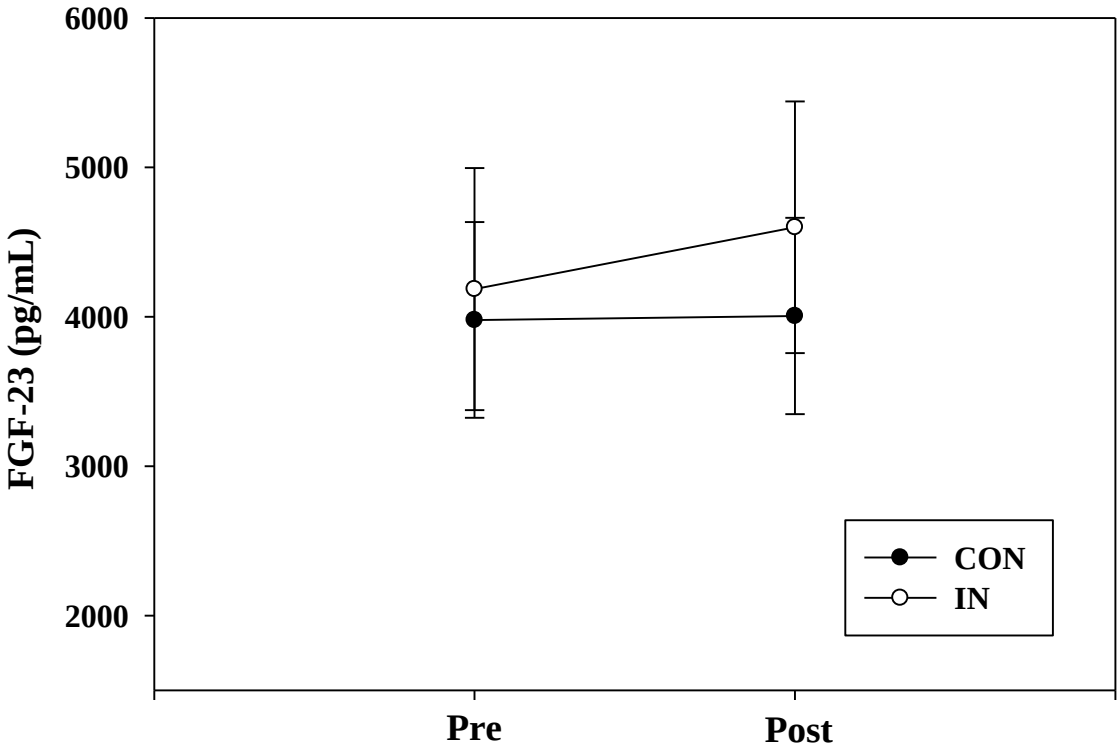
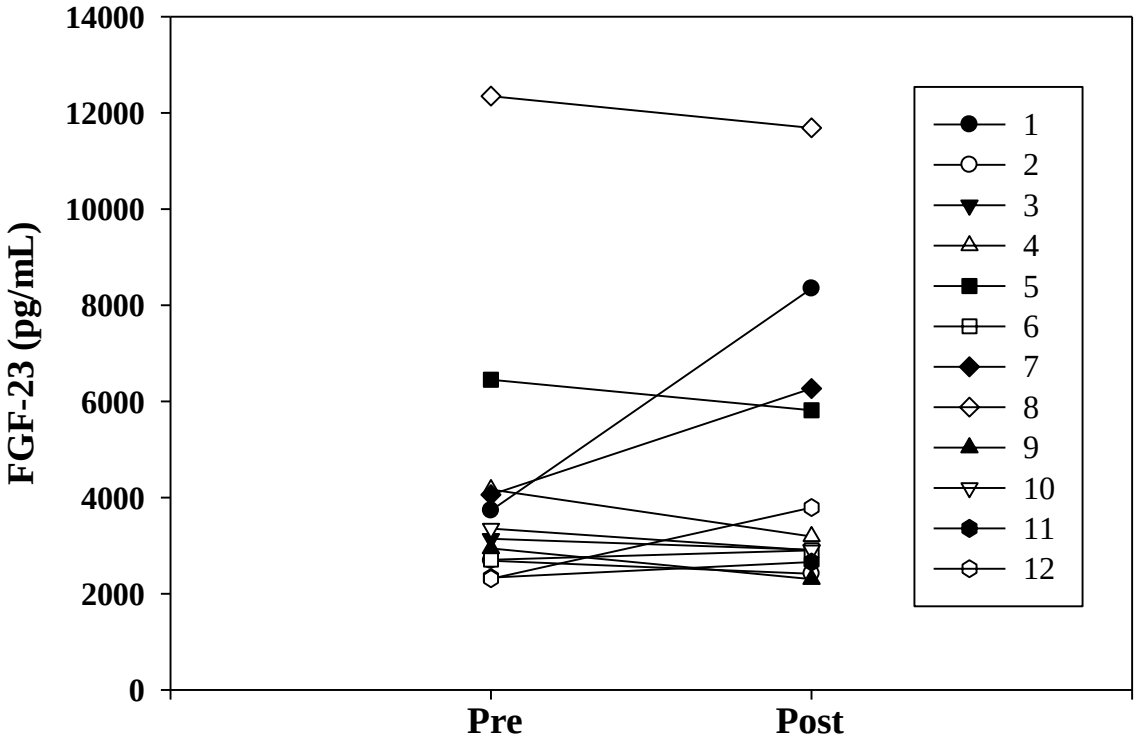


Figure 4.11 No changes in fibroblast growth factor-23 (FGF-23) concentrations after a 4-week supplementation with inulin (IN) or maltodextrin (CON).

Figure 4.12 Individual changes in plasma FGF-23 after 4-weeks with IN or CON

A. IN



B. CON

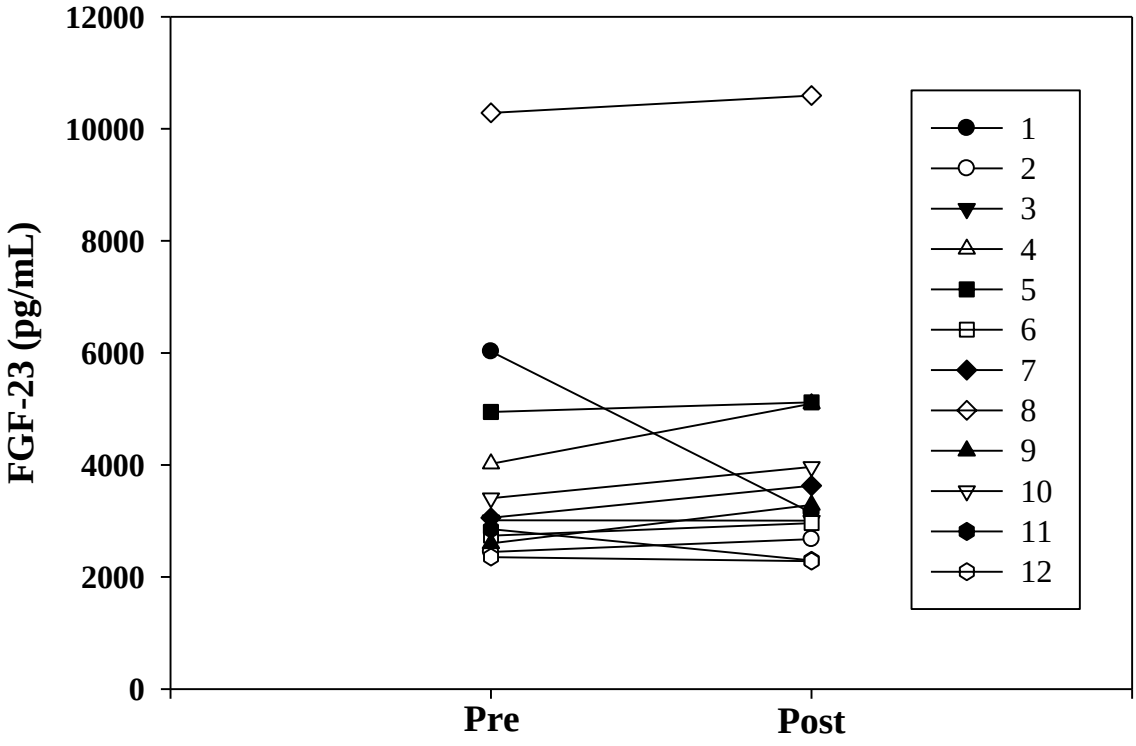


Figure 4.12 Hemodialysis patients show an inconsistent change in plasma fibroblast growth factor-23 (FGF-23) concentration after a 4-week supplementation with A. inulin (IN); and B. maltodextrin (CON).

Figure 4.13 Plasma BALP after 4-week supplementation with IN or CON

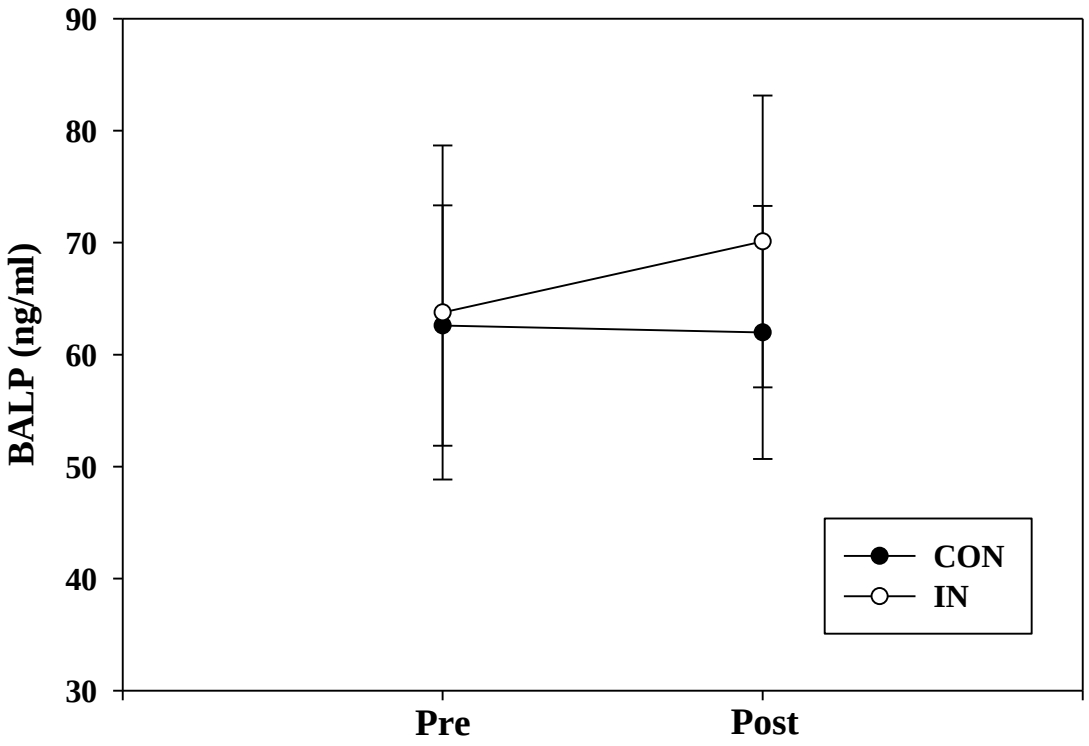
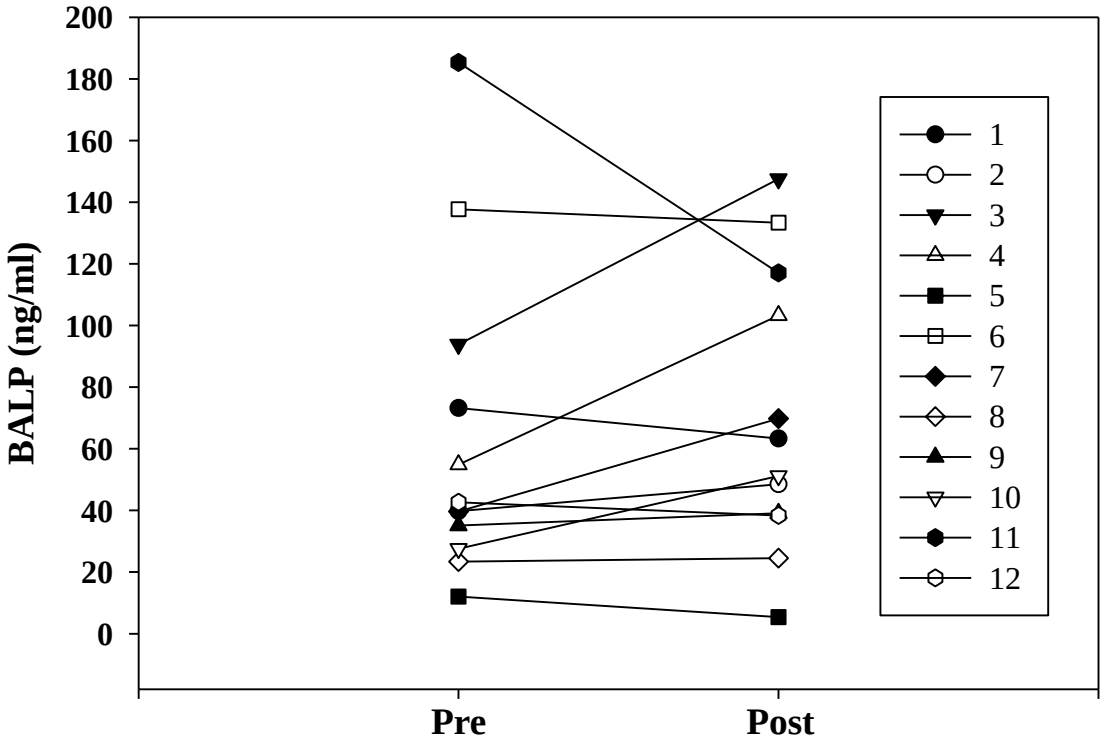


Figure 4.13 No changes in bone-specific alkaline phosphatase (BALP) concentrations after a 4-week supplementation with inulin (IN) or maltodextrin (CON).

Figure 4.14 Individual changes in BALP after 4-weeks with IN or CON

A. IN



B. CON

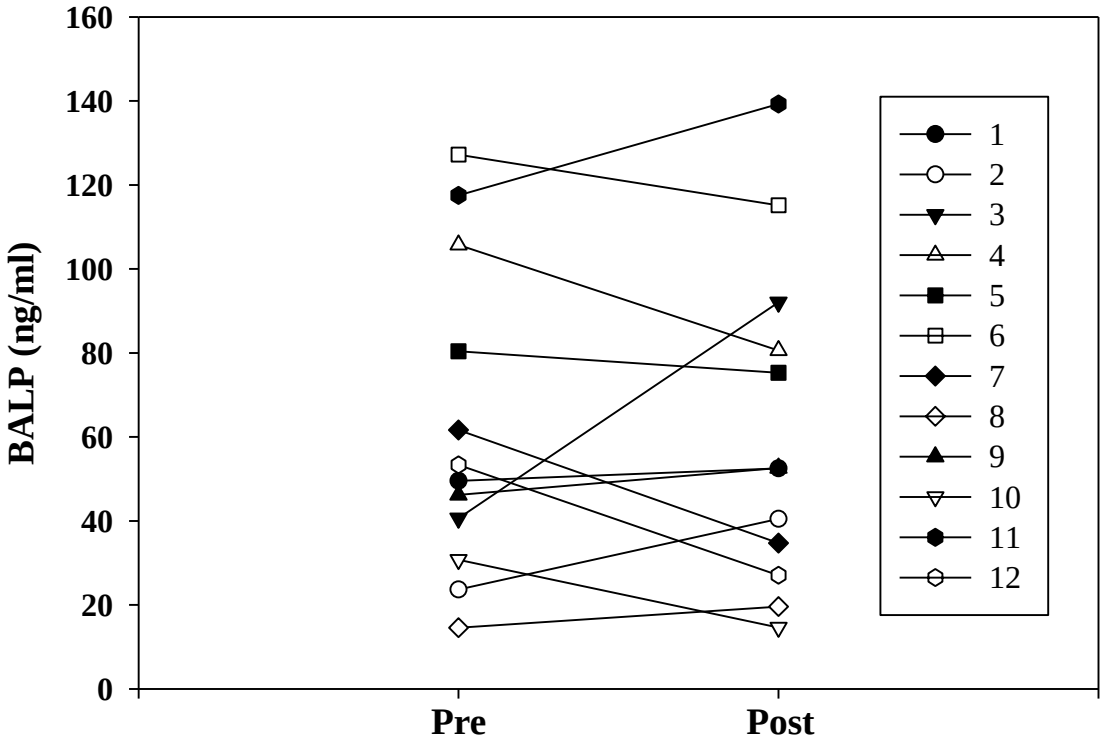


Figure 4.14 Hemodialysis patients show an inconsistent change in plasma bone-specific alkaline phosphatase (BALP) concentration after a 4-week supplementation with A. inulin (IN); and B. maltodextrin (CON).

Figure 4.15 Plasma CTX after 4-weeks with IN or CON

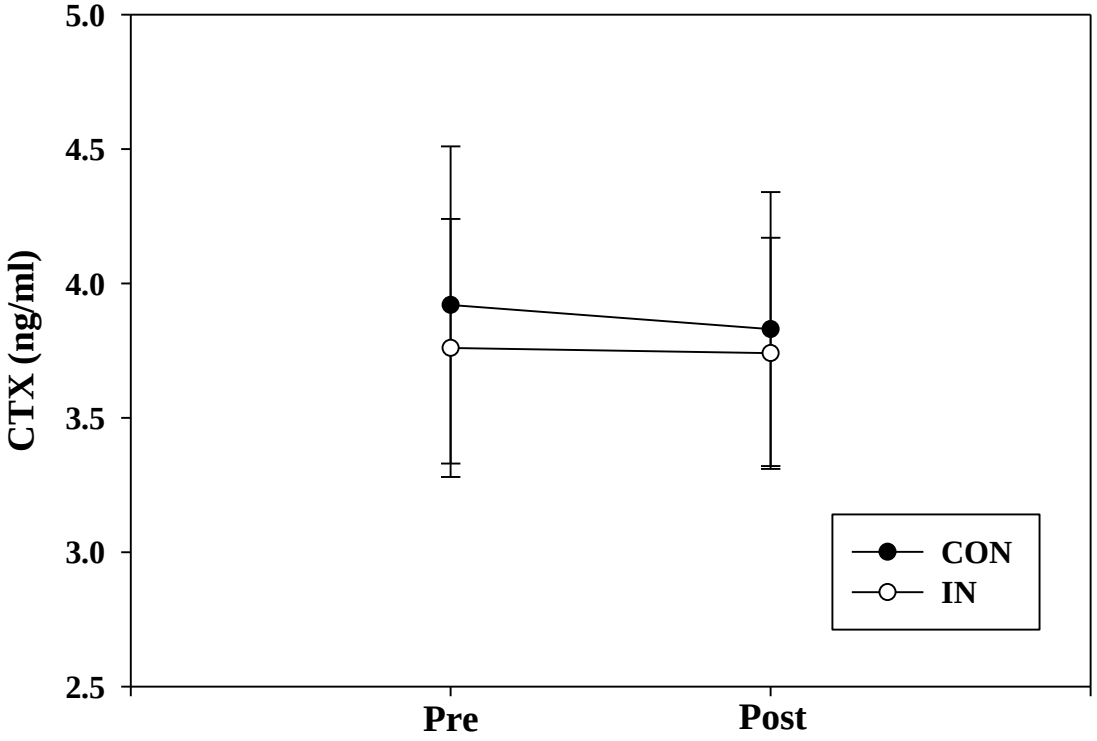
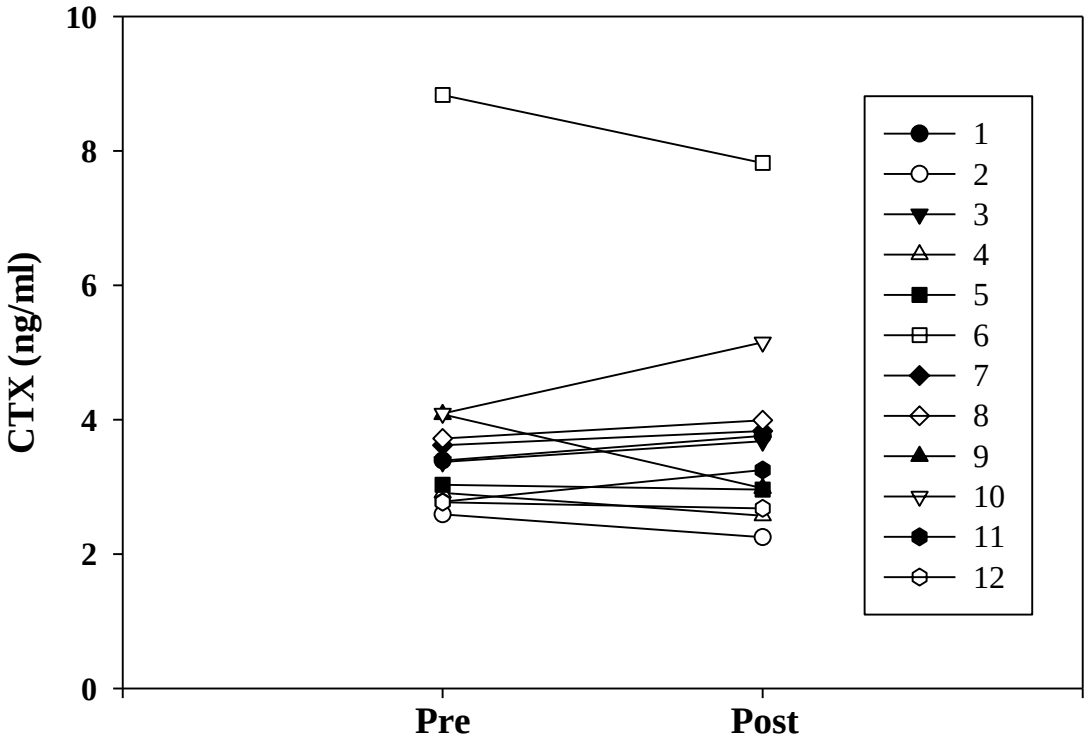


Figure 4.15 No changes in plasma C-telopeptide of type I collagen (CTX) concentrations after a 4-week supplementation with inulin (IN) or maltodextrin (CON).

Figure 4.16 Individual changes in CTX after 4-weeks with IN or CON

A. IN



B. CON

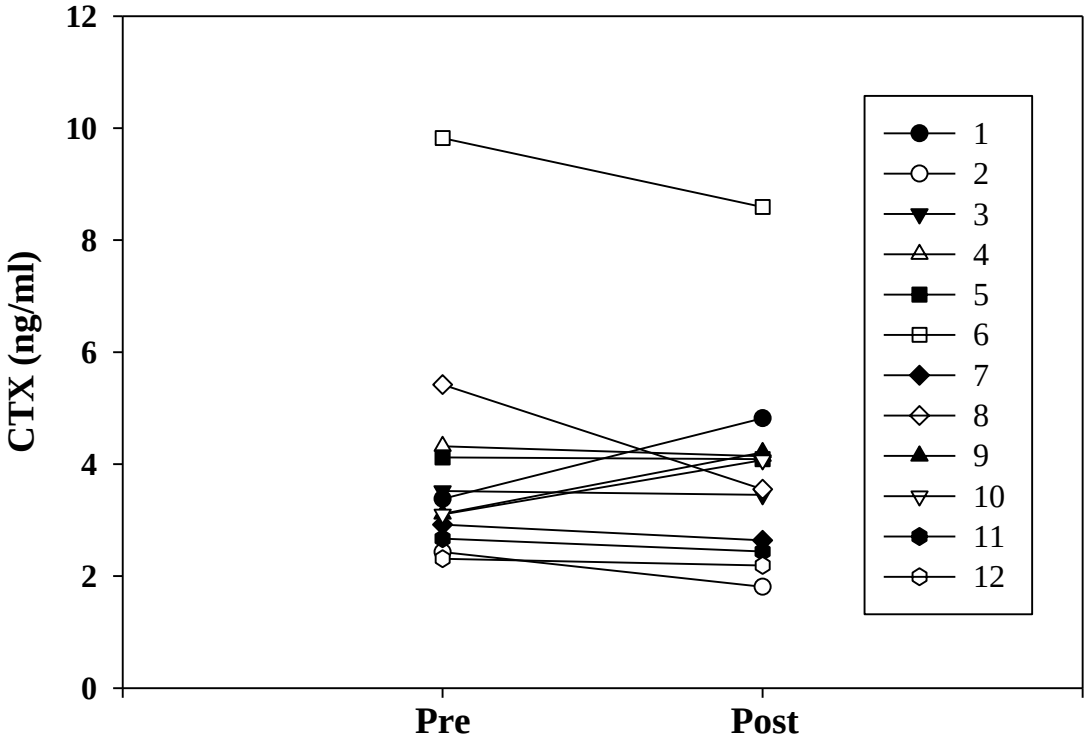


Figure 4.16 Hemodialysis patients show an inconsistent change in plasma c-telopeptide of type I collagen (CTX) concentration after a 4-week supplementation with A. inulin (IN); and B. maltodextrin (CON).

Table 4.5 Fecal mineral excretion after 4-weeks of IN or CON consumption

	IN		CON		Group *P*	Time *P*	G x T *P*/Effect size (n^2)
	Pre	**Post**	**Pre**	**Post**			
Calcium (mg/g DM)	27.05 ± 3.17	26.76 ± 2.72	26.51 ± 2.27	26.48 ± 3.28	0.849	0.943	0.943/ 0.00005
Phosphorus (mg/g DM)	22.73 ± 1.46	21.24 ± 2.0	25.25 ± 1.94	19.49 ± 1.85	0.705	**0.030**	**0.025/** 0.381
Magnesium (mg/g DM)	5.26 ± 0.34	5.42 ± 0.34	5.97 ± 0.48	5.57 ± 0.34	0.059	0.629	0.319/ 0.09

G, group; T, time; G x T, group-by-time; n^2, eta squared (effect size); IN, inulin, CON, maltodextrin; DM, dry matter.

Figure 4.17 Fecal calcium excretion after 4-weeks with IN or CON

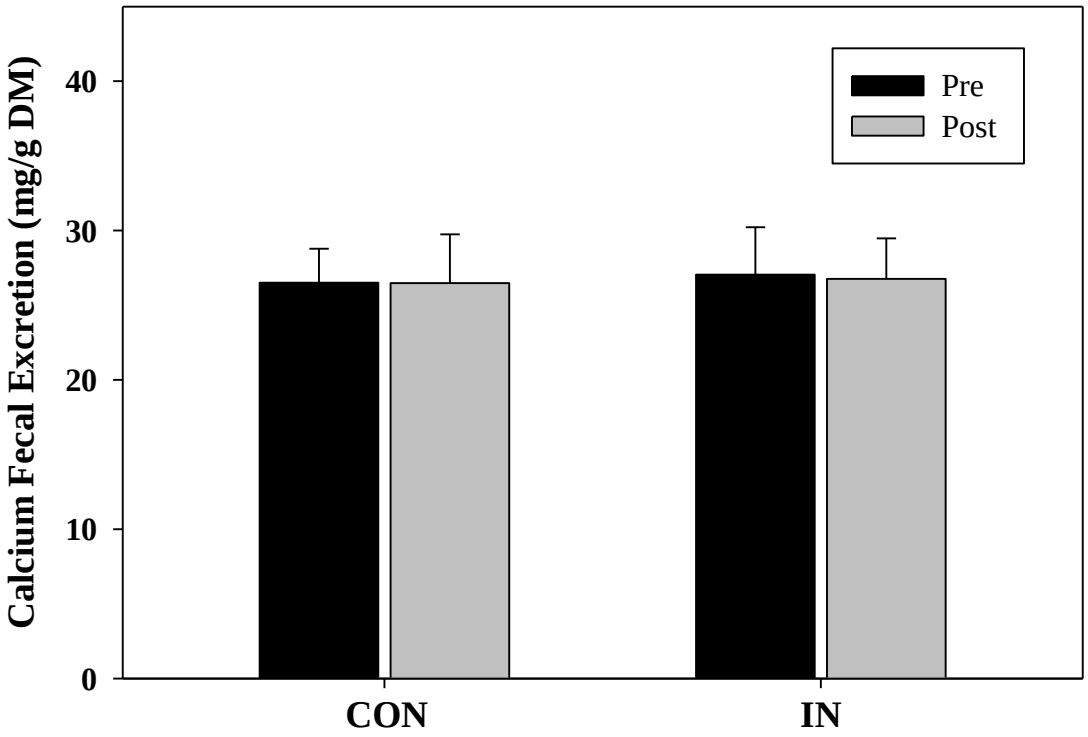


Figure 4.17 No changes in the fecal excretion of calcium after a 4-week supplementation with inulin (IN) or maltodextrin (CON).

Figure 4.18 Fecal phosphorus excretion after 4-weeks with CON

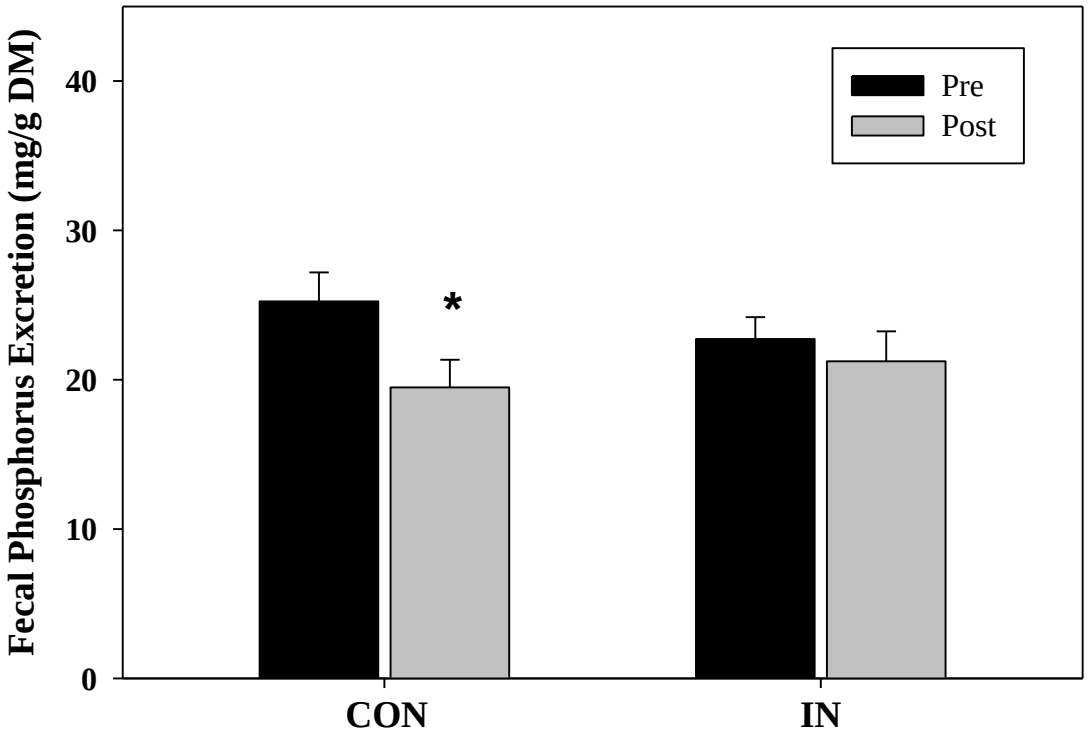


Figure 4.18 There was a group-by-time interaction on the fecal excretion of phosphorus, where it was reduced in the maltodextrin (CON), but not in the inulin (IN) group. * p interaction <0.05

Figure 4.19 Fecal magnesium excretion after 4-weeks with IN or CON

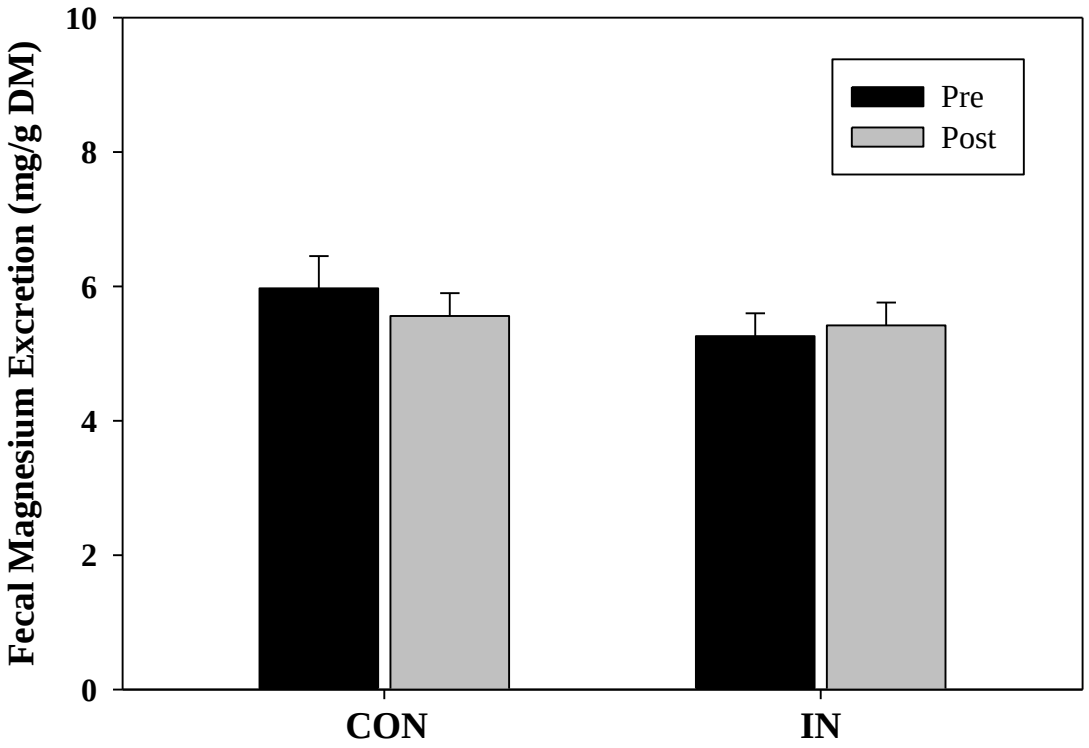


Figure 4.19 No changes in the fecal excretion of magnesium after a 4-week supplementation with inulin (IN) or maltodextrin (CON).

Figure 4.20 MBD composite score after 4 weeks with IN or CON

Variable	Negative (-1)	Neutral (0)	Positive (+1)
Blood Calcium (mg/dl)	<8.2 >10.2	N.A.	8.2-10.2
Blood Phosphorus (mg/dl)	≥5.5	N.A.	<5.5
Blood Magnesium (mg/dl)	<1.7 ≥2.2	N.A.	1.7-2.1
Plasma PTH (% change)	↑25%	-24 to 24%	↓25%
Plasma FGF-23 (% change)	↑25%	-24 to 24%	↓25%
Plasma BALP (% change)	↓10%	-9 to 9%	↑10%
Plasma CTX (% change)	↑10%	-9 to 9%	↓10%
Fecal Calcium (% change)	↑10%	-9 to 9%	↓10%
Fecal Phosphorus (% change)	↓10%	-9 to 9%	↑10%

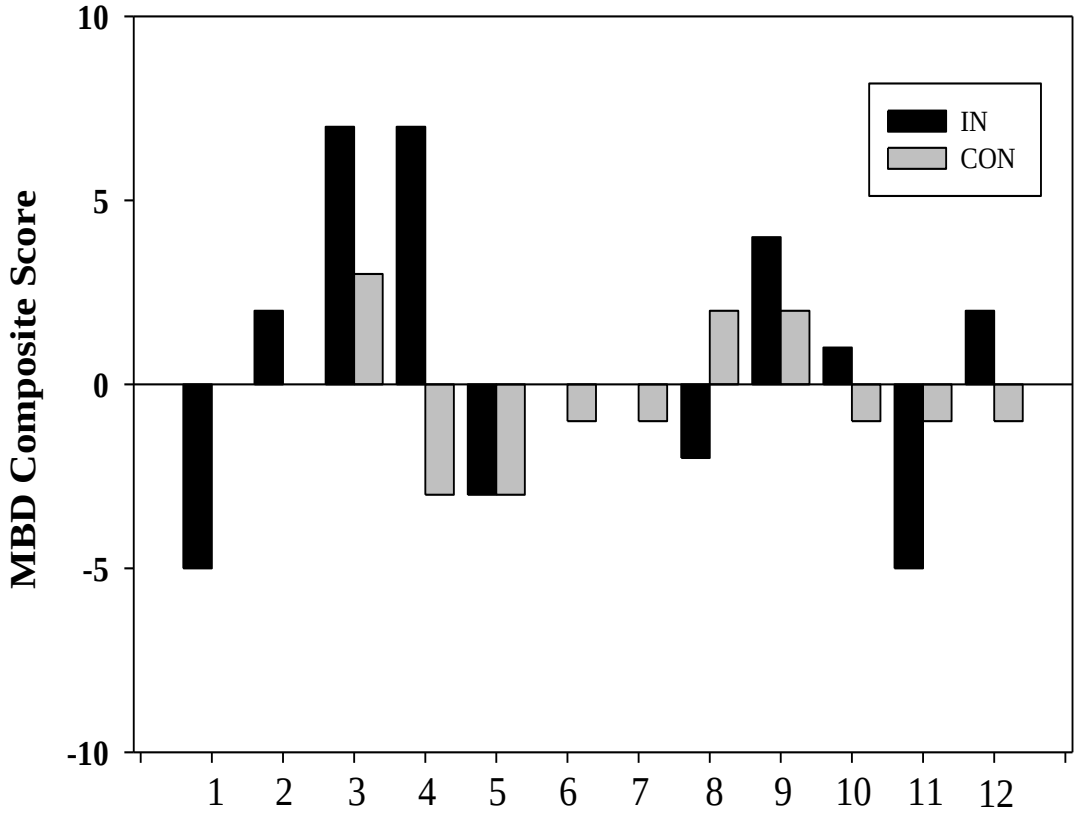


Figure 4.20 Mineral and bone disorder (MBD) composite score based on blood minerals (calcium, phosphorus, and magnesium), biomarkers of mineral and bone metabolism (intact parathyroid hormone, fibroblast growth factor-23, bone-specific alkaline phosphatase, C-telopeptide of collagen type I), and fecal excretion of calcium and magnesium. A neutral (zero) or positive score denotes that most of the measurements were the values desired or had a desired % change (Table 4.3). A negative score denotes that most of the measurements are not on the values desired or an undesired % change after a 4-week supplementation with inulin (IN) or maltodextrin (CON). The subjects are on the x-axis. If a bar is missing for a treatment (IN or CON), the value is zero.

Table 4.6 Dietary intake after 4-weeks of IN or CON consumption

	IN		CON		Group *P*	Time *P*	G x T *P*
	Pre	**Post**	**Pre**	**Post**			
Energy (kcal/kg/d)	23.04 ± 2.71	20.94 ± 2.37	20.22 ± 3.57	19.17 ± 3.20	0.135	0.340	0.756
Protein (g/kg/d)	0.98 ± 0.108	0.92 ± 0.110	0.78 ± 0.113	0.91 ± 0.15	0.206	0.692	0.158
Carbohydrates (% kcal)	41.80 ± 2.03	37.32 ± 1.62	43.48 ± 2.02	37.74 ± 2.84	0.725	**0.044**	0.817
Fat (% kcal)	39.93 ± 2.45	43.68 ± 1.70	38.92 ± 1.72	41.03 ± 2.92	0.403	0.171	0.729
Fiber (g/1000kcal)	7.806 ± 1.36	15.40 ± 0.80	7.17 ± 0.784	7.58 ± 1.21	**0.007**	**0.001**	**0.006**
Calcium (mg/d)	796.46 ± 89.6	754.25±117.4	577.55±81.5	623.97±91.72	**0.042**	0.981	0.493
Phosphorus (mg/d)	1178.7±125.8	1118.7±123.5	914.9±134.7	983.54±152.1	**0.043**	0.966	0.275

G x T, group-by-time; IN, inulin, CON, maltodextrin.

CHAPTER 5

EFFECT OF INULIN SUPPLEMENTATION ON THE GUT MICROBIOTA STRUCTURE AND GUT MICROBIOTA-DERIVED METABOLITES IN HEMODIALYSIS PATIENTS

Abstract

Dietary fiber has been shown to alter the structure of the gut microbiota. Among dietary fibers, inulin-type fructans (ITFs) have been studied extensively due to its ability to modify the gut microbiota. In HD patients, ITFs have been supplemented and have been shown to be successful in reducing protein fermentation byproducts, such as p-cresyl sulfate. However, the effects of the supplementation of ITFs on the gut microbiome in HD has not been explored. Therefore, our objective was to assess the effect of a 4-week supplementation of inulin in the gut microbiota structure, fecal excretion of short-chain fatty acids (SCFA), protein-derived byproducts, and blood concentrations of microbial metabolites in HD patients.

Methods: Twelve HD patients were recruited (56±10 y, 50% M, 58% African American, 31.2±9.2kg/m2). In a randomized, double-blind, placebo-controlled, crossover design subjects consumed inulin (IN) [10g/d for females; 15g/d for males] or maltodextrin (CON) [6g/d for females; 9g/d for males]. Plasma and fecal samples were obtained before and after each supplementation period. DNA was extracted and the V4 hypervariable region of the bacterial 16S rRNA gene was sequenced using Illumina MiSeq. Sequence data was analyzed using QIIME 1.9.1. Fecal SCFA and protein byproducts were quantified by gas chromatography, while plasma metabolites by liquid chromatography-tandem mass spectrometry.

Results: In this randomized, placebo-controlled, cross-over design a 4-week supplementation of IN failed to show changes in the fecal microbiota α- and β-diversities, with no significant changes in gut bacteria at the phyla or genera level. Both IN and CON caused an increase in the fecal excretion of acetate and propionate, with no effect on butyrate. However, there was a positive association between known genera with butyrate producers and fecal excretion of butyrate after supplementation with IN, but not CON. Similarly, there was a trend towards an association between the relative abundance of BCoAT and butyrate excretion after IN supplementation, but not CON. BCFAs and plasma metabolites derived from bacteria were unaffected after the supplementation of IN or CON. Finally, when we assessed the factors that explained the variability of the fecal microbiota in HD patients, sex, BMI category, and prescription of non-calcium-based phosphate binders revealed unique differences at the genera level.

Conclusions: IN supplementation failed to show an effect on the gut microbiota structure. Furthermore, both acetate and propionate were increased after both supplementation periods, raising the question of digestibility of maltodextrin and its use as a control in HD patients. Despite a lack of effect of supplementation on butyrate, known butyrate-producing genera and BCoAT were positively associated with butyrate excretion after IN, but not CON. Interestingly, there was no effect of IN on uremic toxins derived from the gut microbiota. Finally, distinct microbiota structure was seen depending on sex, BMI, and use of calcium-based non-phosphate binders. Future studies should aim to study the long-term effects of IN supplementation on the gut microbiota structure and function and clinical outcomes in HD patients.

Introduction

The gut microbiota is the community of microorganisms that resides in the gastrointestinal tract, especially in the colon.[95–97] The bacteria in the colon can utilize several carbohydrates that escaped digestion (dietary fiber) for energy production, such as resistant starches, cellulose, hemicellulose, glycogen, fructooligosaccharides, galactan, xylan, pectins, and gums.[179] The amount of fiber in the diet has been shown to alter the structure of the gut microbiota.[180] Diets high in dietary fiber have been associated with greater species diversity and a greater abundance of *Prevotella*, while animal-based diets have been associated with greater abundance of *Bacteroides*.[181]

Among dietary fibers, inulin-type fructans (ITFs) have been studied extensively due to their ability to modify the gut microbiota.[182] Inulin is a dietary fermentable fiber derived from plants (chicory root and Jerusalem artichoke, principally) with β (2-1) fructosyl-fructose linkages.[143] Several bacteria genera have been shown to utilize ITFs, including *Bifidobacteria, Lactobacillus, Bacteroides,* and *Faecalibacterium.*[125,183,184] *Bifidobacteria* has the ability to utilize ITFs due to the presence of exo-glycosidases, which hydrolyze monosaccharides at the non-reducing end of the oligosaccharide, and other enzymes to metabolize oligosaccharides intracellularly.[185] Once the oligosaccharides are degraded to monosaccharides, these are converted to intermediates of the hexose fermentation pathway, which involves the pentose-phosphate pathway, ultimately yielding lactate, acetate, and ATP.[185] The lactate and acetate produced are then used by other bacteria for the production of butyrate, principally.[186–188]

When there is a low dietary fiber intake, such as the one observed in hemodialysis (HD) patients,[8] these bacteria may utilize other nutrients, such as protein that escaped digestion and absorption in the small intestine, but also endogenous compounds, such as sloughed intestinal

cells, mucin, hyaluronic acid, sialic acid and heparin.[188–190] The fermentation of protein yields ammonia, branched-chain fatty acids, indoles and phenolic compounds, principally.[187] These protein fermentation byproducts have been associated with higher cardiovascular mortality, endothelial dysfunction, and mineral and bone disorder in kidney disease.[103–106] Furthermore, trimethylamine-oxide (TMAO), a metabolite derived from choline, betaine, and carnitine, has also been associated with the progression of CKD.[99,104] In HD patients, ITFs have been supplemented and have been shown to be successful in reducing protein fermentation byproducts, such as p-cresyl sulfate.[191] However, the effects of the supplementation of ITFs on the gut microbiome in HD has not been studied extensively in HD patients. Therefore, our objective was to assess the effect of a 4-week supplementation of inulin in the gut microbiota structure, fecal excretion of SCFA and protein-derived byproducts, and blood concentrations of microbial metabolites in HD patients.

Methods

HD patients were recruited from local dialysis clinics in Central Illinois. Inclusion criteria for participants included: 1) Received HD therapy 3 days per week and for at least 3 months. Exclusion criteria includes: 1) Sustained hypercalcemia (>3months). 2) Previous major gastrointestinal disease diagnosis (e.g., inflammatory bowel disease and celiac disease). 3) Antibiotic treatment less than two weeks prior the start of the study.

Intervention Protocol

As shown in the previous chapter (Figure 4.1), in a randomized, placebo-controlled, cross-over design, patients were randomized to the intervention group (inulin [IN]) or placebo group (maltodextrin [CON]). Patients consumed IN (90% inulin with a degree of polymerization of 2-60 and 10% short-chain fructooligosaccharides, with a degree of of 2-8; ORAFTI

SYNERGY, Beneo, Belgium) (IN; females: 10g/day; males: 15g/day) or CON (Now Foods Carbogain 100% maltodextrin, Bloomingdale, IL) (females: 6g/day; males: 9g/day) providing the same amount of energy for one month, underwent a two-week washout period, and continue with the other treatment (IN or CON) for another month. The first week of the intervention period was an adaptation week, in which patients consumed half of the dose (5g/day and 7.5g/day for females and males, respectively in the IN group; 3g/day and 4.5g/day for females and males, respectively in the CON group).

Fecal Sample Collection and Gastrointestinal Symptoms

Participants were asked to collect a complete fecal sample (Commode Specimen Collection System Sage Products, Crystal Lake, IL) at the beginning and end of Period 1 and 2 (Previous chapter Figure 4.1). Samples were weighed, homogenized and stored at -80°C within 30 minutes of collection until analysis. Participants were also asked to rate consistency and ease of passage for the bowel movement. Stool consistency was scored according to the Bristol Stool Scale. Ease of stool passage was ranked on a 5-point scale (1=very easy, 2=easy, 3=neither easy nor difficult, 4=difficult, 5=very difficult). Samples were weighed, homogenized and three-2ml aliquots were stored at -80°C within 30 minutes of collection.

DNA Extraction and Fecal Microbiota Analyses

DNA was extracted (Powerlyzer PowerSoil DNA Isolation Kit MO BIO, Carlsbald, CA) and its quantification was performed by Qubit Fluorometric Quantitation (ThermoFisher Scientific, Waltham, MA), while quality was assessed by electrophoresis with agarose 2% Agarose E-gels using the E-Gel iBase (Invitrogen, Grand Island, NY). Primers targeting the V4 hypervariable region of the bacterial 16S rRNA gene amplicons of 250bp were generated as

described previously.[114] Sequencing was performed through Illumina Mi-seq V3 platform. Relative changes in bacterial diversity (α-diversity and β-diversity) and taxonomical changes were analyzed through the open software QIIME (version 1.9.1). In short, sequenced data was demultiplexed and went through quality filtering using split_libraries_fastq.py default parameters. Sequences were clustered into operational taxonomic units (OTU) using closed-reference OTU picking against the Greengenes 13_8 reference OTU database with a 97% similarity threshold.[115] For α- and β-diversity, samples were rarified to 67,614 sequences/sample.

Short-chain Fatty Acids, Branched-chain Fatty Acids, Total Phenols, and Total Indoles

For the quantification of SCFA and branched-chain fatty acids (BCFA), a 2ml aliquot of the fecal sample was acidified with 2N-HCl (10% weight:volume) and frozen at -20°C until analysis, which was performed by gas chromatography . Fecal dry matter was measured according to the methods of the Association of Official Analytical Chemists and SCFA and BCFA, [192] phenols and indoles[193] were measured as previously described.

Plasma Metabolites

Total plasma p-cresyl sulfate, indoxyl sulfate, and trimethylamine-oxide (TMAO) was measured by liquid chromatography-tandem mass spectrometry (UPLC-MS/MS method). The UPLC system was an Acquity H Class (Waters, Zellik, Belgium). Chromatographic separation was performed on an Acquity CSH Fluoro Phenyl column (50 x 2.5 mm; 1.7 mm particle size; Waters, Zellik, Belgium) with an Acquity CSH Fluoro Phenyl VanGuard pre-column (10 x 2.5 mm; 1.7 mm particle size; Waters, Zellik, Belgium). The mobile phase, delivered at a flow rate

of 0.5 ml min^{-1} at 40°C, was a gradient of 0.1% formic acid in MQ water (A) and pure methanol (B) coupled to a Waters 2475 fluorescence detector, as described by de Loor et al.[194]

Statistical Analysis

Mean and standard error of the mean is reported unless otherwise noted. Repeated measures ANOVA was performed in a within-subjects analysis with two groups (IN, CON) and two time-points (Pre, Post), against variables of interest, such as alpha-diversity metrics, phyla and genera relative abundances, fecal excretion of metabolites, and plasma concentration of metabolites with significance at $p<0.05$. Wilcoxon's paired test was performed to assess any potential carryover effects (IN-Pre vs. CON-Pre) in the main variables of interest (e.g., *Bifidobacterium, Faecalibacterium,* and fecal excretion of SCFA).[182] Spearman's correlations were performed between bacteria genera with a relative abundance ≥1% and fecal and blood microbial metabolites. Additionally, the relationship between the bacteria genera, fecal and blood microbial metabolites and variables from Chapter 4 (e.g., blood minerals and mineral and bone metabolism biomarkers) were assessed. All statistical analysis was performed using SPSS version 24. Further statistical analysis was performed through Statistical Analysis of Metagenomic Profiles (STAMP) using categorical variables (e.g., IN vs CON [post-supplementation only], sex, body-mass index [BMI] category, etc.). Analysis in STAMP was corrected for multiple comparisons using Benjamini-Hochberg False Discovery Rate (FDR).[195]

Results

Fecal microbiota α-diversity, β-diversity, and taxonomical analyses

Twelve HD patients (56±10 y, 50% M, 58% African American, 31.2±9.2kg/m2) completed the study. For the fecal microbiota, a total of 4,411,326 bacterial 16S rRNA sequences

were obtained, with a median of 90,240 (range 67,614-146,384) sequences per sample. For α-diversity or species richness, the data was rarified to 67,614 sequences. We did not observe changes in non-phylogenetic metrics of species richness after the supplementation of IN or CON (Chao1 IN Pre 372.26±9.56 species, Post 364.96±13.44 species vs. CON Pre 374.55±12.72, Post 370.15.17 species; $p>0.05$). Additionally, we did not observe any difference in phylogenetic distance (PD), a phylogenetic metric of α-diversity, but there was a trend towards a time effect (INs Pre 24.72±0.89 vs. Post 24.12±0.99 and CON Pre 25.18±1.05 vs. Post 24.37±1.06, time $p=0.093$).

For β-diversity, principal coordinates analyses (PCoA) of unweighted and weighted UniFrac performed on the 97% OTU abundance distance matrix failed to show differences or separation between treatments, time, or treatment-by-time (Figure 5.1). Furthermore, we observed that there was a low intra-individual variation in most subjects at all timepoints in the unweighted UniFrac PCoA, but a higher intra-individual variation in the weighted UniFrac PCoA, suggesting that there was a higher variation in the more abundant OTUs compared to the lower abundance OTUs after four weeks of IN or CON supplementation (Figure 5.2).

At baseline, taxonomic analysis revealed that *Firmicutes* were the most abundant phylum, followed by *Bacteroidetes,* with a relative abundance of 64.01±2.58% and 27.56±2.73% of total sequences, respectively. Furthermore, the *Firmicutes*-to-*Bacteroidetes* ratio was 2.68±0.36 (range 1.13-5.59). *Bacteroides* was the most abundant genera in the *Bacteroidetes* phylum and overall genera (Table 5.2). After the supplementation of IN or CON, there were no differences at the phyla or genera level (Table 5.2). Furthermore, we did not observe any differences in the genera of interest (e.g., *Bifidobacterium* or *Faecalibacterium).* However, there was a trend towards a time effect on *Faecalibacterium,* in which it increased after the

supplementation of IN and CON (IN Pre 5.77±1.45 vs. Post 9.24±2.05 and CON Pre 6.96±1.71 vs. Post 8.42±2.28; p=0.079) (Table 5.2).

Demographic variables determine gut microbiota structure

Principal components analysis (PCA) revealed a distinct fecal microbiota in female and male subjects (Figure 5.3 A). Additionally, there were specific differences at the genera level, where female participants had lower relative abundance of *Faecalibacterium* (FDR-corrected p<0.001) and higher *Ruminococcaceae* (FDR-corrected p=0.03) and *Akkermansia* (FDR-corrected p=0.048) (Figure 5.3 B). Similarly, the fecal microbiota was different depending on body mass index (BMI) category, where subjects with a BMI <25 kg/m^2 clustered together, as well as subjects with a BMI ≥30 kg/m^2 (Figure 5.4 A). These differences were explained at the genera level, where *Ruminococcaceae* (FDR-corrected p=0.00291), *Ruminococcus* (FDR-corrected p=0.025), *Erysipelotrichaceae* (FDR-corrected p=0.027), *Lachnospira* (FDR-corrected p=0.035), and *Clostridium* (FDR-corrected p=0.037) had a higher relative abundance in the subjects with BMI ≥30 kg/m^2, compared to subjects with BMI <25 kg/m^2 (Figure 5.4 B).

Fecal excretion of SCFA and their relationship with the fecal microbiota

Fecal excretion of SCFA was analyzed in only nine out of the twelve subjects that completed the study because we did not obtain the samples within one hour of fecal sample collection. For fecal excretion of SCFA, there was a time effect for the excretion of acetate and propionate, but not butyrate (acetate pre 219.48±39.31 vs. post 298.47±47.41 umol/g of dry matter (DM), time p=0.032; propionate pre 70.29±13.27 vs. post 89.74±15.33 umol/g DM time p=0.027; butyrate pre 40.89±8.05 vs. post 54.58±11.36 umol/g DM, time p=0.12) and a trend towards a group effect for butyrate (IN 43.10±9.19 umol/g DM vs. CON 52.37±9.34 umol/g

DM, p=0.075), but no treatment by time interactions (Table 5.3, Figure 5.5). Additionally, there were no changes in the molar ratios of the three SCFA in both periods (p>0.05). Total SCFA fecal excretion was not different between treatments, but there was a time effect (pre 330.67±58.54umol/g DM vs. post 442.79±68.18umol/g DM; time p=0.03), with no carryover effect for those that were randomized to IN first (p=0.141). In terms of percent change, acetate, propionate, and butyrate increased after both treatments and we did not find significant differences between treatments (acetate IN 39.80±17.67% vs. CON 59.07± 19.71%, p=0.322; propionate IN 30.39± 23.35% vs. CON 68.33± 26.17%, p=0.137; butyrate IN 67.39± 44.63% vs. CON 72.51± 20.61%, p=0.886) (Figure 5.6).

There was a positive association between the sum of genera with known butyrate producers (i.e., *Clostridium, Lachnospiraceae, Coprococcus, Roseburia, Ruminococcus, Faecalibacterium,* and *Oscillospira*) after IN supplementation and fecal excretion of butyrate (ρ=0.810; p=0.015), but not after CON supplementation (ρ=0.490; p=0.162) (Figure 5.7). Similarly, we found trend towards an association between the relative abundance of one of the bacterial enzymes needed for butyrate production, butyryl-CoA-acetyl-CoA transferase (BCoAT), and the fecal excretion of butyrate after IN supplementation (ρ=0.690; p=0.058), but not after CON supplementation (ρ=0.333; p=0.347) (Figure 5.8).

Fecal excretion of BCFA

Similar to SCFA fecal excretion, BCFA were measured in nine out of the twelve subjects. For fecal excretion of total BCFA, isobutyrate, isovalerate, and valerate we did not find any treatment effect, time effect, or treatment-by-time interactions (p>0.05, Table 5.3 and Figure 5.9). However, when we considered the percent change after IN or CON, we observed a numerical increase in isobutyrate (IN 3.81±23.08% vs. CON 128.90±92.14%; p=0.173),

isovalerate (IN -4.41±21.05% vs. CON 422.59±373.99%; p=0.280), valerate (IN 2.48±20.77% vs. CON 109.90±87.02%; p=0.250), and total BCFA (IN -0.94±20.48% vs. CON 169.40±131.73%; p=0.213), although these changes were not statistically significant (Figure 5.10).

Fecal excretion of phenols and indoles

We did not observe differences in the fecal excretion of total phenols and total indoles after the supplementation of IN or CON (Table 5.3 and Figure 5.9). However, there was a trend towards a time effect on the fecal excretion of phenols (IN Pre. 260.01±49.88 umol/g DM vs. Post 158.28±50.87 umol/g DM and CON Pre 199.60±34.29 umol/g DM vs. Post 152.41±42.88 umol/g DM; time p=0.051), but no effect on fecal excretion of indoles (IN Pre 12.76±20.03 umol/g DM vs. Post 176.12±45.37 umol/g DM and CON Pre 259.23±40.93 umol/g DM vs. Post 230.87±44.71 umol/g DM; time p=0.664). Additionally, we did not observe differences between treatments when we considered percent change after IN or CON in the fecal excretion of total phenols (IN -23.30±31.02% vs. CON -26.45±14.43%, p=0.940) or total indoles (IN -9.75±18.73% vs. CON 4.33±22.88%, p=0.560) (Figure 5.10).

Bacteria-derived plasma metabolites and their relationship with fecal excretion of metabolites

We did not observe any changes in the bacteria-derived plasma metabolites indoxyl sulfate, p-cresyl sulfate, TMAO, or phenylacetylglutamine after the supplementation of IN or CON (Table 5.4). When we considered the percent change after the supplementation of IN or CON, we only observed a trend towards significance in indoxyl sulfate (IN -9.89±8.56% vs. CON 8.52±8.99%, p=0.141), but not p-cresyl sulfate (IN -2.22±13.1% vs. CON -12.26± 5.19%, p=0.441), TMAO (IN 12.46±23.50% vs. CON 27.09±18.80%, p=0.591), or

phenylacetylglutamine (IN 0.89±15.62% vs. CON 14.27±14.94%, p=0.539) (Figure 5.11). We did not observe an association between indoxyl sulfate and fecal excretion of total indoles after IN supplementation (ρ=0.183; p=0.637) or CON supplementation (ρ=-0.070; p=0.829). Similarly, we did not observe an association between p-cresyl sulfate and fecal excretion of phenols after IN supplementation (ρ=0.552; p=0.123) or CON supplementation (ρ=0.483; p=0.112).

Gut microbiota and mineral metabolism

Principal component analysis revealed a unique microbiota in those patients that consumed non-calcium-based phosphate binders (e.g., sevelamer hydrochloride and sevelamer carbonate). At the genera level, this was associated with a higher relative abundance of *Ruminococcaceae* (FDR-corrected p=0.0003) and *Clostridiales* (FDR corrected p=0.00335), and a higher relative abundance of *Bacteroides* (FDR-corrected p=0.0002) (Figure 5.12). There were no other associations between blood minerals, plasma biomarkers of mineral and bone metabolism, and fecal excretion of minerals.

Discussion

In this randomized, placebo-controlled, cross-over design a 4-week supplementation of IN failed to show changes in the fecal microbiota α- and β-diversities, with no significant changes in gut bacteria at the phyla or genera level. Both, IN and CON had an increase in the fecal excretion of acetate and propionate, with no effect on butyrate. However, there was a positive association between known genera with butyrate producers and fecal excretion of butyrate after supplementation with IN, but not CON. Similarly, there was a strong trend towards an association between the relative abundance of BCoAT and butyrate excretion after IN

supplementation, but not CON. BCFAs and plasma metabolites derived from bacteria were unaffected after the supplementation of IN or CON. Finally, when we assessed the factors that explained the variability of the fecal microbiota in HD patients, sex, BMI category, and prescription of non-calcium-based phosphate binders revealed unique differences at the genera level.

ITFs have been shown to modify the gut microbiota structure in healthy adults[182,196] and other clinical populations, such as subjects with obesity[197,198] and diabetes.[199,200] Most studies have reported an increase in the relative abundance of *Bifidobacterium* at the genera level, but also butyrate producers, such as *Faecalibacterium, Anaerostipes,* and *Roseburia.*[182,196,198,201] In the present study, we did not observe an increase in the *a priori* genera of interest *Bifidobacterium* or *Faecalibacterium.* Failure to observe an effect on *Bifidobacterium* has been reported as a methodological flaw in 16S rRNA gene sequencing due to the low relative abundance of this genus,[202] which in our subjects was 1.89±0.99% of total sequences throughout the study time points. Furthermore, even though we observed a numerical increase in the relative abundance of *Faecalibacterium* after IN, we did not find a group-by-time interaction compared to CON. Besides these genera, reductions in other genera have been reported, such as *Bacteroides* and *Bilophila.*[182,198] In our study, however, we did not observe any change in those genera.

We hypothesized that the supplementation of IN would increase the fecal excretion of SCFA, especially butyrate, compared to CON. ITFs are fermented in the colon to acetate and lactate by *Bifidobacteria* through the bifidogenic shunt, which are then used by other bacteria to produce other SCFA, especially butyrate.[188,190,202,203] We observed a trend towards a time effect on butyrate, increasing similarly after IN and CON. Despite the lack of effect of IN on butyrate,

we found a positive association between genera with species capable of producing butyrate and butyrate excretion after IN, but not CON. Similarly, we found a positive association between the relative abundance of BCoAT, one of the main enzymes needed for the bacterial production of butyrate, only after IN.[125] These data suggests that gut microbiota of HD patients is capable of degrading IN and produce butyrate.

We observed a time effect in the fecal excretion of acetate and propionate, meaning that these SCFA were increased after the both supplements. In fact, when we looked into the percent change, we observed a numerically higher effect of CON in acetate and propionate fecal excretion. Even without an increase in the dietary fiber intake in the CON group, this increased of fecal excretion of acetate and propionate suggests that maltodextrin may be fermented by the colonic microbiota. We chose to use maltodextrin as our control because it is a completely digestible carbohydrate used extensively as a control for fiber supplementation studies. Maltodextrin is composed by a mixture of amylose and amylopectin, which have α(1-4) and α(1-6) glycosidic bonds, respectively.[204] Maltodextrin digestion starts in the mouth by the action of salivary α-amylose and continues in the proximal small intestine by the action of the pancreatic α-amylase to yield maltose, which then is finally metabolized to glucose by the brush border membrane-enzyme maltase.[69,204] Data regarding abnormalities in carbohydrate digestion in HD patients is scarce. In a study by el-Lakani et al.[205] it was reported that dialysis patients had decreased activity of mucosal maltase and sucrase, but not lactase. However, they concluded that overall HD patients did not have carbohydrate malabsorption. Studies aiming to assess digestion abnormalities in end-stage renal disease patients are needed.

Modulation of the gut microbiota to reduce the production of uremic toxins is a topic of significant interest to Nephrology. In a study by Meijers et al.[191], the supplementation of 20g of

oligofructose-enriched inulin resulted in a 20% reduction in p-cresyl sulfate, without a change in indoxyl sulfate. In our study, we did not observe a difference in plasma p-cresyl sulfate or indoxyl sulfate. Furthermore, we did not observe a decrease in the fecal excretion of total phenols or indoles. It has been suggested that by lowering the ratio of dietary protein-to-dietary fiber, the production of these bacteria-derived uremic toxins may decrease.[109] Despite a higher fiber intake and a sustained protein after IN supplementation we did not find a decrease in these uremic toxins. Furthermore, specific bacterial species within the *Bacteroides* genus, such as *B. thetaiotaomicron* and *B. ovatus* have been shown to express tryptophanase, the enzyme needed for the breakdown of tryptophan to indole, which then will be absorbed and sulfated in the liver for the production of indoxyl sulfate.[206] We did not find an association between the genus *Bacteroides* and the plasma concentration of indoxyl sulfate. However, with our microbial analysis we were not able to assess bacterial species relative abundance. Further studies should aim to assess determinants related to the effectiveness of prebiotic fibers on the reduction of microbiota-derived uremic toxins.

We observed that demographic variables, such as sex and BMI, had a strong effect on the gut microbiota. First, we found a sex effect, in which male subjects had a higher relative abundance of *Faecalibacterium,* whereas female participants had a higher relative abundance of *Ruminococcaceae* and *Akkermansia.* Similarly, Chen et al.[207] studied the fecal microbiota of subjects from the Midwest and found that there were differences in the gut microbiota of males and females. Additionally, we found that the fecal microbiota was different depending on the BMI class, where people with a BMI ≥30 kg/m^2 had a higher relative abundance of SCFA-producing bacteria *Ruminococcaceae, Ruminococcus, Erysipelotrichaceae, Lachnospira, Clostridium.* This may suggest an energy harvesting effect of the gut microbiota of people with

obesity. Similarly, Chen et al.[207] observed an effect of BMI on gut microbiota. However, it remains controversial if gut microbiota contributes to obesity or obesity may cause changes in gut microbiota.[208,209] Due to the reverse epidemiology observed in late stages of CKD,[210] prospective studies should examine the effect of the gut microbiota structure and SCFA production on clinical outcomes in HD patients.

Regarding mineral metabolism, we observed a unique gut microbiota in those subjects that were prescribed non-calcium-based phosphate binders (e.g., sevelamer hydrochloride or sevelamer carbonate). Interestingly, the use of these binders was associated with a higher relative abundance of *Ruminococcaceae* and *Clostridiales.* Sevelamer hydrochloride/carbonate is a polymer that may have the non-selective ability of bind molecules, such as p-cresyl sulfate and indoxyl sulfate.[109] Interestingly, we also observed that people with prescription of sevelamer had a lower relative abundance of *Bacteroides.* As mentioned earlier, tryptophanase is expressed in some of the species within the genus *Bacteroides,* such as *B. thetaiotaomiron* and *B. ovatus.* Future studies should aim to study the effects of non-calcium based phosphate binders on the gut microbiota structure and function.

There are several limitations in our study. First, we assessed supplement compliance verbally, without any biological measurement, such as breath hydrogen. Second, we were not powered to detect differences in the fecal excretion of SCFA and plasma bacteria-derived metabolites. Third, our placebo (maltodextrin) seemed to be acting as a dietary fiber, which may have limited our ability to detect an effect after IN supplementation. Finally, we only collected one fecal sample at each timepoint, which may have limited our ability to detect a more meaningful effect of our intervention. However, we believe our results are valuable as this is the first study using a prebiotic fiber to assess its effects on the gut microbiota in HD.

Conclusion

IN did not produce any changes in the gut microbiota structure. However, acetate and propionate fecal excretion were both increased after four weeks of IN and CON. Though we did not find a treatment-by-time effect on butyrate excretion, we found a positive association between known genera with butyrate producers and butyrate excretion, as well as relative abundance of BCoAT and butyrate excretion after IN supplementation. Furthermore, IN did not produce a change in BCFA or plasma bacteria-derived metabolites. Finally, sex, BMI, and use of non-calcium-based phosphate binders showed differences in the gut microbiota structure of HD patients. Future studies should aim to study the long-term effects of IN supplementation on the gut microbiota structure and function and clinical outcomes in HD patients.

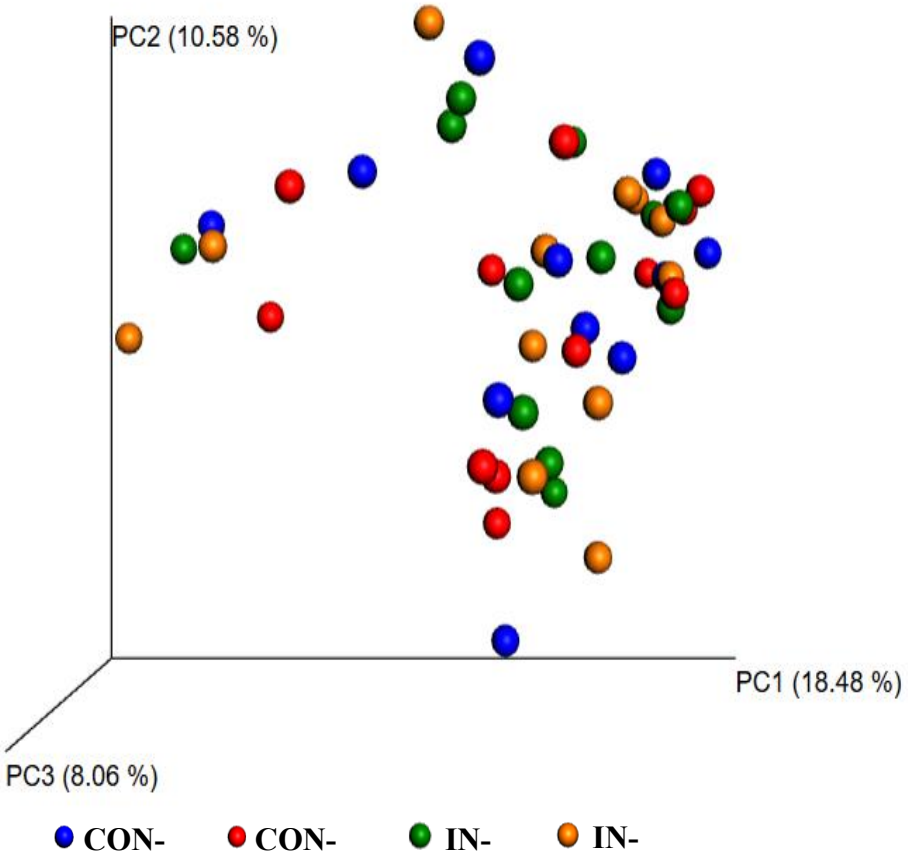
Table 5.1 Patient Baseline Characteristics

Variable	Mean ± SEM/ Median (IQR)
Age (years)	56 ± 9
Gender (M/F)	6/6
African American (%)	58.3%
BMI (kg/m^2)	31.06 ± 8.62
Diabetes (%)	46%
Albumin (g/dl)	3.27 ± 0.27
Energy (kcal/kg/d)	22.43 ± 3.14
Protein (g/kg/d)	0.97 ± 0.14
Carbohydrates (% total kcal)	44.28 ± 2.01
Fat (% total kcal)	37.79 ± 1.77
Dietary Fiber (g/1000kcal)	6.79 ± 0.85
Acetate (umol/g DM)	145.1 (103.53-254.62)
Propionate (umol/g DM)	57.92 ± 10.55
Butyrate (umol/g DM)	35.63 ± 8.42
Indoxyl Sulfate (uM)	110.65 ± 13.94
P-Cresyl Sulfate (uM)	180.82 ± 31.42
Trimethylamine N-Oxide (uM)	58.97 ± 8.84

SEM, standard error of the mean (for normally-distributed data); IQR, interquartile range (for non-normally distributed data); BMI; body mass index.

Figure 5.1 Unweighted and weighted Unifrac does not reveal any difference between treatment and time points

A. Unweighted



B. Weighted

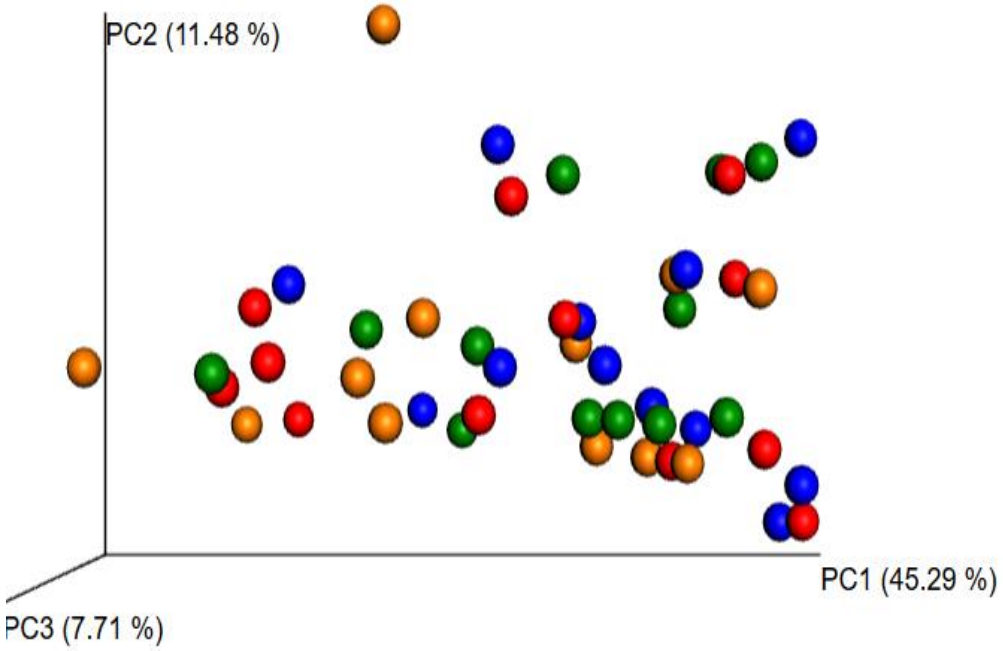
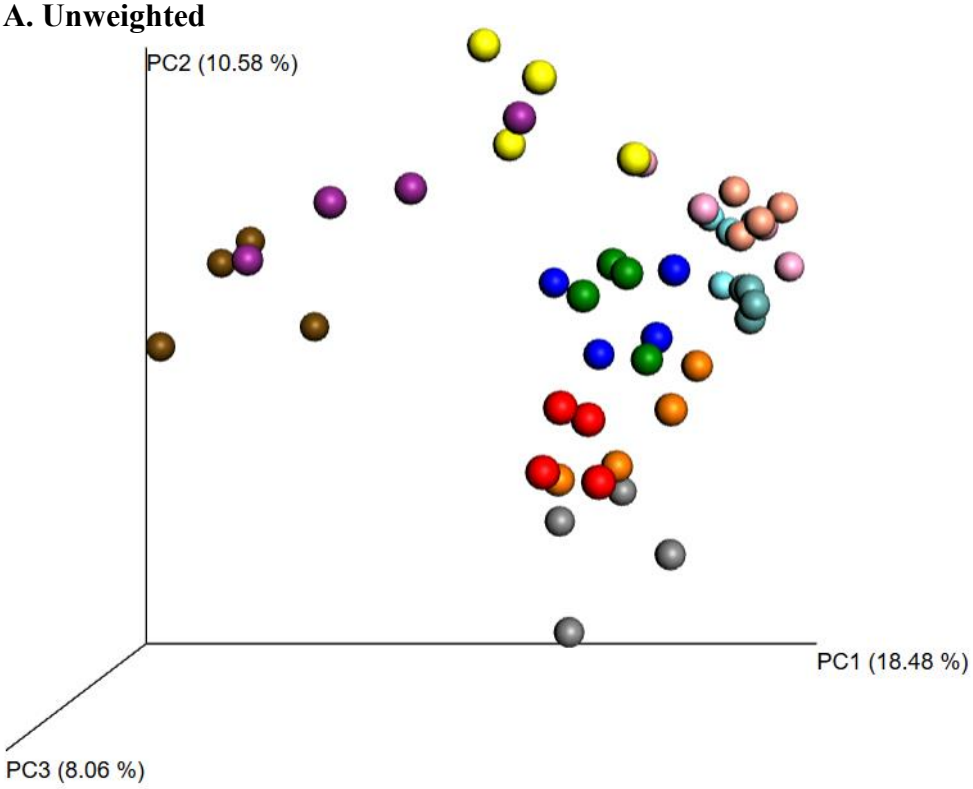


Figure 5.1 Principal coordinate analysis (PCoA) of the unweighted (A) and unweighted (B) UniFrac performed on the 97% OTU abundance distance matrix does not reveal differences between groups (inulin and maltodextrin). Blue, Maltodextrin-Pre; red, Maltodextrin-Post; green, Inulin-Pre; orange, Inulin-Post.

Figure 5.2 PCoA of unweighted and weighted Unifrac at the subject-level

A. Unweighted



B. Weighted

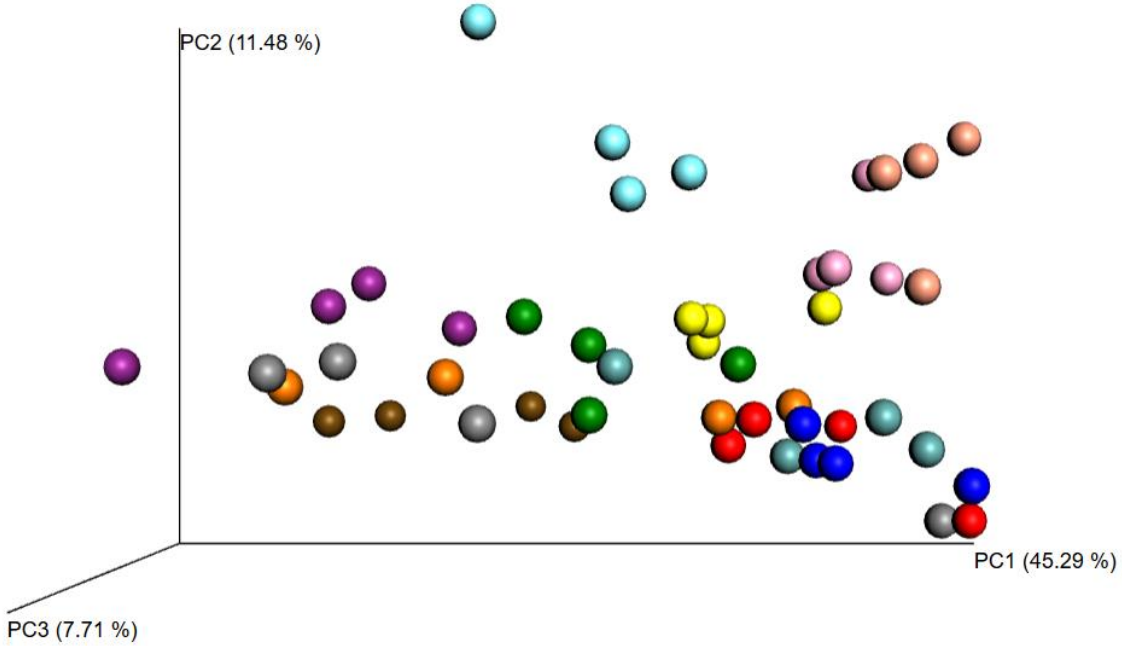


Figure 5.2 Principal coordinate analysis (PCoA) of the unweighted (A) and unweighted (B) UniFrac performed on the 97% OTU abundance distance matrix does reveals a low intra-individual variability within subjects. Every color denotes a different subject.

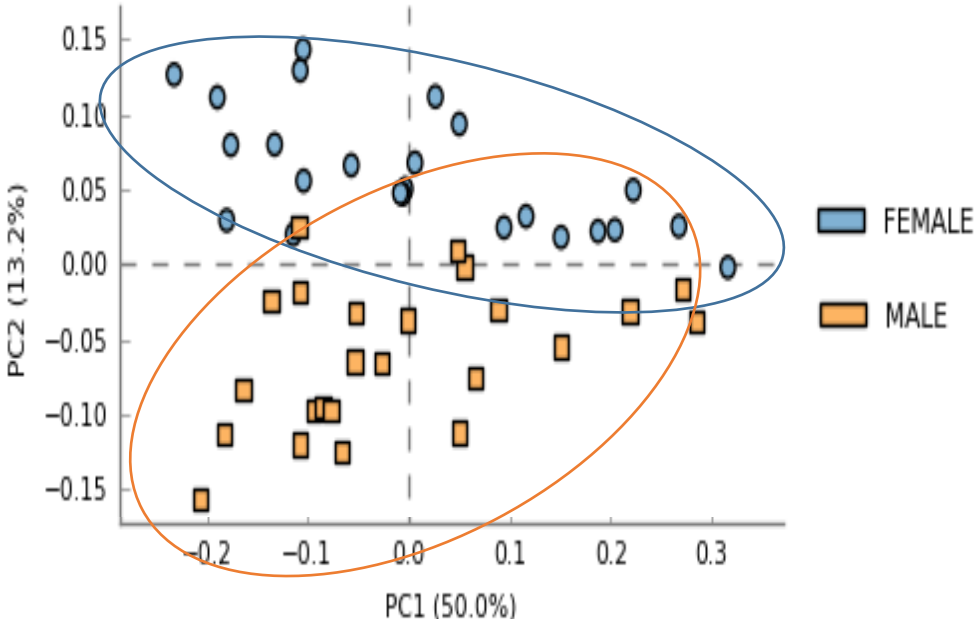
Table 5.2 Relative Abundance of bacteria genera after 4-weeks of IN or CON consumption

Bacteria		IN		CON		Group *P*	Time *P*	G x T *P*
Phyla	***Genera***	**Pre**	**Post**	**Pre**	**Post**			
Bacteroidetes		32.02±3.54	38.05±3.92	27.71±3.07	34.82±4.48	0.079	**0.028**	0.864
	Bacteroides	26.79±3.65	32.08±3.92	23.52±3.23	29.86±4.67	0.122	**0.028**	0.864
Firmicutes		59.75±3.17	54.03±4.19	61.94±3.66	56.47±4.29	0.294	0.084	0.969
	Clostridiales	7.33±0.097	6.39±0.81	9.16±1.29	7.54±1.28	0.106	0.096	0.632
	Faecalibacterium	5.77±1.45	9.24±2.05	6.96±1.71	8.42±2.28	0.848	0.079	0.429
	Lachnospiraceae	7.50±1.005	5.339±1.04	7.23±1.07	7.21±1.06	0.340	0.280	0.166
	Ruminococcus	4.77±0.96	3.75±1.31	3.76±1.31	3.30±0.73	0.182	0.325	0.665
	Streptococcus	3.74±2.13	1.55±0.87	3.45±1.88	2.34±1.00	0.106	0.212	0.630
	Coprococcus	2.10±0.55	1.55±0.31	2.36±0.56	2.04±0.63	0.356	0.258	0.866
	Oscillospira	1.75±0.47	1.01±0.20	1.21±0.23	1.33±0.37	0.730	0.132	0.220
Actinobacteria		3.44±0.87	3.021±0.69	5.69±1.92	4.28±1.48	0.153	0.130	0.436
	Bifidobacterium	1.43±0.64	1.89±0.64	3.3±1.8	2.7±1.47	0.245	0.891	0.249
Verrucomicrobia		0.84±0.485	1.94±1.06	1.95±0.74	0.99±0.52	0.891	0.864	**0.045**
	Akkermansia	1.13±0.67	1.75±0.99	1.65±0.61	1.18±0.67	0.963	0.864	0.322
Proteobacteria		1.20±0.34	2.15±0.77	1.26±0.36	2.04±0.42	0.966	0.099	0.825
	Bilophila	0.175±0.06	0.22±0.07	0.23±0.071	0.322±0.10	0.212	0.347	0.697

G, group; T, time; G x T, group-by-time; IN, inulin, CON, maltodextrin.

Figure 5.3 PCA plot reveals a different gut microbiota in female and male HD patients

A.



B.

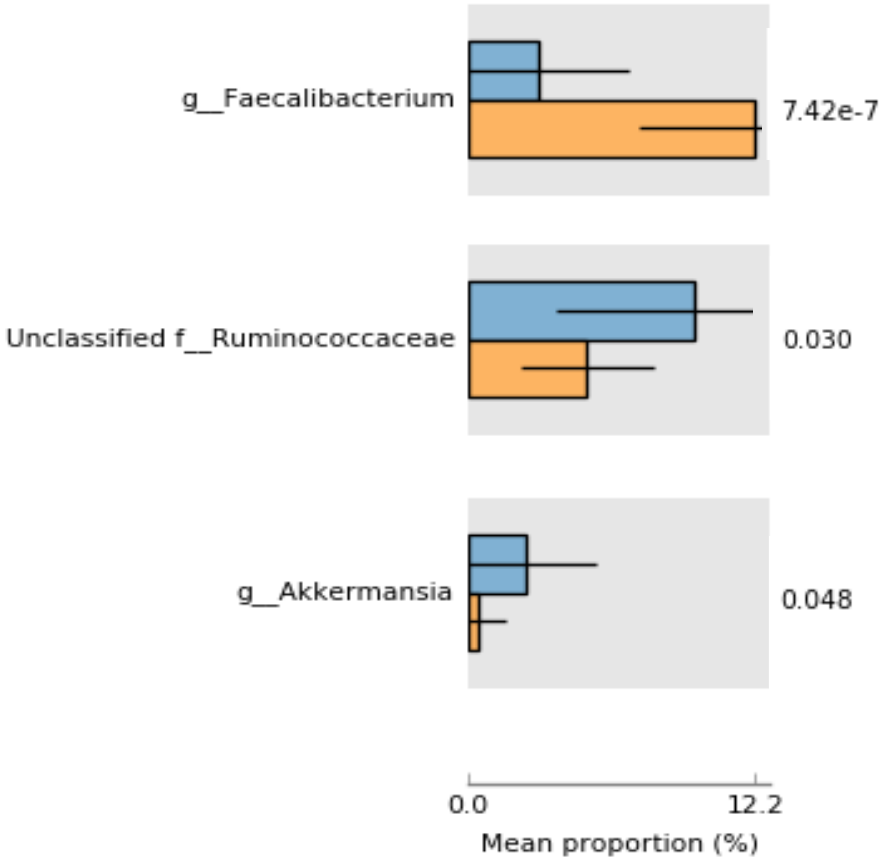
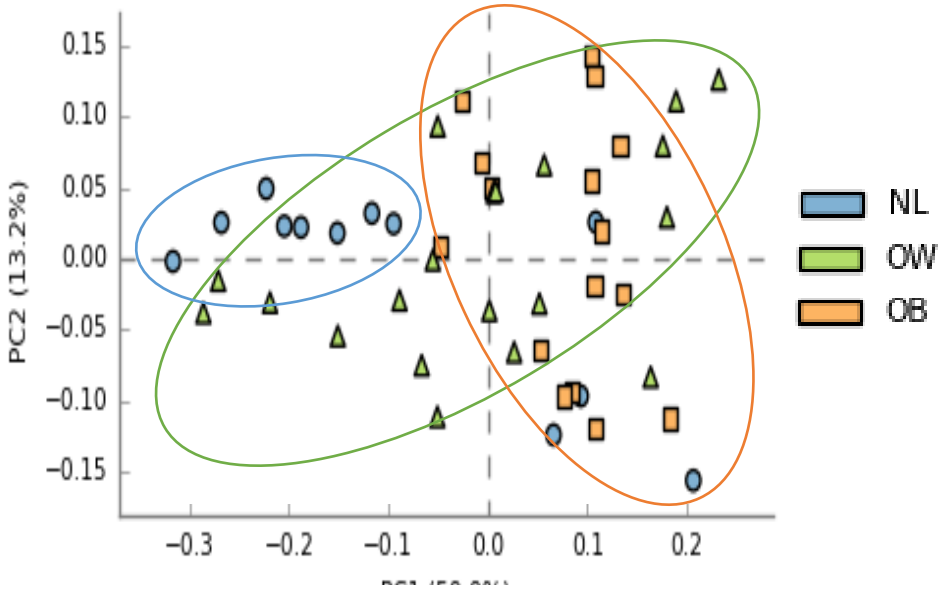


Figure 5.3 Principal component analysis (PCA) performed on the 97% OTU abundance matrix reveals A. a unique microbiota for females and males. B. There was a higher relative abundance of *Faecalibacterium* in males (FDR-corrected $p<0.001$), and *Akkermasia* and *Ruminococcaceae* in females (FDR-corrected $p=0.03$ and 0.048, respectively).

Figure 5.4 PCA plot reveals a different microbiota in lean, overweight, and obese HD patients

A.



B.

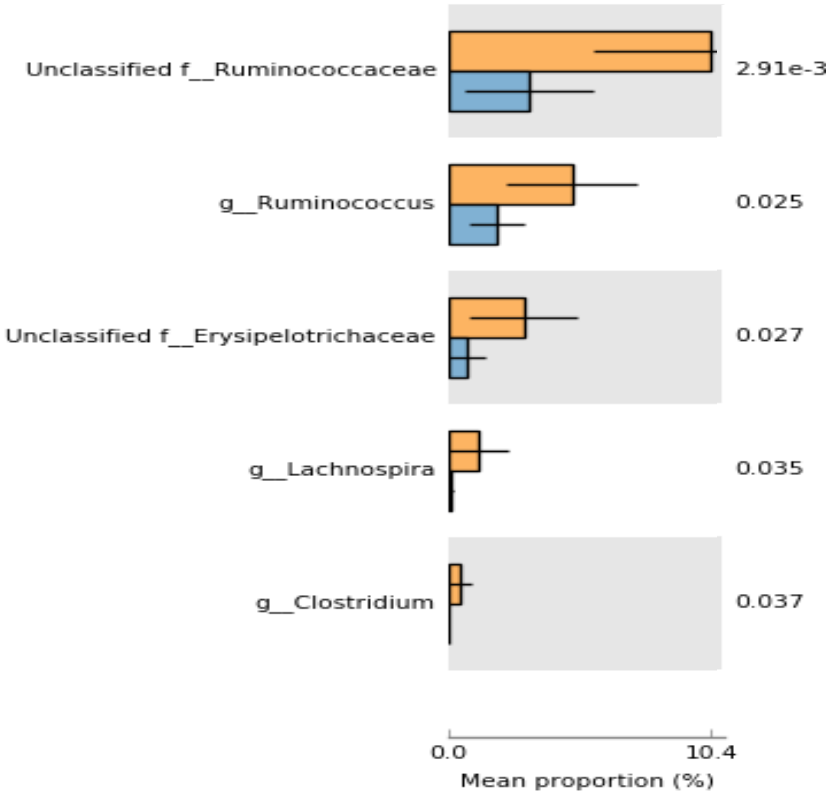


Figure 5.4 Principal component analysis (PCA) performed on the 97% OTU abundance matrix. A. There was a unique microbiota in HD patients with BMI <25kg/m^2 and ≥25kg/m^2. B. There was a higher relative abundance of *Ruminococcaceae, Ruminococcus, Erysipelotrichaceae, Lachnospira,* and *Clostridium* in HD patients with obesity (FDR-corrected p=0.0029, 0.025, 0.027, 0.035, 0.037, respectively).

Table 5.3 Fecal excretion of short-chain fatty acids and branched-chain fatty acids after 4-weeks of IN or CON consumption

	IN		CON		Group *P*	Time *P*	G x T *P*
	Pre	**Post**	**Pre**	**Post**			
Acetate (umol/g DM)	209.26±37.45	263.51±38.89	229.71±43.79	333.42±68.73	0.227	**0.032**	0.401
Propionate (umol/g DM)	75.24±15.71	80.07±14.80	65.35±11.54	99.42±21.74	0.700	**0.027**	0.198
Butyrate (umol/g DM)	39.86±9.73	51.24±11.1	42.84±10.11	66.53±13.52	0.119	0.066	0.266
Total SCFA (umol/g DM)	323.84±59.83	390.44±59.85	337.49±60.90	495.15±93.64	0.215	**0.030**	0.282
Isobutyrate (umol/g DM)	7.09±1.07	6.87±1.42	6.37±0.91	9.84±2.53	0.355	0.433	0.223
Isovalerate (umol/g DM)	10.30±1.50	9.19±1.93	7.91±1.22	13.36±3.90	0.655	0.502	0.148
Valerate (umol/g DM)	7.18±2.17	6.03±1.88	6.83±1.86	8.81±2.41	0.485	0.787	0.245
Total BCFA (umol/g DM)	24.57±4.22	22.11±4.48	21.10±3.55	32.001±8.01	0.500	0.532	0.181
Total Phenols (umol/g DM)	260.01±49.88	158.28±50.87	199.60±34.29	152.41±42.88	0.429	**0.051**	0.495
Total Indoles (umol/g DM)	192.76±20.03	176.12±45.37	259.23±40.93	230.87±44.71	0.129	0.664	0.838

GxT, group-by-time interaction; IN, inulin; CON; maltodextrin; SCFA, short-chain fatty acids; BCFA, branched-chain fatty acids

Figure 5.5 Acetate, propionate, and total SCFA increased after 4 weeks with IN and CON, but not butyrate

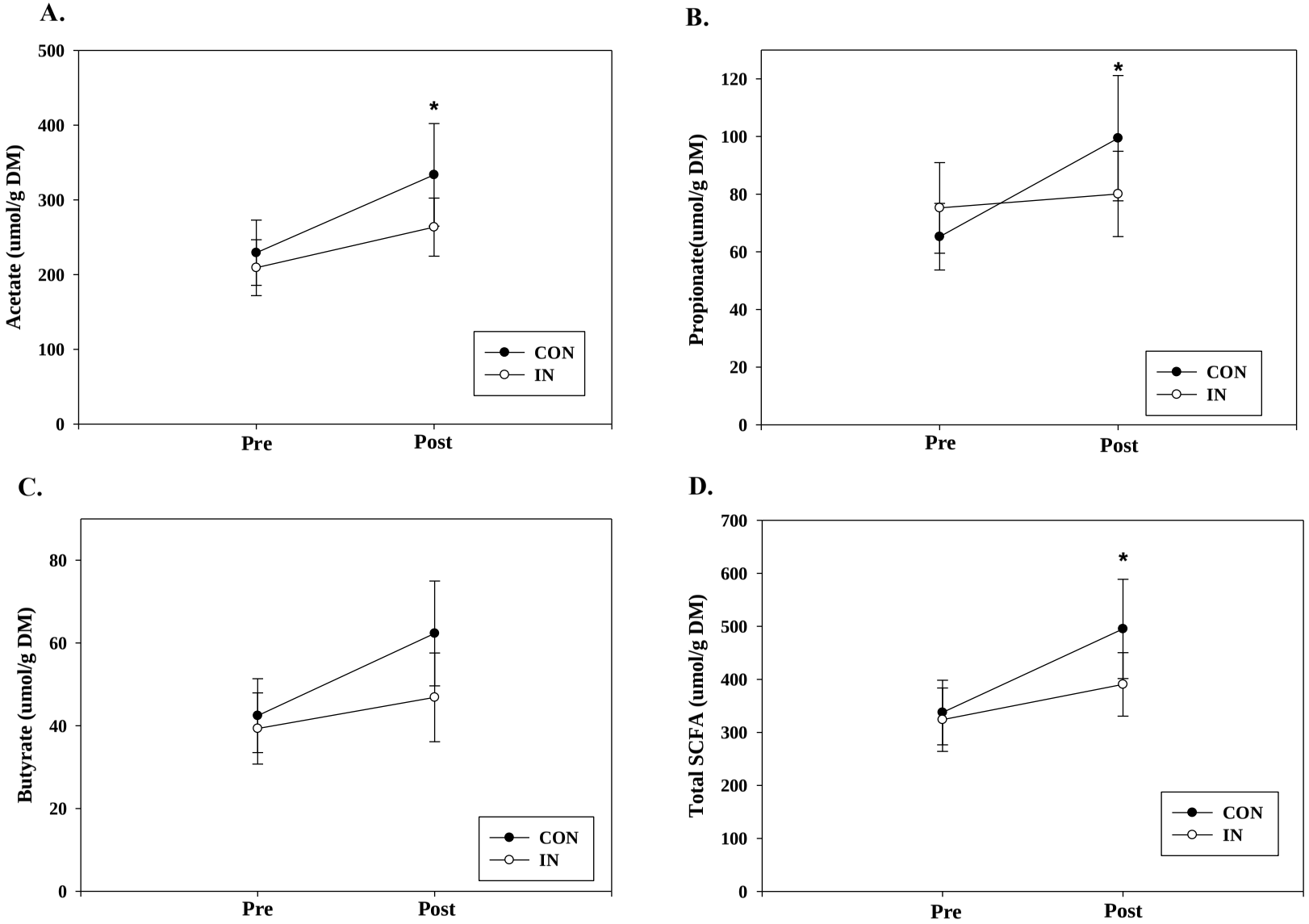


Figure 5.5 Effect of inulin (IN) and maltodextrin (CON) on the fecal excretion of (A) acetate, (B) propionate, (C), butyrate, and (D) total short-chain fatty acids (SCFA). A. There was a time effect on acetate excretion. B. There was a time effect on propionate excretion. C. There was a trend towards a time effect on butyrate excretion. D. There was a time effect on total SCFA excretion. *p time <0.05.

Figure 5.6 Short-chain fatty acids are increased after 4-week of IN and CON, but there were no differences between treatments

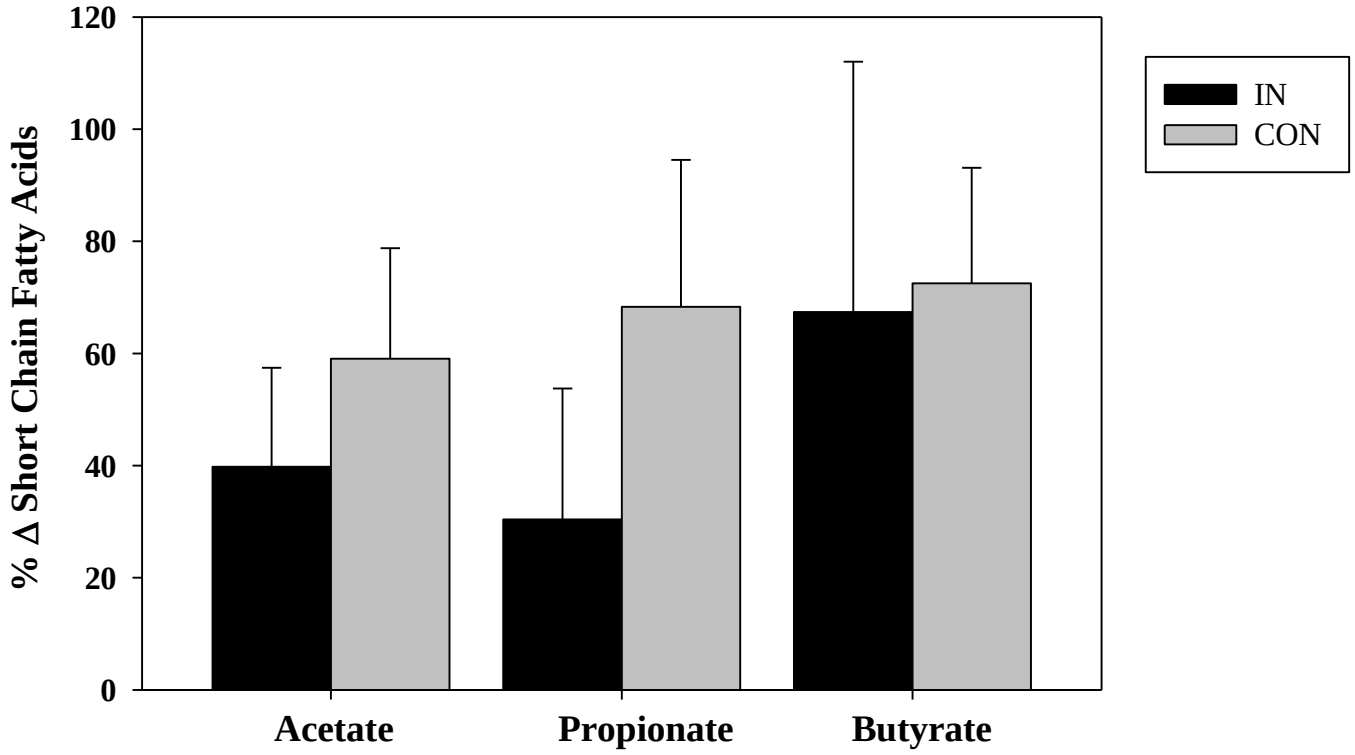
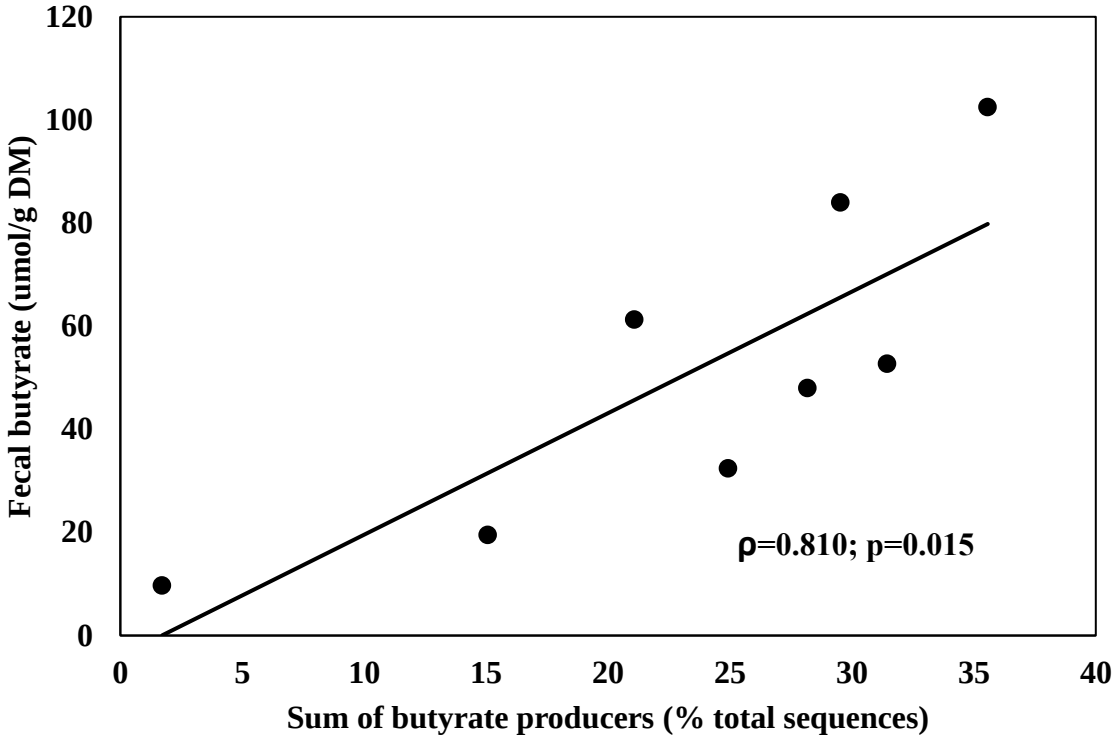


Figure 5.6 Percent change of acetate, propionate, and butyrate after a 4-week supplementation of inulin (IN) and maltodextrin (CON). All short-chain fatty acids were increased after IN and CON supplementation. After a paired t-test analysis, there was no difference between IN and CON.

Figure 5.7 There is a positive relationship between known butyrate producers and fecal butyrate excretion after IN supplementation, but not after CON

A.



B.

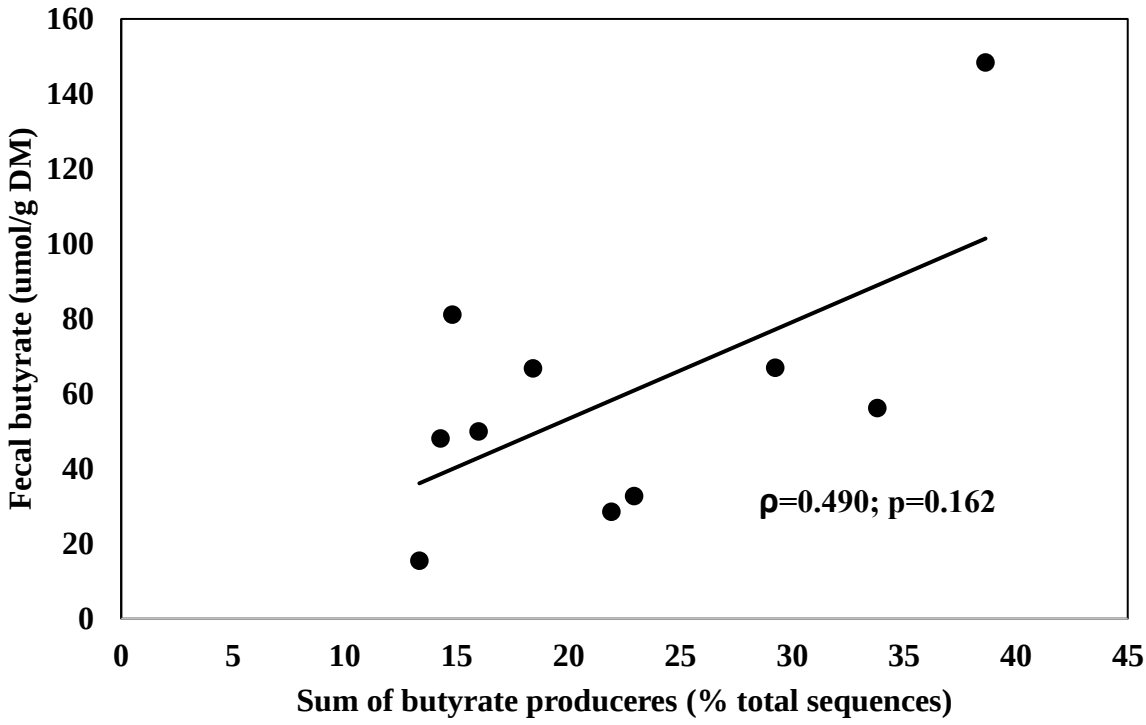
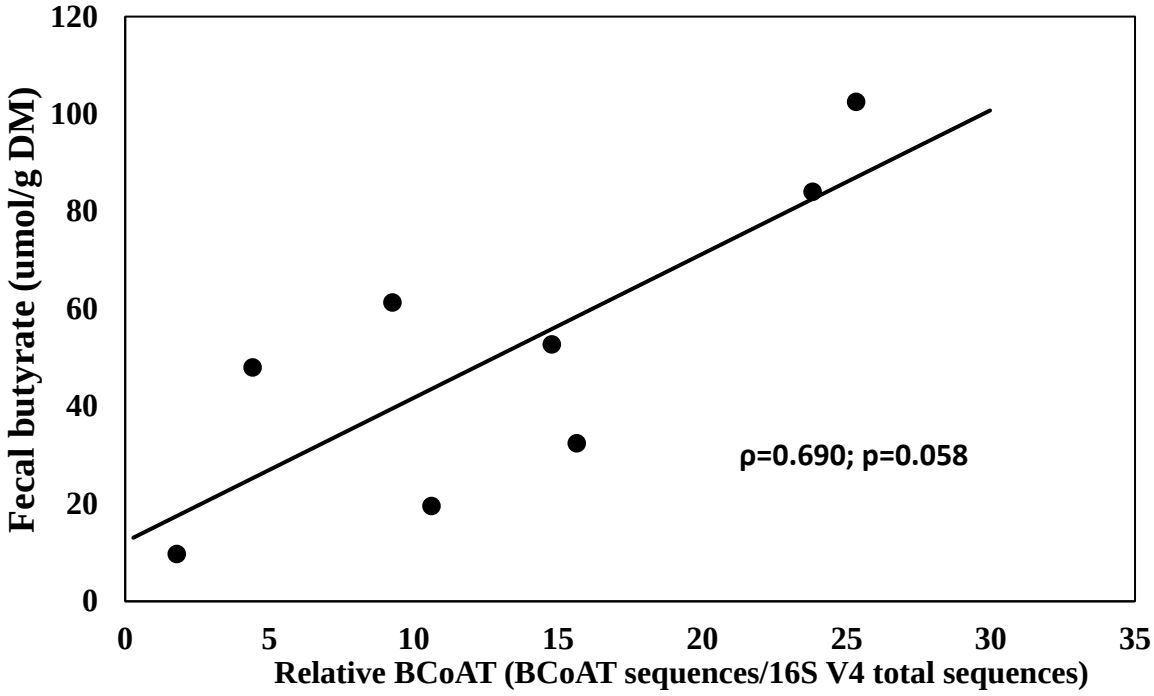


Figure 5.8 There is a positive relationship between relative abundance of BCoAT and fecal butyrate excretion after IN supplementation, but not CON

A.



B.

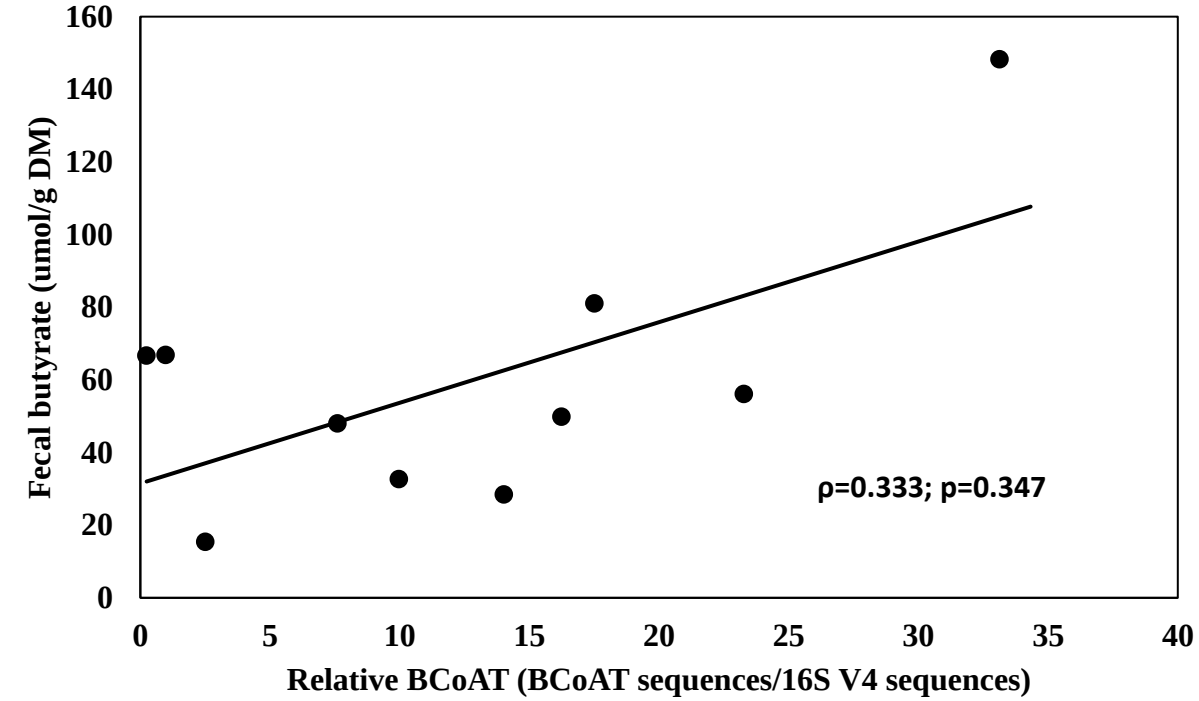


Figure 5.9 Isobutyrate, isovalerate, valerate and total BCFA do not change after 4 weeks with IN and CON

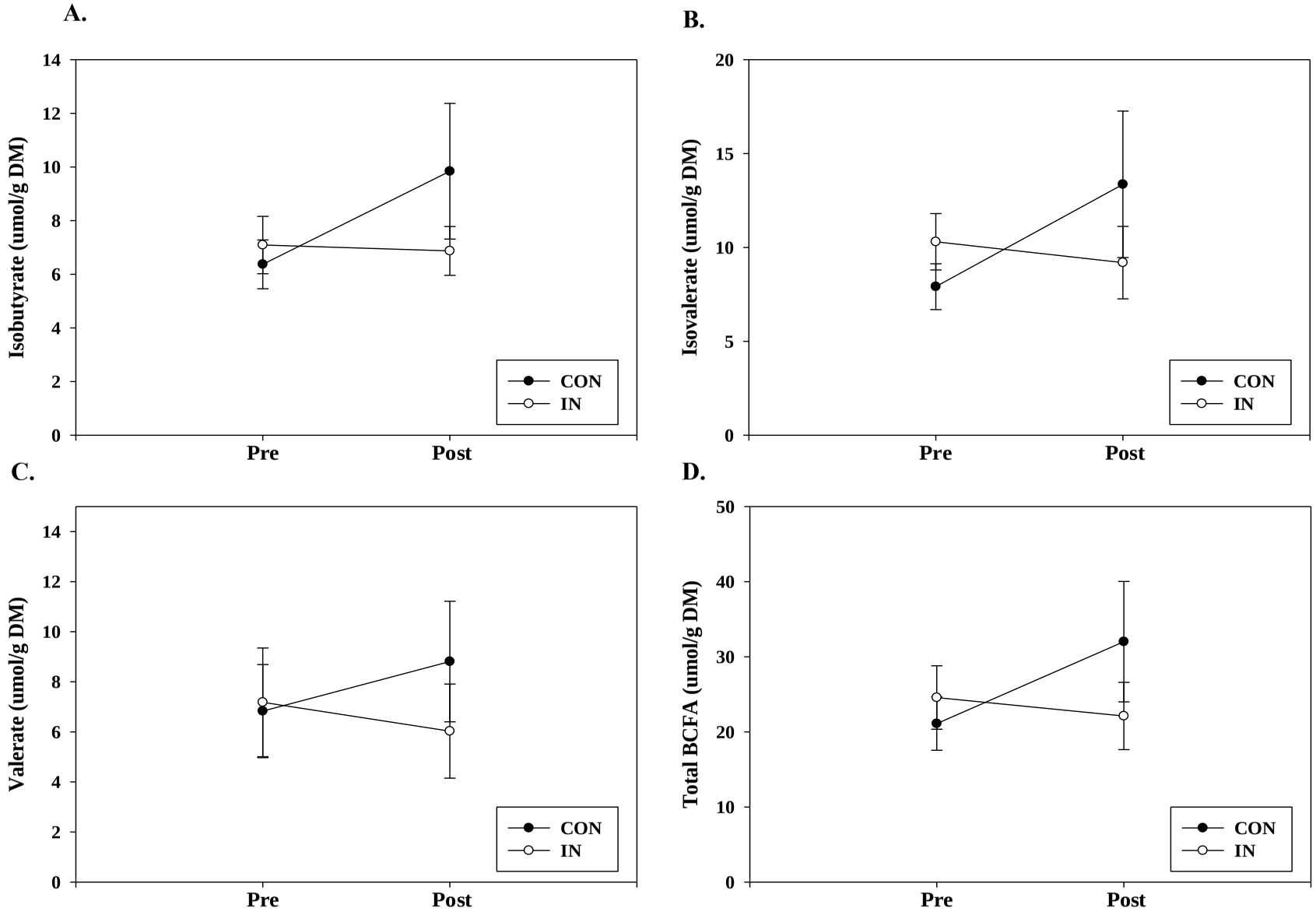


Figure 5.9 There were no effects of inulin (IN) or maltodextrin (CON) on the fecal excretion of (A) isobutyrate, (B) isovalerate, (C) valerate, and (D) total branched-chain fatty acids (BCFA).

Figure 5.10 There were no differences in the fecal excretion of BCFA after 4 weeks with IN or CON

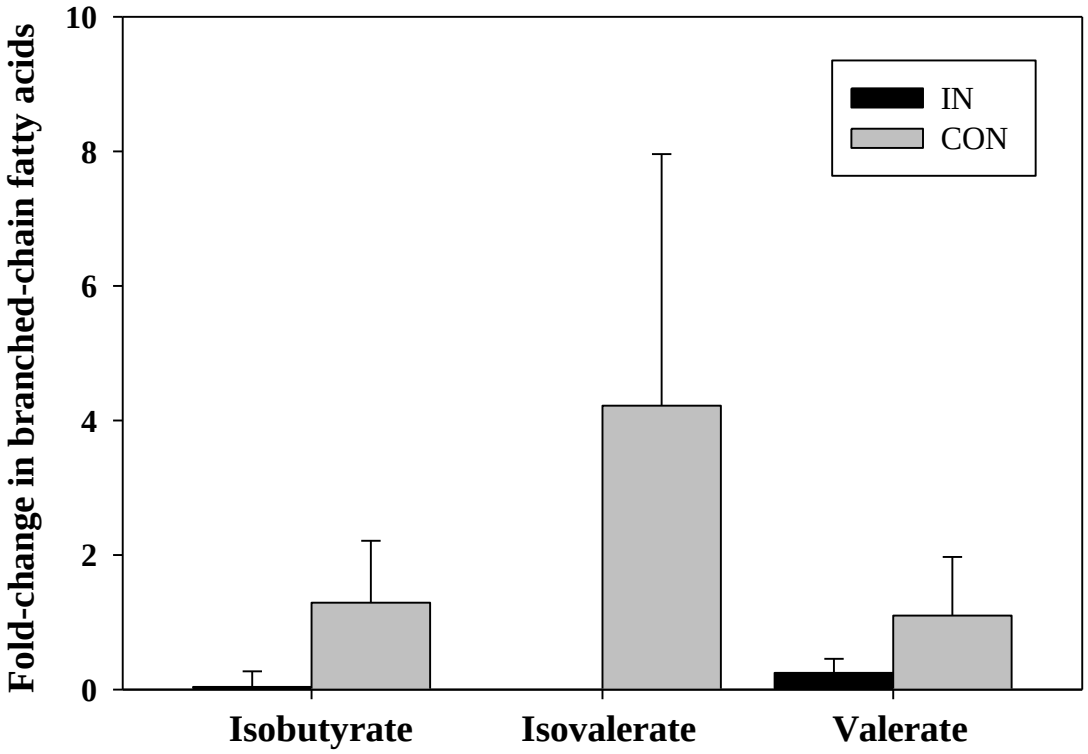


Figure 5.10 Fold change of isobutyrate, isovalerate, and valerate after a 4-week supplementation of inulin (IN) and maltodextrin (CON). After a paired t-test analysis, there was no difference between IN and CON.

Table 5.4 Plasma gut microbiota-derived metabolites after 4-weeks of IN or CON consumption

	IN		CON		Group *P*	Time *P*	G x T *P*
	Pre	**Post**	**Pre**	**Post**			
Indoxyl Sulfate (uM)	125.86±12.29	116.60±15.53	115.78±17.97	121.90±17.90	0.772	0.882	0.210
P-Cresyl Sulfate (uM)	176.57±29.95	176.00±38.37	185.18±33.25	162.63±31.28	0.765	0.427	0.395
TMAO (uM)	66.74±8.29	62.34±8.07	63.72±9.99	71.86±11.10	0.683	0.827	0.284
Phenylacetylglutamine (uM)	155.80±25.63	152.24±33.15	137.05±21.89	142.34±26.97	0.307	0.960	0.681

IN, inulin; CON; maltodextrin; TMAO, trimethylamine N-Oxide.

Figure 5.11 Change in microbial-derived plasma metabolites after 4 weeks of IN or CON

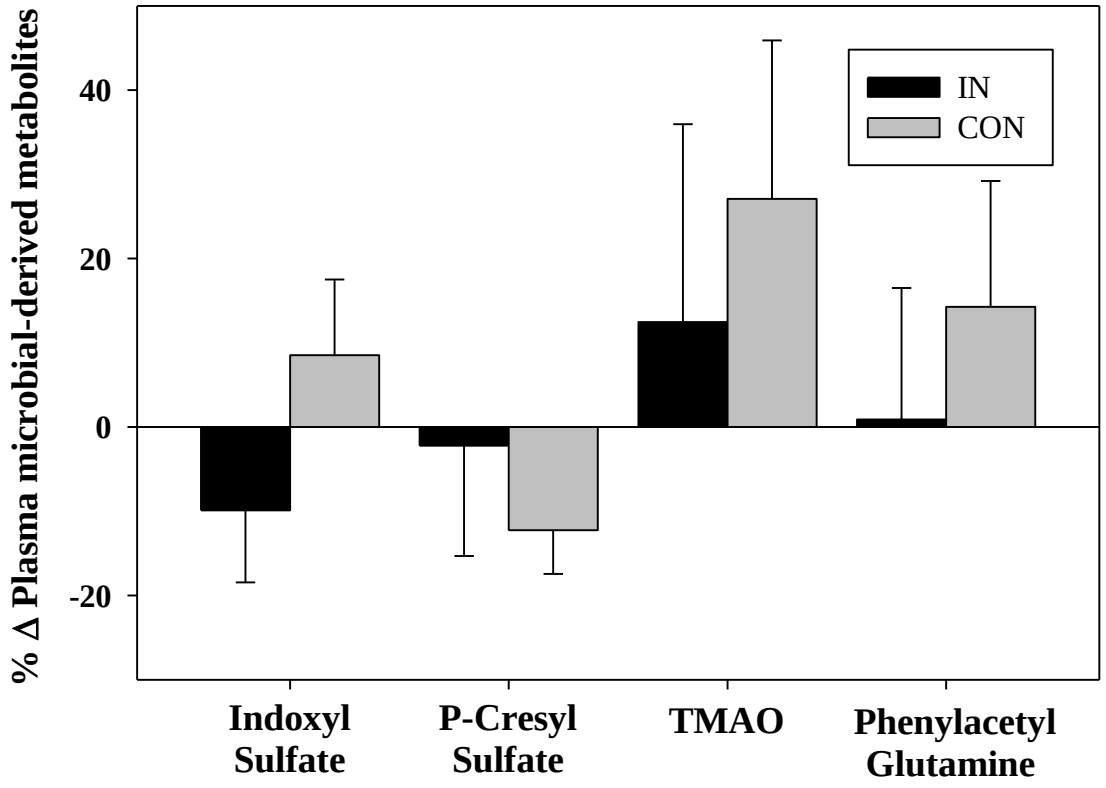
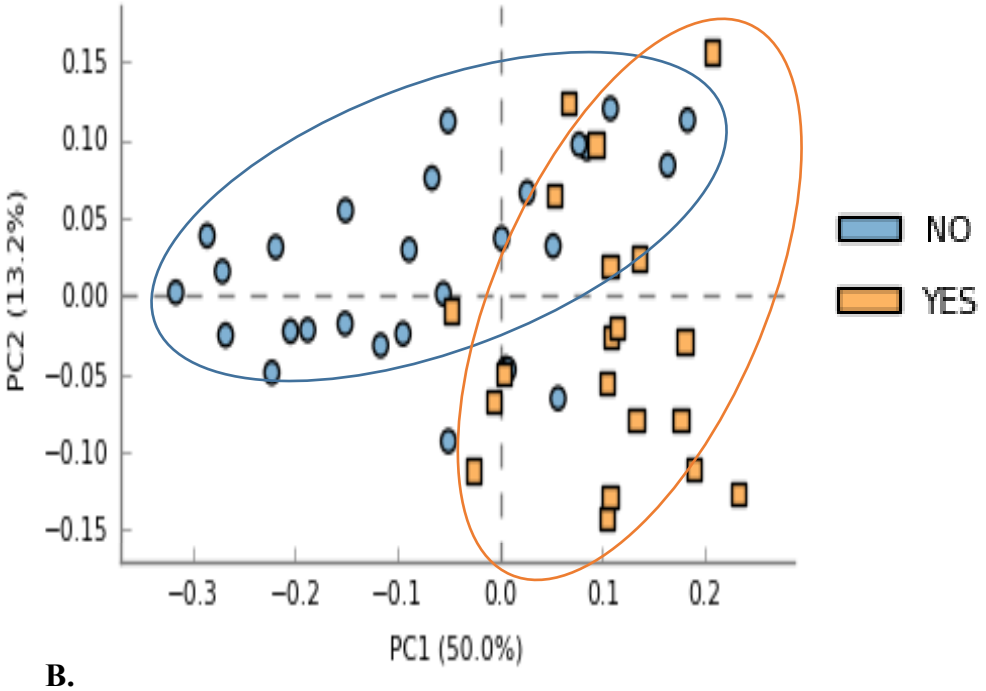


Figure 5.11 Percent change of indoxyl sulfate, p-cresyl sulfate, trimethyl amine N-oxide (TMAO), and phenylacetylglutamine after a 4-week supplementation of inulin (IN) and maltodextrin (CON). After a paired t-test analysis, there was no difference between IN and CON.

Figure 5.12 PCA plot reveals an effect of non-calcium-based phosphate binders on the gut microbiota of HD patients

A.



B.

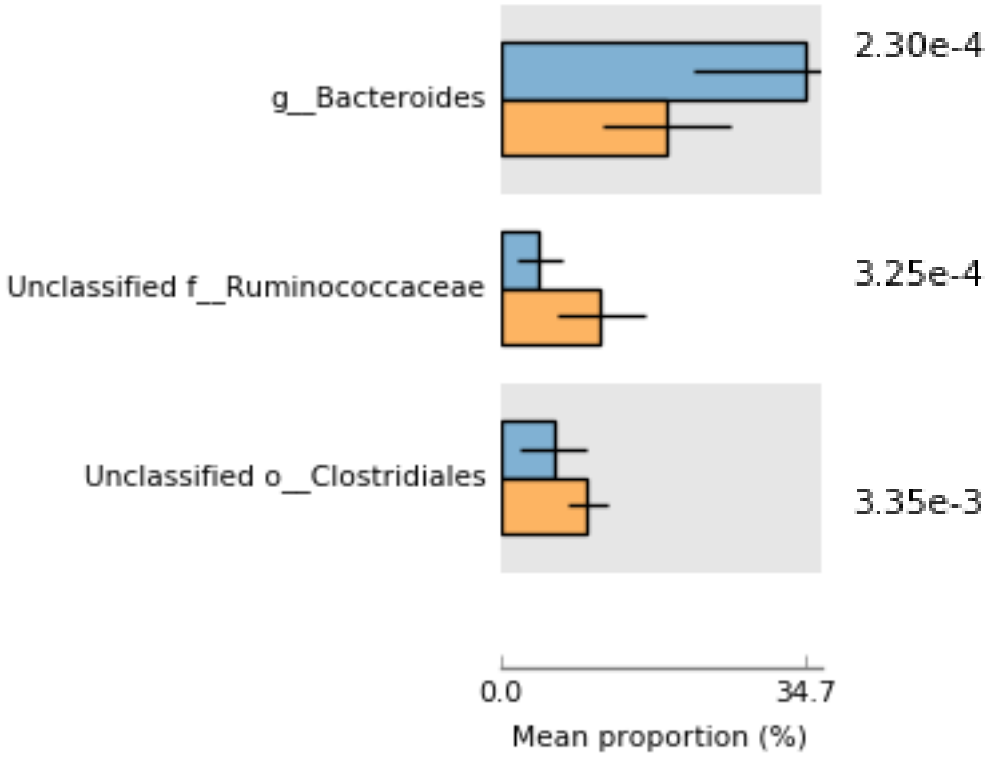


Figure 5.12 Principal component analysis (PCA) performed on the 97% OTU abundance matrix. A. There was a unique microbiota in HD patients that were prescribed non-calcium-based phosphate binders (e.g., sevelamer hydrochloride and sevelamer carbonate). B. There was a lower relative abundance of *Bacteroides* in those prescribed non-calcium-based phosphate binders (FDR-corrected p=0.00023) and a higher relative abundance of *Ruminococcaceae* and *Clostridiales* (FDR-corrected p=0.00032 and 0.00335, respectively).

CHAPTER 6

FUTURE DIRECTIONS

After our randomized, placebo-controlled, cross-over study, contrary to our hypotheses inulin supplementation in hemodialysis (HD) patients did not result in changes in biomarkers of mineral and bone metabolism, fecal excretion of minerals, or the gut microbiota. However, several factors may have affected our ability to observe the effects we hypothesized. First, the variability in the mineral and bone biomarkers was significantly more than what we expected and, therefore, our sample size may have been limited to observe any significant effects. For example, the effect size for blood calcium after inulin and maltodextrin was 0.016. After doing a post hoc power calculation, we would have needed 255 patients. Furthermore, our subjects were prescribed several medications (e.g., phosphate binders, calcimimetics, vitamin D analogs) that affect the mineral and bone metabolism biomarkers of interest. Therefore, the application of a similar approach in earlier stages of chronic kidney disease (CKD) may be more suitable since this patients may not be subject to the prescription of most of the medications aforementioned. Additionally, in earlier stages of CKD, inulin-type fructans may serve as a preventive therapy, rather than a treatment. Finally, as shown in Chapter 4, there have been several animal studies assessing the effects of inulin-type fructans on mineral absorption and mineral and bone metabolism biomarkers. However, to date, there have not been any studies on murine models of CKD, which represents an area of opportunity for future studies.

Regarding the gut microbiome, we observed that demographic variables, such as sex, body mass index (BMI), and a diagnosis of diabetes were determinants of the gut microbiota structure. Additionally, we observed a different microbiota structure in those subjects that were prescribed non-calcium-based phosphate binders. This is particularly interesting because the

most widely used non-calcium-based phosphate binder (e.g., sevelamer hydrochloride/carbonate) may bind to other compounds in addition to phosphate, which may include uremic toxins produced by the colonic bacteria. Therefore, future studies should aim at examining the effects of these non-calcium-based phosphate binders on the gut microbiome structure and function.

Finally, we decided to supplement fermentable fiber in an amount that represents about 40% of the adequate intake (AI) of fiber for females and males. This was due to several studies reporting a low dietary fiber intake in hemodialysis patients,[8,20] as well as previous studies performed in our laboratory (unpublished data). However, we did not recommend any changes in dietary intake or dietary patterns. Dietary patterns have shown to affect the gut microbiome, where subjects that consume a plant-based diet tend to have a different gut microbiota structure than those consuming an animal-based diet.[180] HD patients have a very particular diet, in which they are recommended to increase their energy and protein intake, while limiting the consumption of sodium, potassium, and phosphorus. With these recommendations, patients may be led to consume low amounts of fruits, vegetables, whole grains, legumes, and nuts.[211] These food groups, however, are good sources of dietary fiber. Nevertheless, the effect of current dietary patterns and a more liberalized diet in which patients may be able to consume more of the traditionally-restricted food groups and the effect on the gut microbiome and mineral metabolism has not been studied and should be explored in future studies.

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