METABOLIC IMPLICATIONS OF FIBER CONSUMPTION IN CHILDREN

BY

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B.S. KANSAS STATE UNIVERSITY, 2007
M.S., KANSAS STATE UNIVERSITY, 2009

AN ABSTRACT OF A DISSERTATION

SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE

DOCTOR OF PHILOSOPHY

DEPARTMENT OF HUMAN NUTRITION
COLLEGE OF HUMAN ECOLOGY

KANSAS STATE UNIVERSITY
MANHATTAN, KANSAS

2013

Abstract

Little is known about the impact of dietary fiber (DF) on children. Current recommendations are based on extrapolations from adult studies. Research is needed to provide science based evidence to determine how DF impacts the gut of children. Two studies were conducted to investigate the interactions of DF in the child large intestine. In the first study, the dose response of DF on breath hydrogen, methane, and total hydrogen content was investigated relative to Institute of Medicine (IOM) recommended intakes in free-living preschool children. Only four of the 18 participants were able to comply with the treatment protocol. Although, no significant differences were noted in breath measures of fermentation across fiber consumption levels, there was a numerical pattern for increasing levels of DF to evolved increased gas production in the four compliers. In free-living individuals measures of acute fiber fermentation through breath was not sensitive enough over 6 hours to distinguish a difference in fermentative rate. Children, parents, and child care centers found this approach apparently acceptable. In study two, the impact of DF (10 g) fed over three weeks in children and their parents on metabolic markers of fermentation were evaluated. The body was able to adapt to 10 g/day DF consumption as bloating ($p < 0.05$) and flatulence ($p = 0.06$) decreased each week of the study. Fecal propionic acid was significantly increased over three weeks of DF supplementation. There was also an interaction ($p=0.05$) between time and age for butyric acid. Dietary fiber supplementation (10 g/day) over three weeks via a commercially available extruded cereal was well-tolerated by the participants, with no disturbances in bowel habit in children or adults. Alternatively, there were no improved bowel habit measures with increased DF consumption. This study provides evidence that this tolerable dose of DF supplementation over three weeks had similar impacts in free-living children and adults. However, the presence of increased butyric acid only in children may be reflective of different production or absorptive capacities between children and adults. Although not presented here, the bacterial ecological analysis may shed further insight into the interactions occurring in the large intestine. These are the first studies to my knowledge to have investigated these outcomes in young children. In addition to the gut health outcomes, this research provided a framework into the apparent feasibility of studying children in a gentle, non-invasive, and cost-effective manner.

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A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Human Nutrition
College of Human Ecology

KANSAS STATE UNIVERSITY
Manhattan, Kansas

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Approved by:

Major Professor
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Acknowledgements

Completing my Doctoral Dissertation has been one of the most challenging and rewarding experiences in my educational career! As Isaac Newton said, "If I have seen further it is because I have stood on the shoulders of giants." Behind every successful person there is likely a cast and crew of successful, passionate, and devoted individuals. My case is no different, and in the interest of brevity, I will to attempt to outline all those who have entrusted me to push forward the field of science.

First and foremost, I must thank my major professor, Dr. Mark Haub. Dr. Haub has been a great mentor, patient teacher, and outstanding person, which has permanently molded me for the better. Regardless of my unintended and congenital bias, he has always directed my attention to the evidence leading my quest for, well……more questions (although sometimes I wish I had all the answers and was right!). Unadulterated evidence is truly a "quest" of its own and critical to furthering knowledge and humanity. I need to thank Dr. Haub for innately upholding the Mission of Human Ecology and caring about people first. I have had this luxury bestowed upon me and further witnessed the good it can inspire, despite potential attention to the contrary. Although, this should go without saying, I owe as much to Michelle, Trae, and Trewman for sharing their time and Dr. Haub. A portion of their life has been instrumental in me continuing mine!

Next, I would like to thank my committee Dr. Ric Rosenkranz, Dr. David Dzewaltowski, and Dr. Sandra Procter. They have been instrumental in my success. Long before they were on my committee, they were influencing me. Dr. Ric Rosenkranz, thank you for putting up with me first! Indeed, you were the reason I continued graduate school and pursued a Ph.D. I believe everything happens for a reason, and even though you were not the hand that "fed" me the whole time, your insight, passion, and attention to detail have inspired me to continually improve in all of those areas. Dr. Dzewaltowski, thank you for creating the environment known as the YHBRL and giving me a place to start developing my career. That lab has given me many invaluable friendships and perspectives. Thanks for an intuitive and realistic approach to understanding research, and getting to the nuts and bolts of a study. Dr. Procter, thank you for bringing innate passion for what you do to research, relationships and the department. The passion and eternal

positive spirit has been wonderful to be around. To each of my committee members, thank you to your families who have welcomed me, mentored me, or brought me back to my roots, but also sacrifice a part of their life to improve mine.

Dr. Brian Lindshield – Thank you for your constructive criticism, open door, and devoted example to measured improvement. I can't recall a time when you have been impatient or not calm. You allowed me to teach, become a better teacher, and always focus on the students and improvement. I am not sure that I could have improved and followed through with many things without that mile-wide open door!

Dr. Tona Melgarejo – Thank you for breaking me into my Ph.D. the hard way. I always struggled with your class and wondered what I had gotten into. I think your "get-to-the-point" or the "so-what," both in class and professionally has silently branded me for the better.

Dr. Koushik Adhikari – Thank you for your time, patience, and humor. There is never a moment I have spent with you that is not enjoyable, even when you probably wonder how I made it this far.

Dr. Bronwyn Fees – Thank you for your passion for children and research. Working with you truly made this a natural transition in collaborating with Stone House during my research and working with children in general. In essence, Stone House provided me the opportunity to complete this work. I am very thankful to have had your support.

Janet, Angie and Pam – Collectively, you have patiently taken care of any issues that I have had and always followed through with sincere concern both personally and professionally. Thank you, as you have truly made my time at Kansas State better.

Kate Osei Boadi – You get the only single shout-out from all the graduate students, but your optimism, light heartedness and all the time you spent putting up with me will not be forgotten. Even though I may forget things, you have always been an open and good friend. If I have a regret from graduate school, it is that I never created time for some fried plantains.

Stone House – Stone House was where I spent many days and hours working, playing, and getting to know the staff, children and their families. I was truly fortunate to be able to research in there auspices. Along with Stone House, I have to thank the many facilities in the Manhattan area that afforded me the opportunity to work with them, including: Kindercare, Cultural Academy, Flint Hills Christian School, Bebe Language Academy, and the many others that took the time and effort.

Fellow Graduate Students and lab workers– Thank you, there are too many to go through, and I could write paragraphs about each: Erika, Andi, Christie, Panat, Sara, Soyoung, David (Jianteng), Julie, Tara, Brooke, Andrew, Dr. Lattimer, Alex, Audrey, Ashley, Joe, Kavitha, Dr. Hanson, Dr. Case, Dr. Snyder, Carrie, Jing, Michelle, Dave, J.P., Greg, Kelcie, Brad, Javier, Kelsey, Britany, and many others. Although we never had a formal association and even fewer gatherings, the grad students of this department have been exceptionally altruistic. I don't know if it was a lucky period of time, but I hope that it is a reflection of the wonderful department that brought them in.

Remaining Faculty – I can't think of a single faculty who I have not had a truly personal connection with. Thank you, Dr. Wang, Dr. Kidd, Dr. Higgins, Dr. Medeiros, Dr. Jordan, Dr. Sara Rosenkranz, Dr. Lin, Dr. Edgar Chambers, Dr. Delores Chambers, Dr. Grunewald, John DiCicco, and Diane Challacombe.

Information Technology – Thanks to Marin, Austin, Jordan, Spencer, and the team that provided help in all the technical difficulties and random questions.

There is always a risk of starting a list, and I really hope that I did not forget anyone, but that it was also important enough to take the time and transcribe those moments and people that knowingly and unknowingly have contributed to my success.

Dedication

This Dissertation is dedicated to all those who made this possible and sacrificed to allow me to pursue my Ph.D., my real heroes!!! Thank you to my parents and grandparents (past and present) as they have always been there and made it clear I would go to college (They just didn't know I would stay so long).

Most importantly, this Dedication is to my "new" family, who has sacrificed the most in terms of time and attention. My wife Melissa, who has stood with me for five years and just over two years ago decided she was crazy enough to put up with for the rest of our lives. I know you didn't sign up to live apart for two years, or to only see me on the weekends. I know when school wasn't demanding my attention on the weekends, our 56 hours were never enough and we wished sleep was optional. You never let it show when you sacrificed your weekends with me to see family, whom we had already neglected. I know that this hasn't been easy and you have felt helpless at times, but you have made it possible to see in me what I don't see myself! You have ensured that I not only know what I am capable of, but that I bring that vision to life. All that you have given up for me with the intangibles of time and love to ensure that I am prepared to make a difference in the community. To Eva Clair, who I can't wait to meet, you have been an extra inspiration and a burst of energy for me! Finally, Jet (yes, a dog and he will never hear this), all I can say is you know the difference between a duffle bag and a book bag, I think you are ready for me to be done too.

Preface

This manuscript was written according to the style guidelines of the intended site of journal submission. Journal selection was chosen based on content of each paper and the intended audience, ensured to meet journal coverage criteria. **Chapter 1** is a general introduction, including the overall research objectives and statement of hypotheses. This will be referenced according to the general format of APA. **Chapter 2** is a review of literature pertaining to dietary fiber definition, relationship to children, and metabolic interactions regarding the large intestine. **Chapter 3** is an investigation into the effects and feasibility of dietary fiber intake by children on fermentation in the large intestine, via a non-invasive and gentle methodology. **Chapter 4** is a continuation on Chapter 3, investigating the effects of dietary fiber on fundamental metabolic mechanisms of fermentation in free-living children and their parents, using non-invasive and gentle methodology, with regard to each individual's DF recommendations. **Chapter 5** is a further continuation of Chapter 4 investigating the relationship of the colonic microflora with respect to age, dietary fiber consumption, and non-invasive, gentle metabolic markers of fermentation in free-living individuals. **Chapter 6** draws general conclusions regarding dietary fiber intake in children as assessed by these investigations and where future research should focus resources.

Chapter 1 - General Introduction

Dietary fiber (DF) is a complex molecule that has been described as "unrealistic" (Van Soest, 1994) to define, at least with current methodology and approaches. Fiber has been defined (not measured) traditionally by its presence in plant tissues, which are then consumed (Burkitt, 1974; Hipsley, 1953; Trowell & Burkitt, 1986). More recently, the role of fiber has diverged (Lattimer & Haub, 2010) into both a physiological and a chemical component. This is reflected by the Food and Nutrition Board (FNB) 2002 definition of fiber. The first component DF, is defined as: "Dietary fiber which consists of isolated, non-digestible carbohydrates and lignin that are intrinsic and intact in plants." The second component is composed of functional fiber (FF), which has been defined as: "Functional fiber consists of isolated, non-digestible carbohydrates that have beneficial physiological effects in humans." Total fiber is the sum of DF and FF (IOM, 2002b). Having a two component model recognizes the importance of traditional diets in DF and their health associations, as well as recognizing the interactions that conventionally have been inferred to fiber, and applies to a wider variety of gut active compounds.

Dietary fiber is not well defined and controversy exists in not only which, but how much of each type of fiber should be recommended (Gordon & Goda, 2008). Fiber is only measured by the AOAC International approved methods and thus, fiber content is dependent on those methods (Lupton, 2008). Current Institute of Medicine (IOM) recommendations for both children and adults suggest 14 g/1000 kcal per day (IOM, 2002a). The amount for children and adults is the same relative density. However, this can result in rather large physiological doses in small children. The amount for children and adults is the same relative density. However, this can result in large physiological doses in small children. Current IOM recommendations for children 1-3 years old are 19 g/day as an adequate intake. Reviews are still not conclusive with strong evidence on the relationships due to the types of studies conducted (ADA, 2008a). More evidence contributing to specific contextualization of DF would likely eliminate some of the ambiguous variance with these results and reinforces the need for more research to identify the underlying mechanisms involved with DF consumption.

Increased attention is aimed towards children (IOM, 2011) in order to provide a healthy base, intending to prevent associated problems as children are following the same path as their

adult counterparts (Ogden, Carroll, Curtin, Lamb, & Flegal, 2010). Fiber has been identified to prevent and treat obesity in adults (ADA, 2008c), as well as youth (ADA, 2008b; Gropper & Acosta, 1987; Kimm, 1995). Evidence suggests that children are not consuming enough fiber, both in the United States (ADA, 2008c; S. Kranz, 2006) and around the world (Glackin, Faser, & Neill, 2008; Lee, Ip, Chan, Lui, & Young, 2008) according to current recommendations. This finding is not surprising as most adults are not consuming enough fiber either, both in the US (ADA, 2008c) and elsewhere (Lang & Jebb, 2003; Pohjanheimo, Luomala, & Tahvonen, 2010). Integrating fiber into the diet is recommended for improved overall dietary quality (IOM, 2002b); however it is also recognized that empirical and mechanistic research regarding implications of its consumption are lacking, especially in children (Aggett et al., 2003; Sibylle Kranz, Brauchla, Slavin, & Miller, 2012). The lack of evidence is not new (Dwyer, 1995). Addressing the importance of non-digestible carbohydrates in the diets of young children has been discussed (Aggett, et al., 2003) and currently others have noticed the extrapolation of data from adults to children (Sibylle Kranz, et al., 2012). Common perception is that fiber is necessary for dietary quality and thus, good health. This inference has been noted in the literature (Munoz, Krebs-Smith, Ballard-Barbash, & Cleveland, 1997) that children "should progress to this kind of pattern," (USDA, 1995) as well. However, this is currently a moving target that is not well defined and ambiguity will continue to plague deeper inferential understanding.

Findings in adults show they are not consuming enough fiber, which may be contributing to many conditions, such as: type II diabetes (Ventura et al., 2009), weight status (Cheng et al., 2009), cholesterol metabolism (Queenan et al., 2007), colonic health (Burkitt, 1974), cancer (ADA, 2008c; Rose, Demeo, Keshavarzian, & Hamaker, 2007), cardiovascular disease (Mozaffarian et al., 2003), blood pressure (Burke et al., 2001), and energy intake (Flogan & W, 2010). Children have been hit with similar maladies that only adults were traditionally known to acquire. Two disease of increasing concern in children are obesity (Kimm, 1995)and Type II Diabetes (Fagot-Campagna, 2001). Thus, there has been increasing attention at solving these emerging burdens on health starting in young children all around the world (Gortmaker et al., 2011).

The complexity of the challenges that face DF consumption lend credence to the necessity of further metabolic research to root out mechanistic mechanism that may be occurring to contextualize noted associations. Until recently, studying *in-situ* mechanisms has not been

feasible or unethical to complete. One of the largest metabolic organs in our body was not recognized as such until recently, the large intestine. The role of the large intestine was one solely of water and salt absorption. The term "microbiome" has been coined (Mullard, 2008) to describe the vast interactions that occur between the host, the microflora, and the possible genomic interactions. The vast microflora that inhabit the large intestine offer a major "middle man" to many of the previously observed roles of DF. It is logical to believe that most relationships regarding fiber are conceivably measurable interactions with the microflora and their metabolism of DF (Harris & Kris-Etherton, 2010; Nilsson, Ostman, Preston, & Bjorck, 2008). Understanding the relationships currently known and the interactions of the colon, along with its microscopic host, may provide keys to understanding the "black box" of fiber.

Thus, in order to provide scientifically sound and responsible advice involving the consumption of DF in children, it is likely that the gut and all it entails must be a central component in further analysis. The overall objective of this research was to discover a gap in knowledge and use scientific methods to provide evidence to narrow this gap. In reviewing the literature, it is clear that there is a large gap in basic knowledge of the nutritional component DF and its metabolic consequences, especially those concerning children. Moving forward it is important that we understand basic mechanisms that may drive interactions of DF in a measureable, standardized way. Thus, it was important to study "free-living individuals," in reference to current dietary guidelines. In order to ethically study humans, especially children, non-invasive techniques are central. Therefore, the overall goals with this research were to incorporate existing non-invasive, gentle, and user friendly methods in an applied manner in children, while investigating mechanistic metabolic interactions of DF consumption with regard to the DRI's. Collectively, a basic understanding of DF interactions in the body could have major implications for the future public health.

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Chapter 2 - Considerations in Developing Fiber Recommendations in Children: Implications for Health and Science

Abstract

Given its implications on health, dietary fiber intake is a topic of major interest in nutritional research. Currently, recommendations in children are reflective of and extrapolated from adult data and evidence is lacking to illustrate health outcomes in children. In addition to better understanding the health outcomes of dietary fiber in children, consensus regarding the definitions of fiber is needed as the use of differing definitions creates a challenge regarding how health outcomes are assessed. Furthermore, sound definitions are critical for establishing approved methodologies and to accurately update nutrient databases to significantly enhance our knowledge of fiber's influence on life-long health. While evidence is scarce in children regarding the impacts of fiber on health, there is reason to believe that postulated mechanisms in adults are justified in children. More information regarding fiber's role in glucose and lipid metabolism is necessary, and should be extended to healthy children versus studies in non-healthy populations. Gut bacteria may provide valuable insight as disease indicators or inherent genetic differences that cause diseases, which will offer new prevention and treatment options. The collective genome of the microbiota and the host together create a significant complexity in the potential health effects that can occur. Fiber is considered a principal source of fuel for gut microbiota and thus may drive several health outcomes, through microbial adaption. This is an intriguing concept, but caution must be taken as it is difficult to determine the meaning of new associations. To better understand the role of gut ecology on whole-body health, more research needs to be conducted that investigates mechanistic/causative factors. Elucidating why an association is occurring is a difficult task, but is important if we are to fully understand the necessity of dietary fiber and its specific recommendations for children and their subsequent health status.

Introduction

The prevalence of overweight and obesity in children is a major public health concern (1, 2). In fact, there is great concern over implementing and resolving what scientist have coined, "The Obesity Epidemic," both in the United States and the world abroad for all ages (3, 4). Many researchers have suggested dietary fiber (DF) plays a protective role against obesity in children (5-9). In addition to fighting adult-onset obesity, fiber consumption has been exhorted to benefit other conditions such as: cardiovascular disease (CVD), type II diabetes, cancer (breast and large bowel), constipation, and colon diseases (10). Thus, fiber supplements (8) and functional food supply (11) are also becoming more common in today's food market to help consumers increase their intakes of DF.

Campaigns and recommendations to increase fiber intake for a health benefit are not new and have been around for some time (12, 13). In an upstream approach, children are increasingly becoming the target population of health promotion campaigns as nutrition and food quality is important for proper development (14). Evidence suggests that the proper nutrition from the pre-natal stages into weaning may play a larger role than previously thought in providing an "ideal" base (15). Thus, nutrition during these early years of development has been considered important for a foundation of adult health and a life-course approach should be considered (16, 17). Therefore, it is important to have sound scientific evidence for dietary guidelines for adults and children.

Current recommendations of DF intake in the United States for children older than one is set at 14g/1,000 Kcal, which is considered an adequate intake (AI) (18). However, scientific evidence pertaining to DF intake from children as young as two is scarce and inconsistent, with most data being recorded from adult studies (14). Common perception is that fiber is necessary for dietary quality and thus, good health and children should progress to this kind of pattern (19). It is understood that evidence investigating DF in children is lacking (20, 21), and attempts have been made to try address the paucity of data (22).

Addressing the importance of non-digestible carbohydrates in the diets of young children has been discussed (23) and currently others have noticed the extrapolation of data from adults to children (24). However, the research discriminating inconsistencies and reaching

agreement on issues has been slow. The goal of this review is to address the complexity surrounding DF recommendations, and its consumption and implications to human health, specifically in children. This review will outline the current definitions and challenges in defining DF, sources of DF, DF intake recommendations, metabolic evidence and a discussion on the role microbiota could play in determining health implications.

Definitions

When discussing benefits in relation to health it is critical to first have a consistent definition. One of the most ominous challenges facing policy and dietary advice in regards to DF is defining it. Thus, to accurately describe the impacts of fiber and measure any outcomes associated with fiber, it is important that we better define what classifies as fiber and what does not. The Institute of Medicine (IOM) has summarized the definition of DF, when no less than 20 distinct definitions are currently used around the world (14). Recently, issues defining the consumption of DF for nutritional benefit have included: nutritional status, biochemical functions, physiological functions, dietary quality, nutritional quality, and quality of life (25). Major hurdles in assessing the impact of dietary fiber and analysis in children are contingent on future definitions and subsequent public policies. If an acceptable definition of DF is not established, then assessing clinical and public health implications of DF will remain difficult. This section of the review will not focus on policy as it defines dietary fiber; however, it will cover current understanding of major classes of carbohydrates and analogous compounds used to classify fiber.

Current U.S. Definitions

In the United States, the IOM has currently defined total fiber (TF) as a function of two components. The first component of DF pertains to its physical characteristics and has been defined as: "Dietary Fiber which consists of isolated, non-digestible carbohydrates and lignin that are intrinsic and intact in plants." This first definition is more consistent with philosophies intended by Hipsley (12) and later Burkitt (13). However, understandings of fiber and physiology have warranted additional profiling regarding health outcomes and claims. The second component pertaining to the functional attributes of DF have been defined as: "Functional fiber consists of isolated, non-digestible carbohydrates that have beneficial

physiological effects in humans." Total fiber is the sum of DF and FF (18). Having a two component model recognizes the importance of traditional diets in DF and their health associations, as well as recognizing the interaction that conventionally has been inferred to fiber, and applies to a wider variety of gut active compounds. Currently, the Food and Drug Administration (FDA) has not adopted the IOM's recommended fiber definition. However, current nutritional labeling principles do not incorporate the principles of the bacteria's residence there and the total functionality (26) and thus, are of limited use for public application and incorporation. It is possible these labels may be considered misleading to the public's general perceptions (27) and embodies the importance of setting consistent definitions.

Noncarbohydrate Sources

As defined by the IOM in 2002, there is both DF and FF, which can include noncarbohydrate sources. These substances are not isolated from "DF" but in fact may contribute to their cited effects. Components of fiber including lignin, phytate, oxalates, and saponins would likely be difficult to ever distinguish *in-situ* (28). Furthermore, normal household food preparation parameters, such as cooking, likely increase the production of Maillard products, which are not digested in the small intestine (SI).

There are several sources of functional fiber, which have an impact in the large intestine (LI) and are independent of carbohydrate such as: chitin, chitosan, and endogenous sources (i.e. protein, lipids). Chitin is a non-carbohydrate source that is a component of plant cell walls, insect exoskeleton, and crustacean structure. The structure includes β 1-4 linked glucose and differs from cellulosic and β-glucan structure by the addition of N-acetyl amino group at the C-2 position of the glucose residue (29). A derivative of chitin, chitosan, is a form of chitin in its deacetylated form. Interestingly, chitin is insoluble and viscous, with a high molecular weight. Therefore, it seems logical there are many substances that may demand attention when trying to understand the implications of non-digestible materials to be included in DF.

Mono and disaccharides

Typical discussions surrounding mono and disaccharides are not associated with fiber. In normal gastrointestinal (GI) function monosaccharides such as glucose, fructose, and galactose are considered to be completely absorbed in the SI. In younger children, where the digestive

tract may still be developing monosaccharides have been noted to induce breath hydrogen production after ingestion. Thus, this seems to indicate that a normally absorbed carbohydrate in an adult may actually serve as a functional fiber in young children (30, 31). Dysfunction of the GI tract involving monosaccharide digestion has been described as an issue of "over-load" or "malabsorption" where the monosaccharide passes to the LI and becomes available for fermentation (32). This is indeed the theory behind the common clinical practice of carbohydrate malabsorption in young children (33). The common dietary disaccharides sucrose, lactose, and maltose are completely digested and absorbed in the "normal" SI; however, this varies with population (33). These disaccharides can also contribute to intolerances which are well noted in the scientific literature (34).

Oligosaccharides and Polysaccharides

Oligosaccharides (OG) are carbohydrate polymers that consist of multiple monosaccharide units that vary in definition. Oligosaccharides are consistently characterized by greater than two monosaccharides and nondigestible; but have also been labeled as short chains of monosaccharide units (35), three to ten monosaccharide units (35, 36), two to ten polymers of monosaccharides (37), and three to nine monosaccharides (38). Common short chain OG include raffinose (trisaccharide), stachyose (tetrasaccharide), and verbacose (pentasaccharide). These short chains often are not hydrolyzed in the SI by human digestive enzymes, thus making them available to the LI and available for fermentation by the resident microbiota. These OG serve as prebiotics and have been shown to increase gut bacteria, and are considered to be beneficial and to positively impact young children (39).

In addition to short chain, longer chain OG have been found to be an increasingly important part of infant nutrition (40). Longer chain OG are frequently in the 2 to 60 unit polymer range. Typically, these longer chain OG are chemically speaking laden with fructose, which can be called polyfructans(29). Common specific terms for long chain OG include: polyfructose, inulin, oligofructose, and fructooligosaccharide (FOS). In fact, these longer chain OG seem to provide significant importance in the nutrition spectrum of younger children and infants from natural sources. Polyfructans are generally found in chicory, asparagus, onion, garlic, artichokes, tomatoes, and bananas. In addition, breast milk is an important source of OG that provides many health benefits (41). However, recently it has been observed that human

breast milk may be an important source that preferentially stimulates Bifidobacteria (40). Oligosaccharides processed and collected from plant sources are used in foods as a texture improver or substituted for other ingredients due to their functional properties.

Starches

Traditionally, starches are composed of two distinct groups of alpha-glucan, amylose and amylopectin. As recently as 1987, a food component was measured as nonstarch polysaccharide (NSP), but was a starch fraction and was confirmed in ileostomate models to escape SI digestion (42, 43). These starches were identified as resistant starch (RS), aptly named for their ability to resist digestion in the SI of man. A formal definition of RS was achieved during EURESTA proceedings, "the sum of starch and products of starch degradation not absorbed in the SI of healthy individuals" (44). As such, RS is viewed more as DF or functional fiber as classified in the United States.

Amylose and amylopectin starches are characterized by α 1-4 and α 1-6 glycoside bonds of D-glucose that are readily digested by human SI digestive enzymes. In most conditions amylopectin is present in higher proportion than amylose at 80%-85% and 10-15% respectively. Current opinion suggests that starch structure impacts their ability to be digested and thus their "availability" (45). Amylose has been noted to be less digestible compared to amylopectin, where metabolic response is reduced with increased amylose content in rice (46). This has also been noted with microorganisms' ability to ferment substrate (47). Some mechanisms identified surround the potential ability of amylose to retrograde or complex with other compounds (46).

Resistant starches are present in four groups that are profiled by their presence in the food supply. Presence is a function of source or structure, which can be facilitated by processing (48). Type 1 (RS1) is defined by the physical entrapment of the starch within cell walls that render it indigestible to human digestive enzymes. Sources of RS1 would include grains and seeds that have gone through limited milling or none at all. Type 2 (RS2) can be characterized by granules of starch that are not heated or gelatinized and remain resistant to digestive enzymes. Common sources of RS2 include raw potato starch and unripe (green) bananas. Type 3 (RS3) is referred to as "retrograded starch" which is produced by wet cooking with subsequent cooling. Cooling is important as it allows the starch helices to recrystallize, which portions become unavailable to digestive enzymes. Type 4 (RS4) is synthetically produced resistant starch, which goes through

chemical modification allowing polymers to cross-link and become resistant to normal digestive enzymes. Recently a new RS has been defined which is being labeled RS type V (49). Type V is starch that is complexed by an amylose-lipid interaction (50). According to IOM definitions, RS1 and RS2 would be considered DF with RS3, RS4, and type V, being functional fiber. Resistant starches have received much attention in scientific literature in regard to their potential in prebiotics (51). Thus, RS may provide a functional ingredient for foods that younger children are likely to consume and provide glycemic control.

Analogous Carbohydrates

Carbohydrates that are synthetic or result from processing, but mimic natural DF and retain the physical properties of the natural counterpart are termed "analogous." Typically, these carbohydrates can be produced through food processing, and include: indigestible dextrins (maltodextrins from corn and potato dextrins), synthesized carbohydrate compounds (polydextrose, methyl cellulose, hydroxpropylmethyl cellulose), and resistant starches can be categorized here as well (36). The definition of DF has progressively acknowledged the physiological implications, which may continue to enhance the importance of analogous carbohydrate in the diet. Common uses of these products include thickeners and sweeteners (52) and may represent a significant source of fiber currently not accounted for in young children.

Dietary Fiber Intake

Fiber consumption begins as soon as children start to feed early in infancy. In fact, it is believed that the first sources of DF are lactose, fructose (31), OG and starch (53). The earliest studies reporting intakes in children were recorded almost 30 years ago (54) and have not shown much of a change over time. In data gathered during 1976-1988 for the Bogalusa Heart Study it was found that children 10 y and 13 y maintained intakes of DF and decreased in density with age. Additionally, black children consistently consumed more fiber than white children with an overall mean intake of 12 g/day or 5 g/1000 kcal (54).

Reported fiber intake is universally less than recommended, regardless of assessment method or guideline. Data examined from National Health and Nutrition Examination Survey II (NHANES II) using 24-hour recalls, reported that DF consumption from 3-5 years and 6-11 years, had mean/median intakes of 10.7/9.8, 12.5/11.1, respectively (55). In addition, the 3-5

year old group consumed 1591 kcal/day on average with the 6-11 year old group consuming 1897 on average (56). This corresponded with a DF density of less than 6.7 g fiber/1,000 kcals for 3-5 year olds and 6.6 g of fiber/1,000 kcals for 6-11 year olds. Currently, these are all well below the, "Age + 5", AI, and AAP recommendations.

In 1991, average DF intake for children (4-19 y) was found to be around 12 g/day, with the 4-7 yr. age group reported to eat approximately 10.5 g/day (57). This reflected around 6% of the fiber from cereals and vegetable fiber, which was primarily obtained during snacking, 13% from breakfast and the rest from major meals (57). Current recommendations suggest 14g/1000 kcal, while in this study it was found to be around 6 g/1000 kcal or about 50% below recommendations and it declines with age. In a cross-sectional study of Native American children and non-hispanic white children 1-6 years in age were found to consume similar levels of DF with only 6% coming from fruit. Significant foods contributing to fiber content in the vegetable category were beans and corn (58).

Another large survey, the Continuing Survey of Food Intakes by Individuals (CSFII) has shown similar results as the NHANES data. For instance, dietary data analyzed from the 1989-91 CSFII found that children (4-6 y) consumed 45% of DF recommendations assessed by the Age + 5, guideline (59). As children aged (7-10 y), intakes meeting recommendations were reduced to 32%, and is in accordance with other studies (57), who found a decrease in fiber density with age and approximately a 2 g/day increase up to 19 years of age. A more recent study evaluating pre-school children (2-3 y and 4-5 y) with CSFII 1994-96, 98 surveys found that fruit and legumes were the largest contributor to fiber intake (9.9 g and 11.5 g, repectively) (20). Neither guideline the Age + 5 (which is less than the AI) or the DRI was met for DF intake this population. More recently, data has supported past low intakes, finding children still under-consume recommendations with an average of 11.1 g/day (60).

Interestingly, only 12% of children were found to meet relative recommendations, and lower socio-economic status was actually associated with increased fiber intake and nutrient density, according to DRI's (61). However, not all studies show the same consistent results regarding socioeconomic status. Recently, a study in Flemish children (2.5-6.5 y) found most of their DF intake came from breads and cereals (30%); however, DF from fruits and vegetable sources were found to increase only with maternal education and parental employment status (62).

Data are available in the younger populations assessing the adherence to DRI's, including fiber intake. A national survey of primary caregivers using a 24-hour recall reported that DF was less than recommended (according to CDC median values for EER estimation) in toddlers (12-24 months) and preschoolers (24-47 months), with 9 g and 10 g, respectively (63). This was in accordance with earlier evidence from the same on-going data set indicating that children in the 1-2 yr group were not meeting AI recommendations (19 g/d) of DF intake in 2002 (64).

Children may be more likely to consume fiber if breakfast was consumed. A lack of breakfast consumption has been implicated with reduced fiber intakes in several studies involving children. In young (9-10 y) African American and white girls, eating breakfast was positively correlated with increased fiber intake (65).

Although not a measure of DF, glycemic index (GI) and glycemic load (GL) are sometimes used as a proxy for its consumption. This is based on the premise that lower GI foods tend to be higher in DF. In a study involving young (7-8 y) German children, researchers found that cohorts from 2002 reported food intakes with higher GI values than their predecessors in 1990 (66). In addition, this study found that "tolerated food groups" such as sweets, candies, and refined starches, were largely responsible for the GL of the diet, with potatoes only accounting for ~4% of the GL in the diet. This fits well with the idea that eating lower GI and GL foods may provide increased nutrient consumption including DF. Interestingly, in populations where control of glycemia is important, such as those afflicted with Diabetes Mellitus (DM), increased fiber intake may be important and higher motivation might exist in this population towards adherence to increased fiber consumption. A study in adolescents (13-19 y) with Type 1 DM reported youth with Type I DM consumed more DF (21 g boys, 17 g girls) than non-diseased peers, but still less than recommendations (67). It should be noted that numerically their intakes were higher than other reported studies, but there was a large statistical variation in this population and was only statistically significant compared to national surveys.

While some studies have investigated DF consumption, others have focused on the availability of DF as proxy measure for assessing dietary quality which provides some insight into DF intake. Researchers observing household availability of nutrient status (Nutrient Status Adequacy Ratios) found that most nutrients were available in households at DRI recommendations consistently across weight status except for DF, carbohydrate, and calcium (68). However, this study leads little insight into the flux of foods through the household, which

is a serious limitation. In addition, the authors mention limitations in regards to knowing which foods were consumed or wasted. Thus, measuring the availability of fiber is likely to always be limited by similar pitfalls and makes any kind of inference from the data highly speculative. However, this type of evidence presents an affordable way to add data for helping assess where barriers lie in understanding fiber consumption and the ultimate impact on health.

Dietary Fiber Recommendations

Young children over the age of two do not consume recommended amounts of fiber. This may be setting the stage for DF intake into the adult years (18). Determination of DF intake in children has been evaluated via two guidelines, Adequate Intake (AI) as recommended by FNB (18) and the, "Age + 5," recommendation (53). Each guideline is intended to achieve adult levels of fiber intake by the age of 20, when demarcations to adulthood are set. Other guidelines that are utilized include: the American Association of Pediatrics 0.5 g of DF per kg of weight, the calorie based (21) FDA food label guideline of 25 g/2000 kcal (12.5 g/1000 kcal) and 30 g/2500 kcal (12 g/1000 kcal), and the IOM recommendation of 14 g/1000 kcal.

Physiological Considerations with Fiber Intake

Providing proper recommendations for DF intake in children will require more research in the youth population. As previously noted, most of the recommendations are extrapolated from adult studies and are likely to have similar benefits. However, it is critical to understand the implications specifically within the population studied. The intent of this section is to shed further light on the current available evidence regarding physiological factors of DF on health in children. Information surrounding children, regarding other metabolic considerations are available, but not covered here: obesity (21, 24, 69), laxation (21, 70), and satiety (21). Glucose metabolism, lipid metabolism, and constipation will be the focus of the following portion of the review.

Glucose Metabolism

Evidence regarding the implications of DF on glucose is readily available with adults as subjects (71, 72). Evidence in children is much less prevalent, but seems to be suggesting similar mechanisms as in adults. In adults, meta-analysis through prospective cohort studies

suggest cereal (grains) fiber consumption and magnesium intake decreases the risk of diabetes (73). Furthermore, a review by American Dietetic Association (ADA) (74) shows limited evidence implicating 30-50 g DF/day in reducing serum glucose levels compared to groups with low-fiber diet. In addition, it is suggested that daily intakes of 10 -29 g of fiber supplement may provide beneficial effects for glycemic control. However, the mechanism of action controlling this outcome is debatable and more research is needed to confirm these effects, especially in children at risk for developing insulin resistance. Children are often implicated in the same low-nutrient dense diet of adults (75) and considered at-risk for suffering the same maladies.

Studies examining the effects of DF intake on blood levels of glucose tend to show a general decreased post-prandial response following higher fiber intake, with psyillum fiber being one form of DF reported to decrease post-prandial glucose (76). Specific to children, in overweight Latino adolescents (10-17 years old), an increase in fiber intake elicited increased insulin sensitivity (77). While data do exist, more research needs to be conducted to determine impacts of fiber on glucose metabolism in children, especially longitudinal studies. The research that does exist has typically been focused on those with Type I diabetes (78) and needs to be expanded to the larger population of children to determine the capacity of DF at preventing metabolic disease. This would allow for more information in the assessment of the necessity of fiber in the developing child, or its' lack of consumption, in the development of disease. Furthermore, this could potentially lead to insight into how younger bodies utilize glucose compared to adults. Only a few studies have tried to assess the impact of isolated types of fiber, such as soluble versus insoluble. In a clinical trial, children fed a high dose (~30g, 4 g soluble) experienced decreased fasting serum glucose compared with those eating a low dose (~10 g, 1 g soluble) (79).

Acute consumption of DF suggests a role in controlling post-prandial glycemic response. This has been translated into long-term intake in the DONALD study in German children. Fiber intake was found to significantly decrease across increasing glycemic index (81). Collectively with other measures in that study, it would seem fiber intake displaced rapidly absorbable carbohydrate, which may have impacts on glycemic control. In a study of overweight adolescents, "sugar" (or sucrose) was suggested to be responsible for the adiposity gain and reduced insulin sensitivity (81). When the GI and GL were measured, they did not explain much

of the variation. Conversely, in adults, research has seen fiber as an independent factor regarding glucose metabolism and needs to be accounted for in analysis of glycemic control (82).

Lipid Metabolism

In adults, evidence regarding the implications of fiber in lipid metabolism is abundant and seems to play a significant role (83). Fiber seems to impact the way lipids are metabolized in the body, potentially mediating health outcomes (84). Evidence in children is also indicating that fiber may play a similar role in altering lipid metabolism. In a recent study in Finland, children were found to consume more fiber with increasing food intake, while fat intake was inversely correlated with fiber intake (85). Furthermore, as in adults (83), serum cholesterol was found to be inversely correlated to fiber intake. Children fed high (~30 g, 4 g soluble) versus a low (~10 g, 1 g soluble) fiber diet experienced decreased serum LDL concentrations by approximately 12% compared with the low dose (79). Soluble fiber was defined as pectins, gums and mucilages. Another study of hypercholesterolemic children following a 3 month lead-in low fat, low cholesterol diet, provided psyllium fiber (3 g) for 8 weeks had no significant alterations on blood serum cholesterol from baseline (86). More evidence is needed to confirm associated findings and to translate more specific relationships that exist with level of fiber intake and fiber type.

Constipation

Low fiber intake seems to be associated with several deleterious conditions, suggesting that increased intake may decrease the occurrence of unwanted bowel outcomes such appendicitis (87) or constipation. Constipation is a major cause of children's visits to the hospital, with approximately 5% of outpatient visits being diagnosed as such (88). An estimate of constipation prevalence suggests that it affects from 0.7% to 29.6% of children in different regions throughout the world (89). Complete diagnostic criteria of defecation disorders including constipation can be found under ROME III guidelines, which are more inclusive and have shown a higher prevalence than reported with the previous ROME II criteria (89, 90). Constipation has been defined by the parameters of difficulty passing a stool or by less than 3 bowel movements per week (91, 92). Much research involving children has focused on fiber's role in amelioration of these indicators or symptoms.

Several studies involving Chinese children have tried to assess the impact of fiber level on constipation. In a study in Chinese children (3-7, 8-14 years) recommended DF intake of 10 g and 14.5 g, respectively for the relief or amelioration of constipation (93). Another study involving Chinese children found that mean intake was only 4.1 g/d in pre-school children in Hong Kong (94). However, that intake was about half the level consumed by children in the Chao et al., (2008) study. Furthermore, the American Academy of Pediatrics (AAP) only recommends that 7-10 grams per day should be consumed for these age groups. According to their definition 10 g should be sufficient to prevent constipation. It should be noted that the intake in these children was more than double those in Hong Kong, both occurring in Chinese populations. Conversely, if we use the IOM recommendations then neither group obtained the recommended intake of DF 19 g/day.

In children with constipation, fiber intakes were not meeting the recommendation of "Age + 5" (95). Low fiber intake has been reported in many other countries as well. In Irish children (ages 5-8 years), 76% did not meet recommended guidelines for fiber intake; however, only 13.6% of the children with low DF intake developed constipation compared with 6% of children who reported adequate fiber intake (96). Clearly, there is an association between fiber intake and presence of constipation. However, this association does not necessarily implicate DF in the etiology of constipation nor does DF seem to completely resolve constipation (97). In fact, 63% of individuals who did not consume enough DF were considered as healthy as the 94% who were able to meet recommended doses. Research has also shown that associations exist in constipated children both with DF intake of less than, equal to or higher than other children who are not constipated. Thus, fiber does not seem to be the most significant factor regarding the presence or prevention of constipation.

With that said, DF is often an included in treatment for constipation (98). Considering the mixed results with DF's ability to treat constipation, we must be careful how we characterize fiber and be specific about the types of substrate used to label as "fiber." Indeed, fiber may be a successful treatment option (24, 99, 100). However, this information should be taken in context, as very rarely are fiber treatments (glucomannan, lactulose) given as the first and only treatment and some recommend that DF be ingested concurrently with other treatment options as constipation is a multifaceted condition (98). Moreover, the American Academy of Pediatrics (AAP) seems reluctant to advise a definitive supplement of DF intake to treat constipation.

There are many components that need to be addressed and it would seem logical that every case is different, which should be taken into account when treating constipation.

In Brazilian children (6-8 y) with chronic constipation, daily DF intake was lower than normal controls, 13.8 g versus 17.3 g, respectively (101). In children with chronic constipation it is well known that a cycle may be induced where children are afraid of passing stools and thus, 'hold the stool'. This creates a painful process of large, impacted and dry stool that the child resists passing, further complicating and ensuring the cycle continues (98). Another study of constipated Brazilian children found that children with higher bran intakes tended to have improved bowel conditions compared to those whose conditions worsened (102). This study was not clear, though, on the "acceptance" of bran and its consumption. The only measures of dietary intake were by food frequency questionnaires (FFQ's), which should be considered with their limitations. The authors were not clear as to the participants who were consistent and those who were not as aggregate data seems to be used at each time point instead of paired data. Nevertheless, some children's symptoms did not improve, which again underscores the complexity of constipation management and that changing DF intake may not necessarily affect symptoms.

Fiber alone has been noted to not be sufficient in the treatment of constipation and may require a broader individualized approach. One question that still needs to be addressed is whether low DF intake noted in constipated individuals is, in fact, a response to additional compaction they may feel. If a child is fearful of bowel movements, then higher fiber foods may not be appealing. It would seem logical that children who consume fiber may feel more pressure with increased load to the LI and still be fearful, compounding the eventual voiding and potentially creating a more uncomfortable experience. In addition, children may associate this additional pressure with DF intake and avoid its intake. However, more research should be conducted to determine if indeed these factors could play a role. Due to the complexity of this condition, it is debatable whether an increase of three grams of fiber (the gap most of these studies show) would create a more desirable situation for a functionally constipated child.

Children, Fiber and Gut Microflora

Many reviews are available discussing the metabolic consequences of fiber (8, 14) primarily in adults. However, there are few that discuss DF's relationship in children. It has

been recognized that information regarding the impacts of fiber in children is often an anticipated outcome extrapolated from adult studies (24) and more research should be undertaken to confirm these relationships in children (20, 22, 23, 39). A life-course approach seems to be the agreed upon method of health advice (17, 22), but when making recommendations, credence should be given based on the accumulation of data. Caution should be heeded (103) in order to make a consistent recommendation so that the public can endorse consumption as it meets their individual circumstances.

Still, DF's impact on health is not fully understood or complete, with many reviews covering this topic (104) in adults (8, 103) as well as children (23, 24, 39, 105). It is likely that many health-promoting qualities of fiber are similar in children as adults such as: bulking agent, moisture retention and physical blocking of interaction of substrate and human digestive enzymes. However, one area that warrants further discussion is the belief that fiber acts as a fuel source for colonic bacteria and they may indeed contribute much more too human health than anticipated (106-108). However, it is important to discuss the role each of the mechanisms play in the role of the developing gut of children. As the previous reviews have targeted some metabolic outcomes, it is the goal of this review to focus more towards gut microbiota.

Currently, fiber holds the most intrigue and potential for contributions to the understanding of gut health. This is in regards to its utilization as a fuel for the microflora in the LI. As fiber is not digested by human pancreatic enzymes in the SI, it is made available for utilization by bacteria in the LI and the microbes that reside there (109). Research has determined that the bacteria in the LI need 60-70 g of substrate everyday (110, 111) in order to maintain estimated populations. This can be further broken down into approximately 8-18 grams of fiber in addition to starch (8-40 g) (112). These populations are so large that the bacterial cells outnumber the somatic host cells ten-to-one (113). More notably the collective genome of the resident microflora is estimated to contain in the millions, compared to only 20,000 in the human host genome (106). This makes the potential for interactions from the host to its residents enormous and likely a significant factor.

Most animal species thrive from their mutualistic relationships with the microbiota inhabitants, thus making it very likely the human animal is not completely unique (113, 114). Research regarding the investigation of the microflora and their complete roles in the nutritional world are exceedingly high. Recently, technology has made it possible to further investigate the

impacts of the microbiome (106). So much so that $115 million alone in the U.S. and another $70 million in research funds are being provided for simply the mapping of such genes and potential interest in disease (106). It is logical that if researchers want to begin to understand the role of colonic health, especially in developing child gut it must go through the microbiota that reside there and all the interactions that could take place.

Typical microbial species of interest include: Bacteroides, Clostridium, Desulfovibrio, Lactobacillus, Escherichia coli, Enterococcus, Fusobacterium, Bifidobacteria and the archae Methanobacter. Indeed, evidence indicates that microbial species are substantially influence by dietary habits (115) or substrate available in children (116). When children were studied longitudinally, differences between the rural and the urban children's gut microbial populations were noted (117). These bacteria and their substrates together form a complex interaction that may have health implications such as colon cancer, allergies, obesity and inflammatory bowel disease associated with a Westernized diet (118). Central interest has surrounded the difference in microecology in individuals with metabolic syndrome, obesity, abnormal glucose and insulin responses, cholesterol, and altered immunity (119-122). Thus, this leads to the expectation that modulating diet may provide beneficial alterations in gut microbiota that will lead to improved health outcomes (123).

To better understand the impact of the developing gut, more specifically the LI in children, it is important to consider existing knowledge. Traditional methods were often limited in their ability to detect total microbial populations (113, 124). These limits were based on the ability to culture microorganism and were largely restricted to aerobic cultures. As time progressed, anaerobic models were able to advance these culturing procedures opening new detection opportunities. These resulted in estimates of approximately 400 species in major microflora (125). However, there were still limited in the fact that we still had to have the technology to culture in order to study what exists. With the advent of molecular techniques (126) which have become more cost effective in recent years, it has become quite clear that we only understand fractions of the total microecology that existed. These estimates suggest that approximately 20% of bacterial genomes are known or able to be cultured (127, 128).

Fiber intake in children suggests the when the child is developmentally ready they should begin consume fiber in order to establish important bacteria (39). However, this time frame may be different for every child. They should begin to develop the adult pattern as soon as possible

to prepare them for lifelong health. However, do we want our children following the developmental pattern of most adults? Children do not assimilate carbohydrates similarly to adults (53). Perhaps this could be due to innate functional differences from adults, where children have smaller, SI's which correlate well to body length (129). Furthermore, the flow of colonic contents are an important mitigator of the ability of the bacteria to utilize substrate (113). Evidence also indicates that once substrate makes it to the colon, it is utilized in differentially in all individuals (130) and perhaps children exhibit differential characteristics.

Differences in gut function are not well identified in the literature. However, in children this may translate to a more efficient colon that utilizes more substrate (131). This also appears to be the case in *in-vitro* models as well (132). It is likely to be important then in children and adults alike to understand the role of constipation in alteration in gut microbiota in respect to substrate flow. However, there is currently not enough evidence to elucidate whether current substrates (refined starches) that escape digestion in the young child SI is indeed developmentally necessary. While energy is escaping the large intestine, it is possible that substrate evasion of the SI was necessary for gut development. Allowing simple substrate to escape the SI and develop the gut may prepare the gut for less digestible substrate in the future and an enhance energy supply. Perhaps, the progression of substrate could be important for optimal gut development. As our understanding of the variety of unavailable carbohydrate (DF) increases the amount, as the type, and the outcome desired should be considered in making future DF recommendations. Currently, there is not enough research to develop broad general recommendations that will contribute necessary functions for optimal health.

To find what is necessary for well-functioning or optimal GI health with regard to children (~2 and older), clear goals must be set. To profile and achieve optimal GI function with regard to the microflora several premises must be met: 1) Characterization of an optimal example of gut function; 2) Characterization of optimal microbiota that inhabit this gut; 3) How this microbiota came to be established in the gut (113, 133, 134) and 4) Can we modify an "unhealthy" gut to an "optimal" gut through addition of "optimal" parameters, such as fiber. These premises can be a framework with which to conceptualize the end target. This final premise is of seminal importance if we believe DF to alter this microbiota to the said "optimal" state for life-long health.

Microbiota likely confer specific geno and phenotypical traits that benefit the human host beyond what could be achieved on their own (135). Thus, for optimal health, having this "core microbiome" (136) that could confer the most possible health benefits would be the goal. Savage et al., (1977) extensively discusses the role of autochthonous organism or those that should normally inhabit the host's gut. In newborns (presumably all species) the gut is sterile or microbe free (137), with inoculation occurring to interaction with the environment. These organisms which are first obtained at birth (vaginal, fecal, or cesarean) are most likely to be found in the intestinal tracts of infants (113, 138-141). After initial inoculation dietary modulation appears to be the major factor contributing to development.

Microbiota that are autochthonous are obtained from the environment, which includes microbes present on food that survive digestive forces and substrate for those already surviving that journey. Notable changes in diet with alterations in available substrate have shown to produce variations in colonic microflora (142). Microbial population differences are then noted in breastfed versus formula fed infants (143, 144). Research has shown that formula fed infants are more likely to present an adult like microflora pattern (141). Breastfed infants are more likely to stay centered around the *Bifidobacteria* that largely inhabit their LI (39).

According to overall microecology, formula fed infants are better adapted for the next stage in life, weaning. This is shown by the formula fed infants ability to ferment complex carbohydrates more quickly than breast fed infants (39). This was confirmed by *in-vitro* analysis regarding breast-fed infants in different stages of weaning. As on set of weaning was delayed, the ability of the infant microflora to ferment complex polysaccharides was also delayed (145). Alternatively, infants who were formula fed with a more adult-like microflora showed little trouble fermenting complex carbohydrates (146). This suggests that earlier weaning, which requires consumption of other substrates (such as the formula fed), modifies the LI and prepares it for continual increasing complex substrate load. The authors noted that this is not likely to be a large source of energy in the infant at this time. It is important to recognize the complexity of the developing gut and the need for much more research to determine more optimal recommendations.

In addition to substrate availability affecting our ability to more completely ferment non-digestible carbohydrates (fiber), i.e. altering and modifying colonic microflora, our body seems to have a way to ensure that this population is indeed developed by substrate. Recent evidence

has been able to confirm that infants indeed receive a constant source of fermentative substrate in the form of OS in breast milk (40). As noted above, breast-fed infants are relatively slow in fermenting other complex carbohydrates. Interestingly, FOS from breast milk seems to preferentially target *Bifidobacteria longum* and biovar *infantis*. However, these are not the predominant bacteria in an adult LI (147). This is contrast to Bacteroides, which is considered to have the greatest populations overall (147). In addition, several studies have noted the wide adaptability of Bacteroides to utilize many fermentative substrates (148).

When making specific DF recommendations it is necessary to assess if DF has the potential to alter microbial communities in a controllable fashion for the betterment of a child. Traditional thought has noted that the overall change in microflora composition is not changed by dietary modulation (149). However, this study was conducted before the mass incorporation of molecular techniques. One reason the microecology appear to not change is that *Bacteroides* have proven to be uniquely adept at utilizing a variety of substrates. These substrates are only inclusive of some study outcomes: heparin, pectin, ovomucoid, amylose, amylopectin, dextran, gum tragacanth, gum guar, L. arch arabinogalactan, alginate, laminarin, psyllorium hydrocolloid, xylan, polygalacturonate, gum arabic, and cellulose (148).

Nevertheless, diet is believed to play an important role in the modulation of the gut microbiota. In newborn infants, a shift in the microbiota present can already be detected in 7 days (143) towards *Bacteroides fragilis*. This seems to follow changes from colostrum to more mature milk and substrate changes. Traditional methods have illustrated a shift to Lactobacilli and Bifidobacteria as the predominating cultures towards one month of age (150). When the time for weaning does come, there is some evidence that indeed shows shifts in the outcomes of health towards formula fed infants. Studies have shown that when breast-fed infants are weaned there becomes a period of time where the infant is at risk for diarrhea independent of pathogen introduction (151). This seems to confirm the pancreatic insufficiency of the infant to fully digest substrates and perhaps leading to an osmotic overload of the colon.

What is deemed as an available carbohydrate or un-available in infants and children is different than what can be expected. Excluding malabsorptive disorders for lactose, maltose, fructose, glucose and potentially small intestinal overgrowth; the gut is not as efficient at absorbing all nutrients as those found in adult profiles. Christian et al., (53) reported that not all starch may be absorbed in the infant's SI, thus leaving it available for fermentation in the LI.

This appears to be due to various reasons from processing to physiology. Children do not produce adult levels of enzymes, which is likely a leading contributor to most starches making it to the LI (152-154). In fact, evidence suggests that biology favors efficiency of digestions as some salivary amylase from infant and amylase from the mother (breast-fed) make it to the SI (153, 154). Research suggests children may not reach adult profiles until 12 years of age (155). Further, commercial processing techniques such as canning (156) may decrease the digestibility by infants. This is likely since processing is known to affect glycemic index (as indicator of absorption) (157, 158) and resistant starch formation would be less susceptible in mildly harsh milieu of child gut (49, 159). Understanding how these factors alter substrate availability to survive human digestive mechanisms will be important in understanding which bacteria may be able to utilize and flourish from its consumption, ultimately allowing us to better understand how food choices will impact on going gut health.

Summary

Collectively, it is apparent that more research is needed to better understand the effects of DF on the developing gut of children. More importantly, changes need to be monitored throughout the entire development of the child to begin to understand all of the complex interactions that impact health. There are clear developmental differences of the child, which are not the same as in adults. These differences will be important to determine how optimal dietary recommendations, and specifically DF, may impact these metabolic interactions. Together, the microecology and human metabolism, hold potential to provide significant contribution to improve our understanding of the relationship between food and health. Technology has been uniquely providing new and better methods to study young children using non-invasive and generally mild techniques that allow profiling of health characteristics that may lead to an improved snap-shot of health. Indeed, if we are able to make scientifically sound recommendations for children regarding an optimal DF intake, then we must work towards consensus on the definition of DF and its subsequent methods of measurement. Identifying, the microbiomes associated with health and identifying mechanisms associated with those outcomes will lead to insight on targeted dietary recommendations that will provide flexible and scientifically grounded inferences.

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Case

Chapter 3 - Fiber Fermentation in Children as Measured by Breath Hydrogen and Methane

Abstract

Objective: The objective was to assess the dose of dietary fiber (DF) and the response of breath hydrogen, methane, and total hydrogen content relative to Institute of Medicine (IOM) recommended intakes. **Study Design:** Free-living preschool children were fed oat-based cereal designed to provide 25%, 50%, and 75% of the IOM's recommendation for fiber. Breath hydrogen, methane, and total hydrogens (ppm) were measured via breath collection over approximately 6 hours. The design was a counter-balanced crossover. **Results:** Only four of the 18 participants were able to comply with the treatment protocol. Although no significant differences were noted in breath measures of fermentation across fiber consumption levels, there was a numerical pattern for increasing levels of DF to evolved increased gas production in the four compliers. All children produced hydrogen and methane sometime during the test. In addition, 98% of tests produced breath methane in excess of 1 ppm. **Conclusions:** In free-living individuals measures of acute fiber fermentation through breath was not sensitive enough over 6 hours to distinguish a difference in fermentative rate. Children, parents, and child care centers found this approach apparently acceptable. This study provided a framework into the feasibility of studying young children in gentle, non-invasive, and cost-effective manner.

Introduction

Evidence suggests that children do not consume enough dietary fiber (DF), both in the United States [1, 2] and around the world [3, 4] according to current recommendations. This finding is not surprising as most adults do not consume enough DF either, both in the US [1] and elsewhere [5, 6]. Consumption of low amounts of DF has been found to be associated with conditions including: type II diabetes [7], weight status [8], cholesterol metabolism [9], colonic health [10], cancers [1, 11], cardiovascular disease [12], blood pressure [13], and energy intake [14]. Children have been hit with similar maladies that adults were traditionally known to acquire. Two conditions of increasing concern in children are obesity [15] and Type II Diabetes [16]. Thus, there has been increasing attention at solving these emerging burdens on health starting in young children worldwide [17].

Integrating DF into the diet is recommended for improved overall nutritional quality [18]. However, it is also recognized that empirical and mechanistic research regarding implications of its consumption are lacking, especially in children [19, 20]. A life-course approach to health should be considered, starting with young children [21]. Therefore in children it is important to determine the impact that DF has on the ability of a child to flourish and eventually provide a base for adulthood. Fiber has been recommended as a target to fight obesity, consequently improving health outcomes [22, 23]. Understanding how much fiber each child needs and the mechanisms by which fiber contributes to improving the health of children will help in making appropriate, science-based nutritional recommendations. A major fate of DF in the human body is its fermentation by bacteria in the large intestine [24, 25]. Understanding the impact of DF fermentation may hold keys to understanding the link between diet and measureable metabolic impacts.

Dietary fiber is not well defined and controversy exists in not only which, but how much of each type of DF should be recommended [26]. Dietary fiber has been labeled as “unrealistic” to define [27]. In fact, a precise definition is still being debated [28, 29] with no agreed consensus even within the Food and Drug Administration (FDA) and National Academy of Sciences (NAS) in the United States. To date, fiber content in food is determined by the AOAC approved methods, which makes fiber content dependent on those methods [29]. The

discrepancy between physical and functional properties makes the nutritional implications of eating DF potentially susceptible to conflicting results.

All of these factors have contributed to the difficulty in assessing the implications of fiber consumption in adults, let alone in children, and the downstream affects. Measuring the impact of DF *in-situ* is very difficult and few studies have been carried out in humans [30] regarding any such attempt. Thus, proxy measures have been developed in association with DF intake. Much of our knowledge was gained from animal and *in-vitro* models of fermentation [31, 32] through measures of fecal weight [33], fecal energy [34], short chain fatty acids (SCFA) [35], fecal pH, and more recently fecal bacteria [36]. Further knowledge of indigestible carbohydrate utilization has been limited by its method of study such as: intubation studies [37, 38], ileostomy [39], and hydrogen breath test (HBT) analysis [40-42]. Each method has limitations; however, the HBT is a better non-invasive, reproducible, and sensitive procedure for assessing carbohydrate [42, 43] fermentation in the large intestine.

The purpose of this research is to gather information regarding the impact of DF in free living preschool children by utilizing non-invasive methodologies to assess the fermentation of DF. The objective was to assess the dose of DF and the response of breath hydrogen, methane, and total hydrogen content relative to recommended intakes. The hypothesis was that increased DF consumption by children would result in increased hydrogen and methane evolution as measured by breath gas evolution.

Materials and Methods

Participants

Participants were preschool (3-5 years) children from a university associated childcare facility at Kansas State University. Two classrooms were targeted for recruitment with a total population of 30 children. We were able to acquire informed consent/assents from the parents and/or guardians of 22 children attending the facility. Out of those 22, we were able to obtain data on 18, with 14 providing the target of three measurements. Parents of children were questioned regarding the gastrointestinal health of their child for any symptoms of abnormalities in gastrointestinal function or known confounders. Exclusion criteria would include the presence of clinical constipation, antibiotics use within the last month, inflammatory bowel diseases and

any intestinal distress (recent diarrhea). Inclusion criteria required that children were apparently healthy upon entry into the study, age three to five, and consume commercially available cereal. In addition, on each testing day, fliers were sent home with parents to fill out to ensure conditions upon entry to the study had not changed. This project was approved by the Institutional Review Board of Kansas State University (IRB #6092).

Research Design

The experiment was set-up in a counter balanced cross-over design. Participants were allocated treatments by classroom after signing informed consent/assent to the treatment order. The original design structure though was disregarded as children were not able to completely comply with the treatment structure. Treatments consisted of DF obtained from a commercially available cereal (Kellogg's Fiber Plus, Kellogg Sales Co, Battle Creek, MI) at the local grocery store. The cereal was chosen based internal tests of acceptability with children. In addition, to meet childcare food quality and safety guidelines it was critical to provide a sealed product that was allergen free. The product was primarily whole grain oat flour free of major allergens, making it ideal for use in this environment. As most children do not meet recommendations of DF intake we wanted to minimize potential negative outcomes and keep consistent with dietary advice. Thus, treatment levels consisted of 25, 50, and 75% of DF intakes as recommended by the Institute of Medicine (IOM) with an adequate intake (AI) 14 g/1000 kcal. Estimated energy requirements as documented by IOM's Dietary Referenced Intakes (DRI's), were calculated to assess AI.

Study Protocol

Parents were provided packets the evening prior to the study test day. Packets included treatment condition (cereal level), breath hydrogen collection apparatus (GaSampler™, Quintron, Milwaukee, WI), written instructions for use and a DF informational flier. Parents were verbally instructed on the use of the GaSampler™ kits for collection of baseline breath samples. All children participating in the study were verbally instructed during "instructional time" and allowed to practice with the collection method. We found in most cases the children were able to teach parents in the proper method of collection. This was likely to increase the adherence to test protocol and was an intangible source of pride in this study sample group. In addition, parents were instructed and reminded on common DF containing foods and encouraged

to limit the consumption of these products for supper or late snack as they are known to influence the tests [43]. Parents were given a Kansas State University Research and Extension flier designed for parents and families about DF and common DF containing foods to increase their ability to adhere to protocol.

The morning of the tests, children would wake-up according to the typical morning routine, brush their teeth and then collect a morning breath sample in the fasted state. Children would then consume cereal with 25%, 50%, or 75% of recommended DF intake. Parents would record the time of consumption, changes in stool habits, antibiotic use, DF consumption the night before, and snack consumption the night before. There was also a comments section for recording events that may not have been anticipated. When children reached the child care center, baseline samples were collected, with subsequent breath measurements at half-hour intervals, with one hour intervals for lunch and recess breaks, and a final measurement occurring post-nap. When children were not able to completely eat all of the provided cereal, the extra was returned and weighed to calculate amount of sample consumed.

Samples were collected at regular intervals up to 7 hours with the GaSampler reusable collection bag (Quintron Instrument Co, Milwaukee, WI, QT00841-P). Breath samples were extracted from the GaSampler reusable collection bag with Monoject 35 mL plastic syringe (Quintron Instrument Co, Milwaukee, WI, QT-01735-V) fitted with a 1-way plastic stopcock (Quintron Instrument Co, Milwaukee, WI, QT01727-V). A total of 30 mL was extracted from the reusable collection bag and injected within two-hours of collection into the BreathTracker SC (Quintron Instrument Co, Milwaukee, WI, QT05000-M). Total H_2, CH_4, and CO_2 were recorded for each measure and were normalized according to CO_2 of 5.5%.

Hydrogen and methane values were used to calculate incremental area under the curve (iAUC), total hydrogen, total methane, and total H_2 atoms. The lowest value achieved prior to a sustained rise in breath hydrogen was defined as the basal hydrogen level [42], which was also applied to methane and cumulative H_2 ppm. Cumulative H_2 ppm produced is a novel measure reported here to reflect total hydrogen gas evolution patterns. Methane production consumes 4 mol of hydrogen for 1 mol of methane. This is important because methane production depends on hydrogen availability and does not show the rapidly altering production patterns of hydrogen and is typically more consistent [44]. This may hide the total amount of carbohydrate being

fermented at any given time. Basal hydrogen level was then used as the baseline for iAUC calculation.

Anthropometry

In order to calculate body mass index (BMI), we collected height and weight. Height was measured in cm with a Seca 214 Portable Stadiometer (Seca, Hanover, MD). Children were measure according to standard protocol with four contact points with a horizontal Frankfort Plane. Averages were reported of duplicate measures. Weights were recorded in kg using a Health-O-Meter digital scale (HDL626, Sunbeam Products Inc., New York). Measures were taken in duplicate and reported as the average. Body mass index was calculated using the standard formula $BMI = \frac{Weight\ (kg)}{(Height\ (m))^2}$. In addition, because children under the age of 20 are not classified under the same criteria as adults [45], BMI percentiles were obtained from the CDC growth charts and reported.

Statistics

Graphpad Prism 6 (Graphpad Software, La Jolla, CA) was used to calculate the iAUC for hydrogen, methane and total hydrogen ppm produced. Treatment means for all participants as well as high adheres were analyzed by using the Mixed procedure of SAS version 9.2 (SAS Institute, Cary, NC). High adherers were defined as those who were able to complete the test as instructed by the protocol. The MIXED procedure of SAS was first used to assess the null hypothesis that level of DF consumption is not associated with hydrogen evolution and total gas evolution in a dose-response relationship at significance level of $p = 0.05$. Treatment differences were analyzed using Excel paired t-tests $p = 0.05$ (Microsoft Corporation). The test was also repeated for methane evolution and graphical representation of a pattern justified analysis, which is sometimes reported as not necessary [42] at a $p = 0.05$.

Results

Descriptive analysis of children participating in the study can be viewed in Table 1.1. Evaluation of BMI placed 4.5%, 81.8%, and 13.6% of children in the underweight, normal weight, and overweight, respectively. These numbers were based of BMI percentile for the most recent [45] CDC height and weight charts and current interpretation of percentiles. Overall, 30

children attended the daycare center and 22 participated consented to participate in the DF trial. Out of the 22 who were participants in the trial, 14 were able to provide data on three occasions. Interestingly, only four were able "adhered as intended". "Adhering as intended," was defined as consuming at least 90% of cereal on a particular day assigned and was determined post-hoc. This was necessary as we could only offer and encourage children to consume the cereal. Admittedly, compliance was only achieved at a relatively low percentage of 20% or an n=4 out of 18 participants. Compliance in the high adherers group of near 100% consumption of DF offered, which corresponded to the treatment structure of 25, 50 and 75% DF recommendation. Furthermore, compliance with DF consumption relative to the amount offered was 84, 66, and 46%, with treatments 25, 50, and 75%, respectively. This corresponded to a compliance with treatment structure of 21, 32, 34% for 25, 50, and 75% treatments, respectively (Table 3.2).

Analysis of dose-response of hydrogen evolution analyzed as iAUC revealed no significant relationship with increasing DF consumption ($p = 0.48$) amongst all participants (Figure 3.2). However, a significant difference ($p \leq 0.05$) was observed between individuals for gas evolution. Although not significant, "high adherers" did not show the same between individual significance ($p = 0.125$). In addition, hydrogen gas evolution due to consumption of DF was not found to be significant ($p = 0.30$) in the "high adherers" group, but numerical significance was much closer to the significance required for null hypothesis rejection ($p = 0.05$) (Figure 3.3).

Analysis of dose-response of methane evolution analyzed as iAUC resulted in no significant relationship with increasing DF consumption ($p = 0.25$) amongst all participants. Similar to results obtained with hydrogen evolution, a significant individual effect was found ($p \leq 0.05$). When the "high adherers" were analyzed, similar results were seen as reported with all participants with non-significance due to DF consumption ($p = 0.25$). Individuals were still significant in methane evolution ($p \leq 0.05$).

Total hydrogen ppm produced as calculated from methane and hydrogen production revealed a significant relationship with increasing DF consumption ($p \leq 0.01$) amongst all participants for individual. However, remained highly non-significant for DF consumption ($p = 0.59$). When only the high adherers were analyzed all significant differences were removed for the individual and the treatment ($p = 0.22$ and 0.24, respectively).

In populations there are individuals who are reported as non-producers of hydrogen gas. Typically, they are tested with a known gas producer or fermenter (lactulose) to determine if hydrogen gas is produced. Although, tested as free living individuals in response to cereal DF consumption, 100% participants were able to evolve at least one spike of 10 ppm of hydrogen. Clinically, of more importance is the measure of methane production. Methane was produced 98% of the time, which translates to one occasion. When viewed as average production of methane over time, three children (16%) on 8 occasions (16%) produced less than 1 ppm. These results are interesting given other published studies. However, these results are the first in free-living pre-school children with no known health conditions.

Discussion

To our knowledge, evidence of DF fermentation in healthy free-living pre-school children has not been reported previously. The null hypothesis that increasing DF dose does not have an increasing response with breath hydrogen and methane evolved was accepted. This study shed light on the feasibility of studying the fermentation of DF in the human large intestine and more specifically the developing pre-school child in a gentle, non-invasive method.

Carbohydrate escaping digestion in the large intestine can be detected in as low as 2-5 g [41] and in a dose-dependent manner in tightly controlled experimental settings [46]. Some studies have also suggested that gas produced *in-vitro* showed little distinction between mass of sugar fermented [46, 47], however it is logical that the stoichiometry may influence the fermentation [48] in regards to total hydrogen produced. Studies in adults have found that consumption of non-digestible carbohydrate leads to an increase in breath fermentation detection [49, 50]. In addition, one prior study has found similar results in young children (3-8) who consumed different cereal products [51].

In our study, amongst all the participants, there was a significant individual effect on gas evolution. This agrees with past literature suggesting the detection of gasses is highly variable between subjects [42, 52, 53], but also within subject [53, 54]. However, we did not observe significant increases due to fiber consumption in either light adherers or high adherers groups. Previously, published studies have shown that fiber content the day before may play an important role in next day breath excretion [55] and may have contributed to our inability to detect a dose-response relationship. Measurement time was approximately 7 hours for the

participants in our study, and 6 hours is considered a minimum, but breath evolution may not return to basal values within 14 hours [56]. However, when studying children for this length of time has some practical challenges that must be considered. Due to the fact that our participants were free-living subjects, it is likely that other factors played a role in large variation.

Interestingly, the group labeled "high adherers," meaning they consumed the DF as intended in the protocol did not produce significant results by individual, but treatment variation was much closer for total hydrogens to be significant (p = ~0.22), albeit still not significant. There is an increasing stair-step trend with increasing DF intake for hydrogen, methane and total hydrogens (Figure 3.3). Furthermore, there were no significant differences in the light adherers groups as the iAUC remained relatively constant, despite treatment (Figure 3.2). This was likely due to the fact that the high adherers were not able to comply with the treatment protocol (Table 3.2). There was no measured reason to believe that these individuals were different from that of the larger population in any descriptive traits. It is possible to speculate that the variation due to individual was marginalized relative to an increased precision in treatment variation, but still at relatively high levels. Nevertheless, the numerical trends noted in this study in combination with the reduced p-value with increasing compliance suggest that linear response may exist.

All individuals produced at least 1 ppm methane in our study. There was only one occasion when no methane was produced or 2%. The mean basal methane production was 2.84 ± 2.53 ppm. The methane results are not surprising for studies that we have conducted in our laboratory, but the values are not consistent with previous literature. Previous research indicates that methane is generally produced within a relatively constant percentage of a population [57, 58]. Traditionally, methane production has been consistent around 34% averaging 15.2 ppm [59]. Participants in this study are different than previously reported as 98% of our tests were positive for > 1 ppm produced. Three individuals averaged less than 1 ppm over all time points measured. This still leaves 84% of samples positive for methane production. One published study suggests that ethnicity should be considered [60], it should also be noted that 88% of some populations have been noted to produce methane [61]. However, presence in children is more variable [44, 62]. Taken together, it is possible that children have a different methane evolution patterns than adults. More research needs to be conducted in children to better determine the prevalence to methane producers as it has been linked to health outcomes [63, 64]. Regardless, the measurement of methane with translation to total hydrogens produced (Figure 3.5) illustrated

slightly different fermentative pattern that could be useful in future studies [43], particularly if followed through to baseline [42].

The concept of non-producers, that is those who do not produce over 10 ppm of hydrogen after lactulose load, are also a constant percentage (2-10%) in the population ([60, 61, 65]). All of our participants produced at least 10 ppm. Low hydrogen evolution is considered to be an enhanced utilization of hydrogen in these individuals [48, 66, 67]. In young children, such as those of pre-school age the development of an adult profile is desired. This has been considered a critical stage in gut colonization just after weaning [68]. Measures in this age group could be highly variable based on the early colonization succession. However, our results did not differ from those in the population suggesting that pre-school age children should have no inhibition of hydrogen production. These results suggest that children as young as three are capable of producing hydrogen and thus, have another measure from which to non-invasively study this age group.

This study was limited by the small sample size of participants who completed the study as intended. Post-hoc sample size analysis revealed that increasing the sample size to 30 participants would provide 0.753 power to detect significant differences in a similarly conducted study. In addition, due to the nature of this study in free-living individuals many factors of control was not implemented to avoid potential confounders. Studies with children should consider the development of the child in the planning of the experimental design. Children have three major categories of development to keep in mind that could potentially influence resultant individual variability. In our discussions, children have "commonalities," interindividual variability, and intraindividual variability [69]. A study should be designed with these differences in mind in order to get a precise measure of individual variation. In future studies it would be important to control for, and provide the previous nights' dinner. In addition, the test day's snacks and meals should also be controlled for size and food types, which are in accordance with low fermentation patterns. This should be coupled with an extended period of monitoring. Due to the location of the study (child care center) we were restricted in the amount of control we could provide. This was important to reduce the burden on parents, children, and the child care center and accommodate as "normal" of conditions as possible. Future studies should be conducted to determine the time needed to differentiate DF fermentative patterns in both children and adults. Also, more studies need to be conducted to determine the length and

number of intervals that provide more consistent estimate of changes in fermentative pattern in response to DF.

Summary

Nevertheless, this trial offered some positive outcomes; we found the breath test to be apparently acceptable to preschool children, parents and teachers. In most cases it was an important learning experience and could be integrated with classroom curriculum. There are concerns of safety from parents and teachers when dealing with children; however, we felt the interaction was beneficial for both groups and not detrimental through observation and personal communication, although this was not quantitated. When working with children it is important to invest the time getting familiar with all parties involved and developing clear methods and goals. We did not see significant difference in acute breath hydrogen, methane or total hydrogen breath evolution response to dietary fiber dose. This was a simple non-invasive measure that provided innovative data to a group that has not been traditionally studied, potentially due the many difficulties associated with young child research. Standard methodology for carbohydrate fermentation and interpretation of metabolic outcomes does not exist, but does have potential for helping to characterize fermentation and provide insight into gut health. Our results should be taken with caution as they are the first of their kind, but do highlight the ability to study pre-school children in an apparently feasible manner.

Acknowledgements

I would like to acknowledge all of the parents and teachers involved with Stone House Development Center, Kansas State University who made this possible with their time and help. Thank you, Dr. Bronwyn Fees and Sally Meyer, who were more that welcoming with their time and expertise. Without your help this research would not have been possible. In addition, I would like to acknowledge the College of Human Ecology for awarding of the Doctoral Fellowship Award, which was used in the funding of this research.

Table 3.1. Population Characteristics with Recommended Energy and Dietary Fiber Intakes

	N	Age (SD) y	Ht (SD) m	Wt (SD) kg	Energy Needed (SD) kcal/day	IOM Fiber Rec (SD) g/day	Age + 5 Fiber Rec (SD) g/day
Boys	9	4.3 (0.7)	1.07 (0.08)	18.7(3.2)	1501 (143)*#	21.0 (1.2)*#	9.3 (0.7)~
Girls	9	4.2 (0.8)	1.05 (0.06)	16.9 (2.1)	1469 (89)*#	20.6 (1.2)*#	9.2 (0.8)~
Overall	18	4.3 (0.7)	1.06 (0.07)	17.8 (2.8)	1486 (117)*#	20.8 (1.6)*#	9.3 (0.7)~

***** IOM 2006 Food and Nutrient Guidelines

PA level of low active assumed

Age + 5

~ Guideline

Table 3.2. Participant Compliance by Group

		Average Fiber Consumption (g)	Fiber Consumption (%)	Percentage of Recommended Intake
All Participants (n=18)				
	25%	4.5 ± 1.2	87.9 ± 20.8	22 ± 5.3*
	50%	7.4 ± 3.7	73.2 ± 35.5	35.8 ± 17.5*
	75%	9.3 ± 5.2	59.2 ± 31.5	44.5 ± 23.9*
High Adherers (n=4)				
	25%	5.3 ± 0.4	100 ± 0.0	25.3 ± 0.3*
	50%	10.4 ± 0.4	97.9 ± 4.2	49.4 ± 2.5*
	75%	16.0 ± 1.0	100 ± 0.0	75.7 ± 1.4*
Light Adherers (n=14)				
	25%	4.3 ± 1.3	84.2 ± 22.6	21.0 ± 5.7*
	50%	6.5 ± 3.8	65.6 ± 37.6	31.7 ± 18.1*
	75%	7.0 ± 3.8	45.6 ± 23.4	34.1 ± 17.5*

*As recommended by IOM at 14 g/1000 kcal.

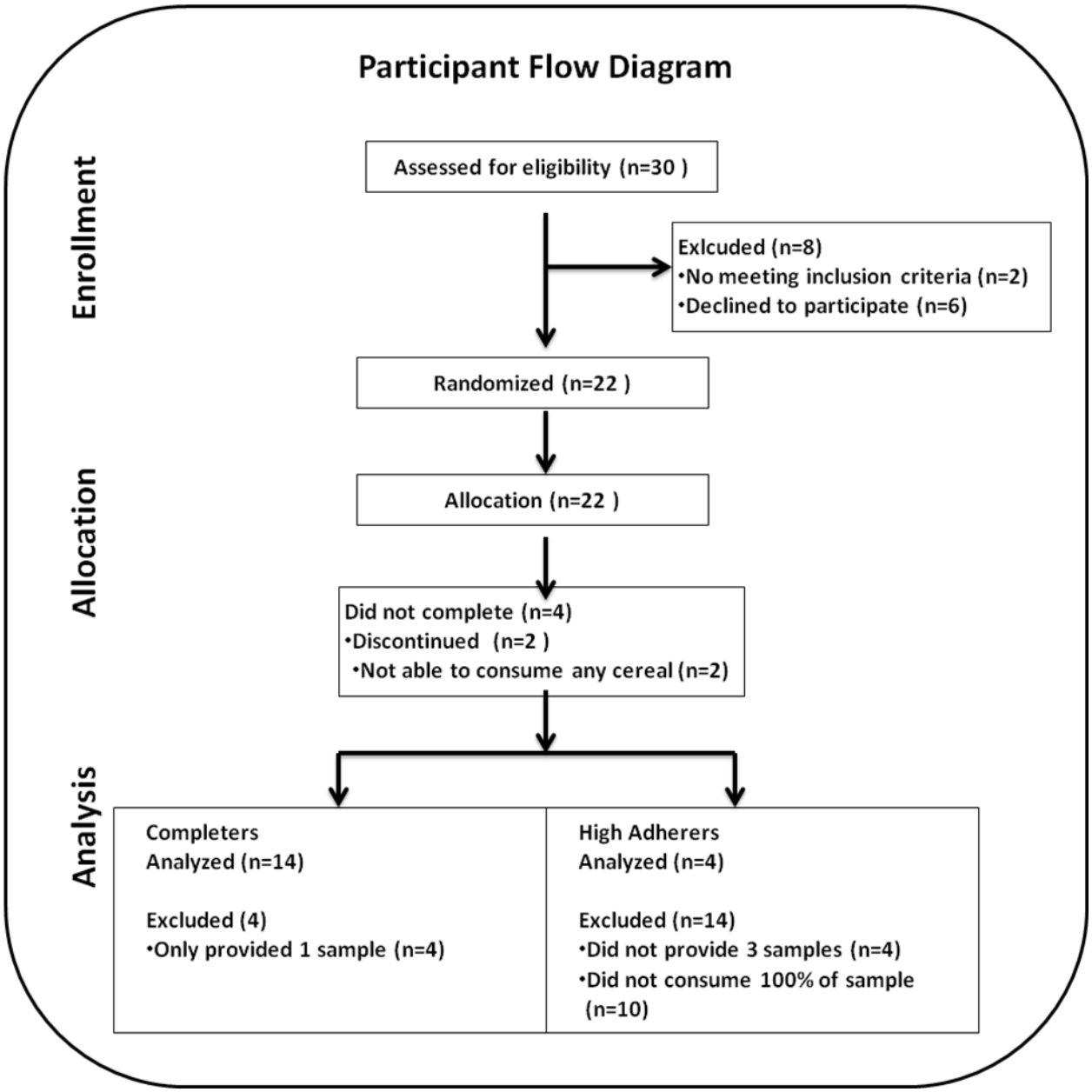

Figure 3.1. Participant Flow Diagram

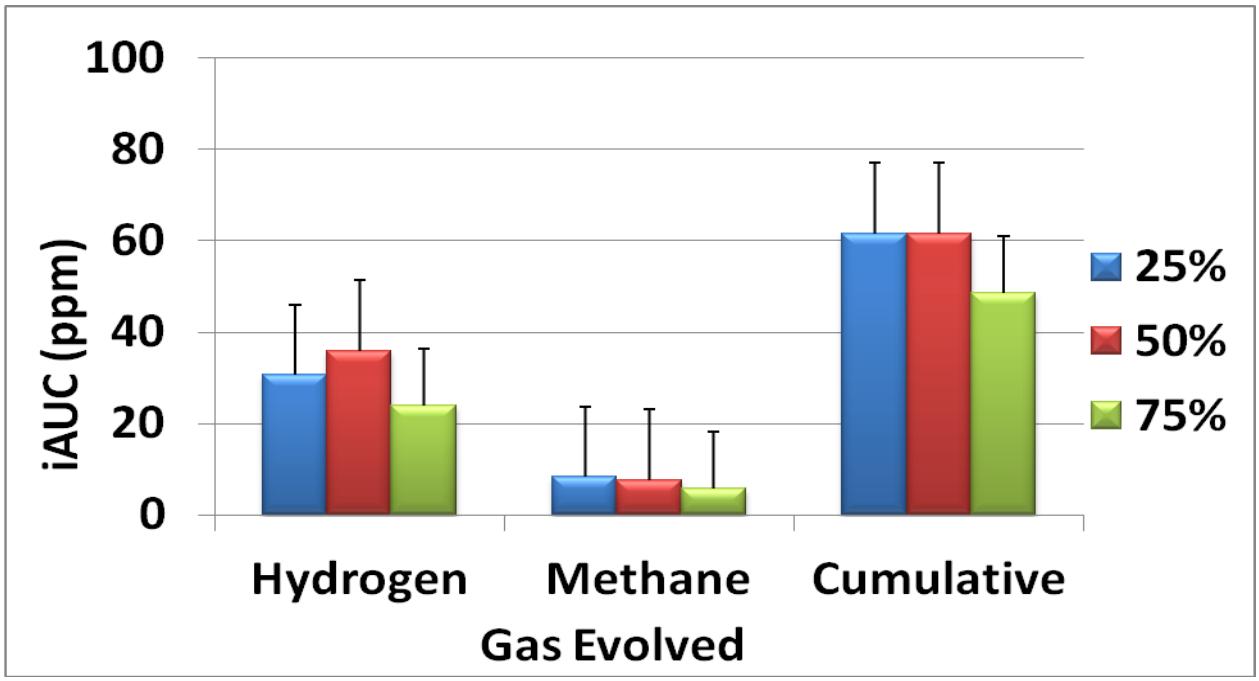

Figure 3.2. iAUC for Hydrogen, Methane, and Cumulative Hydrogen Evolution in Light Adherers

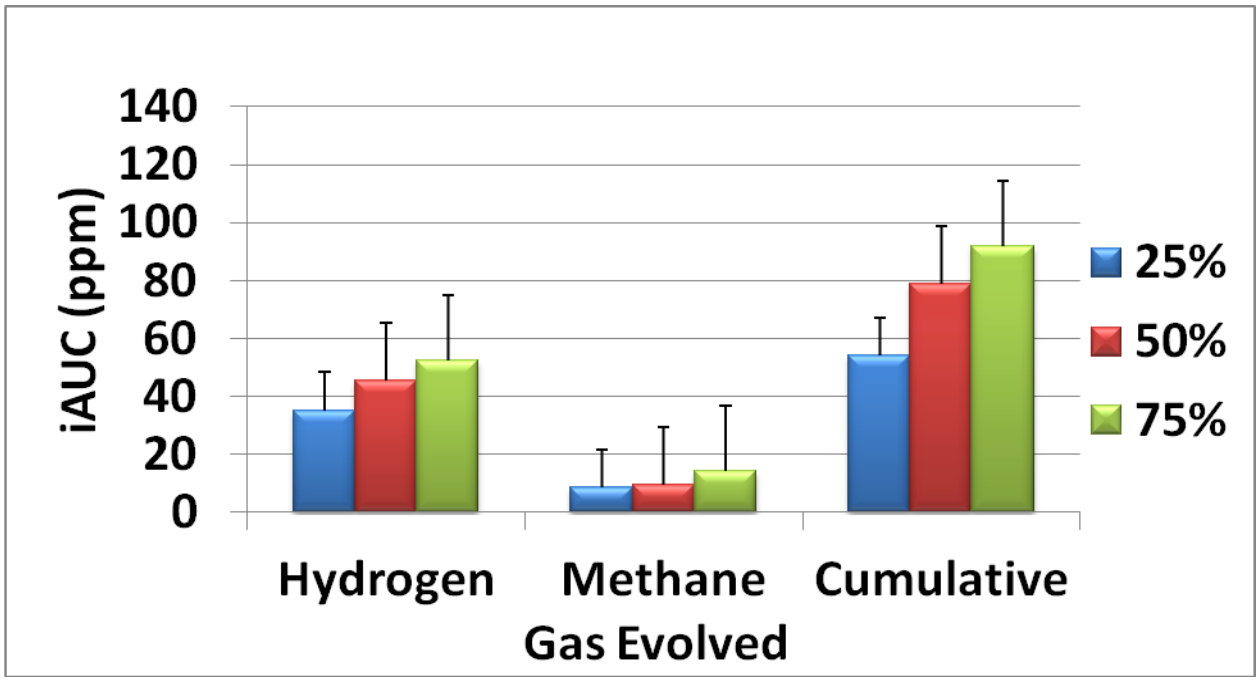

Figure 3.3 iAUC for Hydrogen, Methane, and Total Hydrogen Evolution in Light Adherers

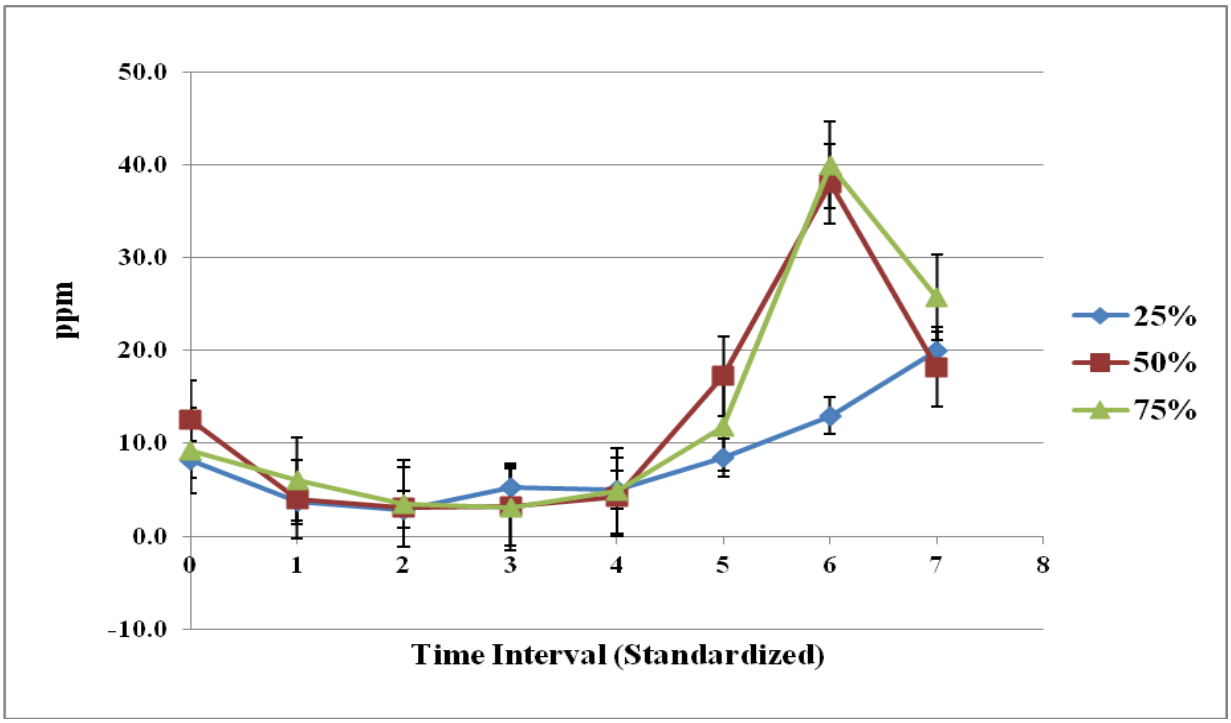

Figure 3.4 Hydrogen (ppm) Breath Evolution over Time in High Adherers

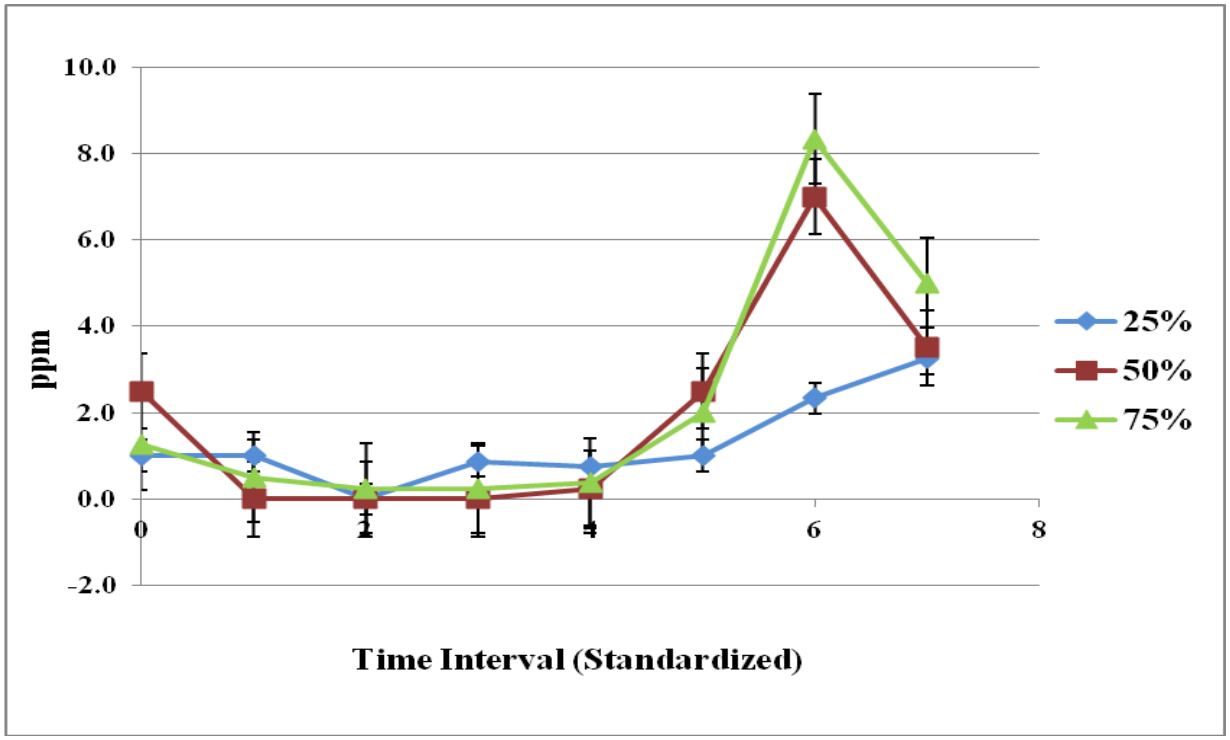

Figure 3.5 Methane (ppm) Breath Evolution over Time in High Adherers

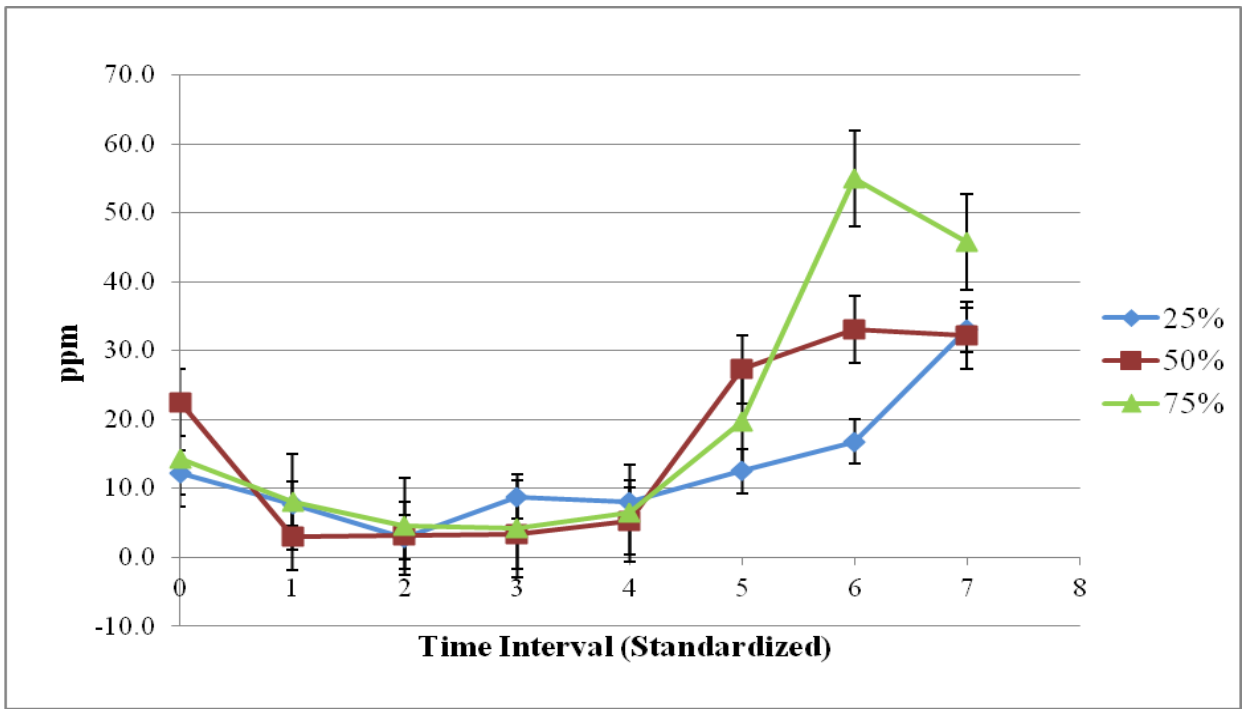

Figure 3.6 Calculated Total Hydrogen (ppm) Breath Evolutin over Time in High Adherers

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Chapter 4 - Metabolic Effects of Fiber Consumption Between Adults and Children

Abstract

Background: Children consume dietary fiber (DF) at less than half the current Institute of Medicine (IOM) recommendations, and guidelines for children are extrapolated from adult studies. Little evidence exists in children to assess the impact of fiber fermentation in the gut. **Objective**: The objective of this research was to investigate the metabolic consequences of DF consumption in children and adults and determine if children and adults utilize DF similarly. **Design:** Children and their parents were fed 10 g of fiber daily for six days a week, over a three week period. Baseline and post measures of breath hydrogen, methane, oro-cecal transit time, time to peak, fecal pH, and fecal short chain fatty acids were taken. Weekly measures of bowel habit and symptoms diaries were also taken. The design was a randomized block with a split plot for time. **Results**: Fecal propionic acid increased (p<0.05) from 106.7 to 127.5 μmol following DF consumption. There was also an interaction (p=0.05) between time and age for butyric acid. Bloating decreased (p=0.05) from week one to week two. **Conclusions:** Dietary fiber consumption as an increase of 10 g/day over three weeks is well-tolerated in this form, with no disturbances in bowel habit in either children or adults. Alternatively, there were no improved bowel habit measures with increased DF consumption. This study yields evidence that adults and children may process breath fermentation measures similarly. Current methodology allows for apparently feasible study of young children in a non-invasive and cost-effective manner, but the current measures did not show distinct child and adult differences.

Introduction

Dietary Fiber (DF) is an indigestible food which contains a unique mixture of bioactive compounds encompassing vitamins, minerals, phytochemicals, antioxidants, and resistant starches (1). Epidemiological and clinical evidence on DF and whole grain indicates that consumption is inversely associated with obesity (2), type-two diabetes (3), cancers (4) and cardiovascular disease (5). Increased intake of DF has been associated with positive health outcomes, suggesting that a lack of consumption may decrease the potential health benefits. In addition, DF is metabolically active in regards to large bowel health, and is associated with outcomes involving constipation and colonic disease (6).; however, due to the observational nature of these studies, conclusive evidence is needed to provide insight on fiber's health benefits (7). In fact, it has been suggested that all of the health benefits associated with DF sources such as whole grains, could be derived from fermentation of DF by the bacteria present in the large intestine (8, 9).

Part of the inconsistent results reported with DF consumption and its health implications can be attributed to its ambiguity in definition and quantification. In a strict technical sense, DF has been labeled as "unrealistic" to define (10). A specific definition is still the source of debate today (11, 12) with no agreed consensus, even within leading health organizations in the United States. Fiber is only measured by AOAC approved methods and thus, fiber content is dependent on those methods (12). These large conceptual issues have contributed to the difficulty in delineating specific effects of DF in adults, let alone in children. Current recommendations suggest that children and adults require the same amount of fiber relative to total energy consumption and evidence is lacking to confirm or refute this idea (13).

Measuring the impact of fiber *in-situ* is very difficult and only a few studies have been carried out in humans (14). Moreover, much of the current knowledge regarding DF implications on metabolism is due to animal and *in-vitro* models of fermentation (15, 16). In humans, limited evidence has been provided through the use of ileostomy and intubation studies (17-19), however these should be considered with their inherent limitations (17). Thus, it is important when investigating mechanisms in humans, especially children, that non-invasive and gentle measures are utilized. Non-invasive metabolic markers that have been associated with health outcomes

exist and can help to provide insight into DF impacts in the human gut. These measures include: fecal weight (20), fecal energy (21), short chain fatty acids (SCFA) (22), fecal pH, breath hydrogen testing (17, 18, 23), and recently genetic identification of gut microflora (24, 25). The importance of microbial fermentation may not be fully understood and has been targeted as the sole benefit of whole grain and DF consumption (8, 26).

Clinical evidence is needed to verify observations regarding DF consumption and to determine any metabolic impacts in the gut. The objective of this research was to investigate the metabolic consequences of DF consumption in children and adults and determine if children and adults utilize DF similarly. The hypothesis was increasing DF consumption by 10 g/day in children and adults would increase breath and fecal measures of fermentation. The null hypothesis was that children and adults would show no differences with respect to maturity or time over these measures.

Materials and Methods

Participants

Parents and children were recruited from the Kansas State University (Manhattan, KS) community through e-mail list-serves and fliers. Fliers were attached to e-mails or handed out at local pre-schools, childcare centers, and through University announcements. Interested parties met with researchers to review complete protocols with both the parents and the children before signing consent/assent forms. Exclusion criteria were treatment with antibiotic within one month of the study, vegan, a history of gastrointestinal disorder, and dislike of cereal products. Eight parent/child combinations consented to participation in the study, with a total participation of 16 individuals. One pair was excluded from analysis for failure to meet study expectations. This study was approved by the Institutional Review Board of Kansas State University (IRB #6091).

Test Sample

Participants were asked to consume a commercially available cereal product (Kellogg's Fiber Plus, Cinnamon Oat Crunch, Kellogg's) that contains approximately 1 gram of fiber as measured by TDF (Nutritional Facts Label) for every 3.5 g of cereal. The cereal is primarily whole grain oat flour with soluble corn fiber and corn bran as the main ingredients. Providing fiber from this source allowed a feasible and appealing method for fiber delivery to young

children that was also suitable for adults. Participants were provided 10 g TDF or 35.5 grams of cereal and asked to consume the amount provided each day and avoid carry-over or "doubling up."

Study Design

The study was designed to investigate the change in colonic microflora fermentation over three weeks between adults and children with increased DF intake of 10 g/day, providing near the IOM recommended dose of fiber. Three weeks has been shown to be sufficient for measuring fermentation changes (25, 27, 28). Parents and children provided baseline measurements and were asked to consume the cereal for twenty-one days. Participants were allowed one "free day" out of every seven days to help ensure compliance. In addition, participants completed weekly symptom diaries each week as self-reported bowel movement, discomfort, flatulence, bloating, stool consistency, and overall general well-being. Responses were marked on a scale of 1 to 5 (1 being optimal/normal and 5 worst/abnormal).

Study Variables

Measurements were taken at the Foods and Nutrition Laboratory at Kansas State University. Participants were measured at baseline for height and weight, in addition to three day diet record (3DR) to record food intake. Measurements collected at baseline and post fiber treatment were oro-cecal transit time (OCTT), fecal short chain fatty acids (SCFA), fecal pH, and total hydrogen/methane production at 4 hours post lactulose load after an overnight fast. Weight was assessed using Ohaus ES 200L/Series scale (Ohaus, Corporation, Parsippany, NJ) with measures occurring in duplicate and a third measurement was taken if readings were more than 0.1 kg apart. Height was assessed via a Seca wall-mounted stadiometer. Measures were taken in duplicate and were repeated if they were more than 5 mm apart. Averages were reported for both height and weight with standard deviation and were used to calculate their body mass index (BMI).

Food Record

Participants were instructed to report their food intake utilizing the 3DR. Intakes were only recorded at baseline to represent typical intake at the time of the study and assumed to be relatively similar throughout the duration of the study period. Parents were instructed to record

the intake of two weekdays and one weekend day for both themselves and their children. They were encouraged to choose consecutive days, but also choose days that would be the most representative of habitual intake. Proper completion of the 3DR was orally discussed with all parents. In addition, written instructions detailing examples of the proper information and way to record foods were given to each parent. An example diet record was provided as an example of details to record about foods. Participants were encouraged to e-mail and call at any time if questions arose. These assessments were primarily used to determine the total macronutrient make-up, caloric intake, and DF consumption of each participant.

Breath Hydrogen Test

Oro-cecal transit time, total hydrogen (ppm), total methane (ppm), and total H^+ (ppm) were measured via the breath hydrogen test (BHT) over 4 hours at 0, 30 45, 60, 75, 90, 105, 120 minutes and every half hour up to four hours. Lactulose was given based on its ability to resist small intestinal digestion, thereby providing a reliable and repeatable source of fermentable substrate (23, 28, 29). Participants were given 0.25 g lactulose / kg body weight, up to a max dose of 10g. We felt it was important, especially in children to provide a size based approach to dosing in order to truly evaluate fermentative capacity. To our knowledge only one other study has provided evidence for this size-based approach and was further evidence for our methodology (30). Determination for OCTT was determined as the first time marked by three consecutive rises in breath hydrogen that cumulatively are $\geq$ 10 ppm, or two consecutive rises where the cumulative is $\geq$ 20 ppm total (31, 32).

Breath samples were extracted from the GaSampler reusable collection bag (Quintron Instrument Co, Milwaukee, WI, QT00841-P) with Monoject 35 mL plastic syringe (Quintron Instrument Co, Milwaukee, WI, QT-01735-V) fitted with a 1-way plastic stopcock (Quintron Instrument Co, Milwaukee, WI, QT01727-V). A total of 30 mL was extracted from the reusable collection bag and injected within two-hours of collection into the BreathTracker SC (Quintron Instrument Co, Milwaukee, WI, QT05000-M). Participants received their own collection bags and were the only individuals to use those bags throughout the duration of the study. Total H_2, CH_4, and CO_2 were recorded for each measure and were normalized according to CO_2 of 5.5%, which is a valid way of correcting for dead space and atmospheric contamination (40). To ensure samples were not diluted, dead-space air was eliminated by calibrated air discard sac.

This allowed for the disposal of up to 750 ml of dead space air. Once the blow-off sac was full, air was automatically diverted through a one-way valve for collection of alveolar air or end-expiratory air (23).

During the test, participants came in after a 12-hour fast. Participants were instructed to consume a low fiber meal and were presented alternatives when necessary. The same meal was consumed the night before each test. When the previous condition was not possible, a low fiber meal was consumed in its place. Participants were also advised to consume light meals with no snacking after supper and no alcohol. During the test, participants were to be awake, refrain from caffeine, smoking and exercise. Due to the age of the participants, easily digestible snacks were provided in less than 200 calories to prevent passing of carbohydrate beyond the small intestine. The same snack was consumed in the pre and post trial to ensure both test were treated equally. Hydrogen and methane values were used to calculate incremental area under the curve (iAUC), hydrogen, total methane, and total H_2 ppm. The lowest value achieved prior to a sustained rise in breath hydrogen was defined as the basal hydrogen level (23), which was also applied to methane and cumulative H_2 atoms. Basal hydrogen level was then used as the baseline for iAUC calculation.

Bowel Habits/Symptoms Diaries

Symptom diaries were self-reported by participants and parents verbally explained the survey for children and reported their responses. Symptoms/habits measured included bowel movement, discomfort, flatulence, bloating, stool consistency and general well-being on a scale from 1 to 5 (1 being optimal/normal and 5 worst/abnormal) as previously reported (25).

Stool Collection and Preparation

S Stool was collected within 3 days prior to the first study day and immediately prior to study completion. Stool was received within one hour of voiding and processed within two hours. If samples were not immediately prepared and stored, they were placed in 7°C until processed within the two-hour time limit. Samples were homogenized manually with a Sterileware (Bel-Art Products) sampling knife. In a 50 mL Corning Centrifuge Tube, 27 ml of Phosphate Buffered Saline (PBS) were added. To the PBS, 3 g of stool were added, and the mixture was homogenized via vortex for approximately 30 seconds to one minute. Cryovials

(Fisherbrand, 2ml) were filled with approximately 1.5 ml homogenized stool/PBS slurry. Samples were stored in -80°C until further processing.

Fecal pH

Fresh fecal samples were packaged with sterileware® plastic knife (Bel-Art Products) in bulk Nalgene® Specimen Collection Cup 15 mL Cryogenic Screw-top vials (Thermo Scientific). Samples were subsequently thawed and two grams were mixed in a 1:5 dilution with distilled deionized (DD) water and homogenized for pH (Accumet pH Meter 25). Measurements were reported as average of triplicate repeated measures.

Fecal SCFA

Fecal SCFA samples were diluted in a 1:5 w/v with double distilled water and homogenized and prepared according to previous works (22). Briefly, samples were homogenized for 3 min and adjusted to pH 2-3 with 5 M HCL, and kept at room temperature for 10 min with gentle agitation. Modifications to match equipment include placing the homogenate in a clean polypropylene tube and centrifuged for 10 min at 3,500 rpm. Supernatant was then transferred to 2.0 ml microcentrifuge vials and centrifuged for 10 min at 8,000 x g. The clear supernatant was then transferred and spiked with 2-ethybutyric acid as an internal standard. The final concentration of 1mM and 1 µL was injected for analysis. Standard stock solutions were prepared with aqueous concentration of 400mM for acetic acid, propionic, and butyric acid. Stock solutions were stored at -20°C.

Chromatographic Conditions

Analysis was carried out using Agilent 6890 GC, equipped with a flame ionization detector (Agilent, USA) and PALcombi-xt (LEAP, Lake Elmo, MN) autosampler. The column was a Nukol™ FUSED SILICA Capillary Column (SUPELCO, Bellefonte, PA, USA) with dimensions of 30 m x 0.53 mm i.d. coated with 0.50 µm film thickness. Methods used have been previously described (22), but nitrogen was replaced with helium as the make-up gas at a total of 20 mL/min. Data recording and anaylsis was carried out with HP ChemStation Plus software (A.10.02, Agilent).

Statistical Analysis

The design was a randomized block design with a split-plot. The split-plot was time and the whole plot was age. The blocking factor was pair or family, which consisted of one parent and one child. Results for all measures, except bowel habits/symptoms diaries, were analyzed with the MIXED procedure in SAS 9.2 (SAS Institute Cary, NC) with a significance of $p = 0.05$. Measures for the bowel habits/symptoms diaries were analyzed as repeated measures for time (pre, middle, post) in the MIXED procedure in SAS 9.2 to a $p = 0.05$. Means, ranges, and standard deviations were reported for descriptive statistics.

Results

Population Characteristics

A summary of baseline characteristics for children and adults used in this study can be found in Table 1.1. Children ranged in age from 3.9 to 7.9 y with an average age of 5.4 y. The BMI for children ranged from 14.5 to 19.5 with an average of 16.4. Energy intake in children was 1312.0 ± 194.0 kcal/day, Estimated Energy Requirement (EER) was 1561.0 ± 77.2 kcal/day, DF intake was 9.9 ± 2.3 g/day, and the AI for DF intake was 21.9 ± 1.1 g/day. Adults ranged in age from 33.2 to 49.8 y with an average age of 38.8 y. Adults had a BMI range from 19.7 to 31.4 with an average of 23.5. Energy intake in adults was 2067.0 ± 106.0 kcal/day, the EER was 2446.1 ± 487.3 kcal/day, DF intake was 20.5 ± 7.3 g/day, and the AI for DF was 34.3 ± 6.8 g/day

Bowel Function and Intestinal Symptoms

Data for bowel symptoms and intestinal function are present in Table 1.2. For bloating there was a main effect for time with bloating being higher ($p < 0.05$) during week one than week two. There was a trend ($p = 0.06$) for flatulence to be affected over time with flatulence being higher during week 1 and 2 than week 3. No other differences were noted for bowel function and intestinal symptoms.

Fecal Short Chain Fatty Acids and pH

Fecal short chain fatty acid and pH main effects are presented in Table 1.3. There was a main effect ($p < 0.05$) for time propionic acid to increase ($p < 0.05$) from pre to post measurement following dietary fiber consumption. No differences were seen for acetic acid or

butyric acid due to time or age. However, there was an age trend ($p = 0.08$) for acetic acid with children possessing numerically higher levels of acetic acid than adults. No differences were observed for pH due to time or age. There was an age by time interaction ($p < 0.05$) for butyric acid. Adults post DF consumption and children pre DF consumption had lower ($p < 0.05$) butyric acid levels than children post DF consumption (Table 1.5).

Breath Fermentation

There were no differences ($p = 0.05$) in breath fermentation tests pre DF to post DF consumption, and there were no significant differences in breath fermentation test between adults and children. However, there was a trend ($p = 0.0565$) for an age by time interaction for fasting breath hydrogen. Adults pre DF consumption had a lower ($p = 0.0565$) fasting breath hydrogen than adults post DF consumption and children in both pre and post DF consumption (Table 1.5).

Discussion

The IOM reports the average DF consumption in the United States to be approximately 15g/day (13). The adults in our study averaged approximately 5 g more per day than the IOM reported average. Initial reports of DF consumption in children were around 12 g/day (33, 34) in older children (>10 y) and approximately 11 g/day (35) in pre-school children (3-5 y). Using data from nationally representative surveys, such as the Continuing Survey of Food Intakes for Individuals (CSFII) and National Health and Nutrition Examination Survey (NHANES), Williams (36) put together a table which shows DF consumption over the last 40 years around 9 g/day for young children (< 3 y) and about 11 g/day when including older children (3-7 y) (37) which has also been recently confirmed in this age group (37). In the present study, children (range 3-7 years old) consumed 9.9 ± 2.2 g/day, which is about on par with past studies. Consumption of 10 g/day extra DF as provided in this study should have put both adults and children near the IOM recommendation for DF intake. However, it should be noted that we did not measure overall DF at the end of the study only at baseline. Participants consumed 10 g/day of DF as provided and was assumed to be additional intake.

In Chinese children (3-7 years old), 10 g/day has been suggested as a "cut-off" amount for amelioration of constipation (38). Improvement in bowel habit has been observed with increasing fiber intake from 11 g/day to ~17 g/day in children consuming a fiber snack. The

addition of 5 g/day of glucomannan in constipated children increased parent rated improvement in "feeling better" than when not on fiber treatment (39). There was no change with the increase of 10 g/day in bowel movements/habits as measured in this study. While there was no improvement there were also no negative outcomes associated with this measure.

Dietary fiber was added to diets at a density of 1 g fiber per 3.5 grams cereal, which equates to 35 grams of cereal or approximately ¾ of a cup (a recommended serving). The cereal provides about 3.44 kcals/g in energy or ~12 kcals/g of energy of fiber. This contrasts the less dense and current recommendation of 14 g/1000 kcals or 71.4 kcals/g energy per g of fiber. Participants in this study likely fell short of recommendations with a relatively dense supplement in an apparently agreeable form. These are important numbers to consider as optimistic estimations of fiber consumption in our study would reveal 19.9 g/day in children, and 34 g/day in adults. It is possible that increased fiber consumption through high DF supplementation in the form of a commercial cereal product contributed to overall dietary intake increases. However, it is also possible that this relatively small amount of cereal displaced DF throughout the rest of the day. This does not seem likely that all DF could have been replaced and indeed DF was increased throughout the trial. Providing DF in this dense form in suggest that supplementation could be important to meeting DF guidelines. Moreover, this also sheds light into the practical implications of consuming the IOM recommended amount by traditional food alone, especially in regards to children in this age group.

Functional differences have been noted between healthy and non-healthy populations, but are not well understood. Furthermore, studies have evaluated the relationship of DF consumption and its changes with development of functional constipation. This is important because constipated children may be consuming less fiber for other reasons (i.e. stomach pain from compaction, other organic nature) than children who are healthy and fiber is less likely to be uncomfortable to consume. In our study, there were no changes in response to an extra 10 g/day of fiber regarding bowel habit and intestinal health, except bloating between week 1 and week 2. Bloating significantly decreased from 1.50 to 1.07 between week 1 and 2, suggesting that by week three participants were able to adapt to increased DF as no differences were noted. In addition, since general well-being or discomfort remained unchanged, this likely did not cause significant distress to the participants. There was only one verbalized or communicated discomfort reported in reference to cereal consumption, which was increased flatulence, which

can be only be classified as a trend in this study and warrants further attention. Indeed, discomfort due to flatulence may be of increased importance that was not conclusively detected in this study. In patients with carbohydrate malabsorptive disorders, the rapid fermentation by the gut microbiota often leads to discomfort (29). However, this amount of fiber did not seem to impart any major discomfort that affected the participans well-being.

Fiber is known to affect laxation as increased intake has been associated with increased bowel movements (1, 39, 40). However, data for the present study did not provide any measureable difference that would contribute to this observation. However, direct measures of stool output or any other objective measure to confirm the self-reported measures were not assessed in the present study. The effects of fiber may not be fully understood and may be different between healthy and children with abnormal GI function. In fact, medical doctors are cautious to prescribe fiber during functional constipation due to the complex nature of the diagnosis and the inconclusive evidence presented (41). Further evidence has shown that the intestinal transit may be a more localized problem leading to constipation than DF consumption and DF may have a longer transit time through the left colon in those with constipation (42). Additionally, it is known that children who have developed functional constipation have abnormal sensitivity of the sigmoid colon (43). In order to determine the interaction of fiber in preventing, treating or other roles it needs to be determine if these differences are a biological determinant of abnormal bowel habits or if abnormal food habits contribute to this development. More evidence is needed to fully understand the interactions of DF intake and the large intestinal response.

There is major emphasis on understanding the role of microbiota in the large intestine and the interactions that occur between the two (44). Bacteria in the large intestine ferment DF when it reaches the large intestine or utilizable substrate that reaches the colon (45). Major microbial fermentation by-products include: hydrogen, methane, carbon dioxide, and short chain fatty acids. These microbial metabolites have been linked to a host of health outcomes with technology rapidly allowing us insight into how the specific bacteria (1) and substrate are interacting to yield measurable outcomes which can be related to health (46). We did not see any significant differences between adults or children in SCFA in response to 10 g/day DF consumption. In this study, fecal propionic acid was the only SCFA to change in response to DF consumption over the three week time period. Increases in propionic acid have been reported in

other studies (34) over time in response to DF supplementation. However, other studies have reported no changes in SCFA due to changes in DF consumption (25).

Although not statistically significant, butyric acid showed a large numerical increase, accompanied with a large standard deviation. It has been previously reported that fecal SCFA seem to be highly dependent on the individual (47). Also, a recent study evaluating gut microbiota and fecal SCFA found that propionate was associated with overweight and obese individuals (48). In the present study, the men's average BMI was approximately 26 kg/m^2. More research should be conducted to verify this association, but perhaps the participants that were overweight contributed to the higher levels of propionate acid. Many factors influence SCFA production such as flow rate of digesta, microflora composition and intrainteraction, and total substrate, thus these variables need to be studied in these populations to understand if there are environmental factors mediating this result. In the present study, inherent biological variation in the sample population could have led to the large variation and potentially this variation could be overcome with larger sampling. Large biological differences in individuals could be driving these differences, as there are many factors influencing the fecal outputs including, colonocyte absorption, pH, microflora, and diet. The present study was designed to observe effects relative to age and over time, which were only significant for propionic acid. This suggests that indeed parents and children may respond the same to fecal outputs of SCFA due to DF consumption.

In addition, there was a significant interaction, where butyric acid in post DF consumption of children was increased above those of adults post DF treatment. The major component of this commercial cereal fiber was whole grain oat flour. Addition of oat fiber, specifically beta-glucan, has been associated with lower cholesterol in both adults and children. In addition, it is the only fiber to be an approved health claim with oats. With fermentation of RS and oat fibers, butyrate has been found to be produced in greater amounts *in-vitro* (59) and *in-vivo (47)*. This is not surprising as the role of butyrate as a source of fuel for the colonocytes is well documented (1, 49). Child profiles of gut microbiota reach those similar to adults at various rates and depend on many variables starting in infancy (50), however, similarities are seen after weaning (51). As the same amount of DF was provided to both adults and children in the present study, it is possible the butyrate remaining for fecal measurement remained due to a relatively larger load, which the child's colonocytes were not able to fully utilize. All SCFAs

seem to be rapidly absorbed, consumed or utilized in first-pass metabolism. Perhaps, propionic and acetic acids were able to be utilized more completely. However, the difficulty of understanding the full scope of metabolic fates of fecal SCFA has been discussed before (52). More research is needed to determine if this interaction is consistent, and if so provides evidence that adults and children may have different uptake capacities, as this cannot be determined from fecal SCFA output.

Fecal pH has been linked to many health outcomes. In our study there were no significant differences between children and parents or pre and post DF consumption. Numerically, there was a separation between pre and post DF consumption. Post fiber consumption resulted in a mean pH of 7.26 ± 0.14 versus 7.03 ± 0.14 ($p = 0.14$). A reduction in colonic pH would coincide with increased SCFA production and fermentative rate. A significant difference would help indicate that fecal SCFA are cumulatively increasing and yield some insight to levels required for colonic absorptive capacity being reached. As increasing fermentation would increase SCFA, they would likely be subsequently absorbed. This absorption is likely traded with HCO_3^-, which would help buffer colonic pH. Thus, it would make sense that absorptive capacity would be reached with increasing SCFA production. At that point, pH would lower and fermentative reactions would start to slow as pH will start to inhibit some metabolic enzymes for the microflora. In order for pH to remain measurably lower it would seem logical that the left colon is less efficient at absorbing SCFA or once a potential absorptive capacity is hit the microflora are well adapted to resume fermentation at the slightest increase in pH as each enzyme is suited. No significant difference in pH suggests that parents and children may not have differences in pH and instead are set by environmental and biologically differences as opposed to developmental differences.

The gut is a continuous reaction of changing inputs and outputs. Decreasing colonic pH from 6.5 to 5.5 has been shown to restrict growth (53) of certain species and promote others. This is thought to be primarily driven through SCFA synthesis. In obese rats, increased energy intake was found to decrease colonic pH (53, 54). Increasing SCFA, such as propionate as in the present study, should decrease pH. This lends credence that increased cecal load, not obesity itself, as a cause of decreased fecal pH. However, this would still have important ramifications if an autochronous microflora exists and we can identify an indigenous microbiome. This also suggests evidence that adjusting nutrient intake and substrate (nutrition) may have significant

public health implications. In our study, energy intake was relatively low compared to recommended intake, as well as fiber. Yet, there was no significant change and overflow of non-digested nutrients into the colon would not seem to be a problem in this case. Indeed, more research is necessary as limited evidence exists and there is likely a large genetic component with each individual.

Many studies have tried to look at the significance of breath hydrogen as a marker of carbohydrate fermentation. Few studies have investigated the fermentation of indigestible carbohydrates in healthy individuals (55, 56). Although breath fermentation has been found to be limited in the ability to quantify exact amounts of carbohydrate fermented (17, 23); it has been found to be an effective relative marker of fermentation (23). In addition, increasing fermentation has been found to be reproducible and repeatable (23). Load of substrate has been found to be proportional to the output of gasses in the breath (57, 58). We did not observe any significant increase in capacity to handle lactulose, an easily fermentatable sugar, in our study with hydrogen, methane, total hydrogen produced, or OCTT. This is similar to other studies, looking at similar outcomes (27), but the design of this particular study restricted the present study to four hours of sampling, which may have limited discrimination between particular fermentation patterns.

A trend in fasting hydrogen values of adults after DF consumption was observed, which was not present in children. This may provide evidence to the hypothesis that children may handle the same fermentative load differently than adults, as they were able to remain stable with fasting concentrations. However, it does seem plausible that the microbiome of the large intestine of adults is more fully capable of fermenting the available fuel, because a larger diversityis typically found (51). Thus, in children residual DF may be escaping fermentation (59). This is likely as children are known to incompletely utilize easily fermentable starch (60) and would have more difficulty utilizing a more chemically protected fermentative substrate such as oat fiber.

This study was exploratory in nature and its relatively small sample size limits generalizability or representativeness, in addition to statistical power. In addition, this study was limited by having few documented studies available to help guide the research methods. Although previously published, the bowel habits/symptoms diaries should be investigated for validity. Alternatively, this was the first study to report metabolic outcomes in children and

adults regarding three weeks of DF consumption. The design of this study allowed for statistical control of uncontrollable environmental factors within familial pair. This study also provides insight to understand the use of non-invasive metabolic markers of health in children that can be used to improve understanding of DF implications to gut health.

Conclusions

This study provides novel evidence of metabolic markers related to DF consumption in children and utilized gentle, relatively rapid, non-invasive measures to characterize the impacts of DF on the child gut. Adding 10 g/day of DF in commercial oat cereal to the participants' diet had no positive or negative bowel habit/intestinal symptoms over three weeks. Free-living adults and their children did not show significant differences in selected markers of DF fermentation iAUC's, or fecal SCFA's (acetate and butyric) and pH. The presence of interactions of DF with butyrate response and a trend for acetic acids warrants further study to determine if these differences are repeatable and perhaps explain biological differences between adults and children. A larger study of these variables is warranted, especially between adults and children to understand potential genetic and environmental variation. This study provided evidence that children and adults in general do not handle DF differently relative to the variables measured. However, there were several trends, both statistical ($0.05 > p < 0.15$) and numerical that do warrant further study in a larger group to elucidate if further differences could be seen. Understanding the relationship between DF fermentation in the child intestinal tract through non-invasive, gentle and relatively inexpensive methods seems to be a viable option for assessing dietary factors on child health outcomes.

Acknowledgements

Thank you to the parents and children who participated in my study, always interested and engaging. Thanks to Jianteng Xu (David) and Kavitha Penugonda for their expertise in related equipment. Funding was provided by the United States Department of Agriculture (Hatch KS#xxx) and donations made to the Food and Metabolism Laboratory.

Table 4.1 Baseline characteristics (n=7) of parent and children pairs participating in the consumption of 10 g commercial cereal fiber with IOM recommended energy and fiber requirements.

	Age (y)	Ht (m)	Wt (kg)	BMI	Energy Intake (kcal)	Fiber Intake(g/day)	Rec EER (kcal)	Rec Fiber (g/day)
Children								
Female (n=1)	4.0	1.05	15.5	14.5	1174.9 kcal	10.7	1467	20.6
Male (n=6)	5.6 ± 1.7	1.14 ± 0.07	21.6 ± 2.5	16.7 ± 1.8	1334 kcal ± 202	9.8 ± 2.5	1576 ± 72	22.1 ± 1.0
Overall	5.4 ± 1.7	1.13 ± .08	20.7 ± 3.2	16.4 ± 1.8	1312 kcal ± 194	9.9 ± 2.3	1561 ± 77	21.9 ± 1.1
Adults								
Female (n=3)	36.3 ± 4.3	1.63 ± 0.06	53.4 ± 2.6	20.1 ± 0.4	2154 kcal ± 395	22.3 ± 11.2	2066 ± 164	28.9 ± 2.3
Male (n=4)	40.7 ± 6.8	1.79 ± 0.10	84.6 ± 24.5	26.0 ± 4.8	1979 kcal ± 545)	18.64 ± 9	2732 ± 451	38.2 ± 6.3
Overall	38.8 ± 5.9	1.72 ± 0.11	71.2 ± 24.1	23.5 ± 4.6	2067 kcal ± 106	20.5 ± 7.3	2446 ± 487	34.3 ± 6.8

* All values represent the mean ± standard deviation.

Table 4.2 Main effect for time of commercial cereal fiber consumption on bowel function and intestinal symptoms

Sympotoms Diaries	Week 1	Week 2	Week 3	St. Error	p-value
General Well Being (1-5)	1.93	1.86	1.93	0.28	0.73
Flatulence (1-5)	2.57	2.36	2.00	0.37	0.06
Abdominal Pain (1-5)	1.21	1.14	1.43	0.16	0.18
Bloating (1-5)	1.50[a]	1.07[b]	1.21[ab]	0.14	0.03
Stool Consistency (1-5)	3.43	3.43	3.36	0.22	0.89
Change in Bowel Movements (1-3)	1.86	1.93	1.93	0.08	0.73

1= excellent or no symptoms, 5= poor, or extreme symptoms
[ab] Values with different superscripts are considered significantly different ($p < 0.05$)

Table 4.3 Main effect for age with commercial cereal fiber consumption on fecal short chain fatty acids and pH

Fecal Measures	Adult	Child	St. Error	p-value
Acetic Acid	28.46	29.59	0.42	0.08
Propionic Acid	112.67	121.54	5.94	0.27
Butyric Acid	133.91	160.48	27.78	0.51
pH	7.11	7.17	0.17	0.17

[ab] *values with p=0.05 are considered significantly different for adult vs child, and pre vs post consumption*
Values of SCFA are in µmol

Table 4.4 Main effect for time with commercial cereal fiber consumption on fecal short chain fatty acids and pH

Fecal Measures	Pre	Post	St. Error	p-value
Acetic Acid	28.77	29.28	0.41	0.38
Propionic Acid	106.71	127.49	5.94	0.02
Butyric Acid	136.11	158.28	21.76	0.26
pH	7.26	7.03	0.14	0.14

[ab] *values with p=0.05 are considered significantly different for adult vs child, and pre vs post consumption*
Values of SCFA are in µmol

Table 4.5 Main effect for age with commercial cereal fiber consumption on breath fermentation and OCTT

Breath Values	Adult	Child	St. Error	P-value
Time Peak Hydrogen	171.43	147.86	0.87	0.37
Time Peak Methane	139.29	121.07	16.33	0.45
Fasting Hydrogen Conc.	3.71	4.43	0.88	0.41
Hydrogen iAUC	4758.04	3283.88	960.89	0.23
Methane iAUC	1304.27	836.93	257.47	0.14
Total Hydrogen ppm iAUC	6115.14	4122.88	1100.52	0.15
OCTT	72.86	64.29	6.19	0.35

Table 4.6 Main effect for time with commercial cereal fiber consumption on breath fermentation and OCTT

Breath Values	Pre	Post	St. Error	p-value
Time Peak Hydrogen	158.57	160.71	15.58	0.87
Time Peak Methane	135	125.36	16.63	0.69
Fasting Hydrogen Conc.	3.5	4.64	0.85	0.12
Hydrogen iAUC	4497.95	3543.96	885.43	0.27
Methane iAUC	1089.63	1051.57	257.47	0.9
Total Hydrogen ppm iAUC	5559.52	4678.5	1050.04	0.4
OCTT	68.57	68.57	4.52.45	1

Table 4.7 Significant interaction effects for age and time following commercial cereal fiber consumption.

Interaction Effects	Adult Pre	Adult Post	Child Pre	Child Post	St. Error	p-value
Butyric Acid	144.85^{ab}	122.97^{a}	127.37^{a}	193.59^{b}	30.77	0.04
**Fasting Hydrogen*	$*2.42^{a}$	$*5^{b}$	$*4.57^{ab}$	$*4.29^{ab}$	*1.0025	*0.0567

[ab] Interactions with different superscripts represent significantly (p = 0.05) different responses

* Fasting Hydrogen values were only a trend, but express what numerical differences might have been present

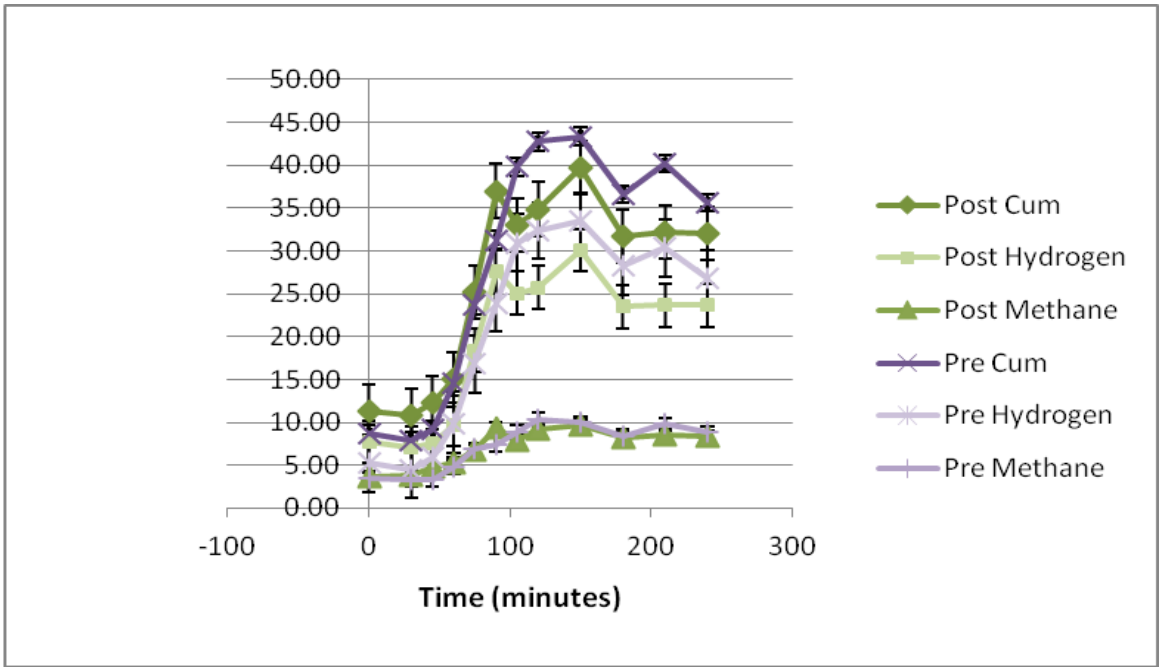

Figure 4.1 Pre-Post Pattern of Breath hydrogen, methane, and total hydrogen's (ppm) produced over time.

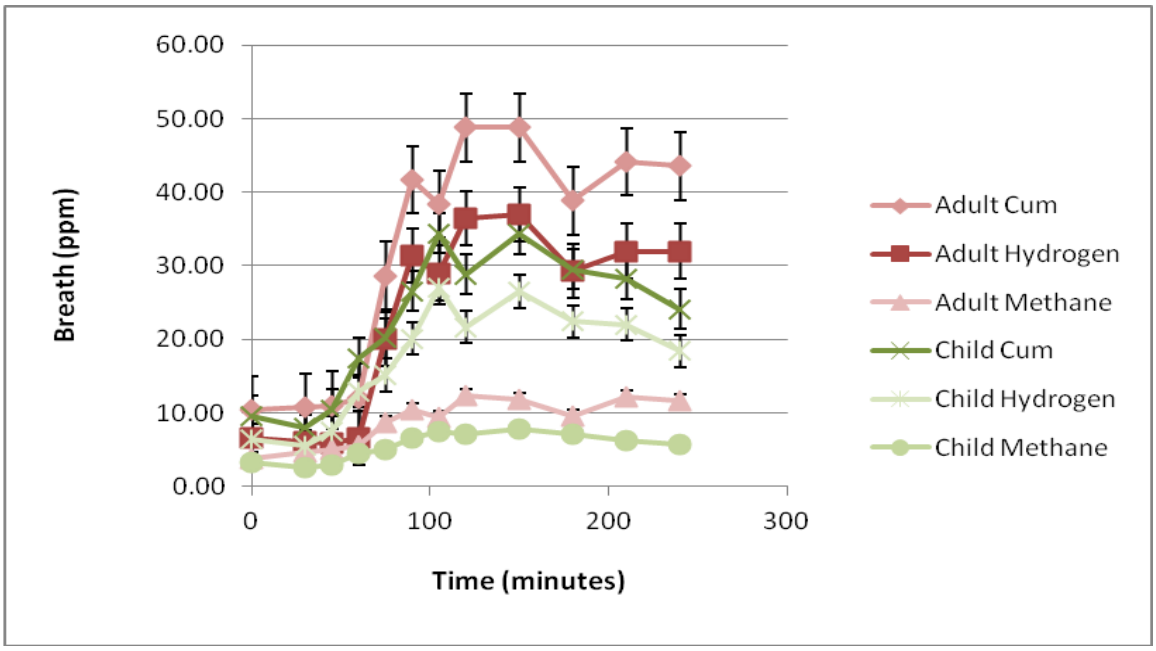

Figure 4.2 Adult vs Child pattern of Breath hydrogen, methane, and total hydrogen's (ppm) produced over time.

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Chapter 5 - Metabolic Implications of Fiber Consumption: The Link Between Gut Microflora and Health

Introduction

Ever increasing attention is being aimed towards children [1] to provide a healthy base in nutrition as recommended by IOM, intending to prevent associated problems as children are following the same path as their adult counterparts [2]. Fiber has been identified to help prevent and treat obesity in adults [3], as well as youth [4-6]. Clinical evidence in children is limited [7-10] allowing for the adjudication of observational studies. However, many hypotheses have been generated looking into the mechanisms to better understand the role DF may play in, "Health." It has been suggested that all of the health benefits due DF sources such as whole grains, could be derived from fermentation of DF by the bacteria present in the large intestine (8, 9).

Although, there are many hypotheses that have been postulated as the mechanisms for fibers' health implications, gut bacteria have played a relatively small one until recently. It seems likely that many mechanisms play a role in fibers health promoting characteristics, but the complexity is only beginning to be understood. Current attributes bestowed upon fiber may indeed be interactions mediated by the gut microflora. A major role for DF, among many others, is to provide fuel for bacterial fermentation in the large intestine. These bacteria possess enzymes that allow for their utilization of materials not processed by the human small intestine. It has been estimated that 60-70 g of carbohydrates are necessary to maintain the mass of colonic microflora found in the large intestine (LI) [11,12]. These bacteria are being researched for their ability to impact health, specifically: metabolism, physiology, immune function, and disease development [13-18].

The Institute of Medicine (IOM) recommends the consumption of 14 g/1000 kcals, which approximates about 38 g for the Reference man of 154 lbs. This represents roughly half (54.3%) of the estimated mass necessary for microflora homeostatis. Research suggests, the rest of that gap is likely filled by starch. Current estimates of starch malabsorption estimate approximately ~10-20% of starch escapes digestion [19,20]. Essentially, any component resistant to digestion, becomes a source of food for the LI [21]. Thus, these indigestible components of food may provide a significant tool for dietary modulation of gut microbiota [22]. The proper categorization and identification of DF to include these components is important to progress towards accurate

measurement and would add greatly to our ability to translate that information into guidelines. It seems likely that DF could be the major direct or indirect contributor to fuel delivery to the large intestine and potentially, the single most important factor of gut modulation.

The idea of altering the gut microbiota has recently been a hot topic in nutrition and public interest. Prebiotics and probiotics have been investigated for their ability to impact health on many levels [23-25]. Prebiotics are the substrates necessary to provide fuel for microbial growth. Probiotics are the delivery of the live biological organisms, themselves. These are not exclusive to adults, as the potential to provide a healthy base via pre and probiotics in infants [26] has also been reviewed. It stands to reason that we could consume a particular food substance; this substance will fuel certain bacteria, and thus, alter our health for the better. To take this a step further, in theory we could prepare children for the best possible nutritional start in life, from breast feeding (a significant source) through weaning and beyond. However, there is still a large gap in knowledge allowing us to delve in this train of thought, including the idea of an "ideal bacterial composition."

The idea of a "normal" or "indigenous flora" is not new [27] and seems difficult to characterize. These bacteria that should reside in our LI historically have been coined autochthonous microbiota [27]. More recently, the complexity of this concept coupled with the human body has taken on another term, cohesive with the technology that has allowed this insight, known as "microbiome" [28]. The name given to the concept that both the human and the bacteria in the LI have a vast genomic diversity that work together (almost one), assumedly for health or lack of it. It is presumed that we can modulate this ecology for the improvement of health. Improvements would come from both direct and indirect effects of the bacteria. Direct effects are likely to result in interactions of the bacterial cells with the host through immunological response. These responses can range from toll-like receptor (TLR) activation[29], to simply preventing pathogens adhesion[30]. Indirect effects are the focus of this study, which include the by-products from bacterial metabolism and their interaction with our LI and body at large.

Research has shown microflora that reside in the LI are detected in different concentrations based on dietary modulation, which in turn would presumably alter the by-products produced. Changes have been noted in response to resistant starch (RS) [31,32] and oligosaccharides (OG) [33,34]. As bacterial populations and genera shift, this results in changes in

the metabolic by-products that are produced. These fermentative products include short chain fatty acids (SCFA), lactate, which concurrently lowers pH. Reducing pH subsequently lowers conversion to secondary bile acids, phenols, and ammonia concentrations[22,35,36]. The SCFA commonly produced are acetate, propionate, and butyrate [36]. In addition to a lower LI pH, they can be utilized by the colonocytes as fuel and absorbed into the portal blood for utilization by the body. Recent research is suggesting a prominent role in SCFA production and absorption affecting blood metabolic markers of disease, such as blood triglycerides (TG) [37].

In addition DF has been implicated with bowel dysfunction. The major dysfunction that has been associated with fiber intake is constipation. Intake of DF has been found to be lower in children presenting with constipation, than unconstipated children [38,39]. In addition, DF intake has been associated with colon cancer, diverticular disease, and oro-cecal transit time (OCTT)[21,40]. This seems logical as increasing intake of DF has been associated with increasing stool weight and evacuation frequency [21]. Recently, the hydrogen breath test (HBT) has been utilized to measure OCTT [41,42]. Measuring the OCTT has been found to be an important indicator of bowel function and motility. Thus, could provide meaningful contribution to the assessment of gut function.

Based on the lack of knowledge towards this area and the presumption of control, we set out to contribute to a base of knowledge to investigate the application of these concepts. Understanding how DF affects the children is important [43-45] and more specifically the gut. Current recommendations for children are the same as adults 14 g/1000 kcal [46]. Children often have higher metabolic requirements and thus make the current level of fiber level quite high, potentially unrealistic. Children are different in regards to nutritional requirements and anatomical considerations. Anatomical consideration may be especially important when considering DF consumption. This study intends to provide information discussing feasibility of non-invasive measures to study DF metabolic interaction in the bodies of children. The objective of this study was to determine the impact of 10 g of DF supplementation for 21 days on gut community analysis by pyrosequencing and their correlation to metabolic markers of fermentation. This will be the first study that we know of to provide a snap-shot of the gut microflora relative to non-invasive metabolic indicators of DF fermentation in the gut. The hypothesis was that gut bacterial communities will shift after three weeks DF treatment and will

show significant differences between child and parent, and pre and post dietary fiber consumption.

Materials and Methods

Participants

Parents and children were recruited from the Kansas State University (Manhattan, KS) community through e-mail list-serves and fliers. Fliers were attached to e-mails or handed out at local pre-schools, child care centers and through University announcements. Interested parties met with researchers to review complete protocols with both the parents and the children before signing consent/assent forms. Exclusion criteria were treatment with antibiotic within one month of the study, vegan, a history of gastrointestinal disorder and dislike of cereal products. Eight parent/children combinations consented to participation in the study, with a total participation of 16 individuals. One pair was excluded for analysis for failure to meet study expectations. This study was approved by the Institutional Review Board of Kansas State University (IRB #6091).

Test Sample

Participants were asked to consume a commercially available cereal product (Kellogg's Fiber Plus, Cinnamon Oat Crunch, Kellogg's) that contains approximately 1 gram of fiber as measured by TDF (Nutritional Facts Label) for every 3.5 g cereal. The cereal is primarily whole grain oat flour with soluble corn fiber and corn bran as the main ingredients. Providing fiber from this source allowed a feasible and appealing method for fiber delivery to young children that was also suitable for adults. Participants were provided 10 g TDF or 35.5 grams of cereal and asked to consume the amount provided each day and avoid carry-over or "doubling up."

Study Design

The study was designed to investigate the change in colonic microflora over three weeks between adults and children with increased DF intake of 10 g/day, providing near the recommended dose of fiber per day by the IOM. Three weeks has been shown to be sufficient for measuring fermentation changes 47-49. Parents and children provided baseline measurements and were asked to consume the cereal for twenty-one days. Participants were allowed one "free day" out of every seven days to help ensure compliance. In addition, weekly

symptom diaries were completed by participants each week as self-reported bowel movement, discomfort, flatulence, bloating, stool consistency and overall general well-being. Responses were marked on a scale of 1 to 5 (1 being optimal/normal and 5 worst/abnormal).

Study Variables

Measurements were taken at the Foods and Nutrition Laboratory and KSU. Participants were measured at baseline for height and weight, in addition to Three Day Diet Record (3DR) to record food intake. Measurements collected at baseline and post fiber treatment were oro-cecal transit time (OCTT), fecal short chain fatty acids (SCFA), fecal pH, fecal bacteria as measured by 454 pyrosequencing and total hydrogen, methane production at 4 hours post lactulose load after an overnight fast. Weight assessed using Ohaus ES 200L/Series scale (Ohaus, Corporation, Parsippany, NJ) with measures occurring in duplicate and a third measurement was taken if readings were more the 0.1kg apart. Height was assessed via Seca wall-mounted stadiometer. Measures were taken in duplicate and were repeated if they were more than 5 mm apart. Averages were reported for both height and weight with standard deviation and were used to calculate their body mass index (BMI).

Food Record

Participants were instructed to report their food intake utilizing the 3DR. Intakes were only recorded at baseline to represent typical intake at the time of the study and assumed to be relatively similar throughout the duration of the study period. Parents were instructed to record the intake of two weekdays and one weekend day for both themselves and their children. They were encouraged to choose consecutive days, but also choose days that would be the most representative of the habitual intake. Proper completions of the 3DR were orally discussed with all parents. In addition, written instructions detailing examples of the proper information and way to record foods were given to each parent. An example diet record as well as sample conversions were also provided. Participants were encouraged to e-mail and call at any time if questions arose. These assessments were primarily used to determine the total macronutrient make-up, caloric intake, and DF consumption.

Breath Hydrogen Test

Oro-cecal transit time, total hydrogen (ppm), total methane (ppm), and total H^+ (ppm) were measured via the breath hydrogen test (BHT) over 4 hours at 0, 30 45, 60, 75, 90, 105, 120, 150, 180, 210, and 240 minutes. Lactulose was given based on its ability to resist small intestinal digestion, thereby providing a reliable and repeatable source of fermentable substrate [50-52]. Participants were given 0.25 g lactulose / kg body weight, up to a max dose of 10g. We felt it was important, especially in children to provide a size based approach to dosing in order to truly evaluate fermentative capacity. To our knowledge, only one other study has provided evidence for this size-based approach and was further evidence for our methodology [53]. Determination for OCTT was determined as the first time marked by three consecutive rises in breath hydrogen that cumulatively are ≥ 10 ppm, or two consecutive rises where the cumulative is ≥ 20 ppm total [54,55].

Breath samples were extracted from the GaSampler reusable collection bag (Quintron Instrument Co, Milwaukee, WI, QT00841-P) with Monoject 35 mL plastic syringe (Quintron Instrument Co, Milwaukee, WI, QT-01735-V) fitted with a 1-way plastic stopcock (Quintron Instrument Co, Milwaukee, WI, QT01727-V). A total of 30 mL was extracted from the reusable collection bag and injected within two-hours of collection into the BreathTracker SC (Quintron Instrument Co, Milwaukee, WI, QT05000-M). Participants received their own collection bags and were the only individuals to use those bags throughout the duration of the study. Total H_2, CH_4, and CO_2 were recorded for each measure and were normalized according to CO_2 of 5.5%, which is a valid way of correcting for dead space and atmospheric contamination [56]. To ensure samples were not diluted, dead-space air was eliminated by calibrated air discard sac. This allowed for the disposal of up to 750 ml of dead space air, once the blow-off sac was full, air was automatically diverted through a one-way valve for collection of alveolar air or end-expiratory air [52].

During the test, participants came in after a 12 hour fast. Participants were instructed to consume a low fiber meal and were presented alternatives when necessary. The same meal was consumed the night before each test. When the previous condition was not possible a low fiber meal was consumed in its place. Participants were also advised to consume light meals with no snacking after supper and no alcohol. During the test, participants were to be awake, refrain from caffeine, smoking and exercise. Due to the age of the participants, easily digestible snacks

were provided in less than 200 calories to prevent passing of carbohydrate beyond the small intestine. The same snack was consumed in the pre and post trial to ensure both test were treated equally. Hydrogen and methane values were used to calculate incremental area under the curve (iAUC), hydrogen, total methane, and total H_2 ppm. The lowest value achieved prior to a sustained rise in breath hydrogen was defined as the basal hydrogen level [52], which was also applied to methane and cumulative H_2 atoms. Basal hydrogen level was then used as the baseline for iAUC calculation.

Bowel Habits/Symptoms Diaries

Symptom diaries were self-reported by participants and parents verbally explained the survey for children and reported their responses. Symptoms/habits measured included bowel movement, discomfort, flatulence, bloating, stool consistency and general well-being on a scale from 1 to 5 (1 being optimal/normal and 5 worst/abnormal) as previously reported (31).

Stool Collection and Preparation

Stool was collected within 3 days prior to the first study day and the same prior to study completion. Stool was received within one hour of voiding and processed within two hours. If samples were not immediately prepared and stored, they were placed in 7°C until processed within the two-hour time limit. Samples were homogenized with Sterileare (Bel-Art Products) sampling knife manually. In a 50 mL Corning Centrifuge Tube, 27 ml of Phosphate Buffered Saline (PBS) were pipetted. To the PBS, 3 g of stool were added, and the mixture was homogenized using vortex for approximately 30 seconds to one minute. Cryovial (Fisherbrand, 2ml) were filled with approximately 1.5 ml homogenized stool/PBS slurry. Samples were stored in -80°C until further processing.

Fecal pH

Fresh fecal samples were packaged with sterileware® plastic knife (Bel-Art Products) in bulk Nalgene® Specimen Collection Cup 15 mL Cryogenic Screw-top vials (Thermo Scientific). Samples were subsequently thawed and two grams were mixed in a 1:5 dilution with distilled deionized (DD) water and homogenized for pH (Accumet pH Meter 25). Measurements were reported as average of triplicate repeated measures.

Fecal SCFA

Fecal SCFA samples were diluted in a 1:5 w/v with double distilled water and homogenized and prepared according to previous works [57]. Briefly, samples were homogenized for 3 min and adjusted to pH 2-3 with 5 M HCL, and kept at room temperature for 10 min with gentle agitation. Modifications to match equipment include placing the homogenate in a clean polypropylene tube and centrifuged for 10 min at 3,500 rpm. Supernatant was then transferred to 2.0 ml microcentrifuge vials and centrifuged for 10 min at 8,000 x g. The clear supernatant was then transferred and spiked with 2-ethybutyric acid as an internal standard. The final concentration of 1mM and 1 µL was injected for analysis. Standard stock solutions were prepared with aqueous concentration of 400mM for acetic acid, propionic, and butyric acid. Stock solutions were stored at -20°C .

Chromatographic Conditions

Analysis was carried out using Agilent 6890 GC, equipped with a flame ionization detector (Agilent, USA) and PALcombi-xt (LEAP, Lake Elmo, MN) autosampler. The column was a Nukol™ FUSED SILICA Capillary Column (SUPELCO, Bellefonte, PA, USA) with dimensions of 30 m x 0.53 mm i.d. coated with 0.50 µm film thickness. Methods used have been previously described [57], but nitrogen was replaced with helium as the make-up gas at a total of 20 mL/min. Data recording and anaylsis was carried out with HP ChemStation Plus software (A.10.02, Agilent).

DNA Extraction from Fecal Samples

Fecal Homogenates were thawed and approximately 2 mL were used to recover by centrifugation (8,000 x g, for 5 min at room temperature). Cells were recovered, approximately 0.50 g and were added to MOBIO PowerSoil Kit (MOBIO Laboratories, Inc., Carlsbad, CA, USA). Manufacturer's instructions were followed to complete the extraction. Extracts were checked for quality and quantity using the NanoDrop ND-1000 (ThermoScientific, UV-VIS, Wilmington, DE). Samples were standardized based on concentration and stored at -20°C. Samples were thawed and prepared in 96 well nuclease free plates for the primary PCR reaction as follows. Reaction mixture consisted of 25 µL of Master Mix (AmpliTaq Gold 360 Master Mix, Applied BioSystems, Life Technologies, Carlsbad, CA), 5 µL template (10 ng/ µL), 5 µL primer forward (IDT), 5 µL primer reverse (IDT), and 10 µL NCF H_2O. Forward primer set was

a FC9F-GAGTTTGATCCTTGGCTCAG and the reverse primer set was FC541R-TTACCGCGGCTGCTGG targeting the bacterial 16s rRNA. Thermocylcer conditions were set with an initial denaturing step at 95°C 8 min and lid temperature of 105°C. Cycle conditions with denaturing step of 30 sec, annealing for 1 min at 54°C, and extension for 2 min at 72°C, for 24 cycles. A final elongation step was held at 72°C for 10 min. Samples were frozen in -20 until further analysis. Secondary PCR reaction was performed under the conditions above for 5 cycles to attach barcode and adapter sequencing with GSFLX Titanium reagents for 454 sequencing. Individual samples were amplified and pooled, allowing for mixing of PCR products into a single run. Templates were pyrosequenced at the K-State Integrated Genomics Facility (IGF).

Samples were cleaned using AMPure XP (Agencourt, Beverly, Massachusetts) according to manufacturer conditions. Samples were checked for quantity and quality using the NanoDrop. Samples templates were concentrated for 55 min at on low heat at 1,500 RPM. Samples were cleaned a second time and check with NanoDrop and standardized according to total DNA concentration, samples were stored at -20°C. Samples were Bioanalyzed with 1 µl of template using the DNA 1000 kit for final quality and quantity check before pyrosequencing.

Sequence Processing

Sequences were processed using the mothur community analysis program [58]. Sequences were extracted from the raw 454 data and extracted for fasta, qual, and flow files. Summarizing our fasta file revealed approximately 233,000 sequence reads. Flow files were "quieted" using the translated de-noising algorithm via mothur. Sequences were then trimmed for removal of the barcode and adapters, as well as removal of homopolymers and sequences less than 200 nucleotides in length. Aligned sequences were checked for coverage, richness, and diversity indicators and approximately 450 sequences were used for taxonomic classification at 97%. Sequences were assigned to a bacterial phylum, family, and genus.

Summary

Although, specific interpretable results cannot confidently be documented at this time, the author felt it was important to provide written documentation up to this point. Provided here

is a framework from which to follow for future work. Appendix B is dedicated to providing the basic framework to all of these procedures to allow for future researchers to have a conglomeration of information from which to move forward within the field of human nutrition. Upon publication of the results, the paper and the dissertation can give a complete picture of the experimental process for future guidance.

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Chapter 6 - General Conclusions

There are apparently feasible ways to characterize the metabolic fate of DF in children that are non-invasive, gentle, and cost-effective manner. There was not an acute fermentative response of breath methane and hydrogen from increasing DF dose in free-living children (3-5 y) in our study. However, based on previous studies and the free-living nature of this study, it is possible a dose-response could be detected with a longer follow-up. Further research is necessary to determine the follow-up time and proper interval sampling required to determine DF fermentative characteristics in a free-living population of children, while controlling for fiber intake. Non-invasive markers of fermentation correlate well in *in-vitro* studies, but interpreting *in-vivo* results is challenging and will require more carefully controlled studies from all disciplines to add to the basic functions of the gut. The results of our studies together suggest that the biological variability is large between individuals. We provided some evidence showing little differences between children and adults as assessed by these methods; however, there was also some evidence pointing to the contrary, and a larger study is warranted with fiber-controlled diets. Overall, it is difficult to apply these findings given the variability in the results and the small sample sizes. In addition, there is limited evidence characterizing measureable outcomes of DF fermentation. In free-living individuals, these findings seem to indicate that there are not many differences based on fiber consumption. Dietary fiber requires clarity on definition, measurement, and basic knowledge of the gut in order to improved recommendations. Currently, there seems to be no serious implications of large amounts of DF consumption, minus intestinal discomfort that can be adapted too. This work provides a framework from which to move forward in understanding not only the child gut, but the gut in general.

Appendix A - Chapter 3 Supplemental Materials

DEPARTMENT OF HUMAN NUTRITION

KANSAS STATE UNIVERSITY

PARENTAL PERMISSION SLIP

Measurement of Fiber Intake Using Breath Samples

Principal Investigator: Mark Haub, PhD

Research Assistants: Casey Weber, MS, James Lattimer, MS, Greg Tanquary

Project Information: The perception of dietary fiber as healthy in adults is common. However, the effects in children are poorly understood. There is limited evidence that requiring fiber intake in children will result in improved health outcomes now or in the future. To better understand how dietary fiber recommendations and subsequent changes affect children it is important to have data illustrating how fiber is interacting in the bodies of children. The goal of this study is to non-invasively determine the dose-response of fiber consumption in children by measurement of breath hydrogen and methane content in commonly provided food. The intent is to obtain data that illustrates how fiber affects the body. Particularly, in the developing human gut and translate that to information researchers and food companies can use to assess the potential healthfulness of a fiber intake.

***What's Involved*:** If you choose to participate, your child will be asked to consume a breakfast cereal before coming to Stone House on four occasions and collect a quick breath sample. We will also require that you fill out a form designating the approximate amount cereal not consumed if this was not possible, time of consumption and some information regarding the meal the night before. We will need to know if your child has had gastrointestinal concerns as this study may not be appropriate for them as it may cause stomach discomfort. All materials will be provided to you in advance. In addition, we will then ask if your child has recently been on any antibiotics or gastrointestinal motility drugs as they may affect the results of the study. We will need to ensure a time, perhaps at drop-off or pick-up your child to explain the project in full. Foods that will be provided for your child to consume are all purchased at a local grocery store (cereal) and will be further discussed with you at the time of enrollment. We ask that if you are aware of any allergies (e.g. peanuts) or intolerances (e.g. lactose) your child may have, that you let us know. You will always have the opportunity to withdraw from the study regardless of the reason.

Description of Breath Test: The breath collection and analysis procedure has been most developed for clinical testing for malabsorptive disorders (lactose, fructose), Small Intestinal Bacterial Overgrowth (SIBO), and oro-cecal transit times (time required for food to reach the colon). Bacteria in your body are the only sources of hydrogen and methane production

occurring from fermentation of food in the large intestine. When the bacteria produce these gases, some are released through your breath in much the same manner as CO_2 is release through your breath. Fiber is typically considered to be "resistant" to digestion and will pass into your large intestine largely unaltered. In the large intestine (where bacteria normally reside) any carbohydrate, including fiber that exist will be available for bacterial digestion.

The test will begin the night before as children will need to be fasted for 8-10 hours. Water is permissible during that time, however, food and other beverage consumption may alter the reliability of the test. A baseline breath test will be necessary, followed by cereal consumption. We will then take measures approximately every 30 minutes for 5 hours while your children are completing a typical day's activity at Stone House. After measurements are completed will we be able to see a graphical representation of the Hydrogen and Methane gas released by bacteria in the colon

Anthropemetric Measurement: For anthropometric measurement, we will be assessing Body Mass Index (BMI), by collecting children's body weight using a digital scale, and height using a standard stadiometer. Children will be measured away from other children's line of sight with two technicians and the parents will be encouraged to be present. Measurement of BMI will only occur at one time point. For body composition, we will use a common measure, the Bioelectrical Impedance Analysis (BIA). Children will lie in a comfortable position and a small electrode will be place on the right ankle and right foot. This device will send a very low current to determine how quickly the charge moves through the body. The charge is not noticeable and does not physiologically interfere with the body (same principal as a common bathroom scale). The procedure will allow for estimation body water for calculation of body composition from this measure. The measure will last 2-3 minutes and will be taken only once at the beginning of the study.

Potential Benefits and Concerns: At the conclusion of this project, you will obtain your child's information related to this study. We will also provide a brief interpretation of the information that we obtain. The results of this study will be the first of their kind and will contribute immensely to the impact of dietary fiber in children. The results provide the opportunity for many more projects and idea generation regarding fiber intake and health.

The potential risks are minimal, but may include the following: Gas production that may result in flatulence, belching, and stomach discomfort. We will be inquiring information about normal fiber consumption and your child will be provided less than the recommended amounts of fiber for consumption, which will reduce risks of stomach discomfort.

***Debriefing*:** After the final trial you will receive all results as well as the interpretations of the information obtained. Also, the investigators would be more than happy to answer your questions over the results or any other part of the study at any time.

Alternative Procedures: None. You and your child may decline to participate or withdraw at anytime from this project without prejudice or penalty.

Time Commitment: There will be a slight additional commitment the four mornings before the study. This commitment will entail obtaining an initial breath sample and remembering to provide the cereal to your child the morning of the study. All materials needed

along with any instruction will be provided to you and we will always be available for questions or concerns. Otherwise your day will be normal and the child's routine will be the same as currently provided at Stone House.

Confidentiality: All records associated with your participation in the study will be subject to confidentiality standards applicable to medical records, and in the event of any publication resulting from the research no personally identifiable information will be disclosed. All medical records will be kept confidential, with the possible exception of review by the University Research Compliance Office at Kansas State University – Dr. Rick Scheidt at (785)532-3224.

Right to ask questions: You have been given the opportunity to ask any questions you may have to your satisfaction. If you have any further questions about the study or your participation in it, please contact the following investigators at your convenience.

Mark D. Haub, Ph.D. (haub@ksu.edu) (785)532-0170 -- (785)776-4282 -- (785)320-3330 or
Casey Weber (cweb81@ksu.edu) (785)410-6490 – (785)532-0170

Compensation: You will be given a gift card to Wal-Mart $20 after completion of this study. In case of complications, physical injury, or illness resulting from the proposed research, only acute and essential medical treatment is available. This institution will not provide monetary compensation for wages lost as a result of injury, hospitalization, and/or professional services.

Voluntary Participation: Please understand that your child's participation in this study is **voluntary**, and that you may withdraw from this study at any time by notifying the investigator. Your withdrawal from this study or refusal to participate will in no way affect your care or access to medical or educational services or participation in future studies. Your participation in this research may be terminated by the investigator without regard to your consent if you are unable or unwilling to comply with the guidelines and procedures explained to you.

I understand this project is research, and that my participation is completely voluntary. I also understand that if I decide to participate in this study, I may withdraw my consent at any time, and stop participating at any time without explanation, penalty, or loss of benefits, or academic standing to which I may otherwise be entitled. I verify that my signature below indicates that I have read and understand this consent form, and willingly agree to participate in this study under the terms described, and that my signature acknowledges that I have received a signed and dated copy of this consent form.

→ Please mark one box, sign, and return to the program as soon as possible:

❑ I will allow my child to participate in having their breath, heigh, weight, and body composition. measured and has no known digestive conditions (list of potential conditions can be found below).

❑ I will not allow my child to participate.

Parent Name _____ _____
(Please print) (Date)

Parent Signature _____ _____

Child's Name: _____ Child Date of Birth: _____/_____/_____

Potential Digestive Conditions:

Malabsorptive Disorders

Lactose Intolerance
Fructose Intolerance
Glucose Intolerance

Intestinal Disorders

Small Intestinal Bacterial Overgrowth
Crohn's Disease
Ulcerative Colitis
Chronic Constipation

Questionnaires

Take-Home Survey

In the Past 1 week:

1. Has your child had normal stool this week?
    a. If inclined, please describe (This will increase our interpretation)

2. Has your child required an antibiotic recently for the treatment of an infection, i.e. ear infection, eye infection,

Last Night:

1. Were food any foods consumed during supper last night that may have had fiber in them?
    a. If so, please elaborate on type of food and amount consumed:

2. Was a snack necessary after supper?
    a. If so, please list the snack and amount consumed:

This morning:

1. What was the approximate time your child provided the breath sample? _____
   Was the cereal able to be consumed shortly after this time? _____

2. Was your child able to completely consume the cereal? _____

3. Was milk necessary? If so, what kind? (i.e. Whole, 2%, skim, soy) _____

Further Comments (Please feel free to list any comments or questions you may have):

Thank you very much!

Recruitment Fliers

Participants needed for study involving the understanding of colonic health in children (3-5)

What is involved: The study would require consumption of cereal for three weeks; accompanied by collection of two stool samples and two visits to Justin Hall. Each visit to Justin Hall would require approximately 6 hours. Children will be asked to breathe into a gas collection bag (which is very close to a normal balloon) at regular intervals of 30 minutes during the 6 hours. During collection (10 seconds) periods children will be interacting with research staff and during non-collection periods (rest of the time) children will be entertained via games, movies and other activities designed for their age. The first visit children will be monitored for height and weight for assessment of Body Mass Index (BMI). In addition we will be conducting an analysis of body water to estimate body composition. Estimations of body water will occur with the use a Bioelectrical Impedance Analyzer (BIA); this works according to the same principles found in the common home weight scale. In addition, we will be collecting height and weight values for calculation of BMI. We will require collection of stool samples before consumption of cereal and after consumption. Parents are welcome and encouraged to stay for the duration of the study.

Where: Human Metabolism Lab, Justin 127, Kansas State University.

Why is this research being conducted: Currently, knowledge involved in understanding the role fiber plays in our large intestine is limited in children. Therefore, it is the intent of this research to help increase that understanding. There are bacteria in the large intestine that ferment fiber, when they undergo this fermentation one effect that can occur is that gas is produced and released through normal breathing. This is the same as breathing in oxygen and release of carbon dioxide in our breath. This research intends to measure the quantity of two gases produced (hydrogen and methane) by collecting breath samples. Obtaining an indication of the way gases are released by fiber fermentation we may have another indicator of health, much like a blood sample can tell our cholesterol levels and relate that to heart health. The stool samples are needed to determine the type of bacteria that are present in the stools and to determine how the fiber from the cereal might have interacted with the bacteria in the large intestine.

Who is involved: Researchers from the Human Metabolism Lab, located on the campus of Kansas State University in Justin Hall.

Participation: This flier is simply inquiring on the interest in participating in a study of this nature; you or your child, are not required to participate in this study. In addition, if you would choose for your child to participate in this study, at no time, would the child be required to participate and would have the option to withdrawal at any time.

Interested in more information or participation?

Contact:

Casey Weber, M.S. (ABD) (cweb81@ksu.edu) (785)410-6490 or (785)-532-0170 or

Mark Haub, Ph.D. (haub@ksu.edu) (785)532-0170 or (785)776-4282 or (785)320-3330

Take-Home Materials Provided

Pre-Day Testing Instructions

The Night Before:

Goal: To avoid high fiber, complex carbohydrate containing foods.
Try to have a moderately sized meal
Try to limit food consumption to before 9:00 PM

Example Foods:
(If there is a food your child absolutely loves and will be difficult to give up, please talk to us)

- Boiled White Rice
- Baked Potatoes
- Meats
- Eggs
- Soup broths
- Fruit snacks, gummy, fruit juice
- Lower fiber foods

The Morning of:

- -Water only (Until, cereal)
- -Brush teeth (This is best done before breath test if possible, but is okay if not)

Treatment BagReminder

Reminder (On Sample to take home

1. Rinse mouth or brush teeth
2. Complete breath test
3. Consume cereal, encourage without milk. However, add milk if necessary.
4. Encourage complete consumption (This may not always be possible.
5. Remember to grab K-State bag, with:
    a. Breath Collector
    b. Extra cereal
    c. Completed Survey

Research and Extension Fiber Information Flier

18 Ways to Increase Fiber

Check at least ONE box to do today!

- ☐ Leave the peeler in the drawer when preparing apple and pear snacks and potatoes.
- ☐ Look for the words "high in fiber" and "good source of fiber" on labels.
- ☐ Buy 100% whole-grain breads, crackers, noodles and pasta.
- ☐ Choose bran cereal or oatmeal for breakfast.
- ☐ Try brown rice instead of white rice.
- ☐ Choose a fruit or veggie snack each day.
- ☐ Sprinkle wheat germ on top of casseroles.
- ☐ Mix dried fruit, nuts and bite-size whole grain cereal for a snack.
- ☐ Choose whole foods instead of processed ones.
- ☐ Substitute half whole-wheat flour for half the white flour in recipes.
- ☐ Buy fresh, frozen or canned fruits which are higher in fiber than juice.

(continued on back)

- ☐ Add extra vegetables, cooked beans or lentils to soups, salads and casseroles.
- ☐ Add cooked beans to casseroles, pizza, and sandwiches.
- ☐ Shred carrots on top of a salad.
- ☐ Eat sliced berries with angel food cake or yogurt.
- ☐ Add dried fruits to cereal, breads, cookies and salads.
- ☐ Fill tacos with canned pinto beans.
- ☐ Use legumes as a meat substitute in chili, meat loaf, soups and casseroles.

This material was funded by USDA's Food Stamp Program through a contract awarded by the Kansas Department of Social and Rehabilitation Services.
The Food Stamp Program provides nutrition assistance to people with low income. It can help you buy nutritious foods for a better diet. To find out more, contact your local SRS office or call (800) 221-5689.

Fiber

A great carbohydrate!

- *fruits*
- *vegetables*
- *legumes*
- *whole grains*
- *nuts and seeds*

Eat a variety every day!

Legumes (LEG Yooms) – What are they?

Legumes are seeds or pods that are an excellent source of fiber, such as:

- garbanzo beans (chickpeas)
- black-eyed peas
- baked beans
- kidney beans
- lentils
- navy beans
- soybeans

Why should I eat more fiber?

- Helps prevent constipation.
- Helps lower cholesterol.
- Lowers risk for developing diabetes.
- Helps prevent some cancers.
- Helps control weight—you feel full longer!

Fiber-rich foods are healthy!

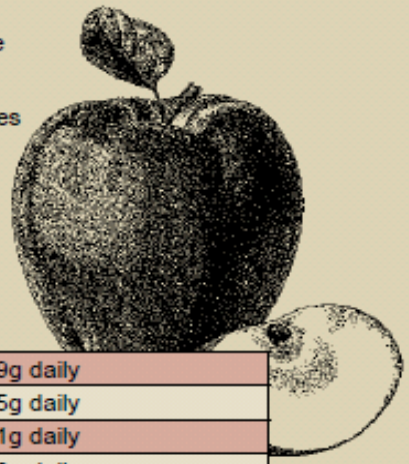
- Eat whole fruit rather than drinking juice.
- Make half your grains whole.

- Eat more fruits and vegetables.
- Try legumes several times each week.
- Eat popcorn, nuts or seeds as snacks.

Circle the food with more fiber:

1 medium unpeeled apple	or	1/2 cup applesauce
1/2 cup mashed potatoes	or	1 baked potato with skin
1 carrot stick	or	1 bread stick
1/2 cup brown rice	or	1/2 cup white rice
1/2 cup strawberries	or	1/2 cup grapes
1/3 cup cornflakes	or	1/3 cup bran flakes
1 ounce cheese	or	1/2 cup peanuts
1/2 cup spinach	or	1/2 cup peas

(answers at bottom)



Fiber Finder–How much fiber do I need?

It depends on your age and gender.

Children	1-3 years	19g daily
	4-8 years	25g daily
Boys	9-13 years	31g daily
Girls	9-13 years	26g daily
Men	up to age 50	38g daily
Women	up to age 50	25g daily
Pregnant and breast-feeding women		slightly more
Men	over 51 years	30g daily
Women	over 51 years	21g daily

Caution: Increase fiber slowly and drink plenty of water to reduce intestinal gas. Too much fiber for young children may take away their appetite for other nutritional foods.

Good news: Fiber acts like a large sponge in the colon. It holds water as it keeps waste moving along. Remember to drink enough fluids so fiber can do it's job. You don't need to eat huge amounts of whole grains, fruits, vegetables and legumes to meet your daily fiber needs. Just follow MyPyramid and your everyday meals and snacks can meet your fiber needs.

Answers to food with more fiber: unpeeled apple, baked potato with skin, carrot, brown rice, strawberries, bran flakes, peanuts, peas

Appendix B - Chapter 4 Supplemental Materials

DEPARTMENT OF HUMAN NUTRITION

KANSAS STATE UNIVERSITY

PARENT & CHILD PERMISSION SLIP

Comparison of Fiber Fermentation between Adults and Children

Principal Investigator: Mark Haub, PhD

Research Assistants: Casey Weber, MS

Project Information: The perception of dietary fiber as healthy in adults is common. However, the effects in children are poorly understood. There is limited evidence that demanding that fiber intake in children will result in improved health. To better understand how dietary fiber recommendations and subsequent changes affect children it is important to have data illustrating how fiber is interacting in the bodies of children. The goal of this study is to non-invasively determine the changes incurred to a set-dose of fiber in both adults and children over the same time period. The intent is to obtain data that illustrates how fiber affects the body of children and adults. Particularly, in the developing human gut and translate that to information researchers and food companies can use to assess the potential healthfulness of a fiber intake, potentially later in life.

***What's Involved*:** If you choose to participate, you and your child and/or adults will be asked to make approximately 2 visits to Justin Hall (Hoffman Lounge) on a prescheduled Thurs, Friday or Saturday. We will need to know if your child (or if you are the participant) has had gastrointestinal concerns as this study may not be appropriate for them (you) as it may cause stomach discomfort. In addition, we will then ask if the potential participant has recently been on any antibiotics or gastrointestinal motility drugs as they may affect the results of the study. We will then set-up a schedule for you to attend two of the above time points for testing. Foods that will be provided for the participants to consume are all purchased at a local grocery store (cereal and milk) and will be further discussed with you at the time of enrollment. We ask that if you are aware of any allergies (e.g. peanuts) or intolerances (e.g. lactose) your child may have, that you let us know. You will always have the opportunity to withdraw from the study regardless of the reason.

Description of Breath Test: The breath collection and analysis procedure has been most developed for clinical testing for malabsorptive disorders (lactose, fructose), Small Intestinal Bacterial Overgrowth (SIBO), and oro-cecal transit times (time required for food to reach the

colon). Bacteria in your body are the only sources of hydrogen and methane production occurring from fermentation of food in the large intestine. When the bacteria produce these gases, some are released through your breath in much the same manner as CO_2 is release through your breath. Fiber is typically considered to be "resistant" to digestion and will pass into your large intestine largely unaltered. In the large intestine (where bacteria normally reside) any carbohydrate, including fiber that exist will be available for bacterial digestion. The test will begin the night before as children will need to be fasted for 8-10 hours. Water is permissible during that time, however, food and other beverage consumption may alter the test. A baseline breath test will be conducted followed by a lactulose drink. We will then take measures every 15 minutes for 2 hours and every 30 minutes for up to 6 hours. After all measurements we will be able to see a hydrogen/methane gas profile and oro-cecal transit time.

Anthropemetric Measurement: For anthropometric measurement, we will be assessing Body Mass Index (BMI), by collecting body weight using a digital scale, and height using a standard stadiometer. Participants will be measured away in Food and Metabolism Lab (FML) with two technicians and the parents will be present in the measurement of the child. Measurement of BMI will only occur at the beginning of study.

***3 Day Diet Records*:** Participants will need to fill out a 3-Day Diet Record one time before the onset of the study. This will be used to assess diet and the amount of fiber each individual receives. The purpose for the use of these extensive forms is because they are the most representative method available to assess food intake. In this way we will assess two weekdays and one weekend day with the goal of obtaining a normal dietary intake pattern.

Symptoms Diary: Symptom diaries will be filled out every week and will not be required to be returned until the final week. Participants will be reminded every week via preferred method to ensure completion of the symptom diary is indeed accurate within reason for said purpose. Symptoms assessed include: bowel movement number, stool consistency, discomfort, flatulence, abdominal pain and bloating, and subjects were asked to score them on a scale from 1 (none, normal, good well-being) to 5 (severe symptoms and discomfort). One additional question will assess the compliance of sample consumption. Should there be any unforeseen symptoms that cause more than mild discomfort participants should stop.

Fecal Collection: Participants will be provided stool collection containers. In the case of children parents will be provided with the container. Fecal samples will be collected immediately before and after the cereal consumption phase. There will be a total 2 collections that will be processed for assessing changes in gut health (colon cells and intestinal bacteria). In addition we will check the pH of the stool and short chain fatty acids, which are both indicators of fermentation.

Potential Benefits and Concerns: At the conclusion of this project, you will obtain your child's information related to this study. We will also provide a brief interpretation of the information that we obtain. The results of this study will be the first of their kind and will contribute immensely to the impact of dietary fiber in children. The results provide the opportunity for many more projects and idea generation regarding fiber intake and health.

The potential risks are minimal, but may include the following: Gas production that may result in flatulence, belching, and stomach discomfort. We will be

inquiring information about normal fiber consumption and your child will be provided less than the recommended amounts of fiber for consumption, which will reduce risks of stomach discomfort.

***Debriefing*:** After the final trial you will receive all results as well as the interpretations of the information obtained. Also, the investigators would be more than happy to answer your questions over the results or any other part of the study at any time.

Alternative Procedures: None. You and your child may decline to participate or withdraw at anytime from this project without prejudice or penalty.

Time Commitment: This study requires visits to the study center over four weeks/weekends. Your total time commitment for this study will be approximately 8-12 hours (4-6 hours for each visit), plus 1 additional hour for potential paper work.

Confidentiality: All records associated with your participation in the study will be subject to confidentiality standards applicable to medical records, and in the event of any publication resulting from the research no personally identifiable information will be disclosed. All medical records will be kept confidential, with the possible exception of review by the University Research Compliance Office at Kansas State University – Dr. Rick Scheidt at (785)532-3224.

Right to ask questions: You have been given the opportunity to ask any questions you may have to your satisfaction. If you have any further questions about the study or your participation in it, please contact the following investigators at your convenience.

Mark D. Haub, Ph.D. (haub@ksu.edu) (785)532-0170 -- (785)776-4282 -- (785)320-3330 or
Casey Weber (cweb81@ksu.edu) (785)410-6490 – (785)532-0170

Compensation: Upon completion of completion of this study participants will receive $100. This represent $50 for each visit to the Food and Metabolism Laboratory. In case of complications, physical injury, or illness resulting from the proposed research, only acute and essential medical treatment is available. This institution will not provide monetary compensation for wages lost as a result of injury, hospitalization, and/or professional services.

Voluntary Participation: Please understand that your child's participation in this study is **voluntary**, and that you may withdraw from this study at any time by notifying the investigator. Your withdrawal from this study or refusal to participate will in no way affect your care or access to medical or educational services or participation in future studies. Your participation in this research may be terminated by the investigator without regard to your consent if you are unable or unwilling to comply with the guidelines and procedures explained to you.

I understand this project is research, and that my participation is completely voluntary. I also understand that if I decide to participate in this study, I may withdraw my consent at any time, and stop participating at any time without explanation, penalty, or loss of benefits, or academic standing to which I may otherwise be entitled. I verify that my signature below indicates that I

have read and understand this consent form, and willingly agree to participate in this study under the terms described, and that my signature acknowledges that I have received a signed and dated copy of this consent form.

→ Please mark one box, sign, and return to the program as soon as possible:

- ❑ I will allow my child to participate in having their breath, height and weight measured and has no known digestive conditions (list of potential conditions can be found, below).
- ❑ I will not allow my child to participate.

Parent Name _____ _____

(Please print) (Date)

Parent Signature _____ _____

Child's Name: _____ Child Date of Birth:

_____/_____/_____

Potential Digestive Conditions:

Malabsorptive Disorders

Lactose Intolerance
Fructose Intolerance
Glucose Intolerance

Intestinal Disorders

Small Intestinal Bacterial Overgrowth
Crohn's Disease
Ulcerative Colitis
Chronic Constipation

Recruitment Flier

Volunteers Needed

In a Nutrition Fiber Study for Parents and Children

This study will determine how related adults and children interact with fiber in their bodies.

Considerations:

1) Age 3-8 Children, Any age for parents
2) Apparently healthy
3) Willing to make two visits to Food and Metabolism Lab in Justin Hall for 4-6 hours
4) Provide two stool samples
5) Time spent in lab will be compensated

If you find yourselves willing and eligible or have questions please contact us:

Food and Metabolism Lab
Casey Weber
cweb81@ksu.edu 785-532-0170 785-410-6490
or
Dr. Mark Haub
haub@ksu.edu 785-532-0159

Three Day Diet Records

Food and Metabolism Lab

Kansas State University

Food Record Instructions

You will be keeping a food record for three or more consecutive days. In order to complete the food record thoroughly, **please make a note of all food and beverages consumed** during the days you have been assigned, this includes gum, breath mints, etc.

It is also important to note the method of preparation (e.g., fried, baked, broiled, etc) and all condiments (ketchup, mustard, mayonnaise, sauces, etc.) or other additives (salt, butter, sugar, nutrasweet®, etc.) that are consumed with the food. So, if you have eggs for breakfast you need to record the amount of eggs, how it was prepared (fried, poached, etc.), what was fried with (vegetable oil, butter, etc.), anything you may have put on the eggs (ketchup, tobacco sauce, salt, pepper, etc.), and the time you ate. Also, reporting the brand of food item is very helpful. If you eat at a restaurant be sure to note if you excluded normal items (e.g., McDonald's cheeseburger without ketchup and onions).

Please do not overlook the importance of reporting the serving size or amount eaten, as this is the most important piece of information for us when calculating your dietary intake. Do not hesitate to use comparisons such as equating the size of the serving to a deck of cards if you do not know the exact portion size. Giving us any information to use will be very helpful – and we understand that most people do not measure their food to the nearest gram.

Over the days when you are keeping your food record, you must tell us everything you eat and drink, even water and diet sodas. Don't forget about breath mints, gum, tobacco products, and vitamins/supplements. All of these items contribute to your daily intake of nutrients and that is what we are interested in knowing – regardless of what it might be.

If you ever have any questions regarding your food record, please contact the study manager (Casey Weber). We will be more than happy to help – it is in everyone's best interest to keep accurate food records for the success of this scientific study.

Thank you Very much for your cooperation and dedication. Your participation will not only provide information for you, but will also benefit science.

Food Intake Record

Date:_____ Phone/Email:_____ Day of Week: _____

Time:	Food Item	Amount/Portion	Additional Information	Dine-In/Out
	MORNING - include snacks			
	AFTERNOON - include snacks			

	EVENING - include snacks			
	EXTRA BEVERAGES			
	SUPPLEMENTS			

A three day food record is designed to get an accurate description of your typical daily diet. Since this food record will be used to help you make appropriate dietary changes it is important that you try *not* to change your usual eating patterns for these three days. Please try to be as accurate as possible by recording all of the foods and beverages you eat and drink. Include the exact amount of food eaten and important variations (ex. skim, 2%, reduced fat, sugar-free, etc). If the food is prepared at home or in a restaurant, please include a description of the preparation techniques (ex. grilled vs. fried).

In order to get an accurate representation of your diet, record your food intake for 2 weekdays and 1 weekend day (ex. Monday, Thursday, & Saturday).

Symptom Diary

Name_____ Subject Number_____ Dates_____

Please rank the following symptoms based on an average for each observation from this week using a 1-5 scale:

General Well Being/Health:

1 Excellent 2 Above Average 3 Average 4 Below Average 5 Extremely Poor

Flatulence:

1 None 2 Little 3 Somewhat 4 Much 5Excessive

Abdominal Pain:

1 None 2 Little 3 Somewhat 4 Much 5Excessive

Bloating:

1 None 2 Little 3 Somewhat 4 Much 5Excessive

Stool Consistency:

1 Watery 2 Fluffy/Soft blobs 3 Smooth/Snake-like 4 Lumpy 5 Small hard pieces

(w/ or w/o cracks)

Have bowel movements in general (Please note if you feel comfortable any chages):

Increased Decreased Stayed about the same?

Comments:

Has the cereal been consumed in one sitting or through-out the day, please note what has worked best?

Please return any cereal not consumed.

Appendix C - Bacterial Community Analysis Procedures

Fecal Sample Collection

1. Collect fecal samples using fecal collection hat (system).
    a. Fecal collection hats –
        i. Fisher Sci – Fisherbrand – Commode Specimen Collection System
        ii. Catolog # - 02-544-208 – 60 for 201.62
2. Ensure that samples are collected within 1 hr and processed with/in 2 hr.
    a. Nechvatal et al., 2010, Martinez et al., 2010

Sample Preparation

1. If multiple samples have been collected please ensure they are in the refrigerated conditions (32 – 37^{o}F). Samples may be removed when preparation is necessary
2. Collect 3 grams of fecal sample and place in 27 g of Phosphate Buffered Saline (PBS) in a Corning 50 mL centrifuge tube
    a. Corning – Centrifuge Tube (or Falcon Tube) – 50 mL
        i. Cat # - 430291 – 25 tubes per bag
    b. Martinez et al., 2010
    c. This creates a 1:10 dilution or ratio for preservation
3. Vortex sample until homogenized
4. Place approximately 2 mL of homogenate into 4 microcentrifuge tubes.
    a. Dispose of extra homogenate in appropriate BioHazard receptacle
    b. Call for pick-up if has been more than 1 week or the last sample.
        i. Environmental Health and Safety -> Medical Waste ->
        ii. 532-5856
5. In additional step, core approximately .2-.25 g fecal homogenate
    a. Bore maybe made modifying a 1-3 mL syringe and validating for weight.
    b. In a 1 mL syringe, approximately .2 mL mark is appropriate.
        i. Fisherbrand - Sterile; Luer Lok; 1.0 mL Centered tip
        ii. Catolog # - 14-823-261 – 1000 – 342.62
6. Place core into 3 cryocontainers.
    a. Cryovials
        i. Fisherbrand – Cryogenic Vial – 2 mL, sterile
        ii. FisherBrand Cat. # - 10-500-26
        iii. *Note these are for extra*
        OR
    b. Micro Tube 2 mL
        i. Sarstedt – Micro tube 2 mL –
        ii. Ref # 72.694.006 – 100 pc
7. Fill an addition 15 mL Cryogenic Vial with stool
    a. Extra storage if necessary
    b. ThermoScientific – Nalgene – 15 mL Cryogenic Specimen Vial
    c. Screw Close – Cat # 5005-0015
8. Use a sterile knife to complete the transfer
    a. Sterileware – Bel-Art Products – Sampling Knife
    b. Cat # - 369650000 – 286.20 Box of 200
    c. Can be a sterile knife to reduce introduction of outside DNA
    d. However, I don't see a problem with simple sterilization of the same knife.
9. Place all samples in -20^{o}F while preparing multiple samples.
   Transfer to the -80^{o}F as soon as all samples are completed.

DNA Extraction Prep

(References to reference for fecal samples include Nechvatal et al, 2008; Martinez et al., 2010)

1) Samples will be thawed only prior to use
    a. Samples will be thawed in 32-37^{o}F conditions
    b. Minimizing freeze thaw is important for DNA integrity and should be avoided
    c. TE buffer is appropriate in our case PBS, perhaps other buffers are appropriate too.
2) Samples should be recovered by centrifugation
    a. 8,000 x g for 5 min
    b. Remove supernatant until 0.5ml remains
    c. Mix with pipette using sterile cut-off tip
        i. Mix approximately 15 times
        ii. Keep each sample mixed and treated identically
        iii. Switch tips between all samples
        iv. Transfer into MoBIO Bead Beating Tubes
3) Follow Procedures as in MoBio kit

MoBio Procedure – PowerSoil Kit

1) Add .25 g of fecal sample from recollection to provided PowerBead Tubes
    a. 0.50 g worked better for me
    b. This tube contains a buffer that will help disperse particles
    c. Begin to dissolve fecal acids
    d. Protect nucleic acids from degradation
2) Gently vortex
    a. Only enough to disperse the solution
3) Add solution C1
    a. If C1 is precipitated heat to 60^{o}C, this will dissolve
    b. Contains SDS and other agents to help disrupt and lyse cells.
    c. SDS is anionic which disrupts FA, especially of cell membrane
    d. Can be used while warm
4) Add 60 µl of Solution C1 and invert several times (vortex briefly will also do)
5) Secure power bead tubes securely to Vortex Adapter.
6) Vortex for 10 minutes
    a. This completes homogenization and lysis.
7) Place Power Bead Tubes in the centrifuge @ 10,000 x g for 30 seconds at room temp.
    a. Tubes may break if 10,000 x g is surpassed
8) Transfer supernatant to clean 2 mL Collection Tube
    a. Should have approximately ½ mL of supernatant at this step.
    b. May be dark solution which is fine. Subsequent steps will help with any carry over.
9) Add 250 µL of Solution C2 and vortex for 5 seconds
10) Incubate for 5 min @ 5°C.
    a. IRT or inhibitor removal technology, removes PCR inhibitors.
    b. Precipitates non-DNA organic and inorganic material including extraneous substances, cell debris, proteins.
11) Centrifuge the tubes at room temp for 1 min at 10,000 x g

12) Being careful to avoid the pellet, transfer up to 600 µL of supernatant to a clean 2 mL Collection Tube
13) Add 200 µL of Solution C3 and vortex briefly. Incubate at 4°C for 5 min.
    a. Another IRT
14) Centrifuge at room temp for 1 min at 10,000 x g
15) Transfer up to 750 µL of supernatant to a clean 2 mL Collection Tube
16) Shake to mix Solution C4
17) Add 1.2 mL of Solution C4 to the supernatant, vortex for 5 sec
    a. BE CAREFUL not to exceed the RIM of the tube
18) Load approximately 675 µL onto a Spin Filter and centrifuge at 10,000 x g for 1 min
19) Discard the flow through and add an addition 675 µL to the Spin Filter and cent for 10,000 x g for 10 min.
20) Load remaining supernatant and essentially repeat.
    a. DNA is selectively binding to the silica membrane in the filter. C
    b. Contaminants pass through leaving only the DNA
21) Add 500 µL of Solution C5 and centrifuge at room temp for 30 sec at 10,000 x g.
    a. Ethanol based wash solution to further clean the DNA passing contaminants through
22) Discard the Flow Through from the 2 mL collection tube.
23) Centrifuge for 1 min at 10,000 x g
    a. Removes all traces of wash
24) Carefully place Spin Filter in clean 2 mL Collection Tube
    a. Be extra careful to avoid splashing of flow through C5 ethanol solution
25) Add 100 µL of Solution C6 to the center of the white filter membrane
    a. Sterile elution buffer (10mM TRIS)
    b. Wet the entire membrane
    c. Sterile DNAase and DNA free PCR grade water may be used to elute
26) Centrifuge for 30 sec @ 10,000 x g.
27) Discard the Spin Filter
28) DNA tube is ready for Downstream application
29) DNA can be stored at -20 or -80°C until further use.

DNA Quantification and Quality Estimate

1) Nanodrop will be the necessary equipment use for this procedure.
    a. Nanodrop – Thermo Scientific – UV-VIS – Wilmington, DE
    b. Need to determine the specific instrument
2) Ensure the computer and Nanodrop on are set to "on"
3) Wash the NanoDrop pedestal
4) Lift the arm and remove the previous wipe (should be present)
5) Add 4-5 µL of purified water to the lower pedestal, then lower the arm
6) Wait 30-60 secs
7) Lift the pedestal arm and use the wipe to vigorously scrub both the upper and lower pedestals
    a. This seems excessive?
8) Open NanoDrop software
9) Initialize
10) Click "Nucleic Acid" button in the NanoDrop software.
11) DO NOT click okay until you've added water
12) Add 2 µL of purified water to the lower pedestal and lower upper arm.
13) Click "Okay," on the omputer and wait ~20 sec while the NanoDrop initializes
14) When it's done, lift the upper arm and dry pedestal with a wipe
15) Blank the Nanodrop
16) Add 2µL of the buffer from your sample. Whatever the suspension is.
17) Lower the upper arm of the NanoDrop and click "Blank"
18) Wait ~20 sec for the blank measurement to be made.
19) When it's done, life the upper arm and dry the pedestal with a wipe.
20) Measure your sample
21) Add 2 µL of your sample to the lower pedestal, then lower the upper arm
22) In the Sample ID box, type in the name of your sample.
23) Click the "Measure" button on the software and wait ~20 sec.
24) Lift upper arm when complete, dry the pedestal
    a. Portions of your samples may be retained if you wish.
25) Collect Data
26) Write down all measurements interested in
27) Click "Print Screen" if interested in the complete spectrum
28) Click "Print Report" to get a table of all the data.
29) Clean the Pedestal
30) Add 4-5 µL of purified water to the lower pedestal and lower arm
31) Wait 30-60 second and scrub.
32) Place a new folded lab wipe on the lower pedestal and close the upper arm
33) Shutdown

Standardizing DNA Concentration

This entails individually adjusted concentrations of all samples to 5 or 10 ng/µL
Helps reduced bias in the steps moving forward.

Dilution should be commensurate with total PCR volume extracted, i.e. a protocol with 25 µL or 50 µL.

Protocol for August 27th Prep.

1. Samples were adjusted to equal 10 ng/µL final concentration.
2. This was calculated based on the follow procedure.
3. Observed Quantity / Amount to be achieved = Dilution Factor (DF)
4. All values were calculated from 100 µL observed sample.
5. Thus the DF X 100 µL = Final Volume
6. Final volume – 100 µL =Dilution Volume
    a. All working solutions are differing amount
    b. But using 100 µL for ease of calculation this is best.
    c. If the extract does not allow for 100 µL, the adjust as necessary
    d. The same steps still apply, just choose a consistent DF

Preform PCR Reactions

1. PCR reactions are performed first by creating a 96 well template to keep samples organized.
    a. Excel is easiest
2. Sterile nuclease free PCR tubes are place in the appropriate location.
3. In one tube be sure to provide a negative control
4. A positive control is not as necessary as, no bands are no bands.
5. The following are for 50 µL RXN
    a. COMPLETE ON ICE, ALWAYS ADD MM last
    b. 25 µL of Master Mix (AmpliTaq Gold 360 Master Mix, Applied BioSystems, Life Technologies, Carlsbad, CA)
    c. 5 µL template (10 ng/ µL)
    d. 5 µL primer forward (IDT) FC9F-GAGTTTGATCCTTGGCTCAG
    e. 5 µL primer reverse (IDT) FC541R-TTACCGCGGCTGCTGG
    f. 10 µL NCF H_2O
6. Basic Procedure (August 27th, 2012 protocol was Shawn's)
    a. Basically, Denaturing Step 95°C 8 min
    b. Cycle Denaturing 95°C for 30 seconds
    c. Annealing 54°C for 1 min
    d. Extension 72°C for 2 min
    e. Repeat 24 cycles (25 cycles total, as you will enter cycle and have to repeat)
    f. Final Elongation at 72°C for 10 min

Repeat technical replicates 2 and 3 after one complete run and gel.

Run Gels for PCR Products

1. Prepare Gel about 1 hour before use (When starting the thermocylcer)
    a. A gel can last covered in fridge overnight at most.
    b. Gel for a mini = .45 g agarose, 3 mL 10x TBE (Tris-Boric Acid-EDTA), 27 mL of Distilled water
    c. Our mix for the 27th, 2012 was .45 g agarose, 6 mL 5x TBE, and 24 mL of distilled water
    d. Remove well Die slowly and consistently to prevent ripping the gel.
    e. Prepare Electrophloretic box, black goes to black and red to red electrodes.
    f. Wells should run from Black to Red or Pos to Neg.
        i. RUN TO THE RED
    g. Run 110 V for 1 hr, with approximately 60 mAmps, if mAmps are 40 or less wash solution may need to be replaced.
    h. Wash Solution is 1x TBE (Usually re-used 5-6 times, then replaced.)
    i. Fill wash solution about 1 cm over the gel
2. To load sample, tape the four sides of a parafilm to the lab bench.
3. Place 2 µL of Loading dye as a dot on the parafilm
    a. Our Loading dye is 6X, so we dilute 2 to 10 µL
    b. I suppose a 1 to 5 is also a reasonable preposition.
4. Place a "dot" for every sample to be analyzed include the ladder.
    a. The ladder includes 100, 200, 300, 500, 750, 1000 bp, I believe
5. 10 µL of sample and ladder will be added to each dot.
6. Be careful to have samples, properly labeled, to keep straight.
7. Load the sample into the loading wells.
    a. Be careful, some do this before the wash solution is added.
    b. I did with the solution in and it worked fine.
    c. I place the pipette tip in the well and
8. After 1 hr cycle, stop machine
9. Pour TBE back into container.
10. Wash for 10 min Ethidium Bromide.
    a. Fill EtBr enough to cover the gel.
    b. Must use lab-coat and gloves always for EtBr.
    c. Must not touch anything other than designated material once glove contaminated with EtBr (Very suspected human carcinogen)
11. Place on stir plate gently for 10 min for large gels, 5 for small.
    a. Never use gloved hand to turn on stir plate as could contaminate with EtBr
12. Dispose of EtBr into storage container.
13. Wash with water, repeat stir plate for10 or 5 respectively, depending on sample.
14. Dump water down drain using funnel (Also, use the designated spatula to hold the gel together and not dump out.)
15. Wash with water and place on stir plate same as above.
16. Once washed and drain take to lab 322 – Carol Ferguson's lab to use camera (Co-owned by Ari).

17. Plan to remove gloves and take extra and be sure to not wear gloved hands to touch places in hallways!!!!!!!!! Prepare for necessary clean-up and contact if dropped or an accident were to happen!!!!
18. Open Camera Shortcut
19. Open camera door, turn on fluorescent light,
20. Remove Tray
21. Place Wells towards the left on the plastic tray, with wing sides of tray facing up.
22. Place in camera
23. Turn off fluorescent light
24. Close door and turn on UV light
25. Adjust settings to see the bands.
26. Integration was used in our case (however, the user was not ultimately sure what the integration was doing)
27. Take picture, store, and verify the presence of the bp appropriate for your amplicon
28. Remove gel and place in same container brought down.
29. Remove one glove throw all wastes in with gel and container, to dispose in Ari's lab. Plus this leaves an open hand for hallway interaction. Don't carry this during busy

periods.

Secondary PCR products

1. **BASIC PREP**
    a. Sterile nuclease free PCR tubes are place in the appropriate location.
    b. In one tube be sure to provide a negative control
    c. Here a TEMPLATE IS A MUST
    d. EACH sample will have its own unique barcode.
    e. A positive control is not as necessary as, no bands are no bands.

2. **BARCODE PRIMERS**
    a. Refer to following page for complete list
    b. Example of decoding the below
    c.

30 - Base Pair Adapter/Linker	10 - Tag - Barcode - MID	Primer
CCATCTCATCCCTGCGTGTCTCCGACTCAG	ACGAGTGCGT	WTTACCGCGGCTGCTGG

    d. The adapter/linker followed by the barcode (unique 10 digit code) the primer excluding the "W".
    e.

3. **PCR REACTION PREP**
4. <u>The following are for 50 µL RXN</u>
    a. COMPLETE ON ICE, ALWAYS ADD MM last
    b. 25 µL of Master Mix (AmpliTaq Gold 360 Master Mix, Applied BioSystems,Life Technologies, Carlsbad, CA)
    c. 5 µL template (10 ng/ µL)
    d. 5 µL primer forward (IDT) FC9F-GAGTTTGATCCTTGGCTCAG
        i. Here an A adapter is added for pyrosequencing identification
    e. 5 µL primer reverse (IDT) FC541R-TTACCGCGGCTGCTGG
        i. Here the B adapter is added to the R primer as this will be the sequencing direction in the 454
    f. 10 µL NCF H_2O

Basic Procedure

5. Basically, Denaturing Step 95°C 8 min
6. Cycle Denaturing 95°C for 30 seconds
7. Annealing 54°C for 1 min
8. Extension 72°C for 2 min
9. Repeat 4 cycles
10. Final Elongation at 72°C for 10 min
11. Repeat technical replicates 2 and 3 after one complete run and gel.
    a. Gels always ensure a product has run.
    b. In this case we are interested in the "jump-up"

c. Here the secondary products should show up about 50 bp longer
d. These are the extra for the Barcodes and Adapters, we assume it worked.

Reverse Primer Barcode and 454 Adapters

	Plate Name:		
	Well Position	Sequence Name	Sequence
1	A1	A-FC-541R-A1	CCATCTCATCCCTGCGTGTCTCCGACTCAGACGAGTGCGTWTTACCGCGGCTGCTGG
2	B1	A-FC-541R-B1	CCATCTCATCCCTGCGTGTCTCCGACTCAGACGCTCGACAWTTACCGCGGCTGCTGG
3	C1	A-FC-541R-C1	CCATCTCATCCCTGCGTGTCTCCGACTCAGAGACGCACTCWTTACCGCGGCTGCTGG
4	D1	A-FC-541R-D1	CCATCTCATCCCTGCGTGTCTCCGACTCAGAGCACTGTAGWTTACCGCGGCTGCTGG
5	E1	A-FC-541R-E1	CCATCTCATCCCTGCGTGTCTCCGACTCAGATCAGACACGWTTACCGCGGCTGCTGG
6	F1	A-FC-541R-F1	CCATCTCATCCCTGCGTGTCTCCGACTCAGATATCGCGAGWTTACCGCGGCTGCTGG
7	G1	A-FC-541R-G1	CCATCTCATCCCTGCGTGTCTCCGACTCAGCGTGTCTCTAWTTACCGCGGCTGCTGG
8	H1	A-FC-541R-H1	CCATCTCATCCCTGCGTGTCTCCGACTCAGCTCGCGTGTCWTTACCGCGGCTGCTGG
9	A2	A-FC-541R-A2	CCATCTCATCCCTGCGTGTCTCCGACTCAGTCTCTATGCGWTTACCGCGGCTGCTGG
10	B2	A-FC-541R-B2	CCATCTCATCCCTGCGTGTCTCCGACTCAGTGATACGTCTWTTACCGCGGCTGCTGG
11	C2	A-FC-541R-C2	CCATCTCATCCCTGCGTGTCTCCGACTCAGCATAGTAGTGWTTACCGCGGCTGCTGG
12	D2	A-FC-541R-D2	CCATCTCATCCCTGCGTGTCTCCGACTCAGCGAGAGATACWTTACCGCGGCTGCTGG
13	E2	A-FC-541R-E2	CCATCTCATCCCTGCGTGTCTCCGACTCAGATACGACGTAWTTACCGCGGCTGCTGG
14	F2	A-FC-541R-F2	CCATCTCATCCCTGCGTGTCTCCGACTCAGTCACGTACTAWTTACCGCGGCTGCTGG
15	G2	A-FC-541R-G2	CCATCTCATCCCTGCGTGTCTCCGACTCAGCGTCTAGTACWTTACCGCGGCTGCTGG
16	H2	A-FC-541R-H2	CCATCTCATCCCTGCGTGTCTCCGACTCAGTCTACGTAGCWTTACCGCGGCTGCTGG
17	A3	A-FC-541R-A3	CCATCTCATCCCTGCGTGTCTCCGACTCAGTGTACTACTCWTTACCGCGGCTGCTGG
18	B3	A-FC-541R-B3	CCATCTCATCCCTGCGTGTCTCCGACTCAGACGACTACAGWTTACCGCGGCTGCTGG
19	C3	A-FC-541R-C3	CCATCTCATCCCTGCGTGTCTCCGACTCAGCGTAGACTAGWTTACCGCGGCTGCTGG
20	D3	A-FC-541R-D3	CCATCTCATCCCTGCGTGTCTCCGACTCAGTACGAGTATGWTTACCGCGGCTGCTGG
21	E3	A-FC-541R-E3	CCATCTCATCCCTGCGTGTCTCCGACTCAGTACTCTCGTGWTTACCGCGGCTGCTGG
22	F3	A-FC-541R-F3	CCATCTCATCCCTGCGTGTCTCCGACTCAGTAGAGACGAGWTTACCGCGGCTGCTGG
23	G3	A-FC-541R-G3	CCATCTCATCCCTGCGTGTCTCCGACTCAGTCGTCGCTCGWTTACCGCGGCTGCTGG
24	H3	A-FC-541R-H3	CCATCTCATCCCTGCGTGTCTCCGACTCAGACATACGCGTWTTACCGCGGCTGCTGG
25	A4	A-FC-541R-A4	CCATCTCATCCCTGCGTGTCTCCGACTCAGACGCGAGTATWTTACCGCGGCTGCTGG
26	B4	A-FC-541R-B4	CCATCTCATCCCTGCGTGTCTCCGACTCAGACTACTATGTWTTACCGCGGCTGCTGG
27	C4	A-FC-541R-C4	CCATCTCATCCCTGCGTGTCTCCGACTCAGACTGTACAGTWTTACCGCGGCTGCTGG
28	D4	A-FC-541R-D4	CCATCTCATCCCTGCGTGTCTCCGACTCAGAGACTATACTWTTACCGCGGCTGCTGG
29	E4	A-FC-541R-E4	CCATCTCATCCCTGCGTGTCTCCGACTCAGAGCGTCGTCTWTTACCGCGGCTGCTGG
30	F4	A-FC-541R-F4	CCATCTCATCCCTGCGTGTCTCCGACTCAGAGTACGCTATWTTACCGCGGCTGCTGG
31	G4	A-FC-541R-G4	CCATCTCATCCCTGCGTGTCTCCGACTCAGATAGAGTACTWTTACCGCGGCTGCTGG
32	H4	A-FC-541R-H4	CCATCTCATCCCTGCGTGTCTCCGACTCAGCACGCTACGTWTTACCGCGGCTGCTGG
33	A5	A-FC-541R-A5	CCATCTCATCCCTGCGTGTCTCCGACTCAGCAGTAGACGTWTTACCGCGGCTGCTGG
34	B5	A-FC-541R-B5	CCATCTCATCCCTGCGTGTCTCCGACTCAGCGACGTGACTWTTACCGCGGCTGCTGG
35	C5	A-FC-541R-C5	CCATCTCATCCCTGCGTGTCTCCGACTCAGTACACACACTWTTACCGCGGCTGCTGG
36	D5	A-FC-541R-D5	CCATCTCATCCCTGCGTGTCTCCGACTCAGTACACGTGATWTTACCGCGGCTGCTGG
37	E5	A-FC-541R-E5	CCATCTCATCCCTGCGTGTCTCCGACTCAGTACAGATCGTWTTACCGCGGCTGCTGG
38	F5	A-FC-541R-F5	CCATCTCATCCCTGCGTGTCTCCGACTCAGTACGCTGTCTWTTACCGCGGCTGCTGG
39	G5	A-FC-541R-G5	CCATCTCATCCCTGCGTGTCTCCGACTCAGTAGTGTAGATWTTACCGCGGCTGCTGG
40	H5	A-FC-541R-H5	CCATCTCATCCCTGCGTGTCTCCGACTCAGTCGATCACGTWTTACCGCGGCTGCTGG
41	A6	A-FC-541R-A6	CCATCTCATCCCTGCGTGTCTCCGACTCAGTCGCACTAGTWTTACCGCGGCTGCTGG
42	B6	A-FC-541R-B6	CCATCTCATCCCTGCGTGTCTCCGACTCAGTCTAGCGACTWTTACCGCGGCTGCTGG
43	C6	A-FC-541R-C6	CCATCTCATCCCTGCGTGTCTCCGACTCAGTCTATACTATWTTACCGCGGCTGCTGG
44	D6	A-FC-541R-D6	CCATCTCATCCCTGCGTGTCTCCGACTCAGTGACGTATGTWTTACCGCGGCTGCTGG
45	E6	A-FC-541R-E6	CCATCTCATCCCTGCGTGTCTCCGACTCAGTGTGAGTAGTWTTACCGCGGCTGCTGG
46	F6	A-FC-541R-F6	CCATCTCATCCCTGCGTGTCTCCGACTCAGACAGTATATAWTTACCGCGGCTGCTGG
47	G6	A-FC-541R-G6	CCATCTCATCCCTGCGTGTCTCCGACTCAGACGCGATCGAWTTACCGCGGCTGCTGG
48	H6	A-FC-541R-H6	CCATCTCATCCCTGCGTGTCTCCGACTCAGACTAGCAGTAWTTACCGCGGCTGCTGG
49	A7	A-FC-541R-A7	CCATCTCATCCCTGCGTGTCTCCGACTCAGAGCTCACGTAWTTACCGCGGCTGCTGG
50	B7	A-FC-541R-B7	CCATCTCATCCCTGCGTGTCTCCGACTCAGAGTATACATAWTTACCGCGGCTGCTGG
51	C7	A-FC-541R-C7	CCATCTCATCCCTGCGTGTCTCCGACTCAGAGTCGAGAGAWTTACCGCGGCTGCTGG
52	D7	A-FC-541R-D7	CCATCTCATCCCTGCGTGTCTCCGACTCAGAGTGCTACGAWTTACCGCGGCTGCTGG
53	E7	A-FC-541R-E7	CCATCTCATCCCTGCGTGTCTCCGACTCAGCGATCGTATAWTTACCGCGGCTGCTGG
54	F7	A-FC-541R-F7	CCATCTCATCCCTGCGTGTCTCCGACTCAGCGCAGTACGAWTTACCGCGGCTGCTGG
55	G7	A-FC-541R-G7	CCATCTCATCCCTGCGTGTCTCCGACTCAGCGCGTATACAWTTACCGCGGCTGCTGG
56	H7	A-FC-541R-H7	CCATCTCATCCCTGCGTGTCTCCGACTCAGCGTACAGTCAWTTACCGCGGCTGCTGG
57	A8	A-FC-541R-A8	CCATCTCATCCCTGCGTGTCTCCGACTCAGCGTACTCAGAWTTACCGCGGCTGCTGG
58	B8	A-FC-541R-B8	CCATCTCATCCCTGCGTGTCTCCGACTCAGCTACGCTCTAWTTACCGCGGCTGCTGG
59	C8	A-FC-541R-C8	CCATCTCATCCCTGCGTGTCTCCGACTCAGCTATAGCGTAWTTACCGCGGCTGCTGG
60	D8	A-FC-541R-D8	CCATCTCATCCCTGCGTGTCTCCGACTCAGTACGTCATCAWTTACCGCGGCTGCTGG
61	E8	A-FC-541R-E8	CCATCTCATCCCTGCGTGTCTCCGACTCAGTAGTCGCATAWTTACCGCGGCTGCTGG
62	F8	A-FC-541R-F8	CCATCTCATCCCTGCGTGTCTCCGACTCAGTATATATACAWTTACCGCGGCTGCTGG
63	G8	A-FC-541R-G8	CCATCTCATCCCTGCGTGTCTCCGACTCAGTATGCTAGTAWTTACCGCGGCTGCTGG
64	H8	A-FC-541R-H8	CCATCTCATCCCTGCGTGTCTCCGACTCAGTCACGCGAGAWTTACCGCGGCTGCTGG
65	A9	A-FC-541R-A9	CCATCTCATCCCTGCGTGTCTCCGACTCAGTCGATAGTGAWTTACCGCGGCTGCTGG
66	B9	A-FC-541R-B9	CCATCTCATCCCTGCGTGTCTCCGACTCAGTCGCTGCGTAWTTACCGCGGCTGCTGG
67	C9	A-FC-541R-C9	CCATCTCATCCCTGCGTGTCTCCGACTCAGTCTGACGTCAWTTACCGCGGCTGCTGG
68	D9	A-FC-541R-D9	CCATCTCATCCCTGCGTGTCTCCGACTCAGTGAGTCAGTAWTTACCGCGGCTGCTGG
69	E9	A-FC-541R-E9	CCATCTCATCCCTGCGTGTCTCCGACTCAGTGTAGTGTGAWTTACCGCGGCTGCTGG
70	F9	A-FC-541R-F9	CCATCTCATCCCTGCGTGTCTCCGACTCAGTGTCACACGAWTTACCGCGGCTGCTGG
71	G9	A-FC-541R-G9	CCATCTCATCCCTGCGTGTCTCCGACTCAGTGTCGTCGCAWTTACCGCGGCTGCTGG
72	H9	A-FC-541R-H9	CCATCTCATCCCTGCGTGTCTCCGACTCAGACACATACGCWTTACCGCGGCTGCTGG
73	A10	A-FC-541R-A10	CCATCTCATCCCTGCGTGTCTCCGACTCAGACAGTCGTGCWTTACCGCGGCTGCTGG
74	B10	A-FC-541R-B10	CCATCTCATCCCTGCGTGTCTCCGACTCAGACATGACGACWTTACCGCGGCTGCTGG
75	C10	A-FC-541R-C10	CCATCTCATCCCTGCGTGTCTCCGACTCAGACGACAGCTCWTTACCGCGGCTGCTGG
76	D10	A-FC-541R-D10	CCATCTCATCCCTGCGTGTCTCCGACTCAGACGTCTCATCWTTACCGCGGCTGCTGG
77	E10	A-FC-541R-E10	CCATCTCATCCCTGCGTGTCTCCGACTCAGACTCATCTACWTTACCGCGGCTGCTGG
78	F10	A-FC-541R-F10	CCATCTCATCCCTGCGTGTCTCCGACTCAGACTCGCGCACWTTACCGCGGCTGCTGG
79	G10	A-FC-541R-G10	CCATCTCATCCCTGCGTGTCTCCGACTCAGAGAGCGTCACWTTACCGCGGCTGCTGG
80	H10	A-FC-541R-H10	CCATCTCATCCCTGCGTGTCTCCGACTCAGAGCGACTAGCWTTACCGCGGCTGCTGG
81	A11	A-FC-541R-A11	CCATCTCATCCCTGCGTGTCTCCGACTCAGAGTAGTGATCWTTACCGCGGCTGCTGG
82	B11	A-FC-541R-B11	CCATCTCATCCCTGCGTGTCTCCGACTCAGAGTGACACACWTTACCGCGGCTGCTGG
83	C11	A-FC-541R-C11	CCATCTCATCCCTGCGTGTCTCCGACTCAGAGTGTATGTCWTTACCGCGGCTGCTGG
84	D11	A-FC-541R-D11	CCATCTCATCCCTGCGTGTCTCCGACTCAGATAGATAGACWTTACCGCGGCTGCTGG
85	E11	A-FC-541R-E11	CCATCTCATCCCTGCGTGTCTCCGACTCAGATATAGTCGCWTTACCGCGGCTGCTGG
86	F11	A-FC-541R-F11	CCATCTCATCCCTGCGTGTCTCCGACTCAGATCTACTGACWTTACCGCGGCTGCTGG
87	G11	A-FC-541R-G11	CCATCTCATCCCTGCGTGTCTCCGACTCAGCACGTAGATCWTTACCGCGGCTGCTGG
88	H11	A-FC-541R-H11	CCATCTCATCCCTGCGTGTCTCCGACTCAGCACGTGTCGCWTTACCGCGGCTGCTGG
89	A12	A-FC-541R-A12	CCATCTCATCCCTGCGTGTCTCCGACTCAGCATACTCTACWTTACCGCGGCTGCTGG
90	B12	A-FC-541R-B12	CCATCTCATCCCTGCGTGTCTCCGACTCAGCGACACTATCWTTACCGCGGCTGCTGG
91	C12	A-FC-541R-C12	CCATCTCATCCCTGCGTGTCTCCGACTCAGCGAGACGCGCWTTACCGCGGCTGCTGG
92	D12	A-FC-541R-D12	CCATCTCATCCCTGCGTGTCTCCGACTCAGCGTATGCGACWTTACCGCGGCTGCTGG
93	E12	A-FC-541R-E12	CCATCTCATCCCTGCGTGTCTCCGACTCAGCGTCGATCTCWTTACCGCGGCTGCTGG
94	F12	A-FC-541R-F12	CCATCTCATCCCTGCGTGTCTCCGACTCAGCTACGACTGCWTTACCGCGGCTGCTGG
95	G12	A-FC-541R-G12	CCATCTCATCCCTGCGTGTCTCCGACTCAGCTAGTCACTCWTTACCGCGGCTGCTGG
96	H12	A-FC-541R-H12	CCATCTCATCCCTGCGTGTCTCCGACTCAGCTCTACGCTCWTTACCGCGGCTGCTGG

Amplicon Cleaning

1. Clean with AMPure per instructions

Procedure 96 Well Format:

2. **Determine whether or not a plate transfer is necessary**
    a. If the PCR reaction volume * 2.8 exceeds the volume of the PCR plate, a transfer to a 300 µL round bottom plate is required. Agencourt recommends the Costar 3795 plate to work with the Agencourt AMPure kit, because the Agencourt SPRIPlate96R was designed specifically for the Costar plate. The PCR Reactions can be set up in polypropylene PCR/ thermal cycling plates. The cleanup reaction can be performed in the same plate, if the volume of the PCR reaction is below 71 µL. A 300 µl plate will hold up to 105 µL of sample and 189 µL of Agencourt AMPure.
3. **Gently shake the Agencourt AMPure bottle to resuspend any magnetic particles that may have settled. Add Agencourt AMPure according to PCR reaction volume chart below:**

PCR Reaction Volume (µL)	Agencourt AMPure Volume (µL)
5	9
7	12.6
10	18
14	25

The volume of Agencourt AMPure for a given reaction can be derived from the following equation: (Volume of Agencourt AMPure per reaction) = 1.8 x (Reaction Volume)

Due to the total volume of PCR reaction plus Agencourt AMPure, it is not possible to purify PCR reactions larger than 14 µL within the well of 384 well plates (14 µL reaction + 25 µL Agencourt AMPure = 39 µL).

4. **Mix the Agencourt AMPure and PCR reaction thoroughly by pipette mixing 10 times or vortexing for 30 seconds.**
    a. This step binds PCR products 100bp and larger to the magnetic beads. Pipette mixing is preferable as it tends to be more reproducible. If vortexing is used, it is recommended to seal the plate with a plate seal before vortexing. The color of the mixture should appear homogenous after mixing. Let the mixed samples incubate for 3 -5 minutes at room temperature for maximum recovery (optional). This is strongly recommended for reactions 50 µl and larger.
5. **Place the reaction plate onto an Agencourt SPRIPlate 96R for 5 - 10 minutes to separate beads from solution.**
    a. The separation time is dependent on the size of the reaction. Wait for the solution to clear before proceeding to the next step.
6. **Aspirate the cleared solution from the reaction plate and discard.**
    b. This step must be performed while the reaction plate is situated on the Agencourt SPRIPlate 96R. Do not disturb the ring of separated magnetic beads.

7. **Dispense 200 µL of 70% ethanol to each well of the reaction plate and incubate for 30 seconds at room temperature. Aspirate out the ethanol and discard. Repeat for a total of two washes.**
    c. See Note below for removal amendment.
    d. It is important to perform these steps with the reaction plate situated on an Agencourt SPRIPlate 96R. Do not disturb the separated magnetic beads. Be sure to remove all of the ethanol from the bottom of the well as it may contain residual contaminants. The ethanol can also be discarded by inverting the plate to decant, but this must be done while the plate is situated on the Agencourt SPRIPlate 96R.
8. **Place the reaction plate on bench top to air-dry. Be sure to allow the plate to dry completely.**
    e. The plate should be left to air-dry for 10-20 minutes on a bench top to allow complete evaporation of residual ethanol. Longer drying times may be required for microarraying. Alternatively the plate can be incubated at 37°C for faster evaporation. If the samples will be used immediately, proceed to Step 8 for elution. If the samples will not be used immediately, the dried plate may be sealed and stored at 4°C or -20°C.
9. **Add 40 µL of elution buffer (TRIS-Acetate, DiH2O, or TE) to each well of the reaction plate and seal to vortex 30 seconds or pipette mix 10 times.**
    a. The liquid level will be high enough to contact the magnetic beads at a 40 µl elution volume. A greater volume of elution buffer can be used, but using less than 40 µL will require extra vortexing (to ensure the liquid comes into contact with the beads) and may not be sufficient to fully elute all of the product. 10 mM Tris-Acetate pH 8.0 (recommended), reagent grade water, or TE buffer may be used for the elution. Recommended elution conditions are sealing and vortexing or 30 seconds or 10 pipette mixes. Elution is quite rapid and it is not necessary for the beads to go back into solution for complete elution to occur. When setting up ownstream reactions, pipette the DNA from the plate while it is situated on the Agencourt SPRIPlate96R. This will prevent bead carry over (however, the beads do not inhibit thermal cycling reactions). For long term freezer storage, Agencourt recommends transferring Agencourt AMPure purified samples into a new plate away from the magnetic particles.

*Note, when removing the alcohol wash, clamp tight to the 96 well plate, be sure to keep pressure between the magnet plate and your template plate. Then simply throw the water out. The final step you lightly slam the plate on paper towels. Very nerve-wrecking, but very quick and efficient.

BioAnalyze

1. Sample were analyzed in Agilent BioAnalyzer2100
2. Using the DNA 1000 Kits
3. Samples were analyzed in 123 Coles @ Vetmed in the COBRE center
    a. Contact was Donald Harbinger – COBRE manager

Follow manufacturer instructions following the plate instructions.

Agilent DNA 1000 Assay Protocol - Edition April 2007

Handling DMSO

Kit components contain DMSO. Because the dye binds to nucleic acids, it should be treated as a potential mutagen and used with appropriate care. Wear hand and eye protection and follow good laboratory practices when preparing and handling reagents and samples. Handle the DMSO stock solutions with particular caution as DMSO is known to facilitate the entry of organic molecules into tissues.

Preparing the Gel-Dye Mix

1 Allow DNA dye concentrate (blue □) and DNA gel matrix (red □) to equilibrate to room temperature for 30 min.

2 Vortex DNA dye concentrate (blue □) and add 25 µl of the dye to a DNA gel matrix vial (red □).

3 Vortex solution well and spin down. Transfer to spin filter.

4 Centrifuge at 2240 g ± 20 % for 15 min. Protect solution from light. Store at 4 °C.

Loading the Gel-Dye Mix

1 Allow the gel-dye mix equilibrate to room temperature for 30 min before use.

2 Put a new DNA chip on the chip priming station.

3 Pipette 9.0 µl of gel-dye mix in the well marked .

4 Make sure that the plunger is positioned at 1 ml and then close the chip priming station.

5 Press plunger until it is held by the clip.

6 Wait for exactly 60 s then release clip.

7 Wait for 5 s. Slowly pull back plunger to 1ml position.

8 Open the chip priming station and pipette 9.0 µl of gel-dye mix in the wells marked .

Loading the Markers

1 Pipette 5 µl of marker (green □) in all 12 sample wells and ladder well. Do not leave

any wells empty.

Loading the Ladder and the Samples

1 Pipette 1 µl of DNA ladder (yellow □) in the well marked .

2 In each of the 12 sample wells pipette 1 µl of sample (used wells) or 1 µl of de-ionized water (unused wells).

3 Put the chip horizontally in the adapter and vortex for 1 min at the indicated setting (2400 rpm).

4 Run the chip in the Agilent 2100 bioanalyzer within 5 min.

Microbial Sequencing

1. Samples will be prepared and Sequenced in the Integrated Genomics Facility
    a. Alina Akhunova is the contact
2. At the IGL samples will be go throw emPCR and then sequenced with Next-generation sequencing or 454, which is a high-throughput sequencing. Sequencing by synthesis with all sequences elongated in parallel allows the technology to produce 1.2 million reads per chip. In our case approximately 300,000 reads or a ¼ reaction.
   s in alphabetical order (A, B, C, etc.).