Processing and Cooking on Lentil Prebiotics to Reduce Obesity

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PROCESSING AND COOKING ON LENTIL PREBIOTICS TO REDUCE OBESITY

A Thesis
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Master of Science
Plant and Environmental Sciences

by
Niroshan Siva
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Accepted by:
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ABSTRACT

Lentil is a rich source of proteins, range of prebiotic carbohydrates including sugar alcohols (SA), raffinose family oligosaccharides (RFO), fructooligosaccharides (FOS), and resistant starch (RS), minerals, and vitamins. Research indicated that foods rich in prebiotics reduce obesity via modulating gut microbiota. The objectives of this thesis were 1) to determine the effects of lentil processing (dehulling, splitting, and cooking) on SA, RFO, FOS, and RS in three lentil market classes (red, green, and pardina), and 2) to determine the effects of lentil diet on rat body weight, percent body fat, plasma triglycerides (TGs) concentration, and change of fecal bacteria. Lentil dehulling and splitting decreased SA, and increased RFO and FOS concentrations. Concentration of SA, RFO, and FOS increased with cooling and reduced after reheating. RS concentration increased with cooling and reheating. For the rat study, lentil diet significantly reduced body weight, percent body fat, plasma TGs concentration within 6 weeks compared to the control diets. Abundance of fecal Firmicutes was relatively low, and abundance of Actinobacteria and Bacteriodetes were relatively high in rats fed with lentil diet than the control diets. In conclusion, processing, and cooking can change the levels of prebiotic carbohydrates however, regular consumption of lentil may tend to reduce obesity risk factors. Further human studies are warrant to determine the potential of lentil to reduce obesity risk.

Key words: lentil, prebiotics, processing, microbiota, obesity
DEDICATION

This thesis is dedicated to
my family and friends
for their early inspiration, coaching, and
enthusiasm.
None of this would have happened
without them
ACKNOWLEDGMENTS

I have been plentifully blessed during my degree program and research. Most of all, I thank my family and friends who have given me better life and shaped me into who I am. I offer my sincere thanks to my advisor, Dr. Dil Thavarajah and members of the graduate committee, Drs. Susan Duckett, Elliot Jesch, Pushparajah Thavarajah, and William Whiteside who gave me tremendous support on this research. I would like to thank Dr. Vincent P. Richards’ research team, Godley-Snell Research Center, and Vegetable/Pulse Quality and Nutrition Laboratory research team at Clemson University. Also, I would like to thank Dhanuska Wijesinghe, Suranga Basnagala, Indika Pathirana, and Anuradhi Wickramasinghe for their support in this research and my life. Finally I would like to thank College of Agriculture, Forestry, and Life Sciences (CAFLS), Clemson University, the American Pulse Association, the USA Dry Pea Lentil Council, Clemson University Research Grant Committee, and CAFLS new faculty startup funds for providing funds for this project.
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1. INTRODUCTION

Obesity is a global health problem. According to the World Health organization, 13% of the world population are obese (WHO, 2016). Obesity is the fifth major risk factor causing death particularly in high income countries (WHO, 2015). For example, 35% of American adults (one in three adults) are obese. By 2030, more than 50% of the American population will be obese (Finkelstein et al., 2012; NCD Risk Factor Collaboration, 2016). Major reason for increasing obesity is the unhealthy food consumption (WHO, 2016). Consumption of high fat, high added sugar diets increase caloric intake which increase obesity (Drewnowski & Popkin, 1997; Kearney, 2010). Therefore, consumption of traditional whole foods (vegetables, fruits, and legumes) are highly recommended to reduce obesity and related non communicable diseases (WHO, 2016).

Lentil (*Lens culinaris* Medikus) is an ancient food legume crop, originating from the Near East approximately 10,000 years ago (Cubero, Pérez de la Vega, & Fratini, 2009; Ladizinsky, 1979). Lentil is a rich source of protein (20-30 g/100 g), carbohydrates (40-60 g/100 g), essential fats (<2 g/100 g), minerals (Iron, Zink, Selenium), vitamins (folate), and dietary fiber (Thavarajah & Thavarajah, 2012). Furthermore, lentil is a good source of prebiotic carbohydrates (Johnson, Thavarajah, Combs, & Thavarajah, 2013). Prebiotic carbohydrates are selectively fermented by beneficial gut microbiome that allow specific
biochemical changes in gastrointestinal environment to increase host well-being and health (Roberfroid, 2007).

The human gut microbiome possess two dominant bacterial groups: *Bacteriodetes* and *Firmicutes*, representing more than 90% of the gut microbial population (Ley, Turnbaugh, Klein, & Gordon, 2006). *Bacteriodetes* to *Firmicutes* ratio changes as a result of host obesity status (Ley et al., 2005). Few studies (Ley et al., 2005; Turnbaugh et al., 2006) observed decreased ratio of *Bacteriodetes* to *Firmicutes* in obese rats and some others (Collado, Isolauri, Laitinen, & Salminen, 2008; Schwertz et al., 2010) provide evident to decreased ratio. These conflicting results were observed due to the differences in animal models, duration of study, and different DNA sequencing approaches.

Prebiotics rich diet increase beneficial gut bacteria (Everard et al., 2011). Extend of beneficial effects of the prebiotics depends on the prebiotic concentration in the diets (Scholz-Ahrens, Schaafsma, van den Heuvel, & Schrezenmeir, 2001). Food processing and cooking operations change prebiotic concentration in foods. For an example, lentil prebiotic carbohydrates (raffinose family oligosaccharides, fructooligosaccharides, and resistant starch) changed after dehulling, cooking, cooling, and reheating (Johnson et al., 2015). Therefore, it is important to know the impact of processing (dehulling, splitting) and thermal treatments on prebiotic carbohydrates to maintain optimum prebiotics concentration in processed food to maintain a healthy gut.
Legumes rich in prebiotic carbohydrates increase gut health via increasing good bacteria. Chick pea (Cicer arietinum L.), pea (Pisum sativum L.), common bean (Phaseolus vulgaris L.), and lentil (Lens culinaris Medikus) diets increase bifidobacteria, a beneficial bacterial group (Queiroz-Monici, Costa, da Silva, Reis, & de Oliveira, 2005). However, few studies were focused on the gut microbial changes and related obesity bio markers changes respect to legume consumption. No efforts have been made to determine the potential of lentil as a food legume to reduce obesity risk via modulating gut microbiome. Therefore, the overall objective of this thesis to study prebiotic rich lentil as a possible whole food source to reduce obesity risk.

1.1. References


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2. HYPOTHESES AND OBJECTIVES

2.1. Study 1

2.1.1. Hypotheses

H₁: Prebiotic carbohydrates concentrations [raffinose family oligosaccharides (raffinose, stachyose, and verbascose), fructooligosaccharides (kestose and nestose), sugar alcohols (sorbitol and mannitol), and resistant starch] of different lentil market classes is affected by dehulling, splitting, cooking, cooling, and reheating.

H₀: Prebiotic carbohydrates concentrations of different lentil market classes is not affected by dehulling, splitting, cooking, cooling, and reheating.

2.1.2. Objective

Determine the prebiotic carbohydrates concentrations [raffinose family oligosaccharides (raffinose, stachyose, and verbascose), fructooligosaccharides (kestose and nestose), sugar alcohols (sorbitol and mannitol), and resistant starch] in three lentil market classes (red, green, and Pardina) subjected to three processing methods (whole, dehulled, and split), cooking, cooling, and reheating.
2.2. Study 2

2.2.1. Hypotheses

H₁: Lentil change rat’s feed and energy intake, body weight, percent body fat, liver weight, blood plasma triglycerides (TG’s), and fecal microbial composition.

H₀: Lentil does not change rat’s feed and energy intake, body weight, percent body fat, liver weight, TG’s, and fecal microbial composition of rats.

2.2.2. Objective

Determine the impact of lentil diet on rat feed and energy intake, body weight, percent body fat, liver weight, and blood plasma triglycerides (TG’s), and the fecal microbiome.
3. CHAPTER ONE

CAN LENTIL (Lens culinaris Medikus) REDUCE THE RISK OF OBESITY?

3.1. Abstract

Lentil (Lens culinaris Medikus), a cool season food legume, provides significant amounts of essential nutrients for healthy living. Lentil is a rich dietary source of low digestible carbohydrates (also known as prebiotic carbohydrates) that stimulate growth and activity of hind gut bacteria. These beneficial bacteria produce short-chain fatty acids that provide an energy source for colonocytes, strengthen the gut mucosal barrier, and suppress colonization of pathogens leading to reduced obesity and related non-communicable diseases. As such, products enriched with prebiotic carbohydrates are becoming popular health-promoting foods in human diets. This paper reviews an overview of current obesity prevalence, lentil production, available data on lentil prebiotic carbohydrates, and the promise of lentil as a whole food solution to combat global obesity. In addition, the effect of prebiotic carbohydrates on the human microbiome is briefly discussed.

3.2. Introduction

Obesity is a global health concern. Millions of deaths occur annually as a result of obesity-related non-communicable diseases. Today, more than 50% of the population in the developed world are obese or overweight; specifically, 13% of adults are obese and 39% are overweight but this varies regionally (Wang, Beydoun, Liang, Caballero, & Kumanyika, 2008; WHO, 2016). For example, 35%
of American adults (one in three adults) are obese, which is significantly higher than corresponding rates for Europe (17%), Africa (10%), or South East Asia (3%) (Abubakari et al., 2008; OECD, 2012; OECD, 2013; Ogden et al., 2014; WHO, 2015). It has been projected that by 2030 more than 50% of the American population and 20% world population will be obese (Finkelstein et al., 2012; NCD, 2016). Therefore, government and non-government organizations not only in the USA but internationally have mandates to prevent the global epidemic of overweight and obesity (“globesity”). Specifically, the World Health Organization (WHO) recommends the following actions: (1) a minimum of 150 min of physical activity per week for regular adults, (2) reduced intake of added sugars and fat, and (3) increased consumption of legumes, vegetables, and fruits by at least 5-6 servings per day (WHO, 2016).

Legumes have been a central part of vegetarian diets since the Paleo era. Lentil (Lens culinaris Medikus) is an ancient food legume crop, originating from the Near East approximately 10,000 years ago (Cubero, Pérez de la Vega, & Fratini, 2009; Ladizinsky, 1979). Lentil is a medium energy food that is recognized for its high nutritional value (Johnson, Thavarajah, Combs, & Thavarajah, 2013; Wang & Daun, 2006; Thavarajah et al., 2011). In particular, lentil is an excellent source of protein (20-30 g/100 g), healthy fat (<2 g/100 g), carbohydrates (40-60 g/100 g), dietary fiber, and a range of micronutrients (Thavarajah & Thavarajah, 2012). A 50 g serving of lentil can provide 3.7-4.5 mg of iron, 2.2-2.7 mg of zinc, 22-34 µg of selenium, 50-250 µg of beta-carotene,
and 216-290 µg of folates (Sen Gupta et al., 2013; Thavarajah et al., 2011; Thavarajah, Thavarajah, Sarker, & Vandenberg, 2009). Unlike other grains, lentil is very low in phytic acid (2.5-4.4 mg/g), which binds iron and zinc and thus renders these nutrients poorly bioavailable (Thavarajah, Thavarajah, & Vandenberg, 2009).

Recent studies indicate that lentil is also a rich source of prebiotic carbohydrates (Johnson, Thavarajah, Combs, & Thavarajah, 2013). Prebiotic carbohydrates are a selectively fermented ingredient that allow specific biochemical changes in gastrointestinal microflora that benefit host well-being and health (Roberfroid, 2007). The human gut microbiome feature two dominant beneficial bacterial groups: Bacteriodetes and Firmicutes (Ley, Turnbaugh, Klein, & Gordon, 2006). Interestingly, Bacteriodetes to Firmicutes ratios increase and a number of metabolic parameters improve in obese mice fed a prebiotic rich diet (Everard et al., 2011). However, these results are still inconclusive and more research is required to measure the true prebiotic effect on obesity and overweight. The objective of this review paper is to provide an overview of current lentil production, available data on lentil prebiotic carbohydrates, and the promise of lentil as a whole food solution to combat global obesity. The effect of prebiotic carbohydrates on the human microbiome is also briefly discussed.

### 3.3. Obesity Prevalence

Obesity is defined as an excess fat accumulation in the body, measured by body mass index (BMI), waist circumference, skinfold thickness, and
bioimpedance (Kopelman, 2000). BMI is the most widely used method, with values between 25.0 and 29.9 kg/m² considered overweight, 30.0 to 39.9 kg/m² obese, and ≥40 kg/m² morbidly obese (Kopelman, 2000). Obesity increases the risk of non-communicable diseases, including cardiovascular diseases, type 2 diabetes, and cancers (Beaglehole et al., 2011). Global obesity has doubled since 1980, and by 2014 more than 1.9 billion adults (>18 y) were overweight and more than 600 million were obese (WHO, 2016). The prevalence of obesity is high in developed regions compared to developing regions; for example, obesity prevalence in Asian and African countries is much lower than in Middle Eastern, European, or North American countries (Figure 3.1) (OECD, 2013; The World Factbook, 2015). The prevalence of overweight, obesity, and extreme obesity in the USA is 33, 36, and 6%, respectively (Ogden & Carroll, 2010); this means that approximately two out of every three adults in the USA are overweight or obese. Within the USA, the state of Arkansas has the highest obesity prevalence (36%) and Colorado the least (21%) (The State of Obesity, 2015). This variation in American obesity prevalence is mainly influenced by social, economic, and demographic factors as well as access to nutritious foods (Caprio et al., 2008; Cummins & Macintyre, 2006; Larson, Story, & Nelson, 2009). For example, the prevalence of obesity among non-Hispanic blacks, non-Hispanic whites, and Hispanics is 36, 24, and 29%, respectively (Ogden, Carroll, Kit, & Flegal, 2013). Non-Hispanic black women have the highest obesity prevalence (39%) compared to non-Hispanic black men (32%), Hispanic women
(29%), Hispanic men (28%), non-Hispanic white men (25%), and non-Hispanic white women (22%) (Ogden, Carroll, Kit, & Flegal, 2013).

However, obesity is preventable. Changes in diet and physical activity are often benefits of changing environmental and societal behaviors (European Food Information Council, 2014). Supportive government policies in health, agriculture, transport, urban planning, environment, food processing, distribution, marketing, and education are also important for preventing obesity and overweight (Robinson & Sirard, 2005; Sallis & Glanz, 2009). Sedentary lifestyles and high intake of energy dense (high in fat and/or sugar) foods increase weight gain and obesity (WHO, 2016).
Figure 3.1. Obesity prevalence in regional countries, 2014 (OECD, 2013; The World Factbook, 2015)
3.4. Lentils

Lentil is a cool season food legume commercially cultivated around the world (Cokkizgin & Shtaya, 2013). Current annual lentil production is approximately 5 million tons with the greatest production attributed to Western Canada (38%) followed by India (23%), Turkey (8%), Australia (7%), and the USA (5%) (FAOSTAT, 2015). More than 90% of the lentil produced in Canada, the USA, and Australia is exported to South East Asia, the Middle East, and Africa (FAOSTAT, 2015). Lentil was first introduced to North America in the early 1980s and it has since become a major pulse crop in the Pacific Northwest and Midwestern regions of the USA, including North Dakota, South Dakota, and Montana. Lentils belong to the genus *Lens* and the tribe *Fabeae* of the *Fabaceae* family (Fikiru, Tesfaye, & Bekele, 2007), and are a self-pollinating dicot with diploid chromosomes (2n=2x=14) (Ford & Taylor, 2003). The size of the lentil genome is approximately 4,063 Mbp (Arumuganathan & Earle, 1991), and genome sequencing is currently underway (Kaur et al., 2011).

Several lentil market class are represented in the North American lentil trade (Table 3.1) (Government of Saskatchewan, 2016; Saskatchewan Pulse Growers, 2000; Thavarajah, Ruszkowski, & Vandenberg, 2008). These market classes are based on consumer preference, seed size, and color. Two market classes are based on seed size: large seeded Chilean type (1000 seed weight >50 g) and small seeded Persian type (1000 seed weight <40 g). Also, 1000 seed weight is used to classify further lentils as extra small (29-32 g), small (33-
45 g), medium (51-52 g), or large (55-75 g). Lentil seed coat color can be green, brown, gray, purple, or black, and seed cotyledon colors range from yellow to red to green (Table 3.1) (Government of Saskatchewan, 2016; Saskatchewan Pulse Growers, 2000; Thavarajah, Ruszkowski, & Vandenberg, 2008).
Table 3.1. Common lentil market classes and consuming countries (Government of Saskatchewan, 2016; Saskatchewan Pulse Growers, 2000; Thavarajah, Ruszkowski, & Vandenberg, 2008).

<table>
<thead>
<tr>
<th>Market Class</th>
<th>Seed size (1000 seed weight)</th>
<th>Genotype</th>
<th>Consuming countries</th>
</tr>
</thead>
<tbody>
<tr>
<td>Red</td>
<td>Extra small (29-32 g)</td>
<td>CDC Impala, CDC Imperial CL, CDC Red bow, CDC Robin, CDC Rosebud, CDC Rosetown</td>
<td>Canada, United States of America, Turkey, Egypt, India, Australia, Sri Lanka, Pakistan, Bangladesh, Syria, Nepal</td>
</tr>
<tr>
<td></td>
<td>Small (33-45 g)</td>
<td>CDC Blaze, CDC Redberry, CDC Rouleau, CDC Impact CL, CDC Red Rider, CDC Maxim CL, CDC Imax CL, CDC Dazil CL, CDC Red coat, CDC Redcliff, CDC Cherie</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Large (55-73 g)</td>
<td>CDC KR-1, CDC-KR-2</td>
<td></td>
</tr>
<tr>
<td>Yellow</td>
<td>Extra small (29-32 g)</td>
<td>CDC Asterix</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Small (33-45 g)</td>
<td>CDC Eston, CDC Milestone, CDC Icery, CDC Invincible CL</td>
<td>Spain, England, United States, Germany</td>
</tr>
<tr>
<td></td>
<td>Large (55-73 g)</td>
<td>CDC Sedley, Laird, Plato, CDC Sovereign, CDC Greenland, CDC Improve</td>
<td></td>
</tr>
<tr>
<td>Green</td>
<td>Extra small (29-32 g)</td>
<td>CDC QG-2</td>
<td>Morocco, Greece, Italy, Egypt, Mexico, Northwestern Europe, Spain, Algeria, United States</td>
</tr>
<tr>
<td></td>
<td>Small (33-45 g)</td>
<td>CDC QG-3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Medium (51-52 g)</td>
<td>CDC Impress, CDC Imigreen, CDC Meteor, CDC Richlea</td>
<td></td>
</tr>
<tr>
<td>Spanish Brown</td>
<td>Small (33-45)</td>
<td>Pardina</td>
<td>Spain</td>
</tr>
</tbody>
</table>
As noted above, lentil is a rich source of protein with a balanced amino acid profile, plentiful low digestible carbohydrates, and a range of human essential human micronutrients (Table 3.2) (de Almeida Costa, da Silva Queiroz-Monici, Pissini Machado Reis, & de Oliveira, 2006; Hefni, McEntyre, Lever, & Slow, 2015; Iqbal, Khalil, Ateeq, & Sayyar Khan, 2006; Johnson, Thavarajah, Thavarajah, Payne, et al., 2015; Johnson, Thavarajah, Combs, & Thavarajah, 2013; Ray et al., 2014; Solanki, Kapoor, & Singh, 1999; Thavarajah, Ruszkowski, & Vandenberg, 2008; Thavarajah et al., 2011). For instance, a single serving of lentils (100 g) contains 2 g of fat, 4-9 g of dietary fiber, 23-27 g of protein, and 64-74 g of total carbohydrates (by difference). As a result of high levels of low digestible carbohydrates, lentils have a low energy density that reduces glycemic response in humans (Chung, Liu, Hoover, Warkentin, & Vandenberg, 2008). Lentil is low in fat (contributes <5% of its energy as fat) compared to other legumes including chickpea (Cicer arietinum L.), field pea (Pisum sativum L.), and soybean (Glycine max L.) that contain >15-45% of their energy as fat (Messina, 1999). Lentil also contains substantial amounts of vitamins and minerals in relative proportions that are much higher than other grain legumes (Messina, 1999).

Lentil starch refers to the non-structural carbohydrates that comprise the 47-52 g of total starch found in 100 g of lentil. Lentil starch is composed of amylose (a linear glucan with few branches) and amylopectin (a larger and highly branched molecule), and the higher levels of amylose starch mean legume
Table 3.2. Nutritional composition of lentil (de Almeida Costa, da Silva Queiroz-Monici, Pissini Machado Reis, & de Oliveira, 2006; Hefni, McEntyre, Lever, & Slow, 2015; Iqbal, Khalil, Ateeq, & Sayyar Khan, 2006; Johnson, Thavarajah, Thavarajah, Payne, et al., 2015; Johnson, Thavarajah, Combs, & Thavarajah, 2013; Ray et al., 2014; Solanki, Kapoor, & Singh, 1999; Thavarajah, Ruszkowski, & Vandenberg, 2008; Thavarajah et al., 2011).

<table>
<thead>
<tr>
<th>Nutrients</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Energy (kcal)</td>
<td>359-362</td>
</tr>
<tr>
<td>Carbohydrates</td>
<td></td>
</tr>
<tr>
<td>Total starch (g/100 g)</td>
<td>45-48</td>
</tr>
<tr>
<td>Total prebiotic carbohydrates (g/100 g)</td>
<td>12-14</td>
</tr>
<tr>
<td>Resistant starch (mg/100 g)</td>
<td>2.8-3.4</td>
</tr>
<tr>
<td>Fiber (g/100 g)</td>
<td>4-9</td>
</tr>
<tr>
<td>Protein (g/100 g)</td>
<td>23-27</td>
</tr>
<tr>
<td>Fat (g/100 g)</td>
<td>2.0-2.3</td>
</tr>
<tr>
<td>Minerals</td>
<td></td>
</tr>
<tr>
<td>Potassium (mg/100 g)</td>
<td>800-1002</td>
</tr>
<tr>
<td>Magnesium (mg/100 g)</td>
<td>94-107</td>
</tr>
<tr>
<td>Calcium (mg/100 g)</td>
<td>27-43</td>
</tr>
<tr>
<td>Iron (mg/100 g)</td>
<td>8-10</td>
</tr>
<tr>
<td>Zinc (mg/100 g)</td>
<td>4-5</td>
</tr>
<tr>
<td>Phosphorus (mg/100 g)</td>
<td>290-298</td>
</tr>
<tr>
<td>Copper (mg/kg)</td>
<td>7-9</td>
</tr>
<tr>
<td>Selenium (µg/100 g)</td>
<td>43-67</td>
</tr>
<tr>
<td>Sodium (mg/100 g)</td>
<td>76-82</td>
</tr>
<tr>
<td>Vitamins</td>
<td></td>
</tr>
<tr>
<td>Folate (µg/100 g)</td>
<td>216-290</td>
</tr>
<tr>
<td>Choline, total (mg/100 g)</td>
<td>176-196</td>
</tr>
</tbody>
</table>
starch digestion is significantly slower than foods high in amylopectin starch (Thorne, Thompson, & Jenkins, 1983). This slower digestion is possibly due to the degree of crystallization or character of the outermost layers of the starch granule. In addition, lentil has approximately 12-14 g of prebiotic carbohydrates per 100 g that pass through the gastrointestinal tract as they are resistant to digestion by human digestive enzymes (Johnson, Thavarajah, Combs, & Thavarajah, 2013). These prebiotic carbohydrates may also lower the rate and extent of starch digestibility that is associated with increased satiety, resulting in improved management of body weight, reduced glycemic response, and insulin resistance (Cani & Delzenne, 2011; Delzenne & Cani, 2010; Kau, Ahern, Griffin, Goodman, & Gordon, 2011).

### 3.5. Lentil Prebiotic Carbohydrates

Most dietary nutrients are metabolized in the human gastrointestinal tract using digestive enzymes. Some nutrients not utilized by digestive enzymes, called colonic nutrients or “prebiotics”, are used by human gastrointestinal microflora. Prebiotics are defined as “selectively fermented components that allows specific changes in the composition and/or activity in the gastrointestinal microflora that confers benefits to host well-being and health” (Roberfroid, 2007). Only two carbohydrates, inulin and trans-galactooligosaccharide, fulfill the original definition of prebiotics; however, several other carbohydrates are now considered prebiotics based on their chemical structure and beneficial impacts on human gut health (Table 3.3). Prebiotic carbohydrates are classified into two
major groups: dietary fiber and sugar alcohols. Dietary fiber is divided into categories of glucose based polymers (e.g., resistant starch and cellulose) and non-glucose based polymers. Non-glucose based polymers are further classified as either (1) fructose based polymers (e.g., kestose, nystose, and inulin) or (2) others, which includes raffinose family oligosaccharides (e.g., raffinose, stachyose, and verbascose), pectin, hemicellulose, guar gum, and polydextrose (Table 3.3). Naturally occurring sugar alcohols include sorbitol, mannitol, and galactinol. Most legumes, cereals, fruits, and vegetables are naturally rich in prebiotic carbohydrates, and have a considerable potential to promote human health and nutrition. Major sources of dietary prebiotic carbohydrates are wheat, onion, and green bananas. These prebiotic carbohydrates have been used in the food industry in the production of dry cereals, beverages, dairy products, chewing gum, candy, and pharmaceuticals (Grabitske & Slavin, 2009).
Table 3.3. Prebiotic carbohydrates in common staple foods (Academy of Nutrition and Dietetics, 2012; Berardini, Knödler, Schieber, & Carle, 2005; Brown & Serro, 1953; Campbell et al., 1997; Cruz & Park, 1982; Fanaro et al., 2007; Figuerola, Hurtado, Estévez, Chiffelle, & Asenjo, 2005; Hidaka, Eida, Takizawa, Tokunaga, & Tashiro, 1986; Johnson, Thavarajah, Combs, & Thavarajah., 2013; Kuo, VanMiddlesworth, & Wolf, 1988; Lo Bianco, Rieger, & Sung, 2000; Loo et al., 1999; Rupérez & Toledano, 2003; Sajilata, Singhal, & Kulkarni, 2006; Slavin, 1987; Wang, 2009; Yang & Keding, 2009).

<table>
<thead>
<tr>
<th>Category</th>
<th>Examples</th>
<th>Food Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Dietary fiber</td>
<td>a. Glucose based polymers</td>
<td></td>
</tr>
<tr>
<td></td>
<td>I. Resistant starch</td>
<td>Potatoes, green banana, corn</td>
</tr>
<tr>
<td></td>
<td>II. Cellulose</td>
<td>Plant based foods</td>
</tr>
<tr>
<td>b. Non-glucose based polymers</td>
<td>1. Fructose based</td>
<td></td>
</tr>
<tr>
<td></td>
<td>I. Kestose</td>
<td>Jerusalem artichoke</td>
</tr>
<tr>
<td></td>
<td>II. Nystose</td>
<td>Onion, Jerusalem artichoke, legumes</td>
</tr>
<tr>
<td></td>
<td>III. Inulin</td>
<td>Leeks, onion, garlic, asparagus, Jerusalem artichokes, chicory</td>
</tr>
<tr>
<td></td>
<td>2. Others (non-fructose based)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Raffinose family oligosaccharides</td>
<td></td>
</tr>
<tr>
<td></td>
<td>I. Raffinose</td>
<td>Legumes, cereals</td>
</tr>
<tr>
<td></td>
<td>II. Stachyose</td>
<td>Legumes, cereals</td>
</tr>
<tr>
<td></td>
<td>III. Verbascose</td>
<td>Legumes, cereals</td>
</tr>
<tr>
<td></td>
<td>Pectin</td>
<td>Apple, pomace, citrus</td>
</tr>
<tr>
<td></td>
<td>Hemicellulose</td>
<td>Wheat bran, legumes</td>
</tr>
<tr>
<td></td>
<td>Guar gum</td>
<td>Guar or Cluster bean</td>
</tr>
<tr>
<td></td>
<td>Polydextrose</td>
<td>Cereals</td>
</tr>
<tr>
<td>B. Sugar alcohols</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sorbitol</td>
<td>Peach, apple, pears, legumes</td>
</tr>
<tr>
<td></td>
<td>Mannitol</td>
<td>Seaweed, celery, legumes</td>
</tr>
<tr>
<td></td>
<td>Galactinol</td>
<td>Beet, legumes</td>
</tr>
</tbody>
</table>
3.5.1. Lentil sugar alcohols

Sugar alcohols include polyols, polyalcohols, and polyhydric alcohols (Bieleski, 1982). Sugar alcohols are formed during plant growth, especially under water stress conditions (Loescher, 1987). In addition to moisture stress tolerance, recent research reveals that sugar alcohols have a prebiotic effect as they generate a low glycemic response similar to resistant starch (Foster-Powell, Holt, & Brand-Miller, 2002). Lentil is a rich source of sugar alcohols, however the type and concentration thereof varies with genotype, growing location, and country (Table 3.4) (Johnson, Thavarajah, Thavarajah, Fenlason, et al., 2015). For example, lentil grown in the USA has average concentrations of 1126-1392 mg/100 g sorbitol and 45-69 mg/100 g mannitol; however, moderate differences are reported as a result of growing location, genotype, and environmental effects (Johnson, Thavarajah, Combs, & Thavarajah, 2013). Quemener et al. reports galactinol concentrations among pulse crops ranging from 50 to 170 mg/100 g, results from Johnson et al. of 46-89 mg/100 g across all countries are within the range (Johnson, Thavarajah, Thavarajah, Fenlason, et al., 2015; Quemener & Brillouet, 1983).
Table 3.4. Lentil sugar alcohol types and concentrations (Johnson, Thavarajah, Thavarajah, Fenlason, et al., 2015).

<table>
<thead>
<tr>
<th>Growing country</th>
<th>Sorbitol (mg/100 g)</th>
<th>Mannitol</th>
<th>Galactitol</th>
</tr>
</thead>
<tbody>
<tr>
<td>USA</td>
<td>1126-1392</td>
<td>45-69</td>
<td>60-78</td>
</tr>
<tr>
<td>Lebanon</td>
<td>1358-1698</td>
<td>95-139</td>
<td>39-65</td>
</tr>
<tr>
<td>Morocco</td>
<td>1657-1991</td>
<td>112-152</td>
<td>49-77</td>
</tr>
<tr>
<td>Syria</td>
<td>1307-1531</td>
<td>69-105</td>
<td>35-57</td>
</tr>
<tr>
<td>Turkey</td>
<td>1230-1426</td>
<td>100-122</td>
<td>45-61</td>
</tr>
<tr>
<td>Ethiopia</td>
<td>1461-1761</td>
<td>97-139</td>
<td>73-105</td>
</tr>
<tr>
<td>Mean</td>
<td>1495</td>
<td>104</td>
<td>62</td>
</tr>
</tbody>
</table>

3.5.2. Lentil raffinose family oligosaccharides

Raffinose family oligosaccharides (raffinose, stachyose, and verbascose) are plentiful in cereals and legumes (Bachmann, Matile, & Keller, 1994). Raffinose is mainly present in cereals whereas stachyose and verbascose are mainly present in legumes (Bachmann et al., 1994). Lentil is a rich source of raffinose family oligosaccharides, with concentrations ranging from 5181-6763 mg/100 g depending on genotype, growing location, and environmental conditions (Johnson, Thavarajah, Combs, & Thavarajah, 2013; Johnson, Thavarajah, Thavarajah, Fenlason, et al., 2015). Raffinose family oligosaccharides vary with lentil growing location and country (Table 3.5) (Johnson, Thavarajah, Thavarajah, Fenlason, et al., 2015). The mean concentration of raffinose family oligosaccharides grown in the USA is 6409 mg/100 g, which is within the range of values for Lebanon, Morocco, Syria, Turkey, and Ethiopia of 5240, 7149, 5225, 5767, and 6046 mg/100 g, respectively (Table 3.5). Processing may also
change lentil raffinose family oligosaccharide concentration. Johnson et al. indicate that cooking, cooling, and reheating of lentil can reduce total raffinose family oligosaccharide concentrations from 5500-6100 to 4300-4900 mg/100 g depending on the lentil market class (Figure 3.2) (Johnson, Thavarajah, Thavarajah, Payne, et al., 2015). In contrast, Wang et al. show cooking significantly reduces the concentration of raffinose and stachyose but increases the concentration of verbascose in eight lentil varieties (Wang, Hatcher, Toews, & Gawalko, 2009). Overall, these results clearly indicate that raffinose family oligosaccharide concentrations are influenced by genotype, growing location, country, and processing conditions, and therefore the careful genetic selection of lentil germplasm may need to consider with those variables.

**Table 3.5.** Raffinose family and fructooligosaccharide concentrations of lentil grown in different countries (Johnson, Thavarajah, Thavarajah, Fenlason, et al., 2015).

<table>
<thead>
<tr>
<th>Growing country</th>
<th>Raffinose family oligosaccharides (mg/100 g)</th>
<th>Fructo oligosaccharides (mg/100 g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Raffinose+Stachyose</td>
<td>Verbascose</td>
</tr>
<tr>
<td>USA</td>
<td>3489-4423</td>
<td>2146-2760</td>
</tr>
<tr>
<td>Lebanon</td>
<td>2915-3713</td>
<td>1634-2218</td>
</tr>
<tr>
<td>Morocco</td>
<td>3913-5691</td>
<td>1712-2982</td>
</tr>
<tr>
<td>Syria</td>
<td>2979-3657</td>
<td>1636-2178</td>
</tr>
<tr>
<td>Turkey</td>
<td>3126-3862</td>
<td>2056-2490</td>
</tr>
<tr>
<td>Ethiopia</td>
<td>3458-4090</td>
<td>2012-2532</td>
</tr>
<tr>
<td>Mean</td>
<td>3776</td>
<td>2196</td>
</tr>
</tbody>
</table>

nd - not detected
Figure 3.1. Concentration of raffinose family oligosaccharides (mg/100 g; dry weight basis) in whole and dehulled lentil grown in the USA (Johnson, Thavarajah, Thavarajah, Payne, et al., 2015).
3.5.3. Lentil fructooligosaccharides

Fructooligosaccharides are a mixture of oligosaccharides linking to fructose units (Hidaka et al., 1986). Compared to raffinose oligosaccharides, very small quantities of fructooligosaccharides (nystose and kestose) are found in US-grown lentils (Bhatta, 1988; Biesiekierski et al., 2011; Johnson, Thavarajah, Combs, & Thavarajah, 2013), and levels are significantly lower still in lentil grown in other countries such as Lebanon and Morocco (Johnson, Thavarajah, Thavarajah, Fenlason, et al., 2015). Unlike sugar alcohols and raffinose family oligosaccharides, fructooligosaccharide concentrations are not affected by genotype but significantly vary with growing location and country (Johnson, Thavarajah, Combs, & Thavarajah, 2013). A 100 g of lentil serving can provide 0-988 mg of fructooligosaccharides, including kestose and nystose (Table 3.5) (Johnson, Thavarajah, Thavarajah, Fenlason, et al., 2015).

3.5.4. Lentil resistant starch

Resistant starches are glucose-based polymers that are resistant to human digestive enzymes. Jernkins et al. report that lentil induces a low-glycemic response as a result of the high resistance of lentil starch to hydrolysis (Jenkins et al., 1981). Lentils, on average, contain 63% carbohydrates calorically (Bhatta, 1988). Among lentil carbohydrates, starch represents about 45-48% of total carbohydrates (Johnson Thavarajah, Combs, & Thavarajah, 2013). Resistant starch accounts for approximately 1.6 to 8.4% of the dry weight of raw lentil and 1.6-9.1% after cooking and freeze-drying (de Almeida Costa et al., 2006). Johnson et al. indicate that resistant starch concentrations in raw, cooked, cooled, and reheated lentil are 3.0, 3.0, 5.1, and 5.1%, respectively, demonstrating cooling-induced synthesis of resistant starch from
gelatinized starch (Johnson, Thavarajah, Thavarajah, Payne, et al., 2015); however, these values vary with lentil market class, genotype, and processing method (Johnson, Thavarajah, Thavarajah, Payne, et al., 2015). Wang et al. show that cooking different lentil varieties can increase resistant starch from 2-4 to 4-5 g/100 mg (Wang, Hatcher, Toews, & Gawalko, 2009); however, de Almeida Costa et al. report a slight reduction in lentil resistant starch after cooking (de Almeida Costa et al., 2006). Other pulse crops including chickpea, field pea, and common bean (*Phaseolus vulgaris* L.) have comparable amounts of resistant starch to lentils (de Almeida Costa et al., 2006).

### 3.6. Prebiotic carbohydrate consumption

Understanding of prebiotic carbohydrate consumption is still limited. A survey of American diets estimates that human consumption of prebiotic carbohydrates ranges from 1 to 10 g/d/capita in the United States (Van Loo, Coussement, De Leenheer, Hoebregs, & Smits, 1995). The Institute of Medicine (IOM) set the Acceptable Macronutrient Distribution Range (AMDR) for carbohydrates at 45-65% of total energy intake. The Adequate Intake (AI) of total fiber is 38 g for men and 25 g for women (The National Academies of Sciences, Engineering and Medicine, 2016). Although many prebiotic carbohydrates are categorized as fiber, no official recommendations have been made specifically regarding their consumption. Several researchers have recommended intakes for fiber components, as follows:

1. Fructooligosaccharide (FOS) – 10 g/day (Hauly & Moscatto, 2002);
2. Galactooligosaccharide (GOS) – 2-3 g/day (Carabin & Flamm, 1999);
3. Xylooligosaccharide (XOS) – 0.7 g/day (Tomomatsu, 1994);
4. Resistant starch (RS) – 4 g/day (Australian National Health and Medical Research Council and New Zealand Ministry of Health, 2006);

5. Inulin – no recommendations are available, but there are no known toxic effects at any level of intake (Hauly & Moscatto, 2002).

Many of these prebiotic carbohydrates are found naturally in legumes, vegetables, and fruits (Table 3.3). However, legumes including lentil can be a major source of prebiotic carbohydrates. A 100 g serving of lentil provides 57% of the daily recommended intake of raffinose family oligosaccharides, which is higher than that provided by chickpea (42%), pea (52%), or common bean (38%); a 100 g serving of lentil also provides 78% of the recommended daily intake of resistant starch, which is also more than that provided by chickpea (56%), pea (47%), or common bean (58%) (de Almeida Costa et al., 2006; Johnson, Thavarajah, Thavarajah, Payne, et al., 2015). Thus, lentil is a possible source of prebiotic carbohydrates to combat obesity and provide moderate amounts of energy.

3.7. Human gut microbiome

The human intestinal tract is home to more than 100 trillion microorganisms (Bäckhed, Ley, Sonnenburg, Peterson, & Gordon, 2005), over a surface area of 300 m² (Bäckhed, Ley, Sonnenburg, Peterson, & Gordon, 2005; Holzapfel, Haberer, Snel, Schillinger, & Huis in’t Veld, 1998). Gut microbes are involved in major physiological activities, acting as a barrier to pathogens attempting to invade gut epithelial cells, stimulating the immune system, increasing nutrient availability, stimulating bowel motility, and reducing cholesterol levels (Holzapfel, Haberer, Snel, Schillinger, & Huis in’t Veld, 1998; Holzapfel & Schillinger, 2002). Studies suggest the intestinal
microbiome and a low-calorie diet can play important roles in combating obesity and related non-communicable diseases (Ley, Turnbaugh, Klein, & Gordon, 2006; Nadal et al., 2009). A complex bacterial community inhabits the human gastrointestinal tract. Three dominant phyla have been identified in fecal flora: *Firmicutes*, *Bacteroides*, and *Actinobacteria* and sub-dominant groups include *Enterobacteria*, *Streptococci*, and *Lactobacilli* (Sghir et al., 2000; Vrieze et al., 2010). The relative proportion of *Bacteroidetes* is decreased in obese individuals compared to lean individuals; however, this relative proportion rebounds with a low-calorie diet (David et al., 2013). Furthermore, consumption of non-digestible, fermentable carbohydrates (or prebiotics) may stimulate the growth and activity of hind gut bacteria by producing short-chain fatty acids that provide an energy source for colonocytes, strengthen the gut mucosal barrier, and suppress colonization of pathogens. Even though several studies reveal that gut microbiome composition or activity is related to both obesity and related non-communicable diseases, none clearly describe the link between microbial composition and obesity prevention (Tremaroli & Bäckhed, 2012).

A diet rich in prebiotic carbohydrates can reduce cholesterol levels by modulating gut microbiomes (Cani et al., 2009). Cani et al. suggest that fructooligosaccharides increase the growth of *Bifidobacteria*, which increases the expression of tight junction proteins (zonula occludens 1 and occludin) and is linked to reduced permeability of the gut epithelium (Cani et al., 2009). As a result, cholesterol formation is reduced via a reduction in blood lipopolysaccharide levels, leading to increased glucose tolerance, insulin sensitivity, and reduced fat storage (Blaut & Bischoff, 2010). A recent human study indicates that fructooligosaccharide supplementation reduces body weight as a
result of increasing satiety hormones including peptide YY (PYY) and ghrelin (Parnell & Reimer, 2009). A diet rich in resistant starch can also reduce food intake as a result of increasing satiety hormones (Willis, Eldridge, Beiseigel, Thomas, & Slavin, 2009). Further, resistant starch can influence long-term energy balance by altering the neuronal pathways associated with gut peptide YY (PYY) and glucagon like peptide (GLP-1) signals (Keenan et al., 2006). Delzenne et al. clearly outline the effect of prebiotics on gut microbiota and metabolic disorders using animal and human models (Delzenne, Neyrinck, & Cani, 2013). They conclude that highly fermented prebiotic carbohydrates are able to counteract several metabolic alterations linked to obesity, including hyperglycemia, inflammation, and hepatic steatosis. Delzenne et al. also discuss how initial mechanistic studies indicated prebiotics could only increase \textit{Bifidobacteria} counts, which are related to regulation of host energy homoeostasis (Delzenne, Neyrinck, & Cani, 2013); however, it is clear now with animal models that these bacteria can promote gut hormone release, change the gut barrier integrity, and release bacterially derived metabolites that can reduce human food intake and obesity. In conclusion, human intervention studies are required to test the ability of ‘colonic’ nutrients to selectively promote beneficial bacteria in the human gut as well as to determine how promoting foods with colonic nutrients can aid in the nutritional management of overweight and obesity.

3.8. Mechanisms of prebiotic effects on metabolism

Adipose tissue, once thought to be largely inactive, is now understood to be a complex and highly interactive endocrine organ, with many secretory functions (e.g. leptin, cytokines, adiponectin) and hormonal responses via receptors (e.g. via insulin,
glucagon, leptin, and catecholamines, among many others) (Kershaw & Flier, 2004). Leptin, for example, is secreted in response to multiple hormonal stimuli and is critical for central feedback of total body energy reserves (Friedman & Halaas, 1998). Cytokines are also produced within adipose tissue, including tumor necrosis factor (TNF)-α, interleukin (IL)-1, and IL-6, all of which are immune activators and markers of inflammation (Amrani et al., 1996; Fried, Bunkin, & Greenberg, 1998; Hrnciar et al., 1999).

Chronic, low-grade inflammation is characteristic of obesity and important in early pathogenesis of the disorder (Cani et al., 2007). Exogenous administration of lipopolysaccharide (LPS) in rats, normally shed from Gram-negative organisms in the gut microbiota, stimulated an inflammatory response and induced weight gain, insulin resistance, and hyperglycemia to a similar extent seen with high-fat diet (Cani et al., 2007). These changes are mediated via activation of toll-like receptors (TLR); specifically, TLR4 responds to microbial LPS and lipids, resulting in increased production of NF-κB gene transcription (Creely et al., 2007). NF-κB signals production of cytokines, such as TNF-α (Hotamisligil, Shargill, & Spiegelman, 1993), IL-1 (Hotamisligil, Shargill, & Spiegelman, 1993; Weisberg et al., 2003), and IL-6 (Weisberg et al., 2003), as well as chemotaxins, such as C3a (Koistinen et al., 2001), which activate and recruit inflammatory cells to adipose tissue in obesity.

The same metabolic disturbances seen in rats injected with LPS (weight gain, insulin resistance, and fasting hyperglycemia) (Cani et al., 2007) are observed with high-fat diet and are associated with changes in gut microbiota, intestinal permeability, and endotoxemia (Moreira, Texeira, Ferreira, Peluzio, & Alfenas, 2012). Specifically,
high-fat diet allows increased permeability to LPS by down-regulation of tight junction proteins, ZO-1 and occludin (Cani et al., 2009). These discoveries and others lent support to the hypothesis that diet can be used to alter microbiota populations and activities, reducing the inflammatory cascade seen in obesity and DM II. Researchers have since uncovered numerous interactions by which prebiotics improve obesity and DM II, including anti-inflammatory and metabolic mechanisms, but, surprisingly, many of the prebiotic effects are moderated via hormones (Carnahan, Balzer, Panchal, & Brown, 2014).

Prebiotic fermentation in the gastrointestinal (GI) tract liberates short-chain fatty acids (SCFAs) (Gibson & Roberfroid, 1995). These microbial products have been studied extensively, and their many physiological actions are well documented (Carnahan, Balzer, Panchal, & Brown, 2014). The presence SCFAs leads to a reduction in TLR4 expression and subsequent inflammatory response in colonocytes (Isono et al., 2007). SCFAs also activate GPR43 receptors, reducing lipolysis and free fatty acids in serum, thus reducing TLR activation by lipids and the ensuing inflammatory cascade (Maslowski et al., 2009). By treating neutrophils with propionate and acetate, the LPS-induced NF-κB and TNF-α inflammatory response was suppressed (Kiens, Alsted, & Jeppesen, 2011). Furthermore, supplementation with 5% butyrate to mice fed a high-fat diet prevented development of obesity and insulin resistance (Gobinath, Madhu, Prashant, Srinivasan, & Prapulla, 2010). Finally, propionate was able to reduce serum cholesterol in rats, indicating a possible interaction with HMG CoA reductase (Arora, Sharma, & Frost, 2011).
Microbial fermentation of prebiotics induces endogenous release of a variety of hormones, controlling gut endothelial barrier functions and metabolic homeostasis (Cani, 2016). Intestinal L cells release glucagon-like peptide (GLP)-1 and GLP-2, controlling metabolic hormone activity and gut permeability, respectively (Cani et al., 2009; Delzenne, Cani, & Neyrinck, 2007). Prebiotic-induced changes in gut microbiota also modulate peptide YY (PYY) and ghrelin, involved in appetite regulation (Cani, Dewever, & Delzenne, 2004).

Systematic review and meta-analysis of randomized controlled trials in humans revealed that prebiotic supplementation reduced total serum cholesterol and triglycerides and increased high-density lipoprotein cholesterol (Beserra et al., 2015). Further, when taken as a symbiotic (i.e. with beneficial microbial species), prebiotics reduced insulin resistance and triglycerides (Beserra et al., 2015). Prebiotic effects on body weight have been controversial, with some studies showing improvement and others insignificant (Barengolts, 2016; Beserra et al., 2015); however, this is unsurprising for several reasons. 1) Obesity is an insidious disease, and short trials of prebiotic carbohydrates are insufficient to produce significant effects. 2) BMI, commonly used in clinical trials, is not an accurate measure of obesity as a disease, because it does not account for changes in lean body mass or distribution of adipose tissue, e.g. subcutaneous vs. visceral. Finally, 3) most trials focus on prebiotic use as a supplement rather than a staple component of diet, as they would have been consumed traditionally by ancient populations. To achieve significant, lasting effects from prebiotic use, prebiotic components must be consumed as part of a balanced, whole-food diet.
3.9. Closing thoughts

Millions of people around the world suffer from health issues as a result of poor nutrition. Obesity has been a severely neglected global public health concern for decades and, today, obesity is taking over many parts of the world. In fact, the global population continues to increase, with more than 90 million people to feed each year; global food demands are expected to double by 2050. Therefore, to combat global obesity, novel ways to produce nutritious foods, beyond calorie-focused approaches, are required. Investigating the potential of traditional food legumes including lentils may be necessary to provide better nutrition solutions towards improved human health.

Recent research demonstrated that prebiotic carbohydrate rich diet may reduce obesity related non communicable diseases via modulation of hind gut bacteria. Lentil is an emerging pulse crop in the USA that is typically grown in rotation with cereal and oil crops. Interestingly, research indicates that lentils may provide over 12-14 g of total prebiotic carbohydrates and a range of micronutrients per 100 g serving. In addition, these levels can further increase two-fold after cooking, cooling, and re-heating.

Therefore, lentils offer new opportunities as a whole food solution to combat obesity and overweight. Finally, obesity is preventable, however holistic systemic approaches are required to combine agricultural production and human health.
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4. CHAPTER TWO

THE IMPACT OF PROCESSING AND COOKING ON PREBIOTIC CARBOHYDRATES IN LENTIL (Lens culinaris Medikus)

4.1. Abstract

Lentil (Lens culinaris Medikus) is a significant food source of prebiotic carbohydrates, including sugar alcohols (SA), raffinose-family oligosaccharides (RFO), fructooligosaccharides (FOS), and resistant starch (RS). The levels of these carbohydrates and, hence, nutritional value, can change during processing and cooking. This study determined changes in prebiotic carbohydrate types and concentrations in lentil from three market classes (red, green, and Spanish Brown) subjected to different processing methods (whole, dehulled, and splitting) and cooking, cooling, and reheating. Dehulling and splitting of lentil decreased SA in red and green market classes but RFO and FOS significantly decreased only in dehulled split red lentil. Further, dehulling and splitting of red lentil significantly decreased RS concentrations compared to the whole seed. In some cases, SA, RFO, and FOS significantly increased with cooling but decreased after re-reheating. Cooling and reheating significantly increased lentil RS concentration for all market classes. Spanish Brown “Pardina” had the highest total prebiotic carbohydrates (9492 mg/100 g) of all market classes tested (range 6935-8338 mg/100 g). Overall, selection of lentil market class, processing, and cooking method should be considered to optimize nutritional value.

Keywords: Lentil, sugar alcohols, raffinose-family oligosaccharides, fructooligosaccharides, resistant starch, dehulling, cooking
4.2. Introduction

Lentil (*Lens culinaris* Medikus) is a cool season pulse crop that is low in fat (<2%) and provides significant quantities of carbohydrate (40-50%), protein (20-30%), a range of minerals [iron (Fe), zinc (Zn), and selenium (Se)], carotenoids, and folates (Bhatty, 1988; Sen Gupta et al., 2013; Thavarajah et al., 2011). Lentil has significant amounts of prebiotic carbohydrates or low digestible carbohydrates, including sugar alcohols (SA), raffinose-family oligosaccharides (RFO), fructooligosaccharides (FOS), and resistant starch (RS) (Johnson et al., 2015a, 2013). A study of 10 lentil cultivars grown in North Dakota, USA for two years reported mean concentrations of SA, RFO, FOS, and RS of 1423 mg, 4071 mg, 62 mg, and 7.5 g/100 g, respectively (Johnson et al., 2013). They also reported significant variations in lentil prebiotic carbohydrate concentrations: RFO concentrations varied with cultivars, RS varied with growing location, and SA varied with both variety and location.

As a result of the increasing incidence of obesity in the USA, lentil is gaining popularity in Western diets to combat systemic gut inflammation via modulating the human gut microbiome. A diet rich in prebiotic carbohydrates promotes human gastrointestinal health by increasing beneficial bacteria and reducing pathogenic bacteria (Roberfroid, 2007). A prebiotic was originally defined as “a *selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microflora that confers benefits upon host well-being and health*” (Roberfroid, 2007). Thus, FOS, galactooligosaccharides, and lactulose were the only bioactive compounds originally classified as prebiotics (Kolida and Gibson, 2008). However, prebiotics are now included under the broad category of low-digestible carbohydrates (Blaut, 2002; Grabitske and
Low-digestible carbohydrates are fermentable carbohydrates and are classified into three groups: (1) SA, (2) non-digestible oligosaccharides, and (3) RS. Sugar alcohols (also known as polyols, polyalcohols, and alditols) include sorbitol, mannitol, and galactinol. Non-digestible oligosaccharides include RFO (raffinose, stachyose, and verbascose) and FOS (kestose and nystose). These low-digestible carbohydrates are poorly digested in the human digestive tract due to lack of specific enzymes, but are fermented in the large intestine by hindgut bacteria and used as a substrate for their growth and activity (Grabitske and Slavin, 2009). Products of bacterial fermentation provide various health benefits including induction of satiety, reduction of serum cholesterol, glucose concentration, and reduce systemic inflammation (Cani et al., 2009; Lee and Mazmanian, 2010; Parnell and Reimer, 2012).

Food processing techniques (dehulling, splitting) and cooking can impact the concentrations of prebiotic carbohydrates that lead to projected human health benefits. Dehulling is a process to remove the seed coat (hull) from the seed and is done in response to consumer preference with respect to taste and shorter cooking time (Kon et al., 1973; Singh and Singh, 1992). For example, dehulled red lentil (also known as “football lentil”) is a popular lentil market class in South East Asia, specifically India, Nepal, and Bangladesh. Dehulled split red lentil is a popular lentil market class in Sri Lanka, the Middle East, and Turkey (Thavarajah et al., 2008). Both dehulling and splitting increase consumer acceptance as a result of increased taste, richer color, and shorter cooking time (Kon et al., 1973; Singh and Singh, 1992). Lentil low digestible carbohydrate concentrations change after processing and cooking for a short time (Johnson et al., 2015b; Wang et al., 2009); however, no data have yet been reported on
corresponding changes in SA and FOS levels in popular lentil market classes. An understanding of changes in lentil prebiotic carbohydrates levels upon processing and cooking is vital with respect to defining consumer nutritional benefits, developing new food products, and other food industry considerations. Therefore, the objective of this study was to determine the concentrations of SA, RFO, FOS, and RS in lentils from three market classes (red, green, and Spanish Brown – variety “Pardina”) subject to three different processing methods (whole, dehulled, and split) as well as cooking, cooling, and reheating.

4.3. Materials and Methods

4.3.1. Materials

Chemicals used for high performance anion exchange chromatography and RS enzymatic assays were purchased from Sigma-Aldrich (St. Louis, MO 63104, USA), Fisher Scientific (Asheville, NC 28804, USA), and VWR International (Satellite Blvd, Suwanee, GA 30024, USA). Water was distilled and deionized (ddH₂O) to a resistance of ≥ 18.2 MΩ (NANO-pure Diamond, Barnstead, IA, USA) prior to use.

4.3.2. Lentil samples

Approximately 2-4 kg of six commercially available lentil seed samples were collected from the Northern Pulse Growers Association, ND, USA (Table 4.1). These six lentil samples belong to three major market classes (red, green, and Spanish Brown – variety “Pardina”) and were selected based on consumer preferences. Red lentil is marketed as whole red (with seed coat), football (whole seed without seed coat), and dehulled split (split seed without seed coat) for local and international markets. Green lentil is marketed as whole green (with seed coat) and dehulled split. Pardina is
marketed as whole seed. Lentil samples were mixed thoroughly, subsampled \((n = 6)\), and stored at \(-20^\circ C\) prior to the cooking experiment. The treatment design was a completely randomized design with six lentil types, three food preparation methods (cooked, cooled, reheated), and three replicates \((n=54)\). The experiment was duplicated for a total of 108 samples analyzed. No raw lentil samples were analyzed for prebiotic carbohydrates as humans only consume cooked lentil; results with respect to increases and decreases relate only to cooked vs. cooled vs. reheated.

**Table 4.1.** Description of lentil market classes used in this experiment.

<table>
<thead>
<tr>
<th>Market class</th>
<th>Commercial form</th>
<th>1000 seed weight (g)</th>
<th>Consuming regions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Red</td>
<td>Whole (with seed coat)</td>
<td>29</td>
<td>South East Asia (India, Sri Lanka, Nepal, Pakistan, Bangladesh), Middle East (Turkey, Egypt, Syria), Europe, Australia, USA</td>
</tr>
<tr>
<td></td>
<td>Dehulled football</td>
<td>35</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dehulled split</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td>Green</td>
<td>Whole (with seed coat)</td>
<td>48</td>
<td>Europe, South/North America, Africa, Asia</td>
</tr>
<tr>
<td></td>
<td>Dehulled split</td>
<td>45</td>
<td></td>
</tr>
<tr>
<td>Pardina</td>
<td>Whole (with seed coat)</td>
<td>34</td>
<td>Spain, Europe</td>
</tr>
</tbody>
</table>

**4.3.3. Cooking, cooling, and reheating procedure**

Samples (~12 g) of lentil seeds were placed in 50 mL round bottom Pyrex tubes with ddH\(_2\)O at a weight ratio of 1:3 (seed:water). Samples were then suspended in a boiling water (100°C) bath and cooked for 60 min. Immediately after cooking, samples were refrigerated (ROPER, Whirlpool corporation, MI, USA) at 4°C for 24 h. Cooled lentil samples were then reheated in a microwave oven (General Electronic Co., Louisville, KY, USA) at high power (950 W) for 1 min. Cooked, cooled, and reheated samples were homogenized using a mortar and pestle prior to analysis for sugar
alcohols, RFO, FOS, and RS. The moisture content of each sample for each step was determined using a previously described method (AACC International, 2000). All data were reported on a wet weight basis (normalized to 14% moisture).

### 4.3.4. Determination of SA, RFO, and FOS concentrations

Homogenized finely ground lentil samples (500 mg) were incubated with 10 mL of ddH₂O for 1 h at 80°C as previously described (Muir et al., 2009). Samples were then centrifuged at 3000 g for 10 min (Fisher Scientific, USA). An aliquot (1 mL) of the supernatant was diluted with 9 mL of ddH₂O, then filtered through a 13 mm × 0.45 µm nylon syringe filter (Fisher Scientific, USA).

Sugar alcohol, RFO, and FOS concentrations were measured using high performance anion exchange chromatography with pulsed amperometric detection (HPLC-PAD; Dionex, ICS-5000, Sunnyvale, CA, USA) as per a previously published method (Feinberg et al., 2009). These compounds were separated by a CarboPac PA1 column (250 × 4 mm; Dionex, CA, USA) connected to a CarboPac PA1 guard column (50 × 4 mm; Dionex, CA, USA). Solvent A (100 mM sodium hydroxide/ 600 mM sodium acetate), solvent B (200 mM sodium hydroxide), and solvent C (ddH₂O) were used as mobile phases with a flow rate of 1 mL/min as follows: 0-2 min, 50% B/ 50% C; 2-20 min, linear gradient change from 2% A/ 49% B/ 49% C to 16% A/ 42% B/ 42% C; final extension, 50% B/ 50% C. Detection was carried out using a pulsed amperometric detector with a working gold electrode and a silver–silver chloride reference electrode at 2.0 µA. Sugar alcohols, RFO, and FOS were identified and quantified based on pure standards (>99%; sorbitol, mannitol, raffinose, stachyose, verbascose, kestose, and nystose). Sugar alcohol, RFO, and FOS concentrations were detected within a linear
range of 3 to 100 μg/g, with a minimum detection limit of 0.2 μg/g. CDC Redberry lentil was used as an external reference to ensure accuracy and reproducibility of detection. Peak areas for the reference sample, glucose (100 ppm), SA (3-100 ppm), RFO (3-100 ppm), and FOS (3-100 ppm) were routinely analyzed for method consistency and detector sensitivity with an error of less than 5%. Linear calibration curves for prebiotic carbohydrate standards had an error of less than 2%. Filtrate concentrations (C) of SA, RFO, and FOS were used to determine oligosaccharides in the samples according to X = (C × V) / m, where X is the concentration of oligosaccharides in the sample, V is the final diluted volume, and m is the mass of the dry sample aliquot (moisture corrected).

4.3.5. Determination of RS concentration

The concentration of RS in the lentil samples was determined using a previously described method (McCleary and Monaghan, 2002; Megazyme, 2012). Homogenized cooked/cooled/reheated lentil samples (500 mg) were incubated with 4 mL of enzyme mixture (3 U/mL amyloglucosidase and 10 mg/mL α-amylase in 100 mM sodium malate, pH 6) at 37°C for 16 h in a water bath with vertical shaking (Orbit shaker bath, Lab Line Instruments Inc., Melrose Park, ILL.). After incubation, samples were diluted with 4 mL of 95% ethanol followed by centrifugation at 1500 g for 10 min at room temperature. Pellets were re-suspended with 6 mL of 50% (v/v) ethanol, centrifuged, and decanted. The remaining pellets were dissolved in 2 mL of 2 M potassium hydroxide at 0°C while stirring with a magnetic stirrer for 20 min. The suspension was then incubated with 8 mL of 1.2 M sodium acetate buffer (pH 3.8) and 0.1 mL of 3300 U/mL amyloglucosidase at 50 °C for 30 min. The suspension was centrifuged at 1500 g for 10 min at room temperature. An aliquot (1 mL) of supernatant containing the resistant starch fraction
was diluted with 19 mL of ddH₂O and filtered through a 13 mm × 0.45 µm nylon syringe filter (Fisher Scientific, Asheville, NC 28804, USA). The concentration of glucose was determined using HPLC-PAD as described above. Starch fraction concentrations were calculated by multiplying the glucose concentration by 0.9 (factor to convert free glucose to anhydro-glucose as occurs in starch) (McCleary and Monaghan, 2002). Data were validated using a standard reference material (regular corn starch; RS concentration 1.0±0.1% (w/w)). Batches were checked regularly to ensure an analytical error of less than 10%.

4.3.6. Statistical analysis

Replicates, runs, lentil types, and processing methods were considered as random factors. Runs, lentil types, food preparation methods, and replicates were included as class variables. Analysis of variance was performed using the General Linear Model procedure (PROC GLM) of SAS version 9.4 (Version 9.4, SAS Institute, 2016). Fisher’s protected least significant difference (LSD) at $P < 0.05$ was used to separate means.

4.4. Results

4.4.1. SA

Total SA concentrations (sum of sorbitol and mannitol) ranged from 353 to 813 mg/100 g in cooked lentil (Table 4.2). Pardina had the highest total SA concentration of all cooked lentil types tested. The total SA concentration significantly increased after cooling ($P < 0.05$ vs. cooked) and decreased after reheating ($P < 0.05$ vs. cooled) in all lentil types except dehulled green and Pardina (data not shown). Further, lentil with seed coat (whole red: 750 mg/100 g; whole green: 572 mg/100 g; Pardina: 813 mg/100 g) had higher concentrations of total SA than dehulled lentil (football red: 394 mg/100 g;
split red: 353 mg/100 g; decorticated green 525 mg/100 g) (Table 4.2). Among SAs, sorbitol was present in higher concentrations (290-710 mg/100 g; Figure 4.1) than mannitol (47 to 102 mg/100 g; Figure 4.2) in cooked lentil. Cooling significantly increased sorbitol concentration in most lentil types except Pardina and dehulled split green lentil (Figure 4.1). Upon reheating, sorbitol concentration significantly decreased in dehulled split red and increased in whole green lentil (vs. cooled); changes noted for other lentil types were not significant. Similar to sorbitol, cooling significantly increased (vs. cooked) and reheating decreased (vs. cooled) mannitol concentration in all lentil types except dehulled split green and Pardina (Figure 4.2).

Table 4.2. Concentrations of SA, RFO/FOS, RS, and total prebiotic carbohydrates (mg) in a 100 g serving of cooked lentil with percent recommended dietary allowance.

<table>
<thead>
<tr>
<th>Lentil market class</th>
<th>SA</th>
<th>RFO+FOS</th>
<th>RS</th>
<th>Total</th>
<th>% of RDA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole red</td>
<td>750</td>
<td>4362</td>
<td>3026</td>
<td>8137</td>
<td>81</td>
</tr>
<tr>
<td>Football red</td>
<td>394</td>
<td>4577</td>
<td>1964</td>
<td>6935</td>
<td>69</td>
</tr>
<tr>
<td>Dehulled split red</td>
<td>353</td>
<td>3901</td>
<td>2922</td>
<td>7176</td>
<td>71</td>
</tr>
<tr>
<td>Whole green</td>
<td>572</td>
<td>5147</td>
<td>2614</td>
<td>8333</td>
<td>83</td>
</tr>
<tr>
<td>Dehulled green</td>
<td>525</td>
<td>5139</td>
<td>2674</td>
<td>8338</td>
<td>83</td>
</tr>
<tr>
<td>Pardina</td>
<td>813</td>
<td>6111</td>
<td>2569</td>
<td>9492</td>
<td>94</td>
</tr>
</tbody>
</table>

RDA (Recommended dietary allowance); 10 g/day (Douglas & Sanders, 2008), RFO (raffinose family oligosaccharides), FOS (fructooligosaccharides), RS (resistant starch). Values are presented on wet weight basis (14% moisture).
Figure 4.1. Sorbitol concentrations of different lentil market class after cooking, cooling, and reheating. Values are presented on wet weight basis (14% moisture). Values within each lentil market class followed by a different letter are significantly different at \(P < 0.05\) (n=108).
Figure 4.2. Sorbitol concentrations of different lentil market class after cooking, cooling, and reheating. Values are presented on wet weight basis (14% moisture). Values within each lentil market class followed by a different letter are significantly different at $P < 0.05$ (n=108).
4.4.2. RFO and FOS

Total RFO and FOS concentrations ranged from 3901 to 6111 mg/100 g in cooked lentil (Table 4.2). Similar to SA, Pardina had higher RFO and FOS concentrations than all other types. Among the RFO and FOS, lentil has higher concentrations of raffinose and stachyose (2523-5157 mg/100 g; Figure 4.3) followed by verbascose and kestose (451 to 1845 mg/100 g; Figure 4.4), and finally nystose (45 to 58 mg/100 g; Figure 4.5). Cooling significantly increased ($P < 0.05$) stachyose and raffinose concentrations in whole red and split red lentil and decreased concentrations in Pardina; no significant change was noted for the other three lentil types. Reheating significantly reduced (vs. cooled) stachyose and raffinose concentrations in dehulled split red lentil (Figure 4.3).

Among lentil types, whole green lentil had the highest concentrations of verbascose and kestose after cooking, and whole red lentil the least (Figure 4.4). After cooling, verbascose and kestose concentrations significantly increased in football red, split red, and whole green lentil, decreased in Pardina, and remained unchanged for dehulled split green and whole red lentil. Reheating significantly ($P < 0.05$) reduced verbascose and kestose concentrations (vs. cooled) in split red and increased concentrations in whole red and Pardina; concentrations in football red and whole green were lower but changes were not significant (Figure 4.4). Processing did not affect nystose concentrations in whole red, whole green, and dehulled split green lentil (Figure 4.5). Cooling significantly increased nystose concentrations in football red, dehulled split red, and Pardina lentil (vs. cooked) and reheating significantly reduced nystose concentration in football red (vs. cooled).
Figure 4.3. Raffinose and stachyose concentrations of different lentil market classes after cooking, cooling, and reheating. Values are presented on wet weight basis (14% moisture). Values within each lentil market class followed by a different letter are significantly different at $P < 0.05$ (n=108).
Figure 4.4. Verbascose and kestose concentrations of different lentil market classes after cooking, cooling, and reheating. Values are presented on wet weight basis (14% moisture). Values within each lentil market class followed by a different letter are significantly different at $P < 0.05$ (n=108).
Figure 4.5. Nystose concentrations of different lentil market classes after cooking, cooling, and reheating. Values are presented on wet weight basis (14% moisture). Values within each lentil market class followed by a different letter are significantly different at $P < 0.05$ (n=108).
4.4.3. RS

Resistant starch concentration ranged from 1964 to 3026 mg/100 g in cooked lentil types (Table 4.2). In all lentil types, cooled lentil had significantly higher ($P < 0.05$) RS concentrations than cooked lentil. In many cases, reheated lentil had significantly higher ($P < 0.05$) RS concentrations than cooled lentil (Figure 4.6).

4.5. Discussion

Processing and cooking changes the concentration of food prebiotic carbohydrates. Our results clearly indicate that dehulling and splitting reduce total prebiotic carbohydrate levels in medium red lentil but do not affect levels in large green lentil. Spanish Brown “Pardina” lentil had the highest concentration of total prebiotic carbohydrates (9492 mg/100 g) of all market classes considered here (range 6935-8338 mg/100 g). All lentil market classes provide a significant percent of the recommended intake (%RDA) of prebiotic carbohydrates from a single serving of cooked lentil, with “Pardina” providing the most (94%) and Football red lentil the least (64%) (Table 4.2).

Variations in concentrations of SA, RFO, FOS, and RS in raw lentil have been reported (Chung et al., 2008; de Almeida Costa et al., 2006; Johnson et al., 2013). For example, lentil has been reported to contain 880-1550 mg/100 g of sorbitol and 48-250 mg/100 g of mannitol prior to processing and cooking (Johnson et al., 2015a, 2013). Data from the present study indicate lower concentrations of sorbitol (300-700 mg/100 g) and mannitol (45-100 mg/100 g), suggesting that cooking may reduce SA levels compared to that of raw seeds. In addition, lentil seeds that are unprocessed – i.e., whole red, whole green, and “Pardina” – have more sorbitol, and whole green and “Pardina” lentil have more mannitol; this indicates the dehulling process removes a
significant amount of SA. The seed coat might also act as a barrier to prevent thermal decomposition of SA during cooking at high temperature. Cooking breaks the chemical form of sorbitol and mannitol at high temperature (100°C), and excess water conditions (Matsumoto et al., 2015; Matsumura, 2016). Overall, dehulling appears to reduce SA concentration and thus the prebiotic nutritional value of cooked lentil.

RFOs are known antinutrients that cause human gastrointestinal discomfort and flatulence (Fleming, 1981). As a result, most conventional lentil breeding programs aim to reduce RFO levels in the seed using plant breeding and selection (Frias et al., 1999). However, regular consumption of RFOs is recommended as an important dietary component to combat chronic diseases (Cani et al., 2009; Parnell and Reimer, 2012), reduce inflammation (Lee and Mazmanian, 2010), eliminate pathogens (Manning and Gibson, 2004; Sousa et al., 2011), and stimulate mineral bioavailability (Coudray and Fairweather-Tait, 1998; Yeung et al., 2005). Our previous work has shown that US-grown lentil contains 4071 mg of RFO and 62 mg of FOS/100 g before processing and cooking and after cooking total RFO ranges from 6000 mg/100 g in whole red to 5900 mg/100 g in football red, 5500 mg/100 g in duhulled green, and 5200 mg/100 g in whole green (Johnson et al., 2015b, 2013). Our current study results do not follow the same trend, and indicate cooked lentil has lower amounts of RFO+FOS (range 3901 mg/100 g in dehulled split red to 6111 mg/100 g in Pardina; Table 4.2). These variations are possibly the result of lentil growing conditions (location, variety, management practices, and weather), processing method, and cooking time.

Lentil RFO and FOS concentrations are known to be affected by processing (Wang et al., 2009), cooking, cooling, and reheating (Johnson et al., 2015b). Johnson et
al. (2015b) indicate that RFO concentrations of whole red and whole green lentil are reduced due to cooking and cooling, but our results do not show the same pattern; rather, cooling increased RFO and FOS concentrations. Regardless, evidence to date suggests higher concentration of raffinose are present in the seed coat of lentil than in the cotyledon, with the reverse being the case for stachyose and verbascose. Our results show reheating reduces RFO and FOS in all lentil market classes except Pardina. Temperatures of 100°C can reduce RFO and FOS concentrations due to thermal hydrolysis; for example, the fructose furanosyl residues and glycosidic bonds in FOS are sensitive to both thermal and acid hydrolysis (Courtin et al., 2009). Therefore, cooking, cooling, and reheating will have impact lentil RFO and FOS concentrations.

The concentration of RS in foods also changes as a result of processing, cooking, and consumer handling. Mishra et al. (2008) indicate that RS levels in cooked potato increase by more than 400% after 2 days of refrigeration. Even the simple heating and cooling of autoclaved cereals, tubers, and legumes increases their RS content by 30 to 70%, and additional heating and cooling further increases RS formation (Yadav et al., 2009). Annealing of lentil increases RS concentrations from 6.5 to 9.5% (Vasanthan and Bhatte, 1998). Our previous study reported RS changes in two commercially available lentil market classes (medium green and small red) after cooking, cooling, and reheating (Johnson et al., 2015b). Mean RS concentrations in raw, cooked, cooled, and reheated lentil were 3.0, 3.0, 5.1, and 5.1% (w/w), respectively, indicating cooling-induced synthesis of RS from gelatinized starch. Our RS results presented here are comparable to these previous reports, with cooling and
Figure 4.6. Resistant starch concentrations of different lentil market classes after cooking, cooling, and reheating. Values are presented on wet weight basis (14% moisture). Values within each lentil market class followed by a different letter are significantly different at P < 0.05 (n=108).
reheating resulting in a two-fold increase in lentil RS concentration. During heating and cooling, starch molecules (i.e., amylose) in foods undergo a retrogradation process that results in the formation of a new type of RS and increases the overall nutritional value (Sievert and Pomeranz, 1989). Our results highlight the impact of temperature on lentil nutritional quality, and show lentil is more nutritious after cooling and reheating.

4.6. Conclusion

An understanding of prebiotic concentrations in different lentil market classes provides key information to improve lentil nutritional quality through processing and cooking. Results of this study clearly show that dehulling, cooking, cooling, and reheating significantly alter the types and levels of prebiotic carbohydrates present in lentil.

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5. CHAPTER THREE

WILL LENTIL (Lens culinaris MEDIKUS) DIET REDUCE THE RISK OF OBESITY? A RAT STUDY

5.1. Abstract

Obesity prevalence is rapidly increasing due to increased consumption of high caloric foods. Research have been focused on dietary compounds including prebiotic carbohydrates to reduce obesity risk. Lentil is rich in prebiotic carbohydrates including sugar alcohols, raffinose family oligosaccharides, fructooligosaccharides, and resistant starch (RS). This study was carried out to assess the potential of lentil to reduce obesity risk in rats. Eight weeks old Sprague Dawley male rats were fed with lentil, RS, and control diet for 6 weeks. Rat feed intake, body weight, fat%, plasma triglycerides (TG) concentration, liver weight, and fecal microbiome were analyzed. Feed intake (22-28 g/week/rat) was not different among rats, but after 6 weeks mean body weight of rats fed with lentil (443 g/rat) significantly lower than rats fed with control (511 g/rat) and RS (502 g/rat) diets. Mean body fat% and plasma TG concentration were lower in rats fed with lentil (20% and 109 mg/dl) than rats fed with control (24% and 133 mg/dl) and RS (29% and 169 mg/dl). Rat liver weight ranged from 12 to 22 g and did not significantly different among treatments. Phylum Actinobacteria and Bacteriodetes abundance increased in rats fed with lentil (5% and 34%) and RS (4% and 37%) diets than rats fed with control diet (2% and 30%). Firmicutes reduced in rats fed with lentil (57%) and RS (53%) than rats fed with control diet
These results show lentil reduces body weight, fat%, and plasma TG and increases beneficial gut microbiome.

**Keywords:** obesity, lentil, prebiotic carbohydrate, gut microbiome

### 5.2. Introduction

Prevalence of obesity is dramatically increasing in developed countries (Ogden et al., 2016). Current adult obesity prevalence in USA ranged from 19-38% (CDC, 2016; The State of Obesity, 2016). It was estimated that more than 50% of people in USA will become obese by 2030 (Finkelstein et al., 2012). Increasing obesity is considered as a serious problem because of obesity related health issues, associated medical cost and economic losses. Obesity often associated with coronary heart disease (CHD), non-insulin dependent diabetes mellitus (NIDDM), hyper tension, osteoarthritis and several cancers (WHO, 1997). Estimated annual medical cost for obese people is $190 billion which is 21% of total medical expenditure in US (Cawley & Meyerhoefer, 2012). It was estimated that total medical cost saving will be $550 billion, if the current obesity prevalence remains same for next 20 years (Finkelstein et al., 2012). Therefore, it is necessary to take preventive actions against increasing obesity risk.

Unhealthy eating behavior is one of the major factors for increasing obesity (Malik, Willett, & Hu, 2012). People increase consumption of high caloric foods and beverages rich in fat and added sugar. High caloric, processed foods are highly available due to low cost compared to legumes, fruits, and vegetables (Popkin, Adair, & Ng, 2012). However, obesity is related to complex factors
including food choice, knowledge, emotional state, and social context (Wardle, 2007). Therefore, multiple approaches including increase vegetable and fruits consumption, increase physical activities, changes in social behaviors, and surgical options are carried out to reduce obesity prevalence (Bischoff et al., 2016). Among these approaches, dietary treatments gain central attention since obesity is highly influenced by dietary pattern (Fung et al., 2001). Therefore, recent research focused on legumes which are rich in proteins, micronutrients, and prebiotic carbohydrates as a dietary approach to reduce obesity risk.

Prebiotic carbohydrates are selectively fermented by gut beneficial microorganisms, resulting products increase host well-being and health (Roberfroid, 2007). Beneficial gut microbiome and low fat, prebiotic carbohydrates rich diet reduce obesity risk (Wu et al., 2011). It was found that bacterial phyla Bacteroidetes and Firmicutes are associated with obesity (Ley et al., 2005). Lean rats had high proportion of Bacteroidetes and low proportion of Firmicutes compare to obese rats (Ley et al., 2005). Similar results were found with human trials where the increased Bacteroidetes correlates with body weight loss (Ley, Turnbaugh, Klein, & Gordon, 2006). However, few studies are controversial to these finding (Collado, Isolauri, Laitinen, & Salminen, 2008; Finucane, Sharpton, Laurent, Pollard, & Zafar, 2014; Schwiertz et al., 2010); hence the relationship between gut microbiome and obesity is still debatable.

Gut microbiota ferment prebiotic carbohydrates releasing short chain fatty acids (SCFA) including butyrate, acetate, and propionate (Cummings, Pomare,
Branch, & Naylor, 1987). SCFA stimulate endocrine L cells and produce gut hormones related to food intake, gut permeability, and insulin resistance (Cani et al., 2009; Everard et al., 2011; Gao, Yin, Zhang, Ward, & Martin, 2009; Keenan et al., 2006; Lin, Frassetto, Jr, & Nawrocki, 2012; Parnell & Reimer, 2009). Additionally, SCFA reduce lipolysis and free fatty acids in serum leads to low adiposity (Samuel et al., 2008). Therefore, supplementation of prebiotic carbohydrates and prebiotic carbohydrates rich foods may modulate gut microbiome and related SCFA; hence reduce obesity risk.

Currently consumption of legumes including lentil (*Lens culinaris* Medikus) is promoted due to theirs nutritional quality to combat obesity. Lentil is a cool season food legume, rich in protein, minerals (Fe, Zn, and Se), vitamins (folate, beta-carotene), and prebiotic carbohydrates (sugar alcohols, raffinose family oligosaccharides, fructooligosaccharides, and resistant starch) (Johnson, Thavarajah, Combs, & Thavarajah, 2013; Sen Gupta et al., 2013; D. Thavarajah et al., 2011). A serving of lentil (16 g) provides 180-223 mg of sugar alcohols, 0-158 mg of fructooligosaccharides, 829-1082 mg of raffinose family oligosaccharides, and 960-1424 mg of resistant starch (Johnson et al., 2013). Therefore, lentil is currently looked as a potential legume to reduce obesity risk.

Dietary pattern can change the composition of gut microbiome (Wu et al., 2011). Beneficial gut microbiome can be increased by incorporating legume foods rich in prebiotics. The effect of chick pea (*Cicer arietinum* L.), pea (*Pisum sativum* L.), common bean (*Phaseolus vulgaris* L.), and lentil (*Lens culinaris* Medikus)
Medikus) diets on bifidobacteria, a beneficial bacterial group have been previously described (Queiroz-Monici, Costa, da Silva, Reis, & de Oliveira, 2005). Addition, Yellow pea (*Lathyrus aphaca* L.) reduce *Clostridium leptum*, a *Firmicute* group in obese rats (Eslinger, Eller, & Reimer, 2014). Few studies were focused on the gut microbial modification and related obesity bio markers changes respect to legume diets. No efforts have been made to determine the potential of lentil as a food legume to reduce obesity risk via modulating gut microorganisms. Therefore, the objectives of the study were to (1) determine the effects of lentil on body weight, body fat%, plasma TG concentration, and liver weight, and (2) determine the changes in fecal microbiome composition in rats after feeding lentil.

**5.3. Materials and methods**

**5.3.1. Diet formulation**

Diets were formulated as 0.5 inch pellets (Teklad Lab Animal Diets, Envigo RMS, 8520, Allison Pointe Boulevard, Suite 400, Indianapolis, IN 46250, US). Control diet was formulated based on AIN-93 M diet suggested by American Institute of Nutrition (Reeves et al., 1993). Resistant starch (3.5% w/w high amylose corn starch) diet and lentil (71% w/w) diet were formulated with substituting high amylose corn starch and red split lentils with control diet respectively (*Table 5.1*) to match other nutrients.
Table 5.1. Composition of standard, corn (3.5% high amylose corn starch), and lentil (71%) diets.

<table>
<thead>
<tr>
<th>Formula</th>
<th>Control diet</th>
<th>Corn starch diet</th>
<th>Lentil diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein (g/kg)</td>
<td>200</td>
<td>200</td>
<td>0</td>
</tr>
<tr>
<td>Lentils (g/kg)</td>
<td>0</td>
<td>0</td>
<td>708</td>
</tr>
<tr>
<td>L-Cysteine (g/kg)</td>
<td>3</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Corn starch (g/kg)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>398</td>
<td>362</td>
<td>0</td>
</tr>
<tr>
<td>Maltodextrin (g/kg)</td>
<td>132</td>
<td>132</td>
<td>76</td>
</tr>
<tr>
<td>Sucrose (g/kg)</td>
<td>100</td>
<td>100</td>
<td>58</td>
</tr>
<tr>
<td>Soybean oil (g/kg)</td>
<td>70</td>
<td>70</td>
<td>61</td>
</tr>
<tr>
<td>Cellulose (g/kg)</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Mineral mix, AIN-93G-MX (94046) (g/kg)</td>
<td>35</td>
<td>35</td>
<td>35</td>
</tr>
<tr>
<td>Vitamin mix, AIN-93-VX (94047) (g/kg)</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Choline bitartrate (g/kg)</td>
<td>3</td>
<td>10</td>
<td>3</td>
</tr>
<tr>
<td>TBHQ, antioxidant (g/kg)</td>
<td>0.014</td>
<td>0.014</td>
<td>0.014</td>
</tr>
<tr>
<td>High amylose corn starch (g/kg)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0</td>
<td>35</td>
<td>0</td>
</tr>
</tbody>
</table>

Calculated composition:
- Protein, N x 6.25, %: 18, 18, 18
- Gross Energy, kcal/kg: 3800, 3700, 3400
- Total Carbohydrate, %: 60, 59, 52
- Resistant Starch, %: 4, 6, 3
- Fat, %: 7, 7, 7

5.3.2. Animals and feed trial

Male, 8 weeks aged Sprague Dawley rats (n=36) were purchased (Charles river, 251, Ballardvale St, Wilmington, MA, 01887-1096). Rats were housed in individual cages with controlled environmental conditions; temperature 25°C, RH 60%, and 12 hour light/dark condition. Rats were randomly selected in
to three groups (n=12) and allowed for one week to adapt the environment prior to start the experiment. Rats were fed ad libitum water and 1 of 3 formulated diet for 6 weeks.

### 5.3.3. Feed intake and body weight measurements

Feed intake was measured on 3 days interval throughout the study period by weighing available feed in the cage and subtracting this weight from the previously measured weight. Body weights were measured using weighing balance (TS2KS, OHAUS Corporation, Parsippany, NJ, USA) from initial week (before the feed trail) to 6th week at one week intervals. The protocol was approved by Clemson University Institutional Animal Care and Use Committee.

### 5.3.4. Fecal sample collection

Every two weeks interval, fresh fecal samples were collected from each rat separately in sterilized conical tubes and immediately transferred to a -80°C freezer and stored until further analysis.

### 5.3.5. Blood collection

End of the 6th week, blood samples (3 ml) were obtained from each rat into sterilized tubes containing heparin as an anticoagulant. Tubes were centrifuged at 1000 x g for 10 minutes at 4°C. Top plasma layer was pipetted off in to 1.5 ml microtube and stored at -80°C until further analysis.

### 5.3.6. Fat% and liver weight measurements

End of the 6th week, rats were euthanized with carbon dioxide in a closed chamber. After euthanization, immediately fat% was determined using dual
energy x-ray absorptiometry technique (Hologic Discovery A, Hologic, Inc. 250, Campus Drive, Marlborough, MA 01752, USA). Liver samples were collected and weighed. All procedures were approved by Clemson University Institutional Animal Care and Use Committee.

5.3.7. Triglyceride (TG) measurements

Blood plasma TG concentration was measured using a colorimetric assay (Cayman TG colorimetric assay kit, Cayman chemicals, 1180 East Ellsworth Road, Ann Arbor, Michigan 48108, USA). An aliquot (10 µl) of blood plasma was added into microwell plate. TG standard series and blank were prepared according to the procedure given in the kit. A volume of diluted enzyme buffer (150 µl) solution contains lipoprotein lipase, glycerol kinase, glycerol phosphate oxidase, peroxidase, 4-aminoantipyrine, N-ethyle-N-(3-sulfopropyl)-m-anisidine, and sodium phosphate buffer were added in to each well. Microwell plate was shaken for few seconds to mix followed by a 15 minutes incubation period at room temperature. Absorbance were measured at 540 nm using a microplate reader (SpectaMax M2 with SoftMax pro software, Molecular Devices Corporation, 1311 Orleans Drive, Sunnyvale, California 94089). Corrected absorbance values were obtained by subtracting blank value from sample value. Standard curve was obtained using corrected absorbance values of standard series. TG concentration of sample were calculated using following equation.

\[
TG \text{ concentration (mg/dl)} = \frac{(\text{corrected absorbance-y intercept})}{\text{slope}}
\]
5.3.8. Fecal 16S rRNA analysis

Fecal DNA was extracted using QIAamp DNA stool mini kit (QIAGEN, Inc. 19300, Germantown Road, Germantown, MD, 20874, USA). Concentration of DNA was checked using Qubit dsDNA HS Assay Kit via Qubit 3.0 fluorometer (Invitrogen Corporation, 5791 Van Allen Way, Carlsbad, CA 92008) to ensure proper DNA extraction from each sample. Extracted fecal DNA samples were stored in -80°C freezer until further analysis.

Gene specific primers were used to amplify the V4 region of the bacterial 16S rRNA gene as previously described by Caporaso et al., 2011. 16S rRNA V4 primers were as follows: 16S forward; GTGCCAGCMGCCGCGGTAA, 16S reverse; GGACTACHVGGGTWTCTAAT (Kozich, Westcott, Baxter, Highlander, & Schloss, 2013). Illumina sequencing libraries were built in a single PCR by adding index and flow cell adaptor sequences to the 16S primers following (Kozich et al. 2013). Each primer consisted of Illumina adaptor, an 8-nt index sequence, a 10-nt pad sequence, a 2-nt linker, and the gene specific primer (Appendix A). Index primers and Illumina primers were purchased from IDT (Integrated DNA Technologies, Inc., 1710 Commercial Park, Coralville, IA, 52241, USA).

Amplicons were generated using PCR (AccuPrime Pfx super mix; Invitrogen), and then quantified using a bioanalyzer (Agilent 2100 Bioanalyzer, Agilent Technologies, 5301 Stevens Creek Blvd, Santa Clara, CA 95051, USA). Amplicons were pooled into equimolar concentrations using a SequalPrep plate
normalization kit (Invitrogen Corporation, 5791 Van Allen Way, Carlsbad, CA 92008). Final concentration of the library was determined using previously published protocol (Kozich et al., 2013). Nucleotide diversity of the pooled sample was increased by spiking with 10% phiX DNA.

5.3.9. Liver tissue slicing, staining, and imaging

Liver samples were thawed at room temperature for 2 hrs. Proximal and distal parts of livers were kept in tissue cassettes to process for slicing. Liver tissues were processed in a tissue processor (Tissue-Tek VIP, Sakura Finetek USA, Inc, Torrance, CA, USA). Following cycles were used to process liver samples, buffered formalin (10%); 2 min, buffered formalin (10%); 30 min, ethanol (70%); 30 min, ethanol (80%); 30 min, ethanol (95%); 45 min, ethanol (95%); 30 min, ethanol (100%); 45 min, ethanol (100%); 45 min, xylene; 20 min, xylene; 40 min, paraffin; 30 min, paraffin; 30 min, paraffin; 30 min, and paraffin; 30 min. Above cycles were performed at 35 °C except paraffin cycles where those were performed at 58 °C. Cassettes with processed liver samples were transferred to Tissue-Tec tissue embedding console system (Sakura Finetek USA Inc., Torrance, CA, USA). Liver samples were kept at 4 °C until solidify paraffin blocks. Liver tissue sections (thickness of 5 µm) were obtained using Leica RM 2155 rotary microtome (Leica Microsystems, Nussloch, Germany). Tissue sections were transferred to slides and kept in a slide warmer (Premiere slide warmer XH-2004, Premiere, 7241, Gabe court, Manassas, VA 20109) at 44 °C for 15 minutes to remove excess water. Then, slides were incubated in an
incubator (12-140E Incubator, Quincy Lab Inc., Chicago, IL, USA) at 55 °C for fix
the tissue to slides. Finally dried slides were stained using Hematoxylin and
Eosin staining process. Liver tissue images were taken using Stereo Microscope
(M125, Leica Microsystems Inc., Buffalo Grove, IL, USA). Extended focus depth
images was taken by focus stacking using Helicon Focus Software (HeliconSoft,
Kharkiv, Ukraine).

5.3.10. Statistical analysis

Diet types, weeks, and replicates were considered as random factors. Diet
types, weeks, and replicates were included as class variables. Analysis of
variance was performed using the General Linear Model procedure (PROC GLM)
significant difference (LSD) at $P < 0.05$ was used to separate means.

Considering microbial analysis, Python scripts within the software package
QIIME version 1.9.0 (Caporaso et al., 2010) were used to analyze sequence reads.
Taxonomic assignments for assembled reads were obtained using the greengenes
database (greengenes.lbl.gov). Normalized taxon counts were used to calculate
beta diversity measures (Bray-Curtis). Sample groups were tested for significant
differences in beta diversity using PERMANOVA tests. $P$ values were corrected
for multiple testing using the false discovery rate (FDR). The frequency of OTUs
among sample groups was tested for significant difference using a Kruskal Wallis
test. $P$ values were generated using 10,000 permutations and corrected for
multiple testing using FDR.
5.4. Results

Rat feed intake ranged from 21 to 30 g/rat/day during the study period (Figure 5.1). At the first week, the feed intake of rats fed with lentil (21-25 g/rat/day) was significantly lower than other two groups (RS, 24-26 g/rat/day; control, 23-29 g/rat/day). Second week onwards, no significant differences ($P > 0.05$) were observed among feed intake of rats fed with lentil, RS, and control diets (Figure 5.1). End of the study period, the feed intake of rats fed with lentil, RS, and control were 22-30, 23-29, and 22-28 g/rat/day respectively. Calculated energy intake of rats fed with lentil is significantly lower (71-87 kcal/rat/day) than rats fed with RS (88-98 kcal/rat/day) and control (84-110 kcal/rat/day) diets at initial week. After third week, energy intake was similar among rats regardless the type of diets (76-108 kcal/rat/day) (Figure 5.1).

Considering body weight, no significant differences ($P > 0.05$) were observed among rats fed with lentil, RS, and control diet at initial week, ranged from 245 g to 291 g/rat (Figure 5.2). After 6 weeks of feeding lentil, RS, and control diets, significantly lower mean body weight was observed in rats fed with lentil (383-522 g/rat) than rats fed with RS (431-555 g/rat) and control (440-609 g/rat) (Figure 5.2). Overall, the growth rate (increased body weight per week per rat) of rats fed with lentil (29 g/rat/week) was significantly lower than rats fed with RS (39 g/rat/week) and control (41 g/rat/week).
Figure 5.1. Feed and energy intakes of rats fed with different diets. Means; vertical bars represent standard deviations. Values within each week followed by the same letter are not significantly different, \( P > 0.05 \).
Figure 5.2. Growth of rats fed different diets. Means; vertical lines represent standard deviations. Values within weeks (n=36) followed by the same letter are not significantly different, P > 0.05.
Lower body fat% was observed in rats fed with lentil (17-23%), but did not significantly different from body fat% of rats fed with control (16-32%). Highest body fat% was observed in rats fed with RS (25-33%) (Figure 5.3). Similar pattern was observed in rat blood plasma TGs concentration. Lower plasma TG concentrations were observed in rats fed with lentil (68-150 mg/dl), but did not significantly different from plasma TGs concentration of rats fed with control (98-168 mg/dl). Highest plasma TGs concentrations were observed in rats fed with RS (128-210 mg/dl) (Figure 5.3). Liver weight of rats fed with lentil, RS, and control ranged from 12-20, 14-22, and 14-20 g respectively. However, liver weight of rats were not significantly different ($P > 0.05$) among rats fed with lentil, RS, and control (Figure 5.3).

Figure 3.4 shows the abundance (percentage of total known gene sequences) of major fecal bacterial phyla throughout the study period. Two major bacterial phyla; *Firmucutes* and *Bacteriodetes* represent the rat fecal microbiome. *Firmucutes* and *Bacteriodetes* represent 46-73% and 25-44% of fecal microbiome respectively. Addition to these two phyla, *Proteobacteria* and *Actinobacteria* represent 0-5% and 1-5% of total fecal microbiome. At the initial week of the study, fecal *Firmicutes* abundance of rats fed with lentil, RS, and control were 56%, 63%, and 58% respectively. After 6th week, the abundance of *Firmicutes* were 57%, 53%, and 65% in rats fed with lentil, RS, and control diets respectively. Abundance of fecal *Bacteriodetes* were 40%, 34%, and 37% in rats fed with lentil, RS, and control diets respectively at initial week. After
Figure 5.3. Body fat (%), liver weight (g), and plasma TGs (triglycerides; mg/dl) of rats fed with different diets.

Means; vertical bars represent standard deviations; values within body fat, liver weight, and plasma TGs followed by the same letter are not significantly different, $P > 0.05$. 
Figure 5.4. Most abundant bacterial phyla (percentage of total) in rat feces. Weekly average samples (n=36).

Abundance were calculated after eliminating unassigned species.
6 weeks, *Bacteroidetes* abundance were 34%, 37%, and 30% respectively. Fecal *Actinobacteria* abundance (1.1-1.2%) did not show significant differences among rats fed with lentil, RS, and control diets at initial week. *Actinobacteria* abundance were increased after 6 week as 5%, 4%, and 2% in rats fed with lentil, RS, and control respectively (*Figure 5.4*). Similarly, abundance of fecal *Proteobacteria* (3-4%) did not significantly different among rats fed with different diets at initial week. Six weeks of feeding lentil, RS, and control diets changed fecal *Proteobacteria* abundance as 3%, 5%, and 4% respectively.

In phylum *Firmicutes*, 8 prominent species including *Lachnospiraceae* sp. and *Peptostreptococcus stomatis* were found (*Figure 5.5*). At the initial week, the abundance of *Lachnospiraceae* sp. was 12.8, 16.9, and 15.4% in rats fed with lentil, RS, and control diets respectively at initial week. After 6 weeks, the abundance was significantly lower in rats fed with lentil (8.7%) than RS (11.7%), and control (14.6%) diets. *Peptostreptococcus stomatis* abundance were ranged from 3.7 to 4.3% at initial week. After 6 weeks, the abundance was reduced in rats fed with lentil (2.9%) compare to RS (3.2%) and control (6.3%). Addition, lentil diet significantly reduce abundance of *Streptococcaceae* sp., and *Peptostreptococcus stomatis* and increase *Lachnospiraceae* sp. and *Shutterworthia satelles*, but abundance are less prominent (data not shown).

Two prominent bacterial species were found in phylum *Bacteroidetes*; *Bacteroides heparinolyticus* sp. and *Tannerella* sp. (*Figure 5.5*). At initial week, abundance of *Bacteroides heparinolyticus* sp. in rats fed with lentil, RS, and
control were 33.8, 27.6, and 29.5% respectively. Six weeks of feeding lentil and control reduced the abundance of *Bacteroides heparinolyticus* sp. to 27.8% and 19.4% respectively. RS diet increase the abundance to 28.1%. The abundance of *Tannerella* sp. were 5.9, 6.0, and 7.2% in rats fed with lentil, RS, and control diets respectively at initial week. After 6 weeks, the abundance were 6.5, 9.4, and 10.5% respectively.

In phylum *Actinobacteria*, two prominent bacterial species; *Bifidobacterium* sp. and *Eggerthella lenta* were found (Figure 5.5). *Bifidobacterium* sp. abundance were 1.1-1.2% at initial week and did not show any significant differences among rats fed with lentil, RS, and control diets. After 6 weeks, the *Bifidobacterium* sp. abundance increased in rats fed with lentil (5.3%) and RS (4.0%) than rats fed with control (1.5%). *Eggerthella lenta* abundance ranged from 1.5-3.5% at initial week. After 6 weeks, the abundance was lower in rats fed with lentil (4.7%) and RS (5.7%) than rats fed with control (14.2%).

In phylum *Proteobacteria*, *Lautropia mirabilis* was the prominent species. At initial week, no significance differences of *Lautropia mirabilis* abundance (0.02-0.05%) were found among rats fed with lentil, RS, and control. After 6 week, *Lautropia mirabilis* abundance was lower in rats fed with lentil (2.84%) than RS (5.02%) and control (3.58%).
Figure 5. Dominant species in rat fecal samples at initial week (0 week) and 6th week. Inner circle represents phyla (*Actinobacteria*, *Proteobacteria*, *Bacteroidetes*, and *Firmicutes*). Outer circle represents dominant species of corresponding phyla: (A) control diet group at 0 week, (B) control diet group after 6 weeks, (C) corn starch diet group at 0 week, (D) corn starch diet group after 6 weeks, (E) lentil diet group at 0 week, and (F) lentil diet group after 6 weeks.
5.5. Discussion

Obesity is an emerging problem in most developed as well as developing countries. Obese people may have increased risk of hypertension, type 2 diabetes mellitus, heart diseases, and several cancers. Obesity also associated with increased medical cost (Finkelstein et al., 2012). Therefore, several approaches have been carried out to reduce obesity risk including increasing consumption of fruits, vegetable, and legumes such as lentil. However, there are lack of evidences to illustrate the true potential of lentil to reduce obesity risk. This study was carried to determine the effects on lentil based diet on rat body weight, percent body fat, plasma TG concentration, and fecal microbiome to evaluate the potential of lentil to reduce obesity risk in rats.

Consumption of legumes increase satiety, therefore reduce food intake (Marinangeli & Jones, 2012). Present study, we did not observe significant reduction in feed intake of rats fed with lentil diet compare to control and high amylose corn starch diets. Several studies report similar results where increasing lentil supplementation did not change the feed intake and did not improve satiety responses (Erickson & Slavin, 2016; Landero, Beltranena, & Zijlstra, 2012). Satiety responses significantly improved by anti-nutrient factors such as trypsin inhibitors and phytic acid, addition to legume fibers (Marinangeli & Jones, 2012). Lentil is low in trypsin inhibitors and phytic acid (Guillamón et al., 2008; P. Thavarajah, Thavarajah, & Vandenberg, 2009), which may explains the low responsiveness of lentil on feed intake. However, a study with several legumes
including pea (Pisum sativum L.), common bean (Phaseolus vulgaris L.), chick pea (Cicer arietinum L.), and lentil revealed that lentil significantly reduce feed intake in rats (Queiroz-Monici et al., 2005). These controversial results may be due to differences in the animal model used in the experiments, lentil variety, type of lentil processing (whole vs dehulled vs split), and amount of lentil in the experimental diet.

Generally, legumes increase the fullness after a meal, therefore recommended to reduce body weight (Hermsdorff, Zulet, Abete, & Martínez, 2011). Increased bean and pea consumption associated with lower body weight (Lambert et al., 2017; Papanikolaou & Fulgoni, 2008). Body weight of rats fed with lentil is significantly lower than rats fed with control and high amylose corn diets in current study. Similarly, inclusion of lentil (300 g/kg) in a starter diet for 3 weeks reduce pig body weight (Landero et al., 2012). Also, this study further explains that 75-225 g/kg lentil supplementation did not show significant weight reduction in pigs revealing the dose dependency of lentil on body weight reduction (Landero et al., 2012). High amylose corn starch diet (RS content; 6 g/100 g) did not reduce rat body weight compare to control diet. Similar results were found in a study with 0.8-9.6 g of RS fed to male rats which had no effects of RS on body weight (Deckere, Kloots, & Van Amelsvoort, 1993). These results highlighting that even though feed intake is same among rats, the body weight is reduced in lentil fed rats may due to lentil’s nutrition profile.
Lentil is a rich source of proteins (24-30%; dry weight basis) (Wang & Daun, 2006) including several bio active proteins such as lectins (10-100 mg/100 g dry weight; Peumans & Van Damme, 1996). At initial stages, these proteins considered as anti-nutrients (Peumans and Damme, 1996). However, recent scientific data demonstrate that these proteins have potential to reduce several cancers and reduce obesity risk (Mukherjee, Kim, Park, Choi, & Yun, 2015; Pryme, Bardocz, Pusztai, & Ewen, 2006). Purified lentil proteins reduce plasma TG concentration and very low density lipoprotein (VLDL). Also, lentil proteins reduce adipose lipoprotein lipase (LPL) activity which leads to hypotriglyceridemia; hence prevent access fat accumulation in adipose tissue (Boualga et al., 2009). A study using 15 week old hypertensive rats fed with 30% bean, pea, lentil, and chickpea revealed that lentil associated with large artery remodeling and reduce the risk of high blood pressure (Hanson, Zahradka, & Taylor, 2014). Also, the lentil flour and lentil proteins bind bile salts such as cholate, taurocholate, glycocholate, and chenodeoxycholate (Barbana, Boucher, & Boye, 2011). Bile salts are biosynthesized from cholesterol in the liver and reabsorbed by the ileum. Therefore, binding bile salts in the ileum leads to more degradation of cholesterol in liver; hence lower cholesterol level in blood (Barbana et al., 2011). Addition to lentil proteins, lentil prebiotic carbohydrates may have potential to reduce body fat (Siva et al., 2017).

Lentil is a rich source of prebiotic carbohydrates (Johnson et al., 2015, 2013). Prebiotic carbohydrates reduce body fat% by decreasing the
lipopolysaccharide uptake, increase fat oxidation, and decreasing adiposity (Keenan et al., 2006; Shen et al., 2009; So et al., 2007). Present study, percent body fat, plasma TG concentration is lower in rats fed with lentil than rats fed with high amylose corn and control diets highlights that lentil prebiotic carbohydrates may reduce body fat.

Research have been found that RS decrease total cholesterol, and TG concentration in rats (Deckere et al., 1993). In present study, body fat% and plasma TG concentration are higher in rats fed with high amylose corn diet which had the highest RS content among used diets (Table 5.1). Further, we observed higher hepatic fat portions in the proximal liver tissues of rats fed with high amylose corn diet than lentil and control (Figure 5.6). This indicates that addition to RS, other prebiotics (SA, RFO, and FOS) in lentil (Johnson et al., 2013) may play vital roles in modifying fat metabolism via gut microbiome (Roberfroid, 2000).

Prebiotic carbohydrates change gut microbiome and improve host’s health status (Roberfroid, 2000). Present study, two dominant bacterial phyla were observed in rat fecal samples (Firmicutes and Bacteriodetes) which is similar to previous studies (Ley et al., 2005). Research have been found that the ratio of Bacteriodetes and Firmicutes is related to obesity status of rats and human models, but the results were controversial (Ley, 2010). Some research found that increased Bacteriodetes and Firmicutes ratio in lean subjects (Ley et al., 2005; Turnbaugh et al., 2006), some others found decreased ratio in lean subjects
Figure 5.6. Light microscopic images of proximal tissues of rat liver after 6 weeks of feeding lentil (6A), corn (6B), and control (6C) diets. Scale bar for all images equal to 100 µm.
(Collado et al., 2008; Schwiertz et al., 2010) whereas some other research revealed that no association between *Bacteroidetes* and *Firmicutes* ratio and obesity (Finucane et al., 2014). Considering the present study, the ratio of *Bacteroidetes* and *Firmicutes* did not associated with body weight, percent body fat, and plasma TG concentration. Addition, we found that lentil diet increase abundance of *Actinobacteria* in rats where lowest body weight and percent body fat, and plasma TG concentration found. This is contrast to previous finding that phylum *Actinobacteria* abundance is high in obese compare to lean (Turnbaugh et al., 2009). It is clear that there are much more complex relationship between gut microbiome and obesity. Therefore, rather looking the connection between obesity and gut microbiota at phylum level, the species level elaboration is more useful.

Few research have been focused to evaluate the relationship of individual bacterial species on obesity risk. Four weeks of feeding pea increased cecal *Bifidobacterium* sp. in rats (Queiroz-Monici et al., 2005). High fat diet reduced *Bifidobacterium* sp., a gram positive bacteria which related to low grade inflammatory tone (Cani et al., 2007). *Bifidobacterium* sp. negatively correlated with endotoxaemia and positively correlated with improved glucose tolerant and inflammatory tone (Cani et al., 2007). Present study, increased abundance of *Bifidobacterium* sp. in lentil fed rats may related to lower body fat%, and plasma TG concentration.
Present study, abundance of potential pathogenic bacterial species were reduced in rats fed with lentil diet compare to control and high amylose corn diets. Eggerthella lenta is a gram positive bacteria, causes abdominal pain and severe ulcers (Gardiner et al., 2015). Lautropia mirabilis is gram negative bacteria which is found in children who effected with HIV (Rossmann et al., 1998). Tanneralla sp. and Bacteroides heparinolyticus are normally found at periodontal disease stage in oral cavity (Okuda, Kato, Shiozu, Takazoe, & Nakamura, 1985; Tanner & Izard, 2006). Lentil diet reduce abundance of above bacterial species (except Bacteroides heparinolyticus) revealing that lentil eliminate potential pathogenic bacteria which is related increased immune responses associated with obesity status (Creely et al., 2007; Marti, Marcos, & Martinez, 2001; Osborn & Olefsky, 2012).

Few studies observed reduction in *Firmicute* species after feeding legumes. Six weeks of feeding yellow pea fiber reduced Clostridium leptum in rats (Eslinger et al., 2014). In present study, lentil diet significantly lower the abundance of Lachnospiraceae sp., Streptococcaceae sp., and Peptostreptococcus stomatis. However, lentil increase abundance of few less prominent *Firmicutes* revealing that all *Firmicutes* may be not responsible for increase body fat accumulation. However, individual mechanism of these species on body fat reduction still need to be revealed (DiBaise et al., 2008).
5.6. Conclusion

Reducing obesity risk via changing dietary pattern is an emerging approach. Legumes including lentil gained central attention due to its' nutritional profile which includes bio active proteins and prebiotic carbohydrates. Six week of feeding lentil diet significantly reduced rat body weight than control and corn diets. Also, lentil diet significantly reduced percent body fat and plasma TG concentration than corn starch diet. Lentil diet reduced abundance of fecal Firmicute species. Thus, Lentil is a potential food source to reduce obesity risk. However, specific compounds in lentil and the related changes in fecal microbiome and fat metabolism is to be discovered. Further research are required to understand the fat lowering effect of lentil.

5.7. References


6. GENERAL DISCUSSION

Health and economic impact of obesity highlights the need of novel obesity preventive actions. Such preventive actions focus on reducing energy accumulation in the body. Increasing physical activities and consumption of low energy foods such as vegetable, fruits, and legumes are widely used methods to reduce obesity risk. Lentil is such a legume which has low energy due to low digestible carbohydrates (prebiotic carbohydrates) including SA, RFO, FOS, and RS (Johnson et al., 2013; Johnson et al., 2015). Therefore, the knowledge about the impact of processing and cooking on the prebiotic carbohydrates concentration and the impact of lentil prebiotic carbohydrates on body weight and body fat may useful to produce novel foods to reduce obesity risk.

Processing change prebiotic carbohydrate concentrations. The first study showed that removal of seed coat reduces SA, raffinose and stachyose concentration highlighting seed coat contains high amount of SA, raffinose, and stachyose. The reverse is true for verbascose. Similarly, cooking decrease SA, and RFO+FOS and cooling increase those compounds. Cooling and reheating doubled the RS concentration. Overall, a serving of 100 g of cooked lentil has 353-813 mg of SA, 4-6 g of RFO and FOS, and 2-3 g of RS, providing 71-94% of prebiotic carbohydrate recommended daily intake. Therefore, lentil is a potential food source to increase intake of prebiotic carbohydrates.

The potential of legumes and associated prebiotic carbohydrates on reduction of obesity risk have not been extensively studied. However, prebiotic
carbohydrates such as fructooligosachcharides, inulin, and RS have studied for their role in reduction of obesity risk. Prebiotic carbohydrates increase satiety, promote weight loss, lower body fat, and lower postprandial glucose level via interacting gut microbiome. The second study revealed that lentil reduce risk factors of obesity including body weight, percent body fat, blood plasma TG concentration, and reduce pathogenic gut bacteria. Lentil diet reduce 20-45% of body fat than control and corn diets. Addition, lentil diet reduce 22-55% of blood plasma TG concentration than control and corn diets. The exact mechanism of lentil on fat lowering effect is unknown. However, the bioactive proteins and prebiotic carbohydrates in lentil may be the reason for lower body fat in rats.

Prebiotic carbohydrates prevent excess body fat accumulation by three ways; reduce appetite, reduce gut permeability to lipopolysaccharides (LPS), and reduce fat deposition in adipose tissues (Figure 6.1). Fermentation of prebiotic carbohydrates produce short chain fatty acids (SCFA). It includes acetate (60%), propionate (20-25%), and butyrate (15-20%) (Cummings et al., 1987). These SCFA stimulate the enteroendocrine cells via G-protein-coupled receptor (GPR) called GPR43. Enteroendocrine cells produce a gut peptide called PYY which can slow down the movement of food through digestive tract (Keenan et al., 2006; Parnell & Reimer, 2009). Also, prebiotic fibers reduce the secretion of hunger hormone “ghrelin” by reducing the expression of ghrelin mRNA in gut (Parnell & Reimer, 2009). Increased concentration of gut peptide PYY and reduced level of ghrelin increase satiety; hence reduce food intake.
Short chain fatty acids increase expression of tight junction mRNA expression in enteroendocrine L cells which produce glucagon like peptides called GLP-1 and GLP-2. Ultimately these peptides reduce the permeability of gut membrane via changing the distribution of tight junction proteins zonula occludens 1 (ZO-1) and occludin. Thus, reduces permeability of gut epithelial cells leads to low absorption of LPS (Cani et al., 2009; Everard et al., 2011). Another mechanism of reduce membrane permeability is involved by endocannabinoid system (eCB). eCB system is a group of endogenous receptors located in mammalian nerve system which control appetite, digestion, and energy balance (Aizpurua-Olaizola et al., 2017). A receptor called CB₁ in eCB system reduce gut permeability via changing the distribution of ZO-1 and occludin. Therefore, it reduce LPS absorption leads to low blood LPS concentration (Muccioli et al., 2010).
Figure 6.1. Mechanisms of prebiotic carbohydrates on host pathophysiology related to obesity. Changes in the gut microbiome and SCFA in the gut change the expression of genes related to food intake, gut motility, and adipogenesis.
Another distinguished mechanism of prebiotic carbohydrates is reduce fat deposition by modifying gene expression in adipose tissues. Dewulf et al., 2010 found that prebiotic carbohydrates led to form small adipose tissues compare to high fat diet. Adipogenesis is controlled by GPR43 expression, adipocyte specific genes including adipocyte P2 gene (aP2) and stimulating factors peroxisome proliferator activated receptor gamma (PPARγ) and CCAAT-enhancer-binding protein α (C/EBPα)(Dewulf et al., 2010). It was found that prebiotic carbohydrates reduce the expression of GPR43 in adipose tissues (Dewulf et al., 2010). Further, prebiotic carbohydrates reduce aP2 and C/EBPα mRNAs level. Also, the expression of cluster differentiation 36 (CD36) and lipoprotein lipase (LPL) mRNA levels controlled by PPARγ were significantly reduced (Dewulf et al., 2010). Thus, the fat deposition in adipose tissues are reduced. However, these research are still in early stages, hence more systematic experiments with large number of replicates are needed to confirm the true prebiotic effects on host.

6.1. References


7. CONCLUSION AND FUTURE DIRECTION

Lentil nutrient compounds including proteins, prebiotic carbohydrates, minerals, vitamins can be increased by conventional plant breeding, processing (whole vs dehulling vs splitting), and cooking operations. However, it is important to know that exactly which compounds in lentil increase human health such as reducing body fat. Also, in the present rat study, rats were fed with lentils only for 6 weeks. It may be not enough to evaluate the long term changes in obesity bio markers. Therefore, the future lentil research will include,

1. Determination of impact of lentil diet on obesity biomarkers and gut microbiomes using human subjects for a long period.
2. Separation of lentil prebiotic carbohydrates (SA, RFO, FOS, and RS) and evaluate the effect of those individual prebiotics on obesity biomarkers.
3. Determination of the impact of processing and thermal treatments on prebiotic carbohydrates of other legumes including chickpea, cowpea and beans.
4. Development of legume based food products such as morning cereals, pasta to provide optimum prebiotic carbohydrates to consumer.

In conclusion, prebiotic carbohydrates are important dietary component for healthy living. Lentil processing and cooking operations manipulate the prebiotic carbohydrate concentrations. Further, lentil significantly reduced body weight, and decrease pathogenic bacteria. Thus, lentil is a potential whole food legume to reduce obesity risk. However, further research with human models are required to confirm this hypothesis.
APPENDICES
Appendix A

Primer design

Primers were designed using a published method (Kozich et al., 2013). Each primer consists of illumina adaptor, an 8-nt index sequence, a 10-nt pad sequence, a 2-nt linker, and the gene specific primer. Primer sequences i5 and i7 are the 8-nt index sequences. The pad is a 10-nt sequence to boost the sequencing primer melting temperatures. Linker is a 2-nt sequence that is anti-complementary to the known sequences. The 16S forward and 16S reverse are the gene specific primer sequences for V4 region of 16S rRNA gene.

V4 region gene specific primers

16S forward: GTGCCAGCMGCCGCGGTAA
16S reverse: GGACTACHVGGGTWTCTAAT

V4 Link

Forward: GT
Reverse: CC

Pad

Forward: TATGGTAATT
Reverse: AGTCAGTCAG

Index primer i5

SA501: ATCGTACG
SA502: ACTATCTG
SA503: TAGCGAGT
SA504: CTGCGTGT
SA505: TCATCGAG
SA506: CGTGAGTG
SA507: GGATATCT
SA508: GACACCGT
SB501: CTACTATA
SB502: CGTTACTA
SB503: AGAGTCAC
SB504: TACGAGAC
SB505: ACGTCTCG
SB506: TCGACGAG
SB507: GATCGTGT
SB508: GTCAGATA

1.5. Index primer i7

SA701: AACTCTCG
SA702: ACTATGTC
SA703: AGTAGCGT
SA704: CAGTGAGT
SA705: CGTACTCA
SA706: CTACGCAG
SA707: GGAGACTA
SA708: GTCGCTCG
SA709: GTCGTAAGT
SA710: TAGCAGAC
SA711: TCATAGAC
SA712: TCGCTATA

1.6. Illumina adapter

P5: AATGATACGGCGACCACCGAGATCTACAC
P7: CAAGCAGAAGACGGCATACGAGAT
## 1.7. Primers used to amplify 144 samples

| SA501  | AATGATACGGCCACCAGGATCTACACATCGTACGTATGGTAATTGTGTGCAACGAGCGCAGGTAA |
| SA502  | AATGATACGGCCACCAGGATCTACACATCGTACGTATGGTAATTGTGTGCAACGAGCGCAGGTAA |
| SA503  | AATGATACGGCCACCAGGATCTACACATCGTACGTATGGTAATTGTGTGCAACGAGCGCAGGTAA |
| SA504  | AATGATACGGCCACCAGGATCTACACATCGTACGTATGGTAATTGTGTGCAACGAGCGCAGGTAA |
| SA505  | AATGATACGGCCACCAGGATCTACACATCGTACGTATGGTAATTGTGTGCAACGAGCGCAGGTAA |
| SA506  | AATGATACGGCCACCAGGATCTACACATCGTACGTATGGTAATTGTGTGCAACGAGCGCAGGTAA |
| SA507  | AATGATACGGCCACCAGGATCTACACATCGTACGTATGGTAATTGTGTGCAACGAGCGCAGGTAA |
| SB501  | AATGATACGGCCACCAGGATCTACACATCGTACGTATGGTAATTGTGTGCAACGAGCGCAGGTAA |
| SB502  | AATGATACGGCCACCAGGATCTACACATCGTACGTATGGTAATTGTGTGCAACGAGCGCAGGTAA |
| SB503  | AATGATACGGCCACCAGGATCTACACATCGTACGTATGGTAATTGTGTGCAACGAGCGCAGGTAA |
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| SB506  | AATGATACGGCCACCAGGATCTACACATCGTACGTATGGTAATTGTGTGCAACGAGCGCAGGTAA |
| SB507  | AATGATACGGCCACCAGGATCTACACATCGTACGTATGGTAATTGTGTGCAACGAGCGCAGGTAA |
| SB508  | AATGATACGGCCACCAGGATCTACACATCGTACGTATGGTAATTGTGTGCAACGAGCGCAGGTAA |
| SA701  | CAAGCAGAAACCCAGGATCTACAGAAGCTCTCGATGAGTCAGCCGACACTACHVGGGTWTCTAAT |
| SA702  | CAAGCAGAAACCCAGGATCTACAGAAGCTCTCGATGAGTCAGCCGACACTACHVGGGTWTCTAAT |
| SA703  | CAAGCAGAAACCCAGGATCTACAGAAGCTCTCGATGAGTCAGCCGACACTACHVGGGTWTCTAAT |
| SA704  | CAAGCAGAAACCCAGGATCTACAGAAGCTCTCGATGAGTCAGCCGACACTACHVGGGTWTCTAAT |
| SA705  | CAAGCAGAAACCCAGGATCTACAGAAGCTCTCGATGAGTCAGCCGACACTACHVGGGTWTCTAAT |
| SA706  | CAAGCAGAAACCCAGGATCTACAGAAGCTCTCGATGAGTCAGCCGACACTACHVGGGTWTCTAAT |
| SA707  | CAAGCAGAAACCCAGGATCTACAGAAGCTCTCGATGAGTCAGCCGACACTACHVGGGTWTCTAAT |
| SA708  | CAAGCAGAAACCCAGGATCTACAGAAGCTCTCGATGAGTCAGCCGACACTACHVGGGTWTCTAAT |
| SA709  | CAAGCAGAAACCCAGGATCTACAGAAGCTCTCGATGAGTCAGCCGACACTACHVGGGTWTCTAAT |
| SA710  | CAAGCAGAAACCCAGGATCTACAGAAGCTCTCGATGAGTCAGCCGACACTACHVGGGTWTCTAAT |
| SA711  | CAAGCAGAAACCCAGGATCTACAGAAGCTCTCGATGAGTCAGCCGACACTACHVGGGTWTCTAAT |
| SA712  | CAAGCAGAAACCCAGGATCTACAGAAGCTCTCGATGAGTCAGCCGACACTACHVGGGTWTCTAAT |
1.8. Schematic diagram of 96 well plates that assigned to dual index primers for 144 rat fecal samples

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<td>R21</td>
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S01-S12; fecal samples of rats fed with standard diet, R13-R24; fecal samples of rats fed with 3.5% high amyllose corn starch diet, L25-L36; fecal samples of rats fed with 70.8% lentil diet.

- Yellow: Initial week
- Green: 2nd week
- Blue: 4th week
- Orange: 6th week