PREBIOTICS IN LENTIL (LENS CULINARIS L.)

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Prebiotics in Lentil (*Lens culinaris* L.)

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ABSTRACT

Prebiotics are food ingredients that contribute to improved health via interactions with microbes in the gut. The objectives of this study were (1) to compare the concentrations of prebiotic carbohydrates in different lentil genotypes and growing locations and (2) to determine any concentration changes due to processing and preparation procedures. All lentil genotypes contained several prebiotic carbohydrates: raffinose-family oligosaccharides (RFO), fructo-oligosaccharides, sugar alcohols, and resistant starch (RS). Significant differences were observed in prebiotic concentrations among genotypes. Modest RFO concentration reductions were observed with cooking, cooling, and reheating. Mean RS concentration in raw, cooked, cooled, and reheated lentil were 3.0, 3.0, 5.1, and 5.1 g/100 g respectively, clearly demonstrating cooling-induced formation of RS. Study results suggest that lentil contains nutritionally significant concentrations of prebiotics and that those concentrations may be enhanced through breeding, locational sourcing, and cooking and preparation procedures.

Key Words: prebiotics, microbiota, lentil, obesity, cooking, dehulling

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TABLE OF CONTENTS

ABSTRACT.....iii

ACKNOWLEDGEMENTS.....iv

LIST OF TABLES.....viii

LIST OF FIGURES.....ix

LIST OF SCHEMES.....x

1. INTRODUCTION.....1

2. LITERATURE REVIEW.....3

2.1. Obesity.....3

2.2. Obesity and the current food system: are they related?.....6

2.3. Gut microbiota and prebiotics.....10

2.4. Lentil.....16

2.5. Lentil prebiotics.....19

2.5.1. Fructooligosaccharides.....20

2.5.2. Raffinose-family oligosaccharides.....22

2.5.3. Sugar alcohols.....23

2.5.4. Resistant starch.....24

2.6. Measurement of prebiotics.....27

2.7. Future directions.....29

2.8. References.....30

3. OBJECTIVES AND HYPOTHESES.....45

3.1. Paper 1.....45

3.1.1. Objective.....45

3.1.2. Hypotheses 45

3.2. Paper 2 45

3.2.1. Objective 45

3.2.2. Hypotheses 45

4. PAPER 1. LENTIL (*LENS CULINARIS* L.): A PREBIOTIC-RICH WHOLE FOOD LEGUME 46

4.1. Abstract 46

4.2. Introduction 46

4.3. Materials and methods 49

4.3.1. Materials 49

4.3.2. Lentil samples 50

4.3.3. Analysis of water soluble prebiotic carbohydrates 50

4.3.4. Resistant starch analysis 52

4.3.5. Statistical analysis 53

4.4. Results 53

4.4.1. Thousand-seed weight 53

4.4.2. Concentrations of water soluble prebiotic carbohydrates 56

4.4.3. Concentrations of resistant starch and total starch 60

4.5. Discussion 62

4.6. Conclusions 67

4.7. Acknowledgements 67

4.8. References 67

5. PAPER 2. PROCESSING, COOKING, AND COOLING AFFECT PREBIOTIC CONCENTRATIONS IN LENTIL (*LENS CULINARIS* L.) 75

5.1. Abstract 75
5.2. Introduction 75
5.3. Materials and methods 79
5.3.1. Materials 79
5.3.2. Lentil seed samples 79
5.3.3. Cooking procedure 80
5.3.4. Determination of RFO concentration 80
5.3.5. Determination of RS concentration 81
5.3.6. Scanning electron microscopy (SEM) 82
5.3.7. Statistical analysis 83
5.4. Results 83
5.4.1. Raffinose-family oligosaccharide concentration 83
5.4.2. Resistant starch concentration 85
5.4.3. Scanning electron microscopy 86
5.5. Discussion 88
5.6. Acknowledgements 92
5.7. References 92
6. CONCLUSION AND FUTURE DIRECTIONS 100
6.1. References 105

LIST OF TABLES

Table — Page

2.1. Trends in the age-adjusted and age-specific prevalence of obesity (BMI ≥ 30) in US adults aged 20 years or older for 1999 – 2008 5

2.2. Calories from major commodities (kcal per capita per day) in developing countries, North America, and world 7

2.3. Composition and characteristics of dominant phyla of human gut microbiota and several subgroups of bacteria and their substrates and products 12

2.4. Main areas of pathophysiological interest in which prebiotics have been investigated 15

2.5. Nutrient concentration data in raw lentil and cereal grains 18

2.6. Various prebiotics in common foods 20

4.1. Market class, major consuming countries, and thousand-seed weight of 10 lentil varieties grown in North Dakota, USA 54

4.2. Combined analysis of variance for seed weight (TSW), sorbitol (Sorb), mannitol (Mann), raffinose (Raff), stachyose (Stach), verbascose (Verb), nystose (Nys), resistant starch (RS), and total starch (TS) for 10 lentil varieties grown in North Dakota, USA in 2010 and 2011 55

4.3. Mean concentration of prebiotic carbohydrates of 10 lentil varieties grown in North Dakota, USA, in 2010 and 2011 57

4.4. Mean concentrations of prebiotic carbohydrates and total starch by year and location 58

4.5. Concentrations of total prebiotic carbohydrates, galactooligosaccharides (GOS), and resistant starch (RS) in a 100 g serving of lentils by market class with dietician-recommended intake values 66

5.1. Raffinose-family oligosaccharide concentrations (%) in raw and processed lentil 84

6.1. Concentrations (g per serving) of raffinose-family oligosaccharides, resistant starch, sugar alcohols, fructooligosaccharides, and total apparent prebiotics[a] in a 1-cup serving of cooked lentils by market class 101

LIST OF FIGURES

Figure Page

2.1. Food availability for the USA, 1910 – 2006. There are two distinct phases: a decrease in food energy supply (postulated to be pulled down by reduced energy expenditure requirements for daily living), followed by an increase in food energy supply (postulated to be pushed up by increasing food access). An energy balance flipping point is proposed, marking the change in how the US population generally achieved energy balance. Reproduced with permission from Swinburn et al. 2011. 8

2.2. Estimates of percentage of childhood population overweight, including obese (with use of International Obesity Taskforce cutoffs) in a selection of countries. Reproduced with permission from (Swinburn et al., 2011).....9

4.1. Mean total starch and resistant starch concentrations of 10 lentil genotypes grown in North Dakota, USA in 2010 and 2011.....61

5.1. Mean resistant starch concentration in raw, cooked, cooled, and reheated lentil products by whole and split market classes. Error bars are based on $p < 0.05$ (n = 96).....86

5.2. Scanning electron micrograph images of the same magnification (length of magnification bar = 10 μm) of lentil starch granules after freeze-fracture under the following treatment conditions: (A) native green lentil cotyledon, (B) cooked green lentil cotyledon, (C) cooked and cooled green lentil cotyledon, (D) native red lentil cotyledon, (E) cooked red lentil cotyledon, (F) cooked and cooled red lentil cotyledon.....87

LIST OF SCHEMES

Scheme Page

6.1. Interactions of lentil prebiotics. The flowchart represents the current relationship between lentil prebiotics and various sectors, e.g., gut microbiota, consumer health, plant breeding, and food processing. Dotted arrowed lines represent a causal or influential relationship. The thickness of the arrowed line is indicative of its current impact on the circle or line it points to 104

1. INTRODUCTION

While for centuries, the greatest disease threats facing humanity were infectious, now, chronic non-communicable diseases (obesity, type 2 diabetes, cardiovascular diseases, hypertension, etc.) account for an unprecedented 63% of global disease burden (United Nations, 2012). Dietary behaviors contribute to the etiology and prognosis of these disorders (Singh et al., 1992). Increased caloric intake and altered diet composition, e.g., refined sugar and vegetable oils, are associated with a drastic increase in the prevalence of obesity and related non-communicable diseases (Austin, Ogden, & Hill, 2011). Furthermore, along with the addition of refined, high-energy foods to the typical diet, traditional foods including pulses have been displaced (Kearney, 2010). A variety of bioactive compounds that exist in traditional foods are now being realized for their capacity to reduce risk factors of obesity and its comorbidities. As the burden of disease escalates, demand for these traditional staple crops – previously a pillar of the food system – will increase.

Lentil (*Lens culinaris* L.), a cool-season food legume and a staple food in many Eastern diets, is an important component of a sustainable food system. Lentil is a good source of protein (20 – 30%), carbohydrates (~60%), essential fatty acids, and a range of vitamins and minerals (Bhatty, 1988; Thavarajah et al., 2011). Contributing to its low glycemic index (Jenkins et al., 1981), lentil also has a unique profile of carbohydrates including several healthful prebiotic compounds: raffinose-family oligosaccharides (RFO), fructooligosaccharides (FOS), sugar alcohols, and resistant starch (RS) (Bhatty, 1988; Wang, Hatcher, Toews, & Gawalko, 2009).

A prebiotic is a component of food which is neither digested nor absorbed in the small intestine, is passed to the large intestine and fermented, and elicits its effects via interactions with the microbial flora (Gibson & Roberfroid, 1995). Diet rich in prebiotics contributes to human

health and well-being through multiple facets, both physiological and pathophysiological, including reduction of risk factors for obesity and non-communicable diseases (Roberfroid et al., 2010). These attributes make prebiotic-rich foods such as lentil an interesting research topic for prevention of obesity.

The following literature review will overview the current obesity epidemic, discuss those prebiotics which are present in lentil and their human health consequences, and review pertinent information required to assess lentil as a dietary source of prebiotics. The need for producing appropriate crops for human nutrition will also be reviewed, addressing lentil and its importance in healthy food systems. The remainder will focus on prebiotic oligosaccharides, polysaccharides, and sugar alcohols in lentil, and the effects of genetics, growing environment, and cooking and processing on their concentration.

2. LITERATURE REVIEW

2.1. Obesity

Obesity is simply defined as having excess body fat (CDC). The most commonly used measurement of excess body fat is body mass index (BMI), a calculation from a person's height and weight (m^2/kg). In the US, BMI is used to characterize overweight (BMI > 25) and obese (BMI > 30) individuals (Expert Panel on the Identification, Evaluation, and Treatment of Overweight in Adults). Essentially, BMI is a tool to be used in epidemiological studies that lead to a better understanding of the accumulation of body fat and the development of obesity.

The accumulation of excess body fat is the result of a metabolic imbalance of energy, *i.e.*, more energy is consumed than is utilized (Horton et al., 1995). The human body naturally stores any available energy exceeding its requirements, most notably in the forms of glycogen and lipid, the latter being preferred for long-term storage (Horton et al., 1995). Lipids are energy-dense and require no water to store, making them ideal as an energy reserve. Fat storage in the body is a survival mechanism: when food is unavailable the body utilizes stored fat reserves to maintain function for extended periods of time (Cahill, 1970). Moreover, adipocytes, or lipid depot cells, are responsible for sequestering circulating glucose and triglycerides and for maintaining plasma insulin concentrations (Gavrilova et al., 2000; Seip & Trygstad, 1996). So, if our body fat is so important for metabolism and general well-being, why is obesity of so much concern?

The death toll associated with obesity is over 300,000 each year in the US (U.S. Department of Health & Human Services). Obesity greatly increases the risk of a long list of health consequences – heart disease, type-2 diabetes, and certain types of cancer, stroke, arthritis, breathing problems, and psychological disorders, such as depression (Popkin, Kim, Rusev, Du,

& Zizza, 2006). Globally, over 500 million people are obese (Finucane et al., 2011); this includes about 36% of US adults (Flegal, Carroll, Kit, & Ogden, 2012). In recent years, the percentage of obese adults (over 20 years) in the US increased by 8% in men and 3% in women **(Table 2.1.)**. The combined prevalence of overweight and obesity has reached about 70% in the US (Flegal et al., 2012). In addition to morbidity and mortality concerns, the estimated economic cost of obesity in the US was $117 billion in the year 2000, seen in medical services and loss of worker income and productivity (U.S. Department of Health & Human Services). To put the consequences of obesity in perspective, in addition to causing a drastic reduction in quality of life and disability-free life years, obesity and related comorbidities account for 63% of global deaths (Lopez, Mathers, Ezzati, Jamison, & Murray, 2006; United Nations, 2012). The situation deserves global attention.

Table 2.1. Trends in the age-adjusted and age-specific prevalence of obesity (BMI ≥ 30) in US adults aged 20 years or older for 1999 – 2008.

	(%) of Adults							
	Age ≥ 20 y[a]	(%) change over 10 y	Ages 20-39 y	(%) change over 10 y	Ages 40-59 y	(%) change over 10 y	Age ≥ 60 y	(%) change over 10 y
Men[b]								
1999-2000	28		24		29		32	
2001-2002	28		22		32		30	
2003-2004	31		28		35		30	
2005-2006	33		28		40		32	
2007-2008	32		28		34		37	
2009-2010	36	29	33	38	37	28	37	16
Women								
1999-2000	33		28		38		35	
2001-2002	33		30		36		35	
2003-2004	33		29		39		32	
2005-2006	35		31		41		34	
2007-2008	36		34		38		34	
2009-2010	36	9	32	14	36	-5	42	20

Abbreviations: BMI, body mass index (calculated as weight in kilograms divided by height in meters squared).

Sources: Adapted from (1) Flegal, K; Carroll, M; Ogden, C; Curtin, L. (2010) Prevalence and trends in obesity among US adults, 1999-2008. *JAMA, 303(3):235-241[20]* and (2) Flegal, K; Carroll, M; Kit, B; Ogden, C. (2012) Prevalence of obesity and trends in the distribution of body mass index among US adults, 1999-2010. *JAMA, 307(5):491-497.*

[a]Age adjused by the direct method to the year 2000 Census population using the age groups 20 – 39 years, 40 – 59 years, and 60 years or older.

[b]Includes racial and ethnic groups not shown separately.

2.2. Obesity and the current food system: are they related?

The question of how obesity and non-communicable diseases came to be a global problem and how they progressed cannot be easily answered. However, it will be valuable to consider a few key elements in order to create a logical framework to solve this global health problem. Two factors that contributed to the high prevalence of obesity will be briefly discussed: (1) increased production and consumption of foods that are energy-dense and deficient in bioactive compounds, and (2) decreased production and consumption of traditional pulse crops.

A significant change in the global food system can be dated back to the start of the agricultural, or so-called 'green', revolution (Welch & Graham, 1999). In an effort to preclude impending famine and starvation, technologically-advanced agricultural practices were implemented in many regions to increase productivity (Evenson & Gollin, 2003). The program successfully increased land productivity and food availability per person. Since then, food availability per person increased by about 350 kcal per capita per day, a 15% increase in energy within 30 years (FAOSTAT). Cereal crops including wheat (*Triticum* spp.), rice (*Oryza sativa* L.), and corn (*Zea mays* L.) were primary contributors to this energy boost **(Table 2.2)**. The green revolution is commended for preventing food shortages in many regions. The next agricultural revolution, however, will need to address hidden consequences of the last revolution – malnutrition and obesity.

Swinburn et al. (2011) emphasize that a global energy overbalance increases the obesity epidemic. As opposed to previous generations where energy expenditure determined energy intake, currently, energy intake is driving energy expenditure **(Figure 2.1.)**. As food availability increased, the world prevalence of obesity surged. Globally, the average BMI has increased significantly since 1980 (Finucane et al., 2011), and the prevalence of overweight and obesity

Table 2.2. Calories from major commodities (kcal per capita per day) in developing countries, North America, and world.

	Year	Pulses	(%) change three decades	Starchy roots	(%) change three decades	Cereals	(%) change three decades	Vegetable oils	(%) change three decades	Sugar & sweeteners	(%) change three decades
Developing countries	1970	119		131		1288		104		147	
	1985	91		122		1316		137		173	
	2000	84	-29	145	11	1355	5	178	71	168	14
North America	1970	28		99		592		347		561	
	1985	29		100		682		523		539	
	2000	43	54	110	11	872	47	627	81	626	12
World											
	1970	72		178		1188		142		222	
	1985	60		131		1309		200		239	
	2000	56	-22	141	-21	1306	10	247	74	228	3

Data from: FAOSTAT (http://faostat.fao.org/site/368/Desktop.Default.aspx?PageID=368#ancor).

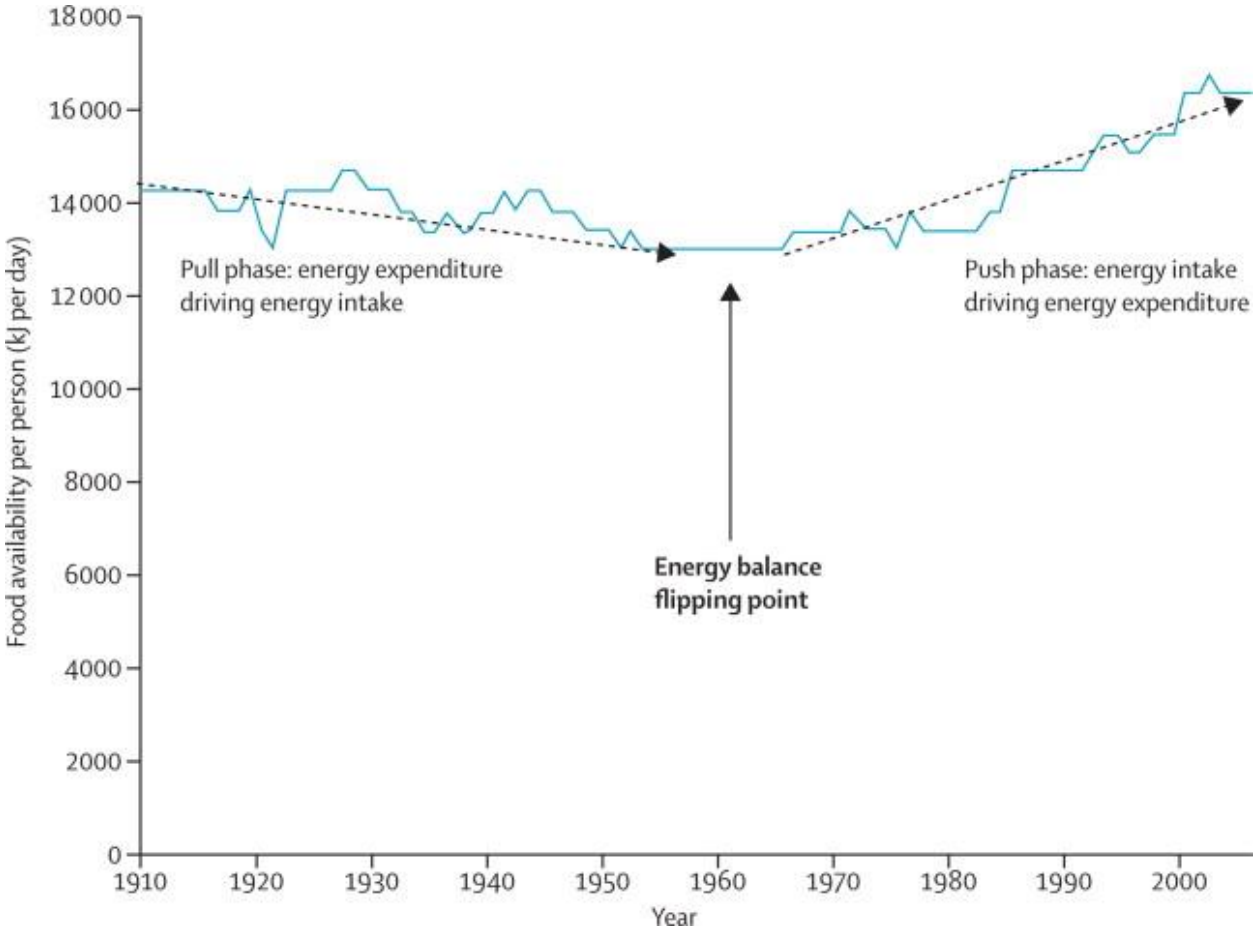

Figure 2.1. Food availability for the USA, 1910 – 2006. There are two distinct phases: a decrease in food energy supply (postulated to be pulled down by reduced energy expenditure requirements for daily living), followed by an increase in food energy supply (postulated to be pushed up by increasing food access). An energy balance flipping point is proposed, marking the change in how the US population generally achieved energy balance. Reproduced with permission from Swinburn et al. 2011.

among children in many countries has more than doubled since the 1970s **(Figure 2.2.)**. While excess food availability is certainly a large contributor to obesity, the question remains, "Does all food contribute to obesity equally?"

During the green revolution, little attention was given to the nutritional quality of the food system as a whole. Certain crops were produced disproportionately: an over-abundance of high yielding cereals and a displacement of micronutrient-rich crops, especially pulses. The world transitioned from traditional food staples – pulses, roots, and tubers – to processed cereal-

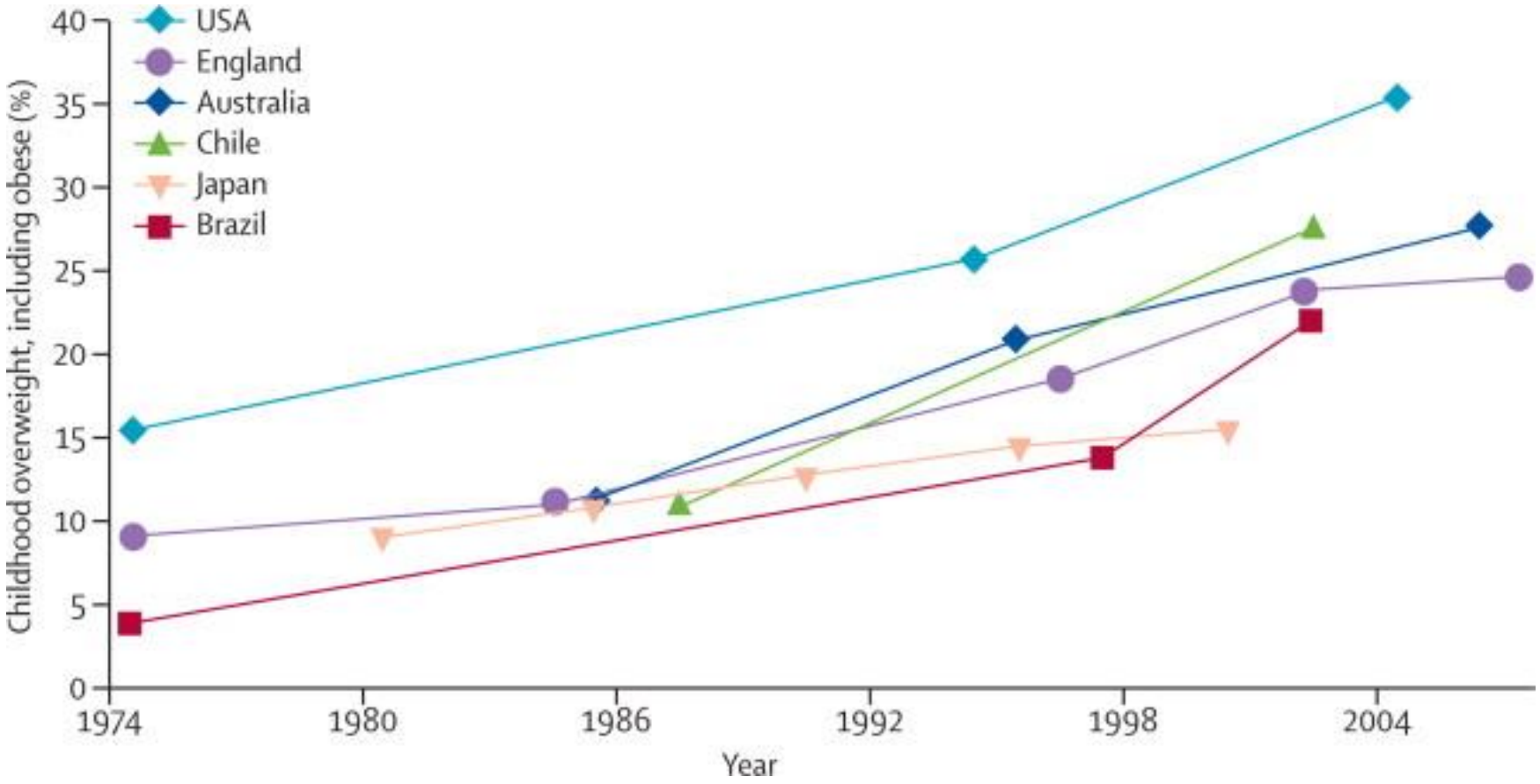

Figure 2.2. Estimates of percentage of childhood population overweight, including obese (with use of International Obesity Taskforce cutoffs) in a selection of countries. Reproduced with permission from (Swinburn et al., 2011).

based foods and foods rich in added fats, vegetable oils, and sugars **(Table 2.2.)**. Over the three decades between the 1970s and 2000s, availability of high-energy food products (cereals, vegetable oils, sugar, etc.) increased in North America and developing countries. Meanwhile, pulses, roots, and tubers availability decreased globally. In North America, although the amount of pulses, roots, and tubers per person increased, availability of these crops remained quite low.

The associations of increased prevalence of obesity with dietary patterns raised many questions. How much of the obesity epidemic can be attributed to the overall increase in calorie consumption? How much to the displacement of traditional foods with their diverse protective agents from the diet? Also, what caused the increase in calorie consumption? Did excess food availability lead to excess consumption, or did altered diet composition lead to excess consumption, or both? These are difficult questions which cannot be fully answered by any single group. Nevertheless, agricultural, nutrition, and food scientists can logically gather some helpful cues on which to focus attention: (1) understanding of the chemistry of healthful bioactive compounds in foods and (2) production of foods that provide appropriate energy and nutrition. Thus, having reviewed the problems to be addressed, we will discuss an important element of the first of these objectives – prebiotics.

2.3. Gut microbiota and prebiotics

Prebiotics emerged in the literature in 1995 with the discovery that certain oligosaccharides could provide host benefits by altering the microbial ecology in the gut (Gibson & Roberfroid, 1995). Soon after, the gut microbiota and prebiotics were hot topics in the area of human nutrition including nutrient absorption, immunology, evolution, and epidemiology (Krajmalnik-Brown, Ilhan, Kang, & DiBaise, 2012; Backhed et al., 2004; Lee & Mazmanian, 2010; Turnbaugh et al., 2006; Roberfroid et al., 2010; Rowland, 2009). Prior to these advances,

surprisingly little was known about the complex relationship between the gut microbiota, its substrates, and the gastrointestinal tract.

The large intestine was, for many years, thought to have only two main functions: (1) waste excretion and (2) water absorption (Welch, 1936). We now understand that without the cooperative role of the gut microbiota in the large intestine human hosts are incapable of performing several vital physiological, metabolic, and immunological functions (Turnbaugh et al., 2007; Gill et al., 2006; Backhed, Ley, Sonnenburg, Peterson, & Gordon, 2005). The gastrointestinal microbiota is also involved in the development of miscellaneous human pathophysiological conditions (Rowland, 2009; Turnbaugh et al., 2006; Rabot et al., 2010). The intestinal epithelium and the gut microbial community function interdependently, cooperatively forming an intricate organ system – a partially external and partially "inhuman" organ system (Backhed et al., 2005). Thus with the wealth of recently generated information, the microbiota is now recognized as a key player in health and well-being.

The human gastrointestinal tract hosts about $10^{12} – 10^{14}$ microorganisms, varying greatly in composition, function, and location of colonization between individuals (Savage, 1977). The concentration of live microorganisms in the stomach is about 10^3 CFU/mL of contents, in the small intestine about $10^4 – 10^6$ CFU/mL of contents, and in the large intestine about 10^{12} CFU/g of contents (Holzapfel, Haberer, Snel, & Schillinger, 1998). Over 1000 commensal species in the human hindgut were identified in a cohort of 124 individuals, with each individual being host to approximately 160 different species (Qin et al., 2010). The dominant phyla present are the Firmicutes and Bacteroidetes, followed by Actinobacteria, and Proteobacteria **(Table 2.3.).** These dominant groups are comprised of various genera, some potentially beneficial, some potentially harmful, and others have the potential to be either harmful or beneficial. For example,

Table 2.3. Composition and characteristics of dominant phyla of human gut microbiota and several subgroups of bacteria and their substrates and products.

Phyla	Bacterial subgroup	Approx. CFU/g of feces	Approx. (%) of microbiota	Mode of action on substrate(s)	Fermentation product(s)
Firmicutes		$3 – 5.3 \times 10^{10}$	30 – 53%		
	Clostridia			saccharolytic, some aa-fermenting species	Ac, Pr, Bu, La, e
	Eubacteriaceae			saccharolytic, some aa-fermenting species	Ac, Bu, La
	Rumminococcus			saccharolytic	Ac
	Lactobacillus	1×10^{8}	1%	saccharolytic	La
	Streptococcus			carbohydrate and aa-fermentation	La, Ac
Bacteroidetes		$0.9 – 4.2 \times 10^{10}$	9 – 42%		
	Bacteroides			saccharolytic	Ac, Pr, Su
Actinobacteria		$0.2 – 2.5 \times 10^{10}$	2 – 25%		
	Bifidobacterium	$0.7 – 1.0 \times 10^{10}$	1 – 14%	saccharolytic	Ac, La, f, e
	Collinsella-Atopobium	$0.3 – 4.0 \times 10^{9}$	0.7 – 10%		
Proteobacteria		$0.7 – 4.0 \times 10^{9}$	1 – 10%		
	Escherichia			carbohydrate and aa-fermentation	Mixed acids
	Desulfovibrio			various	Ac

aa, amino acid; Ac, acetate; Pr, propionate; Su, succinate; Bu, butyrate; La, lactate; f, formate; e, ethanol. Soures: (Pandeya et al., 2012; Roberfroid et al., 2010; Roberfroid, 2008).

many species within these phyla provide energy to the colonocytes in the form of short-chain fatty acids (SCFA) such as acetate, propionate, and butyrate **(Table 2.3.).** Additionally, certain species can produce essential vitamins (e.g., vitamins K_2 and B_{12}) and other beneficial metabolites; other species, however, can produce toxic, genotoxic, or carcinogenic metabolites (Pandeya et al., 2012).

When the composition of commensal groups and their metabolites exist in the right balance, or 'normobiosis,' that is the potentially health-promoting microorganisms predominate over potentially harmful microorganisms, the human host is benefitted (Gibson & Roberfroid, 1995; Cummings & Kong, 2004). On the other hand, an unbalance, or 'dysbiosis,' in the gut microbiota results in a harmful relationship, causing inflammation and disease. The concept of prebiotics is based in the coexistence of these beneficial and harmful bacterial genera. A dietary prebiotic provides the right microbial 'food' to selectively alter the concentrations and functions of the microbial populations leading to 'normobiosis'. The most extensively researched genus that is stimulated by prebiotics and is an important part of the normobiotic phenomenon is *Bifidobacterium* (Roberfroid et al., 2010). *Lactobacillus* is also recognized as a beneficial genus, and other genera will likely be included as more data accrue, e.g. *Eubacterium, Faecalibacterium,* and *Roseburia* (Roberfroid et al., 2010).

Prebiotics have attracted enormous attention (mostly for marketing purposes), and the need for strict criteria to define them became apparent, leading to the establishment of the following requirements to be classified as a prebiotic food ingredient:

- Resist degradation by processes in the upper gastrointestinal tract (acidity, pancreatic enzymes, brush boarder enzymes, etc.)
- Be fermented by intestinal microbiota

- Selectively alter the composition/activity of certain microbes resulting in health benefits to the host

The most recent and widely-accepted definition of a dietary prebiotic is a "selectively fermented ingredient that results in specific changes, in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefit(s) upon host health" (ISAPP, 2008).

A key condition of this definition is 'selectively'. There are many fibers and dietary components that are fermented by the microbiota, but only those which are selectively fermented by certain beneficial microbes are prebiotic. Therefore, though it is likely that more carbohydrates will be considered prebiotic in the future, only several currently have sufficient experimental support to meet the necessary requirements (Roberfroid et al., 2010). Those that have prebiotic 'status' are FOS, galactooligosaccharides (GOS), and lactulose (Kolida & Gibson, 2008).

Prebiotics are included under the broad category of low-digestible carbohydrates (Grabitske & Slavin, 2009). Low-digestible carbohydrates (LDC) are fermentable and are comprised of three groups of compounds: non-starch polysaccharides, sugar alcohols, and RS. Some examples of non-starch polysaccharides are RFO, FOS, and inulin. Sugar alcohols are collectively known as hydrogenated mono-, di-, or polysaccharides. Naturally occurring sugar alcohols include sorbitol and mannitol. The final subgroup, RS, occurs naturally in foods in two forms (RS_1 and RS_2), though other forms exist synthetically. The above mentioned LDCs (discussed in detail under subheading 6) are poorly digested by human enzymes and fermented in the large intestine (Grabitske & Slavin, 2009).

Fermentation of prebiotics and certain LDCs elicits a variety of health effects which can be subdivided into two main groups: functional effects and disease risk reduction **(Table 2.4.)**.

Table 2.4. Main areas of pathophysiological interest in which prebiotics have been investigated.

Effects	Primary model	References
Functional effects		
• Intestinal/colonic functions (e.g., fecal bulking, stool production)	Human	*Causey et al. 2000*
	Human	*Cummings et al. 2002*
• Resistance to intestinal infections	Human	*Gibson et al. 2005*
	Human	*Bosscher et al. 2006*
• Immunostimulation	Dog	*Field et al. 1999*
	Human	*Guigoz et al. 2002*
• Satiety and appetite	Human	*Cani et al. 2009*
	Rat	*Parnell et al. 2012*
• Influence on gastrointestinal peptides (e.g., glucagon-like peptide 1 (GLP-1) and ghrelin)	Human	*Cani et al. 2009*
	Rat	*Parnell et al. 2012*
• Influence on serum lipids and glucose	Human	*Delzenne et al. 2001*
	Rat	*Pereira et al. 2002*
• Bioavailability of minerals, especially Ca and Mg	Human	*Bosscher et al. 2003*
	Human	*Franck 2006*
Disease risk reduction		
• Infectious diarrhea	Human	*Chouraqui et al. 2008*
	Human	*Bosscher et al. 2006*
• Inflammatory bowel diseases	Human	*Furrie et al. 2005*
	Human	*Lindsay et al. 2006*
• Obesity	Rat/human	*Daubioul et al. 2000*
	Rat	*Cani et al. 2007*
	Rat/human	*Delzenne et al. 2010*
• Metabolic syndrome	Rat/human	*Delzenne et al. 2005*
	Rat	*Cani et al. 2007*
	Rat/human	*Delzenne et al. 2010*
• Osteoporosis	Rat	*Roberfroid et al. 2002*
	Human	*Abrams et al. 2005*
• Colon cancer	Rat	*Wollowski et al. 2001*
	Rat	*Le Leu et al. 2010*
	Rat	*Conlon et al. 2012*

Functional effects are physiological effects that can be measured relatively easily, including induction of satiety (Parnell & Reimer, 2012), reduction of caloric intake (Cani et al., 2009), and reduction of serum cholesterol, triglycerides, and glucose concentrations (Pereira & Gibson, 2002). Disease risk reduction, as implied by the name, is the compounding effect over time of one or more functional effects to reduce the risk/severity of chronic diseases. For example, prebiotic-induced satiety, reduced caloric intake, and improved serum lipid profile contribute to reducing the risk and severity of obesity and metabolic syndrome (Delzenne & Kok, 2001; Delzenne, Neyrinck, Backhed, & Cani, 2011; Delzenne, Neyrinck, & Cani, 2013).

2.4. Lentil

Lentil is highly nutritious food crop, often consumed either as a whole food or dehulled and split (ref). The proximate composition of lentil is as follows: moisture (c.a. 10 – 12%), carbohydrate (c.a. 60 – 65%), starch (c.a. 40 – 55%), protein (c.a. 20 – 30%), ash (c.a. 3%), and lipid (c.a. 1 – 3%) (Bhatty, 1988). The seed consists of three parts: the seed coat, cotyledons, and embryo which account for 8%, 90%, and 2% of the seed weight, respectively (Singh, Singh, & Sikka, 1968). Each of these components has a different chemical composition and nutritional quality. The seed coat is formed mostly of fibers – cellulose, hemicellulose, and lignin (Bhatty, 1988). Many of the seed's minerals and free and fiber-bound polyphenolics, flavonoids, and tannins are also present in the seed coat (Duenas, Sun, Hernandez, Estrella, & Spranger, 2003; Xu & Chang, 2010). The cotyledons are the main energy store of the seed, containing the starch fraction and about 90% of the total protein and lipids (Singh et al., 1968). Various sugar alcohols and mono-, di-, and oligosaccharides are also present in the cotyledons (c.a. 5 – 10% of dry matter) including glucose, sucrose, RFO (raffinose, stachyose, and verbascose), FOS (nystose), and various others in lesser concentrations (Tahir, Vandenberg, & Chibbar, 2011; Biesiekierski

et al., 2011; Bhatty, 1988). Minerals and water-soluble vitamins such as ascorbic acid are also concentrated in the cotyledons (Ekinci & Kadakal, 2005). Lipids and fat-soluble vitamins are contained in both the cotyledons and the embryo. The bulk of the lipids in lentil are triacylglycerides: three fatty acid residues bound with ester linkages to a glycerol backbone (Bhatty, 1988). The fatty acid profile is as follows: linoleic acid (37%), oleic acid (16%), palmitic acid (13%), linolenic acid (9%), and less than 1% of stearic, arachadonic, and eicosenoic acids (Salunkhe, Kadam, & Chavan, 1985).

The unique food matrix of lentil leads to a number of desirable nutritional responses (Jenkins et al., 1981; Abeysekara, Chilibeck, Vatanparast, & Zello, 2012). Lentil has a low glycemic index (Jenkins et al., 1981). In other words, after a lentil meal the concentration of glucose in the serum does not increase greatly or rapidly. Jenkins et al. (1980) found that the glycemic responses to pulses in general was about 45% lower than to cereal grains, biscuits, pasta, and tubers. Moreover, consumption of lentil induces a higher degree of satiety after a meal than most foods (McCrory, Hamaker, Lovejoy, & Eichelsdoerfer, 2010). Although it has been suggested that this is accomplished via modulation of gastrointestinal hormones such as cholecystokinin and also through short-chain fatty acid production in the large intestine, a direct causal relationship to satiety has not been firmly established (Sufian, Hira, Asano, & Hara, 2007). Abeysekara et al. (2012) found that lentil diet reduced serum cholesterol in elderly by about 8% compared with regular diet. Comparing the physiological effects of lentil consumption to several effects of prebiotics (**Table 2.4.)**, directly or indirectly, prebiotic components likely play a role in low glycemic, satiating, and cholesterol-reducing responses in lentil (Cani et al., 2009).

Compared with cereal grains, concentrations of vitamins, minerals, protein, and complex carbohydrates are comparatively greater in lentil **(Table 2.5.)**. Especially when consumed as a whole food, lentil is an excellent source of various nutrients; per 100 g, lentil contains ~31g dietary fiber, 8 mg iron, 5 mg zinc, 4 mg vitamin C, 479 µg folate, and 5 µg vitamin K. A ½-cup serving of cooked lentil can provide about one third of the recommended intake of dietary fiber (USDA, 2012). The large quantity of dietary fiber in lentil is a desirable trait for several reasons. First, dietary fiber is associated with reduced incidence of heart diseases and certain types of cancer (Fuchs et al., 1999; Pietinen et al., 1996), and second, some of the components of lentil dietary fiber are prebiotic carbohydrates (Brown, 2004; Martínez -Villaluenga, Frias, Vidal-Valverde, & Gomez, 2005).

Table 2.5. Nutrient concentration data in raw lentil and cereal grains.

Proximates		Value per 100.0g			
		Lentil	Wheat, hard red spring	Brown rice, long-grain	White rice, long-grain, unenriched
Protein	g	26	15	8	7
Total lipid (fat)	g	1	2	3	1
Carbohydrate, by difference	g	60	68	77	80
Fiber, total dietary	g	31	12	4	1
Calcium, Ca	mg	56	25	23	28
Iron, Fe	mg	8	4	2	1
Potassium, K	mg	955	340	223	115
Zinc, Zn	mg	5	3	2	1
Vitamin C	mg	4	0	0	0
Riboflavin	mg	0.2	0.1	0.1	0.1
Niacin	mg	3	6	5	2
Folate, DFE	µg	479	43	20	8
Vitamin A, RAE	µg	2	0	0	0
Vitamin K (phylloquinone)	µg	5	2	2	0

Data obtained from the USDA Nutrient Database (USDA, 2012).

2.5. Lentil prebiotics

A number of prebiotic carbohydrates are widespread in plant-derived foods in varied concentrations; vegetables, roots, tubers, and legumes in particular often contain high concentrations of the one or more prebiotics **(Table 2.6.)**. Concentration of prebiotics in foods ranges from trace amounts, as is the case in white rice, to relatively high amounts in other foods, such as Jerusalem artichoke (van Loo, Coussement, De Leenheer, Hoebregs, & Smits, 1995). In lentil, several groups of prebiotic carbohydrates have been indicated, including certain non-digestible oligosaccharides (FOS and RFO), RS, and sugar alcohols (Wang et al., 2009; Tahir et al., 2011; Bhatty, 1988). There are gaps in our knowledge of these important compounds, however. What is the profile of prebiotic carbohydrates in lentil? How much variation in their concentration exists between lentil genoypes? between growing environments? How much prebiotic carbohydrates are found in commercially available lentils? How does dehulling, cooking, and cooling affect those concentrations? These issues, when pertainent, will be presented for various carbohydrates in the following sections. Additionally, chemical structure of these compounds, as well as their concentration in foods and respective health consequences, will be discussed.

Table 2.6. Various prebiotics in common foods.

Food	Prebiotics[a]	g per 100 g Food			Reference
		Mean	Min	Max	
Lentil (boiled, drained)	RS	3.4	1.6	9.1	*Murphy et al. 2008**
	RFO	0.4	0.2	0.5	*Biesiekierski et al. 2011*
	FOS	0.2	0.1	0.2	*Biesiekierski et al. 2011*
	SA	TR			*Biesiekierski et al. 2011*
Common bean					
(pinto, boiled, drained)	RS	1.9	1.8	2	*Murphy et al. 2008*
(red kidney, boiled, drained)	RFO	1.4			*Biesiekierski et al. 2011*
(red kidney, boiled, drained)	FOS	0.5			*Biesiekierski et al. 2011*
Chickpea (cooked/canned)	RS	2.6	0.8	4.3	*Murphy et al. 2008*
	RFO	0.2			*Biesiekierski et al. 2011*
	FOS	0.2			*Biesiekierski et al. 2011*
White rice (long grain,	RS	1.2	0	3.7	*Murphy et al. 2008*
cooked)	RFO	ND			*Biesiekierski et al. 2011*
	FOS	ND			*Biesiekierski et al. 2011*
White bread	RS	1.2	0.1	4.4	*Murphy et al. 2008*
	RFO	0.2			*Biesiekierski et al. 2011*
	FOS	0.7			*Biesiekierski et al. 2011*
Potato					
(boiled)	RS	1.3	0.3	4.5	*Murphy et al. 2008*
(boiled, cooled 4ºC 24h)	RS	~3 × greater			*Englyst et al. 1987* *Muir et al. 1992*
Jerusalem artichoke	FOS	12.2			*Muir et al. 2007*

RS, resistant starch; RFO, raffinose-family oligosaccharides; FOS, fructooligosaccharides; and SA, sugar alcohols; TR, trace amounts detected only; ND, not detected. [a]Additional prebiotics may be present in selected foods. *Indicates individual reference is a comprehensive review.

2.5.1. Fructooligosaccharides

Often synonymously called oligofructose, FOS are by far the most famous family of prebiotic oligosaccharides. FOS consist of small chains of β (2→1) D-fructose residues of varying length with a terminal α (1→2)-linked D-glucose (Lewis, 1993). The DP of FOS is between 3 and 10 (Kolida & Gibson, 2008). Polymers of β-D-fructofuranosyl units – having a DP greater than 10 – are known as inulin (Roberfroid, 2007). The few investigations of FOS in legumes have focused on the shortest chain length compounds, kestose (β -D-fructofuranosyl-(2→1)- β -D-fructofuranosyl α-D-glucopyranoside) and nystose (β-D- fructofuranosyl -(2→1)-β-

D- fructofuranosyl -(2→1)-β-D- fructofuranosyl -(2→1)-α-D- glucopyranoside) (Biesiekierski et al., 2011).

The human small intestine lacks the necessary enzymes for degradation of FOS (Roberfroid, 1999). Case studies on patients with ileostomies have been the most important in confirming the non-digestibility of FOS (Kolida & Gibson, 2008). These non-digested compounds reach the large intestine intact, where they are fermented by the microbiota to produce SCFAs (Cummings, Macfarlane, & Englyst, 2001). The majority of this fermentation takes place in the cecum and ascending colon (Macfarlane, Gibson, & Cummings, 1992). The capacity of FOS to selectively stimulate microbial populations, especially bifidobacteria and lactobacilli, has been thoroughly demonstrated in recent years (Gibson & Roberfroid, 1995; Kruse, Kleessen, & Blaut, 1999). Supplementation of 15 g of FOS significantly increased counts of *Bifidobacterium* sp., while reducing counts of other prominent bacteria, including bacteroides, fusobacteria, and potentially pathogenic *Clostridium* sp. (Gibson & Roberfroid, 1995). The physiological and disease risk reduction effects of FOS have been widely examined, many of which are included in **Table 2.4.** In addition to maintenance of normal intestinal microbiota and prevention of pathogen colonization (Bouhnik et al., 1999), investigators demonstrated lower levels of circulating glucose and cholesterol in humans after ingestion of FOS (Pereira & Gibson, 2002).

Varying concentrations of FOS occur in over 36,000 plant species (Carpita, Kanabus, & Housley, 1989); high concentrations occur in chicory, Jerusalem artichoke, asparagus, garlic, and onion. Moderate concentrations of FOS have been observed in food legumes (Biesiekierski et al., 2011; Muir et al., 2009). In lentil, only small concentrations of FOS exist: ~100 – 200 mg/100 g food weight (Biesiekierski et al., 2011). However, it may be possible to enhance this

concentration, because FOS is already present in lentil seeds, suggesting that the genetic machinery leading to FOS accumulation, fructosyltransferase (Yun, 1996), is functional in lentil. To our knowledge, no studies have reported concentrations of FOS in lentil genotypes or taken into consideration changes in FOS concentration with growing environment.

2.5.2. Raffinose-family oligosaccharides

The most well-known and studied prebiotics, such as FOS and GOS, are oligosaccarides. Not surprisingly, other oligosaccharides have attracted the attention of researchers for their potential to promote health. Oligosaccarides that are common in legumes include the members of the raffinose family: raffinose, stachyose, and verbascose (Guillon & Champ, 2002). The basic structure of RFO contains a sucrose backbone and one or more α (1→6)-linked galactose residues with a DP of les than 10. This structure differs from trans-GOS in that trans-GOS have a lactose backbone instead of sucrose and β (1→4)-linked galactose residues instead of α (1→6)-linkages (Barreteau, Delattre, & Michaud, 2006). Raffinose, stachyose, and verbascose have chain lengths of 3, 4, and 5 saccharide residues, respectively (Guillon & Champ, 2002).

Due to a lack of α galactosidase activity in the small intestine, RFO are non-digestible (Smiricky et al., 2002). Once RFO reach the large intestine intact, they are fermented by the hindgut microbiota (Desjardins, Roy, & Goulet, 1990). Studies suggest that fermentation of RFO results in the selective increase of bifidobacteria in the large intestine, which is commonly associated with prebiotic compounds (Benno, 1987; Saito, Takano, & Rowland, 1992; Hayakawa et al., 1990). Supplementation of 15g/day raffinose to healthy subjects resulted in increased counts of bifidobacteria (Benno, 1987). Moreover, total bacterial counts remained stable, and *Bacteroides* spp. and *Clostridium* spp. were significantly lesser after raffinose administration than before (Benno, 1987). These observations were confirmed by a double-blind, placebo-

controlled study in which 2.5 to 10 g/day raffinose significantly stimulated bifidogenesis (Bouhnik et al., 2004).

Some of the first reports of RFO in lentil appeared in the late 1970s; total RFO concentrations ranged from 2.5 to 7.2% (Bhatty, 1988; Wang et al., 2009). The profile of individual RFO concentrations has also been reported in lentil [raffinose, 0.1 – 1.0 g ; stachyose, 1.1 – 4.0 g; and verbascose, not detectable – 6.4 g per 100 g dry matter] (Martinez-Villaluenga, Frias, & Vidal-Valverde, 2008). The majority of RFO are concentrated in the cotyledons of lentil; however, multiple investigations have observed significantly raffinose concentrations in the seed coat, but not stachyose or verbascose (Wang et al., 2009; Wang, Hatcher, & Gawalko, 2008). This factor results in raffinose concentration decrease with dehulling (Wang et al., 2009). Owing to the water soluble nature of RFO, boiling results in significant leaching into the cooking water. Discarding cooking water therefore results in significant decreases in RFO concentrations in food (Vidal-Valverde et al., 1994). Onigbinde & Akinyele (1983) observed another interesting effect of cooking – RFO in African legumes were partially hydrolyzed leading to lesser concentrations of higher degree of polymerization (DP) oligosaccharides and greater concentrations of short-chain oligosaccarides and sucrose. The authors attributed this to heat hydrolysis of the α (1→6)-linkages during cooking.

2.5.3. Sugar alcohols

Sugar alcohols are low-digestible, hydrogenated monosaccharides, otherwise known as polyols (Grabitske & Slavin, 2009). They are neither sugars nor alcohols and have a representatively lower energy contribution compared with carbohydrates: sorbitol, 2.6 kcal/g; mannitol, 1.6 kcal/g; and carbohydrates, 4.0 kcal/g (Wolever, Piekarz, Hollands, & Younker, 2002). Sugar alcohols are found naturally in berries, mushrooms, and many higher plants

(Makinen & Soderling, 1980), and are used extensively as artificial sweeteners for their low-calorie properties (Beards, Tuohy, & Gibson, 2010). In addition to the low glycemic index of sugar alcohols (Wolever et al., 2002), research suggests they may also have prebiotic action (de Vaux, Morrison, & Hutkins, 2002). In a mixed bacteria culture, addition of sorbitol to media resulted in the displacement of pathogenic bacteria, *Escherichia coli* O157:H7. Beards *et al.* (2010) assessed the prebiotic capacity of sugar alcohol and other confectionary sweeteners in a human trial and reported beneficial changes in the microflora, based on predominant prebiotic markers: bifidobacteria, lactobacilli, and SCFAs.

2.5.4. Resistant starch

Native starch is made up of two polysaccharides, amylose and amylopectin (Tester, Karkalas, & Qi, 2004). These polymers are acted upon within the upper gastrointestinal tract by a cohort of digestive enzymes (e.g., α-amylase, β-amylase, and amyloglucosidase) (Gray, 1992). Amylose is linear, consisting of α (1→4) linked glucose moieties, and is hydrolyzed by exo- and endo-enzymes (Tester et al., 2004). The average DP of amylose varies among food sources (Zobel, 1988). Amylopectin is highly branched and therefore additionally requires debranching enzymes such as amyloglucosidase for complete hydrolysis (Gray, 1992). The average molecular weight and DP of branches also varies among starch sources (Zobel, 1988).

Resistant starch, as its name suggests, is resistant to hydrolysis by human digestive enzymes. There are a myriad of factors that contribute to this non-digestibility (Hoover & Zhou, 2003). To name but a few, differences in *in vitro* starch digestibility have been attributed to the following: amylose/amylopectin ratio (Hoover & Sosulski, 1985), starch granule size (Snow & O'Dea, 1981), degree of starch crystallinity (Hoover & Sosulski, 1985), starch with B-type crystallinity (Englyst & Macfarlane, 1986), amylose-lipid complexes (Guraya, Kadan, &

Champagne, 1997; Nebesny, Rosicka, & Tkaczyk, 2002), enzyme inhibitors (Lajolo, Finardi Filho, & Menezes, 1991), protein and dietary fiber matrix (Dreher, Dreher, Berry, & Fleming, 1984), physical entrapment in cell structures (Wursch, Del Vedovo, & Koellreutter, 1986), and interactions of starch molecules (Dreher et al., 1984). The high resistance to hydrolysis of legume starch is a cumulative effect of high concentration of amylose, extensive physical entrapment by fibers and other food matrix factors, antinutrients, and stong interactions between amylose chains (Tovar, Francisco, Bjorek, & Asp, 1991; Hoover & Zhou, 2003; Deshpande & Cheryan, 1984).

There are five main types of RS which vary in structure and source (Bird, Conlon, Christophersen, & Topping, 2010). Current categorizations of RS are based on its source or derivation (Cummings, Beatty, Kingman, Bingham, & Englyst, 1996). RS1 refers to starch that is physically encapsulated in food, for example, in a fiber mesh or thick cell wall, and is therefore unavailable to enzymes. RS2 is naturally resistant starch due to crystallinity or tightly-packed and unhydrated nature. RS3 is derived from heating and cooling of gelatinized starch. RS4 has been modified chemically, which may include the formation of cross-linkages and esterification. RS5 is resistant to hydrolysis because of complexation with lipids (Bird et al., 2010).

By definition, RS is "the sum of starch and products of starch degradation not absorbed in the small intestine of healthy individuals" (Asp, 1992). Thus RS can come from any food containing starch, limiting the presence of RS to any starchy food, but its concentration varies greatly (Murphy, Douglass, & Birkett, 2008). The estimated consumption of RS in the United States is 4.9 grams per person per day, on average (Murphy et al., 2008). Even though relative

concentrations of RS are low, bread, pasta, and non-legume vegetables are the major contributors to RS consumption because they are widely eaten.

There has been enormous interest and research emphasis on RS in recent decades because of prebiotic responses and its putative therapeutic and preventative role in obesity and NCDs (Cummings et al., 2001; Johnston, Thomas, Bell, Frost, & Robertson, 2010; Conlon et al., 2012). Highlighted responses to administration of RS include reducing glycemic response, reducing caloric intake, improving bowel health (Brown, 2004), increasing absorption of micronutrients (Scholz-Ahrens et al., 2007), preventing colorectal cancer (Conlon et al., 2012), and improving insulin sensitivity (Johnston et al., 2010). These and other responses related to reducing risk factors of obesity and NCDs have marked it as a target for therapeutic and food applications (Brown, 2004).

Reported concentrations of RS in lentil range widely (**Table 2.6.**). This may be dependent upon lentil cultivars, growing location, and whether or not the material was processed or analyzed as eaten or freeze dried (Skrabanja, Liljeberg, Hedley, Kreft, & Björck, 1999; Wang et al., 2009). Literature reports of RS concentration in cooked lentils have ranged from 1.6 to 5.2% (dry weight) (Chung et al., 2008; Wang et al., 2009) and from 1.6 to 9.1% (food weight) (Murphy et al., 2008). Only limited data is available however on the effect of genotype of RS concentration in the lentil seed. In field pea RS concentrations were found to vary with genotype (Skrabanja et al., 1999). Processing also plays a large role in RS concentration in food. Mishra et al. (2008) nicely demonstrated that cooling of cooked potato increases the RS concentration by a factor of 2 or 3. Yadav et al. (2009) also reported increased RS concentrations in legumes with heating and cooling cycles. To date, demonstrations of changes in RS concentrations in commecially available lentils from the United States have not been reported.

2.6. Measurement of prebiotics

Identification and quantification of prebiotic carbohydrates requires different approaches. Oligosaccharides can be easily analyzed with simple instrument procedures. High performance anion-exchange chromatography with pulsed electrochemical detection (HPAEC-PED), high performance liquid chromatography- refractive index (HPLC-RI), capillary zone electrophoresis (CZE) gas chromatography with flame ionization detection (GC-FID), Matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) and nuclear magnetic resonance (NMR) spectroscopy are the most used instrumental procedures to analyze any non-polysaccharide carbohydrate including RFO, FOS, and sugar alcohols. Identification and quantification of RS, on the other hand, requires enzymatic and/or chemical/physical treatment prior to analysis of resulting carbohydrates. RS, by definition, is the starch fraction that escapes hydrolysis by human digestive enzymes, does require prior enzyme treatment.

The HPAEC-PED could be considered is the most widely used accurate method to quantify RFO. This is mainly due the excellent chromatographic resolution of target compounds from each other and from other comopunds. Furthermore, greater detection sensitivity provides advantages over other methods. The GC-FID and CZE also provide similar advantages as HPAEC-PED. Survey of literature indicates HPAEC-PED is widely used procedure due to greater analytical accuracy and versatility in oligosaccharide separation. The MALDI-TOF-MS and NMR techniques are also powerful analytical chemistry techniques to determine molecular masses and chemical structural details of the carbohydrates. However, these instruments are relatively expensive and requires highly skilled personal to operate and interpretate data.

Many carbohydrates are weak acids. At high pH, hydroxyl groups of carbohydrates are partially or totally transformed to oxyanions depending on the pKa values of those hydroxyl

groups. High pH resistant strong anion-exchange columns with sodium hydroxide and/or sodium acetate mobile phases provide selective elution of carbohydrates based on their number of hydroxyl groups, isomerism, and degree of polymerization (DP). The eluted carbohydrates are then detected by PED. Therefore, HPAEC-PED is a versatile technique to analyze large number of carbohydrates in a single run. The difficulties in analysis of prebiotic carbohydrates with high DP could be overcome by comparison to commerical standards. For those carbohydrate with no commerical standards, those ones could be isolated employing anion exchange chromatography, and then selective acid/enzyme hydrolysing to determine their monosaccharide compositions.

Regarding quantification of RS in foods, one of the greatest obstacles has been validation of data (Champ, Kozlowski, & Lecannu, 2001). This difficulty is largely attributed to the vast amount of factors that lead to resistance to starch hydrolysis mentioned in the previous section. Major advances in RS determination were made with the use of *in vivo* comparisons obtained from ileostomy patients (Muir & O'Dea, 1993). Researchers continued to improve the existing methods, even developing standard reference material of known RS concentrations (Megazyme, 2012).

There are many ways of preparing foods. For example, lentil may be sprouted, boiled, boiled and cooled, ground into a flour. Furthermore, analysis can be done with fresh samples, oven-dried samples, or freeze-dried samples. Changes in RS concentration may accompany any of these changes (Mishra, Monro, & Hedderley, 2008). Therefore, different RS values can be achieved for the same lentil genotype depending on the preparation. To assess RS in lentil, understanding of both native starch resistance and resistance after cooking or processing is informative, but the two may or may not be related, stressing the need for further development of the RS analyical procedures.

2.7. Future directions

Understanding the problem is hard, how much more so the solution? Einstein is quoted saying "We cannot solve our problems with the same thinking we used when we created them." The challenges we face in food security, nutrition, and obesity and its comorbidities are indeed difficult to understand and approach. Taken as a whole, the situation is overwhelming. However, focused efforts from many cooperative disciplines will yield results.

For the food scientist working with lentil, this requires answering several important questions. What is profile of these various prebiotic and low-digestible carbohydrates in lentil genotypes? Are variations in those traits heritable? How much prebiotics are contained in different lentil market classes? Additionally, how are dehulling, cooking, and refrigeration going to affect those concentrations? Furthermore, while it has been demonstrated that both lentil as a whole food and individual carbohydrates found in lentil contribute to reducing risk factors for obesity and NCDs, a causal link still remains to be established using animal and human trials.

In conclusion, although much remains to be elucidated and understood, lentil is a prime candidate as a dietary source of prebiotics and as a potential functional food. Lentil is a popular food in many countries, circumventing the problem of social or cultural rejection associated with many foods. Also, it can also be grown successfully in many regions of the world, so availability (at least under present circumstances) will not be an issue. Finally, prebiotic carbohydrates that are found in lentil have been repeatedly shown *in vitro* and *in vivo* to have beneficial health effects (Brown, 2004; Johnston et al., 2010; Conlon et al., 2012; Koo & Rao, 1991; Benno, 1987). With the necessary questions answered, lentil may be a useful tool in reducing obesity and NCDs (Hermsdorff, Zulet, Abete, & Martínez, 2011).

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3. OBJECTIVES AND HYPOTHESES

3.1. Paper 1

3.1.1. Objective

Compare the concentrations of prebiotic carbohydrates in different lentil genotypes and growing locations.

3.1.2. Hypotheses

H_1: Prebiotic carbohydrate concentrations [fructooligosaccharide (kestose and nystose), raffinose-family oligosaccharides (raffinose, stachyose, and verbascose) sugar alcohols (sorbitol and mannitol), and resistant starch] in commercial lentil will vary with genotype and growing environment.

H_0: Prebiotic carbohydrate concentrations in lentil will not vary with genotype or growing environment.

3.2. Paper 2

3.2.1. Objective

Compare the concentrations of prebiotic carbohydrates in lentil under various processing and preparation procedures.

3.2.2. Hypotheses

H_1: Prebiotic carbohydrate concentrations [fructooligosaccharide (kestose and nystose), raffinose-family oligosaccharides (raffinose, stachyose, and verbascose) sugar alcohols (sorbitoland mannitol), and resistant starch] in lentil change with dehulling, cooking, cooling, and reheating.

H_0: Prebiotic carbohydrate concentrations in lentil do not change with dehulling, cooking, cooling, or reheating.

4. PAPER 1. LENTIL (*LENS CULINARIS* L.): A PREBIOTIC-RICH WHOLE FOOD LEGUME

4.1. Abstract

Prebiotic carbohydrates are important components of healthy diets, supporting healthful hindgut microflora. Lentils grown in North Dakota, USA were evaluated for their prebiotic carbohydrates. Raffinose-family oligosaccharides (RFO), sugar alcohols, fructooligosaccharides (FOS), and resistant starch (RS) carbohydrates were analyzed in 10 commercial lentil varieties grown in Ward and McLean Counties in 2010 and 2011. Mean concentrations of RFO, sugar alcohols, FOS and RS were 4071 mg, 1423 mg, 62 mg, and 7.5 g 100 g^{-1} dry matter, respectively. Significant variations were observed in lentil prebiotic carbohydrate concentrations: RFO concentrations varied with variety, RS varied with location, and sorbitol and mannitol each varied with both variety and location. These results show that lentils contain nutritionally significant amounts of prebiotic carbohydrates and, that it may be possible to enhance those amounts through breeding and locational sourcing.

Key Words: prebiotic, lentil, raffinose, sorbitol, nystose, resistant starch

4.2. Introduction

Obesity and related non-communicable diseases are of global concern, affecting more than one in every ten adults (World Health Organization, 2012). The prevalence of obesity in the United States is estimated to be over 35% among adults (Flegal, Carroll, Kit, & Ogden, 2012). Chronic, non-communicable diseases associated with obesity, including diabetes, cardiovascular diseases, and some types of cancer, result in an estimated 36 million deaths globally each year, claiming more lives than all other causes of death combined (United Nations, 2012). Due to the dietary nature of these metabolic disorders, solutions will necessarily have a focus on diet.

Prebiotics may contribute to dietary strategies to reduce obesity (Cani et al., 2009; Parnell & Reimer, 2009). Roberfroid offered a revised definition of a prebiotic: "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). Such changes among microbial species colonies in the human gut can produce a wide range of positive effects, including increased satiety, regulation of the intestinal motility, production of short-chain fatty acids, prevention of diarrhea and constipation, and reduction of pathogen colonization (Caselato, Freitas, & Sgarbieri, 2011; Manning & Gibson, 2004; Scheppach, Luehrs, & Menzel, 2001). Moreover, consumption of prebiotics may stimulate the immune system (Lee & Mazmanian, 2010), promote mineral absorption, decrease risk of colon cancer (Burns & Rowland, 2000; Conlon et al., 2012; Rowland, 2009), and decrease risk factors associated with obesity and metabolic syndrome (Brugman et al., 2004; Caselato et al., 2011; Rabot et al., 2010). Prebiotics have been shown to reduce excess circulating glucose and cholesterol levels (Kaur & Gupta, 2002) and improve insulin sensitivity (Johnston, Thomas, Bell, Frost, & Robertson, 2010).

Naturally occurring prebiotic carbohydrates are in the larger category of dietary fiber, and, as defined by the Institute of Medicine, dietary fiber is nondigestible carbohydrate and lignin intrinsic to plants (Report of the Panel on Macronutrients Subcommittees on Upper Reference Levels of Nutrients and Interpretation and Uses of Dietary Reference Intakes and the Standing Committee on the Scientific Evaluation of Dietary Reference Intakes, 2005). The European Food Standard Agency set the Dietary Reference Value for dietary fiber at 25 g per day for adults 18 years of age or older to sustain normal bowel function but acknowledged that higher intakes could provide additional benefits (European Food Safety Authority, 2010).

However, a National Health and Nutrition Examination Survey (NHANES) found Americans 20 years of age and older consume only 61% of the indicated level (U.S. Department of Agriculture, 2010). While official recommendations have not been made regarding prebiotic consumption, several investigators have offered suggestions: 10 g per day of fructooligosaccharide (FOS) (Bouhnik et al., 1999) and 7 g per day of galactooligosaccharide (GOS) (Silk, Davis, Vulevic, Tzortzis, & Gibson, 2009). Resistant starch (RS) may elicit effects at low intake levels, but investigators have shown that consumption of up to 45 g per day is well-tolerated (van den Heuvel et al., 2004). Average consumption of prebiotics is estimated to be several grams per day (Moshfegh, Friday, Goldman, & Ahuja, 1999; van Loo, Coussement, De Leenheer, Hoebregs, & Smits, 1995), which is indicative of the low levels of prebiotic compounds in most commonly eaten foods in the Western diet.

An overlooked yet potential source of prebiotic carbohydrates is lentil (*Lens culinaris* L.), a widely grown grain legume and dietary staple in many Middle Eastern, European, South American, African and Asian countries. Lentils are known to contain GOS, which include raffinose-family oligosaccharides (RFO) (Bhatty, 1988). Prebiotic effects of GOS, primarily via bifidogenesis, include increased calcium absorption and pathogen reduction (Brouns & Vermeer, 2000; Scholtens et al., 2006). Resistant starch, which is well-documented in lentil (Chung et al., 2008; de Almeida Costa, da Silva Queiroz-Monici, Pissini Machado Reis, & de Oliveira, 2006; Wang, Hatcher, Toews, & Gawalko, 2009), improved insulin sensitivity in men with metabolic syndrome on a high RS diet (Johnston et al., 2010). Fructooligosaccharides, such as kestose and nystose, are well-known for their prebiotic action (Gibson & Roberfroid, 1995; Scholtens et al., 2006; van Loo et al., 1999). Sugar alcohols have been shown to displace pathogens from rumen and gastrointestinal tract and increase viability of strains of *Bifidobacteria* and *Lactobacilli* (de

Vaux, Morrison, & Hutkins, 2002; Yeo & Liong, 2010). Sorbitol, mannitol, kestose, and nystose were not detected in lentils grown in Australia (Biesiekierski et al., 2011), although sorbitol was reported in varying concentrations among germinated seeds of lentil varieties (Asghar, Stushnoff, & Johnson, 2000). Some prebiotic carbohydrates show significant variation among lentil varieties, suggesting potential for increasing their amounts through conventional plant breeding (Chung et al., 2008; de Almeida Costa et al., 2006; Tahir, Vandenberg, & Chibbar, 2011; Wang et al., 2009).

Though some research has been devoted to prebiotic compounds in lentil, focus has not been toward these compounds as prebiotics, and the scope of the previously analyzed carbohydrates has been narrow. To our knowledge, no study has extensively examined the prebiotic profile in lentil varieties in a replicated field study. The objectives of the present study were to (1) characterize the prebiotic carbohydrate profile [fructooligosaccharide (kestose and nystose), raffinose-family oligosaccharides (raffinose, stachyose, and verbascose), sugar alcohols (sorbitol and mannitol), total starch, and resistant starch] of US grown lentil varieties; and (2) determine the genetic and environment variation in lentil prebiotic carbohydrates.

4.3. Materials and methods

4.3.1. Materials

Standards, reagents, and high-purity solvents used for high-performance liquid chromatographic (HPLC) analyses and enzymatic assays were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA) and VWR International (Radnor, PA, USA) and were used without further purification. Regular maize starch (Megazyme International Ireland Ltd., Bray, Ireland) was used as an external reference sample. Water, distilled and deionized (ddH_20) to a resistance

of ≥ 18.2 MΩ (Milli-Q Water System, Millipore, Milford, MA), was used for sample extractions and preparation.

4.3.2. Lentil samples

Seeds from ten commercial lentil varieties **(Table 4.1.)** were obtained from a regional variety trial conducted in 2010 and 2011 by the Pulse Breeding Program at North Dakota State University, North Dakota, USA. Subsamples of seeds for HPLC analysis of soluble carbohydrates and determination of RS were randomly taken from entire harvested plot of each of three replicated randomized field plots at two locations, Ward (48.2325° N, 101.2958° W, 10.7 inches of rain fall and 17.2 ºC growing season temperature) and McLean (47.5774° N, 101.2360° W, 14.3 inches of rain fall and 17.2 ºC growing season temperature) Counties, for both years. Subsamples (10 – 20 g of seed; 7.3% moisture) were stored at -40°C until analysis. Samples were cleaned of debris and ground to pass through a sieve size of 0.25 mm using a top-loading UD grinder (Unholtz Dickie Corporation, USA).

4.3.3. Analysis of water soluble prebiotic carbohydrates

Water soluble prebiotic carbohydrates for each replicated lentil sample were extracted using a method described by Muir et al. (2009). Each ground sample (500 mg) was weighed into a 15 mL polystyrene conical tube. Samples were dissolved in 10 mL of ddH_2O and incubated in an 80°C water bath for 1 h, then centrifuged at 3,000 × g for 10 min using a Beckman GPR centrifuge (Fullerton, CA, USA). After centrifugation, a 1 mL aliquot of the supernatant was diluted with 10 mL of ddH_2O and passed through a 13 mm × 0.45 μm nylon syringe filter (Chromaotgraphic Specialties, Brockville, ON). Extraction and chemical analysis of oligosaccharides and sugar alcohols was performed on a Dionex system (ICS-5000 Dionex, Sunnyvale, CA, USA) using a method previously described by Feinberg et al. (2009).

Oligosaccharides were separated using a CarboPac PA1 column (250 × 4 mm; Dionex, Sunnyvale, CA, USA) in series with a CarboPac PA1 guard column (50 × 4 mm). The mobile phase flow rate was maintained at 1 mL/min. Solvents used for elution were 100 mM sodium hydroxide/ 600 mM sodium acetate (solvent A), 200 mM sodium hydroxide (solvent B), and 18 MΩ deionized water (solvent C). Solvents B and C at 50% each were used for an initial 2 min, followed by a linear gradient change from 2% A, 49% B, and 49% C at 2 min to 16% A, 42% B, and 42% C at 20 min. The final interval resumed initial conditions of 50% B and 50% C. Detection of oligosaccharides was carried out using a pulsed amperometric detector (PAD) with a working gold electrode with a silver-silver chloride electrode at 2.0 µA. Carbohydrate concentrations reported in the current study were identified based on the pure standards obtained from Sigma Aldrich Chemical Company. The concentrations of those analyzed carbohydrates were detected within a linear range of 3 – 100 µg/g. The minimal detectible limit was 0.2 µg/g. An external lab reference, CDC Redberry, was also used daily to ensure accuracy and reproducibility of detection. Oligosaccharide peak areas for the reference sample were routinely analyzed with an error of less than 5%. Standard solutions of prebiotic carbohydrates were prepared for peak identification and run daily to ensure detection sensitivity. Linear calibration models for oligosaccharide standards had an error of less than 4%. Concentrations of oligosaccharides in the filtrate (C) were calculated from the calibration model used to calculate concentrations in sample dry matter in the expression $X = (C \times V)/m$, where X is the concentration of oligosaccharide in the sample (corrected for moisture), V is the final diluted volume, and m is the mass of the dry sample aliquot.

4.3.4. Resistant starch analysis

Resistant starch analysis was performed by a method approved by AOAC International, previously described (McCleary & Monaghan, 2002; Megazyme, 2012). This involved incubating 50 mg ground lentil seed with 2 mL of a solution containing amyloglucosidase (3 U/mL) and α-amlyase (10 mg/mL) in 100 mM sodium maleate (pH 6.0) at 37°C for 16 h with constant circular shaking. Samples were then washed with 2 mL ethanol (≥ 95% pure), and again centrifuged at 3,000 × g for 13 min at room temperature (RT). Pellets were re-suspended with 4 mL of 50% ethanol (v:v), centrifuged, and decanted two additional times. Washings from the three centrifugations were pooled and brought to a volume of 50 mL with distilled water. Pellets containing the resistant starch fraction were dissolved with 1 mL of 2 M KOH with stirring at 4ºC for 20 min. After dissolution of the RS, 4 mL of 1.2 M sodium acetate buffer (pH 3.8) and 0.5 mL of amyloglucosidase (300 U/mL) were introduced into the tubes, which were incubated at 50°C for 30 min with intermittent stirring. Samples were then centrifuged (3,000 × g for 13 min at RT) and 100 μL aliquots (in duplicate) of both the supernatant containing the RS fractions and the diluted washings containing the soluble starch fractions were transferred to 15 mL polystyrene tubes. A reagent blank was prepared using 100 μL dilute sodium acetate buffer (pH 4.5). Glucose standards (1 mg/mL) were prepared and 100 μL aliquots (in triplicate) were transferred to tubes. A 3 mL aliquot of a reagent containing glucose oxidase (> 12,000 U/L), peroxidase (> 650 U/L), and 4-aminoantipyrine (0.4 mM) at a pH of 7.4 was transferred to each tube. Tubes were incubated in a water bath at 50 °C for 20 min. Absorption at 510 nm was measured using a Shimadzu UV 1800 Spectrophotometer (Shimadzu, Japan).

Starch fractions were calculated using

$$NRS = \frac{x(\Delta A_{sample})}{(\Delta A_{glucose})(W_{sample})}$$

$$RS = \times \frac{y(\Delta A_{sample})}{(\Delta A_{glucose})(W_{sample})}$$

$$TS = RS + NRS$$

where ΔA_{sample} and $\Delta A_{glucose}$ are the change in absorbance of sample and glucose, respectively as measured against reagent blank, W_{sample} is the weight of sample corrected for moisture, *x* is a factor to account for dilutions in determination of RS, *y* is a factor to account for dilutions in determination of non-resistant starch, and total starch (TS) is the sum of RS and non-resistant starch (NRS). Analysis of resistant starch by this method routinely achieves a standard error of ±5% for samples that contain > 2% resistant starch.

4.3.5. Statistical analysis

The experiment was a randomized complete block design with three replicates of ten commercial lentil varieties at two locations over two years (n = 120). Replicates, locations, and varieties were considered as random factors. Years, locations, varieties, and replicates were included as class variables. Data were analyzed in a combined model and separately by year and location. Analysis of variance was performed using the General Linear Model procedure (PROC GLM) of SAS version 9.2 (SAS Institute, 2009). Means were separated by Fisher's protected least significant difference (LSD) at $p < 0.05$.

4.4. Results

4.4.1. Thousand-seed weight

Table 4.1. provides thousand-seed weights of 10 lentil varieties and their respective market classes. Thousand-seed weights of varieties within the large green market class varied from 56 to 62 g per 1000-seed. Thousand-seed weights for varieties of the small red market class

ranged from 34 to 40 g per 1000- seed. The extra small red market class, CDC Rosetown, had a significantly lower thousand-seed weight (26 g per 1000-seed) compared to all other varieties. The medium green lentil, CDC Richlea, and the dark green speckled lentil, CDC Lemay, had thousand-seed weights of 43 and 30 g per 1000-seed, respectively. Combined statistical analysis reveals significant variance of seed thousand-seed weight by year, location, variety, replication, and the year × location interaction **(Table 4.2.)**. Significant replication effect was observed as a result of gradient of soil moisture or fertility or other unknown factors.

Table 4.1. Market class, major consuming countries, and thousand-seed weight of 10 lentil varieties grown in North Dakota, USA.

Market classes	Major consuming countries*	Variety	Thousand-seed weight (g) [a]
Extra small red	Bangladesh, Pakistan, Egypt	CDC Rosetown	26 *h*
Small red	southern Asia, the Middle East, northern Africa	CDC Red Rider	40 *e*
		CDC Redberry	38 *e*
		CDC Rouleau	34 *f*
Small green	Morocco, Greece, Italy, Egypt, Mexico	CDC Viceroy	29 *g*
Medium green	north-western Europe, Spain, Algeria, United States	CDC Richlea	43 *d*
Large green	north-western and southern Europe, Algeria,	Pennell	59 *b*
	South America, and Central America	Riveland	62 *a*
		CDC Greenland	56 *c*
Dark green speckled	France	CDC Lemay	30 *g*

*Data obtained from Thavarajah, Ruszkowski, & Vandenberg, 2008. [a] Means followed by the same letter within a column are not significantly different at $p < 0.05$. Standard error for thousand-seed weight is 0.2 g.

Table 4.2. Combined analysis of variance for seed weight (TSW), sorbitol (Sorb), mannitol (Mann), raffinose (Raff), stachyose (Stach), verbascose (Verb), nystose (Nys), resistant starch (RS), and total starch (TS) for 10 lentil varieties grown in North Dakota, USA in 2010 and 2011.

Source	Mean square[a]								
	Df[b]	TSW	Sorb	Mann	Raff + Stach	Verb	Nys	RS	TS
Year	1	513**	23919**	4023**	5349	40429	237	2	269**
Location	1	941**	92796**	7404**	2656	97652**	40	52*	47**
Variety	8	2027**	14284**	3566**	8834	73239**	142	12	5
Replication (year, location)	9	14*	1534	88	649	3056	7	2	16
Year × location	1	1021**	1441	1796**	60	75883**	136	192**	24
Year × variety	9	46	2573	342	7446**	17598**	150	5	15
Location × variety	9	27	1133	721	2112	4001*	101	11	2
Year × location × variety	9	22**	5429**	306	1215	1549	139	5	11
Error	72	7	1378	94	1122	2100	8	3	4

[a] Mean square was significantly different at $p < 0.05$ (**) and $p < 0.1$ (*). [b] Degrees of freedom based on three replicates.

4.4.2. Concentrations of water soluble prebiotic carbohydrates

Table 4.3. shows mean concentration values of prebiotic carbohydrates and TS. Sorbitol concentrations ranged from 1.0 to 1.3% (dry weight basis) in lentils. The highest sorbitol concentration was observed in the variety Riveland (1349 mg 100 g^{-1}) and the lowest in CDC Red Rider (1036 mg 100 g^{-1}), CDC Lemay (1039 mg 100 g^{-1}), and CDC Greenland (1109 mg 100 g^{-1}). Combined statistical analysis reveals significant variance in sorbitol concentrations by year, location, and variety **(Table 4.2.)**. Mannitol accounted for less than 0.3% of dry lentil weight. The highest concentrations of mannitol were observed in CDC Richlea (294 mg 100 g^{-1}) and Riveland (248 mg 100 g^{-1}) compared to all other tested varieties **(Table 4.3.)**. The lowest concentrations of mannitol were observed in CDC Rosetown (158 mg 100 g^{-1}), CDC Red Rider (160 mg 100 g^{-1}), CDC Lemay (163 mg 100 g^{-1}), and CDC Redberry (176 mg 100 g^{-1}). Mannitol concentrations showed significant variance by year, location, variety, and the year × location interaction **(Table 4.2.)**.

To minimize variation due to weather, agricultural practices, and soil, data were also statistically analyzed by location and year **(Table 4.4.)**. Mean values of carbohydrate concentrations were taken from all samples within a location and year. Mean concentrations of sorbitol and mannitol were higher in lentils grown in McLean County vs. Ward County for both years. Mean sorbitol and mannitol concentrations were significantly higher in 2010 (1267 and 217 mg 100 g^{-1}, respectively) than in 2011 (1172 and 188 mg 100 g^{-1}, respectively).

Verbascose concentrations exhibited substantial variation between varieties, doubling from lowest- to highest-concentration varieties **(Table 4.3.)**. Verbascose levels were highest in Pennell (1968 mg 100 g^{-1}) and lowest in CDC Rosetown (922 mg 100 g^{-1}) and CDC Rouleau (1082 mg 100 g^{-1}). Variance of verbascose concentration was observed by location, variety,

Table 4.3. Mean concentration of prebiotic carbohydrates of 10 lentil varieties grown in North Dakota, USA, in 2010 and 2011.

Variety	Concentration (mg 100 g^{-1})[a]				
	Sorb	Mann	Raff + Stach[*b]	Verb	Nys[*]
CDC Greenland	1109 *c*	211 *c*	2426	1770 *b*	57
CDC Lemay	1039 *c*	163 *d*	2497	1495 *d*	57
CDC Red Rider	1036 *c*	160 *d*	2419	1586 *cd*	52
CDC Redberry	1226 *b*	176 *d*	2349	1481 *d*	61
CDC Richlea	1295 *ab*	294 *a*	2319	1731 *bc*	62
CDC Rosetown	1325 *ab*	158 *d*	2586	922 *e*	62
CDC Rouleau	1304 *ab*	199 *c*	2793	1082 *e*	63
CDC Viceroy	1285 *ab*	215 *c*	2530	1800 *b*	79
Pennell	1231 *b*	204 *c*	2684	1968 *a*	57
Riveland	1349 *a*	249 *b*	2492	1784 *b*	68
Mean	1220	203	2509	1562	62
SE	11.6	2.2	17	18	0.6

*Mean concentration of varieties are not significantly different. [a] Means within a column followed by different letters are significantly different at $p < 0.05$. [b]Raffinose and stachyose are reported as total raffinose and stachyose concentration due to similar elution times for the separation method. SE, standard error of combined data (n = 120). Sorb, sorbitol; Mann, mannitol; Raff, raffinose; Stach, stachyose; Verb, verbascose; Nys, nystose.

Table 4.4. Mean concentrations of prebiotic carbohydrates and total starch by year and location.

Year	Location	mg 100 g^{-1} [a]					g 100 g^{-1}	
		Sorb	Mann	Raff + Stach	Verb	Nys	RS	TS
2010	McLean	1373 *x*	246 *x*	2566 *x*	1710 *x*	61 *x*	9.3 *x*	48. *x*
	Ward	1161 *y*	188 *y*	2524 *x*	1255 *y*	57 *x*	5.5 *y*	49 *x*
	Mean	1267	217	2545	1482	59	7.4	48
	Se	19	3.6	27	26	0.9	0.2	0.3
2011	McLean	1255 *x*	198 *x*	2503 *x*	1656 *x*	67 *x*	7.1 *y*	44 *y*
	Ward	1089 *y*	178 *y*	2444 *x*	1627 *y*	61 *y*	8.3 *x*	47 *x*
	Mean	1172	188	2474	1641	64	7.7	45
	SE	13.4	2.7	18	23.7	0.9	0.2	0.2

[a] Means within a column followed by different letters are significantly different at $p < 0.05$. Sorb, sorbitol; Mann, mannitol; Raff, raffinose; Stach, stachyose; Verb, verbascose; Nys, nystose; RS, resistant starch; TS, total starch. SE, standard error (n = 60).

year × location, year × variety, and variety × location **(Table 4.2.)**. Raffinose and stachyose, reported as a mean, combined total, only showed variance for the interaction between year and variety. Raffinose and stachyose concentrations ranged from 2319 to 2793 mg 100 g^{-1} **(Table 4.3.)**. Analysis of raffinose, stachyose, and nystose did not reveal variation by variety **(Table 4.2.)**.

Within years and locations **(Table 4.4.)**, concentration values of RFO tended to be higher in McLean County than in Ward County. Mean verbascose concentrations were 1710 and 1656 mg 100 g^{-1} in lentils from McLean County and 1255 and 1627 mg 100 g^{-1} from Ward County in 2010 and 2011, respectively. Raffinose and stachyose concentrations were slightly but not significantly higher in lentils from McLean vs. Ward County and in 2010 vs. 2011. Mean verbascose concentrations were significantly higher in 2011 than in 2010.

Nystose, the only observed member of the fructooligosaccharide family, showed no variation that reached statistical significance under the combined model. Nystose concentrations ranged from 52 to 79 mg 100 g^{-1} and variance was only observed for location from 2011 data, when the mean concentration from McLean County (67 mg 100 g^{-1}) was higher than that from Ward County (61 mg 100 g^{-1}). Nystose was slightly higher in lentils from 2011 than those from 2010, but values were not statistically significant. Kestose was not detected.

Mean concentration values of prebiotics for all 10 lentil varieties from both locations and years are derived from **Table 4.3.** data. Total sugar alcohol concentrations, as expressed by the sum of sorbitol and mannitol, accounted for approximately 1.4% of dry lentil flour weight. Total sugar alcohol concentrations varied from 1196 mg 100 g^{-1} in the CDC variety Red Rider to 1598 mg 100 g^{-1} in the Riveland variety. Total RFO accounted for 4%, on average, of dry lentil flour weight. Concentrations of total RFO ranged from 3508 mg 100 g^{-1} in CDC Rosetown to 4652 mg

100 g^{-1} in Pennell. Total FOS comprised approximately 0.06% of dry lentil flour weight, ranging from 52 mg 100 g^{-1} in CDC Red Rider to 79 mg 100 g^{-1} in CDC Viceroy.

4.4.3. Concentrations of resistant starch and total starch

Resistant and total starch concentrations of the 10 lentil varieties are shown in **Figure 4.1.** Mean concentrations of RS and TS for all samples were 7.5 and 47 g 100 g^{-1}, respectively. Resistant starch averages ranged from 6.0 g 100 g^{-1} in CDC Greenland to 8.9 g 100 g^{-1} in Pennell. Total starch ranged from 45 to 48 g 100 g^{-1}. Combined statistical analysis **(Table 4.2.)** showed variance for resistant starch by location and the year × location interaction and for total starch by year and by location.

Starch data were also analyzed by year and location **(Table 4.4.)**. Resistant starch concentrations were higher in McLean County (9.3 g 100 g^{-1}) compared to Ward County (5.5 g 100 g^{-1}) in 2010 but higher in Ward County (8.3 g 100 g^{-1}) compared to McLean County (7.1 g 100 g^{-1}) in 2011. Total starch was higher in Ward County (46.5 g 100 g^{-1}) than in McLean County (44.4 g 100 g^{-1}) in 2011 but mean values were not significantly different in 2010. Overall mean TS concentrations were significantly higher in 2010 (48 g 100 g^{-1}) than in 2011 (45 g 100 g^{-1}).

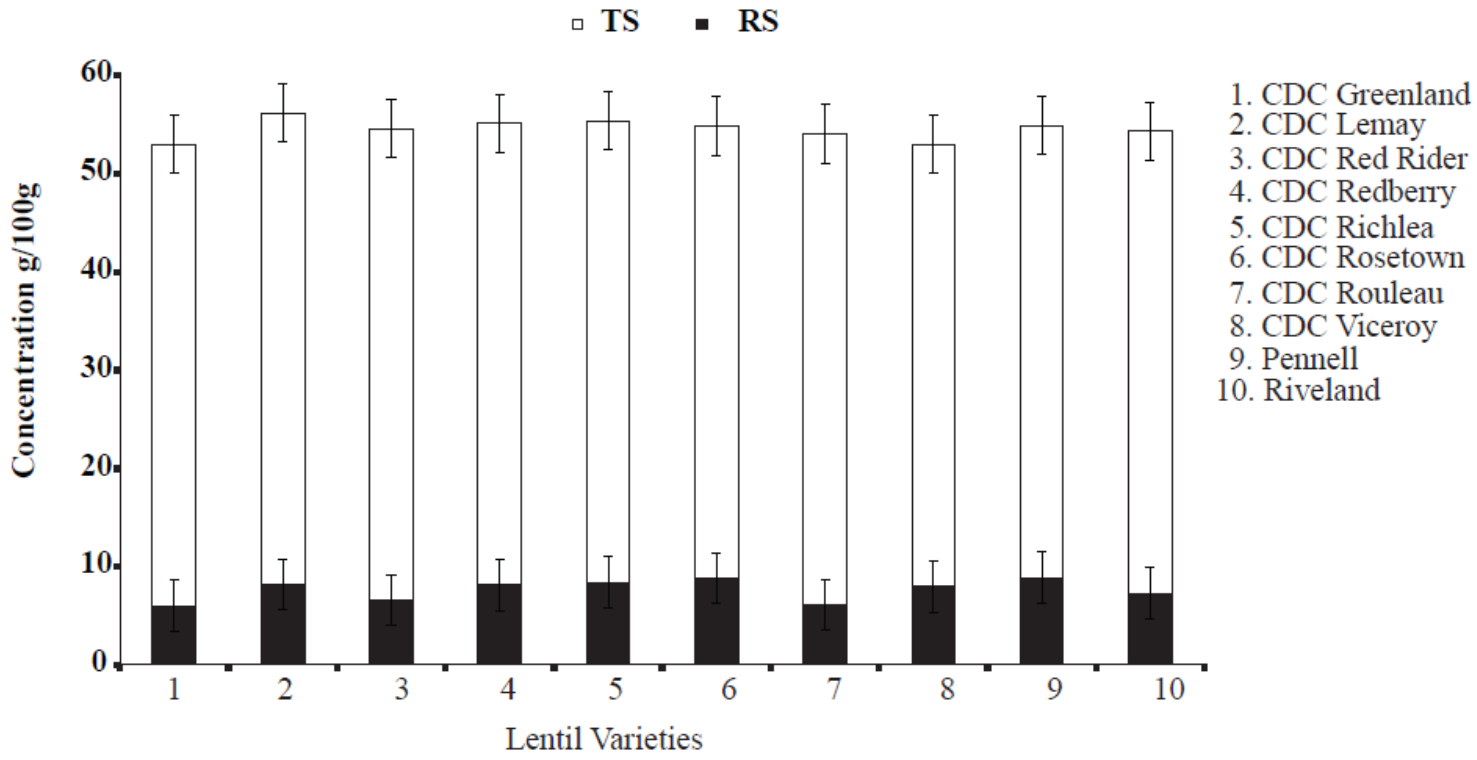

Figure 4.1. Mean total starch and resistant starch concentrations of 10 lentil genotypes grown in North Dakota, USA in 2010 and 2011.

4.5. Discussion

An understanding of prebiotic concentrations in lentils varieties could provide insight to allow for: A) selection of more nutritious lentil market classes; B) an opportunity to further improve overall lentil nutritional quality through breeding and food processing; and C) an understanding of environmental and genetic factors affecting prebiotic carbohydrates, allowing selection of optimal lentil growing locations for mass production. Variation of RFO (Tahir, Lindeboom, Baga, Vandenberg, & Chibbar, 2011; Tahir, Vandenberg, et al., 2011; Wang et al., 2009) and RS (Chung et al., 2008; de Almeida Costa et al., 2006; Wang et al., 2009) concentrations in several commercial lentil varieties have been reported, but these studies have not been designed to assess variation among varieties or environmental influences. Although sorbitol concentrations have been quantified in the shoots and basal leaves for several older lentil varieties not in production, mannitol concentrations were not examined (Asghar et al., 2000). To our knowledge, this is the first study to quantify RFO, RS, FOS, and sugar alcohols in lentils in a replicated field study.

Mean concentrations of various prebiotic compounds have been reported in lentil. Raffinose-family oligosaccharides were first reported in the late 1970s-early 1980s [raffinose, 0.39-1.0% (dry weight basis); stachyose, 1.47-3.1%; verbascose, 0.47-3.1%] (Bhatty, 1988). More recent reports include similar ranges [raffinose, 0.47-2.0%; stachyose, 1.7-2.9%; verbascose, 0.7-1.9%] (Tahir, Vandenberg, et al., 2011; Wang et al., 2009) and compare to values from this study (raffinose + stachyose combined, 2.5%; verbascose, 1.6%). Mean total RFO from our study was 4.1%, which falls in the lower half of the range from previous reports (2.5-7.2%) (Bhatty, 1988; Wang et al., 2009). Other studies analyzing RFO concentrations of CDC Richlea have reported values either similar to (Wang et al., 2009) or higher than (0.5 to

1.5% percent of seed weight, dry; (Tahir, Vandenberg, et al., 2011)) our findings; such differences within the same variety may be due to environmental effects or differences in analytical procedures.

Resistant starch concentrations in raw and cooked lentils have been reported to range from 1.6-5.2% of dry lentil seed weight (Chung et al., 2008; Wang et al., 2009) and 1.6 to 9.1 g 100 g^{-1} of cooked lentils (Yanetz et al., 2008). These values are substantially lower than the present findings for dry lentils **(Figure 4.1.)**. Current methods for quantification of resistant starch include *in vitro* assays performed with amyloglucosidase and α-amylase concentrations at the pH of the duodenum. Due to variability within the human digestive system, resistant starch is difficult to approximate. Concentrations of RS are also affected by cooking, processing, and cooling (Wang et al., 2009). Lentil is cooked before being consumed; making measurement of resistant starch in lentil flour nutritionally irrelevant, but analysis may be useful in comparison between lentil varieties for future breeding and selection.

Prebiotic concentrations in lentils appear to be related to genetic and environmental factors. Location significantly influenced concentrations of various prebiotics carbohydrates **(Tables 4.2. and 4.4.)**. In May of 2011, both Ward and McLean Counties were eligible for public assistance due to flood damage (Federal Emergency Management Agency, 2011). Soil data from Mandan, North Dakota, which lies in the same river basin where the field studies were located, indicates that percent soil moisture increased from 32% saturation (average of top 20 inches of soil) in 2010 to over 36% saturation in 2011 (National Resources Conservation Service, 2011). This was coincident with significant reductions in sorbitol, mannitol, and total starch concentrations in lentil grown in 2011 vs. 2010 **(Table 4.4.)**. Sorbitol and mannitol are humectants which can retain moisture, similar to corn starch that has a water binding capacity of

85 – 92% (Sandhu & Singh, 2006). Together, this information suggests that the lentil plants may decrease production of sugar alcohols and starch under stressful, high moisture conditions to avoid water saturation and decomposition of mature seeds, thus protecting seed viability for the following year.

Locational variance suggests that soil characteristics, moisture, and weather have a greater influence on resistant starch content than genetics. Conversely, the variety effect was significant with respect to concentrations of sorbitol, mannitol, and verbascose. While other studies have indicated significant variety effect on raffinose and stachyose concentrations (Tahir, Vandenberg, et al., 2011; Wang et al., 2009), our study did not reveal significant variation with variety, likely due to their concentrations being expressed as a combined total. Optimization of prebiotics in lentil varieties would necessarily have to consider both hereditary and environmental influences on prebiotic compounds.

Sugar alcohols, although influenced by the environment, also appear to be genetically-linked seed characteristics along with other prebiotics, including the RFOs **(Table 4.2.)**. Seed size, as measured by 1000-seed weight, was positively correlated to total water-soluble prebiotic carbohydrate concentration and inversely correlated to resistant starch (data not shown). Although seed size was positively correlated to the amount of soluble prebiotic carbohydrates, smaller seed sizes within market classes had higher concentrations of total soluble prebiotics than larger varieties. Seed size, therefore, is not a useful indicator of total prebiotic carbohydrate content. Total soluble prebiotic carbohydrates were 5753 mg 100 g^{-1} in green lentil market classes and 5260 mg 100 g^{-1} in red lentil market classes (data derived from **Table 4.3.**). Resistant starch was slightly higher in green lentils (7.8 g 100 g^{-1}) than in red lentils (7.4 g 100 g^{-1}) **(Figure 4.1)**. Relative concentrations of prebiotic carbohydrates may be more closely linked to

green- or red-cotyledon traits than seed size. All commercial lentil market classes were relatively high and uniform in total prebiotic carbohydrate concentrations. Total prebiotic concentrations in lentils ranged from 11.5 g 100 g^{-1} in CDC Rouleau to 15.0 g 100 g^{-1} in Pennell (data not shown). Concentrations of total prebiotic carbohydrates of these two varieties are consistent with their respective market classes, small red and large green, respectively **(Table 4.5.)**.

Our results indicate that lentil may be a good source of prebiotic carbohydrates. Total prebiotic carbohydrate concentrations suggest that a 100 g serving of lentils may provide over 13 g of prebiotics. In wheat (*Triticum spp.*) varieties, fructans range from 0.5 to 1.5% (Huynh et al., 2008) and RS from 1.5 to 2.5% (Bonafaccia et al., 2000). Based on this information, wheat varieties may contain from 2 to 4% prebiotic content as a raw grain. Average consumption of prebiotics is estimated to be several grams per day (Moshfegh et al., 1999; van Loo et al., 1995), which is indicative of the low levels of prebiotic compounds in most commonly eaten foods in the Western diet.

Future studies of the prebiotic carbohydrates in lentils are necessary to understand the physiological and environmental control of prebiotic carbohydrate expression. Of interest would be studies focusing on resistant starch concentrations in relation to soil and moisture characteristics. Moreover, processing, germination, and cooking are essential when evaluating lentil as a dietary source of prebiotics. RFO concentrations change with cooking (de Almeida Costa et al., 2006; Wang et al., 2009), with raffinose and stachyose decreasing and verbascose concentrations increasing; resistant starch may either increase or decrease after cooking. This opens up interesting lines of inquiry including how heating is related to saccharide degradation and synthesis, and if prebiotic efficacy of different fructan constituents varies. Lentils are also consumed as germinated seeds, which Vidal-Valverde & Frias (1992) reported to contain

Table 4.5. Concentrations of total prebiotic carbohydrates, galactooligosaccharides (GOS), and resistant starch (RS) in a 100 g serving of lentils by market class with dietician-recommended intake values.

Market class	Total prebiotic carbohydrate from 100 g serving (g)	Daily GOS intake from 100 g serving (g)	Daily RS intake from 100 g serving (g)
Extra small red	13.9	3.5	8.8
Small red	12.3	3.9	6.9
Small green	13.9	4.3	8.4
Medium green	14.1	4.1	8.0
Large green	13.3	4.4	7.4
Dark green speckled	13.5	4.0	8.2
Recommended prebiotic intake (g per day)	10 – 20 g per day[a]	2 – 7 g per day[b]	≤ 20 g per day[a]

[a] Recommendations for daily total prebiotic intake and resistant starch reported by Douglas & Sanders, 2008. [b] Recommendations for daily galactooligosaccharide intake derived from Carabin & Flamm, 1999; Silk et al., 2009.

reduced concentrations of RFO. Concentrations of other prebiotic compounds throughout germination have not been studied. Finally, prebiotic compounds may function differently depending on the associated food matrix, requiring bio-efficacy studies to determine actual microbiotal and physiological effects of these compounds when consumed as a constituent of lentil.

4.6. Conclusions

Prebiotic carbohydrates are important component of healthy diet, supporting beneficial hindgut microflora. Total prebiotic carbohydrate concentrations suggest that a 100 g serving of lentils may provide over 13 g of prebiotics. In conclusion, our study results clearly show that lentils contain nutritionally significant amounts of prebiotic carbohydrates and, that it may be possible to enhance those amounts through breeding and locational sourcing.

4.7. Acknowledgements

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5. PAPER 2. PROCESSING, COOKING, AND COOLING AFFECT PREBIOTIC CONCENTRATIONS IN LENTIL (*LENS CULINARIS* L.)

5.1. Abstract

Lentil is an important staple food crop in many regions world-wide and is a good source of protein (20 – 30%) and various micronutrients. Lentil contains raffinose-family oligosaccharides (RFO), resistant starch (RS), and other prebiotic compounds essential for maintenance of healthy gastrointestinal microflora. Previously, it was estimated that a one cup serving of row lentil could provide over 7.7 g of prebiotics. The objectives of this study were (1) to assess concentrations of RFO, and RS in two commercially-available lentil market classes, and (2) to determine concentration changes of RFO and RS associated with common processing procedures: dehulling, cooking, cooling, and reheating. Concentrations of RFO and RS were measured in raw, cooked, cooled, and reheated lentil from two different market classes, both with and without the intact hull. Modest RFO concentration reductions were observed with cooking, cooling, and reheating. Mean RS concentration in raw, cooked, cooled, and reheated lentil were 3.0, 3.0, 5.1, and 5.1 g/100 g (dry matter) respectively, clearly demonstrating cooling-induced synthesis of RS from gelatinized starch. These results highlight the importance of processing techniques on lentil nutritional quality for both consumer and food industry use.

Key Words: lentil, prebiotics, raffinose-family oligosaccharides, raffinose, stachyose, verbascose, resistant starch, processing

5.2. Introduction

Chronic non-communicable diseases such as type 2 diabetes and cardiovascular diseases have increased to exaggerated proportions (United Nations, 2012). Overweight and obesity, two major risk factors for non-communicable diseases, result in 2.8 million deaths each year

worldwide (WHO, 2013). In the United States, over 35% of adults suffer from obesity, and the rest of the world's populations are following a similar trend (Ogden, Carroll, Kit, & Flegal, 2012). Overwhelming evidence holds diet partially responsible for the upsurge in obesity and chronic disease prevalence (United Nations, 2012). In a large randomized controlled trial, subjects who were advised to eat more fruits, vegetables, nuts, and grain products had lower incidence of heart attack and death than the control cohort (Singh et al., 1992). Over the last half-century, traditional staple foods such as pulses, tubers, and vegetables have been displaced by refined foods with higher energy density and glycemic response (Kearney, 2010). The result is a large occurrence of micronutrient-poor, energy-dense diets that leads to adverse health consequences, including obesity, diabetes, and cardiovascular diseases, demand global attention (Lopez, Mathers, Ezzati, Jamison, & Murray, 2006).

To better understand the relationship between diet and disease, many researchers have focused on health-beneficial bioactive components present in commonly eaten foods. Prebiotics are an important group of food constituents with positive implications for human health, including reducing risk factors for non-communicable diseases via interactions with the hindgut microbiome (Gibson & Roberfroid, 1995). According to a revised definition by Roberfroid (2007), a prebiotic is 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". Except in the case of sugar alcohols, prebiotic carbohydrates fall under the category of dietary fiber (IOM, 2005). Malabsorption of these fibers in the upper digestive tract contributes to the low glycemic response characteristic of many prebiotic-rich foods (Jenkins et al., 1981). Commonly eaten foods that contain high concentrations of prebiotics include Jerusalem artichoke (*Helianthus tuberosus* L), chicory (*Cichorium intybus* L.), garlic

(*Allium sativum* L.*)*, onion (*Allium cepa* L.), and lentil (*Lens culinaris* L.) (van Loo, Coussement, De Leenheer, Hoebregs, & Smits, 1995; Johnson, Thavarajah, Combs Jr, & Thavarajah, 2013).

Lentil is a cool season food legume grown in many parts of the world with cooler or mediterranean weather conditions. It is an integral component of many food systems as a source of protein rich food and as a means of fixing atmospheric nitrogen to promote sustainable agriculture. Current world lentil production is approximately 4.6 million MT (FAOSTAT, 2012). Lentil is well-suited to growing conditions in western Canada (approximately 30% of world production) and is also an emerging specialty crop in both the Pacific Northwest and the Midwest of the United States. The composition of lentil grown in North Dakota is as follows: 8.3% moisture, 24.9% protein, 2.8% ash, and 51.9% starch (2012 Pulse Quality Survey). In addition, lentil is a good source of mineral micronutrients: a one cup serving of lentil can provide 4.3 – 5.3 mg iron, 1.9 – 3.3 mg zinc, and 25 – 401 µg selenium (Thavarajah, Ruszkowski, & Vandenberg, 2008; Thavarajah, Thavarajah, Sarker, & Vandenberg, 2009). Moreover, several prebiotic fibers are found in lentil including raffinose-family oligosaccharides (RFO) and resistant starch (RS) (Bhatty, 1988; Wang, Hatcher, Toews, & Gawalko, 2009). It was estimated that approximately 7.7 g of prebiotics are contained in one cup of lentil (Johnson et al., 2013); however, to our knowledge, there are no comprehensive reports of the concentration of prebiotics in cooked lentil.

Lentil induces a low-glycemic response (Jenkins et al., 1981), which has been attributed to the lentil starch's high resistance to hydrolysis. High concentrations of low-bioavailable and non-bioavailable RS in lentil relative to other crop starches is a function of many contributing factors: intact tissues and cells, high amylose concentration (20 – 40% of starch), high soluble fiber content, antinutrients, and strong interactions between amylose chains (Piecyk, Woéosiak,

Druynska, & Worobiej, 2012; Hoover & Vasanthan, 1994; Hoover & Sosulski, 1985; Tovar, Francisco, Bjorek, & Asp, 1991; Wursch, Del Vedovo, & Koellreutter, 1986; Siddhuraju & Becker, 2001). Concentrations of RS in raw and cooked lentils ranged from 1.6 to 8.4% (w/w) and from 1.6 to 9.1% (w/w), respectively (Murphy, Douglass, & Birkett, 2008; Johnson et al., 2013; Wang et al., 2009; de Almeida Costa, da Silva Queiroz-Monici, Pissini Machado Reis, & de Oliveira, 2006). In addition, concentrations of RS in other row food legumes are as follow: moth bean (*Vigna aconitifolia* Jacq. (Marechal; 1.2%), horse gram [*Macrotyloma uniflorum* Lam. (Verdc.), previously *Dolichos biflorus*; 2.6%] and black gram (*Vigna mungo* L.; 1.9%) (Bravo, Siddhuraju, & Saura-Calixto, 1998). Therefore, globally lentil is an import source of nutrients and contains significant concentrations of RFO and RS compared to other staple food crops (Johnson et al., 2013).

Raffinose family oligosaccharides have been considered antinutrients because of their involvement in gastrointestinal discomfort and flatulence (Fleming, 1981). Conventional plant breeding programs have aimed to reduce RFO concentration in seeds (Frias et al., 1999); however, current opinion of RFO in staple food crops has changed (Martinez-Villaluenga, Frias, Vidal-Valverde, & Gomez, 2005). Regular consumption of RFO may be an important dietary tool in prevention of chronic diseases (Parnell, Raman, Rioux, & Reimer, 2012; Cani et al., 2009) in addition to providing other health benefits: immunostimulation (Lee & Mazmanian, 2010), pathogen elimination (Caselato, Freitas, & Sgarbieri, 2011; Manning & Gibson, 2004), and stimulation of mineral uptake and deposition (Yeung, Glahn, Welch, & Miller, 2005; Coudray & Fairweather-Tait, 1998). A solid understanding of changes in the concentrations of RFO and RS during processing and cooking is vital prior to further nutritional experiments. Currently, there exists a knowledge gap in the differing concentrations of prebiotics in

commercially available, cooked, and processed lentil market classes. The objectives of this study were (1) to assess concentrations of RFO and RS in two commercially-available lentil market classes, and (2) to determine concentration changes of RFO and RS associated with common processing procedures: dehulling, cooking, cooling, and reheating.

5.3. Materials and methods

5.3.1. Materials

Raffinose, stachyose, and verbascose standards, high-purity solvents, reagents, and enzymes were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA), and VWR International (Radnor, PA, USA). Lentil cultivar, CDC Robin (Saskatoon, SK, Canada), and regular corn starch (Megazyme International Ireland Ltd., Wicklow, Ireland) were used as laboratory reference material to validate data. Water was distilled and deionized to a resistance of ≥ 18.2 MΩ (Milli-Q Water System, Millipore, Milford, MA) for sample extractions and preparation.

5.3.2. Lentil seed samples

Bulk processed lentil seed samples (2 kg) were collected from United Pulse Trading, Inc (Williston, ND, USA). Seeds of two commercially-available market classes were selected, small red and medium green, composed of cultivars CDC Redberry and CDC Richlea, respectively. Selected small red lentil samples were included (1) whole seed with the intact seed coat, and (2) split and decorticated. For medium green lentils, selected samples were (1) whole seed with the intact seed coat, and (2) decorticated only. These two lentil market classes were selected on the local and international consumer preference. Red lentils are generally marketed as split and decorticated for local and international markets. Bulk lentil samples were homogenized and subsampled (n = 6) and stored at -60 °C prior to further cooking. Additional subsamples (n = 6)

of each bulk sample were ground to a particle size of ≤ 1.0 mm and stored for a short period at -60 °C until analysis of RS. The treatment design was a completely randomized design with factorials including whole seed vs. dehulled; and cooking methods include raw, cooked, cooled, and reheated. Following cooking procedure of lentil was done without any prier thermic processing procedures.

5.3.3. Cooking procedure

Approximately 12 g of unground seeds were placed in distilled water at a ratio of 1:3 (*w/w*) in a 50 mL round-bottom test tube. Samples were suspended in a boiling water bath and cooked for 40 min. After cooking, samples were cooled to 4 °C and stored 24 hrs in a refrigerator. Cooled samples were then heated to boiling in a 1300 W microwave oven (Panasonic Electric, Washington, DC, USA) on high for 60 seconds. Cooked lentil samples were then freeze-dried in a VirTis Sentry freeze-dryer (SP Scientific, Gardiner, NY, USA) and hand-ground to a fine powder using a mortar and pestle to measure RFO concentration. Moisture content for each sample was determined gravimetrically (AACC, 2000).

5.3.4. Determination of RFO concentration

Freeze-dried samples (0.5 g) were incubated with ddH_2O for 1 hr at 80 °C to extract RFO, previously described (Muir et al., 2009). After centrifugation at 3,000 *g*, a 1.0 mL aliquot of the supernatant was passed through a 13 mm × 0.45 μm nylon syringe filter (Chromatographic Specialties, Brockville, ON, Canada). Oligosaccharide analysis was conducted using a previously published method (Feinberg, San-Redon, & Assie, 2009), modified for optimal peak separation. Chemical separation and analysis of RFO was performed on a Dionex system (ICS-5000 Dionex, Sunnyvale, CA, USA). Raffinose-family oligosaccharides were separated using a CarboPac PA1 column (250 × 4 mm; Dionex, Sunnyvale, CA, USA) in series with a CarboPac

PA1 guard column (50 × 4 mm). The mobile phase flow rate was maintained at 1 mL/min. Solvents used for elution were 100 mM sodium hydroxide/600 mM sodium acetate (solvent A), 200 mM sodium hydroxide (solvent B), and 18 MΩ deionized water (solvent C). Sample analysis began with a linear gradient change from 50% solvent B and 50% solvent C to 0.5% A, 49% B, and 49% C at a flow rate of 1 mL/min. At 3 min, a gradient change altered solvent concentrations from 0.5% A, 49% B, and 49% C to 3% A, 47% B, and 47% C at 16 min. A gradient change to 16% A, 42% B, and 42% C at 18 min followed. The final interval resumed initial conditions of 50% B and 50% C with a total run time of 20 min. Detection of RFO was carried out using a pulsed amperometric detector with a working gold electrode with a silver-silver chloride electrode at 2.0 μA. Concentrations of RFO were identified and quantified based on the pure standards (> 99%). RFO concentrations were detected within a linear range of 3 – 100 μg/g, with a minimum detection limit of 0.2 μg/g. An external lab reference, CDC Redberry, was also used daily to ensure accuracy and reproducibility of detection. Peak areas for the reference sample, glucose (100 ppm), and RFO (3.125 – 100 ppm) were routinely analyzed for method consistency and detector sensitivity with an error of less than 5%. Linear calibration models for RFO standards had an error of less than 2%. Filtrate RFO concentrations (C) were calculated from the calibration model using the expression $X = (C \times V) / m$, where X is the concentration of RFO in the sample, V is the final diluted volume, and m is the mass of the dry sample aliquot (moisture corrected).

5.3.5. Determination of RS concentration

The concentration of RS in lentil seeds at each stage of processing was determined using a published method, AACC Method 32-40.01 and Megazyme, 2012. Briefly, 200 – 400 mg of cooked lentil seed (~70% moisture), or 100 mg ground raw seed, was placed in a 16 mL round-

bottom test tube with a mixture of amyloglucosidase and α-amylase in sodium maleate buffer (pH 6.0). Tubes were incubated horizontally at 37 °C for 16 hrs with circular shaking (350 rpm). After centrifugation (Eppendorf, Hauppauge, NY, USA) (1,500 *g*), the supernatant was discarded. The pellet was dissolved using 2 M KOH in an ice/water bath for 20 min. Sodium acetate buffer (pH 3.8) and amyloglucosidase were added to the tubes and incubated for 30 min at 50 °C with intermittent vortex mixing (VWR International, Radnor, PA, USA). The suspension was subjected to centrifugation (1,500 *g*), and the supernatant was analyzed for glucose concentration colorimetrically using a UV spectrophotometer (Shimadzu Scientific Instruments, Inc., Columbia, MD, USA). Aliquots of 0.1 mL were transferred to tubes and incubated at 50 °C with 3 mL of glucose oxidase/peroxidase reagent. Absorbance was measured against a reagent blank at 510 nm. Data were validated using a standard reference material (Megazyme International Ireland Ltd., Wicklow, Ireland) (regular maize starch; [RS] = 1.0±0.1% (w/w). Batches were checked regularly to ensure an analytical error of less than 10%.

5.3.6. Scanning electron microscopy (SEM)

Raw, cooked, and cooled lentil samples were prepared for scanning electron microscopy as follows. A freshly boiled lentil was removed from water and immediately placed into an appropriately sized hole drilled into a brass sample-holder cryostub (JEOL USA, Peabody, Massachusetts, USA) with Teflon feet to isolate it thermally from its surroundings and allow it to warm at a slower rate. The lentil was secured in the hole using Tissue-Tek O. C. T. Compound (Sakura Finetek USA, Inc., Torrance, CA). The cryostub and attached lentil were submerged in liquid nitrogen. Once the lentil was completely frozen, the tissue that extended above the surface of the cryostub was fractured by striking it with the edge of a new razor blade cooled in liquid nitrogen; the excess fractured tissue was removed and discarded. The brass holder was inserted

promptly into a low vacuum scanning electron microscope (JEOL JSM-6490LV, JEOL USA, Peabody, MA, USA). The sample was allowed to stand in the SEM for 5 – 10 minutes so that surface moisture/frost could sublimate before the fractured surface was examined. All images were then acquired within a ten-minute window. Backscattered-electron images were taken in low-vacuum mode at a pressure of 30 Pa.

5.3.7. Statistical analysis

The experimental design was a completely randomized design with three replicates of four commercially available lentil products and four processing methods (n = 48). This experiment was repeated twice for data validation. Replicates, runs, and lentil products were considered as random factors. Runs, lentil products, processing methods, and replicates were included as class variables. Data were analyzed both in a combined model and separately by cooking and processing method. Analysis of variance was performed using the General Linear Model procedure (PROC GLM) of SAS version 9.2 (SAS Institute, 2009). Fisher's protected least significant difference (LSD) at $p < 0.05$ was used to separate means.

5.4. Results

5.4.1. Raffinose-family oligosaccharide concentration

Mean total RFO concentrations (sum of the total amount of raffinose, stachyose, and verbascose) ranged from 5.5 to 6.1% (w/w) in raw lentils **(Table 5.1.)**. Raffinose-family oligosaccharide concentrations in raw and processed lentils from different market classes are shown in **Table 5.1.** Total RFO concentration decreased significantly ($p < 0.05$) from raw to reheat seeds in two of the four lentil products, whole red and whole green. Dehulled lentil products tended toward reduced concentrations of RFO in reheated samples but differences were not significant **(Table 5.1.)**. Raffinose concentrations were approximately 0.4% (w/w) in raw

Table 5.1. Raffinose-family oligosaccharide concentrations (%) in raw and processed lentil

	Raffinose (%)			
	Dehulled red	Whole red [a]	Dehulled green	Whole green
Raw	0.4±<0.1*a*	0.4±0.1*a*	0.4±<0.1*a*	0.4 ±<0.1*a*
Cooked	0.4±<0.1*a*	0.4±<0.1*a*	0.4±<0.1*a*	0.4±<0.1*a*
Cooled	0.4±0.1*a*	0.3±<0.1*b*	0.5±<0.1*a*	0.3 ±<0.1*b*
Reheated	0.3±0.1*a*	0.3±<0.1*b*	0.4±<0.1*a*	0.3±<0.1*b*
SE[b]	0.01	0.01	0.01	0.01
	Stachyose (%)			
	Dehulled red	Whole red	Dehulled green	Whole green
Raw	3.1±0.3*a*	3.4±0.3*a*	3.4±0.4*a*	3.0±0.4*a*
Cooked	3.3±0.3*a*	3.4±0.4*a*	3.1±0.2*a*	2.9±0.2*ab*
Cooled	3.3±0.6*a*	2.9±0.3*a*	3.1±0.2*a*	2.6±0.3*bc*
Reheated	2.8±0.6*a*	2.8±0.1*a*	2.9±0.3*a*	2.4±0.3*c*
SE	0.1	0.1	0.1	0.1
	Verbascose (%)			
	Dehulled red	Whole red	Dehulled green	Whole green
Raw	2.1±0.4*a*	2.3±0.3*a*	1.9±0.4*a*	2.1±0.4*a*
Cooked	2.2±0.3*a*	2.2±0.4*a*	2.0±0.3*a*	1.9±0.3*ab*
Cooled	2.2±0.4*a*	1.9±0.2*a*	2.1±0.3*a*	1.8±0.4*ab*
Reheated	1.8±0.4*a*	1.8±0.2*a*	2.0±0.4*a*	1.7±0.3*b*
SE	0.1	0.1	0.1	0.1
	Total raffinose family sugars (%)[c]			
	Dehulled red	Whole red	Dehulled green	Whole green
Raw	5.5±1.1	6.1±1.2	5.7±1.2	5.5±1.1
Cooked	5.9±1.2	6.0±1.2	5.5±1.1	5.2±1.1
Cooled	5.9±1.2	5.2±1.0	5.7±1.1	4.6±0.9
Reheated	4.9±1.0	4.9±1.0	5.4±1.0	4.3±0.9

[a] Mean (±standard deviation) values within a column followed by a different letter are significantly different at $p < 0.05$ (n = 96). [b] SE, pooled standard error of mean (n = 96). [c] Percent total raffinose family sugars were calculated based on the sum of raffinose, stachyose, and verbascose.

lentil products, but significant reductions were observed in whole red and whole green lentil products after cooking, cooling, and reheating **(Table 5.1.)**. From raw to reheated, reductions in raffinose concentration in whole red and whole green were 0.1% (w/w), respectively. This trend was less pronounced in dehulled green lentil and was not observed in dehulled split red.

Stachyose concentrations in raw lentil ranged from 3.0% (w/w) in whole green to 3.4% (w/w) in

whole red and dehulled green. From raw to reheated, a significant reduction of stachyose concentration was observed in whole; other lentil products tended to decrease with processing as well. Across raw lentil products, verbascose concentrations ranged from 1.9 to 2.1% (w/w), and little change in these values was observed with processing. In general, concentrations of RFO were lower in cooked, cooled, and reheated lentil than raw in all lentil products.

5.4.2. Resistant starch concentration

Mean RS concentrations ranged from 3 to over 5% (w/w) in raw and processed lentil. Concentrations of RS in raw and cooked treatments were not significantly different. Both cooled and reheated lentil, however, contained significantly greater RS concentration compared with raw and cooked. Resistant starch concentration in lentil market classes (whole and dehulled) was not observed to change from cooled to reheated treatments.

Mean RS concentration in raw, cooked, cooled, and reheated lentils are shown in **Figure 5.1.** Mean RS concentrations in lentil seed products ranged from 3.7 to 4.8% (w/w). Dehulled green lentil had the greatest concentration of RS followed by whole green, dehulled split red, and whole red, respectively. Significantly greater RS was observed in green lentil (whole and dehulled) than red lentil and in dehulled (red and green) lentil than in whole lentil. Mean RS concentration in processing methods by lentil seed product is shown in **Figure 5.1.** Resistant starch concentrations in lentil seed products were not significantly different between raw and cooked. In all lentil products, RS concentration was significantly greater in cooled and reheated samples than in raw and cooked samples. Increases in RS concentration in lentil from cooked to cooled were as follows: dehulled green, 3. 4 – 5.7% (w/w); whole green, 3.4 – 5.4% (w/w);

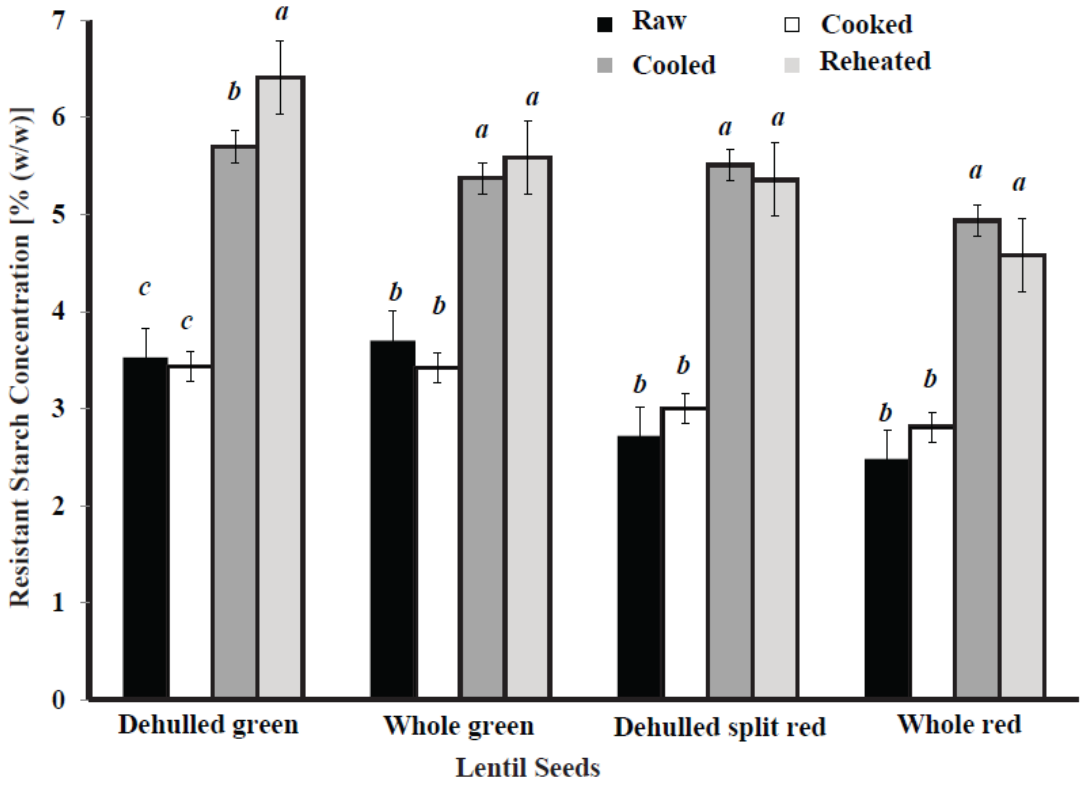

Figure 5.1. Mean resistant starch concentration in raw, cooked, cooled, and reheated lentil products by whole and split market classes. Error bars are based on $p < 0.05$ (n = 96).

dehulled split red, 3.0 – 5.5% (w/w); whole red, 2.8 – 4.9% (w/w), respectively. No significant changes in RS concentration were observed from cooled to reheated lentils except dehulled green lentil was significant **(Figure 5.1.)**.

5.4.3. Scanning electron microscopy

Scanning electron micrograph images of lentil starch granules after cooking and cooling are shown in **Figure 5.2.** These images revealed marked differences in the physical characteristics of red and green lentil starch granules under various processing methods **(Figure 5.2.)**. All micrographs were taken at the same original microscope magnification, and all remain at the same scale (note that the scale indicator provided for each is 10 µm). The oblong starch granules varied in length from approximately 10 to 20 µm and from 5 to 15 µm in width in green lentil

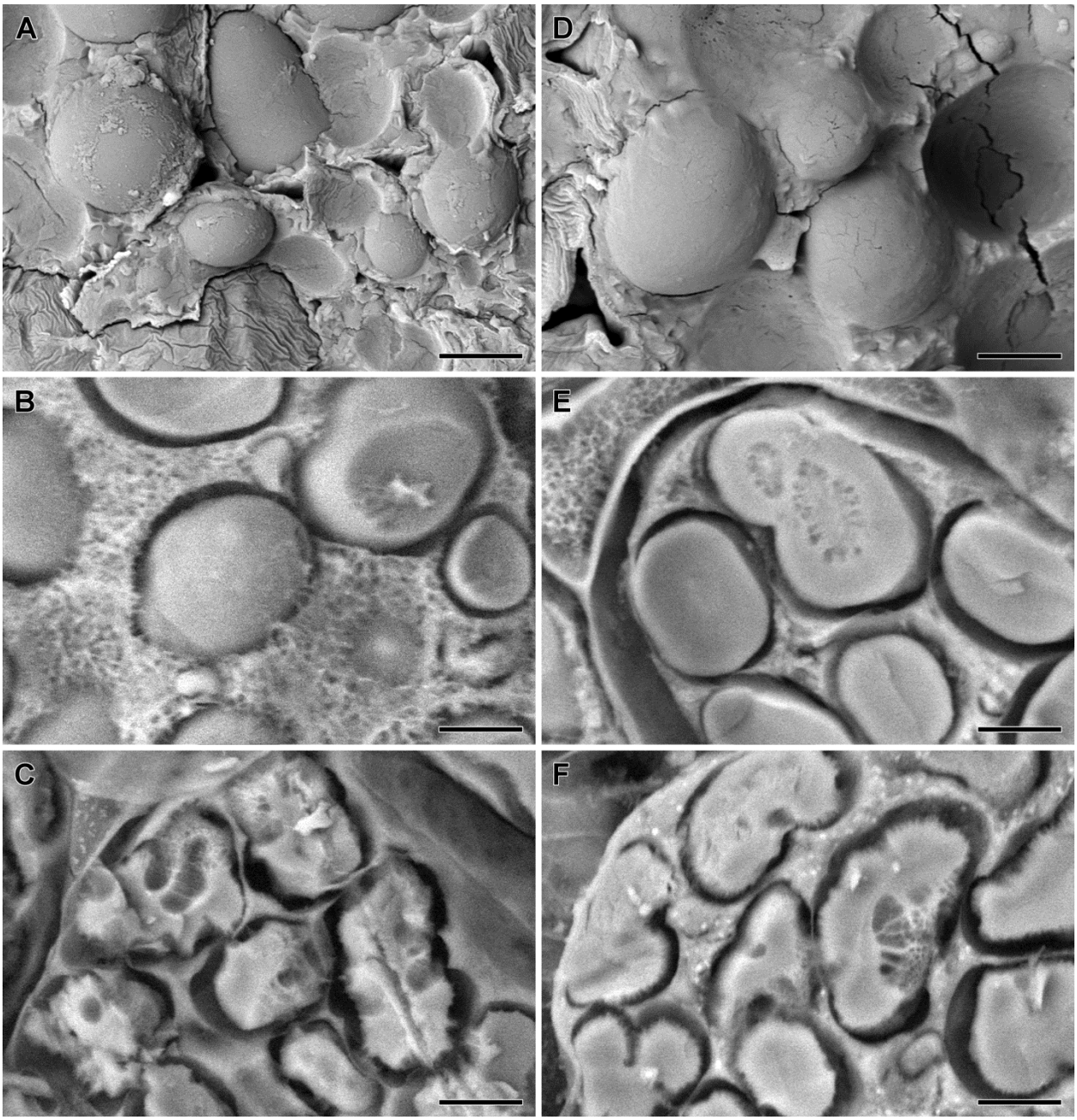

Figure 5.2. Scanning electron micrograph images of the same magnification (length of magnification bar = 10 µm) of lentil starch granules after freeze-fracture under the following treatment conditions: (A) native green lentil cotyledon, (B) cooked green lentil cotyledon, (C) cooked and cooled green lentil cotyledon, (D) native red lentil cotyledon, (E) cooked red lentil cotyledon, (F) cooked and cooled red lentil cotyledon.

cotyledon **(Figure 5.2.A)**: 10 to 25 µm in length and 10 to 20 µm in width red lentil cotyledon **(Figure 5.2.D)**. More starch granules per cell were seen in green lentil than in red. Swelling can readily be seen in the matrix surrounding the granules and, to a lesser extent, in the granules

themselves **(Figures 5.2.B and 5.2.E)**. Fracturation of starch granules, which occurred in cooked and cooled treatments, revealed internal structural changes. Deformation and the existence of cavities were seen to a higher degree in cooled lentil starch granules than in cooked, especially in green lentil.

5.5. Discussion

Prebiotic compounds including RFO and RS in lentil have been described by several researchers, but few have directed attention to the effects of processing and cooking on their concentrations. Processing and cooking procedures used in various studies have been quite different, leading to high variability of data gathered. Considering the many ways in which lentil is utilized domestically, it is important to understand the nutritional implications of all these procedural variations. The present study aimed to not only describe changes in RFO and RS in processing and cooking, but also to develop a general idea of prebiotics in commonly available lentil products.

Concentrations of total RFO in raw lentil products ranged from 5.5 to 6.1% (w/w) **(Table 5.1.)**, which are comparable to values reported for raw lentil seeds in the literature (Wang et al., 2009; Johnson et al., 2013). Dehulling resulted in no significant changes in total RFO concentration, while individual compounds raffinose, stachyose, and verbascose did change in concentration. Dehulling decreased raffinose concentration in red lentil from 0.4 to 0.3% (w/w); changes in green lentil were less pronounced **(Table 5.1.)**. This trend was also identified by Wang et al. (2009) where dehulling reduced raffinose concentrations from 4 to 15%. Dehulling increased stachyose and verbascose concentrations by 4 to 28% and 11 to 30%, respectively. Data from the present study do not adhere strictly to this pattern, suggesting sample variation from the processing facility. Notwithstanding, evidence suggests higher concentration of

raffinose are present in the seed coat of lentil than in the cotyledon, and the reverse trend was observed for stachyose and verbascose. However, further studies are required to test this hypothesis.

Oligosaccharides are soluble in water and are thereby lost when the water used for cooking is discarded (Wang et al., 2009; Ruperez, 1998). In the present study, the water was not discarded. This was to best simulate common uses of lentil in a soup or curry where the water would not be discarded. We observed reductions in RFO with further processing such as cooking, cooling, and reheating. This is consistent with studies that have reported decreases in RFO concentration in lentil over and above losses due to water. However, further experiments are required to understand the actual mechanism of reductions in RFO during lentil processing. Onigbinde and akinyele (1983) proposed that the observed reductions were a function of heat/ acid hydrolysis of higher order oligosaccharides to shorter chains and monosaccharaides. Another factor that could partially explain this phenomenon is intrinsic galactyltransferases which catalyze the transfer of D-galactose units between sucrose and RFO (Peterbauer & Richter, 2001). Bacterial α-galactosidases, which cleave the α-1, 6 linkages of RFO, may also play a role during processing steps that are not enzyme denaturing, e.g. cooling (Slominski, 1994).

The concentration of RS in foods is subjective to processing, cooking, and consumer handling. For example, Mishra et al. (2008) revealed that cooked potato RS increased with cooling by over 400% when stored at refrigeration temperatures for 2 days. Heating and cooling increased RS formation of autoclaved cereals, tubers, and legumes by 30 to 70%, and additional heating and cooling cycles further enhanced RS formation (Yadav, Sharma, & Yadav, 2009). Annealing was used by to increase lentil RS concentration from 6.5 to 9.5% (Vasanthan &

Bhatty, 1998). In the present study, significant changes in RS concentration were observed with processing, cooking and cooling, consistent with other investigations. Cooling of cooked lentil increased RS concentration nearly two-fold from 3.0% (w/w) in cooked lentil to 5.5% (w/w) after cooling **(Figure 5.1.)**. Starch molecules undergo modification with processing, including the formation of RS. Gelatinization of starch upon heating allows for rearrangement or realignment of amylose chains and, to a lesser extent, amylopectin side-chains (Hoover & Zhou, 2003).

Yadav et al. (2009) demonstrated a high correlation between amylose concentration and RS ($y = 0.443x - 5.993$, $r = 0.829$, $p \leq 0.05$, $n = 9$) in cooked/cooled legumes, cereals, and tubers. Other factors which may be contributing to the formation of lentil RS are starch granule size and porosity, physical entrapment by cells and tissues, starch crystallinity, presence of lipids, and enzyme inhibitors, among others (Bird et al., 2000). Quantification and/or assessment of these factors could prove useful in understanding the observed differences in RS concentration between red and green lentil. Dehulling increased RS concentration in both red and green products, which can be accounted for by removal of the starch-free seed coat (8% dry matter), thereby concentrating starch containing organs, i.e. cotyledons. Green lentil contained more RS than red, which may well be explained by differences in amylose concentration. Further increase of RS with reheating lends support to the hypothesis that green lentil contains a greater percentage of amylose than red. It is well understood that amylose can leach out of granules during heating with water and form strong amylase-resistant chain interactions (Haralampu, 2000). Physical evidence of this phenomenon appears to be seen in **Figure 5.2.C**, where disfiguration may have been caused by the loss of amylose from the granule. Starch granule size, as revealed by SEM images, may also be an important factor in RS formation. Scanning electron

micrograph images of field pea starch have revealed a greater resistance to α-amylase hydrolysis in small starch granules than large granules (Bertoft, Manelius, & Qin, 1993). This was attributed to a greater concentration of amylose in small granules. Green lentil, containing smaller granules, may have starch entrapment by cells, tissues, and fibers to a greater degree than red lentil.

The importance of understanding prebiotics in lentil, as well as other pulses, has not likely been fully realized. Yet, as health concerns continue to remain a large problem, demand for foods that promote health will escalate. Apparent dissatisfaction with RFO content in lentil and other pulses, while it may only indicate a transient low density of beneficial species in the gut microflora (Kruse et al., 1999), still needs to be addressed for consumers and marketers. Green lentil, popular in North America, has a lesser concentration of RFO and may be more appropriate for the North American consumers. In the case of all lentil products, refrigeration after a meal may further enhance dietary fiber and prebiotic content of the food. This phenomenon may have implications for "ready-to-eat" lentil products, having been cooked and subsequently cooled. Overall, lentil is a nutritious, high-protein, high-fiber food crop that has supplied nutrients to various populations for centuries.

In conclusion, processing changed overall prebiotic concentrations in lentil. Red lentil contained more RFO than green lentils. Cooking, cooling, and reheating were associated with loss of RFO, possibly because of heat or acid hydrolysis. Resistant starch was present in greater concentrations in green lentil products than in red, and it increased with cooling. Lentil is recommended as a dietary source of prebiotics.

5.6. Acknowledgements

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6. CONCLUSION AND FUTURE DIRECTIONS

The growing concern about obesity and its comorbidities in the world underscores the need for novel therapeutics, especially preventative measures. Lentil is an important component in diet and may help to reduce risk of obesity and chronic disease. Raw lentil genotypes contain nutritionally significant concentrations of prebiotic carbohydrates, and significant genetic and environmental variation in those concentrations allows the possibility for enhancement of those amounts through breeding and locational sourcing. Furthermore, various processing procedures increase concentrations of prebiotics in lentil. Dehulling resulted in an increase in RFO and RS due to greater concentrations in the cotyledons than in the hull. Concentrations of RS increased with cooling via a mechanism related to retrogradation and crystallization of gelatinized starch. Results of the present studies suggest that lentil is a good source of prebiotic carbohydrates **(Table 6.1.)**. A 1-cup serving of cooked lentil may provide 5.0 – 6.2 g of prebiotics, of which RFO, RS, sugar alcohols, and FOS account for 1.7 – 3.6 g, 1.7 – 2.8 g, 0.7 – 1.0 g, and 0.04 – 0.05 g per serving, respectively. Cooled lentil may provide approximately 6.9 – 7.0 g per 1-cup serving.

The exact nutritional significance of these concentrations is still poorly-understood; however, a large body of evidence supports the role of prebiotics (primarily via interactions with the gut microbiota) in reducing risk factors of obesity and metabolic syndrome – weight loss, satiety, serum lipids and glucose, etc. In the future, lentil diet may prove to offer similar benefits to consumers and be important in solutions for the obesity epidemic. Because these effects are mediated through the microbiota, it is important to note that with the high variability of microbiota composition the degree of benefits offered by prebiotics and lentil is also expected to be highly variable.

Table 6.1. Concentrations (g per serving) of raffinose-family oligosaccharides, resistant starch, sugar alcohols, and total apparent prebiotics[a] in a 1-cup serving of cooked lentils by market class.

Market class	RFO								RS							SA	Combined total	
	Raw			Cook.		Cool.	Dehull.		Raw		Cook.		Cool.	Dehull.		Raw	Cook.	Cool.
Reference	b	c	d	c	d	d	c	d	c	d	c	d	d	c	d	b	b,c,d	b,d
Small red	2.3	2.3	3.1	1.9	3.6	3.1	2.6	3.4	1.0	1.5	2.3	1.7	2.9	1.1	1.6	0.8	5.0 - 6.2	6.9
Small green	2.6	2.5		1.7			2.7		1.0		2.4			1.3		0.9		
Medium green	2.5	2.4	2.8	1.7	3.1	2.8	2.7	3.4	2.2	2.2	2.8	2.0	3.2	2.5	2.1	1.0	5.5 - 6.1	7.0
Large green	2.6	2.6		2.0			2.8		1.4		2.6			1.7		0.9		

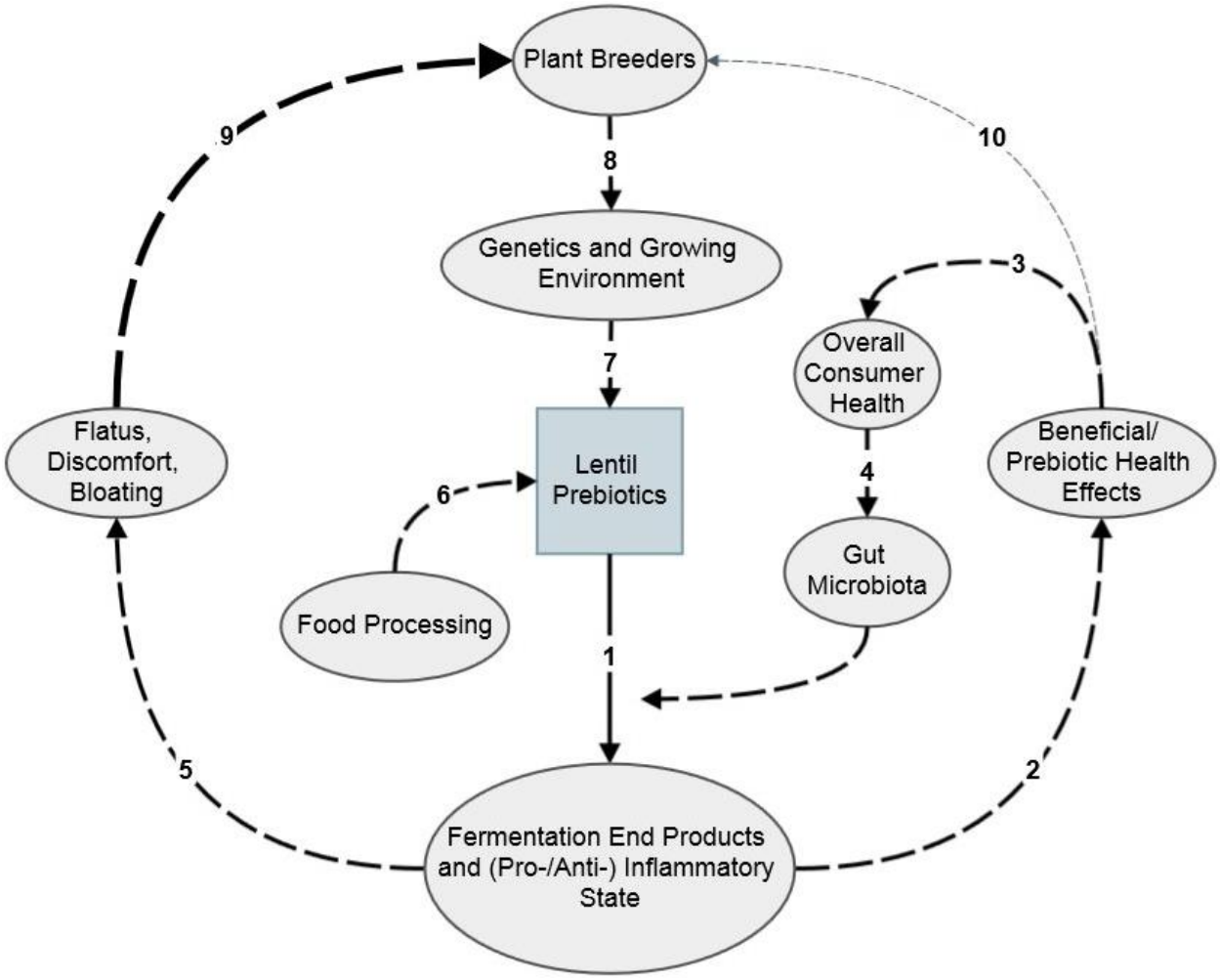
[a]Calculated sum of total RFO, RS, and SA. Conversions from g/100 g (dry matter) to a 1-cup serving (198 g cooked weight) are based on a moisture content of 69.6% in cooked lentil (USDA, 2012). [b]Based on data from Paper 1. [c]Based on data from Wang et al., 2009. [d]Based on data from Paper 2. RFO, raffinose-family oligosaccharides (raffinose, stachyose, and verbascose); RS, resistant starch; SA, sugar alcohols (sorbitol and mannitol); Cook., cooked; Cool., cooled; Dehull., dehulled.

Factors that are associated with lentil prebiotics – which will influence implementation of any changes to the status quo of consumption, agricultural policy or practice, or dietary recommendations of lentil – are summarized in a simplified schematic flowchart **(Scheme 6.1.)**. Fermentation of lentil prebiotics by the commensal gut microbiota leads to the production of various secondary metabolites (e.g., short-chain fatty acids, hydrogen gas, and methane gas) and stimulation of pro- or anti-inflammatory pathways **(Scheme 6.1. arrow 1)**. Prebiotic-induced alterations in the microbiota and their metabolites lead to health-beneficial effects such as satiety, improvement of serum lipid and glucose profile, and decrease in overall gastrointestinal inflammation **(Scheme 6.1. arrow 2)**. These prebiotic effects benefit the lentil consumer temporarily and contribute to improved health status and reduced risk of chronic disease over time **(Scheme 6.1. arrow 3)**. In a kind of feedback loop, the overall health status of the individual then affects the composition and function of the gut microbiota **(Scheme 6.1. arrow 4)**. The effects that are elicited by consuming prebiotics are dependent on the existing (and somewhat transient) composition of the gut microbiota. Depending upon the existing microbiota, consuming lentil prebiotics may result in increasing beneficial prebiotic effects, increasing flatus and bloating, or both **(Scheme 6.1. arrows 2 and 5)**. Adding to the complexity, processing procedures used to prepare lentils (both industrially and domestically) alter the concentrations of prebiotics in lentil, which may further modulate the effects of lentil consumption **(Scheme 6.1. arrow 6)**. For example, food industry may focus on removing certain prebiotic components from lentil products, while various home preparations (such as cooling after cooking) may act to increase their concentrations.

The concentration of prebiotics in lentil seeds is influenced by both genetics and growing environment conditions (i.e., soil, rainfall, temperature, location, etc.) **(Scheme 6.1. arrow 7)**.

Plant breeders can manipulate the genetics of lentil (intentionally or unintentionally) to either increase or decrease the concentration of various prebiotics in lentil seeds **(Scheme 6.1. arrow 8)**. Location sourcing of lentil may also be a viable means of changing the concentrations of prebiotics in lentil. Historically, flatus and bloating have heavily influenced plant breeders to decrease the concentration of prebiotics, especially raffinose-family oligosaccharides **(Scheme 6.1. arrow 9)**; however, as awareness increases of associated health benefits, plant breeders may be motivated to maintain or increase the concentration of prebiotics in lentil instead **(Scheme 6.1. arrow 10)**.

The interrelation of various factors on the final concentrations of prebiotics in lentil and the effects that will result demonstrates the need for further study and careful application to breeding efforts and the food industry of known principles. Significant reductions or eliminations of prebiotics through breeding may be especially detrimental and caution is advised. Although not discussed in the schematic, other factors are critically linked in the system as well including awareness of prebiotics, healthcare, economics, socioeconomics, producer preferences and demands, etc. Data from the present studies highlight the influence of genetics and growing environment on concentrations of prebiotics in raw lentil seeds. Preparation procedures (cooking, cooling, dehulling, etc.) were also shown to influence these concentrations. Lentil prebiotics modulate the gut microbiota which displays various physiological responses including beneficial health effects. These health effects contribute to the overall health status of an individual over time. Depending on the composition and function of the microbiota, lentil prebiotics can also lead to intestinal discomfort. More than any other factor, this consequence, although it is likely only transient in nature (Kruse et al., 1999), has influenced breeding efforts to reduce concentrations of prebiotics in lentil.



Scheme 6.1. Interactions of lentil prebiotics. The flowchart represents the current relationship between lentil prebiotics and various sectors, e.g., gut microbiota, consumer health, plant breeding, and food processing. Dotted arrowed lines represent a causal or influential relationship. The thickness of the arrowed line is indicative of its current impact on the circle or line it points to.

Several avenues of future lentil research will be important:

1. Modifications to the gut microbiota [microbial species density and diversity, type and concentration of microbial metabolites bathing the intestinal lumen, changes in physiological and pathophysiological markers (e.g., inflammation, serum lipid profile, etc.)] in response to lentil diet among diverse individuals and environments;

2. Genetic and phenotypic control/expression of various genes related to prebiotic carbohydrates in lentil genotypes, land races, and breeding lines under multiple environmental conditions; and
3. Interactions of prebiotic carbohydrates with agronomic performance (yield, drought tolerance, disease resistance, etc.).

In conclusion, prebiotics are an important component of healthy diet. Lentils contain nutritionally significant concentrations of several prebiotic carbohydrates. Genetic and environmental control of the expression of these carbohydrates allows for manipulation of their concentrations in the seed. Furthermore, lentil prebiotics likely play a role in the gastrointestinal and overall health of lentil consumers, warranting continued investigation.

6.1. References

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