ABSTRACT

XIE, XUEYING. Oat Avenanthramides Contribute to Attenuation of Intestinal Glucose Absorption. (Under the direction of Dr. Slavko Komarnytsky).

Oat (*Avena sativa* L.) is a popular cereal crop consumed in a form of breakfast porridge, granola, snack bars, and meal replacement formulations. Its favorable nutritional profile enriched with proteins, soluble fibers, vitamins, and phenolic metabolites is an attractive tool for dietary management of chronic and degenerative diseases associated with abnormal glucose metabolism. In this study we quantified free, bound, and *in vitro* digested avenanthramides in 109 oat cultivars grown in two different geographic locations as a part of the AFRI CORE oat worldwide diversity panel. Total free avenanthramide (AVN) content ranged from 3.3 to 227.3 μg/g in Lacombe, AB, and from 1.6 to 65.9 μg/g when grown in Aberdeen, ID. Avenanthramides AVNA (2p) , AVNB (2f), and AVNC (2c) were the most abundant in oat flour, however AVNC was absent from the respective *in vitro* digests. In the gastrointestinal STC-1 cell culture model, oat digests high in avenanthramides showed a prominent reduction of the gastrointestinal glucose uptake and a moderate significant correlation ($p=0.001$) to the total avenanthramide content. Individual avenanthramides reduced intestinal glucose uptake in the potency order of AVNE > AVNC > AVNB > AVNA in a dose range of 0.1-10 μM, and addition of a bitter taste receptor inhibitor p-(dipropylsulfamoyl) benzoic acid (probenecid) abolished these effects. Results suggest that oat avenanthramides reduce intestinal glucose absorption in part by interacting with the gastrointestinal bitter receptors.

Oat Avenanthramides Contribute to Attenuation of Intestinal Glucose Absorption

by
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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

ROLE OF BITTER PHYTOCHEMICALS IN MANAGEMENT OF HUMAN HEALTH

Abstract

Among five basic flavors from food which include sour, sweet, bitter, salty and umami, bitter tasting foods have a very high potential to benefit human health. Since bitter principles in foods are often aversive to the consumer, the food industry routinely removes them from diet through selective breeding and debittering. However, integration of bitter foods in everyday diet can dramatically improve the imbalance of five tastes and play an important role in management of human disease and health outcomes. Bitter taste has the lowest threshold, and small amounts of bitter substances can stimulate the taste buds and salivary glands, increase the production of saliva, stimulate secretion of the gastric juice, promote bile secretion and fat saponification, thus increasing appetite, promoting digestion, enhancing physique, and improving immunity. These effects are achieved by activation of bitter receptors (TAS2Rs) that are expressed not only in the taste buds of the tongue, but also in other tissues of the body and exhibit different biological functions.

Introduction

Phytochemicals are natural chemical compounds in plants. Plants produce them through primary or secondary metabolism, and they function to prevent mold and bacterial growth on plant tissues. Some researchers believe that these phytochemicals are not only good for the plants themselves, but also for human health.[1]

Phytochemicals have been used as medicines throughout human history. Hippocrates in ancient Greece used willow leaf to treat a fever. Salicin, originally extracted from white willow, had potent anti-inflammatory and analgesic effects, and was later chemically modified into the over-the-counter aspirin therapy. Another example of bioactive phytochemicals produced by plants, was a cancer drug paclitaxel, which was extracted from the Pacific yew.[2, 3]

Different phytochemicals may serve different functions not only in a variety of plants, but also have different modulatory or therapeutic effects in humans. Most of the basic types of macronutrients, micronutrients, and phytochemicals can be found in fruits and vegetables consumed as a part of the healthy diet. Antioxidant activity of bioactive molecules from plants is probably most widely used by the consumers to justify their plant-based diets. Certain antioxidants are believed to prevent premature cell death and prevent certain types of cancer and aging. For example, capsaicin, a plant chemical found in red peppers, is known to reduce the size of prostate tumors.[4] Consuming foods that contain capsaicin is therefore commonly regarded as a helpful dietary approach in preventing prostate cancer. Today, many nutritional supplements use these plants as ingredients that can be bought at the natural health food stores. While eating a plant-based diet has benefits in the long term, there is no direct evidence that taking phytochemical supplements has similar benefits.[5]

Most of the essential nutrients can be found in plants, and nutritional quality of plants directly influences human health. One such typical example can be plant protein. Comparing to animal protein, plant protein is easier for humans to digest, and can be successfully used to manage risk factors associated with many human metabolic disorders, such as diabetes. Legumes, a nutrient- and protein-dense food according to the 2010 Dietary Guidelines, can substantially improve the quality and health benefits of any diet when consumed in the amount of 1 to 3 cups

per week. Not only are legumes nutritious, they can also help meet nutrients of concern like fiber, calcium and potassium, which are often missing from a Western diet.[6] The key drivers for consuming different plant-based diets and nutritional interventions, however, are often determined by a combination of hunger, appetite, and taste stimuli. Taste preferences and food aversions develop through experiences, are influenced by attitudes, beliefs and expectations, and have direct effects on human health.

Bitter taste and bitter taste receptors

Taste refers to the sensation that food stimulates and produces in the oral cavity of the taste organ. Taste receptors in the mouth are mainly taste buds, followed by free nerve endings. Most of the taste buds are found in the emulsion bumps on the surface of the tongue, especially in the mucous folds of the tongue. Taste buds generally composed of 40-150 taste cells. There are many taste sensations molecules on the surface of taste buds, and different materials will present different taste with different taste sensations molecules. The tip of the tongue and the edge of the tongue are sensitive to the salty taste. The front of the tongue is sensitive to the sweet taste, while the tongue on both sides of the cheek is sensitive to the sour taste; the root of tongue is sensitive to the bitter and spicy flavor.[7]

A flavor substance stimulates the taste receptor in the mouth, passes through the nerve system and then transmits information to the brain's sensory system conduction taste center. At last, with the analysis of the brain's neural system, a sense of taste is produced. Different tastes have different taste receptors, and the interaction between taste receptors and taste substances is different. T2R membrane proteins, which are also called “TAS2R” proteins, act as bitter receptors. Over the past decade, great progress has been made in understanding the function of

bitter receptors and bitter taste. T2Rs are widely expressed in many parts of human body and they have been shown to be related to the physiology of the respiratory system, gastrointestinal tract and the endocrine system. Empirical evidence indicates that T2Rs are an integral part of the antimicrobial immune response in the upper respiratory tract infection.[8] It has been confirmed that the bitter receptors, located in the nasal respiratory epithelium and the cilia cells of lung epithelium, respond to harmful stimuli and affect respiration. Another site of the TAS2R gene expression is the gastrointestinal tract. Bitter compounds are suspected to possess biological activity by modulating TAS2R-dependent metabolism and digestion.[9]

Putative mechanisms of bitter taste

Brand[7] believed that taste receptors have specificity to the identification of bitter substances, which was related to the molecular arrangement or structure of lipid layer on the taste receptor (space specificity theory). However, Glenn[10] believed that it was related to the surface pressure of the lipid monolayer. When the bitter substance was in contact with a single layer, the surface pressure of the membrane was increased by the formation of the complex, which involved the sensation of bitter taste.

When Kubola[11] performed the study about the biguanide compound in grass, he found that recognition of bitter taste required the presence of AH and B sites. If the distance between the two proteins did not exceed 0.15 nm, hydrogen bonding was formed that defined the specific perception of the bitter taste (internal hydrogen bonding theory). Considering the hydrophobicity of hydrogen molecules, and the ability to form chemical bonds with transition metal chelates, these factors were conformed to the general theory of bitter molecular structure.

Other scholars have suggested that the chemical flavor of bitterness was similar to sweetness. This recognition pattern consisted of three sites: AH (electrophilic site), B (nucleophilic site), and X (hydrophobic site). If the flavoring substance interacted with A', B', and X' sites of the taste receptors, the sweet or bitter sensation was produced. Tamura[12] thought that the condition of bitter taste was that its electrophilic site AH interacted with the A' site, and the hydrophobic site X interacted with the X' location, while the position of B' site remained empty. Temussi[13] proposed that when the position of AH-B sites in the molecules of the flavor substance was opposite in the three-dimensional structure, this placement defined the difference between sweet and bitter tastes (three-point contact theory).

Belitz[14] used three-dimensional space coordinates to show the relative position of electrophilic base (p+), nucleophilic site (p-) and non-polar hydrophobic site (a), and pointed out what kind of material was sweet or bitter. He found that when (p+) site and (p-) site were aligned along the X axis and Y axis, respectively, and the hydrophobic site A was located on the X axis or Z axis, the compound was perceived as bitter. The lack of (p-) site could also produce bitter taste, without consideration of the location of the hydrophobic gene. As for a variety of bitter taste substances, the distances between (p+) and (p-) sites were required to stay in the range of 0.25-0.80 nm.

Distribution and expression patters of bitter receptors

TAS2Rs are expressed not only in the taste buds of the tongue, but also in other tissues of the body and exhibit different biological functions. Studies have shown that some TAS2Rs and homologous α-flavones are expressed in the airway smooth muscles of humans.[15]Shah et al. confirmed that TAS2Rs expressed in the motor cilia of the epithelial cells of the airways could

accelerate the movement of cilia, which might have served as a mechanical defense mechanism that protected the respiratory tract against accumulation of toxic gases.[16]Wu et al. determined the expression of TAS2Rs with known ligands in the gastrointestinal tissues and cells. The results showed that TAS2R108 and TAS2R138 were found in the antrum, fundus and duodenum as well as tongue, but not found in liver, heart and kidney. In addition, the expression of TAS2R134, which was unique in the rodents, was similar from the stomach and duodenum to the tongue. All this indicated that the coding gene of TAS2Rs was expressed throughout the gastrointestinal mucosa of mice.[17]Singh et al. used real-time quantitative PCR (RT-PCR) to detect transcriptional messages of TAS2R104, TAS2R107 and TAS2R138 in C6 rodent glial cells, brain stem, cerebellum, cortex and nucleus accumbens, while TAS2R104 mRNA was also detected in the primary neurons.[18]

The major sites for expression of the T2R genes in mammals are epiglottis taste buds, foliate, palate and taste receptor cells (TRCs) of circumvallate. Patterns of T2R expression often defined the biological effects of bitter taste perception. It appears that some T2Rs are co-expressed in the same TRC, and this co-expression is consistent with identifications of behaviors and generalization data in rats, which indicates a same taste perception for different bitter compounds. On the other hand, the separate expression of different T2Rs is also consistent with neurophysioligical data suggesting that different bitter compounds stimulate different TRCs and guastatory neurons in rats.[19]

Signal transduction mechanism of TSA2Rs activation

Since bitter substances have many chemical structures, the signal transduction pathway of bitterness should also have many different forms. Specificity and multitude of interactions of

various TAS2Rs with a particular bitter compound plays a crucial role in defining its overall bitterness. Activation of TAS2Rs promotes rapid changes in secondary messengers through a shunt signaling pathway containing G-protein-derived elements.[20] For example, a bitter compound such as cycloheximide that binds to TAS2Rs, activates the alpha-subunit of the G protein, which reduces intracellular cyclic adenosine monophosphate (cAMP) via phosphodiesterase activation. A decrease in the level of cAMP leads to the release of the inhibition of cAMP ion channels, the release of Ca^{2+} stored in the cells, the increase in the concentration of Ca^{2+}, and the depolarization of the cell membrane. Other bitter compounds that bind to TAS2Rs activate the β-γ subunits of G protein-coupled receptor, stimulate the synthesis of inositol 1,4,5-triphosphate (IP3) and promote the intracellular release of Ca^{2+} from its stores in the cell.[21]

Humans have different perception of bitter tastes, which are often defined by genetic components.[22] The human TAS2R genes have fairly diverse coding sequences, which suggests that the TAS2R polymorphisms may be responsible for the genetic component of individual differences in bitterness perception. One of the most studied TAS2R proteins with multiple SNP polymorphisms described is TAS2R38, which allows humans to taste PROP (6-n-propyl-2-thiouracil) and PTC (phenylthiocarbamide). Current research has focused on identifying similar taste phenotypes to determine new polymorphism in bitterness perception.[23]

It was previously reported that among the 25 human TAS2Rs (hTAS2Rs) expressed in HEK293T cells, the bitterness of green tea catechins, which are precursors of theaflavins, was perceived by hTAS2R39.[24] Black tea theaflavins are recognized by multiple bitter taste receptors: hTAS2R39 and hTAS2R14 were activated by theaflavin, both hTAS2R39 and hTAS2R14

reacted with theaflavin-3'-O-gallate, and hTAS2R39 was activated by theaflavins and theaflavins-3,3'-O-gallate, but not by theaflavin-3-O-gallate.[25]

Debittering during modern food processing

Since bitter principles in foods are often distasteful to the consumer, the food industry routinely removes them from the diet through selective breeding and debittering. It was reported that Hasegawa and his colleagues had isolated several bacteria and their metabolic enzymes from soil, which can degrade lemon-like bitter tasting substances into their non-bitter metabolites to achieve partial elimination of bitter flavors from foods, in this case limonin dehydrogenase.[26] Inducible enzymes from *Aspergillus niger* such as beta-rhamnosidase and beta-glucosinase decrease the concentration of bitter-tasting naringin glucosides in citrus juices. [27]

Inert adsorption materials can be also used to bind and eliminate bitter tasting substances from foods. The common adsorbents conclude acetate, polyamide, pyrrolidone resin, ion exchange resin S-861, ES-865, SYN42, SYN46, Amberlite XAD-7. It was reported that Amberlite XAD-7 treatment of grapefruit juice removed 63% of naringin, 85% of limonin, and 3% of titratable acids from the final product, while 2% cellulose acetate fibers applied to navel orange juice remove 6% of naringin and 76% of limonin.[28] While the adsorption materials and method are easy to regenerate and apply, their use often reduces the content of other desired nutrients and vitamins in the final preparations.

Inhibiting bitter taste sensation by complexing with bitter food components or binding and inhibiting bitter taste receptors is another viable strategy to reduce bitter taste in foods. Studies on nuclear magnetic resonance and the changes of solubility found that β-cyclodextrin formed inclusion complexes with bitter tasting hesperidin or naringin when added in the range of

0.3-0.5%, thus reducing the bitterness citrus juice. This method was safe and reliable, did not affect nutrition, and significantly improved flavor of the final products.[29]

Major classes of natural bitter compounds

Alkaloids are organic alkali materials containing nitrogen, usually in the form of pyridine, four hydrogenated pyrrole, quinoline, or soquinoline moieties. There are about 6,000 structures of known alkaloids, which can be divided into 59 categories, and almost all of them have bitter taste. In general, the more alkaline alkaloids are, the more bitterness they produce, and they remain bitter after forming the respective salts. Most of alkaloids have significant biological activity closely related with their optical rotation.[30] The bitter substances of tea are mainly alkaloids (such as caffeine, theobromine, theophylline).

Terpenoids are a variety of compounds, and they generally have bitter taste as a result of the structure of chelate moieties, such as lactone, acetal, internal hydrogen bond, and glycoside forms. For example, carrots grown in northern Scandinavia taste more sweet as a result of lower amounts of terpenoids that they produce in this geographical location, and not the increase in their sugar content. During storage, as natural sugar levels are reduced, a harsher more bitter taste slowly develops as carrot terpenoids become unmasked.[31]

Another example of naturally bitter substance is protein hydrolysate. The bitter taste of these mixtures is generally attributed hydrolyzed substances of low molecular weight polypeptides, which is mediated by the hydrophobicity of the amino acid side chains. The bitterness of protein hydrolysates is quantified according to their average hydrophobic value Q ($Q = \Sigma g/n$) as proposed by Nay, with $Q < 5.44$ KJ/mol having no bitterness and $Q > 5.8$ KJ/mol value as an indicator of a bitter taste.[32] Bitter peptides are produced during food processing. They

are present in the fermentation products or generated during aging of proteins and protease treatment of food. One of the earliest reports of bitterness in food processing described bitter and unpleasant taste of a hydrolysate made of gelatin and casein, and suggested a treatment with activated carbon as an approach to reduce bitter taste in the final product. These preliminary observations indicated that bitterness was caused by peptides rather than free amino acids, and the peptides could be adsorbed onto hydrophobic adsorbents to reduce bitter taste. [33] Bitterness of various protein isolates, including soybean and other plant-based proteins, causes a great concern due to the global demand for edible plant protein sources and the important role of plant protein and plant-derived peptides in the prevention of physiology, especially chronic diseases. [34]

Inorganic salt is another constituent of food, and many inorganic salts have bitter taste, which is related to the relative size of salt anions and cations. As the ion diameter increases, the bitterness of salt gradually increases, as it can be seen in a direct comparison of the iodide versus bromide bitterness.[35]

Polyphenols is a group of natural phenolic compounds in plants that are perceived from weekly to moderate bitter, or astringent. The astringency of tea is mainly attributed to tea polyphenols and other flavonoids present in the drink. Astringency is the feeling of dryness, roughness and wrinkles in the mouth. Traditionally, it has been induced by various phenolic compounds (such as tannins), metal cation salts (such as aluminum salts, also known as alum), dehydrating agents (such as ethanol), and organic acids (such as tartaric acid and malic acid). The American Society for Testing and Materials defines astringency as “the complex of sensations due to shrinking, drawing or puckering of the epithelium as a result of exposure to substances such as alums or tannins”. Astringency is generally thought to be triggered by compounds that bind proteins in saliva and oral epithelial cells, or interact directly with oral

tissue possibly through binding to the protein receptors on the surface of mucosal membranes. Due to the shrinkage of the tissue, the astringency can also be used for medicinal purposes outside of the food environment.[36]

Common bitter principles in foods

Fruits like honey pomelo, grapefruit, and orange belong to the citrus fruit family. Studies showed that bitter substances in citrus fruits are flavonoids, mainly include flavanones (naringin), flavones (nobiletin), and flavonols (quercetin).[37] Their relative degree of bitterness is defined both by the structural differences of aglycones and glycoside chains attached to them. For example, naringin (naringenin-7-O-neohesperidoside) is very bitter, while a closely related hesperidin (hesperitin-7-O-rutinoside) is tasteless.[38] Typical concentration of naringin in grapefruit juice is about 400 mg/L, but some early-season juices can have naringenin content of 1200 mg/L, which is less acceptable.[39] Another kind of bitter compounds in citrus fruits are limonoids, derivatives of a triterpenoid backbone, the well-known representatives of which are limonin and nomilin. These compounds are responsible for the delayed bitter taste (aftertaste) of citrus fruits. The odorless limonoid precursors are released when the fruit tissue is damaged and gradually transform into bitter-tasting triterpenoids.[40] Although these bitter substances affect the taste of the citrus products, they also have strong antioxidant and anti-tumor properties,[41] thus having a high applied value in manufacturing of health-promoting food products.

Hops is another example of an important bitter ingredient in beer. The main bitter substances in hops are humulones (alpha-acids) and lupulones (beta-acids). Among alpha-acids, the most common bitter substances are humulone, isohumulone, cohumulone and adhumulone, while beta-acids include lupulone, colupulone, and adluplone. Polyphenols also play a dual role

in beer quality. When the polyphenolic substances have a sufficiently high concentration, they impart bitterness and astringency of the beer, which helps improve flavor stability.[42] Bitterness and astringency of polyphenol substances in beers also varies with the degree of polymerization.[43] Adding hops polyphenol extract to beer can increase bitterness intensity, duration, and astringency compared to beer that contains only alpha-acid acids.[44]

Tea was consumed as a drink from 2000 years ago in China. Tea contains polyphenols, theanine, tea polysaccharides, minerals, vitamins, and other substances. Modern science has proved that the tea has a variety of biological effects on central nervous and cardiovascular systems, including diuresis, spasmolysis, antibacterial, anti-aging, anti-fatigue, antitumor, radiation resistance, resistance to tooth decay, heavy metal poisoning, metabolism, hairdressing, and enhancing memory properties. Zhaopeng Shi has shown that catechin esters from green tea serve as indicators of its bitterness.[45] Scharbert determined the threshold of main flavor substances in tea and showed that black tea astringency is related to its EGCG (epigallocatechin gallate) content, while the bitter taste was defined by a combination of caffeine, EGCG, and rutin tastes.[46]

Recent studies have shown that the bitterness of chocolate may also be due to catechins, which were present in a higher amount in bitter chocolate than milk chocolate.[47] Fermented cocoa contained epicatechin, polyphenols, and anthocyanins according to Arts et al,[48] and it was suggested that cocoa catechins contribute both to bitterness and a sweet aftertaste of chocolate.[49] Initial suggestions that the bitterness of chocolate was caused by caffeine, theobromine, or their interactions with diketopiperazines formed during roasting have not been supported by a more recent study.[50]

The main bitter substance in coffee, however, is the caffeine. Hofmann et al have also found that the chlorogenic acid in the coffee beans can be converted to chlorogenic acid lactone, which can be broken down into phenyldihydrides that are the main causes of the bitter taste of strong coffee.[51]

The phenolics in wine originate from grape skin (30%) and seeds (70%). Wine bitterness and astringency increase in a linear function with the concentration of catechins and tannins.[52] At higher molecular weights, catechin polymers become more astringent. Therefore, wine polyphenols with a molecular weight of > 500, such as grape seed tannins, are more savory than bitter.[53]

Genistin is a bitter insoflavone glucoside found in soybeans, and it was thought to be responsible for the bitter taste of soy protein and soy milk.[54] The concentration of these compounds increased during soaking of soybeans, and the bitter aftertaste of soy milk increased in a linear function with its genistein and daidzein contents.[55]

Functional properties of bitter compounds

Bitter foods are rich in nutrients. Bitter gourd, a common bitter food, contains a bitter-tasting mixture of two steroidal saponins, β-sitosteryl glucoside and 5,25-stigmasteryl glucoside (charantin) that, following an oral ingestion, showed hypoglycemic properties by increasing glucose uptake and glycogen synthesis in liver, muscle, and fat cells. Additionally, fresh bitter gourd is abundant in vitamin C, a powerful natural antioxidants that can help scavenge free radicals from human body.[56] Numerous studies have shown that different components of bitter gourd have anti-inflammatory properties. Peptides extracted from bitter gourd seeds helped to relieve inflammation by inhibiting pro-inflammatory cytokines.[57] Another animal study showed

that the administration of bitter melon polysaccharides reduced inflammation and oxidative stress in the stomach.[58]

Tartary buckwheat has anti-inflammatory and analgesic activity. Karkl et al[59] conducted an analgesic and anti-inflammatory study on Tartary buckwheat malt, using the classical hot plate method to investigate the analgesic effect of the botanical preparation, and examined its anti-inflammatory effect by xylene-induced mouse ear swelling model. The extract of Tartary buckwheat prolonged the latency of the hind paw of the mice, increased the pain threshold, and inhibited the swelling of the auricle caused by xylene. Bitter tasting protein fraction from Tartary buckwheat inhibited the formation of serotonin and reduced the inhibitory effect on the central nervous system. In the anti-fatigue and exercise-enhancing test, Tartary buckwheat protein significantly increased the weight-bearing swimming time, climbing time, and hepatic glycogen content in mice, effectively reducing the amount of serum urea and blood lactate.[60] The flavonoid compound of Tartary buckwheat consisted mainly of rutin, that promoted softening of the blood vessels, improved microcirculation, maintained capillary resistance, reduced permeability and fragility, promoted cell proliferation, and prevented blood cell agglutination. Magnesium found in Tartery buckwheat slowed heart rhythm and excitatory conduction and increased myocardial blood supply.[61]

Additionally, bitter tasting foods promoted the survival and reproduction of intestinal lactobacillus, inhibited the production of harmful bacteria, reduced the production of toxins, and maintained the balanced intestinal environment. Bitter substances also improved hematopoietic functions in intestinal and bone marrow tissues, and improved the state of anemia.[58]

Conclusions

Bitter compounds are widely found in food products, including tea, coffee, wine and chocolates, and have significant health promoting and nutritional values. Modern food processing removes the bitter taste from food products in order to improve flavor and palatability of foods and juices, but consumption of bitter foods can promote healthy and reduce or prevent many disorders and associated risk factors. Cereal grains have been the principal component of human diet, however they are processed to remove the bran and germ, primarily reduce the bitter taste and meet sensory expectations of consumers. Limited studies have demonstrated the presence of bitter phytochemicals in oat and their putative health promoting effects. Through selective introduction of bitter tasting phytochemicals back to the cereal diets, and investigating their corresponding biological activity, there is an increased opportunity to develop novel dietary interventions that promote health outcomes by activating bitter receptor signaling in the body.

REFERENCES

1. Molyneux RJ, Lee ST, Gardner DR, Panter KE, James LF (2007) Phytochemicals: the good, the bad and the ugly? Phytochemistry 68(22-24): 2973-2985
2. W. Sneader (2000) The discovery of aspirin: A Reappraisal BMJ 321(7276): 1591–1594
3. Elizabeth Landau (2010) From a Tree, a 'miracle' Called Aspirin, CNN, http://www.cnn.com/2010/HEALTH/12/22/aspirin.history/index.html.
4. Sophie Malagarie-Cazenave, Olea-Herrero N, Vara D, Diaz-Laviada I (2009) Capsaicin, a component of red peppers, induces expression of androgen receptor via PI3K and MAPK pathways in prostate LNCaP cells, FEBS Letters 583: 141–147
5. Linus Pauling Institute (2014) Phytochemicals,https://lpi.oregonstate.edu/mic/dietary-factors/phytochemicals
6. UnitedHealthcare (2018) Nutrition: The Power of Plant Protein, https://www.uhc.com/health-and-wellness/nutrition/power-of-plant-protein.
7. Elliot Adler, Hoon MA, Mueller KL, Chandrashekar J, Ryba NJ, Zuker CS (2000) "A novel family of mammalian taste receptors, Cell 100(6): 693–702
8. Feroz Ahmed Shaik (2016) Bitter taste receptors: extraoral roles in pathophysiology, The International Journal of Biochemistry & Cell Biology 77: 197–204
9. Wolfgang Meyerhof, U. Beisiegel, and Hans-Georg Joost (2010) Results and problems in cell differentiation, sensory and metabolic control of energy balance, 52
10. Glenn M. Roy (1997) Modifying bitterness: mechanism, ingredients, and applications
11. Kubota T. Kubo I. (1969) Bitterness and chemical structure. Nature23: 97-99.

12. Masahiro Tamura, Takafumi Miyoshi, Naoko Mori, Keisuke Kinomura (1990) Mechanism for the bitter tasting potency of Peptides using O-Aminoacyl Sugars as Model Compounds+, Agricultural Biol. Chemistry 54(6): 1401-1409
13. Piero A. Temussi, Francesco Lelj, and Teodorico Tancredi (1978) Three-Dimensional mapping of the sweet taste receptor site, Journal of Medicinal Chemistry 21, (11): 1154–58
14. Hans‐Dieter Belitz, Herbert Wieser (1985) Bitter compounds: occurrence and structure-activity relationships, Food Reviews International 1(2): 271–354
15. Richard Einstein, Heather Jordan, Weiyin Zhou, Michael Brenner Ester G Moses (2008) Alternative splicing of the G protein-coupled receptor superfamily in human airway smooth muscle diversifies the complement of receptors, Proceedings of the National Academy of Sciences 105(13): 5230–35
16. Alok S. Shah, Ben-Shahar Y, Moninger TO, Kline JN, Welsh MJ (2009) Motile cilia of human airway epithelia are chemosensory, Science 325(5944): 1131–34
17. S. Vincent Wu, Monica C. Chen, and Enrique Rozengurt (2005) Genomic organization, expression, and function of bitter taste receptors (T2R) in mouse and rat, Physiological Genomics 22(2): 139–49
18. Nisha Singh, Vrontakis M, Parkinson F, Chelikani P (2011) Functional bitter taste receptors are expressed in brain cells, biochemical and biophysical research communications 406(1): 146–51
19. Alexander A. Bachmanov, Gary K. Beauchamp, (2007) Taste receptor genes, annual review of nutrition 27 (2007): 389–414

20. Enrique Rozengurt (2006) Taste receptors in the gastrointestinal tract. I. bitter taste receptors and -gustducin in the mammalian gut, AJP Gastrointestinal and Liver Physiology 291(2):G171-7
21. Margolskee RF (2001) Molecular mechanisms of bitter and sweet taste transduction, J boil Chemistry 277(1):1-4
22. Danielle R. Reed, Toshiko Tanaka, Amanda H. McDaniel (2006) Diverse tastes: genetics of sweet and bitter perception, Physiology & Behavior 88(3): 215–26
23. Alexander A. Bachmanov, Gary K. Beauchamp, (2007) Taste receptor genes, Annu Rev Nutrition 2007(27): 389-414
24. Masataka Narukawa, Chiaki Noga, Yoher Ueno, Tsutomu Sato, Takumi Misaka, Tatsuo Watanabe (2011) Evaluation of the bitterness of green tea catechins by a cell-based assay with the human bitter taste receptor HTAS2R39, Biochemical and Biophysical Research Communications 405(4): 620–25
25. Toyomi Yamazaki, Sagisaka M, Ikeda R, Nakamura T, Matsuda N, Ishii T (2014) The human bitter taste receptor HTAS2R39 is the primary receptor for the bitterness of the aflavins, Bioscience, Biotechnology, and Biochemistry 78(10): 1753–56
26. Hasegawa S (1983) Reduction of nomilin bitterness in citrus juice and juice eram with arthrobacter globifomis cells immobilized in acrylamidgel. Food Chemistry 31: 178-180
27. Hasegawa S (1983) Solutions to limonin bitterness problem of citrus juices. Food Technoligy 27(6): 73-77
28. Robert L. Johnson, Bruce V. Chandler (1982) Reduction of bitterness and acidity in grapefruit juice by adsorptive processes, Journal of the Science of Food and Agriculture 33(3): 287

29. Lenggehhager T. (2000) Ultra filtration and adsorb technologies combined produce top – grade concentrates and juice, Food and Machinary 2000(5): 33-34
30. M Dahl, R. P Erickson, S. A Simon (1997) Neural responses to bitter compounds in rats, Brain Research 756(1): 22–34
31. Lars Kjellenberg (2007) Sweet and bitter tate in organic carrot, Faculty of Landscape Planning, Horticulture and Agricultural Science 2007: 2
32. T. K. Murray, B. E. Baker (1952) Studies on protein hydrolysis. I.—preliminary observations on the taste of enzymic protein-hydrolysates, Journal of the Science of Food and Agriculture 3(10): 470–75
33. Guohua Zhao, Zongdao Chen (2000) Advances in research on bitter taste of protein hydrolysate, Science and Technology of Cereals, Oils and Foods 7(2): 57
34. K. Maehashi, L. Huang (2009) Bitter peptides and bitter taste receptors, Cellular and Molecular Life Sciences; Basel 66(10): 1661–71
35. Marie-Luise Blue (2018) What chemical compounds are thought to be responsible for the tastes of bitter, Sour, Salty & Sweet? Sciencing 3(24)
36. Oskar Laaksonen (2011) Astringent food compounds and their interactions with taste properties, Turun ylioposto 12(02)
37. Adam Drewnowski, Carmen Gomez-Carneros (2000) Bitter taste, phytonutrients, and the consumer: A Review, The American Journal of Clinical Nutrition 72(6): 1424–35
38. José Antonio Del Río, Maria Arcas, Obdulio Benavente (1998) Changes of polymethoxylated flavones levels during development of citrus aurantium (Cv. Sevillano) Fruits, Plant Medical 64(6): 575–76

39. Drewnowski A, Gomez-Carneros (2000) Bitter Taste, Phytonutrients, and the consumer, American Journal of Clinical Nutrition 72(6):1424-35

40. Munish Puri, S. S. Marwaha, R.M. Kothari, J.F. Kennedy (1996) Biochemical basis of bitterness in citrus fruit juices and biotech approaches for debittering, Critical Reviews in Biotechnology 16(2): 145–55

41. Shin Hasegawa, Masaki Miyake (1996) Biochemistry and Biological Functions of Citrus Limonoids, Food Reviews International 12(4): 413–35

42. Karl J. Siebert, P. Y. Lynn (2005) Comparison of methods for measuring protein in beer, Journal of the American Society of Brewing Chemists 63(4): 163–70

43. Hanna Peleg et al. (1999) Bitterness and astringency of flavan-3-Ol monomers, dimers and trimers, Journal of the Science of Food and Agriculture 79(8): 1123–28

44. Ian R. McLaughlin, Cindy Lederer, Thomas H. Shellhammer (2008) Bitterness-modifying properties of hop polyphenols extracted from spent hop material, Journal of the American Society of Brewing Chemists 66(3): 174–83

45. Yongxing Zhu, A review of recent studies in China on the possible beneficial health effect of tea, International Journal of Food Science and Technology, 2006(41): 333-340

46. Zhaopeng Shi, Guoben Chen et. al., The relationship between the formation of the bitter taste of summer tea and the composition of the substance.[J] Science of Tea, 1984, 4(1): 61-62.

47. ScharbertS, Hofmann T. (2005) Molecular definition of black tea taste by means of quantitative studies, taste reconstitution and omission experiments. Journal of Agricultural and Food Chemistry 53(13): 5377-5384

48. J. C. Hoskin (1994) Sensory properties of chocolate and their development, The American Journal of Clinical Nutrition 60(6): 1068S-1070S

49. I. C. Arts, P. C. Hollman, D. Kromhout (1999) Chocolate as a source of tea flavonoids, Lancet (London, England) 354(9177): 488

50. J. Serra Bonvehi, F. Ventura Coll (1997) Evaluation of bitterness and astringency of polyphenolic compounds in cocoa powder, Food Chemistry 60(3): 365–70

51. R. L. Rouseff (1990) Bitterness in food products: An overview, Developments in Food Science 25: 1-14

52. Na Xu (2007) Researchers identified two compounds associated with the bitter taste of coffee. Food Science 28(9): 509.

53. Jane L Robichaud, Ann C Noble (1990) Astringency and bitterness of selected phenolics in wine, Journal of the Science of Food and Agriculture 53(3)

54. Hanna Peleg, Karine Gacon, Pascal Schilich, Ann C Noble (1999) Bitterness and astringency of flavan-3-ol monomers, dimers and trimers. Journal of the Science of Food and Agriculture 79(8)

55. Mercedes C. Carrao-Panizzil, Keisuke Kitamura (1995) Isoflavone content in Brazilian soybean cultivars, Japanese Journal of Breeding 45(3): 295–300

56. M. Matsuura, A. Obata, D. Fukushima (1989) Objectionable flavor of soy milk developed during the soaking of soybeans and its control, Journal of Food Science 54(3): 602–5

57. Baby Joseph, D jinni (2013) Antidiabetic effects of Monordica charantia (bitter melon) and its medicinal potency, Asian Pacific Journal of Tropical Disease 3(2): 93-102

58. Lynsey D Jones, Pangloli P, Krishnan HB, Dia VP (2018) BG-4, a novel bioactive peptide from momordica charantia, inhibits Lipopolysaccharide-Induced inflammation in THP-1 human macrophages, Phytomedicine 42: 226–32
59. Mohammad Raish, Ahmad A, Ansari MA, Alkharfy KM, Jan BL (2018) Momordica charantia polysaccharides ameliorate oxidative stress, inflammation, and apoptosis in ethanol-induced gastritis in mucosa through NF-KB signaling pathway Inhibition,International Journal of Biological Macromolecules 111: 193–99
60. Rajendra Karkl, Cheol-Ho Park, Dong-Wook Kim (2013) Extract of buckwheat sprouts scavenges oxidation and inhibits pro-imflammatory mediators in lipopolysaccharide-stimulated macrophages (RAW264.7) Journal of Integrative Medicine 11(4): 246-252
61. Bing L, Changling H (2011) Research progress on chemical constituents and pharmacological effect of Fagopyrum tataricum, Drugs and Clinic, 29(4)
62. Haiyan Bi, Yulu Li, The functional characteristics and research and development of bitter food. Food Research and Development, 2003, 24(1): 57-59.

CHAPTER 2

QUANTIFICATION AND DIGESTIVE RELEASE OF OAT AVENANTHRAMIDES *IN VITRO*

Abstract

Avenanthramides (AVNs) are a group of phenolic alkaloids mainly found in oats. AVNs do not only act as antioxidants but also interact with multiple cellular components to inhibit pro-inflammatory processes and produce a variety of health promoting effects following the appropriate nutritional intervention or dietary supplementation. In this study, we quantified AVNs in 242 oat flour samples consisting of 109 accessions from the AFRI CORE world diversity oat panel grown at two different locations (Lacombe, Alberta, CA and Aberdeen, Idaho, US) and determined their release patterns during the *in vitro* digestion. Total levels of AVNs ranged from 3.3 to 227.3 μg/g in Lacombe location and from 1.6 to 65.9 μg/g when grown in Aberdeen. While total levels of AVNs released from whole flours upon digestion ranged from 1.9 to 67.2 μg/g whole flour in Lacombe and from 1.6 to 14.8 μg/g when grown in Aberdeen, we observed a complete loss of AVNC from in vitro digests. Oat variety and growth location appeared to critically affect both total levels and bioaccessibility of AVNs.

Introduction

Oats are a unique cereal among the whole grains. Evidence has indicated that high intake of whole-grain foods can reduce the risk of CHD (coronary heart disease) and diabetes.[1-2]Consuming oat reduced plasma cholesterol and LDL-cholesterol levels, which are main risk factors for CHD.[3] In 1997, the US Food and Drug Administration approved the heart health

claims for foods containing oat due to high levels of soluble fiber and its impact on the risk factors associated with CHD. Compared with other grains, oat also contains one-third more protein, almost four times as much fat and significantly less carbohydrate. Importantly, oat contains many phytochemicals with a phenolic moiety which have free radical scavenging properties and thus exhibit antioxidant properties *in vitro*.[4]

Avenanthramides (AVNs), which are also called anthranilic acid amides, are a group of phenolic alkaloids mainly found in oat (**Figure 2.1**). AVNs were originally named by Collins in his report about presence of these metabolites in oat kernels.[5] It was later discovered that they were identical to three oat alkaloids (open-chain amides of avenalumins I, II and III) that were previously reported in oat by Mayama and colleagues.[6-7] Research indicated that these compounds have anti-inflammatory, antioxidant, anti-itch, anti-irritant and antiatherogenic activities.[8]

Antioxidants are substances which protect cells from oxidative damage and prevent disorders associated with oxygen species generation.[9] Dimberg et al. reported significantly higher antioxidative potential of AVNs than that of other simple phenolics such as vanillin and caffeic acid. Avenanthramide C (AVNC) was one of three most abundant AVNs found in oat groats, usually accounting for one-third of the total concentration of AVNs (although the relative proportion of AVNs varied greatly) and it showed the highest *in vitro* antioxidant activity.[10] The antioxidant activity of AV-rich oat extracts has been studied in animal studies. A diet supplemented with AV-rich oat extract at 100 mg/kg food (thus providing approximately 20 mg AVNs per kg body weight) increased levels of superoxide dismutase (SOD) in skeletal muscle, liver and kidney of the treated animals. The intervention also enhanced glutathione peroxidase activity in the heart and skeletal muscle.[11] AVNs also inhibited oxidation of low-density

lipoprotein (LDL) in a dose dependent fashion and showed synergistic interaction with vitamin C, thus providing another independent evidence that AVNs acted as potent antioxidant compounds.[12]

AVNs do not only act as antioxidants but also interact with multiple cellular components to inhibit pro-inflammatory processes. Liu et al tested the potential antiatherogenic activity of AVNs in the human aortic endothelial cell (HAEC) culture system, and reported that the beneficial effects of AVNs were mediated through modulation of cellular and molecular processes, which play important roles in arterial inflammation. The reduced adhesion of HAEC cells to U937 cells *in vitro* appeared to be mediated by inhibition of adhesion molecule expression by HAEC, and effect attributed to oat AVNs.[13] In a clinical study, Koenig et al divided women between 50 to 80 years old into two groups in a double-blind fashion, providing them with 0.4 mg or 9.2 mg total AVNs, and collected blood samples between downhill walking sessions. The data indicated that AV supplementation decreased the inflammation induced by downhill walking in postmenopausal women.[14]

Studies revealed that vascular smooth muscle cell (VSMC) is one of the two major factors which lead to atherosclerosis, and the other is impaired nitric oxide (NO). Nie et al conducted a study to examine the effect of AVNs on the VSMC, and the study showed that 120 uM AVNs could inhibit more than half of VSMC cell proliferation. In addition, AVNs also increased NO producing dose-dependently. The results indicated that AVNs had significant antiproliferative properties and could be used to manage atherosclerosis.[15] The same group later continued on their studies to investigate the cell cycle inhibitory mechanism, and the results suggested that AVNs treatment arrested VSMC cell proliferation at the G1 phase. This inhibitory effect could indicate potential health benefit of oat supplementation to prevent CHD.[16]

Oat has long been used to treat different types of skin diseases. Colloidal oat has been found to be very effective in the treatment of atopic dermatitis, drug-induced rashes, psoriasis and other related conditions. It was also suggested that oat extracts containing AVNs have antihistamine and anti-irritation activity.[17] Sur et al showed that low concentrations of AVNs inhibited degradation of inhibitor of nuclear factor kappa B-alpha, and topical application of 1-3 ppm oat amides reduced inflammation in a murine model of allergic and neurogenic inflammation.[18]

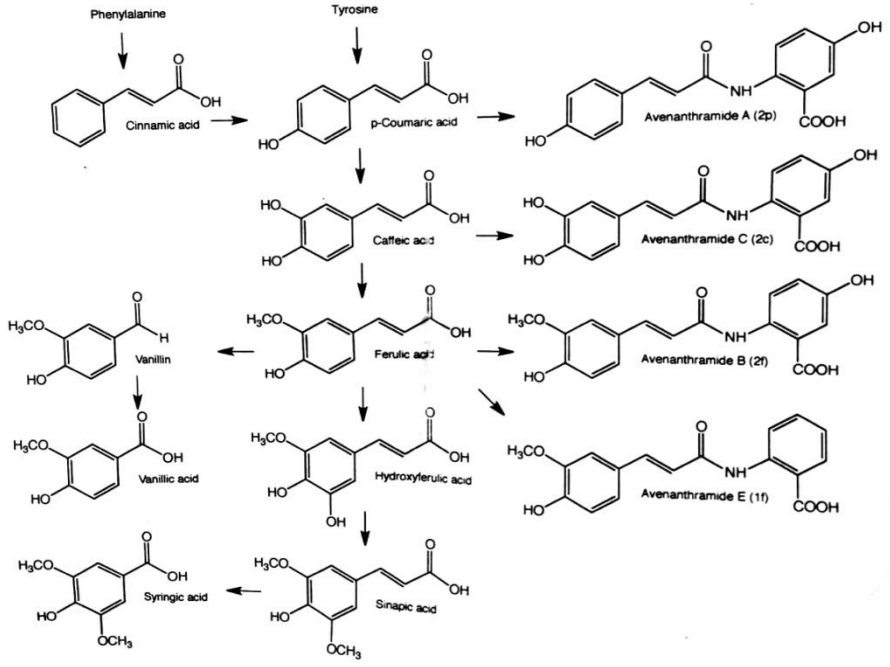

Figure 2.1. Chemical structure of avenanthramides and their precursor phenolic acids.

Studies have shown that AVNs are beneficial to human health because of their multiple health promoting effects. [19] Animal studies and human clinical trials confirmed that the effects of AVNs of lowering cholesterol and inhibiting LDL oxidation can help reduce risks of cardiovascular. [20]Together with two avenacosides and two bidesmosidic saponins, oat AVNs

were described as key components responsible for astringent and bitter taste of oat.[21] Therefore, the objective of this study was to quantify AVNs in oat flour samples and determine their release patterns during the *in vitro* digestion.

Material and methods

Chemicals

General laboratory reagents including all materials used for *in vitro* digestion were purchased from Sigma (Saint Louis, MO). All solvents were of HPLC grade and purchased from VWR (Radnor, PA). Water was purified with a Milli-Q water purification system (Millipore, Burlington, MA). Avenanthramide A (2p), B (2f), C (2c), and E (1f) standards were purchased from SynInnova (Edmonton, AB, Canada).

Oats samples and preparation

A set of 242 samples consisting of 109 accessions from the AFRI CORE world diversity oat panel grown at two different locations – Lacombe, Alberta, CA (1) and Aberdeen, Idaho, US (2) – was kindly provided by Dr. Eric Jackson as a part of the Collaborative Oat Research Enterprise (CORE) initiative[22] in the form of de-hulled and uniformly milled whole grain flour and stored at -20 °C until extraction.

Extraction of free avenanthramides

Oat flours (200 mg) were milled with IKA Analytical Mill into a fine powder and extracted with 1 ml of 95% methanol in a phosphate buffer (pH=2.8) using vertical shaking (20

min, RT, 300 rpm) and ultrasonication (30 min). The samples were centrifuged at 3000 rpm and the exhausted pellets were extracted 2 more times following the same procedure and discarded. Combined supernatants were concentrated under vacuum at a temperature not exceeding 40 °C (Buchi R210 rotavapor, Flawil, Switzerland) and freeze-dried (Labconco Freezone18, Kansas City, MO). Resulting powders were dissolved in methanol, filtered through a syringe filter, and analyzed by HPLC.

Table 2.1. Preparation of stock solutions of stimulated digestion fluids (1.25x concentrate).

			SSF		SGF		SIF	
			pH 7.0		pH 3.0		pH 7.0	
Constituent	**Stock Conc.**		**Vol of stock**	**Conc in SSF**	**Vol of stock**	**Conc in SGF**	**Vol of stock**	**Conc in SIF**
	g/L	**mol/L**	**mL**	**mmol/L**	**mL**	**mmol/L**	**mL**	**mmol/L**
KCl	37.3	0.5	15.1	15.1	6.9	6.9	6.8	6.8
KH2PO4	68.0	0.5	3.7	3.7	0.9	0.9	0.8	0.8
NaHCO3	84.0	1.0	6.8	13.6	12.5	25.0	42.5	85.0
NaCl	117.0	2.0	NIL	NIL	11.8	47.2	9.6	38.4
MgCl2. 6H2O	30.5	0.15	0.5	0.15	0.4	0.1	1.1	0.33
(NH4)2CO3	48.0	0.5	0.06	0.06	0.5	0.5	NIL	NIL
For pH adjustment								
	mol/L		mL	mmol/L	mL	mmol/L	mL	mmol/L
NaOH	1		NIL	NIL	NIL	NIL	NIL	NIL
HCl	6		0.09	1.1	1.3	15.6	0.7	8.4
Distilled water upto			400		400		400	
CaCl2.6H2O is **NOT** added to the stimulated digestion fluids								
	g/L	mol/L		mmol/L		mmol/L		mmol/L
CaCl2.6H2O	44.1	0.3		1.5 (0.75*)		0.15 (0.075*)		0.6 (0.3*)
* in brackets is the corresponding Ca2+ concentration in the final digestion mixture.								

Simulated in vitro digestion

Simulated oral, gastric and small intestinal digestion of oat flours was performed following an established protocol with minor modifications.[23] Briefly, SSF (simulated salivary

fluid), SGF (simulated gastric fluid) and SIF (simulated intestinal fluid) were made up of the respective electrolyte solutions, enzymes, water and calcium chloride as indicated in **Table 2.1.**

A-amylase from human saliva (type IX-A) or porcine pancreas (type VI-B) was prepared as 1500 U/ml stock solution in the SSF fluid. Porcine pepsin was prepared as 25000 U/ml in the SGF fluid. Pancreatin stock solution was prepared based on the trypsin activity as 800 U/ml in the SIF fluid. Fresh bile preparations were made as 160 mM stocks in the SIF solution. Calcium chloride stocks were prepared as 0.3 M solution (0.44 g $CaCl_2x2H_2O$ in 10 ml water).

In the oral digestion phase, 100 mg of oat flour sample was added to 300 µl of water and boiled for 5 minutes, then 280 µl of SSF electrolyte stock solution and 40 µl of salivary α-amylase solution (1500 U/ml) were added. Following the addition of 2 µl of 0.3M $CaCl_2$ and 78 µl water, the reaction was mixed by vortexing to simulate food processing in human mouth for 2 min. The pH of solution was controlled at 6.8 where salivary α-amylase has a highest activity.[24]

In the gastric digestion phase, 600 µl of SGF electrolyte stock solution, 128 µl of porcine pepsin (3850U/mg), 4 µl of 0.3M $CaCl_2$, 16 µl of 1M HCl and 52 µl of water were added to the sample and incubated for 2 hours at 37℃.

In the intestinal digestion phase, 880 µl of SIF electrolyte stock solution, 400 µl of pancreatin (800U/ml), 200 µl of fresh bile (160mM of taurocholic acid), 3.2 µl of 0.3M $CaCl_2$, 12 µl of 1M NaOH and 104.8 µl of water were added to the gastric chyme and digested for additional 2 hours at 37℃.After digestion was completed, the solution was water bathed for 15 minutes in 80℃ to inactivate the enzymes and then centrifuged at 2000 rpm for 10 minutes. Supernatant liquid was transferred to a new vial, frozen at -80℃, and freeze-dried to obtain a powder sample of water-soluble metabolites released from the digested oat flour.

HPLC quantification of avenanthramides

HPLC-UV analysis was performed using a Shimadzu HPLC system equipped with a pump (LC-20AT), an autosampler (SIL-20A), a diode array detector (SPD-M20A) and an automatic column temperature control oven (CTO-20A). Separation was performed on Restek Ultra C18 column (250 x 4.6 mm, 5 μ) at a column temperature of 30°C. The binary mobile phase consisted of 0.1% formic acid in water (Eluent A) and acetonitrile (Eluent B) in a gradient as follows: 0-5 min, 20% acetonitrile; 5-25 min, 20-65% acetonitrile; 25-26 min, 65% acetonitrile; 26-29 min, 65-95% acetonitrile; 29-32 min, 95% acetonitrile; 32-44 min, 95-20% acetonitrile. Each run was followed by an equilibration time of 10 min. Ultraviolet (UV) spectra were monitored at 340 nm, and the flow rate was 1.0 ml/min. The data were collected and analyzed with LC solution (Shimadzu, Nakagyo-ku, Kyoto, Japan) software. Peaks were identified based on comparison of retention times and UV spectra with those of authentic AVNs standards.

Statistical analysis

Statistical analyses were performed using Prism 6.0 (GraphPad Software, San Diego, CA) and expressed as mean ± SEM. Two tailed t-test or one-way ANOVA were applied as necessary at a significance level of $P < 0.05$. Post-hoc analyses of differences between individual experimental groups were made using the Tukey's multiple comparison test.

Results and discussion

Identification and quantification of avenanthramides

Oat cultivars from the AFRI CORE worldwide diversity panel differed markedly not only in total and individual AVNs content, but also in the amounts of AVNs produced depending on the geographical location of the field. Total levels of AVNs ranged from 3.3 to 227.3 μg/g in Lacombe, AB location, and from 1.6 to 65.9 μg/g when grown in Aberdeen, ID. Cultivar UFRGS 881971 grown in Lacombe showed the highest total AVNs content of 227.3 μg/g, with levels of the individual AVNs at 68.1 μg/g (AVNA), 91.4 μg/g (AVNB), and 67.8 μg/g (AVNC). Cultivar Maverick grown in Aberdeen showed the lowest total AVNs content of 1.6 μg/g, with levels of the individual AVNs at 0.8 μg/g (AVNA), 0.2 μg/g (AVNB), and 0.6 μg/g (AVNC) (**Tables 2.2 and 2.3**, free AVNs).

Table 2.2. Content of major individual avenanthramides in flours and *in vitro* digests of the oat cultivars from the AFRI CORE worldwide diversity panel grown in the northern location of Lacombe, Alberta, CA.

Cultivar name	**Location 1 Lacombe, Alberta, CA**					
	Free AVNs (μg/g)			**Digested AVNs (μg/g)**		
	AVNA	**AVNB**	**AVNC**	**AVNA**	**AVNB**	**AVNC**
Aarre	10.5	22.1	10.0	1.4	0.5	nd
Ajax	15.1	19.5	19.9	2.3	1.4	nd
Ajax	--	--	--	--	--	--
Akiyutaka	18.3	12.4	18.9	9.1	4.2	nd
Asencao	12.1	12.7	13.1	5.4	2.9	nd
Assiniboia/S42	27.4	38.8	37.8	9.9	5.9	nd
Baler_CDC	--	--	--	--	--	--
Belinda	6.2	10.5	7.1	2.0	0.8	nd
Bia	6.3	9.6	7.3	2.6	1.0	nd
Blaze	11.9	13.4	16.6	3.0	0.8	nd
Boudrias	25.3	26.3	30.9	6.4	2.7	nd
Bountiful	10.9	11.3	16.0	3.9	2.0	nd
Boyer_CDC	41.8	51.9	39.5	8.2	3.2	nd

Table 2.2 (continued).

Buckskin	23.3	19.3	27.2	5.0	1.7	nd
Buffalo	21.0	22.6	28.6	3.5	1.2	nd
Calibre	8.7	11.3	10.5	2.2	0.3	nd
Centennial	31.0	42.5	50.5	3.0	0.3	nd
Chaps	10.1	10.3	14.7	2.3	0.5	nd
Chernigovskij	3.0	5.7	3.7	1.8	0.7	nd
Cherokee	12.3	14.5	11.7	3.7	1.9	nd
CI 4706-2	2.2	1.3	1.9	2.0	0.4	nd
Clay 6209	25.9	20.0	34.1	11.1	3.8	nd
Clinton	--	--	--	--	--	--
Coker 227	27.2	20.2	24.8	12.3	5.2	nd
Coker 234	21.9	19.2	30.7	9.5	6.2	nd
Dal	13.8	12.8	18.5	9.1	6.2	nd
Dancer CDC	21.1	32.6	18.3	12.3	13.3	nd
Dane	5.3	9.3	7.8	3.3	2.7	nd
Dominik (Bauer)	6.7	15.8	9.7	5.5	8.0	nd
Drummond	23.1	20.7	31.0	16.8	13.9	nd
Exeter	10.8	11.2	9.2	8.6	7.4	nd
Flaemingsnova	22.5	45.7	24.4	18.0	25.3	nd
Florida 501	36.4	34.4	30.3	19.3	16.4	nd
Ford Early Giant	18.7	24.5	31.6	11.2	12.3	nd
Freddy	10.6	31.7	8.8	6.0	11.6	nd
Fulgham	7.2	4.0	8.1	3.5	2.0	nd
Furlong	--	--	--	--	--	--
Gehl	11.2	22.5	5.8	5.7	7.3	nd
Gem	--	--	--	--	--	--
Goslin	24.2	33.9	28.1	12.3	13.7	nd
H927-161XX24	1.4	0.6	1.3	1.6	0.4	nd
Hazel	--	--	--	--	--	--
HiFi	52.0	63.3	57.8	12.4	7.4	nd
Horizon 270	24.6	14.7	39.4	8.3	3.0	nd
Horizon 270	23.6	17.6	35.9	6.7	2.5	nd
Horizon 270	23.3	16.1	36.4	3.8	1.0	nd
Horizon 270	28.0	18.8	43.1	6.1	1.8	nd
Hurdal	2.9	4.7	3.1	2.5	2.2	nd
IL86-5698-3	9.7	20.2	16.4	6.6	10.4	nd
Jay	8.0	6.3	9.9	3.8	1.9	nd
Jerry	9.4	11.2	10.3	8.6	8.5	nd
Kangaroo	14.9	19.9	10.8	10.5	8.5	nd
Kanota	12.7	13.4	12.4	6.0	3.0	nd
Kaufman	23.9	30.0	31.5	11.7	9.3	nd
Lang	8.7	13.7	11.5	11.2	14.5	nd

Table 2.2 (continued).

Leggett	22.0	27.2	26.1	18.3	17.9	nd
Leggett	19.3	23.6	21.3	20.7	16.9	nd
Leggett	19.5	25.7	22.5	14.4	15.3	nd
Leggett	21.5	25.7	24.8	12.6	10.8	nd
Lutz	10.0	16.2	14.2	6.3	6.9	nd
Maldwyn	4.0	5.9	5.4	3.6	3.1	nd
MAM 17-5	18.2	20.0	18.5	10.2	9.1	nd
Marie_AC	12.3	15.7	13.2	12.0	12.7	nd
Matilda	22.3	17.3	31.5	4.2	7.9	nd
Maverick	5.1	5.2	3.9	1.4	1.6	nd
Maverick	7.6	8.6	5.9	1.9	3.0	nd
Maverick	8.0	9.6	7.1	1.7	3.0	nd
Maverick	8.4	8.8	7.5	2.2	3.5	nd
Maverick	4.4	4.6	3.6	1.8	1.6	nd
Melys	4.2	7.0	4.2	3.4	2.5	nd
MF9522-523	26.9	44.7	32.5	10.0	8.5	nd
MN811045	6.3	7.0	8.8	3.7	2.5	nd
MN841801-1	42.4	56.2	21.1	23.1	20.3	nd
Morgan_AC	15.3	24.3	20.7	8.3	8.2	nd
Mortlock	13.8	12.2	18.6	2.1	0.6	nd
Morton	17.7	25.5	9.1	10.7	10.5	nd
Morton	--	--	--	--	--	--
Noble-2	6.0	7.4	7.5	5.2	5.1	nd
Novojatkovo	11.7	10.2	16.5	8.0	5.3	nd
OA1063-8	8.7	6.2	15.5	6.2	4.0	nd
Ogle	3.8	6.6	4.3	3.3	2.6	nd
OT380	15.6	18.3	13.1	2.5	0.9	nd
OT586	27.1	49.3	34.0	6.4	3.7	nd
Otana	13.0	25.5	16.3	10.2	14.8	nd
Pacer_CDC	--	--	--	--	--	--
Pg11	lost	lost	lost	lost	lost	nd
Pg16	lost	lost	lost	lost	lost	nd
Pinnacle_AC	--	--	--	--	--	--
Prescott	--	--	--	--	--	--
ProFi_CDC	43.9	49.9	49.9	30.1	27.6	nd
Provena	9.0	13.4	12.4	4.7	4.8	nd
Pusa Hybrid G	17.8	18.6	28.6	12.1	13.4	nd
Ranch	11.6	21.9	12.6	5.7	6.8	nd
Red Rustproof	5.1	2.5	6.3	4.1	1.3	nd
Rigodon AC	19.0	19.6	26.8	13.6	10.6	nd
Robust	15.2	10.2	18.0	12.3	6.7	nd

Table 2.2 (continued).

Rodgers	18.4	19.5	15.7	11.4	7.7	nd
Rodgers	18.3	19.9	15.0	11.1	7.2	nd
Rodgers	20.4	23.9	15.5	6.9	5.3	nd
Rodgers	19.2	19.6	16.0	8.5	5.7	nd
Ronald_AC	11.1	11.4	13.7	4.6	3.1	nd
Russell	10.1	13.5	11.3	4.1	3.3	nd
Salomon	10.9	19.6	11.4	3.4	2.8	nd
Sang	6.4	15.1	5.5	2.8	2.8	nd
Sesqui	3.6	3.1	3.5	2.2	0.7	nd
Shadow	12.5	22.1	13.2	4.6	4.7	nd
SO-1	25.0	16.7	28.9	11.5	5.3	nd
Sol-Fi CDC	--	--	--	--	--	--
Stout	12.1	20.2	13.3	5.8	5.4	nd
Sunll-1	16.5	24.0	13.5	13.9	16.5	nd
SW Betania	14.6	17.5	12.8	9.7	8.7	nd
TAM O-301	7.1	8.5	7.1	5.4	4.7	nd
TAM O-397	28.9	40.2	34.3	19.8	22.7	nd
TAMO-406	17.9	12.3	23.5	11.2	8.6	nd
TAMO-406	22.8	15.0	32.6	15.4	8.1	nd
TAMO-406	15.0	8.6	19.1	18.0	12.5	nd
Tardis	17.2	12.8	24.0	9.0	6.0	nd
Triple Crown	--	--	--	--	--	--
Troy	7.2	7.0	9.0	4.5	2.7	nd
UFRGS 8	29.3	33.0	28.1	14.2	13.9	nd
UFRGS 881971	68.1	91.4	67.8	31.0	36.2	nd
UFRGS 930605	43.9	32.3	46.1	21.3	12.7	nd
Ukraine res	29.5	42.5	28.3	12.7	12.5	nd
Urano	18.3	13.7	31.7	8.6	4.3	nd
Victoria	4.3	1.6	5.6	3.2	1.1	nd
Vista	50.9	53.4	47.3	22.1	14.8	nd
WAOAT2132	15.2	13.6	16.7	10.2	8.4	nd
Z615-4	7.3	5.2	10.6	9.5	5.2	nd

(--), sample not collected; nd, metabolite not detected in the sample; lost, sample lost during processing.

The total content of AVNs differed markedly across the samples ($p<0.05$) and locations ($p<0.01$), with up to 75-fold difference among the cultivars grown in the northern location, 33-fold difference among the cultivars grown in the southern location, and 5-fold difference among the average means for the cultivars grown in each location (54.9±2.3 versus 11.1±0.8 μg/g whole flour for Lacombe and Aberdeen, respectively). These numbers are consistent with total AVNs

ranges reported for nine oat cultivars from China (5-175 μg/g)],[25] eight oat cultivars from Finland (27-185 μg/g),[26] or four oat cultivars from US (17-116 μg/g) (**Tables 2.2 and 2.3**, free AVNs).[27]

Table 2.3. Content of major individual avenanthramides in flours and *in vitro* digests of the oat cultivars from the AFRI CORE worldwide diversity panel grown in the southern location of Aberdeen, Idaho, US.

Cultivar name	**Location 2 Aberdeen, Idaho, US**					
	Free AVNs (μg/g)			**Digested AVNs (μg/g)**		
	AVNA	**AVNB**	**AVNC**	**AVNA**	**AVNB**	**AVNC**
Aarre	1.4	1.0	1.4	1.7	0.4	nd
Ajax	3.0	7.1	2.5	nd	nd	nd
Ajax	3.5	0.4	0.1	3.7	2.3	nd
Akiyutaka	5.2	4.9	3.0	2.6	1.0	nd
Asencao	2.1	0.7	1.2	1.4	0.6	nd
Assiniboia/S42	18.1	28.8	19.0	1.5	0.7	nd
Baler_CDC	3.9	4.2	4.1	1.7	0.3	nd
Belinda	1.8	1.4	2.3	1.5	0.3	nd
Bia	1.0	1.4	0.6	nd	nd	nd
Blaze	1.7	1.0	2.3	1.6	0.6	nd
Boudrias	3.0	1.4	3.5	1.5	0.4	nd
Bountiful	2.3	1.9	3.4	1.7	0.4	nd
Boyer_CDC	6.8	4.3	6.6	2.5	0.3	nd
Buckskin	5.1	2.6	6.3	1.7	0.9	nd
Buffalo	3.8	3.1	3.6	1.6	0.4	nd
Calibre	1.3	1.4	1.2	1.5	0.6	nd
Centennial	17.3	26.5	19.0	lost	lost	nd
Chaps	2.4	2.0	3.1	1.4	0.6	nd
Chernigovskij	1.7	0.8	0.7	2.0	0.3	nd
Cherokee	7.4	8.1	6.1	4.3	1.1	nd
CI 4706-2	5.2	4.3	6.8	3.4	1.3	nd
Clay 6209	2.1	2.0	2.4	2.3	0.6	nd
Clinton	2.5	2.1	3.8	2.1	0.6	nd
Coker 227	2.4	1.6	2.4	1.6	0.6	nd
Coker 234	9.2	5.9	9.7	3.3	1.2	nd
Dal	1.6	1.5	2.0	1.8	1.1	nd
Dancer CDC	5.1	9.0	7.5	2.9	1.6	nd
Dane	1.9	2.0	2.7	2.5	1.2	nd
Dominik (Bauer)	1.7	2.0	1.9	1.7	1.0	nd

Table 2.3 (continued).

Drummond	1.9	1.5	2.0	1.8	1.0	nd
Exeter	1.8	0.5	0.6	1.5	0.5	nd
Flaemingsnova	4.0	4.5	3.0	2.4	1.5	nd
Florida 501	5.9	6.2	5.3	3.4	2.5	nd
Ford Early Giant	4.2	2.3	6.4	4.3	1.8	nd
Freddy	1.3	2.0	1.0	1.6	1.0	nd
Fulgham	17.9	19.2	16.6	1.7	0.4	nd
Furlong	16.3	16.7	16.4	5.3	3.3	nd
Gehl	1.4	1.7	0.8	1.8	1.2	nd
Gem	2.0	1.2	2.5	1.9	0.9	nd
Goslin	5.6	2.9	7.7	2.8	1.6	nd
H927-161XX24	1.3	0.8	1.4	1.6	0.6	nd
Hazel	2.5	3.5	3.8	1.6	0.9	nd
HiFi	2.8	2.7	3.3	1.5	0.7	nd
Horizon 270	2.3	1.7	2.6	1.6	0.5	nd
Horizon 270	2.1	1.6	2.5	1.5	0.6	nd
Horizon 270	2.2	1.4	2.1	1.6	0.5	nd
Horizon 270	2.3	1.7	2.6	1.5	0.8	nd
Hurdal	1.7	0.3	0.7	1.4	0.5	nd
IL86-5698-3	2.1	2.7	2.6	2.1	1.3	nd
Jay	1.0	0.5	0.6	1.7	1.4	nd
Jerry	1.5	1.5	1.1	1.6	0.7	nd
Kangaroo	7.2	9.6	4.3	4.4	2.5	nd
Kanota	1.8	0.4	0.5	2.3	0.8	nd
Kaufman	4.7	4.3	4.7	4.2	2.4	nd
Lang	2.6	4.1	2.6	3.9	2.0	nd
Leggett	4.1	3.4	4.7	3.6	2.4	nd
Leggett	4.1	3.0	3.9	3.4	1.8	nd
Leggett	5.3	4.9	5.1	4.7	2.5	nd
Leggett	4.8	3.8	5.2	3.3	1.4	nd
Lutz	2.6	3.1	3.6	2.5	1.3	nd
Maldwyn	1.5	0.2	0.5	1.3	0.3	nd
MAM 17-5	3.5	3.6	3.5	2.9	1.5	nd
Marie_AC	2.1	2.4	1.2	2.0	1.6	nd
Matilda	1.7	0.5	0.5	1.3	0.3	nd
Maverick	1.3	0.7	0.9	nd	nd	nd
Maverick	1.4	0.6	1.3	nd	nd	nd
Maverick	2.0	1.5	1.5	nd	nd	nd
Maverick	0.8	0.2	0.6	nd	nd	nd
Maverick	nd	nd	nd	1.3	0.4	nd
Melys	1.5	1.5	1.0	1.6	0.6	nd
MF9522-523	8.3	11.5	9.2	4.3	3.7	nd
MN811045	1.3	1.1	1.2	1.8	0.9	nd
MN841801-1	1.4	1.6	0.4	2.0	1.0	nd

Table 2.3 (continued).

Morgan_AC	2.9	4.0	3.8	2.5	1.6	nd
Mortlock	1.6	0.4	0.5	1.4	0.5	nd
Morton	6.0	7.7	2.5	4.8	4.5	nd
Morton	4.7	5.9	1.9	3.9	2.8	nd
Noble-2	2.8	2.6	2.2	2.4	1.0	nd
Novojatkovo	1.1	1.0	0.9	1.9	1.8	nd
OA1063-8	2.5	2.8	3.0	2.4	1.5	nd
Ogle	2.0	1.7	3.0	2.9	1.2	nd
OT380	6.2	3.3	2.1	4.7	1.9	nd
OT586	9.9	11.7	14.9	3.4	1.4	nd
Otana	0.8	1.2	0.8	1.5	1.5	nd
Pacer_CDC	1.9	1.2	0.9	1.6	0.7	nd
Pg11	3.6	2.3	3.7	3.4	1.4	nd
Pg16	3.8	5.3	3.6	3.0	1.9	nd
Pinnacle_AC	6.2	4.7	8.7	4.1	1.9	nd
Prescott	3.0	3.0	3.1	2.4	1.1	nd
ProFi_CDC	9.4	6.9	10.4	6.5	3.5	nd
Provena	4.0	6.9	5.2	2.4	1.7	nd
Pusa Hybrid G	8.6	6.1	15.4	4.3	2.4	nd
Ranch	1.5	1.3	1.5	1.6	0.5	nd
Red Rustproof	4.5	2.1	5.4	2.8	0.9	nd
Rigodon AC	7.7	6.2	9.1	3.2	1.3	nd
Robust	1.1	0.5	0.9	1.5	0.3	nd
Rodgers	1.4	1.3	0.9	1.5	0.3	nd
Rodgers	1.7	1.7	1.1	1.8	0.9	nd
Rodgers	1.2	1.0	0.9	1.5	0.8	nd
Rodgers	1.1	0.8	0.8	1.6	0.7	nd
Ronald_AC	3.6	2.0	4.1	2.5	0.7	nd
Russell	1.7	1.5	1.4	1.4	0.4	nd
Salomon	1.8	1.8	2.2	1.8	0.5	nd
Sang	1.9	0.6	0.9	1.5	0.4	nd
Sesqui	2.2	1.9	1.5	1.6	0.4	nd
Shadow	12.5	22.1	13.2	1.4	1.2	nd
SO-1	2.3	3.0	2.3	3.5	0.6	nd
Sol-Fi CDC	7.2	3.1	6.4	1.6	0.8	nd
Stout	1.3	1.1	1.7	1.6	0.4	nd
Sunll-1	6.3	8.1	5.7	3.8	3.3	nd
SW Betania	3.9	3.5	3.3	3.4	1.7	nd
TAM O-301	2.0	0.9	0.6	1.6	0.6	nd
TAM O-397	4.9	4.2	5.9	4.8	2.8	nd
TAMO-406	1.9	0.7	0.6	1.3	0.7	nd
TAMO-406	1.7	0.4	0.4	1.5	0.3	nd
TAMO-406	1.7	0.5	0.3	1.6	0.7	nd
Tardis	2.5	2.5	2.4	2.4	0.9	nd

Table 2.3 (continued).

Triple Crown	2.1	1.7	1.5	1.7	0.5	nd
Troy	1.1	0.6	0.8	1.4	0.9	nd
UFRGS 8	3.7	2.8	3.8	3.0	1.4	nd
UFRGS 881971	6.4	5.9	8.1	4.5	2.7	nd
UFRGS 930605	3.8	2.2	4.2	2.3	0.7	nd
Ukraine res	1.9	1.5	1.3	1.5	0.7	nd
Urano	2.0	0.4	0.9	1.5	0.4	nd
Victoria	3.2	1.2	4.1	3.0	1.0	nd
Vista	7.7	7.6	6.1	4.6	3.3	nd
WAOAT2132	10.3	9.7	10.4	8.8	6.0	nd
Z615-4	6.2	4.3	5.1	6.3	4.2	nd

(--), sample not collected; nd, metabolite not detected in the sample; lost, sample lost during processing.

Bioaccessibility of avenanthramides following in vitro digestion

In vitro digestion is widely used in projects focusing on modeling the bioactivity of food components, as well as their structural changes, release, and digestibility in simulated human body conditions of the upper gastrointestinal tract. *In vitro* digestion model is often cheaper and faster than human studies, and it is not limited by an ethical controversy. Digestion fluids used in this study were modeled after Minekus et al[23] protocol that simulates 3 stages of the upper gastrointestinal digestion including SSF (simulated salivary fluid), SGF (simulated gastric fluid) and SIF (simulated intestinal fluid). Although different time intervals have been suggested for each digestion stage in the past in the range of 1-5 minutes,[28] we chose to limit oral stage to 2 minutes. Gastric digestion of a solid meal is usually completed in 3 to 4 hours,[29] and this time range is usually shortened by 1 hour if powder-like meals is simulated because they require shorter mixing times. A liquid meal requires half the volume of gastric juice for digestion,[30] while double the volume of gastric secretion is required for a solid meal.[31] After consuming a meal, pH of the stomach content usually increases to 5 due to the buffering ability of western-type diet, with plenty of vegetable purees, and decreases gradually to about 2. As pepsin activity

is activated in the pH range of 2-4, pH=3 was chosen in this study to simulate a gastric environment. A one to one ratio of gastric chyme to SIF and 2 hour duration are typically applied to simulate intestinal digestion.[28] The pH in duodenum is about pH=6.5 and slightly increases to about pH=7.5 in its distal part, so an average value of pH=7 is applied in order to simulate pH in the entire upper intestinal region.[28, 32]

Bioaccessebility (presence of avenanthramides in the aqueous fraction of the three-stage *in vitro* digestion system following their release from the wet cooked whole oat flour) was quantified in all oat cultivars subjected to free AVNs analysis. Total levels of AVNs released from whole flours upon digestion ranged from 1.9 to 67.2 μg/g whole flour in Lacombe, AB location, and from 1.6 to 14.8 μg/g when grown in Aberdeen, ID. Cultivar UFRGS 881971 grown in Lacombe showed the highest total bioaccesible AVNs content of 67.2 μg/g, with levels of the individual AVNs at 31.0 μg/g (AVNA), 36.2 μg/g (AVNB), and 0 μg/g (AVNC). Cultivar Matilda grown in Aberdeen showed the lowest total bioaccessible AVNs content of 1.6 μg/g, with levels of the individual AVNs at 1.3 μg/g (AVNA), 0.3 μg/g (AVNB), and 0 μg/g (AVNC) (**Table 2.2**, digested AVNs). The complete loss of AVNC from the digested oat samples can be explained both by inherent instability of caffeic acid and the respective avenanthramide AVNC (2c) under both neutral and alkaline conditions, with and without heating, encountered during oat flour processing and digestion.[33]

Therefore, the total content of bioaccessible AVNs differed markedly across the samples ($p<0.01$) and locations ($p<0.001$), with up to 35-fold difference among the cultivars grown in Lacombe, 9-fold difference among the cultivars grown in Aberdeen, and a 5-fold difference among the average means for the cultivars grown in each location (15.3±0.8 versus 3.8±0.2 μg/g whole flour for Lacombe and Aberdeen, respectively). Relative average bioaccessibility of total

AVNs varied in the rage of 40-45% % between locations, and 5-92% among cultivars in the same location.

Conclusions

The selected AFRI CORE world diversity oat panel cultivars differed greatly in their AVNs composition with up to a 75-fold difference among the cultivars grown in the northern location, a 33-fold difference among the cultivars grown in the southern location, and a 5-fold overall difference in favor of the more northern location (Lacombe, AB). Cultivars UFRGS 881971, ProFi CDC, HiFi and Centennial showed the highest content of AVNs. These cultivars could be specifically utilized in the development of novel nutritional and dietary formulations to supplement human diet with high levels of bioactive AVNs. The digestive release of AVNs in the simulated model of upper gastrointestinal digestion, critically depended on cultivar and growth location of the crop. AVNC, however, was completely lost from the target digests, possibly due to its inherent instability under neutral and alkaline conditions.

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REFERENCES

1. J. W. Anderson (2000) Dietary fiber prevents carbohydrate-induced hypertriglyceridemia, Current Atherosclerosis Reports 2(6): 536–41.
2. Joshipura KJ, Hu FB, Manson JE, Stampfer MJ, Rimm EB (2001) The effect of fruit and vegetable intake on risk for coronary heart disease, Annals of Internal Medicine 134(12): 1106–14.
3. Mohsen Meydani (2009) Potential health benefits of Avenanthramides of oats, Nutrition Reviews 67(12): 731–35
4. Emmons CL, Peterson DM, Paul GL (1999) Antioxidant capacity of oat (Avena Sativa L.) extracts. 2. In vitro antioxidant activity and contents of phenolic and tocol antioxidants, Journal of Agricultural and Food Chemistry 47(12):4894-4898
5. F. Willian Collins, W. J. Mullin (1988) High-performance liquid chromatographic determination of Avenanthramides, n-Aroylanthranilic acid alkaloids from oats, Journal of Chromatography A 445: 363-370
6. S. Mayama et al. (1981) The production of phytoalexins by oat in response to crown rust, Puccinia Coronata f. Sp. Avenae, Physiological Plant Pathology 19(2): 217-IN7
7. Leslie Crombie, Jayshree Mistry (1990) The phytoalexins of oat leaves: 4H-3,1-Benzoxazin-4-Ones or amides? Tetrahedron Letters 31(18): 2647–48
8. Ryan T. Koenig et al. (2011) Avenanthramides are bioavailable and accumulate in hepatic, cardiac, and skeletal muscle tissue following oral gavage in rats, Journal of Agricultural and Food Chemistry 59(12): 6438–43
9. Vishwas Tripathi, Anjana Singh, Mohd Tashfeen Ashraf(2018) Avenanthramides of oats: medicinal importance and future perspectives, Pharmacognosy Reviews 12(23): 66

10. David M Peterson, Martha J Hahn, Cheryld L Emmons (2002) Oat avenanthramides exhibit antioxidant activities in vitro, Food Chemistry 79(4): 473–78
11. Li Li Ji et al. (2003) Effects of Avenanthramides on oxidant generation and antioxidant enzyme activity in exercised rats, Nutrition Research 23(11): 1579–1590
12. Vishwas Tripathi, Anhana Singh, Mohd. Tashfeen Ashraf (2018) Avenanthramides of oats: medicinal importance and future perspectives, Pharmacognosy Review 12(23): 66-71
13. Liping Liu et al. (2004) The Antiatherogenic potential of oat phenolic compounds, Atherosclerosis 175(1): 39–49
14. Ryan Koenig et al. (2014) Avenanthramide supplementation attenuates exercise-induced inflammation in postmenopausal women, Nutrition Journal 13: 21
15. Nie L, Wise ML, Peterson DM, Meydani M (2006) Avenanthramide, a polyphenol from oats, inhibits vascular smooth muscle cell proliferation and enhances nitric oxide production, Atherosclerosis 186(2): 260-266
16. Lin Nie et al. (2006) Mechanism by which Avenanthramide-c, a polyphenol of oats, blocks cell cycle progression in vascular smooth muscle cells, Free Radical Biology & Medicine 41(5): 702–8
17. Ellen S. Kurtz, Warren Wallo (2007) Colloidal oatmeal: history, chemistry and clinical properties, Journal of Drugs in Dermatology: JDD 6(2): 167–70.
18. Sur R, Nigam A, Grote D, Liebel F, Southall MD (2008) Avenanthramides, polyphenols from oats, exhibit anti-inflammatory and anti-itch activity, archives of dermatological research 200(10): 569-574
19. H. Boz (2016) Phenolic amides (Avenanthramides) in oats – a Review, Czech Journal of Food Sciences 33(5): 399–404

20. George E. Inglett, Diejun Chen (2012) Antioxidant and pasting properties of oat β-Glucan hydrocolloids, Food and Nutrition Sciences 03(6): 827–35
21. Kirsten Gunther-Jordanland, Corinna Dawid, Maximilian Dietz, Thomas Hofmann (2016) Key phytochemicals contributing to the bitter off-taste of oat (Avena sative L.), Journal of Agricultural and Food Chemistry 64(51): 9639-9652
22. Tinker, Nick & Jackson, Eric & Snyder, Sam & Tiede, Tyler & Azar et al. (2016). Achievements and impact of the Collaborative Oat Research Enterprise (CORE). The Oat Newsletter. 53. 18.
23. Minekus M, Alminger M, Alvito P, Ballance S, Bohn T et al. (2014) A standardised static in vitro digestion method suitable for food - an international consensus, Food Function 5(6): 1113-1124
24. Pedersen AM, Bardow A, Jensen SB, Nauntofte B (2002) Saliva and gastrointestinal functions of taste, mastication, swallowing and digestion, Oral Digestion 8(3): 117-129
25. Chen C, Wang L, Wang R, Luo X, Li Y, Li J, Li Y, Chen Z (2018) Phenolic contents, cellular antioxidant activity and antiproliferative capacity of different varieties of oats. Food Chemistry Jan 15(239): 260-267
26. Multari S, Pihlava JM, Ollennu-Chuasam P, Hietaniemi V, Yang B, Suomela JP (2018) Journal of Agricultural and Food Chemistry. 2018 Mar 21;66(11):2900-2908
27. Li M, Koacher K, Hansen L, Ferruzzi MG (2017) Phenolics from whole grain oat products as modifiers of starch digestion and intestinal glucose transport, Journal of Agricultural and Food Chemistry 2017 Aug 16;65(32):6831-6839

28. Carolien H.M. Versantvoort, Agnes G. Oomen et al. (2005) Applicability of an in vitro digestion model in assessing the bioaccessibility of mycotoxins from Food, Food and Chemical Toxicology 43(1): 31-40
29. Burton DD, Kin HJ, Camilleri M, Stephens DA, Mullan BP (2005) Relationship of gastric emptying and volume changes after a solid meal in humans, American Journal of Physiology-Gastrointestinal and Liver Physiology, 289(2): G261-266
30. Malagelada JR, Go VL, Summerskill WH (1979) Different gastric, pancreatic, and biliary responses to solid-liquid or homogenized meals, Digestive Diseases and Science, 24(2): 101-110
31. Wilsen O, Johansson C (1992) Gastrointestinal function in obesity: motility, secretion, and absorption following a liquid test meal, Metabolism Apr 41(4): 390-395
32. McConnel EL, Fadda HM, Basit AW (2008) Gut instincts: explorations in intestinal physiology and drug delivery, International Journal of Pharmaceutics 364(2): 213-226
33. Lena Hall Dimberg, Kerstin Sunnerheim, Birgitta Sundberg, Kevin Walsh (2001) Stability of oat avenanthramides, Cereal Chemistry Journal 78: 278–281

CHAPTER 3

OAT AVENANTHRAMIDES REDUCE INTESTINAL GLUCOSE ABSORPTION IN STC-1 CELLS BY MODULATING GASTROINTESTINAL BITTER RECEPTORS

Abstract

Oats (*Avena sativa* L.) is a widely consumed cereal and a key component of a healthy diet due to its nutritional benefits on cardiovascular and metabolic health. Bioactive phenolics found in oats such as avenanthramides (AVNs) and phenolic acids are of major interest for the development of new functional foods and dietary interventions for improving glucose metabolism. In this study, we evaluated *in vitro* digests of 242 oat flour samples consisting of 109 accessions from the AFRI CORE world diversity oat panel grown at two different locations (Lacombe, Alberta, CA and Aberdeen, Idaho, US) for their ability to reduce glucose uptake in the STC-1 gastrointestinal cell model. Following a 2 hour exposure, 2-NBDG fluorescent glucose uptake was inhibited by 2-82% relative to baseline (control cells treated with vehicle alone). A weak but statistically significant correlation was established between AVN content of oat digests and the reduction of glucose uptake, suggesting that ANVs are partially responsible for the observed effects. Subsequent dose response studies confirmed glucose absorption lowering effects of the individual AVNs in the order of potency AVNE (1f) > AVNC (2c) > AVNB (2f) >AVNA (2p), as well as bioactivity of the respective hydroxycinnamic acid moieties in a similar potency order of sinapic > ferulic > caffeic > p-coumaric acid in the range of 0.1-10 uM. Diversity of oat cultivars and growth locations provided a wide range of glucose absorption lowering activity *in vitro*, thus yielding a unique tool for future determination of genotypic and environmental contribution of oats to modulation of glucose metabolism.

Introduction

Metabolic disorders affect more than 300 million people worldwide.[1] They are often associated with certain metabolic risk factors such as elevated levels of fasting blood glucose and high postprandial glucose due to insulin insensitivity.[2] Postprandial glucose rise is more reliable in predicting poor glycemic control[3] and correlates better with glycated hemoglobin HbA1C levels, a potential biomarker for the prediction of cardiovascular disease and diabetes.[4] Dietary interventions that acutely lower absorption of glucose in the gastrointestinal tract or enhance tissue uptake of glucose in the body are generally viewed as a better choice for managing postprandial glucose rise than those that work slowly over a long period.[5] Currently high blood glucose is managed with multiple oral antidiabetic agents including acarbose, glibenclamide, metformin, miglitol, rosiglitazone and voglibose, and most of these interventions have undesirable side effects, develop tolerance after continued administration, and/or are too expensive for patients from the developing world.[6]

As the rates of diabetes and obesity grow, the need for foods with improved metabolite profiles that can be used to manage blood glucose level also increases, especially in the category of "substance energy" foods.[7] Among grains, increased levels of soluble fiber and phenolic compounds are considered beneficial to control blood glucose level with dietary adjustments.[8] Phenolic compounds are common in nature as these molecules are often produced as secondary metabolites of plants that act as a primary chemical defense against predators and environmental challenges. Oats is one of the more commonly consumed cereal grain throughout the world.[9] It is an abundant source of macronutrients such as carbohydrates (66%), fats (7%) and proteins (17%).[10] However, lower amounts of bioactive secondary metabolites such as phenolics, organic acids, and phytosterols have an additional and often overlooked beneficial effects on the health

and nutritional values of grains.[11] Among oats secondary metabolites, avenanthramides (phenolic alkaloids), avenacins (triterpenoids), and avenacosides (steroid glycosides) were shown to accumulate in the groats and contribute to bitter or astringent taste of the oat cereal.[12]

Oats have also been observed to have a plethora of positive effects when digested. Several studies illustrated that oats could be to modulate glucose balance and this effect was attributed to presence of soluble β-glucan fibers, leading to the promotion of consuming grains that have high quantities of β-glucan.[10] Other studies showed that oats prevent the alcohol-related increase in intestinal permeability and inhibit endotoxemia and liver damage.[13] Metabolic and anti-inflammatory effects of oats were also often attributed to avenanthramides,[14] thus suggesting their possible use in improving cardiovascular health outcomes.[15] Avenanthramides are generally found in a free phenolic fraction, while their hydroxycinnamic precursor moieties such as ferulic, caffeic, and coumaric acids are reported to be located predominantly in a bound phenolic fraction.[16] Both classes of these phenolic compounds had beneficial functions to human health.[17] For example, it was reported that solution of 1 mg/ml of caffeic or ferulic acid can inhibit glucose transport in the mouse endocrine system.[18] On the other hand, a different report suggested that gastrointestinal Caco-2 cells treated with 100 μM caffeic, p-coumaric, or cholorogenic acids showed no changes in glucose uptake *in vitro*.[19] Application of dihydroandrostane D (DHAvD), a synthetic analog of avenanthramides, to RINm5F cells or islets showed increased resistance of these tissues to cytokine toxicity, inhibition of nitric oxide (NO) production, reduction of NO synthase expression, and improved survival and normalized insulin secretion patterns from beta cells.[20]

Taken together, these results suggested that AVNs and/or their hydroxycinnamic moieties may contribute to maintaining normal levels of blood glucose and insulin secretion, thereby

improving dietary management of chronic metabolic risk factors, but their exact physiological effects and molecular mechanism responsible for these observations remained unclear. In the previous chapter of this thesis, we described accumulation of AVNs in 109 accessions from the AFRI CORE world diversity oat panel grown at two different locations. For this study, we applied in vitro digests obtained from AFRI CORE oat flours and individual AVNs to establish their direct effects on glucose absorption in the gastrointestinal cells and start to characterize their potential molecular targets.

Materials and methods

Chemicals

General laboratory reagents including all materials used for *in vitro* digestion were purchased from Sigma (Saint Louis, MO). All solvents were of HPLC grade and purchased from VWR (Radnor, PA). Water was purified with a Milli-Q water purification system (Millipore, Burlington, MA). Avenanthramide A (2p), B (2f), C (2c), and E (1f) standards were purchased from SynInnova (Edmonton, AB, Canada).

Oats samples and preparation

A set of 242 samples consisting of 109 accessions from the AFRI CORE world diversity oat panel grown at two different locations – Lacombe, Alberta, CA (1) and Aberdeen, Idaho, US (2) – was kindly provided by Dr. Eric Jackson as a part of the Collaborative Oat Research

Enterprise (CORE) initiative[21] in the form of de-hulled and uniformly milled whole grain flour and stored at -20 °C until *in vitro* digestion and cell culture treatments.

Simulated in vitro digestion

Simulated oral, gastric and small intestinal digestion of oat flours was performed following an established protocol with minor modifications.[22] Briefly, SSF (simulated salivary fluid), SGF (simulated gastric fluid) and SIF (simulated intestinal fluid) were made up of the respective electrolyte solutions, enzymes, water and calcium chloride.

A-amylase from human saliva (type IX-A) or porcine pancreas (type VI-B) was prepared as 1500 U/ml stock solution in the SSF fluid. Porcine pepsin was prepared as 25000 U/ml in the SGF fluid. Pancreatin stock solution was prepared based on the trypsin activity as 800 U/ml in the SIF fluid. Fresh bile preparations were made as 160 mM stocks in the SIF solution. Calcium chloride stocks were prepared as 0.3 M solution (0.44 g $CaCl_2x2H_2O$ in 10 ml water).

In the oral digestion phase, 100 mg of oat flour sample was added to 300 µl of water and boiled for 5 minutes, then 280 µl of SSF electrolyte stock solution and 40 µl of salivary α-amylase solution (1500 U/ml) were added. Following the addition of 2 µl of 0.3M $CaCl_2$ and 78 µl water, the reaction was mixed by vortexing to simulate food processing in human mouth for 2 min. The pH of solution was controlled at 6.8 where salivary α-amylase has a highest activity.[23]

In the gastric digestion phase, 600 µl of SGF electrolyte stock solution, 128 µl of porcine pepsin (3850U/mg), 4 µl of 0.3M $CaCl_2$, 16 µl of 1M HCl and 52 µl of water were added to the sample and incubated for 2 hours at 37℃.

In the intestinal digestion phase, 880 µl of SIF electrolyte stock solution, 400 µl of pancreatin (800U/ml), 200 µl of fresh bile (160 mM of taurocholic acid), 3.2 µl of 0.3M $CaCl_2$,

12 µl of 1M NaOH and 104.8 µl of water were added to the gastric chyme and digested for additional 2 hours at 37℃. After digestion was completed, the solution was water bathed for 15 minutes in 80℃ to inactivate the enzymes and then centrifuged at 2000 rpm for 10 minutes. Supernatant liquid was transferred to a new vial, frozen at -80℃, and freeze-dried to obtain a powder sample of water-soluble metabolites released from the digested oat flour.

Gastrointestinal STC-1 cell culture model

The mouse neuroendocrine intestinal cell line STC-1 (CRL-3254) that acts as a model for glucose absorption and hormone secretion was obtained from ATCC (Manassas, VA). Cells were routinely passaged every 3-4 days and maintained in high glucose DMEM containing 10% fetal bovine serum (Life Technologies, Carlsbad, CA) and 1% penicillin-streptomycin (Fisher Scientific, Pittsburg, PA) at 37 °C and 5% CO_2. Cells were sub-cultured into 24-well plates and, once confluent, changed to induction medium that contained glucose-free DMEM supplemented with 2 mM sodium pyruvate to induce fluorescent glucose uptake. Treatments with oat flour digests were administered for 2 hours before cells were exposed to fluorescent 2-NBDG analog of glucose as described below.

Glucose uptake measurements using fluorescent 2-NBDG analog

2-NBDG (2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose) is a fluorescently labeled 2-deoxyglucose analog for monitoring glucose uptake in living cells. It is transported according to Micharelis-Menten kinetics, similar to glucose, and it is compatible with fluorescence techniques such as flow cytometry.[24] Intracellular 2-NBDG shows a strong fluorescence at 542 nm and is excited at 467 nm. It was used in this project to directly detect and

quantify glucose transport in STC-1 cell culture model. Oat flour digests prepared as 50mg/ml stocks in PBS were added into 1-ml batches of STC-1 cells in duplicate and incubated at 37℃ for 30 minutes. Next, 25 µl of 2-NBDG from a 5mM (1.71mg/ml) master stock in PBS were added to the control (baseline vehicle blank) or digest-treated STC-1 cells, incubated for 30 min, centrifuged and washed to removed excessive 2-NBDG, and the fluorescence of intracellular 2-NBDG was measured using flow cytometry (BD Accuri C6, San Jose, CA). Absolute glucose fluorescence intensity values obtained from FL1 fluorescent channel were normalized to baseline blank fluorescence signal and compared between cells in the treatments and baseline blank groups.

Statistical analysis

Statistical analyses were performed using Prism 6.0 (GraphPad Software, San Diego, CA) and expressed as mean ± SEM. Two tailed t-test or one-way ANOVA were applied as necessary at a significance level of $P < 0.05$. Post-hoc analyses of differences between individual experimental groups were made using the Tukey's multiple comparison test.

Results and discussion

Oats have a long history of human use, both as a food and as a nutritional intervention to control glucose metabolism, health of the gastrointestinal system, and inflammation of epithelial tissues of the skin and the gut.[9] This activity is generally attributed to high amounts of the soluble β-glucan fibers that slow down carbohydrate digestion and delay glucose absorption in the gastrointestinal tract.[10] Oat groats, however, also contain a variety of other bioactive

secondary metabolites, including several groups unique to this plant: avenanthramides (phenolic alkaloids), avenacins (triterpenoids), and avenacosides (steroid glycosides), all contributing to bitter or astringent taste of the oat cereal.[12]

Phenolic compounds are common in nature, and many of them have been shown a contribution to bitterness and astringency in foods.[25] They are produced by secondary metabolism of plants, as a chemical defense against predators, and have proven to have beneficial effects on human body.[26] Oats are also a rich source of phenolic acids (caffeic, coumaric, ferulic, vanillioc, and sinapic acid being the most abundant), but these compounds are not unique to oats, and are also available in high concentrations from other plant foods. P-coumaric acid has been perceived as bitter at a threshold of 48 ppm,[27]similar to the ferulic acid that was proven to be more bitter than vanillic acid.[25] Sinapic acid has been perceived both as sour as well as bitter and astringent,[28] and caffeic acid has been shown to easily form esters in the form of the cholorogenic acid found in high concentrations in coffee seed sand partially responsible for the bitter taste of coffee.[29]

Fluorescent glucose uptake in STC-1 cells in response to oat flour digests

Aqueous digest of 109 AFTI CORE phenotypic oats panel grown in two locations (244 oat flour samples overall) were freeze-dried and applied as treatments to the *in vitro* STC-1 cell culture model of the gastrointestinal glucose uptake. Total AVN content showed a weak significant correlation ($r=-0.32$, $p<0.001$) to reduction of fluorescent glucose uptake, suggesting that increase in this trait in oat cultivars is beneficial to enhance their glucose modulation properties (**Figure 3.1**). Weak correlation, however, also suggested a large environmental

(location) influence that drove these responses, as well as the likely presence of other secondary metabolites that contributed to the overall biological activity of the digested oat flours.

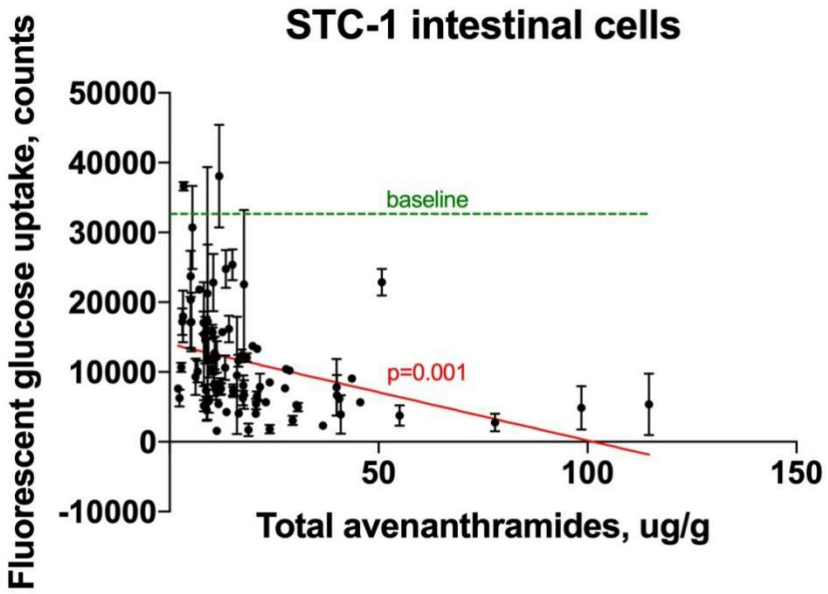

Figure 3.1. Fluorescent 2-NBDG glucose uptake in the STC-1 intestinal cell model.

Exposure to aqueous oat digests reduced fluorescent glucose absorption in the intestinal cells. Cells were incubated with treatments for 2 hours, presented with 2-NBDG for 30 min, and fluorescence was quantified at excitation/emission of 465/540 nm.

Flow cytometry is a technique to detect and measure cell physical and chemical characteristics. This technique combines sample size, data acquisition speed, accuracy and measurement diversity.[30]A flow cytometer is similar to a microscope, but instead of producing the cell images, it provides automated quantifications of specific parameters. It can illuminate cells when they flow in a light source then detect and correlate the signals from them.

A variety of fluorophores can be used as markers in flow cytometry. Fluorophores, which can absorb light of a specific wavelength and re-emit light of longer wavelengths, are usually attached to the target molecule or biomarker under investigation. Every fluorophore has a specific peak excitation and emission wavelength, so the combination of labels depends on the wavelength of lamp and the available detector. [31]Flow cytometry uses fluorescence as a quantitative tool, and a C6 BD Accuri flow cytometer was used to measure the glucose level in this part of project. The data from flow cytometry events was stored as a list mode file, which pointed to associated data file that contained 7 parameters in the list mode file, including forward scatter (FSC) and side scatter (SSC).[32]

Dose range studies with individual avenanthramides

To confirm these observations, a quantitative analysis of glucose absorption in the STC-1 mouse intestinal cell model was performed to determine which individual AVNs are most effective at modulating this process. Decreased glucose uptake was clearly evident from emission intensity data of fluorescent glucose analog 2-NBDG accumulation in STC-1 cells (**Figure 3.2**). Among bioactive constituents tested, all AVNs showed moderate to strong degree of suppression of intestinal glucose intake in the dose range of 1-10 μM and the potency order of AVNE > AVNC > AVNB > AVNA.

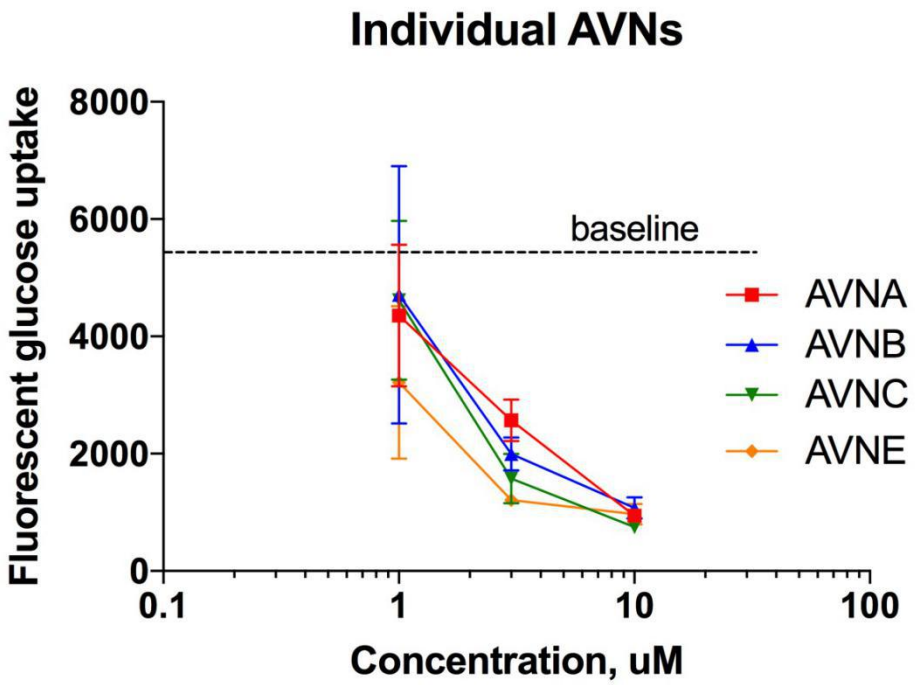

Figure 3.2. Fluorescent 2-NBDG glucose uptake in the STC-1 intestinal cells treated with individual AVNs. Dose-dependent effects of oats AVNs on 2-NBDG glucose absorption in the dose range of 1-10 μM.

Dose range studies with hydroxycinnamic acid moieties

The ability of AVNs treatments to reduce gastrointestinal glucose absorption *in vitro* prompted further analysis of this biological activity in different hydroxycinnamic acids that form the variable part of the AVN molecule. While all AVNs share the anthranilic acid moiety, avenanthramides AVNB (2f) and AVNE (1f) are conjugated to ferulic acid, AVNC (2c) is conjugated to caffeic acid, and AVNA (2p) is conjugated to p-coumaric acid. While another hydroxycinnamic acid such as sinapic acid is also produced in oats, it does not form the amides with the anthranilic acid moiety. All hydroxycinnamic acids used in this study decreased glucose

absorption in the gastrointestinal cells over a dose range of 1–10 μM (**Figure 3.3**), although p-coumaric acid showed a weaker biological activity compared to the rest of the acids tested.

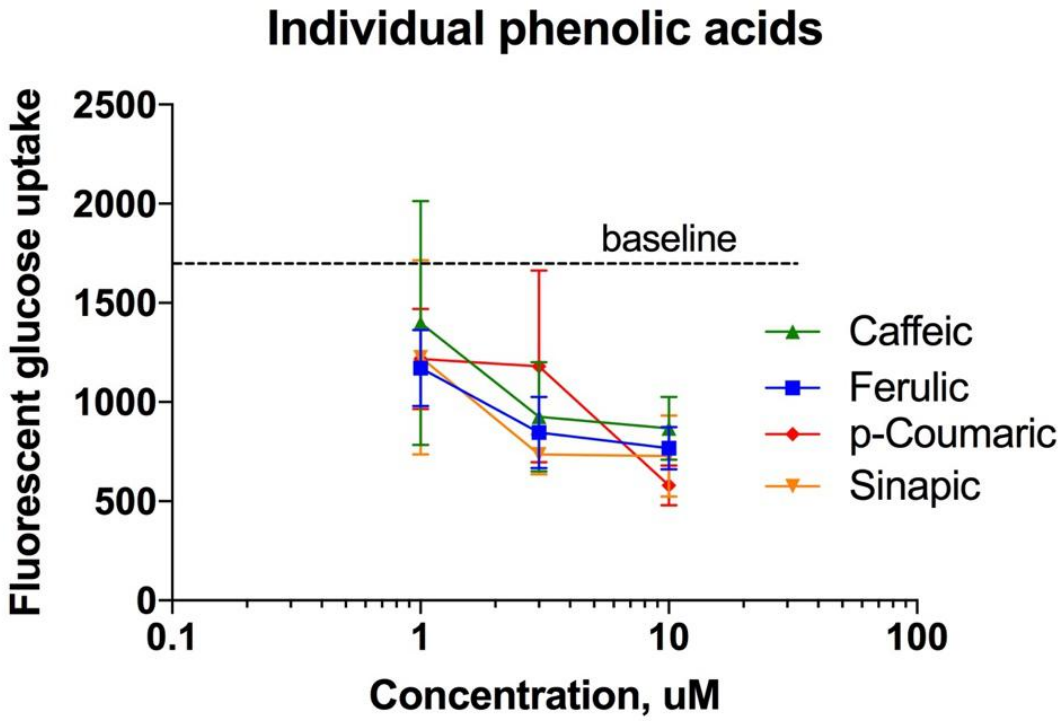

Figure 3.3. Fluorescent 2-NBDG glucose uptake in the STC-1 intestinal cells treated with individual phenolic acids. Dose-dependent effects of oats phenolic acids on 2-NBDG glucose absorption in the dose range of 1-10 μM.

Inhibition of bitter taste receptors with oat flour digests

Next, we took the first step to understand the molecular mechanisms responsible for reduction of glucose absorption by gastrointestinal cells exposed to oat flour digests. Probenecid is an FDA-approved inhibitor of the multidrug resistance protein 1 (MRP1) transporter, clinically used to treat human gout. Greene et al found that it can specifically inhibit cellular response mediated by human bitter taste receptors hTAS2R16, hTAS2R38, and hTAS2R43. [33]Control

experiments in which STC-1 cells were exposed to selected oat flour digests with and without 10 μM probenecid confirmed involvement of bitter receptor signaling in glucose absorption response observed in the gastrointestinal cells. In majority of the treatments, addition of probenecid resulted in increased glucose uptake (**Figure 3.4**).

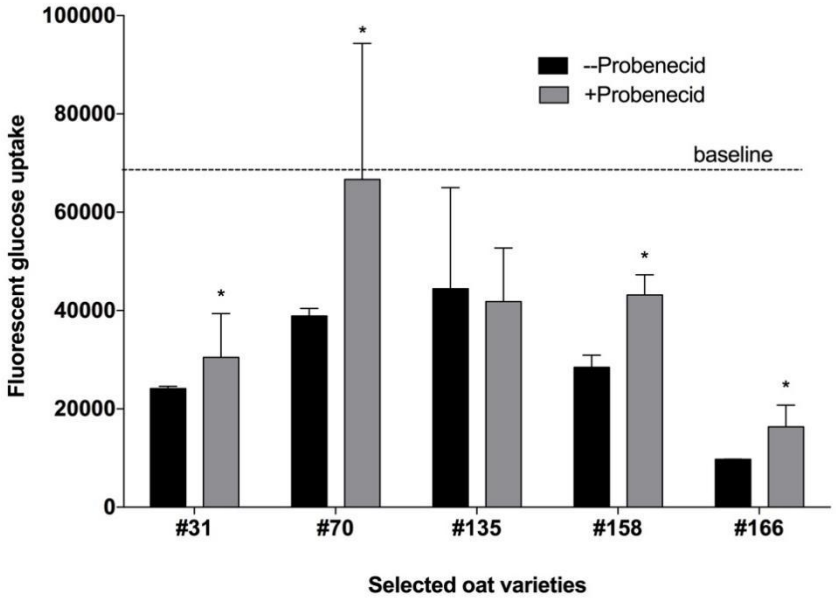

Figure 3.4. Fluorescent 2-NBDG glucose uptake in the STC-1 intestinal cells treated with selected oat flour digests. Glucose absorption effects of oats digests were inhibited by co-exposure to an allosteric inhibitor of bitter receptors probenecid (10 μM).

Inhibition of bitter taste receptors with AVNs and phenolic acids

To further explain this observation, we hypothesized that bitter and/or astringent tasting AVNs and phenolic acids present in oat digests contributed to suppression of gastrointestinal glucose absorption by activating gastrointestinal bitter taste receptors (TAS2Rs). Indeed, co-treatment of STC-1 cells with probenecid, an allosteric inhibitor of TAS2Rs, significantly

decreased efficacy of all AVNs tested (**Figure 3.5**) and decreased the suppressing effects of phenolic acids on glucose uptake in STC-1 cells (**Figure 3.6**).

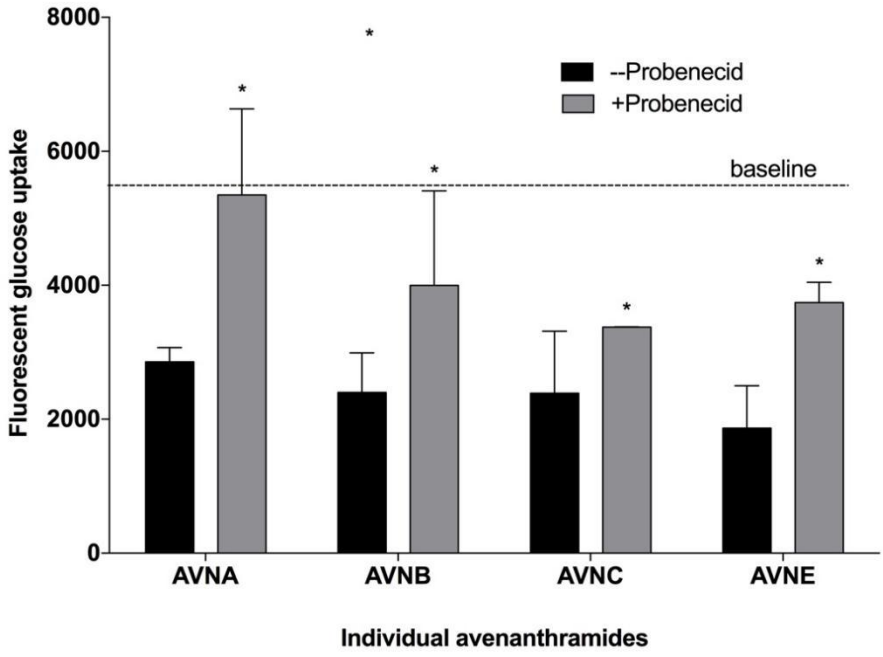

Figure 3.5. Fluorescent 2-NBDG glucose uptake in the STC-1 intestinal cells treated with individual AVNs. Glucose absorption effects of AVNs were inhibited by co-exposure to an allosteric inhibitor of bitter receptors probenecid (10 μM).

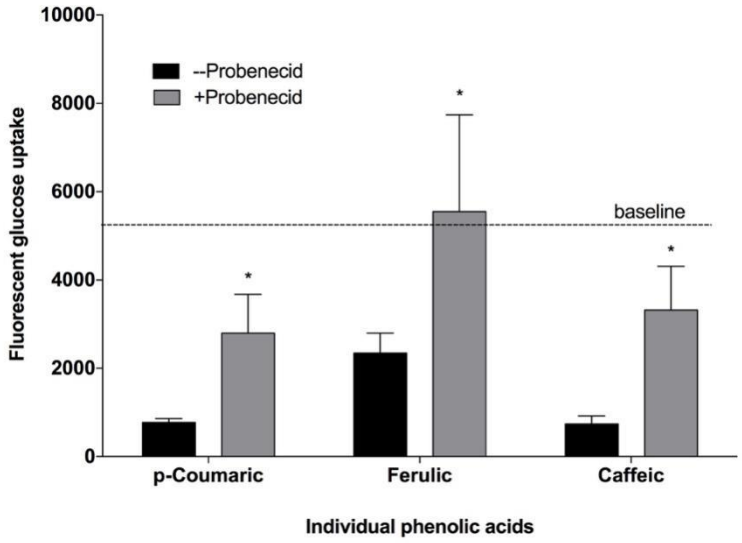

Figure 3.6. Fluorescent 2-NBDG glucose uptake in the STC-1 intestinal cells treated with individual phenolic acids. Glucose absorption effects of phenolic acids were inhibited by co-exposure to an allosteric inhibitor of bitter receptors probenecid (10 μM).

Taste receptors found in the gastrointestinal tract are differentiated into two classes of G-protein coupled receptors (GPCRs): TAS1R and TAS2R. TAS1Rs are responsible for the detection of sweet stimuli and TAS2Rs are responsible for the bitter flavor profile.[34] When stimulated, TAS1R and TAS2R activate phospholipase C β2, which lead to the activation of Inositol triphosphate (IP3) release of Ca2+.[35] These events result in action potential generation allowing the nervous system to communicate with the endocrine system to release hormones and small peptides that control glucose homeostasis. These molecules include: glucagon-like peptide-1 (GLP-1), glucagon, neuropeptide Y, peptide YY (PYY), cholecystokinin (CCK), vasoactive intestinal peptide, insulin, and ghrelin.[35] The exact mechanism following the initial binding of a bitter principle to TAS2R until the receptor signaling cascade is not fully understood. This is due

to the fact that the human genome encodes 25 different TAS2Rs, and this level of genetic variation gives rise to multiple structural and functional differences that are difficult to account for.

TAS2R is of particular interest to our studies because of close human relationship with bitter compounds. This is due to the fact that humans are quite sensitive to bitter tasting foods due to the possible evolutionary advantage of being able to avoid potential toxins, which often have a bitter profile. In another study,[35] a single nucleotide polymorphisms (SNP) and allelic variations in the TAS2R receptor gene markedly affected its affinity for the binding of the ligand, in this case a bitter agonist. Two TAS2R9 receptors, an intact natural receptor and the mutated TAS2R9 with a SNP designation of rs3741845 were directly compared for their ability to bind bitter substances. This SNP corresponds to a missense mutation resulting in a valine residue in place of alanine at the 187th amino acid position. This region has been suggested to have an impact on the binding affinity of the bitter ligand because the mutation occured within the binding pocket of the receptor.[36] With no exceptions, the wild type TAS2R9 bound all three ligands (ofloxacin, procainamide, and pirenzepine) with a much higher affinity than the mutated one. It was also established that NCI-H716 cells expressing TAS2R9 receptors drastically decreased GLP-1 secretion following α-gustducin silencing RNA with siRNA. α-Gustducin is an important intermediary in the molecular signaling cascade that leads from bitter receptor activation to GLP-1 production.[37] Thus, this study gave evidence of the specificity of the TAS2R receptor, examples of appropriate ligands that may bind to it, and evidence of glucose modulating molecules being produced as a result of its activation.

Another interesting example of using a bitter tasting food to control blood glucose levels is bitter melon. [38]Studies showed a significant effect of bitter melon on glucose level by controlling the

levels of fructosamine,[39] therefore providing additional evidence on the potential use of bitter molecules to improve glucose metabolism.

Conclusions

Reducing the glucose absorption in pre-diabetic and diabetic patients to manage hyperglycemia can be a powerful tool in preventing the onset of insulin resistance and associated metabolic disease risks. The selected AFRI CORE world diversity oat panel cultivars inhibited gastrointestinal glucose absorption by 2-82% relative to baseline (control cells treated with vehicle alone). A weak but statistically significant correlation was established between AVN content of oat digests and the reduction of glucose uptake, suggesting that ANVs are partially responsible for the observed effects. Direct inhibition of gastrointestinal glucose absorption by dietary AVNs and phenolic acids via multiple mechanisms, including bitter taste receptor activation, is a very attractive approach to diet- or lifestyle-based modification of metabolic diseases. However, it is not clear how bitter taste receptors transduce this signal to glucose transporters responsible for the glucose uptake in the cell. This question warrants further investigation of dietary AVNs and oat-based foods to understand best dosing and regimen strategies to achieve maximal beneficial effects in controlling glucose metabolism.

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REFERENCES

1. Zimmet P, Alberti KG, Shaw J (2001) Global and societal implications of the diabetes epidemic, Nature 414(6865): 782-787
2. Pfeifer MA, Halter JB, Porte D Jr (1981) Insulin secretion in diabetes mellitus, The American Journal of Medicine 70(3): 579-588
3. Avignon A, Radauceanu A, Monnier L (1997) Nonfasting plasma glucose is a better marker of diabetic control than fasting plasma glucose in type 2 diabetes, Diabetes Care 20(12): 1822-1826
4. Soonthornpun S, Rattarasam C, Leelawattana R, Setasuban W (1999) Postprandial plasma glucose: a good index of glycemic control in type 2 diabetic patients having near-normal fasting glucose levels, Diabetes Research and Clinical Practice 46(1): 23-27
5. P arkin C (2002) Is postprandial glucose control important? Is it practical in primary care settings?, Clinical Diabetes20(2):71–6.
6. Krentz AJ, Bailey CJ (2005) Oral antidiabetic agents: current role in type 2 diabetes mellitus, Drugs 65(3): 385-411
7. Ludwig DS (2002) The glycemic index: physiological mechanisms relating to obesity, diabetes, and cardiovascular disease, The Journal of the American Medical Association 287(18): 2414-2423
8. Fardet A (2010) New Hypotheses for the health-protective mechanisms of whole-grain cereals: What Is beyond Fibre?, Nutrition Research Reviews 23(1): 65-134
9. Singh R, De S, Belkheir A (2013) Avena sativa (Oat), a potential neutraceutical and therapeutic agent: an overview, Critical Reviews in Food Science and Nutrition 53(2): 126-144

10. Bioklund M, van Rees A, Mensink RP, Onning G (2005) Changes in serum lipids and postprandial glucose and insulin concentrations after consumption of beverages with beta-glucans from oats or barley: a randomised dose-controlled trial, European journal of clinical nutrition 59(11): 1272-1281
11. Sladana Zilic, Vesna Hadzi-Taskovic Sukalovic, Dejan Dodig, Vuk Maksimovic, Milan Maksimovic Zorica Basic (2011) Antioxidant activity of small grain cereals caused by phenolics and lipid soluble antioxidants, Journal of Cereal Science, 54(3), 417-424
12. Bekzod Khakimov, Birthe Moller Jespersen, Soren Balling Engelsen (2014) Comprehensive and comparative metabolomic profiling of wheat, barley, oat and rye using gas chromatography-mass dpectrometry and sdvanced chemometrics, Foods, 3(4), 569-585
13. Keshavarzian A, Choudhary S, Holmes EW, Yong S, Banan A, Jakate S, Fields JZ (2001) Preventing gut leakiness by oats supplementation ameliorates alcohol-induced liver damage in rats, Jounal of Pharmacol and Experimental Therapeutics 299(2):442-448
14. Meydani M (2009) Potential health benefits of avenanthramides of oats, Nutrition Reviews 67(12): 731-735
15. H. Boz (2016) Phenolic amides (Avenanthramides) in oats – a Review, *Czech Journal of Food Sciences* 33(5): 399–404
16. Vito Verardo et al. (2011) Free and bound minor polar compounds in oats: different extraction methods and analytical determinations, *Journal of Cereal Science* 54(2): 211–1
17. L. H. Dimberg, O. Theander, H. Lingnert (1993) Avenanthramides---A group of phenolic antioxidants in oats, Cereal Chemistry 70: 637-641

18. Welsch CA, Lachance PA, Wasserman BP (1989) Dietary phenolic compounds: inhibition of Na+-dependent D-Glucose uptake in rat intestinal brush border membrane vesicles, Journal of Nutrition 119(11): 1698-1704
19. Kelly Johnston et al. (2005) Dietary polyphenols decrease glucose uptake by human intestinal Caco-2 cells, FEBS Letters 579(7): 1653–57
20. Na Lv, Mi-Young Song, Young-Rae Lee, Ha-Na Choi (2009) Dihydroavenanthramide D protects pancreatic β-cells from cytokine and streptozotocin toxicity, Biochemical and Biophysical Research Communications 387(1): 97-102
21. Tinker, Nick & Jackson, Eric & Snyder, Sam & Tiede, Tyler & Azar et al. (2016). Achievements and impact of the Collaborative Oat Research Enterprise (CORE). The Oat Newsletter. 53. 18.
22. Minejus M, Alminger M, Alvito P, Ballance S, Bohn T et al. (2014) A Standardised static in vitro digestion method suitable for food – an international consensus - Food & Function5(6): 1113-1124
23. Pedersen AM, Bardow A, Jensen SB, Nauntofte B (2002) Saliva and gastrointestinal functions of taste, mastication, swallowing and digestion, Oral Diseases 8(3): 117-129
24. Tao J, Diaz RK, Teixeira CR, Hackmann TJ (2016) Transport of a fluorescent analogue of glucose (2-NBDG) versus radiolabeled sugars by rumen bacteria and escherichia Coli, Biochemistry 55(18): 2578-2589
25. Duizer LM, Langfried A (2016) Sensory characterization during repeated ingestion of small-molecular-weight phenolic acids, Journal of the Science of Food and Agriculture96(2): 513-521

26. Zhang J, Li L, Kim SH, Hagerman AE, Lu J (2009) Anti-cancer, anti-diabetic and other pharmacologic and biological activities of Penta-Galloyl-Glucose, Pharmaceutical Research 26(9): 2066-2080
27. C.J. Huang, J.F. Zayas (1991) Phenolic acid contributions to taste characteristics of corn germ protein flour products, Journal of Food Science 56(5): 1308–10
28. F. Ismail, M. Vaisey-Genser, B. Fyfe (1981) Bitterness and astringency of Sinapine and its components, Journal of Food Science 46(4): 1241–44
29. Adriana Farah, Carmen Marino Donangelo (2006) Phenolic compounds in coffee, Brazilian Journal of Plant Physiology 18(1): 1677-9452
30. Givan AL (2011) Flow cytometry: An introduction, Methods in Molecular Biology 699: 1-29
31. David A. Basiji, Willian E. Ortyn, Luchuan Liang, Vidya Venkatachalam, Philip Morrissey (2007) Cellular image analysis and imaging by flow cytometry,Clinics in Laboratory Medicine 27(3): 653-viii
32. Zou C, Wang Y, Shen Z (2005) 2-NBDG as a Fluorescent Indicator for Direct Glucose Uptake Measurement, Journal of Biochemical and Biophysical Methods 64(3): 207-215
33. Greene TA, Alarcon S, Thomas A, Berdougo E, Doranz BJ, bRESLIN pa, Rucker, JB (2011) Probenecid inhibits the human bitter taste receptor TAS2R16 and suppresses bitter perception of Salicin,PLoS ONE 6(5): e20123
34. Depoortere I (2014) Taste receptors of the gut: emerging roles in health and disease, Gut 63(1): 179-190
35. Cedrick D. Dotson, Lan Zhang Hong Xu, Yu-Kyong Shin et al. (2008) Bitter taste receptors influence glucose homeoastasis, PLoS ONE 3(12): e3974

36. Lagerstrom MC, Schioth HB (2008) Structural diversity of G protein-coupled receptors and significance for drug discovery, Nature Reviews Drug Discovery 7(4): 339-357
37. Jang HJ, Kokrashvili Z, Theodorakis MJ, Carlson OD et al. (2007) Gut-expressed gustducin and taste receptors regulate secretion of glucagon-like peptide-1, Proc Natl Acad Sci U S A 104: 15069-15074
38. Kathy Abascal, Eric Yarnell (2005) Using bitter melon to treat diabetes, Alternative and Complementary Therapies 11(4): 179–84
39. Anjana Fuangchan et al. (2011) Hypoglycemic effect of bitter melon compared with Metformin in newly diagnosed type 2 diabetes patients, Journal of Ethnopharmacology 134(2): 422–428

SUMMARY AND SUGGESTIONS FOR FUTURE WORK

Since bitter principles in foods are often aversive to the consumer, the food industry routinely removes them from diet through selective breeding and debittering. However, receptors for the bitter taste (TAS2Rs) are widely expressed in many parts of human body including lungs and the gastrointestinal tract, where they may be responsible for regulating immune and metabolic health outcomes. Emerging empirical evidence indicates that TAS2Rs are an integral part of the antimicrobial immune response in the upper respiratory tract infection and possess biological activity by modulating TAS2R-dependent metabolism and digestion (**Chapter 1**).

Oat is a unique cereal among the whole grains. Among oats secondary metabolites, avenanthramides (phenolic alkaloids), avenacins (triterpenoids), and avenacosides (steroid glycosides) were shown to accumulate in the groats and contribute to bitter or astringent taste of the oat cereal. This study quantified AVN composition and variability in 109 AFRI CORE world diversity oat panel cultivars grown in two different locations, and identified target cultivars that could be specifically utilized in the development of novel nutritional and dietary formulations to supplement human diet with high levels of bioactive AVNs (**Chapter 2**). The study also showed that AVNs may contribute to maintaining normal levels of blood glucose by reducing its absorption in the gut, in part by activating bitter tasting receptors on the gastrointestinal cells (**Chapter 3**). The exact physiological effects and molecular mechanism responsible for this biological activity warrant further investigation in preclinical and human studies.

Taken together, this data suggests that dietary interventions based on oat AVNs may lower absorption of glucose in the gastrointestinal tract and may offer an alternative strategy to manage postprandial glucose rise in healthy and metabolically challenged individuals.