UNIVERSITÀ CATTOLICA DEL SACRO CUORE
Sede di Piacenza

Scuola di Dottorato per il Sistema Agro-alimentare

Doctoral School on the Agro-Food System

cycle XXVI

S.S.D: AGR15; AGR18; BIO11

THE ROLE OF DIETARY STARCH AND NON-STARCH POLYSACCHARIDES IN SWINE PERFORMANCE AND NUTRIENT RECEPTORS GENE EXPRESSION

Candidate: Marcin Rzepus
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Academic Year 2013/2014

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γηράσκω δ' αἰεὶ πολλὰ διδασκόμενος

Table of contents

Words index

AA	Amino Acid
AACC	American Association for Clinical Chemistry
ADFI	Average daily feed intake
ADG	Average daily gain;
AUC	Area under the curve
AX	Arabinoxylan
BCFA	Branched-chain fatty acid (sum of iso-butyric and iso-valeric acid)
BG	Beta-glucan
BW	Body weight
C_∞	Final *in vitro* digestion coefficient
CCNFSDU	The Codex Committee on Nutrition and Foods for Special
cDNA	Complementary DNA
C_o	*In vitro* digestion coefficient at 0 min,
CP	Crude protein
C_t	I*n vitro* digestion coefficient of starch at time t
DF	Dietary fibre
DM	Dry matter
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EXP	Exponential model
GC	Gas chromatography
GI	Glycaemic index
HI	Hydrolysis index
k	Process rate
LAG	discrete lag phase;

LCFA	Long-chain fatty acid
MCFA	Medium-chain fatty acid
MM	Mentel model
NS	Not significant
NSP	Non-starch polysaccharide
OM	Organic matter
pGI	Predicted glycaemic index
RNA	Ribonucleic acid
RS	Resistant starch
SCFA	Short-chain fatty acid
SEM	Standard error of mean
T/2	Half-time to asymptote
TS	Total starch
V_f	Final asymptotic gas volume;
VFA	Volatile-fatty acid

1. General introduction

Plant carbohydrates are primary source of energy for both humans and livestock. The need to improve and optimize the efficiency of starch digestion is an important research focus in animal nutrition. In monogastric animals, such as swine, starch from cereals grains and theirs co-products is mainly digested in the small intestine. However, some fractions of carbohydrates known as resistant starch (RS) or fibrous non-starch polysaccharide can escape enzymatic digestion, reach the large intestine to be a source of fermentable material for microbiota.

In vivo studies on digestibility and performance represent the natural methods with appropriate environment to obtain necessary data and information. However, they are time-consuming and expensive and requires a large number of animal subjects, sometimes with specific features. A good alternative for the *in vivo* trials are *in situ* and *in vitro* techniques used to estimate starch digestion potential of feed grains. In swine nutrition, *in vitro* methods, proposed in the last decade, are based on multi-enzymatic reactions mimicking the digestion processes occurring in the gastro-intestinal tract. Besides some limitations, *in vitro* procedures have been shown to accurately and precisely estimate the metabolic response of starch digestion in swine. Efforts have been put to study the complexities of starch digestion kinetics using several mathematical models.

Cereals and their co-products included into diets are choose on the basis of their nutrient composition, usually without taking into account possible differences in palatability. Recent trials proved that feed preference in pigs is affected by cereal type, inclusion rate and diet form. Such approach is strategic, particularly during critical stages like weaning, and could help to improve feed intake. The past several years, since the discovery of taste receptors in the early 2000s, have been an stimulating time for the field of taste biology and the coming years will most likely remain still very rich in research activities. Mammals distinguish the five basic taste qualities, sweet, sour, umami, salty, and bitter, to assess the quality of consumed food. But recent findings challenge the canon of five basic sensations, strongly suggesting significant role of taste in dietary fatty acids perception. The sensory cells that

allow taste perception, the taste receptor cells, are distributed in the oral cavity, with the tongue being the prime taste organ. When the main role of tongue is to taste flavours of food before ingestion, a spread network of receptors are present also along the gastrointestinal system, within enteroendocrine cells population, to sense and transmit the information to regulatory systems, which adjust activities according to the luminal content. Consequently, the composition of the diet can affect the way in which gut hormones, secreted by entero-endocrine, could affect the gastrointestinal physiological mechanisms influencing nutrient uptake, animal growth and development.

The present work has been divided into two parts. At first, research projects attempt to evaluate technologies that increase digestibility of energy and other nutrients in cereal grains and their co-products. Differences in starch digestibility have been attributed to various factors, including the the type of corn endosperm, the presence of proteins and prolamins, the presence of lipids, the amylose-amylopectin ratio, the starch granule structure, the particle size, the conservation and processing methods. Use of various processing techniques and exogenous enzymes could improve the nutritional value of feed. Studies were carried out to select barley verities, which could partially or totally replace corn in swine nutrition. Even if, barley contains anti-nutritional factors such as arabinoxylans and β-glucans (non-starch polysaccharides, NSP), supplementation of exogenous NSP enzymes capable of degrading polysaccharide cage structures around nutrients and reduce the viscosity of the intestinal contents improving the availability and digestibility of nutrients, and at the end the animal performance. Additionally, these trials are in the line with The European Environment Agency indications for sustainability, as Europe's water resources are seriously threatened. Barley belongs to cereals which in contrary to corn do not require long-term irrigation for efficient production. Therefore, agriculture sector could possibly achieve some benefits in terms of water usage performance.

Second part of my work was dedicated to investigate the sensors of diet compounds which could be considered as potential markers to monitor nutrition status of organism. Activation of these receptors is believed to influence the hunger-satiety cycle, and their expression levels may be altered by dietary composition. Thus, the aim was to investigate for the first time the effect of arabinoxylans (AX) and

β-glucans (BG) on the relative level of expression of carbohydrates, amino and fatty acid nutrient sensors genes in porcine oral and non-oral tissues. Moreover a special chapter describe pattern of bitter receptors family (Tas2R) along gastrointestinal tract of pig.

2. Literature review

2.1. Carbohydrates

Carbohydrates are the most important energy source in human nutrition, with the general formula $(CH_2O)_n$. They comprise a group of substances with different structures and varying physical, chemical, and physiological properties. The type and composition of dietary carbohydrates vary greatly among different food products. In this way they have wide range of influence on the food texture and flavor or variability and palatability of diet (Giuberti et al., 2014; Zijlstra and Jha, 2012).

Population studies have shown that general carbohydrates supply in developed countries is approximately 45% of energy requirements and up to 85% in developing countries. They are considered as a fundamental source of nourishment and an inexpensive and versatile staple of the diet. The major sources of carbohydrates are cereals consisting of more than 50% of carbohydrates consumed in both developed and developing countries, followed by sweeteners, root crops, pulses, vegetables, fruits, and dairy products. Cereal grains produce a one seeded dry fruit termed a caryopsis, but usually called grain or kernel. Nutritionally these grains are a perfect source of carbohydrates and other components such as lipids, proteins, vitamins and minerals. Dietary carbohydrates are important in maintaining glycemic homeostasis and gastrointestinal health. The current global emphasis for healthy eating is on increasing the carbohydrate consumption, particularly in the form of rich in fibre whole grains, fruits, and vegetables. Besides, they bind required micronutrients, phytochemicals, and antioxidants. Epidemiological and clinical research have presented a positive association between some carbohydrate consumption and reduced risk of chronic diseases and certain types of cancer (Björck et al., 2012; FAO, 2011).

Dietary carbohydrates can be mainly found in the form of sugar (mono- and disaccharides) and starch or non-starch polysaccharides. Additionally, in the food industry, they can be used in the form of hydrolyzed corn starch, high-fructose corn syrups, modified starches, gums, and sugar alcohols. Depending on their chemical structure and according to their degree of polymerization, carbohydrates

are classified into four categories: monosaccharaides, disaccharides, oligosaccharides, and polysaccharides. For this study two kind of polysaccharides are important – starch and non-starch polysaccharides.

2.1.1. Monosaccharaides

Monosaccharaides are the simplest form of carbohydrate and cannot be further hydrolysed to smaller subunits. According to their chain length, monosaccharaides fall into several categories, the more nutritionally important being the pentoses (5-carbon ring), e.g., ribose, and the hexoses (6-carbon ring), e.g., glucose. They are commonly called "sugars" together with disaccharides.

The presence of asymmetrical carbons in monosaccharaides with different functional groups attached gives rise to optical activity. Monosaccharaides are optically active, which means that if polarized light is passed through a solution of these compounds, the plane of light will be rotated to the left (levorotatory or L-form) or to the right (dextrorotatory or D-form). Consequently, similar structures of the same compound are formed and are called stereoisomers. Monosaccharaides of the D-form are nutritionally important because most naturally occurring monosaccharaides are D-stereoisomers and metabolic and digestive enzymes are specific for them.

Monosaccharaides demonstrate another type of stereoisomerism due to their formation of cyclic structures. The pentoses form furanose and the hexoses form pyranose. Cyclization can produce two stereoisomers of the α and β configuration, and generally an equilibrium mixture of the straight and the cyclic forms exists in monosaccharide solutions. The compounds with different isomerization have different properties and also metabolic importance, as enzymes are specified to bind with particular form.

The most common monosaccharaides are glucose fructose, and galactose. Glucose (or dextrose), is the most nutritionally important and abundant monosaccharide: it is the major cell fuel and the

principal energy source for the brain. Glucose constituite the building block of several polysaccharides and it can also be found unbound in body tissues and fluids.

Glucose is found in fruits, honey, maple syrup and vegetables, but could be also formed from sucrose hydrolysis. The properties of glucose are important for improving food texture, flavour, and palatability. Fructose, on the other hand, is commonly used as a sweetener in soft drinks, bakery products, and candies, in the form of high-fructose corn syrups. Galactose is found primarily in milk and dairy products. Other most important monosaccharaides such as D-Ribose and D-Desoxyribose are constituent of RNA and DNA, respectively (Ernst et al., 2000; FAO/WHO, 1998; Pigman et al., 1970; Stylianopoulos, 2013).

2.1.2. Disaccharides

Disaccharides made up of two monosaccharide units, linked together with glycosidic bonds in the α or β orientation. The most common disaccharides are sucrose, lactose, and maltose. Sucrose is the most abundant and consists of a molecule of α-glucose and β-fructose linked together. Is mostly found in sugarcane and beet, and in lower amounts in honey, maple sugar, fruits, and vegetables. The properties of sucrose are important in improving viscosity, sweetness, and flavour of baked foods, ice cream, and desserts. Maltose is mainly produced by partial hydrolysis of starch and consists of two glucose units linked by an α-1,4-glycosidic bond. It is also produced from the germination of grain for malt liquors. Lactose is found in milk and dairy products and consists of galactose and glucose linked by a β-1,4-glycosidic bond, but is not as sweet as glucose or sucrose.

The term “sugar”, like mentioned earlier, includes monosaccharaides and disaccharides. In the second part of the twentieth century, sugar intake increased markedly in the United States. This is due in particular to increased consumption of added sugars as a result of their greater use in beverages and foods. According to the US Food Supply data, consumption of added sugars has increased from 23 teaspoons per person per day in 1960 to 31 teaspoons per person per day by 2000, which represents a

33% increase (Jensen and Beghin, 2005). A major fraction of this increase has been in the form of high-fructose corn syrup. Soft drinks are the most frequently used form of added sugars and consist of one-third of the total sugar intake. In Europe, the trend of sugar consumption has been a steady one (Connor et al., 2003; Stylianopoulos, 2013).

2.1.3. Oligosaccharides and Polysaccharides

Oligosaccharides consist of a chain of three to nine monosaccharide units, covalently linked to form large units and are named trioses, tetroses, etc., denoting the number of carbons in their molecule. Oligosaccharides are distributed widely in plants and, when digested, yield their constituent mono-saccharides. The major oligosaccharides consist of the raffinose series, formed by the linkage of galactose, sucrose, and glucose units, and the maltose series, formed by the linkage of glucose units.

Polysaccharides consist of long chains of monosaccharide residues (10 or more) linked by glycosidic bonds. These compounds consist of several hundred or even thousands of monosaccharide units. The properties of polysaccharides are determined by the species of monosaccharaides in the polymer backbone, the type of linkages between residues, and the extent and type of chain branching.

Glucans are polymers of glucose and the major polysaccharides in the diet. The most important glucans are starch, glycogen, and cellulose. Glycogen is the short-term storage form of glucose in animal tissues. Starch is the most common digestible storage polysaccharide in plants and cellulose is a major structural component of plant cell walls. Polysaccharides with a linkages have a helical shape, e.g., the amylose starch molecule, whereas those with β linkages generally have a linear or flat ribbon-like molecule, e.g., cellulose. Their molecules can be linear or branched. Branches can be formed through any unlinked hydroxyl group and vary from alternating and consecutive single-unit branches to multiple-unit branches (ramified structure) (Englyst and Englyst, 2007; Gibson, 2012; Keegstra, 2010).

2.1.3.1. Starch

Starch is the most important, abundant, and digestible polysaccharide in nutrition. Starch consists of large chains of α-linked glucose residues and is found in the form of amylose or amylopectin (Figure 1-4). Amylose is a linear, unbranched form of starch, which consists of α-1,4-linked glucose units. Amylopectin is a branched-chain polymer, which consists of lineary α-1,4-linked glucose and brunches of α-1,6-linked glucose. Amylopectin usually represents 80–85% and amylose 15–20% of total starch. It is predominantly derived from plant seed, such as wheat, maize, rice, oats, and rye, and from plant roots, such as potatoes. Bread and pasta are popular products high in starch content.

Starch is classified into three general types based on its rate of digestion: rapidly digestible (RDS), slowly digestible (SDS), and resistant starch (RS).

Figure 1.

Molecular structure of amylose (built with MarvinSketch 14.7.21.0).

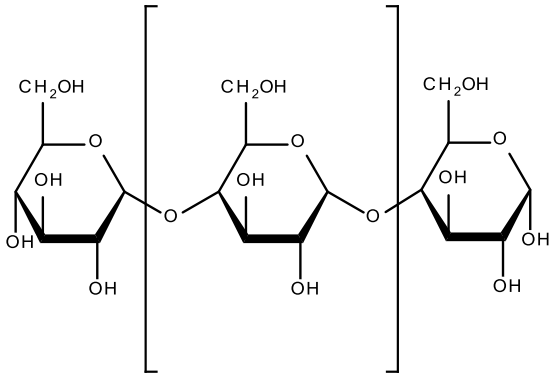


Figure 2.

Molecular structure of glucose unit (built with MarvinSketch 14.7.21.0).

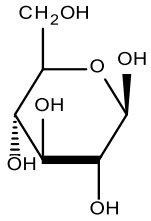
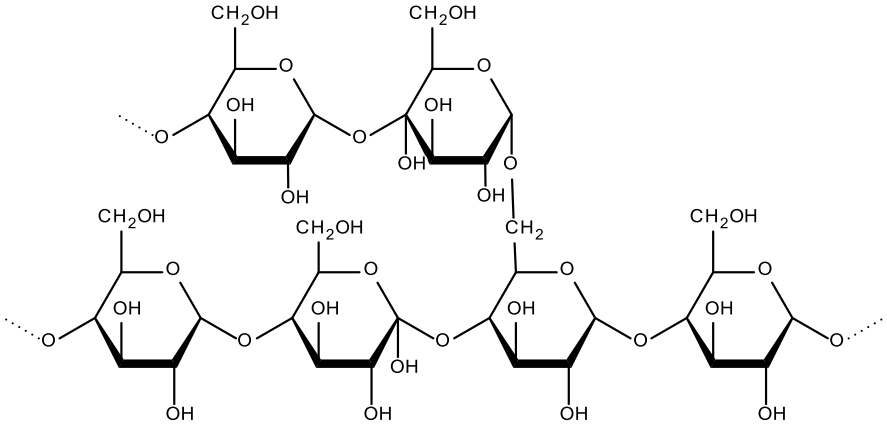


Figure 3.

Molecular structure of amylopectin (built with MarvinSketch 14.7.21.0).



2.1.3.2. Resistant starch

Resistant starch (RS) can be defined as the starch and starch degradation products that resists digestion and absorption in the small intestine. RS is classified into four main types:

- RS1; physically inaccessible starch granules, surrounded by indigestible plant material such as found in partly milled or whole grains;
- RS2; resistant starch granules, this type is the major components of dietary RS and can be found in raw potato, green banana, some legumes and high-amylose starches;
- RS3; retrograded amylase made by alternative cooking–cooling process. Could be found in such as cooked and cooled starchy foods like potato, bread, and cornflakes;
- RS4; chemically modified starch type through esterification, cross-linking or trans-glycosylation and used in processed foods.

RS is a non-viscous, dietary fibre, which is highly fermentable by bacteria when reaches the large intestine. Dietary resistant starch increases the production of short-chain fatty acids (SCFAs) and faecal volume, also reduces faecal pH, and is associated with lower blood glucose and plasma cholesterol levels (Haenen et al., 2013; Hoebler et al., 1999; Jenkins et al., 1998). According to a recent health claim of European Food Standards Agency (EFSA) resistant starch (RS2) lowers glycaemia when it replaces the available carbohydrate portion of a meal (EFSA and NDA, 2011a). Resistant starch fermentation also causes slower emptying of the human gastrointestinal tract when compared with other starch carbohydrates. However, the amount of starch reaching the large intestine will vary between individuals and may be affected by other factors that influence gastrointestinal transit time or inhibit starch hydrolysis, where the gastrointestinal transit time is related inversely to the amount of starch escaping digestion (Silvester et al., 1995).

2.1.3.3. Dietary fibre

It has long been recognized that both animal foodstuffs and human foods contain poorly digestible components that do not contribute to nutrition in the classical sense of providing essential substances or metabolic energy. The term dietary fibre was first used in 1953 by Eben Hipsley in his observation publication noticing diets high in fibre-rich foods tended to also have lower rates of pregnancy toxaemia (Hipsley, 1953). Another term "crude fibre" as the portion of plant foods that escaped solvent, acid and alkali extractions, had been used by Trowell (Trowell, 1976). Dietary fibre proposed to be "unavailable" plant material by Asp, 1987, such as that which escaped digestion and absorption in the human upper GI tract. Most of the recent literature supported that the dietary fibre originate from plants, The Codex Committee on Nutrition and Foods for Special Dietary Uses (CCNFSDU) illustrated that the current definition of dietary fibre should include both edible plant and animal material (FAO/WHO, 2001). After several years of research and debate over the definition of dietary fibre, together with analytical methods for its determination, the classification was important point for public health as well as food industry purposes (Buttriss and Stokes, 2008; McCleary, 2008). The final agreement was reached on a global definition for the Codex Alimentarius (collection of standards, codes of practice, guidelines, and recommendations). At the 2008 meeting of the Codex Committee on Nutrition and Foods for Special Dietary Uses (CCNFSDU), dietary fibre was defined as carbohydrates with 10 or more subunits, which cannot be hydrolysed by endogenous enzymes of the human small intestine and can belong to the following categories:

- naturally occurring edible carbohydrates;
- carbohydrates obtained from food raw material by physical, chemical, or enzymatic methods, and scientifically shown to have a physiological effect or health benefit;
- synthetic carbohydrates scientifically shown to have a physiological effect or health benefit.

This new definition has generated the need for the development of an integrated analytical method for the determination of total dietary fibre. The AACC International (American Association for Clinical

Chemistry) and the AOAC International (Association of Official Analytical Chemists) have previously developed methods for quantify of dietary fibre and its components. Under the supervision of these two international organizations, a new method has been developed for determination of total dietary fibre, using HPLC analytical separation and inter-laboratory evaluations of this method are under way.

Dietary fibre consumption has potential impact all aspects of gut physiology and are a vital part of a healthy diet. Observations linked dietary fibre with important health benefits, including general gastrointestinal health and prevention of several population diseases, through blood cholesterol reduction and regulation of blood sugar levels. However on the other side in case of animal nutrition it is recognized as anti-nutritional factor.

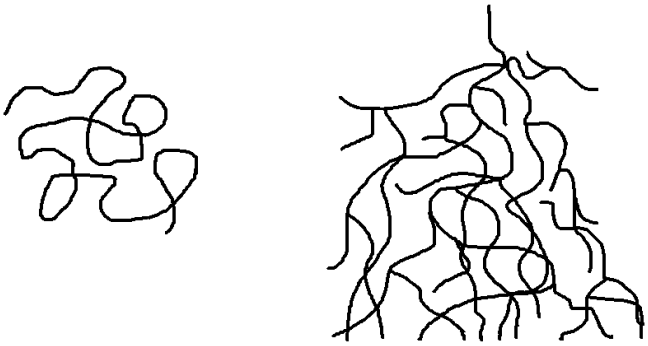


Figure 4.
Native starch types structures: linear amylose (left) and branched amylopectin (right) (built with MS Paint).

2.1.3.4. Non-starch polysaccharides (NSP)

Non-starch polysaccharides (NSP) were formerly referred to as dietary fibre. These fibre types are more digestible than others, and although they cannot be broken down by mammalian enzymes, but they can be fermented by bacteria in the hindgut. Plants cell walls are made up of up to 90% of NSP, that include most abundant: cellulose, hemicellulose, and pectin. Other less abundant NSP include fructans, glucomannans, galactomannans, mucilages, arabinoxylans, β-glucans, and gums. Cellulose is found in tightly bound aggregates in plants, while hemicellulose and pectin have sugar side chains that allow them to be more readily broken down. Lignin is not a polysaccharide, but is a high molecular weight polymer, and is not considered a functional dietary constituent because it is indigestible by monogastric animals. NSP have effects above and beyond simple indigestibility and they can be either soluble or insoluble, on the basis of aqueous solubility. But it have to be highlighted that this solubility may be demonstrated under conditions that do not occur in the human small intestine. Indigestibility is particularly the case for insoluble NSP. Wheat, rice, and maize contain predominantly insoluble NSP, whereas oats, rye, and barley contain predominantly soluble NSP. Vegetables are also a source of NSP and contain equal amounts of insoluble and soluble NSP. Products such as wheat bran are extremely effective causing a stool bulking effect. NSP fractions containing pentosans are particularly effective over other polysaccharides in increasing faecal bulk The reason for this is not clear but may reflect water-holding capacity (Englyst et al., 1992; Izydorczyk and Dexter, 2008; Kumar et al., 2012).

Beta glucans

β-Glucan is the major non-starch polysaccharide of cell walls in cereal grains. Structurally, it consist of long linear chains of glucose having β-1,3 and β-1,4-linkages but these linkages are not arranged in a random and repeating fashion. β-glucan 1,4-linkages occur in groups of two to four while 1,3-linkages occur singly (Figure 6). This leads to a structure that is dominated by β-1,3-linked

cellotriosyl and cellotetraosyl. The rest of the structure consists of longer blocks of 4-15 1,4-linked β-D-glucopyranosyl units. The structure of β-glucan resembles that of cellulose, the only difference being that the β-1,3-linkages establish a twist in the chain. This twist phenomenon gives stability to β-glucan and lessens its affinity to form aggregates, thus the solubility of β-glucan is greatly affected by such a trend. Longer sequences of 1,4-linkages give β-glucans lower solubility because of close intermolecular associations. However, recent data showed assumption that β-1,4-linkages have an insignificant influence on solubility, as compared to that of long blocks of contiguous cellotriosyl residues. Regularity of beta-glucan structure can gives an idea about how a high level of β-1,3-linked cellotriosyl units reduces solubility and increases the tendency to gelatinisation. The property of water solubility is attributed to the introduction of 1,3-linkages in a cellulosic chain. The enzymes endoglycosynthases help in synthesis of β-glucan molecules. However, the mechanism may depend on species and conditions. The major enzyme endo-β-1,3-1,4-glucanase is a thermo-stable and develops during the germination of barley. This enzyme is associated with degradation of the β-glucan molecule after synthesis of β-glucan in that way controlling the length and the molecular weight of β-glucan in cereal crops (Brennan and Cleary, 2005; Buckeridge and Rayon, 2004; Izydorczyk and Dexter, 2008; Lazaridou and Biliaderis, 2007).

Beta-glucan is the principal fibre present especially in barley, oat, and some mushrooms. Some limited amount of the barley is used as a source of β-glucan in various foods for human consumption but the major quantities of barley are used for animal feed, on a worldwide basis (FAO, 2011). Food and Drug Administration (FDA) allowed its use in food products and made it obligatory for labelling requirement to get health claim. It was also recommended that a diet high in soluble fibre from whole oats (oat bran, oatmeal, and oat flour) should be used to reduce the risk of heart diseases. On the basis of FDA evaluated studies for the consumption, was recommended a daily dose of at least 3 g of β-glucans from oats to achieve a clinically relevant decrease in serum total cholesterol. EFSA Panel on

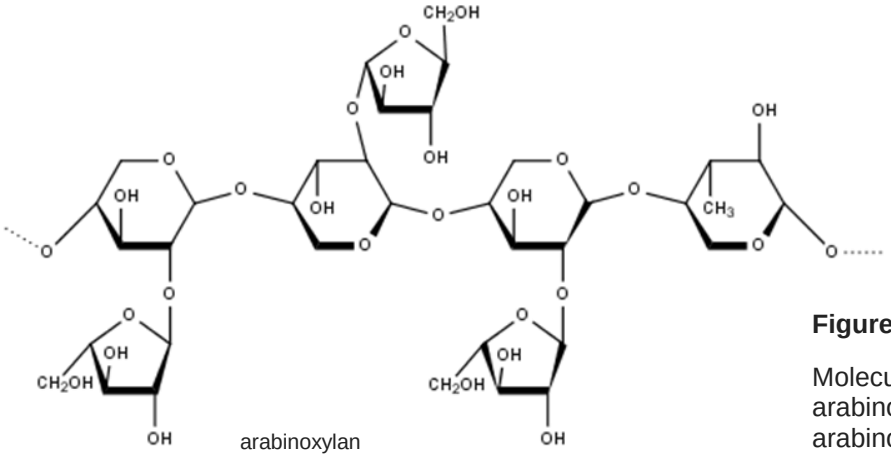
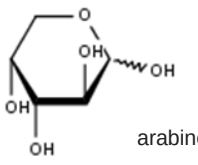
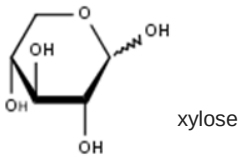


Figure 5.

Molecular structure of cereal arabinoxylan chain, with arabinose and xylose units (built with MarvinSketch 14.7.21.0).





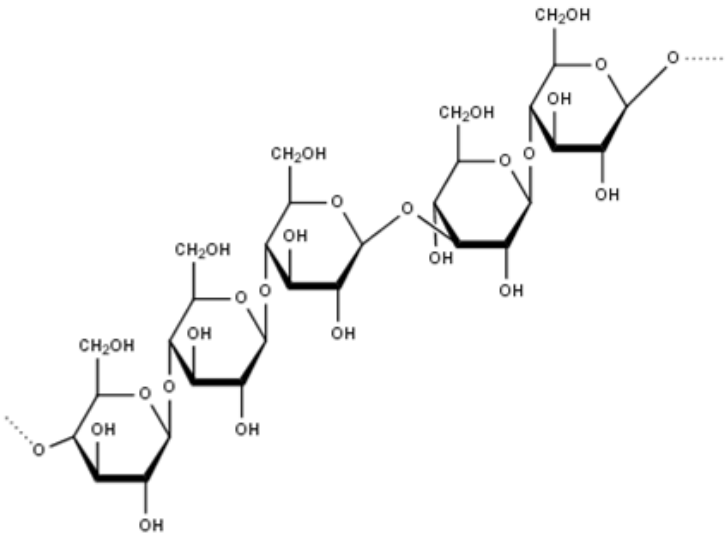


Figure 6.

Molecular structure of cereal β-glucan (built with MarvinSketch 14.7.21.0).

Dietetic Products, Nutrition and Allergies (NDA) did their own research on the health effects. The claimed effects on blood cholesterol refer to the maintenance of normal blood LDL-cholesterol concentrations (EFSA and NDA, 2011b). Individuals who wish to reduce their post-prandial glycaemic responses should consume per meal 4 g of beta-glucans from oats or barley for each 30 g of available carbohydrates. Moreover, the relationship of a sustained increase in satiety leading to a reduction in energy intake and improves digestive function has not been established between the consumption of oats and barley beta-glucans (Brennan and Cleary, 2005; EFSA and NDA, 2011b).

Arabinoxylans

Arabinoxylans (AX) are another important, from nutritional point of view, component of the dietary fibre fraction of cereal grains. Like other heteroxylans (such as β-glucan), the arabinoxylans also have a backbone of 1,4-linked β-D-xylopyranosyl units, that can be either unsubstituted, monosubstituted with a single α-L-arabinofuranoside at either C-(O)-2 or C-(O)-3, or disubstituted with single α-L-arabinofuranoside units at C-(O)-2 and C-(O)-3 (Figure 5). Less abundant substituents can be glucuronic acid, 4-O-methyl glucuronic acid, or short oligomers consisting of L-arabinose, D-xylose, D-galactose, D-glucose or uronic acids, connected to C-(O)-2 position of the xylose residues. Additionally, the C-(O)-5 position of terminal arabinose units could have attached hydroxycinnamic acids, mainly ferulic, dehydrodiferulic, sinapic and *p*-coumaric acid. The degree and frequency of substitution varies greatly amongst AX and depends of the grain as well as the tissue origin (the bran or the aleurone layer). For example, endosperm arabinoxylans from rice, maize, and sorghum contain more arabinose, galactose, and glucuronic acid substituents than those from wheat, rye or barley (Dervilly et al., 2002; Izydorczyk and Dexter, 2008).

AX fibres can be converted into arabinoxylan-oligosaccharides (AXOS) and the non-substituted xylooligosaccharides (XOS) fragments by hydrolytic degradation. Because hydrolysis of AX almost always leads to a mixture of both AXOS and XOS at the same time, they are hereinafter together referred to as (A)XOS. Arabinoxylan-oligosaccharides can be generated in the colon of animals and

humans by microbial degradation. Colonic microbiota can produce some AX-degrading enzymes endoxylanases (endo-β-1,4-xylanases), xylosidases, alpha-L-arabinofuranosidases, feruloyl esterases, acetyl esterases, and alpha-glucuronidases. AXOS can be also prepared and purified from AX-rich sources and used as a food ingredient, for example during bread-making. AXOS resulting from hydrolysis of AX have raised particular interest by the demonstration of their prebiotic properties leading to a potentially beneficially shift in gut bacteria *in vitro* intestinal models (Hughes et al., 2007; Sanchez et al., 2009), poultry studies (Courtin et al., 2008) and human studies (Cloetens et al., 2010).

2.2. Gastrointestinal digestive and absorptive processes

The gastrointestinal tract (GIT) serves as an interface between the external environment and the body. The GIT is a highly specialized organ system that allows animal to consume foodstuffs, in very diverse array, to meet nutrients needs. In the gastrointestinal tract food is converted to compounds that can be absorbed into the body. The GIT system is connected to the vascular, lymphatic and nervous systems. In that way it is possible to regulate the digestive response, delivery of absorbed compounds to organs of the body and the regulation of food intake. A primary function of the gastrointestinal tract is to extract nutrients from the complex mixture of consumed food. Food contain more than essential nutrients, and the GIT has a role in metabolizing and eliminating non-nutrient and toxic compounds as well. Digestibility is the result of several processes including transit, hydrolysis or fermentation, absorption and endogenous secretions. It is known that digestion is affected by the physical and chemical characteristics of the feed, feed processing, animal factors and feeding level (Lahaye et al., 2004; Le Goff et al., 2002; Shi and Noblet, 1993). The importance of each of these aspects depends on the type and quantity of nutrients supplied and on the site of digestion. Ingestion of nutrients induces profound changes in gastrointestinal motility and release of gastrointestinal digestive juices and hormones. Two main features of luminal content chemical profile and luminal bulk appears to control its physiology. The nutrient profile of the gut lumen is sensed by specialised chemosensor enteroendocrine cells within the epithelium, while mechanoreceptors (stretch activated neural cells) activated under the mechanical pressure from luminal contents. All these changes serve to coordinate the digestive process and to adapt it to the nature and composition of the ingested nutrients.

2.2.1. Salivary and gastric digestion

The senses of sight, smell or even thought of food, are associated to trigger the cephalic phase of digestion. Activation of secretion of saliva, gastric acid as well as hormones in the upper parts of gastrointestinal tract is induced through vagal efferent. In the oral cavity food is chewed and mixed with saliva, important role of salivary secretion is lubrication of food passage along the oesophagus. Ingested foods must be mechanically homogenised with digestive fluids in order to allow better hydrolysis of macronutrients, and to allow release of hidden micronutrient. Secreted in saliva alpha-amylase (or ptyalin) hydrolyses alpha-1,4 linkage of starch and converts it to maltose. Human salivary amylase is 94% identical with pancreatic one, but it is inactivated in the acid pH of the gastric lumen and its efficiency depends upon the time spent chewing (Pedersen et al., 2002; Schenkels et al., 1995; Tabak, 1995).

Arrival of food in the stomach activates mechanoreceptors (and probably also chemosensors), which in turn stimulate gastric relaxation and secretion of gastric acid through the reflex pathways. Low pH environment allows denaturation of proteins and solubilisation of other factors. Pepsin and other gastric proteases cut amino bonds in proteins to form a range of shorter peptides and amino acids. On the other hand, dietary lipids digestion is initiate by gastric lipases, accounting for 10–40% of total triglyceride hydrolysis in healthy adult individuals. Before leaving the stomach, its luminal content is processed into a creamy, homogenous slurry, known as chime. The rate of gastric emptying is inversely related to caloric load, limiting the delivery of undigested food to the duodenum, so it do not exceed hydrolytic capacity (Korbonits, 2004; Steinert et al., 2012).

2.2.2. Intestinal phase

The intestinal phase is mainly triggered by chemoreceptor activation in the proximal small bowel. Passage of the acidic chime towards the duodenum (the upper section of the small intestine) will stimulate the release of secretin, which inhibits gastric acid secretion and stimulates bicarbonate-rich secretions from the liver, pancreas and intestinal crypts. Lipids present in luminal content are emulsified by hepatic action secretes bile and other factors, in this way available surface area greatly increase for pancreatic lipase action. Oligopeptides as well as duodenum distensions (mechanical factor) stimulate the release of gastrin from duodenal G cells (Miled et al., 2000; Steinert and Beglinger, 2011; Stewart et al., 2011). While lipid and protein substrates are hydrolysed by more than one gastric and pancreatic enzyme, presence of carbohydrates stimulate pancreatic alfa-amylase (or amylopsin) production delivered into duodenum, that digests major part of starches, around 60%. Starch hydrolysis begins with random cleavage of internal α-1–4 glucose bonds, proceeding to smaller fragments until only small linear glucopolymers (2–8 residues) or small a-limit dextrins (containing alfa 1–6 branch chains) are left. In swine, starch digestion occurs primarily in the intestine, because of the short oral retention time of feed grains the contribution of oral salivary hydrolysis is generally very limited (Hoebler et al., 1999). Intestinal epithelial cell enzymes degrade di- and tr-isaccharides, such as lactose is degraded by lactase into its basic components (6C-carbohydrates) glucose and galactose, sucrose for degradation of sucrose to glucose and fructose, and also maltose is degraded by maltase to two glucose molecules. The 5C-carbohydrates such as xylulose, arabinose and ribose do not need degradation and are easily absorbed by intestinal cells (Dashty, 2013). The distribution of enzymes along the length of the gut is designed to digest in the most efficient way, where in the jejunum the enzyme content peaks and by mid-jejunum digestion process is almost complete. Moreover, enzymatic activity change also along the vertical axis of the villi, where the lower and mid-villus segments showed maximum expression of enzymes which decreases to the villus tip, additionally similar pattern is observed regarding the enzyme content. Small intestinal structure is ideally suited to a large absorptive capacity. The mucosa of the small intestine “traps” the nutrients released by the hydrolytic processes (i.e. glucose, amino acids, peptides, fatty acids or monoglycerol)

(Fan et al., 2001; Hedemann, 2006). Apart of its metabolic activity (secretion of enzymes, bacteria interaction) the epithelial layer is a semipermeable membrane that efficiently regulates the exchange of materials between the body and the luminal contents. Due to intestinal mixing, the epithelium has abundant contact with luminal contents to facilitate absorption. Structural design of mucosa changes along gut regions and accommodates to its function, proximal colon (the region with high nutrient influx) has villi 320–350 μm height which decease towards distal small intestine (220–260 μm) with barely any presented in the colon (Brunsgaard, 1997; Hedemann, 2006; Jin et al., 1994). An approximate surface area of the small intestine is 200 m^2, compared with approximately 0.05 m^2 in the stomach and about 6 m^2 in the large intestine (DeSesso and Jacobson, 2001; Snipes, 1997). Only monosaccharides can be absorbed readily from the human intestine. Disaccharide absorption is very low, but does increase slightly relative to monosaccharides in conditions such as celiac sprue that produce mucosal damage. The absorption of the hexoses from intestinal epithelium happens via passive and active transport systems. In the passive diffusion form, carbohydrates (i.e. glucose or galactose) enter the circulation after phosphorylation in the gut cells to be dephosphorylated before entering the liver. The active transport system utilizes a mobile carrier protein coupled with the ATP dependent ion channel (Gribble, 2012; Shirazi-Beechey et al., 2011).

2.2.3. Colonic (Bacterial) Digestion

Food components that have escaped small bowel digestion, especially the NSP, resistant starches, non-glucan oligosaccharides (short chain carbohydrates), and some polyols or modified starches, reach the colon where will be fermented by the commensal bacteria living there at densities of up to 10^{12}/g (Elia and Cummings, 2007). The rate and overall degree of degradation of fibre polysaccharides is influenced by the chemical nature of the plant fibre, the solubility, and the degree of lignification. Thus, beta-glucan, soluble arabinoxylans (AX), and pectins are all rapidly degraded in the caecum and proximal colon whereas the more insoluble plant fibre (e.g. cellulose and insoluble

AX) are degraded more slowly and at more distal locations in the colon (Bach Knudsen et al., 2012; Glitsø et al., 1998; Lindberg, 2014).

The large intestine is involved mainly in the uptake of water. Absorptive capacities also include microfloral metabolites, together with short-chain fatty acids, ammonia and urea and in retrieve of low levels of other nutrients remaining luminally. The migrating motor complex areas are called haustra, present along the length of the bowel up to the sigmoid colon. Areas of contraction may act as mixing segments to benefit absorption of water, trace nutrients, encourage of fermentation, and also move large volumes of luminal contents distally. Fermentation is an anaerobic process and, therefore, produces unique end products. These include principally the SCFAs (largely acetate, propionate and butyrate), hydrogen gas, and methane (Bindelle et al., 2008; Williams et al., 2001). Butyrate is the major energy source for the colonic epithelial cells and has differentiating properties in the cell, influences gene expression and division of cells, and it is also connected with reduced skatole formation and absorption. After being absorbed propionateis metabolized in the liver and it may slightly influence the hepatic lipid metabolism, however the latter has no significant regulatory propeteries in monogastric animals like humans. At the contrary in ruminant animals where propionate is used to synthesize glucose in the liver. Acetate is the major short chain fatty acid produced in all types of fermentation, its molar ratio is around 60:20:20 (acetate:propionate:butyrate). Acetate is rapidly absorbed, stimulating also sodium absorption. Next, it passes to the liver and then into the blood, from where it is available as an energy source, used principally by skeletal and cardiac muscle and as well as by the brain (Siavoshian et al., 2000; Todesco et al., 1991; Ying et al., 2013).

2.2.4. Effects of dietary fibres on digestion and absorption

Classically, it has been assumed that the presence of any dietary fibre in the upper gastrointestinal tract will result in a decreased nutrient utilization and low net energy values. The negative impact of dietary fibre is determined by its properties and may differ considerably between fibre. However, as that assumption covers a wide range of factors, it is important to state that a minimum level of dietary

fibre has to be included to maintain normal physiological function in the digestive tract in pig. By manipulating the amounts and properties of fibrous material we may obtain positive effects such as the gut health stimulation, increase the satiety, and overall improvement animal behaviour and well-being. The degradability of dietary fibre varies from 0 to 100%, as compared to other macronutrients which are usually over 80%. The variation in fibre fermentability is high due to high diversity in physical structure and in chemical bounds between monomers, such as bulk, viscosity, solubility or water-holding capacity. For example, viscosity may increase the proportion of the diet that is digested in the small intestine due to a reduced digesta passage rate in pigs (Bach Knudsen et al., 2012; Hooda et al., 2011; Knudsen and Bach Knudsen, 2001; Zijlstra and Jha, 2012).

2.2.4.1. Young animals

Digestion in the weaning period is directly affected by an insufficient production of pancreatic enzymes. During first three weeks of age after weaning pig's small intestine produce only maltase and glycoamylase, highly limiting digestive capacity, in comparison with growing pigs and sows. The digestibility of starch in post-weaning animals could be increased by gelatinising it, in this way the available surface area for the digestive enzymes would be grater. Taking into account that gelatinised starch is almost completely digested in growing pigs (Hopwood et al., 2004; Kelly et al., 1991; Knudsen et al., 2005). These conditions have a profound influence on the type of substrate available for the microbial fermentation in the large intestine, de facto starch is the main substrate for microbial growth, during the important first few days after weaning, regardless the dietary fibre concentration. This is in contrast to the situation in growing and adult pigs where fibre (usually NSP) is main factor regulating the flow of nutrients from the small to the large intestine (Knudsen et al., 2008; Zijlstra and Jha, 2012).

During the first weeks post-weaning piglets have limited capacity to ferment fibre which results in the accumulation of non-fermentable material in the gastrointestinal tract, consequently the inclusion of highly fermentable ingredients has been related with the incidence of diarrhoea in this age (Molist et

al., 2014). Recently, Montagne et al. (2012) reported an interaction between highly fermentable fibre content of the diet and sanitary conditions of the farm, what represents an additional risk factor for piglets health and growth. In the same paper authors suggested to first use highly insoluble, low fermentable diet components for post-weaning diet, and than after an adaptation period would be possible to include fermentable fibre, lowering in this way the frequency of post-weaning diarrhoea. The inclusion of soluble fibre sources in diets during the early post-weaning period has been associated with decrease digesta retention time, which in turn may reduce the proliferation of pathogens in the small intestine (Heo et al., 2013; Kim et al., 2012). Also resistant starch type 2 in a moderate amount (about 7% in the diet as fed basis) could favourably prevent post-weaning diarrhoea in piglets, without compromising growth performance (Bhandari et al., 2008). The addition of insoluble NSP ingredients with a low protein content might improve the development of the GIT and restore intestinal transit time and prevent of infections. Wheat straw and oat hulls included into a cereal-based diet during the first two weeks post-weaning enhanced feed intake of the piglets, as compared with control (Gerritsen et al., 2012). Also, Wenk (2001) reported that low performance piglets increase feed intake after the inclusion of insoluble dietary fibre in the diet. Additionally, it was found a positive relationship between the presence of highly fermentable ingredients, such as citrus pulp and soybean hulls, and the occurrence of post-weaning diarrhoea, where the addition of cellulose could reduce that effect (Fonseca et al., 2012). Numerous studies (Bhandari et al., 2008; Hermes et al., 2010; Molist et al., 2011; Montagne et al., 2003) have shown a positive relationship between the fermentation of insoluble fibre sources and butyrate production in the large intestine and improved gut health of the piglets.

Moreover several metabolites such as amines resulting from protein fermentation in the gut was described as potentially harmful to the mucosa of piglets, and could break the integrity of the intestinal barrier increasing the risk of diarrhoea (Kim et al., 2008). However, the inclusion of fibrous ingredients could reduce the negative effects associated with an excess of protein fermentation in the large intestine.

Trials on weaned pigs indicates that insoluble fibre sources might reduce *E. coli* intestine population, as fibrous ingredients have direct physical effect on bacteria, blocking the adhesion to the gut's receptors (Gerritsen et al., 2012; Hopwood et al., 2004). Some ingredients that look a lot like host receptors might bind microorganism, such as *E. coli*, rather than host cells. Most likely, the non-fermented undigested fibrous fraction of these ingredients may interact with pathogens, decreasing its concentration in ileum digesta due to increased excretion via faeces, which in turn will reduce the risk of adhesion and preventt the initiation of the infection process (Becker et al., 2009; Molist et al., 2011; Shoaf-Sweeney and Hutkins, 2009).

2.2.4.2. Growing period and adult pigs

Adult pigs have a more developed and larger GI tract, a lower feed intake per kg body weight, a slower digesta transit time and a higher cellulolytic activity than young pigs. Thus, sows have greater capacity to digest fibrous components like NSP than younger pigs especially in the small intestine (Jørgensen et al., 2007). The inclusion of different RS varieties and types of fibres reduced intestinal fermentation and retention of some nutrients in growing pigs (Rideout et al., 2008). High amylose starch increased the digesta mass in the pig's distal gut, creating a potentially advantageous environment and nutrients flow for microbial growth (Topping et al., 1997). A long-term intake of resistant starch was also associated to a reduction in damage to epithelial cell and could represent a promising strategy for reducing swine odour emission (Nofrarías et al., 2007; Willig et al., 2005). Danielsen and Vestergaard (2001) reported that fibre-rich diets could improve welfare of pregnant sows. Moreover, fibre enriched diets changed also the animal behaviour by reducing aggression and sham chewing, probably because of a higher degree of satiety. Similar effects were also reported by other authors (Meunier-Salaün et al., 2001; Rijnen et al., 1999).

The genetic background of animal can also impact digestibility efficiency of nutrients and energy (Le Gall et al., 2009). Lately, Noblet et al. (2013) performed an interesting trial to evaluate the genetic variability on energy digestibility in growing pigs, suggesting that energy digestibility and

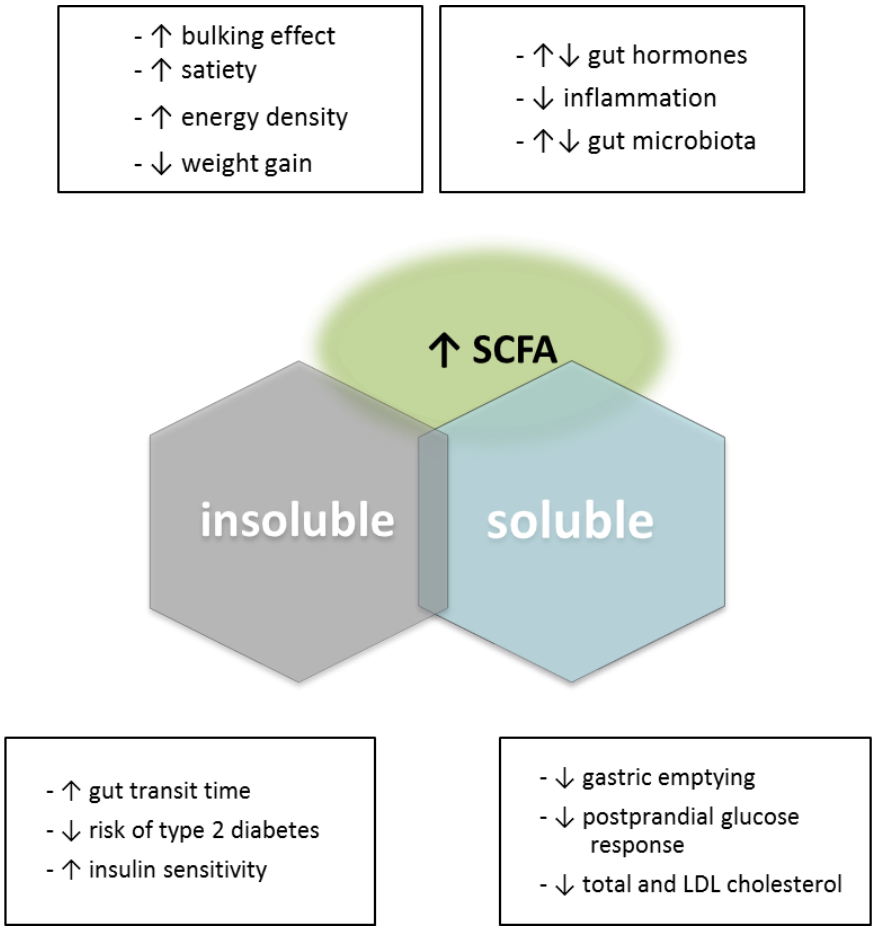


Figure 7.

Potential effects of soluble and insoluble dietary fibre consumption. Colonic fermentation with the production of SCFA can be observed with most types of fibre to some degree, but it tends to be more noticeable with soluble in naturally available foods (modified after Weickert and Pfeiffer (2008)).

digestion in general would be dependent on heritable genetic variability in growing pigs. In this experiment four boars of Large White growing pigs of different origin were fed a high dietary fibre diet (18% NDF). The digestibility coefficients were affected by boar origin and authors noticed that pigs originating from the boar with the highest digestive efficiency consumed slightly less feed and had the better growth rate. Previous studies pointed out differences in digestive efficiency between Asian breeds and commercial European lines, where Asian pigs were more efficient under fibrous feed (Fevrier et al., 1992; Kemp et al., 1991; Len et al., 2009; Morel et al., 2006).

2.2.5. Gut microbial community

The gastrointestinal tract of pigs is colonized with a highly diverse microbiota, comprising 4400 different phenotypes, having a considerable impact on physiology, development and immunology. They make nutrients available to the host organism from fermentation processes, stimulate the mucosal immune system and protect the intestine against adhesion, colonization and overgrowth of pathogens (Chowdhury et al., 2007; Danielsen et al., 2007; Willing and Van Kessel, 2007). The anaerobic bacteria estimated concentration in the swine gut ranges from 7-8 CFU/g (colony-forming unit) in the stomach and the small intestine to log 10-11 CFU/g in the large intestine (Jensen et al., 1994). Approximately 90% of the bacteria are Gram-positive, strict anaerobes belonging predominantly to the *Streptococcus*, *Lactobacillus*, *Eubacterium*, *Clostridium* and *Peptostreptococcus genus*. The Gram-negative bacteria represent remaining 10% of the total flora and belong to the *Bacteroides* and *Prevotella* groups (Jansman and Huisman, 1999; Leser et al., 2002). However, the porcine microbiota is a dynamic ecosystem highly dependent on diet, age or even breed. It is instable especially during the post-weaning period, being easier to modify at this time, giving occasion for pathogenic bacteria to colonize and cause diseases (Pieper et al., 2008). The gut microflora of healthy animals is subjected to modifications in terms of predominant species according to combination of

diet related factors like digesta passage rate, viscosity, digestibility and fermentability (Hooda et al., 2011; Regmi et al., 2011). Microbiota composition could be manipulated by selecting available substrates i.e. cereal grains based on the type and amount of dietary fibre. For example, Leitch et al. (2007) observed different bacterial communities in the primary colonizers of three insoluble colonic substrates (wheat bran, high amylose starch and porcine gastric mucin). The type of dietary fibre and levels of inclusions affect number of bacteria in colonic digesta and *Serpulina hyodysenteriae* connected with swine dysentery (Durmic et al., 1998). In the human large intestine populations of *Bifidobacteria* and *Lactobacilli* clearly increase under oligofructose, galacto-oligosaccharides and lactulose (Macfarlane et al., 2006). Similarly, guar gum or cellulose added to a standard diet showed increase *Bifidobacteria* and *Enterobacteria* in growing pigs ileum (Owusu-Asiedu et al., 2006). Fructo-oligosaccharides (FOS) in diet for neonatal piglets enhanced growth of the *Bifidobacteria* and stimulated cell proliferation (Howard et al., 1995).

Acidic environment and SCFA production could inhibit the growth of some intestinal pathogens such as *Escherichia coli*, *Salmonella* spp. and *Clostridium spp*. (May et al., 1994; Montagne et al., 2003; Wang and Gibson, 1993). Butyrate, in particular, seems to play a selective antimicrobial role, since studies in pigs indicate that *Lactobacillus sp*. and *Streptococcus bovis* are less sensitive to n-butyrate, compared to *Escherichia coli*, *Salmonella spp*., *Clostridium acetobutylicum*, *Streptococcus cremoris*, *Lactococcus lactis* and *Lactococcus cremoris* (Hentges, 1992; Williams et al., 2001). Some oligosaccharides and polysaccharides are recognized as prebiotics for animal nutrition, having similar application as those known for human. According to the definition prebiotics are compounds that have been scientifically proven to resists host digestion, absorption and adsorption processes, are fermented by the microbiota colonizing the GI system and selectively stimulates the growth and/or the activity of one or a limited number of bacteria within the GI system (Bach Knudsen et al., 2012; Björck et al., 2012; Hentges, 1992). Highly fermentable non-enzymatically digestible oligosaccharides (NDO) can decrease gastrointestinal pH creating unfavorable environment to pH-sensitive microbes (Bach Knudsen et al., 2012; Houdijk et al., 2002; Macfarlane et al., 2006). The NDO bring about pre-caeca prebiotic effect without serious affecting nutrient digestion in weaned pigs (Houdijk et al., 1999,

2002). Inclusion of resistant starch, NDO or high amylose grain varieties into feed of older pigs have lower influence on bacteria, probably due to stabilization of the gut environment (Hedemann and Knudsen, 2010; Metzler-Zebeli et al., 2013). Interestingly, Bird et al. (2009) reported that resistant starch and NDO, affect colon and faecal *Bifidobacteria* numbers when fed together to pigs. Soluble and insoluble combined NSP may cause similar effects on ileal *Bifidobacteria* and *Enterobacteria* populations (Owusu-Asiedu et al., 2006). To date there has been some focus on the potential use of cereal β-glucans as functional feed ingredients in pig nutrition. For example barley β-glucans have been shown to exhibit prebiotic properties at the microbiological level, promoting butyrate-producing bacteria or selectively increases colonic lactobacilli and bifidobacteria, thereby having the potential to improve gut health in pigs (Hooda et al., 2011; Metzler-Zebeli et al., 2011; Murphy et al., 2012; Pieper et al., 2008), and decreasing in pathogenic *Enterobacteriacea* population (Smith et al., 2010). β-Glucans derived from *L. hyperborea*, *L. digitata* and *S. cerevisiae* all reduced the *Enterobacteriaceae* population in the ileum and colon without influencing the *Lactobacilli* and *Bifidobacteria* populations. Additionally data suggested soluble and insoluble beta-glucans may act via different mechanisms (Sweeney et al., 2012).

Another factors important in keeping equilibrium of the flora is the existence of the numerous bacterial antagonisms between endogenous and exogenous species, mechanisms of communication bacteria-host, as well as the resistance of gut to colonization by pathogens. Recent research show interesting interactions between nutrient receptors and intestinal flora in monogastric animals (Bourlioux, 1997; Depoortere, 2014; Hooper et al., 2012; Kimura et al., 2013; Pluznick, 2013).

In summary, the presence of dietary fibre modifies the microbial equilibrium in the intestines with a positive or detrimental impact on animal health according to the feed source and the physiological status of the pig.

2.3. Methods to evaluate digestion

Animal *in vivo* studies represent the real methods with natural environment appropriate for characterization of digestion process. However, they are time-consuming, expensive processes that requires many animal subjects, sometimes with specific attributes. Very wide review of the *in vivo* techniques most commonly adopted was provided by Bach Knudsen et al., (2006). It includes the slaughter technique that require sacrifice of animals in order to collect samples at different time points after a meal or the surgical implanted cannulas which provide direct access into specific points of the gastrointestinal tract (stomach, small intestine and caecum). The review includes also the catheterisation technique featuring permanent catheters surgically installed in the portal vein or artery.

On the other hand, *in situ* and *in vitro* approach, used to estimate starch digestion characteristics of feed grains, are a good alternative for the *in vivo* techniques, and since several years are under increasing interests (Frei et al., 2003; Goñi et al., 1997). In swine nutrition, *in vitro* methods proposed in the last decade are based on multi-enzymatic reactions (1, 2, or 3-steps) mimicking the digestion processes occurring in the gastro-intestinal tract (Anguita et al., 2006; Blazek and Gilbert, 2010; Hoffman et al., 2011; Lee et al., 2011; Sun et al., 2006; van Kempen et al., 2010a). Even though all of these methods poses some limitations related to the *in vitro* procedure itself, they have been shown to accurately and precisely estimate the metabolic response of starch digestion in swine. An overview on the starch digestion kinetics highlighting *in vitro* studies was presented in few reviews (Germaine et al., 2008; Hasjim et al., 2010). What is more many efforts have been made to expressed its complexities with various mathematical models used to analyse the data (M. Wang et al., 2011). Strong relationship, nearly 1–1 between an *in vitro* enzymatic hydrolysis index and an *in vivo* glycaemic index in swine was reported by Giuberti et al., (2012) in a work on starches characterized for a wide range of digestion potentials. Others *in vitro* methods used to characterize feed grain starch digestion in swine nutrition require the use of gastric secretions sampled from cannulated pigs (Bauer et al., 2003) or fecal inoculum (G Giuberti et al., 2013; Jha et al., 2011).

2.3.1. Starch fractions

The starch may be classified according to its in vitro digestion into three categories, mentioned in previous paragraph – Carbohydrates, these include rapidly digestible starch (RDS), slowly digestible starch (SDS) and resistant starch (RS). This nutritional classification based on an *in vitro* measure is useful in predicting the likely glycaemic response to foods. This classification system was first developed by Englyst and his colleagues (Englyst et al., 1992) and up to now is popular among authors and is also widely accepted in the food industry. The Englyst method for measuring the nutritive value of starch can be applied to a variety of samples from pure starches to complex food materials. When the sample is a food product with a rather high simple sugars content, each fraction should be called rapidly available glucose (RAG), slowly available glucose (SAG), and dietary fibre rather than the measured values of RDS, SDS, and RS (Englyst et al., 1996). Under standard *in vitro* conditions of substrate and enzyme mixture concentration RDS fraction is assumed to be defined as the amount of starch digested within the first 20 min. fraction digested later but in time not longer than 120 min is SDS, last RS fraction escape enzymatic hydrolysis within 120 min. A coefficient of 0.9 is used for converting glucose to starch, considering the removal of one water molecule per glucose unit. Additionally, Englyst procedure includes the measurement of free glucose and total starch content in order to accurately calculate each fraction (Englyst et al., 1996, 1992). This system implies that starch granules as purified samples or as part of a food matrix, contain fractions that differ in susceptibility to digestion by α-amylase. A recent review (Zhang & Hamaker, 2009) - discussed physical, chemical, and physiological aspects that are important in determining whether a particular starch is digested relatively rapidly or slowly.

Under *in vivo* conditions have been stated that RDS and SDS are fully digested in the small intestine, and RS is the starch fraction escaping digestion in the small intestine, and may be subject to bacterial fermentation in the large intestine. The rate of starch conversion to sugar in experimental analysis of digestion *in vitro,* follows similar kinetics in the animal digestive system. After ingestion of carbohydrates RDS is the fraction of starch granules known to cause a rapid bloodstream increase of glucose concentration, but before the majority of the sugar is absorbed into the bloodstream RDS

must first reach small intestine. Similarly, the SDS part is digested completely but slowly in intestine, therefore it is connected with more stable glucose metabolism, satiety and diabetes management, having potential health benefits. The resistant starch fraction escapes digestion in the small intestine, and may be subject to bacterial fermentation in the large intestine. The beneficial action of RS is similar to that of fibrous polysaccharides that resist endogenous human enzymes (Englyst et al., 1992; Lehmann and Robin, 2007).

2.3.2. Kinetic models of *in vitro* digestion

The digestible starch is mainly hydrolysed by the enzymes into glucose through several steps in gastrointestinal tract, as was previously described above. The *in vitro* digestion of starch or starch-containing foods requires relatively high concentrations of enzymes mixture and long time periods (extended over several hours) (Goñi et al., 1997). In general, the rate of reaction decreases with extended time and quantity of digested starch form a logarithmic plot. The reaction plot has a final point, where even after prolongation of incubation time no further reaction is measurable. The methods predict the response assuming that the concentration of available starch substrate decreases with time as starch is converted to products. There is no back reaction under the conditions of the digestions and so the end point represents a situation in which no more starch substrate is available for digestion. However, reaction rates could be slowed down by maltose and maltotriose, even if that inhibition seems to be insignificant (Dona et al., 2011), the addition of amyloglucosidase enzyme into reaction mixture might avoid it as maltose and maltotiose are converted into non-inhibitory glucose.

To quantitatively determinate starch, it should be hydrolysed and measured as the component glucose monosaccharide units released, applying a 0.9 hydration factor. The factor 0.9 converts monosaccharaides (glucose) to the anhydro sugar (starch polysaccharide, in this case) on a weight basis. The factor can be calculated by dividing the molecular weight of glucose minus one molecule of water (180 - 18) by the molecular weight of glucose (Stevnebø et al., 2006).

A recently published review of (Dona et al., 2010) contains a full picture of various enzyme-based kinetic approaches for prediction of digestibility, including the two most popular trends of Michaelis–Menten kinetic parameters (Butterworth et al., 2011; Michaelis et al., 2011; Slaughter et al., 2001) and also analysis of digestibility curves determined over relatively lengthy periods by fitting to first-order kinetics (Al-Rabadi et al., 2009; Goñi et al., 1997; Mahasukhonthachat et al., 2010).

Michaelis–Menten equation

The Michaelis-Menten (MM) kinetics is the simplest and best-known approach (Cornish-Bowden and Hofmeyr, 2005; Kuchel, 1998; Michaelis et al., 2011). Model show relation of velocity to substrate concentration, where the product forming reaction follows the reversible formation of an enzyme–substrate complex where E is the enzyme, S is the substrate (starch) and P is the product. This system can be represented schematically as follows:

$$E + S \underset{k_{-1}}{\overset{k_1}{\leftrightarrow}} ES \overset{k_2}{\rightarrow} E + P$$

This model works very well in laboratory experiment when an enzyme and its substrate in a test-tube are mixed together for the measurement of the initial rate of product accumulation. However, the Michaelis-Menten model relies on a number of assumptions, such as the presence of the enzyme (E), substrate (S), enzyme-substrate complex (ES), and product (P) in the system; relevant reactions are the reversible association of enzyme and substrate to form the enzyme-substrate complex; and the irreversible breakdown of the enzyme-substrate complex to product. The concentration of the enzyme-substrate complex change much more slowly than the concentration of the substrate:

$$V_0 = \frac{V_{max}\,[S]}{K_m + \,[S]}$$

The kinetic time course of the starch digestion can have very diverse rates between stages, suggesting that a change in the physical nature of the substrate determines the kinetics of digestion. Model works

very well under normal cellular conditions, but, next to E, S, ES and P, cells contain thousands of metabolites. Michaelis–Menten kinetics can describes very well the rate of oligosaccharide hydrolysis by glucoamylase as a function of the concentration of its various substrates, but if case involves for example product inhibition, more complex models have to be used, because the basic MM equation is inconsistent with the data. For that reason it has been stated that the model cannot be applied to *in vitro* studies of α-amylase action, can be rather applied strictly only to the study of initial reaction rates (Mahasukhonthachat et al., 2010; Mulquiney and Kuchel, 2003). This model is not suitable for time extended *in vitro* starch digestion because of commonly α-amylase high enzyme activities, mixtures of enzymes, inhibitory products and possible structural and molecular changes (Duggleby, 2001a; Goñi et al., 1997; Wang et al., 2006). Additionally, Duggleby (2001) described modified and expanded Michaelis–Menten model in terms of digested starch at given time. A simple version described kinetics at low starch concentrations whereas a modified first-order MM model is required at high concentrations (Komolprasert and Ofoli, 2007). Nevertheless, scientists are rather divided regarding usefulness of Michaelis–Menten kinetics, Butterworth et al. (2011) in his work considers a MM kinetics as very useful in providing information about starch–amylase interactions.

Empirical exponential model

An improved *in vitro* method to study starch hydrolysis at different time intervals was suggested by Goñi et al (1997). A first order equation for the hydrolytic process of starch hydrolysis in foods has been proposed:

$$C = C_{\infty}\left(1 - e^{-kt}\right)$$

where C is the concentration of starch hydrolysed at time t, C_{∞} is the equilibrium concentration, k is the kinetic constant and t is the chosen time. Goni equation was modified with additional parameter of C_0 as the initial concentration:

$$C = C_0 + C_{\infty}\left(1 - e^{-kt}\right)$$

This equation is simply the solution of the relevant first order differential equation. The equilibrium concentration C_∞ is somehow misleading as it represents, more realistically, the concentration of starch digested when the reaction has reached a stage where no more product is formed, i.e. when all starch that can be hydrolysed has been exhausted and only so-called resistant starch remains. Various botanical starches generate different values for k that reflect their relative susceptibilities to digestion, and it is reported typically within the range of $10^{-5} – 10^{-3}$ min^{-1}.

2.3.3. Digestibility indexes

In the last decades years the *in vivo* and lately also the *in vitro* digestibility of foods has been classified by a number of metrics, the most popular of which is the glycaemic index (GI). The glycaemic index, indicates the organism response to food containing carbohydrates. According to the definition given by the FAO/WHO experts in 1997, the GI is defined as the incremental area under the blood glucose response curve of a 50g carbohydrate portion of a test food expressed as a percent of the response to the same amount of carbohydrate from a standard food taken by the same subject (FAO/WHO, 1998). The glycaemic index (GI) concept was introduced in the early 1980 by Jenkins and colleagues (Jenkins et al., 1981) and originally designed for people with diabetes as a guide to food selection and advice being given to select foods with a low GI (Jenkins et al., 1984). Food can be ranked based on how they raise blood glucose compared to a standard amount of glucose. The amount of food consumed is a major determinant of postprandial hyperglycaemia, and the concept of glycaemic load (GL) takes account of the GI of a food and the amount eaten. More recent recommendations regarding the potential of low GI and GL diets to reduce the risk of chronic diseases and to treat conditions other than diabetes, should be interpreted in the light of the individual variation in blood glucose levels and other methodological issues relating to measurement of GI and GL (Pavlovich-Abril et al., 2012).

However, GI measurements seem to become less popular perhaps because requires *in vivo* estimation of blood glucose levels at various time points after the meal and not all laboratories are equipped with suitable facilities and with high cost implications. For nutrition purposes, an *in vitro* measured hydrolysis index (HI) may offer an easier alternative to the glycaemic index values, utilized for expressing kinetics of starch digestion by ranking starch-rich materials with respect to their enzymatic hydrolysis raising potential. HI could be obtained from the area under the digestibility curves (AUC) calculated based on reliable estimates of k and C_∞ values obtained by integration of the 1st order rate equation with the bounds of t_0–t, which is similar to standard trapezoidal method.

$$AUC = \left[\frac{C_\infty\, t + \, C_\infty\, e^{-kt}}{k}\right]$$

2.3.3.1. Significance of digestibility indexes

The lower the glycaemic index is, the slower the glucose levels rise in blood will be when the food is consumed. Many factors could affect GI, such as ripeness and storage time, processing and cooking method, variety (i.e. white and brown rice). The effect may also differ from person to person, including genome, age and activity level. Glucose, a monosaccharide, induces a large glycaemic response and is often used as the reference food with a GI equal to 100. In general, fat and fibre tend to lower the GI of food. Meats and fats don't have a GI because they do not contain carbohydrate. Sucrose, a disaccharide of glucose and fructose also has a lower effect on blood glucose (GI=68) than some starches, such as potatoes (GI=85), resulting from the fructose component which has an exceptionally low GI (19). The relationship between GI and GL is not straightforward, for example, a high GI food can have a low GL if eaten in small quantities. On the other hand, a low GI food can have a high GL dependent upon the portion size being eaten. A serving size (120g) of watermelon having a high GI (72), has the same GL (4) as a serving size of high fat ice cream (50g), a low GI food (37). Foods having very different nutrient profiles can have similar GIs (64 and 65, respectively) and GLs (23 and 26) per serving, such as parboiled rice (150g) and a chocolate bar (60g). Although a

food is assigned a fixed GI value, any food could have a low, medium or high GL because GL is dependent upon the amount eaten (FAO/WHO, 1998; McAuley and Mann, 2006; Schaefer et al., 2009).

Prospective studies suggest that low-GI diets may reduce the risk of diabetes, cardiovascular disease, metabolic syndrome, chronic inflammation, and possibly some types of cancer. Clinical trials have shown that low-GI diets improve glycaemic control in diabetes, reduce food intake and body weight, increase insulin sensitivity and β-cell function, influence memory, and may reduce serum cholesterol (Venn and Green, 2007).

An important justification for the claim of an overall health benefit of low GI foods is that may aid weight control because they promote satiety. Studies on weight loss comparing low and high GI diets assessed differences between diets based on ad libitum intake to show that the apparently greater satiating effect of low GI foods led to a reduced energy intake. High GI carbohydrates suppress short-term (1 h) food intake more effectively than low GI carbohydrates, whereas low GI carbohydrates appeared to be more effective over longer periods (6 h) (Anderson and Woodend, 2003; Anderson et al., 2002; Holt et al., 1997).

The glycaemic index of a food is correlated with the rate of absorption of carbohydrate, and also by the rate of glucose removal from the plasma. For example, the lower GI breakfast cereal had induced hyper-insulinaemia earlier than the higher GI cereal, resulting in an earlier increase and more rapid removal of glucose from circulation. Studies indicates that insulin response cannot be predicted based solely on the glycaemic response to food. The effect of glycaemic index on insulin response may also depend upon insulin sensitivity. Excess adiposity, is very important nutrition factor determining insulin resistance. Reducing adiposity by dietary modification and increase in physical activity improves insulin sensitivity and lowers the range of abnormalities associated with insulin resistance (Brites et al., 2011; McAuley and Mann, 2006).

Despite some limitations it does appear that distinguishing between foods with appreciable differences in the indices may produce some benefit in terms of glycemic control in diabetes and lipid

management. Even if some data suggest that the low GI effect is not explained by the dietary fibre content of the foods it remains conceivable that food structure or composition explain some of the health benefits. Finally, GI or GL may be useful indicators to guide food choice, but it should only be done in the context of other nutritional indicators and when values have been measured in a large group of individuals. Diabetes associations in Europe (Connor et al., 2003; EFSA and NDA, 2011a; Venn and Green, 2007), Australia (NHMRC, 2013) and United States (Sheard et al., 2004) indicate GI is a useful tool for differentiating between carbohydrates.

2.3.4. Kinetic models of *in vitro* fermentation

Food material that survived enzymatic digestion in the upper parts of GIT undergoes colon fermentation. Research on ruminant and mono-gastric animals suggest that the fermentation process can be stimulated in laboratory with *in vitro* techniques. The microbial inoculum can be prepared from fresh faeces, making the method easier and ethically acceptable. It has been shown previously, that faeces from pigs has similar *in vitro* fermentative activity to samples from other parts of the colon. *In vitro* models also provide information on different fermentation variables for a large number of ingredients in relatively short time. They are also cost-effective, easy to develop and non-invasive (Bauer et al., 2004; Coles et al., 2005).

Mathematical models are necessary tools to describe and interpret *in vitro* gas production kinetics and, during the last few decades, a great number of models have been proposed. Wang in his detailed report compared several models to describe the kinetics of feed digestion in rumen fluid, which can be also easily applied in mono-gastric animals (M. Wang et al., 2011). It include Logistic–Exponential models (LE), Gompertz model, Logistic model, generalization of the Mitscherlich model (GM), Exponential (EXP) and Michaelis–Menten models.

$$V = V_F \frac{1 - exp(-k(t - LAG))}{1 + exp(ln(1/d) - kt)}$$

The Logistic–Exponential (LE) model with lag time

$$V = V_F(1 - exp(-k(t - LAG)))$$

The Exponential (EXP) model with lag time

Some of those equations were developed to describe microbial growth, and then used to model *in vitro* gas production kinetics, have additional LAG time parameter. The lag phase, is an initial period when no growth is observed, when the cell has to transition from low to high metabolism, but still need more attention, especially its genetic program (Schultz and Kishony, 2013).

2.4. Nutrient perception in gastrointestinal tract

Animals are dependent on food as sources for energy and biochemical components, taste and olfaction - two of our major senses, are dedicated to guides organisms to evaluate the quality and nutritional value of food prior to its ingestion, while avoiding potential harmful substances. In a simple way, it is essential for most animals to determine what to eat (Breslin, 2013; Jiang et al., 2012). When describing food preferences it is easy to confuse taste with flavor. Taste is a chemical sense perceived on contact with substances by specialized receptor cells that make up taste buds. Whereas a flavor is not only a taste but a distinctive fusion of multiple senses, such as smell, tactile and thermal sensations. For mammals as well as for humans, tasting means recognizing and distinguishing each of the basic taste qualities. Up today there are five recognized tastes: sweet, umami, sour, salty, and bitter. Just ten years ago, during my biology classes in high-school I have learned that human has four basic tastes. Recent years have brought a lot of changes in taste science. The definition of taste has been expanded, allowing umami to enter into the original canon of four primary tastes (Chandrashekar et al., 2006; Lindemann, 2002). The tongue map was also challenged - physiological studies showing that distinct regions of tongue may selectively respond to different tastants are no longer valuable. There are additional assets such as fatty or even metallic, that might also be considered as basic tastes in the near future. We all know, food tastes differently to different people - we don't all like the same flavors. Sensation of taste is a very subjective feeling, it depends on our genome, age or diet. In the human population we recognize three kind of tasters: supertaster, medium taster or non-taster. Supertaster is a person who experiences the sense of particular taste with stronger intensity than average, in opposite to the non-taster (Chaudhari and Roper, 2010; Jiang et al., 2012; Smith and Margolskee, 2001).

2.4.1. Gustatory and extra-gustatory taste receptors

The initial step in gustatory process is the detection of tastants by specialized taste cells, clustered in taste buds on tongue and other parts of the mouth, like soft palate and throat. Here, taste receptor cells occur in morphological structures of 50–100 cells, called taste buds. The majority of taste buds are found on the tongue surface grouped in circumvallate, foliate and fungiform taste papillae, which have distinct locations. In the human tongue four types of papillae are present, but the fourth type - filiform has mechanical function and is not involved in gustation (Chaudhari and Roper, 2010; Hoon et al., 1999; Stone et al., 2007). A microphotographs showing a circumvallate papillae on the tongue surface of piglet is presented in figure 8. Once activated by tastants, taste receptor cells transmit the information via sensory afferent fibres to specific brain areas involved in taste perception. Taste cells are classified into four types depending on their morphological features. The salty taste is transduced by some type I glial-like cells. The type II cells express G protein-coupled receptors (GPCRs), and they can be calsified into three different subpopulations each specialized for detecting a single taste quality either sweet or umami or bitter depending on the taste receptor genes being expressed. The type III cells (also called presynaptic cells), which are the only cell type forming synapses with afferent nerve fibres and therefore are thought to fulfill a higher order integrative function, are for the sour sensors. The type IV cells are thought to be taste stem/progenitor cells. Moreover, the fat-sensing receptors, are expressed in type I and type II cells respectively. Alfa-gustducin, the a subunit of the G protein coupled to taste receptors, plays a role in bitter, sweet and at least partially in umami taste transduction (Chaudhari and Roper, 2010; Hoon et al., 1999; Jiang et al., 2012; Stone et al., 2007; Young et al., 2009).

It has become clear, however, that beyond these aware quality assessments the body also evaluates the composition of food at later time points during its passage through the digestive tract, and that this has a major influence on appetite and the coordination of nutrient disposal (Finger and Kinnamon, 2011). Recent findings indicate that they are also expressed outside the gastrointestinal tract, in respiratory systems, smooth muscles, heart, aorta and brain chemosensation in the gut is considered to occur in

specialized epithelial cells, the enteroendocrine (EEC) and tuft cells. EEC are specialized secretory epithelial cells derived from the same crypt-stem cell that produces the four primary epithelial cell types of the mucosal lining, such as absorptive enterocytes, goblet cells, Paneth cells, and enteroendocrine cells (Cheng and Leblond, 1974). EEC comprise less than 1% of the total epithelial cell population, the entirety of the intestinal endocrine cell population makes up the largest endocrine system in the body. It serves an important role as a communicating bridge between the luminal environment and the rest of the gastrointestinal tract to regulate gastrointestinal and metabolic functions. EEC family consist of more than 20 different cell types and secrete a variety of hormones, including gastrin (G cells), ghrelin (P or X/A cells), somatostatin (D cells), cholecystokinin (CCK) (I cells), serotonin (enterochromaffin (EC) cells), glucose-dependent insulinotropic peptide (GIP) (K cells), glucagon-like peptides (GLPs), and peptide YY (PYY) (L cells) (Rindi et al., 2004). We know about two pathways by witch enteroendocrine cells can respond. Many EEC cells are classed as open-type cells, having processes that extend toward the gastrointestinal lumen and apical surfaces located within the absorptive brush border. They make direct contact with ingested and digested nutrients (lipids, carbohydrates, proteins) and this location of cell is perfect to act as sensors of food intake. The second one, such as ghrelin-producing cells in the stomach, are more rarer and buried in the mucosa, they do not make contact with the gut lumen, additionally are believed to respond to mechanical stimuli and neuronal or paracrine factors (Raybould, 2010). Tuft cells, also known as brush or caveolated cells are sparse within the epithelium of the respiratory and gastrointestinal tracts of animals, and are rare in humans. The possibility of tuft cells participating in nutrient signaling was first recognized by Höfer & Drenckhahn, 1998; Hofer, Puschel, & Drenckhahn, 1996. These cells possess a distinct pear shape and apical microvilli similar to lingual taste cells, but differ morphologically from other secretory GI cells, and do not contain secretory granules or transmitter vesicles (Reimann et al., 2012). Many detailed information of the signaling pathways of EEC and tuft cells are still limited because of the scarcity and the difficulty in identifying them for cellular physiological studies.

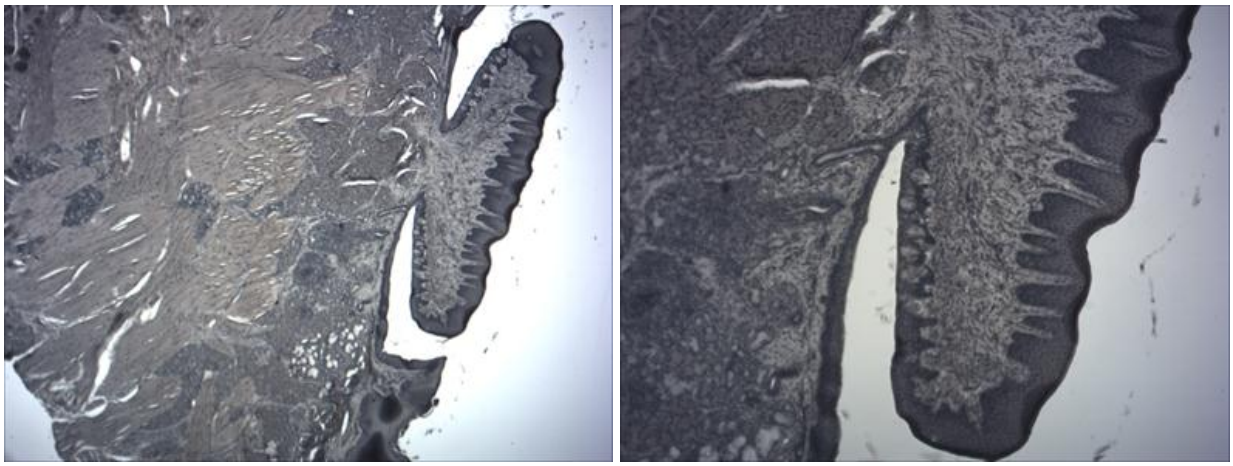


Figure 8.

Microphotographs showing a circumvallate papillae on the tongue surface of piglet, magnification x40 and x100. Each papillae has several taste buds located along their lateral surface (by Nadia de Jager and Mowen Zhan-University of Queensland, CNAFS).

2.4.2. Molecular biology of nutrient sensors

Transmembrane receptors play crucial roles in diverse cell signaling pathways that affect many aspects of cell activities. Their functions in many physiological processes make them targets of a wide range of classes of chemical ligands, activating or modulating downstream signaling pathways of receptors. Several ways of ligand-receptor are known, agonist - lead to activation of downstream pathways, antagonist - do not initiate activation but inhibit agonist-mediated responses, and inverse agonists, which act directly to block activation of pathways. Researchers implied the existence of specific taste receptors for nutrients long time ago, but it was only in the last few years that likely candidate receptors were identified, expressed, and characterized. Molecular biology techniques helped to identify the molecular components of taste transduction during the 1990s, and bring in a large number of genes and proteins potentially involved sensory transduction mechanisms (Kinnamon, 2000). The first proteins identified in taste receptor cells were components of the G-protein complex, involved in signal transduction of many receptors, in particular α-gustducin and α-transducin (McLaughlin et al.,1994; McLaughlin et al., 1992).

The G-protein-coupled receptors (GPCRs) are one of the largest super families of cell-surface receptors and are responsible for signaling responses to a wide variety of hormones, neurotransmitters, sensory stimuli, metabolites, and ions. The receptors cross the cell membrane, so that one N-terminal end of the protein, that can vary considerably in size, projects from the outer surface of the cell (a characteristic seven-transmembrane (7TM) domain) and the other C-terminal tail remains in cytoplasm. 7TM domain allows the receptors to transmit information from outside environment to the interior of the cell. GPCRs can be subdivided, based on their sequence homology, into three classes: A, B, and C (Alexander et al., 2008). Most receptors belong to the rhodopsin-like family A. In this class many receptors are members of the olfactory subgroup and also ones sensing short-chain fatty acids (SCFA). Additionally GPCRs are interesting drug targets for the treatment of several metabolic disorders (osteoporosis) and nervous system diseases (such as migraine, anxiety, and depression). Class C GPCRs, have several structural features that make them unique in the GPCR family, possess a large extracellular N-terminal ligand binding region (>500 amino acids) with a "clamshell" or "venus flytrap" fold, having two ligand binding domains that can open or close. This N-terminal region is linked to the transmembrane segment by a smaller (about 70 amino acids) cysteine-rich domain. They, may function as homodimers or heterodimers, providing a characteristic mode of activation compared to other GPCRs classes. Class C includes several glutamate receptors, sweet and umami (monosodium glutamate) taste receptors, the Ca2þ-sensing receptor, the g-aminobutyric acid type B receptor, and pheromone receptors (Pin et al., 2003).

G proteins associated with GPCRs are heterotrimeric, it means that are build up from three different subunits: alpha, beta, and gamma subunit. Two of these subunits, alpha and gamma, are attached to the plasma membrane. However, exception exists and some G proteins, such as the signaling protein Ras, are small with only a single subunit. Vertebrate genomes contain multiple genes that encode three subunits, so they could be combine in multiple ways producing a diverse family of G proteins. We know at least about 16 different alpha subunit genes, 4 beta subunit genes, and 11 gamma subunit genes. They are primarily classified based upon their alfa subunits and the corresponding downstream signaling pathways they recruit and can be grouped into four families: G_{as}, G_{ai}, G_{aq}, and $Ga_{12/13}$. A G

protein alpha subunit is able to bind either nucleotides guanosine triphosphate (GTP) or guanosine diphosphate (GDP). In the lack of a signal, GDP attaches to the α subunit, and the entire G protein-GDP complex binds to a nearby GPCR. This setup last until a ligand (agonist) interact with the GPCR. Activation of GPCRs generally results in exchange of GTP for GDP bound to the α-subunit, followed by at least partial dissociation of the G protein α-subunit from the β-γ dimer. Both parts, the alpha subunit and the beta-gamma complex, are separated from the GPCR and remain tethered to the plasma membrane. These activated subunits can interact with other membrane proteins such as ion channels, as well as act on cellular enzymes and second messenger molecules that move around the cell. G protein remains active as long as GTP is connected to alpha subunit. When GTP is hydrolyzed back to GDP, the entire G protein complex change conformation into the previous form and re-connect with the inactive GPCR. Basically, G-protein works like a switch, signal-receptor turns it on or off by interactions on the cell's surface (Franco et al., 2010; Hermans and Challiss, 2001; Kristiansen, 2004).

However, even if GPCRs family are major point of research focus, they are not the only candidates for chemosensation. Two basic taste sensations - salty and sour, use different mechanisms, and similar to GPCRs based sensors still need a lot of studies. The sodium-specific salt taste receptor is the epithelial sodium channel, while another is a nonspecific salt taste receptor - the vanilloid receptor 1 (TRPV1, or capsaicin receptor) belongs to the transient receptor potential (TRP) superfamily. Sour taste stimulus is a decrease in the intracellular pH of a subset of acid-sensing taste cells, which serves as the input to separate transduction pathways of the sour neural response. NHE1 (basolateral sodium-hydrogen exchanger isoform-1) activated by an increase in cell Ca_{2+} represents molecular basic of sour sensing. Calcium controls the activity of NHE1, which, in the continued presence of an acidic stimulus, sustains the adaptation level of the sour taste response (Chandrashekar et al., 2006; DeSimone and Lyall, 2006; Törnwall, 2013). An overview of the different taste receptors involved in nutrient sensing is represented in table 1.

2.4.3. Sweet taste receptors

Sweet taste plays a significant role in our lives, first it indicate the presence of carbohydrates that serve as energy source, second we usually have good associations with sweetness. However, no matter how we like it, sweetness is a serious medical issue, as number of people are affected by diseases more or less directly linked to the secondary effects of sugar intake, such as diabetes or hyperlipemia. Recent breakthroughs in molecular biology of sweet taste may suggest strategies to overcome diet-induced diseases. The most commonly known mammalian sweet taste sensor is composed of the two subunits, T1R2 and T1R3, forming a functional heterodimer. Receptor cells expressing the heterodimer T1R2+T1R3 respond to all compounds known to taste sweet to humans (or attractive to rodents) including sugars, artificial sweeteners, D-amino acids and glycine, sweet proteins and plant metabolites such as stevioside. In case of glucose, this pair responds to relatively high concentrations about 30–1000 mM. Of course, the list of compounds able to get response is different among mammalian species (Breslin, 2013; Callaway, 2012; Hashiguchi et al., 2007; Nelson et al., 2001; Young et al., 2009). The best example is the sweet taste receptor of cats which has lost its function due to the pseudogenization of the gene coding for the Tas1R2 subunit, so cats are naturally T1R2 KO animals (Jiang et al., 2012; Li et al., 2005).

Within the intestinal epithelium, similar to the tongue, T1R2 and T1R3 function in association with signaling components, such as Gα subunit alfa-gustducin, TRPM5 (transient receptor potential channel M5) and PLCb2. Gustducin is thought to stimulate phosphodiesterase and thus result in cyclic nucleotide degradation, but in parallel the coreleased Gbg subunits activate PLCb2. Knocking out PLCb2 severely diminishes, but does not eliminate taste sensitivity (Dotson and Spector, 2004; Nelson et al., 2001). PLCb2 stimulates the synthesis of IP3, which opens IP3R3 ion channels on the endoplasmic reticulum, releasing Ca_{2+} into the cytosol of receptor cells. The consequent elevation of cytoplasmic Ca_{2+} activates a taste-selective cation channel TRPM5 triggering membrane depolarization and activation of voltage-gated Ca_{2+} channels. This signaling cascade is similar for all T1Rs group, more details regarding T1R1 and umami taste are available in the next chapter. So far,

receptors T1R2 and T1R3 have been detected in brush cells and endocrine L and X/A cells, but it is still not clear whether all components of that pathway are located in the same cells (Chandrashekar et al., 2006; Nelson et al., 2001; Tomonari et al., 2012).

Several studies showed that stimulated by glucose in gut EEC sweet taste receptors may trigger release of incretin hormones GLP-1 and GIP, from intestinal L and K cells, respectively. Such a response is called "incretin effect" and it was known long before discovery of T1R2 and T1R3 receptors. The idea that the gastro-intestinal tract can sense sweet came from observation that orally administered glucose was more effective than glucose injected intravenously in elevating insulin secretion. Both peptides, GLP-1 and GIP, stimulate pancreatic insulin secretion resulting in blood glucose uptake (Jang et al., 2007; Mace et al., 2007; Steinert et al., 2011). Moreover, that stimulation with sweet compounds, perhaps together with a paracrine mechanism, enhance the sodium–glucose cotransporter 1 (SGLT1) expression, thus modulating the intestinal glucose-uptake capacity (Margolskee et al., 2007). In both human and mouse enteroendocrine cells stimulation with dietary sugars and artificial sweeteners have been shown to stimulate GLP-1 release and increase SGLT1 expression, but in gustducin or T1R3 knockout mice, glucose-mediated actions were significantly reduced (Jang et al., 2007). It was also reported that sucralose, stimulated incretin release from GLUTag cells (Margolskee et al., 2007). Additionally, incretine hormone release from cell lines can be blocked by the sweet receptor antagonists gurmarin, lactisole or siRNA for α–gustducin (Jang et al., 2007; Margolskee et al., 2007). However, other experiments called into question those statements and it looks like sweet taste receptors in gut are not the principal recognition mechanisms responsible for the regulation of endocrine secretion or even perceptions of hunger. *In vitro* experiments on murine primary cultures denied GIP and GLP-1 release by the artificial sweeteners stimulation and suggests that SGLT1, but not sweet taste receptors, plays a key role in glucose-triggered hormone release (Parker et al., 2009; Reimann et al., 2008). In vivo studies on human (Little et al., 2009; Ma et al., 2010) or rodents (Fujita et al., 2009) subjects showed no effect of acute treatments with sweeteners (D-tryptophan, acesulfame K, saccharin, sucralose and stevia), on the rate of intestine

glucose absorption, postprandial blood glucose concentrations, GLP-1 and GIP secretion or gastric empting.

Sodium-dependent glucose transporter (such as SGLT1, SGLT2 and SGLT3) is one of two families of sugar transporters expressed along gastrointestinal mucosa cells. Second family is called facilitated-diffusion glucose transporter (GLUT). Protein-transporters help hydrophilic molecules of sugars cross lipid cell membranes. As mentioned earlier SGLT1 is expressed on the apical part of EEC and supports uphill glucose absorption out of the lumen. Each glucose molecule is transported together with two sodium ions binding to SGLT1. Basolateral Na^+/K^+-ATPases is responsible for maintaining the NA+ and K+ electrochemical potential gradient. SGLT1 mRNA and protein levels are regulated by dietary intake and diurnal rhythms and may involve activity of the sweet taste receptor pathway. D-glucose have a higher affinity among sugars to bound and be transported by SGLT1 (D-glucose > α-methyl-D-glucose > D-galactose > 3-O-methyl glucose > L-glucose and 2-deoxyglucose) (Srichamroen, 2013). SGLT3 properties vary significant among species, human SGLT3, unlike pig SGLT3, had lost its capacity to transport sugars and operate rather as a sugar-sensitive Na+ channel. Rodents, such as mouse have two genes for SGLT, mSGLT3a and mSGLT3b, first is still not well characterized and second one has characteristics intermediate between SGLT1 and human SGLT3. Members of sodium-coupled glucose transporter family may also act as a different mode of sugar sensing, that directly triggers membrane depolarization. The role of SGLT1 activity and glucose sensing was showed in in the GLP-1-secreting cell line, GLUTag (Diez-Sampedro et al., 2003), and in primary murine intestinal cultures (Reimann et al., 2008). The facilitative transporters (GLUT) utilise the diffusion gradient of glucose (and other sugars) across plasma membranes. According to the sequence similarities, the family of 12 members can be divided into three subclasses. Class I is quite well characterized compare to others, can be distinguished on the basis of their distinct tissue distributions (GLUT1 - erythrocytes, brain; GLUT2 - liver, pancreatic islets; GLUT3 - neuronal cells; GLUT4 - muscle, adipose tissue) and their hormonal regulation (e.g., insulin sensitivity of GLUT4). It has been shown that GLUT2 is recruited to the apical membrane surface rapidly after a meal (t½ 3.5 min), allowing the passive absorption of glucose at higher luminal sugar concentrations, and may be

controlled by Ca^{2+}, sweet taste receptors and hormones. Apical GLUT2 insertion probably serve to save energy and prevent the unnecessary run-down of the Na^{+} gradient by SGLT1 when this is not energetically required. Research on animal models for type 2 diabetes showed lower expression levels of GLUT4 in adipose tissue, but not in muscle, which suggest that it is not the causative factor of insulin resistance (Wood and Trayhurn, 2003).

2.4.5. Other nutrients perception relevant for the study

2.4.5.1. Sensors for umami and amino acids

The ingestion of dietary protein is very important, because, their structural units aninoacids are precursors of many molecules and play role in modulating metabolism. Several G protein-coupled receptors, that belong to class C, may serve as amino-acids (AAs) taste receptors. They have been identified in taste cells, mainly in the mouth and gastro-intestinal tract, but also in other parts of the body. The most relevant GPCRs for AA sensing include the extracellular calcium-sensing receptor (CaSR), the heterodimer T1R1/T1R3, glutamate receptors (GluRs) and the GPCR family C subtype 6A (GPRC6A) receptor (Behrens and Meyerhof, 2011; San Gabriel and Uneyama, 2013).

Amongst the GPCRs the important candidate receptors of aromatic and basic amino acid sensing are the calcium-sensing receptor (CaR) and GPRC6A which, as suggested by name are primarily involved in Ca^{2+} signaling. CaSR may plays a significant role in chemosensing given its localization throughout the gut and its ability to change mode of action depending on the cell type. It expression was described in taste tissue of the tongue, in gastrin secreting G cells, somatostatin-secreting D cells and CCK- secreting I cells. Beside absorption of Ca^{2+}, CaSR participate in numerous physiological responses, including secretion of hormones, motility (such as CCK), NaCl and water transport and colonic cell differentiation (Haid et al., 2012; Alice P Liou et al., 2011; Y. Wang et al., 2011). Very little is known about identified recently GPRC6A sensor, which is an evolutionary a close neighbor of

CaSR, and is expressed throughout the gut and in gastric antral D cells and G cells (Nguyen et al., 2012; Wellendorph et al., 2009).

Sensor GPR92 (also named GPR93 or LPAR5) was found to mediate chemosensing in the GI tract in response to peptones, but is not a member of the Venus flytrap family. GPR92 is expressed in taste buds and stomach G and D cells. Peptone, a mixture of enzymatically derived peptide fragments, also stimulate CCK and GLP-1 secretion. In murine taste buds GPR92 is expressed in the majority of T1R1-positive taste cells (Haid et al., 2013).

The receptor that have a particular importance for this study is the heterodimer T1R1/T1R3, known also as umami receptor. Compared to previously mentioned AAs receptors the role of T1R1/T1R3 is understood very well. Umami, means delicious or savory after the Japanese word ‘umai’ and was introduced to the world by Kikunae Ikeda, more than one century ago (Ikeda, 2002). Today umami is widely accepted to elicit a unique taste quality, different from the other basic tastes. These receptors are activated mainly by monosodium glutamate (MSG), 5’-inosinate (IMP) and 5’-guanylate (GMP) compounds found in meat, cheese, tomatoes, fish, and some mushrooms. Ribonucleotide IMP is mainly present in meats and GMP is found in plants (Nelson et al., 2002; Roininen et al., 1996). T1R1/T1R3 has been already found in many model species including swine, human and rodents. Cells expressing T1R1 or T1R3 or both were found in oral and extra-oral tissues (Foster et al., 2013; Roura et al., 2011). It was showed, that receptors respond not only to L-glutamate. Additionally, T1R1 and T1R3 knock-out mice lost their detection and perception to glutamate, aspartate, AP4, IMP, and other amino acids. However, later research demonstrated that T1R1/T1R3 do not have a monopoly for umami taste responses, indicating that also other receptors, such as glutamate receptors (GluRs) can mediate responses to umami compounds (Damak et al., 2003; Nelson et al., 2002; Zhao et al., 2003). Heterodimeric umami taste receptor T1R1/T1R3 play a role in the detection of L-glutamate as a tastant, while the GluRs could also recognize glutamate as a transmitter. Glutamate, is one of the major excitatory neurotransmitter in the central nervous system of mammals, and can acts on metabotropic (mGluR) and ionotropic (iGluRs) receptors (Chandrashekar et al., 2006; Nelson et al., 2002). It was finally confirmed, that the human form of T1R1/T1R3 is very specific to L-glutamate,

whereas the mouse T1R1/T1R3 responds broadly to aliphatic L-amino acids (up to 18 different L-AA). Swine T1R1 sequences are very similar to human and shows smaller gene homologies to rodents, in fact they respond to only eight L-AA (Roura et al., 2011). In humans and other animals L-glutamate, was found to increase the activity of gastric vagal afferents and to stimulate gastric secretion and motility. Ad libitum ingestion of glutamate reduced weight gain in rats without affecting food intake (Toyomasu et al., 2010).

2.4.5.2. Sensors for fatty acids and lipids

Recent findings challenge the canon of five basic tastes, strongly suggesting significant role of taste in dietary fatty acids and lipids perception. Therefore, the molecular mechanisms underlying that sense may put a new light into studies on the preference for fatty foods and, consequently, in the obesity risk (Janssen and Depoortere, 2013). The fat-rich diet derived lipids mostly in the form of triglycerides, other ones, such as phospholipids and cholesterol, are normally found in rather high concentrations in food but are additionally released into the small intestine together with bile. The lipids are cleaved by lipases to release free fatty acids (FFAs), their ingestion is also a potent stimulus for the secretion of a number of enteroendocrine hormones, including CCK, GLP-1, and GIP. In principle, EECs could sense presence of triglycerides directly, however some evidences suggest the need of lipolysis. Among FFAs, long-chain fatty acids (LCFAs) can in particular inhibit gastric emptying and induce satiety, on the other hand, short-chain fatty acids (SCFAs) have also been found to trigger GLP-1 and PYY secretion (Freeland and Wolever, 2010; Raybould et al., 1998; Sakata et al., 1996). Long-chain fatty acids, with 13–21 carbons chain, are absorbed by the fatty acid transporter CD36 and the fatty acid transport protein 4 (FATP-4) whereas, medium-chain fatty acid (MCFA) (6–12 carbons) are absorbed in the intestine by passive diffusion. Monocarboxylate transporter isoform-1 (MCT-1) is involved in absorption off short-chain fatty acids (<6 carbons). FFAs are sensed by nuclear receptors such as PPARs, and also by a many different GPCRs. During the last decade scientist identified four main GPCRs which respond to free fatty acids: FFAR1, FFAR2, FFAR3 and

Table 1.

Overview of the different taste receptors involved in nutrient sensing of carbohydrates, amino-acids and fatty-acids (Janssen and Depoortere, 2013; Foster et al., 2012; Milligan, Ulven, Murdoch, & Hudson, 2014; San Gabriel & Uneyama, 2013).

Chemosensory signaling element			Ligand
Sweet taste receptors / transporters			
▪ **T1R2-T1R3**		Taste receptor type 1 member 2 - Taste receptor type 1 member 3	carbohydrates, artificial sweeteners, sweet proteins
▪ **SGLT**		Sodium-dependent glucose transporter	sugar molecules transporter
▪ **GLUT**		Glucose transporter	sugar molecules transporter
Amino-acids receptors			
▪ **T1R1-T1R3**		Taste receptor type 1 member 1 - Taste receptor type 1 member 3	aliphatic AA
▪ **GRM**	mGluR	Metabotropic glutamate receptor	glutamate
▪ **GPRC6A**		G-protein coupled receptor family C group 6 member A	basic, small, neutral AA
▪ **CaSR**		Calcium-sensing receptor	aromatic AA
Free fatty-acids receptors			
▪ **GPR41**	FFAR3	Free fatty acid receptor 3	SCFAs
▪ **GPR43**	FFAR2	Free fatty acid receptor 2	SCFAs
▪ **GPR40**	FFAR1	Free fatty acid receptor 1	MCFAs, LCFAs
▪ **GPR84**		G-protein coupled receptor 84	MCFAs
▪ **GPR92**	GPR93, LPAR5	G-protein coupled receptor 92, Lysophosphatidic acid receptor 5	peptones, lysophosphatidic acid (LPA)
▪ **GPR120**	O3FAR1, FFAR4	G-protein coupled receptor 120, Omega-3 fatty acid receptor	LCFAs, 0mega-3 FAs

GPR120. First three FFARs are also known as GPR40, GPR43 and GPR41, respectively (Covington et al., 2006; Hirasawa et al., 2005).

The FFAR1 has been identified as a receptor for medium to long-chain free fatty acids (FFAs). Initial studies showed its expression in the pancreatic beta-cells and under FFAs stimulation mediates insulin secretion. Subsequently, FFAR1 was found in endocrine cells throughout the gastro-intestinal tract, indicating that FFAR1 senses the luminal presence of LCFAs and elicits an endocrine response (Edfalk et al., 2008; Alice P. Liou et al., 2011). Although the role of FFAR1 in short-term stimulation of insulin secretion has been confirmed in several studies, inconsistent results on the role of FFAR1 in the long-term effects of fatty acids have been obtained in studies on FFAR1 KO mice (Kebede et al., 2008; Latour et al., 2007; Steneberg et al., 2005).

GPR120, also known as the omega-3 fatty acid receptor (O3FAR1), more specifically binds LCFA of C14 to C22 carbon chain length. Transcripts have been found in the endocrine cell lines, stomach and intestine of mouse and human. It is expressed in brush cells, X/A cells, and L cells. Stimulation of GPR120 by FFAs promotes the secretion of GLP-1 and CCK from STC-1 cells, and the secretion of insulin (Hirasawa et al., 2005; Janssen et al., 2012; Oh et al., 2010; Wellendorph et al., 2009). It remains unclear whether FFAR1 or GPR120 - LCFAs receptors, play the more important role in enteroendocrine cell fatty acid sensing. It was found, in siRNA-mediated knockdown study, that GPR120 could be more important for GLP-1 and CCK secretion than FFAR1, in the STC-1 cell line (Hirasawa et al., 2005). However, experiments with FFAR1 null animals displayed reduced release of hormones in response to dietary fat, so it is unlikely that GPR120 plays a role in direct induced secretion (Edfalk et al., 2008; Alice P. Liou et al., 2011). GPR120 is also expressed in differentiated adipocytes and macrophages (Gotoh et al., 2007; Oh et al., 2010, 2005). Obese individuals had significantly overexpressed GPR120 levels in adipose tissue and stomach. Additionally, a recent study showed that its dysfunction leads to obesity, glucose intolerance, and fatty liver (Ichimura et al., 2012; Widmayer et al., 2012). GPR120 sensor could serve as potential drug target, and its agonists, such as

GW9508 or MEDICA16, may improve insulin sensitivity and also reduce obesity-induced inflammation (Holliday et al., 2011; Oh et al., 2010).

FFAR2 and FFAR3 are expressed in the lower intestine, suggesting that they act as a sensor and mediate stimulated with short chain fatty acids generated by bacterial fermentation of poly-saccharides. FFAR2 preferentially binds C2–C3 fatty acids and FFAR3 reportedly favors C3–C5. These receptors are localized in L cells, but not in 5-HT-containing cells. Both receptors also mediate the effect of SCFAs on immune cells, adipocytes, and pancreatic cells, additionally they initiate the secretion of GLP-1 and PYY in the colonic mucosa which could suggest a role for SCFA in energy homeostasis (Covington et al., 2006; Hirasawa et al., 2005; Tazoe et al., 2008; Xiong et al., 2004). Mice lacking FFAR2 or FFAR3 showed reduced SCFA-triggered GLP-1 secretion and a parallel impairment of glucose tolerance (Tolhurst et al., 2012).

The recent *in silico* analysis, of human and pig alignments (Colombo et al., 2012), showed full homology for exon sequences for 3 genes for fat taste: FFAR1, FFAR2 and GPR120. Moreover, FFAR2 and GPR120 transcripts were detected in GIT of weaned pigs - in jejunum, colon and 3 segments of stomach: oxyntic, pyloric and cardiac to oxyntic transition mucosa; whereas no expression was found for FFAR1. Authors noticed that higher gene expression of GPR120 in the porcine colon, respect jejunum, and could be explained with its major role in the colon in the incretin rise after FFAs stimulation, as observed before in mice (Colombo et al., 2012; Hirasawa et al., 2005). Similar results were obtained very recently by van der Wielen et al., (2014), GPR120 presence was oriented towards large intestine compare with small.

2.4.5.3. Bitter taste receptors

Humans have ~25 functional members of the bitter taste receptor family (T2R) belonging to the class A GPCR superfamily, mouse have ~33 and pig ~15, see table 2 (Behrens & Meyerhof, 2011; Groenen et al., 2012; Meyerhof et al., 2010; Thalmann, Behrens, & Meyerhof, 2013). Each member consists of

Table 2.

Overview of the confirmed bitter family Tas2Rs taste receptor genes of human, mouse and pig (Behrens & Meyerhof, 2011; Groenen et al., 2012; Meyerhof et al., 2010; Thalmann, Behrens, & Meyerhof, 2013). Additionally, the table shows a list of bitter compounds which can stimulate human receptors. Orphan sensors are marked with * (Thalmann et al., 2013).

Human			Mouse		Pig
Gene	**Aliases**	**Ligands**	**Gene**	**Aliases**	**Gene**
Tas2R1		amarogentin, arborescin, cascarillin, chloramphenicol, thiamine, humulone isomers, parthenolide, picrotoxinin, dextromethorphan, diphenidol, diphenylthiourea, sodium cyclamate, sodium thiocyanate	**Tas2r102**	STC9-7	**Tas2R1**
			Tas2r103	Tas2R10, TRB2	**Tas2R3**
Tas2R3		chloroquine	**Tas2r104**	Tas2R45	**Tas2R4**
Tas2R4		amarogentin, arborescin, artemorin, campher, colchicine, quinine, parthenolide, quassin, yohimbine, azathioprine, chlorpheniramine, dapsone, denatonium benzoate, diphenidol	**Tas2r105**	Tas2R5, Tas2R9	**Tas2R7A, -B, -C**
			Tas2r106	Tas2R44	**Tas2R9**
Tas2R5		1,10-phenanthroline	**Tas2r107**	Tas2R43, Tas2R4, STC5-1	**Tas2R10**
Tas2R7		caffeine, papaverine, quinine, diphenidol, cromolyn, chlorpheniramine			
Tas2R8		parthenolide, chloramphenicol, denatonium benzoate	**Tas2r108**	Tas2R8	**Tas2R16**
Tas2R9		ofloxacin, procainamide, pirenzapine	**Tas2r109**	Tas2R62	**Tas2R20**
Tas2R10		caffeine, absinthin, arborescin, arglabin, artemorin, brucine, campher, cumarin, cucurbitacin, quinine, chloramphenicol, erythromycin, papaverine, parthenolide, picrotoxinin, quassin, strychnine, yohimbine	**Tas2r110**	Tas2R57, STC9-1	**Tas2R38**
			Tas2r113	Tas2R58	**Tas2R39**
Tas2R13		denatonium benzoate, diphenidol	**Tas2r114**	Tas2R46	**Tas2R40**
Tas2R14		sodium benzoate, absinthin, caffeine, quinine, arborescin, arglabin, aristolochic acid, artemorin, campher, cascarillin, coumarin, cucurbitacin, falcarindiol, humulone isomers, noscapine, papaverine, parthenolide, picrotoxinin, quassin	**Tas2r115**		**Tas2R41**
			Tas2r116	Tas2R56, TRB1, TRB4	**Tas2R42**
			Tas2r117	Tas2R54,	**Tas2R60**
Tas2R38	PTC, Tas2R61	allylisothiocyanyte, limonin, phenylthiocarbamide (PTC), sinigrin, propylthiouracil, sodium cyclamate, sodium thiocyanate, dimethyl thioformamide, methimazole, methylthiourea, diphenidol, diphenylthiourea, caprolactam, chlorpheniramine	**Tas2r118**	Tas2R16, Tas2R40	**Tas2R134**
			Tas2r121	Tas2R13, Tas2R48	
			Tas2r122		

Table 2.

Continued

Human			Mouse	
Gene	**Aliases**	**Ligands**	**Gene**	**Aliases**
Tas2R39		amarogentin, catechins, chloramphenicol, thiamine, diphenidol, denatonium benzoate, chlorpheniramine, chloroquine, azathioprine, acetaminophen, yohimbine, quinine	**Tas2r124**	Tas2R50
			Tas2r125	Tas2R59
Tas2R40	GPR60, Tas2R59	humulone isomeres, quinine, diphenidol, dapsone, chlorpheniramine, diphenhydramine	**Tas2r126**	Tas2R41, Tas2R12
			Tas2r129	Tas2R60
Tas2R41		*		
Tas2R42	Tas2R55	*	**Tas2r130**	Tas2R7, Tas2R6, Tas2R30, STC7-4, Tas2R42
Tas2R43	Tas2R52	aloin, amarogentin, arborescin, caffeine, arglabin, chloramphenicol, quinine, aristolochic acid, falcarindiol, grossheimin, helicin, acesulfame K, cromolyn, diphenidol, saccharin	**Tas2r131**	
Tas2R44	Tas2R31, Tas2R53	aloin, quinine, aristolochic acid, parthenolide, Acesulfame K, diphenidol, famotidine, saccharin	**Tas2r134**	Tas2R34
			Tas2r135	Tas2R35, Tas2R38
Tas2R45	ZG24P, GPR59	*	**Tas2r136**	Tas2R36, Tas2R52
Tas2R46	Tas2R54	absinthin, amarogentin, andrographolide, arborescin, caffeine, quinine, strychnine, azathioprine, carisoprodol, chlorpheniramine, denatonium benzoate, arglabin, artemorin, brucine, cascarillin, chloramphenicol, cnicin,	**Tas2r137**	Tas2R3, Tas2R41
			Tas2r138	Tas2R38, Tas2R31
Tas2R47	Tas2R30	absinthin, amarogentin, andrographolide, artemorin, denatonium benzoate, campher, cascarillin, quassin, picrotoxinin	**Tas2r139**	Tas2R39, Tas2R34
			Tas2r140	Tas2R40, Tas2R8, Tas2R13, Tas2R64, TRB3, TRB5
Tas2R48	Tas2R19, Tas2R23	cromolyn, diphenidol		
Tas2R49	Tas2R20, Tas2R56	diphenidol	**Tas2r143**	Tas2R43, Tas2R36
Tas2R50	Tas2R51	amarogentin, andrographolide	**Tas2r144**	Tas2R40, Tas2R33
Tas2R60		*		

a short extracellular domain, with relatively divergent amino acid sequences (25% to 90% identity). This variability is thought to correspond with an ability to detect diverse ligands present in the external environment (Behrens and Meyerhof, 2009; Colombo et al., 2012; Drewnowski and Gomez-Carneros, 2000). Furthermore, each T2R functions as a monomer suggesting low ligand specificity, with some members responding to a broad range of bitter compounds, but others being activated only by a selected stimuli. Bitter taste receptors have been suggested to mediate a regulatory role in limiting toxin ingestion and absorption. Intestinal epithelia are in continuous contact with unfamiliar agents, so gut chemosensing may act as preventing mechanism to avoid exposure of the central nervous system to some noxious substances. Distinct mechanisms in the colonic mucosa monitor luminal ambient chemicals. When present, these chemicals induce anion secretion, and by interacting with prostaglandin (PG), it stimulates fluid secretion, an important host defense mechanism, which flushes out harmful agents from the colonic lumen (KAJI et al., 2011). Few studies also linked bitter compounds to the release of ghrelin, CCK, and GLP-1 (Janssen et al., 2011; Jeon et al., 2008). T2R1 has been identified as being sensitive to bitter tasting dipeptides and tripeptides, but its expression and signal transduction in the gut has yet to be explored (Upadhyaya et al., 2010). Moreover, the colonic epithelia may interact with commensal bacteria through their chemical receptors and products, maintaining luminal homeostasis. Investigations of physiological phenomena of the intestinal epithelia and their mechanisms are important in revealing how homeostasis is maintained while interacting with the external environment (KAJI et al., 2011). The recent *in silico* comparison analysis of human and pig's T2Rs found full homology for exon sequences of 7 genes: TAS2R1, TAS2R3, TAS2R7, TAS2R9, TAS2R10, TAS2R16, and TAS2R38. Additionally, it showed that for TAS2R8 alignment from human to pig was not present, and also for TAS2R13 and TAS2R46 the porcine predicted sequence also aligned with several other human bitter genes (Colombo et al., 2012).

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3. Research projects - Part 1

Dietary starch and non-starch polysaccharides in swine performance

3.1. Monocereal diets with hulled or hulless barley varieties. Performances and carcass quality of Italian heavy pigs

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Manuscript submitted to Journal of Animal Science on 4 November 2013.

Abstract

A study was conducted to evaluate the effect of monocereal 26 diets based on hulled or hulless (normal- and low-amylose) barley varieties on growth performance and carcass characteristics in heavy growing-finishing pigs for the production of Protected Designation of Origin (PDO) Italian products. The trial was performed with 40 gilts and 40 barrows (Italian Duroc x Italian Large White). Four treatment diets were formulated: 1) corn based diet (control); 2) control diet with 80% of corn replaced by a normal-amylose hulled barley variety named Cometa (Cometa); 3) control diet with

80% of corn substituted by a normal-amylose hulless barley variety named Astartis (Astartis); 4) control diet with 80% of corn replaced by a low-amylose hulless barley variety named Alamo (Alamo). The diets were formulated according to 3 growth periods (40-80, 80-120 and 120-160 kg BW), with the same Lys:DE (2.60, 2.20 and 1.80, respectively in the three growth periods) according to the NRC requirements for the first two growth phases and according to requirements for high performing pigs for the last growth phase. The diets were analyzed for their *in vitro* starch digestion potentials (predicted glycemic index, pGI) and for their resistant starch (RS) contents. In all three growth periods, Alamo diet had the numerically lowest RS contents and highest pGI values, whereas the control diet had the numerically highest RS contents and the lowest pGI values. Throughout the trial, the pigs fed Cometa and Alamo diets grew faster ($P = 0.01$) than those fed the control diet, whereas animals receiving Astartis diet grew in a similar manner to those receiving all the other diets. Animals fed Cometa and Alamo achieved higher final weights ($P = 0.01$) compared with those fed the control diet. The animals receiving Astartis diet had a mean final weight similar to that of the animals fed other diets. Despite the results observed on ADG and final live weight, when feeding Cometa diet the animals had the lowest dietary efficiency throughout the trial ($P < 0.01$). For the same period, Astartis and Alamo diets had the highest dietary efficiencies, whereas the control was in between. No difference in carcass characteristics was found among treatments. This study showed that monocereal diets based both on hulled and hulless barley might be viable in heavy pig production destined for Italian PDO product manufacturing without adversely affecting growth performance and carcass characteristics.

Keywords: carcass quality, hulled barley, hulless barley, heavy pigs, performance, starch

Acknowledgments: Financial support was provided by the AGROSCENARI project 'Scenari di adattamento dell'agricoltura italiana ai cambiamenti climatici' of MIPAAF (Ministero delle politiche agricole alimentari e forestali – Italy) and by the Doctoral School on the Agro-Food System (Agrisystem) of the Università Cattolica del Sacro Cuore (Italy).

Introduction

Corn is the predominant cereal used in pig diet formulation in Italy and in most countries worldwide, because of high starch and low fiber levels (Prandini et al., 2011). Large amounts of irrigation water are usually provided to corn for maximising yields. However, many researchers warn about possible global climate changes (WWAP, 2009), which might lead to a decrease of water availability for irrigation (Masoero et al., 2013). As a consequence, the evaluation of alternative cereal grains to corn in monogastric diets is currently of great interest.

For instance, barley could represent a possible alternative to corn since it can easily grow in low rainfall areas and does not require irrigation. However, the use of barley in pig production is generally limited because of the high fiber content and lower energy value compared to corn (NRC, 1998). Since a large proportion of crude fiber of barley is contained in the hull fraction (Bell et al., 1983), the absence of hull in barley could substantially improve its nutritive value. Pigs fed hulless barley have shown higher growth performance than pigs fed hulled barley (Thacker, 1999; Thacker and Rossnagel, 2006). Ago, diets based on barley resulted in lower pig performance than diets based on wheat or corn (Hollis and Palmer, 1971; Della Casa et al., 1985). On the contrary, Wu et al. (2000) have obtained similar pig performance with diets based on hulless barley or corn. However, considering the limited available literature, further research is necessary to understand if barley could represent a potential alternative to corn in pig production. The aim of this work was to evaluate the effect of monocereal diets based on hulled or hulless (normal- and low-amylose) barley varieties on growth performance and carcass characteristics in heavy growing-finishing pigs for the production of Protected Designation of Origin (PDO) Italian products.

Materials and Methods

Animals and Experimental Design

Animal care and use practices during the trial conformed to EU Directive 2010/63/EU of 22 September 2010 on the protection of animals used for scientific purposes. The study was conducted with 40 gilts and 40 barrows (Italian Duroc x Italian Large White) of 49 (±2) kg initial BW, housed at the CRA-SUI Pig Research Unit (Modena, Emilia Romagna, Italy). The pigs were reared in 16 pens, 5 pigs per pen (1.8m/pig). The animals were randomly assigned to 1 of the dietary treatments (2 gilt and 2 barrow pens per treatment). The lighting was natural throughout the trial.

Dietary treatments

Four treatment diets were formulated and adapted: 1) corn-based diet (control) where corn was within the maximum level allowed by regulations of PDO Parma and S. Daniele hams (Legislation no. 30 of 14 February 1990; Ministerial Decree no. 253 of 15 February 1993); 2) control diet with 80% of the corn replaced by a normal-amylose hulled barley variety named Cometa (Cometa); 3) control diet with 80% of the corn substituted by a normal-amylose hulless barley variety named Astartis (Astartis); 4) control diet with 80% of the corn replaced by a low-amylose hulless barley variety named Alamo (Alamo). All the barley varieties used in the diet formulation were grown at the Beccastecca CRA-SUI farm (Modena, Emilia Romagna, Italy) and in the same pedo-climatic conditions. For each treatment, the diets were formulated according to 3 growth periods (i.e. 40-80, 80-120 and 120-160 kg BW), with the same Lys:DE (2.60, 2.20 and 1.80, respectively in the three growth periods) according to the NRC requirements (1998) for the first two growth phases and according to the requirements for high performing pigs (Manini et al., 1997) for the last growth phase. The animals had free access to water and the diets were offered twice a day in a liquid form (with a water to feed ratio of 3:1) at average 8.7% of their metabolic weight (BW $^{0.75}$108) until the end of

the trial (153 d). The feed offered was adjusted weekly on the basis of a hypothetical gain verified by previous similar experiences in CRA-SUI Pig Research Unit.

Table 1. Chemical and amino acid composition of corn and barley varieties.

Item	Corn	Cometa	Astartis	Alamo
Chemical composition, % as-fed				
DM	88.00	88.62	89.34	90.16
CP	8.10	11.23	12.53	15.28
Ether extract	2.70	1.83	1.89	2.27
NDF	5.92	15.53	8.23	8.36
ADF	1.97	5.16	2.11	2.02
ADL	0.68	0.99	0.88	0.75
Total starch	64.10	49.58	56.75	54.65
Amylose, % dry starch	33.6	29.8	28.7	8.3
β-glucans	nd[1]	3.81	4.91	5.63
DE, Kcal/kg	3,340	3,058	3,275	3,277
Amino acids, % as-fed				
Lys	0.22	0.40	0.42	0.44
Met + Cys	0.33	0.46	0.48	0.60
Thr	0.27	0.40	0.42	0.50
Leu	0.88	0.78	0.87	1.01
Ile	0.26	0.43	0.46	0.58
Val	0.36	0.56	0.63	0.72
His	0.26	0.29	0.30	0.36
Arg	0.35	0.58	0.61	0.67
Phe + Tyr	0.60	0.89	0.96	1.14

[1]nd, not detected.

Table 2. Ingredients and nutrient composition, and *in vitro* starch characterization of diets for heavy growing-finishing pigs in the 3 growth periods[1]

Item	From 40 to 80 kg				From 80 to 120 kg				From 120 to 160 kg			
	Control	Cometa	Astartis	Alamo	Control	Cometa	Astartis	Alamo	Control	Cometa	Astartis	Alamo
Ingredients, %												
Corn	51.40	9.30	5.80	7.80	53.50	10.90	8.40	9.90	55.00	12.00	9.80	11.50
Barley	22.00	80.00	80.00	80.00	22.00	80.00	80.00	80.00	22.00	80.00	80.00	80.00
Bran	10.00	0.00	0.00	0.00	10.00	0.00	0.00	0.00	10.00	0.00	0.00	0.00
Soybean, 48% CP	13.50	7.50	11.00	9.00	11.50	6.00	8.50	7.00	10.00	5.00	7.20	5.50
L-Lysine HCl	0.20	0.20	0.20	0.20	0.10	0.10	0.10	0.10	0.00	0.00	0.00	0.00
Calcium carbonate	1.30	1.20	1.20	1.20	1.30	1.20	1.20	1.20	1.20	1.20	1.20	1.20
Dicalcium phosphate	0.80	0.90	0.90	0.90	0.80	0.90	0.90	0.90	0.90	0.90	0.90	0.90
Salt	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40
Premix[2]	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Composition, % as-fed												
DM	88.32	89.21	88.83	89.34	88.62	89.91	89.15	89.92	89.14	90.02	89.64	90.16
CP	14.41	13.35	15.72	17.18	13.57	12.70	14.68	16.33	12.90	12.23	14.10	15.67
Crude Fiber	4.39	4.99	2.61	3.29	4.32	4.94	2.51	3.21	4.26	4.90	2.46	3.16
Ether extract	2.87	1.91	1.84	1.88	2.91	1.95	1.90	1.93	2.94	1.97	1.93	1.96
Linolcic acid (C 18:2)	1.33	0.82	0.78	0.80	1.35	0.83	0.81	0.82	1.37	0.85	0.82	0.84
Starch	47.28	46.85	46.28	44.76	48.60	47.98	47.40	45.79	49.92	49.12	48.53	46.83
RS[3], % starch	25.6	17.7	16.6	6.5	25.3	19.2	18.4	7.2	23.6	18.1	17.1	8.3
pGI[4]	56	70	71	80	54	67	69	78	57	66	67	76
Calcium	0.75	0.77	0.78	0.78	0.74	0.77	0.78	0.77	0.76	0.76	0.77	0.77
Phosphorus	0.53	0.50	0.51	0.50	0.52	0.49	0.50	0.49	0.53	0.49	0.50	0.49
Digestible phosphorus	0.22	0.23	0.23	0.23	0.22	0.22	0.23	0.23	0.23	0.22	0.23	0.22
Lys	0.81	0.72	0.83	0.79	0.68	0.60	0.68	0.66	0.56	0.49	0.57	0.54
Digestible Lys	0.71	0.61	0.71	0.67	0.58	0.50	0.57	0.55	0.46	0.39	0.46	0.43
DE, kcal/kg	3,097	2,788	3,196	3,052	3,095	2,786	3,195	3,050	3,090	2,785	3,193	3,049
NE, kcal/kg	2287	2332	2325	2294	2301	2341	2333	2303	2318	2350	2342	2312
Lys:DE[5]	2.61	2.58	2.60	2.60	2.18	2.15	2.14	2.16	1.80	1.77	1.78	1.77

[1]Control, corn-based diet; Cometa, control diet with 80% of corn replaced by a normal-amylose hulled barley variety named Cometa; Astartis, control diet with 80% of corn substituted by a normal-amylose hulless barley variety named Astartis; Alamo, control diet with 80% of corn replaced by a low-amylose hulless barley variety named Alamo.

[2]Provided vitamins and minerals per kilogram of feed (Istituto delle Viatmine S.p.A., Segrate, MI, Italy): vitamin A, 15,000 IU ; vitamin D_3, 2,000 IU; vitamin E, 50 mg; vitamin K_3, 2.5 mg; vitamin B_1, 2 mg; vitamin B_2, 5 mg; vitamin B_6, 4 mg; vitamin B_{12}, 0.03 mg; biotin, 0.15 mg; niacin, 25 mg; D-pantothenic acid, 15 mg; choline chloride, 350 mg; Mn, 25 mg as manganous oxide; Fe, 150 mg as ferrous sulfate; Cu, 15 mg as copper sulfate; Zn, 100 mg as zinc oxide; I, 1.5 mg as calcium iodide; and Se, 0.1 mg as sodium selenite.
[3]RS, resistant starch.
[4]pGI, predicted glycemic index, calculated with the equation proposed by Giuberti et al. (2012b) using white bread as reference.
[5]Calculated as ratio between Lys (g/kg) and DE (Mcal/kg).

Performance measurements

The pigs were individually weighed at the beginning, after 56 days and at the end of the trial. The feed intakes were also recorded to calculate ADG and G:F for each replicate within treatment and for periods 0-56, 57-153 and 0-153 d.

Carcass measurements

The pigs (173 ±3 kg mean final BW) were electrically stunned at slaughter and the hot carcasses were weighed to calculate the dressing percentage. The following determinations were recorded using a Fat-O-Meat'er (Carometec, A/S, Herlev, DK): Thickness of back fat including rind (measured at 8 cm off the midline of the carcass at the level placed between the third and fourth last ribs); thickness of the longissum dorsi muscle (measured at the same time and in the same place as the back fat thickness). Lean meat percentage was then calculated in accordance with EC Commission Decision 2001/468/EC of 8 June 2001 authorizing methods

Chemical analyses

Dried cereal grains and diets collected for each growth period were ground through a 1 mm screen using a Retsch type ZM100 centrifugal grinding mill (Retsch, Haan, Germany). Then, samples were analyzed in duplicates according to AOAC (2000) for dry matter (method 930.15), crude protein (method 976.05), ether extract (method 954.02), and in agreement with Van Soest et al. (1991) for fiber fractions (acid detergent fiber, neutral detergent fiber and acid detergent lignin). The total starch content was measured as detailed by Masoero et al. (2010). The digestible energy of the barley varieties was calculated using the Fairbairn et al. (1999) equation 1, whereas the INRA-AFZ (2002) equation was used for corn. The digestible energy of diets was calculated from digestible energy of single raw material reported by INRA-AFZ (2002), except for barley (Fairbairn et al., 1999). The net

energy was calculated using the equation 4 of INRA-AFZ (2002). Cereal grains were analyzed for β-glucan contents using the Megazyme mixed-linkage β-glucan assay kit (K-BGLU 07/11, Megazyme Int., Wicklow, Ireland) and for the amylose content using the Megazyme amylose/amylopectin assay kit (K-AMYL 07/11, Megazyme Int., Wicklow, Ireland). Amino acids were determined by using a Carlo Erba model 3A29 amino acid analyzer (Carlo Erba Strumentazione, Corsico, Italy; Moore, 1963; Eggum, 1968; Moore et al., 1980). The calcium and phosphorus contents of diets were determined by inductively coupled plasma atomic emission spectrometry (EPA, 2000) after microwave assisted acid digestion (EPA method 3052, 1996). Digestible phosphorus and lysine were calculated according to INRA-AFZ (2002).

Two separate evaluations were performed to characterize the *in vitro* starch digestion potential of diets within each growing period. In particular, diets were characterized in duplicate for their resistant starch (RS) content according to the procedure detailed by Englyst et al. (1996). The *in vitro* starch digestion over time was performed with an enzymatic method simulating gastric and pancreatic phases occurring in pig gastrointestinal tract (Giuberti et al., 2012a). In summary, the procedure was as follows: about 800 mg of each diet were placed in 50 mL screw-top test tubes containing 0.05 M HCl solution (5 mL) plus pepsin (5 mg/mL; Sigma P-7000, Sigma-Aldrich® Co, Milan, Italy) for 30 min at 37 °C under gentle agitation. The pH was then adjusted to 5.2 by adding 20 mL of 0.1 M sodium acetate buffer prior to the inclusion of 5 mL of an enzyme mixture with an amylase activity of about 7000 U/mL (Englyst et al., 1992) given by pancreatin (about 7500 FIP-U/g; Merck 7130, Merck KGaA, Darmstadt, Germany), amyloglucosidase (about 300 U/mL; Sigma A-7095, Sigma-Aldrich® Co, Milan, Italy) and invertase (about 300 U/mL; Sigma I-4504, Sigma-Aldrich® Co, Milan, Italy). Milled white bread (1 mm screen; 72.3 % of starch on a dry matter basis) was added in the *in vitro* digestion assay and used as a reference sample for the hydrolysis index (HI) calculation (details below). Aliquots (0.5 mL) were taken from each tube at 0 (prior to the addition of the enzyme mixture simulating the pancreatic phase) and at 15, 30, 60, 90, 120 and 180 min after the addition, absolute ethanol was then added and the amount of released glucose was determined colorimetrically with a glucose oxidase kit (GODPOD 4058, Giesse Diagnostic snc, Rome, Italy). All the samples were

hydrolyzed in duplicate. The proportion of digested starch at each time interval was calculated using a factor of 0.9 (Stevnebø et al., 2006). The diets were then classified through a predicted glycemic index value (pGI) (Giuberti et al., 2012b). Briefly, a HI was obtained from the ratio between areas under the *in vitro* digestion curve for each diet and the corresponding area for white bread up to 180 min after incubation (considering the HI for white bread 100 by definition). From the obtained HI, a pGI was calculated with the following equation (Giuberti et al., 2012b):

$$pGI = 1.013 \cdot HI.$$

Statistical analysis

Data were tested for normality with the Shapiro-Wilk test before statistical analysis. Then, the data were analyzed as a completely randomized design using the GLM procedure (SAS Inst. Inc., Cary, NC), according to the following model:

$$Y_{ijk}=\mu+\tau_i+\beta_j+ (\tau\beta)_{ij}+\varepsilon_{ij}+\delta_{ijk}$$

Where:

Y_{ijk} is the k^{th} observation, μ is the overall mean, τ_i is the fixed effect of treatment (i = 1 to 4), β_j is the fixed effect of sex (j = 1 to 2), $(\tau\beta)_{ij}$ is the treatment per sex interaction, εij is the residual error and δ_{ijk} is the sampling error.

When not significant, the sex effect and their first interaction were removed from the model. Differences between means were accepted as significant if $P \leq 0.05$.

Results

Chemical and amino acid composition of barley varieties

The chemical and amino acid composition of corn and barley grain varieties used in the experiment are reported in table 1. The three barley varieties were characterized by higher levels of crude protein, NDF and β-glucans, and lower contents of fat, starch and amylose compared to corn. Among the barley varieties, Alamo had the highest contents of crude protein, crude fat and β-glucans followed by Astartis and Cometa. As expected, Alamo barley was characterized by the lowest amylose content and the highest amino acid contents in accordance with its higher crude protein level. Cometa barley had higher levels of NDF, ADF and ADL compared to Astartis and Alamo.

Growth performance and carcass characteristics

Growth performance and carcass characteristics are shown in table 3. In all the three periods (0-56, 57-153 and 0-153 d) ADG was affected by the dietary treatment. In particular, in the 0-56 d period, the pigs fed the Astartis and Alamo diets grew faster than those fed the control diet, whereas the animals receiving the Cometa diet grew in a similar manner to those receiving all the other diets ($P = 0.01$). In the 57-153 d period and throughout the trial (0-153 d period), the pigs fed the Cometa and Alamo diets grew faster ($P = 0.01$) than those fed the control diet, whereas the animals receiving the Astartis diet grew in a similar manner to those receiving all the other diets. The animals fed the Cometa and Alamo diets achieved higher final weights ($P = 0.01$) compared with those fed the control diet. The animals receiving the Astartis diet had a mean final weight similar to that of the animals fed the other diets. Despite the results observed on ADG and final live weight, when feeding the Cometa diet animals had the lowest dietary efficiency throughout the trial ($P < 0.01$). For the same period, the Astartis and Alamo diets had the highest dietary efficiencies, whereas the control was in between. No difference in carcass characteristics was found between treatments.

Table 3. Animal performance and carcass characteristics for the considered growth periods[1]

	Control	Cometa	Astartis	Alamo	√MSE[2]	P =
BW, kg						
Initial	49	49	49	49	2.91	1.00
After 56 days	95	96	97	97	2.46	0.56
Final	169[b]	175[a]	172[ab]	175[a]	2.10	0.01
ADG, kg						
0-56 d	0.82[b]	0.83[ab]	0.85[a]	0.86[a]	0.01	0.01
57-153 d	0.76[b]	0.82[a]	0.77[ab]	0.80[a]	0.02	0.01
0-153 d	0.78[b]	0.82[a]	0.80[ab]	0.82[a]	0.01	0.01
G:F						
0-56 d	0.38[c]	0.35[d]	0.40[a]	0.39[b]	0.01	<0.01
57-153 d	0.26[b]	0.25[b]	0.27[a]	0.27[a]	0.01	<0.01
0-153 d	0.30[b]	0.28[c]	0.31[a]	0.31[a]	0.01	<0.01
Carcass characteristics						
Carcass weight, kg	143	148	146	148	9.86	0.36
Dressing percentage	84.5	84.3	84.6	84.2	1.18	0.76
Longissimus dorsi thickness, mm	63	60	68	65	10.8	0.27
Back fat thickness, mm	27	30	28	29	4.12	0.22
Lean meat, %	49.2	47.6	49.5	48.7	2.95	0.34
Thigh weight, kg	36.0	36.9	36.7	36.8	2.40	0.65
Left loin weight, kg	7.7	7.8	7.7	7.8	0.54	0.88

[1]Control, corn-based diet; Cometa, control diet with 80% of corn replaced by a normal-amylose hulled barley variety named Cometa; Astartis, control diet with 80% of corn substituted by a normal-amylose hulless barley variety named Astartis; Alamo, control diet with 80% of corn replaced by a low-amylose hulless barley variety named Alamo.
[2]√MSE, root of mean-square error.
Within a row, means without a common superscript differ ($P < 0.05$).

The PDO Parma and San Daniele hams are Italian typical products and their production is strictly regulated (Legislation no. 30 of 14 February 1990; Ministerial Decree no. 253 of 15 February 1993). Corn is the base cereal when feeding heavy pigs for the production of PDO Parma and San Daniele hams. Nevertheless, Parma and San Daniele ham disciplinarians establish a maximum limit at 55% on a dry matter basis for the use of corn in pig diets. The limit is related to the high content of linoleic acid (C 18:2) in corn fat, which can negatively affect fat quality in the finished product by increasing its unsaturation level. A fat with a high unsaturation level is more exposed to lipid oxidation, is less compact and white; undesirable fat characteristics according to customers (Toscani et al. 2003). Therefore, the Parma and San Daniele ham disciplinarians fix a maximum level of linoleic acid in pig diet at 2% on a dry matter basis. Although pigs fed on corn-based diets perform well, they may yield a yellow colored fat, a characteristic against which many customers may discriminate (Han et al., 2005). The yellow-colored fat has been reported to be due to the presence of carotenes and xanthophylls in corn (Bauernfeind et al., 1981). Furthermore, in hot and dry areas, such as Italy, problems arise because of corn colonisation by Aspergillus section Flavi, and resulting contamination

with aflatoxins at levels exceeding the maximum levels set by the European Union (Regulation 574/2011 of 16 June 2011) (Piva et al., 2006; Pietri et al., 2012). Barley might represent a valid alternative to corn in diets of heavy pigs for the Parma and S. Daniele ham production since it supplies a lower fat content than corn, with a low linoleic acid level, and without yellow pigments. Moreover, barley being a drought-tolerant crop, the growth of barley in arid and semi-arid areas of Europe (including much of southern France, Greece, Italy, Portugal, Cyprus and Spain) could have a positive impact on the environment because of the reduced water demands. Lastly, starch in barley grains is more available to enzyme action than starch in corn grains (Giuberti et al., 2012a). As expected, the three barley-based diets used in the current experiment (i.e., Cometa, Astartis and Alamo) were characterized by numerically higher starch digestion potentials (expressed as pGI) and lower RS contents when compared to the control corn-based diet. As reviewed by Giuberti et al. (2013), these differences may be ascribed to intrinsic factors associated with the inherent starch architecture in cereal grains, such as the starch amylose level, starch granule sizes and morphologies and the presence of endosperm non-starch organic matter. For instance, the hydrolysis potential of starch in cereal grains is inversely related to the amylose content (Sun et al., 2006; Stevnebø et al., 2006), due to a decreased accessibility of amylose by enzyme action when compared to amylopectin (Giuberti et al., 2013).Throughout the trial, the animals consuming barley-based diets, with the exception of pigs fed the Astartis diet, gained a higher final BW and had a higher ADG compared to pigs fed a corn-based diet (control), without negative effects on carcass quality. There is increasing evidence that dietary starches from different sources, digested and absorbed at different rates and to different extents, can modulate the post-prandial metabolic response (Regmi et al., 2010) with possible implications for feed intake and animal performances (Sun et al., 2006; Black et al., 2009; Zijlstra et al., 2012). Accordingly, pigs fed a high-glycemic index starch source (i.e., gelatinized potato starch) had a greater ADG and energy retention than pigs fed a low-glycemic index source (i.e., native potato starch) (Bolhuis et al., 2008). Lastly, results on the final BW were in agreement with Atakora et al. (2011) reporting that the BW of finished pigs fed a barley-based diet tended to be higher than similar pigs fed a corn-based diet. In swine nutrition, high-RS content is considered as a negative dietary factor since it is not always well suited to optimize animal growth and performances

(Regmi et al., 2011; Gerrits et al., 2012). For instance, pigs fed a diet based on a corn cultivar containing about 45% of RS had a lower feed intake, BW and G:F than pigs fed lower-RS diets formulated with regular corn, brown rice or sticky rice (Li et al., 2007). However, despite a possible negative effect of feeding RS, there is growing interest in including RS in swine diets, due to potential prebiotic properties (Bach Knudsen et al., 2012). Long-term intakes of RS reduced indices associated with damage to epithelial cells and increased the butyrate concentration in colon digesta (Nofrarías et al., 2007). Therefore, starch digestion being an important factor affecting digestive physiology and animal performance (Zijlstra et al., 2012; Giuberti et al., 2013), the classification of feed or complete diets through pGI values and RS contents could represent a promising area of research for pig nutrition that might help in diet formulation for considering intestinal health and whole body nutrient usage in swine.

At present, there seem to be no evidence that the type of starch might affect the carcass composition in pigs. Shelton et al. (2004) observed a slight decrease in the fat percentage of carcasses in pigs fed a low-amylose sorghum compared to pigs fed either normal corn or sorghum. Camp et al. (2003) reported a decrease in the 10th-rib fat thickness when feeding low-amylose corn, whereas similar results were not reported by Shelton et al. (2004) in pigs fed low-amylose sorghum. Camp et al. (2003) reported also an increase in carcass weight and length, but no effects on fatness or leanness. Based on our results it seems that different cereals carrying different contents of amylose did not affect the characteristics of carcasses in pigs. In addition, performance was not affected by hulless varieties in agreement with previous results (Thracker, 1999). However, the presence of the hull affected feed efficiency which was lower for the hulled barley-based diet (Cometa) compared to the hulless barley based (Astartis and Alamo) and corn-based (control) diets. In hulled barley cultivars up to 21% of the total grain weight can be fiber (Juskiw et al., 2011). The Cometa diet had the highest crude fiber content (average in the three growth periods: +12.6, +48.8 and +34.8 % compared to the control, Astartis and Alamo diets, respectively). Increased dietary fiber levels were associated with a reduction of the available energy of feeds (Noblet and Le Goff, 2001) and to a decreased diet digestibility (Wang et al., 2006). Moreover, Pettersson and Lindberg (1997) found higher total tract

digestibility for naked barley-based diet compared to hulled barley-based diet. Bach Knudsen et al. (2012), in their review on the factors associated with polymeric carbohydrates that may influence growth and development of pigs, reported that although the type of starch (i.e amylose or amylopectine) has no effect on the carcass composition, there is enough evidence that fiber (i.e non-starch polysaccharides, NSP) can significantly affect the carcass composition. Just (1984) observed higher muscle and lower subcutaneous fat contents in carcasses when increasing the fiber level of diets. Nevertheless, our results showed no influence of the fiber level on carcass characteristics.

In conclusion, this study showed that monocereal diets based both on hulled and hulless barley might be viable in heavy pig production destined for Italian PDO product manufacturing without adversely affecting growth performance and carcass characteristics. Future research will be aimed at evaluating the nutritional and sensorial characteristics of PDO Piacentina neck and Parma ham.

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2.1.1. *Appendix 1*

Diet based on corn can be replaced by naked barley variety in pig production - *in vitro* approach.

This appendix contain extended version of results and additional material not included into the work of Prandini et al.: Monocereal diets with hulled or hulless barley varieties. Part 1. Performances and carcass quality of Italian heavy pigs.

Materials and Methods

Sample collection

Samples came from study of *Prandini et al.: Monocereal diets with hulled or hulless barley varieties. Part 1. Performances and carcass quality of Italian heavy pigs*, were collected in CRA-SUI Pig Research Unit (San Cesario sul Panaro, Modena, Italy) and analysed in laboratories in Institute of Food Science and Nutrition - Università Cattolica del Sacro Cuore (Piacenza, Italy). Diets and feed stuffs samples were taken for chemical analysis and *in vitro* enzymatic digestion. At the end of trial faeces and eight blood samples per group were collected for further analysis. At the slaughterhouse, after laparotomy and rejection of bellies, large bowel has been separated and we took the five samples of digesta from each group.

Volatile fatty acids analysis

Samples of faeces and digesta collected from the colon of individual pigs were analysed for volatile fatty acids (VFA) contents by gas chromatography (GC) (Varian 3350) according to the method described by Fussell and McCalley (1987).

In vitro starch digestion rate

Corn, barleys and diets underwent an *in vitro* enzymatic hydrolysis simulating the digestion in the upper digestive tract of pigs. The adopted *in vitro* procedure was from van Kempen et al. (2010) and initially proposed by Englyst et al. (1992). About 0.8 g sample was weighed into DURAN 100 ml laboratory bottle and incubated at 37 ^{o}C under constant horizontal agitation. To allow a god mixing, 6 glass balls were added/each bottle. First, 5ml of acid solution, made from porcine pepsin (Sigma P-7000) and HCL 0.05 N, were added. After 30 minutes, 20 ml of acetic buffer and enzyme mixture, consisting of pancreatin (Merck 7130), amyloglucosidase (Sigma A-7095) and invertase (Sigma I-4504), was added. To characterize the starch digestion rate, 0.5 mL sample were collected from bottle after gentle mixing at 0, 15, 30, 60, 90, 120, 240, 360 and 480 minutes of incubation. Collected samples were stabilize by adding 20 ml of absolute ethanol (van Kempen et al., 2010a). The amount of released glucose was determined by a colorimetric method using a glucose oxidase kit (GODPOD 4058, Giesse Diagnostic).

Determination of starch fractions

The total starch (TS) on cereals and diets was characterized as rapidly digestible starch (RDS; % of TS), resistant starch (RS; % of TS) and total digestible starch (TDS; 100 – RS % of TS) according to the procedure of Englyst et al. (1996). The presence of glucose in samples collected after 20 and 120

minutes of hydrolysis was measured by a colorimetric method as described above. The glucose was converted into RDS, SDS and RS indexes as proposed by Englyst et al. (1992).

In vitro gas production

The *in vitro* fermentation simulating the fermentation occurring in the hindgut was performed following the protocol described by Bindelle et al. (2007). The substrate for *in vitro* fermentation was obtained by repeated cycles of enzymatic hydrolysis of test samples (cereals and diets) with pepsin and pancreatin according to the protocol described in Boisen and Fernhdez (1997). Then, samples were passed through a paper filter, washed twice with 96% ethanol and after with 99.5% acetone before being dried at 60 °C overnight. The procedure was repeated few times in order to obtain enough material. Then, obtained substrate was ground to pass a 1 mm screen. Residual starch amount on substrate was measured using the modified enzymatic method (Blasel et al., 2006), and the amount of released glucose was determined by a colorimetric method as described above. 200 mg of hydrolysed material were incubated into 100 ml glass syringes at 39 ^{o}C with 30 ml of faecal inoculum. The faecal inoculum was prepared using fresh faeces collected from three pigs housed at the University experimental farm (CERZOO, Piacenza) and being fed an antibiotics free diet. 28.5 g of the faces were mixed to a buffer solution containing macro- and micro-minerals (Menke et al., 1979). The gas generated by the fermentation process was measured for each syringe at 0, 0.5 1, 2, 4, 6, 8, 24, 36 and 48 h. Then the fermentation was stopped after 48 h of incubation by setting the bottles in boiling water. Samples were collected from each syringe to measure VFA and ammonia contents, as well as in the inoculum used. The ammonia (NH_3) content was determined by a method of Novezamsky et al. (1974) using spectrophotometer.

Denaturing gradient gel electrophoresis (DGGE)

Digesta samples collected at the end of trial from the colon of individual pigs were analysed with DGGE, as described in Bruzzese et al. (2014).

First, 50 mg of samples material was incubated at 37^{o}C in lysozyme buffer (100 mM Tris-HCl, pH 8, 25% sucrose, 10 mM EDTA, and 10 mg/mL of lysozyme) for 1 h and then processed by the MaxwellH 16 System (Promega, Madison, WI, USA) using the MaxwellH 16 DNA purification kit. The extracted bacterial DNA was eluted in 400 mL of elution buffer and stored at 280^{o}C. Next, for the DNA amplification, we used the total bacterial DNA extracted from intestinal digesta was used as a template in the PCR reactions. The V2–V3 region of the 16S rRNA gene was amplified using universal primers Hda.

All DGGE analyses were performed using the INGENYphorU-2x2 system (INGENY International, Amundsenweg, Netherlands). Amplicons were analysed on 8% polyacrylamide (40% acrylamide-bis, 37.5:1) gel with a 40% to 65% denaturing gradient of urea and formamide increasing in the direction of electrophoresis. The gel was run with a constant voltage of 80 V at 60^{o}C for 18 h in Tris-acetate buffer (pH 8.0).

Calculations and statistical analysis

Calculations was performed using R environment (R Development Core Team, 2013). Data were tested for normality using the Shapiro-Wilk test and plots. The results are expressed as mean and standard error of mean (SEM). Statistical significance of differences among the group was evaluated using analysis of variance ANOVA and the Tukey's test. Differences were considered significant for $P<0.05$. There were no significant differences between male and females.

Modelling in vitro hydrolysis kinetics

Using the data obtained from the *in vitro* digestion test an *in vitro* digestion coefficient of starch for each time interval (C_t, as a percentage of TS) was calculated according to Stevnebø et al., 2006 and Giuberti et al., 2012 equation (1):

$$C_t = 100 \frac{0.9(\text{glucose present at time t - } t_0 \text{ glucose release})}{\text{total starch}}$$

In order to describe the kinetics of starch digestion, an exponential equation evaluated by Schofield et al. (1994) and modified by Mahasukhonthachat et al. (2010) was used. The model was applied on the data from *in vitro* test, equation (2):

$$\boldsymbol{C_t = C_0 + C_\infty e^{(-e^{(1-kt)})}}$$

Where C_t corresponds to the *in vitro* digestion coefficient of starch at time t, C_0 is the *in vitro* digestion coefficient at 0 min, C_∞ is the *in vitro* digestion coefficient after 480 min, k is the digestion rate (/min) and t is the chosen time (min).

Kinetic parameters C_0, C_∞ and k was calculated using nls() function (Bates and Chambers, 1992; Bates and Watts, 1998). The area under the *in vitro* digestion curves (AUC) was calculated by AUC function, in MIfuns package (Knebel et al., 2008), using the trapezoidal rule. Hydrolysis index (HI), was obtained from the ratio between the AUC of each diet and the reference AUC 44500.35 of white bread and expressed as its percentage (Goñi et al., 1997), equation (3):

$$HI[\%] = \frac{\text{AUC}}{44500.35} 100 \quad .$$

In vitro degradability (IVD)

In vitro degradability (IVD) was calculated as the percentage of degraded material during the *in vitro* enzymatic hydrolysis, equation (4):

$$IVD = \frac{\text{weight of the digested material} \bullet 100}{\text{weight of the sample before hydrolysis}}$$

Modelling in vitro gas production kinetics

Gas accumulation was modeled according to LAG exponential model with discrete LAG time (Schofield et al.,1994) and described with the following parameters: V_f, final asymptotic gas volume; k, fractional rate of gas production; LAG, discrete lag phase; T/2, half-time to asymptote. Kinetic parameters was calculated using *nls()* function (Bates and Chambers, 1992; Bates and Watts, 1998) in R, equation (4):

$$V = V_f\left(1 - e^{\left(-k(T-LAG)\right)}\right)$$

where

$$T/2 = LAG + \ln(0.5/k)$$

$$T \geq LAG$$

DGGE image analysis

The DGGE profiles were analysed using the Phoretix1D v11.5 software (TotalLab Ltd, UK). After detecting bands and their peak intensities a dendrogram was built to show in a hierarchical manner, the similarities of the lanes matched to the obtained synthetic lane. Neighbour joining dendrogram was constructed according to Saitou and Nei (1987).

Results

Metabolic profile from blood serum

Results of blood test are presented in Table 1. Significant differences between four treatments for protein, glucose, bilirubin with P<0.05 and for Urea with P<0.01, were found. Animals from Control group had the lowest protein (64.75 g/l) an glucose level (5.28 mmol/l) but higher bilirubin level (2.31 qmol/l) The significantly higher levels were almost always recorded in Alamo diet group.

Table 1 *Blood examination of pigs under corn-base Control based and three feeds with different barley varieties.*

	Control		**Cometa**		**Astartis**		**Alamo**		*se*	*P-value*
Albumin [g/l]	37.63		35.75		38		39		1.41	ns
Globuln [g/l]	27.13		28.88		28		28.63		1.32	ns
Haptoglobin [mg/ml]	1.88		2.39		1.62		2.39		0.39	ns
Alb/Gba [ratio]	1.40		1.25		1.36		1.39		0.09	ns
Proteins [g/l]	64.75	b	64.63	b	66	ab	68.25	a	1.27	*
Cholesterol [mmol/l]	2.49		2.40		2.50		2.46		0.14	ns
Glucose [mmol/l]	5.28	b	5.68	ab	6.16	a	5.69	ab	0.26	*
Bilirubin [qmol/l]	2.31	a	1.76	b	2.4	a	2.14	ab	0.21	*
Urea [mmol/l]	3.86	b	3.96	b	4.95	c	6.07	a	0.29	***
AST-GOT [IU/l]	30.25		29.63		32.63		31.13		3.14	ns
ALT-GPT [IU/l]	42.63		45		41.65		43.38		3.31	ns
Phosphorus [mmol/l]	2.79		2.77		2.89		2.98		0.12	ns

se - standard error; levels of significance: *P<0.05* (*), *P<0.01* (**), *P<0.01* (***), ns - not significant. The level of significance between mean treatments is *P<0.05*.

Volatile fatty acid production

The analyzed content of VFC in faeces and digesta are presented in Table 2. The total concentration of VFA did not change in feaces, but in colon digesta it changed significantly (P<0.01) with the highest concentration in Astartis based diet 13.29 % and the lowest in Alamo diet 10.34 %. We can noticed that only amounts of valerianic acid were significantly different in both faeces (P<0.05) and digesta (P<0.01), as well as the levels of significance. In general, differences were easier to see in digesta from colon.

Table 5 *VFA content of faeces took from pigs under corn-base Control based and three feeds with different barley varieties.*

	Control		Cometa		Astartis		Alamo		*se*	*P-value*
Acetic	60.01		59.53		60.33		59.23		1.42	ns
Propionic	21.48		21.44		20.53		22.11		1.47	ns
Isobutyric	2.19		2.32		2.45		2.37		0.21	ns
Butyric	10.99		11.07		10.27		9.40		1.00	ns
Isovalerianic	3.25		3.36		3.80		3.73		0.38	ns
Valerianic	2.08	b	2.28	b	2.63	ab	3.16	a	0.30	*
Total	14.40		12.87		13.11		14.28		0.97	ns

VFA means are expressed in [mmol/100g] and total VFA amount in [mmol%]. se - standard error; levels of significance: *P<0.05* (*), *P<0.01* (**), *P<0.01* (***), ns - not significant. The level of significance between mean treatments is *P<0.05*.

Table 3 *VFA content of digest took from pigs colon fed experimental diets*

	Control		Cometa		Astartis		Alamo		*se*	*P-value*
Acetic	66.27		64.77		64.61		62.92		1.26	ns
Propionic	19.60	ab	21.34	b	18.43	a	18.94	ab	0.94	*
Isobutyric	1.62	a	1.22	a	1.55	a	2.28	b	0.19	***
Butyric	9.00		9.70		11.71		10.58		0.95	ns
Isovalerianic	2.09	a	1.61	a	2.08	a	3.32	b	0.32	***
Valerianic	1.43	b	1.34	b	1.61	ab	1.96	a	0.13	**
Total	11.22	ab	10.99	ab	13.29	a	10.34	b	0.79	**

VFA means are expressed in [mmol/100g] and total VFA amount in [mmol%]. se - standard error; levels of significance: *P<0.05* (*), *P<0.01* (**), *P<0.01* (***), ns - not significant. The level of significance between mean treatments is *P<0.05*.

Starch digestibility

Starch fractions, as percentage of total starch are showed in Table 4. There are significant differences in feed stuff as well as experimental diets. The highest content of RDS and SDS (P<0.001), as fractions easier available for digestion enzymes, are in barley Alamo (97.61%) and also diet based on that variety (93.60%). The level of easy digestible fractions is inversely proportional to resistant starch fraction (P<0.001). Corn has the highest percentage of resistant fraction (32.65%) among prime matters (P<0.001) but within treatments group, diet based on Cometa barley variety has (22.88%) the highest one.

Table 4 *Starch fractions [% of total starch]*

	Feed stuff									
	Corn		Cometa		Astartis		Alamo			
	mean		mean		mean		mean		*se*	*P-value*
RDS	31.56	b	34.36	b	39.88	a	58.43	c	1.03	***
SDS	35.79	a	53.21	b	32.99	a	39.17	c	0.93	***
RS	32.65	c	12.42	b	27.14	a	2.39	d	1.22	***
RDS+SDS	67.35	c	87.58	b	72.86	a	97.61	d	1.22	***
	Experimental diets									
	Control		Cometa		Astartis		Alamo			
RDS	39.17	a	34.18	a	35.92	a	55.92	b	1.78	***
SDS	42.09	ab	42.93	ab	47.75	a	37.68	b	1.77	**
RS	18.74	ab	22.88	b	16.32	a	6.40	c	1.30	***
RDS+SDS	81.26	ab	77.12	b	83.68	a	93.60	c	1.30	***

se - standard error; levels of significance: *P<0.05* (*), *P<0.01* (**), *P<0.01* (***), ns - not significant. The level of significance between mean treatments is *P<0.05*.

Table 5 *Starch content and hydrolysis curve parameters*

	Feed stuff								
	Corn		Cometa		Astartis		Alamo		*se*	*P-value*
IDV	59.63	a	41.69	b	50.59	ab	48.81	b	3.37	**
TS	54.74	a	30.55	b	48.97	a	34.81	b	2.12	***
c_o [%]	6.78	a	1.21	b	6.28	a	6.58	a	1.18	***
c_{oo} [%]	103.45	c	99.36	bc	95.46	ab	91.91	a	1.48	***
k [min^{-1}]	0.01	a	0.01	a	0.01	a	0.02	b	0.00	***
HI [%]	82.58	c	91.08	bc	87.29	ab	95.81	a	1.51	***

	Experimental diets								
	Control		Cometa		Astartis		Alamo			
IDV	38.59	a	22.14	b	45.58	a	46.46	a	4.09	**
TS	31.55	a	30.95	a	32.82	a	24.82	b	1.89	**
c_o [%]	2.19		2.59		0.10		1.83		1.73	ns
c_{oo} [%]	99.45		100.30		101.08		97.94		1.41	ns
k [min^{-1}]	0.01	ab	0.01	b	0.02	a	0.03	c	0.00	***
HI [%]	92.93	ab	90.54	b	95.05	a	99.61	c	1.36	***

IDV – *in vitro* degradability; TS – total starch; se - standard error; levels of significance: *P<0.05* (*), *P<0.01* (**), *P<0.01* (***), ns - not significant. The level of significance between mean treatments is *P<0.05*.

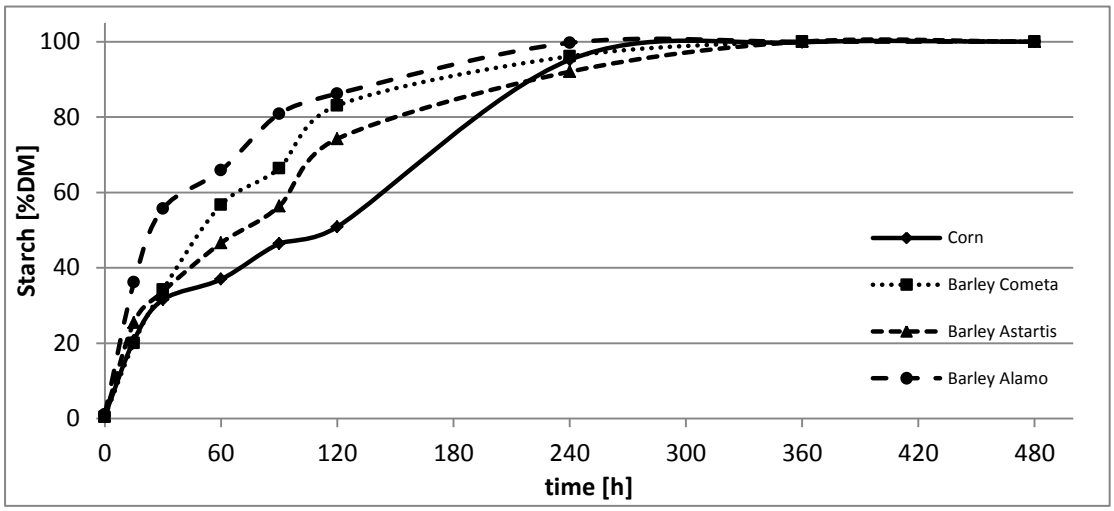


Figure 1

Cumulative mean values of the starch curves recorded during *in vitro* multi-enzymatic hydrolysis of feed stuff.

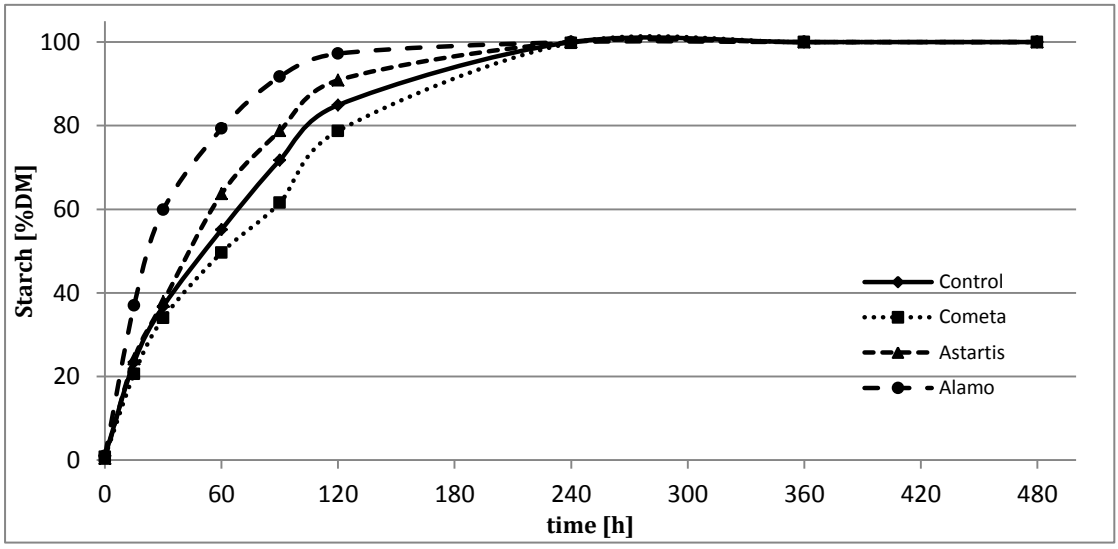


Figure 2

Cumulative mean values of the starch curves recorded during *in vitro* multi-enzymatic hydrolysis of feed of experimental diets.

The curves recorded during the starch multi-enzymatic hydrolysis of feed stuff and experimental diets are illustrated in Figure 1. The parameters of the Mahasukhonthachat et al. (2010) model developed to describe the hydrolysis kinetics and also residual matter results are detailed in Table 5. Amount of material degraded during *in vitro* hydrolysis of feed stuff samples was the highest in corn (59.63%), as we expected. But in case of barley varieties surprisingly, there was no large significant differences. In case of experimental diets, thus based on barley have significantly higher level of degradation than Control and Cometa ($P<0.001$). Percentage of starch inside residual matter obtained after *in vitro* hydrolysis was higher in corn and barley astartis than other feed stuff ($P>0.05$). Treatment based on variety Alamo had only 24.82% of residual starch, other treatments had the same level of significance ($P>0.05$). Different values of digestion coefficient C_0 and C_∞ were recorded for the four prime matters ($P<0.001$) but not for treatments ($P>0.05$). The hydrolysis velocity expressed by parameter k was significantly differ within Alamo group ($P<0.001$), with results for stuff 0.02/min and diet 0.03/min. AUC based hydrolysis index of four cereals and treatments was significantly different ($P<0.001$). The highest HI has barley Alamo (95.81%) as well as its diets (99.61%) respect to Control feed.

In vitro fermentation of residual matter

Table 6 *Gas production curve parameters*

	Feed stuff Corn mean	Cometa mean	Astartis mean	Alamo mean	*se*	*P-value*
V_f [ml]	52.78	38.95	52.52	49.96	3.41	ns
LAG [h]	0.00	0.15	0.05	0.15	0.07	ns
k [h^{-1}]	0.15	0.20	0.17	0.15	0.02	ns
	Experimental diets Control	Cometa	Astartis	Alamo		
V_f [ml]	41.55 b	41.32 b	46.91 a	43.79 ab	1.75	**
LAG [h]	0.07	0.00	0.09	0.12	0.06	ns
k [h^{-1}]	0.19	0.18	0.20	0.18	0.01	ns

se - standard error; levels of significance: *P<0.05* (*), *P<0.01* (**), *P<0.01* (***), ns - not significant. The level of significance between mean treatments is *P<0.05*.

Table 7 *VFA content from in vitro fermentation samples*

	Feed stuff									
	Corn		Cometa		Astartis		Alamo		*se*	*P-value*
Acetic	55.13		58.495		54.845		57.805		1.43	ns
Propionic	29.445		25.295		28.3		25.055		1.22	ns
Isobutyric	1.61		1.22		1.66		1.84		0.21	ns
Butyric	9.385	b	10.36	ab	10.58	a	10.51	a	0.26	*
Isovalerianic	2.51		2.695		2.72		2.46		0.27	ns
Valerianic	1.91	a	1.93	ab	1.91	a	2.33	b	0.08	*
Total	49.84	c	40.99	b	47.15	a	42.90	d	0.36	***
NH_3 [mg/100ml]	16.01	c	14.43	b	12.86	a	15.66	bc	0.45	***

	Experimental diets									
	Control		Cometa		Astartis		Alamo			
Acetic	57.79		56.92		56.84		57.69		1.18	ns
Propionic	26.83		26.39		26.91		24.82		1.83	ns
Isobutyric	1.26		1.87		1.80		2.02		0.38	ns
Butyric	9.00		9.30		9.21		9.70		0.43	ns
Isovalerianic	3.08		3.33		3.08		3.42		0.20	ns
Valerianic	2.04		2.20		2.15		2.35		0.11	ns
Total	42.91	ab	41.40	b	51.61	a	46.29	ab		*
NH_3 [mg/100ml]	18.51		26.73		25.75		24.87		3.59	ns

VFA means are expressed in [mmol/100g] and total VFA amount in [mmol%]. se - standard error; levels of significance: *P<0.05* (*), *P<0.01* (**), *P<0.01* (***), ns - not significant. The level of significance between mean treatments is *P<0.05*.

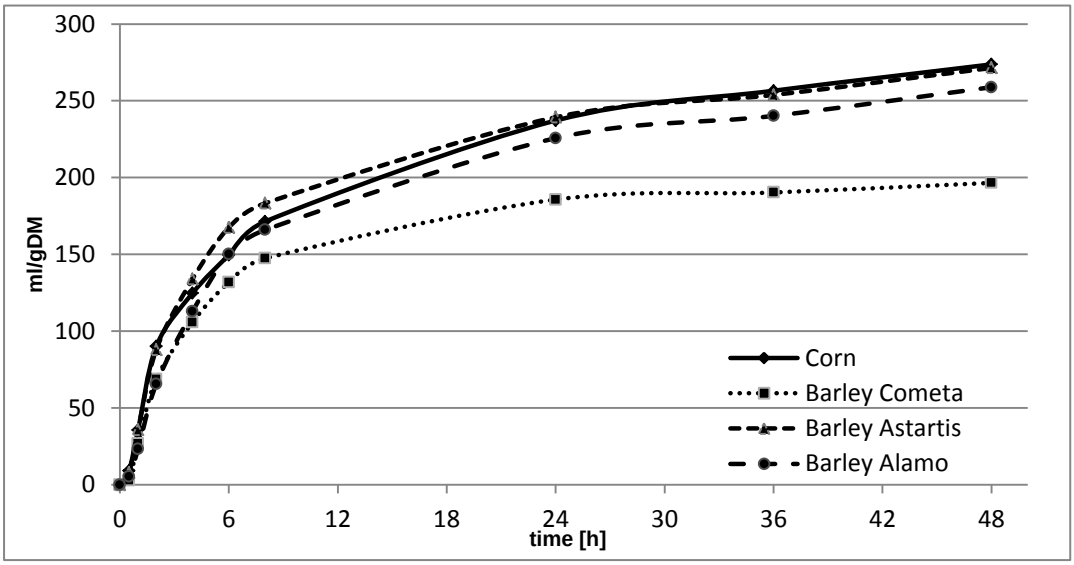


Figure 3

Mean values of the gas production curves recorded during *in vitro* fermentation of feed stuff.

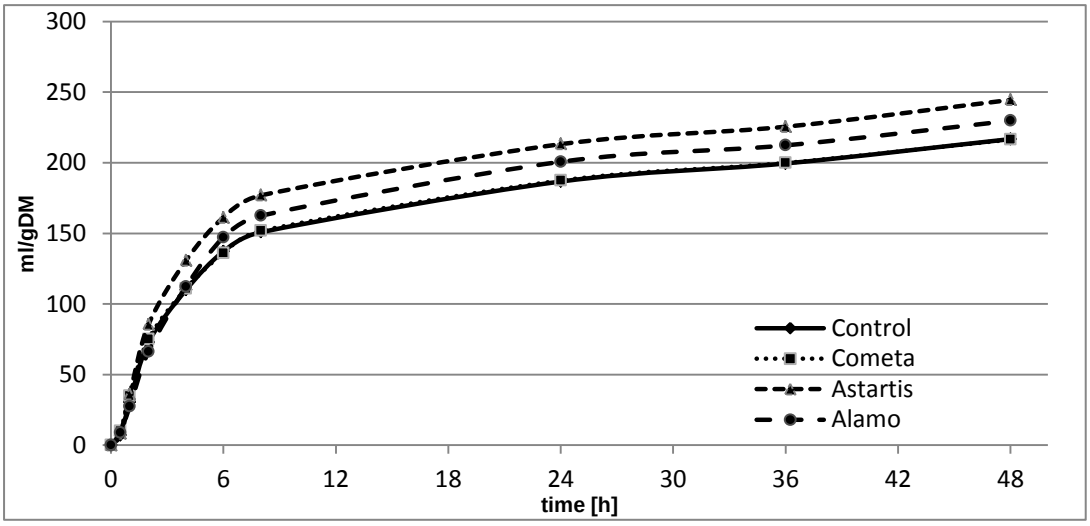


Figure 4

Mean values of the gas production curves recorded during *in vitro* fermentation of experimental diets.

The gas accumulation curves recorded during *in vitro* fermentation of residual matter with pigs inoculum are shown on Figure 3 and 4. The parameters of the exponential model with discrete LAG time are detailed in Table 7. The lag times (LAG [h]) were short and no statistically significant differences were presented ($P>0.05$), same as in velocities (k [h^{-1}]) ($P>0.05$). The final volume (Vf [ml]) was significantly different within diets ($P<0.01$), Astartis based diet has the highest volume (46.91ml), but not in cereals used for their production ($P>0.05$).

Total volatile fatty acids concentration in samples from fermented cereals are significantly different ($P<0.001$). Corn has the highest average total VFA amount (49.84 mmol%) and barley variety Cometa the lowest one (40.99 mmol%). Within, experimental diets group ($P<0.05$), Cometa based feed has also the lowest concentration (41.40 mmol%), but Astartis the highest (51.61 mmol%). Moreover significant differences in percentage of single VFA were found in fermented cereals summary ($P<0.05$), butyric and valerianic acids, but not in treatments summary ($P>0.05$). Ammonium content from *in vitro* fermentation samples is presented in Table 10. Ammonium final content was different among cereals ($P<0.001$). Samples of fermented corn has the highest NH_3 amount (16.01 mg/100ml) as compared to barley cereals. Experimental diets have the similar not significant final concentration.

DGGE

Figure 5
Polyacrylamide gels showing rRNA gene amplicon profiles obtained by denaturing gel electrophoresis of bacterial samples taken from porcine intestine. Lane labels indicate diet type and replicate number. Blue points on the line indicate detected bands.

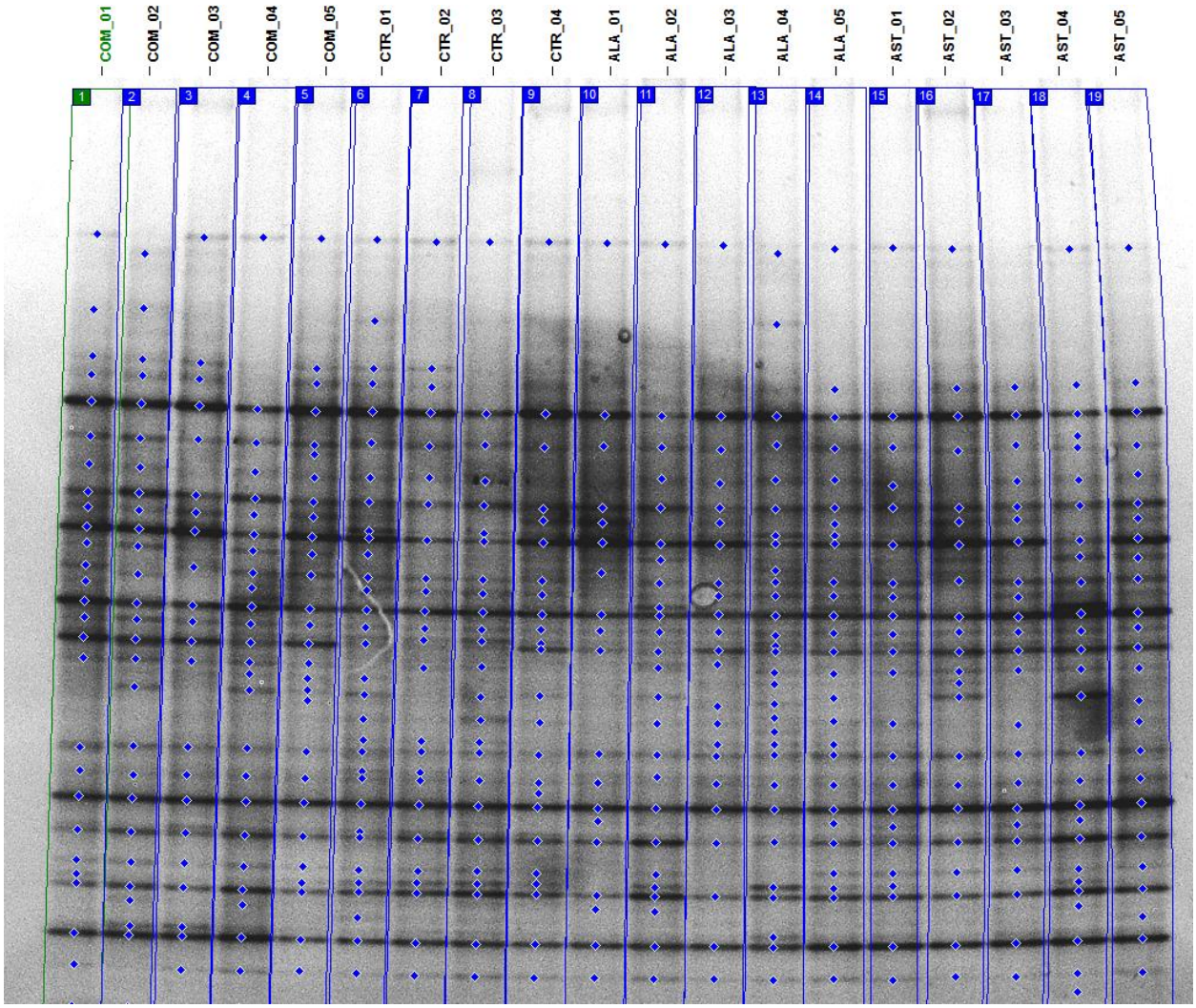
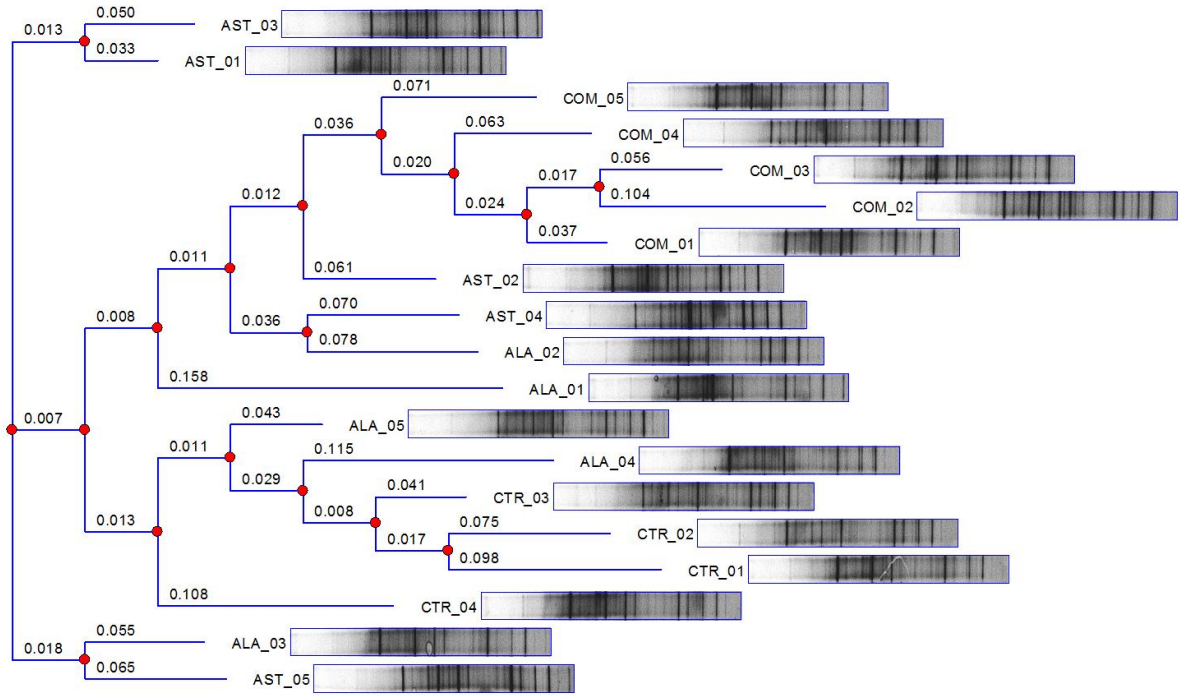


Figure 6
Dendrogram of bacterial DGGE band patterns derived from samples taken from porcine intestine. Lane labels indicate diet type and replicate number. Hierarchical cluster analysis was performed the Phoretix1D v11.5 software (TotalLab Ltd, UK). and distances were calculated from band presence–absence with peak intensities data.



We analysed DGGE gel using presence–absence and peak intensities data (Figure 5). Hierarchical cluster analysis divided bands into three main clusters, the middle could be then divided into another two (Figure 6). Looking from the top, first sub-cluster contain all samples obtained from pigs fed Cometa based diet, second one hold all Control diet samples. Remaining lanes with samples from Alamo and Astaris treatment were attached into different branches of dendrogram.

Discussion

The Mediterranean region is well known from a drier conditions and high temperatures, such clime specifications may lead to lower yields. Crop irrigation especially during dry periods helps improve productivity. Warming increase water requirements with consequences for yield, irrigation is needed to provide water. But irrigation may by also the source of sustainability concerns, such as irrigation erosion, excessive use of subterranean water and increase of soil salinity. European agriculture use, on average, one quarter of total water abstraction. Southern countries use more than 80% of total water, in comparison to northern where water usage is much lower but still accounting for more than 30% in some areas (EEA, 2012; European Council, 2000). One of possible solution is to replace current crop with new ones, resistant to the water scarcity and with no requirements of intensive irrigation (Worch et al., 2011). In Italy cultivation of maize is very widespread. It should be necessary to determine whether the introduction of other crops would not adversely affect animal breeding. So, this study was aimed at evaluating the effect of diets composition based on three different barley varieties on performance, nutrient digestibility and intestinal fermentation in finished pigs in comparison with corn based diet.

Overall, differences in performance and digestibility as well as *in vitro* evaluations can be explained by differences in chemical composition of the cereals used in the diets. Recall that, control diet had around 50% of corn and 20% of barley while other three 60% more barley with only 10% of corn. Pettersson and Lindberg performed ileal and total tract digestibility and found significant improvements in nutrients and energy utilization using diets based on naked as compared with hulled barley varieties, but the performance of pigs on hulled barley based feed is similar to that fed with hulless barley-based diet, according to Thacker, 1999. Our diet based on hulled Cometa variety had higher content of fibre when hulless varieties (Astartis and Alamo) had lower. Use of hulles barley could be probably also more cost-effective than hulled, regarding the de-hulling process. Good performance results of hulled barley-based diets was probably caused also by higher percentage of starch fractions which are easier available for digestive enzymes (RDS and SDS). Lower content of

RS improved digestibility and therefore the optimal weight for slaughter could be reached faster, compared to corn based diet. Performance are in line with *in vitro* starch digestion and fermentation, and gave us useful information about the kinetics. Mathematical models are essential tools in biological sciences to carry out such analysis (Schofield et al., 1994). Feeds based on hulled varieties had starch hydrolysis velocity (k) and its hydrolysis index (HI) higher than remaining ones. The gas production during *in vitro* fermentation of residual matter was surprisingly higher in these two feeds as well as production of VFAs, in comparison to diet based on Cometa variety (Bindelle et al., 2011, 2007b). Lynch et al., 2007 reported linear increase in total colon VFA concentration and in the molar proportions of propionic acid and butyric acid in the pigs fed with increasing levels of dietary barley. In our experiment diet based on standard barley variety Cometa had similar fermentation pattern as diet based on corn. Increased level of fermentation is associated with longer period of satiety, due to gut fill, and with the reduced physical activity of pigs (Jørgensen et al., 2010). Moreover, DGGE analysis reviled great similarity between intestinal bacteria profile of replicates under hulled barley variety Cometa-based diet or corn-based Control diet, in contrary to samples from animals on hulles barley diets (Astartis and Alamo) which had much more various. These patterns are by some means in line with *in vitro* fermentation results and VFA production.

As we discussed above in our article (Prandini et al.), the measurement of body weight is the most important information, but nutritional status and physiological disposition of animal organism is reflected primarily in blood profile. Blood examinations have shown significant differences in total protein, glucose, bilirubin and urine levels. There was no significant differences in cholesterol concentration between our treatments groups. Glucose blood level depends of carbohydrates availability in diet, as we can see pig fed Cometa diet, which has the lowest RDS+SDS percentage, has also lowest glucose concentration. Total proteins levels of significance between treatments are related to diet crude protein content, it was confirmed previously that the blood urea and total protein content increases as the protein content of the diet increases (Eggum, 1970; Pond et al., 1980). The blood urea content is also well known as a rapid and efficient method for determination of protein quality, blood urea decreases with increasing quality of the protein in the diet (Eggum, 1970).

Animals fed diet based on Alamo variety have elevated level of blood urea, suggesting a disruption of normal systematic protein utilization. Control and Cometa based feed have similar levels of CP in diet and also have the same blood protein level of significance. There were no significant differences in albumin, the principal protein in serum, and globulin levels. It was shown that bilirubin is a physiological antioxidant and has anti-inflammatory effects. Its synthesis is induced in response to oxidative stress, but an antioxidant effect is visible only in high concentrations (Ryter and Tyrrell, 2000; Yesilkaya et al., 2000). In human research a low circulating level of total bilirubin is usually associated with increased body mass index (BMI), blood pressure, and insulin resistance (Lin et al., 2010, 2009). Data from study (Conter et al., 1986) indicate that in the prairie dog, carbohydrate feeding resulted in a significant increase in biliary concentrations of phospholipids, calcium, and bilirubin. There was a possible relationship between glucose, protein and bilirubin values. Similar to Préstamo et al., 2002 study on rats we observed that when higher total proteins and glucose, bilirubin level decreased, we can assume that such relation is diet dependent. Animals fed Alamo diet had significantly elevated urea concentration, but it is not high enough to prompt any problems. Other experiments Chiba et al., 1991 suggest that diet high percentage CP and lysine in diet is correlated with elevated urea concentration, but here there is no such effect.

Our findings suggest that hulled barley has no negative effects on growth performance or digestibility of pigs, which was confirmed also by *in vitro* trials. Important thing is the fact that the experiment has been designed primarily to see whether the effective production of pigs can do without corn, used for animal feed, which require irrigation and consuming large amounts of water. It is important to take into consideration that not every barley variety could be successfully used instead of corn. Nutritional characteristics of barley grain can vary across cultivars, the differences may be explained by genetic variations and grow conditions, in terms of the content of NSP, especially β-glucans, and starch. Additionally, the advantage of naked barley is a reduction in water usage, which is widely promoted by the EU and may by a huge step forward for agriculture, toward the water-saving economy.

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3.2. Addition of nonstarch polysaccharides degrading enzymes to two hulless barley varieties fed in diets for weaned pigs

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Manuscript published in Journal of Animal Science on May 2014 vol. 92 no. 5 2080-2086, doi: 10.2527/jas.2012-6199.

Abstract

A study was conducted to evaluate the effect of 2 hulless barley varieties, with or without the addition of a nonstarch polysaccharide (NSP) enzyme complex (β-glucanase and xylanase), on growth performance of weaned piglets in a 42-d feeding study. The study was conducted with 140 piglets (PIC × Duroc). Pigs were allocated to pens (4 castrated males or 4 females per pen) based on BW and sex, and pens were assigned to 5 experimental diets with 4 pens of castrated males and 3 pens of females per treatment. Five isonitrogenous and isoenergetic diets were compared: 1) control corn-based diet (CTR), 2) diet with corn and wheat bran replaced by the Astartis hulless barley variety (AS), 3) diet with corn and wheat bran replaced by the AS supplemented with the NSP enzyme complex (ASE), 4) diet with corn and wheat bran replaced by the Alamo hulless barley variety (AL), and 5) diet with corn and wheat bran replaced by the AL supplemented with the NSP enzyme

complex (ALE). The diets were formulated to meet or exceed nutrient requirements and offered in 2 phases: d 0 to 14 and d 14 to 42. At the end of the study, pigs fed AS and AL had equal weights as pigs fed CTR. Pigs fed the hulless barley diets had greater ($P < 0.05$) ADG during the second phase (P2) and overall phase, BW at d 42, and G:F during the P2 than those fed the CTR. Pigs fed the ASE and ALE had greater ($P < 0.05$) ADFI during the P2 and overall ADG than those fed the AS and AL. The increases in ADG during the P2 and final BW obtained with NSP enzyme supplementation were greater in pigs fed the AS than those fed the AL (barley × enzyme, $P < 0.05$). On the other hand, the NSP enzyme complex increased G:F in pigs fed the AS during the P2 and overall phase, but it had no effect on those fed the AL (barley × enzyme, $P < 0.05$). In conclusion, hulless barley with or without the NSP enzyme complex can be a replacement ingredient for corn and wheat bran in weaned pig diets. Addition of the NSP enzyme complex to AS variety, but not AL variety, improved growth performance of weanling pigs.

Keywords: Corn; growth performance; hulless barley; nonstarch polysaccharides enzymes; weaned piglets;

Acknowledgments: Financial support was provided by the AGROSCENARI project 'Scenari di adattamento dell'agricoltura italiana ai cambiamenti climatici' of MIPAAF (Ministero delle politiche agricole alimentari e forestali – Italy) and by the Doctoral School on the Agro-Food System (Agrisystem) of the Università Cattolica del Sacro Cuore (Italy).

Introduction

Barley is typically produced in areas where corn production is not agronomically feasible. Barley can partially or totally replace corn in swine diets (Thacker, 1999; Wu et al., 2000). Nevertheless, barley contains antinutritional factors such as arabinoxylans and β-glucans, which are classified as nonstarch polysac- charides (NSP; Aman and Graham, 1987; Fleury et al., 1997). Pigs, especially piglets, do not produce enzymes that degrade NSP (Li et al., 2004). Elevated levels of arabinoxylans and β-glucans increase digesta viscosity, which reduces interactions between nutrients and digestive enzymes (Campbell and Bedford, 1992; Kim et al., 2012) and interferes with digestion and absorption of nutrients (O'Connell et al., 2005; Bach Knudsen et al., 2012). Two enzymes, β-glucanase and xylanase, degrade polysaccharide matrixes around nutrients (Hesselman and Åman, 1986) and reduce the viscosity of the intestinal contents. Thus, the availability and digestibility of nutrients are improved (Mathlouthi et al., 2002). Supplementation of exogenous NSP enzymes to barley-based diets has improved animal performance (Inborr et al., 1993; Li et al., 1996). The β-glucan and pentosan contents in whole barleygrain are 4.2 and 6.6%, respectively, with only 1.8 and 1.4% in endosperm (Henry, 1985). Thus, the major pro- portion of NSP content of barley is contained in the hull fraction. Removal of the hull from barley improved its nutritive value (Thacker, 1999; Thacker and Rossnagel, 2006). The aim of the study was to evaluate the potential of 2 hulless barley varieties, with or without the addition of the NSP enzyme complex, as an alternative to corn in weaned pig diets.

Material and Methods

The study was conducted at the Research Centre for Zootechnics and the Environment (S. Bonico, Piacenza, Italy), and animal care and use practices during this study conformed to European Union Directive 2010/63/ EU of 22 September 2010 on the protection of animals used for scientific purposes.

Animals, Housing, and Performance Measurements

The study was performed using 140 weaned pigs (PIC [Hendersonville, TN] × Duroc) with initial BW of 7.7 ± 0.4 kg. Pigs were allocated to pens in 5 rooms based on BW and sex. Each pen contained 4 pigs (0.25 m2/pig) of the same sex, and pens were assigned to 5 experimental diets with 4 pens of castrated males and 3 pens of females per treatment. All 5 treatment groups were present at least once in each room. The temperature was regulated by a thermostatically controlled ventilation system, and the temperature and relative humidity were maintained at 26 ± 2°C and 60 ± 5%, respectively. The illumination during the experimental period was natural. The pigs had ad libitum access to feed and water through 1 feeder and an automatic drinker in each pen. Individual pigs and feeders were weighed at 0, 14, and 42 d to calculate ADFI, ADG, and G:F during the experimental periods: d 0 to 14, d 14 to 42, and d 0 to 42.

Dietary Treatments

After a pre-experimental period of 4 d, pigs were fed their respective diets for 42 consecutive days. During the pre-experimental period, pigs were fed without med- icated feed. All the testing diets were formulated without antibiotics or antibiotic growth promoters. The following experimental diets were compared: 1) control corn-based diet (CTR), 2) diet with corn and wheat bran replaced by the Astartis hulless barley variety (AS), 3) diet with corn and wheat bran replaced by the AS supplemented with the NSP enzyme complex (ASE; DSM Nutritional Product Ltd., Basel, Switzerland), 4) diet with corn and wheat bran replaced by the Alamo hulless barley variety (AL), and 5) diet with corn and wheat bran replaced by the AL supplemented with the NSP enzyme complex (ALE). The 2 barley varieties were grown in the same pedoclimatic conditions (Beccastecca Pig Research Unit farm, Modena, Emilia Romagna, Italy). The NSP enzyme complex (E 1602; DSM Nutritional Product Ltd., Basel, Switzerland) was added to the ASE and ALE at amount of 1 g/kg feed. The NSP enzyme complex

was derived from Tricoderma longibrachiatum and contained endo-1,4-β-glucanase ([European Community] 3.2.1.4), endo-1,3(4)-β-glucanase (EC 3.2.1.6), and endo-1,4-β-xylanase (EC 3.2.1.8).

Table 1. Chemical and amino acid composition of corn and barley varieties (as-fed)

Item	Corn	Hulless barley	
		Astartis variety	Alamo variety
Chemical composition, %			
DM	88.00	89.34	90.16
CP	8.10	12.53	15.28
Ether extract	3.70	1.89	2.27
Crude fiber	1.20	1.70	2.65
Total starch	64.10	56.75	52.34
β-glucans	n.d.[1]	4.91	5.63
DE,[2] kJ/kg	14,194	13,620	12,867
Amino acid, %			
Lys	0.22	0.42	0.44
Met + Cys	0.33	0.48	0.60
Thr	0.27	0.42	0.50
Leu	0.88	0.87	1.01
Ile	0.26	0.46	0.58
Val	0.36	0.63	0.72
His	0.26	0.30	0.36
Arg	0.35	0.61	0.67
Phe + Tyr	0.60	0.96	1.14

[1]n.d. = not detected.

[2]Calculated based on Fairbairn et al. (1999) for barley varieties and Morgan et al. (1987) for corn.

Table 2. Ingredient composition of the diets in the first phase (P1) and second phase (P2)[1]

Ingredient, %	P1 (d 0 to 14)			P2 (d 14 to 42)		
	CTR	AS	AL	CTR	AS	AL
Corn meal	38.09	–	–	37.05	–	–
Barley meal	–	53.00	53.00	–	53.00	53.00
Wheat meal	15.00	11.69	11.08	20.00	16.20	16.37
Wheat bran	10.00	–	–	10.00	–	–
Soy protein[2]	15.00	11.60	10.40	10.00	7.40	7.80
Whey powder	8.00	8.00	8.00	8.00	8.00	8.00
Soybean meal, 44% CP	7.00	8.00	6.30	10.10	10.00	6.00
Calcium carbonate	2.30	2.11	2.12	1.75	1.57	1.55
Monocalcium phosphate	2.30	2.60	2.75	1.30	1.60	1.80
Dextrose	1.00	1.00	1.00	1.00	1.00	1.00
Animal fat	–	0.60	3.80	–	0.30	3.40
Lys HCl (78%)	0.50	0.52	0.64	0.22	0.25	0.37
Premix[3]	0.25	0.25	0.25	0.25	0.25	0.25
Sodium chloride	0.24	0.29	0.30	0.25	0.30	0.30
DL-Met	0.17	0.16	0.15	0.05	0.06	0.04
L-Thr	0.13	0.15	0.15	0.03	0.05	0.08
L-Trp	0.02	0.03	0.06	–	0.02	0.04

[1]CTR = control corn-based diet; AS = diet with corn and wheat bran replaced by the Astartis hulless barley variety; AL = diet with corn and wheat bran replaced by the Alamo hulless barley variety.

[2]Hamlet protein HP200 (Hamlet Protein Inc., Findlay, OH).

[3]Provided vitamins and minerals per kilogram of feed (Unione Veneto Lombarda, Roé, Volciano, BS, Italy): vitamin A, 15,000 IU; vitamin D_3, 1,500 IU; vitamin E, 20 mg; vitamin B_1, 2 mg; vitamin B_2, 0.4 mg; vitamin B_6, 2 mg; vitamin B_{12}, 0.015 mg; biotin, 0.1 mg; vitamin K_3, 2 mg; niacin, 25 mg; D-pantotenic acid, 10 mg; choline cloride, 375 mg; Mn, 60 mg as manganous oxide; Fe, 200 mg as ferrous sulfate; Cu, 20 mg as copper sulfate; Zn, 75 mg as zinc oxide; Co, 0.75 mg as cobalt oxide; I, 2.4 mg as potassium iodide; and Se, 0.3 mg as sodium selenite.

The diets were fed in 2 phases: first phase (P1; d 0 to14) and second phase (P2; d 14 to 42). The diets were formulated to meet or exceed nutrient requirements (NRC, 1998). Within P1 and P2, the diets were isonitrogenous and isoenergetic. The pigs had free access to feed and water for the 42-d experiment. All the diets were fed in meal form. The chemical and AA composition of corn and barley varieties and ingredient composition of the experimental diets are shown in Tables 1 and 2, respectively.

Chemical Analyses

Corn and barley grains were sampled before inclusion in the diets, whereas the diets were sampled before the beginning of each growth phase. Dried cereal grains and diets were ground through a 1-mm screen using a centrifugal grinding mill (Retsch Type ZM100; Retsch, Haan, Germany). The samples were then analyzed for moisture, CP, crude fiber, crude lipid, and ash according to the methods of the

Table 3. Chemical and amino acid composition (% of DM) of the diets in the first phase (P1; d 0 to 14) and second phase (P2; d 14 to 42)[1]

		AS		AL	
Item	CTR	Without enzyme	With enzyme	Without enzyme	With enzyme
P1 (d 0 to 14)					
DM	89.68	90.19	90.05	91.17	91.32
CP	22.83	22.85	22.68	22.57	22.63
Ether extract	5.01	4.59	4.58	5.87	5.96
Crude fiber	3.69	2.75	2.64	3.67	3.33
β-glucans	0.64	2.75	2.75	3.12	3.12
Ash	10.85	9.75	9.57	8.96	8.97
Total starch	47.51	42.02	42.24	36.26	36.30
DE,[2] kJ/kg	16,066	16,107	16,112	16,103	16,116
NE,[3] kJ/kg	11,845	11,849	11,853	11,874	11,874
Lys	1.50	1.49	1.50	1.51	1.50
Met + Cys	0.87	0.87	0.87	0.88	0.87
Thr	0.94	0.94	0.94	0.92	0.93
Trp	0.28	0.27	0.28	0.28	0.27
P2 (d 14 to 42)					
DM	90.60	90.10	90.10	90.96	90.72
CP	21.26	21.41	21.51	21.20	21.30
Ether extract	3.32	2.42	2.59	6.22	6.04
Crude fiber	4.15	2.84	2.76	3.17	3.22
β-glucans	0.69	2.81	2.81	3.16	3.16
Ash	8.20	8.59	8.48	8.38	8.25
Total starch	48.53	45.59	45.46	42.22	42.57
DE, kJ/kg	16,044	16,074	16,082	16,124	16,120
NE, kJ/kg	11,937	11,904	11,908	11,971	11,971
Lys	1.20	1.20	1.20	1.20	1.20
Met + Cys	0.74	0.75	0.75	0.75	0.75
Thr	0.80	0.80	0.80	0.81	0.81
Trp	0.25	0.25	0.25	0.25	0.25

[1]CTR = control corn-based diet; AS = diet with corn and wheat bran replaced by the Astartis hulless barley variety; AL = diet with corn and wheat bran replaced by the Alamo hulless barley variety. Enzyme (1 g/kg) was nonstarch polysaccharides enzyme complex (DSM Nutritional Product Ltd., Basel, Switzerland).

[2]Calculated based on Morgan et al. (1987).

[3]Calculated based on Noblet et al. (1994; Eq. [11]).

Scientific Association of Animal Production (ASPA, 1980) and Martillotti et al. (1987) and for total starch content with an enzymatic method (Blasel et al., 2006) modified by Masoero et al. (2010).

Amino acids were determined using an AA analyzer (Jasco, Inc., Easton, MD) using hydrolysis methods described by Moore (1963), Eggum (1968), and Moore et al. (1980). The DE content of the diets was calculated according to the equation reported by Morgan et al. (1987), whereas the NE content was calculated using Eq. [11] proposed by Noblet et al. (1994). The DE content of the barley varieties and corn was calculated by the equations reported by Fairbairn et al. (1999) and Morgan et al. (1987), respectively. The β-glucan concentrations of the cereal grains and diets were determined using a mixed-linkage β-glucan assay kit (K-BGLU 07/11; Megazyme Int., Wicklow, Ireland). Chemical composition of the diets, including AA, is presented in Table 3.

Statistical Analysis

Data were tested for normality with the Shapiro-Wilk test before statistical analysis. Then, data were analyzed using the GLM procedure of SAS (SAS Inst. Inc., Cary NC) according to the following model:

$$Y_{ijk} = \mu + \tau_i + \beta_j + (\tau\beta)_{ij} + \varepsilon_{ijk},$$

in which Y_{ijk} is the *k*th observation of the *j*th room of the *i*th treatment (j = 1 to 5 and k = 1 to 7), μ is the overall mean, τ_i is the fixed effect of treatment (i = 1 to 5), β_j is the fixed effect of room (j = 1 to 5), $(\tau\beta)_{ij}$ is the treatment per room interaction, and ε_{ijk} is the residual error. The room effect and their first interaction were removed from the model if they were not significant. Contrasts (CTR vs. other treatments, main effect of barley, main effect of enzyme, and barley × enzyme interaction) were used to assess the effect of treatments. Differences were accepted as significant if $P \leq 0.05$.

Results

Chemical and Amino Acid Composition of Barley Varieties

Two barley varieties were characterized by greater levels of CP, fiber, and β-glucans and reduced content of fat and starch in comparison with corn (Table 1). Astartis barley had a greater total starch content and reduced levels of CP, crude fat, crude fiber, and β-glucans than AL Astartis barley was also characterized by lower AA contents, consistent with a lower CP level compared with AL.

Growth Performance

Pigs maintained a general good health status throughout the study. The overall mortality (0.7%) was similarly distributed among treatments. The results for growth performance are given in Table 4. At the end of the study (d 42), pigs fed the barley diets (AS, ASE, AL, and ALE) weighed more ($P < 0.05$) than those fed the CTR. The increase in the final BW obtained with NSP enzyme supplementation was greater in pigs fed the AS than those fed the AL (barley × enzyme, $P < 0.05$). During the P1 (d 0 to 14), the pigs fed the different diets showed similar ADG. However, in the P2 (d 14 to 42) and overall phase (d 0 to 42), pigs fed AS, ASE, AL, and ALE grew faster ($P < 0.05$) than those fed the CTR. During the P2, the increase in ADG obtained with NSP enzyme supplementation was greater in pigs fed the AS than those fed the AL (barley × enzyme, $P < 0.05$). Pigs fed the ASE and ALE had greater ($P < 0.05$) overall ADG than those fed the AS and AL.

The pigs fed the different diets did not show any difference in ADFI during the P1 and throughout the study. However, in the P2, pigs fed the barley-based diets with the addition of the NSP enzyme complex had an increased ADFI ($P < 0.05$) compared to those fed the same diets without enzyme.

There was a barley effect and a barley × enzyme interaction ($P < 0.05$) on G:F during the P1. The NSP enzyme supplementation decreased G:F in pigs fed the AS, but it increased in those fed the AL. On the other hand, the NSP enzyme complex increased G:F in pigs fed the AS during the P2 and

overall phase, but it had no effect on those fed the AL. During the P2, pigs fed the barley-based diets had better ($P < 0.05$) G:F than those fed the CTR.

Discussion

The results of our study showed that hulless barley could totally replace corn and wheat bran in weaned piglet diets without negatively affecting growth performance. These results were in agreement with those obtained by Wu et al. (2000). In their study to compare hulless barley and corn in pig diets, hulless barley had no negative effects on growth performance, carcass traits, or nutrient digestibility in pigs.

Table 4. Growth performance of the pigs fed the different diets[1]

		AS		AL			
Item	CTR	Without enzyme	With enzyme	Without enzyme	With enzyme	SEM	*P*-value
BW, kg							
d 0	7.79	7.63	7.74	7.63	7.81	0.17	NS[2]
d 14	11.86	11.80	11.65	11.93	12.06	0.29	NS
d 42[3,5,6]	21.21	21.25	23.41	22.74	23.26	0.57	<0.05
ADG, g/d							
d 0 to 14	291.1	297.9	279.3	307.4	310.1	14.1	NS
d 14 to 42[3,5,6]	333.9	337.8	420.0	386.1	400.0	16.4	<0.05
d 0 to 42[3,5]	319.6	324.5	373.1	359.9	365.8	12.2	<0.05
ADFI, g/d							
d 0 to 14	423.6	445.4	447.7	440.7	411.9	17.2	NS
d 14 to 42[5]	668.6	676.0	696.5	684.7	739.1	18.1	0.08
d 0 to 42	586.9	599.1	613.6	603.4	621.8	11.9	NS
G:F							
d 0 to 14[4,6]	0.69	0.67	0.62	0.70	0.75	0.03	0.08
d 14 to 42[3,6]	0.50	0.50	0.60	0.56	0.54	0.02	<0.05
d 0 to 42[6]	0.54	0.54	0.61	0.60	0.59	0.02	<0.05

[1]CTR = control corn-based diet; AS = diet with corn and wheat bran replaced by the Astartis hulless barley variety; AL = diet with corn and wheat bran replaced by the Alamo hulless barley variety. Enzyme (1 g/kg) was nonstarch polysaccharides enzyme complex (DSM Nutritional Product Ltd., Basel, Switzerland). $n = 7$ (4 pens of castrated males and 3 pens of females).

[2]NS = not significant.

[3]CTR vs. others, $P < 0.05$.

[4]Main effect of barley, $P < 0.05$.

[5]Main effect of enzyme, $P < 0.05$.

[6]Barley and enzyme interaction, $P < 0.05$.

The use of barley in pig production is generally limited because of its high fiber content. In barley cultivars, in which the hull remains attached to the kernel, up to 21% of the total grain weight can be fiber (Juskiw et al., 2011). Increased dietary fiber level was associated with a reduced available

energy content of feed (Noblet and Le Goff, 2001). The energy value of barley is about 85 to 90% compared with corn. On the other hand, barley contains more CP, Lys, and available P than corn (NRC, 1998). Our results indicate that the nutritional value of barley without the hull was comparable to that of corn in pig diets.

In pig nutrition, evidences indicate that starches from different feed grains are digested and absorbed at different rates and extents, depending on their inherent physicochemical characteristics (Zijlstra et al., 2009; Bach Knudsen, 2011; Giuberti et al., 2012b). As reviewed by Giuberti et al. (2014), several physicochemical characteristics of native starches, including the amylose content, can modulate the postprandial metabolic response in pigs, which can affect pigs performances. Therefore, the greater ADG and G:F in pigs fed the AL than those fed the CTR and AS from d 0 to 42 could be related, at least in part, to the different amylose content of cereal grains in the experimental diets. Corn and AS used in the current work are normal-amylose cereal grains (on average, 20 to 30 g/100 g starch), whereas AL is classified as a low-amylose cultivar (amylose content less than 1 g/100 g starch; Stevnebø et al., 2006). An in vitro study (Giuberti et al., 2012a) showed that low-amylose cereal grains are more susceptible to enzymatic hydrolysis than normal-amylose counterparts. This occurs because amylopectin polymers are digested to a greater extent than amylose polymers (Parada and Aguilera, 2011) because of the decreased accessibility to the molecule for enzyme degradation due to difference in chemical arrangements (Black, 2001).

Amylose in starch consists of long chain of α-(1,4)- linked glucose units that form compact helical structures making the bonds comparatively inaccessible to amylases (Tester et al., 2006). The likely enhanced starch enzymatic digestion of low-amylose cereal grains (i.e., AL) might have contributed to greater energetic efficiency for pigs, thus resulting in greater feed efficiency in pigs consuming the AL. Accordingly, Regmi et al. (2011) reported that diets characterized by starches with wide differences in amylose content (ranging from 0.0 to 63.2 g/100 g of DM) influenced both ADG and feed efficiency in bar- rows. In addition, amylose can enhance the formation of resistant starch (Sajilata et al., 2006), which is associated with a lower in vivo starch digestibility (Sun et al., 2006). As reported by Li et al. (2007), pigs fed diets based on an increased resistant starch corn cultivar had a

reduced G:F compared with pigs fed diets formulated with regular corn or rice. Lastly, the potential for amylose-lipid complex formation before ingestion or during digestion may increase with increasing the amylose level, thus forming a denser molecular network that might reduce enzyme accessibility (Vesterinen et al., 2002; Tester et al., 2004).

The exogenous enzyme supplementation apparently counteracted the negative effects of NSP in barley and improved the availability and digestibility of nutrients. Yin et al. (2001) reported that β-glucanases and xylanase improved growth performance and feed efficiency, if pigs were fed barley-based diets. In our study, only the diet based on AS barley was improved by the supplementation with the NSP enzyme complex. These results were in agreement with those obtained previously (Li et al., 2004; Fan et al., 2009).

A significant interaction was observed between barley source and enzyme supplementation. This result was in agreement with Yin et al. (2001), where different enzyme responses to different varieties of hulless barley were reported in young pigs (initial BW of 15 kg). As discussed before, this interaction could be partially explained by the different amylose content of the 2 barley varieties used in the experimental diets. Likewise, Kim et al. (2005) reported an interaction between wheat (low amylose vs. normal amylose) and enzyme mixture (xylanase and β-glucanase) for ADG in weaned pigs. In addition, the different ingredient composition (i.e., fat addition) between AS and AL could have altered the effect of enzyme supplementation of pigs because the level and the type of fat in the diet are likely to influence pig performance (Pettigrew and Moser, 1991).

In growing pig diets based on barley, low-amylose barley cultivars with an increased starch digestion potential may be added to potentially reduce the need for exogenous NSP enzyme supplementation. Indeed, the effect of the NSP enzyme complex on animal final BW and ADG was more evident for diets based on AS variety compared with AL variety.

In countries where the climate appears to be getting warmer and drier, such as the Mediterranean, there is interest in the application of barley in non-ruminant diets (Moonen et al., 2002; Brunetti et al., 2004; Bonaccorso et al., 2005). Barley is well adapted to areas with lower rainfall and does not

require irrigation to grow. Conversely, efficient corn yields require extensive irrigation. In conclusion, hulless barley with or without the addition of the NSP enzyme complex provides a replacement for corn and wheat bran not only in growing–finishing pig diets but also in weaned pig diets. Moreover, the use of the NSP enzyme complex could improve feed efficiency of normal-amylose barley-based diets with positive effects on growth performance.

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3.2.1. *Appendix 2*

Diets for pigs based on hulles barley varieties supplemented with a non-starch polysaccharides degrading enzymes did not improve starch digestion potential *in vitro*.

This appendix contain extended version of results and additional material not included into the work of Prandini et al.: Addition of nonstarch polysaccharides degrading enzymes to two hulless barley varieties fed in diets for weaned pigs.

Materials and Methods

In vitro starch digestion procedures

Two separate evaluations were conducted to fully characterize the *in vitro* starch digestion potential of samples. In the first evaluation, starch-rich materials were characterized for their time-course starch digestion potential with an extended version (EV) of the method stated by (Englyst et al., 1992) and modified by (Sun et al., 2006). Briefly, the method consists of a two-step enzymatic hydrolysis simulating gastric and pancreatic phases. Ground samples (about 800 mg) were prepared in 50 mL tubes containing glass balls and pre-treated with a 0.05 M HCl solution (5 mL) containing pepsin (Sigma P-7000, Milan, Italy) for 30 min at 37◦C under horizontal agitation. The pH was then adjusted to 5.2 by adding 20 mL of 0.1 M sodium acetate buffer prior to the addition of 5 mL of an enzyme mixture with an amylase activity of about 7000 U/mL given by pancreatin (Merck 7130, Merck, Darmstadt, Germany), amyloglucosidase (Sigma A-7095, Sigma–Aldrich, Milan, Italy) and invertase (Sigma I-4504, Sigma–Aldrich, Milan, Italy). The incubations were carried out up to 240 min. To

fully characterize the starch digestion rate, eight incubations time were employed. In particular, aliquots (0.5 mL) were taken from each tube at 0, 15, 30, 60, 90, 120, 180 and 240 min, then absolute ethanol was added (van Kempen et al., 2010b) and the amount of released glucose was determined colorimetrically in this blend with a glucose oxidase kit (GODPOD 4058, Giesse Diagnostic, Rome, Italy). For the second evaluation, cereals were characterized as rapidly digestible starch (RDS, g/kg DM), slowly digestible starch (SDS, g/kg DM) and resistant starch (RS, g/kg DM) according to the procedure described by Englyst et al. (1996). Concisely, following a pre-incubation with pepsin–HCl solution, the various categories of starch were measured after degradation in an enzyme mixture containing, pancreatin, amyloglucosidase and invertase enzymes as described above. Glucose released from samples after 20 min and 120 min of incubation was measured colorimetrically as described above and then converted to RDS, SDS and RS indexes with the equations proposed by Englyst et al. (1992). Samples were randomized, analyzed in duplicate and in separate runs for a total of 12 runs.

Blood sampling

Blood samples were collected at the end of the trial from 6 animals per treatment, using vacutainer blood collection tubes with EDTA anticoagulant.

Denaturing gradient gel electrophoresis (DGGE)

Samples used for DGGE were collected from large intestine content at the end of the trial, as described in Bruzzese et al. (2014).

First, 50 mg of samples material was incubated at 37oC in lysozyme buffer (100 mM Tris-HCl, pH 8, 25% sucrose, 10 mM EDTA, and 10 mg/mL of lysozyme) for 1 h and then processed by the MaxwellH 16 System (Promega, Madison, WI, USA) using the MaxwellH 16 DNA purification kit. The extracted bacterial DNA was eluted in 400 mL of elution buffer and stored at 280oC. Next, for

the DNA amplification, we used the total bacterial DNA extracted from intestinal digesta was used as a template in the PCR reactions. The V2–V3 region of the 16S rRNA gene was amplified using universal primers Hda.

All DGGE analyses were performed using the INGENYphorU-2x2 system (INGENY International, Amundsenweg, Netherlands). Amplicons were analysed on 8% polyacrylamide (40% acrylamide-bis, 37.5:1) gel with a 40% to 65% denaturing gradient of urea and formamide increasing in the direction of electrophoresis. The gel was run with a constant voltage of 80 V at 60uC for 18 h in 16Tris-acetate buffer (pH 8.0).

Calculations and statistical analysis

Statistical analyses were completed with the R statistical environment (R Development Core Team, 2013). ANOVA with TukeyHSD pair-wise comparison was conducted to compare the mean values. Statistical significance was achieved at P values less than 0.05 and error is presented as ±SEM (standard error mean). Differences were considered significant level lower than 5%.

Modelling in vitro hydrolysis kinetics

Using the data obtained from the *in vitro* digestion test an *in vitro* digestion coefficient of starch for each time interval (C_t, as a percentage of TS) was calculated, by the following Equation 1.

$$C_t = 100\frac{0.9(\text{glucose present at time t - t}_0\text{ glucose release})}{\text{total starch}}$$

Equation 1. Method used to calculate an *in vitro* digestion coefficient of starch for each time interval. C_t is a percentage of total starch (TS). For quantitative determination, the factor of 0.9 was applied to convert mono- to poly-saccharide (Stevnebø et al., 2006).

For quantitative determination, starch is hydrolysed and measured as the component glucose monosaccharide units released, therefore the factor of 0.9 was applied to convert mono- to poly-saccharide (Stevnebø et al., 2006).

To investigate the kinetics of starch digestion for different particle sizes of maize we have been tested graphical representations and differential equations of several digestion models used to determine the disappearance of matter during the rumen fermentation (Tedeschi, 2008; Wang et al., 2011). Some of these nonlinear functions can be modified for *in vitro* starch degradation. We chose the Gompertz exponential nonlinear function, used to compute substrate disappearance on *in situ* experiments, given its the best fit capabilities, see Equation 2. Kinetic parameters C_o, C_{oo} and k was calculated using *nls()* function (Bates and Chambers, 1992; Bates and Watts, 1998).

$$C_t = C_0 + C_\infty e^{\left(-e^{(1-kt)}\right)}$$

Equation 2. Where C_t corresponds to the *in vitro* digestion coefficient of starch at time t, C_0 is the *in vitro* digestion coefficient at 0 min, C_∞ is the *in vitro* digestion coefficient after 480 min, k is the digestion rate (/min) and t is the chosen time (min).

Areas under the digestion curves (AUC) were calculated by AUC function, using MIfuns package (Knebel et al., 2008) in R. Hydrolysis index (HI), was obtained from the ratio between the AUC of each diet and the reference AUC 44500.35 of white bread and expressed as its percentage (Goñi et al., 1997), Equation 3.

$$HI[\%] = \frac{\text{AUC}}{44500.35} 100$$

Equation 3. Hydrolysis index (HI). Where AUC is area under the digestion curve and 44500.35 the reference AUC of white bread.

From the HI obtained *in vitro*, a predicted glycaemic index (pGI) was calculated for each sample using the equation reported by Giuberti et al. (2012), see Equation 4.

$$pGI = 1.013 \cdot HI$$

Equation 4. Predicted glycemic index (pGI).

DGGE image analysis

The DGGE profiles were analysed using the Phoretix1D v11.5 software (TotalLab Ltd, UK). After detecting bands and their peak intensities a dendrogram was built to show in a hierarchical manner, the similarities of the lanes matched to the obtained synthetic lane. Neighbour joining dendrogram was constructed according to Saitou and Nei (1987).

Results and Discussion

Our study was conducted to evaluate the effect of two hulless barley varieties, with or without the addition of a non-starch polysaccharide (NSP) enzyme complex (β-glucanase and xylanase), using *in vivo* and *in vitro* methods. Several studies on post-weaning or growing pigs reported that exogenous enzyme supplementation increases both ileal nutrient digestibility and growth performance (Graham et al., 2013; Li et al., 1994; Nortey et al., 2008, 2007; O'Connell et al., 2006; Omogbenigun et al., 2004; Smith et al., 2010; Yin et al., 2001). The above results suggest that the better swine performance have been mostly linked with the improved nutrient utilization as a result of nutrient releasing after fibrous structure breach. Similar, in our *in vivo* trial we showed that the use of the NSP enzyme complex could improve feed efficiency of normal-amylose barley-based diets with positive effects on growth performance.

We calculated *in vitro* parameters of starch degradation together with three fractions of starch: RDS, SDS and RS, see Table 1. Differences were found between diets in RDS and RS fractions of starch,

but not in SDS. Control feed had the lowest content of RDS and the highest of RS ($P<0.05$). Both Alamo based treatments had the highest levels of RDS. All four Barley based feeds share the same significance level regarding RS content, which was lower than in Control ($P<0.05$). The kinetic time course of the digestion shows very different rates between stages, suggesting changes in the physical nature of the substrate. From previous studies of *in vitro* digestion it is well known that by reducing sugar production from RDS to SDS or by increasing RS fraction we will observe a transition in the smoothness of the progress curves. Statistical analysis reviled no differences in amount of starch digested at 0 min (C_0) and potential digestibility of starch (C_∞), between experimental treatments. However, we found significant differences ($P<0.001$) among diets in rate of starch digestion (K). Alamo treatments had higher rate than Astartis ($P<0.05$), additionally diets supplemented with enzyme had the same rate as these without. Corn based Control had the lowest K index ($P<0.05$). *In vitro* enzyme incubation method might be practical for predicting the responses of pigs to exogenous enzymes, as was showed in previous experiments of Fang et al. (2007) and Li et al. (2004), hence identifying those preparations that possess potential for improvement of the nutritive values of feedstuffs. Superior performance of animals on barley feed was partly in line with our *in vitro* results, as barley diets had much better kinetic parameters compare to corn-based Control. However, in contrast to *in vivo* trial, we did not notice improved kinetic in diets supplemented with the NSP enzyme complex, which improved growth performance of weanling pigs fed AST variety, but not ALA variety. What is more, significant differences were established between diets ($P<0.05$) for hydrolysis index (HI) and predicted glycaemic index (pGI), where Alamo diet with enzyme formula had higher levels (97.41 and 98.68, respectively) than Control. In the study of Bindelle et al. (2011) the addition of the NSP-degrading enzymes (xylanase and β-glucanase) showed a positive influence on the pepsin-pancreatin hydrolysis and the subsequent fermentation profiles with pig faeces, in contrary to Garry et al. (2007) and Pauly et al. (2011), showed lack of response to enzyme supplementation.

	RDS (TS%)		SDS (TS%)	RS (%TS)		C_0 (g/100 g)	C_∞ (g/100 g)	K (/min)		HI (%)		pGI	
CTR	20.66	b	52.03	27.31	b	1.07	96.3	0.0114	c	84.56	b	85.66	b
AST	26.95	ab	59.57	13.48	a	-1.56	103.05	0.0163	a	94.29	ab	95.51	ab
AST+E	28.1	a	59.06	12.84	a	-0.94	101.66	0.0168	a	94.16	ab	95.39	ab
ALA	37.02	c	55.06	7.92	a	0.96	97.3	0.0233	b	95.52	ab	96.76	ab
ALA+E	37.31	c	56.42	6.27	a	0.67	99.4	0.023	b	97.41	a	98.68	a
P-value	<0.001		0.26	<0.001		NS	NS	<0.001		<0.05		<0.05	
SEM	1.38		2.47	2.27		1.66	4.16	9E-04		2.46		2.50	

Table 1

Digestion coefficients and characterising the *in vitro* enzymatic starch digestion at time of incubation 480 min, together with and starch three fractions RDS, SDS and RS.

Abbrevations: C_0, Starch digested at 0 min; C_∞ , Potential digestibility of starch. K, Rate of starch digestion; TS, Total Starch; HI, hydrolysis index; pGI, predicted glycaemic index; SEM, standard error of mean; NS, not significant.

Means sharing the same letter in given column are not significantly different, at the chosen level (5%).

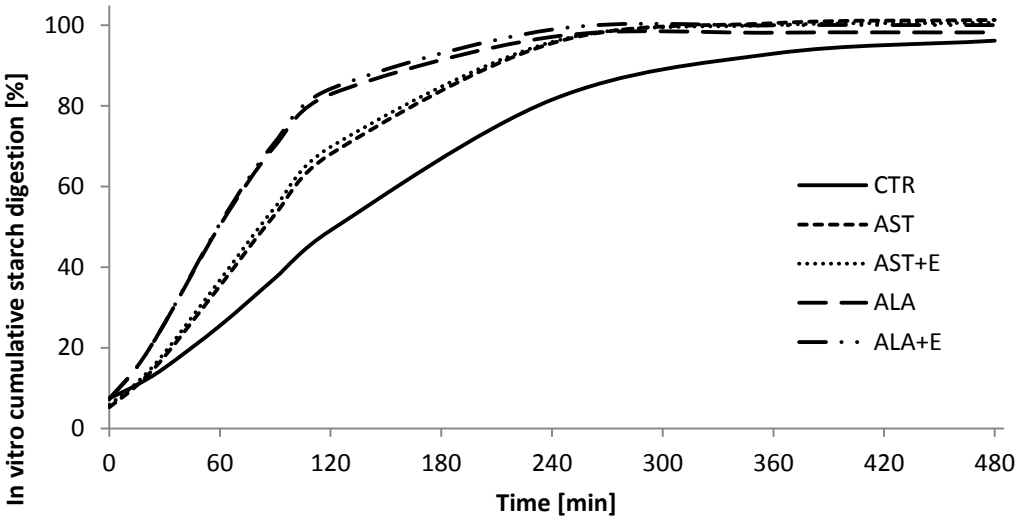

Figure 1. Time course of average cumulative *in vitro* starch digestion (as a fraction of total starch) of five experimental diets: CTR - control corn based diet; AST - diet with Astartis barley variety; AST+E - diet with Astartis barley variety and supplemented with NSP enzyme complex; ALA - diet with Alamo barley variety; ALA+E - diet with Alamo barley variety and supplemented with NSP enzyme complex.

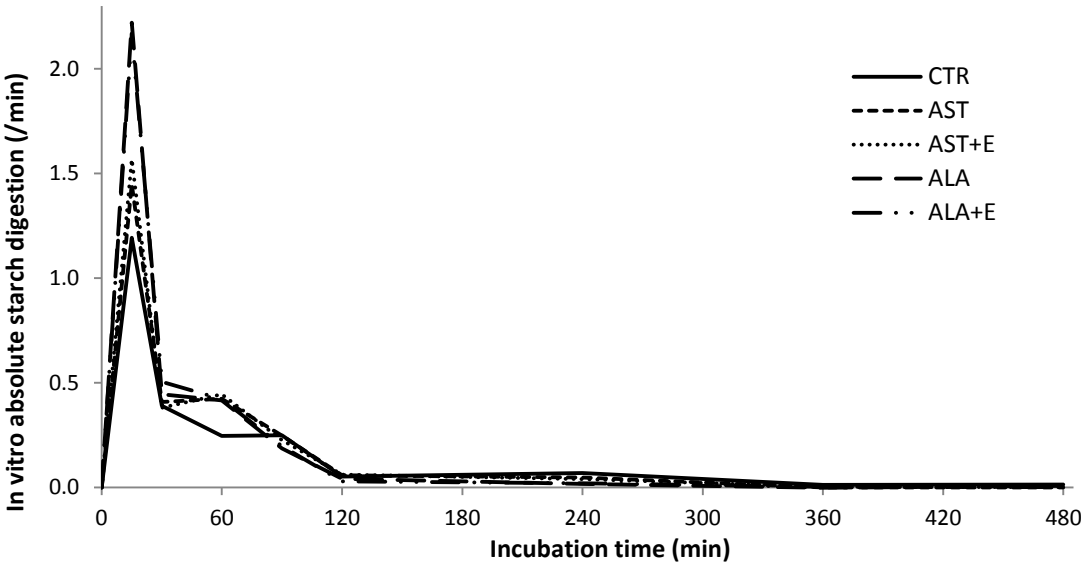

Figure 2. Time course of average absolute *in vitro* starch digestion (as a fraction of total starch/min) of five experimental diets: CTR - control corn based diet; AST - diet with Astartis barley variety; AST+E - diet with Astartis barley variety and supplemented with NSP enzyme complex; ALA - diet with Alamo barley variety; ALA+E - diet with Alamo barley variety and supplemented with NSP enzyme complex. Values were calculated by subtracting the proportion of digested starch at each time point from the value at the next time point for each sample.

Average cumulative (Figure 1) and absolute curves (Figure 2) of time course starch digestion release (given as a fraction of total starch digested after 9 different incubation times) indicated some differences among samples ($P<0.05$). After two hours of process the TS was digested in only 49% regarding Control, when Astartis and Alamo based feeds had around 68% and 83% respectively. Our analysis showed no differences between barley based diets with or without NSP enzyme complex supplementation. The digestion rate (K) was in general the highest during first 15 min and then tended to decrease (Figure 2). Similar to the previous results, also here Control had the lowest rate and both Alamo feeds the highest rate ($P<0.05$).

Metabolic profile of blood serum

Statistical analysis with one-way ANOVA did not record any differences between five feeds among: Proteins, Albumin, Globulin, Urea, ALT-GPT, AST-GOT, ALP, Glucose, Cholesterol, Calcium, Phosphorus and Magnesium, see Table 2ab. Albumin/Globulin ratio had a tendency to be different among diets, but TukeyHSD comparison did not show any significant changes. Total blood bilirubin level was different between feeds ($P<0.001$), where CTR and ALA+E had the lowest level and AST the highest. Animals on diets supplemented with enzyme tended to have a lower bilirubin level respect to ALA and AST feeds. Moreover, results obtained during our earlier experiment (see Appendix 1) on hulled or hulless barley varieties, where both Astartis and Alamo have a similar bilirubin levels as Control, are not in line with our recent outcomes.

	Proteins Tot. [g/l]	Albumin [g/l]	Globulin [g/l]	Alb/Glob [ratio]		Urea [mmol/l]	ALT-GPT [IU/l]	AST-GOT [IU/l]	ALP [IU/l]	Bilirubin Tot. [µmol/l]	
CTR	53.00	25.83	26.60	1.00	a	4.69	39.83	44.60	120.60	2.03	b
AST	55.00	27.57	28.17	1.03	a	4.63	42.43	52.33	131.29	3.26	a
AST+E	55.00	28.29	28.14	0.97	a	4.02	43.71	49.29	136.86	2.66	ab
ALA	51.86	25.38	27.88	0.87	a	4.25	43.75	46.50	113.38	2.97	ab
ALA+E	51.57	25.88	27.50	0.80	a	4.02	43.13	49.86	137.38	1.99	b
p-value	NS	NS	NS	0.05	•	NS	NS	NS	NS	<0.001	**
sem	1.30	1.19	1.69	0.06		0.45	4.47	4.75	13.53	0.27	

Table 2a

	Glucose [mmol/l]	Cholesterol [mmol/l]	Calcium [mmol/l]	Phosphorus [mmol/l]	Magnesium [mmol/l]
CTR	5.14	2.32	2.58	3.00	0.83
AST	5.68	2.53	2.57	2.78	0.84
AST+E	5.81	2.38	2.49	2.97	0.89
ALA	5.26	2.13	2.52	2.99	0.84
ALA+E	5.77	2.32	2.51	2.90	0.81
p-value	NS	NS	NS	NS	NS
sem	0.32	0.13	0.04	0.08	0.03

Table 2b

Blood content of pigs in relation to the diet type.

Means sharing the same letter in given column are not significantly different, at the chosen level (5%).

Abbrevations: CTR - control corn based diet; AST - diet with Astartis barley variety; AST+E - diet with Astartis barley variety and supplemented with NSP enzyme complex; ALA - diet with Alamo barley variety; ALA+E - diet with Alamo barley variety and supplemented with NSP enzyme complex; SEM, standard error of mean; NS, not significant.

DGGE

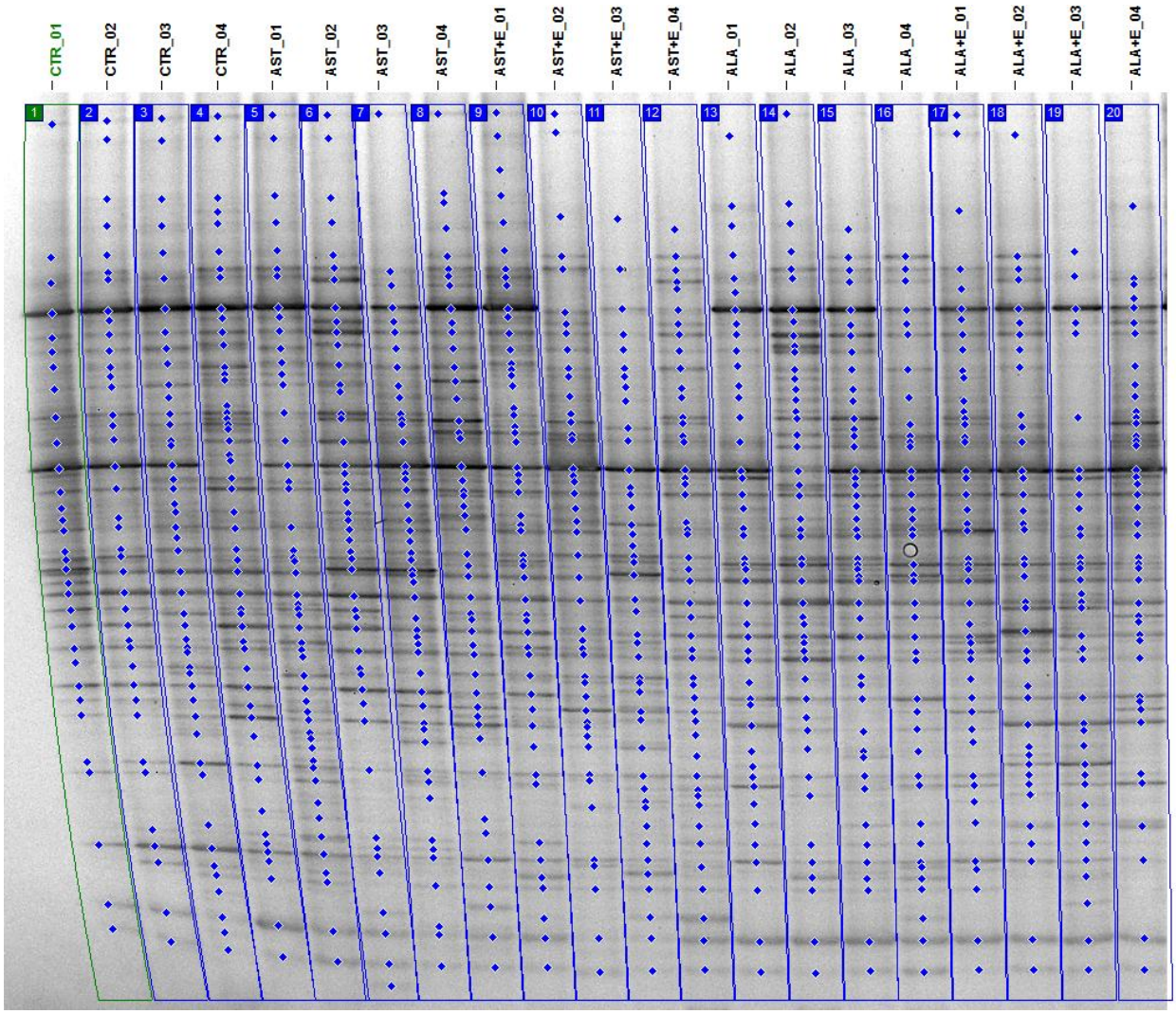

Figure 3
Polyacrylamide gels showing rRNA gene amplicon profiles obtained by denaturing gel electrophoresis of bacterial samples taken from porcine intestine. Lane labels indicate diet type and replicate number. Blue points on the line indicate detected bands. Control (CTR) was a corn based diet; AST - based on Astartis barley variety; AST+E - diet Astartis supplemented with exogenous enzyme mix; ALA - based on Alamo barley variety; ALA+E - diet Alamo supplemented with exogenous enzyme mix.

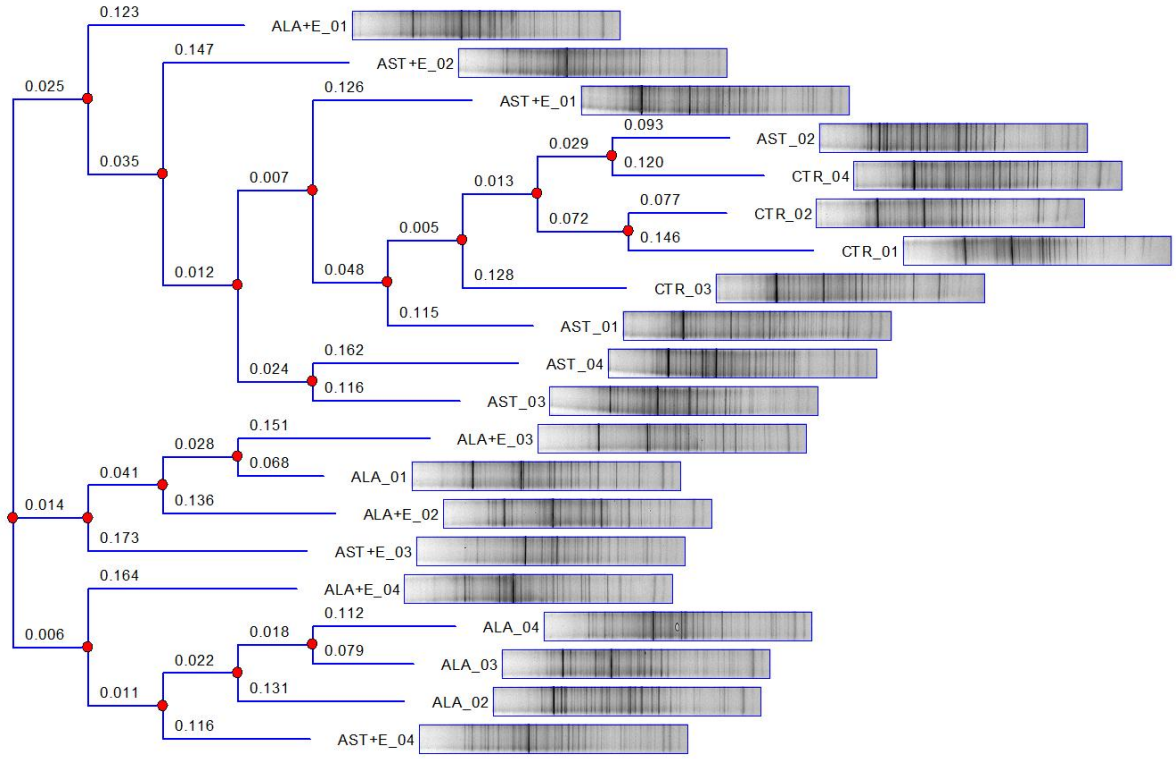

Figure 4
Dendrogram of bacterial DGGE band patterns derived from samples taken from porcine intestine. Lane labels indicate diet type and replicate number. Hierarchical cluster analysis was performed the Phoretix1D v11.5 software (TotalLab Ltd, UK). and distances were calculated from band presence–absence with peak intensities data.

Presence of fibrous substrates reduced feed passage rate and increases the time available for intestinal microorganisms to multiply during fermentation. However, the addition of a exogenous enzyme to feed could reduce total bacterial numbers by 60 % and also reduce the proportion of bacteria with low content of guanidine:cytosine in the total DNA pool (Bedford and Schulze, 1998). In our experiment we analysed samples taken from porcine intestine with DGGE (Figure 3). Hierarchical cluster analysis divided gel lines into three main clusters (Figure 4). All four Control and four out of five Astartis samples were found to be quite close each other. On the other hand samples from animals fed diet supplemented with enzyme are spread among clusters. Previous study Bindelle et al. (2011) implies that NSP-degrading enzymes can affect the nutrient composition entering the large intestine and subsequently alter the bacterial composition. Nevertheless Murphy et al. (2012) showed lowered

microflora and (Reilly et al., 2010) no effect on bacteria population after enzyme inclusion into swine diet.

Concluding, diets for pigs based on hulles barley varieties supplemented with a non-starch polysaccharides degrading enzyme complex did not improve *in vitro* starch digestion potential. We observed positive effects of hulles barley on digestion kinetics compare to corn-based diet. Moreover, minor shift in bacteria patterns were noticed.

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3.3. Inclusion of pasta by-products into diet improve starch degradation, fermentation kinetics and lower production of volatile fatty acids in an in vitro model of the porcine gastrointestinal tract

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Abstract

Feed costs represents around 65 - 75 percent of the variable costs of pork production. Bakery by-products are one of the common alternative feedstuff in animal nutrition, being also a possible way to reduce production costs. They are great ingredients in swine diets due to their comparatively high energy content, which is also unfortunately quite variable, thus need to be under continuous monitoring of their nutritional composition and quality. To complement information gap regarding use of bakery by-products a trial was performed to investigate animal performance, and also an *in vitro* methods were used to facilitate the control of quality and digestibility. Here we examined how wheat pasta by-product supplemented to swine feed in 30%, 60% and 80% affect performance, *in vitro* starch digestion, *in vitro* fermentation and haematological profile. 144 female and castrated male pigs of initial weight of 102.9±3.31 Kg have been subjected into four treatments. Diets have been formulated together with the pasta factory, which provided by-products collected during pasta production, according to actual legislations for animals nutrition and treatment. Control diet was based on corn flour (60%) and barley flour (15%). Remaining three diets D30, D60 and D80 were based on pasta by-products (30%, 60% and 80% respectively), in addition D30 contained 30% of corn flour and 15% of barley flour, and D60 15% of barley flour. Diet composition affected ADFI

(P<0.05), where Control had the lowest (2.87) and D80 the highest index (3.04), but the final weight was similar. D80 had slightly better kinetic during the *in vitro* enzymatic starch digestion compare with CTR (P=0.06). As suspected, addition of pasta by-products lowered production of VFA during *in vitro* fermentation of D80 (P<0.01), but increased gas and NH_3 production (P<0.01). Our results indicate that the introduction of pasta by-products into swine feed means possible reduction of production cost without lowering animal production efficiency, yet high share of pasta may increase odour emission.

Key words: bakery by-products; pasta; in-vitro model; pig;

Introduction

Bakery by-products are one of the common alternative feedstuff in animal nutrition, being also a possible way to reduce production costs (NPB, 2008). Still, only few studies have investigated inclusion of by-products or waste materials originating from food industries (Kwak & Kang, 2006; Slominski, Boros, Campbell, Guenter, & Jones, 2004; Williams, Belyea, Hsieh, & Firman, 1998). Bakery products are great ingredients in swine or poultry diets because of their comparatively high energy content coming from starch, sugars and fat. Pasta based on durum wheat is rich in starch (about 70% DM) and protein content (14-15% DM). What is more, bakery products contain usually high amounts of sugar, and can therefore animals received a pleasant tasting feed (Peter R. Shewry, Tatham, & Bry, 2000). High energy and nutrients content require less special supplements and can easily replace corn or barley (P R Shewry, 2009). However, because of differences in the processing or storage techniques and possible mix of wheat varieties, chemical composition of bakery by-products could be highly variable (Huang et al., 2014; Slominski et al., 2004). Therefore, alternative feedstuff such as wheat pasta by-products need to be under continuous monitoring of their nutritional composition. To fill up information gap regarding use of bakery by-products a trial was performed to

investigate animal performance, and to facilitate the quality control of feed by-products we propose use of *in vitro* procedures. In swine nutrition, *in vitro* methods, recommended in the last decade, mimic the digestion processes occurring in the gastro-intestinal tract, and they have been shown to accurately and precisely estimate the metabolic response of starch digestion (Giuberti, Gallo, Cerioli, & Masoero, 2012). Here we examined how wheat pasta by-product supplemented to swine feed in 30%, 60% and 80% affect *in vitro* starch digestion, *in vitro* fermentation and animals blood profile. We hypothesize that pigs on diet rich in pasta by-products will perform better and *in vitro* starch degradation will proceed faster in experimental diets supplemented with 30%, 60% and 80% of wheat pasta by-product compare to corn-base Control, but in opposite to *in vitro* fermentation kinetics. Additionally, we expect minor hematologic responses, especially in proteins, urea and glucose levels.

Materials and methods

Animal and diets

144 female and castrated male pigs of initial weight of 102.9±3.31 Kg have been subjected into four treatments. Diets have been formulated together with the pasta factory, which provided by-products collected during pasta production, according to actual legislations for animals nutrition and treatment (Table 1). Control diet was based on corn flour (60%) and barley flour (15%). Remaining three diets D30, D60 and D80 were based on pasta by-products (30%, 60% and 80% respectively), in addition D30 contained 30% of corn flour and 15% of barley flour, and D60 15% of barley flour. Water was provided *ad libitum.*

Table 1
Diets composition.

Item (%)	Control	D30	D60	D80
Corn flour	60.000	30.000	-	-
Pasta by-product	-	30.000	60.000	79.991
Barley flour	14.992	14.995	15.000	-
Soy protein extract	11.767	7.767	3.547	3.580
Wheat bran	5.000	11.473	14.994	6.120
Wheat middling	3.343	-	0.143	-
Beetroot dried pulps	2.000	3.000	4.000	4.000
Soybean hulls	-	-	-	4.000
Calcium carbonate	0.980	0.983	0.930	0.807
Bovine tallow	0.633	0.500	-	-
Sodium chloride	0.500	0.372	0.350	0.350
Bi-calcium phosphate 12/24	0.250	0.250	0.250	0.360
Premix [1]	0.200	0.200	0.200	0.200
Lysine HCl	0.172	0.172	0.327	0.335
Finase 500	0.100	0.100	0.100	0.100
Belfeed B 220	0.050	0.050	0.050	0.050
Threonine L	0.012	0.050	0.088	0.087
Methionine DL	-	0.010	0.021	0.020

[1] Premix vitamins and minerals (Fermix – Adisseo, Modena)

Chemical analysis

The proximate composition of each diet was determined according to AOAC, 2000 standards: dry matter content (Method 930.15), protein (Method 990.03), fat (Method 920.39), crude lipid (Method 954.02 without acid hydrolysis) and ash content (Method 942.05). Total starch (TS) content was determined by an enzymatic method (Blasel, Hoffman, & Shaver, 2006) modified according to the procedure described by Masoero et al. (2010). Chemical content of analysed diet are showed in Table 3 and Table 4.

In vitro starch degradation

Starch digestibility of each diet sample was evaluated based on two *in vitro* experiments. First testing was performed to characterize three fractions of starch according to the procedure defined by Englyst, et al., 1996, these are digestible starch (RDS), slowly digestible starch (SDS) and resistant starch (RS). Samples (800 mg) were pre-incubated with 5 ml of pepsin–HCl solution (0.05 M HCl and pepsin Sigma–Aldrich P-7000, 5 mg/ml) in 50 ml tubes containing glass balls for 30 min at 37^{o}C

under horizontal agitation. Next, 20 ml of 0.1 M sodium acetate buffer was added to get pH level of 5.2. Incubation was initiated by addition of 5 ml volume of an enzyme mixture composed by: pancreatin (Merck 7130, Merck KGaA), amyloglucosidase (Sigma A-7095, Sigma–Aldrich) and invertase (Sigma I-4504, Sigma–Aldrich). After 20 and 120 minutes of incubation solution samples (0.5 ml) were collected, and have been measured colorimetrically for glucose presence, using GODPOD (Giesse Diagnostic). Data were then converted using equations developed also by Englyst, Kingman, & Cummings (1992) into RDS, SDS and RS values. Additionally, a sample contain Gelose 80 maize starch (Penford Food Ingredients Co.) was used along as a standard and quality control.

Second, in vitro hydrolysis experiment stimulating gastric and pancreatic phases, was carried out using similar protocol with pre-incubation in pepsin–HCl solution and incubation in enzyme mix up to 480 minutes. To describe starch digestion efficiency, aliquots of digestive solution were taken from each tube at nine times 0, 15, 30, 60, 90, 120, 240, 360, 480 min, and released glucose was determined colorimetrically as described above.

In vitro fermentation

For purpose of *in vitro* fermentation, samples had undergone an *in vitro* enzymatic hydrolysis using pepsin and pancreatin according to protocol described in Boisen and Fernhdez (1997). Samples were passed through a paper filter, next washed twice first with 96% ethanol and once with 99.5% acetone, then dried at 60 °C overnight. The procedure has to be repeated several times in order to obtain enough material for the subsequent *in vitro* fermentation procedure.

The *in vitro* fermentation simulates the fermentation occurring in the hindgut, was performed following the protocol described by Bindelle et al. (2007). Gas accumulation measurements give useful information about the kinetics of digestion process. 200 mg of hydrolysed material were incubated in a 100 ml glass syringes at 39 ^{o}C with 30 ml of inoculum. A faecal inoculum was prepared using fresh faeces of three pigs, from the herd of the CERZOO (Centro di ricerche per la

Zootecnia e l'ambiente, Piacenza) that were fed a antibiotics free diet, mixed to a buffer solution containing macro- and micro-minerals (Menke et al., 1979). The gas generated by the fermentation process were measured at 0, 0.5, 1, 1.5, 2, 3, 4, 6, 8, 24, 30, 36 and 42 hour. Fermentation was stopped at 42nd hour of incubation by setting the bottles in boiled water. Samples were collected from the syringes for measurement of VFAs, as well as samples of the inoculum prior to fermentation.

Volatile fatty acids analysis

Samples from *in vitro* fermentation, were measured for the concentration of volatile fatty acids (VFA). VFA concentrations was determined using gas chromatography (GC) (Varian 3350) according to the method described by Fussell and McCalley (1987).

Blood sampling

Blood samples were collected at the end of the trial from 8 animals per treatment, using vacutainer blood collection tubes with EDTA anticoagulant.

Statistics

Statistical analyses were completed with the R statistical environment (R Development Core Team, 2013). Collected data have been ultimately expressed as means, analysed by one-way ANOVA followed by Dunnet's test for comparison between control (CTR) and treatment groups (D30, D60, D80). The level of significance was set at $P \leq 0.05$ and error is presented as ±SEM (standard error mean).

Modelling in vitro hydrolysis kinetics

Using the data obtained from the *in vitro* digestion test an *in vitro* digestion coefficient of starch for each time interval (C_t, as a percentage of TS) was calculated using Equation 1.

Equation 1
Digestion coefficient of starch for each time interval (C_t , as a percentage of total starch).

$$C_t = 100\frac{0.9(\text{glucose present at time t - } t_0 \text{ glucose release})}{\text{total starch}}$$

For quantitative determination, starch is hydrolysed and measured as the component glucose monosaccharide units released, therefore the factor of 0.9 was applied to convert mono- to poly-saccharide (Stevnebø, Sahlström, & Svihus, 2006).

To investigate the kinetics of starch digestion we used the Gompertz exponential nonlinear function, used to compute substrate disappearance on *in situ* experiments (Equation 2).

Equation 2
Gompertz exponential nonlinear function describing kinetics of starch digestion.

$$C_t = C_0 + C_\infty e^{\left(-e^{(1-kt)}\right)}$$

Where C_t corresponds to the *in vitro* digestion coefficient of starch at time t, C_0 is the *in vitro* digestion coefficient at 0 min, C_∞ is the *in vitro* digestion coefficient after 480 min, k is the digestion rate (/min) and t is the chosen time (min).

Kinetic parameters C_o, C_{oo} and k was calculated using *nls()* function (Bates & Chambers, 1992; Bates & Watts, 1998). The area under the *in vitro* digestion curves (AUC) was calculated by *AUC* function, in MIfuns package (Knebel et al., 2008), using trapezoidal rule. Hydrolysis index (HI), was obtained from the ratio between the AUC of each diet and the reference AUC (Equation 3). HI expressed as a percentage (Giuberti et al., 2012; Goñi, Garcia-Alonso, & Saura-Calixto, 1997).

Equation 3
Hydrolysis index (HI)

$$HI[\%] = \frac{\text{AUC}}{44500.35}100$$

Where, 44500.35 is an area under the curve (AUC) of white bread, as standard to calculate hydrolysis index (HI).

Kinetics of gas production

Gas accumulation was modeled according to LAG exponential model with discrete LAG time (Schofield et al.,1994) and described with the following parameters: V_f, final asymptotic gas volume; k, fractional rate of gas production; LAG, discrete lag phase; T/2, half-time to asymptote. Kinetic parameters was calculated using *nls()* function (Bates & Chambers, 1992; Bates & Watts, 1998) in R (Equation 4).

Equation 4
LAG exponential model with discrete LAG time for kinetics of *in vitro* gas production.

$$V = V_f\left(1 - e^{\left(-k(t-LAG)\right)}\right)$$

Where $t/2 = LAG + \ln(0.5/k)$ and $t \geq LAG$
Abbrevations: V_f, final asymptotic gas volume; k, fractional rate of gas production; LAG, discrete lag phase; t, time; t/2, half-time to asymptote.

Results and discussion

During trial we measured animal performance (Table 3). We found average daily gain (ADG) to be the same across diets. Nevertheless, diet composition affected ($P<0.05$) average daily feed intake (ADFI), where Control had the lowest (2.87) and D80 the highest (3.04). Pigs fed diet supplemented with pasta by-products consumed more feed compare to Control animals, but finally the performance was the same. In this way the incorporation of by-products means possible reduction of production cost without lowering production efficiency. Study of Slominski et al., (2004) support a thesis for the effective use of bakery by-products in the dietary formulation for poultry and swine, although rigorous quality control is necessary due to high nutrients variability. Another study performed on turkeys (Williams et al., 1998) indicated pasta as a good energy source increasing gain. Feeds have been examined in the content of dry matter (DM), proteins, lipids, fat, ash and total starch with its fractions (Table 3). Diets with 60% and 80% of wheat pasta by-products were significantly higher

DM and protein content than Control (P<0.05). Lipid content was lower in all three wheat feeds respect control (P<0.05). D80 had the lowest ash content (P<0.001). Total starch and RDS fraction content was the lowest in Control and gradually increase (P<0.001) together with wheat inclusion (Table 4). Slowly digested starch fraction was the highest in Control and had a tendency do decrease (P<0.001) together with wheat inclusion. Treatments containing pasta by-product were not different than Control regarding RS content (Table 4). Our analysis of time course *in vitro* starch digestion (Figure 1) showed the highest progress in D60 and D80 diets (P<0.05) until 90 minutes time point, when Control was the lowest one. Similar relationship was found in average absolute *in vitro* starch digestion (Figure 2) where the Control diet was the slowest compare to D60 and D80 (P<0.05). Additionally, the highest rate was observed after 15 minutes of digestion. Digestion coefficient C_0 was inversely correlated to the wheat content (P<0.001) and is the lowest in D80, but we found no significant differences in C_∞ (Table 5). Feed with 80% of wheat pasta by-product had the highest hydrolysis index and tended to for the highest rate k compare to Control, in Dunnet's test. Slowly digestible starch (SDS), such as native maize starch, has moderate influence on postprandial increase blood glucose levels, compared to RDS with its fast and high peak and fast decline (Lehmann & Robin, 2007). Moreover, heat processed wheat have a higher HI (76.1) than unprocessed corn (39) (Giuberti et al., 2012). Our results confirmed that higher percentage of easily digestible dietary starch (RDS and SDS) has a significant impact on cumulative *in vitro* starch digestion and its rate.

Table 2
Growth performance.

	Sex											
	Castrated males				Females					Main effects		
	Diets											
	CTR	D30	D60	D80	CTR	D30	D60	D80	SE	Pasta by-products	Sex	Pasta by-products*sex
Initial BW, kg	102	102	102	104	103	104	104	104	2.2	NS	NS	NS
Final BW, kg	165	166	165	170	167	165	168	170	2.3	NS	NS	NS
ADG, kg	0.70	0.71	0.69	0.73	0.71	0.67	0.70	0.73	0.021	NS	NS	NS
ADFI, kg	2.87	2.90	3.00	3.03	2.87	2.88	3.02	3.06	0.022	<0.05	NS	NS
F:G	4.16	4.10	4.35	4.16	4.06	4.31	4.31	4.18	0.137	NS	NS	NS

Marginal means of pasta by-products

ADFI: CTR, 2.87; P30, 2.90; P60, 3.01; P80, 3.04; P<0.05, linear effect.

Abbreviations: BW, Body Weight; ADG, Average Daily Gain; ADFI, Average Daily Feed Intake; SE, standard error; NS, not significant;

Table 3
Chemical composition of the examined feeds (CTR – corn based Control diet; D30, D60 and D80 – contains 30%, 60% and 80% of pasta by-product).

Diet	**DM (%)**		**Proteins (% DM)**		**Lipids (% DM)**		**Fat (% DM)**	**Ash (% DM)**	
CTR	89.21	-	13.33	-	3.54	-	3.27	3.59	-
D30	89.93		13.69		2.93	*	3.31	3.56	
D60	90.55	*	13.80	*	1.62	***	3.37	3.54	
D80	90.51	*	13.83	*	1.20	***	3.49	3.27	***
P-value	0.043		0.040		<0.001		NS	<0.001	
SEM	0.33		0.12		0.13		0.18	0.02	

Data expressed as mean (n=4), analysed by one-way ANOVA followed by Dunnet's test for comparison between control and treatment groups. The level of significance was set at P≤0.05.

Abbreviations: SEM, standard error of mean; DM, dry matter; NS, not significant; •, tendency (0.05<P<0.1); *, P<0.05; **, P<0.01; ***, P<0.001.

Table 4
Total starch (TS) content and three starch fractions RDS (rapidly digested starch), SDS (slowly digested starch) and RS (resistant starch) of experimental feeds (CTR – corn based Control diet; D30, D60 and D80 – contains 30%, 60% and 80% of pasta by-product).

Diet	TS (% DM)		RDS (% TS)		SDS (% TS)		RS (% TS)
CTR	55.87	-	48.51	-	45.04	-	6.45
D30	58.07	•	68.02	•	26.69	*	5.29
D60	59.69	**	87.18	**	10.88	***	1.94
D80	60.72	***	91.17	***	8.46	***	0.37
P-value	<0.001		<0.001		<0.001		NS
SEM	0.64		5.70		4.29		2.06

Data expressed as mean (n=3), analysed by one-way ANOVA followed by Dunnet's test for comparison between control and treatment groups. The level of significance was set at P≤0.05.
Abbreviations: TS, total starch; DM, dry matter; SEM, standard error of mean; NS, not significant; •, tendency (0.05<P<0.1); *, P<0.05; **, P<0.01; ***, P<0.001.

Figure 1
Time course of average cumulative *in vitro* starch digestion (as a fraction of total starch) of the examined feeds (CTR – corn based Control diet; D30, D60 and D80 – contains 30%, 60% and 80% of pasta by-product).

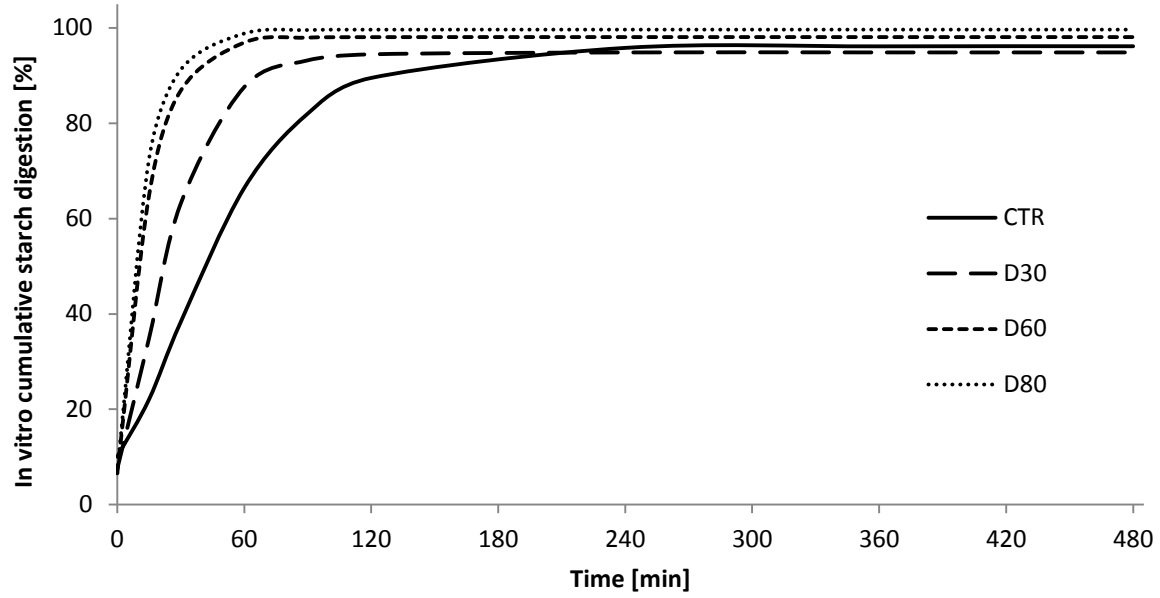
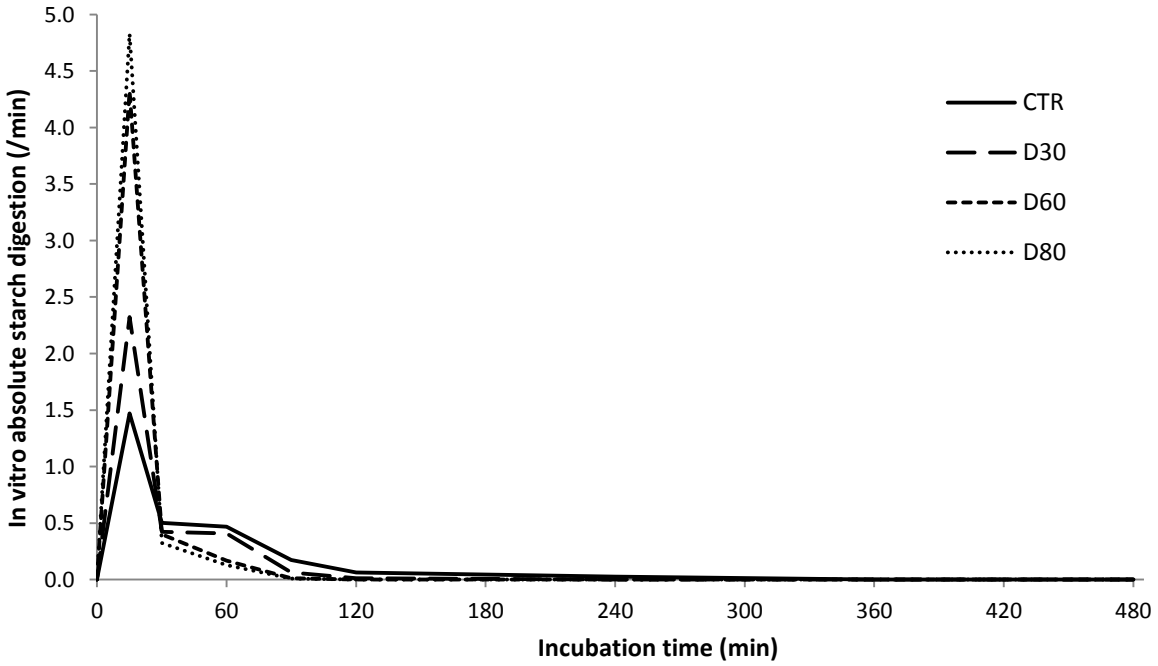


Figure 2
Time course of average absolute in vitro starch digestion of the examined feeds (CTR – corn based Control diet; D30, D60 and D80 – contains 30%, 60% and 80% of pasta by-product).



Results were obtained by subtracting the proportion of digested starch at each time point from the value at the next time point for each sample.

Table 5
Digestion coefficients and hydrolysis index (HI) characterising the *in vitro* enzymatic starch digestion of the examined feeds (CTR – corn based Control diet; D30, D60 and D80 – contains 30%, 60% and 80% of pasta by-product), at time of incubation 480 min.

Diet	C_0 (g/100 g dry starch)		C_∞ (g/100 g dry starch)	K (/min)		HI (%)	
CTR	4.01	-	92.15	0.03	-	97.10	-
D30	1.31	*	93.55	0.06		99.48	
D60	0.10	**	97.98	0.15		103.68	
D80	0.07	***	99.57	0.23	•	105.33	*
P-value	<0.001		0.076	NS		0.07	
sem	0.57		2.06	0.06		2.15	

Data expressed as mean (n=3), analysed by one-way ANOVA followed by Dunnet's test for comparison between control and treatment groups. The level of significance was set at P≤0.05.
Abbreviations: SEM, standard error of mean; NS, not significant; •, tendency (0.05<P<0.1); *, P<0.05; **, P<0.01; ***, P<0.001.

Table 6
Parameters characterizing the *in vitro* fermentation kinetic of the examined feeds (CTR – corn based Control diet; D30, D60 and D80 – contains 30%, 60% and 80% of pasta by-product), at incubation time of 36 hours.

Diet	V_f		LAG		k		T/2	
CTR	191.61	-	0	-	0.20	-	3.50	-
D30	184.79		0.06	***	0.19		3.70	
D60	183.45		0.13	***	0.17	**	3.96	.
D80	213.24	**	0.17	***	0.15	***	4.44	***
P-value	<0.001		<0.001		<0.001		<0.001	
SEM	3.23		0.02		0.01		0.12	

Data expressed as mean (n=2), analysed by one-way ANOVA followed by Dunnet's test for comparison between control and treatment groups. The level of significance was set at P≤0.05.
Abbreviations: V_f, maximum gas volume; LAG, discrete LAG time; k, fractional rate of gas production; T/2, half-time to asymptote; SEM, standard error of mean; NS, not significant; •, tendency (0.05<P<0.1); *, P<0.05; **, P<0.01; ***, P<0.001.

Table 7
Production of volatile fatty acids (VFAs) and ammonia (NH_3) during the *in vitro* fermentation of the examined feeds (CTR – corn based Control diet; D30, D60 and D80 – contains 30%, 60% and 80% of pasta by-product), at incubation time of 36 hours.

Diet	Acetic (mmol/g OM)		Propionic (mmol/g OM)		Butyric (mmol/g OM)		Valerianic (mmol/g OM)		BCFA (mmol/g OM)		TOTAL (mmol/g OM)		NH_3 (mg/100ml)	
Ctr	5.89		1.51		0.75		0.19		0.49		8.84		1.75	-
D30	5.33		1.20		0.62		0.17		0.49		7.81		2.42	
D60	4.47	•	1.08	•	0.39	*	0.16		0.47		6.56	•	2.15	
D80	2.70	***	0.70	**	0.23	**	0.10	**	0.31	*	4.04	***	2.78	*
P-value	<0.001		0.011		0.003		0.015		0.027		0.002		0.059	
SEM	0.44		0.15		0.09		0.02		0.04		0.70		0.24	

Data expressed as mean (), analysed by one-way ANOVA followed by Dunnet's test for comparison between control and treatment groups. The level of significance was set at P≤0.05.
Abbreviations: OM, organic matter; BCFA, branched-chain fatty acid (sum of iso-butyric and iso-valeric acid); VFA, volatile fatty acid; SEM, standard error of mean; NS, not significant; •, tendency (0.05<P<0.1); *, P<0.05; **, P<0.01; ***, P<0.001.

Statistical analysis with one-way Anova reviled significant differences between diets ($P<0.001$) for parameters characterizing the *in vitro* fermentation kinetic such as V_f, LAG, k, T/2 (Table 6). Further investigation with Dunnet's test showed that maximum gas volume of D80 was higher than Control ($P<0.01$). Control LAG time was lower respect treatments with wheat ($P<0.001$). Fractional rate of gas production (k) decrease together with increase of wheat content, CTR-D60 ($P<0.01$) and CTR-D80 ($P<0.001$). Amounts of volatile fatty acids (VFAs) produced during the *in vitro* fermentation have been different ($P<0.05$) for investigated diets (Table 7). Moreover, we observed a trend, confirmed with Dunnet's test, where the production of Acetic, Propionic, Butyric, Valerianic and BCFA was lower usually in D60 or D80 compare to Control. Total amount of produced VFAs was the lowest in D80 ($P<0.001$), D60 tended to be lower, in comparison to CTR diet. Interestingly, the feed D80 had the lowest VFA content but the highest gas production. During the first 8 hours of fermentation D80 had initially lower and next the same gas production compare to Control (data not shown), but then after 24 hours become significantly higher ($P>0.05$). Possibly pasta residues were enough sticky that first enzyme and next bacteria were not able to reach all the substrate. Additionally, production of ammonia could also affect total gas production. Generally, formation of VFA is connected with several factors such as chemical composition of diet, availability and form of specific fermentable substrates (Zijlstra, Jha, Woodward, Fouhse, & van Kempen, 2012), and what is important, on the microbial population (Jiang, Li, Su, & Zhu, 2013). We observed higher VFAs production in samples with Control diet, due to significantly higher amount of more complex carbohydrates (i.e. RS), as was underlined earlier. The interaction between intestinal microbiota and fibrous components of the dietary ingredients in the hind-gut has important implications for the development, maturation and keeping equilibrium in the porcine gastro-intestinal tract, which in turn will influence the effectiveness of the intestinal barrier and the mucosal immune system (Bach Knudsen, Hedemann, & Lærke, 2012; Jin, Reynolds, Redmer, Caton, & Crenshaw, 1994). Statistical analysis showed tendency in the ammonia concentration, at the end of fermentation the highest level was found in D80 samples ($P<0.05$) (Table 7). Ammonia is well known as a cytotoxic agent. Under *in vivo* conditions, NH_3 is freely diffusible and absorbed by colonic epithelial cells or into bacterial cells for growth. Ammonia level is correlated with crude protein content, diets low in crude protein could

reduce ammonia emission (O'Connell, Callan, & O'Doherty, 2006). D80 had slightly higher CP content than Control, additionally pasta by-products may have different form of proteins or other nutrients resulting in further increase of NH_3 production.

Apart of studying *in vitro* starch degradation and *in vitro* fermentation, during animal trial we collected blood samples in the mid-term (Table 8) and at the end (Table 9). Samples have been tested to measure blood total proteins, albumin, globulin, Albumin/Globulin ratio, urea, alanine aminotransferase (ALT-GPT), aspartate aminotransferase (AST-GOT), alkaline phosphatase (ALP), bilirubin, glucose, total cholesterol, haptoglobin, phosphorus, calcium and magnesium content. Mid-term studies reviled blood globulin level in animals on D60 diet was significantly higher than CTR ($P<0.05$), and consequently these samples had also low Albumin/Globulin ratio ($P<0.05$). Bilirubin level was lower in D60 fed pigs compare to Control ($P<0.05$). Anova analysis of samples collected at the end of trial showed only tendency for urea level among diets, where animals fed D80 had higher level of urea than CTR. Lower amount of significant differences within blood metabolites at the end of trial may indicate adaptation of organism into diet. A low plasma urea concentration reflects a high quality of dietary protein (Eggum, 1970; Zervas & Zijlstra, 2002), thus, the low plasma urea in pigs fed Control diet for the low-protein diets indicated a better balance of ingested AA. Corn protein quality is lower but comparable to that of wheat because of its large germ which is high in lysine-rich protein (FAO, 2011), but without considering actual digestibility. Our experimental feeds were supplemented with wheat pasta by-products, heaving probably lower protein quality than corn based Control diet, what was reflected in the blood results. Additionally, first blood collection data showed that animals on D80 feed had lower phosphorus ($P<0.05$), but not in the second one. In general, wheat grains are higher in available phosphorus than corn (Cromwell, 2002), observed small decrease may not be related to diet, as it was not confirm with second blood sampling.

In conclusion, diet supplemented with 30%, 60% or 80% of wheat pasta by-products showed improved starch degradation, fermentation kinetics and lower production of volatile fatty acids in an *in vitro* model of the porcine gastrointestinal tract. However, NH_3 and urea levels had a tendency to increase after pasta inclusion. Additionally, supplementation with bakery by-products may reduce swine production costs, as the performance is similar to animals fed corn based feed.

Table 8
Blood content of pigs in relation to the diet type (CTR – corn based Control diet; D30, D60 and D80 – contains 30%, 60% and 80% of pasta by-product). Samples were collected in the middle time of trial.

Diet	Proteins	Albumin	Globulin		Alb/Glb		Urea	ALT-GPT	AST-GOT	ALP	Bilirubin		Glucose	Cholesterol	Ca	P		Mg	Hp
CTR	65.83	39.17	26.67	-	1.48	-	6.50	38.17	28.50	100.50	1.95	-	4.98	1.91	2.58	2.59	-	0.88	1.68
D30	65.50	39.67	25.83		1.53		5.38	38.50	25.83	77.33	1.24		4.89	1.75	2.49	2.47		0.85	1.72
D60	67.17	37.33	29.83	*	1.25	*	6.25	38.33	24.00	82.33	0.90	*	5.59	2.09	2.51	2.49		0.87	2.13
D80	66.50	39.00	27.50		1.42		6.90	35.50	26.50	82.83	1.52		5.16	1.94	2.51	2.33	*	0.85	2.06
P-value	NS	NS	0.027		0.014		0.085	NS	NS	NS	0.065		NS	NS	NS	0.048		NS	NS
SEM	1.11	0.82	0.89		0.06		0.41	2.69	2.21	13.10	0.26		0.24	0.15	0.03	0.06		0.02	0.37

Data expressed as mean, analysed by one-way ANOVA followed by Dunnet's test for comparison between control and treatment groups. The level of significance was set at P≤0.05.
Abbreviations: ALP, Alkaline phosphatase; Alb/Glb, Albumin/Globulin ratio; ALT-GPT, Alanine Aminotransferase; AST-GOT, Aspartate aminotransferase; ALP, Alkaline phosphatase; Hp, Haptoglobin; P, Phosphorus; Ca, Calcium; Mg, Magnesium; SEM, standard error of mean; NS, not significant; •, tendency (0.05<P<0.1); *, P<0.05; **, P<0.01; ***, P<0.001.

Table 9
Blood content of pigs in relation to the diet type (CTR – corn based Control diet; D30, D60 and D80 – contains 30%, 60% and 80% of pasta by-product). Samples were collected at the end of trial.

	Proteins	Albumin	Globulin	Alb/Glb	Urea		ALT-GPT	AST-GOT	ALP	Bilirubin	Glucose	Cholesterol	Ca	P	Mg	Hp
Diet	**Mean**															
CTR	69.00	39.50	29.50	1.35	4.35	-	41.00	29.00	58.50	1.63	4.38	2.64	2.43	2.24	0.92	2.35
D30	69.50	39.00	30.50	1.30	4.71		40.50	30.17	81.17	1.47	4.41	2.33	2.42	2.42	0.93	1.96
D60	72.00	40.17	31.83	1.27	5.05		39.33	26.67	82.33	1.43	4.46	2.42	2.46	2.23	0.96	1.70
D80	70.50	38.33	32.17	1.20	5.38	*	38.17	27.50	82.33	1.37	4.67	2.54	2.44	2.39	0.91	2.06
P-value	NS	NS	NS	NS	0.097		NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
SEM	1.26	0.81	1.06	0.05	0.29		2.84	3.52	9.51	0.19	0.21	0.17	0.03	0.08	0.02	0.26

Data expressed as mean, analysed by one-way ANOVA followed by Dunnet's test for comparison between control and treatment groups. The level of significance was set at P≤0.05.
Abbreviations: ALP, Alkaline phosphatase; Alb/Glb, Albumin/Globulin ratio; ALT-GPT, Alanine Aminotransferase; AST-GOT, Aspartate aminotransferase; ALP, Alkaline phosphatase; Hp, Haptoglobin; P, Phosphorus; Ca, Calcium; Mg, Magnesium; SEM, standard error of mean; NS, not significant; •, tendency (0.05<P<0.1); *, P<0.05; **, P<0.01; ***, P<0.001.

Conflict of interest

None.

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3.4. Kinetics of starch digestion and *in vitro* gas production affected by maize particle size.

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Manuscript written to be published in Animal Feed Science and Technology

Abstract

The particle size of milled grains is well known to affect availability of nutrients and the starch digestion kinetics of animal feeds. The aim of this experiment was to identify the contributions made by particular size fractions of milled maize to nutritional properties. Three batches of common commercial maize (Zea mays, L.) were ground through a 1, 2 and 4 mm screens and subsequently fractionated on a set of nine sieves ranging from 0.15 mm to 3.5 mm. Obtained fractions were characterized for starch, protein, prolamin, fat content, *in vitro* starch digestibility and fermentation. *In vitro* enzymatic starch digestion was performed using pancreatic alpha-amylase, amyloglucosidase and invertase, followed by *in vitro* fermentation with fresh faeces inoculum, to determine respectively fractional starch digestion and gas production rates. We observed a tendency for change in chemical composition of the examined particle fractions regarding total starch, prolamin and fat, but not proteins. Moreover, resistant starch content decreased with decreasing average particle size. The resulting glucose production rate data were well fitted into exponential nonlinear function and obtained parameters were found to be dependent of sieve screen size ($P<0.001$). Similar predisposition was detected for vitro fermentation parameters ($P<0.001$) and production of single

volatile fatty acids (VFAs). Our finding are consitent with the hypothesis that starch digestion properties of unsieved maize grains are a weighted average of the individual size fractions and *in vitro* methods are related to possibility of enzymes to penetrate grain fragments. We also suggested a simple method to predict Hydrolysis Index (HI) which could potentially help more efficiently asess digestion potential of maize-based feed.

Keywords: particle size, maize, pig, starch digestion, *in vitro* fermentation

Abbreviations: AA, amino acids; DM, dry matter; RDS, rapidly digestible starch; SDS, slowly digestible starch; RS, resistant starch; C_0, Starch digested at 0 min; C_∞ , Potential digestibility of starch; k, Rate of starch digestion or gas production; dgw, geometric mean diameter; SEM, standard error of the mean; Sgw, geometric standard deviation; t, incubation or digestion time; Vf, maximum gas volume; LAG, discrete LAG time; T/2, half-time to asymptote; OM, organic matter; BCFA, branched-chain fatty acid (sum of iso-butyric and iso-valeric acid); VFA, volatile fatty acid; RSE, residual standard error; NS, not significant.

1. Introduction

Dietary starch is the largest constituent in diets for pigs, and cereal grains represent the main starch source (Stevnebø et al., 2006). Particularly, maize is traditionally one of the most common cereal grain included in swine diets (FAO, 2011). The need to improve and optimize the efficiency of starch digestion is an important research focus in pig nutrition (Giuberti et al., 2014). Differences in starch digestibility have been ascribed to various factors, including the the type of corn endosperm, the presence of proteins and prolamins (Giuberti et al., 2013), the presence of lipids (Ai et al., 2013), the amylose-amylopectin ratio (Brewer et al., 2012; Jane et al., 1999), the starch granule structure (Chen and Zhang, 2012), the particle size (Blasel et al., 2006; Jong et al., 2012), the conservation (Humer et al., 2013; Vilariño et al., 2012) and processing methods (Jong et al., 2012). Material that escapes

enzymatic digestion by endogenous enzyme in the upper gastrointestinal tract, passes into the colon where it can be fermented by microbial communities. The level of fermantation is directly related to factors menttioned above, as it state the amount of material available for microbiota (Bach Knudsen et al., 2012).

Several *in vitro* studies have been conducted to investigate and charaterized the kinetics of starch digestion followed by fermentation for different ground grains, demonstrating relation to the increasing particle size, especially its available surface area (Al-Rabadi et al., 2009; Amrein et al., 2003; Blasel et al., 2006; Stewart and Slavin, 2009). For instance, Blasel et al., (2006) reported for each 100 mm increase in particle size in ground maize grain, a reduction in the degree of starch access by a-amylase of about 27g/kg of starch. Similarly, the work of Anguita et al. (2006) reported a significant decrease in the rate of starch hydrolysis after 60 and 240 min of *in vitro* incubation for feed grains grounded through 3.0 mm when compared to values obtained with a 0.8 mm opening screen. On the other hand, *in vitro* 24h long fermentation of small, large particle bran, aleurone, fine and coarse aleurone by-product, showing that small-particle bran and fine by-product produced greater SCFA concentrations than their large-particle or coarse counterparts (Stewart and Slavin, 2009).

Normally, grains are ground before being incorporated in diets for pigs, and agricultural machinery, being widely avaible with with variable screen sizes or milling settings, can create wide distribution of feed grains particle size (Al-Rabadi et al., 2012). Up to date, to the best or our knowledge, only few papers have been conducted with the aim to characterize the kinetics of starch digestion for ground grain fragments after segregation by size, and currently, this kind of information is available only for other commonly useed cereals barley and sorghum (Al-Rabadi et al., 2012; Mahasukhonthachat et al., 2010), however those studies do not include important aspect of *in vitro* gas production kinetics.

Consequently, an *in vitro* experiment consisting of enzymatic digestion followed by fermentation was conducted to investigate the contribution made by individual size fractions of maize grains to the chemical composition and related kinetics. The hyphotesis of this study is that physico-chemical and starch degradability properties of unsieved maize grains are a weighted average of the individual size

fractions. Testing was made by characterizing a range of parameters of separated size fraction obtained from cutting-milled samples at three different opening screen.

2. Materials and methods

Samples of common commercial maize (*Zea mays, L.*) variety grains were obtained from the three Italian farms in separate batches. Grains were ground by passing through a universal cutting mill Pulverisette19 (Fritsch GmbH, Germany) at ambient temperature. The opening sizes of the mill screens used were 1, 2 and 4 mm. The milled grain was collected at a constant motor load during milling. The mill was cleaned in between passes and obtained maize flours were stored at 4^{o}C.

2.1. Sieve analysis

In order to obtain the particle size distribution of the milled maize, samples were sized with a series of nine selected sieves and a pan, fitted into a sieve shaker Octagon Digital (Endecotts Ltd., London, England). Meshes (Laboratory Test Sieve, Octagon, ISO 3310-1:00) having opening dimensions of 3500, 2500, 1500, 1000, 710, 500, 355, 250 and 150 µm. A set of sieves were stacked with decreasing size of openings and procedure was carried out according to the standard ASAE method S319.3. A charge of 100 g of milled sample was put on the top sieve and shaken for 10 min. A total of 9 fraction of particle size range were finally collected for each sample, representing ranges >3500, 3500 to 2500, 2500 to 1500, 1500 to 1000, 1000 to 710, 710 to 500, 500 to 355, 355 to 250 250 to 150 µm and lower (Pan), respectively. In this way, the term particle fraction/size use in this study refers to particles that were retained on a particular sieve. Thus a particle size defined as the "1500" fraction are particles material retained on 1500 µm sieve, so are larger than 1500 µm, next fraction "1000" are particles passed through 1500 µm sieve and accumulated on the 1000 µm sieve. Additionally, particles lower than 250 µm are defined as Pan. The mass of obtained material and the particle size distribution was determined. The geometric mean diameter (dgw) and geometric standard deviation (Sgw) for each

maize were determined with 100 g sample according to (ASAE, 2003). Material obtained from fractions 1500 µm until Pan, was used for further analyses.

2.2. Chemical analysis

The proximate composition of each fraction was determined according to AOAC, 2000 standards: moisture content (Method 930.15), protein content (Method 990.03) and fat content (Method 920.39). Total starch (TS; g/kg DM) content was determined by an enzymatic method (Blasel et al., 2006) modified according to the procedure described by Masoero et al. (2010), whereas glucose content was achieved using the method proposed by Casterline et al. (1999). Prolamins were extracted with the procedure proposed by Giuberti et al. (2013) using *tert*-butyl alcohol. The sieved material obtained this way, for each sieve fraction size, was kept at 4^{o}C until further use. Chemical content of unsieved maize flour is presented in Table 1. The selected sieves, fraction yield on each sieve after sieving and the average particle sizes are listed in Table 2. Chemical content of analysed fractions are showed in Table 3 and Table 4.

2.3. In vitro enzymatic hydrolysis

Starch digestibility of each partcle fraction sample was evaluated based on two *in vitro* experiments. First, starch was characterized by rapidly digestible starch (RDS), slowly digestible starch (SDS) and resistant starch (RS) fractions according to the procedure defined by Englyst, et al., 1996. Samples (800 mg) were pre-incubated with 5 ml of pepsin–HCl solution (0.05 M HCl and pepsin Sigma–Aldrich P-7000, 5 mg/ml) in 50 ml tubes containing glass balls for 30 min at 37^{o}C under horizontal agitation. Next, 20 ml of 0.1 M sodium acetate buffer was added to get pH level of 5.2. Incubation was initiated by addition of 5 ml volume of an enzyme mixture composed by: pancreatin (Merck 7130, Merck KGaA), amyloglucosidase (Sigma A-7095, Sigma–Aldrich) and invertase (Sigma I-4504, Sigma–Aldrich). After 20 and 120 minutes of incubation solution samples (0.5 ml) were

collected, and have been measured colorimetrically for glucose presence, using GODPOD (Giesse Diagnostic). Data were then converted using equations developed also by Englyst, Kingman, & Cummings (1992) into RDS, SDS and RS values. Additionally, a sample contain Gelose 80 maize starch (Penford Food Ingredients Co.) was used along as a standard and quality control.

Second, *in vitro* hydrolysis experiment stimulating gastric and pancreatic phases, was carried out using similar protocol with pre-incubation in pepsin–HCl solution and incubation in enzyme mix up to 480 minutes. To describe starch digestion efficiency, aliquots of digestive solution were taken from each tube at nine times 0, 15, 30, 60, 90, 120, 240, 360, 480 min, and released glucose was determined colorimetrically as described above.

2.4. In vitro microbial fermentation

For purpose of *in vitro* fermentation, samples had undergone an *in vitro* enzymatic hydrolysis using pepsin and pancreatin according to protocol described in Boisen and Fernhdez (1997). Samples were passed through a paper filter, washed twice first with 96% ethanol and next with 99.5% acetone and dried at 60°C overnight. The procedure was repeated few times in order to obtain enough material for the following *in vitro* fermentation procedure.

The *in vitro* fermentation simulates the fermentation occurring in the hindgut, was performed following the protocol described by Bindelle et al. (2007). Gas accumulation measurements give useful information about the kinetics of digestion process. 200 mg of hydrolysed material were incubated in a 100 ml glass syringes at 39 ^{o}C with 30 ml of inoculum. A faecal inoculum was prepared using fresh faeces of three pigs, from the herd of the CERZOO (Centro di ricerche per la Zootecnia e l'ambiente, Piacenza) that were fed a antibiotics free diet, mixed to a buffer solution containing macro- and micro-minerals (Menke et al., 1979). The gas generated by the fermentation process were measured at 0, 0.5, 1, 1.5, 2, 3, 4, 6, 8, 24, 30 and 36 hour. Fermentation was stopped at

36th hour of incubation by setting the bottles in boiled water. Samples were collected from the syringes for measurement of VFA, as well as samples of the inoculum prior to fermentation.

2.5. Volatile fatty acids analysis

Samples from *in vitro* fermentation, were measured for the concentration of volatile fatty acids (VFA). VFA concentrations was determined using gas chromatography (GC) (Varian 3350) according to the method described by Fussell and McCalley (1987).

2.6. Scanning electron microscopy

Degradation progress of corn particles was determined with a scanning electron microscope (Quanta Feg 250; Fei Company; Hillsboro, USA). Samples (pre- and post-hydrolysis) were thinly spread onto metal stubs with double sided adhesive carbon tape and coated with 12 mm gold layer. Selected micrographs are showed in Figure 3.

2.7. Statistics and model used

Statistical analyses were completed with the R statistical environment (R Development Core Team, 2013). ANOVA with TukeyHSD pair-wise comparison was conducted to compare the mean values. Statistical significance was achieved at P values less than 0.05 and error is presented as ±SEM (standard error mean). Additionaly, means sharing the same letter are not significantly different, at the chosen level of 5%.

2.7.1. Modelling in vitro hydrolysis kinetics

Using the data obtained from the *in vitro* digestion test an *in vitro* digestion coefficient of starch for each time interval (C_t, as a percentage of TS) was calculated, by the following equation (1):

$$C_t = 100 \frac{0.9(\text{glucose present at time t - } t_0 \text{ glucose release})}{\text{total starch}}$$

For quantitative determination, starch is hydrolysed and measured as the component glucose monosaccharide units released, therefore the factor of 0.9 was applied to convert mono- to poly-saccharide (Stevnebø et al., 2006).

To investigate the kinetics of starch digestion for different particle sizes of maize we have been tested graphical representations and differential equations of several digestion models used to determine the disappearance of matter during the rumen fermentation (Tedeschi, 2008; Wang et al., 2011). Some of these nonlinear functions can be modified for *in vitro* starch degradation. We chose the Gompertz exponential nonlinear function, used to compute substrate disappearance on in situ experiments, given its the best fit capabilities, equation (2):

$$C_t = C_0 + C_\infty e^{\left(-e^{(1-kt)}\right)}$$

Where C_t corresponds to the *in vitro* digestion coefficient of starch at time t, C_0 is the *in vitro* digestion coefficient at 0 min, C_∞ is the *in vitro* digestion coefficient after 480 min, k is the digestion rate (/min) and t is the chosen time (min).

Kinetic parameters C_0, C_∞ and k was calculated using nls() function (Bates and Chambers, 1992; Bates and Watts, 1998). The area under the *in vitro* digestion curves (AUC) was calculated by AUC function, in MIfuns package (Knebel et al., 2008), using trapezoidal rule. Hydrolysis index (HI), was

obtained from the ratio between the AUC of each diet and the reference AUC (Equation 3). HI expressed as a percentage (Giuberti et al., 2012; Goñi et al., 1997), equation (3):

$$HI[\%] = \frac{\text{AUC}}{44500.35} 100$$

where, 44500.35 is an AUC of bread, as standard to calculate hydrolysis index (HI).

2.7.2 Kinetics of gas production

Gas accumulation was modeled according to LAG exponential model with discrete LAG time (Schofield et al.,1994) and described with the following parameters: V_f, final asymptotic gas volume; k, fractional rate of gas production; LAG, discrete lag phase; T/2, half-time to asymptote. Kinetic parameters was calculated using nls() function (Bates and Chambers, 1992; Bates and Watts, 1998) in R, equation (4):

$$V = V_f\left(1 - e^{\left(-k(t-LAG)\right)}\right)$$

where

$$t/2 = LAG + \ln(0.5/k)$$

$$t \geq LAG$$

2.7.3. Prediction of Hydrolysis Index (HI)

Assuming that each of particle fractions of given maize have the same Hydrolysis Index, like that calculated by us, it is possible to calculate a total HI of that milled maize. First, using sieves with the same diameters like here presented (>1500-1000-750-500-355-250 μm >Pan) calculate percentage distribution of material accumulated within each fraction of given maize. Using obtained fraction percentage (FPi) and weighted HI of fraction (wHIi) it is possible to calculate the share of each fraction in total predicted HI (pHI) of given maize, where *n* is a number of given particle size fractions, equation (5):

$$pHI = \sum_{i=1}^{n}(wHI_i * FP_i /100)$$

3. Results

Chemical composition of the three investigated unsieved maize flours are presented in Table 1 and their geometric parameters (d_{gw} and S_{gw}) together with particle size distributions are given in Table 2. Maize grain milled with 1, 2 and 4 mm screens had d_{gw} factor of 0.48, 0.657 and 1.27 with standard deviation (S_{gw}) of 0.327, 0.537 and 1.00, respectively. Fraction yield on the larger sieve, of maize milled with 1mm screen, between 1500 and 1000 μm was 8.42%, it was also the lowest amount of material obtained for that milling. The highest percentage of fraction yield 29.25%, we obtained within the next range 1000-710 μm. Maize milled with 2 mm screen high yield was distributed between the ranges 1500-710 in total 43.74%, and the lowest yield was in 355-250 range (6.99%). More than a half (54.2%) of total amount of sieved material, ground 4 mm screen, was accumulated on sieves diemnsions larger than 1500 μm.

Table 1
Chemical composition (g/kg dry matter) of the unsieved maize flour.

	Maize milling		
Item	1 mm	2 mm	4 mm
DM (%)	92.0	91.9	91.8
Total Starch (% DM)	70.4	70.6	65.9
RDS (% of TS)	18.0	14.9	14.4
SDS (% of TS)	41.5	41.8	39.6
RS (% of TS)	40.5	43.3	46.0
Proteins (% DM)	11.4	9.8	9.7
Prolamin (% DM)	2.9	2.8	2.7
Fat (% DM)	3.4	3.2	2.6

DM, Dry matter
TS, Toatal Starch

Table 2
Geometric mean particle size diameter (dgw), geometric standard deviation (Sgw), fraction yield (±SEM) for each milling and material retained on each of the selected sieves, of maize.

Milling	1 mm		2 mm		4 mm	
Dgw (mm)	0.48		0.66		1.27	
Sgw (mm)	0.33		0.54		1.00	
Sieve size (µm)	Fraction yield					
	[%]	±sem	[%]	±sem	[%]	±sem
3500	0.00	0.00	0.00	0.00	2.71	0.13
2500	0.00	0.00	0.02	0.00	11.36	0.38
1500	0.00	0.00	10.44	0.62	40.13	0.77
1000	8.42	0.23	22.82	0.45	18.87	0.76
710	29.25	0.31	20.92	0.14	8.78	0.14
500	16.44	0.01	12.21	0.04	5.55	0.08
355	10.46	0.10	7.65	0.07	3.32	0.05
250	8.75	0.04	6.99	0.06	2.77	0.05
150	26.68	0.45	18.94	0.13	6.51	0.05

Maize fractions were analysed for chemical composition regarding DM, protein, prolamin and fat (Table 3). Factorial statistics for two factors Milling and Fractions did not reviled any significance differences for Milling and Milling:Fraction interaction. Fractions means for prolamin content had tendency to be different (P = 0.063). We found no differences in protein content of fractions. Effect of Fraction was visible only in total Fat content (P < 0.001). Pan fraction had the highest significantly (P < 0.05) content of fat (4.565%), together with 355-250 fraction (3.594%). But, fraction 355-250 was also not different from the remaining fractions.

Table 3
Chemical composition (g/kg dry matter) of the examined particle fractions and fraction effect

Fraction (µm)	DM (%) [z]	Prolamin (% DM) [z]	Proteins (% DM) [z]	Fat (% DM) [z]
1500	92.17	2.88 [a]	10.26	2.10 [a]
1000	92.08	3.28 [a]	9.84	2.67 [a]
710	91.81	3.03 [a]	9.65	2.87 [a]
500	91.60	3.06 [a]	10.39	3.17 [a]
355	91.45	2.96 [a]	11.14	3.30 [a]
250	91.21	2.94 [a]	10.10	3.59 [ab]
Pan	91.19	1.96 [a]	8.75	4.57 [b]
SEM	0.60	0.27	0.82	0.22
P	NS	0.06	NS	0.001

Abbrevations: SEM, standard error of mean; NS, not significant; DM, dry matter.
[z] Means sharing the same letter are not significantly different, at the chosen level (5%).

Table 4
Effects of Milling and Fractions on starch content and starch three fractions RDS, SDS and RS.

Item		Total Starch (% DM)	RDS (% TS)	SDS (% TS)	RS (% TS)
Milling	Fraction				
1	1500	n.a.	n.a.	n.a.	n.a.
	1000	72.03	13.87	38.36	47.85
	710	69.34	15.02	46.17	37.74
	500	66.89	16.71	47.35	42.80
	355	67.68	19.28	51.74	35.78
	250	71.16	19.12	52.13	27.80
	Pan	74.16	23.43	53.11	23.46
2	1500	69.85	14.45	32.74	52.96
	1000	73.94	14.89	35.25	51.72
	710	69.97	16.84	38.51	44.65
	500	69.53	17.20	41.49	41.16
	355	71.24	19.26	47.27	33.14
	250	73.49	20.04	49.71	29.21
	Pan	76.89	23.23	55.12	21.65
4	1500	66.25	12.40	27.74	59.85
	1000	65.09	14.72	40.19	45.26
	710	64.66	16.25	43.31	40.56
	500	66.94	17.98	46.09	34.78
	355	68.31	18.06	50.94	31.18
	250	69.73	21.86	54.63	20.79
	Pan	74.92	23.49	60.45	13.55
SEM		2.04	2.33	2.91	4.16
Milling		0.005	NS	NS	NS
Fraction		0.1	0.05	0.01	0.001
Milling:Fraction		NS	NS	NS	NS

Milling effect [y]
Starch [z] - 1: 70.21 [ab]; 2: 72.13 [b]; 4: 67.98 [a]
Fraction effect [y]
Starch [z] - 1500: 68.05 [a]; 1000: 70.35 [ab]; 710: 67.99 [a]; 500: 67.78 [a]; 355: 69.17 [a]; 250: 71.46 [ab]; Pan: 75.32 [b]
RDS [z] - 1500: 13.22 [a]; 1000: 14.38 [a]; 710: 16.03 [ab]; 500: 17.21 [ab]; 355: 19.10 [abc]; 250: 20.15 [bc]; Pan: 23.39 [c]
SDS [z] - 1500: 30.24 [a]; 1000: 37.93 [ae]; 710: 42.66 [de]; 500: 44.68 [bde]; 355: 49.59 [bcd]; 250: 51.84 [bc]; Pan: 55.69 [c]
RS [z] - 1500: 56.40 [a]; 1000: 48.33 [ae]; 710: 41.39 [de]; 500: 39.58 [de]; 355: 33.64 [bd]; 250: 25.94 [bc]; Pan: 20.30 [c]

Abbrevations: SEM, standard error of mean; NS, not significant; TS, Total Starch; n.a.; not analyzed, no material was retained.
[z] Means sharing the same letter are not significantly different, at the chosen level (5%).
[y] Marginal mean with principal effect on factor.

Different RDS, SDS and RS fractions of starch were recorded (P<0.05) (Table 3). Factorial analyses for Milling and Fraction showed no differences for M:F interaction. Results revealed significant differences in Milling (P<0.005) for total starch content, maize milled using 2mm screen had the highest 72.13% one and using 4mm screen the lowest 67.98%. Fraction effect displayed significant differences in RDS (P<0.053), SDS (p<0.004) and RS (P<0.001) content, and also a tendency regarding total starch content (<0.104). The highest level of total starch (P<0.05) was registered in Pan fraction (75.32%), all the others had the same significance level. RDS range from the lowest values (P<0.05) for fractions >1500 and 1500-1000, 13.22 and 14.38 respectively, into the highest (P<0.05) for Pan fraction 55.69%. SDS content range from the lowest 30.24% in >1500 to the highest 55.69% in Pan fraction (P<0.05). Resistant part of starch (RS) in contrary to RDS and SDS was the highest (P<0.05) for particles >1500 (56.40%) and the lowest (P<0.05) for Pan (20.30%).

Average cumulative (Figure. 1) and absolute curves (Figure. 2) of time course starch digestion release (given as a fraction of total starch digested after 9 different incubation times) indicated large differences among samples (P<0.05). Among all seven fractions of particle size of maize, >1500 was digested very slowly and had the lowest digestion curve at all time intervals. In contrary, the finest obtained fraction Pan was digested very fast and after 2 hours of incubation 80% of total starch had been already digested. Time course profile of average absolute *in vitro* starch digestion showed similar results, with high peak after 15 min of incubation.

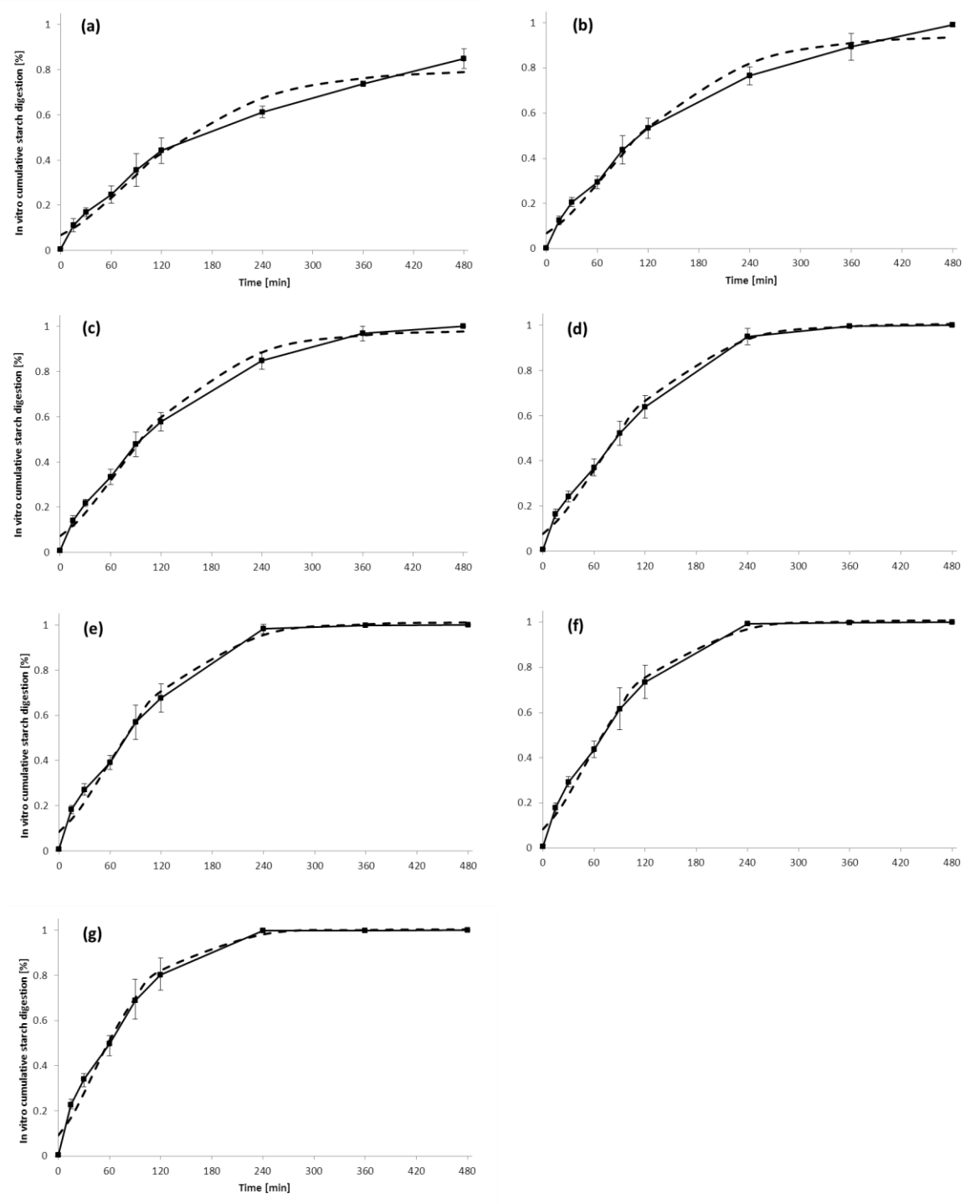

Fig. 1. Time course of average cumulative *in vitro* starch digestion (as a fraction of total starch) of maize particle size fraction. Letters correspond to fractions as: (a) 1500; (b) 1000; (c) 710; (d) 500; (e) 355; (f) 250; (g) Pan. Solid line represents experimental data and short-dashed line prediction model.

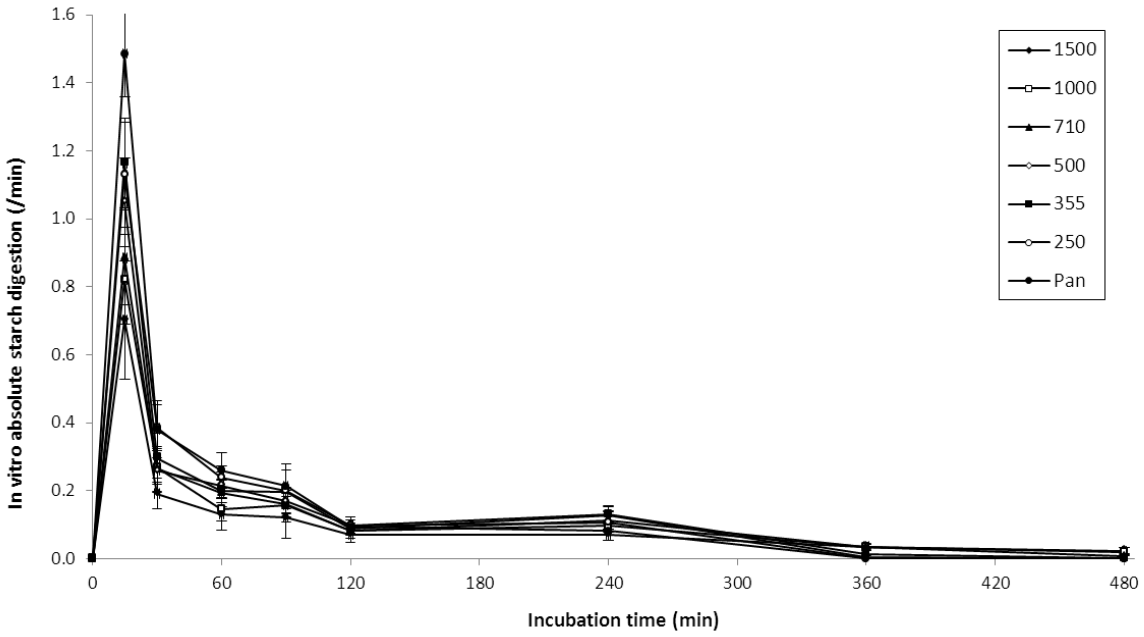

Fig. 2. Time course of average absolute *in vitro* starch digestion (as a fraction of total starch/min) of maize flour and flour fractions, calculated by subtracting the proportion of digested starch at each time point from the value at the next time point for each sample.

The *in vitro* digestion parameters obtained with mathematical model together with the hydrolysis index describe digestion profile of every fraction in more precise way (Table. 4). Factorial analyses with Milling and Fraction as factors, showed no significant effects of Milling and of the Milling:Fraction interaction. The *in vitro* digestion coefficient at 0 min, C_0, was found to be the same among particle size fractions. C_∞ ($P<0.001$), the *in vitro* digestion coefficient after 480 min, was significantly different only for fraction >1500, it was also the lowest level (78.51%). The digestion rate (k/min) parameter ($P<0.001$) range from 0.012 (fraction >1500) to 0.023 (Pan). Particle size fractions larger than 500 had significantly lower digestion rate from Pan ($P<0.05$). Hydrolysis index calculated basing on AUC, was significantly ($P<0.001$) increasing along with decreasing dimensions of particle size and range from 60.57 (>1500) to 91.36 (Pan). Furthermore, we found no significant diference between HI of unsieved 1 mm flour and its predicted value.

Parameters characterizing the kinetic of *in vitro* fermentation and production of volatile fatty acids (VFAs) are presented in Table 5. Particle size 710 and Pan share the same level of means significance of maximum gas volume (V_f) and particle fractions 1000 and 1500 are significantly higher than others ($P>0.05$). *In vitro* fermentation parameter k is inversely correlated with particle size, velocity decrease with particle size increase ($P>0.001$), contrary to half-time to asymptote (T/2) which increase with with particle size increase ($P>0.001$). LAG time was different among particle fractions ($p<0.01$) and the highest was for the largest particle size in fraction 1500 (0.42 h). Production of volatile fatty acids (VFAs) butyric, propionic and BCFAs was significantly different ($P>0.01$), or had tendency regarding acetic ($P=0.1$), among maize grain fragments fractions. However, we found no differences in total production of VFAs. Concentration of acetic and propionic tend to decrease with increase particle size and concentration of BCFAs in oposite. In butyric we didn't observe such change, the lowest concentration was still also for 1500 but the highest ones 500, 355 and Pan share same significance level ($P>0.05$).

Table 4
Digestion coefficients and hydrolysis index (HI) characterising the *in vitro* enzymatic starch digestion of maize for different particle fractions, at time of incubation 480 min.

Fraction	C_0 (g/100 g dry starch) [z]	C_∞ (g/100 g dry starch) [z]	K (/min) [z]	HI (%) [y, z]
1500	1.62	78.51 [a]	0.012 [a]	60.57 [a]
1000	0.70	93.90 [b]	0.014 [a]	73.51 [e]
710	0.82	97.47 [b]	0.015 [a]	79.29 [d]
500	1.08	99.71 [b]	0.016 [a]	84.53 [bd]
355	1.82	99.48 [b]	0.017 [ab]	86.81 [bc]
250	1.84	99.05 [b]	0.019 [ab]	88.86 [bc]
Pan	2.77	97.59 [b]	0.023 [b]	91.36 [c]
SEM	0.61	1.47	0.001	3.27
P	NS	0.001	0.001	0.001
		Unsieved (1mm) [u]		84.87
		Predicted value [v]		84.48
			SEM	0.97
			P	NS

Abbrevations: C_0, Starch digested at 0 min; C_∞ , Potential digestibility of starch. k, Rate of starch digestion; SEM, standard error of mean; NS, not significant.
[z] Means sharing the same letter in given column are not significantly different, at the chosen level (5%).
[y] Calculated by using white bread as reference (HI = 100).
[u] Unsieved maiz flour milled with 1mm screen.
[v] For comparison with unsieved value, calculated based on percentage distribution of material accumulated and hydrolysis index for each fraction of given maize.

Table 5
Parameters characterizing the *in vitro* fermentation kinetic and production of volatile fatty acids (VFAs) of maize for different particle fractions, at incubation time of 36 hours.

Item	Fraction 1500	1000	710	500	355	250	Pan	SEM	P
Kinetic parameters									
V_f [z]	416.05 [a]	363.51 [d]	304.07 [c]	281.36 [bc]	270.90 [bc]	266.61 [b]	301.77 [c]	6.28	0.001
LAG [z]	0.42 [a]	0.26 [ac]	-0.01 [b]	-0.06 [b]	-0.10 [b]	-0.06 [b]	0.05 [bc]	0.04	0.001
k [z]	0.04 [a]	0.07 [ae]	0.10 [e]	0.16 [d]	0.22 [c]	0.26 [b]	0.30 [f]	0.006	0.001
T/2 [z]	16.72 [a]	9.65 [e]	6.91 [d]	4.46 [c]	3.28 [bc]	2.78 [b]	2.22 [b]	0.25	0.001
Production of VFAs									
Total (mmol/g OM) [z]	6.535 [a]	6.661 [a]	6.734 [a]	6.928 [a]	6.994 [a]	7.193 [a]	7.851 [a]	0.342	NS
Acetic [r, z]	0.536 [a]	0.529 [a]	0.553 [ab]	0.568 [b]	0.570 [b]	0.576 [b]	0.570 [b]	0.005	0.002
Propionic [r, z]	0.273 [a]	0.279 [ab]	0.278 [ab]	0.277 [a]	0.282 [ab]	0.287 [ab]	0.298 [b]	0.004	0.021
Butyric [r, z]	0.011 [a]	0.011 [a]	0.012 [a]	0.012 [a]	0.013 [a]	0.011 [a]	0.011 [a]	0.0005	NS
Valerianic [r, z]	0.012 [a]	0.015 [a]	0.016 [d]	0.018 [c]	0.020 [bc]	0.019 [b]	0.018 [b]	0.0005	0.001
BCFA [r, z]	0.169 [a]	0.166 [ae]	0.142 [de]	0.125 [bcd]	0.115 [b]	0.106 [bc]	0.103 [cd]	0.002	0.001

Abbrevations: V_f, maximum gas volume; LAG, discrete LAG time; k, fractional rate of gas production; T/2, half-time to asymptote; OM, organic matter; BCFA, branched-chain fatty acid (sum of iso-butyric and iso-valeric acid); VFA, volatile fatty acid; RSE, residual standard error; NS, not significant

[z] Means sharing the same letter in given row are not significantly different, at the chosen level (5%).

[r] Molar ratio of the individual SCFA.

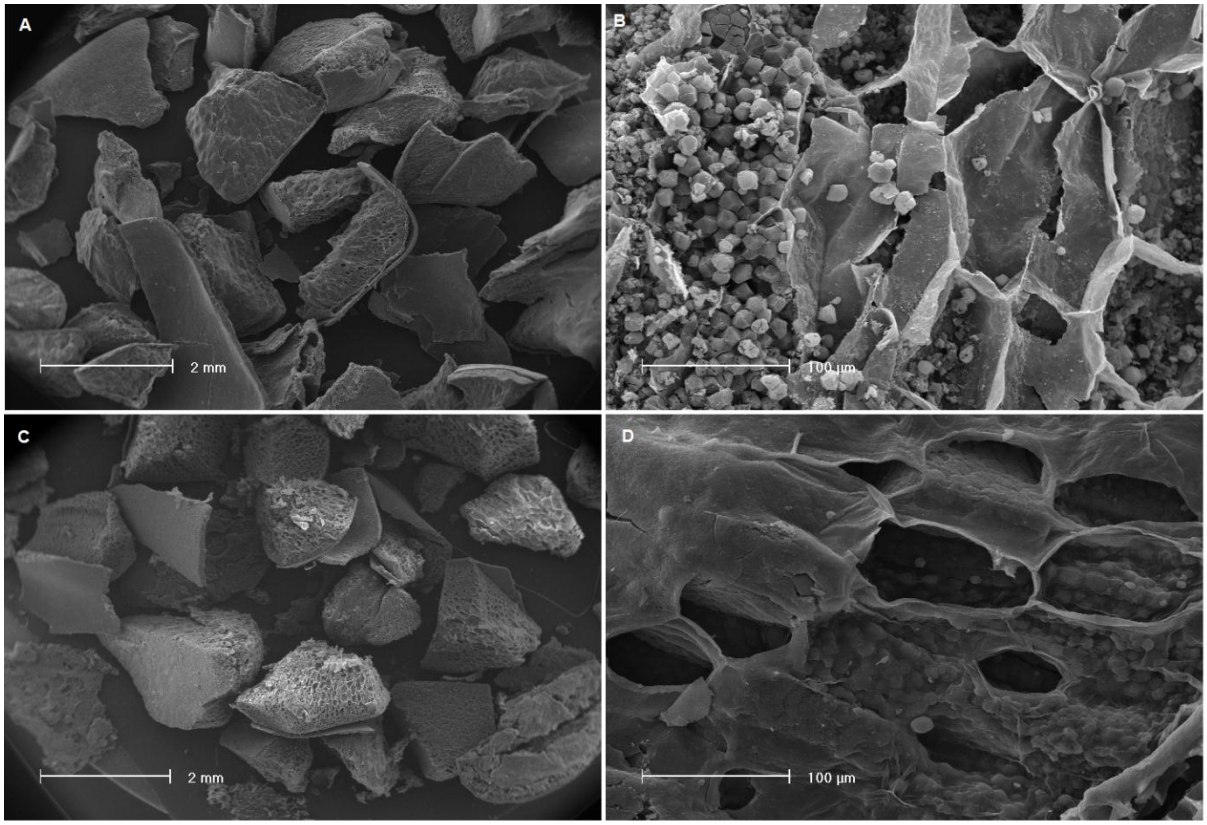

Fig. 3.
SEM micrographs of the maize particles larger than 1.5 mm, before (A and B) and after (C and D) *in vitro* enzymatic digestion process. The ellipsoidal objects imbedded within prolamin-protein matrix are starch granules.

4. Discussion

In the present experiment we were able to determine the milled maize fractions characteristics that could be used in prediction model for feed digestibility. Additionally we confirmed that different fraction of particle size of milled maize affect *in vitro* starch degradation, *in vitro* fermentation and presence of some nutrients. Results shown the strong dependence of digestion time on particle size, the distribution of particle size after grinding is important in determining the rate of digestion of ground (non-fractionated) grain. This is particularly significant for non-laboratory milling applications, such as animal feeds production, where a varied range of screen sizes are used that would outcome in wide distribution of particle sizes. Our results are consistent with work of Blasel et al., 2006 where was pointed out that degree of starch access (DSA) is sensitive to maize grain particle size of maize. Monogastric animals are unable to degrade cell walls, similar enzyme mix used during *in vitro* digestion was unable to penetrate cell walls. The seed coat of dry shelled maize grain is a barrier to enzymatic hydrolysis of intact kernel starch, therfore the whole maize grain or its large parts have significantly decreased enzymatic hydrolysis of starch to glucose in maize, as it was also observed by (Ehrman, 1996). Our SEM micrographs (Fig 3.) showing starch granules imbedded in prolamin-protein matrix and behind thin cell walls, granules visible outside cells have been digested during *in vitro* process. Wondra with his colleagues, whose studied *in vitro* effects in pigs intensively, showed increased apparent total track digestibility (ATTD) of energy in pigs, after grinding to achieve a smaller mean particle size (Wondra et al., 1995a, 1995b, 1995c) and increased energy digestibility (Yáñez et al., 2011). Larger particles provide per unit of mass less surface area for digestive enzymes to interact with their substrates in feed particle, thus require more time for complete digestion but, time during digesta transit in the intestine is limited (Goodband et al., 2002). Grinding feedstuffs may also increase CP and AA digestibility. For example, reduced mean particle size increased CP or AA digestibility of maize protein sources (Wondra et al., 1995c). Stewart and Slavin (2009) reported that small particles of wheat bran produced during *in vitro* fermentation higher ammount of SCFAs than their larger counterparts. However, our results revealed no differences in total production of VFAs, but just between single acids where concentration change along with particle size. *In vitro*

fermentation kinetic model showed that until 8h point of fermentation production of gas was inversely correlated with particle size, and then large particles have significantly increased their gas production to finally line up or even overtake the Pan fraction. We can speculate that microbiota need more time to break tought layers of bigger particle size, such as 1 or 1.5 mm, and enter hidden decks of fermentable material. The wast part of of neutral sugars degradate during first hours of fermentation, but some cross-linking of polysacchardes, an important factor for the stability of cell walls, could significantly limits biodegradability of cell walls by microorganisms (Amrein et al., 2003). Indeed, fraction 1500 reach T/2 after more than 16 hours when in case of Pan it was just around 2 hours. Moreover, research studies have observed negative relationship between the protein and prolamin (vitreousness) content and starch degradability or digestibility (Giuberti et al., 2014, 2013). We did not observe any difference between fractions regarding protein content, but we noticed tendency regarding prolamin, maize particles >1mm had rather higher content than fine ground flour Pan. Hydrolysis index could be predicted from its weighted value of each particle size fraction. Similar implication has been previously reported also for other cereal parameters (Al-Rabadi et al., 2012). Our results (Table 4.) showed that with appropiated database this simple method could be very useful to fast and precise asess nutritional parameters of cereals.

In conclusion, enzyme digestion as well as microbiotic fermentation of maize grain fragments of different sizes can be well fitted into mathematical models, allowing to explain processes kinetics. Overall differences in starch degradability and fermentability among grain fragments of different sizes can be attributed to their matrix complexity, which makes nutrients less accessible to enzymes and microbes for digestion and fermentation (Jha and Leterme, 2011). During *in vitro* starch digestion procedure enzyme mix was not able to reach some parts of starch, this reflects later during *in vitro* fermentation where larger particles had the highest gas production but within longer time period.

Acknowledgments

This study was supported by the AGROSCENARI project "Scenari di adattamento dell'agricoltura italiana ai cambiamenti climatici" of MIPAAF (Ministero delle politiche agricole alimentari e forestali – Italy) and by the Doctoral School on the Agro-Food System (Agrisystem) of the Università Cattolica del Sacro Cuore (Italy).

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4. Research projects - Part 2 –

Porcine taste receptors and nutritional homeostasis

4.1. Sugar, amino and fatty acids receptor gene expression in pigs fed iso-energetic diets enriched with β-glucans or arabinoxylans.

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Abstract

The mammalian gastrointestinal tract is the epithelium responsible to absorb nutrients from foods. Nutrient sensors are expressed in the tongue and along the gastrointestinal tract. Most nutrient sensors known to date belong to the G-protein-coupled receptors (GPCR) superfamily. During recent years scientists have discovered several novel receptors responsible for carbohydrates, amino or fatty acids sensing. Receptors are able to sense the luminal content and trigger adaptive responses that affect gastrointestinal function by release of messengers and regulation of nutrient absorption. Additionally, they seem to be involved in the regulation of energy and glucose homeostasis. Fibrous dietary compounds have the ability to increase the viscosity of digesta which slows down the flow of digestive enzymes and the absorption of nutrients. For example, soluble fibre reduces glucose absorption (and the glycaemic index) and other macronutrient absorption such as fatty acids. We hypothesize that high dietary fibre may limit sugar, amino or fatty acid availability which, in turn, will increase the

expression of their sensing receptors. The purpose of this study was to evaluate the effect of dietary addition of soluble fibre compounds arabinoxylan (AX) or β-glucan (BG), compared to a wheat-based Control (CTR) diet, on the expression level of fatty acids and sugar receptor genes in the porcine gustatory and non-gustatory tissues. For that reason, 18 Large-White male pigs were assigned to three experimental diets during two weeks. The control (CTR) was a standard wheat starch based diet having 17.4 MJ/kg DE. The two fiber rich treatments were iso-energetic compared to CTR, and consisted of 10% soluble wheat arabinoxylans or oat beta-glucans substituting equivalent amounts of .wheat starch. Real time qPCR was performed on cDNA with SYBR green from collected tissues, such as tongue papilla (Circumvallate - CV, Fungiform - FU, Foliateform - FL), stomach antrum (SA) and ridge (SR), duodenum (DD), jejunum (JE), ileum (IL), colon proximal (CP) and distal (CD), caecum (CA), liver (LV). The results showed significant expression of receptor genes along the porcine GIT. To the best of our knowledge, our results showing significant expression of several of the taste and nutrient sensors outside the oral cavity had never been published before. Interaction between Gene and Diet was found exclusively within tongue circumvallate papilla, where GPR120 expression was significantly higher in animals fed the AX enriched diet but not the BG enriched diet suggesting that the interaction between fiber and nutrients might be more specific than previously hypothesized. The fact that the diets were iso-energetic may have influenced the lack of wider differences between energy-related nutrient sensors.

Keywords: taste receptor; gastrointestinal tract; pig; GPCR; gene expression;

Introduction

Cereal non-starch polysaccharide (NSP) influence the digestive processes and gut microbiota composition in both pigs and humans, nevertheless from the animal production point of view, a part of some benefits, it is recognize mostly as an anti-nutritional factor (Bach Knudsen et al., 2012; EFSA and NDA, 2011; Wenk, 2001). Swine are able to utilize moderate, but not high, levels of fibre in the post-weaning and finisher phases of growth (de Lange et al., 2010; Molist et al., 2014). Viscous and gel-forming properties of soluble fibres are involved in delaying of macronutrient absorption, slowing of gastric emptying, reducing postprandial glucose responses and reducing total and low-density lipoprotein (LDL) cholesterol levels. Part of the NSP, such as beta-glucan and arabinoxylan, fermented by intestinal microbiota can mainly attribute in the formation of short-chain fatty acids (SCFAs) (Daniel et al., 1997; Lu et al., 2000; Reilly et al., 2010). Furthermore, some types of NSP may stimulate the activity of the microbial population and a health-promoting bacteria, and thus contribute to the gut health maintenance (Bouhnik et al., 2004; Reilly et al., 2010; Wong et al., 2006). With the many changes in the swine gastrointestinal tract environment connected to high fibre consumption, the maintenance energy requirements may be increased by the extra metabolic demand due the low energy density and increased satiety (Lange et al., 2000; Lu et al., 2000; Zijlstra et al., 2012). Cereals and their co-products included into feed plan are usually chosen on the basis of their nutrient composition, therefore it would be useful to take into account also possible differences in palatability (Liou, 2013). The luminal content in the gastrointestinal tract is under continuous monitor by proper sensory systems, which help adjust appetite to energy homeostasis or the hormone secretion according to the composition of ingested food (Depoortere, 2014a). The major part of nutrient receptors belongs to the group of G proteins associated with GPCRs (G protein coupled receptors). Two of the basic taste qualities, sweet and umami, are mediated by G protein-coupled receptors (GPCRs) belonging to the taste receptor type 1 family (T1R). T1R consists of three proteins (encoded by Tas1Rs genes) that form heterodimers for sweet sensation (T1R2/T1R3), commonly associated with high caloric food and responds to carbohydrates (i.e. mono- and disaccharides), on the other hand T1R1/T1R3 heterodimer is associated with umami or/and aliphatic amino acids. Another relevant

GPCRs for amino acid sensing include the metabotropic glutamate receptor proteins mGluR1 and mGluR4 encoded by the glutamate receptors metabotropic 1 and 4 genes (GRM1 and GRM4) (Brosnan and Brosnan, 2013; Zhang et al., 2013), and the G-protein coupled receptor family C group 6 (GPRC6A) (Haid et al., 2012). mGluRs shares common signal transduction pathways with umami taste, and GPRC6A with basic and small neutral amino acids and extracellular calcium. Recent findings challenge the canon of five basic tastes, strongly suggesting significant role of taste in dietary fatty acids perception. Five main GPCRs which respond to free fatty acids, have been identified during the last decade: GPR40, GPR43, GPR41, GPR120 and GPR84 (Im et al., 2009). Four first are also known as, GPR40, GPR43, GPR41 and GPR120respectively. FFARs are activated by free fatty acids of different chain lengths with varying degrees of specificity, GPR120 more specifically binds LCFA, whereas the GPR40 has been identified as a receptor for medium to long-chain. GPR84 prefer medium-chain fatty acids (such as: capric, undecanoic, and lauric acid). GPR43 and GPR41 preferentially bind short chain fatty acids (SCFA), C2–C3 and C3–C5 respectively (such as, formate, acetate, propionate, butyrate and pentanoate) (Milligan et al., 2014). Additionally as the short-chain fatty acids are produced mainly by anaerobic fermentation of dietary carbohydrate fibres, FFARs could thus be also classified as carbohydrate intake sensors.

Thus, we hypothesize that high dietary fibre may limit amino acids, fatty acids and sugar availability which, in turn, will increase the expression of their sensing receptors. The purpose of this study was to evaluate the effect of dietary addition of soluble fibre compounds arabinoxylans (AX) or β-glucans (BG), on the expression level of sugar, amino and fatty acids nutrient sensor genes in porcine gastro-intestinal tract.

Materials and Methods

Animals and housing

A total of 18-week-old male pigs of a commercial Large White strain were obtained from the University of Queensland piggery. Pigs had an average starting weight of 23.9 (±2.4) kg and were

housed at the Centre for Advanced Animal Science (University of Queensland, Gatton, QLD, Australia). Before the start of the experimental period, pigs were weighted and blocked by litter and randomly assigned to one of the three diets. Experimental procedures involving pigs were approved by the Animal Ethics Committees of the University of Queensland.

Diets and feeding procedures

Three types of diet were formulated. The control (CTR) was a standard wheat starch based diet consisting of 17.4 MJ/kg DE, 197g/kg crude protein and 14.4g/kg digestible Lys. The two treatments were iso-energetic compared to CTR, but contained 10% additional fibre through the replacement of starch in the CTR diet by arabinoxylans (AX) and beta-glucans (BG) source, respectively. Composition of the experimental diets is shown in Table 1 and 2. Animals had a one week adaption period, with gradual introduction to their individual diet. Following adaptation, pigs have been fed with the experimental diets for 14 days, received two meals per day (morning and afternoon) and had free access to water.

Tissues sampling

Tissues were collected from eighteen five-months old pigs of a commercial Large White breed were obtained from the University of Queensland piggery. Prior to the sacrifice animals were receiving wheat based diet with 17.4 MJ/kg digestible energy, 197g/kg crude protein and 14.4g/kg digestible Lys. The following samples from 12 different types of tissue were obtained after euthanasia of pigs: tongue papilla (Circumvallate - CV, Fungiform - FU, Foliate - FL), stomach antrum (SA) and ridge (SR), duodenum (DD), jejunum (JE), ileum (IL), colon proximal (CP) and distal (CD), caecum (CA), liver (LV). Tissues were snap-frozen on liquid nitrogen immediately after collection and stored at -80^{o}C freezer until RNA extraction.

Table 1
Percentage composition of experimental diets

Diet	Wheat starch control (CTR)	Wheat starch + sol AX (AX)	Wheat starch + sol BG (BG)
b-Glucan source	.	.	0.117
AX source	.	0.255	.
Wheat starch	0.429	0.194	0.307
Casein	0.050	0.050	0.050
WPC80	0.100	0.080	0.105
Egg powder	0.150	0.150	0.150
Sucrose	0.050	0.050	0.050
Arbocel	0.060	0.060	0.060
Palm oil	0.060	0.060	0.060
Sunflower oil	0.040	0.040	0.040
Limestone	0.015	0.015	0.015
Dical-phosphate	0.013	0.013	0.013
NaHCO3	0.006	0.006	0.006
NaCl	0.003	0.003	0.003
	1.000	1.000	1.000

Table 2
Chemical compositions of experimental diets.

Diet	Wheat starch control (CTR)	Wheat starch + sol AX (AX)	Wheat starch + sol BG (BG)
	Nutrient composition, g/kg		
DM	902.5	916.6	912.2
Crude ash	45.6	50.8	48.7
Crude protein	197.0	200.9	200.3
Dig crude protein	178.2	181.0	181.2
Crude fat	170.6	169.4	170.9
dig Crude fat	156.3	155.6	156.4
Ca	9.50	9.58	9.57
P	4.20	4.42	4.33
Na	4.58	4.45	4.56
K	1.22	2.19	1.69
Cl	2.40	2.47	2.47
Mg	0.73	0.84	0.79

RNA extraction

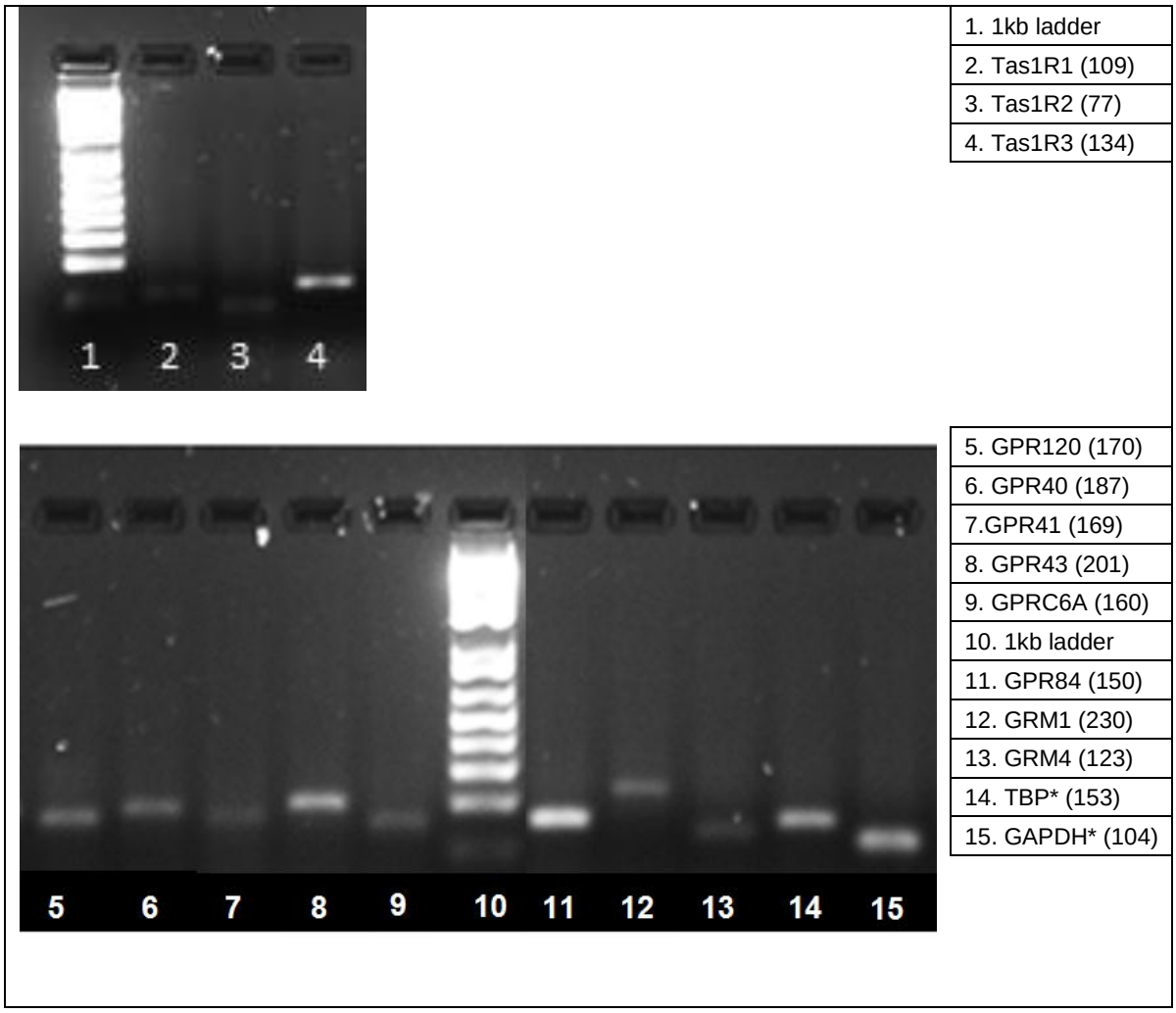
Total RNA was extracted using TRIzol® reagent (Life Technologies – Invitrogen, USA) and RNeasy Mini Kit (Qiagen, USA). Tissue fragments of 5 mg were homogenized using TissueRuptor (Qiagen, USA) in 800 ml TRIzol® reagent. After the addition of 200 ml chloroform, the samples were incubated for 2 min at room temperature, centrifuged for 15 min at 13 000 g (4 ^{o}C). The aqueous RNA phase was separated from DNA/protein fraction and mixed with 500 ml of 70% ethanol, then extraction was continued with RNeasy columns according to instruction. Final purification was performed in 0.1 volume of 3 M sodium acetate and 2.5 volumes of 100% ethanol, sample was incubated for 30 min 4 ^{o}C. RNA was centrifuged for 30 min at 13 000 g (4 ^{o}C), the pellet was washed with 70% ethanol, and then the RNA was resuspended in RNase-free water. Quantification and estimation of purity were controlled using NanoDrop 8000 (Thermo Scientific, USA). All RNA samples were stored at -80 ^{o}C freezer until use.

Reverse transcription and real-time PCR

Experiment was carried out according to guideline for real-time PCR of Bustin et al. 2009. First strand cDNA synthesis was generated by reverse transcription from 1ug of total RNA, using cDNA synthesis kit QuantiTect Reverse Transcription Kit (Qiagen, USA) according to protocol. PCR amplification was performed with 40 cycles, using an ABI 7900 HT Real Time PCR machine (Applied Biosystem, USA) and Power SYBR Green PCR Master Mix (Applied Biosystems, USA), according to the standard manufacturer's instructions. Primer probe sets were designed using gene sequences from the GenBank database and were purchased directly from Sigma-Aldrich. Each set of primers was optimized for annealing temperature and extension times. Primers concentrations were 0.4 mmol/l. Positive and negative controls were run in parallel from master mixes. All quantitative PCR assays were performed in triplicate, and a six independent experiments were conducted. Presence of all amplicons: Tas1R1, Tas1R2 and Tas1R3, GPR40, GPR43, GPR41, GMR1, GPR84, GPRC6A, GRM4 and GPR120, was tested for all amplicons with the conventional PCR, results are shown in Figure 1.

Statistics

To extract CT and efficiency data from run file we used SDSv2.4 software (Applied Biosystems, USA). Threshold line was adjusted automatically by program based on two housekeeping genes TBP and GPDH. Data obtained from real-time PCR experiments was processed using R environment (CRAN) (R Development Core Team, 2013). Fold-change values of expression were calculated using Pfaffl model corrected with efficiency (Pfaffl, 2001). Samples for which the cycle threshold was above 35 were excluded from the analysis. The GOI and HK efficiencies only with $R^2 > 0.99$ were included in our model. Expression levels of genes of interests (GOI) were normalized against two housekeeping genes (HK) transcripts in the same plate. The second step was to calculate fold-change values using average expression and efficiency of chosen gene and tissue in pigs, regarding its low and stable expression across replicates. We chose Tas1R1 expressed in circumvallate papillae (CV). Log2 transformation method was used on fold-change data during two-way ANOVA tests. The differences between single means were calculated by Tukey HSD (honest significant difference) test.



1. 1kb ladder
2. Tas1R1 (109)
3. Tas1R2 (77)
4. Tas1R3 (134)

5. GPR120 (170)
6. GPR40 (187)
7.GPR41 (169)
8. GPR43 (201)
9. GPRC6A (160)
10. 1kb ladder
11. GPR84 (150)
12. GRM1 (230)
13. GRM4 (123)
14. TBP* (153)
15. GAPDH* (104)

Figure 1.
Agarose gels (2%) following electrophoresis of PCR products from the newly designed primers for 11 taste receptors showing the amplification of single products/bands of the predicted size, indicated as the number of base pairs in brackets following the gene name. * indicates 2 housekeeping gene controls.

Results

The pattern of gene expression across all the samples is presented with a heatmap in Figure 2. Using hierarchical clustering we produced horizontally located histogram, which divided samples into 10 main clusters according to pattern similarity. The first seven clusters belong to one bigger group, we can noticed presence of only one gene GPR120 and three tissues CD, CA and SR. Additionally, in two first clusters samples have been divided according to experimental feeds.

To have a look on our results from another point of view, we analysed samples with two-way ANOVA and TukeyHSD test creating a set of bar plots (Figures 3-5). We considered three factors Gene, Tissue or Diet, and interaction effects between them.

Tas1R family members Tas1R1 and Tas1R3 were significantly expressed in all 12 tissue samples (P-value<0.05), see Figure 3. We found that Tissue factor was significant in both Tas1R1 and Tas1R3 expression (P-value<0.001 and 0.05, respectively), however no differences were detected for Diet factor. Basically, regarding Tissue factor, Tas1R1 had the highest fold-change (P-value<0.05) in both stomach tissues (ridge and antrum, fold-change (FC) respectively: 0.00492 and 0.00370). Next, Tas1R3 had the highest fold-change (P-value <0.05) in SR tissue (FC 0.0426) and SA (FC 0.0253) which shared the same significance level with IL (FC 0.0138). The Diet:Tissue interaction was significant for Tas1R1 (P-value<0.01), or showing a tendency (P-value=0.1) for Tas1R3 as well. The Tas1R2 had a tendency (P-value=0.1) to be expressed significantly different by tissues. HSD test reviled significantly high (P-value<0.05) FC level in CV (FC 0.00122) respect other tissues. Surprisingly, Tas1R2 expression was not detected in SR, SA, DD, IL, JE, CA, CD and CP.

In contrast, GRM1 and GRM4 were expressed in all selected tissues showing no Diet or Diet:Tissue interaction effect (Figure 4). Regarding the Tissue effect, GRM1 was the highest in SR (P-value <0.05) FC value of 0.000714, compare to other tissues, except CV (FC 0.000431). GPRC6A was significantly expressed only in CV papillae (P-value <0.05), with no differences between diets.

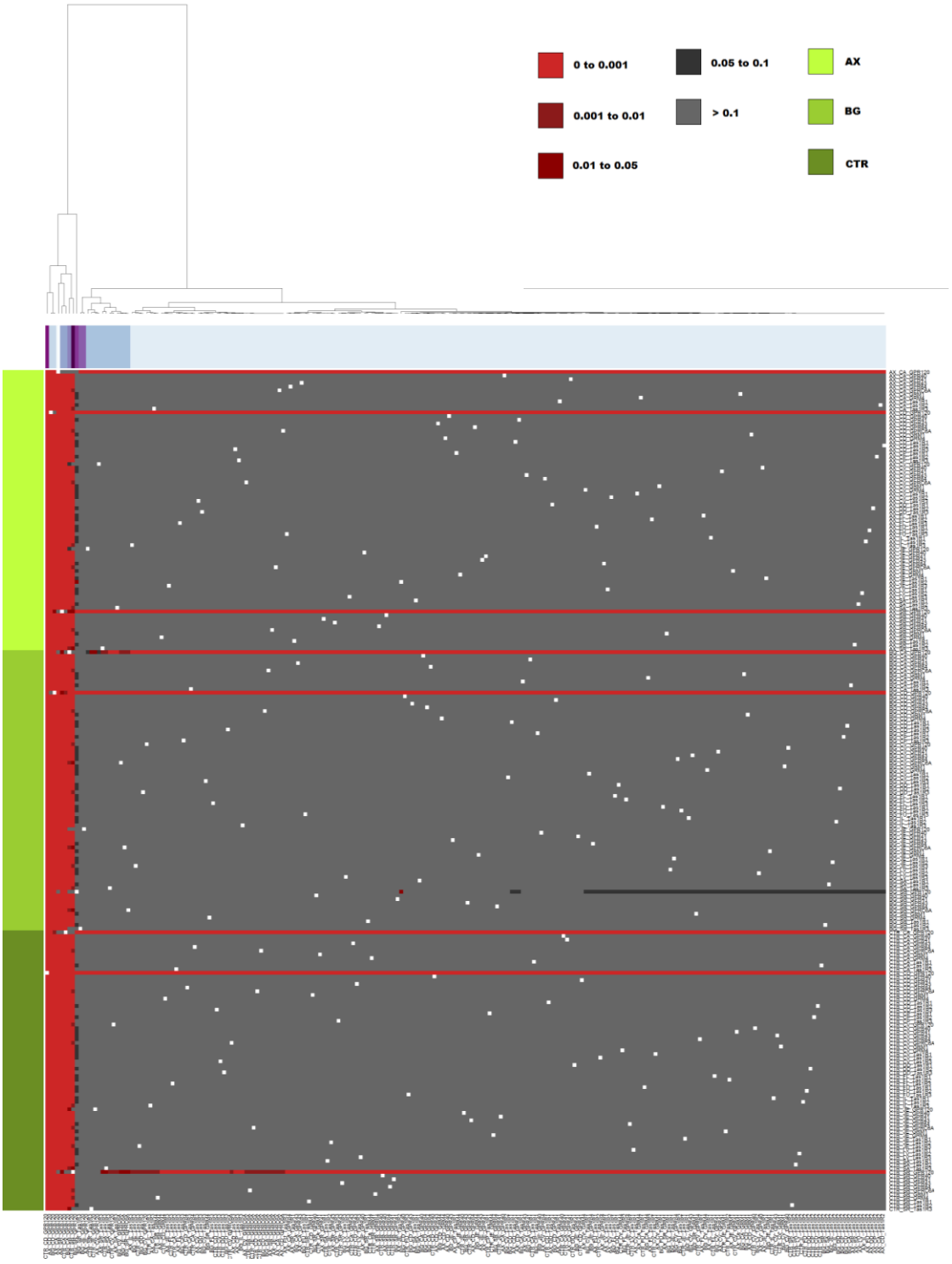


Figure 2
Heatmap presents comparison between means of single samples, three shapes of red denotes significant differences, dark grey - tendency and bright grey – non significant, white color means the same sample. Vertically, samples have been sorted according to Diet factor, biright green represents AX, next BG and darker green CTR. Horizontally, samples have been sorted according to the histogram clustering, different clusters are marked with blue – pourple color pallete. Heatmap was prepared using Tukey HSD and hierarchical cluster analysis.

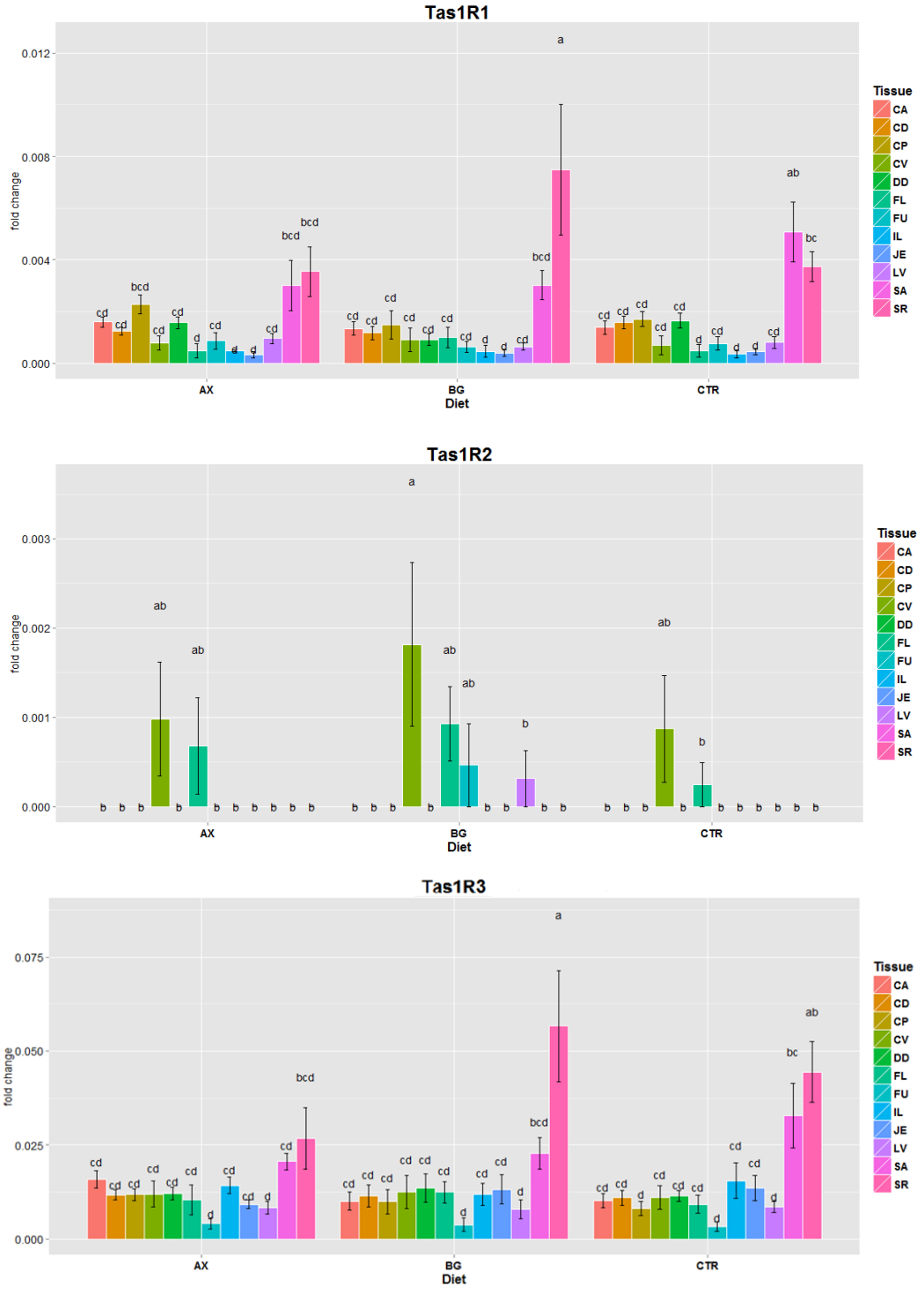


Figure 3
Plots present gene expression stated as fold change of Tas1R family receptors, along selected Tissues of gastrointestinal tract (CV-Circumvallate papilla, FU-Fungiform, FL-Foliateform, SA-stomach antrum and ridge (SR), DD-duodenum, JE-jejunum, IL-ileum, CP-colon proximal and distal (CD), CA-caecum (CA), LV-liver) of pigs fed three Diets (AX-arabinoxylans, BG-beta-glucans, CTR-wheat based control). Means (n=6) with different letters differ significantly (P<0.05).

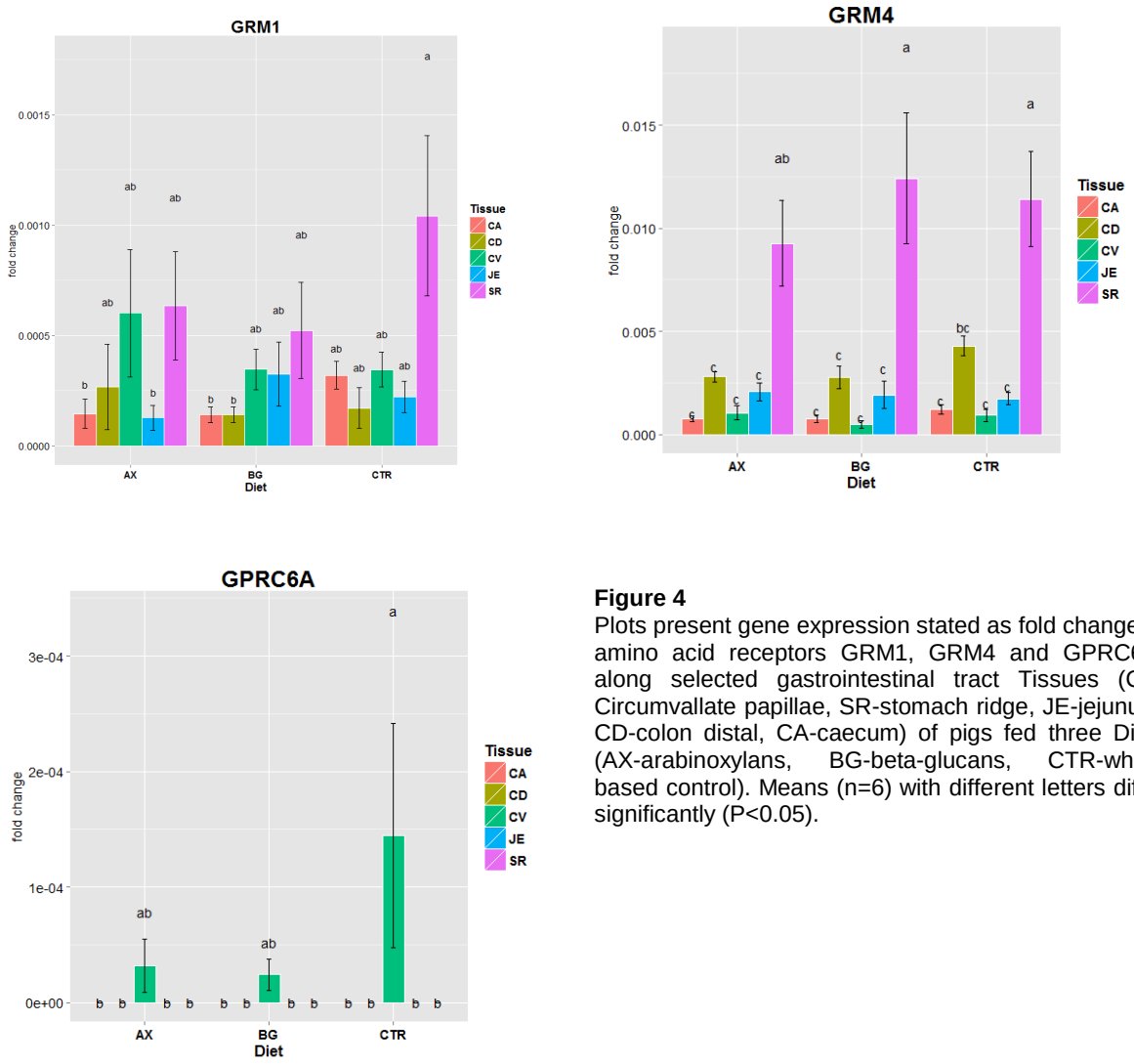


Figure 4
Plots present gene expression stated as fold change of amino acid receptors GRM1, GRM4 and GPRC6A, along selected gastrointestinal tract Tissues (CV-Circumvallate papillae, SR-stomach ridge, JE-jejunum, CD-colon distal, CA-caecum) of pigs fed three Diets (AX-arabinoxylans, BG-beta-glucans, CTR-wheat based control). Means (n=6) with different letters differ significantly (P<0.05).

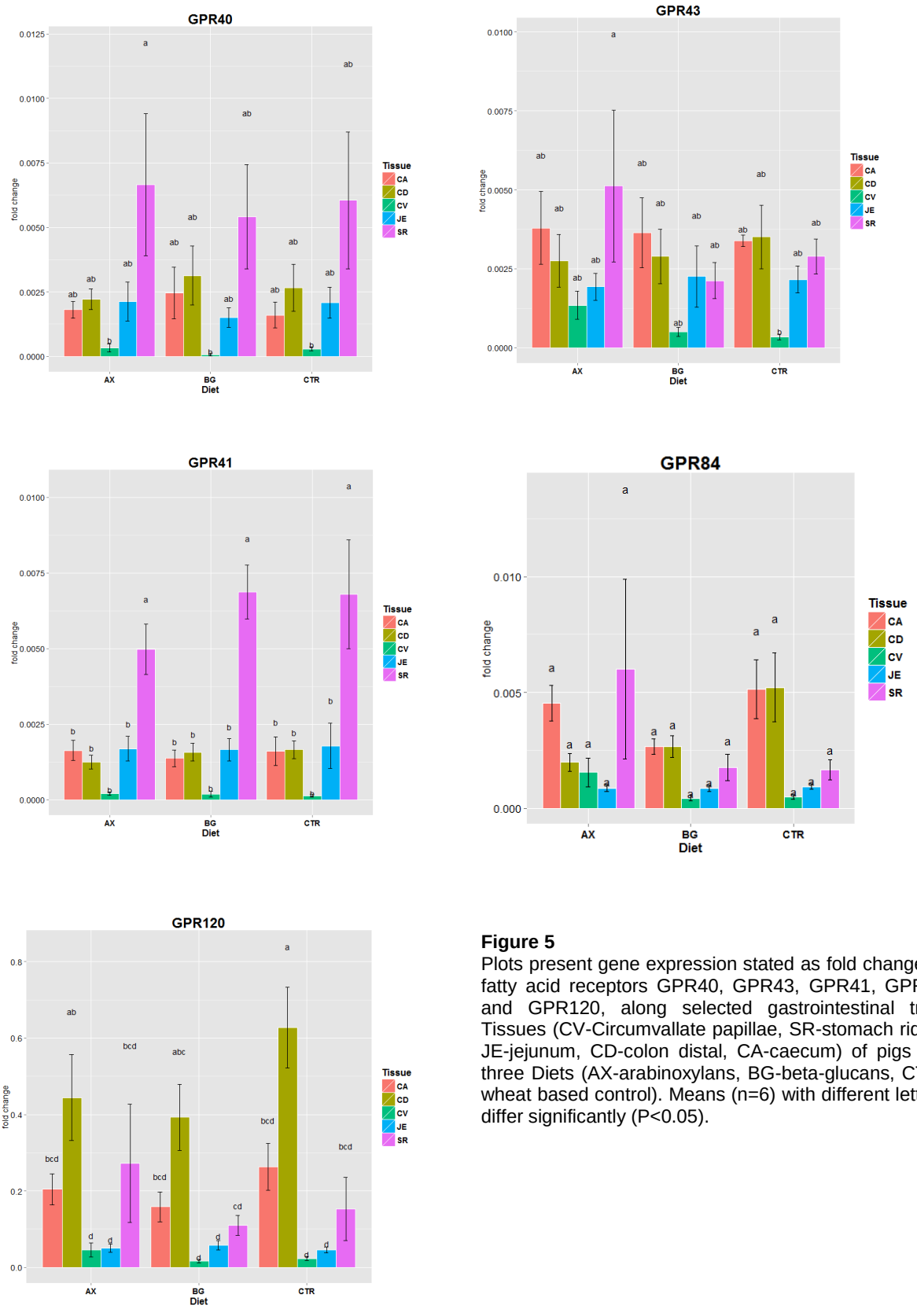


Figure 5
Plots present gene expression stated as fold change of fatty acid receptors GPR40, GPR43, GPR41, GPR84 and GPR120, along selected gastrointestinal tract Tissues (CV-Circumvallate papillae, SR-stomach ridge, JE-jejunum, CD-colon distal, CA-caecum) of pigs fed three Diets (AX-arabinoxylans, BG-beta-glucans, CTR-wheat based control). Means (n=6) with different letters differ significantly (P<0.05).

The GPR40, 41 and 43 belonging to free fatty acid receptor (FFAR) family were expressed in all five Tissues (Figure 5). Statistical analysis revealed no differences regarding Diet effect or Diet:Tissue interaction. GPR40, GPR43 and GPR41 genes expression levels was found to be different across Tissues, with P-value <0.01, <0.05 and <0.001 respectively. GPR40 had the highest level (P-value <0.05) in SR (FC 0.00603). GPR43 was significantly high (P-value<0.05) expressed in CA (FC 0.00362), SR (0.00340) and CD (FC 0.00302), in CV tissue (FC 0.000732) was the lowest (P-value <0.05). Analysis of Tissue effect using the HSD test showed that GPR41 fold-change was the highest in SR (FC 0.00621) followed by JE (FC 0.00171) and the lowest gene expression was found in CV (FC 0.000178). Two remaining genes from this group GPR84 and GPR120 also showed no Diet or Diet:Tissue effects. However a significant (P-value <0.01 and 0.001) Tissue effect was found for GPR84 and GPR120, respectively. GPR84 had the highest (P-value <0.05) fold-change level in CA (0.00405) and the lowest in JE (0.000891) and CV (0.000823). GPR120 had the highest (P-value <0.05) fold-change level in CD (0.479), next also CA (0.205) and the lowest in JE (0.0509) and CV (0.0286).

Statistical analysis of gene expression in circumvallate papillae tissue revealed significant (P-value <0.001) diet effect and a tendency (P-value=0.058) for a Gene by Diet interaction , see Figure 6. The GPR120 gene (0.0285 FC) was found to have the highest fold-change (P-value <0.05) with 0.0118 FC, in comparison to all other genes. A more detailed study of the CV Gene:Diet interaction using HSD test showed that GPR120 expression is significantly higher in AX fed animals compared to Control or BG fed animals. Foliate and fungiform tissues had been investigated using primers just for Tas1R family genes (Figure 6). The level of expression was Gene dependent (P-values respectively <0.001 and <0.01). Tas1R3 had the highest FC level in both FL (FC 0.0106) and FU (0.00366).

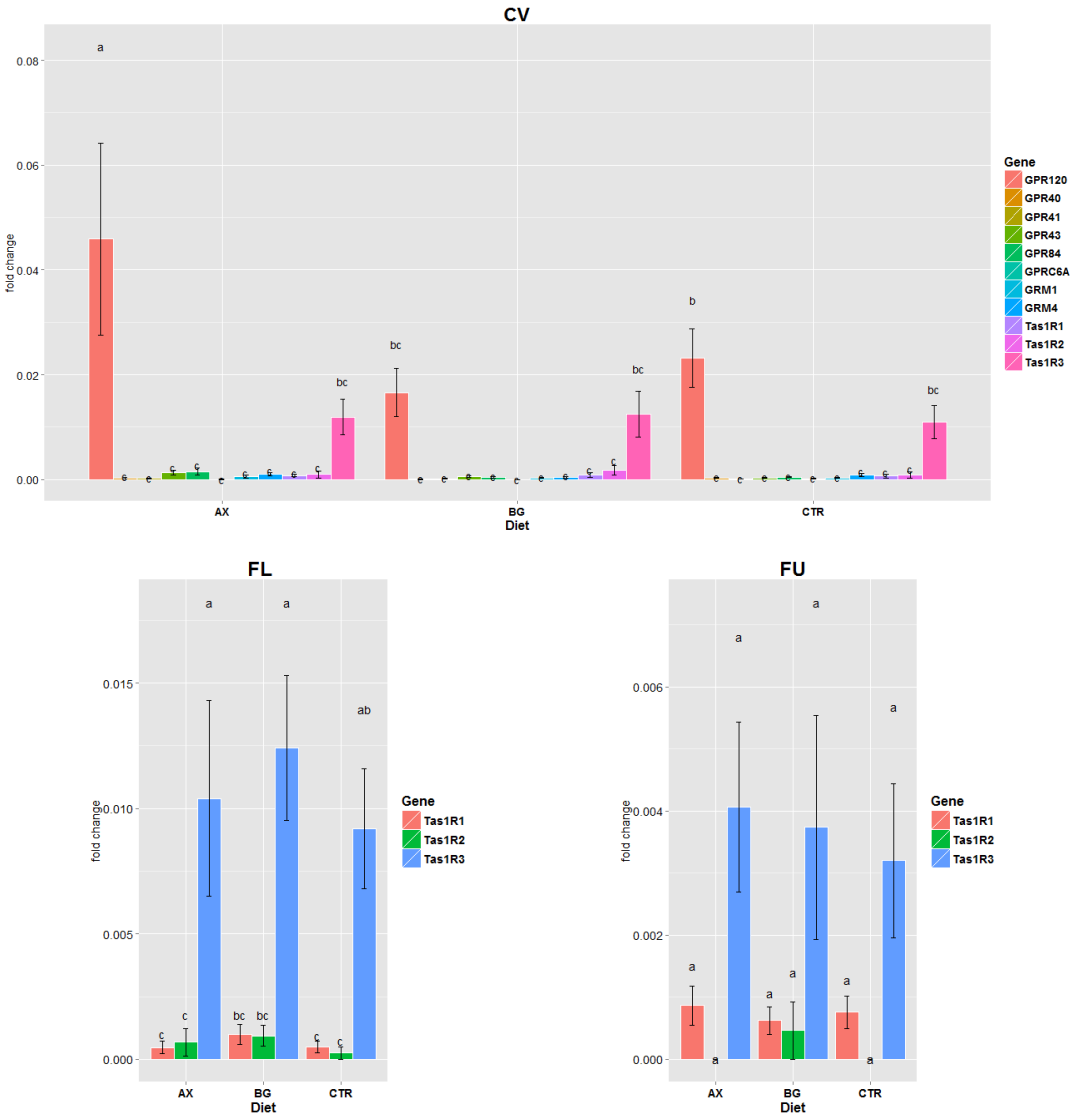


Figure 6
Plots present gene expression as fold change in three tongue papillae (CV-Circumvallate, FU-Fungiform and FL-Foliateform) of pigs fed three diets (AX-arabinoxylans, BG-beta-glucans, CTR-wheat based control). Means (n=6) with different letters differ significantly (P<0.05).

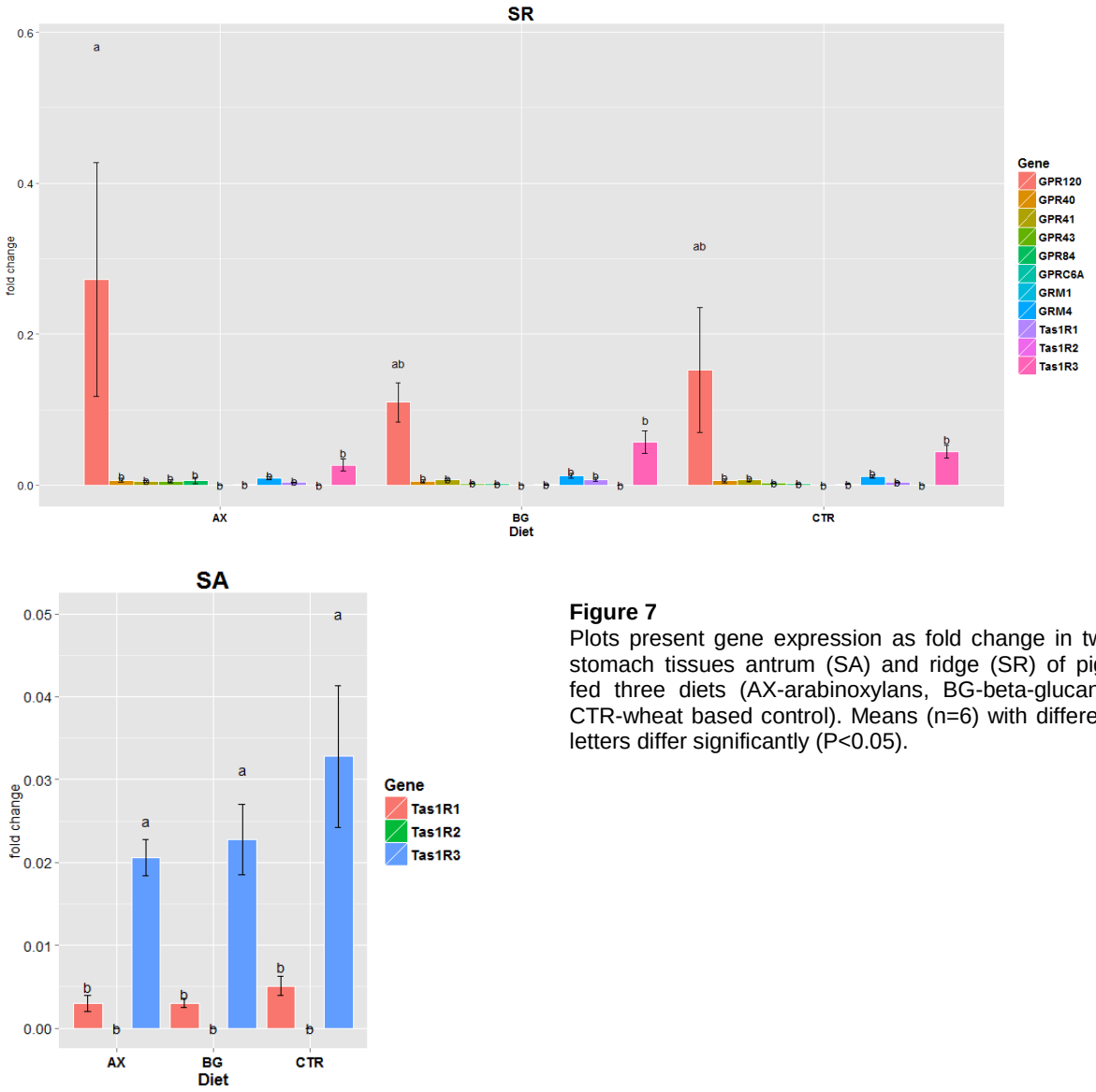


Figure 7
Plots present gene expression as fold change in two stomach tissues antrum (SA) and ridge (SR) of pigs fed three diets (AX-arabinoxylans, BG-beta-glucans, CTR-wheat based control). Means (n=6) with different letters differ significantly (P<0.05).

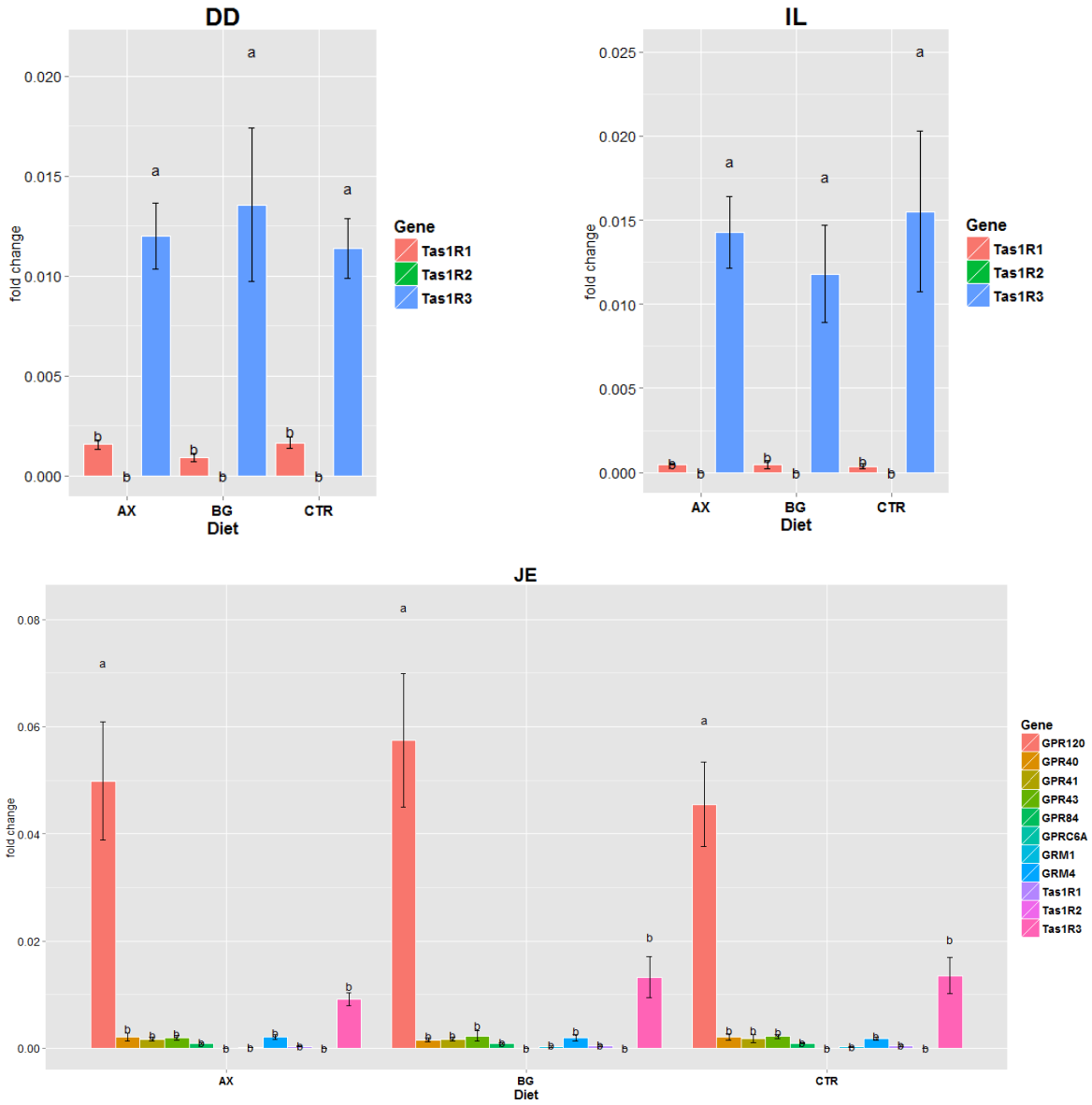


Figure 8
Plots present gene expression as fold change in three small intestine tissues (DD-duodenum, JE-jejunum and IL-ileum) of pigs fed three diets (AX-arabinoxylans, BG-beta-glucans, CTR-wheat based control). Means (n=6) with different letters differ significantly (P<0.05).

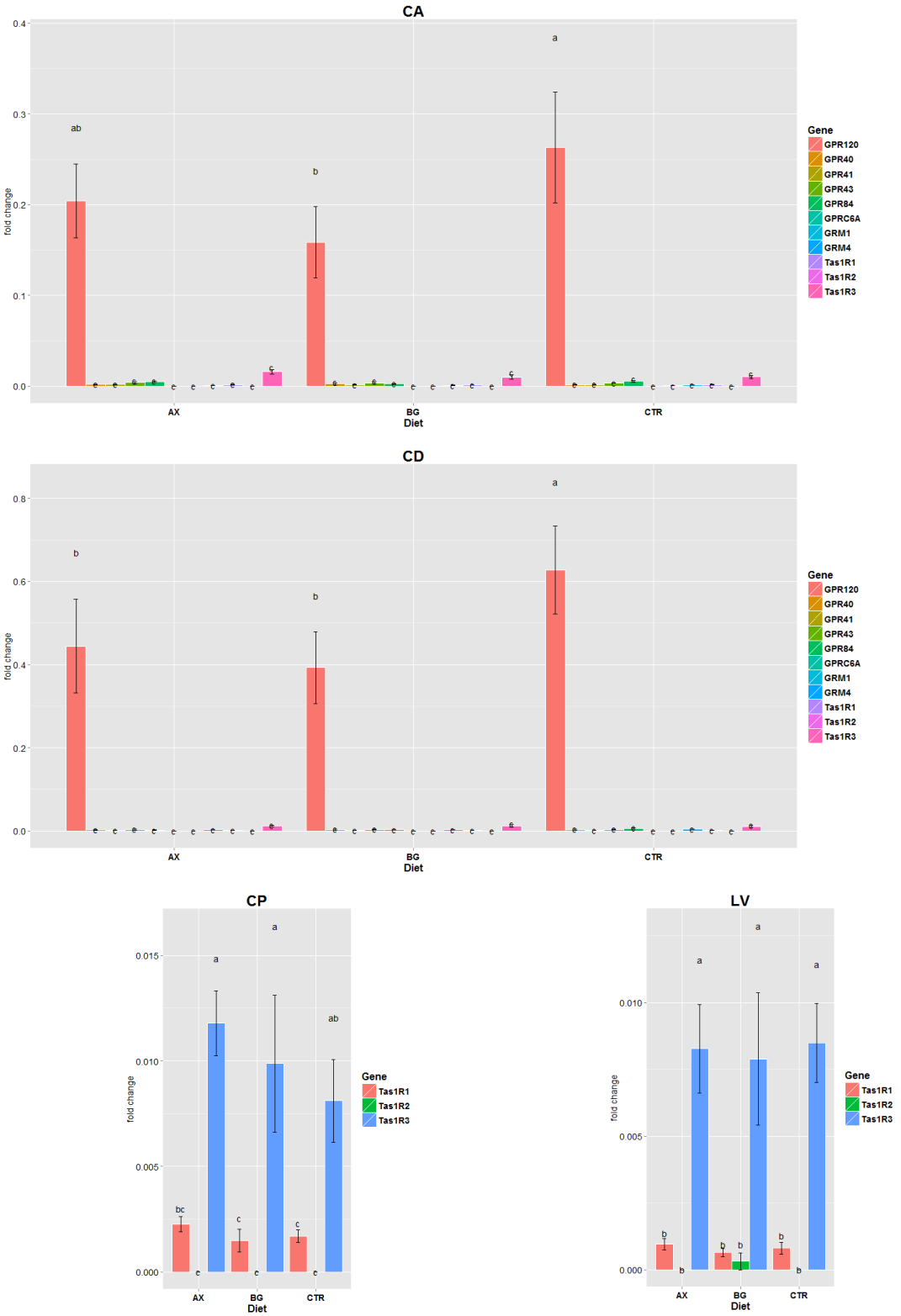


Figure 9
Plots present gene expression as fold change in three intestine compartments (CP-colon proximal and distal (CD), CA-caecum) and in liver tissue (LV) of pigs fed three diets (AX-arabinoxylans, BG-beta-glucans, CTR-wheat based control). Means (n=6) with different letters differ significantly (P<0.05).

Similar to the previous tissues in two stomach samples collected from ridge and antrum (Figure 6) and in duodenum (Figure 8) we detected significant Gene effects (P-value <0.001). Compared to the other genes, GPR120 expression was significantly (P-value <0.05) higher in SR(FC 0.179) and also in SA and DD Tas1R3 had the highest fold-change (P-value <0.05), 0.0253 and 0.0123 respectively.

In both caecum and colon distal (Figure 9) the GPR120 had the highest fold-change values compare to other samples (P-value <0.05), 0.205 and 0.479 respectively. Gene expression levels in liver did not show any Diet or Gene by Diet interaction effect. However, significant gene differences were found showing that Tas1R3 fold-change 0.00821 was the highest (P-value <0.05), compare to other members of Tas1R family.

Discussion

In the present study, we investigated how the addition of two soluble non-starch polysaccharides, arabinoxylan (AX) or β-glucan (BG) into swine feed, will influence the expression level of sugar, amino and fatty acids nutrient sensor genes in gustatory and non-gustatory tissues. We examined circumvallate (CV), fungiform (FU) and foliate (FL) papillae, then stomach antrum (SA) and ridge (SR), duodenum (DD), jejunum (JE), ileum (IL), colon proximal (CP) and distal (CD), caecum (CA), liver (LV). For most of the genes studied, it is the first time that the expression has been reported outside the oral cavity. In addition, it is also novel to report the effect of dietary fiber enrichment on the expression pattern of taste receptor genes in pigs. However, our hypothesis that fibre-rich diets may impact gene expression level has only been partially proved. We hypothesized that dietary fibre would limit nutrient absorption which, in turn, could result in an up-regulation of the taste receptors expression. Many of the nutrient/taste receptors studied were related to energy, consequently, the fact that the experiment was design with iso-energetic diets may have reduced the dietary impact on gene expression (Hayes, 2012; Roura et al., 2011; Shirazi-Beechey et al., 2011). So far most of the studies related to taste receptors gene expression have been performed on laboratory rodents. Non-rodent or

human taste receptors research has been recently reported in horses, dogs, cats (Daly et al., 2012a; Dyer et al., 2009) and humans (Toyono et al., 2007) and pigs (Daly et al., 2012b; Jager et al., 2013).

Positive interaction in circumvallate papillae was caused mostly by difference in GPR120 expression between treatments. Research of (Martin et al., 2012, 2011) revealed relations between GPR120 and CD36, another plasma membrane glycoprotein. This study did not include CD36, which is specifically found in the gustatory papillae and displays a very high affinity for LCFAs. Indeed, CD36 is another possible candidate of a lipid sensor in the oral cavity. What Martin and co-workers found is that CD36 expression is subjected to a short-term lipid-mediated down-regulation in mouse taste buds during food intake, whereas GPR120 gene expression remains unchanged, is consistent with distinct functions. In that contest GPR120 gene expression could be subjected to a long-term lipid-mediated down-regulation. This relation may be a reason why our animals fed Control and beta-Glucan diets had lower GPR120 expression than pigs on arabinoxylan enriched diet. Nutrient receptors located within Circumvallate papillae of animals under Arabinoxylan diet responded to lower amounts of LCFAs.

We observed that expression of the selected genes was often higher in porcine stomach compare to other tissues. It was particularly clear when looking at the expression pattern of Tas1R1 or Tas1R3 where a positive Diet:Tissue interaction was found. Such a high expression in GIT of animals under high fibre diet might be due to an increase in cell proliferation (Jin et al., 1994). However, increased proliferation should also affect housekeeping genes, but in our experiment HK genes were at the similar levels (data not shown). A number of studies associate a viscous meal, i.e. fibre-enriched, with a delay in gastric emptying (Goetze et al., 2007; Kaplan et al., 1994; Park and Camilleri, 2005; Schwartz et al., 1993). Gastric parameters such as gastric distension provide a direct negative feedback signal to inhibit eating and together intestinal signals stomach have been reported to interact to promote satiation (Goetze et al., 2007; Kaplan et al., 1994; Park and Camilleri, 2005; Schwartz et al., 1993). This concept suggest a limited role of stomach in nutrient sensing and higher correlation to volume of meal and fullness effect, however recent findings show chemosensory properties of brush cells in the gastric mucosa of mice (Eberle et al., 2013). We may speculate that similar mechanisms

exist in the porcine stomach. Additionally, different pattern of expression in those regions may explain a different role in monitoring the composition of ingested food (Delzenne et al., 2010). Though it does not entirely explain the situation where pigs under the fibre enriched treatments (BG and AX) have the highest and the lowest respectively gene expression (with Control diet in the middle) of Tas1R3 and Tas1R1 in SR. In addition, that effect has not been observed in other genes, this result is interesting with a need for further investigations.

The presence of nutrients in the gastrointestinal tract is a principal driver in modulating gene expression of nutrient sensors. Our study includes samples from different intestinal compartments, such as duodenum (DD), jejunum (JE), ileum (IL), caecum (CA), colon proximal (CP) and distal (CD). They have been characterized with a low expression of sweet, umami, amino and fatty acids receptors, regardless of the diet. Low level of gene expression is related to a high nutrient availability for a specific taste receptor. Recent studies indicate at an important role of nutrient sensing in energy management (Depoortere, 2014b; Andrew W. Moran et al., 2010; Nakamura et al., 2014; Raybould, 2009). The latest study showed high similarity in receptors genes expression between humans, pigs and mice in the distal ileum and a high similarity between human and pigs in the colon. Additionally, levels of gene expression depends of GIT compartment (van der Wielen et al., 2014). In our case it means that, even with NSP enriched diets, sufficient nutrients (including energy) were available in optimal amounts to provide stable growth and the well-being of animals. However, non-iso-energetic treatments in extended periods may change the nutritional status and the gene expression profiles. When rats were fed treatments with different energy content, the Tas1R3 gene expression in taste buds was decreased by High-Fat diet, and OB-Rb rats gene expression was markedly increased by chronic diet restriction. But, Tas1R2 expression did not change (Chen et al., 2010). Moran et al. 2010a demonstrated that T1R2/T1R3, are part of a sugar-sensing regulatory machinery that regulates transports of dietary sugars expression in response to high dietary CHO. Similar experiments have been done also on rodents and horses (Mace et al., 2007; Merigo et al., 2011). GPR43 and GPR41 are expressed in the lower intestine, suggesting that they act as a sensor and mediate metabolic adaptations to short chain fatty acids generated by bacterial fermentation of polysaccharides (Nøhr et

al., 2013). Moreover, the FFAR4 (GPR120) receptor has been proposed as a sensor of unabsorbed LCFAs reaching the lower intestine, increases in plasma GLP1, and the inhibition of the gastro-intestinal motility (Hirasawa et al., 2005).

Although it is known that Tas1R2 is widely distributed throughout the body (Dotson et al. 2010), we did not find significant levels of Tas1R2 expression in non-gustatory tissues except in the liver. It is widely accepted that T1Rs are expressed in enteroendocrine cells (EEC). However, our data is consistent with previous reports showing no expression of Tas1r2 in non-taste tissues van der Wielen et al. (2014). However, our results are not consistent with previous studies by Toyono et al. 2007 and Roura and Tedó 2009 where Tas1R1, Tas1R2 and Tas1R3 receptor mRNA was expressed in liver.

Our results indicated that expression levels of Tas1Rs changes among papillae. Gene expression of Tas1R2 in tongue taste buds were the highest in CV and lowest in FU, we did not record it in Tas1R1 and Tas1R3. Low expression of Tas1R2 or Tas1R3 in Fungiform papillae could be related to the lower amount of taste cells in that type of papilla, and not with treatment effect. Previous studies showed that members of T1Rs, are expressed in a subset of the taste bud cells, but differ with the expression pattern among papillae. In situ hybridizations and gene expression studies, in subsets of laboratory rodents taste receptor, usually showed Tas1r3 as the most strongly expressed in all fungiform, foliate and circumvallate papillae. Next, Tas1r1 is usually expressed higher in Circumvallate and lower in foliate and fungiform, in contrary to Tas1R2 but its expression vary between studies (Kim et al., 2003; Nelson et al., 2001; Stone et al., 2007). Tas1R3 is expressed higher than Tas1R1 and Tas1R2 (Dyer et al., 2005).

Transcripts of metabotropic glutamate receptors (GMR1 and GMR4) were found in circumvallate papillae, stomach, caecum, jejunum and colon distal. The presence of these receptors may indicate an involvement in sensing products derived from dietary protein. Previously, E. Nakamura, Hasumura, Uneyama, & Torii (2011) in their study characterized the distribution of glutamate and other amino acid sensors on the gastric mucosa of rat. The GPRC6A receptor was expressed in G-cells and D-cells in the gastric antrum of men, swine, and mouse. The GPRC6A expression has been reported in several

tissues such as oral tissues, stomach, pancreas, liver, intestine, kidney, brain, skeletal muscles including hart and bone (Haid et al., 2012; Pi and Quarles, 2012), however, in our study we could only confirm a significant level of expression in pig tongue's CV.

In contrast, the expression of the free fatty acid receptors (FFARs) GPR40, GPR43, GPR41, GPR84 and GPR120 in our pig tissues was positive for circumvallate papillae, stomach, caecum, jejunum and colon distal, their expression was unchanged concerning diet factor. Previous studies identified GPR40 expression in mice taste buds, stomach, pylorus, duodenum, ileum, pancreas and in many endocrine cells of the gastrointestinal tract cells but not in D cells (Cartoni et al., 2010; Edfalk et al., 2008; Hirasawa et al., 2005; Janssen et al., 2012). Quantitative reverse transcription-PCR showed its expression in human brain, pancreas, adipocytes and peripheral blood mononuclear cells (Briscoe et al., 2003; Trayhurn and Denyer, 2012). GPR43 and GPR41 are expressed also in multiple tissues in rodents (adipose tissue, small intestine and colon) and human immune cells, adipose tissue, small intestine, colon, spleen, pancreas and heart. GPR41 is also present in human skeletal muscle, whereas GPR43 not (Layden et al., 2013). GPR84 tissue distribution has not been well characterized, and is not closely related to any of the previously identified fatty acids activated GPCRs (Yonezawa et al., 2013; Yousefi et al., 2001). Studies suggest that GPR84 involvement in fatty acid sensing is linked with immunological regulation, since it is expressed in adipocytes, leukocytes, granulocytes and monocytes/macrophages under inflammatory conditions (Nagasaki et al., 2012; Suzuki et al., 2013). FFARs expression analyses with northern blot revealed that human GPR84 is also expressed in the brain, heart, muscle, colon, thymus, spleen, kidney, liver, intestine, placenta and lungs (Im et al., 2009). Studies on laboratory rodents showed its expression within mouse spleen and liver tissues (Im et al., 2009), in rat, GPR84 was significantly expressed by posterior part of the tongue, probably circumvallate papillae, but was absent in fungiform papillae (Gilbertson et al., 2010). It has been reported that GPR120 mRNA and protein is expressed along the whole gastrointestinal tract and liver in the pig, mouse and human (Ichimura et al., 2012; van der Wielen et al., 2014; Wellendorph et al., 2009; Zhao et al., 2013).

In conclusion, our research shows significant differences in the level of expression of the taste and nutrient sensing genes under study mainly as a function of the gene type and the tissue. In addition, we observed diet (fibre) effects and a Gene by Diet interaction in tongue CV, where GPR120 expression was significantly higher in AX enriched group compared to the control and BG groups. In addition, a Tissue by Diet interaction was observed for Tas1R1 and Tas1R3 with the highest expression in stomach ridge tissue of animals fed beta-glucans diet, indicating a possibly important role of stomach in nutrient chemosensing. Future studies should explore the effect of altering dietary energy density on key nutrient sensors.

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4.2. Identification and expression profile of the porcine bitter taste receptor repertoire along the gastrointestinal tract.

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Abstract

Bitter taste is a basic taste modality, required to safeguard animals against consuming potential toxic substances. Recent progress in animal genomics has identified the gene family responsible for bitter perception, the T2R family which belongs to G-protein-coupled receptors (GPCR) superfamily. In pigs, the most recent genome annotation (*Sus scrofa* 10.2) included 17 functional bitter genes. However, our current knowledge on the porcine Tas2R system is very limited. The present study aimed at identifying the expression profile of the porcine Tas2R gene repertoire along the gastrointestinal tract. Fourteen porcine Tas2R-like genes were identified based on sequence homologies with humans and mouse genes: Tas2R1, Tas2R3, Tas2R4, Tas2R9, Tas2R10, Tas2R16, Tas2R20, Tas2R38, Tas2R39, Tas2R41, Tas2R42, Tas2R60, Tas2R134 and three gene versions for Tas2R7. The sequences obtained "*in silico*" were then used to study their expression by qPCR in five porcine gastrointestinal tissues: circumvallate tongue papilla (CV), stomach ridge (SR), caecum (CA), jejunum (JE) and colon distal (CD). Our results confirmed the presence of members of the porcine tas2r family in all investigated tissues. The Tas2R3, Tas2R4, Tas2R7, Tas2R9, Tas2R10, Tas2R20,

Tas2R38 and Tas2R42 had a similar gene expression pattern, high in CV and SR, and low in JE, CA, CD ($P<0.05$). Compared to all the other genes, the tas2r134 presented the highest ($P<0.05$) expression. In contrast, lowest expressions were found for Tas2R1, Tas2R3 and Tas2R60. Our data represent a first step for more detailed molecular studies on bitter chemosensing, further studies are needed to characterise the functions of single receptors.

Keywords: bitter, Tas2R; bitter, gastrointestinal tract; pig.

Introduction

Recent progress unravelling the pig genome gave researchers better tools to understand the chemosensory mechanisms (Groenen et al., 2012). The mammalian sense of taste provides important information about the quality and nutritional value of food prior to its ingestion in the oral cavity (Behrens and Meyerhof, 2011). Nutrient sensing principally involves the interaction between tastant molecules and receptor cells. Nutrient receptor-expressing (or sensory) cells reside not only in the tongue papillae but also along the rest of the digestive tract and also other organs (i.e. heart, lungs or brain). They seem to be involved in the information pathway to the brain regarding the nutrient content of foods by mediating in endocrine responses. For example they seem to be involved in the maintenance of glucose homeostasis (Dotson et al., 2008). Pigs similar to humans recognize five basic tastes sweet, umami, sour, salty, and bitter. Bitter taste receptors (T2Rs) are a large family of G-protein-coupled receptors (GPCRs) responsible for bitter taste sensations in the mouth and the warning against potential toxic foodstuff. The human genome comprises of 25 different Tas2R genes and in rodents over 30. The members of T2R family could be divided into two main subclasses: first a very specific that detect one or a few bitter compounds; and a second, broadly tuned to respond to multiple ligands (Behrens and Meyerhof, 2009). During the evolution receptor sensitivity has been balanced with specificity. Mutations that increase specialization of receptor for a given agonist may

also decrease attraction for others (Born et al., 2013). The *in silico* analysis revealed several porcine tas2r genes homologous to human TAS2R genes (Groenen et al., 2012).

The goal of this study was initially to identify *in silico* porcine bitter taste receptor candidates by blasting human and mouse gene sequences on the pig genome and assess their expression along the porcine gastrointestinal tract using RT-PCR.

Materials and Methods

In silico analysis

We conducted a data-mining search to identify porcine T2R candidates by blasting (Basic Local Alignment Search Tool - BLAST, NCBI) the pig genome with the known sequences of the Tas2R human, mouse, rat and chicken genes.

Animals and tissue sampling

For the purpose of the study six 4-months-old Large White male pigs (23.9 ±2.4 kg) were housed at the Queensland Animal Science Precinct (University of Queensland, Gatton, Queensland, Australia). Experimental procedures were approved by the Animal Ethics Committees of the University of Queensland. Pigs were humanely sacrificed and samples were collected from tongue circumvallate papilla (CV), stomach ridge (SR), caecum (CA), jejunum (JE) and colon distal (CD). Tissues were immediately snap-frozen in liquid nitrogen and stored at -80^{o}C freezer until RNA extraction.

RNA extraction

Total RNA was extracted using TRIzol® reagent (Life Technologies – Invitrogen, USA) and RNeasy Mini Kit (Qiagen, USA). Tissue fragments of 5 mg were homogenized using TissueRuptor (Qiagen, USA) in 800 ml TRIzol® reagent. After the addition of 200 ml chloroform, the samples were

incubated for 2 min at room temperature, centrifuged for 15 min at 13000 g (4 ^{o}C). The aqueous RNA phase was separated from DNA/protein fraction and mixed with 500 ml of 70% ethanol, and then extraction was continued with RNeasy columns according to instruction. Final purification was performed in 0.1 volume of 3 M sodium acetate and 2.5 volumes of 100% ethanol following by incubation for 30 min at 4 ^{o}C. RNA was centrifuged for 30 min at 13000 g (4 ^{o}C), the pellet was washed with 70% ethanol, and then the RNA was re-suspended in RNase-free water. Quantification and estimation of purity were controlled using NanoDrop 8000 (Thermo Scientific, USA). All RNA samples were stored at -80 ^{o}C freezer until use.

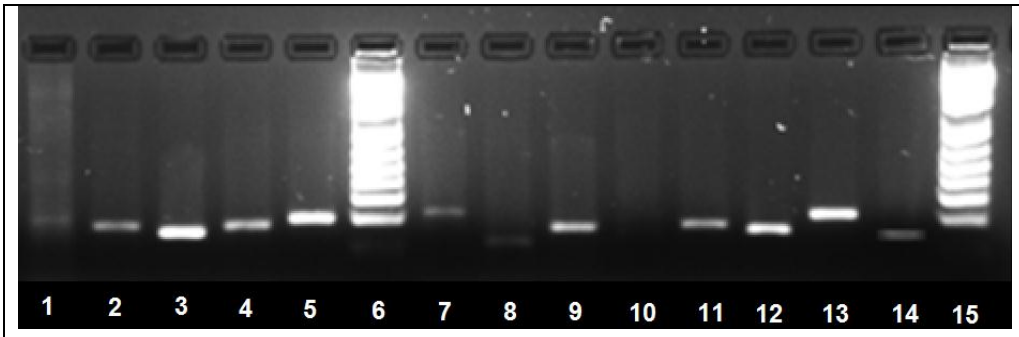
Reverse transcription and real-time PCR

The experiment was carried out according to the guidelines for real-time PCR of Bustin et al. 2009. First strand cDNA synthesis was generated by reverse transcription from 1 µg of total RNA, using cDNA synthesis kit QuantiTect Reverse Transcription Kit (Qiagen, USA) according to protocol. PCR amplification was performed with 40 cycles, using an ABI 7900 HT Real Time PCR machine (Applied Biosystem, USA) and Power SYBR Green PCR Master Mix (Applied Biosystems, USA), according to the standard manufacturer's instructions. Primer probe sets were designed using gene sequences from the GenBank database and were purchased directly from Sigma-Aldrich. Each set of primers was optimized for annealing temperature and extension times. The concentrations used were 0.4 mmol/l. All quantitative PCR assays were performed in triplicate, positive and negative controls were run in parallel from master mixes.

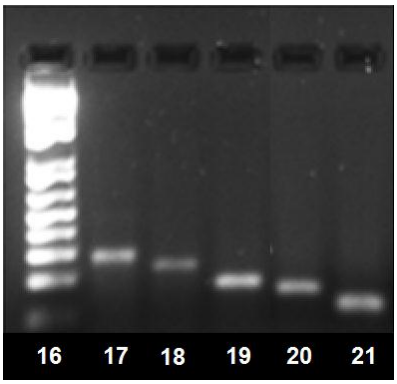
Statistics

To extract CT and efficiency data from run file we used SDSv2.4 software (Applied Biosystems, USA). The threshold line was adjusted automatically by the program based on two housekeeping genes TBP and GPDH. Data obtained from real-time PCR experiments was processed using R environment (RStudio, Inc., Boston, USA). Fold-change values of expression were calculated using

Pfaffl model corrected with efficiency (Pfaffl, 2001). The cut off for significant expression was set at 35 cycles. The GOI and HK efficiencies only with R^2>0.99 were included in our model. Expression levels of genes of interests (GOI) were normalized against two housekeeping genes (HK) transcripts in the same plate. The second step was to calculate fold-change values using average expression and efficiency of chosen gene and tissue in pigs. We chose Tas2R4 expressed in circumvallate papillae (CV) because of its low and stable expression across replicates. The differences between means (n=6) were calculated with Wilcox test (*pairwise.wilcox.test* {stats}). Plots and heatmap were created using *ggplot2* and *gplots* packages.



1. Tas2R1 (215)
2. Tas2R3 (186)
3. Tas2R4 (160)
4. Tas2R7-17 (188)
5. Tas2R7-44 (213)
6. 1kb ladder
7. Tas2R7-46 (236)
8. Tas2R9 (116)
9. Tas2R10 (173)
10. Tas2R16 (138)
11. Tas2R20 (182)
12. Tas2R38 (155)
13. Tas2R39 (224)
14. Tas2R41 (134)
15. 1kb ladder



16. 1kb ladder
17. Tas2R42 (300)
18. Tas2R60 (248)
19. Tas2R134 (184)
20. TBP* (153)
21. GAPDH* (104)

Figure 1
Agarose gels (2%) following electrophoresis of PCR products from the newly designed primers for 14 taste receptors showing the amplification of single products/bands of the predicted size, indicated as the number of base pairs in brackets following the gene name. * indicates 2 housekeeping gene controls. The band for Tas2R16 is too faint to visualise in this format, however it was present.

Results and discussion

Our "in silico" data confirms the findings of Groenen et al (2012) consisting of 14 Tas2R-like genes in the pig genome: Tas2R1, Tas2R3, Tas2R4, Tas2R10, Tas2R9, Tas2R16, Tas2R20, Tas2R38, Tas2R39, Tas2R41, Tas2R42, Tas2R60, Tas2R134 and Tas2R7 with its three gene versions: R7-17, -44 and -46. In addition, we were able to confirm the presence of all the porcine Tas2R genes in all the investigated porcine tissues (circumvallate tongue papilla, stomach ridge, jejunum and colon distal). Previous studies used also RT-PCR to identify Tas2R mRNA in human or rodent gastrointestinal tissue or model cell lines for enteroendocrine cells (Chen et al., 2006; Dotson et al., 2008; Kaji et al., 2009; Rozengurt et al., 2006; Wu et al., 2005, 2002).

Samples similarity is presented at heatmap using p-values, see Figure 2. The histogram showed five main clusters. The first cluster (from the up/left), could be further divided into two sub-clusters containing Tas2R134 and Tas2R16 from all five tissues. The second cluster contained several genes expressed in circumvallate. Cluster number 3 contains 10 samples representing genes expressed within JE, additionally contain all Tas2R60 samples. Remaining two clusters contain predominantly samples from SR, CA and CD.

Looking at the expression pattern of single genes by tissue, Tas2R1 has the same levels of expression along all tissues (Figure 3). Several genes such as Tas2R3, Tas2R4, Tas2R7, Tas2R9, Tas2R10, Tas2R20, Tas2R38, Tas2R42 showed a similar pattern of expression (Figure 3, 4, 5 and 6), where the highest value was in CV and SR, and the lower in JE, CA, CD ($P<0.05$). Regarding Tas2R16 the level of expression in CD was significantly ($P<0.05$) higher than any of the other tissues (Figure 5). The Tas2R39 was highly expressed in JE ($P<0.05$). Tas2R41 in CA, CD and CV tissues (Figure 6) was at the higher level respect SR and JE ($P<0.05$). Tas2R60 was expressed significantly ($P<0.05$) higher in CV than in the remaining tissues (Figure 6). Tas2R134 (Figure 6) was expressed the lowest in CV ($P<0.05$), higher in CA, CD and the highest in SR, JE ($P<0.05$). In the study of Dotson et al. (2008) human Tas2R7 was not amplified from either human caecum cDNA or human enteroendocrine cells cDNA pool, contrary to Tas2R9 detected in both tissues. Wu et al. (2002) showed presence of the

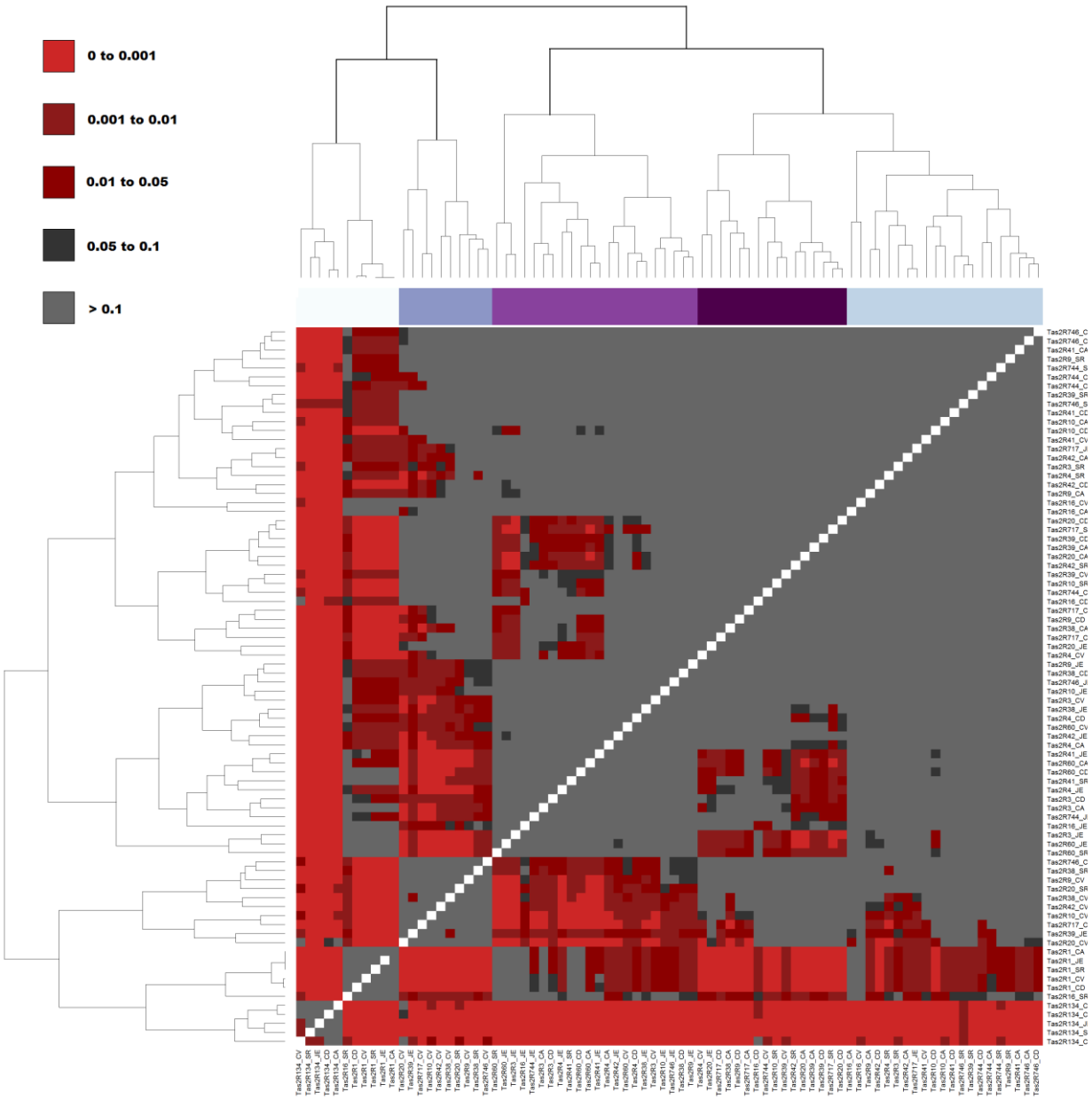


Figure 2.
Hierarchical clustering and heatmap representation of Tas2R genes differentially expressed in the indicated porcine tissues (CV-circumvallate papilla, SR-stomach ridge, JJ-jejunum, CD-colon distal and CA-caecum). Single sample name consists of Gene-Tissue abbreviations. Red and grey colours indicate statistical significance (p-value), see the legend. Samples have been sorted according to dendrogram clusters coloured in blue-violet. The matrix was prepared using Wilcox comparison and heatmap.2 function for hierarchical clustering in R (RStudio, Inc., Boston, USA).

bitter receptor subtypes from Tas2R1 to Tas2R12 in the duodenum and stomach, except Tas2R4 which was absent in the stomach. Looking from a gene as a main effect, regardless of the tissue type a significantly ($P<0.05$) higher, and lower levels of expression were found for Tas2r134 and Tas2R1, Tas2R3 and Tas2R60 respectively (Figure 7, 8 and 9). The latest update to pig genome assembly 10.2 instead of three Tas2R7 versions (Groenen et al., 2012) has only one Tas2R7-46 (LOC100523246). Additionally, later work of Colombo et al. (2012) confirmed presence of seven bitter receptors (Tas2R1, Tas2R3, Tas2R7, Tas2R9, Tas2R10, Tas2R16, and Tas2R38) with only one Tas2R7.

In our work we observed rather high expression of Tas2R genes in stomach tissue and may participate in the control of gastric emptying, as bitter compounds represent an aversive taste stimulus inhibiting feeding (Janssen et al., 2011). However, other studies on human present contrasting conclusions confirming (Wicks et al., 2005) or denying (Little et al., 2009) the influence of bitter compounds on the slowing of gastric emptying, similar to Wu et al. (2002).

In conclusion, our results showed the presence of 14 Tas2R genes in porcine GIT. Moreover, expression patterns of the Tas2Rs seem to be in line with previous reports in humans and laboratory rodents. Our work form the basis for further studies which should be aimed at characterizing the porcine T2R family at a protein level and to assess their genetic diversity.

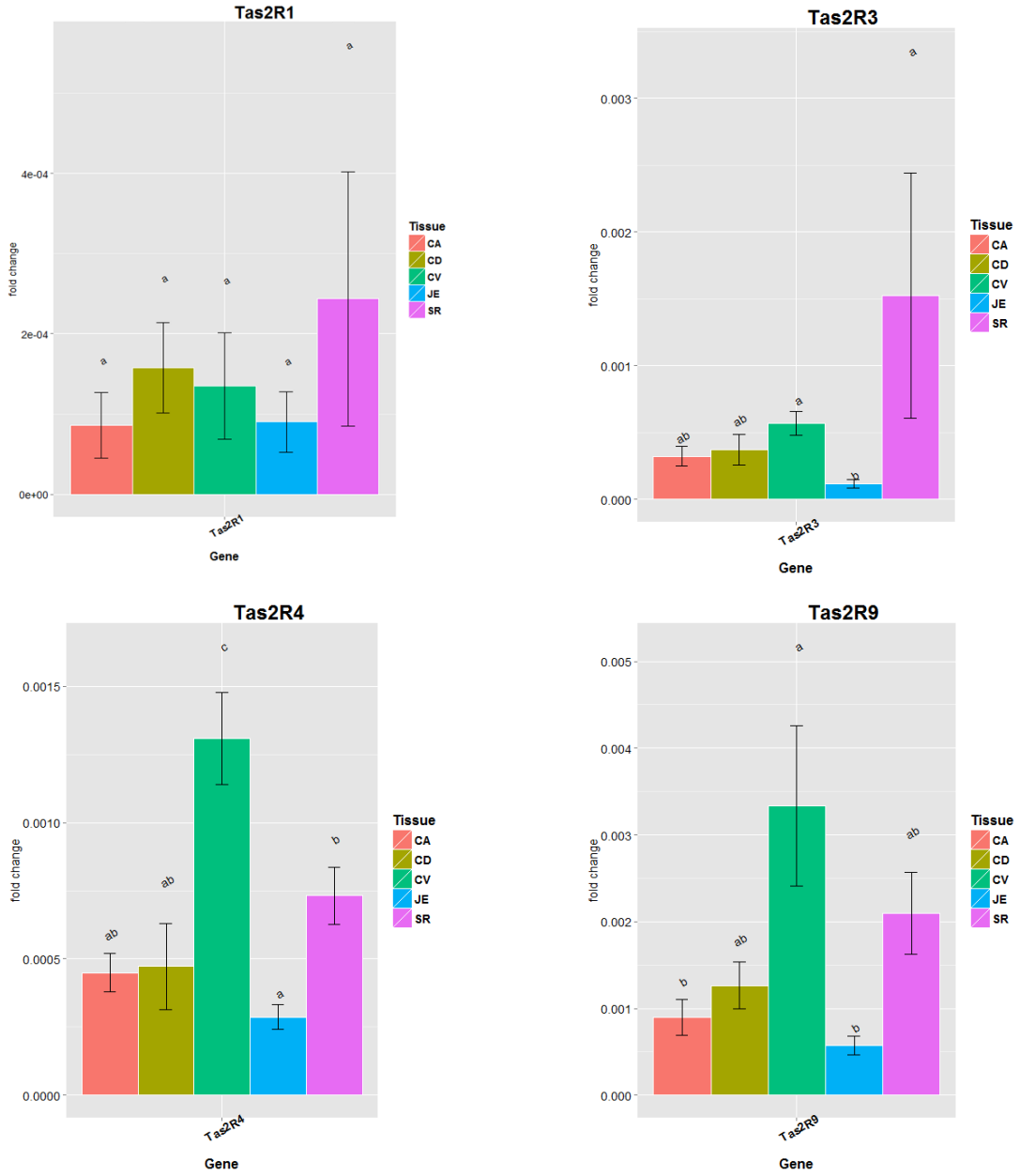


Figure 3.
Plots present the gene expression levels of Tas2R1, Tas2R3, Tas2R4 and Tas2R9 receptors along five porcine tissues (CV-circumvallate papilla, SR-stomach ridge, JJ-jejunum, CD-colon distal and CA-caecum). Gene expression was calculated according Pfaffl method (Pfaffl 2001) with two housekeeping genes (TBP and GPDH) and Tas2R4 expressed in circumvallate papillae (CV) to obtain fold-change values. Data were analysed by Wilcox test for multiple contrasts in R (RStudio, Inc., Boston, USA). Means (±SEM; n=6) with different letters differ significantly (P<0.05).

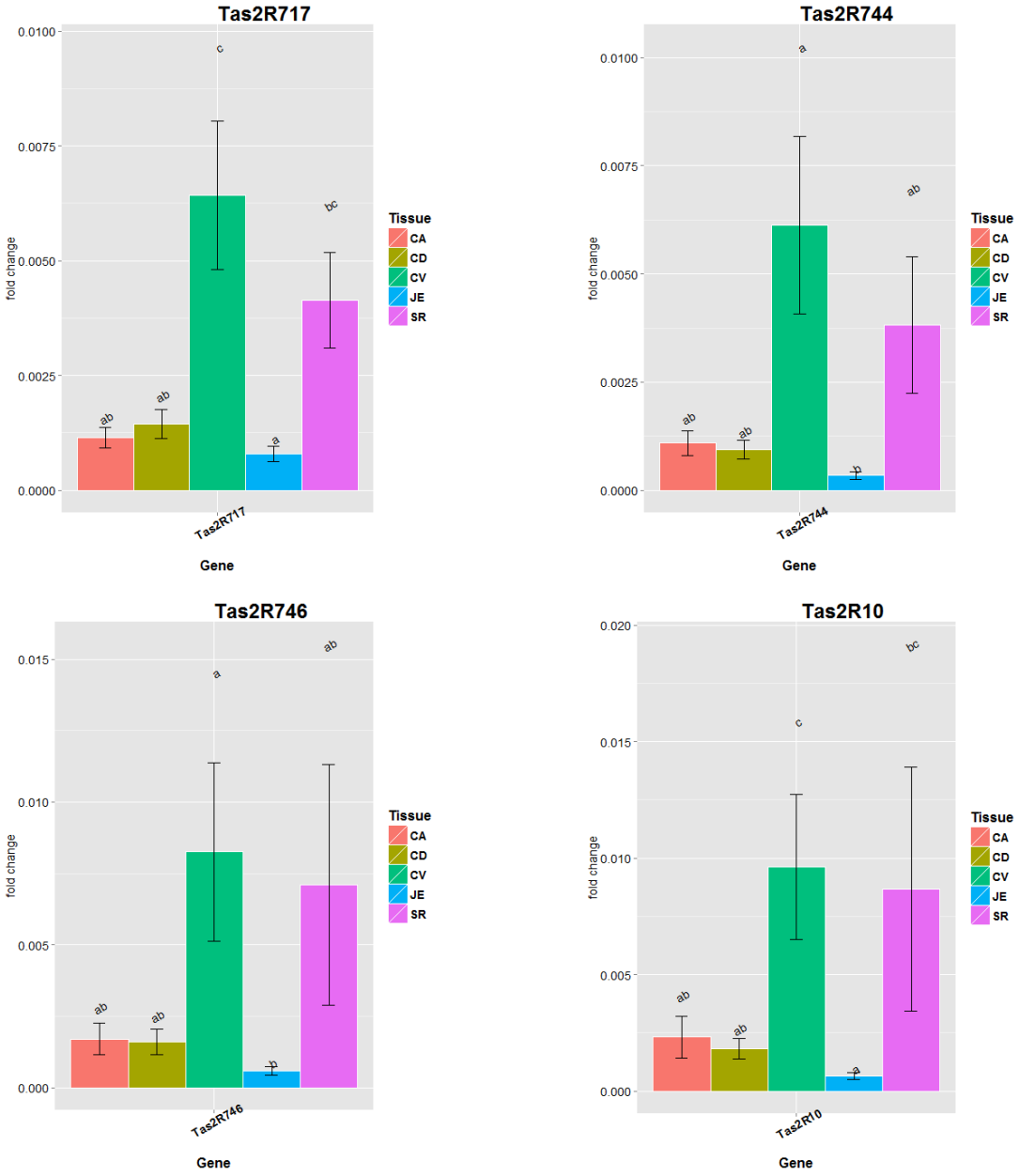


Figure 4.
Plots present the gene expression levels of Tas2R10 and three type of Tas2R7 (-17, -44 and -46) receptors along five porcine tissues (CV-circumvallate papilla, SR-stomach ridge, CA-caecum, JJ-jejunum and CD-colon distal). Gene expression was calculated according Pfaffl method (Pfaffl 2001) with two housekeeping genes (TBP and GPDH) and Tas2R4 expressed in circumvallate papillae (CV) to obtain fold-change values. Data were analysed by Wilcox test for multiple contrasts in R (RStudio, Inc., Boston, USA). Means (±SEM; n=6) with different letters differ significantly (P<0.05).

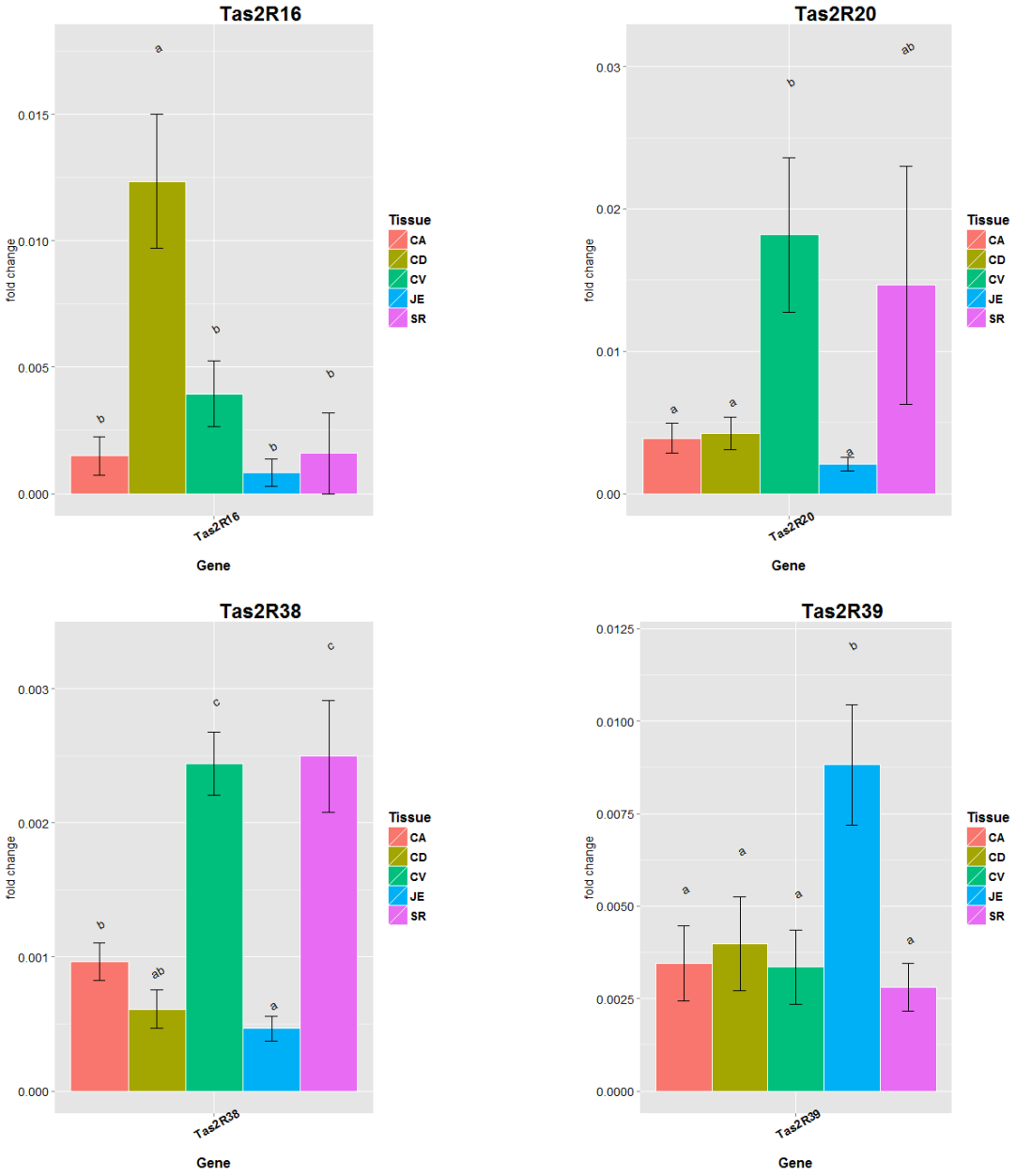


Figure 5.
Plots present the gene expression levels of Tas2R16, Tas2R20, Tas2R38 and Tas2R39 receptors along five porcine tissues (CV-circumvallate papilla, SR-stomach ridge, CA-caecum, JJ-jejunum and CD-colon distal Gene expression was calculated according Pfaffl method (Pfaffl 2001) with two housekeeping genes (TBP and GPDH) and Tas2R4 expressed in circumvallate papillae (CV) to obtain fold-change values. Data were analysed by Wilcox test for multiple contrasts in R (RStudio, Inc., Boston, USA). Means (±SEM; n=6) with different letters differ significantly (P<0.05).

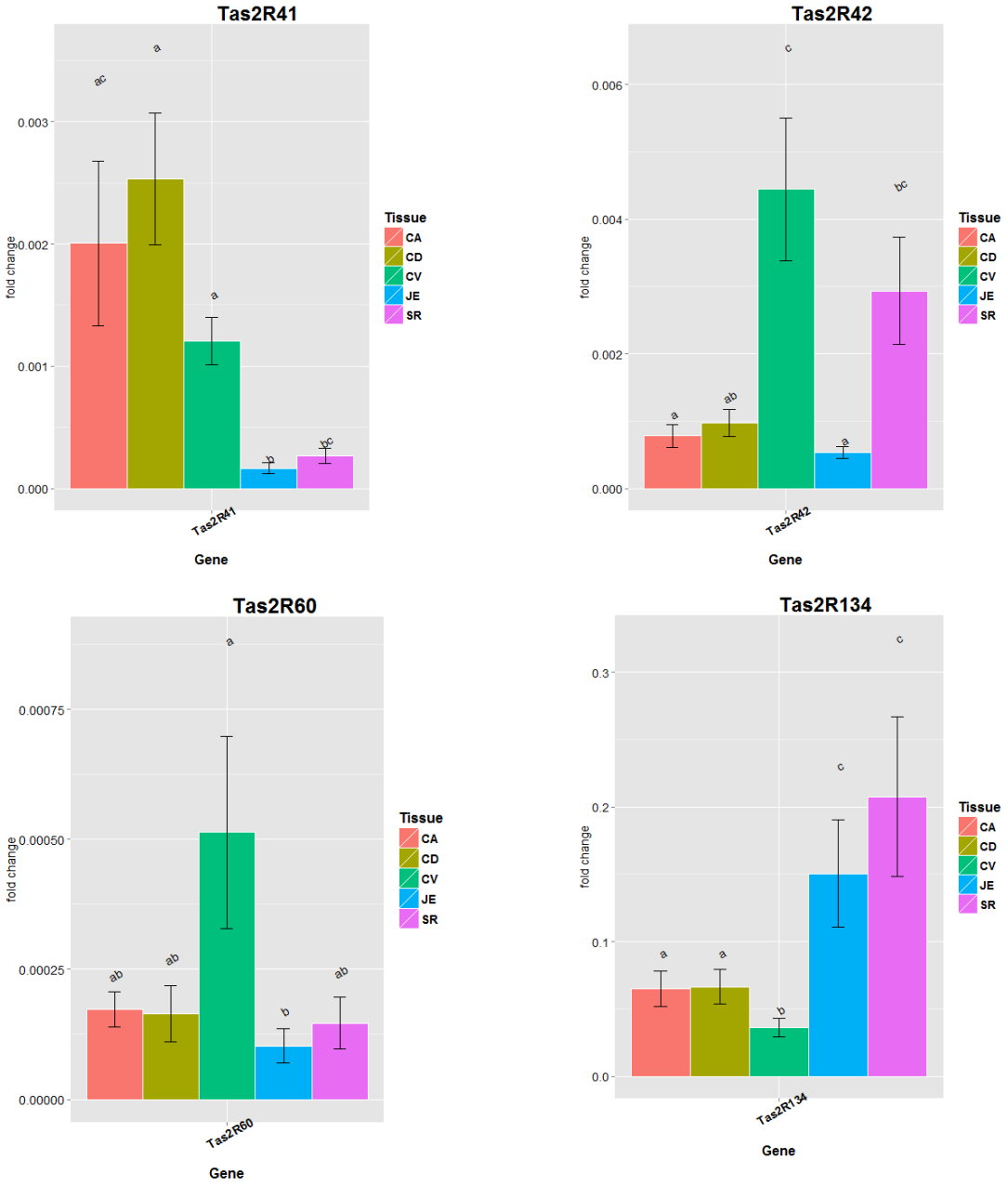


Figure 6.
Plots present the gene expression levels of Tas2R41, Tas2R42, Tas2R60 and Tas2R134 receptors along five porcine tissues (CV-circumvallate papilla, SR-stomach ridge, CA-caecum, JJ-jejunum and CD-colon distal). Gene expression was calculated according Pfaffl method (Pfaffl 2001) with two housekeeping genes (TBP and GPDH) and Tas2R4 expressed in circumvallate papillae (CV) to obtain fold-change values. Data were analysed by Wilcox test for multiple contrasts in R (RStudio, Inc., Boston, USA). Means (±SEM; n=6) with different letters differ significantly (P<0.05).

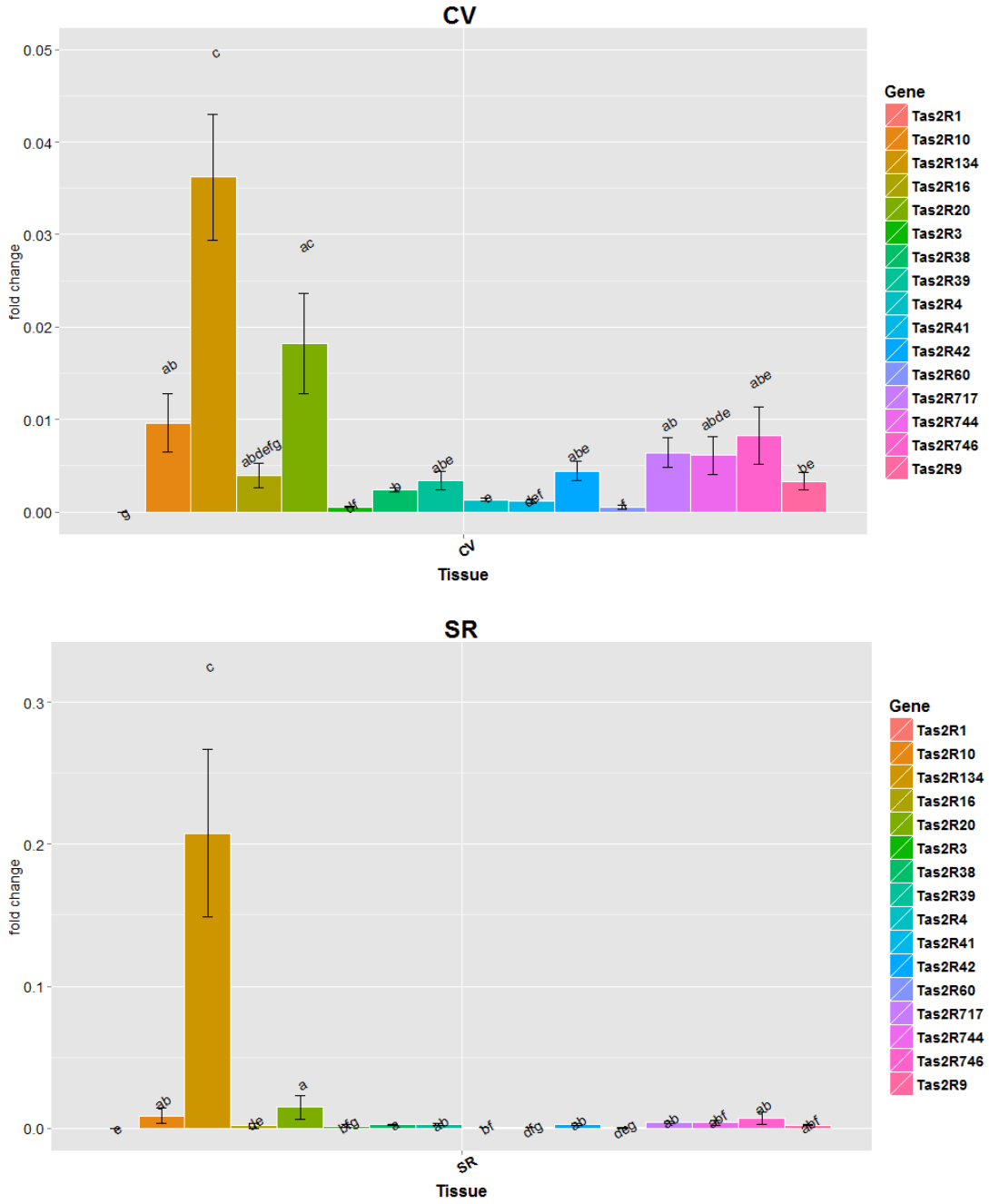


Figure 7.
Plots present pattern of Tas2R genes expression as fold change in two porcine gastrointestinal tissues: tongue circumvallate papilla (CV) or stomach ridge (SR). Gene expression was calculated according Pfaffl method (Pfaffl 2001) with two housekeeping genes (TBP and GPDH) and Tas2R4 expressed in circumvallate papillae (CV) to obtain fold-change values. Data were analysed by Wilcox test for multiple contrasts in R (RStudio, Inc., Boston, USA). Means (±SEM; n=6) with different letters differ significantly (P<0.05).

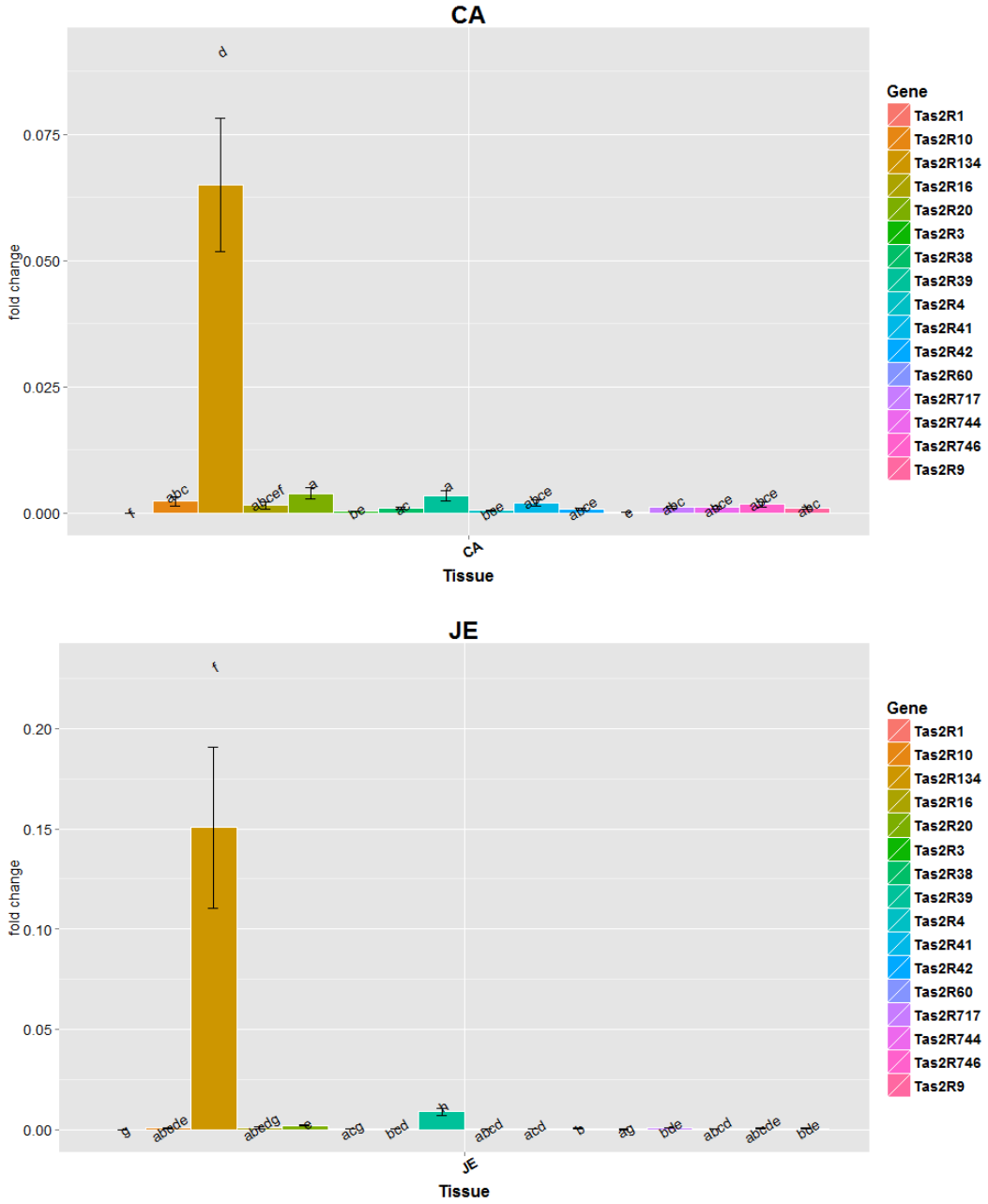


Figure 8.
Plots present pattern of Tas2R genes expression as fold change in two porcine gastrointestinal tissues: caecum (CA) or jejunum (JE). Gene expression was calculated according Pfaffl method (Pfaffl 2001) with two housekeeping genes (TBP and GPDH) and Tas2R4 expressed in circumvallate papillae (CV) to obtain fold-change values. Data were analysed by Wilcox test for multiple contrasts in R (RStudio, Inc., Boston, USA). Means (±SEM; n=6) with different letters differ significantly (P<0.05).

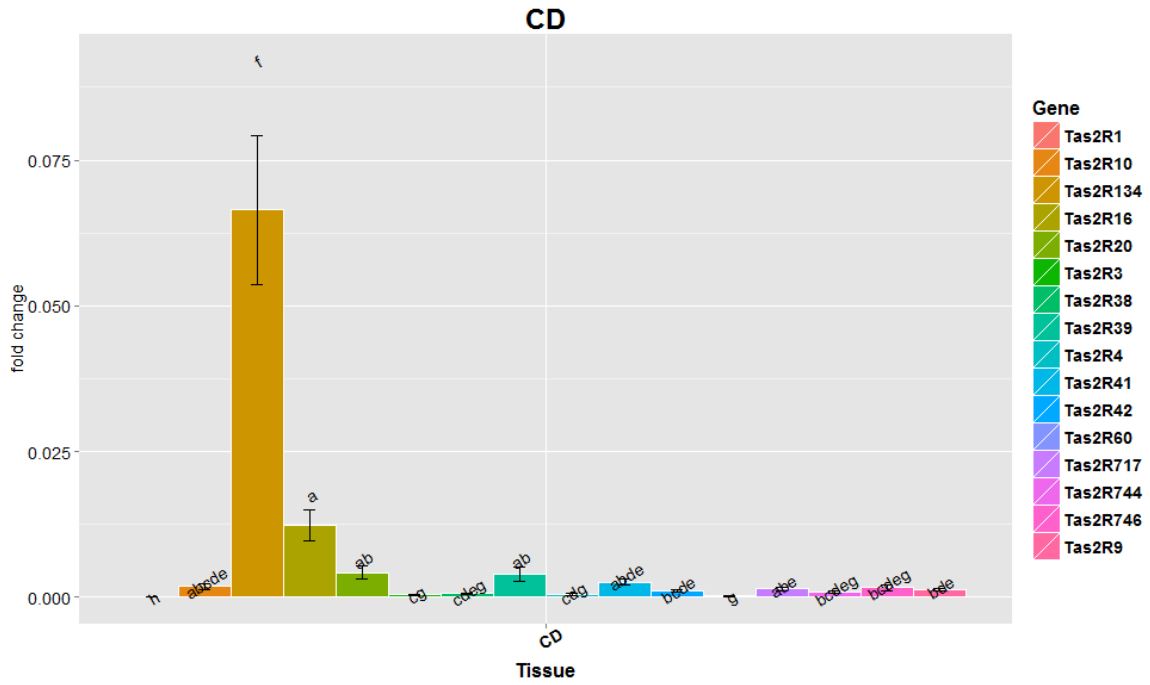


Figure 9.
Plot present pattern of Tas2R genes expression as fold change in porcine colon distal (CD). Gene expression was calculated according Pfaffl method (Pfaffl 2001) with two housekeeping genes (TBP and GPDH) and Tas2R4 expressed in circumvallate papillae (CV) to obtain fold-change values. Data were analysed by Wilcox test for multiple contrasts in R (RStudio, Inc., Boston, USA). Means (±SEM; n=6) with different letters differ significantly (P<0.05).

Acknowledgments

Research funded by the ARC Centre of Excellence in Plant Cell Walls. I would like to express gratitude to Doctoral School on the Agro-Food System – Agrisystem for supporting me with a scholarship, which covered my abroad period at UQ.

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5. Conference papers

5.1. Resistant starch content of cereal grains common utilized for pig nutrition

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Manuscript published in Journal of Animal Science (2012), Volume 90, Supplement 3 / Journal of Dairy Science, Volume 95, Supplement 2. - The 2012 Joint Annual Meeting (JAM) ADSA®, AMPA, ASAS, CSAS, WSASAS on July 15–19, Phoenix, Arizona, USA

Resistant starch (RS) has been defined as the portion of starch that is not hydrolyzed by the enzymes in the small intestine. Therefore it passes to the large bowel, where can be principally fermented to short-chain fatty acids. In pigs, diets containing starch with a high level of RS have been associated with a greater digesta mass that may benefit the diversity and population of gut bacteria and may improve the production of short-chain fatty acids, including butyrate. However, high-RS diets may affect digestion of nutrient, were associated with a lower in vivo starch digestibility and feed efficiency and can exacerbate the expression of swine dysentery. Currently, limited information is available about the RS content of single cereal grains commonly employed in pig nutrition. Consequently a set of 42 raw samples (6 maize, 6 dehulled barley, 6 triticale, 6 rice, 6 high moisture corn (HMC), 6 wheat and 6 sorghum) randomly collected from international feed companies was evaluated for the RS content with a two-step *in vitro* method simulating gastric and pancreatic phases up to 120 min. Data were subject to one-way ANOVA with the GLM procedure of SAS (2003) and significance were declared at $P<0.05$. Minimum significant difference (MSD) was generated from Tukey's test and was used as the basis of the multiple comparisons among means. Cereals were characterized by a wide range of RS content ($P<0.05$) and the MSD was 53 g/kg DM. In particular,

sorghum had the highest RS content (262 ±31 g/kg DM; P<0.05), whereas wheat the lowest (53 ±27 g/kg DM; P<0.05). Intermediate RS values were recorded for dehulled barley (133 ±31 g/kg DM), triticale (138 ±40 g/kg DM), HMC (142 ±25 g/kg DM), rice (165 ±29 g/kg DM) and corn (191 ±22 g/kg DM) samples. A proper evaluation of the RS content of cereal grains could be important for a complete feed evaluation. More research is warranted to increase knowledge about the role of RS on metabolic and productive responses in pigs.

Keywords: starch, *in vitro* method, resistant starch, pig

Acknowledgements: Financial support was provided by the AGROSCENARI project of MIPAAF (Italy)

5.2. Use of monocereal diets based on barley in heavy pigs: the effects on performance

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Manuscript published in Italian Journal of Agrometeorology (2012) p 43-44 – Agroscenari Project Meeting: agricoltori, politiche agricole e sistema della ricerca di fronte ai cambiamenti climatici. Ancona 1-2 March, 2012.

Abstract

Corn is the most used cereal in diets of pigs to be used for PDO products, but requires irrigation. However, barley has a higher concentration of proteins, more fiber, less starch and so, lower energy content but do not require long-term irrigation. Today's EU policy puts emphasis on water conservation and underlined the needs of reducing agriculture water usage. A study was conducted to verify if corn can be replaced by barley in diet for heavy pigs. Four diets were formulated: a control diet using corn as energy feed and three alternative single cereal diets based on different barley varieties (one normal and two hulles). 80 pigs were allotted to the four dietary treatment (2 genders, 5 animals per pen and 2 replicates). The average animal weight at the beginning of the trial was 49.3 kg and animals fed the trial diets for 153 days to a final weight of approximately 173.0 kg. Hams and necks were used for the production of PDO products (data not reported). Based on performance results of current trial both normal and hulles barley could be used as corn replacement in heavy pig diet formulation without negative effects on performance.

Key words: corn, barley, pig

Introduction

Italian heavy pig production is an important sector of the national animal production. The Po valley is one of the major European zones of pig production with over 7 million pigs, of which about 4 million in Lombardy only (Marquer, 2010). The cuts of Italian Heavy pig are used for Protected Designation of Origin (PDO) products as Parma and San Daniele ham, Piacenza neck and belly, as well as for fresh pork. Diets used in pig feeding are based on corn as the main energy source. Another important feedstuff is barley that, according to PDO rules can be used up to the 40% of diet dry matter. Because of the high content of starch and fats highly available to pig and the relative economical convenience, corn is considered as the primary source in pig feeding. However, barley has a lower quantity of fats and lacks the carotenoid pigment, that yellow corn has. These translate into the fat quality of barley-fed pigs, which have more hard and white subcutaneous fat ,more suitable for long term curing.. Because of the higher fiber content, the energy of barley is about 90% of the one of corn, however, the lack of hull in hulles barley reduces considerably the fibre level. The price of barley is rather higher compared to corn. However, the higher price would be partially compensated by the significantly higher concentrations of amino acids and phosphorus (Jurgens, 1993). When considering the growth conditions of both cereals, efficient corn yields need high amounts of water from irrigation, whereas barley can easily be grown without irrigation in the Po valley. EU reports the agricultural sector accounts for about one-quarter of water needs from the natural environment, which could rise to 80% in southern Europe. Meantime, Europe's water resources are seriously threatened, the groundwater resources over-exploit and there is a continuous increase in demand for water. According to the European Environment Agency agriculture is one of the sectors in which water savings can be easily achieved, particularly during irrigation of crops (European Council, 2000; EEA, 2012). Thus, within a progressive reduction of water availability scenario, the aim of our study was to compare the production of Italian Heavy pigs fed diets either based on corn or barley, as cereal with lower water requirements and reduced agronomic requirements compared to corn.

Materials and Methods

Eighty crossbreed Duroc Italian x Italian Large White (females and castrated males) pigs were used. Animals were wet fed (water:feed ratio 3:1) twice a day and had a free access to the water by a nipple drinker. The experimental scheme was: 4 diets x 2 gender x 5 animal for pen x 2 replicates. The trial lasted 153 days. The illumination during the experimental period was natural. The average starting weight of animals was 49.3 ± 2.13 kg. and the slaughtering weight was 173± 3,22 kg.

Tab.1 - Composition and nutrient analysis of experimental diets

		Ingradients [%]					Chem. comp as an. [%]		Energy Content
Diet		**Corn**	**Barley**	**Bran**	**Soybean**	**L-Lys HCl + premix**	**C Proteins**	**C Fiber**	**En. Dig. [kcal/kg]**
From 40 to 80 kg of l. w.	**Control**	51.40	22.00	10.00	13.50	3.10	14.41	4.39	3097
	Cometa	9.30	80.00	0.00	7.50	3.20	13.35	4.99	2788
	Astartis	5.80	80.00	0.00	11.00	3.20	15.72	2.61	3196
	Alamo	7.80	80.00	0.00	9.00	3.20	17.18	3.29	3052
From 80 to 120 kg of l. w.	**Control**	53.50	22.00	10.00	11.50	3.00	13.57	4.32	3095
	Cometa	10.90	80.00	0.00	6.00	3.10	12.70	4.94	2786
	Astartis	8.40	80.00	0.00	8.50	3.10	14.68	2.51	3195
	Alamo	9.90	80.00	0.00	7.00	3.10	16.33	3.21	3050
From 80 to 160 kg of l. w.	**Control**	55.00	22.00	10.00	10.00	3.00	12.90	4.26	3090
	Cometa	12.00	80.00	0.00	5.00	3.00	12.23	4.90	2785
	Astartis	9.80	80.00	0.00	7.20	3.00	14.10	2.46	3193
	Alamo	11.50	80.00	0.00	5.50	3.00	15.67	3.16	3049

Four diets were formulated: a control diet based on corn and three single cereal diets with different varieties of barley: Cometa (the most frequently used for livestock) and two hulless varieties Astartis and Alamo with different levels of beta-glucans and fiber. Composition and nutrient concentrations for each diet are reported in Table 1. The diets were formulated to have the same digestible energy/lysine ratio and fed in different amounts to supply the same digestible energy to all pigs.

Analyses were performed on diets and feed ingredients. The total content of starch was measured and its fractions: RDS (Rapidly Digestible Starch), SDS (Slowly Digestible Starch) and RS (Resistant Starch). Samples were subjected to *in vitro* hydrolysis simulating the enzymatic digestion in the upper digestive tract of pigs and incubated 480 minutes to estimate the rate of digestion (Giuberti et al., 2012). Moreover, the *in vitro* fermentation was performed to simulate the processes occurring in the

large intestine of animals (Bindelle et al., 2011). Statistical analysis was perform using SAS software (SAS, 2003).

Results and Discussion

Animals feeding on the three single cereal diets had (Cometa and Alamo) (P<0.01) or tended to a better growth performance (Astartis) compared to the control group (Table 2). The group fed the diet based on the Cometa variety, which is rich in fiber, had better feed conversion ratio (FCR) calculated on Digestible Energy, but worst FCR basing on kg of feed. Pigs fed Alamo variety, showed improved FCR both on Digestible Energy and kg of feed; pigs fed the Astartis diet showed better FCR calculated on kg of feed but not on Digestible Energy.

Tab.2 - Growth performance

Diet		**Control**	**Cometa**	**Astartis**	**Alamo**		
Average live weight						**p-value**	**EMS**
Initial live weight	Kg	49.3	49.3	49.3	49.3	NS	5.6565
After 56 days	Kg	95.1	95.9	97.1	97.4	NS	4.1686
Final live weight	Kg	169.1 B	175.4 A	172.1 AB	175.3 A	P<0.01	3.8913
Average daily gain							
From 1 to 56 d.	g	817 B	833 AB	853 A	858 A	P<0.01	158.2324
From 57 to 153 d.	g	763 B	819 A	774 AB	804 A	P<0.01	244.3246
From 1 to 153 d.	g	783 B	824 A	803 AB	823 A	P<0.01	132.632
Feed conversion ratio							
From 1 to 56 d.	n	2.66 B	2.87 A	2.48 C	2.57 BC	P<0.01	0.0012
From 57 to 153 d.	n	3.80 AB	3.94 A	3.64 B	3.65 B	P<0.01	0.0055
From 1 to 153 d.	n	3.37 B	3.54 A	3.19 C	3.24 C	P<0.01	0.0021
Feed conversion ratio in Kcal/DE							
From 1 to 153 d.	n	10418 A	9866 B	10190 AB	9873 B	P<0.01	18624.38

Different letters on the same line indicate significant differences at p <0.01 if capital and P <0.05 if sensitive.

Tab.3 - In vitro starch digestion parameters

Diet	Control			Cometa			Astartis			Alamo			
	mean	sd		mean	sd		mean	sd		mean	sd		p-value
k [min-1]	0.01	0	AB	0.01	0	B	0.02	0	A	0.03	0	C	0.0001
HI [%]	92.9	1.7	AB	90.5	2.5	B	95	0.9	A	99.6	0.7	C	0.0009

k - the digestion rate, velocity (/min)

HI – Hydrolysis Index

Moreover, the diet based on the Cometa barley has lower content of crude proteins, and it is commonly known that a reduction of the protein, leads to a better utilization of energy. This could be the main limitation to the use of these hulless barley varieties, taken into consideration that, in contrast to Cometa, they have an increased content of crude proteins and a higher ratio of crude proteins to lysine. Diets were characterized for different hydrolysis index (HI) (Table 3). In particular, greater HI was obtained from the Alamo diet (99.61, $P < 0.001$) compared to control, while the lowest numerically HI, although not different than the control, was obtained with the Cometa diet (90.54). Probably, the HI was affected both by the content of beta-glucans and starch structure of analyzed cereals.

Conclusions

The use of single cereal diets with high barley content may be proposed for the heavy pig feeding. Probably the nutritive value of barley has been underestimated in formulating diets; in fact the equation used (Fairbairn et al. 1999) was developed using light pigs; therefore it would be useful to have equations estimating the nutritive value considering the increased digestibility of fiber in heavy pig compared to those defined for the light weight pig. A more complete comments of results can be presented after analysis of ripened products.

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5.3. Expression of the nutrient sensor family Tas1R along the porcine gastrointestinal tract

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Manuscript published in Chemical Senses (2014) Volume 39, Issue 1Pp. 73-116. - The XXIInd Congress of the European Chemoreception Research Organization, ECRO 2013, 27-29 August 2013, Leuven, Belgium

The 3 members of the GPCR T1R receptor family are known to form heterodimers that are responsible for the sweet (T1R2 + T1R3) or umami (T1R1 + T1R3) tastes. Studies in rodents and humans have established that Tas1R genes are expressed in the tongue, but also outside of the oral cavity. However, only a few studies have reported Tas1R expression in porcine gastrointestinal tract (GIT) tissues. The aim of this study was a systematic analysis of the expression of the Tas1R gene family in 12 different tissue samples (3 tongue papillae, 2 stomach -ridge and antrum-, duodenum, ileum, jejunum, 2 colon -proximal, distal-, caecum and liver) by real-time PCR. Regardless of tissue, the expression level of Tas1R3 was higher than both Tas1R1 and Tas1R2. The Tas1R2 was only significantly expressed in liver and tongue. In particular, the expression of Tas1R2 and Tas1R3 was higher in circumvallate than in fungiform papillae. This suggests that pigs may have a higher capacity to taste sweet in the back of the tongue. Outside the oral cavity, the two stomach tissues had higher expression levels of Tas1R1 and Tas1R3 than the other tissues analysed. Our results demonstrated that pigs may have a specific pattern of expression of Tas1Rs in the GIT.

5.4. Isoenergetic diets differing in arabinoxylans or β-glucans show similar taste receptor expression profile in pig tongue

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Manuscript published in APSA Proceedings 2013 Manipulating Pig Production XIV - The Australasian Pig Science Association (APSA) Biennial Conference, Melbourne, Victoria, Australia, 24th to 27th November, 2013.

The taste receptor type 1 family (T1R) responds to dietary nutrients and consists of three proteins that form dimers for sweet (T1R2/T1R3) and umami (T1R1/T1R3) taste sensing. The sweet dimer responds to carbohydrates (i.e. mono- and disaccharides) whereas the umami dimer responds to amino acids (e.g. glutamic acid). Our current understanding is that inadequate dietary supply of these nutrients (i.e. energy or amino acids) in pigs may cause up regulation of the expression levels of the Tas1R genes (Treesukosol, Smith, & Spector, 2011). Dietary fibre has been reported to impact on the availability and digestibility of some essential nutrients, such as lysine (Lys) and threonine (Thr), or net energy (Dégen, Halas, & Babinszky, 2007). Therefore, we hypothesised that fibre-rich diets will impact on the gene expression level of Tas1Rs. This research studied the effect of arabinoxylans (AX) and β-glucans (BG) on the level of expression of Tas1Rs in porcine taste tissues.

In total, 18 male Large White pigs were included in this study (6 × 3 diets). The control (CTR) was a standard wheat-starch based diet consisting of 17.4 MJ/kg DE, 197 g/kg crude protein and 14.4 g/kg available Lys. The two treatments were isoenergetic compared to CTR, but contained 10% additional

fibre through the replacement of starch in the CTR diet by AX and BG, respectively. Pigs (23.9kg±2.4 kg; mean±SEM) were blocked by litter and randomly assigned to one of the three diets, with a 1 week adaptation. The animals received 2 meals per day (morning and afternoon) for 2 weeks before being sacrificed. Water was offered *ad lib*., Tissue samples from tongue circumvallate-, fungiform- and foliate papillae were collected and immediately frozen in liquid nitrogen, then stored at -80^{o} C pending RNA extraction. Real-time PCR amplification was performed to estimate Tas1R1, Tas1R2 and Tas1R3 mRNA abundance. Data were analysed using the Tukey's Honestly Significant Difference (HSD) test in R.

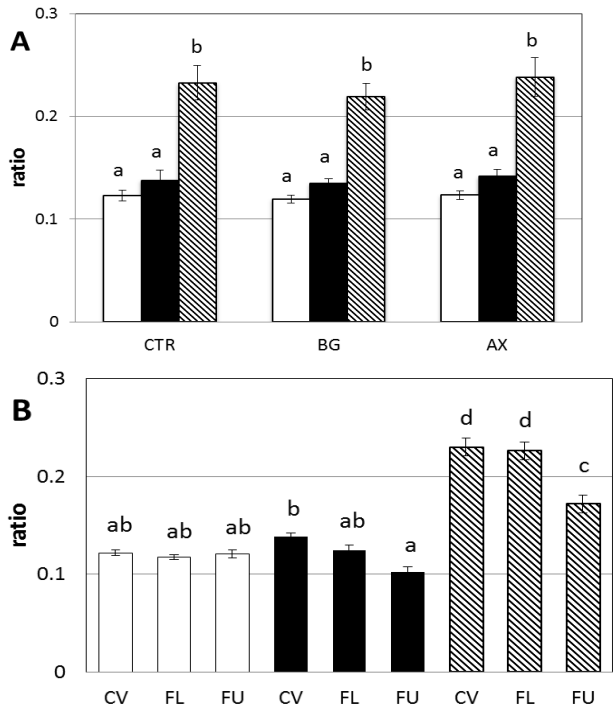

Figure 1. *Relative mRNA abundance (as a ratio to reference genes) of Tas1R genes in porcine tongue. Tas1R1 (solid white bars), Tas1R2 (solid black bars) and Tas1R3 (hatched bars);* ***A.*** *By dietary treatment in circumvallate (CV) papilla: control (CTR), β-glucans (BG) and arabinoxylans (AX).* ***B.*** *Across treatments by type of papillae: CV, fungiform (FU) and foliate (FL). Means with different letters differ significantly (P<0.05).*

The expression of Tas1R3 was higher (P<0.05) than the levels of Tas1R1 and Tas1R2 independent of dietary treatment and type of papillae. This is consistent with existing literature in other mammalian species. Figure 1A shows there were no differences (P>0.05) in the expression level of the three

Tas1R genes across dietary treatments in circumvallate, fungiform or foliate papillae. Since the diets were isoenergetic and pigs had similar growth performance (data not shown), these results suggest that energy homeostasis is a principal driver in modulating Tas1R expression in the tongue. Across treatments, fungiform papillae had lower ($P<0.05$) expression of Tas1R2 and Tas1R3 when compared to the circumvallate papillae (Figure 1B). This may be consistent with the lower number of taste sensory cells in fungiform papillae. In conclusion, the level of expression of Tas1R3 in pig tongue is the highest among the Tas1R genes. High levels of AX and BG administered as part of isoenergetic diets did not affect Tas1R expression in pig tongue.

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Research funded by the ARC Centre of Excellence in Plant Cell Walls. The contributions by Dr Dagong Zhang and Dr Walter Gerrits in experimental and diet design are thankfully acknowledged.

5.5. Towards defining the taste receptor repertoire in the pig

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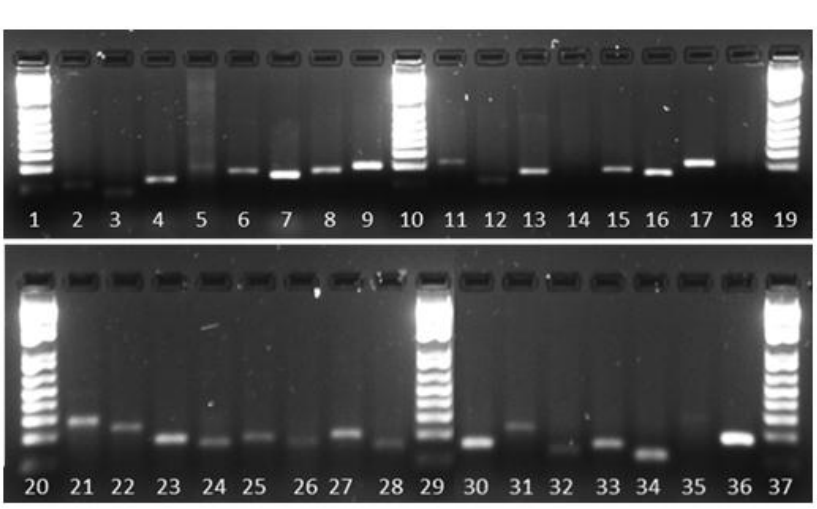
Manuscript published in APSA Proceedings 2013 Manipulating Pig Production XIV - The Australasian Pig Science Association (APSA) Biennial Conference, Melbourne, Victoria, Australia, 24th to 27th November, 2013.

The advent of the pig genome has furnished pig scientists with novel tools to understand pig physiology. Current research is involved in understanding factors controlling feed intake and the chemosensing system seems to play a fundamental role in and outside the oral cavity. For example, it has become apparent from research mainly done in rodents that receptors responsible for detecting taste in the tongue are also present in the stomach, heart, brain and gastrointestinal tract (Roura, 2011). This is suggestive of a nutrient sensing role and could have wide ranging implications. However, to date, there has not yet been evidence of a systematic approach to catalogue the full set of taste receptors in the pig. We hypothesize that the taste receptor repertoire in pigs will be concordant with rodent and human literature.

Six Large White piglets (24 ± 3 days of age) were anaesthetised and sacrificed prior to weaning. Tissues including three sections of the tongue, circumvallate-, fungiform- and foliate papillae were collected and frozen in liquid nitrogen. Total RNA was extracted using a Qiagen RNeasy kit. Candidate genes included receptors for sweet and umami taste (Tas1r1, Tas1r2 and Tas1r3), bitter taste (16 Tas2Rs), fat or fatty acid receptors (GPR120, GPR40, GPR41, GPR43 and GPR84), glutamic/amino acid receptors (mGLUR1, mGLUR4) and an amino acid receptor (GPRC6A). Full length mRNA sequences for these genes were retrieved from the latest pig genome assembly Sscrofa

10.2 in NCBI. Primers were designed to amplify unique regions within each of these genes. *In silico* primer BLASTing confirmed the specificity of the primers to their target transcripts. In addition, real time PCR following the MIQE guidelines (Bustin et al. 2009) and the delta delta normalisation method (Pfaffl 2001) on all genes for the three tissues was carried out on an ABI-7900HT using SYBR green and confirmed single disassociation peaks.



1	1kb ladder	20	1kb ladder
2	Tas1R1 (109)	21	Tas2R42 (300)
3	Tas1R2 (77)	22	Tas2R60 (248)
4	Tas1R3 (134)	23	Tas2R134 (184)
5	Tas2R1 (215)	24	GPR120 (170)
6	Tas2R3 (186)	25	GPR40 (187)
7	Tas2R4 (160)	26	GPR41 (169)
8	Tas2R7.1 (188)	27	GPR43 (201)
9	Tas2R7.2 (213)	28	GPRC6A (160)
10	1kb ladder	29	1kb ladder
11	Tas2R7.3 (236)	30	GPRB4 (150)
12	Tas2R9 (116)	31	mGLUR1 (230)
13	Tas2R10 (173)	32	mGLUR4 (123)
14	Tas2R16 (138)	33	TBP* (153)
15	Tas2R20 (182)	34	GAPDH* (104)
16	Tas2R38 (155)	35	ACTB* (292)
17	Tas2R39 (224)	36	RPLP1* (181)
18	Tas2R41 (134)	37	1kb ladder
19	1kb ladder		

Figure 1. *Agarose gels (2%) following electrophoresis of PCR products from the newly designed primers for 27 taste receptor candidate genes showing the amplification of single products/bands of the predicted size, indicated as the number of base pairs in brackets following the gene name. The white * indicates 4 reference gene controls. Lanes 1, 10, 19, 20, 29 and 37 contain 1kb ladders.*

Conventional PCR results are shown in Figure 1 and confirm the amplification of a single product of the correct size for each candidate gene. The band for Tas2R16 (lane 14) is too faint to visualise in this format; however it is present when viewed under a gel visualiser. Additional evidence of its expression was confirmed by the presence of a single amplification peak in real time PCR. The expression of the remaining candidate genes was confirmed in the tongue by real time PCR, with no differences in abundance between the three types of papillae (data not shown). In conclusion, our research confirms the presence and abundance of taste receptor genes in the pig tongue, concordant with human and rodent literature. Our research provides tools necessary to investigate the role that

taste receptor genes/nutrient sensors play in feed intake, nutrient specific appetite and nutrient absorption in pigs.

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5.6. Taste-related nutrient sensors: analogies between humans and pig

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Published in Australian Medical Journal (2013) 6, 11; Proceedings of the Nutrition Society of Australia (2013) 37. - The Nutrition Society of Australia and Nutrition Society of New Zealand 2013 Joint Annual Scientific Meeting, 4 - 6 December 2013, Brisbane, Queensland, Australia.

Background

The pig is increasingly being used as a model for understanding human nutrition, mainly due to similarities in physiology, the omnivore diet and genomics. The taste system in humans is diverse with taste receptors (TR) identified and confirmed to be expressed not only in the oral cavity, but also in a wide range of tissues including, for example, the tongue, stomach, large and small intestines, and liver. A systematic study of the TR repertoire in pig has not been reported to date and it is hypothesised that the taste machinery will share significant homologies with the human.

Objective

The aim of this study is to determine whether concordance exists between the expression profiles of taste receptor genes in humans compared to pigs in the gastrointestinal tract.

Design

Six Large-White pigs were anaesthetised and sacrificed and various tissues were collected including tongue (3 papillae: circumvallate, fungiform and foliate), stomach antrum, stomach ridge, duodenum, jejunum, ileum, colon proximal, colon distal, caecum and liver. Using real time PCR assays, the

relative gene expression levels for our candidate taste receptor genes Tas1R1, Tas1R2, Tas1R3, 16 Tas2Rs, mGLUR1, mGLUR4, GPR120, GPR40, GPR41, GPR43, GPR84, GPRC6A, were determined for each tissue.

Outcomes

With the exception of Tas1R2 which was only found to be expressed in liver and tongue, all of the other candidate genes were confirmed to be significantly expressed in all of the tissues investigated. In the tongue as well as in non-oral tissues, of the three Tas1R genes, Tas1r3 was the most abundantly expressed. This is expected as Tas1R3 is the common subunit for both sweet and umami taste receptor dimers. Preliminary results suggest that Tas1R3 is most abundantly expressed in the stomach ridge, an area possibly being subjected to high cellular turnover.

Conclusion

These findings further confirm the relevance of using the pig as a model system for human research. Our data should provide the necessary background for further investigations into the potential links that may exist between the expression levels of taste receptor genes and effects from nutritional paradigms.

5.7. High dietary soluble fibre affects taste receptor expression in pig stomach

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Published in Australian Medical Journal (2013) 6, 11; Proceedings of the Nutrition Society of Australia (2013) 37. - The Nutrition Society of Australia and Nutrition Society of New Zealand 2013 Joint Annual Scientific Meeting, 4 - 6 December 2013, Brisbane, Queensland, Australia.

Background

Sweet and umami tastes are perceived by the Taste Receptor Type 1 (T1R) family of GPCRs which includes T1R1, T1R2 and T1R3. Previous data from our lab shows that Tas1R genes are not only expressed in the tongue, but also in non-oral tissues of pigs (e.g. GIT), consistent with other rodent and human studies.

Since the activation of these receptors is believed to influence the hunger-satiety cycle, their expression levels may be altered by dietary composition. Therefore, we hypothesise that fibre-rich diets will impact the expression level of Tas1R genes.

Objective

We aimed to investigate the effect of arabinoxylans (AX) and β- glucans (BG) on the relative level of expression of Tas1R genes in porcine oral and non-oral tissues.

Design

Large-White pigs (n=18; 23.9kg±2.4kg) were used, with 6 pigs allocated to each of 3 diets. The control diet was a wheat starch base (17.4 MJ/kg DE; 197g/kg crude protein; 14.4g/kg dig Lys). The

treatment diets were iso-energetic to the control diet, but with starch being exchanged for 10% soluble arabinoxylan and 10% soluble β-glucan, respectively. Pigs were blocked by litter

and randomly assigned to the diets. Two meals per day were provided for 2 weeks, following a 1 week adaptation period and access to water was ad lib. Following anaesthesia and exsanguination, tongue papillae and a range of other non-oral tissues were collected and frozen in liquid nitrogen. Following RNA extraction and cDNA synthesis, qPCR was perfomed using TBP and GAPDH as reference genes. Statistical analysis using ANOVA and Tukey‘s Honestly Significant Difference test was carried out in R.

Outcomes

Fibre did not affect the expression level of the Tas1R genes in the tongue ($P>0.05$). None of the iso-energetic diets resulted in growth changes (data not shown); suggesting that Tas1R expression in the tongue may rather be associated with energy homeostasis. However, pilot data suggests a lower expression of Tas1R1 and Tas1R3 in the stomach ridge associated with fibre diets.

Conclusion

High levels of soluble arabinoxylans or β-glucans in iso-energetic diets did not affect the expression level of Tas1Rs in the pig tongue, however pilot data suggests that the diets were associated with lower expression levels of the Tas1R1 and Tas1R3 (encoding the umami receptor) in the stomach ridge.

Source of funding

Research funded by the ARC Centre of Excellence in Plant Cell Walls.

5.8. Effects of a high-fibre diet and starvation on sweet and umami taste receptor in porcine tongue and gastrointestinal tract

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Manuscript presented during The 47th Annual AIFST Convention, 22-25 June 2014, Melbourne, Australia.

Taste elicits both pleasurable and warning responses to nutrient and toxic substances, respectively. Different tastes are recognized by taste buds which are clusters of epithelial cells located in the taste papillae on the tongue. These sensory cells express a subfamily of G protein coupled receptors which bind taste receptors (TR) and relays messages to the brain. Previously it was assumed that the expression of TR was confined to the tongue. However, it has since been shown that TR are found along the gastrointestinal tract, possibly performing a nutrient sensing role. Sensing of nutrients is believed to be important in maintaining a healthy lifestyle. We studied taste in the context of two nutritional paradigms using pig as a model and hypothesized that nutrient availability is associated with abundance of TR. Firstly we investigated the effects of a fibre-rich diet using arabinoxylan and β glucan supplementation on changes in the expression of sweet and umami TR. Secondly we tested how sweet and umami gene expression levels vary relatively in tongue and gastrointestinal tract following a 48 hour starvation period. Gene expression levels are quantified relatively using real time quantitative polymerase chain reaction.

5.9. Iso-energetic diets containing β-glucan or arabinoxylan show different expression of Tas1R3 and similar expression of fatty acid sensing genes in the porcine gastrointestinal tract

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Manuscript presented at ECRO 2014 - The XXIVth International Conference of European Chemoreception Research Organization, 10-13 September 2014, Dijon, France.

Recent studies have uncovered several fatty acids (GPR40, GPR41, GPR43, GPR120 and GPR84) and carbohydrate (T1R2/T1R3) receptors which respond to short, medium and long chain fatty acids and simple sugars present in the diet, respectively (Janssen & Depoortere, 2013). Fibrous dietary compounds have the ability to increase the viscosity of digesta which slows down the flow of digestive enzymes and the absorption of nutrients (Bach Knudsen, Hedemann, & Lærke, 2012). For example, soluble dietary fibre reduces glucose absorption (and the glycaemic index) and other macronutrient absorption such as fatty acids (Gunness & Gidley, 2010). Thus, we hypothesize that high dietary fibre may limit fatty acid and sugar availability which, in turn, will increase the expression of their sensing receptors. The purpose of this study was to evaluate the effect of dietary addition of soluble fibre compounds arabinoxylan (AX) or β-glucan (BG), on the expression level of fatty acids and sugar nutrient sensor genes in the porcine gastrointestinal tract (GIT).

For two weeks, 18 Large-White male pigs were assigned to three iso-energetic diets, differing on their fibre type and content. The three diets consisted of: a control (CTR) based on wheat starch, and two experimental diets containing 10% wheat AX or oat BG substituted for some wheat starch. The

mRNA was extracted from collected tissues and qPCR followed by statistical analysis were carried out according to the Pfaffl method (Bustin et al., 2009). The taste and fatty acid sensor gene amplicons were assayed for five selected tissues: circumvallate papilla (CV), stomach ridge, jejunum, colon distal and caecum.

The results showed significant expression of fatty acid receptor genes along the porcine GIT particularly outside the oral cavity. Compared to the other fatty acid sensors, GPR41 and GPR43 had different expression patterns across tissues showing a significantly ($P<0.05$) higher expression in caecum or stomach than in CV. In addition, Tas1R3 was also abundantly expressed in and outside the oral cavity. In contrast, the Tas1R2 expression pattern was found to be site-specific, with higher ($P<0.05$) levels in the tongue compared with the other tissues. The presence of high dietary BG significantly ($P<0.05$) increased the expression of the Tas1R3 in stomach when compared to the AX. Overall, our findings show significant expression of fatty acid and sugars receptors along the porcine GIT which was not affected by the fibre level in iso-energetic diets. However, the effect of fibre type on specific receptor genes (Tas1R3) relevant to carbohydrate sensing, warrants further investigation.

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6. Conclusions

The present work starts with attempts to evaluate technologies that increase digestibility of energy and other nutrients in cereal grains, and their co-products, *in vivo* and *in vitro* trials have been performed. The first objective of the thesis was to study if barley varieties, which do not require intensive irrigation, can replace commonly use corn based-feed in pig production, without adverse effect on animal breeding. Animals were fed three monocereal diets based on hulled (Cometa variety) or hulless (normal- Astartis and low-amylose Alamo) barley varieties, effect on growth performance, *in vitro* digestibility and fermentation model and also carcass characteristics was evaluated in heavy growing-finishing pigs for the production of Protected Designation of Origin (PDO) Italian products. Animals fed Cometa and Alamo attained higher final weights compared with those fed the control diet. Under Cometa diet the animals had the lowest dietary efficiency throughout the trial. For the same period, Astartis and Alamo diets had the highest dietary efficiencies, whereas the control was in between. Diet based on hulled Cometa variety had higher content of fibre when hulless varieties had lower. Use of hulles barley may be more cost-effective than hulled, taking into account the de-hulling process. This study showed that monocereal diets based both on hulled and hulless barley might be useful in heavy pig production destined for Italian PDO product manufacturing without adversely affecting growth performance and carcass characteristics, which was confirmed also by *in vitro* digestibility trials.

Next project continues an investigation on barley, focusing on its higher non-starch polysaccharide (NSP) content compare with corn. The 42-days trial was conducted to evaluate the effect of two hulless barley varieties, with or without the addition of a nonstarch polysaccharide (NSP) enzyme complex (β-glucanase and xylanase), on growth performance of weaned piglets. Moreover, an *in vitro* digestion kinetics of diets was also investigated. The performance study demonstrated that hulless barley with or without the addition of the NSP enzyme complex provides a replacement for corn and wheat bran not only in growing–finishing pig diets but also in weaned pig diets. Besides, the use of the NSP enzyme complex could improve feed efficiency of normal-amylose barley-based diets with

positive effects on growth performance. Though, barley-based feed supplemented with an enzyme complex did not improve *in vitro* starch digestion potential. Positive effects of hulles barley on digestion kinetics compare to corn-based diet were observed. Additionally, minor shifts in bacteria patterns were detected.

Industry by-products, such as these coming from bakery, are among common alternative feedstuff in animal nutrition, being also an another possible way to reduce production costs. To complement the information gap concerning usefulness of by-products, was examined how wheat pasta by-product supplemented to swine feed in 30%, 60% and 80% affect performance, *in vitro* starch digestion and *in vitro* fermentation kinetics. Diet composition positively influenced average daily feed intake of pasta diet D80 compare with corn-based control, but the final animal weight was similar. D80 had slightly better kinetic during the *in vitro* enzymatic starch digestion compare with CTR. As suspected, addition of pasta by-products lowered production of VFA during in vitro fermentation of D80, but increased gas and NH3 production. Obtained outcomes indicate that the introduction of pasta by-products into swine feed means possible reduction of production cost without lowering animal production efficiency, yet high share of pasta may increase odour emission.

Availability of nutrients and the starch digestion kinetics of animal feeds could be affected by many factors, one of them is particle size of milled grains. The aim of the last project from the this part of thesis was to identify the contributions made by particle size fractions of milled corn to nutritional properties. We observed a tendency for change in chemical composition of the examined particle fractions regarding total starch, prolamin and fat, but not protein. Study confirmed that starch digestion properties of unsieved corn grains are a weighted average of the individual size fractions and *in vitro* methods are related to possibility of enzymes to penetrate grain fragments. Moreover, a simple method to predict hydrolysis index (HI) was proposed and which could potentially help more efficiently assess digestion potential of maize-based feed.

Dietary starch and non-starch polysaccharides are important nutrients in animal and human nutrition, consequently, is a must to understand their effects on gastrointestinal physiology. Numerous

publications studied the effects of dietary fibre on nutrient flow and intestinal health. However, evidence explaining results of functional properties on changes in gastrointestinal tract characteristics and mechanisms are still rare. Here we studied only arabinoxylans and beta-glucans but, numerous fibre sources exist and their potential have been not explored. These dietary fibres could affect animal performance or could also be potentially used as functional fibre in animal as well as in human nutrition. Moreover, the advancement of new genomic techniques can be used to explore the potential of different kinds of dietary fibre to manipulate the gut microbiota to improve digestion and gut health. The concept of gut health involves the dynamic equilibrium of diet, gut mucosa and gut microbiota. There is a need of not only exploring the gut microbiology but also the complex interaction of bacteria and host mucosa as influenced by various nutrients.

Regarding *in vitro* digestibility, a number of doubts still remain, particularly because structural features of starch in grains and interactions with other non-starch components which control kinetic of digestion, are connected and sometimes not easily defined. This knowledge can be gained via *in vitro* digestion methods to obtain more information on starch factors as well as on animal factors influencing utilization of nutrients from feed grains. A general standardization for *in vitro* starch digestion methods is consequently crucial to make available laboratory tools for fast nutritional value assessment, even if they show some advantages and disadvantages if compared to *in vivo* techniques.

Second part of my research was dedicated to investigate the nutrient receptors mechanisms, able to collect information about nutritional qualities of ingested food as well as control intake. Data reviled here represent a first step for more detailed molecular studies on pig chemosensing. For the first time taste receptors genes in porcine gastro-intestinal tract have been showed all together. We were able to detect genes responding to carbohydrates (i.e. mono- and disaccharides) (T1R2/T1R3), associated with umami or/and aliphatic amino acids (T1R1/T1R3) and the glutamate receptors metabotropic 1 and 4 genes (GRM1 and GRM4), then also GPCRs which respond to free fatty acids: GPR40, GPR43, GPR41, GPR120 and GPR84. Additionally, 14 porcine Tas2R-like genes were identified and confirmed in gastro-intestinal tract: Tas2R1, Tas2R3, Tas2R4, Tas2R9, Tas2R10,Tas2R16, Tas2R20, Tas2R38, Tas2R39, Tas2R41, Tas2R42, Tas2R60, Tas2R134 and Tas2R7. What is more, another

crucial aim of this project was to evaluate the effect of dietary addition of soluble fibre compounds arabinoxylan (AX) or β-glucan (BG), compared to a wheat-based Control (CTR) diet, on the expression level of fatty acids and sugar nutrient sensor genes in the porcine gustatory and non-gustatory tissues. The results showed significant expression of receptor genes along the porcine gastro-intestinal tract in particular outside the oral cavity. The diet (fibre) effects and a Gene by Diet interaction was observed in tongue CV, where GPR120 expression was significantly higher in arabinoxylans enriched group compared to the control and BG groups. In addition, a Tissue by Diet interaction was detected for Tas1R1 and Tas1R3 with the highest expression in stomach ridge tissue of animals fed beta-glucans diet, indicating a possibly important role of stomach in nutrient chemosensing.

Knowledge of the expression patterns of nutrient receptors genes may contribute to a better understanding of the satiating effects of specific diets and products. We investigated for the first time the gene expression of nutrient sensors in relation to dietary fibre. Surprisingly, our results suggested a great role of stomach in chemosensing, therefore it will require much more attention during upcoming experiments. Nutrient sensors have a great potential which may allow us design rational approaches to feed formulation and additives with the potential for the prevention of nutrient malabsorption and postweaning enteric disorders. However we need to deeply understand molecular and cellular mechanisms underlying processes such as the ability of the gut to sense the concentration of wide selection of nutrients, activation of signaling pathways and the capacity of nutrients absorption.

In this work a research went through the *in vivo* performance trials, the modeling of digestibility *in vitro*, and finally to the latest discoveries in chemosensing. The concept of physiological function of gut nutrient-sensing receptors is still fresh but is evolving quickly, especially in they role in appetite control, which could encourage both universities and industry for stronger cooperation and technology transfer.

Acknowledgments

I would like to express my deepest gratitude to my supervisors Prof. Francesco Masoero and Dr Maurizio Moschini from Universita Cattolica del Sacro Cuore and Dr Eugeni Roura from University of Queensland, for giving me the opportunity to pursue PhD and for a continuous support during research projects.

I am very grateful to Doctoral School on the Agro-Food System for funding my scholarship and travel grants during my PhD.

Thank you Dr Gianluca Giuberti, Dr Antonio Gallo and Prof. Aldo Prandini for your contribution, advice and help, I have learned a lot.

Special thanks to Dr Nadia de Jager – "The Right Hand of Eugeni" – for a great team work and support during my stay in Australia. I like to thank Dr Maxine Preston for introducing me to the laboratory.

I would like to acknowledge the excellent technical help and advice of Dr Paola Fortunati, Dr Sara Bruschi, Dr Alessia Gualla, Dr Annalisa Mulazzi, Dr Silvia Rastelli, Dr Samantha Sigolo, Dr Mauro Varacca, Dr Maria Luisa Callegari, Dr Lorenzo Bomba and Dr Sotirios Vasileiadis.

I would like to express my sincerest gratitude to my parents Ewa and Andrzej Rzepus, and my brother Robert, for understanding and support.

To all my friends and collegues for unforgettable experiences during last few years of my work and travel, thank you and big hugs.