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ROLE OF SOLUBLE AND INSOLUBLE POLYSACCHARIDES IN OMNIVORE AND CARNIVORES NUTRITION

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Chapter 1 - General introduction

In recent years both in human and animal nutrition there is a growing interest towards the beneficial effects of complex polysaccharides in health, as well as the popularity of functional foods. Some fermentable carbohydrates can be defined prebiotics, non-digestible ingredients that play a beneficial role in health, stimulating the growth and/or metabolic activity of desirable bacterial species already resident in the colon (Gibson and Roberfroid, 2008).

Alternative and innovative carbohydrates sources were researched. As a result, less digestible carbohydrates and ingredients with a low glycaemic index and/or rich in slowly fermentable carbohydrates and/or in soluble fiber (whole barley, spelt and oats and fruits) have received attention by human and veterinary diabetologists.

Dietary Fiber definitions

The definition of dietary fiber is flexible because the knowledge on dietary fiber was progressively increased and the entities that are included in the definition changed based on the judgment of the experts working in the field. In 1953 Eben and Hipsley were the first to use the term “dietary fiber”, as a shorthand term for the constituents of plant cell wall. As it is the case with most of the concepts related to the analysis and food composition, the term “dietary fiber” derived from the methodology used for its quantification. Since the beginning of the XX century until 1970, the term “fiber raw or crude fiber” prevailed (Williams and Olmstead, 1935; Saura and García, 2001), referring to the ash-free residue of vegetable samples after a treatment with acids and alkalis (Matissek *et al*., 1998), composed primarily of cellulose, hemicelluloses and lignin (Kirk and Harol, 2002), which is mainly indigestible and has nutritional value equal to zero. When it has been established that the indigestible part is actually larger than the crude fiber, the term “fiber cleansed” was used; in this term are enclosed less nitrogenous substances (Cho *et al*., 1997), known as cellulose, lignin and ash rich in silica. Significant contributions in fiber cleansed are used today in animal nutrition, because it is highly energetic. Between 1972 and 1976, Trowell and Burkitt adopted the term “dietary fiber” in conjunction and developed a medical hypotheses (Burkitt *et al.*, 1972; Trowell, 1972; Trowell, 1974; Truswell and Kay, 1976) according to which all parts of the plant cell indigestible by enzymes, such as gums, cellulose, pectin and mucilage are

considered components of dietary fiber (De Vries *et al*., 1999). These papers describe the physiological consequences related to the indigestibility in small intestine of plant cell wall and the properties of these sources, postulating an inverse relationship between dietary fiber consumption and the incidence of colon cancer and heart disease. From these results several research projects on dietary fiber in human nutrition, analytical, and food technology were developed.

In 1976, the dietary fiber definition was broadened (Truswell and Kay, 1976) to a physio-chemical definition including all indigestible dietary polysaccharides in addition to the other fiber components. These non-digestible polysaccharides were included in the definition because they have the physiological actions similar to dietary fiber, but could not necessarily be chemically identified as having their origins in the cell wall. Subsequent to 1976, consumer interest and public policy on dietary fiber have changed, focusing on its unique physiological functions and potential health benefits. To verify if an update of the current definition of dietary fiber is needed, AOAC Associate and General Referees for dietary fiber (Lee and Prosky, 1995) conducted two international surveys on definition and analyses of dietary fiber. The majority of scientists surveyed support the continued use of the current definition. Before that the definition of dietary fiber was universally accepted, there have been attempts to eliminate or restrict it, limiting dietary fiber to only cell wall materials, or to some compounds such as non-starch polysaccharides. These attempts have been unsuccessful; correlations found between human and/or animal health improvements and dietary fiber consumption do not hold true using these restricted definitions.

In 2001 the American Association of Cereal Chemists (A.A.C.C.) defines dietary fiber as *"the edible parts of plants or analogous carbohydrates that are resistant to digestion and absorption in the human small intestine with complete or partial fermentation in the large intestine. Dietary fiber includes polysaccharides, oligosaccharides, lignin and associated plant substances. Dietary fiber promotes beneficial physiological effects including laxation, and/or blood cholesterol attenuation, and/or blood glucose attenuation".*

Classification of dietary fiber fractions

Dietary fiber components can be classified according to their solubility in water as soluble and insoluble components. The proportion and origin of dietary fiber fractions affect their metabolic properties and physiological effects. The Soluble Dietary Fiber (SDF) is able

to form a viscous gel in the intestinal tract, which delays gastric emptying, generates a great sense of satiety and makes more efficient digestion and absorption of food. The Insoluble Dietary Fiber (IDF) is able to increase up to 20 times its volume and weight, thanks to its ability to retain water that helps in digestive d*isor*ders treatment, such as constipation.

Both types of fiber have the ability to bind molecules of water with certain cations, giving rise to the formation of a network that can act on the colon microflora. The insoluble fiber fraction binds water, swelling and increasing peristaltic movements and faecal volume. On the other hand soluble fiber fraction allows the maintenance and growth of bacterial microflora and epithelial cells (Manrique and Lajolo, 2001) for the production of short chain fatty acids (SCFA, acetic, propionic and butyric acids), which are absorbed (95-99%) and utilised as energy source, reducing cholesterol synthesis in the liver (Larrauri *et al.,* 1996). The SCFAs reduce the pH, causing a vasodilator effect, which increases the absorption of water and salts from the intestine (Calixto *et al.,* 2002).

Insoluble Dietary Fiber (IDF)

The IDF are insoluble in aqueous enzyme solution as representative of the alimentary enzyme solution. The insoluble dietary fiber makes up about 2/3 of the fiber in most feedstuffs. Several compounds such as cellulose, hemicelluloses, resistant starch, lignin, cutin and suberin are constituent of insoluble fraction of dietary fiber.

Cellulose: it is the primary component of IDF, it is insoluble in water (hot and cold) and in weak acids and alkalis. The cellulose is the most common organic compound on the earth. Because it is the structural component of the primary cell walls of green plants, many forms of algae and some fungi (*Omycetes*). About 33% of all plant matter is represented by cellulose, i.e. the cellulose content of cotton fiber is 90%, that of wood is 40–50% and that of dried hemp is approximately 75%. The cellulose monomers are polymerized in β-glucans compacted in aggregates, called microfibrils, superimposed to form different intra and extra-cellular layers held together by hydrogen bonds.

Cellulose is a polymer of β-D-glucose molecules linked in 1-4-positions. The animals do not have hydrolytic able to break β-linkages, but the symbiotic microorganisms present in the rumen and/or in intestine are able to ferment the cellulose with production of

fatty acids, methane and carbon dioxide carbon. Although such degradation is much slower compared to those born by other polysaccharides.

Hemicelluloses: this term is applied to polymers composed by different saccharides. Usually are small molecules (50-200 saccharide/units) with branched chain usually consisting of more than two sugars. It is commonly found in cereal grains, fruits and vegetables. Structurally, hemicelluloses are composed mainly of sugars, such as xylose, arabinose, galactose and mannose, linked in different combinations, the composition and the location in which sugars are positioned influence their degree of solubility and fermentability. Generally hemicelluloses are insoluble in water and soluble in basic solutions, however when glucuronic acid is in lateral position, they are partially soluble in water. Even the hemicelluloses fermentation by microflora is affected by sugars position. For example, the hemicelluloses containing hexoses and uronic acid, are more susceptible to bacterial action compared to those composed by other sugars. The most important biological role of hemicelluloses is their contribution to strengthening the cell wall by interaction with cellulose and lignin.

Lignin: it is an organic substance binding the cells, fibres and vessels, which constitute wood and the lignified elements of plants. It is not possible to define the precise structure of lignin as a chemical molecule. In function of the sources, lignin shows certain variation in chemical composition. However the definition common to all is a dendritic network polymer of phenyl propene basic units. Lignin results when polyfunctional phenols are polymerized with ether and ester linkages during plant growth, intimately forming with and infiltrating the cellulose of cell walls, resulting in a hard, rigid matrix of tremendous strength. At sufficient lignin concentration, plant tissues become “highly lignified” or “woody” to the point of being highly indigestible. Lignin is an important component of dietary fiber, making the fiber hydrophobic, resistant to enzymatic digestion in the small intestine and to bacterial breakdown in the large intestine. It is almost completely recovered in the faeces. Lignified tissue in feedstuffs offers only textural properties, although they are not always considered desirable.

Cutin: it is waxy hydrophobic layer composed by long chain hydroxyl-aliphatic fatty acids polymerized by ester bonds. It is resistant to digestion and can be recovered in faecal material. Cutin is one of the main components of the plant cuticle, which covers all aerial surfaces of plants. Cutin consists of omega hydroxy acids and their derivatives, which are interlinked via ester bonds, forming a polyester polymer of indeterminate

size. Typically the fatty acids which composed cutin are palmitic acid (C16:0), stearic acid (C18:0) or oleic acid (C18:1). Ester linkages also occur between the cutin and other cell wall polymers such as hemicelluloses (Deas and Holloway, 1977).

Suberin: suberin is a highly hydrophobic material; its main function is to prevent water from penetrating the tissue. In roots suberin is deposited in the radial and transverse cell walls of the endodermal cells. This structure is known as the *Casparian strip* or *Casparian band*. Its function is to prevent water and nutrients taken up by the root from entering the stele through the apoplast in order to promote the adsorption by the symplast. This allows the plant to select the solutes that pass further into the plant. It thus forms an important barrier to harmful solutes. Kolattukudy (1981) indicates that the scanty evidence available allows only conjecture regarding the structure of suberin as a highly branched and cross-linked (by ester linkages) combination of polyfunctional phenolics-hydroxyacids and dicarboxylic acids. Like cutin, it is chemically linked to cell wall carbohydrate polymers especially through its lignin forming components (p-coumaric and ferrulic acids). Even if pure preparation of suberin is not available, it is possible that portion of this component can interact during digestion of other plant cell wall components.

Soluble Dietary Fiber (SDF)

The viscous soluble carbohydrates are polysaccharides of low molecular weight such as pectins, gums, β-glucans, mucilage and glucomannan. These compounds are soluble in aqueous enzyme system, but precipitates upon the addition of four parts of alcohol to the aqueous mixture. The main characteristic of these compounds is represented by the capacity to bind water, forming a gelatinous mass in the small intestine. This mass slowly passes through the small intestine, modifying the efficiency of digestive enzymes, the stomach emptying and the nutrients absorption. Soluble fiber is rapidly fermented in the large intestine.

Pectins: the most widespread SDF in feeds are pectins (polygalacturonic acids) found in fruits, vegetables, legumes and roots as storage polysaccharides. Commercial pectins are *iso*lated either from apple or citrus peels (levels up to 30% DM).

Pectins are complex polysaccharide consisting mainly of esterified D-galacturonic acid resides in α 1-4 chain. The acid groups along the chain are largely esterified with methoxy groups in the natural product. The galacturonic acid main chain also has the occasional rhamnose group present, which disrupts the chain helix formation and contain other neutral sugars in side chains. The most common side chain sugars are xylose, galactose and arabinose. Pectins form gel quickly or slowly in function to their methoxy content. The interruptions of pectins chain result in a soft water soluble molecule rather than a linear polymer with high intermolecular hydrogen bonding having properties similar to cellulose. The cellulose provides support to ensure the rigidity of the plant, while the pectins ensure its flexibility. Because of gelling properties, pectins influencing the gastro-intestinal transit of *digesta* and can reduce nutrients digestibility, and post-prandial glucose levels (Jenkins *et al.,* 1978). In dogs, the administration of citrus pectins does increase by 19% the levels of propionate (Sunvold *et al.,* 1995a).

β-glucans: are different polysaccharides of D-glucose monomers linked by β-glycosidic bonds. The differences between β-glucans linkages and chemical structure are significant in regards to solubility, mode of action, and overall biological activity. Diverse β-glucans vary with respect to molecular mass, solubility, viscosity, and three-dimensional configuration. The β-glucans have great shear and tensile resistance, strong rugged, and durable enough to use for clothing and shelter. Thus some forms of β-glucans are useful in food and feed industries as texturing agents and as soluble fiber supplements. The treatments of cooking and freezing may modify the rheological properties of β-glucans, decreasing its solubility (Beer *et al.,* 1997; Anderson *et al.,* 2004). This is a factor that should not be neglected by manufacturers of animal feed, because of the heat treatments (for example, extrusion and canning) applied to their products that may reduce the bioactivity of β-glucans. They occur most commonly as cellulose in plants, bran of cereal grains, cell wall of baker's yeast, certain fungi, mushrooms and bacteria.

Grains are the primary source of β-glucans (Stuart *et. al.,* 1987): barley (2.0-9.0%), oats (2.5-6.6%), rye (1.9-2.9%) are particularly rich in β-glucans, while wheat (0.5- 1.5%), triticale (0.3-1.2%), sorghum (1%), rice (0.6%), and maize (0.1%) have lesser amounts of β-glucans. Ripsin *et. al.* (1992) showed that oat products consistently display hypocholesterolemic effects in controlled human studies. The relationship between

oat consumption and heart health effects is strong enough that the US Food and Drug Administration has proposed to allow a cardiovascular health claim on the label of oat based foods (Anonymous, 1996). The β-glucans may be playing a significant role in the observed effects and are proposed as a marker entity for oats. Oat β-glucans are the only dietary fiber currently recognized by the European Food Safety Authority (EFSA, 2011) to be able to reduce a disease risk.

Gums: are hetero-polysaccharides, which are stems exudates of some plants or seeds extracts. Gums are also called mucilages and are used in industry as thickeners. These gums consist of a polymer backbone to which sugar side chains are attached. The carbohydrates more represented in gums are mannose, glucose, arabinose, xylitol, galactose, dextrane, xantane and uronic acid. The molecular weight of these compounds is highly variable, but generally these compounds are characterised by low molecular weight. Bacteria produce some gums, in particular those composed by dextran and xanthan.

Generally, cellulose, hemicelluloses and lignin are not viscous, and pectins and gums are viscous. Peptic substances and gums are easily fermented, which fermentability of hemicelluloses and cellulose are also dependent on the solubility (Kerley *et al.,* 1988; Guillon *et al.,* 1998).

Sources

Dietary fiber comes mainly from vegetables, fruits, nuts and seeds and also from sources of different nature, such as fungi (McDougall *et al.,* 1996). In animal nutrition the main source of carbohydrates (structural and non-structural) are represented by whole or husked cereal grains. Cereal grains are rich in starch, dietary fiber, trace minerals and vitamin B and E (Fardet, 2010). The whole grains are composed mainly of endosperm (about 80%), with the germ and bran that comprise variable proportions in function of the species and varieties (Slavin *et al.,* 2001). The American Association of Cereal Chemists International (A.A.C.C., 2001) and the Food and Drug Administration (FDA, 2006) have been defined the whole grains as: “intact, ground, cracked or flaked fruit of the cereals whose principal components, the starchy endosperm, germ and bran, are present in the same relative proportions as they exist in the intact grain”. The dietary fiber is principally located in cereals

bran, which represents a variable proportion of grain weight (18-87%) in function of the species, being affected by seeds morphology. Consequently, dietary fiber content varies considerably among cereal species and varieties as well as in function of the processing methods used. For example milled rice and row oats show very different dietary fiber contents: about 10% vs. more than 30%, respectively.

In all cereal bran, the insoluble dietary fiber is largely predominant, particularly in rice and wheat, while oat and barley fiber is richer in β-glucans and consequently in soluble fiber. The physiological function and behaviour of the dietary fiber added to a product depends on the IDF/SDF ratio (Fernández and Rodríguez, 2001), researchers are looking for sources rich in one or both fractions.

Rice (*Oryza sativa*) is the most used cereal in the world (Ryan, 2011), about 631 million tons of rice are harvested annually (Kahlon, 2009) mainly for human nutrition. When rice paddy is milled, the first envelope, which is removed, is the hull and the resulting portion is considered a whole rice grain. The further milling from brown rice to white rice removes the rice bran (Ryan, 2011). Rice is the major starch source in human and companion animal's nutrition, whereas its harvesting and milling by-products were highly utilised in livestock nutrition.

Oats and barley are good sources of β-glucans (Spina *et al.*, 2013), which contribute to reduce lipid and glucose levels in humans and animals serum. As humans, companion animals have a high incidence of metabolic diseases. Therefore, the use of these whole cereals as functional ingredients can be useful in the control or prevention of obesity, diabetes mellitus and dyslipidaemia. Cereal's β-glucans have rheological properties comparable to Guar gum (Wood *et al.,* 1994; Wood 2007; Giacomessi and Jorgens, 2013). In general, the content of β-glucans from oats varies from 3 to 5% as fed (a.f.), while in barley ranges from 7 to 11% a.f. (Skendi *et al.,* 2003).

For example rice bran has a variable Total Dietary Fiber (TDF) content from 21 to 27% and is highly insoluble. It is rich in tocopherols and γ-oryzanol and presents a correct CA/P ratio. In Europe and, in particular in Italy, the most utilised cereal bran is the wheat bran which fiber is highly insoluble (more than 90%) (Duque *et al.,* 1998) and it is moderately fermentable (Cutrignelli, 2007; Calabrò *et al.*, 2012). The wheat bran TDF is composed by cellulose (14-24%) and lignin (5-10%) (Bello, 2000) and the dietary fiber is completely insoluble.

Beet pulp, cereals bran and cellulose were traditionally utilised as fiber supplement in animal nutrition, particularly in omnivores and carnivores. Thanks to their specific physic and chemical properties these by-products affect the diet digestibility and fermentability.

Beet pulp is moderately fermentable, with a mean ratio IDF/SDF of 70/30 (Fahey *et al.,* 1990; Sunvold *et al.* 1995c). Fahey *et al.* (1990) have reported that beet pulp contains 16% of soluble polysaccharides, 31% of hemicelluloses and 25% of cellulose, while Bosch *et al.* (2008) have reported the same cellulose and hemicelluloses contents (22%). These data indicate the high variability that can occurs in this sugar by-product due to the different technological processes. Even if the different chemical characteristics seem to not affect the beet pulp fermentability, several authors (Sunvold *et al.*, 1995a; Bosch *et al.*, 2008; Calabrò *et al.*, 2012) reported similar values of cumulative gas production and dry matter disappearance by *in vitro* gas production using faecal inoculum from dog.

In nature cellulose is insoluble fiber, non-fermentable and not viscous for these characteristics different cellulose extracts were utilised as functional ingredients both in food and feed industries (Sunvold *et al.,* 1995a; Sunvold *et al.,* 1995b). A cellulose-derivative, named carboxymethyl cellulose, in which some hydroxyl groups were substituted by carboxymethyl groups is characterised by TDF content similar to cellulose, but it is richer in soluble fraction. In spite of these physic differences, comparing *in vitro* the fermentation characteristics of several carbohydrates sources, Calabrò *et al.* (2012) have not found any significant differences in cumulative volume of gas between cellulose and carboxymethyl cellulose, confirming that the higher solubility did not alter the fermentability.

Several alternative dietary fiber sources have been studied as potential functional ingredients in animal nutrition, such as fiber fruits and legume fiber. Fiber fruits are by-products of juice or puree, dried and ground as fine particle size (Walter *et al.,* 1985). Usually fruit fiber contains more pectins and hemicelluloses than cellulose (Fischer, 2009). Fruit by-products could have different fiber composition in function of fruit species, fruit product (juice, pulp, marmalade, etc.) and technical process. Fiber legume, such as pea fiber, are by-products of canned or frozen legume grains industry and it is particularly rich in TDF (mean value of 77% a.f.) almost completely insoluble (more than 95% TDF).

There are other types of functional polysaccharides, such as gums. Guar gum is one of the most utilised; it is present in the endosperm of the seeds of an annual leguminous plant (*Cyamopsis tetragonoloba* L.), the Guar germ. The Guar germ is widespread in subtropical Asian areas (India, Pakistan), where it is cultivated for forage and grain. Guar

gum is fermented with a high propionic acid production (Telung *et al.,* 1987). The Arabic gum is the dried exudate of the leaves and stems of the Acacia tree. It is a complex polysaccharide (arabino-galactan with a glycoprotein), characterized by high molecular weight. It is used by industry as additive (emulsifier or stabilizer) in different foodstuffs. The Arabic gum increases the fermentations with high acetic and butyric production.

Physico-chemical and functional characteristics

The properties of the different fiber fractions are studied in order to understand the industrial application and physiological effects in human and animal. These properties are a reflection of the composition and quantity of soluble and insoluble fiber, chemical nature, structure of the fiber, the process to which it has been subjected and the size of the particles (Rosado and Díaz, 1995). The size of the fiber particles establishes the type of process in which it is incorporated. The optimal size of the fiber particles is included in a range from 50 to 500 nm (Fuertes, 1998), while particles of greater dimensions can affect the appearance of the product and provide chewing difficulties, resulting in the formation of lumps and agglomeration and therefore compression of the product.

In a study performed by Sagnark and Noomhorm (2003) specifically with bagasse of sugar cane, it has been found that a reduction of the particle size can affect the increase of density and could reduce the capacity of retention of water and oil holding capacity, probably because the cellulose size affects the matrix structure. Thus could affect intestinal transit decreasing the volume of the faecal mass. This behaviour was also observed by Dreher in 1999 (quoted by Betancur-Ancona, 2004) to decrease the size of the particles of wheat bran.

When an ingredient or a component of food changes the characteristics of composed feed or contributing to the quality of the final product it could be considered a “processing aid” (Badui, 1999). The technological properties which the dietary fiber confer to food are: the ability to retain water, the oil holding capacity, the swellability, the adsorption and water absorption capacity, the emulsifying activity and emulsion stability. During the processing of food industry it is necessary to know the potential of application and the functional properties of each fiber sources as well as the IDF/SDF ratio and product final destination (Zambrano *et al.,* 2001).

The ability to retain water

The ability to retain water expresses the maximum amount of water that can be retained by a sample of dry matter with known weight and under the action of a force model (Tamayo and Bermúdez, 1998). This property affects the fiber physiological effects into the organism and the maximum level of incorporation in food/feed (Zambrano *et al.,* 2001). The water retention determines the viscosity of the mixture, defining the machining of foodstuffs. The factors that influence the ability to retain water in a fiber are the particle size, the pH and the ionic strength (Baquero and Bermúdez, 1998). Fruit-based products for their balanced IDF/SDF have good properties to bond water, and this can be used in the manufacture of foods to control food texture and rheological behaviour (Fischer, 2009). This characteristic can be advantageous in canned pet-food production, where the high water content must be related to low activity water in order to obtain the desirable consistency.

Oil holding capacity

Particles with large surface have a good ability to trap components of oily nature; the fat is mechanically trapped in mainly fiber surface. It has been observed that the insoluble carbohydrates have higher absorption capacity than soluble ones, which were used as emulsifiers. This property is related to size and surface of fiber particles (Cruz, 2002).

Emulsifier activity and emulsion stability

An emulsion is a mixture of two or more liquids that are normally immiscible (eg. water and oil). There are different emulsifier substances, which act according to different chemical and physical processes. Some polysaccharides like Arabic gum and carboxymethyl cellulose increase the viscosity of the medium, helping to create and maintain the suspension of globules of dispersed phase. The emulsifier activity is referred to the volume of oil that a substance could emulsify; the emulsion stability refers to the ability of an emulsion in resisting to the changes in its properties over time and it is compared to the volume of oil retained by the emulsifier after the emulsion collapse (Peraza *et al.,* 2001). This reaction is influenced by the interaction that can occur between proteins, polysaccharides and other compounds involved in the emulsion (Dickinson, 1995). The addition to a product

of emulsifiers capable of forming stable emulsions improves its shelf life, preserving the structure and the visual quality (Peraza *et al.,* 2001).

Swellability

It refers to the ability of a product to increase its volume in the presence of high quantity of water (Tamayo and Bermúndez, 1998). This characteristic is affected by the amount of polysaccharides, the porosity and the fiber particle size (Femenia *et al.,* 1997). The access of water causes a swelling of the vegetable cell wall, which allows the complete relaxation and dispersal of macromolecules present in it. The insoluble polysaccharides, such as cellulose, are characterised by strong and compact structure that determine the cell swelling due to the access and retention of water. Soluble polysaccharides absorb the water until a break point, beyond which they are solubilised (Thibault *et al*., 1992; Roberfroid, 1993).

Water adsorption and absorption

The adsorption of water is the capacity of a substance to retain water on the surface. This characteristic affects the stability of a product and the degenerative changes during its storage. The water absorption is defined as the amount of water that a substrate can absorb when it is placed in sufficient quantity of water. This property is important when the fiber is used into extruded products, since the material must be moistened before and during the process. Both properties are related to chemical nature, origin of related compounds, pH and form in which the sample is prepared (Zambrano *et al.,* 2001).

Nutritional and health effects

Carbohydrates in digestive tract are characterised by different fate as represented in table 1. Mono and disaccharides are rapidly absorbed and transformed in glucose. Starches are one of the most important energy sources for omnivore and carnivore species, usually high proportions of starches are rapidly hydrolysed in more simple carbohydrates into the small intestine and transformed in glucose. However, in function of starch sources and feed

processing, a variable portion of administered starch could result indigestible in the first part of the digestive tube and quickly fermentable into large intestine.

The complex structural carbohydrates (fructans, galactans, mannans, mucillages, pectins, hemicelluloses, cellulose) are fermented into the large intestine. In function of their chemical structure the rate of fermentation of the complex carbohydrates varies considerably. We could difference rapidly (fructans, galactans, mannans, mucillages), moderately (pectins, hemicelluloses) and slowly (cellulose) fermentable carbohydrates. The rate and extent of fiber fermentation are important distinguishing characteristics when discussing physiologic function of fiber. As the fermentation rate of fiber increases gastrointestinal transit time, faecal bulk decrease, while bile acid excretion with faeces increases.

Certain microorganisms into the gut are able to use fiber as nutrients sources producing metabolites (short chain fatty acids, methane and nitrogen), which could alter the gut habitat and represent nutrients for the host. Several fermentable carbohydrates are classified as “prebiotic fiber” with beneficial effects on host health. A prebiotic is a non-digestible ingredient that beneficially affects the host by selectively stimulating in the large intestine the growth and/or activity of one or a limited number of saprophytic bacterial species, and thus improves host health.

The nutritional and health benefits of dietary fiber continue to be the subject of several studies and have been reviewed by many authors (O’Sullivan, 1998; Anderson *et al.*, 2009). There are many epidemiological evidences of the role of dietary fiber in disease prevention (Ahmed, 1995; Marlett *et al.*, 2002). In human medicine inverse relationships between fibre intake and incidence of obesity, heart disease and some cancer (carcinoma of colon and breast) (Bingham *et al.,* 1979; Fuchs *et al.,* 1999; Bingham *et al.,* 2003; Park *et al*., 2005), diabetes and gastrointestinal d*iso*rders have been reported (Chandalia *et al.*, 2000; Slavin, 2008). Despite the difficulty in utilizing health claim on food labels, both the European Food Safety Authority (EFSA) and United States Food and Drug Administration (US FDA) increasingly request documented evidences of structure/effect relationships in order to review health claims. Either in livestock and companion animals the prebiotics fibers have functional properties that improve host production and health (Sunvold *et al.*, 1995a; Sunvold *et al.*, 1995b).

Table 1 - Carbohydrates and their fate in digestive tract.

Carbohydrates	**Fate**
Mono- and disaccharides	Absorbed
Starch	Enzymatically digested
Resistant starch	Moderately fermentable
Fructans, galactans, mannans, mucilages	Rapidly fermentable
Pectins	
Hemicelluloses	Moderately fermentable
	Slowly fermentable
Cellulose	
Lignin	Non digested or fermentable

Since 1940_s antibiotics are administered in livestock to promote animal performance (Cromwell, 2000; Anderson *et al.,* 2000; Gaskins *et al.,* 2000). In 2003 the European Council has banned these molecules as growth promoters (EU Regulation (EC) No 1831/2003), consequently the number of studies carried out to find alternative methods to control and prevent pathogenic bacterial colonization increased. The modulation of gut microbiota with new additives, such as prebiotics, towards host-protecting functions to support animal health, is a topical issue in animal nutrition and creating fascinating possibilities. Carbohydrates are the first limiting nutrient for many bacterial species in the intestinal tract; the type of carbohydrates available influences the bacterial growth, selectively enriching or limiting growth of some bacterial species, directly and indirectly, because the carbohydrates fermentation allows important changes of luminal habitat (SCFAs and nitrogen availability and pH reduction). However, not all the dietary carbohydrates are prebiotics, and clear criteria for classifying them, as prebiotics are not established yet. Also the effects of different carbohydrates on host health vary considerably in function of their chemical

structure and physical characteristics (Jenkins *et al.,* 1975). Measurement of fiber components as well as their individual chemical compositions may be required to interpret the physiological effects of various food and fiber preparation. Several complex carbohydrates such as cellulose and hemicelluloses, which are partially and slowly fermentable, even if they are not classifiable as prebiotics, could represent functional ingredient too. For example the supplementation of cellulose in diets for obese patients could be useful for inducing satiety sensation and reducing nutrient absorption, indeed epidemiological studies show that high intakes of dietary fiber are associated with lower body weight and decreased risk of obesity (Pond *et al.*, 1988; Slavin, 2005; German, 2006). The effects on host health vary in function of several factors related to carbohydrates and/or the host and are resumed in table 2.

Table 2 – Physical properties of different complex carbohydrates and physiological and nutritional effect on host health.

Carbohydrates	Physical Properties	Physiological Effects	Nutritional
Pectins, gums, β-glucans	Viscosity	gel formation	↑ transit rate ↓ insulin, glucose
Pectins, gums, β-Glucans	Water Retention	↑ viscosity, microbial activity	↑ transit rate
Polysaccharides	Microbial Degradation	↑ microbial growth, polysaccharides fermentation, SCFA synthesis	↑ faeces yield, SCFA, ↓ pH
Pectins, gums, Lignin	Organic molecule holding capacity	Interaction with bile acids and digestive enzymes	↑ bile in faeces ↓ enzymes activity

Effect of the fiber on the gastrointestinal tract

In recent years, the microbial fermentation of complex polysaccharides in the gastro-intestinal tract of non-ruminant species has been a subject of considerable interest. In omnivore and carnivore species, where the feed digestion is mainly linked to the action of hydrolytic enzymes, when diets rich in fiber are administered, the role of large intestinal microorganisms population becomes indispensable. The gastrointestinal tract presents a complex variety of fungi and numerous bacterial species (more than 500, about 10^{10} bacteria

per g of gut content), most of them are strictly anaerobic. The composition and distribution of such species varies in function of several host's aspects (species, age, physio-pathological condition) and environment. At the birth gut is sterile and its colonization, in most of case, is completed within 3-4 weeks with the development of "mature" microflora. Gut bacteria are not pathogen and constitute, for the host, a barrier against pathogen bacteria. The interactions between luminal factors (of diets or bacterial origin) and gut wall influence digestion (secretion, absorption and motility), immunologic mechanisms (exclusion of antigens regulation of gastrointestinal immune system, antigen processing, sensitivity and allergic responses) and neuroendocrine processes. The combined actions of bacteria and host factors are defined "resistance to colonization": animal defends itself through the peristaltic movements, the exfoliation of epithelial cells and the production of immunoglobulins form the lymphoid tissue, while bacterial flora combats pathogens both competing for the same sites of attack and nutrients and thanks to the production of extra-cellular enzymes. The fermentation of fiber is guaranteed by the synergic action of different bacterial populations. The polymeric substrates are hydrolysed to glucose, galactose, xylose, arabinose, and uric acid, subsequently fermented to pyruvate and, possibly, to lactic acid, short chain fatty acids (acetic, propionate and butyrate) and gas (methane, hydrogen and carbon dioxide) (Macfarlane and Cummings, 1991). These metabolites play an important role in the host animal ensuring the maintenance of the state of health of the gastrointestinal tract. In particular, short chain fatty acids have also a tropic effect on the intestinal epithelial tissue, promoting cellular turnover. Typically the molar ratio of fatty acids produced may change depending on type of substrate, pH and microflora composition (Williams *et al.*, 2001). In pigs the administration of diets rich in simple sugars and starch reduces the availability of carbohydrates for bacteria, so that the carbohydrate/nitrogen ratio and the bacteria fermentation become principally proteolytic (Piva *et al*., 1995), producing higher proportion of branched fatty acids and catabolities (ammonia, amine, volatile phenol and indole) which could have toxic effects. Excessive ammonia production in the intestine may influence the life cycle of the digestive mucosa cells, increases urea excretion with loss of energy, and could cause colon cancer.

The effect found after administration of diets high in complex carbohydrates vary a lot in function of the fiber solubility. The use of diets rich in soluble fibre stimulates the non-pathogen gut microflora, reducing protein catabolism and diarrhoea incidence. The extent of microbial fermentation of indigested polysaccharides is influenced by carbohydrate

polymers nature and degree of lignification (Bach Knudsen *et al.,* 1991). The SCFAs produced are physiologically important especially in the large intestine, in which butyrate in particular is required to maintain the health of the epithelial cells of gut mucosa (Roediger, 1980). Although the gastrointestinal tract utilizes a large proportion of whole-body energy and protein (Ferrell, 1988), and thereby has a major influence on the efficiency of growth, the role of dietary factors in regulating intestinal growth and development is poorly understood. In several species, weight, volume, and gut capacity have been shown to increase with rising dietary fiber (Coey and Robinson, 1954; Southgate, 1990; Hansen *et al.,* 1992). Complex carbohydrates concentration in the diet affects also intestinal motility in rats (Schneeman and Gallaher, 1985) and gut morphology in pigs (Jin, 1992). Moreover, addition of cellulose to low-fiber diet induced a significant increase in colonic DNA synthesis in rats (Sircar *et al.,* 1983; Jacobs and Lupton, 1984).

The effects of different levels of fiber inclusion in the pigs diets were studied (Just *et al.,* 1983), comparing a low-fiber diet based on barley and wheat starch as a carbohydrates source, and a high-fiber diet based on barley, fiber pea and pectins. After two months of administration, pigs were sacrificed and the contents of each gastrointestinal segment were analysed. The amount of gastrointestinal contents in all segments of the gut was greater in pigs fed with a high-fiber diet than in pigs of the other group (10.7 vs. 3.6 kg for the entire intestine, respectively). The use of diets for pigs with high levels of crude fiber increased the amount of gastrointestinal contents (Bach Knudsen *et al.,* 1991; Low *et al.,* 1978).

In several studies on pigs the prebiotics administration were related to improvements of growth performance, with decrease of mortality and morbidity indexes and reduction of medication costs (Patterson and Burkholder, 2003).

In recent years, companion animal's owners have become more aware of the important relationship between diet and health for their pets. Consumers are now looking for ingredients sources that could support health beyond providing basic nutrition. Prebiotics play an important role in the development of new pet-food. Studies carried out by the 1990^{s} to today on prebiotics have reported interesting effects on: feed intake, stool consistency, faecal output, fermentation end-products, immune indices, intestinal microbial populations.

Stool consistency and faecal quality are important output in companion animal nutrition. Specific microorganisms found in the large intestine of dogs and cats are at various grades capable of degrading fiber (Case *et al*., 1998; Reinhart and Sunvold, 1996).

Zentek (1995) compared the effect of cellulose (IDF and SDF), pectins (soluble fiber) and Guar gum (soluble fiber) in concentration of 10% in the diet: the dry matter (DM) and acid detergent fiber (ADF) apparent digestibility decreased with all the supplementation, and cellulose showed the lowest DM and ADF digestibility values. Muir *et al*. (1996) confirmed these results, administering to dog diets with different concentrations of pectins (source of soluble fiber) and cellulose (insoluble fiber source) in ratio of 2.5% cellulose and 5% pectin vs. 5% cellulose and 2.5% pectins vs. or 7.5% cellulose. The digestibility of total dietary fiber linearly decreased with the increase of the cellulose content in the diet.

On the other hand, excessive fiber intake, especially high-fermentable carbohydrates, can lead to poor quality faeces formation with great gas production, due to the increased peristalsis and higher water retention in stools (Case *et al*., 1998; Reinhart and Sunvold, 1996). Indeed, high fiber concentration in the diets causes high water faecal retention, which stimulates mucosal receptors and increases peristaltic movements, reducing retention time of digesta in the gut (Lewis *et al*., 1994). Fiber is the main nutrient that affect digestibility of crude energy and its inclusion in equations employ the estimate of metabolizable energy of feed (Kienzle *et al.*, 1998). Fiber could also affect feed palatability even if this effect is directly related to fiber source and quantity (Carciofi, 2005).

Similar gastrointestinal diseases affect human, dogs and cats and the fiber supplementation has been advocated as part of the therapeutic protocol against these d*iso*rders. Because the intestine is considered the largest lymphoid organ, disease, stress and starvation can compromise intestinal health and impairing animal immunologic defences (Brunetto *et al.*, 2010).

The principal role of insoluble carbohydrates is to modulate the gastro-intestinal functionality, contributing to increase faecal mass and determining higher intestinal rate. Thanks to these characteristics, they may help in the management of constipation, diverticulitis, haemorrhoids and cancer of the large intestine in dogs (Gumaa *et al.*, 2001). Moreover they increase transit rate and decrease digestibility with worsening of feed conversion in rabbits (Romero *et al*., 2011). The reduction of luminal pH allows changing in composition and metabolic activity of intestinal microbiota. The SCFAs produced, specifically butyrate, are the main energy source for the colonocytes, modulate the absorption of ions and influence bloodstream and intestinal peristalsis (Campbell *et al*., 1997; Roediger, 1982).

The presence of dietary fiber promotes the development of gut bacteria (in particular *Lactobacillus* and *Bifidobacteria* sp.), reducing the risk of pathogenic bacteria colonization

responsible of neonatal diarrhoea in pigs (Edwards and Parrett, 1996), rabbits (Xiccato *et al.,* 2008) and dogs (Havenaar *et al.,* 1992). These infective diseases are the most diffuses causes of neonatal death in all animal species producing high economic losses.

Many authors clearly evidenced that sources of non-starch polysaccharides increase the proliferation of intestinal epithelial cells (Pell *et al.*, 1992; Lupton *et al.*, 1988), although the mechanism by which this effect is expressed is not established yet. Maybe, this phenomenon is attributed to different mechanisms involved simultaneously, as the higher release of organic substances in the large intestine (Mathers *et al.*, 1993), which would stimulate the bacterial fermentation resulting in greater production of SCFAs and the achievement a more acidic pH. These changes, in turn, would induce a greater proliferation of the crypts' cells (Lupton and Kurtz, 1993). Sakata (1987) highlighted as the butyrate infusion in the cecum and/or in the colon stimulates the crypts cells proliferation. Goodiad *et al.* (1989) have reported that the non-starch polysaccharides of the diet increase the cell proliferation in the small intestine and in the colon of conventional rats, while no effects were found in germ-free animals; the authors concluded that some fermentation products increase cell proliferation. Another mechanism through which the polysaccharides affect the intestinal cells development is the formation of inorganic ions (i.e. calcium) into the lumen (James, 1980) that are considered important modulators of cell proliferation (Durham and Walton, 1982). The soluble polysaccharides bind bile acids, which are known to damage mucosal cell walls, causing a high incidence of mutations that may result in a compensatory synthesis (Jacobs and Lupton, 1984). Hormones play an important role in regulating the cell proliferation; Pell *et al.* (1992) and Winsette *et al*. (1986) reported that soluble carbohydrates affect the production of some intestinal hormones, such as entero-glucagon and gastrin.

Obesity

Obesity is a pathological condition characterized by excessive deposition of fat, which leads to changes in various body functions, potentially harmful for health. The prevalence of obesity has increased to endemic proportions (Hulouyang *et al*., 2003). Obesity is a complex syndrome, in which the increase in fat mass is accompanied by profound changes in physiological functions (Gayet *et al*., 2004; Crane, 1991). The bad habits of companion animal's owners have greatly helped to increase obesity incidence as well as the amount of

palatable snacks and high energetic value offered them (Markwell and Butterwick, 1994). Nutrients and ingredients that can favour weight loss are important today and have potential use in diets for pets. The reduction of diet energy using fiber is a common strategy to promote weight loss in dogs (Carciofi *et al*., 2005; Jewell *et al*., 2006). Dobenecker and Kienzle (1998) suggested that high cellulose content in the diet affects feed intake due to the reduction in palatability. Jewel *et al*. (2006) concluded that feed intake and body fat in obese dogs decrease increasing the percentage of fiber in the diet.

The intensity of satiety response is related to the time between two following meals and the amount of consumed food (Burton-Freeman, 2000; Slavin and Green, 2007); understanding the mechanisms involved in the regulation of feed intake has great value for the development of effective approaches to control obesity in dogs. Dietary fiber may be important artifice to calorie restriction and appetite modulation (Jewell and Toll, 1996; Jackson *et al*., 1997; Jewell *et al*., 2000; Jewell *et al*., 2006). The regulation of feed intake in animals is related to a complex system involving dietary energy, energy requirements of animals, nervous and sensory stimuli, neurotransmitters, hormones, such as characteristics of foods and nutrients in the diets (Schwartz *et al*., 2000). The hypothalamus is the main modulator of feed intake. Currently, more than 40 hormones and peptides are known to act in neuroendocrine control of feed intake, determining stimuli for hunger or satiety behaviour in function of dietary nutrient concentration and absorption (Vasconcellos *et al*., 2005). Catecholamine such as adrenaline and noradrenaline, stimulates appetite. The main physiological factor that influences the secretion of catecholamine is hypoglycaemia (Greco and Stabenfeldt, 1999). The chronic administration of noradrenaline induced obesity in laboratory animals due to overfeeding, and preferably increased consumption of carbohydrates (Case *et al*., 1998). Other neurotransmitters can inhibit appetite, i.e. dopamine and serotonin (Mourão, 2007; Schwartz *et al*., 2000). Physiological and environmental signals stimulate the release of two peptides before and after meals, respectively ghrelin and cholecystokinin (CCK). Besides, ghrelin, acting in energy metabolism, stimulates hunger. When there is an increase in fat and protein consumption the CCK has positive feedback effect, inhibiting gastric emptying (Vasconcellos, 2008; Mourão, 2007; Burton-Freeman, 2000).

Bosch *et al*. (2009a, 2009b) evaluated the effect of diet fermentability on voluntary feed intake and activity in dogs. Dogs were fed either a low-fermentable fiber diet (8.5% cellulose) or a high-fermentable fiber diet (8.5% sugar beet pulp + 2% inulin). Compared to

dogs fed low fermentable diet, the high fermentable group tended to have a lower voluntary feed intake (404 ± 70 g and 250 ± 46 g, respectively), it is probably related to the greater fermentability of the diet.

The effect of dietary fiber on feed intake has been measured in several studies using dogs (Fahey *et al*., 1990; Fahey *et al*., 1992; Sunvold *et al*., 1995c) even if the results were contradictory and the different results are only partially justifiable by the observation that diet palatability influences the regulation of feed ingestion.

The nutritional strategies to prevent or treat obesity include reduction of diet energy density and promotion of satiety (Englyst *et al.,* 2007). Dietary fiber fermentation in the large intestine might promote satiety by affecting blood lipid levels (Slavin, 2003; Brighenti *et al.*, 2006). Diez *et al.* (1998), administering to eight Beagle dogs four diets containing different types of fiber, including Guar gum, observed that this polysaccharide reduced blood cholesterol levels in fasting, post-prandial glucose levels and plasmatic urea concentrations. Also in humans has been demonstrated that Guar gum, completely fermented in the colon, reduces the serum levels of cholesterol and triglycerides (Mclean *et al.,* 1983).

Moreover, high fiber diets due to their consistency encourage mastication and stimulate the secretion of digestive juices. The soluble components of dietary fiber, especially from cereals dietary fibers (β-glucans and arabinoxylans) in the stomach form viscous compound that delays gastric emptying. This effect is due to the ability in forming a gelatinous mass by binding water, consequently the food transit along the digestive tract is slowed, limiting the action of digestive enzymes, decelerating the emptying of the stomach (satiety) and influencing lipid and carbohydrate metabolisms (Leclere *et al.,* 1994). The viscosity of a meal is affected by fiber molecular weight, food processing methods, and the combination of feeds and water intake during a meal (Tosh, 2007). No standardized method to monitor the bolus physico-chemical behaviour in gut is still available, thus it is difficult to compare the outcomes of different studies (Salovaara *et al.,* 2007). Viscous polysaccharides represent a valuable aid in the treatment of obesity as well as other metabolic diseases (i.e. diabetes) in dogs as in humans (Aguggini *et al.,* 1992).

Diabetes

The consumption of dietary fiber is associated with a reduction of diabetes, which is characterized by sustained high blood sugar levels due to insufficient insulin production or to improperly use of the insulin produced. There are several important habits useful to reduce diabetes risk, such as maintaining a healthy weight and being physically active. Food and feed processing methods (i.e. milling, cooking, extrusion and freezing) could modify the carbohydrates metabolism and consequently the post-prandial glycaemic response. Modifying the viscosity of extracted β-glucan, probably for the formation of hydrogen bonds intra-and inter-molecular, alter the structure and the viscosity of the feed (Tosh, 2007). Particle size may also influence the post-prandial responses; Behall *et al.* (2005) observed higher plasma glucose and insulin responses after a meal containing finely ground wheat flour than after a meals containing coarse flour.

Variation of response in post-prandial glucose to different sources of starch and nutrient composition of the diets has been observed in human and animals (Nguyen *et al*., 1998; Nelson *et al*., 2000; Murray *et al*., 1999; Carciofi *et al*., 2008; De-Oliveira *et al.*, 2011; Musco *et al.*, 2013; Musco *et al.*, 2014) as represented in figure 1. The addition of fiber to a diet can result in lower post-prandial glycaemic peaks, reducing glucose absorption and insulin requirements in diabetic subjects.

Soluble fiber has also been shown to reduce selectively serum low density lipoprotein (LDL) cholesterol and to improve glucose metabolism and insulin response (Glore *et al*., 1994). Soluble fiber found in beans, oats and flaxseed might help to reduce total blood cholesterol levels by lowering low-density lipoprotein or “bad” cholesterol (figure 2).

Epidemiologic studies have shown that fiber increase in the diet can reduce blood pressure and inflammation and protect heart health. In animal studies, Guar gum incorporated into the hyper-cholesterolemic diets lowered serum cholesterol (Fahrenbach *et al.,* 1966; Riccardi *et al.,* 1967), liver cholesterol and total liver lipid (Riccardi *et al.,* 1967; Ershoff *et al.,* 1962) in rats.

Figure 1 - Possible mechanisms by which complex carbohydrates help to reduce glycaemia.

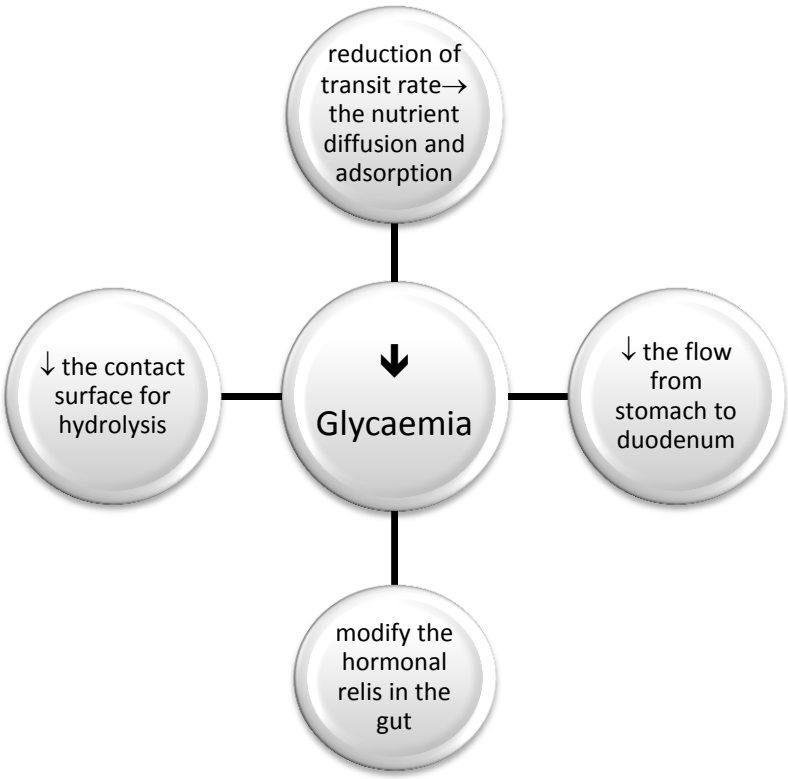
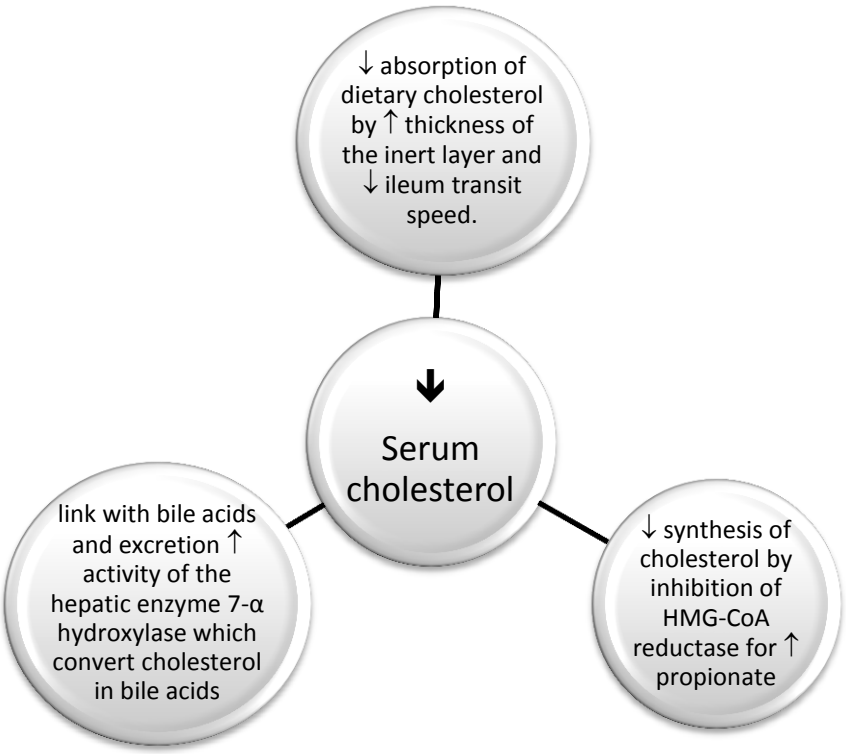


Figure 2 - Possible mechanisms of interaction of non-starch polysaccharides and blood levels of cholesterol.



Cancer

Several studies suggested that diet plays an important role in the etiology of certain cancers, particularly those of the colon and rectum,. In the 1970_s, Burkitt proposed the hypothesis that dietary fiber could reduce the risk of colorectal cancer, based on the observation of low rates of such cancer among rural Africans who eat diet with high fiber content. Several plausible mechanisms have been proposed to explain this hypothesis, including increased stool bulk and dilution of carcinogens in the colonic lumen, reduced transit time, and bacterial fermentation of fiber to short chain fatty acids. The physico-chemical and fermentation characteristics of the fiber seem to cause these effects. Indeed, the inclusion of fermentable and/or moderate fermentable carbohydrates in a diet stimulates the formation of faecal mass and reduces the residence time in the colon-rectum, considered one of the predisposing factors for this type of cancer as, probably increases the exposure time of colonocytes to carcinogens (Ferguson *et al.*, 2000).

Prebiotics, such as fructo-oligosaccharides or inulin, have the ability to influence the intestinal microbial population by favouring the development of beneficial species, causing reduction of those pathogens. The fermentation of carbohydrates by saprophytic bacteria limits the production of putrefactive compounds (i.e. ammonia) that can promote the development of cancer. Hussein *et al.* (1999) showed that the supplementation of oligofructose in dogs diet reduces faecal ammonia concentration and increases SCFAs and the presence of *Bifidobacterium spp*. The production of these fatty acids in turn causes a reduction of intestinal pH and can improve the absorption of cations, in particular calcium ions, that inhibit the proliferation, and consequently differentiation and apoptosis, of mucosa cells (Lamprecht and Lipkin 2003). In particular, Frankel *et al.* (1994) hypothesized that the production of butyrate reduces the risk of tumour development.

In the colon, dietary fiber tends to increase faecal bulking due to increased water retention and the insoluble dietary fiber reduces transit time. This is particularly important since it is known that the conversion of sterols to carcinogenic polycyclic aromatic hydrocarbons occurs with time. Epidemiological evidence suggests that low faecal weights are associated with an increased risk of colon cancer (Burkitt *et al.*, 1972; Cummings *et al.*, 1982). Dietary fiber may also bind toxins, bile acids and carcinogens (Reddy, 1980).

Analysis of dietary fiber

In 1979 Prosky gathered a group of researchers in order to develop analytical methods to quantify the dietary fiber fractions in a way consistent with this definition. The first result was achieved in 1985, when the enzymatic gravimetric method to determine total dietary fiber was proposed. This method was validated in 1990 by the *Association of Official Analytical Chemists* AOAC (ID method 985.29). In 1988 the same research group developed a first method to quantify soluble and insoluble fractions of dietary fiber, based on phosphate buffer. The soluble portion was determined by precipitation in alcoholic solution (95%) followed by filtration in pre-weighed glass crucibles (Scott Duran, porosity #3). The method was validated in 1992 by AOAC (ID method 991.42). Lee and Prosky in 1995 proposed for the same scope a method based on MES-TRIS buffer utilization, which was also validated by AOAC (ID method 991.43). The latter allows a better evaluation of dietary fiber fractions due to an enzymatic digestion, which removes the non-resistant starch before the separation of the fraction.

Table 3 – Carbohydrates and fiber fractions solubility.

<table>
<tr><th>Carbohydrates and fiber fractions</th><th>Fiber solubility</th><th>Total dietary fiber analysis</th><th>Crude fiber analysis</th></tr>
<tr><td rowspan="4">Mono- and di- saccharides
Starch
Resistant starch
Fructans, galactans, mannans, mucilages
Pectins
Hemicelluloses
Cellulose
Lignin</td><td></td><td></td><td></td></tr>
<tr><td>Soluble fiber</td><td rowspan="3">Total dietary fiber</td><td></td></tr>
<tr><td>Insoluble fiber</td><td rowspan="2">Crude fiber</td></tr>
<tr><td></td><td></td></tr>
</table>

Several methods have been proposed to evaluate the dietary fiber content. A lot of them are based on an enzymatic pre-digestion in order to remove free sugars, starch and protein. The quantification of fiber fraction could be performed gravimetrically (Prosky *et al.,* 1990; Lee and Prosky, 1995; Monro, 1993) or by gas chromatograph (Faulks and Timms, 1985). Archibald and Kays (2000) have carried out a study on the total dietary fiber contained in cereals with a method based on the spectroscopic properties of its

components. This method does not require sample preparation and saves a lot of time and reagents. Kays and Barton (2002) have reported that they were able to quantify soluble dietary fiber and insoluble ones with the same method.

Currently the only officially recognized methods to determine total dietary fiber, soluble dietary fiber and insoluble dietary fiber are the methods proposed by the researches group coordinated by Prosky (AOAC ID methods: 985.29, 991.42 and 991.43).

General Aim

The general aim of the present PhD thesis, realised at the Department of Veterinary Medicine and Animal Production (University of Napoli, Federico II, Italia), was to study the functional properties of dietary fiber fractions (soluble and insoluble) in nutrition of different mono-stomached species (swine, dog and cat). With this aim more than 30 different feedstuffs, more of them by-products, were evaluated using several analytic techniques (Weende, Van Soest and Prosky) (Chapter 2). Moreover, in order define the functional activities and the potential health benefits some of these feedstuffs were tested *in vitro* using cumulative gas production technique (IVGPT). Tree different trials (Chapters 3 - 4 - 5) of IVGPT were carried out using as *inoculum* faeces from adult dog, swine and cat, respectively.

The comparison of chemical data and fermentation parameters was important to better define the potential functionality of each tested substrate in order to identify new functional ingredients, which could represent chipper constituents for diets of the tested species to improve animal health and quality of products.

Chapter 2 - Nutritional and functional evaluation of several feedstuffs for omnivore and carnivores species

Abstract

Twenty-eight feedstuffs were analysed in order to evaluate their nutritional value and the potential beneficial effects. Particular attention has been directed to determine the structural carbohydrates composition using the methods proposed by the researcher groups coordinated by Van Soest and by Prosky, respectively. The feedstuffs were divided into six categories according to their nature: legumes, cereals, seeds, roots and tubers, plant and by-products. The concentration of total dietary fiber in legumes resulted highly variable, from 17% (soybean) to 33% (beans) a.f.; cereal grains showed higher variation both for TDF concentration (from 8 to 38% a.f. in rice and oats, respectively) and for the proportion of insoluble/soluble fractions. On the contrary in seeds category the psyllium and hemp were characterised by high TDF contents and low IDF/SDF ratio (TDF: 44 vs 67%; IDF/SDF: 0.52 and 1.39 for psyllium and hemp, respectively). Root and tubers samples showed the highest variability, both in reserve and structural carbohydrates. Instead, carrots showed higher concentration of sugars and SDF than potatoes, while this last presented greater amount of starch and IDF. As expected, the highest variability was observed in the by-products category: TDF varied from 16 to 93% a.f., in faba pod, purified cellulose and sugar cane bagasse, respectively.

The use of alternative feedstuffs in the formulation of feed appears to be useful for prevention and treatment of gastro-intestinal diseases in livestock and companion animals. From these results it is possible to confirm the potential functional effects of several feed ingredients such as oats, peas, beans. In our opinion the obtained results justify the beneficial effects of diets supplementation with aloe, hemp, psyllium, legumes or fruit fiber registered in vivo by several authors, both in livestock and companion animals.

Keywords: complex polysaccharides, insoluble and soluble dietary fiber, by-products.

Introduction

Carbohydrates present in the diet of omnivore and carnivores species belong to the following classes: non-structural polysaccharides (pectins and hemicelluloses); reserve polysaccharides (fructans, glucofructans, mannans and galactomannans); isolated polysaccharides (gums and mucilages, containing a mixture of pentoses, hexoses and uronic acid, Kay, 1982); part of other compounds (lignin, proteins, lipids and cuticular inorganic constituents, like silica, magnesium, calcium and potassium) associated to the plant cell wall polysaccharides (Cummings, 1981); resistant starch, consists of starch portion resisting to the digestion in the small intestine, which becomes the substrate of fermentation in large intestine (Englyst and Cummings, 1987). Generally carbohydrates according to their solubility in water are divided into two main groups: soluble and insoluble. The first ones are very viscous and fermentable (approximately 100%) in the large intestine, while insoluble carbohydrates are not viscous and generally are little and slowly fermentable (Roberfroid, 1993).

Fermentable carbohydrates are important components of diets for omnivore and carnivores species, which reach the colon and are suitable for bacterial fermentation (Gibson and Roberfroid, 1995). Into large intestine they provide an adequate supply of organic matter for growth and maintenance of microbiota. Bacterial fermentation of these compounds results in short chain fatty acids (SCFAs), methane (CH_4), hydrogen (H_2) and carbon dioxide (CO_2), causing a luminal pH reduction. These changes of gut habitat could modify the composition and metabolic activity of the intestinal bacteria population (Campbell *et al.,* 1997). Human and animal nutritionists have long discussed on SCFAs role. In particular, these fatty acids are essential in maintaining the health of the gastro-intestinal tract, favouring the development of beneficial bacteria, predominantly *Lactobacilum* and *Bifidobacterium* spp., as well as of the local immune system. The short chain fatty acids, especially butyric acid, are important energy sources for colonocytes (Roediger, 1982). They lead to suitable ion absorption and act in intestinal blood flow and peristalsis, helping the host to increase the nutrients available for the colonocytes and saprophytic microorganisms (James, 1980). For all these aspects dietary fiber was considered a functional component of the diet.

Kritchevsky (1988) has defined dietary fiber as "*a heterogeneous group of substances with chemical structure, physical properties and specific physiological effects that are indigestible in the small intestine and fermentable by saprophytic bacteria present in the*

cecum-colon", including into the definition carbohydrates of different nature. With the exception of lignin, all included compounds are carbohydrates. Chemically they are "non-starch polysaccharides" (Cummings, 1981), such as cellulose and non-cellulosic polysaccharides (Kay, 1982). Nowadays the edible parts of plants and analogue carbohydrates of different nature, such as those that constitute the cell wall of some fungi, are identified as dietary fiber.

Aim of present study was to describe the nutritional characteristics of several feedstuffs to create a database useful to formulate functional diets for omnivore and carnivores species.

Material and methods

For the study 28 feedstuffs classified into six categories in function of the nature were analysed (table 1). The samples were ground (sieve of 1.1 mm) and analysed for proximate analysis (dry matter, crude protein, ether extract, ash and crude fiber) according to AOAC methods (2006). Starch content was measured after acid hydrolysis and polarimetric detection (Polax L, Atago – Tokyo, Japan) as indicated by Martillotti *et al.* (1987). Structural carbohydrates fractions were determined according the method proposed by Van Soest *et al.* (1991), calculating for difference the hemicelluloses, cellulose and Non Structural Carbohydrates (NSC) amounts. The indications of Prosky (1990) and Lee and Prosky (1995) were utilised to determine total dietary fiber (TDF), insoluble dietary fiber (IDF) and soluble dietary fiber (SDF) contents. For the separation of SDF from IDF fraction the MES-TRIS buffer was utilised and each residual was filtered through pre-weighed glass crucibles (Scott Duran porosity #3). TDF, IDF and SDF values were calculated deducting residual ash and crude protein contents.

For each sample, the gross energy content was estimated as follow:

$$GE = (4 \times CP) + (9.4 \times EE) + (4.15 \times NFE)$$

Where: GE is the gross energy content (kcal/kg), CP, EE and NFE the crude protein, ether extract and nitrogen free extract content (g/kg), respectively.

Table 1- Classification of the samples.

Category	Sample	Origin	Abbreviation
Legume grain	Lentil (*Lens culinaris*)	Italy	L-L
	Bean (*Pheseolus vulgaris* L.)	Italy	L-B
	Pea (*Pisumsativum* L.)	Italy	L-P
	Faba bean (*Viciafaba major* L.)	Italy	L-F
	Soybean (*Glycine max*)	Argentine	L-S
	Whole carob (*Certonia siliqua* L.)	Italy	L-C
Cereals	Rice (*Oryza sativa* L.)	Italy	C-R
	Corn (*Zeamays* L.)	Netherland	C-C
	Oats (*Avena sativa*)	Italy	C-O
	Wheat (*Triticuma estivum*)	Italy	C-W
	Barley (*Hordum vulgare* L.)	Italy	C-B
Seeds	Psyllium (*Plantagopsylium*)	Italy	S-P
	Hemp (*Cannabis sativa* L.)	Russian	S-H
	Linseed (*Linum usitatissmum* L.)	Italy	S-L
Roots and tubers	Carrots (*Daucus carota* L.)	Poland	R-C
	Potatoes flakes (*Solanum tuberum* L.)	Belgium	R-PF
Plant	Aloe (*Aloe arborescens* L.)	Italy	P-A
By-products	Beet pulp	Greece	B-BP
	Sugar cane bagasse	Brazil	B-SB
	Purified cellulose	India	B-C
	Wheat middling	Italy	B-WM
	Pea fibre	Belgium	B-PF
	Pea hulls	Italy	B-PH
	Pea pod	Italy	B-PP
	Faba pod	Italy	B-FP
	Fiber fruit	Brazil	B-FR1, B-FR2, B-FR3

Results

In table 2 the proximate composition, starch content and gross energy level of the tested feedstuffs were reported. As expected, legume grains, with the exception of whole carob (L-C) showed the highest values of crude protein (mean value 22% a.f.) and cereal grains showed moderate protein concentration (mean value equal to 9% a.f.). All seeds samples, aloe plant and several by-products (B-WM, B-PH, B-PP and B-FP) showed crude protein content higher than 10% a.f., whereas cellulose (B-C), sugar cane bagasse (BS-B), faba pod (B-FB) and all the fruits fiber (B-FR) showed the lowest protein values.

All tested seeds showed very high ether extract contents (mean value 35%), the other samples, with the exception of soybean (18% a.f.), were characterised by fat concentration lower than 5%.

Regarding the non-structural carbohydrates five samples (carrots, B-FR2, B-FR1, aloe and whole carob) showed the highest NSC contents (74, 70, 57, 53 and 46% a.f.,

respectively), consisting only in part by starch (6, 10, 0, 0 and 0% a.f., respectively) indicating a high concentration of soluble sugars.

Table 2- Proximate composition, starch content (% as feed) and gross energy (kcal/kg) value of the tested feedstuffs.

Category	Sample	DM	CP	EE	CF	NDF	ADF	ADL	NSC	Starch	Ash	GE
Legumes	L-L	89.9	22.9	0.85	4.91	23.8	7.81	3.12	39.9	36.6	2.51	3527
	L-B	87.5	20.2	1.61	4.65	11.2	6.02	1.43	50.6	28.2	3.82	3414
	L-P	88.8	18.2	0.75	6.28	11.0	7.14	1.63	55.9	44.0	2.90	3390
	L-F	93.6	27.2	2.14	2.54	9.17	4.45	1.72	50.4	30.8	4.60	3768
	L-S	93.4	34.8	18.1	9.07	9.70	9.10	3.4	18.0	6.78	5.00	4332
	L-C	86.6	8.35	1.75	7.30	26.5	18.2	10.7	45.8	-	4.19	3229
Cereals	C-R	87.8	7.59	3.98	0.71	3.33	0.92	0.22	72.1	71.9	0.80	3808
	C-C	88.2	5.80	3.45	2.74	4.17	2.81	0.71	73.9	58.0	0.94	3705
	C-O	92.9	9.40	4.53	12.8	32.8	15.2	2.37	42.3	38.6	3.85	3424
	C-W	87.7	11.8	1.50	2.68	12.0	2.70	0.79	60.6	59.1	1.83	3560
	C-B	89.5	9.41	2.03	6.41	10.5	7.02	1.77	64.6	48.2	2.98	3453
Seeds	S-P	94.3	19.2	28.3	19.4	40.8	19.6	9.64	2.33	-	3.55	4494
	S-H	91.9	17.9	35.9	25.1	34.0	22.8	7.81	0.98	-	3.07	4572
	S-L	93.3	17.1	41.8	7.92	11.9	9.32	0.90	14.3	-	8.18	5443
Roots and tubers	R-C	91.2	4.77	1.48	6.30	7.46	6.75	2.53	73.8	6.26	3.78	3458
	R-PF	90.4	4.93	1.77	1.87	4.17	2.60	1.40	76.2	54.4	3.35	3642
Plant	P-A	90.3	15.3	1.08	11.5	15.9	15.0	4.04	52.6	-	5.40	3138
By-products	B-BP	90.8	7.73	0.79	22.2	54.0	26.4	4.88	24.6	10.6	3.59	2759
	B-SB	98.6	1.27	0.83	46.0	89.2	57.0	9.93	5.85	1.08	1.48	2169
	B-C	96.2	0.30	0.17	72.2	92.1	89.6	2.73	2.68	-	0.92	967
	B-WM	88.7	14.6	4.77	10.6	36.2	12.6	4.32	27.5	20.8	4.91	3324
	B-PF	94.8	4.85	0.75	49.2	62.5	58.4	3.89	25.2	8.69	1.61	1876
	B-PH	92.5	15.8	0.70	14.9	19.8	16.4	3.56	47.4	10.9	4.08	3128
	B-PP	91.0	14.8	0.88	11.5	22.3	19.7	5.04	47.9	15.2	5.10	3170
	B-FP	94.5	13.4	0.76	34.5	30.8	23.0	3.38	35.4	3.80	4.06	2396
	B-FR1	87.4	4.59	0.18	9.83	23.1	12.7	4.93	56.8	-	2.73	3126
	B-FR2	94.7	2.85	2.03	8.72	14.7	11.2	5.16	70.5	10.3	4.72	3487
	B-FR3	91.6	7.47	0.50	17.8	42.8	22.9	6.01	37.2	10.6	3.71	2956

DM: dry matter; CP: crude protein; EE: ether extract; CF: crude fiber; NDF: neutral detergent fiber; ADF: acid detergent fibre; ADL: acid detergent lignin; NSC: non-structural carbohydrates; GE: gross energy.

As expected, the highest starch contents were registered for all cereal seeds and the legume grains (mean value 55 and 35%, respectively), with exception of soybean and whole carob. Both roots and tubers samples showed NSC contents higher than 70%, but their composition was highly different because in potatoes (71% of NSC) it is represented by starch, while in carrots by simple sugars (91% of NSC). The NSC composition of aloe was proportionally similar to carrots one.

Also some by-products are characterised by interesting amounts of starch, in particular pea pod and wheat middling (15 and 21% a.f., respectively), while the other by-products showed starch values included from 1.08 to 10.9% a.f..

Seeds samples did not present starch but all seeds showed the highest ether extract concentrations (28, 36 and 42% a.f., for S-P, S-H and S-L, respectively). In all the other samples the fat contents was almost lower than 5% with the exception of soybean (18% a.f.). This characteristic confers to all tested seeds the highest gross energy density (mean value 4836 kcal/kg). On the contrary the elevated energy content of legumes and cereal grains (mean value 3601 kcal/kg) resulting from high starch and protein levels, and for soybean from ether extract concentration too. Regarding by-products, the gross energy values (included from 1876 to 3487 kcal/kg) were mainly related to the different carbohydrates composition (i.e. starch and other sugars).

Regarding the structural carbohydrates composition, all the by-products showed high NDF values; in particular, four of them (B-C, B-SB, B-PF and B-BP) showed the highest amounts (92, 89, 62 and 54% a.f., respectively). However, in both sugar by-products (B-SB and B-BP) the neutral detergent fiber was similarly distributed between hemicelluloses and cellulose, while in purified cellulose (B-C) and pea fiber (B-PF) it was mainly represented by cellulose (94 and 87% of NDF, respectively). Among the other by-products, wheat middling, faba pod and fiber fruit number 3 seem interesting for their hemicelluloses levels. Two seeds samples hemp and psyllium showed high NDF concentration (34 and 41% a.f., respectively); in particular, hemp seeds fiber was characterised by a higher proportion of hemicelluloses than psyllium seeds, whereas the higher proportion was of cellulose.

Among cereals grain, oats, as expected, showed the highest NDF and lignin contents. Lentils and whole carob were the legumes with the highest NDF and lignin contents, even if these were due to different reasons: because lentil sample is composed only by grains, while carob sample comprised also the pod. All the other legume and cereal grains presented structural carbohydrates content closed to that presented in literature (De Almeida *et al.,* 2006; De Godoy *et al.,* 2013). As a whole, roots and tubers presented very low cell wall content.

As expected, total dietary fiber concentrations (table 3) reflect only in part the NDF amount. However, the four samples characterised by the highest NDF concentrations (B-C; B-SB; B-PF and B-BP) showed also the highest total dietary fiber values, but the samples with

the lowest NDF concentrations (C-R, C-C, R-PF, R-C, L-S and L-F) were not always characterised by the lowest TDF concentrations.

Table 3 - Dietary fiber fraction (% a.f.) of the analysed feedstuffs.

Category	Sample	TDF	IDF	SDF
		% a.f	% TDF	
Legumes	L-L	23.7	99.36	0.64
	L-B	33.5	98.22	1.78
	L-P	27.5	98.48	1.52
	L-F	21.5	97.55	2.45
	L-S	17.4	98.40	1.60
	L-C	29.5	91.99	8.01
Cereals	C-R	7.64	90.19	9.81
	C-C	34.7	85.08	14.92
	C-O	38.3	58.81	41.19
	C-W	12.6	97.67	2.33
	C-B	25.8	90.19	9.81
Seeds	S-P	44.0	34.34	65.66
	S-H	67.2	58.23	41.77
	S-L	32.1	99.60	0.40
Roots and tubers	R-C	20.0	51.83	48.17
	R-PF	14.9	95.06	4.94
Plant	P-A	37.11	72.85	27.15
By-products	B-BP	65.9	80.36	19.64
	B-SB	92.6	99.68	0.32
	B-C	92.9	99.00	1.00
	B-WM	36.9	100.0	0.00
	B-PF	78.8	96.21	3.79
	B-PH	24.5	95.67	4.33
	B-PP	28.0	97.92	2.08
	B-FP	35.6	97.43	2.57
	B-FR1	29.6	76.78	23.22
	B-FR2	32.7	62.37	37.63
	B-FR3	64.0	71.13	28.87

Regarding the proportion between soluble and insoluble dietary fiber all tested feedstuffs, with the exception of psyllium seeds, were characterised by a higher values of insoluble fraction than soluble one. However, differences among categories both for TDF concentration and for IDF/SDF were observed. The concentration of total dietary fiber in legumes samples varied from 17% a.f. (soybean) to 33% a.f. (beans); in all grain samples the proportion of soluble dietary fiber was very low (less than 3% of TDF), only carob showed a high SDF due to the presence of the pod around the seeds.

Cereal grains showed high variation both for TDF concentration (from 8 to 38% a.f., for rice and oats, respectively) and for IDF/SDF ratio; in particular, oats and corn were characterised by high proportion of SDF (41 and 15% of TDF, respectively). The TDF composition of the three seeds samples was particularly interesting, varying from 32 to 67% a.f., in linseed and hemp, respectively. Moreover, dietary fiber of psyllium and hemp seeds was highly soluble (66 and 42% of TDF, respectively), while linseeds fiber was completely insoluble. Both roots and tubers samples showed moderate TDF concentration (15 and 20% a.f., in potatoes and carrots, respectively), but the SDF were highly different: about 50 in carrots and 5% in potatoes on TDF basis. The lyophilised aloe showed a TDF concentration higher than 30%, with a soluble proportion closed to 30% TDF. As expected among all samples, the highest variability was observed in the by-products category: TDF varied from 16 to 93%a.f., in faba pod and cellulose and sugar cane bagasse, respectively. The dietary fiber of these last two samples and that one of wheat middling was completely insoluble, while beet pulp and all fiber fruit samples showed soluble fiber proportion close or higher to 20% of total dietary fiber.

Discussion

From these results it is possible to obtain several indications regarding the potential functionality of feedstuffs usually utilised in diet formulation for non-ruminant species (swine and carnivores), such as cereal and legume grains and most used by-products (beet pulp, wheat middling). They not only showed high and known nutritional values (e.g. crude protein concentration of legume grains; high starch values of cereals and in both case high energy density), but also interesting functional characteristics (e.g. high soluble dietary fiber concentration of oats, beet pulp and corn). Regarding nutritive value, it seems interesting to underline that wheat and peas grains could be considered as good starch and energy sources, and could represent a valid alternative to corn in particular for organic livestock system in pig production.

In our opinion extremely interesting are the evaluations of nutritional and functional characteristics of less known feedstuffs, which needs to be discussed for each category. Regarding seeds samples the nutritional (ether extract, NDF, gross energy values) and functional (TDF, IDF and SDF concentrations) characteristics of hemp seeds seem almost close to the psyllium seeds ones. The high ether extract values registered for all the three

seeds samples and the favourable fatty acids profile (high PUFA and MUFA proportions, and low ω-6/ω-3 ratio), as reported by Callaway (2004) make these seeds useful diet ingredients to modify fatty acids profile in animal products, both in ruminant (Mach *et al*., 2013) and non-ruminant species (Smink *et al.,* 2012; Mašek *et al.,* 2014). Moreover, all seeds samples could be useful in formulating diets for companion animals affected by diabetes (Diez and Nguyen, 2007) gastric (Zentek, 2012) or intestinal infection or renal failure (Elliott and Lefebvre, 2007) both for their dietary fiber proportions and for the favourable fatty acids profile.

The results referred to carrots and potatoes suggest different uses for these samples, in particular carrots could be useful to reduce transit rate, due to its high mucilaginous properties that help to increase ion (Ca, Fe etc.) absorptions (Torre *et al.,* 1991) and to reduce glycaemia and cholesterol haematic levels despite its high sugar concentration. On the contrary potatoes represent primarily a source of starch highly digestible (Singh *et al.,* 2010), which cause rapid post-prandial increasing serum glucose and insulin levels.

The carbohydrates composition of aloe explains partially its phytotherapic effect (Capasso *et al.,* 1998); the complex carbohydrates are equally distributed between pectins plus gums and cellulose, only partially lignified. This composition justifies the laxative effect of aloe, but also the immunostimulant (Infascelli *et al.,* 2010) and antiseptic activity related to the high proportion of soluble fraction (more than 27%) that could be rapidly fermentable in the large intestine with high production of SCFAs. In dietary formulation aloe could be considered a mixed fiber source useful for nutritional treatments of animals affected by gastro-intestinal diseases or during particularly stressing periods (Gonzalez *et al.,* 2012; Bontempo *et al.,* 2000; Dell'Orto *et al.,* 2001).

Tested by-products could be differentiated in function of their NDF content into the following categories: high NDF by-products (>60%) – cellulose, sugar bagasse and pea fiber; medium NDF by-products (30< NDF <60%) – beet pulp, wheat middling, faba pod and FR3. All the high-NDF by-products are characterized by great TDF values (more than 75%), exclusively composed by insoluble fiber. For these characteristics all these by-products could be useful to increase crude fiber and NDF in the diets; however, purified cellulose and pea fiber, due to their production process, resulted more expensive than sugar cane bagasse, which needs only to be dried. The supplementation with these by-products could represent an efficacious nutritional key in the treatment and prevention of several gastro-intestinal pathologies, characterized by constipation, or meteorism (Zentek, 2012). A diet rich in

insoluble fiber could be useful also in weight loss program, in order to decrease energy density and increase satiety sensation (Pond *et al.*, 1988; Slavin, 2005; German, 2006).

The four medium-NDF by-products presented moderate crude protein (from 7 to 15% a.f., for B-FR3 and B-WM, respectively) and starch levels (from 4 to 21% a.f., for B-FP and B-WM, respectively). These samples were characterized by high insoluble fiber portion, but beet pulp and FR3 presented also a considerable soluble fiber concentration (>100 g/kg). These last two samples could be considered mixed fiber supplements, useful to be utilized alone or in association with higher insoluble fiber sources. This kind of supplement could be suitable in order to stimulate satiety sensation, to increase mineral absorption and to reduce glycaemic and cholesterolemic levels both in humans and animals.

The last group of by-products, low-NDF, showed high differences in terms of nutritional characteristics: both samples of legumes origin (pea hulls and pea pod) showed intermediate crude protein values, starch content higher than 10%, and high proportion of NSC and TDF (close to 20%) completely insoluble. Both fruit fiber where characterized by very high concentration of NSC (57 and 70% a.f., for FR1 and FR2, respectively) but by high differences in starch amounts (higher to 100 g/kg for FR2 and equal to 0 for FR1). Regarding TDF concentration, both fruit samples showed amounts close to 300 g/kg, with a proportion of SDF higher to 20%. The inclusion of these four by-products in diets for omnivore and carnivores as source of TDF was obviously made with different targets. Indeed, both legume fiber by-products could represent cheap and easily available not-lignified cellulose sources, useful to modulate the SCFAs production in the large intestine (Roediger, 1980). Both fiber fruit samples could be considered as good SDF supplements to be used in association with products richer in IDF or as major fiber ingredient for the nutritional treatment of metabolic diseases (diabetes, obesity, lipidemia) (Aguggini *et al.*, 1992) or to increase the efficiency of immune system during particular livestock phases (weaning, diets and/or management changes) (Piva *et al.*, 1995).

Conclusion

The combined application of the methods proposed by Van Soest and Prosky to determine the fractions of structural carbohydrates in the feedstuffs allows us to better appreciate the nutritional and functional properties of each tested ingredient. Our results confirmed the nutritional and beneficial role of several ingredients usually utilized in feed

industry (i.e. pea, bean, corn and oat), which combine the high nutritive values (high protein, starch, gross energy amounts) with considerable TDF contents. More interesting in our opinion are the functional properties of the tested by-products, which in different proportion, could contribute to prevent or treat several pathologies. The use of alternative fiber sources as ingredients in feed formulation seems a promising and important area in livestock and companion animals nutrition, because most of them are predominantly composed by carbohydrates and it is evident their beneficial effects in improving the animals health status. These by-products could be utilized as more functional and less expensive alternative to the common ingredients in formulating diets for omnivore and/or carnivores species.

Chapter 3 -Fermentation characteristics of several carbohydrate sources for dog diets using the *in vitro* gas production technique

Abstract

Fermentable carbohydrates are an important part of the canine diet; they can improve gastrointestinal health modifying gut microbial population and metabolic activity. The present study compared the fermentation characteristics and kinetics patterns of 10 carbohydrate sources using the *in vitro* gas production technique (IVGPT) with dog fecal *inoculum*. The substrates tested were: pure cellulose (PC); carboxymethylcellulose (CMC); sugar cane fiber (SCF); beet pulp (BP); wheat bran (WB); fructo-oligosaccharides (FOS); inulin; yeast cell wall (YCW); ground psyllium seed (PS); pea hulls (PH). All substrates were incubated at 39°C under anaerobic conditions with faeces collected from dogs as microbial *inoculum*. Gas production of fermenting cultures was recorded, and after 48 h pH, short-chain fatty acids (SCFAs) and the organic matter disappearance (OMD) were determined. The results confirm high fermentation by dog fecal bacteria of FOS and inulin, that produced high propionate amounts and where fermented very rapidly. Three substrates (SCF, CMC and PC) were not able to support bacterial growth, with low gas and SCFAs productions and high BCFAs formation. The PH and BP showed moderate OMD and SCFAs production. Wheat bran was fermented rapidly and generates high proportion of butyrate. The PS was slowly fermented with delayed gas production, supporting a high formation of SCFAs, with adequate proportion of butyrate for bacterial growth. While YCW, which presented a delayed fermentation, allowed a moderate SCFAs production. The fermentation characteristics of PS and YCW suggest a potential use to promote a more distal fermentation on intestinal tract.

Keywords*:* dietary fiber, prebiotic, digestibility, SCFAs, butyrate.

Introduction

Fermentable carbohydrates are important components of dog diets, including some fibers, starch, non-starch polysaccharides such mannano-oligosaccharides, fructo-oligosaccharides, stachyose and raffinose, and non-absorbed sugars. These nutrients reach the colon and are suitable of bacterial fermentation (Gibson and Roberfroid, 2008). They

allow an adequate organic matter supply for the large intestine. Bacterial fermentation of these compounds results in short-chain fatty acid (SCFAs) production and pH reduction, which could modify the composition and metabolic activity of the intestinal bacteria (Campbell *et al.,* 1997). The short chain fatty acids, especially butyric acid, are important energy sources for the colonocytes (Roediger, 1982), lead to suitable ion absorption and act in intestinal blood flow and peristaltism, helping the animal by increasing the nutrients available for the colonocytes (James, 1980). Some of these fermentable carbohydrates can be classified as prebiotics, defined as non-digestible ingredients that beneficially affect the host by selectively stimulating the growth and/or metabolic activity of limited number of desirable bacterial species already resident in the colon (Gibson and Roberfroid, 2008). On the other hand, protein fermentation by gut microbiota results in the production of putrefactive compounds, including phenols, indoles, ammonia, branched-chain fatty acids (BCFAs) and biogenic amines increasing the quantity of nitrogenous waste materials entering the bloodstream (Smith and Macfarlane, 1997).

The *in vitro* gas production technique (IVGPT) proposed by Theodorou *et al.* (1994) could be useful to assess the fermentation pattern and kinetics of a carbohydrate source by dog intestinal microbiota. The IVGPT is based on the fact that the anaerobic fermentation by gut microorganisms produces many compounds, including gas (CO_2, CH_4 and traces of H_2), SCFAs (acetic, propionate, butyrate, valeriate, iso-valeriate and iso-butyrate) and ammonia. The technique was already used to evaluate feedstuffs with *inocula* from rumen liquor (Calabrò *et al.,* 2008; Cutrignelli *et al.,* 2005; Cutrignelli *et al.,* 2007), content of gastrointestinal tract sections of pigs (Williams *et al.,* 2001), caecal content of rabbits (Bovera *et al.,* 2008), poultry (Williams *et al.,* 1997), and dog faeces (Bosh *et al.,* 2008; Cutrignelli, 2007; Cutrignelli *et al.,* 2009).

The extent of fermentation and the SCFAs profile are related to the interaction between substrate and microbiota. The structure of the carbohydrate (e.g. different sugar compositions and molecular size), the bacterial specie present in the ecosystem and their metabolic collaboration are probably important factors in controlling fermentation (Cummings and Mac Farlane, 2002). Moreover in estimating the fermentation patterns among animal species the morphological and functional characteristics of the hindgut should be considered. In particular, carnivores have a short and relatively simple large intestine where the undigested food residences for approximately 12 hours (Maskell and Johnson, 1993). Notwithstanding the results of *in vitro* tests are not directly transferable *in vivo*,

several advantages (e.g. standardized fermentation conditions, defined fermentation times, ability to measure fermentation end-products, low costs) allows these methods useful also to study fermentable capabilities of intestinal microflora of dogs. The use of faeces as *inocula* source has been largely argued. However, Bosch *et al.* (2008) concluded that because the ranking remained the same for the large intestinal *inocula*, the use of faeces for inoculum preparation appears to be suitable for *in vitro* screening purposes.

Therefore, the aim of this study was to compare by the IVGPT the fermentation characteristics, kinetics and end products of different carbohydrate sources using dog fecal *inocula*, in order to better understand the potential effects of their inclusion in dog diets.

Material and methods

Ten carbohydrate substrates were used: purified cellulose, PC (CPKelco Brazil S.A.); a soluble sodium salt of cellulose, carboxymethylcellulose (CMC; FINNFIX 700 CPKelco Brazil S.A.); a purified by-product of sugar cane (*Saccharum officinarum*) namely sugar cane fiber (SCF); beet (*Beta vulgaris*) pulp (BP); wheat bran (WB), a residue of wheat (*Triticum* spp.) flour production; two different commonly used prebiotics, purified fructo-oligosaccharide (FOS; Actilight Beghin Meiji, France) and inulin (Prebiofeed 95, Socode -Belgium); spray-dried yeast cell wall (YCW; ActiveMOS, Biorigin, Brazil), consisting of purified *Saccharomyces cerevisiae* cell wall, a common source of mannano-oligosaccharides in dog diets; two natural sources of dietary fiber (Dilumix Industrial Ltda., Brazil), namely ground whole seed of psyllium (*Plantago psyllium*, PS) and pea hulls (*Pisum sativum*, PH) obtained after peeling and grinding.

Chemical analyses of substrates were carried out using AOAC (2005) procedures: crude protein (ID 954.01), ether extract (ID 920.39C), ash (ID 942.05) and dry matter (ID 934.01). Prosky *et al.* (1988) methodology was used for total dietary fiber (TDF), soluble and insoluble fiber determinations. All the analysis were carried out in Brazil, at Faculdade de Ciências Agrárias e Veterinárias, (UNESP) Campus de Jaboticabal.

The IVGPT was performed according to Bosh *et al.* (2008). Each substrate was accurately weighed (mean ± SD: 0.502 ± 0.008 g) in triplicate into 120 ml serum flasks and 75 ml of an anaerobic medium was added as described by Williams *et al.* (2005). It was decided to use the whole materials without preliminary treatment, because as reported by Bauer *et*

al. (2003) there is very little change in fermentability as a result of enzyme treatment using carbohydrate sources as substrates.

The medium contains nitrogen in several forms suitable for microbial use because the substrates were mainly a source of energy for the microbial population. Contextually, three flasks were prepared without substrates (blank) to correct degradability, gas and SCFAs productions. All the flasks were immediately sealed with butyl rubber stoppers and aluminum crimp.

Faeces were collected *per rectum* from two healthy adult (4-year-old) German Shepherd dogs (mean body weight 32 kg) fed a commercial dry food without prebiotics (chemical composition, g/kg as fed: crude protein 258; total dietary fiber 66; acid hydrolyzed fat 175; ash 70) for two weeks prior to faeces collection. Fecal samples were immediately transported to the laboratory of the Department of Animal Science and Food Control (Naples, Italy), and processed within 40 min of collection. Fecal samples were pooled, diluted (1:10, v/v) with 0.9% NaCl sterile solution, homogenized and filtered through six layers of gauze. In each prepared flask (including blank), 5 ml of the fecal solution were injected. After inoculation, flasks were placed in a PI400 incubator (Carbolite, Sheffield, UK, model PI400) at 39°C for 48 h under anaerobic condition.

According to other *in vitro* study with inoculum from dogs (Sunvold *et al.,* 2005; Bosch *et al.*, 2008) the fermentation was stopped after 48 hours of incubation, considering the high transit rate of digesta in dog hindgut. Gas production of fermenting cultures was recorded 18 times (at 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 24, 26, 30, 32, 34, 42, 46 and 48 hours) using a manual pressure transducer (Cole and Parmer Instrument Co, Illinois, USA).

Gas volumes obtained for each sample were related to the quantity of incubated organic matter (organic matter cumulative gas volume, OMCV). Fermentation was stopped after 48 h by cooling at 4°C. At the end of incubation, fermenting liquor was analyzed for pH (Alessandrini Instrument glass electrode Jenway, Dunmow, UK, pHmeter model 3030) and 10 ml were collected and frozen at -15°C for SCFAs analysis. The organic matter disappearance (OMD) was measured by filtering the flask residues using pre-weighed sintered glass crucibles (Scott Duran, porosity #2) under vacuum. Residual dry matter was determined by drying the sample to a constant weight at 103°C, residual organic matter was calculated by difference following burning (5 h at 550°C).

For SCFAs determination, fermenting liquors were thawed and centrifuged twice at 12,000 g for 10 min at 4°C (Hettich FurnTech Division DIY, Germany, model Universal 32R).

One ml of supernatant was then mixed with 1 ml of oxalic acid (0.06 mol). Short chain fatty acids were measured by gas chromatography (ThermoQuest Italia SpA, Rodano, Milan, Italy; model 8000top, fused silica capillary column 30 m 0.25 mm 0.25 lm film thickness), using as external standard a solution composed of acetic, propionic, butyric, iso-butyric, valeric and iso-valeric acids as described by Calabrò et al. (2006). In order to evaluate if proteolysis occurs during the fermentation the branched chain fatty acid was calculated according the following equation:

$$BCFAs = [(iso\text{-}butyric + iso\text{-}valeric)/SCFAs].$$

For each flask the gas production profiles were fitted to the sigmoidal model described by Groot *et al.* (1996):

$$G\ (ml/g) = A/(1+B/t)^{C}$$

where G is the total gas produced (ml/g of organic matter) at t (h) time, A is the asymptotic gas production (ml/g of incubated organic matter), B is the time at which one-half of the asymptote is reached (h), and C is the switching characteristic of the curve. The NONLIN package (Sherrod, 1995) was used to fit the data to this equation. Maximum fermentation rate (R_{max}) and the time at which it occurred (T_{max}) were calculated according to the following formulas (Bauer *et al.,* 2001):

$$R_{max}\ (ml/h) = (AxC^{B})xBx[T_{max}^{(-B-1)}]/[(1+C^{B})x(T_{max}^{-B})^{2}]$$

$$T_{max}\ (h) = Cx[(B-1)/(B+1)]^{(1/B)}$$

The fermentation characteristics (OMCV, cumulative volume per g of incubated OM; OMD; SCFAs; BCFAs; pH) and the fitted parameters (A, B, T_{max} and R_{max}) were subjected to analysis of variance to detect the influence of the different substrates. The statistical model was (GLM procedure, SAS, 2000):

$$y_{ij} = \mu + Sub_i + \varepsilon_j$$

where y is the experimental data, μ the general mean, Sub the substrates (i = 1, 2, …10), ε the error term. When significant differences among substrates were found in the analysis of variance, means were compared using the Tukey's test.

Results

Chemical composition of the studied carbohydrate sources was presented in table 1. Substrates may be classified according to their TDF amount in: very high TDF content (TDF≥

850 g/kg; PC, SCF and PH); high TDF content (TDF≥ 600 g/kg; CMC, FOS, Inulin and BP); moderate TDF content (TDF≤ 400 g/kg; YCW, PS and WB). Regarding dietary fiber solubility, CMC, FOS and inulin were completely soluble; on the contrary PC, PH and SCF appeared completely insoluble. The other substrates were intermediate, most with a higher content of insoluble fiber.

Table 1 - Chemical composition of the evaluated carbohydrate sources (g/kg as-fed basis).

Carbohydrate	DM	CP	EE	Ash	TDF	IDF	SDF
PC	936	-	0.4	2.0	920	914	15
CMC	937	-	-	195	740	-	740
SCF	982	17	13	56	908	908	-
BP	905	75	5.5	64	620	371	245
WB	883	152	19.8	43	389	229	151
FOS	959	0.3	0.6	-	705	-	705
Inulin	977	-	0.4	-	683	-	683
YCW	941	288	8	57	550	328	207
PS	931	219	101	27	513	401	102
PH	943	55	13	23	853	830	26

PC: purified cellulose; CMC: carboxymethylcellulose; SCF: sugar cane fiber; BP: beet pulp; WB: wheat bran; FOS : fructo-oligosaccharide; inulin; YCW: spray-dried yeast cell wall; PS: ground whole seed of psyllium; PH: pea hulls. DM: dry matter; CP: crude protein; EE: ether extract; TDF: total dietary fiber; IDF: insoluble dietary fiber; SDF: soluble dietary fiber.

The fermentation characteristics (OMD and OMCV) and kinetics parameters [asymptotic gas production (A), time at which one-half of the asymptote was reached (B), maximum fermentation rate (R_{max}) and time at which it occurred (T_{max})] varied among the studied ingredients (table 2).

As expected, both the cellulose substrates (PC, CMC) and sugar cane fiber showed very low ($P<0.01$) OMCV values on the contrary the OMD values of these substrates resulted significantly ($P<0.01$) different. Pure cellulose and sugar cane fiber showed the lowest OMD (2.20 and 5,41%, respectively) the CMC organic matter disappearance was one of the highest (96.3%). Inulin, FOS and YCW presented high OMD and OMCV, even with the different

kinetic parameters showed by those substrates: YCW resulting in lower A (ml/g) and R_{max} (ml/h) and higher T_{max} (h) than inulin and FOS (P<0.01). Regarding the other natural fiber sources evaluated (WB, BP, PH and PS), they produced moderate (WB and BP) or high (PH and PS) amount of gas (OMCV), even if only for PS the gas produced (214 ml/g) did not correspond to the high OM degraded (28.8%). Only PH did not fit the gas profile model used in the study.

Table 2 - *In vitro* fermentation characteristics of the tested substrates.

Carbohydrate	OMD	OMCV	A	B	T_{max}	R_{max}
	%	*ml/g*	*ml/g*	*h*	*h*	*ml/h*
PC	2.20^{F}	3.55^{E}	3.43^{D}	5.67^{B}	2.83^{D}	0.42^{C}
CMC	96.3^{A}	8.34^{E}	3.83^{D}	3.64^{C}	3.28^{D}	0.69^{C}
SCF	5.41^{F}	16.6^{E}	7.63^{D}	8.36^{B}	7.40^{Cc}	1.02^{C}
WB	64.0^{C}	152^{C}	158^{A}	5.83^{B}	2.17^{D}	18.4^{Aa}
BP	49.8^{D}	116^{D}	137^{B}	19.9^{Ab}	11.8^{B}	4.54^{B}
PH	81.2^{B}	256^{A}	nd	nd	nd	nd
PS	28.8^{E}	214^{B}	113^{C}	23.1^{Aa}	20.6^{Aa}	5.64^{B}
FOS	99.4^{A}	165^{C}	159^{A}	8.39^{B}	5.63^{D}	12.2^{Ab}
Inulin	93.8^{A}	185^{B}	188^{A}	8.88^{B}	6.23^{Cd}	15.4^{Aa}
YCW	99.1^{A}	199^{B}	99.1^{C}	19.6^{Ab}	16.7^{Ab}	5.02^{B}
MSE	54.6	191.07	106.83	3.29	3.63	3.88

OMD: organic matter disappearance; OMCV: cumulative gas production related to incubated organic matter; A: asymptotic value for gas production; B: time at which one-half of the asymptote is reached (h); T_{max}: time at which the maximum rate of gas production is reached; R_{max}: maximum fermentation rate.

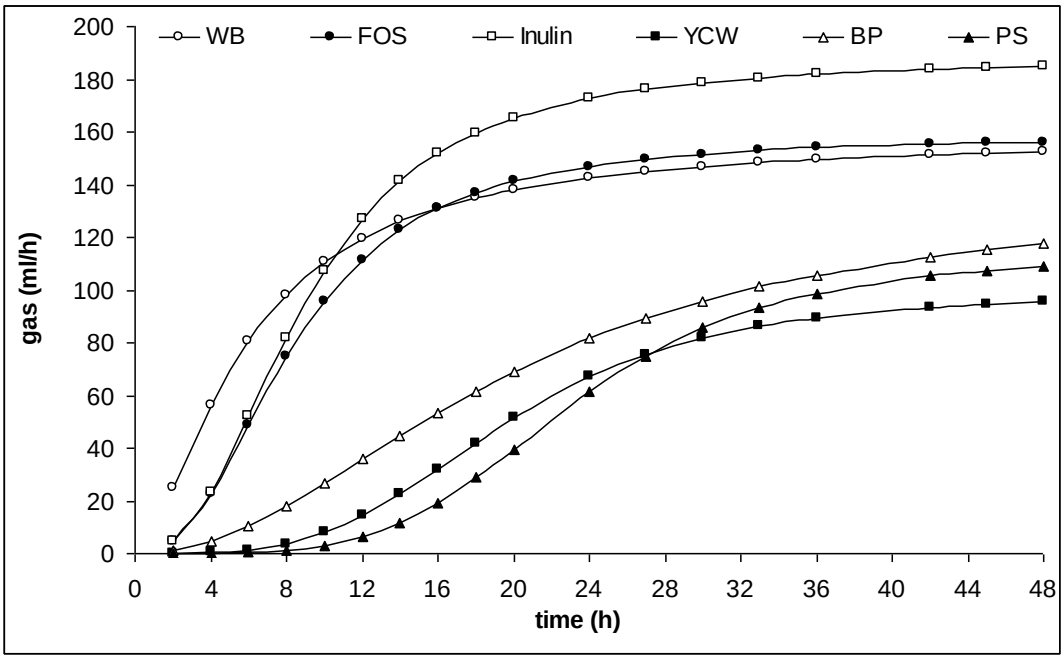
PC: purified cellulose; CMC: carboxymethylcellulose, SCF: sugar cane fiber, WB: wheat bran; BP: beet pulp; PH: pea hulls PS: ground whole psyllium seed; FOS: fructo-oligosaccharides; YCW: spray-dried yeast cell wall.

A-E and a-d within the column: differences statistically significant for P<0.01 and P<0.05, respectively. MSE: mean square error.

The potential gas (A, ml/g) and maximum rate (R_{max}, ml/h) were highest (P<0.01) for inulin, FOS and WB, intermediate for BP, YCW and PS, and lowest (P<0.01) for SCF, CMC and PC.

The PS and YCW needed more time to reach R_{max} (P<0.01) than all the other substrates, with BP presenting intermediate results.

Figure 1 - *In vitro* gas production over time of the tested substrates.



WB: wheat bran; FOS: fructo-oligosaccharides; YCW: spray-dried yeast cell wall; BP: beet pulp; PS: ground whole psyllium seed.

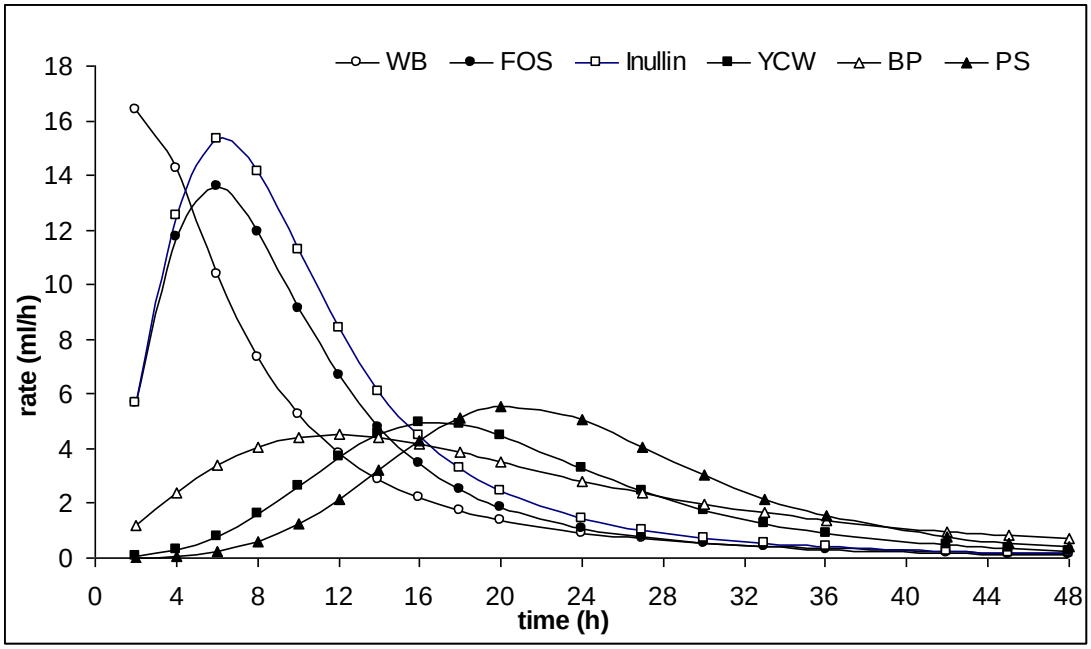
The *in vitro* gas production and fermentation rate over time of WB, FOS, inulin, YCW, BP and PS are illustrated in figures 1 and 2, respectively. For PC, CMC, PH and SCF the curves were almost flat (data not shown). Wheat bran, fructo-oligosaccharides and inulin presented similar gas profiles, which differ from the YCW, BP and PS curves; while the first three samples showed their highest values at the beginning of fermentation BP, YCW and PS, showed a more flat curve with maximum values between 12 to 24 h (figure 2).

Values of pH, determined after 48 h of incubation, were significantly higher (P<0.01) for the low fermentable substrates (PC, CMC, and SCF) compared to the more fermented ones, as presented in Table 3. Particularly low were the pH values of inulin and FOS.

High amount of total SCFAs (Mmol/g OM) were found for BP, PS, inulin and WB (P<0.05); while PC, CMC and FOS showed lower values (P<0.05), the remaining substrates

presented intermediate values. In particular PS showed the highest value (41.24 Mmol/g OM) and PC and CMC the lowest values (8.84 and 7.19 Mmol/g OM, respectively).

Figure 2 - *In vitro* fermentation rate over time of the tested substrates.



WB: wheat bran; FOS: fructo-oligosaccharides; YCW: spray-dried yeast cell wall; BP: beet pulp; PS: ground whole psyllium seed.

Most of tested substrates yielded a SCFAs production with a high proportion of acetic and propionate, which together accounted for more than 50% of SCFAs production; in particular, for FOS and inulin acetic and propionic acids account more than 85% of produced SCFAs. The proportion for butyric acid production was significantly ($P<0.01$) higher for WB (4.49 Mmol/g OM) compared to all the other substrates. As expected for the substrates tested and in according to Bosh *et al.* (2008), the branched chain fatty acids proportion was quite low for all the tested substrates. However the cellulose samples (PC and CMC) and SCFAs produced higher proportions of iso-butyric, iso-valeric, and valeric acids than the other substrates ($P<0.05$). As consequence these substrates showed the highest proportion of branched chain fatty acids [(iso-butyric + iso-valeric)/SCFAs]: 0.33, 0.31 and 0.32 for PC, CMC and SCF, respectively.

Table 3 - *In vitro* fermentation end products after 48 h of incubation.

Carbohydrate	pH	SCFAs	Acetic	Propionic	Iso-butyric	Butiryc	Iso-valeric	Valeric	BCFAs
		mMol/g	% of total SCFAs						mMol/g
PC	7.76^{A}	8.84^{Bd}	32.96^{B}	10.77^{B}	17.40^{Aa}	2.00^{B}	15.85^{Aa}	21.03^{Aa}	0.33^{A}
CMC	7.74^{A}	7.19^{Bd}	34.93^{B}	10.83^{B}	16.92^{Aa}	2.00^{B}	14.15^{Aa}	21.17^{Aa}	0.31^{Ab}
SCF	7.70^{A}	25.73^{Bb}	37.72^{B}	10.90^{B}	16.02^{Aa}	1.72^{B}	15.75^{Aa}	17.89^{Aa}	0.32^{Ab}
WB	6.28^{B}	34.07^{Ba}	48.43^{A}	17.58^{B}	6.94^{Bb}	13.18^{A}	4.20^{Bd}	9.67^{Ab}	0.11^{Bc}
BP	6.22^{B}	37.04^{Aa}	56.15^{A}	25.31^{B}	4.84^{Bb}	4.46^{B}	3.17^{Bd}	6.08^{Bc}	0.08^{C}
PH	6.67^{B}	26.00^{Bb}	57.90^{Aa}	12.89^{B}	8.98^{Ab}	1.64^{B}	8.18^{Bc}	10.42^{Bb}	0.17^{Abc}
PS	6.74^{B}	41.24^{Aa}	44.74^{A}	21.39^{B}	9.68^{Ab}	5.52^{B}	7.02^{Bc}	11.65^{Bb}	0.17^{Abc}
FOS	4.72^{C}	11.72^{Bc}	52.16^{A}	36.01^{Ab}	4.59^{Bb}	2.51^{B}	2.74^{Bd}	2.00^{Bd}	0.07^{C}
Inulin	4.86^{C}	35.99^{Aa}	47.35^{A}	41.52^{Aa}	6.28^{Bb}	1.47^{B}	2.01^{Be}	1.38^{Bd}	0.08^{C}
YCW	6.80^{B}	17.89^{B}	37.32^{B}	26.77^{B}	12.44^{Aa}	2.13^{B}	7.44^{Bc}	13.90^{Ab}	0.20^{Abc}
MSE	0.0104	64.17	76.7	12.7	18.2	10.9	6.32	16.7	0.004

SCFAs: sort chain fatty acids. BCFA: branched chain fatty acid proportion [(iso-butyric + iso-valeric)/SCFAs]

PC: purified cellulose; CMC: carboxymethylcellulose, SCF: sugar cane fiber, WB: wheat bran; BP: beet pulp; PH: pea hulls PS: ground whole psyllium seed; FOS: fructo-oligosaccharides; YCW: spray-dried yeast cell wall.

A-D and a-d within the column: differences statistically significant for $P<0.01$ and $P<0.05$, respectively.

MSE: mean square error.

Discussion

Common methods of fiber analysis, including total dietary fiber, soluble and insoluble fiber provide information on the carbohydrate sources, in terms of fiber amount and physical characteristic (e.g. solubility), but do not inform about their suitability for bacterial fermentation (de-Oliveira *et al.,* 2011). So, the combination of the chemical composition analysis with the *in vitro* gas production technique provides interesting complementary information about the ingredients.

The gas production technique proved to be useful for an *in vitro* screening of non-digestible carbohydrate for dogs, separating them according to fermentation amount as well as characteristics, and SCFAs production.

The short chain fatty acids are the major end-products of bacterial fermentative reactions on carbohydrates in the colon in all the mammals. All the SCFAs are rapidly absorbed and then metabolized by the gut epithelium, liver and muscle. One of the most important SCFAs properties is their trophic effect on the intestinal epithelium, maintaining the mucosal defense barrier against invading organisms. Acetic, propionate and butyrate are trophic when infused into the large intestine although the butyrate seems to be the most effective and propionate the least. Butyrate is the most interesting SCFAs because it is an important energy source for the colonic epithelium and regulates cell growth and differentiation (Salminen *et al.,* 1998).

The *in vitro* data obtained for FOS, PC, inulin and BP are in broad agreement with previous canine studies (Cutrignelli 2007; Cutrignelli *et al.,* 2009).

For PC, CMC and sugar cane fiber, the high values of BCFAs associated with a very low gas production suggest a low availability of nutrients, depressing bacteria fermentation and growth. This situation resulted in bacterial autolysis and the fermentation of dead bacteria by the remaining microorganisms. Fermentation of aminoacids (valine and leucine) released during bacterial death produces iso-butyric and iso-valeric acids, explaining the higher BCFAs values of these substrates (McDonald et *al.,* 2002). The higher values of pH for these substrates can probably be justified by a higher accumulation of ammonia due to protein degradation.

Carboxymethylcellulose is an ingredient characterized by high solubility and viscosity in water. No information about its fermentation was hitherto available. The very low fermentation found in this study suggests the advisability of using CMC in low-

energy dog diets formulated for weight maintenance or weight loss, which require high fiber addition. In particular, the high viscosity of CMC could allow delayed gastric emptying and nutrient absorption, increasing the animal's satiety sensation (Slavin and Green, 2007), aspect that could be explored in future studies. The high OM disapparence of CMC could be related to the extremely high solubility of the ingredient. Carboxymethylcellulose is a sodium salt of cellulose in which some hydroxyl groups were substituted by carboxymethyl groups, its completely soluble in water. At the end of incubation, the filter did not retain the incubated OM that results in "apparent" OMD, whereas it was only solubilized and not fermented, as the gas data demonstrated. Filtration problems must also be considered during the interpretation of OMD results of other high soluble carbohydrates evaluated, including FOS, inulin, and YCW.

The very low fermentation of SCF, comparable with that of PC, suggests the high potentiality of this insoluble fiber source. Instead, the association of a poorly fermentable substrate with high insoluble fiber content (91% of insoluble dietary fiber) suggests that SCF could be a cheaper substitute for purified cellulose in formulating low-energy diets. In these diets, high fiber addition is necessary to reduce energy digestibility, making necessary to use low fermentable insoluble fiber sources. Low fermentable fiber result in less interference with protein and fat digestibility, and better faeces formation than high fermentable ones (Fahey *et al.,* 1990; Sunvold *et al.,* 1995b).

As regards YCW, previous information on its fermentation by gut microbiota of dog is both scant and ambiguous. The IVGPT results for these substrates showed a moderate gas production (OMCV) and slow fermentation (large T_{max}) with fair SCFAs profile. Gomes *et al.* (2008) found *in vivo* an increase in dog fecal SCFAs concentration when the diet was supplemented with YCW. Yeast cell wall is a source of mannano-oligosaccharides and has been considered useful prebiotic for dogs, potentially favoring the dog gut health (Swanson and Fahey, 2006). The prebiotic effect of YCW is largely attributed to the ability of mannano oligosaccharides to attach at type A fimbriae of *Salmonellae* and *Clostridiae* avoiding they adhesion to enterocytes (Vernazza *et al.,* 2006). Our findings obtained here suggest that at least the benefits could also be attributed to its intestinal fermentation and SCFAs production in dog colon. The delayed maximum rate of gas production suggests that YCW is probably more fermented in the distal part of gastrointestinal tract, what might be interesting favoring SCFAs production in the colon.

The oligosaccharides FOS and inulin are readily available energy sources for gut bacteria: they produced a high proportion of propionic acid and significantly ($P<0.01$) lower pH values than the other substrates, probably due to lactic acid production (Böhmer *et al.,* 2005). Previous studies already demonstrated high fermentation of these substrates by canine fecal *inoculum* (Sunvold, *et al.,* 1995a). Besides the high gas production, FOS and inulin presented a short T_{max}, suggesting that these substrates are fermented more cranially in the intestinal tract. These compounds could be considered for diets able to promote the fermentation activities also in small intestine (e.g to treat small intestinal diseases).

Wheat bran and pea hulls showed peculiar characteristics regarding organic matter fermentability and short chain fatty acids production. Wheat bran was readily fermented and produced a high proportion of butyric acid, contrary to its classification by NRC (2006) as a low fermentable fiber source. Also Bosch *et al.* (2008) found that wheat middling is readily fermented by dog fecal *inoculum*, with a fermentation pattern very close to that verified in the present paper, probably due to the high starch and hemicelluloses content of this ingredient. Pea hulls are high fiber ingredient, with 830 g/kg of insoluble fiber and 26 g/kg of soluble fiber; which presented a good fermentation and SCFAs production. Bosch *et al.* (2008) also studied pea fiber that presented similar fermentation characteristics as well as PH in the present study. Notwithstanding, the SCFAs production was different and not comparable due to the differences in chemical composition and incubation time.

Beet pulp and wheat bran are the most used fiber source for dogs nutrition. Results from the present study and also from Sunvold *et al.* (1995b) and Bosch *et al.* (2008) suggest that the *in vitro* fermentation of WB are comparable with that of BP, classifying them also as moderate fermentable fiber sources for dog diets. However the fermentation pattern of BP and WB was different; WB presented a very high maximum fermentation rate and a very short maximum fermentation time, suggesting a more cranial and intense gas production on dog gut than BP. Moreover, even if the total SCFAs production was similar (34.07 vs. 37.04 mMol/g for WB and BP, respectively), the amount of main SCFAs was different (acetic: 16.5 vs. 20.8; propionate: 5.99 vs. 9.37; butyrate: 4.49 vs.1.65 mMol/g for WB and BP, respectively) due to the great structural complexity (i.e., sugar, starch, insoluble fibers and pectins) of both substrates.

Psyllium seed exhibited low OMD (28.8%) but moderate gas (214 ml/g OM) and very high SCFAs (41.24 Mmol/g OM) productions, and a particularly high proportion of butyrate (more than 5% of total SCFAs). Psyllium gum has been widely used in veterinary medicine in order to prevent constipation, obesity and diabetes mellitus (Leib, 2000; Tortola, *et al.,* 2009). However, most pet-food companies use ground psyllium seeds instead of psyllium gum due the cost and the availability. The difference in gum and seed chemical composition must be considered during ingredient evaluation and more data on psyllium seeds are required to improve their use in diet formulation. Psyllium seeds were composed not only by mucilage (approximately 20%; Anderson and Fereman, 1935) as Psyllium gum, but also by starch, triglycerides and protein which alter the substrate fermentation pattern and explain the higher gas production. These differences in chemical composition could explain the disagreements with Sunvold, *et al.* (1995a). The seeds of Psyllium have also a low maximum rate of gas production and a delayed T_{max}, being possible more fermented in the distal part of the gastrointestinal tract.

Conclusions

The *in vitro* gas production technique was useful for an *in vitro* screening of un-digestible carbohydrates for dogs, separating them according to fermentation kinetics, organic matter disappearance and SCFAs production. Un-digestible carbohydrates could be very different ranging from almost un-fermentable (PC, CMC, and SCF) to highly fermentable (inulin, FOS, and WB), fast fermented (inulin, FOS, and WB) or slowly fermented (YCW, BP, and PS), and with different molar ratios of SCFAs and BCFAs profiles. All these outcomes have different consequences on hindgut and dog health. Therefore, ingredients should be chosen which result in higher production of SCFAs, particularly of butyric acid and lower, which suggest reduced protein fermentation. A selection of several ingredients with different kinetic and end-product characteristics can be made to design diets to stimulate carbohydrate fermentation along the entire tract. However, all considerations on fermentability and SCFAs production of the tested substrates should be evaluated in relation to gas production. Indeed, control of intestinal gas production is an important goal during ingredients selection and diets formulation in order to avoid undesirable effects such as flatulence and soft faeces.

Chapter 4 -Influence of fermentable carbohydrate-rich feedstuffs on intestinal microbial activity of adult pigs

Abstract

Nine feedstuffs were tested *in vitro* with faecal *inoculum* from pigs. Sugar beet pulp, wheat bran, soybean hulls, grapecake, chestnuts, glutamic beet pulp, citrus by-product, fructo-oligosaccharides and corn starch were fermented for 96 h. Cumulative gas production was measured as an indicator of the fermentation kinetics. At the end of incubation organic matter disappearance and fermentation end-products (SCFAs and NH_3) were measured. The gas production profiles were fitted with a multi-phasic model. As expected, the carbohydrates composition of the tested samples was particularly different. The fermentation parameter showed a different trend for grapecake and FOS, very low gas production for the first one, probably due to the high lignin and tannins contents of this by-product, on the contrary the higher OMCV and OMD values for the second one, due to the high soluble fiber proportion. Some samples, like chestnuts, soybean hulls and citrus by-product showed similar value of degradability and gas production, but were characterized by different fermentation profiles. SCFAs production reflected the carbohydrates composition of each sample, such as ammonia and BCP value were affected by crude protein content.

The most innovative substrates (chestnuts, grapes hulls and citrus by-product) gave interesting information in terms of gastro-intestinal tract fermentation in pigs. In particular grapecake showed the lower fermentation in terms of gas production, while chestnuts and citrus by-product produced were characterized by more productive fermentation profile (high gas and SCFAs production). These characteristics could be particularly useful to optimize the caecum-colon fermentation, with high butyric acid production.

Keywords: *in vitro* gas production technique (IVGPT), dietary fiber, prebiotic, gut microbial fermentation.

Introduction

Since 2003 when the European Community has prohibited the use of antibiotics as growth promoters (Regulation (EC) No 1831/2003) a lot of studies were carried out in order to find valid alternatives to reduce the enteric diseases in swine breeding.

Several possible alternatives to antimicrobial additives in intensive farming systems were proposed. Particular interest was focused on substances capable to promote the bacterial microflora development in the large intestine, such as probiotics and prebiotics. These ingredients were defined functional or nutraceutical feedstuffs, because they are characterized by beneficial effects behind their nutritional characteristics. The most utilized functional ingredients in animal nutrition were prebiotics, due to their high shelf-life and resistance to the technological procedures, including high temperature. Prebiotics have been linked with improvements in pig growth performance, decreasing of mortality and morbidity, as reported in many studies (Patterson and Burkholder, 2003; Konstantinov *et al.,* 2008). Several fermentable carbohydrates were classified as "prebiotic" (Roberfroid, 1993, Sunvold *et al.,* 1995a; Sunvold *et al.,* 1995b) because they were non-digestible oligosaccharides and promote useful bacterial strains proliferation in the distal part of the small bowel and in the large intestine. The *in vitro* gas production technique (IVGPT), proposed by Theodorou *et al.* (1994), was utilised in order to evaluate the anaerobic fermentation of nine feedstuffs by gut microorganisms, using faeces from adult swine as *inoculum*.

Material and methods

Nine potential ingredients for pig diets were used as substrate. These substrates were chosen for their different carbohydrates composition. Six by-products of different nature were selected: sugar beet pulp (SBP), glutamic acid beet pulp (GBP), wheat bran (WB), soybean hulls (SH), grapecake (GC) and citrus by-product (CBP); and one waste product: chestnuts (CN), composed by broken or too small nuts for human market. The last two substrates were purified carbohydrates: fructo-oligosaccarides (FOS) and starch from corn (CS).

All samples were analysed for crude protein (CP), ether extract (EE) and ash contents according to AOAC (1997) procedures (ID members: 984.13, 920.39 and 942.05, respectively), neutral detergent fibre (NDF), acid detergent fibre (ADF) and lignin (ADL) were determined according to Van Soest *et al.* (1991). Starch content was

determined with polarimetric detection (Polax L, Atago – Tokyo, Japan) as indicated by Martillotti *et al.* (1987).

The substrates were incubated at 39°C under anaerobic conditions in 120 ml serum flasks using as source of *inoculum.* Faecal samples collected *per rectum* from 3 adults neutered finisher pigs (Landrace × Large White) fed a commercial diet (CP: 14.8 %; CF: 4.0 %). Faeces were pooled, diluted (1:6) with an anaerobic NaCl solution, homogenized and filtered. Gas production of fermenting cultures was recorded 26 times using a manual pressure transducer (Cole and Parmer Instrument Co, Illinois, USA) (Theodorou *et al.,* 1994). The fermentation was stopped at 96 h and the pH of each flask was measured (Alessandrini Instrument glass electrode, Jenway, Dunmow, UK; model 3030); the fermentation liquor was analysed for ammonia (NH_3) and SCFAs.

Ammonia was determined according to the method described by Searle (1984). The samples were centrifuged twice at 1900 rpm for 10 min at room temperature (about 22°C), diluted 10 times with water, and then 1 ml of the diluent was deproteinized using 10% trichloroacetic acid. Ammonia and phenol were oxidised by sodium hypochlorite in the presence of sodium nitroprusside to form a blue complex. The intensity was measured colorimetrically at a wavelength of 623 nm. Intensity of the blue is proportional to the concentration of ammonia present in the sample.

For SCFAs determination the samples were centrifuged twice at 12000 × g for 10 min at 4°C and 1 ml of supernatant was taken and mixed with 1 ml of oxalic acid 0.06M. The SCFAs were measured by gas chromatography (ThermoQuest Italia SpA, Rodano, Milan, Italy; model. 8000 top, fused silica capillary column 30m × 0.25mm × 0.25µm film thickness) comparing samples peaks area of each SCFA with the corresponding of an external standard composed by acetate, propionate, iso-butyrate, butyrate, valerate and iso-valerate (Cutrignelli *et al.,* 2009).

The organic matter disappearance (OMD) was determined by filtering the residues using pre-weighed sintered glass crucibles (Scott Duran, porosity #2) under vacuum.

Residual dry matter was determined by drying to a constant weight at 103°C, and OM by difference following incineration (5 h at 550°C).

Gas volumes obtained were related to the quantity of incubated organic matter in order to obtain the cumulative gas volume (OMCV).

The gas profiles were fitted to the multiphasic model described by Groot *et al.* (1996) as follows:

$$OMCV = \Sigma_n A_n/[1 + (C_n/t)^{Bn}]$$

where OMCV: total gas produced (ml/g OM initial substrate weight), A: asymptotic gas production (ml/g OM initial substrate weight), B: switching characteristic of the curve, C: time at which one-half of the asymptote had been reached (h), t: time (h), and *n*: number of phases.

The goodness of fit of monophasic and biphasic models was determined using the mean squared prediction error (MSPE) as described by Bibby and Toutenburg (1977) and where the root MSPE was scaled to the observed mean (Mean Prediction Error, MPE).

Maximum fermentation rate (R_{max}) and time at which it occurs (T_{max}) were also calculated according to the following formula (Bauer *et al.,* 2001):

$$R_{max} = (A \cdot C^B) \cdot B \cdot [T_{max}^{(-B-1)}]/[(1+C^B) \cdot (T_{max}^{-B})^2]$$

$$T_{max} = C \cdot [(B-1)/(B+1)]^{(1/B)}$$

Differences among substrates for all the fermentative characteristics were tested with Student T-test (SAS, 2000).

Results

Generally, the chemical composition of the nine substrates (Table 1) agrees with the data reported in literature (Sunvold *et al.,* 1995c; DePeters *et al.*, 1997).

Seems interesting to underline the differences registered between the sugar beet by-products, which derived from two different technological processes: SBP from sugar extraction and GBP from glutamic acid extraction. Sugar extraction from beet consists in sugar solubilisation in water, while the extraction of glutamic acid is a hydrolysis of the molasses; these different extraction procedures yield different by-products. The main differences between beet by-products was the crude protein content, which in GBP was more than 5 times higher than SBP ones. All tested samples showed low fat amounts (from 0.74 to 5.35 % DM of SBP and GC, respectively).

As expected, the carbohydrate composition was particularly different among the tested substrates. Indeed four samples (FOS, corn starch, chestnuts and citrus by-product) are characterized by NSC values higher or close to 50%. Nevertheless, the composition of non-structural carbohydrates of these four samples was highly different because CS and CN were richer in starch (90 and 43% DM, respectively), while CBP and FOS were really poor in this nutrient (less than 2%). The high evaluation of NSC in these last two samples was due to the high percentage of soluble fiber, deductible by the differences from TDF and NDF contents. The other samples (glutamic acid beet pulp, wheat bran, soybean hulls, sugar beet pulp and grapecake) showed NSC content comprised between 1 and 17% and NDF contents ranged from 45 to 63%. Particularly high was the lignin values registered by GC sample.

Table 1 - Chemical composition (% DM) of the nine feed ingredients.

	DM	CP	EE	Starch	NSC	TDF	NDF	ADF	ADL	Ash
GBP	90.27	34.53	2.87	0.94	1.19	69.45	45.21	38.74	3.41	6.85
SBP	88.04	7.58	0.74	1.03	15.61	65.82	56.93	37.50	9.37	7.18
WB	87.08	17.79	2.96	17.13	14.64	47.49	46.64	15.12	4.34	5.05
CBP	88.44	5.27	1.89	1.51	48.20	70.97	23.67	9.14	2.71	9.41
SH	90.05	12.45	3.22	0.27	6.70	74.88	62.01	52.52	2.47	5.67
GC	94.59	13.91	5.35	1.06	4.75	75.43	62.32	59.23	36.2	8.26
FOS	98.46	-	-	-	97.96	70.31	0.42	0.37	-	0.08
CN	90.47	6.53	3.74	42.81	60.85	20.74	16.83	2.01	0.21	2.52
CS	88.87	-	-	89.53	87.71	1.52	1.07	0.39	-	0.09

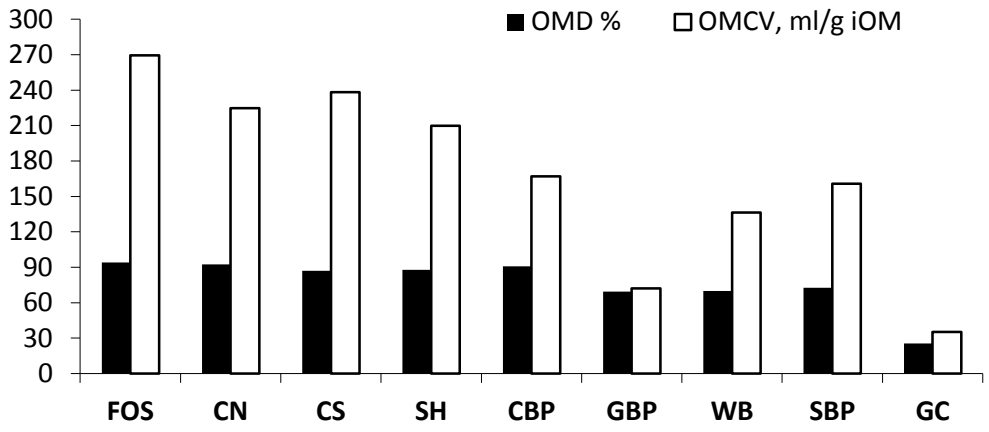
GBP: glutamic acid beet pulp; SBP: sugar beet pulp; WB: wheat bran; CBP: citrus by-product; SH: soybean hulls; GC: grapecake; FOS: fructo-oligosaccharides; CN: chestnuts; CS: corn starch.
DM: dry matter; CP: crude protein; EE: ether extract; NSC: nitrogen free extract; TDF: total dietary fiber; NDF: neutral detergent fibre; ADF: acid detergent fibre; ADL: acid detergent lignin.

In Figure 1 are represented the main fermentation characteristics (OM degradability and gas volume) of the tested substrates. As a whole, they are well correlated between each other (r values comprise between 0.69 and 0.89).

Grapecake showed the lowest fermentation in terms of OM degradability (OMD 24.2 %; $P<0.01$) and gas production (OMCV 38.5 ml/g OM; $P<0.01$). On the other hand FOS, chestnuts, corn starch, soybean hulls and citrus pulp showed the higher OMD (94.0, 92.3, 87.0, 87.9 and 90.7%, respectively) and OMCV values (269, 225, 238, 210 and 167 ml/g iOM, respectively). The other samples (sugar beet pulp, wheat bran and glutamic beet pulp) showed intermediated values for both parameters (OMD 72.6, 69.9 and 69.4

% and OMCV 161, 136 and 72 ml/g iOM, respectively), even if the OMCV values registered for GBP resulted significantly (P<0.01) lower than the other samples.

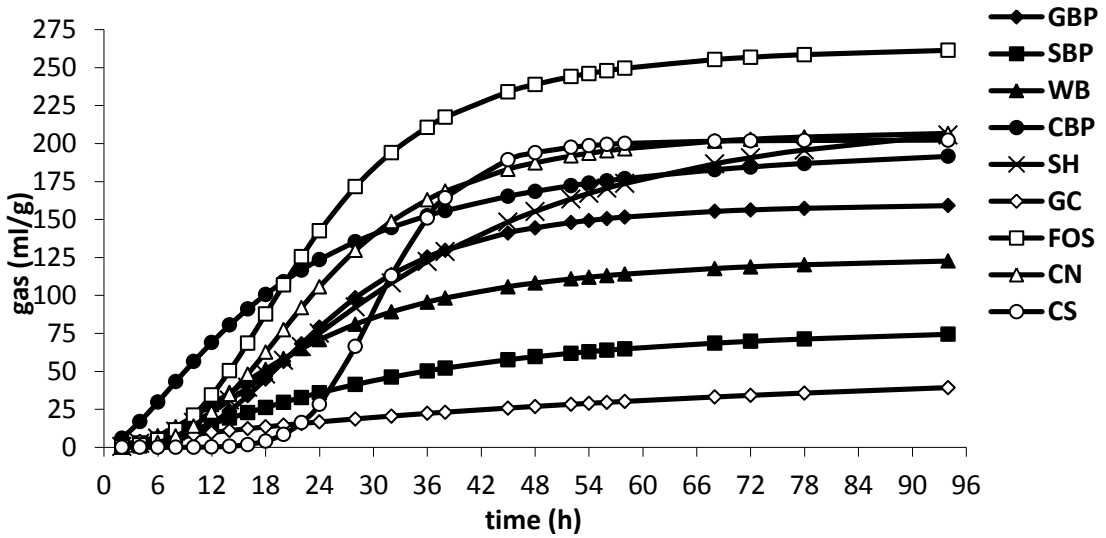
Figure 1 - Fermentation characteristics of the nine feed ingredients.



FOS: fructo-oligosaccharides; CN: chestnuts; CS: corn starch; SH: soybean hulls; CBP: citrus by-product; GBP: glutamic acid beet pulp; WB: wheat bran; SBP: sugar beet pulp; GC: grapecake.
OMD: organic matter degradability; OMCV: cumulative volume of gas related to incubated organic matter.

The registered OMD and OMCV values were in according with that one reported by other authors on *in vitro* studies using faecal inoculum form pigs (Sunvold *et al.,* 1995c; Mauricio *et al.,* 2001; Bauer *et al.,* 2003; Williams *et al.,* 2005).

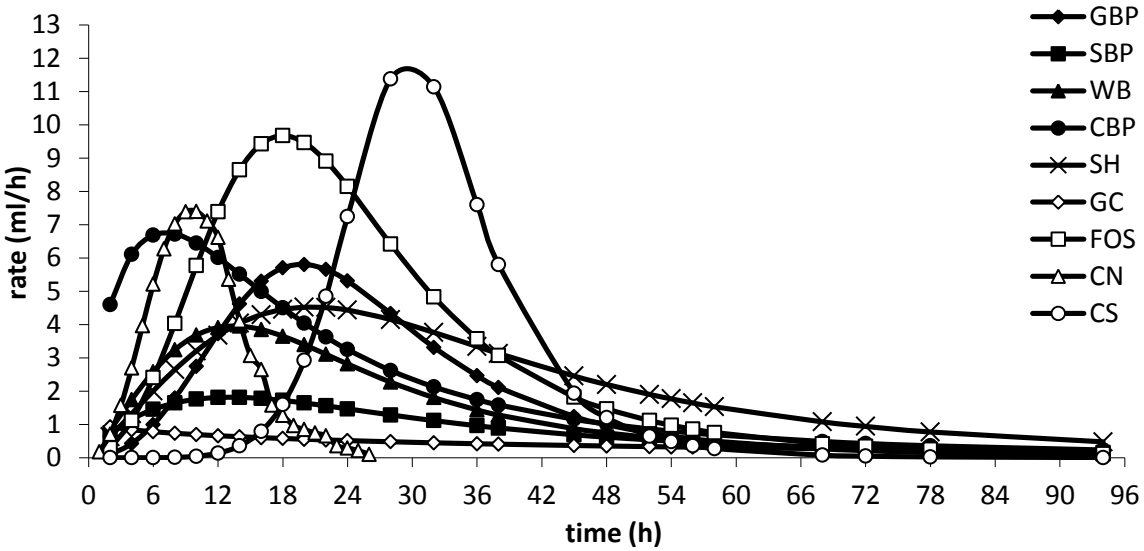
Figure 2 – Gas production profiles of the nine feed ingredients with monophasic model.



GBP: glutamic acid beet pulp; SBP: sugar beet pulp; WB: wheat bran; CBP: citrus by-product; SH: soybean hulls; GC: grapecake; FOS: fructo-oligosaccharides; CN: chestnuts; CS: corn starch.

For brevity and because the use of bi-phasic model contributed to better explain the results only the parameters obtained with this model were reported in table 2.

Figure 3 - Fermentation rate over time of the nine feed ingredients.



GBP: glutamic acid beet pulp; SBP: sugar beet pulp; WB: wheat bran; CBP: citrus by-product; SH: soybean hulls; GC: grapecake; FOS: fructo-oligosaccharides; CN: chestnuts; CS: corn starch.

Fermentation parameters evidenced for each substrate specific characteristics.

- Grapecake and FOS fermentation kinetics showed opposite trends: GC kinetics was characterized by particularly low gas production (A_1 12.3 and A_2 32.0 ml/g OM) and reaching the A/2 volume very fast (B_1 3.9 and B_2 3.0). Such trends was better represented in figure 2 from which is possible to note that the gas production starts very slow without reaching a peak, so the curve assumes the shape of a straight line, equally flat appear the trend of the gas production rate over time (figure 3). Fructo-oligosaccharides showed more intense fermentative process, characterized by very fast kinetics (A_1 168.7 and A_2 93.0 ml/g OM; B_1 6.5 and B_2 11.4) as reported also by other authors (Bauer *et al.,* 2003; Williams *et al.,* 2005).
- Glutamic beet pulp produced low gas over all the incubation and its kinetics was quite slow. The adaptation to the bi-phasic model evidenced two phases with similar amount of gas (A_1 40.7 and A_2 36.1 ml/g OM) characterised by different rate (R_{max1} 2.8 and R_{max2} 1.1 ml/h).

- Wheat bran and sugar beet pulp showed intermediate and similar gas profiles with slower (A_1 48.1 and 62.0, respectively) and lower (R_{max1} 2.6 and 0.6 ml/h) gas production during first phase than the second one (A_2 123.2 and 128.8; R_{max2} 3.9 and 6.7 ml/h, respectively).
- Starch, soybean hulls, chestnuts and citrus by-products showed at the end of the incubation the same gas production with different profiles. Starch started the fermentation quite late (T_{max1} 27.5 h) even if R_{max} was the highest (13.2 ml/h). This trend was similar to that ones observed in a previous experiment (Calabrò *et al.,* 2002) in which fermentation kinetics of starch from different sources were tested using rumen liquor from bovine. The soybean hulls showed a trend that after 96 hours of incubation seems to go toward the asymptote. Its first phase was slower (R_{max1} 1.9 ml/h) and characterised by few gas (A_1 97.7 ml/g OM) the second one was faster (R_{max2} 4.0 ml/h) with more gas (A_2 106.9 ml/g OM). Chestnuts showed a quite good fermentation profile in terms of gas (A_1 158.1 and A_2 78.3 ml/g OM) and time (T_{max1} 12.8 and T_{max2} 9.5 h). Citrus by-product kinetics showed a similar gas production during the two phases (A_1 123.0 and A_2 106.9 ml/g OM), but different production rate (R_{max1} 6.0 and R_{max2} 3.8 ml/h).

Table 2 – Model parameters with the bi-phasic model of the nine feed ingredients.

	1st phase					2nd phase					
	A1	**B1**	**C1**	**T_{max1}**	**R_{max1}**	**A2**	**B2**	**C2**	**T_{max2}**	**R_{max2}**	**MPE**
	ml/g OM	*h*	*h*	*h*	*ml/h*	*ml/g OM*	*h*	*h*	*h*	*ml/h*	*(%)*
GBP	40.7^{cd}	1.7^{c}	11.0^{a}	1.1^{c}	2.8^{b}	36.1^{b}	3.5^{bc}	25.0^{a}	21.6^{c}	1.1^{bc}	2.02^{abc}
SBP	62.0^{abcd}	8.1^{ab}	22.5^{a}	15.4^{b}	0.6^{b}	128.8^{ab}	1.0^{c}	42.2^{a}	20.2^{c}	6.7^{ab}	1.51^{abc}
WB	48.1^{bcd}	6.4^{abc}	20.3^{a}	2.3^{c}	2.6^{b}	123.2^{ab}	1.1^{c}	39.4^{a}	19.4^{c}	3.9^{abc}	1.30^{abc}
CBP	123.0^{abc}	2.8^{c}	19.2^{a}	2.7^{c}	6.0^{ab}	106.9^{ab}	2.6^{bc}	23.8^{a}	18.0^{c}	3.8^{abc}	0.86^{c}
SH	97.7^{abcd}	5.3^{abc}	33.5^{a}	3.9^{c}	1.9^{b}	181.2^{a}	1.0^{c}	65.6^{a}	31.2^{abc}	4.0^{abc}	1.58^{abc}
GC	12.3^{d}	3.9^{bc}	21.5^{a}	6.3^{c}	-0.3^{b}	32.0^{b}	3.0^{bc}	54.7^{a}	42.7^{ab}	0.3^{c}	2.95^{ab}
FOS	168.7^{a}	6.5^{abc}	16.5^{a}	15.7^{b}	17.1^{a}	93.0^{ab}	11.4^{ab}	36.2^{a}	35.2^{abc}	7.0^{ab}	3.25^{a}
CN	158.8^{a}	2.2^{c}	23.3^{a}	12.8^{b}	3.7^{ab}	78.3^{b}	14.8^{a}	31.1^{a}	25.3^{bc}	9.5^{a}	1.24^{bc}
CS	157.4^{ab}	9.3^{a}	28.2^{a}	27.5^{a}	13.2^{ab}	81.7^{ab}	5.0^{bc}	51.5^{a}	47.3^{a}	2.4^{bc}	3.04^{ba}
MSD	110.0	4.9	26.3	6.3	12.3	102.4	9.7	60.9	19.2	5.8	1.95
Root MSE	34.0	1.5	8.1	1.6	3.1	31.6	3.0	18.8	5.9	1.8	0.65

GBP: glutamic acid beet pulp; SBP: sugar beet pulp; WB: wheat bran; CBP: citrus by-product; SH: soybean hulls; GC: grapecake; FOS: fructo-oligosaccharides; CN: chestnuts; CS: corn starch.

A: asymptotic gas production; B: time at which A/2 was reached; C: switching characteristic of the curve; R_{max}: maximum rate of gas production; T_{max}: time at which R_{max} occurs; MPE: Mean prediction error.

a, b, c …: P< 0.01; MSD: minimum significant differences; MSE: mean square of error.

In table 3 there are the pH values determined after 96 h of incubation and the products obtained at the end of the gas production trial.

All the registered pH values were included in the range (5.5 - 7.5) indicated by Younes *et al.* (2001) as the physiological pH of colonic contents and resulting faeces one.

Short chain fatty acids resulted lower in grapecake and higher in the chestnuts and FOS (1.31 vs 5.30 and 6.58 mM/g OM, respectively), the other samples were fairly uniform, ranging from 2.72 to 4.15 in sugar beet pulp and starch, respectively. For all tested substrates the main represented short chain fatty acids were acetate and propionate, which sum represents for all samples more than 80 %, with the exception of GBP and FOS, which showed acetate plus propionate production corresponding to 74 and 78% SCFAs, respectively. FOS and CN showed significantly high butyrate production (0.926 and 0.632 mM/g OM, $P<0.05$, respectively) than GC (0.095 mM/g OM) and SBP (0.139 mM/g OM). All the other samples showed intermediate values.

Regarding nitrogen utilization, the ammonia production was moderate and were not be affected by the substrate composition, varying from 1.20 mM/g OM of corn starch to 2.69 of wheat bran. However, the proportion of branched chain fatty acids was significantly ($P>0.05$) higher for GBP (0.098) and GC (0.071) than the other samples.

Table 3 - Fermentation end-products of the nine feed ingredients.

	pH	SCFAs	Acetic	Propionate	Iso-Butyric	Butyric	Iso-valeric	Valeric	NH_3	BCP
					mM/g OM					
GBP	7.15^{a}	3.36^{bc}	1.77	0.73^{a}	0.086	0.294^{bc}	0.243^{a}	0.242	2.29	0.098^{a}
SBP	6.87^{bc}	2.72^{bc}	1.64	0.79^{b}	0.031	0.139^{c}	0.045^{c}	0.075	1.63	0.028^{de}
WB	6.93^{b}	3.98^{abc}	2.29	0.88^{b}	0.070	0.343^{bc}	0.145^{b}	0.255	2.69	0.054^{bcd}
CBP	6.80^{bcd}	3.30^{bc}	1.92	0.74^{b}	0.030	0.423^{bc}	0.056^{bc}	0.134	1.63	0.026^{de}
SH	6.73^{cde}	3.03^{bc}	1.68	0.88^{b}	0.077	0.214^{bc}	0.083^{bc}	0.096	1.17	0.056^{bc}
GC	7.30^{a}	1.31^{c}	0.68	0.39^{b}	0.047	0.095^{c}	0.044^{c}	0.058	1.66	0.071^{ab}
FOS	6.50^{f}	6.58^{a}	2.86	2.30^{a}	0.034	0.926^{a}	0.042^{c}	0.338	1.22	0.011^{e}
CN	6.66^{def}	5.30^{ab}	2.20	2.17^{a}	0.053	0.632^{b}	0.121^{bc}	0.123	1.41	0.033^{cde}
CS	6.57^{ef}	4.15^{abc}	2.18	1.33^{ab}	0.062	0.417^{bc}	0.065^{bc}	0.095	1.20	0.030^{cde}
MSD	0.18	3.44	2.16	0.96	0.102	0.452	0.093	0.281	1.55	0.028
Root MSE	0.06	0.98	0.69	0.31	0.029	0.145	0.030	0.090	0.50	0.008

GBP: glutamic acid beet pulp; SBP: sugar beet pulp; WB: wheat bran; CBP: citrus by-product; SH: soya bean hulls; GC: grapescake; FOS: fructo-oligosaccharides; CN: chestnuts; CS: corn starch.

SCFAs: total fatty acids; NH_3: ammonia; BCP: bran chain proportion.

a, b, c …: $P< 0.01$; MSD: minimum significant differences; MSE: mean square of error.

Discussion

The use of gas production technique appears useful to evaluate the potential healthy rules of feedstuffs, which may be introduced in diets for pigs. Comparing chemical composition data and fermentation parameters the following observations on tested ingredients could be made.

The fermentative process of the GC characterized by particularly low gas production, and dry matter disappearance with moderate SCFAs and NH_3 values could be related by high lignin concentrations registered, which could be due also by the presence of condensed tannins as reported by Llobera and Canellas (2006). Indeed these substances could interfere into the ADL determination (Calabrò *et al.*, 2012b). The presence of cell wall components such as tannins could affect negatively gas production and dry matter degradability (Guglielmelli *et al.*, 2011).

Fructo-oligosaccharides showed intense and fast carbohydrates fermentation due to the high proportion of soluble fiber, which is almost completely fermentable by gut microorganisms. These polysaccharides have the ability to remain almost intact in the first part of the intestine stimulating the growth of beneficial microorganisms (Manrique and Lajolo, 2001) for the host health, for this reason they are widely used as prebiotics. Our results confirming that FOS are energy source for gut bacteria, which produce butyric, propionic and lactic acid, causing lowering pH during the fermentation.

Despite the similar OMCV values registered for starch, chestnuts and soybean hulls the gas profiles had highly diverged, as indicated by the B_1 values of bi-phasic model: corn starch fermentation started after 9 h of incubation and quickly increased progressively, on the contrary soybean hull and chestnuts fermentation started after 5 and 2 h of incubation. Both samples richer in starch showed higher gas volume during the first phase, demonstrating an intense fermentative activity at the beginnings of incubation, while soybean hulls showed a continuous gas production a long the 96 h of incubation.

Probably the differences between chestnuts and corn starch were related either to starch contents or to the relative amylose/amylopectin (de-Oliveira *et al.,* 2011). Indeed chestnuts starch was more soluble and digestible, while corn starch, probably also for the utilized extraction procedure was more resistant to the digestive attach. For this reason chestnuts could be more useful for the formulation of diets for piglets or for sow immediately after delivery, when the low feed intake represents a limit to satisfy the high energy requirements and the high digestibility of chestnuts starch could compensate this limit. It is interesting to note that the chestnuts have presented high butyric acid content second only to FOS one; this data confirm the ability of this

feedstuff to promote gut health. Thereafter the supplementation of chestnuts in the diets for the last phases of breeding could increase the nutritional characteristics of pork meat. Indeed Pugliese and Franci (2007) reported that grazing under chestnuts tree caused a higher percentage of poly-unsaturated fatty acids (series omega-3) in pig fat, hence, not causing a decline in the characteristics of fat quality compared to traditional diets.

The fermentation kinetics of soybean hulls was affected by the high concentration of un-lignified cellulose and moderate soluble fiber and hemicelluloses contents. These last polysaccharides were fermentable and responsible of the obtained gas production and of the moderate SCFAs production. This heterogeneous composition of SH carbohydrates allows a modulate fermentation rate which could be particularly useful to prevent gastro-intestinal disease in critical livestock phases (Jensen and Jorgensen, 1994). Probably crude protein content affected BCP value of SH.

GBP showed very low OMD and OMCV values due to the high protein concentration, which affected short chain fatty acids production as demonstrated by the high values of BCP and NH_3. Indeed glutamic beet pulp proteins, as reported by Dell'Orto and Savoini (2005), are characterized by a mean value of degradability of 65%.

Citrus by-product kinetics is very intense, characterized by high gas production during both phases. This is due to a mix of soluble and insoluble structural carbohydrates present in this sample. Indeed soluble fiber, highly represented by pectins, is responsible of the first tumultuous fermentation, while the insoluble hemicelluloses were fermented in the second one. This by-product could be particularly useful in formulating diets for all livestock stages, for his mixture of structural carbohydrates with different physical and chemical properties, characterised by high viscosity and high fermentability, these characteristics allow to reduce hematic cholesterol levels, decreasing also the cholesterol concentration at muscular level (Glore *et al*., 1994) and increase mineral absorption (James *et al.,* 1980).

Sugar beet pulp and wheat bran produced similar fermentation kinetics characterised by higher gas production during the second phase and faster rate during the first phase. These results were in our opinion due by different reasons. Notoriously dietary fiber of sugar beet pulps is a mixture of soluble and insoluble fiber: the soluble fraction and the residual sugar are fermented rapidly, while the less soluble components were fermented with high gas production during the second phase. On the contrary in wheat starch and non-structural carbohydrates represent bran the fermentable portion more as demonstrated by the high acetic acid production.

Conclusions

The *in vitro* fermentation data were in line with the chemical composition values and contribute to better characterize the tested feedstuffs. The results obtained *in vitro*, for the already known substrates (FOS, wheat bran, corn starch, soya bean hulls, sugar beet pulp, glutamic beet pulp) agree with that reported by other authors in similar experiments.

The alternative substrates (chestnuts, grapecake and citrus by-products) give interesting information in terms of gut fermentation in pigs. These substrates could be useful to improve swine performance and meat quality. Thereafter seems interesting to underline that main of the tested substrates are by-products and could represent economical and readily available nutrient sources. These results offer good perspectives for the possibility of using some of these raw materials in the formulation of pig diets. In particular, chestnuts, a traditional feed ingredient in family pig production in the South of Italy, showed as a whole very interesting results. Also the citrus by-products showed interesting functional characteristics that deserve to be studied *in vivo* in order to evaluate their effect on growth performance and meat quality.

Chapter 5 -In vitro evaluation of *Aloe arborescens* supplementation in cat diet

Abstract

Aloe spp. has been used for centuries for a multiplicity of unrelated human ills. *Aloe* plant contains anthraquinone glycosides (aloins), mucilages (30%), resinous materials (16-63%), sugars (25%), mucopolysaccharides (acemannan, β-mannan), fatty acids, glycoproteins, enzymes and other compounds. Some of these components possess laxative properties, others anti-inflammatory, immunostimulant and antiseptic activities. Aim of the present study was to evaluate the hindgut effects of *Aloe arborescens* in cat using the *in vitro* gas production technique. Three European adult neutered cats were fed with a commercial diet (ME 3.750 kcal/kg, CP 31.2%; EE 16.7%, CF 3.5%) for 20 days before the collection of their faeces that were used as *inoculum*. The same diet administered to the animals was supplemented with different ratios (0; 0.7; 1.6 and 3.2%) of a commercial product containing *Aloe arborescens* and incubated *in vitro*. Organic matter degradability and gas production were significantly ($P<0.01$ and $P<0.05$, respectively) decreased with the aloe supplementation, as well as total SCFAs production; however, acetic and iso-valeric acids showed an inverse behavior. The acetic acid was highly influenced by aloe dose, with the highest value in the diet without supplementation; on the contrary iso-valeric showed an inverse relationship. A different trend was observed for the parameters related to the protein degradation (BCFAs and NH_3), where any significant differences were observed in relation to the aloe supplementation. The pH values trend was affected by the content of acid and basic end-products proportions. The relative increase of lactic acid production registered in relation to the dosage supplementation of aloe suggests a potential increase of lactic fermentation in the gut related to aloe administration. In this preliminary study the complex *Aloe arborescens* composition surely affected the *in vitro* digestibility and fermentability of the diet. The additional evidences obtained by the IVGPT were particularly useful to better understand the potential beneficial effects of aloe.

Keywords: short chain fatty acids, cats, gas production, faecal inoculum.

Introduction

The genus aloe comprises about 600 species native in southern and eastern Africa, and subsequently introduced into northern Africa, the Arabian Peninsula, China, Gibraltar, the

Mediterranean countries and the West Indies. Four aloe species [*Aloe barbadensis Miller* (syn. *Aloe vera*; Liliaceae), *Aloe ferox* (syn. *Cape Aloe*; Liliaceae), *Aloe arborescens* (syn. *Candelabra Aloe*; Liliaceae) and *Aloe perryi baker* (syn. *Perry's Aloe*; Liliaceae)] have been traditionally applied for the medicinal practice over thousands of years in many countries. Aloe is considered one of the most known herbs; member of *Liliaceae*, it is similar to cactus in appearance and mostly grows in arid regions of Asia and Africa (Boudreau and Beland, 2006). Aloe has been called the "plant of immortality" because it can live and bloom without soil. According to the Roman scholar, Plinio, the plant was used for embalming. The Greek physician, Dioscoride Pedanio described the use of aloe gel for several infections, to treat sores, wounds, hair loss, ulcers, and as a laxative. In India, the whole leaves and the fresh gel have been used as a laxative, to improve appetite and digestion, to promote menstrual flow, and to destroy and expel intestinal worms.

The middle major parts of aloe leaves consist in a gel. Many studies discovered different properties of this gel, including wound healing, anti-parasitic, anti-viral, anti-fungal and anti-bacterial properties (Boudreau and Beland, 2006; Reynolds and Dweck, 1999). Studies performed on broilers performance have shown that *Aloe vera* gel improves the immune response (Chinnah *et al*., 1992; Valle-paraso *et al*., 2005). Aloe gel has demonstrated wound healing (Davis, 1989; Heggers, 1993), anti-inflammatory (Vazquez*,* 1996), antiviral (Saoo*,* 1996), spermicidal (Fahim and Wang, 1996), and gastro-protective (Danhof, 1991) cicatrising (Davis *et al.,* 1994; Heggers, 1993; Heggers *et al.,* 1997) properties. It has also shown immune-stimulating (Zhang and Tizard, 1996; Infascelli *et al.,* 2010; Capasso *et al.,* 1998) and cholesterol-lowering (Tizard *et al.,* 1989) activities.

Aloe plant contains anthraquinone glycosides (aloins), mucilages (30%), resinous materials (16-63%), sugars (25%), mucopolysaccharides (acemannan, β-mannan), fatty acids, glycoproteins, enzymes, vitamins (A, B_1, B_2, B_3, B_6, B_9) and minerals (calcium, potassium, iron, manganese, magnesium, copper, zinc, chromium) (Vogler and Ernst, 1999; Eshun and He, 2010). The acemannan, a mannose polymer, is one important constituent of aloe gel (Reynolds and Dweck, 1999), several beneficial effects of aloe may stem from this compound (Mascolo *et al*., 2004), while the wound-healing activity has been partially attributed to the mannose-6-phosphate (Davis *et al.,* 1994). As regards the healing properties, many researches have demonstrated that the mucilaginous polysaccharides contained in the clear pulp of aloe leaf are the major ingredient responsible for the healing. However, new evidence has shown that emodin, one of the derivatives of anthraquinones produced by superficial pericyclic cells, is also capable of promoting the repair of rats excisional wounds via stimulating tissue regeneration (Eshun and He, 2010; Teng

et al., 2007), supporting evidence to the claim that the healing function of aloe plant is essentially a result of the synergistic mode of action of many bioactive compounds, rather than one single "magic bullet" (Dagne *et al.,* 2000).

The aim of this study was to evaluate the effects of *Aloe arborscens* supplementation on fermentation pathway of a commercial diet for adult cat using *in vitro* gas production technique.

Materials and methods

For the trial three European adult neutered cats were used as faeces donors. The cats were progressively adapted to a commercial diet (ME 3750 kcal/kg, CP 31.2%; EE 16.7%, CF 3.5%) administered in ratio of 100 kcal ME/BW $kg^{0.67}$. After 20 days of diet administration faeces were collected and immediately transported to the laboratory into thermo stated boxes (39°C) under anaerobic condition. To obtain the *inoculum*, the faeces were pooled, diluted with sterile saline solution (1:1), homogenized and filtered according to Bauer *et al.* (2001).

A commercial product (NUTRIZOO s.a.s., Italy) containing the whole lyophilized plant of *Aloe arborescens* was added to the administered diet. The supplementation ratios (0.7; 1.6 and 3.2%) were calculated halving and doubling the oral dosage suggested by the producer for adult cats with a mean body weight of 3.5 kg. The obtained diets were utilized as substrates such as the diet without aloe supplementation, which was incubated as negative control. All substrates were analyzed for chemical composition, including TDF, SDF and IDF content (Prosky *et al.,* 1990; Lee and Prosky, 1995). The *in vitro* procedures were carried out according to Cutrignelli *et al.* (2009). In particular, each diet was weighed in serum flasks with a buffer solution under anaerobic condition for 48 hours at 39°C. Gas production of fermenting cultures was recorded every two hours using a manual pressure transducer (Cole and Parmer Instrument Co, Illinois, USA) and related to the amount of incubated organic matter (OM cumulative volume of gas, OMCV). After 48h of incubation the fermentation was stopped by cooling, pH was measured by pHmeter (model 3030, Alessandrini Instrument glass electrode Jenway, Dunmow, UK) and fermentation liquor was collected for the determination of end-products. The OM disappearance (OMD, %) was measured by filtering through glass crucibles (Scott Duran porosity #2) and burning the residual for 5 h at 550°C.

Short chain fatty acids (SCFAs) were measured by gascromatography (ThermoQuest Italia SpA, Rodano, Milan, Italy; model 8000 top, fused silica capillary column 30 m 0.25 mm 0.25 lm film thickness) according to Calabrò *et al.* (2012). The lactic acid was determined by spectrophotometer using a colorimetric kit (L-Lactic acid – UV method – Boehringer Mannheim/R-Biopharm.

Enzymatic BioAnalysis/Food Analysis). Ammonia (NH_3) content was determined according to the method described by Searle (1984).

The influence of Aloe supplementation on the *in vitro* parameters was statistically tested by ANOVA, using the PROC GLM of SAS (2000).

Results

In table 1 chemical composition and dietary fiber values of tested diets were reported. The inclusion of aloe in the diets moderately decreased concentrations of crude protein, starch and ether extract.

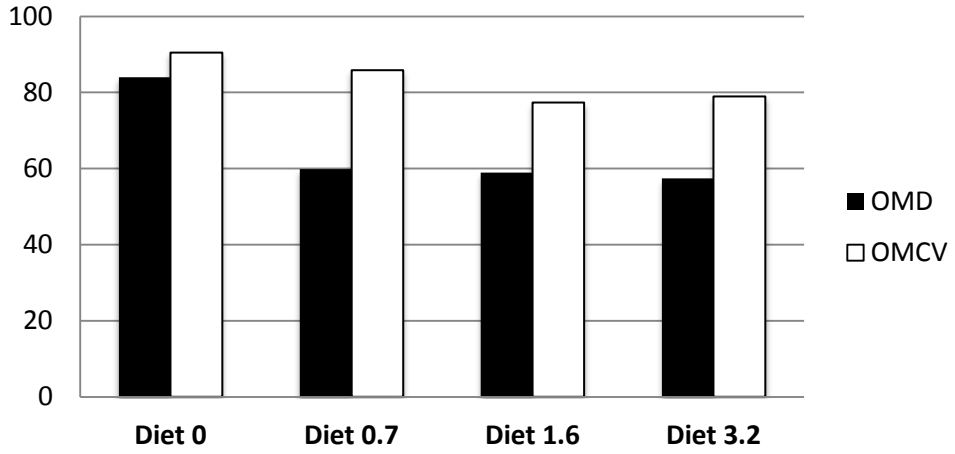
Table 1 - Chemical composition (% a.f.) of tested diets.

	Diet 0	Diet 0.7	Diet 1.6	Diet 3.2
CP	31.21	31.09	30.95	30.69
EE	16.74	16.59	16.45	16.20
CF	3.52	3.56	3.63	3.76
NFE	34.59	34.76	34.96	35.31
Starch	30.99	30.77	30.49	30.00
TDF	29.61	29.65	29.72	29.84
IDF	21.98	22.05	22.06	22.17
SDF	7.58	7.62	7.64	7.81

CP: crude protein; EE: ether extract; CF: crude fiber; NFE: nitrogen free extract; TDF: total dietary fiber; IDF: insoluble dietary fiber; SDF: soluble dietary fiber.

Mean values of the *in vitro* organic matter disappearance (OMD, %) and the cumulative gas production (OMCV, ml/g iOM) were reported in figure 1.

Figure 1 – In vitro organic matter disappearance (OMD,%) and cumulative volume of gas production (OMCV, ml/g iOM) of the tested diets.



The aloe supplementation affects both parameters, which were significantly (OMD P<0.01; OMCV P<0.05) higher for diet 0 compared to the other three diets. In particular, OMD trend resulted inversely proportioned to aloe supplementation dosage, as well as for OMCV excepted for diet supplemented with 3.2% of aloe.

In table 2 the mean values of short chain fatty acids produced after 48 h of incubation were reported. The values showed a clear decreasing in according to aloe supplementation. Particularly relevant seem the aloe effects on acetic and iso-valeric acids production: the first, which represent in all cases more than 45% of SCFAs, resulted highly dosage dependent (P<0.01 and P<0.05) with the highest values in the diets characterized by the absence or very low aloe supplementation. On the other hand, iso-valeric, which was the less representative SCFAs, showed an inverse relation with aloe dose (P<0.01). Propionic, iso-butyric, butyric and valeric acids production were not affected by aloe supplementations.

Table 2 - Short chain fatty acids production.

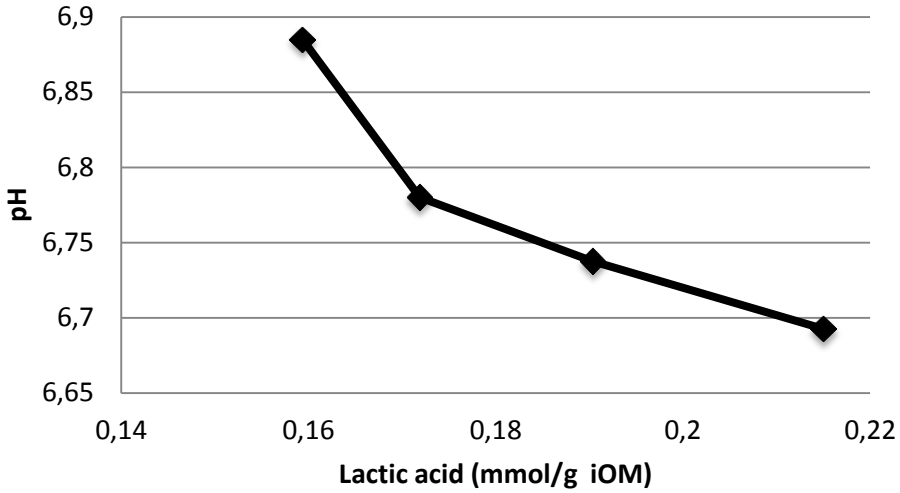
	Acetic	**Propionic**	**Iso-butyric**	**Butyric**	**Iso-valeric**	**Valeric**
	mmol/g SOi					
Diet 0	10.94Aa	3.33	0.135	1.28	0.036B	1.37
Diet 0.7	8.49ABb	3.15	0.110	1.16	0.055AB	1.30
Diet 1.6	7.31B	3.05	0.094	1.22	0.060A	1.35
Diet 3.2	6.63B	3.68	0.115	1.08	0.072A	0.99
MSE	14.38	0.31	0.041	0.041	0.0007	0.124

A, B: P<0.01; a, b: P<0.05

In figure 2 the relation between pH and lactic acid production is represented; it seems interesting to underline that diet 3.2 showed the highest values of lactic acid (0.22 mmol/g iOM) and the lowest pH ones (6.69), while the lowest lactic acid concentration (0.16 mmol/g iOM) and the highest pH (6.88) were obtained incubating diet 0. In each case the pH values were included in the range (5.5 - 7.5) indicated by Younes *et al.* (2001) as the physiological pH of colonic contents and resulting faeces one in several animal species, including dog and cat.

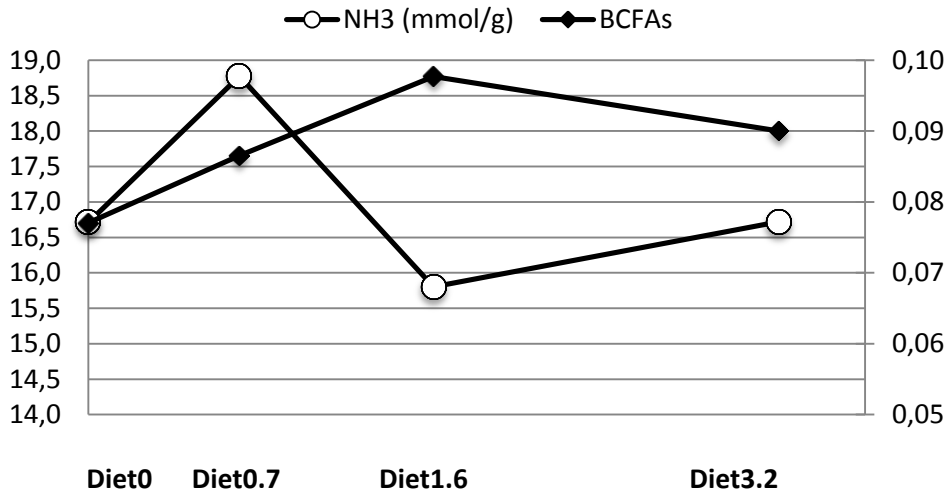
Ammonia production (NH_3, mmol/g) and branched chain fatty acids (BCFAs) proportion, considered the main indicators of protein degradation, were represented in figure 3.

Figure 2 – Mean values of pH and lactic acid.



For both parameters no significant differences were observed in relation to the aloe supplementation and the registered trends were independent to aloe dose, the lowest ammonia concentration was registered with diet 1.6, while the lowest values of BCFAs were observed with diet 0.

Figure 3 – Branched chain fatty acids proportion and ammonia production.



Discussion

The effect of aloe supplementation on fermentation parameters may be partly explained by the presence in aloe plant of miscellaneous bioactive constituents, such as anthraquinone glycosides, acemannan, β-glycosides, mannose-6-phosphate, alkaloids, and triterpenoids. Even if

most of these compounds are soluble carbohydrates, they could be classified as un-fermentable polysaccharides. Instead, acemannan and anthraquinone were transformed by gut microflora in more active substances without gas and/or SCFAs production.

The data obtained in this study were partially in discordance with those obtained in a preview study, where the effect of aloe supplementation was tested *in vitro* using as *inoculum* rumen liquor from buffalo and as substrates specific diets for ruminant (Calabrò *et al.,* 2013). These differences could be due either to the differences in microorganism population or in chemical composition of tested diets. Instead, the increasing of lactic acid values and the decreasing of acetic acid production in function of aloe supplementation, suggest that aloe improves the lactic fermentation, flattening the other fermentation pathways. These results could be indicative of a higher *Lactobacillus* spp. proliferation, which was indicated as one of the main bacterial general present in healthy intestine of carnivores and humans (Maskell and Johnson, 1993). Probably this observation could partially justify the immune-stimulant activity of aloe.

Conclusion

In this preliminary study, aloe supplementation affected most part of *in vitro* fermentation parameters. The complex aloe composition surely interfered on digestibility and fermentability of the diet. In particular, the mucilaginous and resinous compounds inhibited the faecal bacteria activity on fermentable nutrients decreasing digestibility and gas production. These last results partially explain the laxative effect of aloe. The low SCFAs productions appeared to deny the trophic effect on the gut mucosa. However, the direct relation between lactic acid and aloe supplementation suggests an increasing use of lactic fermentation and, consequently, a higher *Lactobacillus* spp. development in the gut.

As suggested by these results to understand all functional activity of innovative diets and/or ingredients, especially carbohydrates ones, it is necessary to study their fermentation characteristics. The *in vitro* gas production technique seems particularly useful for this aim, because it allows to evaluate not only the degradation extent, but also to determine all the products of microorganisms activities.

General Conclusions

In the second chapter twenty-eight feedstuffs (6 legume grain, 5 cereal grain, 3 seeds, 1 plant, 2 roots and tubers and 11 by-products) were analysed in order to evaluate their nutritional value and the potential beneficial effects. Particular attention has been directed to determine the structural carbohydrates composition using the methods proposed by the researcher groups coordinated by Van Soest and by Prosky, respectively. The concentration of total dietary fiber in legumes and cereal grains resulted highly variable, on the contrary in seeds category the psyllium and hemp were characterised by high TDF contents and low IDF/SDF ratio. Roots and tubers samples showed the highest variability, both in reserve and structural carbohydrates. Instead, carrots showed higher concentration of sugars and SDF than potatoes, while this last presented greater amount of starch and IDF. As expected, the highest variability was observed in the by-products category.

The combined application of the methods proposed by Van Soest and Prosky to determine the fractions of structural carbohydrates in the feedstuffs allows us to better appreciate the nutritional and functional properties of each tested ingredient. Our results confirmed the nutritional and beneficial role of several ingredients usually utilized in feed industry (i.e. pea, bean, corn and oat), which combine the high nutritive values (high protein, starch, gross energy amounts) with considerable TDF contents. More interesting in our opinion are the functional properties of the tested by-products, which in different proportion, could contribute to prevent or treat several pathologies. The use of alternative fiber sources as ingredients in feed formulation seems a promising and important area in livestock and companion animals nutrition, because most of them are predominantly composed by carbohydrates and it is evident their beneficial effects in improving the animals health status. These by-products could be utilized as more functional and less expensive alternative to the common ingredients in formulating diets for omnivore and/or carnivores species.

In the third chapter the fermentation characteristics and kinetics patterns of 10 carbohydrate sources using the *in vitro* gas production technique (IVGPT) with dog fecal *inoculum* were compared. The substrates tested were: pure cellulose (PC); carboxymethylcellulose (CMC); sugar cane fiber (SCF); beet pulp (BP); wheat bran (WB); fructo-oligosaccharides (FOS); inulin; yeast cell wall (YCW); ground psyllium seed (PS); pea hulls (PH). All substrates were incubated at 39°C under anaerobic conditions with faeces collected from dogs as microbial *inoculum*. Gas

production of fermenting cultures was recorded, and after 48 h pH, short-chain fatty acids (SCFAs) and the organic matter disappearance (OMD) were determined.

The *in vitro* gas production technique was useful for a screening of non-digestible carbohydrates for dogs, separating them according to fermentation kinetics, organic matter disappearance and SCFAs production. Non-digestible carbohydrates could be very different ranging from almost un-fermentable (PC, CMC, and SCF) to highly fermentable (inulin, FOS, and WB), fast fermented (inulin, FOS, and WB) or slowly fermented (YCW, BP, and PS), and with different molar ratios of SCFAs and BCFAs profiles. All these outcomes have different consequences on hindgut and dog health. Therefore, ingredients should be chosen which result in higher production of SCFAs, particularly of butyric acid and lower, which suggest reduced protein fermentation. A selection of several ingredients with different kinetic and end-products characteristics can be made to design diets to stimulate carbohydrate fermentation along the entire tract. However, all considerations on fermentability and SCFAs production of the tested substrates should be evaluated in relation to gas production. Indeed, control of intestinal gas production is an important goal during ingredients selection and diets formulation in order to avoid undesirable effects such as flatulence and soft faeces.

In chapter four, nine feedstuffs (sugar beet pulp, wheat bran, soybean hulls, grapecake, chestnuts, glutamic beet pulp, citrus by-product, fructo-oligosaccharides and corn starch) were incubated *in vitro* with faecal *inoculum* from pigs for 96 h, under anaerobic condition. Cumulative gas production was measured as an indicator of the fermentation kinetics. At the end of incubation, OM disappearance and fermentation end-products were measured. The gas production profiles were fitted with a multi-phasic model.

The fermentation parameters showed a different trend for grapecake and FOS, very low gas production for the first one, probably due to the high lignin and tannins contents of this by-product, on the contrary the higher OMDV and OMCV values were registered for the second one for its high soluble fiber proportion. Some samples, like chestnuts, soybean hulls and citrus by-product produced during the incubation the some gas amount with different profiles.

The *in vitro* fermentation data were in line with the chemical composition values and contribute to better characterize the tested feedstuffs. The obtained results, for the already known substrates (FOS, wheat bran, corn starch, soya bean hulls, sugar beet pulp and glutamic beet pulp) agree with that reported by other authors in similar experiments. The alternative feedstuffs (chestnuts, grapecake and citrus by-products) give interesting information in terms of

gut fermentation in pigs. These substrates could be useful to improve swine performance and meat quality. These results offer good perspectives for the possibility of using some of the tested raw materials in the formulation of pig diets. In particular, chestnuts, a traditional feed ingredient in family pig production in Italy, showed as a whole very interesting results. Also citrus by-product showed chemical composition and fermentation kinetics, which allow to this feedstuff functional characteristics able to reduce cholesterol and increase mineral levels in meat.

In the last chapter the fermentation kinetics of a commercial product containing *Aloe arborescens* was measured. With this aim a commercial diet was supplemented with different ratios (0; 0.7; 1.6 and 3.2 %) of aloe. The four diets were incubated with faecal *inoculum* from cat under anaerobic condition for 48 hours at 39°C. At the end of the incubation the fermenting liquor was analyzed for pH, lactic acid, SCFAs and NH_3.

In this preliminary study, aloe supplementation affected main part of *in vitro* fermentation parameters. The complex aloe composition surely interfered on digestibility and fermentability of the diets. In particular, the mucilaginous and resinous compounds inhibited the faecal bacteria activity on fermentable nutrients, decreasing digestibility and gas production. These last results partially explain the laxative effect of aloe. The low SCFAs productions appeared to deny the trophic effect on the gut mucosa. However, the direct relation between lactic acid and aloe supplementation suggesting an increasing lactic fermentation and, consequently, a highest Lactobacillus *spp.* development in the gut.

As suggested by these results to understand all functional activity of novel diets ingredients, especially carbohydrates ones, it is necessary to study its fermentation characteristics. The *in vitro* gas production technique seems particularly useful for this aim because it allows to evaluate not only the organic matter disappearance and cumulative volume of gas produced, but also to determine all the products of fermentation.

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Acronyms

ADF: acid detergent fiber.

ADL: lignin detergent fiber.

B-BP: by-products-beet pulp.

B-C: by-products-purified cellulose.

B-FP: by-products-faba pod.

B-FR1; B-FR2; B-FR3: by-products-fiber fruits.

B-PF: by-products-pea fibre.

B-PH: by-products-pea hulls.

B-PP: by-products-pea pod.

B-SB: by-products-sugar cane.

B-WM: by-products-wheat middling.

BCFA: branched chain fatty acids.

BCP: branched chain fatty acids proportion.

BP: beet pulp.

C-B: cereals-barley.

C-C: cereals-corn.

C-O: cereals-oats.

C-R: cereals-rice.

C-W: cereals-wheat.

CBP: citrus by-product.

CF: crude fiber.

CMC: carboxymethylcellulose.

CN: chestnuts.

CP: crude protein.

CS: corn starch.

DM: dry matter.

EE: ether exctract.

FOS: fructo-oligosaccharides.

FOS: fructo-oligosaccharides.

GBP: glutamic acid beet pulp.

GC: grapescake.

GE: gross energy.

HDL: high density lipoprotein.

IDF: insoluble dietary fiber.

L-B: legumes-bean.

L-C: legumes-whole carob.

L-F: legumes-faba bean.

L-L: legumes-lentil.

L-P: legumes-pea.

L-S: legumes-soybean.

LDL: low density lipoprotein.

MUFA: mono-unsatured fatty acids.

NDF: neutered detergent fiber.

NFE: nitrogen free extract.

NSC: non-structural carbohydrates.

P-A: plant-aloe.

PC: pure cellulose.

PH: pea hulls.

PS: ground psyllium seed.

PUFA: poly-unsatured fatty acids.

R-C: roots-carrots.

R-PF: roots-potatoes flakes.

S-H: seeds-heamp.

S-L: seeds-linseed.

S-P: seeds-psyllium.

SBP: sugar beet pulp.

SCF: sugar cane fiber.

SCFA: short chain fatty acids.

SDF: soluble dietary fiber.

SH: soya bean hulls.

TDF: total dietary fiber.

WB: wheat bran.

WB: wheat bran.

YCW: yeast cell wall.