UNIVERSITÀ DEGLI STUDI DI MILANO

DOCTORAL PROGRAMME IN NUTRITIONAL SCIENCE

Former foodstuffs products intended for pig nutrition: *in vitro* and *in vivo* nutritional evaluation, impact on growth performances and gut health

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Abstract

Livestock play a key role in food security, through food provision, agricultural production, and by providing employment and income. However, with the diminishing availability of farmland, climate change and the threat of declining water resources, the goal is to meet the growing demand for food and feed by using fewer resources. Exploiting alternative ingredients for livestock, feed could be one way of increasing livestock sustainability.

This thesis focused on processed and ready-to-eat food products that are no longer suitable for human consumption due to logistical, manufacturing or packaging defects. Such products would normally go to a landfill yet actually have a high potential of being used as sustainable feed ingredients.

The first part of this thesis investigated the chemical composition of six different former foodstuff products (FFPs). Based on the FFP composition data, the digestible energy and metabolisable energy values for pigs were estimated. In addition, the *in vitro* digestibility values of FFPs were evaluated using a multi-step enzymatic technique. The *in vitro* predicted glycaemic index and hydrolysis index of the same samples were examined using a two-step *in vitro* digestion assay.

In the second part, the safety issues linked to the use of FFPs were investigated. FFP samples were thus analysed in relation to the microbial load and the presence of presumed remnants of packaging materials. For this purpose, two different methods were used: stereomicroscopy, according to published methods; and stereomicroscopy coupled with a computer vision system.

The final part addressed the effects of a diet in which common cereal grains were partially replaced by FFPs in post weaning piglet diets. Specifically, pig growth performance and selected plasma biochemical variables were evaluated in twelve post-weaning piglets. The apparent total tract digestibility of dry matter and the faecal microbiota were also characterized.

When compared with common cereal grains used in pig feed formulations, FFPs can be considered a fortified version of cereals, with comparable *in vitro* digestibility values and with higher glycaemic and hydrolysis indexes, thus characterizing them as an excellent source of carbohydrates. All FFP samples were safe from a microbiological point of view, showing a limited microbial load and were always *Salmonella* free. Regarding the presumed remnants of packaging materials, the contamination level was always below the safety threshold set by German authorities, and the validated method demonstrated that packaging remnants were mainly from the 1-mm sieve mesh fraction. In order to find a more rapid and objective method for evaluating the packaging remnants, the innovative computer vision system was a rapid alternative for the detection of packaging remnants in ex-food samples when combined with a stereomicroscope.

The *in vivo* study revealed that both *in vitro* and *in vivo* digestibility values were higher for the diet based on FFPs compared to the control diet. At the end of the experiment, no differences in growth performance were observed, however the plasma glucose increased in piglets fed FFPs compared to piglets fed the control diet, while the urea concentration decreased. The sequencing analysis of the variable regions V3 and V4 of the 16S rRNA gene showed that the use of FFPs in the post-weaning period decreased the bacterial richness and evenness in the large intestine. The unweighted beta diversity analysis also resulted in a statistically significant difference between the two groups in terms of the taxa composition. The linear discriminant analysis of effect size also demonstrated an increased amount of *Proteobacteria* phylum and a decreased amount of *Lactobacillales* genus in the FFP compared to the control group.

The results highlighted the potential of these alternative feed ingredients and their safe use in pig nutrition. This is essential for establishing the best scientific practices for the use of FFPs in animal nutrition and feeding. Given the increasing need to obtain a more sustainable livestock sector, research in animal sciences should focus not only on increasing the efficiency of the animal production chain but also on the efficiency of the entire food system in ensuring sustainable nutrition. By recognizing that former foodstuffs that are not suitable for human consumption are a resource for animal nutrition and not a waste product, food and feed industries could reduce the amount of waste sent to landfill or deposed-off every year, thus saving costs, and reducing the environmental impact of the food production chain.

Riassunto

La produzione animale riveste un ruolo chiave nel garantire la sicurezza alimentare. Tale ruolo viene esercitato soprattutto grazie all'approvvigionamento di prodotti di origine animale e prodotti dell'agricoltura. Tuttavia, a causa delle diminuite disponibilità di terreni destinati all'allevamento ed alla agricoltura, insieme ai cambiamenti climatici e alla riduzione delle risorse idriche, diventa sempre più importante aumentare la sostenibilità e l'efficienza del settore agroalimentare. Per fare ciò, diventa necessario soddisfare le crescenti esigenze utilizzando al tempo stesso una quantità ridotta di risorse. Questa tesi ha avuto come tema principale quello di esaminare a fondo il potenziale utilizzo di scarti della industria alimentare (chiamati "former foodstuffs products", FFPs) come ingredienti alternativi e sostenibili per la nutrizione animale. I prodotti esaminati sono alimenti che vengono scartati dalla grande distribuzione per difetti relativi alla loro forma, al loro colore, al loro packaging ecc. Tali scarti solitamente sono destinati a diventare rifiuto, nonostante il loro elevato potenziale nel poter essere utilizzati come ingredienti sostenibili per mangimi.

La prima parte della tesi si concentra sull'analisi della composizione chimica di sei diversi tipi di FFPs. Inoltre, di questi prodotti sono state anche stimate l'energia digeribile e metabolizzabile con riferimento ai suini, la digeribilità *in vitro,* l'indice glicemico e di idrolisi attraverso tecniche di digestione enzimatica.

La seconda parte della tesi è stata dedicata agli aspetti legati alla sicurezza dei FFPs. Campioni di FFPs sono stati quindi analizzati per la loro carica microbica e la presenza di residui di materiale di imballaggio. Per questo ultimo aspetto, sono stati testati due metodi differenti: il primo, precedentemente validato, basato sull'uso dello stereomicroscopio; il secondo, basato sull'uso dello stereomicroscopio accoppiato ad un sistema digitale di acquisizione di immagine (Computer Vision System).

L'ultima parte, invece, ha investigato gli effetti di una dieta in cui in cui i cereali comunemente utilizzati per la formulazione di diete per suini in post svezzamento, sono stati parzialmente sostituiti dagli FFPs. In particolare, una dieta di controllo e quella contenente FFPs sono state confrontate per quanto riguarda la digeribilità *in vitro* ed *in vivo* della sostanza secca, le performances di crescita di suini in post svezzamento, così come alcuni metaboliti ematici ed il microbiota fecale.

I risultati della tesi hanno dimostrato che gli FFPs possono essere considerati una "versione fortificata" dei cereali tradizionali comunemente utilizzati nel settore suinicolo, con valori di digeribilità *in vitro* comparabili agli stessi, ma con valori di indice glicemico e di idrolisi maggiori, caratterizzandoli come una fonte eccellente di carboidrati. Tutti i campioni di FFP sono risultati sicuri dal punto di vista microbiologico e sempre privi di *Salmonella.* Per quanto concerne la presenza di residui di materiale da imballaggio, il livello di contaminazione è risultata sempre al di sotto delle soglie di tolleranza. Il Computer Vision System si è inoltre rivelato essere una rapida alternativa per rilevare la presenza di materiali di imballaggio nei FFPs se accoppiata allo seteromicroscopio.

Lo studio *in vivo* ha rivelato che sia i valori di digeribilità *in vitro* che *in vivo* delle diete contenenti FFPs sono maggiori rispetto ai valori delle diete di controllo. Alla fine dell'esperimento, non sono state osservate differenze nelle performance di crescita, tuttavia nei suinetti alimentati con la dieta FFP c'è stato un aumento di glucosio plasmatico ed una riduzione nella concentrazione di urea.

Il sequenziamento di nuova generazione delle regioni variabili V3 e V4 del gene che codifica per il 16S rRNA hanno evidenziato come l'utilizzo di FFPs nelle diete per suini in post svezzamento riduca sia la numerosità che la biodiversità dei batteri che costituiscono il microbiota nel largo intestino. L'analisi "unweighted beta diversity" ha anche dimostrato piccole differenze nella composizione dei taxa batterici tra il gruppo FFP e quello di controllo. Inoltre, l'analisi lineare delle discriminanti ha documentato un aumento del phylum *Proteobacteria* ed una diminuzione del genere *Lactobacillales* nel gruppo FFP rispetto al controllo. Questi risultati hanno messo in evidenza il potenziale di questi ingredienti alternativi ed il loro utilizzo sicuro nella nutrizione suinicola. Il loro aumentato utilizzo potrebbe quindi portare ad una riduzione dello spreco alimentare, una riduzione dei costi del mangime, e ad un ridotto impatto ambientale della catena alimentare.

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Chapter 1

GENERAL INTRODUCTION

THE NEED FOR ALTERNATIVE FEED INGREDIENTS

The key goals of sustainable human development are to preserve human welfare by ensuring the long-term viability of natural resources (United Nations, 2015). The global human population is approaching 10 billion people (Springmann, Godfray, Rayner, & Scarborough, 2016) where more people live in urban than in rural areas. As a consequence, an increasing demand for housing, infrastructures and energy, together with changes in dietary patterns and the subsequent impact on the food chain are expected (Thornton, 2010; Crist, Mora, & Engelman, 2017) (Table 1.1).

Table 1.1. *Evolution in the global population and food supply. Source: http://faostat3.fao.org.*

	World		
	1990	**2000**	**2014**
The setting			
Population, total (mln)	5320.8	6127.7	7243.8
Population, rural (mln)	3033	3263,4	3362,5
Production indices (2004-06=100)			
Net food	73	90	121
Net crops	72	89	123
Cereals	82	92	123
Vegetable oils	51	77	141
Roots and tubers	74	94	119
Fruit and vegetables	58	86	127
Sugar	86	93	132
Livestock	76	92	115
Milk	83	89	114
Meat	74	91	118
Fish	72	92	119

According to the Food and Agriculture Organization of the United Nations (FAO), the increase in the food output of an animal origin is estimated to be about 70% above the current production (FAO, 2017). The consumption of meat, fish, milk and eggs is essential for meeting the human requirements for amino acids and important trace nutrients (Flachowsky & Meyer, 2015). Studies have demonstrated that about 20 g of the daily intake of about 60 g of protein should be of animal origin. Today, the average consumption of protein of animal origin (without fish) is about 24 g per capita per day, ranging between 1.7 (Burundi) and 69.0 g, where meat, milk and eggs provide around 13% of the energy and 28% of the protein consumed globally, respectively (Flachowsky & Meyer, 2015; FAO, 2015; Thornton, 2010).

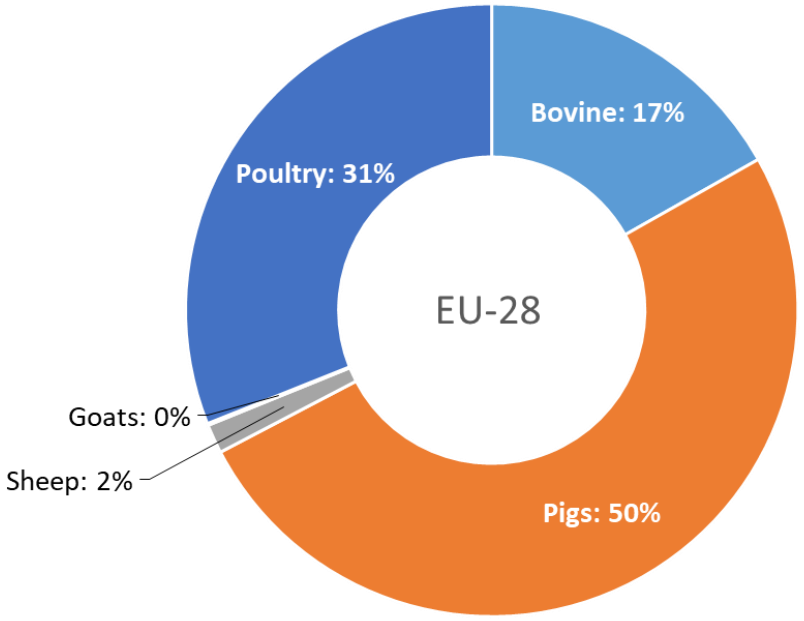
The increasing income of the population and the imitation of western nutritional habits are considered further reasons for the higher demand for food of animal origin in some countries (Flachowsky & Meyer, 2015). Many developing countries continue to consume more animal products than they produce. They will therefore continue to drive the world demand for all agricultural products, including food of animal origin (FAO, 2017). It has been estimated that meat consumption will increase by 76%, and that milk consumption will increase by 62% in the coming decade (Alexandratos & Bruinsma, 2012). As a consequence, the livestock sector is expected to growth rapidly (Herrero, et al., 2015). Whilst the livestock sector plays a key role in containing food insecurity, it is also a source of environmental pressure with impacts on the air, water and soil (Gerber, et al., 2013). Besides climate change, in fact, there is increasing competition for natural resources between human and animal nutrition: both require land, water, raw materials and fossil-energy (De Vries & De Boer, 2010; Leip, et al., 2015).

A total of 30% of the ice-free land on the planet is used by livestock production, including the land used for feed production (Steinfeld, et al., 2006). It has been estimated that, of the 5 billion ha of global land used for agriculture, 70% has been used by the livestock sector (FAO, 2015; Steinfeld, et al., 2006). It is increasingly being argued that improvement in the environmental performance index of livestock systems and the establishment of more sustainable levels of ASF consumption, are crucial for the sustainability of the global food system (Herrero, et al., 2013). Livestock production and land use are closely connected by the feed. Taking into account pig production, the grains that are usually fed as concentrates are mainly consumed by this livestock sector, depending on the specific region of the world and particular commodities. Many of the impacts from livestock production thus include the indirect impacts from the production of feed crop for livestock.

Sustainable agriculture aims to satisfy economic demands without detrimental environmental effects. It is based on the development of sustainable processes, the use of renewable energy sources, the reduction of chemical products and limiting the use of the landscape in order to reduce the environmental footprint of the agriculture sector. Each foodstuff is characterized by its specific environmental impact. Generally, per ton of product, animal products generally have a larger water footprint than crop products. The same is true when we look at the water footprint per calorie. When we look at the water requirements for protein, it has been found that the water footprint per gram of protein for milk, eggs and chicken meat is about 1.5 times larger than for pulses. With the exception of the butter, all other animal products have larger water footprints per gram of fat when compared to oil crops. From a freshwater resource perspective, it is more efficient to obtain calories, protein and fat through crop products than animal products (Vahnam et al., 2013). Regarding the land use and carbon footprint in general, from an analysis of life cycle assessment studies, has been concluded that food products of animal origin have higher climate- and land use related impacts than vegetable products (Nijdam et al., 2012).

For this reasons the feed industry needs to enhance the efficiency of livestock production by the reduction of GHG emissions and other environmental impacts. There should thus be more focus on limiting natural resources per amount of animal product, expressed as footprint per product such as the “water footprint”, “mineral footprint”, “land (arable or total land) footprint”. These footprints are given in kg; L or tons per unit of product and characterize the efficiency of various production processes (Flachowsky & Meyer, 2015).

Several factors can affect these emissions, such as the selection of raw material, the feed formulation, the physical characteristics of the feed and how it is processed, animal genetics, milk, meat and egg production, manure management etc. A huge amount of water is consumed by livestock production in order to grow feed crops which contributes to problems with effects beyond the increasing water scarcity. Water consumption for feed crop production reduces the amount of water available to natural ecosystems, contributing to the degradation of habitats. Nevertheless, compared to 2010, the amount of cereal production in the EU increased (Eurostat, 2017). In Europe, industrialized systems currently account for the production of 31% of poultry meat, 50% of pig meat, 17% of bovine, and 2% of sheep and goat meat (EUROSTAT, 2016) (Figure 1.1). This trend in meat production is strongly influenced by the cost and availability of the feed (Rischkowsky & Pilling, 2007).



	Bovine	Pigs	Sheep	Goats	Poultry
EU-28	7799	23440	713	45	14400

(thousand tonnes of carcass weight)

Figure 1.1. *Production of meat, by species, EU-28, 2016 (in percentage and in thousand tonnes of carcass weight). Source: Adapted from Eurostat. (http://ec.europa.eu/eurostat/statistics-explained/index.php?title=File:Production_of_meat,_by_species,_2016.png)*

Developing countries are expected to greatly increase production with a greater reliance on industrial farm animal production (Humane Society International, 2011). At the same time, there is a need to find strategies to reduce the feed production impact on the environment. Such strategies include sustainable intensification (increasing the yield on existing lands, (Tilman, Balzer, Hill, & Befort, 2011) and improving feed efficiency (Šebek & Temme, 2009). Feed efficiency is commonly measured as the feed conversion ratio (FCR), which is defined as the amount of feed used per kg of animal product (kg feed intake/kg growth). Consequently, a reduction in the FCR will improve the feed efficiency of livestock systems (Patience, Rossoni-Serão, & Gutiérrez, 2015).

However, improving feed efficiency entails a high use of human-edible products in animal diets. In fact, about one billion tons of cereals are fed to livestock annually (Eisler, et al., 2014) resulting in the competition for land and raw materials between human and animal nutrition. Thus, losses should be converted from the food industry into ingredients for the feed industry, thereby keeping food losses in the food chain, (Eisler, et al., 2014; Pinotti, et al., 2016).

In addition, due to the increasing land scarcity and lack of arable land, the industry has relied increasingly on technological advances and new alternative resources to keep up with the demand for increased livestock production. Other changes and innovations must involve the management of waste and food surpluses. Making the food chain more efficient through waste reduction will reduce the need for new resources for food production. It has been estimated that between 30% and 50% of global food products are lost or wasted before and after reaching the consumer. In 2006, the total loss of food in the EU 27 was about 90 million tons, and it is estimated that in 2020 food waste will reach 126 million tons (FAO, 2011). As a result, the European Parliament recommended that the Sustainable Development Goals should be reached by 2030, particularly Goal 12 which is aimed at a sustainable consumption and production pattern (Union Innovation, 2014). Goal 12 of ensuring sustainable consumption and production pattern (Union Innovation, 2014).

FORMER FOODSTUFF PRODUCTS TO REPLACE CONVENTIONAL CEREAL GRAINS

The decreasing availability of natural resources, together with the consequent increase in the cost of traditional feed for the livestock sector, has encouraged farmers, nutritionists, and industries to search for alternative and sustainable resources for feeding animals. There are several products that humans cannot or will not eat, but that are suitable as livestock feed, e.g. co-products, food-waste, and biomass. Feeding co-products, ex-food or food-waste to livestock or using biomass to feed livestock, referred to as 'leftover streams', could be effective options for using resources and reduce food losses from a circular economy point of view (Figure 1.2).

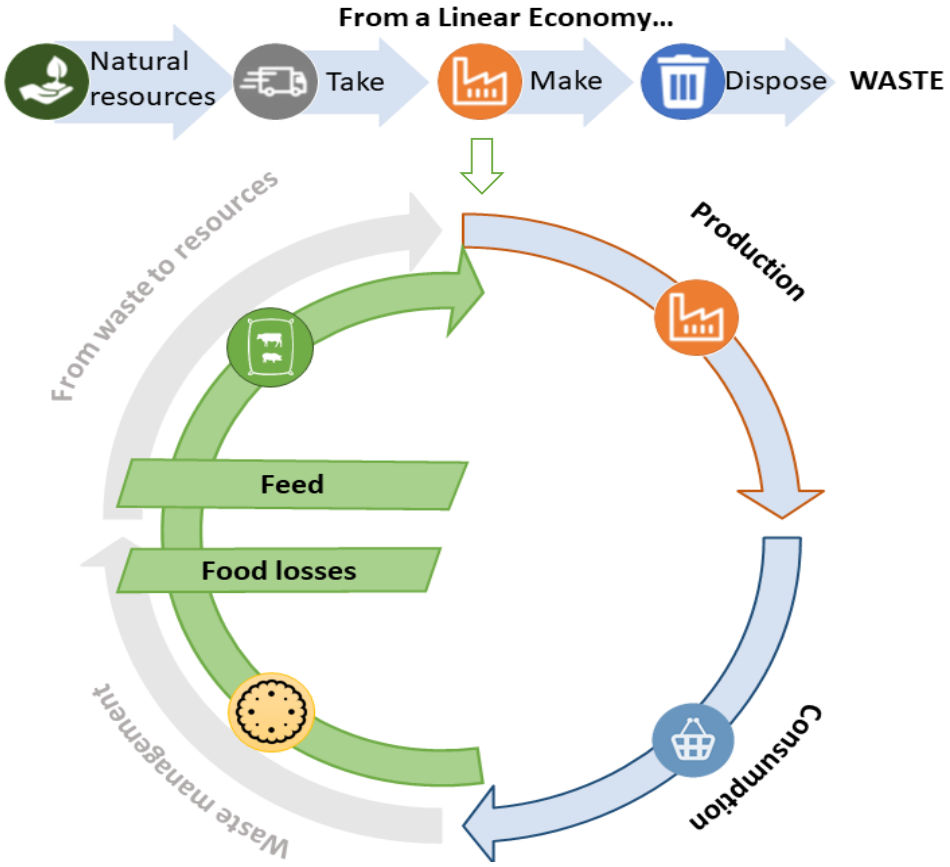


Figure 1.2. *FFPs as link leading to a circular economy from a linear economy approach.*

It has been reported (Pinotti & Dell'Orto, 2011; Pinotti, et al., 2014) that the use of a balanced by-co-product combination could partially replace traditional energy and protein sources in animal diets. This substitution can be obtained without major changes in the diet composition. In addition, the use of these alternative feed ingredients may reduce the cost of the feed, compared to the use of traditional ingredients.

Alternative ingredients for feed are expected to be increasingly used around the globe as replacers for conventional nutrient sources (Spiegel, Noordam, & Fels-Klerx, 2013; Jo & Lee, 2016). The European Commission has focused on foodstuffs that are unsuitable for human consumption as possible animal feed in order to reach the Sustainable Development Goals regarding the reduction of food waste and the environmental impact of livestock production.

These alternative feed ingredients are called Former Foodstuff Products (FFPs) and are defined by the European Commission in the Regulation (EU) No 68/2013 as "foodstuff other than catering reflux, which were manufactured in full compliance with EU food law but are no longer intended for human consumption for practical and logistical reasons or due to problems in manufacturing or packaging which are unlikely to cause any health risks when used as feed" (European Commission, 2013).

Foodstuffs that become unsuitable for human consumption for different reasons, such as production errors leading to broken or intermediate foodstuffs or surpluses caused by the logistical challenges of daily delivery, surpluses caused by discontinuation of a food product line, are all potential FFPs (Pinotti, et al., 2016). Biscuits, bread, cereals, chocolate bars, pasta, savoury snacks and sweets leftovers from the food industry are all FFPs. It is important to clarify that FFPs are not waste and are fully recognised as part of the food production chain. This is in accordance with the European Former Foodstuff Processors Association (EFFPA) and to the revised Waste Framework (DIRECTIVE (EU) 2018/851), which clearly distinguishes former foodstuff products (FFPs) from waste: FFPs are covered by feed legislation, they are placed on the market as safe feed materials and consequently they cannot be downgraded to waste (Waste Framework Directive (EU) 2018/851, Art. 2, e) (Figure 1.3).

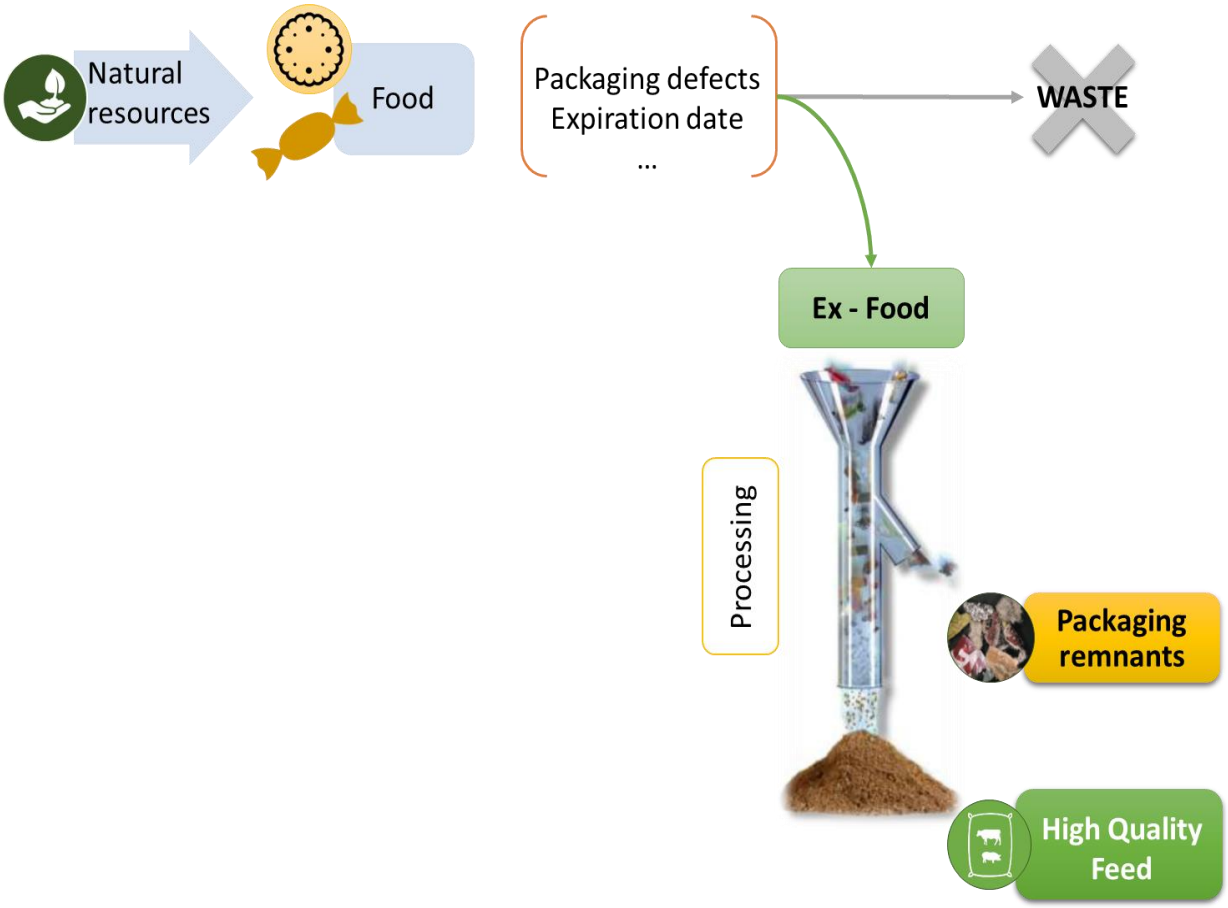


Figure 1.3. *FFP processing plant: from food losses to the final product.*

The exploiting FFPs as high quality animal feed is an active and promising area of feed research both in terms of assessing alternative feed ingredients and biomass and food waste reprocessing. FFPs are a challenging opportunity for the feed sector as they represent innovative sources of energy for feedstuffs (Herrero, et al., 2015; Giromini, et al., 2017; Tretola, et al., 2017). Therefore, FFPs represent a way of converting losses from the food industry into ingredients for the feed industry, thereby reducing overall food losses in the food chain (Featherstone et al. 2014).

Although some alternative feed (such as FFPs) already constitutes the raw material for the production of compound feed for animals, in the European Union the reprocessing of FFPs in feedstuffs is still limited compared to the total amount of food waste. In fact, only five million tonnes/year of FFPs are converted into feed ingredients, compared to 89 million tonnes/year of total food waste produced in EU (EFFPA, 2017). Currently, FFPs are mainly reprocessed for monogastric nutrition (Stenmarck, et al., 2016; Featherstone P. , 2016; EFFPA, 2017).

In terms of the target species, pigs and poultry are ideally suited to convert these food losses as well as other non-human-edible by/co-products, into high-quality animal protein food. Therefore, by/co-products and

other alternative feedstuffs such as FFPs can be included in monogastric diets to reduce/optimize the feed cost per metric ton of feed. One example is the use of candy co-products as an alternative carbohydrate source to lactose for newly weaned pigs in commercial farms (Guo, Phillips, Coffey, & Kim, 2015). Guo at al., (2015) observed that the replacement of up to 45% of lactose with candy co-products did not impair growth performance, feed intake and the feed efficiency of pigs during the nursery period. In addition, the price of candy co-products was 45% cheaper than the price of whey powder, and 68% cheaper compared to the price of whey permeate, commonly used in feed formulations (Guo, Phillips, Coffey, & Kim, 2015). However, as already reported for by-products, the inclusion of alternative feedstuffs in farm animal diets does not necessarily reduce the feed cost per kilogram of gain. Former foodstuff processors typically purchase the food losses from food manufacturing facilities, and a few years ago started to source surplus bread from the retail sector. Increased former food processing is one way of improving the sustainability of FFPs to the EU circular economy and the prevention of food waste. However, to stimulate the processing of former food into feed for food-producing animals, it is essential to guarantee the feed safety, to increase the knowledge regarding its nutritional and functional properties and the effects on growth performance and gut wellbeing. Having former foodstuffs transformed into animal feed can be part of a safe, sustainable business strategy that contributes to food waste prevention. In addition, with continued innovation in processing techniques, the expansion to other food chain sources, and with improved knowledge regarding their use as alternative feed ingredients, the sector is estimated to grow rapidly in the near future.

INNOVATIVE INGREDIENTS FOR ALTERNATIVE DIETS

FFPs can be divided into different categories, according to their origin: leftovers from the food industry mainly composed of bakery products (i.e. bread, pasta, etc.) and confectionery products (e.g. chocolates, biscuits, etc.). Rejected bread, various cookie products, high-quality baked goods and confectionary from industrial cookie bakeries are dried and sorted, unpacked, ground and sieved to create suitable ingredients, which replace some of the existing raw materials in various pig compound feed. Other dry products include sweets and dairy powders where sweets are dissolved and processed in the form of syrup. Finally, also a limited amount of other wet or moist products are used for feed purposes, especially in the case of dairy products and beverages (Figure 1.4).

Figure 1.4. *Examples of packaged and unpackaged former foodstuff products ready to be processed in FFP ingredients for feed production.*

Due to the high amount of digestible carbohydrates and their palatability, young animals, e.g. piglets and calves are the main animal targets for the use of FFPs. Sugar-rich materials (candy syrups) are also being used as the replacement for molasses, which is used as a binding agent during the pelleting of feed (Raamsdonk, et al., 2011). Based on the nutritional facts reported for humans, in general FFPs are extremely rich in carbohydrates, and depending on their origin, also in fat. As a consequence, FFP products still have a high value for feeding animals because they often contain a lot of energy. The high energy content and their digestibility, make FFPs non-traditional but promising ingredients for feeding pigs (Table 1.2).

Table 1.2. *Example of the nutritional values of FFPs (values are expressed as percentage/DM or Mj/kg). (adapted from EFFPA, 2016).*

	Dry Matter	Crude Protein	Lysine	Crude Fat	Crude Fibre	Starch	Sugar	Metabolisable Energy Pig
FFPs	88.0	10.0	0.38	14.5	2.2	41.0	14.0	16.75 MJ/kg

The composition of FFPs may vary, based on the ingredients mixed for their production. The chemical composition of bakery meal reflects the composition of the different food products that were included in the meal, and the reason for the relatively large variability in composition is that different batches of bakery meal may be produced based on different combinations of food ingredients. Due to the variability in raw ingredient inclusion in bakery meal, has been hypothesized that bakery meal collected from different geographical areas may had different nutritional values. However, has been demonstrated that differences among geographical regions in the chemical composition of bakery meals appear to be relatively small. This observation gives confidence that average values may be used to predict concentrations of nutrients in bakery meals (Liu et al., 2018). Consequently, different compositional features (i.e. content in free sugars) could be obtained in order to ensure a proper animal diet. To appreciate their nutritional implications, it is essential to estimate the composition and availability of nutrients among the different types of products. Alternative feed ingredients need to be properly balanced according to their effects on the animal performance and on the product quality intended for human consumption (Baldi & Pinotti, 2014). Based on their original composition, these products may have a high potential in terms of nutrient and energy content. However, due to the recent introduction of FFPs in animal feed, their nutritional potential has not yet been fully exploited and little is known about their chemical composition, digestibility, and energy values. The nutritional content and digestibility index should be further studied, in order to guarantee the appropriate nutrient supply and adequate availability of energy for the target species, such as pigs.

PROCESSING-RELATED PROPERTIES OF FFPS

Former foodstuffs are rich in starch, making them a valuable source of energy for pig diets. In fact, one of the main energy sources for monogastric animals is starch, and in pig diets it commonly found in a ratio of 0.40 to 0.55 (dry matter basis). There are three fractions of starch that can be differentiated based on the rapidity and magnitude of the enzymatic digestion: i) rapidly digestible starch (RDS) which rapidly increases the blood sugar levels; ii) slowly digestible starch (SDS) which causes a prolonged and slow increase in glycaemia; iii) resistant starch (RS) which is resistant to digestion by the mammalian enzymes and therefore does not produce glucose (Van Kempen et al., 2010).

Rapidly digestible starch completes the output of glucose in the upper part of gastrointestinal tract, while slowly digested starch is completed in the large intestine (Englyst et al., 1992). The feed digestibility represents the nutrient concentration lacking in the faeces in a given period of time compared to the quantity of the same nutrients ingested in the same time interval. The digestibility value is therefore extremely variable, depending not only on the feed characteristics, but also on the interaction between the feed and animal (Giuberti et al., 2014). Digestibility is strongly affected by feed dietary factors such as diet composition and feed processing. In addition, feed treatments modify the chemical-physical structure of the feed ingredients affecting their digestibility. For instance, small food particles have a greater surface in contact with digestive enzymes compared to coarser ones, leading to a greater digestion rate. However, the fast intestinal transit of small particles through the intestinal tract can negatively affect their digestibility due to a reduced contact time with digestive enzymes. As a consequence, the right balance between the particle size and their transit speed through the gastrointestinal tract is essential to obtain the highest digestibility value (Klopfenstein, 2018).

Temperature is another processing factor that influences feed digestibility, where high-temperature treatments can improve digestibility values by the protein denaturation of some anti-nutritional elements such as the anti-tryptic factor of raw soybeans (Giuberti et al., 2014). Starches consumed by livestock are usually used in untreated and/or in raw forms (Giuberti et al., 2014). However FFPs, which were originally intended for human consumption are usually subjected to mechanical/heat treatments such as heating (cooking), rolling, pelleting, flaking, extrusion, and expander processing before consumption (Singh et al., 2010). All these processing techniques lead to an alteration in the food structure and also influence the nutritional characteristics of the food, including starch digestibility. Table 1.3 presents the effects of various processing techniques on starch digestibility.

Table 1.3. *Effects of processing on starch digestibility. [a]Expressed as rapidly and slowly digestible starch (%); [b]Expressed as starch digestibility (%); [c]Expressed as hydrolysis index (%). Adapted from Singh et al. (2010).*

Processing	Starch digestibility	Reference
Baking	7.2[a]	(Roopa & Premavalli, 2008)
Frying	11.2[a]	
Toasting	31.8[a]	
Puffing	33.4[a]	
Cooking	34[a]	
Roasting	37.2[a]	
Pressure cooking	42[a]	
Sheeting of pasta (3 passes)	156[a]	Kim et al., (2008)
Sheeting of pasta (45 passes)	217[a]	
Extruded beans	306[b]	Alonso et al., (2000)
Extruded amaranth seeds	93[c]	Capriles et al., (2008)
Cooked amaranth seeds	96[c]	
Popped amaranth seeds	112[c]	
Flaked amaranth seeds	120[c]	

Extrusion cooking significantly increases the digestibility of starches (Alonso, et al., 2000; Altan et al., 2009) probably because of the loss of structural integrity of the starch granules due to the increased shearing and kneading in the extruder barrel, which ultimately increase the starch's susceptibility towards enzymatic attack. Another factor that increases the starch digestibility in foodstuffs used as feed ingredients is the decrease in the particle size of the starch granules after extrusion, resulting in an increase in the surface area and a higher percentage of hydrolysis and digestibility (Anguita et al., 2006). In addition, the processing of cereals such as dehulling, soaking and germination may result in a loss of phytic acid and tannins which are normally considered anti-nutritional factors since they inhibit the activity of a-amylase and thus decrease the starch digestibility (Rehman & Shah, 2005). The removal of tannins and phytic acid, in fact, creates a large space within the matrix, increasing the susceptibility towards enzymatic attack and consequently improves the starch digestibility (Rehman & Shah, 2005).

Other examples of the effects of processing on starch digestibility are the increased degree of starch hydrolysis for wheat, barley and oats when exposed to different technological processes (Anguita, et al., 2006) together with the increased *in vitro* digestibility of starches after extrusion cooking (Alonso et al., 2000; Altan

et al., 2009). Additionally, the *in vitro* starch digestibility can be enhanced by mechanical processes such as repeated passing of a dough through sheeting rolls that affect the protein structure (Kim, et al., 2008). As previously demonstrated (Table 3), processing treatments of FFPs can strongly affect the nutritional characteristics of these alternative feed ingredients and, subsequently, of the resulting complete feed. Starch and starchy food with different degrees of digestibility are also characterized by a different rate and duration of the glycaemic response (Englyst et al., 1999). The glucose response is strongly affected by the kind of cereal and its starch fractions. Table 1.4 compares the resistant starch content and glycaemic index for several starch-based foods.

Table 1.4. *Comparison of resistant starch content and glycemic index for some commonly-consumed starchy foods. Adapted from Birt et al., (2013).*

	Resistent Starch	Glycaemic Index
	(g/100 g)	
Grain and cereals products		
Bread (white)	1.2	69
Bread (whole meal)	1.0	72
Rice (brown)	1.7	66
Rice (white)	1.2	72
Breakfast cereals		
All-bran	0.7	51
Cornflakes	3.2	80
Shredded wheat	1.2	67
Vegetables		
Potatoes (white)	1.3	80
Sweetcorn	0.3	59

As a consequence, a diet containing a high percentage of resistant starch could be detrimental to the animal performance (Giuberti et al., 2012a), by decreasing the blood sugar and the subsequent insulin release (Giuberti et al., 2012a). It is well known that the diet energy level can affect the productive performance of the animal by modulating the dynamics of the feed intake and the accumulation of body fat (Giuberti et al., 2012a).

The glycaemic index was originally introduced in human nutrition to classify starchy foods based on their post-prandial glucose release in the bloodstream (Giuberti et al., 2012b). Menoyo et al. (2011) also introduced this concept for pig nutrition, classifying cereals according to their glycaemic index. In addition, it has been reported that cereals with a high glycaemic index lead to increased insulin production over time and a subsequent increased feed intake.

Beside the starch content, margarine, butter and partially hydrogenated vegetable oils characterize bakery products as main fat source. In this regard, has been demonstrated that bakery and pastry products are often composed by high percentage of saturated fatty acids (Albuquerque et al., 2017). However, the effect of dietary fats differing in fatty acid composition on the metabolism of saturated fatty acids (SFA) and monosaturated fatty acid (MUFA) in growing pigs has been recently investigated (Raj et al., 2017). The study demonstrated that the type of dietary fat affected neither growth performance of pigs nor the final content of whole body protein and fat and also the total accumulation of SFA and MUFA was similar. However, the increasing net intake of SFA was associated with a lower total de novo production of FA in the whole body of pigs and high SFA content and intake lowered the elongation rate of fatty acids (Raj et al., 2017).

FFPS AND SAFETY ISSUES: GUT MICROBIOTA

Since the quality of animal products is directly related to animal feeding practices, it is of paramount importance to study the quality and safety of the alternative ingredients used in animal feed because their quality and safety are strictly related to animal health and welfare and to the quality of the resulting animal-based food. This involves a functional evaluation focusing on the impact of FFPs on animal welfare in general and the gastro-intestinal tract (i.e. gut health), specifically.

An animal's gut health is essential for optimal wellbeing, production and feed efficiency. The gut of piglets, for instance, is a complex environment that changes rapidly in the weaning period with modifications in size, protein turnover rates, microbiota composition and digestive and immune functions. The complex interactions between the microbiota and gut maturation in maintaining a healthy gut environment, are influenced by the host and a wide spectrum of environmental factors; of which feeding strategies and husbandry practices are the most significant. Understanding what a healthy microbiota looks like and how FFPs can influence the composition of the gut microbial population (e.g. improving eubiosis and/or reducing disbiosis) provides fundamental information which can be used to reconvert FFPs into added value products for animal nutrition (Pluske, 2013; Lalles et al., 2007). Because the vast majority of GI bacteria cannot currently be cultured *in vitro*, culture-independent bacterial community analysis (metagenomics) and next-generation sequencing technologies have improved very rapidly and have become the most common methods for characterizing gut microbiota (Weinstock, 2012). These DNA sequencing techniques include profiling the main taxa comprising complex bacterial communities by 16S rRNA gene sequencing or whole-genome sequencing, which can also reveal functional changes within the community (Weinstock, 2012).

From birth, the pig gut is colonized by gastrointestinal microbiota which constitute a complex and dynamic ecosystem that can strongly influence animal health. The main functions of gut bacteria are to provide additional calories to the host, to form a physical barrier against pathogens, to ensure the development of the immunological system, gut morphology, digestion processes, and to modulate the host gene expression.

However, this complex ecosystem is under-exploited. Due to the different environments, pig microbiota differs quantitatively and qualitatively throughout the gastrointestinal tract (Konstantinov, et al., 2004), with the highest bacterial counts in the caecum and the colon. Bacteria start colonizing at birth, from the moment the foetal membranes are ruptured. Contact with the birth canal, faeces and skin of the mother, as well as with the environment, are all factors that mediate the first colonization of the piglet's gut (Thompson, Wang, & Holmes, 2008). However, the microbiota pattern in the piglet changes in a few days, becoming characteristic for each healthy individual (Fouhse et al., 2016). This pattern becomes more complex, increasing its bio-diversity as the animal grows (Fouhse et al., 2016). The evolution and maturation of the pig gut microbiota can be divided into four phases (Swords et al., 1993).

The first phase corresponds to the first week of life, where aerobes and facultative anaerobes from the sow and environment become the predominant bacteria groups, with an extremely fast gut colonization where bacteria reach counts of 10^9 CFU/g of colonic content in the distal colon (Thompson et al., 2008). Due to the consumption of molecular oxygen and reduction in the redox potential, the first aerobic colonizers modify the gastrointestinal environment, making it more favourable for the following colonization by anaerobes. Also the intake of immunoglobulins by colostrum exerts a fundamental function in this period, excluding antigens from entering the gut (Brandtzaeg, 2002). Consequently, 48h after birth, already 90% of aerobic and facultative anaerobic bacteria are supplanted by strict anaerobes in the piglet's gut (Figure 1.5) (Swords et al., 1993). At the end of the first week, the large intestine is dominated by *Lactobacilli* and *Streptococci* genera (Swords et al., 1993).

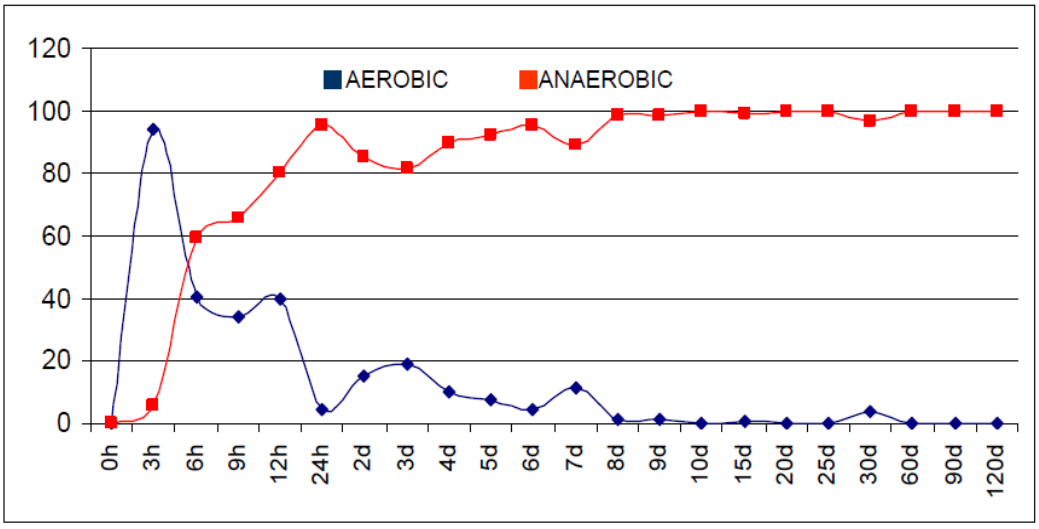


Figure 1.5. *Evolution of aerobic and anaerobic bacteria in piglet faeces from birth to 120 days of life. Adapted from Swords et al., (1993).*

The **second phase** corresponds to the end of the first week until the conclusion of suckling. In this period, the gut microbiota remains stable in terms of species composition (Heo, et al., 2013). However, anaerobic bacteria increase their biodiversity (Inoue et al., 2005) and complete the supplantation of aerobic and facultative anaerobic bacteria. Thanks to their ability to utilize substrate from the milk diet, *Lactobacilli* and *Streptococci* also dominate this second phase. In addition, *Clostridium*, *Bacteroides* and *Bidifobacteria* are usually found in this period (Thompson et al., 2008).

The adaptation to dry food represents the **third phase** which starts from weaning. Modern pig production involves this important step very early in the piglet's life, usually at three or four weeks of life. Separation from its mother and litter mates together with other environmental and nutritional changes, lead to a feed refusal and, simultaneously, to profound changes in the intestinal structure and functionality (Lalles et al., 2007) (Figure 1.6). As a consequence, during the first week post-weaning, the microbiota becomes highly unstable with a strong decrease in biodiversity, which is restored after two or three weeks (Lalles et al., 2007).

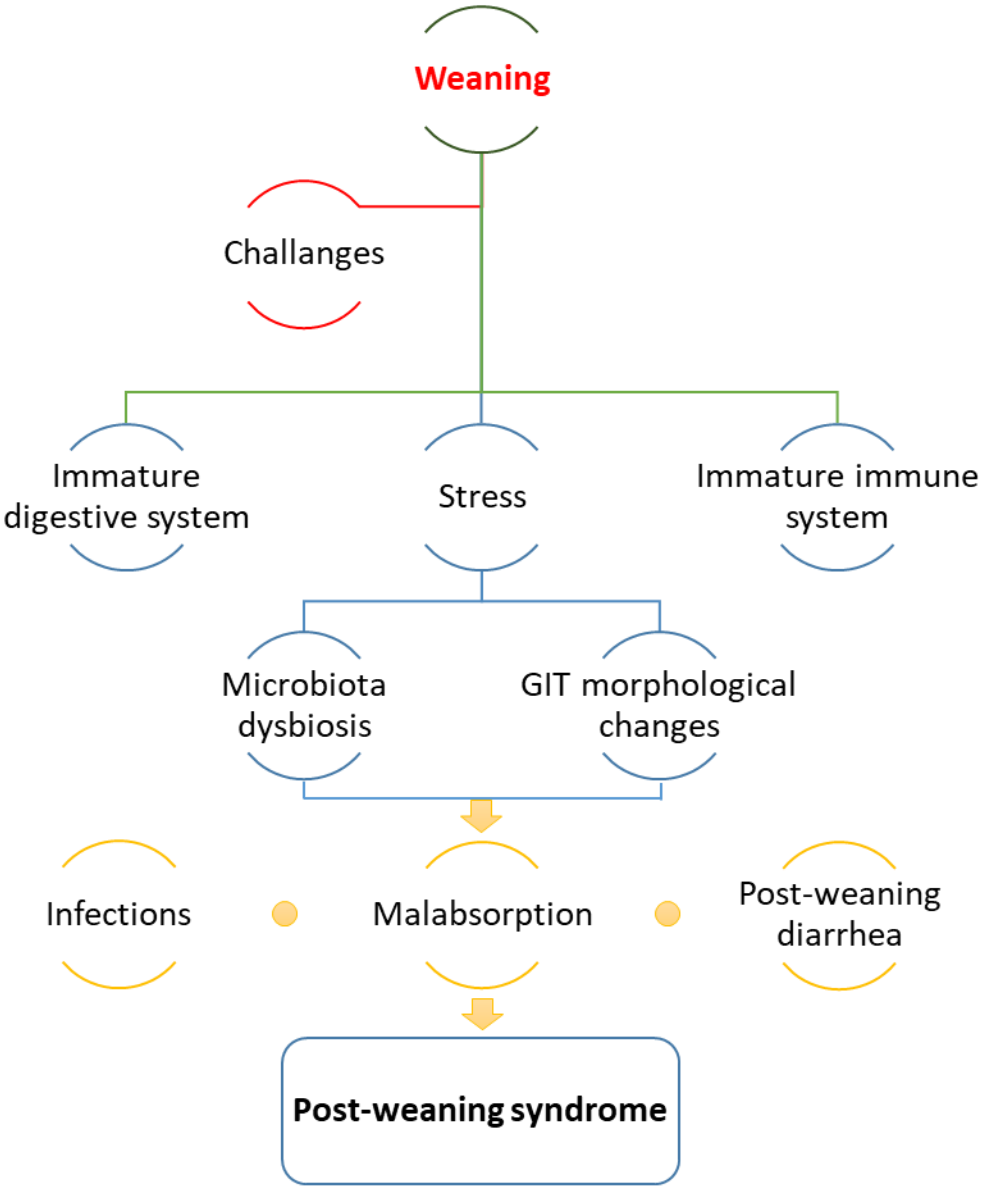


Figure 1.6. *Main challenges of post-weaning piglets.*

The introduction of a carbohydrate-rich feed, instead of lipids, is another important element that cause changes in microbiota composition (Lalles et al., 2007). This step is characterized by the displacement of gram-positive anaerobes by members of the gram-negative genus *Bacteroides*, which are one of the main bacteria populations in adult pigs (Swords et al., 1993). Moreover, *Lactobacilli* and *E. coli* show a 100-fold and 50-fold decrease after weaning, respectively. As a result of all these changes, piglets become more susceptible to the overgrowth of potentially pathogenic bacteria and to diseases (Kim et al., 2011).

In the last phase, the normal adult microbiota develops, becoming stable and characteristic for each healthy adult animal (Kim et al., 2011). In the stomach and small intestine, the number of bacteria is relatively low due to the acidic conditions and the rapid flow of digesta. However, lactic acid bacteria predominate these segments probably thanks to their ability to associate with the squamous epithelial surface of the stomach.

In the distal small intestine, the flow rate is slower, and the greater amount of digesta and pH values result in an increased density of bacteria. In this transition zone, *Lactobacillus*, *Streptococci*, *Clostridium*, *Enterobacteria*, *Bacillus* and *Bacteroides* are the most important culturable bacteria described (Hill et al., 2005). The highest biodiversity and bacteria density have been observed in the cecum and colon, where the high amount of substrate, the slow flow of the digesta, the neutral pH and the low competition with host for the nutrient absorption constitute the perfect environment for the development of a complex and stable microbiota composed of 10^{12} CFU/g of digesta (Gaskins, 2001). The microbiota plays a key role in the digestion of the dietary compounds that are not degradable by the pig endogenous enzymes, especially in the large intestine where the digesta flow rate is low. The bacterial competition for absorbable nutrients in the upper part of the gastrointestinal tract could be detrimental for the host, while the microbial digestion of non-digestible dietary residues that reach the distal gut (mainly carbohydrate polymers) is beneficial to the host, representing an additional calorie extraction from otherwise poorly utilized dietary substrates.

Carbohydrates

Carbohydrates are the main energy substrate for bacteria, as a result of the inability of mammals to produce enzymes capable of degrading dietary fibres. Dietary fibre (DF) has been defined as lignin plus those polysaccharides that cannot be digested by monogastric endogenous enzymes. Initially epidemiological studies linked a lack of DF to constipation, gut and bowel disorders, cardiovascular disease and type 2 diabetes. However, the causes of such diseases are multifactorial and in some cases it is not just DF in itself that has the beneficial effects but other aspects of the diet also (e.g. antioxidants) (McDonald et al., 2011). Nevertheless, DF is a major component related to human health, and it has equally important effects in animals (see below).

The definition of DF has proved difficult, with definitions ranging from physiological/botanical (derived from the cell walls of plants, which are poorly digested); chemical/botanical (non-starch polysaccharides (NSP) of plant cell walls); chemical (NSP and lignin); to nutritional/physiological (NSP not digested in the small intestine). The common features of DF definitions are carbohydrates (polysaccharides, oligosaccharides and lignin) resistant to digestion in the small intestine but which can be fermented in the large intestine and promote beneficial physiological effects.

Due to these varying definitions, DF is difficult to determine in the laboratory. The NSP in most foods, along with lignin, are considered to represent the major components of cell walls (McDonald et al., 2011). The digestion of these compounds totally depends on the activity of different bacteria and their enzymes such as cellulases, hemicellulases, pectinases and xylanases (Tian et al., 2017). Highly cellulolytic and hemicellulolytic bacterial species present in pig gut microbiota are *F. succinogenes*, *F. intestinalis*, *R. albus*, *R. flavefaciens*, *Butyrivibrio spp*. and *P. ruminicola* (Varel & Yen, 1997), while other carbohydrate substrates such as B-glucans and pectin are predominantly fermented by lactobacilli in the hindgut (Hill et al., 2005).

The fermentation of carbohydrates in the pig colon results in the production of high SCFA concentrations, lactic acid and gases (Nielsen, 2014). Whereas lactic acid is the main organic acid in the stomach and small intestine, SCFAs predominate in the colon and cecum (Nielsen, 2014). SCFAs have important functions in the physiology of the host: i) butyrate is the main source of energy for the colonic epithelium; i) acetate and propionate are metabolised by the liver (propionate) or by peripheral tissues and play a key role in modulating glucose and cholesterol metabolism (Wong et al., 2006). In addition, SCFAs–producing bacteria seem to influence the cycle of enterocytes in the colon with the production of butyrate which inhibits epithelial cell proliferation and stimulates their differentiation (Gibson et al., 1992). Beside these functions, SCFAs stimulate the reabsorption of water and sodium thus limiting diarrhoea and inhibiting the growth of some opportunistic pathogens such as *Salmonella*, *C. difficile* and E. *coli* (Nielsen, 2014).

The use of FFPs for ruminants' nutrition could represent a risk factor: because of the high starch and sugar content that characterize FFPs commonly used in pig or poultry diets, they cannot be included in high percentage in ruminant ones. Diets very rich in starch and simple sugars, in fact, can increase the growth rates of all bacteria in the rumen, resulting in an increase in total volatile fatty acid production, and a decrease in ruminal pH. Specifically, when large amounts of starch are added to the diet, the growth of *Streptococcus bovis* is no longer restricted by a lack of this energy source and this population grows faster than other species of rumen bacteria. S. bovis produces lactic acid, which eventually exceed the buffering capacity of rumen fluid. Moreover, simple sugars such as glucose, in which FFPs are rich, are converted to fructose 1,6-diphosphate able to activate the lactate dehydrogenase and, consequently, increasing the lactate production (Lean et al., 2007) . For this reason, FFPs have to be specifically formulated for ruminants, have to be used in low percentage in diet formulations with minor impact on profitability and sustainability. For all these reasons, the main targets in the use of FFPs as alternative feed ingredients for animal nutrition are pigs and poultries.

Protein utilization

Microbiota can use nitrogen from dietary nitrogenous compounds as well as enzymatic secretions of the host, mucin, and sloughed epithelial cells (Pieper, et al., 2016). Bacteria also have the ability to utilize N not only in the form of protein but also from other organic or inorganic sources. In particular, urea from plasma can be efficiently utilized by bacteria for the synthesis of their own proteins (Pieper, et al., 2016). Although degradation of protein by bacteria in the small intestine seems to be scarce, in the large intestine proteolytic fermentation is very important. In this portion of the gastrointestinal tract, numerous bacterial species use peptides and amino acids as a source of carbon, nitrogen and energy. As a result, branched-chain volatile fatty acids (VFAs) are formed by the use of branched-chain amino acids valine, leucine and isoleucine (Lalles, Bosi, Smidt, & Stokes, 2007). However, the proteolytic fermentation can also lead to the formation of potentially toxic metabolites such as NH3, amines, phenols and indoles (Pieper, et al., 2016). The genera

Bacteroides, Clostridium, Enterobacterium, Lactobacillus and *Streptococcus* have the ability to produce amines by the decarboxylation of amino acids (Mac Farlane, 1995). A wide range of intestinal bacteria have urease activity of which an excess can lead to an increase in ammonia, with related impaired mucosa development (Pieper, et al., 2016).

Lipids

Intestinal microbiota also play an important role in the digestion and metabolism of lipids. The metabolism of bile acids is crucial. The microbial deconjugation and dehydroxylation of bile acid, in fact, impair lipid absorption by the host and produce toxic degradation products (Wahlström et al., 2016). Among the bacteria that possess the activity to dehydroxilate bile acids*, E. coli, B. cereus, S. faecalis, Bacteroides spp, Eubacterium spp* and *Clostridium spp* are the most abundant (Wahlström et al., 2016). Several bacteria, such as *Clostridium spp., E. lentum, Peptostreptococcus spp.,* and *Ruminococcus spp.,* also have different dehydrogenases capable of bile acid transformation. The toxicity of some compounds released by the metabolism of bile acid may rely on the growth inhibition of competing bacteria (Wahlström et al, 2016). The deconjugation of bile acids can negatively affect the digestion of dietary fatty acids as they act as emulsifiers, facilitating their absorption. The digestion of lipids in gnotobiotic rats is higher than in normally reared animals (Fuller & Reed, 1998).

In addition, gut microbiota increases the bio-hydrogenation of unsaturated fatty acids, resulting in a relatively high proportion of stearic acid which is less well absorbed (Yen et al., 1991). Cholesterol, dietary sterols, and other lipids are also altered by microbiota in the large intestine (Wahlström et al., 2016). Cholesterol, for example, is reduced to coprostanol and coprostanone by different bacteria belonging to the *Eubacterium* genus such as *Bacteroides, Bifidobacterium* and *Clostriudium* (Ridlon et al., 2016).

The composition of FFPs, i.e. rich sources of simple and cooked carbohydrates, thus clearly has its own set of advantages and disadvantages. As described above, the processing techniques to which FFPs are exposed, can improve starch digestibility and reduce the amount of resistant starch content. However, the ability of resistant starch to modulate colonic pH, SCFA composition, and enzymatic activity associated with bacterial degradative pathways, and the abundance of several bacterial taxa have been demonstrated in both human and animal models (Louis et al., 2007; Phillips et al., 1995; Tomlin & Read, 1990). Furthermore, the production of SCFAs, together with the lower pH in the colon, can exert different protective roles such as preventing the overgrowth of pathogenic bacteria (Roy et al., 2006), protection against mucosal oxidative stress, strengthening of the colonic defence barrier, and also an anti-inflammatory action (Hamer et al., 2008). As a result, a reduction in the amount of slow digestible starch or resistant starch, may lead to a negative alteration in the gut microbial composition and subsequently, in the pig gut health. Consequently, gut health is an essential requirement to ensure feed digestibility, nutrient bioavailability and to achieve optimal growth and production rate. These features imply a rigorous nutritional evaluation in order to increase the potential of FFPs in animal feeding and nutrition.

FFPs, MICROBIOLOGICAL LOAD AND PRESUMED PACKAGING REMNANTS CONTAMINATION

The quality, traceability and safety of FFPs are key in guaranteeing the safe re-use of biomass, according to a biosecurity approach. One such approach involves a systematic assessment of these former foodstuffs, as is done with products already used in animal feed. The safety and sustainability of the process are key to such assessments.

In terms of safety, one of the major concerns in terms of feed ingredients is their microbiological quality. Animal feed, due to its composition, provides a favourable environment for the growth of microorganisms. Salmonella is one of the most frequent foodborne diseases, with poultry and pork being a potential source for humans in many countries (Mainar-Jaime et al., 2018). Live animals can be infected sub-clinically, leading to mild diarrhea and depressed growth performance in the animal, whereas acute infection can bring about septicemia and death in humans (Mainar-Jaime et al., 2018).

The most prevalent form of salmonellosis is sub-clinical infection. Current regulations in many countries have a very strict approach to salmonella tolerance in human foods. Nevertheless, in practical terms, it is unrealistic to expect the complete elimination of *Salmonella* from animal feed, especially at the point when the animal actually consumes the feed. Animal feed ingredients, particularly animal and plant-derived protein meals, are frequently contaminated with Salmonella either from the source or from the processing plant, and recontamination in compounding mills is an additional problem (Yang et al., 2017).

Several complementary strategies have been used to control this feed contamination, which include a range of chemical treatments. Fortunately, these treatments in combination with the current technology can reduce Salmonella contamination to insignificant levels. Moisture, temperature, type of feed, chemical and physical properties of the raw material, pH, storage conditions and many more are factors that can modulate bacterial growth and proliferation (Andreoletti et al., 2008). Current regulations in many countries have a very strict approach to Salmonella tolerance in human foods.

However, feed contamination can occur during processing, transport or storage. The process of removing the food packaging also can be a source of contamination. For all these reasons, the verification of the microbiological quality of these materials is always recommended when they are used as animal feed. A further aspect that has been reported in terms of safety are the packaging material remnants. Processing methods to convert FFPs into feed ingredients do not usually include the pre-removal of packaging materials. Feed processors thus routinely remove the packaging from FFPs mechanically in the feed plant. The typical un-packaging process of FFPs can be summarized as follows: 1) the packaging is broken and reduced in size, 2) the now accessible FFPs are processed to a ready product and 3) the remains of the packaging materials

are finally removed by several procedures such as sieving, magnetic attraction, eddy current separation or based on density (Raamsdonk et al., 2011) (Figure 1.7).

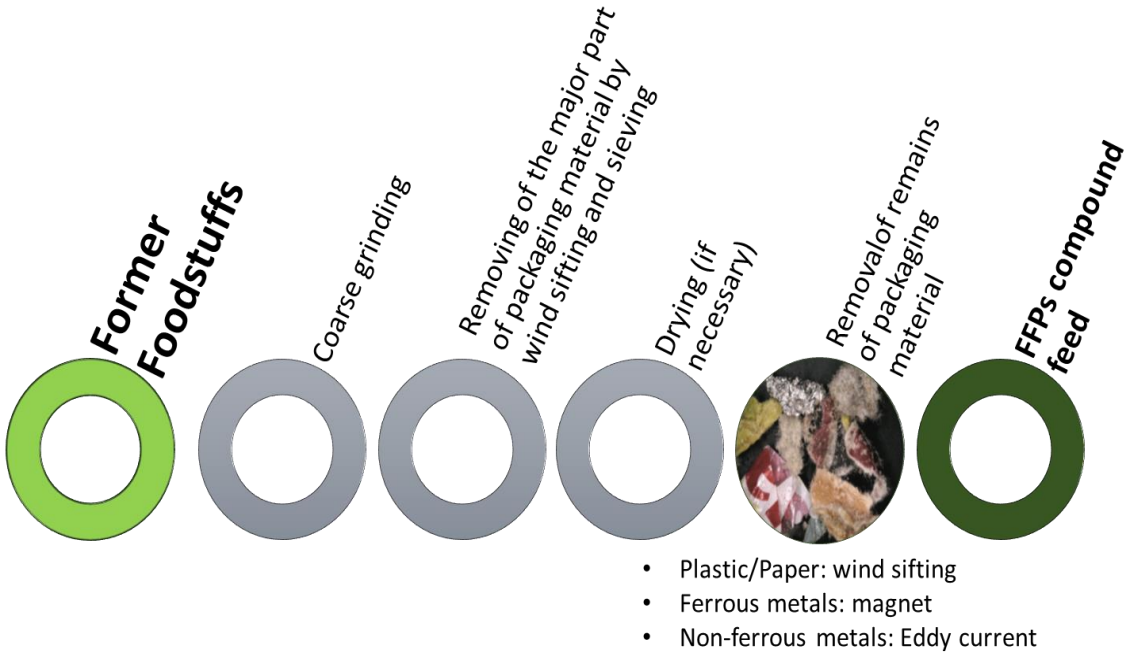


Figure 1.7. *Summary of the mechanical un-packaging process of FFPs.*

Despite these processes, some packaging residue can remain in the final product. The most common packaging materials for food products are plastics, paper, board and aluminium foil. In addition, packaging materials are often manufactured using adhesives with printing on the outside. Plastics are made by the polymerisation of monomers and several additives may be added to obtain physical or chemical properties of the plastics, such as fillers, polymeric additives, light stabilizers, optical brighteners, antistatics etc.

Paper and board are the second most frequently used packaging material after plastics. They are often used in direct contact with dry foodstuffs and, thus need to be treated with different substances and chemicals. Aluminium is used as trays for the packaging of some types of bread and, in the form of thin foil, such as candy bar wrapping. Additionally, aluminium coated paper is frequently used to package chocolate bars and some sweets. Even after processing and cleaning, this type of coated paper can be identified as such in the re-processed FFPs. Although to a lesser extent, metal wires and closing clips are used as packaging materials for several food products. Unlike aluminium foils, ferrous metals can be easily separated from FFPs using magnets. However, some ferro-metal remnants can be found in the final product. The printing inks that cover many food packaging materials are pigments or colorants and cannot be separated from plastics, paper/board and thin aluminium foil.

Packaging materials are not accepted as a feed ingredient according to (Regulation (EC) No 767/2009), which prohibits the use of feedstuffs containing packaging materials from the agri-food industry. The packaging used for food is characterized by a large range of materials, often with complex compositions. The (Regulation (EC) No. 1935/2004) covers the general requirements for all types of packaging materials. It requires that packaging materials should not release their constituents at a level that could endanger human health. Specific EU directives have been published which regulate in detail the composition of plastics and regenerated cellulose. Other packaging materials (i.e. paper, coatings or aluminium foil) are regulated in detail at a national level. Consequently, appropriate methods are needed for detecting possible packaging contaminants and their remnants in FFPs used in the feed chain.

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Chapter 2

NUTRITIONAL EVALUATION OF FORMER FOOD PRODUCTS (EX-FOOD) INTENDED FOR PIG NUTRITION

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NUTRITIONAL EVALUATION OF FORMER FOOD PRODUCTS (EX-FOOD) INTENDED FOR PIG NUTRITION

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ABSTRACT

Ex-food or former food products (FFPs) have been proposed as one of the categories with great promise as alternative feed ingredients. FFPs' nutritional potential is not yet fully exploited. Therefore, the aim of this study was to perform a nutritional evaluation of selected FFPs. In particular, six samples of mixed FFPs, all based on bakery products, were analysed for moisture, crude protein, ether extract, crude fibre, neutral detergent fibre, acid detergent fibre, starch and ash. Nitrogen-free extractives and non-structural carbohydrate were also determined. Based on FFPs' composition data, estimation of digestible energy and metabolisable energy values for pigs were calculated. Further, the *in vitro* digestibility values of FFPs were investigated using a multi-step enzymatic technique. A wheat sample was included as a control feed ingredient in the study. All data were reported on dry matter basis. FFPs have shown a nutrient composition comparable with cereal grains. In the tested FFPs, the average protein content was 10.0% and the average starch content was 52.4%. Nitrogen-free extractive ranged from 61.2% to 74.7%, whereas non-structural carbohydrate ranged from 58.5% to 79.3%. Compared with wheat, FFPs were characterised by a relative high fat content, averaging about 10.1%. The relatively high nitrogen-free extractive/non-structural carbohydrate/starch and fat concentration designated FFPs as valuable energy sources. Digestible energy and metabolisable energy averages were 17.2 and 16.9 MJ kg–1, respectively. The average *in vitro* digestibility value of FFPs samples was 88.2% ± 5.8%, comparable with that of wheat (90.6% ± 1.6%). FFPs are a fat-fortified version of common cereals grains. The high energy content and digestibility values elect FFPs as promising non-traditional ingredients for swine.

INTRODUCTION

The use of alternative feed ingredients in farm animals' diets represents an interesting opportunity from numerous standpoints. In the last decade, industrial by-products, co-products, insect materials and seaweed ingredients have been proposed as alternative feed ingredients (Pinotti, et al., 2014; Spranghers, et al., 2017; Woyengo, Jha, Beltranena, & Zijlstra, 2016). Among these, ex-food or former food products (FFPs) have been proposed as one of the category with great potential as alternative ingredients for the animal diet. The valorisation of ex-food, processing by-products and food waste represents a challenging opportunity for the sustainable and competitive development of industrial food and feed sectors. According to the European Catalogue of Feed

Materials, 'ex-food' or 'FFPs' means foodstuffs, other than catering reflux, that were manufactured in full compliance with European food law but which are no longer intended for human consumption for practical or logistical reasons or due to problems in manufacturing or packaging, which are unlikely to cause any health risks when FFPs are used as feed (European Commission, 2013). Therefore, FFPs represent a way of converting losses from the food industry into ingredients for the feed industry, thereby reducing overall food losses in the food chain (Featherstone P. , 2014). The circular economy package has pointed out that operations behind the FFPs' production process have nothing to do with waste processing or food waste recycling. Typical former foodstuffs are represented by leftovers of the food industry such as biscuits, bread, breakfast cereals, chocolate bars, pasta, savoury snacks and sweets. Thus, they can represent a cheap and abundant source of nutrients of high interest for the feed sector. In the European Union, a part of FFPs is already reprocessed for animal nutrition, but their use as feedstuffs remains limited (3.5 million tonnes/year) (Featherstone P. , 2016) compared with the total food waste (88 million tonnes/year) (Stenmarck, et al., 2016). It has been previously reported (Pinotti & Dell 'Orto, 2011; Pinotti, et al., 2014) that when using a balanced by-products combination it is possible to substitute the traditional energy and protein sources in farm animals' nutrition. This replacement, when supported by a robust nutritional evaluation, can be obtained without major changes in the diet composition, reducing feed cost and expanding the raw material portfolio. In light of this, FFPs may represent valuable alternative ingredients for swine nutrition, in line with the circular economy concept. These products, according to their original composition, can maintain a high nutritional potential in terms of nutrient content and energy values for feeding animals. However, to the best of our knowledge, FFPs' nutritional potential is not yet fully exploited. In particular, limited information are available on FFPs' chemical composition, digestibility and energy values (Bouxin, 2016). Nutrient content and digestibility features should be further evaluated in order to guarantee an accurate supply of nutrients and, in addition, an adequate supply of energy for target species as swine. In light of that, the aim of this study was to perform a nutritional evaluation of selected FFPs. In particular, FFPs' chemical composition, digestible (DE) and metabolisable energy (ME) and *in vitro* digestibility (IVD) were investigated.

MATERIALS AND METHODS

Sample collection

In this study, six FFPs samples, collected in the frame of the IZS PLV 06/14 RC project funded by the Italian Ministry of Health, were analysed. FFPs were produced starting from different ex-food materials (Table 2.1), in an ex-food processing plant based in the north of Italy. In all the experiments performed, a flaked wheat sample was included as a control feed ingredient. Flaked wheat was selected as an experimental control because FFPs considered in this study were obtained from ex-food materials in which starch was previously exposed to food-technology processes (e.g., heat treatments, steam flaking, pelleting). All samples were milled through a 1-mm screen (Model 160-D, Jacobsen Machine Works, Minneapolis, MN, USA) and stored at 4°C until analysis.

Table 2.1. *Former food products (FFPs) ingredients.* [a]*Ingredients are listed in descending order of inclusion.*

Sample[a]	Ingredients
FFP1	Leftover of the food industry (confectionery products, bakery products (cookies, biscuits), products of pastry); wheat by-products (e.g., bran); wheat flour
FFP2	Leftover of the food industry (bakery products, pasta, of pastry products industry, confectionery products); wheat by-products (e.g., bran); wheat flour.
FFP3	Leftover of the food industry (bakery products, pasta, pastry products industry, confectionery products); wheat by-products (e.g., bran); wheat flour
FFP4	Extruded and puffed rice cakes and corn extruded
FFP5	Leftover of the food industry (pasta, bakery products, confectionery products, products of pastry products industry)
FFP6	Bakery products (e.g., biscuits)

Chemicals

All chemicals were purchased from Sigma-Aldrich (Milan, Italy), unless otherwise indicated.

Nutrient composition

All samples were analysed for moisture, crude protein (CP), ether extract (EE), neutral detergent fibre (NDF), acid detergent fibre (ADF), starch and ash as per the Association of Official Analytical Chemists (AOAC) (2005) and the European Commission (2009) regulation. Specifically, the moisture of samples was determined by an oven-drying method (130°C for 2 h) (Commission Regulation (EC) No 152/2009, 2009). All data were presented on a dry matter (DM) basis. CP content was measured according to the Kjeldahl method (proc.

2001.11; AOAC 2005). EE was determined by the Soxhlet method, with prior hydrolysis, as proposed by the European Commission Regulation No. 152/2009. NDF and ADF analyses were performed according to procedures of the AOAC (2005) (methods 2002.04 and 973.18, respectively), using an Ankom 220 fibre analyser (ANKOM™ Technology, Fairport, NY, USA); NDF and ADF were measured using heat-stable amylase and expressed exclusive of residual ash (aNDFom, ADFom). Starch content was determined using a polarimetric method (Commission Regulation No. 152/2009). Ash was measured by using a muffle furnace at 550°C (proc. 942.05; AOAC 2005). Nitrogen-free extractives (NFE) and non-structural carbohydrate (NSC) were obtained by difference, using the following equations:

NFE (%DM) = 100 – (moisture% + ash% + CP% + EE% + CF%)

NSC (%DM) = 100 – (NDF% + CP% + EE% + ash%)

Energy values

Based on FFPs' composition data, the estimation of DE and ME values for pigs was calculated using the following equations:

$$DE\ (Mj\ Kg\text{-}1) = (53.7 * \%CP) + (75.8 * \%EE) + (41.1 * \%\ starch) + (7.6 * \%NDF) + (39.0 * \%\ residue)$$

$$Residue = OM - CP - EE - starch - NDF$$

$$ME\ (Mj\ kg\text{-}1) = DE - (6.8 * \%CP)$$

The DE equation was originally elaborated by Sauvant et al. (2004), while ME was proposed by Noblet and Perez (1993) and further adapted by NRC (2012). Energy and chemical constituents are expressed on a DM basis in all equations.

In vitro digestibility (IVD)

The IVD was serially performed on each FFP sample three times (three biological replicates); in each digestion series, FFPs, control and blank (digestion enzymes alone) samples were included in triplicate (three technical replicates). The IVD of FFPs was realised according to the protocol described by Boisen and Fernandez (1997) and Regmi et al. (2009) with minor adaptations. Briefly, a sample (0.5 ± 0.1 g) was mixed with 0.1 M phosphate buffer (pH 6.0). Subsequently the pH of the solution was lowered with 10 ml of 0.2 M hydrochloric acid solution and adjusted at pH 2.0. A total of 1 ml of freshly prepared pepsin (P7000 Sigma-Aldrich) solution (25 mg ml–1) was added. In order to minimise bacterial fermentations during digestion, 0.5 ml chloramphenicol solution (5 mg ml–1 ethanol) were added to the mixture. The bottle was placed in a shaking water bath at 39°C for 2 h (simulation of the gastric phase). Subsequently, 10 ml of 0.2 M phosphate buffer at pH 6.8 were added, followed by 5 ml of 0.6 M NaOH and the pH was adjusted at 6.8 with 1 M hydrochloric acid or 1 M NaOH. Further, 3 ml of freshly prepared pancreatin (P3292; Sigma-Aldrich) solution (100 mg/3 ml) were added to the mixture. The bottle was placed in a shaking water bath at 39 C for 4 h (simulation of the small intestinal phase). At the end of the second incubation, 10 ml of 0.2 M EDTA were added to the mixture and the pH was lowered to 4.8 with a 30% acetic acid solution. Further, 0.5 ml of Viscozyme (V2010;

Sigma-Aldrich) were added to each bottle and incubated in a shaking water bath at 39 C for 18 h (simulation of the fermentation process). In order to allow undigested soluble proteins precipitation 5 ml of 20% sulfosalicylic acid were added and the bottle was kept at RT for 30 min. The undigested fraction (UF) was then collected in a filtration unit using a porcelain filtration funnel lined with pre-weighed filter paper (Whatman no. 54; Whatman Inc., Florham Park, NJ, USA). The UF, along with the filter paper, was dried overnight at 65°C. *In vitro* DM digestibility (%) was calculated as follows:

$$\text{IVD (\% DM)} = \frac{(\text{sample DM} - \text{sample UF DM})}{sample\ DM} * 100$$

Statistical analysis

Data were analysed using IBM SPSS Statistics version 21 software (SPSS). IVD values for FFPs samples were analysed using one-way analysis of variance (ANOVA) in order to compare means. The analysis was performed using the following model: $y_{ij} = \mu_j + \varepsilon_{ij}$

where y_{ij} is the observations (values); μ_j is the mean of the observations for the j-th group (sample); and ε_{ij} represents the within-sample random variability. Differences with p-values < 0.05 were considered significant. In order to compare the nutrient composition of FFPs and evaluate the overlap of the six withinsample distributions, box plots of these distributions were examined. Accordingly, the box plot analysis was performed in order to calculate the mean, quartiles, minimum and maximum observations and outliers for each single sample. Correlation studies were performed by Pearson's correlation method to study the association between the IVD and nutrient composition estimated DE and ME values. In this case, FFP samples altogether were considered the experimental unit for Pearson's correlation analyses. Pearson's correlation, r, was considered to determine the degree to which a relationship is linear and values < 0.05 were considered significant.

RESULTS

The nutrient compositions of FFPs and control are presented in Table 2.2.

Table 2.2. *Former food products (FFPs) nutrient composition (% DM basis).*

	Moisture	CP	EE	CF	NDF	ADF	Starch	Ash	NFE	NSC
FFP1	7.4	9.2	11.7	2.7	10.1	2.7	45.9	2.2	66.7	66.7
FFP2	8.7	11.5	11.0	4.4	15.7	5.0	42.3	3.2	61.2	586
FFP3	8.5	12.0	9.9	4.5	16.2	5.1	44.6	3.4	61.7	58.5
FFP4	8.1	9.1	4.8	1.9	5.4	2.0	73.4	1.4	74.7	79.3
FFP5	8.7	10.9	10.4	1.5	7.7	1.3	56.4	2.1	66.4	68.8
FFP6	6.2	7.3	12.9	1.3	5.6	1.2	51.5	1.8	70.4	72.4
Wheat	10.9	10.1	1.5	2.7	12.6	4.4	63.0	1.4	73.3	74.4

In addition, a representation of the full range of nutrient variation of all FFP samples is shown in Figure 2.1.

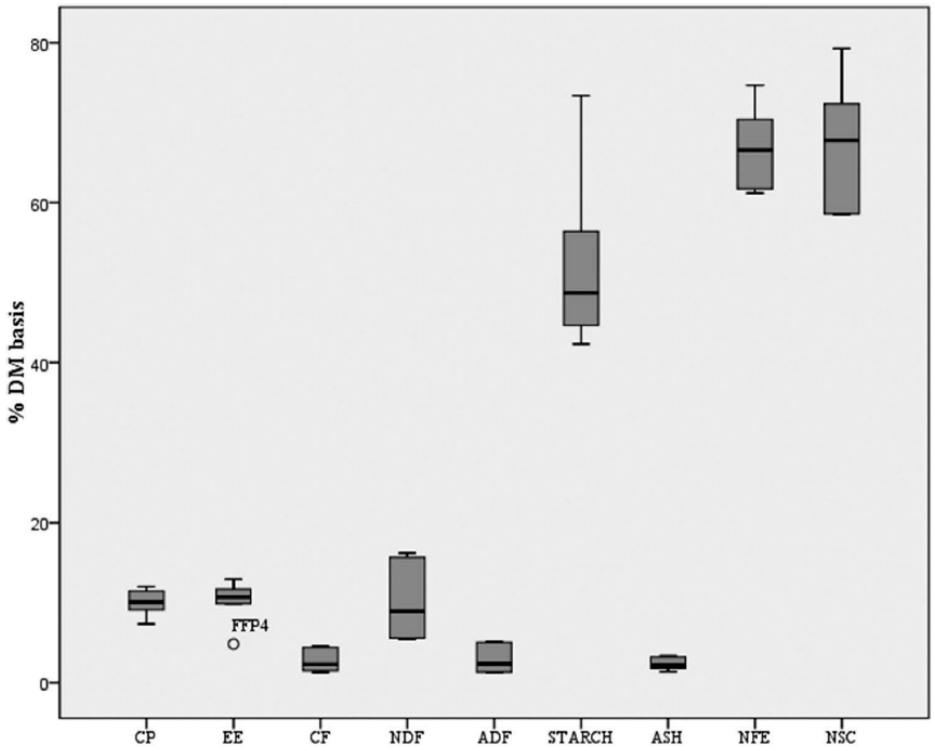

Figure 2.1. *Former foodstuff products (FFPs) nutrient composition (box plot analysis, IBM SPSS Statistics version 21 sof2tware; SPSS Inc.).*

FFPs' DM concentration ranged from 91.3% DM in FFP2 to 93.8% DM in FFP6. The overall mean of protein content in FFPs was about 10.0%, comparable with that of wheat (10.1%). All FFPs had a high fat content,

with an average of 10.1%. Except for FFP4, all the other samples exhibited a fat content six times higher when compared with wheat (1.5%). FFP1, FFP2, FFP3 and wheat were characterised by a fibre content (CF, NDF and ADF) higher than FFP4, FFP5 and FFP6 (Table 2). In particular, NDF content was lower in FFP4, FFP5 and FFP6 (ranging from 5.4% to 7.7%) compared with FFP1, FFP2 and FFP3 (ranging from 10.1% to 16.2%) (Table 2). This feature was also evident considering ADF content, which was lower in FFP4, FFP5 and FFP6 than in samples FFP2, FFP3 and wheat and, to a lesser extent, FFP1. The starch content in FFPs was close to that of the control sample, averaging about 52.4%, with sample FFP4 reaching 73.4%. In FFPs, NFE ranged from 61.2% to 74.7%, whereas NSC ranged from 58.5% to 79.3% (Table 2). FFPs were characterised by an average content of crude ash of 2.3%, while wheat control contained 1.4% of crude ash. The energy values obtained for FFPs ranged from 16.2 to 18.1 MJ kg–1 for DE and from 15.9 to 17.9 MJ kg–1 for ME (Table 2.3); they were comparable with wheat control.

Table 2.3. *Energy values calculated for former food products (FFPs) and control (g kg-1 DM basis).*

	DE (MJ)	**ME (MJ)**
FFP1	17.3	17.1
FFP2	16.5	16.1
FFP3	16.2	15.9
FFP4	17.3	17.0
FFP5	17.7	17.4
FFP6	18.1	17.9
Wheat	15.8	15.5

The greatest energy values were reported in sample FFP6, whereas the lowest values were reported in sample FFP3. IVD values calculated for FFPs and control wheat are shown in Figure 2.2.

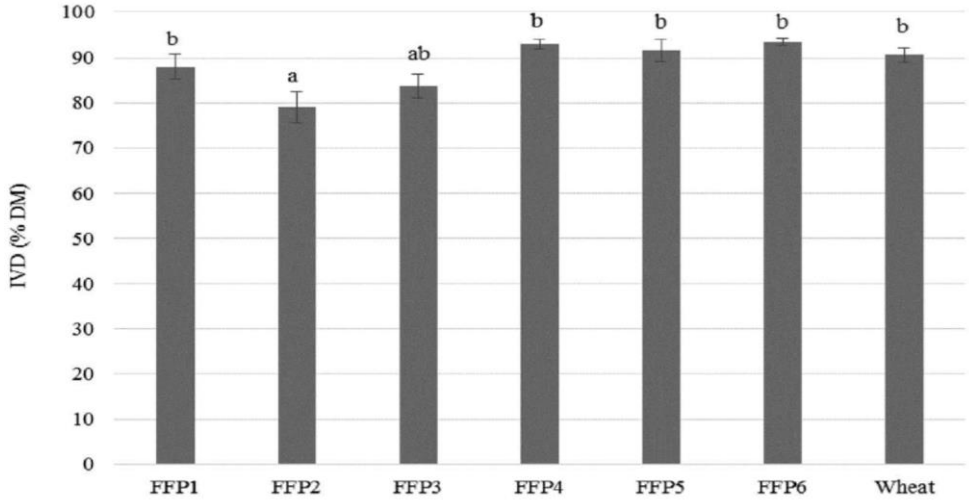

Figure 2.2. *In vitro digestibility (IVD, % DM) of former food products (FFPs) samples. Results are from three independent experiments and presented as least square means ± SEM. Samples identified with different letters are significantly different (P < 0.05).*

The IVD values obtained in FFPs (average of 88.2% ± 5.8%) were largely comparable with that of wheat (90.6% ± 1.6%). The IVD values of FFPs samples were ranked as follows: FFP6 > FFP4 > FFP5 > FFP1 > FFP3 > FFP2 (Figure 2.2). In FFP2 the IVD value was significantly ($p < 0.05$) lower than IVD values of FFP6, FFP4, FFP5, FFP1 and control wheat. Table 2.4 reports correlation coefficients (r) of IVD with nutrients composition and energy values (DE, ME).

Table 2.4. *Correlation coefficients (r) of nutrients composition, energy values with in vitro digestibility (IVD).*

	Mean	SD	CV	r	P values
CP	10.0	1.8	17.7	-0.737	N.S.
EE	10.1	2.8	27.7	-0.210	N.S.
CF	2.7	1.4	53.1	-0.948**	0.004
NDF	10.1	4.8	47.8	-0.955**	0.003
ADF	2.9	1.8	61.0	-0.941**	0.005
Starch	52.4	11.5	22.0	0.720	N.S.
Ash	2.3	0.8	33.4	-0.923**	0.009
NFE	66.9	5.2	7.7	0.875*	0.022
NSC	67.4	8.0	11.9	0.899*	0.015
DE (MJ)	17.2	0.7	4.2	0.865*	0.026
ME (MJ)	17.0	0.8	4.5	0.866	0.026
IVD	88.2	5.8	6.5	-	-

**P <0.05; ** P < 0.01; N.S. P > 0.05*

An inverse relationship ($p < 0.01$) between fibre fractions (CF, NDF and ADF) and ash content versus IVD can been observed. Whereas Pearson correlation coefficients were positive between IVD and DE, ME ($r = 0.865$, $p < 0.05$ and $r = 0.866$, $p < 0.05$, respectively). A positive correlation was also observed for IVD and NFE and NSC ($r = 0.875$, $p < 0.05$ and $r = 0.899$, $p < 0.05$, respectively).

DISCUSSION

Nutrient composition

FFPs have shown a nutrient composition comparable with cereal grains. Nevertheless, compared with wheat control, FFPs were characterised by a relative high energy content. The chemical composition of wheat control was in line with values reported in the literature (Regmi, Ferguson, & Zijlstra, 2009; McDonald, et al., 2012; Sumczynski, Bubelova, Sneyd, Erb-Weber, & Mlcek, 2015) The CP values calculated in this study showed that FFPs cannot be considered a valuable protein source, not only for the low nitrogen concentration observed (less than 10%), but also, coming mainly from cereals based products, for the expected protein quality. FFPs' fat content was generally higher than fat quantity reported in the literature for cereal grains and for the other protein cakes commonly used in swine compound feed formulations (e.g., corn and soya bean meal) (McDonald, et al., 2012). Thus, data reported in this study suggest that FFPs can be considered a fat-fortified version of common cereals used as energy ingredients in swine nutrition. Lipids have nutritive and physiological importance in energy nutrition due to their energy supply potential. From a biochemical point of view, lipids contain carbon and hydrogen in a more reduced state compared with other nutrients (carbohydrates and proteins), and, therefore, these elements can potentially be much more oxidised, with a greater energy yield (Maynard & Loosli, 1969; Pettigrew, Moser, Miller, Ullrey, & Lewis, 1991). For this reason, the supplementation of fat in swine nutrition is a common practise. However, given the proven difficulties in handling fats (Pettigrew & Moser 1991) and in mixing them with other ingredients for the formulation of complete feeds, FFPs can represent a valuable novel opportunity in compound feed production. This aspect, however, needs to be addressed by specific studies in feed technology. Based on fibre values, two groups of samples could be identified: FFP1, FFP2 and FFP3, characterised by a fibre content comparable with other cereal grains commonly used in animal nutrition (McDonald et al. 2012), and a second group including FFP4, FFP5 and FFP6, characterised by a limited fibre content. It is important to note that the analytical methods used to characterise 'fibre' often overlap or may exclude portions of other carbohydrate fractions in a feedstuff and, consequently, our ability to relate adequately analytical measures to fibre utilisation is problematic (Kerr & Shurson, 2013). In this respect, the present study indicated that when FFPs were considered, NDF and ADF fractions can be more effective in describing fibre content than CF alone. This information can be useful for nutritionists who can be aware about the nutritional effect/potential of these ingredients. The pig's ability to digest fibre varies with age. Itis notable that pigs in general can digest dietary fibre even though piglets have limited cellulolytic activity (Noblet & Le Goff, 2001). Additionally, fibre components, especially soluble fractions (NDF-ADF), are fermented in the large intestine of pigs (Wenk, 2001) with subsequently energy losses in methane through fermentation (Knudsen K. , 2001). Increasing fibre in swine diets decreases energy and protein digestibility, with a greater amount of manure excreted (Noblet & Le Goff, 2001; Bindelle, Leterme, & Buldgen, 2008). Nevertheless, a minimum level of dietary fibre is

necessary for the digestive tract, especially for adult animals (Noblet & Le Goff, 2001). This information on fibre digestion in swine, together with the results obtained in this study, lead to the conclusion that FFPs can be used as feed ingredients, without adverse effects. In addition, the variability of fibre content among samples can be useful for selecting the proper FFP that fit with the dietary need of the different target class of pigs. The significant starch concentration in FFPs designated them as valuable energy sources for pig diets. Starch represents one of the major energy sources for monogastric animals in general, being approximately in the proportion of 0.40–0.55 (dry weight) in the diet of pig (Knudsen, Lærke, Steenfeldt, Hedemann, & Jørgensen, 2006). Stevnebø and co-authors (2006) report that cereals represent the main starch source in animal feeds and, for this reason, animal nutrition research is focused in investigating feed factors that may influence the digestion of the starch and its effect on animal performance (Giuberti, Gallo, Cerioli, & Masoero, 2012a). For instance, it is known that starch processing (e.g., heat treatments) can modulate the kinetics of starch digestion (Rehman & Shah, 2005) and the glycaemic index (Giuberti, Gallo, & Masoero, 2012b). Intuitively, due to the origin of raw material (exfood) used for FFP production, it can be speculated that FFPs' starch has been previously heat treated or cooked, and, therefore, they are relatively high digestible. This is of particular interest, considering that piglets have a limited capacity to digest raw starch. The use of cooked or processed cereal-based feed is required in swine nutrition (McDonald, et al., 2012). Starch represented approximately the 77% of NSC present in the FFPs tested, reaching 93% in FFP4. Both NFE and NSC fractions are a heterogeneous mixture of all those components not determined in the other classical constituents, according to methods used (CF versus NDF). Starch is in general included in both NFE and NSC fractions, and it can be one of the main contributors in defining the NFE and NSC content. As expected, both NFE and NSC were very close in absolute values to those measured in wheat, confirming the resemblance of FFPs with cereals grains. Generally, both starch and fat content calculated for FFPs in this study designated them as nutritious feedstuffs, which can be valuable non-traditional feed ingredients for swine nutrition. The expected advantage in using these kind of materials is the fact that they are already embedded in the matrix, which facilitates their manipulation and processing in the compound feed production. FFPs were characterised by a low quantity of ash. It is reported that the ash content of wheat and wheat by products ranges from 2.1% to 6.7% (McDonald, et al., 2012). The addition of wheat milling by-products in some of the tested samples seems to affect the ash content. Ash content in wheat grains is about 1.5%, whereas in wheat flour it is dependent on the milling process and ranges between 0.5% and 1.3%, as reported by Sumczynski et al. (2015). Overall, the specific and variable nutrient composition of the different FFPs offers a flexibility in formulating rations according to nutrient/energy requirements of the target animals. FFPs' features, especially regarding starch and fat content, may promote the inclusion of FFPs in the raw material portfolio for feed industry and farmers.

Energy values

The nutrient composition has provided information used to calculate DE and ME values for pigs, and to set-up the adequate protocol for assessing IVD. The obtained DE and ME values were comparable or even slightly higher (8.8%) than DE and ME calculated for wheat (15.8 versus 15.5 MJ kg–1). Although energy is not a nutrient per se, it originates from the metabolism of carbohydrates, lipids and proteins and is the primary factor to consider in defining a complete diet. Energy, together with protein and phosphorus, accounts for the most important parts of the cost of feed, reaching 64% of the diet cost. Energy in a diet for pig is used for physiological and productive purposes. Energy systems for monogastric animals are largely based on DE or ME. In fact, the efficiency of DE utilisation (or ME) is relatively constant compared with net energy systems (McDonald, et al., 2012). DE is the energy in feed after subtracting the energy lost in faeces, while ME represents the energy consumed by animals, and it is estimated as the DE minus urinary energy and gaseous energy losses (Just, 1982). ME is used to meet different energy requirements as maintenance, growth, protein or lipid gain in pig. In light of this, ME can be considered a better estimator of the available energy in pigs (Velayudhan, Kim, & Nyachoti, 2015). However, DE and ME systems can underestimate the energy value of ingredients high in fat and starch, and overestimate energy value for ingredients characterised by high protein and fibre content (Kil et al. 2013). Although energy systems based on chemical composition cannot calculate the true energy values and predict animal performances of a complete diet, due to missing information such as growth stage/rate of the animal, environmental factors or digestibility potential (Noblet, Fortune, Shi, & Dubois, 1994), the DE and ME system can be used as *in vitro* predictors of the energy content of these non-traditional feed ingredients. FFPs' composition, in fact, fitted perfectly with the proposed equations, especially when DE was considered. Moreover, DE and ME estimated for FFPs make them candidates as energydense feedstuffs, which combine carbohydrates (mainly starch) with high fat in the same ingredient.

In vitro digestibility

The IVD differences observed among the FFPs samples tested can be attributable to the small variation in nutrients' composition, especially fibre content, the type and the amount of non-fibre carbohydrates (both NFC and NFE). Of note, FFP6, FFP4 and FFP5, which were the poorest in fibre with a high starch content, exhibited the highest digestibility. On the contrary, the IVD value of FFP2 was significantly lower than wheat but largely comparable with the IVD of other feed ingredients commonly used in swine nutrition, such as corn (L. Pinotti, unpublished data), and to the IVD value reported by Regmi et al. (2009). This result can be attributed to the higher fibre content (expressed as CF and NDF) that probably limited FFP2 digestion *in vitro* and, presumably, in vivo. Regmi et al. (2009) reported an IVD value of 84.6% ± 0.03% calculated on 20 wheat samples using the same IVD protocol. The small increase in the IVD value of wheat (7%) observed in our study can be ascribed to the flaking process undergone by wheat when included as a reference feed. In the present

study the digestibility evaluation was carried out using a three-step *in vitro* digestion model of the pig digestive tract: as for the other *in vitro* models, they represent a simplification of reality. In spite of that, the IVD assay proposed was originally developed to mimic pig digestive tract by Boisen and Fernández (1997) and was further adapted by Regmi et al. (2009). The results obtained using this IVD procedure have indicated that digestibility values were highly comparable with values obtained in vivo (Noblet & Jaguelin-Peyraud, 2007; National Research Council (NRC), 2012). Therefore, it can be suggested that the IVD assay applied in the present study can be used to predict the IVD of feed samples on a routine basis (Zijlstra, 2006; Regmi, Ferguson, & Zijlstra, 2009), becoming an interesting tool for a preliminary screening of non-conventional feed ingredients as FFPs that must be further evaluated by in vivo trials. The correlation coefficients of IVD with nutrient composition and energy values (DE, ME) of FFP samples were in line with the literature which reported that the quantity and quality of the fibre fraction of a feed have the greatest influence on its digestibility, in particular in non-ruminant species (McDonald, et al., 2012). However, ash represents a consistent portion of fibre in a feed, and it can have properties similar to an indigestible marker. Interestingly, Pearson correlation coefficients were positive between IVD and DE, ME, indicating that a higher energy content of an FFP was positively correlated with its digestibility. The r coefficients confirmed that nutrient composition is crucial in determining the digestibility properties and, thus, the availability of energy to the animal body. Moreover, in general, the correlation coefficients obtained between carbohydrates fractions (NFE, NSC, starch and fibre fractions) and IVD were in line with literature results (Kerr & Shurson, 2013). Simple sugars (accounted in NFE and NSC) and starch are primarily digested in the upper gastrointestinal tract of pigs, although not completely, while structural carbohydrates are only partially degraded by the microflora in the caecum and large intestine, justifying the correlation coefficients obtained with IVD.

CONCLUSIONS

By combining nutrient composition results obtained in the present study, it can be concluded that the FFPs tested are energetic feed ingredients generally characterised by a valuable starch and fat content. These nutritional features allocated them as a fat-fortified version of common cereals used as energy sources in swine. In fact, samples analysed in the present study were characterised by a comparable starch concentration and higher fat content (two to eight times) than conventional cereals grains. Accordingly, these FFPs can be considered as nutritious non-traditional feed ingredients for swine nutrition. The expect advantage in using these kind of ingredients is related to the fact that the lipid fraction is already embedded in the matrix, facilitating its manipulation and processing in the compound feed production. Both DE and ME contents were quite high, confirming their features as energy-dense feed ingredients. Furthermore, the data obtained on FFPs' digestibility are also encouraging: FFPs tested in the present study were characterised by quite high IVD values and, in most cases, comparable with that of wheat. Thus, even though most of these nutritional values need to be verified in vivo and in the context of a balanced diet, data presented herein are promising: FFPs seem to be valuable non-traditional ingredients for a highly focused modern animal diet.

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Chapter 3

CARBOHYDRATE DIGESTION AND PREDICTED GLYCEMIC INDEX OF BAKERY/CONFECTIONARY EX-FOOD INTENDED FOR PIG NUTRITION

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CARBOHYDRATE DIGESTION AND PREDICTED GLYCEMIC INDEX OF BAKERY/CONFECTIONARY EX-FOOD INTENDED FOR PIG NUTRITION

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ABSTRACT

Ex-food or Former Food Products (FFPs), represent a way by which convert losses from the food industry into feed ingredients, which have been proposed as promising sustainable supplies for feeding animals. However, their nutritional potential in terms of carbohydrate digestion and related kinetic parameters is not yet fully exploited. Therefore, this study evaluated the in vitro carbohydrate digestion potential in FFPs intended for pig nutrition. Six samples of FFPs, all based on bakery products, were analysed using an in vitro method based on the Englyst-assay that simulates gastric and small intestinal digestion, and that has been proposed for predicting the glycemic index (pGI) of cereal-based foods entering pig diets. For each sample hydrolysis index (HI), have been also measure. Maize meal, and heat-processed wheat samples were also included as control ingredients, while white bread was used as reference material. Considering control samples, the lowest HI value was observed in maize meal (81.05) the same value increased of 23% and 15% in white bread and flacked wheat, respectively. In the tested FFPs, the average HI value was 104.53. Predicted glycemic index value, increased from maize meal (82.1), to flacked wheat (94.81), and white bread (101.3). When FFPs were considered, pGI ranged from 87.1 to 114.9, indicating a higher glycemic index potential in FFPs compared to common cereals. Combining HI and pGI results it can be suggested that FFPs tested in the present study were characterized by a high glycemic index potential that seems to be linked to the starch/sugars HI.

INTRODUCTION

The valorisation of ex-food, processed by-products, waste and effluents is a challenging opportunity for the sustainable and competitive development of industrial food and feed sectors. Former Food Products (FFPs), also termed ex-food, represent a way by which convert losses from the food industry into ingredients for the feed industry, thereby keeping food losses in the food chain (Featherstone, 2014; Featherstone, 2016). According to the EU Catalogue of Feed Materials "FFPs" or "Ex-food" means foodstuffs, other than catering reflux, which were manufactured in full compliance with the EU food law but are no longer intended for human consumption for practical or logistical reasons or due to problems in manufacturing or packaging which do not determine any health risks when FFPs are used as feed (Regulation (EC) No 68/2013 Bouxin, 2016). Typical former foodstuffs are represented by leftover of the food industry including, but not limited to, biscuits, bread, snacks and pasta (Pinotti et al., 2016), that are reprocessed by former food processor to produce feedingstuffs for animal diets. Part of FFPs is already reprocessed for animal nutrition but, compared with the total food loss, their use as feedstuffs still remains limited (3.3%). Previous studies contributed in characterizing nutrient composition, energy value, digestibility (Giromini et al., 2017) and safety features (Tretola et al., 2017a; Tretola et al., 2017b) of FFPs. These studies have pointed out that FFPs are a valuable energy sources, characterized by a valuable sugar/starch content (Giromini et al., 2017). This characteristic designated them as an excellent energy sources for pig diets. Carbohydrates represent the major energy sources in the diet designed for pigs (Stevnebø et al. 2006). Since carbohydrates, mainly starch, represent such a major part of the diet, characterizing their digestion potentials and improving their utilization are very important. To investigate the kinetics of carbohydrate/starch digestion of cereal-based feeds, several authors (Drew et al., 2011; Menoyo et al., 2011) introduced a novel approach for pig nutrition based on the glycemic index (GI) classification. However, considering the complexity and cost of in vivo GI determinations, an *in vitro* GI prediction (pGI) could be utilized as a valuable alternative (Giuberti et al., 2012b). In this sense, the pGI can be considered as a simple tool, adopted both for human (Capriles et al., 2008) and animal (Solà-Oriol et al., 2010) nutrition purposes, utilized for expressing kinetics of carbohydrate digestion by ranking cereal-based materials with respect to their enzymatic hydrolysis raising potential (expressed as a hydrolysis index; HI). In particular, different responses in productive performance could be expected in pigs fed diets based on diverse cereal-based sources differing in starch digestibility (Giuberti et al., 2012a). Specifically, diets with increased GI (appreciated in animal nutrition), can result in increased feed intake, due to a faster clearance of glucose in blood and then a more rapid return to a hunger state (Menoyo et al. 2011; Giuberti et al., 2012a; Doti et al., 2014). In this sense, it appears clear the importance of determining the GI not only in conventional feeding stuffs (Prandini et al., 2016), but also in FFPs, in which it has been never investigated before. Accordingly, the aim of this study was to evaluate the *in vitro* carbohydrate digestion and related pGI values

in former foodstuffs products intended for pig nutrition. As omnivores, pigs are ideally suited to convert these FFPs as well as other non-human-edible by/co-products, into high-quality food animal protein.

MATERIALS AND METHODS

Samples

In this study, six FFPs samples, collected in the frame of the IZS PLV 06/14 RC project funded by the Italian Ministry of Health, were analysed. FFPs were produced starting from different ex-food materials, as described in Giromini et al. (2017), in which nutritional features are also reported (Giromini et al., 2017). In all the experiments performed, a heat processed wheat and maize samples were included as control feed ingredients. Heat-processed wheat was selected as experimental control because FFPs considered in this study were obtained from ex food materials in which starch was previously exposed to food-technology processes (e.g., heat treatments, steam flaking, pelleting). All samples were milled through a 1-mm screen (Model 160-D, Jacobsen Machine Works, Minneapolis, MN, USA) and stored at 4°C until analysis.

Chemical analysis

All samples were analysed for moisture, crude protein (CP), ether extract (EE), neutral detergent fibre (NDF), acid detergent fibre (ADF), starch and ash as per the Association of Official Analytical Chemists (AOAC) (2005) and the European Commission (2009) regulation. Specifically, the moisture of samples was determined by an oven-drying method (130°C for 2 h) (Commission Regulation No. 152/2009). All data were presented on a dry matter (DM) basis. CP content was measured according to the Kjeldahl method (proc. 2001.11; AOAC 2005). EE was determined by the Soxhlet method, with prior hydrolysis, as proposed by the European Commission Regulation No. 152/2009. NDF and ADF analyses were performed according to procedures of the AOAC (2005) (methods 2002.04 and 973.18, respectively), using an Ankom 220 fibre analyser (ANKOM™ Technology, Fairport, NY, USA); NDF and ADF were measured using heat-stable amylase and expressed exclusive of residual ash (aNDFom, ADFom). Starch content was determined using a polarimetric method (Commission Regulation No. 152/2009). Determination of sugar was performed according to the official method (European commission, 2009). Ash was measured by using a muffle furnace at 550°C (proc. 942.05; AOAC 2005).

In vitro digestion procedure

An *in vitro* technique was conducted to fully characterize the *in vitro* carbohydrate digestion potential of samples. In particular, FFPs were characterized for their time-course carbohydrates digestion potential according to the protocol detailed by Englyst et al. (1992) and Giuberti et al. (2012b) with minor adaptations. The *in vitro* digestion was serially performed on each FFP sample two times (two biological replicates); in each digestion series, FFPs, control and blank (digestion enzymes alone) samples were included in duplicate (two technical replicates). Briefly, the method consists of a two-step enzymatic hydrolysis that simulates

gastric and pancreatic phases. Ground samples (about 800 mg) were placed in 100 mL screw cap bottles containing glass beads and a 0.05 M HCl solution (5 mL). Samples were incubated with a fresh pepsin solution (5 mg/mL; P-7000, Sigma–Aldrich) for 30 min at 39 ◦C in a shaking water bath under horizontal agitation. Subsequently, 10 mL of 0.25 M sodium acetate buffer has been added to adjust the pH at 5.2. Further, 4.5 mL of fresh amylase-activity enzyme mixture was prepared by mixing 0.7 g pancreatin (P7545, Sigma–Aldrich), 0.05 mL amyloglucosidase (A-7095, Sigma–Aldrich) and 3 mg invertase (I-4504, Sigma–Aldrich) as described by Van Kempen and co-authors (2010). Each bottle was placed in a shaking water bath at 39°C under horizontal agitation (simulation of the small intestinal phase) and the incubations were carried out up to 240 min. To fully characterize the carbohydrates digestion rate, seven incubation time points were investigated. In particular, aliquots (0.5 mL) were collected from each tube at 0, 15, 30, 60, 90, 120, and 180 min, then 2mL of absolute ethanol was added and the amount of released glucose was determined by a colorimetric analysis with a glucose oxidase activity assay kit (Megazyme).

Model used

Using the data obtained from the *in vitro* method, the proportion of digested carbohydrates for each time point (DCt) was calculated by the following equation:

$$DCt = (\text{amount of glucose present at time t})/(\text{total carbohydrates})$$

As suggested by Mahasukhonthachat et al. (2010) for *in vitro* techniques with pepsin pre-treatment, the C0 time point was included in the model. The first-order equation Eq. (1) has the form:

$$Ct = C0 + C\infty \cdot (1 - e\text{-}kt)$$

where Ct is the amount of carbohydrates digested at time t (g/100 g total dry carbohydrates), C0 is the amount of carbohydrates digested at 0 min (g/100 g dry carbohydrates), $C\infty$ is the potential digestibility of total carbohydrates (g/100 g total carbohydrates), k is the digestion rate (/min) and t is the incubation time (min). The only modification applied to the original model was the inclusion of C0 that could be utilised for *in vitro* techniques with pepsin pre-treatment (Mahasukhonthachat et al., 2010). For data fitting, C0, $C\infty$ and k values were obtained by the Marquardt method using the PROC NLIN procedure of SAS (2003). Areas under the digestion curves (AUC) were then calculated up to 180 min of incubation and the HI was derived from the ratio between the AUC for each sample and the corresponding area for a reference product. For this purpose, white bread = 100, with a total carbohydrates content of 750 g·kg-1 DM, has been selected. From the HI obtained *in vitro*, a pGI was calculated for each sample using the equation reported by Giuberti et al. (2011a). The equation [Eq. (2)] has the form:

$$pGI = 1.013 \cdot HI$$

Considering HI of white bread 100 by definition.

Statistical Analysis

Data were analysed using IBM SPSS Statistics version 25 software (SPSS). Carbohydrates digestion parameters for FFPs samples were analysed using one-way analysis of variance (ANOVA) in order to compare means. The analysis was performed using the following model:

$$yij = \mu j + \varepsilon ij$$

where yij is the observations (values); μj is the mean of the observations for the jth group (sample); and εij represents the within-sample random variability. The minimum significant difference (MSD) was generated from Tukey's test and it was used as the basis of the multiple comparisons among means. Differences with $P < 0.05$ were considered significant.

RESULTS

The composition of both heat processed wheat and unprocessed maize, and mean value of six FFP are reported in Table 3.1.

Table 3.1. *Chemical composition of the forages, grains and beet pulp.*

Item	HP Wheat	Corn	Average FFP[a]
Dry matter (g/kg)	109	84	79
105°C DM (g/kg)			
Crude Protein	101	103	100
Ether extract	15	42	101
Neutral detergent fibre	126	101	101
Acid detergent fibre	44	36	29
Sugars	28	15	213
Starch	714	700	523
Ash	14	15	23

[a]Giromini et al. 2017.

The model parameters, the HI and pGI values of FFPs, and control samples (maize and wheat) are presented in Table 3.2.

Table 3.2. *Model parameters, hydrolysis index and predicted glycemic index of unprocessed corn and heat processed wheat and former food products (FFP) 1 to 6 and relative standard deviation (SD) (n replicates = 2).*

Item	C_0	C_∞	k	HI	pGI
Unprocessed corn	1.04 (0.14)	74.86 (1.68)	0.05 (0.0021)	81,05 (0,41)	82,10 (0,42)
Heat processed wheat	1.77 (0.00)	80.79 (0.00)	0.12 (0.0001)	93,59 (1,38)	94,81 (1,40)
FFP1	4.13 (0.16)	97.65 (0.05)	0.12 (0.0014)	113,41 (0,33)	114,89 (0,34)
FFP2	4.73 (0.25)	91.67 (3.47)	0.19 (0.0608)	109,53 (2,66)	110,96 (2,70)
FFP3	5.37 (0.05)	87.00 (7.09)	0.09 (0.0205)	100,08 (6,41)	101,38 (6,49)
FFP4	0.92 (0.16)	78.87 (0.09)	0.09 (0.0092)	86,01 (1,07)	87,13 (1,08)
FFP5	2.86 (1.04)	87.11 (1.83)	0.13 (0.0374)	104,70 (1,54)	106,06 (1,56)
FFP6	2.87 (0.23)	99.75 (0.16)	0.12 (0.0099)	113,45 (1,09)	114,92 (1,11)

C0 – carbohydrates digested at 0 min (g/100 g dry carbohydrates); C∞ – potential digestibility of carbohydrates (g/100 g dry carbohydrates); k – rate of carbohydrates digestion (/min); HI – calculated by using white bread as reference (HI = 100); pGI – predicted glycemic index calculated by using the equation proposed by Giuberti et al. (2011b).

In addition, a graphical representation of time courses of average cumulative *in vitro* carbohydrates digestion (as a fraction of total dry carbohydrates) of cereal grains, namely heat processed wheat and unprocessed maize, and six FFPs samples is shown in Figure 3.1.

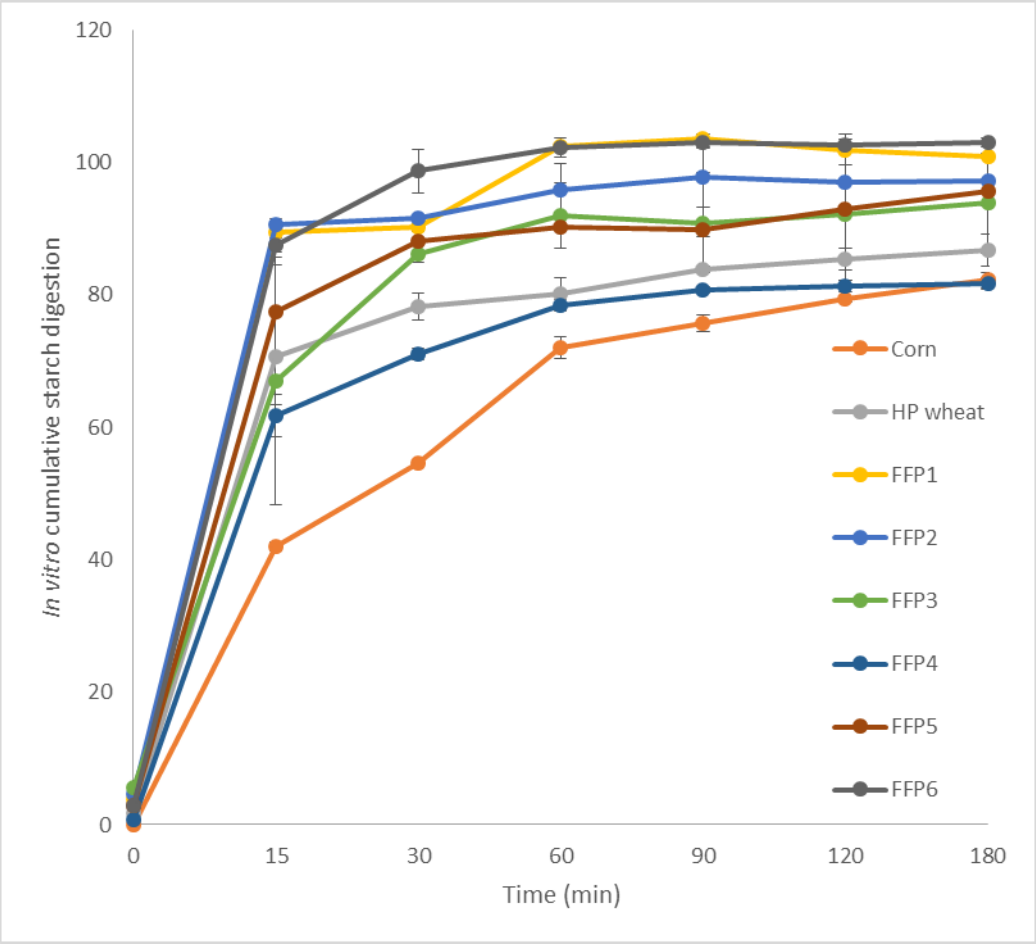

Figure 3.1. *Time course of average cumulative in vitro carbohydrates digestion (as a fraction of total carbohydrates) of cereal grains (unprocessed corn and heat processed wheat) and former food products (FFP) 1 to 6 (n replicates = 2).*

The HI and the pGI for white bread, used as reference material, were by definition 100 and 101.3, respectively. As reported in table 2, maize has shown the lowest HI and pGI values compared to both white bread and other tested samples. Maize HI and pGI indeed, were 20% lower than white bread, while for the same variables wheat has shown values around 90, i.e. higher then maize. With regard to FFPs, the overall mean for HI and pGI was 104.5 and 105.9 respectively. Specifically, five of the six FFPs analysed were characterized by higher values in both HI and pGI, compared to maize, heat processed wheat and white bread; by contrast, one sample has shown HI and pGI, below the control and reference materials considered (i.e. maize, heat processed wheat and white bread). The FFP4, indeed, has shown HI and pGI values similar to maize ($P>0.05$) and lower than heat processed wheat ($P<0.05$). With regard to the digestion kinetic parameters, in both control feed ingredients (i.e. maize and wheat) and FFPs, C0 values ranged from 0.92 for FFP4 to 5.37 for FFP3, while maize and processed wheat scored 1.04 and 1.72, respectively. When the potential digestibility of total carbohydrates (C∞, g/100 g total carbohydrates), was considered C∞ ranged from 74.86 to 99.75 /100 g total carbohydrates, while k values ranged from 0.05 to 0.19/min.

Considering average cumulative (Fig. 3.1) and absolute curves (Fig. 3.2) of time course carbohydrates digestion release (given as a fraction of total carbohydrates digested after 7 different incubation times), considerable differences have been observed.

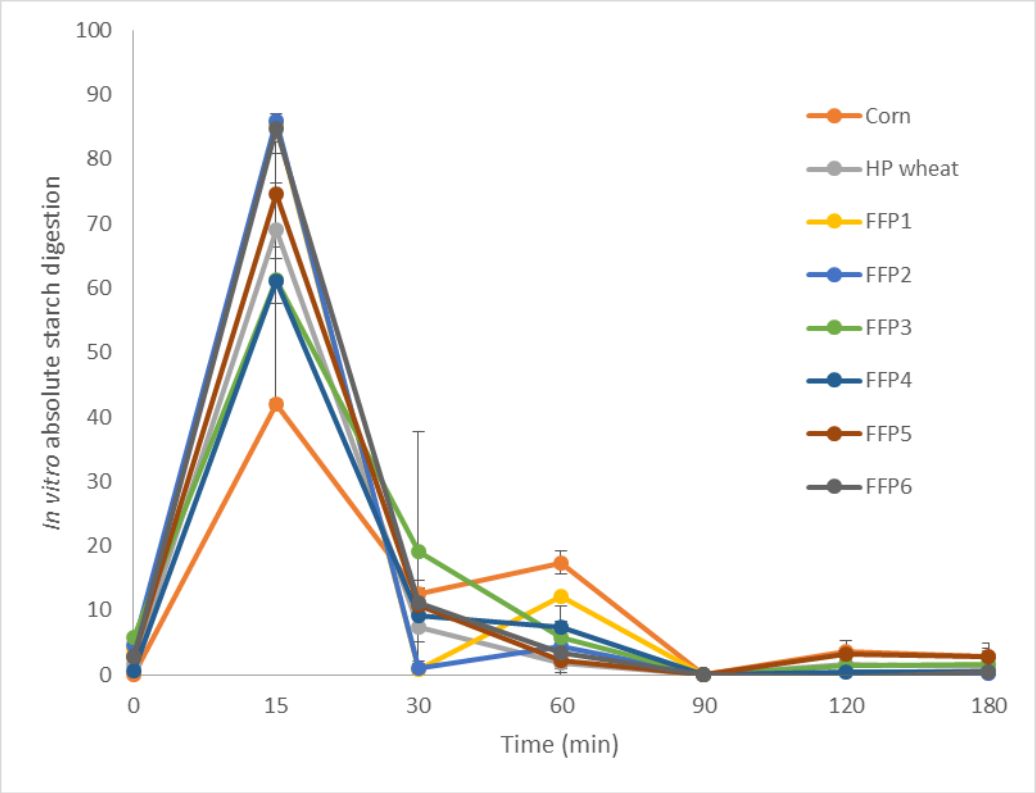

Figure 3.2. *Time course of average absolute in vitro total carbohydrates digestion (as a fraction of total carbohydrates /min) of cereal grains (unprocessed corn and heat processed wheat) and former food products (FFP) 1 to 6 (n replicates = 2).*

Graphical representation reflects evidences observed among samples for HI and pGI. Specifically, maize carbohydrates were digested slowly and have the lowest digestion curve at all-time intervals, whereas all FFPs and heat-processed wheat were digested faster and to a higher extent for almost the entire incubation period. As in the case of HI and pGI, rate of carbohydrates digestion in FFP4 sample was intermediate between maize and heat processed wheat, while in all the other FFPs samples (FFP1, FFP1, FFP3, FFP5, and FFP6) rate of total carbohydrates digestion was faster. For all samples the *in vitro* absolute carbohydrates digestion curves peaked at 15 min after the start of incubation (Fig. 3.2), when fraction of total carbohydrates digested ranged from 42% to 86% for maize and FFP2, respectively. Subsequent time points did not evidence substantial differences among samples. When *in vitro* cumulative carbohydrates digestion has been considered, the main differences among samples have been reported after 15 min of incubation. At this point maize sample exhibited the main difference in term of cumulative *in vitro* glucose release, showing the lowest value of glucose release among the tested samples. Considering the subsequent incubation time points, all

FFP samples showed higher cumulative *in vitro* glucose release compared to both maize and heat processed wheat samples. The only exception was represented by FFP4: in this case, cumulative *in vitro* glucose release was higher than maize but lower than heat processed wheat for the same incubation times (from 30 to 180 min). Irrespective of that, FFP4 has shown the slowest *in vitro* absolute carbohydrates digestion values recorded in all FFPs.

DISCUSSION

As reported elsewhere (Giuberti et al., 2012a), from a compositional point of view, control materials (i.e. maize and wheat) were in line with published data. In the case of FFPs they were characterized by a high starch and sugars concentration, whose are combined with an adequate fat content (Giromini et al., 2017). These results are consistent with previous studies in which FFPs have been considered (Giromini et al., 2017; Tretola et al., 2017a). Their composition, however, might be variable and some nutritional features (i.e. content in free sugars and fat) should be evaluated with caution in order to ensure a proper animal diet inclusion. Moving to carbohydrate digestion and predicted glycemic index of ex-food, the results obtained in the present work merit a specific assessment. Indeed, this is the first study in which former foodstuffs have been considered, and assuming that evaluation of HI and pGI is an *in vitro* technique potentially able to fully characterise the carbohydrates digestion potential of cereal-based feed materials, present data can be considered innovative. As expected, for the two control samples (maize and heat processed wheat) HI and pGI values were lower in maize than in heat-treated wheat. These results are in line with other authors (Giuberti et al., 2012b), who using the same assay have observed great differences between different cereals species/varieties, and also within the same species/varieties. Key distinguishing factors in defying HI and PGI values in differ grains were mainly the amylose content and the technological treatment (Svihus et al. 2005; Wiseman J., 2006). Both HI and pGI, as well as the time trend in carbohydrates digestion, were quite higher in FFPs compared to conventional cereals feed ingredients like maize. These nutritional and functional features are probably linked to the raw materials present in the former foodstuffs products. In fact, as reported elsewhere, typical FFPs are represented by leftover of the food industry such as biscuits, bread, snacks, pasta, characterized not only by a valuable sugar/starch content (Giromini et al., 2017), but also by several heat treatments (cooking, extrusion, etc.) (Svihus et al. 2005). Nowadays more bakery and snack foods include some whole grain flour. However, when used for human consumption, cereals are usually milled and fractioned with the aim, in general, of separating the fibre rich-bran (e.g. in wheat flour) and the hulls (e.g. in rice and oats) from the starchy endosperm (Knudsen and Lærke, 2018). Moreover, cooking of grains generally increases digestibility of nutrients and studies in both animal models and human demonstrated that processed grains are often nutritionally superior to unprocessed grains, probably due to enhanced nutrient bioavailability in processed grains (Slavin et al., 2000). Has been already observed that cooking, processing and fat content of the food are all factors able to affect the glycemic response. In this

regard, has been observed that postprandial blood glucose profiles were higher in subjects that consumed bread made with milled flour compared to breads made with a high proportion of whole cereal grains and that processes that disrupt the physical or botanical structure of food ingredients led to an increased plasma glucose and insulin responses (Slavin et al., 2000). Considering FFPs samples, higher values in HI and pGI, compared to maize and heat processed wheat samples, can be attributed to them. When compared with common cereal grains, Former foodstuffs products are characterized by a similar starch content (Giromini at al., 2017) but by a higher simple sugars amount (more than 200 g·kg-1, Giromini et al. personal communication). These latter's has been linked with higher digestibility potential *in vitro*, that probably has affect the results obtained in the present study. The only exception was represented by FFP4 which was composed by puffed cereals (rice and maize crispbreads), with a limited simple sugar content (124 g·kg-1, Giromini et al. personal communication). Observed HI and pGI are in line with the digestion kinetic parameters. In both control feed ingredients (i.e. maize and wheat), C0 (i.e. carbohydrates digested at 0 min), $C\infty$ (i.e. potential digestibility of carbohydrates), k (i.e. rate of carbohydrates digestion) were in general lower than in almost all the FFPs tested. These values indicate that the quantity of glucose released by maize and wheat was quite limited, as in the case of FFP4 that showed the lowest value compared to the other tested samples. This digestion pattern observed for FFP4 seems to be linked to the material used in its preparation that were extruded and puffed rice and maize cakes. As reported by Giuberti et al., (2012b), the technological treatment probably induced a gelatinization of starch (not measured in the present work), which affect the glucose release. A differ pattern, especially for potential digestibility of carbohydrates, has been recorded for FFP3, FFP5, FFP2 samples in which the $C\infty$ ranged from 87 to 91. Both values were higher than that observed for control feed ingredients and FFP4, but lower than the remaining FFPs. Specifically, potential digestibility of carbohydrates reached almost 100% in FFP1 and FFP6 which were characterized by the highest simple sugar content, according to the confectionary origin of the raw material used for their production. Considering the digestion rate, according to the classification proposed by Giuberti et al. (2012b), maize meal used as control feed ingredient, was gradually digested ($0.021 \leq k \leq 0.070$/min), while heat processed wheat and all the six FFPs were rapidly digested ($k > 0.071$/min). Among ex food, FFP2 has shown the highest value. This indicates that FFP2 was the fastest digested *in vitro*, followed by the others FFPs which scored all $k > 0.071$/min. The main reason for this pattern seems to be related to the fact that all FFPs were produced starting from cooked ex-food, in which therefore starch and other carbohydrates have been heat-treated. A different scenario, as expected, has been observed for maize, which was gradually digested ($0.021 \leq k \leq 0.070$/min). Thus, based on the recorded differences and similarities in term of HI and pGI, as well as in term of kinetics of digestion, the six FFPs samples can be divided in 3 main categories, namely: Group 1, including FFP1 FFP2 and FFP6, which is characterized by materials with the highest HI and pGI values; Group 2, including FFP3 and FFP5, which is characterized by ex-food with intermediate HI and pGI values;

Group 3, which includes only FFP4 that has shown the lowest HI and pGI values. Kinetic values seems to resemble the same categorization. Combining the present results, it can be suggested that kinetics of carbohydrates digestion, as well as HI and pGI, greatly differed among cereal sources and FFPs, even though the proposed assay is able to measure the digestion profiles (Mahasukhonthachat et al., 2010; Giuberti et al., 2012b). Weurding et al. (2001) observed that high starch digestion rates resulted in a more complete starch digestion for broiler chickens. In pigs, higher starch digestion rates could minimize the amount of undigested feed material that reach the distal part of the small intestine or in the proximal of colon (Al-Rabadi et al., 2011), thus influencing feed intake by avoiding the 'ileal brake' mechanism (Black et al., 2009). In addition, higher starch digestion rates could reduce starch fermentation in the small intestine with an increase in the apparent faecal digestibility of protein, due to a minor excretion of microbial biomass (Sun et al., 2006). However most the literature has been focused on starch, while limited information are available on simple sugars that actually seems to be a valuable proportion of the carbohydrates present in FFP. In fact, FFPs contain notable amount of free sugars, electing them as ingredients with high glycemic index. This concept is commonly used in equine nutrition (Kronfeld et al., 2004) where it has application in disorders associated with carbohydrates metabolism as well as in the nutrition of performance horses, but not properly addressed in in other farm species. However, while the potential influence on glycemic index it can be appreciated in some way, e.g. on feed intake, practical experience and research has demonstrated that in pigs soft faeces are produced in any high-sugar diet, imposing a deep functional gut health evaluation of these type of alternative feed ingredients. The case of molasses and other sugar co-products are quite well documented in literature (Becker et al., 1954; Becker and Terrill 1954; Aherne et al., 1969; Low, 1980; Ly, 1996), which recommends an adequate inclusion of these type of ingredients. Recently, Guo and co-authors (2015) demonstrated that the "candy coproducts can be used to replace up to 45% of dietary lactose for nursery pigs without negative effects on growth performance or health status". In addition, different studies reported that performance of nursery pigs was not reduced when less than 50% of dietary lactose was replaced by other carbohydrates from cane sucrose (100% sucrose), carbohydrate products (40 to 75% sucrose), molasses (47% glucose and fructose), or a milk chocolate product (20% lactose and 60% sugar) (Kim and Allee ,2001; Mavromichalis et al., 2001, Naranjo et al., 2010a,b). Similar results have been observed also in newly weaned piglets, where the dietary inclusion of sweeteners such as sucrose, lactose, dextrose or artificial high intensity sweeteners increased feed intake and body weight gain (Schlegel and Hall, 2006). It has been hypothesized (Sterk et al., 2008) that pigs offered sweetened feeds start eating sooner after weaning and also reduce the number of visits to the feeder. In fact, taste modifiers are now regularly used in feed to improve palatability and feed intake in post weaning piglets. Another interesting aspect to consider is the impact of sweeteners on gut ecosystem. It has been shown that the addition of either lactose or artificial sweeteners (saccharin/NHDC) to the piglets' feed dramatically increased the caecal population abundance of Lactobacillus, with concomitant increases in intraluminal lactic acid concentrations (Daly et

al.,2014). In this respect, lactobacilli are characterized by the presence of diverse sugar transport and metabolic systems, specifically induced by substrates like sugars and starches (Daly et al., 2014). The primary metabolic product obtained by the fermentation of these substrates is lactic acid with stimulatory effects on both gut immunity and maturation (Pierce et al., 2006). It is also able to enhance immune protection end reduce gastrointestinal inflammatory responses (Daly et al., 2012, Blum and Schiffrin, 2003). Moreover, lactic acid reduces the colonic pH resulting in protection against mucosal pathogen invasion (Gómez et al., 2016). Consequently, all these observations demonstrate the ability of dietary supplemented lactose or sweeteners to positively influencing the pig gut bacterial composition. This implies a rigorous nutritional and functional evaluation of FFPs, with special emphasis on their impact on gut health. FFPs might contain more than 20% of simple sugars which not only could affect the gut transit, but also its health and ecology (Oliveira et al., 2017). Understanding what a healthy microbiota looks like and how FFPs can influence the composition of the gut microbial population, i.e. improving eubiosis and/or reducing disbiosis, would provide new fundamental insights to efficiently reconvert FFPs into value added products for animal nutrition.

CONCLUSIONS

This study has evaluated for the first time the predicted glycemic index (pGI) in former foodstuffs products intended for pig nutrition. Determination of predicted glycemic index represents a valuable approach for the evaluation of cereal-based feeds rich in starch/sugars, such as FFPs, providing information on rates of carbohydrates digestion potential. In general, FFPs evaluated in this study showed a pGI higher than common cereals and flacked cereals for feed use. However, carbohydrates digestion potential as well as pGI were extremely variable among FFPs hereby studied according to their original composition. Although, the results presented in this study should be analysed carefully, since they are case sensitive, (i.e., they represent the situation of a specific set of samples), they provide some clear nutritional information, namely: i) FFPs have an excellent potential use as alternative feed ingredients for livestock production; ii) this potential is closely linked to their content in “ready to use” carbohydrates, which in turn define their high nutritional/energy value.

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Chapter 4

FORMER FOOD PRODUCTS SAFETY: MICROBIOLOGICAL QUALITY AND COMPUTER VISION EVALUATION OF PACKAGING REMNANTS CONTAMINATION

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FORMER FOOD PRODUCTS SAFETY: MICROBIOLOGICAL QUALITY AND COMPUTER VISION EVALUATION OF PACKAGING REMNANTS CONTAMINATION

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ABSTRACT

The use of alternative feed ingredients in farmanimal's diets can be an interesting choice from several standpoints, including safety. In this respect, this study investigated the safety features of selected former food products (FFPs) intended for animal nutrition produced in the framework of the IZS PLV 06/14 RC project by an FFP processing plant. Six FFP samples, both mash and pelleted, were analysed for the enumeration of total viable count (TVC) (ISO 4833), Enterobacteriaceae (ISO 21528-1), Escherichia coli (ISO 16649-1), coagulase-positive Staphylococci (CPS) (ISO 6888), presumptive Bacillus cereus and its spores (ISO 7932), sulphite-reducing Clostridia (ISO 7937), yeasts and moulds (ISO 21527-1), and the presence in 25 g of Salmonella spp. (ISO 6579). On the same samples, the presence of undesired ingredients, which can be identified as remnants of packaging materials, was evaluated by two different methods: stereomicroscopy according to published methods; and stereomicroscopy coupled with a computer vision system (IRIS Visual Analyzer VA400). All FFPs analysed were safe from a microbiological point of view. TVC was limited and Salmonella was always absent. When remnants of packaging materials were considered, the contamination level was below 0.08% (w/w). Of note, packaging remnants were found mainly from the 1-mm sieve mesh fractions. Finally, the innovative computer vision system demonstrated the possibility of rapid detection for the presence of packaging remnants in FFPs when combined with a stereomicroscope. In conclusion, the FFPs analysed in the present study can be considered safe, even though some improvements in FFP processing in the feeding plant can be useful in further reducing their microbial loads and impurity.

INTRODUCTION

Animal feed is the most important livestock production cost factor and in 2014 represented up to 85% of the farm gate value of poultry (Bouxin, 2016). In the EU-28, farmed animals consume annually around 475 million tons of feed, of which 30% are compound feed. Clearly, ensuring that such high volumes of traded products conform to adequate quality standards is a major undertaking and it is fair to say that the European Union (EU) has made significant progress in defining standards and promoting legislation in this area. As a consequence, the explicit and detailed formulation of the concepts of food/feed safety and food/feed quality has been consolidated within the EU to legislation on the traceability, control and labelling of both feed and food. These concerns are even more important when innovative feed products are considered as alternative feed ingredients. In this context, the former food products' (FFPs) story represents an exhaustive example. Nowadays, in the food production chain, specific demands for quality and excess in production exist due to problems of manufacturing, packaging defects etc. The foods, which are removed from the regular food chain for economic and quality reasons, can be indicated as FFPs or ex-food (Pinotti, et al., 2016; Giromini, et al., 2017). According to the EU Catalogue of Feed Materials (Regulation (EU) No 68/2013), former foodstuffs are: foodstuffs, other than catering reflux, which were manufactured for human consumption in full compliance with the EU food law but which are no longer intended for human consumption for practical or logistical reasons or due to problems of manufacturing or packaging defects or other defects and which do not present any health risks when used as feed. Typical former foodstuffs are represented by raw materials with high energy content in the form of sugars, oils and starch such as biscuits, bread, breakfast cereals, chocolate, pasta, bakery products and snacks. The Catalogue of Feed Materials therefore also specifically stipulates that former foodstuffs are inherently different from catering waste, whose use in animal feed is prohibited in the EU (EFFPA , 2017). Nowadays, over 3 million tons of so-called former foodstuffs are produced in the EU, even though not all are reconverted and revalorised as animal feed, with a consequent waste of highly nutritional material when FFPs are not used as ingredient of animal feed (Featherstone, 2016). The main reason of the limited use of ex-food is their poor characterisation not only from a nutritional point of view but also from a safety standpoint. Prerequisites to improve the use of these innovative feed ingredients consist in a minimisation of risks and in an increased valorisation. The valorisation of FFPs entails considering several safety aspects such as microbiological quality and remnants of packaging material contamination levels. Animal feed, due to its composition, provides a favourable environment for the growth of microorganisms. Moisture, temperature, type of feed, chemical and physical properties of raw material, pH, storage conditions and much more represent factors that can modulate bacterial growth and proliferation (Andreoletti, et al., 2008). Bakery products and cooked materials that mainly represent FFPs can generally be considered microbiologically stable. However, feed contamination may occur during processing, transport or storage. Moreover, the process of removing the food packaging can be a source of contamination. For all

these reasons, verification of the microbiological quality of these materials is always recommended when they are used as animal feed. A further aspect that has been reported in terms of safety is referred to packaging material remnants. Processing methods to convert former food in to feed ingredients do not usually include pre-removal of packaging materials. For this purpose, feed processors routinely remove the packaging from ex-food mechanically in the feed plant but, despite the removal of most part of the packaging, small amounts of wrapping materials can remain in the resulting feed. As a result, a small amount of packaging remnants in the final product (feed) appears to be unavoidable (Marchis et al., personal communications). Classical remnants that residue in former food are plastics, paper and aluminium foils. The remnants of packaging (Regulation (EC) No 1935/2004) are often characterised by complex compositions and large variety of materials. According to the general food law principles (Regulation (EC) No.178/2002, 2002) and the feed hygiene requirements (Regulation (EC) No. 183/2005), traceability and safety should be guaranteed at any stage of the food and feed chain. This scenario imposes a proper approach in defining appropriate methods for detecting possible packaging contaminants and their remnants in ex-food used in the feed chain. Recently, van Raamsdonk et al. (2012) validated a method (RIKILT) for the detection and quantification of packaging materials in bakery products. Nevertheless, this method can be considered very laborious and the basic principle of this method is to separate every particle that is not native to the matrix by examination by the naked eye. In the light of this, the aim of this study was to investigate the safety features, in term of microbiological quality and packaging remnants contamination by different methods, of specific FFPs intended for animal nutrition.

MATERIALS AND METHODS

Sample

Six samples of mixed FFPs, collected in the frame of IZS PLr V 06/14 RC project, were used. All samples were produced starting from different food materials (e.g. broken biscuits and chocolates, confectionery products, such as croissant and chocolate, surplus bread, rice cakes, and breakfast cereals) in an ex-food processing plant based in the north of Italy. Four samples were in meal form whereas two of them were pelleted. Samples were received in paper bags and stored at 4°C until analyses. Microbiological analyses Samples were received in refrigerated conditions. For microbial counts, 10 g of each sample were homogenised in 90 ml of diluent solution (0.85% NaCl and 0.1% peptone), and then serial 10-fold dilutions were made in sterile saline. Total viable count (TVC) was determined on plate count agar (PCA) (Biogenetics, Ponte San Nicolò, Italy) according to the NF EN ISO 4833:2003 method. The number of Enterobacteriaceae was determined on violet red bile glucose agar (VRBGA) (Biogenetics) according to the NF EN ISO 21528-2:2004 method. Escherichia coli counts were determined on TBX (Oxoid, Basingstoke, UK) according to the NF EN ISO 16649-1:2001 method. Coagulase-positive Staphylococci were determined on Baird Parker agar (BP) (Biogenetics)

supplemented with egg yolk tellurite emulsion (Biogenetics) following the NF EN ISO 6888-1:1999 method. Presumptive Bacillus cereus, a spore-forming species, were enumerated on PEMBA medium (Biogenetics) according to the NF EN ISO 7932:2004 method. Spores of presumptive B. cereus were enumerated on the same medium after pasteurisation of the dilutions (80°C, 10 min.). A count of spores of sulphite- reducing Clostridia was performed on tryptose sulphite cycloserine agar (TSC) (Oxoid) according to the ISO 7937-2004 method, after pasteurisation of the dilutions. Yeasts and moulds were enumerated onto sabouraud dextrose agar (SAB) (Scharlau, Barcelona, Spain) supplemented with tetracycline (10 ml l–1) according to the NF EN ISO 21527-1:2008 method. Salmonella spp. detection was performed on 25 g of each sample by the NF EN ISO 6579:2002 method.

Experiment 1: detection and quantification of packaging materials by stereomicroscopy

The presence of undesired ingredients that can be identified as remnants of packaging materials was evaluated in meal and pelleted samples by a stereomicroscopy technique, according to Raamsdonk et al. (2012) and Marchis et al. (2016), with minor adaptations. Briefly, 100 g of each sample were weighed in a 600-ml beaker and sieved at mesh sizes of 4, 2, 1 mm, 800, 630, 400, 250 and 125 µm. For pelleted samples, the wet sieving procedure was performed according to Miladinovic (2009). Specifically, 100 g of each sample were weighed in a 600-ml beaker. The pellet was soaked for 2 h in approximately 500 ml of RT tap water. Pellets were stirred at least once after the first hour for best results. When completely dissolved, the sample was sieved using a water flow to wash all the particles. Sieves were placed on a rack in the oven at 104°C (standardised European procedure EU 71/393) and left to dry-out overnight. Each sieve with the dried material was weighed. For all FFPs, each sieve fraction was investigated separately for remnants of presumed packaging materials with a stereo-microscope (OLYMPUS SZX9) in a large plate with upright borders. All extraneous particles were isolated and collected. The selected particles were picked up by a pair of tweezers. When necessary, a larger magnifying glass was used. The specific sieved portions of remnants of presumed packaging material were kept separated for the entire process. Each sieved portion of selected material was weighted and the amount was used to calculate the percentage (w/w) of remnants of presumed packaging materials per fraction and for the entire sample.

Experiment 2: detection of packaging materials by computer vision

All the samples were analysed using a computer vision system (CVS) (IRIS Visual Analyser VA400). In a preliminary trial, all samples were analysed according to the manufacturer's instructions (IRIS Visual Analyser Alpha Soft V14 Manual), with minor adaptations. In brief, each sample was positioned on the available surface and submitted to top and bottom-controlled white lighting conditions (6500°K) to avoid shadow effects. A picture of the sample surface was taken by the use of a high-resolution CCD camera (16 million colours). In this trial, due to the small particle size of both feed material and packaging remnants, it was not possible to obtain high-resolution pictures for an appropriate image analysis. For this reason, a further

approach was adopted. Specifically, the CVS (IRIS Visual Analyser VA400) technique was coupled to a stereo-microscope image analysis. Briefly, 2 g randomly composed of each FFP were weighted; pelleted samples were previously ground with mortar and pestle. In order to define an internal reference material, i.e., an FFP cleaned sample, the cleanest FFP resulted from experiment 1 was used. For this purpose, in FFP6 all visible packaging remnants were removed. Correspondence w/w packaging remnants levels between experiment 1 (100 g) and the subsamples (2 g) used in experiment 2 was verified. Packaging materials recovered in the samples (by visual inspection) and in subsamples used for CVS had a correlation of $r = 0.997$ ($p < 0.001$). For each sample, about 30 pictures (6.3×) of sample fractions were obtained (CoolSNAP-Pro colour camera). The pictures were uploaded on the CVS AlphaSoft software (Alpha MOS, France) and analysed. Specifically, for colour analysis the image background of the FFPs pictures was first removed by a pre-processing method (AlphaSoft software) in order to limit the analysis to the FFP colours. Afterwards, the pictures were processed as a colour spectrum representing the proportion of each colour (%) on the FFP surface, within a fixed scale of 4096 colours, corresponding to a unique set of three values in the red–green–blue (RGB) space. To automate the visual quality control of FFPs, the visual parameters measured with CVS were processed using statistical quality control (SQC) model.

Statistical analysis

Pearson correlation between packaging remnants levels in experiment 1 samples and the cleaned FFP sample obtained in experiment 2 were determined using SPSS (SPSS/PC Statistics 23 SPSS Inc., Chicago, IL, USA). Regarding the computer vision analyses, starting from the colour spectrum (percentage of the different visible colours) obtained from samples and reference material (cleaned FFP sample) surface, the AlphaSoft software (Alpha MOS, France) was used to build up the SQC chart for the statistical monitoring of sample quality (presence/absence). Using the cleaned FFP, the training phase was performed in order to relate the variability of the sample to the sensor data recorded by the analysis system. In this statistical approach, average value (mj) and a standard deviation (δj) from the responses of each sensor 'j' were calculated. To build the SQC model, the colour-code 4095 sensor was selected as the most discriminating variable. By multivariate analysis, the visual difference between the training sample and FFP samples was estimated. The software expresses the difference as a distance. The resulting chart was constructed with an acceptability area defined by the upper and lower limits (horizontal lines). To set limits of the SQC graph, the AlphaSoft software uses the following acceptability index (or rejection probability):

$$I_{acceptability} = (1 - P_{rejection}) * 100 \text{ with } 0 < P_{rejection} < 1$$

Samples mapped into acceptability area were recognised ***as*** uncontaminated. Conversely, samples mapped outside this area were recognised as contaminated by presumed packaging remnants.

RESULTS AND DISCUSSION

Microbiological quality

Microbiological analyses showed that all six samples examined were of a high microbiological standard and free from pathogenic organisms (Table 4.1). For the evaluation of microbial counts, commonly used to evaluate the general hygienic condition of food and feedstuffs, as no official microbiological guidelines for feed samples are available, those for food for human consumption were considered (Health Protection Agency, 2009). Mean TVC, a parameter commonly used to evaluate the general hygienic condition of food and feedstuff, was 4.92 ± 0.25 log CFU g–1. None of the samples exceeded the microbial loads of 6 log CFU g–1, generally recognised in food as the threshold limit above which spoilage could occur (Health Protection Agency, 2009). These results were expected as the analysed feedstuffs are characterised by low moisture content and a very high temperature reached during the production process. Mean count of Enterobacteriaceae, used as a faecal contamination index for feedstuff, was 3.61 ± 0.55 log CFU g–1, confirming the low level of bacterial contamination; the product FFP3 showed counts below the detection limit (2 log CFU g–1) with all samples within the threshold safety limit for the animal feed sector of 10 log CFU g–1 established by (Regulation (EU) No 142/2011). E. coli was always under the detection limit, while Staphylococci count was extremely low (> 2 log CFU g–1), except for FFP2 in which it reached 2 log CFU g–1. Levels of B. cereus and its spores, a potential pathogenic aerobic microorganism occurring ubiquitously in several natural habitats and food products, were on average below 3 log CFU g–1 (2.84 ± 0.73). Generally, these bacteria never exceed the level of 5 log CFU g–1, recognised as the starting concentration from which toxin production may occur. Not all B. cereus strains produce toxins and are able to produce gastrointestinal disease, but levels > 5 log CFU g–1 are considered indicators of poor processing, poor quality of raw materials or poor temperature control. Clostridia were found to be countable just in two of the six samples and in very low loads (1.7 and 1.0 log CFU g–1 in FFP2 and FFP3 samples, respectively); levels < 1 log CFU g–1 are considered satisfactory and commonly < 4 log CFU g–1 not of particular apprehension. Clostridia count is used as a marker of the effectiveness of thermal treatments during the production process. Yeasts and moulds, which are among the most critical organisms for this type of feedstuff, were detected in all the samples, with loads around 3–4 log CFU g–1 (means of 4.03 ± 0.56 and 3.30 ± 0.41 log CFU g–1 respectively). Moulds can negatively affect feed quality, reducing dry matter and nutrients contents. Mouldy raw materials are not appetising and can considerably reduce feed consumption (Liu, et al., 2011). However, levels found should be considered not of particular concern since yeasts may cause alteration at levels around 6–7 log CFUg–1 and the loads of moulds do not indicate spoilage of the product. The Panel on Biological Hazards identified Salmonella spp. as the major hazard for microbial contamination of animal feed. Transmission of Salmonella from animal feed to animals consuming the feed and to food products derived from the animals

was shown in several studies. In all FFPs tested in the present study, Salmonella spp. was never detected, matching the requirements included in (Regulation (EU) No 142/2011) for the animal feed sector.

Table 4.1. *Microbiological quality of FFPs.*

	Contaminant/threshold limit (log CFU g-1)									
Sample	TVC/ 6[a]	Enterobacteriaceae/10[b]	E. coli /2[a]	Coagulase-positive Staphylococci/4[a]	B. cereus /5[a]	B. cereus spores /5[a]	Clostridia/4[a]	Yeasts/ 7[b]	Moulds /7[b]	Salmonella spp./ absent[b]
FFP1	4.98	3.65	< 2	< 2	3.20	< 2	< 1	4.38	3.40	Absent
FFP2	5.11	3.67	< 2	2	2.00	< 2	1.70	4.12	3.26	Absent
FFP3	4.90	< 2	< 2	< 2	2.00	2	1	3.00	3.88	Absent
FFP4	4.81	4.20	< 2	< 2	3.86	3.70	< 1	4.21	3.30	Absent
FFP5	4.51	3.81	< 2	< 2	3.08	2	< 1	4.60	3.40	Absent
FFP6	5.20	2.70	< 2	< 2	2.90	< 2	< 1	3.88	2.60	Absent

Sources: [a]Health Protection Agency (2009)*; [b]Regulation (EC) No. 142/2011.*

Packaging remnants analysis

In this study, FFPs have shown a mean level of packaging materials remnants of 0.02% (w/w). Specifically, in mash FFPs samples the remnants' contamination was below 0.08% (w/w), whereas in pelleted samples it was 0.02% (w/w). The possible effects of feed processing (i.e., pelleting) are unknown. FFP2 showed the highest packaging remnants level (0.07% w/w), whereas FFP4 and FFP6 showed the lowest one (0.005% w/w) (Figure 4.1).

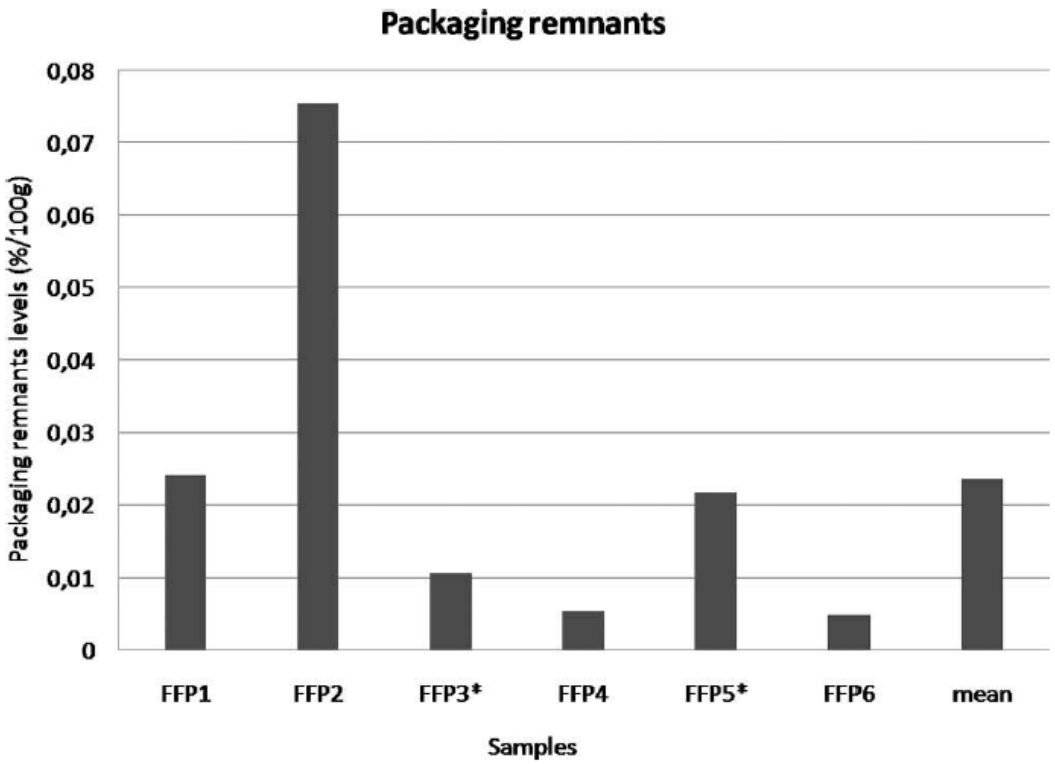

Figure 4.1. *Packaging remnants contamination (% w/w) in all FFPs samples. The percentage levels of contamination in FFPs samples were determined by weighing packaging remnants by the sieving procedure and manual selection. *Pelleted samples.*

However, despite the low quantity of packaging remnants recovered, in FFP4 an abundant amount of light spots that look like plastic fragments were observed (Figure 4.2). These presumed remnants materials were too small for a systematic (manually) collection and their subsequent quantification. In this respect, it can be suggested that the methods tended to underestimate the packaging remnant contamination, even though as reported elsewhere (van Raamsdonk, Pinckaers, Vliege, & van Egmond, 2012) these small particles in terms of quantity are insignificant. Furthermore, the German Federal Ministry of Food, Agriculture and Consumer Protection (Bundesministerium für Ernährung, Landwirtschaft und Verbraucherschutz – BMELV) acknowledged that the presence of packaging material in bakery products and confectionery should not result in significant risks within a tolerance level of 0.125% w/w (Kamphues, 2005).

Figure 4.2. *Light spots detected in FFP4 by the use of stereomicroscope Olympus SZX9 (6.3X).*

In this study, all FFPs tested showed presumed packaging remnants level below the tolerance level established by BMELV. Of note, the distribution of packaging remnants was heterogeneous among different sieve meshes in all samples. In particular, remnants were observed mainly from the 1-mm sieve mesh fraction (Figure 4.3). Quantification of particles smaller than the sieve mesh size of 400 µm would be too laborious and, according to Raamsdonk et al. (2012), these small particles present in the two smallest fractions were excluded from the quantification, since the share in the total weight is insignificant. Furthermore, the contribution of the particles larger than 2 mm to the total amount of material in the samples was insignificant. Based on these results, it can be concluded that FFPs are safe, even though some improvements in FFP processing, at the feeding plant, can be useful in further reducing their impurity.

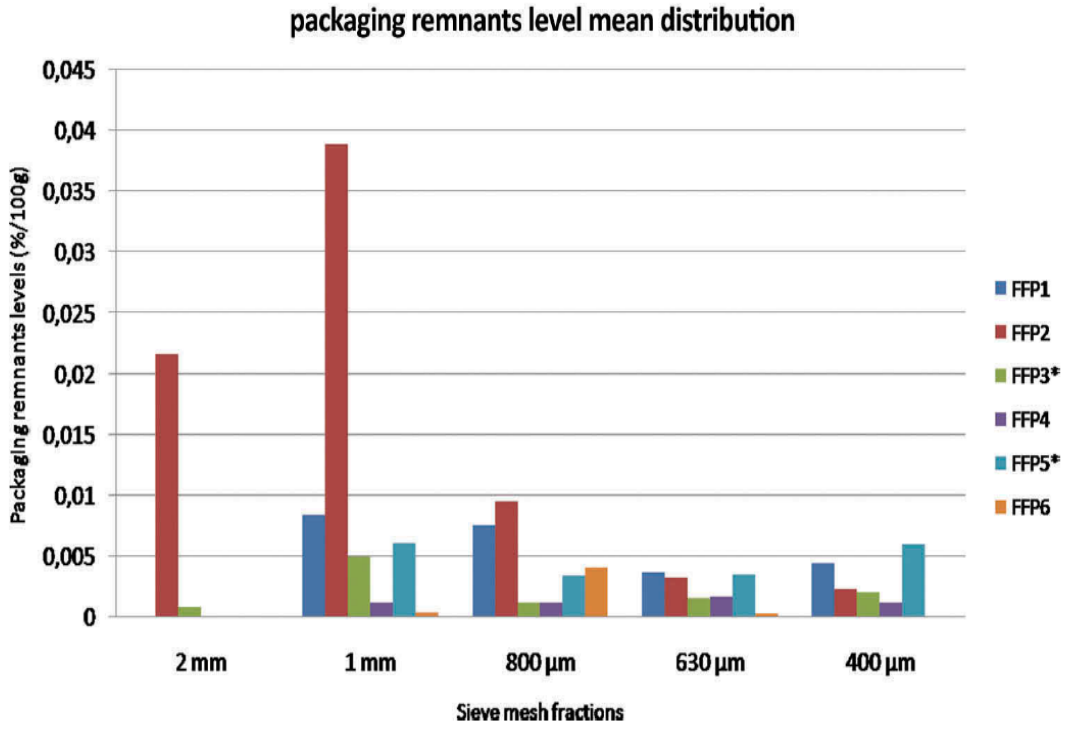

Figure 4.3. *Packaging remnants level mean distribution. *Pelleted samples.*

Computer vision analyses

The CVS allowed a visual assessment of sample appearance, determining the proportion of each visible colour across the surface of the samples (i.e., FFPs). Moreover, the advanced data processing software (Alpha MOS, France) offered the possibility to perform a SQC among FFPs by comparison of their colour spectrum (percentage of the different visible colours). Its approach, however, should be considered qualitative (presence/absence). Preliminary colorimetric analysis of FFPs pictures showed that a specific colour code, i.e., 4095, was always present in samples' pictures where presumed packaging remnants were present. The same colour code was absent in samples' pictures without packaging remnants. The occurrence of 4095 colour code in pictures where packaging remnants were observed could be due to the particular reflecting properties of some packaging materials, such as aluminium foil and plastics. Although the definition of 'plastic' refers to several types of packaging materials (van Raamsdonk, et al., 2011), such as polyethylene (PE), polypropylene (PP), polyvinyl chloride rigid (PVC), polystyrene (PS), polyethylene terephthalate and its copolymers (PET) (Marchis et al., personal communication), this result is in agreement with Raamsdonk et al. (2012) who reported that fibres of papers, fragments of plastic, aluminium foil and chips of plastic clips are most of the recovered types of material detectable in FFPs. Therefore, the 4095 colour code could be associated with the presence of packaging remnants in FFP samples. In light of these findings, a further step in the study was the SQC analysis. Through the SQC model, the presence of the colour code 4095 in the six FFP samples, in comparison with a cleaned FFP, was performed. As reported in Figure 4.4, in samples FFP1–

FFP5, the presence of packaging remnants was recorded, even though there were some differences between samples. In fact, with the exception of FFP4 and FFP6, all the other samples (FFP1–FFP3 and FFP5) have shown a presence, at a very low level, of packaging material.

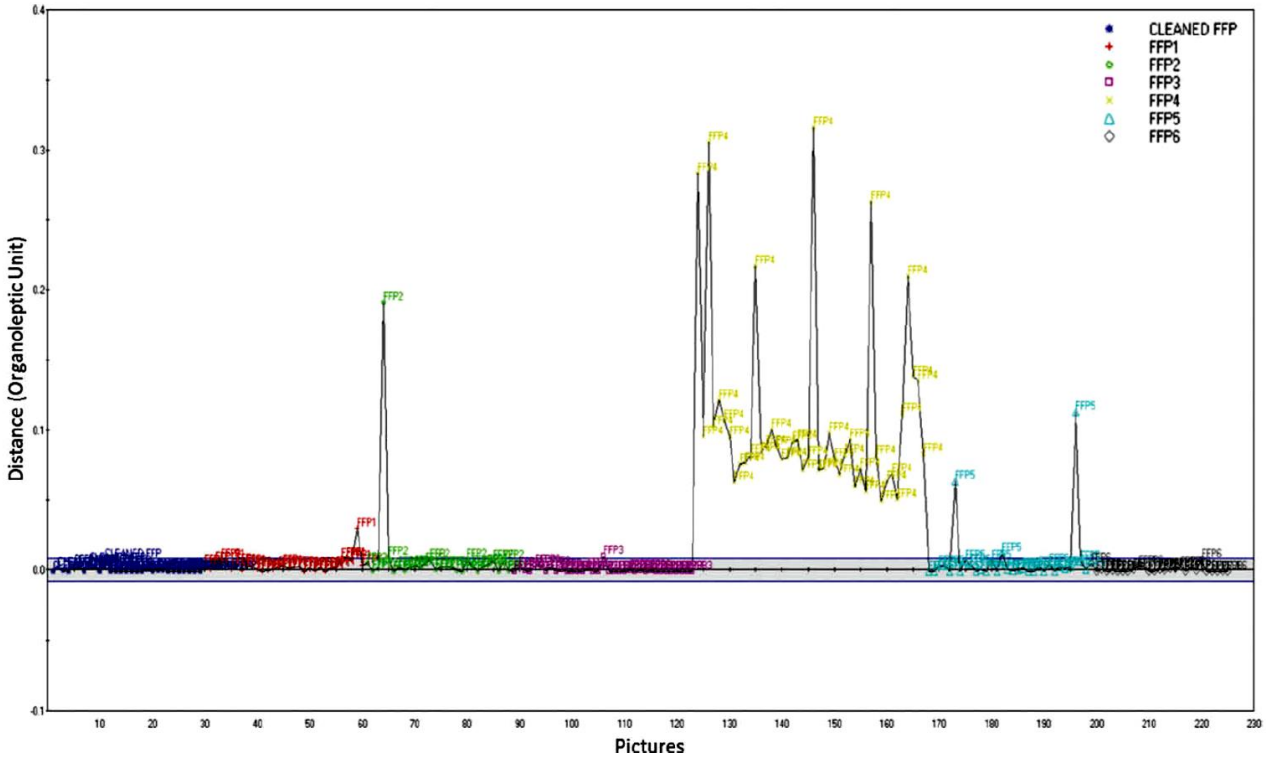

Figure 4.4. *Statistical quality control (SQC) chart. Each symbol represents a picture of the corresponding sample, based on its colour. The grey area indicates the range distance obtained by the use of a cleaned FF sample in the training phase. Symbols plotted outside the grey band indicate sample pictures characterised by the presence of packaging remnants, specifically percentage of the colour code 4095.*

The four samples were plotted outside this grey band (area of tolerance), indicating that they were contaminated by packaging remnants, as shown in Figure 4.5. A completely different pattern in the SQC analysis was obtained for FFP4, which was plotted as very distant from the cleaned FFP sample in the CVS analysis. The reason for this is unknown, even though the nature of these presumed packaging materials, especially in terms of colour attribute, could be implicated. This discrepancy between methods can be attributed to the presence of abundant reflecting light spots in the sample (Figure 2), presumably originating from the packaging materials, that were detected by the CVS, but too small for a systematic measurement by the classical method. A further exception, in comparison with the conventional method, was sample FFP6. It has shown a SQC pattern very close to the cleaned sample FFP, i.e., within the area of tolerance, probably due to the fact that it was the lowest contaminated sample.

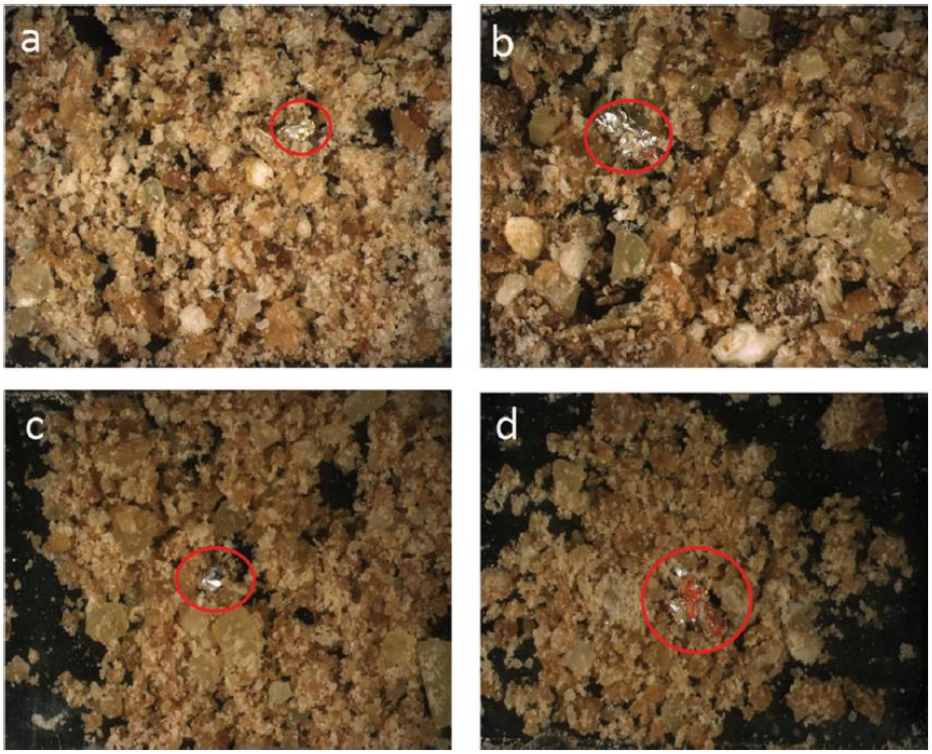

Figure 4.5. *Presumed packaging remnants detected by a visual analyser in selected samples' pictures (Olympus SZX9), namely: FFP1 (A), FFP2 (B) and FFP 5 (C, D) samples.*

Combining these results, it can be concluded that although detailed studies will be necessary to clarify the nature of this material, CVS and the SQC analysis seems to be informative in distinguishing between contaminated and uncontaminated samples. This method even though qualitative for the moment, can be considered effective in defining further analysis or investigations in FFPs.

CONCLUSIONS

This study investigated the two main safety issues linked to the use of ex-food in animal nutrition. All FFPs analysed were found to be safe from a microbiological point of view, and among the samples collected, Salmonella spp. were always absent. All the loads of the other microbiological contaminants were moderate or not detectable, always within tolerance levels established by the Health Protection Agency and European Regulation No. 142/2011. These results indicate a high quality of these former foodstuffs, suggesting the low risk level of the production process. When remnants of packaging materials were considered, the contamination level measured in the present study was below 0.08% (w/w). Of note, presumed packaging remnants showed a heterogeneous distribution among different sieve meshes fractions in all samples. In particular, the packaging remnants were observed mainly from the 1-mm sieve mesh fraction. These results indicated that all samples were significantly below the tolerance level of 0.125% (w/w) proposed by a EU member state authority (Germany's BMELV). Moreover, contamination levels of up to 0.15% w/w are considered not only unavoidable in bakery products but also not significant in terms of safety risks. In terms of methods of analysis, even though a validated (RIKILT) method for the detection and quantification of packaging materials in bakery products exists, innovative complementary method based on a CVS was proposed. Results suggest a possible application of this method to perform the rapid detection of the presence/absence of presumed packaging remnants in ex-food that can prelude or integrate the validated method. This method has shown a big potential in detecting efficiently the presence/absence of presumed packaging remnants also in low contaminated ex-food. Based on these results, it can be concluded that FFPs can be considered safe feed ingredients, even though some improvements in FFP processing, at the feeding plant, and specific implementations in innovative methods of analysis can be useful in further reducing their microbial loads and impurity.

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Chapter 5

FORMER FOOD PRODUCTS HAVE NO DETRIMENTAL EFFECTS ON DIET DIGESTIBILITY, GROWTH PERFORMANCE AND SELECTED PLASMA VARIABLES IN POST-WEANING PIGLETS.

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FORMER FOOD PRODUCTS HAVE NO DETRIMENTAL EFFECTS ON DIET DIGESTIBILITY, GROWTH PERFORMANCE AND SELECTED PLASMA VARIABLES IN POST-WEANING PIGLETS.

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ABSTRACT

The effect of a diet based on former food products (FFP) as alternative feed ingredients on in vitro and in vivo digestibility of DM, pig growth performance, and selected plasma biochemical variables was evaluated. After an adaptation period (7d), twelve post-weaning piglets (28 days old) were housed for 16 days in individual pens and assigned to two experimental groups: CRT (n=6), received a standard diet and FFP (n=6), received a diet in which conventional cereals (wheat, barley, corn), plasma powder and whey powder, were partially replaced by 30% FFP. During the experiment, stool and blood samples were also collected for DM in vivo digestibility and for plasma biochemical measurements, respectively. The results revealed that both in vitro and in vivo digestibility values were higher ($P<0.05$) for FFP diets compared to the control diets. At the end of the experiment, no differences in BW were observed between groups ($P=0.61$). ADG and ADFI were not affected by dietary treatment. Conversely, piglets on the FFP diet showed a lower FCR ($P<0.01$). Finally, dietary treatment also affected plasma glucose and urea, which in pigs fed FFPs increased and decreased, respectively. Taken together, these results suggest that the use of FFPs up to a level of 30% in post-weaning diets has no detrimental effects on pig growth performance, although more studies are needed to confirm these results.

INTRODUCTION

The livestock sector is highly dynamic worldwide. Human population growth, increasing urbanization and rising incomes are predicted to double the demand for, and production of livestock and livestock products in developing countries over the next decades. By contrast, in developed countries, many production systems have been increasing their efficiency and environmental sustainability (Pinotti et al. 2016). Sustainability includes the rational use of non-renewable and renewable resources (i.e. energy, raw materials) to combat climate changes, a lower load on the environment due to manure production and contaminant excretion by animals, and enhanced animal welfare and profitability (Pinotti et al. 2016; Renaudeau et al. 2014). Animal feed is the most important livestock production cost factor and represents up to 85% of the farm gate value of poultry (FEFAC 2017). Appropriate feeding and nutrition have thus become increasingly important as livestock systems strive to become more efficient (Tretola et al. 2017b). Converting losses from the food industry into ingredients for the feed industry, thereby keeping food losses in the food chain (Pinotti et al. 2016; Tretola et al. 2017b), can be considered a virtuous practice that should be implemented globally. Our research group (Pinotti and Dell'Orto 2011; Pinotti et al. 2014) has found that by using a balanced by-co-product combination it is possible to partially substitute the "classic" traditional source of energy and protein in animal diets. This substitution can be obtained without major changes in the composition of the diet, and may reduce feed costs, compared to traditional ingredients. In this respect former food products (FFPs), represents a "case study" in facilitating the transition to more sustainable material management and to a circular economy model. These concepts are also reported in the revised Waste Framework (European Commission, 2018a), which clearly discerns FFPs from waste. FFPs are covered by feed legislation, they are placed on the market as safe feed materials and consequently they cannot be downgraded to waste (European Commission, 2018a). A further milestone is the European Commission Notice (2018b) "for the feed use of food no longer intended for human consumption", which highlights the importance of FFPs for the European Union (European Commission 2018b), not only in terms of implementing the circular economy but also in mitigating the competition for natural resources between human and animal nutrition (EFFPA 2018). The conversion of three million tons of losses from the food industry into ingredients for the feed industry has been estimated to save more than 350,000 hectares of wheat (EFFPA 2018). This data seems to be in line with various recent works (Giromini et al. 2017; Tretola et al. 2017a; Tretola et al. 2017b; Pinotti et al. 2016) which defined FFPs as a "fortified version of cereals", with valuable energy content thanks to their sugar and high digestible starch content. An adequate fat content can also contribute to conditioning the energy density of these materials. Dietary starch represents one of the major energy sources for monogastrics, and starch effective digestion has a great impact on the animal energy status. If an animal's digestion occurs in the small intestine, the final product for absorption is represented by glucose, resulting in improved energy-yielding pathways. The lower the starch digestibility due to the high resistant starch content, the higher the

fermentation of the starch in the large intestine, leading to the production of volatile fatty acids, which are energetically less efficient than glucose (Gerrits et al. 2012). In general, the site, extent and rate of starch digestion are affected by intrinsic and external factors that may be interrelated and are thus not easily defined. It has been demonstrated that starch digestibility can be greatly altered by mechanical and/or thermal processing such as rolling, pelleting, flaking, extrusion, and expander processing due to changes in physicochemical starch characteristics (Anguita et al. 2006; Alsaffar 2011). Starchy food for human nutrition is usually subjected to heat treatments before consumption, resulting in an edible product, with increased nutritive values (Alsaffar 2011). In fact, the processing techniques applied during the manufacture of cereal products tend to destroy the food matrix and lead to the gelatinisation of starch, thus increasing the susceptibility of starch to enzyme hydrolysis (Alsaffar 2011). Nevertheless, starches consumed by livestock are still fed largely untreated and/or in raw forms (Giuberti et al. 2014). A higher starch digestion rate can increase the plasma level of metabolites and nutrients, as well as the energy intake (Yin et al. 2010). Van Kempen et al. (2010) and Giuberti et al. (2012) measured the post-prandial blood glucose response in swine that were fed diets containing purified starches or different starch sources. The authors highlighted that high digestible starch diets (based on normal and low amylose purified rice starches) were associated with an increased blood glucose response over time, which can lead to a higher insulin secretion (Giuberti et al. 2014). In this scenario, treated foodstuffs no longer intended for human consumption represent an attractive opportunity to introduce innovative and sustainable ingredients in pig nutrition. However, their use is still limited because of lack of information on their effects on pig growth and wellbeing. Nowadays, over many years of analysis of in-bound products, former foodstuffs producers owns enough experience to obtain consistent and standardized final products starting from a mixture of different substances with variable composition provided by different manufacturers. In order to increase the knowledge, and consequently the use of FFP in pig diets, this study evaluated for the first time the effects of a diet based on 30% FFP in pig diets, by assessing the impacts on in vitro and dry matter (DM) apparent total tract digestibility, growth performance, and hematological parameters in post-weaning piglets.

MATERIALS AND METHODS

Former food evaluation

A former foodstuffs producer based in the north of Italy has provided the complementary feed used for the formulation of the complete FFP diet. The complementary feed was composed by, in descending order of inclusion: leftover of the food industry (bakery products, pasta, confectionary products); wheat by-products (e.g. bran); wheat flour. Table 5.1 reports the nutrient composition of the complementary FFP product used in this study. Dry matter (DM), ash, ether extract (EE), crude fibre (CF), neutral detergent fibre (NDF), crude protein (CP), starch and sugar are all expressed in g/100g on DM and have been analysed as described below in the experimental diets section. The sugar content in the complementary feed has been evaluated as proposed by the Commission Regulation (EU) No. 152/2009. Additional information on the pure FFP product used in this study (namely FFP2) can be found in Giromini et al., (2017).

Table 5.1. *Analysed composition in g/100g on DM of the pure FFP product.*

	DM	Ash	EE	CF	NDF	CP	Starch	Sugar
Complementary FFP	91.3	3.22	11.0	4.41	15.7	11.5	42.3	20.2

DM= Dry matter; EE = Ether extracts; CF = Crude fibre; NDF = Neutral detergent fibre, CP = Crude protein

Experimental diets

Experimental feed ingredients are reported in Table 5.2. In FFPs complete feed, 30% of conventional cereals (wheat, barley, corn) and whey powder were substituted for 30% FFPs. Feed samples of CTR and FFPs diets were analysed for moisture, ash, CP, EE, CF, NDF and starch, as proposed by the Association of Official Analytical Chemists (AOAC) (2005) and the Commission Regulation No. 152/2009. The content of glucose, fructose and sucrose in the two diets has been analysed according to the method PT 119 NA-2017. Based on CTR and FFPs' composition data, the estimation of DE and ME values for pigs was calculated using the following equations:

$$DE\ (kcal/kg) = (53.7 * \%\ CP) + (75.8 * \%EE) + (41.1 * \%\ starch) + (7.6 * \%NDF) + (39.0 * \%\ residue)$$

$$Residue = OM - CP - EE - starch - NDF$$

$$ME\ (kcal/kg) = DE - (6.8 * \%CP)$$

The DE equation was originally elaborated by Sauvant et al. (2004), while ME was proposed by Noblet and Perez (1993) and further adapted by NRC (2012). Energy and chemical constituents are expressed on a DM basis in all equations. In Table 4.3, the analysed chemical composition of the two experimental diets is

presented. The diets were iso-energetic (16.0 MJ/kg DM) and iso-nitrogenous (20.5 % DM), and met NRC (2012) requirements (Table 5.3).

Table 5.2. I*ngredient compositions in g/100g of diet of the two experimental diets.*

Ingredients	**CTR[1]**	**FFPs[2]**
Former Foodstuffs	-	30
Barley	22.8	22.1
Dextrose	5	4,5
Flaked decorticated barley	4	0
Corn	6,5	4
Flaked corn	6,5	1
Vegetable fibres	1	1
Wheat	12,33	10,1
Flaked wheat	6	1
Wheat bran	3	2,48
Vegetable oil	1,5	0,5
Soy oil	1,5	0,5
Fish meal (65% protein)	2,5	2,6
Plasma powder	3,5	3,8
Whey powder	11	4,5
Soy f.e. 50[1]	3,5	3,5
Soycomil R[2]	5,5	4,55
L-lysine HCl	0,55	0,55
DL-methionine	0,23	0,23
L-threonine	0,25	0,25
L-tryptophan	0,08	0,08
Vitamin-mineral premix[3]	2,76	2,76
Total	100	100

CTR = Control diet; FFPs = Former foodstuffs products diet; [1]Soy extraction flour 50%; [2]High quality soy protein concentrate; [3]Provided per 100g of complete diet: 0.25g Vitaminic premix, 0.4g Benzoic acid, 0.5g Hydrated dicalcium phosphate, 0.4g Calcium carbonate, 0.15 Sodium Chloride, 0.8g Acidifying mixture, 0.06g Copper sulphate, 0.2g Sodium butyrate.

Table 5.3. *Analysed composition in g/100g or Mj/kg on DM of the CTR and FFPs diets.*

Dietary treatments	CTR	FFPs
Dry matter	90.9	90.3
Ash	5.62	4.94
CP	20.9	20.6
EE	5.94	5.92
Crude fibre	4.23	2.79
NDF	13.07	9.56
Starch	36.2	42.6
Glucose	6.00	6.17
Fructose	0.13	0.41
Sucrose	1.07	3.11
Calculated energy content		
Metabolisable energy	16.0	16.2

[1]CTR = Control diet: [2]FFPs = Former foodstuffs products diet; [3]CP = Crude Protein; [4]NDF = Neutral Detergent Fiber

Animal, housing and treatment

The in vivo trial was performed at the Experimental Animal Research and Application Centre in Lodi, at the University of Milan. The in vivo trial, which complied with Italian regulations on animal experimentation and ethics (DL 26/2014), was authorized by the Italian Health Ministry (number 711/-PR) in accordance with European regulation (Dir. 2010/6) and according to the principles of the 3Rs (Replacement, Reduction and Refinement). A conventional herd was selected to supply piglets. The herd was free from diseases according to the list of the International Office of Epizootic and from Aujeszky's disease, atrophic rhinitis, transmissible gastroenteritis, porcine reproductive and respiratory syndrome and salmonellosis, without history of PWD and Oedema Disease (OD).

A total of 12 piglets (8.52 ±1.73kg of BW) (Large White × Landrace) born from the selected sows, were used for this study. On arrival, individual faecal samples were collected in order to microbiologically verify the absence of haemolytic E. *coli* strains. After an adaptation period of one week, post-weaning piglets (28d old) were housed for 16 days in individual pens and randomly assigned to two experimental groups (CTR and FFPs) with similar initial body. Six piglets were assigned to the CTR group and received a standard diet, while six other piglets were assigned to the FFP group and received a diet in which conventional cereals (wheat, barley, corn) were partially substituted for 30% former food (Table 5.2). All pens were in the same room with the same environmental conditions. All pigs always had ad libitum access to the feed and water. Individual feed intake (FI) was recorded daily, piglet bodyweight (BW, kg) was recorded on days 0, 5, 9 and 16 of the experiment. Both individual feed intake and BW were used to calculate the average daily feed intake (ADFI

kg/day), average daily gain (ADG kg/day), and Feed Conversion Ratio (FCR kg/kg). Specifically, ADFI, ADG and FCR were calculated as the means of the entire experimental period (16 days).

In vitro digestibility

The *in vitro* digestibility (IVD) was serially performed on each experimental diet sample three times (three biological replicates); in each digestion series, CTR diet, FFP diet, internal control (flaked wheat) and blank (digestion enzymes alone) samples were included in triplicate (three technical replicates). A flaked wheat sample was included as an internal control feed ingredient because of similar undergone food-technology processes (e.g., heat treatments, steam flaking, pelleting) compared to FFPs and in order to compare IVD values obtain in previous studies (Giromini et al., 2017). The IVD of experimental diets was realised according to the protocol described by Boisen and Fernandez (1997) and Regmi et al. (2009) with minor adaptations. Of note, both protocol do not include mouth digestion since it is known that in vivo food is quickly swallowed and passed along the oesophagus to the stomach, where the pH is unfavorable for salivar alpha-amylase activity (McDonald et al., 2011). Briefly, a sample (0.5 ± 0.1 g) was mixed with 0.1 M phosphate buffer (pH 6.0). Subsequently, the pH of the solution was lowered with 10 ml of 0.2 M hydrochloric acid solution and adjusted at pH 2.0. A total of 1 ml of freshly prepared pepsin (P7000 Sigma-Aldrich) solution (25 mg ml–1) was added. In order to minimise bacterial fermentations during digestion, 0.5 ml chloramphenicol solution (5 mg ml–1 ethanol) were added to the mixture. The bottle was placed in a shaking water bath at 39°C for 2 h (simulation of the gastric phase). Subsequently, 10 ml of 0.2 M phosphate buffer at pH 6.8 were added, followed by 5 ml of 0.6 M NaOH and the pH was adjusted at 6.8 with 1 M hydrochloric acid or 1 M NaOH. Further, 3 ml of freshly prepared pancreatin (P3292; Sigma-Aldrich) solution (100 mg/3 ml) were added to the mixture. The bottle was placed in a shaking water bath at 39 °C for 4 h (simulation of the small intestinal phase). At the end of the second incubation, 10 ml of 0.2 M EDTA were added to the mixture and the pH was lowered to 4.8 with a 30% acetic acid solution. Further, 0.5 ml of Viscozyme (V2010; Sigma-Aldrich) were added to each bottle and incubated in a shaking water bath at 39 °C for 18 h (simulation of the fermentation process). In order to allow undigested soluble proteins precipitation 5 ml of 20% sulfosalicylic acid were added and the bottle was kept at RT for 30 min. The undigested fraction (UF) was then collected in a filtration unit using a porcelain filtration funnel lined with pre-weighed filter paper (Whatman no. 54; Whatman Inc., Florham Park, NJ, USA). The UF, along with the filter paper, was dried overnight at 65°C.

In vitro DM digestibility (%) was calculated as follows:

$$IVD(\%\ DM) = \frac{sample\ DM - sample\ UF\ DM}{sample\ DM} * 100$$

Apparent total tract digestibility of DM

The apparent total tract digestibility (ATTD) of DM was determined using the acid-insoluble ash (AIA) method for the determination of DM digestibility coefficients, which is a reliable technique for the measurement of the digestibility of pig diets (Kavanaghn et al. 2001). Faeces were thus collected before the morning feeding on days 4, 5 and 6, for the first monitoring period (initial ATTD), and on days 10, 12 and 16, i.e. at the end of the treatment (final ATTD). After collection, the faeces were immediately stored in a plastic bag and frozen pending analysis (-20°C). Fecal samples were pooled per pig per period, ground to pass through a 1-mm sieve, and sampled for analyses.

In accordance with the AIA analytical procedure reported below and based on Van Keulen and Young (1977), faeces were analyzed as follows:

(i) Each duplicate 5g sample of feed or faeces (dried and ground) was weighed in a 50 ml crucible, dried (2 h) in a forced air oven (135° C), cooled in a desiccator to room temperature, re-weighed (Ws), and then ashed overnight at 450° C. (ii) The ash was transferred to a 600 ml Berzelius beaker (without a spout) and 100 ml of 4N HCI were added. The mixture was then boiled for 5 min on a hotplate. (iii) The hot hydrolysate was filtered (Whatman No. 41) and washed free of acid with hot distilled water (85 to 100° C). The ash and filter paper were then transferred back into the crucible and ashed overnight at 450 C. (iv) The crucible and content were cooled in a desiccator to room temperature, weighed while containing ash (Wf) and re-weighed immediately after emptying (We). Percentage AIA was calculated from the equation:

$$AIA = (Wf - We)/Ws \times 100$$

where Wf = weight of crucible with ash, We = weight of empty crucible and Ws = weight of sample dry matter. All weighings were made to the nearest 91 milligrams.

Blood sampling and analysing

For the plasma biochemical analyses, blood was collected in EDTA containing and heparinized Vacutainers (Vacuette, Greiner Bio-One GmbH, Kremsmünster, Austria) after puncture of the jugular vein on day 0 and 16 of the trial. Blood samples were centrifuged (14 000 rpm for 15min at 10°C) to obtain plasma, which was stored at -20°C pending analysis. Plasma total proteins, albumin, globulins, urea, as well as Alanine aminotransferase (ALT-GPT), aspartate aminotransferase (AST-GOT), alkaline phosphatase (ALP), glucose, cholesterol, calcium, potassium and magnesium were measured via standard enzymatic colorimetric analysis using multiparametric autoanalyzer for clinical chemistry (ILab 650; Instrumentation Laboratory Company, Lexington, MA, USA) at the temperature of 37°C.

Statistical analysis

Data were analysed using IBM SPSS Statistics version 24 software (SPSS). In the case of in vivo data, the pig was considered the experimental unit. Data were tested for normality with the Shapiro-Wilk test before statistical analysis. *In vitro* and in vivo digestibility values, growth performance data (BW, ADFI, ADG and FCR) and plasma biochemical values were analysed using one-way analysis of variance (ANOVA) in order to compare means. The analysis was performed using the following model:

$$yij = \mu j + \varepsilon ij$$

where yij is the observations (values); μj is the mean of the observations for the j-th group (sample); and εij represents the within-sample random variability. The d 0 plasma Mg and K concentrations differed among treatments and were used as a covariate in the model. Differences with p-values < 0.05 were considered significant. *In vitro* digestibility data are presented as means of triplicate stimulations from three independent experiments ± standard error of means (±SEM), while in vivo data are presented as least squares means and standard errors.

RESULTS

In-vitro digestibility, apparent total tract digestibility and growth performance

The IVD values recorded for FFPs diet (86.5% ± 1.21) and Wheat (88.7% ± 0.7) were comparable (P>0.05); by contrast IVD values were significantly lower (P<0.05) for CTR diet (83.8% ± 1.02).

There were no differences in ATTD of the two experimental diets at the beginning of the trial, but the final ATTD was higher (P<0.05) in piglets fed FFPs. Data are reported in table 5.4.

Body weight measured at the piglets' arrival and at the end of the trial did not differ between diets (Table 5.4). Despite similar FI, piglets fed FFPs diet showed a lower feed conversion ratio (P<0.01) compared to piglets fed control diet.

Table 5.4. *Effects of partial replacement of conventional cereals by FFPs on growth performances and apparent total tract digestibility.*

Item	CTR[1]	FFPs[2]	SE	P values
Initial BW[3] (kg)	8.30	8.76	0.52	0.68
Final BW[3] (kg)	14.1	13.7	0.37	0.61
Growth Rate (%)	5.99	5.09	0.57	0.45
Average Daily Gain (kg)	0.29	0.31	0.01	0.52
Average daily Feed Intake (kg)	0.45	0.43	0.02	0.81
FCR[4] (kg/kg)	1.55^{x}	1.39^{y}	0.02	0.002
Initial ATTD[5] (%)	78.0	81.2	0.92	0.06
Final ATTD[5] (%)	78.6^{a}	83.3^{b}	1.10	0.02

[1]CTR = Control diet; [2]FFPs = Former foodstuffs products diet; [3]BW = Body weight; [4]FCR = Feed conversion ratio; [5]ATTD = Apparent total tract digestibility; ^{x,y} Means within a row with different superscripts differ significantly at P<0.01; a,b Means within a row with different superscripts differ significantly at P<0.05.

Plasma biochemical variables

The plasma biochemical variable measured in piglets are presented in Table 5.5. After 16 days of FFPs diet, no differences in the analysed haematological parameters have been observed, with the exception of increased (P<0.05) glucose and decreased (P<0.05) urea concentration in piglets fed FFPs.

Table 5.5. *Plasma biochemical variables in post-weaning piglets fed CTR and FFPs diet on day 0 and 12.*

Item	CTR		FFPs			
	d 0	d 12	d 0	d 12	SE	P value
Total Proteins (g/L)	49.8	51.5	49.7	49.6	0.74	0.45
Albumin (g/L)	27.7	26.9	26.5	24.8	0.55	0.22
Globulins (g/L)	22.1	24.6	23.2	24.7	0.83	0.96
A:G	1.31	1.16	1.16	1.09	0.06	0.72
Urea (mmol/L)	2.03[a]	1.58[a]	1.51[a]	1.03[b]	0.14	<0.05
ALT:GPT[1] (IU/L)	26.7	27.3	24.3	30.7	1.55	0.34
AST:GOT[2] (IU/L)	45.5	45.0	70.8	51.7	5.66	0.50
ALP[3] (mmol/L)	2.21	1.78	1.90	1.81	0.06	0.80
Glucose (mmol/L)	4.82[a]	5.08[a]	5.65[a]	6.18[b]	0.19	<0.05
Colesterol (mmol/L)	2.05	2.33	1.71	1.97	0.08	0.10
Calcium (mmol/L)	2.54	2.56	2.58	2.64	0.02	0.26
Fosforo (mmol/L)	3.00	3.16	2.46	2.88	0.06	0.13
Magnesium (mmol/L)	0.96	0.91	0.79	0.80	0.02	0.69

CTR = Control diet; FFPs = Former foodstuffs products diet; [1]ALT: Alanine aminotransferase, GPT: Glutammate Piruvate Transaminase; [2]AST: Aspartate Amino Transferase, GOT: Glutammate-Ossalacetate Transaminase; [3]ALP: Alkaline Phosphatase; [a,b] Values within a row with different superscripts differ significantly at $P<0.05$

DISCUSSION

Defining nutritional and functional proprieties of FFPs intended for pig nutrition is essential for establishing the best practices for their sustainable use (Pinotti and Dell'Orto 2011; Pinotti et al. 2014). However, the lack of knowledge regarding their effects on growth performance and animal wellbeing has limited their use in practise.

A concern in the use of FFP could be the variability in the composition, since they are a mixture of different substances. One load of product received by Former Foodstuffs Producers, in fact, can vary from the next even from the same manufacturer (e.g. biscuits may be plan on one day and chocolate covered on another), making a significant different in oil and energy. However, over many years of analysis of in-bound products, the former foodstuffs processors are able to predict the range of variation in analysis between different sources of product and between the same source and different loads. Moreover, has been demonstrated that differences among geographical regions in the chemical composition of bakery meals appear to be relatively small (Liu et al. 2018). This enables the processors to produce an average analysis of raw materials with very low statistical coefficients of variation, where this average values may be used to predict concentrations of nutrients in bakery meals (Liu et al., 2018). Consequently, the finished feed produced use raw materials whose nutrient data the producers are very confident with, and this is validated by finished product analysis. Summarizing, a good nutrient analysis of in-bound FFP ensures a good mix on similar materials to ensure consistent analysis.

Previous studies have demonstrated that candy co-products, and the related sugar-rich diet (total sugar content about 20% DM), can be used in a high percentage as an alternative carbohydrate source in diets for nursery pigs up to 12 days post-weaning (Guo et al. 2015). The present study provided data on the effects of partial replacement of conventional cereals with 30% FFPs on in vitro and in vivo digestibility, growth performance and selected plasma biochemical variables in post-weaning piglets. The study indicated improved feed digestibility and no detrimental effect on growth performance and animal wellbeing in piglets fed FFP, although further evaluations are necessary.

In vitro and apparent total tract digestibility

The FFP-based diet showed increased values of both in vitro and in vivo digestibility compared to the conventional diet. In fact, the differences between the tested diets in absolute values were smaller (Δ 2.7%) in the case of IVD, compared to 4.7% for ATTD. The reason for this is unknown, although the "maturation" of the digestive system in post weaning piglets, as well as the presence of highly digestible nutrients (probably starch) cannot be ruled out. An FFP-based diet contains a relative high concentration of FFPs (30%) characterized by a higher digestibility rate compared to the conventional ingredients commonly used in feed

formulation. These results are in line with a previous study performed by Giromini et al. (2017) in which the authors observed that IVD values of FFP ingredients not included in a complete diet were more digestible than flaked wheat, particularly regarding FFP samples with low fibre and a high starch content. Consequently, the differences in the digestibility values could be explained by the higher starch and lower fibre fractions and ash content, which differentiate the FFP from the CTR diet. Another possible hypothesis is related to the nature of FFPs. In contrast to ingredients commonly used in feed production, FFPs are originally intended for human consumption and thus are subjected to a wide range of processing techniques to improve starch digestibility. Compared to the untreated starch consumed by livestock (Giuberti et al. 2014), small food particles have a greater surface in contact with digestive enzymes, leading to a higher digestion rate (Alsaffar 2011). Also processing such as extrusion can significantly increase the digestibility of starches (Altan et al. 2009). This is probably because of the loss of structural integrity of starch granules due to the increased shearing action and kneading in the extruder barrel, which increase starch susceptibility towards enzymatic attack. It has also been demonstrated that the processing of starchy food can result in a decrease in anti-nutritional factors such as phytic acid and tannins (Rehman and Shah 2005). These authors suggested that the removal of tannins and phytic acid creates a large space within the matrix, which increases the susceptibility towards enzymatic attack and consequently improves starch digestibility (Rehman and Shah 2005). As reported by Singh et al. (2010), the nutritional characteristics of the food and in vitro starch hydrolysis are strongly influenced by processing. Anguita et al. (2006) observed an increased degree of starch hydrolysis, with values higher than 90% (at the end of incubation with pancreatin) for wheat, barley and oats when subjected to different technological processes. Extrusion cooking has also been demonstrated to significantly increase the in vitro digestibility of starches (Altan et al. 2009). Starch and starchy food with different levels of digestibility are characterized by a different rate and duration of the glycaemic response (Giuberti et al. 2012). Several studies have proved a good relationship between the rate of in vitro and in vivo digestion and the glycaemic response (Bellmann et al. 2017). This evidence is in accordance with our hematological data which showed an increased plasmatic glucose concentration in pigs fed FFPs compared to CTR, together with a reduction in urea concentration, as detailed below.

Growth Performance

The growth performance was within the expected range for the commercial breed chosen for the study. All animals remained in good health throughout the experiment and there were no morbidity or mortality issues. Although there was no significant difference in the growth rate ($P = 0.45$), in pigs fed FFPs, the feed conversion ratio (-10.3 %, $P<0.01$) was significantly improved. The reduction in feed conversion ratio was a combination of the small but non-significant changes in the average daily gain and variability within groups. However, these results could also be due to the different chemical composition of the diets, with special emphasis on starch content and its digestibility. Doti et al. (2014) argued that a high rate of starch digestion results in a sharp but short increase in blood glucose after feeding, facilitating fat deposition. In contrast, a gradual increase in

blood glucose from the digestion of slowly-digestible starch gives rise to a prolonged release of insulin in the blood, resulting in an increased lean deposition (Li et al. 2017). In line with our findings, previous studies have demonstrated that other former food sources have no detrimental effects on growth performance in newly weaned pigs in commercial farm conditions. Gou et al. (2015) investigated the effects of supplemental candy co-products as an alternative carbohydrate source to lactose in nursery pigs. Similarly to our study, in which most of the whey powder was replaced by simple sugars present in FFPs, Gou et al. (2015) partially replaced lactose in nursery diets with candy co-products containing about 51% of sugars from the food and candy industries without impairing growth performance, feed intake and feed efficiency. The authors (Guo et al. 2015) speculated that partially replacing lactose with carbohydrates from candy co-products could increase feed intake in early nursery phases but may cause a decrease in weight gain in later nursery phases. In fact, as demonstrated by earlier studies, whey powder with sucrose or other sweeteners may increase the feed palatability compared to diets containing only whey powder. However, it may be less efficient in the production of lactic acid by lactic acid bacteria in the gut and in the protein accretion in tissues, resulting in a reduced weight gain (Guo et al. 2015). Further evaluations are thus necessary to clarify the role of FFPs on carcass composition and gut microbiota community.

Haematological parameters

According to Addass et al. (2012), nutrition affects the blood values of animals. The analysis of haematological parameters represents a readily available assessment of nutritional health status of animals on feeding trials and may be considered as appropriate measure of nutritional status (Olabanji et al. 2007) or to distinguish normal state from stress which could be nutritional stress (Isaac et al. 2013). Blood analyses showed that the FFP diet increased ($P < 0.05$) glucose and decreased ($P < 0.05$) urea concentrations compared to the CTR diet, which is indicative of changes in metabolism associated with the diet. We speculate that these changes are due to the higher digestibility of the starchy food present in FFPs and their higher glycemic index. Ottoboni et al demonstrated that FFP ingredients are characterized by a higher hydrolysis index and predicted glycemic index, as well as the time trend in carbohydrate digestion compared to conventional cereal feed ingredients such as corn (Ottoboni et al. 2018). The high availability of glucose can lead to a reduction in the use of other energy sources such as a decreased deamination of amino acids via the urea cycle, as observed in an opposite scenario by Newman et al. (2017). It has also been demonstrated that, compared to less digestible starch sources such as trans-glycosylated starch, highly digestible starch is associated with reduced serum urea levels, which clearly suggests the decreased utilization of amino acids (Newman et al. 2017). In addition, glucose, irrespectively of insulin levels, decreases hepatic amino nitrogen conversion resulting in a decreased plasma nitrogen urea concentration (Newman et al. 2017). However, these aspects merit further evaluation.

CONCLUDING REMARKS

The increasing global need to find alternative ingredients to counteract food insecurity and the environmental impact of livestock sector, has promoted research in the field of non-conventional energy sources for feed production. In this study, for the first time FFPs were included up to a level of 30% in a complete diet. The results showed that FFPs can replace up to 30% of conventional cereal grains commonly used in pig nutrition without impairing growth performance and nutritional status in post-weaning piglets. Furthermore, the partial replacement of untreated starch with processed starch may increase the feed digestibility as a consequence of the nature of FFPs, which were originally intended for human consumption and subjected to a wide range of processing techniques. In conclusion, this study suggests that conventional cereal grains could be partially replaced by FFPs in pig production as a sustainable alternative energy and nutrient source to traditional feed ingredients. However, further studies are necessary to evaluate their effects on carcass composition and gut health.

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Chapter 6

FORMER FOOD PRODUCTS DECREASE THE ABUNDANCE AND BIODIVERSITY OF THE BACTERIAL COMMUNITY IN THE LARGE INTESTINE WHEN USED IN HIGH PERCENTAGE AS ALTERNATIVE FEED INGREDIENTS IN POST-WEANING PIGLETS.

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FORMER FOOD PRODUCTS DECREASE THE ABUNDANCE AND BIODIVERSITY OF THE BACTERIAL COMMUNITY IN THE LARGE INTESTINE WHEN USED IN HIGH PERCENTAGE AS ALTERNATIVE FEED INGREDIENTS IN POST-WEANING PIGLETS.

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ABSTRACT

In this study, Former Foodstuffs Products (FFPs) partially replaced common cereal grains in post-weaning piglet's diets in order to investigate the effects of these alternative feed ingredients on faecal microbiota richness, evenness, taxa composition and temporal changes in post weaning period. Twelve post-weaning piglets were housed for 16 days in individual pen and were fed two diets, including a standard wheat-barley-corn meal control diet and a diet containing 30% FFPs partially substituting for conventional cereals. Stool samples have been collected for the next generation sequencing of the variable regions V3 and V4 of the 16S rRNA gene in order to characterize the faecal microbiota composition. The FFP diet decreased the bacterial richness and evenness in the large intestine. Only minor differences have been observed in the taxa composition. Specifically, at the end of the trial FFP diet increased the amount of the Proteobacteria phylum and decreased the abundance of Lactobacillales genus, compared to the control diet. The core microbiota composition was slightly affected, while no differences between the two groups over time in the gut microbiota composition at the phylum level have been observed. Feeding post-weaning piglets with 30% FFPs diet affected the abundance and the evenness of bacteria in the large intestine, but with minor effects on taxa composition. Consequently, when FFPs are used in post weaning period, the chemical composition of the diet and the subsequent degree of microbial growth and fermentation in the large intestine need to be considered.

INTRODUCTION

The use of alternative feed ingredients in farm animal's diet can be an interesting choice from several standpoints, including efficiency and environmental sustainability (Giromini et al. 2017). Sustainability includes the rational use of non-renewable and renewable resources (i.e. energy, raw materials) under climatic changes, a lower load on the environment due to manure production and contaminants excretion by animals, but also deals with animal welfare and profitability (European Commission 2018). There is thus a growing need to characterize the nutritive and functional values of novel feedstuffs with the potential to be used as alternatives to traditional feedstuffs in livestock diets. Exploiting leftover streams is one way to decrease the environmental impact on the livestock sector. In particular, former foodstuffs products (FFPs) have a great potential to be used as alternative feed ingredients in livestock production. FFPs are mainly characterized by leftovers from the bakery and confectionary industry, such as bread, biscuits, snacks and broken pasta (European Commission 2018). The nutritional and functional characteristics of several categories of FFPs have been recently investigated (Giromini et al. 2017) and classified as "fortified version of common cereal grains". They also have no detrimental effects on the apparent total tract digestibility, growth performances and hematological parameters in post-weaning piglets when used in a complete diet to replace 30% of common cereal grains (Tretola et al. 2018)

The composition of FFPs, however, varies. The chemical composition of bakery meal, in fact, reflects the composition of the different ingredients included in the meal. Thanks to many years of experience and analysis of several loads of products received, former food processors are able to predict the range of variation in analysis between different sources of product and between the same source and different loads. For this reason, the finished feeds are produced starting from an appropriate mix of similar materials in order to result very consistent in the composition, validated by finished product analysis.Has ben also demonstrated that the chemical composition of bakery meals among different geographical regions is small (Liu et al., 2018). Dietary fibre is one of the main components the diets of pigs and is present in a variety of feedstuffs of plant origin, including cereal grains. Although a high dietary fibre content is associated with decreased nutrient utilization and low net energy values (Noblet and Le Goff 2001), it plays an important role in pig diets as it maintains normal physiological function in the digestive tract (Wenk 2001). Dietary fibre is the main substrate for bacteria in the gastrointestinal tract (GIT), and its inclusion in the diet may improve animal well-being as well as stimulating gut health and promoting bacterial growth (Wenk 2001; Knudsen et al. 2012). FFP ingredients are also characterized by a high starch content compared to conventional cereal grains, such as wheat (Giromini et al. 2017). In pig nutrition, starch represents the primary source of energy by providing at least 50% of the total cost of pork production, and it accounts for 80-90% of all polysaccharides in pig and human diets (Yin et al. 2004). In addition, resistant starch is an important substrate for bacterial species that reside in the colon (Louis et al. 2007).

Gut bacteria have several essential functions for the host. For example, they provide additional calories through different digestion processes, they also form a physical barrier against pathogens, ensure the development of the immunological system and gut maturation and modulate host gene expression (Konstantinov et al. 2004). The adaptation to dry food represents the third phase of the evolution and maturation of pig gut microbiota and it starts from the weaning (Fouhse et al. 2016). Modern pig production involves very early this important step in the piglet's life, usually at three or four week of life. Consequently, during the first week post-weaning, the microbiota becomes highly unstable with a strong decrease in bio-diversity, which will be restored after two or three weeks (Lalles et al. 2007). However, to the best of our knowledge the functional evaluation of FFPs with special emphasis on their impact on gut health has never been described. Its potential for an efficient animal production has therefore not yet been documented. The aim of this study was thus to evaluate the impact of a diet on the microbial community and biodiversity in the gut of post-weaning piglets using 30% of FFPs instead of conventional cereal grains.

MATERIALS AND METHODS

Former food product ingredients

In this study, the FFP diet was formulated by the use of a standard FFP product provided by an FFP-processing plant based in the north of Italy. Details on the ingredients used by the Italian former food processing plant to obtain the complementary FFP product (namely FFP2), together with its chemical composition, are reported in Giromini et al. (2017).

Animals and treatment

The *in vivo* trial was performed at the Experimental Animal Research and Application Centre in Lodi, at the University of Milan and it complied with Italian regulations on animal experimentation and ethics (DL 26/2014). The trial received the authorization by the Italian Health Ministry (number 711/-PR) in accordance with European regulation (Dir. 2010/6) and according to the principles of the 3Rs (Replacement, Reduction and Refinement). In this study have been used a total of 12 piglets (8.52 ±1.73kg of BW) (Large White × Landrace) born from selected sows free from diseases according to the list of the International Office of Epizootic. At arrival, individual faecal samples were analyzed to verify the absence of hemolytic E. *coli* strains. After an adaptation period of 7 days, post-weaning piglets (28 days old) were housed for 16 days in individual pens and randomly assigned to two experimental groups (CTR and FFPs) such that the initial body weight was similar. In total, piglets (n=6) assigned to the CTR group received a standard diet, while piglets (n=6) assigned to the FFP group received an FFP-based diet (Table 6.1). All piglets were in the same room with the same environmental conditions. All piglets always had ad libitum access to the feed and water.

Experimental diets

Experimental feed ingredients are reported in Table 1. In FFPs complete feed, conventional cereals (wheat, barley and corn) plasma powder and whey powder were partially substituted with 30% FFPs. The feed samples of CTR and FFP diets were analysed for moisture, ash, crude protein (CP), ether extracts (EE), crude fibre (CF), neutral detergent fibre (NDF), total dietary fibre (TDF), soluble and insoluble dietary fibre and starch, as proposed by the Association of Official Analytical Chemists (AOAC) (2005) and the European Commission Regulation No. 152/2009. The content of glucose, fructose and sucrose in the two diets has been analysed according to the method PT 119 NA-2017. In addition, metabolisable energy (ME) values for pigs were estimated by the use of equations formulated by Noblet and Perez (1993), further adapted by NRC (2012). To estimate the ME, digestible energy (DE) has been also estimated using the equation formulated by Sauvant et al., (2004). Energy and chemical constituents are expressed on a DM basis in all equations.

Table 6.1. *Ingredient composition (g/100g of diet) of the two experimental diets.*

Ingredients	CTR	FFPs
Former Foodstuffs	-	30
Barley	22.8	22.1
Dextrose	5	4,5
Flaked decorticated barley	4	0
Corn	6.5	4
Flaked corn	6.5	1
Vegetable fibres	1	1
Wheat	12.33	10.1
Flaked wheat	6	1
Wheat bran	3	2.48
Vegetable oil	1.5	0.5
Soy oil	1.5	0.5
Fish meal (65% protein)	2.5	2.6
Plasma powder	3.5	3.8
Whey powder	11	4.5
Soy f.e. 50[1]	3.5	3.5
Soycomil R[2]	5.5	4.55
L-lysine HCl	0.55	0.55
DL-methionine	0.23	0.23
L-threonine	0.25	0.25
L-tryptophan	0.08	0.08
Vitamin-mineral premix[3]	2.76	2.76
Total	100	100

CTR = Control diet; FFPs = Former foodstuff product diet. [1] Soy extraction flour 50%; [2]High quality soy protein concentrate; [3]Provided per 100g of complete diet: 0.25g Vitaminic premix, 0.4g Benzoic acid, 0.5g Hydrated dicalcium phosphate, 0.4g Calcium carbonate, 0.15 Sodium Chloride, 0.8g Acidifying mixture, 0.06g Copper sulphate, 0.2g Sodium butyrate

Sample collection

Faecal samples were collected from rectal ampulla, immediately frozen in liquid nitrogen and stored at -80°C until further analysis. Specifically, samples were collected on days D0, D8, D16 after a one week adaptation period. In total, 36 faecal samples were sent for Illumina sequencing: 6 samples per experimental group x 2 experimental groups x 3 sampling times (36 samples).

DNA extraction and sequencing

In order to extract bacterial DNA from stool samples, the QIAamp Fast DNA Stool Mini Kit (QIAGEN) was used, starting with 200 µg of stool following the manufacturers' procedure. The extracted DNA was quantified using Nanodrop ND2000 with a final concentration ranging from 3-10 ng/ul. Variable regions V3 and V4 of the 16S rRNA were amplified by PCR. Both the DNA quality assessment and the next generation sequencing (NGS) of the extracted amplicons were performed by BMR Genomics (Pavia, Italy) with universal primers (Takahashi, Tomita, Nishioka, Hisada, & Nishijima, 2014) on an Illumina MiSeq 300PE platform in order to obtain raw paired-end reads 2x300 bp.

NGS Data Analysis

The 16S rRNA gene sequences, quality control and operational taxonomic unit (OTU) binning were performed using the open source pipeline QIIME version 1.9.1 (Caporaso, et al., 2010; Bokulich, et al., 2013). Sequences were binned into OTUs based on 97% identity against the Greengenes reference database v. 13-8 (McDonald, et al., 2012). Calculations were performed after rarefying to an equal number of reads for all samples and for all time points to control for an unequal sampling effort. Microbial composition at each taxonomic level was defined using the 'summarize taxa' function in QIIME. Alpha and Beta-diversity (unweighted and weighted UniFrac metrics) were calculated using QIIME.

Analysis of similarity (ANOSIM) was used to evaluate whether gut microbiota differed ($P < 0.05$) among diets, also considering the effect size of the test (R). The LDA Effect Size (LEfSe) (Segata, et al., 2011) algorithm using the Galaxy online tool was performed to identify taxa differences between the two groups at each time point. Specifically, LEfSe uses the non-parametric factorial Kruskal-Wallis (KW) sum-rank test to detect features with significant differential abundance with respect to the class of interest. In addition, LEfSe uses linear discriminant analysis to estimate the effect size of each differentially abundant feature. The "microbiome" library in R, version 2.5.0, was used to estimate the core microbiota, which is the number of common microbes within an arbitrary set of samples. A two-way repeated measures ANCOVA was used to compare proportions of taxa as well as diversity indexes (Chao1, OTUs, PD-whole tree and Shannon indexes) between the two groups of pigs during the different sampling time points using a GLM procedure in SPSS (SPSS/PC Statistics 24 SPSS Inc., Chicago, 207 IL).

RESULTS

Experimental diets

The analysed composition of the control (CTR) and FFP based diets is reported in Table 6.2. The diets were iso-energetic (16.0 MJ/kg DM) and iso-nitrogenous (20.5 % DM), and met NRC (2012) requirements (Table 6.3). Specifically, the FFP diet was characterized by a lower CF, NDF and TDF content, but a higher starch content compared to the CTR diet.

Table 6.2. *Analysed composition (g/100g or Mj/kg on DM) of the CTR and FFP diets.*

Dietary treatments	CTR	FFPs
Dry matter	90.9	90.3
Ash	5.62	5.40
CP	20.9	20.6
EE	5.94	5.92
NDF	13.1	9.56
Total dietary fibre	16.4	13.9
Insoluble dietary fibre	14.6	11.6
Soluble dietary fibre	1.89	2.33
Glucose	6.00	6.17
Fructose	0.13	0.41
Sucrose	1.07	3.11
Starch	36.2	42.6
Calculated energy content		
Metabolisable energy	16.0	16.0

CTR = Control diet; FFP = Former foodstuff product diet; CP = Crude protein; EE= Ether Extracts; NDF = Neutral detergent fibre; o.m. = Organic matter

Gut microbiota

A total of 506,725, 491,093 and 557,399 sequences were acquired for days 0 (D0), 8 (D8) and 16 (D16), respectively. The alpha diversity analysis showed that the time did not affect either the number of OTUs or the diversity within a group. However, from D8 onwards, the FFPs decreased ($P < 0.05$) Shannon's index, together with a decrease ($P<0.05$) in the OTU average by D16. A summary of several alpha diversity indexes of all experimental periods is reported in Table 6.3.

Table 6.3. *Summary of next generation sequencing data and effect of FFP-based diet on diversity and abundance indexes at each sampling time (± standard deviation of the mean) in post-weaning piglets.*

	D0		D8		D16		P values[1]		
	CTR	FFPs	CTR	FFPs	CTR	FFPs	T	G	TxG
Shannon's	6.19±0.5^{AB}	6.39±0.4^{AB}	6.46±0.12^{A}	5.96±0.49^{B}	6.25±0.27^{A}	5.76±0.31^{B}	0.57	0.001	0.71
Chao1	549.71±53.2	556.7±92.7	603.9±43	581.2±56.3	609.3±28.6	541.3±55.7	0.67	0.10	0.55
OTUs	470.6±51.3^{ab}	489.±86.4^{ab}	529.3±37.4^{ab}	500.0±62.5^{ab}	534.1±31.7^{a}	435.1±46.6^{b}	0.78	0.03	0.16
PD-whole tree	35.3±2.82	36.5±4.1	38.1±2.45	36.4±2.62	37.8±1.9	33.2±2.6	0.70	0.10	0.28

CTR= Control diet, formulated to meet NRC requirements for post-weaning piglets; FFPs = Experimental diet in which 30% of FFPs partially replaced conventional cereal grains. D0, D8, D16 = day 0, day 8, day 16 of sampling, respectively.

[1] Probability values for the effects of Time (T), Group (G) and T X G.

[a,b] Values within a row with different superscripts differ significantly at P<0.05.

[A,B] Values within a row with different superscripts differ significantly at P<0.01.

Considering the diversity between samples (beta-diversity), the UniFrac distance metric incorporates information on the phylogenetic distances between observed organisms in the 16S rRNA dataset. In this study, this metric showed that the use of FFPs led to a qualitative modification in the gut microbial community over time. Specifically, while no changes were observed in D0 and D8, the unweighted UniFrac beta-diversity analysis showed a slight clusterization ($P < 0.05$, $R = 0.2$) in the microbial community between the two dietary groups (Figure 6.1) in D16.

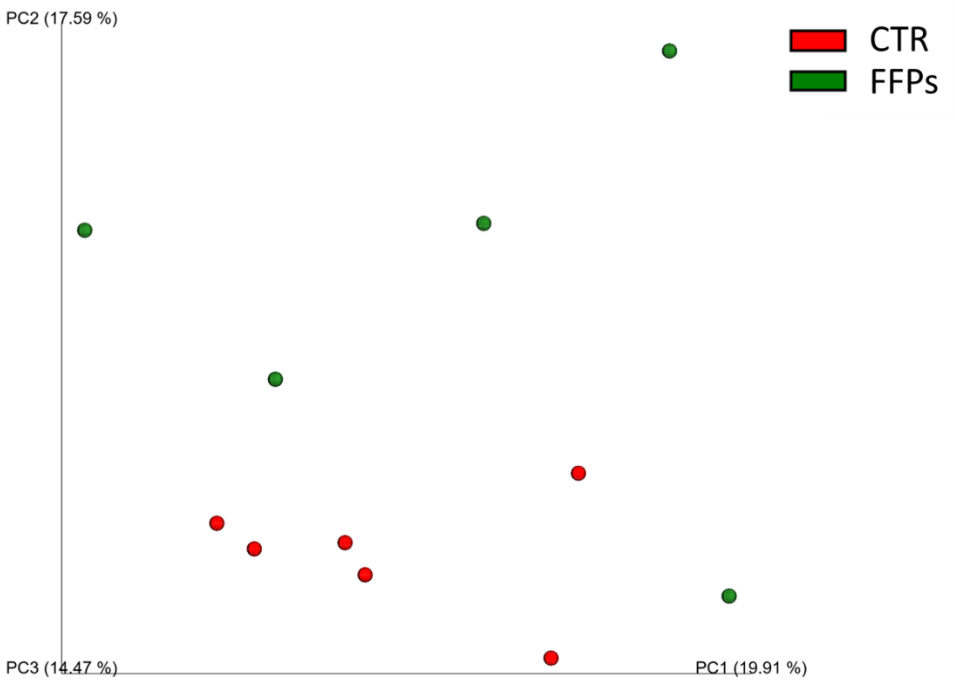

Figure 6.1. *PCoA of unweighted UniFrac beta-diversity of gut microbial communities from stools collected at the end of the experimental period. The first three principal coordinates (PC) from the PCoA are plotted. Symbols represent data from individual piglets, color-coded by the metadata indicated.*

Regarding the gut microbiota composition in D16, LefSe analysis showed that enriched phylotypes from the CTR group predominantly belonged to the class Bacilli and genus Lactobacillus, whereas phylotypes from the FFP group belonged to the phylum Proteobacteria (Figure 6.2A, B).

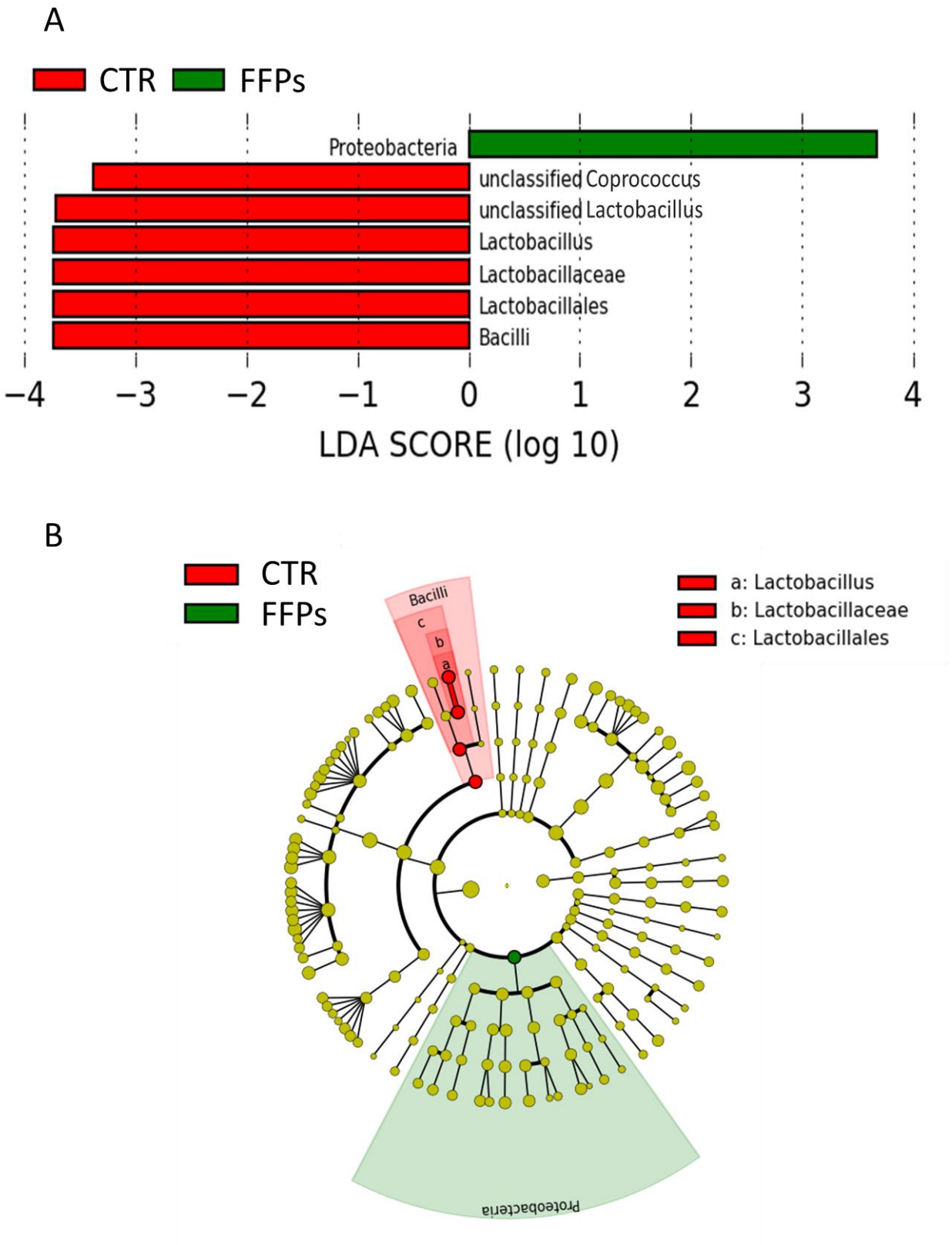

Figure 6.2. *A) Plot from LefSe analysis indicating enriched bacterial taxa associated with post weaning piglets fed with the FFP (green) or CTR (red) diet; B) Different representation of LefSe analysis in the form of cladogram, which is one way of representing significance and phylogeny. The red and green colors represent the branch of the phylogenetic tree that most significantly represents the CTR and FFP groups, respectively.*

After 16 days of the experiment, the core microbiota - defined as operational taxonomic units (OTUs) found in all samples - were comprised of 66 and 69 OTUs for the CTR and FFP groups, respectively. Despite the fact that these OTUs represented only 12.4% and 15.3% of the total OTUs for the CTR and FFP groups, they contained 68% and 59%, respectively, of the total sequences ($P > 0.05$, data not shown). The main core microbiota OTUs in CTR and FFPs groups at day 16 are illustrated in Figure 6.3.

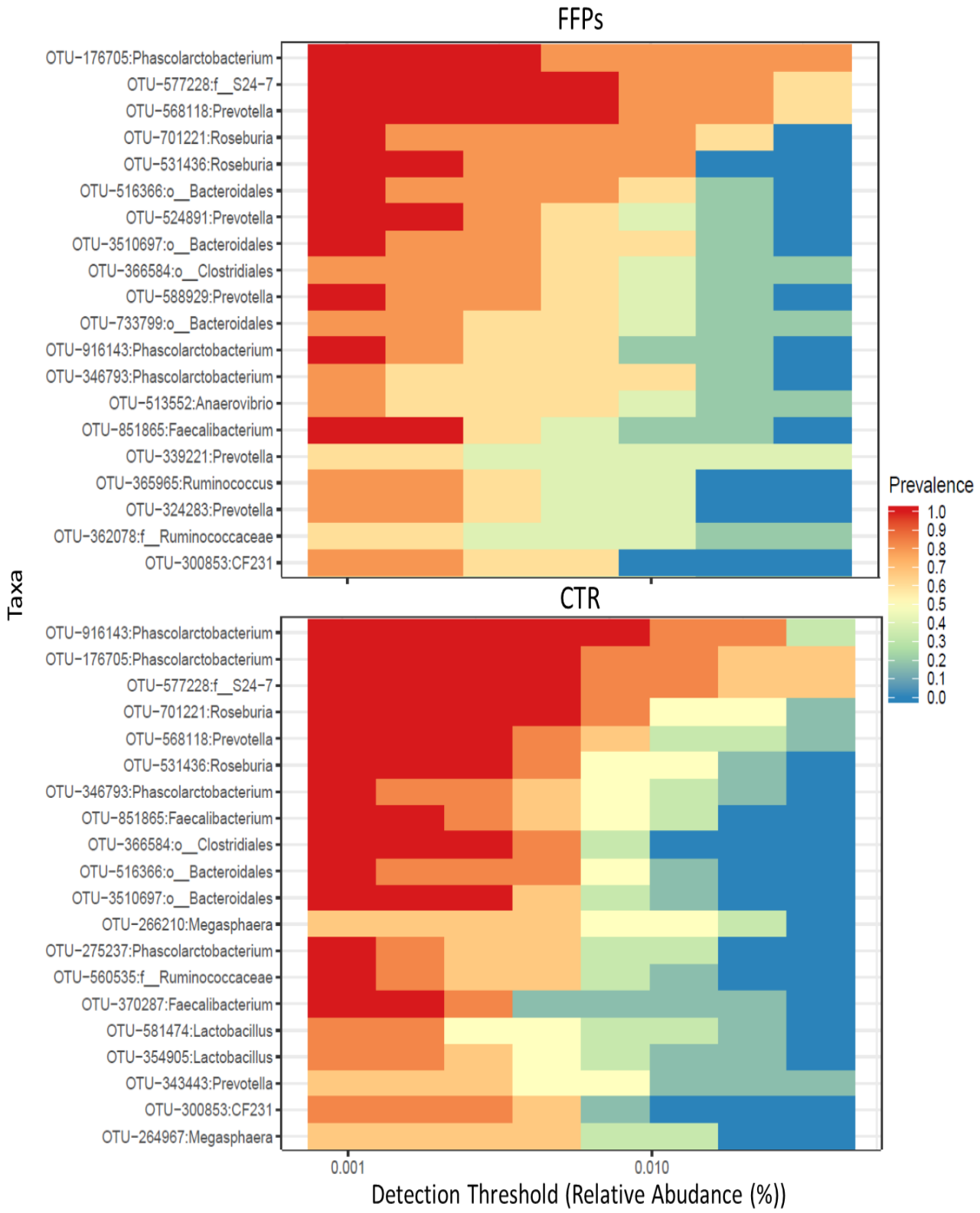

Figure 6.3. *Cross-sectional comparisons of the fecal microbiota in the FFP and CTR groups indicating specific keystone taxa detected at the end of the trial (D16).*

However, when temporal changes between CTR and FFPs are taken into account, repeated measurement ANCOVA analysis showed no differences between the two groups over time in the gut microbiota composition at the phylum level. Figure 6.4 shows an overview of the temporal changes in the bacterial community at the phylum level.

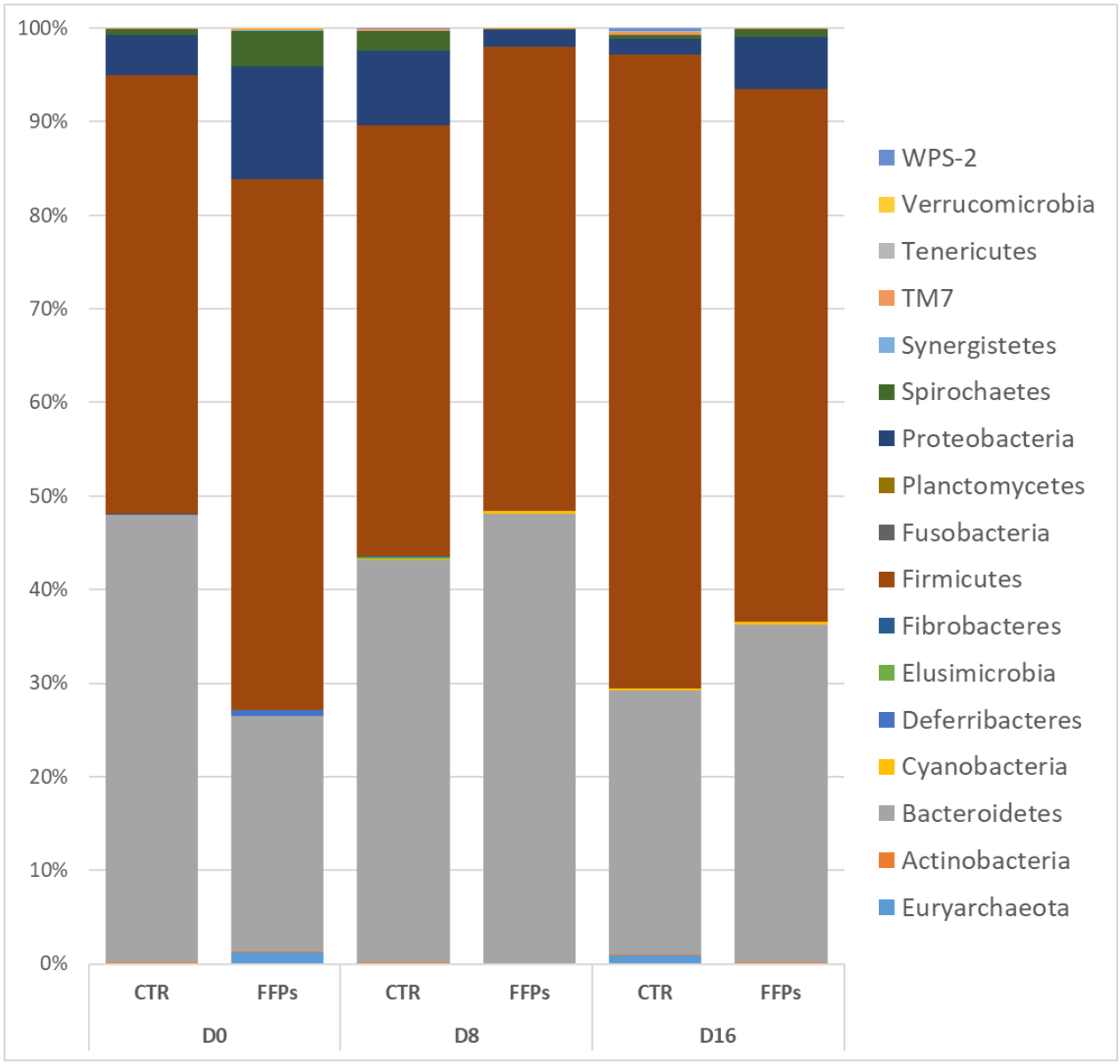

Figure 6.4. *Classification of gene sequences at the phylum level for the control and FFP pigs at each sampling time.*

Looking at each experimental group, in the CTR group, the main changes over time affect the proportion of Firmicutes, which increased significantly ($P < 0.01$) between D8 and D16 (45.9 ± 2.9% and 65.4 ± 3.5%, respectively). Similarly, the main changes in the FFP group over time affected the Bacteroidetes, which increased ($P = 0.007$) during the first period (27.7 ± 2.56% and 48.2 ± 3.49% in D0 and D8, respectively), and

decreased again to the original values (P = 0.008) (29.7 ± 6.01%) in the last sampling time D16. The time-trend of Firmicutes and Bacteroidetes in the two groups is shown in Figure 6.5.

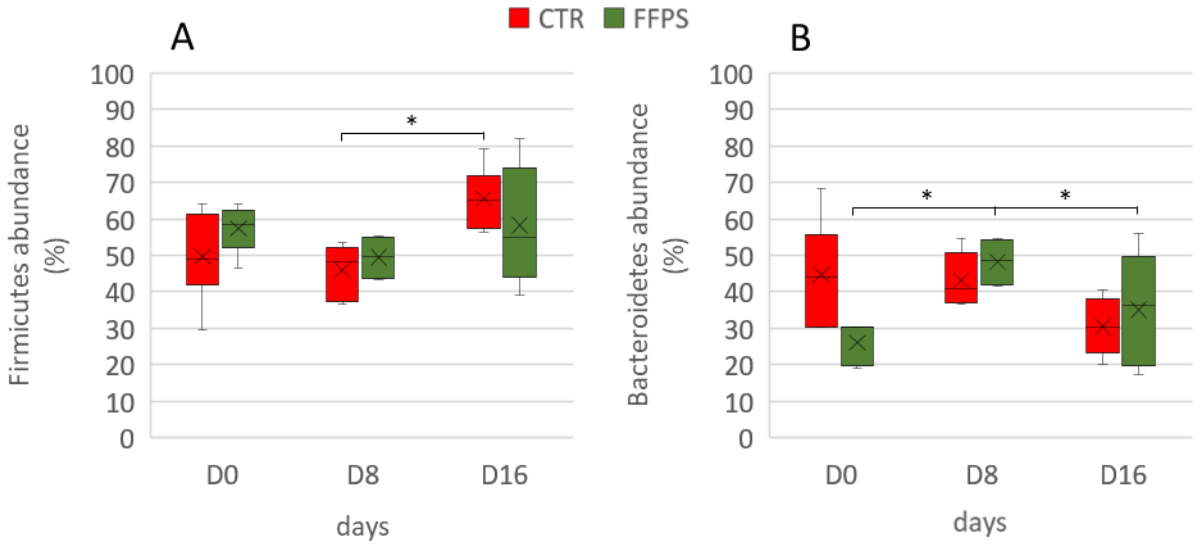

Figure 6.5. *Temporal changes in Firmicutes and Bacteroidetes phyla in CTR (red boxes) and FFP (green boxes) groups. The proportions of Firmicutes (A) and Bacteroidetes (B) are shown for post-weaning piglets at days 0, day 8 and day 16 of the experiment. The lower boundary of the box is the 25th percentile, the line and the cross within the box mark the mean indicators, and the upper boundary of the box indicates the 75th percentile. Whiskers (error bars) above and below the box indicate the 90th and 10th percentiles; the symbol * indicates P-values < 0.01.*

DISCUSSION

The livestock industry is an ideal platform to reintroduce high-value food leftover from the bakery and confectionary industry into the food chain in a circular economy. Current swine production systems, however, need to be very efficient and safe, where animal wellbeing is key. The use of alternative feed ingredients to replace conventional cereal grains can improve the sustainability of livestock. Accurate formulation of diets for pigs depends on accurate values for the nutritional composition of feed ingredients. Despite the variability of the ingredients used, the chemical composition of FFP products can be monitored and validated by the former food processors. However, due to the characteristics and properties of FFP products, such as the high digestibility, a diet containing 30% FFP could differently affect the bacterial population in the large intestine, compared to the standard diets.

Compared to a traditional diet, in our study the FFP diet showed a reduced TDF content, and an increased concentration of starch. We also found that at the end of the trial, the bacterial abundance and its biodiversity, indicated by the OTU number and Shannon's index respectively, decreased in the post-weaning piglets fed the FFP diet, compared to the CTR diet.

These results could be explained by the different nutritional characteristics of the diets, particularly the dietary fibre and starch content. Total dietary fibre is the sum of the dietary carbohydrates that are resistant to digestion by mammalian enzymes in the small intestine, but can be partially or completely fermented in the hindgut (IOM 2006). In the lower part of the digestive tract, the undigested compounds from the diet undergo further degradation by gut bacteria. After microbial degradation, the resulting products are above all volatile fatty acids which can be absorbed by the host cells (Metzler and Mosenthin 2008). This results in additional calories that can represent up to 30% of the pig maintenance energy requirements (Adesehinwa 2008). As a consequence, the bacterial abundance in the colon is higher compared to the small intestine, reaching a density of 1010 or higher per g^{-1} digesta.

The growth of bacteria depends on several factors such as temperature, pH and the substrates that can be metabolized (Wenk 2001). The chemical composition of the feed, as well as the technological processing and digestion process of the diets are all factors that can affect the availability of the substrates for bacterial growth (Wenk 2001). In accordance with our results, it has been observed that the content of dietary fibre can affect microbial growth and activity. As described by Jørgensen and Just (1994), in pigs fed a low fibre diet, the maximum microbial activity was observed at the end of the small intestine due to the lack of substrates in the large intestine that lead to a rapid reduction in microbial activity. Conversely, Jorgensen and Just observed that the maximum microbial activity was in the large intestine of pigs fed a high fibre diet, where the activity decreased slowly due to the presence of substrates throughout the distal intestine (Jørgensen and Just 1994).

These assumptions are of particular interest in post-weaning piglets, since dietary fibre digestibility is lower in young animals compared to adults (Noblet and Knudsen 1991). Although the TDF was lower in the FFP compared to the CTR diet, the FFP diet showed a higher soluble fraction of dietary fibre (DF). However, soluble fibres, such as beta-glucans are highly degradable in the upper gut and does not reach the lower gut (Jha et al. 2011). In contrast, insoluble DF is less accessible to the action of the endogenous enzymes in the upper gut, reaching the large intestine where it can be fermented by gut bacteria (Jha et al. 2011).

Another aspect of the FFP diet is the high starch content compared to the CTR diet. Previous studies have already classified FFPs as a "fortified version of common cereal grains" because of their high starch content (Giromini et al. 2017). Starch and starchy food products can be classified according to their digestibility, which is generally characterized by the rate and duration of the glycaemic response (Singh et al. 2010). Specifically, the starch can include rapidly digestible starch, slowly digestible starch and RS, which is defined as a portion that cannot be digested by the host enzymes (Englyst et al. 1999). Resistant starch occurs basically in all starchy foods and, since it can resist digestion by pancreatic amylase in the small intestine, it reaches the colon where it is fermented by endogenous bacteria.

Despite the higher starch content of the FFP diet, Ottoboni et al recently found a faster glucose release in the FFP diet used in this study, compared to the CTR, suggesting a lower RS content when FFPs partially replace common cereal grains in the diet of post-weaning piglets (Ottoboni et al. 2018). Their observations could be due to the nature of FFPs, which are composed of starchy food for human nutrition and subjected to processing techniques that increase the degree of starch hydrolysis (Anguita et al. 2006). In fact, the amount of RS can be affected by food processing, which can result in an increase or decrease in the RS values from those found in the raw product (Englyst et al. 2007). Nevertheless, starches consumed by livestock are still fed largely in untreated and/or raw forms (Giuberti et al. 2014). The physiological effects of RS in monogastrics are very similar to those attributed to dietary fibre. As for TDF, RS can be fermented by gut microbiota, thus providing an optimal substrate for more than 500 bacteria species, and thus altering their metabolic activities (Fuentes-Zaragoza et al. 2011).

We speculate that in our study, the smaller amount of fermentable substrates for the bacteria led to an impaired colonization of gut microbiota during the post-weaning period, as highlighted by the alpha diversity analysis. Early-life gut microbiota is a key factor for growth, immune system development and long-lasting health (Rodrìguez et al 2015). The richness and evenness of the bacterial community structure have been related to the stability of the gut ecosystem, and reduced bacterial abundance could be associated with an increased expression of pro-inflammatory cytokines in pigs (Mulder et al. 2009).

The biodiversity of the faecal bacterial community of FFPs and CTR pigs differed as early as day 8, with a decrease in the relative proportion of dominant bacterial species in FFP pigs, as indicated by the lower value of the Shannon index. A decrease in microbiota abundance and diversity is often related to a decreased ability of bacterial ecosystem to respond to gastrointestinal perturbations (McCann 2000) and to an increased

probability of pathogen colonization in the gut (Dillon et al. 2005). Stecher et al. (2010) showed that both an ecosystem with a low diversity and the abundance of commensal bacteria could reduce the ability of the gut ecosystem to resist to pathogens. On the other hand, Werner et al. (2014) demonstrated that the reduction in biodiversity might be associated with ecosystem instability, in the case of abiotic aggression.

Thus, in our study the replacement of 30% of traditional cereal grains with FFPs resulted in a decreased abundance and biodiversity of gut microbiota, which may reflect a less robust ecosystem, and an impaired complexity of the bacterial community after 8 days of the FFP diet. In addition, an increased number of intestinal bacteria results in an enhanced hindgut fermentation and production of volatile fatty acids (VFAs), which decreases the pH of the gut content (Suiryanrayna and Ramana 2015). A decrease in pH promotes the growth of beneficial bacteria, such as *Lactobacilli spp*., at the expense of potential pathogenic ones such as *Proteobacteria* (e.g. *Salmonella*) (Suiryanrayna and Ramana 2015).

In line with the literature, in our study the different TDF content of the experimental diets affected *Lactobacillu*s and *Proteobacteria* richness. Specifically, after 16 days of the experiment, LefSe analysis highlighted a decreased number of bacteria belonging to the genus Lactobacillus and an increased number of bacteria belonging to *Proteobacteria* in the FFP group, compared to the CTR group. Nevertheless, pigs fed the CTR and FFP diets showed a similar core microbiota, composed of a similar number of OTUs and both groups showed *Phascolarctobacterium* as the most representative OTU of the core microbiota.

Phascolarctobacterium belongs to the phylum of *Firmicutes* and produces short-chain fatty acids, including acetate and propionate. It is commonly found in the human gut, providing beneficial effects to the host (Wu et al. 2017). Other key taxa observed in both CTR and FFPs are the family S24-7 belonging to the order *Bacteroidales* which is a common component of mammals' gut microbiota (Ormerod et al. 2016); the genus *Roseburia*, belonging to the *Lachnospiraceae* family which is part of the commensal bacteria producing short-chain fatty acids, with positive effects on colonic motility, immunity maintenance and anti-inflammatory properties (Tamanai-Shacoori et al. 2017); and the genus *Prevotella*, a fibre-utilizing bacteria belonging to Bacteroidetes and associated with high fibre diets.

Of the OTUs characterizing the core microbiota of FFP pigs, the absence of the genus *Lactobacillus*, a member of *Firmicutes* (Figure 6.3), is of particular interest. Bacteria belonging to the genus *Lactobacillus spp*. are members of lactic acid bacteria because they produce lactic acid as the main end product of carbohydrate metabolism. They are considered as beneficial bacteria for the gut function and health of the host (Harmsen et al. 1999), and have been reported as the most prominent probiotics from the lactic acid bacteria group (Azad et al. 2018). *Lactobacillus* species found in the GIT have received notable attention due to their health-promoting properties such as improved gastrointestinal barrier function, intestinal permeability, and protection against inflammatory diseases (Harmsen et al. 1999).

In our study, however, CTR and FFPs followed the same temporal variation in the gut microbiota composition at the phylum level, without major alterations in taxa distributions throughout the whole experimental

period. To our knowledge, this study is the first to address the effects of former foodstuff products on gut microbiota when used to replace 30% of traditional cereal grains in the feed formulation for post-weaning piglets. Previous studies (Tretola et al. 2018) have demonstrated that this substitution has no detrimental effects on growth performance and apparent total tract digestibility. However, our results showed that the different nature of FFPs compared to untreated cereals used in feed formulation can result in a reduced TDF and, consequently, in a reduction in fermentable substrates for gut bacteria growth.

In fact, we observed a reduction in the abundance and biodiversity in gut microbiota of piglets fed the FFP diet for 16 days. However, with the exception of *Lactobacillus* genus and *Proteobacteria,* no significant differences were observed between the two groups and no diarrhea events were recorded. Nevertheless, further investigations are necessary to evaluate the impact of FFPs on the gut microbiota ecosystem and the effects of the lack of the *Lactobacillus* in the core gut microbiota of piglets fed FFPs in growing and finisher pigs.

CONCLUSIONS

The use of FFPs as alternative feedstuffs to convert food losses into animal protein food can mitigate the impact of the livestock industry on the environment and can reduce the competition between humans and pigs for food. Former foodstuff products could benefit animal health and performance; however, they also have various challenges. First, when used in high percentage, FFPs affect the nutrient profile of the feedstuff matrix beyond the variability intrinsic to the crops. An appropriate feed quality evaluation using modern feed evaluation techniques is therefore important. Second, there is wide variation in the composition of DF in different FFP ingredients, which has a major impact on their physico-chemical properties and digestion and fermentation characteristics in the GIT. When FFPs are used in pig diets, the DF content and the subsequent degree of microbial fermentation in the large intestine and the extent of absorption and utilization of the volatile fatty acids produced thus need to be considered.

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Chapter 7

GENERAL DISCUSSION

The livestock industry is an ideal platform to convert high-value food losses into high quality animal protein, reintroducing these unintentional losses into the food chain in a circular economy context. Ex-Food or Former Foodstuffs Products (FFPs) like rejected bread, different cookie products, high-quality baked goods and confectionary from industrial cookie and biscuit bakeries, no longer suitable for human consumption due to logistical, manufacturing or packaging defects (European Parliament, 2018), are the best candidates for these purpose. As omnivores, pigs are ideally suited to convert these non-human edible feedstuffs into high quality food animal protein. Dietary inclusion of leftover from bakery and confectionary industry can improve the livestock sustainability, reduce competition between human and animal nutrition, and reduce the waste of energy-rich ingredients. The main characteristics of FFPs is that they are generally rich in starch, since wheat flour is the main ingredient in all bakery products. Of note, when cooked, this starch is already thermally processed, making it highly digestible increasing its nutritive values. Moreover, if candy bars, snacks, cakes and other high-fat ingredients comprise a large part of the product mix, then FFPs will also be rich in fat concentration, increasing the energy content of the diet. Sugar is another ingredient highly used in most bakery products, and its final concentration in these products can be up to 25 percent. Consequently, the sugar content represents another factor to take into account in the final diet formulation for young piglets. In fact, an excess of sugar can lead to a nutritional (non-pathological) diarrhoea (Knudsen, Hedemann, & Lærke, 2012). For this reason, FFPs can be considered also as a valid option to replace lactose in diet formulation for post-weaning piglets. Despite FFPs can be considered a fortified version of common cereal grains due to the high energy content, their protein content is not of the best quality possible. In fact, the thermal processing and the overcooking can destroy part of these proteins. Consequently, FFPs cannot be considered an optimal alternative protein source. For all these reasons, feed quality and safety evaluation of FFPs, as well as their impact on gut health, is essential for the successful introduction of these innovative feed ingredients in diet formulations for livestock. Indeed, this thesis aimed to obtain more data on feed ingredient composition, especially their available nutrient and energy content, digestibility, effects on growth performance and metabolic status of piglets and on safety aspects related to their use to replace traditional cereal grains.

Nutritional properties

The outcome of the study reported in the **second chapter** of this thesis demonstrated that FFPs have a nutrient composition comparable to that of cereal grains but with a higher fat and energy content. However, compared to feedstock, the nutrient profile of FFPs is more variable according to the specific FFPs tested. The starch content, for example, has fluctuated within a range of 52.4% to 73.4%, as well as the digestible energy, ranged from 16.2 to 18.1 MJ kg^{-1} and the metabolisable energy, ranged from 15.9 to 17.9 Mj kg^{-1}. In addition, FFPs can be classified in low-fibre and high-fibre, according to their NDF content, whit averaged values of 6.55% and 13.2% respectively. Finally, also the FFPs *in vitro* digestibility can vary with a large extent, which can be comparable or lower to the flaked wheat one, according to their composition. It is known that crops

can also vary in quality due to genetic variation, agronomic, weather, harvest, and storage conditions. For FFPs, however, processing is an extra source of variation (Zijlstra, 2006). Former Food Products are mainly composed by leftover of bakery products and pasta, usually based on cereal flours (mainly wheat flour), with addition of eggs, sugar and fats (Bushuk & Scanion, 1993; Bushuk W. , 1986). These ingredients are mixed with water to form a dough or batter and then subdivided into portions for the second stage of processing by the application of heat. Industrial and domestic cooking induce severe physical modifications in food, resulting in a generalized increase of macro- and micronutrient bioavailability. Extruded wheat flours, due to their increased water absorption capacity, constitute an opportunity to increase bread output in bakery production. However, it can results in starch gelatinization, increased damaged starch content, together with a reduction in lipid oxidation due to enzyme inactivation, an increase in soluble fiber and a reduction in thermolable vitamins, anti-nutritional factors, and microbial load (Camire, Camire, & Krumhar, 1990). Extrusion also causes higher levels of mechanical damage in starch than traditional cooking methods (Wolf, 2010). Protein denaturation is another example of heat-induced food modification, and it usually facilitates their digestion, especially for plant proteins. However, in some cases protein digestibility can be reduced by thermal protein aggregation (Ercolini & Fogliano, 2018). Moreover, oil extraction of oilseeds using a range of processing techniques (solvent-extraction, expeller-press, and cold press) causes a range of residual oil content and therefore variability in energy value of the resulting meal or cake (Spragg & Mailer, 2007). As a consequence, extruder/expander conditions such as degree of cooking, preconditioning, and temperature can alter the nutritional value of feeds (Alonso, Aguirre, & Marzo, 2000; Altan, McCarthy, & Maskan, 2009). An overview of different FFPs ingredients and their chemical composition reported in literature is summarized in Table 7.1.

A standard FFP product does not exist and nutritional values vary depending upon the origin of the food leftover. For example, averaged values of ether extract (EE) content of FFPs assessed in the second chapter of this thesis is about 10%, although these values can reach the 7.5% in the bakery waste valuated by De Peters et al., (1997). Similarly, the neutral detergent fiber (NDF) content can be extremely variable with values ranging between 17.9% in a bakery waste product analysed by DePeters et al., (1997) and 5.4% in one of the FFPs analysed in this thesis. Another example is the crude fiber (CF) content, ranging from 1.3% in the bakery by products assessed by Kwak et al., (2006) and 4.5% in one of FFPs samples analysed by Giromini et al., (2017).

This variability offers a flexibility in formulating ratios according to nutrient/energy requirements of the target animals, but it is important to predict this variation in order to allow the feed industry to mitigate this variability and consider it during feed formulation. Therefore, it is possible to obtain diets with an equal planned nutrient density resulting in a predictable growth performance. For all these reasons, FFPs could effectively enlarge the raw material portfolio for feed industry and farmers, offering them a valuable and high quality alternative to common cereal grains.

Table 7.1. *Chemical composition in g/100g on DM of different Former Foodstuffs Products used as compound feed ingredients for pig nutrition.*

	DM	CP	EE	CF	NDF	ADF	Starch	Ash	NFE	NSC	ME (Mj/kg)	Source
FFP1	92.6	9.2	11.7	2.7	10.1	2.7	45.9	2.2	66.7	66.7	17.1	Giromini et al., (2017)
FFP2	91.3	11.5	11.0	4.4	15.7	5.0	42.3	3.2	61.2	586	16.1	
FFP3	91.5	12.0	9.9	4.5	16.2	5.1	44.6	3.4	61.7	58.5	15.9	
FFP4	91.9	9.1	4.8	1.9	5.4	2.0	73.4	1.4	74.7	79.3	17.0	
FFP5	91.3	10.9	10.4	1.5	7.7	1.3	56.4	2.1	66.4	68.8	17.4	
FFP6	93.8	7.3	12.9	1.3	5.6	1.2	51.5	1.8	70.4	72.4	17.9	
FFP7	88.0	10.9	9.8	2.2	-	-	41.9	-	-	-	15.48	Bouxin (2016)
Bakery bp	89.0	9.5	9.3	1.3	-	-	-	-	77.9	-	-	Kwak et al., (2006)
Eco-feed	90.0	12.9	9.6	0.4	-	-	-	1.9	65.2	-	-	Takahashi et al., (2012)
BW1	-	11.9	4.46	-	17.9	7.5	-	3.8	-	61.9		DePeters et al. (1997)
BW2	-	11.4	6.35	-	15.3	6.4	-	3.5	-	63.4		
BW3	-	12.9	11.7	-	7.5	4.9	-	3.5	-	64.3		
Wheat	10.9	10.1	1.5	2.7	12.6	4.4	63.0	1.4	73.3	74.4	15.5	Giromini et al., (2017)

DM= dry matter; CP= crude protein; EE= ether extract; CF= crude fibre; NDF= neutral detergent fibre; ADF= acid detergent fibre; NFE= nitrogen free extractive; NSC= non-structural carbohydrates; ME= metabolisable energy; FFP= former foodstuffs products; Bakery bp= bakery by products; BW= bakery waste.

Assuming that the variability issue can be overcome at industrial level (during the former food product preparation) a further step in FFP's nutritional evolution, it is to know their functional/dietetic properties. In this context, concerns can be related also to the carbohydrate digestion and the predicted glycaemic index of FFPs. The **third chapter** of this thesis assessed for the first time this issue relative to the FFPs ingredients. Specifically, the study observed that FFPs are characterized by a higher Hydrolysis Index (HI) and predicted Glycaemic Index (pGI) compared to the unprocessed corn. As for the chemical composition, also the HI and pGI of FFPs seem to be related to the nature and the processing of the different FFPs, with a high variability among different samples. In fact, according to their HI and pGI, FFPs can be divided into 3 main categories (high, medium and low pGI values) (Ottoboni et al., 2018). As expected, this variability in HI and pGI can be attributed to the different total sugar content (averaged values of 23.2%, 18.4% and 11.4% for high, medium and low HI and pGI values groups, respectively) (Giromini et al., personal communication). However, also the different treatments (cooking, extrusion, etc.) can affect the glucose release (Giuberti, Gallo, & Masoero, 2012; Slavin, Jacobs, & Marquart, 2000). Since carbohydrates represent the main energy source of a pig's diet, taking into account the variability in sugar content, HI and pGI in the different FFPs is crucial for a balanced diet formulation. The concept of pGI is commonly used in equine nutrition (Kronfeld, Treiber, &

Geor, 2005) where it has application in disorders associated with carbohydrates metabolism as well as in the nutrition of performance horses, but it is not properly addressed in pigs. Several studies in humans (Ludwig, et al., 1999; Holt, Brand, Soveny, & Hansky, 1992; Lavin & Read, 1995) suggested that ingestion of high-GI meals increases hunger and promotes overeating in a subsequent meal relative to low-GI meals, positive effects when pig nutrition is considered. However, while the potential impact on glycemic index it can be appreciated in some way, practical experience and research has demonstrated that in pigs soft feces are produced when animal are fed high-sugar diet (Mavromichalis, 2012). To note that this lassative effect is comparable to the lactose one and is never the cause of pathogenic diarrhea (Mavromichalis, 2012). If secretory scours occur, by adjusting the fiber content of the diet, the electrolyte balance could be restored and gut liquidity can be reduced.

Microbial load and presumed packaging remnants in FFPs: the safety issue

Beyond the nutritional evaluation, also the use of FFPs in animal feeding imply the evaluation of safety issues. Quality, traceability and safety of former foodstuffs represent key points to guarantee a safe re-use of biomass, according to a biosecurity approach. Like with products already used in animal nutrition, one such approach involves a systematic assessment at different levels of these former foodstuffs. In this sense, the safety aspect, as well as those related to the sustainability of the process, are among the most important. In term of safety, one of major concern that are reported for feed ingredients is their microbiological quality. In this respect, in the **fourth chapter** of this thesis, has been evaluated the microbiological load in different FFPs. Results obtained indicated that all FFPs analysed resulted not only safe from microbiological point of view, but also Salmonella spp. free. Moreover, all other microbiological contaminants resulted to be moderated or not detectable, and therefore always within the safeguard levels established by Health Protection Agency and European Regulation No 142/2011 (Regulation (EU) No 142/2011). These results highlighted the high quality of the analyzed FFP pointing out a low microbiological risk of the production process.

A further aspect that has been reported in term of safety is referred to the packaging material remnants. Processing methods to convert former foodstuffs into feed ingredients do not usually include pre-removal of packaging materials. For this purpose, feed processors routinely remove the packaging from ex-food mechanically in the feed plant. Former foodstuffs, in fact, are un-packaged though automatic methods in order to process a larger amount of product. The typical un-packaging process of FFPs can be summarized as follow: 1) the packaging is broken and reduced in size, 2) the now accessible FFPs are processed to a ready product and 3) remains of packaging materials are finally removed by several procedures such as sieving, magnetic attraction, Eddy current separation or based on density (van Raamsdonk, et al., 2011). Despite these processes, some packaging remnants such as plastic, resin, aluminium and pressed paperboard can residue in the final product and their presence appear to be unavoidable (Tretola, et al., 2017). Findings

reported in the chapter four confirmed the presence of presumed packaging remnants in FFPs. The contamination level, however, was always significantly below the tolerance level proposed by a EU member state authority (Germany's BMELV). In the same study has been also observed that the packaging remnants were distributed mainly from the 1-mm sieve mesh fraction. Packaging materials are not accepted as a feed ingredients according to Regulation (EC) No 767/2009 (Regulation (EC) No 767/2009, 2009), which prohibits the placing on the market and the use of feedstuffs containing packaging materials from the agri-food industry. This scenario imposes a proper approach in defining appropriate methods for detecting possible packaging contaminants and their remnants in former foodstuffs used in the feed chain. Different research groups have proposed progress in this way. Amato and co-authors (Amato, et al., 2017) have validated a gravimetric method for routine official controls, for the determination of packaging residues in feed. The proposed method can be summarised as follow: (1) visual selection of the undesired ingredients, which can be identified as remnants of packaging materials; (2) weighing of the selected materials; (3) defatting; (4) dehydration; (5) final weighing and (6) reporting of weight and percentage. Similarly, the study presented in the chapter four evaluated the possibility to apply computer sensing for the packaging remnants visualization (Tretola, et al., 2017). Results showed that the computer vision, when coupled with stereomicroscope for images acquisition, could be considered a rapid qualitative screening approach to estimate the presence of foreign materials in food and feed allowing the reduction of tedious and subjective human visual involvement. EFSA has evaluated few of these type of components concluding that the risk is limited (EFSA, GMO, 2008), however improvements in the methods for their detection and quantification are still necessary. The effects of a diet in which conventional cereal grains are partially replaced by FFPs have been addressed in the chapter four in order to evaluate the effect and the safe use of these alternative feed ingredients on feed efficiency, feed digestibility and growth performance in post weaning piglets.

FFPs have no detrimental effects on pig performance.

The use of alternative feedstuffs in the pig industry is not new. Traditionally, pigs were housed in small numbers and obtaining high growth performance was a less important goal. In small swine industries, pigs were fed feedstuffs that currently are regarded as alternative, such as leftover human food products (Pond & Lei, 2001). Such traditional production is still common practice in global small-scale swine production, particularly in Asia (Chen, 2009). Nowadays, the modern swine-production systems need to be more efficient, with high growth rates, safe, and consistent in pork products. These results can be obtained by the supply of affordable feed grains and a few protein sources to produce pork competitively (Pond & Lei, 2001). Results reported in the **fifth chapter** showed that FFPs can replace up to 30% the conventional cereal grains commonly used in pig nutrition without impairing growth performance and hematological parameters in post-weaning piglets. Furthermore, the study showed that the partial replacement of unprocessed starch with processed starchy FFPs may increase the feed digestibility as a consequence of the nature of FFPs,

subjected to a wide range of processing techniques. Currently, in some countries the inclusion of alternative feedstuffs in pig nutrition is considered advantageous only during period in which the price of common cereal grains increases, probably because the lack of a consistent supply of co-products. Across the world, few regions have a solid logistical system in place for the commercial swine industry to rely on co-products as main feedstuffs in swine diets. However, some European countries with a small land base, such as The Netherlands, have historically been heavily dependent on a large array of alternative feedstuffs (FEFAC , 2005). The introduction of alternative feed ingredients is a challenge for consistent growth performance and predictable pork quality. The presence of high amounts of free sugars in FFPs may negatively affect the animal gut health (Knudsen, Hedemann, & Lærke, 2012). Likewise, optimal gut health is an essential requirement to ensure food digestibility, and nutrients bioavailability. Several other challenging aspects need to be considered when adding alternative feed ingredients to conventional feeds. However, as demonstrated by the in vivo trial in the chapter four, by the use of modern feed formulation and feed processing is possible to achieve predictable swine growth performance and wellbeing.

The importance of feeding the gut microbiota

In the **chapter six** of this thesis, has been reported that the replacement of common cereal grains with 30% of a specific FFP ingredient resulted in a diet for post-weaning piglets with a reduced dietary fibre (DF) content and increased starch concentration. Dietary fibre consists of plant carbohydrates that are indigestible by endogenous animal enzymes (AACC, 2001) and includes cell wall compounds like cellulose, hemicelluloses, mixed linked β-glucan (βG), pectins, gums and mucilages (Davidson & McDonald, 1998). The most common classification of DF is based on its physico-chemical characteristics.

Classification of Dietary Fibre based on solubility in water

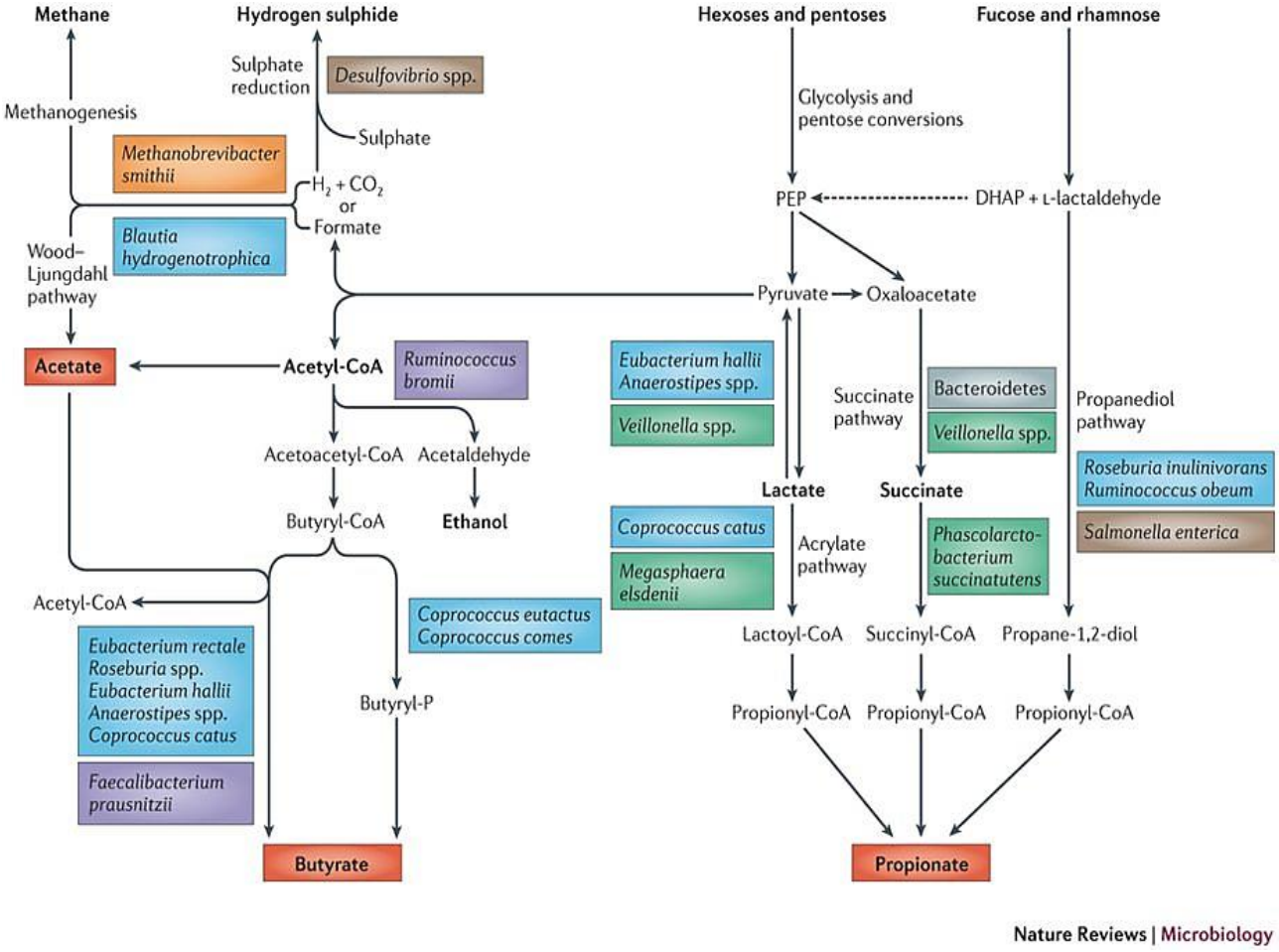
- Soluble Dietary Fibres: they can increase the viscosity of digesta and can lead to a reduced glycaemic response, by delaying gastric emptying and nutrient release as well as by inhibiting the action of alpha-amylase, thus regulating blood glucose. Soluble fibres include pectin, arabinoxylan, mixed-Linkage Glucans, Xyloglucans, and others (Davidson & McDonald, 1988).
- Insoluble Dietary Fibres: are characterised by a reduced accessible surface area and hydrogen-bonding networks which hold the carbohydrate chains together. These characteristics make insoluble fibres more difficult to be fermented by bacteria. Cellulose, lignin and beta-glucans are example of insoluble dietary fibres (Davidson & McDonald, 1988).

Specifically it can be classified in soluble and insoluble, according to its solubility in water. The chemical structures of specific soluble and insoluble fibres are showed in Figure 7.1.

The gut of piglets is a complex environment. In particular, in new-borns and around the time of weaning, the pigs' gut rapidly changes in size, has high protein turnover rates, undergoes rapid changes in gut bacterial communities, and quickly alters its digestive and immune functions.

The entire gastrointestinal tract (GIT) is populated by GIT microbiota, which includes bacteria, fungi, viruses and archea. Among several functions exerted by gut bacterial communities, they are able to break down complex cell wall polymers by different bacterial enzymes such as polysaccharides, glycosidase, proteases and peptidases (Salyers, Reeves, & D'Elia, 1996), degrading them into smaller components like sugar or amino acid. Thanks to the so-called “cross-feeding”, several species act together on a complex molecule to complete the process (Hugenholtz, Mullaney, Kleerebezem, Smidt, & Rosendale, 2013). Because of the bacterial fermentation, short chain fatty acids (SCFAs) together with other carboxylic acids, CO2, H2, and other end products such as ammonia and branched-chain fatty acids (BCFA) are produced, exerting several positive effects the GIT health. An example of microbiota “cross feeding” that results in the production of SCFAs is showed in Figure 7.1.

Figure 7.1. *Pathways that are responsible for the biosynthesis of the major microbial metabolites that result from carbohydrate fermentation and bacterial cross feeding (Louis, Hold, & Flint, 2014).*



Of the three main short-chain fatty acids (SCFAs; shown in red), acetate can be produced by many enteric bacteria from pyruvate via acetyl-CoA and via the Wood–Ljungdahl pathway by acetogens, such as Blautia hydrogenotrophica. Butyrate is formed from two molecules of acetyl-CoA by several Firmicutes, and the butyryl-CoA:acetate CoA-transferase is usually used to catalyse the last enzymatic step41. The main propionate production pathway is the succinate pathway, which is used by Bacteroidetes to generate propionate from carbohydrates and by some Firmicutes to produce propionate from lactate or succinate. Two other propionate formation pathways are found in some gut bacteria: the acrylate pathway, which uses lactate, and the propanediol pathway, which uses deoxyhexose sugars (such as fucose and rhamnose). Pathways that are involved in hydrogen metabolism and ethanol production are also shown. The bacterial species that are shown are based on studies of cultured isolates of dominant species and metagenomic analyses and are thus not exhaustive. Archaea are shown in orange, Bacteroidetes are shown in grey, Lachnospiraceae (Firmicutes) are shown in blue, Ruminococcaceae (Firmicutes) are shown in purple, Negativicutes (Firmicutes) are shown in green and Proteobacteria are shown in brown. DHAP, dihydroxyacetonephospate; PEP, phosphoenolpyruvate.

Host and a wide spectrum of environmental factors with feeding strategies and husbandry practices being the most significant factors influence the gut health and the complex interactions between microbiota and gut maturation, to maintain a healthy gut environment through life. Different studies demonstrated that a complex diet containing a wide range of DF molecules increases diversity of faecal microbiota in monogastrics (Rogowski, et al., 2015; Martens, Kelly, Tauzin, & Brumer, 2014). Taking into account all these findings, difference in the diet composition between traditional and FFPs-based diet used in the **chapter six** probably has led to a reduced bacterial growth and biodiversity in the cecum of the piglets fed FFPs-based diet. In fact, after 16 days feeding post-weaning piglets with FFPs diet, we observed a strong decrease in OTU number and Shannon's index values, compared to piglets fed control diet. It is well known that, despite the anti-nutritional properties of DF, it improves gut health (Williams, Verstegen, & Tamminga, 2001) and reduces post-weaning diarrhoea in pigs (Mateos, Martin, Latorre, Vicente, & Lazaro, 2006) by modulating the gut microbiota. More specifically, DF modify the digestion site and gut environment, thereby affecting the conditions for the proliferation of microbiota in the gut (Högberg & Lindberg, 2006). Moreover, DF provides energy for microbes supporting their proliferation. These properties of DF are also termed "prebiotic effect" (Gibson & Roberfroid, 1995), since it increases the number of cellulolytic bacteria, enhance the hindgut fermentation and production of VFA, decreases the pH of the gut content, promoting in this way the growth of beneficial bacteria (e.g. Lactobacilli spp.) instead of potential pathogenic ones (e.g. Clostridium or Salmonella) (Slavin J. , 2013). A diverse microbiota comprises a wide range of potential functions and a much larger gene pool, able to fulfil a wider variety of functions. Consequently, it is becoming clear that high diversity in gut bacterial population can be considered an indicator of high microbiota stability against potential perturbation (Lozupone, Stombaugh, Gordon, Jansson, & Knight, 2012). Gorham and co-authors (2017) found that the DF content of the diet can strongly affect the stability of the GIT microbiota within a 16-day period, with a high DF diet leading to a more rapid stabilization compared to low DF diet, even if the microbiota was still not completely stable by the day 16. Due to the effects on the food and feed nutritional characteristics, also the food processing can significantly affect the gut microbiota composition. Results reported in chapter 5 showed a decreased Lactobacillus and increased Proteobacteria abundance in piglets fed FFPs diet compared to the control one. We speculate that these differences are due to the origin of FFPs, composed by foodstuffs intended for human nutrition and subjected to heat and mechanical processing, while unprocessed grains mainly compose the conventional diet. According to our results, in a recent study (Costabile, et al., 2008) has been observed a decreased amount of Bifidobacterium and Lactobacillus bacteria, considered beneficial for the gut health, for humans fed processed wheat bran compared to whole grain supplement. In a similar study, Lappi and co-authors compared the effect of whole grains versus their processed components on gut microbiota, reporting a 37% reduction in Bacteroidetes within the GIT microbiota of human faecal sample, when fed refined wheat bread versus a whole grain rye bread (Lappi, et al., 2013). It can be summarized that the presence of DF in the gut significantly affects the gut microbial environment, creates a more favourable

conditions for gut health by stimulating the growth of 'beneficial bacteria' at the cost of 'harmful bacteria', which can have some negative impact on gut health. As a consequence, when alternative feed ingredients are used in pig diets, the DF content and the subsequent degree of microbial fermentation in the large intestine and the extent of absorption and utilization of the volatile fatty acid (VFA) produced have to be considered. Understanding what a healthy microbiota looks like and how FFPs can influence the composition of the gut microbial population improving eubiosis and/or reducing disbiosis, provides fundamental information to efficiently reconvert FFPs into value added products for animal nutrition (Nilsson, Johansson, Nilsson, Björck, & Nyman, 2008; Hoffman, et al., 2005).

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GENERAL CONCLUSION

The use of FFPs as alternative feedstuffs to convert food losses into animal protein food represents a promising opportunity to mitigate the impact of the livestock industry on the environment, but also to reduce the competition between humans and pigs for raw materials such as wheat.

By combining results about the chemical composition, the *in vitro* digestibility, together with predicted glycaemic index and hydrolysis index, this thesis demonstrated that the tested FFPs can be considered a valid alternative to cereal grains commonly used in pig nutrition. In fact, they are generally characterized by a valuable starch and fat content but they can be also considered an interesting source of carbohydrates with high glycaemic and hydrolysis indexes values. The particular nature of FFPs, originally intended for human nutrition, resulted also in high *in vitro* digestibility. This thesis also showed that FFPs are safe from a microbiological point of view and that all the loads of the microbiological contaminants were moderate or not detectable, always within tolerance levels established by the Health Protection Agency and European Regulation. All samples analysed in this thesis were also safe from the packaging remnants contamination point of view. Specifically, the presence of presumed plastic, board and aluminium foils was always significantly below the tolerance level proposed by an EU member state authority. Moreover, the innovative method tested in order to evaluate the presence of presumed packaging remnants has been found to have big potential in an efficient detection of presumed packaging remnants also in low contaminated ex-food.

All these results have been confirmed *in vivo*, in the context of a balanced diet. In this regard, the thesis demonstrated that FFPs can partially replace wheat, oat and corn in a balanced diet for post-weaning piglets without impairing growth performance and haematological parameters. Furthermore, the partial replacement of untreated starch with the heat-processed one present in FFPs increased the apparent total tract digestibility of the feed. Of note, no gastrointestinal disorders were recorded in piglets fed FFP diet, however the replacing of cereals affected the number and the evenness of bacteria in the large intestine, but with no important detrimental effects on taxa composition.

Concluding, this study implies that conventional cereal grains could be partially replaced by FFPs in pig production as a sustainable alternative energy and nutrient source to traditional feed ingredients. However, when FFPs are used, the chemical composition of the diet need to be considered to avoid detrimental effects on gut ecosystem.

Further studies are necessary to evaluate the effects of diet containing 30% FFPs on carcass composition and gut health in growing and finisher pigs, together with an assessment of sustainability features associated to the use of FFPs as sustainable and alternative feed ingredients in pig nutrition.

SATELLITE ACTIVITIES

HYDROLYSABLE TANNINS INHIBIT MICROBIAL GROWTH IN THE CECUM THEREBY AFFECTING SKATOLE AND INDOLE PRODUCTION

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ABSTRACT

Background

Bioactive substance such as hydrolysable tannins (HT) are of great interest in livestock production due to their proposed positive impact on health. In this regard the interaction between HT, gut microbiota and host needs to be considered to understand their respective biological functions and the bidirectional link between the biotransformation of HT into their metabolites by gut microbiota and the modulation of gut microbiota composition by HT and their degradation products, which could contributes to positivegut health outcomes. In this study, control and HT enriched (3%) diets were fed to 44 Swiss Large White entire male pigs.

Results

Hydrolysable tannins reduced the number of bacteria without affecting the within-community bacterial biodiversity, confirming the overall antibacterial effects of HT with no detrimental effects on the gut ecosystem. At the same time, due to the inhibition of the microbial growth, together with qualitative changes in the microbiota population between samples, HT supplementation could lead to a lower apoptosis of intestinal epithelial cells. This limits the availability of L-tryptophan from cell debris and consequently microbial mediated production of skatole production, the latter being a main component of boar taint in pork from entire males.

Conclusions

Hydrolysable tannins are a promising alternative to conventional antibacterial additives with no detrimental effects on pig gut health and appealing properties for reducing synthesis of main components of boar taint.

Keywords: Hydrolysable tannins, gut microbiota, gut health, boar taint.

BACKGROUND

Condensed tannins (CT) have long been considered 'anti-nutritional' factors in monogastric nutrition. Their presence in diets of pigs has been associated with impaired palatability and, consequently, impaired feed intake and lower protein digestibility [1]. The main cause of these negative effects is their ability to form insoluble complexes with dietary proteins, carbohydrates [2], digestive enzymes [3] and the bacterial cell envelope [4]. Furthermore, it has been shown that they can also affect the intestinal mucosa [5]. On the other hand, health-enhancing properties such as antibacterial [6, 7, 8], antidiarrhoic [9] and antioxidant [10] effects of both CT and hydrolysable tannins (HT) have been demonstrated. Recent studies showed that dietary CT and hydrolysable tannins (HT) supplementation reduced risk of livestock diseases and transmission of zoonotic pathogens [11] making these sources of bioactive compounds an interesting alternative to conventional antibacterial additives.

Recently, it has been demonstrated that compared to CT, which are not degraded in the digestive tract, dietary HT and their hydrolysis product like urolithins [14, 15] have lesser adverse impact on pig growth performance [12, 13]. In line with these findings, it appears that pigs tolerate certain quantities of both CT and HT-rich feedstuffs without suffering from toxic side effects [14] but instead being beneficial for intestinal health [15]. For instance, 3% HT in the pig diet increased height, perimeter and surface area of villi by up to 23, 20 and 38%, respectively [15] and 1% dietary HT increased mucosal thickness by 14% in the duodenum [15]. Furthermore, dietary HT supply reduced apoptotic cell count, with the most notable effect observed in the ascending colon [15].

A recent trial with entire male (EM) pigs revealed that dietary HT supplementation decreased skatole production in the hindgut [12]. These findings are in line with the lower apoptotic cell count, which is believed to be the main source of tryptophan for the microbial production of indolic compounds [16, 15]. In fact, it has been hypothesized that degradation products of HT reduce the production of skatole by affecting either the gut microflora or the process of enterocyte proliferation and apoptosis [12]. Given the above, in this study we hypothesize that dietary supplementation of HT to the finisher diet of EM reduce the development of boar taint by a quantitative and qualitative modification of gut microbiota.

MATERIALS AND METHODS

Animals and Diets

Forty-four Swiss Large White EM originating from 11 litters weighing 26.0 ± 4.95 kg (average ± standard deviation) were randomly selected and assigned to four dietary treatments: High amount of polyunsatured fatty acids (PUFA) without chestnut extract containing HT (H-); High PUFA with 3% chestnut extract containing HT (H+); low PUFA without chestnut extract containing HT (L-) and low PUFA with 3% chestnut extract containing HT (L+). The chestnut extract (Castanea sativa) (Silvateam, San Michele Mondovì Italy) contained approximately 50% HT [13]. All diets were formulated to be isocaloric and isonitrogenous and to meet nutrient requirements according to the Swiss feeding recommendations for pigs (Agroscope 2015). The experimental diets were offered for 92 to 104 d ad libitum in a pelleted form. All pigs were reared in group pens, equipped with automatic feeders and individual pig recognition system (Schauer Maschinenfabrik GmbH. & Co KG, Prambachkirchen, Austria) as described previously by Bee et al. [63] The pigs were switched from the grower to the finisher diet when the average BW of all 48 pigs was on average 60 kg.

Slaughter procedure, carcass measurements, tissue sampling and meat quality assessment

Following the procedure described in detail by Bee et al. [13], pigs were slaughtered at 170 d of age at the research abattoir of Agroscope Posieux (Switzerland). Within 30 min after exsanguination, the content of the caecum, ascending and descending colon was collected and immediately frozen in liquid nitrogen and stored at -80°C until analysis.

DNA extraction and sequencing

In order to extract bacterial DNA from caecum samples, the QIAamp Fast DNA Stool Mini Kit (QIAGEN) was used starting from 200 µg of samples following the manufacturers' procedure. The DNA quality was assessed by capillary electrophoresis using a Fragment Analyzer (Advanced Analytics) and DNF-487 kit (Advanced Analytics). The extracted DNA was quantified using Nanodrop ND2000. The variable regions V3 and V4 of the 16S rRNA were amplified by PCR. The Next Generation Sequencing (NGS) of the extracted amplicons was performed by Microsynth AG (Balgach, Switzerland) in order to obtain raw paired-end reads.

NGS Data Analysis

The 16S rRNA gene sequences, quality control and operational taxonomic unit (OTU) binning were performed using the open source pipeline Quantitative Insights Into Microbial Ecology (QIIME) version 1.9.1 [65, 66]. Sequences were binned into OTUs based on 97% identity against the Greengenes reference database [67]. Microbial composition at each taxonomic level was defined using the summarize taxa function in QIIME.

Alpha and Beta-diversity (unweighted and weighted UniFrac metrics) were calculated using QIIME package. Analysis of similarity (ANOSIM) was used to evaluate whether gut microbiota and HT supplementation and/or PUFA level differed ($P < 0.05$) among diets, also considering the effect size of the test (R). Since the dietary PUFA level had no effect on microbiota composition, thus only HT levels were considered in the analysis. As Shannon's index data are not normally distributed, the non-parametric Kruskal-Wallis test was applied using SPSS (SPSS/PC Statistics 23 SPSS Inc., Chicago, 207 IL). The same statistical software was used to perform Mann-Whitney test to evaluate the most significant different phylotypes between the Ctrl and HT groups.
The heatmap figure was implemented by the QIIME package 267 at the species level. To identify bacterial taxa whose sequences were differentially abundant between the Ctrl and HT groups, emphasizing both statistical significance and biological relevance, LEfSe online tool has been used [68]. The differential features were identified on the genus level, LEfSe analysis was performed under the following conditions: the alpha value for the non-parametric factorial Kruskal-Wallis sum-rank test among classes was < 0.05 and the threshold on the logarithmic LDA score for discriminative features was > 4.0. The heatmap of Figure 4 was constructed using the heatmap 2 function of the R gplots package [69].

RESULTS

A total of 5,172,998 paired-end 250-bp reads and 1,786 Operational taxonomic units (OTUs) were acquired. On average, 117,568.136 sequences per sample were obtained by 16S rRNA sequencing from caecum content collected from each pig. Alpha diversity analysis showed that the Chao1 index, based upon the number of OTUs found in a sample, was lower (P = 0.001; Table1), with average OTUs being consequently lower (P = 0.001) in the Ctrl than in the HT group (Table S.1). On the contrary, the analysis of the Shannon's index revealed that dietary HT supplementation did not (P = 0.167) affect the microbiota biodiversity (Table 1).

Table S.1. *Summary of Next Generation Sequencing data.*

	Ctrl (n=22)	HT (n=22)
Sequences	1196186 ± 201114	1155176 ± 168900
Chao1	1124 ± 130*	1020 ± 70*
OTUs	986 ± 113*	886 ± 62*
Shannon	4.99 ± 0.34	5.02 ± 0.11

* The number of OTUs and the richness estimator Chao1 between the Ctrl and the HT supplemented group differed (P=0.001).

The 16S rRNA datasets were then analyzed using UniFrac, an algorithm that measures similarity between microbial communities based on the degree to which their component taxa share branch length on a bacterial tree of life [17]. For unweighted UniFrac Beta diversity analysis, a clear ($P < 0.05$, $R = 0.8$) clusterization between the Ctrl and the HT group was found (Figure 1A). The amount of variation explained by the principal axes was 33.66% for PC1, 11.46% for PC2 and 5.22% for PC3. Similarly, the weighted UniFrac analysis revealed a significant ($P < 0.05$) but, based on the lower R value (0.2), a less distinct clusterization of microbiota composition between the Ctrl and the HT group (Figure S.1B). In this case, the amount of the full variation explained by the principal axes was greater than that observed in the unweighted analysis (43.17%, 17.19% and 7.79% for PC1, PC2 and PC3, respectively).

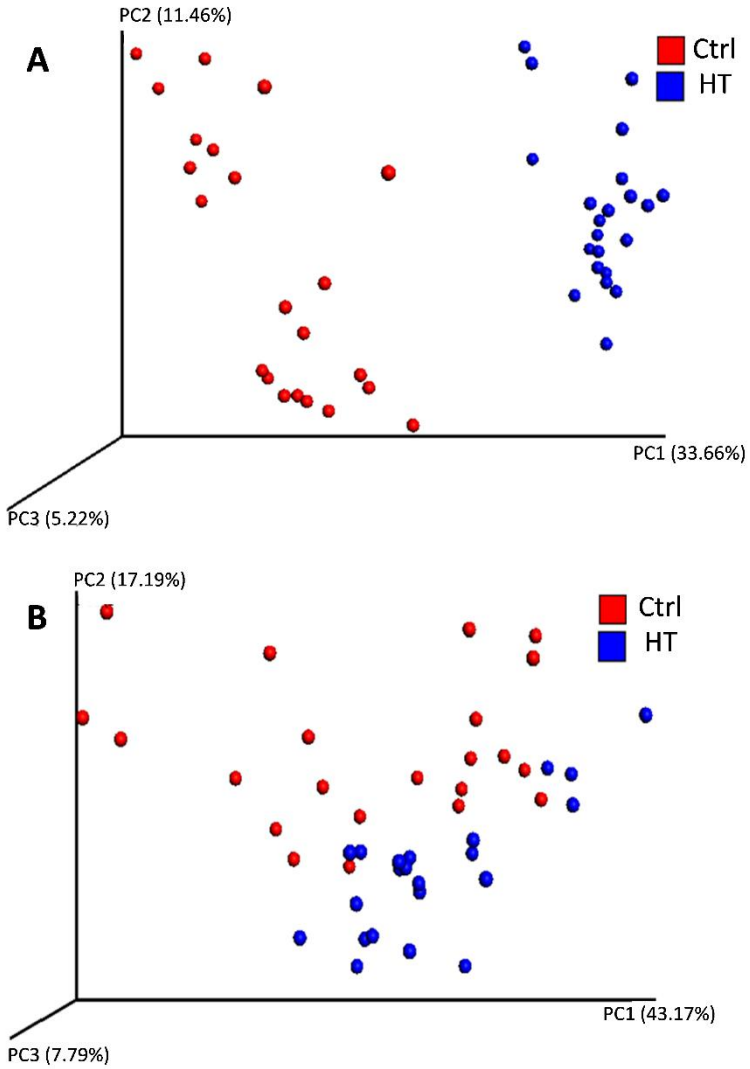

Figure S.1. *Beta diversity analysis: Unweighted (A) and weighted (B) UniFrac principal component analysis (PCA) of caecum microbiota collected from pigs fed the control (Ctrl) or the diets supplemented with 3% chestnut extract containing hydrolysable tannins (HT).*

The taxa summary plot at the phylum level, showed that compared to the Ctrl group, dietary HT supplementation decreased the amount of Proteobacteria and increased the amount of Spirochaetes and Verrucomicrobia (Table S.2; Figure S.2).

Table S.2. *The most significant different phylotypes between the control (Ctrl) and the hydrolysable tannin (HT) group.*

Taxonomic rank		Ctrl	HT	P-value
Phylum	*Bacteroidetes*			
Family	*Unclassified Bacteroidales*	0.106	0.182	< 0.001
Phylum	*Spirochaetes*	0.058	0.096	< 0.001
Order	*Spirochaetales*	0.026	0.051	< 0.001
Family	*Spirochaetaceae*	0.025	0.050	< 0.001
Genus	*Treponema*	0.025	0.049	< 0.001
Phylum	*Firmicutes*			
Class	*Bacilli*	0.053	0.014	< 0.001
Family	*Veillonellaceae*	0.047	0.025	< 0.001
Genus	*Unclassified Ruminococcaceae*	0.077	0.052	0.002
Genus	*Oscillospira*	0.022	0.042	< 0.001
Order	*Lactobacillales*	0.051	0.011	< 0.001
Family	*Streptococcaceae*	0.045	0.007	< 0.001
Genus	*Streptococcus*	0.045	0.007	< 0.001
Phylum	*Proteobacteria*	0.051	0.028	< 0.001

Data are expressed as the relative abundance of all sequences in each group expressed as percentage

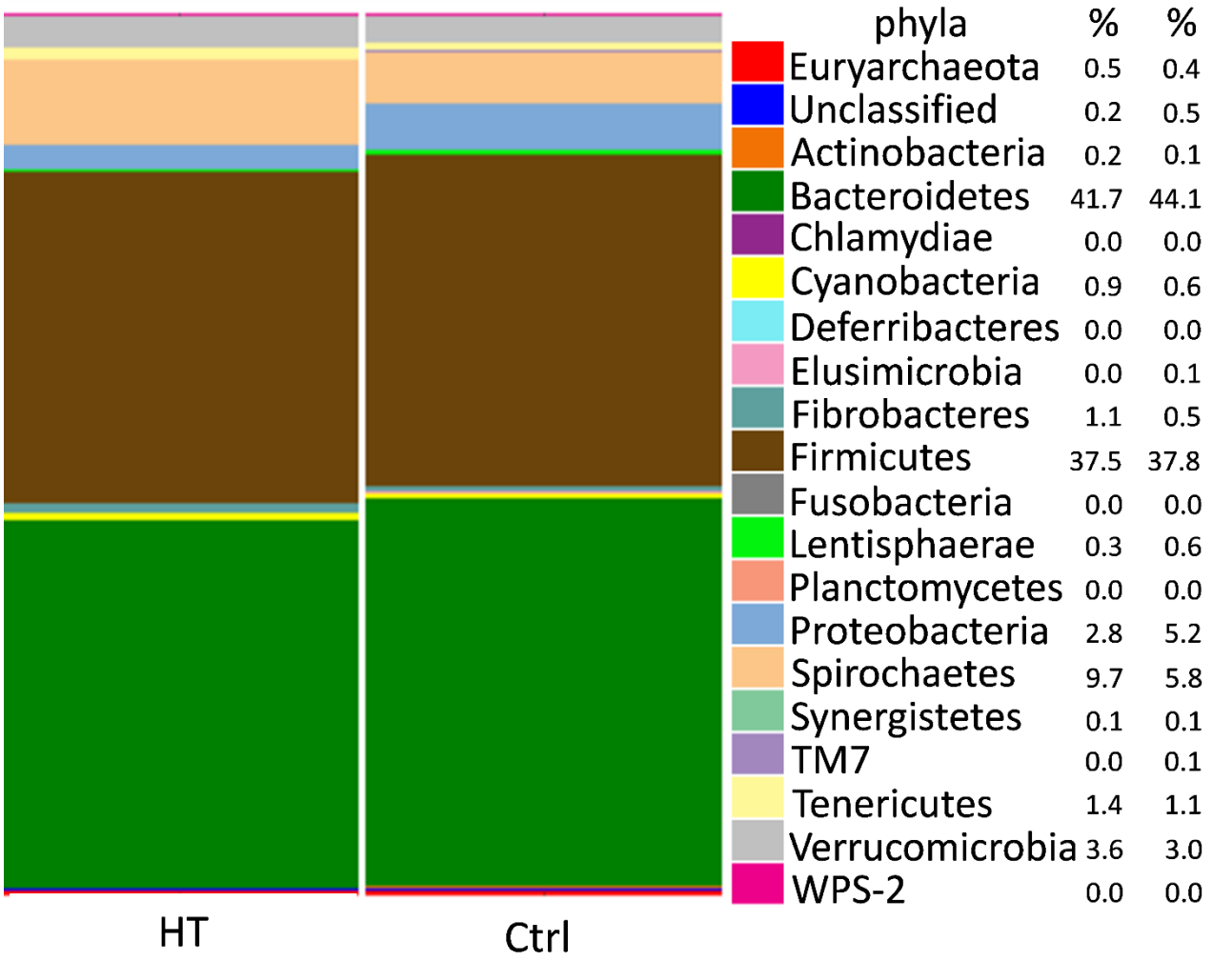

Figure S.2. *Taxa level plots: Phylum-level classification of bacteria identified in caecum samples from pigs fed the control (Ctrl) or the diets supplemented with 3% chestnut extract containing hydrolysable tannins (HT). Each bar represents the percent contribution of phylum-level profiles grouped for the Ctrl and the HT group. The phyla represented by the different colors are shown on the side of the figure.*

In accordance, the OTU heat map shows also substantial differences between the two dietary groups with respect to the abundance of several OTUs (Figure 3). The linear discriminant analysis coupled with effect size measurements (LEfSe) revealed that members of the Firmicutes phylum are affected.

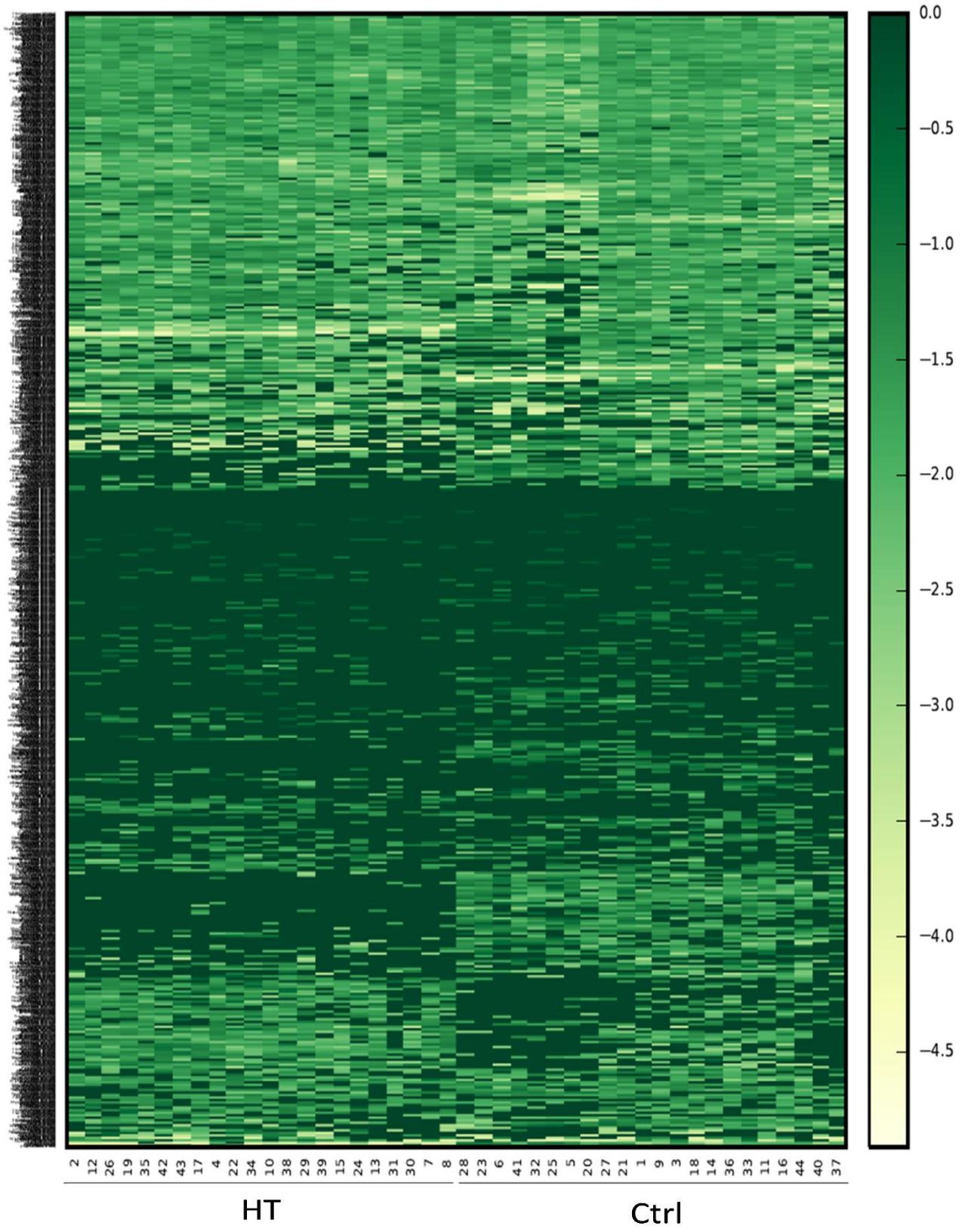

Figure S.3. *Heatmap of OTU data: The Heatmap of OTU data from samples originating from pigs fed the control (Ctrl) or the diets supplemented with 3% chestnut extract containing hydrolysable tannins (HT). Each row in the heatmap represents a different OTU, and the color of the OTU for each sample is scaled between dark green (low abundance) and white (high-abundance) according to the relative abundance of that OTU within the sample.*

Dietary HT supplementation resulted in a greater proportion of Oscillospira genus of the Ruminococcaceae family and a lower amount of the Lactobacillales order, Streptococcaceae and Veillonellaceae families (Figure S.4, Table S.2). Moreover, dietary HT increased the number of Treponema and Sphaerochaeta genera, which are members of the Spirochaetes phylum but reduced the Proteobacteria phylum abundance (Figure S.4, Table S.2).

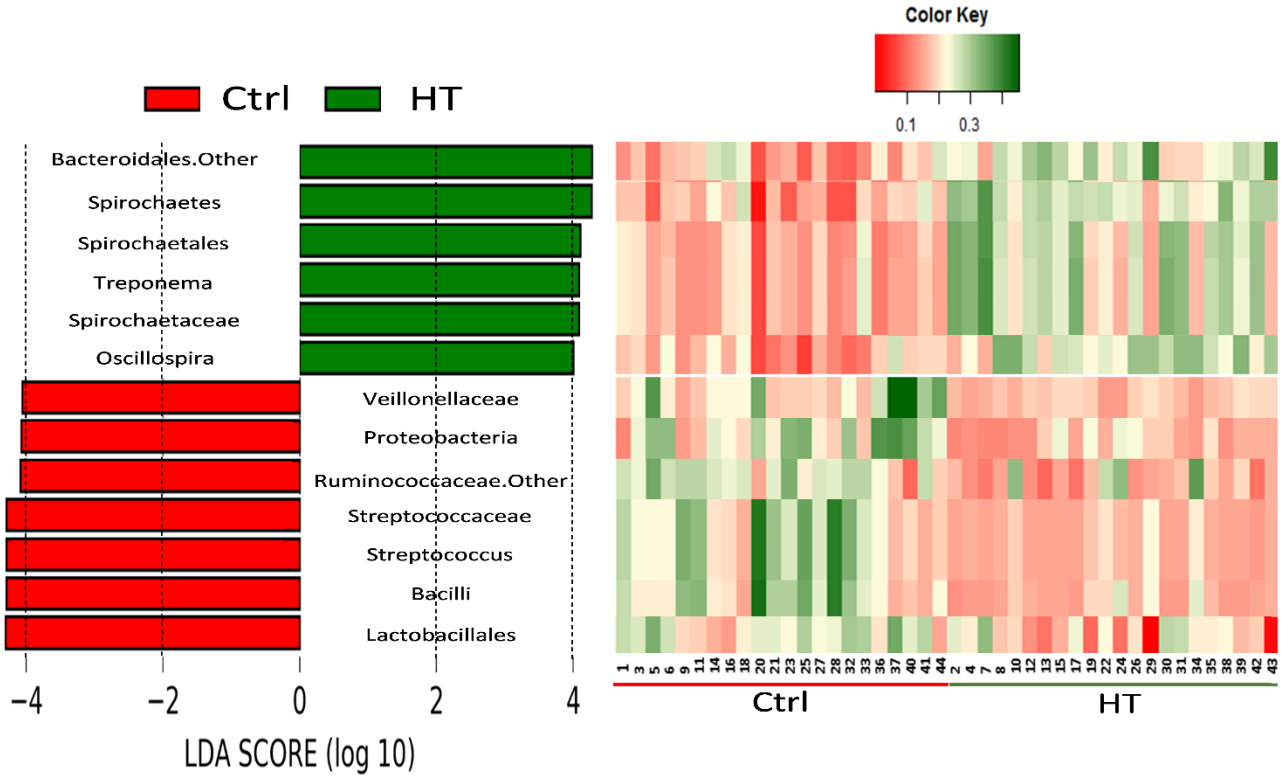

Figure S.4. *The linear discriminant analysis coupled with effect size measurements (LefSe): The most differentially abundant genus level taxa determined in caecum samples from pigs fed the control (Ctrl; in red) or the diets supplemented with 3% chestnut extract containing hydrolysable tannins (HT; in green). The heatmap shows the scores of these relative abundances.*

DISCUSSION

The use of plant extracts appears to be an attractive alternative to the use of antimicrobial growth promoters. The HT and CT from chestnut and Quebracho, respectively, are probably the most readily available commercial products that are being used in livestock [18]. Hydrolysable tannins are composed of esters of gallic (gallotannins) and ellagic acid (ellagitannins) with a sugar core and are readily hydrolyzed by acids and enzymes into monomeric products [19]. Tannases are part of relevant enzymes distributed throughout the animal, plant and microbial kingdoms [20]. Tannases produced by bacteria can degrade tannic acid and natural tannins like chestnut [21]. An important number of data support the beneficial dietary role of HT metabolites: results of various studies demonstrated the ability of HT to reduce the number of gastrointestinal parasites in mammals [22, 23], their antimicrobial activity [24, 25] and effects on gastrointestinal bacteria colonization in chickens and pigs [26,121 27] especially regarding potential pathogens like Campylobacter and Salmonella [28,122 18]. There is clear evidence pointing towards the influence of the diet on microbial communities and the subsequent health status [29]. However, the effects of this microbial shaping on pig health, on skatole and indole metabolism-microbiota interaction and growth performance remain unclear. In light of this, the aim of this study was to evaluate the effects of dietary HT supplementation on gut microbiota composition in entire male pigs, to clarify which taxa are mainly affected by dietary HT and/or its metabolites and to estimate the associations between specific microbial taxa, skatole and indole metabolism and growth performance.

Effects of HTs on the gut microbial structure

The degree of the gut microbiota biodiversity characterizes the health status of the gut ecosystem [30]. In the present study, dietary HT had an antimicrobial effect but without affecting the biodiversity of microbial community, as shown by the lower OTUs and the similar Shannon' index in the HT compared to Ctrl group, respectively. These findings suggest that no detrimental effects originated from the dietary HT supplementation on the microbiota ecosystem. It is noteworthy that despite a similar feed intake, dietary HT inclusion in the finisher period reduced average daily gain and consequently feed efficiency, presented by Maghin and co-workers [31]. A possible explanation for the impaired growth performance caused by dietary HT intake could be that lower OTUs might negatively affect nutrient digestibility [32]. Intestinal microbiota is estimated to contain more than 100 times the number of functional genes than genes encoded in the respective host genome and it can be considered as a virtual organ able to increase caloric harvest from complex nutrients that are indigestible by the animal's own enzymes. In addition, the microbiota can also synthesize vitamins, contributing to the overall nutritional status of the host [33, 32, 34, 35]. Generally, about 85% of carbohydrates, 66 to 95% of proteins and approximately 100% of the triglycerides are absorbed before entering the large intestine [36, 37]. The indigestible portion of the carbohydrates and proteins

represent 10 to 30% of the total ingested gross energy [38,149 39] and, without the activity of the colonic microbiota, would generally be eliminated via the feces. In the colon, methanogens, acetogens and sulfate reducers microorganisms work in symbiosis to ferment starch (including resistant starch), unabsorbed sugars, cellulosic and non-cellulosic polysaccharides [40] and mucins [41] into short-chain fatty acids (SCFAs) [42] and CO2, CH4, and H2. [43, 44]. Consequently, a lower number of gut microbiota could result in an impaired balance of these bacteria, thereby affecting the nutritional status and ultimately weight gain of the growing pig. In support of this relationship, we observed a negative correlation ($p = 0.021$) between OTUs number and feed efficiency. Similarly, Stanley et al. [45] observed similar evidence in chicken, where the feed efficient birds showed greater biodiversity with markedly greater chao1 indexes than in less feed efficient birds. In addition we observed in this study that HT supplementation not only affected the OTUs number in the microbial ecosystem, but also changed the bacterial taxa composition. Beta diversity analysis determined by the weighted and unweighted UniFrac analysis showed a clear clusterization due to HT supplementation. Specifically, at the phylum level the HT group showed a decrease in Bacteroidetes and Proteobacteria and an increase in Spirochaetes. Moreover, differences between the 165 two treatment groups can be observed considering all OTUs, demonstrating that HT supplementation not only affected the number of bacteria but also influenced the gut ecosystem composition. At the microbial taxa level, dietary HT increased the abundance of Oscillospira genus. Furthermore, the abundance of Treponema and Sphaerochaeta genera was also greater in the HT group [46]. Conversely, HT supplementation decreased the abundance of Streptococcus genus and Proteobacteria, their abundance being associated with an unhealthy gut [47]. In addition, HT decreased the amount of Veillonellaceae family. The latter is known to efficiently produce high amounts of short chain fatty acids like acetate and propionate [48, 49], where acetate represent a substrate for cholesterol and fatty acid synthesis [50]. Surprisingly in the HT group the abundance of Lactobacillales order was lower compared to the Ctrl group. Lactic acid bacteria are generally considered beneficial by exerting protective functions such as antagonistic effect on the gastroenteric pathogens such as Clostridium difficile, Campylobacter jejuni, Helicobacter pylori and rotavirus [51]. Despite the decrease in the abundance of Lactobacillales, no visible impairment in the health status of the HT compared to the Ctrl pigs was detected. However, as discussed below, a reduction in Lactobacillales could lead to lower skatole production.

Effects of HT on the gut microbial structure and boar taint

Boar taint is one of the major problems occurring when entire male pigs are raised [52]. Recent studies suggested that the supplementation of HT in the diet reduced bacteria mediated skatole and indole production in the colon, resulting in lower skatole and indole levels in the adipose tissue [53, 54]. Skatole results from a multistep degradation of tryptophan by microbial activity, mainly in the hind gut of the pigs [55]. Many types of intestinal bacteria are able to synthesize indole from L-tryptophan. As demonstrated in this study, the addition of HT inhibits microbial growth that could result in reduced microbial degradation of

tryptophan to indole and skatole. 191 A schematic model for a possible mode of action of dietary HT on skatole synthesis and degradation is proposed in the Figure S.5. It has been proposed that gut cell debris are the major source of tryptophan for the microbial mediated synthesis of indolic compounds [56, 57]. In this regard, feed additives (antibiotics and Chinese herbs), which reduce the number of pathogenic bacteria in the intestine are effective in reducing atrophy of villi in piglets after weaning [58]. Accordingly, the current results showed a decrease in Streptococcus genus and Proteobacteria, often associated to unhealthy gut and inflammation status [47] and an increase of Oscillospira genus which abundance is negatively correlated to intestinal inflammatory diseases [59, 60]. All these factors could lead to a lower apoptosis of intestinal epithelial cells, limiting the availability of L-tryptophan from cell debris and consequently microbial mediated skatole production [61]. Bacteria that degrade tryptophan often are capable of degrading other aromatic amino acids [62]. Several strains of Lactobacillus that produce skatole has been isolated from a bovine rumen and has been partially characterized; these organisms are able to produce skatole not directly from L-tryptophan but by the decarboxylation of indole acetic acid [62]. Moreover, apart from skatole, these Lactobacillus strains also produce by the same mechanism of decarboxylation p-cresol (4-methylphenol) another major phenolic component of pig odor [62]. Consequently, the reduction in Lactobacillales order observed in this study could be another effect of HT on the gut microbial structure that could lead to a reduced intestinal indole and skatole production.

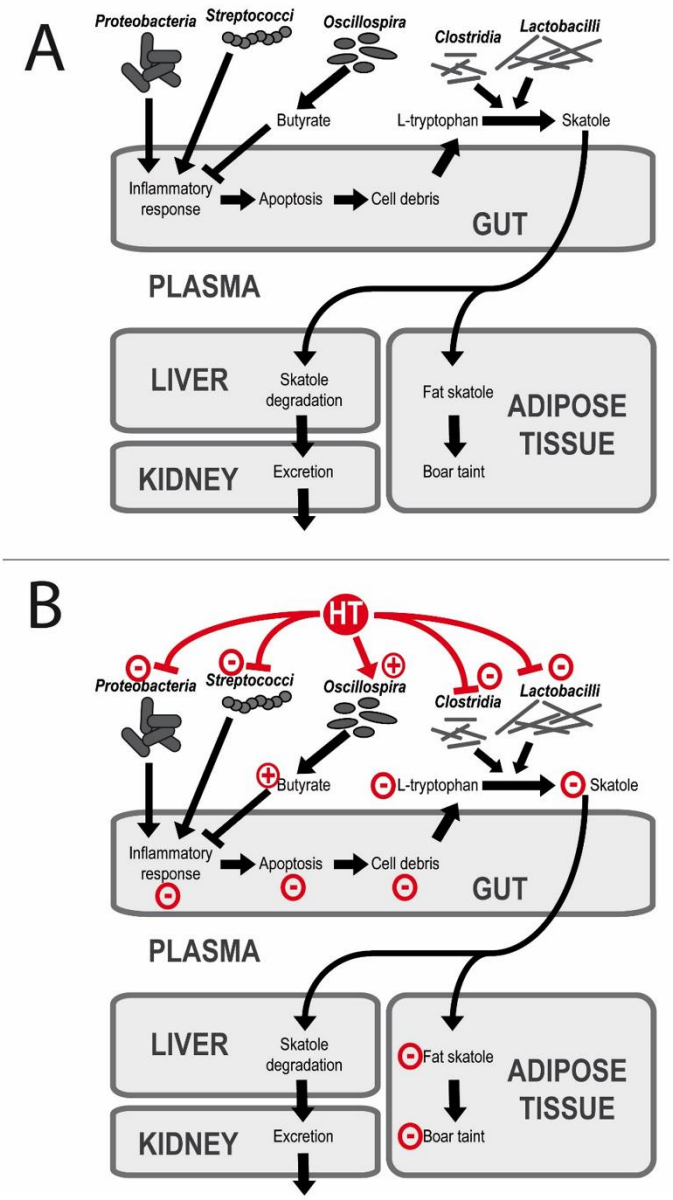

Figure S.5. *Proposed model for the actions of dietary hydolysable tannins (HT) on skatole synthesis and degradation. Panel A: Skatole is produced via the fermentation of L-tryptophane (Trp) by the gut microbiota. The last step of the pathway is performed by decarboxylases of several Clostridiales (C. scatologenes, C. nauseum) and several Lactobacillales (L. helveticus, L. sp 11201) among others [55]. Tryptophan originates from the naturally occurring cell debris of apoptotic cells and its amount is increased by the inflammatory response in the gut. Skatole is then transferred from the gut to the plasma where it can be either incorporated into adipose tissues or degraded in the liver and subsequently excreted by the kidney. Panel B: Dietary HT and/or their metabolites positively enhances the proportion of Oscillospira species in the gut. Oscillospira mediated butyrate inhibits the inflammatory response in the gut, thus leading to fewer cell debris and less Trp available for skatole production. On the contrary, dietary HT reduces the proportion of Streptococci and Proteobacteria in the gut, thus limiting the Streptococci and Proteobacteria-born inflammatory response. Moreover, dietary HT are decreasing the amounts of Lactobacillaceae, which are involved in skatole production from Trp.*

CONCLUSIONS

Supplementing the diet of EM with 3% of HTs from chestnut wood extract affected both, qualitatively and quantitatively the pig gut microbiota without having an impact on its biodiversity, confirming its antibacterial activity with no negative effects on the bacterial community. Further investigations are needed in order to clarify the nature of association between the gut microbial ecosystem and the 217 synthesis of skatole and indole.

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ABOUT THE AUTHOR

Curriculum Vitae

Acknowledgments

List of publications

Training

CURRICULUM VITAE

Marco Tretola was born on June 13rd, 1988 in Benevento, Italy. In 2007 he started his BSc education in Life Sciences at the University of Sannio. Following his BSc, Marco worked in a medical laboratory of cytogenetics, histology and hematology in 2012. In the same year, Marco joined the MSc program in Biology at the University of Sannio. During this period, Marco served his traineeship at the University of Naples, Federico II where, for his MSc thesis, investigated the intestinal microbiota in different pathological subjects using high throughput sequencing technique under the supervision of Prof. P. Mancini. Marco graduated in 2014 and in 2015 he received a post graduate grant entitled "Metabolic regulation and intestinal microbiota" from the University of Rome. In 2015, Marco started his PhD research under the supervision of Prof. L. Pinotti. His PhD programme was focused on different *in vitro* and *in vivo* features related to the use of food industry leftover as alternative feed ingredients in pig nutrition at the department of Health, Animal Science and Food Safety (VESPA, University of Milan) within the project IZS PLV 06/14 RC funded by the Italian Ministry of Health. During his PhD, in 2016 Marco attended an international training school "Contemporary feed production – technological and nutritional aspects" at the Institute of Food Technology, Center "Feed to Food" in Novi Sad (Serbia). In 2017 he was a visiting PhD student in Agroscope (Swiss federal research institute for agri-food sector, Posieux, Switzerland) where he investigated the interaction between dietary polyphenols and microbial colonization of the gastrointestinal tract of pigs. In the same year, Marco performed a training school funded by the PiGutNet network at the department of Animal Science, Aarhus University (Foulum, Denmark). In 2018 Marco was a visiting PhD student at the Animal Nutrition Group (ANU), Wageningen University and Research (Wageningen, the Netherlands) where he was involved in the project "Ileal amino acid digestibility in dietary protein sources commonly used by humans", focused in microbiota DNA analyses on samples from ileo-cannulated pigs to characterize differences in microbiota composition among various protein sources. In 2018 Marco got a PosDoc position in Agroscope (Posieux, Switzerland) for a project entitled "implementation of *in vitro* technologies for animal nutrition research" where he will continue to work after his graduation.

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LIST OF PUBLICATIONS

Peer reviewed journals

- Caprarulo, V., Ottoboni, M., **Tretola, M**., Demartini, E., Gaviglio, A., Agazzi, A., Rossi, L., Pinotti, L., Levic, J. (2016). A survey on the potential research and development tendency in the Italian and Serbian feed industry= Anketa o potencijalnim tendencijama vezanim za istraživanje i razvoj u kompanijama za proizvodnju hrane za životinje iz Italije i Srbije. FOOD & FEED RESEARCH, 43(2), 69-82.

- Cheli F., Pinotti L., M. Ottoboni, **M. Tretola**, V. Dell'Orto (2016). Cereal Industry: e-Nose for Real Time and Online Quality and Safety Control and Management. SENSORS & TRANSDUCERS, vol. 201, p. 52-58, ISSN: 2306-8515.

- Amato, G., Desiato, R., Giovannini, T., Pinotti, L., **Tretola, M**., Gili, M., Marchis, D. (2017). Gravimetric quantitative determination of packaging residues in feed from former food. Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment, pp. 1-5. DOI: 10.1080/19440049.2017.1337277

- Giromini, C., Ottoboni, M., **Tretola, M**., Marchis, D., Gottardo, D., Caprarulo, V., Baldi, A., Pinotti, L. (2017). Nutritional evaluation of former food products (ex-food) intended for pig nutrition. Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment, pp. 1-10. Article in Press.DOI: 10.1080/19440049.2017.1306884

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Book Chapters

- Pinotti L., V. Caprarulo, M. Ottoboni, C. Giromini, A. Agazzi, L. Rossi, **M. Tretola**, A. Baldi, G. Savoini, R. Čolović, O. Đuragić, D. Vukmirović, J. Lević (2016). FEEDNEEDS: trends in R&D in the Italian and Serbian feed sectors. In: P. Battinelli;J. Striber. Serbia - Italia : Italian - Serbian Bilateral Cooperation on Science, Technology and Humanities. vol. 3, p. 21-25, Belgrade: Museum of Yugoslav History, Belgrade, ISBN: 9788675220480.

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- Cheli, L. Pinotti, M. Ottoboni, **M. Tretola**, V. Dell'Orto (2016). On line E-nose technology for safety and quality evaluation in cereal processing. In: P.E. Cruvinel; S. Yurish;S.S. Compte, ALLSENSORS 2016. p. 15-17, IARIA, ISBN: 9781612085234

- Cheli, F., Pinotti, L., Novacco, M., Ottoboni, M., **Tretola, M**., & Dell'Orto, V. Mycotoxins in Wheat and Mitigation Measures (2017). Mycotoxins in Wheat and Mitigation Measures, Wheat Improvement, Management and Utilization, Ms. Ruth Wanyera (Ed.), InTech, Chapter 11, DOI: 10.5772/67240. Available from: https://www.intechopen.com/books/wheat-improvement-management-and-utilization/mycotoxins-in-wheat-and-mitigation-measures

- Pinotti, L., Ottoboni, M., **Tretola, M**., Varotto Boccazzi, I., Epis, S., & Eeckhout, M. (2018). Insect biomass quality and safety: basic concepts, recent issues, and future challenges. In Annual Meeting of the European Federation of Animal Science (pp. 398-398). Wageningen Academic Publishers.

Conference and technical papers

- Pinotti L., C. Giromini, M. Ottoboni, **M. Tretola**, F. Cheli, A. Baldi (2016) Ex-food4feed: quality & safety. Proceedings, FoodTech2016, Novi Sad, Serbia, from 25th to 27th October 2016.

- Pinotti L., M. Ottoboni, **M. Tretola**, C. Giromini, F. Cheli, A. Baldi (2016). Quality & Safety features of Former Food Products intended for animal nutrition. Abstract FoodTech2016, Novi Sad, Serbia, from 25thto 27th October 2016.

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- Ottoboni M., **M. Tretola**, V. Caprarulo, D. Marchis, P. Veys, V. Baeten, L. Pinotti (2016). Light microscopy technique for the discrimination of insect processed animal proteins versus marine arthropods. 5th International Feed Conference 2016: "Present and Future Challenges ",19 & 20 October 2016 in Geel Belgium.

- **Tretola M**., Tirloni E., Bernardi C., Giromini C., Ottoboni M., Castrica M., Baldi A, Pinotti L. (2016). Former food products safety evaluation: microbiological quality and packaging material residuals. 5th International Feed Conference 2016: "Present and Future Challenges “,19 & 20 October 2016 in Geel Belgium.

- Ottoboni, M., Giromini, C., **Tretola, M**., Gottardo, D., Marchis, D., Caprarulo, V., Cheli, F., Baldi, A., & Pinotti, L. (2017). Nutrients content and *in vitro* digestibility of ex-food as feed ingredient for pig diets. ITALIAN JOURNAL OF ANIMAL SCIENCE – ISSN: 1828-051X vol. 16, p. 63 (suppl.1). ABSTRACT

- **Tretola, M.,** Ottoboni, M., Luciano, A., Rossi, L., Baldi, A., & Pinotti, L. (2018). Effects of former food products as cereal substitute on growth performance in post-weaning pig. In Annual Meeting of the European Federation of Animal Science (pp. 137-137). Wageningen Academic Publishers.

- Pinotti, L., Ottoboni, M., **Tretola, M.**, Varotto Boccazzi, I., Epis, S., & Eeckhout, M. (2018). Insect biomass quality and safety: basic concepts, recent issues, and future challenges. In Annual Meeting of the European Federation of Animal Science (pp. 398-398). Wageningen Academic Publishers.

- Ottoboni, M., **Tretola, M.,** Luciano, A., Fusi, E., Giromini, C., & Pinotti, L. (2018). Evaluation of predicted glycemic index in bakery/confectionary former food products and in former food based pig diet. In Feed Conference: Present and Future Challenges (pp. 31-31). Institute of Marine Research Bergen, Norway.

- Ottoboni, M., **Tretola, M.**, & Pinotti, L. (2018). Microscopic methods and computer image analysis for distinguishing fish meals containing pelagic and farmed fish vs sea mammals (no target species). In International Feed Conference: Present and Future Challenges (pp. 59-59). Institute of Marine Research Bergen.

Disseminating publications

- Luciano, P., **Tretola, M**., Ottoboni, M., Caprarulo, V., Giromini, C., Baldi, A., & Dell’Orto, V. (2017). EX ALIMENTI: innovazione e sostenibilità nel settore agroalimentare = Ex-food as innovation and sustainability source in the agri-food sector. TECNICA MOLITORIA. Vol. 68

- **Tretola, M**., Gottardo, D., Ottoboni, M., Caprarulo, V., Giromini, C., Cheli, F., Baldi, A., Dell’Orto V., & Pinotti, L. (2016). L'alimentazione animale oggi: animal & human prospective = Past and present insights into animal nutrition: animal & human prospective. TECNICA MOLITORIA, 67(10), 754-765.

FURTHER ACTIVITIES

Conferences/Seminars

- 1st World Conference on the Mediterranean Diet. REVITALIZING THE MEDITERRANEAN DIET. From a healthy dietary pattern to a healty Mediterranean sustainable lifestyle. 6-7-8 July 2016
- Seminar: *In vitro* methods for feed evaluation. Milano, May, 19, 2016
- NutriMi. 10° forum di Nutrizione Pratica. Milano. April, 21-22th, 2016-10-12
- La formazione trasversale nei dottorati di ricerca: ricerca e mondo del lavoro. Milano, March, 2, 2016
- 22nd Congress of the Animal Science and Production Association. Perugia, June 13th – 16th, 2017, presenting "former food products safety: stereomicroscopy and computer vision for evaluation of packaging remnants contamination" (oral presentation).
- 6th International Feed Conference: Present and Future Challenges. Institute of Marine Research Bergen, Norway. 25th - 26th October 2018, presenting "Effects of former food products as cereal substitute on growth performance in post-weaning pig" (oral presentation).

Training schools/Summers schools

- Parma Summer School 2016. "IN SILICO/*IN VITRO* APPROACHES FOR FEED SCIENCE". Parma, September, 9th, 2016
- Corso Teorico-Pratico: "Dalla Cellula al QSAR: Modelli predittivi alternativi in tossicologia". Genova, 18-20 Aprile 2016-10-12
- Seminar METTLER TOLEDO Good Pipetting Practice Training. Milano, March, 8th, 2016
- 2nd Training School of COST Action FA1401 (PiGutNet). "Contemporary feed production – technological and nutritional aspects". University of Novi Sad, Serbia. February 22-26, 2016
- Course SAS UNIVERSITY EDITION. Milano, 26 June 2017.
- Course and PiGutNet Training School: Gut Biology and Health - Dept. Animal Science, AU-Foulum, Aarhus University. 14th - 25th August 2017.
- Summer school "Open & reproducible microbiome data analysis" Wageningen University and Research, Wageningen, The Netherlands. 28th – 30th May 2018.