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PhD Thesis

Valorisation of Mediterranean agro-industrial by-products in pig production as feed and anaerobic co-digestion of slurry

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Abstract

Nowadays the sustainability of the pig sector relies on its capability to respond to the increasing demands for livestock products that are arising from population growth, adapting to changes in the economic and policy contexts, and improving its environmental performance through the mitigation of its impact on climate. In this framework, the use of the agro-industrial by-products offers potential alternative raw materials for animal production with a lower associated environmental burden in the form of feedstuffs for livestock, source of bioactive compounds or raw materials useful in bioenergy production.

This PhD thesis aims to evaluate the use of Mediterranean agro-industrial by-products as feed ingredients for pigs or co-substrates for biogas production. To fulfil these objectives, four trials were designed and conducted to evaluate the use of olive oil and orange juice industry by-products in swine nutrition, assessing its nutritional value and the consequences of its inclusion in the diet on animals' performance and health, final product quality traits and gas emissions associated to the pig slurry. Additionally, one more trial was conducted to evaluate the effect of four agricultural substrates (tomato, pepper, peach and kaki) on the biochemical methane potential (BMP) in anaerobic co-digestion with pig slurry, focusing on the type of substrate and its inclusion level on the final substrate's mixture.

The results presented in this PhD Thesis from the nutritional value assays indicate that the olive cakes and orange pulps tested can be potentially included in pig diets to replace part of the cereals of the diet with associated changes in urine and faeces nutrients excretion that leads to modifications in the potential ammonia and BMP emissions from slurries. Concerning olive cake by-products, the nutritional value assay designed to test the crude (COC) and partially defatted (PDOC) olive cakes showed that olive cakes are appreciable sources of insoluble fibre, but have limited energy value (11.2 and 7.4 MJ/kg DM for COC and PDOC respectively) and a low value as protein source. On the contrary, the dehydrated (DOP) and ensiled sun-dried (ESDOP) orange pulps tested are a relevant energy source (14.2 and 13.2 MJ/kg DM for DOP and ESDOP respectively) with added value in terms of SF concentration. With respect to the *in vitro* potential ammonia and BMP emissions assays, the by-products tested led to a decreased N excretion in urine and, in the case of the OC, increased DM excretion in faeces. The ammonia emission per kg

of slurry decreased with the inclusion of olive cake and orange pulp, whereas the BMP per animal and per day was negatively affected by the inclusion of olive cake obtaining higher BMP with these by-products.

Regarding the performance assays, the PDOC and the DOP may be included in balanced pig diets at rates of up to 120 and 240 g/kg respectively, without negative effects in the case of PDOC and minor effects for DOC on growth performance, body composition and carcass quality traits. Contrary to what was expected, the inclusion of PDOC and DOP did not affect microbial counts nor excreta volume, composition and global gas emission from the slurry. Additionally, beneficial effects on subcutaneous fat were observed with the inclusion of PDOC, improving its oleic acid concentration.

The anaerobic co-digestion of agricultural by-products and pig slurry improves the BMP from the mixture compared to only pig slurry anaerobic digestion. Higher BMP values were obtained with increasing addition of agricultural substrate, confirming the better performance of co-digestion systems at adequate inclusion levels. In fact, combinations with tomato, pepper and peach at inclusion level 3 (50% of VS) achieved the highest BMP. This resulted in an increase in BMP of 41% with tomato, 44% with pepper, 28% with peach and 12% with kaki. Vegetables substrates (pepper and tomato) showed higher lipid, protein, lignin and cellulose content than fruit substrates (kaki and peach).

Resumen

Actualmente, la sostenibilidad del sector porcino depende de su capacidad para responder a la elevada demanda de productos ganaderos derivadas del crecimiento de la población, adaptándose a los cambios en los contextos económico y político, y mejorando su rendimiento medioambiental mediante la mitigación de su impacto en el clima. En este contexto, el uso de los subproductos agroindustriales ofrece potenciales materias primas alternativas para la producción animal, con una menor carga ambiental asociada, en forma de piensos para el ganado, fuente de compuestos bioactivos o materias primas útiles en la producción de bioenergía.

Esta tesis doctoral pretende evaluar el uso de subproductos agroindustriales mediterráneos como ingredientes en piensos para el ganado porcino o como co-substratos para la producción de biogás. Con este objetivo, se diseñaron y realizaron cuatro ensayos para evaluar el uso de subproductos de la industria del aceite de oliva y del zumo de naranja en la alimentación del ganado porcino, evaluando su valor nutricional y las consecuencias de su inclusión en la dieta sobre el rendimiento y la salud de los animales, la calidad del producto final y las emisiones de gases asociadas a los purines. Además, se realizó un ensayo para evaluar el efecto de cuatro sustratos agrícolas (tomate, pimiento, melocotón y caqui) sobre el potencial bioquímico de metano (BMP) en co-digestión anaerobia con purines de cerdo, centrándose en el tipo de sustrato y su nivel de inclusión en la mezcla final de sustratos.

Los resultados presentados en esta Tesis Doctoral a partir de los ensayos de valor nutricional indican que las tortas de aceituna y las pulpas de naranja ensayadas pueden ser potencialmente incluidas en las dietas de los cerdos reemplazando parte de los cereales de la dieta con cambios asociados en la excreción de nutrientes en la orina y las heces que conducen a modificaciones en las emisiones potenciales de amoníaco y BMP de los purines. En cuanto a los subproductos de la torta de aceituna, el ensayo de valor nutricional con tortas de aceituna crudas (COC) y parcialmente desgrasadas (PDOC) mostró que ambas tortas son fuentes apreciables de fibra insoluble, pero tienen un valor energético limitado (11.2 y 7.4 MJ/kg MS para COC y PDOC respectivamente) y un bajo valor como fuente de proteínas. En cambio, las pulpas de naranja deshidratadas (DOP) y ensilada secada al sol (ESDOP) ensayadas son una fuente de energía relevante (14.2 y 13.2 MJ/kg MS para DOP y ESDOP

respectivamente) con valor añadido debido a su contenido en fibra soluble. En cuanto a los ensayos de emisiones potenciales de amoníaco y BMP *in vitro*, los subproductos ensayados generaron una disminución en la excreción de N en la orina y, en el caso de la pulpa de aceituna, un aumento de la excreción de materia seca en heces. La emisión de amoniaco por kg de purín disminuyó con la inclusión de torta de aceituna y pulpa de naranja, mientras que el BMP por animal y por día se vio negativamente afectado por la inclusión de torta de aceituna obteniendo una mayor BMP con estos subproductos.

En cuanto a los ensayos de rendimientos productivos, la PDOC y la DOP pueden incluirse en dietas equilibradas para cerdos en proporciones de hasta 120 y 240 g/kg respectivamente, sin efectos negativos en el caso del PDOC y efectos menores para el DOP sobre los rendimientos productivos, la composición corporal y la calidad de la canal. Al contrario de lo que se esperaba, la inclusión de PDOC y DOP no afectó a los recuentos microbianos ni al volumen de excrementos, la composición y la emisión global de gases de los purines. Además, se observaron efectos beneficiosos sobre la grasa subcutánea con la inclusión de PDOC, mejorando su concentración de ácido oleico.

La co-digestión anaerobia de subproductos agrícolas y purines mejora el BMP de la mezcla de sustratos en comparación con la digestión anaerobia de purines únicamente. Se obtuvieron mayores valores de BMP con el aumento de la adición de sustrato agrícola, lo que confirma el mejor rendimiento de los sistemas de co-digestión a niveles de inclusión adecuados. De hecho, las combinaciones con tomate, pimiento y melocotón al nivel de inclusión 3 (50% de SV) alcanzaron el mayor BMP. Esto supuso un incremento de la BMP del 41% con tomate, 44% con pimiento, 28% con melocotón y 12% con caqui. Los sustratos vegetales (pimiento y tomate) mostraron un mayor contenido en lípidos, proteínas, lignina y celulosa que los sustratos frutales (caqui y melocotón).

Resum

Actualment, la sostenibilitat del sector porcí depèn de la seua capacitat per a respondre a l'elevada demanda de productes ramaders derivades del creixement de la població, adaptant-se als canvis en els contextos econòmic i polític, i millorant el seu rendiment mediambiental mitjançant la mitigació del seu impacte en el clima. En aquest context, l'ús dels subproductes agro-industrials ofereix potencials matèries primeres alternatives per a la producció animal, amb una menor càrrega ambiental associada, en forma de pinsos per al bestiar, font de compostos bioactius o matèries primeres útils en la producció de Bioenergia.

Aquesta tesi doctoral pretén avaluar l'ús de subproductes agro-industrials mediterranis com a ingredients en pinsos per al bestiar porcí o com co-substrats per a la producció de biogàs. Amb aquests objectius, es van dissenyar i realitzar quatre assajos per a avaluar l'ús de subproductes de la indústria de l'oli d'oliva i del suc de taronja en l'alimentació del bestiar porcí, avaluant el seu valor nutricional i les conseqüències de la seua inclusió en la dieta sobre el rendiment i la salut dels animals, la qualitat del producte final i les emissions de gasos associades als purins. A més, es va realitzar un assaig addicional per a avaluar l'efecte de quatre substrats agrícoles (tomaca, pimentó, bresquilla i caqui) sobre el potencial bioquímic de metà (BMP) en co-digestió anaeròbia amb purins de porc, centrant-se en el tipus de substrat i el seu nivell d'inclusió en la mescla final de substrats.

Els resultats presentats en aquesta Tesi Doctoral a partir dels assajos de valor nutricional indiquen que les tortes d'oliva i les polpes de taronja assajades poden ser potencialment incloses en les dietes dels porcs reemplaçant part dels cereals de la dieta amb canvis associats en l'excreció de nutrients en l'orina i les femtes que condueixen a modificacions en les emissions potencials d'amoníac i BMP dels purins. Pel que fa als subproductes de la torta d'oliva, l'assaig de valor nutricional amb tortes d'oliva crues (COC) i parcialment desengreixades (PDOC) va mostrar que les tortes d'oliva són fonts apreciables de fibra insoluble, però tenen un valor energètic limitat (11.2 i 7.4 MJ/kg MS per a COC i PDOC respectivament) i un valor baix com a font de proteïnes. En canvi, les polpes de taronja deshidratades (DOP) i ensitjada assecada al sol (ESDOP) assajades són una font d'energia rellevant (14.2 i 13.2 MJ/kg MS per a DOP i ESDOP respectivament) amb valor afegit a causa del seu contingut en fibra soluble. Quant als assajos d'emissions potencials

d'amoníac i BMP *in vitro*, els subproductes assajats van generar una disminució en l'excreció de N en l'orina i, en el cas de la polpa d'oliva, un augment de l'excreció de matèria seca en femtes. L'emissió d'amoníac per kg de purí va disminuir amb la inclusió de torta d'oliva i polpa de taronja, mentre que el BMP per animal i per dia es va veure negativament afectat per la inclusió de coca d'oliva obtenint un major BMP amb aquests subproductes.

Quant als assajos de rendiments productius, la PDOC i la DOP poden incloure's en dietes comercials per a porcs en proporcions de fins a 120 i 240 g/kg respectivament, sense efectes negatius en el cas de la PDOC i efectes menors per a la DOP sobre els rendiments productius, la composició corporal i la qualitat de la canal. Al contrari del que s'esperava, la inclusió de PDOC i DOP no va afectar els recomptes microbians ni al volum d'excrements, la composició i l'emissió global de gasos dels purins. A més, es van observar efectes beneficiosos sobre el greix subcutani amb la inclusió de PDOC, millorant la seua concentració d'àcid oleic.

La co-digestió anaeròbia de subproductes agrícoles i purins millora el BMP de la mescla de substrats en comparació amb la digestió anaeròbia de purins únicament. Es van obtenir majors valors de BMP amb l'augment de l'addició de substrat agrícola, la qual cosa confirma el millor rendiment dels sistemes de co-digestió a nivells d'inclusió adequats. De fet, les combinacions amb tomaca, pimentó i bresquilla al nivell d'inclusió 3 (50% de SV) van aconseguir el major BMP. Això va suposar un increment del BMP del 41% amb tomaca, 44% amb pimentó, 28% amb bresquilla i 12% amb caqui. Els substrats vegetals (pimentó i tomaca) van mostrar un major contingut en lípids, proteïnes, lignina i cel·lulosa que els substrats fruiters (caqui i bresquilla).

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Glossary of terms

ADG: Average Daily Gain

ADICP: Acid Detergent Insoluble CP

ADL: Acid Detergent Lignin

BF: Back Fat and

BMP/B_0: Biochemical Methane Potential

BW: Body Weight

C: Carbon

CFU: Colony Forming Units

CH_4: Methane

COC: Crude Olive Cake

CP: Crude Protein

CTTAD: Coefficient of Total Tract Apparent Digestibility

DE: Digestible Energy

DFD: Dark Firm and Dry

DM: Dry Matter

DOC: Defatted Olive Cake

DOP: Dehydrated Orange Pulp

EE: Eter Extract

ESDOP: Ensiled Sun-Dried Orange Pulp

FA: Fatty Acid

FCR: Feed Conversion Ratio

GE: Gross Energy

N: Nitrogen

NDF: Neutral Detergent Fibre

aNDFom: Stable Amylase Neutral Detergent Fibre

NDICP: Neutral Detergent Insoluble Crude Protein

NH_3: Ammonia

OC: Olive Cake

OM: Organic Matter

OP: Orange Pulp

PDOC: Partially Defatted Olive Cake

PSE: Pale Soft and Exudative

PUFA: Polyunsaturated Fatty Acids

qPCR: quantitative real-time TaqMan Polymerase Chain Reaction

SEM: Standard Error of Means

SF: Soluble Fibre

SFA: Saturated Fatty Acids

TAN: Total Ammonia Nitrogen

TDF: Total Dietary fibre

TKN: Total Kjeldahl Nitrogen

TS: Total Solids

UE: Urinary Energy

VFA: Volatile Fatty Acids

VS: Volatile Solid

Chapter 1

General Introduction

1.1 Environmental and production challenges of pig production

The pig sector is of worldwide importance in terms of census and meat production. According to the data published by the MAPA (2019), China is the world's biggest producer of pig followed by the EU. In terms of exports, the EU is the exporter of pigs and pig products of major importance worldwide. Within the EU, as Figure 1.1 shows, Germany and Spain are the main producer countries with 59.4 and 47.7 million of pigs slaughtered in 2016 respectively. They were followed by France (23.8 million, 9%), representing these three countries a half of the EU's total production. In the EU, the pig market exports about 13% of its total production, being the main destination the East of Asia, in particular China.

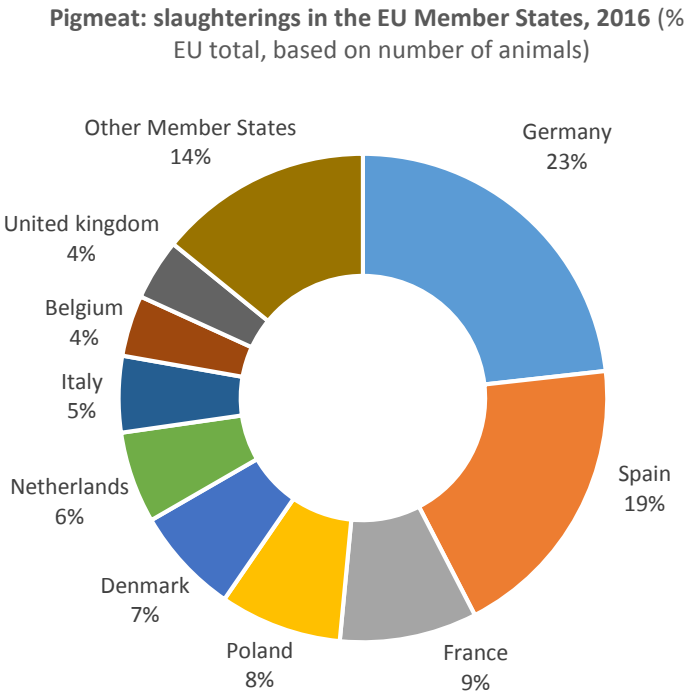

Fig 1.1. Percentage of the pigs slaughterings in the EU Member States in 2016. Source: Eurostat, 2018.

In Spain, pig production is a leading agricultural sector, as it accounts for around 14% of Final Agricultural Production. Within livestock production, the pig sector occupies the first place in terms of economic importance, reaching around 39% of Final Livestock Production. In recent years, the

pig sector has grown considerably. The Spanish pig census has increased in the last years to more than 30 million, and almost 50 million of pigs were slaughtered in 2018 (MAPA, 2019). Exports have also increased during the last years.

Associated to pig rearing, several environmental impacts have been identified related with compound feed production and provision and slurry management. The pig sector is the main consumer of compound feed in Spain (44%; FEFAC, 2016), which means consuming high amounts of resources as feedstuffs and a high environmental impact related to raw materials. Additionally, pig slurry management is the main direct environmental impact of pig production related to nutrient loads, and emissions of ammonia (NH_3) and greenhouse gases (GHG) to the atmosphere in animal houses, slurry storage and land application.

Therefore, it is expected that a key for the pig sector to being competitive in the coming years will be to produce "more with less", following a strict legal framework also in terms of food safety, environment and welfare. In other words, it will be necessary to produce food more efficiently and sustainably, contributing to the "Low carbon and nitrogen economy" policy and the so-called circular economy enacted by the European Commission (COM, 2011). This is particularly challenging in the current world growing population and climate change scenario, in which the resources needed in terms of food, water, land and fossil fuel, among others, to meet this demand are limited (Flachowsky et al., 2017).

In this scenario, the pig sector has to face the major challenge of meeting the food requirements of an exponentially growing world population (estimated at approximately 9 billion people in 2050; FAO, 2009) and, in turn, the commitment to produce safe and quality food based on a legal framework in terms of the environment, animal welfare and sustainability which is increasingly demanding.

1.2 Role of nutrition and slurry management on the sustainability in the pig sector

Nutrition plays an essential role on the sustainability of pig production for three main reasons. First, feedstuffs are the main nutrient input in intensive pig production; second, nutrition has direct implications on animal health and productivity; and third, the quality of feedstuffs determines the efficiency of animals in the use of nutrients, and therefore also the amount and composition of effluents of the animals.

Recent life cycle assessment studies suggest that obtaining feedstuffs such as cereals and soya for animal feeding, together with the slurry management, are key contributors to the carbon footprint associated with animal products for human consumption (Roy et al., 2009; Hermansen and Kristensen, 2011; Nijdam et al., 2012).

The supply of feed ingredients coming from crops for animal production is a global concern worldwide, because of the increasing demand of animal products, the limited availability of crops and the dependence of imported feed resources (grains and soybean meal) which affect its sustainability. Furthermore, the expected scenarios of climate change show increased risk for crop production due to climate variations, and a more frequent occurrence of extreme climate episodes. Additionally, changes in land uses through the conversion of forest lands and grasslands to cropland and pastures for animal feed production leads to a loss of carbon dioxide (CO_2) sinks which results in higher GHG emissions attributable to livestock production (Nijdam et al., 2012). Also, crops usually compete with human feeding and in a growing world population and scarcity scenario, their availability and competitiveness for its use as animal feeds will decrease. In this regard, the replacement of part of the cereals used in feeds by agro-industrial by-products has been proposed to reduce the environmental impact of pig production (Ajila et al., 2012; Zijlstra and Beltranena, 2013; Makkar and Ankers, 2014; Schader et al., 2015; Salemdeeb et al., 2017).

Additionally, the livestock sector is an important source of NH_3 emission, mainly associated to slurry management, and a key actor on global climate change throughout its associated GHG emissions and nutrient release into the environment, since it contributes to 14.5% of global GHG (Gerber et al., 2013). Overloading of nutrients (nitrogen and phosphorus), NH_3 and GHG emission into the atmosphere through pig slurry management are major concerns in certain areas where intensive livestock rearing has led to high livestock-density production systems. The mismanagement of pig slurry in these high livestock-density areas can result in soil and water eutrophication and acidification, gas emissions and accumulation of heavy metals in soils (González-Fernández et al., 2008). In that respect, proper pig slurry management has been demonstrated to be an efficient mitigation strategy (Sáez et al., 2017).

Animal feeding has been recognised as an essential tool for controlling gas emissions from manure in the livestock sector (Gerber et al., 2013).

Several studies have reported an effect of nutritional management of pigs on gas emission from slurry. These changes on gas emission are based on the assumption that diet composition affects nutrient digestibility, metabolism and the associated fermentation rates in the hindgut, which in turns can modify slurry characteristics (Møller et al., 2004a, Møller et al., 2004b; Dinuccio et al., 2008; Beccaccia et al., 2015; Ferrer et al., 2018).

Therefore, the high dependence of crops for feed production in the pig sector and the intensive pig production poses a challenge for achieving nutrient circularity. International nutrient flows have increased dramatically in the last decades, and more than half the nitrogen used for crop fertilization is currently lost into the environment (Lassaletta et al., 2014). As recognized in the EU Farm to Fork Strategy (European Comision, 2020), it is necessary to enhance a circular bio-based economy which minimizes the excess of nutrients (especially nitrogen and phosphorus) and reduces the dependency on critical feed materials. Exploring alternative feed ingredients is essential to ensure the economic, social and environmental sustainability of intensive pig production.

1.3 Agro-industrial by-products in animal production

Agro-industrial wastes constitute a major proportion (almost 30%) of worldwide agricultural production (Ajila et al., 2012). They are human-inedible products that generally do not compete with human feeding. At the food manufacturing level, there are always unintentional and unavoidable food losses, which were manufactured for human consumption but finally do not reach the human consumption market or are generated as by-products during the industrial process (Luciano et al. 2020). Worldwide food waste ranges from 194 to 389 kg per person and year globally. At the EU level, it accounts for 158 to 290 kg per person and year (Corrado and Sala, 2018).

Within the agro-industrial wastes, crop waste and residues constitute the agricultural by-products which are non-marketable fruits, plants and vegetables that have been rejected for commercialization, generated as by-product or are withdrawn from the market with the aim of regulating the markets within the framework of the common organisation of agricultural markets (MAGRAMA, 2012).

Besides these non-marketable fruits and vegetables, the agricultural sector generates an important volume of agro-industrial by-products from the agri-food industry. The agri-food by-products are of very diverse

and variable origin, and include fruit and vegetables from the processing sector such as by-products from sugar, starch and confectionary industry, the cereal grain milling and legume by-products, oil extraction, brewery, malt production, juice production, and products derived from canning industry.

As a whole, agro-industrial by-products pose a serious environmental and public health risk if they are not properly managed with several implications in terms of the sustainability and profitability of the food system (Pinotti et al., 2020). They have practical and environmental limitations to their use such as:

i) Challenging management and transport system among plants and farms, that increases transport costs.
ii) High moisture (<20% dry matter, DM) and organic matter (OM) content which difficult its storage and preservation due to the production of leachates and the elevated OM degradation rate.
iii) High variability related to the obtaining process (Belyea et al., 2010).
iv) Possible content of anti-nutritional factors or toxic elements (Bernard, 2010; Ajila et al., 2012; Zijlstra and Beltranena, 2013).
v) Reduced nutrient digestibility, especially protein, due to its high fibre content (Schofield et al., 2001).

Most of these agro-industrial by-products contain compounds that are interesting from a nutritional point of view in human or animal food, and in other sectors such as cosmetics. They also constitute a good raw material for obtaining new compounds or energy in the form of biogas when is not possible to recycle it as animal feed (Galanakis, 2013; Kasapidou et al., 2015 ;Schader et al., 2015; Pinotti et al., 2019). Far from be considered these by-products as a problem, they should be considered as a resource and therefore as an alternative to the commercialization of fresh products.

Thus, there is a need to take further actions on exploring the potential valorisation of agro-wastes (Lai et al., 2017). Different approaches have been proposed for agro-industrial by-products utilization in animal production. The EU has established guidelines with the preferable disposal technologies in order to aid with the selection of the adequate food disposal alternative. This so-called food waste hierarchy (Fig. 1.2), stipulates that governments should prioritise efforts (in order of most to least preferable) to (i) reduce food waste (use in the food chain), (ii)

redistribute it (e.g. to the homeless), (iii) recycle it as animal feeding and (iv) compost, (v) recover energy through anaerobic digestion, and finally, (vi) landfill the remainder (Salemdeeb et al., 2017; EPA, 2020).

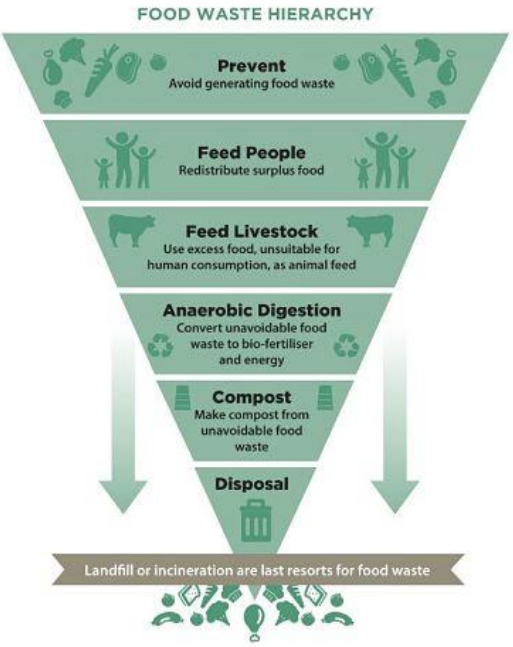

Fig 1.2. The food waste hierarchy. Source: Environmental Protection Agency, Ireland (EPA, 2020)

In this sense, the use of agro-industrial by-products for animal feeding offers an alternative for the by-products disposal from the industries, which is essential to comply with legislative pressure related with the environmental protection. Although recycling these by-products into animal feeding should be a primary use, there are alternative uses, as it has been stated previously, such as obtaining biofuel, anaerobic digestion or fertilisation for which other types of substrates not suitable for animal feeding may be useful. This is of special importance, for example, in high-density pig production areas where slurry management can become a problem. In these areas, the anaerobic co-digestion of pig slurry with by-products can help to increase the energy generally obtained during the treatment of slurries.

In all, in a framework of economic efficiency and sustainability, the management of agro-industrial by-products as alternative sources of

nutrients for animal feeding, and in co-digestion with pig slurry constitutes a challenge and, at the same time, an opportunity. Its benefits as local resources will contribute to close the nutrient cycles at a local level.

1.4 Main agro-industrial by-products in Spain

The most important agrarian sectors in Spain and therefore the most commonly available local by-products for animal production come from the fruit and vegetable (15%), olive oil (40%), sugar-beet pulp (27%) and cereal (18%) sectors (MAPAMA, 2017). One of the few studies published on the amount of by-products generated by the sector in Spain, estimates that 10% of the total production is lost in the fruit selection (GPA, 2006), whereas more than 50% of the original material is generated as by-product in processed vegetables (MAPA, 2006). Therefore, it can be assumed that the amount of agricultural by-products from fruit and vegetables generated in Spain is considerable. Spain produces 18,4 million MT agro-industrial waste/year and 67% of them are from vegetal origin (MAPAMA, 2017).

The Mediterranean region, due to its agricultural tradition, is a major producer of agro-industrial by-products such as vegetable and fruit wastes, olive cake and citrus pulp that can be potentially used in pig production. Fruit and vegetable by-products are generated as a result of the marketing and/or processing of agricultural products, being highly variable depending on the type of raw material to be processed. We can distinguish between different types of agricultutal by-products; raw product which are non-marketable pieces or came from the withdrawal operations, pulps originated as a result of the industrial processing of whole fruits and cooked product from the canning industry. In Spain, the most commonly produced agricultural by-products came from the citrus and olive industry, tomato, artichokes, onion and peppers cultivars. Generally, vegetable cultivars have a higher water content than fruits and pulps, so they also present problems for their preservation during the out season. Their use as animal feed is limited since the dehydration process needed to favour their conservation increase their cost. The most commonly used for animal nutrition are those from the canning industry, which are cooked products with added water.

Among vegetables, tomato is the most important vegetable crop in Europe and one of the main components of the Mediterranean diet (EUROSTAT, 2018). The main by-products generated from the industrial

processing of the tomato for production of pastes, juice, and sauce are seeds and peels, representing 10–40% of total processed tomatoes (Al-wandawi et al., 1985). With respect to artichoke cultivars, the agri-food industry produces two types of by-products from the canning or the fresh-handling industries. Artichoke by-products come from the packaging of artichoke hearts and are composed by the outer bracts and setms, either cooked or raw (Martínez Teruel et al., 1998). Another major vegetable crop in Europe is the onion cultivar which generates more than 500.000 tonnes of onion wastes, mainly in UK, Holland and Spain and are not suitable for animal feeding due to their strong aroma (Benítez et al., 2011; Roldán et al., 2008). Onion industry produces by-products that yield approximately 38 % of the fresh weight of processed onion and are composed of different parts of the bulb in variable proportions and include the skin, outer scales, roots and tops (Mandura, 2020). Pepper wastes amount to approximately 65% of the raw material and are discarded as non-marketable products or by-products constituted by seeds, skin leftovers and stems (15.9%, 34.7% and 49.4% percentage in weight respectively), (Romo-Hualde et al., 2012). In addition to the waste generated during the fresh trade of peppers, part of the by-products generated from pepper processing come from the canning industry and spices (Sandoval-Castro et al., 2017).

Regarding pulps by-products from fruits, they are highly variable products whose composition are affected by the type of product, climate, state of maturity and by the type of industrial processing used. As an example, in the Mediterranean region are generated pulps by-products from citrus, apples, and olives.

Olive oil production is a major industry in Mediterranean countries such as Spain, and generates large amounts of waste. Olive cakes (OC) consist of a mixture of olive pulp, skin, stone, water and residual oil, in variable proportions, which are often dried to facilitate further use. The OC contains a relevant amount of oil that can be extracted with hexane and the resulting extracted product is called defatted OC. For its management and stabilization, a previous dehydration of the product is necessary. The final yield of the process is 25-40 kg of OC per 100 kg of olive (Eraso et al., 1978), which implies a potential production in Spain of around 2.000.000 Tm/year of dehydrated OC.

The chemical composition of OC varies widely depending on the characteristics of the olive, the climate and the manufacturing process

used. According to the databases consulted (CIHEAM (1990), FEDNA (2010), Feedipedia database), defatted OC is characterised by a high proportion of fibre (highly lignified) and a low protein content (around 10%), being in a great extent linked to fibre. Partially defatted OC, however, has an appreciable concentration of fat (between 10 and 20% on dry matter (DM)) of high quality (rich in oleic), which makes it of interest for its inclusion in pig diets.

Due to its variability, information on the nutritional value (digestibility and energy and protein value) of partially defatted OC in pigs is very scarce and variable. Furthermore, due to its high oleic acid content, its inclusion in pig diets could improve the carcass quality traits. In addition to its nutritional value and acceptance by the animals, OC contains an appreciable and variable amount of phenolic compounds (0.3-5% DM) mainly composed of orthophenols such as oleuropein (Vázquez et al., 1974). These compounds have an antimicrobial and antioxidant capacity (Obied et al., 2007) that can be beneficial when used in animal feed.

On the other hand, citrus production is widespread worldwide (131 million Tm per year; FAO, 2012), and is usually concentrated in specific geographical areas (Mediterranean regions, Sao Paulo state in Brazil and Florida in the USA). This market generates a huge amount of citrus pulp in these areas, which often represent an environmental problem. A relevant fraction of citrus production is transformed into juice, and citrus pulp is produced as a by-product. In Spain, the country with the highest citrus production within Europe, approximately a 20% of production is transformed (MAPAMA, 2017).

Citrus pulp represents an important proportion of the original weight (around 50-70 %) of the fresh fruit and is generally composed of a mixture of the skin (60-65 %), segments of the fruit (30-35 %) and seeds (0-10 %) (Martínez-Pascual and Fernández-Carmona, 1980; FEDNA, 2010; Feedipedia database). In general, citrus pulp has a high water content (80-83%) and low content in crude protein (6-9% on DM basis) and ether extract (1-4% on DM basis). It is characterized by a high amount of easily fermentable fibre (30% pectins) and sugars (20% soluble sugars over DM) (FEDNA, 2010; own data). Some juice industries incorporate technologies to reduce the humidity of the citrus pulp such as pressing or dehydration and thus reduce the volume of waste.

1.5 Agro-industrial by-products as a source of nutrients in pigs feeding

The study of agro-industrial by-products as alternative sources of nutrients for animals has an increasing interest not only by its impact on the economic sustainability of the pig industry (Jha et al., 2013), but also due to environmental implications. The former is based on the facts that production is generally local and prices are usually lower. Likewise, they require no additional land for its production resulting in no additional nutrient and pesticides surplus, and have lower demand for non-renewable energy, water, and GHG emissions (Ajila et al., 2012; Del Prado et al., 2013; Zijlstra and Beltranena, 2013; Makkar and Ankers, 2014; Salemdeeb et al., 2017). Otherwise, apart from the economic and environmental benefits, in the recent years the fibre and bioactive compounds that are usually present in agro-industrial by-products, with proved effects on metabolism and health, have increased its interest in the animal feeding sector (Jha et al., 2019; Georganas et al., 2020).

Nonetheless, the use of agro-industrial by-products in pig diets present some nutritional and practical limitations due to (Cerisuelo and Calvet, 2020): i) lack of knowledge about their nutritional value, ii) high variability on their composition, iii) their generally high fibre content and the presence of anti-nutritional factors that can affect nutrient digestibility; iv) the limited availability of some by-products due to their seasonality or low production (for example in the case of new protein sources) and v) the need for transformation or adaptation of many of them to be used by feed mills (e.g. dehydration, pelleting...).

In this regard, some agro-industrial by-products such as oilseed meals or DDGS are commonly used in pig feeds. Their nutritional value is well known and they even have prediction equations that estimate it according to their composition. However, there are other by-products that are highly available in Spain during most part of the year for which the information on composition, nutritional value and limits of inclusion in feeds is scarce.

This lack of knowledge about some agro-industrial by-products can lead to an improper use and the refusal of producers to use them in practical diets. This is the case of typically Mediterranean agro-industrial by-products such as olive and citrus fruit by-products. These by-products have been traditionally used in ruminant diets (Bampidis and Robinson, 2006; Gharbi and Benarif, 2011). However, their composition and the

incorporation of further dehydration systems during its management into the food industries make their use increasingly interesting in monogastrics, especially in pigs. Furthermore, taking into account the importance of the Spanish pig sector, the use of these agro-industrial by-products in animal feeding is an easy way to valorise them, contributing to the sustainability and profitability of the production system

With respect to their fibre content, the fibre fraction of a feed or a diet has been traditionally associated with a decreased energy density and nutrient digestibility (Bindelle et al., 2008). This effect results from a decreased gastrointestinal transit time in the case of insoluble fibre (lignocellulose materials such as brans or ingredients rich in lignin). In the recent years, far from being considered an anti-nutritional factor with negative consequences on nutrient digestibility, several benefits on gut health, metabolism and animal welfare have been attributed to fibre (Agyekum and Nyachoti, 2017). These benefits will depend on the type of fibre, which is highly variable among by-products. In the case of soluble fibre, it has been described by Jha et al. (2019) to have the ability to increase the viscosity of intestinal digesta and the transit time, which in turns favours the proliferation of selective microbiota.

In this context, during the last years have been carried out several studies about the introduction of fibrous by-products into practical diets with different conclusions to be taken out from the results. While Zijlstra and Beltranena (2013) state that fibrous by-products could negatively affect not only nutrient utilization and growth performance, but also carcass and meat quality, other studies (Wu et al., 2016; Smit et al., 2017, 2018) reported no adverse effects of feeding high-fibre diets in the growing-finishing phases compared with low-fibre diets. Likewise, Laitat et al. (2015) state that the detrimental effect on growth performance is restricted to the grower rather to the finisher period, whereas Clarke et al. (2018) reported that overall performance is decreased when including 350 g/kg maize DDGS and 210 g/ kg of rapeseed meal in pig diets. Overall, the ingestion of high-fibre diets has been associated to reduce carcass yield and traits because of the enlargement of the secretory organs. However, the detrimental effect of feeding high-fibre diets with the inclusion of agro-industrial by-products would need to be re-evaluated in terms of the cost of kg meat produced per unit of net energy consumed, which will favour the use to less expensive net energy fibrous feeds in diets.

On the other hand, nutrient diet modification is one of the main strategies used to reduce gas emissions from livestock production (Canh et al., 1998; Philippe and Nicks, 2015), from both enteric fermentation and slurry management. Worldwide organizations such as the European Commission, the Intergovernmental Panel on Climate Change (IPCC) or the Food and Agriculture Organization of the United Nations have recommended different strategies to mitigate NH_3 and GHG emissions from livestock production, such as improving feed efficiency and reducing protein concentration. These strategies have been included as best available techniques in intensive pig and poultry production in a reference document published by the European Commission (Giner Santonja et al., 2017).

The relationship between the diet and gas emissions in livestock production is based on the assumption that the composition of the diet can modify the animal's digestive behaviour, including factors such as nutrients utilisation (digestibility) and the fermentation rates from the different ingredients at the intestinal level. Both will affect the composition and characteristics of the slurry and thus the associated emissions of gases such as NH_3 or GHGs such as methane (CH_4) or nitrous oxide (N_2O) (Canh et al., 1998; Moset et al., 2012; Jarret et al., 2011a; Cerisuelo et al., 2012; Philippe and Nicks, 2015).

In this regard, the inclusion of fibrous by-products in pig diets has been shown to affect the composition of pig excreta (Rigolot et al., 2010, Jarret et al., 2011a; Jarret et al., 2012), with a clear indirect effect on NH_3 emission, which decrease as the fermentable fibre content of the diet increases (Canh et al., 1998; Jarret et al., 2012). Similar results have been reported by Beccaccia et al. (2015) in an assay conducted to assess the inclusion of fermentable fibre (citrus pulp) and lignified fibre (carob meal) in pig diets on NH_3 and CH_4 emissions from pig slurry. The results reported by those authors confirm that increasing the percentage of fibre in the diets reduces the associated NH_3 independently of the type of fibre (more or less lignified).

With respect to the diet effect on GHG emissions, the reported data in the literature is not consistent. While some studies do not find clear differences in the potential CH_4 emission using fibrous by-products such as DDGS or rapeseed meal (Jarret et al., 2011a; Jarret et al., 2011b), others observe a reduction in the potential CH_4 emission when these by-products are included in diets. Differences in the amount of fat in these feedstuffs

could affect the potential CH_4 emission from the excreta (Torres-Pitarch et al., 2014). However, when this emission is expressed by animal, the increase in the volume of excreta caused by the inclusion of fibre results in higher amounts of CH_4 emitted by animal and day (Jarret et al., 2012; Torres-Pitarch et al., 2014). According to Beccaccia et al. (2015) increasing fibre content reduces the potential CH_4 emission from faeces. However, while the inclusion of a lignified fibre source such as carob meal in diets increased OM excretion and CH_4 emission by animal and day, the inclusion of citrus pulp did not change the amount of OM excreted and CH_4 emission by animal and day was even lower than that derived from animals consuming a commercial feed.

In all, the use of agro-industrial by-products in pig diets is recognised as a viable strategy to mitigate the environmental impact of intensive pig production through the formulation of environmentally sustainable feeds and the implementation of measures to reduce the volume of pig slurry, nutrient excretion, and gas emissions. However, the effects of the by-products fibre content on animal's health, nutrient digestibility, emissions, production, and volume of excreta, need to be in-depth studied since the results from the last studies published are not consistent.

1.6 Agro-industrial by-products in anaerobic co-digestion of industrial by-products

Anaerobic digestion of pig slurry is an interesting process in environmental terms. The advantages of pig slurry treatment trough anaerobic digestion are clear, since it reduces the amount of volatile solids (VS) from the substrates, odours and pathogens (González-Fernández et al., 2008). Alternatively, it avoids CH_4 emission into the atmosphere from pig slurry storage and is a source of renewable energy from the biogas produced. Furthermore, the nutrients (N and phosphorus) from the slurry remain practically constant in the digestate after the anaerobic digestion process, so they can be used for land application as fertilizer to crops.

Nonetheless, anaerobic digestion of single substrates present some drawbacks linked to substrate properties such as organic loads, N and heavy metals concentrations, seasonality and availability or inhibitory volatile fatty acids (VFA) levels (Mata-Alvarez et al., 2014). Single digestion of pig slurry faces several challenges. First, it has low CH_4 yields compared with other organic substrates such as energy crops (Ward et

al., 2008) due to its high water. Second, its OM has fibre fractions which are slowly biodegradable. And third, there can be inhibitions due to excessive N (especially NH_3) content (Moller et al., 2004). In order to overcome these limitations and enhance CH_4 production from slurry, anaerobic co-digestion of pig slurry with easily-fermentable carbohydrates is being widely used (Ward et al., 2008).

Anaerobic co-digestion is a feasible option to overcome the drawbacks of single substrate digestion and to improve the economic viability of anaerobic digestion plants (Mata-Alvarez et al., 2014) through the joint treatment of two or more substrates with the aim of:

i) Taking advantage of the complementarity of the substrates composition and to compensate the deficiencies of each one of the substrates separately, allowing more efficient process profiles.
ii) Sharing treatment facilities, reducing investment and operating costs.
iii) Unifying management methodologies.
iv) Absorbing temporary variations in composition and production of each waste separately.

The agro-industrial by-products are characterised by their high content in easily degradable OM, having higher potential for biogas production than substrates of livestock origin. Overall, agro-industrial by-products can reach biogas productions of up to 1000 m^3 of biogas per ton of substrate, improving the economic viability of plants (Angelidaki and Ahring, 1997a). However, these substrates can present limitations during the anaerobic digestion process, such as deficiency in the nutrients needed for the development of microorganisms, low alkalinity or an excessive DM content (Banks and Humphreys, 1998). Livestock substrates, and in particular pig slurry, can be a good basis for co-digestion with complementary substrates as it generally has higher water content than most agro-industrial by-products, a higher buffer capacity and provides all the nutrients needed for the growth of anaerobic microorganisms (Angelidaki and Ahring, 1997b).

The main aspect when choosing two substrates for the co-digestion process is based on the balance of different parameters in the mixture regarding macro and micronutrients, carbon-nitrogen (C:N) ratio, pH, presence of toxic/inhibiting compounds and OM biodegradability (Hartmann and Ahring, 2005). Optimal values for the C:N ratio around 20

have been proposed for the stable performance of anaerobic digesters (Burton and Turner, 2003; Chen et al., 2008). However, lower values of the C:N ratio (between 6 and 9) are considered adequate for the anaerobic digestion of nitrogen-rich wastes (Mshandete et al., 2004). Regarding the N content from substrates, it has been reported that inhibiting concentrations by free NH_3 and total NH_3 are 1.1 and 4 g N/L in pig and cattle manure, respectively (Hansen et al., 1998; Chen et al., 2008). On the other hand, the alkalinity of slurries is necessary to avoid pH drops due to VFA accumulation when high organic loads are used. Anaerobic digesters work in a wide range of alkalinity levels depending on the substrate to be degraded, from 2.000 to 18.000 mg $CaCO_3$/L (Mshandete et al., 2004).

Taking into account the overall parameters that must be considered in the anaerobic digestion of substrates, agricultural by-products pose a viable option for co-digestion with pig slurry due to their composition, since they have high amounts of easily biodegradable carbohydrates and low protein and water contents. Therefore, they allow increasing the C:N ratio of the slurry without affecting aspects such as the concentration of NH_3 coming from protein degradation into the digester. However, their inclusion in anaerobic co-digestion digesters depends on the potential alternative uses of the substrate. Those substrates coming from the agricultural sector which can be managed thorough other environmentally justified and economically viable disposal technologies should not be considered as substrates for anaerobic co-digestion. In this regard, animal feed, biomass or composting could be mentioned as possible final disposal for these substrates following the food waste hierarchy established by the EU represented in the Figure 1.2. However, some of the agricultural by-products generated cannot be managed by these more preferable options. In those cases, the co-digestion of both substrates (pig slurry and agricultural by-products) can overcome the negative environmental impacts associated to their mismanagement.

Several anaerobic co-digestion studies have been carried out mixing substrates of different origins, which almost always confirm the expectations of a better performance from the mixture. Agro-industrial by-products such as brewer's spent grain and pasteurized slaughterhouse waste has been co-digested with pig slurry achieving higher biogas productions than pig slurry solely (Goberna et al., 2013; Rodríguez-Abalde et al., 2017). Other authors such as Aboudi et al. (2020), Ferreira et al. (2007) or Campos, (2001) determined the biogas production of different

mixtures of sugar beet by-products and fruit tree pulps (pear and apple) with pig slurry, obtaining higher potential for biogas production from the joint digestion of both substrates. Agelidaki et al. (1997b) evaluated the effectiveness of the process of co-digestion of by-products from the olive oil industry and cattle manure as well as Riggio et al. (2015) who co-digested cow slurry, apple pulp and olive pomace. These authors concluded that such co-digestion makes possible the anaerobic treatment of olive oil by-products, increasing in turn the production of biogas from the manure. Mixing pig slurry and sewage sludge, both in thermophilic and mesophilic regime, has also provided positive results (Flotats et al., 1999). Bouallagui et al. (2009) reported positive results with the anaerobic co-digestion of agricultural by-products and poultry slaughterhouse waste. Improvements in biogas production have also been obtained with mixtures of pig slurry or bovine manure and vegetable by-products (Dar and Tandon, 1987), mixtures of tomato and rabbit manure residues (Trujillo et al., 1993), fruit and vegetable by-products (Callaghan et al., 2002), or dairy industry by-products (Gavala et al., 1996).

In all, improving the environmental sustainability of the pig sector involves reducing the environmental impacts linked to feed production and slurry management. The valorisation of agro-industrial by-products through animal feeding or anaerobic co-digestion has been identified as a valuable strategy to mitigate the environmental impact of livestock and included in the food waste hierarchy stablished by the EU. Thus, the use of agro-industrial by-products in the pig sector can reduce the negative environmental impacts associated to food-competing feeds and, as well as NH_3 and GHG emissions from pig slurry storage.

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Chapter 2

Objectives and thesis structure

The studies presented in this PhD thesis are part of a project funded by the Spanish Ministry of Science and Innovation entitled "Valorización integral de subproductos agroindustriales mediterráneos (Orujo graso de aceituna y pulpa de cítricos) para reducir el impacto ambiental de la producción porcina" (AGL2014-56653), carried out in the Centro de Investigación y Tecnología Animal from the Instituto Valenciano de Investigaciones Agrarias (CITA-IVIA) and the Universitat Politècnica de València (UPV) in coordination with Universidad Politécnica de Madrid (UPM) and Universidad CEU-Cardenal Herrera.

This PhD thesis aims to evaluate the use of Mediterranean agro-industrial by-products as feed ingredients for pigs or co-substrates for biogas production. It addresses the consequences of using two major Mediterranean agro-industrial by-products such as olive pulp and orange pulp in animal feeding from an integrative point of view, and the potential valorisation in anaerobic co-digestion of minor agro-industrial by-products with pig slurry. This integrative perspective is essential to overcome the disposal limitations of both agricultural by-products and pig slurry.

The general objectives of this PhD Thesis are:

1. To evaluate the use of olive oil industry by-products in swine nutrition, assessing its nutritional value and the consequences of its inclusion in the diet on animals' performance and health, final product quality traits and gas emissions associated to the pig slurry.
2. To evaluate the use of orange juice industry by-products in swine nutrition, assessing its nutritional value and the consequences of its inclusion in the diet on animals' performance and health, final product quality traits and gas emissions associated to the pig slurry.
3. To assess the anaerobic co-digestion of pig slurry and agricultural by-products as an alternative disposal technology to animal nutrition.

To fulfil these objectives, five trials were designed and conducted. Each trial corresponds to a different chapter in this PhD Thesis.

The partial objectives of **Chapters 3 and 5** are to determine the nutritional value for fattening pigs of different by-products from the olive oil and orange juice industries respectively, and evaluate their effects on nutrient

balance, slurry composition and gaseous emissions from slurry, when included in pig diets. For this purpose, the study from Chapter 3 was designed to test a basal diet and another four experimental diets produced by substituting the basal diet by increasing levels of the olive oil industry by-products to estimate its nutritional value by linear regression. The study from Chapter 5 was designed to evaluate a basal diet and two experimental diets produced by substituting 500 g/kg of the basal diet with the two different orange pulps to subsequently estimate its nutritional value by difference, assuming additivity between the basal diet and the ingredients tested.

Chapters 4 and 6 are focused on assessing the effects of the inclusion of defatted olive cake and dehydrated orange pulp in balanced finishing pig diets on growth performance, carcass quality, faecal microbiology, slurry composition and gas emission. To fulfil these objectives, two performance assays were designed in which such fibrous by-products were included at different levels in commercial pig diets during the finishing period from about 60 kg BW until slaughter. Additionally, the slurry excreted by the animals was used to perform a gaseous emissions assay during its storage.

Finally, **Chapter 7** is focused on evaluating the effect of four agricultural substrates (tomato, pepper, peach and caki) on the biochemical methane potential (BMP) in anaerobic co-digestion with pig slurry, focusing on the type of substrate and its inclusion level on the final substrate's mixture. The BMP was determined in a batch assay where the anaerobic state indicators were monitored and microbial composition of the sludge was also studied.

The overall results of this thesis concerning the use of agro-industrial by-products in swine productions are discussed in **Chapter 8** with the aim to identify the best management strategies of by-products disposal to ensure the economic, social and environmental sustainability of intensive pig production. The main conclusions of this PhD thesis are drawn in **Chapter 9**.

Chapter 3

Nutritional value of crude and partially defatted olive cake in finishing pigs and effects on nitrogen balance and gaseous emissions

Nutritional value of crude and partially defatted olive cake in finishing pigs and effects on nitrogen balance and gaseous emissions

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Abstract: By-products from the food industry can be valuable ingredients in animal feeds. One example is olive cake (OC), generated in large amounts by the olive oil industry, which contains oil with a high proportion of oleic acid and polyphenols. An experiment was performed using pigs to determine the nutritional value of crude (COC) and partially defatted (PDOC) olive cake, and to evaluate their effect on nutrient balance, slurry properties and potential ammonia (NH_3) and methane (CH_4) emissions. Five experimental feeds were designed; a basal diet and another four diets produced by substituting 100 or 200 g/kg of the basal diet with either COC or PDOC. Thirty finishing male pigs (76.1 ± 4.2 kg initial BW) were used in the experiment (6 animals/treatment). After a 14-day adaptation period, faeces and urine were collected separately for 7 days to measure nutrient digestibility and the excretory patterns of nitrogen. Potential NH_3 and CH_4 emissions were measured in reconstituted slurry samples over 11 and 100 days, respectively. The dry matter (DM), crude protein (CP), cellulose, starch and energy coefficients of total tract apparent digestibility (CTTAD) were negative and linearly ($P < 0.05$) affected by OC inclusion level. However, the type of OC did not influence any of the digestion efficiencies studied. The energy digestibility of the ingredients tested, estimated by substitution, were 0.479 (± 0.040, SEM) and 0.327 (± 0.049) for COC and PDOC, respectively. Overall, the results indicate a digestible energy (DE) value from COC and PDOC that account respectively for around 80 or 60% of the DE provided by barley grain in pigs. Faecal content of cellulose, polyphenols and gross energy (GE) increased linearly with OC inclusion, whereas ash content decreased. The total N content of urine decreased linearly with OC inclusion, but benzoic and hippuric acid contents increased, which resulted in lower pH values for the OC diets. The ratio between faecal and urine N excretion decreased from 2.48 in the basal diet to 1.01 on average in the 200 g/kg OC diets. As a result, increasing both COC and PDOC levels in diets resulted in lower NH_3 emissions per volume of slurry and in a lower biochemical CH_4 potential. Although slurry excretion increased with OC inclusion, daily NH_3 emissions still decreased with increasing OC inclusion. However potential CH_4 emissions per animal increased. A global perspective throughout the production chain is needed to assess the impact of including OC in pig diets on gaseous emissions.

Keywords: ammonia emission, digestion efficiency, methane emission, nitrogen balance, olive cake

3.1 Introduction

Worldwide pork production reached 115 Tg in 2014 (Food and Agriculture Organization Corporate Statistical Database FAOSTAT, 2017), and is expected to rise in the coming decades (Alexandratos and Bruinsma, 2012). However, increasing intensification of pig production raises concerns about its sustainability, especially in terms of nutrient use. Nearly two thirds of the EU's cereals are used in animal feeds (European Commission, 2017), but, on average, only 25–30% of global animal dietary gross energy is retained in meat and milk products. Consequently, a relevant proportion of nitrogen and organic matter intake is excreted, which has important potential environmental impacts. According to the European Environment Agency (2017a,b), pig slurries in the EU are responsible for about 15% of ammonia (NH_3) and 4% of total methane (CH_4) emissions. In the future, the feed industry will need to find alternative feedstuffs and minimize their eco-footprints.

By-products from the food industry often constitute a serious management problem both in economic and environmental terms, but they also involve great losses of valuable nutrients (Mirabella et al., 2014). Olive oil production is a major industry in Mediterranean countries and generates large amounts of waste. Olive cakes (OC) consist of a mixture of olive pulp, skin, stone, water and residual oil, in variable proportions, which are often dried to facilitate further use (Uribe et al., 2013). Although OC is widely used as biofuel (Casanova-Peláez et al., 2015), its nutrient content and phenolic makeup may be useful for animal nutrition (Uribe et al., 2013).

Oil extraction procedures affect the amount and composition of olive by-products (Vlyssides et al., 1998; De Blas et al., 2015). Crude OC has an appreciable oil content with a high proportion of oleic acid (Joven et al., 2014). When cost-efficient, it can be partially or totally extracted, based on variability in the market price of olive oil. The by-products are generally dried and available throughout the year. The OC can potentially be used as a source of energy in diets for finishing pigs and sows, which would contribute to circular economy in Mediterranean countries such as Spain, the most important olive oil producer and the fourth biggest pig producer worldwide (FAOSTAT, 2017). Previous research (e.g. Canh et al., 1997; Bindelle et al., 2009; Galassi et al., 2010; Beccaccia et al., 2015) has shown that including fibrous by-products (such as sugar beet pulp, orange pulp or carob meal) in diets for growing pigs can help to reduce NH_3 and CH_4

emissions from slurry, per unit of nitrogen (N) or organic matter (OM), respectively. The objective of this study was to determine the nutritive value for fattening pigs of crude (COC) and partially defatted (PDOC) olive cake without stones and evaluate their effects on nutrient balance, slurry composition and gaseous emissions from slurry, when included in pig diets.

3.2 Material and methods

3.2.1 Animals, diets and experimental design

The experimental procedure was approved by the Ethics Committee of the Universitat Politècnica de València (registration number 2016/VSC/PEA/00024). Thirty finishing male pigs, progeny of Pietrain x (Landrace x Large White) with 76.1 ± 4.2 kg initial BW were used in the experiment (6 animals per treatment) in three batches of 10 animals each. Crude and partially defatted OC without stones were provided by a local processor DCOOP, Antequera, Spain (see Table 3.1 for their chemical composition). Five experimental feeds were designed; a basal diet and another four diets produced by substituting 100 or 200 g/kg of the basal diet with either COC or PDOC.

Table 3.1. Chemical composition of the olive cakes (OC) studied (g/kg, as fed basis).

	Crude OC	**Partially defatted OC**
Dry matter	932	937
Ash	83.4	89.5
HCl insoluble ash	6.66	6.37
Crude protein	96.0	79.9
NDICP[a]	59.7	72.1
ADICP[b]	33.7	38.0
Ether extract	162	119
aNDFom	356	367
ADFom	227	247
ADL	121	131
Total polyphenols	15.9	19.8
Sugars	97.0	100
Gross energy (MJ)	21.7	21.2

[a] Neutral detergent insoluble CP.

[b] Acid detergent insoluble CP.

The basal diet was formulated with common ingredients (barley, corn, wheat, and soybean meal) in commercial feeds to meet or exceed net energy, protein and essential amino acid levels in order to avoid an excessive imbalance in diets supplemented with OC with respect to the recommendations of FEDNA (2013) for fattening pigs (20–100 kg). Ether extract (EE) and fibre contents were kept low in the basal diet to compensate for the high content of these components in OC. The ingredients and chemical composition of the experimental diets are shown in Tables 3.2 and 3.3, respectively.

Table 3.2. Ingredient composition of the experimental diets (g/kg, as fed basis).

	Diets[a]				
	Basal	**100_COC**	**200_COC**	**100_PDOC**	**200_PDOC**
Barley grain	300	270	240	270	240
Corn grain	300	270	240	270	240
Wheat grain	163	147	131	147	131
Soybean meal 48	205	184	164	184	164
Crude olive cake	0	100	200	0	0
Partially defatted olive cake	0	0	0	100	200
Calcium carbonate	7	6.3	5.6	6.3	5.6
Dicalcium phosphate	12.8	11.5	10.2	11.5	10.2
Sodium chloride	4.2	3.8	3.4	3.8	3.4
DL-methionine	0.4	0.4	0.3	0.4	0.3
L-lysine HCL	2.3	2.1	1.9	2.1	1.9
L-threonine	0.5	0.5	0.4	0.5	0.4
Premix[b]	5	4.5	4	4.5	4

[a] 100_COC = 100 g/kg crude olive cake; 200_COC = 200 g/kg crude olive cake; 100_PDOC = 100 g/kg partially defatted olive cake; 200_PDOC = 200 g/kg partially defatted olive cake.
[b] Vitamin and mineral premix supplied per kg complete diet: 5000 IU of vitamin A; 1000 IU of vitamin D3; 3 mg of vitamin B2; 20 mg of vitamin B12; 10 mg of niacin; 4 mg of pantothenic acid; 48 mg of betaine; 30 mg of manganese oxide; 110 mg of zinc oxide; 10 mg of copper sulphate; 0.75 mg of potassium iodide; 0.1 mg sodium selenite; 90 mg of iron carbonate

Table 3.3. Chemical composition of the experimental diets (g/kg, as fed basis).

	Diets[a]				
	Basal	**100_COC**	**200_COC**	**100_PDOC**	**200_PDOC**
Dry matter	896	897	898	895	898
Ash	50.4	56.4	61.0	54.8	59.2
Crude protein	176	169	161	172	156
Ether extract	23.4	37.3	51.1	33.0	39.8
aNDFom	110	130	155	139	164
ADFom	42.5	61.6	85.0	67.5	89.8
ADL	8.1	19.1	33.7	23.9	39.1
Total polyphenols	0.36	1.44	3.15	1.41	3.11
Starch	434	408	350	383	361
Sugars	72.5	66.9	63.5	73.8	73.5
Calciumb	6.7	7.0	7.4	7.0	7.4
Phosphorous	5.8	5.4	5.0	5.4	5.0
Gross energy (MJ/kg)	16.4	16.7	17.2	16.7	17.1
Net energy (MJ/kg)b	9.7	9.4	9.0	9.0	8.4
Ileal standardized digestible amino acids[b]					
Lysine	9.1	8.2	7.3	8.2	7.3
Methionine	2.8	2.5	2.2	2.5	2.2
Total sulphur	5.4	4.8	4.3	4.8	4.3
Threonine	5.8	5.2	4.6	5.2	4.6
Tryptophan	1.8	1.6	1.4	1.6	1.4
Isoleucine	6.1	5.5	4.9	5.5	4.9
Valine	7.1	6.4	5.7	6.4	5.7

[a] 100_COC = 100 g/kg crude olive cake; 200_COC = 200 g/kg crude olive cake; 100_PDOC = 100 g/kg partially defatted olive cake; 200_PDOC = 200 g/kg partially defatted olive cake.
[b] Values calculated according to FEDNA (2010).

The experimental period consisted of a 14-day adaptation period to diets, followed by 7 consecutive days during which faeces and urine were collected individually, as described in Beccaccia et al. (2015a). The total amount of faeces and urine excreted per animal was collected separately every 24 h and pooled by animal at the end of the collection period. At the beginning of the experiment, pigs were assigned to one of five dietary treatments and placed in conventional pens until day 9 of the adaptation period. After that, they were housed individually in metabolism pens (1.2

× 2 m^2) until the end of the experiment. The collection period (the last 7 days of the experimental period) was divided in two parts to facilitate collections for energy and nutrient balance (days 1–4) and gaseous emissions (days 5–7). Feed and water were provided *ad libitum* throughout the experimental period. Feed was provided in dry form (pelleted). Pigs were weighed individually at the beginning of the adaptation period.

3.2.2 Experimental procedures and sample preparation

The experimental procedures and sample preparation for the energy and nutrient balance and emissions period followed the procedure described in Beccaccia et al. (2015a). Briefly, during the energy and nutrient balance (4 days), feed consumption was measured and total urine and faeces excreted per animal were collected daily in separate buckets, weighed and stored in a chamber at 4 °C until the end of the collection period. Urine was collected under sulphuric acid (120 mL of H_2SO_4 at 10% per bucket and day). Upon final collection the faeces and urine were pooled per pig, mixed, subsampled and stored at −20 °C until laboratory analyses were performed. During the next 3 days (days 5–7), urine and faeces were collected in a similar way, but without any addition of sulphuric acid to urine. On day 7, slurries were reconstituted by mixing urine and faeces from each animal in the same proportion as excreted. A part of these slurries was used in fresh for pH and NH_3 emission measurements and another one was subsampled and frozen (–20 °C) for determination of slurry characteristics and biochemical methane potential (BMP).

3.2.3 Chemical analysis of feeds and effluents

Feeds and faeces from the nutrient balance period were analysed for DM (930.15), ash (923.03), EE (920.39) and total dietary fibre (985.29) according to the Association of Official Analytical Chemists (AOAC, 2000) procedures. Ether extract content was determined by AOAC methods (920.39). Total sugars were analysed according to the method of Yemm and Willis (1954). The concentrations of aNDFom, ADFom and ADL were determined sequentially using the filter bag system (Ankom Technology Corp., Macedon, NY, USA) according to Mertens (2002), AOAC (2000 and Van Soest et al. (1991), using heat stable amylase (FAA, Ankom Technology Corp., Macedon, NY, USA), and expressed without residual ash. The contents in soluble fibre were estimated from the difference between total dietary fibre and aNDFom corrected by crude protein (CP) content in the residue. The contents in hemicelluloses and cellulose were

estimated, respectively, from the differences between aNDFom and ADFom and ADFom-ADL concentrations. Feed and faeces were defatted with petroleum ether prior to fibre analysis. The gross energy (GE) concentration was measured in an isoperibol bomb calorimeter (Parr 6400, Parr Instruments Co., Moline, IL, USA). Total N was measured by combustion (method 986.06; AOAC, 2000) using Leco equipment (model FP-528, Leco Corporation, St. Joseph, MI, USA) and CP estimated as N content × 6.25. The proportion of neutral and acid detergent insoluble CP in feed and faeces samples was determined following the standardized procedures in Licitra et al. (1996). The polyphenolic compounds present in OC, diets and faeces were determined after extraction with methanol/acetone/water following the procedure described by Chamorro et al. (2012).

Urine was freeze-dried to obtain its DM content and mixed with benzoic acid before GE analysis to make sure that the whole sample was burned. Total N was determined by steam distillation (APHA, 2005) using an automatic analyser (2300 Kjeltec, Foss Analytical, Hilleroed, Denmark). Hippuric and benzoic acids were analysed directly in urine samples via high performance liquid chromatography (HPLC) on a Varian Pro Star 310 HPLC system (Varian Inc., Palo Alto, CA, USA) following the procedure described by Sánchez-Martín et al. (2017).

Additionally, the pH of faeces, urine and slurry was measured in duplicate using a glass electrode (Crison Basic 20+, Crison, Barcelona, Spain). The pH of faeces was determined by mixing samples with deionized water at a 1:1 proportion. Slurry pH was measured immediately after reconstitution. Slurry samples were analysed for DM and ash following the same methodology used for faeces analyses, and total ammonia N (TAN) and total Kjeldahl N (TKN) using that for urine samples. To avoid N volatilization, the subsample used for TAN analyses was acidified with HCl immediately after reconstitution. Volatile fatty acids (VFA) concentration was determined by gas chromatography equipped with a flame ionization detector (HP 68050 series Hewlett Packard, USA) following the method described by Jouany (1982) with the addition of an internal standard (4-metil valeric).

3.2.4 Gaseous emissions monitoring

The procedure for gas emission monitoring was previously described in Beccaccia et al. (2015a). Briefly, two replicate slurry samples of 0.5 kg from each animal were placed in a 1 L closed container maintained at 25

°C in a thermostatic water bath (Selecta, Barcelona, Spain), and 50 mL of distilled water was added to prevent surface crust formation in each container. Containers were used as dynamic chambers and were connected to an air pump which extracted air at a constant airflow rate of 1.2 L/min. During 11 consecutive days, the air was forced to pass through 2 absorption flasks (impingers) in serial containing 100 mL of sulphuric acid 0.1 N. The acid solution was replaced daily during the first 5 days, and every 48 h until the end of the assay (day 11). The NH_3 trapped in the impingers was analysed following 4500 NH3-D procedure (APHA, 2005) using a detection electrode (Orion High Performance NH3 Electrode, model 9512HPBNWP, Thermo Scientific, Waltham, MA, USA).

Biochemical CH_4 potential from slurry was measured as the cumulative CH_4 production per gram of OM in a batch assay, using 120 mL glass bottles incubated at a mesophilic range (35 °C ± 1 °C) for 100 days, following the methodology described by Angelidaki et al. (2009). Anaerobic inoculum was collected from an anaerobic digester that treated pig slurry, and pre-incubated during 15 days at 35 °C in order to deplete the residual biodegradable organic material (degasification). An inoculum to substrate ratio of 1 on OM basis was used. Slurry samples from each animal were tested by triplicate and three blank bottles containing only anaerobic inoculum were used in order to determine its endogenous CH_4 production. After filling, each bottle was sealed with butyl rubber stoppers and aluminium crimps and the headspace was flushed with pure N_2 for two minutes. During incubation, biogas volume in each bottle was regularly monitored (from 1 to 10 days depending on biogas production) by pressure measurement of the headspace using a manometer (Delta Ohm, HD 9220, Padova, Italy). Methane concentration in the biogas was further analysed using a Focus Gas Chromatograph (Thermo, Milan, Italy) equipped with a split/splitless injector and a flame ionization detector.

3.2.5 Calculations and statistical analysis

The apparent total tract digestibility (CTTAD) of energy, CP, aNDFom and EE of the two OCs studied was determined by difference, assuming additivity between the basal diet and the ingredients tested in the experimental diets. Calculations were made by correcting the CTTAD of energy at the 200 g/kg level of inclusion of OC with the energy contribution of the basal diet, as described in Bolarinwa and Adeola (2012). The CTTAD of energy was also determined by extrapolation to a

total substitution (100%) using linear regression (REG procedure of SAS, 2008) between the CTTAD of energy and the proportion of basal diet substituted with OC at the 100 and 200 g/kg levels of inclusion. The digestible energy (DE) content of OC was estimated from the product between the estimates of CTTAD of energy and its GE concentration (Table 3.1). Each animal was the experimental unit for all the traits studied. The whole data set derived from the five dietary treatments was analysed in a two factors ANOVA as a completely randomized block design, with diet as the main effect and batch as a block factor. The effects of diet were analysed using specific contrasts to test for the effects of type and level of inclusion of OC and of its interaction. The effects of increasing levels of OC were tested using linear and quadratic orthogonal contrasts (Steel et al., 1997).

3.3 Results

3.3.1 Coefficients of total tract apparent digestibility

The influence of trial batch and of its interaction with dietary treatments was not significant for any of the traits studied (data not shown). The CTTAD of the different nutrients analysed in the experimental diets is presented in Table 3.4. The values for DM, cellulose, starch and GE were negative and linearly ($P < .05$) affected by the inclusion level of OC. An interaction ($P = 0.024$) between type and level of OC was observed for CTTAD of CP, as a greater decrease (0.084) was observed in the case of PDOC than for COC (0.028). Moreover, energy losses in urine, expressed as a proportion of DE, increased linearly with level of inclusion for both types of OC (from 0.028 in the control diet to an average of 0.046 in the diets containing 200 g/kg of OC).

Ether extract CTTAD was quadratically affected ($P < .001$) by dietary OC addition, with higher increments being observed at 100 g/kg in the basal diet than from 100 to 200 g/kg. Otherwise, the total polyphenols CTTAD increased linearly ($P < .001$) with dietary OC level. Type of OC did not influence any of the digestion efficiencies studied. A significant interaction between the level and type of OC was observed for CP, as the decrease in CTTAD with the inclusion of OC was greater in the case of PDOC than with COC. The CTTAD of energy of the ingredients tested was determined by the difference method at the 200 g/kg level of inclusion of OC. The results obtained were 0.479 (±0.040, SEM) and 0.327 (±0.049) for COC and PDOC, respectively. Using the GE contents shown in Table 3.1, the DE

concentrations of COC and PDOC were estimated, respectively, to be 11.2 (±0.89) and 7.40 (±1.11) MJ/kg DM.

In the case of CP, the same method led to a greater ($P < .05$) CTTAD for COC than for PDOC (0.375 ± 0.046 vs 0.173 ± 0.060). The estimated apparent digestibilities of EE were greater than 100% and again higher for COC than for PDOC (1.31 ± 0.094 vs 1.04 ± 0.095). The value obtained for aNDFom apparent digestibility in COC was also higher than for PDOC (0.340 ± 0.215 vs 0.282 ± 0.209) although in this case the difference did not reach statistical significance.

The CTTAD of energy and CP of the OC studied were also estimated from regression equations between the values obtained for the experimental diets and the substitution levels of OC (S). The equations obtained were:

CTTAD of energy_COC = 0.871 (±0.0069) − 0.397 (±0.053) × S; $P < .001$; RSD = 0.020; $R^2 = 0.745$.

CTTAD of energy_PDOC = 0.878 (±0.0080) − 0.549 (±0.062) × S; $P < .001$; RSD = 0.023; $R^2 = 0.805$.

dCP_COC = 0.847 (±0.0091) − 0.478 (±0.071) × S; $P < .001$; RSD = 0.026; $R^2 = 0.706$.

dCP_PDOC = 0.859 (±0.012) − 0.680 (±0.093) × S; $P < .001$; RSD = 0.035; $R^2 = 0.736$.

Extrapolating these equations to S = 1, the CTTAD of energy of COC and PDOC was estimated to be 0.474 ± 0.90 (SE of prediction) and 0.329 ± 0.92 and those of CP in 0.369 ± 1.17 and 0.179 ± 1.42, respectively.

3.3.2 Composition of effluents

The effects of type of diet on the composition of faeces and urine are shown in Table 3.5. Inclusion of OC in the diet did not modify starch or aNDFom contents in faeces (averaging 50.4 and 473 g/kg DM respectively), but linearly increased those of ADFom, ADL, hemicelluloses and cellulose, polyphenols and GE concentration. On the contrary, ash faecal content decreased linearly and that of EE linearly and quadratically with dietary OC level.

Table 3.4. Effect of type and level of inclusion of olive cake (OC) on the apparent total tract digestibility coefficients and energy balance of the experimental diets.

	Diets[a]						Significance[b]			
	Basal	**100_COC**	**200_COC**	**100_PDOC**	**200_PDOC**	**SEM[c]**	**1**	**2**	**3**	**4**
Dry matter	0.875	0.826	0.808	0.831	0.781	0.0081	< 0.001	0.44	0.17	0.054
Ash	0.569	0.522	0.586	0.527	0.458	0.040	0.33	0.57	0.13	0.10
Crude protein	0.854	0.786	0.758	0.802	0.718	0.012	< 0.001	0.85	0.33	0.024
Ether extract	0.576	0.720	0.785	0.686	0.709	0.021	< 0.001	< 0.001	0.44	0.075
aNDFom	0.518	0.496	0.482	0.477	0.418	0.034	0.12	0.83	0.39	0.37
Hemicelluloses[d]	0.538	0.490	0.489	0.497	0.430	0.047	0.18	0.81	0.60	0.51
Cellulose[e]	0.567	0.504	0.512	0.486	0.395	0.035	0.013	0.67	0.076	0.19
Total polyphenols	0.663	0.791	0.855	0.774	0.810	0.144	< 0.001	0.31	0.38	0.70
Starch	0.986	0.980	0.973	0.980	0.974	0.0034	0.013	0.94	0.85	0.92
Gross energy	0.877	0.821	0.797	0.825	0.767	0.0087	< 0.001	0.44	0.14	0.054
UE/DE[f]	0.028	0.036	0.045	0.041	0.048	0.0032	< 0.001	0.68	0.19	0.80

[a] 100_COC = 100 g/kg crude OC; 200_COC = 200 g/kg crude OC; 100_PDOC = 100 g/kg partially defatted OC; 200_PDOC = 200 g/kg partially defatted OC.

[b] Contrasts: 1 = linear effect of level of OC, 2 = quadratic effect of level of OC, 3 = type of OC, 4 = interaction type * level of OC.

[c] Standard error of means (n = 6).

[d] Calculated as the difference between ADFom and aNDFom.

[e] Calculated as the difference between ADFom and ADL.

[f] Proportion of digestible energy lost in urine.

Table 3.5. Effect of type and level of inclusion of olive cake (OC) on faeces and urine composition (g/kg DM).

	Diets[a]						Significance[b]			
	Basal	100_COC	200_COC	100_PDOC	200_PDOC	SEM[c]	1	2	3	4
Faeces										
Dry matter	400	383	381	402	401	13.5	0.54	0.80	0.15	0.96
Ash	195	175	147	172	160	1.05	0.003	0.97	0.63	0.46
Crude protein	230	233	226	224	224	5.45	0.48	0.83	0.29	0.59
Ether extract	88.5	68.4	63.9	66.8	59.0	3.12	< 0.001	0.009	0.078	0.82
aNDFom	474	460	464	485	482	19.1	0.99	0.94	0.29	0.86
ADFom	196	225	258	246	269	11.8	< 0.001	0.64	0.21	0.68
ADL	63	82	113	97	114	7.91	< 0.001	0.91	0.37	0.41
Hemicelluloses[d]	278	235	207	239	213	16.3	0.002	0.68	0.75	0.93
Cellulose[e]	133	142	144	149	154	6.03	0.039	0.48	0.19	0.81
Polyphenols	0.099	0.176	0.242	0.180	0.274	0.30	< 0.001	0.87	0.44	0.61
Starch	50.7	52.3	52.3	50.7	46.0	6.14	0.85	0.79	0.59	0.71
Gross energy, MJ	18.0	19.2	20.2	19.4	20.2	0.084	< 0.001	0.055	0.45	0.28
pH	6.56	6.51	6.49	6.42	6.43	0.079	0.28	0.53	0.33	0.80
Urine										
Dry matter	5.58	5.73	6.16	5.25	5.83	0.659	0.58	0.61	0.51	0.93
Total Kjeldahl N, mg/mL	11.3	8.71	7.20	8.34	7.31	0.48	0.008	0.46	0.99	0.82
Benzoic acid, mg/mL	0.408	0.659	0.796	0.432	0.779	0.123	0.005	0.28	0.82	0.46
Hippuric acid, mg/mL	0.647	0.774	1.73	0.918	1.46	0.273	0.013	0.65	0.33	0.40
pH	8.20	8.12	7.68	8.09	7.80	0.184	0.047	0.45	0.80	0.69
Gross energy, MJ/kg DM	8.26	9.36	10.9	10.1	11.1	0.313	< 0.001	0.70	0.09	0.35

[a] 100_COC = 100 g/kg crude OC; 200_COC = 200 g/kg crude OC; 100_PDOC = 100 g/kg partially defatted OC; 200_PDOC = 200 g/kg partially defatted OC.

[b] Contrasts: 1 = linear effect of level of OC, 2 = quadratic effect of level of OC, 3 = type of OC, 4 = interaction type * level of OC.

[c] Standard error of means (n = 6).

[d] Calculated as the difference between aNDFom and ADFom.

[e] Calculated as the difference between ADFom and ADL

Urine composition was also affected by type of diet, as adding OC led to a linear decrease in TKN (by 35.8% at the highest levels of inclusion), whereas benzoic acid, hippuric acid and GE concentration all increased (by 96, 146 and 33%, respectively, comparing controls and the average from 200 g/kg OC). Urine pH decreased linearly (P = .047) with level of OC, from 8.20 in the control diet to 7.74 on average for diets with the highest levels of OC. Neither source nor the interaction level × source of OC affected any of the effluent composition traits studied.

3.3.3 Dry matter and nitrogen flows

Type of diet did not affect DM intake expressed per $kg^{0.75}$ (Table 3.6). However, inclusion of OC led to a linear increase (P < .001) in faecal DM excretion, with values tending (P = .07) to be higher as average for PDOC than for COC diets (16.9 vs 12.1 $g/kg^{0.75}$, respectively), and a trend (P = .09) for a higher urine DM excretion (by 25.9% on average at the highest OC levels with respect to the control diet). In the case of N balance, no differences among treatments were observed either in N intake or N retention (that averaged 2.04 and 0.99 $g/kg^{0.75}$, respectively). However, adding any OC in diets linearly increased N excretion in faeces and decreased it in urine. Consequently, the ratio between faecal and urine N excretion decreased from 2.48 in the basal diet to 1.01 on average in the 200 g/kg OC diets. No differences were observed for DM or N balance between OC sources or for the interaction among sources and levels of inclusion.

3.3.4 Slurry characteristics and gaseous emissions

Table 3.7 shows the effects of including different levels of COC and PDOC in the diets on slurry (faeces + urine) excretion, composition and emissions. The inclusion of either COC or PDOC increased fresh slurry excretion. Additionally, both the DM and OM content of slurry increased, by approximately 18 and 24% per each 10% increase of OC in diet, respectively. On the contrary, a linear reduction of TKN content was observed, mainly because of a reduction in the total ammonia N content. An interaction (P = .04) of type and level of OC was observed for slurry pH, which decreased greatly for COC than for PDOC. The concentration and profile of VFA was not affected by the inclusion of either COC or PDOC. There was no effect of OC type on any of the slurry properties. The inclusion of OC also reduced NH_3 emissions per kg slurry by more than 40% for the highest inclusion rates of both COC and PDOC.

Table 3.6. Effect of type and level of inclusion of olive cake (OC) on DM and nitrogen (N) balance.

	Diets[a]						Significance[b]			
	Basal	**100_COC**	**200_COC**	**100_PDOC**	**200_PDOC**	**SEM[c]**	**1**	**2**	**3**	**4**
Mean body weight (kg)	83.0	80.8	82.0	84.2	77.9	1.49	0.10	0.47	0.80	0.02
DM balance g/kg$^{0.75}$										
Intake	63.1	67.5	66.4	72.9	73.3	4.36	0.21	0.37	0.16	0.86
Faeces	7.91	11.6	12.7	12.2	16.2	1.08	< 0.001	0.47	0.07	0.19
Urine	3.51	3.88	4.15	4.58	4.69	0.18	0.09	0.48	0.14	0.84
N balance, g/kg$^{0.75}$										
Intake	1.98	2.03	1.90	2.24	2.04	0.13	0.94	0.18	0.18	0.77
Faeces	0.293	0.430	0.461	0.442	0.581	0.04	< 0.001	0.46	0.13	0.20
Urine	0.726	0.568	0.485	0.706	0.566	0.03	0.02	0.85	0.12	0.67
Retained	0.963	1.04	0.957	1.09	0.891	0.04	0.58	0.15	0.83	0.50

[a] 100_COC = 100 g/kg crude OC; 200_COC = 200 g/kg crude OC; 100_PDOC = 100 g/kg partially defatted OC; 200_PDOC = 200 g/kg partially defatted OC.
[b] Contrasts: 1 = linear effect of level of OC, 2 = quadratic effect of level of OC, 3 = type of olive cake, 4 = interaction type * level of OC.
[c] Standard error of means (n = 6).

Table 3.7. Effect of type and level of inclusion of olive cake (OC) on slurry (faeces + urine) excretion, initial characteristics and derived ammonia (NH_3) emission and Biochemical Methane (CH_4) Potential.

	Diets[a]						Significance[b]			
	Basal	100_COC	200_COC	100_PDOC	200_PDOC	SEM[c]	1	2	3	4
Slurry excretion (kg/day)	2.23	2.54	2.77	2.98	3.17	0.25	0.01	0.48	0.10	0.92
Slurry characteristics										
Dry matter (g/kg)	111	129	151	116	152	13.0	0.01	0.49	0.63	0.61
Organic matter (g/kg)	81.6	111	121	90.0	122	10.4	0.001	0.90	0.33	0.26
Total ammonia nitrogen (g/L)	9.11	6.32	3.88	5.72	5.19	0.73	< 0.001	0.25	0.64	0.21
Total Kjeldahl nitrogen (g/kg)	11.8	9.49	8.58	9.09	8.84	0.88	0.01	0.23	0.93	0.71
pH	8.61	8.26	7.68	8.07	7.90	0.09	< 0.001	0.70	0.85	0.04
Volatile fatty acids (mmol/L)										
Total	52.8	56.1	60.9	48.3	54.5	5.72	0.47	0.57	0.23	0.91
Acetic acid	39.3	42.5	46.0	37.3	41.0	4.14	0.40	0.69	0.22	0.98
Propionic acid	6.56	6.27	6.80	5.45	5.47	0.86	0.68	0.55	0.22	0.77
Butyric acid	2.87	3.01	4.26	2.38	3.59	0.68	0.20	0.27	0.35	0.98
Gas emissions										
g NH_3/kg slurry	1.76	1.22	0.97	1.35	1.01	0.10	< 0.001	0.34	0.40	0.67
g N-NH_3/kg initial TKN	163	138	116	149	119	11	0.002	0.72	0.49	0.71
mg NH_3/animal and day	359	356	269	393	303	33	0.08	0.10	0.29	0.97
Biochemical methane potential										
B_0, mL CH_4/g OM	300	286	266	280	274	13.7	0.05	0.87	0.93	0.58
L CH_4/animal and day	52.6	76.7	92.4	77.2	110	6.11	< 0.001	0.98	0.12	0.14

[a] 100_COC = 100 g/kg crude OC; 200_COC = 200 g/kg crude OC; 100_PDOC = 100 g/kg partially defatted OC; 200_PDOC = 200 g/kg partially defatted OC.

[b] Contrasts: 1 = linear effect of level of OC inclusion, 2 = quadratic effect of level of OC inclusion 3 = type of OC, 4 = interaction type * level of OC.

[c] Standard error of means (n = 6)..

Despite the higher slurry excretion rates of pigs fed OC diets, NH_3 emissions also tended to decrease when they were expressed per animal and on a daily basis.

In terms of available TKN, the emissions of NH_3 were also lower for COC and PDOC diets compared with the basal diet. Biochemical methane potential (mL CH_4/g OM) decreased linearly (P = .05) with OC inclusion, but as slurry excretion and OM content in slurries increased with the inclusion of OC, higher CH_4 emissions were estimated (with OC) when expressed as L of CH_4 per day.

3.4 Discussion

3.4.1 Nutritional value of olive cake sources

The CTTAD of energy and DE values determined either by difference or by regression (extrapolation to S = 1) were similar for both OC samples, but the SE of the estimations was much lower when they were calculated by difference. The determined energy values were higher for COC than for PDOC, mostly due to a higher EE content (0.162 vs. 0.119, on an as fed basis). The CTTAD of energy for COC (0.479) was close to that assigned (0.446) by Heuzé et al. (2015) for a COC with a similar EE content to that used in the current study (171 g/kg DM). Otherwise, the increase in energy losses in urine in both OC might be related to the higher excretion of benzoic and hippuric acids. Overall, the results indicate an appreciable energy value for this ingredient, especially for COC (around 75% of the DE value assigned to barley grain by FEDNA, 2010). The estimated DE content for COC fits well with the decrease (12%) of feed efficiency observed by Joven et al. (2014) when replacing directly 150 g/kg of barley with COC in the diet of growing pigs. Estimations of CTTAD for EE in both OC were higher than 1. This result might be explained by a decrease in the relative contribution of endogenous lipids to total faecal output with OC addition, which led to a linear, but also quadratic, increase in the dietary CTTAD of EE. Also, the fatty acid profile of OC is characterized by a high proportion (0.679) of highly digestible oleic acid (Joven et al., 2014). The relatively low CTTAD of CP (mainly in the case of PDOC) might be related to the high proportion of insoluble CP in aNDFom (0.672 and 0.902 in COC and PDOC, respectively) and in ADFom (0.351 and 0.476). This low efficiency together with a relatively low CP concentration in the ingredients tested implied that the amount of digestible CP provided by COC and PDOC (36.0 and 13.8 g/kg, respectively) was quite lower than the present

recommendations in this species. The high degree of lignification of aNDFom (0.348 as average of both OC) would explain the low CTTAD of aNDFom observed. Otherwise, the appreciable supply of insoluble fibre with this ingredient can be valuable to meet the current allowances of NDF in pig diets, especially in the case of pregnant and lactating sows and in growing pigs (180, 150 and 110 g/kg respectively, according to FEDNA, 2013).

3.4.2 Nutrient balance and effluent composition

The inclusion of OC affected the composition of effluents and the N balance. The intake of DM and N did not change among treatments, but the inclusion of OC affected the amount excreted and the composition of faeces and urine, which had a direct impact on NH_3 emission. An increase of the dietary levels of OC led to a greater total DM and N excretion in faeces, mainly because of the reduced DM and N CTTAD. On the contrary, the N concentration and total excretion of urine N decreased with increasing OC inclusion rates. As a result, the urine:faecal N ratio decreased from 2.48 in the control diet to 1.01 in the diets containing 200 g/kg of either COC or PDOC. This ratio has been proposed as a main indicator of NH_3 emission, and the reported values in the literature range between 1.2 and 3.8 (Canh et al., 1997, 1998).

Although there were no significant changes in slurry VFA, both benzoic and hippuric acid contents in urine increased with increasing OC inclusion levels. In several species, benzoic acid and its metabolite hippuric acid have been described as secondary metabolites when animals are fed polyphenol-rich diets (Gonthier et al., 2003). The current results show that the total excretion of benzoic and hippuric acids increased from 1.05 mg/mL in the control diet to 2.38 mg/mL in the 200 g/kg OC diets, in parallel to increasing levels of intake, absorption and excretion of polyphenols and fibre. These values are within the range of values reported in the literature for growing pigs using fibrous ingredients (Sánchez-Martín et al., 2017), but are lower than in ruminants, where hippuric acid accounts for about 5% of the N excreted (Bristow et al., 1992, Bussink and Oenema, 1998). Recent studies have demonstrated that both benzoic and hippuric acids may inhibit nitrification in soils, and therefore reduce nitrous oxide emissions from slurry applied to soil (Kool et al., 2006; Sánchez-Martín et al., 2017), which seems to be related with changes in pH and the corresponding reduction of NH_3 emission. Benzoic acid has been tested as a feed additive to reduce NH_3 emissions. Adding

1% benzoic acid to the diet reduces urine pH by about 1 unit, and increases urinary hippuric acid content to more than 10 mg/L, compared to about 1 mg/L in control diets (Bühler et al., 2006). In the current study, slurry pH decreased in parallel with an increase of benzoic + hippuric acid associated with OC supplementation. That finding, along with the lower urine:faecal N ratio and the lower ammonia N concentration in the slurry, would help to explain the decrease (by 28%) of NH_3 emission per kg of slurry when adding 200 g/kg of OC. The decrease was lower (by 20.4%) when expressed per animal and day, because of the parallel increment in the amount of slurry excretion.

Biochemical methane potential expressed as mL CH_4/g OM decreased with OC inclusion. Although higher BMP values were reported when supplementing diets with low digestible fat sources (Antezana et al., 2015) the CTTAD of EE of the ingredients used in the current study was high, so the faecal concentration of EE decreased (by 30.6% on average) with dietary addition of 200 g OC/kg. Moreover, faecal lignin content increased (by 80% on average) with 200 g/kg of OC inclusion, whereas faecal concentration of aNDFom did not vary with type of diet. Previous studies (Angelidaki et al., 2009; Triolo et al., 2011 and Beccaccia et al., 2015b) have shown that lignin concentration in OM is negatively correlated with BMP. Polyphenols have been reported to potentially inhibit methanogenesis, but the content of polyphenols in the faeces (ranging from 99 g/kg DM for the basal diet to 274 g/kg DM in the 200 g/kg inclusion of PDOC) were lower than levels found by Akassou et al. (2010) as potentially causing inhibitions.

When taking into account the higher slurry excretion in OC diets, as well as the higher OM content in those slurries, the overall effect of OC inclusion was to increase BMP (around 100%), when expressed per animal and day. Thus, it is necessary to evaluate whether the potential increase in CH_4 emissions from the slurry could be compensated by the potential mitigation of greenhouse gas emissions gained by using industrial by-products instead of crops, and considering the potential implications on animal performance.

3.5 Conclusions

The nutritional value of the OC tested indicates an appreciable energy content, especially for COC; they also provide significant amounts of oleic acid and insoluble fibre. However, CTTAD of CP was rather low. Otherwise, the inclusion of OC led to a decrease of the NH_3 emission and

BMP per kg of slurry. Nevertheless, slurry excretion increased with OC inclusion, leading to higher BMP when expressed per animal and per day. Although these results indicate a potential for increased CH_4 emissions, a global perspective is needed based on carbon footprint analysis to assess the impact over the whole production chain.

3.6 Acknowledgements

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Chapter 4

Partially defatted olive cake in finishing pig diets: implications on performance, faecal microbiota, carcass quality, slurry composition and gas emission

Partially defatted olive cake in finishing pig diets: implications on performance, faecal microbiota, carcass quality, slurry composition and gas emission

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Abstract: One of the key factors to improve swine production sustainability is the use of agro-industrial by-products in feeds, such as olive by-products. However, it is necessary to assess its effects on the overall production process, including the animal and the environment. With this aim, an experiment was conducted to determine the effects of including a partially defatted olive cake (PDOC) in pig diets on growth performance, faecal microbiota, carcass quality and gas emission from the slurry. Two finishing diets were formulated, a control (C) diet and a diet with PDOC included at 120 g/kg. Eighty finishing male pigs Duroc-Danbred × (Landrace × Large White) of 60.4 ± 7.00 kg BW were divided between these two treatments. During the finishing period (60 to 110 kg BW, 55 days) average daily gain, average daily feed intake and feed conversion ratio were recorded. Faecal samples from the rectum of 16 animals per treatment were incubated for bacteria enumeration. At the end of finishing period, backfat thickness and loin depth (LD) were measured. Animals were slaughtered to obtain carcass weight and carcass composition parameters, and subcutaneous fat was sampled to analyse the fatty acid (FA) profile. In addition, greenhouse gas and ammonia emissions were measured during pig slurry storage using the methodology of dynamic flux chambers. An initial slurry characterisation and biochemical methane potential (B_0) were also determined. No significant differences between treatments were found in performance, carcass quality and microbial counts with the exception of LD, which was lower in PDOC compared with C animals (45.5 v. 47.5 mm, SEM: 0.62; $P = 0.020$). The FA profile of the subcutaneous fat did not differ between treatments, but the monounsaturated FA (MUFA) concentration was higher and the polyunsaturated FA was lower in the animals fed PDOC (50.9 v. 48.3, SEM: 0.48, $P < 0.001$; 17.6 v. 19.3, SEM: 0.30, $P < 0.001$ in mg/100 g of Total FA, for PDOC and C animals, respectively). The initial pig slurry characterisation only showed differences in ADF concentration that was higher ($P < 0.05$) in the slurry from PDOC treatment. Regarding gas emission, slurries from both treatments emitted similar amounts of ammonia (NH_3), carbon dioxide (CO_2), methane (CH_4) and nitrous oxide (N_2O), as well as B_0 values. The results obtained suggest that PDOC may be included in balanced pig diets at rates of up to 120 g/kg without negative effects on performance, carcass quality, gut microflora and slurry gas emission, while improving the MUFA concentration of subcutaneous fat.

Keywords: olive by-products, swine, growth performance, carcass traits, gaseous emissions

Implications

The use of olive cake (OC) in animal feed can be of interest for the livestock sector, increasing its profitability and sustainability. Moreover, its oleic acid and polyphenols' content might positively affect carcass traits and gut health. From the results obtained in the present work, partially defatted olive cake can play a role in pig nutrition, since neither performance or carcass quality traits nor the environmental impact of slurries were negatively affected by its inclusion in diets. Moreover, its use improves the monounsaturated fatty acid concentration in subcutaneous fat. This knowledge is essential to implement the use of OC in animal feeding and to find more sustainable feeding strategies in the livestock sector.

4.1 Introduction

The use of agro-industrial by-products in animal feed can be economically and environmentally beneficial to the livestock sector, increasing its profitability and sustainability. Olive cake (OC) is one of the most relevant agro-industrial by-products in the Mediterranean area, and a major pollutant from olive oil production. The combination of environmental concerns of OC management and the economic interest of components such as phenols has raised the research activity on this by-product (García-González and Aparicio, 2010). Animal feeding is considered one of its possible end uses and this would contribute to circular economy in Mediterranean countries such as Spain, the most important olive oil producer and the fourth biggest pig producer worldwide (FAOSTAT, 2017). Olive cake consists of olive pulp, skin, stone and water. Stones represent about 18 to 32% of the product and are generally removed and used as biomass (FEDNA, 2010). In general, OC without stones show a high fibre and lignin content (from 160 to 557 g lignin/kg DM) and a low but variable CP content (44 to 115 g/kg DM) (Alburquerque et al., 2004; Molina-Alcaide and Yañez-Ruiz, 2008; De Blas et al., 2015a). Its oil content depends on the oil extraction procedure. The crude OC contains about 120-140 g/kg ether extract (EE) and it can be partially or totally extracted, based on variability in the market price of olive oil (Molina-Alcaide and Yañez-Ruiz, 2008; De Blas et al., 2015b). The fatty acid (FA) composition of OC reveals a high proportion of oleic acid (Joven et al., 2014) and thus a possible positive effect on the quality of animal products when used in diets. In main producing areas of olive oil in Spain, these by-products are generally dried and available throughout the year and, thus, potentially used as a source of energy in finishing pigs and sows. Recent studies in growing-finishing pigs show that its digestible energy (DE) content is variable depending on its oil content (around 60 to 80% of the DE provided by barley grain; Ferrer et al., 2018) and that its inclusion up to 100 g/kg of OC in diets replacing barley grain on a weight basis does not impair feed intake and growth but decreases dietary DE concentration and carcass conformation and backfat thickness (Joven et al., 2014). Additionally, its use as a feed ingredient might modify slurry production, composition and gas emission (Ferrer et al., 2018). These effects might be related to its high fibre and phenolic content. Previous research has shown that including fibrous by-products (such as sugar beet pulp, orange pulp, carob meal or rapeseed meal) in diets for growing pigs can help to reduce ammonia (NH_3) and occasionally methane (CH_4) emission from

faeces or slurry, per unit of nitrogen (N) or organic matter (OM), respectively (Canh et al., 1997; Torres-Pitarch et al., 2014; Beccaccia et al., 2015). In the case of OC its inclusion in diets might also decrease NH_3 emission from slurry (Ferrer et al., 2018). On the other hand, olive by-products are rich in phenolic components (3.0-50.0 g/kg DM) with a high antimicrobial and antioxidant capacity (Leouifoudi et al., 2015). When included in diets this antimicrobial capacity might also affect animal health and bacterial-dependent gas emission from slurry.

The objective of the present study was to determine the effects of the inclusion of a partially defatted olive cake (PDOC) in balanced finishing pig diets on growth performance, carcass quality, faecal microbiology, slurry composition and gas emission.

4.2 Material and methods

4.2.1 Animals, diets and experimental design

Eighty growing males, progeny of Duroc-Danbred x (Landrace x Large White) at 25.1±3.6 kg initial BW were used in the experiment. At arrival, pigs were identified and distributed according to BW in 16 pens and two rooms (8 pens per room). The slurry pit from one of the rooms was divided into four different pits that allowed the collection of the slurry excreted by the animals housed in two consecutive pens and fed with the same diet. All the animals were phase-fed two common commercial feeds before the beginning of the experimental period (phase 1: from 25 to 34 kg BW; phase 2: from 34 to 61 kg BW). At 60.5 kg BW pens were assigned to two different treatments (8 pens/treatment according to average pen weight and standard deviation within pen). These treatments consisted of a control feed (C-diet) or a feed with 120 g/kg of PDOC (PDOC-diet) formulated to be isocaloric and isoaminoacidic by adjusting the added fat, soybean meal and synthetic amino acids. Minerals were also adjusted to requirements in both diets. The OC inclusion level in the PDOC diet was chosen from the results obtained in the study of Joven et al. (2014) and our previous results (Ferrer et al., 2018) reporting no differences in average daily feed intake (ADFI) up to 200 g/kg inclusion level of PDOC. Detailed OC and experimental diets composition are given in Tables 4.1 to 4.4. The dehydrated OC was obtained from an olive pomace industry (DCOOP, Antequera, Spain) and added in the PDOC-diet at the expense of barley and sunflower meal. The coefficient of total tract apparent digestibility (CTTAD) of energy for OC was previously determined in an *in vivo* study (Ferrer et al., 2018). Experimental feeds were offered *ad*

libitum in dry form (pelleted) for 55 days, until slaughter (118 ± 10.6 kg BW). Free access to water was provided during all of the experimental period.

Table 4.1. Chemical composition of the partially defatted olive cake used in the swine trial (g/kg DM, unless otherwise specified).

Analysed chemical composition	**OC**
Dry matter (g/kg FM)	914
Ash	121
Gross energy, MJ/kg	23.0
Digestible energy, MJ/kg	8.45
Crude protein	92.3
Digestible crude protein	21.0
Ether extract	122
NDF[1]	415
ADF[1]	290
Lignin	171
NDICP[2]	61.7
ADICP[3]	37.6
Total polyphenols[4]	8.6
Sugars	83.2

[1]Ash-free
[2]Neutral detergent insoluble CP
[3]Acid detergent insoluble CP
[4]Expressed as acid gallic

Table 4.2. Fatty acid profile of the of the partially defatted olive cake used in the swine trial (g/kg DM).

Analysed chemical composition	**OC**
Total Fatty Acids	121
Saturated fatty acids	19.0
Lauric Acid (C12:0)	0.51
Myristic Acid (C14:0)	0.29
Palmitic Acid (C16:0)	12.9
Heptadecanoic Acid (C17:0)	1.22
Stearic Acid (C18:0)	4.11
Monounsaturated fatty acids	88.0
Palmitoleic Acid (C16:1n7)	0.92
Heptadecenoic Acid (C17:1)	1.36
Oleic Acid (C18:1n9)	83.8
Vaccenic (C18:1n7)	1.64
Eicosenoic Acid (C20:1n9)	0.20
Docosadienoic Acid (C22:1n9)	0.07
Polyunsaturated fatty acids	13.6
Linoleic Acid (C18:2n6)	10.5
Linolenic Acid (C18:3n3)	1.20
Estearidonic Acid (C18:4n3)	0.24
Eicosatrienoic Acid (C20:3n9)	0.04
Arachidonic Acid (C20:4n6)	0.08
Eicosapentaenoic Acid (C20:5n3) EPA	0.10
Docosatetraenoic Acid (C22:4n6)	0.12
Docosapentaenoic Acid (C22:5n3)	0.87
Docosahexaenoic Acid (C22:6n3) DHA	0.46

OC: olive cake

Table 4.3. Ingredient content and chemical composition of the experimental pig diets (g/kg as fed, unless otherwise specified).

	Treatments[1]	
	C-diet	**PDOC-diet**
Ingredients		
Barley	455	332
Triticale	50.0	50.0
Wheat	150	150
Hominy feed	86.0	86.0
Glycerol	10.0	10.0
Rapeseed meal	86.0	86.0
Sunflower meal	60.0	20.0
Soybean meal	30.0	60.0
Partially defatted olive cake	0	120
Fat	43.0	58.0
Calcium carbonate	11.5	8.3
Sodium chloride	3.6	3.3
Monocalcium phosphate	0	1.2
L-lysine	6.4	6.0
Methionine	0.7	1.1
Threonine	1.4	1.5
Tryptophan	0.1	0.2
Valine	0	0.1
Phytase	0.2	0.2
Liquid acid	0.15	0.15
Vitamin-mineral premix[2]	0.50	0.50
Analysed chemical composition, g/kg DM		
Dry matter (g/kg FM)	906	908
Ash	43.6	51.9
Crude protein	171	161
Ether extract	85.9	119
NDF[3]	182	215
ADF[3]	59.2	74.3
Lignin	20.4	32.5
NDICP[4]	24.5	33.2
ADICP[5]	1.6	4.4
Total polyphenols	0.38	0.98

	Treatments[1]	
	C-diet	**PDOC-diet**
Gross energy, MJ/kg	17.7	18.4
Calculated chemical composition[6]		
Digestible energy, kcal/kg[7]	3341	3277
Net energy, kcal/kg[7]	2411	2412
Calcium	0.58	0.59
Phosphorus	0.42	0.41
Ileal standardized ileal amino acids		
Lysine	0.78	0.78
Methionine	0.26	0.28
Methionine+Cystine	0.48	0.48
Threonine	0.51	0.51
Tryptophan	0.14	0.14
Valine	0.51	0.51

[1]C-diet = control diet; PDOC-diet = diet with 120 g/kg partially defatted olive cake
[2]Vitamin–mineral premix in the finishing phase provided per kilogram of feed: retinol, 6500 IU (E672); cholecalciferol, 1860 IU (E671); α-tocopherol, 10 mg; menadinone, 0.6 mg; thiamine, 0.8 mg; riboflavin, 3.2 mg; pyridoxin, 1.0 mg; cobalamin, 0.02 mg; niacin, 12 mg; pantothenic acid, 9.60 mg; choline clorure, 116 mg; Fe, 72 mg as $FeSO_4 \cdot 7H_2O$; Cu, 16 mg as $CuSO_4 \cdot 5H_2O$; Zn, 80 mg as ZnO; Mn, 40 mg as MnO; I, 1.44 mg as KI and Se, 0.20 mg as Na_2SeO_3.
[3]Ash-free
[4]Neutral detergent insoluble CP
[5]Acid detergent insoluble CP
[6]Calculated values based on De Blas et al., (2015b)
[7]Calculated from the coefficient of total tract apparent digestibility of energy previously determined in Ferrer et al. (2018)

Table 4.4. Fatty acid profile of the experimental pig diets (g/kg DM).

	Treatments[1]	
	C-diet	**PDOC-diet**
Total fatty acid	64.4	89.6
Saturated fatty acids	20.9	31.6
Capric Acid (C10:0)	0.02	0.03
Lauric Acid (C12:0)	0.03	0.06
Myristic Acid (C14:0)	0.65	1.29
Pentadecanoic Acid (C15:0)	0.06	0.17
Palmitic Acid (C16:0)	13.8	18.9
Heptadecanoic Acid (C17:0)	0.22	0.54
Stearic Acid (C18:0)	5.74	10.2
Arachidic Acid (C20:0)	0.17	0.21
Behenic Acid (C22:0)	0.10	0.11
Lignoceric Acid (C24:0)	0.05	0.07
Monounsaturated fatty acids	23.3	38.4
Palmitoleic Acid (C16:1)	1.01	1.33
Myristoleic Acid(C14:1)	0.03	0.09
Heptadecenoic Acid (C17:1)	0.11	0.24
Oleic Acid (C18:1n9c)	19.1	31.6
Vaccenic Acid (C18:1n7)	2.21	3.09
Elaidic Acid (C18:1n9t)	0.47	1.57
Eicosenoic Acid (C20:1)	0.38	0.42
Erucic Acid (C22:1n9)	0.03	0.08
Polyunsaturated fatty acids	20.1	19.5
Linoleic Acid (C18:2n6c)	18.6	17.8
Linolenic Acid (C18:3n3)	1.16	1.41
Eicosadienoic Acid (C20:2)	0.19	0.14
Eicosatrienoic Acid (C20:3n6)	0.03	0.04
Arachidonic Acid (C20:4n6)	0.08	0.08
Docosatetraenoic Acid (22:4n6)	0.03	0.03
Docosapentaenoic Acid (22:5n3)	0.02	0.03
Docosahexaenoic Acid (C22:6n3)	0	0.03

	Treatments[1]	
	C-diet	**PDOC-diet**
PUFA[2]/SFA[3]	0.96	0.62
MUFA[4]/SFA	1.11	1.22
Oleic Acid/Total Fatty Acids	0.297	0.353

[1] C-diet = control diet; PDOC-diet = diet with 120 g/kg partially defatted olive cake
[2] PUFA: polyunsaturated fatty acids
[3] SFA: saturated fatty acids
[4] MUFA: monounsaturated fatty acids

4.2.2 Growth performance, carcass and meat quality

Pigs were individually weighed fortnightly from the start of trial until slaughter. Feed consumption was recorded and the average daily gain (ADG), ADFI and feed conversion ratio (FCR) were then calculated. *In vivo* backfat (BF) and loin depth (LD) were measured at the P2 position, using a B-mode ultrasound device (Agroscan A16, Angoulême, France) as described by Cerisuelo et al. (2010) on days 53-54. At the end of the experimental period pigs were slaughtered. Fasting was practiced for approximately 12 h before slaughter in all animals. Carcass weight (hot carcass weight) and carcass composition was measured using an ultrasonic automatic carcass grading device (AutoFomTM, Carometec food technology, Denmark) following the methodology described by Torres-Pitarch et al. (2014). At approximately 2 h *post-mortem* (during the chilling process) pH in the *Splenius* muscle and meat colour components at the *Gracillis* muscle were recorded using a pH meter (model pH25+, Crison, Barcelona, Spain) and a portable CR300 Minolta Chromameter (Konica Minolta, Osaka, Japan) respectivelly. Additionally, subcutaneous fat was sampled at the level of the second cervical vertebrae to analyse the FA profile at the left side of the carcass of 20 animals per treatment as described by Torres-Pitarch et al. (2014).

4.2.3 Faecal microbiota by culture-based methods

Faecal samples were aseptically removed directly from the rectum of 16 animals per treatment (2 animals per pen) at days 35 and 36 of the experimental period for bacterial (total anaerobic bacteria, *enterobacteria, lactobacilli and bifidobacteria*) enumeration. The samples of each pen were pooled and treated as a pen sample. Within the 2 hours after collection, faecal samples were diluted 1:10 (1 g faeces in 9 mL of peptone water) and decimal dilutions were prepared. The number of

colony forming units per gram (CFU/g faeces) of total anaerobic bacteria and *bifidobacteria* were isolated onto Thiogliolato Agar (Liofichem, Roseto degli Abruzzi, Teramo, Italy) and BD Bifidobacterium Agar, Modified (Becton Dickinson GmbH, Germany), respectively, following anaerobic incubation at 37ºC for 72 h. *Enterobacteria* were isolated on McConkey agar (Liofichem, Roseto degli Abruzzi, Teramo, Italy), following aerobic incubation at 37ºC for 24 h. *Lactobacilli* were cultured on Man, Rogosa, Sharp agar (MRS, Liofilchem, Roseto degli Abruzzi, Teramo, Italy) following incubation at 37ºC for 48 h. All colonies were counted immediately after removal from the incubator.

4.2.4 Slurry measurements and gas emission

At the end of the fattening period, the slurry excreted from each individualised pit (2 pits per treatment) was quantified by measuring the level of the slurry achieved in the pit. Afterwards, the slurry in each pit was homogenised with a pump and a representative sample was pumped to two tanks of 120 L of capacity per pit (470 mm diameter and 800 mm height) leaving a 200 mm of headspace between the slurry surface and the top of the tank. Overall, eight tanks were filled with 90 L of slurry each and sampled during the fill-in for slurry chemical characterization. The tanks were placed in a mechanically ventilated room for eight successive weeks simulating outdoor slurry storage. The gas emissions (NH_3, CH_4, carbon dioxide (CO_2) and nitrous oxide (N_2O) from slurry over the storage period was measured by using the methodology described by Calvet et al. (2017). In brief, tanks were set as a dynamic chamber by fitting specially adapted lids which had a central circular hole connected to a fan with an extraction duct to draw air from the tank headspace. The lids were only placed on the tanks over the gas measurement periods, remaining open the rest of the time to simulate natural storage conditions. Gas concentrations were measured at the outlet duct of each tank and at the room ambient by means of a photoacoustic gas monitor (INNOVA1412, Air Tech Instruments, Ballerup, Denmark) connected to a multi-point sampler. Every week, emissions were measured continuously during 48 h for the 8 tanks.

Ammonia concentration measurements were also verified using acid wet traps. A subsample from the exhausted air from the headspace of the tanks was forced to pass with an air pump through absorption flasks filled with 100 mL of 0.05N H_2SO_4.The quantity of total ammonia N (TAN) trapped in the absorption flasks was analysed following 4500 NH3-D

procedure (APHA, 2005) using a detection electrode (Orion High Performance NH3 Electrode, model 9512HPBNWP, Thermo Scientific, USA).

Additionally, the biochemical CH_4 potential (B_0) from the initial pit slurry sampled during the fill in of the tanks was measured.

4.2.5 Chemical analysis

The PDOC and experimental feeds were analysed for DM, ash and EE according to the Association of Official Analytical Chemists (AOAC, 2000) procedures. Total sugars were analysed according to the method of Yemm and Willis (1954). The concentrations of NDF, ADF and ADL were determined sequentially according to Van Soest procedure (Van Soest et al., 1991). The gross energy (GE) concentration was measured in an isoperibol bomb calorimeter (Parr 6400, Parr Instruments Co., Moline, IL, USA). Total N was measured by combustion using Leco equipment (model FP-528, Leco Corporation, St. Joseph, MI, USA) and CP estimated as N content x 6.25. The proportion of neutral and acid detergent insoluble CP (NDICP and ADICP, respectively) was determined following the standardized procedures in Licitra et al. (1996). Feeds and PDOC samples were defatted with petroleum ether prior to fibre analysis. The polyphenolic compounds were determined after extraction with methanol/acetone/water following the procedure described by Chamorro et al. (2012) and results expressed as gallic acid equivalent.

Slurry samples were analysed for pH in duplicate using a glass electrode (Crison Basic 20+, Crison, Barcelona, Spain) and for DM, ash and OM, EE, fibre and GE following the same methodology used for PDOC and feeds. The TAN and total Kjeldahl N (TKN) were analysed by steam distillation (4500 NH3-B and 4500 NH3-C procedures; APHA, 2005) using an automatic analyser (2300 Kjeltec, Foss Analytical, Hilleroed, Denmark). To avoid N volatilization, the subsample used for TAN analyses was acidified with HCl immediately after the samples were collected.

The B_0 from the slurry was measured in a batch assay, using 120 mL glass bottles incubated at a mesophilic range (35°C±1ºC) for 100 days, following the methodology described by Ferrer et al. (2018). Anaerobic inoculum was collected from an anaerobic digester that treats domestic and industrial wastewater from the wastewater treatment plant in Sagunto (Spain), and pre-incubated for 15 days at 35ºC in order to deplete the

residual biodegradable organic material (degasification). An inoculum to substrate ratio of 1 on OM basis was used.

The FA profile of the PDOC, experimental feed samples and the subcutaneous fat was measured by gas chromatography. Fatty acid methyl esters (FAME) were prepared according to O'Fallon et al. (2007) and were analysed in a Focus Gas Chromatograph (Thermo, Milan, Italy). The FA profile was calculated as the proportion of saturated, monounsaturated and polyunsaturated FA (SFA, MUFA and PUFA, respectively) in grams per 100 mg of FA.

4.2.6 Statistical analysis

Data were analysed using SAS® (Statistical Analysis System) System Software (Version 9.1, SAS Institute Inc., Cary, North Carolina, EEUU). Differences in BW, ADG, ADFI, FCR, BF, LD and carcass and meat quality traits between experimental treatments were tested by one-way analysis of variance using the GLM procedure of SAS in a completely randomized block design, with the dietary treatment (C-diet and PDOC-diet) as the main effect and room as a block factor in the models. Microbial counts were log10 transformed before analysis. For ADG, ADFI, FCR, initial and final weight, and microbial counts, the experimental unit was the pen and for the carcass and meat quality measurements the individual pig was considered the experimental unit. Gas emission results (mg/ L and h) are presented as the average emission rate during the experiment. These data were also calculated in mg/animal day and h (considering the total amount of slurry excreted) and both were statistically analysed, together with initial slurry characterization, by a one-way analysis of variance using the GLM procedure of SAS, where the pit was the experimental unit and the dietary treatment was considered the source of variation.

4.3 Results

The statistical analysis performed showed no significant influence ($P>0.10$) of the room and its interaction with the dietary treatment for any of the traits studied (data not shown), so that this effect was excluded from the model.

4.3.1 Partially defatted olive cake and experimental diets composition

The chemical composition of the OC used in the study is summarized in Tables 4.1 and 4.2. This OC presented a high EE (122 g/kg DM), sugar (82.2

g/kg DM) and DE (8.45 MJ/kg DM) content. Its fibre concentration was also high, particularly its ADL level. The analysed FA profile of OC revealed that oleic acid was the main FA (83.8 g/kg), followed by palmitic and linoleic acids. Regarding the chemical composition of the experimental diets (Tables 4.3 and 4.4), PDOC-diet showed a 39% higher EE, fibre (18 and 26% higher content of the NDF and ADF fractions), and lignin and polyphenol content compared with C-diet. The concentration of almost all FA (especially oleic acid) was also greater in the PDOC-diet compared with C-diet.

4.3.2 Growth performance, carcass and meat quality

The results on growth performance are summarized in Table 4.5. At the end of the study, BW was not significantly different between treatments. No significant differences were obtained in ADG or in ADFI. However, FCR tended to be higher (0.12 units; P=0.059) and LD was significantly lower (2.02 units, P=0.02) in the group of animals offered PDOC-diet. The carcass and meat quality traits measured are shown in Table 4.6. The inclusion of PDOC in pig diets had no significant effect on carcass characteristics, except the pH that was lower (P<0.001) and the red colour (a*) that tended (P=0.086) to be lower in the meat of pigs offered PDOC-diet. Regarding the FA profile of the subcutaneous fat (Table 4.7), total MUFA concentration was higher and total PUFA concentration lower in the fat tissue of animals offered the diet with 12% PDOC compared with that of the C-diet (P<0.001). The ratio MUFA/SFA was higher and the ratio PUFA/SFA lower in the pigs offered PDOC compared with the pigs offered C-diets (P<0.05). Taking into account individual FA, the biggest differences between treatments were found for the MUFA acids, especially palmitoleic, heptadecenoic, oleic and vaccenic acids (P<0.05), in the fat of animals offered PDOC compared with that of the animals offered C-diet. Also, the oleic vs total FA ratio was higher (P<0.05) in the fat from animals offered PDOC than C-diet.

Table 4.5. Effect of the inclusion of partially defatted olive cake in diets on pig performance traits.

	Treatments[1]			
	C-diet	**PDOC-diet**	**SEM[2]**	***P-value***
Initial body weight, kg	60.0	60.4	2.24	0.892
Final body weight, kg	119	117	2.27	0.656
Average daily gain, kg/d	1.06	1.03	0.02	0.221

	Treatments[1]			
	C-diet	**PDOC-diet**	**SEM[2]**	***P-value***
Average daily feed intake, kg/d	2.88	2.93	0.05	0.509
Feed conversion ratio	2.73	2.85	0.04	0.059
Backfat thickness, mm	12.5	12.1	0.351	0.400
Loin depth, mm	47.5	45.5	0.617	0.020

[1]C-diet = control diet; PDOC-diet = diet with 120 g/kg partially defatted olive cake
[2]SEM: standard error of the mean

Table 4.6. Effect of partially defatted olive cake inclusion in pig diets on carcass and meat quality

	Treatments[1]			
	C-diet	**PDOC-diet**	**SEM2**	**P-value**
Carcass characteristics				
Carcass weight, kg	85.8	84.7	1.39	0.580
Carcass yield, %	72.2	72.3	0.268	0.752
Fat depth at GM, mm[3]	1.59	1.59	0.077	0.952
Lean meat percentage, %	58.7	58.3	0.445	0.521
Ham lean meat, mm	71.6	71.6	0.371	0.954
Ham fat, mm	11.5	11.6	0.377	0.970
Bacon lean meat, mm	56.0	55.7	0.578	0.736
Loin lean meat, mm	59.2	58.4	0.621	0.325
Shoulder lean meat , mm	65.8	65.3	0.360	0.390
Lean meat in 3-4 rib, mm	52.7	51.5	0.628	0.157
Fat in the 3-4 rib, mm	16.8	17.2	0.413	0.424
Meat quality				
pH[4]	6.55	6.01	0.079	<0.001
Meat colour[5]				
Lightness (L*)	37.4	37.4	0.400	0.979
Redness (a*)	8.76	8.10	0.277	0.086
Yellowness (b*)	3.00	2.74	0.203	0.347

[1]C-diet = control diet; PDOC-diet = diet with 120 g/kg partially defatted olive cake
[2]SEM: standard error of the mean
[3]GM = *Gluteus medius* muscle
[4]Measured at the *Splenius* muscle level
[5]Measured at the *Gracillis* muscle level

Table 4.7. Effect of partially defatted olive cake inclusion in pig diets on fatty acid (FA) content and FA profile in subcutaneous fat of pigs (mg/100 mg fresh tissue, unless otherwise specified).

	Treatments[1]			
	C-diet	**PDOC-diet**	**SEM[2]**	***P-value***
Total fatty acid (FA)	65.8	68.8		
SFA[3], mg/100 mg Total FA	32.4	31.5	0.42	0.116
Capric Acid (C10:0)	0.308	0.284	0.0185	0.360
Lauric Acid (C12:0)	0.248	0.205	0.0188	0.100
Myristic Acid (C14:0)	7.98	8.80	0.190	0.003
Pentadecanoic Acid (C15:0)	0.830	0.819	0.0729	0.913
Palmitic Acid (C16:0)	137	140	3.69	0.600
Heptadecanoic Acid (C17:0)	3.90	4.08	0.180	0.472
Stearic Acid (C18:0)	63.1	63.2	3.11	0.983
MUFA[4], mg/100 mg Total FA	48.3	50.9	0.48	<0.001
Palmitoleic Acid (C16:1)	2.62	2.95	0.084	0.006
C16:1 (n-7)	16.0	16.8	0.53	0.321
Heptadecenoic Acid (C17:1)	3.23	3.56	0.092	0.015
Oleic Acid (C18:1n9c)	273	301	6.5	0.004
Vaccenic Acid (C18:1n11)	16.0	19.1	0.81	0.011
Eicosenoic Acid (C20:1)	6.52	6.92	0.197	0.146
PUFA[5], mg/100 mg Total FA	19.3	17.6	0.30	<0.001
Linoleic Acid (C18:2n6c)	103	97.5	2.76	0.129
Linolenic Acid (C18:3n3)	6.46	6.93	0.183	0.067
Eicosadienoic Acid (C20:2)	5.37	5.03	0.145	0.096
Eicosatrienoic Acid (C20:3n6)	1.08	1.04	0.030	0.428
Arachidonic Acid (C20:4n6)	2.42	2.39	0.077	0.765
Docosadienoic Acid (C22:2)	5.43	5.75	0.281	0.416
Docosatetraenoic Acid (22:4n6)	1.35	1.10	0.116	0.125
Docosapentaenoic Acid (22:5n3)	0.928	0.814	0.1247	0.511
Docosahexaenoic Acid (C22:6n3)	0.218	0.348	0.0567	0.104
PUFA[5]/SFA[3]	0.596	0.559	0.0125	0.037
MUFA[4]/SFA[3]	1.50	1.62	0.034	0.009
Oleic Acid/Total FA	4.15	4.37	0.039	<0.001

[1]C-diet = control diet; PDOC-diet = diet with 120 g/kg partially defatted olive cake
[2]SEM: standard error of the mean
[3]SFA: saturated fatty acids
[4]MUFA: monounsaturated fatty acids
[5]PUFA: polyunsaturated fatty acids

4.3.3 Microbial counts

Bacterial counts from faeces did not show any significant differences (P>0.05) between treatments. The ratio *lactobacilli* : *enterobacteria* was also similar in both treatments (Table 4.8).

Table 4.8. Effect of partially defatted olive cake inclusion in diets on faecal bacteria counts (Log10 CFU/g fresh faeces) in finishing pigs

	Treatments[1]			
	C-diet	**PDOC-diet**	**SEM[2]**	***P-value***
Total anaerobic bacteria	8.37	8.11	0.179	0.316
Bifidobacteria	8.75	8.50	0.116	0.154
Enterobacteria	6.92	6.62	0.175	0.237
Lactobacilli	9.11	8.73	0.211	0.228
Ratio *Lactobacilli:Enterobacteria*	1.32	1.33	0.033	0.847

[1]C-diet = control diet; PDOC-diet = diet with 120 g/kg partially defatted olive cake
[2]SEM: standard error of the mean

4.3.4 Slurry composition and gas emission

The amount of slurry produced by the animals offered PDOC tended to be 23% higher than that produced by the animals fed the C-diet (P=0.088) (Table 4.9). Regarding slurry composition, the ADF concentration in the slurry of animals offered PDOC was significantly higher than that of the animals offered C-diet (P<0.05). In addition, ADL concentration in the slurry tended to be higher (P=0.06) and the EE concentration in the slurry tended to be lower (P=0.08) in the group of animals offered PDOC. Concerning the gas emissions, no significant differences were obtained either on the B_0 values or on the amount of gas emitted expressed per L of slurry or per animal and day.

4.4 Discussion

The OC used in the present study shows an intermediate EE level and a high sugar content compared with other OC in the literature (EE ranging from 70 to 170 and sugar content ranging from 10 to 19 g/kg, De Blas et al., 2015b). In particular, it is known that the sugar and fat content can be very variable among dried OC sources (Abo Omar et al., 2012). While the variability observed in terms of fat content among OC sources is mainly related to the olive oil extraction system, the variability in sugar content of OC is attributed to the time of OC storage before being dried due to

microbial fermentation that takes place during its storage (De Blas et al., 2015b). In terms of fibre, as expected, OC is a fibrous product with a relatively high lignin content. Compared with other fibrous feedstuffs such as rapeseed meal, alfalfa or sunflower meal its fibre and particularly ADL content, in this study was high (INRA 2004; FEDNA, 2010). All this led to higher EE and fibre fractions content in the PDOC-diet compared with the control diet. The PDOC-diet showed also a higher oleic acid and polyphenol content, compared with the control-diet (+1.42 and +0.6 g/kg, respectively) due to the high amount of oleic acid (83.8 g/kg DM) and polyphenols (8.6 g/kg DM) in the PDOC.

Table 4.9. Effect of partially defatted olive cake inclusion in pig diets on slurry characteristics and gas emission[1]

	Treatments[2]			
	C-diet	**PDOC-diet**	**SEM[3]**	***P-value***
Slurry production, L/animal and day	4.18	5.14	0.216	0.088
Slurry characteristics				
DM, g/kg	83.9	93.9	3.70	0.196
Organic matter (OM), g/kg	67.3	77.1	3.63	0.197
Total ammonia nitrogen, g/L	5.22	3.88	0.430	0.158
Total Kjeldahl nitrogen, g/kg	6.88	5.72	0.302	0.113
NDF, g/kg of DM	413	422	2.90	0.153
ADF, g/kg of DM	209	233	2.53	0.022
ADL, g/kg of DM	76.6	97.7	3.83	0.060
Ether extract, g/kg of DM	159	144	3.29	0.080
pH	6.70	6.61	0.099	0.568
B_0, ml CH_4/g of OM	394	333	25	0.226
Concentration of gases emitted, mg/L and h				
Ammonia	0.586	0.634	0.053	0.587
Carbon dioxide	4.59	5.29	0.764	0.583
Methane	0.273	0.191	0.063	0.456
Total gas emission, mg/animal and day				
Ammonia	59.3	78.3	8.46	0.253
Carbon dioxide	454.1	653.1	67.5	0.172
Methane	26.8	23.6	4.99	0.695

[1] n = 2
[2] C-diet = control diet; PDOC-diet = diet with 120 g/kg partially defatted olive cake
[3] SEM: standard error of the mean

The inclusion of fibrous by-products in pig diets, such as PDOC, has been related with poorer performance traits especially during the growing phase (Jarret and Ashworth, 2018). However, in the current study, diets were formulated to be isonutritive and, accordingly, the inclusion of a 12% PDOC did not lead to significant differences in growth performance, although a trend to a slight decrease of feed efficiency (FCR 0.12 units higher; P=0.06) was detected in pigs offered PDOC. This might indicate that the net energy value for PDOC was overestimated in the current study, since pigs offered PDOC-diet increased feed consumption to meet their energy requirements. On the other hand, *in vivo* BF measurement was similar between treatments but LD was about 2 mm lower (P<0.05) on average in the group of pigs fed PDOC-diet. Joven et al. (2014) also showed a linear decrease of fat depots in the carcass as the level of OC increased from 0 to 15% in finishing pigs. Mas et al. (2011) and González et al. (2012) described no differences in carcass characteristics from pigs offered diets enriched with oleic acid and olive pomace oil (65 g/kg), respectively. Differences in carcass fat content are expected when pigs consume more energy than required with high energy diets or when the indispensable amino acid or protein levels in diets are lower than required (Cámara et al., 2016). With respect to meat quality traits, Serra et al. (2018) showed similar pH levels and lower yellowness (b*) with the inclusion of olive pomace in pig diets. Despite this our results are in accordance with those authors, only the pH values were affected by feeding OC. Pigs fed OC showed a lower meat pH that could be associated with higher muscle glycogen stores. Although a statistical difference has been observed in the muscle pH at 2h *post-mortem*, it has no practical implications on quality traits (no differences were found in colour parameters). Besides, pH values in the range of 6.6-6.0 after 2h post-mortem are not associated with the development of pale, soft and exudative (PSE) or dark, firm and dry (DFD) meats (Rosenvold and Andersen, 2003).

As expected, PDOC addition led to differences in FA profile of subcutaneous fat, with a higher proportion of MUFA and lower proportion of PUFA with respect to total FA. These differences were caused by the oleic acid increment in the diet, since the deposition of FA in pigs is known to be primarily influenced by the FA composition of the diet (Cava et al., 1997). The modification of FA profile with the addition of olive by-products has been described by numerous authors (González et

al., 2012, Joven et al., 2014 and Serra et al., 2018), being of interest due to the improved sensory quality of meat.

Polyphenols are able to modulate the intestinal ecology, influencing host health through the bioactive compounds generated by the colonic microbiota (Marin et al., 2015). *In vitro* animal and human studies conducted with a selection of polyphenols at a determinate concentration reported modifications in the gut microbiome by the inhibition of pathogenic bacteria and the stimulation of the growth of beneficial bacteria due to modifications of gut ecosystem (Cardona et al. 2013). On the other hand, the inclusion of insoluble fibre in diets can have a prebiotic effect in the gut of pigs and modify gut microbiota (Pieper et el., 2015). In the present study *Lactobacillus, Bidfidobacterium* and *Enterobacteria* values were similar to those obtained by Zhao et al. (2013). However, no significant effects were found when including PDOC in feeds (a fibre-rich ingredient) on gut microbiology. This could indicate, an acclimation of the bacteria to the inclusion of PDOC in the diet or a limitation of cultured-based technique to assess the diversity and dynamics of the gastrointestinal microbiota.

In terms of slurry production and composition, the inclusion of PDOC tended to increase the volume of slurry excreted by the animals due to its high fibre (ADF) content as it has been reported by Morazán et al., (2015) in a study conducted to evaluate the effects of reducing dietary CP and increasing NDF. These changes in slurry excretion are probably induced by the increased intake of lignified dietary fibre with PDOC diet since the slurry from the animals offered PDOC diet showed higher ADL and a numerically higher DM content. The higher fibre content from PDOC diet probably resulted in an increase in faecal dry matter and bulk by virtue of its physical presence and water-holding capacity (Bach Knudsen and Hansen, 1991). However, neither the amount of fibre nor the especially high polyphenol content in diets lead to changes in gas emission from slurry. Although numerically lower B_0 values were observed with the inclusion of PDOC that possibly can be related to the high ADF proportion in the slurry, no statistical difference was obtained as was in CH_4 emission during storage. Accordingly, slurry composition was also similar between treatments. This result increases the interest of PDOC in pig nutrition, since neither growth performance or carcass quality traits nor the environmental impact of slurries were negatively affected by its inclusion in balanced diets. Fibrous by-products such as OC, which is a low cost by-product from the olive oil industry, represents an opportunity to value

wastes from food industries to create further value and thus contribute to the economic, social and environmental sustainability of the animal feeding sector. This study demonstrates that using a relevant proportion of OC in balanced feeds for growing pigs (12%) does not have significant negative effects on performance traits favouring the circular economy strategy, with potential positive effects on pig gut health and meat quality.

4.5 Acknowledgements

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Chapter 5

Effects of orange pulp conservation methods (dehydrated or ensiled sun-dried) on the nutritional value for finishing pigs and implications on potential gaseous emissions from slurry

Effects of orange pulp conservation methods (dehydrated or ensiled sun-dried) on the nutritional value for finishing pigs and implications on potential gaseous emissions from slurry

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Simple Summary: Utilization of local by-products in pig nutrition can reduce the environmental impact of feeds and contribute to the sustainable development of the livestock sector. Orange pulp (OP) is the most abundant citrus by-product worldwide, but its seasonal production and perishable nature requires storage and drying procedures that might affect its nutritive value. Conservation process by fuel drying is expensive and can impair feed sustainability. Instead, in the Mediterranean countries, OP is sun dried in the open air. This procedure often implies a previous silage (during storage) which occurs naturally, because OP has a high level of sugars available for fermentation. Orange pulp is also rich in soluble fibre, which is highly fermentable at the pig's caecum and may reduce gas emissions from slurry. In this study, the nutritive value of conventional fuel dehydrated (DOP) or ensiled sun dried (ESDOP) was determined for pig diets. Sugars fermentation during ensiling increases fibre level in ESDOP and decreases energy digestibility compared to DOP, but both OP have an appreciable digestible energy content for pigs, around 87 and 94% that of barley, respectively. In addition, they do not differ in the amount of slurry excreted and contribute to reduce potential derived ammonia and methane emissions.

Abstract: The inclusion of orange pulp (OP) in pig diets may promote the circular economy, but drying procedures might influence its nutritional value and environmental impact. The purpose of this study was to determine the energy value and nutrient digestibility of dehydrated (DOP) and ensiled sun dried (ESDOP) orange pulp. The potential ammonia (NH_3) and methane (CH_4) emissions derived from slurry were also measured. Digestible energies of 14.2 and 13.2 MJ/kg DM for DOP and ESDOP, respectively, were estimated by difference after a 500 g/kg substitution of a basal diet with OPs. A high fibre digestion efficiency was observed for both OPs. Pigs fed the basal diet showed a higher intake and a greater excretion of urine N than pigs fed with OP, but faecal N excretion did not differ among diets. A higher benzoic and hippuric acid content in urine was observed in OP than in basal diet. Alltogether, these findings explained a lower pH in slurry of OP diets and a reduction of potential NH_3 emissions. The biochemical CH_4 potential also decreased, especially with ESDOP. Overall, both OP are relevant sources of energy for pig diets. Their inclusion in feeds generate favourable changes of slurry characteristics that reduce potential NH_3 and CH_4 emissions.

Keywords: energy value; potential gas emission; nutrient balance; orange pulp; conservation method

5.1 Introduction

The efficient use of agro-industrial by-products in animal diets can reduce the environmental footprint of feed production by using non-edible resources, enhancing the circular economy and the sustainable development of the livestock sector.

Citrus fruits are the main fruit crop worldwide, with a production, over 124 million metric tons. The 22% of orange world production comes from Mediterranean countries (Food and Agriculture Organization of the United Nations FAO, 2017). Around 30% of world orange production is transformed into juice (FAO, 2017), and orange pulp (OP) containing peels, rag and seeds of the original fruit is obtained as a by-product after juice extraction (49 to 69% of the weight of fresh oranges according to orange cultivars (Martínez-Pascual and Fernández-Carmona, 1980). In Spain, the country with the highest citrus production within Europe, approximately 17% of orange production is transformed into juice (MAPA, 2019), but this proportion increases up to 70% in Brazil and USA, the first and fourth largest orange producer countries in the world, respectively (FAO, 2017). The seasonal production of oranges results in large amounts of OP available in short periods of time and elevated costs derived from waste disposal for industrial plants that without feed market would not be able to remain competitive (Bampidis and Robinson, 2006) and to produce in a sustainable manner (Beccali et al., 2009).

Orange pulp chemical composition can vary depending on cultivars and climate, conditions of harvesting season, fruit ripeness and the different industrial process used for juice extraction (Martínez-Pascual and Fernández-Carmona, 1980; Bampidis and Robinson, 2006; de Blas et al., 2018). Orange pulp is typically rich in sugars and soluble fibre (pectins) but low in crude protein (CP) and phosphorus (Bampidis and Robinson, 2006; Feedipedia, 2017; De Blas et al., 2018). Because of the high moisture content (90% after juice extraction) and elevated transportation costs, wet OP can only be used in livestock farms near to the processing plants or stored as silage. In order to increase its usefulness by the feed industry and to reduce transport costs, wet OP is usually pressed after adding lime to reduce humidity by 10%, and either artificially dehydrated by fuel-drying in conventional rotatory dryers or naturally dried in the open air by solar heat. Zema et al. (2018) estimate that around 40–50% of citrus pulp production in Mediterranean countries is used for animal feeding after solar drying during the warmest months of the year (May—September).

Storage conditions and drying temperatures may influence OP nutritional value due to losses of soluble nutrients and volatile organic acids during ensiling (Moset et al., 2015; Calabrò and Panzera, 2017), and dry matter losses and Maillard reactions between reducing sugars and amino groups when high temperatures (>130 °C, Martínez-Pascual and Fernández-Carmona, 1980) are applied in the drying process. The fuel cost and the environmental impact derived of using rotatory dryers make advisable to evaluate other eco-friendlier drying procedures for the valorisation of citrus pulp in animal feeding.

The most widespread use of OP is ruminant feeding (Bampidis and Robinson, 2006). Its inclusion in monogastric feeds generally requires drying and is more challenging because of its high fibre content. However, the nutritional value of dried citrus pulp for pigs reported in different databases (INRA, 2002; NRC, 2012; Brazilian Tables for Poultry and Swine, 2017; de Blas et al., 2019) suggests an appreciable digestible energy content from 12.8 to 14.0 MJ/kg dry matter (DM), although the information available from *in vivo* assays is scarce (O'Sullyvan et al., 2003; Watanabe and Thomaz, 2010; Ruiz et al., 2012) and shows discrepancies (11.7, 13.3 and 15.6 MJ DE/kg dry matter, respectively). Additionally, a previous study with wet ensiled citrus pulp in growing pigs reported a very low energy value (7.0 MJ DE/kg DM (Moset et al., 2015)), compared with other sources of citrus pulp.

Citrus pulp has been also proposed as a valuable source of soluble fibre for finishing pigs with potential benefits on gut microbiota (Cerisuelo et al., 2010; Moset et al., 2015) and controversial effects on its potential for reduction of gaseous emissions from slurry according to the dietary fibre level assayed (Antezana et al., 2015; Beccaccia et al., 2015a).

In light of a recent screening of citrus pulps by de Blas et al. (de Blas et al., 2018) and the interest on alternative drying technologies, the objective of this work has been to determine the nutritional value of two dried OP, either dehydrated (DOP) or ensiled sun-dried (ESDOP), for growing pigs. In addition, the implications of these two ingredients on effluents' composition and derived potential gas emissions have been evaluated.

5.2 Materials and methods

Ethics Committee of the Universitat Politècnica de València approved the experimental procedure (with the registration number 2016/VSC/PEA/00024).

5.2.1 Experimental design and diets

Twenty-four finishing male pigs (Pietrain × (Landrace × Large White)) of 62.3 ± 2.8 kg BW were used in the experiment in two batches of 12 animals each. Fresh and silage orange pulps were dried by conventional fuel-trommel process or in the open air by solar heat, to obtain dehydrated orange pulp (DOP) or ensiled sun-dried orange pulp (ESDOP), respectively. Dried OPs were provided by two local fruit juice producers Zuvamesa and Agriconsa (Valencia, Spain) for DOP and ESDOP, respectively, both processing only orange fruits harvested at the same season. The analysed nutrient composition of these two sources of OP is summarized in Table 5.1. Three experimental feeds were formulated: a basal diet including corn, wheat and soybean meal and two more diets in which 500 g/kg of the complete basal diet were replaced by either DOP or ESDOP. The basal diet was formulated to exceed energy and nutrient requirements for growing-finishing pigs according to Fundación Española para el Desarrollo de la Nutrición Animal (de Blas et al., 2013). Additionally, fibre content in the basal diet was maintained low to compensate for the high content of fibre in the diets with OP. The ingredient and nutrient composition of the experimental diets are shown in Tables 5.2 and 5.3, respectively.

Table 5.1. Analysed nutrient composition of the two sources of orange pulp used in the present study (g/kg, dry matter basis).

Item	Dehydrated	Ensiled Sun-Dried
Dry matter	877	860
Ash	59.9	83.7
HCl insoluble ash	1.09	2.03
Crude protein	64.5	79.3
NDICP [1]	22.6	8.10
ADICP [2]	5.40	1.30
Ether extract	22.8	35.3
Total sugars	355	101
Soluble fibre	287	271
aNDFom [3]	206	247
ADFom [4]	145	176
ADL [5]	24.1	18.9
Total polyphenols	3.59	3.27
Lactic acid	8.30	63.2
Citric acid	21.4	47.0
Acetic acid	2.51	1.60
Propionic acid	3.40	2.28
Butyric acid	0	1.25
Calcium	16.6	22.3
Phosphorous	1.26	1.17
Gross energy (MJ/kg)	17.4	17.4

[1] Neutral detergent insoluble crude protein.
[2] Acid detergent insoluble crude protein.
[3] Neutral Detergent Fibre with heat stable amylase and expressed exclusive of residual ash.
[4] Acid Detergent Fibre expressed exclusive of residual ash.
[5] Acid Detergent Lignin.

Table 5.2. Ingredients of the basal diet (g/kg).

Ingredient	Proportion
Corn	520
Wheat	180
Soybean meal 45.5	270
Calcium carbonate	9.7
Dicalcium phosphate	10.0
Sodium chloride	4.2
DL-methionine	0.5
L-lysine HCL	2.0
L-threonine	0.6
Premix [1]	3.0
Total amount	1000

[1] Vitamin and mineral premix supplied per kg complete diet: 5000 IU of vitamin A; 1000 IU of vitamin D3; 110 mg of zinc oxide; 90 mg of iron carbonate; 48 mg of betaine; 30 mg of manganese oxide; 20 mg of vitamin B12; 10 mg of niacin; 10 mg of copper sulphate; 4 mg of pantothenic acid; 3 mg of vitamin B2; 0.75 mg of potassium iodide; 0.1 mg sodium selenite.

Table 5.3. Nutrient content of the diets (g/kg, dry matter basis).

		Orange Pulp Diets [1]	
	Basal Diet	Dehydrated	Ensiled Sun-Dried
Dry matter	888	881	876
Ash	53.2	55.2	64.8
Crude protein	214	149	157
NDICP [2]	25.6	23.0	17.4
Ether extract	28.5	25.2	29.3
Total sugars	48.2	208	72.6
Soluble fibre	53.6	162	157
aNDFom [3]	108	156	183
ADFom [4]	32.9	79.3	100
ADL [5]	8.86	16.5	13.9
Total polyphenols	0.25	1.27	1.27
Calcium [6]	7.20	11.9	14.9
Phosphorous [6]	6.12	3.71	3.73
Benzoic acid (mg/kg)	<10	14	27
Gross energy (MJ/kg)	18.1	17.6	17.6

	Orange Pulp Diets [1]		
	Basal Diet	**Dehydrated**	**Ensiled Sun-Dried**
Standardized ileal digestibility of amino acids [6]			
Lysine	10.9	5.92	6.04
Methionine	3.42	1.88	1.92
Methionine + Cysteine	6.40	3.37	3.77
Threonine	7.20	4.19	4.34
Tryptophan	2.11	1.25	1.30
Isoleucine	7.54	4.36	4.52
Valine	8.48	5.17	5.41

[1] Substitution of 500 g/kg of complete basal diet with either dehydrated or ensiled sun-dried orange pulp.
[2] Neutral detergent insoluble crude protein.
[3] Neutral Detergent Fibre with heat stable amylase and expressed exclusive of residual ash.
[4] Acid Detergent Fibre expressed exclusive of residual ash.
[5] Acid Detergent Lignin.
[6] Values calculated according to FEDNA tables (de Blas et al., 2019).

The experimental period included an adaptation period to diets of 14 days to allow animals adjust their consumption of experimental feeds, followed by a 7-day collection period during which the total production of faeces and urine were collected individually, as described in (Beccaccia et al., 2015a). At the beginning of the study, pigs were weighed (62.3 ± 2.8 kg BW) and divided, according to body weight, into three dietary treatments. Pigs were housed in conventional pens until day 9 of the adaptation period and individually in metabolism pens (1.2 × 2 m^2) during the last 12 days of the study. Body weight was recorded on day 9 of the adaptation period and at the end of the experimental period, averaging 68.0 ± 3.0 and 76.4 ± 6.2 kg BW, respectively. During the 7-day collection period, feed intake was also recorded. The excreta collection in this period was divided in two phases, one for energy and nutrient digestibility (days 1–4) and another one for gaseous emissions measurements (days 5–7). During the energy and nutrient digestibility period, urine was collected under sulphuric acid (120 mL of H_2SO_4 solution at 10% per bucket and day) to avoid nitrogen (N) losses due to ammonia (NH_3) volatilization. Only on days 2 and 3 of the digestibility period, urine was acidified after collection to permit pH measurement before acidification. At the end of the gaseous emissions collection period, artificial slurries were reconstituted by mixing the fresh urine and faeces from each animal in the same proportion as excreted. Feed and water were provided *ad libitum* throughout the

experimental period and feed was distributed in dry form (mashed). Pigs were individually weighed, again, at the end of the study.

5.2.2 Chemical analysis of feeds and excreta

Representative samples of dried OP, feeds and faeces from the digestibility period were analysed for DM (930.15), ash (923.03), ether extract (920.39), total dietary fibre (TDF, 985.29), CP (986.06), calcium (927.02), phosphorus (964.06) and gross energy (GE) according to the Association of Official Analytical Chemists procedures (AOAC, 2000). Additionally, the proportion of neutral and acid detergent insoluble CP was analysed according to standardized procedures (Licitra et al., 1996). Total sugars from OP and feeds were analysed using the method of Luff–Schoorl (Lees, 1975). The concentrations of neutral acid detergent fibre (aNDFom), acid detergent fibre (ADFom) and lignin (ADL) were determined sequentially (Ankom Technology Corp., Macedon, NY, USA) according to AOAC 973.187 procedure (Mertens, 2002) and Van Soest et al. (1991) using heat stable amylase (FAA, Ankom Technology Corp., Macedon, NY, USA). Fibre concentrations were expressed without residual ash. Both, feeds and faeces were defatted (petroleum ether) prior to fibre analysis. Acid-insoluble ash was analysed according to the technique described by Van Keulen and Young (1977). Soluble fibre content was calculated as the difference between TDF and aNDFom corrected by CP content in the residue. Hemicellulose and cellulose concentrations were calculated from the difference between aNDFom and ADFom and the difference between ADFom and ADL concentrations, respectively. The GE concentration was measured in a bomb calorimeter (Parr 6400, Parr Instruments Co., Moline, IL, USA). Total N was measured using Leco equipment (model FP-528, Leco Corporation, St. Joseph, MI, USA) and CP estimated as N content × 6.25. In addition, the polyphenolic compounds present in OP, diets and faeces were determined after extraction with methanol/acetone/water as described by Chamarro et al., (2012).

Urine was freeze-dried to obtain its DM content. Before GE analysis, samples (around 0.5 g) were mixed with 0.5 g of benzoic acid as an adjuvant to allow its complete combustion. Total Kjeldahl N (TKN) was determined using an automatic analyser (2300 Kjeltec, Foss Analytical, Hilleroed, Denmark) according to Chamorro et al. (2012). Hippuric and benzoic acids were analysed directly in urine via high performance liquid

chromatography on a WATERS Alliance system (model 2695) following the procedure described by Sánchez-Martín et al. (2017).

Additionally, the pH of slurry was measured immediately after reconstitution and by duplicate using a glass electrode (Crison Basic 20+, Crison, Barcelona, Spain). Slurry samples were analysed for DM and ash as for the faecal samples, and total ammonia N (TAN) and TKN as for the urine. To avoid N volatilization, the subsample used for TAN was acidified with HCl 37% immediately after reconstitution. Volatile fatty acids (VFA) concentration was also determined by gas chromatography, equipped with a flame ionization detector (HP 68050 series Hewlett Packard, Palo Alto, CA, USA) and following the method described by Jouany (1982) with the addition of an internal standard (4-metil valeric).

5.2.3 Gaseous emission measurements

The reconstituted slurries were used in fresh for pH, VFA and NH_3 emission measurements and frozen at −20 °C for determination of slurry composition and biochemical methane potential (BMP). The procedure for NH_3 and CH_4 potential emission measurements was previously described by Beccaccia et al. (2015a). Briefly, NH_3 emission was monitored for 11 days using acid wet traps. A subsample of 0.5 kg of reconstituted slurry per animal was placed in a closed container of 1 L-capacity used as dynamic chamber. Containers were then connected to an air pump, and a constant airflow rate of 1.2 L/min was fixed. The exhausted air was forced to pass through glass flasks filled with 100 mL of 0.1N H_2SO_4. The quantity of the TAN trapped in the glass flasks was analysed following 4500 NH3-D procedure (APHA, 2012), using a detection electrode (Orion High Performance NH_3 Electrode, model 9512HPBNWP, Thermo Scientific, Waltham, MA, USA).

Biochemical CH_4 potential from slurry was measured in a batch assay as previously described by Angelidaki et al. (2009). Briefly, slurries were mixed with an anaerobic inoculum at a ratio of 1:1 on organic matter (OM) basis, and incubated in 120 mL glass bottles at 35 ± 1 °C for 100 days. Inoculum was collected from an anaerobic digester of a wastewater treatment plant (Sagunto, Spain) and pre-incubated during 15 days at 35 ± 1 °C in order to deplete the residual biodegradable organic material (degasification). Each slurry was tested by triplicate. During incubation, the biogas produced in each bottle was monitored and the CH_4 concentration in the biogas were measured using a manometer (Delta Ohm, HD 9220, Padova, It- aly) and a Focus Gas Chromatograph (Thermo,

Milan, Italy) equipped with a split/splitless injector and a flame ionization detector, respectively.

5.2.4 Statistical analysis and calculations

The coefficient of apparent total tract digestibility (CATTD) of energy and other nutrients of the two OPs studied was determined by the difference method, assuming additivity between the basal diet and the OPs in the experimental diets as described by Bolarinwa and Adeola (2012). Briefly, the energy digestibility of the test ingredient (OP, Dti) was determined by correcting the substitution rate for the energy contributions of basal ingredients and OP to the total dietary energy, using the following equation:

$$Dti\ (\%) = Dbd + \frac{Dtb - Dbd}{Pti}$$

where Dbd, Dtd, and Pti are the digestibility (%) of the energy in the basal diet and test diet, respectively, and Pti is the proportion of the energy contributed by the test ingredient to the test diet.

The digestible energy (DE) content of OPs was then estimated by multiplying CATTD of energy and the GE concentration of each OP. Each animal was the experimental unit for all the traits analysed in the present study. Results were analysed as a randomized complete block design using one-way ANOVA (GLM procedure of SAS version 9.4; SAS Inst. Inc. Cary, NC, USA), considering the type of diet as the main effect and batch as a block factor. Orthogonal contrasts were used to test for the effects of OP inclusion and type of OP.

5.3 Results

The batch and its interaction with type of diet were not statistically significant ($P > 0.10$) for any of the traits studied (data not shown). Therefore, these effects were excluded from the model.

5.3.1 Orange pulps chemical composition

The main differences in chemical composition between DOP and ESDOP were due to sugars and organic acids levels, fibrous fractions and ash content (Table 5.1). As a result of the silage fermentation, the ESDOP had lower total sugars (a 64% lower) but higher lactic (63.2 vs 8.3 g/kg DM) and citric (47.0 vs 21.4 g/kg DM) acids than DOP. In addition, CP, aNDFom and ADFom concentrations increased (by 20.0–23.0%) in ESDOP with respect to DOP. Regarding the ash content, ESDOP had more acid insoluble ash

and a 39.7% higher total ash content than DOP. Furthermore, low drying temperatures in ESDOP resulted in 64% less insoluble CP (NDICP) and a lower degree of lignification of aNDFom (7.65 vs. 11.7%) than in DOP. Total polyphenols concentration was similar in both OP.

5.3.2 Apparent digestibility of diets and orange pulps

The CATTD of the experimental diets are shown in Table 5.4. Diets including OP showed significantly ($P < 0.05$) lower digestibilities of DM, CP, ether extract, GE and DE by 4.43, 15.6, 17.3, 5.43 and 8.43%, respectively, compared with the basal diet. At the same time, the CATTD of all the fibrous fractions analysed increased with OP inclusion in the basal diet, with the lowest increase for hemicelluloses (by 3.90%; $P = 0.09$) and the greatest for cellulose (by 28.4%; $P < 0.001$). Otherwise, the CATTD of DM, ash, GE and DE was lower (by 4.93, 4.20, 6.01 and 3.36%; $P < 0.05$) or tended to be lower ($P = 0.068$) for CP and soluble fibre in the ESDOP compared with the DOP diet. The CATTD of other fibrous components was similar between the two OP diets. The proportion of energy in urine with respect to DE content was greater in the OP diets compared with the basal diet (0.049 vs. 0.022, $P < 0.001$).

The CATTD calculated by the difference method for the two OP tested are shown in Table 5.5. The CATTD of GE tended to be greater for DOP (by 7.48%; $P = 0.058$) than for ESDOP. From these coefficients and the GE of the OPs tested (Table 5.1), the DE values derived were 14.2 and 13.2 MJ/kg DM for DOP and ESDOP, respectively. Additionally, greater ($P < 0.05$) CATTD values of DM and ash (by 8.43 and 41.3%) were obtained for DOP compared with ESDOP, but no differences between the two sources of OP were observed for CP and fibrous component CATTD with the exception of soluble fibre that tended ($P = 0.075$) to be more digestible in DOP with respect to with ESDOP.

Table 5.4. Coefficients of apparent total tract digestibility of nutrients and energy balance of the experimental diets.

	Orange Pulp [1]				Significance [2]	
Item	**Basal**	**Dehydrated**	**Ensiled Sun-Dried**	**SEM [3]**	**1**	**2**
Dry matter	0.892	0.864	0.832	0.0073	<0.001	0.006
Ash	0.639	0.586	0.509	0.019	<0.001	0.001
Crude protein	0.859	0.738	0.694	0.016	<0.001	0.070
Ether extract	0.450	0.308	0.394	0.031	0.016	0.065
Soluble fibre	0.807	0.902	0.856	0.017	0.002	0.068
aNDFom [4]	0.697	0.749	0.752	0.016	0.013	0.875
ADFom [5]	0.656	0.775	0.767	0.031	0.006	0.869
Hemicelluloses [6]	0.705	0.722	0.743	0.011	0.092	0.427
Cellulose [7]	0.636	0.817	0.816	0.038	<0.001	0.988
Gross energy	0.882	0.843	0.815	0.008	<0.001	0.026
Energy balance, MJ/kg DM						
Digestible energy	16.0	14.9	14.4	0.145	<0.001	0.021
UE/DE [8]	0.022	0.052	0.046	0.0026	<0.001	0.147

[1] Substitution of 500 g/kg of basal diet with either dehydrated or ensiled sun-dried orange pulp.
[2] Contrasts: 1 = inclusion of OP, 2 = type of OP.
[3] Standard error of means (n = 8).
[4] Neutral Detergent Fibre with heat stable amylase and expressed exclusive of residual ash.
[5] Acid Detergent Fibre expressed exclusive of residual ash.
[6] Calculated as the difference between aNDFom and ADFom.
[7] Calculated as the difference between ADFom and ADL.
[8] Proportion of urinary energy (UE) on digestible energy (DE).

Table 5.5. Coefficients of apparent total tract digestibility and digestible energy of dried orange pulps.

	Orange Pulp			
Item	**Dehydrated**	**Ensiled Sun-Dried**	**SEM[1]**	**Significance**
Dry matter	0.836	0.771	0.017	0.019
Ash	0.534	0.378	0.043	0.025
Crude protein	0.617	0.530	0.039	0.133
Soluble fibre	0.997	0.904	0.034	0.075
ANDFom [2]	0.800	0.807	0.015	0.733
ADFom [3]	0.892	0.878	0.028	0.719
Hemicelluloses [4]	0.739	0.764	0.020	0.383
Cellulose [5]	0.997	0.996	0.033	0.973
Gross energy	0.804	0.748	0.019	0.058
Digestible energy, MJ/kg DM	14.2	13.2	0.338	0.058

[1] Standard error of means (n = 8).
[2] Neutral Detergent Fibre with heat stable amylase and expressed exclusive of residual ash.
[3] Acid Detergent Fibre expressed exclusive of residual ash.
[4] Calculated as the difference between aNDFom and ADFom.
[5] Calculated as the difference between ADFom and ADL.

5.3.3 Daily nutrient intake and composition of effluents

Dry matter and GE intake of pigs fed the basal diet was higher (by 27.7 and 32.6%, respectively; $P < 0.001$) than that of pigs fed the OP diets (Table 5.6). The N intake was 78.8% greater for animals fed the basal diet, reflecting the higher level of CP in this diet compared with the OP diets (214 vs 153 g/kg DM, respectively).

Daily faecal and urine excretion of DM and OM was similar between the basal and OP diets. No differences were either observed for excretion of ether extract, aNDFom and ADFom, with the exception of ADL that was lower (by a 47.9%; $P < 0.001$) in the basal diet. The amount of N excreted in faeces did not differ among diets, but pigs fed the basal diet showed a greater excretion of NTK in urine (by 71.5%; $P < 0.001$) compared with pigs fed the diets containing OP. On the opposite, concentrations of benzoic and hippuric acids and GE excreted in urine were greater (by 183, 334 and 56.5%, respectively; $P < 0.002$) in pigs fed diets containing OP compared with those fed the basal diet.

Table 5.6. Effect of experimental diets on daily nutrient intake and composition of effluents (g/day).

		Orange Pulp [1]			Significance [2]	
Item	**Basal**	**Dehydrated**	**Ensiled Sun-Dried**	**SEM [3]**	**1**	**2**
Dietary intake						
Dry matter	1922	1488	1522	73.4	<0.001	0.752
Nitrogen	65.8	35.5	38.1	2.01	<0.001	0.371
Gross energy, MJ	34.9	26.3	26.9	1.31	<0.001	0.768
Faecal excretion						
Dry matter	209	202	259	18.9	0.359	0.049
Organic matter	172	168	210	15.9	0.382	0.080
Nitrogen	9.34	9.29	11.8	1.01	0.329	0.093
Ether extract	30.1	25.8	27.1	1.80	0.107	0.638
aNDFom [4]	69.3	58.2	69.3	4.34	0.305	0.089
ADFom [5]	25.9	25.6	36.0	2.83	0.134	0.031
ADL [6]	5.42	9.48	11.4	0.642	<0.001	0.052
Total polyphenols	0.316	0.488	0.458	0.050	0.019	0.688
Gross energy, MJ	4.13	4.12	5.03	0.371	0.332	0.103
Urine excretion						
Dry matter	84.5	97.3	85.6	6.06	0.353	0.190
Organic matter	56.8	68.7	61.4	4.16	0.120	0.235
Total Kjeldahl N	15.3	9.94	7.90	0.850	<0.001	0.110
Benzoic acid, mL	575	1585	1677	140	<.0001	0.653
Hippuric acid, mL	713	3076	3115	350	0.002	0.939
Gross energy, MJ	0.69	1.15	1.01	0.077	<0.001	0.222

[1] Substitution of 500 g/kg of basal diet with either dehydrated or ensiled sun-dried orange pulp.
[2] Contrasts: 1 = inclusion of OP, 2 = type of OP.
[3] Standard error of means (n = 8).
[4] Neutral Detergent Fibre with heat stable amylase and expressed exclusive of residual ash.
[5] Acid Detergent Fibre expressed exclusive of residual ash.
[6] Acid Detergent Lignin.

When comparing DOP with ESDOP diets, nutrient intake was similar between pigs fed the two OP diets, but DM and ADFom faecal excretion were greater (by 28.2, P = 0.049 and 40.6%, P = 0.031), and tended (P < 0.10) to be higher for the rest of nutrients except ether extract, for pigs fed the ESDOP than the DOP diet. There were no differences (P > 0.10) in GE, NTK and analysed components of urine excretion between the two diets containing OP.

5.3.4 Slurry excretion and gaseous emissions

The results from the artificial slurry characterization and potential gaseous emissions are presented in Table 5.7. Dietary treatments did not influence neither slurry excretion (kg/day) nor its DM and OM concentrations. The N fractions and pH values from slurry were affected by the inclusion of OP, but no differences were observed between OP diets. The slurry from animals fed with OP diets was characterized by a lower TAN and TKN content (4.23 g/L and 9.74 g/kg on average, respectively) than the basal diet (7.88 g/L and 13.4 g/kg respectively, P < 0.002), which means a reduction of TAN and TKN content by 46.2% and 27.2%, respectively. A parallel effect (P < 0.001) was observed for slurry pH with the lowest values observed for diets supplemented with OP (7.62 as average) compared with the basal diet (8.69). With respect to total VFA concentrations in slurry, no differences were found with the inclusion of OP, but propionic and butyric acid concentrations were higher (P < 0.05) in slurries from ESDOP than from DOP diets, with a similar trend being detected for total VFA (P = 0.055).

Concerning gaseous emissions from the reconstituted slurries, the potential NH_3 emission from the slurry of animals fed the diets containing OP (expressed in g NH_3/kg of slurry) was as average a 58.2% lower (P < 0.001) than that from the basal diet. When expressing potential NH_3 emissions in terms of initial TKN of slurry, the decrease in NH_3 emission with the OP supplementation was 41.7% (P < 0.001) with respect to basal diet. No differences were found between both OP studied in these traits.

The BMP was also negatively affected (P = 0.01) by the inclusion of OP, with a trend (P = 0.07) for a lower BMP from the slurry excreted by animals fed the ESDOP compared to the animals fed with DOP. When expressing the BMP in terms of L of CH_4 per animal daily, no differences were found neither between treatments.

Table 5.7. Effect of experimental diets on reconstituted slurry excretion, composition and derived potential ammonia (NH_3) and methane (CH_4) emissions.

	Orange Pulp Diets [1]				Significance [2]	
	Basal	**Dehydrated**	**Ensiled Sun-Dried**	**SEM [3]**	**1**	**2**
Slurry excretion (kg/day)	2.11	2.15	2.20	0.15	0.720	0.810
Slurry composition						
Dry matter (g/kg)	135	137	162	12.6	0.370	0.190
Organic matter (g/kg)	103	108	128	10.8	0.280	0.220
Total ammonia nitrogen (g/L)	7.88	4.07	4.40	0.85	0.002	0.790
TKN (g/L) [4]	13.4	10.1	9.40	0.61	<0.001	0.450
pH	8.69	7.79	7.45	0.15	<0.001	0.130
Total volatile fatty acids (mmol/L)	79.2	70.0	107	12.7	0.520	0.055
Acetic acid (mmol/L)	58.2	57.8	72.2	7.07	0.390	0.150
Propionic acid (mmol/L)	8.97	5.47	10.7	1.13	0.470	0.004
Butyric acid (mmol/L)	5.63	3.26	6.29	1.03	0.440	0.045
Gas emissions						
g NH_3/kg slurry	2.82	1.13	1.03	0.17	<0.001	0.210
g N-NH_3/kg initial TKN	212	135	112	14.5	<0.001	0.270
mg NH_3/animal and day	543	261	201	42.8	<0.001	0.330
mL CH_4/g organic matter	396	344	276	26.0	0.010	0.070
L CH_4/animal and day	82.0	71.4	73.9	6.04	0.200	0.770

[1] Substitution of 500 g/kg of basal diet with either dehydrated or ensiled sun-dried orange pulp.
[2] Contrasts: 1 = inclusion of OP, 2 = type of OP.
[3] Standard error of means (n = 8).
[4] Total Kjeldahl Nitrogen(TKN).

5.4 Discussion

5.4.1 Nutritional value of dried orange pulp sources

Citrus pulp is characterized by high levels of sugars and soluble fibre and can become a relevant energy source for pig diets. Dehydrated OP analysed in this study contains a higher level of sugars (355 g/kg DM) than the values assigned to this nutrient in dried citrus pulp by several databases (from 195 to 312 g/kg DM; CVB, 2019; de Blas et al., 2019; INRA, 2004, NRC, 2012;) and the review (210 g/kg DM) published by Bampidis and Robinson (2006). This might be related to the usual addition of citrus molasses to the citrus pulp in most of the Spanish processor plants. However, the soluble fibre content of DOP and ESDOP in the present study were lower than the average value (329 g/kg DM) reported by Bampidis and Robinson (2006), which confirms the inverse relationship between both components (sugars and soluble fibre) in citrus pulp reported by de Blas et al. (2018). Besides both OPs are rich in soluble fibre (near to 30% in the current study), which has been related to a potential prebiotic effect in pigs through the modulation of gut microbiome and the gut associated immune system (Lindberg, 2014).

Fermentation and drainage losses occurring during ensiling lead to loss of soluble organic components (sugars, organic acids and soluble fibre) that explains the increase of the ash and insoluble fibrous content in ESDOP compared to DOP. Nevertheless, the sugar (101 g/kg DM) and lactic acid (63.2 g/kg DM) content of ESDOP was appreciable, indicating that, as described by Megías et al. (1993) and Grizzotto et al. (2020), the rapid decrease of pH during OP ensiling might also rapidly stop fermentation and maintain nutrient levels in OP high and constant thereafter. In relation to the drying procedure, high temperatures used in rotatory dryers for DOP (135–155 °C; (de Blas et al., 2018)) may explain the greater content of ADL and NDICP compared to solar heat drying in ESDOP (Martínez-Pascual and Fernández-Carmona, 1980).

The estimated DE value for DOP (14.2 MJ/kg DM) is similar to that assigned (13.9 MJ/kg DM) in Brazilian tables (2017) for citrus pulp with similar drying procedures and composition. However, it was higher than the reported by Watanabe et al. (2010) for barrows (11.7 MJ /kg DM) or those assigned by FEDNA (de Blas et al., 2019) and INRA (2002) tables: 13.4 and 13.2 MJ/kg DM, respectively, associated to DOP containing more ash and NDF and less sugars than the DOP used in the current study. On the other hand, the estimated DE content of OP in the present study is

lower than the reported by Ruiz et al. (2012) (15.6 DE/kg DM) for an OP of undisclosed composition, and with a GE content higher than that measured in the current study (18.7 vs. 17.4 g/kg DM). In all, the differences in DE estimates for DOP might be attributable to differences in chemical composition, since it varies according to the type of citrus fruits and the manufacturing process used.

In the present study, DE was higher for DOP compared to ESDOP. To our knowledge, there is no previous research work to compare DE values obtained in the current study for ESDOP. The ensiling process implies the fermentation of some sugars and its conversion into volatile organic acids that could be lost during the sun drying, whereas concentration of the other nutrients (mainly insoluble fibrous components) increased. The greater fibre (aNDFom and ADFom) contents of ESDOP than in DOP relates to its lower digestibility and DE content. Moreover, CATTD of soluble fibre estimated for DOP tends to be greater than for ESDOP, which in addition to its higher OM content also contribute to explain the higher DE content found for DOP than for ESDOP. In general terms, both dried OP are appreciable sources of energy for pigs that might substitute part of the cereals (such as barley, with 15.1 MJ DE/kg DM; (de Blas et al., 2019)) in pig diets.

5.4.2 Gaseous emissions from slurry

Dry matter balance showed a lower DM intake when including OP. This effect was also reported by other studies including levels around 10–15% of OP in commercial diets (O'Sullyvan et al., 2003; Cerisuelo et al., 2010), suggesting negative effects on palatability or positive effects on satiety, due to its high content on soluble fibre.

Total N intake and excretion are main factors determining NH_3 emission from slurry (Portejoie et al., 2004). In the current study N intake in the OP diets was 45% lower than in the basal diet, which greatly contributed to explain the parallel reduction of total N concentration (TAN and TKN; by 46 and 28%, respectively) in the slurry. This effect resulted in lower potential NH_3 emissions per kg of slurry (by 58%) in OP diets, which means a decrease of 8.8% per each unit of reduction of CP in feed. Otherwise, when potential NH_3 emission is referred to initial TKN in the slurry, a 42% reduction in NH_3 emissions is still observed in the present study in animals fed OP diets. This suggests that not only total TKN excretion but also other factors such as the N partition between faeces and urine and the slurry pH are involved in this reduction of NH_3 emission. In this way, previous

work stated that TDF (especially soluble fibre) can modify the partitioning of N from urine to faeces, increasing the N excreted in faeces mainly of bacterial origin, and lowering the N excreted in urine in the form of urea (Canh et al., 1997; Patráš et al., 2012; Beccaccia et al; 2015a). Consequently, the slurry excreted by animals fed TDF rich diets contains less volatile N content, which is associated to lower NH_3 emission.

In the case of our study, differences in NDF intake also explain the decrease of the ratio of urine to faecal N excretion between OP diets (1.09 and 0.755 for ESDOP and DOP, respectively; $P = 0.059$) and the decrease observed with OP inclusion (1.67 vs 0.923 in the basal and the average of the OP diets, respectively; $P < 0.001$). Similar modifications in the N partitioning have been reported by previous studies in pigs (Hansen et al., 2007; Antezana et al., 2015; Beccaccia et al., 2015a; Ferrer et al., 2018) addressed to evaluate the nutritional value of fibrous by-products or the effect of different type of fibre sources in diets. Furthermore, the largest part of the TDF is fermented by microorganisms in the hindgut with a subsequent production of VFA (Noblet and Le Goff, 2001), which decreases the pH of slurry in the OP diets, another mechanism involved in the reduction of NH_3 emission from slurry.

Urine composition was also affected in terms of benzoic and hippuric acid concentration by the inclusion of OP. Hippuric and benzoic acid present in urine are metabolites of undigested polyphenols of dietary origin (from OP in this case) that reach the colon, are metabolized by the microbiota and absorbed through the colonic barrier (Gonthier et al., 2003). Similar increases of these urine components were reported in previous studies in response to OP (Sánchez-Martín et al., 2017) and olive cake supplementation (Ferrer et al. 2018). The increments in benzoic and hippuric acid excretion in urine contribute to explain the lower pH in slurry related to the inclusion of OP in this study. Compared to the basal diet, a mean decline of 1.07 units in the pH values of slurry from pigs fed diets containing OP was observed.

Total slurry excretion did not differ among diets in our study. Although the decrease of N intake (by 21.7%) in OP diets was counteracted by a lower N digestion efficiency, potential NH_3 emission expressed on daily basis also decreased in this study with OP inclusion (by 57%). In this regard, previous work including moderate levels of OP in pig diets did not show differences in the amount of slurry excreted (Antezana et al., 2015; Beccaccia et al., 2015a). Instead, the use of high levels of highly lignified

fibrous by-products in pig diets as carob meal (Beccaccia et al., 2015a) or olive cake (Ferrer et al., 2018), led to an increase of the quantity of slurry excreted compared to basal diet, counteracting the positive effect on NH_3 emission per kg of slurry.

The BMP from slurry expressed as mL CH_4/g OM decreased with OP diets, especially in the case of ESDOP. Differences in BMP potentials might be explained by the amount and composition of OM excreted expressed in g/day. In this way, faecal ADL content increased by 74.9 and 110% with DOP and ESDOP compared with faeces of pigs fed the basal diet, whereas faecal concentration of ADF increased by 41% with ESDOP compared to DOP diet. Previous studies (Angelidaki et al., 2009; Triolo et al., 2011; Beccaccia et al., 2015a, b) have shown that ADL concentration in OM is negatively correlated with BMP, and lignified fibrous components have a low biochemical CH_4 potential (Angelidaki and Sanders, 2004).

5.5 Conclusions

We can conclude that the OP generated as a by-product from the juice industry can be included in pig diets as a relevant energy source that can potentially replace a part of cereals enhancing the sustainability of pig production. After fermentation in the silage, ESDOP contains lower sugar and greater fibre concentration than DOP, which leads to a slightly lower DE content. However, no major differences due to drying procedures on the nutritive value of OP were evident and heat-solar drying might be of interest in future works. Furthermore, the inclusion of OP in diets is able to reduce the potential NH_3 emissions per unit of NTK excreted, probably due to the effect of the TDF on N partitioning between faeces and urine and the lower pH; in addition, it has the benefit to decrease potential CH_4 emission expressed per unit of OM excreted. Future works focused on determining the maximum inclusion level of these ingredients in commercial conditions through the evaluation of growth performance, carcass composition, meat quality and health and environmental aspects are required to optimize its use in pig production.

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Chapter 6

Partial replacement of cereals by dehydrated orange pulp in finishing pig diets does not affect performance, health and gaseous emission from slurry

Partial replacement of cereals by dehydrated orange pulp in finishing pig diets do not affect performance, health and gaseous emission from slurry

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Abstract: In a climate change, growing world population and resources scarcity scenario, the use of agricultural by-products such as dehydrated orange pulp (DOP) in animal feeds is of interest to increase pig sector sustainability. With this aim an assay was carried out to assess the optimal inclusion levels of DOP in pig diets in terms of productivity, environmental impact and health. Four experimental diets were designed, a control diet (T1) and three more diets with increasing levels of DOP with 80, 160 and 240 g/kg of DOP for diets T2, T3 and T4, respectively. One hundred and sixty growing pigs of 25.3±3.0 kg initial BW were used in the experiment. The performance (average daily gain (ADG), average daily feed intake (ADFI) and feed conversion ratio (FCR)) and *in vivo* backfat (BF) thickness and loin depth (LD) gain were recorded during the finishing phase (from 70 to 130 kg BW). Faecal samples were incubated for bacteria enumeration and faecal metabolites (ammonia (NH_3), volatile fatty acids (VFA) and pH) were analysed for T1 and T4 treatments. At the slaughtering, carcass characteristics and meat quality traits were measured, and subcutaneous fat was sampled to analyse the fatty acid (FA) profile. Additionally, the slurry excreted by the animals was measured, characterised and subjected to a gaseous emission assay during its storage. The animals fed DOP diets tended to show lower ADFI, however the final BW and overall ADG and FCR were similar among treatments. No statistical differences were observed in *in vivo* final BF, LD and on carcass characteristics with the addition of DOP but the higher levels of DOP diets tended to decrease BF thickness and LD and achieve lower carcass weight. Regarding the FA profile of the subcutaneous fat, total SFA concentration was lower ($P<0.05$) and total MUFA concentration was higher ($P<0.001$) in the fat tissue of animals fed DOP diets. Bacterial counts and metabolites from faeces did not show any significant difference among treatments, as well as slurry excretion and characterisation. The inclusion of the higher levels of DOP lead to increased CO_2 and CH_4 emissions in mg per L of slurry and hour whereas these differences were not observed when expressed in mg per animal and day. In all, it has been demonstrated that the inclusion of DOP up to 24% in pig diets had minor effects on growth performance, body composition or carcass quality traits, favouring the circular economy strategy and pig sector sustainability.

Keywords: by-products, citrus pulp, swine, growth performance, carcass traits, gaseous emissions.

6.1 Introduction

The supply of feed ingredients for animal production is a global concern worldwide, because of the increasing demand of animal products and the limited availability of crops in a climate change scenario. Furthermore, these crops usually compete with human feeding and, in a growing world population scenario, their availability for its use as animal feeds will decrease (Makkar and Anders, 2014). In this regard, one of the strategies proposed to increase livestock sustainability in the future is reducing the use of crops and food-competing feed components.

The replacement of part of the cereals used in feeds by agro-industrial by-products such us citrus pulp has been proposed to reduce the environmental impact of pig production (Ajila et al., 2012; Zijlstra and Beltranena, 2013; Makkar and Ankers, 2014; Schader et al., 2015; Salemdeeb et al., 2017). Citrus is one of the major fruit products worldwide, covering a relatively stable surface of almost 10 million hectares and a production over 150 million tonnes per year. The main producers are China and Brazil, but a relevant production also comes from areas with Mediterranean climate (FAOSTAT, 2017). The transformation of citrus into juice generates a huge amount of by-products in the form of citrus pulp. The chemical composition of citrus pulp has been reported in several studies (Martínez-Pascual y Fernández-Carmona, 1980; Bampidis and Robinson, 2006; de Blas et al., 2018) and is influenced by the type of fruit, seasonal conditions, maturity, juice extraction system and further processing, such as dehydration. In general terms, citrus pulps are typically rich in sugars and fibre but low in crude protein (CP) and phosphorus (Bampidis and Robinson, 2006; FEDNA, 2019; Heuzé et al., 2017; De Blas et al., 2018).

Dehydrated citrus pulp has been successfully included in fattening pig diets replacing cereals, but the maximum level at which this by-product does not affect growth performance is variable (from 100 g/kg to 240 g/kg; Watanabe et al., 2010a; Amorim et al., 2014; Moset et al., 2015). Additionally, its effects on carcass and meat quality are inconsistent. While Watanabe et al. (2010b) and Crosswhite et al. (2013) described no deleterious effects upon meat quality with the inclusion of 300 and 150 g/kg of citrus pulp, respectively, Strong et al. (2015) reported negative effects on carcass characteristics, and lean quality with the inclusion of 150 g/kg of dietary citrus pulp in finishing pig diets. Contrary to other fibrous by-products, in spite of its high fibre content (26% of neutral

detergent fibre (NDF); FEDNA, 2019), the concentration of soluble fibre (SF) in citrus pulp is particularly high (30%, approx.). This makes it a relevant energy source for pigs (Ferrer et al., 2021), with reported small effects on nutrient digestibility when included at moderate levels (up to 15%; Beccaccia et al., 2015a). However, as it has been extensively reported in animals and humans, fibre can affect feed intake at high inclusion levels because its effects on satiety (Souza da Silva et al., 2012; Ratanpaul et al., 2019).

Furthermore, recent studies report that the inclusion of citrus pulp in pig diets might affect gas emission from slurry and from soil (Beccaccia et al., 2015a; Sánchez-Martín et al., 2017; Ferrer et al., 2020b), also probably due to its type of fibre and/or the presence of bioactive compounds such as polyphenols. In this regard, short-term studies with inclusions of 15 to 50% of DOP (Beccaccia et al., 2015a; Ferrer et al., 2020b) showed a decrease in the ratio nitrogen (N) in urine:N in faeces and a decrease of ammonia nitrogen ($N\text{-}NH_3$) emitted per kg of initial N in the pig slurry. Additionally, a 65% reduction of nitrous oxide (N_2O) emissions was observed when the slurry from animals fed 15% citrus pulp was applied to soils (Sanchez-Martín et al., 2017). However, its effects on methane (CH_4) emission from slurry are not clear.

Besides, far from being considered an antinutritional factor, some beneficial effects have been recently recognized for dietary fibre (DF), especially soluble and fermentable fibre like the one present in citrus pulp, in terms of energy provision, gut health, immune function and gas emission in pigs (Beccaccia et al., 2015a; Pieper et al., 2015; Jha et al., 2019). Recent studies suggest that the inclusion of fibrous agro-industrial by-products in pig diets may improve gut health through its fermentation in the distal gastro intestinal tract and the promotion of beneficial gut bacteria, which supports intestinal integrity and a proper immune function (Moset et al., 2015; Agyekum and Nyachoti, 2017 ; Jha et al., 2019).

The objective of the present work was to evaluate the consequences of including high levels of dehydrated orange pulp (DOP; up to 240g/kg) in balanced finishing pig diets from an integrative point of view, including its effects on growth performance, carcass quality, faecal microbiology and metabolites, and gas emissions from slurry. This information will help to assess the optimal inclusion levels of this by-product in terms of productivity, health and sustainability.

6.2 Material and methods

All the methodology used in this study is similar to that described in a previous study published by Ferrer et al. (2020).

6.2.1 Animals, diets and experimental design

One hundred and sixty growing males, progeny of Duroc-Danbred x (Landrace x Large White) of 25.3±3.0 kg initial BW were used in the experiment. At arrival, pigs were identified and distributed according to BW in 32 pens and two rooms (16 pens per room). All the animals were phase-fed two common commercial feeds before the beginning of the experimental period (phase 1: from 25 to 35 kg BW; phase 2: from 35 to 70 kg BW). At 71.2 ± 7.32 kg BW pens were assigned to four different treatments (8 pens/treatment) according to average pen weight and standard deviation within pen. These treatments consisted in a control feed (T1) and three more experimental diets with increasing levels of DOP: 80, 160 and 240 g/kg of DOP for diets T2, T3 and T4, respectively. A local fruit juice producer (Zuvamesa, Sagunto, Spain) provided the DOP. Dehydrated orange pulp was included in replacement of cereals (barley and wheat) in diets T2, T3 and T4. Diets were formulated to be isocaloric and isoaminoacidic using the coefficient of total tract apparent digestibility (CTTAD) of energy for the same DOP source previously determined by Ferrer et al. (2021) and by adjusting the addition of barley, wheat, oil, soybean meal and synthetic amino acids. Minerals were also adjusted to requirements in both diets. The maximum DOP inclusion level in the experimental diets was chosen from the results obtained in previous studies (Amorim et al., 2014; Beccaccia et al., 2015a; Moset et al., 2015; Ferrer et al., 2021). Detailed DOP and experimental diets composition are given in Tables 6.1 to 6.3. Experimental feeds were offered *ad libitum* in dry form (pelleted) and provided until slaughter (128 ±9.75 kg BW), a total of 50 days. Free access to water was provided during all of the experimental period.

Table 6.1. Chemical composition of dehydrated orange pulp used in the swine trial (g/kg DM, unless otherwise specified).

Analysed chemical composition	**DOP**
Dry matter (g/kg FM)	882
Ash	61.4
Gross energy, MJ/kg DM	17.4
Digestible energy, MJ/kg DM	13.9
Crude protein	81.0
Ether extract	15.5
NDF[1]	195
ADF[1]	130
Lignin	17.9
Total Dietary Fibre	531
Soluble Fibre	336
Sugars	274

[1]Ash-free

Table 6.2. Ingredient content and chemical composition of the experimental diets (g/kg as fed, unless otherwise specified).

	Treatments[1]			
	T1	**T2**	**T3**	**T4**
Ingredients				
Barley	240	160	80	0
Corn	200	200	200	200
Wheat	350	343	336	330
Soybean meal	159	163	167	172
Dehydrated orange pulp	0	80	160	240
Palm oil	17.4	21.8	26.2	30.6
Calcium carbonate	11.9	8.9	6.0	3.1
Monocalcium phosphate	8.9	9.6	10.3	11.1
Sodium cloride	3.3	3.2	3.1	3.0
Sodium bicarbonate	2.0	2.0	2.0	2.0
Methionine	0.34	0.42	0.50	0.58
Sulphate L-Lysine	3.48	3.59	3.69	3.80
L-Threonine	0.54	0.59	0.64	0.69
Choline cloride	0.6	0.6	0.6	0.6

	Treatments[1]			
	T1	**T2**	**T3**	**T4**
Vitamin-mineral premix[2]	3.0	3.0	3.0	3.0
Analysed chemical composition, g/kg DM				
Dry matter (g/kg FM)	892	891	885	886
Ash	50.6	53.8	52.1	51.3
Crude protein	180	176	176	175
Ether extract	30.7	32.9	35.3	38.4
NDF[3]	131	133	142	141
ADF[3]	33.9	39.4	54.3	49.3
Lignin	3.95	4.11	4.10	3.90
NDICP[4]	10.9	19.0	16.8	24.5
ADICP[5]	1.40	1.70	1.90	2.70
Total Dietary Fibre	162	188	212	220
Soluble Fibre	31.2	55.7	69.8	79.5
Sugar	46.0	69.0	89.0	97.0
Starch	449	413	369	370
Gross energy, MJ/kg	18.4	18.4	18.5	18.7
Calculated chemical composition[6]				
Digestible energy, kcal/kg[7]	3417	3416	3415	3414
Net energy, kcal/kg[7]	2400	2400	2400	2400
Calcium	7.0	7.0	7.0	7.0
Phosphorus	5.3	5.3	5.3	5.3
Ileal standardized ileal amino acids				
Lysine	8.6	8.6	8.7	8.7
Methionine	2.6	2.7	2.7	2.7
Methionine+Cystine	5.4	5.3	5.3	5.2
Threonine	5.5	5.8	5.8	5.8
Tryptophan	1.8	1.8	1.8	1.8
Isoleucine	6.0	5.9	5.9	5.9
Valine	7.0	6.9	6.8	6.7

[1]T1 = 0g/kg dehydrated orange pulp; T2 = 80g/kg dehydrated orange pulp; T3 = 160g/kg dehydrated orange pulp; T4 = 240g/kg dehydrated orange pulp.

[2]Vitamin–mineral premix in the finishing phase provided per kilogram of feed: retinol, 6500 IU (E672); cholecalciferol, 1860 IU (E671); α-tocopherol, 10 mg; menadinone, 0.6 mg; thiamine, 0.8 mg; riboflavin, 3.2 mg; pyridoxin, 1.0 mg; cobalamin, 0.02 mg; niacin, 12 mg; pantothenic acid, 9.60 mg; choline clorure, 116 mg; Fe, 72 mg as $FeSO_4 \cdot 7H_2O$; Cu, 16 mg as $CuSO_4 \cdot 5H_2O$; Zn, 80 mg as ZnO; Mn, 40 mg as MnO; I, 1.44 mg as KI and Se, 0.20 mg as Na_2SeO_3

[3]Ash-free

[4]Neutral detergent insoluble CP

[5]Acid detergent insoluble CP
[6]Calculated values based on De Blas et al. (2015b)
[7]Calculated from the coefficient of total tract apparent digestibility of energy previously determined in Ferrer et al. (2021)

Table 6.3. Fatty acid (FA) profile of the experimental pig diets (g/kg as fed).

	Treatments[1]			
	T1	**T2**	**T3**	**T4**
Total FA (mg/100g feed)	3.48	3.78	3.91	4.24
Saturated fatty acids (SFA; mg/g total FA)	307	321	330	342
Monounsaturated fatty acids (MUFA; mg/g total FA)	297	314	327	342
Polyunsaturated fatty acids (PUFA; mg/g total FA)	396	365	343	316
PUFA/SFA	1.29	1.14	1.04	0.924
MUFA/SFA	0.965	0.981	0.992	1.00

[1]T1 = 0g/kg dehydrated orange pulp; T2 = 80g/kg dehydrated orange pulp; T3 = 160g/kg dehydrated orange pulp; T4 = 240g/kg dehydrated orange pulp.

6.2.3 Growth performance, carcass and meat quality

Pigs were individually weighed and *in vivo* backfat (BF) and loin depth (LD) measured at the beginning and at the end of the administration of the experimental diets. Feed consumption by pen was recorded at the same time as BW and the average daily gain (ADG), average daily feed intake (ADFI) and feed conversion ratio (FCR) were then calculated. *In vivo* BF and LD were measured at the P2 position, using a B-mode ultrasound device (Agroscan A16, Angoulême, France) as described by Cerisuelo et al. (2010). Additionally, BF and LD gain was calculated from initial and final measurements. Pigs were slaughtered at the end of the experimental period. Fasting was practiced for approximately 12 h before slaughter in all animals. Carcass weight (hot carcass weight) was measured following the methodology described by Torres-Pitarch et al. (2014). At approximately 2 h *post-mortem* (during the chilling process) meat colour components at the *Gracillis* muscle were recorded using a portable CR300 Minolta Chromameter (Konica Minolta, Osaka, Japan). Additionally, subcutaneous fat was sampled at the level of the second cervical vertebrae to analyse the FA profile at the left side of the carcass of 20 animals per treatment as described by Torres-Pitarch et al. (2014).

6.2.4 Faecal microbiota by culture-based methods and metabolites

Faecal samples were aseptically removed directly from the rectum of 16 animals per treatment (2 animals per pen) at days 42 and 43 of the experimental period for bacterial (total anaerobic bacteria, *enterobacteria, lactobacilli and bifidobacteria*) enumeration. The samples of each pen were pooled and treated as a pen sample. Within the 2 hours after collection, faecal samples were diluted 1:10 (1 g faeces in 9 mL of peptone water) and decimal dilutions were prepared. The number of colony forming units per gram (CFU/g faeces) of total anaerobic bacteria and *bifidobacteria* were isolated onto Thiogliolato Agar (Liofichem, Roseto degli Abruzzi, Teramo, Italy) and BD Bifidobacterium Agar Modified (Becton Dickinson GmbH, Germany), respectively, following anaerobic incubation at 37ºC for 72 h. *Enterobacteria* were isolated on McConkey agar (Liofichem, Roseto degli Abruzzi, Teramo, Italy), following aerobic incubation at 37ºC for 24 h. *Lactobacilli* were cultured on Man, Rogosa Sharp agar (MRS, Liofilchem, Roseto degli Abruzzi, Teramo, Italy) following anaerobic incubation at 37ºC for 48 h. All colonies were counted immediately after removal from the incubator.

Additionally, a subsample of faeces from 16 animals in treatments T1 and T4 were analysed for pH, total ammonia N (TAN) and volatile fatty acid (VFA) content of faeces.

6.2.5 Slurry measurements and gas emission

In one of the experimental rooms, the slurry pit was divided into eight different pits that allowed the collection of the slurry excreted by the animals housed in two consecutive pens, independently from the rest. During the last 20 days of the trial, the slurry excreted by the animals housed in this room (4 pens/treatment and 2 slurry pits/treatment) was quantified according to its height in the pit at the end of this period. Afterwards, the slurry in each pit was homogenised with a pump and a representative sample was pumped to two tanks of 120 L of capacity per pit (470 mm diameter and 800 mm height) leaving a 200 mm of headspace between the slurry surface and the top of the tank. Overall, 16 tanks were filled with 90 L of slurry each and sampled during the fill-in for slurry chemical characterization. The tanks were placed in a mechanically ventilated room for eight successive weeks simulating outdoor slurry storage. Ammonia emissions were measured as described by Calvet et al. (2017). In brief, tanks were set as a dynamic chamber by fitting specially adapted lids which had smaller circular holes for inlet air and a 6-cm

diameter tube for forced outlet air circulation. A subsample from the exhausted air from the headspace of the tanks was forced to pass with an air pump (flow rate 1 L per minute) through absorption flasks filled with 100 mL of 0.05N H_2SO_4. The quantity of TAN trapped in the absorption flasks was analysed following 4500 NH3-D procedure (APHA, 2005) using a detection electrode (Orion High Performance NH_3 Electrode, model 9512HPBNWP, Thermo Scientific, USA).

Methane, carbon dioxide (CO_2) and N_2O emissions were measured once per week using the static chamber method following the methodology described by Hassouna and Engling (2015). To that aim, airtight lids were placed on the tanks during 3 min for the 16 tanks, alternating room air measurement for another 3 minutes between consecutive tanks. The static flux chamber method is based on the determination of the increase of gas concentration within the headspace of the tanks, avoiding any air replacement by the placement of the lids during the measurement periods. The gas concentration over the time shows a linear increasing trend followed by a saturation phase. The emission factor of the gas, expressed in mg/m^2 h, was calculated as the slope of the linear part of the saturation curve, multiplied by the chamber volume to emitting area surface ratio.

6.2.5 Chemical analysis

The DOP and experimental feeds were analysed for DM, ash, total dietary fibre (TDF) and EE according to the Association of Official Analytical Chemists (AOAC, 2000) procedures. Total sugars were analysed according to the method of Yemm and Willis (1954). The concentrations of NDF, acid detergent fibre (ADF) and acid detergent lignin (ADL) were determined sequentially according to Van Soest procedure (Van Soest et al., 1991). The contents in SF were estimated from the difference between TDF and NDF corrected by CP content in the residue. The gross energy (GE) concentration was measured in an isoperibol bomb calorimeter (Parr 6400, Parr Instruments Co., Moline, IL, USA). Total N was measured by combustion using Leco equipment (model FP-528, Leco Corporation, St. Joseph, MI, USA) and CP estimated as N content x 6.25. The proportion of neutral and acid detergent insoluble CP (NDICP and ADICP, respectively) was determined following the standardized procedures in Licitra et al. (1996).

The FA profile of the experimental feed samples and the subcutaneous fat was measured by gas chromatography. Fatty acid methyl esters (FAME)

were prepared according to O'Fallon et al. (2007) and were analysed in a Focus Gas Chromatograph (Thermo, Milan, Italy). The FA profile was calculated as the proportion of saturated, monounsaturated and polyunsaturated FA (SFA, MUFA and PUFA, respectively) in grams per 100 g of FA.

The VFA from faeces were determined by gas chromatography following the method described by Jouany (1985) with the addition of an internal standard (4-metil valeric) from an extraction of 2 g of faeces and 8 mL of distilled water. For faeces pH and TAN analyses, 2 g of homogenized samples were diluted (1:1) and (1:7) with distilled water respectively. The (1:1) extract was handled for pH determination using a glass electrode (Crison Basic 20+, Crison, Barcelona, Spain). Additionally, the dilution (1:7) for TAN analyses was centrifuged for 20 min (4,750 × g) (Heinritz et al., 2016b) and the supernatant acidified and freeze prior to steam distillation (4500 NH3-B and 4500 NH3-C procedures; APHA, 2005) using an automatic analyser (2300 Kjeltec, Foss Analytical, Hilleroed, Denmark).

Slurry samples were analysed for pH in duplicate using a glass electrode (Crison Basic 20+, Crison, Barcelona, Spain) and for DM, ash and OM, EE, fibre and GE following the same methodology used for DOP and feeds. The TAN and total Kjeldahl N (TKN) were analysed by steam distillation as for the faeces. To avoid N volatilization, the slurry subsample used for TAN analyses was acidified with HCl immediately after the samples were collected.

6.2.6 Statistical analysis

Data were analysed using SAS® (Statistical Analysis System) System Software (Version 9.1, SAS Institute Inc., Cary, North Carolina, EEUU). Differences in BW, ADG, ADFI, FCR, BF, LD and carcass and meat quality traits were analysed as a completely randomized design, with type of diet as main effect. The influence of DOP inclusion was studied by a contrast between the control diet and the average of the three DOP supplemented diets. In addition, the effect of DOP inclusion was studied by linear regression. The initial BW, BF and LD were used as covariates in the analysis of final BW, final BF and final LD, respectively. Microbial counts were $\log_{10}$ transformed before analysis. For ADG, ADFI, FCR, initial and final weight, microbial counts and faeces metabolites, the experimental unit was the pen, whereas for the carcass and meat quality measurements the experimental unit was the individual pig. Gas emission results (mg/L and h) are presented as the average emission rate during

the experiment. These data were also calculated in mg/animal day and h (considering the total amount of slurry excreted) and both were statistically analysed, together with initial slurry characterization, where the pit was the experimental unit.

6.3 Results

The statistical analysis performed showed no significant influence (P>0.05) of the room and its interaction with the dietary treatment for any of the traits studied (data not shown), so that this effect was excluded from the model.

6.3.1 Dehydrated orange pulp and experimental diets composition

The DOP used in the study (Table 6.1) shows a high content in TDF (it accounts for over than 50% of the DM, 531 g/kg DM) and sugars (274 g/kg DM). Regarding the different fractions of fibre partitioning, DOP presents a high content in SF (336 g/kg DM) and ADF (130 g/kg DM), and a low content of ADL (17.9 g/kg DM). With respect to the chemical composition of the experimental diets (Tables 6.2 and 6.3), treatments 2 to 4 showed a greater SF, TDF and sugars content than the control diet (T1), as a result of the increasing inclusion rates of DOP from 8 to 24%. The T4 diet showed the highest levels of fibre fractions (124 and 30% higher content of SF and TDF), and sugars (111%), compared with T1 diet. Regarding the concentration of FA in the diets, MUFA and SFA were greater whereas PUFA levels were lower in the diets with DOP compared with T1 diet, as a result of the greater addition of palm oil in T2, T3 and T4 diets, compared with T1.

6.3.2 Growth performance, carcass and meat quality

The results on growth performance are summarized in Table 6.4. The final BW and overall ADG and FCR were similar among treatments with no statistical differences, despite lower numerical values in FCR were observed with the inclusion of DOP (T2 to T4). At the end of the study, the ADFI tended (P=0.082) to decrease as the inclusion level of DOP increased. Regarding the *in vivo* final BF and LD measured at the P2 position, no statistical differences were observed with the addition of DOP. However, a tendency (P=0.087) to a reduction in BF and LD at the end of the study was observed as the inclusion level of DOP increases. With respect to BF and LD gain over the experimental period, the BF gain decrease with the addition of DOP (P=0.062) and with the inclusion level (P=0.022), whereas no statistical differences were observed in LD gain.

Table 6.4. Effect of the addition (Add) and inclusion level (L) of dehydrated orange pulp in pig diets on growth performance.

	Treatments[1]					Significance	
	1	**2**	**3**	**4**	**SEM[2]**	***Add***	***L***
Initial BW, kg	71.8	70.6	71.8	70.8	2.16	0.763	0.798
Final BW, kg	128	128	128	127	1.46	0.719	0.628
ADG[3], kg/d	1.16	1.16	1.15	1.14	0.029	0.848	0.766
ADFI[4], kg/d	3.33	3.20	3.19	3.18	0.068	0.074	0.082
FCR[5], g feed/g gain	2.85	2.78	2.78	2.85	0.071	0.432	0.464
Final backfat thickness, mm	13.4	13.5	12.4	12.5	0.388	0.221	0.075
Final loin depth, mm	52.6	51.6	51.2	50.6	0.836	0.136	0.087
Backfat gain, mm	5.78	5.46	4.49	4.49	0.443	0.062	0.022
Loin gain, mm	14.1	13.2	12.9	12.1	0.837	0.136	0.106

[1]T1 = 0g/kg dehydrated orange pulp; T2 = 80g/kg dehydrated orange pulp; T3 = 160g/kg dehydrated orange pulp; T4 = 240g/kg dehydrated orange pulp.
[2]SEM: standard error of the mean
[3]ADG: Average daily gain
[4]ADFI: Average daily feed intake
[5]FCR: Feed conversion ratio

The results of the carcass and meat quality traits measured and the FA profile of the subcutaneous fat analysed are shown in Table 6.5. The inclusion of DOP in pig diets had no significant effects on carcass characteristics with respect to T1 diet. However, the animals fed with DOP showed a tendency to a lower ($P<0.10$) carcass weight as the inclusion of DOP in diets increased. Regarding the FA profile of the subcutaneous fat (Table 6.5), total SFA concentration was lower ($P<0.05$) and total MUFA concentration was higher ($P<0.001$) in the fat tissue of animals fed DOP diets, compared with T1. Likewise, the SFA values decreased ($P<0.05$) and the MUFA increased ($P<0.001$) as the addition of DOP in diets was increased. Therefore, the ratio MUFA/SFA was higher ($P<0.001$) in the pigs offered the diets supplemented with DOP compared with the pigs offered the control diet (T1), and this ratio increased ($P<0.001$) as the level of DOP in the diets increased (treatments T2 to T4). The concentration of PUFA in the subcutaneous fat was similar among treatments.

Table 6.5. Effect of the addition (Add) and inclusion level (L) of dehydrated orange pulp in pig diets on carcass, meat quality and fatty acid (FA) in the subcutaneous fat.

	Treatments[1]					Significance	
	1	**2**	**3**	**4**	**SEM[2]**	***Add***	***L***
Carcass characteristics							
Carcass weight, kg	95.38	94.38	93.02	92.09	1.280	0.135	0.063
Carcass yield, %	74.60	74.55	73.08	73.00	1.370	0.502	0.355
Fat depth at the GM[3], mm	1.77	1.71	1.55	1.68	0.074	0.148	0.154
Meat quality							
Meat colour[4]							
Lightness (L*)	36.73	36.22	36.16	37.56	0.521	0.486	0.638
Redness (a*)	8.43	8.77	8.86	8.00	0.411	0.818	0.777
Yellowness (b*)	3.18	2.91	3.04	3.12	0.132	0.291	0.597
Fatty acids (FA) in subcutaneous fat							
Total FA (mg/100g subcutaneous fat)	68.25	69.70	70.10	67.00	0.753	0.508	0.802
Saturated FA (mg/g total FA)	329	328	316	323	2.831	0.026	0.012
Monounsaturated FA (mg/g total FA)	480	486	494	500	2.958	<0.001	<0.001
Polyunsaturated FA (mg/g total FA)	189	187	189	183	2.624	0.345	0.204
Ratio PUFA[7]/SFA[5]	0.572	0.571	0.597	0.566	0.010	0.618	0.730
Ratio MUFA[6]/SFA[5]	1.45	1.49	1.57	1.55	0.021	<0.001	<0.001

[1]T1 = 0g/kg dehydrated orange pulp; T2 = 80g/kg dehydrated orange pulp; T3 = 160g/kg dehydrated orange pulp; T4 = 240g/kg dehydrated orange pulp.
[2]SEM: standard error of the mean
[3]GM = *Gluteus medius* muscle
[4]Measured at the *Gracillis* muscle level
[5]SFA: saturated fatty acids
[6]MUFA: monounsaturated fatty acids
[7]PUFA: polyunsaturated fatty acids

6.3.3 Microbial counts and faecal metabolites

Bacterial counts from faeces did not show any significant differences (P>0.05) among treatments. The ratio *lactobacilli:enterobacteria* was also similar in all treatments (Table 6.6).

The metabolite content in faeces from treatments T1 and T4 is summarized in Table 6.7. No significant differences were found in VFA and TAN content neither on pH between treatments.

Table 6.6. Effect of the addition (Add) and inclusion level (L) of dehydrated orange pulp in pig diets on faecal bacteria counts (Log_{10} CFU/g fresh faeces).

	Treatments[1]					Significance	
	1	**2**	**3**	**4**	**SEM[2]**	***Add***	***L***
Total anaerobic bacteria	7.97	7.51	7.40	7.41	0.289	0.124	0.128
Bifidobacteria	8.21	8.62	8.30	8.69	0.166	0.101	0.105
Enterobacteria	6.22	6.32	6.34	6.14	0.233	0.865	0.963
Lactobacilli	9.51	9.69	9.50	9.28	0.156	0.919	0.426

[1]T1 = 0g/kg dehydrated orange pulp; T2 = 80g/kg dehydrated orange pulp; T3 = 160g/kg dehydrated orange pulp; T4 = 240g/kg dehydrated orange pulp.
[2]SEM: standard error of the mean

Table 6.7 Effect of dehydrated orange pulp inclusion in pig diets on faecal volatile fatty acids (VFA; g/kg faeces), total ammonia nitrogen (TAN; mg NH_4/g faeces) and pH.

	Treatments[1]		SEM[2]	*P-value*
	1	**4**		
Total VFA	6.57	6.79	0.313	0.850
Acetic acid	2.92	3.17	0.123	0.267
Propionic acid	1.36	1.34	0.072	0.992
Isobutiric acid	0.180	0.158	0.011	0.174
Butiric acid	1.36	1.08	0.076	0.185
Isovaleric acid	0.284	0.292	0.019	0.706
Valeric acid	0.389	0.347	0.026	0.241
Caproic acid	0.156	0.177	0.017	0.392
Heptanoic acid	0.029	0.029	0.003	0.965
TAN	576	652	46	0.236
pH	6.46	6.42	0.077	0.747

[1]T1 = 0g/kg dehydrated orange pulp; T4 = 240g/kg dehydrated orange pulp.
[2]SEM: standard error of the mean

6.3.4 Slurry composition and gas emission

The amount of slurry produced by the animals varied from a minimum of 8.06 L/ animal and day in T2 and a maximum of 9.85 L/ animal and day in T1, with no statistical differences among treatments (Table 6.8).

Table 6.8. Effect of the addition (Add) and inclusion level (L) of dehydrated orange pulp in pig diets on slurry characteristics and gas emission.

	Treatments[1]					Significance	
	1	**2**	**3**	**4**	**SEM[2]**	***Add***	***L***
Slurry production, L/animal and day	9.85	8.06	9.02	9.51	2.085	0.703	0.856
Slurry characteristics							
Dry Matter (DM), g/kg FM	13.0	19.3	25.0	17.5	10.55	0.566	0.622
Organic matter, g/kg FM	8.28	12.2	17.3	11.39	7.90	0.588	0.633
TAN, g/L	2.66	2.43	3.01	2.38	0.821	0.956	0.947
TKN, g/kg	3.05	3.52	3.73	3.04	1.178	0.793	0.889
NDF, g/kg of DM	28.5	29.8	27.4	19.51	6.510	0.716	0.477
ADF, g/kg of DM	7.02	7.18	8.12	4.28	2.830	0.887	0.700
ADL, g/kg of DM	2.69	2.65	3.07	1.50	1.087	0.831	0.647
EE, g/kg of DM	14.8	10.9	16.8	9.64	4.167	0.650	0.620
pH	7.06	7.10	7.26	7.03	0.165	0.720	0.829
Concentration of gases emitted, mg/L and h							
Ammonia	0.155	0.137	0.176	0.139	0.0506	0.937	0.951
Carbon dioxide	3.47	3.48	4.72	5.20	0.219	0.017	0.005
Methane	0.11	0.11	0.17	0.15	0.013	0.071	0.036
Total gas emission, mg/animal and day							
Ammonia	35.5	26.7	31.9	34.1	9.89	0.705	0.867
Carbon dioxide	820	672	1013	1204	249.8	0.647	0.363
Methane	25.1	20.3	35.6	33.5	5.909	0.523	0.282

[1]T1 = 0g/kg dehydrated orange pulp; T2 = 80g/kg dehydrated orange pulp; T3 = 160g/kg dehydrated orange pulp; T4 = 240g/kg dehydrated orange pulp.

[2]SEM: standard error of the mean

Regarding the slurry composition at the beginning of the gas emission study, none of the traits analysed were significantly different among treatments. Similar values were observed for DM and OM (with high variability between replicates from the same treatment, as we can observe from the standard error), and for N, fibre and EE content and pH among treatments. Concerning gas emission measurements, no significant differences were observed on the amount of NH_3 emitted, whereas CO_2 emission increased ($P< 0.05$) with the inclusion of DOP, being a 43% higher in T3 and T4 on average, with respect to T1 in mg per L of slurry and hour. Similarly, CH_4 emission tended ($P<0.01$) to be higher with the inclusion of DOP and increased significantly with the inclusion level being 55 and 36% higher in T3 and T4, respectively, compared to T1. When the emissions were expressed in mg per animal and day, considering the total amount of slurry produced, these differences followed the same tendency but the statistical differences among treatments disappeared.

6.4. Discussion

The DOP used in this study contained a high amount of TDF, SF and sugars, averaging 53.1%, 33.6% and 27.4% DM, respectively, indicating that this ingredient is rich in highly available carbohydrate fractions and, therefore, energy. These values are similar to those published in the literature (Bampidis and Robinson, 2006; De Blas et al., 2018; FEDNA, 2019), with the exception of sugars, which concentration is especially high in the DOP used. During the processing of the DOP used in this study, a re-addition of citrus molasses to the lime takes place before the drying operation, which may explain its higher sugar content compared with other DOP reported in the literature. In all, its high sugar content and the quality of its fibre makes the DOP used in the present study an excellent ingredient to be included in heavy pig diets from a nutritional point of view. In fact, contrary to most of the studies with citrus pulp that reported maximum inclusion levels of 150 g/kg in growing pig diets (O'Sullivan et al., 2003; Amorim et al., 2014; Strong et al., 2015), in the present study, the inclusion of 240 g/kg of DOP was possible without any negative effects on growth performance, including feed efficiency. Then both, the good quality of the DOP tested, together with the fact that experimental diets were formulated with data from the same DOP obtained from *in vivo* trials could be some of the reasons explaining these favourable results.

The above-mentioned studies point out to a reduction in feed intake, as the main reason for limiting the inclusion of citrus pulp, related to the unpalatability of the by-product. In the present study, although not significant, the inclusion of DOP tended to reduce feed intake suggesting that, probably, this could become a limiting factor for performance at higher inclusion levels. It is known that the inclusion of fibre sources in diets can limit voluntary feed intake due to an increase in the satiety feeling (Souza da Silva et al., 2012; Ratanpaul et al., 2019). Although it is not clear, fibres sources like pectin (high levels in citrus pulp), can increase satiety in pigs due to its high water retention capacity and its effects on delaying gastric emptying (Drochner et al., 2004). This effect can also depend on the age of the animals, since this can affect its ability to ferment fibre and the potential negative effects of fibre in feed intake (Sterk et al., 2008). Thus, the optimum level of DOP inclusion in diets in terms of performance seems to be highly dependent on DOP quality (amount and type of fibre, sugars or even dehydrating method), the precision in feed formulation and the age of the animals to be feed with DOP.

Regarding carcass traits, it is well known that an increase in the fibre fraction in pig diets can led to a lower carcass yield due to the increase in the weight of both content and tissue of stomach, large intestine, colon and cecum (Whitney et al., 2006; Salyer et al., 2012; Coble et al., 2018). In the present study, carcass weight tended to decrease with the inclusion of DOP, but carcass yield was not different among treatments, suggesting that our DOP did not modify organs' weight, contrary to other studies with citrus pulp (Watanabe et al., 2010b). The tendency for a decrease in carcass weight is in accordance with the tendency observed for a lower final BF and LD measurements, and a lower BF and LD gain during the experimental period, when increasing DOP level in diets. This suggest that, although not relevant in terms of body weight gain, probably greater inclusion levels than 240g/kg will end on a lower ADG, due to a lower ADFI, as reported before.

Depth at the *gluteus medius* and meat colour were not affected by the addition of DOP to diets in this study. Watanabe et al. (2010b) reported a linear reduction of L*, a*, and b* values as the dietary levels of citrus pulp increased. The authors associated this effect to the reduction of corn levels in diets when including citrus pulp. Nonetheless, in the present study, DOP replaced barley, and the effects on meat colour are less evident.

On the other hand, the FA profile of the subcutaneous fat was affected by the experimental diets. The proportion of MUFA increased and the proportion of SFA decreased, leading to an increase of the MUFA/SFA ratio with DOP inclusion. Moset et al. (2015) reported similar results with the inclusion of ensiled citrus pulp at levels up to 100 g/kg diet (higher MUFA). However, the reason why DOP in diets can affect the FA profile of the subcutaneous fat is unknown. Dietary effects on FA profile of the subcutaneous fat might be explained by the negative influence of DOP inclusion level on dietary fat digestibility as shown by Antezana et al. (2015). This effect has been related to the binding of bile acids by pectins, resulting in a lower capacity to emulsify dietary fat (Bach Knudsen and Hansen, 1991; Drochner et al., 2004). The reduced digestibility should be greater in the case of SFA with respect to unsaturated FA because of its lower polarity and digestibility.

Citrus pulp contains high levels of bioactive compounds (flavonoids, polyphenols, carotenoids and vitamin C) with antioxidant properties (de Moraes Crizel et al., 2013; Kasapidou et al., 2015) that can affect metabolism and enhance animal health. In terms of faecal metabolites and microbiota, the results from our study did not show differences in VFA, TAN and pH from pigs fed DOP diets with respect to T1 diet, neither on faecal microorganisms. As previous studies report, fibre from the diet is generally fermented in the proximal colon, producing lactic acid and SCFA, promoting the growth of beneficial bacteria in the gut (Heinritz et al., 2016a; Jha et al., 2019), improving the gut mucosal health as well as the immune system of pigs (Jha et al., 2019). Previous studies using ensiled citrus pulp showed clear decreases in faecal *Enterobacteria* counts when this was included at levels of 10-15% in pig diets (Cerisuelo et al., 2010; Moset et al., 2015). However, neither of these effects were observed in the present study, and the reasons for this lack of effects are unknown. The possible bacterial acclimation to the ingredient in the diet and the high limitation of cultured-based techniques to assess the possible changes induced by animal feeding on the diversity and dynamics of the gastrointestinal microbiota, could explain the lack of effects observed in this case.

In terms of slurry production and composition, the inclusion of DOP did not showed differences in the volume and composition of the slurry excreted by the animals. Previous studies from Ferrer et al. (2020a) and Morazán et al. (2015) reported increases in slurry excretion when including ingredients with considerable amounts of lignified fibre, which

resulted in higher faecal dry matter production. These results are in line with those reported by Beccaccia et al. (2015) in which the inclusion of moderate levels of DOP did not severely affect nutrient digestibility and improved DF digestibility from DOP, compared to other by-products with higher content in ADL. With respect to gas emission from slurry, the inclusion of fermentable fibre like the fibre of citrus pulp (pectin and hemicellulose), has been reported as effective in the reduction of NH_3 lost during manure storage (Kreuzer et al., 1998; Beccaccia et al., 2015a; Ferrer et al., 2021). A possible explanation is that DF fermentation affects the N utilization pattern in the intestines shifting the N excretion from urine to faeces, reducing the excretion of N in the form of ammonia (Bindelle et al., 2009; Beccaccia et al., 2015a; Sánchez-Martín et al., 2017; Ferrer et al., 2020). Additionally, the presence of bioactive compounds in DOP and its derivates such as tannins in the slurry could also affect CH_4 and N_2O emission (Sánchez-Martín et al., 2017; Whitehead et al., 2013). In the present study, contrary to the expected, NH_3 emission from the slurry measured in mg per L and hour was not affected, and CO_2 and CH_4 emissions increased as the inclusion level of DOP increased ($P<0.05$).

These results could be explained due to differences in diet composition and methodology used for *in vitro* and storage emission assays (reconstituted slurry) and the variability observed in slurry production when animals are housed in commercial conditions (conventional pens). In the nutritional assays, the diets are designed to estimate the DE of the by-products tested by the regression or substitution method, usually conducted when testing fibrous by-products (Woyengo et al., 2014). In this methodology the digestibility values from the different mixtures fed are regressed against the proportion of feedstuff included in the diets. As a result, the experimental diets are unbalanced in terms of nutrients content which in turns lead to significant differences in slurry composition. However, in the present assay, diets are designed to be isonutritive and the inclusion of the DOP modifies the proportion of the other feedstuffs of the diet, condition that could reduce the potential effect of the by-product inclusion in the slurry composition. Moreover, when emissions are expressed in mg per animal and day, the statistical differences disappeared due to the high variability found in slurry production, as it depends on the individual behaviour of the animals (water and feed intake and spillage)

Thus, the results obtained in the present study may increase the interest of using DOC in pig nutrition from a growth performance point of view,

since it has been demonstrated that its inclusion up to 24% in diets did not negatively affect growth performance, body composition or carcass quality traits. Additionally, the DOP used in the present study, did not affect microbial counts nor faecal metabolites but the results on FA deposition suggest that DOP could alter fat digestibilty. At an environmental point of view, high levels of this by-product did not affect excreta volume but increased CO_2 and CH_4 emission per L of slurry, although these differences disappeared when expressed per pig and day. Then, the use of DOP in pig diets can favour the circular economy strategy and contribute to the economic, social and environmental sustainability of the animal feeding sector.

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Chapter 7

The use of agricultural substrates to improve methane yield in anaerobic co-digestion with pig slurry: Effect of substrate type and inclusion level

The use of agricultural substrates to improve methane yield in anaerobic co-digestion with pig slurry: Effect of substrate type and inclusion level

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Abstract: Anaerobic co-digestion of pig slurry with four agricultural substrates (tomato, pepper, kaki and peach) was investigated. Each agricultural substrate was tested in co-digestion with pig slurry at four inclusion levels: 0%, 15%, 30% and 50%. Inclusion levels consisted in the replacement of the volatile solids (VS) from the pig slurry with the VS from the agricultural substrate. The effect of substrate type and inclusion level on the biochemical methane potential (BMP) was evaluated in a batch assay performed at 35 °C for 100 days. Agricultural substrate's chemical composition was also analysed and related with BMP. Additionally, Bacteria and Archaea domains together with the four main methanogenic archaeal orders were quantified using quantitative real-time TaqMan polymerase chain reaction (qPCR) at the end of the experiment to determine the influence of agricultural substrate on sludge's microbial composition. Results showed that vegetable substrates (pepper and tomato) had higher lipid and protein content and lower carbohydrates than fruit substrates (kaki and peach). Among substrates, vegetable substrates showed higher BMP than fruit substrates. Higher BMP values were obtained with increasing addition of agricultural substrate. The replacement of 50% of VS from pig slurry by tomato and pepper increased BMP in 41% and 44%, respectively compared with pig slurry only. Lower increments in BMP were achieved with lower inclusion levels. Results from qPCR showed that total bacteria and total archaea gene concentrations were similar in all combinations tested. Methanomicrobiales gene concentrations dominated over the rest of individual archaeal orders.

Keywords: biogas, swine manure, agricultural substrate, qPCR

7.1 Introduction

Anaerobic digestion is an environmentally friendly and could be cost-effective pig slurry treatment process since, in turn of degrading organic matter, it produces energy in the form of methane (CH_4) (González-Fernández and García-Encina, 2009). However anaerobic digestion of pig slurry typically has low solids content (<10% total solids (TS)), and thus, leads to low CH_4 yields in volume basis compared with other organic substrates such as energy crops. In order to overcome these limitations and enhance CH_4 production from slurry, anaerobic co-digestion of pig slurry with different substrates is being widely used (Ward et al., 2008).

Agricultural substrates such as fruits and vegetables with easily biodegradable organic matter content and low nitrogen concentration, are appropriate for co-digestion and can enhance CH_4 production with animal organic wastes (Bouallagui et al., 2009; Dinuccio et al., 2010; González-González et al., 2013). Moreover, the use of agricultural substrates in co-digestion is a viable solution for its disposal in areas of high agricultural production. Therefore, the combination of pig slurry and carbon-rich substrates can result in better digestion performance compared with digestion of pig slurry-only. In addition, co-digestion can reduce the concentration of certain compounds found in the slurry which can be toxic to the anaerobic digestion process such as ammonia (Murto et al., 2004) and volatile fatty acids (VFA).

Agricultural substrates might have adverse effects when added to a stable digester or used in conjunction with other types of residues (Fountoulakis et al., 2008). Gelegenis et al. (2007) stated that olive mill wastewater may inhibit certain microbial groups in anaerobic reactors in co-digestion with diluted poultry manure. These authors reported that the presence of aromatic oils or polyphenols in agricultural substrates could inhibit the development of archaea populations within the methanogenic consortia. Furthermore, methanogen populations and their domains are poorly understood when adding a substrate for co-digestion with pig slurry (Traversi et al., 2011; Yue et al., 2013; Zhang et al., 2011). In pig slurry anaerobic digesters, hydrogenotrophic methanogenesis is the main metabolic pathway for organic matter conversion to CH_4, due to unfavourable environmental factors for acetoclastic methanogenes (Song et al., 2010). However, acetogenic methanogenesis is recommended to enhance CH_4 production due to its higher CH_4 yield (Garcia et al., 2000). The addition of a co-substrate could

promote acetogenic methanogenesis. Nevertheless, there are few studies that explore the changes in methanogenic population structure of an agricultural substrate to pig slurry in co-digestion. Consequently, it is key to determine the adequate amount of each substrate in pig slurry and agricultural substrate's mixtures to maximize CH_4 production and minimize adverse antimicrobial effects.

Specialist knowledge on not only on the type of agricultural substrates which can enhance CH_4 production with pig slurry, but also on their amount in the substrate's mixture could improve pig slurry management in high livestock-density areas; promoting the energetic value of the slurry and the agricultural by-products derived from the fruit and vegetable industry. This information could help farmers to select the best combinations using agricultural substrates and pig slurry in anaerobic co-digestion in these areas and to generate an additional revenue and diversity of the agricultural activity.

The objective of this study was to evaluate the effect of four agricultural substrates (tomato, pepper, peach and kaki) on the biochemical methane potential (BMP) in anaerobic co-digestion with pig slurry, focusing on the type of substrate and its amount on the final substrate's mixture (inclusion level). The influence of agricultural substrate on the sludge's microbial composition was also studied. This study aims to finally identify the combination that optimizes CH_4 production and can serve as useful input to develop integrated agricultural by-products and pig slurry management systems in high livestock-density areas.

7.2 Material and methods

7.2.1 Substrates and inoculum

Pig slurry was created from fresh faeces and urine to avoid the effect of external factors (management, storage time, feeding system or amount of added water) on pig slurry composition (Canh et al., 1998). Pig urine and faeces were collected directly after excretion from six dry sows housed individually. Sows were fed 2.5 kg day^{-1} of a conventional diet for pregnant sows containing,on average, 2.8 Mcal net energy kg^{-1}, 14% crude protein, 2.5% crude fat and 3.5% crude fibre. After collection, the faeces and urine were mixed with distilled water following the procedure described in Møller et al. (2004a), to obtain a fresh slurry with a TS content of 40 g L^{-1}.

The four agricultural substrates assayed were tomato, pepper, peach and kaki, selected due to their availability and rich content in easily biodegradable carbohydrates. They were obtained from a local market and stored at room temperature for 3 weeks in order to simulate non-marketable products. Before putting into the vials were grounded to homogenize them.

As inoculum, anaerobic sludge was used from an anaerobic digester that treats domestic and industrial wastewater from the wastewater treatment plant in Sagunto, Spain. The inoculum was incubated during 15 days at 35 °C in order to deplete the residual biodegradable organic material (degasification).

7.2.2 Experimental design and biochemical methane potential determination

The biochemical methane potential (BMP) of each combination of agricultural substrate and pig slurry was determined in a batch assay according to the protocol defined by Angelidaki et al. (2009). The experiments were performed in 120-mL glass bottles, incubated at mesophilic range (35 ± 1 °C) for 100 days.

Bottles were prepared to achieve homogeneity in total volatile solids (VS) (1.3 g VS from manure + agricultural substrates + inoculum), as well as the same amount of inoculum (33.6 mL per bottle). The ratio of inoculum to substrate (pig slurry + agricultural substrates) in all combination was 0.7 on a VS basis (Møller et al., 2004b). Therefore, variations in VS composition were solely attributable to the agricultural substrate.

Each agricultural substrate was tested at four inclusion levels in combination with pig slurry. The inclusion levels consisted of the replacement of the VS from the pig slurry with the VS from the agricultural substrate, expressed as a percentage of the VS coming from the agricultural substrate from the total VS of the mixture (pig slurry + agricultural substrate). The agricultural substrate's inclusion levels tested were: no agricultural substrate addition (inclusion level 0), 15% (inclusion level 1), 30% (inclusion level 2) and 50% (inclusion level 3). Each tested combination was carried out in triplicate. Additionally, three blank bottles containing anaerobic sludge-only were also used in order to determine the anaerobic sludge endogenous CH_4 production which was subtracted

from the CH_4 produced by the tested combination at each biogas sampling.

After filling the bottles, they were flushed with nitrogen (99.9%) for one minute to prevent oxygen inhibition, closed with butyl rubber stoppers and then sealed with aluminum crimps. The biogas volume in each bottle was monitored weekly by measuring pressure in the headspace using a manometer (Delta Ohm, HD 9220, Italy). Additionally, a representative sample from the head space gas volume of each bottle was taken weekly to measure CH_4 content in the biogas. After sampling, the remaining overpressure of the bottle was removed to restore atmospheric pressure.

7.2.3 Chemical analyses

A representative sample of each separate substrate (agricultural substrate, pig slurry, inoculum) and an initial (day 0) and final (day100) sample from each tested combination (pig slurry + agricultural substrate + inoculum) was analysed to determine TS, VS, pHand total ammonium (TAN) (4500 NH_3-B and 4500 NH_3-C procedures) (APHA, 2005). Volatile fatty acids were determined by gas chromatography following the method described by Jouany (1985) with the addition of an internal standard (4-metil valeric). Additionally from agricultural substrates and pig slurry, neutraldetergent fibre (NDF), acid detergent fibre (ADF) and acid detergent lignin (ADL) and total kjeldahl nitrogen (TKN) were determined according to the Van Soest procedure (Van Soest et al., 1991) and APHA (2005) respectively. The lipid content was also analysed from agricultural substrates and pig slurry (AOAC, 2000). Methane concentration in the biogas from the batch assay was further analysed using a Focus Gas Chromatograph (Thermo, Milan, Italy) equipped with a split/splitless injector and a flame ionization detector. The separation was performed in a capillary column GS-Q (J&W Scientific, USA) (30 m, 0.32 mm internaldiameter). The carrier gas was helium at a constant flow of 1 mL min^{-1}. Samples were injected with a split ratio of 1:100. The initial oven temperature was set at 50 °C held for 1 min. The temperature was then increased at a rate of 50 °C min^{-1} until 150 °C, which was finally maintained for 2 min. Both detector and injector temperatures were set at 200 °C. An external standard (41.2% carbon dioxide, CO_2 and 58.8% CH_4) was employed for the quantification of CH_4 content in samples.

7.2.4 Extraction of genomic DNA

On day 100 of the experiment, one sample of each combination under agitated conditions was taken and placed into 20 mL sterile Falcon tubes. After sampling, the samples were immediately frozen with liquid nitrogen and stored at -80 °C. After thawing, the total DNA isolation from 250 µL of anaerobic sludge was carried out using QIAamp DNA Stool Mini kit (Qiagen, Germany) and following the manufacturer's instructions.

DNA concentration, quality, and integrity were evaluated by using a NanoDrop 2000C Spectrophotometer (Fisher Scientific SL, Spain). The extracted DNA was diluted with nuclease-free water (Ambion, USA) until 0.5–1.0 ng DNA μL^{-1} and used as a template. The results were expressed as copies per mg of DNA.

7.2.5 Bacterial and archaeal absolute quantification (qPCR)

In order to measure bacterial and archaeal population, quantitative real-time TaqMan polymerase chain reaction (qPCR) assays were developed and expression analyses performed using a StepOne Plus (Applied Biosystems, Spain) thermocycler. Each 16S rRNA gene sequence from total bacteria and archaea (Bacteria and Archaea domains), furthermore four main methanogenic archaeal orders, were amplified using real-time PCR with the corresponding primers and probes described by Yu et al. (2005). Genomic DNA of four type of the methanogenic archaeal orders *Methanobacteriales (M. byantii DSM 863), Methanomicrobiales (M. mobile DSM 1539), Methanosarcinales (M. barkeri DSM 800) and Methanoccocales (M. voltae DSM 1537);* additionally, *Bacteria* (*E. coli DSM 1607*) and *Archaea (M. mobile DSM 1539*) domains were provided by the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ Braunschweig, Germany) to construct the standard curve.

PCR amplification was performed in an ABI GeneAmpTM system 2700 thermo cycler. The reaction mixture of 25 ll contained 1xPCR buffer (Invitrogen), 200 lM dNTPs (Invitrogen), 0.1 IU of Taq DNA polymerase (Invitrogen), 500 nM of each primer and 1 ll of cDNA template. The first PCR amplification was run as follows: denaturation at 94 ºC for 3 min, followed by 40 cycles of 94 ºC for 30 s, 55 ºC for 30 s, and 72 ºC for 30 s, and finally an extension step at 72 ºC for 10 min. PCR products were visualized in 2% agarose gel stained with ethidium bromide and bands of

expected size were purified using Qiaquick Gel Extraction kit (Qiagen) and ligated into the pGEM-T easy vector (Promega, WI, USA). Cloning was performed in competent *E. coli* JM109 cells (Promega). Positive colonies were isolated and plasmids were extracted by Qiagen Plasmid Mini Kit (Qiagen). Plasmids with inserts were sequenced using an ABI 3100 DNA analyzer (Applied Biosystems, Polytechnique University of Valencia Ibmcp sequencing service, Valencia, Spain). The resulting PCR products were purified and the corresponding copy concentration was calculated using Eq.(7.1).

$$DNA\ (copy) = \frac{(6.02 * 10^{23}(copy/mole) \times DNA\ amount(g))}{DNA\ lenght\ (BP) \times 660((g/mole)/bp)}$$

After an initial Taq activation of polymerase at 95 °C for 10 min, 45 cycles of PCR were performed using the Master Mix solution (Applied Biosystems) with the following cycling conditions: 95 °C for 10 s, 60 °C for 30 s and 73 °C for 30 s in the study of Total bacteria, *Methanosarcinales* and *Methanoccocales*; 95 °C for 10 s and 60 °C for 60 s in the study of Total archaea; 95 °C for 10 s, 63 °C for 30 s and 73 °C for 30 s *Methanomicrobiales*; 95 °C for 10 s, 57 °C for 30 s and 73 °C for 30 s in the study of *Methanobacteriales*.

The total volume for every PCR reaction was 12 µL, performed from cDNA template (2 µL), forward and reverse primers (500 nM each), 6 µL of Master Mix solution (Applied Biosystems) and 0.18 µL of the TaqMan probe (150 nM). The quantification of the results were carried out using an absolute standard curve method by serial dilutions in the range of 10^{-4}–10^{-11} copies μL^{-1} and directly used as a template for qPCR with the corresponding primer and probe sets in triplicate. Average slope and intercepts used for 16S rRNA genes concentration of target microbial orders in the samples are shown in Table 7.1.

Table 7.1. Average slope and intercepts used for 16S rRNA genes concentration of target microbial orders in the samples.

Domain/order	Slope	Intercept	R^2
Total bacteria	-3.41	-45.28	0.995
Total archaea	-3.03	-49.30	0.971
Methanobacteriales	-3.29	-45.30	0.994
Methanosarcinales	-3.77	-49.31	0.995
Methanomicrobiales	-4.59	-60.11	0.989
Methanoccocales	-3.13	-52.06	0.997

7.2.6 Data analyses and calculations

In order to compare agricultural substrate's chemical composition on the same basis, total carbohydrates, hemicellulose, cellulose, protein, lipids and lignin content were calculated from each substrate (each agricultural substrate and pig slurry) and expressed as percentage of total VS. The hemicelluloses were calculated as the difference between NDF and ADF; cellulose as the difference between ADF and ADL; and lignin content was assumed to be equalto ADL according to Van Soest et al. (1991). The protein content was determined by multiplying (TKN-TAN) by 6.25. Total carbohydrates were calculated following Eq. (7.2), as the fraction of VS left after subtraction of VS in proteins, lipids, lignin and VFA expressed per 100 g of VS (Galí et al., 2009; Gelegenis et al., 2007).

$$VS_{carbohydrates}(\%) = 100 - VS_{protein} - VS_{lipids} - VS_{lignin} - VS_{VFA}$$

The VS removal at the end of the experiment was calculated as the difference, in percentage, between initial and final VS content of each combination tested, subtracting the amount of initial VS provided by the inoculum (blank bottles) (Eq. (7.3)).

$$VS_{removal}(\%) = \frac{(VS_{initial\ bottle} - VS_{final\ bottle})}{(VS_{initial\ bottle} - VS_{blanks})} \times 100$$

The BMP was calculated as the cumulative CH_4 production for each combination tested at each sampling day, expressed as the CH_4 production (mL) per g of VS from the substrates introduced in the bottles (pig slurry + agricultural substrate). The average of bottles with 0% of agricultural substrate (inclusion level 0) was considered the control in order to calculate the increase in BMP achieved with the replacement of VS from pig slurry with VS from the agricultural substrate. The increase in BMP achieved with the inclusion of the agricultural substrate was expressed as the increment in percentage of the tested combination (inclusions levels 1,2 and 3) with respect to control BMP following Eq. (7.4).

$$BMP_{increase}(\%) = \frac{BMP_{combination\ tested} - BMP_{control}}{BMP_{control}} \times 100$$

Data were statistically analysed using SAS Software (SAS, 2001). The BMP and cumulative biogas production were analysed by an ANOVA (GLM procedure of SAS®) in which substrate's inclusion level (clustered by substrate), type of substrate (clustered by inclusion level) and its

interactions were the main sources of variance. Logarithmic transformation was applied to those parameters which were not normally distributed. Parameters with a kurtosis coefficient between [-2, 2], were assumed to be normally distributed.

7.3 Results and discussion

7.3.1 Substrate's chemical composition

The chemical composition of each substrate and inoculum used in this study is shown in Table 7.2. Pig slurry and inoculum showedthe highest pH, being equal to 8.7 (pig slurry) and 7.2 (inoculum).On the contrary, agricultural substrates showed pH's below neutrality ranging from 3.8 for peach to 5.9 for kaki.

Table 7.2. Chemical composition of agricultural substrates, pig slurry and inoculum used in this study expressed per kg of fresh matter (FM) or 100 g of volatile solids (VS).

Parameters	Tomato	Pepper	Kaki	Peach	Pig slurry	Inoculum
pH	4.56	5.06	5.94	3.76	8.66	7.22
TS (g kg^{-1} FM)	62.5	113.9	204.4	132.9	41.4	20.9
VS (g kg^{-1} FM)	56.2	106.6	198.6	125.9	29.2	15.9
TKN (g kg^{-1} FM)	1.74	2.37	1.11	0.89	1.19	-
TAN (mg N kg^{-1} FM)	n.d.	n.d.	n.d.	n.d.	488.6	827.7
VFA (mg kg^{-1} FM)	n.d.	n.d.	n.d.	n.d.	484.66	6.10
Carbohydrates (g 100 g^{-1} VS)	74.19	77.77	96.66	93.18	61.29	-
Hemicellulose (g 100 g^{-1} VS)	3.81	2.50	2.45	2.66	37.18	-
Cellulose (g 100 g^{-1} VS)	12.43	8.07	3.19	4.71	23.33	-
Lipid (g 100 g^{-1} VS)	3.81	4.41	0.18	0.73	9.62	-
Protein (g 100 g^{-1} VS)	19.30	13.91	2.80	5.52	14.97	-
Lignin (g 100 g^{-1} VS)	2.70	3.91	0.35	0.66	12.47	-

n.d.: not detected.

As shown in Table 7.2, the pig slurry presented higher content in hemicellulose (37.2 g 100 g^{-1} VS), cellulose (23.3 g 100 g^{-1} VS) and lignin (12.47 g 100 g^{-1} VS) than the agricultural substrates, but a lower content in total calculated carbohydrates. The sum of hemicellulose and cellulose in pig slurry practically explained the total amount of carbohydrates (98%), whereas in agricultural substrates, the sum of

hemicellulose and cellulose explained less than 20% of the total carbohydrates.

Møller et al. (2004b) calculated easily-biodegradable carbohydrates content of 38.9 g 100 g^{-1} VS and slowly-biodegradable carbohydrates of 14.8 g 100 g^{-1} VS in the sow faeces. These values are comparable to pig slurry hemicellulose content (easily-biodegradable carbohydrates) and cellulose content (slowly-biodegradable carbohydrates) reported in our study. The presence of high ratios of slowly-biodegradable carbohydrates and lignin in pig slurry is due to feed degradation processes in the animal's gastrointestinal tract.

The pig slurry showed higher lipid content than agricultural substrates, and higher protein content than the pepper, kaki and peach. The lipid and protein content in the pig slurry were slightly lower to those reported by Møller et al. (2004b), who obtained a lipid and protein content in sow's faeces of 16.3 and 20.2 g 100 g^{-1} VS, respectively. These components have been described to be easily biodegradable and lead to higher CH_4 yields than carbohydrates (Angelidaki and Sanders, 2004).

According to their chemical composition, agricultural substrate can be divided in two groups: vegetables substrates including tomato and pepper, and fruit substrates including peach and kaki. According to Table 7.2, vegetables substrates showed higher lignin, cellulose and hemicelluloses content but lower content in total calculated carbohydrates than fruit substrates. Among vegetables substrates, tomato showed the highest cellulose and hemicellulose content, while pepper showed the highest lipid and lignin content. The higher content in lignin, cellulose and hemicelluloses in vegetables substrates compared with fruits could be explained by the seeds, peel and stems proportions with respect to the pulp, are higher in vegetables substrates than in fruits. The higher content in pulp, attributable to fruits, could imply higher content in non-structural carbohydrates like sugars which have not been determined in this work. For this reason, the sum of hemicelluloses and cellulose in vegetables substrates explained higher amount of carbohydrates than in fruit substrate (18% *vs*. 7%). Dembitsky et al. (2011) also found a high sugar concentration in kaki (about 12.5 g 100 g^{-1} fresh weight), being fructose, glucose and sucrose the major components.

Additionally, vegetables substrates showed higher lipid and protein content than fruit substrates. Among vegetables substrates, tomato

showed the highest protein content while pepper showed the highest lipid content. Tomato composition showed similar protein, lipid and carbohydrates content than those reported by Elbadrawy and Sello (2011), who obtained 10.5 g protein, 4.0 g lipids and 78.6 g carbohydrates per 100 g on dry weight basis inthe chemical composition of tomato peel.

7.3.2 Combination's chemical composition

Initial (day 0) TAN and VFA levels of each tested combination(data not shown) were below 900 mg N-NH_3 $L^{-1}$and 250 mg L^{-1},respectively. Initial TAN levels ranged from 716.7 to 802.9 mg N- NH_3 L^{-1} in all combinations tested. These levels were below 1100 mg N-NH_3 L^{-1}, reported by Hansen et al. (1998) to be the threshold level that may cause NH_3 inhibition in anaerobic digestion. The TAN was also in the range of the values reported in González-Fernández and García-Encina (2009) treating swine slurry. Concerning VFA, combinations with inclusion level 0 (pig slurry-only) showed the highest initial VFA content, since as shownin Table 7.2, pig slurry was the substrate with highest initial VFA. Inaccordance with the inhibition thresholds of the anaerobic digestion process reported by Chen et al. (2008), final pH levels (varied from 7.21 to 7.62) and VFA (Table 7.3) in our study were not in the range to cause anaerobic process failure, therefore no inhibition caused by VFA and TAN during the BMP assay could be expected.The final (day 100) TAN, VFA content and VS removal of each tested combination are shown in Table 7.3.

Concerning TAN levels,similar final TAN content among combinations for the same inclusion level were observed. Inclusion level 0 combinations showed similar final TAN to the other inclusion levels. Final VFA and VS removal values obtained at inclusion level 0 were within the lower ranges of the tested combinations obtained in this work. Final TAN levels did not show any clear pattern among substrates, being in general final TAN levels higher than the initials as a result of the urea and protein hydrolysis and a low nitrogen utilization rate by bacteria (Sung and Liu, 2003). Ammonia accumulation in the digester indicates that proteins and urea are being degraded in the reactors faster than the ratio of microbial nitrogen utilization. In fact, combinations with tomato, the substrate with the highest proteincontent per g of VS, showed the highest final TAN levels.

Table 7.3. Average final concentration ± standard deviation of total ammonia nitrogen (TAN) and volatile fatty acids (VFA) (at day 100), and volatile solids (VS) removal percentage of the tested combinations, expressed per kg of fresh matter (FM).

Combination	Inclusion level	Final TAN (mg N L^{-1})	Final VFA (mg L^{-1})	VS removal (%)
Pig slurry	0	994.06 ± 53.17	3.68 ± 0.16	51.30 ± 1.78
Tomato	1	994.70 ± 57.95	4.25 ± 0.41	71.98 ± 10.10
Pepper		1059.18 ± 2.90	3.97 ± 0.65	64.92 ± 0.83
Kaki		978.96 ± 20.63	6.53 ± 2.18	57.47 ± 2.70
Peach		999.18 ± 14.20	4.10 ± 0.25	58.85 ± 8.63
Tomato	2	998.91 ± 42.86	3.81 ± 0.10	64.99 ± 2.76
Pepper		995.61 ± 21.93	5.21 ± 1.24	53.79 ± 7.87
Kaki		962.59 ± 16.30	4.27 ± 1.21	47.54 ± 13.81
Peach		981.89 ± 26.54	4.41 ± 1.48	49.13 ± 2.87
Tomato	3	1100.53 ± 66.45	6.14 ± 2.28	72.78 ± 2.41
Pepper		976.86 ± 108.12	5.56 ± 1.27	57.34 ± 3.80
Kaki		885.39 ± 44.05	5.34 ± 2.30	58.82 ± 0.91
Peach		946.31 ± 37.31	4.44 ± 1.28	57.76 ± 4.51

Final VFA were low and similar among combinations, indicating a complete degradation of biodegradable organic matter to CO_2 and CH_4 in all cases. No differences among substrates were observed with respect to VFA levels.

Relating to VS removal, average VS removal per inclusion level was the lowest for inclusion level 0 (51%), whereas on average, inclusion level 3 showed the highest VS removal percentage (62%). Although all combinations contained the same amount of VS, different percentage of VS removal were achieved dependingon substrate's biodegradability. The VS removal for inclusion level 0 obtained in this study is in accordance with the values reached by Hill and Bolte (2000) on treating pig slurry in anaerobic digestion. The lower values for VS removal for pig slurry compared with agricultural substrates indicated that organic matter from pig slurry was less biodegradable than the other substrates, probably attributable to the higher lignin and cellulose content compared with agricultural substrates. The percentage of VS removal in agricultural substrates varied from 48% (kaki at inclusion level 2) to 73% (tomato at inclusion level 3), achieving higher VS removal with vegetables substrate than with fruit substrates. Combinations with tomato presented the highest VS removal percentages regardless inclusion level. Kaki and peach combinations presented similar VS removal per inclusion level, ranging from 48 to 59%, being the lowest VS removal rate at inclusion level 2 for kaki. Differences in VS removal are attributable to substrate's biodegradability, indicating that vegetables substrates with higher VS removal are easier biodegradable than fruit substrates and thus higher BMP are expected with them.

7.3.3 Effect of the type of agricultural substrate on the biochemical methane potential

The BMP evolution during the experimental period (100 days) clustered by agricultural substrate is shown in Fig. 7.1. Each line represents the average of the four inclusion levels per substrate type. The BMP production was low during the first 7 days in all substrates and increased thereafter. From day 7 onwards, BMP presented an exponential growth phase (from day 7 to 20). Finally, there was a plateau phase until the end of the experiment, where the steady state conditions were achieved. Average final BMP (100 days) was equal to 279.8 ± 42.26 mL CH_4 g VS added^{-1} (pepper), 276.9 ± 37.74 mL CH_4 g VS added^{-1} (tomato), 261.1 ± 30.39 mL CH_4 g VS added^{-1} (peach) and 241.8 ± 18.52 mL CH_4 g VS added^{-}

[1] (kaki). Combinations containing kaki showed the lowest final BMP values ($P < 0.001$), whereas combinations containing pepper showed the highest final BMP ($P < 0.001$). Combinations containing vegetables substrates (tomato and pepper) showed higher final BMP compared with fruit substrates (kaki and peach). Nevertheless, tomato and pepper showed very similar final BMP ($P > 0.05$).

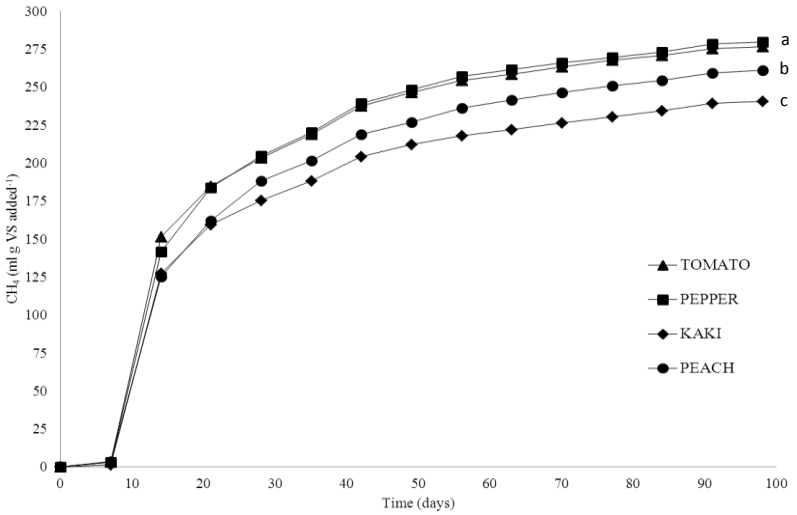

Fig. 7.1. Evolution of the ultimate methane yield (B_0) (mL CH_4 g VS added^{-1}) per agricultural substrate. Each line represents the average from 12 bottles. ^{a,b,c}: Different letters in different curves indicate averages statistically different.

Biogas's CH_4 content when the steady state conditions were achieved (from day 20 onwards) varied from 70% for tomato, 67%for pepper, and 64% for kaki and peach (data not shown). Average percentage of CH_4 from the biogas was higher in the combinations containing tomato than in the others substrates, whereas highest biogas production was achieved with pepper.

Differences observed in this study in BMP production per substrate, could be explained by substrate composition. The BMP depends on the relative amounts of the following four main components: lipids, proteins, carbohydrates and lignin (Neveset al., 2008). The CH_4 potential of these components equals 1014 L CH_4 kg VS^{-1} (lipids), 496 L CH_4 kg VS^{-1} (proteins) and 415 L CH_4 kg VS^{-1} (carbohydrates) (Angelidaki and Sanders,

2004). Little research has been conducted focusing on the effects of substrate's chemical composition and its effects on BMP, as the study published by Triolo et al. (2011) focused on lignin as the predominant variable to predict BMP from biomass. Thus improvements in this area are necessary in order to optimize co-digestion mixtures.

In our study, pepper and tomato presented higher lipid and protein content and lower calculated carbohydrates content than fruit substrates. Consequently, combinations with pepper and tomato presented the highest BMP. Combinations with kaki, which showed the lowest lipid and protein content and the highest calculated carbohydrates content, presented the lowest BMP. Besides kaki's chemical composition, its low BMP could be attributable to the presence of some inhibitory substance that could disable the development of archaea populations. Indeed, kaki has been reported to be rich in polyphenols (Jeong et al., 2009). The antimicrobial activity of kaki has been confirmed in intestinal microorganisms (Jeong et al., 2009) and in archaea populations in sheep's rumen (Shin et al., 2010).

7.3.4 Effect of substrate's inclusion level on biochemical methane potential

Table 7.4 shows final BMP obtained with the different agricultural substrates in co-digestion with pig slurry at inclusion levels 1, 2 and 3, and the increase in BMP of each tested combination with respect to control BMP of pig slurry (inclusion level 0). Control BMP was 239.24 ± 6.45 mL CH_4 g VS added^{-1}.

No statistical differences were observed between control BMP and the combinations tested at inclusion level 1 (15% of total VS) in any agricultural substrate and between control BMP and kaki at inclusion level 2.

The BMP was the highest for pepper at inclusion level 3 ($P < 0.001$) and the lowest for kaki at inclusion level 2. According to Table 7.4, tomato, pepper and peach substrates showed statistical significant differences with respect to control BMP at inclusion levels 2 and 3 ($P < 0.001$), however kaki substrate only showed differences with control BMP at inclusion level 3. Combinations with tomato, pepper and peach at inclusion level 3 achieved a BMP equal to 337.96 mL CH_4 g VS added^{-1} in tomato, 343.46 mL CH_4 g VS added^{-1} in pepper and 306.63 mL CH_4 g VS added^{-1} in peach ($P < 0.001$).

Table 7.4. Average ultimate methane yield (B_0) ± standard deviation (mL CH4 g VS added^{-1}) and B_0 increase (%) with each agricultural substrate and inclusion level tested.

Inclusion level	Tomato		Pepper		Kaki		Peach	
	B_0	Increase	B_0	Increase	B_0	Increase	B_0	Increase
0	239.24±6.45^{c}	-	239.24±6.45^{c}	-	239.24±6.45^{b}	-	239.24±6.45^{c}	-
1	260.21±14.96^{c,u}	9	256.02±7.67^{bc,u}	7	230.39±4.82^{b,v}	-4	232.82±5.03^{c,v}	-3
2	290.58±11.99^{b,u}	21	280.78±11.89^{b,uv}	17	226.65±11.20^{b,w}	-5	264.92±6.23^{b,v}	11
3	337.96±4.25^{a,u}	41	343.46±13.37^{a,v}	44	267.06±14.80^{a,w}	12	306.63±4.00^{a,v}	28

^{a,b,c}: Different letters in the same column indicate averages statistically different (P<0.05)

^{u,v,w}: Different letters in the same row indicate averages statistically different (P<0.05)

Table 7.5. Final 16S rRNA gene concentration of the microorganism determined by qPCR from the different combinations tested expressed as logarithm of the copies mg DNA^{-1} clustered by agricultural substrate.

Domain/Order	Tomato	Pepper	Kaki	Peach	Inoculum	Pig slurry
Bacteria	11.90	12.29	12.19	12.22	11.83	12.12
Archaea	11.16	11.60	11.48	11.61	9.42	11.36
Methanobacteriales	8.97	9.37	9.26	9.39	7.49	9.16
Methanosarcinales	7.24	7.86	7.43	7.61	7.77	7.79
Methnaomicrobiales	10.76	10.96	11.07	11.05	9.66	10.90

This resulted in an increase in BMP of 41% with tomato, 44% with pepper, 28% with peach and 12% with kaki with respect to pig slurry only (control BMP). Combinations with kaki at inclusion level 1 and 2 and peach at inclusion level 1 showed a decrease of 3% to 5% in BMP compared with control BMP although these differences were not statistical significant ($P > 0.05$).

These results are in accordance with Campos (2001) who reported that only inclusion levels of 12.5% and 20% on weight basis of pear waste in co-digestion with pig slurry significantly enhanced BMP in mesophilic range for 80 days, compared with lower inclusions levels (5% on weight basis). Molinuevo-Salces et al. (2010) obtained an increase in BMP of 27% using pig slurry in co-digestionwith fresh maize, carrots, peas and leaks at inclusion levels from 15% to 85% measured in terms of percentage of TS of vegetables substrates in relation to the TS of the initial pig slurry. Similarly, Riaño et al. (2011) increased BMP by 49% (from 233 ± 8 mL CH_4 g COD added^{-1} to 348 ± 18 mL CH_4 g de COD added^{-1}) by adding winery wastewater in co-digestion with pig slurry at inclusion levels from 15% to 85% measured in terms of COD of winery wastewater in relation to the COD of the feed. Although in absolute terms BMP values are lower than those achieved in this study, our results showed an increase in BMP of 28% from inclusion levels 1–3 in all agricultural substrates except in the case of kaki which only showed an increase in BMP of 12% from inclusion levels1–3.

The increase in BMP with increasing agricultural substrate inclusion level can be explained by the adaptation of the co-substrate mixture to the optimum characteristics for digestion as regards to biodegradable organic matter (Hartmann and Ahring, 2005). This has been confirmed with the co-digestion of animal manures and agricultural substrate in continuous experiments using conventional stirred tank reactors or similar configurations (Callaghan et al., 2002; Kaparaju and Rintala, 2005).

7.3.5 Microbial community structure and dynamics

Table 7.5 shows final 16S rRNA gene concentration of the microorganism determined by qPCR from the different combinations tested expressed as copies per mg DNA^{-1} clustered by agricultural substrate. The order *Methanococcales* could not be detected in any combination tested and thus data are not shown.

As shown in Table 7.5, microorganism belonging to total bacteria showed the highest gene concentration. Although no differences were found in the quantity of total bacteria population among combinations, blank bottles and combinations with tomato showed a slightly lower total bacteria gene concentration than the rest of combinations. Gene concentration of total archaea fromthe blank bottles was lower than gene concentration of total archaea from the rest of combinations. No differences were observed in gene concentration of total archaea among the different combinations with agricultural substrates and pig slurry-only.

The percentage of 16S rRNA gene concentration of total archaea to total microbial community (bacteria and archaea) ranged from 0.4% in blank bottles to 20% in peach combination, being in all combinations tested (agricultural substrates and pig slurry) higher than 10%. It is reported that in well operating digesters methanogens account for about 8–12% of the total microbial community (Yu et al., 2006). This indicates that at the end of the experimental period, archaea were well balanced in all bottles except in the blank bottles where total archaea gene concentration was very low.

As previously stated, kaki could contain polyphenols which can inhibit methanogenic activity (Jeong et al., 2009). Fresh kaki has been reported to have total polyphenols equal to 1.3 mg 100 g^{-1} of fresh matter (Dembitsky et al., 2011). However, no differences in total archaea concentration were observed in combinations with kaki compared to the rest of combinations tested. The PCR and other molecular techniques are being used to determine microbial indicators of CH_4 production (Yu et al., 2005, 2006), nevertheless it is difficult to withdraw reliable conclusions about the activity or viability of microorganism using only molecular techniques since it is not possible to distinguish among living, non-living, dormant or extremely slow-growing cells (physiological state) and free DNA present in samples (Solera et al., 2001) which could cause an overestimation of the microbial population counts.

Concerning individual archaeal orders, the 16S rRNA gene concentration of *Methanobacteriales* was lower in the blank bottles than in the rest of combinations tested; the 16S rRNA gene concentration of *Methanobacteriales* was around 9 log copies mg DNA^{-1} in the different combinations. The 16S rRNA gene concentration of *Methanosarcinales*

was the lowest among the individual archaeal orders (on average 7.6 log copies mg DNA^{-1}), in fact *Methanosarcinales* order contributed less than 0.1% of 16S rRNA gene concentration of total *archaeal* orders quantified in all combination tested except for the blank bottles, where the percentage of 16S rRNA gene concentration of *Methanosarcinales* with respect to the sum of the three *archaeal* orders determined was higher than 1%. This could indicate that the addition of pig slurry and agricultural substrate caused a decrease in the *Methanosarcinales* population.

Methanomicrobiales dominated in all combination tested over the rest of individual *archaeal* orders. In fact, *Methanomicrobiales* represented more than 90% of 16S rRNA gene concentration of total archaeal orders quantified in all combinations tested. The predominance of *Methanomicrobiales* over *Methanosarcinales* in this work indicates that hydrogenotrophic methanogenesis was the main metabolic pathway for organic matter conversion to CH_4. The predominance of hydrogenotrophic *vs*. aceticlastic methanogenesis has also been observed in anaerobic digesters working with animal slurry in the literature (Nettmann et al., 2008; Song et al., 2010). This could be related to the fact that hydrogenotrophic methanogenesis is favoured at high concentrations of TAN (Karakashev et al., 2005) in detriment of *Methanosarcinales*, and these substances are typically found in high concentrations in animal slurries.

7.4 Conclusions

The effect of tomato, pepper, kaki and peach agricultural substrates at different inclusion levels in the BMP in anaerobic co-digestion with pig slurry was investigated. From our results we can conclude that:

- Vegetables substrates (pepper and tomato) showed higher lipid, protein, lignin and cellulose content than fruit substrates (kaki and peach). Fruit substrates showed higher calculated carbohydrates content than vegetables substrates.
- The highest BMP were achieved with those substrates with protein content between 14 and 20 g 100 g^{-1} VS, lipids between 4 and 5 g 100 g^{-1} VS, and carbohydrates between 74 and 78 g 100 g^{-1} VS (corresponding to tomato and pepper). Further research on substrate composition, particularly on specific chemical components and BMP are necessary in order to optimize co-digestion mixtures.

- Agricultural substrates improved BMP, from pig slurry co-digestion; higher BMP values were obtained with increasing addition of agricultural substrate. In fact, combinations with tomato, pepper and peach at inclusion level 3 (50% of VS) achieved the highest BMP. This resulted in an increase in BMP of 41% with tomato, 44% with pepper, 28% with peach and 12% with kaki.
- No differences were found in total bacteria and total archaea gene concentrations among combinations tested. *Methanomicrobiales* gene concentrations dominated over the rest of individual archaeal orders indicating that hydrogenotrophic methanogenesis was the main metabolic pathway for organic matter conversion into CH_4.

Knowledge on the type of substrate and its inclusion level that best optimize CH_4 production can serve as useful inputs to develop integrated agricultural by-products and pig slurry management, in high livestock-density areas.

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Chapter 8

General Discussion

The use of agro-industrial by-products in pig production offers a potential alternative to the use of raw materials in feeds, as a source of bioactive compounds or substrates for bioenergy production. Optimizing their use contributes to the economic, social and environmental sustainability of this sector. The present PhD Thesis is aimed at studying the valorisation of Mediterranean agro-industrial by-products from two different approaches: as ingredients replacing other common feedstuffs in feeds and as substrates for anaerobic co-digestion with pig slurry. This approach is in accordance with the food waste hierarchy established by the EU for waste disposal (figure 1.2). In this chapter, the main results from this PhD Thesis are discussed in a broad and integrative context and their implications for pig production systems and future research are identified.

8.1 Characterization of agro-industrial Mediterranean by-products

The Spanish Mediterranean region has an important agro-industrial tradition which generates high amounts of by-products of very diverse and variable origin available for the livestock sector. These include wastes from cultivation of fruits and vegetables, by-products from the cereal grain milling, oilseed extraction, brewery, malt and juice production, and products derived from the canning industry. These by-products, which represent a potential source of feed ingredients or a substrate for bioenergy in pig production systems, are currently not fully exploited in monogastric species because there is not enough information on their nutritional value and potential effects on the animals and biogas production in co-digestion with slurry.

In this PhD Thesis eight agro-industrial by-products have been selected for their valorisation in pig production due to their local availability and suitability for this purpose. With respect to their origin, we selected four dried by-products from the agri-food industry (two of them from the olive oil industry and the other two from the orange juice industry), and four agricultural by-products (two vegetables: tomato and pepper and two fruits: kaki and peach). Table 8.1 summarizes the characterization of the agro-industrial by-products evaluated in this PhD Thesis.

Table 8.1. Chemical composition of the agro-industrial by-products studied (g/kg DM)

	Crude OC	Partially Defatted OC	Dehydrated OP	Ensiled Sun-Dried OP	Tomato	Pepper	Kaki	Peach
Dry matter	932	926	880	860	62.5	114	204	133
Ash	89.5	108	60.7	83.7	10.1	6.41	2.84	5.27
HCl insoluble ash	7.15	6.80	1.09	2.03	n. a.	n. a.	n. a.	n. a.
Crude protein	103	89	72.8	79.3	174	130	27.2	52.3
NDICP[a]	64.1	69.3	n. a.	n. a.	n. a.	n. a.	n. a.	n. a.
ADICP[b]	36.2	39.1	n. a.	n. a.	n. a.	n. a.	n. a.	n. a.
Ether extract	174	125	19.2	35.3	34.3	41.3	1.75	6.92
Crude fibre	n. a.	n. a.	114	136	n. a.	n. a.	n. a.	n. a.
NDF	382	403	201	247	170	136	58	76
ADF	244	277	138	176	136	112	34	51
ADL	130	155	21.0	18.9	24.3	36.6	3.40	6.25
Soluble fibre	n. a.	n. a.	312	271	n. a.	n. a.	n. a.	n. a.
Total polyphenols	17.1	14.9	3.59	3.27	n. a.	n. a.	n. a.	n. a.
Total sugars	104	95.0	274	101	n. a.	n. a.	n. a.	n. a.
Gross energy (MJ)	23.3	22.8	17.4	17.4	n. a.	n. a.	n. a.	n. a.

n. a.: not analysed
OC: olive cake; OP: orange pulp
[a]Neutral detergent insoluble crude protein
[b]Acid detergent insoluble crude protein

The agri-food industry by-products were evaluated for their inclusion in pig diets, whereas the agricultural by-products were used in anaerobic co-digestion with pig slurry. The final aim was to overcome the current challenges that pig sector has to face, related to nutrient and energy recycling. This is discussed and compared in this first section of the general discussion. In general, the nutritional composition of the different by-products strongly depends on their origin. The two OC used in our studies differ in the management system carried out after the extraction of the olive oil. The system mostly used in Spain for olive oil production generates OC as a by-product that is stored in ponds until further processing. Crude OC (COC) is obtained when the by-product from the ponds is dehydrated without any further treatment and partially defatted OC (PDOC) is obtained when OC in the ponds is partially extracted with solvents before the dehydration process. Both, COC and PDOC used in our studies show high EE (ranging from 125 to 174 g/kg DM) and variable sugar (95-104 g/kg DM) contents as a result of the time of storage. In terms of fibre, as expected, both OC are fibrous by-products with a relatively high FND, FAD and lignin content (40%, 24-28% and 13- 16% on DM basis, respectively). Compared with other fibrous feedstuffs such as rapeseed meal, barley or sunflower meal their fibre and, particularly, ADL content is high (INRA 2004; FEDNA, 2019). The analysed fatty acid (FA) profile of OC (data shown in chapter 3) revealed that oleic acid was the main FA, followed by palmitic and linoleic acids. It is known that the sugar and fat content can be very variable among dried OC sources (Abo Omar et al., 2012). While the variability observed in terms of fat content among OC sources is mainly related to the olive oil extraction system, the variability in sugar content of OC is attributed to the time of OC storage before being dried, due to the microbial fermentation that takes place during its storage (De Blas et al., 2015). Due to their different oil extraction processes and by-product management, the COC used in our studies showed higher EE and sugar contents than PDOC.

Likewise, OP has a seasonal production and a perishable nature. Therefore, wet OP requires the application of a conservation method (ensiling or dehydrating) in order to increase its usefulness. For dehydrating, wet OP is usually pressed after adding lime to reduce humidity, and either artificially dehydrated by fuel-drying in conventional rotatory dryers or naturally dried in the open air by solar heat. When citrus pulp is dried in the open air, it is previously stored during a variable period in ponds where, depending on the time of storage, they can

become in an ensiled product. In the present PhD thesis, conventional fuel-dehydrated (DOP) and ensiled sun dried (ESDOP) were tested. The OP is characterised by its high content in sugar (19% DM) and fibre fractions. Regarding the different fractions of fibre partitioning, OP presents a high content in soluble fibre (averaging 29% DM) but also in ADF (16 % DM) and a low content of ADL (2% DM), compared to other fibrous by-products. On the other hand, taking into account the type of OP, the DOP used in our studies shows a higher content in sugars than ESDOP, probably due to the ensiling process in the second, which implies the fermentation of sugars and its conversion into VFA, with a relevant production of lactic acid (Megías et al., 1993). During the DOP dehydratation process with trommel, lime is added and citrus pulp is pelletized, adding citrus molasses which are produced by concentration of the water and sugars recovered from the dehydration. To obtain ESDOP, the fresh orange pulp was stored during the winter season, where the ensiling process takes place, and afterwards extended in thin layers on the soil during the summer season in order to sundry the ensiled orange pulp. These differences in the management process lead to changes in OP composition with respect to sugars, ash and fibre fractions.

On the other hand, the agricultural by-products studied in this PhD Thesis are non-marketable pieces that are collected from the primary production fields and used as unprocessed residue which leads to by-products with lower DM content than the agri-food by-products that has been previously dehydrated and described. According to the chemical composition of the agricultural by-products studied in this PhD Thesis, two groups of by-products can be distinguished; vegetables substrates, including tomato and pepper, and fruit substrates, including kaki and peach. Vegetable substrates showed higher content in fibrous fractions, especially lignin, than fruit substrates. This could be attributed to the proportion of seeds, peel and stems with respect to the pulp, which are higher in vegetables by-products than in fruits. The higher content in pulp attributable to fruits could imply higher content in non-structural carbohydrates, like sugars, and could explain their lower content in protein and lipids.

Knowing the chemical composition of agro-industrial by-products is essential when considering their use in animal production, since it can be crucial when deciding its final use. However, there are other important limitations that have to be taken into account when they are used either in animal nutrition or slurry co-digestion such as the heterogeneity of

these products, the seasonality, their needs for conservation, and the limited connection between suppliers and consumers of these by-products.

Regarding the heterogeneity in agro-industrial by-products, this is mainly due to the heterogeneity of the original fruits and vegetables, which then will be processed. In most cases, this lack of uniformity implies that it is difficult to implement a common management sustainable strategy of the use of these by-products (Waldron et al., 2003; Waldron, 2004). Heterogeneity is an intrinsic characteristic of by-products. Fruits and vegetables include different tissues with variable composition: storage tissues are normally rich in starch, protein or oil, while structural tissues tend to be more fibrous and lignified. This composition is affected by plant variety and maturity. Also, post-harvest treatments and further industrial processing lead to very variable composition in practice. In summary, this lack of uniformity is the main obstacle to implement a generalised management strategy of by-products.

In addition, most of these by-products are subjected to seasonal availability, determined by the type of crops and agricultural processing plants, and have a high moisture content that leads to product perishable (Kasapidou et al., 2015). Indeed, such material constitutes a breeding ground for fungi and bacteria that cause OM degradation and potential contamination, affecting to its potential as feedstuffs for animal feed. It happens when the by-products are kept during few hours at ambient temperature, requiring a stabilization and preservation trough processes such as silage, drying, refrigeration or freezing.

This PhD Thesis provides deeper knowledge about the use of the most common Mediterranean by-products identified to potentially be used for pig nutrition and anaerobic treatment of slurries, according to these criteria.

8.2 Valorisation of agro-industrial by-products in pig production

As it has been stated before, the agro-industrial by-products offer different alternatives of use and valorisation due to their composition, possibility of stabilization and storage and availability. Such alternatives range from recycling them into human or animal feeding to their utilisation in the renewable energy sector to obtain biofuel or in anaerobic co-digestion.

In the recent years, the EU has established the food waste hierarchy with the preferable disposal technologies in order to aid with the selection of the adequate food disposal option. These alternatives consist in, from the most to the least preferable, (i) reduce food waste, (ii) redistribute it, (iii) recycle it as animal feeding and (iv) compost, (v) recover energy through anaerobic digestion, and finally, (vi) landfill the remainder (Salemdeeb et al., 2017). The "Farm to Fork Strategy" of the EU also identifies that using by-products from the bio-economy reduces the dependency of critical feed materials.

8.2.1 Uses as feed ingredients in pig nutrition

The OC and OP evaluated in this PhD thesis as feed ingredients are two Mediterranean agro-industrial by-products which, due to their characteristics and availability, can be used in pig diets. However, these have not been tested in-depth as feed ingredients for pigs in the case of OC, whereas the information about OP characterization is variable as regards of their origin or method of being processed. Therefore, before using these by-products in pig nutrition, it is necessary to assess their variability, nutritional value, and its effects on performance and end product's characteristics (Zijlstra and Beltranena, 2013). Due to their composition (high levels of fibre, moderate levels of fat and polyphenol content), these by-products would initially be more adequate in diets for fattening pigs and pregnant sows, in which they can exert significant effects on feed consumption, intestinal microbiota and animal welfare. In the case of OC, its high oleic acid content can also positively affect meat quality, increasing the percentage of this fatty acid in the carcass (Ferrer et al., 2020).

From an integrative point of view, in order to evaluate the suitability of these by-products to be included in pig diets, it is also important to determine the environmental impact associated to its use in diets. On the one hand, their inclusion in livestock diets may have a lower associated environmental burden due to its condition of by-product (van Zanten et al., 2014). However, its composition (high fibre) might affect diet digestibility, slurry composition and thus the associated potential emissions during its storage. These issues must be taken into account when exploring the inclusion of agro-industrial by-products in pig production.

The studies conducted in the present PhD Thesis regarding the valorisation of OC and OP included all these aspects: product variability,

in vivo nutritional value, effects on performance and final product quality, and also the associated environmental impact when used in practical feeds for pigs. From the results of these studies, we identified several potentialities but still some limitations for the use of these by-products in pigs' feeding.

In the case of OC, three different dry by-products were identified (COC, PDOP and defatted OC) depending on its crude fat content (FEDNA, 2019; Marcos et al., 2019). *In vivo* nutritional value trials also demonstrated differences in energy digestibility, being this higher in COC (0.479 ± 0.040) compared with PDOC (0.327 ± 0.049), due to the differences found in ether extract and sugar contents between these two sources of OC. In all, although their fat content can be relatively high (up to 13-17%), the DE of these by-products is still low in pigs (2675 kcal/kg DM for COC and 1767 kcal/kg DM for PDOC), compared with other ingredients commonly used as sources of energy and fibre (such as sugarbeet pulp). This is due to its high insoluble fibre and lignin content. However, this can be a favourable quality for heavy pigs (such as Iberian pigs) or sows in order to modulate feed consumption during the finishing phase and in terms of animal welfare.

In the case of OP, we identified important differences in composition depending on the dehydration process. Particularly, lime addition and dehydration method, affect the composition of dried citrus pulp, particularly the sugar and calcium contents (de Blas et al., 2018). In terms of energy, the results from the *in vivo* nutritional assay showed a slightly higher energy digestibility for DOP with respect to ESDOP (0.804 ±0.0089 and 0.748 ±0.023 respectively), although the nutritional value of both sources of OP is acceptable, achieving DE values of 3392 kcal/kg DM for DOP and 3129 kcal/kg DM for ESDOP.

Despite both OC and OP are considered fibrous agro-industrial by-products, their nutrient composition and nutritional value can be very different, and therefore the consequences of its use in pig diets on nutrient digestibility, growth performance, final product quality and effluents' amount and composition and derived gas emission also differed. As an example, in terms of energy digestibility, the inclusion of OC decreases but OP does not affect energy digestibility of diets when included at moderate levels (10-15%) (Beccaccia et al., 2015a; Ferrer et al. 2021). In terms of acceptability by the animals and growth performance, the maximum inclusion levels assayed for OC and OP in this

PhD thesis, 12% and 24% respectively, did not cause feed refusal by the animals or impair growth traits during the finishing phase. However, because of the tendency observed in the results of both studies, we believe that it is probable that higher inclusion levels of the tested by-products can negatively affect voluntary feed intake of animals and, therefore, performance. This effect on intake may be important when using fibrous by-products, especially in summer season, but it is not always negative. As previously described for OC, limiting feed intake with fibre in pregnant sows and heavy pigs such as Iberian pigs at the end of their growing period has benefits on welfare and growing rates to achieve commercial standards (Watanabe et al., 2010; Matías et al., 2018)).

Regarding the effects of including these by-products on *in vivo* final back fat (BF) and loin depth (LD) measured at the P2 position and meat quality traits, a similar effect was observed with the addition of both by-products. Pigs fed OC showed about 2 mm lower LD ($P < 0.05$) with respect to control diet, and pigs fed OP diets tended ($P<0.10$) to decrease BF thickness and LD depth at the end of the fattening period, as the inclusion level of DOP increases. In the light of the results obtained in this PhD Thesis, the addition of fibrous by-products to the diet can be of interest in the finishing period and in qualitative feed restriction programs in order to obtain carcasses with lower fat deposition due to a dilution of the energy in the diet (Watanabe et al., 2010). Additionally, the inclusion of OC increased the oleic acid content in the subcutaneous fat of the animals, which can be used as a mean to develop functional meat products, while the differences in the FA profile with the addition of OP are unknown. Probably, changes in the proportion of other ingredients in the diet when including OP are affecting the FA profile of the diet and thus can affect the FA profile of the subcutaneous fat.

Finally, regarding the environmental impact of the use of these by-products in terms of slurry production and derived gas emission, the results obtained in the nutritional assays (chapters 3 and 5) showed a decrease in the potential NH_3 emission (NH_3, g/kg of slurry) with OC and OP inclusion (by 28% and 58%, respectively). Although the by-products tested have different fibre profile, the effects observed in the potential NH_3 emission are similar due to two main reasons. Firstly a decrease of the total urinary N content and a decrease in the urine:faecal N, and secondly an increase in the benzoic and hippuric acid content in urine, associated to lower urine pH, when OC and OP are included in the diets. A decrease in the urine:faecal N has been traditionally associated with

sources of fermentable fibre in diets such a in OP diets (Canh et al., 1998; Portejoie et al., 2004; Jarret et al., 2012). However, Beccaccia et al. (2015a) already suggested that ingredients rich in insoluble fibre could also reduce this N excretion in urine and therefore NH_3 emission. The reduction in the digestibility of nutrients, especially protein, when including high proportions of ingredients with less digestible fibre like in the case of OC increases the presence of N in faeces by reducing N in urine and, therefore the urine:faecal N. Furthermore, OC and OP are rich in phenolic and other bioactive compounds such as triterpene substances, essential oils and carotenoids (Bermejo et al., 2011; Romero et al., 2017) which can contribute to explain these reductions in NH_3 when OC and OP are included in pig diets. In all, the results obtained from the *in vitro* NH_3 emissions assay need to be confirmed with additional studies with isoproteic diets since we cannot hypothesize the effect of the inclusion of these by-products in such conditions.

In the case of CH_4, results from the *in vitro* assays in our studies showed the same pattern that in the NH_3 assays. It is remarkable that the inclusion of both by-products decrease the potential CH_4 (mL/g of OM) emission from the slurry as a result of higher fibre faecal content in animals fed OC and OP compared with the control diet. Methane production from slurry involves a biochemical process with multiple stages and many different microorganisms. In this process, complex OM contained in the slurry is hydrolysed, fermented in the intermediate stages of the process, and eventually reduced to CH_4 and carbon dioxide (Kim et al., 2012). The CH_4 final yield will depend mainly on the amount and composition of OM. The inclusion of OC and OP by-products in pig diets from the *in vitro* assays led to the excretion of faeces with higher ADL and lower EE as a result of diet and nutrients apparent total tract digestibility coefficients modifications. Thus, lower BMP were obtained with the slurries from the animals fed these by-products. Previous studies (Angelidaki et al., 2009; Triolo et al., 2011 and Beccaccia et al., 2015a, 2015b) have shown that ADL concentration in OM is negatively whereas EE is positively correlated with BMP. Furthermore, polyphenols contained in OC and OP have been associated with antimicrobial effects (Daglia, 2012), and several works also report that they reduce CH_4 production in anaerobic digestion plants (Milanesea et al., 2014). However, when the CH_4 emission are calculated as potential emission expressed per animal and day, two different results are observed with the by-product tested with regards of the effect of fibre on faeces bulk density and the volume of the slurry excreted. While in the

case of OC, CH_4 emissions increased with dietary OC inclusion as a result of the increase in the volume of slurry excreted due to the lignified by-product, no effect was observed with the inclusion of OP when expressed as L CH_4/animal and day. The OP is rich in SF that did not affect the volume of the slurry excreted among treatments since this nutrient is more digestible than the ADL present in OC. Consequently, pigs fed OP diets showed a similar BMP compared to control pigs, when expressed in L CH_4/animal and day.

With respect to the gaseous emissions from the pig slurry storage assays (chapters 4 and 6), the results obtained did not show the same pattern as the *in vitro* assays. The inclusion of OC and OP in commercial diets did not affect the slurry composition, only the ADF fraction of pig slurry from OC diets increased. Thus no differences in gaseous emissions from the slurry during its storage along two months were observed, when the emissions were expressed per animal and day.

Such differences between the *in vitro* (chapters 3 and 5) and the storage assays (chapters 4 and 6) can be explained by the differences in the diets designed for *in vitro* and storage assays and the variability observed in slurry production when animals are housed in commercial conditions (conventional pens). In the *in vitro* nutritional assays, the diets were designed to estimate the DE of the by-products tested by the regression or substitution method, usually conducted when testing fibrous by-products (Piano et al., 2012; Bolarinwa and Adeola, 2012; Woyengo et al., 2014). Under this methodology, the digestibility values from the different mixtures fed are regressed against the proportion of feedstuff included in the diets. As a result, the experimental diets are unbalanced in terms of nutrients content which in turns lead to significant differences in slurry compositions. However, in performance assays, diets are designed to be isonutritive and the inclusion of the by-product modifies the proportion of the other feedstuffs of the diet, condition that could reduce the potential effect of the by-product inclusion in the slurry composition. Additionally, housing the animals in commercial conditions lead to greater variability in animal behaviour causing high variability in slurry composition and contributing to the difficulty of detecting significant differences. As it has been stated before, the emissions from slurry storage are mainly affected by the slurry composition and environmental conditions (Canh et al., 1998; Moset et al., 2012; Snoek et al., 2014). In the storage assays from this PhD Thesis, the environmental conditions (temperature, air speed and time of storage) were the same for all the

treatments tested so differences in gaseous emissions were only attributable to the effect of slurry composition. The results from the chemical analyses of the slurry did not show differences between treatments and thus gaseous emissions from pig slurry storage were similar for OC and OP compared to the control diets.

Overall, some practical implications can be drawn from the results of the present PhD Thesis with respect to the use of OC and OP in pig diets during the finishing period. Taking into account the maximum inclusion levels studied, the OC and OP can be included in finishing pig diets replacing barley up to 12% and 24% of total inclusion level, respectively. This different inclusion level is caused by their different characteristics in terms of nutrient content, palatability and acceptability by the animals.

In terms of economic impact in diets, we have estimated the cost of including the by-products taking into account the average feed compound costs for pigs in Spain in 2019 (252.44 €/Tm) published by the MAPA (2020). In the case of PDOC diets, the substitution of 12% of barley from the control diet by the by-product in the experimental diet from chapter 4 also lead to an increase in the percentage of soybean meal and fat, and a decrease in the amount of sunflower meal in order to be isonutritives. Assuming an average cost in 2019 of 184.9, 250, 323.6, 150 and 575.2 €/Tm for barley, sunflower meal, soybean meal, PDOC and fat, respectively, the inclusion of PDOC in commercial diets lead to an increase in feed costs of 3.6 €/Tm, which accounts for a 1.4% increase. However, such increase in feed costs can be balanced with the potential positive effects on pig gut health and the improved sensorial characteristics that allow an increase in the final price of the carcass. With respect to the inclusion of OP in the diets, the replacement of 24% of barley by DOP in the study from chapter 6 could lead to a reduction in the price of the diet of 8.4 €/Tm, which accounts for a 3.3% decrease, assuming an average cost for barley in 2019 of 184.9 €/Tm and an average cost for DOP of 150 €/Tm. Such results indicate that from the economic point of view, these by-products modified the cost of the diet without negatively affecting the performance, carcass quality and the environmental impact of slurries in terms of slurry excretion and associated gaseous emissions

In all, the inclusion of fibrous by-products such as OC and OP from the olive oil and juice industry represents an opportunity to value wastes from the food industries contributing to the economic, social, and environmental sustainability of the animal feeding sector. Currently, the

cost of these by-products is high due to the dehydration process, which is one of the main challenges to be overcome in order to be competitive and requires further research and technological development.

This PhD Thesis demonstrates that using OC and OP in pig diets at an adequate inclusion level, does not have relevant negative effects on performance traits and favours the circular economy strategy, with potential positive effects on meat quality and associated environmental benefits, not only in terms of gaseous emissions. These benefits have not been quantified in the studies from the PhD Thesis but need to be undertaken in future projects through life cycle analyses (LCA). The application of LCA to pig production system reveal that the use of by-products can lead to environmental benefits as it has been reported by reported by Ali et al. (2017) with the inclusion of macaúba kernel cake, rapeseed meal (Van Zanten et al., 2015) or waste-fed larvae (van Zanten et al., 2018) in pig diets. As a result, the dependence of the feed industry from cereals and soybean can be partially alleviated by the use of these by-products, reducing the competition for cropland between the feed and food sectors and thus the reduction of land occupation, water consumption, use of fertilizers and the fossil energy demanded for the cultivation of crops which in turns affects to the acidification and eutrophication of the ecosystems and to the climate change.

8.2.2 Anaerobic co-digestion of agricultural substrates

Traditionally, the anaerobic digestion of organic wastes have been associated with the treatment of sewage sludge from aerobic wastewater treatment and animal manure (Esposito et al., 2012). However, due to the environmental, public health and economic concerns about waste disposal and the limitations of anaerobic digestion for the treatment of one-single substrate, the application of anaerobic digestion shifted over the years from municipal wastewater to liquid (mainly industrial) wastewater, then to the municipal solid waste and agricultural residues (Jimenez et al. 2015). Thus a wide range of substrates have been used for biogas production that include pig slurry, food industry by-products, energy crops and harvesting residues (Mirabella et al., 2013).

In this PhD Thesis four agricultural by-products (tomato, pepper, kaki and peach) have been co-digested with pig slurry as an alternative treatment to direct use as animal feed, in order to obtain added value products and to contribute to the economic, social and environmental sustainability of the pig sector. In the following lines, the effects of the type of substrate

and levels of inclusion in co-digestion with pig slurry on the CH_4 potential are discussed and compared with BMP values obtained in the nutritional assays.

8.2.2.1 Substrates composition for anaerobic digestion

Commonly used substrates for feeding anaerobic digesters include different organic material that range from animal slurries, agro-industrial by-products, organic wastes from the meat, dairy and fish industries, food waste or energy crops. The biogas yield of the individual substrates varies considerably dependent on the feedstock origin, OM content and substrate composition (Lohri et al., 2017). Regarding substrates composition, the theoretical biogas yields depends on the relative amounts of lipids, proteins, carbohydrates and cellulose present in the substrate (Neves et al., 2008), being the lipids the nutrient with the highest potential, followed by proteins and carbohydrates (Angelidaki and Sanders, 2004). Among carbohydrates, cellulose and hemicelluloses are more difficult to degrade than sugar monomers. In this context, Gunaseelan (2009) developed a regression model to predict BMP from individual OM components in which a strong positive relationship between BMP and total carbohydrates, protein and lipids was found (R^2=0.94).

Lipids are present in food wastes and in some industrial wastewaters, such as those produced by slaughterhouses, dairies or fat refineries (Li et al. 2002), whereas carbohydrates are the main components of agro-industrial by-products and food waste. Whit respect to protein rich substrates, they are mainly produced in livestock farms (slurries and manure) and, in the slaughterhouses and meat processing industries. The BMP of some of these substrates vary between the 492 $mLCH_4$ g VS added^{-1} for the sludge from olive oil mill, the 442 $mLCH_4$ g VS added^{-1} for the vegetable wastes, the 334 $mLCH_4$ g VS added^{-1} for the molasses and the 299 $mLCH_4$ g VS added^{-1} for the pig slurry according to the study published by Schievano et al. (2009). The data reported by these authors confirm that substrates with high lipid content (such as olive oil mill) have the highest potentials compared to substrates with lower VS content such as pig slurry, characterised by its slowly biodegradable carbohydrates and N content.

The results from the anaerobic co-digestion study of pig slurry and agricultural substrates of this PhD Thesis showed differences in average BMP with respect to the type of substrate that could be explained by the

substrate composition. The combinations containing kaki showed the lowest BMP values (241.8±18.52 $mLCH_4$ g VS $added^{-1}$), whereas combinations containing pepper showed the highest BMP (279.8±42.26 $mLCH_4$ g VS $added^{-1}$). Considering the type of substrate, the co-digestion of pig slurry with vegetable substrates (tomato and pepper) showed higher BMP compared with fruit substrates (kaki and peach). The chemical characterization from the agricultural substrates studied in the present PhD Thesis showed that pepper and tomato contain the highest lipid and protein and lower carbohydrates proportion compared to fruit substrates. Consequently, the co-digestion of pig slurry with pepper and tomato presented the highest BMP whereas combinations with kaki showed the lowest BMP.

Compared with co-digestion assays, the one-single substrate (pig slurry) BMP assays performed in the *in vitro* studies of this PhD Thesis (chapters 3 and 5) with fresh reconstituted slurries varied between 266 and 396 $mLCH_4$ g VS $added^{-1}$. Overall, higher BMP values were obtained in the one-single substrates (chapters 3 and 5) than in the anaerobic co-digestion studies (chapter 7), although the addition of a co-substrate in the anaerobic co-digestion assays enhanced the BMP production with respect to the reference single pig slurry anaerobic digestion potential. The slurry from the co-digestion BMP assays included in chapter 7 of this PhD Thesis came from gestating sows whereas in the one-single substrate assays from chapters 3 and 5 came from growing pigs, thus differences in pig slurry BMP from the co-digestion and the *in vitro* studies could be explained by animal categories as it has been reported by Antezana et al. (2016), who observed that slurries from sows present more lignin and less lipid content than those from growing pigs. Moreover, those results are in accordance with Møller et al. (2004), who reported an average BMP of 275 and 356 $mLCH_4$ g VS $added^{-1}$ from faeces taken directly after excretion from sows and growing pigs respectively, assuming that the BMP of slurry comes mainly from the OM in the faeces.

It is important to highlight that the by-product inclusion in pig diets lead to slurries with higher fibre content and lower BMP values compared to the slurries from the animal fed the control diet. Therefore, two different patterns can be observed with the utilisation of the by-products studied in this PhD Thesis. While in the case of the anaerobic co-digestion assay, the addition of the by-product led to higher BMP values as a result of the complementarity of the mixtures and the OM composition of the by-product, in the *in vitro* assays lower BMP potentials were obtained with

the inclusion of the by-product in the diet due to modifications in the animal faeces composition.

8.2.2.2 Inclusion level of substrates in the co-digestion assay

The anaerobic co-digestion of different substrates can improve the process producing a higher CH_4 yield than is obtained from individual treatment of substrates due to significant synergistic interactions (Pagés-Díaz et al., 2014). This improvement can be explained by the adaptation of the co-substrate mixture to the optimum characteristics for anaerobic digestion as regards: macro and micronutrients, C:N ratio, pH, inhibitors/toxic compounds, biodegradable OM and DM (Murto et al., 2004; Hartmann et al., 2005; Mata-Alvarez et al., 2014). This fact has been confirmed in several studies with the co-digestion of manures with high buffering capacity, and carbon rich substrates such as the agricultural by-products in Conventional Stirred Tank Reactors or similar configurations (Callaghan, 2002; Murto et al., 2004; Kaparaju and Rintala, 2005; Ferreira et al., 2008).

Nonetheless, the inclusion level of the substrates in anaerobic co-digestion are of key importance since an excessive loading rate of one of the substrates can lead to organic overloading or exposure to toxic compounds resulting in the digester acidification that may induce to process failure (Murto et al., 2004). The results from the study conducted in this PhD Thesis confirm the improvement in BMP with the inclusion of the substrates assayed with the exception of kaki, which did not show differences in CH_4 yield between levels 1 and 2 (15 and 30% of VS from kaki respectively).

Tomato, pepper and peach substrates co-digested with pig slurry showed statistical significant differences with respect to reference BMP (pig slurry) at inclusion levels 2 and 3 (30 and 50% of VS from agricultural substrate respectively), however kaki substrate only showed differences with reference BMP at inclusion level 3. Combinations with tomato, pepper and peach at inclusion level 3 resulted in an increase in BMP of 41% with tomato, 44% with pepper, and 28% with peach and 12% with kaki with respect to pig slurry only.

Similar results were reported by Campos (2001), who obtained enhanced BMP values with the inclusion of 12.5 and 20%, on weight basis, of pear waste and pig slurry co-digested at mesophilic range for 80 days, compared with inclusions of 5% on weight basis. Likewise, Riaño et al.

(2011) and Molinuevo et al. (2010) reported better digestion performance with the co-digestion of pig manure and agricultural by-products. Molinuevo et al. (2010) improved average BMP by 49% with the inclusion levels from 15% to 85% on fresh weight of maize, carrots, peas and leaks in a mixture with pig slurry. In the same way, Riaño et al. (2011) obtained an increase in BMP of 27% using the same inclusion levels as Molinuevo et al. (2010), but in co-digestion of pig slurry with winery wastewater.

In all, agricultural substrates are suitable co-substrates for pig slurry anaerobic co-digestion since they are carbon-rich substrates, with poor buffer capacity, opposite to pig slurry, with high buffer capacity and low C:N ratio. Therefore, the mixture of both substrates allow to overcome the limitations of single digestion of manures due to ammonia concentrations, maintaining a stable pH, within the methanogens range (Mata-Alvarez et al., 2014). Additionally, from an environmental point of view anaerobic co-digestion of pig slurry and agricultural by-products have many environmental benefits such as the decrease in atmospheric CH_4 and NH_3 emissions from pig slurry storage, the production of a renewable energy, the possibility of nutrient recycling and the stabilization and mineralization of the OM from substrates.

8.3 The role of fibrous agro-industrial by-products in the future

In view of the results obtained in this PhD thesis, the use of agro-industrial by-products in animal feeding and anaerobic co-digestion could be an interesting alternative for their disposal. Some recent studies support the hypothesis that the use of fibrous agro-industrial by-products not only is possible, but also can lead to potential benefits on pig production and the environment. However, there are still some challenges that need to be face with respect to:

- Pre-treatment technologies: enforce innovative solutions based on the drying technologies applied to agricultural substrates in order to easily manage these by-products in a cost-effective way and improve their nutritional value.
- Effect of fibre on microbiome and metabolome: explore the prebiotic effect of fibre and bioactive compounds on gut health and metabolism stimulating the growth of commensal gut bacteria and preventing the colonization of opportunist pathogens, as a result of its fermentation (Lindberg, 2014; Jha and Berrocoso, 2016).

- Environmental impact: undertake studies based on LCA in order to assess the overall effect of the use of agricultural by-products in the pig sector and to deepen in the understanding of anaerobic co-digestion of pig slurry and other by-products.

Regarding the pre-treatment technologies, as it has been stated previously in this chapter, the agro-industrial by-products are generally high in moisture content (<20% DM) and seasonal in most cases, which derive in practical and environmental limitations (concentration of production in short periods of time). Dehydration is necessary to allow its use in pig feeding. Conventional dehydration methods (trommel) have high associated costs which derive in less cost-effective by-products (such as the case of OC in the present PhD Thesis). Nowadays, new technologies are developing to dry the by-products in a sustainable and cost-effective manner, maintaining or increasing their nutritional value. As an example can be cited the superheated steam (SHS) or the solar tunnel and greenhouse type dryers. These technologies have been proved to be an effective method for drying sugar beet pulp and spent cereal grains in the case of SHS (Pronyck et al., 2004, Tang et al., 2000, 2004), or fruits and vegetable foods with the solar tunnel and greenhouse type dryers (El-Sebaii and Malaby, 2012; Kumar et al., 2016; Patil and Gawande, 2016). The SHS treatment produces a physicochemical effect due to the high temperature achieved (150ºC) that helps to break cell wall structures and releases phenolic compounds, fermentable simple sugars, oligo and polysaccharides (Lama-Muñoz et al., 2014), increasing the nutrient availability in the treated materials. In the tunnel dryer system, a solar collector and a drying tunnel are connected in parallel and forced air is circulated over the product in order to dry the product at moderated temperatures (never exceed 60ºC), thus preventing harmful reactions between sugars and protein, with low energy requirements.

In terms of the impact of fibrous by-products on gut ecology and metabolism, fibre has recently received a considerable amount of attention because some of its components have recognized beneficial effects on gut health in pigs (Lindberg, 2014; Celi et al., 2017), therefore increasing the interest of fibrous by-products in pig diets. Some studies report that not only microbiome but also metabolome can be affected by fibre. A preliminary study conducted by our research group in the framework of the project in which this PhD Thesis has been developed (AGL2014-56653) revealed that the inclusion of high levels of OP in pig diets has relevant effects on faecal metabolites and microbiome. The

results from the faecal metabolome multivariate analysis after seven weeks of the administration of OP diet showed that the main discriminating variables between treatments are the metabolites associated with bile salts and phenolic metabolites (Ferrer et al., 2019). A large part of the flavonoids in the diet are not absorbed in the small intestine and reach the colon, where they exert their modulating effect on the microbiome (Dueñas et al., 2015). The polyphenols identified in the metabolism of pigs fed the OP diet are found in the citrus peel and therefore their presence would be directly attributable to the OP of diet. Regarding the results of the faecal microbiome, significant differences were also observed in the bacterial genera, highlighting the reduction of *Clostridium* and the increase of *Lactobacillus* with the inclusion of OP in the diet (Ferrer et al., 2019). These changes in the faecal microbiome are associated with an increase in the production of VFA and a reduction in the production of NH_3 in the intestine of pigs, which in turn favours the growth of beneficial bacteria such as lactobacilli (Jha and Leterme, 2012; Pieper et al., 2008).

The modulating effect of OP on gut microbiome and metabolome can be linked with animal health benefits that are attributed to dietary fibre. The development and availability of high-throughput techniques like 16S sequencing (phylogenetic composition), metagenomics (functional capability), metatranscriptomics (functional intent) and metabolomics (metabolic impact) are providing a more comprehensive understanding of the changes in the composition of the intestinal microbiota and their secreted metabolites (Celi et al., 2017). The combination of these new technologies will provide valuable information on the gastro-intestinal tract microbial networks and on their metabolic activities related with animal feeding, which is imperative to address the mechanisms linking animal nutrition (fibre) and health in farm animals (Agyekum and Nyachoti, 2017).

Finally, regarding to the environmental impact, it is important to notice that nowadays the sustainability of the pig sector relies on its capability to respond to the increasing demands for livestock products that are arising from population growth, adapting to changes in the economic and policy contexts, and improving its environmental performance through the mitigation of its impact on climate. In this framework, the use of agro-industrial by-products has been demonstrated to be effectively used in the pig sector, especially taking into account the farm to fork strategy, the food waste hierarchy and the changes in the economic model promoted

by governments favouring the circular economy strategy. Nonetheless, from the results obtained in this PhD Thesis we can conclude that is necessary to evaluate the use of agro-industrial by-products from an integrated perspective, requiring the development of multidisciplinary knowledge and innovative technology in the various fields mentioned above. As an example, the LCA offers the most complete and widely used methodologies for assessing the environmental impact of products and processes, identifying the areas or processes that can be improved integrating the research and development achievements that can contribute to significantly reduce the environmental impact of the pig sector. An LCA of pig production takes into account three modules from the production system relating to the life-cycle stages: i) feed production, including processing, milling and storage; ii) animal production, including breeding; and iii) primary processing. As a result, the different approaches identified for the use of agro-industrial by-products in this PhD Thesis (as feed or as substrate for anaerobic co-digestion) can be evaluated and assessed in terms of their environmental impact aiding in the decision of the most-preferable solution for its disposal.

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Chapter 9

Conclusions

Under the experimental conditions of this PhD Thesis, the following conclusions can be drawn:

1. Olive cakes are appreciable sources of insoluble fibre, but they have limited energy value and a low value as protein source for growing pigs. Their energy value, however, depends on its fat content. The estimated values for DE were 11.2 and 7.4 MJ/DM for the crude olive cake and the partially defatted olive cake respectively. Thus, the use of olive cake in pig diets can be limited to those sources with higher fat content.
2. The substitution of a basal diet with olive cake leads to modifications in N partitioning between faeces and urine, with lower N excretion in urine and increased total DM in faeces, and lower pH values from slurry with an increase of benzoic+hippuric acid contents. Associated to pig slurry modifications, NH_3 emission and BMP per kg of slurry decreases with the inclusion of OC. Nevertheless, slurry excretion increases with OC inclusion, leading to higher CH_4 emission when expressed per animal and per day.
3. Partially defatted olive cake can be included in commercial finishing pig diets at rates up to 120 g/kg without negative effects on performance, carcass quality, gut microflora and slurry gas emission, while improving the oleic acid concentration of subcutaneous fat.
4. Orange pulps are a relevant source of SF and energy for growing pigs, being the DE values obtained 14.2 and 13.2 MJ/kg DM for the dehydrated orange pulp and ensiled sun-dried orange pulp, respectively.
5. The substitution of a basal diet with OP is able to reduce the potential NH_3 emission per unit of NTK excreted, probably due to the effect of the TDF on N partitioning between faeces and urine and the lower pH; in addition, it has the benefit to decrease potential CH_4 emission expressed per unit of OM excreted.
6. Dehydrated orange pulp can be included in commercial finishing pig diets up to 240 g/kg with minor effects on growth performance, body composition, carcass quality traits, microbial counts nor faecal metabolites. Also, this level of OP inclusion did not affect excreta volume, composition and global gas emission from the slurry.

7. The anaerobic co-digestion of agricultural by-products and pig slurry improves the BMP from the mixture, compared to only pig slurry anaerobic digestion. Combinations with vegetables substrates (pepper and tomato) showed higher lipid and protein content, and BMP potentials than fruit substrates (kaki and peach).
8. Higher BMP values were obtained with increasing addition of agricultural substrate, confirming the better performance of co-digestion systems at adequate inclusion levels. Combinations with tomato, pepper and peach at inclusion level 3 (50% of VS) led to the highest BMP. This resulted in an increase in BMP of 41% with tomato, 44% with pepper, 28% with peach and 12% with kaki.