UNIVERSIDAD DE MURCIA

FACULTAD DE VETERINARIA

Study of the inclusion of crude glycerin from biodiesel production in pig diets

Estudio de la inclusión de la glicerina procedente de la elaboración de biodiesel en la alimentación de la especie porcina

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"Study of the inclusion of crude glycerin from biodiesel production in pig diets"

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Tesis doctoral presentada por la licenciada

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INDEX

ABBREVIATIONS

AA	Amino acids
ADF	Acid detergent fiber
ADFI	Average daily feed intake
ADG	Average daily gain
ANOVA	Analysis of variance
AOAC	Association of Official Analytical Chemists
ATP	Adenosine triphosphate
BOE	Boletín Oficial del Estado
BW	Body weight
CNS	Central nervous system
CP	Crude protein
CV	Coeficiente of variation
DE	Digestible energy
DLG	Deutsche Landwirtschafts Gesellschaft
DM	Dry matter
EBB	European Biodiesel Board
FAO	Food and Agricultural Organization of the United Nations
FAS	Foreing Agricultural Service
FDA	Food and Drug Administration
FEDNA	Fundación Española para el Desarrollo de la Nutrición Animal
FFA	Free fatty acids
G:F	Gain to feed ratio
GE	Gross energy
GLM	General linear model
IB	Iberian
IGF-1	Insulin-like growth factor 1
LM	*Longissimus dorsi* muscle
MAPA	Ministerio de Agricultura, Pesca y Alimentación
ME	Metabolic energy
MJ	Milijoules
NDF	Neutral detergent fiber
NE	Net energy
NE_L	Net energy for lactation
NRC	National Research Council

OM	Organic matter
PDI	Pellet durability index
SAS	Statistical analysis software
SEM	Standard error of mean
TCA	Tricarboxylic acid
USDA	United States Departament of Agriculture

LIST OF TABLES

LITERATURE REVIEW

CHAPTER 1

CHAPTER 2

CHAPTER 3

LIST OF FIGURES

LITERATURE REVIEW

SUMMARY

The high demand for biodiesel has resulted in an increased availability of its main byproduct, crude glycerin, at a competitive price. Crude glycerin produced in biodiesel plants contains variable amounts of glycerol and impurities. The low purity of crude glycerin limits its use in the food, pharmaceutical and cosmetic industries. Since process to purify glycerin is very expensive, research activities have focused on new uses for crude glycerin. During recent years, there has been much interest in using crude glycerin as a feed ingredient for livestock to reduce feeding costs. Moreover, several studies have reported that crude glycerin can be effectively used as an energy source by pigs, poultry and ruminants.

Feeding crude glycerin to pigs has yielded variable results. Some studies found improvements in performance or carcass characteristics, while others reported no significant effects as a result of feeding glycerin at moderate levels (5-10%). Differences in the composition and supplementation level of crude glycerine may contribute to the variability of the results, as will the age, sex, genetics or physiological condition of pigs. A better understanding of glycerin metabolism, as well as of its effect on dietary digestibility and its potential relation with metabolic and feed intake-regulating hormones, will help explain the differences observed in previous results and provide valuable insights for optimizing feeding levels.

The general aim of this PhD Thesis was to evaluate the inclusion of crude glycerin from biodiesel manufacturing process in swine diets during the different phases of pig production. To achieve this objective, three experimental chapters were designed.

In **chapter one**, we studied the effects of dietary addition of crude glycerin on pellet production efficiency and growth performance and digestibility in growing-finishing pigs. Three dietary treatments were created by addition of 0, 2.5 or 5% crude glycerin to barley-soybean meal-based diets.

In the manufacturing process, crude glycerin supplementation linearly increased the feeder speed and production rate ($P < 0.05$), resulting in a 20 to 29% improvement in the feed production rate compared with the control. Production efficiency (kg/kWh) increased linearly ($P < 0.05$) as the level of crude glycerin in feed increased, reducing energy costs.

A performance trial was conducted with 240 barrows (30 ± 1.0 kg initial BW) using a 2-phase feeding program over a 12-wk period. On the last day of the growth experiment, blood samples were collected to determine circulating glucose, fructosamine and IGF-1 concentrations. Overall growth performance was not affected by dietary treatment ($P > 0.05$), and there was no effect of dietary treatment on any plasma metabolite measured ($P > 0.05$).

Additionally, nine male pigs were housed in metabolic cages to determine the coefficients of apparent fecal digestibility and nitrogen and mineral balances. Animals were assigned to one of the three diets in each feeding phase following a 3 × 3 Latin square arrangement of treatments (43 ± 3.1 and 74 ± 3.3 kg initial BW in the growing and finishing periods, respectively). In both feeding periods, fecal digestibility of organic matter and ether extract were affected by dietary treatment, increasing linearly ($P < 0.05$) with increasing crude glycerin levels. However, nor CP digestibility or N retention were affected by the glycerin content in either for the growing or finishing period ($P > 0.05$). Digestibilities and balance of Ca and P showed opposite tendencies with the variations in crude glycerin content, which either decreased or increased depending on the feeding phase.

In conclusion, adding crude glycerin to the diet before pelleting improved feed mill production efficiency. Up to 5%, the addition of crude glycerin to the diet of

growing-finishing pigs had no effect on growth performance, blood metabolites, nutrient digestibility and nitrogen balance.

In **chapter two**, we determined the effect of crude glycerin addition on the growth performance, nutrient digestibility and blood hormone levels of Iberian crossbred pigs kept under intensive conditions. The study was carried out with 80 crossbred pigs (Iberian gilts × Duroc boars) of both sexes over a 101-d period (54 ± 3 kg initial BW). Treatments were arranged in a 2 × 2 factorial design, the factors being dietary treatment (control or 10% glycerin) and gender (barrow or gilt). Crude glycerin was included as a replacement for wheat in diets formulated to provide equal net energy and digestible lysine levels.

Glycerin-fed pigs had higher average daily gain and average daily feed intake than pigs fed the control diet ($P < 0.05$). No differences were found in the gain to feed ratio. In regards the gender, barrows consumed more feed, grew more and reached a higher final BW compared with gilts ($P < 0.05$). Nutrient digestibility was not affected by the glycerin content or the gender. However, there was a tendency for acyl-ghrelin levels to be higher in glycerin-fed pigs ($P = 0.058$). Also, gilts showed increased concentrations of acyl-ghrelin and lower insulin compared with barrows ($P < 0.05$).

In conclusion, 100 g/kg of glycerin can partially replace wheat without affecting feed efficiency or nutrient digestibility in Iberian crossbred pigs.

In **chapter three**, an experiment was conducted to evaluate the effect of dietary addition of crude glycerin on sow and litter performance, and to determine the plasmatic levels of hormones related to energy metabolism and feed intake in sows during gestation and lactation. Sixty three sows were assigned randomly to one of three dietary

treatments, containing 0, 3 and 6% crude glycerin, added to a barley-soybean meal-based diet as a replacement for cereals.

During gestation, there was no effect of dietary treatment on any performance variable ($P > 0.05$), while during lactation, glycerin-fed sows consumed less feed than those fed the control diet ($P < 0.05$). Lactating sows fed 3% crude glycerin diet had a higher body weight loss ($P < 0.05$), but these differences were not reflected in litter performance ($P > 0.05$).

In gestation, the inclusion of glycerin did not affect blood levels of insulin or cortisol ($P > 0.05$). However, pregnant sows fed diets supplemented with glycerin showed lower and higher levels of acyl-ghrelin and leptin, respectively ($P < 0.01$). In lactating sows, there were no differences between dietary treatments for any of the hormones measured ($P > 0.05$). Before feeding, the acyl-ghrelin concentration was positively correlated with the cortisol during gestation ($r = 0.81$; $P < 0.01$) and lactation ($r = 0.61$; $P < 0.05$).

In conclusion, the inclusion of up to 6% crude glycerin in the diet can partially replace corn without affecting the performance of pregnant sows, but not during lactation. Our results also suggest a relationship between glycerin inclusion in the diet and the serum levels of some feed intake regulating hormones, but more studies are needed to increase our understanding of hormone concentration-diet composition interactions.

RESUMEN

La alta demanda de biodiesel ha generado un importante incremento en la disponibilidad de su principal subproducto, la glicerina bruta. La glicerina producida en las plantas de biodiesel contiene cantidades variables de glicerol e impurezas, por lo que requiere de un proceso de purificación y refinamiento para su uso como materia prima en la industria alimentaria, farmacéutica o cosmética. Sin embargo, el coste de purificación resulta muy elevado, lo que ha provocado el desarrollo de numerosos estudios sobre nuevas aplicaciones para la glicerina procedente de la elaboración del biodiesel. Durante los últimos años, y con el objetivo de reducir costes de alimentación, ha crecido notablemente el interés en el uso de la glicerina como materia prima para la fabricación de piensos. Así, varios estudios han demostrado que la glicerina puede ser usada eficazmente por cerdos, aves y rumiantes como fuente de energía.

El uso de glicerina en la alimentación porcina ha generado resultados variables. Algunos estudios señalan mejoras en el rendimiento productivo o en las características de la canal, mientras que otros no han obtenido efectos significativos como resultado de la inclusión de glicerina en los piensos de cerdos a unos niveles moderados (5-10%). Las diferencias en la composición y en el nivel de suplementación de la glicerina, así como en la edad, sexo, genética o condición fisiológica de los animales, podrían explicar la gran variabilidad de los resultados observados. Una mejor comprensión del metabolismo de la glicerina, así como de sus efectos sobre la digestibilidad de los nutrientes, y su potencial relación con las hormonas que regulan la ingestión de alimento durante las distintas fases de la producción porcina, ayudará a explicar las diferencias observadas en previos estudios y proporcionará una valiosa información para optimizar los niveles de inclusión de la glicerina.

El objetivo principal de esta Tesis Doctoral se centró en evaluar la inclusión de glicerina bruta procedente de la elaboración de biodiesel en los piensos de cerdos

durante las distintas fases de la producción porcina. Con el fin de alcanzar este objetivo se diseñaron tres capítulos experimentales.

En el **capítulo uno** se estudió el efecto de la adición de glicerina en la fabricación del pienso, así como su influencia sobre el rendimiento productivo y digestibilidad de los nutrientes en cerdos de crecimiento-cebo. Se formularon tres piensos con 0, 2.5 y 5% de glicerina bruta incorporada a piensos base de cebada y harina de soja.

En el proceso de fabricación, la inclusión de glicerina incrementó linealmente la velocidad de alimentación (rpm) y la tasa de producción del pienso (kg/h) ($P < 0.05$), resultando en una mejora de entre un 20 a 29% en la tasa de producción respecto al pienso control. La eficiencia de producción (kg/kWh) aumentó linealmente ($P < 0.05$) a medida que se incrementaba el nivel de glicerina en el pienso, reduciendo así los gastos de energía.

El estudio de los efectos de la glicerina sobre el rendimiento productivo se realizó con 240 machos castrados (30 ± 1.0 kg de peso inicial), usando un programa de alimentación en dos fases (crecimiento y cebo) durante 12 semanas. El último día del periodo experimental se recogieron muestras de sangre para determinar las concentraciones de glucosa, fructosamina e IGF-1. En conjunto, la inclusión de glicerina en el pienso no afectó al rendimiento productivo ($P > 0.05$), y tampoco se observaron efectos sobre ninguno de los metabolitos plasmáticos controlados ($P > 0.05$).

Además, nueve cerdos machos fueron alojados en jaulas metabólicas para determinar los coeficientes de digestibilidad fecal, así como los balances de nitrógeno y minerales. Los animales fueron asignados a uno de los tres tratamientos (0, 2.5 y 5% de glicerina bruta) en cada fase de alimentación, siguiendo un diseño cuadrado latino 3 x 3

(43 ± 3.1 y 74 ± 3.3 kg de peso inicial en el periodo de crecimiento y de cebo, respectivamente). En ambos periodos, el tratamiento afectó a la digestibilidad fecal de la materia orgánica y a la del extracto etéreo, aumentando linealmente ($P < 0.05$) al incrementarse el nivel de glicerina en el pienso. Sin embargo, ni la digestibilidad de la proteína bruta ni la retención de nitrógeno se vieron afectadas en ninguno de los periodos ($P > 0.05$). La digestibilidad y el balance de Ca y P mostraron tendencias opuestas al variar el contenido de glicerina, disminuyendo o aumentando dependiendo de la fase de alimentación.

En conclusión, la inclusión de glicerina antes de la granulación mejoró la eficiencia en la fabricación del pienso. La inclusión de hasta un 5% de glicerina bruta en el pienso de cerdos de crecimiento-cebo no tuvo efectos sobre el rendimiento productivo, metabolitos sanguíneos, digestibilidad de nutrientes y balance de nitrógeno.

En el **capítulo dos** se determinó el efecto de la inclusión de glicerina sobre el rendimiento productivo, digestibilidad de los nutrientes y nivel de hormonas sanguíneas en cerdos de cruce de ibérico criados en régimen de explotación intensivo. El estudio se llevó a cabo con 80 cerdos cruzados (hembras ibéricas x machos Duroc) de ambos sexos durante un periodo de 101 días (54 ± 3 kg de peso inicial). Se utilizó un diseño factorial 2 x 2, con los factores nivel de inclusión de glicerina en el pienso (0 o 10% de glicerina bruta) y sexo (macho o hembra). La glicerina se incluyó en sustitución de trigo en los piensos formulados para ser isoenergéticos y con los mismos niveles de lisina digestible.

Los cerdos alimentados con glicerina tuvieron mayor ganancia media diaria y consumo medio diario que los del grupo control ($P < 0.05$), sin encontrarse diferencias en el índice de conversión. En relación con el género de los animales, los machos consumieron mayor cantidad de pienso, crecieron más y alcanzaron un mayor peso final

que las hembras ($P < 0.05$). La digestibilidad de los nutrientes no se vio afectada por el contenido de glicerina ni por el sexo. Sin embargo, se observó una tendencia para la grelina acilada, siendo mayor en los cerdos alimentados con glicerina ($P = 0.058$). Asimismo, las cerdas presentaron mayores concentraciones de grelina acilada y menores de insulina, en comparación con los machos ($P < 0.05$).

En conclusión, 100 g/kg de glicerina bruta pueden reemplazar parcialmente al trigo en el pienso de cerdos de cruce de ibérico sin afectar a la eficiencia del pienso ni a la digestibilidad de los nutrientes.

En el **capítulo tres**, se evaluó el efecto de la adición de glicerina sobre los parámetros productivos de cerdas y camadas, y se determinaron las concentraciones plasmáticas de hormonas relacionadas con el metabolismo y la ingestión de pienso, en cerdas durante la gestación y la lactación. Sesenta y tres cerdas fueron asignadas aleatoriamente a uno de los tres tratamientos, que contenían 0, 3 o 6% de glicerina bruta en piensos base de cebada y harina de soja.

Durante la gestación, no hubo efecto de la inclusión de la glicerina sobre ninguna variable de rendimiento ($P < 0.05$), mientras que las cerdas alimentadas con glicerina en lactación consumieron menos pienso que las del grupo control ($P < 0.05$). Las cerdas lactantes alimentadas con el pienso que contenía 3% de glicerina tuvieron mayor pérdida de peso ($P < 0.05$), pero estas diferencias no se vieron reflejadas en el rendimiento de la camada ($P > 0.05$).

En la gestación, la inclusión de glicerina no afectó a los niveles sanguíneos de insulina o cortisol ($P > 0.05$). Sin embargo, las cerdas gestantes alimentadas con glicerina mostraron menor nivel de grelina acilada y mayor nivel de leptina ($P < 0.01$). La inclusión de glicerina en el pienso de las cerdas lactantes no afectó a los niveles de ninguna de las hormonas medidas ($P > 0.05$). Antes de la distribución del alimento, la

concentración de grelina acilada estuvo positivamente correlacionada con el cortisol, tanto en gestación ($r = 0.81$; $P < 0.01$) como en lactación ($r = 0.61$; $P < 0.05$).

En conclusión, la inclusión de hasta un 6% de glicerina bruta en el pienso puede reemplazar parcialmente al maíz sin afectar el rendimiento de las cerdas en gestación, pero no en lactación. Nuestros resultados también sugieren la existencia de una relación entre la inclusión de glicerina en el pienso y los niveles sanguíneos de algunas hormonas reguladoras del apetito. Sin embargo, más estudios son necesarios para conocer mejor las interacciones entre la concentración hormonal y la composición del pienso.

LITERATURE REVIEW

1. GENERAL INTRODUCTION

The increasing demand for transportation fuels has attracted considerable attention in recent years. High fossil fuel prices and political instability in petroleum producing regions, coupled with negative environmental impacts has increased global interest in biofuels, mainly bioethanol and biodiesel, which derived from renewable sources. Biofuel production and use is not new. German mechanical engineer Rudolph Diesel developed in the 1890´s the first diesel engine run on pure vegetable peanut oil. However, due to the wide availability and low cost of petroleum diesel fuel, vegetable oil-based fuels remained of minor interest, regaining important attention during the last decade.

In 2003, the European Commission published the Directive 2003/30/EC for promoting the use of biofuels or other renewable fuels for transport, in order to reduce greenhouse gas emissions. The European Union established the goal of reaching a 5.75% share of renewable energy sources in the transport sector by 2010. Under the Directive 2009/28/EC on the promotion of the use of energy from renewable sources, this target rose to a mandatory 10% minimum in each Member State in 2020. The application of tax incentives, grants and capital costs for setting up biofuel facilities have lead to a very rapid growth of the biofuel sector in the European Union, dominated by biodiesel.

Biodiesel is a clean-burning alternative fuel for diesel engines produced from renewable sources such as vegetable oils, animal fats and recycled cooking oils. The transesterification of oils and fats to produce biodiesel generates a considerable quantity of crude glycerin as byproduct, representing approximately 10% by weight of the biodiesel produced (Thompson and He, 2006). Crude glycerin produced in biodiesel

plants contains impurities and a lower purity, which limits its usual application as feedstock in pharmaceutical, cosmetic or food industries. Conventionally, crude glycerin has been purified to achieve the quality specifications for these purposes. However, the purification process is costly, and refine the increasing amounts of glycerin would be economic unfeasible, especially for small and medium producers. Consequently, alternative uses for crude glycerin are being explored to enhance profitability of biodiesel industries, and various reviews on value-added opportunities for direct utilization of crude glycerin have been published (Pachauri and He, 2006; Pluske, 2007; Yang et al., 2012).

One interesting option for using crude glycerin directly is as animal feedstuff. The rising price of cereals, coupled with volatile prices in the agricultural market, continually challenge farmers to achieve profits in their transactions, thus inexpensive byproducts are widely used for diet formulation. With the global expansion of biodiesel industry, it may be more and more glycerin available with a low price, and its inclusion in animal diets would reduce feeding cost.

During recent years, several authors have studied the inclusion of crude glycerin from biodiesel production in animal feed and concluded that it is a good alternative ingredient for swine (Kijora et al., 1995; Lammers et al., 2008b; Schieck et al., 2010b), poultry (Cerrate et al., 2006) and ruminants (Bartoň et al., 2013; Terré et al., 2011). Regarding to pig production, energy values of crude glycerin (Lammers et al., 2008c; Kerr et al., 2009; Mendoza et al., 2010) and its effect on growth performance, carcass composition, and meat quality (Mourot et al., 1994; Lammers et al., 2008b; Della Casa et al., 2009) have been determined.

2. BIODIESEL AND GLYCERIN PRODUCTION

Biodiesel production worldwide has increased considerably in recent years due to energy and environmental concerns, and associated policies. Biodiesel is a clean-burning alternative fuel for diesel engines, which is better for the environment because it is made from renewable resources, is biodegradable and produces less air pollutants and greenhouse gas emissions compared to conventional fossil fuels (Beer et al., 2007). The European Directive 2009/28/EC states that biofuels should account for 20% of the energy produced from renewable sources in the EU's final consumption of energy and a 10% of final energy consumption in the road transport by 2020. For these reasons, biodiesel has become increasingly interested and experienced a rapid expansion in its production capacity.

In the past decade biodiesel production has grown exponentially from 500,000 to 8 million metric tonnes in the European Union (USDA-FAS, 2013) (Figure 1), which is the world's largest biodiesel producer. The number of existing plants all over the EU is 256. Germany and France are by far the main biodiesel producer countries, followed by Benelux and Poland, which have incremented the production afterward 2011 (Table 1). In contrast, Spain and Italy dropped out of the top producers list.

Figure 1. Trend in EU biodiesel market 2006-2014. Source: USDA-FAS, 2013.

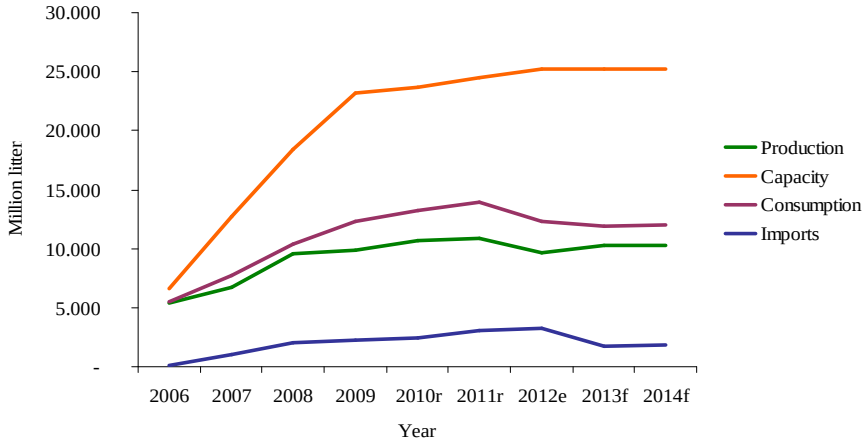


Table 1. EU biodiesel production by country (million liters). Source: USDA-FAS, 2013

	2006	2007	2008	2009	2010[r]	2011[r]	2012[e]	2013[f]	2014[f]
Germany	2,730	3,280	3,250	2,600	2,880	3,400	3,180	3,180	3,180
France	650	1,090	2,000	2,610	2,270	2,060	2,040	2,040	2,040
Benelux	50	290	430	840	910	950	1,000	1,050	1,090
Poland	100	60	310	420	430	410	670	720	740
Italy	680	530	760	900	830	700	570	570	570
Spain	140	170	280	700	1,370	740	510	400	400
Others	1,060	1,250	2,520	1,790	2,020	2,660	1,695	2,320	2,260
Total	5,410	6,670	9,550	9,860	10,710	10,920	9,665	10,280	10,280

r=revised/e=estimated/f=forecast

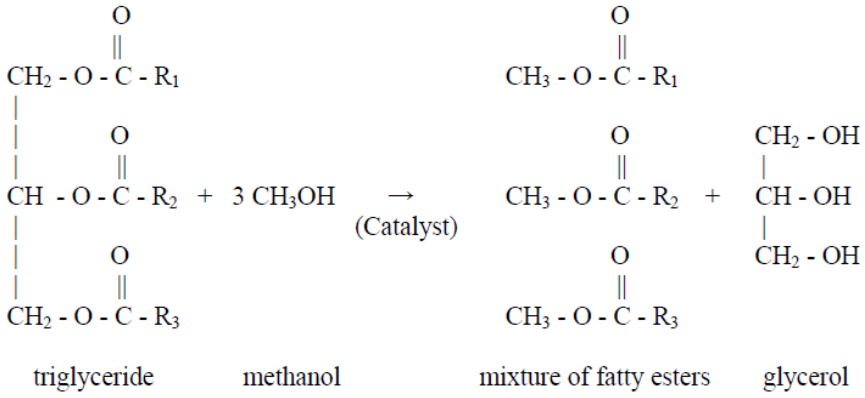
In July 2011, the European biodiesel production capacity reached 22 million tonnes, but currently only 41% of the capacity is in use. The rapid expansion seems to be decelerating from 2011 onwards. Increasing imports from third countries such as Argentina or Indonesia, circumvention measures from North America and the financial crisis had negatively impacted the biodiesel market (USDA-FAS, 2013). In addition, on January 2014, the European Commission published a communication regarding to policy framework for climate and energy in the period from 2020 to 2030, where new targets for biodiesel use in transport sector have not been established after 2020. For these reasons, biodiesel production in the EU is expected to remain stagnant in coming years.

Biodiesel is produced from a variety of renewable sources, mainly vegetable oils, but also recycled frying oils and animal fats. Sources of vegetable oils vary worldwide due to geographical and climate reasons. Thus, rapeseed oil is the primary biodiesel feedstock in the EU, followed by soybean oil, palm oil and sunflower oil, which are majority used in the Mediterranean countries. In the USA, Brazil and Argentina, biodiesel is predominantly produced by soybean oil, while in Southeast Asia, palm oil and coconut oil are the primary sources utilized (FAO, 2012).

Biodiesel can be produced by three different processes: reaction of the triglycerides with an alcohol, using a base catalyst; reaction of the triglycerides with an alcohol, using a strong acid catalyst; or conversion of the triglycerides to fatty acids, and a subsequent reaction of the fatty acids with an alcohol using a strong acid catalyst. Commonly, biodiesel is obtained using the base-catalyzed transesterification of the oil with alcohol. Methanol is currently the main alcohol used due to its lower cost, shorter reaction times and easily and economically way to be recycled, compared to other alcohols (FAO, 2012).

The transesterification reaction is shown in Figure 2. During the process, triglycerides from the oil, stimulated by the catalyst, react with methanol to produce methyls esters (biodiesel) and ethyl ester (glycerin). Stoichiometrically, 100 kg of triglycerides react with 10 kg of alcohol, in the presence of a base catalyst, to produce 100 kg of biodiesel and 10 kg of glycerin (Van Gerpen, 2005). Usually, the alkaline catalyst is sodium or potassium hydroxide, and a molar ratio 3:1 of alcohol to oil is required for the reaction. However, most biodiesel facilities use a 6:1 molar ratio, which is an excess of 100% of alcohol, to complete the reaction more rapidly. The excess alcohol (up to 80%) remains in the glycerin layer after the reaction, and can be later recovered for reuse (Quispe et al., 2013).

Figure 2. Transesterification reaction for biodiesel production.



3. GLYCERIN COMPOSITION

Glycerin, also known as glycerol, is an alcohol formed by three-carbon chain with a hydroxyl group attached to each carbon. In the European Union, glycerol is registered as feed additive E 422 (Anonymus, 1995). Physically, pure glycerin is a clear, colourless, odourless, hygroscopic, viscous liquid, with sweet taste. However, crude glycerin from biodiesel production generally contains glycerol and impurities such as ash, free fatty acids, and methanol in variable quantities (Swiatkiewicz and Koreleski, 2009).

Thomson and He (2006) analyzed the chemical composition and nutritional value of crude glycerin obtained from different vegetable oil sources and recycled cooking oils, using the same operating conditions and without further refinement. The results showed little variation between crude glycerin samples derived from neat oils, but glycerin obtained from waste oils had considerably lower glycerol and higher fat and ash content (Table 2). Hansen et al. (2009) characterized crude glycerin samples from different biodiesel plants in Australia. In contrast with the findings of Thompson and He (2006), the data obtained showed a large variation in the elements measured,

probably due to the different manufacturing processes used in the different biodiesel facilities. For example, pH varied between 2 and 10.8, glycerol content ranged from 38 to 96%, and some samples contained up to 29% ash and 14% methanol. Thus, crude glycerin composition is determined by the feedstock used, the production process involved, the recovery efficiency of the biodiesel and whether methanol and catalysts are recovered (Yang et al., 2012).

Table 2. Nutrient analyses for crude glycerin samples (Thompson and He, 2006).

	IdaGold Mustard	PacGold Mustard	Rapeseed	Canola	Soybean	Crambe	Waste vegetable oil
Fat (%)	2.03	1.11	9.74	13.10	7.98	8.08	60.10
Carbohydrates (%)	82.80	83.80	75.50	75.20	76.20	78.60	26.90
Protein (%)	0.14	0.18	0.07	0.06	0.05	0.44	0.23
Calories (MJ/kg)	14.60	14.50	16.30	17.50	15.80	16.30	27.20
Ash (%)	2.80	1.90	0.70	0.65	2.73	0.25	5.50

The methanol content in the crude glycerin must be monitorized because of its toxicity, especially with the expectation to be included into the animal diet. Methanol toxic effect is due to its metabolite, formate, which disrupts mitochondrial electron transport and energy production (Treichel et al., 2003). Dorman et al. (1993) observed mild CNS depression, tremors, ataxia, and recumbency in minipigs with acute methanol intoxication. Thus, the USDA Food and Drug Administration decided to limit methanol content at 0.15% in the crude glycerin as feed ingredient (USDA-FDA, 2006), while German regulations allow up to 0.2% methanol (DLG, 2012). Methanol has a boiling point of 64.5ºC; consequently, residual methanol will be evaporated during processing (pelleting) of feeds. Thus, Schröder and Südekum (1999) found no negative effects on rumen fermentation or energy retention by using crude glycerin that contained 26.7% methanol in a pelleted concentrate for ruminants. However, methanol vapours can pose a hazard in the feed mill.

In addition, sodium and potassium salts are commonly used as catalysts in the transesterification and trace amounts of them are found in the crude glycerin. For this reason, it is also important to consider the content of mineral salts in the crude glycerin for diet formulation, in order to meet the recommendations and maintain the electrolyte balance.

4. USE OF CRUDE GLYCERIN AS ANIMAL FEEDSTUFF

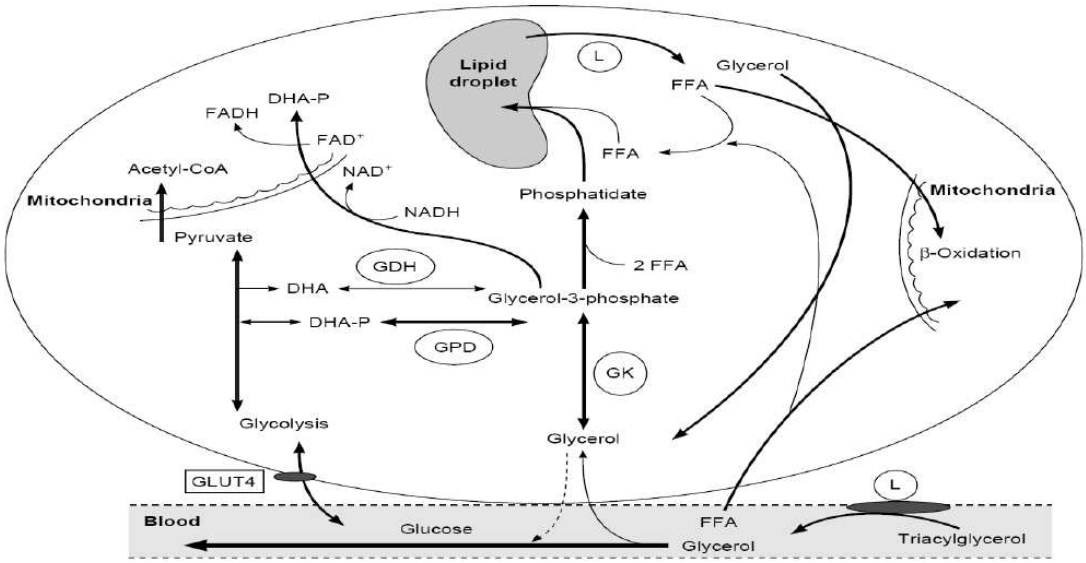
4.1. Glycerol metabolism

Glycerol is a sugar alcohol that exists naturally in food and living tissues. It is an essential structural component of triacylglicerides and phospholipids, thus it plays an important role in energy metabolism. Glycerol is naturally produced in the body and appears in and around all cells in low concentrations (<0.1 mmol/L) (Robergs and Griffin, 1998). Normally glycerol is made available from lipolysis in adipose tissues, from hydrolysis of the triglycerides in blood lipoproteins, and, to a minor extent, from dietary fat (Lin, 1977). Dietary triglycerides are hydrolyzed by pancreatic lipase to form free fatty acids and glycerol (Berg et al., 2002). The resulting glycerol, as hydrophilic solute, is believed to be absorbed mainly by paracellular passive diffusion, but there is also evidence for the presence of a Na[+]-dependent carrier-mediated transport system for glycerol in the rat small intestine (Kato et al., 2004). Glycerol also can be absorbed by the stomach, but at a rate that is slower than that of the intestine (Lin, 1977). Experiments on rats revealed that the rate of glycerol absorption by the intestine range from 70-89% (Hober and Hober, 1937). In laying hens, the intestinal absorption of glycerol has been shown to be more than 97% (Bartelt and Schneider, 2002).

The metabolism of glycerol is closely associated with that of carbohydrates. Since glycerol is a three carbon alcohol, it is metabolized quite readily into

glyceraldehyde 3-phosphate, a key component in the metabolism of glucose for energy generation (Lin, 1977; Tao et al., 1983; Brisson et al., 2001). Once glycerol is absorbed, it can either form glucose via gluconeogenesis or be oxidized for energy production via the tricarboxylic acid (TCA) cycle (Tao et al., 1983), by the shuttling of protons and electrons between the cytosol and mitochondria depicted in Figure 3 (Robergs and Griffin, 1998). When oxidized as an energy substrate, glycerol is converted to carbon dioxide and water, yielding 22 moles of ATP/mol.

Figure 3. Biochemical reactions involved in glycerol synthesis and metabolic conversation to glycerol-3-phosphate, phosphatidate and triacylglycerol.



DHA= dihydroxyacetone; DHA-P = dihydroxyacetone phosphate; FAD+ = oxidised from flavin adenine dinucleotide; FADH = reduced from of flavin adenine dinucleotide; FFA = free fatty acid; GHD = glycerol dehydrogenase; GK = glycerol kinase; GLUT4 = glucose transport protein; GPD = glycerol phosphate dehydrogenase; L = lipase; NAD+ = oxidised from of nicotinamide adenine dincleotide; NADH = reduced from of nicotinamide adenine dinucleotide.

On the other hand, glycerol can serve as a glucose precursor to provide energy for cellular metabolism. The amount of glucose carbon arising from glycerol depends upon metabolic state and level of glycerol consumption (Lin, 1977; Baba et al., 1995). The liver is the principal responsible for this process, accounting for at least three fourths of the total body capacity (Krebs et al., 1966). The kidney cortex can also form glucose from glycerol and this organ accounts up to one fifth of the total glycerol-

utilizing capacity of the body (Krebs and Lund, 1966). Other organs and tissues like brain, intestine, muscle, leukocytes, lungs and spermatozoa have shown to utilize glycerol at lower rates (Lin et al., 1977). Additionally, several studies have shown the beneficial effects of glycerol on amino acid or nitrogen retention in rats (Chan et al., 1981) and humans (Brennan et al., 1975). Glycerol is an inhibitor of important enzymes like phosphoenolpyruvate carboxykinase (Cryer and Bartley, 1973) and glutamate deshydrogenase (Steele et al., 1971), which take part in the gluconeogenic amino acid pathway. Thus, glycerol avoids the conversion of amino acids into glucose through gluconeogenesis, and can prevent protein catabolism.

Glycerol is highly gluconeogenic and some studies have revealed a complete metabolization of all dietary glycerin. Chambers and Deuel (1925) recovered, as extra glucose in the urine, practically all of an 8.5 g dose glycerol administered to two dogs poisoned with phloridzin to block renal sugar reabsorption. Mourot et al. (1994) found no differences in plasma glycerol concentrations when dietary levels of 5% glycerol were fed to pigs. However, others have found that high dietary glycerol is related with increased serum glycerol concentration. In an experiment with rats fed diets containing a high level of glycerol (53.4% w/w), serum glycerol concentrations were elevated when compared to controls (0.26-1.2 *vs.* 0.07-0.12 mg/ml) (Cryer and Bartley, 1973). Hansen et al. (2009) also observed that increasing crude glycerin from 4 to 16% in pig diets increased linearly serum glycerol levels in the end of their trial, but not in the beginning. This difference in plasma glycerol levels has been related with glycerol kinase, a key liver enzyme in glycerol metabolization which catalyzes the phosphorilation of glycerol to glycerol-3-phosphate (Vernon and Walker, 1970). This enzyme might be saturated when increasing levels of glycerin are fed or there is a long-term feeding program. Nevertheless, Papadomichelakis et al. (2012) found that glycerol

kinase mRNA expression in liver of post-weaned pigs increased linearly when the glycerin increased from 0 to 15% in the diet. In a recent study, Bernardino et al. (2014) also found no evidence of saturation of the glycerol kinase activity when feeding up to 7% crude glycerin to broilers.

When gluconeogenic capacity of the liver is exceeded, the excess of glycerol accumulates in blood and is most likely excreted via urine (Kijora et al., 1995). These authors reported that increasing levels of dietary glycerin induced greater glycerol concentration in blood and urine. Moreover, glycerin can have a diuretic effect. Various digestibility studies have found greater urine output and urinary energy excretion in pigs fed high levels of glycerin than in pigs fed control diets (Lammers et al., 2008c; Mendoza et al., 2010). Those authors added 20 and 30% glycerin to diet, respectively, which may have been above the upper limit for glycerin metabolism and could explain the increased energy excretion in the urine. Additionally, various authors have demonstrated feeding glycerin leads to increased water consumption (Johnson et al., 1933; Cryer and Bartley, 1973). As glycerol stimulates water intake, crude glycerin would be an effective hydrating agent and help improve heat stress tolerance, especially for lactating sows. Additionally, increasing water intake would supply the mammary gland with the water necessary for milk synthesis.

4.1.1. Glycerol metabolism in ruminants

In ruminants, dietary carbohydrates are mainly fermented to short-chain volatile fatty acids in the rumen and often less than 10% of the glucose requirement is absorbed from the ruminant digestive tract (Young, 1977). Thus, gluconeogenesis must provide up to 90% of the necessary glucose in ruminants. Much of the carbon used to support ruminant gluconeogenesis is derived from either propionate (40 to 70%) or glucogenic amino acids (Bergman, 1973). However, supply of ruminal propionate as substrate for

gluconeogenesis may not be sufficient when ruminants are in undernutrition or a situation of high-energy demand. Then, up to 23% of the glucose may be synthesized from liberated glycerol from the adipose tissue (Bergman et al., 1968; Weekes and Webster, 1975). Along with glycerol, a larger amount of fatty acids is released into the circulation, which may give rise to an increased rate of ketone body formation and consequently ketosis. For many years, this metabolic disorder has been effectively treated with drenching of propylene glycol and glycerol (Johnson, 1951, 1954; Goff and Horst, 2001).

The fate of glycerol entering the rumen is not fully understood. Glycerol disappears rapidly from the rumen either by microbial digestion, absorption or outflow through the omasal orifice. An in vitro study where 15 to 25% glycerol was added showed that most glycerol disappeared within 6 h (Bergner et al., 1995). Kijora et al. (1998) used glycerol-adapted steers and observed that the rate of disappearance was more rapid, 85% of glycerol disappeared within 2 h of administration, suggesting that microorganisms adapt to glycerol feeding. In this study, elevated serum glycerol was found but only small amounts of glycerol could be detected in the duodenal digesta. More recently, Krehbiel (2008) indicated values of 44% fermentation, 43% absorption and 13% passage.

Glycerin that is directly absorbed from the rumen acts as a precursor for gluconeogenesis in the liver (Remond et al., 1993; Krehbiel, 2008), while the remaining part is fermented by ruminal microbes into volatile fatty acids. Some early reports of glycerol fermentation indicated that glycerol is almost entirely fermented to propionate (Johns et al., 1953; Garton et al., 1961) while others reported increased acetic and propionic acids (Wright, 1969). However, later studies indicated increased propionate at the expense of acetate, but also greater butyrate production (Czerkawski and

Breckenridge, 1972; Remond et al., 1993; DeFrain et al., 2004; Trabue et al., 2007; Ferraro et al., 2009). For example, Schröder y Südekum (1999) used cannulated steers to evaluate ruminal effects of feeding glycerin (0 and 15%). They observed pronounced postprandial decline in pH when diets contained glycerin, indicating ruminal degradation of glycerol was rapid. Feeding glycerin did not affect nutrient digestibility, but decreased the acetate:propionate ratio and increased butyric acid and water intake. As indicated by Hippen et al. (2008), these changes may be beneficial for dairy cows because ruminal increment of propionate would increase the supply of this gluconeogenic substrate to the liver and butyric acid provides energy requirements for growth of ruminal epithelium.

Glycerin in the diet also might affect other aspects of ruminal fermentation. For example, glycerol can negatively affect cellulolytic activity in the rumen (Roger et al., 1992; Parsons et al., 2009). Likewise, reductions in proteolytic activity, bacterial protein synthesis or branched chain volatile fatty acids production have been observed after glycerin administration (Kijora et al., 1998; Paggi et al., 1999).

4.2. Caloric value of crude glycerin

Several studies have evaluated the energy content of glycerin for pigs and poultry. Pure glycerin contains approximately 4,100 kcal of gross energy (GE) per kg (Brambila and Hill, 1966). However, crude glycerin can range from 3,000 to 6,000 kcal GE/kg (Lammers et al., 2008a, 2008c; Kerr et al., 2009; Jung and Batal, 2011), depending upon the different composition of the samples. Various experiments evaluating glycerin have assumed the metabolizable energy (ME) to be approximately 95% of its GE in dietary formulation (Brambila and Hill, 1966; Lin et al., 1977; Cerrate et al., 2006; Dozier et al., 2011).

Bartlet and Schneider (2002) reported ME values of refined glycerin in swine diets and showed that the ME value of glycerin decreased as the level of dietary glycerin fed to 35-kg barrows increased (4,180, 3,439 and 2,256 kcal ME/kg for 5, 10 and 15% dietary glycerin, respectively). Lammers et al. (2008c) examined crude glycerin (86.95% glycerol) fed to starter and finishing pigs, which shown to have a ME of 3,207 kcal/kg. This value, when placed on an equivalent glycerol basis, is marginally greater than the one determined by Bartlet and Schneider (2002) for pure glycerin (3,688 *vs.* 3,439 kcal ME/kg), but similar to the 3,656 kcal ME/kg as reported by Mendoza et al. (2010) when using a 30% inclusion level of glycerin. In the experiment with starter pigs (11 kg initial BW), Lammers et al. (2008c) observed that the ME of crude glycerin declined with increasing levels of supplementation, particularly when starter pigs were fed with a diet containing 20% glycerin. In contrast, ME determination in finishing pigs was not affected when level of crude glycerin increased. In a recent study, Kovács et al. (2011) obtained a ME value of 3,218 kcal/kg for crude glycerin (86.76% glycerol content) added to the diet of growing pigs (25 to 85 kg BW). Results from their experiment showed that ME of glycerin did not decline when the application rate raised from 5% to 10%.

In the study of Kovács et al. (2011), the ratio of DE:GE for crude glycerin was 90.3%, which is slightly lower than the values reported by Lammers et al. (2008c) and Kerr et al. (2009) (92 and 96.6%, respectively). These high values of DE:GE indicate that glycerin is well digested. Supporting this, Bartelt and Schneider (2002) previously found that the ileal digestibility of pure glycerin was higher than 97%, even at an application level of 15%. Additionally, the ME content of the diet expressed as a percentage of the DE content indicates how well energy is utilized once digested. The ratio of ME:DE for crude glycerin obtained by Lammers et al. (2008c) and Kovács et al.

(2011) were 96 and 97.5%, respectively. When compared to corn and soybean oil, ingredients used to provide energy in pig diets, the ME:DE ratio for crude glycerin is very similar to the ratio ME:DE for soybean oil (96%) and corn (97%) (NRC, 1998). Those findings support the assertion that crude glycerin is well utilized by the pig as a source of energy.

The energy value of crude glycerin in poultry has also been recently evaluated. The ME content for pure glycerin was 3,929 and 3,993 kcal/kg for laying hens and broilers, respectively (Bartelt and Schneider, 2002). Cerrate et al. (2006) reported an energy value of 3,527 kcal ME/kg for crude glycerin in broilers, calculated as 98% of the analyzed GE content. Crude glycerin from biodiesel production, containing 86.95% glycerol, was evaluated by Lammers et al. (2008a) using laying hens and Dozier et al. (2011) using broilers. The ME of glycerin obtained in laying hens was 3,805 kcal/kg, being not very different from its GE value. When determined in broilers, it was 3,434 kcal ME/kg. These results indicate that the energy in crude glycerin is used efficiently by poultry.

Schröder and Südekum (1999) explored the potential of crude glycerin to be included in ruminant diets. Wethers were fed 40% forage, 50% concentrate (low or high-starch) and 10% crude glycerin of different purity. In addition, the glycerin of the highest purity (100% glycerol) was included in diets containing 40% forage and 5, 10, 15 or 20% glycerin. Concentrations of net energy for lactation (NE_L) were higher when glycerin was fed with the low-starch concentrate (2,317 kcal NE_L/kg). When fed with a low-starch concentrate, pure glycerin at dietary inclusion levels up to 20% had either no effect or positive effects on nutrient digestibilities. When included in diets containing high-starch concentrates, glycerin had markedly lower NE_L concentrations (1,982 Kcal NE_L/kg) and reduced cell-wall digestibilities were found. Therefore, the economic value

of energy from glycerin is equal to or even greater than that of corn grain (1,980 kcal NE_L/kg; FEDNA, 2010)

4.3. Crude glycerin and feed manufacturing

When a new ingredient is considered to be included in the animal diet, it is important to evaluate its impact on feed processing efficiency. For example, hard-to-handle ingredients might entail additional costs in labour and equipments, countering the benefits of utilizing a cheap feedstock. Thus, the study of the physical properties of the new ingredient, which will determine its flow behaviour, is essential to establish the storage and handling requirements, especially of wet ingredients.

Crude glycerin is a viscous liquid with limited flow properties that may difficult storage and handling operations, especially with low temperatures because tends to thicken and form crystals (Pagliaro and Rossi, 2008). Regarding to storage requirements, Mader (2011) studied the conduct of crude glycerin stored and handled under common feed mill storage operations. Both, ferrous metal and stainless steel deposits, showed any evidence of corrosion after two months containing crude glycerin, which indicates they are suitable for a short-term storage of this product. In addition, mix the crude glycerin before using must be taken into account due to its predisposition to separate into layers in the tank (Crawshaw, 2001).

The use of crude glycerin in diets may improve feed texture and palatability, in addition of reducing dust and fines. Moreover, glycerin can contribute to maintain the hygienic quality of pelleted concentrates during storage, possibly due to the hygroscopic properties of glycerol (Schröder and Südekum, 1999). These authors also observed a positive effect of glycerin on pellet hardness with inclusion level of 10%. Groesbeck et al. (2008) reported glycerin addition up to 12% without affecting pellet quality. In this

study, increasing levels of crude glycerin (3, 6, 9, 12 and 15%) were added prior pelleting to a corn-soybean meal diet. Pellet manufacturing process was enhanced by decreasing production efficiency (kWh/t), which would result in energy savings and potential cost reductions at the feed mill. Additionally, pellet durability index (PDI) increased up to 9% added crude glycerin. Pellet durability index was developed as a predictor of pellet fines produced during mechanical handling. Greater PDI can be related to lower feed wastage and reduced selective feeding, which improves the efficiency of feed utilization in pigs (Behnke, 2001). Shields et al. (2009) observed similar results when glycerin (2.5 and 5%) was added to nursery and finishing pig diets. In this study, crude glycerin improved pellet mill efficiency and PDI, as well as pellet mill flow of mash feed. However, mash diets containing more than 8% crude glycerin formed a firm aggregate within 24 h after mixing and compromised diet uniformity (Hansen et al., 2009). Thus, pelleting seems to be the most appropriate process when crude glycerin is included as feed ingredient.

4.4. Effects of crude glycerin addition on performance and animal products

During recent years there has been increasing interest in using crude glycerin due to its potential to reduce feeding costs. Crude glycerin is an attractive energy source for animal feed because of its similar energy value in comparison with commonly used feed ingredients such as corn. Dairy cows have been the main consumers of glycerin historically. However, the use of this byproduct in rations for nonruminant animals has been growing in recent years.

4.4.1. Swine

Few studies from middle 1990s appeared in the literature detailing the role of glycerin as feed ingredient for pigs. However, with the recent and marked increase in global biodiesel production and the abundance of glycerin, new approaches are being investigated for the use of glycerin in pig diets.

4.4.1.1. Growth performance, carcass composition and meat quality

Mourot et al. (1994) indicated that growth performance of pigs from 35 to 102 kg was not affected by the addition of 5% glycerin to a wheat-soybean meal-based diet. They reported reduced 24-h drip and cooking losses in *Longissimus dorsi* muscle (LM) and *Semimembranosus* muscles in pigs fed 5% added glycerin, suggesting that glycerin could increase water holding capacity of the muscles. In addition, pigs fed 5% glycerin had increased oleic acid and decreased linoleic and linolenic acid in backfat, which resulted in a decline in the PUFA:MUFA ratio in backfat.

German researchers (Kijora et al., 1995, 1997; Kijora and Kupsch, 1996) suggested that up to 10% crude glycerin can be fed to pigs with little effect on pig performance. Kijora et al. (1995) conducted two experiments to test glycerin as a component in barley-soybean meal-based diets of growing-finishing pigs. In their first experiment, pigs fed diets containing 5 and 10% glycerin had increased feed intake (FI) and daily weight gain compared with controls, suggesting that the sweet taste and the better feed structure of diets with glycerin might be the reason for a higher feed intake. Similar results were obtained in a subsequent trial when fed growing-finishing pigs a diet containing 0%, 5% or 10% of pure or crude glycerin (Kijora and Kupsch, 1996). In the second experiment the effect of 5, 10, 20 and 30% inclusion levels of glycerin was tested, with no significant improvement in performance. However, adding 30% glycerin to the diet impaired feed:conversion ratio. Kijora et al. (1995) and Kijora and Kupsch

(1996) reported no consistent effect of 5 or 10% crude glycerin addition to the diet on carcass composition or meat quality, even though they found a tendency toward a reduction in drip and press water loss in loin chops (Kijora and Kupsch, 1996). However, in an additional study, pigs fed 10% crude glycerin showed a significant reduction in linoleic acid (Kijora et al., 1997).

More recently, Della Casa et al. (2008) observed no effect on pig performance when feeding pigs from 43 to 160 kg with diet containing 5% pure glycerin in substitution of corn. However, pigs fed 10% glycerin had reduced growth rate and feed efficiency compared to pigs fed the control or 5% glycerin supplemented diets. Dietary glycerin affected neither meat or fat quality, nor sensory characteristics of LM.

Lammers et al. (2008b) fed pigs (8 to 133 kg BW) with corn-soybean meal-based diets containing 0, 5, or 10% crude glycerin (84.51% glycerol) and reported no effect of dietary treatment on ADG or G:F at any phase of production. Carcass composition, meat quality and sensory attributes were not affected, although loin ultimate pH was slightly increased in pigs fed crude glycerin compared with pigs fed control diets. Moreover, fatty acid profile of the loin from pigs fed 10% crude glycerin had less linoleic acid and more eicosapentaenoic acid. In addition, blood metabolites were not affected by diet or sex.

Some studies have used higher inclusion levels of crude glycerin and found no detrimental effects on pig production. For example, Hansen et al. (2009) fed up to 16% crude glycerin to pigs from 51 to 105 kg pigs with no effects on growth performance or meat quality. However, these authors did not recommend using levels above 8% glycerin in mash diets because they tended to form a firm aggregate within 24 h of mixing, presenting some feeding difficulties. Mendoza et al. (2010) fed heavy pigs (93

to 120 kg) up to 15% refined glycerin in corn-based diets and reported no effect on growth performance, carcass characteristics, or meat quality.

Schieck et al. (2010b) performed a study to test the effects of long- and short-term feeding of crude glycerin on growth performance, carcass traits, and pork quality of growing-finish pigs. They fed pigs either control corn-soybean meal-based diet (16 weeks, 31 to 128 kg), 8% crude glycerin during the last 8 weeks (45 to 128 kg) or 8% crude glycerin for the entire 16 week period (31 to 128 kg). Feeding crude glycerin during the last 8 weeks before slaughter enhanced ADG and ADFI compared to control group, and improved carcass and belly firmness. Longer term feeding (16 weeks) resulted in a slight improvement in growth rate and a small depression in feed efficiency, while carcass composition and pork quality were not affected.

In summary, although contradictions exist in the literature in regard to the potential benefits of adding glycerin to pig diets, no negative effects on performance or pork quality have been reported as a result of including crude glycerin in moderate levels (5-10%).

4.4.1.2. Nursery pigs

Crude glycerin is a valuable energy source for piglets because it is easily digested and metabolized (Kerr et al., 2009). Moreover, its sweet taste could increase the palatability of the feed when fed to nursery pigs (Wapnir et al., 1996). Various studies have evaluated the inclusion of crude glycerin in diets for nursery pigs. Lammers et al. (2008b) reported no differences in pig performance between weaning and slaughter (8 to 133 kg BW) when 10% crude glycerin was included in the diet. In contrast, Groesbeck et al. (2008) fed glycerin at 0, 3, and 6% to nursery pigs (11 to 27 kg BW) and observed a linear improvement in ADG, as a result of increased feed intake of diets containing crude glycerin. Similar results have been observed by Zijlstra et al.

(2009) when feeding 0, 4, and 8% crude glycerin in wheat-based diets. Piglets fed 8% glycerin had greater ADG than pigs fed diet without glycerin. Thus, crude glycerin could be included in substitution of cereals to nursery pig diets without compromising the growth performance of nursery pigs.

More recently, Shields et al. (2011) studied the inclusion of crude glycerin in nursery pig diets as a partial replacement for lactose. Their results exhibit that crude glycerin supplementation at 10% enhanced growth performance. Oliveira et al. (2014) substituted sweet milk whey with crude glycerin at 9 and 18% without affecting ileal digestibility or plasma metabolites. However, urinary glycerol increased when high levels of crude glycerin were used, suggesting the saturation of the metabolic pathways of glycerol utilization.

4.4.1.3. Sows

Only one study has been reported relative to feeding crude glycerin to lactating sows. Schieck et al. (2010a) evaluated the effect of dietary crude glycerin in lactating sow diets on sow and litter performance under heat stress conditions. Treatments consisted on corn-soybean meal-based diet with 0, 3, 6 and 9% crude glycerin. Sow and litter performance were similar for control and glycerin added diets, although sows fed 6% crude glycerin had lower ADFI and BW gain of litters.

On the other hand, it is assumed that glycerol may contribute to increase milk production of sows (Doppenberg and Van der Aar, 2007). However, this assumption was not confirmed by Schieck et al. (2010a), probably due to the decrease in number of pigs weaned per litter with increasing glycerin, which may reduced the number of functional glands and consequently, milk yield. Nevertheless, increased dietary crude glycerin increased lactose concentration in the milk. Blood glucose is the primary precursor for lactose synthesis in milk (Boyd et al., 1995). In lactating sows, plasma

glycerol concentrations linearly increased with glycerin supplementation up to 9%, but no effect on blood glucose was found (Schieck et al., 2010a). This suggests that that sows metabolize a portion of the excess plasma glycerol to glucose via gluconeogenesis, which was ultimately used in production of lactose by the mammary gland.

4.4.2. Poultry

Several studies have determined that glycerin is an acceptable feed ingredient for poultry diets. The addition of glycerin up to 10% of the diet had no adverse effects on growth performance or carcass yield in broilers (Simon et al., 1996, 1997; Cerrate et al., 2006; Min et al., 2008; Mandalawi et al., 2014). Moreover, broilers fed 2.5 to 5% glycerin showed improved feed conversion (Cerrate et al., 2006; McLea et al., 2011) and breast yield (Cerrate et al., 2006). However, increasing dietary glycerin above 10% negatively affected performance and chickens health (Simon et al., 1996). Cerrate et al. (2006) also observed reduced performance when feeding 10% glycerin, although this was probably related to problems with feed flow. More recently, Kim et al. (2013) reported increased total apparent tract digestibility of GE and DM when broilers were fed a diet with 5% crude glycerin supplementation.

In an experiment evaluating AMEn value of crude glycerin in laying hens, Lammers et al. (2008a) observed no impact on egg production during the 8-day experiment. Swiatkiewicz and Koreleski (2009) demonstrated that 6% crude glycerin could be fed to laying hens without any detrimental effect on nutrient retention, egg production and egg quality. The inclusion of crude glycerin at the level of 5 and 7.5% in diets increased egg yolk cholesterol content and decreased the ratio MUFA:SFA (Yalçin et al., 2010).

4.4.3. Ruminants

The use of glycerol in the treatment of ketosis was reported as early as the 1950s. Glycerol was used to treat ketosis in dairy cows via drenching orally, feeding with concentrates, or both, with a relatively large dose (Johnson, 1951, 1954; Fisher et al., 1973); but at that time, the use of glycerol was economically unfeasible due to its high cost. More recently, surplus production of crude glycerin from biodiesel production has generated renewed interest in the study of glycerin as a preventative aid for metabolic problems associated with transition cows. Goff and Horst (2001) administered 1, 2 or 3 L of glycerol via an esophageal pump and found an increase in plasma glucose by 16, 20 and 25% over pretreatment values, respectively. DeFrain et al. (2004) evaluated glycerol (430 and 860 g/d) top dressed in the diet of transition cows. They observed greater total VFA, greater molar proportions of propionate and decreased acetate:propionate ratio, but no differences in glucose plasma concentrations of glycerin-fed cows compared to controls. These findings suggest that glycerin administered by feeding is predominantly used as an energy substrate by the rumen microorganisms instead of entering the gluconeogenic pathway.

More recently, Chung et al. (2007) fed 162.5 g/d of glycerol in a dry product to transition cows without observing effects on feed intake, milk yield and components, blood metabolites or serum composition, although milk production tended to increase three weeks after cessation of feeding, which suggests a potential benefit of dry glycerin on energy status and subsequent milk production. Previously, Bodarski et al. (2005) found an increase in milk yield and milk protein content when feeding 300 ml/d glycerin from 3 weeks prior to calving to 10 weeks after calving. Recent studies indicated that glycerin can replace corn grain in diets for lactating dairy cows to as

much as 15% of the ration DM without deleterious effects on milk production or composition (Donkin et al., 2009).

During recent years, the use of glycerin in feedlot diets has also been evaluated in several studies with cattle and lambs. Normally, increasing levels of glycerin in the diets caused a linear decrease in DMI (Musselman et al., 2008; Parsons et al., 2009; Gunn et al., 2010). However, positive effects on daily gains and feed efficiency have been reported when dietary glycerin was included at less than 10% DM of finishing heifer and steer diets (Pyatt et al., 2007; Parsons et al., 2009). Mach et al. (2009) found no detrimental effect on intake and performance of beef cattle fed concentrate containing up to 12% of DM crude glycerin. Likewise, Bartoň et al. (2013) reported any effect on growth performance, blood, and rumen metabolites of finishing bulls fed 10% of DM crude glycerin in replacement of barley meal. The replacement of 7.5% of alfalfa hay with glycerin in growing steers diets showed beneficial effects in performance (Hales et al., 2013).

Slaughter characteristics, carcass composition and meat quality have not been affected by the inclusion of low to moderate concentrations of crude glycerin (Mach et al., 2009; Bartoň et al., 2013). However, glycerin levels above 12% of DM decreased LM area and marbling scores (Parsons et al., 2009). Recently, Eiras et al. (2014) and Carvahlo et al. (2014) have demonstrated that diets with up to 18% glycerin can be fed to finishing bulls with no effect on carcass characteristics and meat quality. However, they found dietary glycerin decreased saturated fatty acids and increased monounsaturated and polyunsaturated fatty acids in LM, which have been also observed by Egea et al. (2014), with lower inclusion levels (2 and 4% glycerin). Additionally, glycerin might attenuate the effects of long distance transportation by maintaining body

water, decreasing the energy deficit, and sparing muscle protein degradation (Parker et al., 2007).

Musselman et al. (2008) reported negative effects on growth and intake when high levels of crude glycerin (30-45% of DM) was included to the diet of finishing lambs from 33 to 55 kg BW. However, the inclusion of glycerin up to 15% of dry matter during the first 14 d of the feeding period improved feedlot performance variables and had no effects on carcass characteristics (Gunn et al., 2010). In light lambs (25 kg of BW at sacrifice), glycerin can be included up to 10% without adversely affecting feed intake, blood metabolites and meat composition (Terré et al., 2011).

There is little information available on feeding glycerin to sheep and goats. Meale et al. (2013) substituted wheat grain with crude glycerin at 12% DM in the diet of Merino ewes and no effects on feed intake, BW, or wool yield and production characteristics were detected. Regarding to goats, glycerin levels up to 20% DM could be efficiently utilized for this animals (Chanjula et al., 2014).

JUSTIFICATION AND OBJECTIVES

The main objective of this PhD thesis was to evaluate the inclusion of crude glycerin from biodiesel production as a feed ingredient in different phases of pig production. With this purpose, specific objectives have been proposed in three experimental chapters that comprise this thesis:

- **Chapter 1:**
    1. To determine the effect of crude glycerin on pellet production efficiency.
    2. To evaluate the effect of crude glycerin on growth performance, plasma metabolites, nutrient digestibility, and retention in growing-finishing pigs.
- **Chapter 2:**
    3. To study the effect of dietary crude glycerin and gender on the performance, digestibility and blood hormone levels of Iberian crossbred pigs.
- **Chapter 3:**
    4. To evaluate the effect of dietary crude glycerin on sow and litter performance.
    5. To determine the plasmatic levels of hormones related to energy metabolism and feed intake in pregnant and lactating sows fed crude glycerin.

CHAPTER 1

Effect of crude glycerin on feed manufacturing, growth performance, plasma metabolites and nutrient digestibility of growing-finishing pigs

ABSTRACT

Three experiments were conducted to determine the effects of dietary addition of crude glycerin on pellet production efficiency and to evaluate its effect on growth performance and digestibility in growing-finishing pigs. Three dietary treatments were created by addition of 0, 2.5 or 5% crude glycerin to barley-soybean meal-based diets, and four batches of each dietary treatment (two each for grower and finisher diets) were prepared. In the manufacturing process, crude glycerin supplementation linearly increased the feeder speed and production rate ($P < 0.05$), resulting in a 20 to 29% improvement in the feed production rate compared with the control. Production efficiency (kg/kWh) increased linearly ($P < 0.05$) as the level of crude glycerin in feed increased. A growth experiment was performed with 240 barrows (30 ± 1 kg initial BW) using a 2-phase feeding program over a 12-wk period with 4 pens per treatment and 20 pigs per pen. On the last day of the growth experiment, blood samples were collected to determine circulating glucose, fructosamine and IGF-1 concentrations. Overall growth performance was not affected ($P > 0.05$) by dietary treatment, and there was no effect ($P > 0.05$) of dietary treatment on any plasma metabolite measured. A digestibility experiment involving nine male pigs housed in metabolic cages was used to determine the coefficients of apparent fecal digestibility and N and mineral balances. Pigs were assigned to one of the three diets in each feeding period using a 3 × 3 Latin square arrangement of treatments (43 ± 3 and 74 ± 3 kg initial BW in the growing and finishing periods, respectively). In both feeding periods, fecal digestibility of OM and ether extract were affected by dietary treatment, increasing linearly ($P < 0.05$) with increasing crude glycerin levels. However, neither CP digestibility nor N retention was affected by the glycerin content in either for the growing or finishing period. Digestibilities and balance of Ca and P showed opposite tendencies with the variations

in crude glycerin content, which either decreased or increased depending on the feeding period. In conclusion, adding crude glycerin to the diet before pelleting improved feed mill production efficiency. The addition of crude glycerin up to 5% in the diet of growing-finishing pigs had no effect on growth performance, blood metabolites, nutrient digestibility and N balance, but more studies are needed to determine how crude glycerin affects mineral metabolism and balance.

INTRODUCTION

Worldwide production of biodiesel has increased considerably in recent years, and biodiesel is a clean-burning alternative fuel produced from renewable resources such as vegetable oils, animal fats, and used cooking oils. In the past decade, biodiesel production has grown exponentially from 500,000 to 9 million tonnes in the European Union (EBB, 2010), which is the largest producer of biodiesel in the world. The European Directive 2009/28/EC states that biofuels should account for 20% of the energy produced from renewable sources by 2020 (European Union, 2009). The main byproduct of the biodiesel industry is crude glycerin, also known as glycerol. As the production of biodiesel grows, a considerable quantity of crude glycerin will be generated, representing approximately 10% by weight of the biodiesel produced (Thompson and He, 2006).

Several experiments were performed to test the inclusion of crude glycerin as an energy source for replacing cereals in swine diets with variable results. Crude glycerin generally contains glycerol, water, ash, FFA, and methanol in variable quantities (Swiatkiewicz and Koreleski, 2009), which may affect animal performance. Several authors have determined the energy values of crude glycerin (Lammers et al., 2008c; Kerr et al., 2009; Mendoza et al., 2010) and its effect on growth performance, carcass composition, and meat quality (Mourot et al., 1994; Lammers et al., 2008b; Della Casa et al., 2009). However, there is little information about how crude glycerin affects feed processing efficiency (Groesbeck et al., 2008; Shields, 2009) and, to our knowledge, few or no data on its effect on the metabolism and N balance in fattening pigs fed barley-soybean meal-based diets. Therefore, the objectives of this research were to determine the effect of crude glycerin on pellet production efficiency, as well as to

evaluate its effect on growth performance, plasma metabolites, nutrient digestibility, and retention in growing-finishing pigs.

MATERIAL AND METHODS

All procedures involving animals were approved by the Murcia University Ethics Committee, and the animal care and experimental procedures used in this study conform to national and European Union regulations and guidelines (Spanish Royal Decree RD 1201/2005 and EU Directive 86/609/CEE as modified by 2003/65/CE).

General Procedures and Experimental Diets

This research was performed to test the performance of glycerin on the feed production efficiency in the feed mill (Exp. 1). For pig performance, a growth evaluation (Exp. 2) and a digestibility trial (Exp. 3) with growing-finishing pigs were conducted in commercial and experimental conditions, respectively.

Each experiment included three barley-soybean meal-based diets containing 0, 2.5 and 5% crude glycerin (G0, G2.5, and G5, respectively). Diets were formulated to be isoenergetic and iso-AA for each feeding period (growing and finishing). Feed formulation was based on ME and ileal digestible AA according to the recommendations of Fundación Española para el Desarrollo de la Nutrición Animal (FEDNA, 2006). The crude glycerin was included as a replacement for corn. The energy value of crude glycerin included in the feed formulation matrix was based on FEDNA (2010). The crude glycerin used in this study was obtained from a biodiesel production facility (Abengoa Bioenergía San Roque, Cadiz, Spain), which used vegetal oils as feedstock. All experiments used the same batch of crude glycerin. The composition of the diets is shown in Table 3.

Table 3. Ingredients and composition of diets (as-fed basis).

	Growing			Finishing		
	Crude glycerin, %			Crude glycerin, %		
Item	0	2.5	5	0	2.5	5
Ingredient, %						
Barley	30.88	30.88	30.53	38.59	38.14	37.46
Wheat	30.00	30.00	30.00	25.00	25.00	25.00
Soybean meal (47% CP)	17.02	17.44	18.16	15.06	15.59	16.17
Corn	15.00	12.50	10.00	15.00	12.50	10.00
Animal fat	3.70	3.59	3.51	3.04	3.13	3.23
Crude glycerin[1]	-	2.50	5.00	-	2.50	5.00
Calcium carbonate	1.16	1.15	1.15	1.31	1.55	1.54
L-Lysine 50 (50% Lys)	0.63	0.61	0.59	0.39	0.38	0.36
Monocalcium phosphate	0.46	0.46	0.46	0.49	0.49	0.49
Sodium bicarbonate	0.39	0.20	-	0.38	0.18	-
Sodium chloride	0.30	0.20	0.15	0.40	0.20	0.40
Vitamin-trace mineral premix[2]	0.30	0.30	0.30	0.30	0.30	0.30
L-threonine	0.09	0.09	0.09	0.02	0.02	0.02
DL-methionine	0.07	0.07	0.07	0.03	0.03	0.03
Calculated composition[3]						
ME, kcal/kg	3,250	3,250	3,250	3,225	3,231	3,231
Ileal digestible Lys, %	0.95	0.95	0.95	0.79	0.79	0.79
Analyzed composition, %						
DM	90.47	90.57	90.62	89.32	88.99	89.21
CP	15.62	15.86	16.01	14.74	14.99	14.90
Ca	0.65	0.66	0.68	0.80	0.83	0.82
P, total	0.54	0.49	0.47	0.48	0.46	0.46
Na	0.25	0.20	0.18	0.26	0.25	0.23
Lys	0.96	0.97	0.97	0.90	0.89	0.89
Met	0.30	0.32	0.32	0.31	0.31	0.32
Cys	0.28	0.24	0.26	0.23	0.21	0.24
Thr	0.65	0.64	0.63	0.59	0.63	0.65

[1]Analyzed composition: glycerol, 87.42%; methanol, 0.05%; moisture, 7.98%; ash, 5.87%; Ca, 0.04%; P, 0.01%; Na, 2.01%; Cl, 3.06%; and K, 0.05%.
[2]Provided (per kilogram of complete diet): 8,000 IU of vitamin A; 1,100 IU of vitamin D_3; 20 IU of vitamin E; 1 mg of vitamin K_3; 1 mg of vitamin B_1; 3 mg of vitamin B_2; 1 mg of vitamin B_6; 0.015 mg of vitamin B_{12}; 17 mg of niacin; 10 mg of pantothenic acid; 0.08 mg of biotin; 0.02 mg of folic acid; 50 mg of choline; 50 mg of Mn (manganous sulfate); 0.5 mg of I (potassium iodide); 90 mg of Zn (zinc oxide); 10 mg of Cu (copper sulfate); 90 mg of Fe (ferrous carbonate); 0.3 mg of Se (sodium selenite); and 10 IU of endo-1,4-beta-xylanase (CE 3.2.1.8) from *Bacillus subtilis* (LMG s-15136).
[3]According to FEDNA (2010).

Feed Manufacturing Process (Exp. I)

Diets were designed and manufactured by a commercial company (Alia, Lorca, Spain). Raw materials were ground through a 2-mm screen using three horizontal hammer mills (Rosal VRE220 and VRE150 models; Rosal S.A. and Mabrik S.A., Barcelona, Spain) working at 3,000 rpm. Ingredients were mixed with crude glycerin in a horizontal mixer (Rosal MHR 10,000 L, 75 CV, 1,500 rpm; Rosal S.A. and Mabrik S.A.) for 3 min. The mash was conditioned at a temperature of 60ºC and then conveyed to a pellet press (pellet mill motor load at 80%). Diets were pelleted in a pellet mill (Mabrik PV220G; Rosal S.A. and Mabrik S.A.) equipped with a die that had a wall thickness of 65 mm and 3.5 mm diameter holes. Pellets were cooled using a horizontal counter flow pellet cooler (Rosal S.A. and Mabrik S.A.). Four batches of each dietary treatment (two per period: grower and finisher diets) were manufactured (approximately 4 t per batch).

Pellet mill electrical consumption (kWh), average motor load (%), feeder speed (rpm), production rate (kg/h), production efficiency (kg/kWh), and pellet durability (%) data were collected for all batches of the diets. Pellet production efficiency was calculated by dividing kilograms of feed manufactured by electrical consumption in each batch. The pellet durability index (PDI) was determined using a durability tester (Holmen NHP100 Portable Pellet Tester; Texpro Ltd, Coleraine, UK) into which 100 g of cold pellets were inserted. Additionally, pellet samples were collected for each batch and stored at -20ºC for further analysis.

Growth Performance Experimental Design (Exp. II)

This experiment was performed in a commercial operation (Lorca, Spain) over a 12-wk period. The 240 Large White × Landrace barrows (30 ± 1 kg BW) were assigned

to three dietary treatments. Pigs used in the experiment were housed in a grow-finishing facility consisting of 12 pens (20 pigs per pen and 4 pens per treatment) with natural ventilation and a partially slatted floor (30% of the pen floor area). Each pen (5 m × 4 m) was equipped with two feeders and a cup waterer. Pigs were sorted by initial BW and randomly allotted to the pens.

Pigs were fed using a 2-phase feeding program. The growing and finishing phases comprised periods of 45 and 39 d, respectively. Pigs were allowed *ad libitum* access to feed and water throughout the experiment. All animals were weighed individually at the beginning of the trial, at the beginning of the finishing period and at the end of the experiment. The individual BW of the pigs within each pen were used to calculate ADG on a pen basis. Feed intake per pen was recorded by weighing feed added to the feeders and feed remaining at the end of each period to determine ADFI and G:F.

On day 84, after a 12 h fasting period, five animals were randomly selected from each pen (4 pens per treatment) to determine the concentration of circulating glucose, fructosamine and IGF-1. Blood samples were collected via jugular venipuncture into 5 mL tubes containing heparin. The blood samples were centrifuged at 3,000 × *g* for 10 min at 4ºC to recover the plasma, which was stored at -20ºC until analysis. Plasma concentrations of glucose, fructosamine and IGF-1 were analysed at the Clinical Pathology Laboratory (Murcia Veterinary University, Murcia, Spain) by procedures commonly used for domestic animals (Kaneko, 1989). Plasma glucose was measured using the laboratory hexokinase method. Fructosamine was determined by a nitroblue tetrazolium colorimetric test, based on the ability of the ketoamine group of glycated proteins to reduce tetrazolium salts under alkaline conditions. These assays were adapted to an automatic chemistry analyzer (Olympus AU400; Olympus, Tokyo,

Japan). The IGF-1 was analyzed with an automated solid-phase, enzyme-labeled chemiluminescent immunometric assay (Immulite System; Siemens Health Diagnostics, Deerfield, IL). The intra- and inter- assay CV were less than 15%.

Digestibility Experimental Design (Exp. 3)

This experiment was conducted in the Animal Nutrition Experimental Unit at the Veterinary Farm (University of Murcia, Murcia, Spain). Nine male pigs (Landrace × Large White gilts × Duroc boars) were assigned to three diets per period according to a replicated 3 × 3 Latin square arrangement of treatments to determine coefficients of apparent fecal digestibility (DM, OM, ether extract, CP, Ca and P) and N and mineral balances (with three replicates and three pigs in each square). Pigs were fed using a 2-phase feeding program. All pigs were allotted to the three dietary treatments in each feeding period, providing a total of nine individual observations per diet and feeding phase.

All pigs were housed in individual metabolism cages in an environmentally controlled room that allowed the feed intake to be monitored and the separate collection of urine and feces. The length and width of cages were adjustable to the actual size of the pigs. Room temperature was kept at 20ºC. All pigs were fed 2.4 times the energy requirement for maintenance, which was assumed to be 106 kcal of ME/kg of metabolic weight ($BW^{0.75}$; NRC, 1998). The initial BW of the pigs was 43 ± 3 and 74 ± 3 kg in the growing and finishing periods, respectively. Pigs had free access to water from nipple drinkers throughout the experiment.

For each replicate, the experimental periods lasted 10 d, consisting of an adaptation period of 5 d followed by a total collection of urine and feces for 5 d. The feces of each individual pig were collected once a day, weighed, and frozen (-20ºC).

The urine was collected individually in a 20-L plastic container, containing 50 mL of sulfuric acid (25% H_2SO_4) to lower the pH and avoid N volatilization and microbial growth. The daily urine was filtered through a mesh to remove any contaminants, weighed, and the volume was recorded; a 5%-aliquot was saved and frozen at -20ºC. At the end of the collection period, the feces of each pig were homogenized for further analysis using the same procedure as the urine samples.

Chemical Analyses

The DM content of feces was determined by drying a sample (20% of the total amount of feces produced) in a forced-air drying oven (60ºC) until it reached a constant weight. The DM content of the diets was determined by drying at 105ºC for 8 h. The moisture content of crude glycerin was determined by the Karl-Fischer method. Diet and fecal samples were ground through a 1-mm screen (Retsch ZM 200 Ultra Centrifugal Mill; Retsch, Haan, Germany) and analyzed for the ether extract, and N by Kjeldahl method (AOAC, 2005). Urine samples were also analyzed for their N content following the same procedure.

The mineral content of crude glycerin, feed, feces and urine was determined by dry ashing using a muffle furnace at 550ºC. Ashes were solubilized with 50 mL of 0.6 N nitric acid and subsequently filtered. The P content of feces, urine, diets and crude glycerin was determined by the Molibdate-Vanadate method (MAPA, 1998). The Ca, K, and Na concentrations in samples were determined by atomic absorption spectroscopy (Solaar M Series; Unicam, Cambridge, UK), and the chloride ion concentration was determined by Mohr tritation (MAPA, 1998).

The total glycerol and methanol contents of crude glycerin were analyzed by gas chromatography (TRACE GC Ultra; Thermo Electron Corporation, Rodano, Italy),

using a 30 m × 0.25 mm × 0.25 μm capillary column (Tracsil TR-FFAP; Teknokroma, Barcelona, Spain) equipped with a flame ionization detector. The injector and detector temperatures were kept at 220ºC and 250ºC, respectively. Helium was used as a carrier gas at a flow of 2 mL/min with a split ratio of 1:25. Hydrogen, air, and N fluxes were 30 mL/min, 300 mL/min, and 30 mL/min, respectively. Calibration was performed using standard samples of methanol and glycerol. Acetronitrile was added as internal standard. The data handling system was used to acquire and store data from gas chromatograph (Chrom Card; Thermo Electron Corportion). The glycerol content was also determined in urine samples collected in the digestibility trial.

Amino acids in feeds were determined by hydrolyzing the samples with 6 N HCl for 22 h at 112 ± 2ºC in glass tubes under a N atmosphere. Cystine and methionine were analyzed as cysteic acid and methionine sulfone, respectively, by oxidation with performic acid for 16 h at 0ºC (Llames and Fontaine, 1994). Tryptophan was not determined. The AA were separated by reverse-phase HPLC column (Waters, Milford, MA) controlled by a Breeze 2 system (Waters). The hydrolysates were derivatized with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate to determine primary AA, and AA were recorded (ACCQ.Tag 3.9 mm × 150 mm; Waters). Regarding the mobile phases used, solvent A was a 1:10 ratio [AccQ.Tag Eluent A Concentrate Commercial (Waters) to deionized water] and solvent B 60% (vol/vol) acetonitrile solutions. The fluorescent detector was adjusted to an excitation wavelength of 250 nm and an emission wavelength of 395 nm. Amino acids were quantified by comparing peak area of the samples with those of an internal standard (α-aminobutyric acid).

Statistical Analysis

All data were analyzed using SAS (SAS Inst. Inc., Cary, NC). Data for the manufacturing process, growth performance, and plasma metabolites concentrations

were analyzed by 1-way fixed model with dietary treatment as the main effect. The manufacturing batch or pen was considered as the experimental unit.

Grower and finisher digestibility data were analyzed separately, using the mixed model procedure (PROC MIXED), to account for effects of dietary treatment, replicate, animal and residual. The crude glycerin inclusion level was considered as a fixed effect, whereas replicate, animal, and residual were considered random effects. The model for digestibility analysis of Ca and P also included intake of Ca and P, respectively, as a covariate. All reported means are least square means, and orthogonal contrasts were conducted to determine linear and quadratic effects of dietary glycerin level. The significance level and tendencies were set at $P \leq 0.05$ and $P \leq 0.10$, respectively.

RESULTS AND DISCUSSION

Experimental Diets and Manufacturing Process (Exp. 1)

The crude glycerin used in the experiments contained 87.42% glycerol and 0.05% methanol. Moisture (7.98%) and glycerol and methanol contents were similar to those reported by other authors (Lammers et al., 2008b, 2008c; Schieck et al., 2010b; Shields et al., 2011). The ash content of crude glycerin was 5.87% (Ca: 0.04%, P: 0.01%, Na: 2.01%, Cl: 3.06%, and K: 0.05%). Mineral content should be considered in a diet formulation (Groesbeck et al., 2008; Kerr et al., 2009) since a high NaCl content could affect some characters like water-holding capacity of meat (Schieck et al., 2010b). Consequently, diets were formulated to have the same electrolyte balance in each feeding period (205 and 195 mEq/kg for grower and finisher diets, respectively). Analyzed composition indicated that the diets were prepared correctly, and only Na slightly decreased when crude glycerin was added (Table 3).

The effects of glycerin on pellet mill production efficiency are presented in Table 4. Electrical consumption was not affected by glycerin addition, and there was no effect on average motor load, which was initially set at 80%. However, crude glycerin supplementation at 0, 2.5 and 5% increased the feeder speed and production rate linearly ($P < 0.01$), resulting in a 20 to 29% improvement in the production rate compared with the control. Production efficiency improved with the addition of glycerin ($P < 0.01$), indicating that the energy required to manufacture one tonne of feed decreased as the level of crude glycerin increased in the feed. The PDI was numerically greater for diets containing glycerin, but the difference was not statistically significant.

Table 4. Effect of glycerin addition to feed on pellet mill production efficiency (Exp. 1)

	Diet[1]				P-value		
Item	G0	G2.5	G5	SE	Diet	Linear	Quadratic
No. of batches[2]	4	4	4				
Electrical consumption, kWh	125.7	111.7	106.3	6.29	0.13	0.06	0.59
Average motor load, %	77.3	75.5	78.0	1.20	0.36	0.67	0.18
Feeder speed, rpm	27.5	32.0	34.8	0.94	<0.01	<0.01	0.46
Production rate, kg/h	6,904	8,293	8,884	252.1	<0.01	<0.01	0.22
Production efficiency[3], kg/kWh	31.9	39.0	40.1	1.48	<0.01	<0.01	0.13
Pellet durability index, %	75.7	80.9	83.2	4.95	0.57	0.31	0.82

[1]Dietary treatments were 0, 2.5, or 5% crude glycerin inclusion for G0, G2.5, and G5, respectively.
[2]Two batches of each dietary treatment were manufactured per feeding period (growing and finishing). Preliminary results indicated that there were no dietary treatment and period interactions.
[3]Production efficiency was calculated by dividing kilograms of feed manufactured by electrical consumption in each batch.

Other studies have shown similar results. For example, Groesbeck et al. (2008) added crude glycerin (0 to 15%) to a corn-soybean meal diet and found that the addition of crude glycerin linearly increased the production efficiency and improved the pellet durability index. However, the addition of crude glycerin decreased the production rate despite an attempt to hold it constant, perhaps because of slight adjustments in steam pressure and temperature. Previous research conducted by Shields (2009) showed that the addition of crude glycerin improved pellet mill flow because of a reduction of

friction of the feed passing through the pellet die. Shields (2009) also reported an improvement in pellet mill efficiency and PDI as glycerin levels increased (0, 2.5, and 5%).

Growth Performance and Plasma Metabolites (Exp. 2)

Table 5 shows the effect of crude glycerin addition to feed on growth performance (ADG, ADFI and G:F) for each feeding period and for the whole period. For the growing period, ADG and ADFI were affected by dietary treatment. Glycerin-fed pigs had decreased ADG (linear, $P \leq 0.05$) and ADFI (quadratic, $P \leq 0.05$) compared with pigs fed the control diet. However, these differences were not reflected in the G:F. For the finishing period and for the whole period, growth performance was not affected by the dietary treatment, regardless of the trait studied. Final BW did not differ among groups.

Table 5. Effect of glycerin addition to feed on growth performance (Exp. 2).

	Diets[1]				
Item	G0	G2.5	G5	SE	P-value
No. of pens[2]	4	4	4		
Growing period					
ADG, [3] kg	0.77	0.72	0.72	0.013	0.04
ADFI, [4] kg	1.95	1.82	1.88	0.033	0.05
G:F, kg/kg	0.39	0.40	0.39	0.006	0.53
Finishing period					
ADG, kg	0.75	0.77	0.80	0.035	0.64
ADFI, kg	2.63	2.67	2.69	0.065	0.80
G:F, kg/kg	0.29	0.29	0.30	0.013	0.79
Growing-finishing period					
ADG, kg	0.75	0.74	0.76	0.018	0.90
ADFI, kg	2.26	2.21	2.25	0.046	0.73
G:F, kg/kg	0.34	0.34	0.34	0.008	0.92
Final BW, kg	95.2	94.1	96.1	1.74	0.73

[1] Dietary treatments were 0, 2.5, or 5% crude glycerin inclusion for G0, G2.5 and G5, respectively.
[2] Each pen housed 20 barrows. The initial BW was 30 ± 1.0 kg.
[3] Linear effect for diet factor ($P \leq 0.05$).
[4] Quadratic effect for diet factor ($P \leq 0.05$).

The variability and interpretation of results for growth performance reported by different authors is confusing. Although some studies found no effect of glycerin supplementation on the growth rate of growing-finishing pigs fed corn-soybean meal diets (Lammers et al., 2008b; Mendoza et al., 2010; Schieck et al., 2010b) or wheat-based diets (Hansen et al., 2009), others have identified a negative effect (Della Casa et al., 2009; Kerr et al., 2009). Moreover, Groesbeck et al. (2008) and Zijlstra et al. (2009) reported improvements in the ADG of nursery pigs after the inclusion of crude glycerin in corn- and wheat-based diets, respectively.

It is important to note that the inconsistency between current and previous results could be related to the precision of the estimates and the power of each study to detect statistically significant differences. When comparing our findings with those from previous studies, differences between the levels of glycerin in the diets and the degree of purity (crude or refined), as well as differences between the ingredients replaced by glycerin and interactions with other feed ingredients, should also be taken into account. From a productive point of view, our results showed that growth performance over the whole growing-finishing period was not affected by dietary treatment.

The effect of adding glycerin to feed on plasma metabolites is presented in Table 6. There was no effect of diets on any plasma metabolites. The results of the present study indicate that including glycerin at relatively low levels (less than or equal to 5% of the diet) did not affect plasma metabolite concentrations. Glycerol can be converted to glucose in the liver, and if the gluconeogenetic capacity is exceeded, the surplus of glycerol is most likely to be excreted in the urine (Kijora et al., 1995). After a 12-h fasting period, plasma glucose concentrations were, on average, 59 mg/dL less than those measured in animals fasted overnight (Hansen et al., 2009) and much less than the

levels reported by Lammers et al. (2008b) in animals fed their respective diets before bleeding. Generally, no differences in glucose and plasma metabolites because of the effect of dietary glycerin have been found in previous studies (Lammers et al., 2008b; Hansen et al., 2009). On the other hand, Shieck et al. (2010a) found a linear increase of lactose in milk, as dietary crude glycerin increased (up to 9%) in sow diets, indicating that sows metabolize a portion of the excess plasma glycerol, without affecting plasma glucose concentrations. No references to the concentrations of fructosamine or IGF-1, examined as glucose metabolism markers, are available for pigs fed diets including crude glycerin. In our experiment, there was a relevant inter-pen variability in plasma concentrations of IGF-1. The experimental CV was close to 35%; although, intra- and interassay CV for IGF-1 quantification were less than 15%.

Table 6. Effect of glycerin addition to feed on plasma metabolites (Exp. 2).

	Diets[1]				
	G0	G2.5	G5	SE	P-value
No. of pens[2]	4	4	4		
Glucose, mg/dL	60.5	62.5	53.6	2.18	0.27
Fructosamine, µmol/L	91.6	85.7	78.5	3.14	0.28
IGF-1, ng/mL	165.9	129.2	141.9	8.27	0.17

[1] Dietary treatments were 0, 2.5, or 5% crude glycerin inclusion for G0, G2.5 and G5, respectively.
[2] Blood samples for plasma analysis were collected from 5 barrows per pen.

Digestibility Experiment (Exp. 3)

The effects of added glycerin on nutrient digestibility, and N and mineral retention in each feeding period are presented in Table 7. The average feed intake was 1.18 and 1.65 kg DM/d for the growing and finishing period, respectively. The addition of crude glycerin tended to affect the coefficient of apparent fecal digestibility of DM during the growing period (linear, $P \leq 0.10$). In addition, fecal OM digestibility was affected by the glycerin content (linear, $P \leq 0.05$) when crude glycerin was added in both the growing and finishing periods. Also, the fecal digestibility of ether extract

increased linearly ($P \leq 0.05$) with increasing dietary glycerin in both periods. For both the growing and finishing periods, digestibility coefficients for DM, OM, and ether extract were greater than 87%. These results provide evidence that the inclusion of crude glycerin to replace 5% of corn in the grower and finisher diets for pigs could improve or, at least, maintain nutrient digestibility.

Table 7. Effect of crude glycerin addition on nutrient digestibility and N and mineral balance in growing and finishing periods (Exp. 3).

	Growing period					Finishing period				
	Diet[1]					Diet[1]				
Item	G0	G2.5	G5	SE	P-value	G0	G2.5	G5	SE	P-value
No. of pigs[2]	9	9	9			9	9	9		
ADFI, kg DM/d	1.15	1.17	1.20	0.04	0.66	1.65	1.66	1.66	0.03	0.98
Fecal digestibility, %										
DM	87.2	88.1	88.0	0.36	0.08	88.1	89.0	89.1	0.86	0.16
OM[3,4]	88.4	89.3	89.3	0.34	0.03	89.1	90.0	90.2	0.78	0.08
Ether extract[3,4]	88.1	88.0	90.1	0.73	0.02	91.3	92.1	92.6	0.56	0.05
CP	86.3	86.5	86.7	0.78	0.93	89.5	89.4	89.9	0.71	0.72
Ca[3,4]	59.8	62.0	67.6	1.98	0.04	77.7	70.2	62.9	2.15	<0.01
P[3,4]	66.4	60.7	60.0	1.20	<0.01	67.8	65.4	67.7	4.60	0.35
N and mineral balance, %										
N retention	65.9	65.5	68.7	5.25	0.75	64.7	63.6	68.1	6.44	0.63
Ca retention[4]	57.1	59.4	63.2	2.29	0.14	76.0	68.7	57.6	2.14	<0.01
P retention[3,4]	64.5	59.9	59.3	1.33	0.03	52.3	64.2	69.1	2.19	<0.01
Urine output, L/d	3.35	2.69	2.57	0.75	0.41	4.60	4.11	4.65	0.96	0.73

[1]Dietary treatments were 0, 2.5, or 5% crude glycerin inclusion for G0, G2.5, and G5, respectively.
[2]The initial BW of the male pigs was 43 ± 3.1 and 74 ± 3.3 kg in the growing and finishing periods, respectively.
[3]Linear effect for diet factor in growing period ($P \leq 0.05$).
[4]Linear effect for diet factor in finishing period ($P \leq 0.05$).

Fecal digestibility of CP and N retention were not affected by dietary treatment in either the growing or finishing period. The results show that crude glycerin addition had no effect on N retention because N ingestion and N excretion were not affected (data not shown). Few studies have examined the effects of crude glycerin on N digestibility in pigs. Groesbeck et al. (2008) found that the percentage of N digested

tended to decrease in pigs fed diets including crude glycerin compared with pigs fed diets containing soy oil. In previous studies conducted with rats (Chan et al., 1981), broilers (Simon et al., 1997) and humans (Brennan et al., 1975), the use of glycerin resulted in an improvement in N retention.

Glycerol is an inhibitor of important enzymes like phosphoenolpyruvate carboxykinase (Cryer and Bartley, 1973) and glutamate deshydrogenase (Steele et al., 1971), which take part in the AA gluconeogenic pathway. Thus, glycerol avoids the conversion of AA into glucose through gluconeogenesis, and can prevent protein catabolism. On the other hand, N retention depends on many factors, including nutrient availability, genetics, age, etc. (Simon et al., 1996, 1997). Thus, the efficiency of protein utilization by pigs depends on the dietary composition and the physiological status or the growing stage of the animals (Hernández et al., 2011).

The dietary level of crude glycerin affected the digestibility and balance of Ca and P (Table 7). In general, greater digestibility figures were associated with greater retention rates. Within the same feeding period, the data showed inverse relationship between Ca and P. Moreover, digestibility of Ca and P showed opposite trends with respect to the feeding period. In the growing period, the addition of crude glycerin increased Ca digestibility (linear, $P \leq 0.05$), whereas Ca digestibility decreased with greater levels of glycerin in the finishing phase ($P \leq 0.05$). In contrast, the digestibility of P decreased in growing period ($P \leq 0.05$), but no effect was found in the finishing period.

In regard to the mineral balance, no glycerin effect on Ca retention was found for the growing period, but the retention of Ca decrease with the addition of crude glycerin during the finishing period ($P \leq 0.05$). In contrast, P retention after glycerin

addition depended on the feeding period (i.e., linear decrease ($P \leq 0.05$) in the growing period and linear increase ($P \leq 0.05$) in the finishing period).

Studies examining the effects on the mineral balance of diets supplemented with crude glycerin are limited. In a study with laying hens, Swiatkiewicz and Koreleski (2009) concluded that the inclusion of crude glycerin up to 6% in the diets of layers had no effect on the retention or excretion of Ca and P. They also indicated that there was no experimental data reporting a negative influence of dietary glycerin on the availability of minerals. On the other hand, previous digestibility studies found greater urinary energy excretion and urine output in pigs fed high levels of glycerin than in pigs fed control diets (Lammers et al., 2008c; Mendoza et al., 2010). These findings suggest that glycerin could have a diuretic effect, which might affect the mineral balance, perhaps because glycerol excretion via urine increases with increasing glycerin doses in feed. Kijora et al. (1995) also demonstrated that glycerol level in urine increased as the level of dietary glycerin increased. However, at relatively low levels of crude glycerin, we found no diuretic effect of glycerin because there were no differences for urine output between dietary treatments (Table 7). In addition, glycerol was not detected in any analyzed urine samples.

CONCLUSION

Based on the current results, we conclude that adding crude glycerin to the diet before pelleting may improve feed mill production efficiency. From a productive point of view, our results showed that growth performance over the whole growing-finishing period was not affected by the inclusion of crude glycerin. Moreover, the addition of crude glycerin up to a level of 5% in the diet of growing-finishing pigs had no effect on nutrient digestibility and N balance. However, more research is needed on how crude glycerin may affect the mineral metabolism and balance.

CHAPTER 2

Effect of dietary crude glycerin on growth performance, nutrient digestibility and hormone levels of Iberian crossbred pigs from 50 to 100 kg body weight

ABSTRACT

The aim of this study was to determine the effect of crude glycerin addition on the growth performance, nutrient digestibility and blood hormone levels of Iberian crossbred pigs kept under intensive conditions. The study was carried out with 80 crossbred pigs (Iberian gilts × Duroc boars) of both sexes over a 101-d period (54 ± 3 kg initial BW). Treatments were arranged in a 2 × 2 factorial design, the factors being dietary treatment (control or 10% glycerin) and gender (barrow or gilt). Crude glycerin was included as a replacement for wheat in diets formulated to provide equal net energy and digestible lysine levels. Glycerin-fed pigs had higher average daily gain and average daily feed intake than pigs fed the control diet ($P < 0.05$). No differences were found in the gain to feed ratio. In regards the gender, barrows consumed more feed, grew more and reached a higher final BW compared with gilts ($P < 0.05$). Nutrient digestibility was not affected by the glycerin content or the gender. However, there was a tendency for acyl-ghrelin levels to be higher in glycerin-fed pigs ($P = 0.058$). Also, gilts showed increased concentrations of acyl-ghrelin and lower insulin compared with barrows ($P < 0.05$). In conclusion, 100 g/kg of glycerin can partially replace wheat without affecting feed efficiency or nutrient digestibility in Iberian crossbred pigs. However, further research is needed to clarify the potential relationship between glycerin inclusion levels in the diet and the plasmatic levels of hormones related to feed intake and energy balance control.

INTRODUCTION

Environmental concerns and biofuel policies have contributed to a significant increase in global biodiesel production. Biodiesel is produced by the transesterification of vegetable oils or animal fats with an alcohol, usually methanol, in the presence of a catalyst. In the reaction a large amount of glycerin is obtained as a byproduct, approximately 10% by weight of the biodiesel produced (Thompson and He, 2006). In recent years, the surplus of crude glycerin has led researchers to look for new ways of using this byproduct, one of which may be as a cost-competitive feedstuff. In fact, several researchers have determined that crude glycerin is a good alternative as a feed ingredient for swine (Kijora et al., 1995; Lammers et al., 2008b; Schieck et al. 2010b).

The Iberian (IB) pig is a native breed from the southwest of the Iberian Peninsula, traditionally reared under free range conditions and fed acorns and grass. The IB sector is of great economic importance in Spain due to the high acceptance of premium Iberian cured products. The increasing production of pigs of the Iberian type has allowed the sector to expand internationally to satisfy consumer demand for top quality meat. However, only 20% of the IB pigs slaughtered in Spain are produced under traditional systems (Ventanas et al., 2007). The use of non-conventional raw materials could provide higher flexibility in the formulation of the diets, especially when these pigs are reared under intensive growing systems.

Some studies on the use of glycerin in pig fattening have shown that up to 10% of crude glycerin can be added to the diet with no effect on performance, carcass composition and meat quality (Della Casa et al., 2009; Lammers et al., 2008b; Mendoza et al., 2010). However, no information has been published concerning feeding glycerin to IB pigs and its crosses. Therefore, the objective of the present work was to study the

effect of dietary crude glycerin and gender on the performance, digestibility and blood hormone levels of growing-finishing Iberian crossbred pigs.

MATERIAL AND METHODS

All experimental procedures were in compliance with the European Union regulations concerning the protection of animals used for experimental and other scientific purposes (Directive 2003/65/CE).

Animals, diets and experimental design

This experimental trial was carried out in a commercial fattening farm located in south-east Spain (Puerto Lumbreras, Spain) over a 101-d period. A total of 80 crossbred pigs (Iberian gilts x Duroc boars) of both sexes (50/50) with an average initial BW of 54 ± 3 kg were used. According to a 2 × 2 factorial design (diet × gender), pigs of the same sex were sorted by weight into eight pens of five pigs each, and then randomly assigned to one of two dietary treatments. Each pen represented one replicate (4 replicates per dietary treatment and sex). Pigs were fed either a barley-wheat meal-based diet without glycerin (G0; control) or a diet containing 10% crude glycerin (G10). The crude glycerin was included as a replacement for wheat. The crude glycerin used in this experiment (glycerol, 87.42%; methanol, 0.05%; moisture, 7.98%; ash, 5.87%; Ca, 0.04%; P, 0.01%; Na, 2.01%; Cl, 3.06%; and K, 0.05%) was obtained from a biodiesel production facility (Abengoa Bioenergía San Roque, Cadiz, Spain), which used vegetable oils as feedstock. Both diets were manufactured using the same batch of ingredients, and the crude glycerin was added, in equal amounts in mixer and molasse mixer, to the G10 diet. The ingredients and nutrient content of the diets are presented in Table 8. The diets were formulated to contain 9,420 kJ NE and 4.5 g ileal digestible lysine per kg, according to the recommendations for crossbred growing-finishing Iberian pigs (FEDNA, 2006). The diets contained titanium dioxide (5 g kg^{-1}) as an

indigestible marker to calculate the digestibility of nutrients. Both diets were offered in a mash form. Pigs were allowed *ad libitum* access to feed and water throughout the experiment (average final BW of 105 ± 8 kg).

Table 8. Ingredients and composition of diets (as-feed basis).

	Diet[1]	
Item	G0	G10
Ingredient, %		
Barley	45.00	45.00
Wheat	34.00	19.50
Wheat bran	5.00	7.78
Soybean meal (47% CP)	12.79	14.50
Crude glycerin	-	10.00
Animal fat	0.50	0.50
Titanium dioxide	0.50	0.50
L-Lys 50 (50% Lys)	0.10	0.11
Salt	0.35	0.35
Calcium carbonate	1.13	1.13
Monocalcium phosphate	0.33	0.33
Vitamin-trace mineral premix[2]	0.30	0.30
Calculated composition[3]		
NE, kJ/kg	9,420	9,420
CP, %	15.20	15.00
Ca, %	0.65	0.65
P total, %	0.47	0.46
Ileal digestible AA, %		
Lys	0.65	0.68
Met + Cys	0.45	0.43
Thr	0.42	0.43
Analyzed composition, % on DM basis except DM		
DM	91.96	91.30
CP	15.22	15.10
Crude fiber	3.42	3.76
NDF	14.10	13.61
ADF	6.53	4.72
Starch	52.07	44.83
Ash	3.95	4.22
Ca	0.63	0.67
P total	0.46	0.42

[1]Dietary treatments: 0 or 10% crude glycerin included in G0 and G10, respectively.
[2]Provided (per kg of complete diet): 7000 IU of vitamin A; 1500 IU of vitamin D_3; 20 mg of vitamin E; 4 mg of vitamin B_2; 1,5 mg of vitamin B_6; 0.020 mg of vitamin B_{12}; 20 mg of niacin; 8 mg of calcium pantothenate; 100 mg of choline chloride; 75 mg of zinc oxide; 40 mg of manganese (II) oxide; 75 mg of ferrous sulfate heptahydrate; 12 mg of cupric sulfate pentahydrate; 0,15 mg of sodium selenite; 1 mg of potassium iodate; 0,1 mg of basic cobaltous carbonate monohydrate; 500 FTU of phytase (EC 3.1.3.8).
[3]According to FEDNA (2010).

Data recording and sampling

All animals were weighed individually at the beginning and at the end of experimental period (on day 101). The individual BW of the pigs within each pen was used to calculate ADG on a pen basis. Average daily feed intake per pen was recorded by weighing feed added to the feeders and feed remaining at the end of the experiment. Also, G:F was calculated. To calculate the digestibility coefficients of nutrients, fecal grab samples were collected directly from the anus of three randomly selected pigs in each pen on d-58. The feces were pooled by pen and frozen at -18ºC for further analysis.

On d 95 of the experimental period, two animals were randomly selected from each pen for analysing hormones related to energy metabolism: ghrelin (two forms: acylated and unacylated) and insulin. Blood samples were collected via jugular venipuncture into 5 mL tubes without additives. After centrifugation at 3,000 × *g* for 10 min at 4ºC, the serum was collected and stored at -85ºC until further analyses.

Chemical analysis

The DM content of the diets was determined by drying a sample in a convection oven at 105ºC for 8 h (AOAC, 2005). Fecal samples were dried at 60ºC for 72 h. The moisture content of crude glycerin was determined by the Karl-Fischer method (AOAC, 2005). Diet and fecal samples were ground through a 1-mm screen (Retsch ZM 200 Ultra Centrifugal Mill; Retsch, Haan, Germany) and analyzed for crude protein (AOAC, 2005). Diets were also analyzed for crude fibre (AOAC, 2005), and acid and neutral detergent fibre according to the methods described by Van Soest et al. (1991). The starch content was measured polarimetrically using the official analytical method (BOE, 2000).

The mineral content of diets and crude glycerin was determined by dry ashing using a muffle furnace at 550ºC. Ashes were solubilized with 50 mL of 0.6 N nitric acid and subsequently filtered. The P content was determined by the Molibdate-Vanadate method (MAPA, 1998). The Ca, K, and Na content was detected by atomic absorption spectroscopy (Solaar M Series; Unicam, Cambridge, UK), and the chloride ion concentration was determined by Mohr tritation (MAPA, 1998).The total glycerol and methanol content of crude glycerin were analyzed by gas chromatography, as described by Madrid et al. (2013).

The titanium dioxide content of the diet and fecal samples was measured by the colorimetric method described by Myers et al. (2004), and the digestibility coefficients of nutrients were calculated from these data.

Serum insulin and acylated and total ghrelin were quantified using commercial RIA kits (PI-12K, GHRT-88HK, and GHRT-89HK, respectively; Linco Research, Saint Charles, MO, USA). The interassay CVs were less than 10%, and the intraessay CVs were less than 6% for total and acylated ghrelin, and less than 11% for insulin. Sensitivity for total ghrelin, acylated ghrelin, and insulin was 46.5 pg/mL, 7.8 pg/mL, and 0.28 µU/mL, respectively. The unacylated ghrelin levels were calculated by subtracting the acylated ghrelin from the total ghrelin.

Statistical analysis

All data were analyzed using the SPSS program (SPSS Inc., Chicago, IL, USA). Growth performance and digestibility data were analyzed by two-way ANOVA. The model included the effect of dietary treatment and sex as main effects, and the first order interaction. The pen was considered as the experimental unit. Individual blood data were analyzed using a mixed model to account for effects of dietary treatment, sex, pen, and residual. The dietary treatment, sex, and their interaction were considered as

fixed effects, whereas pen and residual were considered random effects. For blood data, normality was previously checked by using normal distribution plots. All reported means are least square means. The significance level and tendency were set at $P \leq 0.05$ and $P \leq 0.10$, respectively.

RESULTS

Growth performance and nutrient digestibility

Glycerin addition affected ADG and ADFI ($P < 0.05$) (Table 9). Glycerin-fed pigs had higher growth and feed consumption than pigs fed the control diet (G0). As a consequence, pigs fed the G10 diet showed higher final BW (107 kg *vs*. 103 kg), but the difference was not significant. The G:F was similar for both treatments. Barrows consumed more feed, grew more and reached a higher final BW compared with gilts ($P < 0.05$). Moreover, G:F was poorer for gilts than for barrows ($P = 0.042$). No interaction between glycerin addition and sex was found, regardless of the trait studied. Glycerin inclusion and sex had no effect on the digestibility of DM, OM and CP (Table 9). Moreover, no dietary treatment and gender interactions were found.

Blood hormone levels

At 95d, no differences for ghrelin and insulin were found between diets (Table 10), although there was a tendency for acyl-ghrelin levels to be higher in pigs fed the G10 diet ($P = 0.058$). Serum concentrations of acylated ghrelin and insulin were affected by the gender ($P < 0.05$), gilts showing higher acylated ghrelin and lower insulin concentrations than barrows. Diet and gender interactions were not found.

Table 9. Effect of sex and glycerin addition to feed on growth performance and nutrient digestibility of Iberian crossbred pigs.

	Diet[1]		Sex			P-value		
Item	G0	G10	Barrows	Gilts	SEM	Glycerin	Sex	G x S
Growth performance								
Replicates, pen	8	8	8	8				
Initial BW, kg	54	54	55	53	0.8	0.940	0.348	0.905
Final BW, kg	103	107	109	101	1.7	0.203	0.030	0.159
ADG, kg	0.47	0.51	0.53	0.46	0.009	0.041	0.002	0.180
ADFI, kg	2.32	2.52	2.52	2.33	0.035	0.013	0.017	0.406
G:F, kg/kg	0.203	0.203	0.209	0.197	0.002	0.939	0.042	0.363
Digestibility, %								
Replicates, pen	8	8	8	8				
DM	74.5	74.3	75.6	74.1	1.05	0.604	0.487	0.888
OM	79.9	77.4	78.3	77.0	0.93	0.768	0.503	0.913
CP	67.5	71.3	69.5	69.3	1.74	0.299	0.946	0.805

[1]Dietary treatments: 0 or 10% crude glycerin included in G0 and G10, respectively.

Table 10. Effect of sex and glycerin addition to feed on ghrelin and insulin concentration in serum.

	Diet[1]		Sex			P-value		
Item	G0	G10	Barrows	Gilts	SEM	Glycerin	Sex	G x S
Nº of pigs	16	16	16	16				
Des-acyl ghrelin, pg/ml	687	705	642	750	58	0.808	0.362	0.130
Acyl ghrelin, pg/mL	128	185	122	191	14.3	0.058	0.023	0.677
Insulin, µU/mL	5.9	6.5	8.1	4.3	0.85	0.732	0.035	0.436

[1]Dietary treatments: 0 or 10% crude glycerin included in G0 and G10, respectively.

DISCUSSION

Growth performance and nutrient digestibility

Crossbred pigs of IB are mainly reared under intensive conditions. These pigs have a high potential for fat accumulation and are slaughtered at higher weights (140-160 kg BW) than conventional pig genotypes. The IB pig is a rustic breed showing lower performance in comparison with industrial genotypes. For these reasons, to

improve feed efficiency and reduce production costs, new feeding strategies need to be developed.

In this study, pigs fed diets containing glycerin had higher ADFI and ADG than those fed the G0 diet, but these differences were not reflected in feed efficiency due to a proportional increase in both variables. Therefore, the inclusion of glycerin did not affect feed palatability and, furthermore, it may reduce dust problems when a mash form is used.

No references are available for IB pigs fed diets including glycerin. However, in other swine genotypes and breeds, previous studies have shown that crude glycerin can be used as a source of dietary energy (Lammers et al., 2008c; Mendoza et al., 2010), although with variable results in the growth performance (Della Casa et al., 2009; Lammers et al., 2008b). The differences between this and previous studies could be related to particular breeds or genotypes (lean *vs.* fat) of pigs, or to differences between the degree of purity (crude or refined) of the glycerin used and inclusion levels in the diets.

On the other hand, our results also showed that IB crossbred pigs present significant gender differences in growth, with barrows growing faster than gilts. The gender effect was in line with the literature in IB pigs (Peinado et al., 2008; Serrano et al., 2009) due to differences in the body composition between sexes.

Our results show that the inclusion of glycerin to replace 10% of wheat in diets for IB pigs did not influence nutrient digestibility. Few studies have examined the effects of glycerin on digestibility. Recently, Madrid et al. (2013) determined the coefficients of apparent fecal digestibility and nitrogen balance using Landrace × Large White pigs from 43 and 74 kg BW in metabolic cages. These authors found that

coefficients of digestibility of OM and ether extract increased linearly as crude glycerin increased, whereas there was a tendency for higher DM digestibility. However, neither CP digestibility nor N retention was affected by glycerin inclusion. In contrast, Groesbeck et al. (2008) found that the percentage of N digested tended to decrease in piglets fed diets including glycerin compared with diets containing soy oil.

Blood hormone levels

Ghrelin is a fast-acting hormone that increases appetite, operating as a meal initiation signal for the short-term regulation of energy balance (Klok et al., 2007, Steinert et al., 2013). Ghrelin has two major molecular forms: acylated and unacylated. The acylated form is considered the biologically active form, although recent studies point to different roles for these two forms (Delhanty et al., 2012). Our results showed that the coefficient of variation for acylated ghrelin levels (55%) was higher than that for unacylated ghrelin levels (44%). Moreover, there was a tendency for acyl-ghrelin levels to be higher in pigs fed the G10 diet, where these higher levels were associated with a higher feed intake.

Insulin is a hormone whose levels usually increase after feeding to provide energy to the cells (Vieira et al., 2010). Glycerin in the diet increases plasmatic glycerol, which can be converted to glucose in the liver, where insulin promotes the conversion of glucose into glycogen and fat. The results showed that the inclusion of glycerin did not affect blood levels of insulin. Previous studies including glycerin at relatively low levels also found no differences in plasma glucose concentrations (Hansen et al., 2009; Lammers et al., 2008b). In addition, Schieck et al. (2010a) found a linear increase of lactose in milk but no effect on blood glucose as the level of crude glycerin was increased in sow diets.

The regulation and relationship between these hormones have previously been described in mice, rats and humans. Several studies indicate that insulin may inhibit ghrelin secretion (Flanagan et al., 2003; Saad et al., 2002) and *vice versa*, with ghrelin having an inhibitory role on insulin release (Egido et al., 2002; Reimer et al., 2003). Moreover, plasma ghrelin levels were higher in women than in men (Makovey et al., 2007). In the current study, gilts also showed increased concentrations of acyl-ghrelin and lower insulin compared with barrows.

Some factors influencing the circulating concentrations of these hormones have been studied in pigs. For instance, Reynolds et al. (2010) reported how plasma concentrations of ghrelin and insuline changed according to the feeding pattern (*ad libitum vs.* restricted feeding). In addition, relatively little is known about interactions between these hormones and the diet composition. Yin et al. (2009) found that dietary supplementation with zinc increased plasma concentrations of ghrelin, as well as feed intake and piglet growth. Also in pigs, oral ingestion of tryptophan increased circulating levels of ghrelin (Zhang et al., 2007). The addition of feedstuffs, such as glycerin, may help increase feed intake and improve the efficiency of IB pig production. Our results showed that pigs fed diets containing glycerin had higher ADFI and ADG, a finding that was associated to a tendency for higher acyl-ghrelin levels.

CONCLUSION

The addition of crude glycerin up to 10% had no effect on either feed efficiency or nutrient digestibility of Iberian crossbred pigs from 50 to 100 kg BW. However, more research is needed to clarify the potential relationship between glycerin inclusion levels in the diet and the plasmatic levels of hormones related to energy metabolism.

CHAPTER 3

Addition of crude glycerin in pregnant and lactating sow diets: individual and litter performance, and metabolic and feed intake regulating hormones

ABSTRACT

An experiment was conducted to evaluate the effect of the dietary addition of crude glycerin on sow and litter performance, and to determine the serum levels of hormones related to energy metabolism and feed intake in sows during gestation and lactation. Sixty three sows were assigned randomly to one of three dietary treatments, containing 0, 3 or 6% crude glycerin (G0, G3, and G6, respectively) added to a barley-soybean meal-based diet as a replacement for cereals. During gestation, there was no effect of dietary treatment on any performance variable ($P > 0.05$), while during lactation, glycerin-fed sows consumed less feed than those fed the control diet ($P = 0.002$). Lactating sows fed the G3 diet had a higher body weight loss ($P = 0.017$), but these differences were not reflected in litter performance ($P > 0.05$). In gestation, the inclusion of glycerin did not affect blood levels of insulin or cortisol ($P > 0.05$). However, pregnant sows fed diets supplemented with glycerin showed lower and higher levels of acyl-ghrelin and leptin, respectively ($P < 0.01$). In lactating sows, there were no differences between dietary treatments for any of the hormones measured ($P > 0.05$). Before feeding, the acyl-ghrelin concentration was positively correlated with the cortisol during gestation ($r = 0.81$; $P < 0.01$) and lactation ($r = 0.61$; $P = 0.02$). In conclusion, the inclusion of up to 6% crude glycerin in the diet can partially replace corn without affecting the performance of pregnant sows, but not during lactation. Our results also suggest a relationship between glycerin inclusion in the diet and the serum levels of some feed intake regulating hormones, but more studies are needed to increase our understanding of hormone concentration-diet composition interactions.

INTRODUCTION

Biodiesel production from renewable sources has increased steadily over the past decade in the European Union (USDA-FAS, 2013), due to energy and environmental concerns and policies. The main byproduct of the biodiesel industry is crude glycerin, which represents about 10% (w/w) of the biodiesel produced (Thompson and He, 2006). Traditionally, glycerin has been refined and used to manufacture many value-added products, including drugs, foods and cosmetics. However, the quantity of glycerin produced nowadays may exceed the level of demand by traditional users. Moreover, given continued growth in biofuel production, refining all the crude glycerin will simply become non-viable, especially for smaller producers, who will not be able to assume the high costs of purification. This has led to a search for alternative ways of using this byproduct, including an evaluation of its nutritive value for animal feeding. However, the use of crude glycerin in feeds is limited by the glycerol content, which is usually higher than 80%.

Glycerol can be converted to glucose in the liver (Krebs and Lund, 1966). During gestation**,** glucose is the major energetic substrate for placental and fetal growth, and the glucose supplied to the placenta and fetus is entirely dependent upon its supply from the maternal circulation. During lactation, glucose is the principal precursor of lactose in the mammary gland (Boyd et al., 1995). Glucose availability to the mammary gland is the limiting source for the synthesis of lactose, playing a key role in milk production. Additionally, glycerol could stimulate water intake and improve heat stress tolerance, especially for lactating sows (Shieck et al., 2010a). Therefore, dietary crude glycerin incorporated in pregnant and lactating diets may improve sow and litter performance, although little information is available in this respect. Most studies to date have determined the energy values of crude glycerin in growing-finishing pig diets and

its effect on growth performance, carcass composition, and meat quality (Kijora et al., 1995; Della Casa et al., 2009; Lammers et al., 2008b; Mendoza et al., 2010).

On the other hand, the physiological status of sows during gestation and lactation involves different energy balances, which, in turn, can affect those hormones related to feed intake (Valros et al., 2003; Pére and Ettiene, 2007; Dong et al., 2009). Feed intake during gestation must provide the energy needed for fetal growth, particularly during late gestation, whereas the first half of gestation is considered an anabolic phase (Noblet et al., 1990). During lactation, the daily energetic requirements of sows are particularly high and, in general, are not met by voluntary feed intake (Noblet et al., 1990). Sow performance could depend on its ability to regulate its metabolism and feed intake, which is thought to be regulated by hormones such as insulin, ghrelin, leptin and cortisol (Satou et al., 2011).

The objective of this research was to determine the effect of dietary addition of crude glycerin on sow and litter performance, as well as to evaluate the serum levels of hormones related to energy metabolism and feed intake in sows during gestation and lactation.

MATERIAL AND METHODS

The experimental procedures were in compliance with the European Union regulations concerning the protection of animals used for experimental and other scientific purposes (EU Directive 2010/63/EU).

General Procedures and Experimental Diets

This experimental trial was performed from December 2011 to March 2012 on a commercial farm located in Huércal-Overa (Almería, Southeastern Spain). A total of 63 pregnant sows (Landrace × Large White) of mixed parity (parity 2-5) were used in the

experimental period, which covered gestation, lactation and the pre-weaning period. After confirmation of gestation by ultrasonography at 28 d post-insemination, the sows were randomly assigned to one of three dietary treatments, with an average initial BW of 190 kg. Experimental diets contained 0, 3, and 6% crude glycerin (G0, G3, and G6, respectively (Table 11). All diets were manufactured using the same batch of ingredients, and crude glycerin was added to a barley-soybean meal-based diet as a replacement for cereals. The crude glycerin used in this experiment (glycerol, 87.42%; methanol, 0.05%; moisture, 7.98%; ash, 5.87%; Ca, 0.04%; P, 0.01%; Na, 2.01%; Cl, 3.06%; and K, 0.05%) was obtained from a biodiesel production facility (Abengoa Bioenergía San Roque, Cadiz, Spain), which used vegetable oils as feedstock. Gestation and lactation diets were formulated to be isoenergetic and iso-AA. Feed formulation was based on NE and ileal digestible AA according to the recommendations of Fundación Española para el Desarrollo de la Nutrición Animal (FEDNA, 2006). In the formulation of both diets, the energy value of glycerin for sows was 12.05 MJ NE/kg (FEDNA, 2010).

Table 11. Ingredients and composition of diets (as fed-basis).

	Gestation			Lactation		
	Crude glycerin, %			Crude glycerin, %		
Item	0	3	6	0	3	6
Ingredient, %						
Barley	57.90	56.92	55.93	49.19	45.30	41.51
Wheat bran	19.57	20.08	20.60	10.00	10.00	10.00
Corn	10.00	7.00	4.00	12.00	12.00	12.00
Soybean meal, 440 g CP/kg	8.93	9.43	9.93	20.19	21.11	21.91
Animal fat	0.50	0.50	0.50	3.80	3.80	3.80
Crude glycerin	-	3.00	6.00	-	3.00	6.00
Calcium carbonate	1.65	1.64	1.62	1.59	1.62	1.66
Monocalcium phosphate	0.75	0.76	0.78	0.95	0.95	0.95
Sodium chloride	0.40	0.37	0.34	0.50	0.46	0.42
L-Lysine 50 (50% Lys)	-	-	-	0.08	0.06	0.05
VTM premix[1]	0.30	0.30	0.30	1.70	1.70	1.70
Calculated composition[2]						
NE, MJ/kg	8.97	8.95	8.93	9.67	9.64	9.67
CP, %	14.00	14.00	14.00	17.00	17.00	17.00
Ileal digestible Lys, %	0.57	0.58	0.59	0.84	0.84	0.84
Ca, %	0.87	0.86	0.86	0.98	1.05	1.01
P, %	0.58	0.58	0.57	0.63	0.63	0.62
dEB[3], mEq/kg	0.65	0.64	0.63	0.59	0.63	0.65
Analyzed composition, % on DM basis except for DM						
DM	89.84	89.81	88.14	90.20	90.11	89.84
CP	14.80	14.73	14.57	18.48	18.35	17.80
CF	7.71	8.18	7.99	6.78	7.37	6.82
Ca	0.86	0.88	0.84	0.98	1.04	1.01
P, total	0.55	0.54	0.54	0.66	0.64	0.62
AA, total						
Lys	0.71	0.67	0.63	1.06	0.91	0.97
Met	0.24	0.23	0.26	0.28	0.28	0.31
Cys	0.33	0.30	0.33	0.35	0.34	0.33
Thr	0.57	0.55	0.61	0.74	0.78	0.80

[1]For gestation, supplied per kg of diet: vitamin A (trans-retinyl acetate), 12,500 IU; vitamin D_3 (cholecalciferol) 2,000 IU; vitamin E (dl-alpha tocopheryl acetate), 20 mg; vitamin K_3 (bisulfite menadione complex), 2 mg; vitamin B_1 (thiamine-mononitrate), 1 mg; riboflavin , 5 mg; pyridoxine (pyridoxine HCl), 2.5 mg; vitamin B_{12} (cyanocobalamin), 0.020 mg; niacin, 25 mg; pantothenic acid (D-Ca pantothenate), 12.5 mg; choline (choline chloride), 300 mg; D- biotin 0.1 mg; Zn (ZnO), 100 mg; Mn ($MnSO_4.H_2O$), 80 mg; Fe ($FeSO_4.7H_2O$), 100 mg; Cu ($CuSO_4.5H_2O$) 10 mg; Se (Na_2SeO_3), 0.22 mg; I (KI) 0.5 mg; Co ($2CoCO_3$. 3 $Co(OH)_2$. H_2O), 0.5 mg, 3-phytase (EC 3.1.3.8) 500 FTU.
For lactation, supplied per kg of diet: vitamin A (trans-retinyl acetate), 12,000 IU; vitamin D_3 (cholecalciferol) 2,000 IU; vitamin E (DL-alpha tocopheryl acetate), 40 mg; vitamin K_3 (bisulfite menadione complex), 2 mg; vitamin B_1 (thiamine-mononitrate), 1 mg; riboflavin , 5 mg; pyridoxine (pyridoxine HCl), 2.5 mg; vitamin B_{12} (cyanocobalamin), 0.030 mg; folic acid, 2.5 mg; niacin, 25 mg; pantothenic acid (D-Ca pantothenate), 12.5 mg; choline (choline chloride), 250 mg; D- biotin 0.3 mg; Zn (ZnO), 100 mg; Mn ($MnSO_4.H_2O$), 80 mg; Fe ($FeSO_4.7H_2O$), 100 mg; Cu ($CuSO_4.5H_2O$) 10 mg; Se (Na_2SeO_3), 0.22 mg; I (KI) 0.5 mg; Co ($2CoCO_3$.3 $Co(OH)_2$. H_2O), 0.5 mg, 3-phytase (EC 3.1.3.8) 500 FTU. Saccharomyces cerevisiae NCYC Sc 47 5.10^{12} UFC.
[2]According to FEDNA (2010).
[3] dEB, dietary electrolyte balance.

Housing, management and data collection

During gestation, sows were housed in individual stalls (2.2 m long × 0.6 m wide). A few days before farrowing, sows were moved into farrowing rooms and placed in individual crates (2 m long × 1.5 m wide) to prevent piglets being crushed. Each gestation and farrowing crate was equipped with a feeder and a nipple drinker. The piglet area was heated by under floor heating.

All experimental diets were offered in restricted amounts during gestation (from 28 days post-insemination). Sows were fed 2.5 kg of their respective dietary treatments once a day (at 08:00 h). From the second postpartum day, the sows were fed lactation diet twice a day (at 08:00 and 16:00 h). The initial amount of feed was adjusted daily for each lactating sow by increasing the amount supplied by 0.5 kg when no refusal was observed until reaching the maximal feed intake. The refused feed was weighed and removed every morning, and feed intake was recorded by subtracting the refused feed from the feed offered.

Sows were weighed at days 28 and 110 of gestation, within 24 h of farrowing, and at weaning. During weighing, backfat thickness was measured at the P2 position (last rib, 65 mm from the center line of the back) to assess body condition and BW changes. At farrowing, the number of piglets born and born alive per litter was recorded immediately. Within the first 48 h, litters were cross-fostered within each dietary treatment to adjust litter size to 11 piglets per sow. All subsequent piglet deaths were recorded daily for each litter. From 7 days of age, suckling piglets had access to a commercial pre-starter diet. Piglets were weaned at 21 days. The litter weight was recorded at birth, after cross-fostering and weaning.

On day 100 of the gestation and on day 11 post-partum, eight sows were randomly selected from each dietary treatment to analyze ghrelin (total and acylated),

insulin, leptin, and cortisol levels. Blood samples were collected via jugular venipuncture into 4 mL tubes (BD Vacutainer) at 0 min and 30 min after feeding. After centrifugation at 3000 × *g* for 10 min at 4ºC, the serum was collected and stored at −85ºC until further analysis.

Chemical Analyses

The DM content of the diets was determined by drying a sample in a convection oven at 105ºC for 8 h (AOAC, 2005). Diet samples were ground through a 1-mm screen, and analyzed for crude protein and crude fiber content (AOAC, 2005).

The mineral content of diets was determined by dry ashing using a muffle furnace at 550ºC. Ashes were solubilized with 50 mL of 0.6 N nitric acid and subsequently filtered. The P content was determined by the Molibdate-Vanadate method (MAPA, 1998). The Ca content was detected by atomic absorption spectroscopy (Solaar M Series; Unicam, Cambridge, UK)

Amino acids in feeds were determined by hydrolyzing the samples with 6 N HCl for 22 h at 112 ± 2ºC in glass tubes under an N atmosphere. Cystine and methionine were analyzed as cysteic acid and methionine sulfone, respectively, by oxidation with performic acid for 16 h at 0ºC (Llames and Fontaine, 1994). Tryptophan was not determined. The AA were separated by reverse-phase HPLC column (Waters, Milford, MA, USA) controlled by a Breeze 2 system (Waters, USA). The hydrolysates were derivatized with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate to determine primary amino acids, which were recorded on a Waters ACCQ.Tag (3.9 mm × 150 mm). Regarding the mobile phases used, solvent A and B were a 1:10 ratio [ACCQ.Tag Eluent A Concentrate Commercial (Waters) to deionized water] and a 60% (vol/vol) acetonitrile solutions, respectively. The fluorescent detector was adjusted to an

excitation wavelength of 250 nm and an emission wavelength of 395 nm. Amino acids were quantified by comparing the peak area of the samples with those of an internal standard (α-aminobutyric acid).

The moisture content of crude glycerin was determined by the Karl-Fischer method (AOAC, 2005). The mineral composition of crude glycerin was analyzed by atomic absorption spectroscopy as described for diets. The total glycerol and methanol content of crude glycerin were analyzed by gas chromatography (TRACE GC Ultra, Thermo Electron Corporation, Rodano, Italy), using a 30 m x 0.25 mm x 0.25 μm capillary column (Tracsil TR-FFAP; Teknokroma, Barcelona, Spain) equipped with a flame ionization detector, and as described by Madrid et al. (2013).

Serum insulin and acylated and total ghrelin were quantified in duplicate using commercial RIA kits (PI-12K, GHRT-88HK, and GHRT-89HK, respectively; Linco Research, Saint Charles, MO, USA). Leptin concentration was measured by the Linco Multi-species assay Kit (Linco Research) previously validated for porcine plasma (Govoni et al., 2005). Cortisol was analyzed by chemoluminiscence as described by Escribano et al. (2012). The intra-assay coefficients of variation were less than 15% in all the assays. Pig serum pool dilutions resulted in linear regression equations for all the kits, with correlation coefficients close to 1. Runs test revealed no deviation from linearity.

Statistical Analysis

Sow and litter performance data were analyzed by one way ANOVA using the General Linear Model (GLM) procedure of SPSS software (SPSS Inc., Chicago, IL). Hormone-related data were analyzed using a mixed model to account for effects of dietary treatment, sampling time (before -0 min- and 30 min after feeding), animal, and residual. The dietary treatment, sampling time, and the first order interaction were

considered as fixed effects, whereas animal, and residual were considered random effects. The normality of blood data was previously checked by using normal distribution plots. For each period (gestation and lactation), Pearson's correlation coefficients were calculated between the concentrations of the hormones studied within each sampling time. Comparisons between groups were assessed using a Bonferroni post-hoc test. All reported means are least square means. The significance level and tendency were set at $P \leq 0.05$ and $P \leq 0.10$, respectively.

RESULTS

Table 12 shows the effect of incorporating crude glycerin in feed on sow performance. Neither gestation length nor lactation length differed between dietary treatments. Body weight at 28 d and 110 d of gestation, and BW change during gestation were not affected by dietary treatment ($P > 0.05$). Dietary treatment had also no effect on backfat depth or the feed intake of pregnant sows ($P > 0.05$). The feed intake was similar for all treatments, averaging 2.2 kg DM/d. During lactation, there were no differences in BW at farrowing or backfat depth ($P > 0.05$). However, there was a tendency for BW at weaning to differ between dietary treatments ($P = 0.075$). Sows fed 3% glycerin showed lower BW at 21 d of lactation than sows fed the control diet. The tendency for BW was also reflected in BW change of lactating sows. Sows fed the G3 diet showed a greater loss of live weight ($P < 0.05$); although the BW change was not different between glycerin-fed sows, or between sows fed 6% glycerin and those fed the G0 diet. In addition, glycerin-fed sows had a lower feed intake than those fed the control diet ($P < 0.05$). The ADFI for lactating sows fed the G3 and G6 diets was 12 and 7% lower, respectively, than for the G0 diet.

Table 12. Effect of glycerin addition to feed on sow performance.

Item	Diets[1]				
	G0	G3	G6	SE	P-value
No. of sows	21	21	21		
Gestation length, d	114.4	114.6	115.4	0.20	0.157
Lactation length, d	21.3	21.6	21.0	0.20	0.387
Sow BW, kg					
Early gestation, at 28 d	188.9	189.1	190.3	2.62	0.971
Late gestation, at 110 d	229.1	228.0	231.8	2.30	0.787
Farrowing, within 24h	207.6	204.7	205.9	2.30	0.876
Weaning, at 21 d	198.9[b]	184.1[a]	190.5[ab]	2.63	0.075
Gestation BW change	40.3	38.9	41.5	1.42	0.757
Lactation BW change	-8.7[a]	-20.6[b]	-15.4[ab]	1.67	0.017
Sow backfat thickness, mm					
Early gestation, at 28 d	14.0	13.3	13.0	0.28	0.320
Late gestation, at 110 d	15.4	14.2	13.8	0.32	0.105
Farrowing, within 24h	14.2	13.9	13.3	0.31	0.494
Weaning, at 21 d	11.9	11.4	10.9	0.22	0.226
Gestation backfat change	1.4	1.0	0.8	0.28	0.625
Lactation backfat change	-2.3	-2.5	-2.4	0.19	0.951
Sow ADFI, kg DM/d					
Gestation	2.2	2.2	2.2	0.01	0.538
Lactation	4.2[a]	3.7[b]	3.9[b]	0.06	0.002

[1] Dietary treatments were 0, 3, or 6% crude glycerin inclusion.
[a,b] Within a row, means without a common superscript differ significantly ($P < 0.05$).

The effects of glycerin on litter performance are presented in Table 13. The average number of piglets born and born alive per litter was 13.7 and 11.7, respectively, with no difference between dietary treatments ($P > 0.05$). At farrowing, litters were cross-fostered to adjust litter size and avoid initial differences among dietary treatments ($P > 0.05$). There was also no effect on litter size at weaning ($P > 0.05$). In addition, the amount of glycerol in the diet did not affect pre-weaning mortality of the piglets ($P > 0.05$), the weight of litters after cross-fostering ($P > 0.05$), or weight of litters at weaning ($P > 0.05$). The pre-weaning weight gain of litters, corrected by the weight of dead piglets, and the ADG of piglets were similar for all treatments ($P > 0.05$), with an average of 41.1 kg and 0.2 kg/d, respectively.

Table 13. Effect of glycerin addition to feed on litter performance.

Item	Diets[1]				
	G0	G3	G6	SE	P-value
No. of litters	21	21	21		
Litter size					
Total piglets born	12.8	13.8	14.4	0.42	0.302
Born alive	11.3	11.6	12.2	0.36	0.605
After cross-fostering, within 48h	10.8	11.1	11.7	0.19	0.178
Weaning, at 21 d	9.9	10.3	10.3	0.19	0.602
Piglet preweaning mortality[2], %	7.6	7.2	11.2	1.27	0.374
Litter weight, kg					
Total born litter wt, kg	18.1	18.8	19.6	0.53	0.511
After cross-fostering, within 48h	17.5	17.0	16.7	0.49	0.767
Weaning, at 21 d	56.3	56.8	56.1	1.30	0.973
Gain[3]	40.5	40.8	41.9	0.95	0.802
Piglet initial BW[4], kg	1.64	1.54	1.44	0.04	0.141
Piglet weaning BW, kg	5.67	5.53	5.47	0.10	0.692
Piglet ADG, kg	0.20	0.19	0.20	0.00	0.604

[1]Dietary treatments were 0, 3, or 6% crude glycerin inclusion.
[2]Calculated for each litter from the number of piglets after cross-fostering.
[3]Corrected by the weight of dead piglets.
[4]Piglet weight after cross-fostering.

The effect of adding glycerin to feed on the serum levels of hormones related to energy metabolism and feed intake is presented in Table 14. At 100 d, pregnant sows fed diets supplemented with glycerin showed lower and higher levels of acyl-ghrelin and leptin, respectively ($P < 0.01$). Insulin and cortisol were not affected by dietary treatment ($P > 0.05$). In lactation, there were no differences between dietary treatments for any of the hormones measured ($P > 0.05$). The measurement time effect, before or after feeding, was also examined. Serum concentration of insulin and leptin changed 30 min after feeding ($P < 0.05$). Insulin concentrations were higher after feeding in both pregnant and lactating sows ($P < 0.001$). The behaviour of leptin, before and after feeding, depended on the physiological status of the sows. Feeding led to an increase in leptin concentration in pregnant sows ($P < 0.05$) and a decrease in lactating animals ($P < 0.05$). No differences in concentration for the other hormones studied were found,

although there was a tendency for cortisol levels to be lower in lactating sows after feeding ($P = 0.052$). In general, diet and sampling time interactions were not significant, except in the case of acylated ghrelin in lactating sows ($P < 0.001$).

Pearson's correlation coefficients were calculated between each of the hormones studied in pregnant and lactating sows within sampling time: before (0 min) and 30 min after feeding. Before feeding, the total ghrelin concentration of sows during gestation was negatively correlated with acyl-ghrelin ($r = -0.47$; $P < 0.05$), and the concentration of acyl-ghrelin was positively correlated with the cortisol ($r = 0.81$; $P < 0.01$). Thirty minutes after feeding, total ghrelin still showed a negative correlation with acyl-ghrelin ($r = -0.48$; $P = 0.05$), and the concentration of acyl-ghrelin was negatively correlated with leptin ($r = -0.73$; $P < 0.01$). During lactation, total ghrelin and acyl-ghrelin concentrations were correlated with insulin ($r = 0.50$; $P < 0.01$) and cortisol ($r = 0.61$; $P < 0.05$) at 0 min, respectively. After feeding, the concentration of leptin in lactating sows was negatively correlated with total ghrelin ($r = -0.59$; $P < 0.01$) and positively correlated with insulin ($r = 0.46$; $P < 0.05$).

Table 14. Effect of glycerin addition and sampling time on plasmatic concentration of some hormones in sows during gestation and lactation.

	Diets[1]			Sampling time[2]		SEM	P-value		
Item	0G	3G	6G	0 min	30 min		P	T	PxT
No. of sows	8	8	8	24	24				
Gestation									
Total ghrelin, pg/mL	755.1	811.2	832.8	783.2	816.3	18.08	0.209	0.110	0.183
Acyl-ghrelin, pg/mL	113.0[a]	50.3[b]	49.6[b]	76.7	65.3	8.65	0.006	0.224	0.985
Insulin, µU/mL	15.14	15.84	21.80	4.63[a]	30.55[b]	2.973	0.607	<0.001	0.174
Leptin, ng/mL	2.57[b]	3.87[a]	4.77[a]	3.50[a]	3.97[b]	0.199	<0.001	0.025	0.553
Cortisol, µU/mL	4.25	3.41	2.19	3.20	3.36	0.449	0.253	0.750	0.710
Lactation									
Total ghrelin, pg/mL	653.7	698.6	639.8	666.1	662.0	29.70	0.701	0.848	0.788
Acyl-ghrelin, pg/mL	37.7	53.3	49.2	44.6	48.8	6.59	0.608	0.322	<0.001
Insulin, µU/mL	15.65	10.05	15.38	8.83[a]	18.56[b]	1.718	0.338	<0.001	0.384
Leptin, ng/mL	3.19	2.73	3.26	3.25[b]	2.86[a]	0.253	0.645	0.031	0.785
Cortisol, µU/mL	3.96	4.78	3.95	4.61	3.85	0.547	0.785	0.052	0.582

[1] Dietary treatments were 0, 3, or 6% crude glycerin inclusion.
[2] Measurement time with respect to feeding time: before (0 min) and 30 min after feeding.
[a,b] Within a row, means without a common superscript differ significantly ($P < 0.05$).

DISCUSSION

Several authors have evaluated the effects of feeding crude glycerin to pigs during weaning (Groesbeck et al., 2008; Zijlstra et al., 2009) and growing-finishing periods (Kijora et al., 1995; Della Casa et al., 2009; Lammers et al., 2008b; Mendoza et al., 2010). However, studies with sows fed diets containing crude glycerin have rarely been conducted (Schieck et al., 2010a).

In our study, the inclusion of up to 6% crude glycerin in diets for pregnant sows did not affect any performance variable. In addition, ADFI did not differ among treatments, taking into account that all diets were offered in restricted amounts during gestation, and there was no leftover feed from the feed offered. To our knowledge, there are no previous studies regarding the effects of glycerin supplementation in diets for sows during gestation with which to compare these results. However, it is important to highlight that the amount of crude glycerin added to diets was relatively low compared with other studies in piglets and growing-finishing pigs. The inclusion levels tested in this study did not exceed the maximum level recommended by FEDNA (2010) for pregnant sows (6%).

During lactation, glycerin-fed sows had a lower ADFI than those fed the control diet. In contrast, Schieck et al. (2010a) reported that lactating sows fed a 3% glycerin diet had greater feed intake than those fed 6% glycerin, although no differences on ADFI were found when glycerin-supplemented diets were compared to a control diet. Crude glycerin inclusion in the diets of growing-finishing pigs has also provided conflicting results as regards growth performance. Some studies found enhanced ADG and ADFI in pigs fed glycerin (Kijora et al., 1995; Schieck et al., 2010b), while others reported negative effects (Della Casa et al., 2009) or no effects on the growth rate and feed efficiency (Lammers et al., 2008b; Mendoza et al., 2010). The variability of these

results may be attributed partially to differences in the chemical composition and energy value of crude glycerin, and the ingredients replaced by it.

When feeding lactating sows, the aim is to minimize the loss of body mass by providing them with energy and nutrients**.** Crude glycerin has an energy content similar to that of corn, and can be used by the pig as a source of energy when included in the diet (Lammers et al., 2008c; Kovács et al., 2011). Moreover, glycerol is a precursor of glucose, which participates in lipogenesis and can prevent protein catabolism (Tao et al., 1983). However, in our study, lactating sows fed G3 diet showed a higher body weight loss, although this difference was not observed in backfat thickness during lactation, which was similar among treatments. The lower BW at weaning of sows fed 3% glycerin could be attributed to the lower ADFI of these sows. Conversely, Schieck et al. (2010a) found that dietary crude glycerin did not affect either BW changes or backfat depth during lactation. Moreover, these authors evaluated the incorporation of up to 9% crude glycerin, which exceeds the upper level of 5% recommended by FEDNA (2010) for lactating sows.

Glycerol is highly gluconeogenic, glucose being the limiting source for the synthesis of lactose, and consequently for milk production. In fact, dietary crude glycerin has been shown to increase the milk lactose content in sows, although it was not reflected in milk yield (Schieck et al., 2010a). Additionally, glycerol stimulates water intake, and and increased water intake supplies the mammary gland with the water necessary for milk synthesis. These effects led us to hypothesize that the dietary supplementation of crude glycerol during lactation might improve litter performance. Our results showed that the addition of up to 6% crude glycerin to sow diets has no detrimental effects on litter traits, which did not differ from those obtained with the control diet. Schieck et al. (2010a) reported a linear decrease in litter BW gain as the

glycerol content of the diet increased from 0 to 6%, probably related to the lower ADFI of sows fed 6% glycerin. However, in our study, litter performance was not affected by the dietary addition of crude glycerin, despite the lower ADFI observed in sows fed glycerin.

Several hormones related to metabolism and feed intake were examined in gestating and lactating sows, when different energy balances are to be expected. Our results showed that dietary glycerin affected acyl-ghrelin and leptin levels in pregnant sows, while no differences between dietary treatments were found for insulin and cortisol, or any of the hormones measured in lactation.

In general, previous studies including dietary glycerin in growing-finishing pigs found no differences in insulin (Orengo et al., 2014), cortisol (Lammers et al., 2008b) or glucose (Hansen et al., 2009) levels between treatments. Likewise, Schieck et al. (2010a) found no effect on plasma glucose as the level of crude glycerin was increased in sow lactation diets. Insulin reduces blood glucose levels by enhancing glucose uptake and use by the cells as an energy source, while cortisol increases plasma glucose in response to stress and its low level in blood (Koopmans et al., 2005). In this study, pregnant sows fed diets containing glycerin had lower levels of acyl-ghrelin and higher levels of leptin at 0 min, suggesting that dietary supplementation of glycerin may exert a satiating effect. Ghrelin is a fast-acting hormone that stimulates appetite by acting as a meal initiation signal for the short-term regulation of the energy balance (Klok et al., 2007; Steinert et al., 2013), and whose the acylated form is considered the biologically active form (Kojima et al., 1999). On the other hand, leptin is involved in the long-term regulation of the energy balance, and is capable of inhibiting feed intake (Barb et al., 1998).

The circulating concentrations of some of these hormones were also dependent on the measurement time (0 min and 30 min after feeding). The postfeeding serum concentrations of insulin in sows during gestation and lactation were higher at 30 min. This was not unexpected taking into account that insulin usually increases after feeding to provide energy to the cells (Koopmans et al., 2005). Regarding leptin, the postprandial concentrations depended on the physiological status of the sows: while feeding led to an increase in leptin concentration in sows during gestation, there was a decrease during lactation. This interaction effect has also been described by Martínez et al. (2014), and suggests a higher satiety in sows during gestation, a period in which the energy balance is more likely to be positive. Leptin levels increase when an individual has a positive energy balance, acting as a satiety signal that decreases appetite and food intake in humans (Klok et al., 2007). Also, De Rensis et al. (2005) concluded that plasma leptin in sows is associated with backfat depth.

The regulation and interactions between these hormones have previously been described in humans (Klok et al., 2007) and other species (Ma et al., 2012). Most studies on the relationship between food intake-regulating hormones and diet have focused on understanding the underlying mechanisms involved in obesity and its related disorders in humans (Schwarz et al., 2011). In this study, the strongest correlation was found between the concentration of acyl-ghrelin and cortisol in both pregnant and lactating sows at 0 min. In this sense, several studies in mice and humans have found that serum ghrelin concentrations increase in stress conditions (Chuang et al., 2011; Stengel et al., 2011), which also increase the plasma levels of cortisol. Our results showed that pregnant sows fed diets containing glycerin had lower levels of acyl-ghrelin at 0 min, associated to a numerically lower concentration of cortisol. In addition to the strong linear correlation between both hormones, these findings suggest that dietary

supplementation of the gestation diet with glycerin may also minimize hunger pangs and improve welfare, although relatively little is known about any interactions between these hormones and diet composition. However, in this line of research, Yin et al. (2009) found that dietary zinc supplementation increased plasma concentrations of ghrelin, as well as feed intake and growth in piglets. Also in pigs, the oral ingestion of tryptophan increased ghrelin expression in gastric fundus and the circulating levels of ghrelin (Zhang et al., 2007).

CONCLUSION

Based on the results of the present study, it can be concluded that up to 6% crude glycerin can be used to partially replace corn in diets for pregnant sows, but not for lactating animals. Furthermore, the addition of crude glycerin seems to affect the serum levels of some feed intake-regulating hormones. However, further research is needed to determine the effects of crude glycerin on the energy balance in relation to the physiological status of the animal.

CONCLUSIONS

1. The addition of crude glycerin to the diet before pelleting increased feeder speed (rpm) and production rate (kg/h), which resulted in improved feed mill production efficiency (kg/kWh).

2. Feeding up to 5% crude glycerin to growing-finishing pigs (Large White × Landrace) had no effects on growth performance and plasma metabolites. Crude glycerin inclusion in replacement of corn slightly improved organic matter and ether extract digestibilities, and did not affect N balance and mineral metabolism.

3. Iberian crossbreed pigs (Iberian gilts × Duroc boars) can be fed with 10% crude glycerin in replacement of wheat without affecting performance and nutrient digestibility. Pigs fed 10% crude glycerin had higher levels of acyl-ghrelin, which were related to a greater ADFI and ADG.

4. Crude glycerin can replace up to 6% of corn in diets for pregnant sows without any detrimental effects on sow and litter performance. However, during lactation, sows fed glycerin had lower ADFI, and consequently a greater body weight loss.

5. Before feeding, pregnant sows (Landrace × Large White) fed diets containing glycerin had lower levels of acyl-ghrelin, with a strong positive linear correlation between acyl-ghrelin and cortisol, suggesting that the inclusion of glycerin in gestating diets may exert a satiating effect, and even minimize hunger pangs and improve welfare

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APPENDIX

Curriculum Vitae

Education:

- PhD. Departament of Animal Production, University of Murcia, Spain. Since 2010.
- Bachelor Degree in Food Science and Technology. University of Murcia, Spain. 2012.
- Master Degree in "Professional and Scientific Pig Farming". University of Murcia, Spain. 2011.
- Bachelor Degree in Veterinary Science. University of Murcia, Spain. 2009.

Research experience:

Research projects as Co-PI or Collaborator:

- **La glicerina procedente de la elaboración del biodiesel en la alimentación porcina y de vacuno de carne** (Glycerin as a co-product of biodiesel production in feeding pigs and beef cattle). CDTI program, Spanish Ministry of Sciences and Innovation. University of Murcia - six companies. 2011-2013.
- **Ecoalga**. Abengoa Bioenergía Nuevas Tecnologías – Universidad de Murcia. 2011-2012.

Fellowships:

- Jan 2013 – Jul 2014. **Institut für Tierernährung. Freie Universität Berlin.**
  Erasmus Training Programme (6 months).
  Doctoral Fellowship funded by SFB 852 (1 year).
- Jan 2010 – Dec 2012. **Department of Animal Nutrition, University of Murcia and SAT 2439 - ALIA.**
  Research Grant funded by Fundación Séneca (Agencia de Ciencia y Tecnología de la Región de Murcia).

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- Orengo, J., Villodre, C., Madrid, J., Martínez, S., López, M.J., Megías, M.D., Valera, L., Hernández, F. Effect of dietary crude glycerin on growth performance, nutrient digestibility and hormone levels of Iberian crossbred pigs from 50 to 100 kg body weight. Livest. Sci. 2014 Jul; 165: 95-99. doi: 10.1016/j.livsci.2014.04.033
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- Martínez, S., Orengo, J., Madrid, J., Villodre, C., Megías, M.D., López, C. López, M.J., Hernández, F. La glicerina en piensos para cerdas en lactación con estrés térmico: efectos sobre los parámetros de las cerdas. XXIV Congreso Panamericano de Ciencias Veterinarias. La Habana, Cuba. 2014. Oral presentation.
- Villodre, C., Boudry, C., Vahjen, W., Zentek, J., Pieper, R. Influence of dietary protein and fermentable carbohydrate level on microbial metabolites and regulation of the monocarboxylate transporter 1 in the colon of pigs. 68. Tagung der Gesellschaft für Ernährungsphysiologie. Göttingen, Germany. 2014. Oral presentation.
- Madrid, J., Martínez, S., Orengo, J., Pelegrín, A.F., López, C. López, M.J., Valera, L., Villodre, C., Megías, M.D., Hernández, F. Monitorización de la concentración de CO2 en naves de cerdos de cebo en climas cálidos. I Workshop sobre mitigación de emisión de gases de efecto invernadero provenientes del sector agroforestal. Bilbao, Spain. 2012. Basque Centre for Climate Change. Poster.
- Madrid, J., Valera, L., Villodre, C., Martínez, S., López, M.J., Orengo, J., Hernández, F. Effects of crude glycerol at various levels of addition on diet digestibility by growing and finishing pigs. 15th Congress of the European Society of Veterinary and Comparative Nutrition. Zaragoza, Spain. 2011. Universidad de Zaragoza. Poster.

- Villodre, C. Determinación de la tasa de ventilación y concentración de CO_2 en instalaciones porcinas con ventilación natural en fase de cebo. I Congreso Nacional Científico de Alumnos de Veterinaria. Murcia, Spain. 2011. Universidad de Murcia. Facultad de Veterinaria. Oral presentation.
- Orengo, J**.,** Villodre, C., Hernández, F., Pelegrín, A.F. Efecto de la actividad animal en el cálculo de la tasa de ventilación horaria. II Congreso de la Asociación Nacional de Veterinarios de Porcino. Lérida, 2010. ANAVEPOR. Poster.