

# Replacement of Soybean Meal with Lupin or Chickpea Seed Meal in Diets for Fattening Iberian Pigs Promotes a Healthier Ileal Microbiota Composition

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## Abstract

Five castrated male Iberian pigs ( $100 \pm 2$  kg b.w.) fitted with T-shaped ileal cannulas at the terminal ileum were used to determine the effects of legume feeding on intestinal microbiota composition. The diets were based on defatted soybean (*Glycine max*), lupin (*Lupinus angustifolius*) or chickpea (*Cicer arietinum*) seed meals and contained similar amounts of digestible energy ( $14.2 - 15.1$  MJ·kg<sup>-1</sup>) and protein ( $107$  g·kg<sup>-1</sup>). A hydrolyzed casein diet was used to determine the bacterial counts in pigs fed on a vegetable-free diet. The composition of the intestinal microbiota at the terminal ileum was analysed by q-PCR. Higher ( $P < 0.05$ ) lactobacilli log<sub>10</sub> number of copies was determined in the ileal contents of pigs fed on lupin- or chickpea-based diets with respect to those fed on the soybean-based diet. Bacteroides and the *Clostridium coccooides/Eubacterium rectale* group log<sub>10</sub> number of copies was lower ( $P < 0.01$ ) than that of soybean in the ileal contents of chickpea-fed pigs. Enterobacteria and the *Escherichia/Shigella* group log<sub>10</sub> number of copies was lower ( $P < 0.01$ ) than that of soybean in pigs fed on diets based on lupin or chickpea. The number of copies of the different bacterial groups in animals fed on the casein-based diet was lower ( $P < 0.01$ ) than that of soybean for lactobacilli and bacteroides, but was higher than that of soybean for bifidobacteria, enterobacteria and the *Escherichia/Shigella* group. This information suggests that lupin or chickpea feeding might induce a benefit in the microbiota composition of Iberian pigs in their final productive stages.

## Keywords

Defatted Soybean, Lupin, Chickpea, Iberian Pig, Ileal Microbiota

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## 1. Introduction

Surplus pig meat production and growing public concern on animal welfare and soil pollution problems have resulted in the encouragement of the production of native pig breeds such as the Iberian pig which are well adapted to local feed resources and provide high quality products [1] [2]. Iberian pig breeding is peculiar in that in order to achieve higher organoleptic quality, and its final fattening stage (from 100 to about 160 kg body weight) is carried out in free range conditions. During that period, pigs are fed basically on acorns and grass, which are the predominant feedstuffs available in the Mediterranean woodland prairies. In these conditions, the use of local feed resources is of paramount importance for the sustainability of the system. However, this regime has been shown to be lower in protein, and particularly in lysine, than that required by the pig [2]. As a consequence, other protein supplements such as legume seed meals, which are grown in the same areas where this breed is produced, might be used to equilibrate its diet and achieve better productive results, even though this practice might alter the final product quality [3] if used in the final fattening period in extensive conditions. Locally grown legume seed meals have traditionally been, and at present in some cases are, incorporated into formulas for Iberian pigs up to 100 kg body weight. In addition, cereals and legumes are ingredients among those legally authorized in Spain in the fattening period of Iberian pig production stages [4]. Therefore, it is important to determine not only the digestibility of nutrients in these feedstuffs, but also the potential effects on the intestinal physiology to establish their practical usefulness in Iberian pig dietary formulas. The amount of information available on the digestive physiology of the Iberian pig is scarce, particularly if compared to lean pig breeds.

Problems associated with intestinal disorders in different production stages are among those of greatest economic impact at present for the pig production industry, and it holds true not only for lean breeds but also for Iberian pig production systems, where costs derived from gastrointestinal disorders sometimes exceed market prices (Sánchez Romero Carvajal Jabugo S.A., personal communication). As a consequence, there is at present the need to look for alternatives that could enhance the natural defense mechanisms of animals and reduce the massive use of antibiotics. In pigs, the distal small intestine is known to harbor more than  $10^8$  bacteria/g or ml of gut contents [5]. As a consequence, one way to improve productive parameters and health in the pig industry is to use specific feed additives and/or dietary raw materials able to modulate the gut microbiota which plays a critical role in maintaining host health [6]. One of the main putative effects of certain additives or feedstuffs might be the improved resistance to potentially pathogenic bacteria colonization and enhanced host mucosa immunity, thus resulting in a reduced pathogen load, an improved health status of the animals and a reduced risk of food-borne pathogens in foods [7].

Accordingly, the present work was designed to study the effects of legume feeding on ileal microbiota composition of Iberian pigs. Pigs in the final fattening period (aprox. 100 kg body weight) were used because it is the period when the animals are fed with the ingredients which naturally grow in the Mediterranean prairies, and could more easily benefit from vegetable local ingredients. Lupins (*Lupinus angustifolius*) and chickpeas (*Cicer arietinum*) were chosen in this work due to their low content in antinutritional factors, and to differences in the composition of their constituent carbohydrates [8] [9].

## 2. Materials and Methods

### 2.1. Animals, Diets and Feeding Regime

Male castrated pigs from the Iberian breed were purchased from Sanchez Romero Carvajal S.A. (Huelva, Spain). Five animals ( $100 \pm 2$  kg mean live b.w.) fitted with T-shaped ileal cannulas surgically implanted [10] about 15 cm anterior to the ileocecal junction were used. They were housed individually in 4 m<sup>2</sup> pens in a temperature controlled ( $20^\circ\text{C} \pm 1^\circ\text{C}$ ) room and allowed to recover from surgery. Feed energy and protein composition were calculated according to ingredients values in [11], and given at about twice their calculated energy maintenance needs ( $\text{MEM} = 458 \text{ kJ per kg}^{0.75} \text{ b.w.}$ ; NRC, [11]) and offered in two meals (at 9:00 a.m. and 18:00 p.m. respectively) of 1 kg each. Water was freely available from low pressure drinking nipples. The design was as previously described [8] [9]. The five animals were fed the same experimental diet for 7 day adaptation and 3 day collection periods. The sequence of feeding the experimental diets was soybean, lupin, chickpea and casein. Samples from the cannulas were taken from day 8 to 10 in plastic bags attached to them. The bags were withdrawn at time intervals <30 min from 10:00 to 18:00 h. The bags were immediately frozen in liquid N and freeze-dried. Fistulated pigs remained in good condition throughout the whole experimental period, and digesta

flowed through the cannulas without blockages. The diets (**Table 1**) were based in defatted soybean, lupin or chickpea seed meals as only protein sources, and contained similar amounts of digestible energy (14.2 - 15.1 MJ·kg<sup>-1</sup>) and protein (107 g·kg<sup>-1</sup>). A diet based in hydrolyzed casein was used to determine the bacterial counts in pigs fed on a vegetable-free diet. The diets were also supplemented with vitamins and minerals to meet requirements [11]. Defatted soybean (*Glycine max*) meal and commercial varieties of lupin (*Lupinus angustifolius*) and chickpea (*Cicer arietinum*) seeds were purchased locally. Hydrolyzed casein was from Sigma (Alcobendas, Madrid, Spain). All management and experimental procedures carried out in this study were done in strict accordance with the appropriate practices for management of experimental animals in Spain (Act no. 223/88 of 18 March 1988) and done by staff trained to carry out such procedures.

## 2.2. q-PCR Analysis of Bacterial Groups

Total DNA was isolated from freeze-dried intestinal samples of ileal mucosa (40 mg) using the QIAamp DNA stool kit (Qiagen, West Sussex, UK) by following manufacturer's instructions. In order to increase its effectiveness, the lysis temperature was increased to 95°C and an additional step with lysozyme (10 mg·mL<sup>-1</sup>, 37°C, 30 min) incubation was added. Eluted DNA was treated with RNase and the DNA concentration and purity assessed spectrophotometrically (260 - 280 nm) by using a NanoDrop ND-100 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Purified DNA samples were stored at -20°C until use [12]. Bacterial log<sub>10</sub> number of copies was determined in by using q-PCR. The 16S rRNA gene-targeted primers and PCR conditions used in this study were as previously described [13].

## 2.3. Statistical Analysis

Individual pigs were considered the experimental unit. The experimental data were subjected to a Two-way Analysis of Variance as a randomized design with diet composition, period of feeding and experimental replicate (block) as factors using a computer software package (Minitab Statistical Software Package, Minitab, New York, NY). In this design, the effects due to period of feeding and the interaction diet composition × period of feeding were first checked, and found not significant in all cases. The effects due to diet composition were studied by pairwise comparisons using the Tukey multiple comparison test.

**Table 1.** Composition (g·kg<sup>-1</sup>) of the diets.

| Diet <sup>1</sup>                        | Soybean | Lupin | Chickpea | Hydrolyzed casein |
|--|---------|-------|----------|-------------------|
| Maize starch                             | 700     | 530   | 250      | 680               |
| Oil (sunflower)                          | 30      | -     | -        | 30                |
| Vit + Min <sup>2</sup>                   | 3       | 3     | 3        | 3                 |
| Ca carbonate                             | 2.8     | 2.7   | 7.1      | 5                 |
| Ca diphosphate                           | 11.7    | 12.6  | 3.7      | 25                |
| Cellulose                                | -       | -     | -        | 30                |
| Sucrose                                  | -       | -     | -        | 100               |
| Sodium chloride                          | -       | -     | -        | 5                 |
| Soybean meal 46%                         | 252.5   | -     | -        | -                 |
| Lupin meal                               | -       | 451.7 | -        | -                 |
| Chickpea meal                            | -       | -     | 736.2    | -                 |
| Hydrolysed casein                        | -       | -     | -        | 122               |
| Digestible energy (MJ·kg <sup>-1</sup> ) | 14.8    | 14.2  | 14.2     | 15.1              |
| Protein <sup>3</sup>                     | 107     | 107   | 107      | 73                |

<sup>1</sup>For details see materials and methods. <sup>2</sup>The mineral-vitamin mix contained (per kg): thiamin, 50 mg; piridoxine, 50 mg; riboflavin, 1000 mg; nicotinic acid, 7500 mg; calcium pantothenate, 5000 mg; folic acid, 100 mg; biotin, 100 mg; inositol, 8000 mg; retinol, 1125 mg; cholecalciferol, 750,000 IU; all-rac- $\alpha$ -tocopherol, 1250 IU; menadione bisulfite, 500 mg; cyanocobalamin, 5 mg; folic acid, 5 mg; Mg, 5 g; Fe, 25 g; Zn, 40 g; I, 150 mg; Cu, 20 g; Co, 100 mg. <sup>3</sup>N determined by Kjeldahl. Protein in diets calculated as N × 5.5 in legume-based diets [23], and N × 6.25 in the hydrolyzed casein diet.

### 3. Results and Discussion

As shown in **Table 2**, higher ( $P < 0.01$ ) lactobacilli  $\log_{10}$  number of copies was determined in the ileal contents of pigs fed on lupin or chickpea-based diets with respect to those fed on the soybean-based diet. Bacteroides  $\log_{10}$  number of copies was lower ( $P < 0.01$ ) in ileal contents of chickpea-fed pigs. Enterobacteria and *Escherichia/Shigella*  $\log_{10}$  number of copies was lower ( $P < 0.01$ ) than that of soybean in pigs fed on diets based on lupin or chickpea. The  $\log_{10}$  number of copies of the *Clostridium coccooides/Eubacterium rectale* group was lower than that of soybean in pigs fed on the chickpea diet. Bifidobacteria and *Clostridium leptum* number of copies was not affected by the legume seed meal incorporated to the diet. The number of copies of the different bacterial groups analysed in the animals fed on the casein-based diet was lower ( $P < 0.01$ ) than that of soybean for lactobacilli and bacteroides, while they were higher than that of soybean for bifidobacteria, enterobacteria and the *Escherichia/Shigella* group.

While information on the use of lupin seed meal for pigs is more abundant [14], works with chickpea meal are scarce in the literature, and even less has been reported in Iberian pig feeding. In a previous work by our group, it was concluded that true ileal nitrogen and amino acid digestibilities of lupin and chickpea meals are comparable to those of defatted soybean in Iberian pigs [7], and higher total amounts of lupin NSP and/or lupin and chickpea oligosaccharides were digested up to the terminal ileum compared to defatted soybean [9]. Also, previous works in rats and pigs [15]-[17] indicate that the main reason for the higher faecal N excretion found in legume fed animals is that intestinal, and particularly lower gut microflora, increases its growth at the expense of undigested carbohydrates coming from the small intestine and urea supplied mainly through the large intestinal wall. It is known that the digestibility and fermentation of legume carbohydrates (mainly starch, NSP and oligosaccharides) in the gut have both healthy and productive implications [18].

In normal conditions, the main role of the intestinal microbiota is to provide a barrier against the invasion of potentially harmful bacteria [18]. Accordingly, a lot of research has been devoted in recent decades to the study of the potential therapeutic and productive uses of dietary additives/supplements able to limit the growth of potentially harmful bacteria such as enterobacteria, clostridia and *E. coli* [6]. Lactobacilli and bifidobacteria are among the most studied species out of those with alleged probiotic effects. The mechanism by which those species may play a beneficial role is related to the production of specific antibiotics, but particularly acetic and lactic acids, which lower the pH of the intestinal contents make it less favorable for the growth of coliforms [6] [18]. Also, the *C. coccooides/E. rectale* cluster includes bacteria producing butyric acid, a metabolite seen to be beneficial for gut functionality in pigs through regulation of epithelial cell growth, induction, differentiation and apoptosis in the small intestine, increase of intestinal cell proliferation and improvement of digestive and absorptive capacities of the small intestine [18].

In the present work, higher  $\log_{10}$  counts of lactobacilli, and lower enterobacteria and *Escherichia/Shigella* counts, were determined with respect to soybean and casein-fed pigs in the intestinal contents of pigs fed on either lupin or chickpea diets (**Table 2**). Previous work in rats [19] and broiler chickens [20] fed on purified lupin fractions indicated that the NSP fraction was mainly responsible for similar effects on intestinal microbiota composition. As already mentioned, lupin and chickpea meals were chosen for the experiments described here, among other reasons, because of the different chemical composition of their constituent carbohydrates. Thus,

**Table 2.** Bacterial  $\log_{10}$  number of copies/mg of freeze-dried ileal contents in cannulated pigs fed on diets based in defatted soybean, lupin or chickpea seed meals as the only protein source.

| Diet                                  | Soybean           | Lupin              | Chickpea          | Casein            | Pooled SD |
|---------------------------------------|-------------------|--------------------|-------------------|-------------------|-----------|
| Lactobacilli                          | 4.18 <sup>a</sup> | 4.93 <sup>b</sup>  | 5.73 <sup>c</sup> | 2.69 <sup>d</sup> | 0.41      |
| Bifidobacteria                        | 2.36 <sup>a</sup> | 2.61 <sup>a</sup>  | 2.46 <sup>a</sup> | 3.52 <sup>b</sup> | 0.56      |
| <i>C. coccooides/E. rectale</i> group | 4.10 <sup>a</sup> | 3.76 <sup>ab</sup> | 3.61 <sup>b</sup> | 4.03 <sup>a</sup> | 0.39      |
| <i>C. leptum</i>                      | 3.62              | 3.51               | 3.45              | 4.16              | 0.30      |
| Enterobacteria                        | 4.26 <sup>a</sup> | 3.52 <sup>ab</sup> | 2.82 <sup>b</sup> | 6.92 <sup>c</sup> | 0.77      |
| <i>Escherichia/Shigella</i> group     | 4.36 <sup>a</sup> | 3.63 <sup>ab</sup> | 3.20 <sup>b</sup> | 5.50 <sup>c</sup> | 0.70      |
| Bacteroides                           | 5.96 <sup>a</sup> | 5.81 <sup>a</sup>  | 4.74 <sup>b</sup> | 3.90 <sup>c</sup> | 0.58      |

<sup>a,b,c</sup>Means (n = 5) in the same row with different superscript letters differ ( $P < 0.01$ ).

while the main carbohydrate fraction in chickpea meal is starch, lupin meal contains only very low amounts of it (405 and 16 g·kg<sup>-1</sup>, respectively), and the reverse situation occurs with respect to NSP contents (86 and 319 g·kg<sup>-1</sup>, respectively). These differences in NSP contents are mainly due to high concentrations of galactose, glucose and uronic acids in lupin meal compared with other legumes. Soybean concentrate NSP values (168 g·kg<sup>-1</sup>) were intermediate between lupin and chickpea meals [9]. Amounts of lupin NSP digested (94.1 g·kg<sup>-1</sup> feed) within the small intestine were higher than those for soybean and chickpea, which were not different from each other (24.3 and 27.1 g·kg<sup>-1</sup> feed, respectively). However, not only NSP sugars, but also oligosaccharides have been associated with prebiotic effects, as legumes are known to contain substantial amounts of these compounds. Higher proportions of total oligosaccharides in lupin and chickpea diets were also digested compared with the soybean diet within the small intestine (10.4, 21.7 and 23.7 g·kg<sup>-1</sup> diet ingested for soybean, lupin and chickpea diets, respectively) [9]. Therefore, since only whole seed meals were fed in the present investigation, and if not  $\alpha$ -galactosides or NSP in separated diets, it is not possible from the results reported here to assign the effects on microbial growth to either NSP and/or  $\alpha$ -galactosides in lupin or chickpea meals. The increase in bifidobacteria log<sub>10</sub> counts in pigs fed on the casein-based diet might be related to the bifidogenic effect of casein fractions [21], while the increase in enterobacteria and the *Escherichia/Shigella* group is probably related to the low amount of dietary fibre, as higher enterobacteria and coliforms counts have been previously found in pigs fed on low fibre diets [22].

#### 4. Conclusion

It is concluded that, in addition to economic and legal considerations, lupin and/or chickpea meal feeding might induce a more protective intestinal microbiota composition of Iberian pigs in their final productive stages.

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