Dietary Effect of a Mixture of Alfalfa Hay: Peas Powder on Growing Pigs. Certain Plasma and Faeces Microbiological Analyses

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Abstract
A 25-day trial was conducted to evaluate the effects of dietary mixture alfalfa hay: peas powder as partial replacement of sunflower meal (SFM) on plasma energetic profile and microbial populations of growing pigs. Ten barrows (30.28±0.53 kg, body weight; 81±3 aged) were allocated to two diets. Control (C) based on corn-SFM and soybean meal, and experimental (LP) where the dietary mixture partially replaces the SFM. At 7 and 25-days, the blood samples (N=10) were used to determine the plasma energetic profile (Spotchem EZ SP-4430, Arkray, Japan). The feed and faeces microbial populations were established by counting the colonies on selected media for each microorganism. Results showed that the LP diet maintains appropriate glucose, triglycerides and total cholesterol content to C diet (P>0.05) while improved the HDL-Cholesterol (13%, P=0.91) and LDL-Cholesterol (9%, P=0.08) content *vs.* C diet. In the pig's faeces, the LP diet increased the *Lactobacillus* (+23.33%; P<0.05) while decreased the abundance of *Escherichia coli*; as an effect, the total germ counts increased (+11%; P<0.05) while the *Lactobacillus: Escherichia coli* ratio was significantly improved *vs.* C diet. In conclusion, the dietary mixture alfalfa hay: peas powder had positive effects on gut health and plasma metabolite profiles of growing pigs.

Keywords: alfalfa hay, energetic profile, growing pigs, peas, microbial populations

1. Introduction

The pig sector is nowadays in continuously evolving and improved. Nutritionally, protein and energy requirements of growing pigs are covered preponderantly by cereals and oilseed meals such as soybean meal, canola meal and sunflower meal [1, 2]. However, due to the great demand for these feedstuffs for livestock feed, high market price and the environmental impact, there is an increasing interest in the use of alternatives sources of nutrients in pig diets. Thereby, expending the range of usable alternative protein feeds will help to reduce feed dependence on imported protein sources [2]. Furthermore, the interest for supplementation of leguminous such as pea (*Pisum sativum*) or alfalfa hay (*Medicago sativa*) in growing pigs nutrition has increased due to its beneficial metabolic and physiological effects of the gastrointestinal tract [3-8]. For instance, probiotic effect in the lower gut (e.g. increased amount of *Bifidobacterium* and *Lactobacillus* spp.) or positive effect on the short-chain fatty acids produced during leguminous fermentation and consequently maintenance of a healthy gut has been reported [9-13]. The peas are a plant seed rich in protein and carbohydrate components, including starches and sugars [13]. Pea is an affordable source of not only protein but also the starch, which has an advantage of being resistant starch and slowly fermented by the microorganisms to subsequently produce important metabolites [13-15]. Moreover,

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bioactive compounds (starch, non-starch polysaccharides and plant sterols) are mainly considered responsible for the effects observed on lipid metabolism [16].
The alfalfa is a widely used nutritional feedstuff source due to its high adaptability, forage yield production and good forage quality characteristics [17] and provides protein, mineral and fibre for animal nutrition. Previous data showed that dietary alfalfa extracts rich in flavonoids, cause lowering of cholesterol levels in the blood of birds [18, 19]. However, dietary mixing locally legume–cereal in the modern-day domestic pig, coming from rigorously selected animals, high productively and less adapted to use uncommon feeds, is not a common practice in Romania. Thereby, the use of a mixture of alfalfa hay: peas powder in growing pigs diet requires the evaluation of plasma parameters to accurately assess their nutritional status.
Therefore, this study aimed to evaluate the effects of dietary mixture alfalfa hay: peas powder as partial replacement of sunflower meal on plasma energetic profile and microbial populations of growing pigs.

2. Materials and methods

Animals, experimental design and diets
Animals were treated following the Romanian Law 43/2014 for handling and protection of animals used for experimental purposes. This trial protocol was approved by the Ethical Committee (no. 6124/2018) of The National Research-Development Institute for Animal Nutrition and Biology - Balotesti, Romania.
A total of 10 growing barrows Topigs (♀ Large White × Hybrid (Large White × Pietrain) × ♂ Talent, mainly Duroc) aged 83.00 ± 2.00 days and weighing, on initial average body weight, 30.28 ± 1.23 kg, were individually housed in metabolism cages. The healthy growing pigs were randomly allotted to two pellet diets: control diet (C) based on corn-sunflower meal and experimental (LP) that contained the same basal diet and a mixture of alfalfa hay: peas powder (1:1) that replaced 50% of the sunflower meal. The experimental period consisted of 7-day adaptation and three periods of determinations. Briefly, the trial period lasted for 25 days. Food and water were available *ad libitum* to the animals. Diets were formulated to contain g kg^{-1} (as-fed basis): 165.8 crude protein, 34.8 crude fat, 49.5 crude fibre, 8.0 calcium, 5.0 phosphorus and 12.6 MJ metabolizable energy. Synthetic forms of methionine (DL-methionine) and lysine (L-Llysine HCl) were added to both diets to ensure adequate amounts of limiting amino acids for pigs (lysine and methionine + cysteine).
The dried field alfalfa was harvested at the third cut and subsequently grounded at 0.2-0.5 mm. Finally, the alfalfa hay powder was briefly incorporated into the feed formulas in the pelletized form.

Chemical analyses
The chemical analyses of the feed compounds used in the study were established using the following standardized methods [20]: dry matter (SR ISO 1442:2010), crude protein (SR ISO 937:2007), crude fat (SR ISO 1444:2008), crude ash (SR ISO 936:2009), calcium (SR ISO 6490-2:1983) and phosphorus (spectrophotometry method).

Feed and faecal microbiological analysis
Samples of pigs feed and faeces were collected on days 7, and 25, in Petri dishes, and used for microbiological analyses. Finally, fungal contamination (total fungal count, TFC; yeasts, *Aspergillus niger*, *Cladosporium* sp.) from feed and faeces and faeces bacterial populations (total germ count, TGC; *Coliforms* spp., *Escherichia coli*, *Salmonella sp.*, *Staphylococcus* spp., *Lactobacillus* spp.,) were determined on selected media using a colony counter. The results were expressed as a logarithm (base 10) of colony-forming units per gram of sample.

Plasma analyses
Blood samples were collected in-heparinized tubes at the beginning and the end of the experimental period. Blood samples (6 mL) were collected in the morning from the jugular vein of the pigs. The blood was allowed to clot and was then centrifuged at 3000 rpm for 15 min at room temperature. The plasma was then separated and stored into Eppendorf safe-lock tubes at −20°C until analysis. The assessment of plasma energetic profile (glucose, Glu; triglyceride, TG; total cholesterol, TC; high-density-lipoprotein cholesterol, HDL-C and low-density-lipoprotein cholesterol, LDL-C) was determined by a

chemistry analyser Spotchem EZ SP-4430, Arkray, Japan).

Statistical analysis

The experiment was designed as a single factor study with two treatments (diets). Results are presented as mean values and standard error of the mean (SEM). The treatment effect was determined using the analysis of variance, the general linear model procedure of SPSS Statistics version 20.0 (2011) program [21]. Differences were significant if P<0.05, highly significant when P<0.001 or P<0.0001 and a trend was considered if 0.05≤P<0.10.

Results and discussion

The obtained results for mycological and bacteriological quality of feed compound were presented in Table 1.

Table 1. The microbiological ($\log_{10}$ CFU/g) analyses of pelletized compound feed used for hybrid Topigs growing pigs

Items	C	LP
Fungal contamination		
TFC[3]	3.02	3.44
Yeasts	3.02	0.34
Aspergillus niger	ND	1.40
Cladosporium spp.	ND	1.70
Bacterial contamination		
TGC[4]	4.77	4.73
Coliforms spp.	2.58	0.74
Escherichia coli	2.58	1.74
Salmonella spp.	ND	ND

[1]C: control diet; LP: alfalfa hay: peas powder (1:1) diet.
[3]TFC=total fungal count; [4]TGC=total germ count; ND=not detectable.

The total fungal count analyses from the feed compound show that the C diet (3.02 $\log_{10}$ CFU/g) obtained the lowest values comparing to the LP diet (3.44 $\log_{10}$ CFU/g). In the C diet fungi occur in feed compound as yeasts while in the LP diet as yeasts and molds. The molds associated with feed compound include various species of *Aspergillus* and *Cladosporium*. Due to their potential to produce harmful mycotoxins, the occurrence of these fungi in the feed compound is of particular concern [22]. However, the relatively low density of fungus (1.40 $\log_{10}$ CFU/g *Aspergillus* and, respectively 1.70 $\log_{10}$ CFU/g *Cladosporium*), under the maximal limit admitted by the EU (2.70 $\log_{10}$ CFU/g), are not considered to represent a major animal health risk.

The total germ count in the C diet was higher (4.77 $\log_{10}$ CFU/g) than that of the LP diet (4.73 $\log_{10}$ CFU/g) while coliform levels were lowest (0.74 $\log_{10}$ CFU/g) in the LP diet. *Escherichia coli* was also identified in C diet (2.58 $\log_{10}$ CFU/g) and LP diet (1.74 $\log_{10}$ CFU/g). *Salmonella* sp. was not isolated from any of the diets. The faecal microbial population at the beginning and the end of the experiment ($\log_{10}$ CFU/g) is given in Table 2.

At day 7, there was no statistically significant difference in the faecal bacterial populations *Staphylococcus* spp., *Escherichia coli, Lactobacillus* spp. and TGC identified from both diets. However, at the end of the experimental period, no significant changes were found in the population of *Staphylococcus* spp. among the two groups. The *Escherichia coli* decreased in the LP faeces samples (-4.44%; P<0.05) comparing to C. We also found that the *Lactobacillus* spp. were positively influenced by the dietary mixture alfalfa hay: peas powder (+23.33%; P<0.05). As an effect, the TGC in the pig's faeces fed LP diet were significantly increased (+11%; P<0.0001) than pigs fed the C diet. While the intestinal site of digestion of carbohydrates was not examined in the present study, previous research has shown that dietary carbohydrate sources (starch and other fermentable carbohydrates) from legumes are partially digested in the small intestine and subsequently reaches the pigs large intestine were provided with a carbon source and energy for the resident microbiota [14, 23, 24]. As an effect, increase distal digesta mass and commensal microbial populations in the gut, including *Lactobacillus* [10, 25, 26], while decreasing the abundance of *Escherichia coli* [27, 28] was observed.

Table 2. Effects of dietary mixture alfalfa hay: peas powder (1:1) on faecal microbial populations (log_{10} CFU/g) of growing pigs

Faecal microbial populations	C	LP	SEM	P-value
		Day 7		
Staphylococcus spp.	16.43	16.38	0.041	0.666
Escherichia coli	4.33	4.34	0.003	0.447
Lactobacillus spp.	37.64	37.70	0.027	0.675
TGC[2]	58.39	58.40	0.035	0.910
		Day 21		
Staphylococcus spp.	25.81	25.65	0.030	0.377
Escherichia coli	4.94	4.73	0.022	<0.0001
Lactobacillus spp.	30.09	37.11	0.163	<0.0001
TGC[2]	60.84	67.49	0.175	<0.0001
Lactobacillus:Escherichia coli ratio	7.71	7.53	0.030	<0.0001

[1]C: control diet; LP: alfalfa hay: peas powder (1:1) diet; [2]TGC: total germ count; [3]SEM: standard error of the mean; [4]*P*<0.05 significant difference; *T*–the differences tend to be influenced by treatment (P≤0.10); P>0.10 not significant difference.

Moreover, in our study improving the *Lactobacillus/Escherichia coli* ratio in the LP group compared to C group, indirectly indicates beneficial effects on piglets gut health, as was previously reported [29, 30]. Plasma biochemical parameters are essential indicators of animal health status and provide us with useful information on the overall metabolism of nutrients [31]. To verify that the pigs were in good nutritional status, we also evaluated plasma energetic profile at the beginning and at the end of the experiment. The results are reported in Table 3.

Table 3. The plasma energetic profile of growing pigs

Item	Limits*	Initial		SEM	P-value
		C	LP		
Glu, mg/dl	85-150[1]	120.0	121.2	2.19	0.795
TG	33-50[2]	32.8	32.2	4.03	0.946
T-Chol, mg/dl	67-367[3]	117.4	109.8	3.96	0.368
HDL-C		58.4	49.0	3.33	0.170
LDL-C		96.8	93.6	2.98	0.616
		Final			
Glu, mg/dl	85-150[1]	116.20	114.80	4.71	0.428
TG	33-50[2]	29.20	20.40	4.21	0.324
T-Chol, mg/dl	67-367[3]	115.80	88.60	10.38	0.207
HDL-C		51.20	45.40	3.58	0. 091
LDL-C		103.96	95.28	2.52	0.082

[1]Radostits et al. [32]; Merck Veterinary Manual [33]; Perri et al. [34].

The levels of Glu, TG, T-Chol, HDL-C and LDL-C at the beginning of the study showed means that were not significantly different. However, factors such as genetic variation and environmental conditions, including nutrition, as well as their interactions, may produce changes in blood lipid levels [31]. Furthermore, the values of energetic profile lay within the standard range for pigs [32-34]. The effect of dietary mixture alfalfa hay: peas powder (1:1) on plasma energetic profile of pigs at 25 days, is given in Table 3. The results of this experiment showed that dietary mixture alfalfa hay: peas powder (1:1) added in diet maintain appropriate blood Glu level to C diet (P>0.05).

Moreover, the LP diet insignificantly decreased the plasma components of blood fat TG (43%, P>0.05) and T-Cho (31%, P>0.05) while improved the HDL-C (13%, P=0.91) and LDL-C (9%, P=0.08) content compared to C diet. Moreover, the values for blood lipid levels range in normal limits that indicate a good health condition. The use of dietary mixture alfalfa hay: peas powder (1:1) in pigs feed on plasma energetic profile has been poorly studied. The majority of the data are on the utilization of either alfalfa or peas as nutritional sources for animals. Thus, similar to our results [35] observed that feeding pigs with dietary raw peas lowered the concentrations total and LDL-C in pigs plasma. As was previously reported this effect on cholesterol metabolism was attributed to increasing faecal bile acid output and an increased biliary bile acid concentration [23, 36]. Concomitantly, peas non-starch polysaccharides lowering of cholesterol levels by stimulates excretion of bile acids *via* faeces and increasing the turnover rate of blood and liver cholesterol [16, 36, 37]. However, in a study which investigated the effect of whole peas on the pig's lipid metabolism, and increased levels of cholesterol fractions was observed [36]. Similarly, a recent study of [18, 19] an alfalfa flavonoid added in Yangzhou geese or broiler diet decreased the serum levels of TG, TC and LDL and improved the HDL content. The authors suggest that a certain level of alfalfa flavonoids could promote cholesterol metabolism. However, it is still a subject of controversy which components of legume-rich diets are responsible for their effects on the lipid metabolism. Furthermore, in our study, by analyzing the above observations on the faecal bacterial population and the metabolic efficiency reflected by the level of plasma energetic parameters we considered that the LP diet has, also a positive repercussion on the pig’s health.

4. Conclusions

The results of the present study suggest that dietary mixture alfalfa hay: peas powder used in modern-day domestic pig diets as local sources of nutrients maintains the important marker of health in the normal reference ranges for growing pigs category and had positive effects on gut health. The dietary mixture could beneficially influence the lipid profile; however, it might be an effect of some bioactive components, but further investigations are needed to confirm this positive effect on lipid markers.

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