Research Article

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Inclusion of limited amounts of extruded legumes plus cereal mixes in normocaloric or obesogenic diets for rats: effects on intestinal microbiota composition

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Abstract

BACKGROUND: Differences in the composition of the intestinal microbiota and energetic metabolism between lean and obese populations have been described. Legume consumption has been reported to modulate intestinal microbiota composition. However, to the best of our knowledge, no information can be found in the literature on the effects of consumption of diets containing extruded legume plus cereal mixes on the intestinal microbiota composition of rats. Our purpose was to evaluate the effects on lipids profile (see the accompanying paper) and intestinal microbiota composition (current paper) of incorporating this new food ingredient in normocaloric and obesogenic diets.

RESULTS: Intestinal and fecal qPCR-based microbial composition of rats fed the extruded legumes plus cereal mixes differed ($P < 0.05$) from controls. Obesogenic diets did not affect bacterial counts. However, the inclusion of the extruded mixes reduced ($P < 0.05$) $\log_{10}$ counts in some bacterial groups and increased ($P < 0.05$) counts of Lactobacilli, while others remained unaffected. PCoA at the genus level grouped together *Lactobacillus reuteri*, *Akkermansia miciniphila* and species from *Parabacteroides, Prevotella, Rikenellaceae*, and *Lactobacillus* with extruded legume plus cereal diets. Feeding on extruded legumes plus cereal mixes was associated with increased mRNA expression of the cytokines IL6 and TNF-α and decreased expression of TLR4.

CONCLUSIONS: Our results show that the inclusion in the feed of limited amounts of extruded legumes plus cereal mix, providing a diet that is closer to a normal human one, did modulate the intestinal microbiota composition. Taken together, these results point to the protective, health-promoting properties of extruded legume plus cereal mixes.

Supporting information may be found in the online version of this article.

Keywords: extrusion; legume; microbiome; obesity; rat

INTRODUCTION

Intestinal microbiota are currently regarded as a true bioreactor, capable of degrading a large number of otherwise unusable substances (polysaccharides, some protein and amylaceous fractions, etc.) to provide in return nutrients such as fatty acids, and vitamins to the host.[1] Although some eukaryotes and archaea are present, four bacterial phyla (Firmicutes, Bacteroidetes, Actinobacteria and Proteobacteria) dominate the human intestinal microbiota, accounting for more than 90% of the total.[2,3] The microbiota ferments indigestible components of food, synthesizes vitamins and other essential micronutrients, transforms cholesterol and bile acids, ensures maturation of the immune system, regulates intestinal angiogenesis and protects against pathogens.[4] The composition and development of the intestinal microbiota are closely linked to a particular type of diet,[5] mainly because the non-absorbed compounds in the small intestine reach the large intestine to be fermented by the local bacteria.[6] A clear indication of this is that those countries whose food is more similar show more similar microbiota among their inhabitants, whereas it is not so similar in countries with different diets.[7] In general, the intake of diets with a high fat content leads to a reduction in the

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amount and bacterial diversity.[8] In studies conducted in humanized gnotobiotic mice, which started from a diet low in fat and rich in plant polysaccharides and later changed to a Westernized diet, with high fat and sugar content, modifications were observed in the microbiota composition, metabolic pathways and gene expression only 1 day after the change, and increased adiposity developed during the next 2 weeks.[9] It has been observed that different communities that consume high levels of fiber have a greater abundance of *Prevotella* spp (together with *Roseburia* spp, *Eubacterium rectale* and *E. bromii*), whereas dietary fat and the protein better correlate with increased *Bacteroides* abundance.[10] So there is a complex interaction between the intestinal microbiota and the diet: the diet can modulate the microbiota, which in turn also regulates gene expression and metabolism in response to nutrient availability.[11] Portune *et al*.[12] discussed in a quite comprehensive review the relationships between gut microbiota, diet, and obesity-related disorders.

Differences in the composition of the intestinal microbiota and energetic metabolism between lean and obese populations have been described.[13] In particular, obese individuals have been reported to register changes in the proportions of Firmicutes and Bacteroidetes within the intestine.[14,15] The main justification for this is that the increase in this ratio results in a greater fermentation of carbohydrates and, consequently, a greater production of their end products (mainly short chain fatty acids, SCFA), resulting in a greater energy utilization of the diet.[5,16] A 20% increase in Firmicutes and a corresponding decrease in Bacteroidetes have been associated with an increase in energy absorption of about 150 kcal/d in humans.[17] However, although many studies corroborate this relationship, there are others that do not support this observation,[18–20] and others that claim an increase in Proteobacteria[21] or Actinobacteria[13] in obese individuals.

Legumes are good sources of slow-release carbohydrates and proteins. With exceptions such as lupin (*Lupinus* spp), starch is the main constituent of legume seeds, which becomes partially resistant starch during processing (usually cooking). This resistant starch has been found to possess beneficial properties, such as stimulation of the growth of favourable intestinal bacterial strains and increase of SCFA production during the fermentation process in the colon.[22] The total concentration of SCFA in the feces of obese volunteers exceeded by more than 20% that found in samples of lean volunteers; the greatest difference was observed in propionate (41%), followed by butyrate (28%).[21] Legumes contain a high level of non-starch polysaccharides (NSP),[23] which are the main components of dietary fiber. Some dietary fibers are fermentable, and their catabolism in the gastrointestinal tract leads to the generation of various bioactive substances, such as SCFA, which can increase the biomass of the gastrointestinal tract markedly and change the microbiota composition of the intestine.[24] Kidney bean (*Phaseolus vulgaris*) and pea (*Pisum sativum*) seeds (the legumes used in this study) are considered excellent sources of proteins, mono-, oligo- and polysaccharides and several micronutrients, including minerals, dietary fiber, and starch.[25] Peas also contain galactose oligosaccharides that may exert beneficial prebiotic effects in the large intestine.[26] Many oligosaccharides have a positive impact on the composition of the intestinal microbiota[27] and on metabolic syndrome markers.[28] Bibi *et al*.[29] recently found that green pea supplementation reduced the severity of DSS-induced colitis in mice challenged with a high fat diet by reducing inflammation, mucosal loss, and the endoplasmic reticulum-stress signaling. Finally, an extract of pea albumins led to an anti-inflammatory effect at the colon level in mice, accompanied by a modulation in the composition of the intestinal microbiota,[30–31] and dark kidney beans or cranberry bean supplements suppressed colonic inflammation and reduced the severity of DSS-induced colitis in mice.[32–33]

As explained in the accompanying paper,[34] new highly palatable extruded mixes of legumes plus cereals similar to a snack food have been produced[35] and incorporated in limited amounts into normocaloric or obesogenic diets for rats. Our purpose was to evaluate the effects of dietary incorporation of this new food ingredient on lipids (accompanying paper[34]) and intestinal microbiota composition (current paper). The inclusion in the diet of extruded mixes induced significant temporal and spatial changes in microbiota composition and diversity indices as determined by quantitative reverse transcription polymerase chain reaction (RT-qPCR) and Illumina analysis. Extruded legumes plus cereal mix feeding was associated with differences in the immune response in comparison with the casein-fed controls.

MATERIALS AND METHODS

All management and experimental procedures carried out in this rat trial were in strict accordance with current European regulations (86/609 EEC) regarding laboratory animals. The bioethics committee for animal experimentation at our institution (EEZ-CSIC) approved the study protocol.

Preparation of the extruded mixes and diets

Two extruded legumes plus cereal mixes were produced by using a Clextral Evolum25 twin-screw extruder (Clextral, Firminy, France). These were rice (*Oryza sativa*) + pea (*Pisum sativum*, cv Cartouche) or kidney bean (*Phaseolus vulgaris*, cv Almonga) + carob tree (*Ceratonia siliqua*) fruits. The composition of extruded mixes – pea extruded mix (PEM) and kidney bean extruded mix (KEM), respectively – was rice meal / pea or kidney bean meal/carob fruits meal (50/40/10, w:w:w). All procedures were as described in Arribas *et al*.[35]

The diets were based on casein (CAS) or casein plus PEM or KEM, and were formulated to contain the same amount of digestible energy and protein taking into account the analyzed composition of the ingredients (Table 1). In order not to be too far from practical conditions in human nutrition, the addition of PEM or KEM was limited to a low inclusion level (25%) to provide no more than 20% (two servings) of the total recommended daily protein intake. Another relevant point on formulation in the present work was that diets contained no added cholesterol. Appropriate amounts of synthetic amino acids were added to the extruded mix-based diets taking into account their amino acid composition to equalize them with control (casein) values. The diets were supplemented with vitamins and minerals to target requirements. Obesogenic diets (CAS-OB, PEM-OB, and KEM-OB) were the same previously described diets (CAS, PEM and KEM) with added amounts (5.48 g d^{-1}) of Ideal® commercial condensed milk (Table 2).

Biological assays

Seventy two (72) male weaned Wistar rats (Charles River Laboratories, Barcelona, Spain), matched by weight (80.0 ± 2.7 g, mean ± SE), were individually housed in metabolism cages throughout the experiment. Rats were fed a control casein diet between weaning and the start of the experiment. They were then randomly distributed into six groups (n = 12), and each group was assigned to one of the dietary treatments (see above). The animals were individually housed in metabolic cages in an

Table 1 Analyzed composition (g kg^{-1}) of casein and legume + cereal extruded mixes

	CAS[a]	Pea extruded mix	Kidney bean extruded mix
Dry matter	920.0	944.4	947.6
Protein	813.3	128.2	132.3
Ether extract	ND	1.2	1.4
Ash	ND	34.6	38.4
Crude fiber	ND	32.1	24.6
Dietary fiber	ND	94.1	114.5
α-galactosides	ND	32.8	25.8
Total carbohydrates[b]	ND	653.5	635.2
Crude energy (Kcal g^{-1})	3.940	3.600	3.620
Amino acids			
Aspartate	58.9	15.1	15.8
Glutamate	184.1	25.4	26.2
Serine	49.1	6.0	7.3
Histidine	16.5	3.3	4.1
Glycine	27.5	6.1	6.1
Threonine	39.6	4.4	5.0
Arginine	37.4	12.5	11.4
Alanine	32.6	6.8	7.0
Tyrosine	49.0	5.5	5.7
Cystine	3.4	1.5	1.4
Valine	53.3	7.0	7.5
Methionine	24.5	1.8	2.5
Phenylalanine	46.9	7.2	8.1
Isoleucine	40.9	5.9	6.3
Leucine	74.9	10.9	11.9
Lysine	97.9	7.2	7.5
Proline	82.8	6.6	6.1

[a]CAS, casein; Pea (*Pisum sativum*) extruded mix (rice/pea/carob tree bean, 50/40/10); Kidney bean (*Phaseolus vulgaris*) extruded mix (rice/kidney bean/carob tree bean, 50/40/10).
[b]Total solids excluding protein, fat, ash, dietary fiber and α-galactosides.
ND, not determined.

Table 2 Composition (g kg^{-1}) of the diets

	CAS[a]	PEM	KEM
Extruded mix	-	250	250
Casein	209	160	160
Maize starch	261.28	209.58	209.58
Potato starch	150	-	-
Cellulose	50	50	50
Sunflower oil	70	70	70
Sacarose	150	150	150
DL-Methionine	3	3	3
Tryptophane	-	0.70	0.70
Coline	1.60	1.60	1.60
Cystine-HCl	1.80	1.80	1.80
Vitamins + minerals	45	45	45
Ca diphosphate	55	55	55
Citric acid	0.12	0.12	0.12
Iron sulfate heptahydrate	0.20	0.20	0.20
Cr_2O_3	3	3	3
Calculated composition			
Energy (kcal/g)	3.23	3.22	3.21
Protein	170	170	170
Fat	70	70	70
Carbohydrates	486.3	531.3	525.8
Dietary fiber	50.0	73.5	78.6

[a]CAS, casein; KEM, kidney bean (*Phaseolus vulgaris*) extruded mix diet; PEM, pea (*Pisum sativum*) extruded mix diet. Diets CAS-OB, KEM-OB and PEM-OB were the same diets described above with added 5.48 g d^{-1} condensed milk (composition: energy 13.75 KJ g^{-1}; protein 74 g Kg^{-1}; fat 85 g Kg^{-1}; carbohydrates 560 g Kg^{-1}; NaCl 2 g Kg^{-1}).

environmentally controlled room under standard conditions (temperature: 20–22 °C with a 12 h light–dark cycle and 55–70% humidity). The rats had *ad libitum* access to their diets and tap water, and were fed the different diets for 21 days. Rats ate all feed offered and there were no leftovers. On day 21, after an overnight fast they were refed at defined time intervals with 11 g of diet and euthanized 60 min after refeeding so that all rats were in the same feeding situation. Animals were anaesthetized with sodium pentobarbital (50 mg kg^{-1} of body weight) (Abbott Laboratories, Granada, Spain) and terminal exsanguination was performed by cannulation of the carotid artery.

RT-qPCR microbiota composition analysis

Once the animals were sacrificed, the small intestine (last 20 cm) and the colon from all rats (n = 72) were aseptically extracted, and the contents collected in sterile vials and stored at −80 °C for microbiological studies. Total DNA was isolated from intestinal contents (ileum, colon), tissues (ileum, colon) or fecal freeze-dried samples (40 mg)[36] using the FavorPrep Stool DNA Isolation Mini Kit (Favorgen-Europe, Vienna, Austria) by following the manufacturer's instructions. Eluted DNA was treated with RNase and the DNA concentration was assessed by using a NanoDrop ND-100 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Bacterial $\log_{10}$ number of copies was determined by using quantitative polymerase chain reaction (q-PCR) (iQ5 Cycler, Bio-Rad Laboratories, Alcobendas, Spain). The 16S rRNA gene-targeted primers and polymerase chain reaction (PCR) conditions used in this study were as in the supporting information, Table S1. The different microbial groups quantified included total bacteria, *Bacteroides / Prevotella* spp, *Lactobacillus* spp, *Bifidobacterium* spp, *Blautia coccoides / Eubacterium rectale* group, *Clostridium leptum / Ruminococcus* spp, *Faecalibacterium prausnitzii*, *Enterobacteriaceae*, and *Escherichia / Shigella*. Samples for q-PCR analysis were run in duplicate.

High-throughput analysis of microbial community

The bacterial diversity of the samples was determined using Illumina technology (MiSeq). Total DNA was isolated from freeze-dried colon content (20 mg) from four rats per group (n = 24) as described above. Libraries preparation was performed by amplification of the V4–V5 region of the 16S rRNA gene. The first amplification was performed by using primers Mi_U515 (5′-GTGCCAGCMGCCGCGGTAA-3′) and Mi_E786 (5′–GGACTACHVGGGTWTCTAAT-3′) including partially Illumina adapters Mi_E786 (50–GGACTACHVGGGTWTCTAAT-30), and PCR conditions were: initial denaturalization at 98 °C 30 s; 25 cycles with denaturalization at 98 °C 10 s, annealing at 52 °C 20 s and extension at 72 °C 10 s; and a final extension at 72 °C 5 min. Second amplification included barcodes and the rest of the Illumina

Table 3 Effect of the diet on the bacterial counts (log_{10} copies of the 16 S-rRNA gene mg^{-1} dry content) in the ileal content of rats fed a control diet (CAS) or diets supplemented with two extruded mixes of legumes plus cereals, normocaloric or obesogenic

	Diet[a]						*P values*[b]		
	CAS	CAS-OB	PEM	PEM-OB	KEM	KEM-OB	E	O	E × O
Ileal content									
Lactobacilli	5.47	5.51	5.43	5.67	5.67	5.78	0.964	0.897	0.604
Bifidobacteria	6.82	7.10	5.65	5.41	5.06	4.67	<0.001	0.787	0.434
Blautia coccoides /Eubacterium rectale	5.30	5.92	4.91	4.81	4.79	4.49	<0.001	0.205	0.124
Clostridium leptum/Ruminococcus spp	4.84	5.75	4.22	3.27	3.76	3.81	<0.001	0.297	0.001
Faecalibacterium prausnitzii	4.77	4.84	4.50	4.32	4.55	4.69	0.097	0.331	0.779
Enterobacteria	5.95	6.07	5.81	4.60	5.26	4.60	0.004	0.183	0.085
Escherichia/Shigella	4.88	5.02	4.58	4.26	4.43	4.15	0.004	0.688	0.275
Bacteroides/Prevotella	3.57	4.06	4.37	3.85	3.96	3.92	0.371	0.673	0.108
Total bacteria	6.91	6.97	7.02	7.16	7.27	7.03	0.263	0.985	0.706
Colon content									
Lactobacilli	6.48	6.46	7.36	6.82	7.46	6.74	0.027	0.244	0.263
Bifidobacteria	8.22	8.11	6.04	5.85	6.25	6.07	<0.001	0.482	0.867
Blautia coccoides/Eubacterium rectale	7.79	7.63	7.52	7.39	7.35	7.43	<0.001	0.155	0.284
Clostridium leptum/Ruminococcus spp	7.51	7.75	6.99	6.77	6.72	6.74	<0.001	0.361	0.031
Faecalibacterium prausnitzii	6.39	6.85	6.24	5.85	6.15	5.87	<0.001	0.636	0.007
Enterobacteria	6.16	6.04	5.54	5.63	5.15	5.43	<0.001	0.838	0.396
Escherichia/Shigella	5.93	5.91	4.87	5.42	5.17	5.44	<0.001	0.241	0.200
Bacteroides/Prevotella	7.15	6.94	7.30	7.07	6.83	7.20	0.600	0.492	0.187
Total bacteria	9.14	9.03	8.85	8.55	8.50	8.55	<0.001	0.085	0.897
Feces									
Lactobacilli	5.40	5.54	7.36	6.76	7.44	6.87	<0.001	0.405	0.176
Bifidobacteria	8.87	8.99	6.67	5.93	6.89	6.68	<0.001	0.790	0.412
Blautia coccoides/Eubacterium rectale	7.15	7.26	7.09	7.43	7.45	7.65	0.023	0.031	0.351
Clostridium leptum/Ruminococcus spp	7.36	7.73	6.86	6.85	6.89	6.81	<0.001	0.118	0.050
Faecalibacterium prausnitzii	6.98	7.16	6.43	6.76	6.68	6.56	0.001	0.331	0.779
Enterobacteria	6.22	6.02	6.13	6.20	6.09	6.02	0.976	0.406	0.505
Escherichia/Shigella	5.75	5.65	5.57	5.84	5.87	5.73	0.700	0.921	0.547
Bacteroides/Prevotella	7.85	7.62	7.19	7.58	7.15	7.84	<0.001	0.039	<0.001

Values are means (n = 12).
[a]CAS, casein; KEM, kidney bean (*Phaseolus vulgaris*) extruded mix; PEM, pea (*Pisum sativum*) extruded mix. Diets CAS-OB, KEM-OB and PEM-OB were the same diets described above with added 5.48 g d^{-1} condensed milk.
[b]E, effect of inclusion of extruded legume/rice/carob tree bean; O, effect of the obesogenic diet: E × O, interaction of both effects.

adapters, and PCR conditions were: initial denaturalization at 98 °C 30 s; 25 cycles with denaturalization at 98 °C 10 s, annealing at 52 °C 20 s and extension at 72 °C 10 s; and a final extension at 72 °C 5 min. All amplifications were performed in duplicate. Total DNA was isolated from freeze-dried colon content (20 mg) as described above. Aliquots of 10 μL of each DNA were sent to the CGEB IMR (Dalhousie University; Halifax, Nova Scotia, Canada) for sequencing.

Gene expression analysis in colonic tissue

The analysis of gene expression in the colonic samples was performed by RT-qPCR. For this purpose, the total RNA of colonic samples from all rats (n = 72) was isolated using TRI Reagent® following the manufacturer's protocol. All RNA samples were quantified with the Thermo Scientific NanoDrop™ 2000 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA) and 2 μg of RNA were reverse transcribed using oligo(dT) primers (Promega, Southampton, UK). The RT-qPCR amplification and detection were performed on optical-grade 48-well plates in a IQ5 Real-Time PCR System (Bio-Rad Laboratories, Alcobendas, Madrid, Spain) with 20 ng of cDNA, the KAPA SYBR® FAST qPCR Master Mix (Kapa Biosystems, Inc., Wilmington, MA, USA) and specific primers at their annealing temperature (supporting information, Table S2). To normalize mRNA expression, the expression of the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was measured. The mRNA relative quantitation was calculated using the ΔΔCt method.

Statistical analysis

The RT-qPCR results were tested for statistical significance using a two-way (+/− extruded mix addition, +/− obesogenic diet) analysis of variance (ANOVA) with a Tukey post hoc test. Statistical significance was set at $P < 0.05$.

Results from high throughput sequencing analyses were performed by using *Quantitative Insights in Microbial Ecology* (QIIME2[37–38]). Due to the low quality of the reverse sequences, only forward sequences were employed in subsequent analyses. Quality filtering was performed by using default parameters in QIIME2. A sub-OTU (sub-operational taxonomic units) table in biom format[39] was created using Deblur,[40] and alignment and

Table 4 SIMPER analysis at different taxonomic levels of colon bacterial community in rats fed a control diet (CAS) or diets supplemented with the extruded mix of legumes plus cereals, normocaloric or obesogenic by Illumina analysis

Family	Average dissimilarity	Contribution, %	Cumulative, %
Ruminococcaceae	15.56	37.12	37.12
Lachnospiraceae	10.02	23.91	61.02
Bacteroidaceae	3.68	8.78	69.80
S24-7	3.60	8.59	78.39
Bifidobacteriaceae	2.65	6.33	84.72
Genus			
Ruminococcus	15.31	27.70	27.70
Lachnospiraceae; g_	7.05	12.76	40.46
Ruminococcaceae; g_	6.27	11.34	51.80
Blautia	6.25	11.30	63.10
Bacteroides	3.68	6.66	69.76
Bacteroidales; f_S24-7;g_	3.60	6.51	76.27
Bifidobacterium	2.65	4.80	81.08
Species			
Lactobacillus;s__	7.75	12.94	12.94
Ruminococcus bromii	6.94	11.58	24.51
Allobaculum;s__	4.80	8.01	32.53
Ruminococcaceae; g__;s__	4.16	6.94	39.47
Blautia;s__	4.06	6.77	46.24
Clostridiales;f__; g__;s__	3.51	5.856	52.10
Lachnospiraceae; g__;s__	3.31	5.52	57.62
f__S24-7;g__;s__	2.94	4.90	62.52
Parabacteroides; s__	2.49	4.16	66.68
Bacteroides;s__	2.25	3.76	70.44
Prevotella;s__	1.84	3.07	73.51
Lactobacillus reuteri	1.79	2.99	76.49
Rikenellaceae;g__; s__	1.64	2.74	79.23
Akkermansia muciniphila	1.63	2.73	81.96

'f__', 'g__' and 's__' indicate unknown family, genus and species, respectively.

taxonomic assignation was performed by fragment insertion script[41] and against the Greengenes database.[39]

Sub-OTUs obtained by Illumina analysis of samples from the colon contents of 24 rats (four per treatment) were grouped by bacterial species (obtained from the bar plots produced by QIIME2). Multivariate statistical techniques[42-43] explored the similarities in rat colon microbiota, and identified species accounting for differences observed in these bacterial communities. Bray–Curtis measures of similarity[44] were calculated to examine similarities between gut microbial communities of rats from the high throughput and qPCR data matrices, following standardization, and square-root transformation. The Bray–Curtis similarity coefficient[44] is a reliable measure for biological data on community structure, and is not affected by joint absences, which are commonly found in microbial data.[45] Analysis of similarity (ANOSIM)[45] was performed to test whether gut microbial communities were significantly different between treatments. Analysis of similarity percentages (SIMPER)[46] was done to determine the overall average similarity in colon microbial community compositions. Principal component analysis (PCA) was used to study the relationships between bacterial groups. It has been reported that PCA is not appropriate where data contain many 'zeros' or where observations (species) exceed the total number of samples.[47] To avoid this limitation, SIMPER analysis was used prior to PCA to select those OTUs responsible for larger dissimilarities, and only those OTUs were used for the PCA analysis. Discriminant analysis (DA) was used to check if the groups to which observations belong are distinct. The Shannon index (H) was calculated as $H = -\sum$ (pi * ln pi), where pi is the abundance of each species. The evenness (E) of the bacterial community was further estimated as E = H/lnS, where S is the total number of species. The Chao1 index is a bias-corrected estimate of total species richness.

RESULTS

For results on growth and feed intake, organs and visceral fat relative weights, bile acids in feces, liver and plasma cholesterol and triglycerides, and long-chain fatty acid composition of liver and visceral fat, see the accompanying paper.[34]

RT-qPCR analysis of microbiota profile in intestinal contents and feces

Data on small intestinal (ileum) bacteria $\log_{10}$ copy numbers after consumption of the experimental diets are collected in Table 3. Obesogenic diets did not affect bacterial counts, while the inclusion of the extruded mixes gave place to significantly lower ($P < 0.005$) values in the counts of most of the bacterial groups studied (bifidobacteria, *Blautia coccoides / Eubacterium rectale*, *Clostridium leptum / Ruminococcus* spp, enterobacteria and *Escherichia / Shigella*). Discriminant analysis (supporting information, Fig. S1) showed that the ileal microbial composition of rats fed the extruded legumes plus cereal mix differed ($P < 0.05$) from the CAS and CAS-OB diets.

Data on colon bacterial numbers are collected in Table 3. Obesogenic diets did not affect bacterial counts, while the inclusion of the extruded mixes gave place to significantly ($P < 0.05$) lower values in the counts of all bacterial groups (bifidobacteria, *Blautia coccoides / Eubacterium rectale*, *Clostridium leptum / Ruminococcus* spp, *Faecalibacterium prausnitzii*, enterobacteria and *Escherichia / Shigella*) except Lactobacilli, which increased, and *Bacteroides / Prevotella*, which was unaffected. Discriminant analysis (supporting information, Fig. S2) showed that the colon microbial composition of rats fed the extruded legumes plus cereal mix was different from CAS and CAS-OB diets.

Data on fecal bacteria numbers after consumption of the experimental diets are collected in Table 3. The replacement of part of the casein for extruded legumes plus cereal mix lowered ($P < 0.001$) bifidobacteria, *Clostridium leptum / Ruminococcus*, *Faecalibacterium prausnitzii* and *Bacteroides / Prevotella* spp fecal counts and increased ($P < 0.001$) *Lactobacillus* spp ($P < 0.018$)

Table 5 Proportions of Illumina sequencing reads at different taxonomic levels of the colon bacterial community of rats fed a control diet (CAS) or diets supplemented with the extruded mix of legumes plus cereals, normocaloric or obesogenic

	Diet[a]						*P* values[b]		
	CAS	CAS-OB	PEM	PEM-OB	KEM	KEM-OB	E	O	E × O
Phylum									
Actinobacteria	8.07	6.81	0.20	0.11	1.07	0.37	<0.001	0.743	0.533
Bacteroidetes	23.50	16.57	22.82	22.08	29.85	39.02	0.054	0.745	0.191
Cyanobacteria	0.07	0.05	0.04	0.13	0.09	0.29	0.199	0.353	0.161
Firmicutes	66.36	73.69	70.98	75.05	64.93	51.87	0.402	0.782	0.255
Proteobacteria	1.29	0.74	1.32	0.48	0.78	1.05	0.746	0.194	0.680
Tenericutes	0.31	0.04	0.59	0.44	0.57	0.80	0.096	0.651	0.530
Verrucomicrobia	1.66	0.84	4.04	1.70	2.71	6.60	0.141	0.991	0.633
Firmicutes/Bacteroidetes	3.01	7.07	3.44	4.27	2.32	1.37	0.012	0.020	0.017
Family									
Bifidobacteriaceae	6.63	7.54	0.20	0.11	1.07	0.36	<0.001	0.835	0.896
Bacteroidaceae	10.37	11.75	2.85	1.71	3.12	3.46	<0.001	0.810	0.661
Bacteroidales;f_S24-7[c]	10.05	3.92	7.29	6.53	9.05	14.73	0.270	0.398	0.057
Lachnospiraceae	35.66	17.35	7.33	8.71	12.62	13.54	<0.001	0.056	0.032
Ruminococcaceae	22.80	53.37	19.39	14.34	16.52	8.81	<0.001	0.025	0.001
Genus									
Bifidobacterium	6.63	7.54	0.20	0.11	1.07	0.36	<0.001	0.835	0.596
Bacteroides	10.37	11.75	2.58	1.71	3.12	3.46	<0.001	0.810	0.661
*Bacteroidales;f_S24-7;g_*[d]	10.05	3.92	7.29	6.53	9.05	14.73	0.270	0.398	0.057
*Lachnospiraceae;g_*	14.57	2 0.88	4.48	5.97	5.08	4.78	0.419	0.225	0.180
Blautia	16.43	10.36	0.37	0.77	4.77	4.77	0.006	0.414	0.384
*Ruminococcaceae;g_*	11.95	15.48	8.92	7.63	10.29	4.23	0.073	0.981	0.265
Ruminococcus	7.47	36.49	3.85	4.65	3.76	2.14	<0.001	<0.001	<0.001
Species									
*Bacteroides;s_*	5.98	10.19	2.59	1.52	2.66	2.99	0.009	0.336	0.254
*Parabacteroides;s_*[e]	2.70	0.82	6.66	7.44	4.69	6.66	0.014	0.886	0.355
*Prevotella;s_*	0.0	0.0	1.74	2.68	5.36	7.46	<0.001	0.432	0.432
*Rikenellaceae;g__;s_*	0.30	0.07	2.59	1.85	6.42	5.04	<0.001	0.518	0.676
*f_S24-7;g_;s_*	10.05	3.92	7.29	6.53	9.05	14.73	0.270	0.398	0.057
*Lactobacillus;s_*	1.21	0.79	19.78	16.71	20.14	11.17	0.014	0.596	0.645
Lactobacillus reuteri	1.65	0.43	2.44	2.48	3.54	8.40	0.033	0.663	0.200
*Clostridiales; f_;g_;s_*	0.28	0.49	9.72	12.59	7.14	4.18	0.003	0.973	0.957
*Lachnospiraceae;g_;s_*	14.57	2.88	4.48	5.97	5.08	4.78	0.419	0.225	0.180
*Blautia;s_*	16.30	9.69	0.37	0.62	4.53	4.59	0.007	0.370	0.348
*Ruminococcaceae;g_;s_*	11.95	15.48	8.92	7.63	10.29	4.23	0.073	0.981	0.265
Ruminococcus bromii	6.67	35.93	1.73	2.49	1.80	0.97	<0.001	<0.001	<0.001
*Allobaculum;s_*	3.07	0.45	9.31	17.99	2.45	2.78	0.287	0.872	0.549
Akkermansia muciniphila	1.66	0.84	5.36	1.7	2.71	2.83	0.039	0.136	0.600

Only groups selected after SIMPER analysis were included.
[a]CAS, casein; KEM, kidney bean (*Phaseolus vulgaris*) extruded mix; PEM, pea (*Pisum sativum*) extruded mix. Diets CAS-OB, KEM-OB and PEM-OB were the same diets described above with added 5.48 g d^{-1} condensed milk.
[b]E, effect of inclusion of extruded legume/rice/carob tree bean; O, effect of the obesogenic diet: E × O, interaction of both effects.
[c,d,e]'f__', 'g__' and 's__' indicate unknown family, genus and species, respectively.

and *B. coccoides/E. rectale* counts. Discriminant analysis (supporting information, Fig. S3) showed that the fecal microbial composition (T = 21 d) of rats fed the extruded legumes plus cereal mix differed from CAS and CAS-OB diets.

Finally, discriminant analysis showed that fecal microbial populations of rats fed the control (CAS) diet differed ($P < 0.01$) with time (T = 0, 10 and 21 d) (supporting information, Fig. S4) and at different points of the intestinal tract (ileum, colon, feces) (supporting information, Fig. S5).

High throughput analysis of microbiota profile in intestinal contents and feces

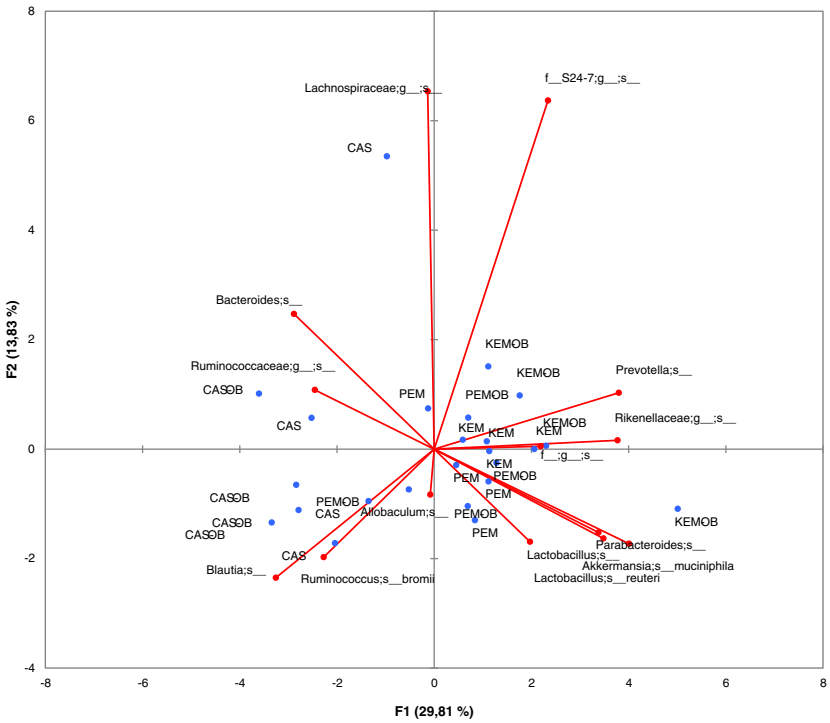
A total of 1 215 064 reads were obtained from the 24 colon contents samples processed through Illumina MiSeq technology. After Deblur, 376 232 sequences belonging to 1217 OTUs and 49 bacterial species were retained for subsequent analyses.

A similarity percentages breakdown (SIMPER analysis) (Table 4) was used to select those bacterial groups with higher contribution to dissimilarity. Thus, five families (*Ruminococcaceae*,

Table 6 ANOSIM (distance measure: Bray–Curtis, Bonferroni corrected *P* values) on sequencing results at different taxonomic levels of samples from the colon bacterial community of rats fed a control diet (CAS) or diets supplemented with the extruded mix of legumes plus cereals, normocaloric or obesogenic.

Family	CAS[a]	CAS-OB	PEM	PEM-OB	KEM	KEM-OB
CAS	0	0.112	0.031	0.030	0.028	0.028
CAS-OB		0	0.027	0.033	0.028	0.030
PEM			0	0.884	0.057	0.057
PEM-OB				0	0.138	0.053
KEM					0	0.198
KEM-OB						0
Genus						
CAS	0	0.060	0.031	0.030	0.030	0.030
CAS-OB		0	0.026	0.028	0.030	0.031
PEM			0	0.734	0.027	0.057
PEM-OB				0	0.093	0.029
KEM					0	0.208
KEM-OB						0
Species						
CAS	0	0.088	0.029	0.031	0.029	0.028
CAS-OB		0	0.027	0.028	0.027	0.027
PEM			0	0.858	0.056	0.058
PEM-OB				0	0.056	0.030
KEM					0	0.083
KEM-OB						0

[a]CAS, casein; KEM, kidney bean (*Phaseolus vulgaris*) extruded mix; PEM, pea (*Pisum sativum*) extruded mix. Diets CAS-OB, KEM-OB and PEM-OB were the same diets described above with added 5.48 g d^{-1} condensed milk.



Square cosines of variables after Varimax rotation			
	D1	D2	D3
Bacteroides;s__	0,138	0,014	**0,313**
Parabacteroides;s__	**0,726**	0,001	0,000
Prevotella;s__	**0,271**	0,127	0,156
Rikenellaceae;g__;s__	**0,254**	0,064	0,222
f__S24-7;g__;s__	0,020	**0,876**	0,009
g__Lactobacillus;s__	0,015	0,004	**0,318**
Lactobacillus;s__reuteri	**0,424**	0,001	0,222
f__;g__;s__	**0,362**	0,010	0,023
Lachnospiraceae;g__;s__	0,065	**0,659**	0,020
Blautia;s__	**0,182**	0,132	0,000
Ruminococcaceae;g__;s__	0,000	0,004	**0,699**
Ruminococcus;s__bromii	0,093	**0,252**	0,187
Allobaculum;s__	0,105	0,004	**0,232**
Akkermansia;s__muciniphila	**0,577**	0,000	0,022

Figure 1 Principal components analysis at the genus level (after SIMPER analysis and varimax rotation) of the sequencing analysis results of the bacterial community of colon samples from rats fed with different diets. CAS, casein; KEM, kidney bean (*Phaseolus vulgaris*) extruded mix; PEM, pea (*Pisum sativum*) extruded mix. Diets CAS-OB, KEM-OB and PEM-OB were the same diets with added 5.48 g d^{-1} condensed milk.

Table 7 Simpson, Shannon, Evenness and Chao1 indexes at different taxonomic levels of sequencing analysis of colon samples from rats fed a control diet (CAS) or diets supplemented with the extruded mix of legumes plus cereals, normocaloric or obesogenic

	Diet[a]						*P* values[b]		
Family	CAS	CAS-OB	PEM	PEM-OB	KEM	KEM-OB	E	O	E × O
Simpson	0.71	0.64	0.78	0.76	0.86	0.87	0.002	0.302	0.417
Shannon	1.70	1.42	2.07	1.91	2.29	2.36	<0.001	0.195	0.359
Evenness	0.30	0.29	0.37	0.37	0.41	0.42	0.001	0.815	0.612
Chao1	19.0	15.25	22.0	18.75	24.50	25.0	0.001	0.912	0.421
Genus									
Simpson	2.09	1.94	2.36	2.14	2.62	2.65	0.013	0.446	0.872
Shannon	0.32	0.31	0.39	0.35	0.44	0.44	0.053	0.651	0.976
Evenness	1.85	1.73	2.08	1.89	2.31	2.33	0.019	0.487	0.903
Chao1	26.5	22.75	29.25	25.25	32.0	32.75	0.009	0.147	0.557
Species									
Simpson	0.79	0.78	0.83	0.79	0.91	0.91	0.084	0.681	0.939
Shannon	2.21	2.09	2.46	2.23	2.77	2.79	0.023	0.485	0.952
Evenness	0.32	0.28	0.38	0.34	0.42	0.42	0.035	0.517	0.858
Chao1	31.5	28.75	32.5	28.5	38.25	38.75	0.059	0.315	0.821

[a]CAS, casein; KEM, kidney bean (*Phaseolus vulgaris*) extruded mix; PEM, pea (*Pisum sativum*) extruded mix. Diets CAS-OB, KEM-OB and PEM-OB were the same diets described above with added 5.48 g d^{-1} condensed milk.
[b]E, effect of inclusion of extruded legume/rice/carob tree bean; O, effect of the obesogenic diet: E × O, interaction of both effects.

Table 8 Expression levels of inflammation parameters[a] in the colon tissue of rats fed a control diet (CAS) or diets supplemented with the extruded mix of legumes plus cereals, normocaloric or obesogenic

	Diet[b]						*P values*[c]		
	CAS	CAS-OB	PEM	PEM-OB	KEM	KEM-OB	E	O	E × O
IL6	1.000	0.867	1.726	1.668	2.061	1.742	0.012	0.344	0.644
IL10	1.000	0.741	1.661	1.011	2.587	0.855	0.369	0.256	0.774
TNF-α	1.000	0.692	1.601	1.296	1.278	1.659	0.036	0.618	0.589
TLR4	1.000	0.892	0.588	0.710	0.490	0.577	0.014	0.979	0.474
IL12b	1.000	1.115	1.616	2.054	2.109	1.386	0.642	0.800	0.494
TJP1	1.000	0.787	0.640	0.990	0.852	0.810	0.812	0.997	0.212
PPARγ	1.000	0.735	0.940	0.756	0.945	0.724	0.757	0.176	0.971
NF-kB65	1.000	0.425	0.990	0.803	1.295	1.037	0.124	0.133	0.346

Values are means (n = 12).
[a]IL6, IL10 and IL12b (interleukins 6, 10 and 12b, resp.), TNF- α (tumour necrosis factor), TLR4 (toll like receptor 4), TJP1 (tight junction protein 1), PPARγ (peroxisome proliferator-activated receptor γ) and NF-kB65 (nuclear factor kB65).
[b]CAS, casein; KEM, kidney bean (*Phaseolus vulgaris*) extruded mix; PEM, pea (*Pisum sativum*) extruded mix. Diets CAS-OB, KEM-OB and PEM-OB were the same diets described above with added 5.48 g d^{-1} condensed milk.
[c]E, effect of inclusion of extruded legume/rice/carob tree bean; O, effect of the obesogenic diet; E × O, interaction of both effects.

Lachnospiraceae, Bacteroidaceae, S24-7 and Bifidobacteriaceae), seven genera (*Ruminococcus, Blautia, Bacteroides, Bifidobacterium* and unidentified genera from *Ruminococcaceae, Lachnospiraceae* and *S24-7*) and 14 species (*Ruminococcus bromii*, *Lactobacillus reuteri*, *Akkermansia muciniphila* and unidentified species from *Lactobacillus, Allobaculum, Ruminococcaceae, Blautia, Clostridiales, Lachnospiraceae, Bacteroidales, Parabacteroides, Bacteroides, Prevotella* and *Rikenellaceae*) were responsible for >80% of the dissimilarity.

Firmicutes, Bacteroidetes and Actinobacteria were the most abundant phyla in all treatments, although Proteobacteria, Verrucomicrobia, Tenericutes and Cyanobacteria were also found at the different taxonomic levels (Table 5). The groups given extruded diets had lower proportions ($P < 0.001$) of Actinobacteria, and ($P < 0.001$) Actinobacteria, and tended to have lower proportions of Bacteroidetes ($P < 0.054$) than the control (casein-based) diets. The Firmicutes / Bacteroidetes ratio was higher ($P < 0.05$) for extruded and obesogenic diets and a significant ($P < 0.05$) interaction between extrusion and obesogenic factors was determined. However, the behavior of PEM and KEM seemed to be different. At the family level, Lachnospiraceae (35.66%), Ruminococcaceae (22.80%), f_S24-7 (10.05%, class Bacteroidales), Bacteroidaceae (10.37%) and Bifidobacetriaceae (6.63%) were the most abundant in the control group. The groups fed the extruded diets had lower (P < 0.001) Lachnospiraceae, Ruminococcaceae, Bacteroidaceae and Bifidobacteriaceae proportions than casein

controls. At the genus level, *Blautia* (16.43%), *Bacteriodes* (10.37%), *Bifidobacterium* (6.63%), *Ruminococcus* (7.47%) and genera from the Lachnospiraceae, Ruminococcaceae and S24-7 families were the most abundant. Rats fed the extruded mixes presented lower ($P < 0.001$) proportions of *Blautia, Bacteriodes, Bifidobacterium* and *Ruminococcus* than casein controls. Animals fed the obesogenic diets had higher ($P < 0.001$) proportions of *Ruminococcus* and this effect depended on the extrusion (extrusion × obesogenic diet interaction, $P < 0.001$). At the species level, *Blautia* spp (16.30%) and species from Ruminococcaceae (11.95%), Lachnospiraceae (14.57%) and S24-7 (10.05%) families were the most abundant. Lower ($P < 0.01$) proportions than control casein diets were determined for *Bacteroides* spp, *Blautia* spp and *Ruminococcus bromii*, while proportions of *Parabacteroides* spp, *Prevotella* spp, a species from the *Rickenellaceae* family, *Lactobacillus* spp, *Lactobacillus reuteri*, a species from class Clostridiales and *Akkermansia muciniphila* increased in comparison with controls ($P < 0.05$). Animals fed the obesogenic diets had higher ($P < 0.001$) proportions of *Ruminococcus bromii* and this effect depended of the extrusion (extrusion × obesogenic diet interaction, $P < 0.001$).

High throughput analysis showed that the microbiota composition of the colon contents of rats fed the extruded mixes, either obesogenic or not, was in all cases different ($P < 0.05$) from that of the rats fed control casein diets (CAS and CAS-OB) at the family (A), genus (B) or species (C) levels (Table 6). The PCA of the Illumina results (Fig. 1) also separated extruded diets from casein controls. On the other hand, PCA at the genus level grouped together *Lactobacillus reuteri*, *Akkermansia muciniphila*, species from *Parabacteroides, Prevotella, Rikenellaceae*, and *Lactobacillus*. Those groups were linked to extruded legume plus cereal diets, while *Ruminococcus bromii*, and species from *Bacteroides, Blautia, Ruminococcaceae* and *Allobaculum* were linked to the casein control diets.

The Simpson, Shannon, Evenness, and Chao1 indexes at the family, genus and species levels tended to be ($P < 0.1$) or were higher ($P < 0.05$) than casein controls for rats fed extruded diets (Table 7).

Gene expression analysis in colonic tissue

Feeding with extruded legumes plus cereal mixes was associated with differences in the immune response compared with the casein-fed controls, as evidenced by increased mRNA expression of the cytokines IL6 and TNF-α and decreased expression of TLR4 (Table 8). Other cytokines (IL10 and IL12b), TJP1, PPARγ and NF-kB65 were not significantly affected by the extruded diets. The obesogenic diets did not affect the expression of any of the genes tested. Figure S6 in the supporting information depicts the discriminant analysis of the expression levels of genes associated with inflammation parameters in the colon of rats fed a control diet (CAS) or diets supplemented with the extruded mix of legumes plus cereals. It showed that rats fed diets containing pea- or kidney-bean based extruded mixes (PEM and KEM) differed ($P < 0.05$) from the controls (CAS and CAS-OB) but not from the extruded mix-based obesogenic diets (PEM-OB and KEM-OB).

DISCUSSION

Legume consumption has been reported to modulate intestinal microbiota composition.[48] However, to the best of our knowledge no information can be found in the literature on the effects of the consumption of extruded legume-based materials on the intestinal microbiota composition of rats, although one reference has appeared recently in pigs.[49] As mentioned above, obese people are associated with changes in the composition and metabolic function of the intestinal microbiota that allow the 'obese microbiota' to extract more energy from the diet.[50] Although controversial, it has been argued that the Firmicutes / Bacteroidetes ratio is affected in those individuals with obesity, although there are several explanations on how this relationship is modified. In our case, the Firmicutes / Bacteroidetes ratio increased in comparison with controls in rats fed obesogenic or extruded diets (Table 5). As mentioned above, most authors report an increase in Firmicutes content[14,51] in diets that favor weight gain, and justify this because the species that belong to this phylum facilitate a greater recovery of energy from the diet ingested, thereby promoting greater weight gain.[5] Ley *et al.*[14] showed that obese subjects present a lower proportion of the Bacteroidetes phylum in comparison with lean subjects, particularly in *Bacteroides* spp, the majority genus of this phylum. Our current high-throughput sequencing results in colon contents are in line with these previous findings (Tables 5). In contrast, other studies report a higher proportion of Bacteroidetes in overweight and obese subjects.[19] It should be borne in mind, however, that, as shown here, population differences can be very marked and even significant at different sampling times (supporting information, Fig. S4) and among the different intestinal tract sections and feces (supporting information, Fig. S5). This might result in substantial variations in this ratio depending on the sampling time and intestinal tract studied. Since most of the studies have been carried out at one only time point and in feces,[21] it seems clear that, if it is to be taken as a reliable marker, a more detailed study of this ratio is necessary taking into account times, intestinal sections and type of diet.

Although there were no significant differences, either for enterobacteria or for the *Escherichia* / *Shigella* subgroup, between the different obesogenic and non-obesogenic treatments, there was a significant and consistent decrease respect to casein controls of both groups in the intestinal contents of rats consuming the extruded mixes (Tables 1 and 2). Drops in the intestinal enterobacteria counts have been also reported earlier in rats[53] and pigs[54] fed legume-containing diets. Enterobacteria are among the gram-negative groups present within the intestine whose overgrowth is a characteristic trait of an abnormal microbiota such as in the course of antibiotic therapy, certain dietary changes or inflammation.[55] Enterobacteria counts may be a relevant factor to modulate the inflammatory process within the intestine. For example, enterobacteria lipopolysaccharide (LPS) activated TLR2- and TLR4-mediated signaling pathways involving myeloid differentiation primary response 88 (MyD88) adaptor and nuclear factor-kappa B (NF-κB) transcription factor, and enhanced reactive oxygen species (ROS) production and the expression of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX) 2 and 4, and inducible nitric oxide synthase (iNOS) genes.[56–57] On the other hand, legume feeding has been reported to decrease the concentrations of LPS and various circulating pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6 in mice, and inhibit induction of iNOS, cyclooxygenase (COX)-2, and activation of NF-κB in the colon.[32,33,58] The administration of a pea protein extract increased lactobacilli counts and reduced both colonic enterobacteria counts and pro-inflammatory markers in mice,[30–31] which is in line with higher lactobacilli counts in colon contents and feces here observed in rats fed the extruded mixes (Tables 2 and 3). The beneficial effects of intestinal lactobacilli have been summarized by Zakostelska *et al.*[59]

Surprisingly, although $\log_{10}$ counts in ileal and colonic content differed between treatments, fecal enterobacteria counts were not different from controls (Table 3), which supports the idea that the sampling point may be a quite relevant factor when studying intestinal microbiota variations. Jumpertz *et al.*[60] suggested that changes in the intestinal microbiota composition observed in obese individuals are associated more with diet composition than obesity status. Wit *et al*. (2012) reached equivalent conclusions with mice fed diets with 45% fat, and suggested that an excess of saturated fat in distal regions of the intestine, rather than obesity, was the major trigger for dysbiosis in the intestinal microbiota.[8] However, it should be noted that the literature does not always indicate from which intestinal tract the counts come. This is a point that may become very relevant, because, as shown in this work, the composition of the microbiota may vary greatly among the different intestinal sections (supporting information, Fig. S8). There are also hardly any studies using condensed milk as obesogenic factor, and even less reporting information on the intestinal microbiota composition, which is a peculiarity of this work.

As far as we are aware, this is the first report on the effects of an extruded mix on the intestinal microbiota composition by using high throughput analysis. High throughput sequencing analysis at the species level (Table 5) of the colon contents confirmed and expanded qPCR results. Thus, lower levels than control casein diets were determined in rats fed extruded mixes for *Bacteroides* spp, *Blautia* spp, and *Ruminococcus bromii*, while proportions of *Parabacteroides* spp, *Prevotella* spp, *Lactobacillus* spp, *Lactobacillus reuteri*, and *Akkermansia muciniphila* increased respect to controls ($P < 0.05$). This is in keeping with the enterotypes concept, which establishes that enterotypes were strongly associated with long-term diets, particularly protein and animal fat (*Bacteroides*) versus carbohydrates (*Prevotella*).[61] Similar results were previously found in mice fed diets supplemented with a pea (*Pisum sativum*) seed extract.[31] Of particular interest is that PCA at the genus level (Fig. 1) linked specifically *Lactobacillus reuteri* and *Akkermansia muciniphila*, two species of recognized probiotic activity,[62–63] with extruded legume plus cereal diets. These species were also among those that contributed most to dissimilarity among groups (Table 4). It is also relevant to note that diets containing the extruded mixes in most cases had higher Simpson, Shannon, Evenness and Chao1 indexes at the family, genus, and species levels than casein controls (Table 7). This is important because biodiversity contributes to the stability and resiliency of ecosystems as highly diverse ecosystems likely contain organisms with redundant functions, making the loss of a single species tolerable because a functionally redundant species can rapidly take over this niche and replenish that function. On the basis of this contention, it is often accepted that, within any given ecosystem, a more diverse community is preferred.[64] Although recent studies in humans and rats have shown that obesity and metabolic syndrome are associated with decreased gut microbial diversity,[65] we have not corroborated it here, probably due to differences in the type of diets used.

Legume (PEM and KEM) diets contained 32.8 and 25.8 g kg^{-1} of α-galactosides (see accompanying paper[34]), which are oligosaccharides of the raffinose family (raffinose, stachyose, and verbascose) present in legume seeds in proportions of at least 7% of the dry matter.[66] Many oligosaccharides, including fructan inulin, fructo-oligosaccharides, and galacto-oligosaccharides have shown prebiotic activity.[28] In particular, legume flour[56,67] and legume oligosaccharides[68] have been reported to increase lactobacilli and decrease enterobacteria intestinal presence, as found in the current investigation. However, dietary fiber[48] and isolated legume (lupin) fiber[69] have also been shown to give place to similar effects on intestinal microbiota composition. As only whole seed meals were fed in the present investigation, it is not therefore possible from the results reported here to assign the effects on microbial growth to either fiber and / or α-galactosides in extruded mixes. Anyway, it is quite relevant to notice that extruded legume plus cereal mixes added in limited amounts to the diet are able to induce beneficial changes in intestinal microbiota composition.

The expression of inflammation parameters was affected in rats fed extruded mixes. Thus, there was an increased mRNA expression of the cytokines IL6 and TNF-α and decreased expression of TLR4 in rats fed the extruded mixes (Table 8), while other inflammatory markers tested were unaffected. As already indicated, previous work by our group showed that the administration of a pea protein extract increased lactobacilli counts and reduced both colonic enterobacteria counts and pro-inflammatory markers in mice.[30–31] Only the effect on TLR4 expression was repeated here, although the current trial was performed in rats and the animals ingested much lower amounts of legume proteins. Although the underlying stimulus for the metabolic derangements[70] in obesity are not fully elucidated, recent evidence in rodents and humans suggests that systemic, low level elevations of gut-derived endotoxin LPS may play an important role in obesity related, whole-body and tissue specific metabolic perturbations. Lipopolysaccharide is the major glycolipid component of the outer membrane of gram-negative bacteria (mostly enterobacteria), which may comprise up to 70% of the total bacteria in the gut.[71] It initiates a well-characterized signaling cascade that elicits many pro- and anti-inflammatory pathways when bound to its receptor, TLR4, which exists in both adipocytes and monocytes, other immune cells, and various other cell types like skeletal muscle, adipose tissue, and liver.[72–74] The downregulation of TLR4 expression is likely to be linked to the lower enterobacteria counts here found in rats fed extruded composites, which suggests an anti-inflammatory effect of legume containing extruded mixes. Finally, a more detailed discussion on the relationship between inflammatory parameters and intestinal microbiota can be found in Aranda-Olmedo and Rubio (2020).[75]

CONCLUSIONS

Our results show that the inclusion in the diet of limited amounts of an extruded legumes plus cereal mix did modulate the intestinal microbiota composition. Differences in microbiota composition were found, depending on the sampling point (ileum, colon, feces) and time of sampling. Except for *Ruminococcus bromii*, obesogenic diets did not affect specific bacterial counts, although the Firmicutes / Bacteroidetes ratio was higher for extruded and obesogenic diets, and a significant interaction between extrusion and obesogenic factors was found. However, the inclusion of the extruded mixes gave place to significant changes in the counts in all relevant bacterial intestinal groups, particularly *Lactobacillus reuteri* and *Akkermansia miciniphila*, which have a recognized probiotic effect. Finally, diversity indexes were in most cases higher than casein controls for rats fed extruded diets, and the inclusion in the diet of extruded legumes plus cereal mixes was associated with differences in the immune response respect to the casein-fed controls. Taken together, these results point to protective, health-promoting properties of

extruded legume plus cereal mixes, and would therefore deserve closer attention in human intervention studies, particularly with adolescents.

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SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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