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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Abbreviations: CAS, casein; PEM and KEM, diets containing pea and kidney bean extruded mixes, respectively; CAS-OB, PEM-OB and KEM-OB, obesogenic diets containing casein or pea or kidney bean extruded mixes, respectively.

Keywords: Extrusion; Legume; Microbiome; Obesity; Rat

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ABSTRACT

BACKGROUND: Differences in the composition of the intestinal microbiota and energetic metabolism between lean and obese population 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 (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 muciniphila* and species from *Parabacteroides*, *Prevotella*, *Rikenellaceae*, and *Lactobacillus* with extruded legume plus cereal diets. Extruded legumes plus cereal mixes feeding 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 diet of limited amounts (closer to practical human eating behaviour) of an extruded legumes plus cereal mix did modulate the intestinal microbiota composition. Taken together, these results point to protective, health promoting properties of extruded legume plus cereal mixes.

49 **INTRODUCTION**

50 The intestinal microbiota is currently regarded as a true bioreactor capable of degrading a large
51 number of otherwise unusable substances (polysaccharides, some protein and amylaceous fractions,
52 etc.) to provide in return nutrients such as fatty acids, vitamins, etc. to the host.¹ Although some
53 eukaryotes and archaea are present, four bacterial phyla (*Firmicutes*, *Bacteroidetes*, *Actinobacteria*
54 and *Proteobacteria*) dominate the human intestinal microbiota accounting for more than 90% of the
55 total.^{2,3} The microbiota ferments indigestible components of food, synthesizes vitamins and other
56 essential micronutrients, transforms cholesterol and bile acids, ensures maturation of the immune
57 system, regulates intestinal angiogenesis and protects against pathogens.⁴ The composition and
58 development of the intestinal microbiota is closely linked to a particular type of diet,⁵ mainly because
59 the non-absorbed compounds in the small intestine reach the large intestine to be fermented by the
60 local bacteria.⁶ A clear indication of this is that those countries whose food is more similar show a
61 more similar microbiota among their inhabitants, whereas it is not so similar in countries with a
62 different diet.⁷ In general, the intake of diets with a high fat content leads to a reduction in the amount
63 and bacterial diversity.⁸ In studies conducted in humanized gnotobiotic mice that started from a diet
64 low in fat and rich in plant polysaccharides and later changed to a westernized diet with high fat and
65 sugar content, modifications were observed in the microbiota composition, metabolic pathways and
66 gene expression only one day after the change, and increased adiposity developed during the next
67 two weeks.⁹ It has been observed that different communities that consume high levels of fiber have a
68 greater abundance of *Prevotella* spp (together with *Roseburia* spp, *Eubacterium rectale* and *E.*
69 *bromii*), whereas dietary fat and the protein better correlate with increased *Bacteroides* abundance.¹⁰
70 Therefore, there is a complex interaction between the intestinal microbiota and the diet: the diet can
71 modulate the microbiota, which in turn also regulates its gene expression and metabolism in response
72 to nutrient availability.¹¹ Portune *et al.*¹² discussed in a quite comprehensive review the relationships
73 between gut microbiota, diet, and obesity-related disorders.

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

84 Legumes are good sources of slow-release carbohydrates and proteins. With exceptions such
85 as lupin (*Lupinus* spp), starch is the main constituent of legume seeds, which becomes partially
86 resistant starch during processing (usually cooking). This resistant starch has been found to possess
87 beneficial properties, such as stimulation of the growth of favourable intestinal bacterial strains and
88 increase of SCFA production during the fermentation process in the colon.²² The total concentration
89 of SCFA in the feces of obese volunteers exceeded by more than 20% that found in samples of lean
90 volunteers; the greatest difference was observed in propionate (41%), followed by butyrate (28%).²¹
91 Legumes contain a high level of non-starch polysaccharides (NSP),²³ which are the main components
92 of dietary fiber. Some dietary fibers are fermentable, and their catabolism in the gastrointestinal tract
93 leads to the generation of various bioactive substances, such as SCFA, which can markedly increase
94 the biomass of the gastrointestinal tract and change the microbiota composition of the intestine.²⁴
95 Kidney bean (*Phaseolus vulgaris*) and pea (*Pisum sativum*) seeds (the legumes used in this study) are
96 considered excellent sources of proteins, mono-, oligo- and polysaccharides and several
97 micronutrients, including minerals, dietary fiber and starch.²⁵ Peas also contain galactose
98 oligosaccharides that may exert beneficial prebiotic effects in the large intestine.²⁶ Many

99 oligosaccharides have a positive impact on the composition of the intestinal microbiota²⁷ and on
100 metabolic syndrome markers.²⁸ Bibi *et al.*²⁹ recently found that green pea supplementation reduced
101 the severity of DSS-induced colitis in mice challenged with a high fat diet by reducing inflammation,
102 mucosal loss, and the endoplasmic reticulum-stress signalling. Finally, a pea albumins extract gave
103 place to an anti-inflammatory effect at the colon level in mice, accompanied by a modulation in the
104 composition of the intestinal microbiota,³⁰⁻³¹ and dark kidney beans or cranberry bean supplements
105 suppressed colonic inflammation and reduced the severity of DSS-induced colitis in mice.³²⁻³³

106 As explained in the accompanying paper,³⁴ new highly palatable extruded mixes of legumes
107 plus cereals similar to a snack food have been produced³⁵ and incorporated in limited amounts into
108 normocaloric or obesogenic diets for rats. Our purpose was to evaluate the effects of dietary
109 incorporation of this new food ingredient on lipids (accompanying paper³⁴) and intestinal microbiota
110 composition (current paper). The inclusion in the diet of extruded mixes induced significant temporal
111 and spatial changes in microbiota composition and diversity indices as determined by RT-qPCR and
112 Illumina analysis. Also, extruded legumes plus cereal mixes feeding was associated with differences
113 in the immune response respect to the casein-fed controls.

114

115 **MATERIALS AND METHODS**

116 All management and experimental procedures carried out in this rat trial were in strict accordance
117 with current European regulations (86/609 E.E.C.) regarding laboratory animals. The Bioethics
118 Committee for Animal Experimentation at our institution (EEZ-CSIC) approved the study protocol.

119

120 **Preparation of the extruded mixes and diets**

121 Two extruded legumes plus cereal mixes were produced by using a Cleextral Evolum25 twin-screw
122 extruder (Cleextral, Riez 42702 Firminy Cedex, France). These were rice (*Oryza sativa*) + pea (*Pisum*
123 *sativum*, cv Cartouche) or kidney bean (*Phaseolus vulgaris*, cv Almonga) + carob tree (*Ceratonia*

124 *siliqua*) fruits. Extruded mixes (PEM and KEM, respectively) composition was rice meal / pea or
125 kidney bean meal / carob fruits meal (50/40/10, w:w:w). All procedures were as in Arribas *et al.*³⁵

126 The diets were based on casein (CAS) or casein plus pea or kidney bean extruded mixes and
127 were formulated to contain the same amount of digestible energy and protein taking into account the
128 analysed composition of ingredients (Table 1). In order not to be too far from practical conditions in
129 human nutrition, pea or kidney bean extruded mixes addition was limited to a low inclusion level
130 (25%) to provide no more than 20% (two servings) of the total recommended daily protein intake.
131 Another relevant point on formulation in the present work was that diets contained no added
132 cholesterol. Appropriate amounts of synthetic amino acids were added to the extruded mix-based
133 diets taking into account their amino acid composition to equalize them to control (casein) values.
134 The diets were supplemented with vitamins and minerals to target requirements. Obesogenic diets
135 (CAS-OB, PEM-OB and KEM-OB) were the same previously described diets (CAS, PEM and KEM)
136 with added amounts (5.48 g d⁻¹) of IDEAL® commercial condensed milk (Table 2).

137

138 **Biological assays**

139 Seventy two (72) male weaned Wistar rats (Charles River Laboratories), matched by weight (80.0 ±
140 2.7 g, mean ± SE), were individually housed in metabolism cages throughout the experiment. Rats
141 were fed a control casein diet between weaning and the start of the experiment. They were then
142 randomly distributed into six groups (n = 12), and each group was assigned to one of the dietary
143 treatments (see above). The animals were individually housed in metabolic cages in an
144 environmentally controlled room under standard conditions (temperature: 20–22 °C with a 12 h
145 light–dark cycle and 55–70% humidity). The rats had *ad libitum* access to their diets and tap water,
146 and were fed the different diets for 21 days. Rats ate all feed offered and there were no leftovers. On
147 day 21, after an overnight fast they were refed at defined time intervals with 11 g of diet and
148 euthanized 60 min after refeeding so that all rats were in the same feeding situation. Animals were

149 anaesthetized with sodium pentobarbital (50 mg Kg⁻¹ of body weight) (Abbott Laboratories, Granada,
150 Spain) and terminal exsanguination was performed by cannulation of the carotid artery.

151

152 **RT-qPCR microbiota composition analysis**

153 Once the animals were sacrificed, the small intestine (last 20 cm) and the colon from all rats (n = 72)
154 were aseptically extracted, and the contents collected in sterile vials and stored at -80 °C for
155 microbiological studies. Total DNA was isolated from intestinal contents (ileum, colon), tissues
156 (ileum, colon) or fecal freeze-dried samples (40 mg)³⁶ using the FavorPrep Stool DNA Isolation Mini
157 Kit (FAVORGEN-Europe, Shuttleworthstraße 19, A-1210 Vienna) by following manufacturer's
158 instructions. Eluted DNA was treated with RNase and the DNA concentration assessed by using a
159 NanoDrop ND-100 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Bacterial
160 log₁₀ number of copies was determined by using q-PCR (iQ5 Cyclor, Bio-Rad Laboratories,
161 Alcobendas, Spain). The 16S rRNA gene-targeted primers and PCR conditions used in this study
162 were as in Table S1. The different microbial groups quantified included total bacteria, *Bacteroides* /
163 *Prevotella* spp, *Lactobacillus* spp, *Bifidobacterium* spp, *Blautia coccoides* / *Eubacterium rectale*
164 group, *Clostridium leptum* / *Ruminococcus* spp, *Faecalibacterium prausnitzii*, *Enterobacteriaceae*
165 and *Escherichia* / *Shigella*. Samples for q-PCR analysis were run in duplicate.

166

167 **High throughput analysis of microbial community**

168 The bacterial diversity of the samples was determined using Illumina technology (MiSeq). Total
169 DNA was isolated from freeze-dried colon content (20 mg) from 4 rats per group (n = 24) as
170 described above. Libraries preparation was performed by amplification of the V4-V5 region of the
171 16S rRNA gene. First amplification was performed by using primers Mi_U515 (5'-
172 GTGCCAGCMGCCGCGGTAA-3') and Mi_E786 (5'-GGACTACHVGGGTWTCTAAT-3')
173 including partially Illumina adapters, and PCR conditions were: initial denaturalization at 98 °C 30 s;

174 25 cycles with denaturalization at 98 °C 10 s, annealing at 52 °C 20 s and extension at 72 °C 10 s; and
175 a final extension at 72 °C 5 min. Second amplification included barcodes and the rest of the Illumina
176 adapters, and PCR conditions were: initial denaturalization at 98 °C 30 s; 25 cycles with
177 denaturalization at 98 °C 10 s, annealing at 52 °C 20 s and extension at 72 °C 10 s; and a final
178 extension at 72 °C 5 min. All amplifications were performed in duplicate. Total DNA was isolated
179 from freeze-dried colon content (20 mg) as described above. Aliquots of 10 µL of each DNA were
180 sent to the CGEB IMR (Dalhousie University; Halifax, Nova Scotia, Canada) for sequencing.

181

182 **Gene expression analysis in colonic tissue**

183 The analysis of gene expression in the colonic samples was performed by RT-qPCR. For this purpose
184 total RNA of colonic samples from all rats (n = 72) was isolated using TRI Reagent[®] following the
185 manufacturer's protocol. All RNA samples were quantified with the Thermo Scientific NanoDrop[™]
186 2000 Spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA USA) and 2 µg of RNA were
187 reverse transcribed using oligo(dT) primers (Promega, Southampton, UK). RT-qPCR amplification
188 and detection was performed on optical-grade 48well plates in a IQ5 Real-Time PCR System (Bio-
189 Rad Laboratories, Alcobendas, Madrid, Spain) with 20 ng of cDNA, the KAPA SYBR[®] FAST qPCR
190 Master Mix (Kapa Biosystems, Inc., Wilmington, MA, USA) and specific primers at their annealing
191 temperature (Table S2). To normalize mRNA expression, the expression of the housekeeping gene,
192 glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was measured. The mRNA relative
193 quantitation was calculated using the $\Delta\Delta C_t$ method.

194

195 **Statistical analysis**

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

199 Results from high throughput sequencing analyses were performed by using the *Quantitative*
200 *Insights In Microbial Ecology* (QIIME2,³⁷⁻³⁸). Due to the low quality of the reverse sequences, only
201 forward sequences were employed in subsequent analyses. Quality filtering was performed by using
202 default parameters in QIIME2. Sub-OTU (sub-Operational Taxonomic Units) table in biom format³⁹
203 was created using Deblur,⁴⁰ and alignment and taxonomic assignation was performed by fragment
204 insertion script⁴¹ and against Greengenes database.³⁹

205 Sub-OTUs obtained by Illumina analysis of samples from the colon contents of 24 (4 per
206 treatment) rats were grouped by bacterial species (obtained from the bar plots produced by QIIME2).
207 Multivariate statistical techniques⁴²⁻⁴³ explored the similarities in rats' colon microbiota, and
208 identified species accounting for differences observed in these bacterial communities. Bray-Curtis
209 measures of similarity⁴⁴ were calculated to examine similarities between gut microbial communities
210 of rats from the high throughput and qPCR data matrices, following standardization, and square root
211 transformation. The Bray-Curtis similarity coefficient⁴⁴ is a reliable measure for biological data on
212 community structure, and is not affected by joint absences that are commonly found in microbial
213 data.⁴⁵ Analysis of similarity (ANOSIM)⁴⁵ was performed to test whether gut microbial communities
214 were significantly different between treatments. Analysis of similarity percentages (SIMPER)⁴⁶ was
215 done to determine the overall average similarity in colon microbial community compositions.
216 Principal Components Analysis (PCA) was used to study the relationships between bacterial groups.
217 It has been reported that PCA is not appropriate where data contain many "zeros" or where
218 observations (species) exceed the total number of samples.⁴⁷ To avoid this limitation, SIMPER
219 analysis was used prior to PCA to select those OTUs responsible for larger dissimilarities, and only
220 those OTUs were used for the PCA analysis. Discriminant Analysis (DA) was used to check if the
221 groups to which observations belong are distinct. The Shannon index (H) was calculated as $H = -\sum$
222 $(p_i * \ln p_i)$, where p_i is the abundance of each species. The Evenness (E) of the bacterial community

223 was further estimated as $E = H/\ln S$, where S is the total number of species. Chao1 index is a bias
224 corrected estimate of total species richness.

225

226 **RESULTS**

227 For results on growth and feed intake, organs and visceral fat relative weights, bile acids in feces,
228 liver and plasma cholesterol and triglycerides, and long chain fatty acids composition of liver and
229 visceral fat, see accompanying paper.³⁴

230

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

232 Data on small intestinal (ileum) bacteria log₁₀ copy numbers after consumption of the experimental
233 diets are collected in Table 3. Obesogenic diets did not affect bacterial counts, while the inclusion of
234 the extruded mixes gave place to significantly lower ($P < 0.005$) values in the counts of most of the
235 bacterial groups studied (bifidobacteria, *Blautia coccoides* / *Eubacterium rectale*, *Clostridium leptum*
236 / *Ruminococcus* spp, enterobacteria and *Escherichia* / *Shigella*). Discriminant Analysis (Figure S1)
237 showed that the ileal microbial composition of rats fed the extruded legumes plus cereal mix differed
238 ($P < 0.05$) from CAS and CAS-OB diets.

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

246 Data on fecal bacteria numbers after consumption of the experimental diets are collected in
247 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 ($P < 0.018$) and *B. coecoides* / *E. rectale* counts. Discriminant analysis (Figure 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) (Figure S4) and at different points of the intestinal tract (ileum, colon, feces) (Figure 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 1,217 OTUs and 49 bacterial species were retained for subsequent analyses.

SIMPER analysis (Table 4) was used to select those bacterial groups with higher contribution to dissimilarity. Thus, 5 families (*Ruminococcaceae*, *Lachnospiraceae*, *Bacteroidaceae*, S24-7 and *Bifidobacteriaceae*), 7 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 ($P < 0.001$) *Actinobacteria* and tended to have lower ($P < 0.054$) *Bacteroidetes* proportions respect to the control

273 (casein-based) diets. The *Firmicutes* / *Bacteroidetes* ratio was higher ($P < 0.05$) for extruded and
 274 obesogenic diets and a significant ($P < 0.05$) interaction between extrusion and obesogenic factors
 275 was determined. However, the behavior of PEM and KEM seemed to be different. At the Family
 276 level, *Lachnospiraceae* (35.66 %), *Ruminococcaceae* (22.80 %), f_S24-7 (10.05 %, class
 277 *Bacteroidales*), *Bacteroidaceae* (10.37 %) and *Bifidobacteriaceae* (6.63 %) were the most abundant
 278 in the control group. The groups fed the extruded diets had lower ($P < 0.001$) *Lachnospiraceae*,
 279 *Ruminococcaceae*, *Bacteroidaceae* and *Bifidobacteriaceae* proportions than casein controls. At the
 280 Genus level, *Blautia* (16.43 %), *Bacteriodes* (10.37 %), *Bifidobacterium* (6.63 %), *Ruminococcus*
 281 (7.47 %) and genera from *Lachnospiraceae*, *Ruminococcaceae* and S24-7 families were the most
 282 abundant. Rats fed the extruded mixes presented lower ($P < 0.001$) proportions of *Blautia*,
 283 *Bacteriodes*, *Bifidobacterium* and *Ruminococcus* than casein controls. Animals fed the obesogenic
 284 diets had higher ($P < 0.001$) proportions of *Ruminococcus* and this effect depended of the extrusion
 285 (interaction extrusion x obesogenic diet, $P < 0.001$). At the Species level, *Blautia* spp (16.30 %) and
 286 species from *Ruminococcaceae* (11.95 %), *Lachnospiraceae* (14.57 %) and S24-7 (10.05 %) families
 287 were the most abundant. Lower ($P < 0.01$) proportions respect to control casein diets were
 288 determined for *Bacteroides* spp, *Blautia* spp and *Ruminococcus bromii*, while proportions of
 289 *Parabacteroides* spp, *Prevotella* spp, a species from *Rickenellaceae* family, *Lactobacillus* spp,
 290 *Lactobacillus reuteri*, a species from class *Clostridiales* and *Akkermansia muciniphila* increased
 291 respect to controls ($P < 0.05$). Animals fed the obesogenic diets had higher ($P < 0.001$) proportions of
 292 *Ruminococcus bromii* and this effect depended of the extrusion (interaction extrusion x obesogenic
 293 diet, $P < 0.001$).

294 High throughput analysis showed that of the microbiota composition of the colon contents of
 295 rats fed the extruded mixes, either obesogenic or not, was in all cases different ($P < 0.05$) from rats
 296 fed control casein diets (CAS and CAS-OB) at the family (A), genus (B) or species (C) levels (Table
 297 6) PCA of the Illumina results (Figure 1) also separated extruded diets from casein controls. On the

other hand, PCA at the Genus level grouped together *Lactobacillus reuteri*, *Akkermansia muciniphila* and 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.

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

Extruded legumes plus cereal mixes feeding was associated with differences in the immune response respect to the casein-fed controls, as evidenced by an increased mRNA expression of the cytokines IL6 and TNF- α and decreased expression of TLR4 (Table 8). Other cytokines (IL10 and IL12b), TJP1, PPAR γ 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 depicts the Discriminant Analysis of the expression levels of genes related 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 diet 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 consumption of extruded legume-based materials on the intestinal microbiota composition of rats, although one reference has appeared recently in pigs.⁴⁹ As already mentioned, obese people are

323 associated with changes in the composition and metabolic function of the intestinal microbiota that
324 allow the “obese microbiota” to extract more energy from the diet.⁵⁰ Although controversial, it has
325 been argued that the *Firmicutes* / *Bacteroidetes* ratio is affected in those individuals with obesity,
326 although there are several explanations on how this relationship is modified. In our case, the
327 *Firmicutes* / *Bacteroidetes* ratio increased respect to controls in rats fed obesogenic or extruded diets
328 (Table 5). As mentioned above, most authors report an increase in *Firmicutes* proportions^{14,51} in diets
329 that favour weight gain, and justify this because the species that belong to this phylum facilitate a
330 greater recovery of energy from the diet ingested, thereby promoting greater weight gain.⁵ Ley *et*
331 *al.*¹⁴ showed that obese subjects present a lower proportion of the *Bacteroidetes* phylum in
332 comparison with lean subjects, particularly in *Bacteroides* spp, the majority genus of this phylum.
333 Our current high throughput sequencing results in colon contents are in line with these previous
334 findings (Tables 5). In contrast, other studies report a higher proportion of *Bacteroidetes* in
335 overweight and obese subjects.¹⁹ It should be borne in mind, however, that, as shown here,
336 population differences can be very marked and even significant at different sampling times (Figure
337 S4) and among the different intestinal tract sections and feces (Figure S5). This might result in
338 substantial variations in this ratio depending on the sampling time and intestinal tract studied. Since
339 most of the studies have been carried out at one only time point and in feces,²¹ it seems clear that, if it
340 is to be taken as a reliable marker, a more detailed study of this ratio is necessary taking into account
341 times, intestinal sections and type of diet.

342 Although there were no significant differences, either for enterobacteria or for the *Escherichia*
343 / *Shigella* subgroup, between the different obesogenic and non-obesogenic treatments, there was a
344 significant and consistent decrease respect to casein controls of both groups in the intestinal contents
345 of rats consuming the extruded mixes (Tables 1 & 2). Drops in the intestinal enterobacteria counts
346 have been also reported earlier in rats⁵³ and pigs⁵⁴ fed legume containing diets. Enterobacteria are
347 among the Gram-negative groups present within the intestine whose overgrowth is a characteristic

348 trait of an abnormal microbiota such as in the course of antibiotic therapy, certain dietary changes or
349 inflammation.⁵⁵ Enterobacteria counts may be a relevant factor to modulate the inflammatory process
350 within the intestine. For example, enterobacteria lipopolysaccharide (LPS) activated TLR2- and
351 TLR4-mediated signalling pathways involving Myeloid differentiation primary response 88 (MyD88)
352 adaptor and nuclear factor-kappa B (NF-κB) transcription factor, and enhanced reactive oxygen
353 species (ROS) production and the expression of NADPH Oxidase (NOX) 2 and 4, and inducible
354 nitric oxide synthase (iNOS) genes.⁵⁶⁻⁵⁷ On the other hand, legume feeding has been reported to
355 decrease the concentrations of LPS and various circulating pro-inflammatory cytokines such as
356 tumour necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6 in mice, and inhibit induction of iNOS,
357 cyclooxygenase (COX)-2, and activation of NF-κB in the colon.^{32,33,58} Even more, the administration
358 of a pea protein extract increased lactobacilli counts and reduced both colonic enterobacteria counts
359 and pro-inflammatory markers in mice,³⁰⁻³¹ which is in line with higher lactobacilli counts in colon
360 contents and feces here observed in rats fed the extruded mixes (Tables 2 & 3). The beneficial effects
361 of intestinal lactobacilli have been summarized by Zakostelska *et al.*⁵⁹

362 Surprisingly, although log₁₀ counts in ileal and colonic contents differed between treatments,
363 fecal enterobacteria counts were not different from controls (Table 3), which adds more to the idea
364 that the sampling point may be a quite relevant factor when studying intestinal microbiota variations.
365 Jumpertz *et al.*⁶⁰ suggested that changes in the intestinal microbiota composition observed in obese
366 individuals are associated more with diet composition than obesity status. Wit *et al.* (2012) reached
367 equivalent conclusions with mice fed diets with 45 % fat, and suggested that an excess of saturated
368 fat in distal regions of the intestine, rather than obesity, was the major trigger for dysbiosis in the
369 intestinal microbiota. However, it should be noted that it is not always indicated in the literature
370 which intestinal tract the counts come from. This is a point that may become very relevant, since, as
371 shown in this work, the composition of the microbiota may vary greatly among the different
372 intestinal sections (Figure S8). In addition, there are hardly any studies using condensed milk as

373 obesogenic factor, and even less reporting information on the intestinal microbiota composition,
374 which is a peculiarity of this work.

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

397 Legume (PEM and KEM) diets contained 32.8 and 25.8 g/kg of α -galactosides (see
398 accompanying paper³⁴) which are oligosaccharides of the raffinose family (raffinose, stachyose and
399 verbascose) present in legume seeds in proportions of at least 7 % of the dry matter.⁶⁶ Many
400 oligosaccharides, including fructan inulin, fructo-oligosaccharides, and galacto-oligosaccharides have
401 shown prebiotic activity.²⁸ In particular, legume flour^{56,67} and legume oligosaccharides⁶⁸ have been
402 reported to increase lactobacilli and decrease enterobacteria intestinal presence, as found in the
403 current investigation. However, dietary fiber⁴⁸ and isolated legume (lupin) fiber⁶⁹ have also been
404 shown to give place to similar effects on intestinal microbiota composition. Therefore, since only
405 whole seed meals were fed in the present investigation, it is not possible from the results reported
406 here to assign the effects on microbial growth to either fiber and/or α -galactosides in extruded mixes.
407 Anyway, it is quite relevant to notice that extruded legume plus cereal mixes added in limited
408 amounts to the diet are able to induce beneficial changes in intestinal microbiota composition.

409 Expression of inflammation parameters was affected in rats fed extruded mixes. Thus, there
410 was an increased mRNA expression of the cytokines IL6 and TNF- α and decreased expression of
411 TLR4 in rats fed the extruded mixes (Table 10), while other inflammatory markers tested were
412 unaffected. As already indicated, previous work by our group showed that the administration of a pea
413 protein extract increased lactobacilli counts and reduced both colonic enterobacteria counts and pro-
414 inflammatory markers in mice.³⁰⁻³¹ Only the effect on TLR4 expression was repeated here, although
415 the current trial was performed in rats and the animals ingested much lower amounts of legume
416 proteins. Although the underlying stimulus for the metabolic derangements⁷⁰ in obesity are not fully
417 elucidated, recent evidence in rodents and humans suggests that systemic, low level elevations of gut
418 derived endotoxin LPS may play an important role in obesity related, whole-body and tissue specific
419 metabolic perturbations. LPS is the major glycolipid component of the outer membrane of Gram-
420 negative bacteria (mostly enterobacteria) which may comprise up to 70 % of the total bacteria in the
421 gut.⁷¹ LPS initiates a well-characterized signalling 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 Therefore, 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 & Rubio (2020)⁷⁵.

CONCLUSIONS

Our results show that the inclusion in the diet of limited amounts (closer to practical conditions) 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 muciniphila*, which have a recognised 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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449

450 The authors declare no conflicts of interest.

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Figure legends

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.

Figure S1. Discriminant Analysis of microbial qPCR counts in samples from the ileum contents of rats fed CAS (yellow); CAS-OB (grey); KEM (orange); KEM-OB (dark blue); PEM (light blue); PEM-OB (green). 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. Values for rats fed diets based in casein (CAS and CAS-OB) differed ($P < 0.01$) from those fed diets based in extruded mixes.

Figure S2. Discriminant Analysis of microbial qPCR counts in samples from the colon contents of rats fed CAS (yellow); CAS-OB (grey); KEM (orange); KEM-OB (dark blue); PEM (light blue); PEM-OB (green). 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. Values for rats fed diets based in casein (CAS and CAS-OB) were different ($P < 0.01$) from those fed diets based in extruded mixes.

Figure S3. Discriminant Analysis of microbial qPCR counts in samples from the feces at T = 21 d of rats fed CAS (yellow); CAS-OB (grey); KEM (orange); KEM-OB (dark blue); PEM (light blue); PEM-OB (green). 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. Values for rats fed diets based in casein (CAS and CAS-OB) were different ($P < 0.01$) from those fed diets based in extruded mixes.

Figure S4. Discriminant Analysis of microbial qPCR counts in samples from the feces at three different times (T=0 d, T=10 d, T=21 d) of rats fed the control casein (CAS) diet. T = 0 d (blue); T = 10 d (green); T = 21 d (orange). Differences between sampling times were significant ($P < 0.001$).

671 **Figure S5.** Discriminant Analysis of microbial qPCR counts in samples from the ileum (orange), colon (blue)
672 and feces (green) at T = 21 d) of rats fed the control (CAS) diet. Differences between intestinal sections were
673 significant ($P < 0.001$).

674 **Figure S6.** Discriminant Analysis of the expression levels of inflammation parameters in samples from the
675 colon of rats fed CAS (yellow); CAS-OB (grey); KEM (orange); KEM-OB (dark blue); PEM (light blue);
676 PEM-OB (green). CAS, casein; KEM, kidney bean (*Phaseolus vulgaris*) extruded mix; PEM, pea (*Pisum*
677 *sativum*) extruded mix. Diets CAS-OB, KEM-OB and PEM-OB were the same diets with added 5.48 g d⁻¹
678 condensed milk. Rats fed pea or kidney bean based extruded diets (PEM and KEM) differed ($P < 0.05$) from
679 the controls (CAS and CAS-OB) but were not different from the extruded mix- based obesogenic diets (PEM-
680 OB and KEM-OB).

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697 **Table 1.** Analysed composition (g kg⁻¹) of casein and legume + cereal extruded mixes

	CAS ¹	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 ²	1.2	1.4
Ash	ND	34.6	38.4
Crude fibre	ND	32.1	24.6
Dietary fibre	ND	94.1	114.5
α-galactosides	ND	32.8	25.8
Total carbohydrates ³	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

698 ¹CAS, casein; Pea (*Pisum sativum*) extruded mix (rice/pea/carob tree bean, 50/40/10); Kidney bean
699 (*Phaseolus vulgaris*) extruded mix (rice/kidney bean/carob tree bean, 50/40/10). ²ND, not
700 determined. ³Total solids excluding protein, fat, ash, dietary fibre and α-galactosides.

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Table 2. Composition (g kg⁻¹) of the diets

	CAS ¹	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 ₂ O 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 fibre	50.0	73.5	78.6

¹CAS, 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).

710 **Table 3.** Effect of the diet on the bacterial counts (log₁₀ copies of the 16 S-rRNA gene mg⁻¹ dry content) in the ileal content of rats fed a control
711 diet (CAS) or diets supplemented with two extruded mixes of legumes plus cereals, normocaloric or obesogenic.

	Diet ¹						<i>P values</i> ²		
	CAS	CAS-OB	PEM	PEM-OB	KEM	KEM-OB	E	O	E x 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
<i>Blautia coccoides</i> / <i>Eubacterium rectale</i>	5.30	5.92	4.91	4.81	4.79	4.49	<0.001	0.205	0.124
<i>Clostridium leptum</i> / <i>Ruminococcus spp</i>	4.84	5.75	4.22	3.27	3.76	3.81	<0.001	0.297	0.001
<i>Faecalibacterium prausnitzii</i>	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
<i>Escherichia/Shigella</i>	4.88	5.02	4.58	4.26	4.43	4.15	0.004	0.688	0.275
<i>Bacteroides/Prevotella</i>	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
<i>Blautia coccoides</i> / <i>Eubacterium rectale</i>	7.79	7.63	7.52	7.39	7.35	7.43	<0.001	0.155	0.284

<i>Clostridium leptum</i> / <i>Ruminococcus spp</i>	7.51	7.75	6.99	6.77	6.72	6.74	<0.001	0.361	0.031
<i>Faecalibacterium prausnitzii</i>	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
<i>Escherichia/Shigella</i>	5.93	5.91	4.87	5.42	5.17	5.44	<0.001	0.241	0.200
<i>Bacteroides/Prevotella</i>	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
<i>Blautia coccoides</i> / <i>Eubacterium rectale</i>	7.15	7.26	7.09	7.43	7.45	7.65	0.023	0.031	0.351
<i>Clostridium leptum</i> / <i>Ruminococcus spp</i>	7.36	7.73	6.86	6.85	6.89	6.81	<0.001	0.118	0.050
<i>Faecalibacterium prausnitzii</i>	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
<i>Escherichia/Shigella</i>	5.75	5.65	5.57	5.84	5.87	5.73	0.700	0.921	0.547
<i>Bacteroides/Prevotella</i>	7.85	7.62	7.19	7.58	7.15	7.84	<0.001	0.039	<0.001

712 Values are means (n = 12). ¹CAS, casein; KEM, kidney bean (*Phaseolus vulgaris*) extruded mix; PEM, pea (*Pisum sativum*) extruded mix. Diets
713 CAS-OB, KEM-OB and PEM-OB were the same diets described above with added 5.48 g d⁻¹ condensed milk. ²E, effect of inclusion of extruded
714 legume / rice / carob tree bean; O, effect of the obesogenic diet: E x O, interaction of both effects.

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

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

721 **Table 5.** Proportions of Illumina sequencing reads at different taxonomic levels of the colon bacterial community of rats fed a control diet (CAS)
722 or diets supplemented with the extruded mix of legumes plus cereals, normocaloric or obesogenic. “f__”, “g__” and “s__” indicate unknown
723 family, genus and species, respectively.

	Diet ¹						P values ²		
	CAS	CAS-OB	PEM	PEM-OB	KEM	KEM-OB	E	O	E x 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	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__	10.05	3.92	7.29	6.53	9.05	14.73	0.270	0.398	0.057
Lachnospiraceae;g__	14.57	2.88	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_	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

724 Only groups selected after SIMPER analysis were included. ¹CAS, casein; KEM, kidney bean (*Phaseolus vulgaris*) extruded mix; PEM, pea
725 (*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
726 milk. ²E, effect of inclusion of extruded legume / rice / carob tree bean; O, effect of the obesogenic diet: E x O, interaction of both effects.

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

Family	CAS	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

732 ¹CAS, casein; KEM, kidney bean (*Phaseolus vulgaris*) extruded mix; PEM, pea (*Pisum*
 733 *sativum*) extruded mix. Diets CAS-OB, KEM-OB and PEM-OB were the same diets
 734 described above with added 5.48 g d⁻¹ condensed milk. ²E, effect of inclusion of extruded
 735 legume / rice / carob tree bean; O, effect of the obesogenic diet: E x O, interaction of both
 736 effects.

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

	Diet ¹						P values ²		
Family	CAS	CAS-OB	PEM	PEM-OB	KEM	KEM-OB	E	O	E x 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

739 ¹CAS, casein; KEM, kidney bean (*Phaseolus vulgaris*) extruded mix; PEM, pea (*Pisum sativum*) extruded mix. Diets CAS-OB, KEM-OB and
740 PEM-OB were the same diets described above with added 5.48 g d⁻¹ condensed milk. ²E, effect of inclusion of extruded legume / rice / carob
741 tree bean; O, effect of the obesogenic diet: E x O, interaction of both effects.

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743 **Table 8.** Expression levels of inflammation parameters¹ in the colon tissue of rats fed a control diet (CAS) or diets supplemented with the extruded
 744 mix of legumes plus cereals, normocaloric or obesogenic.

	Diet ²						<i>P values</i> ³		
	CAS	CAS-OB	PEM	PEM-OB	KEM	KEM-OB	E	O	E x 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
PPAR γ	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

745 Values are means (n = 12). ¹IL6, IL10 and IL12b (interleukins 6, 10 and 12b, resp.), TNF- α (tumour necrosis factor), TLR4 (toll like receptor 4),
 746 TJP1 (tight junction protein 1), PPAR γ (peroxisome proliferator-activated receptor γ) and NF-kB65 (nuclear factor kB65). ²CAS, casein; KEM,
 747 kidney bean (*Phaseolus vulgaris*) extruded mix; PEM, pea (*Pisum sativum*) extruded mix. Diets CAS-OB, KEM-OB and PEM-OB were the same
 748 diets described above with added 5.48 g d⁻¹ condensed milk. ³E, effect of inclusion of extruded legume / rice / carob tree bean; O, effect of the
 749 obesogenic diet; E x O, interaction of both effects.

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Supplementary Material S1.

The q-PCR assays were performed in polypropylene 96-well plates using an iQ5 Cycler Multicolor PCR detection system (BioRad laboratories, Hercules, CA, USA). The reaction mixture (25 µl) comprised of 12.5 µl of SensiFast SYBR Supermix (Bioline), 0.75 µl of each of the specific primers (10 µM; Roche Diagnostics, Barcelona, Spain), 9µl of sterile distilled water and 2 µl of DNA template. The fluorescent products were detected in the last step of each cycle. A melting curve analysis was carried out after amplification to distinguish the targeted PCR product from non-targeted PCR product (Wittwer *et al.*, 1997). The melting curves were obtained by slow heating at temperatures from 55 to 95 °C at a rate of 0.5 °C / 10 s, with continuous fluorescence collection. A plasmid standard containing the target region (a fragment of the 16S ribosomal RNA gene) was generated. For this purpose, DNA was amplified by PCR using specific primers. The amplified product was run on a 2 % agarose gel, purified by NucleoSpin Gel and PCR clean-up kit (Macherey-Nagel), cloned using the pGEM[®]-T Easy Vector System I (Promega), and transformed into Escherichia coli JM109 cells (Zymo Research). Plasmids were eluted by Isolate II Plasmid Mini Kit (Bioline), and sequences were obtained by the sequencing service of the Instituto de Parasitología y Biomedicina López-Neyra (CSIC, Granada, Spain). The concentration of the resulted products was determined by NanoDrop One^C spectrophotometer (Thermo-Scientific) and the copy number was calculated in terms of the product size. For quantification of target DNA copy number, a standard curve was generated using serial 10-fold dilutions of the extracted product by using at least six nonzero standard concentrations per assay. The bacterial concentration in each sample was measured as log₁₀ copy number by the interpolation of the Ct values obtained by the faecal samples into the standard calibration curves. Each plate included duplicate reactions per DNA sample and the appropriate set of standards.

777 *Sequences of the primers used and q-PCR conditions for SYBR green 16 rDNA determination of microbial groups in the chicken faecal freeze-dried*
 778 *samples of the in vivo experiment.*

779				
780	Bacterial group	Primer	Reference	q-PCR conditions
781				
782	Total bacteria	F-Eub 338 (ACTCCTACGGGAGGCAGCAG)	Guo <i>et al.</i> , 2008	Initial denaturation: 1 cycle at
783		R-Eub 518 (ATTACCGCGGCTGCTGG)		50 °C 2 min and 95°C 10 min. Primer
784				annealing and product elongation:
785				45x (95°C 15s, 60 °C 1 min, 72°C 30s),
786				95 °C 1 min, 55 °C 1 min, 81x (55 °C 10s).
787				
788	Lactobacilli	F-lacto (GAGGCAGCAGTAGGGAATCTTC)	Delroisse <i>et al.</i> , 2008	Idem total bacteria
789		R-lacto (GGCCAGTTACTACCTCTATCCTTCTTC)		
790				
791	Bifidobacteria	F-bifido (CGCGTCYGGTGTGAAAG)	Delroisse <i>et al.</i> , 2008	Initial denaturation: 1 cycle at 50°C 2 min
792		R-bifido (CCCCACATCCAGCATCCA)		and at 95°C 10 min. Primer annealing and
793				product elongation: 45x (95°C 15s, 60°C 1
794				min, 83°C 30 s), 95°C 1 min, 55°C 1 min,
795				81x (55°C 10s)

796	Enterobacteria	F5 (ATGGCTGTCGTCAGCTCGT)	Castillo <i>et al.</i> , 2006	Idem total bacteria
797		R5 (CCTACTTCTTTTGCAACCCACTC)		
798				
799	<i>Bacteroides</i> /	AllBac296F (GAGAGGAAGGTCCCCCAC)	Layton <i>et al.</i> , 2006	Idem total bacteria
800	<i>Prevotella</i>	AllBac412R (CGCTACTTGGCTGGTTCAG)		
801				
802	<i>C. coccoides</i> /	g-Ccoc-F (AAATGACGGTACCTGACTAA)	Matsuki <i>et al.</i> , 2004	Initial denaturation: 94°C for 5 min.
803	<i>E. rectale</i>	g-Ccoc-R (CTTTGAGTTTCATTCTTGCGAA)		Primer annealing and product
804				elongation: 40 cycles at 94°C 20s,
805				50°C 20s, and 72°C 1 min.
806				
807	<i>C. leptum</i> /	sg-Clept-F (GCACAAGCAGTGGAGT)	Matsuki <i>et al.</i> , 2004	Idem <i>C coccoides</i> / <i>E rectale</i>
808	<i>Ruminococcus</i>	sg-Clept-R3 (CTTCCTCCGTTTTGTCAA)		
809				
810				
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813	<i>Escherichia-Shigella</i>	F5 (GTTAATACCTTTGCTCATTGA)	Malinen <i>et al.</i> , 2003	Initial denaturation: 95°C for 10 min.
814		R5 (ACCAGGGTATCTAATCCTGTT)		Primer annealing and product
815				elongation: 35 cycles at 95°C 15s,
816				60°C 20s, and 72°C 30s.
817				
818	<i>Faecalibacterium</i>	Fprau-F (GGAGGATTGACCCCTTCAGT)	Heinritz <i>et al.</i> , 2016	Initial denaturation: 95°C 10 min.
819	<i>prausnitzii</i>	R-praust (ACCTTCCTCCGTTTTGTCAAC)	Forssten <i>et al.</i> , 2011	Primer annealing and product
820				elongation: 40 cycles at 95°C 15s,
821				60°C 60s, and 72°C 30s.
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