Pepsin egg white hydrolysate modulates gut microbiota in Zucker obese rats

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Abstract

There is limited information that relates the intake of food-derived bioactive peptides and the gut microbiota. We have previously described a pepsin hydrolysate of egg white (EWH) that ameliorates fat accumulation and dyslipidemia, while reducing oxidative stress and inflammation markers in obese Zucker rats. The aim of this study was to associate the beneficial effects of EWH with gut microbiota changes in these animals. Obese Zucker rats received daily 750 mg/kg EWH in drinking water for 12 weeks and faeces were analysed for microbial composition and metabolic compounds in comparison with Zucker lean rats and obese controls. EWH supplementation modulated the microbiological characteristics of the obese rats to values similar to those of the lean rats. Specifically, counts of total bacteria, *Lactobacillus*/*Enterococcus*~~,~~ and *Clostridium leptum* ~~, *Bifidobacterium* and *Blautia coccoides*/*Eubacterium rectale*~~ in EWH fed obese Zucker rats were more similar to the lean rats than to the obese controls. Besides, feeding the obese Zucker rats with EWH reduced ($P < 0.05$) the faecal concentration of lactic acid. The physiological benefits of EWH in the improvement of obesity associated complications of Zucker rats could be associated with a more lean-like gut microbiota and a tendency to diminish total short-chain fatty acids (SCFA) production and associated obesity complications. The results warrant the use of pepsin egg white hydrolysate as a bioactive food ingredient.

Keywords: Obesity; Egg white hydrolysate; Gut microbiota; Short chain fatty acids; Oxidative stress.

33 **1. Introduction**

34 The potential of dietary modifications using specific ingredients to control energy
35 metabolism and/or modify body weight has been documented.[1] Also, dietary
36 components are described to modulate the multiple clinical complications associated
37 with obesity, including insulin resistance, hypertension, inflammation, oxidative stress
38 and dyslipidemia.[2] Appetite suppression, lipid metabolism regulation and increase of
39 energy expenditure are the main mechanisms by which anti-obesity effects are exerted.
40 Dietary proteins may influence body weight by affecting four targets: satiety,
41 thermogenesis, energy efficiency and body composition.[3] In particular, bioactive
42 peptides derived from milk and marine sources have shown potential anti-obesity
43 effects.[1,4,5]

44 The obese Zucker rat, which presents a mutation of the leptin receptor (*fa*/*fa*), has
45 been one of the most commonly used murine models to study obesity over the past three
46 decades.[6] Many of the metabolic features that characterize the obese Zucker rat when
47 compared with Zucker lean (*fa*/+) or (+/+) phenotypes relate to energy metabolism and
48 gut microbiota composition.[7] Experiments with genetically obese (homozygous for an
49 aberrant leptin gene, *ob/ob)* rodents showed more Firmicutes and correspondingly less
50 Bacteroidetes in their gut compared with heterozygous (*ob*/+) or lean wild-type (+/+)
51 animals,[8] pointing out a potential link between the obese-phenotype and the gut
52 microbiota. In fact, the gut microbiome ability to recover energy from the diet has been
53 suggested to have a role in the obese host phenotype.[9] However, as far as we know,
54 there are no studies that relate the intake of food-derived peptides, the amelioration of
55 symptoms associated to obesity-related metabolic dysfunctions and the gut microbiota.
56 Only recently Monteiro *et al.* have reported that dietary whey proteins can preserve a
57 balanced intestinal microbiota profile in mice consuming a high-fat diet.[10]

In a previous work, we carried out an *in vitro* screening of egg white hydrolysates produced with food-grade enzymes from different sources.[11] The results indicated that a hydrolysate of egg white with pepsin presents potential hypocholesterolemic properties, estimated as its bile acid binding capacity, prevents oxidative damage and can ~~block the degradation of~~inhibit dipeptidyl peptidase IV, the enzyme responsible for the degradation of the incretin hormones that stimulate~~s~~ glucose-dependent insulin secretion. Moreover, this hydrolysate significantly ameliorates obesity-related fat accumulation, hepatic steatosis and dyslipidemia, reducing oxidative stress and inflammation markers in obese Zucker rats.[12] In the present work we aimed to evaluate whether the beneficial effects of the hydrolysate of egg white with pepsin could be associated with gut microbiota changes. For this purpose, we have assessed microbial composition and metabolic compounds in the faeces of these obese rats fed with egg white pepsin hydrolysate in comparison with lean and obese controls.

2. Materials and Methods

2.1. Experimental protocol in Zucker rats

Twenty male 8 week-old Zucker fatty (*fa*/*fa*) rats, weighing 250–275 g, and ten 8 week-old male Zucker lean (+/+) rats, weighing 150–175 g, all purchased from Charles River Laboratories (Barcelona, Spain), were used in the study. The experimental design was published by Garcés-Rimón *et al.*[12] In brief, animals were housed in a conventional animal room in transparent cages (40 × 28 × 25 cm; n= 5 per cage) at a stable room temperature of 23 ºC and 60% humidity on 12 h:12 h light:dark cycles. From the 6th week of the experimental period onwards, due to their severe overweight, the obese animals were redistributed in smaller groups (n= 2 per cage). The rats were fed *ad libitum* with a solid standard diet. The obese Zucker rats were randomly divided into

82 two groups of ten animals that received for 12 weeks, as drinking fluids, tap water or
83 egg white hydrolysed with pepsin (EWH). Preparation of EWH, as well as the peptide
84 sequences contained in the hydrolysate, have been previously described by Garcés-
85 Rimón *et al.*[11] Dose of EWH was 750 mg/kg/day dissolved in tap water. The lean
86 Zucker rats received the standard diet and tap water until the 20th week of life. At the
87 end of the 12th week of the experimental period, the animals were placed individually in
88 metabolic cages and faeces were collected for 16 h, weighted and frozen at -80 °C until
89 further analyses.

90 The experiments were designed and performed in accordance with the European
91 and Spanish legislation on care and use of experimental animals (2010/63/EU; RD
92 53/2013), and were approved by the Ethics Committee of the University Rey Juan
93 Carlos (Madrid, Spain).

94 **2.2. DNA extraction and quantitative PCR (qPCR)**

95 Faecal samples were thawed at room temperature, weighted (0.1 g) and suspended in 1
96 mL 0.1% peptone solution with 0.85% NaCl. The homogeneous faecal suspension was
97 centrifuged at 12000 rpm for 5 min at 4 °C. The pellets were used for DNA extraction
98 and the supernatants were stored for short chain fatty acid (SCFA) and ammonium
99 analyses. Bacterial DNA extraction was performed as described by Moles *et al.*[13]
100 Briefly, the pellet was resuspended in an extraction buffer that contained the lytic
101 enzymes lysozyme (20 mg/mL) and lysostaphin (5 μg/mL), followed by mechanical
102 lysis with glass beads and extraction with phenol/chloroform/isoamyl-alcohol. The
103 DNA was precipitated by adding isopropanol and quantified using a NanoDropH ND-
104 1000 UV spectrophotometer.

105 The quantitative microbiological analysis of samples was carried out by qPCR
106 using SYBR green methodology in a ViiA7 Real-Time PCR System (Life
107 Technologies, Carlsbad, CA, USA). Primers, amplicon size, and annealing temperature
108 for the bacterial groups analysed are listed in Table 1. The targeted bacterial groups
109 represent the predominant Gram-positive bacteria belonging to clostridial clusters XIVa
110 and IV (Firmicutes) and Gram-negative bacteria related to Bacteroidetes. Other groups
111 such as lactic acid bacteria, bifidobacteria and *Akkermansia* are commonly health-
112 related bacteria. DNA from *Escherichia coli* DH5α, *Lactobacillus plantarum* IFPL935,
113 *Bifidobacterium breve* 29M2 and *Bacteroides fragilis* DSM2151 was used for
114 quantification of total bacteria,[14] *Lactobacillus*/*Enterococcus*,[15] *Bifidobacterium*[16] and
115 *Bacteroides*,[17] respectively. For the other groups analysed,[17-22] samples were quantified
116 using standards derived from targeted cloned genes using the pGEM-T cloning vector
117 system kit (Promega, Madison, WI, USA), as described by Barroso *et al.*[23] The
118 correctness of the inserts was confirmed by sequence analysis.

119 **2.3. Short Chain Fatty-Acid (SCFA) determination**

120 Supernatants from the faecal homogenates were filtered and 0.2 µL were injected on a
121 HPLC system (Jasco, Tokyo, Japan) equipped with a UV-975 detector. SCFA were
122 separated using a Rezex ROA Organic Acids column (Phenomenex, Macclesfield, UK)
123 following the method described by Sanz *et al.*[24] The mobile phase was a linear gradient
124 of 0.005 M sulphuric acid in HPLC grade water, and flow rate was 0.6 mL/min. The
125 elution profile was monitored at 210 nm and peak identification was carried out by
126 comparing the retention times of target peaks with those of standards. Calibration
127 curves of acetic, propionic, butyric, formic, succinic and lactic acids were built up in the
128 concentration range of 1 to 100 mM.

129 **2.4. Ammonium determination**

130 Ammonium was determined directly from the supernatant fraction of faecal samples
131 (13000 ×*g*, 15 min, 4 ºC) using an ammonium ion selective electrode (NH500/2; WTW,
132 Weilheim, Germany) and following the manufacturer's instructions. Results are
133 expressed as mM using an ammonium standard solution.

134 **2.5. Statistical analysis**

135 The results are expressed as mean values ± standard error of the mean (SEM), and were
136 analyzed by one-way analysis of variance (ANOVA), using the IBM SSPS Statistics
137 software Version 23 (IBM-SPSS Inc., Chicago, IL, USA). Differences between the
138 groups were assessed *post-hoc* by the Tukey test. A value of $P < 0.05$ was fixed for the
139 level of significance of the tests.

140

141 **3. Results and discussion**

142 In this article we describe the microbiological composition of faeces and the products of
143 microbial fermentative and proteolytic metabolism of obese and lean Zucker rats, after a
144 12 week nutritional intervention in the obese animals with egg white hydrolysed with
145 pepsin (EWH). No differences were observed in the appearance and consistence of
146 faeces, although the amount of faeces excreted was higher in the obese rats regardless of
147 the intake of hydrolysate.[12]

148 **3.1. Microbiological differences between obese and lean Zucker rats**

149 Faecal material was analysed for microbiota composition by qPCR, targeting the
150 specific bacterial groups shown in Table 1. The microbiological results shown in Table

151 2 indicate that obese and lean rats differed in several microbiological parameters.
152 Counts per g of faeces of total bacteria, *Lactobacillus*/*Enterococcus*, *C. leptum*,
153 *Roseburia*, *Akkermansia* and *Ruminococcus* were significantly higher ($P < 0.05$) in the
154 obese rats than in their lean counterparts. Overall, most of the bacterial groups whose
155 counts were comparatively higher in the obese rats belong to the phylum Firmicutes (the
156 only exception was *Akkermansia*), whereas groups from Bacteroidetes (genus
157 *Bacteroides*), Actinobacteria (genus *Bifidobacterium*) and Proteobacteria (family
158 Enterobacteriaceae) showed no significant differences between obese and lean Zucker
159 rats (Table 2). The higher Firmicutes/Bacteroidetes ratio found in the obese rats
160 compared with their lean controls is a general trend in murine genetic obese
161 models.[8,25,26] In humans, some studies have also associated obesity with a higher
162 intestinal Firmicutes/Bacteroidetes ratio in comparison with lean individuals.[27,28]
163 However, other human trials have reported no differences or opposite results in obese
164 individuals.[29-31]

165 In the present work the differences found in the specific microbial groups
166 analysed are in agreement with published data. Thus, the counts of
167 *Lactobacillus*/*Enterococcus* in obese rats have been reported to outnumber those of their
168 lean counterparts.[32,33] Remarkably, there were higher counts of *Akkermansia* in the
169 obese rats than in their lean controls (Table 2) in agreement with the results of Noratto
170 *et al.* in obese Zucker rats.[34] However, obesity induced in rats by a high fat diet is often
171 inversely correlated with numbers of *Akkermansia muciniphila*,[35] the only currently
172 known species within genus *Akkermansia*. This species is a usual inhabitant of the
173 intestinal mucus layer and its decrease in dietary-induced obese rats could be related to
174 disturbances in the mucosa barrier function caused by high fat diets.[36]

The comparison of microbial metabolism between obese and lean rats (Fig. 1 and Table 3) shows that the total of SCFA and lactate concentrations were higher ($P < 0.05$) in the obese rats than in their lean counterparts. A study of energy metabolism comparing obese and lean Zucker rats by Phetcharaburanin *et al.* also showed higher concentrations of faecal lactate and SCFAs in obese rats compared with lean animals.[7] Research on SCFA content in human faeces has also indicated a higher proportion of SCFA in overweight and obese subjects compared to lean controls.[30] A similar trend was observed in this work regarding microbial proteolytic metabolism (Fig. 1), with higher amount of ammonium in the faeces of the obese animals than in those of the lean rats. The genetic background of both rat groups and the fact that they were fed with the same diet point to the amount of food ingested as the keystone for the observed microbial and metabolic changes. Zucker obese rats lack the gene corresponding to leptin receptors and are affected by impaired satiety perception during feeding. Therefore, these rats are hyperphagic and have reduced energy expenditure, leading to development of pronounced obesity at an early stage in life.[6] Indeed, food intake was significantly higher in the obese than in the lean animals during the first 6 weeks of the study, which led to severe obesity at the end of the experimental period (547 g versus 414 g in the lean animals).[12] The higher fermentative and proteolytic metabolism that characterised the obese animals as compared with the lean controls, together with the higher total microbial counts in faeces of the former, suggest that the different metabolic phenotypes of both types of rats could be linked to their particular microbiomes.

3.2. Microbiological effects of pepsin egg white hydrolysate in obese Zucker rats

The counts of *Lactobacillus*/*Enterococcus*, *C. leptum* and total bacteria of the obese rats supplemented with EWH were similar ($P > 0.05$) to those of the lean rats (Table 2). ~~Furthermore, rats fed with EWH showed counts of *Bifidobacterium* and *B. coccoides*/*E.*~~

200 ~~*rectale* that were closer to the lean rats than to the obese ones.~~ Besides, feeding the
201 obese Zucker rats with EWH tended to reduce the faecal concentration of total SCFA in
202 comparison with the control obese rats (Fig. 1; $P = 0.08$). Particularly, the ANOVA test
203 indicated that the lactic acid concentration was similar in the obese rats treated with
204 EWH and the control lean rats (Table 3). On the other hand, treatment with EWH did
205 not reduce microbial proteolytic activity, measured as the ammonium concentration in
206 faeces, of the obese rats (Fig. 1).

207 An association can be established between the higher counts assessed for
208 *Lactobacillus*/*Enterococcus* (Table 2) and the higher concentrations of lactic acid in the
209 faeces of the obese rats, as compared with those of the lean rats (Table 3), and the
210 observation that both, microbial counts and metabolite concentration, tended to be
211 reduced by the treatment with EWH. *Lactobacillus* and *Enterococcus* are genera
212 characterized by the production of lactic acid as the principal end metabolite from
213 carbohydrate fermentation. Moreover, an increased acetic acid production has been
214 observed during growth of *Lactobacillus* species in non-digestible carbohydrates.[37]
215 Results of physiological markers measured in these animals and published by Garcés-
216 Rimón *et al.* showed that the levels of free fatty acids (FFA) in plasma of the obese rats
217 were higher than those in plasma of the lean rats and of the rats treated with EWH.[12]
218 This observation matches the comparatively higher level of acetic acid in the faeces of
219 the obese rats (Table 3) and indicates a higher production and absorption of this SCFA.
220 Acetic and propionic acids, which are 90% absorbed in the intestine, are involved in
221 lipid metabolism and energy storage in the adipose tissue. Particularly, acetic acid is
222 responsible for increased *de novo* lipogenesis and fat accumulation in the epididymal
223 white adipose tissue.[38] In this regard, the obese rats of this study showed increased
224 absolute and relative epididymal adipose tissue weights and a substantial liver steatosis,

225 together with dyslipidaemia (high plasma concentrations of cholesterol, triglycerides
226 and FFA), compared with the lean rats.[12] The intake of EWH significantly decreased the
227 epididymal adipose tissue, improved hepatic steatosis and reduced oxidative stress.[12]

228 The results of this work on microbiota composition and microbial metabolism of
229 obese rats fed with EWH showed not only improvement of the aforementioned
230 physiological markers but also changes in microbial parameters towards those typical of
231 lean rats. However, the lean-like microbial composition observed after intake of EWH
232 was not accompanied by a reduction of the final body weight of obese rats. This
233 observation points out the overall difficulty to elucidate the potential link between
234 specific dietary nutrients, changes in the abundance or phylogenetic composition of the
235 gut microbiota, metabolic consequences and impact on health. It seems unlikely that
236 EWH, supplied in the drinking water to the obese rats, reached the large intestine and was
237 directly responsible for changes in microbiota composition and decrease of SCFA levels.
238 In fact, while an increase of dietary protein usually results in a marked increase in total
239 ammonia formed via bacterial deamination of amino acids in the colon, which produces
240 the majority of ammonia in the body,[39] no enhanced microbial proteolytic activity was
241 observed after administration of EWH (Fig 1). Therefore, it is more likely that the
242 bioactive peptides contained in EWH are already absorbed in the small intestine and reach
243 the target tissues and organs via blood system, causing improvements in physiological
244 markers of lipid metabolism, inflammation and oxidative stress[12] that promoted the
245 changes in composition and metabolism of the gut microbiota found in this work. It is
246 known that obesity and diabetes are two disorders that have in common inflammation and
247 oxidative stress[40] and are repeatedly associated to microbial dysbiosis and changes in
248 composition and functionality of gut microbiota.[41] It could be hypothesized that, by virtue
249 of their antioxidant and anti-inflammatory effects shown in this as well as in other rat

250 models of oxidative stress,[12,42,43] the bioactive peptides contained in EWH would have
251 the potential to modulate gut microbiota in place of, or in addition to, any change effected
252 by their unlikely direct microbial metabolism of the peptides. Moreover, reversion of
253 microbial dysbiosis in obese rats by reduction of inflammation and oxidative stress would
254 turn in favour of a reduction of microbial fermentation, SCFA production and,
255 consequently, less energy recover and associated lipogenesis. Anti-oxidative
256 phytochemicals, such as resveratrol and, particularly, pterostilbene have demonstrated
257 their efficiency as antiobesity dietary supplements for obese Zucker rats[44,45] and
258 oligomeric cocoa procyanidins prevent the development of obesity in high fat fed rats.[46]
259 Moreover, moderate physical exercise can modulate the gut microbiota due to the
260 promotion of antioxidant enzymes and anti-inflammatory cytokines.[47], The increased
261 capacity to tolerate oxidative stress represents a sign of microbial dysbiosis in the
262 anaerobic gut environment , since it is indicative of the presence of aerobic bacteria and/or
263 activation of host inflammatory responses.[41,48]

264 **4. Conclusion**

265 The current study suggests that the ingestion of a pepsin hydrolysate of egg white by
266 Zucker obese rats has the potential to revert the microbial dysbiosis that characterizes
267 these animals. Changes in gut microbiota were accompanied by a trend to diminish
268 faecal SCFA levels and occurred simultaneously with a previously reported
269 amelioration of markers of oxidative stress and inflammation.[12] It is likely that the
270 hydrolysate, by virtue of its antioxidant activity and its capacity to reduce inflammation
271 could have modulated gut microbiota towards a more balanced scenario that lowered
272 SCFA production and associated lipogenesis, contributing to reduced fat accumulation
273 and liver steatosis.

274

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Legend to Figure

Fig. 1. Concentration (mM) of total SCFA and ammonium in the faeces of the Zucker rats: lean, obese and obese treated with egg white hydrolysed with pepsin (EWH). Different letter (a,b) indicate significant differences ($P < 0.05$) between rat groups using one factor Anova analysis.

Table 1. Primer sets used for quantitative PCR.

Bacterial group	Primer sequence 5'-3'	Amplicon size	Annealing temperature	Standard
Bacteroides[17]	GAAGGTCCCCCACATTG CGCKACTTGGCTGGTTCAG	103	60	*Bacteroides fragilis* DSM2151
Bifidobacterium[16]	CTCCTGGAAACGGGTGG GGTGTTCTTCCCGATATCTACA	593	55	*Bifidobacterium breve* 29M2
Lactobacillus/Enterococcus[15]	TGGAAACAGRTGCTAATACCG GTCCATTGTGGAAGATTCCC	192	55	*Lactobacillus plantarum* IFPL935
Clostridium leptum[18] (Cluster IV)	GCACAAGCAGTGGAGT CTTCCTCCGTTTTGTCAA	239	55	Clone
Blautia coccoides/Eubacterium rectale[19] (Cluster XIVa)	CGGTACCTGACTAAGAAGC AGTTTYATTCTTGCGAACG	429	55	Clone
Ruminococcus[17] (Cluster IV)	GGCGGCYTRCTGGGCTTT CCAGGTGGATWACTTATTGTGTTAA	157	60	Clone
Roseburia[17] (Cluster XIVa)	GCGGTRCGGCAAGTCTGA CCTCCGACACTCTAGTMCGAC	81	60	Clone
Faecalibacterium[20] (Cluster IV)	CCATGAATTGCCTTCAAAACTGTT GAGCCTCAGCGTCAGTTGGT	141	60	Clone
Akkermansia[21]	CAGCACGTGAAGGTGGGGAC CCTTGCGGTTGGCTTCAGAT	329	58	Clone
Enterobacteriaceae[22]	ATGGCTGTCGTCAGCTCGT CCTACTTCTTTTGCAACCCACTC	385	58	Clone
Total bacteria[14]	AACGCGAAGAACCTTAC CGGTGTGTACAAGACCC	489	55	*Escherichia coli* DH5α

Table 2. Mean ± SEM of quantitative PCR counts (log copy number/g) for the different microbial groups analysed in the faeces of the Zucker rats: lean (L), obese (O) and obese treated with egg white hydrolysed with pepsin (EWH).

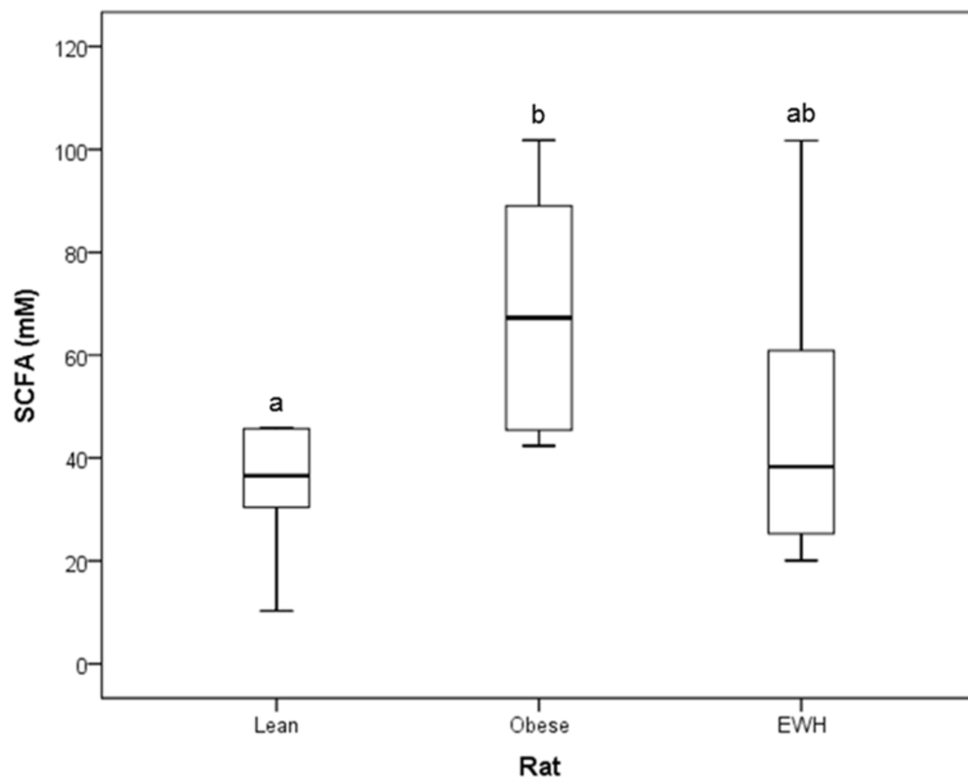
Bacterial group	Lean (L)	Obese (O)	Obese + EWH	P value	
				O *vs.* L	EWH *vs.* L
Bacteroides	7.06^{a} ± 0.22	7.42^{a} ± 0.10	7.00^{a} ± 0.07	0.196	0.958
Bifidobacterium	8.97^{a} ± 0.28	9.69^{ab} ± 0.16	9.05^{ab} ± 0.13	0.043	0.948
Lactobacillus/Enterococcus	9.00^{a} ± 0.32	9.97^{b} ± 0.15	8.89^{a} ± 0.08	0.007	0.910
Clostridium leptum	6.34^{a} ± 0.08	7.42^{c} ± 0.06	6.84^{b} ± 0.06	0.000	0.001
B. coccoides/E. rectale	6.78^{a} ± 0.27	7.84^{a} ± 0.30	7.53^{a} ± 0.32	0.080	0.750
Ruminococcus	5.84^{a} ± 0.16	7.01^{b} ± 0.26	7.12^{b} ± 0.13	0.003	0.001
Roseburia	6.99^{a} ± 0.17	7.97^{b} ± 0.23	7.98^{b} ± 0.10	0.002	0.001
Faecalibacterium	5.33^{a} ± 0.22	5.62^{a} ± 0.16	5.82^{a} ± 0.15	0.553	0.191
Akkermansia	7.15^{a} ± 0.35	9.65^{b} ± 0.20	9.16^{b} ± 0.27	0.000	0.000
Enterobacteriaceae	4.84^{a} ± 0.29	5.52^{a} ± 0.62	5.61^{a} ± 0.09	0.341	0.138
Total bacteria	9.69^{a} ± 0.26	10.33^{b} ± 0.12	9.54^{a} ± 0.12	0.048	0.827

Different letter (a,b,c) in the same row indicate significant differences (P < 0.05) between rat groups using one-way Anova analysis.

Table 3. Concentration (mM; mean ± SEM) of SCFA in the faeces of the Zucker rats: lean (L), obese (O) and obese treated with egg white hydrolysed with pepsin (EWH).

Acid	Lean (L)	Obese (O)	Obese + EWH	P value	
				O *vs.* L	EWH *vs.* L
Acetate	$15.62^{a} \pm 3.03$	$23.67^{a} \pm 2.40$	$18.82^{a} \pm 2.50$	0.108	0.669
Propionate	$3.27^{a} \pm 1.39$	$6.03^{a} \pm 1.81$	$3.95^{a} \pm 1.49$	0.451	0.948
Butyrate	–	$0.50^{a} \pm 0.17$	$0.51^{a} \pm 0.15$		
Lactate	$15.17^{a} \pm 2.86$	$38.99^{b} \pm 6.26$	$21.62^{ab} \pm 5.48$	0.009	0.647
Succinate	$2.43^{a} \pm 0.79$	$1.29^{a} \pm 0.80$	$2.47^{a} \pm 0.81$	0.594	0.999

Different letter (a,b) in the same column indicate significant differences ($P < 0.05$) between rat groups using one-way ANOVA analysis.



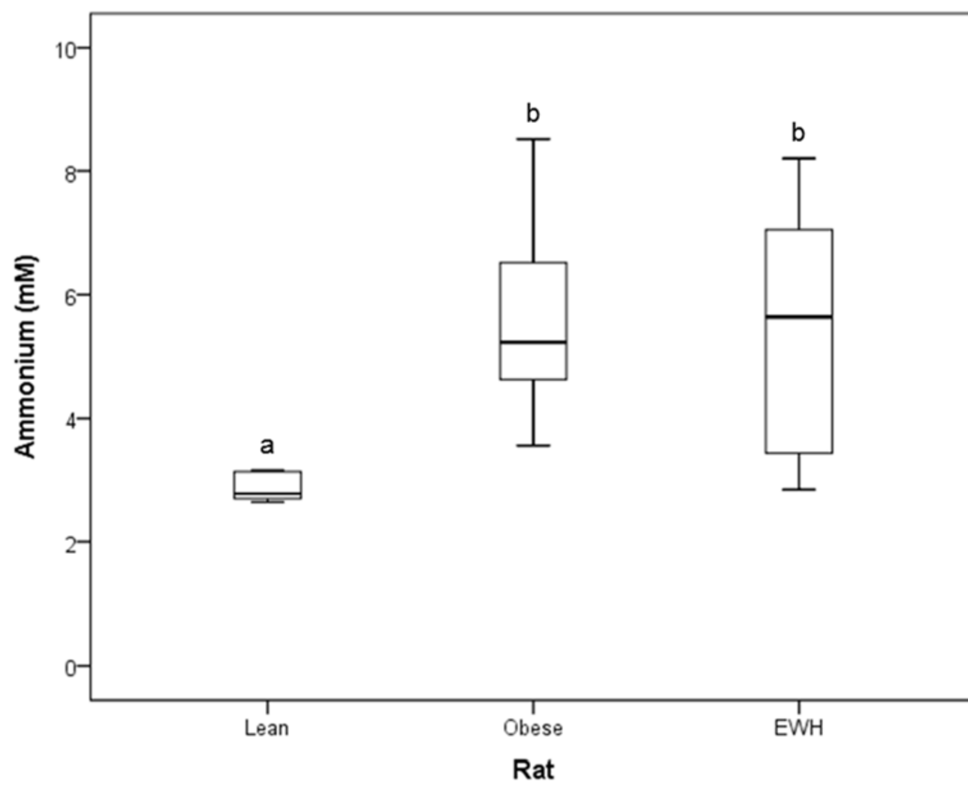


Fig. 1