Eubiotic effect of buckwheat D-fagomine in healthy rats

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ABSTRACT

Diversity and balance of gut microorganisms is fundamental for health throughout life. The aim of this study is to explore the possible eubiotic effect of the buckwheat iminosugar D-fagomine (0.096% w/w in standard feed) in growing healthy Wistar Kyoto rats. Feed and energy intake, residual energy in feces, and body weight gain were independent of D-fagomine supplementation throughout the intervention (24 weeks). The populations of significant bacterial subgroups and species were determined in fecal and cecal DNA by quantitative real-time PCR. D-Fagomine increased the Bacteroidetes:Firmicutes ratio and partially counteracted the loss of Lactobacilliales and Bifidobacteriales over time. The supplementation reduced the levels of excreted short-chain fatty acids (SCFAs) as determined by gas chromatography. This paper provides preliminary evidence that D-fagomine has the capacity to promote microbial functional diversity by increasing the Bacteroidetes:Firmicutes ratio and to mitigate the age-related reduction in populations of the putatively beneficial Lactobacilliales and Bifidobacteriales.

KEYWORDS: Gut microbiota; lactobacillus; bifidobacteria; iminosugar; iminocyclitol; buckwheat

1. INTRODUCTION

Human gut microbiota is formed of some 10^{14} bacteria: more than 10 times the number of eukaryotic cells in a healthy person. It mainly consists of 9 bacterial phyla encompassing over 1,000 species, and more than 15,000 strains. Most of these bacteria belong to the two most abundant phyla in the gut: Bacteroidetes (40% of the gut microbiota) and Firmicutes (60%) (Ley et al., 2005). Their main biological function in the host is the optimization of energy harvesting through the degradation of indigestible biopolymers (e.g. polysaccharides) in the large intestine, and their conversion into smaller species that can be internalized and used as building blocks for lyposynthesis (Thomas, Hehemann, Rebuffet, Czjzek, & Michel, 2011). Bacteroidales is the major order among Bacteroidetes while Clostridiales is the major order among Firmicutes. Other quantitatively minor yet important subgroups of the gut microbiota are the orders: Lactobacillales, Bifidobacteriales and Enterobacteriales, which belong to the phyla Firmicutes, Actinobacteria and Proteobacteria, respectively. Lactobacillales and Bifidobacteriales may confer health benefits on their host, including resistance to infection, amelioration of allergic symptoms and protection against inflammatory processes (Roberfroid et al., 2010). *Lactobacillus acidophilus* is one of the major species of its genus found in the gut, and together with *Lactobacillus plantarum*, it contributes to the maintenance of the normal barrier function of the intestinal epithelium (Gareau, Sherman, & Walker, 2010). Enterobacteriales is composed of non-pathogenic and opportunistic bacteria such as *Escherichia coli*, a facultative anaerobic microorganism. Most *E. coli* strains can coexist inside a healthy host; but they may cause enteric diseases and extra-intestinal infections in immunocompromised hosts or when the normal gastrointestinal barriers are breached (Kaper, Nataro, & Mobley, 2004).

Microbiota products can be either protective or harmful, depending on their concentration and on the metabolic status of the host. These products include lipopolysaccharides (LPS: a component of the bacterial cell wall), angiopoietin-like protein 4 (a protein involved in lipid metabolism), bile acids and short-chain fatty acids (SCFAs) (Janssen & Kersten, 2017). SCFAs are the end products of the fermentation of dietary fiber by anaerobic intestinal bacteria (den Besten et al., 2013; Tan et al., 2014). Bacteroidetes and Actinobacteria are known to produce acetate and propionate; whereas

71 butyrate is mainly generated by bacterial groups in the Firmicutes phylum (e.g.
72 Clostridiales) (Mackie & White, 2012).

73 SCFAs are building blocks for *de novo* lyposynthesis as well as mediators of biological
74 responses in the host. They interact with signaling pathways through activities such as
75 inhibition of histone deacetylases (HDACs) and activation of G-protein-coupled
76 receptors (GPCRs) (Tan et al., 2014).

77 The preservation of microbial diversity and balance is fundamental for host health
78 (Nicholson et al., 2012). Many factors can produce disruptions in gut microbiota and
79 lead to dysbiosis, which consequently increases the susceptibility of the host to contract
80 diseases (Iebba et al., 2016). Physiological changes in the gastrointestinal tract,
81 modifications in lifestyle, and functional alterations of the host immune system over
82 time ultimately affect the bacterial ecosystem (Biagi et al., 2010). In humans, age-
83 related differences in gut microbiota composition include an increase in the total
84 number of facultative anaerobes, mainly Enterobacteriales; and a reduction in the
85 populations of species belonging to the phylum Bacteroidetes, as well as of the health-
86 promoting Lactobacillales and Bifidobacteriales (Woodmansey, 2007). *E. coli* and other
87 opportunistic bacteria tightly adhere to mucosal surfaces (Svanborg, Agace, Hedges,
88 Lindstedt, & Svensson, 1994) and may prevent gut colonization by the more loosely
89 bound species belonging to the Lactobacillales and Bifidobacteriales orders.

90 Nutritional strategies to avert dysbiosis or to restore a normobiotic/eubiotic state include
91 the administration of probiotics (putatively beneficial microorganisms) and prebiotics
92 (ingredients that promote the growth/activity of beneficial microorganisms) (Roberfroid
93 et al., 2010). Other food components may have the capacity to preserve gut microbial
94 diversity through different mechanisms; together with probiotics and prebiotics these
95 may generally be called eubiotics. Iminocyclitols, also called iminosugars, are
96 carbohydrate analogues with a nitrogen atom in place of the endocyclic oxygen. D-
97 Fagomine (1,2-dideoxynojirimycin) is a six-ring iminocyclitol first isolated from seeds
98 of buckwheat (*Fagopyrum esculentum*) and also present in other plant sources, such as
99 mulberry (*Morus alba)* leaves, and gogi (*Lycium chinense)* roots (Amézqueta et al.,
100 2012). D-Fagomine is partially absorbed and then rapidly (8 h) excreted in urine. It is
101 partially metabolized into methyl-D-fagomine (about 10% in urine and 3% in feces)
102 (Amezqueta et al., 2017). D-Fagomine inhibits intestinal disaccharidases *in vitro*,

reduces the post-prandial blood glucose concentration in healthy rats and inhibits the adhesion of *E. coli* and *Salmonella enterica* serovar *Typhimurium* to pig intestinal mucosa (Gómez et al., 2012). D-Fagomine also maintains the glycemic status in pre-diabetic animals (Molinar-Toribio et al., 2015), it reduces fat-induced weigh gain (Ramos-Romero et al., 2014) and there is preliminary evidence that it may elicit these effects through an action on gut microbiota, particularly on Enterobacteriales (Ramos-Romero et al., 2014).

To evaluate the possible use of D-fagomine as a functional food component for the maintenance of balanced gut microbiota, here we explore the changes it induces in the populations of major microbial phyla and selected putatively beneficial minor orders in healthy rats over time.

2. MATERIALS AND METHODS

2.1. Animals

A total of 18 male Wistar-Kyoto rats from Envigo (Indianapolis, IN, USA), aged 8-9 weeks, were used. All animal handling was carried out in the morning, to minimize the effects of circadian rhythms. All the procedures strictly adhered to the European Union guidelines for the care and management of laboratory animals (directive 2010/63/EU) under license from the regional Catalan authorities (reference no. DAAM7921), and were approved by the Spanish CSIC Subcommittee of Bioethical Issues.

2.2. Experimental design and sample collection

The rats were housed under controlled conditions of humidity (60%), and temperature (22 ± 2 ºC) with a 12 h light-12 h dark cycle. To reduce the variation in microbiota between rats, the animals were accommodated in their cages (n = 3 per cage) for 4 weeks before the nutritional intervention. Then, they were randomly divided into 2 groups (n = 9/group): control group (STD), fed a standard diet of 2014 Teklad Global 14% Protein chow from Envigo; and a group fed the standard diet supplemented with 0.96 g D-fagomine/kg feed (> 98% from Bioglane SLNE, Barcelona, Spain) per kg feed (FG). The composition of the diets is provided in Table 1. The proportion of D-fagomine in the feed (2 mg/g carbohydrates) was defined in accordance with the results of previous studies *in vitro* (Gómez et al., 2012) and *in vivo* (Ramos-Romero et al., 2014). The mean daily dose of D-fagomine was 3.9 mg per 100 g body weight, calculated from a mean feed consumption of 4.1 g feed per day per 100 g body weight. The animals were fed *ad libitum* with free access to water (Ribes, Barcelona, Spain).

Feed consumption was monitored daily and body weight was measured three times per week throughout the experiment. Energy intake was calculated as estimates of metabolizable energy, based on the Atwater factors, assigning: 4 kcal/g protein, 9 kcal/g fat, and 4 kcal/g available carbohydrate.

Fecal samples were collected by abdominal massage after weeks 0, 1, 3, 9 and 24. The energy content in the feces collected after week 20 was determined by differential scanning calorimetry (25-600 ºC in an O_2 atmosphere, 10 ºC/min) by means of a TGA/SDTA851e thermogravimetric analyzer (Mettler-Toledo, Columbus, OH) with integrated SDTA signal.

After 24 weeks of supplementation, the rats were fasted overnight and anesthetized intraperitoneally with ketamine from Merial Laboratorios (Barcelona, Spain) and xylacine from Quimica Farmaceutica (Barcelona, Spain) (80 and 10 mg/kg body weight, respectively). The cecal content was collected, weighed and immediately frozen in liquid N_2. All the samples were stored at –80 ºC until analysis.

2.3. Measurement of microbial populations

The relative populations of selected bacterial phyla, orders and species were estimated in fecal and cecal DNA by quantitative real-time PCR (qRT-PCR). Total DNA was extracted from both feces and cecal content using a QIAamp® DNA Stool Mini Kit from QIAGEN (Hilden, Germany) and quantified using a Nanodrop 8000 Spectrophotometer (ThermoScientific, Waltham, MA, USA). All DNA samples were diluted to 20 ng/µL. The qRT-PCR experiments were carried out using a LightCycler® 480 II (Roche, Basel, Switzerland) in 96-well plates. Each qRT-PCR well was run in triplicate and contained DNA (2 µL) and a master mix (18 µL) consisting of 2X SYBR (10 µL), the corresponding forward and reverse primer (1 µL each), and water (6 µL). All the reactions were paralleled by a non-template control (water) and a positive control (Table 2) from DSMZ (Braunschweig, Germany). Water was purified using a Milli-Q system (Millipore Corporation, Billerica, MA, USA). The qRT-PCR cycling conditions were as follows: 10 s at 95 ºC, then 45 cycles of 5 s at 95 ºC, 30 s at the primer-specific annealing temperature (Table 2), and 30 s at 72 ºC (extension). Following amplification, to determine the specificity of the qRT-PCR, melting curve analysis was carried out by heating for 2 s at 95 ºC, then cooling for 30 s at 60 ºC, and a temperature gradient from 30 ºC to 95 ºC at a rate of 0.11 ºC/s, with five fluorescence recordings per ºC.

The relative DNA abundances for the different sequences were calculated from the second derivative maximum of their respective amplification curves (C*p*, calculated in triplicate) by considering C*p* values to be proportional to the dual logarithm of the inverse of the specific DNA concentration, following the equation: $[DNA_a]/[DNA_b] = 2^{Cpb-Cpa}$ (Pfaffl, 2001). Total bacteria was normalized as 16S rRNA gene copies per mg of wet feces (copies/mg).

2.4. Short-chain fatty acids

SCFAs were analyzed in feces after 12 weeks of supplementation and in the cecal content at the end of the study, by gas chromatography using a previously described method (Schwiertz et al., 2009) with some modifications. Briefly, the feces were freeze-dried and weighed (~50 mg dry matter) and a solution (1.5 mL) containing the internal standard 2-ethylbutiric acid (6.67 mg/L) and oxalic acid (2.97 g/L) in acetonitrile/water 3:7 was added. Then, SCFAs were extracted for 10 min using a rotating mixer. The suspension was centrifuged (5 min, 12,880 g) in a 5810R centrifuge (Eppendorf, Hamburg, Germany) and the supernatant passed through a 0.45 µm nylon filter. An aliquot of the supernatant (0.7 mL) was diluted to 1 mL with acetonitrile/water 3:7. SCFAs were analyzed using a Trace2000 gas chromatograph coupled to a flame ionization detector (ThermoFinnigan, Waltham, MA, USA) equipped with a Innowax 30 m × 530 µm × 1 µm capillary column (Agilent, Sta Clara, CA, USA). Chrom-Card software was used for data processing. This method has shown good selectivity for six different SCFAs (acetic acid, propionic acid, butyric acid, isobutyric acid, valeric acid and isovaleric acid), sensitivity, linearity in the working concentration range (acetic and butyric acids 3-750 ppm; propionic acid 1-250 ppm; isobutyric acid 0.3-75 ppm; isovaleric and valeric acids 0.2-40 ppm) and accuracy (trueness and precision). To check the method trueness and precision, a recovery study at three concentration levels and on three different days was performed. Precision (RSD < 15%) and recovery (> 70%) were adequate and intra-day reproducible.

2.5.Statistical analysis

The results are expressed as mean values with their standard errors (SEM). Normal distribution and heterogeneity of data were evaluated by Shapiro-Wilk test and F-tests, respectively. Intra-group statistical significance throughout the study was determined by repeated-measures ANOVA. Statistical significance between groups was determined by Student's *t*-test. Differences were considered significant when $P < 0.05$. All data calculations and statistical analysis were performed using Graph Pad Prism 5 (Graph Pad Software, Inc., San Diego, CA, USA).

3. RESULTS

3.1. Body weight, and feed and energy intakes

Body weight was similar in the STD and FG groups, both before and after the nutritional intervention (Table 3). There were no differences between groups in either water, feed or energy intakes throughout the experiment (Table 3); nor were there in the energy excreted in feces at the end of the study (Table 3).

3.2. Major microbiota phyla and orders

The relative proportions of the two predominant bacterial phyla: Bacteroidetes and Firmicutes, and orders within these phyla: Bacteroidales and Clostridiales in the gut microbiome, were evaluated at time 0 and after 1, 3, 9 and 24 weeks of supplementation in feces, and also at the end of the study (24 weeks) in cecal content (Figure 1).

Intragroup variations in the percentages of Bacteroidetes and Firmicutes over the entire experiment were not significant; while supplementation with D-fagomine clearly increased the populations of Bacteroidetes in feces, already after one week of supplementation, except at week 3 (Figure 1A). This effect was also detected in the cecal content at the end of the study (Figure 1A, B). The populations of Bacteroidales (the main order within Bacteroidetes) presented a similar pattern (Figure 1D). No significant differences were observed in the populations of Firmicutes or its major order, Clostridiales, throughout the study (Figure 1B, E).

3.3. Minor microbiota orders and species

The relative proportions of the orders Lactobacillales, Bifidobacteriales, and Enterobacteriales, as well as *L. acidophilus*, *L. plantarum*, and *E. coli* in the gut microbiota, were evaluated at time 0 and after 1, 3, 9 and 24 weeks of supplementation in feces, and at the end of the study (24 weeks) in cecal content (Figure 2).

The relative populations of Lactobacillales at the end of the study (week 24) were significantly lower ($P < 0.001$) than those at time 0 in the STD group (Figure 2A). D-Fagomine partially counteracted this age-related loss, as after 24 weeks the population of Lactobacilliales in the supplemented group was significantly ($P < 0.01$) greater than that in the STD group (Figure 2A). The same effect was detected for *L. acidophilus*

(Figure 2D). There were no differences between the groups in the percentage of *L. plantarum* (Figure 2E).

The relative populations of Bifidobacteriales also significantly decreased ($P < 0.05$) over time in the feces of animals in the STD group (Figure 2B); at the end of the experiment (week 24) the population was almost undetectable. Supplementation with D-fagomine also had an effect on these proportions of Bifidobacteria over time. Already after 9 weeks of intervention, the population of Bifidobacteriales was significantly higher ($P < 0.05$) in the supplemented group than in the STD group; and at the end of the study (week 24), the differences between the groups were still significant ($P < 0.05$).

The populations of Enterobacteriales and *E. coli* in the STD and FG groups were similar throughout the study except after week 9 of supplementation when a significant ($P < 0.05$) increase was recorded for Enterobacteriales in the FG group (Figure 2C). At the end of the study, the group supplemented with D-fagomine presented higher amounts of Enterobacteriales ($P < 0.05$) and *E. coli* ($P < 0.01$) in the cecal content (Figure 2C, F).

3.4. Short-chain fatty acids

The concentrations of SCFAs were measured in feces after week 12 of the study and in the cecal content at the end (24 weeks) (Table 4).

D-Fagomine significantly ($P < 0.05$) reduced the concentration of acetic and isobutyric acids, and also the total content of SCFAs in feces (Table 4). There were no differences between groups in any SCFA determined in the cecal content (Table 4).

4. DISCUSSION

The present study focuses on the effect of D-fagomine on gut microbiota of healthy WKY rats over a period of 24 weeks (from age 8-9 weeks to 32-33 weeks). The intragroup differences in the populations of Bacteroidetes, Firmicutes and their respective major orders, Bacteroidales and Clostridiales, were not significant. This result roughly agrees with a previous report of fecal microbiota variation in healthy Sprage-Dawley rats over a period of two years (Flemer et al., 2017). In that study, the populations of the two phyla and the Bacteroidetes:Firmicutes ratio showed a non-significant tendency to increase during the first year (Flemer et al., 2017). Now we have shown here that the feces of WKY rats supplemented with D-fagomine contains significantly higher populations of Bacteroidetes and Bacteroidales than those of rats given the STD diet, already after one weak and over the entire experiment, with the exception of Bacteroidetes at week 3 (Figure 1A, D). As the level of functional diversity in the gut microbiome has been linked to the relative abundance of Bacteroidetes (Turnbaugh et al., 2009), D-fagomine may contribute to the maintenance of intestinal health in ageing rats by preserving diversity.

We have also recorded some intergroup differences in fecal SCFAs. The total SCFA content in the group supplemented with D-fagomine showed a tendency to be lower than in the STD group; this difference was only significant in the cases of acetate and isobutyrate (Table 4). This reduction in excreted SCFAs might be related to the increase in the Bacteroidetes:Firmicutes ratio (Figure 1C), in agreement with studies that associate a reduced Bacteroidetes:Firmicutes ratio in obese *vs* lean mice with increased concentrations of acetate and butyrate (Turnbaugh et al., 2006) or acetate and propionate (Murphy et al., 2010). In humans, the transfer of intestinal microbiota from lean donors can improve insulin sensitivity of patients suffering from metabolic syndrome, while increasing the populations of butyrate-producing bacteria and reducing fecal SCFAs (acetate and butyrate) (Vrieze et al., 2012). This apparent contradiction may be explained by considering the host/microbiome ecosystem as a whole, in which the capacity to absorb bacterial metabolites by the host plays a determinant role and the fecal concentration of these metabolites may not be directly related to their generation rate. Fecal SCFAs may still be markers of the host's metabolic status. Hence, lower levels of excreted SCFAs together with higher Bacteroidetes:Firmicutes ratios are

consistently associated in the literature with a lean healthy phenotype, compared to metabolically altered phenotypes (Canfora, Jocken, & Blaak, 2015).

The action of D-fagomine is also evident in the case of the putatively beneficial Lactobacillales and Bifidobacteriales, particularly *Lactobacillus acidophilus* (Figure 2A, B, D). The fecal populations of these bacteria steadily and significantly decreased from week 3 until the end of the study in non-supplemented animals. There is little information in the literature about changes in the populations of putatively beneficial bacteria in healthy rats over time. In Wistar rats, Lactobacilliales show a slight tendency to increase during the first year of life, while species of the *Bifidobacterium* genus are detected only in the second year (Flemer et al., 2017). In humans, the populations of Bifidobacteriales remain relatively stable during adulthood and decrease considerably in old age (Arboleya, Watkins, Stanton, & Ross, 2016). This decline has been associated with the development of intestinal disorders, including diarrhea, irritable bowel syndrome, and inflammatory bowel disease (Gareau et al., 2010). In the present study, the supplemented group presented significantly higher populations of Lactobacilliales and Bifidobacteriales than those in the STD group at the end of the intervention (animals of 32-33 weeks of age). D-Fagomine might counteract the loss of beneficial bacteria by inhibiting the adhesion of opportunistic species such as *E. coli*, as previously reported (Gómez et al., 2012).

The feces of the Wistar-Kyoto rats in this study did not contain elevated percentages of Enterobacteriales, whether they were supplemented with D-fagomine or not. In the supplemented group, a significant increase of Enterobacteriales, and particularly *E. coli*, was recorded after 9 weeks of intervention (Figure 2C, F). Yet these levels (up to 0.2%) fall within the normal range for healthy individuals and they are much lower than those triggered by an obesogenic diet (4%) (Ramos-Romero et al., 2014). The population of Bifidobacteriales also increased significantly at the same time point (Figure 2B). At this particular time in the experiment, a singular event may have occurred. The combination of the standard diet and D-fagomine may have induced changes in the intestinal ecosystem when the rats were 17-18 weeks old. As commented before in the case of SCFAs, the increase in excreted Enterobacteriales and *E. coli* does not necessarily imply an increase of these populations in contact with the intestinal wall. In fact, the opposite might be the case in the supplemented group. The results at week 9 suggest that D-fagomine was eliminating Enterobacteriales and *E. coli* while favoring colonization by

Bifidobacteriales. This assertion is supported by previous results which show that D-fagomine inhibits the adhesion of *E. coli*, but not of Bifidobacteria, to the intestinal mucosa (Gómez et al., 2012) and it reduces the populations of enterobacteria triggered by an obesogenic diet (Ramos-Romero et al., 2014). This explanation is also consistent with the recorded increased populations of *E. coli* in cecum content at the end of the study (Figure 2F).

5. CONCLUSIONS

This paper provides preliminary evidence that the iminosugar D-fagomine has the capacity to promote diversity in gut microbiota and to mitigate the age-related reduction in the populations of some putatively beneficial bacteria in healthy rats. D-Fagomine increased the Bacteroidetes:Firmicutes ratio, reduced the loss of Lactobacilliales and Bifidobacteriales with aging and reduced the levels of excreted SCFAs. A comprehensive metagenomic study should shed more light on the changes in gut microbiota induced by iminosugars such as D-fagomine and their functionality. D-Fagomine may have a eubiotic effect on the composition of intestinal microbiota that may be complementary to that of probiotics and prebiotics.

CONFLICTS OF INTEREST

There are no conflicts of interest to declare

ACKNOWLEDGEMENTS

The language revision by Christopher Evans is appreciated.

This research was supported by the Spanish Ministry of Economy and Competitiveness (Grant AGL2013-49079-C2-1,2-R and a graduate fellowship to M. Hereu: BES2014-068592)

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458 TABLES AND FIGURES

459 Table 1.- Composition of the experimental diets

	Standard [a]	Standard plus D-fagomine
Composition (g/kg)		
Protein	143.00	143.00
L-cystine	3.00	3.00
Available carbohydrate	480.00	480.00
Crude fiber	41.00	41.00
Fat	40.00	40.00
Mineral	28.37	28.37
Vitamins	1.20	1.20
Ash	47.00	47.00
Choline bitartrate	1.00	1.00
D-Fagomine [b]	-	0.96
Total energy (ks ºC/g) [c]	704.3	627.0

460 [a] Teklad Global 14% protein rodent maintenance diet (2014) from Harlan.

461 [b] D-Fagomine (Batch: FG1008E) from Bioglane (Barcelona, Spain).

462 [c] Integrated SDTA signal proportional to energy in diets.

463

464 Table 2.- Quantitative real-time PCR primers and conditions

Target bacteria	Annealing temperature (ºC)	Sequences (5'-3')	Positive Control[a]	Reference
Total Bacteria	65	F: ACT CCT ACG GGA GGC AGC AGT R: ATT ACC GCG GCT GCT GGC	(b)	(Hartman et al., 2009)
Bacteroidetes	62	F: ACG CTA GCT ACA GGC TTA A R: ACG CTA CTT GGC TGG TTC A	*Bacteroides fragilis*	(Abdallah Ismail et al., 2011)
Firmicutes	52	F: CTG ATG GAG CAA CGC CGC GT R: ACA CYT AGY ACT CAT CGT TT	*Ruminococcus productus*	(Haakensen, Dobson, Deneer, & Ziola, 2008)
Bacteroidales	61	F: GGT GTC GGC TTA AGT GCC AT R: CGG AYG TAA GGG CCG TGC	*Bacteroides fragilis*	(Hartman et al., 2009)
Clostridiales	60	F: CGG TAC CTG ACT AAG AAG C R: AGT TTY ATT CTT GCG AAC G	*Ruminococcus productus*	(Hartman et al., 2009)
Lactobacilliales	60	F: AGC AGT AGG GAA TCT TCC A R: CAC CGC TAC ACA TGG AG	*Lactobacillus acidophylus*	(Walter et al., 2001)
Bifidobacteriales	55	F: CTC CTG GAA ACG GGT GG R: GGT GTT CTT CCC GAT ATC TAC A	*Bifidobacterium longum*	(Queipo-Ortuno et al., 2013)
Enterobacteriales	60	F: ATG GCT GTC GTC AGC TCG T R: CCT ACT TCT TTT GCA ACC CAC T	*Escherichia coli* M15	(Hartman et al., 2009)
Lactobacillus acidophylus	64	F: AGC TGA ACC AAC AGA TTC AC R: ACT ACC AGG GTA TCT AAT CC	*Lactobacillus acidophylus*	(Walter et al., 2001)
Lactobacillus plantarum	55	F: GCC GCC TAA GGT GGG ACA GAT R: TTA CCT AAC GGT AAA TGC GA	*Lactobacillus plantarum*	(Walter et al., 2001)
Escherichia coli	61	F: GTT AAT ACC TTT GCT CAT TGA R: ACC AGG GTA TCT AAT CCT GTT	*Escherichia coli* M15	(Malinen, Kassinen, Rinttila, & Palva, 2003)

465 [a] All strains of positive controls were from Deutsche Sammlung von Mikroorganismen
466 und Zellkulturen (DSMZ).

467 [b] Positive control for total bacteria was the same as that for each individual reaction.

468

469 Table 3.- Body weight, feed and energy intake, and energy in feces of rats supplemented
470 (or not) with D-fagomine for 24 weeks.

	STD[a]		**FG**[b]	
	Mean	SEM	Mean	SEM
Initial body weight (g)	224.9	3.9	237.8	4.1
Final body weight (g)	416.4	12.9	435.7	11.15
Water intake (mL/day/100 g body weight)	7.4	0.2	7.3	0.2
Feed intake (g/day/100 g body weight)	4.8	0.7	4.1	0.3
Energy intake[c] (kcal/day/100 g body weight)	14.3	0.2	14.6	0.2
Excreted energy[d]	306.6	19.5	253.6	21.7

471

472 [a] STD (Control group): rats fed a standard diet (2014 Teklad Global 14% Protein chow
473 from Envigo).

474 [b] FG (D-Fagomine group): rats fed the standard diet supplemented with 0.96 g D-
475 fagomine/kg feed.

476 [c] Estimated as metabolizable energy based on Atwater factors, which assign: 4 kcal/g to
477 protein, 9 kcal/g to fat, and 4 kcal/g to available carbohydrates.

478 [d] Integrated STD signal (ks ºC/g) proportional to energy in feces from week 20.

479

480

481 Table 4.- Short-chain fatty acids determined in feces from rats supplemented (or not)
482 with D-fagomine for 12 weeks and at the end of the study (24 weeks) in cecal content.

	FECES				CECAL CONTENT			
	STD[a]		FG[b]		STD[a]		FG[b]	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Acetic acid	310.94	61.1	142.07*	25.7	96.12	5.3	88.07	6.0
Propionic acid	27.42	6.2	16.70	2.7	25.41	2.0	19.95	1.3
Isobutyric acid	1.25	0.2	0.45*	0.1	3.66	0.3	3.00	0.1
Butyric acid	17.58	3.3	10.62	2.0	15.28	1.9	10.59	0.9
Isovaleric acid	1.00	0.3	0.44	0.1	3.92	0.3	3.02	0.2
Valeric acid	0.69	0.1	0.64	0.1	3.00	0.3	2.32	0.2
TOTAL SCFA	356.86	66.1	170.92*	28.5	136.71	10.8	125.77	7.2

483

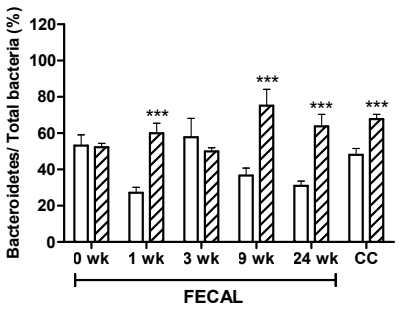
484 Comparisons were made using Student's *t*-test. * $P < 0.05$ *vs* STD group.

485 [a] STD (Control group): rats fed a standard diet (2014 Teklad Global 14% Protein chow
486 from Envigo).

487 [b] FG (D-Fagomine group): rats fed the standard diet supplemented with 0.96 g D-
488 fagomine/kg feed.

489

490 A)



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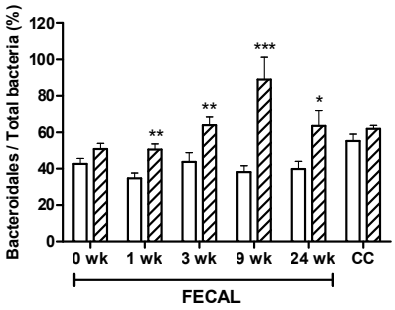
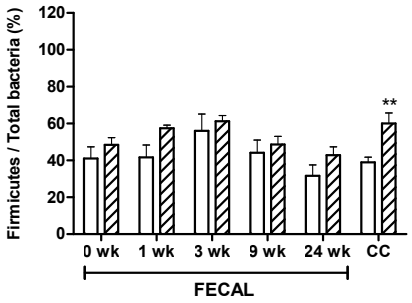


491

492 B)



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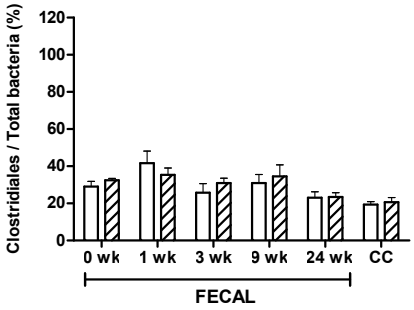

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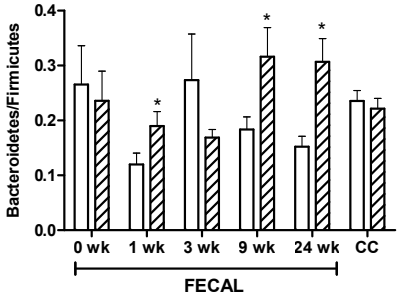
494 C)



495

496 Figure 1.- Bacteroidetes (A), Firmicutes (B), Bacteroidetes:Firmicutes ratio (C)
497 Bacteroidales (D) and Clostridiales (E) in fecal samples from rats fed a standard diet
498 (STD, empty bars), or supplemented with D-fagomine (FG, striped bars) at different
499 times, and in cecal content (CC) at the end of the study. Data are presented as means
500 with their standard error. Comparisons were made using Student's *t*-test. * $P < 0.05$ **
501 $P < 0.01$ *** $P < 0.001$

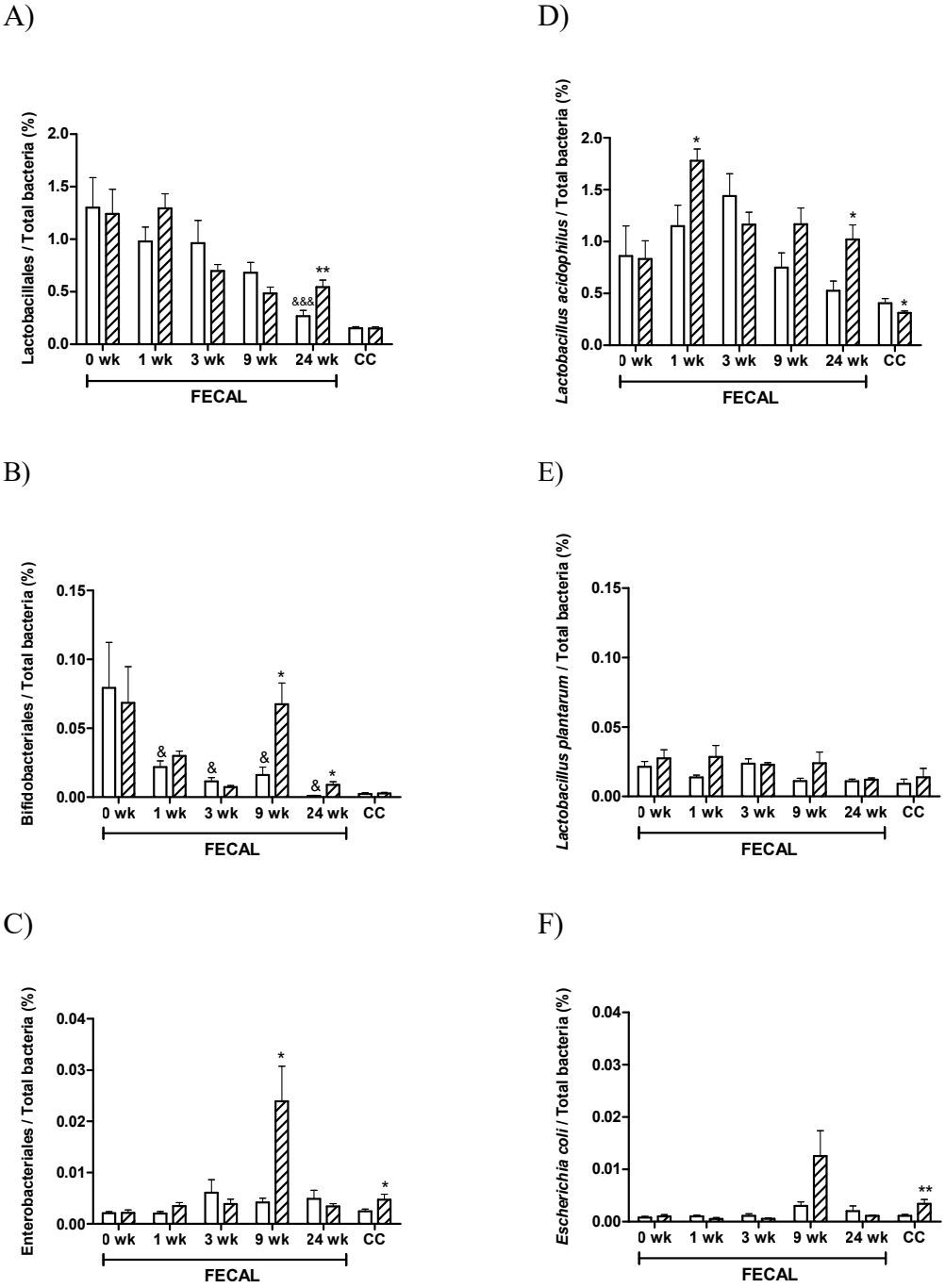


Figure 2.- Lactobacillales (A), Bifidobacteriales (B), Enterobacteriales (C), *Lactobacillus acidophilus* (D), *Lactobacillus plantarum* (E) and *E. coli* (F) in fecal samples from rats fed a standard diet (STD, empty bars), or supplemented with D-fagomine (FG, striped bars) at different times, and in cecal content (CC) at the end of the study. Data are presented as means with their standard error. Comparisons were made using Student's *t*-test or repeated-measures ANOVA. * $P < 0.05$ *vs* STD ** $P < 0.01$ *vs* STD; $^{\&}$ $P < 0.05$ *vs* wk 0 $^{\&\&\&}$ $P < 0.001$ *vs* wk 0