Low-dosage antibiotic intake can disturb gut microbiota in mice

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ABSTRACT

The proportion of different microbial populations in gut microbiota (GM) is an important factor that in recent years has been linked to obesity and numerous metabolic diseases. Antibiotics are one of the factors that can dramatically alter GM at therapeutic dosages, but their effects at subtherapeutic doses have been less investigated. Here, a mouse model using a total of 60 C57BL/6J mice was used to compare the evolution of total microbiota, four phyla and two genera considered as probiotics in control mice, and mice exposed to 50 µg/kg of ampicillin, 100 µg/kg of tetracycline or 100 µg/kg of sulfadiazine. The results obtained found that the presence of antibiotics in foods, even at trace concentrations, can disturb mouse GM, causing in all antibiotics significant increases of Proteobacteria (about 2 log CFU/g) or decreases of Bifidobacterium and Lactobacillus (about 1 log CFU/g) for the cases of ampicillin and sulfadiazine.

La ingesta de bajas dosis de antibióticos es capaz de alterar la microbiota intestinal en ratones

RESUMEN

La composición de las diferentes poblaciones microbianas presentes en la microbiota intestinal humana es un aspecto importante que en los últimos años ha sido relacionado tanto con la obesidad como con muchas patologías metabólicas. Los antibióticos son uno de los agentes que pueden alterar de manera radical la composición de la microbiota intestinal cuando se utilizan a dosis terapéuticas, pero sus efectos a dosis sub-terapéuticas han sido menos investigados. Para este fin, se ha realizado un ensayo empleando 60 ratones C57BL/6J para comparar la evolución de la microbiota total, así como 4 filos y 2 géneros bacterianos beneficiosos en ratones control, ratones expuestos a 50 µg/kg de ampicilina, ratones expuestos a 100 µg/kg de tetraciclina y ratones expuestos a 100 µg/kg de sulfadiazina. Los resultados obtenidos mostraron que la presencia de antibióticos en los alimentos, incluso a concentraciones traza, pueden alterar la microbiota intestinal de los ratones, causando a todos los antibióticos un incremento significativo de Proteobacteria (aproximadamente 2 log ufc/g), o descensos en los géneros Bifidobacterium y Lactobacillus (aproximadamente 1 log ufc/g) en los casos de la ampicilina y la sulfadiazina.

Introduction

The human digestive tract is colonized by trillions of microbes, collectively termed gut microbiota (GM) that comprise approximately $10^{12}$ colony-forming units/ml in the colon and can influence gastrointestinal physiology as well as the function of distant organs of the host (Fröhlich et al., 2016). This ecosystem can be readily modified by multiple factors, including the genotype and immunity of the host, environmental influences, diet or the use of therapeutic agents (Cox & Blaser, 2015).

In recent years, it has been demonstrated that GM play an important role in human health, with a relevant influence in obesity, diabetes, metabolic syndrome, cancer, cardiovascular diseases or even in psychiatric disorders (Conlon & Bird, 2015; Roca-Saavedra et al., 2018). Regarding the relation of GM to obesity, it has been reported that alteration in the populations of the GM may change intra-community metabolic interactions, modify caloric intake by using carbohydrates such as cellulose that are otherwise indigestible by the host and globally affect host metabolic, hormonal and immune homeostasis (Conlon & Bird, 2015). More recently, it has been reported that imbalance in GM can result in a pro-inflammatory luminal environment that can contribute to the progression of low-level chronic inflammation and metabolic disorders (Roca-Saavedra et al., 2018).

One factor that can dramatically affect GM composition and function is the effect of antibiotics. Epidemiological studies have confirmed the positive relationship, especially at early ages, between antibiotic use and weight gain in humans and indicate that even prenatal antibiotic exposure predisposes to childhood overweight (Cox & Blaser, 2015; Korpela & de Vos, 2016). Additionally, it has been reported that associations between obesity and exposure to antibiotics are different depending on the antibiotic spectrum (Mikkelsen, Allin, & Knop, 2016), subject age (Cox & Blaser, 2015; Korpela & de Vos, 2016) and even between boys and girls (Azad, Bridgman, Becker, & Kozyrsky, 2014; Mikkelsen et al., 2016).
Antibiotic use has reached enormous proportions around the world, although it is not consistent across geographical areas and countries (Nobel et al., 2015). However, little attention has been paid to the potential effect of subtherapeutic doses that can reach humans (Roca-Saavedra et al., 2018). Based on this matter, Ternak (2005) was the first to propose that human exposure to low-dose antibiotics may contribute to weight gain in humans. Since the 1940s, farmers have added low doses of antibiotics to the food or water of livestock to promote growth of farm animals (Nobel et al., 2015). Even in the European Union (EU, 2003), in which antibiotics are banned to act as growth promoters (EC Regulation 1831/2003), the use of antibiotics in veterinary medicine is common. As a consequence, antibiotic residues can reach humans at low concentrations through the food supply chain or through drinking water (Andersson & Hughes, 2014; Cox & Blaser, 2015).

In addition to livestock sources, antimicrobial agents are released into the environment from aquaculture, spraying of fruit orchards and vegetables, and from discarded expired drugs, hospital effluents and other human activities. Thus, there is ample evidence that antibiotics can enter our food chain from a variety of sources, that humans are chronically exposed to these drugs (Riley, Raphael, & Faerstein, 2013).

Considering this common exposure of humans to low dosages of antimicrobials, mainly carried by foods, the aim of the present work was to investigate the effects of low dosages of antimicrobials carried by foods at concentrations allowed by European Union Regulation 37/2010 for human consumption on the GM of mice (EU, 2010a).

Materials and methods

Animals and treatments

Sixty female C57BL/6J mice were obtained at 8 weeks of age from the Universidade de Santiago de Compostela central animal facility and allowed to adjust to the Lugo animal facility for a week for adaption to the new environment. Mice were weighed at the beginning of the experiment and distributed five per cage so that mean weights in each cage were similar. Each study group (control or antibiotic) was composed of 15 mice (3 cages of 5 mice) which were maintained at stable light conditions (12 h light/dark cycle), maximal light intensity (100 lx), temperature (set point 22°C) and relative air humidity (set point 50%) and a controlled cycle of filtered ventilation.

Throughout the experiments, tap water and standard laboratory chow were provided ad libitum. Because it was reported, possible alterations in ingestion have been reported if antibiotics are administered via the drinking water (Fröhlich et al., 2016) and antibiotics were administered mixed into feed. Mice received Harlan Global Diet 2018 S (Harlan Laboratories, Madison, WI, USA) mixed to contain 50 μg/kg of ampicillin, 100 μg/kg of sulphadiazine, 100 μg/kg of tetracycline or no antibiotics (control) for 16 weeks. The antibiotics employed were chosen on the basis that tetracyclines (tetracycline), β-lactams (ampicillin) and sulphonamides (sulphadiazine) are those most employed for veterinary purposes in Europe (Grave et al., 2014). Dosages were chosen as the maximum residue limits allowed in the European Union for meat and/or milk destined for human consumption by European Union Regulation 37/2010.

Antibiotics employed for mixing with the feed (ampicillin sodium salt, catalogue number A9518, sulphadiazine catalogue number S8626 or tetracycline catalogue number T3258) were obtained from Sigma-Aldrich (St. Louis, MO, USA).

The experimental procedures and number of animals used were approved by the Institutional Animal Care and Use Committee of Xunta de Galicia (Authorization Code MR110250) and conducted according to the Directive of the European Parliament and the Council of 22 September 2010 (EU, 2010b). The experiments were designed in such a way that animal suffering was minimized.

Each week, the mice were serially weighed on an electronic scale tared between all measurements. Because of their coprophagic behaviour, all cage batches received the same treatment. Feed was changed once daily to supply fresh antibiotics.

Collection of stool samples and DNA extraction

Stool samples were collected twice a week in a sterile container (10–30 g/cage) using sterilized surgical material and anaerobiosis condition generated using a GENbox system (GENbox, Biomérieux, Marcy L’Etoile, France) and were treated within 2 h of collection. DNA extraction consisted of a first step in a 1:10 dilution of phosphate-buffered saline (Invitrogen, Paisley, UK) adjusted to pH 7.2–7.4, and subsequent homogenization in a Masticator® (AES, Coubourg, France) for 5 min. Samples were afterwards frozen until use, and DNA extraction was performed using 1 mL of each sample using a commercial kit (Realpure Microspin, Durviz S., Valencia, Spain), as was previously employed in recent works (Castro-Penalonga et al., 2018; Rodriguez-Costa et al., 2015). Following this protocol, stool samples were diluted in isolation buffer, vortex-agitated and incubated at 80°C for 30 min. Afterwards, samples were centrifuged at 14,000 rpm for 10 min. Pellets were taken, placed in microtubes and diluted with 600 μL of binding buffer and 20 μL of proteinase K. The mix was vortex-agitated and then incubated at 70°C for 10 min, when 300 μL of isopropanol was added and mixed. After this, the lysates were pipetted into a special flask and centrifugated at 12,000 rpm for 60 s to obtain pellets. Afterwards, the pellets were washed with disinhibition buffer, wash buffer and elution buffer, following the volumes and centrifugation times established by the manufacturer’s instructions to obtain genomic DNA.

Bacterial quantification by real-time PCR from faecal samples

A real-time quantitative PCR (qPCR) procedure was used for the quantification of the bacterial groups investigated in human faecal samples by using specific primers based on previously reported methods (Murri et al., 2013) (Table 1). Thus, the first step consisted of a denaturation step at 95°C for 10 min and was followed by 45 denaturation cycles at 95°C for 10 s and an elongation step for 1 min at the temperature optimal for each bacterial group. Finally, analysis of the obtained curves (0.05°C per cycle) was performed by measurement of the intensity of the Syber Green® fluorochrome (Applied Biosystems, Foster City, CA, USA), according to previously reported by Guarddon et al. (2011). All reactions were carried out in triplicate, the final volume being 20 mL and containing 2 mL of DNA and a concentration of 100 nM of each of the probes. PCR amplifications
were performed using ABI PRISM 7000 equipment (Applied Biosystems, Warrington, UK) associated ABI PRISM 7000 Software (Applied Biosystems). Reference strains employed for the standard curves were Enterobacter cloacae CECT 194, Clostridium perfringens CECT 376, Bifidobacterium longum CECT 4503, Bacteroides vulgatus DSMZ 17767, Enterobacter cloacae CECT 194, Lactobacillus reuteri DSMZ 20016 (Table 1). All strains were grown in liquid medium under their optimal growth conditions and decimal dilutions were made for subsequent plate-counting. Once counted, the DNA was extracted from the tube, and the DNA was quantified by fluorescence using a Qubit Fluorometer (Invitrogen, Oregon, USA). Each of the curves was normalized to the copy number of the 16S rRNA gene for each of the species for which the copy number of the 16S rRNA operon was not published, the copy number was calculated by averaging the operon numbers of the closest bacterial taxa from the ribosomal RNA database rrnDB (Rodriguez-Costa et al., 2018). Negative controls containing all the elements of the reaction mixture except template DNA were performed in every analysis and no product was ever detected. The data presented are the mean values of triplicate real-time qPCR analyses.

**Results and discussion**

After 16 weeks of exposure of mice to control or antibiotic-mixed feed, the observed weights were within the expected range of growth for female C57BL/6J mice. In accordance with previous works (Cho et al., 2012), there were no significant differences in overall weight between control mice and mice exposed to tetracycline or sulphadiazine, while a smaller weight gain increase was found for mice exposed to ampicillin (P = 0.034) (Table 2). Additionally, as was previously found by Cho et al. (2012), there were no significant differences in calculated feed efficiency, expressed as weight gained per unit of feed consumed, between the treated and control mice.

As can be seen in Table 3, the evolution of total microbiota in the control subjects was quite stable throughout the study period, oscillating between values of 11 and 11.5 log CFU/g faeces. These values are compatible with those previously published by other authors, both for the total microbiota of mice (Okubo et al., 2013) and of humans (Aurumugan et al., 2011).

In all cases, after administration of the antibiotic (either ampicillin, sulphadiazine or tetracycline), evolution of the total GM of mice exposed to antibiotics was statistically different to that of the controls (P > 0.001) (Table 3). In this way, after the administration of all antibiotics, there was an

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**Table 1. Bacterial species-specific primers and reference strains employed for real-time PCR.**

| Target                     | Primer sequence (5'-3') | Reference strain                  | Product size (bp) |
|----------------------------|-------------------------|-----------------------------------|-------------------|
| Total microbiota           | F: ACTCTTACGGGAGCCGACG  |
|                           | R: ATTACCGGGCGTCTGGG    | Clostridium perfringens CECT 376  | 200               |
| Firmicutes                 | F: ATGTTGTTAAATGCAGAAGA |
|                           | R: AGTGCAGGCAACACATGCA  | Clostridium perfringens CECT 376  | 126               |
| Bacteroidetes              | F: CATGTGTTATTCCGATGAT  | Bacteroides vulgatus DSMZ 17767    | 126               |
|                           | R: AGTGCAGGCAACACATGCA  |                                    |                   |
| Actinobacteria             | F: GGKRCCTATACGCTTGT    | Bifidobacterium longum CECT 4503   | 333               |
|                           | R: CGCCTTACGAGCTTTACC   |                                    |                   |
| Proteobacteria             | F: CATGACGTTACCCGCGAGAAGA| Enterobacter cloacae CECT 194      | 195               |
|                           | R: CTCTACGAGACTCAAGTCTGC| Bacteroides vulgatus DSMZ 17767    | 106               |
| Bacteroides spp.           | F: CTCCTGGAAAACGGGTGTCG | Bifidobacterium longum CECT 4503   | 550               |
|                           | R: GGTGTTCTCCGATATCTACA |                                    |                   |

**Table 2. Weight gain and feed efficiency of control and exposed to antibiotics mouse.**

| Treatment       | Day 0       | 4 weeks     | 8 weeks     | 12 weeks    | 16 weeks    | Weight gain/feed efficiency |
|-----------------|-------------|-------------|-------------|-------------|-------------|----------------------------|
| Control         | 18.43 ± 1.06| 19.83 ± 0.98| 21.56 ± 1.24| 23.23 ± 1.43| 23.83 ± 1.21| 5.4 ± 0.42/0.019 ± 0.02*   |
| Ampicillin      | 18.93 ± 0.93| 20.12 ± 0.89| 21.63 ± 0.99| 23.43 ± 0.93| 24.04 ± 1.48*| 5.11 ± 0.24/0.018 ± 0.04a   |
| Sulphadiazine   | 18.56 ± 1.30| 19.86 ± 1.23| 21.21 ± 1.35| 22.94 ± 1.30| 24.06 ± 1.29*| 5.5 ± 0.37/0.020 ± 0.03*    |
| Tetracycline    | 18.41 ± 1.25| 19.76 ± 1.21| 21.89 ± 1.42| 23.54 ± 1.25| 23.94 ± 1.37*| 5.53 ± 0.31/0.021 ± 0.03*   |

Results are expressed as average ± standard deviation. * Values in the same column with different letters are significantly different.

Los resultados se expresan como media ± desviación estándar. **Los valores en la misma fila con diferentes letras son significativamente diferentes.**
initial decrease in the total microbiota, which after a period in which it was below that of control subjects experienced an increase.

It is an accepted fact that a large amount of bacteria per gram in the colon is related to a better state of health (Abdou, Zhu, Baker, & Baker, 2016). However, this is not always the case, because as well as the quantity of bacteria, the variety of species is also very important for human health (Clemente et al., 2015; Singh, Yeon, & Vijay-Kumar, 2016). Consequently, if few species of bacteria are resistant to an antibiotic, application of the same could eliminate the flora sensitive to the antibiotic. As a consequence, a resistant species can increase its number significantly and even accommodate more units per gram, without this implying any advantage for the host (Riley et al., 2013).

The Firmicutes phylum is usually the most frequent in the GM of people in countries with Western-type diets (Panda et al., 2014; Power, O’Toole, Stanton, Ross, & Fitzgerald, 2014); so, it is logical that it is the group that reaches higher levels within the GM. Table 3 shows the results obtained both in the control mice and in those exposed to the different antibiotics, reaching in some cases values close to those of the total microbiota, higher than 11 log CFU/g faeces. In all cases, exposure to antibiotics caused a significant increase in the Firmicutes population (P < 0.001). This increase in the amount of Firmicutes per gram of colonic content, and therefore in its relative proportion within the GM, has previously been observed by other authors in studies in which experimental mice were exposed to different types of antibiotics (Cho et al., 2012; Ellekilde et al., 2014; Russell et al., 2015). However, this result was not always consistent, since some authors have also found a decrease in the amount of Firmicutes resulting from administration of antibiotics (Vrieze et al., 2014). In general, increases in the amount of Firmicutes are usually considered as an obesogenic factor (Arumugan et al., 2011).

With respect to the phylum Bacteroidetes, all antibiotics used modified GM with respect to the control group, in the case of ampicillin (P < 0.001), tetracycline (P < 0.001) and sulphadiazine (P = 0.003), with a tendency to increase with respect to the start of the experiment and to the control group, as indicated in Table 3. When we compared this increase in relation to that obtained for the Firmicutes phylum, the results showed that in the control mice, the tendency in the frame of diet, age and environment in which the test was conducted favoured an increase of the Bacteroidetes with respect to the Firmicutes. In fact, in the control group, surprisingly, the proportion of Bacteroidetes in GM was even higher that of Firmicutes at week 16. In contrast, in mice exposed to antibiotics, this tendency was much less evident, and in any case, the proportion of Bacteroidetes in the GM exceeded that of Firmicutes, as occurred in the controls. The proportion of these two phyla, expressed as Firmicutes to Bacteroidetes ratio, has been described as higher in obese mice than in normoweight mice, even in obese mice lacking leptin which plays a crucial role in the regulation of appetite (Murphy et al., 2013; Turnbaugh et al., 2009).

### Table 3. Evolution of the faecal microbiota of the experimental control mice and with antibiotics in the feed for 16 weeks.

|                     | Day 0     | Week 4    | Week 8    | Week 12   | Week 16   |
|---------------------|-----------|-----------|-----------|-----------|-----------|
| **Firmicutes**      |           |           |           |           |           |
| Control             | 11.49 ± 0.01 | 11.47 ± 0.02 | 11.38 ± 0.10 | 11.41 ± 0.05 | 11.31 ± 0.01 |
| Ampicillin          | 11.58 ± 0.01 | 11.37 ± 0.01 | 11.26 ± 0.01 | 11.23 ± 0.02 | 11.70 ± 0.03 |
| Sulphadiazine       | 11.60 ± 0.02 | 11.39 ± 0.01 | 11.51 ± 0.02 | 11.68 ± 0.05 | 11.60 ± 0.09 |
| Tetracycline        | 11.35 ± 0.02 | 11.42 ± 0.02 | 11.30 ± 0.01 | 11.27 ± 0.02 | 11.49 ± 0.08 |
| **Bacteroidetes**   |           |           |           |           |           |
| Control             | 10.69 ± 0.07 | 10.65 ± 0.06 | 10.41 ± 0.05 | 10.64 ± 0.11 | 10.49 ± 0.09 |
| Ampicillin          | 10.84 ± 0.07 | 10.40 ± 0.01 | 10.71 ± 0.01 | 10.84 ± 0.02 | 11.11 ± 0.01 |
| Sulphadiazine       | 10.55 ± 0.09 | 10.70 ± 0.01 | 11.00 ± 0.01 | 11.17 ± 0.03 | 11.00 ± 0.00 |
| Tetracycline        | 10.96 ± 0.02 | 10.76 ± 0.01 | 10.83 ± 0.01 | 11.30 ± 0.01 | 11.20 ± 0.01 |
| **Actinobacteria**  |           |           |           |           |           |
| Control             | 10.09 ± 0.04 | 10.57 ± 0.05 | 9.87 ± 0.07 | 10.68 ± 0.04 | 10.54 ± 0.04 |
| Ampicillin          | 10.48 ± 0.01 | 10.46 ± 0.01 | 10.28 ± 0.02 | 10.55 ± 0.09 | 10.87 ± 0.01 |
| Sulphadiazine       | 10.08 ± 0.03 | 10.49 ± 0.03 | 10.60 ± 0.02 | 10.89 ± 0.01 | 10.79 ± 0.02 |
| Tetracycline        | 10.40 ± 0.05 | 10.18 ± 0.04 | 10.30 ± 0.01 | 10.96 ± 0.00 | 11.00 ± 0.02 |
| **Proteobacteria**  |           |           |           |           |           |
| Control             | 10.35 ± 0.04 | 9.50 ± 0.05 | 9.89 ± 0.07 | 9.86 ± 0.04 | 10.08 ± 0.04 |
| Ampicillin          | 10.67 ± 0.03 | 9.47 ± 0.02 | 9.38 ± 0.01 | 9.56 ± 0.09 | 9.89 ± 0.02 |
| Sulphadiazine       | 10.72 ± 0.02 | 9.62 ± 0.03 | 9.44 ± 0.02 | 9.86 ± 0.01 | 9.76 ± 0.01 |
| Tetracycline        | 10.40 ± 0.03 | 9.80 ± 0.02 | 9.95 ± 0.01 | 10.14 ± 0.03 | 9.90 ± 0.02 |
| **Bifidobacterium**|           |           |           |           |           |
| Control             | 5.58 ± 0.06 | 5.35 ± 0.01 | 5.71 ± 0.04 | 6.84 ± 0.07 | 6.55 ± 0.05 |
| Ampicillin          | 5.70 ± 0.13 | 5.40 ± 0.33 | 6.26 ± 0.03 | 6.00 ± 0.23 | 7.38 ± 0.47 |
| Sulphadiazine       | 5.68 ± 0.09 | 6.24 ± 0.03 | 6.83 ± 0.04 | 6.05 ± 0.02 | 7.76 ± 0.03 |
| Tetracycline        | 5.45 ± 0.01 | 6.37 ± 0.02 | 6.52 ± 0.02 | 7.88 ± 0.03 | 7.64 ± 0.02 |
| **Lactobacillus**   |           |           |           |           |           |
| Control             | 5.54 ± 0.09 | 4.75 ± 0.08 | 5.49 ± 0.12 | 5.52 ± 0.11 | 5.42 ± 0.03 |
| Ampicillin          | 5.76 ± 0.06 | 4.63 ± 0.03 | 5.23 ± 0.04 | 5.24 ± 0.04 | 4.98 ± 0.02 |
| Sulphadiazine       | 5.96 ± 0.02 | 4.83 ± 0.11 | 5.09 ± 0.01 | 5.14 ± 0.02 | 4.75 ± 0.02 |
| Tetracycline        | 5.28 ± 0.12 | 4.88 ± 0.14 | 5.73 ± 0.03 | 5.73 ± 0.03 | 5.31 ± 0.05 |

* Evolution of bacterial groups in the same column with different letters is significantly different.

a,b La evolución de los grupos bacterianos en la misma fila con diferentes letras son significativamente diferentes.
With respect to the phylum Actinobacteria, the results obtained can be found in Table 3. Only in the case of mice exposed to sulphadiazine were Actinobacteria counts significantly different to those of control mice ($P = 0.041$), while neither ampicillin nor tetracycline caused significant changes in evolution with respect to control mice. This different behaviour suggests different susceptibility on the part of the bacteria that form the Actinobacteria phylum to the antibiotics tested. Previous works related a greater presence of this phylum in the GM of obese people (Arumugan et al., 2011; Castro-Penalonga et al., 2018; Turnbaugh et al., 2009).

In the case of the phylum Proteobacteria, the results obtained are represented in Table 3, reaching significant increases ($P < 0.001$) for the treatment of the three antibiotics used with respect to the control group. This increase is one of the most important factors from the point of view of the generation of dysbiosis, due to the ability of Proteobacteria to cause low-grade inflammation in the large intestine, because they can generate a large amount of toxins and potentially toxic metabolites (Abdou et al., 2016; Morgan et al., 2012). The result obtained is also consistent with the fact that, as this phylum includes numerous pathogenic bacterial species, when they proliferate in the intestine, it is necessary to use antibiotics to reduce or eliminate them (Murphy et al., 2013). Perhaps for this reason, this phylum is the one with the most resistance genes accumulated in GM (around 35%) (Hu et al., 2013), even though in a normal situation, it does not represent more than 2–3% of GM (Caporaso et al., 2011). In fact, it is a common trend that in studies in which mice are exposed to antibiotics, the Proteobacteria phylum is one of the groups most affected, producing very notable increases with in some cases reaching up to 65 times the initial population (Leclercq et al., 2017).

The results obtained in relation to the amount of Bifidobacterium per gram of faeces were lower than those previously published by Okubo et al. (2013), who found Bifidobacterium counts in faeces of the same strain of mice in the range 8–10 log CFU/g. All antibiotics used in the trials caused changes in the Bifidobacterium genus, reaching significant differences ($P < 0.001$) in all three cases with respect to the control group during the exposure time. Thus, in the case of tetracycline, Bifidobacterium counts initially showed higher values, which then eventually decreased, even below those of the controls. However, in the case of both ampicillin and sulphadiazine, antibiotic exposure caused more pronounced decreases in the amount of Bifidobacterium in the GM of the mice.

The human health benefits of a large amount of Bifidobacterium have already been amply documented (Georgieva et al., 2015; Gueimonde, Sánchez, De Los Reyes-Gavilán, & Margolles, 2013). Therefore, the decreases experienced in mice exposed to ampicillin and sulphadiazine reflect changes that are detrimental to the host. The results obtained are compatible with previously published data, such as those of Gueimonde et al. (2013), who showed that many different strains of Bifidobacterium usually used as probiotics are sensitive to very small concentrations of β-lactams, whereas in the case of tetracyclines, many tet genes are commonly found in this genus, which confers intrinsic resistance (Gueimonde et al., 2013). These results were also confirmed by other studies in which the level of resistance of Bifidobacterium strains commonly used as probiotics to different types of antibiotics was verified (Georgieva et al., 2015; Moubareck, Gavini, Vaugien, Butel, & Doucet-Populaire, 2005). According to these authors, in all the cases tested, the strains of Bifidobacterium were found to be sensitive to ampicillin (hence the clear decrease in the counts of the mice exposed to this antibiotic), while some strains show total or intermediate resistance to tetracycline.

In the case of the genus Lactobacillus spp., the counts obtained in the control mice were compatible with those previously published by Okubo et al. (2013), who found faecal counts ranging from 8.5 to 10 log CFU/g. It is also normal that Lactobacillus counts in mice are superior to those that can usually be found in human faeces (Nguyen, Vieira-Silva, Liston, & Raes, 2015). The antibiotics ampicillin and tetracycline modified the GM showing significant decreases with respect to control mice ($P < 0.001$). On the contrary, sulphadiazine exposure did not cause any significant change with respect to the control group. As in the case of Bifidobacterium, this bacterial genus is considered beneficial for health and is also commonly used as a probiotic (Georgieva et al., 2015), although in some cases, a significantly higher level of Lactobacillus was found in obese patients than in thin subjects (Arumugan et al., 2011).

The exposure of the mouse GM to the different types of antimicrobials showed different results, since no significant effect was found in the case of exposure to tetracycline and sulphadiazine, while exposure to ampicillin caused a clear decrease in the population of Lactobacillus spp. This result is reasonable taking into account that many strains of Lactobacillus spp. are sensitive to ampicillin (Georgieva et al., 2015). Other previous studies showed that both ampicillin and other β-lactams decreased the counts of Lactobacillus spp. in GM, even at very small doses (Leclercq et al., 2017). However, this does not occur with all antibiotics since, for example, Thuny et al. (2010) found an increase in the population of Lactobacillus subsequent to the administration of a vancomycin–gentamicin combination. This is related to the use of vancomycin in some countries as a growth promoter in animals. Other previous work (Temmerman, Pot, Huys, & Swings, 2003) found that about 30% of the strains of Lactobacillus studied were resistant to tetracycline. Perhaps for this reason, the effects of tetracycline in this study were not as clear or categorical as those of ampicillin.

**Conclusion**

The roles of microbiota in human health and interference with GM by antibiotics are widely documented. This study has demonstrated that even at low concentrations allowed by European Union Regulation 37/2010, antibiotics can affect GM. In global terms, all antibiotics employed caused a marked reduction in total microbiota, but this reduction was not homogeneous in the different bacterial groups investigated. Thus, the maintenance or even increase in microbial load of Firmicutes or Proteobacteria suggests that eviction of microorganisms sensitive to these groups of antibiotics provides space for resistant strains to overgrow and dominate the niche. Therefore, the systematic use of these antibiotics could reshape the microbiota and create an obesogenic microbiota in the long-term and, as a consequence, create a major problem afflicting global health.
Disclosure statement
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