The Increase of Lactobacillus Species in the Gut Flora of Newborn Broiler Chicks and Ducks Is Associated with Weight Gain

Emmanouil Angelakis, Didier Raoult*

Unité des Rickettsies, CNRS UMR 6020, IFR 48, Faculté de Médecine, Université de la Méditerranée, Marseille, France

Abstract

Background: A bacterial role in the obesity pandemic has been suspected based on the ingestion of probiotics that can modify the gut flora. The objective of our study was to determine if increased Lactobacillus sp. in the gut flora of newborn broiler chicks and ducks could result in weight gain increase.

Methodology: Female broiler chicks (Gallus gallus domesticus) and ducks (Anas platyrhynchos domestica) were separated into one control and two experimental groups, and inoculated once or twice with 4 x 10^10 Lactobacillus spp. per animal in PBS, or with PBS alone. Fecal samples were collected before and at 24 hours, 2, 4, 8, 16 and 30 days after the inoculation. DNA was extracted from the stools, and qPCR assays were performed on a MX3000TM system for the detection and quantification of Lactobacillus sp., Bacteroidetes and Firmicutes, using a quantification plasmid. Animals were measured and sacrificed 60 days after the beginning of the experiment, and livers were collected and measured.

Principal Findings: Chicks inoculated once and twice with Lactobacillus weighed 10.2% (p = 0.0162) and 13.5% (p = 0.0064) more than the control group animals, respectively. Similarly, ducks inoculated once and twice weighed 7.7% (p = 0.05) and 14% (p = 0.035) more than those in the control group, respectively. Liver mass was also significantly higher in inoculated animals compared to the control group. Inoculation with Lactobacillus sp. increased the DNA copies of Lactobacillus spp. and Firmicutes in the stools. Bacteroidetes remained stable, and only the second Lactobacillus sp. inoculation significantly decreased its population in chicks. The ratio of DNA copies of Firmicutes to those of Bacteroidetes increased to as much as 6,4 in chicks and 8,3 in ducks.

Conclusions: Differences in the intestinal microbiota may precede weight increase, as we found that an increase of Lactobacillus sp. in newborn ducks and chicks preceded the development of weight gain.

Introduction

The manipulation of the gut microbiota through the administration of probiotics and antibiotics has been used for growth promotion in farm animals for 50 years and is regulated by the Food and Drug Administration (FDA) in the United States [1] and by the European Commission in Europe [2]. Microorganisms used in animal food in the European Union (EU) are mainly strains of gram-positive bacteria belonging to the Bacillus, Enterococcus, Lactobacillus, Pedococcus, Streptococcus species and strains of yeast belonging to the Saccharomyces cerevisiae and Kluyveromyces species [2]. The manipulation of the gut microbiota by growth promoters has had a large impact on the livestock and poultry industries [3]. Probiotics were initially used to prevent episodic diarrhea in poultry, as they reduce the intestinal colonization by Salmonella [4] and Clostridium perfringens [5]. However, it was found that they promote weight gain even in the absence of diarrheal outbreaks [6].

Recently, we and others hypothesized that bacteria may play a role in the obesity pandemic due to the ingestion of probiotics that modify the gut flora [7,8], and we stressed the necessity for further investigation of the effects of routinely adding bacteria to food [9]. Recently, type 2 diabetes mellitus was associated with compositional changes in the intestinal microbiota, as diabetics presented a significantly lower proportion of Firmicutes and a higher proportion of Bacteroidetes and Proteobacteria [10]. Obese diabetic subjects also presented significantly higher levels of Lactobacillus sp. [10]. In another study, obese patients presented significantly higher concentrations of Lactobacillus sp. in their feces than lean controls [11]. Moreover, we found that treatment with vancomycin in humans resulted in major and significant weight gain [12]. We speculated that the weight gain was induced by the growth-promoting effect of Lactobacillus sp. in patients who had been treated by vancomycin, as these bacteria are known to be resistant to glycopeptides [12]. Functional foods, such as yogurts and cheese, that are commonly consumed by adults and children
contain the same Lactobacillus sp. in about the same concentrations as used for decades to promote growth in agriculture [13,14]. In our study, we intragastrically administered a single dose of Lactobacillus sp. in broiler chicks and found that this inoculation was associated with significant weight gain [6]. The objective of our study was to determine whether the increase of Lactobacillus sp. in the gut flora of newborn broiler chicks could result in weight gain and to test the effects of such an inoculation on ducks.

**Results**

Animals had the same weight prior to the experiment, as there were no significant differences between the experimental and the control groups for chicks and ducks (p>0.05) (Table 1).

**Chicks**

Chicks inoculated with Lactobacillus spp. showed a faster increase in body weight compared to the control animals. On day 60, the body weight of the control animals (mean gram ± SD) was 1623±145, whereas the body weight of the animals inoculated once and twice was 1809±185 (p = 0.0162) and 1878±255 (p = 0.0064), respectively. On day 60, the liver weight of the control group animals (mean gram ± SD) was 47.7±8.7, whereas the liver weight of the chicks inoculated once and twice with Lactobacillus sp. was 60.3±6 (p = 0.026) and 63.5±8 (p = 0.011), respectively.

Before the inoculation there was no difference between the experimental and the control groups with respect to the mean number of DNA copies of Lactobacillus spp., Firmicutes and Bacteroidetes, and the numbers remained constant in the control group till day 30 (p>0.05) (Figure 1). On day 2, a significant difference was found in the number of DNA copies of Lactobacillus spp. between the control group and the two experimental groups (p = 0.046 and p = 0.041, respectively). Similarly, on day 2, the numbers of DNA copies of Firmicutes were significantly higher in the two experimental groups (p = 0.029 and p = 0.039, respectively) compared to the control group. Animals inoculated twice on day 2 presented significantly more DNA copies of Lactobacillus sp. than did the control group (p = 0.013) and the animals inoculated only once (p = 0.04). Animals inoculated twice on day 8 presented significantly more DNA copies of Firmicutes compared to the control group (p = 0.042), whereas no significant changes on day 8 were observed for DNA copies of Firmicutes between animals inoculated once and twice (p = 0.086). Between the control group and the chicks inoculated once, no changes were found in the amount of DNA copies of Lactobacillus spp. after day 16 (p = 0.3) or in the amount of Firmicutes after day 30 (p = 0.9). The mean number of DNA copies of Bacteroidetes was not significantly different between animals in the control group and animals inoculated once during the 30 days of the experiment. Between the control group and animals inoculated twice, a significant difference in the number of DNA copies on day 8 was only found for Bacteroidetes (p = 0.047).

The ratio of the mean number of DNA copies of Firmicutes to those of Bacteroidetes in the control group remained constant during the 30 days of the experiment. However, in the experimental groups, Lactobacillus spp. inoculation increased this ratio. In chicks inoculated once, the largest difference between Firmicutes and Bacteroidetes with respect to the amount of DNA copies was observed on day 2, in which the Firmicutes/Bacteroidetes ratio was 5.49-fold greater than that in the control group. After day 8, the Firmicutes/Bacteroidetes ratio was similar between chicks inoculated once and the control group. We found the largest difference between Firmicutes and Bacteroidetes in animals inoculated twice on day 8, as the Firmicutes/Bacteroidetes ratio was 6.4-fold greater than that in the control group. On day 30, no difference was observed in the Firmicutes/Bacteroidetes ratio between animals inoculated twice and the control group.

**Ducks**

Lactobacillus spp. inoculation had the same growth-promoting effects in ducks as it did in chicks. On day 60, the body weight (mean gram ± SD) of control animals was 2472±357, whereas the body weight of ducks inoculated once and twice with Lactobacillus sp. was 2679±266 (p = 0.005) and 2876±468 (p = 0.0035), respectively. The liver weight on day 60 of the control group was 79.6±16, whereas that of ducks inoculated once and twice with Lactobacillus sp. was 110.6±26 (p = 0.0068) and 120.3±36 (p = 0.0054), respectively.

qPCR revealed that before the inoculation, there was no difference between the experimental and control groups in the mean numbers of DNA copies of Lactobacillus spp., Firmicutes and Bacteroidetes, and the numbers remained constant in the control group during the 30 days of the experiment (Figure 2). A significant difference was observed in the number of DNA copies of Lactobacillus spp. on day 2 after inoculation between the control group (1.22×10^10 DNA copies of Lactobacillus spp.) and the two experimental groups (p = 0.032 and p = 0.02, respectively). The mean number of DNA copies of Firmicutes on day 2 was significantly different between the control and the two experimental groups (p = 0.03 and p = 0.04, respectively). Ducks inoculated twice on day 8 displayed significantly more DNA copies of Lactobacillus spp. than did the control group (p = 0.01).

**Table 1.** Animals’ body weight and liver mass at the beginning and at the end of the experiment.

| Body weight | Day | Control gr±SD | 1 inoculation gr±SD | P   | 2 inoculations gr±SD | p   |
|-------------|-----|---------------|---------------------|-----|---------------------|-----|
| Broiler chicks | 0   | 94.2±6.7      | 86.5±11             | 0.65| 88.5±8              | 0.71|
|              | 60  | 1623±145      | 1809±185            | 0.0162| 1878±255           | 0.0064|
| Ducks        | 0   | 82.8±16       | 85.7±10             | 0.61| 85.2±11.7           | 0.86|
|              | 60  | 2472±357      | 2679±266            | 0.05| 2876±468            | 0.035|
| Liver mass   |     |               |                     |     |                     |     |
| Broiler chicks | 0   | 47.7±8.7      | 60.3±6              | 0.026| 63±5.8              | 0.011|
| Ducks        | 0   | 79.6±16       | 110.6±26            | 0.0068| 120.3±36           | 0.0054|

doi:10.1371/journal.pone.0010463.t001
and chicks inoculated once ($p = 0.05$). At that time point, the amount of DNA copies of Firmicutes were also significantly different between ducks inoculated twice and those inoculated once ($p = 0.04$) and between ducks inoculated twice and the control group ($p = 0.02$). Between the control group and the ducks inoculated once, no changes were observed in the amount of DNA copies of Lactobacillus and Firmicutes after day 8 ($p = 0.08$ and $p = 0.7$, respectively). Between the control group and the ducks inoculated twice, no changes were found in the amount of DNA copies of Lactobacillus and Firmicutes on day 30 ($p = 0.08$ and $p = 0.7$, respectively). The mean number of DNA copies of Bacteroidetes was not significantly different between animals in the control group and animals inoculated once (largest difference on day 4, $p = 0.098$) or twice (largest difference on day 8, $p = 0.065$).

The Firmicutes/Bacteroidetes ratio remained constant in the control group during the 30 days of the experiment. Although no significant changes were observed in the population of Bacteroidetes, the ratio of DNA copies for Firmicutes/Bacteroidetes increased in the experimental groups. The greatest difference between Firmicutes and Bacteroidetes with respect to DNA copies for ducks inoculated once was observed on day 2, when the number of Firmicutes was 7.9-fold higher than that of Bacteroidetes. In ducks inoculated twice, the highest ratio was observed on day 8, when the Firmicutes/Bacteroidetes ratio was 8.3. No difference was found in the Firmicutes/Bacteroidetes ratio after day 8 or day 30 between the control group and ducks inoculated once, or between the control group and ducks inoculated twice, respectively.

Discussion

Using this experimental model, we found that even one dose of Lactobacillus spp. in newborn chicks accelerated weight gain and resulted in significant differences in body weight. Weight gain and differences in body weight were greater when a second dose of Lactobacillus spp. was administered. The chicks inoculated with Lactobacillus spp. displayed a significant increase not only in their body weight but also in their liver mass. Our results confirmed and extended the previous study of Khan et al. [6], who inoculated a single dose of either L. fermentum or of a strain of Lactobacillus sp. named Autruche 4 in 1-week-old female broiler chicks. Inoculation with the Lactobacillus spp. led to significantly greater weight gain and liver mass on day 29 [6]. In the present study, we found that Lactobacillus spp. inoculation presented the same growth-promoting effects on body weight and liver mass in ducks. In an independent study (unpublished) in collaboration with INRA (Institut National de la Recherche Agronomique) in ducks with free food access inoculated with the same Lactobacillus spp. ($4 \times 10^{10}$ bacteria/animal) we did not find evidence of weight gain although inoculated ducks presented a significant increase in liver mass.

Lactobacillus spp. probiotics are widely used as growth promoters in poultry, and Jin et al. found that the addition of 0.05%, 0.10% or 0.15% of twelve strains of Lactobacillus (1 to $10^9$ per gram) belonging to four species (L. acidophilus, L. fermentum, L. crispatus, and L. brevis) to the basal diet of 1-day-old Arbor Acres broiler chicks resulted in a significantly increased body weight compared to the control [15,16]. The supplementation of $10^6$ CFU/gram of a transformed L. reuteri Pg4 strain in the food of broiler chicks from 0
to 21 days of age increased body weight and ileal villus height [17]. In another study, the daily weight gain of chickens was increased by feeding them with a diet containing a probiotic (0.1% L. casei) during the first 3-wk, but the average quantity of food intake was not increased [18]. In other studies, treatment with Lactobacillus sp. had the same growth-promoting effects as treatment with avilamycin [19,20] and even better growth-promoting effects than chloroxytetracycline [18] or oxytetracycline [21].

We found that inoculation with Lactobacillus spp. in both chicks and ducks increased the population of Firmicutes, whereas the population of Bacteroidetes remained stable or slightly decreased. As a result, the Firmicutes/Bacteroidetes ratio increased after Lactobacillus spp. inoculation. In previous studies, a probiotic formula containing L. reuteri, E. faecium, B. animalis, Pediococcus acidilactici and L. salivarius displayed growth-promoting effects and significantly increased the concentrations of bacteria belonging to Bifidobacterium spp., Lactobacillus spp., and gram-positive cocci [19]. Lan et al. also found that 1×10^6 L. agilis and L. salivarius enriched the diversity of Lactobacillus flora in the chicken jejunum and cecum by increasing the abundance and prevalence of Lactobacillus spp. inhabiting the intestine [22]. The same probiotic treatment, when used for 40 days in chickens, reduced the number of Enterobacteriaceae, whereas the number of lactobacilli and enterococci remained stable [23].

To the best of our knowledge, this is the first demonstration that just one Lactobacillus spp. inoculation early in life is capable of changing the gut flora and result in a weight increase. Analysis of the gut flora in genetically obese (leptin deficient ob/ob) mice and obese humans showed that obesity was associated with a reduction in gram-negative bacteria, specifically Bacteroidetes, and an increase in gram-positive Firmicutes bacteria [8,24]. Kalliomaki et al. showed that differences in the intestinal microbiota may precede the development of an overweight phenotype [25]. It was found that the number of bifidobacterial species in fecal samples during infancy was higher in children with normal weight than in children becoming overweight, who also presented a greater number of Staphylococcus aureus than children with a normal weight [25]. Moreover, Membrez et al. found that a combination of norfloxacin and ampicillin, at a dose of 1 g/L, maximally suppressed the numbers of cecal aerobic and anaerobic bacteria in ob/ob mice and improved fasting glycemia and oral glucose tolerance [26]. The same group identified that a 4-week antibiotic treatment with amoxicillin and neomycin resulted in a reduction of Lactobacillus spp., Bifidobacterium spp., and Bacteroides-Prevotella spp. and reduced metabolic endotoxemia and the cecal content of LPS in both high-fat-fed and ob/ob mice [27]. Altogether, these studies support the idea that the increase of Lactobacillus in the gut flora is associated with weight gain [28,29]. In our animal experiment, we found that the increase of Lactobacillus sp. in the intestinal microbiota preceded the development of a weight increase. However, this link remains to be established for other animal species and humans.

**Materials**

After ethical approval, 30 individually weighed 4-day-old broiler chicks (female, Gallus gallus domesticus, Kabir strain) and 30 individually weighed 4-day-old ducks (female, Anas platyrhynchos domestica, Pekin strain) purchased from a small rural hatchery (R. Ivaldi Elevage, Font Trouvade, Saint Maximin La Sainte, Baume,
France) were randomly allocated to one control group and two experimental groups (10 animals/group). Animal procedures were conducted according to local regulations of animal welfare. The light/dark schedule was 14 hours of light and 10 hours of darkness; the room temperature was maintained at 22.2 ± 2°C and the humidity at 55.2 ± 5.8%.

Animals in the experimental groups were inoculated on day 0 according to a previously described method with Lactobacillus sp. (4×10^10 bacteria/animal) originally isolated from an ostrich, suspended in 1 mL of phosphate-buffered saline (PBS) (pH 7.0) [6]. This Lactobacillus sp., is closely related (i.e., 96% similarity in 16S rRNA gene sequence) to L. fermentum (CIP 102930). For the second experimental group, a second inoculation with the same Lactobacillus dose (4×10^10 bacteria/animal) was repeated on day 7. Animals in the control group were inoculated with PBS alone. The food quantity was the same for all groups. Fecal samples were collected from the anus by the use of a swab before the Lactobacillus sp. inoculation, and at 24 hours as well as 2, 4, 8, 16 and 30 days after inoculation. Animals were sacrificed 60 days after the beginning of the experiment, and livers were collected and measured.

DNA was extracted from stools using a NucleoSpin® Tissue Mini Kit (Macherey Nagel, Hoerdt, France) according to the manufacturer’s instructions. Next, DNA was eluted in 100 μL of elution buffer and stored at −20°C until use. A negative control extraction of 250 μL of sterile water was introduced in each series of DNA extractions. Real-time PCR assays were performed on an MX3000™ system (Stratagene Europe, Amsterdam). The detection and quantification of Lactobacillus sp. were performed as described by Menard et al. [30]. Bacteroidetes and Firmicutes were quantified using a quantification plasmid constructed as previously described by Carcopino et al. [31].

For data comparison, we used EpiInfo version 6.0 software (Centers for Disease Control and Prevention, Atlanta, GA, USA). p<0.05 was considered as significant.

Acknowledgments

The authors thank Claude Nappaz for his assistance on animal manipulation, and Isabelle Combe for helping in formatting the manuscript.

Author Contributions

Conceived and designed the experiments: DR. Performed the experiments: EA. Analyzed the data: EA. Contributed reagents/materials/analysis tools: EA. Wrote the paper: EA.

References

1. Vanderhoof JA, Young R (2008) Probiotics in the United States. Clin Infect Dis 46 Suppl 2: S67–S72.
2. Anadon A, Martinez-Larranaga MR, Aranzazu MM (2006) Probiotics for animal nutrition in the European Union. Regulation and safety assessment. Regul Toxicol Pharmacol 45: 91–95.
3. Anadon A, Martinez-Larranaga MR, Aranzazu MM (2006) Probiotics for animal nutrition in the European Union. Regulation and safety assessment. Regul Toxicol Pharmacol 45: 91–95.
4. Bailey JS, Stern NJ, Cox NA (2000) Commercial field trial evaluation of mucosal starter culture to reduce Salmonella incidence in processed broiler carcasses. J Food Prot 63: 867–870.
5. Craven SE, Stern NJ, Cox NA, Bailey JS, Bergan M (1999) Celiac carriage of Clostridium perfringens in broiler chickens given Masucosal Starter Culture. Avian Dis 43: 484–490.
6. Khan M, Raoult D, Richet H, Lepidi H, La Scola B (2007) Growth-promoting effects of single-dose intragastrically administered probiotics in chickens. Br Poult Sci 48: 734–735.
7. Raoult D (2008) Obesity pandemics and the modification of digestive bacterial flora. Eur J Clin Microbiol Infect Dis 27: 631–634.
8. Ley RE, Turnbaugh PJ, Klein S, Gordon JI (2006) Microbial ecology: human gut microbes associated with obesity. Nature 444: 1022–1030.
9. Raoult D (2008) Human microbiome: take-home lesson on growth promoters? Nature 454: 690–691.
10. Larsen N, Vogensen FK, van den Berg FW, Nielsen DS, Andreasen AS, et al. (2010) Gut Microbiota in Human Adults with Type 2 Diabetes Differs from Non-Diabetic Adults. PLoS ONE 5: e9085.
11. Armoogum F, Henry M, Vialetes B, Raccach D, Raoult D (2009) Monitoring bacterial community of human gut microbiota reveals an increase in Lactobacillus in obese patients and Methanogens in anorexic patients. PLoS ONE 4: e7125.
12. Thuny F, Richet H, Casalta JP, Angelakis E, Habib G, et al. (2010) Vancomycin treatment of infective endocarditis is linked with recently acquired obesity. PLoS ONE 5: e9074.
13. Zhang YH, Kim JK, Kim HJ, Kim WY, Kim YB, et al. (2001) Selection of a potential probiotic Lactobacillus strain and subsequent in vivo studies. Antonie Van Leeuwenhoek 80: 193–199.
14. Saulnier DM, Spindler JK, Gibson GR, Versalovic J (2009) Mechanisms of probiosis and prebiotic considerations for enhanced functional foods. Curr Opin Biotechnol 20: 135–141.
15. Jin LZ, Ho YW, Abdullah N, Jalaludin S (1996) Growth performance, intestinal microbial populations, and serum cholesterol of broilers fed diets containing Lactobacillus cultures. Poult Sci 75: 1258–1265.
16. Jin LZ, Ho YW, Abdullah N, Jalaludin S (2000) Digestive and bacterial enzyme activities in broilers fed diets supplemented with Lactobacillus cultures. Poult Sci 79: 836–841.
17. Liu JR, Lai SF, Yu B (2007) Evaluation of an intestinal Lactobacillus ruminis strain expressing rumen fungi xylanase as a probiotic for broiler chickens fed on a wheat-based diet. Br Poult Sci 48: 507–514.