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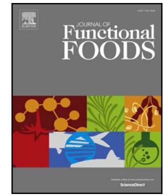

An intact gut microbiota may be required for lactoferrin-driven immunomodulation in rats

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ABSTRACT

Lactoferrin can modulate both the host immunity and gut microbiota. However, whether the immune modulation requires the gut microbiota has not been directly shown. Thus, our study compared (1) lactoferrin-driven immunomodulation profiles and (2) changes in fecal phylogenic metagenome with and without antibiotics-induced dysbiosis in rats. Rats receiving only lactoferrin but not both lactoferrin and antibiotics had a Th-1 type cytokine serum profile. Significant differences were detected between the fecal microbiota of the lactoferrin and control groups at day 19 and/or day 33 but not initially, with a shift in the major contributors for community dissimilarity to *Clostridium*, *Lactobacillus*, and *Oscillibacter valericigenes*. The antibiotics-induced dysbiosis enriched the proinflammatory phyla, Proteobacteria and Deferribacteres, together with the anti-inflammatory species, *Akkermansia muciniphila*, while suppressed some butyrate-producers from the Firmicutes phylum. Our study shows that an intact microbiota is necessary for lactoferrin-driven immunomodulation.

1. Introduction

Lactoferrin is a naturally secreted iron-binding glycoprotein found in milk, colostrum, tears, and saliva (Van der Strate, Beljaars, Molema, Harmsen, & Meijer, 2001). Bovine lactoferrin is around 77 kDa, consisting of a single polypeptide chain of 689 amino acids (EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA), 2012). The protein was first commercially produced by Oleofina Co. Ltd. in 1985. In 1986, a bovine lactoferrin-containing infant formula was marketed by the Japanese company, Morinaga & Co. Ltd. (Tomita et al., 2009). Currently, lactoferrin-containing products are sold also in Indonesia and Korea, as yogurt, dairy drinks, supplemental tablets, pet food, and cosmetics (Wakabayashi, Yamauchi, & Takase, 2006). The EFSA panel on Dietetic Products, Nutrition and Allergies announced that bovine lactoferrin is safe for food use (EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA), 2012).

The health maintenance capacity of lactoferrin mainly relies on its iron-binding ability and molecular interaction with the host and/or pathogens. Lactoferrin inhibits bacterial growth by sequestering surrounding iron (Velliyagounder et al., 2003). It also exerts antibacterial activity by permeabilizing Gram-negative bacteria via interacting with the bacterial lipopolysaccharide (LPS) to destabilize bacterial membrane. Moreover, by neutralizing free LPS, lactoferrin downregulates toll-like receptor 4 pathway activation thus reduces pro-inflammatory mediator release, and ultimately avoids sepsis and tissue damages of the host (Drago-Serrano, de la Garza-Amaya, Luna, & Campos-Rodríguez, 2012; Siqueiros-Cendón et al., 2014). Through different mechanisms e.g. releasing the antimicrobial peptide, lactoferricin, and immunomodulation, ingesting bovine lactoferrin confers host protection in clinical studies (Ochoa, Pezo, Cruz, Chea-Woo, & Cleary, 2012; Tomita et al., 2009). Besides bacteria, it protects the host from viral, fungal, protozoan infections (Liu & Newburg, 2013), and cancer formation (Tomita et al., 2009).

The gut microbiota helps maintain the host health; and gut dysbiosis increases the risks of developing multiple diseases, e.g. cancer, autoimmunity, and infections (Dzutsev, Goldszmid, Viaud, Zitvogel, & Trinchieri, 2015; Kamada, Seo, Chen, & Núñez, 2013). Oral lactoferrin administration can improve the gut microbiota in healthy infants (Roberts et al., 1992), and infants with necrotizing enterocolitis (Vongbhavit & Underwood, 2016). Bovine lactoferrin displays a significant and dose-dependent *in vitro* inhibitory effect on selected pathogens (e.g. *Escherichia coli* by about 20%) but fourfold less for probiotics, meanwhile enhances *Lactobacillus rhamnosus* (ATCC 7469) and *Lactobacillus acidophilus* (BCRC 14065) growth by

Abbreviations: ANOVA, analysis of variance; ELISA, enzyme linked-immunosorbent assay; IFN, interferon; Ig, immunoglobulin; IL, interleukin; Lf, lactoferrin; LPS, lipopolysaccharide; MANOVA, multivariate analysis of variance; OTU, operational taxonomic unit; PCoA, principal coordinate analysis; PCR, polymerase chain reaction; QIIME package, Quantitative Insights Into Microbial Ecology package; RDP, Ribosomal Database Project; SIMPER, similarity percentage; SMRT, single-molecule real-time sequencing; TNF, tumor necrosis factor

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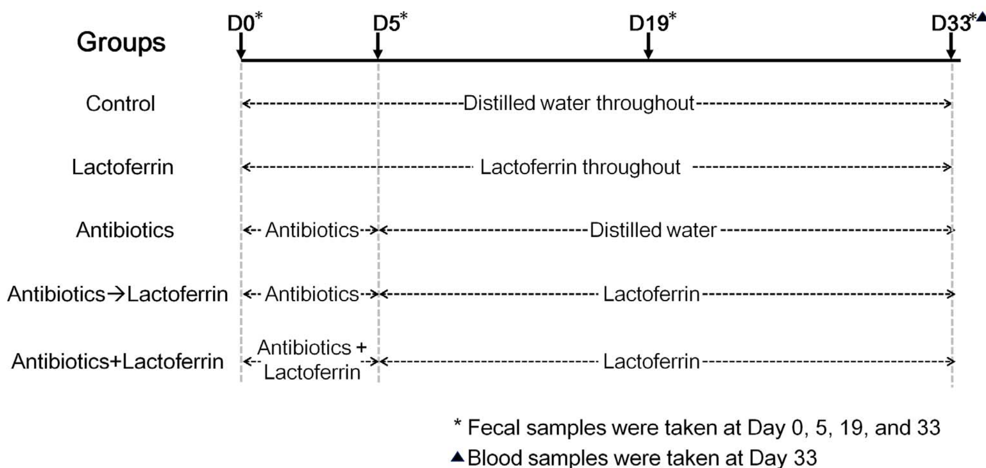

Fig. 1. Schematic diagram showing the experimental design.

40–200% (Chen, Ku, & Chu, 2014; Tian, Maddox, Ferguson, & Shu, 2010). These observations together suggest that lactoferrin can improve the host gut microbiota and hence overall health. However, till now, the effect of lactoferrin on gut microbes has only been studied with cultivation methods and/or real-time polymerase chain reaction (PCR). They do not reflect the global gut microbiota change. The third generation single-molecule real-time sequencing (SMRT) technology enables the depiction of the entire gut microbiota profile by a non-cultivation-based approach.

Although many studies have separately shown the impacts of oral lactoferrin administration on the host immunity and certain gut microbes, it is unclear if there is any causal relationship between the two effects. Thus, to explore if the modulation of gut microbiota is a mechanism of lactoferrin-driven immunomodulation, we compared (1) lactoferrin-driven immunomodulation profiles, and (2) changes in fecal phylogenic metagenome with and without antibiotics-induced dysbiosis in rats.

2. Material and methods

2.1. Animals housing

The rat experiment was approved by the Ethics Committee of Inner Mongolia Agricultural University. Fifty-eight 5-week old male Sprague-Dawley rats (Vital River Lab Animal Technology Co., Ltd., Beijing) were used. The rats acclimatized to the laboratory environment for 1 week before the experiment. During this week, they had free access to food and water. The standard rat feed was purchased commercially (Beijing Keoxieli Feed Co., Ltd.). Each rat was caged individually to avoid any direct contact between animals.

2.2. Antibiotics and lactoferrin preparation

The antibiotic mix was prepared fresh daily, containing neomycin (250 mg/kg body weight), metronidazole (50 mg mg/kg body weight), and polymyxin B (9 mg/kg body weight) (Sigma-Aldrich). Antibiotics were dissolved in sterile distilled water with sonication for 10 min to help dissolve the drugs. The antibiotic mix targeted to the entire gut microbiota (Davey et al., 2013). Lactoferrin was supplied by Hilmar Ingredients (Hilmar, USA); it was diluted with sterile distilled water before giving to the appropriate groups by oral gavage (300 mg/kg body weight/day) (Ishikado, Imanaka, Takeuchi, Harada, & Makino, 2005; Kimoto, Nishinohara, Sugiyama, Haruna, & Takeuchi, 2013).

2.3. Treatment groups

The rats were divided into 5 groups after considering the weight of each group. The 5 groups were orally gavage daily of sterilized distilled water, antibiotics mix and/or lactoferrin (Fig. 1). 1. Control: sterile distilled water throughout (n = 12), 2. Lactoferrin: lactoferrin throughout (n = 12), 3. Antibiotics: antibiotics for first 5 days, then sterile distilled water until day 33 (n = 11), 4. Sequential antibiotics and lactoferrin application initially: antibiotics for the first 5 days, then lactoferrin until day 33 (n = 12), and 5. Initial concomitant antibiotics and lactoferrin application: both antibiotics and lactoferrin for the first 5 days, then lactoferrin until day 33 (n = 11).

2.4. Fecal and blood sample collection

Fecal samples were collected at Day 0, 5, 19, and 33. Samples were stored with cryoprotectant at −80 °C until genomic DNA extraction. Blood samples were collected at day 33 in heparin-containing blood sampling tubes and centrifuged at 3000*g* at 4 °C for 10 min. The supernatants were stored at −80 °C until use.

2.5. Determination of immune factors by enzyme linked-immunosorbent assay (ELISA)

The plasma immunoglobulin A (IgA), IgG, IgM, interferon (IFN)-γ, interleukin (IL)-1β, IL-2, IL-6, IL-10, tumor necrosis factor (TNF)-α, and complement C4 concentrations were estimated by ELISA (Bethyl Laboratories, Inc. and Dakewe Biotech Co., Ltd).

2.6. Fecal DNA extraction and 16S rRNA profiling by SMRT

Fecal DNA was extracted using the Qiagen DNA Stool Mini Kit (Qiagen, Hilden, Germany). The DNA quality was checked by 1% agarose gel electrophoresis and spectrophotometry. The final DNA concentration was above 100 ng/μL and 260 nm/280 nm ratio at 1.8–2.0. All extracted DNA samples were stored at −20 °C until PCR.

The 16S rRNA were amplified by PCR using the forward 27F (5′-GAGAGTTTGATCCTGGCTCAG-3′) and reverse 1492R (5′-AAGGAGG TGATCCAGCCGCA-3′) primers (Mosher, Bernberg, Shevchenko, Kan, & Kaplan, 2013). The Agilent DNA 1000 Kit and an Agilent 2100 Bioanalyser (Agilent Technologies) were used for PCR product quantification. The amplification program consisted of 1 cycle of 95 °C for 5 min, followed by 28 cycles of 95 °C for 1 min, annealing at 58 °C for 1 min, and 72 °C for 2 min, followed by 1 cycle of 72 °C for 10 min. The amplicons (2 μg) were used for constructing the DNA library with the

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