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Characterization of the effects of three *Lactobacillus* species on the function of chicken macrophages

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ABSTRACT

Lactobacillus acidophilus, *Lactobacillus reuteri* and *Lactobacillus salivarius* can influence the adaptive immune responses in chickens but vary in their ability to do so. The present study attempted to identify how these three bacteria alter the innate immune system. A chicken macrophage cell line, MQ-NCSU, was co-cultured with the three live *Lactobacillus* species, alone or in combination, grown at different temperatures for various durations of time. Late exponential growth phase bacteria were more immunostimulatory, while bacterial growth temperature had little effect. *L. acidophilus* and *L. salivarius* significantly increased nitric oxide (NO) production and phagocytosis, while *L. reuteri* did not. In fact, *L reuteri* was shown to inhibit NO production of macrophages when co-cultured with the other bacteria or when cells were pre-treated with LPS. The results demonstrate a possible molecular mechanism for the immunomodulatory effects of *L. acidophilus* and *L. salivarius*, and a unique immunomodulatory ability of *L. reuteri*.

1. Introduction

The intestine is home to a complex community of bacteria that are engaged in a dynamic interaction with the host immune system. This gastrointestinal environment is the primary site where interactions between the host and the microbiota take place. In the chicken, *Lactobacillus* species are normal residents of the microbiota with some isolates having demonstrated probiotic properties, such as the ability to increase the antibody- and cell-mediated immune responses to experimental or vaccine antigens (Dalloul et al., 2005; Dunham et al., 1993; Haghighi et al., 2005; Karimi Torshizi et al., 2010; Koenen et al., 2004a, 2004b; Sato et al., 2009).

Although the molecular mechanisms of how the microbiome alters the host immune system are not completely understood, it is generally accepted that cells of the innate immune system, such as macrophages, after coming in contact with these bacteria influence the rest of the immune system. Typically, there are several barriers between intestinal macrophages and intestinal microbes, however, it has become apparent that certain subsets of intestinal macrophages can interact with these microbes or their microbial associated molecular patterns. For instance, CX3CR1+ macrophages can extend their dendrites through the epithelial barrier to sample the intestinal lumen (Bain and Mowat, 2011). In addition, resident macrophages present in the lamina propria of the intestine are exposed to Toll-like receptor ligands released by commensal microbes (Mowat and Bain, 2011). As a result, macrophages either directly or indirectly interact with commensal microbes and their microbial products. Macrophages eliminate microorganisms and regulate immune responses through phagocytosis, the production of nitric oxide (NO), the development of inflammation, the production of complement proteins and cytokines and through antigen presentation. The ability of probiotic bacteria to directly alter macrophage activation and/or functional ability has been examined. Ivec et al. (2007) demonstrated that a *Lactobacillus*-based probiotic increased the amount of NO and inflammatory cytokines produced by pig macrophages and that these increases led the macrophages to have increased anti-viral activity. More recent studies have provided insight into how probiotic bacteria affect the adaptive immune system. Marranzino et al. (2012) confirmed that in addition to the ability to stimulate intestinal macrophages, probiotic lactobacilli given orally to mice increased the phagocytic and microbicidal activity of alveolar and peritoneal macrophages resulting in better control of an intraperitoneal challenge with pathogenic *C. albicans*. Work performed in chickens directly linked the functional ability of macrophages, namely NO production and phagocytosis ability, to high antibody responses (Guimarães et al., 2011), thus providing one possible mechanism of how the microbiome alters the host immune response.

In previous studies with *Lactobacillus acidophilus*, *Lactobacillus reuteri* and *Lactobacillus salivarius*, we demonstrated that by treating chickens weekly with these bacteria, the antibody- and cell-mediated immune responses can be altered (Brisbin et al., 2011). More specifically, treatment of chickens with *L. salivarius* generated a consistent increase in antibody responses and a decrease in

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cell-mediated immune responses. Administration of *L. acidophilus* increased the antibody response to one of the antigens tested and decreased cell-mediated immune responses, while treatment with *L. reuteri* had little effect on either the antibody- or cell-mediated immune responses. Given the important role of macrophages in initiating and regulating downstream adaptive immune responses, we examined the response of macrophages to these three probiotic bacteria individually or in combination in order to elucidate some of the possible molecular mechanisms behind their probiotic effects on chickens.

2. Material and methods

2.1. Cells

MQ-NCSU cells, a macrophage-like cell line (Qureshi et al., 1990), were provided by Dr. Rodriguez-Lecompte (University of Prince Edward Island). The cells were maintained in LM-HAHN medium containing Lebovitz with glutamine (L-15 medium; Sigma Ca # L11518), McCoy's 5A modified medium (Invitrogen; 16600-082), 8% heat inactivated fetal bovine serum (Invitrogen; 12483020), 10% heat inactivated chicken serum (Invitrogen; 16110-082), 200 U/ml penicillin, 800 μg/ml streptomycin, 1% tryptose phosphate broth (Sigma; T8159), 1% sodium pyruvate (Sigma; S8636), 1% β-mercaptoethanol (1 mM), and 0.2% fungizone. RPMI (Sigma) containing 200 U/ml penicillin and 800 μg/ml streptomycin was used as the experimental medium. Cells were grown at 40 °C at 5% CO_2.

2.2. Bacterial cultures

L. acidophilus, *L. reuteri* and *L. salivarius*, isolated previously (Brisbin et al., 2010), were grown in de Man, Rogosa and Sharpe (MRS) medium at either 37 °C or 41 °C for various times (3 hr, 5 hr, 8 hr, or overnight) without shaking. Heat killed samples of each *Lactobacillus* species were produced by boiling for 30 minutes and verifying death by plating 100 μl of the heated samples on MRS plates followed by incubation at 37 °C in an anaerobic chamber for 24 hr.

2.3. Experimental design

MQ-NCSU cells were seeded at 1×10^6 cells/ml in 24 well plates for 2 hours in RPMI (Gibco) containing 10% heat inactivated fetal bovine serum (Invitrogen). The bacteria were grown at 37 °C and 41 °C for 8 hr, 5 hr, 3 hr and overnight. Live and heat killed cultures of *L. acidophilus*, *L. reuteri*, and *L. salivarius* were centrifuged at $5000 \times g$, washed with PBS and used to treat MQ-NCSU cells at 100:1 bacteria to cell ratio (determined to be the optimal dose). Cells were pulsed with the various bacterial treatments for 20 minutes, the medium discarded and replaced with fresh medium containing 200 U/ml penicillin and 800 μg/ml streptomycin. Culture supernatants were collected following 24 hours incubation at 40 °C in a humidified 5% CO_2 environment.

For experiments examining the various combinations of the three lactobacilli, MQ-NCSU cells were seeded as earlier and the cells were stimulated with *L. acidophilus*, *L. reuteri*, or *L. salivarius*, individually or in combination (grown at 37 °C or 41 °C for 5 hours). The bacteria were combined such that the final number of bacteria (1×10^8 CFU/well) contained equal amounts of the different bacteria. This experiment was repeated three times each with 6 replicates.

For experiments where cells were pre-treated with LPS, MQ-NCSU cells were seeded as earlier and the cells were stimulated with 0.1 μg, 1 μg, or 10 μg of LPS (Sigma) for 2 hours prior to the addition of the three *Lactobacillus* isolates individually or in the various combinations. This experiment was repeated twice each time with 6 replicates.

For experiments in which cells were pre-treated with mitogen-activated protein kinase (MAPK) inhibitors, MQ-NCSU cells were seeded as earlier and the cells were pretreated with DMSO, PD98059 (50 μM; Sigma), or SB23580 (10 μM; Sigma) for 1 hour prior to being treated with LPS (1 μg), *L. acidophilus*, *L. reuteri*, or *L. salivarius* (grown at 41 °C for 5 hours). This experiment was repeated twice each time with 4 replicates.

2.4. Nitric oxide production

Culture supernatants of treated or untreated macrophages were used in order to determine nitric oxide production using a Griess assay (Promega) according to the manufacturer's instructions.

2.5. Phagocytosis assay

A change in phagocytic activity of macrophages after treatment was determined using the Phagocytosis Assay Kit (IgG FITC; Cayman Chemical Company). MQ-NCSU cells were seeded at 5×10^5 cells/ml in 96 well plates for 2 hours in RPMI (Gibco) containing 10% heat inactivated fetal bovine serum (Invitrogen). *L. acidophilus*, *L. reuteri*, and *L. salivarius* were grown at 41 °C for 5 hr, centrifuged at $5000 \times g$, washed with PBS and used to treat MQ-NCSU cells at 100:1 bacteria to cell ratio along with the Latex Beads-Rabbit IgG-FITC complex (according to manufacturer). Cells were incubated for 3 hr at 40 °C in a humidified 5% CO_2 environment, centrifuged ($400 \times g$ for 10 min), and the supernatant was discarded. Subsequently, 50 μl of the Trypan Blue Solution was added, the cells were incubated for 1–2 min at room temperature, centrifuged as $400 \times g$ for 10 min and the excess Trypan Blue aspirated. The fluorescence intensity of each sample was read in a fluorescence plate reader using an excitation of 485 nm and an emission of 535 nm. This experiment was repeated twice with 6 individual replicates.

2.6. Statistical analysis

Data represent mean values plus/minus standard error of the mean (SEM). Significant differences between treatment groups were examined using a general linear model (SAS). Tukey's post-hoc comparison test was used to identify group differences at $P < 0.05$.

3. Results

3.1. Lactobacillus species differentially alter the production of NO

We assessed the immunomodulating capacity of three species of *Lactobacillus* on chicken macrophages by measuring the production of nitrite as a breakdown product of NO. To determine which growth phase of the bacteria was most stimulatory to macrophages, bacteria were grown for 3 h (early exponential), 5 h (late exponential), 8 h (early stationary), or overnight (late stationary) prior to co-culture with cells. Bacteria in late exponential phase to early stationary phase consistently induced macrophages to produce more NO than bacteria in early exponential or late stationary phase (Fig. 1). To determine if temperature has an impact on the immunomodulatory ability of lactobacilli, bacteria were grown at 37 °C or 41 °C prior to being co-cultured with the macrophages. Temperature had little effect on the immunomodulatory ability of the live bacteria (Fig. 2). At both temperatures, live *L. acidophilus* and *L. salivarius* increased nitrite production significantly ($P < 0.001$), while *L. reuteri* did not (Fig. 2). However, the heat-killed bacteria induced significantly ($P < 0.001$), more nitrite production when the bacteria were grown at 41 °C rather than 37 °C (Fig. 2).