Identification of Gut Bacteria such as Lactobacillus johnsonii that Disseminate to Systemic Tissues of Wild Type and MyD88−/− Mice

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ABSTRACT
In healthy hosts the gut microbiota is restricted to gut tissues by several barriers some of which require MyD88-dependent innate immune sensor pathways. Nevertheless, some gut taxa have been reported to disseminate to systemic tissues. However, the extent to which this normally occurs during homeostasis in healthy organisms is still unknown. In this study, we recovered viable gut bacteria from systemic tissues of healthy wild type (WT) and MyD88−/− mice. Shotgun metagenomic-sequencing revealed a marked increase in the relative abundance of L. johnsonii in intestinal tissues of MyD88−/− mice compared to WT mice. *Lactobacillus johnsonii* was detected most frequently from multiple systemic tissues and at higher levels in MyD88−/− mice compared to WT mice. Viable *L. johnsonii* strains were recovered from different cell types sorted from intestinal and systemic tissues of WT and MyD88−/− mice. *L. johnsonii* could persist in dendritic cells and may represent murine immunomodulatory endosymbionts.

Introduction
The current paradigm regarding sampling of luminal material in the gut by mononuclear phagocytes is that migratory intestinal dendritic cells (DCs) can acquire bacteria directly or indirectly from epithelial cells or macrophages and then migrate in a CCR7-dependent manner only as far as the gut draining lymph nodes for initiation of appropriate adaptive immune responses. This has led to the current consensus that bacterial members of the gut microbiota are separated from systemic compartments by multiple molecular, cellular, and tissue barriers or ‘firewalls’. However, the interaction of members of the gut microbiota with the host is not restricted to the gastrointestinal tract (GIT) and can also occur after translocation of selected gut bacterial strains to systemic tissues. There is emerging evidence of the existence of extraintestinal tissue microorganisms in human disease states and human gut symbionts were reported to disseminate from the gut in the absence of an inflammatory response in germ free (GF) mice. Such interactions can have multiple physiological and pathophysiological effects on the host. For example, *Achromobacter spp.*, *Bordetella spp.* and *Ochrobactrum spp.* can translocate from the gut to lymphoid tissues or the spleen to influence adaptive immunity and prevent dissemination of other luminal bacteria. Translocation of gut bacteria might also contribute to the vertical transfer of bacterial species from the mother to the neonate prenatally by crossing the maternal placental barrier or postnatally through maternal breast milk transfer.

However, it is not known if systemic dissemination of gut bacteria occurs in the absence of perturbation to the host or to the gut barrier. Host MyD88-dependent pattern recognition receptor (PRR) pathways are essential for the containment of gut bacteria in the gut lumen and their exclusion from the systemic compartment. Studies in MyD88−/− mice have shown increased dissemination of gut resident bacteria to systemic tissues. However, it has not been investigated if bacteria also...
translocate in WT mice and the specific bacteria that translocate in these mice have not been identified. The main objective of this study was to systematically investigate this dissemination phenomenon and its underpinning mechanisms in mice during the basal homeostatic state. Using a validated aseptic culture-based approach we recovered gut-associated bacteria from systemic tissues and from sorted tissue cells of WT and MyD88\(^{-/-}\) mice. Lactobacillus johnsonii was identified as the most abundant bacterial species in systemic tissues of mice with higher abundance observed in MyD88\(^{-/-}\) mice in comparison to WT mice. L. johnsonii was able to persist intracellularly in DCs and induce cytokine responses in these cells. We also found that L. johnsonii was able to migrate to systemic tissues in monocolonized germ-free WT mice and studies in specific-pathogen-free (SPF) CCR7 knockout (KO) mice indicated that this is CCR7 independent.

**Results**

**Viable gut-associated bacteria such as Lactobacillus johnsonii are present in systemic tissues of WT and MyD88\(^{-/-}\) mice**

To identify gut-associated bacteria residing in systemic tissues of mice, we initially used a workflow involving standard sterile operating procedures, called workflow 1 (Supplementary material 1). However, this was not sufficient to avoid contamination of tissues isolated from germ free mice by microbes derived from the immediate environment (Supplementary material 2 and 3). Therefore, we used a modified workflow – which included an antibacterial wash step for euthanized mice before dissection and a gentamicin wash step for tissues – to ensure the absence of environmental (skin, air, and work surfaces) microbial contaminants. Using this modified workflow – workflow 2 – we isolated only 1 colony of Bacillus *sp*. from a total of 108 plated whole tissue homogenates (Supplemental material 1) from 9 germ free mice (Figure 1a-h). Subsequently, workflow 2 was used for all further experiments.

We recovered several colonies of distinct gut-associated bacterial species from the different systemic tissues of WT and MyD88\(^{-/-}\) mice (Figure 1a-h). We also recovered specific bacteria that are classified here as “uncharacterized bacteria”. This is based on previous reports where they were found at increased levels in the intestine of mice with Salmonella-induced enterocolitis\(^{21}\) (Figure 1a,d); in the intestine of mice with diet induced obesity\(^{22}\) (Figure 1b); and in colonic tissues of WT and NOD2\(^{-/-}\) mice with Dextran Sodium Sulfate (DSS)-induced colitis\(^{23}\) (Figure 1h). Notably, L.
Johnsonii were recovered from both WT and MyD88−/− mice, but more frequently from systemic tissues of MyD88−/− mice (Figure 1a-f). Bifidobacterium pseudolongum and Lactobacillus reuteri were recovered from MLNs (Figure 1g) and “uncharacterized bacteria” were recovered from WAT (Figure 1h) of MyD88−/− mice. To assess if bacteria translocated from gut to systemic tissues, germ-free mice were gavaged with L. johnsonii isolated from liver of WT mice. This strain colonized intestinal tissues (stomach, small intestine, cecum, colon, and stool) and translocated, albeit at lower extent, to liver, pancreas, kidney, MLN and WAT tissues of germ-free mice monoclonized with L. johnsonii (Supplementary material 4). The overall recoverability of bacterial colonies and the extent of systemic dissemination was significantly higher in MyD88−/− mice in comparison to WT mice (Figure 1i).

Higher systemic dissemination of Lactobacillus johnsonii in MyD88−/− mice is a reflection of its higher relative abundance in intestinal tissues

To investigate if the higher recoverability of L. johnsonii from the systemic tissues of MyD88−/− mice might be due to its higher relative abundance in the GIT of these mice in comparison to WT animals, total tissue DNA was isolated from the stomach, small intestine (SI), cecum, colon and stool of both mouse strains and subjected to shotgun metagenomic sequencing (Figure 2a-f). In addition, as controls total DNA was isolated from the gut tissues of GF mice and DNA extraction kits to identify potential environmental/reagent contaminants (Figure 2a-f). The raw sequencing reads from tissue samples were at least 75-fold higher than the kit-reagent control (Supplementary material 5). The average microbial sequence reads in intestinal tissues and stool samples were higher in WT and MyD88−/− mice in comparison to germ-free mice (Supplementary material 5). Extraction reagents used in genomic DNA extraction (referred here as reagent control) have been reported to consist of contaminating microbial reads.24 We identified shotgun reads positive for bacteria such as Bordetella bronchiseptica, Streptococcus suis, Staphylococcus aureus, Methylobacterium sp., Alteromonas mediterranea in all of the tested samples including reagent control and germ-free tissues suggesting they represent the reagent microbiome (Figure 2a-f). However, WT and MyD88−/− mice tissue samples consisted of more diverse and distinct microbial reads (Figure 2a-f) which were positive for different gut-resident bacteria (Supplementary material 6). The proportion of sequencing reads corresponding to L. johnsonii was substantially higher in the GIT tissues (stomach, SI, cecum, and colon) and stool of MyD88−/− mice in comparison to their proportions in WT mice (Figure 2f). In addition, higher systemic dissemination of L. johnsonii was observed in MyD88−/− mice compared to WT mice.

Gut-associated bacteria are located intracellularly and can be cultured from cells sorted from intestinal tissue of WT and MyD88−/− mice

Since all tissues isolated for recovery of viable bacteria had been washed in 20 µg/mL of the cell-impermeable antibiotic gentamicin before homogenization and plating, any bacteria recovered from these tissues were most likely either resistant to gentamicin or located inside host cells. Indeed, none of the culturable bacteria recovered from systemic tissues were able to grow in bacteriological broth media supplemented with gentamicin at concentrations of 20 µg/mL or higher (Supplementary material 7). We reasoned that these bacteria were most likely located intracellularly in the systemic tissues. Therefore, we investigated if gut-associated bacteria could be recovered from different cell types: DCs (CD45+ CD64+CD11C+MHCII+), macrophages (CD45+ CD64+), epithelial cells (CD45 EpCAM+), leukocytes (CD45+CD64−MHCII+), remaining cells minus leukocytes- CD45 negative (CD45−) sorted from single cell suspensions of SI, colon, MLNs, spleen and thymus incubated in gentamicin (Supplementary material 8). Several gut-resident bacteria including L. johnsonii, L. reuteri, L. murinus, Cutibacterium acnes, Bifidobacterium pseudolongum, E. hirae, and Faecalibaculum rodentium were recovered from sorted cells of SI and colon of WT mice. L. johnsonii alone was recovered from sorted cells from small intestine and colon of MyD88−/− mice (Table 1). No gut-resident bacteria were recovered from sorted cells
of MLNs and spleen in both mouse strains. However, *Cutibacterium acnes* was recovered from epithelial cells, macrophages and leukocytes from thymus of WT mice. *L. johnsonii* was recovered from the percoll fraction of WT thymus that was enriched for leukocytes and T cells. *Stenotrophomonas* spp., a bacterium previously recovered from splenic DCs\(^25\) (closely related to low virulence pathogen *Stenotrophomonas malthophilia*\(^26\)) was recovered from MLN leukocytes of MyD88\(^{−/−}\) mice (Table 1).

**Lactobacillus johnsonii isolated from murine systemic tissues can persist and induce cytokine production in innate immune cells**

*L. johnsonii* was cocultured with the murine intestinal epithelial cell line CMT93, murine bone-marrow derived macrophages (BMDMs) and murine bone-marrow derived dendritic cells (BMDCs) in order to test their ability to persist intracellularly in these cells. The intracellular load of *L. johnsonii* reduced gradually from 0 to 24 h in cocultured epithelial cells (Figure 3a) and macrophages (Figure 3b) but not in DCs (Figure 3c). *L. johnsonii* triggered cytokine (interleukin (IL)-6, IL-10, tumor necrosis factor (TNF)-α, mouse keratinocyte chemotactant (mKC)) responses in cocultured macrophages (Supplementary material 9A) and DCs (Supplementary material 9B) but they did not trigger mKC (CXCL1) chemokine response in cocultured epithelial cells (Supplementary material 9C). Since *L. johnsonii* strains were able to persist in cocultured DCs for longer time periods, we investigated if dissemination of *L. johnsonii* from the gut to systemic tissues required migration of DCs from the gut to MLNs using CCR7\(^{−/−}\) mice.
Table 1. Culturable gut-associated bacteria isolated from FACS sorted cell subsets from the small intestines, colons, and systemic tissues of WT and MyD88−/− mice. Tissues were isolated from six WT and six MyD88−/− mice (all males, age: 23–27 weeks). CFU/1000 indicates colony forming units recovered for every 1000 sorted cells and was calculated by dividing the total number of colonies recovered per cell type with the total no. of cells sorted.

| Cell type | Bacteria recovered | No. of cells sorted | CFU/1000 cells | WT mice | MyD88−/− mice | CFU/1000 cells |
|-----------|--------------------|---------------------|---------------|---------|---------------|---------------|
| Small intestine | CD45− | L. reuteri, L. murinus, C. acnes | 964,678 | 0.051 | L. johnsonii | 685,943 | 0.04 |
| | Epithelial | L. reuteri, B. pseudolongum | 517996 | 0.011 | L. johnsonii | 285,739 | 0.01 |
| | Macrophage | L. reuteri | 106,358 | 0.084 | - | 504 | - |
| | Dendritic cells | L. reuteri, E. hirae, B. pseudolongum | 105,191 | 0.076 | L. johnsonii | 110,319 | 2.9 |
| Colon | L. johnsonii, L. murinus, C. acnes | 500,000 | 0.02 | L. johnsonii | 249,568 | 0.02 |
| | CD45− | L. johnsonii, B. pseudolongum, E. hirae, F. rodentium | 514,304 | 0.04 | L. johnsonii | 218,912 | 0.027 |
| | Epithelial | L. murinus, L. reuteri | 500,000 | 0.026 | L. johnsonii | 3,026 | 0.013 |
| | Macrophage | Uncultured bacterium, L. murinus | 41,869 | 0.047 | - | 11,772 | - |
| | Dendritic cells | F. rodentium | 51,566 | 0.019 | L. johnsonii | 54,426 | 0.018 |
| | Epithelial | L. murinus, L. reuteri | 500,000 | 0.026 | L. johnsonii | 54,426 | 0.018 |
| | Macrophage | Uncultured bacterium, L. murinus | 41,869 | 0.047 | - | 11,772 | - |
| | Dendritic cells | F. rodentium | 51,566 | 0.019 | L. johnsonii | 54,426 | 0.018 |
| | Lympocyte | B. pseudolongum | 100,997 | 0.019 | L. johnsonii | 120,401 | 0.018 |
| Thymus | CD45− | 819,698 | - | - | 1,000,000 | - |
| | Epithelial | C. acnes | 20,659 | 0.086 | - | 40,848 | - |
| | Macrophage | C. acnes | 69,376 | 0.048 | - | 27,330 | - |
| | Dendritic cells | C. granulosum | 51,566 | 0.019 | L. johnsonii | 54,426 | 0.018 |
| | T cells | L. johnsonii, C. acnes, C. granulosum | 100,997 | 0.019 | L. johnsonii | 120,401 | 0.018 |
| MLN | DC | - | 104,543 | - | - | 134,838 | - |
| | Lymphocyte | - | 927,516 | - | Stenotrophomonas spp. | 1,128,704 | 0.007 |
| | Macrophage | - | 16,197 | - | - | 31,035 | - |
| | CD45− | - | 175,642 | - | - | 525,151 | - |
| Spleen | DC | - | 660,229 | - | - | 44,835 | - |
| | Lymphocyte | - | 337,012 | - | - | 34,160 | - |
| | CD45− | - | 30,000 | - | - | 34,160 | - |
After cohousing WT and CCR7−/− mice for 12 weeks, to allow potential exchange of the microbiota, no significant difference was observed in the isolation and recovery of bacteria from systemic tissues (Supplementary material 10). *L. johnsonii* was recovered from both groups (Supplementary material 11) indicating that CCR7-dependent migratory DCs were not required for systemic dissemination of *L. johnsonii*.

**Discussion**

The main aim of this study was to investigate if gut-associated bacteria are capable of translocating to systemic tissues during homeostasis or are restricted to the gut lumen and associated intestinal tissues. In our current conceptual framework, bacterial members of the gut microbiota are thought to be separated from systemic tissues by multiple molecular, cellular, and tissue barriers. However, this framework is at odds with emerging data which indicates that gut-associated bacteria can be found intracellularly in a wide range of systemic cells and tissues under normal physiological and pathophysiological contexts.

Previously, meta-analysis of 16S rDNA metagenome sequencing datasets of murine fecal samples had identified *Lactobacillus* as one of most abundant species in the murine gut when compared to human gut. Specifically, *L. johnsonii* has been reported to inhabit the forestomach, ileum and cecum of mice. In our study, *L. johnsonii* was the most prominent bacteria recovered from both intestinal and systemic tissues of both WT and MyD88−/− mice. The recovery of *L. johnsonii* was substantially higher in MyD88−/− mice compared to WT mice and this appeared to be a direct reflection of their higher abundance in all intestinal tissues from MyD88−/− mice. *L. johnsonii* was recovered from different sorted cells from intestinal and systemic tissues of WT and MyD88−/− mice suggesting a broad cell tropism. *L. johnsonii* was able to persist for prolonged time periods within BMDCs, suggesting that DCs might be able to retain viable bacteria for longer time periods due to their reported lower phagolysosomal activity compared to macrophages. Indeed, *Achromobacter spp.*, *Alcaligenes spp.*, *Bordetella spp.* and *Ochrobactrum spp* can persist in DCs, and *E. cloacae*, that can translocate from the gut to MLNs, can be recovered intracellularly from DCs even 60 h after bacterial administration to WT mice. These observations suggest that DCs might represent an alternative intracellular niche for gut associated bacteria and support the existence of possible molecular mechanisms underpinning the ability of *L. johnsonii* and other bacteria to persist and replicate in DCs.

Many of the bacteria that we recovered from systemic tissues, have been reported to have beneficial host immunomodulatory effects. *L. johnsonii* in particular has been reported to reduce proinflammatory responses in murine liver, restore normal levels of CD4+ and CD8+ T cells in spleen, and trigger differentiation of splenic CD4+ T cells into tumoricidal Th17 cells in cyclophosphamide treated tumor mice.- Given that we have also found viable *L. johnsonii* residing in cells and tissues systemically it is tempting

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**Figure 3.** *L. johnsonii* persist in primary DCs but not macrophages or epithelial cells in vitro. *L. johnsonii* was cocultured with (a) CMT93 mouse epithelial cells; (b) BMDMs; and (c) BMDCs at MOI of 10:1, for up to 24 h and cell lysates and cell supernatants were plated separately for the indicated time points. Data are from three independent experiments. Statistical analyses were performed by two-tailed Student’s *t* test. *p* < .05 (denoted by *) was considered statistically significant.
to speculate that gut symbionts, such as \textit{L. johnsonii},
could also function as endosymbionts in order to exert their immunomodulatory effects. For the first
time we show that, different bacterial members of the
murine gut microbiota, predominantly \textit{L. johnsonii},
can translocate from the GIT to systemic tissues in
WT and MyD88\(^{-/-}\) mice. Our stringent and validated
culture-dependent approach will be beneficial to iden-
tify the cellular and molecular mechanisms underpin-
ing the gut and systemic immunomodulatory effects
of gut symbionts and pathobionts, their systemic dis-
semination, and their contribution to health and
disease.

\textbf{Materials and methods}

\textit{Mice}

Wild type (C57BL/6 J) (cat. number 000664),
MyD88\(^{-/-}\) (cat. number 009088), and CCR7\(^{-/-}\) (cat.
number 006621) male mice were purchased from
Jackson laboratories. They were housed in UCC
Biological Service Units’ animal facility under specific
pathogen-free (SPF) conditions, fed a standard pellet
diet (Envigo, Cambridgeshire, UK) and tap water ad
libitum. The GF mice were bred in-house and main-
tained in sterile flexible film isolators at UCC
Biological Service Units and “Washington University
Gnotobiotic Research, Education and Transgenic
(GREaT)” animal facility. Germ-free mice were fed
SDS RM1 A (P) (Special Diet Services, UK) as their
maintenance diet and RM3 A (P) (Special Diet
Services UK) as their breeder diet and deionized
drinking water ad libitum. All food, water bedding
and other supplies needed for GF mice in the isolator
were sterilized by autoclaving before being introduced
into the isolator. Standard housing and environ-
mental conditions were maintained (temperature 21\(^\circ\)C,
12 h light, and 12 h darkness with 50\% humidity) in
the animal housing facility. All animal experiments
were performed in accordance with EU legislation
(Directive 2010/63/EU) and the Institutional Animal
Care and Use Committee at Washington University
School of Medicine for the protection of animals used
for scientific purposes. The study was carried out
under ethical approval (Euthanasia Only: Application ID 2018/009 and AE19130/P085) from
the Animal Experimentation Ethics Committee of
University College Cork.

\textbf{Isolation of mouse systemic tissues for the
identification of gut-associated bacteria}

Mice were euthanized by cervical dislocation prior to
dissection and isolation of tissue. For more informa-
tion on tissue isolation and plating see Supplementary
materials (Supplementary Material 1).

\textbf{Preparation of single cell suspensions from mouse
tissues for fluorescent activated cell sorting (FACS)}

Single cell suspension was obtained from SI, colon,
MLNs, spleen and thymus. Single cell suspension
from SI and colon tissue of euthanized mice were
obtained using the lamina propria dissociation kit,
mouse (Cat# 130–097–410, Miltenyi) following the
manufacturer’s instructions. MLN and spleen tis-
se were physically homogenized and washed with
1X PBS supplemented with 1\% FCS. Red blood cells
in spleen were lysed by 10 min incubation at 37\(^\circ\)C
with 5 mL of 1X Lysebuffer (eBioscience).
Furthermore, spleen cells were enriched for den-
dritic cells (DCs) using mouse pan dendritic cell
isolation kit (Miltenyi Biotec). Immune cells were
resuspended in 1X PBS supplemented with 1\% FCS.
Samples were then centrifuged 5 min at 300 \(\times\) g.
Thymi were removed and finely chopped and
placed in Roswell Park Memorial Institute medium
(RPMI 1640, Sigma) supplemented with 1 mg/mL
Collagenase D (Roche), DNase (Sigma), and
Dispase (Roche). Pieces were incubated for
30 min at 37\(^\circ\)C with gentle shaking. Cells were
filtered through 100 \(\mu\)m strainers and cell suspens-
ion was gently layered on top of tubes with 52.7\%
Percoll (Gibco) layered on top of 92.4\% Percoll.
Tubes were centrifuged at 3000 \(\times\) g for 30 min at
4\(^\circ\)C with brake off. The top fraction of cells was
gently collected and resuspended in 1X PBS sup-
plemented with 1\% FCS.

\textbf{Immunostaining and sorting of single cell
susensions}

Monoclonal antibodies were used to stain single
cell suspension from different tissues. Prior to
FACS analysis isolated cells were incubated with
gentamicin (20 \(\mu\)g/ml) for 10 min at 37\(^\circ\)C in order
to kill potential extracellular bacteria and then
washed twice in sterile 1 \(\times\) PBS. Single cell
suspensions were blocked with the monoclonal antibody 2.4 G2 directed against the FcgRIII/II CD16/CD32 (0.5 ng mAb per 106 cells) (Fc block, BD Biosciences) for 15 min followed by immunostaining. 1 × 10⁶ cells were incubated with 0.5 ng of the relevant mAbs (Supplementary material 12) for 20 min at 4°C, and washed again twice.

**Bacterial identification from culture plates by Sanger sequencing**

Colony PCR was carried out on the resulting colonies from plated tissue homogenates or from sorted cell populations (lysed in ice cold sterile water) plated in BHI or YCFA, using primers which target full length of bacterial 16S rRNA gene. PCR products were purified using High Pure PCR product purification kit (Sigma) and identified by full length Sanger-sequencing (Eurofins Genomics).

**Genomic DNA isolation and shotgun metagenomic sequencing of murine intestinal tissues and stool**

Genomic DNA was extracted from the intestinal tissues by using Qiagen DNeasy Blood & Tissue kit (Qiagen) and extracted from stool samples by using QIAamp Fast DNA Stool mini kit (Qiagen). DNA concentration was estimated using Qubit® (Invitrogen) and metagenomic libraries were prepared using the Nextera XT kit (Illumina) with minor modifications. Briefly, the tagmentation time was increased to 7 min and following addition of indices and purification as described in the manufacturer’s protocol, the average size of each sample was assessed using a High Sensitivity DNA assay on an Agilent bioanalyzer(Agilent) and quantified by Qubit (Invitrogen). The samples were then pooled equimolarly. The concentration of the final pool was determined by qPCR using the Kapa qPCR kit for Illumina (Roche) and sequenced in the Teagasc Next Generation Sequencing Facility using a NextSeq™ 500/550 High Output Kit v2 (300 Cycles) kit (Illumina) on the Illumina NextSeq 500 using standard Illumina guidelines. DNA from intestinal tissues of six GF mice and DNA extraction reagents and library preparation (RC – reagent control) were also sequenced as controls.

**Analysis of shotgun metagenomic sequencing data**

Taxonomic classifications of Resulting FastQ reads were determined using Kraken 2 and Bracken. Resulting FastQ reads from sequencing were quality checked by first removing contaminating mouse derived reads using NCBI Best Match Tagger (BMTagger). Resulting reads were trimmed and poor quality and duplicate reads removed using a combination of SAMtools and Picard tools. Taxonomic classifications of trimmed reads were determined using Kraken 2 and Bracken.

**In vitro persistence assay of L. johnsonii**

*L. johnsonii* strains were cocultured with CMT93 murine intestinal epithelial cell line (ATCC® CCL-223®), BMDCs (bone-marrow-derived macrophages) and BMDCs were prepared as previously described. L. johnsonii strains recovered from systemic tissues were cultured overnight in MRS (DIFCO) under micro-aerobic conditions (5% CO₂) at 37°C. Following 2 h of incubation of *L. johnsonii* with the cells, cells were resuspended in 20 μg/mL gentamicin supplemented media for 20 min and then washed with PBS three times and resuspended in fresh cell culture media. At this time point (0 h) cell supernatants were plated in MRS agar. Cells were lysed in sterile ice-cold water and whole cell lysates plated separately as well in MRS agar. Plating of supernatants and whole cell lysates was repeated at 6, 16, and 24 h. Plates were incubated under microaerobic (5% CO₂) conditions for 48 h before enumerating CFU (colony forming units). All experiments were repeated three times.

**Cytokine secretion of cocultured cells with L. johnsonii**

Cytokines were measured in the supernatant of CMT93 murine intestinal epithelial cell line, BMDCs or BMDCs cocultured with confluent overnight cultures of *L. johnsonii* strains at an MOI of 10:1 for 24 h. Analysis of mKC was carried out using murine CXCL1 ELISA DuoSet®(R & D systems, Bio-Technne) and analysis of TNF-α, IL-10, IL-12p70, IL-1β, and mKC/CXCL1 cytokines was carried out using 7-
plex Uplex MSD assays (MesoScale Discovery, Gaithersburg, MD).

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Disclosure statement

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