

Characterizing how probiotic *Lactobacillus reuteri* 6475 and lactobacillic acid mediate suppression of osteoclast differentiation

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ABSTRACT

Osteoporosis is a disease that impacts over 200 million people worldwide. Taking into consideration the side effects stemming from medications used to treat this illness, investigators have increased their efforts to develop novel therapeutics for osteoporosis. In a previous study, we demonstrated that ovariectomy-induced bone loss in mice was prevented by treatment with the probiotic bacterium *Lactobacillus reuteri* 6475 (*L. reuteri*), an effect that correlated with reduced osteoclastogenesis in the bone marrow of *L. reuteri* treated mice. We also demonstrated that *L. reuteri* directly inhibited osteoclastogenesis *in vitro*. To better understand how *L. reuteri* impacts osteoclast formation, we used additional *in vitro* analyses to identify that conditioned supernatant from *L. reuteri* inhibited osteoclastogenesis at the intermediate stage of fused polykaryons. To elucidate the effect of *L. reuteri* treatment on host cell physiology, we performed RNAseq at multiple time points during *in vitro* osteoclastogenesis and established that *L. reuteri* downregulated several KEGG pathways including osteoclast differentiation as well as TNF- α , NF- κ B, and MAP kinase signaling. These results were consistent with Western Blot data demonstrating that NF- κ B and p38 activation were decreased by *L. reuteri* treatment. We further identified that lactobacillic acid (LA), a cyclopropane fatty acid produced by *L. reuteri*, contributed significantly to the suppression of osteoclastogenesis. Additionally, we demonstrated that *L. reuteri* is signaling through the long chain fatty acid receptor, GPR120, to impact osteoclastogenesis. Overall, these studies provide both bacterial and host mechanisms by which *L. reuteri* impacts osteoclastogenesis and suggest that long chain fatty acid receptors could be targets for preventing osteoclastogenesis.

1. Introduction

Osteoporosis remains a growing problem worldwide. According to the International Osteoporosis Foundation, over 200 million people have low bone mass and are at increased risk for sustaining bone fractures. Unfortunately, several medications used to treat osteoporosis have undesirable side effects. For example, the first line use of bisphosphonates to suppress osteoclast activity can result in the disruption of normal bone remodeling, which can result in atypical femur fractures or osteonecrosis of the jaw in a small number of patients (Rasmusson and Abtahi, 2014; Khan et al., 2015; Crandall et al., 2014). Hormone replacement therapy, while highly effective for preventing bone loss, can increase risk of invasive breast cancer and venous thromboembolism (Grossman et al., 2017). While these responses are rare, the fear of such side effects has reduced the number of patients taking osteoporosis medications. Thus, there is an impetus for the

development of novel therapeutics with few side effects to combat osteoporosis.

One potential option is to utilize our understanding of the role that bacteria, especially in the intestine, play in shaping bone health. The intestinal microbiota is a collection of microbes, including bacteria, archaea, viruses, helminths, fungi, and their genetic material, that inhabit our gut. Investigating host microbe interactions at this interface has led to recognizing microbiota as a critical determinant of health and disease. In particular, discoveries demonstrating the impact of the microbiota on bone health have led to the emergence of the term *osteomicrobiology*, which refers to the study of the role of microbes in bone health and disease (Jones et al., 2017; Yan and Charles, 2017). Furthermore, increased attention has been given to the use of probiotics, defined as live microorganisms that confer health benefits when consumed according to the Food and Agriculture Organization (FAO) and World Health Organization (WHO), for the treatment of bone diseases

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(Morelli and Capurso, 2012). The efficacy of several probiotic strains in improving bone health has been demonstrated in multiple animal models (Narva et al., 2004; Narva et al., 2007; Mutuş et al., 2006; Chiang and Pan, 2011; Tomofuji et al., 2012; McCabe et al., 2013; Britton et al., 2014; Collins et al., 2016; Ohlsson et al., 2014; Schwarzer et al., 2016).

In our previous studies, we showed that treating OVX mice with the immunomodulatory probiotic strain *Lactobacillus reuteri* PTA 6475 (*L. reuteri*) resulted in decreased osteoclast formation from primary bone marrow outgrowth studies (Britton et al., 2014). Moreover, using the *in vitro* RAW264.7 cell line, we demonstrated that the differentiation of this monocyte/macrophage cell line into osteoclasts was arrested by the addition of a < 3 kDa cell culture supernatant (CCS) fraction from *L. reuteri* 6475. Other studies involving probiotics and bone health have highlighted the efficacy of different bioactive compounds in preventing bone loss and this prevention has often times been attributed to suppression of osteoclastogenesis (Narva et al., 2007; Rahman et al., 2006; Ewaschuk et al., 2006; Li et al., 2016; Tyagi et al., 2018). Together, these studies strongly suggest that the identification of bioactive molecule(s), produced by bacteria that target osteoclastogenesis, may lead to understanding how bacteria contribute to bone health and optimize probiotic strain selection for treating bone disease.

In this study, we expanded the initial findings on *L. reuteri* suppression of osteoclastogenesis and characterized this interaction by describing the host response following *L. reuteri* stimulation. Through a guided RNA sequencing experiment, we identified that *L. reuteri* modulates genes involved in osteoclastogenesis as well as TNF α and NF κ B pathways. We further demonstrate that *L. reuteri* suppression of osteoclastogenesis is in part mediated by lactobacillic acid (LA) interaction with the GPR120 receptor. Together, our studies identify specific host mechanisms as well as bacterial mechanisms by which *L. reuteri* modulates osteoclastogenesis. These findings have important implications in future drug development for bone disease.

2. Materials and methods

2.1. Chemicals and reagents used

The GPR40 and GPR120 antagonists, DC260126 (Cat. No. 5357) and AH7614 (Cat. No. 5256), respectively, were purchased from Tocris Biosciences. Lactobacillic acid (LA, also known as phytomonic acid) was purchased from Cayman chemical. The antibodies, p38 (Cat. No. 8610), phosphorylated p38 (Cat. No. 4511), ERK (Cat. No. 3552), phosphorylated ERK (Cat. No. 4377), JNK (Cat. No. 9258), phosphorylated JNK (Cat. No. 9251), p65 (Cat. No. 8242), phosphorylated p65 (Cat. No. 3033), and beta actin (Cat. No. 4970) used for this study were purchased from Cell Signaling Technology.

2.2. Bacterial strains used in this study and growth conditions

L. reuteri ATCC PTA 6475 was provided to the Britton laboratory by Biogaia inc. (Sweden) and was cultured anaerobically in deMan, Rogosa, Sharpe media (MRS, Difco) for 18 h at 37 °C. To generate *L. reuteri* cell-free conditioned supernatant (CCS), the overnight culture was subcultured into fresh MRS and grown until log phase ($OD_{600} = 0.4$) and cells were pelleted by centrifugation at 4000 rpm for 10 min. The pellet was washed twice with sterile PBS to remove any residual MRS. The *L. reuteri* CCS was generated by resuspending the bacterial cell pellet in Minimum Essential Medium (MEM- α , Invitrogen) to an $OD_{600} = 3.0$ (5 ml in 50 ml BD conical tube) and incubated for 3 h at 37 °C with gentle orbital shaking (60 rpm). The bacterial cells were pelleted and the supernatant collected, filter-sterilized using a PVDF membrane filter (0.22 μ m pore size, Millipore), and fractionated (Amicon filter, Millipore) to include only the < 3 kDa fraction. The *L. reuteri* CCS were pipetted into 96-well plates (deep well, 3 ml volume) in 250 μ L aliquots, lyophilized and stored at -80 °C. Sterile MEM- α

also underwent processing in parallel to serve as the vehicle control for each experiment. This was also performed with the bacterial strains: *E. coli* DH5- α .

2.3. Culture conditions and osteoclastogenesis differentiation assay

The murine macrophage cell line, RAW264.7, was obtained from ATCC and maintained in phenol red-free medium (MEM- α , Invitrogen) supplemented with charcoal stripped fetal bovine serum (Invitrogen) at 37 °C with 5% CO₂. In 24-well tissue culture grade plates (Costar), 2×10^4 cells were seeded. Following one day of incubation, cells were stimulated for differentiation with the addition of receptor activator of NF-kappa B ligand (RANKL, 100 ng/ml, R&D systems). Lyophilized *L. reuteri* CCS or CCS from other bacterial strains were resuspended in culture medium and used to treat the cells. For all experiments, each well contained 20% (0.2x) of the $OD_{600} = 3.0$ CCS (50 μ L) unless otherwise noted. For dose response experiments, a CCS of 100% is equivalent to containing 250 μ L of CCS ($OD_{600} = 3.0$) in each well. Fresh medium is replenished after every 2 days for a week. On day 7, cells were fixed and stained for tartrate-resistant acid phosphatase (TRAP) using a commercially available staining kit (Sigma Cat. No. 387A). Giant cells with ≥ 3 nuclei that stained positive were considered osteoclasts.

2.4. Pharmacological inhibition studies

For the antagonist studies, the osteoclastogenesis assay was performed as previously described (Britton et al., 2014) with an additional preincubation step with the inhibitor. Prior to the addition of *L. reuteri* or LA, 1 μ M of GPR40 or GPR120 antagonist was added for 1 h. The fresh medium that was replenished every 2 days contained 1 μ M of GPR40 or GPR120 antagonist. Following 7 days of culture, the cells were fixed, stained for TRAP, and the enumeration of osteoclasts was performed as previously described (Britton et al., 2014).

2.5. Cell viability or metabolic activity assay

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was used to assess cell viability as a function of metabolic activity. This is signified by the NAD(P)H-dependent oxidoreductase enzymatic activity of a cell. In the presence of these enzymes, MTT becomes reduced to formazan, which is an insoluble product possessing chromogenic properties. Briefly, 1×10^4 RAW264.7 cells are seeded on a 96-well culture plate (Cat. No. 07-200-760, Fisher Scientific) and incubated at 37C with 5% CO₂. Following 24 h, the media is exchanged with fresh media and the cells are stimulated with the vehicle control or cell culture supernatant from *L. reuteri*. The cells are co-incubated with the different treatment conditions for 4 and 24 h. Upon completion of each treatment, 0.02 ml of MTT solution (5 mg/ml) dissolved in MEM α + 10% FBS was added to each well and the plate is incubated at 37 °C with 5% CO₂ for 4 h. The plate is washed twice with PBS and then DMSO is added to each well to dissolve the formazan formed. The plate is shaken for 5 min and absorbance readings at 570 nm are taken to measure the amount of metabolic activity for each condition. The data is expressed as a ratio between the specific treatment groups to that of the control group containing untreated cells.

2.6. RNA extraction from RAW264.7 cells and sequencing

RNA extraction was performed using TRIzol Reagent (Cat. No. 15596018, Thermo Fisher Scientific) according to the manufacturer's instructions. Library preparation and sequencing of *Mus musculus* transcriptome was performed by the Baylor Human Genome Sequencing Center. Libraries were prepared from 500 ng of total RNA with the TruSeq Stranded Total RNA Library Prep Kit according to the manufacturer's directions (Illumina). Sequencing was performed on an

Illumina HiSeq 2000 platform. Sequencing data were mapped onto the *Mus musculus* 10 genome available from UCSC Genome Bioinformatics.

2.7. Analysis of RNA sequencing data

On average, there were 31,060,000 high quality bases sequenced per sample with a mean fragment length of 225 bases per read. There was an average of 51,905 transcripts detected per sample. The count table signifying the amount of reads that fell into each gene was generated with using Bioconductor with Rsamtools and GenomicAlignments in R. Differential expression analysis was performed by the DESeq package in R (Anders et al., 2013). Pathway analysis was performed by GAGE in R (Luo et al., 2009).

2.8. Quantitative reverse transcriptase (RT) polymerase chain reaction (qPCR)

Following RNA extraction, cDNA synthesis was performed with Superscript III Reverse Transcriptase (Cat. No. 8080093, Thermo Fisher Scientific). A total of 1 µg of RNA was reverse transcribed. Briefly, an Eppendorf Mastercycler EP S was preheated to 65 °C. The mixture containing RNA, 100 ng of random hexamers, (Cat. No.C1181, Promega) and 1 µl of 10 nM dNTPs (Cat. No. 18427088) was placed into the thermocycler for 5 min. Following 1 min on ice, a mixture containing the reverse transcriptase and RNaseOUT was added to complete the cDNA synthesis. A cycle of 10 min at 25 °C, 50 min at 50 °C, and then 85 °C for 5 min was used. The cDNA was used immediately or stored at -20 °C. The qPCR reactions contained 1 µl of cDNA, 1 µl of each forward and reverse primer (10 µM), 7 µl of nuclease free water, and 10 µl of Power SYBR Green PCR Master Mix (Cat. No. 4367659, Thermo Fisher Scientific). A 2-step PCR amplification protocol was used with acquisition at the annealing and melting curve steps. The protocol included an initial denaturation at 95 °C for 30 s, followed by 40 cycles of denaturing at 95 °C for 10 s, annealing at 51 °C for 20 s. A melting curve was performed at the end at 95 °C for 15 s and ramping up from 60 °C to 95 °C at a rate of +0.2 °C/sec. Data analysis was performed according to the method described by Pfaffl (2001).

2.9. Primer sequences for qPCR

The following primer pairs were used for the RNA sequencing validation studies:

Primers	Forward	Reverse
HPRT	5' GCTATAAAATCTTTGCTGACCTGCT 3'	5' AATTACTTTTATGTCCCTGTTGACTG 3'
NFATc1	5' CATGCGAGCCATCATCGA 3'	5' TGGGATGTGAACTCGGAAGAC 3'
TRAP	5' GACAAGAGTTCCAGGAGACC 3'	5' GGGCTGGGGAAGTTCCAG 3'
MMP9	5' CTGGACAGCCAGACACTAAAG 3'	5' CTCGCGCAAGTCTTCAGAG 3'
CtsK	5' GAAGAAGACTACCAGAAGCAG 3'	5' TCCAGTTATGGCAGAGATT 3'
DC-STAMP	5' GGCTGACGGGAAACCGAGCC 3'	5' ACAGAAGCAGCAGTTGGCCAG 3'
H+ ATPase	5' ACGGTGATGCACAGCAGACGT 3'	5' CCTCTGGATAGAGCTGCCCA 3'

2.10. Western blot analysis

The osteoclastogenesis assay was performed as previously described and proteins were extracted using the commercially available Cell Extraction Buffer (Cat. No. FNN0011, Thermo Fisher Scientific). It was used according to the manufacturer's protocol. Timepoints were taken at 15, 30, 45, and 60 min. Chemiluminescent imaging was performed with the ProteinSimple FluorChem E system. Analysis was performed by comparing densitometry measurements obtained using the ImageJ software package (Girish and Vijayalakshmi, 2004). The following antibodies were used from Cell Signaling Technology: 1) Phospho-NF-κB

p65 (Ser536) (93H1) Rabbit mAb (Cat No. 3033), 2) Phospho-p38 MAPK (Thr180/Tyr182) (D3F9) XP® Rabbit mAb (Cat. No. 4511), 3) p38 MAPK (D13E1) XP® Rabbit mAb (Cat. No.8690), 4) Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (D13.14.4E) XP® Rabbit mAb (HRP Conjugate) (Cat. No. 8544), 5) Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (D13.14.4E) XP® Rabbit mAb (HRP Conjugate) (Cat. No. 8544), 6) Phospho-SAPK/JNK (Thr183/Tyr185) (81E11) Rabbit mAb (Cat. No. 4668), 7) SAPK/JNK Antibody (Cat. No. 9252), 8) β-Actin (13E5) Rabbit mAb (Cat. No. 4970).

2.11. Bone resorption assay

Osteoassay (Cat. No. 3988) plates coated with inorganic crystalline calcium phosphate were purchased from Corning Inc. The osteoclastogenesis assay was performed as previously described. Resorption pits were measured using the NIH imaging software, ImageJ (Girish and Vijayalakshmi, 2004).

2.12. Statistical analysis

The results presented are as means +/- SEM. An unpaired Student's *t* test was used to assess differences between groups. One-way ANOVA analysis was applied when more than 2 groups were compared. The cutoff for significance was $p \leq 0.05$. The post-hoc Tukey HSD test was used as a follow-up to experiments with statistically significant ANOVA results.

3. Results

3.1. *L. reuteri* suppressed osteoclastogenesis in a concentration-dependent manner

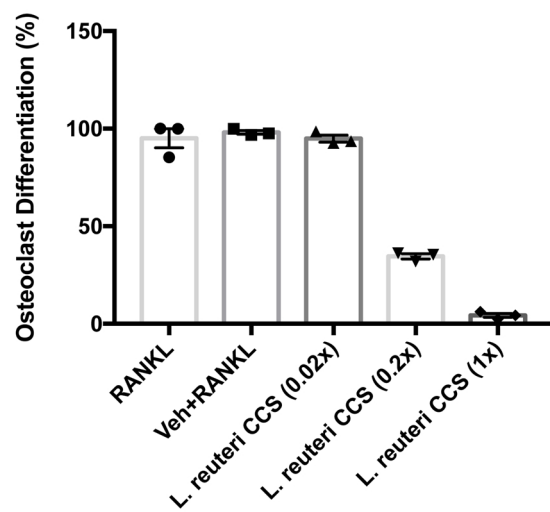
In a previous study we showed that concentrated conditioned supernatant (CCS) from *L. reuteri* inhibits osteoclastogenesis *in vitro* from RAW264.7 cells (Britton et al., 2014). This was also demonstrated in primary murine bone marrow macrophages, as osteoclast differentiation could be suppressed in cells treated with conditioned media from *L. reuteri* (Supplementary Fig. 1). Further characterization of this response was performed using the RAW264.7 cell line. We confirm here that *L. reuteri* CSS inhibited osteoclastogenesis in a concentration-dependent

manner (Fig. 1). We further show specificity of this response by demonstrating that *Escherichia coli* CSS did not affect osteoclast formation in the same cell culture model (Supplementary Fig. 2).

3.2. *L. reuteri* halts osteoclastogenesis at the polykaryon stage

Osteoclast differentiation is a multi-step process consisting of osteoclast precursor activation, fusion, and differentiation before fully mature osteoclasts are developed (Yang et al., 2008a; Yagi et al., 2005; Cuetara et al., 2006; Asagiri and Takayanagi, 2007; Ikeda and Takeshita, 2015). To gain a better understanding at which stage(s) *L.*

A)



B)

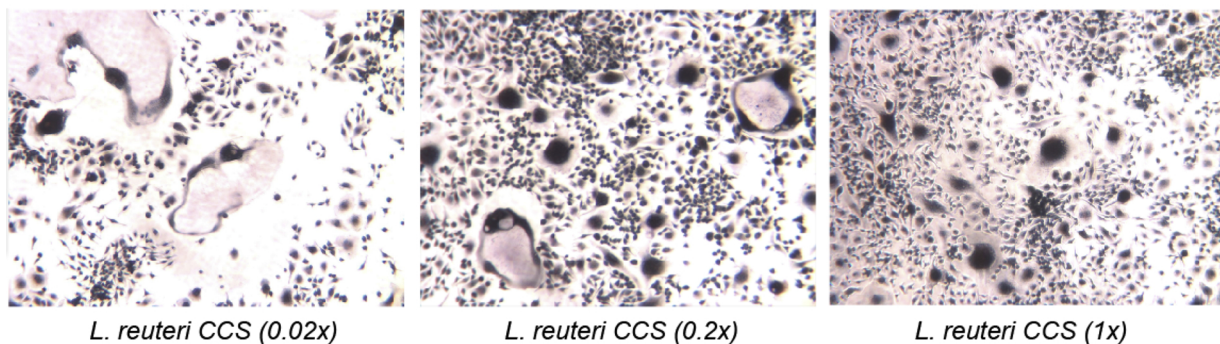


Fig. 1. Dose-dependent inhibition of osteoclast formation by *L. reuteri*. Osteoclast differentiation was induced with the addition of 100 ng/mL RANKL. Giant multinucleated cells that stained positive for TRAP and with ≥ 3 nuclei were considered osteoclasts (A). Light microscopy images (B) were taken and demonstrate an increase in osteoclast formation with decreasing levels of *L. reuteri* CCS used. This was performed three times in total and the reported result is a representative experiment with associated standard deviation, * $p < 0.05$ compared to untreated and MEM- α (vehicle control) conditions as determined by one-way ANOVA.

reuteri CSS inhibits osteoclastogenesis, the phenotypic progression was observed for the duration of the assay by microscopy (Supplementary Fig. 3). During an intermediate stage of osteoclastogenesis, mononuclear preosteoclasts fuse to form giant multinucleated cells known as polykaryons (Teitelbaum et al., 1997; Teitelbaum et al., 1997; Zhang et al., 2000; Weinberg et al., 1984); these cells display cytoskeletal actin rearrangement and increased cytosolic space (Kwon et al., 2015; Aharon and Bar-Shavit, 2006). Interestingly *L. reuteri* treatment resulted in an accumulation of fused polykaryons by days 5 and 7 (Fig. 2a, b) suggesting that differentiation was halted at this stage (Boyle et al., 2003; Brodbeck and Anderson, 2009; Miyamoto et al., 2012).

To test whether the osteoclastogenesis process was delayed rather than inhibited, additional experiments were performed where the duration of the experiment was extended to 17 days. Following the final treatment on day 5, there was a washout period where only fresh medium containing RANKL was replenished without *L. reuteri* CCS treatment. At the end of day 17, cells treated with *L. reuteri* still had suppressed osteoclastogenesis with an accumulation of polykaryons (Fig. 2c). Taken together, these results indicated that *L. reuteri* halted osteoclastogenesis at a fused polykaryon stage and that the effects were long lasting.

3.3. *L. reuteri* suppresses mineral resorption in RANKL-stimulated RAW264.7 cells

To address if inhibition of osteoclast formation by *L. reuteri* at the polykaryon stage impacted osteoclast function, we performed bone resorption assays using Osteoassay plates coated with calcium phosphate to mimic bone resorption. These plates were incubated with RAW264.7 cells for 24 h and they stimulated with RANKL. To assess the impact of *L. reuteri* on calcium phosphate resorption, we further added 0.2X CSS or equivalent vehicle control. Consistent with the ability to suppress osteoclastogenesis, *L. reuteri* CCS significantly decreased the amount of mineral resorption compared to the controls by $\sim 75\%$ (Fig. 3A, B).

3.4. Treatment with *L. reuteri* CCS early during osteoclastogenesis is required for inhibition of osteoclast formation

Because osteoclastogenesis was inhibited at a late stage, we were interested in identifying when CCS treatment was required for inhibition. To test this idea, we performed a time course experiment where

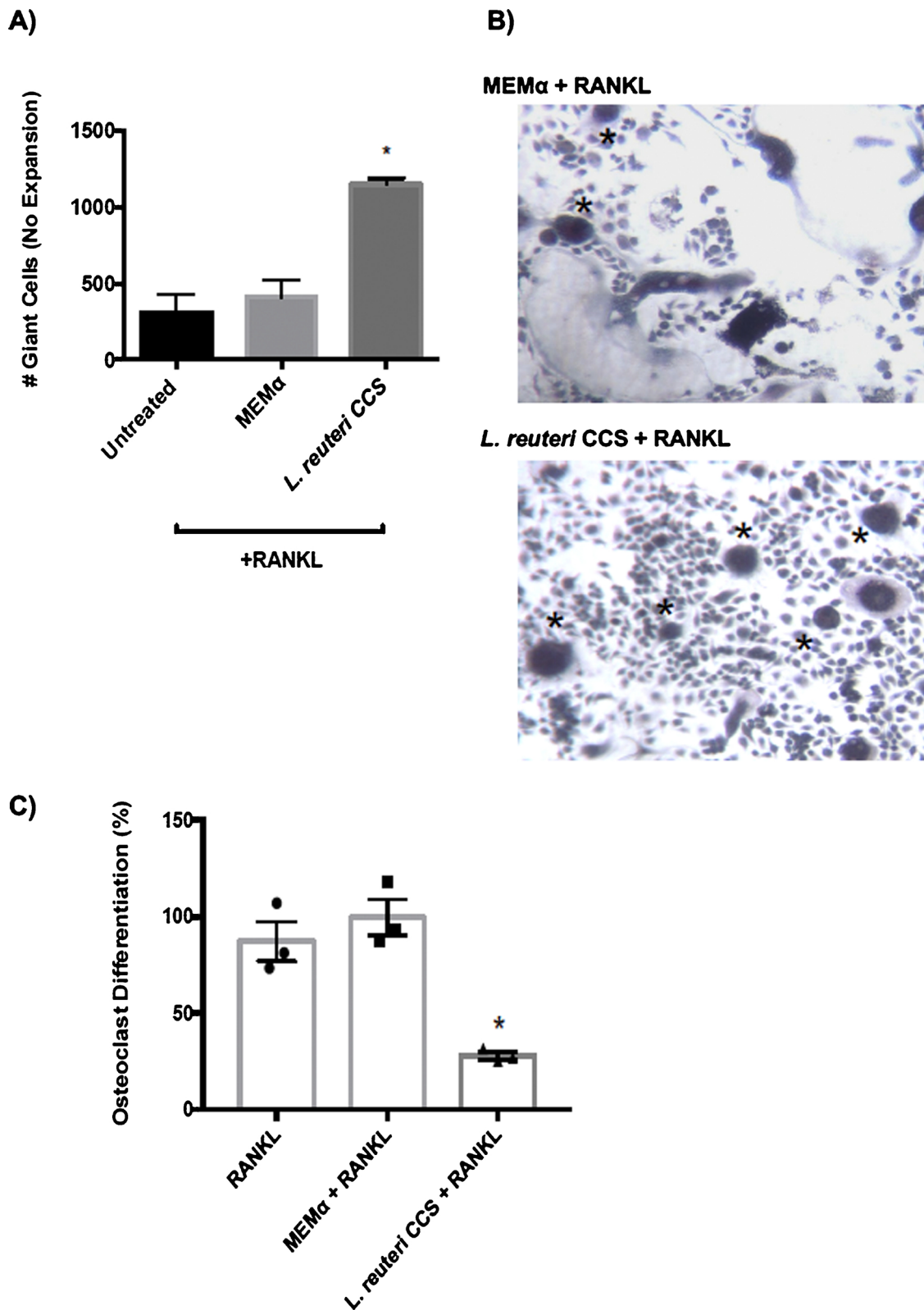


Fig. 2. Progression of osteoclastogenesis over time and accumulation of polykaryons. RAW264.7 cells were stimulated for osteoclast differentiation with RANKL (100 ng/ml) and treated with a vehicle (MEM- α) or *L. reuteri* CCS. (A, B) Fused polykaryons (asterisks) were present in both conditions but *L. reuteri* treatment led to an accumulation of them by day 7. (C) After a 10 day washout period, osteoclast differentiation was still inhibited in cells treated by *L. reuteri* CCS at day 17. * $p < 0.05$ compared to untreated and MEM- α (vehicle control) conditions as determined by one-way ANOVA. $n = 3$.

RAW264.7 cells were stimulated by RANKL for osteoclast differentiation and treated with *L. reuteri* 0.2X CCS throughout the duration of the assay (Fig. 1A) or added just once on day 1, 3, or 5. The addition of *L.*

reuteri CCS on day 1 alone inhibited osteoclast differentiation while treatment at day 3 ($p = 0.07$) or day 5 had only a modest to no impact on osteoclastogenesis (Fig. 4). These results, coupled with the

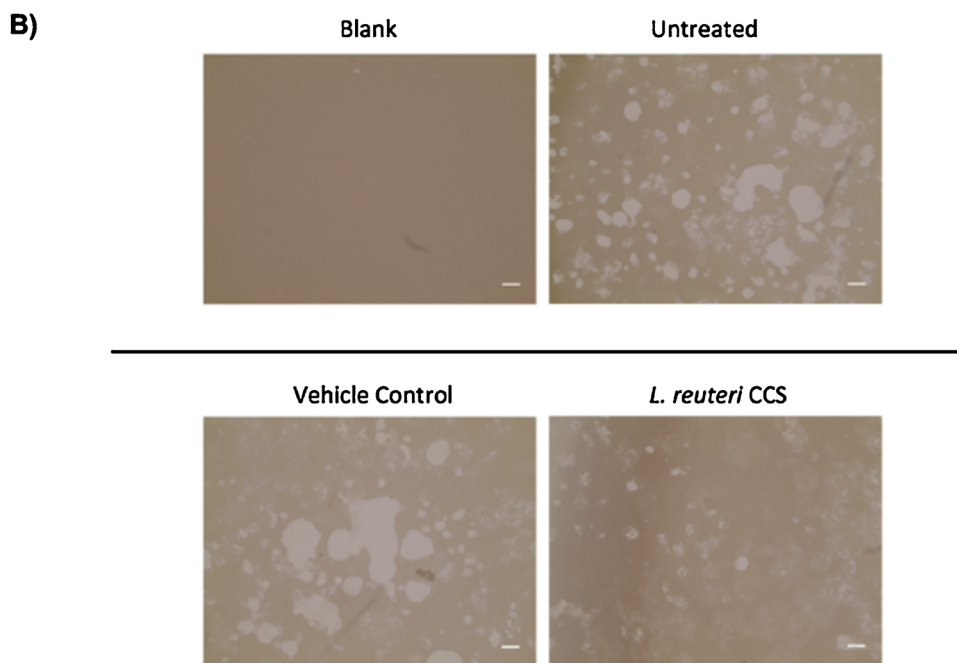
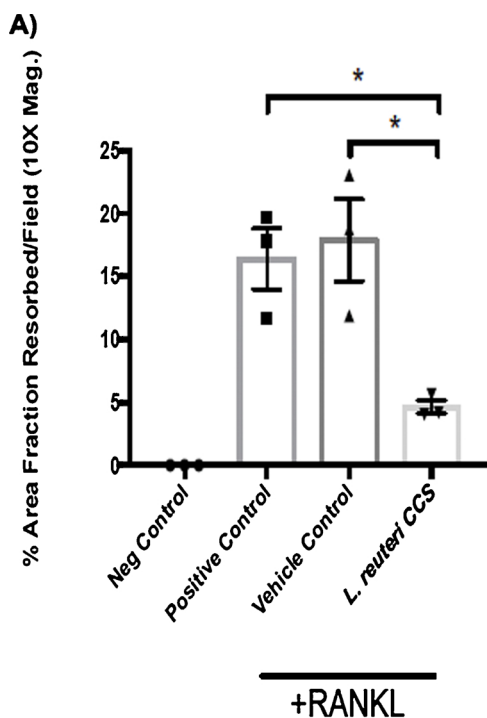


Fig. 3. Suppression of mineral resorption by *L. reuteri*. RAW264.7 cells were plated for on Osteoassay plates for 1 day and then stimulated concurrently with RANKL (100 ng/ml) and either the vehicle control (MEM- α) or *L. reuteri* CCS. Fresh medium was replenished every 2 days. The analysis was performed using the Image J software package to measure densitometry. (A) *L. reuteri* CCS significantly decreased the amount of absorption from calcium and phosphate coated plates. (B) The microscopy images also support that less absorption had taken place in the presence of *L. reuteri* CCS. Three biological replicates are depicted with associated standard error of mean, * $p < 0.05$ compared to untreated and MEM- α (vehicle control) conditions as determined by one-way ANOVA. Scale bars signify 100 μ m increments. n = 3.

phenotypic studies (Fig. 2b, c), suggested that *L. reuteri* targeted an early signaling event during osteoclastogenesis that resulted in halting development at the late polykaryon stage.

3.5. Lactobacillic acid is involved in *L. reuteri* suppression of osteoclastogenesis

It was previously shown that the ability of *L. reuteri* to suppress TNF- α production from a human monocyte line was related to the presence of lactobacillic acid (LA), a long chain cyclopropyl fatty acid (Jones et al., 2011). Other long chain fatty acids have been shown to suppress in vitro osteoclastogenesis (Kwon et al., 2015; Drosatos-Tampakaki

et al., 2014). Therefore, to test if LA is able to directly inhibit osteoclastogenesis, RAW264.7 macrophages were incubated without or with varying concentrations of LA, during osteoclastogenesis. Our results demonstrate that LA caused a concentration-dependent inhibition of osteoclast formation (Fig. 5a). A concentration of 1 μ M LA was sufficient to suppress osteoclast formation by 50% whereas 10 μ M suppressed 80%. These result show that LA is important for the inhibition of osteoclast formation.

LA is formed from vaccenic acid by the action of the enzyme cyclopropyl fatty acid synthase that is encoded by the *cfa* gene in *L. reuteri* 6475. To directly test whether *L. reuteri* CCS inhibits osteoclastogenesis via LA, we used a *L. reuteri* Δ *cfa* mutant, in which the cyclopropyl fatty

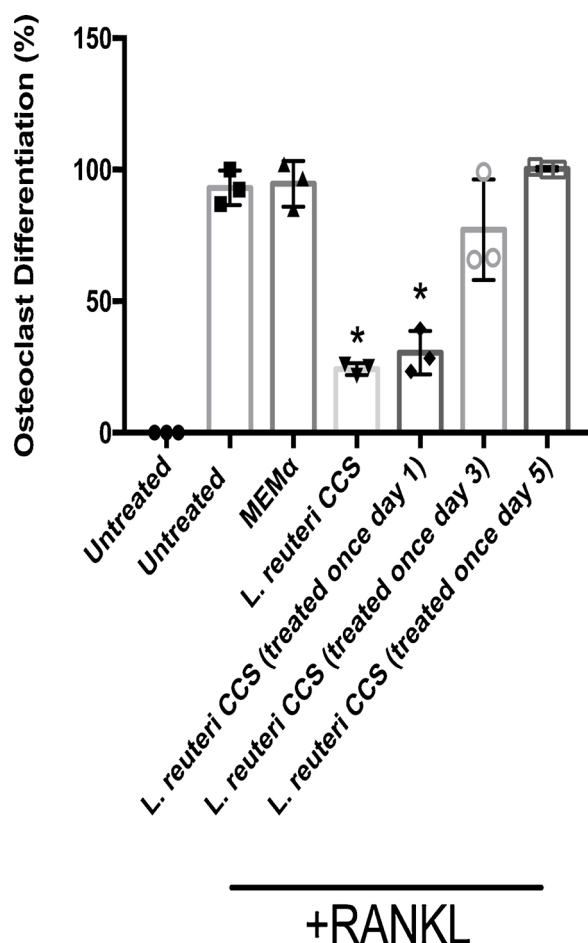


Fig. 4. Impact of *L. reuteri* CCS on osteoclast differentiation at different time points. RAW264.7 cells were plated and osteoclast differentiation was induced with the addition of 100 ng/mL RANKL. *L. reuteri* CCS was added normally when the media was replenished (days 1, 3, and 5) or just once on day 1, 3, or 5. After 7 days, the number of giant multinucleated (≥ 3 nuclei) cells staining positive for TRAP were quantified. Treatment by *L. reuteri* CCS on day 1 was sufficient to suppress osteoclastogenesis. Three biological replicates are depicted with associated standard error of mean, * $p < 0.05$ compared to untreated and MEM- α (vehicle control) conditions as determined by one-way ANOVA.

acid synthase (*cfa*) gene in disrupted and is unable to produce LA (Jones et al., 2011). Interestingly, we found that CCS from the Δcfa mutant was less effective (by more than 50%) in suppressing osteoclastogenesis in comparison with WT *L. reuteri* (Fig. 5b). Though, the *L. reuteri* Δcfa mutant retained some ability to suppress osteoclastogenesis.

To identify possible mechanisms by which LA produced by *L. reuteri* mediates anti-osteoclastogenic effects, we focused on the free fatty acid receptor 4 (FFAR4; aka GPR120), a G protein-coupled receptor that has been shown to be a receptor for long chain fatty acids (Oh et al., 2014; Oh et al., 2010). Interestingly, activation of GPR120 in RAW264.7 cells and bone marrow macrophages suppresses osteoclastogenesis (Kim et al., 2015). Therefore, we hypothesized that LA from *L. reuteri* CCS inhibits osteoclastogenesis potentially via GPR120 activation. To test this, we induced osteoclast differentiation of RAW264.7 cells and treated them with *L. reuteri* CCS in the absence or presence of a GPR120 antagonist (AH7614). Inhibition of GPR120 with 1 μ M AH7614 completely abolished the ability of *L. reuteri* CCS to inhibit osteoclast formation (Fig. 6a). GPR40 is another receptor for long chain fatty acids and has been shown to have a positive impact on bone health (Covington et al., 2006; Wauquier et al., 2013). Blocking GPR40 signaling with an antagonist (DC260126) however, did not impact the

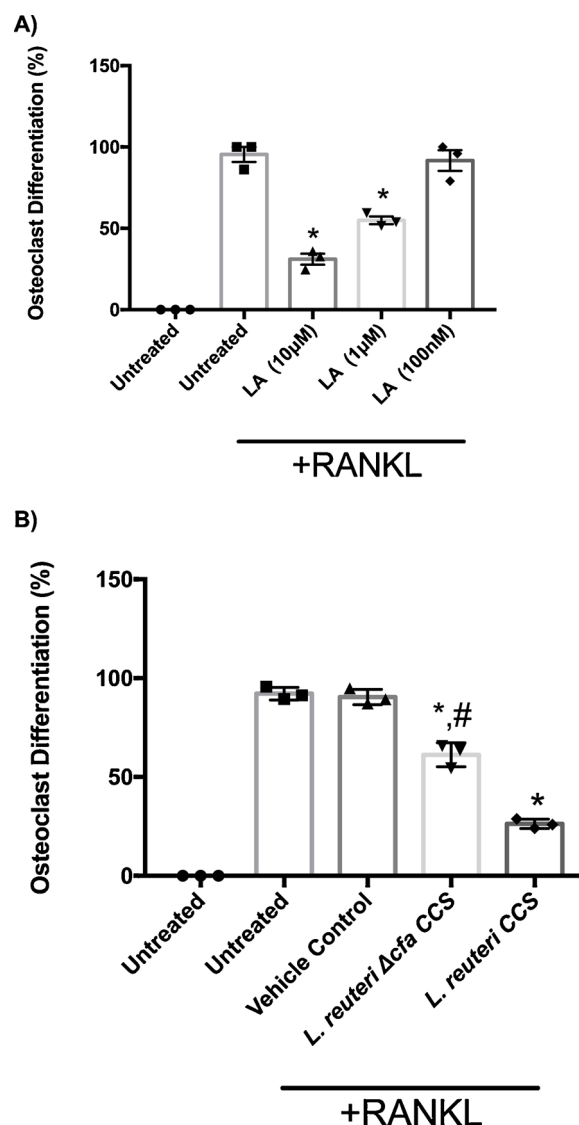


Fig. 5. Lactobacillic acid (LA) involved with the suppression of osteoclastogenesis. RAW264.7 cells were plated and osteoclast differentiation was induced with the addition of 100 ng/mL RANKL. (A) Dose-dependent suppression of osteoclastogenesis was observed with LA at the concentrations of 10 μ M, 1 μ M, and 100 nM. (B) A mutant unable to produce LA in the *L. reuteri* genetic background was not as effective as the WT strain in suppressing osteoclastogenesis. Three biological replicates are depicted with associated standard error of mean, * $p < 0.05$ compared to untreated and MEM- α (vehicle control) conditions, # $p < 0.05$ compared to *L. reuteri* as determined by one-way ANOVA.

ability of *L. reuteri* to suppress osteoclastogenesis.

To directly demonstrate LA inhibition of osteoclast formation occurs via GPR120, we induced osteoclastogenesis of RAW 264.7 cells in the absence or presence of AH7614 (GPR120) antagonist as well as LA. Again, we observed that 1 μ M LA reduced osteoclast formation by 50% and that addition of AH7614 was able to partially block this suppression (Fig. 6b). However, it is worth noting that attempts to increase the concentration of the antagonist past 2.5 μ M resulted in cellular toxicity. Nevertheless, these results support the hypothesis that *L. reuteri* inhibits osteoclastogenesis via LA stimulation of GPR120.

3.6. Transcriptomic profiling of RAW264.7 cells during *L. reuteri* treatment supports the suppression of osteoclastogenesis

To gain a better understanding of how *L. reuteri* impacts

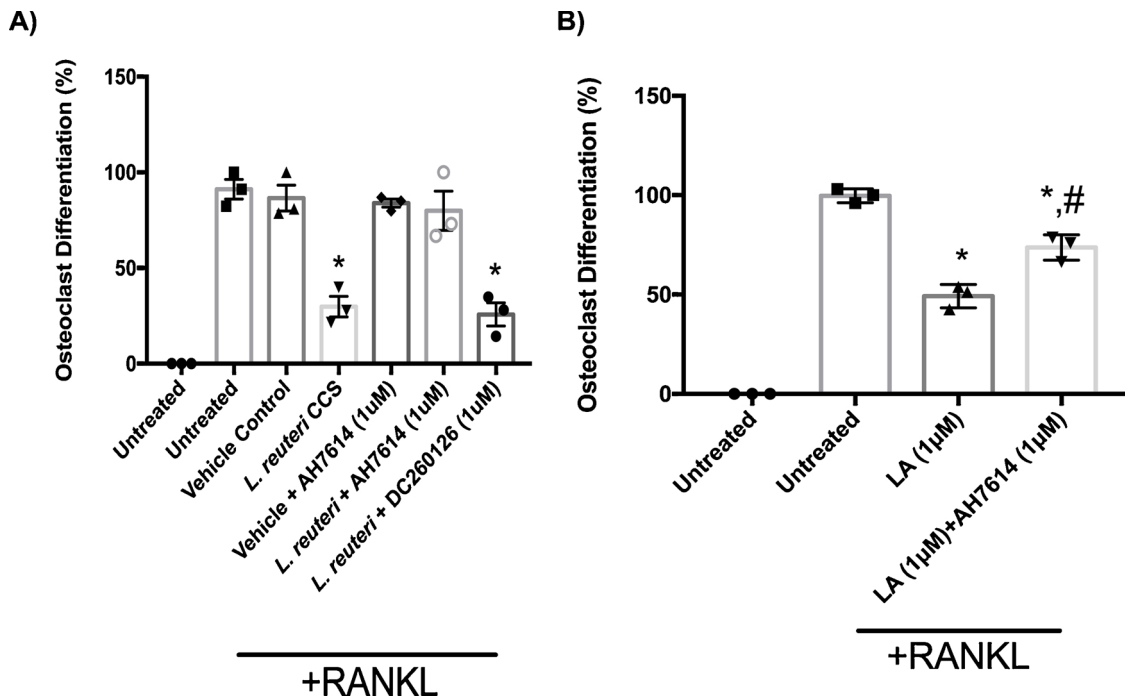


Fig. 6. Suppression of osteoclastogenesis by *L. reuteri* was mediated through GPR120 signaling. RAW264.7 cells were plated. After 24 h, the cells were pretreated with 1 µM of AH7614 for 1 h. Then, osteoclast differentiation was induced with the addition of 100 ng/mL RANKL. Cell differentiation medium (containing RANKL and the inhibitor) was replenished every 2 days. (A) The presence of the inhibitor attenuated the suppression of osteoclastogenesis by *L. reuteri*. (B) Pharmacological inhibition of GPR120 decreased the ability of LA to suppress osteoclast formation. Three biological replicates are depicted with associated standard error of mean, **p* < 0.05 compared to untreated and MEM-α (vehicle control), #*p* < 0.05 compared to LA conditions as determined by one-way ANOVA.

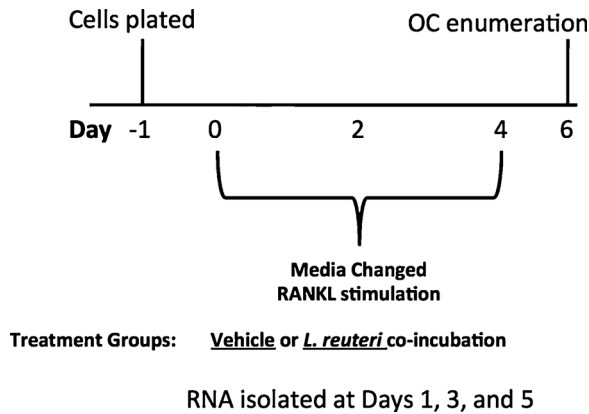


Fig. 7. Experimental layout for gene expression analysis. RAW264.7 cells were plated for 1 day and then stimulated concurrently with RANKL (100 ng/ml) and either the vehicle control (MEM-α) or *L. reuteri* CCS. RNA was extracted at days 1, 3, and 5.

osteoclastogenesis, we performed an RNA sequencing experiment to measure the levels of gene expression from the entire transcriptome at days 1, 3 and 5 after induction of osteoclast formation by RANKL. Cells were also treated with the vehicle control or *L. reuteri* CCS, with biological replicates for each condition at each timepoint (Fig. 7). Differential gene expression analysis was performed using the Bioconductor software package DESeq2 (Love et al., 2014). Using a fold change cutoff of 3, we observed 2,993 genes were differentially regulated by *L. reuteri* in comparison to the vehicle control when combining all time points (Table 1). However, there were substantially more genes differentially regulated at the day 3 and 5 timepoints.

The gage software package was used to gain a better understanding of pathways that were differentially regulated by *L. reuteri* (Luo et al., 2009). The cutoff for statistical significance was determined using a *q*

Table 1

Genes with significantly changed expression between vehicle and *L. reuteri* treatment (Fold change > 3). V denotes Vehicle (MEM-α), T denotes treatment, and the numbers signify time points day 1, 3, and 5.

	V1T1	V3T3	V5T5
Up-regulated	24	1334	1635
Down-regulated	21	3097	2611

Table 2

Pathways differentially regulated between vehicle and *L. reuteri* treatment. V denotes Vehicle (MEM-α), T denotes treatment, and the numbers signify time points day 1, 3, and 5.

	V1T1	V3T3	V5T5
Up-regulated	4	2	4
Down-regulated	4	43	41

value < 0.05 (*p value* that takes into account the false discovery rate based off of multiple comparisons). Similar to the gene expression data, more KEGG pathways were differentially regulated by *L. reuteri* at days 3 and 5 with 90 out of the total 98 pathways being significant at those 2 time points (Table 2).

At day 1, few changes were observed. There was an activation of 4 pathways and down-regulation of 4 pathways, but none that were involved with osteoclast differentiation (Supplementary Table 1). Out of the up-regulated pathways, one was associated with innate immunity (Complement and coagulation cascades, mmu04610) and another associated with the detection of fatty acids (PPAR signaling pathway, mmu 03320). Notably, the down-regulated pathways suggested that cell cycle and/or proliferation were affected by *L. reuteri* treatment (Supplementary Table 1). However, light microscopy and trypan blue exclusion staining did not reveal any discernible phenotypic or viability differences (Fig. 5). Additionally, an MTT assay was utilized to detect

metabolic activity and no differences were observed up to 3 days following *L. reuteri* treatment (data not shown).

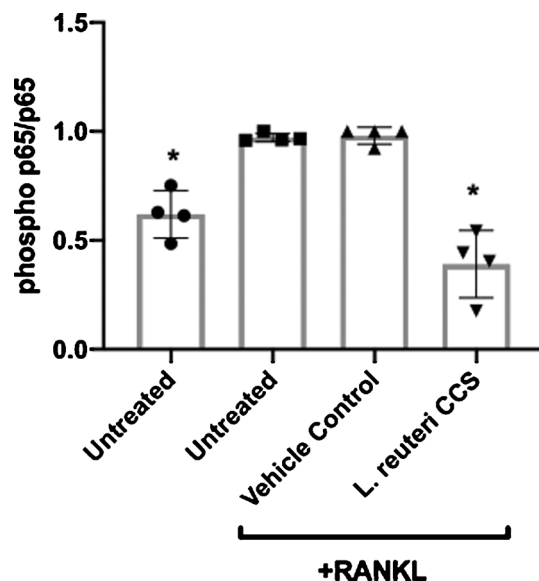
By day 3, there were 2 pathways that were up-regulated and 43 that were down-regulated (Supplementary Table 2). Notable pathways that were down-regulated included osteoclast differentiation (mmu04380), NF-kappa B signaling (mmu04064), MAPK signaling (mmu04010), and TNF signaling (mmu04668). Pathway analysis from the day 5 timepoint suggested that the majority of the processes being impacted involved cellular metabolism and immune signaling (Supplementary Table 3). Consistent with day 3, osteoclast differentiation, NF-kappa B signaling, MAPK signaling, and TNF signaling were down-regulated.

To validate the data set, we performed quantitative PCR analysis of genes that were well characterized in the osteoclast differentiation pathway. The genes measured were *NFatC1*, *TRAP*, *MMP9*, *CtsK*, *DC-STAMP*, and *ATP6v0d2* (Zhang et al., 2008; Yang et al., 2008b). For all the genes tested, the RNA sequencing results closely paralleled the quantitative PCR results (Supplementary Fig. 4). Taken together with previous studies showing that *L. reuteri* has immunomodulatory activity, the transcriptional analysis suggests that immune signaling may be targeted to impact osteoclastogenesis (Jones et al., 2011; Iyer et al., 2008; Thomas et al., 2012).

3.7. Activation of NF- κ B and p38 in RAW264.7 cells was inhibited by *L. reuteri*

Based on the RNAseq results, we tested the impact of *L. reuteri* CCS treatment during osteoclastogenesis on NF- κ B activation. RANKL initiates a cascade of intracellular signaling that culminates in the activation of NF- κ B that is essential for osteoclastogenesis (Strait et al., 2008; Boyce et al., 2015). We observed that RANKL treatment of RAW264.7 cells resulted in the induction of NF- κ B/p65 phosphorylation and treatment with *L. reuteri* CCS significantly inhibited this stimulation (Fig. 8). This was consistent with the transcriptional analyses that indicated the NF- κ B signaling pathway being down-regulated by *L. reuteri*.

Mitogen-activated protein kinases (MAPK) signaling pathways have also been shown to be important for various facets of host physiology such as the immune response and cellular metabolism (Eriksson et al., 2006; Li et al., 2015; Arthur and Ley, 2013; Alam et al., 2015).



Additionally, their role in osteoclast differentiation has also been heavily studied (Sharma et al., 2007; Huang et al., 2006; Ihn et al., 2015; Nie et al., 2016; Chen et al., 2016). To corroborate our transcriptomics findings, we performed Western blot analysis to assess the impact of *L. reuteri* on MAPK signaling. The MAPKs p38, ERK, and JNK were activated with RANKL and treated with the vehicle control or *L. reuteri* CCS for 15 min. Interestingly, our results suggested that while ERK and JNK were unaffected by *L. reuteri* treatment, p38 activation is inhibited (Fig. 9). Taken together, our data suggests that *L. reuteri* impacts NF- κ B and p38 to suppress RANKL-induced differentiation of RAW264.7 cells.

4. Discussion

Recent work has indicated that probiotics and the microbiota have a key role in regulating bone health. Not only do probiotics inhibit bone loss in localized inflammatory reactions such as bone infections or periodontitis, but they have also been shown to regulate bone mass from a distal site such as the gut (Britton et al., 2014; Ohlsson et al., 2014; Maekawa and Hajishengallis, 2014). Despite establishing this link between gut bacteria and bone health, our mechanistic understanding of how gut bacteria regulates bone health is only beginning to emerge. In previous studies, our group has shown that *L. reuteri* can protect against bone loss or accelerate bone formation under different scenarios using a mouse model of estrogen deficiency, an inflammatory model mediated by dorsal surgical incision, and under healthy conditions in a male murine model (McCabe et al., 2013; Britton et al., 2014; Collins et al., 2016). Under estrogen deficiency, the data suggests that *L. reuteri* can protect against bone loss by inhibiting osteoclast activity and bone resorption, and altering the gut microbiota (Britton et al., 2014). In healthy male mice, *L. reuteri* treatment lead to decreased intestinal inflammation and increased osteoblast activity and bone formation (McCabe et al., 2013). Lastly, under inflammatory conditions mediated by dorsal surgical incision in female mice, *L. reuteri* treatment also lead to decreased intestinal inflammation and increased levels of bone formation in the distal femur metaphyseal trabecular region (Collins et al., 2016). Given that *L. reuteri* has been shown to improve bone health in several different ways, it is likely that targeting the process of osteoclast differentiation is just one of several probiotic effects mediated by *L.*

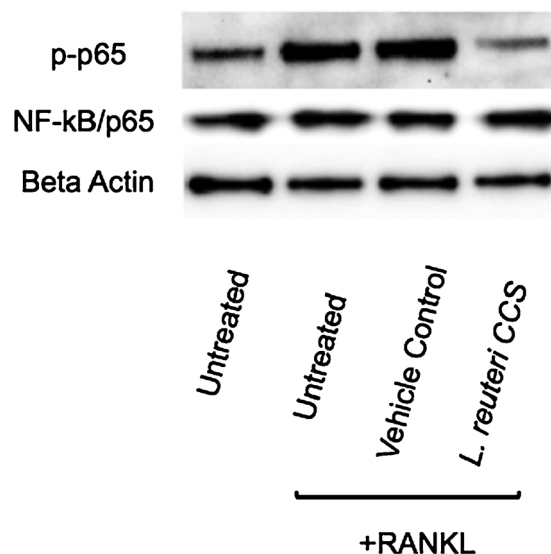


Fig. 8. Effect of *L. reuteri* on RANKL-induced NF- κ B/p65 phosphorylation. RAW264.7 cells were concurrently treated with RANKL (100 ng/ml) and MEM- α or *L. reuteri* CCS for 60 min. Total intracellular contents were prepared and an equal amount of protein was analyzed. Western blot analysis using antibodies to the phosphorylated NF- κ B/p65 subunit was performed. Band intensity was measured by densitometry using the ImageJ software package. Results were normalized to levels of total p65. Four biological replicates are depicted with associated standard error of mean, * $p < 0.05$ compared to untreated and MEM- α (vehicle control) conditions as determined by one-way ANOVA.

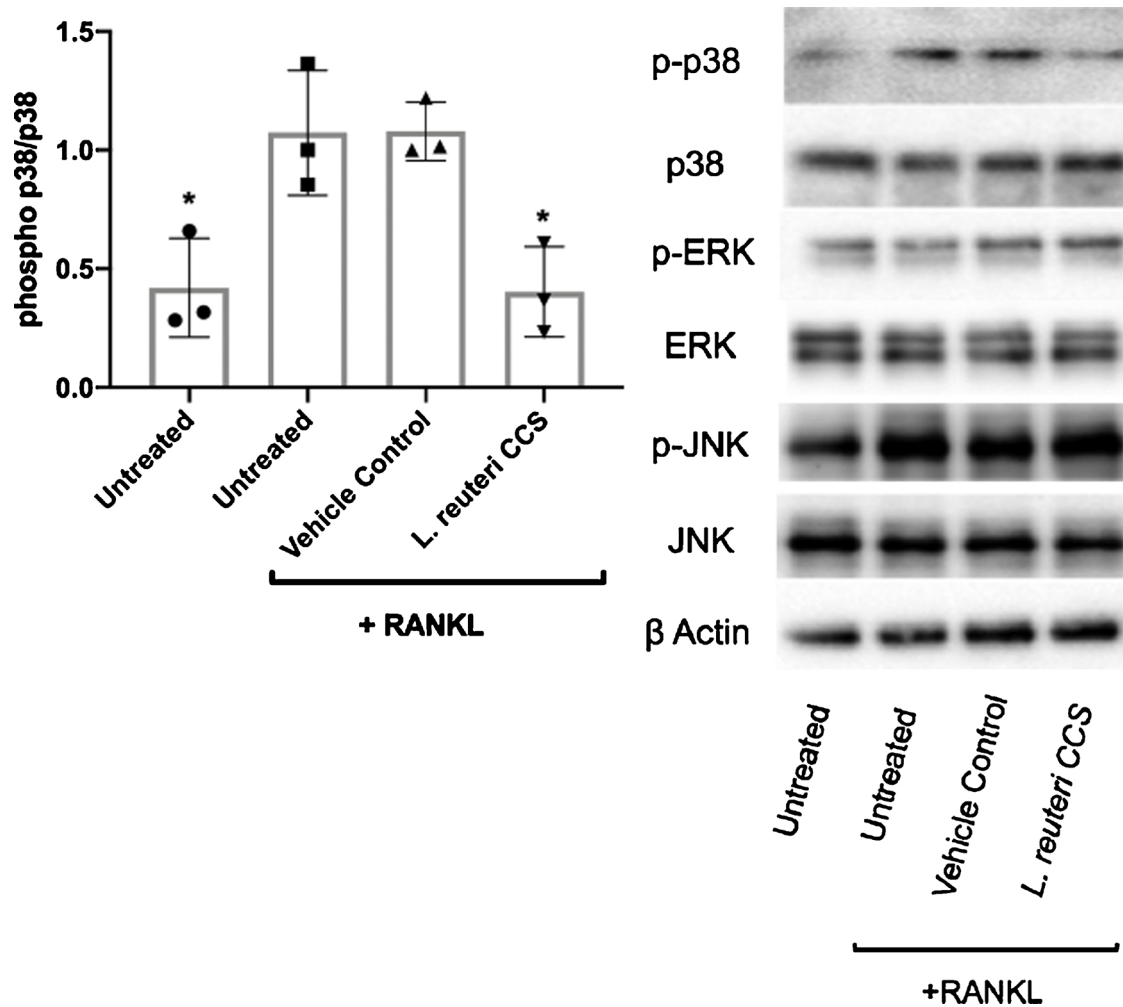


Fig. 9. Impact of *L. reuteri* on MAPK signaling. RAW264.7 cells were concurrently treated with RANKL (100 ng/ml) and MEM- α or *L. reuteri* CCS for 60 min. Total intracellular contents were prepared and an equal amount of protein was analyzed. Western blot analysis using antibodies to the p38, phosphorylated p38, ERK, phosphorylated ERK, JNK, phosphorylated JNK. Band intensity was measured by densitometry using the ImageJ software package. Results were normalized to levels of total p38. Three biological replicates are depicted with associated standard error of mean, * $p < 0.05$ compared to untreated and MEM- α (vehicle control) conditions as determined by one-way ANOVA.

reuteri supplementation. This study aimed at further characterizing this response and identifying the factors involved.

Our results indicated that *L. reuteri* conditioned medium contains factor(s) that targeted an early stage of osteoclastogenesis since a single treatment with *L. reuteri* at the beginning of RAW264.7 cell culture was able to suppress RANKL-induced osteoclastogenesis by 75%. However, phenotypic changes were not apparent until days 5 and 7 where *L. reuteri* treatment resulted in an accumulation of fused polykaryons. A majority of the changes in the transcriptional profile also were apparent later in the assay (days 3 and 5) as very few genes were differentially regulated by *L. reuteri* at the day 1 time point. Although, it is worth noting that the transcriptional differences evident in later time points would be more pronounced as cells that were not treated by *L. reuteri* CCS would be farther down the path of osteoclastogenesis as compared to the treatment group. Together, the results indicated that while early stimulation by *L. reuteri* did not impact initial phenotypic changes, the early transcriptional changes that were present likely play a significant role in the suppression of osteoclastogenesis and arrest in a state of fused polykaryons. As a result, we focused our attention on early signaling pathways involved in osteoclastogenesis.

Pathway analysis revealed 3 pathways up-regulated by *L. reuteri* that may account for its activity in suppressing osteoclastogenesis. First, it has been previously shown that the complement cascade plays a role in

bone metabolism. For example, C3a has been demonstrated to stimulate osteoblast formation (Matsuoka et al., 2014), while other studies have shown the importance of C3 in osteoclast formation (Ignatius et al., 2011; Tu et al., 2010). It is possible that the early promotion of monocyte fusion ultimately leads to the suspension in the polykaryon stage. The Jak-STAT pathway was another early pathway that was up-regulated by *L. reuteri* CCS treatment. It was previously shown that STAT3 activation was important for RANKL-induced osteoclastogenesis (hong Li et al., 2013; Li, 2013), while the phosphorylation and activation of STAT6 resulted in the inhibition of NFATc1 (Yamada et al., 2007). Additionally, the PPAR signaling pathway was also up-regulated by *L. reuteri* treatment. While PPAR- γ signaling has been shown to promote osteoclastogenesis and decreased bone mass under certain circumstances (Zou et al., 2016; Duque et al., 2013), activation of PPAR signaling has also been shown to inhibit osteoclastogenesis (Kasonga et al., 2019; Hounoki et al., 2008; Cho et al., 2012). These results, coupled with the analysis of decreased p65 and p38 signaling activation after *L. reuteri* treatment, suggested that these pathways are involved with the mediated suppression of osteoclastogenesis by *L. reuteri*.

The next issue we addressed was whether this inhibition of osteoclastogenesis by *L. reuteri* was a direct or indirect effect. It has been shown that osteoclast differentiation can be impacted indirectly by preventing the interaction between RANKL and its receptor, RANK

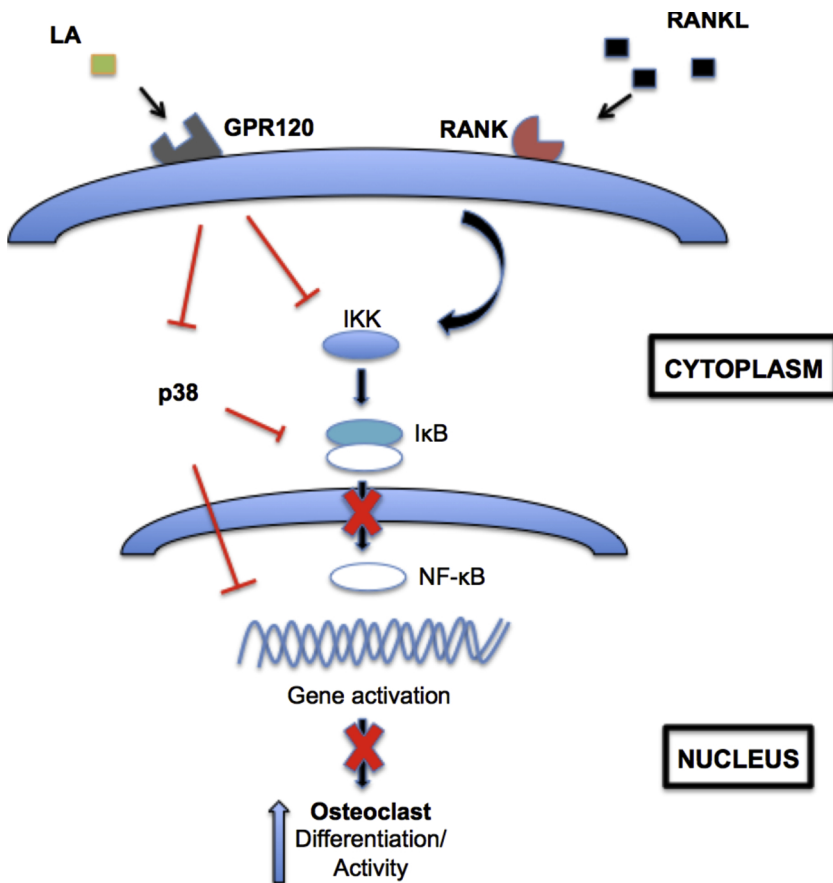


Fig. 10. Working model of osteoclastogenesis suppression by *L. reuteri* and LA. RAW264.7 cells are stimulated for osteoclastogenesis by RANKL. *L. reuteri* and LA activated GPR120 or a receptor yet to be identified to suppress osteoclastogenesis. GPR120 receptor antagonism partially inhibited the ability of *L. reuteri* and LA to suppress osteoclastogenesis. MAPK and NF- κ B signaling has been shown to be downstream of GPR120 signaling and we demonstrated that *L. reuteri* impacts several arms of these pathways.

(Zhao et al., 2015). However, the time course study suggested that *L. reuteri* does not interact with RANKL as an early pulse of RAW264.7 cells with CCS was sufficient to prevent osteoclast formation (Fig. 3). One potential candidate is lactobacillic acid (LA, also known as phytomonic acid), which is a long chain fatty acid containing a cyclopropane ring. LA is produced by *L. reuteri* and a mutant defective in the production of LA is deficient in the suppression of TNF- α production from a human monocyte cell line (Jones et al., 2011). The immunomodulatory activity of this strain contributed towards strain selection in the original bone health studies in the OVX osteoporosis model since TNF- α plays such an important role in promoting osteoclastogenesis and subsequent bone loss during estrogen deficiency (Kimble et al., 1997; Cenci et al., 2000; Srivastava et al., 1999; Weitzmann and Pacifici, 2006). LA is a 19-carbon long chain cyclopropane fatty acid derived from the conversion of vaccenic acid by cyclopropane fatty acid synthase (Jones et al., 2011). Dihydrostercolic acid, another CFA, is also present in *L. reuteri* and is derived from oleic acid. Interestingly, oleic acid has been shown to suppress osteoclastogenesis (Drosatos-Tampakaki et al., 2014; Cornish et al., 2008). Other fatty acids have also been shown to inhibit osteoclastogenesis through the inhibition of NF- κ B activation, which is a crucial signaling event in osteoclast differentiation (Rahman et al., 2006; Rahman et al., 2011; Zwart et al., 2010). However, LA has yet to be studied in this context and warranted further investigation. We demonstrated that LA impacted osteoclastogenesis in two ways (Fig. 5). Using purified LA, we obtained a concentration-dependent inhibition of osteoclastogenesis. Additionally, CCS isolated from a mutant strain of *L. reuteri* incapable of producing LA was not as effective in suppressing osteoclastogenesis. Taken together, these results indicate that exogenous LA directly impacts osteoclastogenesis and its presence in *L. reuteri* contributes to its suppression of osteoclast differentiation. However, *L. reuteri* still maintained partial inhibition of osteoclastogenesis despite the absence

of LA, suggesting that other bacterial product(s) that have not been identified also contribute to this process.

While LA production is not exclusive to *L. reuteri*, its presence in bacteria and potential for bone health suggests an evolutionary relationship that may elucidate the purpose of receptors that detect fatty acids of different carbon chain lengths. G-protein-coupled receptors (GPCR) signaling systems have been evolutionarily conserved over time (Krishnan and Schiöth, 2015; Corrêa-Oliveira et al., 2016; Mushegian et al., 2012) and their importance in regulating diseases such as cancer, obesity, and development has been the topic of much research (Oh et al., 2014; Oh et al., 2010; Marivin et al., 2016; Yu and Brown, 2015). More recently, the roles of the long chain fatty acid receptors, GPR40 and GPR120, have been shown to be important in bone health and regulating osteoclastogenesis (Kim et al., 2015; Wauquier et al., 2013; Philippe et al., 2016; Ahn et al., 2016). Although studies have demonstrated the potential impact of long chain fatty acids on bone health, LA has yet to be investigated in the same context and remains a promising candidate as a novel therapeutic (Bonnet and Ferrari, 2011; Chen et al., 2013). In our studies, we established that GPR120, but not GPR40, was important for the suppression of osteoclastogenesis by *L. reuteri* or LA. Future studies directed at testing the impact of LA on *in vivo* osteoclastogenesis and bone density will be crucial for determining whether it can potentially be a novel therapeutic for bone health.

In the present study, we have established that early signaling events in osteoclastogenesis are targeted by *L. reuteri*. Additionally, we have uncovered a role for the fatty acid LA in osteoclastogenesis. In addition to suppressing osteoclastogenesis, we also demonstrated that *L. reuteri* inhibited osteoclast function, as indicated by reduced mineral resorption of calcium and phosphate coated surfaces. Together, our studies suggest that *L. reuteri* impacts osteoclast differentiation by targeting p38 and NF- κ B activation. LA produced by *L. reuteri* is important for this inhibition and GPR120 signaling also plays an important role in the

suppression of osteoclastogenesis (Fig. 10). However, there are many interesting observations that are made. Since osteoclast differentiation is not completely suppressed using a mutant deficient in LA production, another mechanism mediated by *L. reuteri* is suggested to be involved. Moreover, the fact that inhibition of GPR120 was unable to completely block the ability of LA to suppress osteoclast formation either suggests that LA has another target receptor or the concentration of LA used could overcome inhibition by AH7614. As previously mentioned, increasing the concentration of AH7614 past 2.5 μM lead to cellular toxicity. Thus, the concentration of 1 μM was used for the studies possibly rendering it not 100% effective.

5. Conclusion

With the emergence of studies demonstrating the beneficial impact that gastrointestinal microbes can have on bone health, additional studies will be required to validate its true therapeutic potential. The pathogenesis of bone diseases is multifactorial, spanning influences from several organ systems, and a better understanding of the interactions between the different systems will be crucial for this purpose. Specifically, when it comes to osteomicrobiology, the increasing use of genetically tractable organisms in murine models of disease will aid in uncovering potential probiotic mechanisms of action. Moreover, prospective clinical trials establishing the efficacy of probiotics to promote bone health in humans are also warranted. Novel treatment options that take advantage of microbes that co-evolved and co-existed with humans may develop as we continue filling in these gaps of knowledge.

Data

The RNAseq data reported in this manuscript has been deposited in NCBI's Gene Expression Omnibus (GEO) database. Accession number: [GSE135755](https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE135755).

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Declaration of Competing Interest

The authors report that they have no competing interests.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.bonr.2019.100227>.

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