Extracellular Proteins Isolated from *L. acidophilus* as an Osteomicrobiological Therapeutic Agent to Reduce Preformed Pathogenic Biofilm, Regulate Chronic Inflammation, and Augment Bone Formation *In Vitro*

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Introduction. Periprosthetic joint infection (PJI) is a challenging complication that can occur following joint replacement surgery and is one of the leading causes of total knee, and total hip arthroplasty failure. Further, the emergence of antimicrobial resistance exacerbates the difficulty in treating PJI. As the demand for primary and revision joint surgeries continues to rise at an accelerated rate, an increasing future PJI burden is anticipated, which will have significant healthcare and economic implications. However, efficacious approaches to prevent and treat PJI and its recurrence remain elusive, predominantly due to bacterial adhesion, and subsequent biofilm formation on the implant and surrounding tissue surfaces. Commensal bacteria within the gut microbiota are reported to convey beneficial effects by contributing to a defense named "colonization resistance"; a strategy against pathogenic infection and mucosal overgrowth, where dense, stable, and diverse microbial communities occupy an array of niches along the length of the surface of the intestinal tract. Colonization-regulating activities involve microbe-microbe, and microbe-host interactions, inducing competition over nutrients, inhibition by antimicrobial peptides, stimulation of the host immune system, and the promotion of mucus and intestinal epithelial barrier integrity. This blueprint may be applicable to PJI. Here, the aim was to investigate *Lactobacillus acidophilus spp.* and primarily, isolated extracellular-derived *L. acidophilus* proteins (LaEPs) on PJI relevant *S. aureus*, MRSA, and *E. coli* planktonic growth and biofilm disassembly. The effect of LaEPs on macrophages and osteogenic, and adipogenic hBMSC differentiation was also analyzed.

Methods. *L. acidophilus* (ATCC 4356, Manassas, VA, USA) was cultured in de Man, Rogosa and Sharpe (MRS) broth (BD Difco Laboratories, NJ, USA). *Staphylococcus aureus* (*S. aureus*) ATCC 6538, methicillin resistant *S. aureus* (MRSA) ATCC 33592, and Gram-negative *Escherichia coli* (*E. coli*) ATCC 43888 were cultured in Luria-Bertani (LB) (Sigma, Germany). All strains were grown at 37 °C for 16 h under 200 rpm of agitation. Bacterial co-aggregation was assessed using Transmission Electron Microscopy, and aggregate size, polydispersive index, and zeta (ζ) potential measured using a Zetasizer (Malvern instrument, Malvern, UK). To isolate the LaEPs (20, 40, and 60 µg/mL), *L. acidophilus* was cultured in MRS broth for 48 h at 37 °C, and cultures harvested in a refrigerated centrifuge at 10,000 × g for 15 min at 4 °C. The extracellular proteins were precipitated from the culture supernatant by the addition of solid ammonium sulphate at 60% saturation. LaEP-induced alterations in macrophage and hBMSC metabolic activity, morphology, and DNA damage were assessed. qRT-PCR was used to measure the gene expression of pro- (*TNF-α, IL-6, IL-1β and iNOS*) and anti- (*TGFβ, IL-10, CD163, CD206*) inflammatory markers under ambient, and lipopolysaccharide (LPS)-induce chronic inflammatory conditions, as well as osteogenic (*Col-I, Runx2, SPP*1, *ALPL, BMP-7, BSP, OCN, OPN, BGLAP, SP7*), and adipogenic (*C/EBPα, PPARγ, LEP, FASN, FABP4, SREBF1, LRP4, TRARSF11B, ADIPOQ, LPL and PLIN1*) markers. Macrophage and osteoclastic activation were confirmed *via RANKL*, and *Cathepsin K* (*CTSK*) expression as well as $TRAP^+$ staining. Statistical analysis was carried out using GraphPad Prism (version 8.0, US) and groups compared using the nonparametric Mann-Whitney test. p values < 0.05 were considered significant.

Results. Although widespread, bacterial auto-aggregation is poorly understood, and considered to provide pathogenic bacteria with the ability to maintain mobility whilst also evading host defenses and antimicrobial treatment. However, co-aggregation with probiotic bacteria may neutralize pathogenic strains, preventing their motility and adhesion to host cells, as well as the mucosal surface thereby creating a barrier to colonization within the host. Our ζ potential results reveal *L. acidophilus* species were more anionic and the %aggregation was immediately, and significantly higher in the *L. acidophilus-S. aureus* co-cultured group when compared with a monoculture of each bacterial species alone. Data show this electrostatically-induced probiotic-pathogen species co-aggregation resulted in pathogenic growth inhibition. This is of relevance as probiotic adherence to host cells and tissue, or metal implant surfaces will theoretically limit sites available for pathogenic adherence thereby reducing the incidence of pathogenic infection. Further, data showed the LaEP-induced disassembly of preformed biofilm. Additionally, LaEPs activated macrophages priming them for enhanced microbial phagocytosis *via CTSK*, while also reducing LPS-induced DNA damage and *RANKL* expression and promoted a reparative M2 macrophage morphology when under chronic inflammatory conditions. These antimicrobial and pro-repair properties are likely highly beneficial following orthopaedic implant surgery. Notably, a trend was seen where *ALP*, *Runx2*, and *SPP7* together with *COL1*, *BGLAP*, *SPP1*, *ALP*, *BMP7*, *BSP*, *OCN*, and *OPN* were upregulated primarily when supplemented at a LaEP concentration of 20 µg/mL. The osteoinductive properties of LaEPs were investigated and remarkably, results showed significantly increased ALP activity, and thus osteoblastogenesis, at a concentration of 20 µg/mL and when in the absence of osteogenic media. However, no increase in mineral deposition was measured. Further work is needed to clarify its ability to selectively osteoinduce dose-dependent osteoblastogenesis and not mineral formation.

Discussion. Our results reveal LaEPs confer bacteriostatic properties and disassemble preformed biofilm. Further, LaEPs induced the activation of macrophages priming them for enhanced microbial phagocytosis *via CTSK* upregulation, reduce LPS-induced DNA damage and *RANKL* expression, increase anti-inflammatory gene expression, and promote a reparative M2 morphology when under chronic inflammatory conditions. Our data also show LaEPs significantly augment osteogenesis while downregulating adipogenesis and lipid formation. Notably, a trend was observed where the higher doses of LaEPs were more effective in disassembling pathogenic biofilm, reducing LPS-induced DNA damage, increasing overall anti-inflammatory gene expression parallel to pro-inflammatory expression, *CTSK* expression, and in reducing adipogenesis and lipid accumulation. A lower dose was more effective at promoting cell metabolic activity, osteogenesis, and mineralization. Finally, the secretome characteristics of different Lactobacilli are distinct, and therefore their capacity to interact and influence varying factors encountered within host bone tissue and on an abiotic implant surface will also be diverse. As such, further work is warranted to investigate the effect of LaEPs *in vivo*, and to also analyze varying Lactobacilli exoproteomes as this may aid in identifying candidate effector molecules, and thus, potentially advance the future development of a multi-modal strategy to improve the prevention and outcome of PJIs. As such, LaEPs hold promise as a potential multimodal therapeutic strategy.

Significance. Microbial biofilm is associated with ~1.7 million hospital acquired infections per year in the United States, incurring an annual economic burden of ~$11 billion. Presently, no available efficacious approaches definitively prevent and treat PJI. Our results suggest that LaEPs hold promise as a potential bacteriostatic therapeutic agent that induces pathogenic biofilm disassembly, beneficially regulates chronic inflammation, promotes a reparative macrophage phenotype, and augments bone deposition over adipogenesis *in vitro*.