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Colonization of breastfed infants by *Bifidobacterium longum* subsp. *infantis* EVC001 reduces virulence gene abundance

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ABSTRACT

The infant gut microbiome is rapidly colonized by bacteria from the environment after birth, and this gut ecosystem can facilitate expansion of potential pathogens. Human milk shapes the infant gut microbiome and has evolved to foster the growth of specific bacteria. Breastfed infants fed the coevolved infant gut symbiont *Bifidobacterium longum* subsp. *infantis* EVC001 had significant modifications to their gut metagenome, including a decreased number of virulence factor genes.

Virulence factors (VFs) enable bacterial survival and infection in the host [1] and exhibit a broad spectrum of functions that are indispensable for microbes to achieve colonization, evade the host immune system and obtain nutrients from the host [2,3]. The advancement of molecular techniques, particularly metagenomics, has allowed extensive characterization of the VF mechanisms, thus enabling a deeper understanding of bacterial pathogenesis [2].

Neonates are particularly susceptible to microbial infections since the infant gut has low microbiome stability and colonization resistance [4]. In this environment, microbes with VFs can easily establish persistent reservoirs and colonize newborn infants [5]. A rising global incidence of bacteria resistant to several classes of antibiotics, limits effective therapies [6,7], and infections are a leading cause of death in infant intensive care units [8].

Recent studies have shown how commensal bacteria play a key role in the evolution and dissemination of VFs, even if they do not directly express virulence genes [9,10]. There are limited ways by which VFs and the organisms that harbor them can be restricted without the use of antibiotics. In a recent clinical trial, we demonstrated how a single-strain probiotic containing *Bifidobacterium longum* subsp. *infantis* EVC001 (*B. infantis* EVC001) fed to breastfed infants changed the gut microbiome composition to improve its stability and function [11].

In the present study, we extended our findings from our previous clinical trial [11] using shotgun metagenomic sequencing to examine whether colonization by *B. infantis* EVC001 significantly reduces the abundance of potential pathogens, and their VFs, in the healthy breastfed-infant gut microbiome. Shotgun metagenome sequencing was performed on 60 fecal samples collected from infants at day 21 postnatal. The mothers of 29 breastfed infants were provided lactation support, and the infants were fed *B. infantis* EVC001 daily (EVC001-fed) from day 7 postnatal. Another 31 mothers received only lactation support and their infants were not fed *B. infantis* EVC001 (controls). *B. infantis* is a well-characterized organism for which there is extensive evidence of evolutionary adaptation to the breastfed infant gut [12,13]. However, the infant gut microbiome in resource-rich countries has experienced a progressive loss of *B. infantis*, likely due to high rates of Cesarean section delivery, formula feeding and antibiotics usage over the last three generations [14].

Metagenomic analysis using clade-specific marker genes to unambiguously assign reads to functional genes confirmed our previous 16S rRNA-based analysis [15] (Supplementary Methods, Supplementary Table 1). Particularly, feeding *B. infantis* EVC001 increased *Bifidobacteriaceae* abundance in feces of breastfed infants, whereas no *B. infantis* was detected among control samples. After two weeks of supplementation, *Bifidobacteriaceae* was significantly increased ($p = 7.18E-07$), whereas *Enterobacteriaceae* ($p = 0.0001$) and *Clostridiaceae* ($p = 0.007$) families were significantly decreased (Fig. 1A). To profile the VF gene composition in samples, we used a non-redundant database obtained by merging three well-known VF gene databases (Supplementary Methods). A total of 2,832 VF genes were identified in controls, representing nearly twice the number of VF genes identified in EVC001-fed infant samples (Supplementary Table 2), which contained significantly fewer VF genes ($p = 0.0001$). On average, EVC001-fed infant samples had an 85% lower relative abundance of VF genes in the overall metagenome than control samples (Fig. 1B).

Hierarchal clustering based on the overall abundance of VF gene profiles revealed a marked separation of the individual samples based

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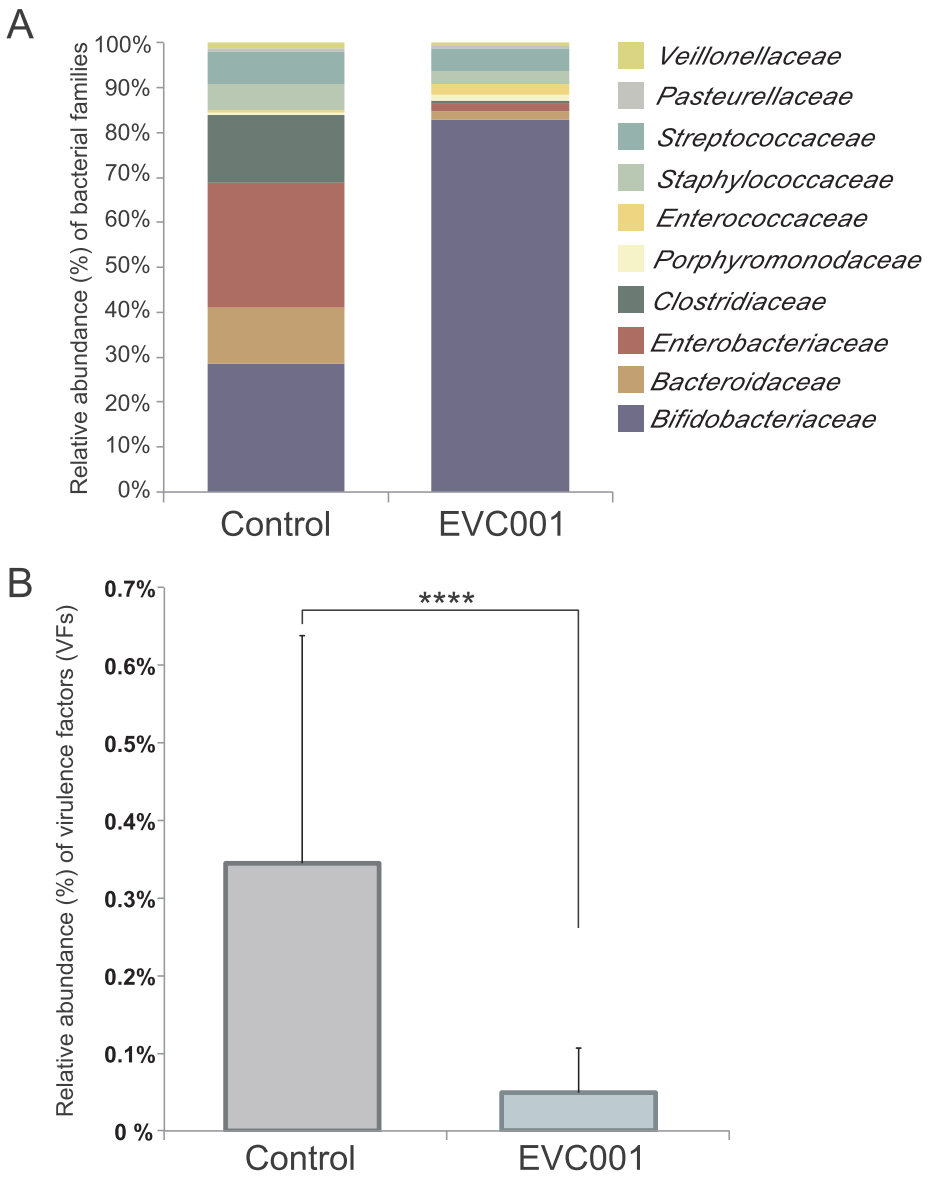

Fig. 1. A) Average relative abundance of top 10 bacterial families identified within the EVC001-fed and control groups. B) Average relative abundance of virulence factors in the entire metagenomes. ($p < 0.0001$, Mann–Whitney test).

on supplementation status (Fig. 2A). This suggested similarities in VF type and respective abundances in samples within the same treatment group, with the majority of those in the EVC001-fed group clustering with the lower VF gene abundance (Fig. 2A). Furthermore, 146 individual VF genes were lower in the EVC001 group (FDR-$p < 0.05$). These genes were mapped to the corresponding KEGG orthologs and pathways (Fig. 2B). Contextually, many of the gene functions in the database identified in samples from EVC001-fed infants as virulence factors are housekeeping genes that are necessary but not sufficient, or are repurposed from their known housekeeping function, for infection by pathogenic bacteria [16].

Conserved among the genes for many of the potentially pathogenic organisms identified were those for glutathione reductases, arginine *N*-succinyltransferases, stress response regulators (Hsp90, OmpR/EnvZ, rpoS), Fe(III) and Zn permeases, and flagellar proteins (FliN/FliY) (Supplementary Table 3). These gene functions are all associated with response to stress. Host inflammation creates an environment that favor certain taxa who thrive under differentially oxidative states [17,18], and a picture of inflammation and dysbiosis is emerging based on this understanding [19].

Although ideal for functional classification, VF databases are usually built with genes identified in model organisms, thus limiting taxonomic classification. To infer the proper taxonomic assignment of bacteria contributing to the identified VFs, we performed individual metagenome assemblies of five representative samples with the most abundant and diverse VF gene profiles (Supplemental methods). Assembled metagenomes were converted into local databases used to retrieve taxonomic information coupling previously identified clade-specific VFs amino acids sequences. Higher levels of *Bifidobacteriaceae* (i.e., *B. infantis*) were associated with a lower abundance of VFs, whereas higher abundance of *Enterobacteriaceae* (e.g., *Escherichia coli, Klebsiella*), *Clostridiaceae* (e.g., *Clostridium*), *Pasteurellaceae* (e.g., *Haemophilus*), *Staphylococcaceae and Streptococcaceae* families, as well as different potential pathogens belonging to the Proteobacteria and Firmicutes phyla, were related to a higher abundance of VFs (Fig. 2C). Multivariate linear modeling identified associations between supplementation status and global relative abundance of bacterial species. Particularly, *Haemophilus parainfluenzae*, *Escherichia coli* and

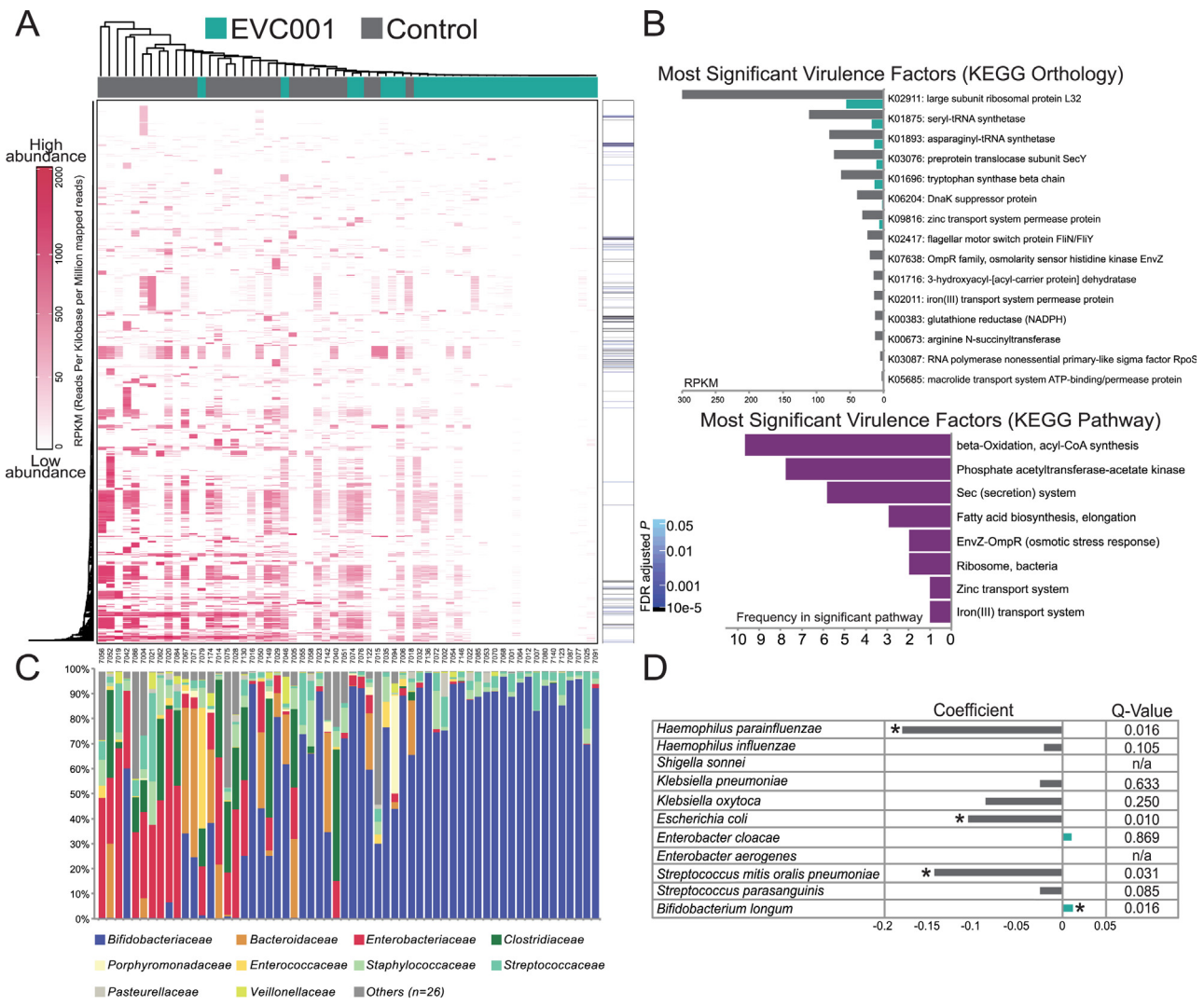

Fig. 2. A. Heatmap showing virulence factor (VF) abundance across samples in Reads Per Kilobase per Million mapped reads (RPKM). Samples were hierarchically clustered based on similar VF profiles and colored by treatment, with the p-value bar highlighting only significant VFs ($p < 0.05$; Kruskal–Wallis with FDR correction). B. Abundance among treatments (RPKM) of most significant VF ($p < 0.05$) mapped to 15 KEGG orthologs and 8 KEGG pathways. Higher frequencies are associated with pathway completeness based on gene presence. C. Relative abundance of bacterial families identified across samples. "Others" refers to several bacterial families for which individual relative abundance was lower than 1%. D. Differences in the gut microbiome composition at the species level between the EVC001-fed and control groups. Bar plot of γ-coefficients from multivariate association with linear models (MaAsLin) statistical analysis assessing associations between microbial species and supplementation status. Positive (teal bars) and negative (grey bar) coefficient values represent taxa enriched in the EVC001-fed group and the control group, respectively. Q-values are FDR-adjusted p-values as computed by MaAsLin.

Streptococcus mitis were significantly higher in controls. *Bifidobacterium longum* was significantly increased in the EVC001-supplemented group (Fig. 2, D).

This is the first study using shotgun metagenomic sequencing to report a direct effect of VF gene reduction in the infant gut bacterial community in response to colonization by a probiotic organism. Colonization of breastfed infants with *B. infantis* EVC001 may offer an attractive approach to reduce the number of VFs and the relative abundance of potential pathogenic gut bacteria that harbor them. Future work will be needed to determine whether colonization by *B. infantis* EVC001 relates to increased resilience in the face of pathogen challenges.

Ethics approval and consent to participate

The study was conducted under the approval of the University of California, Davis Institutional Review Board and registered at ClinicalTrials.gov under study NCT02457338. All experiments were performed in accordance with relevant guidelines and regulations.

Consent for publication

Consent for participation and publication of the study results was obtained from subjects or their legal guardians.

Availability of data and material

Human filtered sequencing libraries were deposited on the Sequence Read Archive (SRA; https://www.ncbi.nlm.nih.gov/sra) with number PRJNA390646.

Conflict of interest

GC and SAF are employed by Evolve BioSystems, Inc., Davis California.

Authors' contributions

GC and SAF contributed to the study design, experimental design and conducted the analysis. All authors contributed to writing, editing and approving the manuscript.

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Competing interests statement

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.humic.2018.05.001.

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