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RESEARCH

The Influence of Protein Secretomes of *Enterococcus durans* on ex vivo Human Gut Microbiome

Carolina Baldisserotto Comerlato[1] · Xu Zhang[2] · Krystal Walker[2] · Janice Mayne[2] · Daniel Figeys[2] · Adriano Brandelli[1]

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Abstract

The gut microbiome plays a critical role to all animals and humans health. Methods based on ex vivo cultures are time and cost-effective solutions for rapid evaluation of probiotic effects on microbiomes. In this study, we assessed whether the protein secretome from the potential probiotic *Enterococcus durans* LAB18S grown on fructoligosaccharides (FOS) and galactoligosaccharides (GOS) had specific effects on ex vivo cultured intestinal microbiome obtained from a healthy individual. Metaproteomics was used to evaluate changes in microbial communities of the human intestinal microbiome. Hierarchical clustering analysis revealed 654 differentially abundant proteins from the metaproteome samples, showing that gut microbial protein expression varied on the presence of different *E. durans* secretomes. Increased amount of Bacteroidetes phylum was observed in treatments with secretomes from *E. durans* cultures on FOS, GOS and albumin, resulting in a decrease of the Firmicutes to Bacteroidetes (F/B) ratio. The most functionally abundant bacterial taxa were *Roseburia*, *Bacteroides*, *Alistipes* and *Faecalibacterium*. The results suggest that the secretome of *E. durans* may have favorable effects on the intestinal microbial composition, stimulating growth and different protein expression of beneficial bacteria. These findings suggest that proteins secreted by *E. durans* growing on FOS and GOS have different effects on the modulation of gut microbiota functional activities during cultivation.

Keywords *Enterococcus*; Human fecal microbiome · FOS · GOS · Probiotics · Ex vivo culture

Introduction

The human gut harbors a diverse and complex microbial community and plays important roles in host health. Changes in the intestinal microbiota composition have been associated with diseases such as obesity, inflammatory bowel disease (IBD), diabetes mellitus and colorectal cancer (CRC). Modulations of the gut microbiota mediated by dietary interventions or using probiotics and/or prebiotics were also described to improve host metabolic phenotypes [1, 2]. Moreover, the recent discovery that small-molecule metabolites causing DNA damage in host cells may be produced by bacterial strains isolated from CRC and IBD patients reinforce the importance of gut microbiome in shaping host biology and disease susceptibility [3].

Next generation sequencing (NGS) techniques, such as metagenomics and metatranscriptomics, are used to examine microbiota composition and predict possible functions. However, the effective translation of genes into proteins is not accomplished by these methodologies [4]. Metaproteomics can provide valuable evidence about the microbiome functional activities through direct profiling of protein expression levels. In contrast to metagenomics, the metaproteomic approach has been employed with less frequency in studies of the intestinal microbiota [5, 6].

The term "probiotics" first appeared in 1974 and its definition refers to living microorganisms that confer health

✉ Daniel Figeys
dfigeys@uottawa.ca

✉ Adriano Brandelli
abrand@ufrgs.br

[1] Laboratório de Bioquímica e Microbiologia Aplicada, Instituto de Ciência e Tecnologia de Alimentos, Universidade Federal do Rio Grande do Sul, 91510-970 Porto Alegre, Brazil

[2] School of Pharmaceutical Sciences, Ottawa Institute of Systems Biology and Department of Biochemistry, Microbiology and Immunology, Faculty of Medicine, University of Ottawa, Ottawa, ON K1H 8M5, Canada

benefit when consumed in adequate quantities [7]. Probiotics are currently one of the most popular dietary supplements consumed worldwide, being widely supported by doctors, specifically gastroenterologists [8, 9]. The ability of probiotics to promote health benefits has fueled a growing scientific interest for several decades. The use of these microorganisms is often associated with beneficial microbiota modulation and normalization of intestinal dysbiosis, obtaining favorable results alone or triggering mechanisms by which protect the host against disease [10].

Although the positive health effects of probiotic use are described in many studies, most of the published data is obtained from populations with specific health conditions. Evidence supporting the benefits of probiotics in healthy adults is limited and less reliable [11]. Despite this, the widespread consumption of probiotics by healthy individuals is strongly promoted by manufacturers as compared with the consumer market for diseased populations [10, 11].

Enterococcus durans LAB18S was isolated from a soft cheese, showing several desirable characteristics of a probiotic strain [12, 13]. The production of clinically relevant enzymes for cancer treatment was recently observed when *E. durans* LAB18S was cultivated on fructooligosaccharides (FOS). Moreover, proteins associated with cellular proliferation, cell wall integrity and H_2O_2 detoxification were induced under anaerobic growth [14]. These features of strain LAB18S could be helpful for the maintenance of intestinal health. Therefore, this study aimed to evaluate the effect of the secretome (proteins secreted in the extracellular environment) of *E. durans* LAB18S growing on two widely used prebiotics (FOS and GOS) on the intestinal microbiota of a healthy individual using an ex vivo microbiome assay [15] as described previously.

Materials and Methods

Bacterial Cultivation and Secretome Extraction

E. durans LAB18S was grown in Synthetic Medium (SM) containing 10 g/L of either fructooligosaccharides (FOS), galactooligosaccharides (GOS) or glucose (GLU, used as a control carbon source). The composition of the SM was previously defined [16] as 15 g/L casamino acids, 6.7 g/L yeast nitrogen base, 10 g/L ascorbic acid, 10 g/L sodium acetate, 5 g/L $(NH_4)_2SO_4$, 2 g/L urea, 0.2 g/L $MgSO_4{\cdot}7H_2O$, 0.01 g/L $FeSO_4{\cdot}7H_2O$, 0.007 g/L $MnSO_4{\cdot}7H_2O$, 0.01 g/L NaCl, 1 g/L Tween 80, 0.5 g/L cysteine (pH adjusted to 7.0 and autoclaved for 30 min at 110 °C). In addition to these treatments, the isolate was cultured in SM medium containing 7 g/L bovine serum albumin (BSA) as control protein. *E. durans* obtained from 24 h cultivation in Luria–Bertani (LB) broth was inoculated (2%, v/v) in triplicate in 30 mL SM with an initial $OD_{600\ nm}$ at 0.8–0.9 (approximately 6.9×10^8 cells/mL). Incubation was carried out under anaerobic conditions (5% H_2, 5% CO_2 and 90% N_2) at 37 °C until the mid-log phase (8 h).

Extraction of proteins secreted by *E. durans* after incubation was performed as described elsewhere [17]. Proteins were extracted from culture supernatants after centrifugation at $14{,}000 \times g$ for 20 min at 4 °C. Supernatants were removed and filtered through 0.22 µm membranes into new tubes. Protein precipitation was achieved by adding 20% (w/v) trichloroacetic acid and centrifugation at $16{,}000 \times g$ for 20 min at 4 °C. The resulting supernatants were discarded, and the pellets were washed twice with acetone. The extracted proteins were kept at − 20 °C until further use.

Ex Vivo Gut Microbiome Culturing

The protocol for stool sample collection was approved by the Ottawa Health Science Network Research Ethics Board at the Ottawa Hospital (Ottawa, Canada). The RapidAIM workflow, a culture- and metaproteomics-based rapid assay of individual microbiome responses, was performed as described elsewhere [15]. The culture of human intestinal microbiome in vitro was prepared from the fecal sample obtained from a healthy adult volunteer. Fresh stool samples (∽3 g) were mixed in pre-reduced PBS with 0.1% (w/v) L-cysteine hydrochloride, weighted in an anaerobic workstation where the tube was uncapped to remove O_2, filtered with sterile gauze to eliminate bulky particles and attain the microbiome inoculum. The microbiome inoculum was adjusted to a final concentration of 2% (w/v) into 96-well polypropylene deep well plates of 2 mL containing 1 mL culture medium. The proteins secreted by *E. durans* were added to reach two different working concentrations: 10 and 50µg/mL, as the secretome protein concentration at logarithmic growth phase was around 10µg/mL. Considering the high metabolic activity during this growth phase, a 5 times higher concentration was also tested for comparison. The microbiome culture media contained 2.0 g/L peptone, 2.0 g/L yeast extract, 0.5 g/L L-cysteine hydrochloride, 2 mL/L Tween 40, 5 mg/L hemin, 10 µL/L vitamin K1, 1.0 g/L NaCl, 0.4 g/L K_2HPO_4, 0.4 g/L KH_2PO_4, 0.1 g/L $MgSO_4{\cdot}7H_2O$, 0.1 g/L $CaCl_2{\cdot}2H_2O$, 4.0 g/L $NaHCO_3$, 4.0 g/L porcine gastric mucin, 0.25 g/L sodium cholate, and 0.25 g/L sodium chenodeoxycholate. The sterile medium was preconditioned overnight in an anaerobic workstation. A control was prepared using PBS in place of proteins secreted by *E. durans*. The plate was incubated on a digital shaker (MS3, IKA, Germany) operating at 500 rpm into an anaerobic chamber at 37 °C.

Metaproteomic Sample Processing and LC–MS/MS Analysis

Briefly, culture debris was removed from a 1 mL culture by centrifugation at 300 × *g* for 5 min and 4 °C. The resulting supernatants subjected to two additional centrifugations, and then transferred to 2 mL tubes and centrifuged at 14,000 × *g* for 20 min and 4 °C. Microbial cell pellets were suspended in 150 µL lysis buffer (freshly prepared, containing 8 M urea and 4% SDS in 100 mM Tris–HCl buffer pH 8.0, and Roche cOmplete™ Mini tablets). After lysis using an ultrasound processor (Q125 Qsonica, USA) on ice bath, the protein lysates were precipitated overnight at − 20 °C with a solvent mixture of acetone/ethanol/acetic acid at 50:50:0.1 (v/v/v) ratios. The pellets were washed three times with ice-cold acetone and centrifuged at 16,000 × *g* for 25 min. Protein concentrations of the samples were measured in triplicate using the DC Protein Assay (Bio-Rad, USA). BSA was utilized to generate standard curves. Trypsin digestion and desalting were carried out according to standard protocols [18]. Protein samples (50µg) were reduced with 10 mM dithiothreitol (DTT), alkylated with 20 mM iodoacetamide (IAA), diluted 10 × with 100 mM Tris–HCl (pH 8.0) and hydrolyzed at 37 °C for 18 h using 1µg trypsin per sample (Worthington Biochemical Corp., Lakewood, NJ, USA). Tryptic peptides were desalted by C18 cartridges and eluents dissolved in 0.1% (v/v) formic acid. Samples (1µg) were subjected to liquid chromatography-tandem mass spectrometry (LC–MS/MS) analysis with an Q Exactive mass spectrometer (Thermo Electron, Waltham, MA, USA). Briefly, peptides were loaded on a 50 cm analytical column packed with reverse phase C18 beads (1.9µm; 120-Å pore size; Dr. Maisch GmbH, Ammerbuch, Germany). The LC separation was performed with 90-min gradient from 5 to 35% acetonitrile (v/v) at a flow rate of 200 nL/min. The instrument method consisted of one full MS scan from 300 to 1800 *m/z* followed by data-dependent MS/MS scan of the 12 most intense ions with a dynamic exclusion repeat count of 2 and repeat exclusion duration of 30 s. The MS data were recorded in Xcalibur software tool and exported as RAW format for further data processing as described in the following section. Mass spectrometry data are available via ProteomeXchange Consortium under the identifier PXD040751.

Metaproteomics Data Processing

Protein/peptide identification and quantification, taxonomic assignment and functional annotations were done using the MetaLab software (version 1.1.0) [19]. MetaLab is a software that automates an iterative database search strategy, i.e., MetaPro-IQ [18]. The search was based on a human gut microbial gene catalog comprising 9,878,647 sequences from http://meta.genomics.cn/. Database construction was based on a spectral clustering strategy from all raw files. Peptide and protein lists were created by applying strict filtering based on an FDR of 0.01, and quantitative information of proteins were achieved with the maxLFQ algorithm on MaxQuant (version 1.5.3.30). Oxidation (M) and N-terminal acetylation (Protein N-term) were set as variable modifications, while carbamidomethyl (C) was set as a fixed modification. Instrument resolution was established as "High-High".

The quantified protein groups were first filtered following the criteria that the protein should be identified by ≥ 1 unique peptide in ≥ 50% of the samples (Q50). LFQ intensities of the filtered protein groups were $\log_{10}$ transformed for further statistical analysis. Functional annotations of protein groups, including COG and KEGG information, were obtained in the MetaLab software.

Protein groups were annotated with Gene Ontology (GO) terms using Unipept [20] (https://unipept.ugent.be), and these annotations were used for GO enrichment analysis. GO relative abundance analysis was done for Q50-filtered protein groups.

Statistical Analysis

All missing values of the $\log_{10}$ transformed and Q50-filtered protein group abundance data were assigned using the KNN software [19]. The discrimination of differentially abundant proteins in response to *E. durans* secretomes was carried out by partial least-squares discriminant analyses (PLS-DA) using the MetaboAnalyst algorithm [21] (http://www.metaboanalyst.ca/). The performance of the PLS-DA models was cross-validated with evaluation of R^2 and Q^2 values. Differential proteins in response to *E. durans* secretomes were identified using the variable importance in projection (VIP), where proteins with VIP > 1.0 were considered as significant attribute for discrimination in the model. Hierarchical clustering of samples was performed by Pearson's correlation of the normalized data.

Results

Metaproteomic Responses of ex vivo Microbiome to *E. durans* Secretomes

A metaproteomics investigation was performed with ex vivo human intestinal microbiome treated with secretomes from *E. durans* previously cultivated on FOS, GOS or glucose. The analysis of metaproteomics data resulted in a total of 21,519 peptides corresponding to 6470 protein groups being identified across 27 samples with a false discovery rate (FDR) threshold of 1%. An

accurate assessment of the effects of *E. durans* secretomes on the human microbiome was obtained by using data filtering criteria to identify 3149 protein groups quantified in more than 50% of the samples.

Principal component analysis (PCA) using the log-transformed LFQ intensity of protein groups revealed a secretome concentration-dependent effect on human microbiome cultures (Fig. 1A). This indicates that the microbial communities could be separated according to the exposure to different secretome concentrations. These findings suggest that proteins secreted by *E. durans* LAB18S growing on prebiotics may have a differential modulatory effect on functional human gut microbiota during in vitro cultivation.

In addition, the treatment with PBS (used as a control) in a high concentration was clearly isolated from the other groups (Fig. 1), showing that the human microbiome culture displays specific responses by influence of *E. durans* secretomes. The use of BSA as a control protein showed that the response of the human microbiome was depending on the concentration, forming a separate cluster at low concentration, but clustering with GOS and glucose groups at higher concentrations.

The differentially expressed proteins related to the presence of different *E. durans* secretomes were identified by a PLS-DA approach (Fig. 1B), which resulted in 654 differential proteins with the threshold of VIP > 1.0 in the first component of the PLS-DA (Table S1). The performance of PLS-DA model was considered appropriate by analysis of cross validation parameters ($R^2 > 0.98$ and $Q^2 > 0.43$; Fig. S1).

Hierarchical clustering analysis of the identified 654 differential proteins showed that different concentrations of proteins secreted by *E. durans*, named as high (50µg/mL) and low (10µg/mL), resulted in differences in protein expression of the human fecal microbiome (Fig. 2). Overall, a similar pattern of differentially regulated proteins can be observed when comparing the samples treated with low concentration and the control PBS. However, several proteins that appeared as upregulated in control and LOW treatments were downregulated in HIGH treatments, and vice-versa (Fig. 2). Thus, the results obtained from this analysis indicate that a higher concentration of *E. durans* secretome substantially modifies the protein expression profile of the intestinal microbiome.

Gene Ontology Responses of ex vivo Microbiome to *E. durans* Secretomes

The results of gene ontology (GO) analysis provide a comprehensive description and association of the proteins uniquely regulated in the human fecal microbiome in response to different *E. durans* secretomes. The 654 differentially regulated proteins were allocated according to GO annotation, being assigned to biological processes and molecular functions (Fig. S2). In biological processes,

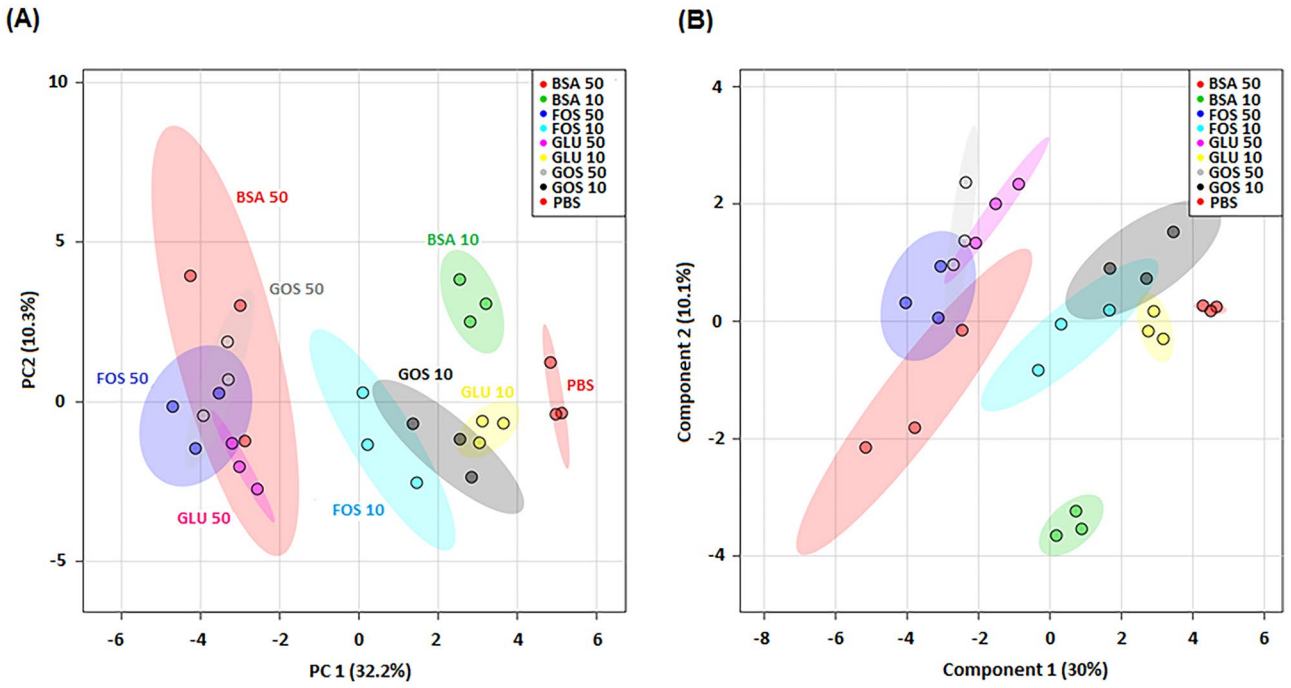

Fig. 1 **A** PCA and **B** PLS-DA score plots with LFQ intensities of protein groups of all samples showing trends of protein changes over high 50µg/mL and low 10µg/mL concentrations of *E. durans* secretomes treated in different carbon sources

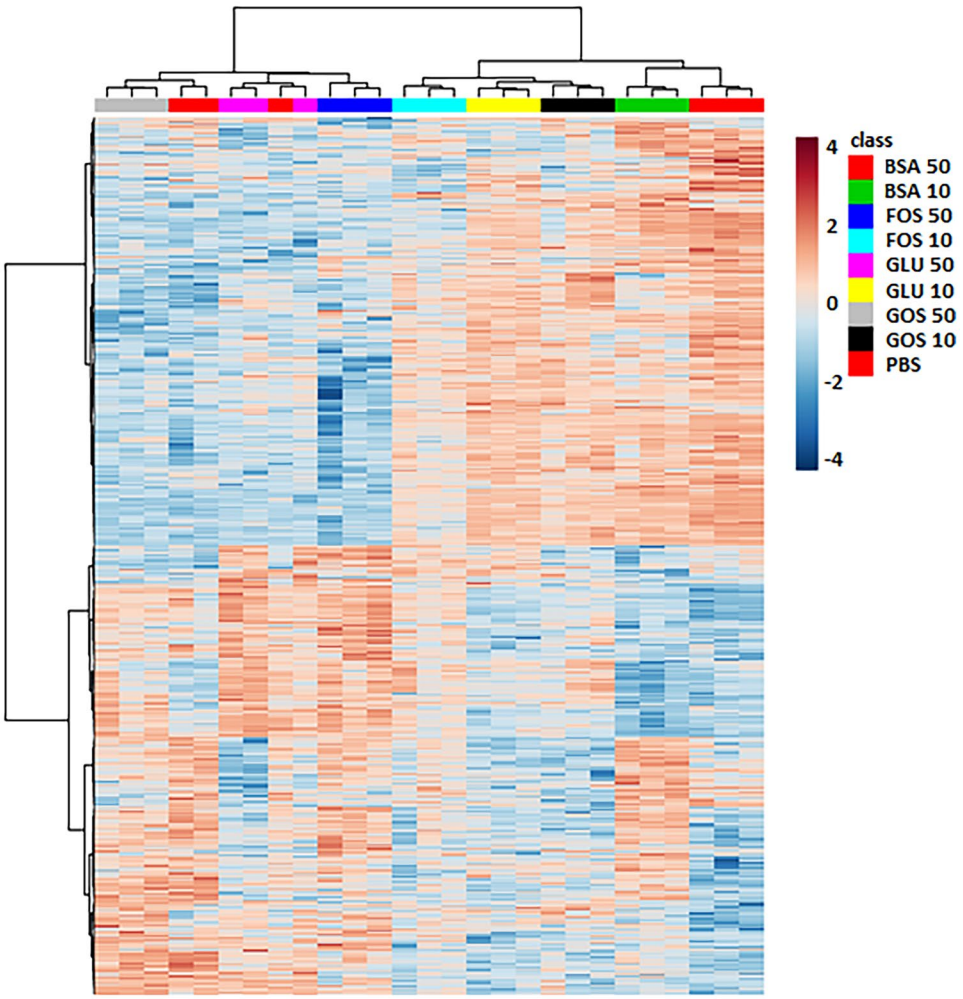

Fig. 2 Hierarchical clustering of the differentially abundant gut microbiome proteins, with samples in columns and proteins in rows. Log_{10}- and *z*-score transformed LFQ intensities of each protein were used for clustering analysis. The two-color scale ranging from the lowest intensity (*z*-score of -4) in blue to the highest intensity (*z*-score of 4) in red were used

a prevalence of the terms can be evidenced: translation (GO:0006412), glycolytic process (GO:0006096), gluconeogenesis (GO:0006094), and carbohydrate metabolic process (GO:0005975). Other terms are electron transport chain (GO:0022900), cellular amino acid metabolic process (GO:0006520), pyruvate metabolic process (GO:0006090) and glucose metabolic process (GO:0006006). The most common terms in molecular functions include ATP binding (GO:0005524), structural component of ribosome (GO:0003735), metal ion binding (GO:0046872), rRNA binding (GO:0019843) and oxidoreductase activity (GO:0016491).

Although proteins with the same function have differences in their amino acid sequences, their functional domains are normally highly conserved. Thus, the InterPro database provides functional analysis based on similar functional domains of proteins by classifying them into families and predicting domains and important sites. The result of InterPro analysis of differentially abundant proteins in the human fecal microbiome in response to the treatment with *E. durans* secretomes is summarized in Fig. S3. It could be observed that the most abundant InterPro entry was NAD(P)-binding domain superfamily, which agrees with the number of biological processes associated with carbohydrate metabolism in GO analysis. Several enzymes in these metabolic pathways use NAD^+ or $NADP^+$ as electron acceptors in the redox reactions they catalyze. In all cases, the main enzymes were pyruvate synthase (E.C. 1.2.7.1), oxidoreductases with NAD(+) or NADP(+) as acceptor (E.C. 1.2.1), pyruvate phosphate dikinase (E.C. 2.7.9.1), oxidoreductases with iron-sulfur protein as acceptor (E.C. 1.2.7), and phosphoenolpyruvate carboxykinase (E.C. 4.1.1.49). Phosphoglycerate kinase (E.C. 2.7.2.3) also appeared as a major protein in BSA and GLU treatments.

Taxonomic Composition of ex vivo Cultured Human Fecal Microbiota

The taxonomical assignment obtained from metaproteomics data revealed that Firmicutes, Bacteroidetes, Actinobacteria and Proteobacteria were the four major bacterial phyla in the fecal microbiome. Regardless of the treatment, it can

be clearly observed that Firmicutes and Bacteroidetes were the dominant phyla, ranging from 60 to 70% and 28 to 32%, respectively. The treatments with *E. durans* secretomes FOS (50µg/mL) and GOS (50µg/mL), in addition to BSA (10µg/mL), resulted in an increased amount of Bacteroidetes phylum as compared with control PBS treatment (Fig. 3A,B). The amount of Actinobacteria also increased in GOS (50µg/mL). This increased amount of Bacteroidetes observed for treatments with BSA and *E. durans* secretomes from cultivation with FOS and GOS in higher concentration resulted in significant decrease ($P < 0.05$) of the Firmicutes to Bacteroidetes (F/B) ratio (Fig. 3C).

As the higher concentration of *E. durans* secretomes caused remarkable changes in the metaproteomic profile and a significant decrease of F/B ratio for BSA50, FOS50 and GOS50, a more detailed comparison on the microbial taxa was performed for these treatments. The dominant phylum Firmicutes (Fig. 4A) was mostly represented by the families Lachnospiraceae, Ruminococcaceae and Clostridiaceae (Fig. 4B). The abundance of Lachnospiraceae and Ruminococcaceae was lower and higher, respectively, in BSA and GOS groups as compared with those in the control PBS. In the phyla Bacteroidetes and Actinobacteria, Bacteroidaceae and Coriobacteriaceae were the dominant

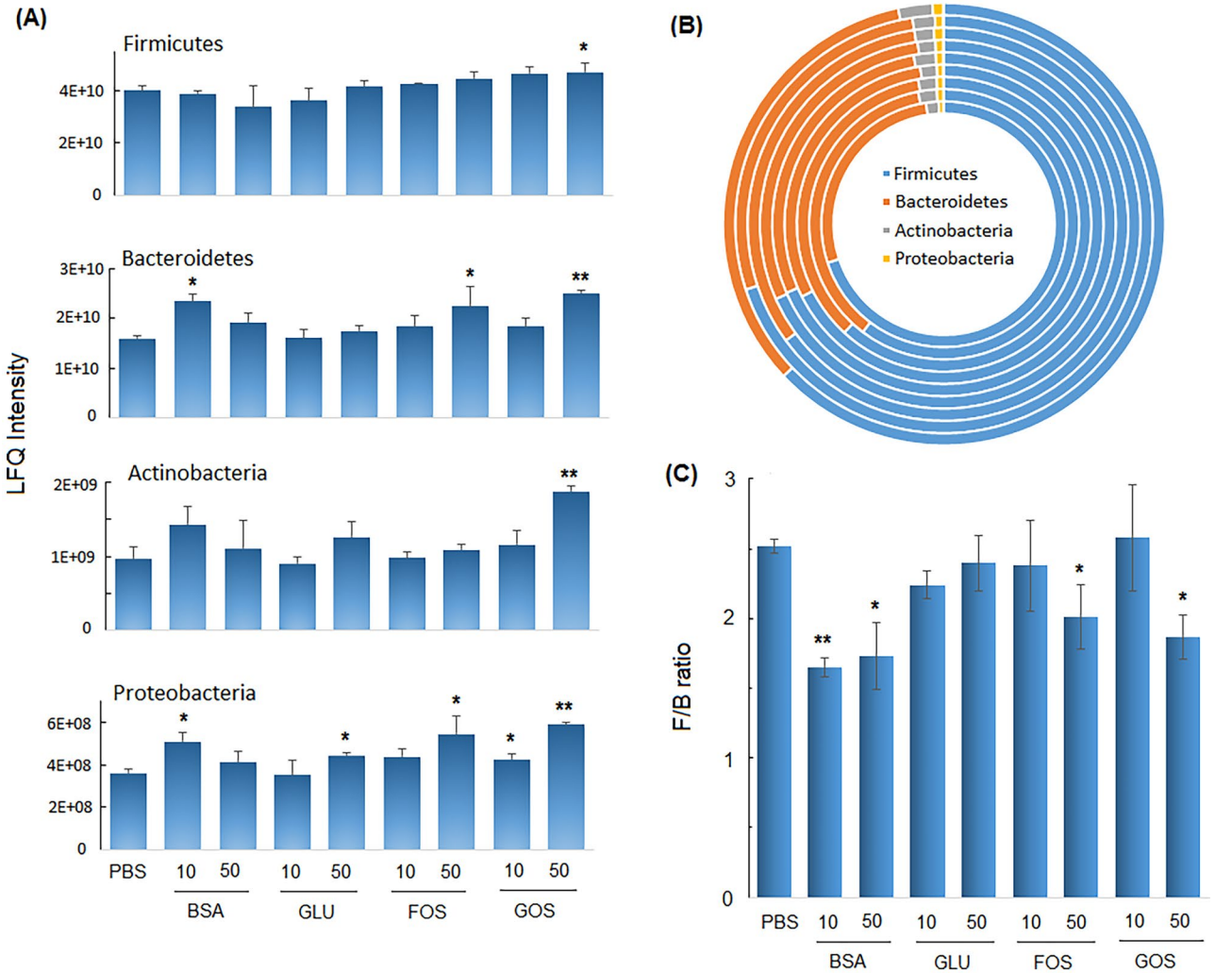

Fig. 3 Influence of *E. durans* secretomes on taxonomic composition of ex vivo cultured human gut microbiota. **A** LFQ intensity of major bacterial phyla and **B** histogram showing the relative abundance of major bacterial phyla in human gut microbiota after incubation with different *E. durans* secretomes. Inner to outer rings represent PBS, BSA10, BSA50, GLU10, GLU50, FOS10, FOS50, GOS10 and GOS50 secretomes. **C** Firmicutes to Bacteroidetes (F/B) ratio of different treatments. Values are the means ± standard deviation of three biological replicates. Asterisks denote significant differences as compared with control PBS treatment at $*P < 0.05$ and $**P < 0.01$ (paired Student's *t*-test)

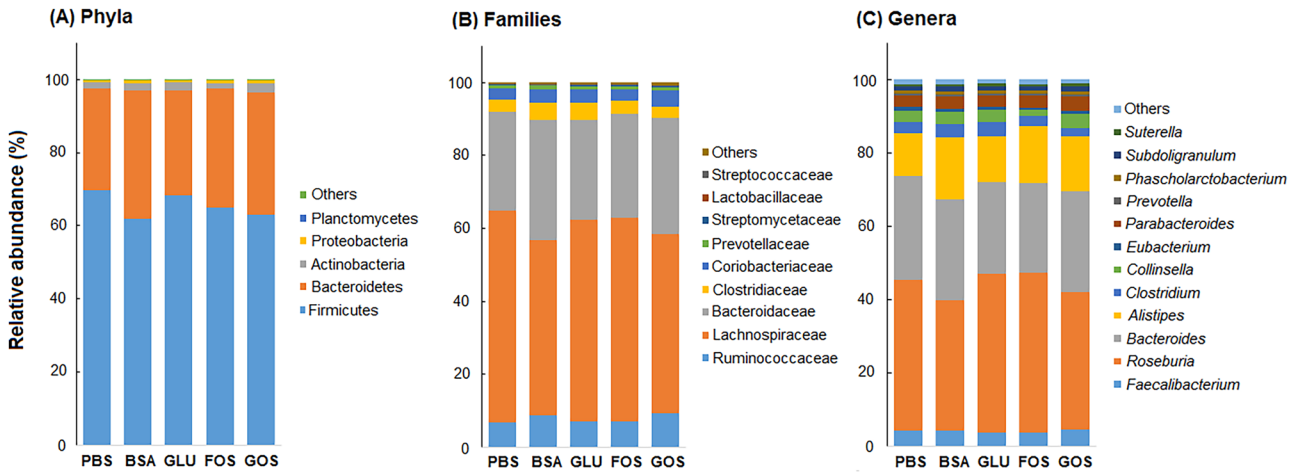

Fig. 4 Relative abundance of the most abundant phyla in the human intestinal microbiome subjected to the treatment with three different oligosaccharides (glucose, fructooligosaccharide and galactooligosaccharide). PBS was used as a negative control for the microbiome treatments and BSA as a protein control

families, respectively (Fig. 4B). The relative abundance of Bacteroidaceae was higher than control samples in BSA and GOS samples, whereas Coriobacteriaceae was increased in GOS groups. The Enterobactereaceae family was not detected in groups with treatments with PBS and GLU secretomes.

Roseburia, *Bacteroides* and *Alistipes* were the most abundant genera in all treatment groups (Fig. 4C). Decreased *Roseburia* and increased *Alistipes* were observed in BSA and GOS samples, while decreased *Collinsella* was found in FOS group. The LFQ values for *Lactobacillus*, *Leuconostoc*, *Staphylococcus* and *Lawsonella* were considerably lower as compared to those intensities related to dominant genera like *Roseburia* and *Bacteroides*.

The LFQ intensities of the dominant bacterial species were compared, and some significant differences were observed among treatments (Fig. 5). *Roseburia intestinalis* and *Roseburia innulinivorans* were the species showing higher intensities in control PBS, followed by *Faecalibacterium prausnitzii*. In general, samples treated with the secretomes of *E. durans* showed increased abundances for bacteria often associated with healthy gut, such as *F. prausnitzii*, *R. intestinalis*, *Roseburia hominis*, *Anaerostipes caccae*, *Subdoligranulum variable*, and *Alistipes* and *Bacteroides* species as well. The secretomes obtained from FOS or GOS cultivations showed increased abundances for *Lachnoclostridium asparigiforme*, *Oscillibacter valericigenes* and *Phascolarctobacterium succinatutens* as compared with control PBS, while potential pathobionts *Prevotella* sp. and *Lawsonella clevelandensis* showed decreased abundances. Decreased abundances for *Blautia wexlerae*, *Coprococcus comes*, *Eubacterium hallii* and *Hungatella hathewayi* were also observed for FOS samples.

Discussion

The assembled data from several studies revealed that the human microbiota encompasses 12 different phyla, and Firmicutes, Bacteroidetes, Actinobacteria and Proteobacteria are the most abundant phyla. Among these, Firmicutes and Bacteroidetes dominate the gut microbiota in healthy individuals [22]. In this study, the taxonomic profiles of fecal microbiota outlined changes in the bacterial composition and the ratio of Firmicutes to Bacteroidetes when exposed to *E. durans* secretomes. Changes and unbalance in the composition of the intestinal microbiota can lead to various diseases in humans and animals [23, 24]. The high abundance of the phylum Firmicutes and the decrease of Bacteroidetes have been associated with the unbalanced composition of microbiomes (intestinal dysbiosis) and several diseases are associated with dysbiosis, including obesity, diabetes, cancer, and irritable bowel diseases [25, 26]. Accordingly, in the last decade, the F/B ratio has been recurrently considered as a possible marker for obesity and related diseases. However, some studies did not detect any changes of F/B ratio or even observed this parameter decreased in obese animals and humans, suggesting that other compositional modifications at the different taxonomic levels might be more relevant than the F/B ratio itself [2, 27].

E. durans LAB18S has been characterized as a potential probiotic bacterium [12, 28]. Many studies show the ability of probiotic strains to change the composition and control microbial population of the intestinal microbiota [26, 29, 30]. Moreover, synbiotic mixture of FOS, GOS and probiotics promoted the diversity and positively influenced the development of the gut microbiota in neonates [31, 32]. In this

Bacterial taxa	LFQ Intensity (x 10^6) PBS	BSA	GLU	FOS	GOS
Firmicutes					
Acidaminococcus fermentans	5.7	7.0	4.8	7.4	9.4 *
Anaerostipes caccae	28.4	44.7 *	45.2 *	46.6 *	69.1 **
Blautia wexlerae	54.1	39.9	59.2	28.3 **	48.4
Butyrivibrio fibrisolvens	31.9	23.7	34.7	36.8	36.6
Clostridium sp. 7-2-43FAA	101.8	157.7	173.1	169.4 *	236.9 **
Clostridiales bacterium 1-7-47FAA	5.2	45.9	18.2 **	47.5 *	41.9 *
Coprococcus comes	54.6	22.5 *	87.9	25.8 *	51.2
Dorea longicatena	35.3	34.9	47.0 **	36.5	32.3
Eubacterium hallii	140.3	135.5	63.5 *	24.0 **	104.0 *
Faecalibacterium prausnitzii	1017.8	1154.1 *	1081.9	1302.5 **	1617.6 **
Hungatella hathewayi	30.5	38.8	46.2	16.3 *	73.9
Lachnoclostridium asparagiforme	26.8	39.7	35.7	45.9 *	49.2 *
Lachnospiraceae bacterium 2-1-46FAA	58.1	62.7	74.8	70.8 *	70.0 *
Lachnospiraceae bacterium 3-2	29.2	27.9	44.0 **	27.0	50.2
Oscillibacter valericigenes	23.8	37.8	31.3	33.0 *	64.1 **
Phascolarctobacterium succinatutens	164.8	167.3	173.3	237.8	228.3 *
Roseburia hominis	764.3	2152.6 *	2892.2 **	3314.1 **	2911.2 **
Roseburia inulinivorans	2828.7	897.3	664.9	1471.9	1459.3
Roseburia intestinalis	3937.7	3922.7	5164.2 *	5835.7 *	5719.6 *
Subdoligranulum variable	257.0	353.6	228.5 *	376.1 *	521.0 **
Ruminococcaeae bacterium D16	38.3	113.9 **	61.2	87.7 *	151.7 *
Streptococcus uberis	18.7	35.4 **	62.4 *	78.4 *	106.3 *
Bacteroidetes					
Alistipes shahii	64.3	209.7 **	152.1 **	217.4 *	204.2 **
Alistipes putredinis	117.5	105.9	116.3	137.2	165.0 *
Alistipes finegoldii	958.2	1589.6 **	1245.9 **	1880.4 *	1835.7 **
Bacteroides fluxus	70.7	107.9 *	84.1	101.3	130.1 *
Bacteroides caccae	541.2	719.8	591.4	811.3 *	875.5 *
Bacteroides vulgatus	321.4	328.1	376.3 *	390.3 *	647.4 *
Bacteroides salyersiae	99.4	115.1	103.5	102.4	155.3 *
Odoribacter splanchnicus	12.4	30.3	7.6	17.6	21.7 **
Parabacteroides merdae	296.4	389.4	409.8	529.9 *	625.3 *
Parabacteroides goldsteinii	46.7	72.6 *	67.8 *	81.0	97.9 **
Prevotella sp. oral taxon 299	33.6	20.7 *	9.6 *	0 **	0 **
Actinobacteria					
Collinsella aerofaciens	84.6	95.9	88.8	64.7	204.1 **
Lawsonella clevelandensis	29.3	30.5	32.4	11.6 *	10.9 *
Streptomyces viridochromogenes	27.9	27.3	20.4 *	33.8	34.9
Proteobacteria					
Deltaproteobacteria bacterium DG-8	6.3	8.3	8.8	8.6	13.2 *
Suterella waspsworthensis	3.1	5.3	1.4	20.8 **	16.0 *
Succinatimonas hippei	14.3	11.4	15.3	6.6	14.9

Fig. 5 LFQ intensity of the dominant bacterial groups of the human gut microbiota treated with *E. durans* LAB18S secretomes. The human gut microbiota was exposed to control treatment (PBS), or secretomes (50µg/mL protein) of *Enterococcus durans* cultivated in bovine serum albumin (BSA), glucose (GLU), fructooligosaccharides (FOS) or galactooligosaccharides (GOS). Group average LFQ intensities were shown and significant differences in comparison with control are denoted in green (increased) or red (decreased) boxes, respectively. *$P < 0.05$; **$P < 0.01$

study, the fecal microbiota exposed to *E. durans* secretomes obtained from BSA, FOS and GOS cultures showed an increased abundance of the phylum Bacteroidetes, which was mostly represented by *Bacteroides* and *Alistipes*. Although a "gold standard" reference of intestinal microbiota related with the capacity of promoting human health does not exist, the reduced abundance of *Bacteroides*, *Alistipes* and *F. prausnitzii* have been associated with some illnesses, such as obesity, metabolic liver disease, and type-2 diabetes [2, 33]. These bacterial taxa produce short-chain fatty acid (SCFA) like butyrate, which are largely recognized as relevant to maintain a balanced gut microbiota and host health [34, 35]. In particular, *F. parusnitzii* is recognized as an important producer of butyrate, showing anti-inflammatory effects that contribute to intestinal homeostasis. Increasing the abundance of *F. prausnitzii* through dietary modulation or fecal microbiota transplantation may protect from inflammatory diseases such as IBD, *Clostridiales difficile* infection (CDI), viral infection like COVID-19, and chronic inflammation as in cases of obesity [36, 37].

Furthermore, species from the two major bacterial phyla, Bacteroidetes and Firmicutes, have been identified as groups that actively hydrolyze non-digestible and complex oligosaccharides [38]. Several constituents of the intestinal microbiota are capable to obtain carbon and energy from the fermentation of non-digestible dietary fibers. Particularly, bacterial strains that thrive on these non-digestible carbohydrates play an important role in human health by producing SCFA [34], especially butyrate, which has been described as the main nutritional source for colonic epithelial cells [39]. Overall, the host-microbiota crosstalks are mediated by numerous metabolic products (often called postbiotics), including vitamins, immunomodulatory peptides and SCFA [40, 41].

Lachnospiraceae and Bacteroidaceae were the most abundant families in all treatment groups. Lachnospiraceae is included in the core of human intestinal microbiota, colonizing the gut from birth and increasing during the host's life, in terms of species richness and relative abundances [42]. The integration of Bacteroidetes and Lachnospiraceae into the early gut microbiota has been pointed out as a relevant aspect for optimal developmental outcomes. Bacteroidetes could affect neurodevelopment by influencing the integrity of intestinal barrier and systemic metabolite disposal, whereas Lachnospiraceae may affect neurodevelopment by modulating energy resources and immunity [43]. Members of Lachnospiraceae frequently linked to a healthy state, such as *Blautia* and *Roseburia*, are some of the key producers of SCFA. These genera are associated with the control of gut inflammation and maturation of the immune system, which are processes often mediated by the end products of microbial metabolism, particularly butyrate [39]. Metabolism of major bacterial taxa observed in this study, namely *R. inulinivoans*, *R. intestinalis* and *F. prausnitzii*, utilizing dietary polysaccharides like starch, inulin and arabinoxylan and prebiotic oligosaccharides, produces butyrate as the main SCFA [42, 44]. In addition, *Bacteroides* was the most abundant genus of the Bacteroidaceae family. Several species of *Bacteroides* metabolize polysaccharides and oligosaccharides, providing nutrition and vitamins to the host and other intestinal microorganisms, acting as major players in the maintenance of gut microbial food web [45].

The fecal microbiota treated with *E. durans* secretomes showed increased abundance of *Alistipes*. The genus *Alistipes* embodies emerging gut bacteria with anti-inflammatory properties and positive implications in health, although it could be increased in cases of unbalanced fecal microbiota associated with some diseases [46]. Patients with metabolic liver diseases showed reduced abundance of *Alistipes* spp. and *Alistipes finegoldii* and reduced acetate and propionate levels, which has been associated with inflammatory process [47]. An exploratory analysis merging data from two human cross-sectional datasets, revealed that 7 bacterial genera, including *Alistipes* and *Roseburia*, correlated with healthier eating behavior and lower subjective hunger ratings in young overweight adults, suggesting the host metabolism could be benefited by these bacterial taxa [48]. Higher *Alistipes* correlated with increased levels of fecal butyrate and with acetate levels in both serum and feces, but with reduced levels of fecal propionate. This relatively new genus may have exclusive functional features that may qualify them to play unique physiological roles compared to other Bacteroidetes, thus additional studies are necessary to understand their effective influence as a leading, co-inducer or bystander agent in health and disease [46].

The genus *Collinsella* was the main representative of Actinobacteria phylum, showing lower abundance in FOS treatment, while increased values of *C. aerofaciens* were observed in GOS. Overgrowth of *Collinsella* was correlated with low dietary fiber intake in overweight pregnant women [49], and with unhealthier eating traits, higher subjective hunger ratings and higher body fat mass in young overweight adults [48]. Despite the molecular mechanisms by which *Collinsella* influences the host metabolism are unknown, some studies suggest that this microbial genus may influence triglyceride synthesis and cholesterol absorption [49, 50]. However, *Collinsella* produces ursodeoxycholate, which inhibits SARS-Cov2 binding to angiotensin-converting enzyme 2 and may suppress the cytokine storm syndrome in COVID-19 [51].

Besides selective carbohydrate utilization, cross-feeding mechanisms allow homogeneous microbial populations to promote increased diversity, where metabolites from one strain provide a niche for the other [52, 53]. Substrate availability tests revealed that subdominant members of the GI tract, like *Phascolarctobacterium succinatutens*, may adapt to the gut environment by specializing to utilize succinate produced by other bacterial species [54]. In this regard, the increased LFQ values observed for *P. succinatutens* in FOS and GOS could be associated with the concomitant increase of some important succinate producers, such as *F. prausnitzii* and *Bacteroides vulgatus*. Besides its role as precursor of propionate, succinate has receiving attention as a signaling molecule important for host-microbiota crosstalk [55].

The recognition of beneficial proteins secreted by commensal or probiotic bacteria may result in the development of innovative postbiotic products. The potential use of some beneficial secreted proteins as postbiotics has been already proposed, such as the protein Amuc-1100 from *A. muciniphila* and protein HM0539 from *Lactobacillus rhamnosus* GG [56]. In an experimental mouse colitis model, both the postbiotic and prebiotic derived from *Bifidobacterium adolescentis* B8598 showed the ability to ameliorate the disease phenotype, but the postbiotic showed stronger modulating effect on the microbiota [57].

Overall, the results of this study suggest favorable effects of the *E. durans* secretomes in improving the intestinal microbial

composition, stimulating the growth of some beneficial bacterial groups. The exposure to *E. durans* secretomes had different results compared to the control treatment with PBS. In addition, the different concentrations of the secretome used showed important changes in the expression of gut microbial proteins. Considering the overwhelming complexity of the intestinal microbiome and the relatively limited research on the effect of postbiotics on gut microbiota, this study contributes to provide a better understanding on the potential of *E. durans* LAB18S as probiotic. Moreover, further studies on the effect of *E. durans* secretomes on fecal microbiota of unhealthy individuals would be also relevant.

In conclusion, the secretomes of *E. durans* LAB18S cultivated on prebiotic oligosaccharides showed differential effects on the modulation of gut microbiota, stimulating growth and different protein expression of beneficial bacteria. Although the positive health benefits of this strain should be validated, the results of this work could be translated into the development of postbiotic and/or probiotic formulations based on *E. durans* LAB18S.

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Author Contribution CBC, XZ, JM, AB and DF contributed to the study conception and design. Material preparation, data collection, CBC and KW; data analysis and statistical analysis were performed by CBC, XZ, JM. The manuscript was written by CBC, and critically revised by AB. All the authors read and approved the final manuscript.

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Availability of Data and Material The proteomics mass spectrometry data are available via ProteomicXchange under the identifier PXD040751 (Username: reviewer_pxd040751@ebi.ac.uk; Password: glc8SRky).

Declarations

Ethics Approval and Consent to Participate The protocol for stool sample collection was approved by the Ottawa Health Science Network Research Ethics Board at the Ottawa Hospital (Ottawa, Canada).

Competing Interests DF is a co-founder of MedBiome Inc., a personalized microbiome therapeutic company. The other Authors declare no conflicts of interest.

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