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Enterococcus durans Secretome Modulates Interleukins Gene Expressions in Intestinal Epithelial Cells Challenged by *Staphylococcus aureus* Secretome: In Vitro Study on the HT-29 Cell Line

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Abstract

The present study examined the effect of *Enterococcus durans* cell free supernatant (CFS) on interleukin (IL) 8, 10 and 1β gene expressions in the intestinal cell line HT-29 treated with *Staphylococcus aureus* CFS. HT-29 cells were incubated with *E. durans* CFS or *S. aureus* CFS, or *S. aureus* CFS plus *E. durans* CFS. All concentrations of *E. durans* CFS did not show cytotoxicity, while the highest treatment (44.9 μg/mL) with *S. aureus* CFS induced significant cell death. *S. aureus* CFS did not modify IL-1β gene expression, while *E. durans* CFS alone or in combination with *S. aureus* CFS reduced it. Treatment with *S. aureus* CFS induced greater expression of the IL-8 gene compared to *S. aureus* CFS plus *E. durans* CFS. *S. aureus* CFS alone or in combination with *E. durans* CFS increased the expression of the IL-10 gene, while *E. durans* CFS alone did not modify it. These results suggest a potential protective role of the *E. durans* secretome in mitigating the inflammatory environment in intestinal cells. This treatment could be useful to protect against possible contact with dangerous soluble microbial products present in food.

Keywords: cell-free supernatants; *Enterococcus*; homeostasis; HT-29 cells; inflammation; *Staphylococcus*

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1. Introduction

One of the most recurring and current public health issues is gastrointestinal tract diseases. Frequently, many pathological processes are accompanied by an inflammatory intestinal state, such as inflammatory bowel disease or simply irritable bowel syndrome, in which cells of different nature are involved. In the intestinal ecosystem, the microbiota plays an essential role in both digestive processes and in the production of active components that contribute to human health [1].

Food composition influences the intestinal microbiota [1], so its safety must be ensured to prevent the introduction of harmful pathogens and / or their products. To prevent this, attention is needed to (I) pathogenic bacteria in raw materials, (II) microorganisms used as starter cultures, or (III) any other bacterial contamination during food processing. *Staphylococcus aureus*–is part of the latter group, as responsible for clinical syndromes associated with the production of toxic molecules [2], which are not destroyed by acids, proteases, or heat treatments, remaining biologically active during all food processing [3–5]. Contaminated or inefficiently processed foods can generate a highly variable spectrum of diseases [6,7], resulting in serious public health and economic damage. Natural defenses against foodborne pathogens and their products include cytokines and chemokines, which can communicate with neighboring inflammatory cells and the immune mucosa cell system [8]. *S. aureus* enterotoxins, under particular conditions, could induce the expression of pro-inflammatory cytokines [9,10]. Interleukin 8 (IL-8) is a neutrophil chemoattractant [11,12], which amplifies the acute immune response of macrophages and neutrophils. and it is expressed and secreted by epithelial cells [13]. *S. aureus*, through the toll-like receptor 2, triggers the signaling cascade for inflammatory responses in which IL-8 is involved [14]. The intestinal epithelial tight junction (TJ) barrier controls the paracellular permeation of contents from the intestinal lumen into the intestinal tissue and systemic circulation [15]. IL-1β increase this TJ permeability, contributing to the intestinal inflammatory process [15].

S. aureus has been shown to manipulate host immunoregulatory mechanisms to facilitate persistence during infection and IL-10 appears to be at the forefront of this evasion strategy[16] Intestinal epithelial cells also secrete IL-10 and its expression can be modulated by commensal microorganisms, although the ability to stimulate cells to produce mediators is related to bacterial species and/or directly to specific strains [17,18]. Lactic acid bacteria (LAB) are commensal microorganisms of the gastrointestinal tract that can be introduced from the diet. *Enterococcus* spp. is an ancient genus of LAB that represents normal intestinal commensals and plays an important role in maintaining intestinal homeostasis through continuous immune system activities [19]. Among Enterococcaceae, the strain of *E. durans* is widely found in the production of cheese and yoghurt, showing antimicrobial properties [20] and probiotic characteristics [21,23].

Beyond live probiotics, interest is growing in probiotic-driven metabolites, known as ‘postbiotics’, soluble factors such as products or metabolic by-products released in the medium by living and growing bacteria [24]. They include organic acids, short-chain fatty acids, peptides and proteins, enzymes, cofactors, immune modulating compounds, as well as different components of bacterial lysis [25,26] that could represent bioactive components involved in a beneficial effect against some intestinal disorders [27]. Therefore, postbiotics are considered a good alternative to live bacteria with some beneficial effects on gut microbiota homeostasis, offering advantages compared to the most known ‘parent’-live probiotics, such as stability, resistance to environmental stress, and safety, as they do not carry antibiotic resistance genes.

Numerous studies have analysed the impact of metabolic compounds derived from probiotic fermentation on hazardous microorganisms or systems under dysbiosis conditions. The secretome of various intestinal bacteria was shown to exert strong antibacterial activity against *Escherichia coli, Candida albicans,* and *Clostridium perfringens* infection [28,30]. These activities are dependent on concentration and strain and are also influenced by the experimental conditions of the models used [7,31].

Enterococci strains were frequently isolated from fermented food, where they contributed to the ripening and aroma development of certain cheeses or fermented sausages. [32], these bacteria are nevertheless involved in probiotic activities [33]. Therefore, even though there is no extensive research on the immunomodulatory properties of *Enterococcus* spp., some strains are receiving increasing interest [34,35].

The HT-29 cell line is an in vitro model that is used to simulate cellular responses, making it suitable for the study of inflammatory signaling [36]. These cells are characterized by multiple functions of their normal counterparts, such as the expression of a constitutive common pattern of cytokines [7]. Ohtsuki et al. [37] reported that in vitro liposaccharides increased IL-8 secretion from HT-29, while the curative secretome derived from human amniotic mesenchymal stromal cells decreased IL-8 production by HT-29 [38].

The secretome is defined as the set of molecules and biological factors that cells release, also by vesicles, into the extracellular space [39], in in vitro cultured bacteria it corresponds to the soluble components naturally released in the medium (cell-free supernatant, CFS) [40]. The secretome of a Gram-positive bacterium can be easily extracted from growth medium and separated from other cellular contaminants [41].

In an attempt to investigate the regulation of inflammatory conditions in intestinal epithelial cells induced by the secretome of *S. aureus,* we tested the regulation of inflammatory responses in intestinal epithelial cells by a strain of *E. durans* isolated from dairy products. In particular, we have evaluated the in vitro effects of *E. durans* CFS on the gene expressions of IL-8, -10 and -1β in the HT-29 cell line pretreated with *S. aureus* CFS.

2. Materials and Methods

2.1. Bacterial Isolation and Identification

The bacteria strains were from the collection of our laboratory and classified as *E. durans* (internal reference #79) [4] and *S. aureus* (ATCC 29213, internal reference #239) [5].

sBefore the test, freeze-dried *E. durans* (internal ref. 79) was grown in Tryptic Soy Broth (TSB, BD Difco, Franklin Lakes NJ, USA) at 37 ° C for 48 h in air and then spread on mENTagar (mENT, BD Difco) at 37 ° C for 48 h to verify purity. Instead, *S. aureus* was grown in Mueller Hinton broth (MH, BD Difco, Franklin Lakes NJ, USA) at 37 ° C for 48 h on air and then spread on Baird Parker agar (BP, BD Difco) at 37 ° C for 48 h to check for purity. After incubation, the concentration for all strains was approximately 1x109 cfu/mL.

All microorganisms used in this study were kept as stocks in a freeze-dried state (-80 ° C containing 10% DMSO) until use.

2.2. Bacterial Cell Free Supernatant Preparation

E. durans was incubated for 24 hours at 37 ° C in MRS broth (de Man, Rogosa, Sharpe medium, Oxoid) and grown to a stationary phase. *S. aureus* was grown in brain heart infusion broth (BHI, Oxoid) at 37 ° C to a concentration of 108-109 cfu/mL.

Bacterial cells were removed by centrifugation (4000 xg, 15 ’at 4 ° C). Each supernatant was then filtered to sterilize and remove any bacteria using a 0,22 mm pore size filter (Millipore, USA). To ensure that the filtrates were bacteria-free, we tested the *E. durans* cell-free supernatant (Ed-CFS) and the *S. aureus* cell-free supernatant (Sa-CFS), previously introduced as secretome, on plates with the relative culture medium. CFSs were kept at -80 °C until use.

The protein content of different bacterial CFS samples was determined by a Biorad assay (Biorad Laboratories. Inc.) based on a standard curve performed by using BSA solutions (Sigma-Aldrich, MO, USA) under the same experimental conditions.

The bacterial CFS used for the experiments was diluted to optimal protein concentrations in the cell culture medium.

2.3. Cell Cultures

The human epithelial colorectal adenocarcinoma cell line HT-29 was obtained from American Type Culture Collection (ATCC) (Manassas, VA, USA). They derived from adenocarcinoma, showing characteristics of human large intestine colonocytes and human small intestine enterocytes, respectively.

HT-29 are cultured in supplemented RPMI (Roswell Park Memorial Institute) 1640 (Microtech. S.R.L. Europe, Italy, Naples) with 10% fetal bovine serum (FBS) (GIBCO Burlington, ON, Canada, BRL), 100 U/mL penicillin and 100 mg/mL of streptomycin and 1% di glutamine obtained from Sigma Chemical. Cells were kept at 37 °C in a 5% CO2 humidified incubator. The medium was changed every 3-4 days. The cells grew to 80% of confluence. Then they were detached from the flask using

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0,05% trypsin—0,1% EDTA solution from MICROGEM (Microtech. S.R.L. Europe, Italy, Naples) and after washing, the cellular pellets were resuspended in a relative supplemented medium. Cells were counted by the Trypan Blue assay on the automated cell counter Countess 3 instrument (ThermoFisher Scientific) to seed a suitable number of cells for further experiments.

2.4. Determination of Enterococcus durans and Staphylococcus aureus CFSs Cytotoxicity in HT-29 Cells

A range of protein concentrations for each secretome was established using serial two-fold dilutions of the basal CFS (from 1:2 to 1:32 dilution range) in cell medium.

The protein content recovered from the medium depended on the bacterial concentration and the growth conditions used in the experiments. The growth of the *E. durans* strain was up to 1 × 108 cfu/mL and the production of protein concentrations of 123 µg/mL, so the protein concentrations of the *E. durans* secretome ranged from 3.8 to 61.5 µg/mL. Sa-CFS obtained from *S. aureus* cultured at 1 × 10^8 cfu/mL, corresponding to 89.9 µg of proteins/mL (dilution from 2.8 to 49.9 µg/mL).

Briefly 1X 104 cells/well were seeded in 96-well plates and incubated in their complete medium for 48 hours until 80-85% of confluence. After removing the medium, cells were treated with Ed-CFS (123 g / ml base concentration), Ef-CFS (104 µg/ml basal concentration) or Sa-CFS (89,8 µg/ml basal concentration) appropriately filtered and diluted in cell medium medium for 24 h.

Following bacterial treatments, the viability was determined as reported by the manufacturer's instructions of the MTT-based in vitro toxicology assay kit (Sigma-Aldrich).

Non-cytotoxic concentrations of the tested CSFs were used for all subsequent experiments.

2.5. HT-29 Cells Treatments

HT-29 cells were detached from T75 cm^2 tissue culture flasks (NuncTM EasYFlaskTM Cell Culture Flasks), counted, resuspended in complete culture medium, seeded at a concentration of $5X10^5$ cells/mL/well, and used for the following four experimental groups:

a) incubation of HT-29 cells (2 hr) with culture medium only (Control);

b) incubation of HT-29 cells (2 hr) with culture medium plus Ed-CFS (61.5 µg protein/mL);

c) incubation of HT-29 cells (2 hr) with culture medium plus Sa-CFS (22,45 µg protein/mL);

d) incubation of HT-29 cells (2 hr) with culture medium plus Sa-CFS (22,45 µg protein/mL) followed by the addition of Ed-CFS (61.5 µg protein/mL), further 2 h of incubation.

This latter experimental group was used to determine the effects of Ed-CFS in cells that have previously been challenged by Sa-CFS.

2.6. Interleukin 8, 10, and 1β Gene Expression

Total RNA from HT-29 cells from each well was extracted using TRI reagent solution from Ambion (Applied Biosystem) according to the manufacturer's instructions. RNA concentration and purity (OD260/OD280 absorption ratio > 1.9) was assessed by spectrophotometry analysis determined using a NanoDropTM (TermoFisher Scientific, Wilmington, DE, USA). Total RNA (1 µg of each sample) was then reverse transcribed into cDNA using High-Capacity cDNA reverse transcription kit (ThermoFisher Scientific). Finally, quantitative real-time PCR (qRT-PCR) was performed with the SYBR Green method using the PowerUp SYBR Green Master Mix (ThermoFisher Scientific).

The primer sequences used for the analysis of the genes IL8, IL10, IL1β, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are listed in Table 1. GAPDH represented the housekeeping gene. It was used as an internal control for the normalisation of gene expression. The specificity of each primer pair was verified by the presence of a single melting temperature peak. The reactions were performed in a Bio-Rad i-Cycler thermal cycler and conducted in triplicate.

The level of mRNA expression of each gene was analysed according to the Ct values of the target genes and the endogenous control (GAPDH) of each experiment and calculated using the $2^{-\Delta\Delta Ct}$ method.

Table 1. Primer sequences.

Gene	Primer	Sequence (5′ → 3′)
IL-8	forward	GACATACTCCAAACCTTTCCA
	reverse	AACTTCTCCACAACCCTCT
IL-10	forward	GCTTCTGGTGAAGGAGGATC
	reverse	TCTTGGTTCTCAGCTTGGGG
IL-1β	forward	GGACCTGGACCTCTGCCCTCTGG
	reverse	GCCTGCCTGAAGCCCTTGCTGTAG
GAPDH	forward	TGGTATCGTGGAAGGACTCATGAC
	reverse	ATGCCAGTGAGCTTCCCGTTCAGC

2.7. Statistical Analysis

Statistical analyses were performed using GraphPad Prism (version 8.4.3 for Mac OS). Differences among groups were evaluated by one--way ANOVA followed by Tukey's multiple comparison test. Data are expressed as mean ± SD of three independent experiments, each performed in quadruplicate, unless otherwise stated. For all tests, a two--tailed significance level of $p<0.05$ was considered statistically significant.

3. Results

3.1. Cytotoxicity of Enterococcus durans and Staphylococcus aureus in HT-29 Cells

All concentrations of *E. durans* CFS did not show significant cytotoxic effects after 24 hours of supplementation with HT-29 and (Figure. 1a).

Only 24 hours with 44.9 μg/mL of *S. aureus* CFS induced significant ($p<0.05$) cell death by up to 48.8% (Figure 1b).

3.2. Effect of Enterococcus durans and Staphylococcus aureus CFS on HT-29 Interleukin 8, 10, and 1β Gene Expression

Interleukin 8. Treatments with Ed-CFS and Sa-CFS alone or in combination did not modify IL-8 gene expression compared to the control (Fig. 2a). The expression of the IL-8 gene was higher ($P<0.001$) in Sa-CFS alone than in Sa-CFS plus Ed-CFS (Figure 2a).

Interleukin 1β. Treatments with Sa-CFS alone did not modify IL-1β gene expression compared to control (Figure 2b), whereas Ed-CFS alone or in combination with Sa-CFS reduced ($P<0.05$) this gene expression (Figure 2b). IL-1β gene expression was higher ($P<0.001$) in Sa-CFS alone than in Ed-CFS alone or in Sa-CFS plus Ed-CFS (Figure 2b).

Interleukin 10. Ed-CFS treatments did not modify IL-10 gene expression compared to the control, while Sa-CFS alone ($p<0.001$) or Sa-CFS plus Ed-CFS ($p<0.05$) increased this gene expression (Figure 2c), this increase was greater ($p<0.001$) in Sa-CFS alone than in Sa-CFS plus Ed-CFS (Figure 2c). IL-10 gene expression was higher ($p<0.001$) in Sa-CFS alone and in Sa-CFS plus Ed-CFS than in Ed-CFS alone (Figure 2c)

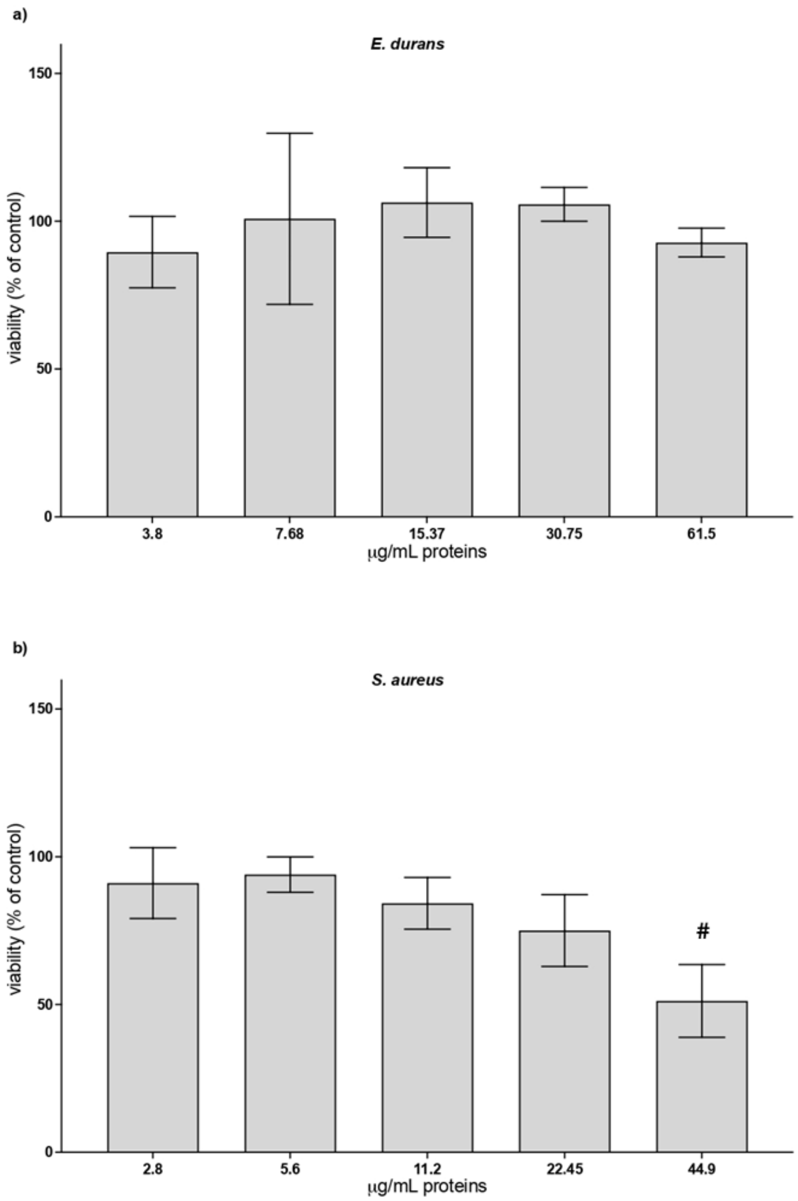

Figure 1. Cell viability of HT-29 cells after treatment with *E. durans* CFS (a panel) or *S. aureus* CFS (b panel) treatment. The X axis represents the CFS proteins expressed in µg/mL. Values are reported with respect to control cells set as 100%. All results are expressed as mean ± S.D. of 4 samples. Statistical significance: #$p < 0.05$ *vs* control.

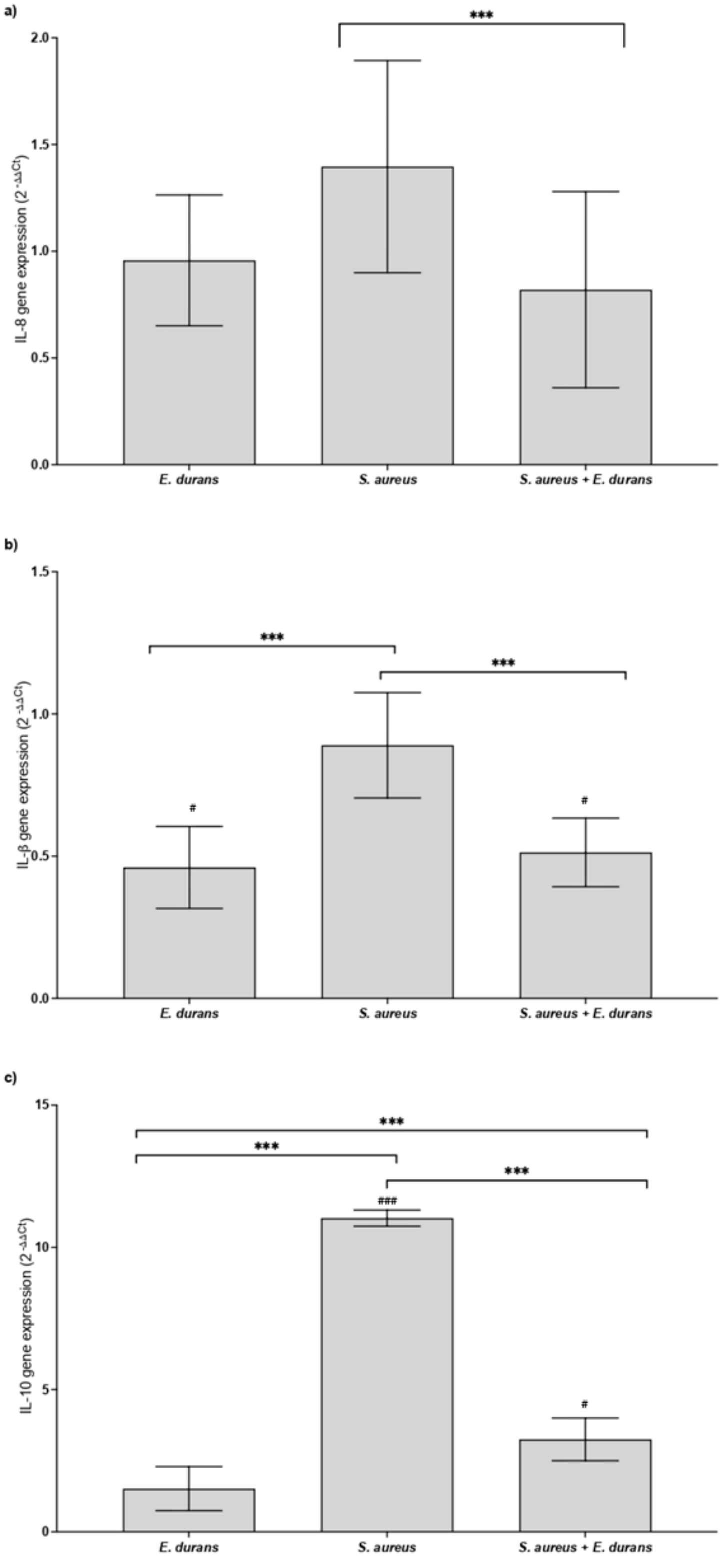

Figure 2. Effects of *E. durans* CFS alone, *S. aureus* CFS alone or *E. durans* CFS plus *S. aureus* CF on gene expression levels of IL-8 (a panel), IL-1β (b panel), IL-10 (c panel) in HT-29 cells. Gene expression values were normalized to GAPDH and presented as $2^{-\Delta\Delta Ct}$. Data represent mean ± S.D of 3 samples. Statistical significance: #p< 0.05 *vs* control, ###p < 0.001 *vs* control, *p< 0.05, ***p < 0.001.

4. Discussion

The present study suggests that the *E. durans* secretome represents a potentially reducing agent the inflammatory state of the intestinal epithelium, particularly the one induced in the HT-29 intestinal cell line by the *S. aureus* secretome.

The strain of *E. durans*, isolated from Umbrian dairy production and used in the present investigation, was chosen because its isolation from fermented foods has ensured the evaluation of the virulence of Ed-CFS [4], as confirmed by our data on the non-cytotoxicity of *E. durans* in HT-29 cells. Treatment with the *E. durans* secretome for 24 h did not show cytotoxic effects in HT-29 epithelial cells, demonstrating that all secreted products were not hazardous. Viability was always maintained at each concentration of the CFS protein used and was greater than 90% in HT-29 compared to control cells.

These data are consistent with a previously published study on the safe in vitro use of the enterococci secretome in Caco-2 cells [42]. On the contrary, the present data reported that 24 h administration of the *S. aureus* secretome (45 µg/mL) has induced significant cell death in HT-29 cells, up to 47%. Regarding the effects on IL8 gene expression, all Ed-CFS and Sa-CFS added alone or in combination maintained the same level of gene expression of control cells, demonstrating that there was no induction of this interleukin in colonocytes and confirms previous data in Caco-2 cells [43]. Despite this, interestingly, our results show a significant difference in gene activity between treatments with Sa-CFS alone (higher levels) and those with Sa-CFS plus Ed-CFS (lower levels); this could suggest an inhibitory effect of the secretome of *E. durans* on the activity of the IL8 gene affected by *S. aureus*.

Various authors [44,45] reported that IL-8 expression is very low in normal tissue and is tightly regulated; in particular, HT-29 cells show a low level of constitutive IL-8 secretion [46]. Mc Cracken et al. [47] showed that IL-8 was not detectable in HT-29 cells and that it was not altered after coculture with *Lactobacillus planctarum*. In this context, Ma et al. [46] reported that IL-8 synthesis did not increase when intestinal epithelial cells HT-29 and Caco-2 were stimulated with *L. reuteri*. Subsequently, Jeffrey et al. [48] demonstrated that exposure of host epithelial cells to *L. rhamnosus* and *L. helveticus*, without any innate immune stimulants, did not induce alteration in constitutive IL-8 production of IL-8 in HT-29 cells. In our study, the significant difference between the experimental groups of Sa-CFS and Sa-CFS plus Ed-CFS appears to suggest that the secretome of *E. durans* affects Il-8 secretion only after Sa-CFS exerted its effects.

Intestinal epithelial cells of the mucosa do not produce a significant amount of IL-1β under physiological conditions IL-1β [45]. In our experimental model, we found that its basal gene expression was significantly reduced by the secretome of *E. durans*, both alone and in combination with those of *S. aureus*. The latter alone, in turn, did not show an effect on IL-1β gene expression, suggesting that the two secretomes do not interact in the regulation of IL-1β.

The most intriguing data from the study concern IL-10 gene expression: the administration of Ed-CFS alone did not affect the level of gene expression; instead, this gene activity showed a massive and significant increase after the administration of Sa-CFS alone, which was reduced, but not cancelled, with subsequent treatment with Ed-CFS; in fact, the value of mRNA decreased significantly compared to Sa-CFS alone, but remained significantly higher than the baseline value. There is a large body of literature on the ability of *S. aureus* to escape the immune system [49], and the fact that the production of IL-10 can facilitate bacterial persistence [50,51]. Recently, it has been attributed to staphylococcal lactate the increase in IL-10 gene transcription [52]. Consistent with the above findings, we observed a significant increase in IL-10 gene expression in HT 29 cells in the presence of Sa CSF, confirming the ability of *S. aureus* to inhibit the onset or repress the hostile inflammatory environment through its natural secretions, including food-related enterotoxins [53]. Moreover, our data suggest that this activity of enterotoxins is drastically reduced by the presence of *E. durans*. As previously demonstrated, the decreased level of IL-10 expression after *E. durans* secretome supplementation did not return to baseline levels, suggesting a persistent state of tolerance with a concomitant inflammatory alert [54].

The data from the present study evidence the promising ability of the *E. durans* secretome to modulate the inflammatory responses of the eukaryotic host, representing a good alternative to mimicking and reproducing the activities of the corresponding lived or inactivated bacteria while avoiding the possibility of side effects. Overall data, even in their complexity, substantially support the potential postbiotic properties of the *E. durans* strain.

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Conflicts of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationship that could be construed as a potential conflict of interest.

Abbreviations

The following abbreviations are used in this manuscript:

CFS	cell-free supernatant
Ed	*Enterococcus durans*
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
IL	interleukin
LAB	lactic acid bacteria
MTT	3-(4,5-dimethylthiazol-2-thiazolyl)-2.5-diphenyl-2H-tetrazolium bromide
PCR	polymerase chain reaction
Sa	*Staphylococcus aureus*

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