ORIGINAL ARTICLE

AG-2021-23
doi.org/10.1590/S0004-2803.202100000-79

Synergistic immunomodulatory activity of probiotics *Bifidobacterium animalis* and *Lactobacillus casei* in Enteroaggregative *Escherichia coli* (EAEC)-infected Caco-2 cells

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Received: 3 February 2021
Accepted: 3 May 2021

ABSTRACT – **Background** – Enteroaggregative *Escherichia coli* (EAEC) is an *E. coli* pathotype that presents aggregative adhesion patterns on in vitro cultivated cells, mainly related to persistent diarrhea cases in children. EAEC virulence factors are important for host colonization and pathogenicity. Intestinal epithelial cells (IECs) recognize pathogen-associated molecular patterns (PAMPs) and initiate an immune response. Several studies using in vivo and in vitro models emphasize the probiotic activity and immunomodulatory capacity of *Lactobacillus* and *Bifidobacterium* species. **Objective** – To evaluate the modulation of cytokine production by probiotics *Bifidobacterium animalis* and *Lactobacillus casei* in human intestinal Caco-2 cells exposed to different strains of EAEC. **Methods** – Caco-2 cells were incubated with EAEC strains in the presence or absence of probiotics. The production of cytokines IL-8, TNF-α, IL-1β and IL-10 was evaluated in the supernatants by a sandwich enzyme-linked immunosorbent assay (ELISA). **Results** – Cytokine production did not change when uninfected and EAEC-infected Caco-2 cells were exposed to probiotics separately. All EAEC induced a significant increase in IL-8 production by Caco-2 cells, but the probiotics, even together, could not reduce its production. On the other hand, the synergic activity of probiotic strains significantly increased TNF-α production but decreased the basal production of IL-1ß. Also, probiotics induced a significant increase in the production of the anti-inflammatory cytokine IL-10 during EAEC infection. **Conclusion** – Our results reinforce the synergistic immunomodulatory activity of probiotics during EAEC infection.

Keywords – *Escherichia coli*; probiotics; *Bifidobacterium animalis*; *Lactobacillus casei*; cytokines; epithelial cells; immunity.

INTRODUCTION

Intestinal infections caused by *Escherichia coli* are set due to infection by pathotypes of diarrheagenic *E. coli* (DEC) strains, which includes Enteroaggregative *Escherichia coli* (EAEC)[1]. This pathotype is recognized as the cause of persistent diarrhea in children, foodborne outbreaks, traveler's disease and intestinal infections of people infected with human immunodeficiency virus (HIV)[2,3].

EAEC strains are characterized by manifesting aggregative adherence (AA) pattern ("stacked bricks") on HEp-2 cells and may present the aggregative adherence plasmid (pAA)[1,4]. The pathogenesis of infection follows up three stages: (i) initial adherence to the mucosa and early colonization with biofilm formation; (ii) toxins secretion; and (iii) inflammatory response induction, cytokine secretion and mucosal damage[2]. Virulence genes, such as aggregative adherence fimbriae (AAF) located on pAA and cytotoxins are essential for EAEC colonization, which assure adherence and invasion, mediating inflammatory responses as well[5-7]. The inflammatory response includes increased secretion of pro-inflammatory cytokines, leukocyte migration to the infected site and tissue damage, being close to diarrheal disease symptoms[8,9]. EAEC strains can promote IL-8, IL-1β, IL-6, and TNF-α production by intestinal epithelial cells (IECs) as shown in studies using in vitro models and infected patients' analysis. However, the production of anti-inflammatory cytokines such as IL-10 has not been detected[6,10,11].

IECs play an essential role in the recognition of microorganisms in the intestinal lumen through pattern recognition receptors (PRRs) that recognize pathogen-associated molecular patterns (PAMPs), such as molecules expressed on the bacterial surface. Toll-like receptors (TLRs), which can be expressed on cell membranes, are important proteins included in the PRRs group. PAMPs are recognized by PRRs located on IECs membrane, resulting in intestinal cell activation. This process leads to different immune responses, managing colonization or infection progress[12,13].

Probiotics are live non-pathogenic microorganisms found on different surfaces such as the urogenital and gastrointestinal mucosa that confer health benefits to the host as protection against

Declared conflict of interest of all authors: none
Disclosure of funding: this work was supported by *Coordenação de Aperfeiçoamento de Pessoal de Nível Superior* – Brasil (CAPES) – Finance Code 001; *Conselho Nacional de Desenvolvimento Científico e Tecnológico* (CNPq), *Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro* (FAPERJ) and *Sub Reitoria de Pós Graduação e Pesquisa* (SR-2/UERJ).
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colonization by pathogens[14]. Probiotics are also found in fermented foods such as dairy products[15,16]. The group of lactic acid bacteria (LAB) includes *Lactobacillus* and *Bifidobacterium* genera and their species. These microorganisms are widely used in probiotic activity studies[17].

Several studies report LAB probiotic activity against foodborne pathogens such as DEC, including their ability to reduce bacterial biofilm formation, reduce adherence, inhibit damage to in vitro cultured cells and prevent diarrhea on in vivo models. LAB also present antimicrobial properties and immunomodulatory capacity[17,18]. This study investigated cytokines' production by Caco-2 cells exposed to different EAEC strains and their modulation by probiotics *Bifidobacterium animalis* and *Lactobacillus casei.*

METHODS

Bacterial strains

Four EAEC strains were included in this study. The prototype EAEC 042, EAEC I49 and EAEC H92/3 strains were previously isolated and characterized for putative virulence factors and adherence pattern[11,19-21]. EAEC 1500 strain was isolated from faeces of hospitalized HIV$^+$ patients with persistent diarrhea in Rio de Janeiro. All EAEC strains were stored at -80°C in Luria Broth (LB; Difco Laboratories) supplemented with 20% glycerol (Merck).

Probiotic strains *Bifidobacterium animalis* DN 173 010 (Activia – Danone®) and *Lactobacillus casei* Shirota (Yakult®), respectively identified as *Ba* and *Lc*, were kindly provided by Paulo de Góes Microbiology Institute (Rio de Janeiro Federal University – UFRJ) or obtained from commercial products. Probiotic strains were stored at -80°C in Man, Rogosa and Sharpe broth (MRS; Difco Laboratories) supplemented with 20% glycerol (Merck) and maintained on MRS agar (Difco Laboratories) at 4°C.

Cell culture and infection

The human intestinal colon adenocarcinoma Caco-2 cell line (ATCC HTB37) was cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 2% Fetal Bovine Serum (FBS) and antibiotics in 24-well plates for 14 days, when cell monolayers were polarized and differentiated. Infection with EAEC and probiotic strains was performed as previously described by Rosa et al.[22] and Braga et al.[11] with modifications.

Probiotic strains were cultured in 3 mL of MRS broth (Difco) and incubated for 48 hours at 37°C under anaerobic conditions. Bacterial EAEC strains were cultured in 3 mL of LB (Difco) and incubated for 18 hours at 37°C. Aliquots of 100 µL of EAEC and probiotics standardized suspensions (approximately 10^7 CFU.mL^{-1}) were inoculated on the cell monolayers in triplicates. Caco-2 cells were exposed to EAEC strains, probiotics only, and EAEC+probiotics (each probiotic and both probiotics together). The plates were centrifuged at 2500 rpm for five minutes at 15°C for synchronization and incubated for three hours. After incubation, the wells were washed and added DMEM medium supplemented with 2% of FBS, 1% D-mannose and 100 µg/mL amikacin (Teuto, Brazil). Plates were then cultured for additional 21 hours. Following a total of 24 hours of incubation, supernatants were collected, centrifuged, transferred to microtubes and stored at -20°C.

The production of cytokines IL-8, IL-1β, TNF-α and IL-10 was measured in the supernatants by a sandwich enzyme-linked immunosorbent assay (ELISA), according to the manufacturer's instructions (R&D Systems – Wiesbaden, Germany).

Statistical analysis

Cytokine concentrations were expressed as mean and standard error of mean (S.E.M) from three experiments performed independently. Statistical analyses were performed using Graph-Pad Prism version 7.0. Mann-Whitney test was performed to compare differences between different culture conditions and $P<0.05$ was considered statistically significant.

RESULTS

There was no significant change in the production of the cytokines IL-8, IL-1β, IL-10 and TNF-α by Caco-2 cells exposed to each probiotic alone or to EAEC in co-cultivation with probiotics *Ba* and *Lc* separately when compared to basal production by non-infected cells (data not shown).

IL-8 production by Caco-2 was significantly increased by EAEC strains 042 (P=0.0004, FIGURE 1.A), I49 (P=0.0008, FIGURE 1.B), H92/3 (P<0.0001, FIGURE 1.C) and 1500 (P=0.0004, FIGURE 1.D) but not by the probiotics *Ba+Lc* (FIGURE 1). The EAEC 042 strain (FIGURE 1.A) co-cultivated with probiotics also significantly increased IL-8 production when compared to non-infected cells (P=0.0004) and cells exposed to both probiotics (P=0.0496). Similar results were observed to EAEC I49 strain (P=0.0008 and P=0.0319, respectively, FIGURE 1.B) and EAEC 1500 (P=0.0004 and P=0.039, respectively, FIGURE 1.D), but the amounts of IL-8 induced by EAEC H92/3 in the presence of both probiotics were higher only when compared to basal production (P=0.0003, FIGURE 1.C). Interestingly, both probiotics, together with each EAEC strain, did not reduce the IL-8 production induced by the EAEC strains (FIGURE 1).

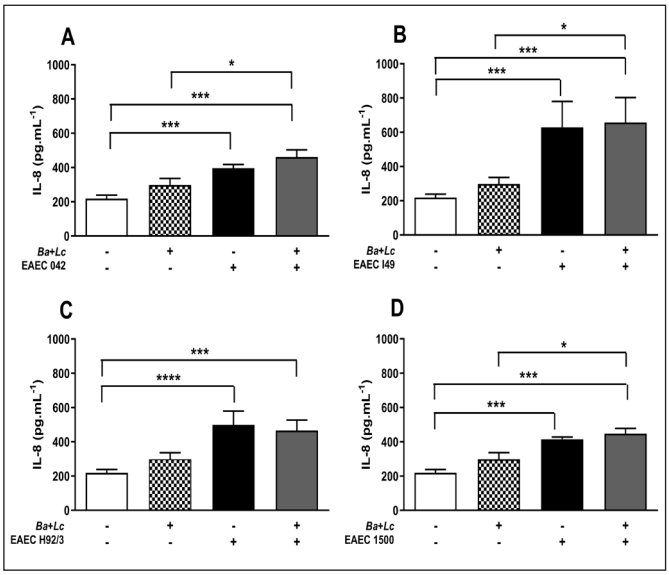

FIGURE 1. IL-8 production by uninfected Caco-2 cells (basal production), exposed to both probiotic strains (*Ba+Lc*) and to EAEC 042 (A), I49 (B) H92/3 (C) and 1500 (D) strains in the absence and in the presence of both probiotics. Co-cultivation lasted for three hours and cells were cultured for additional 21 hours. Results are expressed as mean and S.E.M from three experiments performed independently. EAEC: Enteroaggregative *Escherichia coli*; *Ba: Bifidobacterium animalis*; Lc: *Lactobacillus casei*. Differences between culture conditions were calculated using Mann-Whitney test. *P<0.05; ***P<0.001; ****P<0.0001.

The infection of Caco-2 cells by EAEC strains used in this study did not induce IL-1β production (FIGURE 2). Importantly, Caco-2 cells exposed only to *Ba+Lc* probiotic strains reduced IL-1β production significantly compared to basal levels (P=0.0411, FIGURE 2). Moreover, co-cultivation of the EAEC I49 (FIGURE 2.B) with both probiotics reduced significantly IL-1β production compared to cells infected only (P=0.0095) and compared to basal production (P=0.0216). Besides, probiotics, even in the presence of H92/3 (FIGURE 2.C) or 1500 strain (FIGURE 2.D), also reduced the production of IL-1β compared to basal levels (P=0.0238 and P=0.0303, respectively).

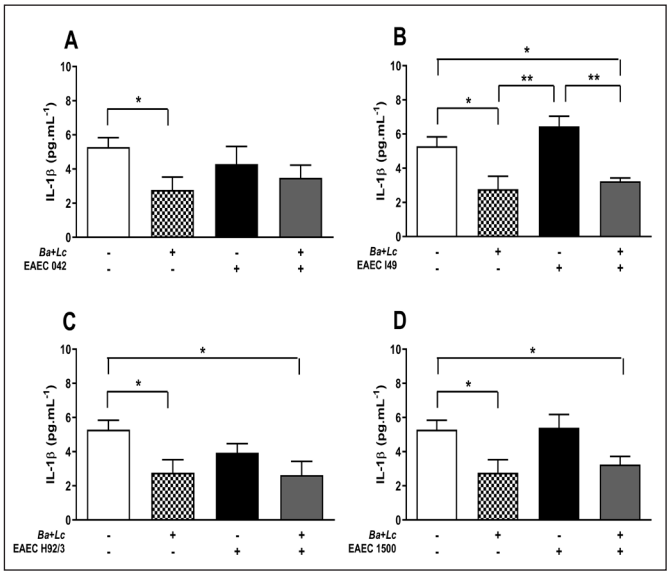

FIGURE 2. IL-1β production by uninfected Caco-2 cells (basal production), exposed to both probiotic strains (*Ba+Lc*) and to EAEC 042 (A), I49 (B) H92/3 (C) and 1500 (D) strains in the absence and in the presence of both probiotics. Co-cultivation lasted for three hours and cells were cultured for additional 21 hours. Results are expressed as mean and S.E.M from three experiments performed independently. EAEC: Enteroaggregative *Escherichia coli*; *Ba: Bifidobacterium animalis*; Lc: *Lactobacillus casei*. Differences between culture conditions were calculated using Mann-Whitney test. *P<0.05; **P<0.01.

As observed to IL-1β, none of EAEC strains significantly modified TNF-α production (FIGURE 3). However, Caco-2 cells exposed to both probiotics showed higher levels of TNF-α production compared to basal ones (P=0.026) and to EAEC strains 042 (P=0.026, FIGURE 3.A), H92/3 (P=0.0087, FIGURE 3.C) and 1500 (P=0.0087, FIGURE 3.D). Furthermore, co-cultivation of EAEC strains 042 (FIGURE 3.A), I49 (FIGURE 3.B), H92/3 (FIGURE 3.C) and 1500 (FIGURE 3.D) with both probiotics induced significantly higher levels of TNF-α compared to the production by Caco-2 cells alone (P=0.0043, P=0.0022, P=0.0411 and P=0.011, respectively) or to the EAEC infected ones (P=0.0043, P=0.0022, P=0.0108 and P=0.0087, respectively).

The production of anti-inflammatory cytokine IL-10 was not altered by exposition to both probiotics only. However, except for the H92/3 strain, EAEC strains' infection reduced IL-10 levels compared to baseline production, although with no statistical difference (FIGURE 4). The presence of both probiotics held IL-

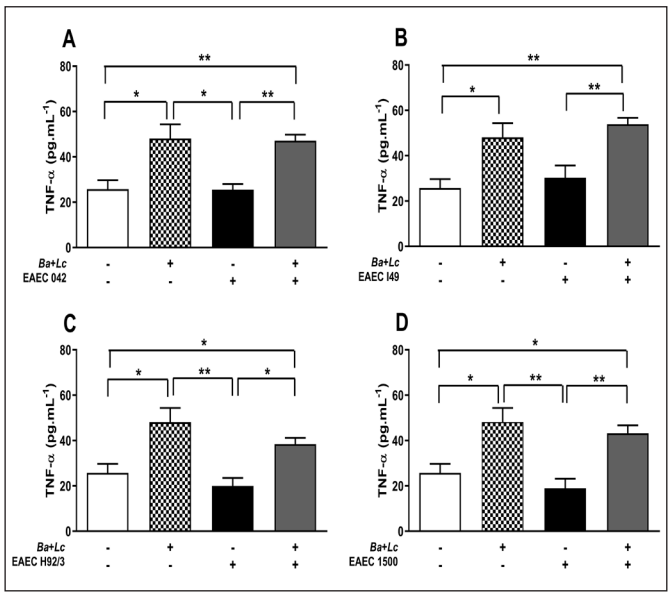

FIGURE 3. TNF-α production by uninfected Caco-2 cells (basal production), exposed to both probiotic strains (*Ba+Lc*) and to EAEC 042 (A), I49 (B) H92/3 (C) and 1500 (D) strains in the absence and in the presence of both probiotics. Co-cultivation lasted for three hours and cells were cultured for additional 21 hours. Results are expressed as mean and S.E.M from three experiments performed independently. EAEC: Enteroaggregative *Escherichia coli*; *Ba: Bifidobacterium animalis*; Lc: *Lactobacillus casei*. Differences between culture conditions were calculated using Mann-Whitney test. *P<0.05; **P<0.01.

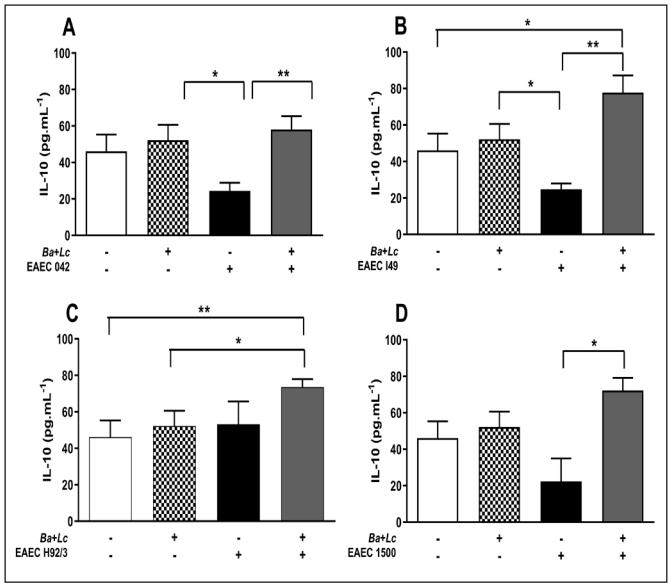

FIGURE 4. IL-10 production by uninfected Caco-2 cells (basal production), exposed to both probiotic strains (*Ba+Lc*) and to EAEC 042 (A), I49 (B) H92/3 (C) and 1500 (D) strains in the absence and in the presence of both probiotics. Co-cultivation lasted for three hours and cells were cultured for additional 21 hours. Results are expressed as mean and S.E.M from three experiments performed independently. EAEC: Enteroaggregative *Escherichia coli*; *Ba: Bifidobacterium animalis*; Lc: *Lactobacillus casei*. Differences between culture conditions were calculated using Mann-Whitney test. *P<0.05; **P<0.01.

10 levels similar to basal levels but statistically higher than that induced by the infection with 042 strain (P=0.0152, FIGURE 4.A) and I49 strain (P=0.0346, FIGURE 4.B). Notably, co-cultivation of both probiotics in the presence of EAEC strains induced a significant increase in the amount of IL-10 compared to that produced only in the presence of 042 (P=0.0065, FIGURE 4.A), I49 (P=0.0043, FIGURE 4.B) and 1500 (P=0.0216, FIGURE 4.D) EAEC strains. The association of both probiotics and EAEC H92/3 strain increased IL-10 levels compared to basal production and cells exposed to both probiotics only (P=0.0065 and P=0.026, respectively, FIGURE 4.C).

DISCUSSION

Diarrhoeagenic *Escherichia coli* (DEC) strains are highlighted as the most common intestinal infections cause among enteric pathogens. As a common characteristic, *E. coli* pathotypes included in DEC group colonize mucosa by adhering to IECs, often evading host defenses and causing tissue damage. These events are often associated with alteration in water absorption and electrolytes, causing diarrhea. EAEC is the most frequently detected pathotype in acute and persistent diarrhea in children cases worldwide[1,23,24].

The mucosa is an essential component of natural immunity, acting as a barrier to microorganisms. When an enteric pathogen overcomes this host defense, IECs can induce an inflammatory response, resulting in intestinal damage and symptoms associated with the infection[9,12,13]. Studies have shown the association between virulence factors and stimulation of cytokine production in EAEC infection. Increased levels of the cytokines IL-1β, IL-8, IFN-γ and other inflammatory markers in stool samples, such as lactoferrin, gross mucus, leukocytes and occult blood, are related to some virulence factors, including AggR, AAFs, dispersin and flagellin[2,11,25-28].

Several studies have adopted in vitro cell infection model to investigate cytokine production induced by EAEC infection. Braga et al.[11] studied three of the four strains used in this work (042, I49 and H92/3). The authors observed that all EAEC strains induced IL-8 production by differentiated T84 cells. Other studies also show that Caco-2 cells produce IL-8 in vitro after EAEC infection[2,26]. IL-8 is associated with the inflammatory response of EAEC infection and is a central interleukin involved in polymorphonuclear (PMN) leukocyte chemotaxis[2]. In our study, EAEC strains were evaluated for inducing cytokine production in polarized and differentiated Caco-2 cells. Our results corroborate previous studies, once all EAEC strains induced IL-8 production and EAEC 042, I49 and H92/3 strains present AAFs and aggR transcriptional activator. These virulence factors are related to inflammatory response, which could explain the high IL-8 production by infected Caco-2 cells[11,25,26]. We will further perform the genotypic characterization of EAEC 1500 strain.

The production of other pro-inflammatory cytokines besides IL-8 has been reported. Using the cell line HCT-8 derived from colonic adenocarcinoma, Cennimo et al.[27] detected increased IL-8, IL-6 and TNF-α production induced by EAECs expressing AggR. Using the same cell line and measuring mRNA expression, Medeiros et al.[29] observed that the strain EAEC 042 induced the expression of IL-8 and TNF-α, which did not occur with IL-6. Also, this strain reduced the expression of TGF-β. Braga et al.[11] also observed the stimulation of IL-6 and TNF-α production by T84 cells infected with EAEC 042 and I49 strains. Although we observed an increase in IL-8 levels, we did not detect an increase in the production of IL-1β and TNF-α by Caco-2 cells infected with EAEC strains, which may be associated with low TLR expression at IECs apical membrane under homeostatic conditions[30] or due to the different IEC used in our system.

Probiotics are microorganisms that colonize surfaces such as mucosa and confer benefits to the host and their prophylactic or therapeutic use is related to action against intestinal dysbiosis, especially antibiotic-associated diarrhea and infectious diarrhea[31,32]. Probiotic bacteria, especially *Bifidobacterium* and *Lactobacillus* species, may inhibit growth, toxin production and other virulence factors expression of pathogenic microorganisms[33].

Probiotics also play an important role in the development of mucosal immunity. Ogawa et al.[34] identified high levels of anti-Stx 1 and 2 IgA in Shiga toxin-producing *E. coli*-infected rabbits after *L. casei* treatment, decreasing intestine toxin concentration of the animals. Ashraf and Shah[35] also associated immunological mechanisms, including higher numbers of IgA+ cells in animals' intestines, to the administration of probiotic bacteria *B. animalis*, *L. johnsonii*, *B. lactis* and the yeast *Saccharomyces boulardii*. The immunomodulatory properties of probiotics have been linked to TLR and NF-ϰB activation pathways. Karlsson et al.[36] found that a *L. rhamnosus* strain increases NF-ϰB expression of uropathogenic *E. coli*-infected bladder cells by TLR-4 activation, with increased TNF-α production. Jung et al.[37] observed that a *L. sakei* strain increased phagocytic activity and induced nitric oxide (NO) production, IL-6 and TNF-α by probiotic-exposed macrophages through activation of NF-ϰB. This inflammatory response was related to TLR-2 activation since the authors observed that the blockage of TLR-2 inhibited NO production. Rocha-Ramírez et al.[38] also obtained similar results in which lactobacilli strains led to NF-ϰB activation and the production of activated macrophage-related cytokines as a TLR-2-dependent inflammatory response.

Multispecies synergism is an essential factor in the action of probiotics against pathogens[39]. Lee et al.[16] demonstrated that yogurt consumption containing *B. animalis*, *L. paracasei* and *L. plantarum* together is related to increased NK cell action, suggesting that multiple probiotic sources may have a positive effect on host immune response. In this study, *B. animalis* and *L. casei* separately were unable to modulate cytokine production by Caco-2 cells exposed to different EAECs strains, suggesting that these probiotics strains better act synergistically.

In our study, EAEC strains induced IL-8 production, and probiotics could not reduce its production. Probiotics induced a reduction in IL-1β production when compared to non-infected Caco-2 cells. Additionally, probiotics were able to reduce IL-1β levels induced by EAEC I49 strain. Interestingly, EAEC infected and non-infected Caco-2 cells exposed to probiotics produced higher amounts of TNF-α.

The absence of the modulation in IL-8 production and the induction of increased TNF-α levels by probiotic-exposed Caco-2 cells conflict with some results of previous studies using co-cultivation in vitro models[40,41]. Jiang et al.[40] demonstrated that *L. plantarum* decreased the expression of pro-inflammatory cytokines induced by *Salmonella typhimurium* in Caco-2 cells but had no effect on IL-10 expression. Recently, Kim et al.[41] have shown that *L. acidophilus* reduced the expression of TNF-α while increasing IL-10 expression in RAW-macrophages stimulated with LPS. On the other hand, different studies have shown that TNF-α production is at least in part dependent on NF-ϰB activation and that TLR expression is induced in IECs exposed to probiotics. In this way, the increase in TNF-α production by probiotic-exposed

and infected Caco-2 cells observed in our system may be related to the signaling pathway of TLR responsive to probiotics and surface molecules present in enterobacteria, such as LPS[30,36-38,42].

IL-10 has a regulatory function of the immune response by inhibiting the inflammatory response. We found that Caco-2 cells infected with three of the four EAEC strains and treated with probiotics produced higher levels of IL-10 than untreated cells, highlighting the immunomodulatory properties of probiotics as previously described[40,41].

This study demonstrates the synergistic activity of probiotics *B. animalis* and *L. casei* in modulating inflammation induced by EAEC in Caco-2 cells.

Authors' contribution

Ferreira AF: survey execution, data analysis, writing of text. Braga RLL: survey execution. Andrade MF: survey execution. Rosa ACP: designed the research, data analysis, writing of text. Pereira-Manfro WF: designed the research, data analysis, writing of text.

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Ferreira AF, Braga RLL, Andrade MF, Rosa ACP, Pereira-Manfro WF. Atividade imunomoduladora sinérgica de probióticos *Bifidobacterium animalis* e *Lactobacillus casei* em células Caco-2 infectadas com *Escherichia coli* enteroagregativa (EAEC). Arq Gastroenterol. 2021;58(4):433-8.

RESUMO – **Contexto** – *Escherichia coli* enteroagregativa (EAEC) é um patotipo de *E. coli* que apresenta o padrão de aderência agregativa em células cultivadas in vitro, sendo comumente relacionada a casos de diarreia persistente em crianças. Fatores de virulência presentes em EAEC são importantes para a colonização do hospedeiro e patogenicidade. As células epiteliais intestinais (IECs) reconhecem padrões moleculares associados a patógenos (PAMPs) e iniciam uma resposta imune. Vários estudos usando modelos in vivo e in vitro enfatizam a atividade probiótica e a capacidade imunomoduladora de espécies de *Lactobacillus* e *Bifidobacterium*. **Objetivo** – Este estudo avaliou a modulação da produção de citocinas pelos probióticos *Bifidobacterium animalis* and *Lactobacillus casei* em células intestinais humanas Caco-2 expostas a diferentes cepas de EAEC. **Métodos** – As células Caco-2 foram incubadas com as cepas de EAEC na presença ou ausência dos probióticos. A produção das citocinas IL-8, TNF-α, IL-1β e IL-10 foi avaliada nos sobrenadantes por ELISA sanduíche. **Resultados** – Não houve alteração na produção de citocinas quando as células não infectadas e as células infectadas com EAEC foram expostas aos probióticos separadamente. Todas as cepas de EAEC induziram aumento significativo na produção de IL-8 pelas células Caco-2, mas os probióticos, ainda que em conjunto, não foram capazes de reduzir a produção desta citocina. Por outro lado, as cepas de probióticos aumentaram significativamente a produção de TNF-α mas diminuíram a produção basal de IL-1ß. Além disso, os probióticos induziram um aumento significativo na produção da citocina anti-inflamatória IL-10 durante a infecção por EAEC. **Conclusão** – Nossos resultados reforçam a atividade imunomodulatória sinérgica dos probióticos durante a infecção de EAEC.

Palavras-chave – *Escherichia coli*; probióticos; *Bifidobacterium animalis*; *Lactobacillus casei*; citocinas; células epiteliais; imunidade.

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