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The immunomodulating effect of bifidobacteria is modified by the anticoagulant acenocoumarol

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ABSTRACT

Bifidobacteria are health promoting microorganisms that can interact with drugs in the intestinal tract thus modifying the activation of immune cells and host homeostasis. To study the effect of acenocoumarol on the interaction between bifidobacteria and THP-1 cells or intestinal epithelial cells. *Bifidobacterium bifidum* CIDCA 5310 and *B. adolescentis* CIDCA 5317 were grown in MRS broth in anaerobic conditions. The experiments were done in the presence of acenocoumarol. FITC-labeled bacteria were incubated with THP1 cells differentiated with PMA (THP1-PMA), and phagocytosis and intracellular localization of bacteria were assessed by flow cytometry and confocal laser microscopy, respectively. Also, expression of macrophage surface molecules in THP1 and THP1-PMA cells was assessed by flow cytometry. By using the Caco-2 ccl20:luc reporter system innate immune response was analysed by determining luciferase expression induced by flagellin. Acenocoumarol decreased phagocytosis of bifidobacteria but intracellular routing was not modified. Also, anticoagulant reduced the expression of HLA-DR and CD64 induced by bifidobacteria in both THP1-PMA and THP1 cells. Instead, the decrease in the expression of CD206 and CD23 was dependent on differentiation status of THP1 cells. Acenocoumarol increased CCL20 expression and it was partially abrogated by strain CIDCA 5310. Presence of acenocoumarol modifies the interaction between bifidobacteria and eukaryotic cells thus suggesting that the expected modulation of immune response by probiotic foods can be different in persons under anticoagulant treatment.

1. Introduction

Probiotics are preparations based on viable microorganisms that lead to beneficial effects on the host (Pflughoeft & Versalovic, 2012; Quigley, 2011; Shah, 2007). Both genera *Bifidobacterium* and *Lactobacillus*, are widely used in probiotic products for humans (Sharma, Wasan, & Sharma, 2021). The beneficial properties of probiotic microorganisms has been related to, among others, the ability to provide enzymes, the prevention of the effects of intestinal pathogens and the modulation of the immune response (Ashraf & Shah, 2014; Bermudez-Brito, Plaza-Diaz, Muñoz-Quezada, Gomez-Llorente, & Gil, 2012; de LeBlanc, & Perdigón, 2014; Donkor et al., 2012; Lemme-Dumit, Polti, Perdigón, & Galdeano, 2018; Núñez, Galdeano, de Moreno; Trejo, De Antoni, & Pérez, 2013; Trejo, Minnaard, Perez, & De Antoni, 2006; Verdu, 2009; Yousefi et al., 2019). In addition, probiotics modify the bioavailability of some drugs either by inducing changes in the expression of cytochromes, by affecting the expression of transporters, by modulating cellular permeability or by changing the enzymatic potential in the intestinal environment (Al-Salami et al., 2008, 2012; Matuskova et al., 2014; Stojančević, Bojić, Al Salami, & Mikov, 2014).

The relevance of probiotic microorganisms in the gut-associated immune system has been demonstrated. This role has been associated to bacterial components such as nucleic acids, polysaccharides and cell wall fractions (Delgado, Sánchez, Margolles, Ruas-Madiedo, & Ruiz, 2020; Dogi, Weill, & Perdigón, 2010; Kapila et al., 2012; Kaushal & Kansal, 2014; Lemme-Dumit et al., 2018; Li et al., 2005; Tahoun, Masutani, Sharkawy, El Gillespie, & Honda, 2017; Van Bergenhenegouwen et al., 2014). In addition, secreted metabolic products such as lactic acid and short chain fatty acids have also shown immunomodulatory activity (Bengoa, Dardis, Gagliarini, Garrote, & Abraham, 2020; Iraporda, Romanin, Rumbo, Garrote, & Abraham, 2014; LeBlanc et al., 2017).

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Macrophages play a pivotal role in the innate immune response, and their polarization driven by environmental factors determines functional diversity that is crucial for the host (Mantovani et al., 2004; Mosser & Edwards, 2008; Murray, Allen, Fisher, & Lawrence, 2015; Orecchioni, Ghosheh, Pramod, & Ley, 2019). Differentiation of macrophages is relevant not only in physiological but also in pathological conditions such as infectious, metabolic and autoimmune diseases (Mosser & Edwards, 2008; Shapouri-Moghaddam et al., 2018; Sica, Erreni, Allavena, & Porta, 2015). Thus, these cells can be biomarkers of diseases and they can be used for assessing effects of different treatments or even vaccination schedules (Mosser & Edwards, 2008).

Several strains of *Lactobacillus* and *Bifidobacterium* have an effect on immune response through the induction of distinctive patterns of cytokines (Ashraf & Shah, 2014; Azad, Sarker, & Wan, 2018; Dogi et al., 2010; Donkor et al., 2012; Esmaeili et al., 2018; Rolny, Tiscornia, Racedo, Perez, & Bollati-Fogolin, 2016) and macrophage polarization (Christoffersen et al., 2014; Wang, Liu, & Zhao, 2019). It has been described that these microorganisms can modulate the innate and adaptive immune defence mechanisms (Borchers, Selmi, Meyers, Keen, & Gershwin, 2009) through the modification of the Th1/Th2 balance (Azad et al., 2018; Hua et al., 2010; Mohamadzadeh et al., 2005).

The microecology of the intestinal tract involves complex interactions between microbiome, nutrients incorporated with food and xenobiotics. These interactions impact on the host's health. Maintaining intestinal homeostasis is important since imbalances can lead to increased inflammation. Considering that both probiotic microorganisms and macrophage coexist with medications in the intestinal tract, studies that evaluate the interactions between these three players are necessary. In spite that effects of antibiotic imbalance on intestinal homeostasis are widely known, there is little knowledge on the effect of other drugs such as those used in chronic hypertension or cardiac diseases. These studies are even more relevant given the high demand of probiotic foods by consumers.

Acenocoumarol, a coumarin derivative, is one of the most used oral anticoagulants for the treatment of thromboembolic disorders (Gschwind et al., 2013; Militaru, Vesa, Pop, & Buzoianu, 2015). It is a vitamin k antagonist that exerts its function by inhibiting vitamin K epoxide reductase (Holmes, Hunt, & Shearer, 2012). Therapeutic dosing of acenocoumarol is influenced by diverse factors such as genetic variability in metabolic enzymes of patients, age, stress and ingestion of food and beverages (Bachmann & Hoffmann, 2004; Gschwind et al., 2013; Holmes et al., 2012; Militaru et al., 2015; Warzecha et al., 2017). Noteworthy, the role of the intestinal microbiota on the metabolism of acenocoumarol (Thijssen, Baars, Hazen, & Van Den Bogaard, 1984) and the closely related warfarin have been reported (Giuliano et al., 2010).

In addition to its anticoagulant properties, acenocoumarol has immunomodulatory properties (Schroecksnadel et al., 2013). Indeed, in peripheral blood mononuclear cells stimulated with phytohemagglutinin, acenocoumarol reduced the concentration of INFγ and TNFα and prevented the degradation of tryptophan. In addition, the anticoagulant decreased tryptophan degradation in Caco-2 cells stimulated with INFγ (Schroecksnadel et al., 2013). Knowledge of the interactions of probiotic with drugs as acenocoumarol help to avoid inappropriate activation of the immune cells and disruption of host homeostasis.

Bifidobacterium strains included in the present study have shown immunomodulatory properties (Assad, Rolny, Minnaard, & Pérez, 2020) and also the ability to modify acenocoumarol (Fragomeno et al., 2021). These properties along with the relevance of both bifidobacteria and macrophages in intestinal homeostasis (Bermudez-Brito et al., 2013) prompted us to assess the effect of acenocoumarol on the interaction between bifidobacteria and cells relevant in the context of the modulation of the intestinal immune response: phagocytic and enterocyte-like cells.

2. Materials and methods

2.1. Eukaryotic cells culture

Caco-2 ccl20:luc (Nempont, Cayet, Rumbo, Bompard, & Sirard, 2008) was grown at 37 °C in a 5% CO_2/95% air atmosphere in Dulbecco's Modified Eagle's Medium (DMEM) (25 mM glucose) (GIBCO Life Technologies, Grand Island, USA) supplemented with 1% v/v nonessential amino acids (GIBCO Life Technologies, Grand Island, USA), streptomycin (12 μg mL^{-1}) and penicillin (12 UI mL^{-1}) (Life Technologies, Cergy, France), and 15% (v/v) heat-inactivated (30 min, 56 °C) fetal calf serum (FCS) (GIBCO Life Technologies, Grand Island, USA). For the experiments, cells monolayers were prepared in 24-well tissue culture plates (JetBiofilm, China) by seeding 7×10^4 cells per well. Assays were performed with cells at confluence (7 days in culture).

The same culture condition was used for monocytic THP-1 cells line (Daigneault, Preston, Marriott, Whyte, & Dockrell, 2010) except the DMEM which was supplemented with 10% (v/v) FCS. To differentiate THP-1 cells, they were incubated with 200 nM phorbol myristate acetate (PMA) (Sigma, St. Louis, USA) (THP1-PMA) in DMEM containing 10% (v/v) FCS for 2 days at 37 °C in 5% CO_2 atmosphere. Cells were cultured in 24 or 48-well tissue culture plates (JetBiofilm, China) by seeding 2.53×10^5 cells cm^{-2}. For confocal microscopy cells were grown on glass coverslips in 24-well tissue plates.

2.2. Bacterial strains and growth conditions

Bifidobacterium bifidum CIDCA 5310 and *Bifidobacterium adolescentis* CIDCA 5317 were isolated from breast fed infant's faeces (Pérez, Minnaard, Disalvo, & de Antoni, 1998). Bacteria were stored at −80 °C with 20% (v/v) glycerol as cryoprotectant. Reactivation of strains was carried out in MRS broth (BIOKAR, Biokar Diagnostics, Beauvais, France) supplemented with 0.05% (w/v) cysteine (Laboratorios ANEDRA, Argentina) in anaerobic conditions for 48 h at 37 °C. Afterwards, microorganisms were inoculated in the same medium (1×10^7 CFU mL^{-1}) and further incubated for 24 h in the above indicated conditions.

For the experiments, bacteria were washed twice with phosphate buffered saline (PBS) and suspended in DMEM. Bacterial concentration was assessed by determining absorbance values at 600 nm (OD = 1 is equivalent to corresponds to 10^8 CFU mL^{-1}) (METROLAB 330, Argentina).

2.3. Fluorescent labelling of bacteria

Microorganisms were washed twice in PBS and labeled with fluorescein isothiocyanate (FITC) (Sigma, St. Louis, USA) 0.3 mg mL^{-1} for 20 min at room temperature. Then, bacteria were washed twice with PBS and suspended in 500 μL of DMEM. Labelling was evaluated by fluorescent microscopy (Leica Microscopy Systems Ltd., Microsystems, Germany) and bacterial concentration was assessed as described above.

2.4. Acenocoumarol

The stock solution of acenocoumarol (Lab Bago, Argentina) was prepared in N,N-dimethyl formamide (10 mg mL^{-1}) and filtered (0.22 μm). For the experiments, dilutions were prepared in DMEM.

2.5. Phagocytosis assay

Fluorescent bacteria were incubated with THP1-PMA cells at multiplicity of infection (MOI) 10 bacteria/cell for 18 h at 37 °C 5% CO_2 in the presence or not of acenocoumarol 475 μM (final concentration) (Fragomeno et al., 2021).

Phagocytosis was evaluated by flow cytometry (FACSCalibur™ CellQuest™ software) by recording percentages of FL1(+) cells. For quenching of non-internalized bacteria trypan blue 0,4% w/v was used.

Analysis was done by using FlowJo™ V10.4.

2.6. Fate of internalized bacteria in the presence of acenocoumarol

With the aim of evaluating the cellular fate of internalized bacteria in the presence of the anticoagulant, Lysotracker DND-99 was used to detect bacteria in acidic compartments. FITC-labeled strain CIDCA 5310 was incubated in the presence of acenocoumarol 475 μM (final concentration) for 1 h at 37 °C with THP1-PMA cells (MOI = 10) cultured on glass coverslips in 24-well tissue plates. Samples without drug were used as controls. Next, cells were stained with Lysotracker DND-99 200 nM (Life Technologies, Carlsbad, California) for 5 min. Afterwards macrophages were washed twice with PBS and fixed with 3% (v/v) paraformaldehyde (PFA) for 1 h at 4 °C in the dark. As antifading agent, mounting medium (DakoCytomation, USA) was used.

A confocal laser-scanning microscope (LEICA TCS SP5, Leica Microsystems, Germany) at excitation wavelengths of 488 nm (argon laser) and 594 nm (helium-neon laser) with a HCX PL APO CS 63.0×1.40 OIL UV objective (Leica Microsystems, Germany) was used. The resulting images had resolution of 8 bits (1024 × 1024 pixels). For analysis at least 200 cells per sample were assessed by means of the Leica LAS AF Lite software. The percentage of colocalization was determined as the ratio between the number of bacteria co-localizing with the marker and the total number of internalized bacteria.

2.7. Expression of macrophage surface molecules

In order to evaluate the expression of surface markers in the presence of the anticoagulant, bifidobacteria were incubated with THP-1 or THP1-PMA cells at MOI 10 in the presence or not of 475 μM acenocoumarol for 18 h at 37 °C 5% CO_2 in DMEM with 10% (v/v) FCS. Positive controls of expression were stimulated with 375 ng mL^{-1} INF-γ (THERMO Scientific, Rockford, USA) for HLA-Dr, 375 ng mL^{-1} IFN- γ plus 0,5 μg mL^{-1} LPS (Sigma-Aldrich, St. Louis, USA) for CD16 and CD64, 10 ng mL^{-1} IL-4 for CD163, CD23 and CD206 and heat treated (60 °C for 3 min) *Staphylococcus aureus* for TLR-2. Unstimulated macrophages were used as controls of basal expression. Besides, bacteria or anticoagulant alone were also included for comparisons.

After incubation, cells were washed with PBS and non-specific binding of antibodies was blocked with PBS supplemented with 20% (w/v) inactivated human serum. Staining was performed for 20 min at 4 °C in the dark with PE-conjugated anti-human HLA, FITC-conjugated anti-human CD16, PE-conjugated anti-human CD64, FITC-conjugated anti-human CD163, FITC-conjugated anti-human CD206, PE-conjugated anti-human CD23 or FITC-conjugated anti-human TLR2 (BD Pharmigen, San Jose, CA, USA). Isotype controls were included.

After being labeled, samples were washed twice with PBS and analysed by flow cytometry. The expression index (EI) was calculated as the percentage of positive cells x mean fluorescence intensity.

2.8. Evaluation of cellular response in the Caco-2 ccl20:luc reporter system

The Caco-2 ccl20:luc reporter system allows to determine activation of innate response by induction of firefly luciferase as a reporter of the activation of the ccl20 gene (Nempont et al., 2008).

Caco-2 ccl20:luc monolayers grew on 24-well tissue culture plates were incubated 16 h at 37 °C in 5% CO_2 atmosphere with suspension of strain CIDCA 5310 or CIDCA 5317 in DMEM FCS-free medium at MOI = 10 and 471 μM acenocoumarol. Wells with bifidobacteria or anticoagulant alone were also included. Then, cells were stimulated with flagellin (FliC) (1 μg mL^{-1}) from *Salmonella enterica* serovar Enteritidis for 5 h. Epithelial cells non stimulated with FliC were used to assess basal expression. After removing the supernatants, cells were lysed and luciferase activity was measured in a Labsystems Luminoskan TL Plus luminometer (Thermo Scientific, USA) by using the kit from Promega (Promega, Madison WI, USA) (Nempont et al., 2008). The values obtained were normalized to FliC control (stimulated control) to obtain the normalized average luminescence (NAL) and the percentage of the mean of stimulated control was calculated for each condition (Romanin et al., 2010).

2.9. Statistical analysis

Statistical analysis of the variations was performed by paired Student's *t*-test. The ratios of colocalization were compared by means of the Fisher's exact test at the 0.05 level of significance. All statistical tests were conducted by using INFOSTAT software (Di Rienzo, Casanoves, Balzarini, Gonzalez, & Tablada, 2020Di Rienzo J.A., Casanoves F., Balzarini M.G., Gonzalez L., Tablada M., 2020)

3. Results

3.1. Effect of acenocoumarol on phagocytosis of bifidobacteria

Considering that interaction of intestinal bacteria with phagocytic cells is crucial for the host immune response and that drugs present in the intestinal tract could modify such interaction, the phagocytosis of *Bifidobacterium* strains by THP-1 cells was evaluated in the presence of acenocoumarol. The results showed a significant decrease ($p < 0.05$) in the phagocytosis of both strains under study in the presence of 471 μM of anticoagulant (Fig. 1).

3.2. Fate of internalized bacteria in the presence of acenocoumarol

To assess the effect of the anticoagulant on the intracellular fate of bacteria, confocal microscopy was used. These experiments were conducted with strain CIDCA 5310 because this strain was highly phagocytosed by THP-1 cells. The microscopic evaluation confirmed the decrease in phagocytosis of strain CIDCA 5310 in the presence of the drug after 1 h of incubation. Interestingly, intracellular localization was not modified by the drug and most bacteria were found in non-acidic compartments (Fig. 2).

3.3. Expression of macrophage surface molecules

Different macrophage surface markers were evaluated (Mantovani et al., 2004; Martinez & Gordon, 2014). In addition, TLR2 was analysed within the M1 profile since it is relevant in the inflammatory response (Martinez & Gordon, 2014).

The results obtained here showed that acenocoumarol modified the expression induced by bifidobacteria in the M1 markers, irrespectively that THP1-PMA or THP1 cells were stimulated (Figs. 3, 4A and 4B). In THP1-PMA cells, acenocoumarol reduced the expression induced by bifidobacteria on HLA-DR (Fig. 3A), TLR-2 (Fig. 3B) and CD64 (Fig. 3D) and increased the expression of CD16 (Fig. 3C). The same results were observed when HLA-DR and CD64 were evaluated in THP-1 cells (Fig. 4A and B).

On the other hand, when the expression of CD206, a M2 marker, was studied, differences depended on the stimulated cells i. e. monocytes (Fig. 4C) or macrophages (Fig. 5). Acenocoumarol decreased the expression of CD206 in THP-1 cells induced by *B. bifidum* strain CIDCA 5310 (Fig. 4C) whereas no changes were observed in THP1-PMA cells (Fig. 5B). In addition, in THP1-PMA cells the expression of CD23, another M2 marker, was also diminished in the presence of acenocoumarol (Fig. 5C).

3.4. Cell response in the Caco-2 ccl20:luc reporter system

Several stimuli such as LPS, flagellin or cytokines like IFNγ and TNF-α can trigger the expression of the chemokine CCl20 in a NF-kB-dependent manner (Romanin et al., 2010; Rumbo, Sierro, Debard,

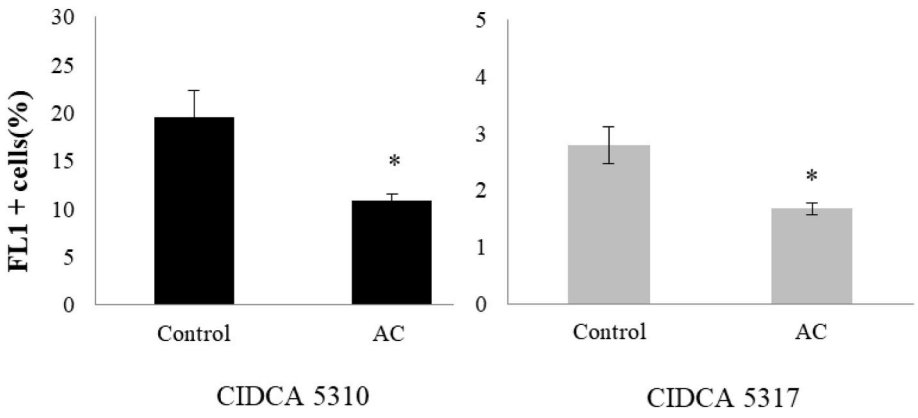

Fig. 1. Effect of acenocoumarol (AC) on the internalization of *Bifidobacterium bifidum* CIDCA 5310 and *Bifidobacterium adolescentis* CIDCA 5317 by THP1-PMA cells. Internalization was determined by flow cytometry after 18 h incubation and quenching of exocellular bacteria with Trypan blue. Results are expressed as percentage of FL1(+) cells. Results show a representative of three independent experiments. Bars indicate standard deviation (n = 3).
* = P < 0.05 with control for CIDCA5310 and CIDCA5317, respectively.

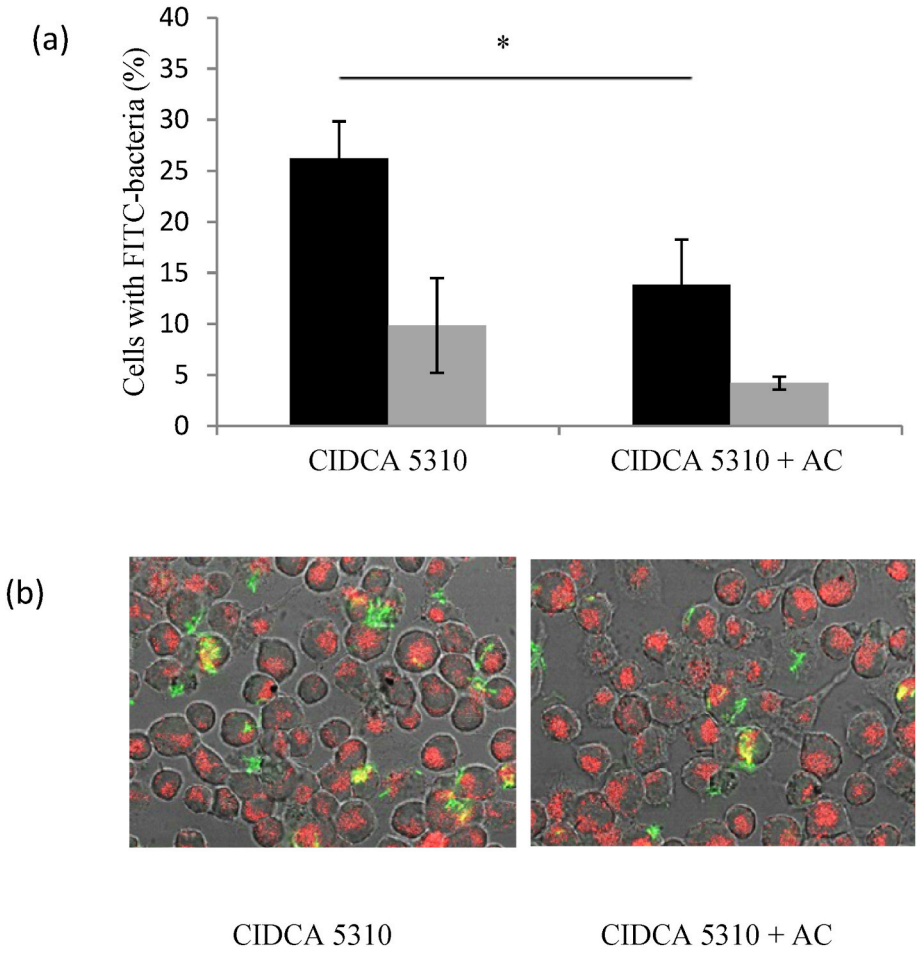

Fig. 2. Intracellular trafficking of strain *B. bifidum* CIDCA 5310 in THP1-PMA cells. Colocalization with Lysotraker with FITC-labeled bacteria was evaluated by confocal laser scanning microscopy after 1 h incubation with cells. Results are expressed as percentage of internalized bacteria co-localizing with marker (grey columns) in relation to total internalized bacteria (black columns) (a). Representative images with yellow areas indicating colocalization of FITC-labeled bacteria (green) and the lysosomal marker Lysotracker (red) (b). Results show a representative experiment from at least 3 independent experiments and are expressed as means (n = 3). Bars indicate standard deviation. *P < 0.05. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Kraehenbuhl, & Finke, 2004). This is detected as surrogate luciferase activity in the Caco-2 ccl20:luc reporter system.

Acenocoumarol increased luciferase expression of Flic treated cells, as compared with cells stimulated with Flic or Flic + bifidobacteria (Fig. 6). Noteworthy, this stimulatory effect is partially abrogated by coincubation with strain CIDCA 5310 that is also able to modulate Flic-dependent induction of luciferase activity in absence of acenocoumarol (Fig. 6).

4. Discussion

In the present work the effect of acenocoumarol on the modulation of immune response by *Bifidobacterium* was demonstrated. Using THP-1 cells in the presence of the anticoagulant, a decrease in phagocytosis of bifidobacteria and modification of the expression of surface markers after stimulation with bacteria were observed. Also, an acenocoumarol-dependent enhancement on the activation of the NF-κB pathway was demonstrated. This effect can be modulated by *Bifidobacterium*.

Selection of probiotic bacteria is a crucial step in the formulation of functional foods. In this context, the probiotic potential of bacterial strains deserves great interest and it has been addressed in many research studies. In a previous work, we demonstrated that *Bifidobacterium* strains used in the present study interact differently with monocyte-derived macrophages. In addition, a strain-dependent host

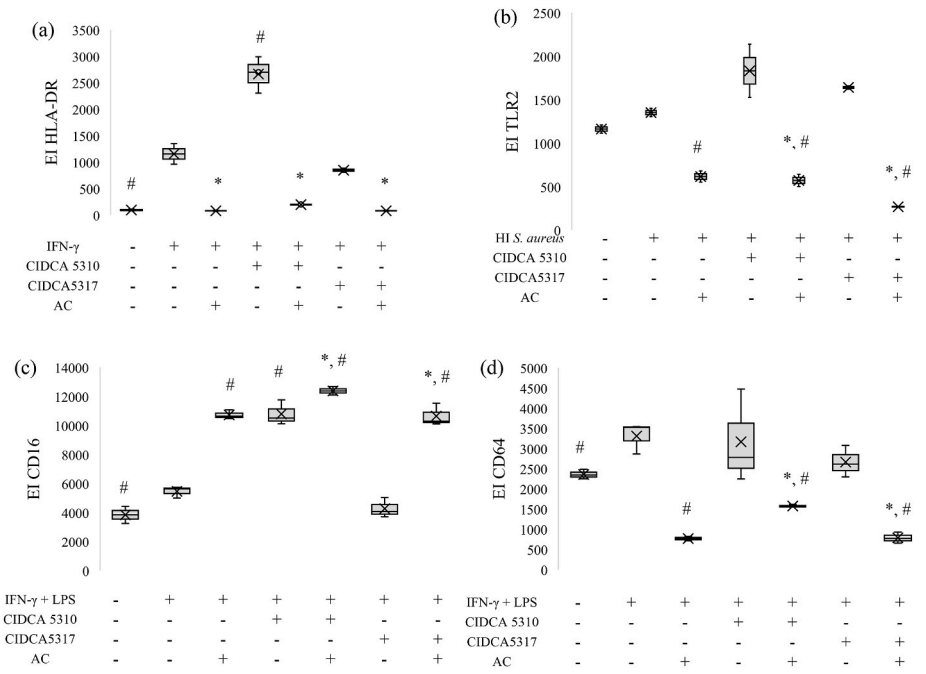

Fig. 3. Expression of M1 surface markers in THP1-PMA cells. HLA-DR (a), TLR2 (b), CD16 (c) and CD64 (d) in THP 1-PMA cells co-incubated with strains CIDCA 5310 or CIDCA 5317 (at MOI 10) and acenocoumarol (AC) 471 μM for 18 h. Values are expressed as expression index (EI). IFN-γ, heat-inactivated *Staphylococcus aureus* or IFN-γ and LPS were used as a positive control as indicated in graphics. Results show a representative experiment from at least 3 independent experiments.
*: $P < 0.05$ with strain without AC; #: $P < 0.05$ with positive control.

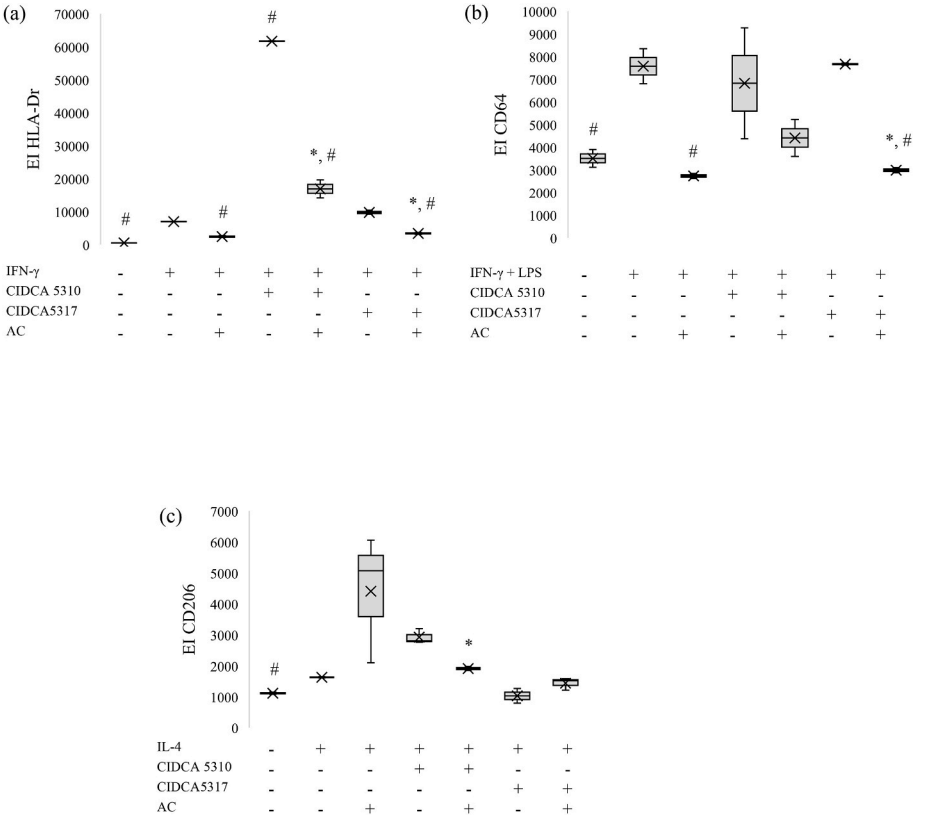

Fig. 4. Expression of M1 and M2 surface markers in THP1 cells. HLA-DR (a), CD64 (b) and CD206 (c) in THP-1 cells incubated with strains CIDCA 5310 or CIDCA 5317 (at MOI 10) and acenocoumarol (AC) 471 μM for 18 h. Values are expressed as expression index (EI). Positive control is indicated in graphics. Results show a representative experiment from at least 3 independent experiments.
*: $P < 0.05$ with strain without AC, #: $P < 0.05$ with positive control.

response was observed when opsonic or non-opsonic receptors were evaluated (Assad et al., 2020). In the present work we demonstrated that the above mentioned effects can be modified by acenocoumarol, a widely used anticoagulant.

Both *in vitro* and *in vivo* models have demonstrated the relevance of probiotic microorganisms in the innate immune response through the increase of phagocytic activity (Dogi et al., 2010; Jia et al., 2020; Kapila et al., 2012; Kaushal & Kansal, 2014; Klein, Friedrich, Vogelsang, & Jahreis, 2008; Lemme-Dumit et al., 2018; Rocha-Ramírez et al., 2017, 2020). In relation to this, it was observed that a strain of *Lactobacillus jensenii* with high immunomodulatory capacity was more internalized by mononuclear porcine Peyer's patches phagocytes than a non-immunomodulatory strain (Tsukida et al., 2016). These findings are of interest since the decrease in phagocytic activity could be relevant in

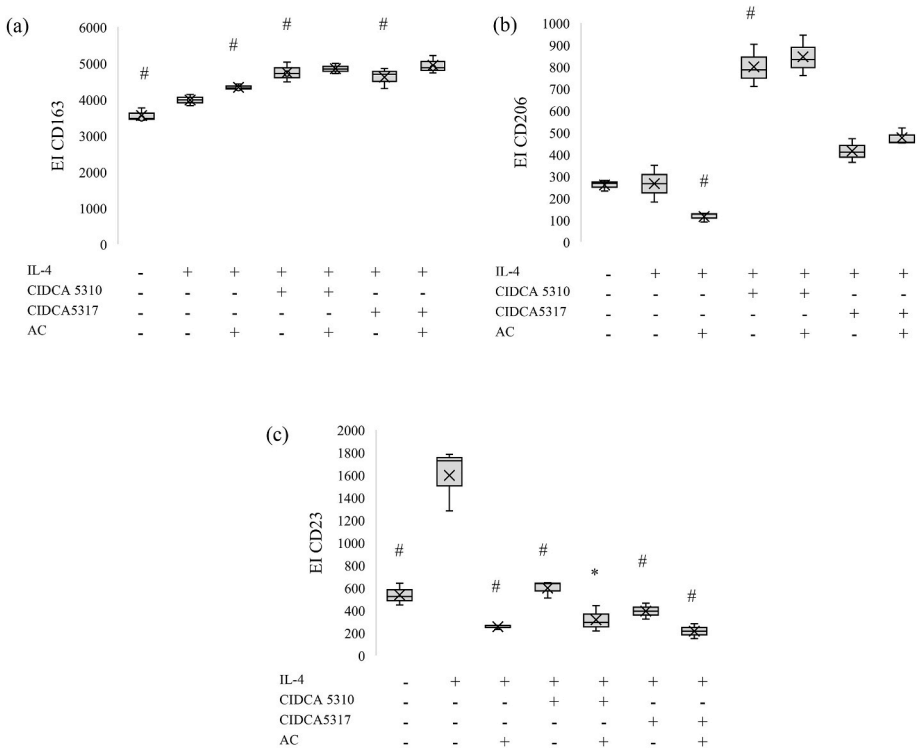

Fig. 5. Expression of M2 surface markers in THP1-PMA cells. CD163 (a), CD206 (b) and CD23 (c) in THP-1-PMA cells co-incubated with strains CIDCA 5310 or CIDCA 5317 (at MOI 10) and acenocoumarol (AC) 471 μM for 18 h. Values are expressed as expression index (EI). IL-4 was used as a positive control. Results show a representative experiment from at least 3 independent experiments.
*: $P < 0.05$ with strain without AC, #: $P < 0.05$ with positive control.

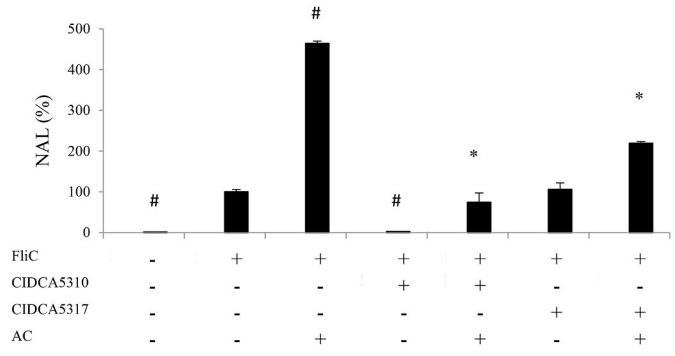

Fig. 6. Luciferase activity in the Caco-2 ccl20: luc reporter system. Luciferase activity in the presence of the positive stimulus flagellin (flic) (5hs) after co-incubation with the bifidobacteria strains CIDCA 5310 or CIDCA 5317 and acenocoumarol (AC) 471 μM for 18 h. Results are expressed as normalized average luminescence (NAL) and show a representative experiment from 3 independent experiments. Bars indicate standard deviation (n = 3).
*: $P < 0.05$ with strain without AC, #: $P < 0.05$ with positive control.

pathologies such as chronic obstructive pulmonary disease (Mortaz et al., 2015) thus impacting on the ability in controlling pathogen microorganisms (Franken, Schiwon, & Kurts, 2016).

In the present work, we demonstrated that acenocoumarol modifies the phagocytosis of *Bifidobacterium*. This effect could affect expected outcomes for probiotic strains present in foods since activity of macrophages is essential for the clearance of pathogen or cell debris in the intestine (Smith et al., 2011). It is important to note that the decrease of phagocytosis produced by acenocoumarol is not a general effect since no alteration of the internalization of latex beads was observed (data not shown). Furthermore, the interaction with other cell lineages (e. g. enterocyte-like cells) was not modified by acenocoumarol (data not shown).

Acenocoumarol decreased HLA-DR expression in THP-1 cells in the presence of IFN-γ thus demonstrating the ability to modify the expression of a key molecule for both innate and adaptive immune responses. Noteworthy, in phagocytic cells the receptor HLA-DR allowed for the detection of antigens by regulatory T-cells (Shapouri-Moghaddam et al., 2018).

Strain CIDCA 5310 was able to increase expression of HLA-DR in THP-1 cells. This correlates with the localization of bifidobacteria in acidic compartments (Assad et al., 2020) similarly to that was observed in *Bacillus cereus* (Rolny, Racedo, & Pérez, 2017). However, the decrease in expression of HLA-DR in the presence of AC, could not be fully explained by a decrease in routing to acidic compartments since no significant differences in internalized bacteria was found in the presence of the anticoagulant. It is possible that the lower number of microorganisms in acidic compartments is a direct consequence of the decrease in phagocytosis.

For resident intestinal macrophages the acquisition of phagocytic activity is related to the expression of surface markers such as CD163 and CD206 (Bain et al., 2013). In the present study, we demonstrated that acenocoumarol diminished expression of receptors related to phagocytosis (e. g. TLR-2 and CD64). In addition, acenocoumarol also decreased the expression of another receptor related to phagocytosis (mannose receptor; CD206) when PMA-stimulated monocytes were evaluated, while no changes were observed in macrophages treated with bifidobacteria and the drug (Fig. 5).

To contribute to the homeostasis, resident intestinal macrophages down-regulate expression of CD64, CD16 and TLR3-9 (Smith et al., 2011). However, the activation of TLRs by the commensal microbiota also plays an important role in homeostasis (Rakoff-Nahoum, Paglino, Eslami-Varzaneh, Edberg, & Medzhitov, 2004) and the participation of TLR2 in the immunomodulatory effect of probiotic microorganisms such as *L. sakei* was observed (Jung et al., 2015). In the present study acenocoumarol decreases expression of HLA-DR, TLR2 and CD64 but increases CD16 expression. These results agree with a dynamic modulation of surface markers (Ferreira da Mota N; Colo Brunialti M; Sousa Santos S; Ribeiro Machado F; Assuncao M; Pontes de Azevedo L; Salomao R., 2018). These apparently controversial results in macrophage polarization have been previously observed for probiotic bacteria

(Fu et al., 2019). Instead, other probiotic strains triggered a shift to M2 macrophage activation (Id, Ohshio, Sugamata, & Morita, 2020).

It is important to note that CD23 (a M2 marker) decreased in macrophages stimulated with bifidobacteria and acenocoumarol. This marker (also known as FcεRII) plays a role in phagocytosis mediated by antibodies in macrophages and monocytes and contributes to the clearance of parasites via nitric oxide (Sutton, Davies, Bax, & Karagiannis, 2019).

The ability of acenocoumarol to decrease the activation of proinflammatory responses triggered by LPS and IFN-γ as well as the production of proinflammatory cytokines in THP-1 cells has been reported (Schroecksnadel et al., 2013). These findings are in agreement with the results presented here that show a decrease in inflammatory markers in THP-1 cells stimulated with bifidobacteria. Also, the immunomodulatory ability of acenocoumarol was described in a model of acute pancreatitis in rats with an inhibition of the development of inflammation was observed at low doses of the drug (Warzecha et al., 2017).

There are several studies that report the ability of potentially probiotic microorganisms and their metabolites to decrease the innate immune response caused by flagellin (Jia et al., 2020; Kaci et al., 2011). In this way, the ability of isolated kefir yeasts to inhibit the innate response in intestinal epithelial cells triggered via NF-κB has been observed (Romanin et al., 2010). Furthermore, the immunomodulatory properties of lactate was ascribed to the binding of this metabolite with specific GPR81 G protein-coupled receptors in enterocytes (Iraporda et al., 2014, 2015). In addition, sodium butyrate downregulates NF-κB signalling (Sun et al., 2020). Related to this, we observed that *B. bifidum* CIDCA 5310 is able to inhibit the response induced by flagellin (Fig. 6). Interestingly, the anticoagulant in turn was a potent response activator, in coincidence to previous reports that have shown an increase in the transcription factors NF-κB/AP-1 in THP-1 Blue cells stimulated with LPS, a TLR4 ligand (Schroecksnadel et al., 2013). The effect of the anticoagulant seems to be synergic with TLR5-dependent NF-kB activation, since no activation was observed when acenococumarol was studied without flagellin (data not shown). The strain CIDCA 5310 was able to modulate the increase in Caco2-CCL20-luc reporter cells triggered by flagellin + acenocoumarol activation. In contrast, the strain CIDCA 5317 showed lower capacity to modulate the innate immune response induced by flagellin. It is evident that acenocoumarol can impact the modulatory effect of bifidobacteria in the context of an inflammatory stimulus such as flagellin. It could be pertinent to consider these findings in the context of metaflammation/inflammation in the gastrointestinal tract, where the inhibition of NF-kB pathway related to TLR4 was demonstrated for commensal bifidobacteria (Ghadimi et al., 2019).

5. Conclusion

Our results demonstrated *in vitro* that acenocoumarol impacts on immunomodulatory effects of bifidobacteria strains from human origin. These findings encourage further studies to assess the relevance of the observed effects on the modulation of immune response by probiotic foods in persons under anticoagulant treatment.

Author statement

Sabrina E Assad: Formal analysis, Investigation **Melisa Fragomeno:** Investigation **Martin Rumbo,** Writing - Review & Editing, Resources; **Jessica Minnaard:** Project administration, Visualization, Writing - Original Draft **Pablo F Pérez:** Funding acquisition, Supervision, Writing - Review & Editing.

Declaration of competing interest

The authors have no conflict of interest in publishing this article.

Data availability

Data will be made available on request.

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References

Al-Salami, H., Butt, G., Tucker, I., Golocorbin-Kon, S., & Mikov, M. (2012). Probiotics decreased the bioavailability of the bile acid analog, monoketocholic acid, when coadministered with gliclazide, in healthy but not diabetic rats. *European Journal of Drug Metabolism and Pharmacokinetics, 37*(2), 99–108. https://doi.org/10.1007/s13318-011-0060-y

Al-Salami, H., Butt, G., Tucker, I., Skrbic, R., Golocorbin-Kon, S., & Mikov, M. (2008). Probiotic pre-treatment reduces gliclazide permeation (ex vivo) in healthy rats but increases it in diabetic rats to the level seen in untreated healthy rats. *Archives of Drug Information, 1*(1), 35–41. https://doi.org/10.1111/j.1753-5174.2008.00006.x

Ashraf, R., & Shah, N. P. (2014). Immune system stimulation by probiotic microorganisms. *Critical Reviews in Food Science and Nutrition, 54*(7), 938–956. https://doi.org/10.1080/10408398.2011.619671

Assad, S. E., Rolny, I. S., Minnaard, J., & Pérez, P. F. (2020). Bifidobacteria from human origin: Interaction with phagocytic cells. *Journal of Applied Microbiology*. https://doi.org/10.1111/jam.14861. jam.14861.

Azad, M. A. K., Sarker, M., & Wan, D. (2018). Immunomodulatory effects of probiotics on cytokine profiles. *BioMed Research International*. https://doi.org/10.1155/2018/8063647, 2018.

Bachmann, H. U., & Hoffmann, A. (2004). Interaction of food supplement L-carnitine with oral anticoagulant acenocoumarol. *Swiss Medical Weekly, 134*(25–26), 385, 2004/25/smw-10631.

Bain, C. C., Scott, C. L., Uronen-Hansson, H., Gudjonsson, S., Jansson, O., Grip, O., et al. (2013). Resident and pro-inflammatory macrophages in the colon represent alternative context-dependent fates of the same Ly6C hi monocyte precursors. *Mucosal Immunology, 6*(3), 498–510. https://doi.org/10.1038/mi.2012.89

Bengoa, A. A., Dardis, C., Gagliarini, N., Garrote, G. L., & Abraham, A. G. (2020). Exopolysaccharides from lactobacillus paracasei isolated from kefir as potential bioactive compounds for microbiota modulation. *Frontiers in Microbiology, 11*, 1–13. https://doi.org/10.3389/fmicb.2020.583254. October.

Bermudez-Brito, M., Plaza-Diaz, J., Muñoz-Quezada, S., Gomez-Llorente, C., & Gil, A. (2012). Probiotic mechanisms of action. *Annals of Nutrition and Metabolism, 61*(2), 160–174. https://doi.org/10.1159/000342079

Borchers, A. T., Selmi, C., Meyers, F. J., Keen, C. L., & Gershwin, M. E. (2009). Probiotics and immunity. *Journal of Gastroenterology, 44*(1), 26–46. https://doi.org/10.1007/s00535-008-2296-0

Christoffersen, T. E., Hult, L. T. O., Kuczkowska, K., Moe, K. M., Skeie, S., Lea, T., et al. (2014). In vitro comparison of the effects of probiotic, commensal and pathogenic strains on macrophage polarization. *Probiotics and Antimicrobial Proteins, 6*(1), 1–10. https://doi.org/10.1007/s12602-013-9152-0

Daigneault, M., Preston, J. A., Marriott, H. M., Whyte, M. K. B., & Dockrell, D. H. (2010). The identification of markers of macrophage differentiation in PMA-stimulated THP-1 cells and monocyte-derived macrophages. *PLoS One, 5*(1). https://doi.org/10.1371/journal.pone.0008668

Delgado, S., Sánchez, B., Margolles, A., Ruas-Madiedo, P., & Ruiz, L. (2020). Molecules produced by probiotics and intestinal microorganisms with immunomodulatory activity. *Nutrients, 12*(2), 1–14. https://doi.org/10.3390/nu12020391

Di Rienzo, J. A., Casanoves, F., Balzarini, M. G., Gonzalez, L., & Tablada, M. R. C. W. (2020). *InfoStat v2020 (No. 2020)*. Argentina: Centro de Transferencia InfoStat, FCA, Universidad Nacional de Córdoba.

Dogi, C. A., Weill, F., & Perdigón, G. (2010). Immune response of non-pathogenic Gram(+) and Gram(-) bacteria in inductive sites of the intestinal mucosa. Study of the pathway of signaling involved. *Immunobiology, 215*(1), 60–69. https://doi.org/10.1016/j.imbio.2009.01.005

Donkor, O. N., Ravikumar, M., Proudfoot, O., Day, S. L., Apostolopoulos, V., Paukovics, G., et al. (2012). Cytokine profile and induction of T helper type 17 and regulatory T cells by human peripheral mononuclear cells after microbial exposure. *Clinical and Experimental Immunology, 167*(2), 282–295. https://doi.org/10.1111/j.1365-2249.2011.04496.x

Esmaeili, S. A., Mahmoudi, M., Rezaieyazdi, Z., Sahebari, M., Tabasi, N., Sahebkar, A., et al. (2018). Generation of tolerogenic dendritic cells using Lactobacillus rhamnosus

and Lactobacillus delbrueckii as tolerogenic probiotics. *Journal of Cellular Biochemistry, 119*(9), 7865–7872. https://doi.org/10.1002/jcb.27203
Ferreira da Mota, N., Colo Brunialti, M., Sousa Santos, S., Ribeiro Machado, F., Assuncao, M., Pontes de Azevedo, L., et al. (2018). Immunophenotyping of monocytes during human sepsis shows impairment in antigen- presentation, a shift towards non-classical differentiation and up-regulation of FcγRI- receptor. *Shock, 50* (3), 293–300. https://doi.org/10.1097/SHK.0000000000001078
Fragomeno, M., Assad, S., Mobili, P., Peruzzo, P. J., Minnaard, J., & Pérez, P. F. (2021). Biomodification of acenocoumarol by bifidobacteria. *FEMS Microbiology Letters, 368* (18), 1–10. https://doi.org/10.1093/femsle/fnab125
Franken, L., Schiwon, M., & Kurts, C. (2016). Macrophages: Sentinels and regulators of the immune system. *Cellular Microbiology, 18*(4), 475–487. https://doi.org/10.1111/cmi.12580
Fu, A., Mo, Q., Wu, Y., Wang, B., Liu, R., Tang, L., et al. (2019). Protective effect of Bacillus amyloliquefaciens against Salmonella via polarizing macrophages to M1 phenotype directly and to M2 depended on microbiota. *Food & Function, 10*(12), 7653–7666. https://doi.org/10.1039/c9fo01651a
Ghadimi, D., Farghaly, M., Hassan, Y., Fölster-Holst, R., Röcken, C., Ebsen, M., et al. (2019). *Regulation of hepcidin/iron-signalling pathway interactions by commensal bifidobateria plays an important role for the inhibition of metaflammation-related biomarkers*. https://doi.org/10.1016/j.imbio.2019.11.009
Giuliano, V., Bassotti, G., Mourvaki, E., Castellani, D., Filippucci, E., Sabatino, G., et al. (2010). Small intestinal bacterial overgrowth and warfarin dose requirement variability. *Thrombosis Research, 126*(1), 12–17. https://doi.org/10.1016/j.thromres.2009.11.032
Gschwind, L., Rollason, V., Daali, Y., Bonnabry, P., Dayer, P., & Desmeules, J. A. (2013). Role of P-glycoprotein in the uptake/efflux transport of oral vitamin K antagonists and rivaroxaban through the caco-2 cell model. *Basic and Clinical Pharmacology and Toxicology, 113*(4), 259–265. https://doi.org/10.1111/bcpt.12084
Holmes, M. V., Hunt, B. J., & Shearer, M. J. (2012). The role of dietary vitamin K in the management of oral vitamin K antagonists. *Blood Reviews, 26*(1), 1–14. https://doi.org/10.1016/j.blre.2011.07.002
Hua, M. C., Lin, T. Y., Lai, M. W., Kong, M. S., Chang, H. J., & Chen, C. C. (2010). Probiotic Bio-Three induces Th1 and anti-inflammatory effects in PBMC and dendritic cells. *World Journal of Gastroenterology, 16*(28), 3529–3540. https://doi.org/10.3748/wjg.v16.i28.3529
Id, T. Y., Ohshio, K., Sugamata, M., & Morita, Y. (2020). *Lactic acid bacterium, Lactobacillus paracasei KW3110, suppresses inflammatory stress-induced caspase-1 activation by promoting interleukin-10 production in mouse and human immune cells*. https://doi.org/10.1371/journal.pone.0237754
Iraporda, C., Errea, A., Romanin, D. E., Cayet, D., Pereyra, E., Pignataro, O., et al. (2015). Lactate and short chain fatty acids produced by microbial fermentation downregulate proinflammatory responses in intestinal epithelial cells and myeloid cells. *Immunobiology, 220*(10), 1161–1169. https://doi.org/10.1016/j.imbio.2015.06.004
Iraporda, C., Romanin, D. E., Rumbo, M., Garrote, G. L., & Abraham, A. G. (2014). The role of lactate on the immunomodulatory properties of the nonbacterial fraction of kefir. *Food Research International, 62*, 247–253. https://doi.org/10.1016/j.foodres.2014.03.003
Jia, G., Liu, X., Che, N., Xia, Y., Wang, G., Xiong, Z., et al. (2020). Human-origin: Lactobacillus salivarius AR809 protects against immunosuppression in S. aureus -induced pharyngitis via Akt-mediated NF-κB and autophagy signaling pathways. *Food & Function, 11*(1), 270–284. https://doi.org/10.1039/c9fo02476j
Jung, J. Y., Shin, J. S., Lee, S. G., Rhee, Y. K., Cho, C. W., Hong, H. D., et al. (2015). Lactobacillus sakei K040706 evokes immunostimulatory effects on macrophages through TLR 2-mediated activation. *International Immunopharmacology, 28*(1), 88–96. https://doi.org/10.1016/j.intimp.2015.05.037
Kaci, G., Lakhdari, O., Doré, J., Ehrlich, S. D., Renault, P., Blottière, H. M., et al. (2011). Inhibition of the NF-kappaB pathway in human intestinal epithelial cells by commensal Streptococcus salivarius. *Applied and Environmental Microbiology, 77*(13), 4681–4684. https://doi.org/10.1128/AEM.03021-10
Kapila, R., Kapila, S., Kapasiya, M., Pandey, D., Dang, A., & Saliganti, V. (2012). Comparative evaluation of oral administration of probiotic lactobacilli-fermented milks on macrophage function. *Probiotics and Antimicrobial Proteins, 4*(3), 173–179. https://doi.org/10.1007/s12602-012-9107-x
Kaushal, D., & Kansal, V. K. (2014). Dahi containing lactobacillus acidophilus and Bifidobacterium bifidum improves phagocytic potential of macrophages in aged mice. *Journal of Food Science & Technology, 51*(6), 1147–1153. https://doi.org/10.1007/s13197-012-0637-8
Klein, A., Friedrich, U., Vogelsang, H., & Jahreis, G. (2008). Lactobacillus acidophilus 74-2 and Bifidobacterium animalis subsp lactis DGCC 420 modulate unspecific cellular immune response in healthy adults. *European Journal of Clinical Nutrition, 62*(5), 584–593. https://doi.org/10.1038/sj.ejcn.1602761
LeBlanc, J. G., Chain, F., Martín, R., Bermúdez-Humarán, L. G., Courau, S., & Langella, P. (2017). Beneficial effects on host energy metabolism of short-chain fatty acids and vitamins produced by commensal and probiotic bacteria. *Microbial Cell Factories, 16* (1), 1–10. https://doi.org/10.1186/s12934-017-0691-z
Lemme-Dumit, J. M., Polti, M. A., Perdigón, G., & Galdeano, M. M. (2018). Probiotic bacteria cell walls stimulate the activity of the intestinal epithelial cells and macrophage functionality. *Beneficial Microbes, 9*(1), 153–164. https://doi.org/10.3920/BM2016.0220
Li, Y., Qu, X., Yang, H., Kang, L., Xu, Y., Bai, B., et al. (2005). Bifidobacteria DNA induces murine macrophages activation in vitro. *Cellular and Molecular Immunology, 2*(6), 473–478.
Mantovani, A., Sica, A., Sozzani, S., Allavena, P., Vecchi, A., & Locati, M. (2004). The chemokine system in diverse forms of macrophage activation and polarization. *Trends in Immunology, 25*(12), 677–686. https://doi.org/10.1016/j.it.2004.09.015
Martinez, F. O., & Gordon, S. (2014). The M1 and M2 paradigm of macrophage activation: Time for reassessment. *F1000Prime Reports, 6*, 1–13. https://doi.org/10.12703/P6-13. March.
Matuskova, Z., Anzenbacherova, E., Vecera, R., Tlaskalova-Hogenova, H., Kolar, M., & Anzenbacher, P. (2014). Administration of a probiotic can change drug pharmacokinetics: Effect of E. coli Nissle 1917 on amidarone absorption in rats. *PLoS One, 9*(2), 3–7. https://doi.org/10.1371/journal.pone.0087150
Militaru, F., Vesa, S., Pop, T., & Buzoianu, A. (2015). Pharmacogenetics aspects of oral anticoagulants therapy. *J Med Life, 8*(2), 171–175.
Mohamadzadeh, M., Olson, S., Kalina, W. V., Ruthel, G., Demmin, G. L., Warfield, K. L., et al. (2005). Lactobacilli active human dendritic cells that skew T cells toward T helper 1 polarization. *Proceedings of the National Academy of Sciences of the United States of America, 102*(8), 2880–2885. https://doi.org/10.1073/pnas.0500098102
Mortaz, E., Adcock, I. M., Ricciardolo, F. L. M., Varahram, M., Jamaati, H., Velayati, A. A., et al. (2015). Anti-inflammatory effects of Lactobacillus rahmnosus and Bifidobacterium breve on cigarette smoke activated human Macrophages. *PLoS One, 10*(8), 1–17. https://doi.org/10.1371/journal.pone.0136455
Mosser, D. M., & Edwards, J. P. (2008). Exploring the full spectrum of macrophage activation. *Nature Reviews Immunology, 8*(12), 958–969. https://doi.org/10.1038/nri2448.Exploring
Murray, P. J., Allen, J. E., Fisher, E. A., & Lawrence, T. (2015). Macrophage activation and polarization nomenclature and experimental guidelines. *Immunity, 41*(1), 14–20. https://doi.org/10.1016/j.immuni.2014.06.008.Macrophage
Nempont, C., Cayet, D., Rumbo, M., Bompard, C., Sirard, V. V., & J, C. (2008). Deletion of flagellin's hypervariable region abrogates antibody-mediated neutralization and systemic activation of TLR5-dependent immunity. *The Journal of Immunology, 181*, 2036–2043. https://doi.org/10.4049/jimmunol.167.5.2529
Núñez, I. N., Galdeano, C. M., de Moreno de LeBlanc, A., & Perdigón, G. (2014). Evaluation of immune response, microbiota, and blood markers after probiotic bacteria administration in obese mice induced by a high-fat diet. *Nutrition, 30* (11–12), 1423–1432. https://doi.org/10.1016/j.nut.2014.03.025
Orecchioni, M., Ghosheh, Y., Pramod, A. B., & Ley, K. (2019). Macrophage polarization: Different gene signatures in M1(Lps+) vs. Classically and M2(LPS-) vs. Alternatively activated macrophages. *Frontiers in Immunology, 10*, 1–14. https://doi.org/10.3389/fimmu.2019.01084. MAY.
Pérez, P. F., Minnaard, Y., Disalvo, E. A., & de Antoni, G. L. (1998). Surface properties of bifidobacterial strains of human origin. *Applied and Environmental Microbiology, 64* (1), 21–26.
Pflughoeft, K. J., & Versalovic, J. (2012). Human microbiome in health and disease. *Annual Review of Pathology, 7*, 99–122. https://doi.org/10.1146/annurev-pathol-011811-132421. December 2011.
Quigley, E. M. (2011). Gut microbiota and the role of probiotics in therapy. *Current Opinion in Pharmacology, 11*(6), 593–603. https://doi.org/10.1016/j.coph.2011.09.010
Rakoff-Nahoum, S., Paglino, J., Eslami-Varzaneh, F., Edberg, S., & Medzhitov, R. (2004). Recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis. *Cell, 118*(2), 229–241. https://doi.org/10.1016/j.cell.2004.07.002
Rocha-Ramírez, L. M., Hernández-Ochoa, B., Gómez-Manzo, S., Marcial-Quino, J., Cárdenas-Rodríguez, N., Centeno-Leija, S., et al. (2020). Impact of heat-killed lactobacillus casei strain imau60214 on the immune function of macrophages in malnourished children. *Nutrients, 12*(8), 1–17. https://doi.org/10.3390/nu12082303
Rocha-Ramírez, L. M., Pérez-Solano, R. A., Castañón-Alonso, S. L., Moreno Guerrero, S. S., Ramírez Pacheco, A., García Garibay, M., et al. (2017). Probiotic lactobacillus strains stimulate the inflammatory response and activate human macrophages. *Journal of Immunology Research*. https://doi.org/10.1155/2017/4607491, 2017.
Rolny, I. S., Racedo, S. M., & Pérez, P. F. (2017). Fate of Bacillus cereus within phagocytic cells. *International Microbiology, 20*(4), 27–29. https://doi.org/10.2436/20.1501.01.291
Rolny, I. S., Tiscornia, I., Racedo, S. M., Perez, P. F., & Bollati-Fogolin, M. (2016). Lactobacillus delbrueckii subsp lactis CIDCA 133 modulates response of human epithelial and dendritic cells infected with Bacillus cereus. *Beneficial Microbes*, 1–12. https://doi.org/10.3920/BM2015.0191
Romanin, D., Serradell, M., González Maciel, D., Lausada, N., Garrote, G. L., & Rumbo, M. (2010). Down-regulation of intestinal epithelial innate response by probiotic yeasts isolated from kefir. *International Journal of Food Microbiology, 140* (2–3), 102–108. https://doi.org/10.1016/j.ijfoodmicro.2010.04.014
Rumbo, M., Sierro, F., Debard, N., Kraehenbuhl, J. P., & Finke, D. (2004). Lymphotoxin β receptor signaling induces the chemokine CCL20 in intestinal epithelium. *Gastroenterology, 127*(1), 213–223. https://doi.org/10.1053/j.gastro.2004.04.018
Schroecksnadel, S., Gostner, J., Jenny, M., Kurz, K., Schennach, H., Weiss, G., et al. (2013). Immunomodulatory effects in vitro of vitamin K antagonist acenocoumarol. *Thrombosis Research, 131*(6), e264–e269. https://doi.org/10.1016/j.thromres.2013.02.012
Shah, N. P. (2007). Functional cultures and health benefits. *International Dairy Journal, 17*(11), 1262–1277. https://doi.org/10.1016/j.idairyj.2007.01.014
Shapouri-Moghaddam, A., Mohammadian, S., Vazini, H., Taghadosi, M., Esmaeili, S. A., Mardani, F., et al. (2018). Macrophage plasticity, polarization, and function in health and disease. *Journal of Cellular Physiology, 233*(9). https://doi.org/10.1002/jcp.26429

Sharma, M., Wasan, A., & Sharma, R. K. (2021). Recent developments in probiotics: An emphasis on Bifidobacterium. *Food Bioscience, 41*. https://doi.org/10.1016/j.fbio.2021.100993. March.

Sica, A., Erreni, M., Allavena, P., & Porta, C. (2015). Macrophage polarization in pathology. *Cellular and Molecular Life Sciences, 72*(21), 4111–4126. https://doi.org/10.1007/s00018-015-1995-y

Smith, P. D., Smythies, L. E., Shen, R., Greenwell-Wild, T., Gliozzi, M., & Wahl, S. M. (2011). Intestinal macrophages and response to microbial encroachment. *Mucosal Immunology, 4*(1), 31–42. https://doi.org/10.1038/mi.2010.66

Stojančević, M., Bojić, G., Al Salami, H., & Mikov, M. (2014). The influence of intestinal tract and probiotics on the fate of orally administered drugs. *Current Issues in Molecular Biology, 16*(1), 55–67.

Sun, X., Luo, S., Jiang, C., Tang, Y., Cao, Z., Jia, H., et al. (2020). *Sodium butyrate reduces bovine mammary epithelial cell inflammatory responses induced by exogenous lipopolysaccharide, by inactivating NF-ĂŽÂ°B signaling*. https://doi.org/10.3168/jds.2020-18189

Sutton, B., Davies, A., Bax, H., & Karagiannis, S. (2019). IgE antibodies: From structure to function and clinical translation. *Antibodies, 8*(1), 19. https://doi.org/10.3390/antib8010019

Tahoun, A., Masutani, H., Sharkawy, H., El Gillespie, T., & Honda, R. P. (2017). Capsular polysaccharide inhibits adhesion of Bifidobacterium longum 105 - a to enterocyte - like Caco - 2 cells and phagocytosis by macrophages. *Gut Pathogens*, 1–17. https://doi.org/10.1186/s13099-017-0177-x

Thijssen, H. H. W., Baars, L. G. M., Hazen, M. J., & Van Den Bogaard, A. E. J. (1984). The role of the intestinal microflora in the reductive metabolism of acenocoumarol in man. *British Journal of Clinical Pharmacology*, 247–249.

Trejo, F. M., De Antoni, G. L., & Pérez, P. F. (2013). Protective effect of bifidobacteria in an experimental model of Clostridium difficile associated colitis. *Journal of Dairy Research, 80*(3), 263–269. https://doi.org/10.1017/S0022029913000216

Trejo, F. M., Minnaard, J., Perez, P. F., & De Antoni, G. L. (2006). Inhibition of Clostridium difficile growth and adhesion to enterocytes by Bifidobacterium supernatants. *Anaerobe, 12*(4), 186–193. https://doi.org/10.1016/j.anaerobe.2006.03.004

Tsukida, K., Takahashi, T., Iida, H., Kanmani, P., Suda, Y., Nochi, T., et al. (2016). Immunoregulatory effects triggered by immunobiotic Lactobacillus jensenii TL2937 strain involve efficient phagocytosis in porcine antigen presenting cells. *BMC Immunology*, 1–12. https://doi.org/10.1186/s12865-016-0160-1

Van Bergenhenegouwen, J., Kraneveld, A. D., Rutten, L., Kettelarij, N., Garssen, J., & Vos, A. P. (2014). Extracellular vesicles modulate host-microbe responses by altering TLR2 activity and phagocytosis. *PLoS One, 9*(2), 1–11. https://doi.org/10.1371/journal.pone.0089121

Verdu, E. F. (2009). Probiotics effects on gastrointestinal function: Beyond the gut? *Neuro-Gastroenterology and Motility, 21*(5), 477–480. https://doi.org/10.1111/j.1365-2982.2009.01297.x

Wang, Y., Liu, H., & Zhao, J. (2019). Macrophage polarization induced by probiotic bacteria: A concise Review. *Probiotics and Antimicrobial Proteins*. https://doi.org/10.1007/s12602-019-09612-y

Warzecha, Z., Sendur, P., Ceranowicz, P., Cieszkowski, J., Dembiński, M., Sendur, R., et al. (2017). Therapeutic effect of low doses of acenocoumarol in the course of ischemia/reperfusion-induced acute pancreatitis in rats. *International Journal of Molecular Sciences, 18*(4). https://doi.org/10.3390/ijms18040882

Yousefi, B., Eslami, M., Ghasemian, A., Kokhaei, P., Salek Farrokhi, A., & Darabi, N. (2019). Probiotics importance and their immunomodulatory properties. *Journal of Cellular Physiology, 234*(6), 8008–8018. https://doi.org/10.1002/jcp.27559