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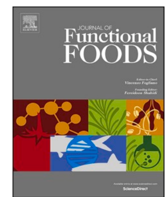

# *In vitro* examination of antibacterial and immunomodulatory activities of cinnamon, white thyme, and clove essential oils

Magdalena Valdivieso-Ugarte [a], Julio Plaza-Diaz [b,c], Carolina Gomez-Llorente [a,b,c,d,*], Eduardo Lucas Gómez [e], Maria Sabés-Alsina [e], Ángel Gil [a,b,c,d]

[a] *Institute of Nutrition and Food Technology "José Mataix", Center of Biomedical Research, University of Granada, Avda. del Conocimiento s/n, 18016 Armilla, Granada, Spain*
[b] *Department of Biochemistry and Molecular Biology II, School of Pharmacy, University of Granada, 18071 Granada, Spain*
[c] *ibs.GRANADA, Instituto de Investigación Biosanitaria, Complejo Hospitalario Universitario de Granada, 18014 Granada, Spain*
[d] *CIBEROBN (CIBER Physiopathology of Obesity and Nutrition), Instituto de Salud Carlos III, 28029 Madrid, Spain*
[e] *Preparados Aditivos y Materias Primas S.A, c/Carrasco i Formiguera,5, Pol.Ind. Can Llobet, 08192, Sant Quirze del Vallès, Barcelona, Spain*

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ABSTRACT

The present study was conducted to evaluate the *in vitro* antimicrobial effect of three essential oils (cinnamon, white thyme, and clove) on selected intestinal pathogen bacteria, probiotic strains, and commensal bacteria. Additionally, their effects on inflammatory gene expression were also evaluated in a well-known intestinal human cellular model (Caco-2 cells). Cinnamon showed the highest antipathogenic microbial activity, followed by white thyme and clove. In the case of the probiotic strains, the inhibitory effect was observed at 0.1% (v/v), although growth recovery was seen after 24 h of incubation. Besides, cinnamon down-regulated the expression of genes related to the Toll-like receptor, nitric oxide, and apoptosis pathways. Similarly, secretion of interleukin 2 was inhibited by cinnamon. Results support the potential use of essential oils in the food industry for their antimicrobial and immunomodulatory effects; however, the underlying mechanisms are unknown and warrant further research.

## 1. Introduction

The World Health Organization (WHO) has estimated that 1 in 10 people fall ill every year due to eating contaminated food (WHO, 2015). Food contamination with pathogenic microorganisms is one of the main factors in developing foodborne disease (Valdivieso-Ugarte, Gomez-Llorente, Plaza-Diaz, & Gil, 2019). The ingestion of contaminated food with pathogenic microorganisms can cause diarrhea, vomiting, or a more severe alteration such as hemorrhagic colitis (Munekata et al., 2020). In this sense, *Escherichia coli* and *Salmonella* strains are two common pathogens associated with foodborne disease. To avoid food contamination with these or other pathogenic microorganisms, food industry use preservative agents for some foodstuffs. In recent years, the demand for the development of new, safe, and natural preservatives agents has grown. In this regard, special attention has been paid to the use of plant-derived essential oils (EOs), or their mains components, as natural, safe, and biodegradable food preservative agents (Valdivieso-Ugarte et al., 2019). Several authors have worked on developing successful strategies for the bio-preservation by using EOs in different types of food, such as meat products, milk, dairy products, salad dressing, mayonnaise, bread, and baked foods (Teneva et al., 2020).

EOs are responsible for the odor of aromatic plants, although they

*Abbreviations:* BCL2, B-cell lymphoma 2; CASP8, Caspase 8; DMSO, Dimethylsulfoxide; EOs, Essential oils; FDA, Food and Drug Administration; GRAS, Generally Recognized as Safe; HPRT1, Hypoxanthine phosphoribosyltransferase 1; HSP90, Heat shock protein 90; IL, Interleukin; IRAk-4, Interleukin-1 receptor-associated kinase 4; IRF-3, Interferon regulatory factor 3; LPS, Lipopolysaccharide; MRS, de Man, Rogosa and Sharpe; MYD88, Myeloid-differentiation primary response; NFKB-1, Nuclear factor-kappa-B subunit 1; NFKBIA, nuclear factor-kappa B inhibitor alpha; NF-κB, nuclear factor-kappa B; NOS, Nitric oxide synthase; O.D, Optical density; PTGS2, Prostaglandin G/H synthase and cyclooxygenase; SEM, Standard error mean; TBK-1, TANK-binding kinase 1; TBS, Tryptic soy broth; TLR, Toll-like receptor; TNF-α, Tumor necrosis factor alpha; TOLLIP, Toll interacting protein; TRAM1, Translocation associated membrane protein 1; WHO, World Health Organization.

* Corresponding author at: Dept. Biochemistry and Molecular Biology II. School of Pharmacy. Campus de Cartuja s/n 18071 Granada, Spain.
*E-mail address:* gomezll@ugr.es (C. Gomez-Llorente).

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have other important plant functions such as attracting beneficial insects and pollinators or protecting plants from microorganisms (Falleh, Ben Jemaa, Saada, & Ksouri, 2020). EOs exhibit important biological functions that make them suitable in cosmetics, sanitary products, and agriculture. According to this, many EOs or specific components are classified as Generally Recognized As Safe (GRAS) by the U.S Food and Drug Administration (FDA). Among them, cinnamon, clove, and thyme are considered GRAS. It must be noted that EO effects, as preservative agents, depend on their sources and composition (Falleh et al., 2020). The antimicrobial activity of EOs have been tested in several foodborne pathogens (Valdivieso-Ugarte et al., 2019). In this regards, cinnamon EO has been shown to exert the highest antimicrobial activity against saprophytic and pathogenic microorganism (Denkova-Kostova et al., 2020).

EOs are volatile secondary metabolites synthesized by aromatic and medicinal plants, and in general, they are poorly soluble in water. Chemically, they are a mixture of numerous bioactive components such as terpenes, terpenoids, and phenolic compounds. These bioactive compounds are responsible for their biological antimicrobial, antioxidant, and immunomodulatory properties (Falleh et al., 2020; Valdivieso-Ugarte et al., 2019). Some of the proposed mechanisms for their activity compromise the irreversible damage of the bacterial cell wall and membrane, together with their capacity to modulate the secretion of important cytokines in a cell culture challenged with lipopolysaccharide (LPS). EOs have shown to exert an effect on tumor necrosis factor (TNF-α), interleukins (ILs), thromboxane and leukotriene production (Valdivieso-Ugarte et al., 2019). These activities make EOs suitable for their use as bio-preservative agents and ingredients in functional foods.

The current study was conducted to determine the antibacterial activities of tree selected EOs (cinnamon, clove, and white thyme) out of ten screened EOs, against common intestinal pathogens, intestinal commensal bacteria, and probiotics. The effect of EOs on the three different kinds of intestinal bacteria will add new information of their possible influence on intestinal growth bacteria that will be useful in the food industry for the design of new bio-preservative agents. Additionally, to their well-known antibacterial activity, the EOs have also been shown to possess immunomodulatory properties. For that, we studied its antibacterial effect and we determined its immunomodulatory effect on a well-known *in vitro* cellular model (human intestinal cells -Caco-2 cells) to understand their possible mechanisms of action and support its use in the food industry.

## 2. Materials and methods

### *2.1. Evaluation of the antimicrobial activity of the essential oils*

#### *2.1.1. Bacterial strains*

Gram-negative and Gram-positive bacteria were tested. Four recognized pathogen species commonly involved in intestinal diseases, four commensal strains belonging to each of the described enterotypes (Arumugam et al., 2011), and four probiotics were selected. The pathogen strains *E. coli* CECT 729, *E. coli* CECT 501, *Salmonella typhi* CECT 725, and *Salmonella typhimurium* CECT4594) were obtained from the Spanish Type Culture Collection (CECT). The probiotic strains *Lactobacillus paracasei* CNCM I-4034, *Bifidobacterium breve* CNCM I-4035, and *Lactobacillus rhamnosus* CNCM I-4036 have been characterized and are described elsewhere (Munoz-Quezada et al., 2013) and are available from the Collection of Bacteria of the Institut Pasteur (CIP), together with the *Lactobacillus plantarum* Lp3547 strain provided by Preparados Aditivos y Materias Primas S.A. (PAYMSA). The commensal species *Bacteroides* spp. DSM 107544 (Enterotype 1), *Prevotella bryantii* DSM 11371 (Enterotype 2), and *Trichococcus pasteurii* DSM 2381 (Enterotype 3) were obtained from the German Collection of Microorganisms and Cell Cultures GmbH (DSMZ).

Regarding the growth media, *Salmonella* and *E. coli* were grown in tryptic soy broth (TSB) medium (Sigma-Aldrich, St. Louis, MO) aerobically; probiotics were grown anaerobically in the de Man, Rogosa and Sharpe (MRS) agar medium (Oxoid, Hampshire, United Kingdom) whereas MRS broth medium was used for preparing the inoculate anaerobically; and the commensal species *Bacteroides* spp., *Prevotella bryantii,* and *Trichococcus pasteurii* were grown in Cooked Meat Medium and Muller Hinton (Oxoid, Hampshire, United Kingdom) anaerobically using a Whitley DG250 Anaerobic workstation (Don Whitley Scientific, Yorkshire, United Kingdom).

#### *2.1.2. Determination of antibacterial growth activity of the essential oils by disc diffusion assay*

Ten common EOs were initially tested and screened for their antibacterial properties by disc diffusion: Garlic, onion, clove, cinnamon, black pepper, bay leaf, nutmeg, cardamom, rosemary, and white thyme. The screened EOs were purchased from LLUCH ESSENCE (Barcelona, Spain) (Cinnamon, onion and white thyme), INDUKERN F&F INGREDIENTS (Barcelona, Spain) (Clove and cardamom,) and from VENTOS (Barcelona, Spain) (Garlic, rosemary, bay leaf, black pepper, and nutmeg). All oils were obtained from steam distillation. The corresponding information about the main chemical components has been included as a supplementary Table S1 for the three selected oils and as supplementary Table S2 for the rest of the EOs studied (Table S1 and Table S2).

We selected three EOs (cinnamon, white thyme, and clove) out of 10 screened EOs, based on their antibacterial growth effect measured by disk diffusion. The latter is a good approximation method; however, for a better determination of the antibacterial growth, a microdilution method was performed only in the tree selected EOs.

The disc diffusion method was conducted to evaluate the antibacterial activity of the 10 EOs according to the method previously described by Bauer et al. (1966). The suspension was prepared from an overnight culture for each microorganism. TSB agar plates (Sigma-Aldrich, St. Louis, MO), MRS agar plates (Oxoid, Hampshire, United Kingdom), and Columbia CNA agar with 5% sheep blood plates (Sigma-Aldrich, St. Louis, MO) were swabbed with 100 mL of the bacterial suspension in the case of pathogenic, probiotic, and commensal bacteria, respectively. Subsequently, sterile paper discs (d = 6 mm) were soaked with 10 μL of crude EO and placed on the inoculated surface of Petri dishes. Plates were incubated at 37 °C for 24 h for *E. coli* and *Salmonella*, and for 48 h in anaerobiosis (Oxoid, Hampshire, United Kingdom) for the probiotics and the commensal bacteria. After incubation, the diameters of the inhibition zones were measured. Disk impregnated with sterile distilled water served as a negative control.

#### *2.1.3. Determination of the antibacterial growth activity of the essential oils by the microdilution method*

The microdilution broth method was used to determine the Minimum Inhibitory Concentration (MIC) for the cinnamon, white thyme, and clove EOs. This EOs were selected based on the disc diffusion results. Three serial dilutions of EOs (0.1%, 0.01%, and 0.001%) (v/v) were prepared with dimethylsulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO). The *E. coli* and *Salmonella* strains were grown in TSB and incubated aerobically for 24 h at 37 °C. The *Lactobacillus* and *Bifidobacterium* strains were grown in MRS and MRS broth (Oxoid, Hampshire, United Kingdom), supplemented with 0.05% (w/v) cysteine (Sigma-Aldrich, St. Louis, MO), respectively, and incubated anaerobically for 24 h at 37 °C. These assays were performed in polystyrene 96-well (volume, 200 mL/well) plates (Maxisorp, Sigma-Aldrich, St. Louis, MO). Tryptone soy (*E. coli* and *Salmonella*) or MRS and MRS supplemented with 0.05% (w/v) cysteine (Oxoid, Hampshire, United Kingdom), (*Lactobacillus* and *Bifidobacterium*) broth was inoculated at 5% (v/v) with a concentrated microbial cell solution grown overnight. The inhibition of bacterial growth was evaluated by monitoring bacterial growth at 37 °C in tryptone soy (Oxoid, Hampshire, United Kingdom), or nutrient medium (Oxoid, Hampshire, United Kingdom), in 96-well plates according to the

methodology of Chenoll (Chenoll et al., 2011). Bacterial growth was determined using optical density data (O.D) at 620 nm using a Multiskan microplate reader (Thermo Fisher Scientific). For each EO and condition, three independent experiments were performed. In each case, the percentage of resistance was calculated by comparing the final optical densities at 620 nm obtained with different concentrations of EOs with those of the corresponding control samples. An estimated concentration of each bacteria utilized in the present study were, *E. coli* CECT 729 ($6.5 \times 10^{10}$ colony-forming units (CFU)), *E. coli* CECT 501 ($4.5 \times 10^{9}$ CFU), *Salmonella typhi* CECT 725 ($3.6 \times 10^{10}$ CFU), *Salmonella typhimurium* CECT4594 ($2.6 \times 10^{10}$ CFU), *Lactobacillus paracasei* CNCM I-4034 ($8.0 \times 10^{8}$ CFU), *Bifidobacterium breve* CNCM I-4035 ($1.8 \times 10^{9}$ CFU), and *Lactobacillus rhamnosus* CNCM I-4036 ($2.6 \times 10^{8}$ CFU).

#### 2.1.4. Determination of the antibacterial growth activity of the EOs against the commensal bacteria

Because of the insufficient bacterial growth in small volumes, the commensal bacteria, *Bacteroides* spp., *Prevotella bryantii,* and *Trichococcus pasteurii,* were grown in 10 mL of Cooked Meat medium using a Whitley DG250 Anaerobic workstation (Don Whitley Scientific, Yorkshire, United Kingdom) and incubated anaerobically for 72 h at 37 °C (*Bacteroides* spp. DSM 107544, *Prevotella bryantii* DSM 11371) and at 30 °C *Trichococcus pasteurii* DSM 2381. Tubes of 15 mL of Cooked Meat Medium broth (Oxoid, Hampshire, United Kingdom) with selected EOs at different concentrations (0.1%, 0.01% and 0.001%) were inoculated with a concentrated microbial cell solution. Paraffin was added to each tube to favor the anaerobic conditions. Tubes were placed in anaerobic jars and incubated at the appropriate temperature for each strain. After 5 days of incubation, the O.D. was measured at 620 nm. Before 5 days, no growth was observed.

### 2.2. Evaluation of the immunomodulatory activity of the essential oils

For the evaluation of the immunomodulatory effect of the three selected EOs, a well-known intestinal human cell line was used (Caco-2 cells). Many studies have described the Caco-2 cell line (derived from human colon adenocarcinoma) as a reliable and high-throughput *in vitro* model of human intestinal colonocytes. Differentiated Caco-2 cells express functional tight junctions, brush border characteristics, biotransformation enzymes (Pinto et al., 1983; Van de Walle et al., 2010), and secrete many immunomodulatory molecules such as TNF-α and IL-8 (Perez del Palacio et al., 2016).

#### 2.2.1. CaCo-2 cells culture and incubation conditions

CaCo-2 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich, St. Louis, MO) supplemented with 10% inactivated fetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, MO), 1% glutamine (Sigma-Aldrich, St. Louis, MO), penicillin G (0.1 U/mL) (Sigma-Aldrich, St. Louis, MO), and streptomycin (0.1 mg/mL) (Sigma-Aldrich, St. Louis, MO). Cells were cultured at 37 °C in an atmosphere of 5% $CO_2$ and 95% air. Caco-2 cells were incubated for 15–21 days. Cells were grown to confluence (until the transepithelial resistance (TER) reached 300 $\Omega.cm^2$).

In order to test the appropriate EO concentration, the cells were treated with different EO concentrations (0.1, 0.01 and 0.001%) and the cellular viability was determined by Trypan blue staining (Perry, Epstein, & Gelbard, 1997). Based on these results, the EO concentration selected was 0.01% in DMSO (v/v) (Sigma-Aldrich, St. Louis, MO). As a negative control, the cells were incubated with 0.01% of DMSO (Sigma-Aldrich, St. Louis, MO). Caco-2 cells were cultured separately with the three selected EOs (cinnamon, white thyme, and, clove) in the presence and absence of 20 ng/μL of LPS (a potent inflammatory agent of

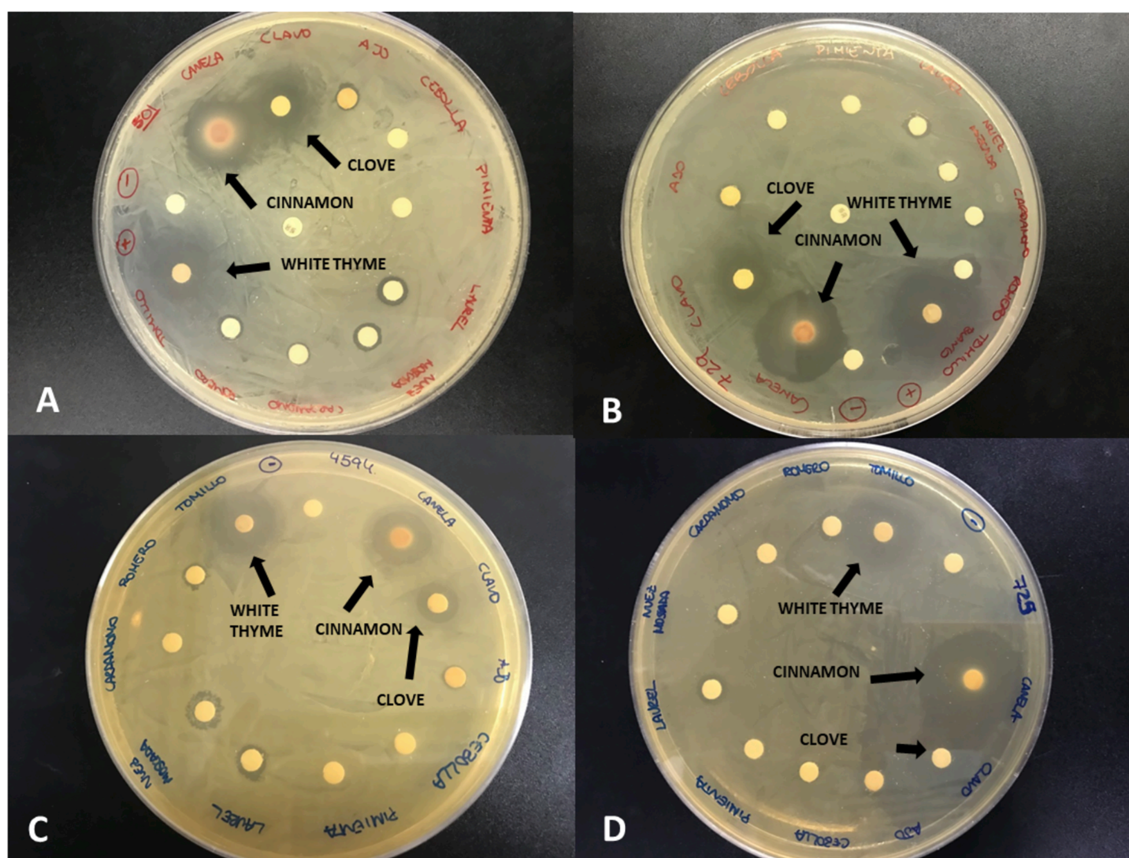


**Fig. 1.** Evaluation of the antimicrobial effect by disk diffusion of crude essential oils on the growth of the pathogenic strains *Escherichia coli* CECT 501 (A), *Escherichia coli* CECT 729 (B), *Salmonella typhimurium* CECT4594 (C), and *Salmonella typhi* CECT 725 (D).

**Table 1**
Effect of essential oils on the growth of *Escherichia coli* CECT 729, *Escherichia coli* CECT 501, *Salmonella typhi* CECT 725, and *Salmonella typhimurium* CECT4594 tested by the microdilution method. (+) Growth inhibition of pathogenic bacteria with significant difference $P < 0.05$; (−) no inhibitory effects or growth inhibition of pathogenic bacteria with no significant difference $p < 0.05$ (cc = concentration).

| Essential Oil | *E. coli* CECT 501 | | | *E. coli* CECT 729 | | | *S. typhi* CECT 725 | | | *S. typhimurium* CECT 4594 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| cc (%) | 0.1 | 0.01 | 0.001 | 0.1 | 0.01 | 0.001 | 0.1 | 0.01 | 0.001 | 0.1 | 0.01 | 0.001 |
| Cinnamon | + | − | − | + | + | + | + | + | + | + | − | − |
| Clove | + | + | − | + | + | − | + | + | − | + | − | − |
| White thyme | + | − | − | + | + | − | + | + | + | + | − | − |

bacterial origin). Cells were incubated for 4 h at 37 °C in an atmosphere of 5% $CO_2$ and 95% air. For each EO three independent experiments were performed.

#### 2.2.2. Gene expression assay

CaCo-2 cells incubated in the presence of 0.01% of each selected EO were lysed and total RNA was extracted using the RNeasy Mini Kit (Qiagen, Barcelona, Spain) according to the manufacturer's recommendations. Isolated RNA was then treated with the RNase-Free DNase Set (Qiagen, Barcelona, Spain). Final RNA concentration and quality were determined spectrophotometrically using a NanoDrop 2000 spectrophotometer (NanoDrop Technologies, Winooski, Vermont, USA). The cDNA was synthesized from total RNA with an $RT^2$ First-Strand Kit (SABiosciences, Barcelona, Spain). The cDNA was then subjected to real-time PCR with an $RT^2$ Real-time PCR SYBR Green/ROX Kit (SABiosciences) on an ABI Prism 7900 sequence detector (Applied Biosystems, Foster City, CA). The PCR conditions were 1 cycle of 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Real-time qRT-PCR analysis of the samples was performed using a PCR array (SABiosciences), including primer pairs specific for 19 genes involved in TLR-mediated signaling pathways: toll-like receptor (*TLR)-1*, *TLR2*, *TLR4*, *TLR5*, *TLR6*, myeloid differentiation primary response 88 (*MYD88*), interleukin-1 receptor-associated kinase 4 (*IRAK-4*), toll interacting protein (*TOLLIP*), nuclear factor-kappa-B inhibitor alpha (*NFKBIA*), nuclear factor-kappa-B subunit 1 (*NFKB-1*), TANK-binding kinase 1 (*TBK-1*), translocation associated membrane protein 1 (*TRAM1*), and interferon regulatory factor 3 (*IRF-3*); the nitric oxide synthase (NOS) pathway: *NOS1*, *NOS2*, and *NOS3*; and genes involved in the inflammatory pathway: prostaglandin G/H synthase and cyclooxygenase (*PTGS2*), and the apoptosis pathway: B-cell lymphoma 2 (*BCL2*) and caspase 8 (*CASP8*). The housekeeping genes were hypoxanthine phosphoribosyltransferase 1 (*HPRT1*) and heat shock protein 90 (*HSP90*) used as controls.

The expression level of each gene was analyzed with $RT^2$ Profiler PCR Array Data Analysis software (version 3.4; SABiosciences). The changes in expression or activity levels were expressed as fold changes (Fc).

#### 2.2.3. Cytokine and chemokine quantification assay

IL-1β, IL-2, IL-4, IL-6, IL-7, IL-8, and TNF-α concentrations in the cell supernatant were measured by immunoassay with a MILLIplex™ kit HSTCMAG-28SK (Linco Research Inc, Missouri, USA) using the Luminex 200 system according to the manufacturer's instructions.

### 2.3. Statistical analysis

All results are expressed as the mean ± SEM of three independent experiments unless otherwise indicated. A two-sided Mann–Whitney *U* test was used to determine changes in the inhibitory growth effect as well as for the gene expression differences between treatments and their respective control. The cytokine release's significant differences were analyzed using one-factor ANOVA, which was corrected by an a posteriori test (least significant difference). Statistical calculations were performed using IBM SPSS Statistics version 25.0 (IBM Corp., Armonk, NY, USA). Differences between treated cells and controls were considered statistically significant when the *P* values were less than 0.05.

## 3. Results

### 3.1. Antibacterial effect of essential oils

To determine the antibacterial effect of the EOs (garlic, onion, clove, cinnamon, pepper, bay leaf, nutmeg, cardamom, rosemary, and white thyme), first, a disc diffusion assay was performed as a screening method. Fig. 1 shows that cinnamon, white thyme, and clove showed the highest growth inhibitory effect of the pathogenic strains. In the case of probiotic bacteria, cinnamon, white thyme, and clove also showed the highest growth inhibitory effect. However, in the case of the commensal bacterial, no growth was observed in Petri dishes. Based on these results, the three EOs selected for the antibacterial and immunomodulatory study were cinnamon, white thyme, and clove.

The antibacterial effect of the three selected EOs was confirmed by the microdilution method. Fig. S1 shows the cinnamon, white thyme, and clove EOs growth curves for each bacterial group (pathogenic and probiotic strains). Tables 1 and 2 depict the inhibitory effect of each EO at the different tested concentrations. In the case of the pathogenic intestinal bacteria, the three studies EOs were able to inhibit bacterial growth (probiotic and pathogenic) at the 0.1% concentration. Cinnamon is the EOs with the highest antibacterial effect, followed by clove and white thyme. Indeed, cinnamon inhibited *E. coli* CECT 729 and *S. thyphi* CECT725 growth at all studied concentrations.

In the case of the probiotic bacteria, no inhibitory effect was observed at 0.01 and 0.001% concentrations. At 0.1% concentration, cinnamon and white thyme presented antibacterial effect in all tested probiotic bacteria. However, this observed effect was attenuated after 10–15 h of incubation. In the case of clove EO, we only found a significant inhibition of the *L. paracasei* and *L. rhamnosus* growth, although this inhibition was attenuated after 5–10 h of incubation.

**Table 2**
Effect of essential oils on the growth of *Lactobacillus. plantarum* 3547, *Lactobacillus. paracasei* CNCM I-4034, *Bifidobacterium breve* CNCM I-4035, and *Lactobacillus. rhamnosus* CNCM I-4036 tested by the microdilution method. (+) Growth inhibition of probiotic bacteria with significant difference $p < 0.05$; (−) no inhibitory effects or growth inhibition of pathogenic bacteria with no significant *difference* $P < 0.05$ (cc = concentration).

| Eos | *L. plantarum* 3547 | | | *L. paracasei* CNCM I-4034 | | | *B. breve* CNCM I-4035 | | | *L. rhamnosus* CNCM I-4036 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| cc (%) | 0.1 | 0.01 | 0.001 | 0.1 | 0.01 | 0.001 | 0.1 | 0.01 | 0.001 | 0.1 | 0.01 | 0.001 |
| Cinnamon | + | − | − | + | − | − | + | − | − | + | − | − |
| Clove | − | − | − | + | − | − | − | − | − | + | − | − |
| White thyme | + | − | − | + | − | − | − | − | − | + | − | − |

**Table 3**
Effect of essential oils on the growth of *Bacteroides* spp. DSM 107544, *Prevotella bryantii* DSM 11371, and *Trichococcus pasteurii* DSM 2381. (+) Growth inhibition of commensal bacteria with significant difference $p < 0.05$; (−) no inhibitory effects or growth inhibition of pathogenic bacteria with no significant difference $P < 0.05$ (cc = concentration).

| EOs | *Bacteroides* spp. DSM 107544 | | | *Prevotella bryantii* DSM 11371 | | | *Trichococcus pasteurii* DSM 2381 | | |
|---|---|---|---|---|---|---|---|---|---|
| cc (%) | 0.1 | 0.01 | 0.001 | 0.1 | 0.01 | 0.001 | 0.1 | 0.01 | 0.001 |
| Cinnamon | + | + | − | + | − | − | + | + | − |
| Clove | + | + | − | + | + | − | + | + | − |
| White thyme | + | − | − | + | − | − | + | − | − |

**Table 4**
Differential gene expression mediated by cinnamon, clove, and white thyme essential oils in CaCo-2 cells. Control was CaCo-2 cells with the presence of 0.01% DMSO. Data are expressed as fold change. **Abbreviations:** toll-like receptor (*TLR)-1*, *TLR2*, *TLR4*, *TLR5*, *TLR6*, myeloid differentiation primary response 88 (*MYD88*), interleukin-1 receptor-associated kinase 4 (*IRAK-4*), toll interacting protein (*TOLLIP*), nuclear factor-kappa-B inhibitor alpha (*NFKBIA*), nuclear factor-kappa-B subunit 1 (*NFKB-1*), TANK-binding kinase 1 (*TBK-1*), translocation associated membrane protein 1 (*TRAM1*), and interferon regulatory factor 3 (*IRF-3*); nitric oxide synthase (NOS) pathway: *NOS1*, *NOS2*, and *NOS3*; and genes involved in the inflammatory pathway: prostaglandin G/H synthase and cyclooxygenase (*PTGS2*), and the apoptosis pathway: B-cell lymphoma 2 (*BCL2*) and caspase 8 (*CASP8*). DMSO, Dimethylsulfoxide.

| Gen symbol | CaCo-2 cells | P-value | CaCo-2 cells + Cinnamon 0.01% | P-value | CaCo-2 cells + Clove 0.01% | P-value | CaCo-2 cells + White Thyme 0.01% | P-value |
|---|---|---|---|---|---|---|---|---|
| *MYD88* | 1.3 | 0.27 | 1.10 | 0.31 | 1.09 | 0.57 | −1.08 | 0.37 |
| *IRF3* | 1.53 | 0.04 | 1.57 | 0.09 | 1.44 | 0.22 | 1.03 | 0.90 |
| *TLR2* | −1.09 | 0.45 | −1.58 | 0.10 | −1.01 | 0.64 | −1.14 | 0.36 |
| *TLR6* | 1.76 | 0.12 | −1.04 | 0.79 | 8.79 | 0.13 | −1.09 | 0.80 |
| *NOS2* | −1.02 | 0.82 | −9.09 | 0.01 | −1.04 | 0.94 | −2.12 | 0.09 |
| *BCL2* | −1.35 | 0.14 | 1.04 | 0.82 | 5.57 | 0.14 | −1.47 | 0.05 |
| *TRAM1* | 1.22 | 0.14 | −1.40 | 0.23 | −1.25 | 0.20 | 1.04 | 0.64 |
| *NFKBIA* | −1.13 | 0.84 | 1.33 | 0.18 | −1.37 | 0.61 | 1.28 | 0.28 |
| *IRAK4* | 1.15 | 0.44 | 1.54 | 0.01 | 2.80 | 0.11 | −1.05 | 0.66 |
| *TLR4* | 2.12 | 0.03 | 4.73 | 0.00 | 20.90 | 0.13 | 1.32 | 0.32 |
| *TOLLIP* | −1.81 | 0.17 | −4.26 | 0.00 | −2.22 | 0.02 | −1.33 | 0.09 |
| *NOS3* | 1.07 | 0.64 | 2.96 | 0.01 | 16.45 | 0.13 | 1.25 | 0.32 |
| *TBK1* | 1.64 | 0.11 | 1.59 | 0.01 | 4.24 | 0.15 | 1.00 | 0.90 |
| *NFKB1* | −1.16 | 0.17 | −4.00 | 0.00 | −1.11 | 0.79 | −1.21 | 0.02 |
| *TLR1* | 1.73 | 0.16 | 6.40 | 0.00 | 27.67 | 0.13 | −1.19 | 0.06 |
| *TLR5* | −1.47 | 0.01 | −1.17 | 0.77 | 2.11 | 0.17 | −1.51 | 0.02 |
| *NOS1* | −1.21 | 0.99 | 2.19 | 0.33 | 6.11 | 0.14 | −1.29 | 0.35 |
| *PTGS2* | −1.2 | 0.94 | −1.08 | 0.41 | 1.86 | 0.00 | −1.14 | 0.27 |
| *CASP8* | 1.17 | 0.083 | −1.47 | 0.0008 | 1.91 | 0.137 | 1.06 | 0.358 |

**Table 5**
Differential gene expression mediated by cinnamon, clove, and white thyme essential oils in CaCo-2 cells in the presence of lipopolysaccharides. Control was CaCo-2 cells with the presence of 0.01% DMSO and LPS. Data are expressed as fold change. LPS, lipopolysaccharide. **Abbreviations:** toll-like receptor (*TLR)-1*, *TLR2*, *TLR4*, *TLR5*, *TLR6*, myeloid differentiation primary response 88 (*MYD88*), interleukin-1 receptor-associated kinase 4 (*IRAK-4*), toll interacting protein (*TOLLIP*), nuclear factor-kappa-B inhibitor alpha (*NFKBIA*), nuclear factor-kappa-B subunit 1 (*NFKB-1*), TANK-binding kinase 1 (*TBK-1*), translocation associated membrane protein 1 (*TRAM1*), and interferon regulatory factor 3 (*IRF-3*); nitric oxide synthase (NOS) pathway: *NOS1*, *NOS2*, and *NOS3*; and genes involved in the inflammatory pathway: prostaglandin G/H synthase and cyclooxygenase (*PTGS2*), and the apoptosis pathway: B-cell lymphoma 2 (*BCL2*) and caspase 8 (*CASP8*). DMSO: Dimethylsulfoxide, LPS: lipopolysaccharide.

| Gen symbol | CaCo-2 cells | P-value | CaCo-2 cells + LPS + Cinnamon 0.01% | P-value | CaCo-2 cells + LPS + Clove 0.01% | P-value | CaCo-2 cells + LPS + White Thyme 0.01% | P-value |
|---|---|---|---|---|---|---|---|---|
| *MYD88* | 2.02 | 0.114 | −1.08 | 0.523 | 1 | 0.779 | 1.62 | 0.127 |
| *IRF3* | 2.95 | 0.036 | 1.26 | 0.846 | 2.13 | 0.235 | 1.92 | 0.325 |
| *TLR2* | 2.01 | 0.083 | 1.47 | 0.47 | 1.43 | 0.544 | 1.4 | 0.574 |
| *TLR6* | −1.63 | 0.254 | −10 | 0.051 | −1.92 | 0.191 | −2.17 | 0.232 |
| *NOS2* | 1.24 | 0.91 | −4.34 | 0.113 | 1.77 | 0.898 | 1.25 | 0.637 |
| *BCL2* | −3.57 | 0.138 | −6.66 | 0.115 | −1.69 | 0.237 | −2.22 | 0.184 |
| *TRAM1* | 2.53 | 0.02 | 1.34 | 0.606 | 1.71 | 0.156 | 1.8 | 0.109 |
| *NFKBIA* | 1.03 | 0.469 | 2.21 | 0.718 | 3.68 | 0.109 | 3.03 | 0.276 |
| *IRAK4* | −1.08 | 0.659 | −1.29 | 0.2 | 1.09 | 0.819 | −1.23 | 0.276 |
| *TLR4* | −2.63 | 0.162 | −4.34 | 0.154 | −2.27 | 0.175 | −4.34 | 0.144 |
| *TOLLIP* | 1.68 | 0.697 | −1.08 | 0.402 | 2.39 | 0.337 | 2.7 | 0.202 |
| *NOS3* | −3.84 | 0.144 | −5.26 | 0.134 | −3.7 | 0.156 | −3.22 | 0.161 |
| *TBK1* | 1.11 | 0.583 | −2.94 | 0.005 | −1.14 | 0.499 | −1.26 | 0.175 |
| *NFKB1* | 1.42 | 0.903 | −1.25 | 0.323 | 1.52 | 0.968 | 2.08 | 0.428 |
| *TLR1* | −3.33 | 0.156 | −7.1 | 0.136 | −2.78 | 0.172 | −3.22 | 0.153 |
| *TLR5* | −1.36 | 0.283 | 1.58 | 0.589 | −2.43 | 0.115 | −1.49 | 0.224 |
| *NOS1* | −2.22 | 0.217 | −5.26 | 0.104 | −2.08 | 0.176 | −1.99 | 0.218 |
| *PTGS2* | −2.27 | 0.059 | −2.17 | 0.009 | −1.4 | 0.435 | −1.47 | 0.26 |
| *CASP8* | −1.11 | 0.494 | −2.38 | 0.034 | −1.16 | 0.408 | −1.39 | 0.184 |

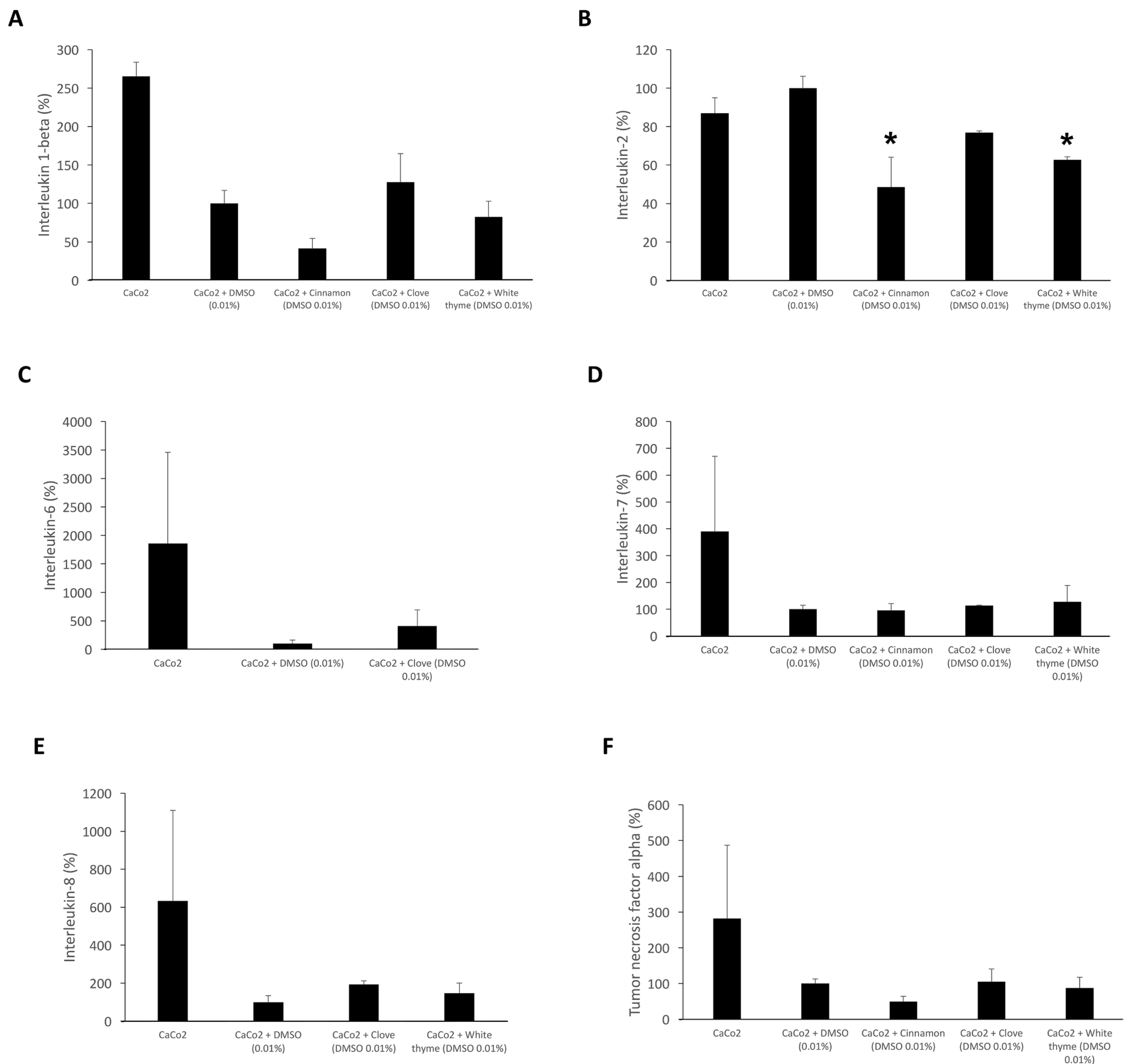


**Fig. 2.** Cytokine and chemokine release of Caco-2 cells in the presence of 0.01% cinnamon, 0.01% clove, and 0.01% white thyme essential oils. Values are expressed as mean and standard deviation percentage according to the CaCo-2 cells value; this value was used as 100%. The number of experiments were 3 per sample. A. Interleukin 1-beta (%), B. Interleukin-2 (%), C. Interleukin-6 (%), D. Interleukin-7 (%), E. Interleukin-8 (%), F. Tumor necrosis factor alpha (%).

Finally, the inhibitory effect of the three EOs was also observed against the three commensal strains tested (Table 3). The inhibitory effects against the growth of *Trichococcus pasteurii* are shown in Fig. S2. In terms of concentration, the most significant inhibitory effect was that corresponding to 0.1% of EOs. However, in the case of 0.01% of cinnamon and clove, their inhibitory effect against the growth of *Bacteroides* spp. and *Trichococcus pasteurii* was significant.

### 3.2. Essential oils effects over gene expression and cytokine release in CaCo-2 cells

The gene expression modulatory effects of cinnamon, white thyme, and clove EOs were tested on 19 genes involved in TLR-mediated signaling and NOS pathways, and inflammation and apoptosis. Cinnamon significantly decreased *NOS2*, *TOLLIP*, *NFKB1*, and *CASP8* gene expression and significantly increased the expression of *IRAK4*, *TLR4*, *NOS3*, and *TLR1*. Similarly, clove significantly decreased *TOLLIP* and significantly increased *PTGS2* gene expression. Finally, white thyme significantly decreased the *NFKB1* gene expression (Table 4). In the presence of LPS, only cinnamon EO significantly decreased *PTGS2*, *CASP8*, and *TBK1* gene expression (Table 5). Although the gene expression of inflammatory genes was affected by the EOs, in the case of cytokine secretion, only cinnamon and clove EOs significantly decreased IL-2 in the absence of LPS (Fig. 2). However, in the presence of LPS no significant effect was observed for any of the studied EOs (Fig. 3).

## 4. Discussion

In recent years, there has been a growing interest in researching and developing new antimicrobial agents from EOs to use against foodborne bacterial pathogens. In this context, our study shows that cinnamon, white thyme, and clove EOs have significant antibacterial activity

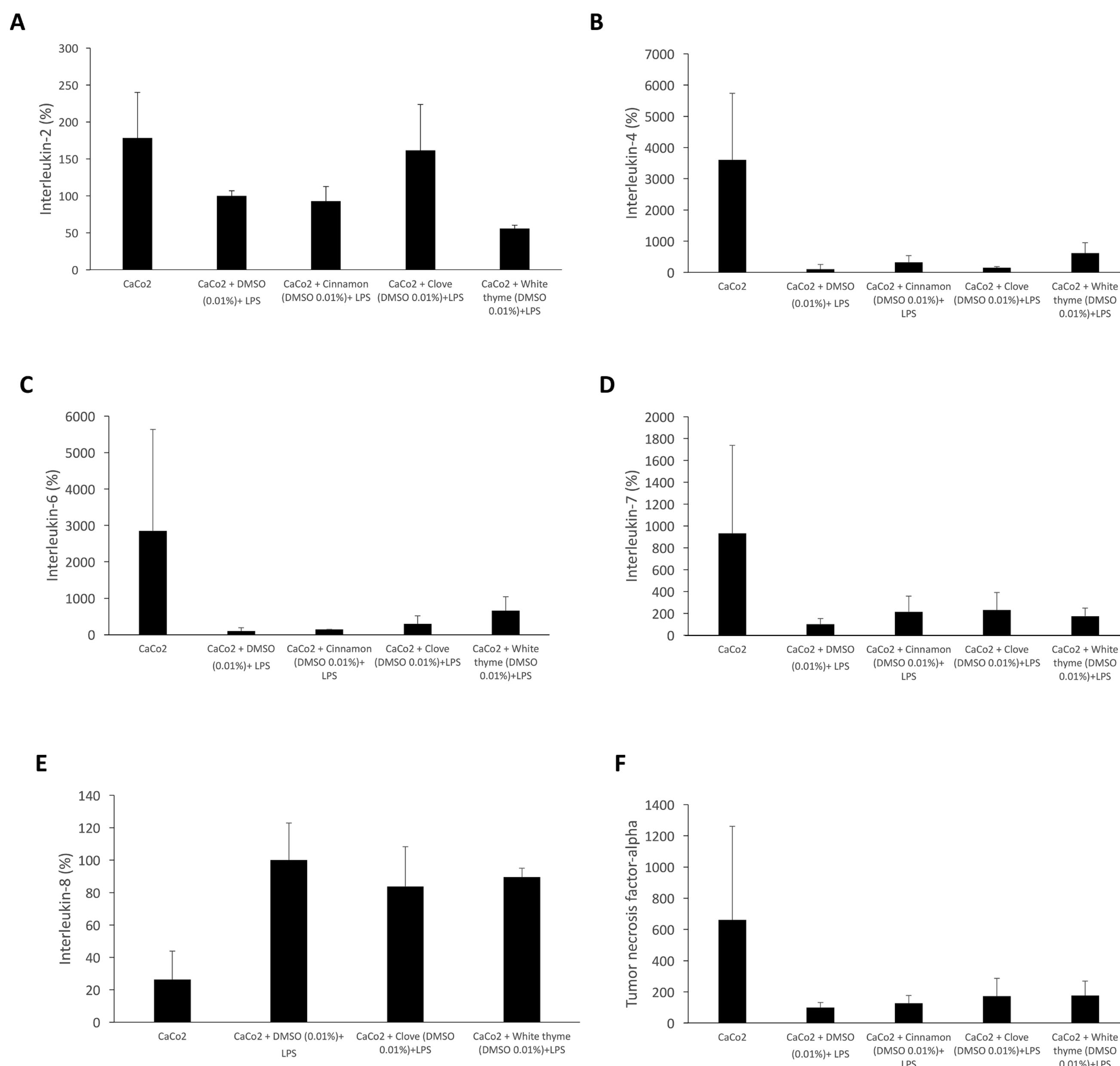


**Fig. 3.** Cytokine and chemokine release of the activated Caco-2 cells with lipopolysaccharides in the presence of the 0.01% cinnamon, 0.01% clove, and 0.01% white thyme essential oils. Values are expressed as mean and standard deviation percentage according to CaCo-2 cells plus DMSO and LPS values; this value was used as 100%. The number of experiments was 3 per sample. A. Interleukin 2 (%), B. Interleukin-4 (%), C. Interleukin-6 (%), D. Interleukin-7 (%), E. Interleukin-8 (%), F. Tumor necrosis factor alpha (%). LPS, lipopolysaccharide; DMSO, Dimethylsulfoxide.

against intestinal pathogenic strains, whereas this effect is less acute in probiotic strains. Regarding the commensal bacterial, an inhibitory growth effect was also observed in the presence of the EOs. These results indicate that the studied EOs could inhibit pathogenic bacteria without affecting gastrointestinal probiotics bacteria, although they have an inhibitory effect on commensal bacteria. Additionally, this study revealed that EOs have an immunomodulatory effect on intestinal Caco-2 cells.

Cinnamon is known as one of the most common spices, with anti-emetic, anti-diarrheal, anti-flatulent, and stimulant properties (Nabavi et al., 2015), whereas clove and white thyme have been used traditionally as savoring agents in food (Thosar, Basak, Bahadure, & Rajurkar, 2013; Xu, Liu, Hu, & Cao, 2016). Different studies have described the activity of the EOs against important intestinal pathogenic bacteria such as *E. coli* and *S. typhimurium* (Man, Santacroce, Jacob, Mare, & Man, 2019; Valdivieso-Ugarte et al., 2019). The antimicrobial effect of these oils was correlated to the occurrence of the major compounds such as cinnamic aldehyde, thymol, and eugenol (Swamy, Akhtar, & Sinniah, 2016). In general terms, pathogenic species are more sensitive to EOs than most commensal bacteria; however, the sensitivity of bacterial species to EOs in the mixed intestinal population may differ from that of the pure culture (Thapa, Louis, Losa, Zweifel, & Wallace, 2015).

Although different studies described that Gram-positive bacteria are more susceptible to EOs than Gram-negative bacteria, we did not find any differences between Gram-positive and Gram-negative bacteria. Numerous studies have shown the antibacterial activity of cinnamon, clove, and white thyme against Gram-negative and Gram-positive bacteria (Nabavi et al., 2015; Valdivieso-Ugarte et al., 2019). This is also in agreement with previous results on the antibacterial activity of *Cinnamomun verum, Thymus vulgaris,* and *Eugenia caryophyllata* (Alibi et al.,

2020).

In our study, cinnamon and clove EOs showed the highest activity against the pathogenic bacteria at almost all the tested concentrations, followed by thyme. This is in agreement with a recent study that reported that cinnamon EO showed the highest inhibitory activity, followed by grapefruit zest oil, tangerine zest oil and lemon zest oil (MIC ranging from 6 to 60 ppm) against saprophytic and pathogenic microorganisms (*E. coli*, *S. abony*, *S. aureus*, *P. aeruginosa* and *Candida albicans*) (Denkova-Kostova et al., 2020). In the present study, variable susceptibility of different strains belonging to the same pathogenic species to the EOs has been observed. This effect could be explained by different antibacterial mechanisms, including selective pressure, cross-breeding, and adaptation of a strain to the ecological environment (Salas, Calvo, & Martinez-Martinez, 2008). Regarding the probiotic bacteria, the EOs could inhibit their growth, mainly at the highest tested concentration (0.1%). Cinnamon showed activity against all the probiotic strains, whereas clove and thyme showed activity against 3 and 2 out of the 4 probiotic strains tested, respectively. Growth inhibition was observed in the first hours of incubation, while after 24 h a growth recovery was seen.

Another result of the present study was the immunomodulatory activity of the EOs in Caco-2 cells. EOs are also of interest in the food industry for their anti-inflammatory properties, which are potential sources for the development of functional foods. During a chronic inflammation response, different signaling pathways are activated and an overexpression of pro-inflammatory genes and proteins can be observed (Valdivieso-Ugarte et al., 2019; First of all, we showed that incubation with 0.01% of EOs did not produce any cytotoxic effect or cell growth inhibition. Cinnamon and white thyme were able to inhibit the secretion of IL-2 by the Caco-2 cells. Similarly, gene expression of the TLR, nitric oxide, and apoptosis pathways were affected mainly by cinnamon in Caco-2 cells. The transcription factor NF-κB may be a common center point for the immunomodulatory effect of different EOs, such as eucalyptol and camphor (Borges, Ortiz, Pereira, Keita, & Carvalho, 2019). In experimentally-induced inflammation models EOs have shown promising effects as agents against inflammation; however, we did not find any significant immunomodulatory effect on LPS-stimulated Caco-2 cells.

Based on our results, it appears that the most promising EO is cinnamon. It is noteworthy to mention that cinnamon is safe to be used as a spice and/or flavoring agent; however, in large doses or when used over long periods, it could lead to adverse effects (Hajimonfarednejad et al., 2019). Although EOs are a new promising strategy for the food industry, the organoleptic impact of these EOs in foodstuffs needs to be evaluated.

## 5. Conclusions

Considering the results of the present study, cinnamon, clove, and white thyme EOs could be potential candidates to be used as natural alternatives for application in food preservation to retard or inhibit bacterial growth, and to extend the shelf life of the food products. Furthermore, at a 0.01% concentration, these EOs appear to be safe for the intestinal enterocytes and seem to exert some immunomodulatory effects. However, the underlying mechanisms are unknown and warrant further research.

## Ethics statements

This work does not involves the use of human subjects or animal experiments.

## CRediT authorship contribution statement

**Magdalena Valdivieso-Ugarte:** Investigation, Formal analysis, Writing - original draft, Writing - review & editing. **Julio Plaza-Diaz:** Investigation, Formal analysis, Writing - review & editing. **Carolina Gomez-Llorente:** Conceptualization, Formal analysis, Writing - original draft, Writing - review & editing. **Eduardo Lucas Gómez:** Conceptualization, Writing - review & editing. **Maria Sabés-Alsina:** Conceptualization, Writing - original draft, Writing - review & editing. **Ángel Gil:** Conceptualization, Formal analysis, Writing - original draft, Writing - review & editing.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jff.2021.104436.

## References

Alibi, S., Selma, W. B., Ramos-Vivas, J., Smach, M. A., Touati, R., Boukadida, J. & Mansour, H. B. (2020). Anti-oxidant, antibacterial, anti-biofilm, and anti-quorum sensing activities of four essential oils against multidrug-resistant bacterial clinical isolates. Current Research in Translational Medicine.

Arumugam, M., Raes, J., Pelletier, E., Le Paslier, D., Yamada, T., Mende, D. R., & Bork, P. (2011). Enterotypes of the human gut microbiome. *Nature, 473*(7346), 174–180. https://doi.org/10.1038/nature09944.

Bauer, A. W., Kirby, W. M., Sherris, J. C., & Turck, M. (1966). Antibiotic susceptibility testing by a standardized single disk method. *American Journal of Clinical Pathology, 45*, 493–496.

Borges, R. S., Ortiz, B. L. S., Pereira, A. C. M., Keita, H., & Carvalho, J. C. T. (2019). Rosmarinus officinalis essential oil: A review of its phytochemistry, anti-inflammatory activity, and mechanisms of action involved. *Journal of Ethnopharmacology, 229*, 29–45. https://doi.org/10.1016/j.jep.2018.09.038.

Chenoll, E., Casinos, B., Bataller, E., Astals, P., Echevarria, J., Iglesias, J. R., & Genoves, S. (2011). Novel probiotic Bifidobacterium bifidum CECT 7366 strain active against the pathogenic bacterium Helicobacter pylori. *Applied and Environment Microbiology, 77*(4), 1335–1343. https://doi.org/10.1128/AEM.01820-10.

Denkova-Kostova, R., Teneva, D., Tomova, T., Goranov, B., Denkova, Z., Shopska, V., Slavchev, A., & Hristova-Ivanova, Y. (2020). Chemical composition, antioxidant and antimicrobial activity of essential oils from tangerine (Citrus reticulata L.), grapefruit (Citrus paradisi L.), lemon (Citrus lemon L.) and cinnamon (Cinnamomun zeylanicum Blume). Zeitschrif Für Naturforschung doi: 10.1515/znc-2020-0126.

Falleh, H., Ben Jemaa, M., Saada, M., & Ksouri, R. (2020). Essential oils: A promising eco-friendly food preservative. *Food Chemistry, 330*, Article 127268. https://doi.org/10.1016/j.foodchem.2020.127268.

Hajimonfarednejad, M., Ostovar, M., Raee, M. J., Hashempur, M. H., Mayer, J. G., & Heydari, M. (2019). Cinnamon: A systematic review of adverse events. *Clinical Nutrition, 38*(2), 594–602. https://doi.org/10.1016/j.clnu.2018.03.013.

Man, A., Santacroce, L., Jacob, R., Mare, A., & Man, L. (2019). Antimicrobial activity of six essential oils against a group of human pathogens: a comparative study. *Pathogens, 8*(1), 15. https://doi.org/10.3390/pathogens8010015.

Munekata, P. E. S., Pateiro, M., Rodriguez-Lazaro, D., Dominguez, R., Zhong, J., & Lorenzo, J. M. (2020). The role of essential oils against pathogenic Escherichia coli in food products. *Microorganisms, 8*(6), 924. https://doi.org/10.3390/microorganisms8060924.

Munoz-Quezada, S., Chenoll, E., Vieites, J. M., Genoves, S., Maldonado, J., Bermudez-Brito, M., & Gil, A. (2013). Isolation, identification and characterisation of three

novel probiotic strains (Lactobacillus paracasei CNCM I-4034, Bifidobacterium breve CNCM I-4035 and Lactobacillus rhamnosus CNCM I-4036) from the faeces of exclusively breast-fed infants. *British Journal of Nutrition, 109*(Suppl 2), S51–S62. https://doi.org/10.1017/S0007114512005211.

Nabavi, S. F., Di Lorenzo, A., Izadi, M., Sobarzo-Sanchez, E., Daglia, M., & Nabavi, S. M. (2015). Antibacterial effects of cinnamon: From farm to food, cosmetic and pharmaceutical industries. *Nutrients, 7*(9), 7729–7748. https://doi.org/10.3390/nu7095359.

Perez del Palacio, J., Diaz, C., De la Cruz, M., Annang, F., Martin, J., Pérez-Victoria, I., … Gálvez, J.(2016). Hight-throughput screening platform for the discovery of new immunomodulatory molecules from natural products extract libraries. Journal of Biomolecular Screening; 21: 567–578.

Perry, S. W., Epstein, L. G., & Gelbard, H. A. (1997). In situ trypan blue staining of monolayer cell cultures for permanent fixation and mounting. *BioTechniques, 22*(6), 1020–1024.

Pinto, M., Robineleon, S., Appay, M. D., Kedinger, M., Triadou, N., Dussaulx, E., et al. (1983). Enterocyte-like differentiation and polarization of the human-colon carcinoma cell-line Caco-2 in culture. *Biolology of the Cell, 47*, 323–330.

Salas, C., Calvo, J., & Martinez-Martinez, L. (2008). Activity of tigecycline against coryneform bacteria of clinical interest and listeria monocytogenes. *Antimicrobial Agents and Chemotherapy, 52*(4), 1503–1505. https://doi.org/10.1128/AAC.01129-07.

Swamy, M. K., Akhtar, M. S., & Sinniah, U. R. (2016). Antimicrobial properties of plant essential oils against human pathogens and their mode of action: an updated review. Evidence-Based Complementary and Alternative Medicine, 2016.

Teneva, D., Denkova, Z., Denkova-Kostova, R., Goranov, B., Kostov, G., Slavchev, A., & Degreaeve, P. (2020). Biological preservation of mayonnaise with Lactobacillus plantarum LBRZ12, dill and basil essential oils. *Food Chemistry*.

Thapa, D., Louis, P., Losa, R., Zweifel, B., & Wallace, R. J. (2015). Essential oils have different effects on human pathogenic and commensal bacteria in mixed faecal fermentations compared with pure cultures. *Microbiology, 161*(2), 441–449.

Thosar, N., Basak, S., Bahadure, R. N., & Rajurkar, M. (2013). Antimicrobial efficacy of five essential oils against oral pathogens: An in vitro study. *European Journal of Dentistry, 7*(Suppl 1), S071–S77. https://doi.org/10.4103/1305-7456.119078.

Valdivieso-Ugarte, M., Gomez-Llorente, C., Plaza-Diaz, J., & Gil, A. (2019). Antimicrobial, antioxidant, and immunomodulatory properties of essential oils: A systematic review. *Nutrients, 11*(11), 2786. https://doi.org/10.3390/nu11112786.

Van de Walle, J., Hendrickx, A., Romier, B., Larondelle, Y., & Schneider, Y. J. (2010). Inflammatory parameters in Caco-2 cells: Effect of stimuli nature, concentration, combination and cell differentiation. *Toxicology in vitro, 24*, 1441–1449.

WHO (2015) Retrieved 2020-27-07, 2020, from https://www.who.int/news-room/detail/03-12-2015-who-s-first-ever-global-estimates-of-foodborne-diseases-find-children-under-5-account-for-almost-one-third-of-deaths.

Xu, J.-G., Liu, T., Hu, Q.-P., & Cao, X.-M. (2016). Chemical composition, antibacterial properties and mechanism of action of essential oil from clove buds against Staphylococcus aureus. *Molecules, 21*(9), 1194.