A cocoa (*Theobroma cacao* L.) extract impairs the growth, virulence properties, and inflammatory potential of *Fusobacterium nucleatum* and improves oral epithelial barrier function

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Abstract

*Fusobacterium nucleatum* is associated with many conditions and diseases, including periodontal diseases that affect tooth-supporting tissues. The aim of the present study was to investigate the effects of a cocoa extract (*Theobroma cacao* L.) on *F. nucleatum* with respect to growth, biofilm formation, adherence, and hydrogen sulfide (*H*₂*S*) production. The anti-inflammatory properties and the effect on epithelial barrier function of the cocoa extract were also assessed. The cocoa extract, whose major phenolic compound is epicatechin, dose-dependently inhibited the growth, biofilm formation, adherence properties (basement membrane matrix, oral epithelial cells), and *H*₂*S* production of *F. nucleatum*. It also decreased IL-6 and IL-8 production by *F. nucleatum*-stimulated oral epithelial cells and inhibited *F. nucleatum*-induced NF-κB activation in monocytes. Lastly, the cocoa extract enhanced the barrier function of an oral epithelial model by increasing the transepithelial electrical resistance. We provide evidence that the beneficial properties of an epicatechin-rich cocoa extract may be useful for preventing and/or treating periodontal diseases.

Introduction

Periodontitis is a multifactorial chronic inflammatory disease that is initiated and maintained by a dysbiotic biofilm and that is characterized by the progressive destruction of the periodontal ligament and alveolar bone [1]. A limited number of Gram-negative anaerobic bacteria with the ability to express virulence factors have been associated with the various stages of periodontitis [2, 3]. Of these periodontal pathogens, *Fusobacterium nucleatum* is believed to play a central role in periodontal biofilm maturation through its ability to act as a bridge between the early (streptococci, actinomyces) and late (*Porphyromonas gingivalis*, *Treponema denticola*, *Tannerella forsythia*) colonizers of oral biofilms [4]. *F. nucleatum* has also been associated with extra-oral infections, including appendicitis, osteomyelitis, atherosclerosis, pericarditis, and brain abscesses [5], and may play a role in preterm/low birth weight and colorectal cancer development [5, 6].
*F. nucleatum* is known to increase in numbers in diseased periodontal sites [7]. It expresses a number of adhesins that are involved in adherence to salivary proteins, bacteria, and host cells [8–10]. Additional virulence properties associated with *F. nucleatum* include hemolytic activity [11] and hydrogen sulfide (H$_2$S) production [12]. *F. nucleatum* can induce an inflammatory response in different cell types, including oral epithelial cells and macrophages [13, 14]. More specifically, oral epithelial cells challenged with *F. nucleatum* respond by producing high levels of cytokines, including interleukin (IL)-6 and IL-8 [15, 16].

The gingival epithelium protects the connective and bone tissues from bacterial invasion and thus plays an important role in the innate immune response [17, 18]. In the course of periodontal disease, inflammation caused by bacteria leads to the disruption of epithelial tight junctions, which facilitates bacterial invasion [19]. Translocation of bacteria into the underlying connective tissue promotes a destructive inflammatory response, leading to bone loss [20].

Various therapeutic approaches for treating periodontal disease include surgical intervention, mechanical therapy (ultrasonic debridement, scaling and root planning) and use of pharmacological agents, with the objective to clean the periodontal pockets, polish the root surfaces and kill or remove bacteria. The pharmacological agents, by exerting an antibacterial effect or modulating the host response, are known to enhance treatment outcomes [21]. In this context, plant polyphenols have attracted the interest of several research groups. Cocoa (*Theobroma cacao* L.) is a plant with a high phenolic content and over 300 different constituents [22]. The beneficial effects of cocoa on human health have been mainly attributed to its phenolic compounds, including monomeric flavanols (catechin, epicatechin), dimer procyanidins B2 and B1, and polymeric flavanols [23]. These cocoa phenolic compounds have been described as bioactive natural molecules because of their antioxidant, anti-inflammatory, anticarcinogenic, and cardio-protective properties [22, 24]. While evidence has been brought that cocoa polyphenols may have benefits for cardiovascular diseases, endocrine disorders, and cancers, their impact with respect to periodontal disease has been poorly investigated [22–24].

Given that *F. nucleatum* is thought to play a key role in periodontal disease development by modulating the formation of a pathogenic subgingival biofilm that induces chronic inflammation, the aims of the present study were to investigate a cocoa extract for its ability to inhibit the growth, biofilm formation, adherence properties, and H$_2$S production of *F. nucleatum*, and to attenuate the *F. nucleatum*-induced inflammatory response in oral epithelial cell and monocyte models. The ability of the cocoa extract to enhance epithelial barrier function was also assessed.

**Materials and methods**

**Cocoa extract and phenolic characterization**

A cocoa extract prepared by ethanol/water extraction of cocoa beans (*Theobroma cacao* L.) was kindly provided by Naturex (Avignon, France). A stock solution (20 mg/mL) was prepared in 10% dimethylsulfoxide (DMSO), sterilized by filtration (0.22 µm pore size), and kept at 4°C protected from light. Anthocyanins and procyanidins in the extract were characterized by high-performance liquid chromatography (HPLC) as described previously [25]. Delphinidin 3-glucoside was used as a standard for anthocyanins. Procyanidins, which were eluted on the basis of their degree of polymerization, were quantified using epicatechin monomer as a standard. Phenolic acids and flavonoids were characterized as previously described [25] using an Acquity® ultra-performance liquid chromatography-tandem mass spectrometer (UHPLC–MS/MS) coupled to a triple quadrupole (TQD) mass spectrometer equipped with a Z-spray electrospray interface (Waters Ltd., Mississauga, ON, Canada). External phenolic and flavonoid standards were analyzed using the same parameters and were used for quantification purposes.
Bacteria and growth conditions

*F. nucleatum* ATCC 25586 was grown under anaerobic conditions (80% N<sub>2</sub>, 10% CO<sub>2</sub>, 10% H<sub>2</sub>) at 37˚C in Todd-Hewitt broth (THB; Becton Dickinson and Company, Sparks, MD, USA) supplemented with 0.001% hemin and 0.0001% vitamin K.

Cell cultures

The previously characterized human oral epithelial cell line GMSM-K [26] was kindly provided by V. Murrah (University of North Carolina, USA). The cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 100 μg/mL of penicillin G-streptomycin. The previously characterized human oral epithelial cell line B11 [27] was kindly provided by S. Groeger (Justus-Liebig-University Giessen, Germany). The cells were cultured in keratinocyte serum-free medium (K-SFM) supplemented with 50 μg/mL of bovine pituitary extract, 5 ng/mL of recombinant epidermal growth factor, and 100 μg/mL of penicillin G-streptomycin. The human monoblastic leukemia cell line U937 3xκB-LUC, a subclone of the U937 cell line stably transfected with a luciferase gene coupled to a promoter of three NF-κB- binding sites [28], was kindly provided by R. Blomhoff (University of Oslo, Norway). These cells were cultured in Roswell Park Memorial Institute 1640 medium (RPMI-1640) supplemented with 10% FBS, 100 μg/mL of penicillin G/streptomycin, and 75 μg/mL of hygromycin B. The cultures were incubated in a humidified incubator with a 5% CO<sub>2</sub> atmosphere at 37˚C. All the culture media and supplements were obtained from Life Technologies Inc. (Burlington, ON, Canada).

Determination of minimum inhibitory and minimum bactericidal concentrations

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of the cocoa extract were determined using a broth microdilution assay [29]. A 24-h culture of *F. nucleatum* was diluted in fresh medium to an optical density at 660 nm (OD<sub>660</sub>) of 0.1, which corresponds to a concentration of 8 x 10<sup>7</sup> colony forming units (CFU)/mL. Equal volumes (100 μL) of the bacterial culture and two-fold serial dilutions of the cocoa extract (31.25 to 4,000 μg/mL) in fresh medium were mixed in the wells of a 96-well tissue culture treated, flat-bottom, microplate (Sarstedt, Newton, NC, USA). Wells without bacteria or cocoa extract (but with carrier solvant) were used as controls. The microplate was incubated for 24 h under anaerobic conditions prior to assessing bacterial growth by recording the OD<sub>660</sub> using a Synergy 2 microplate reader (BioTek Instruments, Winooski, VT, USA). The MIC value was defined as the lowest concentration of the cocoa extract that completely prevented bacterial growth. The MBC value was determined by spreading 5-μL aliquots from each well that exhibited no visible growth on sheep blood-supplemented THB agar plates. The plates were then incubated at 37˚C for 48 h in an anaerobic chamber. The MBC value was defined as the lowest concentration at which no colonies formed. The MIC and MBC assays were performed in triplicate in two independent experiments.

Biofilm formation

The effect of the cocoa extract on biofilm formation by *F. nucleatum* was assessed using the 96-well microplate described above after recording bacterial growth. Planktonic and poorly-attached bacterial cells were removed by aspiration with a 26G needle. Biofilms were stained with 100 μL of 0.01% crystal violet for 15 min, washed with distilled water, and dried at 37˚C for 2 h. To release biofilm-embedded crystal violet, 100 μL of 75% ethanol was added, and the
plate was shaken for 15 min. The biofilm biomass was estimated by recording the absorbance at 550 nm (A<sub>550</sub>) using a Synergy 2 microplate reader. Assays were performed in triplicate in two independent experiments.

**Adherence to oral epithelial cells and to a basement membrane model**

The effect of the cocoa extract on the adherence of *F. nucleatum* to oral epithelial cells (GMSM-K cell line) and a basement membrane model (Matrigel<sup>TM</sup>; BD Biosciences, Bedford, MA, USA) was investigated, as previously described by Ben Lagha and Grenier [30]. Briefly, bacterial cells from a 24-h culture of *F. nucleatum* were labeled with fluorescein isothiocyanate (FITC, 0.03 mg/mL) and were suspended in DMEM medium supplemented with 10% heat-inactivated FBS or 50 mM phosphate-buffered saline (PBS; pH 7) to assess adherence to the epithelial cells or the Matrigel<sup>TM</sup>, respectively. Epithelial cells (1.5 x 10<sup>6</sup> cells/mL) were seeded at confluence in a 96-well clear bottom black microplate (Greiner Bio-One North America, Monroe, NC, USA), which was incubated (37˚C/5% CO<sub>2</sub>) overnight to allow cell adherence. Cell monolayers were pre-incubated (30 min) with two-fold serial dilutions of the cocoa extract (15.63 to 500 μg/mL in DMEM medium) prior to adding FITC-labeled *F. nucleatum* cells at a multiplicity of infection (MOI) of 100. The microplate was incubated for a further 4 h at 37˚C in a 5% CO<sub>2</sub> atmosphere. Unbound bacteria were then removed by aspiration, and adhering epithelial cells were washed twice with PBS. The number of bacteria adhered to the epithelial cell monolayers was estimated by determining relative fluorescence units (RFU; excitation wavelength 495 nm, emission wavelength 525 nm) using a Synergy 2 microplate reader. Matrigel<sup>TM</sup>, a basement membrane extract containing several extracellular matrix components, including laminin, type IV collagen, heparin sulfate proteoglycans, and entactin, was diluted 1:10 in ice-cold PBS, was added (100 μL) to the wells of 96-well clear bottom black microplates and was maintained at room temperature for 2 h to allow gellification. The Matrigel<sup>TM</sup> was then washed twice with PBS, and two-fold serial dilutions of the cocoa extract (15.63 to 500 μg/mL in PBS) were added to the wells. After a 30-min incubation at room temperature, FITC-labeled *F. nucleatum* cells (OD<sub>660</sub> = 0.5) were added to each well (100 μL), and the microplate was incubated at 37˚C for 4 h. The number of bacterial cells that adhered to the basement membrane model was determined as described above for the oral epithelial monolayers. Wells without *F. nucleatum* were used as controls to measure basal autofluorescence, while wells with no cocoa extract were used to determine 100% adherence values. All the above assays were performed in triplicate in two independent experiments.

**Production of hydrogen sulfide**

The production of H<sub>2</sub>S from cysteine and homocysteine by *F. nucleatum* was assessed by monitoring the precipitation of bismuth sulfide, according to the procedure described by Yoshida et al. [12], with slight modifications. Cells from a 24-h culture of *F. nucleatum* were harvested by centrifugation and were suspended in oxygen-free 0.1% Tryptone to an OD<sub>660</sub> of 1.0. Aliquots (50 μL) of the bacterial suspension were pipetted into the wells of a 96-well microplate. The cocoa extract (125, 250, 500, 1000 μg/mL) was added (50 μL) to the wells along with 100 μL of a reaction mixture containing 400 mM triethanolamine-HCl, 20 μM pyridoxal-5'-phosphate, 10 mM bismuth III chloride, 10 mM L-cysteine, 10 mM DL-homocysteine, and 20 mM EDTA (pH 8). The plate was sealed with a plastic sheet and was incubated under anaerobic conditions for 1 h at 37˚C. H<sub>2</sub>S production was quantified by measuring the absorbance at 620 nm (A<sub>620</sub>) using a BioTek Synergy 2 microplate reader. The control values (without bacteria) were subtracted from the corresponding reactions to compensate for background absorbance by the medium. The assay was performed in triplicate in two independent experiments.
**Determination of cell viability**

With the objective to identify non-cytotoxic concentrations of the cocoa extract for the cell lines used below, an MTT (3-[4,5-diethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) colorimetric assay (Roche Diagnostics, Laval, QC, Canada) was performed according to the manufacturer’s protocol. Cell lines were treated (24 h) with two-fold serial dilutions of the cocoa extract (31.25, 62.5, 125, 250, and 500 μg/mL), prior to assess cell viability.

**Cytokine secretion by oral epithelial cells**

The human oral epithelial cell line GMSM-K was used to investigate the effect of the cocoa extract on the secretion of the pro-inflammatory cytokines interleukin-6 (IL-6) and IL-8. Epithelial cells were seeded (10^6 cells/well) in a 6-well microplate and were cultured overnight at 37˚C in a 5% CO₂ atmosphere. The epithelial cells were then pre-treated with non-cytotoxic concentrations of the cocoa extract for 30 min prior to being stimulated with *F. nucleatum* at an MOI of 100. After a 24-h incubation, the culture medium supernatants were collected and were stored at -20˚C until used. Cells incubated in the absence of the cocoa extract and stimulated or not with *F. nucleatum* were used as controls. IL-6 and IL-8 concentrations were determined using ELISA kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer’s protocol. Assays were performed in triplicate in two independent experiments.

**Activation of the NF-κB transcription factor in monocytes**

To determine the effect of the cocoa extract on *F. nucleatum*-induced NF-κB activation, the U937 3xxB-LUC monocytes were seeded (10^5 cells/well) in the wells of a black wall, black bottom, 96-well microplate (Greiner Bio-One North America) and were pre-incubated with non-cytotoxic concentrations of cocoa extract for 30 min. Thereafter, the monocytes were stimulated with *F. nucleatum* at an MOI of 100 for 6 h. Wells containing monocytes but no *F. nucleatum* or no cocoa extract were used as controls. A commercial inhibitor (BAY-11-7082 [25 μM], EMD Millipore Canada, Mississauga, ON, Canada) of the NF-κB signaling pathway was used as a positive control. Bright-Glo reagent (Promega Corporation, Durham, NC, USA) was used according to the manufacturer’s protocol to measure luciferase activity and determine NF-κB activation. Luminescence was monitored using a Synergy 2 microplate reader. Assays were performed in triplicate in two independent experiments.

**Oral epithelial barrier function**

The human oral epithelial cell line B11 was used to investigate the effect of the cocoa extract on epithelial barrier function using the protocol described by Ben Lagha and Grenier [30]. Epithelial cells were seeded on Transwell™ clear polyester membrane inserts (6.5 mm diameter, 0.4 μm pore size; Corning Co., Cambridge, MA, USA) at a concentration of 3 x 10^5 cells per insert. The basolateral and apical compartments were filled with 0.6 mL and 0.1 mL of culture medium, respectively. After a 72-h incubation (37˚C/5% CO₂), the culture medium was replaced with fresh antibiotic-free K-SFM, and the cells were incubated for a further 16 h. Non-cytotoxic concentrations of the cocoa extract were added to the apical compartment, and the integrity of the epithelial tight junctions was determined by monitoring the transepithelial electrical resistance (TER) using an ohmmeter (EVOM2, World Precision Instruments, Sarasota, FL, USA). TER was recorded after a 0-, 2-, 4-, 8-, 24-, or 48-h incubation (37˚C/5% CO₂). Resistance values were calculated in Ohms (Ω)/cm² by multiplying the resistance values by the surface area of the membrane filter. Results were expressed as a percentage of the basal control value measured at time 0 (100% value). Assays were performed in triplicate in two independent experiments.
**Statistical analysis**

All experiments were performed in triplicate in two independent experiments, and the means ± standard deviations (SD) were calculated. A one-way ANOVA with a post hoc Bonferroni multiple comparison test was used to analyze the data. Results were considered statistically significant at $p < 0.01$.

**Results**

**Polyphenolic composition**

The polyphenolic composition of the cocoa extract, as determined by the chromatographic and MS analyses, is reported in Table 1. The fraction contained 30.93% of polyphenols. Phenolic acids, flavonols, anthocyanins, flavan-3-ols, and procyanidins made up 0.15%, 0.72%, 0.12%, 2.19%, and 96.82% of the total polyphenols, respectively. Monomers, dimers, trimers, and tetramers made up 75% of the total procyanidin content.

**Effects on *F. nucleatum* growth and virulence properties**

The antibacterial activity of the cocoa extract was assessed using a broth microdilution assay. As shown in Fig 1, the cocoa extract had a MIC value of 2000 μg/mL against *F. nucleatum* while a concentration of 1000 μg/mL reduced bacterial growth by 73.6%. No MBC value was obtained, suggesting that the cocoa extract has a bacteriostatic mode of action, although concentrations > 2000 μg/mL may have a bactericidal effect. Interestingly, the cocoa extract prevented biofilm formation by *F. nucleatum*. When cultivated in the presence of 500 μg/mL of the cocoa extract, the growth of *F. nucleatum* was not affected while the biofilm was reduced by 94.3% (Fig 1).

The ability of the cocoa extract to reduce the adherence of *F. nucleatum* to oral epithelial cells was investigated. As shown in Fig 2A, the cocoa extract dose-dependently inhibited bacterial adherence to oral epithelial cells. At the lowest (15.63 μg/mL) and the highest (500 μg/mL) concentrations of the cocoa extract tested, the adherence of *F. nucleatum* was reduced by 17.8% and 65.7%, respectively. We then evaluated the capacity of the cocoa extract to attenuate the adherence of *F. nucleatum* to the Matrigel™ basement membrane model. The cocoa extract also exerted a dose-dependent inhibitory effect on adherence to oral epithelial cells. At 15.63 μg/mL, the cocoa extract reduced the adherence of *F. nucleatum* to the Matrigel™ basement membrane model by 27.9%, while a concentration of 500 μg/mL inhibited adherence by 89.7% (Fig 2B).

The effect of the cocoa extract on H$_2$S production by *F. nucleatum* was determined with a colorimetric assay using cysteine and homocysteine as substrates. As reported in Table 2, the extract dose-dependently reduced H$_2$S production. At the highest concentration tested (1000 μg/mL), the cocoa extract reduced the production of H$_2$S by 71.4%.

In summary, the cocoa extract, in addition to inhibit the growth of *F. nucleatum*, can impair its virulence determinants, including biofilm formation, adherence properties and H$_2$S production.

**Effects on *F. nucleatum*-induced inflammatory response**

In order to explore the anti-inflammatory property of the cocoa extract, we investigated its ability to attenuate the secretion of IL-6 and IL-8 by oral epithelial cells (GMSM-K cell line) challenged with *F. nucleatum*. At concentrations up to 250 μg/mL, the cocoa extract did not significantly reduce cell viability (Table 3). As shown in Fig 3, *F. nucleatum* induced a significant increase in the secretion of both cytokines by oral epithelial cells. This increased secretion
was dose-dependently reduced in the presence of the cocoa extract. More specifically, 250 μg/mL of the cocoa extract reduced the secretion of IL-6 and IL-8 by 71.9% and 57.6%, respectively.

We then used a second model to confirm the anti-inflammatory property of the cocoa extract. Given that the NF-κB signalling pathway is a transcription factor involved in inflammatory processes leading to the production of cytokines and chemokines, we evaluated the

Table 1. Phenolic composition of the cocoa extract.

| Compound                        | Amount (mg/100 g dry weight) |
|---------------------------------|------------------------------|
| PHENOLIC ACIDS                  | 47.42                        |
| Caffeic acid                    | 2.29                         |
| Caffeoyl glucoside              | 0.42                         |
| Chlorogenic acid                | 1.42                         |
| m-Coumaric acid                | 0.29                         |
| p-Coumaric acid                | 0.79                         |
| Cryptochlorogenic acid          | 0.27                         |
| p-Hydroxybenzoic acid          | 0.96                         |
| Protocatechuic acid            | 35.56                        |
| γ-Resorcylic acid              | 4.48                         |
| Shikimic acid                  | 0.94                         |
| FLAVONOLS                       | 223.71                       |
| Kaempferol glucoside / galactoside | 0.89                      |
| Phlorizin                       | 2.65                         |
| Quercetin                       | 6.45                         |
| Quercetin 3-arabinoside         | 0.43                         |
| Quercetin 3-galactoside         | 85.50                        |
| Quercetin 3-glucoside           | 34.59                        |
| Quercetin 3-xyloside            | 91.66                        |
| Rutin                           | 1.54                         |
| ANTHOCYANINS                    | 36.66                        |
| Cyanidin 3-arabinoside / xyloside | 21.92                      |
| Cyanidin 3-galactoside / glucoside | 14.35                    |
| Cyanidin 3-sambubioside         | 0.39                         |
| FLAVAN-3-OLS                    | 676.36                       |
| Catechin                        | 150.23                       |
| Epicatechin                     | 526.13                       |
| PROCYANIDINS                    | 29943                        |
| Monomers                        | 6119                         |
| Dimers                          | 7838                         |
| Trimers                         | 4823                         |
| Tetramers                       | 3794                         |
| Pentamers                       | 2027                         |
| Hexamers                        | 1759                         |
| Heptamers                       | 887                          |
| Octamers                        | 647                          |
| Nonamers                        | 656                          |
| Decamers                        | 1074                         |
| Polymers (DP > 10)              | 319                          |

DP: Degree of polymerization.

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effect of the cocoa extract on NF-κB activation using the U937-3xκB monocytic cell line. The cocoa extract at concentrations up to 125 μg/mL did not significantly reduce cell viability (Table 3). As shown in Fig 4, the cocoa extract reduced F. nucleatum-induced NF-κB activation, although less efficiently than the commercial positive inhibitory control (BAY-11-7082). Significant inhibitory effects (33.1% to 45.8%) were observed at concentrations ranging from 15.63 to 125 μg/mL.

In summary, the cocoa extract exerts anti-inflammatory properties towards two cell types challenged with F. nucleatum. It attenuates cytokine secretion by oral epithelial cells and decreases the activation of the NF-κB signalling pathway in monocytes.

Effects on oral epithelial barrier function
As the tight junctions of oral epithelial cells likely play a key role in protecting the underlying connective tissues from invasion by periodontopathogens, we investigated the ability of the
We monitored, over a 48-h period, the TER values of a monolayer of epithelial cells (B11 cell line) exposed to the cocoa extract at concentrations (≤ 125 μg/mL) that did not affect cell viability (Table 3). As shown in Fig 5, the cocoa extract caused a significant time- and dose-dependent increase in TER values. The TER values reached their maximum after a 4-h treatment of the cells and tended to decrease thereafter. Despite this decrease, the TER values of the treated cells remained significantly higher than the untreated control cells. Following a 4-h exposure to 31.25, 62.5, and 125 μg/mL of the cocoa extract, the TER values increased 2.5-, 3.2-, and 3.5-fold, respectively, compared to the untreated control cells.

### Discussion

Periodontal disease is initiated by a synergistic and dysbiotic polymicrobial community colonizing the subgingival areas [31]. *F. nucleatum* is a key pathobiont involved in periodontal biofilm formation by bridging the early and late colonizers [4]. It expresses several virulence factors that allow it to adhere to and invade oral epithelial cells and trigger an immune response [5, 6]. The focus of the present study was to evaluate the antimicrobial and anti-inflammatory activities of a cocoa extract against *F. nucleatum*. Given the important role of the oral epithelium as a physical barrier to prevent bacterial invasion of gingival connective tissues [17], we also investigated the ability of the cocoa extract to enhance epithelial barrier function. To the best of our knowledge, these aspects have never previously been studied.

### Table 2. Effect of the cocoa extract on H₂S production by *F. nucleatum.*

| Cocoa extract (μg/mL) | H₂S production (%)* |
|----------------------|----------------------|
| 0                    | 100 ± 9.7            |
| 125                  | 64.3 ± 7.7 †         |
| 250                  | 51.7 ± 14.3 †        |
| 500                  | 35.5 ± 9.8 †         |
| 1000                 | 28.6 ± 11.4 †        |

A value of 100% was assigned to H₂S production by *F. nucleatum* in the absence of the extract.

*: Results are expressed as the means ± SD of triplicate assays from two independent experiments. A value of 100% was assigned to the control (no cocoa extract).

†: Significantly different (p < 0.01) from the control (no cocoa extract).

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The cocoa extract to enhance oral epithelial barrier function. We monitored, over a 48-h period, the TER values of a monolayer of epithelial cells (B11 cell line) exposed to the cocoa extract at concentrations (≤ 125 μg/mL) that did not affect cell viability (Table 3). As shown in Fig 5, the cocoa extract caused a significant time- and dose-dependent increase in TER values. The TER values reached their maximum after a 4-h treatment of the cells and tended to decrease thereafter. Despite this decrease, the TER values of the treated cells remained significantly higher than the untreated control cells. Following a 4-h exposure to 31.25, 62.5, and 125 μg/mL of the cocoa extract, the TER values increased 2.5-, 3.2-, and 3.5-fold, respectively, compared to the untreated control cells.

### Table 3. Effects of the cocoa extract on viability of B11 oral epithelial cells, GMSM-K oral epithelial cells, and U937 3xB-LUC monocytic cells.

| Cocoa extract (μg/mL) | % Viability (Mean ± standard deviation) |
|----------------------|----------------------------------------|
|                      | B11 cell line | GMSM-K cell line | 3xB-LUC cell line |
| None                 | 100 ± 3.5     | 100 ± 4.4        | 100 ± 6.6         |
| 500                  | 61.4 ± 0.2*   | 46.1 ± 15.6*     | 39.7 ± 8.7*       |
| 250                  | 79.5 ± 11.1*  | 89.8 ± 12.5      | 53.4 ± 9.6*       |
| 125                  | 121.2 ± 8.8   | 109.7 ± 17.7     | 94.5 ± 8.3        |
| 62.5                 | 151.3 ± 9.5   | 130.2 ± 5.5      | 111.7 ± 6.5       |
| 31.25                | 153.8 ± 8.5   | 122.2 ± 16.8     | 113.4 ± 10.2      |

Cell viability following a 24-h treatment was assessed using an MTT (3-[4,5-diethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) colorimetric assay.

*: Significant decrease (p < 0.01) in cell viability compared to untreated control cells.

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Cocoa phytochemical constituents, particularly polyphenols, exhibit beneficial health properties and thus have the capacity to improve and prevent many human diseases [22–24]. However, the potential oral health-promoting benefits of cocoa have been poorly studied to date. In terms of the antibacterial effects of cocoa polyphenols against cariogenic bacteria, Percival et al. [32] reported that, while the growth of *Streptococcus sanguinis* was inhibited, a cocoa bean extract did not affect the growth of *Streptococcus mutans*. Cocoa polyphenols have also been reported to inhibit acid production and biofilm formation by and the glycosyltransferase activity of *S. mutans* [32, 33]. Hirao et al. [34] showed that a crude cocoa polyphenol extract can reduce the viability of *F. nucleatum*, *P. gingivalis*, and *Prevotella intermedia*. In the present study, we showed that a cocoa extract completely prevents the growth of *F. nucleatum* when added to the culture medium at a concentration of 2000 μg/mL. The exact antibacterial
mechanism of action of the cocoa extract on *F. nucleatum* is difficult to elucidate given that the fraction contains a variety of polyphenols, which may act on different targets. However, growth inhibition may be related to the ability of epicatechin, which is the major phenolic compound in the cocoa extract, to strongly bind to the lipid bilayer of the bacterial membrane, resulting in a loss of cell structure and function, leading to cell death [35].

The adherence of bacteria to oral surfaces is the first step in biofilm formation and host colonization. In the present study, we showed that *F. nucleatum* adheres to oral epithelial cells as well as to the Matrigel™ basement membrane model. The cocoa extract significantly inhibited the ability of *F. nucleatum* to adhere to both surfaces. The results of a colorimetric microplate assay showed that the cocoa extract almost completely prevented biofilm formation by *F. nucleatum* at a concentration (500 μg/mL) that did not reduce bacterial growth. As *F. nucleatum* is a critical bacterium involved in subgingival biofilm formation, the above observation suggests that the cocoa extract may prevent or slow periodontal disease initiation and progression since biofilms enable bacteria to evade immune defenses and resist mechanical removal and chemotherapeutic agents.

*F. nucleatum* is known to produce large quantities of H2S and consequently has been associated with halitosis [12]. Moreover, H2S is a metabolite that can induce an inflammatory response and that has a toxic effect on oral epithelial cells and gingival fibroblasts [36–39]. The cocoa extract significantly reduced H2S production by *F. nucleatum*, suggesting that it may be of interest for reducing unpleasant oral odors as well as gingival inflammation.

*F. nucleatum* induced the production of IL-8 and IL-6 by the GMSM-K cell line, which is in agreement with previous studies [40, 41]. An imbalance in IL-8 levels can contribute to the transmigration of neutrophils from the submucosa to the periodontal pocket and can, consequently, lead to exaggerated inflammation and periodontal tissue destruction [42, 43]. On the other hand, prolonged and elevated secretion of IL-6 may induce periodontal bone loss in patients with chronic periodontitis [44] by osteoclast activation caused by the overexpression of RANKL [45, 46]. Given this, the modulation of cytokine expression in gingival tissues during periodontal disease may be key for maintaining periodontal health. Interestingly, the cocoa
extract dose-dependently reduced the secretion of both IL-6 and IL-8. Consistent with these results, previous studies have shown that cocoa causes a reduction in IL-8 levels in inflammatory models of colonic epithelial cells [47, 48].

The NF-κB pathway is a key transcription factor that plays a crucial role in the innate immune response and in chronic inflammation [49, 50]. It can induce the expression of inflammatory mediators, and its dysregulation contributes to the chronic inflammatory process [51, 52]. We used the U937-3xx B-LUC cell line to show that the cocoa extract significantly inhibits the activation of NF-κB. In agreement with our results, polyphenols identified

\[ Theobroma cacao \]

\[ \downarrow \]

Beneficial properties against periodontal diseases

\[ \downarrow \]

- Inhibits *F. nucleatum* growth and H₂S production
- Impairs biofilm formation and adherence properties of *F. nucleatum*
- Attenuates *F. nucleatum*-induced inflammatory response of oral epithelial cells and monocytes
- Improves oral epithelial barrier function

**Fig 6. Beneficial effects of the cocoa extract against periodontal diseases.**

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in cocoa-related products exhibit beneficial effects with respect to cardiovascular inflammatory disorders and changes in the levels of pro-inflammatory cytokines associated with preeclampsia by inhibiting NF-κB activation [53, 54]. In this respect, the (−)-epicatechin, (+)-catechin, and dimeric procyanidins B1 and B2 in T. cacao seem to play an anti-inflammatory role due to their capacity to modulate NF-κB binding to DNA [55–57].

The ability of periodontal pathogens to damage the epithelial barrier and translocate into gingival connective tissues contributes to the destruction of the periodontal support [58, 59]. In the present study, we used an in vitro epithelial barrier model to investigate the capacity of the cocoa extract to enhance epithelial barrier function. Our results showed that the cocoa extract has a positive effect on tight junction integrity by increasing the TER values. This is in agreement with Bitzer et al. [47], who reported that polymeric cocoa procyanidins can prevent epithelial inflammation and the loss of gut barrier function in an in vitro model of colonic inflammation.

In summary, this study brought evidence that the cocoa extract under investigation acts on multiple targets related to the etiology of periodontal diseases (Fig 6). On the one hand, while at a high concentration it inhibits the growth of F. nucleatum, lower concentrations that do not reduce growth, it reduces biofilm formation and adherence properties. This is of interest given that the objective of anti-adherence therapies is to inhibit the host colonization without affecting bacterial viability, thus minimizing the emergence of resistant strains. On the other hand, the cocoa extract decreases the inflammatory response of mucosal and immune cell types and can thus attenuate periodontal tissue destruction.

Within the limitations of the present study, cocoa polyphenols, incorporated into mouthrinses, toothpastes, gels, or local drug delivery systems, can be considered as a promising therapeutic agent for the prevention and/or treatment of periodontal disease due to its antimicrobial and anti-inflammatory properties as well as its ability to enhance the epithelial barrier protective function. Interestingly, Tomofuji et al. [60] used a rat-periodontitis model and reported that a cocoa-enriched diet could inhibit alveolar bone loss and polymorphonuclear leukocyte infiltration. Further research to evaluate the clinical efficacy of cocoa polyphenols is needed.

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