In vitro assessment of probiotics, blueberry and food carbohydrates to prevent *S. pyogenes* adhesion on pharyngeal epithelium and modulate immune responses

Valentina Taverniti[1], Alessandro Dalla Via[1], Mario Minuzzo[2], Cristian Del Bo'[1], Patrizia Riso[1], Hanne Frøkiær[3], Simone Guglielmetti[1,#]

[1]Department of Food, Environmental and Nutritional Sciences (DeFENS), and [2]Department of Biosciences, University of Milan, Italy.

[3]Department of Veterinary and Companion Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, Frederiksberg, Denmark.

[#]Correspondence to: simone.guglielmetti@unimi.it

Abstract

Group A streptococci (GAS) cause 20-30 % of pediatric pharyngitis episodes, which are a major cause of ambulatory care visits. Therefore, a strategy to prevent GAS dissemination in children could significantly benefit public healthcare. Contextually, we assessed the possibility to employ alternative food-grade strategies to be used with the oral probiotic *L. helveticus* MIMLh5 for the prevention of pharyngeal infections. First, we demonstrated through an antagonism-by-exclusion assay that guaran may potentially prevent the adhesion on pharyngeal cells by *S. pyogenes*. Subsequently, we showed that the anthocyanin-rich fraction extracted from wild blueberry (BbE) exerts anti-inflammatory properties on the human macrophage cell line U937. Finally, we showed that the BbE reduces interferon-β expression in MIMLh5-stimulated murine dendritic cells, resulting in the reduction of the pro-inflammatory cytokines IL-12 and TNF-α. In conclusion, this proof-of-concept study indicates that different food-grade strategies may be concomitantly adopted to potentially prevent GAS colonization and modulate local immune defenses.

Keywords: *Lactobacillus helveticus* MIMLh5, blueberry, probiotic, functional food, *Streptococcus pyogenes*, pharyngitis, guar gum

1. Introduction

Nose and oral cavity are the main gate of entrance for microorganisms from the external environment. The primary site for the potential colonization is then the pharyngeal mucosa, which is in fact a frequent location for bacterial and viral infections. To manage such risk, the host developed a complex system of immunological defenses, which are collectively known as the nasal-associated lymphoid tissue or Waldeyer ring (WR). The WR is mainly composed by lingual tonsil, adenoid, and palatine tonsils. The epithelium of palatine tonsils extends into a complicated network of invaginations constituting crypts, where molecules and microorganisms constantly diffusing from the oropharyngeal cavity meet a rich and abundant set of immune cells such as monocytes, macrophages and dendritic cells, which respond to stimuli affecting both local and systemic immunity [1].

Streptococcus pyogenes is a beta-hemolytic bacterium of the Lancefield serogroup A that can efficiently elude the immune barrier of the WR. *S. pyogenes* may be the cause of a wide range of pathological conditions, ranging from local (e.g. pharyngitis; [2]) to invasive systemic infections (e.g. endocarditis; [3]). Notably, *S. pyogenes* is the most common bacterial etiological agent of acute pharyngeal infections especially in school-age children [4, 5], and it is, consequently, a primary reason for antibiotic prescription in the pediatric population [6].

The use of live microorganisms for the prevention and treatment of pharyngeal infections (i.e. the probiotic approach) has been investigated, leading to the development of probiotic products already available on the market. The most successful example is represented by the BLIS products based on *Streptococcus salivarius* K12, a bacterium originally isolated from the oral cavity of a healthy child, demonstrated in several studies to colonize the oral cavity [7], inhibit pathogenic streptococci through the production of the lantibiotic bacteriocin salivaricin [8], and modulate the immune responses [9]. Another bacterium that has been selected as a probiotic candidate for the

pharyngeal mucosa is represented by the dairy strain *Lactobacillus helveticus* MIMLh5, which possesses the ability to antagonize *S. pyogenes* [10] and to modulate the immune response in epithelial cells [10], macrophages [11] and dendritic cells [12] also independently from its viability, due to the presence of an abundant immune-active surface layer (S-layer) protein on the outer surface of the bacterium [12].

In this study, we assessed the possibility to use alternative food-grade strategies for the prevention of pharyngeal infections, which can be adopted concomitantly to the use of the oral probiotic *L. helveticus* MIMLh5. Specifically, since *S. pyogenes* adhesion on epithelial cells *in vivo* is mediated by its capsule polysaccharide hyaluronate[13], here we tested the ability of several food carbohydrates (*i.e.*, alginate, aloe, arabinogalactan, chitosan, guar gam, inulin, pectin, starch and xylan) to inhibit the adherence of this pathogen *in vitro* to FaDu pharyngeal cells. In addition, we investigated an anthocyanin (ACN)-rich wild blueberry extract, since this food component was demonstrated to exert anti-inflammatory properties on epithelial cells [14] and, therefore, could contribute to the management of inflammation occurring during the pharyngeal infections.

2. Material and methods

2.1 Food carbohydrates, bacterial strains and blueberry extract

All carbohydrates used in antagonism experiments on FaDu cells were from Sigma-Aldrich (Steinheim, Germany) except for aloe extract, which was from ESI (Aloe Vera Esi Gel 99.9 %; ESI S.p.A., Albissola Marina, Italy). In the study, we used *Streptococcus pyogenes* C11, belonging to the emm-type 77, which was originally isolated from a pharyngitis patient. *S. pyogenes* was grown in Brain Heart Infusion broth (BHI; Difco, Detroit, USA) supplemented with 0.3 % yeast extract at 37°C for 18-24 h. For the cultivation of the recombinant *S. pyogenes* strain C11^{LucFF} [15], 5 µg ml^{-1} of chloramphenicol was added to the medium. *Lactobacillus helveticus* MIMLh5 (from the Food

Microbiology culture collection of the Department of Food, Environmental and Nutritional Sciences, University of Milan) was cultivated in de Man, Rogosa and Sharpe medium (MRS; Difco) at 37°C overnight. The blueberry extract (BbE) used in the study was prepared as previously described [14] using a freeze-dried, powdered, *Vaccinium angustifolium* berries preparation standardized at 1.5 % total ACNs by FutureCeuticals (Momence, IL, USA) [16, 17]. Specifically, the BbE corresponds to the ACN-fraction obtained and characterized as previously reported by Taverniti et al. [14]. The ACN-fraction was dissolved and stabilized in methanol acidified with 0.05 mM HCl (MetOH:HCl). The BbE contained 14 different ACNs with malvidin and delphinidin glycosides as dominant compounds followed by cyanidin, petunidin and peonidin glycosides [14].

2.2 Experiments on FaDu cell layer

The human pharynx carcinoma cell line FaDu (from the American Type Culture Collection, ATCC HTB-43) was cultivated at 37°C in an atmosphere of 95 % air and 5 % carbon dioxide, until confluent monolayer was formed. The culture broth was Dulbecco's Modified Eagle's Medium (DMEM; Lonza, Basel, Switzerland), supplemented with 10 % (v/v) heat-inactivated (at 56°C for 30 min) fetal calf serum, 100 U ml^{-1} penicillin, 100 µg ml^{-1} streptomycin, 0.1 mM non-essential amino acids, 2 mM L-glutamine (Sigma-Aldrich).

Antagonism-by-exclusion assay was performed with the recombinant bioluminescent reporter strain *S. pyogenes* $C11^{LucFF}$, which was previously developed by introduction of the reporter vector pCSS945, carrying a phage T5 promoter-lac operator upstream of the insect luciferase gene *lucGR* [15]. In brief, the experiment consisted of an hour pre-incubation of the FaDu layer with 10 g l^{-1} of the carbohydrates under study, followed by a washing step with PBS (pH 7.3) and 1-h incubation with approximately 2×10^8 *S. pyogenes* $C11^{LucFF}$ cells ml^{-1} resuspended in PBS (bacterial concentration determined microscopically by means of Neubauer Improved counting chamber, Paul Marienfeld

GmbH & Co. KG). After two washes with PBS, D-luciferin (Sigma-Aldrich) was added at the concentration of 12.5 µM (in citrate buffer, pH 5) and the luminescence signal was immediately measured with a Victor 3 luminometer (PerkinElmer, Monza, Italy).

For bacterial adhesion experiments, FaDu cells were grown as defined above on microscopy cover glasses following a previously described method [18]. In brief, FaDu layer was incubated for 1 h in PBS supplemented with 10 g l^{-1} of the following carbohydrates: alginate, aloe, arabinogalactan, chitosan, guar gam, inulin, pectin, starch and xylan. In addition, hyaluronate, which constitutes the exopolysaccharide capsule of *S. pyogenes*, has been used as positive control (10 g l^{-1}). Afterwards, the cell layer was gently washed (three times) with PBS and incubated for 1 h with approximately 2×10^8 cells of an overnight culture of *S. pyogenes* resuspended in PBS. Adherent bacteria and FaDu cells in 10 randomly selected microscopic fields were counted and averaged; results were expressed as adhesion index, i.e. number of bacterial cells per 100 FaDu cells.

2.3 Experiments with U937 cell line

The U937 cells (from the American Type Culture collection, ATCC CRL-1593.2) were seeded at a density of 5×10^5 cells $well^{-1}$ in 24-well plates and cultivated at 37°C in 5 % CO_2 humidified atmosphere in RPMI 1640 medium (Lonza) supplemented with 10 % (v/v) fetal bovine serum (FBS) (Gibco-BRL, Life Technologies, Milan. Italy), 2 mM L-glutamine, 100 U ml^{-1} penicillin and 100 µg ml^{-1} streptomycin (Sigma-Aldrich). Differentiation into adherent, non-replicative macrophage-like cells [19, 20] was induced for 48 h by the addition of phorbol myristate acetate (PMA; Sigma-Aldrich) into the cellular medium at a final concentration of 0.1 mM. Afterwards, cells were washed once with sterile PBS buffer to remove all non-adherent cells. One hour before stimulation, the culture medium was replaced with RPMI 1640 supplemented with 1 % (v/v) FBS to enable cell adaptation. Lipopolysaccharide (LPS, final concentration 1 µg ml^{-1}) from *Escherichia coli* 0127:B8 (Sigma-

Aldrich) was used as pro-inflammatory stimulus. BbE was tested at three different concentrations (1, 10 and 25 μg ml^{-1}). U937 cells were also treated with MetOH:HCl to evaluate the possible interference of the BbE solvent on U937 responses. In all tested conditions, the final concentration of MetOH:HCl was lower than 0.1 % (v/v). Two different experimental settings were used: 1 h of pre-incubation with LPS followed by BbE supplementation for 3 h, and 1 h of pre-incubation with the BbE followed by the treatment with LPS for 3 h.

After incubating macrophages for a total of 4 h (1 h preincubation + 3 h treatment) according to the two experimental settings described above, the supernatant was carefully removed from each well and the total cellular RNA was isolated from the adhered cells and converted to cDNA as previously described [11]. The expression levels of TNF-α gene was then analyzed with a quantitative PCR using SsoFast EvaGreen Supermix (Bio-Rad Italia, Segrate, Italy) on a Bio-Rad CFX96 system as previously described [11, 12]. Reference gene coding for glyceraldheyde-3-phosphate dehydrogenase was used to normalize gene expression. Gene expression levels are reported as the fold of induction (FOI) in comparison to the control (namely unstimulated U937 cells), to which we attributed a FOI of 1.

2.4 Experiments with murine bone marrow derived dendritic cells (BMDCs)

All animals used as source of bone marrow cells were housed under conditions approved by the Danish Animal Experiments Inspectorate (Forsøgdyrstilsynet), Ministry of Justice, Denmark and experiments were carried out in accordance with the guidelines of 'The Council of Europe Convention European Treaty Series 123for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes'. Since the animals were employed as a source of cells, and that no live animals were used for the experiments, no specific approval was required for this study. Hence, the animals used for this study are included in the general facility approval for the

157 faculty of Health and Medical Sciences, University of Copenhagen. BMDCs were prepared as
158 described previously [21]. Briefly, bone marrow from C57BL/6 mice (Tactonic, Lille Skensved,
159 Denmark) was flushed out from the femur and tibia and washed. Bone marrow cells (3×10^5) were
160 seeded into 10 cm Petri dishes in 10 ml RPMI 1640 (Sigma- Aldrich, St. Louis, MO, USA)
161 containing 10 % (v/v) heat inactivated fetal calf serum supplemented with penicillin (100 U ml^{-1}),
162 streptomycin (100 mg ml^{-1}), glutamine (4 mM), 50 mm 2-mercaptoethanol (all from Cambrex Bio
163 Whittaker) and 15 ng ml^{-1} murine GM-CSF (harvested from a GM-CSF transfected Ag8.653
164 myeloma cell line). The cells were incubated for 8 days at 37°C in 5 % CO_2 humidified atmosphere.
165 On day 3, 10 ml of complete medium containing 15 ng ml^{-1} GM-CSF was added. On day 6, 10 ml
166 were removed and replaced by fresh medium. Non-adherent, immature DC were harvested on day
167 8. Afterwards, immature DCs (2×10^6 cells ml^{-1}) were resuspended in fresh medium supplemented
168 with 10 ng ml^{-1} GM-CSF, and 500 µl $well^{-1}$ were seeded in 48-well tissue culture plates (Nunc,
169 Roskilde, Denmark).
170 To prepare fresh cultures to be used in immunological experiments, bacterial cells from an
171 overnight culture were collected, washed twice with sterile PBS, counted with Neubauer Improved
172 counting chamber, and resuspended at known concentration in the same medium used to culture the
173 BMDCs. *L. helveticus* MIMLh5 was tested at a multiplicity of infection (MOI) of 5. In co-
174 incubation experiments, the BbE was used at the concentrations 1, 3 and 10 µg ml^{-1}, and added the
175 BMDCs 30 min before the addition of *L. helveticus*. When tested alone, the BbE was used at
176 concentration of 5, 25 and 50 µg ml^{-1}. Cell supernatants were harvested 20 hrs after bacterial
177 stimulation and tested for cytokine concentration by sandwich ELISA duosets (RnD Systems,
178 Abingdon, UK) according to the manufacturer's protocol. For the gene expression analysis,
179 BMDCs were harvested for RNA extraction after 4 h. Total RNA was extracted using the
180 MagMAX sample separation system (Applied Biosystems, Foster City, CA), including a DNAse

181 treatment step for genomic DNA removal. RNA concentration was determined by Nanodrop
182 (Thermo, Wilmington, DE). Then, 500 ng of total RNA was reverse transcribed by the TaqMan
183 Reverse Transcription Reagent kit (Applied Biosystems, Foster City, USA) using random hexamer
184 primers according to the manufacturer's instructions. The thermic cycle used for retrotranscription
185 was: 10 min at 25° C, 120 min at 37° C, and 5 s at 85° C. The obtained cDNA was stored in
186 aliquots at -80°C. Primers and probes were obtained as previously described [22]. The probes were
187 labelled with the 5′ reporter dye 6-carboxy-fluorescein (FAM) and the 3′ quencher dye NFQ-MGB
188 (Applied Biosystems). The amplifications were carried out in a total volume of 10 µl containing
189 1×TaqMan Universal PCR Master Mix (Applied Biosystems), forward and reverse primer, TaqMan
190 MGB probe and purified target cDNA (6 ng in reaction). The cycling parameters were initiated by
191 20 s at 95° C, followed by 40 cycles of 3 s at 95° C and 30 s at 60° C using the ABI Prism 7500
192 (Applied Biosystems). Amplification reactions were performed in triplicates, and DNA
193 contamination controls were included. The amplifications were normalized to the expression of the
194 β-actin encoding gene. Relative transcript levels were calculated applying the $2^{(-\Delta\Delta CT)}$ method. Data
195 were collected from three independent experiments carried out in duplicate.
196
197 *2.5 Statistical Analysis*
198 The experiments were carried out at least twice in duplicate. Unpaired Student's t-test or one-
199 way analysis of variance (ANOVA) with Dunnet's post test were run for statistically significant
200 differences.
201
202 **3. Results**
203 *3.1* S. pyogenes *adhesion to FaDu hypopharyngeal carcinoma cell layer may be inhibited by food*
204 *carbohydrates*

205 The ability of 10 different carbohydrates to inhibit *S. pyogenes* adhesion was studied by means of
206 an antagonism-by-exclusion assay based on a three-component system consisting of the FaDu cell
207 layer, the bioluminescent mutant *S. pyogenes* C11^{LucFF}, and the selected carbohydrate molecule. In
208 this experiment, bioluminescence reduction was measured to assess the potential ability of a
209 carbohydrate to prevent epithelial layer colonization by the pathogen. All carbohydrates were tested
210 at 10 g l^{-1} since this concentration was found during the set-up of the experiment to determine about
211 80 % reduction of the luminescence by hyaluronate (the polysaccharide that mediates *S. pyogenes*
212 adhesion to epithelial cells[13]), ensuring its appropriateness as positive control. Besides hyaluronate,
213 four other carbohydrates significantly modified the bioluminescence emitted by *S. pyogenes*;
214 specifically, xylan and chitosan induced a significant increase, whereas guar and aloe gel
215 determined a significant reduction of the relative luminescence units (RLU) of *S. pyogenes* C11^{LucFF}
216 (Fig. 1A).
217 Subsequently, to verify whether the ability of guar and aloe gel to inhibit bioluminescence was
218 associated to the reduced adhesion of *S. pyogenes* to the FaDu cell layer, we performed adhesion
219 experiments. The analysis was carried out counting at the optical microscope the number of adhered
220 *S. pyogenes* cells after incubation of the FaDu layer with the carbohydrate. In specific, we tested the
221 two potential inhibitory carbohydrates identified in the previous experiment (i.e. guar and aloe gel),
222 a low-viscosity oligosaccharide (i.e. inulin) and a high-viscosity polysaccharide (i.e. alginate). We
223 found that, in addition to the positive control hyaluronate, only guar significantly inhibited *S.*
224 *pyogenes* adhesion. On the contrary, the reduction of pathogen's adhesion by aloe gel, although
225 demonstrated to drastically reduce the bioluminescence, was not significant (Fig. 1B and 1C). In a
226 separate set of adhesion experiments, we also ascertained that the blueberry extract used alone or in
227 combination with guar did not affect the adhesion of *S. pyogenes* on FaDu cells (Data not shown).
228 Overall, these results suggest that the guar gum may potentially prevent pharyngeal epithelial

229 colonization by the pathogen *S. pyogenes*.

230

231 *3.2 Blueberry extract possesses anti-inflammatory properties on U937 human macrophages*

232 In the subsequent part of the study, we investigated the potential ability of the ACN-rich fraction
233 from freeze-dried wild blueberry to prevent an inflammatory response. To this aim, we used an
234 immunological model based on PMA-differentiated U937 human macrophages, stimulated with *E.*
235 *coli* LPS molecule to induce the pro-inflammatory response. The presence of MetOH:HCl, which
236 was used to solubilize the blueberry extract (BbE), had only a slight effect on TNF-α expression
237 level induced by LPS (Fig. 2). Specifically, the BbE was added to U937 cells both before or after
238 the treatment with the inflammatory stimulus LPS. We found that the highest concentration tested
239 of BbE (25 μg ml^{-1}) reduced the LPS-induced induction of TNF-α gene expression; however, the
240 statistical significance was reached only when U937 cells were pre-stimulated with the BbE
241 ($p<0.05$), whereas only a trend towards a reduction of the inflammatory induction ($p=0.085$) was
242 observed when U937 cells were treated with the BbE after LPS stimulation (Fig. 2).

243 Overall, these results indicate that the ACN-rich fraction extracted from freeze-dried wild
244 blueberry (i.e. the BbE) exerts anti-inflammatory activity on macrophages *in vitro*.

245

246 *3.3 Blueberry extract affects the* L. helveticus *MIMLh5 effects on dendritic cell*

247 In the last set of experiments, we tested the immunomodulatory properties of the BbE on BMDCs in
248 presence of the immune-active probiotic strain *L. helveticus* MIMLh5. Specifically, BMDCs were
249 co-stimulated with MIMLh5 cells (MOI 5) and increasing BbE concentrations, and the resulting
250 cytokine production was assessed. MIMLh5 alone resulted in 3.5 ng ml^{-1} of TNF-α and 250 pg ml^{-1}
251 of IL-12. MIMLh5 alone or in combination with BbE did not induce the proinflammatory IL-1β in
252 the BMDCs (data not shown). The addition of the BbE determined a significant reduction of both

253 TNF-α and IL-12 at the highest tested concentrations (Fig. 3A). Particularly, we observed a strong
254 reduction of the MIMLh5-induced production of the pro-inflammatory cytokine IL-12 (about –89
255 %) when 50 µg ml^{-1} of BbE were used (Fig. 3A).
256 It was previously shown that upon stimulation of BMDCs with various lactobacilli, IL-12 can be
257 primarily induced indirectly through a transient up-regulation of IFN-β around 4-6 h after addition
258 of bacteria, which in turn stimulates the induction of IL-12 and to a lesser extend TNF-α by binding
259 to the IFN activating receptor (IFNAR) [23]. To study how addition of the blueberry extract affected
260 the IL-12 inducing pathway, we harvested cells after 4 h of stimulation and extracted RNA for RT-
261 PCR analysis of *Il12* and *Infβ* expression. At 4 h of stimulation, the expressions of both *Ifnβ* and
262 *Il12* was affected only by the addition of 50 µg ml^{-1} BbE (Fig. 3B).
263 Overall, these experiments demonstrate that the ACN-rich fraction extracted from freeze-dried
264 wild blueberry reduced the expression of the cytokine IFN-β in murine bone marrow-derived
265 dendritic cells that have been stimulated by *L. helveticus* MIMLh5, and that this in turn leads to a
266 reduction of the pro-inflammatory cytokines IL-12 and TNF-α.
267
268 **4. Discussion**
269 Pharyngitis is a major cause of pediatric ambulatory care visits and often leads to antibiotic
270 prescription [24]. Up to 20 % of children are carriers of group A streptococci (GAS) [24], which are
271 estimated to cause 20 % to 30 % of acute pharyngitis episodes in this population. Therefore, a
272 strategy to prevent GAS dissemination in children could result in significant benefits for public
273 health care. Contextually, in this study we tested the *in vitro* effectiveness of food-grade strategies
274 that can be potentially used together to prevent pharyngeal infections and, specifically, GAS
275 colonization of the oropharyngeal mucosa. Particularly, we focused on strategies that can be
276 compatible with food applications, since food products may be consumed on a larger scale than

277 pharmaceutical formulations.

278 The first step of colonization of the oropharyngeal mucosa by *Streptococcus pyogenes* includes
279 the recognition of the hyaluronate bacterial capsule by CD44 proteins exposed on the outer surface
280 of oral epithelial cells [5]. Accordingly, *in vivo* and *in vitro* experiments supported the potential
281 efficacy of disrupting the interaction between the *S. pyogenes* hyaluronic acid capsule and CD44 to
282 prevent pharyngeal infection [13]. Contextually, the initial part of the present study was devoted to the
283 selection of an edible carbohydrate possessing the ability to antagonize *S. pyogenes* colonization
284 similarly to hyaluronate. Out of nine different carbohydrates tested, only aloe gel and guar gum
285 demonstrated a significant inhibition of *S. pyogenes* colonization *in vitro* on FaDu cell layer,
286 assessed by determining intracellular ATP as a measure of bacterial cell wellbeing. Nonetheless,
287 only guar could significantly inhibit *S. pyogenes* adhesion, indicating that plausibly the observed
288 intracellular ATP reduction induced by aloe gel was due to the toxic activity of the extract on the
289 bacterial cells rather than the preclusion of pathogen adhesion.

290 Guar gum, also called guaran, is a polysaccharide composed of the sugars galactose and
291 mannose, which is obtained from the maceration of the seed of plants *Cyamopsis tetragonoloba*
292 (Linne) Taub. or *Cyamopsis psoraloides* (Lam.) D.C. Guar gum is an ingredient that meets the
293 specifications of the "Food Chemicals Codex" (3rd edition, 1981, page 141) and has been generally
294 recognized as safe (GRAS) by the U.S. Food and Drug Administration under the provisions of the
295 Code of Federal Regulations (title 21 CFR 121.101). In Europe, the Food Safety Authority (EFSA)
296 stated that "*there is no safety concern for the general population at the refined exposure assessment*
297 *of guar gum (E 412) as a food additive*" [25]. In this study, we used a concentration of guar of 10 g
298 per liter, which is compatible with the use in several food matrices, from bread to yogurt or fruit
299 juice [26, 27, 28].

300 Increasing evidence suggests the ability of plant polyphenols to affect the immune regulation [29].

For instance, we recently demonstrated the ability of an anthocyanin-rich extract from wild blueberry to exert anti-inflammatory activity on intestinal epithelial cells *in vitro* [14]. Nonetheless, the ability of food polyphenols to modulate immune responses *in vivo* can be greatly hindered at intestinal level by limited diffusion through the mucin layer, reduced absorption, and rapid degradation by intestinal microorganisms. On the contrary, the same bioactive polyphenols may plausibly have much increased chances to interact with the immune system at oral and pharyngeal level, where immune cells, such as macrophages and dendritic cells, may come into direct contact with the food molecules in structures specifically evolved for this purpose, such as the tonsillar crypts. The immune system associated to the oral cavity and the pharynx, in fact, is continuously challenged by antigens from air and food and has the primary role of avoiding pathogen entry while preserving immune homeostasis [30]. Therefore, plausibly, specific food components may activate and modulate the immune responses occurring at the WR. Contextually, in this study we tested the ability of the same anthocyanin-rich blueberry extract previously used on intestinal epithelial cells [14] to exert immunomodulatory properties also toward antigen presenting cells. Specifically, here we used both macrophage and dendritic cell models because macrophages are directly involved in a rapid resolution of local inflammation, whereas dendritic cells are specialized antigen presenting cells, which can convert a local response into a systemic effect by differently activating T cells populations depending on the stimuli they received and processed[31]. Consequently, the anthocyanin-rich blueberry extract (BbE) was used to stimulate PMA-treated U937 cells, which were employed as an *in vitro* model of macrophages. U937 cells were treated with BbE before the addition of the pro-inflammatory stimulus LPS to investigate a possible preventive role towards an incoming inflammatory agent (inflammation-preventing experiment); in addition, the experiment was also performed supplementing the BbE after LPS stimulation to assess the potential anti-inflammatory activity (inflammation-reducing experiment). The obtained results evidenced the

ability of the BbE to reduce the LPS-dependent expression of TNF-α, a cytokine induced within the NF-κB pathway and involved in inflammatory responses [32]; nonetheless, the statistical significance was reached only in the inflammation-preventing experiment. Reportedly, anti-inflammatory properties have been shown for different categories of polyphenols. For instance, the pretreatment of BV-2 microglial cells *in vitro* with a blueberry extract before LPS stimulation showed a concentration-dependent reduction in TNF-α release [33]. Our results are also in accordance with those by Pergola et al. [34] who demonstrated that a 2 h pre-treatment with an anthocyanin fraction from blackberry extract before LPS stimulation could inhibit iNOS protein expression in murine J774 macrophage cell line. Furthermore, it was reported that supplementation with a grape powder extract reduced the LPS-induced production of inflammatory cytokines in U937 macrophages [35] and a proanthocyanidin-rich cranberry fraction decreased the LPS-induced cytoxicity in macrophages and oral epithelial cells [36]. Finally, we recently documented that the supplementation with an ACN-rich fraction, single ACNs and their gut metabolites were able to reduce lipid accumulation in THP-1 derived macrophages [37] and to counteract their adhesion to endothelial cells in a TNF-α stimulated pro-inflammatory environment [38].

In this study, we also assessed the immunomodulatory potential of the anthocyanin-rich BbE on mouse bone marrow-derived dendritic cells (DCs). DCs constitute a link between the innate and adaptive immunity, since they act as professional antigen-presenting cells (APCs). This function is crucial in initiating the adaptive immune response, as T cells do not respond to free antigens but only to antigens that are presented by APCs. Several dendritic cells subsets have been identified in tonsils, where they can play different roles in regulating immunity or tolerance to antigenic material coming from the mouth and the nose [39]. It has been proposed that DCs in the tonsils should mainly possess the default role to maintain tolerance/ignorance towards the numerous harmless food and microbial antigens coming from the nasopharynx [40]. An exacerbated activation toward harmless

stimuli would determine the enhanced production of pro-inflammatory cytokines such as TNF-α and IL-12, leading to (low-grade) inflammation, which has been associated with susceptibility to infection [41], [42]. In this context, we can speculate that the observed properties of the blueberry preparation are potentially beneficial, since BbE does not trigger the production of pro-inflammatory cytokines by DCs and, notably, gives rise to a more balanced/less stimulatory cytokine profile in combination with MIMLh5, and mitigates the stimulatory attitude of *L. helveticus* MIMLh5 toward TNF-α and IL-12 in DCs. *L. helveticus* MIMLh5 was included in this study since it is a bacterial strain that was selected in previous studies for its ability to efficiently adhere on FaDu epithelial cell line [10, 11] and antagonize *S. pyogenes* on FaDu and HaCat keratinocyte in *in vitro* models [10]. In addition, this strain was shown to inhibit IL1β-induced activation of NF-κB in FaDu human pharyngeal cells [10] and Caco-2 human intestinal epithelial layer [12], and attenuate LPS-induced TNF-α gene expression in U937-derived human macrophages [12]. On the other hand, *L. helveticus* MIMLh5 was demonstrated to trigger the secretion of TNF-α and IL-2 once in contact with murine BMDCs [10].

It was reported that elderberry fruit extracts may increase the ability of *L. acidophilus* NCFM to induce interferon (IFN)-β production by BMDCs [43]. Inspired by this research, here we investigated the effect of BbE on the stimulatory potential of MIMLh5 strain on BMDCs. However, in contrast to the results presented by Frøkiær et al. [43], we found that the BbE slightly but significantly reduced the ability of MIMLh5 to induce the expression of IFN-β by BMDCs, which may explain the reduced production of IL-12 as well as TNF-α. The different results can be plausibly due to different content in extracts which must be expected as other plant materials were used here [43].

Conclusions

In this study, we demonstrated that different food-grade strategies may be potentially adopted to

prevent infections and inflammation of the pharyngeal mucosa. The proposed strategies can be employed in a food formulation (e.g. candy lozenge), whose functional ingredients may potentially prevent GAS colonization (through the effect of guar gum and MIMLh5 probiotic cells) and modulate local immune defenses (through the activity of MIMLh5 probiotic cells and blueberry extract). Such food preparations may be particularly effective for the protection of the pharyngeal mucosa and easily acceptable in school-age children. Nonetheless, this was a proof-of-concept study, and further investigations, both *in vitro* and *in vivo*, are needed to define, primarily, the most proper concentrations of polysaccharide, probiotic and berry extract to use in combination.

Acknowledgments

Part of the study was financially supported by the "piano di sviluppo UNIMI 2014/2017 (Linea B)" (MAGIC-MAMPs project) of the University of Milan.

Conflict of interest

There are no conflicts of interest to declare.

References

1. M. E. Perry, The specialised structure of crypt epithelium in the human palatine tonsil and its functional significance, *J Anat*, 1994, **185 (Pt 1)**, 111-127.
2. A. L. Bisno, Acute pharyngitis, *N Engl J Med*, 2001, **344**, 205-211.
3. D. R. Weidman, H. Al-Hashami and S. K. Morris, Two cases and a review of Streptococcus pyogenes endocarditis in children, *BMC Pediatr*, 2014, **14**, 227.
4. B. M. Schroeder, Diagnosis and management of group A streptococcal pharyngitis, *Am Fam Physician*, 2003, **67**, 880, 883-884.
5. M. W. Cunningham, Pathogenesis of group A streptococcal infections, *Clin Microbiol Rev*, 2000, **13**, 470-511.
6. J. L. Fierro, P. A. Prasad, A. R. Localio, R. W. Grundmeier, R. C. Wasserman, T. E. Zaoutis and J. S. Gerber, Variability in the diagnosis and treatment of group a streptococcal pharyngitis by primary care pediatricians, *Infect Control Hosp Epidemiol*, 2014, **35 Suppl 3**, S79-85.
7. D. A. Power, J. P. Burton, C. N. Chilcott, P. J. Dawes and J. R. Tagg, Preliminary investigations of the colonisation of upper respiratory tract tissues of infants using a paediatric formulation of the oral

probiotic Streptococcus salivarius K12, *Eur J Clin Microbiol Infect Dis*, 2008, **27**, 1261-1263.
8. C. Barretto, P. Alvarez-Martin, F. Foata, P. Renault and B. Berger, Genome sequence of the lantibiotic bacteriocin producer Streptococcus salivarius strain K12, *J Bacteriol*, 2012, **194**, 5959-5960.
9. C. Cosseau, D. A. Devine, E. Dullaghan, J. L. Gardy, A. Chikatamarla, S. Gellatly, L. L. Yu, J. Pistolic, R. Falsafi, J. Tagg and R. E. Hancock, The commensal Streptococcus salivarius K12 downregulates the innate immune responses of human epithelial cells and promotes host-microbe homeostasis, *Infect Immun*, 2008, **76**, 4163-4175.
10. S. Guglielmetti, V. Taverniti, M. Minuzzo, S. Arioli, I. Zanoni, M. Stuknyte, F. Granucci, M. Karp and D. Mora, A dairy bacterium displays in vitro probiotic properties for the pharyngeal mucosa by antagonizing group A streptococci and modulating the immune response, *Infect Immun*, 2010, **78**, 4734-4743.
11. V. Taverniti, M. Minuzzo, S. Arioli, I. Junttila, S. Hamalainen, H. Turpeinen, D. Mora, M. Karp, M. Pesu and S. Guglielmetti, In vitro functional and immunomodulatory properties of the Lactobacillus helveticus MIMLh5-Streptococcus salivarius ST3 association that are relevant to the development of a pharyngeal probiotic product, *Appl Environ Microbiol*, 2012, **78**, 4209-4216.
12. V. Taverniti, M. Stuknyte, M. Minuzzo, S. Arioli, I. De Noni, C. Scabiosi, Z. M. Cordova, I. Junttila, S. Hamalainen, H. Turpeinen, D. Mora, M. Karp, M. Pesu and S. Guglielmetti, S-layer protein mediates the stimulatory effect of Lactobacillus helveticus MIMLh5 on innate immunity, *Appl Environ Microbiol*, 2013, **79**, 1221-1231.
13. C. Cywes, I. Stamenkovic and M. R. Wessels, CD44 as a receptor for colonization of the pharynx by group A Streptococcus, *J Clin Invest*, 2000, **106**, 995-1002.
14. V. Taverniti, D. Fracassetti, C. Del Bo, C. Lanti, M. Minuzzo, D. Klimis-Zacas, P. Riso and S. Guglielmetti, Immunomodulatory effect of a wild blueberry anthocyanin-rich extract in human Caco-2 intestinal cells, *J Agric Food Chem*, 2014, **62**, 8346-8351.
15. S. Guglielmetti, V. Taverniti, M. Minuzzo, S. Arioli, M. Stuknyte, M. Karp and D. Mora, Oral bacteria as potential probiotics for the pharyngeal mucosa, *Appl Environ Microbiol*, 2010, **76**, 3948-3958.
16. S. Vendrame, S. Guglielmetti, P. Riso, S. Arioli, D. Klimis-Zacas and M. Porrini, Six-week consumption of a wild blueberry powder drink increases bifidobacteria in the human gut, *J Agric Food Chem*, 2011, **59**, 12815-12820.
17. S. Guglielmetti, D. Fracassetti, V. Taverniti, C. Del Bo, S. Vendrame, D. Klimis-Zacas, S. Arioli, P. Riso and M. Porrini, Differential modulation of human intestinal bifidobacterium populations after consumption of a wild blueberry (Vaccinium angustifolium) drink, *J Agric Food Chem*, 2013, **61**, 8134-8140.
18. S. Guglielmetti, I. Tamagnini, D. Mora, M. Minuzzo, A. Scarafoni, S. Arioli, J. Hellman, M. Karp and C. Parini, Implication of an outer surface lipoprotein in adhesion of Bifidobacterium bifidum to Caco-2 cells, *Appl Environ Microbiol*, 2008, **74**, 4695-4702.
19. P. Harris and P. Ralph, Human leukemic models of myelomonocytic development: a review of the HL-60 and U937 cell lines, *J Leukoc Biol*, 1985, **37**, 407-422.
20. H. J. Radzun, M. R. Parwaresch, C. Sundstrom, K. Nilsson and M. Eissner, Monocytic origin of the human hematopoietic cell line U-937 and its convertibility to macrophages evidenced by isoenzyme mapping, *Int J Cancer*, 1983, **31**, 181-186.
21. L. Boye, I. Welsby, L. D. Lund, S. Goriely and H. Frokiaer, Plasma membrane Toll-like receptor activation increases bacterial uptake but abrogates endosomal Lactobacillus acidophilus induction of interferon-beta, *Immunology*, 2016, **149**, 329-342.
22. G. Weiss, S. Rasmussen, L. Nielsen Fink, H. Jarmer, B. Nohr Nielsen and H. Frokiaer, Bifidobacterium bifidum actively changes the gene expression profile induced by Lactobacillus acidophilus in murine dendritic cells, *PLoS One*, 2010, **5**, e11065.
23. G. Weiss, H. R. Christensen, L. H. Zeuthen, F. K. Vogensen, M. Jakobsen and H. Frokiaer, Lactobacilli and bifidobacteria induce differential interferon-beta profiles in dendritic cells, *Cytokine*, 2011, **56**,

520-530.
24. S. T. Shulman, A. L. Bisno, H. W. Clegg, M. A. Gerber, E. L. Kaplan, G. Lee, J. M. Martin, C. Van Beneden and A. Infectious Diseases Society of, Clinical practice guideline for the diagnosis and management of group A streptococcal pharyngitis: 2012 update by the Infectious Diseases Society of America, *Clin Infect Dis*, 2012, **55**, e86-102.
25. E. Panel o. F. Additives, F. Nutrient Sources added to, A. Mortensen, F. Aguilar, R. Crebelli, A. Di Domenico, M. J. Frutos, P. Galtier, D. Gott, U. Gundert-Remy, C. Lambré , J.-C. Leblanc, O. Lindtner, P. Moldeus, P. Mosesso, A. Oskarsson, D. Parent-Massin, I. Stankovic, I. Waalkens-Berendsen, R. A. Woutersen, M. Wright, M. Younes, L. Brimer, P. Peters, J. Wiesner, A. Christodoulidou, F. Lodi, A. Tard and B. Dusemund, Re-evaluation of guar gum (E 412) as a food additive, *EFSA Journal*, 2017, **15**, e04669-n/a.
26. R. Numfon, Effects of different hydrocolloids on properties of gluten-free bread based on small broken rice berry flour, *Food Sci Technol Int*, 2017, **23**, 310-317.
27. W. J. Pasman, W. H. Saris, M. A. Wauters and M. S. Westerterp-Plantenga, Effect of one week of fibre supplementation on hunger and satiety ratings and energy intake, *Appetite*, 1997, **29**, 77-87.
28. T. P. Rao, Role of guar fiber in appetite control, *Physiol Behav*, 2016, **164**, 277-283.
29. M. del Corno, B. Scazzocchio, R. Masella and S. Gessani, Regulation of Dendritic Cell Function by Dietary Polyphenols, *Crit Rev Food Sci Nutr*, 2016, **56**, 737-747.
30. R. Q. Wu, D. F. Zhang, E. Tu, Q. M. Chen and W. Chen, The mucosal immune system in the oral cavity-an orchestra of T cell diversity, *Int J Oral Sci*, 2014, **6**, 125-132.
31. F. Geissmann, M. G. Manz, S. Jung, M. H. Sieweke, M. Merad and K. Ley, Development of monocytes, macrophages, and dendritic cells, *Science*, 2010, **327**, 656-661.
32. F. Belardelli, Role of interferons and other cytokines in the regulation of the immune response, *APMIS*, 1995, **103**, 161-179.
33. A. N. Carey, D. R. Fisher, A. M. Rimando, S. M. Gomes, D. F. Bielinski and B. Shukitt-Hale, Stilbenes and anthocyanins reduce stress signaling in BV-2 mouse microglia, *J Agric Food Chem*, 2013, **61**, 5979-5986.
34. C. Pergola, A. Rossi, P. Dugo, S. Cuzzocrea and L. Sautebin, Inhibition of nitric oxide biosynthesis by anthocyanin fraction of blackberry extract, *Nitric Oxide*, 2006, **15**, 30-39.
35. A. Overman, A. Bumrungpert, A. Kennedy, K. Martinez, C. C. Chuang, T. West, B. Dawson, W. Jia and M. McIntosh, Polyphenol-rich grape powder extract (GPE) attenuates inflammation in human macrophages and in human adipocytes exposed to macrophage-conditioned media, *Int J Obes (Lond)*, 2010, **34**, 800-808.
36. V. D. La, J. Labrecque and D. Grenier, Cytoprotective effect of proanthocyanidin-rich cranberry fraction against bacterial cell wall-mediated toxicity in macrophages and epithelial cells, *Phytother Res*, 2009, **23**, 1449-1452.
37. C. Del Bo, Y. Cao, M. Roursgaard, P. Riso, M. Porrini, S. Loft and P. Moller, Anthocyanins and phenolic acids from a wild blueberry (Vaccinium angustifolium) powder counteract lipid accumulation in THP-1-derived macrophages, *Eur J Nutr*, 2016, **55**, 171-182.
38. C. Del Bo, M. Roursgaard, M. Porrini, S. Loft, P. Moller and P. Riso, Different effects of anthocyanins and phenolic acids from wild blueberry (Vaccinium angustifolium) on monocytes adhesion to endothelial cells in a TNF-alpha stimulated proinflammatory environment, *Mol Nutr Food Res*, 2016, **60**, 2355-2366.
39. J. Plzak, Z. Holikova, K. Smetana, Jr., F. Riedel and J. Betka, The role of dendritic cells in the pharynx, *Eur Arch Otorhinolaryngol*, 2003, **260**, 266-272.
40. C. M. Hallissey, R. S. Heyderman and N. A. Williams, Human tonsil-derived dendritic cells are poor inducers of T cell immunity to mucosally encountered pathogens, *J Infect Dis*, 2014, **209**, 1847-1856.
41. N. M. Moutsopoulos and P. N. Madianos, Low-grade inflammation in chronic infectious diseases: paradigm of periodontal infections, *Ann N Y Acad Sci*, 2006, **1088**, 251-264.

503 42. X. Zhang and M. N. Starnbach, An Excess of the Proinflammatory Cytokines IFN-gamma and IL-12
504 Impairs the Development of the Memory CD8+ T Cell Response to Chlamydia trachomatis, *J*
505 *Immunol*, 2015, **195**, 1665-1675.
506 43. H. Frokiaer, L. Henningsen, S. B. Metzdorff, G. Weiss, M. Roller, J. Flanagan, E. Fromentin and A.
507 Ibarra, Astragalus root and elderberry fruit extracts enhance the IFN-beta stimulatory effects of
508 Lactobacillus acidophilus in murine-derived dendritic cells, *PLoS One*, 2012, **7**, e47878.

509

510 **Figure legends**

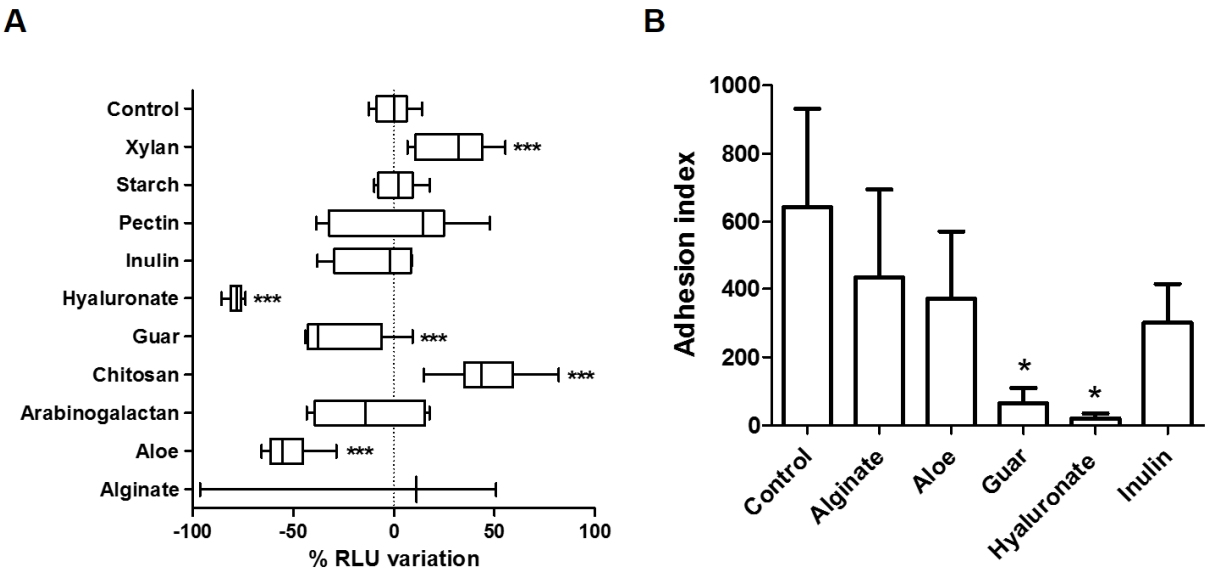
511 **Fig. 1. Effect of food carbohydrates on *S. pyogenes* colonization of FaDu hypopharyngeal**
512 **epithelial cell layer**. **A**. Antagonistic exclusion activity of carbohydrates against bioluminescent
513 *Streptococcus pyogenes* C11^{lucFF} on FaDu cells. Data are reported as percentage variation of light
514 emission (RLU, relative luminescence units) as referred to the cell layer treated with only PBS
515 (control) before incubation with *S. pyogenes*. Data are represented as Tukey box and whiskers and
516 derive from at least three independent experiments performed in duplicate. **B**. Adhesion of *S.*
517 *pyogenes* C11 to FaDu cell layer after treatment of the cell layer with carbohydrates. Data are
518 expressed as adhesion index (i.e. number of adhered bacteria per 100 FaDu cells) and represent the
519 mean (+ standard deviation) of at least two independent experiments conducted in duplicate. **C**. *S.*
520 *pyogenes* C11 adhesion to FaDu cell monolayers as observed with Giemsa staining under a light
521 microscope at 1000x magnification. Statistically significant differences compared to control are
522 according to unpaired Student's *t*-test (*** $P < 0.001$; * $P < 0.05$).

523 **Fig. 2**. **Effect of the blueberry extract on U937 macrophages**. Quantitative analysis of TNF-α
524 gene expression in U937 cells stimulated for 4 h with the blueberry extract (BbE). Expression levels
525 of TNF-α gene are indicated as fold of induction (FOI) relative to the control (unstimulated U937
526 cells), which was set at a value of 1. LPS was used at a concentration of 1 μg ml^{-1}. LPS+Met:HCl
527 refers to U937 cells stimulated with LPS supplemented with methanol + 0.05 mM HCl
528 (MetOH:HCl) at a concentration corresponding to the concentration used for BbE samples. The
529 BbE was tested at the concentrations of 25, 10 and 1 μg ml^{-1}. MetOH:HCl was added in all samples
530 to reach the concentration present in the highest quantity of BbE used. 1st stimulation refers to 1-h
531 pre-stimulations of U937 cells; 2nd stimulation refers to 3-h stimulation of U937 cells. Data from at
532 least three independent experiments are reported as mean (+ standard deviation). Horizontal square
533 brackets indicate statistically significant differences (*, P<0.05; **, P<0.01) according to unpaired

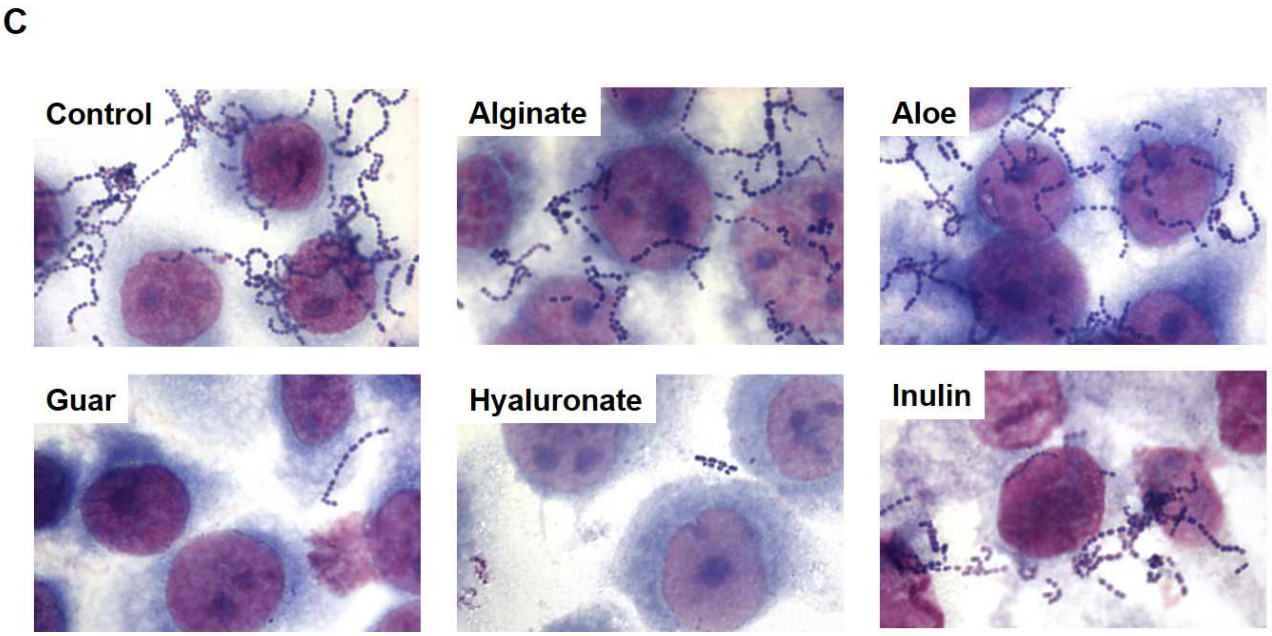
534 Student's *t*-test; p values between 0.05 and 0.1 are also indicated.

535 **Fig. 3. Modulation of the cytokine production in murine bone marrow derived dendritic**
536 **cells. A**. Murine bone marrow derived dendritic cells (BMDCs) stimulated for 30 min with BbE
537 before the addition of media or *Lactobacillus helveticus* cells at a multiplicity of infection (MOI) of
538 5, with subsequent 20-h stimulation and harvest of cell supernatant. The BbE was tested at the
539 concentrations of 0, 1, 10 and 50 μg ml^{-1}. Methanol + 0.05 mM HCl (MetOH:HCl) was added in all
540 samples to reach the concentration present in the highest quantity of BbE used. Cytokine
541 concentrations were measured in the supernatant and expressed in relative values. **B.** BMDCs were
542 harvested 4 h after addition of bacteria and RNA extracted for gene expression analysis by RT-
543 qPCR. FOI, fold of induction. Data from three independent experiments conducted in duplicate are
544 reported as mean +/- standard deviation. Asterisks indicate results from stimulation with MIMLh5
545 and BbE that are statistically different from cells only stimulated with MIMLh5 (*, $P<0.05$; **,
546 $P<0.01$, ***, $P<0.001$) according to one-way ANOVA with Dunnet's post test.
547

Fig. 1



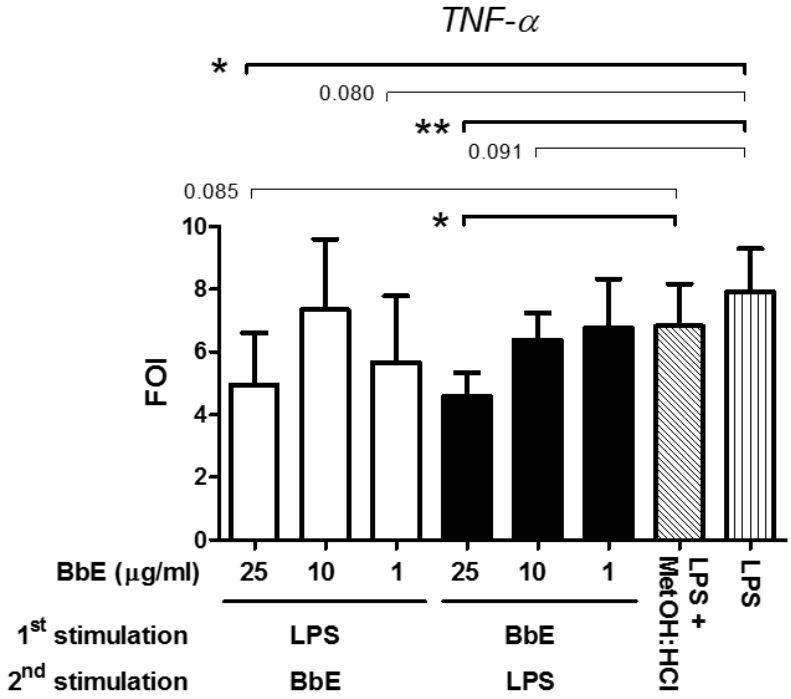
548



549

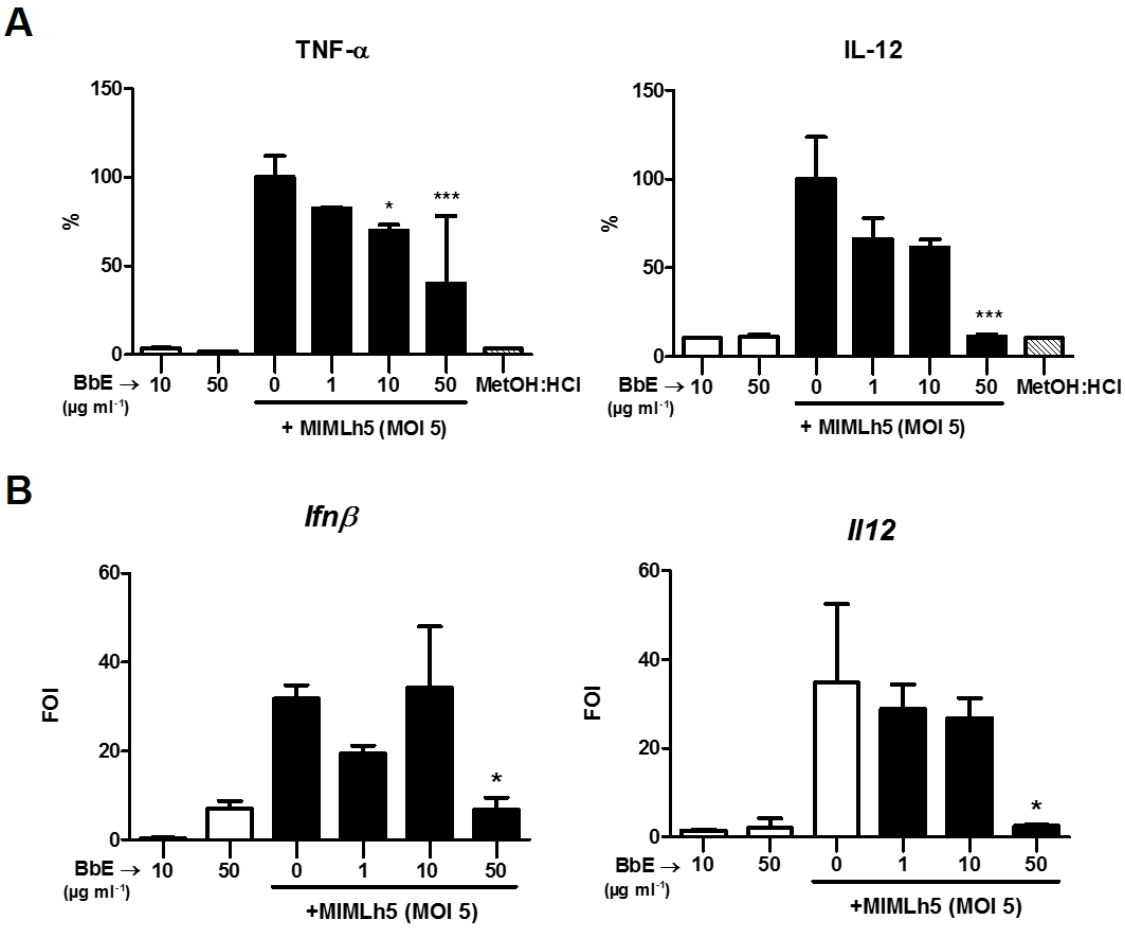
550

Fig. 2



551

Fig. 3



552