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Short Communication

# Chocolate consumption modulates cytokine production in healthy individuals

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ABSTRACT

Epidemiological studies suggest that chocolate increases the incidence and severity of acne. Here we demonstrate that chocolate consumption primes human blood mononuclear cells from volunteers to release more interleukin-1β (IL-1β) and IL-10 upon stimulation with *Propionibacterium acne* or *Staphylcoccus aureus*, the two microorganisms involved in the pathogenesis of acne. In contrast, production of the Th17-derived cytokine IL-22 was inhibited by chocolate. Modulation of inflammation could represent an important mechanism through which chocolate consumption influences acne.



## 1. Introduction

Acne vulgaris, a skin infection mainly caused by *Propionibacterium acnes* (*P. acnes*), has different incidences in various populations [1]. In addition to genetic differences, diet has been proposed to play a role in this phenomenon, and recent studies suggested that chocolate consumption might worsen acne in adolescents [2,3]. However, the mechanisms that drive the effects of chocolate on acne are not known. Chocolate contains a large number of flavonoids that have been shown to have important antioxidant properties [4], and thus provide beneficial effects on vascular diseases such as hypertension and atherosclerosis [5]. Moreover, chocolate flavonoids have been shown to have modulatory effects on inflammation and cytokine production [6,7], as well as on intracellular reactive oxygen species [8].

From a mechanistic point of view, the effect of chocolate on acne may be mediated through a direct effect on *P. acnes* growth, or it may indirectly influence acne through the modulation of the inflammation induced by *P. acnes*. This latter effect of chocolate has been shown when cells were stimulated with purified microbial ligands [4,7], yet it is not known whether chocolate would have the same effect if cells are stimulated by *P. acnes* or other microorganisms that often colonize the skin of acne patients such as *Staphylococcus aureus (S. aureus)*.

In the present study we investigated the mechanisms through which chocolate may influence the development of acne. We studied the effect of chocolate on the growth of *P. acnes*, and we investigated whether chocolate had modulatory effects on cytokine production stimulated by *P. acnes* or *S. aureus*.

## 2. Materials and methods

### 2.1. In vitro production of cytokines by peripheral blood mononuclear cells (PBMCs)

PBMCs were isolated by differential centrifugation with Ficoll-Paque (Amersham Biosciences) from blood collected from seven healthy volunteers, after written informed consent (approved by Ethical Committee of Arnhem–Nijmegen Region). $5 \times 10^5$ of PBMCs in 100 μL culture medium were stimulated with 100 μL of the culture medium RPMI-1640 supplemented with glutamax, 0.02 mM sodium pyruvate, 100 U/mL Penicillin, 100 μg/mL Streptomycin, as negative control (all from Gibco, Paisley, UK). In addition, cells were stimulated with heat-killed *P. acnes* or *S. aureus* $10^7$/mL or $10^6$/mL (prepared from clinical strains).

In two separate sets of experiments, the cells were stimulated with microbial stimuli without or with a combination of 2 μg/mL OmniCoA flavonoid preparation (OmniCoA, Ajinomoto OmniChem, Louvain-la-Neuve, Belgium) using two different protocols: either simultaneous stimulation with ligands and flavonoids, or in a subsequent manner with 24 h preincubation with flavonoids, followed by stimulation with microbial stimuli (*P. acnes* or *S aureus*). After 24 h stimulation, the TNF and IL-1β concentrations in the supernatants were measured using specific ELISA (Sanquin, Amsterdam).

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### 2.2. In vivo effects of chocolate consumption on cytokine production

Blood samples were collected by venipuncture from seven healthy volunteers, after written informed consent, before and after the consumption of 50 grams of chocolate (Milka, Switzerland, containing 30% of cocoa solids) for four days. Before the start of the study, the volunteers had not consumed chocolate for at least one week. The PBMCs were stimulated with RPMI, *P. acnes* $10^7$/mL or $10^6$/mL, or *S. aureus* $10^7$/mL. ELISAs were performed on supernatants after 24 h (TNFα and IL-1β), 48 h (IFNγ and IL-10) and 7 days of stimulation (IL-17 and IL-22). The time points after which each cytokine was measured was chosen based on extensive previous experiments in our laboratory [9]. IFNγ, IL-10, IL-17 and IL-22 ELISA kits were purchased from R&D Systems (Minneapolis, USA).

### 2.3. Statistical analysis

Cytokine production was compared between the groups using a paired Student *t*-test (Excell software). Data are presented as individual values before and after chocolate consumption. *p*-Values lower than 0.05 were considered statistically significant.

## 3. Results

### 3.1. In vitro effects of chocolate flavonoids on cytokine production

The TNFα and IL-1β production induced by *P. acnes* or *S. aureus* in PBMCs was not influenced by simultaneous incubation with chocolate flavonoids (not shown). Interestingly however, 24 h preincubation of PBMCs with chocolate flavonoids, followed by stimulation with microbial ligands, significantly primed the cells for TNF and IL-1β production secondary to stimulation with *S. aureus* or *P. acnes* (Fig. 1). *S. aureus*-induced IL-10 production was also primed twofold (64 vs 30 pg/mL) when PBMCs were preincubated with flavonoids, while *P-acnes*-induced IL-10 was not influenced. Chocolate flavonoids had no effects on IFNγ production (not shown). These data argue that chronic exposure of immune cells to chocolate can prime their production of proinflammatory cytokines.

### 3.2. In vivo effects of chocolate consumption on cytokine production

In order to assess the relevance of these effects in vivo, cytokine production was assessed in cells isolated from seven healthy volunteers before and after consumption of chocolate. Although chocolate had no effect on TNFα production induced by low concentrations of *P. acnes* ($10^6$/mL), a tendency towards a higher TNFα production stimulated by *P. acnes* $10^7$/mL was apparent after chocolate consumption (Fig. 2a). More strikingly, after stimulation of PBMCs with *P. acnes* ($10^6$/mL), there was a significant 2-fold increase in IL-1-production after chocolate consumption ($p = 0.03$, Fig. 2b).

No significant differences were measured in the production of lymphocyte-derived cytokines IL-10, IFNγ, IL-17 and IL-22 (Fig. 2c and d, and not shown). In contrast, we documented a significant 16-fold increase of IL-10-production after stimulation of cells with *S. aureus* after chocolate consumption ($p = 0.0007$, Fig. 2e). Moreover, chocolate consumption decreased by 34% the IL-22-production after stimulating the PBMCs with *S. aureus* ($p = 0.08$, Fig. 2f). Finally, TNFα, IL-1β, IFNγ and IL-17-production induced by *S. aureus* was not modulated by the chocolate consumption (data not shown).

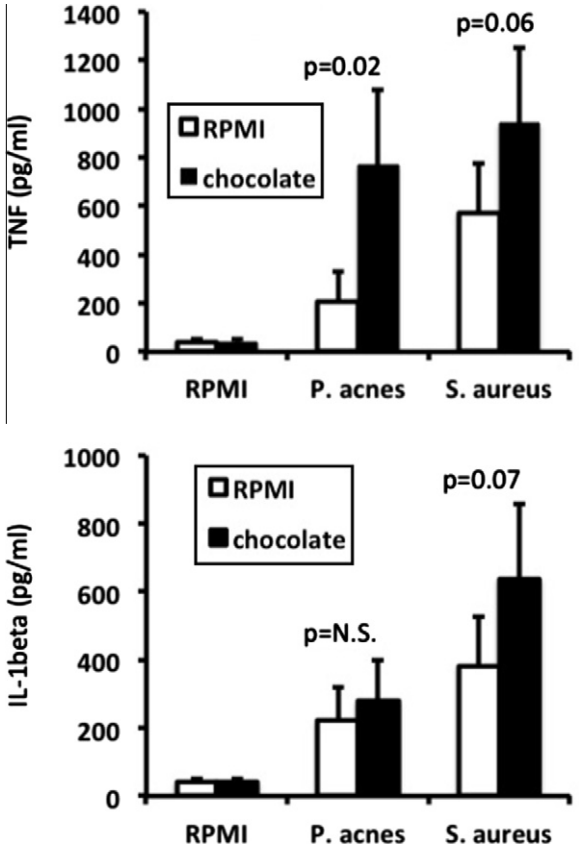


**Fig. 1.** In vitro effects of chocolate consumption on cytokine production. After 24 h preincubation of PBMCs with culture medium (RPMI) or chocolate flavonoids (chocolate), cells were stimulated for an additional 24 h with *P. acnes* ($10^7$ colony forming units-CFU/mL) or *S. aureus* ($10^7$ CFU/mL). After 24 h, the TNFα and IL-1 concentrations were measured in the supernatants. $n = 7$, $^*p$-values calculated by paired Student *t*-test.

## 4. Discussions

In the present study we investigated the immunomodulatory effects of chocolate on cytokine production induced by *P. acnes* and *S. aureus*, two microorganisms involved in the pathogenesis and complications of acne. No direct effects of chocolate could be seen on the growth of *P. acnes* (data not shown). In contrast, chocolate had stimulatory effects of proinflammatory cytokines such as TNF and IL-1β induced by *P. acnes*. Interestingly, chocolate increased production of the anti-inflammatory cytokine IL-10 induced by *S. aureus*, while decreasing the release of IL-22.

In a direct stimulation assay in vitro, we have not observed significant effects of chocolate flavonoids on *P. acnes*-induced cytokines. In contrast however, preincubation with chocolate flavonoids primed immune cells to produce higher amounts of proinflammatory cytokines upon secondary stimulation with bacterial ligands such as LPS. The studies that investigated the effects of chocolate on inflammation until now have used standard stimuli of mononuclear cells, such as lipopolysaccharide (LPS) or phytohemaglutinin [4,7,10]. However, these stimuli are less relevant for a well-defined clinical condition such as acne, which is induced mainly by *P. acnes*. Moreover, in vitro studies of the inflammatory reaction cannot be easily extrapolated to explain the effects of chocolate in humans, and therefore in vivo studies are needed. In our study performed in human healthy individuals, volunteers have abstained from chocolate consumption for one week before the start of the study. At the beginning of the study (before chocolate was administered) and after 4 days consumption of 50 g of