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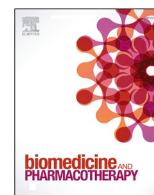

Evaluating the effects of a standardized polyphenol mixture extracted from poplar-type propolis on healthy and diseased human gut microbiota

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A R T I C L E I N F O

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A B S T R A C T

Introduction: A large body of evidence suggests that propolis exerts antioxidant, anti-inflammatory, and antimicrobial activities, mostly ascribed to its polyphenol content. Growing evidence suggests that propolis could modulate gut microbiota exerting a positive impact on several pathological conditions. The aim of this study was to determine the in vitro impact of a poplar-type propolis extract with a standardized polyphenol content, on the composition and functionality of gut microbiota obtained from fecal material of five different donors (healthy adults, and healthy, obese, celiac, and food allergic children).
Methods: The standardized polyphenol mixture was submitted to a simulated in vitro digestion-fermentation process, designed to mimic natural digestion in the human oral, gastric, and intestinal chambers. The antioxidant profile of propolis before and after the digestion-fermentation process was determined. 16 S rRNA amplicon next-generation sequencing (NGS) was used to test the effects on the gut microbiota of propolis extract. The profile of the short-chain fatty acids (SCFA) produced by the microbiota was also investigated through a chromatographic method coupled with UV detection.
Results: In vitro digestion and fermentation induced a decrease in the antioxidant profile of propolis (i.e., decrease of total polyphenol content, antiradical and reducing activities). Propolis fermentation exhibited a modulatory effect on gut microbiota composition and functionality of healthy and diseased subjects increasing the concentration of SCFA.
Conclusions: Overall, these data suggest that propolis might contribute to gut health and could be a candidate for further studies in view of its use as a prebiotic ingredient.

Abbreviations: ALT, alanine aminotransferase; ANOVA, one-way analysis of variance; ASD, autism spectrum disorder; AST, aspartate aminotransferase; BMI, Body Mass Index; DPPH, 2 Diphenyl-1–1-picrythydrazul hydrate; FRAP, ferric reducing ability of plasma; HPLC–MSn, high-performance liquid chromatography-mass spectrometry; M.E.D., Multi Dynamic Extraction; NGS, next-generation sequencing; PCA, Principal Components Analysis; PCoA, Principal Coordinates Analysis; SCFA, short-chain fatty acids; TEAC, Trolox equivalent antioxidant capacity; TPTZ, 2,4,6-tri(2-pyridyl)-s-triazine; URTI, upper respiratory tract infections; UV, ultraviolet.

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1. Introduction

The term microbiota refers to the entire population of microorganisms colonizing a specific location, which is not limited to bacteria but includes viruses, fungi, archaea, and protozoans [1]. The concept of a human microbiota was first coined by Joshua Lederberg as "*the ecological community of symbiotic and pathogenic microbes that literally share our body space and have been all but ignored as determinants of health and disease*". Some bacteria are well known for their advantageous effects, while other components of the human microbiota (viruses, fungi, archaea, and bacteria) are less known for their beneficial role in relation to their host [2]. The microbiota offers a range of physiological benefits to the host such as strengthening the integrity of the intestinal epithelium, protection of the gut barrier against disruption, regulating the immunity of gastro-intestinal mucosa, and protection against pathogenic microorganisms [3]. The gut microbiota is also involved in vitamin synthesis, nutrient absorption, metabolism of xenobiotics, and production of short chain fatty acids (SCFAs) and polyamines [4]. Gut microbiota primarily consists of obligate anaerobes, dominating facultative aerobes and facultative anaerobes by two to three orders of magnitude. To date, 50 bacterial phyla have been identified in the gut, though Bacteroidetes and Firmicutes are the most common. Other phyla present in minor proportions include Proteobacteria, Actinobacteria, Verrucomicrobia, Fusobacteria, and Cyanobacteria [5].

The homeostatic balance of intestinal microflora is extremely advantageous to the host, as a change in microbial composition may cause a drastic imbalance between beneficial and pathogenic microorganisms, leaving the gut more vulnerable to pathogenic insult with gut microbial alterations associated with unhealthy outcomes. This imbalance is termed as dysbiosis and categorized into three classes: (a) loss of beneficial microbes, (b) overgrowth of pathogenic microbes, and (c) loss of overall microbial diversity [6]. In recent decades, the possible role of gut dysbiosis in a plethora of diseases including both intestinal and extra-intestinal disorders have become steadily apparent [3]. Gut dysbiosis may lead to dysregulation of immune and inflammatory pathways, resulting in leaky gut syndrome, systemic inflammation, and autoimmune reactions, as well as increased susceptibility to infection [2]. Many factors are associated with the alteration of normal microbiota, including dietary changes (changes in intake of gluten, salts, saturated fatty acids, alcohol, and artificial sweeteners), antibiotic use, and physiological and physical stresses [2,4]. The role of dysbiosis has been implicated in a wide range of diseases in both animal models and humans, including inflammatory bowel disease (Crohn's disease and ulcerative colitis), asthma, allergic conditions, autism spectrum disorder (ASD), obesity, metabolic syndrome, diabetes mellitus, and colorectal cancer [6,7]. The effects of dysbiosis on human health make remedies aimed at maintaining eubiosis an innovative approach in the prevention of many diseases. Nutraceuticals have been recently proposed as a potentially beneficial approach towards chronic disorders, through restoration of intestinal eubiosis [8].

Propolis has been used as a traditional remedy worldwide since ancient times, especially in Europe, China, Brazil, Japan, Australia, and in North and Sub-Saharan Africa [9]. Propolis is considered to be a traditional medicine in certain countries such as Germany and Switzerland, while in other parts of Europe, Japan, and the United States, it is regulated as a food supplement. In Australia, it is regulated as a complementary medicine [10]. In addition, propolis is listed under Schedule 14 of the Commonwealth Therapeutic Goods Act 1989 [10]. The most common applications of propolis include formulations for upper respiratory tract infections, common colds, flu-like infections, and dermatological formulations useful in acne, burns, herpes simplex infections and neurodermatitis [11]. Raw propolis is composed of resins (50%), waxes (30%), essential oils (10%), pollen (5%), and several organic compounds (5%). More than 300 constituents have been identified through HPLC–MSn analysis in different samples of propolis extracts [11], with polyphenolic compounds presenting the main interesting bioactive components of propolis extracts, ranging from about 40–50% w/w depending on the origin and extraction methods. The main identified polyphenols are flavones (chrysin, apigenin), flavonols (galangin, quercetin) and flavonones (pinocembrin, pinobanksin) [12].

Growing evidence from experimental animals suggests that gut microbial modulation induced by treatment with propolis extract exerts a positive impact on several pathological conditions [13–17]. In particular, Cai et al. reported that in high fat diet-fed mice the ethanol extract of propolis reduced weight gain, liver fat accumulation, proinflammatory cytokines, and insulin resistance and improved glucose tolerance and lipid profile, through the modulation of gut microbiota, especially those bacterial taxa associated with the metabolic parameters of obesity, insulin resistance, and lipid profile. Similar results were obtained by Xue et al. that showed in diabetic rats the improvement of gut mucosal damage,which in turn induced positive effects on gut microbiota functionality.

Nevertheless, it is not easy to compare the results obtained thus far due to the high variability in the different varieties of propolis extracts used in these studies. To overcome the problem of propolis variability, our previous investigations [12,18,19] used a new poplar-type propolis extract with a standardized polyphenol content, especially with regards to the six main flavonoids, namely pinocembrin, apigenin, galangin, chrysin, pinobanksin, and quercetin, obtained using a patented extraction method called Multi Dynamic Extraction (M.E.D.® propolis) [20]. M.E.D.® propolis is a strong antioxidant and anti-inflammatory agent [19] with an epigenetic mechanism of action, able to modify the expression levels of microRNAs and mRNA targets coding for antioxidant enzymes and pro-inflammatory cytokines. In addition, in adult male mice (C57BL/6), the oral administration of M.E.D.® propolis is followed by the rapid absorption and metabolism of galangin and an induced adaptation of the antioxidant first line defense system [18]. More recently, an oral spray based on M.E.D.® propolis was found to be active against bacterial and viral uncomplicated upper respiratory tract infections (URTI), being able to relieve URTI symptoms in a smaller number of days without the use of pharmacological treatments [10,12].

This study aims to evaluate in vitro the impact of M.E.D.® propolis on the composition and functionality of gut microbiota obtained from the fecal material of healthy and diseased subjects using a simulated in vitro digestion-fermentation process, designed to mimic natural digestion in the human oral, gastric, and intestinal chambers. [21,22].

2. Materials and methods

2.1. Chemicals and propolis sample

2.1.1. In vitro digestion and fermentation

Salivary α-amylase, pepsin from porcine gastric mucosa, porcine bile acids, tryptone, sodium dihydrogen phosphate, resazurin, cysteine and sodium sulfide were provided by Sigma-Aldrich (Darmstadt, Germany). Pancreatin from porcine pancreas was obtained from Alpha Aesar (United Kingdom).

2.1.2. Antioxidant assays

2,4,6-tri(2-pyridyl)-s-triazine (TPTZ), iron (III) chloride hexahydrate, sodium acetate, 2 Diphenyl-1–1-picrythydrazul hydrate 95% (DPPH), hydrochloric acid, methanol, 2Trolox ((±)– 6-hydroxy-2,5,7,8- tetramethylchromane-2-carboxylic acid), Folin–Ciocalteu reagent, sodium carbonate and gallic acid were provided by Sigma-Aldrich (Darmstadt, Germany).

2.1.3. Propolis sample

A standardized polyphenol mixture extracted from poplar-type propolis (B Natural S.r.l, Corbetta, Italy) was prepared using the patented extraction method called M.E.D. [20] as reported in Curti et al. [18] and analysed to ensure that it has the same standardized

composition, as previously reported [10,12]. The sample used in the experiments was a water dispersible powder (PROPOLIS DRY EXTRACT ESIT 12®) in which the propolis extract was combined with arabic gum, sucralose, and silicon dioxide, used as excipients. The taste was bitter, and the microbiological, heavy metal and allergen tests were within regulatory limits. The blank sample consisted of the same ingredients excepting the propolis extract, namely arabic gum, sucralose, and silicon dioxide.

2.2. In vitro digestion

Propolis (PROPOLIS DRY EXTRACT ESIT 12®) was subjected to in vitro gastrointestinal digestion to simulate physiological human intestinal processes as described by Pérez-Burillo et al. [22]. Five grams of sample were weighed into centrifuge tubes in triplicate for in vitro digestion. The in vitro digestion was carried out in three steps: oral, gastric and intestinal steps. First, five grams of sample were mixed with 5 mL of simulated salivary fluid containing α-amylase 150 U/mL, for 5 min at 37 °C and in oscillation. Right after, 10 mL of simulated gastric fluid containing 4000 U/mL were added into the tube, the pH lowered to 3 and kept at 37 °C for 2 h in oscillation. Finally, 20 mL of simulated intestinal fluid containing 200 U/mL of pancreatin and 20 mM bile salts were added into the tube, the pH increased to 7 and kept at 37 °C for 2 h. Enzyme activity was stopped by immersion in ice for 15 min. Afterwards, tubes were centrifuged at 4000 xg for 10 min and the supernatant (fraction available for absorption at the small intestine) collected and the pellet (fraction not digested that would reach the colon) used for in vitro fermentation. A tube no containing samples to account for the enzymes (control) was also run.

2.3. In vitro fermentation

This process was carried out following a protocol described by Pérez-Burillo et al. [22], using fecal material from different 5 donors: 3 healthy adults (Body Mass Index [BMI] = 21.3), and 12 children (5–10 years old): 3 healthy children (percentile 95), 3 obese children (percentile 90), 3 celiac children and 3 children with a food allergy. None of the donors had taken antibiotics in the last 3 months. Fecal material from the same population was pool together to account for inter-individual variability [22]. *In vitro* fermentation was carried out using 15 mL centrifuge tubes. To each tube, 500 mg of the solid digestion residue, plus 10% of the final digestion volume, were added. A control tube was also run, which did not contain propolis. Each of the tubes also contained 7.5 mL of fermentation growth medium (14 g/L of peptone, cysteine 312 mg/L, hydrogen sulfide 312 mg/L and resazurin 0.1% v/v) and 2 mL of fecal inoculum (fecal material in phosphate saline buffer, 33% w/v). Nitrogen was bubbled into the tubes until anaerobic conditions were reached and kept at 37 °C in oscillation for 20 h. Microbial activity was halted by immersion in ice for 15 min. Tubes were centrifuged at 4000 xg for 10 min, supernatant aliquoted for antioxidant capacity and SCFA analysis and pellet stored for 16 S amplicon sequencing.

2.4. Antioxidant assays

The antioxidant capacity was studied in two stages: the supernatant obtained from in vitro digestion and the supernatant obtained from in vitro fermentation. The sum of both was considered the total antioxidant capacity. The respective blanks (chemicals, enzymes and inoculum) were considered to correct the antioxidant capacity values of each method.

2.4.1. $TEAC_{FRAP}$ assay (Trolox equivalent antioxidant capacity referred to reducing capacity)

The protocol of Benzie and Strain [23] was followed to measure the ferric reducing capacity of each sample. It was carried out on a microplate reader (FLUOStar Omega, BMG Labtech, Germany). Twenty μL of each sample (digestion and fermentation supernatant) together with 280 μL of FRAP reagent (prepared daily) were introduced into each well of the plate. The FRAP reagent was composed of 25 mL of 0.3 M sodium acetate buffer pH 3.6, 2.5 mL of ferric chloride and 2.5 mL of 40 mM 2,4, 6-tri(2-pyridyl)-s-triazine (TPTZ). Absorbance readings were taken every 30 s for 30 min at 595 nm. The assay was performed at 37 °C and each sample was studied in triplicate. A calibration curve was prepared with Trolox from 0.01 to 0.4 mg/mL. Results were expressed as mmol Trolox equivalent/Kg of propolis.

2.4.2. $TEAC_{DPPH}$ assay (Trolox equivalent antioxidant capacity against DPPH radicals)

The Yen and Chen protocol was followed to perform a $TEAC_{DPPH}$ assay [24]. Twenty μL of digestion or fermentation supernatant plus 280 μL of DPPH reagent were mixed in a 96-well plate. The DPPH reagent was prepered freshly each day by dissolving 74 mg of DPPH in 1 L of methanol. The reaction was monitored for one hour at 37 °C. Each sample was studied in triplicate. A calibration curve was prepared with Trolox (0.01–0.4 mg/mL). The results were expressed as mmol Trolox equivalent/kg propolis.

2.4.3. Folin-Ciocalteu assay (Determination of total phenolic content)

The protocol followed was that of Moreno-Montoro et al. [25]. Thirty μL of the digestion or fermentation supernatant were added in triplicate to the 96-well plate and mixed with 60 μL of sodium carbonate 10% (w/v), 195 μL of ultra-pure water and 15 μL of Folin-Ciocalteu reagent. The antioxidant reaction was monitored for 60 min at 37 °C. The calibration curve was prepared with gallic acid at concentrations from 0.01 to 1.00 mg/mL. The results obtained were expressed as mg gallic acid equivalents/kg of propolis.

2.5. Short-Chain fatty acid determination

Determination of SCFA was carried out according to the procedure described in Panzella et al. [26] with few modifications. The HPLC was an Accela 600 (Thermo scientific) equipped with a quaternary pump, an autosampler and a UV-Vis photo diode array detector (PDA) that was set at 210 nm. The analysis was carried out isocratically with a mobile phase composed of a mixture of 99% of A and 1% of B delivered at a flow rate of 0.250 mL/min. Solution A was ultra-pure water acidified with 1% of formic acid and solution B was acetonitrile acidified with 1% of formic acid. The column was a reversed phase Accucore™ C18 (Thermofisher) with particle size of 2.6 μm and 150 mm long. Briefly, fermentation supernatant was centrifuged, filtered through a 0.22 μm nylon filter, and analyzed by means of a HPLC system. The analysis was performed in duplicate, and the data presented are the mean values expressed as millimolar concentration (mM) of each SCFA.

2.6. DNA extraction and sequencing

DNA extraction was performed using a NucliSENS easy MAG platform (Biomerieux) following the standard protocol. Microbial genomic DNA was used at a concentration of 5 ng/μL in 10 mM Tris (pH 8.5) for the Illumina protocol for 16 S rRNA gene Metagenomic Sequencing Library Preparation (Cod. 15044223 Rev. A). PCR primers targeting the 16 S rRNA gene V3 and V4 regions were designed as in Klindworth et al. [27]. Primer sequences were Forward 5′TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG3′ and Reverse 5′GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC3′. Primers contained adapter overhang sequences added to the gene-specific sequences, making them compatible with the Illumina Nextera XT Index kit (FC-131–1096). After 16 S rRNA gene amplification, amplicons were multiplexed, and 1 mL of amplicon pool was run on a Bioanalyzer DNA 1000 chip to verify amplicon size (~ 550 bp). After size verification, libraries were sequenced in an Illumina MiSeq sequencer according to the manufacturer's instructions in a 2 × 300 cycle paired-end run (MiSeq Reagent kit

v3MS-102–3001).

Quality assessment of sequencing reads was performed with the prinseq-lite program, applying the following parameters: a minimal length (min_length) of 50 nt and a quality score threshold of 30 from the 3′-end (trim_qual_right), using a mean quality score (trim_qual_type) calculated with a sliding window of 10 nucleotides (trim_qual_window). Read 1 and read 2 from Illumina sequencing were joined using fastq-join from the ea-tools suite. Taxonomic affiliations were assigned using the RDP_classifier from the Ribosomal Database Project (RDP). Reads that had an RDP score below 0.8 were assigned to the next higher taxonomic rank, leaving the last rank as unidentified. We assigned six taxonomic levels: kingdom, phylum, class, order, family, and genus.

2.7. Statistical analysis

The statistical significance of the data was tested by one-way analysis of variance (ANOVA), followed by the Duncan test to compare the means that showed significant variation ($P < 0.05$). Principal Components Analysis (PCA) was performed as an exploratory method to study the distribution of samples according to SCFA production. Principal Coordinates Analysis (PCoA) with Bray-Curtis distance was performed for the exploratory analysis of 16 S rRNA data. Coinertia analysis was carried out as an interpretative method. Coinertia analysis tries to find associations between two sets of variables: the microbial community and SCFA, in this case, by finding the correlation between the metabolites PCA and the microbial PCoA. The strength of the association found with coinertia analysis is measured via the RV coefficient. It is a number between 0 and 1, and the higher the stronger the association. We also calculated a log2 ratio of each genus relative abundance in order to show which ones were favored by propolis and, secondly, to make the figure easier to read. Log2 ratio was calculated by dividing each genus relative abundance by the corresponding blank and then applying the log2. For instance, log2 (*Bacteroidies* rel abundance obese with propolis/ Bacteroides rel abundance obese with no propolis). Statistical significance was calculated by the two-sided Wilcoxon test at 95% confidence. All analyses were carried out in R version 3.6.3.

3. Results

The propolis sample was prepared in a water-dispersible powder delivery form by combining a standardized polyphenol mixture extracted from poplar-type propolis, with arabic gum used as high molecular weight carrier in spray drying and adding sucralose and silicon dioxide excipients. The blank sample consisted of arabic gum, with sucralose and silicon dioxide in the same delivery form. These samples were submitted to a simulated in vitro digestion-fermentation process, designed to mimic the natural digestion in the human oral, gastric, and intestinal chambers. Propolis samples before and after digestion and fermentation were analyzed to determine their antioxidant profiles. Moreover, the ability of digested-fermented propolis to modify gut microbiota composition and functionality (production of SCFAs) was determined by comparing the propolis sample with the blank sample.

3.1. Antioxidant profile of propolis before and after digestion-fermentation process

To evaluate the influence of digestion and fermentation on propolis, the total polyphenol content and the antioxidant capacity were determined before and after in vitro digestion and fermentation [28]. In particular, the Folin-Ciocalteu assay was used to assess the effect of in vitro digestion and fermentation on total polyphenol content in propolis. In addition, two different methods were used to estimate the antioxidant capacity of digested and fermented samples (FRAP, which measures Fe^{3+} reduction, and DPPH, which measures antiradical activity). The Folin-Ciocalteu assay showed that the total polyphenol content of propolis extract before the digestion process corresponds to 70.0 g of gallic acid equivalents/kg of propolis (corresponding to 111.2 g of galangin equivalents/kg of propolis). After the in vitro digestion, propolis polyphenols underwent almost total degradation (Table 1), with a total polyphenol content corresponding to 15.0 g of gallic equivalents/kg of propolis. As far as the fermentation process is concerned, the total polyphenol content decreased to 2.5, 2.9, 3.7, 2.1, and 0.9 g of gallic acid equivalents/kg of propolis, when gut microbiota from healthy adults, and healthy, allergic, obese, and celiac children were used for the fermentation, respectively. In this sense, the decrease on total phenolics was statistically larger ($P < 0.05$) for obese and celiac children (Table 1).

The radical scavenging capacity of propolis extract was tested against DPPH, a stable nitrogen synthetic radical, and was expressed as $TEAC_{DPPH}$. The DPPH assay showed that before the in vitro digestion-fermentation process, the propolis sample exerted a radical scavenging ability corresponding to 158 g of Trolox/kg of propolis. After the in vitro digestion, $TEAC_{DPPH}$ decreased to a value corresponding to 8 g of Trolox/kg of propolis (Table 1). Finally, $TEAC_{DPPH}$ decreased to 7.2 g of Trolox/kg of propolis and 6.9 g of Trolox/kg of propolis after in vitro fermentation with the gut microbiota isolated from fecal material of healthy adults and healthy children, respectively. $TEAC_{DPPH}$ corresponded to 7.5 g of Trolox/kg of propolis, 4.6 g of Trolox/kg of propolis, and 8.5 g of Trolox/kg of propolis (Table 1), after fermentation with fecal materials from allergic, obese, and celiac children, respectively. Only a lower antioxidant capacity was obtained after fermentation with obese microbiota ($P < 0.05$).

The reducing power, expressed as $TEAC_{FRAP}$, was determined through a FRAP assay. Before the digestion-fermentation process, $TEAC_{FRAP}$ was 142 g of Trolox/kg of propolis. After the digestion process, it decreased to 16 g of Trolox/kg of propolis (Table 1). After the fermentation $TEAC_{FRAP}$ decreased in all the samples ($P < 0.05$) to 7.3 g of Trolox/kg of propolis with fecal materials from healthy adults, 9.2 g of Trolox/kg of propolis for healthy children, 9.3 g of Trolox/kg of propolis for allergic children, 9.1 g of Trolox kg of propolis for obese children, and 9.5 g of Trolox/kg of propolis for celiac children.

3.2. SCFA production after digestion-fermentation process

SCFA are metabolites produced by gut microbiota, mainly following the fermentation of dietary fibers. After the digestion-fermentation process of the propolis and blank samples, SCFA were determined in the fermentation supernatant by a chromatographic method coupled with UV detection (HPLC-UV). The SCFA identified in the samples were acetic, propionic, and butyric acids, while succinic, isovaleric and pentanoic acids were not detected at concentrations within the limit of detection (LOD) of the applied method (data not shown). Acetate, propionate and butyrate were quantified, and the concentrations (mM) determined in the supernatant obtained from propolis fermentation were compared with blank concentrations. The results are reported in Table 2.

The results suggest that propolis significantly increased the production of SCFA in comparison to the blank sample, exerting a booster effect on SFCA producing bacteria. Generally, the increase in butyric acid production was lower than the increase induced for acetic and propionic acid concentrations, irrespective to the gut microbiota used for fermentation. In particular, the presence of propolis boosted the production of acetic acid in diseased children ($P < 0.05$) while for propionic acid, just for allergic and celiac children (Table 2). Lower increases were obtained for butyric acid, being the larger increases obtained for obese and celiac children ($P < 0.05$).

Once studied the production of SCFA, a PCA was carried out to explore the potential relationships between the samples (Fig. 1). The addition of propolis altered the production of SCFA, since blank samples were grouped in a different sector that those of fermented samples. A similar production of SCFA was obtained for healthy children and adults, while the samples of celiac and allergic children behaved similar between them. The obese children however, showed a rather different

Table 1
Antioxidant capacity of propolis after in vitro digestion-fermentation in healthy and diseased subjects.

Assay	*In vitro* Digestion	*In vitro* Fermentation				
		Healthy adults	Healthy children	Allergic children	Obese children	Celiac children
FOLIN-CIOCALTEU (g of gallic acid equivalents/kg of propolis)	15.0 ± 2.3[a]	2.5 ± 0.4[b]	2.9 ± 0.3[b]	3.7 ± 0.6[b]	2.1 ± 0.2[c]	0.9 ± 0.1[d]
DPPH (g of Trolox/kg of propolis)	8.0 ± 1.7[a]	7.2 ± 1.4[a]	6.9 ± 1.1[a]	7.5 ± 1.2[a]	4.6 ± 0.5[b]	8.5 ± 1.3[a]
FRAP (g of Trolox/kg of propolis)	16.0 ± 2.1[a]	7.3 ± 1.0[b]	9.2 ± 1.8[b]	9.3 ± 1.7[b]	9.1 ± 1.6[b]	9.5 ± 1.8[b]

Different letters within the same row indicates statistically significant differences (P < 0.05).

Table 2
SCFA concentrations (mM) in healthy and diseased subjects and percent increase (%).

Subjects	Sample	Acetic acid (mM)	Increase (%)	Propionic acid (mM)	Increase (%)	Butyric acid (mM)	Increase (%)
Healthy adults	Propolis	316.3 ± 10.3[a]	119%	381.2 ± 12.8[a]	832%	29.3 ± 0.4[a]	142%
	Blank	144.4 ± 4.7		40.9 ± 0.9		12.1 ± 0.1	
Healthy children	Propolis	223.5 ± 7.6[b]	33%	198.7 ± 6.6[b]	904%	15.9 ± 0.2[b]	47%
	Blank	167.7 ± 5.4		19.8 ± 0.3		10.8 ± 0.1	
Allergic children	Propolis	335.9 ± 11.9[b]	3226%	694.6 ± 21.7[c]	7289%	10.2 ± 0.2[c]	9%
	Blank	10.1 ± 0.2		9.4 ± 0.1		9.4 ± 0.1	
Obese children	Propolis	135.5 ± 3.9[c]	692%	921,3 ± 30.9[d]	7%	113.2 ± 3.9[d]	116%
	Blank	17.1 ± 0.3		859.3 ± 29.6		52.4 ± 1.8	
Celiac children	Propolis	112.9 ± 3.9[d]	5842%	235.5 ± 7.3[e]	1194%	7.1 ± 0.3[e]	145%
	Blank	1.9 ± 0.1		18.2 ± 0.2		2.9 ± 0.1	

Different letters within the same column indicates statistically significant differences (P < 0.05).

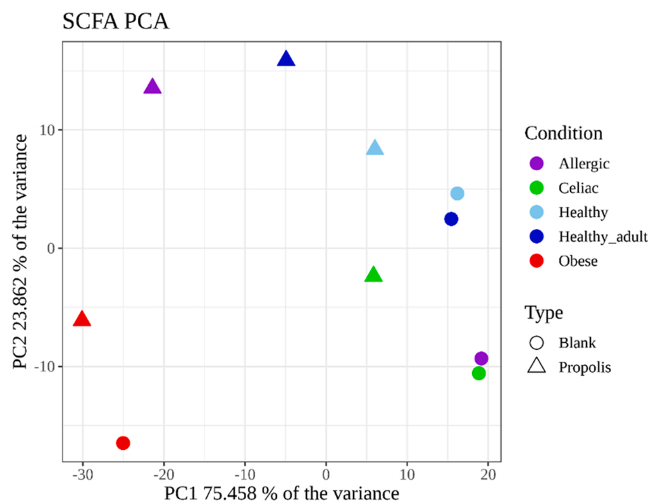

Fig. 1. exhibits the output scatter plot of an Euclidean-based principal component analysis (PCA) of SCFA with the aim of visualizing dissimilarity and possible clustering between samples. Each color represents a population from where the fecal material used for in vitro fermentation came. Shapes represent blanks (in vitro fermentation tubes with no propolis added – Control) and propolis (in vitro fermentation tubes with propolis added).(For interpretation of the references to colour in this figure, the reader is referred to the web version of this article.)

SCFA profile than the others (Fig. 1).

3.3. *Gut microbiota composition*

After the fermentation, supernatants were submitted to antioxidant assays and to the determination of SCFA, while the solid residues of each sample were analyzed using 16 S rRNA sequencing for the determination of gut microbiota composition after treatment with the propolis sample and compared with the blank sample. Given the large amount of data obtained, different kinds of analyses were performed in order to evaluate the modification of gut microbiota, such as PCoA with the Bray-Curtis dissimilarity index, which was carried out for the exploratory analysis of 16 S rRNA sequencing data (Fig. 2).

As it can be observed from the PCoA (Fig. 2), variations in microbial communities were greater between samples than as a consequence of adding propolis, except in the case of celiac and allergic children. In both cases, the addition of propolis caused a deeper change in the microbial community than that for the other groups.

A Coinertia analysis was also carried out as an interpretative method. This method tries to find associations between two sets of variables, in

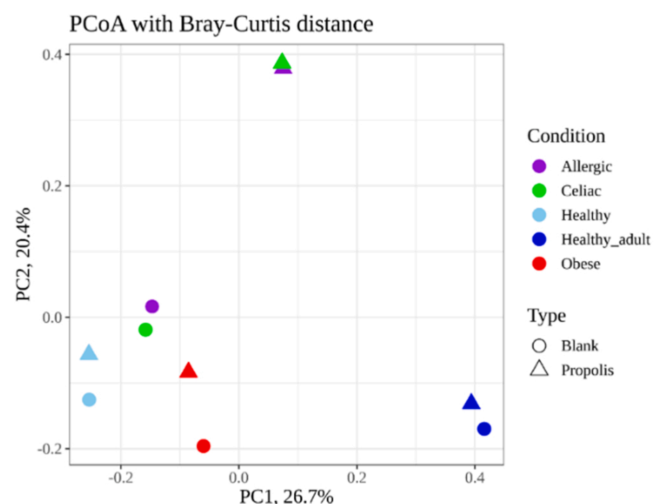

Fig. 2. shows the output scatter plot of a Bray-Curtis based principal co-ordinates analysis (PCoA) of the gut microbial community structure. Each color represents a population from where the fecal material used for in vitro fermentation came. Shapes represent blanks (in vitro fermentation tubes with no propolis added – Control) and propolis (in vitro fermentation tubes with propolis added).(For interpretation of the references to colour in this figure, the reader is referred to the web version of this article.)

this case the microbial community structure and SCFA. In this sense, Fig. 3 shows a correlation between the SCFA PCA and the microbial PCoA. The strength of the association found through Coinertia analysis is measured via the RV coefficient. It is a number between 0 and 1, with higher values representing a stronger association (Fig. 3).

There are two sets of samples for each group: SCFA vs microbial population for blanks, and SCFA vs microbial population for propolis. As depicted in Fig. 3, there was a mildly strong, though not statistically significant, correlation (RV = 0.503; p = 0.06) between the SCFA-based PCA and the genus- based PCoA ordinations, since the distance between the same samples (depicted with an arrow) is usually lower than that between different samples. This indicates that SCFA and gut microbial composition analyses support each other.

Finally, Fig. 4 represents the increase or decrease of specific bacterial strains after administration of propolis. A log2 fold increase of each bacterium (with respect to the blank) was calculated as in the following example: log2 (Bacteroides with propolis/Bacteroides blank), or in other words, only those bacteria with a log2 fold increase greater than 2 were considered. Thus, a green signal means that the propolis increased the relative abundance of that specific bacteria, whereas a red signal means a decrease in the relative abundance of specific bacteria with propolis.

4. Discussion

In this study, a standardized polyphenol mixture extracted from poplar-type propolis, combined with arabic gum, sucralose and silicon dioxide (propolis sample), was submitted to simulated in vitro digestion, followed by the determination of the antioxidant capacity. The digested sample was then submitted to fermentation using fecal material from five different donors: healthy adults, and healthy, obese, celiac and food allergic children. Then the digested-fermented propolis sample was analysed to determine its bioactivities, such as antioxidant capacity, ability to modify gut microbiota composition and functionality (in terms of SCFA production) and compared with those of the blank sample (i.e. arabic gum with sucralose and silicon dioxide) submitted to the same in vitro digestion-fermentation process.

As far as the TPC is concerned, our results are in accordance with

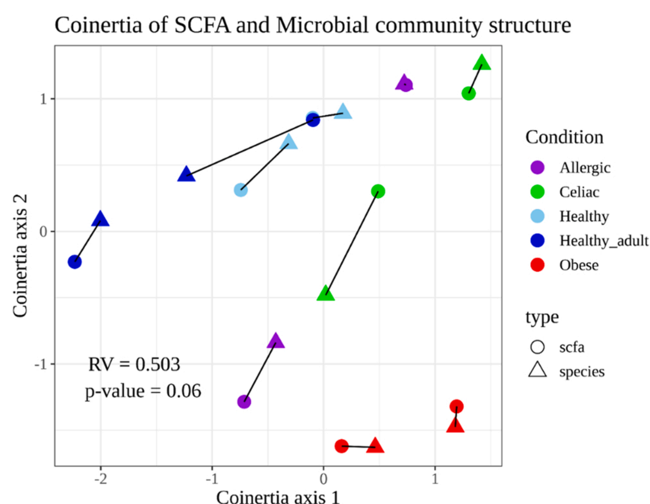

Fig. 3. represents the output scatter plot of the Coinertia analysis carried out using as input the SCFA based PCA and the microbial community structure based PCoA. Whereas colors represent different populations, shapes represents both inputs: SCFA PCA and microbial community PCoA. Each pair of samples (PCA and PCoA) are linked by an arrow which length tells how far or close to each other are in the previous ordination plots; shorter arrows indicate similar sample positioning in each plot. The RV coefficient measures the relative fit between both ordination plots and the significance of that fit is assessed by the p-value.(For interpretation of the references to colour in this figure, the reader is referred to the web version of this article.)

those obtained by Ozdal et al. [29], who studied 11 raw propolis samples from various geographical areas in Turkey, by Shi et al. [30], who studied 15 poplar-type Chinese propolis samples, and by Ahn et al. [31] who measured the total phenolic contents of 20 poplar-type Chinese propolis samples. The polyphenols available after in vitro digestion decreased by about 80%. This result is also in accordance with that obtained by Ozdal et al. [29] who found that the degradation of polyphenols after digestion ranged from 67% to 96% with an average degradation of 87%. With regards to the effect of fermentation on propolis polyphenol bioaccessibility, to the best of our knowledge no data are available on the fermentation of propolis by human gut microbiota obtained from fecal materials of healthy and diseased subjects. In addition, limited data are available regarding the biotrasformation of propolis through its bioconversion by some strains of *Lactobacillus helveticus* and *Lactobacillus plantarum*, showing the degradation of the polyphenol components [32–34]. Our results showed that fermentation induces further polyphenol degradation, with slight variation based on the origin of the fecal inocula, probably associated with the different capacity of the microorganisms occurring in the fecal materials to use propolis polyphenols as substrates for their metabolism. This result is partly expected, as it is known that the gut microbiota can transform polyphenols into bioactive metabolites such as benzoic acid, phenylacetic acid, and phenylpropionic acid, which may affect intestinal ecology, impacting host health [35].

With regard to the antiradical activity of propolis and the reducing power of propolis before digestion, our results confirmed the high antioxidant potential reported by a large body of evidence. In particular, Ahmed et al. evaluated the antioxidant activities of Malaysian propolis by DPPH and FRAP assays [36]. After digestion, the radical scavenging activity and reducing power of propolis decreased as shown by previous studies [29] and a further decrease to a different extent was measured after fermentation, depending on the origin of the gut microbiota used to perform the fermentation. Numerous in vivo studies have supported the antioxidant potential of propolis that resulted in protection against cardiac diseases, endothelial dysfunction, vascular damage, and hepatic pathologies [37–41]. Accordingly, the ability of propolis to exert such protective actions could indeed be mediated by gut microbes and be variable depending on its which of the are present.

As far as SCFAs are concerned, fermentation of dietary polysaccharides (that are not otherwise digested) is the pivotal role of gut microbiota, where enzymes derived from microbiota metabolize soluble fibers into SCFA (acetate, propionate, and butyrate). SCFA are absorbed in the intestine and are used as energy source by the host. They exert regulatory functions on gut physiology, metabolism, and immunity [42] and act as regulators of energy intake and inflammation [43,44]. Butyrate also facilitates the regeneration and protection of intestinal cells, mucin production, regulates blood cholesterol levels, and the release of neurotransmitters and hormones essential for the regulation of intestinal motility and insulin resistance [45]. It is known that dysbiosis leads to a reduction in SCFA production [46]. As expected, our results showed that the total concentration of SCFAs was higher in the supernatants of blank samples obtained from the fermentation induced by fecal materials of healthy adults and children (197.4 mM and 198.3 mM, respectively), while it was lower in the supernatants corresponding to allergic and celiac children (28.9 mM and 23.0 mM, respectively). In addition, as shown by PCA, SCFA production by healthy adults and children was similar, as was between diseased subjects (celiac and allergic children), while obese children showed a different trend. In particular, the concentrations of propionic and butyric acids were 20–40 times higher for propionic and about 5 times higher for butyric, in comparison with the concentration determined in healthy subjects. These results are in agreement with those of Jaimes et al. [47] who demonstrated that butyric acid concentration was higher in the feces of overweight/obese children compared to those of normal-weight children. In addition, previous studies reported that high levels of total SCFAs, propionic acid and butyric acid concentrations in feces have been

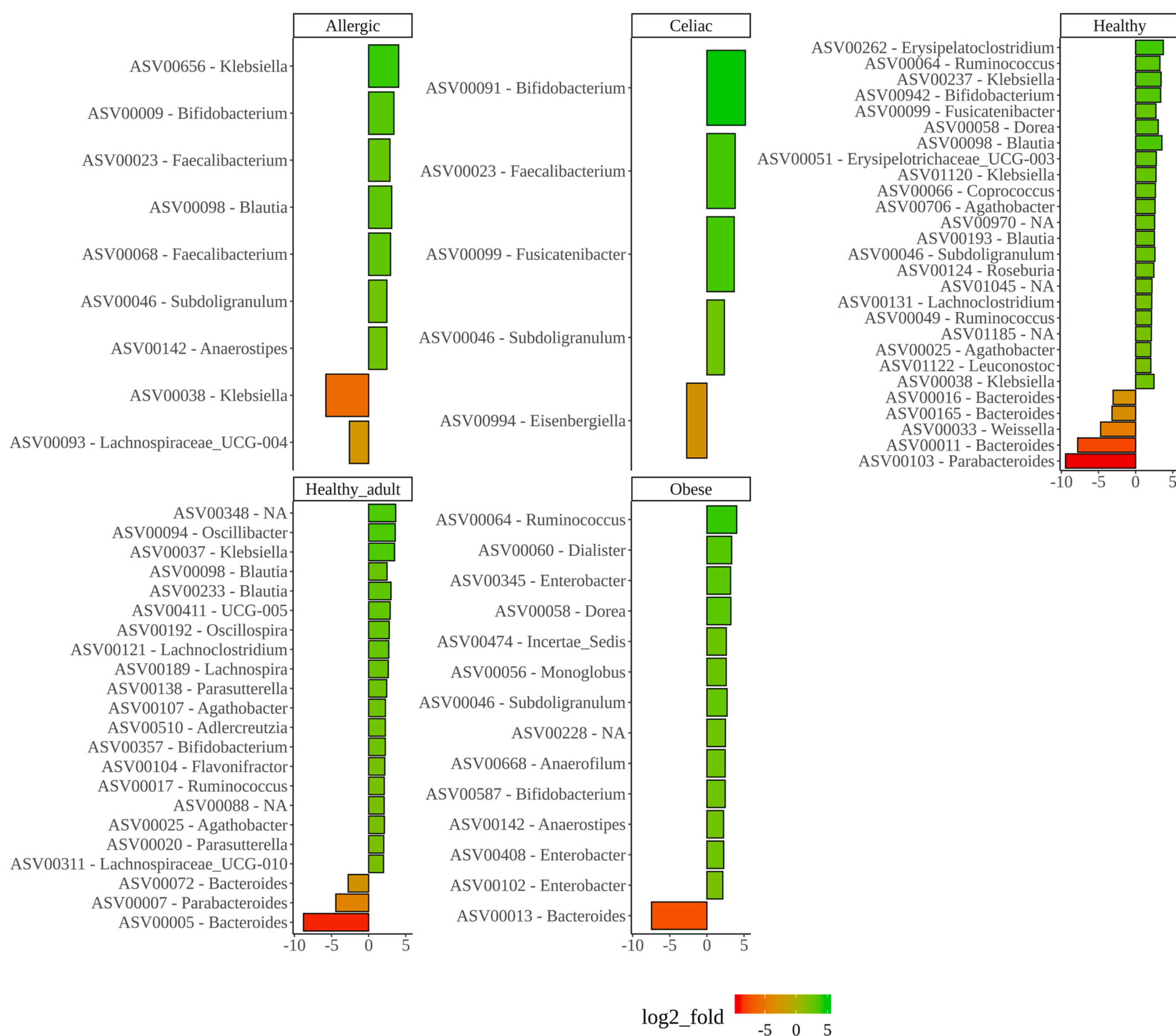

Fig. 4. Bar plot representing the log2 fold change of specific genera in comparison to each respective blank. Green color indicates that such genus was found in higher abundance in the in vitro fermentation tube with propolis than in its control (tube with no propolis and same fecal material). Red color indicates the opposite, such genus was found in lower abundance than in its control.(For interpretation of the references to colour in this figure, the reader is referred to the web version of this article.)

positively correlated with high BMI and fat-to-lean mass ratio [46,48,49].

With regards to the influence of propolis on SCFA production, propolis exerted a beneficial effect, leading to an increase in total SCFA production, especially in allergic and celiac children. These results lead to the conclusion that different bacterial strains are influenced by propolis in different ways, releasing SCFAs to different extents. This increase could be related to the synthesis of SFCAs using propolis components as substrates, and/or to the increased relative abundance and/or activity of SCFA–forming bacteria. It is noteworthy that while propolis generally had a great booster effect on the production of SCFAs, it did not induced a significant increase in propionic acid concentration when interacting with the gut microbiota of obese children. As previous studies have shown that a higher concentration of propionic acid has been associated with a higher android/gynoid fat ratio, which in turn is a risk factor for metabolic and cardiovascular diseases in children, the fact that propolis did not induce a further increase in propionic acid concentration leads to the hypothesis that propolis could exert a protective role in obese subjects, or at least does not aggravate the situation [50].

The eubiotic state of gut microbiota is characterized by the preponderance of the beneficial species of the phyla Firmicutes and Bacteroidetes, which together represent approximately 70–90% of the total microbiota, and a low percentage of potentially pathogenic species of Proteobacteria [51,52]. In addition, the gut microbiota plays an important role in maintaining human health and in the prevention of many pathologies such as inflammatory bowel disease, celiac disease, obesity, metabolic disorder, etc. [53]. The composition of the gut microbiota is influenced by many endogenous and exogenous factors (i.e., age, body mass index, healthy status, environmental factors, diet, stress, drugs). To evaluate the effect of propolis on gut microbiota composition, microbiota community structure was determined in fermented propolis by comparison to blank samples, using high-throughput sequencing of 16 S rRNA gene. The results suggest that propolis fermented with gut microbiota obtained from healthy subjects (i.e., adults and children) and obese children decreased the abundance of Bacteroidetes and increased the level of a variety of beneficial microorganisms, including Firmicutes (*Ruminococcus*, *Dorea*, *Roseburia*) and Actinobacteria (*Bifidobacterium spp.*). Although it is not entirely correct to speak about beneficial and harmful gut microorganisms, because some

species could be beneficial or harmful depending on the habitat, which may determine different behaviors [53], the modifications of gut microbiota composition induced by propolis (especially with obese children) suggest that propolis could improve the dysbiosis state. In fact, a large body of evidence supports that dysbiosis consists in an overall reduction of beneficial bacterial species (i.e., Lactobacilli and Bifidobacteria), which use fiber and increase the production of SCFA, and an increase in Bacteroides and other putrefactive bacteria (from a high-protein rich diet) which produce metabolites (i.e., ammonia, amines, and phenols) that negatively affect gastrointestinal and systemic health [53].

At the genus level, propolis fermented with the gut microbiota of obese children resulted in the increase of some genera, such as *Dialister, Subdoligranulum* and *Anaerostipes*. As far as the level of *Dialister* is concerned, high levels are associated with lower BMI and weight reduction [54]. In fecal samples, Chen et al. [55] evaluated gut microbial biodiversity between obese (23 subjects) and normal weight (28 subjects) children of both sexes (27 males and 24 females), aged between 6 and 11 years old, using 16 S rRNA gene sequencing, and showed that the fecal microbiota of children in the obese group had lower proportions of the *Dialister* genus compared to the normal weight group. This finding was confirmed by Quiroga et al. [56] who determined the effect of a 12-week strength and endurance combined training exercise on gut microbiota composition in 39 obese pediatric patients, randomly distributed between the control and training groups. These authors found that at the baseline, the levels of some genera (including *Dialister*) were low, but after exercise the levels increased leading to a microbiota profile similar to that of healthy children. In our study, further beneficial bacteria belonging to the *Subdoligranulum* genus were increased by propolis fermented by gut microbiota of obese children. Many studies have shown a positive association between high *Subdoligranulum* abundance and a healthier metabolic status (i.e., fecal microbiota richness, high HDL-cholesterol and adiponectin levels, and low-fat mass, adipocyte diameter, leptin levels, and inflammation markers such as CRP and IL-6) [57]. Moreover, when propolis was fermented by the gut microbiota of obese children, it increased the level of genus *Anaerostipes*, which is one of the butyrogenic bacteria in the healthy microbiota that decreases in type 2 diabetes patients [58,59].

As far as the influence of propolis on the gut microbiota composition of celiac children is concerned, the most interesting results were the increase in the levels of *Bifidobacterium, Faecalibaterium* (which belongs to the Firmicutes Phylum, Ruminococcaceae Family), and F*usicatenibacter* (belonging to Firmicutes Phylum, Lachnospiraceae Family). Celiac disease is an autoimmune disorder triggered by ingestion of gluten in genetically susceptible individuals, causing a chronic intestinal inflammation associated with the destruction of small intestinal villi. Growing evidence suggests that changes in gut microbiome composition and its metabolic activity are associated with a number of chronic inflammatory diseases including obesity, inflammatory bowel disease, and cancer. Recent findings have shown that there are specific bacteria that are biomarkers of celiac disease [60]. Although the studies on the association between celiac disease and gut microbiota composition began about 15 years ago, limited data are available on human fecal microbiota of celiac subjects using next-generation sequencing. Recent evidence confirms that a reduction in *Bifidobacteria* in celiac subject fecal materials is a result of the gluten-free diet, more than the cause of celiac disease, due to the decreased intake of nondigestible carbohydrates from cereals, which in turn affects the abundance of fiber-fermenting species and SFCA production [60]. Anyway, by increasing *Bifidobacteria*, propolis leads to a microbiota profile and SCFA production similar to those of healthy children. Moreover, the increase in the levels of butyrate producers (i.e., *Faecalibaterium* and F*usicatenibacter*) can reduce the chronic inflammation caused by this autoimmune disorder.

Finally, with regards to the influence of propolis fermented by gut microbiota isolated from allergic children, the increase in the levels of *Bifidobacterium, Faecalibaterium, Anaerostipes,* and *Blautia*, and a decrease in the level of the genus *Klebsiella* were observed. Wopereis et al. [61] showed that infants with eczema have low levels of lactate-utilizing bacteria producing butyrate, (*Eubacterium* and *Anaerostipes* species) and infant formula with partially hydrolyzed protein and specific prebiotics modulated the gut microbiota closer to that of breast-fed infants, increasing *Bibidobacteria* levels. Moreover, at the onset of allergic symptoms, Icpi et al. [62] showed lower counts of *Faecalibacterium prausnitzii* among the other *Clostridium spp.* with increased stool lactate and decreased butyrate levels. Recent findings [63] have shown that in atopic eczema, the infant gut microbiota has an enrichment of *Escherichia coli* and *Klebsiella pneumonie*, with increased gene expression of virulence factors (such as invasin, adhesin, flagellin and lipopolysaccharides). As growing evidence suggests that gut dysbiosis precedes food allergy and plays an essential role in the development of said allergy [64], the modulation of gut microbiota composition with propolis treatment aimed at restoring eubiosis before the onset of allergic symptoms, should be further investigated.

In conclusion, the standardized polyphenol mixture used in this study, extracted from poplar-type propolis, is a bioactive product that exerts a range of protective effects including modulation of the gut microbiota. This study analyzed the TPC and antioxidant effects of propolis following in vitro digestion and fermentation. The in vitro digestion induced a decrease in the antioxidant profile of propolis. The propolis extract was also fermented with different fecal samples, keeping in mind the fact that undigested food matrices reach the colon where they are exposed to the colonic bacteria. TPC and antioxidant activities were decreased in the fermented extract, probably due to the metabolic shift of polyphenols by gut microbiota, while the biological effects of the extract were retained in part. Propolis fermentation exhibited a modulatory effect on gut microbiota composition and functionality in healthy and diseased subjects. Propolis increased the concentration of SCFA, reflecting an increase in the growth of SCFA producing bacteria. Despite propolis is used for centuries, is considered a healthy bee product, and the present promising results, it should be considered that propolis is a potential allergen, as few cases of contact dermatitis have been reported among beekeepers. As far as in vivo studies, the oral administration of propolis (500 mg/kg/day) for 8 weeks did not affect the body weight and serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatinine, and urea in rodents. Moreover, few studies also reported cases of oral mucositis with the use of propolis candies or lozenges and clinical trials have reported no serious adverse events in adult subjects up to 2.87 g/day dose. Moreover, the adverse events reported in clinical trials were not serious including parasitic infections, gastroenteritis, and mild skin reactions (rash) [65–69].

Therefore, in the light of the safety of propolis and these promising results on gut microbiota composition and functionality, this standardized polyphenol mixture extracted from poplar-type propolis deserves to be further investigated in humans to show the possible effects on gut microbiota and dysbiosis-based diseases.

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CRediT authorship contribution statement

Emanuele Ugo Garzarella: Methodology, Data analysis, Data curation, Software; **Beatriz Navajas-Porras:** Research design, Methodology, Data analysis, Software; **Sergio Pérez-Burillo:** Writing manuscript, Research design, Methodology, Data analysis; **Hammad Ullah:** Writing manuscript; **Cristina Esposito:** Methodology, Data analysis; **Cristina Santarcangelo:** Methodology, Data analysis; **Daniel Hinojosa-Nogueira:** Research design, Data analysis; **Silvia Pastoriza:** Research design; **Vincenzo Zaccaria:** Research design; **Jianbo Xiao:**

Writing manuscript; **José Angel Rufian-Henares:** Writing manuscript, Research design, Obtained funding; **Maria Daglia:** Conceptualization, Writing manuscript.

Author Contributions

S.P.B., H.U., J.A.R.H., J.X., and M.D. wrote or contributed to the writing of the manuscript. S.P.B., J.A.R.H., B.N.P., D.H.N., V.Z., and S.P. participated in research design. S.P.B., E.U.G., C.E., C.S., B.N.P., D.H.N. conducted experiments and performed data analysis. J.A.R.H. obtained funding for the study. All authors have read and agreed to the published version of the manuscript.

Conflicts of interest

V.Z. is a B Natural srl employee. This does not alter the author's adherence to all the journal policies on sharing data and materials. None of the academic researchers listed as co-authors served as consultant for B Natural srl or received any personal compensation. The other authors declare no conflict of interest.

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