Anaerobe 43 (2017) 56–60

Contents lists available at ScienceDirect

Anaerobe

journal homepage: www.elsevier.com/locate/anaerobe

Anaerobes in the microbiome

Pomegranate ellagitannins stimulate the growth of *Akkermansia muciniphila* in vivo

Susanne M. Henning [a], Paula H. Summanen [b, *], Ru-Po Lee [a], Jieping Yang [a], Sydney M. Finegold [b, c, d], David Heber [a], Zhaoping Li [a, b, d]

[a] Center for Human Nutrition, David Geffen School of Medicine, University of California Los Angeles, Los Angeles, CA 90095, USA
[b] VA Medical Center West Los Angeles, 11301 Wilshire Blvd., Los Angeles, CA 90073, USA
[c] Department of Microbiology, Immunology and Molecular Genetics, University of California Los Angeles, CA 90095, USA
[d] Department of Medicine, University of California Los Angeles, CA 90095, USA

ARTICLE INFO

Article history:
Received 6 November 2016
Received in revised form
1 December 2016
Accepted 4 December 2016
Available online 7 December 2016

Handling Editor: Elisabeth Nagy

Keywords:
Akkermansia muciniphila
Ellagic acid
Ellagitannins
Intestinal bacteria
Pomegranate extract
Prebiotic
Urolithin A

ABSTRACT

Results from our previous human pomegranate extract (POM extract) intervention study demonstrated that about seventy percent of participants were able to form urolithin A from ellagitannins in the intestine (urolithin A producers). Urolithin A formation was associated with a high proportion of *Akkermansia muciniphila* in fecal bacterial samples as determined by 16S rRNA sequencing. Here we investigated whether *A. muciniphila* counts increased in stool samples collected after the POM extract intervention compared to baseline stool samples using real-time PCR. In addition, we performed in vitro culture studies to determine the effect of POM extract and ellagic acid on the growth of *A. muciniphila* and to analyze ellagic acid metabolites formed in the culture broth by high-performance liquid chromatography. Supplementation of culture broth with 10 μM of ellagic acid did not change *A. muciniphila* growth while the addition of 0.18 mg/ml and 0.28 mg/ml of POM extract to the culture broth inhibited the growth of *A. muciniphila* significantly. Incubation of *A. muciniphila* with POM extract resulted in formation of ellagic acid and incubation of *A. muciniphila* with ellagic acid demonstrated hydrolysis of ellagic acid to metabolites different from urolithin A. The in vitro culture studies with *A. muciniphila* partially explain our in vivo findings that the presence of *A. muciniphila* was associated with breakdown of ellagic acid for further metabolism by other members of the microbiota. This is the first report of the role of *A. muciniphila* in ellagitannin hydrolysis. However, we conclude that enzymes from other bacteria must be involved in the formation of urolithin A in the human intestine.

1. Introduction

The gut microbiota is an important contributor to human health and has been implicated in the development of obesity and obesity-related diseases such as diabetes [1–3]. A recent study demonstrated a prebiotic effect of pomegranate in high-fat diet induced obese mice, and suggested that the gut microbiota is involved in the management of host metabolism of pomegranate polyphenolic compounds [4]. Most health benefits associated with the consumption of pomegranate have been attributed to the presence of ellagitannins, mainly punicalagins and ellagic acid [5–9]. Although pomegranate ellagitannins are highly bioactive in vitro, they are not absorbed intact into the blood stream. They undergo hydrolysis by stomach acid and intestinal enzymes to yield ellagic acid, which can be absorbed [8]. The unabsorbed ellagitannins and remaining ellagic acid are further metabolized to the urolithins by the microbiota in the large intestine. While the ultimate evidence for prebiotic effects must be obtained in clinical studies, pomegranate polyphenols have been shown to alter bacterial populations in mixed cultures while also being metabolized by the bacteria to smaller metabolites such as urolithin A [10].

Akkermansia muciniphila is a mucin-degrading bacterium that

* Corresponding author. Anaerobic Bacteriology Research Laboratory, Veterans Administration, 11301 Wilshire Blvd., Building 304, rm E3-241, Los Angeles, CA 90073, USA.
E-mail address: carlsonph@aol.com (P.H. Summanen).

http://dx.doi.org/10.1016/j.anaerobe.2016.12.003
1075-9964/

has been described to reside in the mucus layer [11] and contributes to 3–5% of the microbial community in healthy subjects [11,12]. A decreased abundance of *A. muciniphila* has been shown to correlate with increased body weight in mice and humans [13–16]. A recent study also associated *A. muciniphila* with a healthier metabolic status and better clinical outcomes after calorie restriction in overweight and obese adults [17]. Similarly, it was demonstrated that the abundance of *A. muciniphila* decreased in obese and type 2 diabetic mice and that demonstrated that *A. muciniphila* treatment reversed high-fat diet-induced metabolic disorders, including fat-mass gain, metabolic endotoxemia, adipose tissue inflammation, and insulin resistance [18]. The administration of prebiotic oligofructose has been shown to increase the abundance of *A. muciniphila* in obese mice [16,18] and we discovered that prebiotic xylooligosaccharide significantly increased the proportion of *Akkermansia* in healthy humans [19]. Another investigation demonstrated a marked increase in proportion of *Akkermansia* in addition to a reduction in weight gain and increase in insulin resistance after cranberry extract consumption in mice fed high fat/high sucrose diet [20]. These data support the hypothesis that *A. muciniphila* may play an important role in the healthy gut microbiome.

We recently studied the effect of pomegranate extract (POM extract) on the intestinal microbiota utilizing metagenomic 16S rRNA sequencing [21]. In this study, healthy participants consumed 1000 mg of POM extract daily for four weeks. Based on urinary and fecal content of urolithin A, the subjects divided into urolithin A producers (stimulation of urolithin A formation by POM extract consumption) and urolithin A non-producers. A finding of this study was that the genus *Akkermansia* was significantly higher in stool samples of urolithin A producers compared to non-producers. The aim of the present study was to quantitatively study the amount of *A. muciniphila* in the stool samples of subjects before and after POM extract intervention utilizing real-time PCR. We also investigated the utilization of pomegranate polyphenols by *A. muciniphila* in vitro. These studies contribute to the concept that pomegranate polyphenols may have prebiotic activity and contribute to human gut health.

2. Materials and methods

2.1. Phytochemical preparations

The pomegranate extract was from POM Wonderful, Inc., Los Angeles, CA. POM extract was prepared by dissolving 7 mg of POM extract (capsule powder) per ml water and vortex for 10 min. Stock solution was centrifuged for 10 min at 2800 × g and supernatant was filtered (MillexGP, PES membrane filter 0.22 μm, EMD Millipore, Billerica, MA) and frozen in aliquots. The stock solution was diluted in thioglycollate broth (Thioglycollate Broth, Anaerobe Systems) 1:25 for 0.28 mg/ml POM extract and 1:40 for 0.18 mg/ml POM extract. Ellagic acid was obtained from Sigma-Aldrich (E2250 from tree bark, St Louis, MO). A 50 mM stock solution (15.1 mg/ml) of ellagic acid was prepared in dimethylsulfoxide (DMSO) (Sigma-Aldrich). The stock solution was sonicated for 10 min at room temperature and centrifuged for 10 min at 2800 × g. The supernatant was diluted 1:1000 in thioglycollate broth for culture analyses.

2.2. Stool specimens

The specimens were obtained from 18 healthy volunteers who were recruited into a POM extract intervention study as described [21]. There were two study phases including a 2-week run-in period (low-flavonoid diet) and a 4-week intervention period. Subjects were instructed to take a daily dose of 1000 mg of the POM extract, which delivers pomegranate polyphenols in an amount equivalent to one glass (about 8 ounces or 236 ml) of pomegranate juice. Stool specimens were collected before (baseline) and after (4 weeks) of the POM extract consumption. The stool specimens were collected and processed as described [21]. Approximately 1 g of the stool was weighed and dried in a vacuum drying oven (15 in Hg) at 80 °C for 48 h, then weighed again to establish the moisture content so that all counts could be corrected to dry weight. Urolithin A content of the stool specimens was assayed by high-performance liquid chromatography (HPLC) method as previously described [21].

2.3. Bacterial strain

The reference strain of *A. muciniphila* ATCC BAA-835 was obtained from the American Type Culture Collection (ATCC). *A. muciniphila* ATCC BAA-835 was grown on Brucella blood agar plates (Brucella Blood Agar, Anaerobe Systems, Morgan Hill, CA) under anaerobic conditions inside an anaerobic chamber at 37 °C for 48 h [22]. Anaerobic conditions consisted of a gas mixture of 5% CO_2, 5% H_2, and 90% N_2; the residual oxygen was removed by palladium catalysts.

2.4. DNA extraction and real-time PCR

Fecal DNA was extracted using a commercial extraction system (QIAamp® Stool DNA Extraction Kit, Qiagen, Valencia, CA) according to manufacturer's instructions. Two specific primers, AM1 (5′-CAG CAC GTG AAG GTG GGG AC-3′) and AM2 (5′-CCT TGC GGT TGG CTT CAG AT-3′), were used for the detection of *A. muciniphila* species as described by Collado et al. [11]. The primers were obtained from Eurofins MWG Operon (Huntsville, AL). All assays were performed in 96-well MicroAmp optical plates with MicroAmp optical plate seals (Applied Biosystems, Foster City, CA). The amplification reactions were carried out in a total volume of 25 μl, containing 2 μl of DNA sample, 0.3 μl SYBR green (Applied Biosystems), and 0.25 μM each primer. The assays were performed with the ABI 7500 real-time PCR instrument (Applied Biosystems) in a protocol comprised of 2 min at 55 °C and 10 min at 95 °C, followed by 40 cycles of 95 °C for 15 s and finally by 60 °C for 1 min. Assay results were calculated by using the manufacturer's software as signal threshold cycle (C_T) values, where C_T is the PCR cycle number at which fluorescence of the released reporter dye reaches a threshold level. Standard curves were generated by plotting the C_T versus the corresponding serial 10-fold dilutions of template DNA extracted from the reference culture of *A. muciniphila* ATCC BAA-835 as described previously [23]. Briefly, *A. muciniphila* ATCC BAA-835 was grown on Brucella blood agar plates under anaerobic conditions at 37 °C for 48 h. Growth was harvested from these plates in sterile saline using cotton swabs. A quantitative culture of the bacterial suspension was performed by standard 10-fold serial dilution method to determine the cfu/ml. 1 ml aliquot of the suspension was subjected to DNA extraction by using QIAamp DNA Mini Kit. Tenfold serial dilutions of the DNA were prepared. The corresponding cfu was calculated based on plate counts. All determinations were performed in duplicate. All counts were corrected to cfu/g dry weight stool.

2.5. Growth of A. muciniphila in the presence of POM extract and ellagic acid

To investigate the effect of POM extract and ellagic acid on *A. muciniphila* growth in vitro we estimated that potential intestinal concentration could be ~0.25 mg/ml from 1000 mg daily dose,