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Grape proanthocyanidin-induced intestinal bloom of *Akkermansia muciniphila* is dependent on its baseline abundance and precedes activation of host genes related to metabolic health

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Abstract

We previously showed that C57BL/6J mice fed high fat diet (HFD) supplemented with 1% grape polyphenols (GP) for 12 weeks developed a bloom of *Akkermansia muciniphila* with attenuated metabolic syndrome symptoms. Here we investigated early timing of GP-induced effects and the responsible class of grape polyphenols. Mice were fed HFD, low-fat diet (LFD), or formulations supplemented with GP (HFD-GP, LFD-GP) for 14 days. Mice fed HFD-GP, but not LFD-GP, showed improved oral glucose tolerance compared to controls. *A. muciniphila* bloom occurred earlier in mice fed LFD-GP than HFD-GP; however, timing was dependent on baseline *A. muciniphila* levels rather than dietary fat. Mice gavaged for 10 days with GP extract (GPE) or grape proanthocyanidins (PAC), each delivering 360 mg PAC/ kg body weight, induced a bloom of fecal and cecal *A. muciniphila*, the rate of which depended on initial *A. muciniphila* abundance. Grape PAC were sufficient to induce a bloom of *A. muciniphila* independent of specific intestinal

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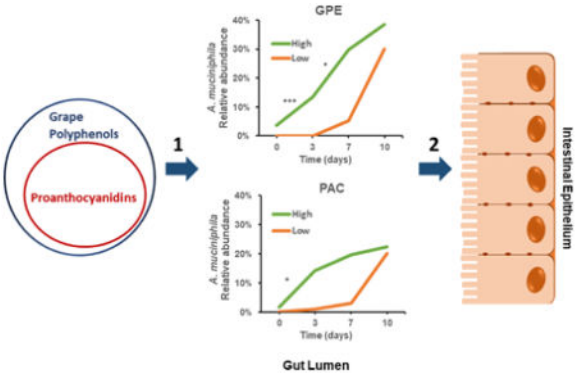
gene expression changes. Gut microbial community analysis and *in vitro* inhibition of *A. muciniphila* by GPE or PAC suggest that the *A. muciniphila* bloom *in vivo* occurs via indirect mechanisms.

Graphical abstract



Keywords

Akkermansia; grape; gut; microbes; polyphenols; proanthocyanidins

1. Introduction

Metabolic syndrome (MetS) defines co-occurrence of at least three of five symptoms (hyperglycemia, insulin resistance, hypertension, dyslipidemia, and central obesity [1]), which develop due to a combination of poor diet, sedentary lifestyle, and genetic predisposition. Underlying MetS is a state of chronic low grade inflammation, possibly associated with an increase in pro-inflammatory gut microbe-derived lipopolysaccharide (LPS), which leaks into systemic circulation due to impaired gut barrier integrity [2]. MetS is a precursor to type-2 diabetes (T2D), which an estimated 10% of adults worldwide will develop by 2040 [3]. Polypharmacological interventions that treat individual symptoms of MetS have not curtailed T2D prevalence, therefore lifestyle and dietary adjustments may be a more effective approach to preserving metabolic health.

Dietary polyphenols present in fruit, vegetables, nuts, teas, and spices, are associated with reduced risk of metabolic and cardiovascular disease [4], despite generally poor absorption into circulation [5-7]. Recent evidence suggests that poorly absorbed fruit polyphenols mediate host systemic effects in association with alterations in gut microbial composition, although cause-effect relationships remain to be established. Compared to high fat diet (HFD) controls, our prior studies showed that mice fed HFD formulated with Concord grape polyphenols (GP) for 12 weeks had increased abundance of the gut Verrucomicrobium *Akkermansia muciniphila* in association with leaner phenotype, less intestinal and systemic inflammation, improved oral glucose tolerance, and intestinal gene expression related to improved gut barrier and metabolic resilience [8]. Similar results were demonstrated in mice fed a high-fat, high sucrose (HFHS) diet supplemented with cranberry extract for 8 weeks

[9] and in mice fed a HFHS diet supplemented with polymeric proanthocyanidins (pentamers and larger) from apple juice for 20 weeks [10].

Major compounds contained in grape berries, cranberries, and apples include flavan-3-ols (catechins and epicatechin), proanthocyanidins (PAC; polymers of flavan-3-ols), anthocyanin pigments, flavonols, and phenolic acids [11, 12]. Mice fed HFD supplemented with a polyphenol-rich red lettuce variety rich in chlorogenic acid, anthocyanins, and flavonols, but undetectable levels of PAC, demonstrated improved oral glucose tolerance after 9 weeks of supplementation, but did not show a bloom in *A. muciniphila* after 12 weeks [13] further suggesting that PAC may be the compounds responsible for the GP-induced bloom. Here we investigated the timing of the GP-induced *A. muciniphila* bloom and identify the polyphenol class sufficient to promote this effect.

2. Materials and Methods

2.1 Diets

Polyphenol-protein complexes allow stable and concentrated delivery of polyphenols in a food-based formulation while permitting release of bioactive polyphenols from the protein matrix [11, 14, 15]. Grape polyphenol-soy protein isolate (GP-SPI) complex containing 10% total polyphenols extracted from frozen grape pomace (Welch Foods Inc, Concord, MA) was prepared as previously described [8, 11]. Mice were fed the following ingredient-matched diet formulations (Research Diets, New Brunswick, NJ): 1) high fat diet containing 10% SPI (HFD); 2) HFD formulated with 10% GP-SPI delivering 1% GP (HFD-GP); 3) low fat diet containing 10% SPI (LFD); and 4) LFD formulated with 10% GP-SPI delivering 1% GP (LFD-GP) (Supplementary Tables 1 & 2).

2.2 Mice

All mice (male C57BL/6J) were purchased from Jackson Laboratory (Bar Harbor, ME) at age 5 weeks and fed *ad libitum* with free access to water in a room with a temperature of 24 ± 1 °C and a 12:12 h light-dark cycle (7 am – 7 pm). Protocols were approved by Rutgers University Institutional Care and Use Committee and followed federal and state laws.

2.2.1. 14-day time course experiments—Two independent studies were performed. **1)** Pair-housed mice were fed LFD for one week and at age 6 weeks switched to receive HFD or HFD-GP (12 mice/ group) for 14 days. **2)** Pair-housed mice (n = 24), purchased several weeks later, were fed LFD for one week then at age 6 weeks evenly split into two groups that received LFD or LFD-GP.

2.2.2. GPE vs. PAC 10-day time course experiment—Purified oligomeric proanthocyanidins from grape seeds (PAC standard; Sigma, catalog no. 1298219) were solubilized in 0.5% aq. ethanol (100 mg/mL) on day of gavage. Grape polyphenol extract (GPE) was prepared from Concord grape pomace (Welch Foods Inc, Concord MA). Individually housed mice (n = 30) were fed LFD *ad libitum*. At age 6 weeks, mice were randomized to three groups (10 mice/ group) and gavaged daily for 10 days with: 1) GPE

delivering 360 mg PAC/kg body wt.; 2) PAC dissolved in 0.5% aq. ethanol delivering 360 mg /kg body wt. or; 3) 0.5% aq. ethanol (100 μL dose).

2.3. *A. muciniphila* in vitro experiments

A. muciniphila (ATCC, BAA-835) was cultured overnight in liquid medium prepared as previously described [16-18]. Diluted cultures were plated on solid agar medium containing increasing concentrations SPE column-purified GPE (GPE^{CP}) or PAC standard delivering equivalent amounts of proanthocyanidins.

2.4. Data deposition

DNA sequences encoding bacterial and archaeal 16S rRNA V4 region reported in this paper have been deposited in the Sequence Read Archive (SRA) under the accession number SRP119480.

Detailed experimental procedures are provided in online supporting materials.

3. Results

3.1. GP supplementation promotes metabolic resilience

Two cohorts of mice were fed either HFD or LFD with and without GP supplementation to investigate short-term effects of GP on host phenotypes and microbial community structure. In each case, mice were fed LFD during a one-week acclimation period and data from baseline oral glucose tolerance tests (OGTT, Fig. 1A-B day 0) were used to assign 6-week old male mice (n = 12 per group) to the control or GP-supplemented group to ensure groups had similar baseline OGT ($p > 0.05$). Compared to the HFD group, mice in HFD-GP group showed significantly better OGT after 14 days (Fig. 1A day 14 blood glucose area under the curve, unpaired, 2-tailed t-test, $p = 0.023$), which is consistent with our previous study where mice fed HFD-GP had improved OGT at 3 weeks [8]. Mice in LFD and LFD-GP groups showed no difference in OGT (Fig. 1B, day 14 blood glucose area under the curve, unpaired t-test, $p = 0.25$). Consistent with improved metabolic status, after 14 days serum insulin was significantly decreased in HFD-GP group compared to HFD group ($p = 00066$), but similar to serum insulin levels in the LFD and LFD-GP groups (T-test, $p > 0.05$; Table 1). With respect to serum LPS, there were no significant differences between HFD and HFD-GP groups ($p = 0.9$) or LFD and LFD-GP groups ($p = 0.2$; Table 1).

Food consumed per day was not significantly different between HFD and HFD-GP groups of mice ($p= 0.10$) or LFD and LFD-GP groups of mice ($p= 0.30$), indicating diets with and without GP had comparable palatability (Table 2). The average daily dose of total polyphenols (TP, as gallic acid equivalents) consumed by mice in the HFD-GP group was not significantly different from that consumed by mice in the LFD-GP group ($p= 0.17$; Table 2). Compared to HFD or LFD controls, GP supplementation did not affect body weight gain over the 14-day period (Supplementary Fig. 1A). Except for fat mass in the LFD-GP group, fat and lean mass significantly increased within all groups from day 0 to day 14 (Supplementary Fig. 1B), as determined by EchoMRI. Mice receiving HFD, but not HFD-GP, had significantly greater adiposity than mice on LF-based diets (Supplementary Fig.

1B). Lean mass was similar among the four diet groups, both before and after 14 days of GP supplementation (Supplementary Fig. 1B).

Diet energy content (kCal/g) was measured by bomb calorimetry to confirm diets were isocaloric; measured values were somewhat higher than the values derived by calculation (Supplementary Tables 2 & 3). Bomb calorimetry of fecal samples collected after 14 days on diets showed that compared to LFD controls, the fecal samples from the LFD-GP group had greater energy content (t-test, $p = 0.012$). As studies were not performed with single housed mice in metabolic cages to allow collection of total feces produced over a fixed time period, it is unclear whether GP can reduce energy absorbed from diet, or affect other factors such as intestinal transit time. HFD and HFD-GP groups showed no differences in fecal energy content (Supplementary Table 3).

3.2. Short-term GP exposure promotes intestinal gene expression changes independent of dietary fat

In our prior study, mice fed HFD-GP for 12 weeks demonstrated several gene expression changes consistent with improved energy metabolism, improved gut barrier integrity, and decreased tissue inflammation compared to HFD-fed animals [8]. In the present study, a subset of previously observed gene expression changes was observed in the jejunum and ileum, but not in colon tissues ($p > 0.05$), after mice were fed HFD-GP for 14 days (Fig. 2A-C). Compared to HFD-fed controls, mice in the HFD-GP group had significantly decreased gene expression of Glut-2 in ileum tissue ($p = 0.0038$; Fig. 2B), but elevated Glut-2 in jejunum tissue ($p = 0.033$; Fig. 2A). Polyphenols are known to inhibit SGLT1 and Glut-2 transporters responsible for glucose absorption [19-21]; elevated jejunal Glut-2 gene expression could be a compensatory mechanism in response to such inhibition. Mice fed HFD-GP diet had lower expression of cytokine IL-6 in jejunum tissues ($p = 0.014$; Fig. 2A), and lower expression of iNOS in ileum tissues ($p = 0.0094$; Fig. 2B), suggesting attenuation of HFD-induced intestinal inflammation. Tight junction proteins ZO-1 (encoded by Tjp1) and occludin control the permeability of the intestinal epithelium [22]. Mice fed HFD-GP showed significant increase in ileal Tjp1 ($p = 0.0009$; Fig. 2B), suggesting improved gut barrier integrity. GP exposure for 14 days did not induce gene expression changes in occludin, GCG, TNFα, or Angptl4/Fiaf ($p > 0.05$; Fig. 2A-C) that were previously observed after 12 weeks of HFD or HFD-GP feeding [8].

Compared to LFD-fed animals, mice fed LFD-GP showed decreased IL-6 expression in jejunum, ileum, and colon tissues (Fig. 2A-C). Mice fed LFD-GP displayed increased ileal gene expression of Angptl4 ($p = 0.016$) and GCG ($p = 0.0003$; Fig. 2B), markers consistent with metabolic health. Angptl4 stimulates fatty acid oxidation and limits fat deposition in peripheral tissue [23] while GCG encodes pre-proglucagon, which is cleaved to glucagon-like peptide-1 (GLP-1), an incretin that promotes insulin production, and GLP-2, which promotes the integrity of the mucosal and intestinal epithelium barrier [24]. The LFD-GP group had lower occludin expression in ileum ($p = 0.02$; Fig. 2B), inconsistent with improved gut barrier, but this result may be due to two individuals with unusually high expression in the LFD group. The LFD-GP group had lower Glut-2 expression in colon ($p = 0.0038$; Fig. 2C).

3.3. GP supplementation rapidly reshapes gut microbial community structure

16S rRNA gene sequencing was performed on fecal samples collected on days 0, 2, 5, 7, 9, and 14 from 6 mice (one cage-mate per cage), along with matched cecal samples. Ceca from mice that consumed HFD-GP or LFD-GP were enlarged and twice the mass of ceca from mice fed LFD or HFD (Figure 2D), while liver weights were similar between all four diet groups (Supplementary Fig. 1C). GP supplementation of HFD or LFD significantly decreased OTU richness (Figure 3A) in fecal samples within 2 days and in cecal samples at endpoint, suggesting the *A. muciniphila* bloom occurs at expense of other taxa. Shannon index is a microbial diversity measure that scales OTUs based on community evenness. Regardless of the GP-mediated decrease in OTU richness, GP supplementation did not change evenness of fecal microbial communities, except for day 14 where Shannon index was higher for HFD-GP group ($p < 0.05$, Figure 3B). Shannon index diversity results suggest that in fecal samples, while GP reduced overall OTU richness, there are also changes in the remaining OTUs that contribute to overall evenness of the community. In contrast, GP supplementation decreased evenness of cecal microbial communities regardless of dietary fat, as indicated by a lower Shannon index ($p < 0.01$, Figure 3B).

Principal coordinate analysis (PCoA) showed that baseline fecal microbial communities of mice, all on LFD, clustered together (Figure 3C, Baseline); however, dissimilarities in microbiotas were detectable between the cohort of mice used for the HFD-based study and the cohort used for the LFD-based study (Supplementary Table 4, ADONIS; overall effects with respect to assigned diet base: $R^2 = 0.15$, $p < 0.01$ and effects of diet base by supplementation status: HFD vs. LFD, $R^2 = 0.285$, $p < 0.01$; HFD-GP vs. LFD-GP, $R^2 = 0.184$, $p < 0.05$). Two days into the intervention period, microbial communities were distinguishable by diet group (significance considered at $p < 0.05$) and they continued to differentiate over the 14-day supplementation period (Fig. 3C and Supplementary Table 4). Over the time course the microbial communities from LFD-GP and HFD-GP groups clustered together on the PCoA plot (Fig. 3C); after 14 days dissimilarities were detectable between fecal microbial communities, but not cecal microbial communities (Supplementary Table 4, ADONIS, day 14 fecal samples LFD-GP vs. HFD-GP: $R^2 = 0.229$, $p < 0.05$; cecal samples LFD-GP vs. HFD-GP: $R = 0.230$, $p = 0.054$).

Relative abundance of bacterial taxa by diet and day of fecal sample collection are illustrated in Fig. 3D and details of significant differences within diet group (i.e. change from baseline) and between group differences (i.e. comparisons at matched time points) are presented in Supplementary Fig 2. Regardless of diet base, GP supplementation increased abundance of the Verrucomicrobia phylum, of which *Akkermansia muciniphila* is the sole member in mice (Fig. 3D and Supplementary Fig. 2). Baseline fecal samples of mice used for the HFD-based experiment exhibited very low relative abundances of *A. muciniphila* (HFD at baseline: 0.003% ± 0.0006%; HFD-GP at baseline: 0.003% ± 0.0005%); however, these increased measurably by day 7-14, particularly in the GP supplemented group (mean ± SEM across days 7-14; HFD: 0.2% ± 0.17%; HFD-GP: 5.7% ± 2.1%). The *A. muciniphila* bloom was most notable in cecal samples (HFD-GP: 22.1% ± 10.32%; HFD: 0.45% ± 0.23%; $p < 0.0001$; Fig. 3B and Supplementary Fig. 2A). Relative abundance of Firmicutes increased in fecal samples collected from HFD (baseline: 52.3% ± 3.5% vs. day 14: 91.1% ± 2.6%, $p <$

0.05) and HFD-GP (baseline: 39.7% ± 5.7% vs. day 14: 69.0% ± 9.9%; p< 0.05) groups; however, GP supplementation significantly suppressed the HFD-induction of Firmicutes in both fecal and cecal samples (Fig. 3B and Supplementary Fig. 2B). Indeed, levels of Firmicutes in fecal samples were indistinguishable ($p > 0.05$) at all time points between HFD-GP and LFD-based treatments, which was not the case for the HFD control group. Levels of Bacteroidetes were suppressed in HFD-fed mice compared to baseline, regardless of GP supplementation (Fig. 3B and Supplementary Fig. 2C). Compared to time-matched HFD samples, the HFD-GP group had increased levels of Proteobacteria in day-2 fecal samples as well as cecal samples and increased levels of Actinobacteria (Fig. 3B and Supplementary Fig. 2C, F).

Starting from day 9 through 14, the average relative abundance of *A. muciniphila* in fecal and cecal samples from the LFD-GP group exceeded 17% (mean ± SEM across days 9-14: 20.2% ± 2.5%), a significant increase compared to baseline levels (3.2% ± 1.9%) and compared to time-matched samples from the LFD group (across days 9-14: 2.3% ± 0.8%; at baseline: 7.4% ± 2.7%) (Fig. 3B and Supplementary Fig. 2A). Increased *A. muciniphila* in the LFD-GP group was accompanied by significant reduction in Bacteroidetes compared to LFD group and significant increase in Proteobacteria compared to baseline (Fig. 3B and Supplementary Fig. 2A-C). Compared to LFD group, relative abundance of Firmicutes was decreased in LFD-GP cecal samples, but similar in fecal samples of LFD and LFD-GP groups (Fig. 3B and Supplementary Fig. 2D).

Independent of dietary fat and as early as 2 days post-treatment, GP supplementation induced dynamic genus level changes, with functional relevance to fiber digestion and gut barrier integrity (Supplementary Fig. 3). Consistent with improved gut barrier function, GP supplementation lowered *Oscillibacter*, which has been correlated with HFD-induced weight gain, decreased transepithelial resistance, and lower ZO-1 gene expression in proximal colon [25]. GP decreased *Clostridium IV*, *Intestinimonas,* and *Acetatifactor* genera, associated with metabolism of fiber and short chain fatty acid (SCFA) production [26-28]; however, this was accompanied by increases in *Blautia* and *Akkermansia*, which also produce SCFAs [29]. GP was associated with decreased *Gemella*, which inhabit mucus and were reported to be enriched in colorectal cancer patients [30] and contribute to salivary dysbiosis in subjects with inflammatory bowel disease [31]. GP correlated with decreased *Romboutsia*, which has been isolated from human colon [32], but functional characteristics remain undetermined. Genera *Enterococcus* and *Streptococcus* include commensal probiotic, lactic acid producing strains in addition to strains associated with antibiotic resistance and disease [33]. Genus *Weissella*, originally classified as members of genus *Lactobacillus*, include strains of probiotic, lactic acid producing bacteria isolated from fermented foods while some strains are associated with infection [34].

C57BL/6J mice (n= 3) were fed LFD-GP for 7 days and then returned to LFD to determine if the GP-induced *A. muciniphila* bloom could be reversed. Relative abundance of *A. muciniphila* significantly increased within 7 days of GP supplementation and was returned to pre-supplementation levels by 21 days after GP removal (Supplementary Fig. 4A). Total bacteria and archaeal 16S rRNA gene counts per gram of feces were similar ($p > 0.05$) at all

time points (Supplementary Fig. 4B) suggesting that GP supplementation does not affect total fecal bacterial and archaeal load.

3.4. Proanthocyanidins promote intestinal bloom of *A. muciniphila* before specific changes in host gene expression

The grape pomace extract (GPE) used as the source of GP is a mixture of several classes of compounds including proanthocyanidins (PACs), catechins, flavonols, anthocyanins, and hydroxycinnamic acids [11], in addition to extractable, soluble fiber [35]. As the most abundant class of polyphenols, we hypothesized that PACs, which are concentrated in seeds and skin, could be sufficient to induce a bloom in *A. muciniphila*. This was bolstered by our previous finding of no *Akkermansia* bloom with a high-polyphenol lettuce variety that did not contain PACs [13], as well as evidence of an *Akkermansia* bloom with cranberry extract rich in PAC [12] and apple PAC [10]. We therefore compared the ability of GPE or a standard of purified oligomeric PACs from grape seeds (PAC standard) to promote the intestinal bloom of *A. muciniphila* in a 10-day dosing study. We also hypothesized that this *A. muciniphila* bloom would occur independently and prior specific host gene expression changes associated with metabolic health.

GPE was prepared as described in methods. Total polyphenols in GPE accounted for 11.2 % of the dry extract weight as determined by Folin-Ciocalteu assay [36]. PACs accounted for 90 % of the total polyphenols in GPE (or 10% of dry weight), as determined by the DMAC method [37]. Anthocyanins contributed 0.23 % of total polyphenols in the GPE, as determined by the pH differential method [38]. Using same colorimetric method, total polyphenols quantified in the PAC standard accounted for 80% of the dry weight, 84% of total polyphenols could be attributed to PAC compounds, and monomeric anthocyanins were not detected. To identify and compare the levels of the most abundant proanthocyanidin compounds in GPE and PAC standard, samples were separated by UPLC followed by high resolution mass spectrometry. Supplementary Figs. 5A-B show the total ion current (TIC) chromatogram and individual chromatograms depicting the relative abundance of ions with mass/charge ratios (m/z) corresponding to the most abundant PAC monomers, oligomers (2 – 5 degrees of polymerization, DP), and their gallate derivatives detected during full scan mode of GPE and PAC samples. For each chromatogram, areas of individual peaks were integrated and summed to give total peak area as an estimate of ion abundance. Based on total peak area, the PAC standard had 2.5 times more monomeric flavan-3-ols (catechin/epicatechin) and 13 – 38 times more proanthocyanidins compared to GPE. Supplementary Fig. 5C illustrates the comparative abundance of catechin/epicatechin monomers and the most abundant type B proanthocyanidins (*i.e.* dimers, trimers, dimer gallates, trimer gallates, tetramers and pentamers) detected in GPE and PAC samples.

After a one-week acclimation period on LFD, six-week old mice were randomly assigned to be gavaged daily for 10 days with GPE (delivering 360 mg total PACs/ kg), PAC standard (360 mg/kg), or vehicle (0.5% ethanol in water). The relative abundance of fecal *A. muciniphila* started increasing as early as day 3 for individual animals treated with GPE or PAC standard; however, *A. muciniphila* was significantly increased for GPE ($p = 0.0005$)

and PAC standard-treated ($p = 0.035$) groups at day 10 in comparison to the control group (Fig. 4A).

There appeared to be marked baseline (day 0) variations in relative abundance of *A. muciniphila,* more easily observed when data are plotted on a log scale as opposed to linear scale, which emphasized differences at later time points (Supplementary Figs. 6A-B). We hypothesized that mice with higher levels of *A. muciniphila* at baseline would develop a GPE- or PAC-induced bloom more rapidly. qPCR analysis of day 0 fecal samples revealed that mice with high (> 0.01%) and low baseline levels of *A. muciniphila* were evenly distributed among control, GPE, and PAC standard groups (Supplementary Table 7). *A. muciniphila* bloom rates for each mouse was calculated for day 0 – 3, day 4 – 7 and day 8 – 10 time periods and within each treatment group the bloom rates were compared between subsets of mice having high and low levels of *A. muciniphila*. Compared to mice with a low relative abundance of *A. muciniphila* at baseline, mice starting with a high relative abundance showed a more rapid GPE- induced *A. muciniphila* bloom (0% per day vs. 3.3 % per day, $p < 0.0079$) and PAC-induced *A. muciniphila* bloom (0.3% per day vs. 4.2% per day, $p < 0.016$) within the first three days (Supplemental Table 7). There was one super-responder that started with a low (0.00005%) relative abundance *A. muciniphila* and increased to 4.9 % by day 3 (Supplemental Table 7). Subsets of mice with low and high baseline levels of *A. muciniphila* within the control group showed no differences in bloom rate (Supplemental Table 7).

Total bacterial load, estimated as total bacteria and archaea 16S rRNA gene counts per gram of fecal sample extracted (Supplementary Fig. 6C), was not significantly different between GPE, PAC and control groups on day 0 or day 7, consistent with our previous data [8]. Non-*A. muciniphila* 16S rRNA gene counts per gram of fecal sample, representing non-*A. muciniphila* bacterial load, was similar between treatment groups on day 0, but significantly decreased ($p = 0.02$) in the GPE group on day 7 (Supplementary Fig. 6C). Cecal weights of PAC-treated mice were significantly higher than that of control-treated mice, while the GPE-treated group showed higher cecal weight than both control and PAC groups (Fig. 4B). Body weights, cumulative food consumption, and liver weights were similar among control and treated groups after 10 days of treatment (Supplementary Fig. 6D-F).

Based on the rapid GP-induced gut microbial changes we hypothesized that the *A. muciniphila* bloom would occur before host gene expression changes. PACs caused greater increase in IL-6 ($p = 0.033$) and TNF ($p = 0.048$), relative to GPE treatment, in jejunum and colon tissues respectively (Supplementary Fig. 7 A & C). This increase in inflammatory mediators may be an effect of the purified PAC standard compared to PAC within a mixture of other GPE compounds including soluble fibers that may have opposing effects. Increased Glut-2 ($p = 0.011$) was detected in ileum tissues of GPE-treated mice relative to the control group (Supplementary Fig. 7 B). Compared to control, ten days of GPE or PAC standard treatments did not induce significant changes in gene expression of iNOS, GCG, Angptl4, Tjp-1, occludin, TNFα, Muc-2, or Muc-3 ($p > 0.05$; Supplementary Fig. 7 A-C). These data indicate that the GPE and PAC standard-induced bloom in *A. muciniphila* precedes, and possibly directly or indirectly promotes, these host gene expression changes associated with metabolic resilience.

3.5. Effect of GPE or PACs on cultured *A. muciniphila*

The observed bloom in *A. muciniphila* could be due to a direct growth-promoting effect of GPE or the PAC standard. Alternatively, *A. muciniphila* growth may be indirectly promoted by GPE- or PAC-mediated suppression of microbes that limit growth of *A. muciniphila*, either through competition for growth substrate or other means, or via cross-feeding activities. To directly evaluate the effect of GPE and PAC on *A. muciniphila* growth, two dilutions of pure culture were plated on agar medium containing increasing concentrations of C18 column-purified GPE^{cp} or PAC, adjusted to pH 7.1. Compared to control, *A. muciniphila* growth was inhibited with concentrations of 0.03 mg – 1.0 mg proanthocyanidins/mL (Fig 5).

4. Discussion

We previously showed that feeding mice HFD supplemented with 1% GP for 12 weeks resulted in a bloom of gut bacterium, *A. muciniphila,* in association with intestinal gene expression changes consistent with metabolic health [8]. Here we characterized the timing of GP effects, and showed that GP can promote an *A. muciniphila* bloom within two weeks (Fig. 3, Supplementary Figs. 2 and 4). Genus level analysis showed that GP supplementation altered the abundance of microbes associated with fermentation of resistant fibers, gut barrier function, and probiotic activity(Supplementary Fig. 3). Furthermore, the GPE- or PAC-induced *A. muciniphila* bloom occurred prior to significant changes in the expression of several host genes (Fig. 4A and Supplementary Fig. 7). The speed of *A. muciniphila* bloom induction was dependent on its baseline levels (Supplementary Fig. 6A-B and Supplementary Table 7). Mice were sourced from the same vendor and came from the same breeding facility (Jax East); however, different cohorts of animals had different starting levels of *A. muciniphila* (Fig. 3B) and, furthermore, animals received within a single shipment also showed vastly different baseline levels of *A. muciniphila* (Supplementary Figs. 6A-B and Supplementary Table 7) ranging from 0.00005% - 6.19% relative abundance. While such variations may be better controlled by using litter mates, achieving adequate numbers of mice of same gender and age for all groups is not feasible as mice generally have a litter size of 8-10 pups (4-5 males/litter) therefore pups are typically pooled at age 21 days to fulfill orders (personal communication). Such differences in baseline *A. muciniphila* abundance, in addition to plant source and extraction methods, may explain why the bloom is not consistently observed in other recent studies of grape pomace extract [39].

GP supplementation of HFD for 14 days induced major changes in gut microbial community structure; however, changes in host gene expression consistent with improved metabolic status observed over this period were much less pronounced than changes observed in our previous 12-week study [7]. Limited changes in the intestinal gene expression were seen after 14 days of GP supplementation of HFD or LFD (Figs. 2A-C). Another study reported increased gene expression of Muc2 in mice administered grape seed extract in drinking water for 12 weeks [40], which we have not observed in our studies. Relative timing of GP effect on gut microbiota and molecular markers of gut health, suggests that the bloom in *A. muciniphila* precedes and possibly promotes beneficial changes in the gut.

Dietary intake of polyphenols in the human diet is estimated at greater than 2 g per day from food and supplements [41] and total PAC intake from all food sources was recently estimated to be 68 – 192 mg/day [42] or 1.0 mg/kg/day – 2.9 mg/kg/day for human males assuming average weight of 75 kg. Mice in our studies ingested an estimated 500 mg PAC/kg body weight/day and using 12.3 as species conversion factor [43] this murine dose converts to a human dose of 40 .6 mg/kg/day, or 14 - 40 times the estimated range for human intake. The present dose was chosen to remain consistent with our previous study [8], but future work will determine whether lower doses of PAC show similar gut microbiota and host effects. In a recent clinical study where subjects consumed sweetened dried cranberries daily for 2 weeks there was a trend towards increased fecal *Akkermansia* indicating that effects in mice relate to human subjects [44].

Biochemical characterization by LC-MS confirmed that the GPE used in this study contained the broad range of polyphenols previously reported [11]. In contrast, the PAC standard (derived from grape seeds) was purified to contain mainly catechin/epicatechin monomers and PAC oligomers (dimers to pentamers) whose relative quantities decreased with increasing degrees of polymerization (Supplementary Fig. 5C). The GPE used for oral administration to the mice (10 % proanthocyanidins relative to dry weight) was prepared the same way as for production of GP-SPI ingredient, to approximate the range and levels of polyphenols incorporated in HFD-GP and LFD-GP. In contrast, for testing growth of *A. muciniphila* in vitro, the GPE was further subject to SPE column-purification to obtain GPE^{cp}, enriched for PAC compounds (50 % PAC relative to dry weight). Standard phytochemical methods were used to standardize the levels of PAC delivered in GPE or GPE^{cp} vs. the PAC standard; however, due to the complex chemistries of these mixtures the levels of individual PAC are never equimolar. While PAC were identified as sufficient to induce the *A. muciniphila* bloom, there may be other grape-derived compounds that contribute to the effect as levels of *A. muciniphila* achieved with PAC were somewhat lower than those achieved with GPE, but still significant compared to control. In addition to polyphenols, soluble fibers such as pectins, inulin, gums, and water-soluble hemicellulose (e.g. arabinoxylan) can be extracted from grape pomace [35]. Soluble fibers in GPE could therefore explain the trend of its greater activity with respect to the *A. muciniphila* bloom in comparison to purified PAC (Fig. 4A).

A recent study of grape seed polyphenol extract (GSPE) gavaged to mice over 7 weeks showed no change in *A. muciniphila*; however, compositional analysis to confirm presence and levels of PACs in this GSPE test material was not reported [45]. In a study where mice were fed HFHS diet in conjunction with oligomeric PAC (OP, monomer - tetramer) or polymeric PAC (PP, pentamer to undecamer and larger) for 20 weeks, only mice supplemented with PP showed significant increase in *A. muciniphila* [10]. It is unclear why oligomeric PAC from grape and apple showed different effects on *A. muciniphila* in mice. Nevertheless, the present demonstration of the powerful prebiotic effects of dietary PAC provides an important mechanistic clue as to how these poorly bioavailable antioxidant compounds abundant in fruits and berries may benefit human health, as demonstrated in several epidemiological studies [4, 46-49].

A. muciniphila levels inversely correlated with onset of inflammation and insulin resistance in HFD-fed mice [50], while administration of live *A. muciniphila* to HFD-fed mice attenuated symptoms of metabolic syndrome [51]. It was recently reported that administration of pasteurized, non-replicative *A. muciniphila* enhanced the metabolic benefits of the bacterium in HFD-fed mice [17, 52]. Amuc_1100*, a thermostable outer-membrane protein of *A. muciniphila,* was demonstrated to partially recapitulate the beneficial effects *A. muciniphila* in HFD-fed mice through interaction with host toll-like receptor 2 (TLR2) [17, 52]. These results help to explain how the *A. muciniphila* bloom may produce beneficial metabolic outcomes, while our data link dietary polyphenols, such as grape PACs, to the *A. muciniphila* bloom. Although compositionally defined LFD has a fat content similar to murine chow, LFD promotes metabolic stress as it is formulated with cellulose rather than soluble fibers, which undergo fermentation by gut bacteria resulting in production of bacterial metabolites (e.g. short chain fatty acids) that decrease intestinal inflammation and support intestinal health [53]. Compared to mice fed LFD, mice fed LFD-GP had lower expression of inflammatory IL-6 in all intestinal segments, indicating that GP can attenuate LFD-induced metabolic stress (Figure 2).

A. muciniphila metabolizes mucin and has been localized to the loose outer layer of mucus, suggesting a separate bacterial niche. Mucus, however, is continually and rapidly shed into the intestinal lumen therefore bacteria associated with the outer mucus layer, such as *A. muciniphila*, will ultimately end up among the luminal bacterial population and must compete for resources in the lumen. A recent study that compared microbial communities in mucus layer and luminal content from caecum and colon of stable defined medium density microbiota (sDMDM) gnotobiotic mice found that the microbial community of the outer mucus layer differed in composition from the lumen; however, all constituents of isobiotic microbiota were present at some level in both compartments [54]. Furthermore, *Akkermansia* abundances were similar in cecal content vs. cecal mucus as well as in colon content vs. colon mucus [54]. In vitro growth of *A. muciniphila* was inhibited by GPE^{cp} or PAC standard delivering at least 0.03 mg/mL of PAC (Fig. 5A-B) suggesting that PAC do not directly induce *A. muciniphila* bloom.

There are several plausible explanations for the *A. muciniphila* bloom that warrant further investigation. PAC as well as other GPE compounds may confer a selective growth advantage to *A. muciniphila* by suppressing competitor microbes within the gut microbial community. GP-supplementation rapidly decreased OTU richness (Fig. 3A) and GPE administration decreased non-*A. muciniphila* 16S rRNA gene counts/g feces (Supplementary Fig. 6C), but further work is needed to understand how specific alterations may promote the *A. muciniphila* bloom. Alternatively, cross-feeding activities among the community may favor growth of *A. muciniphila*. Powerful antioxidant activity of PAC may give *A. muciniphila* an ecological advantage over more oxygen-tolerant bacteria in the gut. PAC-induced change in expression of host genes other than those investigated in the study may also play a role. Understanding how PAC can influence the gut microbiota and intestinal milieu represents a novel approach to treating and preventing metabolic disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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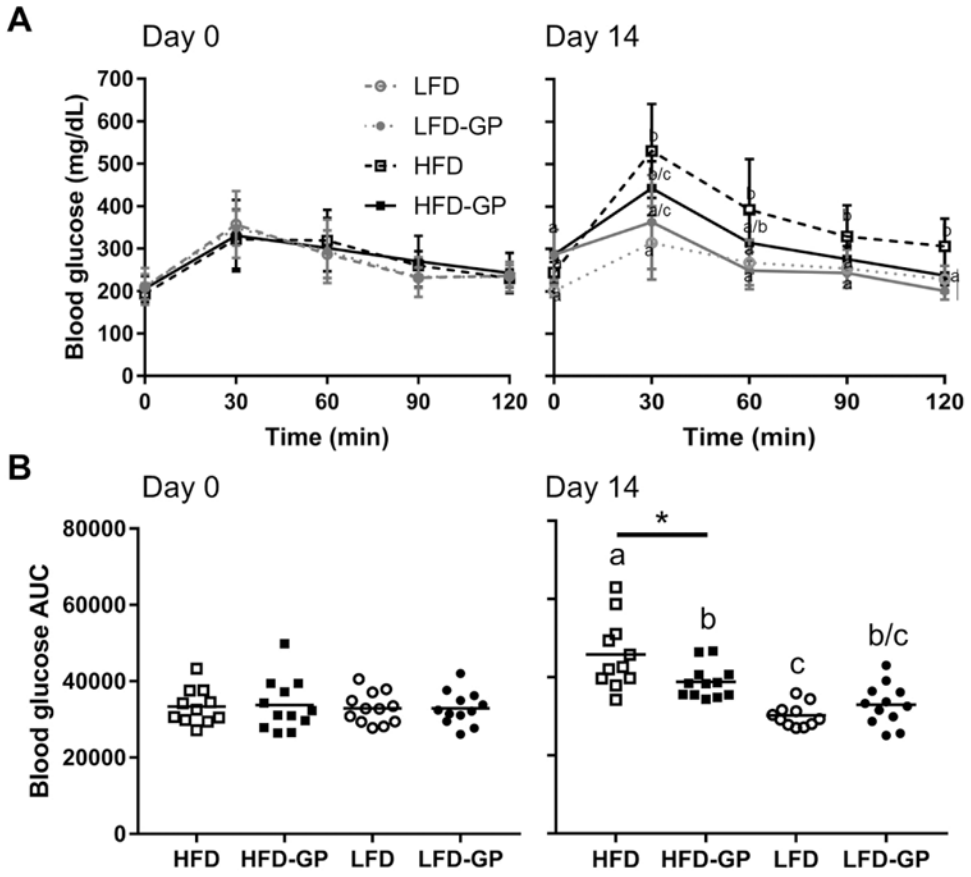

Figure 1. Effect of 14 days of GP supplementation on oral glucose tolerance

A Oral glucose tolerance tests were performed at baseline (day 0) and after 14 days on diets. Blood glucose concentrations (mg/dL) expressed as mean ± SD (n= 11 – 12 mice per group) were measured at the indicated time points (0-120 min) following oral administration of 2 g/kg glucose. **B.** Area under the curve (AUC) representation of data in A. was determined for individual mice, horizontal bar represents mean for each group. Between-group difference by diet base was determined by unpaired t-test (* = $p < 0.05$), while difference across all four groups was determined by one-way ANOVA followed by Tukey's multiple comparisons test. Different letters indicate significant difference between groups ($p < 0.05$) and the same letter indicates no difference.

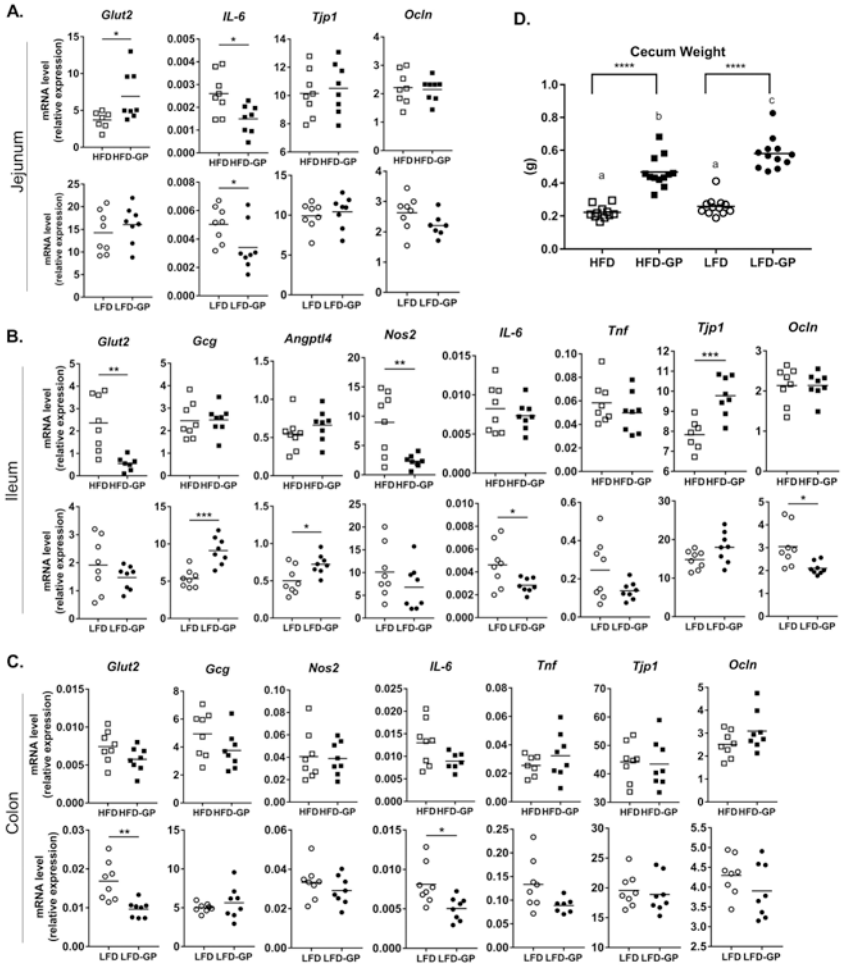

Figure 2. Effect of 14 days of GP supplementation on intestinal gene expression and cecal weights

Relative mRNA levels of selected genes expressed in **A.** jejunum, **B.** ileum, and **C.** colon tissues was determined by qPCR. Target mRNA was normalized to HMBS as endogenous control and data were analyzed according to the $2^{-\Delta CT}$ method. Data are mean ± SD (n=8 samples per group) and the average of technical duplicates are used for each sample. Between-group difference was determined by unpaired, 2-tailed t-test: *p<0.05; **p<0.01; ***p<0.001. **D.** Cecal weights (g) of individual mice. Significant difference between diet groups in panels A - C is signified by letters a, b, or c; different letters indicate significant difference (p<0.05) between groups and the same letter indicates no difference. **** = p < 0.001.

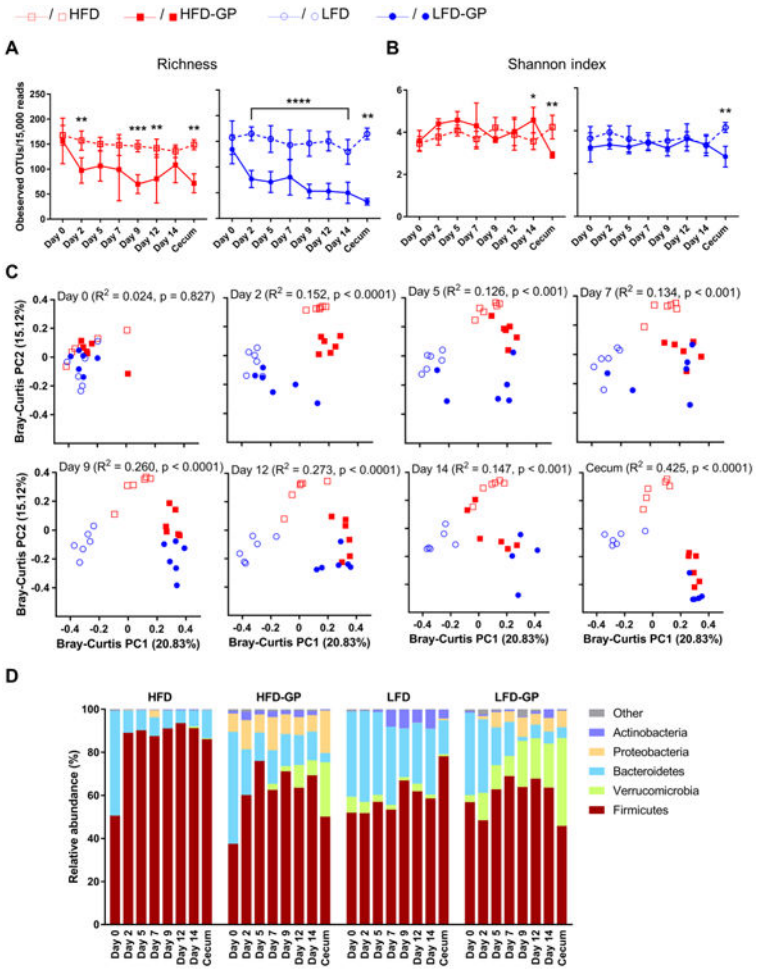

Figure 3. GP altered microbial composition in both HFD and LFD conditions. A-B

Microbiota α diversity as calculated by (**A**) OTU richness and (**B**) Shannon index of fecal and cecal samples by diet and day of study. Asterisks represent significant differences between GP-supplemented groups and control groups determined by repeated measurement two-way ANOVA followed by Sidak's multiple comparisons tests (for fecal samples from Day 0-Day 14) or Mann-Whitney tests (for cecal samples from Day 14), *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. **C.** Bray-Curtis principal coordinate plots of gut microbial communities by day of study. ADONIS tests were performed to assess differential clustering caused by GP supplementation and diet base. R^2 and p values representing the percentages of overall variation explained by GP supplementation and corresponding significance are listed, while the effect of diet base and other analyses by diet base/ supplementation can be found in Supplementary Table 4. **D.** Relative abundance of the five dominant bacterial phyla. Low-abundance phyla (< 0.3%) were combined into the Other category. In panels A-C, analyses were performed on 15,000 sequences per sample, while non-rarefied data was used in panel D. n = 6 mice per group.

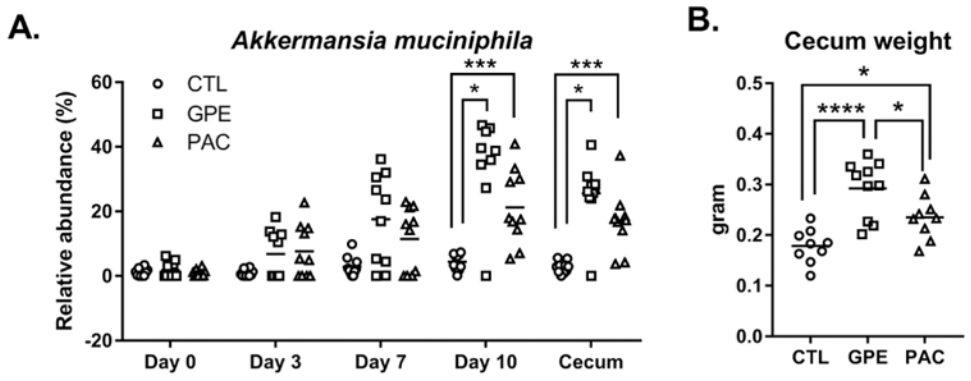

Figure 4. Comparison of PAC vs. GPE treatment on *A. muciniphila* bloom and cecal weight

A qPCR quantification of *A. muciniphila* relative to total bacteria in fecal samples collected on days 0, 3, 7, and 10 during oral gavage with PAC, GPE, or vehicle as well as cecal content after euthanasia on day 10. Kruskal-Wallis test was used to detect differences between groups followed by pair wise comparisons using Dunn's multiple comparison test to detect differences between vehicle vs. GPE and vehicle vs. PAC groups. **B.** Comparison of cecal weights (g) of individual mice in each group. One-way ANOVA was performed to evaluate differences between the 3 groups followed by Tukey post-hoc test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

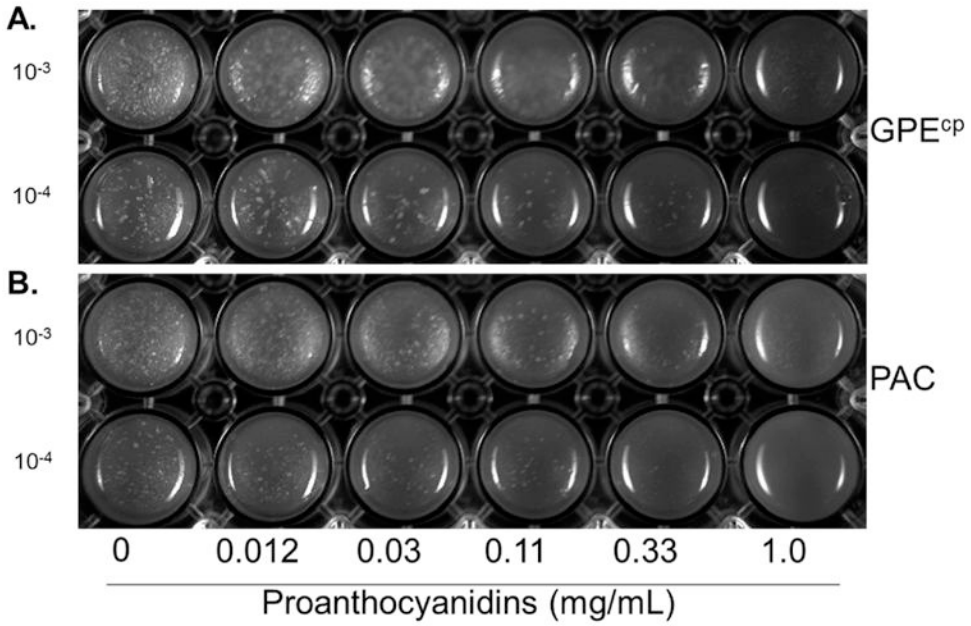

Figure 5. Grape polyphenols and proanthocyanidins inhibit the *in vitro* growth of *A. muciniphila*

GPE^{cp} and PAC standard were adjusted to pH 7.1, dilutions were prepared and added to molten agar to achieve 1.0, 0.33, 0.11, 0.03, or 0.012 mg of proanthocyanidin (B2 equivalents) per mL medium. *A. muciniphila* culture (OD = 0.875) was diluted 10^{-3} and 10^{-4} and 30 μL aliquots were spread on the surface of solid medium impregnated with **A.** GPE^{cp} or **B.** PAC standard. Photograph (representative of two independent experiments) shows 24-well plates incubated under anaerobic conditions at 37°C after 3 days.

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Table 1

Serum Biochemistry

	HFD	HFD-GP	LFD	LFD-GP
Insulin (pg/mL)	1884 ± 434	1207 ± 378 ***	1423 ± 862	1198 ± 873
LPS (ng/mL)	0.51 ± 0.26	0.74 ± 0.49	0.56 ± 0.27	0.58 ± 0.24

T-test, unpaired, two tailed:

*** $p = 0.00066$

Table 2

Food and Total Polyphenols Consumed

	HFD (n= 11)	HFD-GP (n= 12)	LFD (n= 12)	LFD-GP (n= 12)
Food consumed (g/day/mouse)	2.6 ± 0.5	3.2 ± 0.3	2.8 ± 0.2	2.9 ± 0.1
Total Polyphenols consumed (mg/day/mouse)	-	32.5 ± 3.1	-	29.3 ± 1.2

Unpaired, 2-tailed t-test performed on HFD or LFD control vs. supplemented groups Mice housed two per cage, six cages per group