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Dietary prophage inducers and antimicrobials: toward landscaping the human gut microbiome

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ABSTRACT

The approximately 10^{11} viruses and microbial cells per gram of fecal matter (dry weight) in the large intestine are important to human health. The responses of three common gut bacteria species, and one opportunistic pathogen, to 117 commonly consumed foods, chemical additives, and plant extracts were tested. Many compounds, including *Stevia rebaudiana* and bee propolis extracts, exhibited species-specific growth inhibition by prophage induction. Overall, these results show that various foods may change the abundances of gut bacteria by modulating temperate phage and suggests a novel path for landscaping the human gut microbiome.

Introduction

The human adult gut microbiome is numerically dominated by viruses, particularly the bacteriophages (a.k.a., phages), and bacteria belonging to the Bacteroidetes and Firmicutes phyla.[1–4] The relative abundances of particular gut bacteria strains and species are regulated through bottom-up (e.g., food) and top-down mechanisms like phage predation.[5,6] Consumption of foods such as cocoa,[7] tea, wine, soy, and plant polyphenols;[8] nondigestible carbohydrate substrates such as resistant starches, plant cell wall material, and oligosaccharides;[9] specific fibers such as inulin,[10] and hemicellulose such as arabinoxylan,[11] have significant effects on the relative abundances of microbes in the gut and the metabolites they produce.

In the lumen of the gut, there is approximately 1 virus-like particle (VLP) for every bacterial cell.[12] Most of these VLPs are dsDNA phage. These phages play a protective role by adhering to mucus that lines the intestinal epithelium.[13,14] Most phages in the human gut are temperate and integrated into the genomes of their bacterial hosts as prophage.[4] In turn, prophages are induced by certain foods and chemicals, including soy sauce,[15] nicotine, other cigarette chemicals,[16] sunscreen,[17] and antibiotics such as ciprofloxacin.[18]

Prophage induction in gut bacteria may result in the horizontal transfer of genes to other bacterial strains or species. For example, prophages that encode Shiga exotoxin are induced by prophylactic antibiotics, like carbadox, and are known to move these exotoxins between *E. coli* strains in cattle.[19] Prophage induction can also alter the relative abundances of bacteria species/strains. This can lead to dysbiosis if pathogens fill the niche generated by lysed commensals. For example, benzo[*a*]pyrene-diol-epoxide in cigarette smoke induces multiple prophages that lyse beneficial lactobacilli, thereby enabling anaerobic pathogens to persist and cause bacterial vaginosis.[16] Different foods and diet types are associated with varying microbiome compositions[20] and may shift

gut community composition.[3,21,22] Thus, there is strong evidence that many common food and/or environmental compounds alter gut community composition, sometimes through prophage activation.

To identify additional compounds that induce prophages and/or affect gut community composition, ingestible compounds were tested for their effects on growth of human-associated Bacteroidetes (*Bacteroides thetaiotaomicron* strain VPI-5482), two Firmicutes (*Enterococcus faecalis* and *Staphylococcus aureus*), as well as the opportunistic pathogen *Pseudomonas aeruginosa* (Phylum Proteobacteria). To determine whether antimicrobial effects were due to prophage induction, compounds that inhibited bacterial growth were assayed for their ability to induce VLPs. Numerous compounds exhibited antimicrobial activity and several were identified as novel prophage-induction agents. Most of the compounds displayed differential activity on the test bacterial species, introducing the possibility of using diet to intentionally landscape the human gut microbiome via prophage induction.

Results

Effects of compounds on the growth of gut bacteria

B. thetaiotaomicron, E. faecalis, and *S. aureus* were tested for their growth in the presence of 117 consumable compounds (Supplementary Table S1). These taxa are common in human gut metagenomes, being detected in 84-94% of the 2,229 human gut metagenome datasets. The average relative abundances of *B. thetaiotaomicron, E. faecalis*, and *S. aureus* in these datasets were 8.04%, 0.55%, and 0.32%, respectively. Although not a typical member of the gut microbiota, *P. aeruginosa* was also screened because it is a common, opportunistic pathogen.

To identify compounds with antimicrobial properties, bacterial growth in the presence of each tested compound was compared to growth without any added compound (growth curves are shown in Supplementary Figure S3). Clustering of the growth curves identified general categories of antimicrobial activity (Figure 1). The compounds with the strongest and most widespread bactericidal effects were Tabasco (TAB), glycolic acid (GLY), N-acetyl-cysteine (NAC), citric acid (CIT), toothpaste (TOO), and vinegar

(VIN). Cinnamon (CIN), neem (NEE), licorice (LIC), Fernet (FER), and clove (CLO) were antibacterial in most cases. Aspartame (ASP), coffee Arabica (COFa), stevia (STE), trigonelline (TRI), and uva ursi (UVA) were antibacterial in some cases. Cottage cheese (COT), cayenne (CAY), miso (MIS), fish sauce (FIS), and reuterin (REU) had potentially prebiotic effects. See Supplementary Table S2 for a complete list of compounds and their effects.

Prophage induction

Bioinformatics analyses predicted functional prophages in all four bacterial species (Figure 2 & Supplementary Table S3), opening the possibility that some of the bactericidal effects might be due to prophage induction. Flow cytometry was used to count VLPs when bacteria were incubated with antimicrobial compounds. Because lysogens produce some phage spontaneously, the VLPs in the treatments were compared to controls without the treatment compound. Compounds were chosen for flow cytometry analysis based on the following criteria: (1) growth curves that suggested a decline in logarithmic growth of at least 1.19 (peak to trough ratio) that might indicate prophage induction, and 2) growth curves where the shifted area under the curve ($auc_{shifted}$) was less than the control, but not so low as to indicate the majority of bacteria were killed (see Supplementary Figure S5b). These criteria identified 28 compounds for counting VLPs via flow cytometry (Table 1). *P. aeruginosa* was excluded because its mucoid polysaccharides prevent efficient VLP counting in the flow cytometer.

Of the 28 compounds tested with flow cytometry, eleven induced VLPs to levels higher than the no-compound control in at least one species (Figure 3). For *B. thetaiotaomicron*, the strongest inductor was stevia, which displayed a 410% increase in VLPs in the treatment versus the control. Clove and propolis increased the number of VLPs by +185% and +115%, respectively, in *B. thetaiotaomicron*. The highest inducers for *E. faecalis* were uva ursi (+842%), propolis (+695%), and aspartame (+579%). For *S. aureus*, stevia (+321%), grapefruit seed extract (+103%), and toothpaste (+79%) were the strongest inducers (Figure 3).

Several compounds reduced the number of VLPs relative to the control. Rhubarb (−62%),
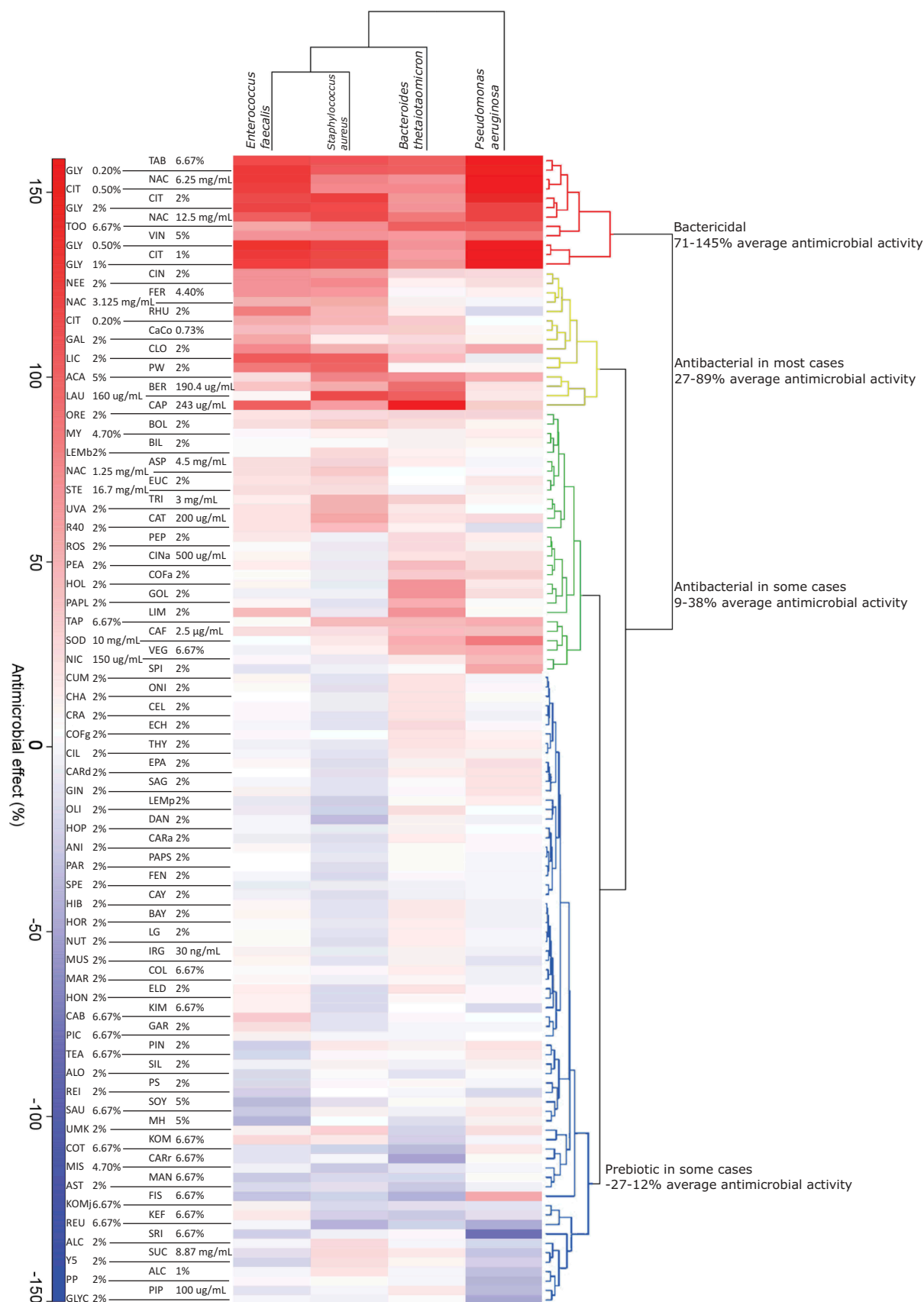
Figure 1. Heat map of clustering based on the antimicrobial effect of each substrate, calculated as described in the Materials and Methods. Percent change in growth is indicated by color: red indicates less growth and blue indicates more growth compared to negative controls. Clusters are classified as bactericidal (red), antibacterial in most cases (yellow), antibacterial in some cases (green), and sometimes prebiotic (blue). Prebiotic compounds can only be digested and utilized by Bacteria, thereby facilitating their growth.
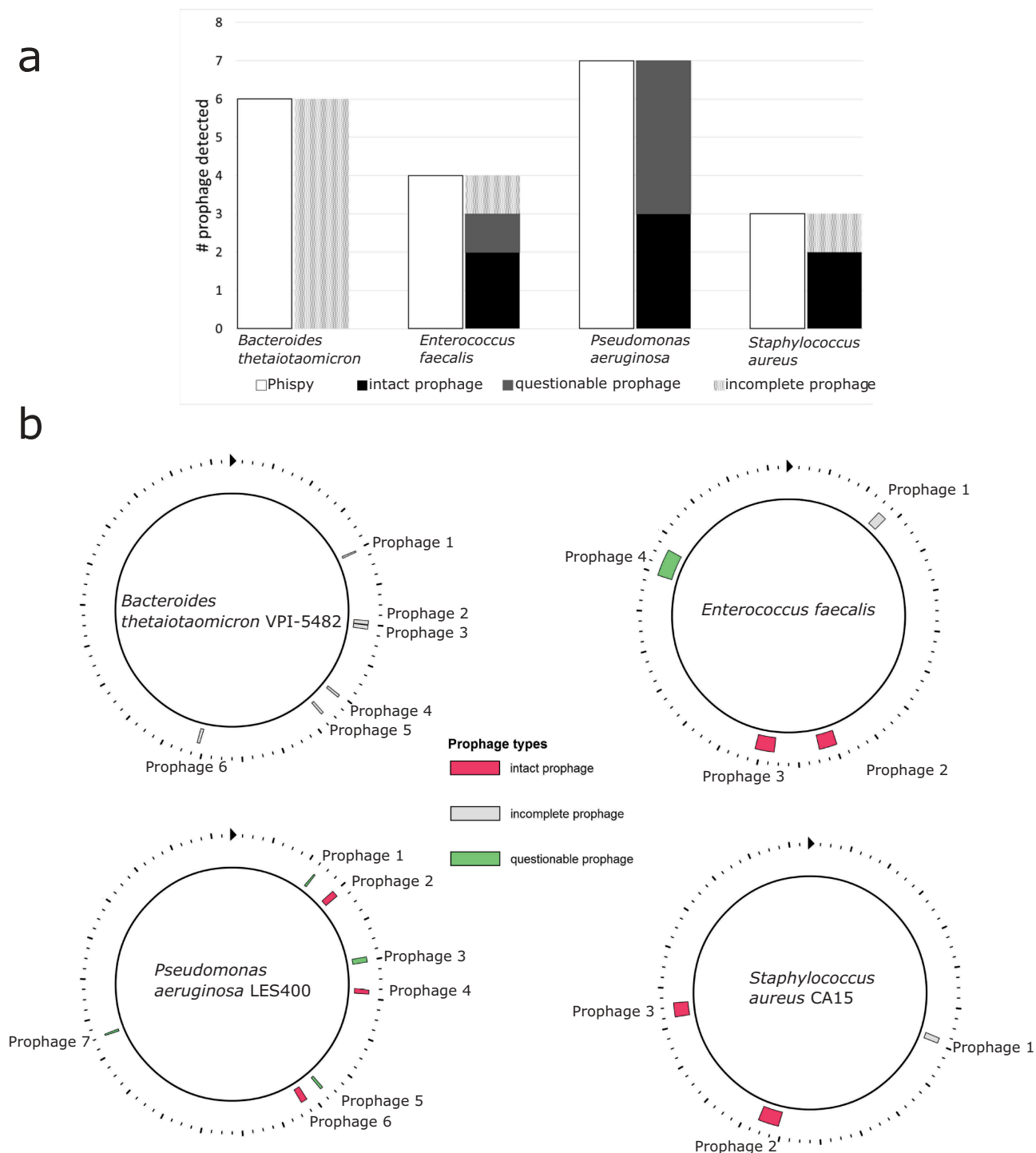
Figure 2. (a) A comparison of the number of putative prophages found by Phispy and PHAST. (B) Putative prophage in bacterial genomes calculated with PHAST. Each prophage's length, genomic position, and most closely related phage are listed in Supplementary Table S3.

Fernet (57%), coffee Arabica (−49%), and oregano (−44%) reduced the number of VLPs in all bacterial species. Several other compounds suppressed VLPs in individual species. Pomegranate (−89%), grapefruit seed extract (−89%), toothpaste (−88%), and cinnamon (−88%) reduced VLP production in B. thetaiotaomicron. Kombucha reduced E. faecalis VLPs by 44%.

Uva ursi increased VLP production in E. faecalis by +842%, while markedly reducing VLP production in B. thetaiotaomicron (−83%) and S. aureus (−68%; Figure 3).

Table 1. Reagents and concentrations chosen for flow cytometry testing based on the growth curve and heat map results. Concentrations were conserved across species where possible but sometimes increased to improve prophage induction or decreased to reduce cytotoxicity. The value next to each concentration is the ratio of the maximum OD of the growth curve to the lowest OD measured after this peak. If the concentration was adjusted for flow cytometry, the initial concentration is provided. Ratios above the 1.19 cutoff are bolded.

Compound	[Optimal] BT	Ratio	[Optimal] EF	Ratio	[Optimal] SA	Ratio
ASP (aspartame)	4.5 mg/mL	1.13	4.5 mg/mL	1.01	4.5 mg/mL	**1.23**
BER (berberine)	190.4 ug/mL	**1.20**	190.4 ug/mL	1.12	190.4 ug/mL	1.02
CAB (cabbage)	6.67%	1.01	6.67%	**1.72**	6.67%	1.12
CAF (caffeine)	2.5 mg/mL	**1.25**	2.5 mg/mL	1.03	2.5 mg/mL	**1.19**
CAP (capsaicin)	243 ug/mL	NA	243 ug/mL	**1.42**	243 ug/mL	1.12
CAT (catechins)	400 ug/mL	**1.19 @ 200 ug/mL**	200 ug/mL	1.03	200 ug/mL	NA
CIN (cinnamon)	2%	1.10	2%	**1.72**	2%	NA
CLO (clove)	2%	1.08	2%	**1.32**	2%	**1.30**
COFa (coffee arabica)	4%	1.12 @ 2%	4%	NA @ 2%	4%	1.08 @ 2%
EUG (egenol)	0.006%	**1.35 @ 0.05%**	0.10%	NA @ 0.05%	0.009%	NA @ 0.005%
FER (Fernet)	4.40%	1.01	4.40%	**2.00**	4.40%	**1.33**
GAL (Galangal)	2%	1.11	2%	**1.73**	2%	**1.50**
GRA (grapefruit seed extract)	0.10%	1.09	0.10%	**1.39**	0.10%	NA
KOM (kombucha)	13.30%	1.051 @ 6.67%	13.30%	**1.638 @ 6.67%**	13.30%	**1.38**
LAU (lauric acid)	40 ug/mL	**1.43**	160 ug/mL	NA	40 ug/mL	**1.32**
LIC (licorice)	1%	NA @ 2%	1%	NA	0.50%	NA
NAC (N-acetyl-cysteine)	3.13 mg/mL	**1.27**	3.13 mg/mL	**1.32**	3.13 mg/mL	**1.27**
NEE (neem)	2%	1.11	2%	**1.68**	2%	1.15
ORE (oregano)	2%	1.09	2%	1.11	2%	**1.62**
POM (pomegranate)	6.67%	NA	6.67%	**1.66**	6.67%	**1.88**
PROg (propolis)	1%	NA	1%	NA	1%	NA
PW (pine)	1%	1.166 @ 2%	1%	1.01	1%	**1.244 @ 2%**
RHU (rhubarb)	2%	**1.22**	2%	**1.40**	2%	1.15
STE (stevia)	33.3 mg/mL	1.19 @ 16.7 mg/mL	16.7 mg/mL	**1.30**	33.3 mg/mL	NA
TAB (Tabasco)	0.50%	1.15	1%	**1.20 @ 0.67%**	1%	1.04 @ 0.67%
TOO (toothpaste)	1%	1.08 @ 0.83%	1%	1.10 @ 6.67%	1%	1.10 @ 0.67%
TRI (trigonelline)	3 mg/mL	**1.34**	3 mg/mL	1.08	3 mg/mL	1.08
UVA (uva ursi)	4%	1.10 @ 2%	2%	**1.29**	2%	**1.57**
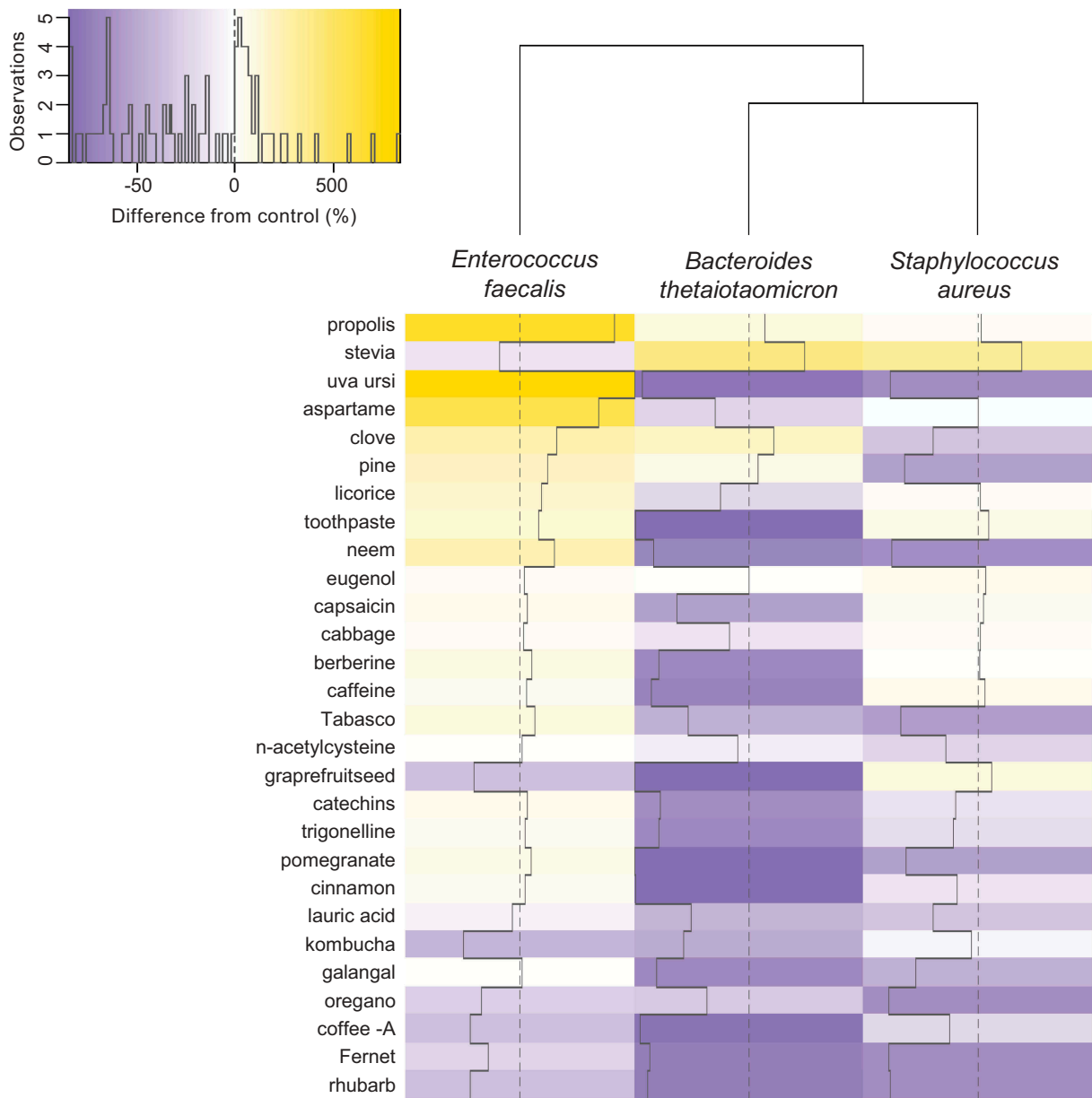
Figure 3. Induction of virus-like-particles (VLPs) by experimental compounds as detected by flow cytometry. VLPs were induced in *B. thetaiotaomicron*, *E. faecalis*, and *S. aureus* separately incubated with 28 compounds and the percent difference in abundances of VLPs were calculated relative to controls that lacked addition of any compound. Solid lines within cells represent deviations from 0 difference (dotted vertical line through columns).

Discussion

Foods, herb and dietary supplements are potentially powerful tools to manipulate the relative abundances of gut bacteria. Most recent studies of diet and gut community composition compared prebiotic diets such as high plant polysaccharide vs. high fat/high sugar, [11] or high-fat/low fiber vs. low-fat/high fiber.[3] Our research complements these studies by identifying reductive modulators of bacterial abundances that may act by inducing prophages.

Hot sauces

Tabasco sauce at a final concentration of 3.3% (v/v) reduced the growth of all three GI species, except the opportunistic pathogen *P. aeruginosa*, by an average of 92% (Supplementary Table S2). Tabasco's antimicrobial activity was not solely due to the vinegar, as the vinegar test reduced bacterial growth in all species by an average of 71%. The additional bactericidal activity may be capsaicin[23] – a known antimicrobial in hot peppers. Capsaicin had antibacterial effects in

our experiments at a concentration of 486 µg ml^{-1}. Since this concentration is much higher than that found in Tabasco, the vinegar and capsaicin may be synergistically interacting to decrease bacterial growth. VLPs were not detected when any species was incubated with Tabasco, vinegar, or capsaicin, so these antimicrobial effects were not due to prophage induction. Kurita *et al.* found that genes contributing to DNA repair mechanisms were not induced by capsaicin, and that antimicrobial activity was due to membrane damage and osmotic stress.[24]

Tapatío, a hot sauce similar to Tabasco, reduced bacterial growth by an average of 37% at a final concentration of 6.7% (v/v). Conversely, Sriracha sauce, which contains very similar ingredients as the Tapatío and Tabasco increased growth at a concentration of 6.7% (v/v). This is probably due to the sugar in the Sriracha. The overall antimicrobial effect of Tabasco suggests that an ingredient or a combination of ingredients make it a potent modulator of gut community structure. The consumption of Tabasco and other hot sauces could potentially have similar effects as low dose antibiotics,[25,26] due to their powerful antimicrobial properties and high frequency of consumption in some populations.

Hot sauces carry eukaryotic viruses like pepper mild-mottled virus (PMMV), which was the primary RNA virus found in human fecal samples.[27] PMMV was found in 4 of 7 chili sauces, indicating that some viruses survived food production processes.[27] However, in a flow cytometry analysis of Tabasco diluted in BHIS media, there were no detectable viruses (Supplementary Figure S4).

N-acetyl-cysteine (NAC)

NAC had a strong antimicrobial effect in all species. NAC is a synthetic amino-acid supplement that is converted to glutathione in the body.[28] NAC is used as a supplement to treat acetaminophen toxicity[28] and Parkinson's disease,[29] to reduce influenza symptoms and viral replication,[30] and as a mucolytic agent for cystic fibrosis and chronic obstructive pulmonary disease patients.[25] NAC is also used to treat *Helicobacter pylori* infection in the stomach,[26] which implies that NAC may affect the gut microbiome despite stomach acids. NAC is bacteriostatic and inhibitory against biofilm formation in *P. aeruginosa*[31] and *E. faecalis*.[32] In the case of *E. faecalis*, NAC inhibits infections and biofilm formation following root canal treatment. NAC is thought to be antimicrobial due in part to the effect of its sulfhydryl groups reacting with essential proteins.[33] This is consistent with the absence of prophage induction by NAC despite its strong antimicrobial activity.

Several other compounds featured in folklore and world medicine – cinnamon, neem, rhubarb, licorice, pine, and clove – as well as the bitter aromatic herbal spirit Fernet Branca, demonstrated differential antimicrobial effects in our study (Figure 1).

Prophage inducers

Stevia, an artificial sweetener extracted from the plant species *Stevia rebaudiana*, was the most potent prophage inducer with an increase of 410% and 321% VLPs detected from *B. thetaiotaomicron* and *S. aureus*, respectively. Several studies have demonstrated the antimicrobial potential of stevia extracts,[34–36] but none has proposed a mechanism of action. Abundant terpenes may be responsible for the antimicrobial properties of stevia (as reviewed by[37]). Our results agree with this possibility, in that terpenes have been shown to be more effective against gram-positive bacteria (*E. faecalis* and *S. aureus*). Tomita et al.[36] showed that fermented stevia is effective against food-borne pathogenic bacteria, with no significant killing of gut microbes.[36] A stevoside mixture from *Stevia rebaudiana* has also been shown to be anti-tumorigenic,[38,39] which may involve proviral induction. Together, these studies and our results suggest stevia could be used to manipulate the gut microbiome.

Artificial sweeteners such as saccharin, sucralose, and aspartame have been shown to have detrimental effects, such as decreased glucose tolerance, that are initiated by alterations of gut flora.[40,41] Suez et al.[41] showed that saccharin supplementation triggered gut dysbiosis and increased *Bacteroides* spp. abundance in mice. Our results showed a decrease in growth for all species challenged with aspartame, and differential effects from sucralose[42] found that recent consumers of aspartame or acesulfame-K had significant differences in bacterial diversity compared to non-consumers.[42] While we only measured the effects of artificial sweeteners on three gut microbes, the data presented here may fall into the

dysbiosis paradigm. Additionally, the increased VLP expression in *E. faecalis* when challenged by aspartame provides mechanistic insight into the potential cause of dysbiosis.

A propolis glycerite increased VLP abundances by 115% and 695% for *B. thetaiotaomicron* and *E. faecalis*, respectively. This and other bee products were hypothesized to increase prophage expression based on the antimicrobial effects of honey, which are a product of hydrogen peroxide (a known prophage inducer) and phenolic activity.[43,44] It showed that in mice, a 2 or 5-week high-fat diet containing 0.2% w/ w of crude propolis extract mitigated the community composition shift toward an increased Firmicutes: Bacteroidetes ratio and subsequent inflammatory markers that result from high-fat diets. This suggests that propolis could be another supplement for shifting or maintaining gut community composition. We were unable to measure whether any bee products were antimicrobial because they formed precipitates that obfuscated $OD_{600\ nm}$ readings. Nevertheless, increased VLP production was observed in the Firmicute *E. faecalis*, suggesting that prophage induction may be partly responsible for the equalizing effect of propolis on the Firmicutes:Bacteroidetes ratio.

Some of the compounds tested for prophage induction had significantly fewer VLPs in their supernatants relative to the mean, and for some compounds, the effect varied between species (Figure 3). Compounds with the greatest reduction in VLPs are strongly supported in the literature as antivirals – namely rhubarb,[45–47] coffee,[48–50] oregano,[51,52] and pomegranate.[53–55]

Caveats

For the most part, growth curves did not produce the characteristic pattern of a prophage induction. The lack of a characteristic "induction" growth curve means that future studies of potential prophage inducers show a direct measure of VLPs like flow cytometry or epifluorescence microscopy. Future work needs to determine which prophage is induced by the test treatments. Our study was conducted on one strain of each bacterial species, so these results may not be generalizable to other strains of each species. Therefore, further studies are needed to test the effects of these compounds on additional gut bacteria *in vivo*, particularly because enzymatic

and/or pH alterations of the compounds may occur before they reach the large intestine. Input into a simulator of the intestinal microbial ecosystem (SHIME)[56] would be a logical next step. *In vivo* investigations could be performed in gnotobiotic mice or humans, considering most compounds are recognized as safe food products.

Conclusions

Here we expanded the possibility of microbiome landscaping through intentional ingestion of particular dietary compounds that modulate bacterial abundances in the gut. We found that some compounds act as reductive modulators by inducing prophages, and because some of these are surprisingly common ingredients (e.g., aspartame, toothpaste), diet-induced prophage activation is likely a regular occurrence in the gut ecosystem. As these ingredients are consumed by populations around the world, these mechanisms could further our understanding of how particular foods shape gut microbiomes. They could also provide experimental tools for identifying novel mechanisms of prophage induction; these tools are essential for probing the dark matter of viral functional diversity, and for determining how phages influence and are affected by their environments.

Materials and methods

Bacterial strains and media

B. thetaiotaomicron VPI-5482 (NCBI Taxonomy #226186, Genbank #AE015928.1) and *E. faecalis* (ATCC #19433, NCBI Taxonomy #1169286) were used for this study. The *P. aeruginosa* strain RM1 was isolated on cetrimide media from the lung biopsy of a cystic fibrosis donor and *S. aureus* was isolated from a human fecal sample.[1] Bacteria from frozen glycerol stocks were streaked onto supplemented brain–heart infusion (BHIS) plates that were incubated at 37°C for 24 h unless otherwise described. *B. thetaiotaomicron* VPI-5482 was incubated for 48 h on Schaedler Blood Agar OxyPlates (Oxyrase – Mansfield Ohio).

S. aureus, *E. faecalis*, and *P. aeruginosa* were cultured in 3–5 mL of Brain-Heart Infusion Broth (Becton, Dickson, and Company – Sparks,

Maryland) supplemented with the following per liter (BHIS): hemin 5 mg, menadione 1 mg, yeast extract 5 g, L-cysteine HCl 50 mg, MgSO$_4$ 120 mg, and CaCl$_2$ 50 mg. For *B. thetaiotaomicron*, Oxyrase for Broth (Oxyrase – Mansfield Ohio) was added to BHIS at a concentration of 4% v/v, and media was pre-incubated at 37°C for at least 1 h to ensure complete removal of oxygen (O$_2$). Mineral oil (~1 mL) was floated over the top of inoculated media to maintain anaerobiosis. All species were incubated until the late exponential phase, for up to 24 h at 37°C and shaken at 250 RPM.

Predicting prophages in genomes

To verify prophages are present in these bacterial strains, each genome was screened using Phage Search Tool (PHAST)[57] and PhiSpy.[58] Because the strains of *P. aeruginosa* and *S. aureus* used in this study do not have genome sequences available, genomes from strains also isolated from humans (*P. aeruginosa* LES400 and *S. aureus* CA15) were screened for prophage content.

Screening compounds and master plate preparation

One hundred seventeen compounds were screened for antimicrobial activity (Supplementary Table S1). Compounds were selected to include those with known, presumed, or anecdotal antimicrobial activity and test concentrations were obtained from peer-reviewed literature when possible. All compounds were formulated (see Supplementary Table S1), filter-sterilized and then stored in two master 96-well plates (MP1 and MP2) so that a common volume (10 or 20 μL depending on total well volume) could be added to each test-well.

Bacterial growth and prophage-induction assays

Bacteria were cultured in the presence of each of the 117 test compounds for 24 h in 96-well plates. A single colony from each species was used to inoculate a 3 mL liquid BHIS overnight culture, and 140 μL of diluted overnight culture (1:10 in BHIS) was then mixed with 10 μL of test compound solution. For *B. thetaiotaomi-cron*, volumes were doubled and 96-well plates sealed with SealPlate Film (Excel Scientific) to promote

anaerobic conditions. The following controls were included: BHIS alone (media control), bacteria in BHIS without test compound (bacterial viability control), and each diluent used for the various test compounds (Supplementary Table S1). Using a SpectraMax M2 plate reader/incubator, optical density (OD) at 600 nm (OD$_{600\ nm}$) was measured every 10 min for *E. faecalis*, *S. aureus*, and *P. aeruginosa*, and every 60 min for *B. thetaiotaomicron*.

Preliminary tests showed that known prophage inducers, mitomycin-C and carbadox, [59] reproducibly affected the growth of *P. aeruginosa* and *E. faecalis*: logarithmic growth was followed by a rapid decline in OD$_{600\ nm}$ as host cells were lysed (Supplementary Figure S2). This pattern of bacterial growth served as a guide for identifying prophage induction; those test compounds eliciting a similar growth profile were further analyzed at varying concentrations. Each test was accompanied by a positive "induction" control of carbadox (Sigma-Aldrich) at a range of final concentrations (Supplementary Figure S3).

Growth curve analyses

The PMAnalyzer pipeline was used to analyze the growth curves (Supplementary Figure S3A).[60,61] The PMAnalyzer pipeline receives OD files from the microtiter plate reader as an input and uses the SciPy Python numerical library to model each growth curve.[62] Each growth curve was fitted with the Zwietering model to predict the y_0 (OD$_{600\ nm}$ where growth begins), lag time, maximum growth rate, and biomass yield.[63] For this study, several new metrics were added to the pipeline. First, the area under the curve (*auc*) was calculated using the SciPy integrate library to perform integration on the growth curve using the composite trapezoidal rule. Second, the shifted area under the curve (*auc$_{shifted}$*) was calculated using the formula:

$$auc_{shifted} = auc - y_0 t_f$$

where *auc* is the area under the curve, y_0 is the starting OD$_{600\ nm}$ value predicted by the Zwietering model and t_f is the final time point of the experiment. By using the *auc$_{shifted}$* calculation, the y-axis cutoff of y = 0 becomes the actual starting optical density, providing a method of normalization

necessary for downstream analysis (Supplementary Figure S3B). Raw growth curves were plotted in R using ggplot2.[64]

Differences between growth curves were analyzed by comparing experimental wells to controls using a method similar to one developed by Firsov[65] who determined that the antimicrobial effect of any compound could be measured by directly comparing the growth of cells when the compound is present or absent. For each growth curve the $auc_{shifted}$ was calculated and then compared to the $auc_{shifted}$ from negative control wells (Supplementary Figure S3B) to derive the antimicrobial effect (%) of each compound:

$$antimicrobial\ effect = -\left(\frac{C - E}{C}\right)x100$$

where C is the shifted area under the growth curve for control (no test compound added) and E is the shifted area under the growth curve for experimental (test compound added).

For each microorganism, the mean antimicrobial effect (Supplementary Table S2) of each compound was used to generate a clustering heatmap in R using the gplots library.[66,67] Dendrograms were created in gplots and used to re-order the heatmap, clustering by the compound response and similar bacterial response.

The following compounds were excluded from the heatmap analysis because they formed precipitates in the wells or were too opaque to accurately measure $OD_{600\ nm}$: apple skin (APP), eugenol (EUG), grapefruit seed extract (GRA), pomegranate (POM), propolis glycerite (PROg) and turmeric (TUR). Although these compounds could not be used for growth curve analyses, they were suitable for flow cytometry.

VLP quantification with flow cytometry

Each compound listed in Table 1 was tested for its ability to induce prophages in B. thetaiotaomicron, E. faecalis and S. aureus using flow cytometry. These compounds were chosen based on the ratio of the maximum $OD_{600\ nm}$ of the growth curve to the lowest $OD_{600\ nm}$ measured after this peak. The cutoff ratio was set to 1.19, which is the ratio observed when B. thetaiotaomicron was incubated with 1 ug ml^{-1} of carbadox. Some special cases were included that did not meet the ratio criteria. Licorice (LIC) and

toothpaste (TOO) had strong antimicrobial/inhibitory effects without typical induction growth-curves. Although propolis could not be used for growth curve analyses, it was included in flow cytometry analysis based on our hypothesis that bee products' peroxide-mediated antimicrobial effect[43,68] may trigger prophage induction as does peroxide.[69,70] An extract of coffee Arabica was included since pure caffeine had a ratio above 1.19 in two of the three species tested.

Overnight liquid cultures were diluted 1:100 in BHIS media and an aliquot (130 μL) of each dilution (260 μL for B. thetaiotaomicron) was transferred to plate wells in duplicate and grown in a SpectraMax M2 plate reader, as described above, until cultures reached early log growth phase. Turbidity of approximately 0.25, 0.35, 0.45 was reached for S. aureus, E. faecalis, and B. thetaiotaomicron, respectively. Upon reaching log growth, antimicrobial test solutions were added to each well, at concentrations optimized for each species based on data from growth curves and heat maps (Table 1). Optimal concentrations of traditional prophage inducers slightly inhibit bacterial growth without causing excessive cytotoxicity;[71] therefore, the concentration of each antimicrobial compound was optimized to be similar for each species while maximizing prophage induction and minimizing cytotoxicity (Table 1). Bacteria were incubated for 4–5 h with each test compound before well contents were removed. Duplicate wells were not sampled but were returned to the plate reader/incubator to determine the growth curve (Supplementary Figure S5a). Growth curves were similar to those in Supplementary Figure S3, with few minimal differences due to compound concentrations being optimized for flow cytometry. The antimicrobial effect of each compound was defined as the negative percent difference between the control and test shifted area under the curve (Supplementary Figure S5b).

Samples were prepared for flow cytometry with the following procedure adapted from Brussaard et al.[72] First, bacteria incubated alone or with food and herbal extracts were transferred from 96-well plates to epi-tubes and centrifuged for 5 min at 4000 RPM. A portion (10 μL) of the supernatant was then combined with 970 μL of sterile SM buffer. The purpose of the bacteria grown alone in the plate was to create bacteria controls so that VLP production between bacteria grown with and without experimental

compounds could be compared. All samples were then stored overnight at 4°C. Samples were fixed by adding 0.5% (v/v) glutaraldehyde (final concentration) to each sample, vortexing and then incubating at 4°C for 30 min. To stain samples, 10 µL of a 10X solution of SYBR Green I (Thermo Fisher Scientific) was mixed with 200 µL of each fixed sample and then this mixture was heated at 80°C for 10 min, then held at 4°C for 5 min. As an internal flow rate standard, 5 µL of Fluoresbrite yellow-green 0.75 µm microspheres (starting concentration of 10^7 beads mL^{-1}, Polysciences Inc.) were added to each experimental well containing 195 µL of the sample. Prior to analysis, the BD FACSCanto flow cytometer sheath fluid was replaced with Milli-Q water to reduce the background noise of the instrument and to aid in the separation of stained VLPs. To ensure cleanliness of the machine, 10% bleach followed by Milli-Q water were acquired for 20 min each or until Milli-Q water reached ≤30 events/second, while thresholding on side scatter 200. All samples were analyzed in standard mode utilizing the high-throughput sampler (HTS) 96-well plate reader. For enumeration, 30 µL of each sample was analyzed at a flow rate of 0.5 µL s^{-1} followed by Milli-Q water to wash any residual products or dye. The 488 nm blue laser was used to excite the SYBR green dye detected in the FITC channel (530/30 nm bandpass filter preceded by a 502 nm long pass mirror). Threshold gating was applied in the FITC channel above the background noise of the Milli-Q water controls. As a positive control, *E. coli* phage T4 virions (10^6 VLPs mL^{-1}) were used to confirm that VLPs were detectable in this region. *Salmonella enterica* serovar Typhimurium strain LT2 [78, 79] incubated with the known prophage inducers mitomycin C and carbadox was used as a positive control (Supplementary Figure S4).

Bivariate plots displaying the FITC channel versus side scatter were created to differentiate beads and microbes from samples so that VLPs could be measured. These values were compared to background controls to determine which conditions had elevated VLPs compared to the control. This comparison was calculated as a percent difference between the test condition and the control (Supplementary Table S4). All data were collected and analyzed on FACSDiva 6.1.1 software (BD Biosciences – San Diego, CA).

Species abundance in environmental samples

To determine ubiquity and abundance of these four species in the human gut, 2,299 human gut metagenomes were chosen from the Sequence Read Archive that were identified to be shotgun sequenced by PARTIE.[73] These datasets were then scanned using SearchSRA.[74] When a species was found to be present in a metagenome, the number of reads assigned to that species per 10,000 reads was used to calculate its relative abundance. For quality control, reads less than 50 bp or 90% identity were excluded.

Conflict of Interest

The authors report no conflict of interest.

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