Degradation of the incretin hormone Glucagon-Like Peptide-1 (GLP-1) by *Enterococcus faecalis* metalloprotease GelE.

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Abstract

Metabolic diseases, including Type 2 Diabetes and obesity, have become increasingly prevalent global health concerns. Studies over the past decade have established connections between the gastrointestinal microbiota and host metabolism, but the mechanisms behind these connections are only beginning to be understood. We were interested in identifying microbes that have the ability to modulate the levels of the incretin hormone glucagon like peptide 1 (GLP-1). Using a human derived cell line that is capable of secreting GLP-1 in response to stimulatory ligands (NCI-H716), we identified supernatants from several bacterial isolates that were capable of decreasing GLP-1 levels, including several strains of *Enterococcus faecalis.* We further identified the secreted protease GelE, an established virulence factor from *E. faecalis,* as being responsible for GLP-1 inhibition via direct cleavage of GLP-1 by GelE. Finally, we demonstrated that *E. faecalis* supernatants can disrupt a colonic epithelial monolayer and cleave GLP-1 in a *gelE* dependent manner. This work suggests that a secreted factor from an intestinal microbe can traverse the epithelial barrier and impact levels of an important intestinal hormone.

Importance

Humans have a complex and interconnected relationship with their gastrointestinal microbiomes, yet our interest in the microbiome tends to focus on overt pathogenic or probiotic activities, leaving the roles that commensal species may have on host physiology and metabolic processes largely unexplored. Commensal organisms in the microbiome produce and secrete many factors that have an opportunity to interact

35 with the gastrointestinal tract and host biology. Here we show that a secreted protease
36 from *E. faecalis*, GelE, is able to degrade the gastrointestinal hormone GLP-1, which is
37 responsible for regulating glucose homeostasis and appetite in the body. The disruption
38 of natural GLP-1 signaling by GelE may have significant consequences for maintaining
39 healthy blood glucose levels and in the development of metabolic disease. Furthermore,
40 this work deepens our understanding of specific host-microbiome interactions.

Introduction

The human gastrointestinal (GI) tract is home to trillions of microorganisms, collectively referred to as the GI microbiome (1). Because of the direct physical proximity that the gut microbiome has with its human host, it is no surprise that the microbiome plays a role in multiple aspects of health, the best characterized of these being immune tolerance, pathogen resistance, and digestion (2). Less understood are the interactions between the microbiome and human metabolism. Despite limited mechanistic insight into the cross-section of microbiome and host metabolism, it is of great interest as both an etiology of disease and for potential therapeutic applications, and some understanding is beginning to emerge. The first insights into microbial influence over human metabolism came from studies demonstrating that the simple absence of a microbiome resulted in decreased total body fat in germ-free mice, compared to conventional mice, independent of food intake; further, the decrease in fat mass could be gained back by colonizing the germ-free mice with bacterial communities from conventionally raised mice (3). The observed ability of the microbiome to help harvest energy from the diet sparked a variety of research studies over the next decade, with a focus on the interaction of the microbiome with GI hormone peptides, in particular the nutrient-stimulated incretin Glucagon-like Peptide-1 (GLP-1). Already a therapeutic target for Type 2 Diabetes (T2D), GLP-1 is an integral signaling hormone responsible for promoting insulin secretion and satiety, while decreasing glucagon secretion and gastric emptying. Early work showed that addition of the prebiotic oligofructose to the diets of rats on a high-fat diet increased GLP-1 levels measured from the portal vein, in addition to protecting from weight gain (4). Additional studies demonstrated that

64 administration of *Akkermansia muciniphila* could reverse high-fat diet induced metabolic
65 disorders, and that this activity was mediated at least partially by an outer membrane
66 protein purified from *A. muciniphila* interacting with Toll-like receptor 2 (5, 6). These
67 studies strikingly demonstrate that a specific bacterial species and factor are capable of
68 impacting metabolic disease phenotypes. Despite a heightened research interest,
69 additional mechanisms behind how the microbiome and host metabolism influence each
70 other still remain largely undescribed.

71 Some of the obvious suspects to investigate for host-microbiome interactions are
72 the many secreted proteins and metabolites that bacteria release as part of their natural
73 lifecycle. While these external products are often part of bacterial cellular metabolism or
74 provide beneficial function to the bacterial cell, they also have an opportunity to interact
75 with their environment, in this case the human GI tract. Here, our screening for bacterial
76 modulators of GLP-1 revealed multiple bacterial strains that can inhibit GLP-1 levels in
77 an in vitro assay. We further characterize this inhibition as direct cleavage from *E.*
78 *faecalis* strains by its secreted protease GelE, revealing a novel cleavage target of
79 GelE. Finally, we suggest a role for GelE in disrupting natural GLP-1 signaling and
80 metabolic processes.

81

82 **Results**

83 *Screening a human-derived bacterial library for GLP-1 modulatory activity*

84 Bacterial strains capable of modulating GLP-1 levels were identified by an in vitro
85 screening pipeline using the GLP-1 secreting human cell line NCI H716 (7). Over 1500
86 cell-free supernatants collected from individual bacterial isolates were prepared and

applied to NCI H716 cell monolayers for 2 h, and secretion of GLP-1 into the medium was measured by ELISA. NCI H716 cell viability was also monitored by PrestoBlue Cell Viability Reagent to ensure no significant increase in NCI H716 cell lysis or death (data not shown). The majority of bacterial isolates screened had no impact on GLP-1 levels; however, approximately 20 isolates showed a marked decrease in GLP-1 levels, many of them below the limit of detection of the ELISA (**Figure 1A**). We also identified 45 isolates that dramatically increase GLP-1 levels; these were further characterized in a separate study (8).

To identify the species of each isolate, the 16S rRNA gene was sequenced. The majority of the isolates identified as *Enterococcus faecalis*, as well as *Clostridium perfringens*, *C. bifermentans*, and *C. butyricum*. The *E. faecalis* isolates exhibited a stronger GLP-1 inhibitory effect, ranging between 0% ± 0.0 and 5.44 ± 9.1% GLP-1, compared to media controls ($p < 0.0001$, **Figure 1B**). The *Clostridium* species, while still inhibitory, consistently show a slightly weaker inhibitory effect, ranging between 8.0% ± 4.6 and 15.2% ± 21.0 GLP-1, compared to media controls ($p < 0.0001$, **Figure 1B**). Because of this difference in the GLP-1 inhibitory activities of *E. faecalis* and *Clostridium* species, we decided to further characterize the activity of the *E. faecalis* isolates.

Identifying the factor secreted from E. faecalis responsible for GLP-1 inhibition

Size fractionation experiments showed that GLP-1 inhibitory activity from *E. faecalis* was contained within the 30-50 kDa size fraction of supernatant (**Figure S1**). We also found that the GLP-1 degradation activity was sensitive to heat and the metal

ion chelator N,N,N',N'-tetrakis(2-pyridylmethyl)ethane-1,2-diamine (TPEN) (**Figures S2 and S3**). These data suggest the factor responsible for GLP-1 inhibitory activity is a secreted, metal-dependent protein. Two secreted proteases from *E. faecalis* are well known, the serine protease SprE and the metalloprotease GelE, both of which are regulated by the two-component, quorum-sensing *fsr* (*faecalis* system regulator) operon (9) (**Figure 2A**). We obtained strains of *E. faecalis* containing null mutations in *sprE* (TX5243), *gelE* (TX5264), *fsrB* (TX5266), and a *gelE, sprE* double mutant (TX5128), all generated in the commonly used wild-type OG1RF *E. faecalis* strain (10). To test the GLP-1 inhibitory activity of these strains, supernatants were incubated directly with recombinant GLP-1 (Tocris) (**Figure 2B**). Of the strains tested, only the Δ*sprE* strain maintained GLP-1 inhibitory activity equal to that of wild-type OG1RF (OG1RF -0.06% ± 0.76, Δ*sprE* -0.14% ± 0.74, $p < 0.0001$), while the Δ*gelE*, Δ*fsrB*, and Δ*gelE;*Δ*sprE* strains no longer showed decreased GLP-1 levels (95.19% ± 4.48, 97.8% ± 7.37, 88.41% ± 17.47, respectively). Taken together, the loss of GelE, either directly by knock-out or indirectly by dysregulation through FsrB, results in a loss of GLP-1 inhibitory activity, suggesting direct cleavage of GLP-1 by the metalloprotease GelE.

To further characterize GLP-1 cleavage by GelE, the expression of *gelE* in five *E. faecalis* isolates was measured by quantitative PCR in relation to their ability to cleave GLP-1 (**Figure 3**). During the initial screen, we identified one *E. faecalis* isolate that did not inhibit GLP-1, a clinical isolate *E. faecalis* S613 (from Cesar A. Arias's laboratory, University of Texas, Health Science Center) (11). The four GLP-1 degrading isolates highly express *gelE*, with expression levels at least 10-fold higher and up to 380-fold

132 higher than the non-GLP-1 degrading isolate S613, demonstrating a correlation
133 between *gelE* expression and GLP-1 degradation.
134
135 *GelE specificity for human metabolic substrates*
136 Previously, it has been shown that GelE degrades a range of substrates (12);
137 thus, we wanted to better understand the range and specificity of GelE cleavage targets
138 relevant to GLP-1 and other proteins involved in human metabolism. Supernatants from
139 *E. faecalis* strains 30054A, 30002, OG1RF, Δ*gelE*, Δ*sprE*, and S613 were incubated
140 with a panel of recombinant protein substrates (GLP-1, Glucose-dependent
141 insulinotropic peptide (GIP), Peptide YY (PYY), leptin, glucagon, pancreatic peptide,
142 insulin, IL-6, tumor necrosis factor alpha (TNFα), and monocyte chemoattractant
143 protein-1 (MCP-1)) and quantified by a Luminex assay. From our findings, GelE is
144 capable of degrading, to some extent, nearly all the substrates tested in the metabolic
145 panel (**Figure 4**); however, some patterns of degradation emerged. Recapitulating our
146 previous findings, GLP-1 levels were reduced to 0.57% ± 0.13, down to the limit of
147 detection of the assay. Similar degradation was observed for GIP, glucagon, leptin,
148 PYY, and pancreatic peptide. For insulin, MCP-1, and TNFα, degradation was less
149 striking, but still statistically significant. Finally, we did not observe consistent
150 degradation of IL-6 by GelE.
151 To gain a better understanding of the substrate preference of GelE, we also
152 tested the ability of diluted supernatants of *E. faecalis* 30054A, from 1-0.0001X, to
153 degrade the same panel of metabolic substrates (**Table 1**). Most prominently,
154 supernatant from *E. faecalis* 30054A diluted to 0.01X still degraded the majority of GLP-

1 present, leaving only 10.38% ± 3.52 GLP-1 remaining. GIP, glucagon, and leptin also show high degradation with diluted supernatants (1.55% ± 1.09, 3.08% ± 3.25, and 7.95% ± 4.72 substrate remaining, respectively, for 0.1X supernatants). This level of degradation does not hold true for all substrates tested: pancreatic peptide and PYY maintain fairly high levels of degradation with undiluted supernatants (10.35% ± 2.4 and 10.45% ± 3.1 substrate remaining, respectively, for undiluted supernatants), but degradation lessens upon dilution (47.49% ± 17.15 and 54.61% ± 13.14 substrate remaining, respectively, for 0.1X supernatants). Finally, MCP-1, TNFα, and insulin show less degradation even with undiluted supernatants (35.14% ± 14.1, 39.73% ± 8.0, and 57.29% ± 7.7 substrate remaining, respectively, for undiluted supernatants).

Interaction of GelE and GLP-1 through an epithelial layer

While GelE from *E. faecalis* may readily cleave GLP-1 and other substrates in vitro, it is important to consider the GI epithelium separating these two molecules in vivo. Previous work has implicated *E. faecalis*, and specifically GelE, in contributing to intestinal epithelium disruption (13-15). We aimed to model the ability of GelE to contact GLP-1 through an epithelial layer using T84 epithelial cells in a transwell format, to mimic the microbial interface on the apical side and the presence of GLP-1 on the basolateral side of the epithelium (**Figure 5A**). The integrity of the T84 epithelial layer, as measured by transepithelial electrical resistance, decreased by approximately half with the apical addition of cell-free *E. faecalis* supernatants expressing GelE, 30054A and OG1RF (52.2% ± 17.97, p = 0.0022 and 65.5% ± 9.47, p = 0.0439, respectively, compared to DMEM/F12 T84 cell culture media control); while *E. faecalis* supernatants

178 lacking GelE, Δ*gelE* and S613, increased the integrity of the epithelial layer (156.6% ±
179 24.76, p = 0.0045 and 153.7% ± 18.93, p = 0.0084, respectively, compared to
180 DMEM/F12 T84 cell culture media control) (**Figure 5B**).
181 The basolateral compartment of each transwell contained approximately 500 pM
182 GLP-1 supplemented into the DMEM/F12 media. When GelE containing supernatants
183 30054A or OG1RF were added apically to the T84 cell epithelial layer, GLP-1 in the
184 basolateral compartment was cleaved nearly completely (3.1% ± 2.68, p < 0.0001 and
185 1.6% ± 0.46, p < 0.0001 respectively, compared to DMEM/F12 T84 cell culture media
186 control), while GLP-1 in the basolateral compartment of apical supernatants from Δ*gelE*
187 and S613 *E. faecalis* strains, remained intact (**Figure 5B**). Together, these data
188 demonstrate that GelE causes moderate damage to an epithelial layer, allowing access
189 to the basolateral side where it can cleave GLP-1.
190
191 **Discussion**
192 Bacterial cells secrete a wide range of proteins and metabolites during their
193 lifecycle, and one such class of secreted molecules are proteases, enzymes that break
194 down other proteins or peptides. A well-studied example of this is *Enterococcus faecalis*
195 and its gelatinase GelE. A secreted metalloprotease, GelE serves *E. faecalis* by
196 degrading misfolded surface proteins and decreasing chain length for dissemination
197 (16). A role for GelE in preventing biofilm formation has also been described by multiple
198 research groups (17, 18).
199 Because *E. faecalis* has been implicated in various infections, including
200 endocarditis, bacteremia, and urinary tract infections (19), GelE has been studied for its

interaction with human proteins. A thorough characterization of GelE revealed multiple host cleavage targets, including glucagon and cholecystokinin, among several other substrates (12). More recently, GelE has been shown to cleave the C3-α chain of the human complement system, promoting immune evasion (20). Additionally, GelE can degrade the tight junction protein E-cadherin, contributing to intestinal inflammation and impaired barrier integrity (13). Our work adds GLP-1, among other metabolic factors, to the list of GelE targets.

Our data from Luminex assays using a panel of metabolic substrates show that GelE is able to degrade more substrates than previously suspected, and furthermore, that GelE has an enhanced ability to degrade some substrates over others, including GLP-1, GIP, glucagon, and leptin. If occurring in the human body, degradation of these various substrates could have differing, sometimes contradictory, effects on host metabolic processes. GLP-1, GIP, and PYY are secreted basolaterally by enteroendocrine cells and have beneficial functions for host metabolism, and their absence would likely permit a change in glucose homeostasis, leading to hyperglycemia, and an increase in food intake. Made and secreted in the pancreas, degradation of insulin by GelE would likely result in similar hyperglycemia, and a reduction in pancreatic peptide might allow for increased food intake. Conversely, degradation of glucagon from alpha cells of the pancreas might result in hypoglycemia. Leptin is produced in adipocytes, and its degradation would likely cause dysregulation of fat accumulation and processing. Finally, MCP-1 and TNFα are pro-inflammatory cytokines, and disruption of their signaling by degradation from GelE might delay an immune response during pathogen invasion. Importantly, whether these cleavage

events are occurring and have relevant consequences in a whole biological system needs to be confirmed in animal studies. Based on proximity, GelE crossing the epithelial layer would first encounter molecules secreted into the lamina propria, and so we suspect GI hormones such as GLP-1, GIP, and PYY would be primary targets for degradation by GelE.

Our study supports the findings that *E. faecalis* and its protease GelE can compromise an epithelial layer and gain access to the basolateral environment (13-15). This is not surprising from a clinical perspective, as *E. faecalis* is implicated in cases of bacteremia and sepsis (23). For the topic of this study, this access could allow GelE to contact vital substrates responsible for host metabolic homeostasis as they are secreted into the GI lamina propria. Even mild inflammation often observed in individuals with metabolic syndrome, coupled with bacterial instigators like GelE from *E. faecalis*, could create a weakened epithelium (24). Once the integrity of the epithelium is damaged, luminal contents have an opportunity to move from the lumen of the GI tract and into the lamina propria just on the other side of the epithelial layer, where many hormones and metabolites are secreted before moving into the circulatory system. Furthermore, there is little indication regarding whether GelE could also be capable of diffusing into circulation as most hormones and nutrients do. Additionally, others have demonstrated by proof of concept that the microbiome encodes DPP-4 like activity that can traverse the epithelium, and further propose that this activity is capable of modulating protein digestion and ultimately host metabolism and behavior (25); while DPP-4 and GelE work via different proteolytic mechanisms, the idea of bacterial proteases modulating host proteins and peptides is gaining traction.

Interestingly, the *Enterococcus* genus has been linked to obesity in children and adolescents, as well as to mice consuming a western diet (21, 22). While not characterized in this study, we also identified several *Clostridium* isolates whose supernatants are capable of decreasing GLP-1 levels in vitro. We suspect this activity is also the result of a secreted protease cleaving GLP-1, supporting the idea that the microbiome produces a suite of proteases capable of interfering with host metabolism. Although *E. faecalis* and *Clostridium* have been implicated in infection, but they can also behave as commensal organisms and often live inconspicuously in our GI tracts. It is important to understand all the interactions of these organisms with their host, not just the overt pathogenic functions.

In summary, the results of this study demonstrate that GelE, a recognized virulence factor of *E. faecalis*, can degrade the human GI hormone GLP-1, among other metabolic substrates. The degradation of GLP-1 likely occurs by slight damage to the intestinal epithelium, allowing GelE to translocate across the epithelial layer and access GLP-1. While it would be reckless to assume this activity is an etiology of metabolic disease, we do believe that interference with natural GLP-1 signaling by microbial degradation of GLP-1 could be a contributing factor to the development of disease. An important next step for this work is to assess the contribution of GelE to intestinal barrier permeability and the development of metabolic syndrome in vivo. Finally, this study adds a novel mechanism of action to the ever-growing list of host-microbe interactions.

Materials and methods

Bacterial strain isolation

Bacterial strains for screening were isolated previously from fecal, breast milk, and ileum biopsy samples (8). Genetic mutant strains of *Enterococcus faecalis* (OG1RF, TX5266 (*fsrB*), TX5264 (*gelE*), TX5243 (*sprE*), TX5128 (*gelE;sprE*)) were generously gifted from the Danielle A. Garsin Laboratory (University of Texas, Health Science Center) (10). *E. faecalis* S613 was generously gifted from the Cesar A. Arias Laboratory (University of Texas, Heath Science Center) (11).

Bacterial growth and preparation of cell-free supernatants

Bacterial isolates were streaked from frozen glycerol stocks onto GM17 agar plates and incubated anaerobically overnight at 37°C. One colony was inoculated into 5 mL of GM17 broth and incubated overnight at 37°C followed by one more subculture into GM17 broth, and incubation overnight at 37°C. Once grown, bacterial cultures were centrifuged at 5000 x g for 20 min. Supernatants were collected and lyophilized (Labconco Freezone), followed by storage at -80°C until used for subsequent assays.

16S rRNA gene sequencing of isolates

To identify the bacterial isolates, bacteria were streaked on GM17 (M17 + 0.5% (w/v) glucose) agar plates from frozen glycerol stocks and incubated at 37°C for 24-48 h. Bacterial colony mass was then resuspended in 800 µL of sterile water and transferred to sterile bead beating tubes and homogenized for 2 min in a mini-beadbeater-96 (Biospec Products). Tubes were centrifuged at 8000 xg for 30 sec and supernatants were used for 16S rRNA gene PCR amplification with Phusion High-Fidelity DNA Polymerase (New England Biolabs) in a 20 µL reaction according to the

293 manufacturer's protocol, with sequencing primers 8F and 1492R. The amplification
294 cycle consisted of an initial denaturation at 98°C for 30 sec, followed by 26 cycles of 10
295 sec at 98°C, 20 sec at 51°C, and 1 min at 72°C. Amplification was verified by agarose
296 gel electrophoresis. For sample cleanup, DNA was treated with Exo-SAP-IT
297 (ThermoFisher) and incubated at 37°C for 15 min followed by a 15 min incubation at
298 80°C to inactivate the enzyme. The product was cooled and sent to Genewiz for
299 sequencing according to company protocol. Upon return of sequencing data, sequences
300 were compared to the NCBI BLAST database.
301
302 *Screening for GLP-1 stimulatory activity using NCI H716 cells*
303 NCI H716 (American Type Culture Collection (ATCC) CCL-251) cells were grown
304 in Roswell Park Memorial Institute (RPMI, ATCC) medium supplemented with 10% (v/v)
305 heat inactivated newborn calf serum (NBCS, Gibco). Cultures were maintained at a
306 concentration of 2-8 x 10^5 cells/mL and used at passages 15-40 for cell studies. For cell
307 studies, 96-well plates were coated with 100 µL of 10 mg/mL Matrigel (BD Biosciences)
308 for 2 h at room temperature. Following coating, NCI H716 cells were seeded at a
309 concentration of 1 x 10^5 cells/well in Dulbecco's Modified Eagle's Medium (DMEM)
310 supplemented with 10% (v/v) NBCS, as determined by trypan blue staining using a
311 hemocytometer. Two days later, lyophilized bacterial supernatants were resuspended in
312 Krebs buffer (Sigma) containing bovine serum albumin (BSA, 0.2% w/v) and bovine bile
313 (0.03% w/v) and incubated on the NCI H716 cells at 37°C with 5% CO_2. 4-phorbol 12
314 myristate 13-acetate (PMA, 2 µM) was used as a positive control as it is a potent
315 stimulator of GLP-1 secretion through activation of protein kinase C (PKC). Following a

316 2 h incubation, supernatants were collected and analyzed for total GLP-1 levels by
317 ELISA (Millipore Sigma) according to the manufacturer's protocol. Cell viability was
318 monitored using PrestoBlue Cell Viability Reagent (ThermoFisher Scientific) following
319 the manufacturer's instructions.
320
321 *Characterization studies (TPEN, heat, size)*
322 For metalloprotease inhibitor studies, *E. faecalis* strains were subcultured 0.1%
323 (v/v) from an overnight culture into GM17 containing the indicated concentration of
324 N,N,N',N'-tetrakis(2-pyridylmethyl)ethane-1,2-diamine (TPEN). Cultures were grown
325 overnight, supernatants were collected as described above, and incubated with 500 pM
326 GLP-1 (GenScript) for 4 h at room temperature, followed by storage at -80°C until ready
327 for GLP-1 quantification by ELISA.
328 For heat treatment studies, bacterial supernatants were heated to 90°C for 30
329 min. Samples were then used in an NCI H716 cell assay as described above, followed
330 by storage at -80°C until ready for GLP-1 quantification by ELISA.
331 For size fractionation studies, bacterial supernatants were separated by size
332 using centrifugal filter units (Amicon) and centrifuged as described above. Samples
333 were then used in an NCI H716 cell assay as described above, followed by storage at -
334 80°C until ready for GLP-1 quantification by ELISA.
335
336 *Protease knock-out studies*
337 Supernatants from *E. faecalis* protease mutants and controls (OG1RF, TX5266
338 (*fsrB*), TX5264 (*gelE*), TX5243 (*sprE*), TX5128 (*gelE;sprE*)) were collected from an

339 overnight culture grown in GM17 aerobically at 37°C by centrifugation as described
340 above. Supernatants were incubated with 500 pM GLP-1 (Tocris or GenScript) for 4 h at
341 room temperature, followed by storage at -80°C until ready for GLP-1 quantification by
342 ELISA, as described above.
343
344 *RNA collection and quantitation of gelE expression*
345 *E. faecalis* were subcultured 1% (v/v) from an overnight culture into GM17. After
346 5 h incubation, cells were collected by centrifugation, resuspended in RNALater solution
347 (Invitrogen), and stored at -80°C. Cells were washed in 1X PBS, resuspended in 1 mL
348 RTL buffer (Qiagen RNeasy Kit) and lysed by bead beating (2 x 1 min) at 4°C followed
349 by RNA extraction according to the manufacturer's instructions. cDNA was synthesized
350 using Superscript III reverse transcriptase (Invitrogen) following the manufacturer's
351 recommended protocol. Quantitative PCR reactions were performed using Power SYBR
352 Green Master Mix (Applied Biosystems) with either *E. faecalis* 16s RNA (f: 5′-
353 CCGAGTGCTTGCACTCAATTGG-3′, r: 5′-CTCTTATGCCATGCGGCATAAAC-3′) or
354 gelE (f: 5′-CGGAACATACTGCCGGTTTAGA-3′, r: 5′-TGGATTAGATGCACCCGAAAT-
355 3′) specific primers (Wang et al. 2011). The expression of gelE was normalized to that of
356 the 16S RNA and the data were analyzed using the $2^{-\Delta\Delta CT}$.
357
358 *Cell culture growth and assays of T84 monolayers*
359 Growth and assays of T84 cells were performed by methods described
360 previously, with slight modifications (Zeng 2004, Hopper 2000). T84 human colonic
361 epithelial cells (ATCC CCL-248) were propagated in tissue culture-treated T75 flasks

(CELLSTAR) as indicated by ATCC. When between 90-100% confluency, T84 cells were treated with 0.25% trypsin and plated onto 24-well, 3.0 μm polycarbonate membrane transwell filters (Costar, 3415) at a density of 8 x 10^4 cells/well. The electrical resistance of the monolayer was monitored over the course of 2-3 weeks, and monolayers with a transepithelial electrical resistance (TEER, Millipore Millicell ERS-2) >800 Ω/cm^2 were used for GLP-1 cleavage assays.

To prepare bacteria, on the day of the assay *E. faecalis* strains were subcultured 1% (v/v) from an overnight culture into GM17 and grown for 5 h as described above. Whole culture (cells + supernatant) samples were diluted in DMEM/F12 (Gibco) to a concentration of 1 x 10^7 CFU/mL, and supernatant samples were diluted 50% in DMEM/F12. All samples were neutralized to a pH of 6.8-7 using 3M NaOH.

Once prepared, the TEER was measured for each monolayer, followed by 100 μL of *E. faecalis* sample added to the upper chamber of the transwell, and 500 μL of tissue culture media containing 500 pM GLP-1 (GenScript) added to the lower chamber. After a 16 h incubation at 37°C in 5% CO_2, 200 μL of media from the lower chamber was removed and stored at -80°C until ready for GLP-1 quantification by ELISA, as described above. A final TEER measurement was taken for each monolayer.

Luminex

A Milliplex Multiplex assay was performed using a Metabolic Luminex kit (Millipore Sigma) to measure total GLP-1, glucagon, Gastric Inhibitory Peptide/Glucose Insuliotropic Peptide (GIP), leptin, Peptide YY (PYY), pancreatic peptide, insulin, monocyte chemoattractant protein-1 (MCP-1), interleukin-6 (IL-6), and tumor necrosis

385 factor alpha (TNFα). Bacterial supernatants were collected from an overnight culture by
386 centrifugation as described above, diluted in bacterial culture media as indicated, and
387 incubated with recombinant protein (GenScript, Sigma, Tocris) of each analyte for 4 h at
388 room temperature, followed by storage at -80°C until ready for analyte quantification.
389 The Milliplex Multiplex assay was run according to the protocol provided by the
390 manufacturer.
391
392 *Statistical analysis*
393 Statistical analyses were performed using GraphPad Prism version 8.0 (San
394 Diego, CA, USA). Experimental results are expressed as means ± standard deviation.
395 Statistical significance was set at $p < 0.05$. One-way statistical comparisons were
396 carried out using one-way analysis of variance (ANOVA), followed by multiple
397 comparisons of the means using Tukey's post-hoc analysis, for the GLP-1 secretion
398 experiments in NCI-H716 cells, protease knock-out mutation assay, and degradation
399 preference of GelE using a Luminex assay (undiluted supernatants). Two-way ANOVA
400 analysis, followed by multiple comparisons of the means using Tukey's post-hoc
401 analysis, was performed for the degradation preference of GelE using a Luminex assay
402 (diluted supernatants) and T84 cell experiments.

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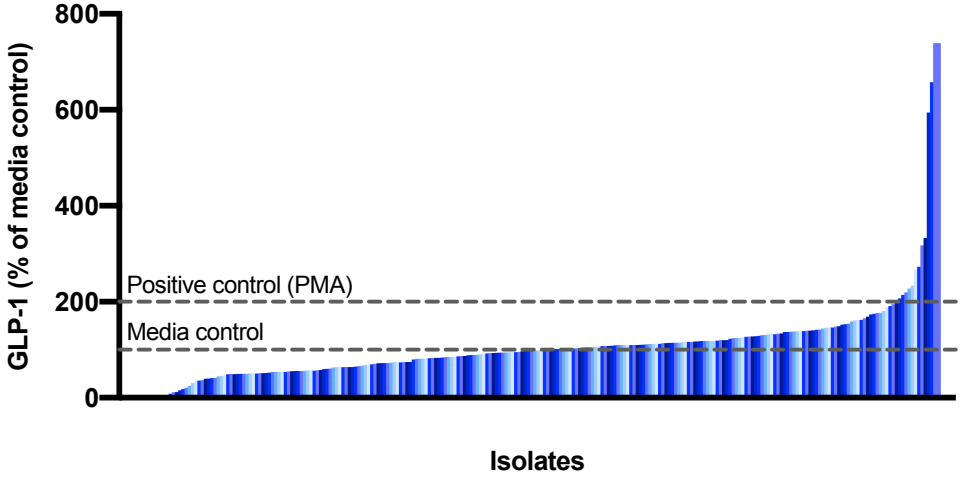
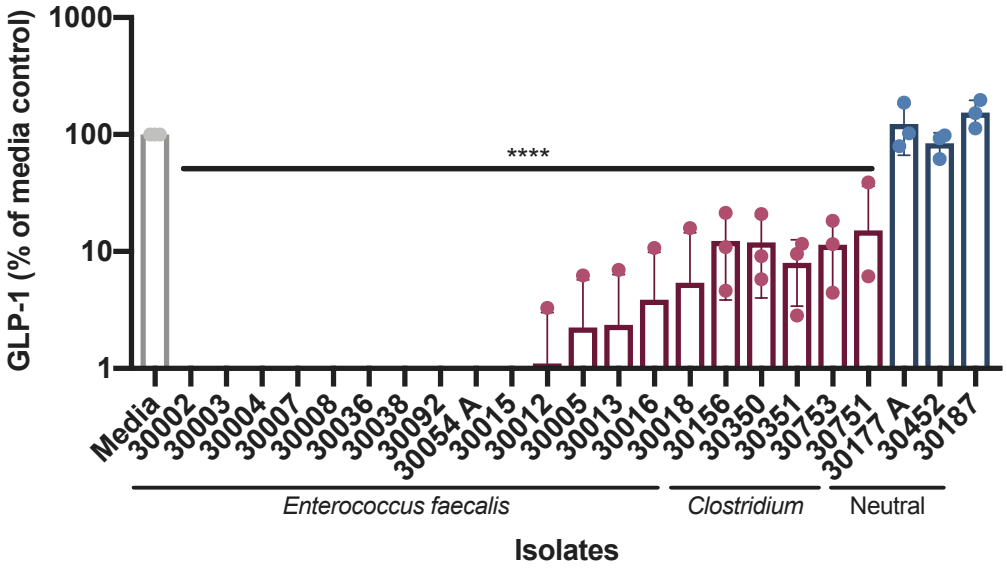
411

Figures and tables

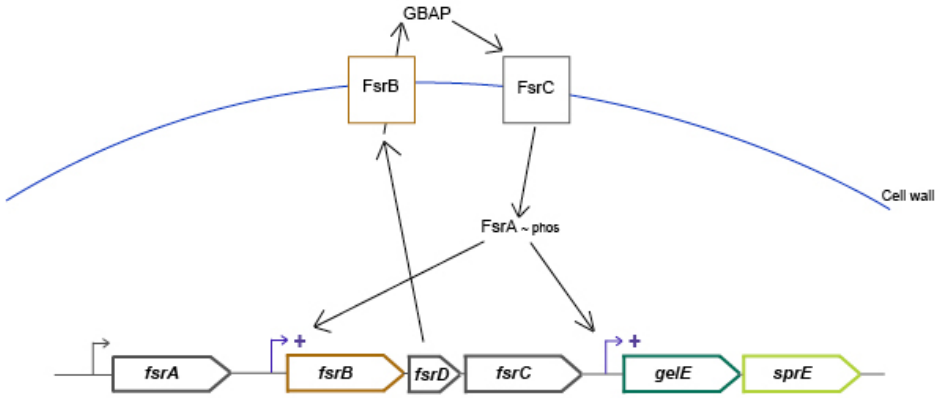


A.

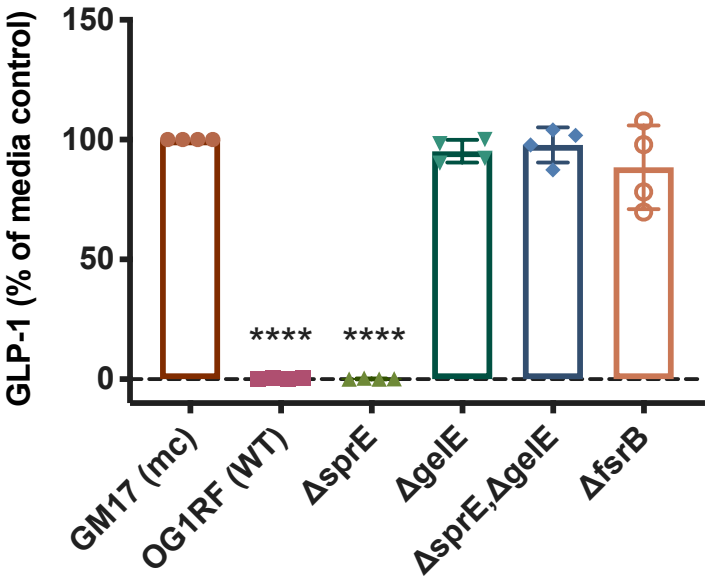


B.

Figure 1. Screening for modulation of GLP-1 Bacterial supernatants have a wide range of effects on GLP-1 secretion from NCI H716 cells (A). Multiple isolates of *Enterococcus faecalis* and *Clostridium* decreased GLP-1 levels compared to media control (B). Data were obtained from three independent experiments (n = 3) and expressed as mean values ± SD (****p < 0.0001).



A.



B.

Figure 2. GelE is the factor responsible for GLP-1inhibition Genetic pathway for production of *E. faecalis* proteases *gelE* and *sprE* (A). Activation of the quorum sensing system *fsr* activates transcription of *gelE* and *sprE*, immediately downstream of the *fsr* operon. *E. faecalis* strains unable to produce a functional GelE no longer deplete GLP-1 levels in an in vitro co-incubation of bacterial supernatants with GLP-1 (B). Data were obtained from four independent experiments (n = 4) and expressed as mean values ± SD (****p < 0.0001). GM17 (mc), bacterial culture media control.

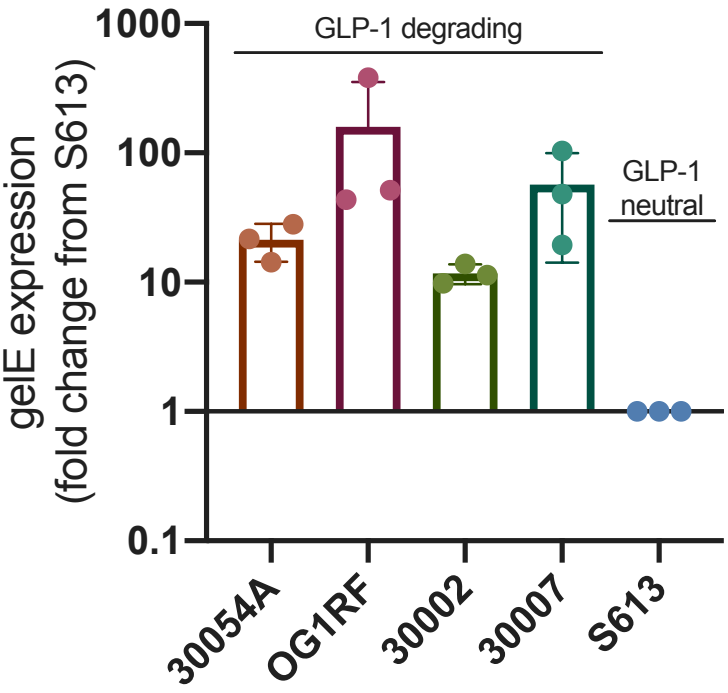


Figure 3. Expression of gelE correlates with GLP-1 degradation GLP-1 degrading strains of *E. faecalis* have significantly higher expression levels of *gelE* compared to a GLP-1 neutral strain S613. Data were obtained from three independent experiments (n = 3).

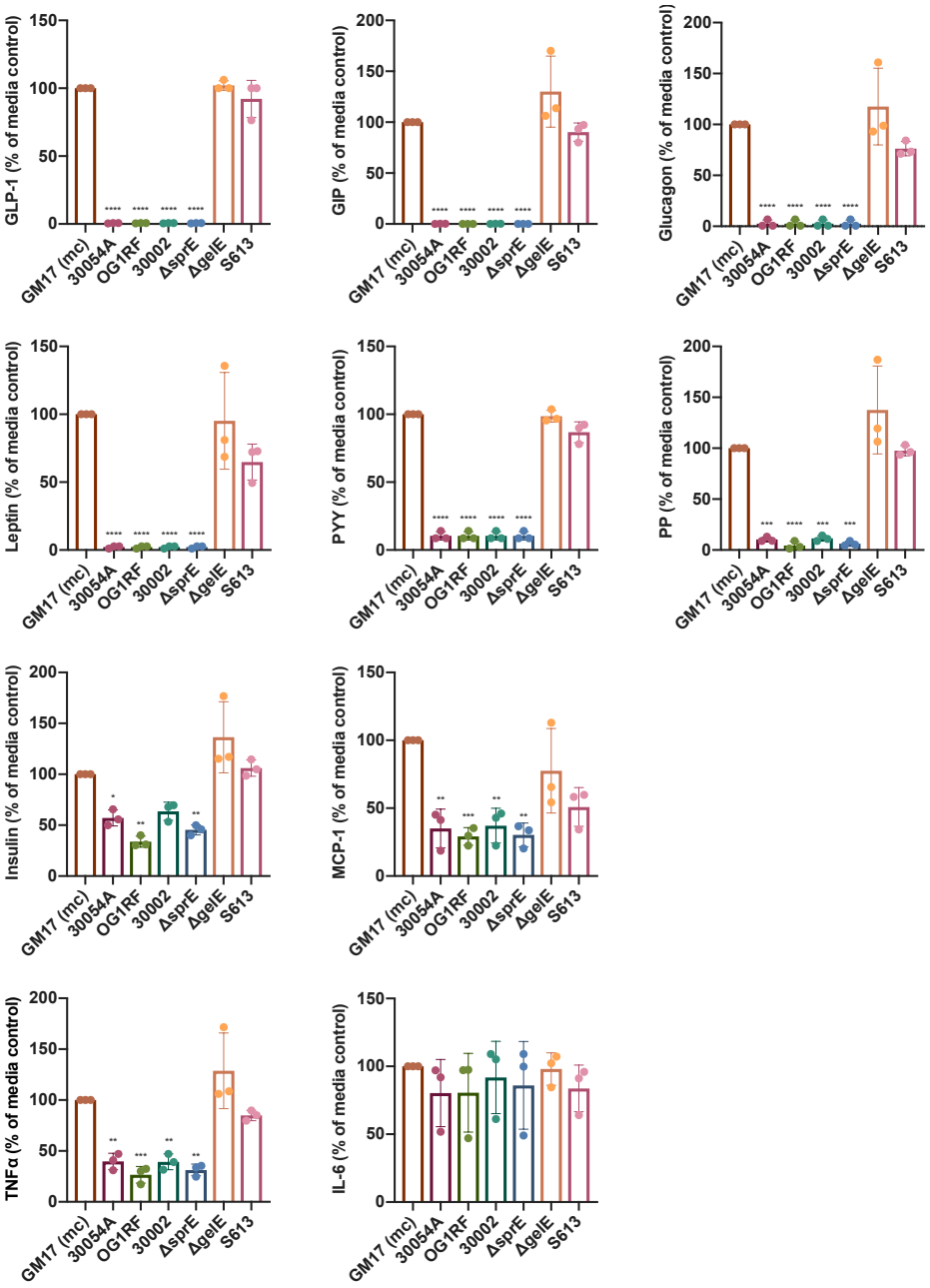


Figure 4. GelE specificity for human metabolic substrates GelE is capable of degrading many human metabolic substrates Data were obtained from three independent experiments (n = 3) and expressed as mean values ± SD (GLP-1, GIP, Glucagon, Leptin, PYY, and PP: ****p < 0.0001; Insulin: 30054A *p = 0.0351, OG1RF *p = 0.001, ΔsprE **p = 0.0058; MCP-1: 30054A **p = 0.0021, OG1RF ***p = 0.0009, 30002 **p = 0.0029, ΔsprE **p = 0.0011; TNFα: 30054A **p = 0.0038, OG1RF ***p = 0.0006, 30002 **p = 0.0036, ΔsprE **p = 0.0012). GM17 (mc), bacterial culture media control.

Table 1: Degradation of metabolic substrates by E. faecalis 30054A diluted supernatant Dilutions of supernatant from *E. faecalis* 30054A show an uneven distribution of degradation. Values represent the quantity of substrate remaining after incubation with supernatants. Data were obtained from three independent experiments (n = 3) and expressed as mean values ± SD.

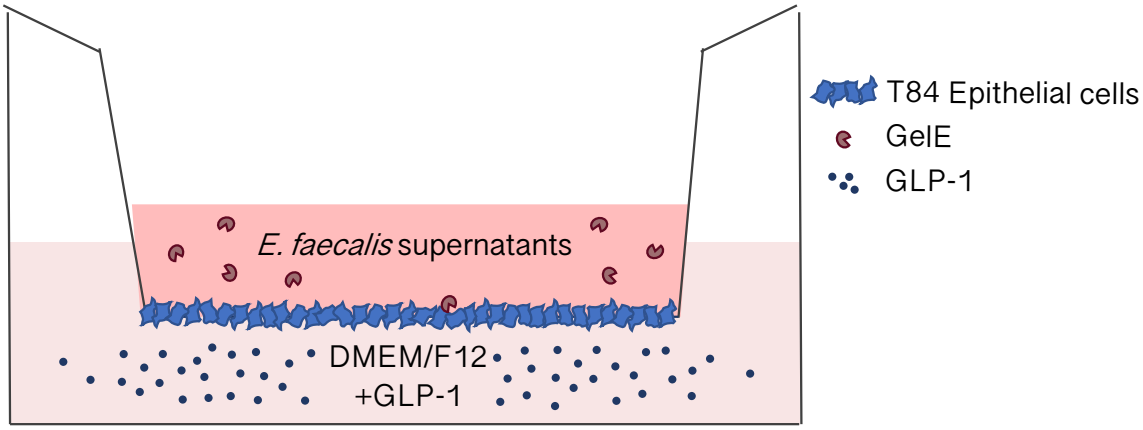
Substrate	Supernatant dilution				
	1x	0.1x	0.01x	0.001x	0.0001x
GLP-1	0.57 ± 0.13	0.71 ± 0.33	10.38 ± 3.52	90.12 ± 17.10	96.25 ± 6.50
GIP	0.17 ± 0.06	1.55 ± 1.09	43.34 ± 1.19	96.32 ± 2.55	98.40 ±1.40
Glucagon	2.66 ± 3.59	3.08 ± 3.25	31.98 ± 6.09	86.37 ± 3.87	96.74 ± 2.83
Leptin	2.15 ± 0.96	7.95 ± 4.72	72.29 ± 1.72	99.78 ± 0.39	97.77 ± 3.87
Pancreatic peptide	10.35 ± 2.36	47.49 ± 17.15	90.51 ± 3.41	99.19 ± 0.91	98.02 ± 1.70
PYY	10.45 ± 3.08	54.61 ± 13.14	94.26 ± 0.43	98.82 ± 1.30	99.02 ± 0.88
MCP-1	35.14 ± 14.14	74.03 ± 44.98	99.92 ± 0.14	99.99 ± 0.01	98.33 ± 2.89
TNFα	39.73 ± 8.05	87.87 ± 19.41	99.72 ± 0.48	99.28 ± 1.25	98.12 ± 1.67
Insulin	57.29 ± 7.69	82.24 ± 14.59	99.77 ± 0.40	99.97 ± 0.05	99.23 ± 1.33
IL-6	80.28 ± 24.77	99.78 ± 0.37	99.91 ± 0.15	95.75 ± 7.36	95.74 ± 6.94

<15% (high) degradation

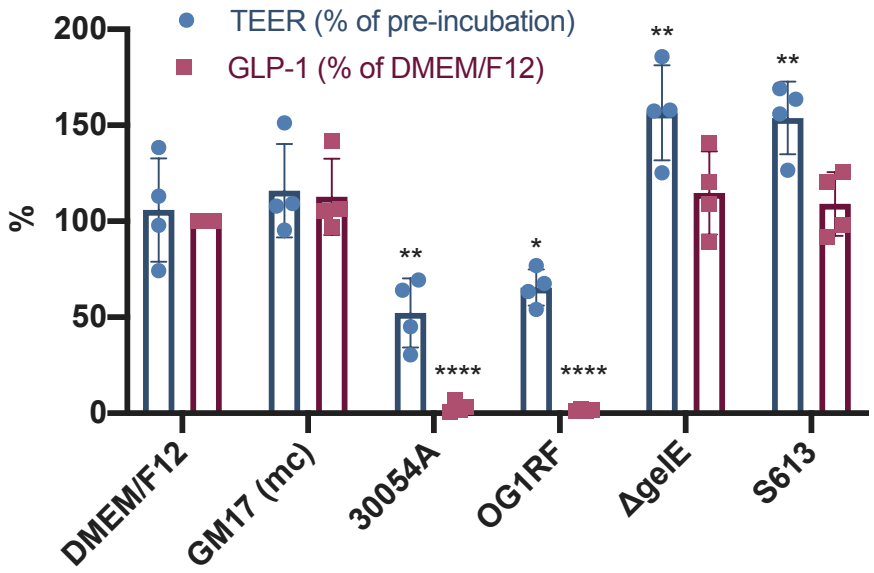
15-40% (moderate) degradation

40-60% (mild) degradation

>60% (low) degradation



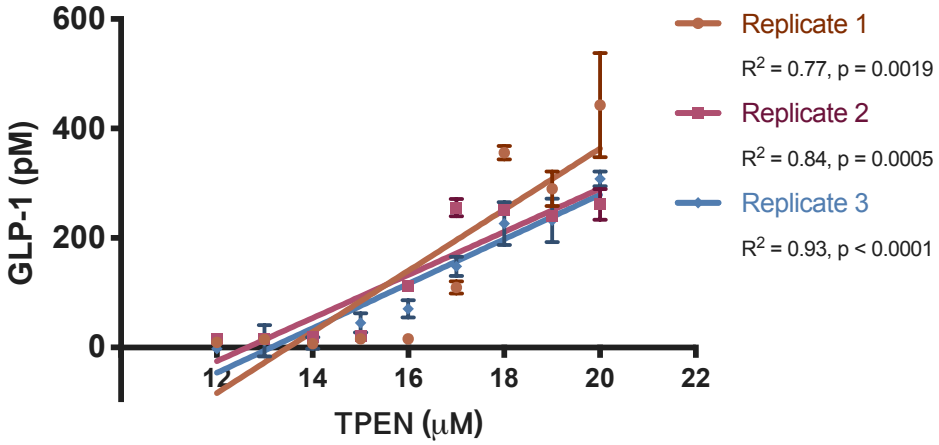
A.



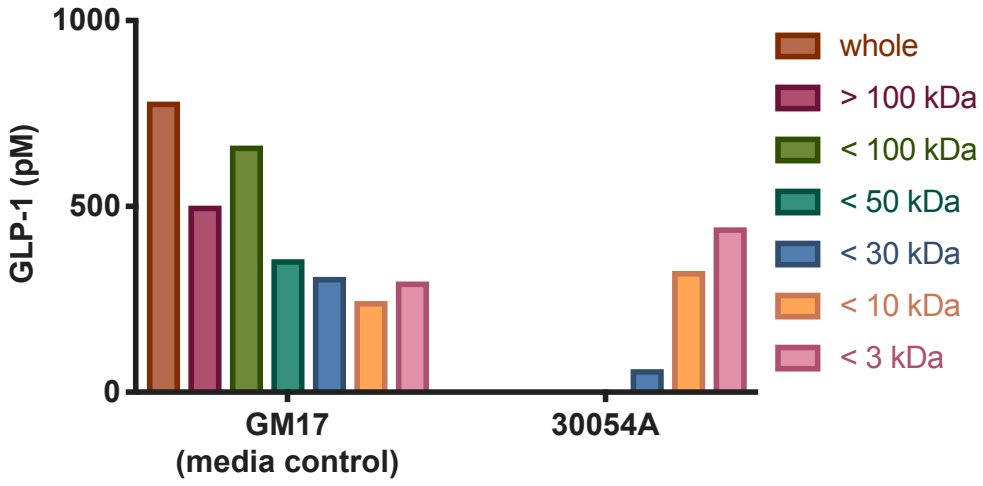
B.

Figure 5. Degradation of GLP-1 through T84 epithelial layer Schematic of experimental setup: T84 epithelial cells seeded onto transwell insert, with *E. faecalis* supernatants added to the upper chamber and GLP-1 in cell culture media (DMEM/F12) to the lower chamber (A) Schematic not drawn to scale. GelE producing supernatants decrease the integrity of a T84 epithelial cell layer while degrading GLP-1 on the basolateral side of the epithelium, compared to DMEM/F12 T84 cell culture media control (B). Data were obtained from four independent experiments and expressed as mean values ± SD (TEER: 30054A **p = 0.0022, OG1RF *p = 0.0439, ΔgelE **p = 0.0045, S613 **p = 0.0084; GLP-1: 30054A and OG1RF ****p < 0.0001). GM17 (mc), bacterial culture media control.

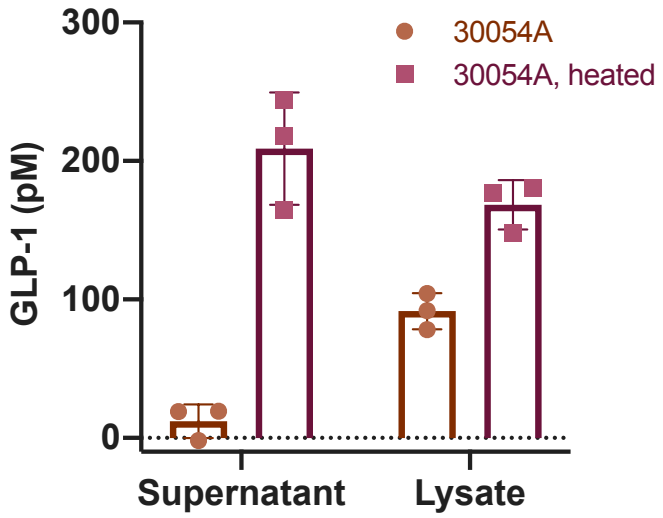
Supplemental figures:



S1. GLP-1 inhibitory activity is sensitive to TPEN. Supernatnats from *E. faecais* 30054A grown overnight with increasing amounts of metalloprotease inhibitor TPEN (higher specificity for zinc) result in a reduction of GLP-1 degradation when incubated with recombinant GLP-1, in a dose-dependent manner.



S2. GLP-1 inhibitory activity is contained in the >30 kDa size fraction. Supernatants from *E. faecalis* 30054A were fractioned using centrifugal filters of various kDa cutoffs, and then incubated on NCI H716 cells. GLP-1 inhibitory activity is maintained in all fractions >30 kDa.



473

474 ***S3. GLP-1 inhibitory activity is lost with heat treatment.*** Supernatants and whole cell lysates from *E.*

475 *faecalis* 30054A treated to 30 min at 100°C result in a reduction of GLP-1 inhibitory activity when

476 incubated with NCI H716 cells. GLP-1 inhibitory activity is primarily contained in the bacterial supernatant

477 as opposed to the cellular lysate.

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