Short chain fatty acids and microbiota metabolites attenuate ghrelin receptor signaling

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Abstract

The gastrointestinal microbiota is emerging as a unique and inexhaustible source for metabolites with potential to modulate G-Protein-coupled receptors (GPCRs). The ghrelin receptor (GHSR-1a), is a GPCR expressed throughout both the gut and the brain, with a crucial role in maintaining energy balance and metabolism as well as in central modulation of food intake, motivation, reward and mood. To date, no studies have investigated the potential of the gastrointestinal microbiota and its metabolites to modulate GHSR-1a signaling. Here we investigate the effects of short-chain fatty acids (SCFAs), lactate, and different commensal bacterial supernatants on GHSR-1a signaling, using calcium mobilization and imaging assays, receptor internalization, β-arrestin recruitment, and further downstream mTOR, ERK 1/2 and eEF2 signaling. We identify, for what is to our knowledge the first time, a potent effect of microbiota-derived metabolites on GHSR-1a signaling with potential significant consequences for host metabolism and physiology. We suggest a novel route of communication between the gut microbiota and the host via modulation of GHS-R-1a receptor signaling. Together, this study highlights, the vast and emerging therapeutic potential in the exploration of the microbiota metabolome in the specific targeting of key GPCRs, with pleiotropic actions that span both the central nervous system and periphery.

Keywords: Ghrelin, GHSR-1a, Microbiota, SCFA, Lactate, Probiotics

Introduction

Recent evidence is positioning the gut microbiota as a rich source for bioactive metabolites able to specifically target G-protein coupled receptor (GPCR) within the gut brain axis (1–6). These studies not only reveal the gut microbiome as a rich reservoir to mine ligands with a high potential to modulate several GPCRs, but also provide a targeted platform to identify the molecular mechanism by which gut microbiota can manipulate its host. Here, we investigate the potential of microbiota derived metabolites to modulate the growth hormone secretagogue receptor (GHSR-1a), also known as the ghrelin receptor. The ghrelin receptor is ubiquitously expressed throughout the gut-brain axis, and represents a major therapeutic target for several different disorders ranging from metabolic and eating disorders, including obesity, diabetes and cachexia, to affective disorders, including anxiety and depression, to even cancer (7–9). The peripheral and central effects of GHSR-1a signaling, are mediated by the endogenous ligand, ghrelin, a 28-amino acid peptide hormone, which is synthesized and secreted from the stomach (10). The gut microbiome has been demonstrated to play a crucial role in a plethora of host functionalities, many of which are overlapping with the therapeutic areas for which the GHSR-1a is being investigated, including metabolism, obesity, mood disorders and stress-vulnerabilities (11, 12). Moreover, the GHSR-1a is expressed throughout the small and large intestine (13), making it a likely target for the direct interaction with the gut microbiota and microbiota-derived metabolites, such as short chain fatty acids (SCFAs). Evidence suggests that SCFAs are able to reach the brain, highlighting the potential to modulate both peripheral and central functions mediated by GPCRs (14–17). Interestingly, a recent study showed that gut-microbiota derived acetate was able to stimulate ghrelin secretion and obesity (18) and a few studies have shown changes in circulating ghrelin levels when the microbiota is altered, reinforcing a potential association of the gut microbiota with the ghrelinergic system (19–21).

The pleiotropic physiological effects of the ghrelinergic system are both down to the prevalent distribution of the GHSR-1a and likely also due to its complex intracellular signaling, involving G-protein–dependent and –independent pathways, high basal constitutive activity and biased modulation of GHSR-1a signaling (22). Indeed, G-protein–dependent signaling of the GHSR-1a includes Gαq-dependent activation of Phospholipase C (PLC), inositol trisphosphate (IP3) and calcium release as well as Gαi-dependent activation of phosphatidylinositol 3-kinase (PI3K) while G-protein-independent signaling involves the recruitment of β-arrestin leading to receptor internalization (22). As a result, binding of ghrelin to GHSR-1a can trigger the activation of protein kinase C (PKC), AMP-activated protein kinase (AMPK), mammalian Target of Rapamycin (mTOR) and extracellular signal–regulated kinases (ERK) 1/2 signaling, stimulating pro-synthetic pathways and gene expression, cellular metabolism and proliferation (22–24).

To date, no studies have investigated the potential of gut microbial components to modulate GHSR-1a signaling in spite of its central role in gut-brain axis signaling. Therefore, in this study, we investigate different bacterial-derived metabolites from different bacterial strains, most of them belonging to the most common species often used as probiotics, *Bifidobacterium* spp. and *Lactobacillus* spp., for their capability to modulate the GHSR-1a. Regarding bacterial metabolites, we focus on SCFAs (acetate, propionate and butyrate) and lactate, because these represent some of the most common gut microbiota-derived metabolites and have been shown to exert multiple effects on host physiology including modulation of cell homeostasis, neuronal activity and immune system phenotype (11, 25, 26). SCFAs are of particular interest as they constitute the major products from bacterial fermentation of dietary fiber in the gut (27). They are present at high levels (mM) in the gut lumen (25), where they maintain gut health and play a key role in promoting intestinal epithelial barrier integrity and mucus production, and protect against intestinal inflammation (28). Some studies have also shown that SCFAs influence gastrointestinal motility (29, 30). Moreover, it has recently been demonstrated that SCFAs may influence brain function via interactions with GPCRs or histone deacetylases exerting humoral effects, indirect hormonal and immune pathways and neural routes (28).

To address the complexity of GHSR-1a signaling, we used human embryonic kidney cells (Hek293a), engineered in-house to stably express the human GHSR-1a, and tested selected bacterial supernatants along with SCFAs and lactate for their capacity to modulate inositol phosphate, mTOR, ERK1/2 and the recently identified as a ghrelin target Eukaryotic Translation Elongation Factor 2 (eEF2) signaling pathways, as well as β-arrestin recruitment and GHSR-1a internalization. Furthermore, we probed bacterial supernatants and SCFAs for their capacity to alter constitutive GHSR-1a signaling (in the absence of ghrelin) and ghrelin-induced activity of the GHSR-1a.

Methods

Short chain fatty acids (SCFAs) and lactate preparation

SCFA (sodium acetate, sodium propionate and sodium butyrate) and lactate (Sigma-Aldrich, USA) were dissolved in assay medium (HBSS containing 20mM HEPES) and pH was adjusted to 7.4 with NaOH 1 M prior to filtration through 0.2 µm membrane filters. All compounds were previously tested for cytotoxic effects in order to establish a safe dose (supporting **Figure S1**). Final doses were as follows: SCFA were tested at 1 mg/mL (acetate: 17 mM; propionate: 13.7

mM; butyrate: 11.5 mM) and lactate at 0.3 mg/mL (3.3 mM), which corresponds with the physiological concentration range as can be found in the gut lumen (28)).

Bacterial strains supernatants preparation

Different bacterial strains (*B. longum* APC1472, *B. breve* APC6331, *L. rhamnosus* DPC6118, *L. gasseri* DPC6106, *L. brevis* DPC6108 and *P. avidum* APC6544) from the APC Microbiome Institute and Teagasc Research Center (Cork, Ireland), were maintained in anaerobic culture conditions in Man Rogosa Sharpe (MRS) agar medium (Difco Laboratories, USA) (for *Bifidobacterium* strains the medium was supplemented with cysteine hydrochloride (0.05% w/v)). A single colony from each strain was inoculated in MRS broth medium and incubated overnight under anaerobic conditions at 37°C. A subculture was carried out by adjusting the OD_{600} to 0.05 and incubated overnight under anaerobic conditions at 37°C for 15 hours approx. Bacterial cell pellets were collected by centrifugation at 4500 g for 15 min at 4°C, washed twice in assay medium (1x Hanks balanced salt solution (HBSS) containing 20mM HEPES) (Gibco, UK) and incubated for 4 hours under culture conditions in assay medium. Finally, bacterial supernatants were collected by centrifugation at 4500 g for 15 min at 4°C, passed through a 0.2 µm membrane filter (Minisart®, Fisher Scientific, USA) and pH adjusted to 7.4 with NaOH 1 M. All supernatants were diluted 1:2 in assay medium. Suitability of assay medium to prepare bacterial supernatants containing microbial-derived metabolites was validated by gas chromatography analysis of SCFAs (for details see supplementary information and **Figure S2**).

Hek-GHSR-1a-EGFP cell culture

Human embryonic kidney cells (Hek293a) (Invitrogen, Ireland) were transfected with a plasmid construct expressing the human GHSR-1a receptor with a C-terminal enhanced green fluorescent protein (EGFP) tag and cultured in high glucose Dulbecco's modified Eagle's medium (DMEM, Invitrogen) containing 10% heat-inactivated fetal bovine serum (FBS) (Sigma-Aldrich), 1% non-essential amino acids (NEAA) (Gibco) and 300 ng/µl G418 (Calbiochem, Merck KGaA, UK) at culture conditions (37°C and 5% CO_2 in a humidified atmosphere) as previously described (31). Cells were grown to a confluence of >85% and subsequently split to a lower density for continued culturing.

Calcium mobilization assay

Changes in intracellular calcium (Ca^{2+}) influx were investigated using a FlexStation® 3 multiplate fluorometer (Molecular Devices Corporation, Sunnyvale, CA, USA) as previously described (32–34). Briefly, Hek-GHSR-1a-EGFP cells were seeded in black 96-well microtiter plates ($2.8*10^4$ cells/well) and maintained at culture conditions. Once the monolayer reached full confluence (~24 h), cells were incubated for a further ~24h at culture conditions in serum-free DMEM media. Cells were incubated for 90 min with assay medium (HBSS containing 20mM HEPES) and 1x Ca4 dye (Molecular Devices Corporation) (1:1 v/v), according to the manufacturer's instructions. SCFAs and lactate were dissolved in assay medium as described above. Finally, compounds were automatically added by the FlexStation® 3 during continuous fluorescent measurements for a total of 80 sec at 37°C in flex mode with an excitation wavelength of 485 nm and an emission wavelength of 525 nm. The relative increase in intracellular calcium [Ca^{2+}] was calculated as the difference between maximal and baseline fluorescence (Vmax-Vmin) and depicted as percentage relative fluorescent units (RFU) normalized to a maximal response (100% signal) obtained with the GHSR-1a agonist ghrelin (0.3 µM) (Innovagen, Sweden). Background fluorescence was obtained by cells in assay medium alone and subtracted from RFUs. Exposure to the inverse agonist [D-Arg1, D-Phe5, D-Trp7,9, Leu11]-substance P (SP-analog) (0.5 µM) (Tocris, R&D Systems, UK), which also has antagonist properties, was also carried out. Cells were treated with SCFAs and lactate in the absence or presence of the agonist ghrelin (0.3 µM) in order to study the effects on ghrelin-independent and ghrelin-dependent responses. Thus, cells were exposed to compounds alone, or to a combination of compounds with ghrelin (co-treatment) or to a pre-treatment with compounds during the 90 min of dye incubation before the automatic addition of ghrelin. SCFAs were tested at 1mg/mL and lactate at 0.3 mg/mL (see sample preparation section above). Wild type Hek293a cells were included as a control for unspecific calcium responses (see supplementary information). Data were normalized to the maximum calcium influx obtained upon treatment with the agonist ghrelin.

Inositol Phosphate assay

Inositol phosphate (IP) accumulation assay was carried out in Hek-GHSR-1a-EGFP cells using an IP-One Tb assay kit (Cisbio, USA) according to the manufacturer’s instructions. Briefly, Hek-GHSR-1a-EGFP cells were seeded in a 96-well microtiter plate at $3x10^4$ cells/well and incubated for 48 hours at culture conditions. For the last 24 hours, the medium was replaced with serum-free DMEM-high glucose medium. Cells were incubated for 1 hour with samples prepared as described above in samples preparation section. Untreated cells (cells exposed to assay medium alone) or cells exposed to ghrelin (0.5 µM) or SP-analog (0.5 uM) alone were used as controls. SP-analog (0.5 µM), was also included as control. In addition, cells were exposed to samples in presence of ghrelin in order to elucidate the effects of samples on ghrelin-mediated signaling. Thus, cells were co-exposed to samples and ghrelin for one hour or to a pre-treatment with samples for 1 hour followed by exposure to ghrelin for another hour. The ratio of two fluorescence intensities 665/620 (acceptor/donor) was read in a FlexStation® 3 reader with

Cisbio setting recommendations. Data were normalized to the maximum expected IP-one accumulation obtained upon treatment with the agonist ghrelin.

GHSR-1a internalization assay

The potential of SCFAs, lactate and bacterial supernatants to modulate the GHSR-1a was analyzed by measuring their effect on receptor internalization using Hek-GHSR-1a-EGFP cells according to previous publications (32–34). Briefly, cells were seeded in a 96-well microtiter plate at $3x10^4$ cells/well and incubated for 48 hours at culture conditions. For the last 24 hours medium was replaced with serum free DMEM-high glucose medium. Cells were exposed to the same treatments as in IP assay described above. Untreated cells (cells exposed to assay medium alone) or cells exposed to ghrelin (0.5 µM) alone were used as controls. The inverse agonist SP-analog (0.5 µM) was also included as control. Finally, cells were fixed with 4% paraformaldehyde in phosphate buffer saline (PBS) (Sigma-Aldrich) for 20 min and washed with 1x PBS. Cells were imaged in PBS using the GE Healthcare IN Cell Analyzer 1000 (GE Healthcare, UK). Receptor GHSR-1a-EGFP trafficking towards the perinuclear region was quantified by analyzing the EGFP fluorescence intensity using the INCell Analyzer Developer Toolbox V1.6 software (GE Healthcare) as previously described (33). A total of 15 fields were taken across 3 independent images in each independent experiment. Data were normalized to the maximum expected receptor internalization obtained upon treatment with the agonist ghrelin.

β-Arrestin-1 recruitment analysis

PathHunter® eXpress GHSR-1a U2OS β-Arrestin-1 GPCR Assay (Discoverx, UK) was used to investigate the effect of SCFAs, lactate and bacterial strains supernatant on GHSR-1a activation by monitoring β-Arrestin-1 proteins recruitment. The assay was performed according to the manufacturer's protocol with some modifications. Briefly, cells were incubated for 48 hours at culture conditions. Cells were exposed to same treatments as for the IP assay detailed above. Finally, detection reagents were added, cells were incubated for 60 min at room temperature and luminescence was read with the Synergy 2 (Biotek, UK). Data were normalized to the maximum expected β-Arrestin-1 proteins recruitment obtained upon treatment with the agonist ghrelin (0.5 µM).

RPPA screening of GHSR-1a signaling pathways activated by ghrelin

A reverse phase protein array (RPPA) (35) of over 300 proteins was conducted by Functional Proteomics Group of MD Anderson Cancer Centre (University of Texas, USA), as described (Zhdanov et al, 2019, unpublished). Hek-GHSR-1a-EGFP cells were seeded in a 6-well plate at 1.0 x 10^6 cells/well and incubated for 48 hours at culture conditions. For the last 16 hours, the medium was replaced with serum free DMEM-high glucose medium. Cells were treated with 0.5 µM ghrelin for 1 h. Protein extracts were prepared as described (36) with modifications. Briefly, cells were lysed in RIPA buffer (ThermoFisher Scientific) with protease and phosphatase inhibitors' cocktails (cOmplete™ ULTRA and PhosSTOP™ tablets, Roche). After a brief sonication, the lysates were cleared by centrifugation at 14000 g (15 min, +4°C). Protein yield was quantified with BCA assay (Pierce), adjusted to 1.0 mg/mL in a dye-free Laemmle buffer and heated at 95°C for 5 min. Samples were shipped for the analysis on dry ice. Three independent experiments were performed for RPPA assay. Within each experiment, the signal in ghrelin-treated cells was normalized to non-stimulated cells. Proteins displaying larger or equal to 10% change were selected for the construction of Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways in the Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.8.

Western Blot analysis of mTOR, ERK and eEF2 phosphorylation

Hek-GHSR-1a-EGFP or WT cells were seeded in a 6-well plate at 2.5 x 10^6 cells/well and incubated for 48 hours at culture conditions. For the last 24 hours, the medium was replaced with serum free DMEM-high glucose medium. Cells were treated with SCFAs, lactate and bacterial supernatants (prepared as described above) for 1 h either with or without the addition of agonist ghrelin (0.5 µM). SP-analog (0.5 µM) was also included as a control. Additionally, cells were exposed to a 1 h pre-treatment with SCFAs, lactate, bacterial supernatants and SP-analog, followed by exposure to ghrelin for another 1 h. Protein extracts were prepared as for the RPPA analysis. Protein concentration was adjusted to 1.5 – 2.0 mg/mL with 5X Sample Buffer (Genscript). Western blot analysis was conducted as described (36). Briefly, 50 μg of protein were loaded onto 4-20% Bis-Tris Gels in Tris-MOPS running buffer (Gensript) and wet-transferred to Immun-Blot® PVDF membrane (BioRad). The membranes were blocked with 5% BSA, and then incubated overnight at 4°C with primary antibodies against phospho-mTOR (Ser2448) (Cell Signalling, #2971), phospho-ERK1/2 (Thr202/Tyr204) (Cell Signalling, #9101) and phospho-eEF2 (Thr56) (Cell Signalling, #2331). Incubation with the HRP-conjugated secondary antibodies (Sigma) was done in 5% milk for 1 h at room temperature. Protein bands were visualized using Amersham ECL Prime detection system (GE Healthcare) on the LAS-3000 Imager (FujiFilm). The membranes were further re-stained against alpha-tubulin as a loading control (Sigma, T5168). Quantitative analysis was done in ImageJ-FijI software (NIH). The density of target protein bands was normalized to alpha-tubulin. Three independent experiments were performed for each treatment. Within each experiment, the signal in treated cells was normalized either to non-stimulated control cells (if cells were treated with compounds in the absence of ghrelin) or to ghrelin-stimulated cells (if cells were treated with compounds in the presence of ghrelin). These values were used for the statistical analysis. Western Blot data were plotted as Median and IQR (box) with minimal and maximal values as whiskers.

Statistical analysis

Data were plotted using GraphPad Prism software (PRISM 5.0; GraphPAD Software Inc., San Diego, CA, USA) and statistical analysis was performed using SPSS software (IBM SPSS statistics 22). Normality of the data was tested by Shapiro-Wilk test. Normally distributed data was analyzed with One-Way ANOVA followed by Dunnett post-hoc test for multiple comparisons. Non-normally distributed data was analysed with non-parametric multiple comparisons Kruskal-Wallis test followed by Bonferroni adjustment of p-values. Western blot data were analyzed with non-parametric Mann-Whitney U test; the Benjamini-Hochberg adjustment procedure was applied with the false discovery rate (FDR) set at 10% to correct for multiple testing. *indicates significant differences vs untreated control (comparisons between controls and non-ghrelin treated samples) (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$); \$indicates significant differences vs ghrelin control (comparisons between samples and ghrelin control) (\$$p \leq 0.05$, \$\$$p \leq 0.01$, \$\$\$$p \leq 0.001$).

Results

Effects of SCFAs and lactate on ghrelin receptor signaling

Hence, we investigated their capability to modulate GHSR-1a signaling by testing their effects on GHSR-1a-mediated intracellular calcium influx in our well stablish *in vitro* screening platform using Hek293a cells stably overexpressing this receptor. Cells were exposed to SCFAs (acetate, propionate and butyrate) and lactate (at 1 mg/mL and 0.3 mg/mL respectively), or to SP-analog (0.5 μM) in presence or absence of the endogenous ligand ghrelin (0.3 μM). First, calcium mobilization was analyzed on Hek293a WT cells without overexpression of the GHSR-1a, to control and rule out any potential unspecific responses mediated by SCFAs. No effects were observed on Hek293a WT cells (Supporting **Figure S3**). As expected, exposure to ghrelin (0.3 µM) led to a significantly ($p \leq 0.001$) increased calcium influx compared to untreated cells (**Figure 1A**). Exposure to SCFAs, lactate or to SP-analog in the absence of the agonist ghrelin, did not lead to any significant change in intracellular calcium influx compared to untreated cells, indicating that none of these compounds act as agonist of the GHSR-1a (**Figure 1A**). However, when cells were exposed to propionate, butyrate or lactate in presence of ghrelin (co-treatment), a significant decrease ($p \leq 0.001$, $p \leq 0.05$ and $p \leq 0.001$ respectively) in ghrelin-mediated calcium influx was observed (**Figure 1A**). Exposure to acetate also showed a strong trend ($p=0.06$) towards reduced ghrelin-mediated calcium influx. The inverse agonist SP-analog did not demonstrate an antagonist effect on GHSR-1a-mediated calcium mobilization (**Figure 1A**). Interestingly, when cells were exposed to SCFAs or lactate for 1 hour prior to the addition of ghrelin (pre-treatment), no antagonist effect could be observed on GHSR-1a-mediated calcium influx (**Figure 1A**). In contrast, pre-treatment with SP-analog led to a significantly ($p \leq 0.001$) increased ghrelin-mediated calcium influx (**Figure 1A**) as previously described (32, 34). The intracellular calcium release from the endoplasmic reticulum stores (ER) is characteristic of the activation of Gαq protein coupled receptors, and it is triggered by D-myo-inositol (1,4,5) tri-phosphate (IP3) following the activation of phospholipase C β (PLC-β) and second messengers diacyl glycerol (DAG) (37). The lifetime of IP3 is very short (less than 30 sec), and rapidly degraded to IP2 and then IP1 (38, 39). Therefore, to further investigate the effects of SCFAs and lactate on GHSR-1a-mediated calcium signaling, IP1 accumulation assay was also carried out in these cells (**Figure 1B**). Exposure to the agonist ghrelin (0.5 µM) led to a significantly increased

IP1 accumulation indicating activation of the GHSR-1a (**Figure 1B**). Treatment with the inverse agonist SP-analog for one hour in the absence of ghrelin decreased significantly ($p \leq 0.001$) the accumulation of IP1 compared to untreated cells (**Figure 1B**), which is in agreement with previous publications (40, 41). Lactate showed the same effect as SP-analog ($p \leq 0.05$) and decreased IP1 accumulation, indicating its potential to act as an inverse agonist for this specific pathway (**Figure 1B**). However, exposure to SCFAs did not affect IP1 accumulation, although in the case of butyrate, a non-significant reduction ($p=0.07$) was observed towards a reduction of IP1 accumulation compared to untreated cells (**Figure 1B**). In addition, none of the SCFAs nor lactate were able to modulate IP1 accumulation in the presence of ghrelin. Finally, using calcium imaging, it was also shown that the SCFAs, acetate, butyrate and to a lesser extent, propionate, were able to inhibit ghrelin-mediated calcium response either when following a co-treatment or after a pre-treatment for 1 hour prior to the addition of ghrelin (Supporting **Figure S4**). This is in line with the calcium mobilization assay (**Figure 1A**).

The GHSR-1a has a very high ligand-independent constitutive activity (~50%) (41–43) and, as a consequence of this high basal activity, is internalized into endosomes. This is a mechanism to prevent receptor overstimulation, which following β-arrestin recruitment, desensitizes and internalization the GHSR-1a and marks it for degradation or recycling back to the membrane (44). Therefore, we next investigated the effect of bacterial metabolites and supernatants on GHSR-1a internalization. Treatment with ghrelin (0.5 µM) for one hour led to a significantly ($p \leq 0.001$) increased internalization compared to untreated cells (**Figure 2A, 2B**). In contrast, exposure to SP-analog (0.5 µM) showed a level of GHSR-1a internalization below ($p \leq 0.05$) the basal levels observed in untreated cells, which is characteristic of inverse agonists (45) (**Figure 2A, 2B**). Interestingly, exposure to acetate (1 mg/mL) also showed inverse agonist properties on this signaling pathway as it significantly ($p \leq 0.01$) reduced GHSR-1a internalization compared to untreated cells (**Figure 2A, 2B**). No effects were observed when cells were treated with the other bacterial-derived metabolites used here (**Figure 2A, 2B**). In order to elucidate if SCFAs or lactate may have antagonist activity, ghrelin-mediated receptor internalization was also investigated in the presence of these compounds. Remarkably, compounds showed capability to inhibit ghrelin-mediated receptor internalization (**Figure 2A, 2B**) either when cells were exposed to these compounds and ghrelin for 1 hour (SP-analog: $p \leq 0.001$; acetate: $p \leq 0.001$; propionate: $p \leq 0.01$; lactate: $p \leq 0.001$) or when cells were first pre-treated with these compounds for one hour followed by addition of ghrelin for another hour (acetate: $p \leq 0.001$; propionate: $p \leq 0.01$; butyrate: $p \leq 0.05$; lactate: $p \leq 0.001$) (**Figure 2A, 2B**), indicating potential GHSR-1a antagonist properties, as seen in calcium mobilization assays also (**Figure 1**). Finally, SCFA and lactate-mediated effects on β-arrestin-1 recruitment, which is one of the main proteins involved in the endocytosis process (42), were also investigated (**Figure 2C**). Exposure to the agonist ghrelin (0.5 µM) for one hour led to significantly ($p \leq 0.01$) increased β-arrestin-1 recruitment compared to untreated cells (**Figure 2C**). However, exposure to the inverse agonist SP-analog at 0.5 µM did not show any effect but it did block ghrelin-mediated β-arrestin-1 recruitment when incubated as a co-treatment with ghrelin for one hour (**Figure 2C**). Pre-treatment with acetate showed a potentiation effect ($p \leq 0.001$) of ghrelin-mediated β-arrestin-1 recruitment while lactate and ghrelin co-treatment led to a significant reduction in ghrelin-mediated β-arrestin-1 recruitment at the tested concentrations (**Figure 2C**). Exposure to propionate and butyrate did not show any effect (**Figure 2C**).

Effects of bacterial supernatants on ghrelin receptor signaling

Next, we aimed to elucidate whether SCFAs and/or lactate-producing bacterial strains may have effects on GHSR signaling similar to those observed when testing these compounds directly on the cells. Hence, six supernatants from different bacterial strains, most of these belonging to common probiotic genus such as *Bifidobacterium* and *Lactobacillus*, were investigated. We first tested their effects on the GHSR internalization assay. We identified a bacterial strain, *Bifidobacterium longum* APC1472, that was able to reduce ($p \leq 0.001$) the basal levels of GHSR-1a internalization with a similar efficacy than the inverse agonist SP-analog (**Figure 3A, 3B**). Moreover, this strain also showed a strong ($p \leq 0.001$) inhibition of ghrelin-mediated GHSR-1a internalization (**Figure 3A, 3B**). In contrast, another *Bifidobacterium* strain, *Bifidobacterium breve* APC6331, did not show capability to reduce basal levels of receptor internalization, but was able to inhibit ghrelin-mediated GHSR-1a internalization either when added together with ghrelin (co-treatment, $p \leq 0.001$) or when added 1hour prior addition of ghrelin (pre-treatment, $p \leq 0.01$). *Lactobacillus strains* (*Lactobacillus rhamnosus* DPC6118, *Lactobacillus gasseri* DPC6106 and *Lactobacillus brevis* DPC6108) were, overall, less active towards GHSR signaling compared to the *Bifidobacterium* strains. Thus, the only significant effect observed was an inhibition of ghrelin-mediated receptor internalization when cells were exposed to ghrelin and the supernatants from the strains *L. rhamnosus* DPC6118 (co-treatment, $p \leq 0.001$) and *L. gasseri* DPC6106 (co-treatment, $p \leq 0.05$) (**Figure 3A, B**). Propionate-producing bacterial strain, *Propionibacterium avidum* APC6544, was also able to inhibit ghrelin-mediated internalization in both assay settings (co-treatment, $p \leq 0.001$; pre-treatment, $p \leq 0.01$) (**Figure 3A, B**). The most active strains, *B. longum* APC1472, *B. breve* APC6331, *L. rhamnosus* DPC6118 *P. avidum* APC6544, were selected for further investigations. In contrast to the internalization assay, the effects observed on β-arrestin-1 recruitment were less prominent (**Figure 3C**). Here, the only observed effect was an inhibition of ghrelin-mediated β-arrestin-1 recruitment after pre-treatment with bacterial strains supernatants (*B. longum* APC1472: p≤0.001; *B. breve* APC6331: p≤0.05, *L. rhamnosus* DPC6118: p≤0.05; *P. avidum* APC6544: p≤0.05). In terms of calcium signaling, only *L. rhamnosus* DPC6118 showed a significant (p≤0.05) increased ghrelin-mediated IP1 accumulation when exposed to cells together with ghrelin for 1 hour (co-treatment) (**Figure 3D**).

Effects of SCFAs and lactate on ghrelin-induced mTOR and ERK signaling

According to gene expression and functional analyses, Hek293a cells are often considered as a neural cell lineage (46). On the other hand, the pattern of protein phosphorylation induced by ghrelin can vary even for closely related neuronal cells. Therefore, before addressing the question how SCFA modulate the effect of ghrelin on intracellular signaling, we selected the best responders to ghrelin treatment in our Hek-GHSR-1a-EGFP cell line, using the RPPA analysis. Among 340 proteins tested (both total and phosphorylated), 42 were affected by 1 h treatment with ghrelin (Supporting **Table S1**). This data helped to build a cumulative map of ghrelin-induced signaling based on KEGG pathways analysis, which was conducted using DAVID v6.8 online bioinformatics resource (**Figure 4**). We found that pro-synthetic pathways were overall activated upon ghrelin-mediated GHSR-1a signaling stimulation, while processes up-regulating apoptosis, autophagy and cell cycle control, were inhibited. In particular, mTOR (including mTOR, Akt, Rictor, S6K) and ERK1/2 (including ERK1/2, Raf, RSK) pathways, the hallmarks of the signaling response to ghrelin, were activated in Hek-GHSR-1a-EGFP cells (**Figure 4; Supporting Figures S5**, **S6**). Hence, phosphorylation levels of mTOR and ERK were selected as signaling readouts for further analysis, together with phosphorylation status of eEF2, which has been recently shown to be involved in ghrelin-dependent increase in protein synthesis (Zhdanov et al, 2019, under review). Using Western blotting analysis, we examined effects of SP-analog, SCFAs and lactate on ghrelin-mediated mTOR signaling activation. Treatment with ghrelin increased ($p \leq 0.01$) p-mTOR levels compared to untreated cells (**Figure 5A,** middle and lower panel). However, none of the tested compounds affected mTOR phosphorylation in either the presence or the absence of ghrelin. Next, stimulation with ghrelin induced a dramatic increase ($p \leq 0.01$) in ERK1/2 phosphorylation levels (**Figure 5B**, middle and lower panel). While SP-analog itself demonstrated a negative effect ($p \leq 0.01$) on the activity of ERK, all three SCFAs increased ERK1/2 phosphorylation, suggesting pre-disposition of cells to a stronger response to ghrelin (**Figure 5B**, upper panel). Indeed, when applied to cells together with or prior to ghrelin addition, acetate and propionate significantly ($p \leq 0.05$) potentiated the effect of ghrelin on ERK1/2 phosphorylation (**Figure 5B**, middle and lower panel). In cells pre-treated with butyrate or lactate, ghrelin was able to increase ($p \leq 0.05$) p-ERK1/2 levels (**Figure 5B**, lower panel). Treatment with ghrelin activated eEF2 by decreasing ($p \leq 0.05$) its phosphorylation levels (**Figure 5C**, middle and lower panel**)**. Unlike ghrelin, SP-analog ($p \leq 0.01$) and lactate ($p \leq 0.05$), when acting alone, inhibited eEF2 by increasing its phosphorylation (**Figure 5C**, upper panel**)**. Moreover, ghrelin was unable to re-activate eEF2 in the presence of lactate (**Figure 5C**, middle and lower panel**)** (**Figure 5C**, middle and lower panel**)**. SCFAs did not change eEF2 phosphorylation and did not modulate the effect of ghrelin on it. Unexpectedly, SP-analog potentiated ($p \leq 0.01$ and $p \leq 0.05$ for co-treatment and pre-treatment respectively) the decrease in p-eEF2 levels induced by ghrelin (**Figure 5C**, middle and lower panel**)**.

Effects of bacterial supernatants on ghrelin-induced mTOR and ERK signaling

Bacterial strains supernatants were also tested as potential modulators of the effect of ghrelin on mTOR, ERK and eEF2 phosphorylation in Hek-GHSR-1a-EGFP cells (**Figure 6**). While exposure to ghrelin led to significant ($p \leq 0.01$) increased levels of p-mTOR (**Figure 6A,** middle and lower panel), none of the supernatants nor SP-analog showed any effect on this pathway signaling (**Figure 6A**). SP-analog showed a strong inhibition ($p \leq 0.01$) of ERK1/2 phosphorylation compared to untreated cells (**Figure 6B,** upper panel) and a significant reduction ($p \leq 0.05$) of ghrelin-mediated ERK1/2 phosphorylation when added together with ghrelin (**Figure 6B**, middle panel). Exposure to *L. rhamnosus* supernatant led to a significant reduction ($p \leq 0.05$) on ghrelin-mediated ERK1/2 phosphorylation when doing either co-treatment or pre-treatment prior ghrelin addition (**Figure 6B,** middle and lower panel). The opposite effect was observed following pre-treatment with *B. longum* and *B. breve* supernatants. Here, the ghrelin-mediated activation of ERK was significantly enhanced ($p \leq 0.05$) compared to cells treated with ghrelin alone, as demonstrated by increased p-ERK1/2 levels (**Figure 6B,** middle and lower panel). Finally, increased p-eEF2 levels were observed following exposure to SP-analog ($p \leq 0.01$) and *P. avidum* supernatant (**Figure 6C,** upper panel). Exposure to SP-analog potentiated ghrelin-mediated reduction in p-eEF2 levels either in co-treatment ($p \leq 0.01$) or following pre-treatment prior to ghrelin addition ($p \leq 0.05$) (**Figure 6C,** middle and lower panel). Strikingly, the attenuating effect of ghrelin on eEF2 phosphorylation was blocked following pre-treatment with the four bacterial strain supernatants (**Figure 6C**).

Discussion

The ghrelinergic system, through the activation of the GHSR-1a, is crucial for a diversity of peripheral functions ranging from gastric motility, gut hormones secretion, glucose and lipid metabolism, pancreatic function, immune function, cardiac output, to even bone formation (8, 47–54). Moreover, ghrelin and the GHSR-1a also modulate several signaling processes in the brain, such as appetite regulation, reward and neuropeptide release (including growth hormone, adrenocorticotropic hormone, cortisol, and prolactin), mood, memory, learning, and stress response (7, 9, 54–58). Interestingly, changes in circulating levels of ghrelin have been linked to changes in gut microbiota composition, suggesting that the ghrelinergic system may be under regulation of the gut commensal microbes (20, 21). Here, we show, to our knowledge for the first time, that bacteria-derived SCFAs (acetate, propionate and butyrate) and lactate, as well as bacterial supernatants of the *Bifidobacterium* and *Lactobacillus* genera, are able to affect GHSR-1a signaling. Butyrate, propionate, acetate and lactate were all able to inhibit ghrelin-mediated calcium mobilization in cells expressing the GHSR-1a (**Figure 1A and Figure S4**). This points to an important novel functionality for microbiota-derived SCFAs in gut-brain axis signaling and demonstrates the potential of commensal bacteria metabolites as key components of host interactions through the modulation of host GPCR signaling. In line with this, is the recent finding that bacteria-derived N-acyl amides fatty acids can mimic human GPCR signaling molecules also (2). In addition, a forward chemical genetic screen, recently demonstrated an orthogonal approach to uncover biologically relevant host-microbiota metabolome interactions (1). They screened the human microbiota metabolome against the GPCRome and showed that bacterial metabolites can indeed function as agonists for GPCRs, reinforcing the potential powerful effect that commensal gut microbes can exert on host physiology. Moreover, SCFA have already been shown to be able to modulate the free fatty acid receptor 2 (FFAR2) and FFAR3, which are also GPCRs (60). They are mainly expressed in the periphery but evidences exist for FFAR3 to be also expressed in the central (CNS) and peripheral nervous system (PNS) (28). Indeed, the effect of SCFAs on appetite suppression via the vagus nerve has been suggested to be mediated by their interaction with the FFAR3 receptor present in nodose ganglion neuros (61). Together, these findings suggest that SCFAs may indeed affect the central and peripheral nervous system via modulation of GPCR signaling within the gut-brain axis (28).

Interestingly, in our assay, the antagonistic activity of SCFAs on GHSR-1a-induced calcium influx were not observed when cells were pre-treated with SCFAs before addition of ghrelin (**Figure 1A**). Likewise, calcium imaging also demonstrated that the inhibitory effect of the SCFAs was less pronounced when cells were pretreated with SCFAs compared to the inhibitory effect observed upon simultaneous addition of SCFAs and ghrelin (Supplementary **Figure S4**). This may suggest that during the pre-incubation time, bacterial-derived metabolites are used as energy source and become exhausted (57). Moreover, the inhibitory effect of SCFAs on ghrelin-mediated signaling was not observed when looking at IP accumulation (**Figure 1B**). This discrepancy in calcium mobilization and IP1 accumulation assays may be explained by the source of calcium measured in these two different assays. Indeed, the IP1 accumulation assay measures calcium release from endoplasmic reticulum (ER) only (38, 39). In contrast, the calcium mobilization assays measure the total intracellular calcium influx, derived from both the ER stores as well as calcium influx produced by the direct interaction of GPCRs with calcium channels (63, 64). This may suggest that SCFAs specifically inhibit GHSR-1a-mediated modulation of calcium channels. This inhibitory effect may have significant relevance for neuronal function as constitutive and agonist-dependent GHSR-1a activation has previously been shown to inhibit voltage-gated calcium channels CaV2.1 and CaV2.2 currents in rat and mouse hypothalamic neurons leading to inhibitory effects on depolarization (65, 66). In addition, all SCFAs and lactate also showed inhibitory effects on subsequent ghrelin-mediated GHSR-1a internalization, reinforcing their potential as attenuators of ghrelin-mediated GHSR-1a trafficking (**Figures 2A, 2B**). Moreover, acetate showed capability to decrease the basal levels of receptor internalization, indicating inverse agonist properties. Overall, this was in accordance with what we observed with the bacterial strains supernatants (**Figure 3**), where *B. longum* APC1472 was the most active strain since its supernatant showed both a decrease in the high basal GHSR-1a internalization and a complete inhibition of ghrelin-mediated receptor internalization which correlates with its higher content in acetate (**Figure S3**) and indicates a potent inhibition of ghrelinergic signaling. Interestingly, a positive correlation was observed between acetate content in bacterial supernatants that showed an inhibitory effect on GHSR-1a internalization (unpublished data**).** In contrast, while SP-analog was equally able to decrease basal internalization, it was unable to block ghrelin-mediated receptor internalization. Interestingly, pre-incubation with acetate or bacterial strains supernatants potentiated the ghrelin-mediated β-arrestin recruitment while co-treatment with lactate inhibited it (**Figures 2C**, **3C**). No others effects were observed with the others bacterial metabolites. The observed differences in β-arrestin recruitment may also suggest involvement of β-arrestin independent GHSR-1a internalization via interactions with other proteins like μ-AP2 (41, 43).

In agreement with previously published data, the results of RPPA analysis (**Figure 4**) showed that activation of GHSR-1a strongly affects the pattern of cellular protein phosphorylation (67, 68). This suggests that ghrelin-induced signaling cascades may affect transcription and translation factors such as NFkB, ERK, mTOR and eEF2. Thus, we next investigated effects of

SCFAs on the phosphorylation and activation of these factors. In our experimental conditions, phosphorylation of mTOR, a master-regulator of protein synthesis and the key responder to ghrelin treatment, remained unaffected by SCFA, lactate or bacterial supernatants (**Figures 5A, 6A**). A possible explanation is the fact that mTOR lies on a crossroad of many positive and negative regulatory pathways (69), which could compensate for each other, suggesting that all tested metabolites and, particularly, bacterial supernatants of unknown composition may trigger numerous pathways. In contrast, ERK1/2 demonstrated a strong response following SCFAs exposure, and increased ERK phosphorylation (in agreement with (70)), as well as increased ghrelin-mediated ERK activation was observed, with acetate and propionate as the most effective mediators (**Figure 5B**). This demonstrates, that the interplay between ghrelin and SCFAs is complicated and further detailed analysis is warranted, including various timing and concentration ranges. We believe that signaling pathways involving ERK, and in particular, upstream kinases driving ERK1/2 phosphorylation, are the most important targets for further analysis of SCFA / ghrelin crosstalk.

The effects of bacterial supernatants were more ambiguous, most likely because they represent complex cocktails containing SCFAs and / or lactate among other bioactive molecules. In general, being neutral towards ERK phosphorylation on their own, bacterial supernatants decreased or tended to decrease the effect of ghrelin when added together with ghrelin (co-treatment). Exposure to supernatants from *Bifidobacterium* spp. for 1hour prior addition of ghrelin potentiated the effect of ghrelin on p-ERK levels, in contrast to that of *L. rhamnosus* DPC6118 (**Figure 6B, lower panel**). Further analysis is required to reveal actual driving forces of these effects at molecular levels.

As we have recently shown, mTOR/p70S6K and, potentially, ERK/RSK axes are involved in ghrelin-induced activation of e-EF2 (via inhibition of its only kinase eEF2K) with a sequential rapid activation of protein synthesis (Zhdanov et al, 2019, under review). The eEF2K activity is regulated by numerous factors (e.g. AMPK, ERK, mTOR, Ca^{2+}), which are triggered by ghrelin and many other stimuli, with an unknown outcome for eEF2 phosphorylation when they applied in combinations. Here we observed no effect of SCFAs on ghrelin-driven activation on eEF2, while lactate suppressed it (**Figure 5C**). Similarly, only lactate acted as an inhibitor of eEF2 phosphorylation when used alone. While we cannot fully explain these phenomena, together with the observed differences between effects of SCFAs and lactate on IP1 and β-arrestin (**Figures 1B, 2C**), they may suggest that lactate can trigger unique signaling and metabolic pathways. On the other hand, phosphorylation status of eEF2 is very sensitive to any stress or metabolic perturbation imposed to cells, and therefore pre-treatment with lactate, as well as with bacterial supernatants (**Figure 6C**) could alter metabolic equilibrium in the treated cells.

In this study, the analysis of mTOR, ERK and eEF2 phosphorylation status suggests that SCFAs, lactate and bacterial supernatants mediate ghrelin-dependent signaling cascades through the GHSR-1a, which can rapidly affect cellular functions and gene expression at the mRNA translation level (exemplified by eEF2). Moreover, RPPA analysis showed that a one-hour treatment with ghrelin activates a number of transcriptional factors (e.g. FOXO, NFkB, c-Jun) (**Figure 4**, **Table S1**), which in turn can trigger a slower gene expression response, acting at DNA transcription level. Future analysis of these factors is of a particular interest for biological models with a long-term / steady increase in ghrelin levels (e.g. cancer).

In conclusion, we have identified for the first time the ability of bacterial-derived metabolites (i.e. butyrate, propionate, acetate and lactate) and bacterial strain supernatants to affect the complex signaling cascades of ghrelin-mediated GHSR-1a activation. Together, these results highlight the promising therapeutic potential of the gastrointestinal microbiota as an extensive source for metabolites targeting specific GPCRs within the gut-brain axis, affecting both the central nervous system and periphery. Further investigation is needed to clarify the mechanism at molecular levels following SCFA-mediated modulation of the ghrelinergic system and the functional consequences on energy balance, metabolism, food intake, motivation, reward and mood. Together, this study highlights the promising potential of the gastrointestinal microbiota and its metabolites in the development of novel therapeutic strategies to treat several metabolic and affective disorders involving GHSR-1a signaling.

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Author Contributions

HS, JFC, CTF, BLR, AVG and TGD were involved in the conception of the research; CTF, AVG, AVZ, SW and SA performed experiments and analyzed data; CTF, HS and AVG wrote the manuscript; and BLR, DBP, CS and PR contributed with reagents and analytical tools. HS led the conception of all experiments, supervised the work and edited the manuscript.

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Figure Legends

Figure 1. SCFAs and lactate effects on calcium signaling in Hek-GHSR-1a-EGFP cells. (A) Intracellular calcium influx and (B) inositol phosphate (IP) accumulation in Hek cells stably overexpressing the GHSR-1a. Cells were exposed to the endogenous ligand ghrelin (0.3 µM, ghrl), GHSR-1a inverse agonist, [D-Arg1, D-Phe5, D-Trp7,9, Leu11]-substance P (0.5 µM, SP-analog), SCFAs (1 mg/mL) or lactate (0.3 mg/mL) following different treatments: SCFAs/lactate alone (1 h incubation), co-treatment with ghrelin (1 hour incubation) or pre-treatment with SCFAs/lactate (1 hour) before ghrelin addition (1 hour). Untreated cells and cells exposed to ghrelin alone were included as controls. Graphs represent the mean ± SEM from at least three independents experiments with each sample performed at least in duplicate. Data is depicted as a percentage of maximal response as elicited by the positive control ghrelin. *$p \leq 0.05$ vs untreated control (comparisons between controls and non-ghrelin treated samples); \$$p \leq 0.05$ vs ghrelin control. For incubation times and statistical details for each assay see methods section.

Figure 2. SCFAs and lactate effects on GHSR-1a internalization. (A) Representative images are depicted following different treatments: SCFAs/lactate alone (1 h incubation), co-treatment with ghrelin (1 hour incubation) or pre-treatment with SCFAs/lactate (1 hour) before ghrelin addition (1 hour) and **(B)** their quantified fluorescence intensity (15 pictures per treatment) of perinuclear GHSR-1a-EGFP receptor. **(C)** β-arrestin-1 recruitment analysis expressed as relative light units (RLU). Graphs represent the mean ± SEM from at least 2 independent experiments with each treatment performed in triplicates. Untreated control (assay buffer), ghrelin (0.5 µM), inverse agonist, [D-Arg1, D-Phe5, D-Trp7,9, Leu11]-substance P (0.5 µM, SP-analog), SCFAs

(1 mg/mL) and lactate (0.3 mg/mL). *$p \leq 0.05$ vs untreated control (comparisons between controls and non-ghrelin treated samples); \$$p \leq 0.05$ vs ghrelin control. For statistical details, see methods section.

Figure 3. Bacterial strains supernatants effects on GHSR-1a signaling. (A) Representative images are depicted following different treatments: bacterial supernatants alone (1 h incubation), co-treatment with ghrelin (1 hour incubation) or pre-treatment with supernatants (1 hour) before ghrelin addition (1 hour) and **(B)** their quantified fluorescence intensity (15 pictures per treatment) of perinuclear GHSR-1a-EGFP receptor from at least 5 independent experiments with each treatment performed in triplicates. **(C)** β-arrestin-1 recruitment analysis expressed as relative light units (RLU) from 2 independent experiments with each treatment performed in triplicates. Graphs represent the mean ± SEM. Untreated control (assay buffer), ghrelin (ghrl, 0.5 µM), inverse agonist [D-Arg1, D-Phe5, D-Trp7,9, Leu11]-substance P (SP-analogue, 0.5 µM) and diluted bacterial strain supernatants (1:2 v/v in assay buffer; bacterial strains: *B. longum* APC1472, *B. breve* APC6331, *L. rhamnosus* DPC6118, *L. gasseri* DPC6106, *L. brevis* DPC6108 and *P. avidum* APC6544). *$p \leq 0.05$ vs untreated control (comparisons between controls and non-ghrelin treated samples); \$$p \leq 0.05$ vs ghrelin control. For statistical details see methods section.

Figure 4. GHS-R1a-mediated activation of mTOR and ERK1/2 signalling pathway induced by ghrelin in Hek293a-GHSR-1a-EGFP cells. Stimulation of GHSR-1a with agonist ghrelin (0.5 µM) induced numerous changes in protein expression and phosphorylation levels (see also Suppl. Table X for details); many of the observed changes merged as components of mTOR and

ERK1/2 signalling network. The pathway network was constructed in the Database for Annotation, Visualization and Integrated Discovery (DAVID) based on the results of RPPA analysis.

Figure 5. Investigation of SCFAs and lactate potential for mTOR/ERK/eEF2 signalling modulation in Hek-GHSR-1a-EGFP cells. Phosphorylation of mTOR_Ser2448 **(A)**, ERK1_Thr202 **(B)** and eEF2_Thr56 **(C)** was analysed in cells treated with SCFAs/lactate alone (1 h incubation), co-treatment with ghrelin (1 hour incubation) or pre-treatment with SCFAs/lactate (1 hour) before ghrelin addition (1 hour). Graphs represent median and IQR (box) with minimal and maximal values (whiskers) of three independent experiments. Untreated control (assay buffer), ghrelin (ghrl, 0.5 µM), inverse agonist [D-Arg1, D-Phe5, D-Trp7,9, Leu11]-substance P (SP-analogue, 0.5 µM), SCFAs (1 mg/mL) and lactate (0.3 mg/mL). * $p \leq 0.05$ vs untreated control and \$ $p \leq 0.05$ vs ghrelin-treated cells, Mann-Whitney U test.

Figure 6. Investigation of bacterial strain potential for mTOR/ERK/eEF2 signalling modulation in Hek-GHSR-1a-EGFP cells. Phosphorylation of mTOR_Ser2448 **(A),** ERK1_Thr202 **(B)** and eEF2_Thr56 **(C)** was analysed in cells following different treatments: bacterial supernatants alone (1 h incubation), co-treatment with ghrelin (1 hour incubation) or pre-treatment with supernatants (1 hour) before ghrelin addition (1 hour). Graphs represent median and IQR (box) with minimal and maximal values (whiskers) of three independent experiments. Untreated control (assay buffer), ghrelin (ghrl, 0.5 µM), inverse agonist [D-Arg1, D-Phe5, D-Trp7,9, Leu11]-substance P (SP-analogue, 0.5 µM) and diluted bacterial strain supernatants (1:2 v/v in assay buffer; bacterial strains: *B. longum* APC1472, *B. breve* APC6331,

L. rhamnosus DPC6118, *L. gasseri* DPC6106, *L. brevis* DPC6108 and *P. avidum* APC6544). * $p \leq 0.05$ vs untreated control and \$ $p \leq 0.05$ vs ghrelin-treated cells, Mann-Whitney U test.

Figure 1

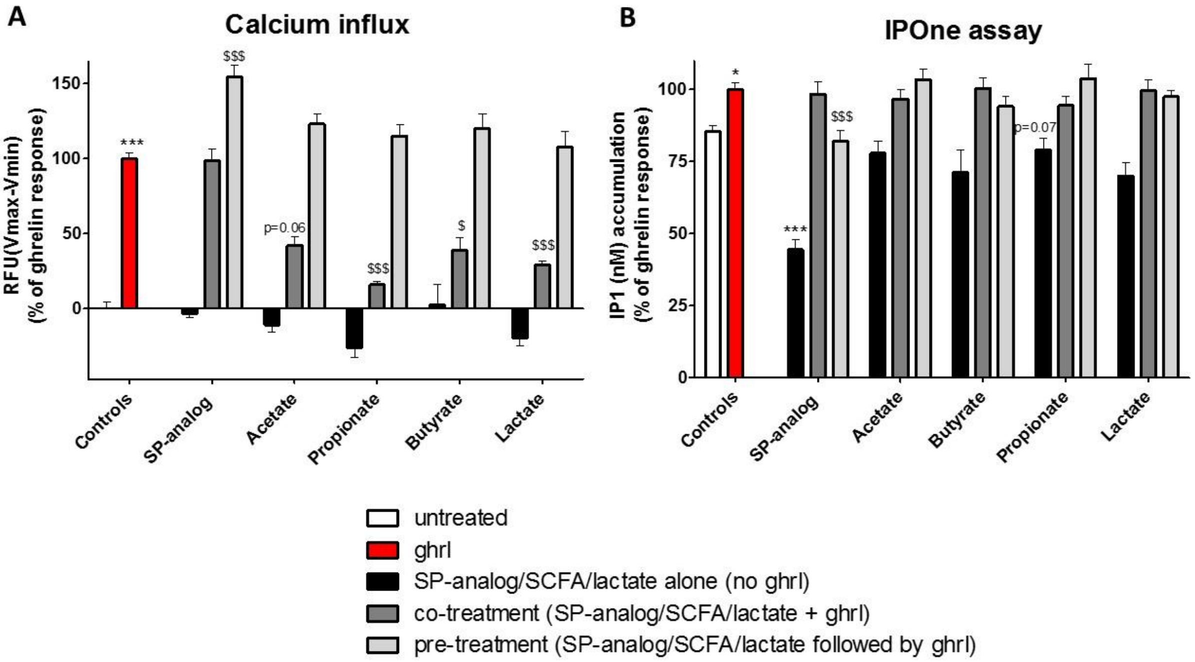

A
Calcium influx
RFU(Vmax-Vmin)
(% of ghrelin response)
150
100
50
0
***
$$$
p=0.06
$$$
$
$$$
Controls
SP-analog
Acetate
Propionate
Butyrate
Lactate
B
IPOne assay
IP1 (nM) accumulation
(% of ghrelin response)
100
75
50
25
0
*
***
$$$
p=0.07
Controls
SP-analog
Acetate
Butyrate
Propionate
Lactate
☐ untreated
🟥 ghrl
■ SP-analog/SCFA/lactate alone (no ghrl)
▨ co-treatment (SP-analog/SCFA/lactate + ghrl)
☐ pre-treatment (SP-analog/SCFA/lactate followed by ghrl)

Figure 2

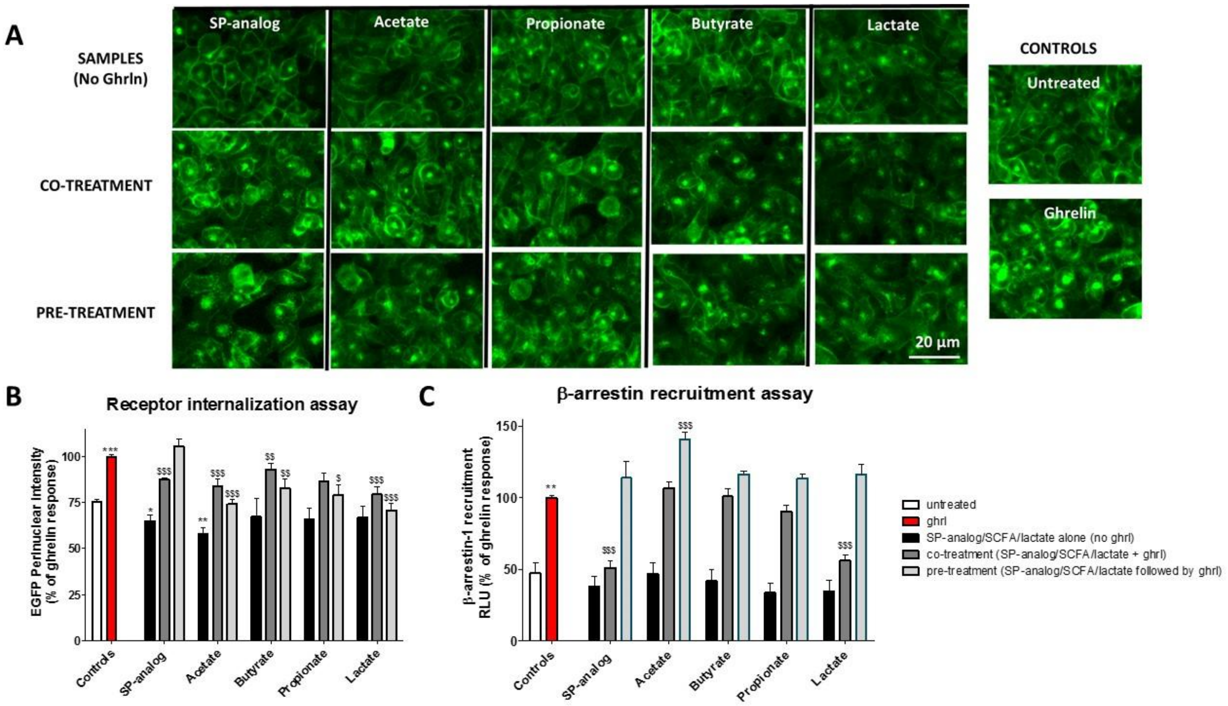

A
SP-analog
Acetate
Propionate
Butyrate
Lactate
SAMPLES (No Ghrln)
CO-TREATMENT
PRE-TREATMENT
20 µm
CONTROLS
Untreated
Ghrelin
B
Receptor internalization assay
EGFP Perinuclear Intensity (% of ghrelin response)
0
25
50
75
100
***
*
**
$$$
$$
$
Controls
SP-analog
Acetate
Butyrate
Propionate
Lactate
C
β-arrestin recruitment assay
β-arrestin-1 recruitment RLU (% of ghrelin response)
0
50
100
150
**
$$$
Controls
SP-analog
Acetate
Butyrate
Propionate
Lactate
untreated
ghrl
SP-analog/SCFA/lactate alone (no ghrl)
co-treatment (SP-analog/SCFA/lactate + ghrl)
pre-treatment (SP-analog/SCFA/lactate followed by ghrl)

Figure 3

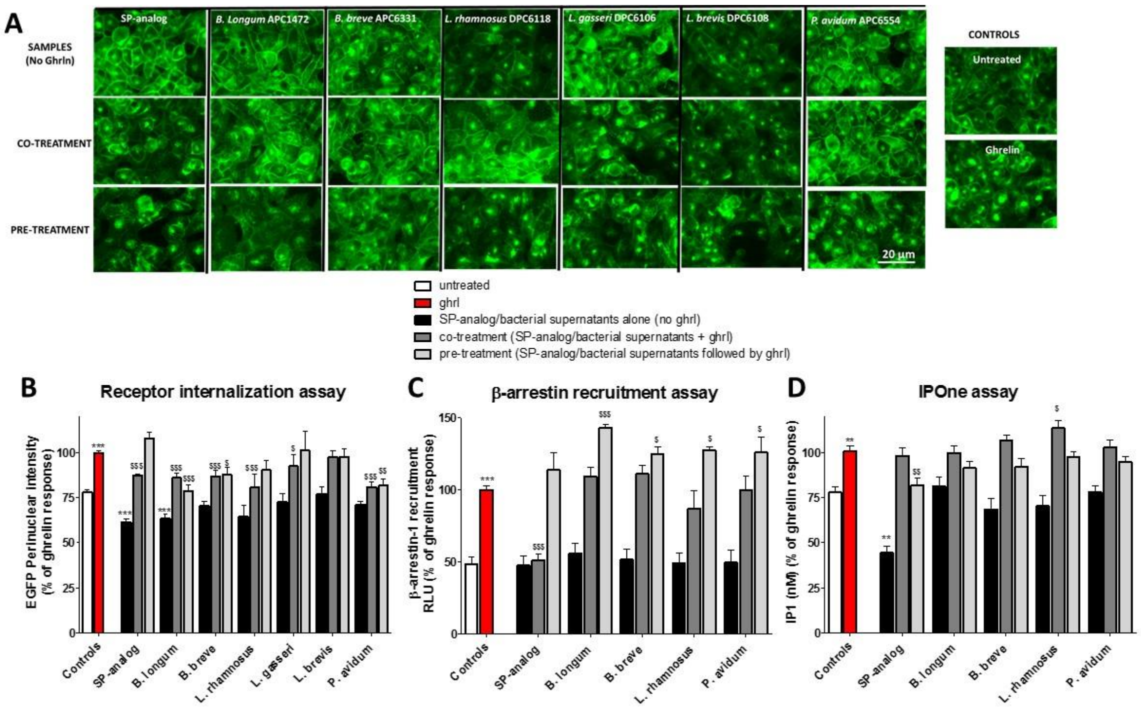

A
SAMPLES (No Ghrln)
CO-TREATMENT
PRE-TREATMENT
SP-analog
B. Longum APC1472
B. breve APC6331
L. rhamnosus DPC6118
L. gasseri DPC6106
L. brevis DPC6108
P. avidum APC6554
CONTROLS
Untreated
Ghrelin
20 µm
untreated
ghrl
SP-analog/bacterial supernatants alone (no ghrl)
co-treatment (SP-analog/bacterial supernatants + ghrl)
pre-treatment (SP-analog/bacterial supernatants followed by ghrl)
B
Receptor internalization assay
EGFP Perinuclear intensity (% of ghrelin response)
0
25
50
75
100
Controls
SP-analog
B. longum
B. breve
L. rhamnosus
L. gasseri
L. brevis
P. avidum
C
β-arrestin recruitment assay
β-arrestin-1 recruitment RLU (% of ghrelin response)
0
50
100
150
D
IPOne assay
IP1 (nM) (% of ghrelin response)

Figure 4

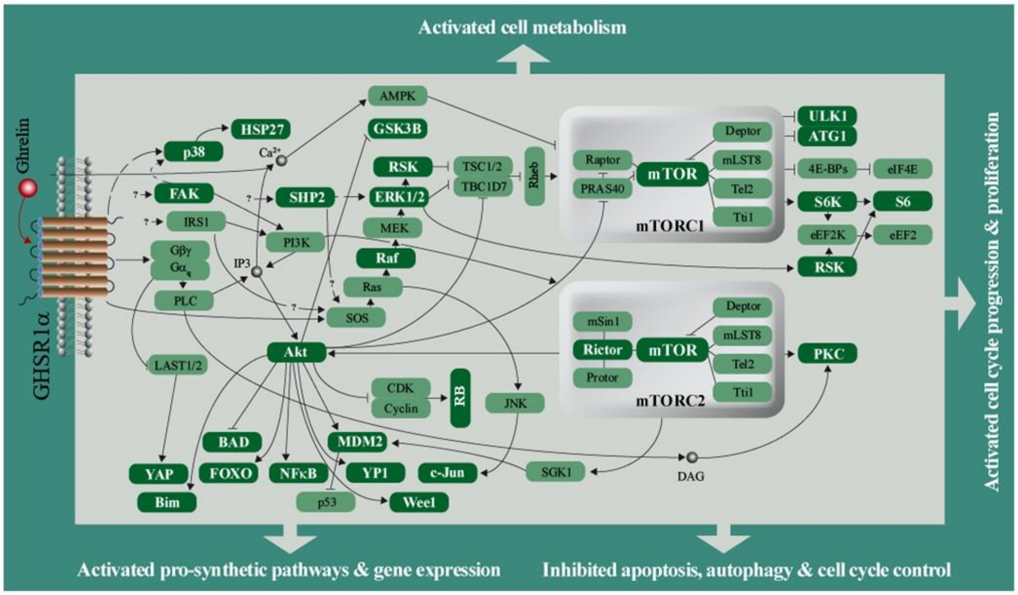

Activated cell metabolism
Ghrelin
GHSR1α
p38
HSP27
Ca2+
AMPK
GSK3B
RSK
TSC1/2
TBC1D7
Rheb
? FAK
? SHP2
ERK1/2
? IRS1
PI3K
MEK
Gβγ
Gαq
IP3
Raf
PLC
?
Ras
?
SOS
Akt
LAST1/2
CDK
Cyclin
RB
JNK
BAD
MDM2
YAP
FOXO
NFκB
YP1
c-Jun
SGK1
DAG
Bim
p53
Wee1
Raptor
PRAS40
mTOR
Deptor
mLST8
Tel2
Tti1
mTORC1
ULK1
ATG1
4E-BPs
eIF4E
S6K
S6
eEF2K
eEF2
RSK
mSin1
Rictor
mTOR
Protor
Deptor
mLST8
Tel2
Tti1
mTORC2
PKC
Activated cell cycle progression & proliferation
Activated pro-synthetic pathways & gene expression
Inhibited apoptosis, autophagy & cell cycle control

Figure 5

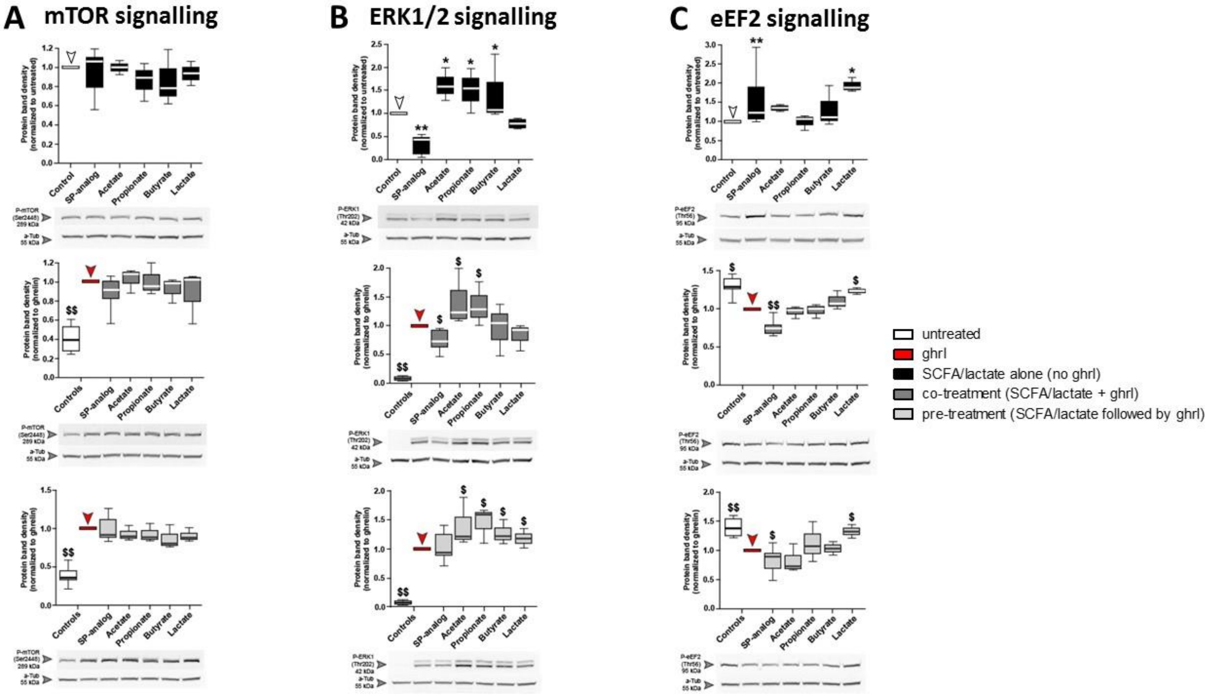
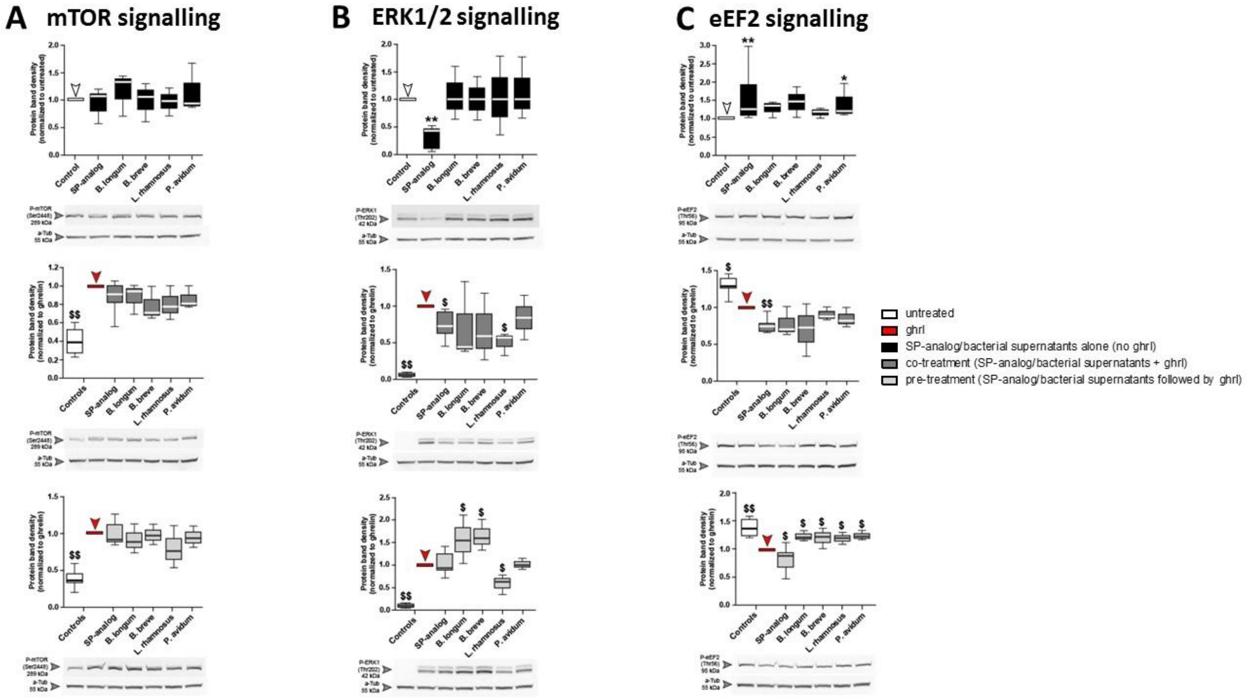

A mTOR signalling
B ERK1/2 signalling
C eEF2 signalling
Protein band density (normalized to untreated)
Protein band density (normalized to ghrelin)
Control
Controls
SP-analog
Acetate
Propionate
Butyrate
Lactate
P-mTOR (Ser2448) 289 kDa
a-Tub 55 kDa
P-ERK1 (Thr202) 42 kDa
P-eEF2 (Thr56) 95 kDa
*
**
$
$$
☐ untreated
🟥 ghrl
■ SCFA/lactate alone (no ghrl)
▨ co-treatment (SCFA/lactate + ghrl)
▨ pre-treatment (SCFA/lactate followed by ghrl)

Figure 6



A mTOR signalling
B ERK1/2 signalling
C eEF2 signalling
Protein band density (normalized to untreated)
Protein band density (normalized to ghrelin)
Control
Controls
SP-analog
B. longum
B. breve
L. rhamnosus
P. avidum
**
*
$
$$
P-mTOR (Ser2448) 289 kDa
P-ERK1 (Thr202) 42 kDa
P-eEF2 (Thr56) 95 kDa
a-Tub 55 kDa
untreated
ghrl
SP-analog/bacterial supernatants alone (no ghrl)
co-treatment (SP-analog/bacterial supernatants + ghrl)
pre-treatment (SP-analog/bacterial supernatants followed by ghrl)

Supplementary information

Resazurin assay

Cytotoxicity was analyzed using the resazurin assay (R&D systems, Inc.) as previously described (Torres-Fuentes et al., 2018, 2014). Briefly, Hek293a-GHSR-1a cells were seeded at 2.8*104 cells/well and maintained for 48 h at culture conditions. For the last 24 h of this time period, media was replaced with serum free DMEM media containing 1% NEAA. Cells were exposed for 4 h to SCFA and lactate at different concentrations containing 10% resazurin dye. Absorbance was measured at 570 nm. Cell viability was calculated as percentage of control (cells in 1x Hanks balanced salt solution (Gibco), supplemented with 20 mM HEPES (Sigma-Aldrich)). Values above 90% are not considered cytotoxic.

Gas chromatography analysis of SCFAs in bacterial supernatants

The concentration of SCFA was analyzed using a Varian 3500 GC flame-ionization system, fitted with a Nukol-FFAP column (30m x 0·32mm x 0·25mm; Sigma). The initial oven temperature was set at 100ºC for 0·5min, raised to 180ºC at 8ºC/min and held for 1min, then increased to 200ºC at 20ºC/min, and finally held at 200ºC for 5min. The temperatures of the injector and the detector were set at 240 and 250ºC, respectively. He gas was used as a carrier at a flow rate of 1·3 ml/min.(Marques et al., 2015). 270ul of the supernatant was filtered and transferred to a clear GC vial. 2-Ethylbutyric acid (Sigma) was used as the internal standard (30 μl). A standard curve was built with different concentrations of a standard mix containing acetate, propionate, isobutyrate and n-butyrate (Sigma). Peaks were integrated by using the Varian Star Chromatography Workstation version 6.0 software. All SCFA data are expressed as mM. Butyrate was not detected in the tested bacterial supernatants.

Calcium imaging

Hek-GHSR-1a-EGFP cells were seeded in 12-well plate at $2*10^5$ cells/well 48 hours prior to the assay and maintained at culture conditions. On the day of the assay media was removed and cells were carefully washed with phosphate buffered saline and incubated for 1 hour at 37ºC with 7 µM Fura 2-AM (Thermo Fisher Scientific, Massachusetts, United States) in assay buffer (1x Hanks balanced salt solution, HBSS, containing 20mM HEPES). Following incubation, Fura 2-AM was removed and replaced with assay buffer. Cells were monitored using an Olympus BX50W1 set up with a Mercury arc burner (MT20 illumination system, Cell R, Olympus). An excitation spectrum of 380 nm and 340 nm were recorded with a fixed emission of 510 nm in a real time following manual pipetting of the compounds. Cells were exposed to same treatments as per IP assay (see methods section). Pre-treatments were done during the dye incubation. Regions of interest of single cells were set using a digital epiflourescent system and its corresponding computer software (Cell R, Olympus). The ratio of excitation/emission positively correlates to calcium released from intracellular stores. Data were analyzed using GraphPad Prism software (PRISM 5.0; GraphPAD Software Inc.).

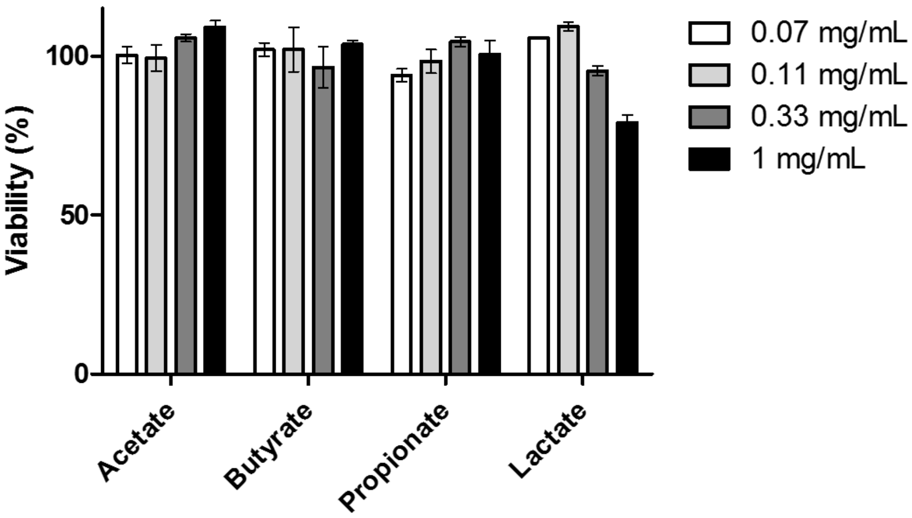


Figure S1. SCFA and lactate cytotoxicity effects on Hek-GHSR-1a-EGFP cells. Results are expressed as percentage of viability compared to untreated cells (cells in 1x HBSS containing 20 mM HEPES). Graph represents the mean ± SEM with each treatment in triplicate.

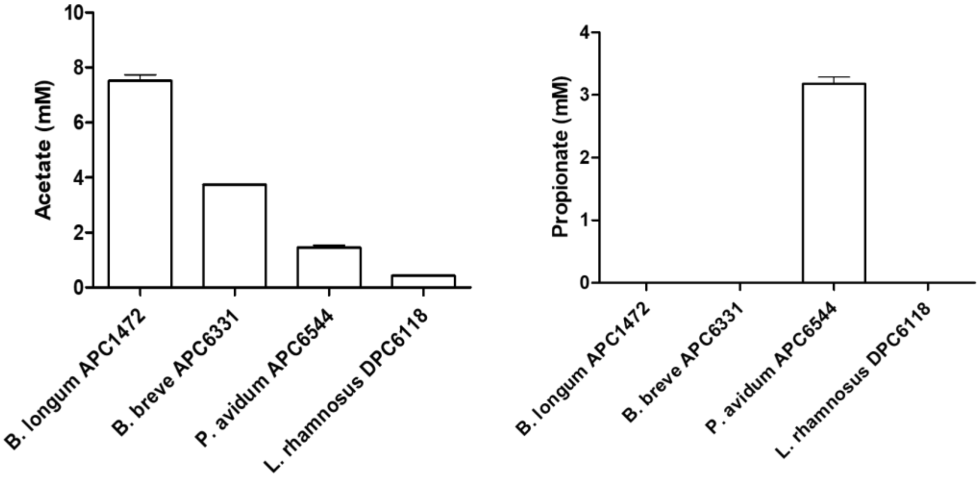


Figure S2. Bacterial strains supernatants SCFAs analysis by GC.

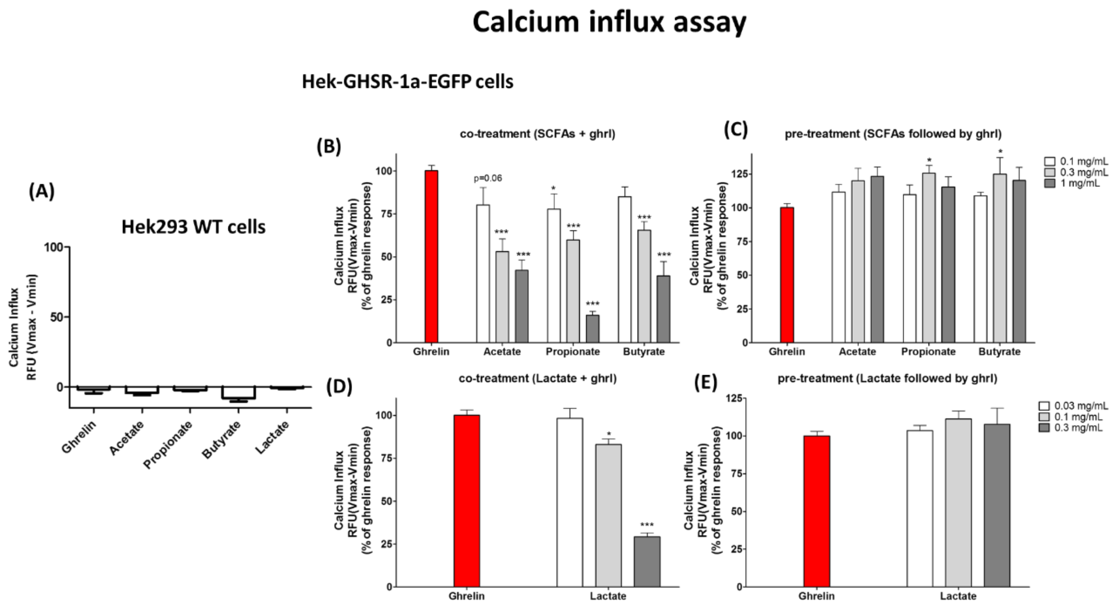


Figure S3. SCFAs and lactate effects on calcium signaling. **(A)** No response was observed in Hek293 WT cells. Data is depicted as a percentage of maximal response as elicited by the positive control FBS (3.3%). SCFA (1 mg/mL); lactate (0,3 mg/mL). SCFAs **(B, C)** and lactate **(D, E)** DRCs in Hek-GHSR-1a-EGFP cells for both assays modes: co-treatment with ghrelin (1 hour incubation) or pre-treatment with SCFAs/lactate (1 hour) before ghrelin addition (1 hour), Data is depicted as a percentage of maximal response as elicited by the positive control ghrelin. Graphs represent the mean ± SEM from at least three independents experiments with each sample performed in triplicate. *p ≤ 0.05 vs ghrelin control.

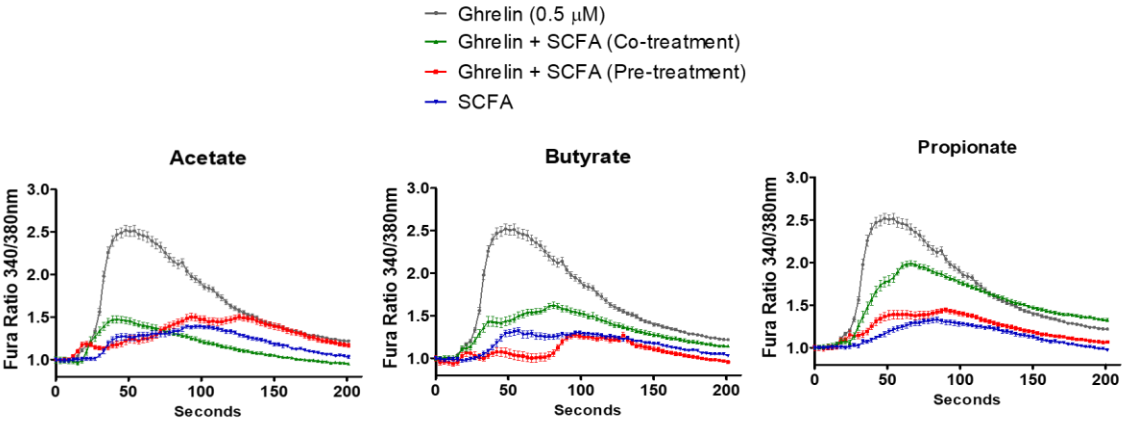


Figure S4. Calcium imaging analysis in Hek-GHSR-1a-EGFP cells. Cells were exposed to different treatments: the agonist ghrelin (grey bars), co-treatment with ghrelin and SCFAs (green), pre-treatment with SCFAs before ghrelin addition (red) or SCFAs alone (blue). Graphs represent the mean ± SEM from at least four independents experiments. Ghrelin: 0.5 μM; SCFAs: 1 mg/mL

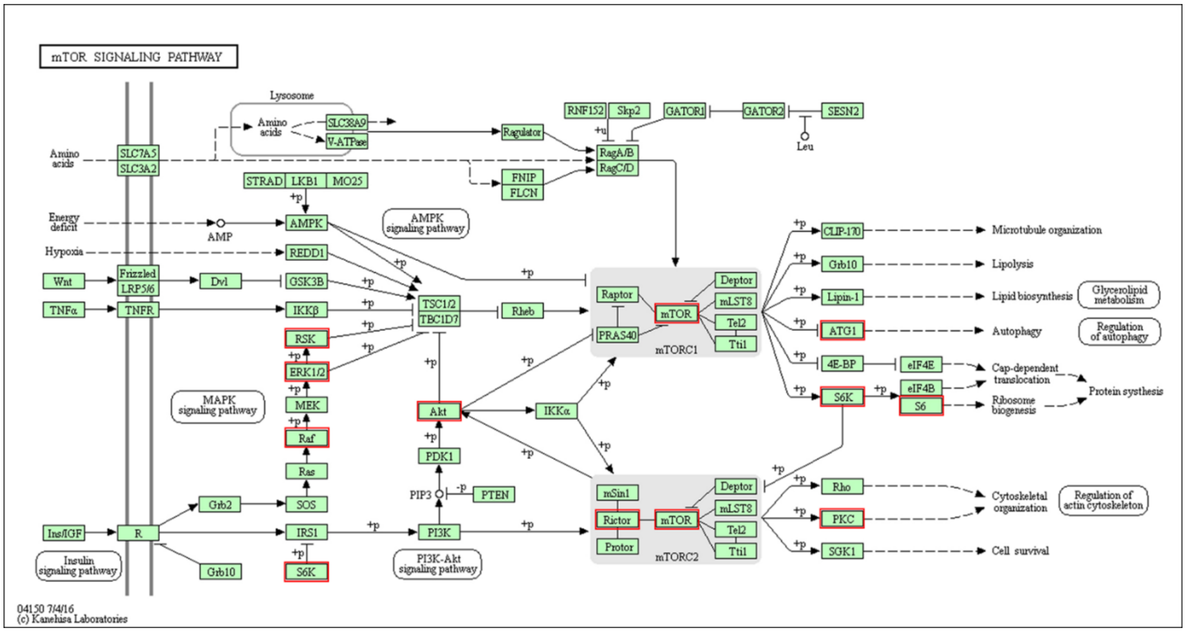


Figure S5. GHS-R1a-mediated activation of mTOR & ERK1/2 signalling pathway in Hek293a-GHSR-1a-EGFP cells. Cells were treated for 1 h with GHS-R1a agonist ghrelin (0.5 µM), and subsequent changes in protein expression and phosphorylation levels were screened with RPPA assay. Proteins displaying ≥ 10% change vs. untreated control in three independent experiments (in red rectangles) were selected for the construction of KEGG pathways using Functional Annotation Clustering in the Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.8.

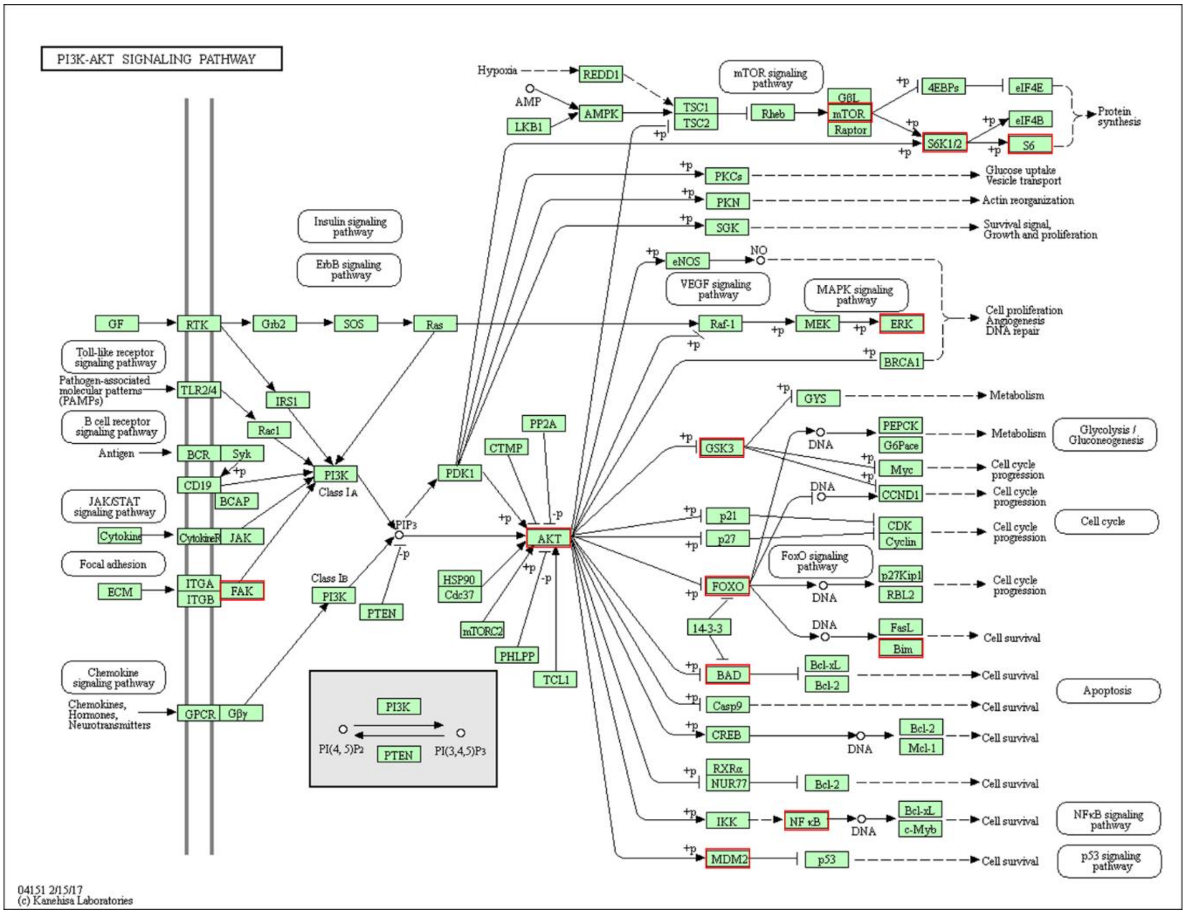


Figure S6. GHS-R1a-mediated activation of PI3K/Akt signalling pathway in Hek293a-GHSR1a-EGFP cells. Cells were treated for 1 h with GHS-R1a agonist ghrelin (0.5 µM), and subsequent changes in protein expression and phosphorylation levels were screened with RPPA assay. Proteins displaying ≥ 10% change vs. untreated control in three independent experiments (in red rectangles) were selected for the construction of KEGG pathways using Functional Annotation Clustering in the Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.8.

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