Degree project

GLP-1-SECRETING L-CELL SIGNALLING IN RESPONSE TO BIFIDOBACTERIUM BREVE NCFB2258

Bachelor Thesis Project in Biomedicine
30 ECTS
Spring term 2024

Lauryn Adewale
b21lauad@student.his.se

Supervisor: Dervla O'Malley
d.omalley@ucc.ie

Examiner: Homa Tajsharghi
homa.tajsharghi@his.se

Abstract

The relationship between gut microbiota and intestinal epithelial cells, particularly specialized enteroendocrine cells, such as GLP-1-secreting L-cells, is a critical aspect of gastrointestinal homeostasis and the gut-brain axis. This study delves into the molecular signalling pathways activated by the interaction between *Bifidobacterium breve ncfb2258,* a putative probiotic, and GLP-1-secreting L-cells. *Bifidobacterium breve ncfb2258* produces conjugated linoleic acids (CLAs), which activate peroxisome proliferator-activated receptors (PPARs) and free fatty acid receptors (FFARs). This study aims to shed light on whether L-cells function as cross-barrier signal transducers and the mechanisms with which they do. Significant findings emerged through immunofluorescent labelling and calcium imaging, revealing differential expression patterns of these molecules in response to exposure to *Bifidobacterium breve ncfb2258* supernatants. A significant response was observed when the human colorectal cancer cell line, NCI-H716 cells, a model of human GLP-1-secreting L-cells, were exposed *to Bifidobacterium breve ncfb2258* supernatants ($p = 0.003$). PPARα and FFAR4 were both detected on NCI-H716 cells and while exposure to *Bifidobacterium breve ncfb2258* supernatants increased the intensity of their cellular expression ($p = <0.001; 0.042$), expression only decreased with the addition of PPARα antagonist ($p < 0.001$) and remained elevated after the addition of FFAR4 antagonist ($p = 0.999$). These findings suggest the NCI-H716 cell line is suitable for exploring human GLP-1-secreting L-cells as potential cross-barrier signal transducers for the putative probiotic, *B. Breve ncfb2258,* and identify PPARα as potentially being involved in the molecular mechanisms of this interaction.

Popular scientific summary

Human bodies provide a home for over a trillion microbes. Among these microbes is the bacteria, *Bifidobacterium breve ncfb2258* (*B. breve ncfb2258*) which stands out for its ability to produce conjugated linoleic acid (CLA), an essential fatty acid, with health-promoting properties that may also act as a messenger for the bacteria.

This research delved into how *B. Breve ncfb2258* interacts with Glucagon-like peptide-1 secreting L cells, a type of gut cell known for its role in regulating digestion, blood sugar and communication between the gut and brain. Using a human cell line that mimics L-cells to explore this interaction, we found that these L-cells, upon exposure to *B. Breve ncfb2258,* were activated suggesting their involvement as cells that can translate messages from the bacteria in the gut to the host. Through advanced laboratory techniques, the pathways activated when *B. Breve ncfb2258* interacts with GLP 1 secreting L-cells were also explored.

Evidence was revealed about the molecular pathways that underpin how L-cells respond to bacterial stimuli. Receptors for CLAs (PPARα and FFAR4) are found on L-cells and respond to the bacterial secretory products. Understanding these mechanisms is crucial for understanding the interactions between gut microbiota and host cells, providing insights into microbe-host communication. This knowledge may pave the way for targeted therapeutic interventions to modulate these pathways to promote gut health and potentially alleviate conditions associated with impaired gut-brain axis communication, such as metabolic syndrome, diabetes, obesity, and gastrointestinal disorders.

In summary, this study offers insights into how bacteria in the inner environment of the gut signal across the gut barrier and on towards the brain. Probiotics are widely available, but their effects on human physiology is not fully understood. This research could potentially lead to the development of supplements that support gut health, aiding digestion and potentially preventing diseases like diabetes and obesity.

List of Abbreviations

Abbreviation	**Description**
IEC	Intestinal epithelial cells
EEC	Enteroendocrine cells
GLP-1	Glucagon-like peptide-1
PYY	Peptide tyrosine tyrosine
GI	Gastrointestinal
IBS	Irritable bowel syndrome
B. breve	*Bifidobacterium breve*
B. breve ncfb2258	*Bifidobacterium breve ncfb2258*
PUFA	Polyunsaturated fatty acids
CLA	Conjugated linoleic acid
ALA	Alpha-linoleic acid
LA	Linoleic acid
AA	Arachidonic acid
EPA	Eicosapentaenoic acid
DHA	Docosahexaenoic acid
IL-6	Interleukin-6
IL-6R	Interleukin-6 receptor
FFAR	Free fatty acid receptor
PPAR	Peroxisome-proliferator activated receptor
IBS	Irritable bowel Syndrome
FBS	Fetal bovine serum
HBSS	Hepes-buffered saline solution
TRITC	Tetramethyl rhodamine
PBS	Phosphate-buffered saline
DAPI	4′,6-diamidino-2-phenylindole
FITC	Fluorescein isothiocyanate
PFA	Paraformaldehyde
GLP1-R	Glucagon-like peptide-1 receptor

Table of Contents

Introduction - 1 -
Intestinal Function and the Role of GLP-1 Secreting L-Cells - 1 -
Bifidobacterium Breve - 3 -
Gaps in Research - 4 -
Aims - 4 -
Materials and Methods - 4 -
Cell Culture - 4 -
Cell Plating - 4 -
Calcium Imaging - 5 -
Immunofluorescence - 5 -
Statistical Analysis - 6 -
Results - 6 -
Discussion - 12 -
Ethical aspects and impact of the research on society - 15 -
Conclusions/Future Perspectives - 15 -
Acknowledgements - 16 -
References - 16 -
Appendices - 22 -
Appendix A – Recipes - 22 -
Appendix B - B. breve ncfb2258 Culture - 24 -

Introduction

Intestinal Function and the Role of GLP-1 Secreting L-Cells

The intestine's main purpose is digestion, which is carried out by the breakdown and absorption of food and its nutrients and the solidification of waste. Although the principal function of the intestine is digestion, that is not its only function; it is also involved in other processes (Ogobuiro et al., 2023). The intestine has exocrine and endocrine glands that produce hormones and enzymes. Some of the hormones produced in the small intestine include gastrin, cholecystokinin, and secretin, while some of the enzymes produced include trypsin and chymotrypsin for peptide digestion (Ellingsgaard et al., 2011; Liu et al., 2021). However, hormone release is not only important in the context of food breakdown and assimilation but also important for signalling. Another function of the intestine is both paracrine and autocrine action locally and on the brain through various pathways, collectively known as the gut-brain axis. For example, hormones such as peptide YY (PYY), glucagon-like peptide 1 (GLP-1) and cholecystokinin play key roles in regulating appetite and satiety (Wren et al., 2007), GLP-1 also plays a role in regulating metabolism, specifically glucose metabolism (Lewis et al., 2020). Cholecystokinin has anti-inflammatory properties and can modulate immune responses in the gut (Dlugosz et al., 1988) while certain hormones such as serotonin are involved in the body's response to stress (Bellono et al., 2017).

The gut-brain axis is bidirectional and complex. The autonomic nervous system (ANS) and enteric nervous system (ENS) are among the pathways through which afferent and efferent neurons facilitate the connection of neuronal pathways (Karaosmanoglu et al., 1996). The intrinsic innervation of gastrointestinal functions is mostly carried out by the ENS, which is made up of the myenteric and submucosal plexuses, two ganglionated plexuses that control gut motility, secretion, and absorption (Savidge et al., 2007). The ENS transmits sensory data via primary afferent neurons that follow vagal afferent pathways in gut-brain signalling to the ganglia of the sympathetic nervous system (SNS) (Suarez et al., 2018). Through the central nervous system (CNS), the ANS directly induces neurological reactions in the gut that modify the gut's physiology (Levenstein et al., 2000). Additionally, the ANS can directly modify the way that gut immune cells respond to bacteria or indirectly impact the immune system by regulating the way that microbes interact with gut immune cells (Alonso et al., 2008).

The internal environment of the gastrointestinal (GI) system comprises the GI tract and accessory organs. The mouth, throat, oesophagus, stomach, small and large intestines, and anal canal make up the GI tract, while the tongue, teeth, and glandular organs such the pancreas, liver, gallbladder, and salivary glands are examples of accessory organs (Ogobuiro et al., 2023). The external environment on the other hand simply refers to the environment outside the intestinal lumen or barrier. The internal environment of the GI system is linked to interoceptive signalling in the intestines, which is the transmission of sensory information from the GI tract to the central nervous system. This is collectively known as the gut-brain axis (Osadchiy et al., 2019). The interoceptive signalling pathways in the intestine are crucial to understanding the gut-brain axis and the link between the gut and the brain can be accomplished through direct or indirect chemical pathways that may involve microbial metabolites and certain specific neurotransmitters, as well as neuroanatomical pathways like the vagus nerve (VN) or spinal cord, the neuroendocrine system like the hypothalamic-pituitary-adrenal axis (HPA axis), and cytokines. More interestingly, cells known as intestinal epithelial cells (IECs) are active participants in interoceptive signalling in this context (Erny et al., 2015). IECs preserve intestinal

homeostasis by segregating host tissue and commensal bacteria with a physical and biochemical barrier (Vaishnava et al., 2011). This homeostasis is dependent on the various roles of intestinal epithelial cells (IECs), such as the physical segregation of commensal bacteria, mostly in the colon, and the incorporation of microbial signals (Lee et al., 2010; Berer et al., 2011). An important class of specialised IECs are enteroendocrine cells (EECs) (Barker et al., 2007). EECs, found in the small and large intestines, are specialized cells with a secretory function. They release hormones and peptides in response to the contents of the intestinal lumen, such as nutrients. These signalling molecules can affect nearby cells and, as components of the endocrine system, can circulate throughout the body (Christiansen et al., 2018). On a local level, EECs play a significant role in functions such as gastrointestinal motility through the release of peptides, including ghrelin and motilin during the interdigestive phase and cholecystokinin, glucagon-like peptide-1 (GLP-1), and peptide YY (PYY) following meals (Stolk et al., 2001). They are also a significant component of the gut-brain axis, where they establish direct synaptic connections with peripheral neurons through particular axon-like structures called pseudopods and hormone release to transmit signals about nutrition, pain, and information from the gut microbiome. (Bohorquez et al,2014, 2015; Bellono et al.,2017).

Glucagon-like peptide 1 (GLP-1) secreting L-cells are an interesting example of EECs that have been found to play a role in the gut-brain axis (Buckley et al., 2020). These cells are mostly found in the distal small intestine and colon, where they influence appetite, sense nutrients present in the lumen, regulate gastric emptying and gastrointestinal transit, and promote β-cell survival and proliferation by secreting GLP-1, an incretin hormone (Orskov et al., 1996;). Depending on where they are located, L-cell activity varies. In the proximal gut, nutrients in the lumen stimulate L-cells, leading them to secrete GLP-1 and Peptide YY (PYY) (Reiman et al., 2008; Gorboulev et al., 2011). On the other hand, in the distal intestine, bacterial lipopolysaccharides (LPS), indole, secondary bile acids, and metabolites from bacterial fermentation stimulate this activity (Edfalk et al., 2008; Chimerel et al., 2014; Nguyen et al., 2014). L-cells respond to several luminal components, including digestive products of carbohydrates, proteins, and lipids, which are polarized and excitable and in response to this stimulation, L-cells secrete gut hormones such as GLP-1 and PYY (Buckley et al., 2020).

The concentration of L-cells increases along the length of the gut with the greatest concentration of GLP-1 expressing L-cells in the colon, but nutrients do not reach the colon until long after a meal, thus it is crucial to note that even though the colon contains many L-cells, ingesting food is likely to directly excite only the L-cells in the small intestine. This is because most macronutrients are absorbed in the proximal small intestine, preventing colonic L-cells from being directly stimulated by nutrients from the lumen. Instead, colonic L-cells may be exposed to significant amounts of secondary bile acids, fatty acids, and other microbial metabolites, which can trigger or modify colonic GLP-1 production (Kuhre et al., 2021). This variation in direct exposure is likely to influence the expression of nutrient sensors and secretory responses of L cells in the small intestine and L cells in the colon (Kuhre et al., 2021). For example, studies have shown that glucose is a strong stimulator of GLP-1 secretion in the small intestinal segment, whereas GLP-1 secretion in the colon is only subtly stimulated by glucose and is instead strongly stimulated by bile acids (Christiansen et al., 2018). Therefore, it is reasonable to hypothesise that colonic L-cells are sensors for microbial products rather than for primary macronutrients. In the pancreas, GLP-1 increases insulin secretion and β-cell proliferation (Hou et al., 2016), while in the gut it inhibits gastric emptying and food intake, which it does through interactions with the CNS. Through binding to GLP-1 receptors (GLP1R) in enteric or vagal sensory neurons, the actions of GLP-1 are indirectly communicated. The nodose ganglion and vagal nerve terminals that

innervate the portal vein both express GLP-1Rs, and the brainstem and hypothalamic circuits regulate the effect of GLP-1 (Van Bloemendaal et al., 2014).

Bifidobacterium Breve

Some microbes live symbiotically in the intestine, establishing an ecosystem referred to as the gut microbiome. However, intestinal microbiota does not simply remain inactive in the gut; rather, it provides significant advantages to the host (Furusawa et al., 2013) such as host nutrition and drug metabolism, immunomodulation, and preservation of the gut mucosal barrier's structural integrity (Johansson et al., 2008). The gut microbiota produces various kinds of molecules that regulate EECs and immune cells, ultimately, secretion of signalling molecules that can modify gut function and interact with other organs, including the CNS, forming the microbiota-gut-brain axis (Zeng et al., 2023).

Probiotics are live bacterial organisms that in suitable doses, produce a health benefit on the host, and Bifidobacterium is a representative example of such. Bifidobacteria are prevalent in the GI tract of a variety of hosts, and their presence has been linked to good gut health (Bottacini et al., 2014) for instance, they promote intestinal barrier function, intestinal homeostasis, immunomodulation, and regulate the synthesis and secretion of small reactive molecules and metabolites linked to the gut flora (Engevik et al., 2021; Zhang et al., 2022; Zhao et al., 2022. *Bifidobacterium breve (B. breve)* is a strain of bifidobacteria that is the most common species in the gut of breastfed newborns and has been isolated from human milk. It is non-cytotoxic, displays immunostimulant properties, lacks the features indicative of resistance to antibiotics, and holds antibacterial action against human infections, by preventing harmful and drug-resistant bacteria from growing or adhering to cells, and by increasing the effectiveness when coupled with certain antibiotics (Turroni et al., 2012; Choi & Shin, 2021).

Gut microbiota metabolites stimulate GLP-1 secretion by L cells (Zeng et al., 2023). These microbiota metabolites include fatty acids (Kuhn et al., 2020). The interaction between the microbiota and dietary fibres, fatty acids stimulation of L-cells and the subsequent increase in plasma GLP-1 levels have been the subject of research (Arora et al., 2018). Previous studies have demonstrated that *B. breve* stimulated the release of GLP-1 from intestinal L-cells through indigestible carbohydrate fermentation which produces fatty acids, which are further sensed by L-cells (Alisi et al., 2014). These carbohydrates undergo conversion into intermediates of the fructose-6-phosphate shunt, also known as the "bifid" shunt and ultimately into short-chain fatty acids (SCFAs) (de Vries & Stouthamer, 1967). *Bifidobacterium Breve ncfb2258 (B. breve ncfb2258)* produces conjugated linoleic acids (CLAs) (O'Connell et al., 2013), a subfamily of polyunsaturated fatty acid (PUFAs). PUFAs are known to activate peroxisome proliferator-activated receptors (PPARs) (Poirier et al., 2001; Yang et al., 2018), free fatty acid receptors (FFARs) (Schmidt et al., 2011), and modulate immune molecules including interleukin-6 (IL-6) (Yang et al., 2018), an inflammatory cytokine with neuromodulatory effects in the colon, which are all associated with GLP-1 secreting L-cells. PUFAs are bioactive lipids which modulate inflammation and immunity and have more than one double bond in their structure. The main members of these groups are n-6 PUFAs, such as linoleic acid (LA, 18:2), arachidonic acid (AA, 20:4), and n-3 PUFAs, such as alpha-linolenic acid (ALA, 18:3), eicosapentaenoic acid (EPA, 19:5), and docosahexaenoic acid (DHA, 22:6). LA and ALA are referred to as essential fatty acids, since the human body lacks the enzymes for their production (Glaser et al., 2010).

Gaps in Research

The importance of this study stems from the established knowledge that *B. breve ncfb2258* produces CLAs, which have been shown to activate specific receptors and modulate immune molecules associated with GLP-1 secreting L-cells and the further knowledge that GLP-1 secreting L-cells are involved in neuromodulatory processes. This revelation highlights the need to explore how *B. Breve ncfb2258* interacts with GLP-1-secreting L-cells to facilitate trans-membrane signalling. This knowledge is important for comprehending the implications of the gut-microbiota interaction, particularly in the context of the gut-brain axis and possibly leading to advancements in gastrointestinal health.

Aims

This study aims to investigate:

- if the NCI-H716 human cell line is an appropriate model to explore GLP-1-secreting L-cells as potential cross-barrier signal transducers for colonic microbes, specifically the putative probiotic, *B. Breve ncfb2258.*
- the molecular mechanisms involved in signalling induced by PUFA-producing *B. Breve ncfb2258* in human GLP-1-secreting L-cells.

Research Question: How does *B. Breve ncfb2258* interact with GLP-1-secreting L-cells to facilitate trans-cellular signaling?

Hypothesis: *B. Breve ncfb2258* interacts with GLP-1-secreting L-cells through interaction with specific receptors or signalling molecules to facilitate trans-cellular signalling.

Materials and Methods

Cell Culture

NCI-H716 cells (LGC Standards, Ireland), a human colonic cancer cell model for GLP-1-secreting L-cells, were cultured in T75 flasks. The cell culture suspension medium used was RPMI-1640 (Sigma-Aldrich), supplemented with 1% penicillin-streptomycin (Sigma-Aldrich) and 10% fetal bovine serum (FBS) (Sigma-Aldrich). The cells were incubated in a controlled environment of 37°C and 5% CO_2. The cells were passaged every three to four days to allow for proliferation and viability.

Cell Plating

NCI-H716 cells were prepared for calcium imaging and immunofluorescence experiments. For calcium imaging, the cells were plated onto a glass coverslip in 1 ml of Matrigel Growth Factor Reduced Basement Membrane Matrix (Corning) coated dishes, while for immunofluorescence studies, cells were plated onto two glass coverslips 1 ml of Poly-D-lysine hydrobromide (Sigma-Aldrich) coated dishes. The plated cells were then further incubated in a controlled environment at 37°C with 5% CO_2 and passaged triweekly until use in subsequent experiments.

Calcium Imaging

NCI-H716 cells were loaded with 1 ml of the calcium indicator, Fluo-2 AM (4 µM, Ion Biosciences) mixed with 1X Hepes -Buffered Saline Solution (HBSS, see Appendix A) in the dark and incubated for one hour at 37°C with 5% CO_2. Imaging was conducted using a calcium imager (Integrating Camera (Hamamatsu ORCA-ER); DAQ device (USB6229; Frame grabber) and the Winfluor Imaging software (John Dempster, University of Strathclyde). For the control experiment, during imaging, the cells were exposed to HBSS, *B. Breve ncf2258* (1:50, 5 minutes, see Appendix B), and a 50 mM K^+ solution (3 minutes) at appropriate intervals. For the FFAR4 antagonist experiment, the cells were exposed to HBSS, *B. Breve ncfb2258*, FFAR4 antagonist (10 µM, 20 minutes, AH7614, Tocris Bioscience) dissolved in HBSS then FFAR4 antagonist with *B. Breve ncfb2258* and a 50mM K^+ solution also at appropriate intervals. All reagents were added to the experimental chamber using a perfusion system (1ml / minute). The experiment was repeated in cells from at least three different cultures. The change in fluorescence and change in peak amplitude were obtained from the fluorescence values at appropriate time points using Microsoft Excel before further analysis.

Immunofluorescence

NCI-H716 cells in polylysine-coated dishes were pre-incubated with either 1 ml FFAR antagonist (10 µM), PPARα antagonist (1 µM, GW6471, Tocris Bioscience) or HBSS at room temperature for 20 minutes. The dishes were then incubated with *B. Breve ncfb2258* for 15 minutes except for the control dish. Dishes were washed for five minutes with Phosphate Buffered-Saline (PBS, Sigma-Aldrich), fixed with 4 % Paraformaldehyde (PFA) (Sigma-Aldrich) for 15 minutes and then washed three times for five minutes each with PBS. The cells were blocked for one hour at room temperature with a blocking permeabilization (see Appendix A) buffer. Table 1 shows primary antibodies that cells were incubated against, their dilution factors and their origin. Cells were incubated overnight at 4°C.

Table 1. Primary antibodies

Primary antibody	Dilution Factor	Origin
IL-6Rα mouse monoclonal IgG	1:100	Santa Cruz Biotechnology
GLP-1 goat polyclonal IgG*	1:100	Santa Cruz Biotechnology
GLP-1R (D-6) mouse monoclonal IgG	1:100	Santa Cruz Biotechnology
GLP-1R (H-55) rabbit polyclonal IgG	1:100	Santa Cruz Biotechnology
GPR120/FFAR4 rabbit polyclonal IgG	1:100	Invitrogen (Thermo Fisher Scientific)
PPARα mouse monoclonal IgG	1:100	Santa Cruz Biotechnology
c-Fos rabbit polyclonal IgG**	1:200	Santa Cruz Biotechnology

* Was used in control experiments. **Co-stained with PPARα.

The cells were washed three times with PBS and incubated with appropriate secondary fluorophores (1:250, 1 hour), Tetramethyl rhodamine (TRITC)- conjugated AffiniPure donkey anti-rabbit IgG (Jackson ImmunoResearch), Fluorescein isothiocyanate (FITC)- conjugated AffiniPure goat anti-mouse IgG

(Jackson ImmunoResearch) or Alexa Fluor 488 conjugated AffiniPure donkey anti-sheep IgG (Jackson ImmunoResearch). Slides were subsequently mounted with 4',6-diamidino-2-phenylindole (DAPI) mounting medium (Abcam). Cells were imaged using cellSens Dimension software (Olympus Life Science), Olympus BX53 upright light microscope and Olympus DP74 camera. Image J (Wayne Rasband) was used to obtain the corrected total cell fluorescence (integrated density – (area of selected cell x mean fluorescence of background readings) of the cells.

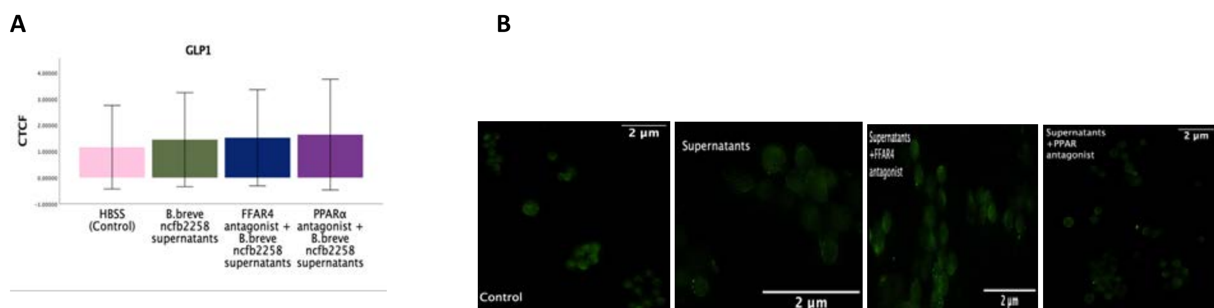
Statistical Analysis

The Statistical Package for the Social Sciences (SPSS Mac OS, Version 28) was used to analyze the data. The paired t-test, one-way ANOVA, followed by Tukey's post-hoc multiple comparisons test was used where appropriate. Significance level (α) = 0.05.

Results

GLP-1R expression was increased by exposure to *B. Breve ncfb2258*

No non-specific fluorescence was detected in control experiments where cells were incubated with primary antibodies or secondary antibodies alone. NCI-H716 cells secrete GLP-1 in response to bacterial stimulation so the expression of GLP-1 in these cells was examined to see if they responded to *B. breve ncfb2258*. GLP-1 staining was visible in the cytosol of cells in the semi-quantitative assessment of GLP-1 expression under control conditions and following exposure to *B. breve ncfb2258* supernatants alone and with PPARα antagonist or FFAR4 antagonist. There was no significant difference in GLP-1 staining after exposure to *B. breve ncfb2258* supernatants alone ($p = 0.316$) compared to control conditions nor exposure in the presence of either PPARα antagonist ($p = 0.670$) or FFAR4 antagonist ($p = 0.974$). GLP1R expression was also examined in these cells. GLP1Rs were visible in both the cytosol and at the cell membrane of cells in the semi-quantitative assessment of GLP1R expression under control conditions and following exposure to *B. breve ncfb2258* supernatants alone and with PPARα antagonist or FFAR4 antagonist. There was a non-significant increase in GLP1R expression following exposure to B. breve ncfb2258 compared to the control conditions ($p = 0.265$), an increase in expression following exposure to *B. breve ncfb2258* supernatants in the presence of PPARα antagonist ($p = 0.001$), and a non-significant increase when exposed to *B. breve ncfb2258* supernatants and FFAR4 antagonist ($p = 0.138$). Figure 1 contains representative graphs and images which depict the relative expression of GLP-1 in the cytosol and GLP1R in the cytosol and at the cell membrane when unstimulated (incubated with HBSS) or following exposure to *B. breve ncfb2258* (1:50, 15 minutes) alone or in the presence of either FFAR4 antagonist (10 µM, 20 min) or PPARα antagonist (1 µM, 20 minutes).



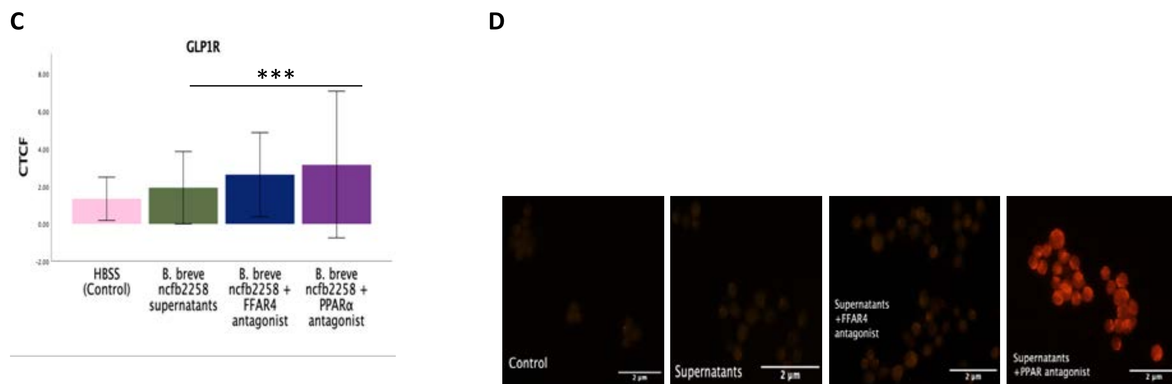


Figure 1. **A.** Illustrates the mean corrected total cell fluorescence of GLP1 from immunofluorescence carried out on NCI-H716 cells exposed to HBSS (control), *B.breve ncfb2258* supernatants, FFAR4 antagonist and *B.breve ncfb2258* supernatants and PPARα antagonist and *B.breve ncfb2258* supernatants. Bars represent mean value ± SD. Statistical significance was determined by one way-ANOVA ($F_{3,236}$ = 2.94, p = 0.034) N = 60 cells for each condition, from 3 experiments. **B.** Representative fluorescence images for control (incubated with HBSS), *B. breve ncfb2258 supernatants, B. breve ncfb2258 supernatants* and FFAR4 antagonist, *B. breve ncfb2258 supernatants* and PPARα antagonist stained with GLP-1 antibody. **C.** Illustrates the mean corrected total cell fluorescence of GLP1R from immunofluorescence carried out on NCI-H716 cells exposed to HBSS (control), *B.breve ncfb2258* supernatants, FFAR4 antagonist and *B.breve ncfb2258* supernatants and PPARα antagonist with *B.breve ncfb2258* supernatants. Bars represent mean value ± SD. Statistical significance was determined by one way-ANOVA ($F_{3,475}$ = 11.94, p < 0.001) N = 120 cells for each condition, from 3 experiments. *** indicates p < 0.001. **D.** Representative fluorescence images for control (incubated with HBSS), *B. breve ncfb2258 supernatants, B. breve ncfb2258 supernatants* and FFAR4 antagonist, *B. breve ncfb2258 supernatants* and PPARα antagonist stained with GLP1R antibody.

B. breve ncfb2258 supernatants increased nuclear c-Fos in NCI-H716 cells

In Fluo-2-AM-loaded GLP-1-secreting NCI-H716 human cells, the *B.breve ncfb2258* supernatant containing PUFAS resulted in a small increase in intracellular calcium in 45% of cells tested. In responding cells, fluorescence (AUs) (211.5 ± 5.9) was higher than baseline fluorescence (211.03 ± 6.1) (95% Cl 0.18 to 0.72), t_8 =3.5, p = 0.003, N = 9 cells from 4 experiments, Figure 2). A paired t-test was conducted to determine whether there was a significant mean difference in fluorescence between responses compared to the baseline (HBSS) from the calcium imaging control experiments. Cellular activation in response to B. breve ncfb2258 was also examined using c-Fos, an early transgene. C-Fos was visible in the nucleus in the semi-quantitative assessment of c-Fos expression under control conditions and following exposure to *B.breve ncfb2258*. C-Fos staining was significantly increased when cells were exposed to *B.breve ncfb2258* supernatants (p = 0.008). Figure 2 contains representative graphs and images which depict the relative expression of nuclear c-Fos when unstimulated (incubated with HBSS) or following exposure to *B. breve ncfb2258* (1:50, 15 minutes).

A **B**

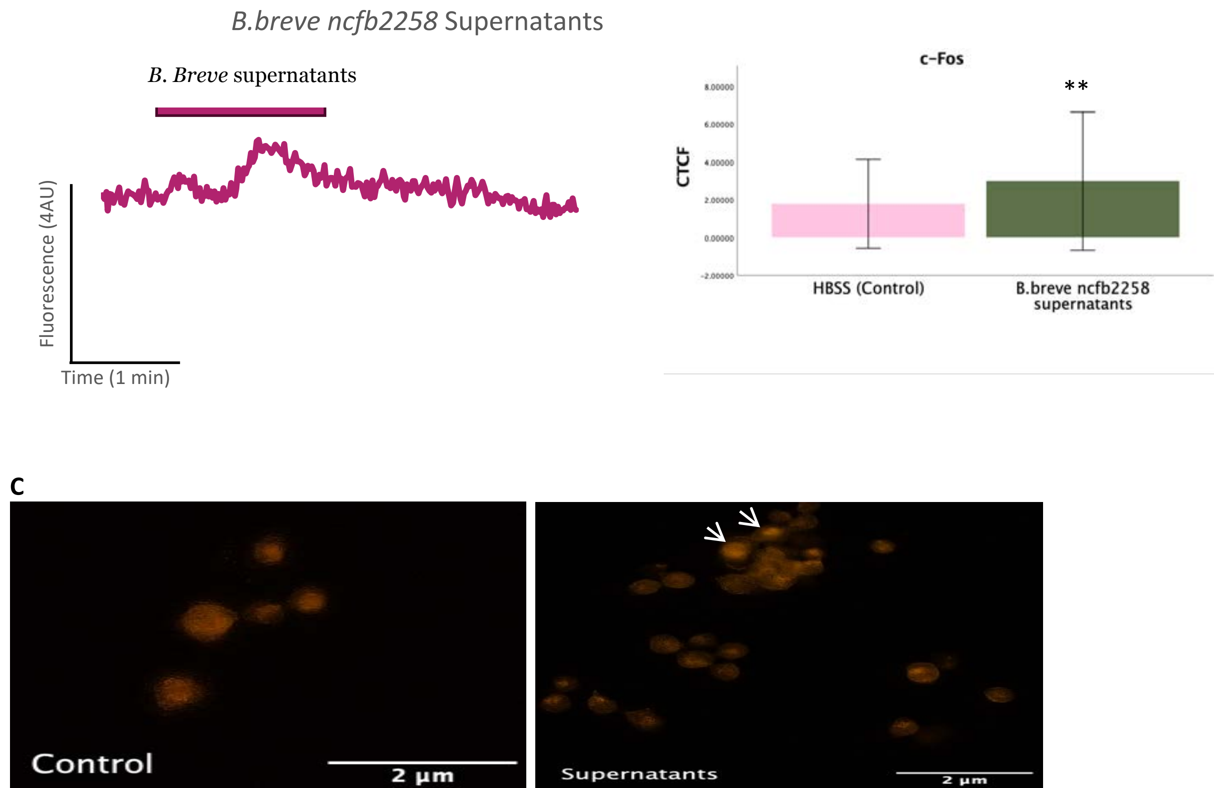
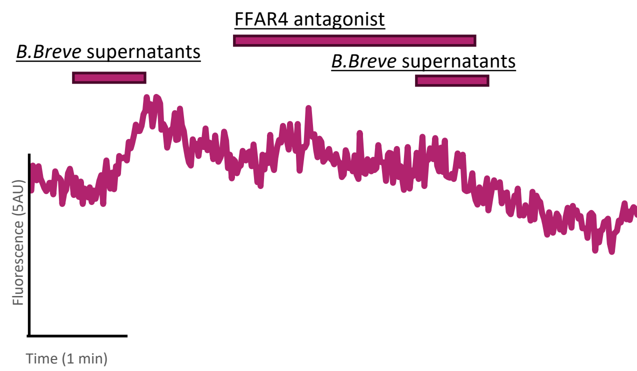


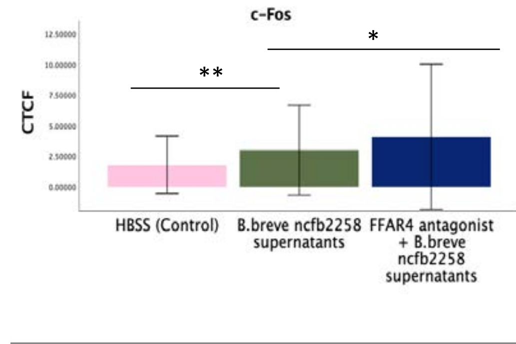
Figure 2. **A**. *B. Breve ncfb2258* supernatants stimulate a calcium response in human GLP-1-secreting NCI-H716 cells **B.** Illustrates the mean corrected total cell fluorescence of c-Fos from immunofluorescence carried out on NCI-H716 cells exposed to HBSS (control) and *B.breve ncfb2258* supernatants. Bars represent mean value ± 1 SD. Statistical significance was determined by one way-ANOVA ($F_{3,236} = 12.9$, p = 0.008), followed by Tukey's post-hoc test. N=60 cells for each condition, from 3 experiments. ** indicates p < 0.01 between groups. **C.** Representative fluorescence images for control (incubated with HBSS) and *B. breve ncfb2258* supernatants stained with c-Fos antibody (arrows).

FFAR4 expression in response to *B. breve ncfb2258* on NCI-H716 cells

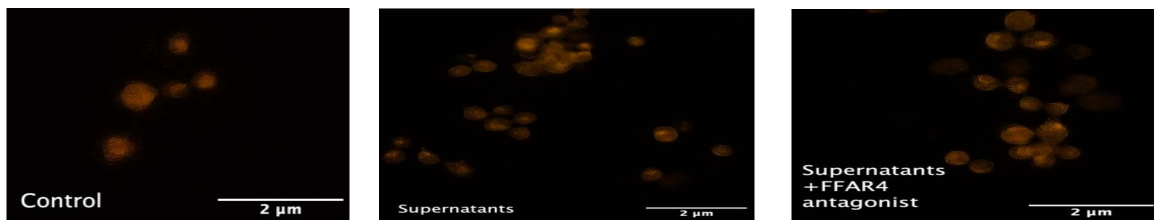
To explore if FFAR4 expression on the GLP-1-secreting NCI-H716 human cells facilitated signalling, the *B. breve ncfb2258* supernatant was reapplied in the presence of an FFAR4 antagonist (AH7614, 10 uM, 20 minutes) with a non-significant but lower calcium response observed (N = 7 cells from 6 experiments, p = 0.432. Figure 5) in 18.75% of cells tested. FFAR4 was visible at the cell membrane and cytosol in the semi-quantitative assessment of FFAR4 expression under control conditions and following exposure to *B.breve ncfb2258* supernatants alone or with FFAR4 antagonist. FFAR4 expression was significantly increased when cells were exposed to *B.breve ncfb2258* supernatants (p = 0.042) but remained elevated when exposed to FFAR4 antagonist and *B.breve ncfb2258* supernatants (p= 0.999). C-Fos staining remained elevated when cells were exposed to B.breve ncfb2258 supernatants in the presence of the FFAR4 antagonist (p = 0.021). Figure 3 contains representative graphs and images which depict the relative expression of FFAR4 in the cytosol and at the cell membrane and nuclear c-Fos when unstimulated (incubated with HBSS) or following exposure to *B. breve ncfb2258* (1:50, 15 minutes) alone or in the presence of FFAR4 antagonist (10 uM, 20 minutes).

A **B**

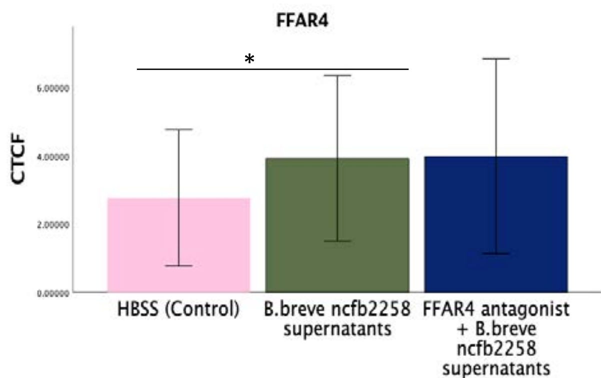




C



D



E

Figure 3. **A**. A sample trace illustrating calcium release when NCI-H716 cells were exposed to FFAR4 antagonist and *B.breve ncfb228* supernatants. **B.** Illustrates the mean corrected total cell fluorescence of c-Fos from immunofluorescence carried out on NCI-H716 cells exposed to HBSS (control), *B. breve ncfb2258* supernatants alone and in the presence of FFAR4 antagonist. Bars represent mean value ± 1 SD. Statistical significance was determined by one way-ANOVA ($F_{3,236}$ = 12.9, p = 0.021) followed by Tukey's post-hoc test. N=60 cells for each condition, from 3 experiments. * indicates p < 0.05, ** indicates p < 0.01 **C.** Representative fluorescence images for control, *B. breve ncfb2258* supernatants and *B. breve ncfb2258* supernatants with FFAR4 antagonist stained with c-Fos antibody. **D.** Illustrates the mean corrected total cell fluorescence of FFAR4 from immunofluorescence carried out on NCI-H716 cells exposed to HBSS (control), *B.breve ncfb2258* supernatants and FFAR4 antagonist with *B.breve ncfb2258* supernatants. Bars represent mean value ± 1 SD. Statistical significance was determined

by one way-ANOVA ($F_{3,236}$ = 9.45, p = < 0.001), followed by Tukey's post-hoc test. N=60 cells for each condition, from 3 experiments. * indicates p < 0.05. **E**. Representative fluorescence images for control (incubated with HBSS), *B. breve ncfb2258* supernatants and *B. breve ncfb2258* supernatants with FFAR4 antagonist stained with FFAR4 antibody.

Supernatants increase PPARα expression on NCI-H716 cells

The expression of PPARα was examined to determine if it expressed receptors for bacterial products. PPARα was visible at the cell membrane and cytosol. In the semi-quantitative assessment of PPARα expression under control conditions and following exposure to *B.breve ncfb2258* supernatants alone and with PPARα antagonist. PPARα expression was significantly increased when exposed to *B.breve ncfb2258* supernatants (p < 0.001). It was also significantly decreased after exposure to PPARα antagonist with *B.breve ncfb2258* supernatants (p < 0.001). PPARα antagonist blocked cFos activation evoked by B.breve ncfb2258 supernatants such that there was no significant difference between the control dish and the dish exposed to *B.breve ncfb2258* supernatants in the presence of the PPARα antagonist (p = 0.749). Figure 4 contains representative graphs and images which depict the relative expression of PPARα in the cell body and cytosol and nuclear c-Fos when unstimulated (incubated with HBSS) or following exposure to *B. breve ncfb2258* (1:50, 15 minutes) alone or in the presence of PPARα antagonist (1 uM, 20 minutes).

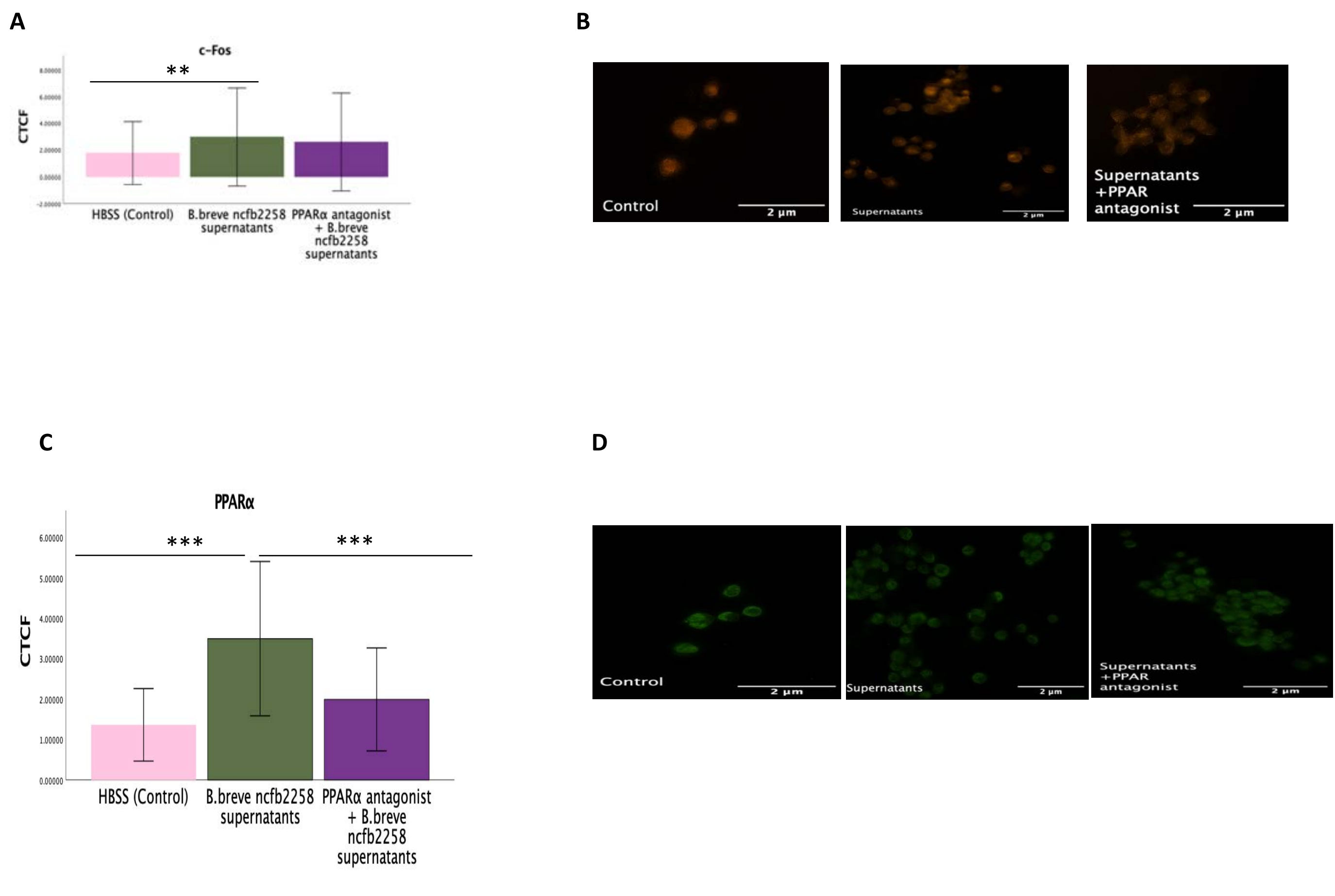


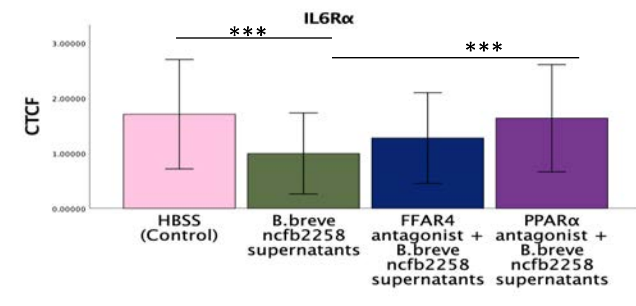
Figure 4. **A**. Illustrates the mean corrected total cell fluorescence of c-Fos from immunofluorescence carried out on NCI-H716 cells exposed to HBSS (control), *B.breve ncfb2258* supernatants and PPARα antagonist with *B.breve ncfb2258* supernatants. Bars represent mean value ± 1 SD. Statistical significance was determined by one way-ANOVA ($F_{3,236}$ = 12.9, p = 0.749), followed by Tukey's post-hoc test. N=60 cells for each condition, from 3

experiments. ** indicates $p < 0.01$. **B.** Representative fluorescence images for control (incubated with HBSS), *B. breve ncfb2258* supernatants and *B. breve ncfb2258* supernatants with PPARα antagonist stained with c-Fos antibody. **C.** Illustrates the mean corrected total cell fluorescence of PPARα from immunofluorescence carried out on NCI-H716 cells exposed to HBSS (control), *B.breve ncfb2258* supernatants and PPARα antagonist with *B.breve ncfb2258* supernatants. Bars represent mean value ± 1 SD. Statistical significance was determined by one way-ANOVA ($F_{3,235} = 30.79$, $p = < 0.001$), followed by Tukey's post-hoc test. N=60 cells for each condition, from 3 experiments. *** indicates $p < 0.001$. **D**. Representative fluorescence images for control (incubated with HBSS), *B. breve ncfb2258* supernatants and *B. breve ncfb2258* supernatants and PPARα antagonist stained with PPARα antibody.

IL6R-α expression is inhibited by *B. breve ncfb2258* supernatants

The cells were examined to determine the expression of receptors for cytokines that are activated by bacterial products and involved in neuromodulatory processes. IL6Rα was visible at the cell membrane of these cells. In the semi-quantitative assessment of IL6Rα expression was significantly decreased when exposed to *B.breve ncfb2258* supernatants ($p < 0.001$). The suppression of staining was blocked by antagonists for PPARα ($p < 0.001$) and FFAR4 (0.309). Figure 5 contains representative graphs and images which depict the relative expression of IL6Rα at the cell membrane when unstimulated (incubated with HBSS) or following exposure to *B. breve ncfb2258* (1:50, 15 minutes) alone or in the presence of either FFAR4 antagonist (10 uM, 20 min) or PPARα antagonist (1 uM, 20 minutes).

A



B

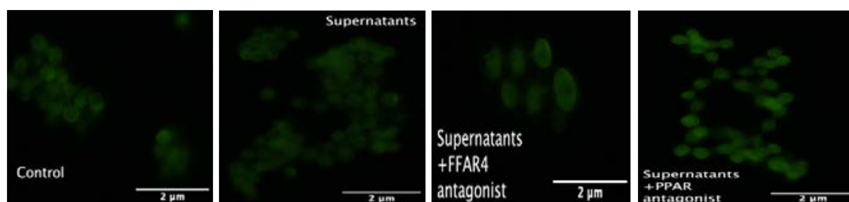


Figure 5. **A**. Illustrates the mean corrected total cell fluorescence of IL6Rα from immunofluorescence carried out on NCI-H716 cells exposed to HBSS (control), *B.breve ncfb2258* supernatants, FFAR4 antagonist and *B.breve ncfb2258* supernatants and PPARα antagonist and *B.breve ncfb2258* supernatants. Bars represent mean value ± 1 SD. Statistical significance was determined by one way-ANOVA ($F_{3,236} = 8.37$, $p = < 0.001$), followed by Tukey's post-hoc test. N=60 cells for each condition, from 3 experiments. *** indicates $p < 0.001$. **B.** Representative fluorescence images for control (incubated with HBSS), *B. breve ncfb2258* supernatants, *B. breve ncfb2258* supernatants and FFAR4 antagonist, *B. breve ncfb2258* supernatants and PPARα antagonist stained with IL6Rα antibody.

Discussion

This study aimed to investigate whether the NCI-H716 human cell line was appropriate to explore GLP-1-secreting L-cells as potential cross-barrier signal transducers for *B. Breve ncfb2258* and the molecular mechanisms involved in signalling induced by PUFA-producing *B. Breve ncfb2258* in human GLP-1-secreting L-cells. The findings from this research could contribute to the understanding of gut-brain signalling pathways, metabolic regulation, and potential therapeutic interventions for metabolic disorders. The results indicated that the NCI-H716 human cell line was, in fact, appropriate to explore GLP-1-secreting L-cells as potential cross-barrier signal transducers for *B. Breve ncfb2258.* Moreover, the results support a mechanism by which PPARα is the receptor that mediates cellular activation induced by the bacterial products.

The calcium imaging experiments revealed that *B. breve ncfb2258* supernatants caused a significant response in NCI-H716 cells, suggesting that the cell line was responsive and an appropriate model for exploring GLP-1 secreting L-cells as potential cross-barrier signal transducers. Furthermore, the immunofluorescence study provided additional confirmation by showing elevated levels of c-Fos when these cells were exposed to B. breve ncfbb2258 supernatants. The elevation of c-Fos, a well-known marker of cellular activation, reinforces the idea that *B. breve* can indeed stimulate GLP-1 secretion in these cells. These findings are consistent with earlier studies that demonstrated the ability of B. breve to stimulate GLP-1 secreting L-cells (Alisi et al., 2014). Additionally, they align with other studies indicating that NCI-H716 cells are excellent models for studying the regulation of GLP-1 and the mechanisms involved (Gagnon et al., 2015).

After cells were exposed to *B. breve ncfb2258* supernatants, c-Fos staining remained elevated even when exposed to the FFAR4 antagonist along with B. breve ncfb2258 supernatants. This sustained elevation indicates that FFAR4 is not involved in the signalling pathway mediating the cellular response to *B. breve ncfb2258*. In contrast, when the PPARα antagonist was introduced, it blocked the activation of c-Fos that was otherwise evoked by *B. breve ncfb2258* supernatants. In this scenario, there was no significant difference in c-Fos activation between the control dish and the dish exposed to B. breve ncfb2258 supernatants in the presence of the PPARα antagonist. This observation suggests that the PPARα antagonist inhibited the c-Fos activation induced by B. breve ncfb2258 supernatants, implying that PPARα likely plays a crucial role in this cellular response. The inhibition of c-Fos activation by the PPARα antagonist highlights the importance of PPARα in the signalling pathway, as its inhibition prevented the expected cellular response to the supernatants.

These results are consistent with previous findings suggesting that PPARα is vital in GLP-1 secretion in these cells (Daoudi et al., 2011) and aligns with the notion that PPARα plays a major role in the regulation of GLP-1 secretion, reinforcing the idea that PPARα is an important part of the cellular mechanisms regulating the interaction between GLP-1 secreting L-cells and *B. breve ncfb2258*. In contrast, the results do not support earlier hypotheses which suggested that FFAR4 might be involved in the interaction between GLP-1-secreting L-cells and *B. breve ncfb2258* (Schmidt et al., 2011). This deviation from previous hypotheses regarding FFAR4's role helps narrow the search for the molecular mechanisms involved. Furthermore, these findings potentially identify a specific molecular mechanism involved in the signalling induced by PUFA-producing *B. breve ncfb2258* in human GLP-1-secreting L-

cells. The role of PPARα in this context suggests a connection between bacterial metabolites and host cellular receptors, where PPARα acts as a key mediator.

GLP-1 was visible in vesicles in the cytoplasm of these cells in immunofluorescence studies, yet the expression levels remained unchanged when treated with the bacterial supernatants. Notably, GLP-1 was visibly moved towards the cell membrane and appeared more punctate, indicating vesicles on the verge of being released from the cells after stimulation of the cells by *B. breve ncfb2258* supernatants. This observation suggests a potential preparatory stage for secretion, where GLP-1 is mobilized to the cell membrane in response to the bacterial supernatants. Additionally, there was visibly less fluorescence when cells were exposed to *B. breve ncfb2258* supernatants in the presence of a PPARα antagonist. This decrease in fluorescence further supports the notion that PPARα plays a significant role in the signalling pathway mediating GLP-1 release. As the statistical analysis did not yield substantial results, this experiment must be repeated as there is promise in the results from the immunofluorescence images.

The redistribution of GLP-1 towards the cell membrane and the reduction in fluorescence under the influence of the PPARα antagonist both indicate that PPARα is crucial for the process of GLP-1 secretion. This result aligns with studies that have shown B. breve stimulates the release of GLP-1 from intestinal L-cells (Alisi et al.,2014; Izumi et al., 2019). Furthermore, the involvement of PPARs, particularly in L-cells, has been documented and has shown that conjugated linoleic acids (CLAs) activate PPARs found in L-cells (Poirier et al., 2001; Yang et al. in 2018) and these findings are also consistent with evidence which demonstrated that enteroendocrine L-cell GLP-1 secretion is positively regulated by PPAR activation (Daoudi et al., 2011). This positive regulation reveals the role of PPARs in the metabolic functions of L-cells, where their activation leads to enhanced GLP-1 secretion.

The immunofluorescence studies also provide a visual confirmation of the intracellular dynamics of GLP-1, where the movement towards the cell membrane upon exposure to *B. breve ncfb2258* supernatants suggests a preparatory phase for secretion. This observation is critical as it highlights the cellular readiness to secrete GLP-1, influenced by bacterial metabolites. This finding confirms what is already known and supports previous evidence of GLP-1 secretion from L-cells (Buckley et al., 2020), also confirming that NCI-H716 cells are a suitable model for exploring this interaction as earlier revealed in this study and an earlier one by Gagnon et al. in 2015. The decrease in fluorescence intensity in the presence of the PPARα antagonist implies a direct regulatory effect, where inhibition of PPARα disrupts the secretion of GLP-1.

GLP1R was slightly but not significantly increased after exposure to B. breve nfcb2258 supernatants and increased significantly after exposure to PPARα antagonists. The upregulation of GLP1R suggests that the cells are becoming more responsive to GLP-1, which could enhance GLP-1 secretion. This potential increase in cellular responsiveness to GLP-1 indicates a heightened sensitivity of the cells to this important incretin hormone. Previous research has suggested that certain signalling pathways triggered by bacterial stimuli are mediated by GLP-1R activation, implicating L-cells as bacterial cellular transducers (Buckley et al., 2020). This makes it interesting to study the interaction between bacterial supernatants and GLP-1R activation. However, the current findings partially differ from previous studies, as the increase in GLP1R expression was more pronounced with PPARα antagonists than with bacterial supernatants alone. This deviation from previous research shows the complexity of the signalling mechanisms involved and suggests that multiple pathways might be involved in mediating the effects of bacterial stimuli on GLP-1 secretion. There is still a possibility that GLP1R is involved in

the signalling processes, perhaps through signalling crosstalk with PPARα. The increase in GLP1R expression in response to PPARα antagonists suggests that the receptor may play a role in modulating GLP-1 secretion. The observed upregulation of GLP1R upon PPARα antagonism could indicate a compensatory mechanism or a feedback loop in response to the blockade of these receptors.

This feedback mechanism might function to maintain cellular homeostasis by upregulating GLP1R expression when PPARα activity is inhibited, thereby ensuring that the cells remain responsive to GLP-1. Such compensatory upregulation could enhance the sensitivity of the cells to GLP-1, potentially offsetting the reduced signalling through PPARα pathways. This interplay between PPARα and GLP1R highlights the regulatory pathways that control GLP-1 secretion and responsiveness in L-cells. Evidence has pointed to PPARα being involved in the stimulation of GLP-1 (Daoudi et al., 2011), supporting the idea that PPARα and GLP1R may interact. PPARα activation has been shown to influence various metabolic processes, including lipid metabolism and energy homeostasis, which could indirectly affect GLP-1 secretion (Vargas-Sanchez et al., 2020). The potential crosstalk between PPARα and GLP1R could represent a regulatory axis, where modulation of one receptor impacts the expression and function of the other.

Another finding of this study was the absence of a significant difference in the peak amplitude between *the B. breve ncfb2258* supernatant response and the FFAR4 antagonist, in the presence of *B. breve ncfb2258*, response. The findings suggest that FFAR4 does not mediate the effects of the supernatants, however, additional experiments are warranted to bolster this finding, due to the small sample size and image acquisition faults encountered while carrying out this experiment. Also, immunofluorescence studies of FFAR4 showed a significant increase in FFAR4 expression observed when cells were exposed to *B. breve ncfb2258* supernatants indicating that FFAR4 expression is increased in GLP-1-secreting L-cells by bacterial supernatants. However, the absence of a significant difference in FFAR4 expression between cells exposed to *B. breve ncfb2258* supernatants alone and those treated with FFAR4 antagonist and *B. breve ncfb2258* supernatants suggests that inhibiting FFAR4 does not influence FFAR4 expression in this circumstance. This finding partially contrasts previous evidence suggesting the involvement of FFAR4 in regulating the effects of microbial stimuli on cellular responses, and hypotheses suggesting its direct involvement in this interaction between *B. breve ncfb2258* and GLP-1 secreting L-cells (Schmidt et al., 2011; Hirasawa et al., 2005), but it also supports earlier results in this study.

This study also alluded to PPARα being involved in the molecular mechanisms involved in signalling induced by PUFA-producing *B. Breve ncfb2258* in human GLP-1-secreting L-cells as PPARα expression significantly increased following exposure to *B. breve ncfb2258* supernatants. Exposure to PPARα antagonist led to a substantial decrease in PPARα levels, again suggesting PPARα's involvement in regulating cellular responses to *B.breve ncfb2258* stimulation. This result was expected and serves as supporting evidence for the involvement of PPARα-mediated pathways in the interaction between *B. breve ncfb2258* and GLP-1-secreting L-cells. This corresponds to evidence found in previous studies that enteroendocrine L-cell GLP-1 secretion is positively regulated by PPAR activation (Daoudi et al., 2011) and that PPAR is activated by CLAs (Poirier et al., 2001; Yang et al., 2018), a product of *B.breve ncfb2258* (O'Connell et al., 2013) and presents the possibility of PPARα being involved in the molecular signalling pathway activated by the interaction between GLP-1-secreting L-cells and *B. Breve ncfb2258*.

Additionally, the suppression of IL6Rα expression observed when cells were exposed to *B. breve ncfb2258* supernatants demonstrates the impact of bacterial supernatants on IL6Rα expression. This

implies that elements found in *B. breve ncfb2258* supernatants might affect the expression of IL6Rα, hence linking IL6Rα to the regulation of responses to B. breve ncfb2258. It deviates from earlier results that *B. breve* activates IL-6 (Yang et al., 2018). The significantly higher expression of IL6Rα after PPARα antagonist administration in the presence of B. breve ncfb2258 supernatants suggests a role for PPARα in modulating IL6Rα levels. This is in line with previous findings that PPARα regulates immunity and lowers plasma concentrations of IL-6 (Delerive et al., 1999). It is interesting to note that IL-6 and PPARα work together to increase liver fatty acid binding protein (LFABP) expression in the liver (Vida et al., 2013) and a similar pathway might be activated between them by the interaction between *B. breve ncfb2258* and L-cells. No significant difference in IL6Rα expression was observed when cells were exposed to *B.breve ncfb2258* supernatants compared to FFAR4 antagonist and B.breve ncfb2258 supernatants, indicating that FFAR4 signalling may not play a major role in modulating IL6Rα expression in these circumstances, however, this is a deviation from other studies that have shown FFAR4 activation reduces IL-6 expression both directly and indirectly through different pathways (Tomita et al., 2020; Ren et al; 2019; Sulijaya et al; 2018). However, it is important to note that IL6Rα was imaged at a relatively higher exposure time than others, due to it not showing up on the microscope and these results might therefore be unreliable. This initial lack of visibility could be attributed to non-specific binding of the antibody to other antigens or background staining could distort true differences in IL-6Rα levels.

Ethical aspects and impact of the research on society

The use of NCI-H716 cells raises ethical concerns regarding the sourcing and handling of cell lines. It is important to ensure that cells used in research are obtained ethically. However, there was no need for ethical permits when using this cell line. Additionally, the handling and maintenance of cell cultures need careful attention to minimize contamination. Moreover, the use of chemical reagents such as Fluo-2 AM dye, Paraformaldehyde, and blocking permeabilization buffer in calcium imaging and immunofluorescence experiments raises concerns about potential hazards to health and safety of researchers. It is important to handle these reagents per safety protocols, including appropriate personal protective equipment, waste disposal procedures, and adherence to relevant regulations on chemical handling and disposal.

Understanding the signalling mechanisms employed by GLP-1-secreting L-cells in response to *B. breve ncfb2258* can have important implications for human health. Knowing how these specialized cells interact with specific bacterial strains and modulate immune molecules provides the potential to deepen the understanding of gut microbiota-host interactions, immune modulation, and gastrointestinal physiology, all of which are essential for developing targeted interventions to promote gut health and mitigate conditions associated with impaired gut-brain axis communication. By manipulating the signalling pathways involved in this interaction, researchers may be able to regulate immune responses, enhance metabolic regulation, and potentially alleviate conditions such as metabolic syndrome, diabetes, obesity, and gastrointestinal disorders.

Conclusions/Future Perspectives

In conclusion, this study has provided valuable insights into the complex interaction between GLP-1-secreting L-cells and *B. breve ncfb2258*, indicating that NCI-H716 cells are suitable for exploring this interaction and PPARα is involved in the molecular signaling pathways activated by this interaction.

However, some results were either unreliable or deviated from previous hypotheses and require further investigation to provide more evidence or completely rule out the findings. This study also suggests the existence of additional regulatory mechanisms yet to be discovered.

Moving forward, future research should explore these pathways by investigating potential signalling crosstalk and identifying additional factors that may influence cellular responses to bacterial stimuli. Efforts should also be made to address variability in experimental outcomes, such as image acquisition parameters, antibody incubation protocols or small sample sizes to ensure the reliability and reproducibility of findings. Overall, this study lays the groundwork for further exploration of the relationship between gut microbiota and host cells and paves the way for potential therapeutic interventions targeting the gut-brain axis and gastrointestinal health.

Acknowledgements

I would like to express my deepest gratitude to my supervisor, Dr Dervla O'Malley, for her support and guidance throughout the process of completing this bachelor thesis. I would also like to extend my appreciation to Cliona O'Connor, who assisted me with various aspects of my research. A special note of thanks goes to Qiao Xiao and Dr Mark Rae. Finally, I would like to thank the staff and students at University College Cork and the University of Skövde.

References

Alisi, A., Bedogni, G., Baviera, G., Giorgio, V., Porro, E., Paris, C., Giammaria, P., Reali, L., Anania, F., & Nobili, V. (2014, April 16). Randomised clinical trial: the beneficial effects of VSL#3 in obese children with non-alcoholic steatohepatitis. *Alimentary Pharmacology & Therapeutics*, 39(11), 1276–1285.

Alonso, C., Guilarte, M., Vicario, M., Ramos, L., Ramadan, Z., Antolín, M., Martínez, C., Rezzi, S., Saperas, E., Kochhar, S., Santos, J., & Malagelada, J. R. (2008, July). Maladaptive Intestinal Epithelial Responses to Life Stress May Predispose Healthy Women to Gut Mucosal Inflammation. *Gastroenterology*, 135(1), 163-172.e1. https://doi.org/10.1053/j.gastro.2008.03.036

Arenas-Gómez, C. M., Garcia-Gutierrez, E., Escobar, J. S., & Cotter, P. D. (2022). Human gut homeostasis and regeneration: the role of the gut microbiota and its metabolites. *Critical Reviews in Microbiology*, 1–22. https://doi.org/10.1080/1040841x.2022.2142088

Arora, T., Akrami, R., Pais, R., Bergqvist, L., Johansson, B. R., Schwartz, T. W., Reimann, F., Gribble, F. M., & Bäckhed, F. (2018). Microbial regulation of the L cell transcriptome. *Scientific Reports*, 8(1), 1207. https://doi.org/10.1038/s41598-017-18079-2

Barker, N., Van Es, J. H., Kuipers, J., Kujala, P., Born, M. P., Cozijnsen, M., Haegebarth, A., Korving, J., Begthel, H., Peters, P. J., & Clevers, H. (2007). Identification of stem cells in small intestine and colon by marker gene Lgr5. *Nature*, 449(7165), 1003–1007. https://doi.org/10.1038/nature06196

Bellono, N. W., Bayrer, J. R., Leitch, D. B., Castro, J., Zhang, C., O'Donnell, T. A., ... & Julius, D. (2017). Enterochromaffin cells are gut chemosensors that couple to sensory neural pathways. *Cell*, *170*(1), 185-198.

Berer, K., Mues, M., Koutrolos, M., Rasbi, Z. A., Boziki, M., Johner, C., Wekerle, H., & Krishnamoorthy, G. (2011, October 26). Commensal microbiota and myelin autoantigen cooperate to trigger

autoimmune demyelination. *Nature*, 479(7374), 538–541. https://doi.org/10.1038/nature10554

Bohórquez D. V., Samsa L. A., Roholt A., Medicetty S., Chandra R., Liddle R. A. (2014). An enteroendocrine cell–enteric glia connection revealed by 3D electron microscopy. *PLoS One* 9: e89881. doi: 10.1371/journal.pone.0089881

Bohórquez, D. V., Shahid, R. A., Erdmann, A., Kreger, A. M., Wang, Y., Calakos, N., Wang, F., & Liddle, R. A. (2015, January 2). Neuroepithelial circuit formed by innervation of sensory enteroendocrine cells. *Journal of Clinical Investigation*, 125(2), 782–786. https://doi.org/10.1172/jci78361

Bottacini, F., O'Connell Motherway, M., Kuczynski, J., O'Connell, K. J., Serafini, F., Duranti, S., Milani, C., Turroni, F., Lugli, G. A., Zomer, A., Zhurina, D., Riedel, C., Ventura, M., & Sinderen, D. V. (2014, March 1). Comparative genomics of the Bifidobacterium brevetaxon. *BMC Genomics*, 15(1). https://doi.org/10.1186/1471-2164-15-170

Buckley, M. M., O'Brien, R., Brosnan, E., Ross, R. P., Stanton, C., Buckley, J. M., & O'Malley, D. (2020, April 30). Glucagon-Like Peptide-1 Secreting L-Cells Coupled to Sensory Nerves Translate Microbial Signals to the Host Rat Nervous System. *Frontiers in Cellular Neuroscience*, 14. https://doi.org/10.3389/fncel.2020.00095

Chimerel, C., Emery, E., Summers, D., Keyser, U., Gribble, F., & Reimann, F. (2014, November). Bacterial Metabolite Indole Modulates Incretin Secretion from Intestinal Enteroendocrine L Cells. *Cell Reports*, 9(4), 1202–1208. https://doi.org/10.1016/j.celrep.2014.10.032

Christiansen, C. B., Gabe, M. B. N., Svendsen, B., Dragsted, L. O., Rosenkilde, M. M., & Holst, J. J. (2018, July 1). The impact of short-chain fatty acids on GLP-1 and PYY secretion from the isolated perfused rat colon. *American Journal of Physiology-Gastrointestinal and Liver Physiology*, 315(1), G53–G65. https://doi.org/10.1152/ajpgi.00346.2017

Choi, Y. J., & Shin, H. S. (2021, September 1). Antibacterial Effect of Eight Probiotic Strains of Bifidobacterium against Pathogenic Staphylococcus aureus and Pseudomonas aeruginosa. *Journal of Bacteriology and Virology*, 51(3), 128–137. https://doi.org/10.4167/jbv.2021.51.3.128

Daoudi, M., Hennuyer, N., Borland, M. G., Touche, V., Duhem, C., Gross, B., Caiazzo, R., Kerr–Conte, J., Pattou, F., Peters, J. M., Staels, B., & Lestavel, S. (2011). PPARβ/δ Activation Induces Enteroendocrine L Cell GLP-1 Production. *Gastroenterology*, 140(5), 1564–1574. https://doi.org/10.1053/j.gastro.2011.01.045

Delerive, P., De Bosscher, K., Besnard, S., Vanden Berghe, W., Peters, J. M., Gonzalez, F. J., Fruchart, J. C., Tedgui, A., Haegeman, G., & Staels, B. (1999, November). Peroxisome Proliferator-activated Receptor α Negatively Regulates the Vascular Inflammatory Gene Response by Negative Cross-talk with Transcription Factors NF-κB and AP-1. *Journal of Biological Chemistry*, 274(45), 32048–32054. https://doi.org/10.1074/jbc.274.45.32048

DLUGOSZ, J., FÖLSCH, U. R., CZAJKOWSKI, A., & GABRYELEWICZ, A. (1988, June). Feedback regulation of stimulated pancreatic enzyme secretion during intraduodenal perfusion of trypsin in man. *European Journal of Clinical Investigation*, 18(3), 267–272. https://doi.org/10.1111/j.1365-2362.1988.tb01257.x

Edfalk, S., Steneberg, P., & Edlund, H. (2008, September 1). Gpr40 Is Expressed in Enteroendocrine Cells and Mediates Free Fatty Acid Stimulation of Incretin Secretion. *Diabetes*, 57(9), 2280–2287. https://doi.org/10.2337/db08-0307

Ellingsgaard, H., Hauselmann, I., Schuler, B., Habib, A. M., Baggio, L. L., Meier, D. T., Eppler, E., Bouzakri, K., Wueest, S., Muller, Y. D., Hansen, A. M. K., Reinecke, M., Konrad, D., Gassmann, M., Reimann, F., Halban, P. A., Gromada, J., Drucker, D. J., Gribble, F. M., . . . Donath, M. Y. (2011, October 30). Interleukin-6 enhances insulin secretion by increasing glucagon-like peptide-1 secretion from L cells and alpha cells. *Nature Medicine*, 17(11), 1481–1489. https://doi.org/10.1038/nm.2513

Engevik, M. A., Herrmann, B., Ruan, W., Engevik, A. C., Engevik, K. A., Ihekweazu, F., … Versalovic, J. (2021). Bifidobacterium dentium-derived y-glutamylcysteine suppresses ER-mediated goblet cell stress and reduces TNBS-driven colonic inflammation. *Gut Microbes*, 13(1). https://doi.org/10.1080/19490976.2021.1902717

Erny, D., Hrabě de Angelis, A., Jaitin, D. et al. Host microbiota constantly control maturation and function of microglia in the CNS. *Nat Neurosci* 18, 965–977 (2015). https://doi.org/10.1038/nn.4030

Furusawa, Y., Obata, Y., Fukuda, S., Endo, T. A., Nakato, G., Takahashi, D., Nakanishi, Y., Uetake, C., Kato, K., Kato, T., Takahashi, M., Fukuda, N. N., Murakami, S., Miyauchi, E., Hino, S., Atarashi, K., Onawa, S., Fujimura, Y., Lockett, T., . . . Ohno, H. (2013, November 13). Commensal microbe-derived butyrate induces the differentiation of colonic regulatory T cells. *Nature*, 504(7480), 446–450. https://doi.org/10.1038/nature12721

Gagnon, J., & Brubaker, P. L. (2015, January 1). NCI-H716 Cells. *Springer*. https://doi.org/10.1007/978-3-319-16104-4_20

Glaser, C., Heinrich, J., & Koletzko, B. (2010, July). Role of FADS1 and FADS2 polymorphisms in polyunsaturated fatty acid metabolism. *Metabolism*, 59(7), 993–999. https://doi.org/10.1016/j.metabol.2009.10.022

Gotze, H., Adelson, J. W., Hadorn, H. B., Portmann, R., & Troesch, V. (1972, June 1). Hormone-elicited enzyme release by the small intestinal wall. *Gut*, 13(6), 471–476. https://doi.org/10.1136/gut.13.6.471

Gorboulev, V., Schürmann, A., Vallon, V., Kipp, H., Jaschke, A., Klessen, D., Friedrich, A., Scherneck, S., Rieg, T., Cunard, R., Veyhl-Wichmann, M., Srinivasan, A., Balen, D., Breljak, D., Rexhepaj, R., Parker, H. E., Gribble, F. M., Reimann, F., Lang, F., . . . Koepsell, H. (2011, December 12). Na+-d-glucose Cotransporter SGLT1 is Pivotal for Intestinal Glucose Absorption and Glucose-Dependent Incretin Secretion. *Diabetes*, 61(1), 187–196. https://doi.org/10.2337/db11-1029

Hevia, A., Milani, C., López, P., Cuervo, A., Arboleya, S., Duranti, S., Turroni, F., González, S., Suárez, A., Gueimonde, M., Ventura, M., Sánchez, B., & Margolles, A. (2014, October 31). Intestinal Dysbiosis Associated with Systemic Lupus Erythematosus. *MBio*, 5(5). https://doi.org/10.1128/mbio.01548-14

Hirasawa, A., Tsumaya, K., Awaji, T., Katsuma, S., Adachi, T., Yamada, M., Sugimoto, Y., Miyazaki, S., & Tsujimoto, G. (2004, December 26). Free fatty acids regulate gut incretin glucagon-like peptide-1 secretion through GPR120. *Nature Medicine*, 11(1), 90–94. https://doi.org/10.1038/nm1168

Hou, Y., Ernst, S. A., Heidenreich, K., & Williams, J. A. (2016, January 1). Glucagon-like peptide-1 receptor is present in pancreatic acinar cells and regulates amylase secretion through cAMP. *American Journal of Physiology-Gastrointestinal and Liver Physiology*, 310(1), G26–G33. https://doi.org/10.1152/ajpgi.00293.2015

Izumi, H., Ehara, T., Sugahara, H., Matsubara, T., Mitsuyama, E., Nakazato, Y., Tsuda, M., Shimizu, T., Odamaki, T., Xiao, J. Z., & Takeda, Y. (2019, February). The Combination of Bifidobacterium breve and Three Prebiotic Oligosaccharides Modifies Gut Immune and Endocrine Functions in Neonatal Mice. *The*

Journal of Nutrition, 149(2), 344–353. https://doi.org/10.1093/jn/nxy248

Johansson, M. E. V., Phillipson, M., Petersson, J., Velcich, A., Holm, L., & Hansson, G. C. (2008, September 30). The inner of the two Muc2 mucin-dependent mucus layers in colon is devoid of bacteria. *Proceedings of the National Academy of Sciences*, 105(39), 15064–15069. https://doi.org/10.1073/pnas.0803124105

Karaosmanoglu, T., Aygun, B., Wade, P.R. and Gershon, M.D. (1996), Regional differences in the number of neurons in the myenteric plexus of the guinea pig small intestine and colon: An evaluation of markers used to count neurons. *Anat. Rec.*, 244: 470-480. https://doi.org/10.1002/(SICI)1097-0185(199604)244:4<470::AID-AR5>3.0.CO;2-Z

Kuhre, R. E., Deacon, C. F., Holst, J. J., & Petersen, N. (2021). What Is an L-Cell and How Do We Study the Secretory Mechanisms of the L-Cell? *Frontiers in Endocrinology*, 12. https://doi.org/10.3389/fendo.2021.694284

Lee, Y. K., Menezes, J. S., Umesaki, Y., & Mazmanian, S. K. (2010, July 26). Proinflammatory T-cell responses to gut microbiota promote experimental autoimmune encephalomyelitis. *Proceedings of the National Academy of Sciences*, 108(supplement_1), 4615–4622. https://doi.org/10.1073/pnas.1000082107

Levenstein, S., Prantera, C., Varvo, V., Scribano, M. L., Andreoli, A., Luzi, C., Arcà, M., Berto, E., Milite, G., & Marcheggiano, A. (2000, May). Stress and Exacerbation in Ulcerative Colitis: A Prospective Study of Patients Enrolled in Remission. *American Journal of Gastroenterology*, 95(5), 1213–1220. https://doi.org/10.1111/j.1572-0241.2000.02012.x

Lewis, J. E., Miedzybrodzka, E. L., Foreman, R. E., Woodward, O. R., Kay, R. G., Goldspink, D. A., Gribble, F. M., & Reimann, F. (2020). Selective stimulation of colonic L cells improves metabolic outcomes in mice. *Diabetologia*, 63, 1396–1407. https://doi.org/10.1007/s00125-020-05149-w

Liu, X., Lyu, W., Liu, L., Lv, K., Zheng, F., Wang, Y., Chen, J., Dai, B., Yang, H., & Xiao, Y. (2021, June 14). Comparison of Digestive Enzyme Activities and Expression of Small Intestinal Transporter Genes in Jinhua and Landrace Pigs. *Frontiers in Physiology*, 12. https://doi.org/10.3389/fphys.2021.669238

McQuilken, S. A. (2021, May). The mouth, stomach and intestines. *Anaesthesia & Intensive Care Medicine*, 22(5), 330–335. https://doi.org/10.1016/j.mpaic.2021.04.001

Nguyen, A. T., Mandard, S., Dray, C., Deckert, V., Valet, P., Besnard, P., Drucker, D. J., Lagrost, L., & Grober, J. (2014, January 16). Lipopolysaccharides-Mediated Increase in Glucose-Stimulated Insulin Secretion: Involvement of the GLP-1 Pathway. *Diabetes*, 63(2), 471–482. https://doi.org/10.2337/db13-0903

O'Connell, K. J., Motherway, M. O., Hennessey, A. A., Brodhun, F., Ross, R. P., Feussner, I., … van Sinderen, D. (2013). Identification and characterization of an oleate hydratase-encoding gene from Bifidobacterium breve. *Bioengineered*, 4(5), 313–321. https://doi.org/10.4161/bioe.24159

Ogobuiro, I., Gonzales, J., Shumway, K. R., & Tuma, F. (2023, April 8). *Physiology, Gastrointestinal*. StatPearls - NCBI Bookshelf.

Ørskov, C., Wettergren, A., & Holst, J. J. (1996). Secretion of the Incretin Hormones Glucagon-Like Peptide-1 and Gastric Inhibitory Polypeptide Correlates with Insulin Secretion in Normal Man Throughout the Day. *Scandinavian Journal of Gastroenterology*, 31(7), 665–670.

https://doi.org/10.3109/00365529609009147

Osadchiy, V., Martin, C. R., & Mayer, E. A. (2019, January). The Gut–Brain Axis and the Microbiome: Mechanisms and Clinical Implications. *Clinical Gastroenterology and Hepatology*, 17(2), 322–332. https://doi.org/10.1016/j.cgh.2018.10.002

Penas, F., Mirkin, G. A., Vera, M., Cevey, G., González, C. D., Gómez, M. I., Sales, M. E., & Goren, N. B. (2015, May). Treatment in vitro with PPARα and PPARγ ligands drives M1-to-M2 polarization of macrophages from T. cruzi-infected mice. *Biochimica Et Biophysica Acta (BBA) - Molecular Basis of Disease,* 1852(5), 893–904. https://doi.org/10.1016/j.bbadis.2014.12.019

Poirier, H., Niot, I., Monnot, M. C., Braissant, O., Meunier-Durmort, C., Costet, P., Pineau, T., Wahli, W., Willson, T. M., & Besnard, P. (2001, April 6). Differential involvement of peroxisome-proliferator-activated receptors α and δ in fibrate and fatty-acid-mediated inductions of the gene encoding liver fatty-acid-binding protein in the liver and the small intestine. *Biochemical Journal*, 355(2), 481–488. https://doi.org/10.1042/bj3550481

Reimann, F., Habib, A. M., Tolhurst, G., Parker, H. E., Rogers, G. J., & Gribble, F. M. (2008, December). Glucose Sensing in L Cells: A Primary Cell Study. *Cell Metabolism*, 8(6), 532–539. https://doi.org/10.1016/j.cmet.2008.11.002

Ren, Z., Chen, L., Wang, Y., Wei, X., Zeng, S., Zheng, Y., Gao, C., & Liu, H. (2019, February 1). Activation of the Omega-3 Fatty Acid Receptor GPR120 Protects against Focal Cerebral Ischemic Injury by Preventing Inflammation and Apoptosis in Mice. *The Journal of Immunology*, 202(3), 747–759. https://doi.org/10.4049/jimmunol.1800637

Savidge, T. C., Newman, P., Pothoulakis, C., Ruhl, A., Neunlist, M., Bourreille, A., Hurst, R., & Sofroniew, M. V. (2007, April). Enteric Glia Regulate Intestinal Barrier Function and Inflammation Via Release of S-Nitrosoglutathione. *Gastroenterology*, 132(4), 1344–1358. https://doi.org/10.1053/j.gastro.2007.01.051

Schmidt, J., Liebscher, K., Merten, N., Grundmann, M., Mielenz, M., Sauerwein, H., Christiansen, E., Due-Hansen, M. E., Ulven, T., Ullrich, S., Gomeza, J., Drewke, C., & Kostenis, E. (2011, April). Conjugated Linoleic Acids Mediate Insulin Release through Islet G Protein-coupled Receptor FFA1/GPR40. *Journal of Biological Chemistry*, 286(14), 11890–11894. https://doi.org/10.1074/jbc.c110.200477

Stolk, M., Erpecum, K.V., Peeters, T. et al (2001). Interdigestive Gallbladder Emptying, Antroduodenal Motility, and Motilin Release Patterns Are Altered in Cholesterol Gallstone Patients. *Dig Dis Sci* 46, 1328–1334. https://doi.org/10.1023/A:1010635901414

Stone, V. M., Dhayal, S., Brocklehurst, K. J., Lenaghan, C., Sörhede Winzell, M., Hammar, M., Xu, X., Smith, D. M., & Morgan, N. G. (2014, March 25). GPR120 (FFAR4) is preferentially expressed in pancreatic delta cells and regulates somatostatin secretion from murine islets of Langerhans. *Diabetologia*, 57(6), 1182–1191. https://doi.org/10.1007/s00125-014-3213-0

Suarez, A. N., Hsu, T. M., Liu, C. M., Noble, E. E., Cortella, A. M., Nakamoto, E. M., Hahn, J. D., de Lartigue, G., & Kanoski, S. E. (2018, June 5). Gut vagal sensory signaling regulates hippocampus function through multi-order pathways. *Nature Communications*, 9(1). https://doi.org/10.1038/s41467-018-04639-1

Sulijaya, B., Takahashi, N., Yamada, M., Yokoji, M., Sato, K., Aoki-Nonaka, Y., Nakajima, T., Kishino, S., Ogawa, J., & Yamazaki, K. (2018, April 23). The anti-inflammatory effect of 10-oxo-trans-11-

octadecenoic acid (KetoC) on RAW 264.7 cells stimulated with Porphyromonas gingivalis lipopolysaccharide. *Journal of Periodontal Research*, 53(5), 777–784. https://doi.org/10.1111/jre.12564

Tomita, Y., Cakir, B., Liu, C. H., Fu, Z., Huang, S., Cho, S. S., Britton, W., Sun, Y., Puder, M., Hellström, A., Talukdar, S., & Tomita, Y. (2020, March 5). Free fatty acid receptor 4 activation protects against choroidal neovascularization in mice. *Angiogenesis*. https://doi.org/10.1007/s10456-020-09717-x

Turroni, F., Peano, C., Pass, D. A., Foroni, E., Severgnini, M., Claesson, M. J., Kerr, C., Hourihane, J., Murray, D., Fuligni, F., Gueimonde, M., Margolles, A., De Bellis, G., O'Toole, P. W., van Sinderen, D., Marchesi, J. R., & Ventura, M. (2012, May 11). Diversity of Bifidobacteria within the Infant Gut Microbiota. *PLoS ONE*, 7(5), e36957. https://doi.org/10.1371/journal.pone.0036957

Vaishnava, S., Yamamoto, M., Severson, K. M., Ruhn, K. A., Yu, X., Koren, O., Ley, R., Wakeland, E. K., & Hooper, L. V. (2011, October 14). The Antibacterial Lectin RegIIIγ Promotes the Spatial Segregation of Microbiota and Host in the Intestine. *Science*, 334(6053), 255–258. https://doi.org/10.1126/science.1209791

Van Bloemendaal, L., IJzerman, R. G., ten Kulve, J. S., Barkhof, F., Konrad, R. J., Drent, M. L., Veltman, D. J., & Diamant, M. (2014, November 13). GLP-1 Receptor Activation Modulates Appetite- and Reward-Related Brain Areas in Humans. *Diabetes*, 63(12), 4186–4196. https://doi.org/10.2337/db14-0849

Vargas-Sánchez, K., Vargas, L., Urrutia, Y., Beltrán, I., Rossi, A. B., Lozano, H. Y., Guarín, J., & Losada-Barragán, M. (2020, October 31). PPARα and PPARβ/δ are negatively correlated with proinflammatory markers in leukocytes of an obese pediatric population. *Journal of Inflammation*, 17(1). https://doi.org/10.1186/s12950-020-00264-2

Vida, M., Serrano, A., Romero-Cuevas, M., Pavón, F. J., González-Rodriguez, A., Gavito, A. L., Cuesta, A. L., Valverde, A. M., Rodríguez de Fonseca, F., & Baixeras, E. (2013). IL-6 cooperates with peroxisome proliferator-activated receptor-α-ligands to induce liver fatty acid binding protein (LFABP) up-regulation. *Liver international: official journal of the International Association for the Study of the Liver*, 33(7), 1019–1028. https://doi.org/10.1111/liv.12156

Wren, A., & Bloom, S. (2007, May). Gut Hormones and Appetite Control. Gastroenterology, 132(6), 2116–2130. https://doi.org/10.1053/j.gastro.2007.03.048

Yang, B., Chen, H., Gao, H., Wang, J., Stanton, C., Ross, R. P., Zhang, H., & Chen, W. (2018, October). Bifidobacterium breve CCFM683 could ameliorate DSS-induced colitis in mice primarily via conjugated linoleic acid production and gut microbiota modulation. *Journal of Functional Foods*, 49, 61–72. https://doi.org/10.1016/j.jff.2018.08.014

Zeng, Y., Wu, Y., Zhang, Q., & Xiao, X. (2023). Crosstalk between glucagon-like peptide 1 and gut microbiota in metabolic diseases. *MBio*. https://doi.org/10.1128/mbio.02032-23

Zhang, W., Huang, J., Gao, F., You, Q., Ding, L., Gong, J., Zhang, M., Ma, R., Zheng, S., Sun, X., & Zhang, Y. (2022, December). Lactobacillus reuteri normalizes altered fear memory in male Cntnap4 knockout mice. *EBioMedicine*, 86, 104323. https://doi.org/10.1016/j.ebiom.2022.104323

Zhao, J., Wang, L., Cheng, S., Zhang, Y., Yang, M., Fang, R., Li, H., Man, C., & Jiang, Y. (2022, January 16). A Potential Synbiotic Strategy for the Prevention of Type 2 Diabetes: Lactobacillus paracasei JY062 and Exopolysaccharide Isolated from Lactobacillus plantarum JY039. *Nutrients*, 14(2), 377. https://doi.org/10.3390/nu14020377

Appendices

Appendix A – Recipes

Table 1. HBSS

Component	Concentration	10X (1 L)	1X (1 L)
NaCl	130 mM	75.90 g	
KCl	5.4 mM	4 g	
HEPES	10 mM	23.80 g	
$CaCl_2$	2 mM		0.3 g
Glucose	10 mM		1.8 g
$MgCl_2$	1 mM		0.2 g

Table 2. HBSS K+ (50 mM)

Component	Concentration	10X (1 L)
NaCl	85.4 mM	49.9 g
KCl	50 mM	37.27 g

Everything is the same as normal HBSS except these.

Table 3. Blocking permeabilization buffer

Items	Concentration (w/v)	Amount in 50 ml
Fetal Bovine Serum	10 %	5 ml
BSA	1 %	0.5 g

Triton-X 100	0.1 %	50 µl
Sodium Azide (25% w/v stock)	0.05 %	100 µl

Appendix B - *B. breve ncfb2258* Culture

Bifidobacterium Breve ncfb2258 (*Bif. Breve* ncfb2258) were cultured at 1% (v/v) in de Man, Rogosa and Sharpe broth (Difco, VWR, Philadelphia, PA, US) for ~ 17 h at 37°C under anaerobic conditions (anaerobic jars with Anaerocult A Gas Packs (Merck, Darmstadt, Germany)) until stationary phase and centrifuged (16,900 x *g* for 15 min, at 4 °C; SLA-3000 rotor, Sorvall RC B5-Plus). The cell pellet was washed twice with phosphate-buffered saline (PBS; Sigma Aldrich, UK), re-suspended at ~2×10^{10} CFU.ml^{-1} in sterile 15% trehalose (Sigma Aldrich), which acted as a cryoprotectant and 1ml aliquots were dispensed into 2ml lyophilisation vials. The vials were lyophilised on a 24 h program (freeze temperature −40 °C, additional freeze 1 min, condenser set point −60, vacuum set point 600 mTorr; VirTis AdVantage Wizard 2.0) and stored at 4 °C. Bacteria were re-suspended in distilled water each day to deliver $1x10^{10}$ CFU.ml^{-1} for each exposure.