DISCOVERIE

Project No. 848228

DISCOvERIE

Development, dIagnostic and prevention of gender-related Somatic and mental COmorbitiEs in iRritable bowel syndrome In Europe

Workpackage 5 - Microbiome and Metabolome

Deliverable D5.4:
Mechanistically oriented sex-specific molecular signature of IBS comorbidities following animal and human microbiota transfer

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Table of Contents

List of Tables

List of Figures

List of abbreviations

MS: Maternal Separation

NS: Non-separated

IBS: Irritable bowel syndrome

Faecal microbiota transplantation: FMT

AEEC: Animal Experimentation Ethics Committee

HPRA: Health Products Regulatory Authority

PND: Postnatal day

CRD: Colorectal distension

FST: Forced swim test

PBS: phosphate buffered saline

EPM: Elevated plus maze

ELISA: Enzyme-linked immunosorbent assay

qRT-PCR: Quantitative reverse-transcriptase polymerase-chain-reaction

HPA: Hypothalamic-Pituitary-Adrenal Axis

TRPV1: Transient receptor potential cation channel subfamily V member 1

TAC1: Tachykinin Precursor 1

TEER: trans-epithelial electrical resistance

ISC: short-circuit current

Executive Summary

Introduction

Gut microbiota alterations are a feature of IBS. However, the functional implications of these compositional reconfigurations remains unclear. The signalling pathways of the microbiota-gut-brain axis include a number of important routes of communication that may be relevant in this regard. These bidirectional pathways include neuronal, endocrine, immune and microbial signalling pathways that may be associated with specific symptom sets.

Aim

in Deliverable 5.4, we successfully confirmed the transfer of comorbid behavioural phenotypes from rodent to rodent and from human to rodent via the gut microbiome. The aim of this deliverable was to study the biological pathways underpinning these phenotypes.

Methods

ELISA assays were used to assess corticosterone as an index of HPA axis activation. Immunohistochemistry was used to evaluate microglia number in spinal cord tissue. qRT-PCR was used to assess genes important for tryptophan metabolism, barrier function and nociception/sensation. Ussing Chambers were used to assess colonic permeability and secretory function.

Results

This was a sex-specific alteration in HPA axis activation following an acute stressor, where only male animals in receipt of an FMT from the donor depression-like cluster exhibited differential corticosterone profiles. At the molecular level in the colon, there was a sex- and cluster-specific expression of genes important for tight junctions in the gut barrier, with decreases only evident in male rats in the depression-like cluster for Cldn3 and Ocldn. Meanwhile male animals in the pain cluster showed increased expression of TRPV1, a receptor involved in nociception. Female rats in the pain cluster showed increased expression of TAC1 in the resilient cluster. This gene relates to Substance P, a peptide primarily produced by sensory neurons. We found no evidence for spinal cord alterations in microglia, and human to rodent FMT did not modify colonic permeability or secretory function.

Conclusion

Our study is the first to identify a role of the gut microbiota in driving the physiological and molecular profile of the behavioural phenotypes manifesting as a consequence of early life stress. These results confirm the presence of a mechanistically-oriented sex-specific molecular signature of IBS comorbidities following rodent to rodent FMT. These signatures are present in key signalling pathways of the gut-brain axis, including the HPA axis, barrier function and nociception/sensation. Taken together, these results establish a potential causal role for the gut microbiota in driving the expression of important molecular and physiological characteristics relevant to the behavioural features of IBS. Improving our understanding of the role of gut microbiota in driving specific gut-brain axis signalling

pathways important for the cardinal gastrointestinal and psychological features of IBS will be critical to accrue therapeutic benefits.

Short introduction: what is the aim of the deliverable?

Gut microbiota alterations are a feature of IBS. However, the functional implications of these compositional reconfigurations remains unclear. The signalling pathways of the microbiota-gut-brain axis include a number of important routes of communication that may be relevant in this regard. These bidirectional pathways include neuronal, endocrine, immune and microbial signalling pathways that may be associated with specific symptom sets. Improving our understanding of the role of gut microbiota in driving specific gut-brain axis signalling pathways important for the cardinal gastrointestinal and psychological features of IBS will be critical to accrue therapeutic benefits. These pathways were assessed in order to reveal candidate biomarkers of comorbidity as well as potential causative mechanisms arising from dysfunctional host-microbe interactions.

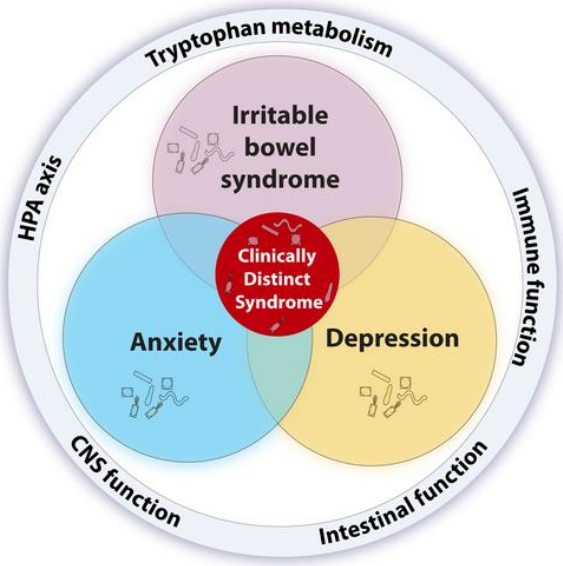

Figure 1 - Intersections between IBS, depression, and anxiety at the level of the gut microbiota. Figure reproduced from (Wilmes et al. 2021).

There is currently a poor understanding of the nature of the clinical entities that sit at the intersections between IBS, depression, and anxiety. One possibility is that a comorbid gut microbiota drives aberrant signalling along the gut-brain axis, leading to the manifestation of both gastrointestinal and behavioural symptom sets. Increased research efforts are required to understand why specific microbiota configurations lead in some cases to IBS and in others IBS with psychiatric comorbidity (In D5.4, we aimed to understand the biological pathways underpinning the pathophysiology of IBS with and without psychiatric comorbidity.

One of the gold standard approaches for gaining mechanistic insight about disease-associated microbiota configurations is via faecal microbiota transplantation (FMT) (Gheorghe et al. 2021; Secombe et al. 2021). Using this approach in Deliverable 5.4, we successfully confirmed the transfer of comorbid behavioural phenotypes from rodent to rodent and from human to rodent via the gut microbiome. The aim of this deliverable was to study the biological pathways underpinning these phenotypes, using both samples from

animal models of IBS (Maternal separation), and samples from rodent to rodent and human to rodent FMT. This was achieved using three interconnected approaches:
A: Early life stress is an important risk factor for IBS. Preclinically, this is modelled using maternal separation (O'Mahony et al. 2011). Animals subjected to this early-life stress exposure exhibit a behavioural phenotype reminiscent of IBS in adulthood, including both gastrointestinal dysfunction and depression- and anxiety-like behaviours. In deliverable 5.4, we stratified animals according to sex-specific vulnerability and resilience to identify translationally relevant subgroups consistent with the clinical presentation of comorbid phenotype development. Here, we evaluated these subgroups at a physiological and molecular level.
B. In deliverable 5.4, we successfully demonstrated the transfer of early life stress-induced behavioural phenotypes using rodent to rodent FMT. The next step described below was the assessment of physiological and molecular characteristics of the behavioural phenotypes transferred in each of these subgroups post-FMT.

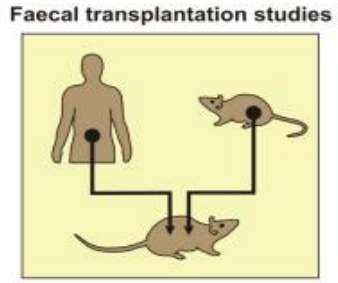

Figure 2 - Faecal microbiota transplantation (FMT) studies are used to establish if the gut microbiota could have a causal role in symptom generation (Gheorghe et al. 2021; Secombe et al. 2021).

In D5.3, we used both rodent to rodent, and human to rodent FMT to successfully transfer phenotypes the gut microbiota. In this deliverable, we evaluated the physiological and molecular alterations associated with these transferred phenotypes.
In particular, in this report we successfully demonstrated the transfer of IBS phenotypes using human to rodent FMT. The final step in this deliverable was an evaluation of the physiological and molecular alterations associated with these transferred phenotypes.

Detailed description of the methods used & the work performed

Methods:

Evaluation of stratified subgroups according to sex-specific vulnerability and resilience to maternal separation at a physiological and molecular level.

Animals and Housing

All procedures were conducted with approval from the Animal Experimentation Ethics Committee (AEEC) at University College Cork and the Health Products Regulatory Authority (HPRA), under project authorization number AE19130/P127, in accordance with the recommendations of the European Directive 2010/63/EU. Male and female Sprague Dawley rats (approximately 6 weeks of age) were purchased from Envigo, UK and were mated in the Biological Services Unit, Western Gateway Building, University College Cork. Two females were mated with one male per cage. The male was removed after one week, and the females were separated into individual cages 1–3 days prior to giving birth. The day of birth was designated as postnatal day 0 (PND 0). Dams and littermates were housed in large plastic breeding cages (45 × 28 × 20 cm), after weaning animals were housed in RC2F type cages (56 x 38 x 22 cm) in a humidity- and temperature-controlled room set to 55±10% and 21^{o}C ± 1^{o}C. The light/dark cycle was set to 12 hours (light phase 7am-7pm).

Experimental Design

After birth, rat pups were randomly assigned to maternal separation (MS) or control (non-separated, NS) groups. Rat pups underwent the MS paradigm between PND 2-12. At weaning (PND25), littermates were randomly housed in groups of 3-4 rats/cage/sex. In adulthood NS (NS, N=30 rats [4 rat/litter]) and MS (N=90 rats [4-5 rat/litter] rat offspring were assessed for visceral sensitivity and stress-coping/depression-like behaviour by undergoing colorectal distension (CRD; PND 59-61) and forced swim test (FST; PND 63-65), respectively. The animals were culled and samples were harvested for the evaluation of physiological and molecular characteristics. Experimental design and timeline are shown in Figure 3.

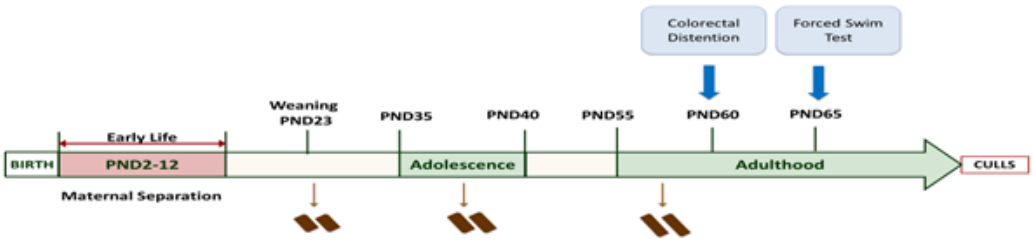

Figure 3 - Timeline showing the early-life stress exposure of maternal separation followed by the culling of animals and harvesting of samples in adulthood for the assessment of physiological and molecular characteristics.

Maternal separation paradigm

Early life stress was induced by MS as described previously (Collins et al. 2022). Briefly, at postnatal day (PND) 0 litters were randomly assigned to MS or NS groups. At PND 2, the litters assigned to MS were moved from the main colony room to an adjacent room maintained at the same temperature (21 ± 2◦C) and lighting conditions. The dam was first removed from the home cage and placed into a smaller holding cage, following which, the pups (entire litters) were gently transferred together into a small cage and kept there for 3 hours. Cages containing the pups were placed on heating pads set to 30–33◦C and were filled with 3 cm of bedding for thermoregulation. The dam was returned to the home cage and transferred back to the main colony room without her pups for this period to avoid communication between the dam and her pups. After the 3-hours, dams were again brought into the adjacent room and pups were returned to their original home cages. NS litters were also transported to the same room as the MS groups to avoid the confound of transportation stress. NS groups were left undisturbed in their home cages with their dams except for weekly cage cleaning. This procedure was repeated daily from PND 2 to PND 12 inclusive. The period of separation was carried out at the same time each day (9am–12pm). At weaning (PND 25), rat offspring were sexed, weaned and both male and female offspring were used for the remainder of the study.

Visceral sensitivity assessment through colorectal distension

The colorectal distension (CRD) protocol was carried out as previously described at PND 59-61 (with matching oestrous cycle for the females). Rat offsprings were fasted for 16 hours prior to the start of the procedure. Animals were lightly anaesthetised with isoflurane and a 6-cm long polyethylene balloon with a connecting catheter was inserted into the colon, 1 cm proximal to the anus. The catheter was secured to the tail of the animal with surgical tape to prevent displacement. Animals were allowed to recover from the anaesthesia for 10 minutes prior to the start of the procedure. The CRD paradigm used was an ascending phasic distension from 0 to 80 mmHg over an 8-minute period. Air inflation and pressure were monitored during the procedure using a customised barostat (Distender Series II, G and J Electronics, Toronto, ON, Canada). Pain behaviours were identified as abdominal retraction, withdrawal and stretching. A trained observer, blinded

to the experimental groups, scored each animal for the threshold pressure, when the first pain behaviour was observed, as well as the total number of pain behaviours displayed across all pressure ranges by each animal.

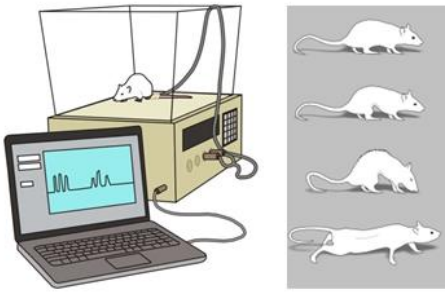

Figure 4 - Visceral sensitivity was assessed via a customised barostat and pain behaviours recorded.

Forced swim test

FST was used to assess stress-coping/depression-like behaviour. The test was carried out in two sessions as described previously (Slattery and Cryan 2012). Briefly, on the first day rats were carefully placed in a transparent glass cylinder (H: 45 cm; D: 20 cm) filled with 24±0.2°C water at a depth of 30 cm for a period of 15 minutes. 24 hours later, the rat was placed again in the same plexiglass cylinder for a 5-minute test swim. This test was recorded by a video camera placed above the cylinder. Following both swims, rats were thoroughly hand-dried with a towel and then moved to a recovery cage before being replaced in their home cage. The water was changed between each animal. Behavioural scoring was performed by trained personnel blinded to experimental condition. The 5-minute sessions were scored using a time-sampling technique, whereby the predominant behaviour in each 5 seconds of the 300-second trial was recorded. Climbing behaviour is defined as the upward movements of the animal to escape the cylinder. Swimming behaviour is defined as the vertical movement in the cylinder. Immobility behaviour consists in the absence of any movements that would not strictly ensure floating and/or keeping the head above water level.

Figure 5 - The forced swim test was used to assess stress coping/depression-like behaviours and as an acute stressor.

Two-step cluster analysis of behavioural data

The clusters were identified using IBM SPSS Statistics 27. To detect subgroups within the dataset, a two-step cluster analysis was performed for both sexes separately. The key behavioural readouts were used as input (continuous) variables from each animal. To ensure that all variables are independent, only one metric was used per behavioural task. For the FST, the input was the number of immobile behaviours. For the CRD, the two readouts (threshold, number of pain behaviours) were summarised using the z-score method to account for both allodynia and hyperalgesia according to the following formula (Becker et al. 2023):

$$Z = \frac{x_i - \bar{x}}{\bar{\sigma}}$$

In which x_i represents the test score of each individual animal, while $\bar{x}$ and $\bar{\sigma}$ represent the mean and standard deviation of the control population respectively. The directionality of scores was adjusted by multiplying with –1 so that an increased z-score indicated increased pain sensitivity. Lastly, a single CRD z-score was calculated using following equation (Becker et al. 2023):

$$Z_{CRD} = \frac{Z_{threshold} + Z_{Total\ pain}}{2}$$

For the identification of clusters, Log-likelihood was used as a distance measure for the pre-clustering step, while the Akaike information criterion (AIC) was used as a cluster criterion to estimate the most appropriate number of clusters. The main behavioural readouts from each rat were fed into the cluster analysis without predetermining the number of clusters, thereby avoiding bias in terms of identifying the number of cluster numbers. Four clusters were revealed for both sexes, and this formed the basis for the evaluation of molecular and physiological characteristics.

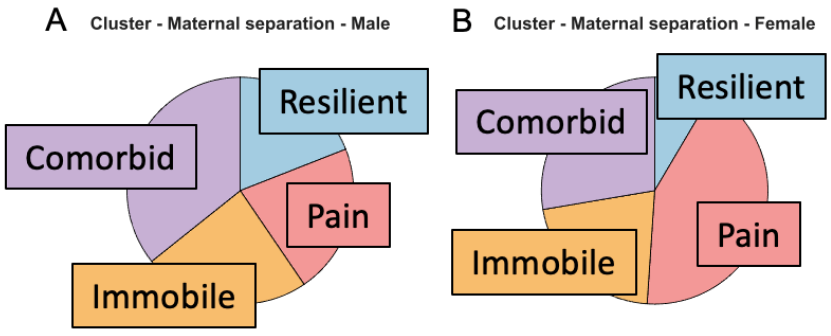

Figure 6 - Behavioural clusters

In D5.4, our analysis indicated the presence of behavioural clusters related to resilience, pain, comorbid and depression-like behaviour (Immobility) in male and female animals.

HPA Axis Evaluation

Plasma samples were collected for the evaluation of corticosterone as an index of HPA axis function, both at the end of the experiment and/or during the FST to assess the acute stress response. Plasma samples obtained on the day of the FST were from tail-tip incisions at five different time points: immediately before performing the test (T0), 15 minutes, 45 minutes, 90 minutes and 120 minutes post-test completion (T15, T45, T90, T120; Fig 6). To separate the blood plasma, the samples were centrifuged at 3500g for 10 minutes. Enzyme-linked immunosorbent assay (ELISA) was used to assess plasma corticosterone concentrations. The ELISA was performed using the Corticosterone ELISA kit ADI-901-097 (Enzo) according to the manufacturer's instructions.

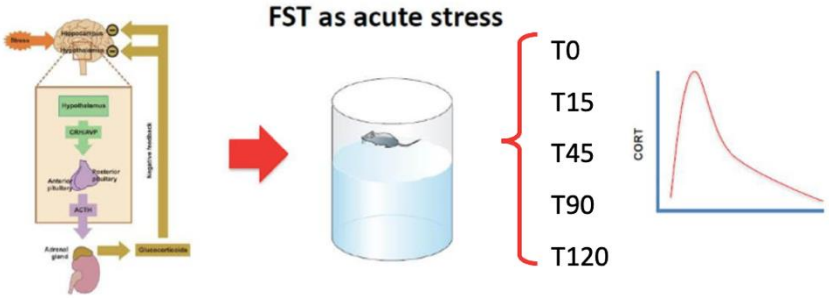

Figure 7 - Plasma samples were harvested at baseline and during the FST to measure HPA axis function using corticosterone as a readout.

Colon Tissue Analysis

Colon tissue was dissected and stored at -80°C for qRT-PCR analysis. RNA was extracted from the Colon tissue using the mirVana™ miRNA Isolation Kit according the manufacturers instructions. The Thermo Fischer High Capacity cDNA Reverse Transcription Kit was used to produce and transcribe the RNA in cDNA. qRT-PCR was carried out LightCycler® 480 II (Roche) with primer sequences designed using the Eurofins Genomics website.

Table 1 - Gene targets and rationale

Gene	Rationale
Cldn2	Barrier Function
Cldn3	Barrier Function
Ocldn	Barrier Function
Zo-1	Barrier Function
JAM1	Barrier Function
IL-17	Immune Marker
MUC2	Mucosal marker
TRPV1	Visceral Pain Marker
Tac1	Pain Marker
Tph1	Tryptophan metabolism
Calca	Visceral Pain Marker
Calcb	Visceral Pain Marker
Gapdh	Housekeeper

Spinal Cord Immunohistochemistry

Immunohistochemistry was used to stain activated microglia in spinal cord tissue. Fixed spinal tissues were cut in twenty-micrometer-thick sections at the level of the lumbar-sacral spinal cord using a freezing cryostat LEICA CM1520 (Leica Biosystems, Germany) and mounted onto SuperFrost plus microscope slides. They were then stored at 4°C until immunohistochemical analysis. Sections were rinsed 3 times x 5 minutes in 10mM phosphate-buffered saline (PBS) and incubated with 50mM NH_4Cl in PBS for 10 minutes. The sections were washed again two times and blocked by using 5% normal donkey serum (NDS) in PBS with 0.3% Triton X-100 for 2 hours. Samples were incubated overnight at 4°C in 2% NDS diluted in 0.1% Tween plus PBS (PBS-T) and 1:250 primary antibody (Goat anti-iba1: Abcam; ab5076). Control sections were incubated in 0.1% PBS-T and NDS only. The day after, the sections were washed 3 times x 10 minutes in 0.1% PBS-T, then incubated in the secondary antibody (donkey anti-goat; Invitrogen; A11057) plus 2% NDS and 0.1% Tween-PBS for 2 hours, at room temperature. The antibodies were diluted following a ratio of 1:500, directed toward the host of primary antibody, and conjugated to AlexaFluor 568. Samples were washed 3 times x 10 minutes in PBS-T again before counterstaining with 1:5000 DAPI diluted in PBS. Followed 3 more washing steps (2 times in PBS and 1 time in dH_2O) and the slices were finally mounted onto cover slips using Dako Fluorescence Mounting medium (Agilent Technologies, Unites States). Slices were stored in the dark at -20°C. All concentrations were chosen after thorough optimization.

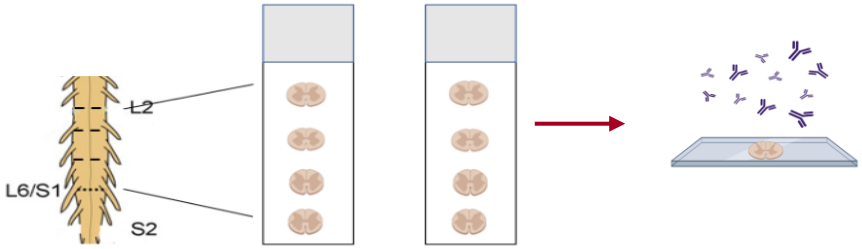

Figure 8 - Fixed spinal tissues were cut in twenty-micrometer-thick sections at the level of the lumbar-sacral spinal cord using a freezing cryostat LEICA CM1520 (Leica Biosystems, Germany) and mounted onto SuperFrost plus microscope slide for immunohistochemist

Image Collection and Analysis for Immunohistochemistry

For each animal, four segments of the spinal cord were cut transversally, immunolabeled, and then analyzed using the 20X objective of a fluorescent microscope (BX43, Olympus, Japan) for the presence of microglial cells. For every section, both left and right dorsal horns (lamina I-II) were z stacked with 1-µm steps in the z direction using the software accompanying the DP 74 digital camera (Olympus, Japan). Quantitative analyses were performed by a blinded person who did not know the history of the animals. To determine the microglial activation, microglia were counted using the Image J software (Fiji, W.Rasband). White and grey matters displayed different fluorescence levels, which allowed for the measurement of the area of each dorsal horn. Iba1+ cells were identified by Iba1 immunoreactivity surrounding the nuclear marker DAPI and counted only in the dorsal horn areas using the manual tag tool. The numbers of cells were reported as a function of their respective dorsal horn area and then normalized to number of cells per µm². Iba1-positive cells were not observed in the negative control in the absence of primary antibody.

Assessment of transfer of molecular and physiological characteristics of the subgroups identified using this stratification approach via rodent-to-rodent FMT

Faecal Sample Harvesting from Rodents

Faecal inocula were collected and prepared for FMT. Samples were prepared within an anaerobic cabinet, mixed with sterile reduced phosphate buffered saline (PBS 50 mM)/20% glycerol as cryoprotectant (final faecal inocula concentration: 100 mg/mL), and manually passed through a 70-µm stomacher filter to remove large particulates. Then, samples were aliquoted and stored at −80 °C until administered to animals.
Naïve adult rats were exposed to an antibiotic cocktail for 1 week to deplete the gut microbiota (see table below for concentration). Animals were randomized to donors in

which 2 recipient receive the FMT inocula from the same donor to account for inter-donor variability. After a three-day washout period, animals received the respective FMT treatment via oral gavage (300ul) daily for three successive days followed by two booster FMTs per subsequent weeks for the remainder of the study (10 total FMTs). Visceral sensitivity and stress-coping/depression-like behaviour was assessed using CRD and FST as described above.

Assessment of physiological/molecular phenotypes following human to rodent FMT

Faecal Sample Harvesting from humans with IBS and Healthy Controls

The sterile reduced phosphate buffered saline 1% (PBS 50mM)/20% glycerol used as cryoprotectant in this step was prepared in UCC an shipped to WP4 collaborators, Farre and Van Oudenhove. IBS (with and without psychiatric comorbidity) and HC faecal inocula were collected and prepared for FMT by WP4 collaborators within four hours of production by collaborators Farre and Van Oudenhove from WP4 . Samples were stored in an airtight container with an AnaeroGen sachet (Oxoid AGS AnaeroGen Compact, Fischer Scientific, Ireland) to maintain an anaerobic environment during transport. Upon arrival at the laboratory, samples were immediately placed within an anaerobic cabinet, mixed with sterile reduced phosphate buffered saline 1% (PBS mM)/20% glycerol as cryoprotectant (final faecal inocula concentration: 100 mg/mL), and manually passed through a 70-µm stomacher filter to remove large particulates. Then, samples were aliquoted and stored at −80 °C, and shipped to UCC for administration to animals. Animals were randomized to donors in which 3 recipients receive the FMT inocula from the same donor to account for inter-donor variability. Animals were randomized to a specific donor and received their respective FMT treatment via oral gavage (3100 µL) daily for three successive days followed by two booster FMTs per subsequent week for the remainder of the study (10 total FMTs). Visceral sensitivity and stress-coping/depression-like behaviour was assessed using CRD and FST as described above. In addition, we evaluated anxiety-like behaviour using the open field and elevated plus maze.

Ussing Chambers

Animals were euthanized by decapitation and distal colon samples (3cm proximal to the anus) were collected for analysis of gut barrier permeability (TEER) and secretory and secretory/absorptive function (basal I SC). Sensitivity of short circuit current (I SC) to serotonin (50nM – 26250nM) and histamine (1250nM – 26250nM) ex-vivo. Samples were carefully rinsed of any faecal matter and the circular and longitudinal muscle layers, along with the myenteric plexus, removed by blunt dissection. Colon segments were then placed in NaviCyte vertical Ussing chambers (Harvard Apparatus, Kent, United Kingdom), and each side of the tissue was bathed in 5 ml of circulating oxygenated (95% O2/5% CO2) Krebs buffer (1.2 mM NaH2PO4 , 117mM NaCl, 4.8mM KCl, 1.2mM MgCl2, 25mM NaHCO3, 11mM CaCl2, 10mM glucose) maintained at 37°C. were then voltage clamped at 0 mV (EVC-4000 or DVC-1000; World Precision Instruments, Sarasota, Florida, United

States of America) and short-circuit current (Isc) digitally recorded (LabTrax 4/16; World Precision Instruments) and analysed using DataTrax 2 software (World Precision Instruments). Tissues were allowed to stabilise for 30 minutes after which either serotonin or histamine are administered in ascending concentrations. All drug additions were to the basolateral half of the Ussing chamber.

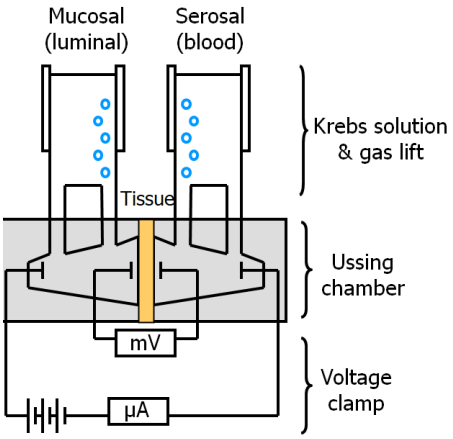

Figure 9 - Ussing Chambers were used to evaluate colonic permeability and secretory/absorptive function.

Results

Evaluation of stratified subgroups according to sex-specific vulnerability and resilience to maternal separation at a physiological and molecular level.

Maternal Separation and Cluster Analysis for HPA Axis function

As expected, maternal separation resulted in increased baseline HPA axis activity as indicated by increased corticosterone concentrations in both male and female animals. However, the behavioural clusters identified did not exhibit differential corticosterone concentrations.

Table 2 - Corticosterone concentrations following maternal separation and in the behavioural clusters identified.

Parameter	Male Maternal separation Vs Control	Female Maternal separation Vs Control
Baseline Corticosterone	↑	↑

Parameter	Male - Resilient	Male Pain	Male Depression	Male Comorbid
Baseline Corticosterone	↔	↔	↔	↔

Parameter	Female - Resilient	Female Pain	Female Depression	Female Comorbid
Baseline Corticosterone	↔	↔	↔	↔

Colon Tissue Analysis

Maternal Separation Effects

Tight junction protein markers were evaluated at the gene expression level. Maternal separation disrupted gut barrier integrity in both male and female MS rats at the transcriptional level as indicated by reduced Cldn3 and Ocldn expression. The expression of MUC2 was reduced only in the female animals exposed to MS.

CGRP, encoded by Calca, is a neuromodulator, which has multiple functions including an involvement in nociception in the gut. Calca expression was reduced following MS, primarily in the female animals.

The expression of TPH1, an enzyme responsible for the metabolism of tryptophan into serotonin, was reduced in MS rats and this was primarily due to the effects in male animals.

Table 3 - Colonic gene expression alterations in male and female maternal separation groups

Parameter	Male Maternal separation Vs Control	Female Maternal separation Vs Control
Cld2	↔	↔
Cld3	↓	↓
Ocldn	↓	↓
Zo-1	↔	↓
Jam-1	↔	↔
IL-17	↔	↔
MUC-2	↔	↓
TRPV1	↔	↔
Tac1	↔	↔
TPH1	↓	↔
Calc1a	↔	↓
Calc1b	↔	↔

Cluster Specific effects

Male rats in the pain cluster showed increased expression of TRPV1, a receptor involved in the transmission and modulation of pain (nociception).

Male rates in the depression-like cluster decreased tight junction gene expression including Cldn3 and Ocldn.

Table 4 - Colonic gene expression alterations in behavioural clusters

Parameter	Male - Resilient	Male Pain	Male Depression	Male Comorbid
Cld2	↔	↔	↔	↔
Cld3	↔	↔	↓	↔
Ocldn	↔	↔	↓	↔
Zo-1	↔	↔	↔	↔
Jam-1	↔	↔	↔	↔
IL-17	↔	↔	↔	↔
MUC-2	↔	↔	↔	↔
TRPV1	↔	↑	↔	↑
Tac1	↔	↔	↔	↔
TPH1	↔	↔	↔	↔
Calc1a	↔	↔	↔	↔
Calc1b	↔	↔	↔	↔

Female rats in the pain cluster showed increased expression of TAC1 in the resilient cluster. This gene relates to Substance P, a peptide primarily produced by sensory neurons.

Table 5 - Colonic gene expression alterations in female behavioural clusters

Parameter	Female - Resilient	Female Pain	Female Depression	Female Comorbid
Cld2	↔	↔	↔	↔
Cld3	↔	↔	↔	↔
Ocldn	↔	↔	↔	↔
Zo-1	↔	↔	↔	↔
Jam-1	↔	↔	↔	↔
IL-17	↔	↔	↔	↔
MUC-2	↔	↔	↔	↔
TRPV1	↔	↔	↔	↔
Tac1	↑↑	↔	↔	↔
TPH1	↔	↔	↔	↔
Calc1a	↔	↔	↔	↔
Calc1b	↔	↔	↔	↔

Assessment of transfer of the physiological and molecular characteristics of the behavioural phenotypes of the subgroups identified using this stratification approach via rodent-to-rodent FMT.
The samples harvested from each of the behavioural clusters identified in deliverable 5.4 were transferred via FMT and the recipient animals assessed for physiological and molecular characteristics.

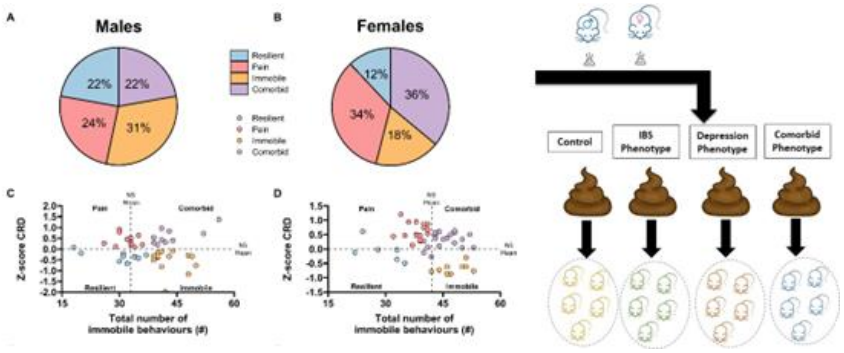

Figure 10 - Analysis of gut-brain axis signalling pathways in recipient animals following rodent to rodent FMT from donor animals in behavioural clusters

HPA Axis activation

Both male and female FMT recipient animals exhibited a corticosterone response to an acute stressor (FST). However, male animals in the depression-like cluster had a lower physiological stress response at the 45 minute timepoint. This male behavioural cluster also exhibited a reduced increase in corticosterone concentration from baseline to peak (T45 for males).

Table 6 - Male corticosterone profile following acute stress (FST)

Parameter	Male - Resilient	Male Pain	Male Immobile	Male Comorbid
Baseline	↔	↔	↔	↔
Minute 15	↔	↔	↔	↔
Minute 45	↔	↔	↓	↔
Minute 90	↔	↔	↔	↔
Minute 120	↔	↔	↔	↔

Table 7 - Female corticosterone profile following acute stress (FST)

Parameter	Female - Resilient	Female Pain	Female Immobile	Female Comorbid
Baseline	↔	↔	↔	↔
Minute 15	↔	↔	↔	↔
Minute 45	↔	↔	↔	↔
Minute 90	↔	↔	↔	↔
Minute 120	↔	↔	↔	↔

Colon Tissue Analysis

As previously shown, the male donor depression-like (immobile) cluster showed lower Ocldn expression in the donor rats. In the recipient male group, a trend for lower Ocldn expression could be seen in the immobile group in the recipient animals. However, this effect appears to be non-specific as expression was reduced across behavioural clusters, which was inconsistent with the donor molecular characteristics. There was no evidence of a transfer of any other profile of colonic gene expression from donor to recipient animals Assessment of transfer of physiological phenotypes following human to rodent FMT.
The faecal samples harvested from healthy controls and IBS (+/- psychiatric comorbidity) were transferred to recipient rodent animals, and colonic permeability and secretory/absorptive function were subsequently assessed.

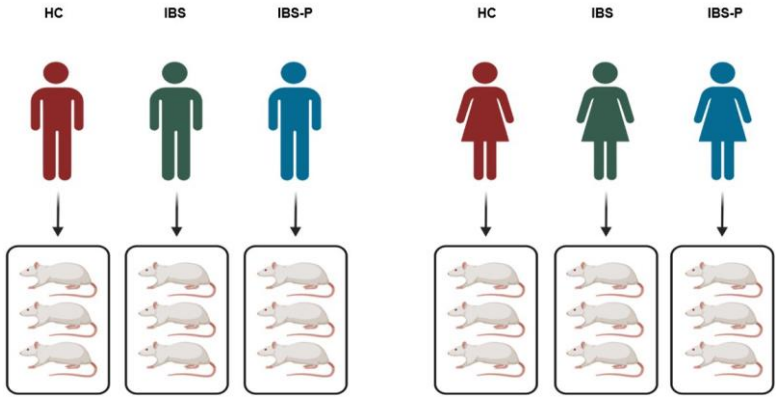

Figure 11 - Samples from male and female healthy controls and IBS (+/- psychiatric comorbidity) were assessed for colonic permeability and secretory/absorptive function.

Human to Rodent FMT Ussing Chamber Results

No baseline changes in gut permeability, or secretory/absorptive function were observed between animals receiving FMT material from healthy controls or IBS patients with and without psychiatric comorbidities. There was no impact of histamine or serotonin exposure on colonic secretory function, indicating that these neuromodulators were not implicated in the visceral hypersensitive phenotype reported in D5.4.

Table 8 - Colonic permeability and secretory/absorptive function following human to rodent FMT.

Parameter	Female - IBS	Female IBS + Psy	Male - IBS	Male – IBS + Psy
TEER (Permeability)	↔	↔	↔	↔
Basal I SC (Secretory/absorptive function)	↔	↔	↔	↔
Histamine induced I SC	↔	↔	↔	↔
Serotonin induced I SC	↔	↔	↔	↔

Conclusion

This deliverable identifies a potential role for the gut microbiota in driving important gut-brain axis signalling pathways associated with the behavioural and gastrointestinal phenotypes that manifest as a distinct consequences of early life stress. This is evident in a sex-specific way at the physiological level for HPA axis activation following an acute stressor, where only male animals in receipt of an FMT from the donor depression-like cluster exhibited differential corticosterone profiles.

At the molecular level in the colon, we have reported a sex- and cluster-specific expression of genes important for tight junctions in the gut barrier, with decreases only evident in male rats in the depression-like cluster. Meanwhile male animals in the pain cluster showed increased expression of TRPV1, a receptor involved in nociception. Expression of this receptor was reduced in all recipient clusters post-FMT, confirming microbial regulation. Female rats in the pain cluster showed increased expression of TAC1 in the resilient cluster. This gene relates to Substance P, a peptide primarily produced by sensory neurons.

These results confirm the presence of a mechanistically-oriented sex-specific molecular signature of IBS comorbidities following rodent to rodent FMT. These signatures are present in key signalling pathways of the gut-brain axis, including the HPA axis, barrier function and nociception/sensation. Taken together, these results establish a potential causal role for the gut microbiota in driving the expression of important molecular and physiological characteristics relevant to the behavioural features of IBS.

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