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

Workpackage WP5 - Microbiome and Metabolome

Deliverable D5.3
Confirmation of transfer of comorbid IBS phenotypes from animal models and from human samples

Start date of project: 2020-01-01
End Date: 2024-12-31
Duration: 60 months

Organisation name of lead contractor for this deliverable:
University College Cork, Partner 4: John F Cryan/Gerard Clarke

Project co-funded by the European Commission within the Horizon 2020 research and innovation programme under Grant Agreement No 848228		
Dissemination Level		
PU	Public	X
PP	Restricted to other programme participants (including the Commission Services)	
RE	Restricted to a group specified by the consortium (including the Commission Services)	
CO	Confidential, only for members of the consortium (including the Commission Services)	

Disclaimer

This document is an output of a project grant (Grant Agreement nº: 848228) funded under H2020 Research Programme of the European Commission. The content of this document represents the views of the author only and is his/her sole responsibility; it cannot be considered to reflect the views of the European Commission or any other body of the European Union. The European Commission do not accept any responsibility for use that may be made of the information it contains.

Consortium

Partcicipant Nº	Participant Legal Name	Country	Partner's Acronym
1	Fundacio Hospital Universitari Vall d'Hebron-Institut de recerca	ES	VHIR
2	Goeteborgs Universitet	SE	UGOT
3	Stichting Katholieke Universiteit	NL	SKU
4	University College Cork – National University of Ireland, Cork	IE	UCC
5	Johann Wolfgang Goethe – Universitatfrankfurt Am Main	DE	GUF
6	Katholieke Univeriteit Leuven	BE	KUL
7	Universitatsklinikum Heidelberg	DE	UKL-HD
8	Alma Mater Studiorum – Universita Di Bologna	IT	UNIBO
9	VIB VZW	BE	VIB
10	Universiteit Maastricht	NL	UM
11	Semmelweis Eguetem	HU	SU
12	Universitatea de Medicina Si Farmacie Iuliu Hatieganu Cluj-Napoca	ROU	UMF
13	Anaxomics Biotech, S.L.	ES	AX
14	Hycult Biotechnology BV	NL	HB
15	Emergentec Biodevelopment GMBH	AU	EMTEC
16	Nanostring Technologies Inc	USA	NSTG
17	Cliclab Technologies Inc	ES	Cliclab
18	Followhealth SL	ES	hITc
19	BYTE Factory Systems SL	ES	BF

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

Executive Summary

Introduction:

Early-life stress is a risk factor irritable bowel syndrome (IBS), a disorder of gut-brain interaction showing high levels of psychiatric comorbidities. The maternal separation (MS) rodent model recapitulates many prominent features of IBS. This includes perturbation of the microbiota-gut-brain axis and behavioural changes in adulthood that encompasses both abdominal pain and depression-like behaviours. Similar to the clinical population, not all animals exposed to MS present both gastrointestinal and behavioural phenotypes.

Aim

The aim of this deliverable is to study the transfer of comorbid behavioural phenotypes via the gut microbiome, using both samples from animal models of IBS (maternal separation) and samples from human studies supplied by WP4.

Methods:

Rat offspring was subjected to maternal separation. Adult offspring visceral pain sensitivity and depression-like behaviour was assessed. Behavioural data were processed in a two-step cluster analysis to identify natural groupings. Faecal microbiota transplantation (FMT) into naïve animals was performed, to establish a causal link between gut microbiota configurations and behavioural response profiles in each cluster, and from samples harvested from healthy humans and those with IBS provided by partners in WP4.

Results:

A cluster analysis revealed four clusters within the behavioural dataset representing distinct pathophysiological domains as a consequence of early life stress: resilient, pain, depression-like and comorbid. FMT from the different clusters into naïve animals partially transfers depression-like and pain behaviours in the recipient animals. Similarly, FMT from human donors into naïve animals recapitulated the abdominal pain phenotype in both male and female animals, with evidence for transfer of anxiety-like behaviours from IBS with psychiatric comorbidity in female animals only.

Conclusion:

Our study is the first to identify a role of the gut microbiota in gut-brain phenotypes showing susceptibility to the distinct consequences of early life stress. The role of the microbiota in a clinical IBS population was also confirmed with a transfer of visceral hypersensitivity in both males and females. Our results thus confirm the role of the microbiota in explaining the susceptibility and resilience of distinct pathophysiological consequences of early-life stress in adulthood. The transfer of comorbid behavioural phenotypes from human to rodents was via the gut microbiome was also confirmed, indicating the translational utility of this approach to study the common biological pathways underpinning pathophysiology of IBS with psychiatric comorbidity.

Short introduction: what is the aim of the deliverable?

The gastrointestinal microbiota is a key regulator of gut-brain axis signalling, a role that comes with important implications for neurogastroenterology. There is continuous bidirectional communication between the gut and the brain facilitated by neuronal, endocrine, metabolic, and immune pathways. The microbiota influences these signalling pathways via several mechanisms. Studies have shown compositional and functional alterations in the gut microbiota in stress-related gastrointestinal and psychiatric disorders. Gut microbiota reconfigurations are thus a notable feature of irritable bowel syndrome (IBS), a disorder of gut-brain interactions sharing high levels of psychiatric comorbidity including both anxiety and depression. It remains unclear how the gut microbiota alterations in IBS align with both core symptoms and these psychiatric comorbidities (Wilmes et al. 2021).
One of the gold standard approaches for establishing a casual role for disease-associated microbiota configurations is via faecal microbiota transplantation (FMT) (Gheorghe et al. 2021; Secombe et al. 2021). The aim of this deliverable is to study the transfer of comorbid behavioural phenotypes via the gut microbiome, using both samples from animal models of IBS (Maternal separation) and samples from human studies. 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. The first step described below was thus the stratification of animals according to sex-specific vulnerability and resilience to identify translationally relevant subgroups consistent with the clinical presentation of comorbid phenotype development.

Figure 1 - The early life stress model of maternal separation results in a phenotype in adulthood reminiscent of IBS, with both gastrointestinal disturbances and behavioural abnormalities
Figure reproduced from Moloney et al. 2015

In D5.3, we aimed to first stratify animals according to sex-specific vulnerability and resilience to subgroups for later FMT.
B. The next step described below was the assessment of transfer of the behavioural phenotypes of the subgroups identified using this stratification approach via rodent-to-rodent FMT.

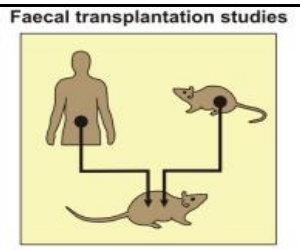

Figure 2 - Faecal microbiota transplantation (FMT) studies are used to establish if the gut microbiota could have a causal role in symptom generation
Figure reproduced from Gheorghe et al. 2021; Secombe et al. 2021.

In D5.3, we used both rodent to rodent, and human to rodent FMT to establish if comorbid behavioural phenotypes could be transferred via the gut microbiota.
C. The final step in this deliverable was a human to rodent FMT, using human samples collected by WP4 collaborators from individuals with IBS with and without psychiatric comorbidity compared to healthy controls.

Detailed description of the methods used & the work performed

Methods:

Stratification of animals according to sex-specific vulnerability and resilience to maternal separation

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 (PND 25), 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 behavioural data were incorporated into a two-step cluster analysis to identify natural groupings within the dataset. Experimental design and timeline are shown in Figure 3.

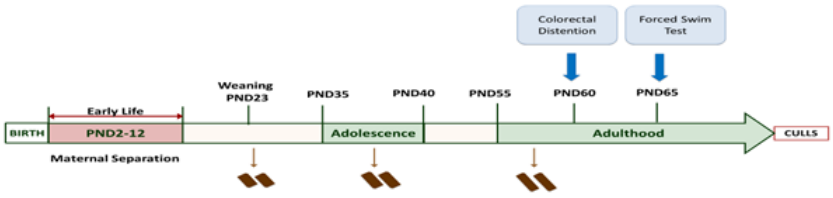

Figure 3 - Timeline showing the early-life stress exposure of maternal separation followed by behavioural assessment of animals in adulthood for depression-like behaviour and visceral hypersensitivity (visceral pain), both key features of IBS.

Maternal separation paradigm

Early life stress was induced by MS as described previously (Collins et al. 2022). Briefly, at 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 PND59-61 (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.

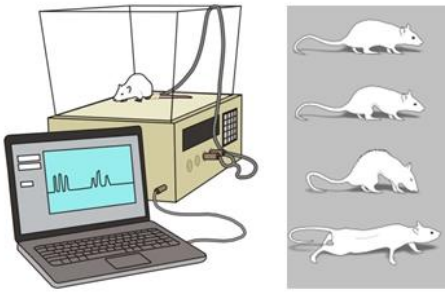

Figure 4 - Visceral sensitivity was assessed via a customised barostat and pain behaviours recorded.

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.

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.
Assessment of transfer of the behavioural phenotypes 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.

Table 1 - Composition of Antibiotic Cocktail

Solution	Concentration
Ampicillin	1 g/L
Vancomycin	500 mg/L
Ciprofloxacin HCl	200 mg/L
Imipenem	250 mg/L

Assessment of transfer of the behavioural phenotypes of the subgroups identified using this stratification approach via human to rodent FMT.

Faecal Sample Harvesting from humans with IBS and Healthy Controls

The sterile reduced phosphate buffered saline (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 Farre and Van Oudenhove from WP4. Samples were stored in an airtight container with AnaeroGen sachets (Oxoid AGS AnaeroGen Compact, Fischer Scientific, Ireland), which was also shipped from UCC to WP4 collaborators, 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 (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 and received their respective FMT treatment via oral gavage (300 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.

Open field test

The open field test was used to investigate locomotion and anxiety-like behaviour. The rat is habituated to the behavioural room for up to 1 hour before the test. The rat is then placed in the centre of an open brightly lit (up to 1000 lux) arena made of plastic (90 cm diameter) and are allowed to explore the environment for a 10 min period during which time his behaviour will be recorded by a video camera mounted above the apparatus. After the test, the rat is removed and returned to its home cage and the open field is cleaned with 70% alcohol and allowed to dry between tests to remove odour cues. The number of entries into and the amount of time spent in the central (aversive) area of the arena, the locomotor activity (total distance moved in the arena and velocity of movements), and the faecal pellets production are evaluated as readouts of anxiety-like behaviour.

Elevated plus maze

The EPM is used to assess anxiety-like behaviour. The maze has two opposed open (50 cm length x 10 cm width x 25 cm height) and two opposed closed arms (50 cm length x 10 cm width x 40 cm height) mounted at a 90° angle, all facing a central platform (10 cm x 10 cm), elevated 50 cm above the floor. The rat is habituated to the behavioural room for 1 hour before the test. Afterwards each rat is placed in the centre of the EPM facing the open arm. Time spent in the open areas reflect less anxiety whereas increased time in closed areas represent higher levels of anxiety and their behaviour is monitored, and video tracked for 5 minutes. After 5 minutes the animal is returned to its home cage.

Results

Stratification of animals according to sex-specific vulnerability and resilience to maternal separation

Cluster Analysis

A two-step cluster analysis of behavioural data identified four clusters for both sexes.

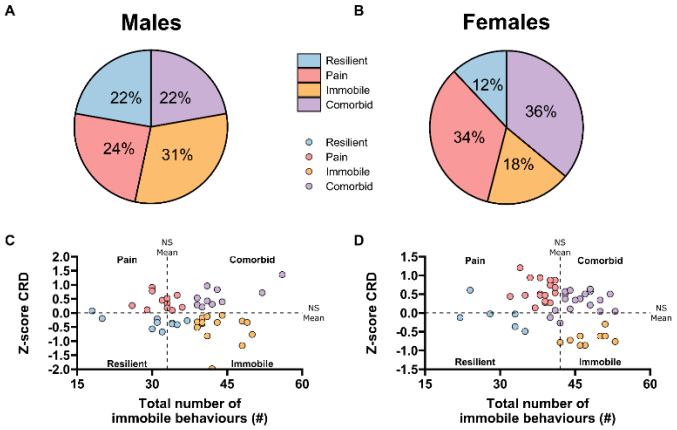

Figure 6 - Stratification of maternally separated animals into behavioral subgroups.
(A, B) Pie charts illustrating the distribution of clusters across the offspring in percentage in both sexes. (C, D) Scatterplots illustrating the behavioural characteristic of each animal in both sexes. Y-axis showing the outcome of colorectal distension and the x-axis showing the outcome of the forced swim test. Dotted lines represent the mean outcome of the non-separated control group. Males N = 10 (Resilient), 11 (Pain), 14 (Immobile), 10 (Comorbid). Females N = 6 (Resilient), 17 (Pain), 9 (Immobile), 18 (Comorbid).

Assessment of transfer of the behavioural phenotypes of the subgroups identified using this stratification approach via rodent-to-rodent FMT.
The faecal samples harvested from each of the clusters identified in (A) above were transferred to recipient rodent animals, whose behaviour was subsequently assessed.

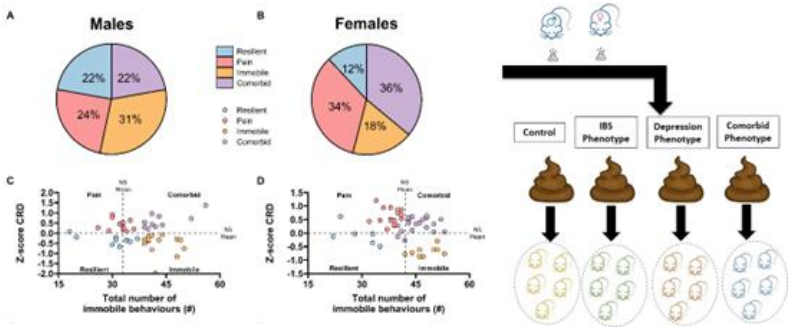

Figure 7 - Samples from male and female animals in each cluster (Control, IBS, comorbid, Immobile) were transferred to recipient animals.

Male animals

The male recipient animals in receipt of a FMT from the comorbid cluster exhibited a reduced visceral pain threshold compared to the other groups. The male recipient animals in receipt of a FMT from the immobile (depression-like) cluster exhibited an increased number of immobile behaviours compared to the control animals.

Table 2 - Summary of behavioural alterations in the male recipient animals compared to controls

Male Donor Groups/Behaviour	Pain	Depression-like	Comorbid
Depression-like behaviour in recipient animals compared to control	↔	↑	↔
Visceral hypersensitivity in recipient animals compared to control	↔	↔	↑

Female Animals

The female recipient animals in receipt of a FMT did not exhibit alterations in visceral sensitivity or depression-like behaviour post-FMT from any of the clusters.

Table 3: Table 3 - Summary of behavioural alterations in the female recipient animals compared to controls

Female Donor Group/Behaviour	Pain	Depressive-like Vs Control	Comorbid Vs Control
Depression-like behaviour in recipient animals	↔	↔	↔
Visceral hypersensitivity in recipient animals	↔	↔	↔

Assessment of transfer of the behavioural phenotypes of the subgroups identified using this stratification approach via human to rodent FMT.
The faecal samples harvested from healthy controls and IBS (+/- psychiatric comorbidity) were transferred to recipient rodent animals, whose behaviour was subsequently assessed.

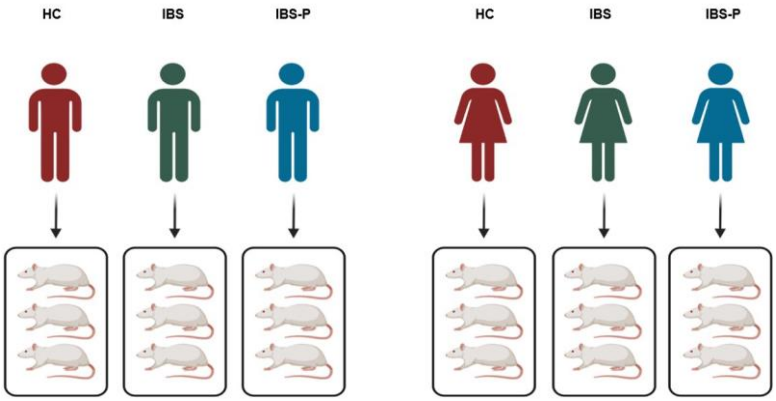

Figure 8 - Samples from male and female healthy controls and IBS (+/- psychiatric comorbidity) were transferred to recipient animals.

Human to Rodent FMT Results

Recipient animals in receipt of a FMT from humans with IBS exhibited a reduced visceral pain threshold compared to those in receipt of a FMT from healthy controls. This was observed in both male and female animals. Overall, there was no evidence of a transfer of anxiety-like or depression-like behaviours, although female animals in receipt of a FMT from humans with IBS with psychiatric comorbidity displayed a trend for increased anxiety-like behaviour.

Table 4 - Summary of behavioural alterations following FMT from humans to rodents in the recipient animals compared to controls

Donor Groups/Behaviour	Male IBS	Male IBS with psychiatric comorbidity	Female IBS	Female IBS with psychiatric comorbidity
Visceral hypersensitivity	↑	↑	↑	↑
Depression-like behaviour	↔	↔	↔	↔
Anxiety-like behaviour	↔	↔	↔	(↑)

Conclusions

This deliverable identifies a role of the gut microbiota in gut-brain phenotypes showing susceptibility to the distinct consequences of early life stress. The role of the microbiota in a clinical IBS population was also confirmed with a transfer of visceral hypersensitivity in both males and females. Our results thus confirm the role of the microbiota in explaining the susceptibility and resilience of distinct pathophysiological consequences of early-life stress in adulthood. The transfer of gastrointestinal and some behavioural phenotypes from human to rodents via the gut microbiome was also confirmed, highlighting the translational utility of this approach to study the common biological pathways underpinning the pathophysiology of IBS with psychiatric comorbidity. Taken together, these results establish a potential causative role for the gut microbiota from both humans and animal samples in driving the expression of the prominent gastrointestinal as well as some of other behavioural features of IBS.

References

Becker, L. J., et al. (2023), 'The basolateral amygdala-anterior cingulate pathway contributes to depression-like behaviors and comorbidity with chronic pain behaviors in male mice', *Nat Commun,* 14 (1), 2198.

Collins, J. M., et al. (2022), 'Supplementation with milk fat globule membrane from early life reduces maternal separation-induced visceral pain independent of enteric nervous system or intestinal permeability changes in the rat', *Neuropharmacology,* 210, 109026.

Gheorghe, C. E., et al. (2021), 'Investigating causality with fecal microbiota transplantation in rodents: applications, recommendations and pitfalls', *Gut Microbes,* 13 (1), 1941711.

Moloney, R. D., et al. (2015), 'Stress-induced visceral pain: toward animal models of irritable-bowel syndrome and associated comorbidities', *Front Psychiatry,* 6, 15.

O'Mahony, S. M., et al. (2011), 'Maternal separation as a model of brain-gut axis dysfunction', *Psychopharmacology (Berl),* 214 (1), 71-88.

Secombe, K. R., et al. (2021), 'Guidelines for reporting on animal fecal transplantation (GRAFT) studies: recommendations from a systematic review of murine transplantation protocols', *Gut Microbes,* 13 (1), 1979878.

Slattery, D. A. and Cryan, J. F. (2012), 'Using the rat forced swim test to assess antidepressant-like activity in rodents', *Nat Protoc,* 7 (6), 1009-14.

Wilmes, L., et al. (2021), 'Of bowels, brain and behavior: A role for the gut microbiota in psychiatric comorbidities in irritable bowel syndrome', *Neurogastroenterol Motil,* 33 (3), e14095.