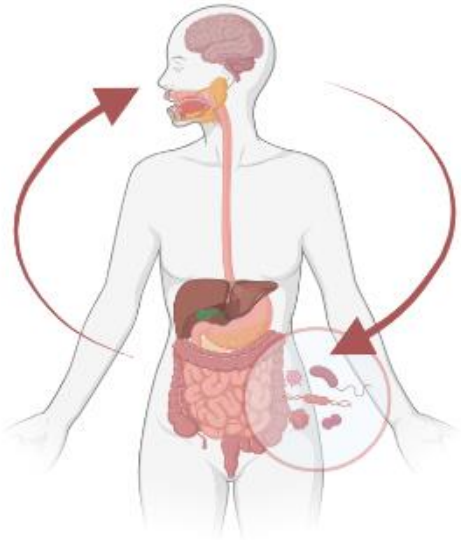
THE INFLUENCE OF GUT TRACTS, SEX AND MICROBIOTA ON THE EXPRESSION OF OXYTOCIN AND ITS RECEPTOR

Alejandra Marín Ruiz

FINAL DEGREE PROJECT

Biotechnology



Academic Supervisor: Dra. Begoña Muguerza

In cooperation with: APC Microbiome Ireland, University Collage Cork.

Professional Supervisor: Dra.Friederike Uhlig

TARRAGONA, JUNE 2023

I, Alejandra Marín Ruiz, with ID 21796981L, am aware of the URV Plagiarism prevention guide Prevention, detection, and treatment of plagiarism in teaching: guide for students (approved in July 2017) (http://www.urv.cat/ca/vidacampus/serveis/crai/que-us-oferim/formacio-competencies-nuclears/plagi/) and I affirm that this TFG does not constitute any of the behaviours considered as plagiarism for the URV.

Tarragona, 7 June 2023

ALEJANDRA|MARIN|RUIZ

Firmado digitalmente por ALEJANDRA|MARIN|RUIZ
Fecha: 2023.06.05 22:16:51 +02'00'

INDEX

1. CENTER DETAILS

The APC Microbiome Ireland was the research center where I did my internship for four months. It was founded in 2003 and now is a national institute with an international reputation as a leading global microbiome institute, with a broad range of academic and commercial partnership.

The APC aims consist in investigate the influence of intestinal microbiota in health and diseases and develop new therapies for lifelong debilitating gastrointestinal conditions. Their research is subdivided into four Research Themes. The main areas in which they work are “Microbes to Molecules”; “Diet and Microbes”; “Gut Brain Microbe Axis” and “Host-Microbe Dialogue”.

Of all the groups that can be found in the APC Microbiome Ireland, my work focused on the “Gut Brain Microbiome Axis”. They address the communication between the brain and the gut and if it may be influenced by the gastrointestinal microbiota. This is an area of focus in links between diet, microbiota, and cognition. This is the group in which I was doing my internship, on which I have based this project. It is led by Dr. Harrïet Schellekens.

- **Department:** Gut Brain Microbiome Axis
- **Adress:** Biosciences Institute, Biosciences Research Institute, Collage Rd, University Collage, Cork
- **Professional mentor during the internship**: Friederike Uhlig

2. ABSTRACT

The human gut microbiota is implicated in multiple diseases and conditions throughout the lifespan of the host due to the bidirectional relationship between the gut and the brain.

The largest amount of microbial biomass is found primarily in the gastrointestinal tract, where the release of gut neuropeptides, including oxytocin (OXT), occurs. These small fragments are released in the colon and ileum and travel from the gastrointestinal tract via neuronal or humoral pathways to different regions of the brain, where they bind with their receptors, thus establishing a reciprocal connection between the brain and the gastrointestinal tract.

OXT and its receptor play central roles in reproduction and social and emotional behaviors in animals and humans. Many of the actions in which OXT is involved are sex-dependent, such as mate preference, memory modulation, emotion regulation and trust.

The following study focuses on the effect of microbiota on oxytocin expression as a function of sex and in different sections of the gut, specifically in the ileum and colon. To perform the different comparisons, the colon and ileum of male and female mice were initially removed. Subsequently, to determine the effect of the microbiota, the ileum and colon of *germ-free* (GF) and conventional (CONV) male mice were compared.

To study this expression, the methodology was based on the extraction of RNA from each of the samples and its subsequent analysis by RT-PCR *(Reverse Transcription Polymerase Chain Reaction)*. This analysis allows us to determine the variation on OXT and OXTR expression as a function of sex and the influence of the microbiota of the ileum and colon.

KEYWORDS: Oxytocin, Oxytocin receptor, male mice, female mice, colon, gut tram, ileum, *germ-free* mice, and conventional mice.

ABBREVIATIONS

BCAA	Branched-chain amino acids
CCK	Cholecystokinin
CONV	Conventional mice
Cp	Crossing point
CRF	Corticotropin-releasing Factor
dCt	Delta Threshold point method
ENS	Enteric Nervous System
EST	Estrogen
EST	Estrogen receptor
GBA	Gut-Brain Axis
GF	Germ-free mice
GLP-1	Glucagon-like peptide-1
OXT	Oxytocin
OXTR	Oxytocin Receptors
PVN	Paraventricular nuclei
PYY	Peptide tyrosine-tyrosine
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SCFA	Short-Chain fatty acids
SON	Supraoptic nuclei

3. BACKGROUND

In recent years, there has been a growing interest in how the microbiota influences human behavior and different metabolic disorders. In fact, different studies have demonstrated that the human gut microbiota participates in multiple interactions that affect the health of the host throughout life (Clapp et al. 2017; Welch et al. 2014b; Milani et al. 2017).

The occurrence of some pathologies, such as metabolic disorders, are due to the bidirectional relationship between the gut and the brain. This relationship is influenced by the microbiota and different hormones and humoral signaling molecules (Socała et al. 2021).

3.1. Gut microbiota

The microbiota is a set of microorganisms that live in our body such as in the vagina, skin, saliva, and gastrointestinal tract. However, the main part of our microbiota is found in the gastrointestinal tract, mainly in the colon (Del Campo-Moreno et al. 2018).

It has been discovered that over trillions of microbes compose the microbiota (Wang et al. 2022). More than 90% of the gut community is represented by the bacterial phylum *Firmicutes* (60%), including the genera *Lactobacillus*, *Clostridium* and *Enterococcus*, and *Bacteroidetes* (25%), but also *Actinobacteria* and *Proteobacteria (Figure 1)* (Wang et al. 2022).

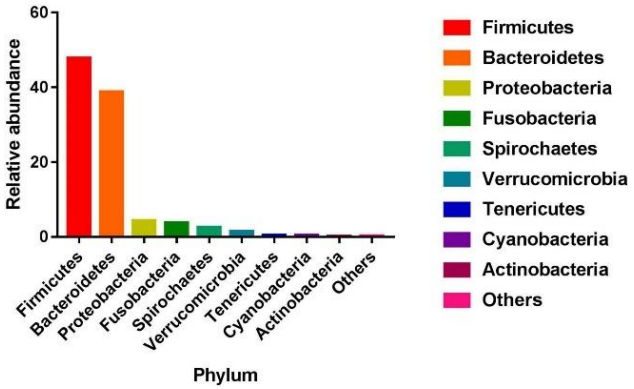


Figure 1. Relative abundance of the different bacterial phyla (Wang et al. 2022).

Some of the functions of the gut microbiota are the metabolization of certain foods, the production of vitamins, the degradation of toxins or protection against pathogens (Wernroth et al. 2022).

The gut microbiota also exerts a significant influence on an individual's physical and mental health. It also plays an important role in the prevention of some disorders such as anxiety, depression, or irritable bowel disease and modulates the expression of hormones due to the bidirectional communication of microbiota and brain along the gut-brain axis (GBA) (Adak and Khan 2019).

3.2. Gut-brain axis

GBA consists of bidirectional communication between the central and the enteric nervous system (ENS), linking emotional and cognitive centers of the brain with peripheral intestinal function (Carabotti et al. 2015). The GBA ensures proper maintenance of gastrointestinal homeostasis and has multiple effects on affect, motivation, and higher cognitive functions.

The microbiota and the brain communicate with each other via several pathways, including the immune system, the vagus nerve, and the ENS. This crosstalk involves microbial metabolites such as short-chain fatty acids (SCFA), branchened-chain amino acids (BCAA), peptidoglycans and gut peptides (Cryan et al. 2019).

Gut neuropeptides are small molecules, formed by linking three or more amino acids, and show action on the nervous system such as providing nervous mechanisms of learning and memory, regulation of food and drink intake or sexual behaviour (Crespo et al. 2009). A considerable proportion of these gut peptides play a vital role in the central regulation of the control of feeding behavior (Lawson et al. 2015). Among them we can distinguish the anorexigenic, which decrease food intake and orexigenic that increase food intake (Lorraine Jaimes 2005).

These gut neuropeptides related to the control of feeding behavior include glucagon-like-receptor 1 (GLP-1), peptide tyrosine-tyrosine (PYY), cholecystokinin (CCK), corticotropin-releasing factor (CRF), OXT and ghrelin (Skonieczna-żydecka et al. 2020).

3.3. Oxytocin

OXT is an anorexigenic neuropeptide that is synthesized the hypothalamus, specifically in the supraoptic (SON) and paraventricular nuclei (PVN) (Kingsbury and Bilbo 2019).

Its structure is composed of nine amino acids so, it is a nonapeptide, CYIQNCPLG *(Figure 2).*

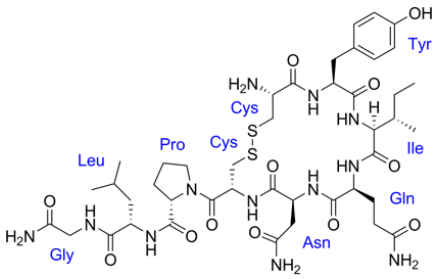


Figure 2. Amino acids sequence of oxytocin (Elsayed Azab 2022).

Once synthesized in the hypothalamus, it passes into the bloodstream via the neurohypophysis, thus having hormonal actions.

However, due to its diffusibility in the interstitial space, OXT can reach other areas of the brain, influencing our behavior and conduct (Kingsbury and Bilbo 2019). This neuropeptide has been implicated in a wide range of biological functions mediated through central OXTR (sociability, bonding, stress response) or peripheral OXTR (uterine contraction, intestinal contractility and secretion, visceral pain)(Cuesta-Marti et al. 2023).

OXT expression can also be produced in the myenteric plexuses myenteric (MP) and submucosal plexuses (SMP) of the gastric body *(Figure 3).* This expression was located mainly, in the proximal and distal colon but also in the jejunum and ileum (Yu et al. 2011).

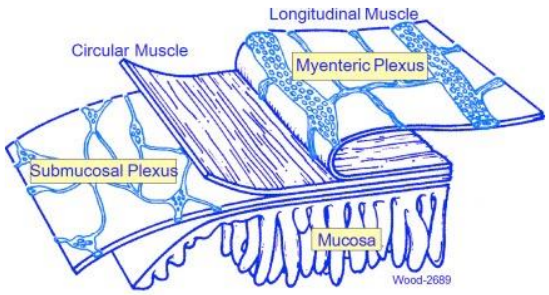


*Figure 3. The myenteric plexus is situated between the circular and longitudinal muscle coats; the submucous plexus is between the mucosa and circular muscle coat (Orlando and Orlando 2004)***.**

3.3.1. Oxytocin and the gut-brain axis

OXT plays an important role in the GBA as part of the signaling by the vagus nerve. The vagus nerve is the tenth cranial nerve, which links the viscera and the brain via afferent and efferent sensory neurons in the ENS. Gut peptides can bind to cognate receptors in vagus nerves terminals (Lach et al. 2018).This signaling is the fastest and most direct route of communication between the gut and the brain (Sue Carter et al. 2020).

The central and peripheral effects of OXT are mediated by the OXTR (Cuesta-Marti et al. 2023).OXTRs act as neurotransmitters or neuromodulators in higher functions and modulate peripheral physiological hormonal activity *(Figure 4)* (Aspé-Sánchez et al. 2016).

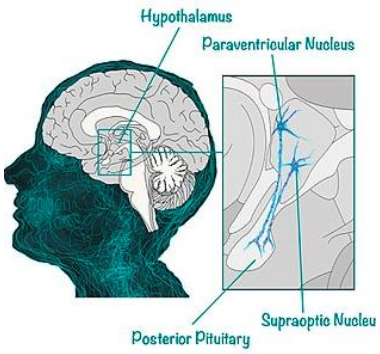


Figure 4. OXT are synthesized in the paraventricular nucleus (PVN) and the supraoptic nucleus (SON) of the hypothalamus. The peptidergic neurons, capable of synthesising peptides that act as neurotransmitters or neuromodulators of the electrical or hormonal activity of other neurons, project axons to the posterior pituitary, from where the peptides are released into the bloodstream(Aspé-Sánchez et al. 2016)

It has been studied that there is expression of OXTR in regions of the stomach, intestinal tract nerve fibers and MP plexus in the ileum and colon. Therefore, there is expression of the OXTR throughout the intestinal tract (Monstein et al. 2004). This expression is part of the GBA as it performed the interaction between the visceral organs of the gut tract with the brain (Vaidyanathan and Hammock 2017).

OXT and OXTR expression is influenced by life stage, species, and sex because of biological factors such as the influence of sex hormones (Dumais and Veenema 2016).

Regarding the difference in OXT and OXTR expression that exists between the sexes, the modulation of OXT-mediated behavioral effects, such as uterine contraction, mentioned above, has been linked to the involvement of estrogen (EST) (Dumais and Veenema 2016; Lu and Hu 2021). EST is a steroid hormone that is present in both females and males, in smaller proportions in males. It is responsible for sexual character differentiation and plays a role in other functions, from bone health to cognitive function (Adan and Burbach 1992). Estrogen receptors (ESTRs) induce OXT production by binding in a dimerized form to the hormone response element OXT promoter gene compound in the hypothalamus. Although there is evidence that EST and OXT may act antagonistically, it has been shown that increasing EST levels can control possible OXT-dependent effects (McCarthy 1996).

Once synthesized, OXT can pass into the bloodstream via the neurohypophysis, so it has hormonal actions, although, due to diffusibility in the interstitial space, oxytocin can reach other areas of the brain, influencing our behavior and conduct (Kingsbury and Bilbo 2019).

4. WORKING HYPOTHESISIS AND OBJECTIVES

Considering these antecedents, the aim of this project is to study the influence of different sections of the intestinal tract, sex, and the influence of the microbiota on the expression of OXT and OXTR.

The objectives of this work are:

- To study the different expression of OXT and OXTR in the colon and small gut in different portions. For this purpose, different portions of the gut are used, at 0 cm, 15 cm and 30 cm and the proximal, middle, and distal part of the colon.
- Compare the difference in the expression of OXT and OXTR in different sexes.
- Studying the difference in OXT and OXTR expression in GF mice compared to CONV mice. To find out the influence of the microbiota on this expression. In this study, a portion of colon and ileum was extracted.

5. METHODOLOGY

5.1. Sacrifice and sampling procedures

5.1.1. Conventional male and female mice dissection

For sample collection, 10–18-week-old male (n= 3) and female (n =3) C57BI/6 mice (Envigo, UK) were housed in open cages in groups of 3 animals. Theses boxes were enrichened with cardboard tubes and shredded paper. Mice were allowed to acclimatize to the animal unit under these conditions: 21 ± 1°C, 55 ± 10% humidity, 12 h light/dark cycle for 1-2 weeks with *ad libitum* access to food and water.

On the day of tissue collection, mice were removed from the animal unit approximately 1 hour before euthanasia. Mice were euthanized by decapitation between 8 and 10.30 am.

5.1.2. Germ-free mice dissection

For the gene expression analysis in ileum and colon of GF animals, male F1-generation offspring from GF (n=8) and CONV raised (n=8) *C57/BL6* mice breeding pairs previously obtained from Taconic (Germantown, New York, USA) were used. Mice were sacrificed at 16 weeks old (GF) and 13.3 week old (CONV) with similar body weights *(CONV:Mean = 25.96, Standard deviation= 1.85 g; GF, Mean = 27.76, Standard deviation = 2.33 g).* GF mice were housed in gnotobiotic isolators (two to four mice per cage). All mice were housed under controlled conditions: 20–21ºC and 55%–60% humidity; under a strict 12 h light/dark cycle with *ad libitum* access to autoclaved chow *(Special Diet Services, product code 801010)* and water.

The experiment was conducted in accordance with the European Directive 2010/63/EC, the requirements of the SI Nº 543 of 2012, and approved by the Animal Experimentation Ethics Committee of Cork and the Health Products Regulatory Authority (HPRA AE19130 P047).

For our study, all the samples taken from the gut of the mice, we only used the first parts of the small gut: at 0 cm (i.e immediately distal to the stomach) the middle part, at 15 cm and the most distal final part (approximately 30 min, ileum). In addition, the proximal, medium, and distal colon had been extracted (*Figure 5).*

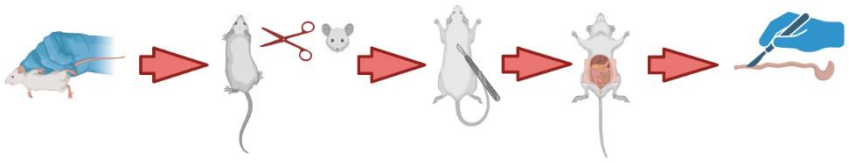


Figure 5. Summary of the procedure

With these six portions, we could clearly study he expression along the gut.

Once the different extractions were obtained, these samples were put on ice and kept the freezer at -80ºC for further analysis.

5.2. RNA extraction

RNA extraction was conducted from each one of previously extracted samples. The samples taken belong to colon and ileum tissue extracted from mice and their analysis was conducted individually. To find out which was the best extraction kit, different kits were tasted.

To conduct the different RNA extractions, we combine several procedures because, after different tests, we conclude that "High Pure RNA Tissue Kit- Sigma Aldrich" was the most efficient method for our samples. First, we started to homogenize with Triazole (TRI Reagent) in a suitable homogenizer and then we followed the protocol of "High Pure RNA Tissue Kit- Sigma Aldrich" to do the RNA precipitation *(Figure 6)*.

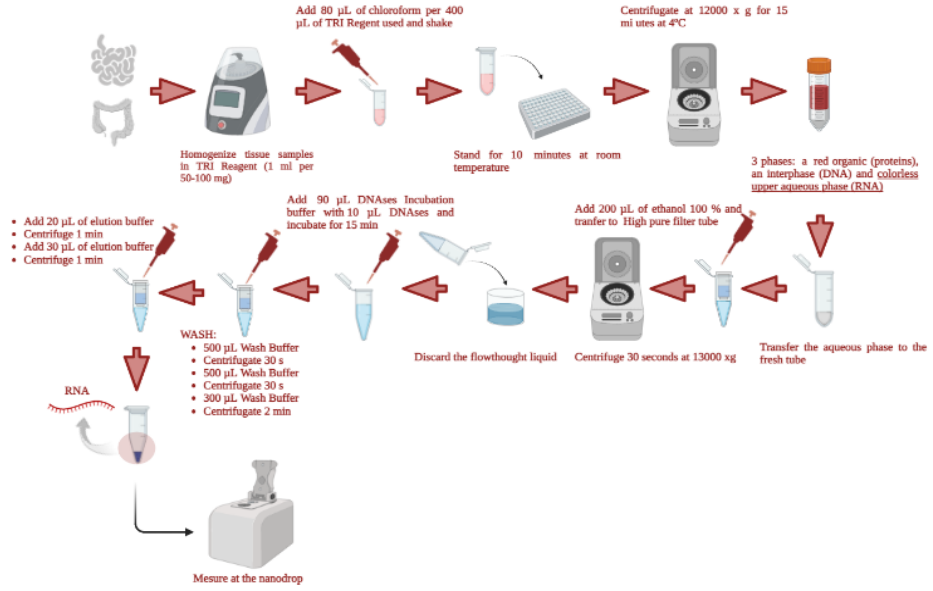


Figure 6. Summary of the procedure

5.3. DNA amplification via RT-PCR

Once the RNA had been extracted from the different samples, the genetic material was amplified using the RT-PCR technique to study the expression of oxytocin in the samples.

5.3.1. CDNA Synthesis

To do this, we conduct different dilutions based on the RNA concentrations obtained. These dilutions based on achieving the same concentration of RNA in each of the wells, using two of the wells as calibrations to check that the total concentration was correct.

For the realization, the *MiniAmp Plus* thermal cycler (*Figure 7*) was used, in which a 4D cDNA synthesis was conducted and the "*High-Capacity cDNA Reverse Transcription Kit*" (Henson et al. 2008) was used. The program applied to conduct the cDNA synthesis is detailed in the figure below *(Figure 7).*

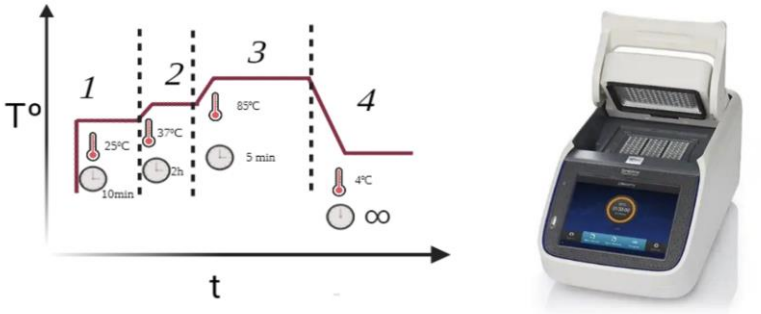


Figure 7. Cycle program to carry out cDNA synthesis in the MiniAmp plus thermal cycler (Henson et al. 2008)

As shown *Annex 3,* the RNA extractions good quality and concentration from most samples. The RNA concentrations were high, and the 260/280 and 260/230 ratios were correct and valid. A260/280 absorbance ratio were between 2.0-2.2 so, it was considered indicative of RNA optimal, and RNA is generally considered acceptable because A260/230 was higher than 1.5. Because of this, we continued with the procedure to perform the RT-PCR.

5.4. RT-PCR

From the synthesized cDNA, the expression of both genes was analyzed using the PCR technique. For this, cDNA amplification was performed on the *Roche LightCycler® 480 system.*

Table 1. Concentrations used in the different mixtures for the RT-PCR.

For 10 µL reaction	**Water**	**2x SensiFAST SYBR Lo-ROX Mix**	**10 µM Primer Reverse**	**10 µM Primer Forward**	**cDNA**
Concentration(x sample)	2,2 µL	5 µL	0,4 µL	0,4 µL	2 µL

The table lists the kit *SensiFAST SYBT-Lo-ROX kit* reagent (Hawkins and Guest 2017) and the concentrations used in the different mixtures for this PCR. The sequences of the OXT and OXTR primers are listed in the table 2.

Table 2. Primers sequences

actb_R	5'-AAGCAATGCCACCTTCC-3'
actb_F	5'- AAGTCCCTCACCTCTCCCAAAAG-3'
oxtr_R	5'-TAAACAAGGGGTGGAGGGA-3'
oxtr_F	5'-GACTCAAGTGTTCCTGGGGA-3'
oxt_R	5'-AFAGCCAGTAAGCCCAAGCAG -3'
oxt_F	5'-CGGATCTCAGACTGAGCAGC-3'

actb: actin beta; R: Reverse primer and F: Forward primers. Actin beta (actb) was used as housekeeping.

The process was conducted using the Sybr-Green technique, so that gene expression was detected using the SensiFAST SYBT-Lo-ROX, which detects the PCR product as it accumulates in each of the cycles *(Figure 8).*

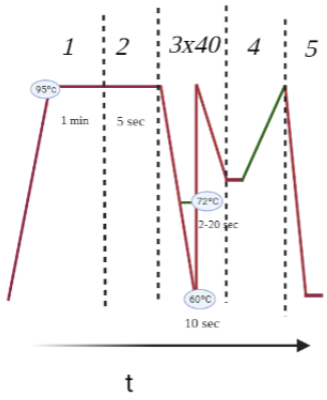


Figure 8. Cycle program to carry out RT-PCR. Step 3 is repeated 40 times. (Hawkins and Guest 2017)

For amplification, each sample was run in triplicates so that the data could be amplified more accurately. Due to the considerable number of samples, it was necessary to make two plates so that, to calibrate the results, a triplicate of one calibration was made. This calibration had an exact RNA concentration on both plates to serve as a reference. It is a mixture of all the samples where they are homogeneously represented. This concentration was 426.9 ng/µL of first RNA.

Operations are performed through the delta Threshold point (dCt), in which the amplification cycles are studied. dCT method makes one important assumption about the PCR, namely that the amplification efficiencies of the reference gene.

5.5. Statistical analysis

The analysis of all the results was performed through the *GraphPad Prisme 9* software.

Results were analyzed by two-way ANOVA (Male and female mice comparation) and by a T-student test (GF and CONV mice comparation). A significance level of alpha = 0,05 was used. Although, pair-wise comparison of means (*Bonferroni*) was used to determine the significance between treatment means $p < 0.05$) for the type I error.

6. RESULTS

6.1. Expression of oxytocin and oxytocin receptor in the colon and ileum in male and female mice.

No significant differences were found in the expression of OXT in CONV female and male mice *(Figure 9)*. There was an increased expression in the colon of male mice compared to female mice. This does not support the influence of EST on OXT expression (Liu et al. 2021; Lach et al. 2018). If it did, the expression would have been higher in females because they have higher levels of EST.

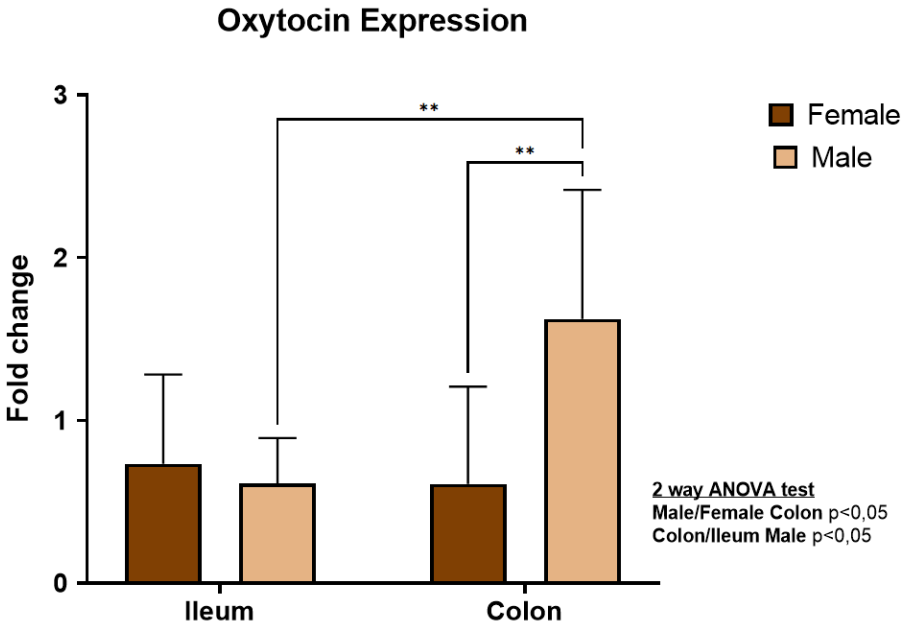


*Figure 9. Oxytocin expression between male and female mice in ileum and colon. The differences were determined by using 2-Way ANOVA test. N=9. **: p<0,05 (significant differences between the different samples)*

As for the expression of OXT along the gut tram, no differences were found in the different female sections analyzed *(Figure 9)*. However, there were significative differences in males, between the ileum and the colon, with a higher expression in the colon. These findings are supported by a previous study that determined that the main expression of OXT is in the colon (Yu et al. 2011).

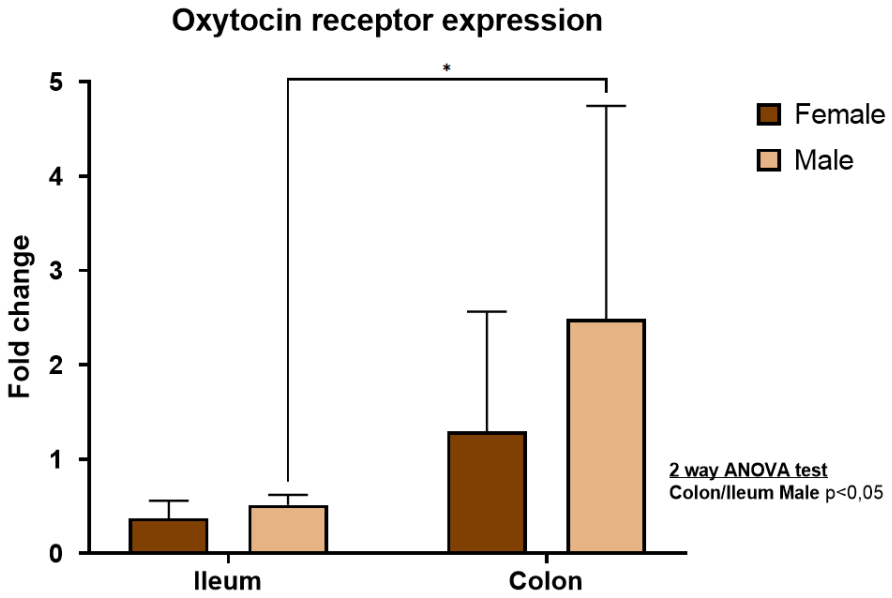


*Figure 10. Oxytocin receptor expression between male and female mice in ileum and colon. The differences were determined by 2-Way ANOVA test. N=9. *: p<0,05 (significant differences between the different samples)*

The results of OXTR expression in male and female mice showed that no significant differences were observed between the sexes but within males *(Figure 10)*. In this case, there was an increase in OXTR expression in the colon of male mice compared to the ileum.

The results obtained follow the evidence that their higher expression in the colon than in the ileum in both cases as stated in a previous article (Yu et al. 2011). Since the greatest amount of microbiota was found in the colon, it is possible that there is an influence of the type of microbiota inhabiting the colon on the expression of OXT and OXTR. To this end, the effect of the microbiota was further studied.

As differences in expression appeared in both cases along the length of the intestinal tract in male mice, the effect that the microbiota might have on the expression of OXT and OXTR in male mice only was studied. For this purpose, GF male mice were compared to male CONV mice.

6.2. Expression between germ-free mice and conventional mice

When comparing individually both parts of the gut tram between GF and CONV mice in the case of OXT expression, no significant differences were found in the ileum and colon between the different mice (*Figure 11).*

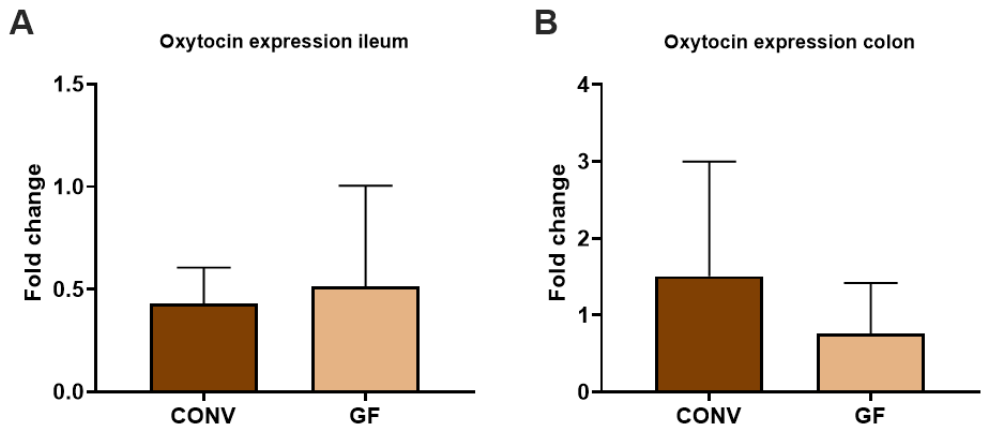


Figure 11. Oxytocin expression in germ-free mice against Conventional mice in ileum (A) and colon (B) GF= Germ-free and CONV= Conventional. N= 16 The differences were determined by t-student test.

These data would indicate that the microbiota is not essential for the expression of this hormone due to the expression in mice without microbiota are OXT expression in animals without microbiota and with microbiota is similar.

On the other hand, significant differences have been observed in the case of OXTR expression. No differences were seen in the colon but there were differences in the ileum between GF and CONV mice *(Figure 12).* This result does not follow the expected data as the highest amount of microbiota is found in the colon.

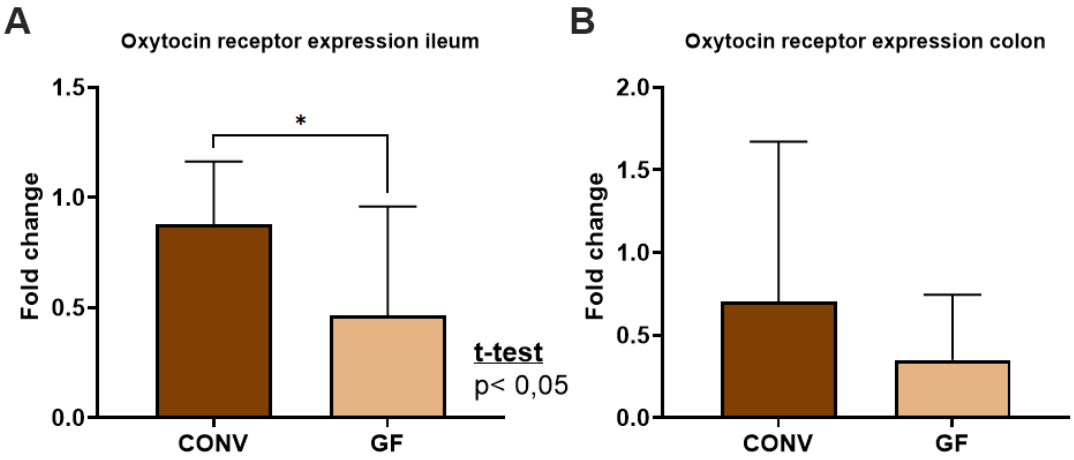


*Figure 12. Oxytocin expression in germ-free mice against Conventional mice in ileum (A) and colon (B) GF= Germ-free and CONV= Conventional. N= 16 The differences were determined by t-student test. *: p<0,05 (significant difference between the different samples).*

There was a continuous expression throughout the gut tract in conventional mice as previous study demonstrated (Yu et al. 2011). However, there may be some influence of ileum microbiota on OXTR expression.

Ultimately, all the data obtained showed that there is a continuous expression of OXT and OXTR throughout the gut tract in line with the perspective of previous studies (Yu et al. 2011; Welch et al. 2014).

7. CONCLUSION

Based on the results obtained, the following conclusions were reached:

- Differences in the expression of OXT and its receptor between ileum and colon are found only between males, with higher expression of OXT in the colon.
- Differences are found in colon expression of OXT but not of its receptor, between males and females, being higher in males.
- There are no differences in oxytocin expression in male animals between CONV mice and GF mice.
- Differences are found in the expression of the OXTR in the ileum but not in the colon between GF and CONV. There was higher expression in the ileum in CONV mice.

8. FUTURE PERSPECTIVES

During this work, there has been a focus on differences in oxytocin expression by the microbiota between sexes. To continue in this line of research, we could use other ways of eliminating the microbiota. Instead of using GF mice, we could treat mice with antibiotics. Different studies could be carried out, with antibiotics specific to certain bacteria or with a cocktail of antibiotics. This other way of eliminating the gut microbiota could give us more detailed information about which bacteria are responsible for this.

Furthermore, extraction of different portions of the gastrointestinal tract to study the expression of some genes through RT-PCR would allow us to study the expression, not only of oxytocin, but also of the other neuropeptides such as ghrelin. This neuropeptide has been studied and it can also modify their expression depending on the type and quantity of microbiota.

To conclude this work, I would like to reaffirm the influence of our microbiota on the occurrence of diseases and metabolic disorders. Therefore, to have a better control of these occurrences, further research is needed.

9. ACKNOWLEDGEMENTS

First, I wanted to thank my parents for giving me the opportunity to get out of my comfort zone throughout my degree and especially these last few months, for all their help, love, and support always. Without them I could not have made it this far. On the other hand, I wanted to thank my family and friends for always giving me the push I need to keep going.

Secondly, I would like to thank Dr. Begoña Muguerza, my academic tutor, for her patience and immediate collaboration during these months of work.

I would also like to thank the whole department where I did my internship and where APC Microbiome Ireland has given me the tools to carry out this work. Above all, thanks to Dr. Harret Schelleckens for giving me an excellent welcome in the "gut-brain axis" department and allowing me to work with them for four months. I would also like to thank Dr. Friderike Uhlig, my professional tutor during the whole stay, for her help and professionalism during this period.

On behalf of the professional Centre, I would like to thank each of the colleagues who have been with me during these months and for making me feel part of this great team from the first day.

10. AUTOEVALUATION

Thanks to the international internship I have learnt techniques, procedures, and ways of working different from those I had acquired previously during the four years of my degree. On the other hand, I have improved other techniques I already had. Regarding the project, I had the opportunity to be involved in more than one experiment and therefore in more than one project. This set of experimental techniques and acquired knowledge has allowed me to base my final degree project.

The first thing we learnt is the different skills of dissection. Being able to dissect different parts of organisms such as the brain or the gut tram of both mice and rats has been a privilege. This is a very complex procedure but very important when it comes to in vivo studies. For this reason, improving in this aspect has seemed to me a great advantage for the future.

Continuing with the knowledge acquired regarding RNA extraction to measure the expression of the different genes to be studied, in my case oxytocin, has allowed me to learn about different methods. I have also had the opportunity to use machinery that I had not previously used, such as the nanodrops, to quantify the amount of RNA obtained, or the thermal cycler to carry out the PCR, the process of which is very precise. In addition, I have been able to apply the knowledge acquired in bioinformatics by having the opportunity to carry out different statistical analyses.

In conclusion, I think that being able to do the internship in an international stay has helped me to improve the different biotechnological techniques that I had already acquired by studying different subjects such as Biochemistry and Molecular Biology Techniques, Gene Expression and Replication, to understand the different stages of the procedures to follow and at the same time, it has helped me to see another part of the research such as the case of animal experimentation.

Therefore, I can say that I really enjoyed having the opportunity to apply all the knowledge acquired during the four years of my studies in a field of work in which I was previously interested, and which has increased. I am grateful to all the research staff who have given me the opportunity to learn from them and have helped me to improve.

11. BIBLIOGRAFY

Adak, Atanu, and Mojibur R. Khan. 2019. “An Insight into Gut Microbiota and Its Functionalities.” *Cellular and Molecular Life Sciences*. https://doi.org/10.1007/s00018-018-2943-4.

Adan, Roger A.H., and J. Peter H. Burbach. 1992. “Chapter 10 Regulation of Vasopressin and Oxytocin Gene Expression by Estrogen and Thryroid Hormone.” *Progress in Brain Research* 92 (C). https://doi.org/10.1016/S0079-6123(08)61169-3.

Aspé-Sánchez, Mauricio, Macarena Moreno, Maria Ignacia Rivera, Alejandra Rossi, and John Ewer. 2016. “Oxytocin and Vasopressin Receptor Gene Polymorphisms: Role in Social and Psychiatric Traits.” *Frontiers in Neuroscience*. https://doi.org/10.3389/fnins.2015.00510.

Campo-Moreno, Rosa del, Teresa Alarcón-Cavero, Giuseppe D’Auria, Susana Delgado-Palacio, and Manuel Ferrer-Martínez. 2018. “Microbiota En La Salud Humana: Técnicas de Caracterización y Transferencia.” *Enfermedades Infecciosas y Microbiología Clínica* 36 (4). https://doi.org/10.1016/j.eimc.2017.02.007.

Carabotti, Marilia, Annunziata Scirocco, Maria Antonietta Maselli, and Carola Severi. 2015. “The Gut-Brain Axis: Interactions between Enteric Microbiota, Central and Enteric Nervous Systems.” *Annals of Gastroenterology* 28 (2).

Clapp, Megan, Nadia Aurora, Lindsey Herrera, Manisha Bhatia, Emily Wilen, and Sarah Wakefield. 2017. “Gut Microbiota’s Effect on Mental Health: The Gut-Brain Axis.” *Clinics and Practice* 7 (4). https://doi.org/10.4081/cp.2017.987.

Crespo, M A, l.C. González Matías, M G Lozano, S F Paz, M R Pérez, E V Gago, and F M Ferrer. 2009. “Gastrointestinal Hormones in Food Intake Control [Las Hormonas Gastrointestinales En El Control de La Ingesta de Alimentos].” *Endocrinologia y Nutricion* 56 (6).

Cryan, John F., Kenneth J. O’riordan, Caitlin S.M. Cowan, Kiran V. Sandhu, Thomaz F.S. Bastiaanssen, Marcus Boehme, Martin G. Codagnone, et al. 2019. “The Microbiota-Gut-Brain Axis.” *Physiological Reviews* 99 (4): 1877–2013. https://doi.org/10.1152/physrev.00018.2018.

Cuesta-Marti, Cristina, Friederike Uhlig, Begoña Muguerza, Niall Hyland, Gerard Clarke, and Harriët Schellekens. 2023. “Microbes, Oxytocin and Stress: Converging Players Regulating Eating Behavior.” *Journal of Neuroendocrinology*. https://doi.org/10.1111/jne.13243.

Dumais, Kelly M., and Alexa H. Veenema. 2016. “Vasopressin and Oxytocin Receptor Systems in the Brain: Sex Differences and Sex-Specific Regulation of Social Behavior.” *Frontiers in Neuroendocrinology*. https://doi.org/10.1016/j.yfrne.2015.04.003.

Elsayed Azab, Azab. 2022. “An Overview of Oxytocin: Chemical Structure, Receptors, Physiological Functions, Measurement Techniques of Oxytocin, and Metabolism.” *Journal of Clinical Research and Reports* 11 (4): 01–11. https://doi.org/10.31579/2690-1919/256.

Hawkins, Steve F.C., and Paul C. Guest. 2017. “Multiplex Analyses Using Real-Time Quantitative PCR.” In *Methods in Molecular Biology*. Vol. 1546. https://doi.org/10.1007/978-1-4939-6730-8_8.

Henson, Maile A., Adam C. Roberts, Kayvon Salimi, Swarooparani Vadlamudi, Robert M. Hamer, John H. Gilmore, L. Fredrik Jarskog, and Benjamin D. Philpot. 2008. “Developmental Regulation of the NMDA Receptor Subunits, NR3A and NR1, in Human Prefrontal Cortex.” *Cerebral Cortex* 18 (11). https://doi.org/10.1093/cercor/bhn017.

Kingsbury, Marcy A., and Staci D. Bilbo. 2019. “The Inflammatory Event of Birth: How Oxytocin Signaling May Guide the Development of the Brain and Gastrointestinal System.” *Frontiers in Neuroendocrinology*. https://doi.org/10.1016/j.yfrne.2019.100794.

Lach, Gilliard, Harriet Schellekens, Timothy G. Dinan, and John F. Cryan. 2018. “Anxiety, Depression, and the Microbiome: A Role for Gut Peptides.” *Neurotherapeutics*. https://doi.org/10.1007/s13311-017-0585-0.

Lawson, Elizabeth A., Dean A. Marengi, Rebecca L. Desanti, Tara M. Holmes, David A. Schoenfeld, and Christiane J. Tolley. 2015. “Oxytocin Reduces Caloric Intake in Men.” *Obesity* 23 (5). https://doi.org/10.1002/oby.21069.

Liu, Clarissa M., Mai O. Spaulding, Jessica J. Rea, Emily E. Noble, and Scott E. Kanoski. 2021. "Oxytocin and Food Intake Control: Neural, Behavioral, and Signaling Mechanisms." *International Journal of Molecular Sciences*. https://doi.org/10.3390/ijms221910859.

Lorraine Jaimes. 2005. "Péptidos Anorexigénicos y Su Participación En La Conducta Alimentaria." *Revista de Endocrinología y Nutrición*. Vol. 13.

Lu, Qiaoqiao, and Shaohua Hu. 2021. "Sex Differences of Oxytocin and Vasopressin in Social Behaviors." In *Handbook of Clinical Neurology*. Vol. 180. https://doi.org/10.1016/B978-0-12-820107-7.00005-7.

McCarthy, M. M. 1996. "Estrogen Modulation of Oxytocin and Its Relation to Behavior." *Advances in Experimental Medicine and Biology* 395.

Milani, Christian, Sabrina Duranti, Francesca Bottacini, Eoghan Casey, Francesca Turroni, Jennifer Mahony, Clara Belzer, et al. 2017. "The First Microbial Colonizers of the Human Gut: Composition, Activities, and Health Implications of the Infant Gut Microbiota." *Microbiology and Molecular Biology Reviews* 81 (4). https://doi.org/10.1128/mmbr.00036-17.

Monstein, Hans Jürg, Niclas Grahn, Mikael Truedsson, and Bodil Ohlsson. 2004. "Oxytocin and Oxytocin-Receptor MRNA Expression in the Human Gastrointestinal Tract: A Polymerase Chain Reaction Study." *Regulatory Peptides* 119 (1–2). https://doi.org/10.1016/j.regpep.2003.12.017.

Orlando, Lori A., and Roy C. Orlando. 2004. "Esophagus, Anatomy." *Encyclopedia of Gastroenterology*, 763–66. https://doi.org/10.1016/B0-12-386860-2/00253-7.

Skonieczna-żydecka, Karolina, Karolina Jakubczyk, Dominika Maciejewska-Markiewicz, Katarzyna Janda, Karolina Kaźmierczak-Siedlecka, Mariusz Kaczmarczyk, Igor Łoniewski, and Wojciech Marlicz. 2020. "Gut Biofactory—Neurocompetent Metabolites within the Gastrointestinal Tract. A Scoping Review." *Nutrients*. https://doi.org/10.3390/nu12113369.

Socała, Katarzyna, Urszula Doboszewska, Aleksandra Szopa, Anna Serefko, Marcin Włodarczyk, Anna Zielińska, Ewa Poleszak, Jakub Fichna, and Piotr Wlaź. 2021. "The Role of Microbiota-Gut-Brain Axis in Neuropsychiatric and Neurological

Disorders." *Pharmacological Research*. https://doi.org/10.1016/j.phrs.2021.105840.

Sue Carter, C., William M. Kenkel, Evan L. Maclean, Steven R. Wilson, Allison M. Perkeybile, Jason R. Yee, Craig F. Ferris, et al. 2020. "Is Oxytocin 'Nature's Medicine'?" *Pharmacological Reviews* 72 (4). https://doi.org/10.1124/pr.120.019398.

Vaidyanathan, Radhika, and Elizabeth A.D. Hammock. 2017. "Oxytocin Receptor Dynamics in the Brain across Development and Species." *Developmental Neurobiology*. https://doi.org/10.1002/dneu.22403.

Wang, Yuan, Ping Zhou, Xiang Zhou, Ming Fu, Tengfei Wang, Zuhong Liu, Xiaolei Liu, Zhiquan Wang, and Bang Liu. 2022. "Effect of Host Genetics and Gut Microbiome on Fat Deposition Traits in Pigs." *Frontiers in Microbiology* 13. https://doi.org/10.3389/fmicb.2022.925200.

Welch, Martha G., Kara G. Margolis, Zhishan Li, and Michael D. Gershon. 2014a. "Oxytocin Regulates Gastrointestinal Motility, Inflammation, Macromolecular Permeability, and Mucosal Maintenance in Mice." *American Journal of Physiology - Gastrointestinal and Liver Physiology* 307 (8). https://doi.org/10.1152/ajpgi.00176.2014.

———. 2014b. "Oxytocin Regulates Gastrointestinal Motility, Inflammation, Macromolecular Permeability, and Mucosal Maintenance in Mice." *American Journal of Physiology - Gastrointestinal and Liver Physiology* 307 (8): G848–62. https://doi.org/10.1152/ajpgi.00176.2014.

Wernroth, Mona Lisa, Sari Peura, Anna M. Hedman, Susanne Hetty, Silvia Vicenzi, Beatrice Kennedy, Katja Fall, et al. 2022. "Development of Gut Microbiota during the First 2 Years of Life." *Scientific Reports* 12 (1). https://doi.org/10.1038/s41598-022-13009-3.

Yu, Qiang, Ruihua Ji, Xiaofei Gao, Jiqiang Fu, Wei Guo, Xianmin Song, Xiaolin Zhao, et al. 2011. "Oxytocin Is Expressed by Both Intrinsic Sensory and Secretomotor Neurons in the Enteric Nervous System of Guinea Pig." *Cell and Tissue Research* 344 (2). https://doi.org/10.1007/s00441-011-1155-0.

12. ANNEXES

12.1. TABLE WITH RNA EXTRACTION RESULTS

Table 3. Table of results obtained during the RNA extraction process. Data provided by the nanodrop used. Columns 260/230 and 280/230 provide the validity of the results since both are around 2, which ensures a correct purification of the RNA from the male and female samples.

Sample ID	Date	Time	ng/ul	A260	A280	260/280	260/230	Constant	Cursor Pos.	Cursor abs.	340 raw
F1_0	04/05/2023	1:07 PM	304.30	7.608	3.548	2.14	2.29	40.00	230	3.329	0.041
F1_15	04/05/2023	1:09 PM	691.95	17.299	8.176	2.12	2.28	40.00	230	7.575	0.004
F1_30	04/05/2023	1:10 PM	175.21	4.380	2.090	2.10	2.06	40.00	230	2.129	0.215
F1_P	04/05/2023	1:11 PM	375.20	9.380	4.497	2.09	2.20	40.00	230	4.266	0.169
F1_M	04/05/2023	1:12 PM	108.37	2.709	1.309	2.07	1.95	40.00	230	1.387	0.164
F1_D	04/05/2023	1:13 PM	413.34	10.333	4.936	2.09	2.24	40.00	230	4.610	0.034
M1_0	04/05/2023	1:13 PM	579.92	14.498	6.820	2.13	2.29	40.00	230	6.343	0.166
M1_15	04/05/2023	1:17 PM	518.08	12.952	6.120	2.12	2.23	40.00	230	5.807	0.025
M1_30	04/05/2023	1:17 PM	412.46	10.312	4.971	2.07	2.11	40.00	230	4.879	0.424
M1_P	04/05/2023	1:19 PM	397.78	9.944	4.768	2.09	2.21	40.00	230	4.505	0.186
M1_M	04/05/2023	1:19 PM	241.62	6.040	2.845	2.12	2.27	40.00	230	2.666	0.035
M1_M	04/05/2023	1:20 PM	213.23	5.331	2.489	2.14	2.33	40.00	230	2.286	0.013
M1_D	04/05/2023	1:21 PM	220.20	5.505	2.570	2.14	2.30	40.00	230	2.399	0.017
M2_0	04/13/2023	4:16 PM	561.44	14.036	6.570	2.14	2.17	40.00	230	6.476	0.168
F2_0	04/13/2023	4:18 PM	930.35	23.259	10.866	2.14	2.27	40.00	230	10.243	-0.026
M2_15	04/13/2023	11:45 AM	877.12	21.928	10.244	2.14	2.27	40.00	230	9.671	0.071
M2_30	04/13/2023	11:46 AM	401.00	10.025	4.814	2.08	2.32	40.00	230	4.318	-0.050
M2_P	04/13/2023	11:48 AM	388.15	9.704	4.605	2.11	2.33	40.00	230	4.158	-0.023
M2_M	04/13/2023	11:49 AM	456.54	11.413	5.509	2.07	2.30	40.00	230	4.959	-0.059
M2_D	04/13/2023	11:49 AM	119.68	2.992	1.385	2.16	2.50	40.00	230	1.198	-0.030
F2_15	04/13/2023	11:50 AM	769.10	19.227	8.980	2.14	2.26	40.00	230	8.505	0.023
F2_15	04/13/2023	11:51 AM	234.52	5.863	2.760	2.12	2.35	40.00	230	2.492	-0.052
F2_30	04/13/2023	11:51 AM	241.79	6.045	2.847	2.12	2.37	40.00	230	2.553	-0.024
F2_P	04/13/2023	11:52 AM	202.31	5.058	2.400	2.11	2.44	40.00	230	2.077	-0.038
F2_M	04/13/2023	11:52 AM	574.96	14.374	6.754	2.13	2.25	40.00	230	6.388	0.011
F2_D	04/13/2023	11:53 AM	72.10	1.803	0.838	2.15	2.65	40.00	230	0.681	-0.040
M3_0	04/13/2023	3:57 PM	397.54	9.938	4.785	2.08	2.26	40.00	230	4.393	0.008
M3_15	04/13/2023	3:58 PM	268.70	6.717	3.191	2.10	2.25	40.00	230	2.986	-0.007
M3_30	04/13/2023	3:58 PM	340.35	8.509	4.058	2.10	2.28	40.00	230	3.731	0.000
M3_P	04/13/2023	3:59 PM	482.52	12.063	5.812	2.08	2.27	40.00	230	5.304	-0.010
M3_M	04/13/2023	4:00 PM	191.66	4.791	2.275	2.11	2.31	40.00	230	2.076	0.004
M3_D	04/13/2023	4:00 PM	128.02	3.200	1.531	2.09	2.27	40.00	230	1.413	-0.005
F3_0	04/13/2023	4:01 PM	292.23	7.306	3.473	2.10	2.26	40.00	230	3.238	-0.004
F3_15	04/13/2023	4:02 PM	359.93	8.998	4.313	2.09	2.23	40.00	230	4.033	-0.021
F3_30	04/13/2023	4:03 PM	477.84	11.946	5.795	2.06	2.25	40.00	230	5.303	0.004
F3_P	04/13/2023	4:04 PM	167.64	4.191	2.033	2.06	2.07	40.00	230	2.020	0.010
F3_M	04/13/2023	4:05 PM	117.73	2.943	1.397	2.11	2.36	40.00	230	1.246	-0.026
F3_D	04/13/2023	4:05 PM	130.27	3.257	1.571	2.07	2.33	40.00	230	1.399	-0.022

F(X)= Female;M(X)= Male ; 0= 0 cm; 15= 15 cm ; 30= 30 cm; P=Proximal colon; M=Medium colon and D= Distal colon

Table 4. Table of results obtained during the RNA extraction process. Data provided by the nanodrop used. Columns 260/230 and 280/230 provide the validity of the results since both are around 2, which ensures a correct purification of the RNA from the GF and CONV mice samples.

Sample ID	Date	Time	ng/ul	A260	A280	260/280	260/230	Constant	Cursor Pos.	Cursor abs.	340 raw
ILE GF_7	4/26/2023	12:01 PM	392.00	9.800	4.741	2.07	2.25	40.00	230	4.351	0.014
ILE GF_8	4/26/2023	12:01 PM	467.06	11.677	5.662	2.06	2.22	40.00	230	5.260	0.024
ILE GF_9	4/26/2023	12:02 PM	658.87	16.472	7.784	2.12	2.19	40.00	230	7.527	0.394
ILE GF_10	4/26/2023	12:03 PM	692.02	17.300	8.062	2.15	2.27	40.00	230	7.620	0.099
ILE GF_11	4/26/2023	12:03 PM	451.99	11.300	5.327	2.12	2.20	40.00	230	5.147	0.278
ILE GF_12	4/26/2023	12:04 PM	182.82	4.571	2.185	2.09	2.23	40.00	230	2.051	0.024
CONV_13	4/26/2023	12:05 PM	309.41	7.735	3.706	2.09	2.21	40.00	230	3.506	0.028
ILE CONV_14	4/26/2023	12:06 PM	876.91	21.923	10.238	2.14	2.27	40.00	230	9.647	0.073
ILE CONV_15	4/26/2023	12:06 PM	360.32	9.008	4.314	2.09	2.23	40.00	230	4.032	0.026
ILE CONV_16	4/26/2023	12:07 PM	395.14	9.878	4.758	2.08	2.26	40.00	230	4.369	0.011
ILE CONV_17	4/26/2023	12:08 PM	396.06	9.902	4.806	2.06	2.08	40.00	230	4.750	0.290
ILE CONV_18	4/26/2023	12:08 PM	636.82	15.921	7.484	2.13	2.19	40.00	230	7.284	0.154
ILE CONV_19	4/26/2023	12:09 PM	379.50	9.487	4.553	2.08	2.26	40.00	230	4.194	0.004
ILE CONV_20	4/26/2023	12:09 PM	99.12	2.478	1.223	2.03	2.08	40.00	230	1.192	0.063
COL_GF_5	4/27/2023	4:42 PM	397.10	9.927	4.707	2.11	2.42	40.00	230	4.109	0.029
COL_GF_6	4/27/2023	4:42 PM	547.98	13.700	6.459	2.12	2.26	40.00	230	6.061	0.161
COL_GF_7	4/27/2023	4:43 PM	228.63	5.716	2.757	2.07	2.12	40.00	230	2.697	0.295
COL_GF_8	4/27/2023	4:44 PM	235.25	5.881	2.775	2.12	2.34	40.00	230	2.516	0.007
COL_GF_9	4/27/2023	4:44 PM	198.94	4.974	2.355	2.11	2.51	40.00	230	1.980	0.023
COL_GF_10	4/27/2023	4:45 PM	253.92	6.348	2.995	2.12	2.44	40.00	230	2.601	0.024
COL_GF_11	4/27/2023	4:45 PM	381.40	9.535	4.510	2.11	2.41	40.00	230	3.963	0.019
COL_GF_12	4/27/2023	4:46 PM	108.29	2.707	1.288	2.10	2.32	40.00	230	1.169	0.008
COL_CON_13	4/27/2023	4:47 PM	109.38	2.734	1.320	2.07	2.44	40.00	230	1.121	0.071
COL_CON_18	4/27/2023	4:47 PM	109.50	2.737	1.308	2.09	2.39	40.00	230	1.146	0.100
COL_CON_17	4/27/2023	4:47 PM	219.26	5.482	2.571	2.13	2.12	40.00	230	2.583	0.024
COL_CON_16	4/27/2023	4:48 PM	227.48	5.687	3.215	1.77	1.12	40.00	230	5.072	3.230
COL_CON_15	4/27/2023	4:49 PM	312.23	7.806	3.710	2.10	2.36	40.00	230	3.308	0.044
COL_CON_14	4/27/2023	4:49 PM	297.52	7.438	3.511	2.12	2.44	40.00	230	3.043	0.001
COL_CON_13	4/27/2023	4:50 PM	469.91	11.748	5.545	2.12	1.94	40.00	230	6.046	0.156
COL_CON_19	4/27/2023	4:51 PM	470.50	11.763	5.563	2.11	2.28	40.00	230	5.162	0.025
COL_CON_20	4/27/2023	4:51 PM	134.09	3.352	1.613	2.08	2.48	40.00	230	1.352	-0.000

F(X)= Female;M(X)= Male ; 0= 0 cm; 15= 15 cm ; 30= 30 cm; P=Proximal colon; M=Medium colon and D= Distal colon