SOCIETÀ ITALIANA DI FARMACOLOGIA

MODELLO PER INVIO RELAZIONE DI METÀ E FINE PERIODO

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TIPOLOGIA DI BORSA RICEVUTA: BORSE DI RICERCA SIF PER BREVI PERIODI ALL'AESTERO

TIPOLOGIA DI RELAZIONE (es.: metà periodo o finale): Finale

TITOLO DELLA RELAZIONE: "Study of microbiota involvement in the development of post-inflammatory visceral pain"

RELAZIONE:

Background and aims

Visceral pain management is a major clinical problem, since the lack of effective and safe drugs (*Camilleri and Boeckxstaens, 2017*). The development of a chronic visceral hypersensitivity frequently occurs in patients with a history of intestinal damage. Indeed 20-50% of patients affected by Inflammatory Bowel Diseases (IBDs) manifest a chronic abdominal pain also in the remission phase of colitis. On the other hand, an acute gut illness increases the risk of developing the Irritable Bowel Syndrome (IBS) by 7-fold (*Stern and Brenner, 2018*). Despite the large number of sufferers, the underlying pathophysiology of visceral hypersensitivity in these common disorders remains unclear. Several mechanisms have been proposed to contribute to the initiation, exacerbation and persistence of visceral pain. Among them there is the dysbiosis of gut microbiota (*De Palma et al., 2014; Hyland et al., 2014; O'Mahony et al., 2017; Foster et al., 2017*). The most well-documented line of evidence linking disruption of gastrointestinal microbial homeostasis to the development of chronic visceral hypersensitivity comes from the literature on post-infectious IBS (*Thabane et al., 2007; Klem et al., 2017*). In line with this evidence, it has been demonstrated that antibiotics administration early in life is able to induce long-lasting effects on visceral pain responses in the animals (*O'Mahony et al., 2014*). Moreover, disturbance of the gut microbiota in adult mice and rats affects the local immune response and enhances pain signalling (*Verdu et al., 2006; Hoban et al., 2016; Luczynski et al., 2017*). Although alterations in intestinal microbial composition have long been associated with chronic inflammation, a definitive cause–effect relationship between dysbiosis and post-inflammatory visceral pain has not yet been clarified. The aims of the present project are:

1) To evaluate the effect of transplanting the microbiota from animals with post-inflammatory visceral pain to naïve animals;
2) To study of the mechanisms by which the microbiota influences visceral pain perception.

Materials and Methods

Experimental Set. Sprague Dawley rats, weighing about 300 g, were used. To obtain a model of persistent post-inflammatory visceral pain 2,4-dinitrobenzene-sulfonic acid (DNBS, 30 mg in 0.25 ml EtOH 50%) was intrarectally injected in the animals (*Antonioli et al., 2007*; *Adam et al. 2013*). The controls were administered with saline solution. Between day 14 and 21 after the damage induction the faeces of DNBS- and vehicle-treated animals were collected and administered in naïve rats. Visceral sensitivity was assessed in these animals by measuring the viscero-motor response and the abdominal withdrawal reflex to colo-rectal distension (*Christianson and Gebhart, 2007*; *Chen et al., 2014*). Before performing the FMT, the gut microbiota of rats was depleted by treating them with a combination of antibiotics for 7 days, control group was treated with vehicle. On day 7 the antibiotics-treated animals were divided into 3 groups and respectively administered per os for five consecutive days with control-derived faecal material, DNBS-derived faecal material or vehicle (*Luczynski et al. 2017*; *Yan et al., 2018*). One week after the administrations were repeated. Behavioural tests were performed at the end of the antibiotic treatment, 24h and 7 days after each Feacal Microbiota Transplantation (FMT) set and once week after the last treatment. On Day 7 (the end of the antibiotic treatment), Day 32 (when the effect of FMT on pain was well-estabilished) and Day 46 (when the effect of FMT disappeared) the animals were sacrificed and faecal pellet, colon and plasma where collected from each experimental group. *Histological Analysis.* The evaluation of colon damage was performed both macroscopically and histologically, in accordance with the criteria previously reported by Antonioli et al. (2007). The macroscopic criteria were: presence of adhesions between colon and other intra-abdominal organs (0-2); consistency of colonic faecal material (indirect marker of diarrhoea; 0-2); thickening of colonic wall (mm); presence and extension of hyperaemia and macroscopic mucosal damage (0-5). Microscopic evaluations were carried out on haematoxylin/eosin-stained sections of samples obtained from the distal colon. Full-thickness colonic samples were fixed in 4% formalin for 24 hours, dehydrated in alcohol, embedded in paraffin and cross-sectioned (5 µm). Histological evaluations were performed paying attention to: structure of the mucosa, presence of crypt abscess and goblet cell depletion, cellular infiltration and the thickening of the muscle tissue. Light microphotographs were captured using an optic a microscope Nikon Olympus BX40 equipped with the NIS F3.00 Imaging Software®. *Assessment of gut permeability.* The Lipopolysaccharides Binding Protein (LBP) was measured in the plasma by Elisa Immunoassay (Enzo®).
Gene expression analysis. Gene expression analysis by quantitative RT-PCR (qRT-PCR) was performed on colon samples. Total RNA was extracted using the mirVana™ miRNA Isolation kit (Ambion, Life Technologies) according to the manufacturer's recommendations. For each group, 4–6 animals were used. RNA concentration and quality were determined using a Nanodrop 1000 (Thermo Scientific). Quantitative PCR was carried out in a LightCycler480 System using PowerUp™ SYBR® Green Master Mix (Applied Biosystems) and specific probes designed by Applied Biosystems to rat occludin, ZO-1, TNF-α, IL6, IL10 and Tgf-β, while using β-Actin as an endogenous control. Experimental samples were run in triplicate with 4 µL cDNA per reaction. To check for amplicon contamination, each run contained no template controls in triplicate for each probe used. Cycle threshold (Ct) values were recorded. Data was normalised using β-Actin and transformed using the $2^{-\Delta\Delta Ct}$ method. No significant differences were observed in the mRNA expression levels of β -Actin between groups. *High Performance Liquid Chromatography (HPLC).* HPLC with electrochemical detection was used to measure the levels of 5-HT and 5-HIAA in the colon of animals. Rat colons were placed into chilled mobile phase spiked with N-methyl-serotonin, an internal standard (N-methyl-5-HT; 2 ng/20 µl; Sigma-Aldrich, Ireland). Samples were weighed, individually sonicated in 500 µl buffer and centrifuged (14 000 rpm, 4°C, 20 min). Supernatant was collected and diluted 1/2 in HPLC mobile phase. The mobile phase contained 0.1 M citric acid, 0.1 M sodium dihydrogen phosphate, 0.01 mM EDTA (Sigma-Aldrich, Ireland), 5.6 mM octane-1-sulphonic acid (Sigma) and 11% (v/v) methanol (Sigma-Aldrich, Ireland) and was adjusted to pH 2.8 using 1M sodium hydroxide (Sigma-Aldrich, Ireland). Twenty microlitres of supernatant was injected onto the HPLC system which consisted of a SCL 10-Avp system controller, LC-10AS pump, SIL-10A autoinjector (with sample cooler maintained at 4°C), CTO-10A oven, LECD 6A electrochemical detector (Shimadzu) and an online Gastorr Degasser (ISS, UK). A reverse-phase column (Kinetex 2.6u C18 100× 4.6 mm, Phenomenex) maintained at 30°C was employed in the separation (flow rate 0.9 ml/min), the glassy carbon working electrode combined with an Ag/AgCl reference electrode (Shimdazu) was operated at +0.8 V and the

chromatograms generated were analysed using Class-VP 5 software (Shimadzu). 5-HT and 5-HIAA were identified by their characteristic retention times as determined by standard injections which were run at regular intervals during the sample analysis. Concentrations were calculated using analyte:internal standard peak height ratios and expressed as nanograms of neurotransmitter per gram of fresh tissue weight.

Results (First period)

DNBS is a sensitizing agent which induces colitis in the animals once intrarectally administered. This local immune-inflammatory response has a peak 3 day after the injection and a progressive remission starting from the day 7 (*Ippolito et al., 2015*). On Day 14 and 21 the intestinal damage appears significantly reduced, though visceral sensitivity was still altered in these animals (data not shown).

In previous studies we found that a long antibiotic treatment (28 consecutive days) was able to significantly alter visceral sensitivity in the animals. The visceral hypersensitivity induced by microbiota depletion was almost reverted by treating the animals with the feaces of controls. By contrast, the administration of the faecal material derived from DNBS-treated animals led to a partial and slower recovery from the initial visceral hypersensitivity. In order to reduce the impact of the antibiotic treatment on visceral sensitivity and to allow a better evaluation of the effect of FMT, we decide to apply a shorter antibiotic treatment to deplete the gut microbiota before applying the FMT. We observed that a seven days-antibiotic treatment is enough for depleting microbiota in the animals and that the shortening of the antibiotic protocol should permit to reduce its impact on visceral sensitivity. Hence, we repeated the experiment adopting the new protocols.

As expected, the antibiotic treatment induced an increase in visceral sensitivity in the animals, but it did not result significative (Fig. 1, Day 7). The antibiotic effect was completely reverted by the re-colonization of intestine with a healthy microbiota, as well as by administering the vehicle. By contrast, the transplant of the faecal material from DNBS-treated animals led to a further increase in visceral sensitivity in the animals (Fig. 1, Day 32). Anyway, tree weeks after the interruption of the treatments this hypersensitivity progressively decrease again, as if the “innate healthy” condition was able to restore itself (Fig. 1, Day 46). We collected the intestine and the nervous tissues of animals at different key points to further investigate the presence of biological or morphological alterations. The preliminary analysis on colon showed the presence of hyperaemia and a slight thickening of wall after the antibiotic treatment, no other macroscopic alterations were observed (Fig. 2, Day 7). The same alterations were found also in the animals treated with DNBS-derived microbiota on day 32 (when they showed visceral hypersensitivity) and then disappeared on day 46 (when the normal pain threshold was restored; Fig. 2).

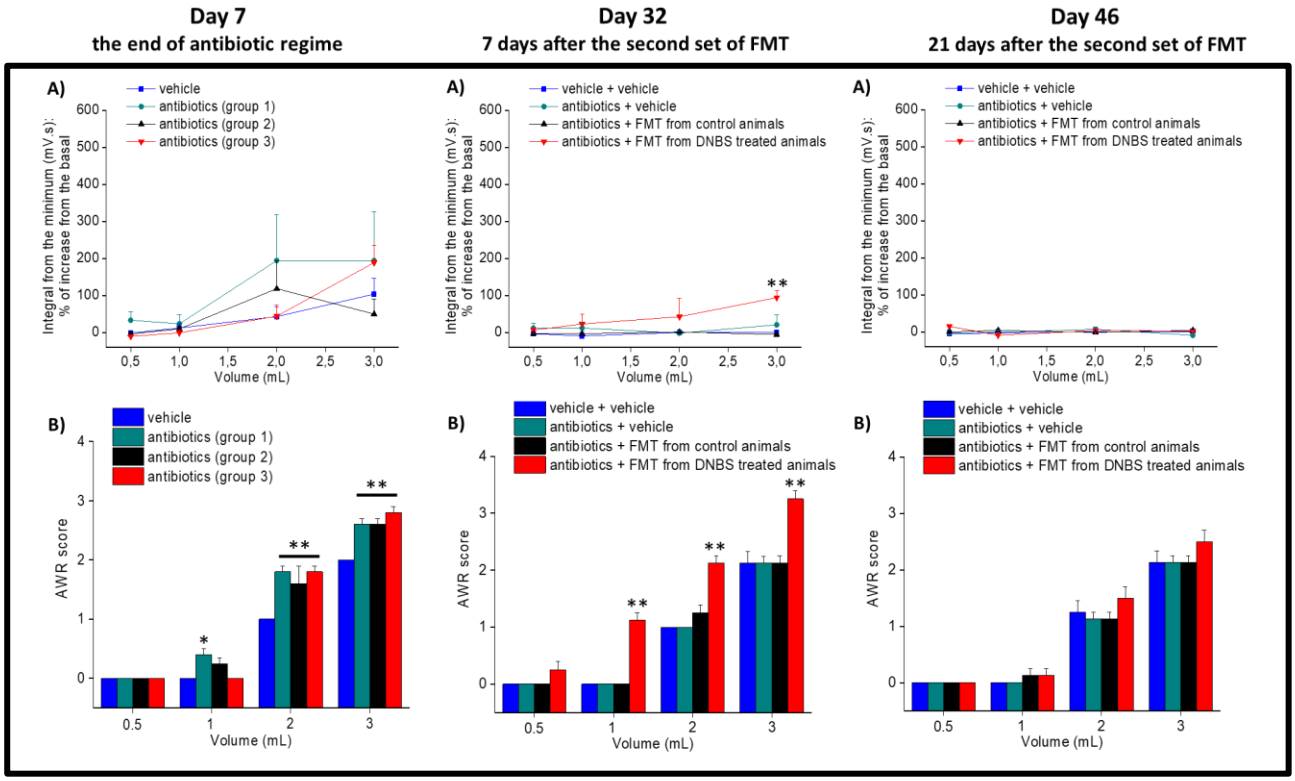

Figure 1. Effect of microbiota depletion and FMT from DNBS treated to naïve rats on visceral sensitivity. A) Measurament of VMR in response to CRD (animals under anesthesia); B) Measurament of AWR in response to CRD (awake animals). *P<0.05 and **P<0.01 vs vehicle or vehicle + vehicle treated animals.

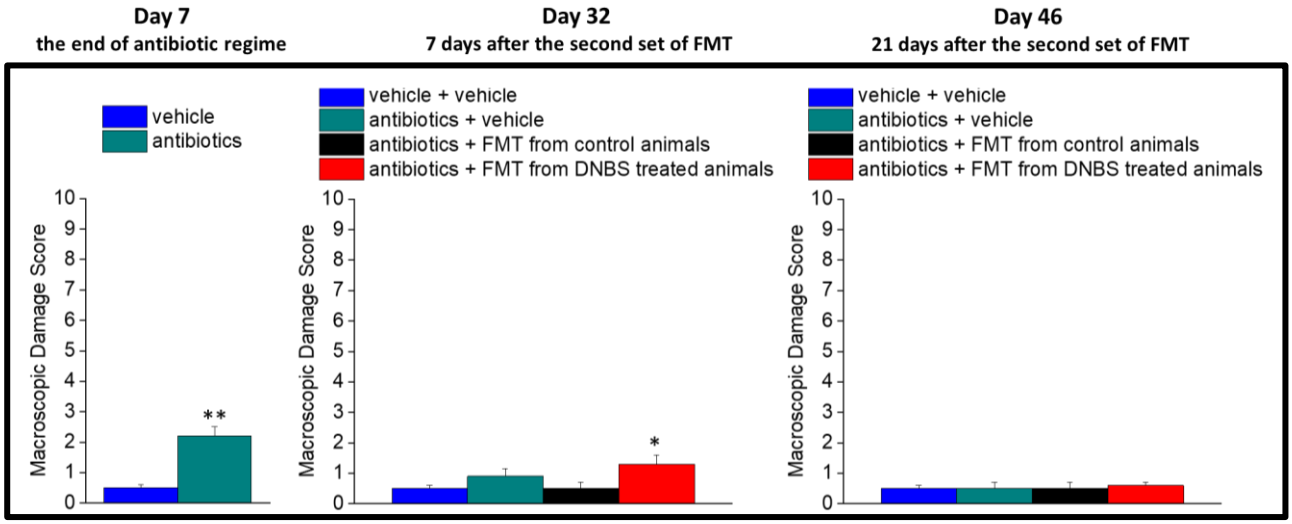

Figure 2. Effect of microbiota depletion and FMT from DNBS treated to naïve rats on colon - macroscopic damage. Assessment of Macroscopic Damage Score (MDS). *P<0.05 and **P<0.01 vs vehicle or vehicle + vehicle treated animals.

Results (Second period)

We performed a morphological analysis on the colon by haematoxylin/eosin staining for exploring further the presence of an intestinal damage in the animals underwent the antibiotic treatment and FMT. The microscopic analysis on colon showed the presence of an infiltrate of neutrophils after the antibiotic treatment (Day 7), while no significant tissue alterations were found in any of the experimental groups on Day 32 (Fig 3). Then, to evaluate if there was an impairment of the intestinal barrier as a consequence of the antibiotic treatment and the FMT, we measured the levels of Lipopolysaccharides Binding Protein (LBP) in the plasma by Elisa technique and the expression of tight junctions-related proteins in the colon by RT-qPCR. Regarding the levels of LBP in the plasma, I found no difference among the experimental groups at the different experimental time point (Fig 4). In accordance, analysing the gene expression in the colon, I found no difference in the expression of occludin, with exception of a significative increase in the animals treated with the antibiotic and the FMT at day 46 (Fig 5). On the other hand, the expression of Zo-1 resulted significantly upregulated only at the end of the antibiotic treatment, on Day 7 (Fig 5). In parallel we used the quantitative RT-PCR to evaluate the expression of pro- and anti-inflammatory markers in the gut of FMT recipient animals. This analysis aims to understand if the immuno-inflammatory response could have a role in the increase of visceral sensitivity resulting from FMT. The expression of the pro-inflammatory cytokines, TNF-alfa and IL-6, resulted increased at the end of the antibiotic treatment. The levels of TNF resulted significantly augmented in the animals treated with the antibiotics + vehicle and in the animals receiving antibiotics + FMT from CTR. Interestingly, the levels of TNF-α drastically decrease in the group receiving antibiotics + FMT from DNBS on both Day 32 and 46. By contrast, IL-6 expression resulted upregulated in both the groups treated with the FMT on Day 32 (Fig 6). We observed also a general increase in the expression of anti-inflammatory cytokines among the groups. In particular we found an augmented expression of both IL-10 and Tgf-β at the end of the antibiotic treatment and 7 days after the FMT (Day 32). Interestingly, we found a peak of IL-10 in the animals receiving the microbiota from DNBS on Day 32 which disappears on Day 46. Tgf-β expression resulted significantly upregulated as a consequence of both the antibiotic treatment and the FMT, no difference where observed between the animals receiving the FMT from CTR in respect to the animals receiving the FMT from DNBS (Fig 6). These data confirmed that the microbiota transplantation causes a dysregulation in the immune response, but they don't explain the difference observed at the level of pain perception among the groups.

Finally, through HPLC, we assessed the levels of serotonin (5-HT) and its main metabolite 5-hydroxyindoleacetic acid (5-HIAA) in the colon. Serotonin is the main neurotransmitter produced in the gut where it is involved in the regulation of gut motility as well as of visceral sensitivity. Anomalies in the production or release of serotonin were suggested as one of the possible mechanisms by which the microbiota influences the neurotransmission (*Clarke et al., 2012; O'Mahony et al., 2015; Kennedy et al., 2017*). Anyway, this neurotransmitter does not seem involved in the modulatory effect mediated by

microbiota on visceral pain. In fact, we did not find any significative alteration in serotonin and 5-HIAA after the antibiotic treatment and the FMT (Fig 7).

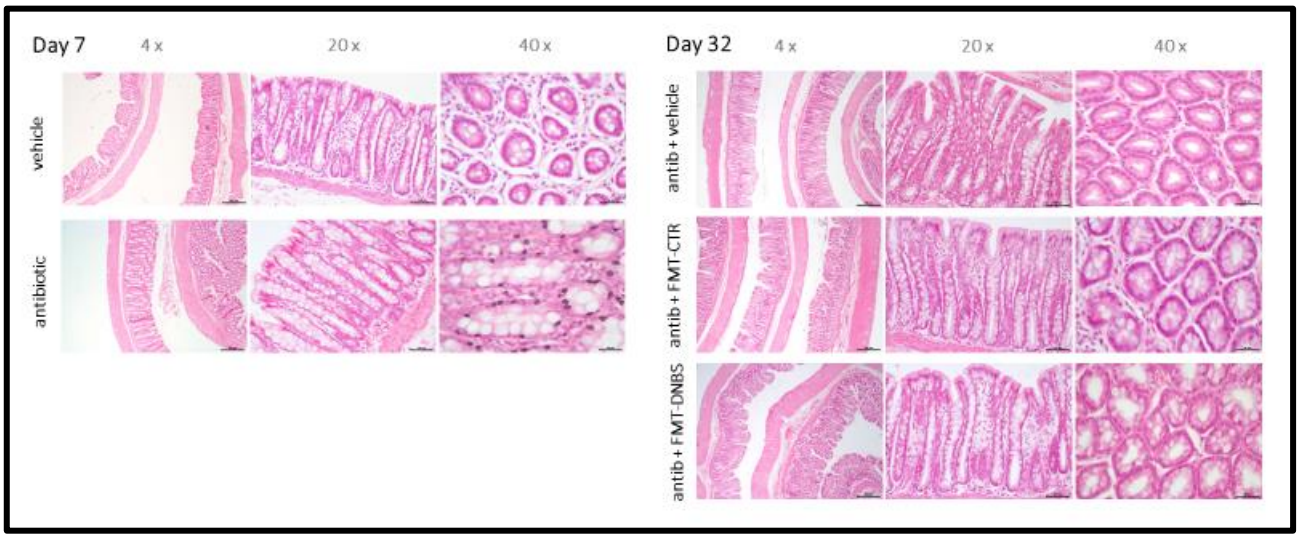

Figure 3. Effect of microbiota depletion and FMT from DNBS treated to naïve rats on colon - histological analysis. Histological analysis on haematoxylin/eosin stained colon slices.

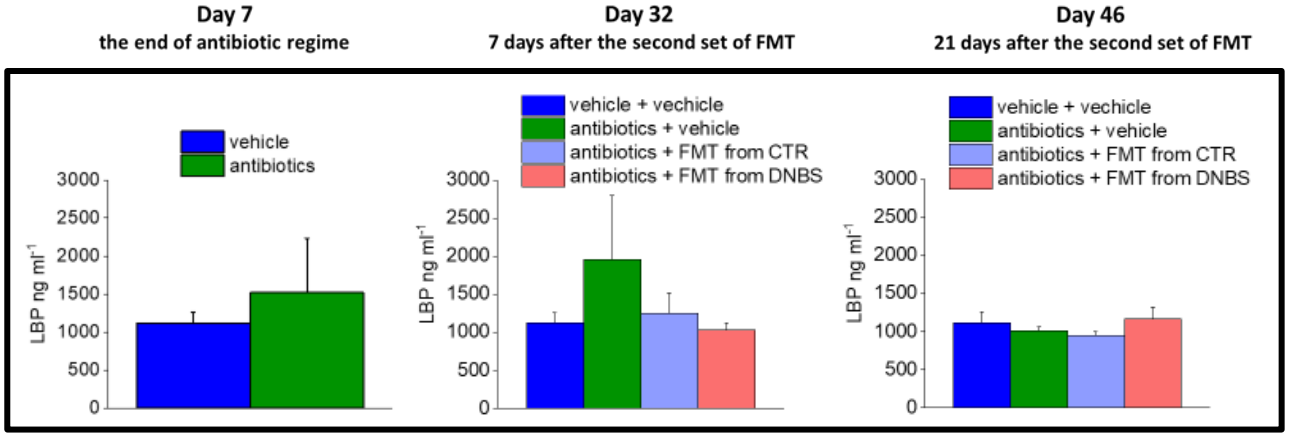

Figure 4. Effect of microbiota depletion and FMT from DNBS treated to naïve rats on gut permeability – assessment of Lipopolysaccharides Binding Proteins (LBP) in plasma. LBP was measured in plasma by Elisa Immunoassay.

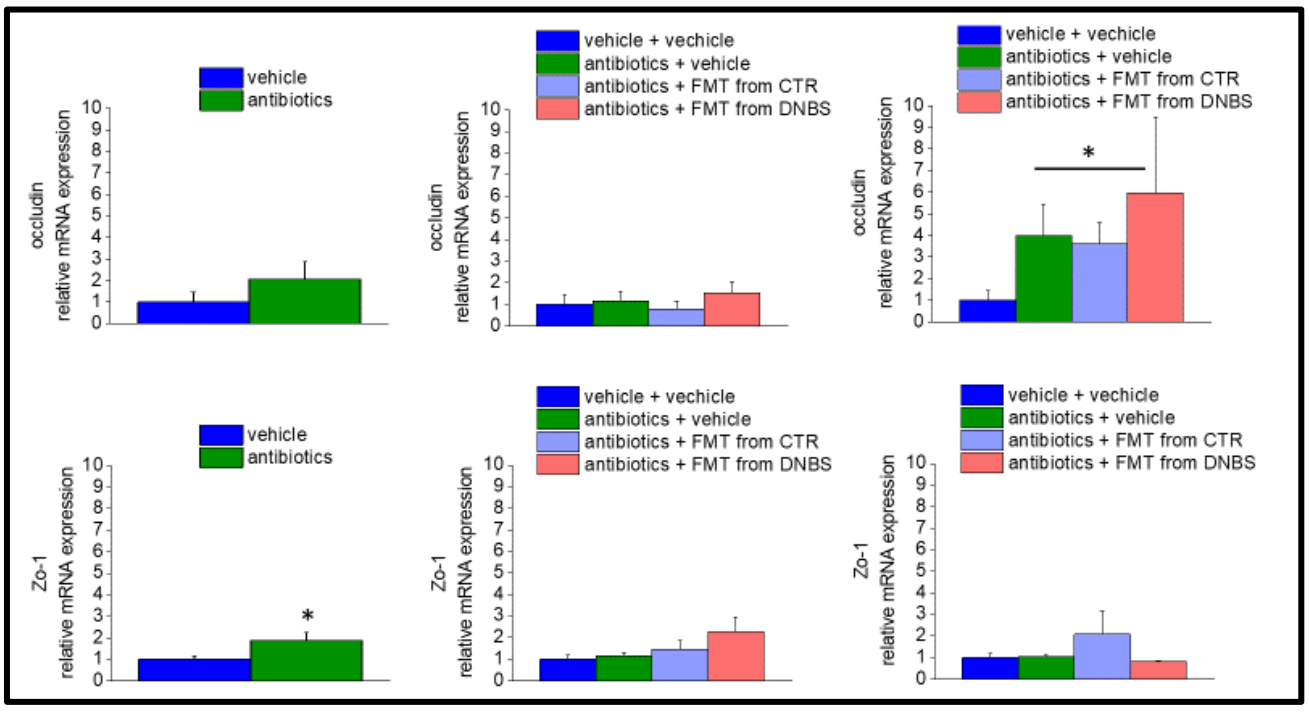

Figure 5. Effect of microbiota depletion and FMT from DNBS treated to naïve rats on gut permeability – assessment of tight junction proteins gene expression. Gene expression of occludin and Zo-1 in colon was quantified by RT-qPCR. *P<0.05 and **P<0.01 vs vehicle or vehicle + vehicle treated animals.

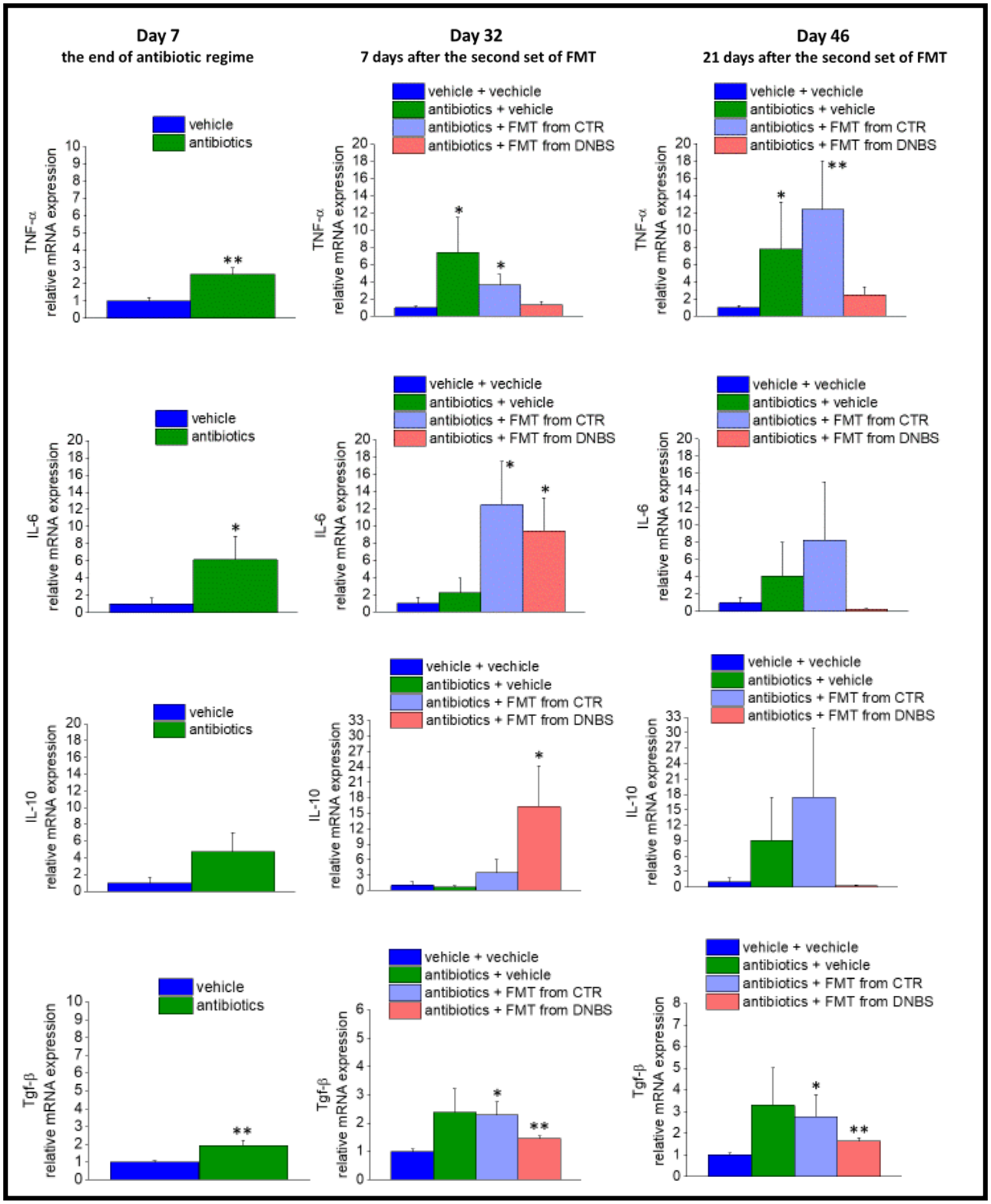

Figure 6. Effect of microbiota depletion and FMT from DNBS treated to naïve rats on immune response – assessment of cytokines gene expression. Gene expression of TNF-α, IL-6, IL-10 and Tgf-β in colon was quantified by RT-qPCR. *P<0.05 and **P<0.01 vs vehicle or vehicle + vehicle treated animals.

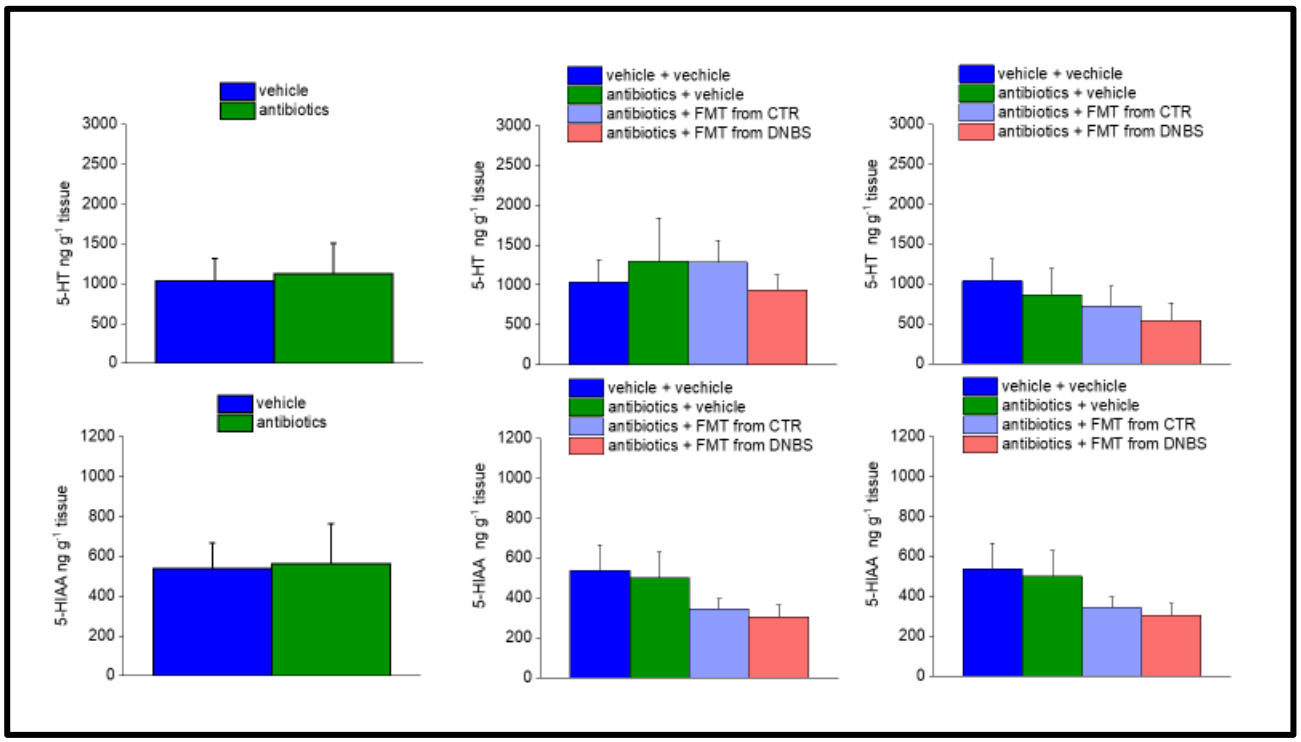

Figure 7. Effect of microbiota depletion and FMT from DNBS treated to naïve rats on neurotrasmitters levels in the colon. The concentrations of serotonin (5-HT) and its metabolite 5-hydroxyindoleacetic acid (5-HIAA) were quantified by HPLC.

Conclusions

The transplant of microbiota derived from DNBS animals is able to directly transfer visceral hypersensitivity to naïve animals. This effect is not imputable to an inflammatory response, neither to changes in gut permeability or serotonin increase. Anyway, the results obtained in vivo strengthen the hypothesis of a direct involvement of microbiota in post-inflammatory visceral pain and so encourage to further study the therapeutic effect of microbiota transplantation on post-inflammatory visceral pain. In order to make the microbiota a suitable target for the treatment of visceral pain, it is however necessary to continue investigating the mechanisms by which it can modulate visceral pain perception. The knowledge I have acquired in these months at the University College Cork with the supervision of Dr. S. O'Mahony and Prof. J.F. Cryan allowed me to optimize the experimental procedures and to deepen further my knowledge about the physiology of the microbiota-gut-brain axis. During the laboratory work, I became familiar with new experimental techniques, as the High Potency Liquid Chromatography (HPLC) and the analysis of genes expression by quantitative RT-PCR.

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Data: 17/09/2019 Firma: