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Early life stress in mice is a suitable model for Irritable Bowel Syndrome but does not predispose to colitis nor increase susceptibility to enteric infections

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Running title: Long-term effects of maternal separation on gastrointestinal diseases susceptibility

Abstract: Neonatal period is characterized by an immature intestinal barrier. Scattered evidence suggests that early life stressful events induce long lasting alterations of intestinal homeostasis mimicking Irritable Bowel Syndrome (IBS). Those observations highlighting defect of intestinal barrier by early life stress questioned its potential role as a risk factor for gastrointestinal disorders such as colitis and infections. In this study, we aimed to analyze if maternal separation (MS) in mice gathers IBS main features. We next addressed whether MS could trigger or exacerbate colitis in genetically predisposed mice and/or enhance susceptibility to gastrointestinal infections in wild type mice. MS induced main features of IBS in adult wild type male mice i.e. intestinal hyperpermeability, visceral hypersensitivity, microbiota dysbiosis, bile acid malabsorption and low grade inflammation in intestine associated with a defect of Paneth cells and the ILC3 population. This breach in mucosal barrier functions in adults was associated with a systemic IgG response against commensal *E. coli* and increased IFNγ secretion by splenocytes. However, in $IL10^{-/-}$ mice, MS did not trigger nor worsen colitis. Furthermore, wild type mice submitted to MS did not show increase susceptibility to gastrointestinal infections (*S.* Typhimurium, *L. monocytogenes* or *T. gondii*) compared to controls. Altogether, our results identify MS in mice as a good experimental model for IBS mimicking all the main features. In addition, early life stress, even though it has long lasting consequences on intestinal homeostasis, does not constitute a facilitating factor to colitis in predisposed individuals nor to gastrointestinal infections in wild type mice.

Keywords: Maternal separation, humoral and cellular response toward microbiota, pro-inflammatory T cell response, innate and adaptive immune responses.

Abbreviations: AMP, antimicrobial peptides; CD: Crohn's disease; GALT, Gut Associated Lymphoid Tissue; IBD, Inflammatory Bowel Disease; IBS, Irritable Bowel Syndrome; Ig, Immunoglobulin; ILC, Innate Lymphoid Cells; MS, Maternal Separation.

Highlights:

- Early life stress in mice is a unique model mimicking IBS features i.e. visceral hypersensitivity, intestinal hyper-permeability, lowgrade inflammation, microbiota dysbiosis and bile acid malabsorption.
- Early life stress does not precipitate nor exacerbate colitis in mouse models genetically predisposed to IBD
- Early life stress does not increase susceptibility to gastrointestinal pathogen infections.
- Early life stress induces long lasting consequences on intestinal barrier that do not impair the ability of intestinal barrier to prevent enteric infections or contain colitis. This result is consistent with epidemiological studies.

1. Introduction

Irritable Bowel Syndrome (IBS) is a functional gastrointestinal disorder newly called "Disorder of the gut-brain interaction" according to Rome IV classification (Drossman, 2016). It is characterized by abdominal pain and altered bowel habits (Drossman, 2006). It is highly prevalent (~11%) in Europe and in the United States (Lovell and Ford, 2012). The occurrence of stressful events is considered as a contributing factor triggering and/or maintaining IBS (Mayer et al., 2001; Wood, 2011), suggesting that dysfunctional interactions in the brain-gut axis contribute to the pathophysiology of the disease (Bonaz and Bernstein, 2013) and as such justifying it's new classification as Disorder of the brain-gut interaction (Drossman, 2016). Beside visceral pain, IBS is also characterized by increased intestinal permeability (Bischoff et al., 2014), microbiota dysbiosis (for review (Collins, 2014)) and increased state of activation of immune cells (Barbara et al., 2011) even though this last observation is still under debate. Early life events draw particular attention since they were associated with IBS susceptibility (Hislop, 1979; Lowman et al., 1987; Videlock et al., 2009). The perinatal period is a crucial and critical window where the intestinal barrier, the immune system and the gut microbiota mature and establish an appropriate complex relationship (Renz et al., 2012). Maternal separation (MS) is an established model of early life stress in rodent (for review (Marco et al., 2015; O'Mahony et al., 2011)). Data from various studies show that maternal separation (MS) induces in rats many features of IBS i.e. visceral hypersensitivity (Barreau et al., 2004), intestinal hyperpermeability (Barreau et al., 2004) and microbiota dysbiosis (O'Mahony et al., 2009; Zhou, 2016). However, so far there is no study showing all IBS features in a single experimental model. Furthermore, as MS appears to weaken the intestinal barrier and exacerbates intestinal and systemic immune responses at adulthood, we questioned its role as a risk factor for susceptibility to inflammatory bowel diseases (IBD) or gastrointestinal infections.

In this study, we analyzed the consequences of adverse stressful events occurrence in neonatal mice on the development of IBS symptoms, i.e. visceral hypersensitivity, intestinal hyperpermeability, microbiota dysbiosis and immune cells activation. Next, we hypothesized that if early stressful events impair intestinal barrier functions, it might be associated with a loss of immune tolerance toward luminal contents. Then, we assessed if the MS-induced alteration of intestinal barrier and exacerbation of immune response may trigger or exacerbate colitis in the IBD model of $IL10^{-/-}$ mice or make wild type mice more susceptible to gastrointestinal infectious challenge with bacterial pathogens (*Listeria monocytogenes, Salmonella typhimurium*) or parasites (*Toxoplasma gondii*). More generally, the question of wherever early life adverse events are weakening the organism or not is of particular interest. It is admitted that neonate respond to an adverse environment by making developmental adaptations that are likely to increase survival and resilience in the anticipated deprived environment. Therefore perinatal adversity may affect immunity in such a way to allow the organism to better cope with a stressful environment (for review (Avitsur et al., 2015)). This statement is counterintuitive and needs scientific evidence.

2. Material and Methods

All experimental protocols described in this study were approved by the local Animal Care Use Committee (Comité d'Ethique de Pharmacologie-Toxicologie de Toulouse - Midi-Pyrénées, France) registered as N°86 at the Ministry of Research and Higher Education (N° 0029/SMVT), and conducted in accordance with the European directive 2010/63/UE. All experiments were performed on mice aged of 50 days (D50). All animals were euthanized by cervical dislocation.

2.1 Animals

In all experiments, mice were kept at a constant temperature (22+/-1°C) and maintained on a 12:12h light/dark cycle (light on at 7h30 am). Food (Harlan, Gannat, France) and water were available *ad libitum*. After delivery (D1), litters were adjusted to 6±1 pups. Weaning was performed on D21, siblings were sex matched and the animals were kept under Specific and Opportunistic Pathogen Free (SOPF) conditions.

Maternal separation

Nulliparous female C3H/HeN mice (Janvier, Roubaix, France) or C57BL/6 IL10$^{-/-}$ (kindly provided by Dr Manuela Büttner) were mated with males for 4 days. Maternal separation (MS) was performed daily for three consecutive hours (from 9 am to 12 pm). Pups were kept at a controlled temperature (27+/-1°C). MS was repeated for 10 working days, weekend excluded, between D2 and D15 as already published (Barreau et al., 2004). Repeated maternal deprivation during Hypothalamic Pituitary Axis development (PND2 to PND15) have been shown to have long lasting consequences in offspring behaviors and physiology (for review (O'Mahony et al., 2011)). Control pups were left with their dam and were not manipulated. From D15 to D21, all pups were maintained with their dam. Only male mice were kept for the following experiments to avoid female hormonal cycle interference.

Weight was monitored every week between D2 and D50.

Infections with Salmonella enterica subsp. *enterica serotype* Typhimurium (*S.* Typhimurium), *Listeria monocytogenes or Toxoplama gondii*

We analyzed three models of intestinal pathogens: *S.* Typhimurium (bacterial pathogen), *L. monocytogenes* (intracellular bacterial pathogen) and *T. gondii* (intracellular pathogen

parasite). PND50 C3H/HeN male mice were orally challenged by intra-gastric gavage with 10^9 CFU of *Salmonella* Typhimurium 14028s (pACYC177), or 10^9 CFU of *Listeria monocytogenes* Lmo-InIA^m expressing the internalin recognized by the murine E-cadherin (Wollert et al., 2007) or 35 cysts of *Toxoplasma gondii* 76K. Mice were weighted and euthanatized seven or five days post infection respectively with *S.* Typimurium and *T. gondii* or *L. monocytogenes*. Then small intestine, ileum, liver and/or spleen were mashed through a 70 µm nylon mesh and plated on McConkey supplemented with 50µg/ml kanamycin for *S.* Typhimurium or ChromID Lmono for *L. monocytogenes* and incubated at 37°C for 24 and 48H respectively. For *T. gondii*, DNA extraction was performed with the kit Genomic DNA from Tissue (Macherey-Nagel, Germany). Real time PCR was performed on 1 µg of total DNA in a total volume of 25 µl containing Platinium® Quantitative PCR Super Mix-UDG (Uracil-DNA glycosylase) (Invitrogen, USA), 4 mM $MgCl_2$, 0.5 µM of each primer specific for the 18s rDNA gene of *T. gondii* TG III (5′-CCT TGG CCG ATA GGT CTA GG-3′); TG IIb (5′-GGC ATT CCT CGT TGA AGA TT-3′), and 180 nM of the probe 5′-TGC AAT AAT CTA TCC CCA TCA CGA TGC ATA CTC AC-3′ modified with 5′-FAM and 3′-TAMRA as reporter and quencher, respectively (Kupferschmidt et al., 2001). After initial UDG incubation for 2 min at 50°C and denaturation for 5 min at 95°C, the two-step amplification conditions were 50 cycles of 20 s at 95°C and 60 s at 65°C with the LightCycler® 480 Real-Time PCR System (Roche Applied Science, Germany). A standard curve for parasite number equivalence was generated in parallel.

2.2 Intestinal Permeability

In vivo oral gavage with FITC-Dextran 4kDa

Mice received per oral gavage a solution of FITC-Dextran 4kDa (Sigma, St Quentin, France) at a concentration of 750mg/kg of body weight. Intracardiac blood collection after opening of thoracic cavity was performed 4 hours after FITC-Dextran 4kDa gavage. FITC-Dextran 4kDa concentration in plasma was assessed by measuring the fluorescence intensity (FI) excitation 485nm / emission 525nm using an automatic Infinite M200 microplate reader (Tecan). Results were expressed as µg per ml of plasma.

Ex vivo jejunal fragments mounted in Ussing chambers

Jejunal fragments were mounted in Ussing chambers (Easy Mount, Physiologic Instruments) exposing a surface area measuring 0.1 cm^2. They were bathed on each side with 1 ml of oxygenated thermostated Kreb's solution (Sigma). Electrical parameters, including electrical resistance (R), were recorded at regular intervals during the 2-hour period of experimentation. A change in electrical resistance was considered an index of altered paracellular permeability. Horseradish peroxidase (HRP 44kDa) (Sigma) transport was measured as an index of macromolecular permeability, and FSS (376Da) (Sigma) epithelial passage was measured as a marker of paracellular permeability to small molecules. After equilibration of electrical parameters, HRP was added to the mucosal compartment at a final concentration of 0.4 mg/ml, and FSS at a final concentration of 40µg/ml. The two markers were applied simultaneously in the mucosal compartment. Epithelial permeability to total HRP was determined by an ELISA. Briefly, 96-wells flat-bottomed black plates (Greiner, Thermo Scientific, Dutcher, Brumath, France) were coated overnight at +4°C with 50µl of 10µg/ml mouse polyclonal to HRP (Abcam) in PBS. Plates were blocked with PBS-1% bovine serum albumin (BSA) before incubation with serosal compartments of Ussing chamber. Rabbit polyclonal anti HRP biotin (Abcam) was added at a concentration of 10µg/ml before FITC-conjugated streptavidin (Becton Dickinson; BD, Le Pont de Claix, France) was added for 20 minutes and fluorescence intensity measured 485nm/525nm using an automatic Infinite M200

microplate reader (Tecan). Epithelial permeability to FSS was determined by measuring the fluorescence intensity (FI) 485nm/525nm using an automatic Infinite M200 microplate reader (Tecan). Permeability was calculated as the ratio of flux/concentration, and expressed as cm/second.

2.3 Visceral sensitivity in response to colorectal distension

Mice were anesthetized with a mixture of ketamine 100mg/kg of body weight and xylazine 10mg/kg of body weight, equipped with 3 NiCr wire electrodes implanted into the abdominal external oblique muscle at D47 and kept individually after surgery. The electromyographic (EMG) activity was recorded and analyzed with a Powerlab Chart from AD instrument. EMG recordings began 3 days after surgery. Mice were placed in polypropylene tunnels. A balloon consisting of an arterial embolectomy catheter (Fogarty, 4F, Edwards Laboratories, Santa Ana, CA) was introduced into the rectum at 2.5 cm from the anus and fixed at the base of the tail. The balloon was progressively inflated during 15 seconds by step of 0.02 ml, from 0.02 to 0.1 ml, with 10 minutes wait between each step. The Fogarty embolectomy catheter balloon was calibrated using an electronic caliper gauge and the maximal pressure applied (corresponding to 0.1 ml) was calculated as 63.1±1.7 mmHg meaning that volume progressive distension corresponds to a range of pressures between 0 and 63.1±1.7 mmHg . Basal EMG activity was subtracted from the EMG activity registered during the periods of distension. The use of Forgaty probe and volumes rather than barostat and pressures was selected to obtain reliable VMR at low volumes.

2.4 Enteric lysozyme analysis

Lysozyme expression in Paneth cells

Ileal samples were fixed in 4% formalin, dehydrated through graded ethanol and embedded in paraffin. Sections (5µm) were rehydrated and submerged in antigen retrieval solution (citrate buffer, 10mM, pH6, 99°C) for 30 minutes. After incubation in blocking solution (PBS 0,01% Tween 20, 1% bovine serum albumin and 2% donkey normal serum) for 15 min, sections were incubated with rabbit anti-mouse lysozyme antibody (1/100, overnight, +4°C) (Abcam, Paris, France) followed by a Alexa fluor 488-conjugated donkey anti-rabbit IgG (0.75µg/ml, 1H, Room temperature) (Jackson, Sullfolk, UK). After washing, sections were incubated with Alexa fluor 594-conjugated Wheat Germ Agglutinin (WGA) (10µg/ml, 45 min) (Invitrogen, Life Technology, Cergy Pontoise, France). Sections were mounted in Prolong gold antifade mounting medium with DAPI (Invitrogen) and examined under a Nikon 90i fluorescence microscope. Lysozyme fluorescence intensity in Paneth cells was quantified employing the software Nis-Elements Ar (Nikon, Champigny sur Marne, France) and results were expressed in fluorescence intensity per cell. Analyses were done on five crypts per animal and on five animals from each group.

Lysozyme activity in fecal content

Feces were collected and frozen at -80°C. Activity of lysozyme against the peptidoglycan was determined using the EnzChek® Lysozyme Assay Kit (Molecular probes, life technology, St Aubin, France).

2.5 Fecal microbiota composition analysis

Total community DNA was extracted from stool samples and adjusted to 1 ng/µl prior to use as described in (Yvon et al., 2016). Changes in the relative abundance of 20 relevant microbial 16S rRNA gene targets (supplementary table) were obtained using the GULDA

platform approach (Bergström et al., 2014, 2012) with minor adaptations (Yvon et al., 2016). The normalized No-values were reported per g feces, log10-transformed and processed by MixOmics package (5.2.0 version) with RStudio software (0.99.902 version) to build a partial least-squares discriminant analysis (PLS-DA) (dejean omics data integration project. R package version 2012). PLS-DA is a multivariate supervised approach that operates by projecting the samples (X) onto a low-dimensional space of so-called latent variables that maximizes the separation between different groups of samples according to their class labels (Y= mouse treatments). Missing normalized No-values were reconstituted using the NIPALS algorithm and leave-one-out cross-validation was used to select the optimal number of latent variables for PLS-DA models with minimal error rate. Variable Importance in Projection (VIP, weighted sum of squares of the PLS loadings) scores were estimated and allowed to classify the microbial amplicon groups according to their explanatory power of class label (Tenenhaus, 1998).

2.6 ^{1}H NMR metabolomics and data analysis

Fecal extracts for NMR spectroscopy were prepared by mixing 50 mg of fecal samples with 500 µL of phosphate buffer (0.2 M, pH = 7.4) in D2O, containing 1% (w/v) of sodium 3-(trimethylsilyl) propionate (TSP). After vortex mixing, the samples were subjected to a freeze−thaw cycle in liquid nitrogen and subsequently homogenized with a tissue lyser (MP Biomedicals, Illkirch Graffenstaden, France) at 20 Hz during 40 s, followed by centrifugation at 10 000g for 10 min at 4 °C. Supernatants were collected, and the remaining pellet was further extracted once as described above. Supernatants obtained from two runs of extraction were combined and centrifuged at 10 000g for 10 min at 4 °C. A total of 600 µL of supernatant was transferred into NMR tubes with an outer diameter of 5 mm pending NMR analysis.

All ^{1}H NMR spectra were obtained on a Bruker DRX-600-Avance NMR spectrometer (Bruker, Wissembourg, France) operating at 600.13 MHz for ^{1}H resonance frequency using an inverse detection 5 mm 1H-13C-15N cryoprobe attached to a cryoplatform (the preamplifier cooling unit). The ^{1}H NMR spectra were acquired at 300K using the Carr-Purcell-Meiboom-Gill (CPMG) spin-echo pulse sequence with water presaturation. A total of 128 transients were collected into 64,000 data points using a spectral width of 20 ppm, a relaxation delay of 2 sec, and an acquisition time of 2.72 sec. Metabolites were assigned using previously published data (Zhao et al., 2012) and additional two-dimensional NMR experiments on selected samples.

Data were analyzed by applying an exponential window function with a line broadening of 0.3 Hz prior to Fourier transformation. The resultant spectra were phased, baseline corrected and calibrated to TSP (δ 0.00) manually using Mnova (Mestrelab Research S.L.). The spectra were subsequently imported into MatLab (R2014a, The MathsWorks inc.). All data were analyzed on full-resolution spectra (33 600 data points). The region containing the water resonance (δ 4.6-5.2) was removed. Spectra were normalized to the probabilistic quotient (Dieterle et al., 2006) and aligned using an alignment function (Veselkov et al., 2009). Data were mean-centred and scaled using the unit variance scaling prior to analysis with orthogonal projection on latent structure-discriminant analysis (O-PLS-DA). ^{1}H NMR data were used as independent variables (X matrix) and regressed against a dummy matrix (Y matrix) indicating the class of samples (*control* or MS) (Trygg and Wold, 2002). The O-PLS–derived model was evaluated for goodness of prediction (Q^2Y value) using 8-fold cross-validation. The reliability of each model was established using a permutation test of the Y vector (1000 permutations) to determine a p-value for each Q^2Y, as previously described (Eriksson et al., 2008). Parameters of the final models are indicated in the figure legends.

To identify metabolites discriminating the animal groups, the O-PLS-DA correlation coefficients (r^2) were calculated for each variable and back-scaled into a spectral domain, so that the shape of the NMR spectra and the sign of the coefficients were preserved (Cloarec et al., 2005). The weights of the variables were color-coded according to the square of the O-PLS-DA correlation coefficients. Correlation coefficients extracted from significant models were filtered so that only significant correlations above the threshold defined by Pearson's critical correlation coefficient ($p<0.05$; $|r|>0.49$) were considered significant. For illustration purposes, the area under the curve of several signals of interest was integrated, and statistical significance was tested using Mann-Whitney test.

2.7 Spleen and Lamina propria cell characterization

Splenocyte isolation

Spleens were removed and cells were isolated through a 70 µm nylon mesh in PBS/1% KnockOut™ SR (KO SR) (GIBCO). Splenocytes were stained with antibodies to the following markers: anti-CD4 (BD), anti-CD44 (BD), anti-CD62L (BD), anti-CD25 (BD), anti-foxp3 (ebioscience, Rennes, France) prior analysis by flow cytometry.

Small intestines were washed in cold PBS, cut into 0.5 cm pieces, incubated four times in 30 ml of PBS-3 mM EDTA (Sigma) for 10 min at 37°C and digested in 30 ml of RPMI 1640 added with 20% BSA and 100 U/mL of collagenase (Sigma) for 40 min at 37°C. *Lamina propria* cells were purified on a 40%–80% Percoll gradient run for 15 min at 2,000g and cells were stained with anti-MHC II and anti-RORγt antibodies (BD).

Flow cytometry data collection was performed on MACSQuant® Analyzers (Miltenyi Biotec, Paris, France). Data were analysed using VenturiOne® (ApplliedCytometry) software.

Measurement of splenocytes activation

After washing, cells were seeded on 24-well plates at $1x10^6$ cells per well for cytokines assays in Cerrotini culture medium (Dulbecco modified Eagle medium supplemented with 8% KO SR (GIBCO), 36 mg/l asparagine, 116 mg/l arginine, 10 mg/l folic acid, 1 g/l 4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid, 0.05 mmol/l β-mercaptoethanol, 100 U/ml penicillin, 100 Kg/ml streptomycin and 1 µg/ml fungizone) in the presence or absence of 5µg/ml hamster anti-mouse CD3 and hamster anti-mouse CD28 (BD) coated wells. After 3 days of stimulation, culture supernatants were collected and frozen at -80°C prior cytokines measurement.

2.8 Cytokine measurement

Cytokines were measured in supernatant of primary cell culture of spleen, on ileal fragments or in feces previously treated as follow. Frozen ileal fragments or feces were suspended in RIPA buffer (0.5% deoxycholate, 0.1% SDS and 1% Igepal in TBS) containing complete anti protease cocktail (Roche), protein concentrations were measured using BCA uptima kit (Interchim).

TNFα, IFNγ, TGFβ or lipocalin present in primary cell culture supernatant, ileal tissues or feces were assayed using commercial enzyme linked immunosorbent assays (ELISA kits; Duoset R&D Systems, Lille, France). Concentration of TNFα in ileal tissue and lipocalin in feces were normalized per mg of proteins and results expressed in pg of cytokine per mg of proteins.

2.9 Commensal E. coli and food lysate preparations

E. coli lysate for humoral response

Pellet of an overnight culture of one of the *E. coli* isolates previously characterized (Riba et al., 2017) was washed in 0,9% NaCl, suspended in 1 ml of distilled water, sonicated for 1 hour, frozen in liquid nitrogen, melt and centrifuged. Supernatant was filtered in 0,22 µm and conserved at -20°C as the *E. coli* lysate used for humoral and cellular response. Lysate protein concentration was measured using BCA (bicinchoninic acid) protein Assay kit, Uptima (Interchim, Montluçon, France).

Food lysate for humoral response

Food was suspended in PBS, incubated and vortexed. Supernatant was filtered on 0.22 µm filter and kept at -20°C as the food lysate used for humoral response. Lysate protein concentration was measured using BCA protein Assay kit, Uptima (Interchim, Montluçon, France).

2.10 Humoral response in plasma and feces.

Intracardiac blood was recovered with heparined needle and plasma was kept at -80°C.

Fecal proteins were extracted mechanically in complete antiprotease cocktail (Roche Diagnostic, Meylan, France) and frozen at -80°C.

Quantification of IgG and IgA concentrations

Plasma and fecal IgG or IgA concentrations were measured by ELISA in MaxiSorp 96-wells flat-bottomed plates (NUNC, Thermo Scientific, Dutcher, Brumath, France). Plates were coated overnight at +4°C with 50µl of 5µg/ml sheep anti-mouse IgA (Sigma) or goat anti-mouse IgG (SoutherBiotech, Cliniscience, Nanterre, France) in PBS. Plates were blocked with PBS-5% fetal calf serum (FCS) (GIBCO, Invitrogen, Life Technology, St Aubin,

France) before incubation with diluted samples or purified IgA or IgG (SoutherBiotech). Plasma was diluted at 1:20,000 for IgG and fecal extracts at 1:6,000 for IgA. Horseradish-peroxidase (HRP)-conjugated goat anti-mouse IgA (Sigma) or goat anti-mouse IgG (SoutherBiotech) were added, HRP was revealed using TMB (Becton Dickinson; BD, Le Pont de Claix, France) and the reaction was stopped with 25 µl of H_2SO_4 2N before reading at 450 nm using an automatic Infinite M200 microplate reader (Tecan). Results were expressed as µg/ml for plasma and µg/mg of fecal proteins for IgA.

Immunoglobulin specificity against luminal content

Maxisorp 96-well plates (NUNC) were coated overnight at +4°C with 50µl of a) 5 µg/ml of *E. coli* lysate or b) 20µg/ml of food lysate. After blocking with PBS 5%-FCS, plates were incubated for 2 hours at 37°C with 50 µl of plasma samples diluted with PBS 5%-FCS to adjust IgG concentration to 20 µg/ml. HRP-conjugated goat anti-mouse IgG (SoutherBiotech) was incubated for 2 hours at 37°C and revealed as above-mentioned. Results were expressed as optical density (OD) at 450 nm and were considered positive if above two blanks.

2.11 Histology

Colon samples were collected, fixed with 10% formalin for 24 h, dehydrated and embedded in paraffin according to standard histological procedures. Sections (5 µm) were mounted on SuperFrost® Plus Slides then dewaxed in a xylene bath for 10 min and rehydrated in graded alcohol baths. Slides were stained with hematoxylin and eosin (H&E) and analyzed for intestinal morphometry. Erben and el. histomorphological scoring was used (Erben et al., 2014).

2.12 Statistical analysis

Statistical analysis was performed using GraphPad Prism version 6.04 (GraphPad Software, San Diego, California, USA). Results were expressed as box and whisker plots. Mean comparisons were performed using two-way ANOVA analysis followed by post test or t tests. Differences were considered significant for $p<0.05$.

3. Results

Of note, no difference in weight was observed between control and stressed mice.

3.1 MS increased intestinal permeability and visceral sensitivity to colorectal distention

MS significantly increased *in vivo* intestinal permeability to FITC-Dextran 4kDa at D50 (17.76±1.18 vs. 8.09±0.79 µg/ml of plasma, t_{10}=2.275 p=0.0462 Two-tail t-Test, , Figure 1A). MS also significantly increased *ex vivo* transcellular permeability to HRP (4.168±0.7273 vs. 2.542±0.198 cm/s $.10^{-8}$ t_{18}=2.158 p=0.0447, Two-tail t-Test) but not para cellular permeability to FSS in jejunal fragments mounted in Ussing chambers (Figure 1B). Furthermore, MS increased visceral sensitivity to colorectal distention at low volumes (3.99±0.59 vs. 0.9±0.1 mV.s at 0.02 ml; 5.24±1.06 vs. 2.03±0.44 mV.s at 0,04 ml; 6.34±0.87 vs. 3.07±0.62 mV.s at 0.06ml compared to control p<0.05, MS versus control $F_{4,141}$=2.158, p<0.0001 Two-way ANOVA followed by Holm-Sidak's post test) (Figure 1C). This result reflects MS-induced allodynia i.e. pain response at non nociceptive stimulation.

3.2 MS decreased ileal expression and fecal activity of lysozyme

Production of antimicrobial compounds by Paneth cells represents an important defense mechanism of the epithelium and shapes intestinal microbiota. MS decreased ileal expression of lysozyme in Paneth cells (65803±17448 vs.160960±27224 fluorescence intensity/cell, t_6=2.943 p=0.0259Two-tail t-Test) without modification of the number of crypts producing lysozyme (Figures 2A-C). Moreover, fecal anti-peptidoglycan activity of lysozyme was decreased in MS mice compared to controls (155.5±35.28 vs. 332.2±45.43 U/mg of fecal proteins t_{23}=3.099, p=0.0051 Two-tail t-Test, Figure 2D). The defect of lysozyme expression

in ileal Paneth cells was associated with higher TNFα concentration in ileum (13.7±2.47 vs. 6±1.17 pg/mg of proteins, t_{11}=2.653, p=0.0225 Two-tail t-Test) (Figure 2E).

3.3 MS induced fecal microbiota dysbiosis associated with bile acid malabsorption.

We next investigated if the alterations of intestinal barrier functions induced by MS resulted from a change in microbiota composition. A score plot of PLS-DA built from the relative quantitative abundances of the 20 phyla/groups/taxa or species analyzed, showed a clear separation of mouse microbiota according to stress exposure. Based on the variable importance on projection (VIP) of the PLS-DA model, a decrease of *Bifidobacterium* spp., *Eubacterium hallii* and *Roseburia* spp. associated with an increase of *Clostridium butyricum* and *Desulfovibrio* spp. were selected as the 5 most important bacterial contributors that characterize the fecal bacterial profile of MS group (Figure 3A).

We then used ^{1}H-NMR-based metabolomics to further investigate differences in metabolic activity of gut microbiota in feces from MS and control. The fecal metabolome of the mice comprised many metabolites derived from the host-microbiota co-metabolism such as short-chain fatty acids (propionate, succinate, acetate), amino acids (leucine, valine, isoleucine…), keto-acids (5-aminovalerate…) and bile acids among others (Supplementary Figure 1 A and B). MS significantly increased the fecal cholic acid content (t_{14}=4.537, p=0.0005 Two-tail t-Test) and decreased the fecal lactate content (t_{14}=2.649 p=0.0191 Two-tail t-Test) (Figure 3B-C).

3.4 MS increased IgA in feces but decreased ILC3 in lamina propria associated with increased IgG against microbial components

Beside intestinal epithelium and microbiota intestinal barrier is also composed of immune factors. Therefore, we focused on the changes in mucosal and systemic immunity following MS. MS increased IgA in feces (t_{20}=3.686 p=0.0015Two-tail t-Test, Figure 4A). MS decreased ILC3 ($MHCII^+$, $ROR\gamma t^+$) percentage in the *lamina propria* of the small intestine (0.41±0.003% vs. 0.74±0.12%, t_8=3.109, p=0.0145 Two-tail t-Test, Figure 4B). MS did not induce any modifications of total IgG concentration in plasma (Figure 4C). However, decrease of ILC3 was associated with an increase of anti-*E. coli* IgG in plasma (0.046±0.012 vs. 0.035±0.012 OD450nm, t_{20}=3.686, p=0.0015 Two-tail t-Test)Figure 4D) but not IgG directed against hydrosoluble food antigens (Figure 4E).

3.5 MS altered T cell populations and functionality in spleen

MS induced splenomegaly (Supplementary Figure 2). A detailed analysis of splenocyte populations showed that MS increased the percentage of $CD4^+$ $CD44^{high}$ $CD62L^{low}$ activated T lymphocytes (13.8±0.4% vs. 12.4±0.4%, t_{21}=2.542 p=0.019 Two-tail t-Test, Figure 5A) and decreased the percentage of $CD4^+$ $CD25^+$ $Foxp3^+$ regulatory T lymphocytes (1.07±0.08 vs. 1.35±0.06%, t_7=2.7, p=0.0306, Two-tail t-Test, Figure 5B). We next investigated functionality of T lymphocytes by anti-CD3/CD28 stimulation. Without stimulation, MS modified neither IFNγ nor TGFβ secretion by splenocytes (Figures 5C-D). Anti-CD3/CD28 antibody stimulation induced similar levels of TGFβ secretion in both controls and MS mice but significantly higher IFNγ secretion in MS mice ($F_{1,11}$=5.042 p=0.0463 MS versus control Two-way ANOVA followed by Holm-Sidak's post test Figure 5C-D). In summary, MS not only altered splenic T cell populations but also their functionality.

3.6 MS did not precipitate nor exacerbate colitis in IL10$^{-/-}$ C57BL/6 genetically predisposed mice.

As we showed that MS induced intestinal barrier impairment associated with systemic immune response, we then wondered if MS could precipitate or worsen colitis in genetically predisposed mice, housed on SOPF sanitary status. At PND170, IL10$^{-/-}$ mice that were exposed to MS had a non-significant reduction in weight gain compared to control IL10$^{-/-}$ mice (Figure 6A). We analyzed further the consequences of MS on colitis features on IL10$^{-/-}$ mice. We observed no significant decrease of colon length (Figure 6B), no increase of histology damage score (Figure 6C) nor lipocalin (fecal marker for inflammation) concentration in feces (figure 6D) in MS mice compared to control.

3.7 MS did not induce susceptibility to gastrointestinal infections

Finally, we investigated if the consequences of MS on intestinal barrier function and systemic immune response might impair the defense against pathogens known to enter the host via the intestine. Three enteric pathogens were evaluated: *Salmonella* Typhymurium, *Listeria monocytogenes* and *Toxoplasma gondii*. *Samonella* Typhymurium and *Listeria monocytogenes* are bacteria whereas *Toxoplasma gondii* is a parasite. Both *Listeria monocytogenes* and *Toxoplama gondii* are intracellular pathogens whereas *Salmonella* Typhymurium is a facultative intracellular bacterium. Infection was confirmed by measuring bacterial/parasite titers in liver, spleen and MLN. However, MS had no effect on mouse weight nor on bacterial (*S. typhimurium* or *L. monocytogenes*) burden in small intestine, liver or spleen or *T. gondii* parasite load in the ileum or MLN (Figure 7).

4. Discussion

Previous studies in rats demonstrated that MS increased intestinal permeability (Barreau et al., 2004) or visceral sensitivity (Barreau et al., 2004) to colorectal distension in young-adults. Other studies revealed an increased permeability in response to MS in mice (Varghese et al., 2006). However, to our knowledge, this study described for the first time an experimental model of male mice reproducing all main features of IBS: visceral hypersensitivity, intestinal hyperpermeability, microbiota changes, activated immune system and bile acid malabsorption.

A comparative study showed that MS increased visceral sensitivity in both male and female rats with a higher visceral sensitivity in male submitted to MS at PND56 (Yi et al., 2017). In our study, male mice were used to avoid female hormonal cycle interference. Also in rats previous work of our laboratory in rats demonstrated that nerve growth factor induced by MS was responsible for visceral hypersensitivity and intestinal hyperpermeability (Barreau et al., 2004). The same mechanism may apply in mice but this needs to be further investigated. Instead, in this study, we further characterized the consequences of early life stress on male mice on factors contributing to intestinal barrier integrity. MS decreased lysozyme expression in Paneth cells and its antimicrobial activity. This defect of lysozyme is associated with microbiota dysbiosis characterized by a decrease of health associated *Bifidobacteria spp., E. hallii and Roseburia spp.*. Interestingly, *Bifidobacteria* were decreased in IBS patients (Rajilić-Stojanović et al., 2011). In addition, MS induces an increase in fecal cholic acid, thereby mimicking the bile acid malabsorption described in IBS patients (Peleman et al., 2017; Shin et al., 2013). This defect in bile acid absorption could be linked to microbiota dysbiosis as both *Bifidobacterium spp.* and *Eubacterium spp.* are involved in bile acid metabolism (Gérard, 2013). In addition, MS increased TNFα production in the ileum that

might trigger and/or maintain the increase of intestinal permeability (Gitter et al., 2000) and Paneth cell dysfunction (Van Hauwermeiren et al., 2014).

Furthermore, MS induced a decrease of RORγt^{+} MHCII^{+} ILC3 cells in *lamina propria* associated with increase IgG response toward microbiota, *E. coli* lysate being used as representative bacteria of the intestinal microbiota. Those results are in accordance with literature showing that ILC3 contain immune response toward bacteria locally (Hepworth et al., 2013; Sonnenberg et al., 2012).

Interestingly, increase of antibodies toward bacterial antigens was described in IBS plasma samples (Cremon et al., 2008; Schoepfer et al., 2008). Furthermore, stress during childhood was associated with higher antibody titers against cytomegalovirus (Dowd et al., 2012), herpes simplex virus-1 (Shirtcliff et al., 2009) and Epstein-Barr Virus (Slopen et al., 2013). As an increase in intestinal trans-cellular permeability was observed, one can speculate that higher amounts of bacterial antigens may cross the intestinal epithelium of MS mice and affect the immune response. Interestingly, a defective intestinal barrier led to increased humoral response toward colibiota but not food antigens, in line with the recent observation reported by Frehn et al in IBD (Frehn et al., 2014), which excludes a general loss of tolerance toward luminal content as observed in our model.

MS induced splenomegaly was associated with an increase of activated T cells ($CD4^{+}CD44^{high}CD62L^{low}$) and a decrease of regulatory T cells (Treg) ($CD4^{+}CD25^{+}Foxp3^{+}$). *Bifidobacteria* which are decreased in MS mice, are known to markedly induce Foxp3^{+} Treg in mice (O'Mahony et al., 2008). Those changes in T cell populations were associated with functional modifications as reflected by the increase in IFNγ secretion by splenocytes after anti-CD3/CD28 stimulation. This increase in the pro-inflammatory profile of T cells can be a consequence of bacterial translocation occurring during the neonatal period when maternal

separation was applied. In agreement with this, repeated stress during neonatal period in mice is associated with long-term release of IFNγ by splenocytes in later life (Loizzo et al., 2002). In IBS, anti-CD3/CD28 stimulation of PBMC also induced a pro-inflammatory profile as reflected by higher amount of pro-inflammatory cytokine IL1β compared to controls (Ohman et al., 2009). Only one paper studied Treg in IBS but did not observe any modifications (Holmen et al., 2007). Nevertheless, further investigations are needed to better understand a potential role of Treg in IBS physiopathology.

Interestingly, both intestinal hyperpermeability and visceral hypersensitivity induced in adult after MS in early life and observed herein are common features of IBS and IBD pathophysiology (Halpin and Ford, 2012; Piche et al., 2009; Wyatt et al., 1993). Furthermore, an increase of antibodies against bacterial antigens was not only observed in IBS plasma samples (Cremon et al., 2008; Schoepfer et al., 2008) but also in CD patients (Landers et al., 2002; Lodes et al., 2004). Finally, a defect of antimicrobial peptides production by Paneth cells has been described in CD patients (HD5) (Wehkamp, 2005) and is observed in mice submitted to MS. All those similarity between MS mouse model and CD questioned a potential role of MS in triggering or exacerbating colitis in mice genetically predisposed. The $IL10^{-/-}$ mouse is a well-described model of IBD that develops spontaneous Crohn's disease-like intestinal inflammation in the presence of conventional intestinal microbiota (Schultz et al., 2002; Sellon et al., 1998) but develop a mild, patchy colitis with incomplete disease penetrance under SOPF conditions (Berg et al., 1996). Therefore, $IL10^{-/-}$ in SOPF represents an ideal model to study the role of MS on the initiation and exacerbation of colitis. In this model, MS did not precipitate nor exacerbate colitis suggesting that the observed defect of intestinal barrier and immune response is not sufficient to induce a breach in intestinal defense and predispose the mice to colitis. However, in $IL10^{-/-}$ mice under conventional sanitary status, Lennon and al demonstrated that a longer MS protocol (18 days) does not

induce but exacerbates the severity of colitis in 10-12 weeks old mice (Lennon et al., 2013) suggesting that MS alone is not sufficient to increase colitis but is a risk factor factor under conventional sanitary status. Our results are in accordance with epidemiological studies (Bonaz and Bernstein, 2013; Shiga et al., 2013) and experimental studies (Lennon et al., 2013; Schultz et al., 2015) showing that stress does not induce de novo IBD. A susceptibility of IBS patients to develop IBD, especially CD, has been suggested but only in case of Post Infectious (PI)-IBS (Gradel et al., 2009; Porter et al., 2012; Ternhag et al., 2008). In addition to genetic models of IBD, much of the research concerning the role of stress in intestinal inflammation has been conducted in chemically-induced models and shows an aggravation of colitis following stress (Barreau et al., 2004; Maunder, 2000; Qiu et al., 1999). Although the oral or rectal administration of different chemicals reliably induces colitis, the administration itself is a stressor adding to the several shortcomings of these models in the study of stress on intestinal inflammation.

Even though MS could not trigger nor worsen colitis in IL10$^{-/-}$ mice, it did not mean that it had no consequences on host defense against gastrointestinal infection. MS did not increase susceptibility to *L. monocytogenes, S.* Typhimurium or *T. gondii* infections inoculated orally. Previous studies showed that rats submitted to perinatal MS are more susceptible to *N. brasiliensis* infection at adulthood. However, the inoculation route was systemic (subcutaneous) and not intestinal (Barreau et al., 2006). It has been well described that MS in mice induced a more rapid onset and a more severe sickness to influenza virus (Avitsur et al., 2015). However, evidence is lacking on the influence of stress on enteric infections. Our study is of particular interest in the context of epidemiological studies in humans. Indeed, psychosocial stress is associated with increased viral load in HIV and HPV (Ironson et al., 2005; Lu et al., 2016) as well as elevated risk of diagnosis or severity of other infections (upper and lower respiratory tract) (Biondi and Zannino, 1997; Cohen et al., 2012).

Nevertheless, there was either inverse or no association of stress with enteric infections (Melinder et al., 2017).

5. Conclusion

Altogether, our results identify MS in mice as a suitable experimental model for IBS including all the main features i.e. intestinal hyperpermeability, visceral hypersensitivity, microbiota dysbiosis, bile acid malabsorption and low grade inflammation. In addition, even though they have long lasting consequences on intestinal homeostasis early life events do not constitute a sufficient risk factor for colitis in genetically predisposed mice nor to gastrointestinal infections in wild type mice. This observation is in accordance with epidemiological studies showing that only post infectious IBS are at risk to develop IBD and that stress does not induce de novo IBD. Therefore, it appears that early life stress does not induce a detrimental effect on the organism, and preserves its ability to cope with intestinal challenges.

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Author contributions

Conceived and designed the experiments: AR, SM

Performed the experiments: AR, MO, SLL, CL, VB, CH, NVL, MG, CC, MB, CS, VM, MZ, HR, SES, SM

Analyzed the data. Contributed materials/analysis tools: AR, MO, SLL, SES, SM

Wrote the paper: MO, SLL, FL, VT, SM

Conflict of interest

The authors declare no conflict of interest.

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LEGENDS

Figure 1: Effects of Maternal Separation (MS) in mice on Intestinal Permeability to various markers and visceral sensitivity in young-adult male mice. (A) Representative box plots of Dextran-FITC 4kDa (FD4) concentration measured in plasma 4 hours after gavage, in MS group (grey histogram) and control group (white histogram) (n = 6 mice per group) *p<0.05 compared to control mice (Mann-Whitney) (B) jejunal permeability of Horse Radish Peroxidase (HRP) or Fluorescein Sodium Salt (FSS) measured after 2H bathing in Ussing chambers in MS group (grey) and control group (white) (n = 10 to 14 mice per group) *p<0.05 compared to control mice (Mann-Whitney). **(C)** Representative curves of abdominal contractions in response to colorectal distension with increasing volumes (0.02 to 0.1ml) in MS (grey scares plain line) compared to control (white circles dashed line) (n = 17 to 19 mice per group).* p<0,05 compared to identical volumes for controls (Two-way ANOVA followed by Holm-Sidak's post-test).

Figure 2: Lysozyme expression and activity. (A) Immunostaining of ileum paraffin section with anti-lysozyme-FITC, Wheat germ agglutinin (WGA)-Texas red and DAPI. **(B)** Lysozyme fluorescence intensity per crypt cell in MS (grey) compared to control (white) (n = 4 mice per group). * p<0,05 compared to controls (Mann-Whitney test) **(C)** Number of crypts producing lysozyme on small intestine in MS (grey) compared to control (white) (n = 4 to 5 mice per group). **(D)** Lysozyme enzymatic activity against peptidoglycan measured in feces of MS (grey) compared to control (white) (n= 12 to 13 mice per group). ** p<0,01 compared to controls (Mann-Whitney test). **(E)** TNFα concentration in the ileum fragment measured by ELISA in MS (grey) compared to control (white) (n = 6 to 7 mice per group). * p<0,05 compared to controls (Mann-Whitney test).

Figure 3: Fecal microbiota population and metabolites analysis on maternally separated (MS) adult mice. (A) PLS-DA score plot of the relative quantitative abundances (Log10 No) of 20 microbial taxa in feces of MS and control mice (n= 10 mice per group, confidence ellipse level =0.5). VIP plot representing important features (microbial taxa) identified by PLS-DA in a descending order of importance (Relative enriched taxa appeared in red and depletion in green) in MS and control mice (n = 10 mice per group). **(B)** Coefficient plots related to the O-PLS-DA model discriminating between ^{1}H-NMR-based spectra from fecal extracts of control (top) and MS (bottom) animals. Metabolites are color-coded according to their correlation coefficient, red indicating a very strong positive correlation. The direction of the metabolite indicates the group with which it is positively associated as labeled on the diagram. Parameters of the PLS-DA model with one predictive component and no orthogonal component: R^2Y=0.38, Q^2Y=0.31, p=0.02 **(C)** The areas under the curve of the ^{1}H-NMR spectra were integrated for the signals of cholic acid (singulet at 0.73ppm) and lactate (doublet at 1.33ppm) in MS (grey) compared to control (white) (n = 8 mice per group). * p<0,05, *** p<0.001 compared to controls mice (white) (Mann-Whitney test).

Figure 4: IgA in feces, ILC3 populations in small intestine and IgG response in plasma. (A) IgA concentration in feces of MS (grey) compared to control (white) (n=8 per group) * p<0,05 compared to controls mice (Mann-Whitney test)**. (B)** Flow cytometry analysis of ILC3 population by immunostaining with anti-RORγt and anti-MHC II on *lamina propria* cells from small intestine (n = 4 to 6 mice per group). * p<0.05 compared to control (Mann-Whitney test) **(C)** Plasma IgG concentrations measured by ELISA (n = 13 to 17 mice per group) in MS (grey) compared to control (white)**. (D)** IgG specificity against *E. coli* lysate assessed by ELISA after normalizing IgG concentrations in MS (grey) compared to control (white) (n = 10 to 12 mice per group) ** p<0,01 compared to controls (Mann-Whitney test)**.**

(E) IgG specificity against food lysate (hydrosoluble fraction) assessed by ELISA after normalizing IgG concentration in MS (grey) compared to control (white) (n =15 to 17 mice per group).

Figure 5 – Immune response in spleen. (A) Flow cytometry analysis of activated T cells $CD4^+$ $CD44^{high}$ $CD62L^{low}$ in splenocytes (n = 9 to 14 mice per group). * p<0,05 compared to controls mice (Mann-Whitney test) **(B)** Flow cytometry analysis of regulatory T cells $CD4^+$ $CD25^+$ $Foxp3^+$ in splenocytes (n = 4 to 5 mice per group). * p<0,05 compared to controls mice (Mann-Whitney test) **(C)** IFNγ, and **(D)** TGFβ secretion by splenocytes in basal condition, or after anti-CD3/anti-CD28 stimulation in MS group (grey histogram) and control group (white histogram) (n = 5 to 10 mice per group). ** p< 0.01 compared to control (Two-way ANOVA followed by Holm-Sidak's post test).

Figure 6 – MS and susceptibility to colitis in C57BL/6 $IL10^{-/-}$ mice. (A) Weight, **(B)** colon length, **(D)** histological score in colon and **(D)** lipocalin 2 concentrations in feces of MS mice (grey) and control (white) (n = 7 to 17 mice per group).

Figure 7 – MS and susceptibility to gastrointestinal infections. Weight and tissues colonization after oral inoculation by **(A)** *Salmonella typhimurium* (10^9 CFU/mouse), **(B)** *Listeria monocytogenes* Lmo-$InlA^m$ (10^9 CFU/ml) or **(C)** de *Toxoplasma gondii* 76K (35 cysts) in MS mice (grey) and control (white) (n = 6 to 12 mice per group).

Supplementary Figure 1 – (A) Typical 600 MHz ^1H NMR spectra of the fecal extracts from a control animal. **(B)** A spike-in experiment was performed in a representative fecal extract sample using a standard solution of cholic acid and confirmed an increase of the singulet resonating at 0.735 ppm in the spiked sample.

Supplementary Figure 2 – Spleen weight. Spleen weight in control (white) and MS (grey) mice. * p< 0.05 compared to control (Mann-Whitney test).

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Highlights:

- Early life stress in mice is a unique model gathering IBS features i.e. visceral hypersensitivity, intestinal hyper-permeability, low grade inflammation, microbiota dysbiosis and bile acid malabsorption.
- Early life stress do not precipitate nor exacerbate colitis in mouse models genetically predisposed to IBD
- Early life stress do not increase susceptibility to gastrointestinal pathogen infections.
- Early life stress induces long lasting consequences on intestinal barrier that do not impair the ability of intestinal barrier to prevent enteric infections or contain colitis. This result is consistent with epidemiological studies.

Figure 1

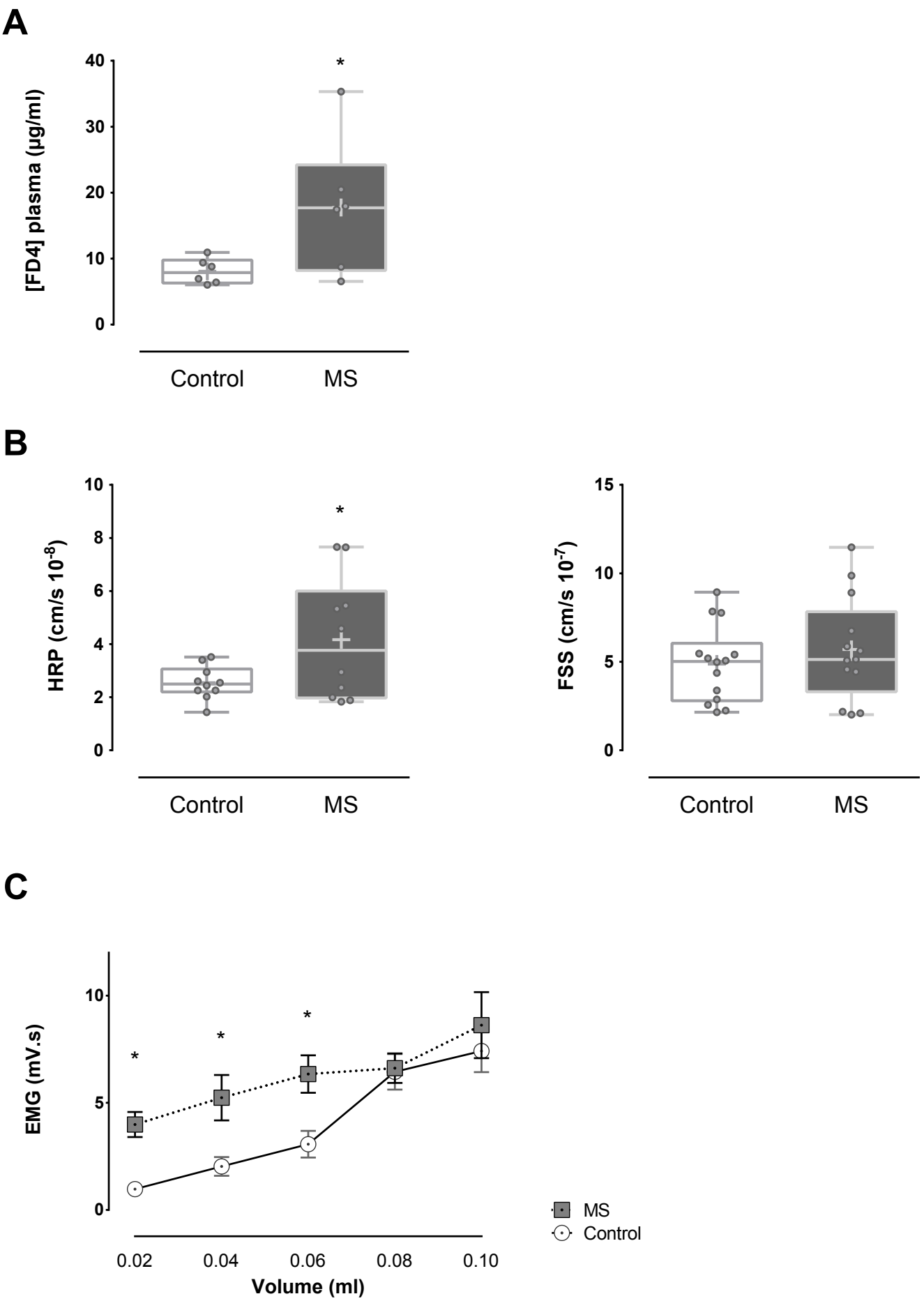

A
[FD4] plasma (µg/ml)
40
30
20
10
0
*
Control
MS
B
HRP (cm/s 10-8)
10
8
6
4
2
0
*
Control
MS
FSS (cm/s 10-7)
15
10
5
0
Control
MS
C
EMG (mV.s)
10
5
0
*
*
*
0.02
0.04
0.06
0.08
0.10
Volume (ml)
MS
Control

Figure 2

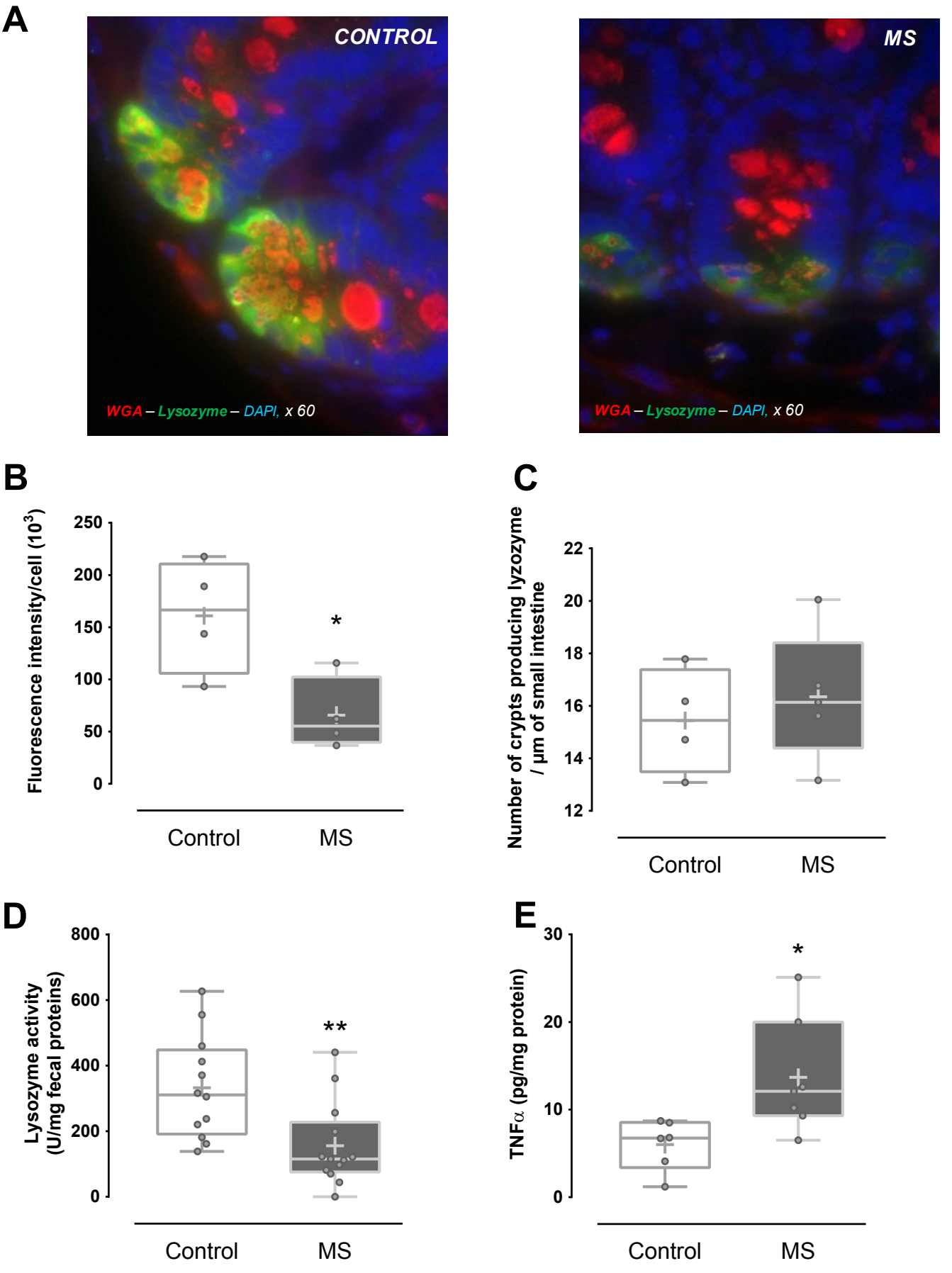

A
CONTROL
WGA – Lysozyme – DAPI, x 60
MS
WGA – Lysozyme – DAPI, x 60
B
Fluorescence intensity/cell (10³)
250
200
150
100
50
0
*
Control
MS
C
Number of crypts producing lyzozyme / µm of small intestine
22
20
18
16
14
12
Control
MS
D
Lysozyme activity (U/mg fecal proteins)
800
600
400
200
0
**
Control
MS
E
TNFα (pg/mg protein)
30
20
10
0
*
Control
MS

Figure 3

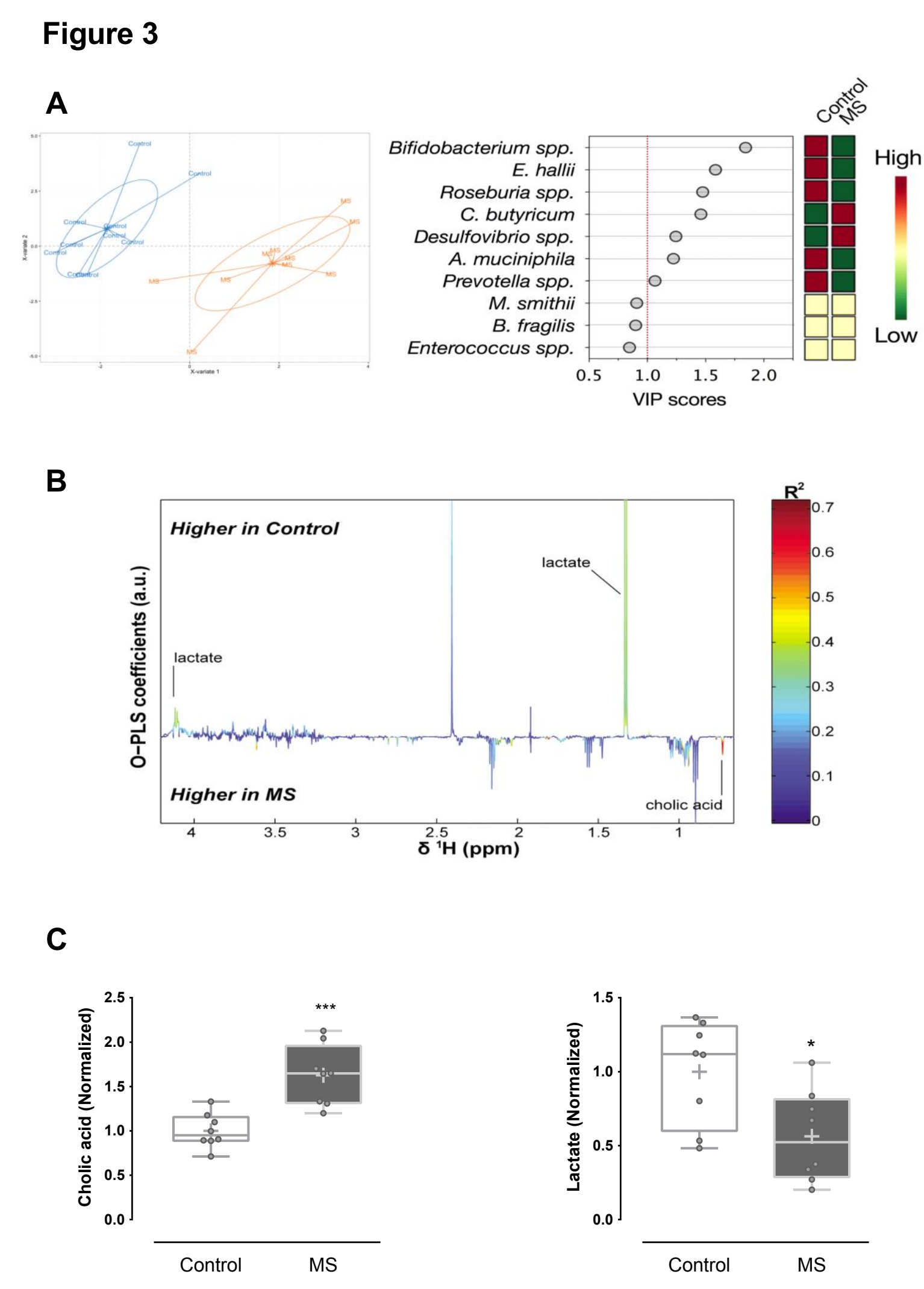

A
Control
MS
Bifidobacterium spp.
E. hallii
Roseburia spp.
C. butyricum
Desulfovibrio spp.
A. muciniphila
Prevotella spp.
M. smithii
B. fragilis
Enterococcus spp.
0.5
1.0
1.5
2.0
VIP scores
High
Low
B
Higher in Control
Higher in MS
lactate
lactate
cholic acid
O-PLS coefficients (a.u.)
δ ¹H (ppm)
R²
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
C
Cholic acid (Normalized)
***
Control
MS
Lactate (Normalized)
*
Control
MS

Figure 4

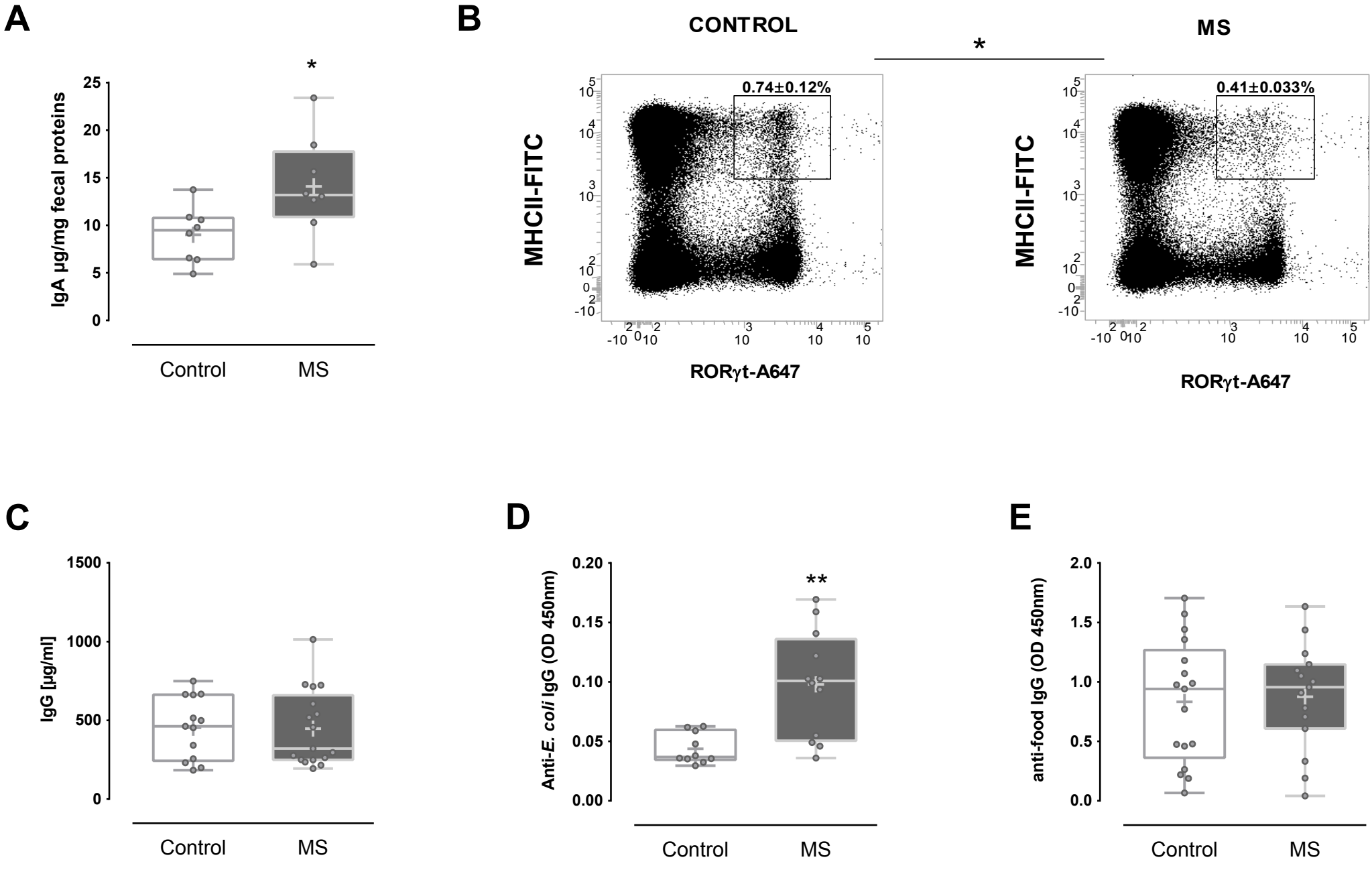

A
IgA µg/mg fecal proteins
25
20
15
10
5
0
*
Control
MS
B
CONTROL
MS
*
MHCII-FITC
0.74±0.12%
0.41±0.033%
RORγt-A647
C
IgG [µg/ml]
1500
1000
500
0
Control
MS
D
Anti-E. coli IgG (OD 450nm)
0.20
0.15
0.10
0.05
0.00
**
Control
MS
E
anti-food IgG (OD 450nm)
2.0
1.5
1.0
0.5
0.0
Control
MS

Figure 5

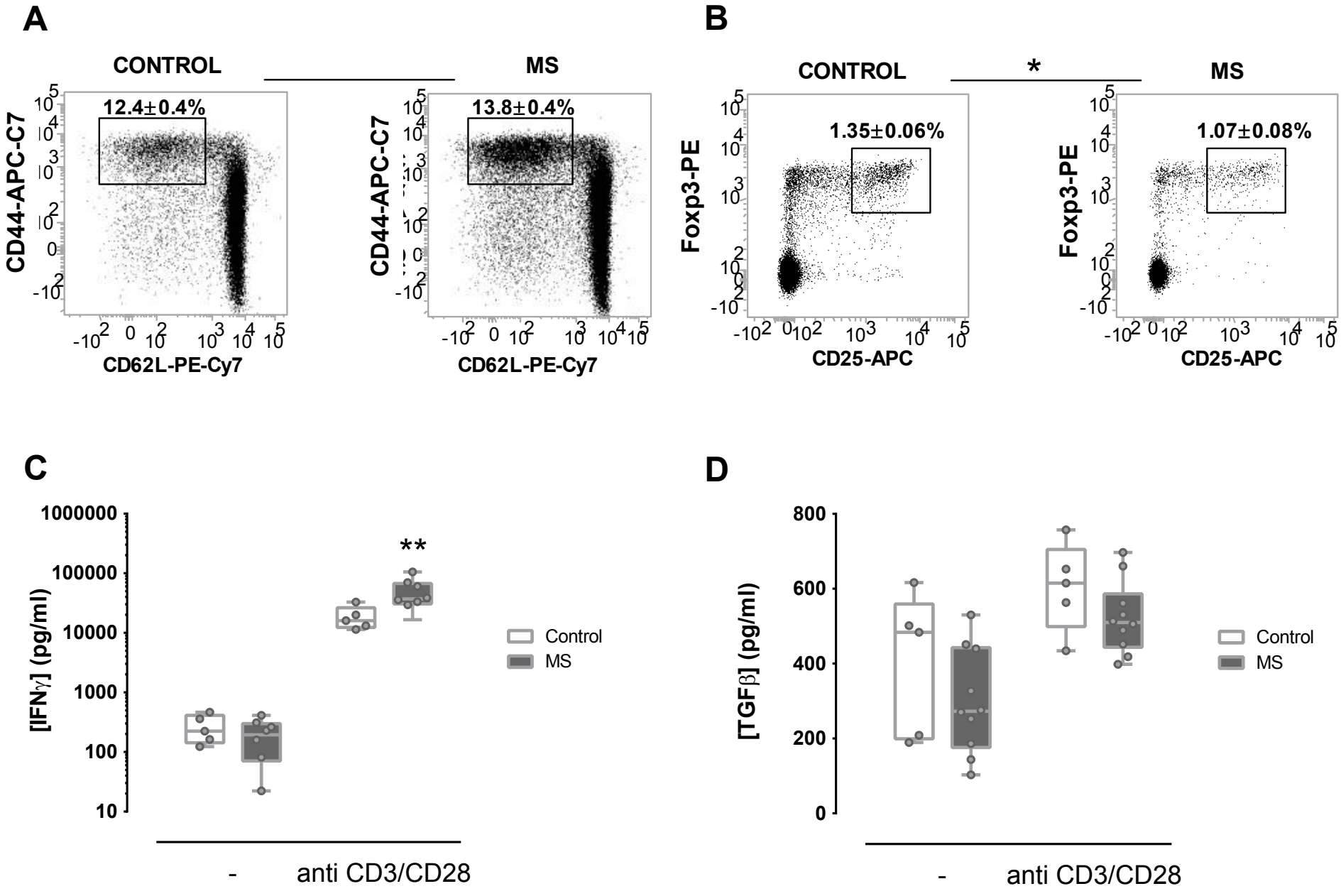

A
CONTROL
MS
12.4±0.4%
13.8±0.4%
CD44-APC-C7
CD62L-PE-Cy7
B
CONTROL
*
MS
1.35±0.06%
1.07±0.08%
Foxp3-PE
CD25-APC
C
[IFNγ] (pg/ml)
1000000
100000
10000
1000
100
10
**
Control
MS
-
anti CD3/CD28
D
[TGFβ] (pg/ml)
800
600
400
200
0
Control
MS
-
anti CD3/CD28

Figure 6

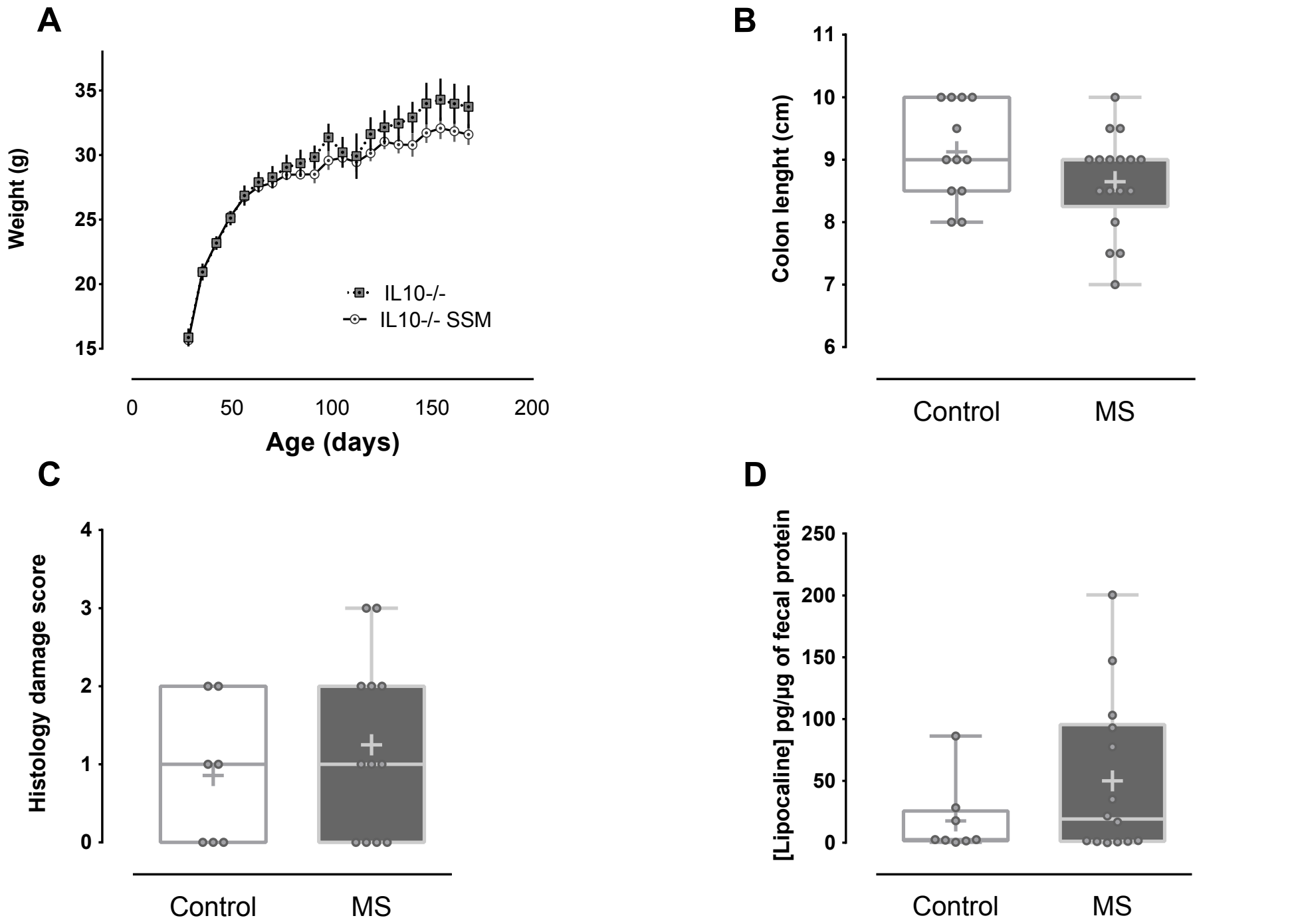
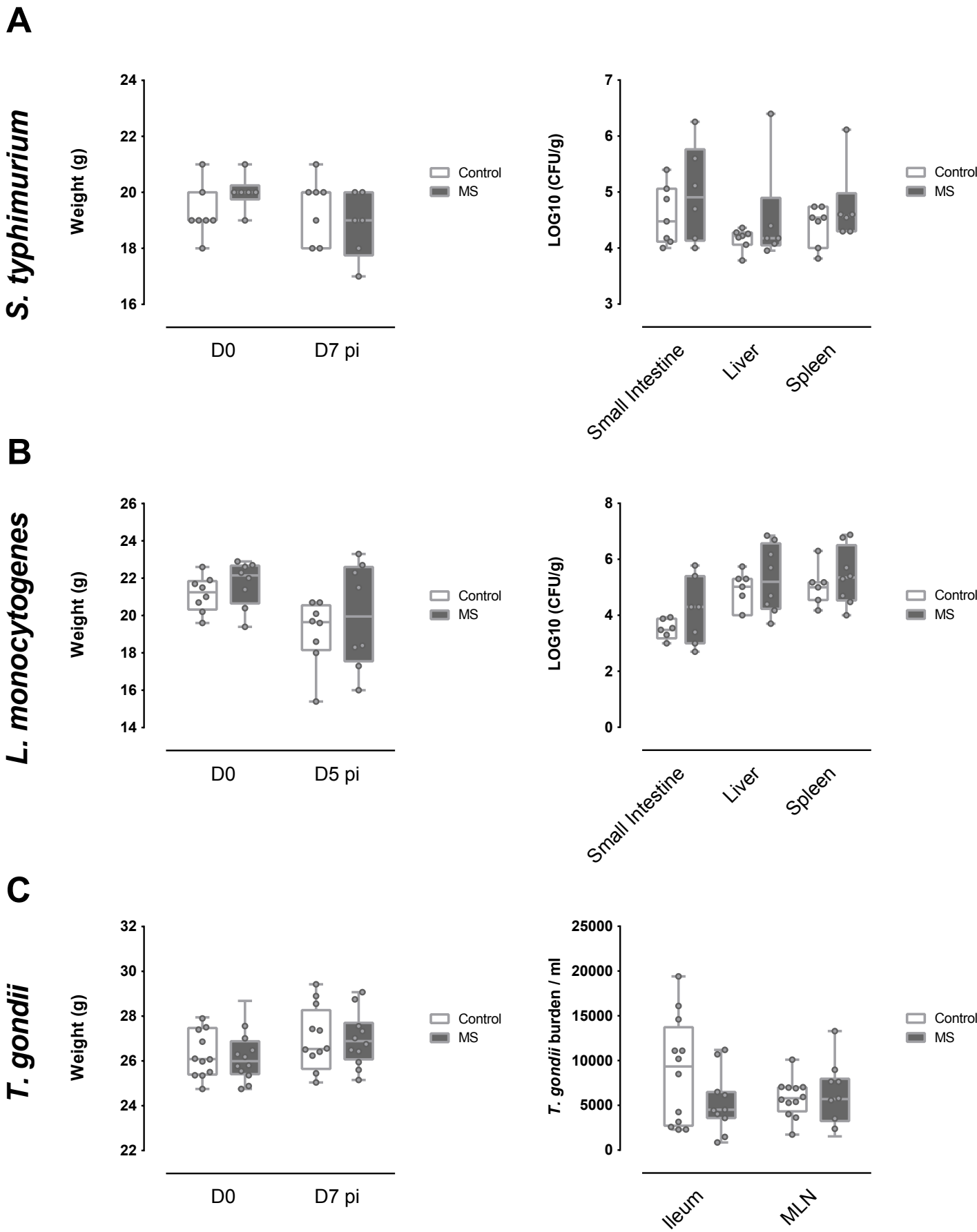

A
Weight (g)
35
30
25
20
15
IL10-/-
IL10-/- SSM
0
50
100
150
200
Age (days)
B
Colon lenght (cm)
11
10
9
8
7
6
Control
MS
C
Histology damage score
4
3
2
1
0
Control
MS
D
[Lipocaline] pg/µg of fecal protein
250
200
150
100
50
0
Control
MS

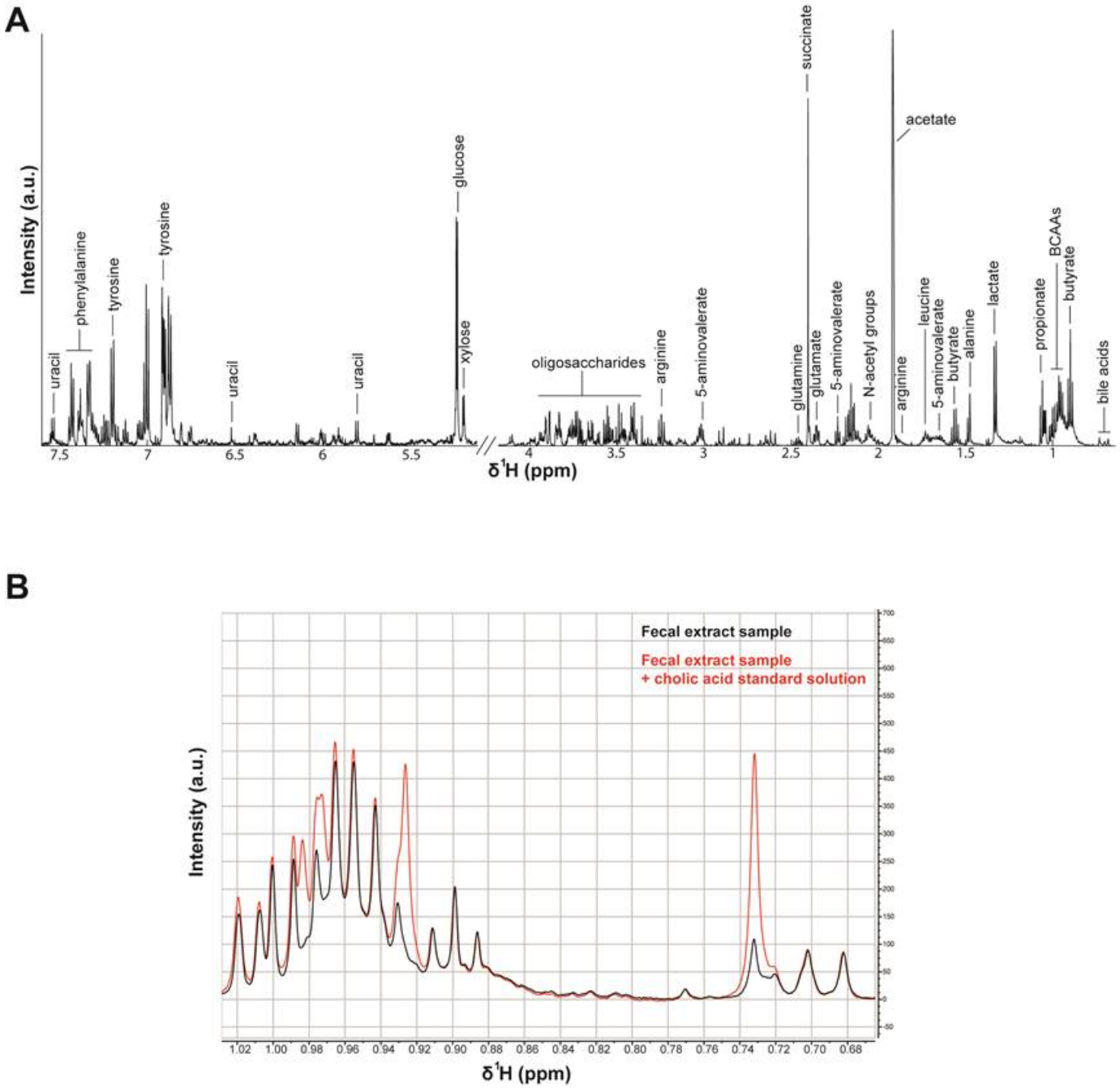
Figure 7


A
S. typhimurium
Weight (g)
24
22
20
18
16
D0
D7 pi
Control
MS
LOG10 (CFU/g)
7
6
5
4
3
Small Intestine
Liver
Spleen
Control
MS
B
L. monocytogenes
Weight (g)
26
24
22
20
18
16
14
D0
D5 pi
Control
MS
LOG10 (CFU/g)
8
6
4
2
0
Small Intestine
Liver
Spleen
Control
MS
C
T. gondii
Weight (g)
32
30
28
26
24
22
D0
D7 pi
Control
MS
T. gondii burden / ml
25000
20000
15000
10000
5000
0
Ileum
MLN
Control
MS

Supplementary Table. Primers used in this study

Phylum	Class/Family/ Genus	Species /Group	Primers sequences (5′-3′)
All phyla	All	All	ACTCCTACGGGAGGCAGCAGT GTATTACCGCGGCTGCTGGCAC
Actinobacteria	*Bifidobacterium*	spp.	CGCGTCYGGTGTGAAAG CCCCACATCCAGCATCCA
Bacteroidetes	All	All	GGARCATGTGGTTTAATTCGATGAT AGCTGACGACAACCATGCAG
Bacteroidetes	*Bacteroides /Prevotella*	spp.	GAGAGGAAGGTCCCCCAC CGCTACTTGGCTGGTTCAG
Bacteroidetes	*Bacteroides*	spp.	CGATGGATAGGGGTTCTGAGAGGA GCTGGCACGGAGTTAGCCGA
Bacteroidetes	*Bacteroides*	*fragilis group*	CTGAACCAGCCAAGTAGCG CCGCAAACTTTCACAACTGACTTA
Bacteroidetes	*Bacteroides*	*thetaiotaomicron*	GGCAGCATTTCAGTTTGCTTG GGTACATACAAAATTCCACACGT
Bacteroidetes	*Prevotella*	spp.	CACCAAGGCGACGATCA GGATAACGCCYGGACCT
Firmicutes	All	All	TGAAACTYAAAGGAATTGACG ACCATGCACCACCTGTC
Firmicutes	*Lactobacillus*	spp.	AGCAGTAGGGAATCTTCCA CACCGCTACACATGGAG
Firmicutes	*Clostridium*	*butyricum* (Cluster I)	GTGCCGCCGCTAACGCATTAAGTAT ACCATGCACCACCTGTCTTCCTGCC
Firmicutes	*Clostridia*	Cluster IV (*C. leptum* group)	GCACAAGCAGTGGAGT CTTCCTCCGTTTTGTCAA
Firmicutes	*Clostridia*	Cluster XIVa (*C. coccoides–E. rectale* group)	ACTCCTACGGGAGGCAGC GCTTCTTAGTCAGGTACCGTCAT
Firmicutes	*Clostridia/Lachnospiraceae*	*Ruminococcus gnavus* (*Blautia*)	GGACTGCATTTGGAACTGTCAG AACGTCAGTCATCGTCCAGAAAG
Firmicutes	*Eubacterium*	*hallii*	GCGTAGGTGGCAGTGCAA GCACCGRAGCCTATACGG
Firmicutes	*Roseburia*	spp.	TACTGCATTGGAAACTGTCG CGGCACCGAAGAGCAAT
Firmicutes	*Enterococcus*	spp.	CCCTTATTGTTAGTTGCCATCATT ACTCGTTGTACTTCCCATTGT
Proteobacteria	*Escherichia*	*coli*	CATGCCGCGTGTATGAAGAA CGGGTAACGTCAATGAGCAAA
Proteobacteria	*Desulfovibrio*	spp.	CCGTAGATATCTGGAGGAACATCAG ACATCTAGCATCCATCGTTTACAGC
Verrucomicrobia	*Akkermansia*	*muciniphila*	CAGCACGTGAAGGTGGGGAC CCTTGCGGTTGGCTTCAGAT
Euryarchaeota	*Methanobrevibacter*	*smithii*	CCGGGTATCTAATCCGGTTC CTCCCAGGGTAGAGGTGAAA

Supplementary Figure 1



Supplementary Figure 2