Effects of gut microbiota perturbation on Th1- and Th2-biased mice following treatment with Vancomycin

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Abstract

Use and abuse of antibiotics not only cause microbial resistance but also influence abundance and diversity of microbiota. Gut, in mammals, harbour a large number of diverse microbiota. Gut microbiota are a) the largest reservoir of beneficial microbes in mammals and b) play a major role in maintaining health and homeostasis. To understand how do gut microbiota maintain health, one major tool is to perturb the microbial population. Antibiotics are important and potent perturbing agents for microbiota to study the role of gut microbes in maintaining various host physiology. We selected vancomycin, used mainly for the treatment of intestinal infection due to *Clostridium difficile*, for further studies to establish the effects on host immunity and metabolism in Th2-(BALB/c) and Th1- (C57BL/6) biased mice models. We found that during early days (until day 4) following treatment with vancomycin the abundance of phyla Firmicutes and Bacteroides are reduced in contrast to significant increase in the phylum Proteobacteria in the gut. These changes in microbial profile could be correlated with the observed increase in a) inflammation, b) permeability of gut, and c) cecal indices opposed to a decrease in the rate of a) glucose tolerance, and b) change in Short Chain Fatty Acid (SCFA) metabolic profile of the host. In addition, we observed significant increase in the phylum Verrucomicrobia for the genus Akkermansia following treatment with vancomycin for 5 days and more. Increased Akkermansia may be suggestive of the restoration of gut environment as is observed with a) decreased inflammation and b) increased rate of glucose tolerance. The effects of Akkermansia was more pronounced in C57BL/6 than BALB/c.

Introduction:

While progressive use of antibiotics is raising cautions and warnings, the role of commensal microbiota, in maintaining health and perhaps establishing itself as a potential solution in combating antibiotic resistance, is becoming more confluent with newer reports and insights (Andoh, 2016; Jandhyala et al., 2015; Young, 2012). The mammalian gastrointestinal tract is inhabited by a hundred trillions of highly diverse and complex microbial cells (Guinane and Cotter, 2013). A large number of these microbes have co-evolved with the host over thousands of years (Thursby and Juge, 2017). In fact, these microbes have at least hundred times more genomic content than the human genome (O'Hara and Shanahan, 2006; Shanahan, 2002) . The abundance and diversity of gut microbiota play significant roles in maintaining various host physiology – for example, harvesting energy (Dalby et al., 2017), maintaining immune homeostasis (Wu and Wu, 2012), regulating metabolism (Cani and Delzenne, 2009), and protecting host against pathogen (Ubeda et al., 2017). While microbiota profile may be diverse and abundant, reports suggested that only a handful of major phyla of gut microbes that include Firmicutes, Bacteroidetes, and Proteobacteria are accounting for at least 90– 95% of the gut microbiome. Among which, the anaerobic genera that constitute the majority of gut microbe include Bacteroides, Eubacterium, Clostridium, Ruminococcus, and Faecalibacterium (Palmer et al., 2007).

More reports have started revealing that perturbation of gut microbiota can be used as an effective tool to understand its role in the host. Abundance and diversity of gut microbiota changes with different factors like age (Hill et al., 2017), diet(Singh et al., 2017), geography (Falony et al., 2016; Morton et al., 2015), stress (Bendtsen et al., 2012), pathogen (Khan, 2014), and antibiotics (Lange et al., 2016). Since, treatment with antibiotics is still a most important and major avenue of dealing with many diseases, it is reported that antibiotics could perturb the general taxonomy of the abundant and diverse gut microbes. Antibiotics are, therefore, being

used as one of the most potent agents to study the role of gut microbiota (Jernberg et al., 2010). Because of frequent and long term usage, various commonly used antibiotics can change the composition, diversity, and richness of commensal gut microbiota to result in alteration of different metabolism of the host (Dethlefsen et al., 2008; Fujisaka et al., 2016) and may lead to different metabolic syndromes like diabetes, obesity, and Inflammatory Bowel Disease (Bosi et al., 2006; Dunlop et al., 2006). Intensity of the perturbation of commensal microbes, by antibiotics, depends on different factors like a) the mode of action of a particular antibiotic and b) the presence of various resistant group of microbes in gut against the antibiotic (Jernberg et al., 2010).

Among various antibiotics, vancomycin is majorly prescribed orally against the infection of two multi-drug resistant strains; i.e., *Clostridium difficile* and *Staphylococcus* (Bernard et al., 2003; Pepin, 2008; Tang et al., 2015). Besides the effectiveness of vancomycin in Clostridium Difficile Infection (CDI) patients, it can cause drastic changes in the human gut microbiota by increasing pathogens and decreasing the commensal healthy microbes (Isaac et al., 2016). It has also been reported that following treatment with vancomycin in humans for 7 days, genes for oxidative pathways were upregulated and the genes for immune pathways were downregulated (Reijnders et al., 2016).

However, the dose and time dependent effects of vancomycin on gut microbiota are still unknown. The present study focuses on the effect of a clinical dose of vancomycin on mouse gut microbiota and its effect on different physiology of mice. The current study reveals that the changes, that happen in gut microbiota following treatment of mice with vancomycin, are not as evident as reported before. Although initially, vancomycin treatment causes an increase in abundance of specific pathogens and a decrease in native microbes, but at the later stage of the treatment, some healthy microbes start appearing and pathogens started declining, that could provide health benefits to the host.

The current laboratory reported earlier the effects of probiotics on the gut microbiota of Th1 and Th2 biased mice (Pradhan et al., 2018). There are reports that elucidated the intrinsic differences in immune and immune-metabolic responses between C57BL/6 (Th1) and BALB/c (Th2) mice (Jovicic et al., 2015; Watanabe et al., 2004), we reported the differences in gut microbiota from phylum to genus between the two strains (Th1- and Th2-biased mice) (Pradhan et al., 2018). In the current study, we reported the role of vancomycin on gut microbiota in Th1 and Th2 mice. The microbiota present in the gastrointestinal tract is important for the development of a mature immune system of the host (Hooper et al., 2012). It has been reported that the gut microbiota influences the immune system of the host through interaction of their molecular patterns (lipopolysaccharide, peptidoglycan) with toll-like receptors (TLR4, TLR2) present in the epithelial cell of the gut. This interaction produces various cytokines and chemokines for immune regulation (Christiansen et al., 2018). In this study, following vancomycin treatment, we observed and correlated the kinetics of immunological changes of host at both transcriptional and protein level with metagenomic changes in the gut of C57BL/6 and BALB/c mice.

Microbiota present in the gut controls the metabolism of major nutrients like carbohydrates, amino acids, fatty acids, and vitamins. Moreover, it was already reported that some microbes could convert complex carbohydrates into short chain fatty acids (SCFAs) like acetate, propionate, and butyrate, and these SCFAs play important roles in regulating inflammatory response and metabolism of the host by binding to free fatty acid receptors (e.g. FFAR2/3) present in intestinal epithelial cells, immune cells, adipocytes (Morrison and Preston, 2016).

Intestinal epithelium acts as a barrier to the entry of different pathogens, inflammatory molecules and toxins into the mucosal tissue (Turner, 2009). The commensal microbes present on intestinal epithelial cell maintain the integrity of the gut permeability by controlling the expression of various tight junction proteins such as by occludin, claudin, and mucin (Ulluwishewa et al., 2011). The defects in barrier due to perturbation of gut microbiota leads to different metabolic diseases like Inflammatory Bowel Disease, obesity, and diabetes (Bosi et al., 2006; Dunlop et al., 2006). In the current study, we reported that the variation in the abundance and diversity of different groups of microbes, in Th1 and Th2 mice, could differentially regulate the gut barrier integrity, host inflammation and glucose tolerance.

Material and methods:

Animal Used in study: All mice of same strain used in the present study were co-housed in polysulfone cage, and corncob was used as bedding material. Food and water were provided ad libidum. Animals were co-housed in a pathogen-free environment with 12h light-dark cycle (lights on from 7:00 am – 7:00 pm), at temperature 24 ± 3° with the humidity around 55%. Guidelines for animal usage was as per CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals, Govt. of India), and all protocols were approved by the Institute Animal Ethics Committee constituted by CPCSEA. Two mice strains C57BL/6 (Th1) and BALB/c (Th2) of 6-8 weeks of age were used in the present study. Unless otherwise stated, we used 3 mice per treatment condition per time point.

Antibiotic treatment: Both Th1-(C57BL/6) and Th2- (BALB/c) biased mice were treated (n=3 per group) twice daily for 6 consecutive days by oral gavaging with vancomycin (Cat#11465492) (at 50 mg per kg of body weight) (Erikstrup et al., 2015). Vancomycin was of molecular biology grade and was purchased from MP Biomedical, France. All concentrations used were as per FDA approved guidance. For vancomycin, the antibiotic that is majorly used for the current study was 125 mg for 4 times daily for adult and 40 mg/kg of body weight for 3-4 times daily for pediatric use (Patel et al., 2019).

Mice treatment and sample collection: Following screening of 4 antibiotics by plating the fecal samples for culturable microbes, we decided to continue the study with vancomycin. Mice were separated in two different groups: untreated (control) and treated with antibiotic. Treated group of mice were orally gavaged with vancomycin twice daily for 6 days. Each day both time-matched control (n=3) and treated (n=3) mice were euthanized by cervical dislocation as per protocols approved by the Institutional Animal Ethics Committee. Duodenum, jejunum, ileum, colon, spleen, liver, serum and fecal samples were collected from each mice and used as described in following sections. Serum and fecal samples were processed and analyzed right away. Tissue samples, not used immediately, were stored either by snap freezing for protein analysis or in RNAlater for RNA analysis until further used.

RNA extraction: RNA was extracted from gut wall tissue by using RNeasy mini kit (Cat# 74104, Qiagen, India) following manufacturer's protocol. 20-23 mg of tissue were processed using liquid nitrogen followed homogenization in 700 µl of RLT buffer. An equal volume of 70% ethanol was added and mixed well. The solution was centrifuged at 13,000 rpm for 5 minutes at room temperature. The clear solution containing lysate was passed through RNeasy mini column (Qiagen, Germany), which leads to binding of RNA to the column. The column was washed using 700 µl RW1 buffer and next with 500 µl of RPE buffer. RNA was eluted using 30 µl of nuclease-free water. RNA was quantified using NanoDrop 2000 (ThermoFisher Scientific, Columbus, OH, USA).

cDNA preparation: cDNA synthesized by using AffinityScript One-Step RT-PCR Kit (Cat# 600559, Agilent, Santa Clara, CA, USA). RNA was mixed with random nonamer primer, Taq polymerase, and NT buffer, the mixture was kept at 45 °C for 30 min for the synthesis of cDNA and temperature increased to 92 °C for deactivating the enzyme.

Real time PCR (qRT-PCR): Real time PCR performed in 96 well plate, using 25 ng cDNA as template, 1 μM of each of forward (_F) and reverse (_R) primers for genes mentioned in Table 2, SYBR green master mix (Cat#A6002, Promega, Madison, WI, USA), and nuclease free water. qRT-PCR was performed in Quantstudio 7 (Thermo Fisher Scientific, Columbus, OH, USA. All values were normalized with cycle threshold (Ct) value of GAPDH (internal control) and fold change of the desired gene was calculated with respect to the control C_t-value.

Cytokine Analysis at protein level

Colon tissues were collected on day 3 and day 6 following treatment with vancomycin and on day 0 untreated (control) mice. After washing the colon tissues thoroughly, lysis buffer (Tris-hydrochloric acid, sodium chloride, and Triton X-100 in distilled water) containing 1X protease inhibitor cocktail (PIC) (Cat#ML051, Himedia, India) was used to churn the tissue (Kim et al., 2012). The supernatant was collected following centrifuging the churned mixture at 20,000g for 20 minutes. ELISA (BD Biosciences, San Diego, CA, USA) was performed using manufacturer's protocol for TNFα (Cat#560478) and IL10 (Cat#555252) expression (Kim et al., 2012). Protein concentration was normalized through Bradford assay (BD Biosciences, San Diego, CA, USA). Absorbance was taken through Multiskan Go (Thermo Fisher Scientific, Columbus, OH, USA).

Genomic DNA extraction: Cecal sample was collected from mice of both strains and gDNA was extracted using phenol-chloroform method. 150-200 mg of cecal sample was used to homogenize using 1ml of 1X PBS and centrifuged at 6700g for 10 minutes. The precipitate was lysed by homogenizing it in 1ml of lysis buffer (containing Tris-HCl 0.1M, EDTA 20 mM, NaCl 100 mM, 4% SDS (at pH 8) and thereafter heating it at 80 °C for 45min. Lipid and protein were removed from the supernatant using an equal volume of phenol-chloroform, this process was repeated until the aqueous phase becomes colorless. DNA was precipitated overnight at -20 °C with 3 volumes of absolute chilled ethanol. Finally, it was washed with 500 μl of 70% chilled ethanol and briefly air dried. The gDNA was dissolved in nuclease-free water and quantified using NanoDrop 2000.

Serum collection: Mice were anesthetized and blood was collected by cardiac puncture. Blood was kept on ice for 30 mins and centrifuged at 1700 rpm for 15 min at 4°C, and serum was collected for further analysis. If required, serum was stored at -80°C till further used.

16S-rRNA sequencing (V3-V4 Metagenomics):

Using cecal DNA samples, V3-V4 regions of 16S rRNA gene was amplified. For this amplification, V3F (Forward primer): 5'-CCTACGGGNBGCASCAG-3' and V4R (Reverse primer): 5'-GACTACNVGGGTATCTAATCC-3' primer pair was used. In Illumina Miseq platform, amplicons are sequenced using paired end (250bpX2) with sequencing depth of 500823.1 ± 117098 reads (mean ± SD). Base composition, quality and GC content of fastq sequence were checked. More than 90% of the sequences had Phred quality score above 30 and GC content nearly 40-60%. Conserved regions from the paired end reads were removed. Using FLASH program, a consensus V3-V4 region sequence was constructed by removing unwanted sequences (Mysara et al., 2017; Purcell et al., 2017). Pre-processed reads from all the samples were pooled and clustered into Operational Taxonomic Units (OTUs) by using de novo clustering method based on their sequence similarity using UCLUST program. QIIME was used

for the OTU generation and taxonomic mapping (Caporaso et al., 2010; Kastenberger et al., 2012). Representative sequence was identified for each OTU and aligned against Greengenes core set of sequences using PyNAST program (DeSantis et al., 2006b, 2006a; Frank et al., 2007; Zhou and Zhi, 2016). Alignment of these representative sequences against reference chimeric data sets was done and RDP classifier against SILVA database was used for taxonomic classification to get rid of hybrid sequences.

CMT (cecal microbiota transplantation): Cecal sample was collected from sixth day vancomycin treated mice and diluted with PBS (1gm/10ml) to make stock. 400 µl of the stock of cecal material was orally gavaged to each of third day vancomycin treated mice.

Glucose tolerance test (GTT): GTT was assayed on days 0, 3 and 6 following treatment of both types of mice with vancomycin. Following 6h of starvation of mice from each treatment group, fasting blood glucose level (considered as control glucose level at 0 minute) was measured by a glucometer by tail vein bleeding. Mice, fasted for 6h, were orally gavaged with glucose at a dose of 1 mg/g body weight of mouse. Blood glucose levels were measured at intervals of 15-, 30-, 60- and 90-minutes post-glucose gavaging by using blood glucose monitoring system (ACCU-CHEK Active, Roche Diabetes Care GmbH, Mannheim, Germany).

Same procedure was adopted for oral glucose tolerance test was done for third day CMT recipient mice following 24h of CMT procedure.

Sample preparation and NMR data acquisition for metabolomics study

Serum was isolated from blood of vancomycin treated and control mice as described before. Proteins in the serum were removed by passing it through pre-rinsed (7 times washed) Amicon Ultra-2ml 3000 MWCO (Merck Millipore, USA) column. Centrifugation was done at 4°C at 12,000g. Total 700 µL solution (containing serum sample, D_2O, pH maintenance buffer and DSS) was taken in 5 mm Shegemi tubes. NMR for all samples were performed at 298K on a Bruker 9.4 T (400 MHz) AVANCE-III Nanobay liquid-state NMR spectrometer equipped with 5 mm broadband (BBO) probe. Pre-saturation technique was used with a moderate relaxation delay of 5 second to ensure complete water saturation. Offset optimization was performed using real-time 'gs' mode for each sample. Topspin 2.1 was used to record and process the acquired spectra.

Metabolomic Analysis of NMR data

ChenomX (Canada) was used for analysis of NMR data. Bayesian approach is used to derive metabolite concentration in serum. The phase and baseline of raw spectrum was corrected and different concentration of metabolites was obtained through profiler. Through Metaboanalyst, (Hapfelmeier et al., 2005; Vazquez-Torres et al., 2004; Xia et al., 2015; Xia and Wishart, 2011) concentration files were analyzed. Then, for normalization purpose, the samples were log transformed and by comparing with control sample, fold change analysis was performed for each treated samples. More than 2-Fold change values (above or below reference value) with $p \leq 0.05$ were considered for further analysis.

Calculation of Cecal index: Body weight of individual mice was measured and recorded. The whole cecal content was collected in a microfuge tube and weighed for each individual mouse. The cecal index was measured by taking the ratio of cecal content to the body weight of respective mice and used by normalizing the data with respect to average body weight of mice used (Vazquez-Torres et al., 2004).

Gut permeability test by FITC dextran: Mice used for this experiment were water-starved for overnight. Next day FITC-dextran (Cat#F7250, Sigma-Aldrich, Missouri, US) at a concentration of 100mg/ml was dissolved in PBS and oral gavaged. After 4h, mice were anesthetized by

isoflurane inhalation and blood was collected by cardiac puncture. Serum was collected from blood and concentration of FITC in serum was measured by spectro-fluorometry with an excitation wavelength of 485 nm (20 nm bandwidth) and emission of 528 nm (20 nm bandwidth) (Woting and Blaut, 2018).

Endotoxin detection assay from serum: Mice were sacrificed on days 0, 3 and 6 following treatment of mice with vancomycin and blood was collected by cardiac puncture in an endotoxin-free vial. Toxinsensor chromogenic LAL endotoxin assay kit from GeneScript (Cat#L00350, Piscataway, NJ, USA) was used for detecting endotoxin level in serum of mice using the manufacturer's protocol.

Acetate detection assay in serum: Acetate level was measured in the serum samples of untreated (control day 0) and day 6 following treatment of BALB/c and C57BL/6 mice with vancomycin by using acetate colorimetric assay kit (EOAC-100, San Francisco, USA). Both control and treated mice were anesthetized and blood was collected through cardiac puncture. Blood was kept on ice for 30 mins followed by centrifugation at 1700 rpm for 15 min at 4 °C. The supernatant was collected and 10 µl of serum from each sample was used to detect acetate level and after substrate-enzyme coupled reaction formed color, absorbance was taken at 570 nm.

Hormonal assay: Leptin (Cat# ELM-Leptin), and Insulin (Cat# ELM-Insulin) hormone levels were assayed in serum samples and PYY (Cat# EIAM-PYY) in gut tissue samples for both mouse strains (BALB/c and C57BL/6) on day 3 and day 6 following treatment with vancomycin and compared with untreated day 0 control mice by using Raybiotech mouse hormonal assay kit (Norcross, Georgia, USA).

Statistical Analysis

All the graphs were plotted using GraphPad Prism 7.0a. Statistical package in Prism was used for statistical analysis for the data to perform 't'-test (to compare any 2 data sets) or ANOVA (to compare more than two datasets) as described in the text.

Result:

Vancomycin treatment alters the abundance and diversity of gut microbiota

Two important parameters, in microbiota research, are abundance and diversity that account for various physiological functions of the microbes. Metagenomic (16S rRNA) analysis of the cecal content in vancomycin treated mice showed a decrease in major phyla of bacteria like Firmicutes and Bacteroidetes and an increase in proteobacteria level (Figs. 1B, and 1D) compared to untreated time matched mice (Figs. 1A, and 1C). Comparing the control phyla (Figs. 1A and 1C) with vancomycin treated phyla (Figs 1B and 1D) for both mouse strains, the results revealed that Proteobacteria level in vancomycin treated mice increased from 1-2% (with respect to no treatment group) to 90% (on day 4 following treatment with vancomycin). On the contrary, Firmicutes level decreased from 70-80% (untreated group) to below 10% (fourth day following treatment with vancomycin) and Bacteroidetes level from 25-30 % (untreated group) to 1% (fourth day following treatment with vancomycin) (Figs 1B and 1D).

In addition, we further determined the diversity of the cecal microbiota. Shannon equitability index for diversity at phylum level showed a decrease in diversity up to the fifth day of treatment in BALB/c (Fig.1E) and fourth day of treatment in C57BL/6 mice (Fig.1F). Diversity increased on day 6 for BALB/c and day 5 for C57BL/6 following treatment with vancomycin. Changes in abundance and diversity in cecal microbiota opened up avenues for newer explorations some of which is reported here later.

Comparative analysis of time dependent changes in genus level data in time matched BALB/c and C57BL/6 specific controls (Fig. 2A and Fig. 2C) and in vancomycin treated BALB/c and C57BL/6 (Figs 2B and 2D) further corroborated with phylum level results (Fig. 1). When Shannon equitability index for measuring diversity between phylum and genus level data were compared, it was observed that the trend of the data at both levels until day 5 were similar but absolute values were different (Table 1). Day 6 values for phylum and genus were similar for either BALB/c or C57BL/6 (Table 1). The phylum level results are also confirmed by showing an increase in *Escherichia* and *Shigella* group of bacteria that belong to Proteobacteria phylum and decrease in Clostridia (Blautia, Instentimonas, Roseburia) group of bacteria of Firmicutes phylum in vancomycin treated mice following the day 4 treatment in comparison to time matched control mice (Figs. 2E and 2F). Increase in Proteobacteria is concomitant with increased inflammation (Holzheimer, 2014). It is also important to note that the Firmicutes (F) from 60-70% and Bacteroidetes (B) from 30-35% on day zero turned to 10% (F) and 1% or less for B for both BALB/c and C57BL/6 on day 5 or day 4, respectively. This suggested increase in Firmicutes/Bacteroidetes (F/B) ratio is correlated with enhanced body mass index and inflammation (Koliada et al., 2017). Current results showed increase in F/B ratio of 2-fold on day 0 to 10 fold or more following treatment with vancomycin on day 5 for BALB/c or day 4 for C57BL/6. The F/B ratio significantly decreased after day 4 or 5 for C57BL/6 or BALB/c. It is further observed that the phylum, Verrucomicrobia level increased (20-25%) in BALB/c mouse (Fig. 1D) on the sixth day following treatment with vancomycin and Verrucomicrobia increased significantly (>80%) from fifth day following vancomycin treatment in C57BL/6 mice (Fig. 1F). Akkermansia genus from Verrucomicrobia phylum increased from 2-3% (untreated group) to 70-75% by fifth day following treatment with vancomycin in C57BL/6 mice (Fig. 2D). This result is significant to understand the differential response exhibited in two different mice species (C57BL/6 and BALB/c) used in this study following treatment with vancomycin. The differences observed may be because of the immune bias (Th1 and Th2) of two species leading to different beneficial effects of Akkermansia to regulate glucose tolerance efficiency.

It is reported that *Akkermansia muciniphila* in the Verrucomicrobia phylum is the major known species that contributes positively to enhance insulin sensitivity and glucose metabolism by reducing inflammation and body-weight gain (Cani and de Vos, 2017; FUJISAKA et al., 2018; Khan et al., 2014). We, therefore, tested for *A. muciniphila* on day 6 following treatment with vancomycin of BALB/c and C57Bl/6. Species specific primer (Table 2) of *Akkermansia muciniphila* was used to confirm its abundance (Collado et al., 2007). Results revealed that there was a 6-fold increase in *A. muciniphila* abundance in vancomycin treated BALB/c on day 6 compared to time matched untreated control while the abundance of *A. muciniphila* was around 16384-fold and 24833-fold higher in vancomycin treated C57BL/6 on day 5 and day 6 compared to its time matched control. The current study revealed a hitherto unknown fact that abundance of *A. muciniphila* increases significantly following treatment with Vancomycin. The increase of *A. muciniphila* may have beneficial roles in physiology.

The inflammatory response in gut changes during vancomycin treatment in terms of dosage and with time

Pro- and anti-inflammatory responses are two important classes to check out the outcome of various treatments. Current results revealed that expression of pro-inflammatory cytokine genes (tnfα, il6, il1a, il17) in the gut at transcriptional level, increased till day 5 for BALB/c mice (Fig. 3A) and till day 4 for C57BL/6 mice (Fig. 3B) following treatment with vancomycin. Concentration of anti-inflammatory cytokines (tgfb, and il10) started increasing and pro-

inflammatory cytokines started decreasing from day 6 in BALB/c mice (Fig. 3C) and from day 5 in C57BL/6 mice (Fig. 3D) following treatment with vancomycin. Concomitant to the increase in pro-inflammatory cytokines we observed increase in expression of tlr4 at transcriptional level till day 4 and tlr2 till day 6 in both BALB/C and C57BL/6 mice following treatment with vancomycin (Fig. 3E and Fig. 3F).

Major changes happened in the expression of tnfα and il10 at the transcriptional level, as revealed from qRT PCR data, on day 3 and day 6 following treatment with vancomycin in both Th1- and Th2-mice. Validation of qRT PCR results was done, at the protein level expression of gut wall tissues, by ELISA (Fig. 4). ELISA results revealed that, on the third day following vancomycin treatment, TNFα level was significantly more in both BALB/C (Fig. 4A) and C57BL/6 (Fig. 4B) mice with respect to day 3 time-matched untreated control group of mice. Similarly, IL10 cytokine level was more in BALB/c (Fig. 4C) and C57BL/6 (Fig.4D) on the day 6 following treatment with vancomycin compared to the day 6 time-matched untreated control mice. While changes in TNFα expression corresponds well for both mice strain between transcriptional and protein level validation, we noted that the transcriptional changes for IL10 in BALB/c does not corroborate with protein level validation but it is validated for C57BL/6. The reason for such mismatch needs to be explored further. It is important to note that the time kinetics (day 6 post vancomycin treatment), observed for enhanced anti-inflammatory (il10) response in either of the mouse strains, matches with the rise of *A. muciniphila*. The results might indicate hitherto unknown finding that increase in *A. muciniphila* may lead to transition from pro-inflammatory to anti-inflammatory response irrespective of initial immune bias of the mice. The observed transition from pro- to anti-inflammatory responses may be a combined effects of decreased level of Proteobacteria and elevated abundance of Verrucomicrobia.

Gut permeability increased following vancomycin treatment:

Gut permeability gets affected as a direct consequence of altered gut microbiota. Alteration of abundance and diversity of gut microbiota is measured in terms of the cecal index of host (a measure of normalized cecal mass over body mass). The cecal index was calculated and the ratio was significantly more in vancomycin treated C57BL/6 and BALB/c mice than their respective time-matched control groups (Fig. 5A). Cecum is known to be a better representation than fecal sample for understanding intestinal microbiota profile (Donaldson et al., 2016) and cecum usually gets enlarged following treatment with antibiotic by accumulating fluid and solid matter (Reikvam et al., 2011). The changes in the bacterial composition due to antibiotic treatment in cecum may have led to accumulation of significantly high volume of fluid in its lumen. The fluid volume following centrifugation of cecal sample was significantly more in vancomycin treated mice than respective time-matched control mice (Fig. 5B). In vancomycin treated mice, a larger cecum with more fluid accumulation was observed than control mice (Fig. 5C). For further confirmation of gut permeability, FITC conjugated dextran was gavaged on day 3 following treatment with vancomycin and in respective control mice. FITC dextran Concentration was detected significantly higher in the serum of mice on day 3 following vancomycin treatment (BALB/c 341.6±25.9 ng/ml, C57BL/6 367.5±25.3 ng/ml) than corresponding time-matched control mice (BALB/c 217.1±24 ng/ml, C57BL/6 229.8±22 ng/ml). (Fig. 5D). The preceding observations on fluid accumulation and FITC-dextran results on gut permeability prompted us to check for respective gene expression. The results from qRT-PCR revealed compromised expression of tight junction genes (occludin and claudin) at transcriptional level in the gut of vancomycin treated mice at different time point with respect to the time-matched control mice. Their expression of claudin gene decreased continuously from day one to day six following

treatment with vancomycin in BALB/c (from 1.2-fold activation following vancomycin treatment with respect to time matched control on day zero to 3.4-fold repression on day 6 by treatment with vancomycin when compared to time matched untreated control) and C57BL/6 (From two-fold activation on day zero to 1.5-fold repression on day six when vancomycin treated mice re compared to untreated time matched controls) mice (Fig. 5E and Fig. 5F). Similarly, expression of occludin gene decreased in BALB/c mice (from 2.5-fold activation on day zero to 6.5-fold repression on day six) and C57BL/6 (from 2.3- fold activation on day one to no-change on day six) following vancomycin treatment. It is important to note that the expression of claudin 1in C57BL/6 and occludin in both C57BL/6 and BALB/c mice, that were activated on day 1 following vancomycin treatment but significantly repressed following treatment with vancomycin on subsequent days. The results presented in Figs. 5E and 5F, perhaps suggested that effects of vancomycin on expression of gut permeable genes are very rapid. The results of gene expression corroborated with the FITC-dextran data to confirm reduced gut permeability following vancomycin treatment in both C57BL/6- and BALB/c mice. While results from FITC dextran studies on day 6 suggested restoration of gut barrier for both BALB/c and C57BL/6 expression of claudin 1 and occludin genes remained repressed with respect to time matched control mice. Further studies are required to understand this apparent discrepancy which may be due to a different mechanism, independent of these two genes, that may exist to re-establish the gut barrier function.

The reduced gut permeability following treatment with vancomycin may lead to increased level of systemic endotoxin level. The endotoxin level in serum correlates with increase in pathogenic gram-negative bacteria (proteobacteria) in gut and compromised permeability and inflammation of gut tissue (Holzheimer, 2014). The endotoxin level was measured in the serum of control and vancomycin treated mice. On the third day of vancomycin treatment, endotoxin level was the highest in the serum of both BALB/c (0.82±0.07 EU/ml) and C57BL/6 (0.67±0.12 EU/ml) mice but on the sixth day, its level decreased (0.44±0.07 EU/ml in BALB/c and 0.16±0.05 EU/ml in C57BL/6 mice) and became similar with the control group of mice (Fig.5G). The results may be corroborating with either enhanced level of beneficial bacteria on day 6 following vancomycin treatment as described below or due to decreased level of proteobacteria or both.

Alteration of blood glucose level with changes in Proteobacteria and Verrucomicrobia level in gut:

Glucose is an important metabolite to keep the energy requirement and balance in the body. It is also easy to measure in the host blood to determine the glucose metabolism status. Kinetics of oral glucose tolerance test (OGTT) from 0 to 90 minutes was done following 12h fasting of either strain of mice. From glucose tolerance test, it was revealed that glucose metabolism was different in control and vancomycin treated mice (Fig. 6A and 6B). Important to note that the results from OGTT studies for control animals for both BALB/c and C57BL/6 remain unchanged on days 0, 3 and 6. On the day 3 following vancomycin treatment, fasting serum glucose levels in the Th2- and Th1-biased mice (BALB/c 194.6± 6.3 mg/dl and C57BL/6 186±6 mg/dl) were significantly higher than their time-matched control (BALB/c 115±3 mg/dl and C57BL/6 126±4 mg/dl). On day 6 following vancomycin treatment glucose levels in BALB/c was 148.6±7 mg/dl and in C57BL/6 was 103±5 mg/dl. On the sixth day of vancomycin treatment, BALB/c mice (VB6) had similar fasting glucose level in blood like control (CB) and C57BL/6 mice (VC6) had lower fasting glucose level than control (CC) mice. At fifteen-minute post glucose gavage, the elevation of glucose level in the blood of treated mice of the third day (VB3 295±14 mg/dl and VC3 333±8 mg/dl) was significantly higher than control (CB 215±12 mg/dl, CC 216±7 mg/dl)

and sixth day post-vancomycin (VB6 235±5 mg/dl, VC6 200±9 mg/dl) mice (Figs. 6A and 6B). The metabolism rate of glucose in the blood of the sixth day vancomycin treated mice (VB6, VC6) was faster than the third day treated mice (VB3 and VC3). This rate was higher for VC6 than VB6. In VC6 group of mice, the glucose level in the blood came to normal rapidly and its concentration at ninety-minute post glucose gavage was similar with the control group in BALB/C mice and lower than the control group in C57BL/6 mice. In VB3 and VC3 group of mice, the glucose concentration in blood at ninety minutes was still higher than control.

Day 3 vancomycin treated mice were transplanted with cecal microbiota (CMT) of day 6 vancomycin treated mice (more abundant with *A. muciniphila*) to observe its efficacy on glucose metabolism. In CMT treated group of day 3 (following vancomycin treatment) mice, glucose level in serum decreases drastically in C57BL/6 mice (Fig. 6B) and became equivalent to control groups of mice but in BALB/c mice the effect was insignificant (Fig. 6A).

Metabolite level in serum changes following vancomycin treatment

It is believed that the short chain fatty acids (SCFA) are the microbes associated molecular patterns that could regulate innate immunity of the host. The current results revealed that the ratio of abundance of acetate/lysine (Fig. 6C), butyrate/lysine (Fig. 6D) and propionate /threonine (Fig. 6E) in serum are reduced significantly on day 6 following treatment with vancomycin. The results depicted in Figs. 6C-6E might indicate that conversion of SCFAs such as acetate and butyrate from lysine and propionate from threonine were perhaps significantly decreased in the gut of vancomycin-treated mice. The results revealed that the concentrations of acetate, butyrate, and propionate (Table 3) in the blood of vancomycin treated mice on day 6 were lower but their respective substrate concentrations (lysine, and threonine) were higher than control on day 6 post vancomycin treatment. Important to note here that concomitant with the Th1-(C57BL/6) and Th2-(BALB/c) bias of the mice one should expect lower level of abundance of SCFAs in control (untreated) C57Bl/6 (CC) and control BALB/c (CB) on day 0 and our results supported such proposition (Table 3). The results, however, further suggested accumulation of the substrate in blood and less detection of SCFA in the vancomycin treated day 6 group of mice (VB and VC on D6). Among vancomycin treated groups, BALB/c (VB) mice had lower SCFA production rate than C57BL/6 (VC) mice. We further validated the abundance of acetate in the serum of both BALB/c and C57BL/6 mice by using acetate detection kit (EOAC-100, San Francisco, USA). The results validated the abundance of acetate following vancomycin treatment in VB6 (Vancomycin treated BALB/c on day 6) and VC6 (Vancomycin treated C57BL/6 on day 6). The concentrations of acetate in VB6 was 23.9±1.4 μM while the concentration in CB6 (time matched untreated control of BALB/c) was 35.3±1.6 μM. The same in VC6 (Vancomycin treated C57BL/6 on day 6) was 22.2±4 μM while the concentration of acetate in respective time-matched untreated (control) group CC6 was 41.3±1.02 μM (Fig. 6F). The results implied for the first time the role of vancomycin on SCFA production in Th1- and Th2-biased mice. Results also supported the intrinsic SCFA production (more in Th2- compared to Th1-biased mice) as per the immune bias of mice strain.

Gut hormonal level during vancomycin treatment

We already reported the rate of glucose metabolism that increased and production of SCFAs that decreased on day 6 following treatment with vancomycin. Considering the changes in glucose and SCFAs we investigated further the roles of other associated hormones such as insulin and Peptide tyrosine tyrosine (PYY) in serum and also a control hormone leptin which is mostly activated in adipocytes (Larraufie et al., 2018; Woods and D'Alessio, 2008; Xiong et al., 2004). Results (Fig. 7A) revealed that the concentration of insulin in serum on the third day of

vancomycin treated a) BALB/c (VB3) was 1.16±0.25 ng/ml and b) C57BL/6 (VC3) was 1.43±0.15 ng/ml. Similarly, the concentration of insulin in serum on the sixth day of vancomycin treated a) BALB/c (VB6) was 0.98±0.1 ng/ml and b) C57BL/6 (VC6) was 0.83±0.06 ng/ml. Fig. 7A revealed that insulin level decreased significantly on day 6 compared to day 3 following treatment with vancomycin for both BALB/c and C57BL/6. However, insulin levels for both mice strains on day 3 and day 6 post-vancomycin were significantly higher than day 0 (untreated controls CB and CC). As hypothesized, no significant changes were observed in serum leptin concentrations on any day following vancomycin treatment of BALB/c and C57BL/6 mice with respect to respective untreated controls (Fig. 7B). The results revealed that the concentration of PYY hormone in the gut decreased following vancomycin treatment in BALB/c (8836±311 pg/mg of tissue on day 0 and 4843.9±149 on day 6) and in C57BL/6 mice (7883±155pg/mg of tissue on day 0 to 6283.8±209 on day 6) (Fig.7C). As documented recently that SCFA regulates PYY production is validated by the current results (Larraufie et al., 2018). Vancomycin treatment reduces SCFA level following deceased abundance of PYY.

Discussion

The extent of perturbation of gut microbiota varies differently following treatment with different antibiotics. We selected vancomycin for continued studies, because metagenomic (16S rRNA) analysis of cecal samples of mice showed that vancomycin altered the gut microbiota most extensively by decreasing the phylum like Firmicutes and Bacteroidetes and by increasing the Proteobacteria level. As described before the dosage of vancomycin used was as per FDA guidance. By correlating the NGS data with plating data, it might be concluded that the group of bacteria, that overpopulated on the plate following day 2 but decreased following day 5 of vancomycin treatment, might belong to Proteobacteria phylum. Proposition was further validated by plating the cecal samples of day 3 vancomycin treated mice on specific media such as, salmonella-shigella specific agar plate and EMB agar plate. Results from media specific growth suggested overgrown colonies were mostly Shigella and *E.coli* and it was proven by metagenomic data of cecal samples at genus level. Metagenomic analysis further suggested that following day 5 and day 6 treatment with vancomycin, Verrucomicrobia phylum replaced the Proteobacteria phylum in the gut. The Verrucomicrobia phylum consists of Akkermansia genus that is showing promise for future therapeutics and prophylaxis (Maier et al., 2014).

During vancomycin treatment, Proteobacteria phylum started increasing significantly up to day 4 post-vancomycin treatment and it replaced other phyla like Firmicutes, Bacteroidetes, Actinobacteria, Tenericutes, and Verrucomicrobia (Figs. 1D and 1F). This increase in only one phylum (Proteobacteria) caused decrease in the diversity of other gut microbiota; therefore, gut microbiota diversity was the lowest on day 4 following treatment with vancomycin for C57BL/6 and on day 5 following treatment with vancomycin for BALB/c mice. During treatment with vancomycin till day 4, abundance of Proteobacteria was over 90% while abundance of other phyla was significantly low. Starting from day 5 following treatment with vancomycin, abundance of other phyla such as, Firmicutes, Tenericutes, and Verrucomicrobia started increasing in the gut resulting into greater diversity that culminated on the sixth day of treatment (Fig. 1G and Fig. 1H).

In Proteobacteria phylum, Escherichia and Shigella genera were increased in mice gut during vancomycin treatment. These genera belong to gram-negative groups of bacteria containing LPS, that increases the endotoxin level in blood. These bacteria activate the tlr4 receptor present in the gut epithelial cell, and this increases the expression of pro-inflammatory cytokines.

Combinatorial effect of the increase in Proteobacteria and decrease in Firmicutes in mice gut caused the elevation in inflammation of the gut tissue. Firmicutes, specifically Clostridium group present in the gut produces short chain fatty acid like acetate, butyrate, propionate from complex carbohydrate foods (Boutagy et al., 2016). Instentimonas bacteria (Firmicutes phylum) produces butyrate from lysine (Bui et al., 2015) and Bacteroidetes produces propionate from threonine in the (Bui et al., 2015; Neis et al., 2015). The production of these SCFA in the gut suppresses the LPS and pro-inflammatory cytokines like TNF-α, IL-6 level and enhances the release of the anti-inflammatory cytokine like IL10. (Morrison and Preston, 2016; Vinolo et al., 2011). Vancomycin treatment causes the decrease in Firmicutes and Bacteroidetes level significantly in mice gut, which results in less SCFA production and increases level of inflammation in vancomycin treated mice than control.

Overexpression of Inflammatory cytokine like TNFα is associated with higher gut permeability by suppressing the expression of tight junction proteins like occludin and claudin 1 (Al-Sadi et al., 2013; Rios-Arce et al., 2017). Lower expression of tight junction protein in vancomycin treated mice caused the increase in gut permeability resulting in higher cecal index and fluid accumulation of cecal sample (Fig. 5). On the sixth day of vancomycin treatment, results revealed a decrease in Proteobacteria and increase in Verrucomicrobia which might have caused an anti-inflammatory effect in the gut. While it is known that increased abundance of Verrucomicrobia causes a decrease in inflammation and enhances glucose metabolism of the host, it is also known that increase in abundance of proteobacteria causes inflammation (Fujio-Vejar et al., 2017; Hakansson and Molin, 2011; Plovier et al., 2017). During obesity and diabetic condition, metabolic endotoxemia had been observed where endotoxin (LPS) level increased in the blood to cause inflammation and impaired glucose metabolism in the host (Boutagy et al., 2016; Hawkesworth et al., 2013). The increase in Proteobacteria phylum in gut during vancomycin treatment elevated the endotoxin level of blood in mice. This resulted in impaired glucose metabolism and insulin resistance (highest level of both insulin and glucose on the day 3 following treatment with vancomycin. On the sixth day following vancomycin treatment, in C57BL/6, replacement of Proteobaceria by Verrucomicrobia caused significant improvement in glucose metabolism in mice; fasting glucose (Fig.6A and 6B) and insulin level (Fig. 7A) in blood came to normal. This effect is more prevalent in C57BL/6 than BALB/c mice. Following successful transfer of cecal sample from sixth day of vancomycin treated C57BL/6 mice (*A. muciniphila* level is above 70%) to third day of vancomycin treated mice, blood glucose level decreased significantly on the third day mice, which showed the effective role of *Akkermansia muciniphila* in controlling the blood glucose level. Higher fasting Insulin concentration in serum of the third day vancomycin treated BALB/c and C57BL/6 than control and sixth day vancomycin treated C57BL/6 mice (Fig.7A) showed the insulin resistance following vancomycin treatment and its correlation with *A. muciniphila* level in mice gut. On the sixth day of vancomycin treatment, *A.muciniphila* level is significantly higher than in C57BL/6 mice than BALB/c, which might cause more prominent effect in decreasing glucose and insulin level in blood of C57BL/6 mice than BALB/c mice (Fig.6A and 6B).

It is also important to note that the SCFA production was more in Th2- compared to Th1-biased mice. More SCFA usually leads to anti-inflammatory response or Th2-bias. Current observation further confirms the fact as per the immune bias of mice strain used. SCFA stimulates PYY hormone production by activating Gq coupled receptor FFA2 of endocrine cells present in the gut (Larraufie et al., 2018). The current results revealed that following treatment with vancomycin the level of SCFA decreased, which cause a reduction in serum PYY production

(Fig.7C) that might have an effect of observed enhanced glucose metabolism or increased insulin sensitivity (Cahill et al., 2014). We observed decrease in insulin level in serum on day 6 was less than day 3 following treatment with vancomycin but blood glucose level was better managed on day 6 compared to day 3 perhaps suggesting enhanced insulin sensitivity.

The clinical dose of vancomycin treatment perturbs the gut microbiota of mice by depleting the healthy microbes and increasing the proteobacteria group microbes that include various pathogens. The increased proteobacteria, until day 3 following vancomycin treatment, could disrupt the homestasis of various physiological processes (such as, immunological and metabolic) and as a result it perhaps increased serum endotoxin level, blood glucose level, and enhanced pro-inflammatory responses. Physiology, in terms of endotoxin, glucose or inflammation status, got restored at the later stages of treatment (day 5 or 6). The restoration of these physiological parameters may be because of decrease in harmful proteobacteria and increase in certain type of healthy microbes like Verrucomicrobia. Generally, the Verrucomicrobia phylum that appeared in huge number at the later stages of vancomycin treatment are present very less number in control group, but their functional role is similar to a major group of healthy microbes of the gut to restore the equilibrium by alleviating the adverse effects of Proteobacteria. Proetobacteria belong to gram-negative groups of bacteria containing LPS, that increases the endotoxin level in blood. These bacteria activate the tlr4 receptor present in the gut epithelial cell, and this increases the expression of pro-inflammatory cytokines. Combinatorial effect of the increase in Proteobacteria and decrease in Firmicutes in mice gut caused the elevation in inflammation of the gut tissue.

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Author Contribution Statement

PR performed all experiments drafted the manuscript. PR and PA designed the experiments. PA supervised the studies in general and also finalize the manuscript.

Contribution to the Field Statement

Anti-biotics play important role in our society in terms of regulating and maintaining our health. Overuse of the antibiotics yielded a major threat and we are looking for better ways to alleviate the problem of antibiotic resistance. Microbiota can provide the solution that is required along the line. However, understanding the problems associated with antibiotic usage and the help that could be conferred by the microbiota needs more studies. The current study is providing a small leap in that direction by showing the usage of antibiotic like vancomycin that may boost certain beneficial microbes that can confer genuine health benefits. The current study in immunologically differently biased mice revealed that the treatment with vancomycin elevated abundance of *A. muciniphila* may provide health related significant benefits more in mice with proinflammatory (C57BL/6) bias over tolerogenic mice (BALB/c). To the best of our knowledge the current report is the first of its kind where it is showing the importance of perturbation of gut microbiota to exert on their roles and also how a treatment with an antibiotic may become beneficial for health manipulation.

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Figure Legends

Fig 1.

Phylum level changes in the gut microbiota. Time dependent changes in the phyla of gut microbiota, in the control A. BALB/c, and C. C57BL/6 and in vancomycin treated B. BALB/c, and E. C57BL/6 mice, are shown. In the figure, only major phyla are shown to avoid clutter. Data shown are average of 3 biological replicates. To avoid clutter, standard deviation (SD) calculated using 2-way ANOVA is not shown. However, SD was less than 10% on average. Kinetics of changes in phylum level Equitability index (E) (diversity) of the gut microbiota following treatment with vancomycin in E. BALB/c and F. C57/BL6 mice. Statistical significance of diversity in panels G. and H. are shown by the letters a, and b among the treatment conditions that are significantly ('a', and 'b' correspond to $P \leq 0.001$) different. Error bars are one standard deviation of the mean value and determined from the average values of three biological replicates..

Fig 2.

Metataxonomic studies of genus level variation of gut microbiota in vancomycin treated and its respective control group. Kinetics of changes in genera of gut microbiota, in the control A. BALB/c, and C. C57BL/6 and in vancomycin treated B. BALB/c, and D. C57BL/6 mice are

shown. Data shown are average of 3 biological replicates and standard deviation was less than 10% on average. Percentage abundance of different genera for various treatment conditions are shown on the 'Y'-axis and the days elapsed post treatment or for time matched control are shown on the 'X'-axis. Colony of culturable Proteobacteria by plating of cecal samples from both strains of mice on selective and differential media. Evidence of E. Shigella colonies growth on Salmonella-Shigella specific media agar plate on day 4 (CC) control C57BL/6, (VC) vancomycin treated C57BL/6, (CB) control BALB/c and (VB) vancomycin treated BALB/c and F. Growth of *E. coli*. colonies on EMB (Eosin methylene blue agar plate) on day 4 (VC) vancomycin treated C57BL/6 and (CC) control C57BL/6.

Fig 3.

Transcriptional gene expression profile. Kinetics of transcriptional (by qRT-PCR) expression of genes categorized as, pro-inflammatory in A. BALB/c, and B. C57BL/6, anti-inflammatory in C. BALB/c, and D. C57BL/6 as Toll like receptors tlr4 and tlr2 in E. BALB/c, and F. C57BL/6 mice. Statistical significance was calculated by two-way ANOVA and changes are denoted by the letters a, b, and c among the genes that are significantly ('a', and 'b' correspond to $P \leq 0.001$ while 'c' corresponds to $P \leq 0.01$) different. Error bars are one standard deviation of the mean value and determined from the average values of three biological replicates.

Fig 4.

Protein level gene expression and comparative analysis of qRT PCR and ELISA data for TNFα and IL10. A. Mean values (n=3) of protein level concentration (in pg/mg) with standard deviation of TNFα (blue) and IL10 (red) expression on days 0, 3 and 6 for control and vancomycin treated BALB/c (CB and VB) and C57BL/6 (CC and VC) are shown. Statistical significance was calculated by two-way ANOVA and changes are denoted by the letters a, b, and c among the genes that are significantly ('a', and 'b' correspond to $P \leq 0.001$ while 'c' corresponds to $P \leq 0.01$) different. The genes that are compared for significance have the same letter. Fold change values of expression of TNFα (blue) and IL10 (red) to compare the values obtained from qRT-PCR and ELISA studies are shown for B. BALB/c, and C. C57BL/6. Error bars of the data are already shown in preceding figures.

Fig 5.

Measurement of intestinal integrity of BALB/c and C57BL/6 mice following treatment with Vancomycin. Changes in A. Cecal index (total cecal mass/total body mass), and B. Cecal liquid content in control (untreated) and vancomycin treated BALB/c and C57BL/6 mice are shown as bar plot. C. Representative images of various sizes of Cecum from both BALB/c and C57BL/6 mice on day 6 following vancomycin treatment for 1. C57BL/6, and 2. BALB/c and corresponding time matched (day 6) untreated control for 3. C57BL/6 and 4. BALB/c are shown. D. Gut permeability data by measuring FITC dextran concentration in serum of BALB/c and C57BL/6 on day 0 (control) and on day 3 and day 6 following treatment with vancomycin. Transcriptional expression levels of tight junction genes, claudin 1 and occluding, in gut tissue by qRT-PCR are shown in vancomycin treated and untreated (control) groups for E. BALB/c and F. C57BL/6 mice. Endotoxin concentration in the serum for both strains of mice are shown in panel G., where CB, VB3 and VB6 implies untreated (control) and day 3 (D3) and day 6 (D6) post vancomycin treated BALB/c and CC, VC3 and VC6 denote the same for C57Bl/6 mice. The colors corresponding to different days are shown above the panels E. and F. Comparisons among the groups were calculated by two-way ANOVA. In the figure for panels A. B. D. and G., '***' corresponds to $P \leq 0.001$, '**' corresponds to $P \leq 0.01$, '*' corresponds to $P \leq 0.05$

level of significance). In panels, E. and F., significance among groups are indicated by 'a' and 'b' to avoid clutter. Error bars shown are one standard deviation from the mean value of four replicates (n=4).

Fig 6.
Glucose tolerance and abundance of select metabolites in serum. Kinetics of fasting blood sugar in A. BALB/c B. C57BL/6 mice following treatment with Vancomycin on days 0, 3 and 6 and following treatment with CMT from day 6 vancomycin treated mice transferred to vancomycin treated day 3 group of mice. Ratio of abundance, from chemometric ^{1}H-NMR studies for major short chain fatty acids, of C. acetate production over lysine, D. butyrate production over lysine and E. propionate production over threonine in untreated control BALB/c (CB) and C57BL/6 (CC) and Vancomycin treated BALB/c (VB) and C57BL/6 (VC) are compared for Day 0 and Day 6 following treatment with vancomycin. In addition, F. acetate concentration in the serum by using acetate detection kit on day 6 in vancomycin treated groups of mice (VB6, VC6) along with the time matched control mice (CB6, CC6) of BALB/c and C57BL/6, respectively. In the figure, '***' corresponds to $P \leq 0.001$, '**' corresponds to $P \leq 0.01$, level of significance). For panels 'C', 'D' and 'E', significant difference are shown by letters 'a' and 'b' as applicable and both letters indicated $P \leq 0.001$. Error bars shown are one standard deviation from the mean value of four replicates (n=4).
Fig 7.
Changes in select hormones in serum. Abundance of A. Insulin (ng/ml), B. Leptin (ng/ml) and C. PYY (pg/mg) in the serum of control BALB/c (CB) or C57BL/6 (CC) and vancomycin treated mice on third day (VB3, VC3) and sixth day (VB6, VC6) of BALB/c and C57BL/6 mice respectively. Comparisons among the groups were calculated with two-way ANOVA. In the figure, '***' corresponds to $P \leq 0.001$, '**' corresponds to $P \leq 0.01$, '*' corresponds to $P \leq 0.05$ level of significance). Error bars shown are one standard deviation from the mean value of four replicates (n=4).

Tables

Table 1. Diversity assessment of Phylum and Genus for BALB/c and C57BL/6 following treatment with vancomycin.

Days post treatment	Phylum		Genus	
	Evenness[a] (± SD)			
	BALB/c	**C57BL/6**	**BALB/c**	**C57BL/6**
0	0.4(±0.05)	0.4(±0.06)	0.7(±0.01)	0.8(±0.03)
2	0.4(±0.07)	0.3(±0.03)	0.5(±0.05)	0.6(±0.04)
3	0.3(±0.06)	0.3(±0.01)	0.7(±0.03)	0.6(±0.08)
4	0.2(±0.04)	0.3(±0.04)	0.5(±0.04)	0.7(±0.06)
5	0.1(±0.06)	0.3(±0.01)	0.6(±0.08)	0.8(±0.05)

6	0.7(±0.04)	0.5(±0.08)	0.6(±0.06)	0.3(±0.12)

[a] Evenness, calculated based on diversity from Shannon Index

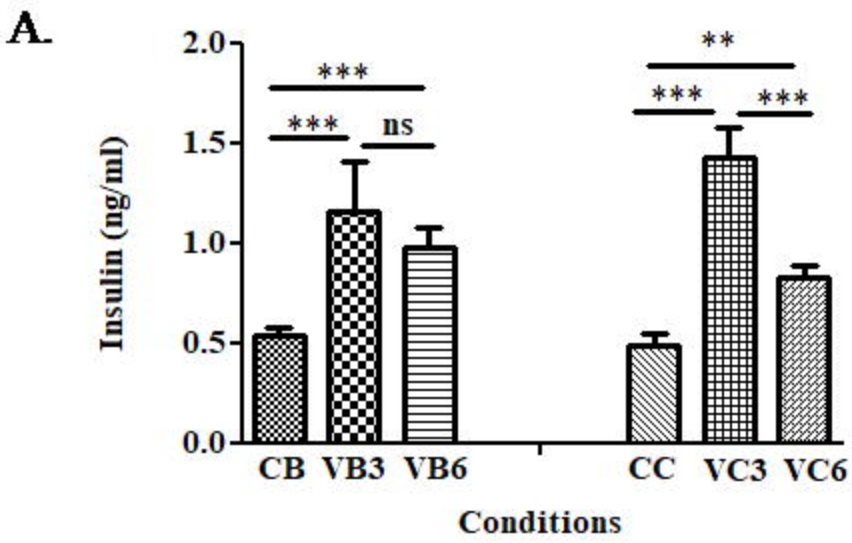
Table 2: Sequences of forward (_F) and reverse (_R) primers for PCR studies to confirm presence and expression level of various genes used in this study.

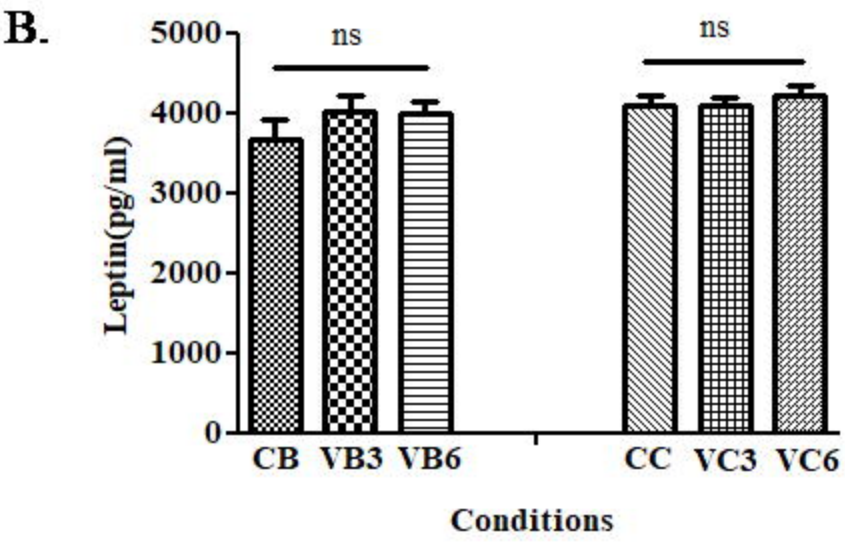
Genes specific for	Sequence of the primers used
***A. muciniphila*_F**	5’-CAGCACGTGAAGGTGGGGAC-3’
***A. muciniphila*_R**	5’- CCTTGCGGTTGGCTTCAGAT-3'
***il10*_F**	5’-AGGCAGTGGAGCAGGTGAAGAGTG-3’
***il10*_R**	5’-GCTCTCAAGTGTGGCCAGCCTTAG-3’
***tnf*_F**	5’-CCACGTCGTAGCAAACCACCAAAG-3’
***tnf*_R**	5’- TGCCCGGACTCCGCAAAGTCTAAG-3’
***cldn1*_F**	5’-TGCCCCAGTGGAAGATTTACT-3’
***cldn1*_R**	5’-CTTTGCGAAACGCAGGACAT-3’
***tlr4*_F**	5’- CGCTGCCACCAGTTACAGAT-3’
***tlr4*_R**	5’-AGGAACTACCTCTATGCAGGGAT-3’
***ocln*_F**	5'- GTTGAACTGTGGATTGGCAG -3'
***ocln*_R**	5'- AAGATAAGCGAACCTTGGCG -3'
***il6*_F**	5'-AGACAAAGCCAGAGTCCTTCAGAG-3’
***il6*-R**	5’-CCACAGTGAGGAATGTCCACAAAC-3’
***tlr2*-F**	5’-GCCCGTAGATGAAGTCAGCTCACC-3’
***tlr2*-R**	5’-CGGGCATCTACTTCAGTCGAGTGG-3’
***il17*_F**	5’-TCCAGAAGGCCCTCAGACTA-3’
il17_R	5’-ACACCCACCAGCATCTTCTCA-3’
***tgfb*_F**	5’-CCCAGCATCTGCAAAGCT-3’
***tgfb*_R**	5’-GTCAATGTACAGCTGCCGCA-3’
***il1a*_F**	5’-ATCAGTACCTCACGGCTGCT-3’
***il1a*_R**	5’-TGGGTATCTCAGGCATCTCC-3’

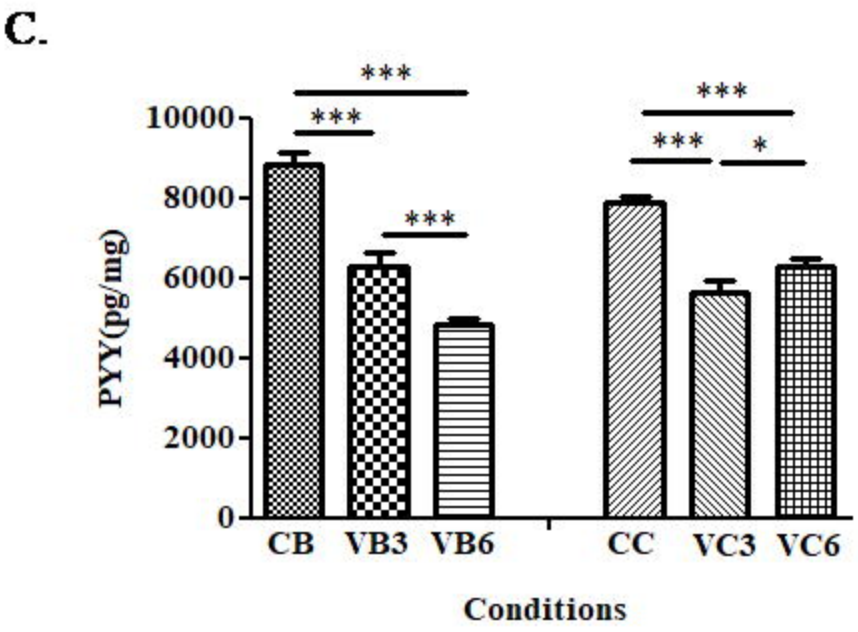
Table 3. Abundance of various SCFAs and associated metabolites in untreated (control) and vancomycin treated BALB/c and C57BL/6

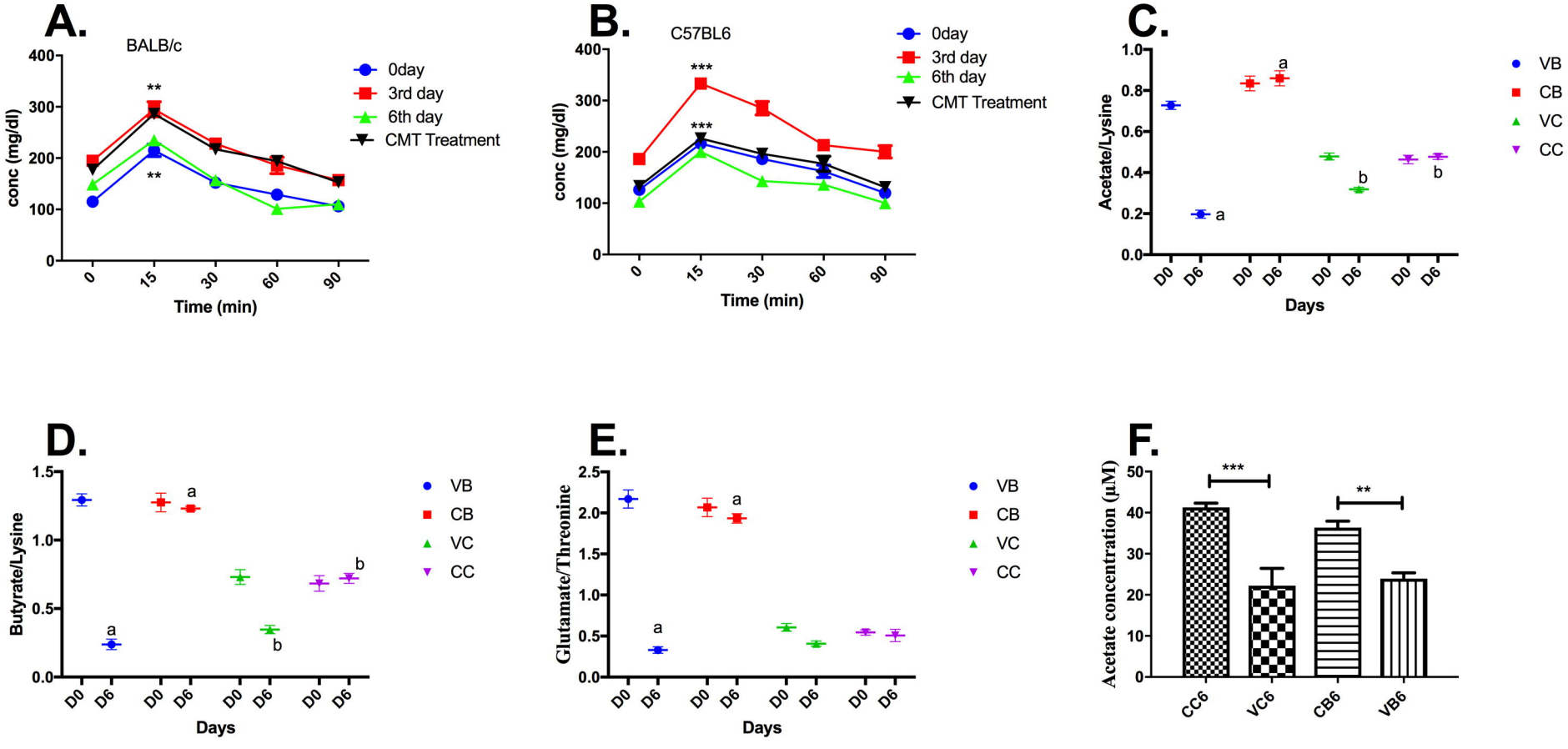
Days post treatment	**Mouse**	**Treatment**	**Condition**	**Acetate**	**Butyrate**	**Lysine**	**Propionate**	**Threonine**
				Mean concentration [μM] (± SD)				
0	BALB/c	None (Control)	CB0	149(±9.5)	231.4(±11.4)	183.8(±7.8)	216.6(±2)	105.1(±6.9)
0	BALB/c	Vancomycin	VB0	136.2(±7.4)	242(±13.6)	187.1(±1.3)	221.5(±5.7)	102.5(±10)
6	BALB/c	None (Control)	CB6	157.5(±4.9)	223.2(±18.3)	179(±8.5)	223.2(±5.9)	115.7(±7.6)
6	BALB/c	Vancomycin	VB6	54.3(±5.7)	65.9(±17.1)	275.6(±9.1)	119.8(±10.6)	293.9(±7.1)
0	C57BL/6	None (Control)	CC0	93.2(±3)	147.7(±3.5)	197.3(±1.6)	144.1(±4.3)	265.4(±14.2)
0	C57BL/6	Vancomycin	VC0	90.4(±2.1)	135.3(±14.3)	189(±4.9)	145.6(±9.3)	240.7(±11.6)
6	C57BL/6	None (Control)	CC6	94.6(±3.5)	130.8(±6.3)	199.6(±2.6)	132.6(±3.7)	248(±19.8)
6	C57BL/6	Vancomycin	VC6	70(±0.6)	76.6(±12.2)	219.8(±7.8)	91.8(±12.6)	278.4(±8.7)

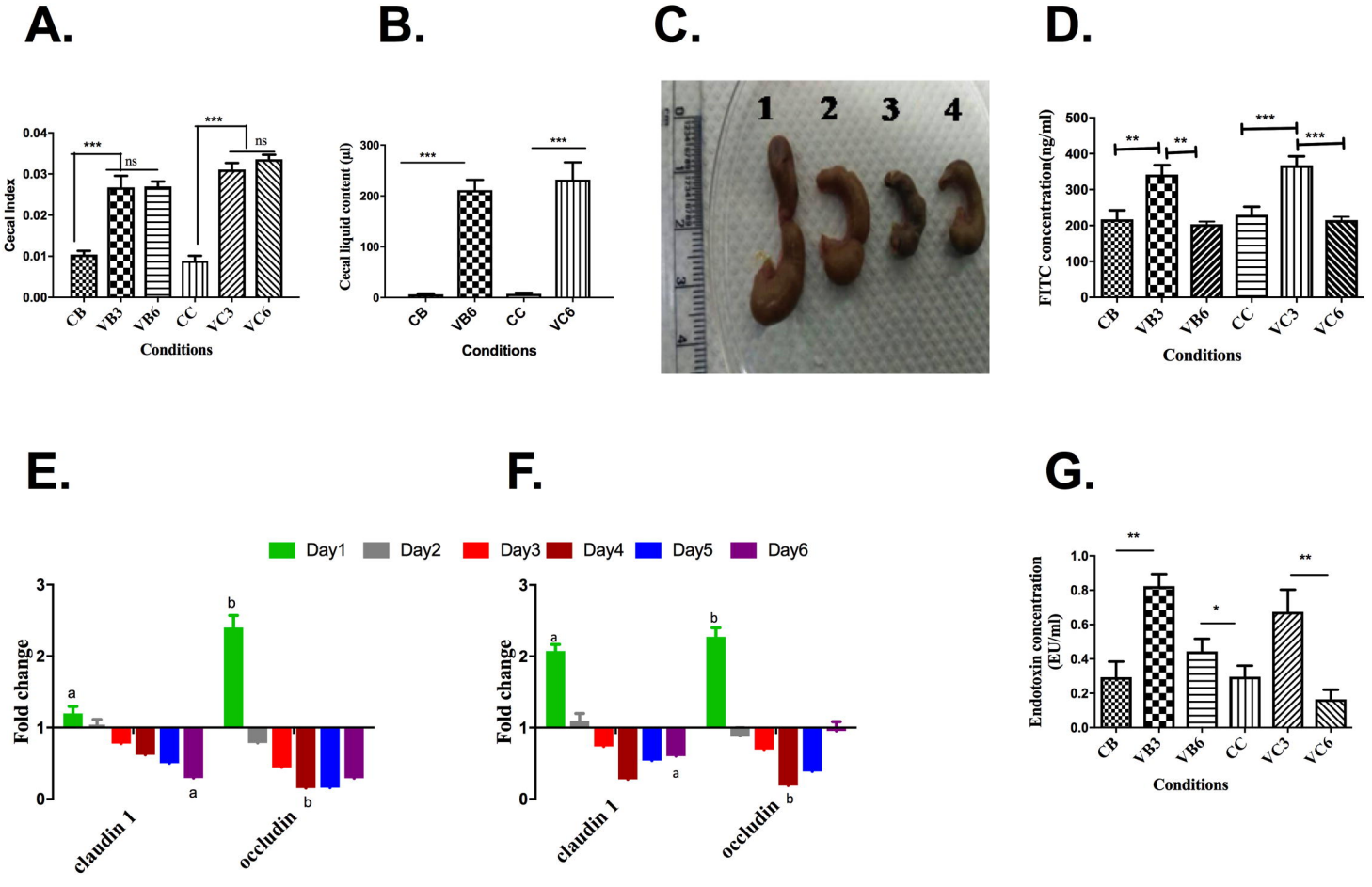
Results for day 0 and day 6 for control and treated mice are shown

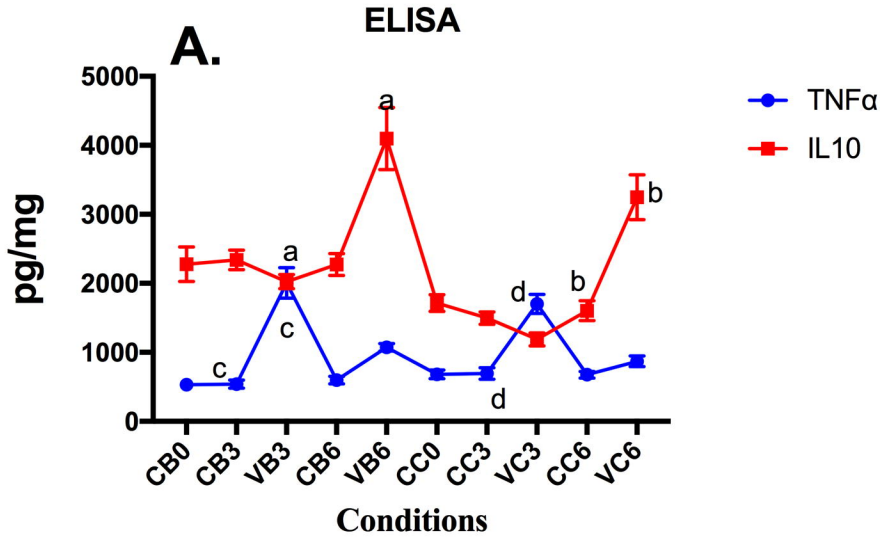

A.
Insulin (ng/ml)
2.0
1.5
1.0
0.5
0.0
***
***
ns
**
***
***
CB
VB3
VB6
CC
VC3
VC6
Conditions

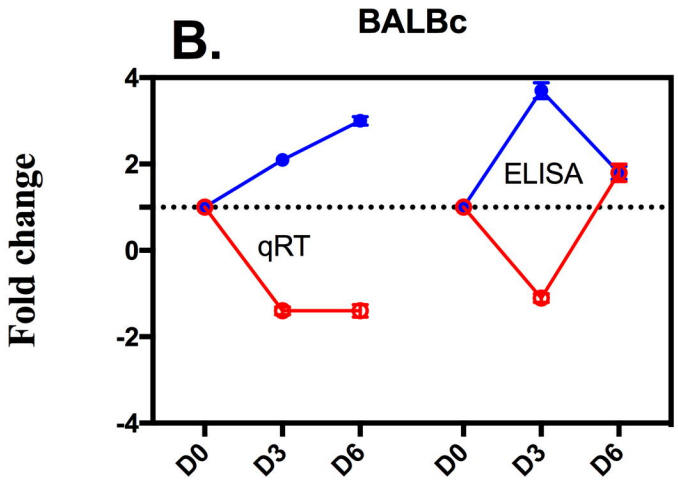

B.
Leptin(pg/ml)
5000
4000
3000
2000
1000
0
ns
ns
CB
VB3
VB6
CC
VC3
VC6
Conditions

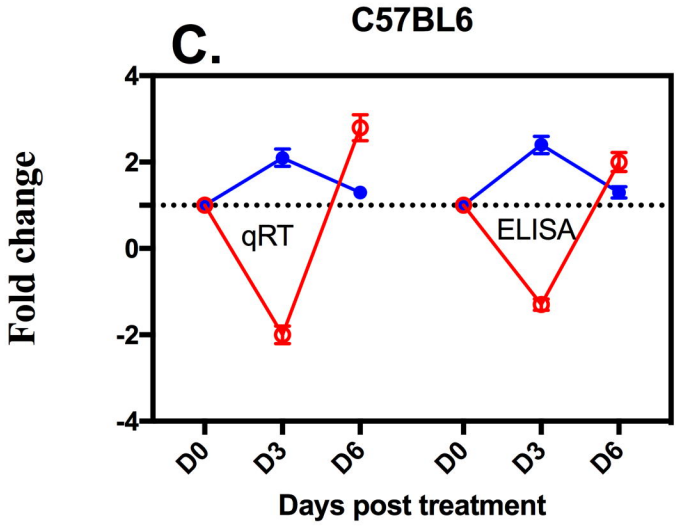

C.
PYY(pg/mg)
10000
8000
6000
4000
2000
0
***
***
***
***
***
*
CB
VB3
VB6
CC
VC3
VC6
Conditions

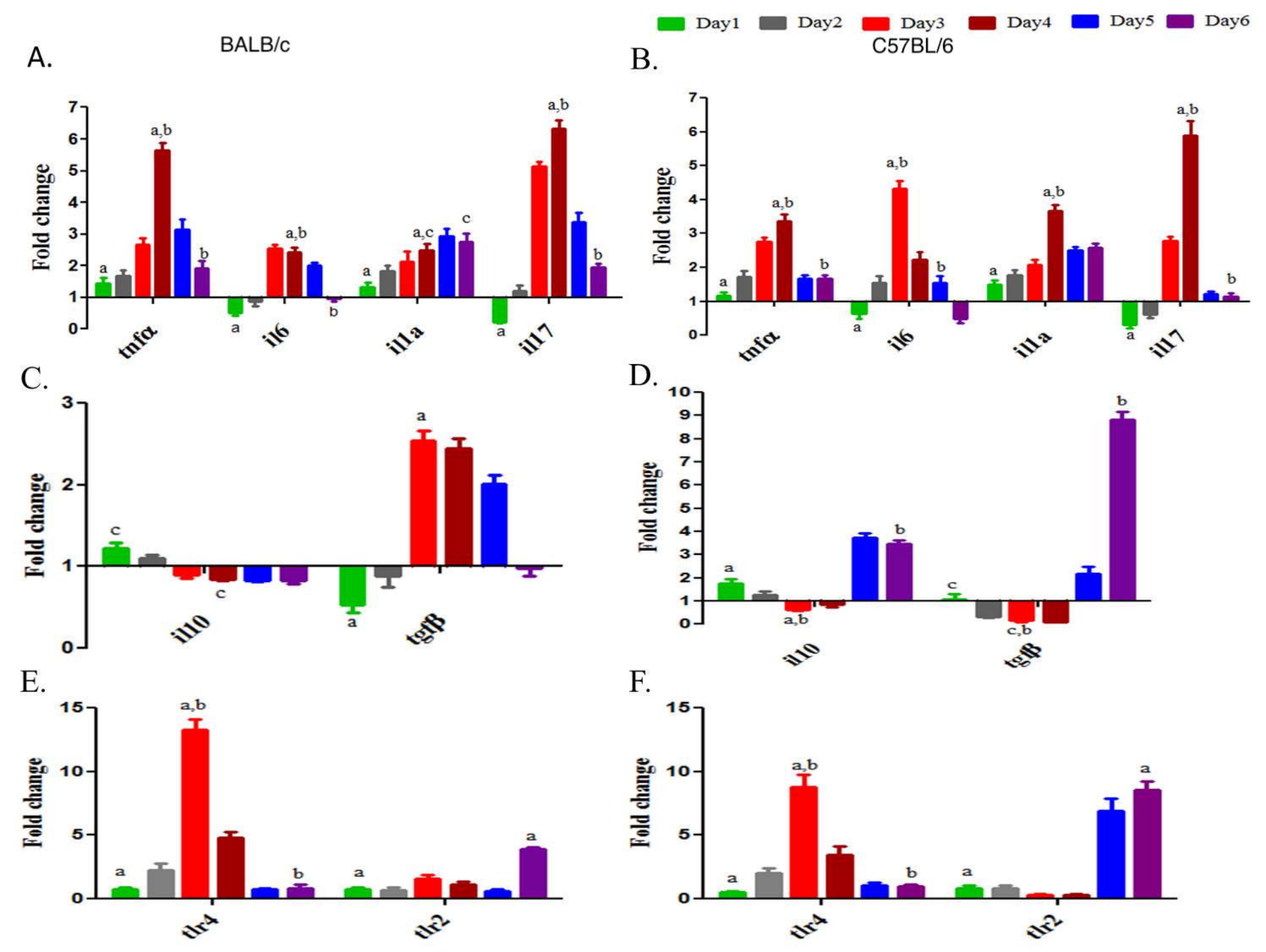

A.
BALB/c
conc (mg/dl)
Time (min)
0day
3rd day
6th day
CMT Treatment
B.
C57BL6
C.
Acetate/Lysine
Days
VB
CB
VC
CC
D.
Butyrate/Lysine
E.
Glutamate/Threonine
F.
Acetate concentration (μM)
CC6
VC6
CB6
VB6

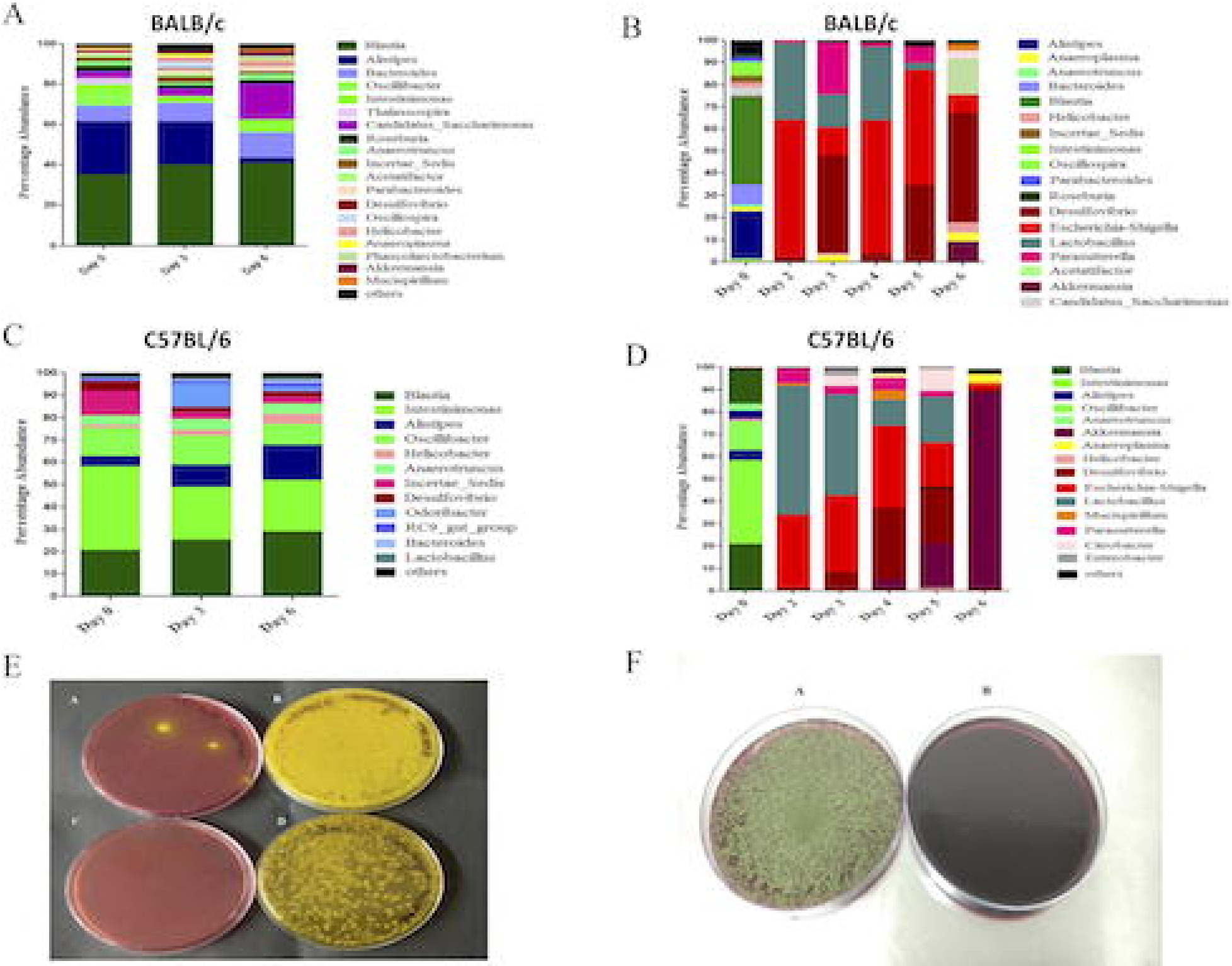

A.
Cecal Index
0.04
0.03
0.02
0.01
0.00
***
ns
***
ns
CB
VB3
VB6
CC
VC3
VC6
Conditions
B.
Cecal liquid content (μl)
300
200
100
0
***
***
CB
VB6
CC
VC6
Conditions
C.
1
2
3
4
D.
FITC concentration(ng/ml)
500
400
300
200
100
0
**
**
***
***
CB
VB3
VB6
CC
VC3
VC6
Conditions
E.
Day1
Day2
Day3
Day4
Day5
Day6
Fold change
3
2
1
0
a
b
a
b
claudin 1
occludin
F.
Fold change
3
2
1
0
a
b
a
b
claudin 1
occludin
G.
Endotoxin concentration (EU/ml)
1.0
0.8
0.6
0.4
0.2
0.0
**
*
**
CB
VB3
VB6
CC
VC3
VC6
Conditions

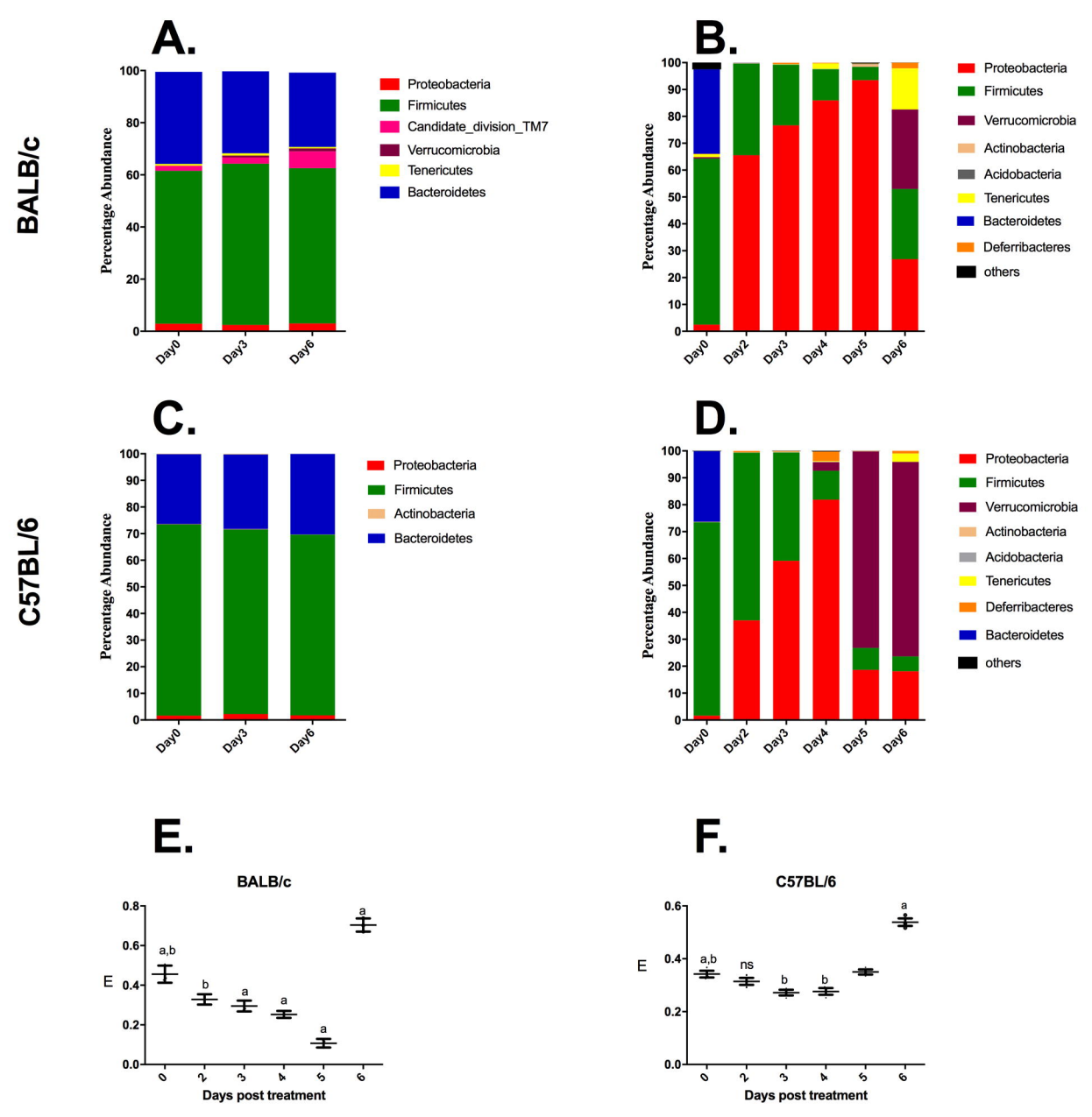

A.
ELISA
pg/mg
5000
4000
3000
2000
1000
0
TNFα
IL10
a
a
b
b
c
c
d
d
CB0
CB3
VB3
CB6
VB6
CC0
CC3
VC3
CC6
VC6
Conditions


B.
BALBc
Fold change
4
2
0
-2
-4
ELISA
qRT
D0
D3
D6
D0
D3
D6


C.
C57BL6
Fold change
4
2
0
-2
-4
qRT
ELISA
D0
D3
D6
D0
D3
D6
Days post treatment


Day1
Day2
Day3
Day4
Day5
Day6
A.
BALB/c
B.
C57BL/6
Fold change
tnfα
il6
il1a
il17
C.
D.
il10
tgfβ
E.
F.
tlr4
tlr2


A
BALB/c
Percentage Abundance
Day 0
Day 3
Day 6
Blautia
Alistipes
Bacteroides
Oscillibacter
Intestinimonas
Thalassospira
Candidatus_Saccharimonas
Roseburia
Anaerotruncus
Incertae_Sedis
Acetatifactor
Parabacteroides
Desulfovibrio
Oscillospira
Helicobacter
Anaeroplasma
Phascolarctobacterium
Akkermansia
Mucispirillum
others
B
BALB/c
Percentage Abundance
Day 0
Day 2
Day 3
Day 4
Day 5
Day 6
Alistipes
Anaeroplasma
Anaerotruncus
Bacteroides
Blautia
Helicobacter
Incertae_Sedis
Intestinimonas
Oscillospira
Parabacteroides
Roseburia
Desulfovibrio
Escherichia-Shigella
Lactobacillus
Parasuterella
Acetatifactor
Akkermansia
Candidatus_Saccharimonas
C
C57BL/6
Percentage Abundance
Day 0
Day 3
Day 6
Blautia
Intestinimonas
Alistipes
Oscillibacter
Helicobacter
Anaerotruncus
Incertae_Sedis
Desulfovibrio
Odoribacter
RC9_gut_group
Bacteroides
Lactobacillus
others
D
C57BL/6
Percentage Abundance
Day 0
Day 2
Day 3
Day 4
Day 5
Day 6
Blautia
Intestinimonas
Alistipes
Oscillibacter
Anaerotruncus
Akkermansia
Anaeroplasma
Helicobacter
Desulfovibrio
Escherichia-Shigella
Lactobacillus
Mucispirillum
Parasuterella
Citrobacter
Enterobacter
others
E
A
B
F
A
B
F
A
B


A.
B.
C.
D.
E.
F.
BALB/c
C57BL/6
Percentage Abundance
Proteobacteria
Firmicutes
Candidate_division_TM7
Verrucomicrobia
Tenericutes
Bacteroidetes
Actinobacteria
Acidobacteria
Deferribacteres
others
Day0
Day2
Day3
Day4
Day5
Day6
E
Days post treatment
a,b
b
a
ns