

1 Comparative analysis of beneficial effects of Vancomycin treatment on Th1- and Th2-biased  
2 mice and role of gut microbiota

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12 **Running Headline:** Vancomycin and gut microbiota

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14 **Abstract**

15 **Aims:** Vancomycin, an antibiotic, is used to treat infection of multi-drug resistant strains of  
16 *Clostridium difficile* and *Staphylococcus*. Post-usage effects of vancomycin may lead to many  
17 unwanted effects including perturbation of gut microbiota. Perturbation of the gut microbiota, by  
18 Vancomycin, was used to understand the altered metabolic and innate immune profile of  
19 C57BL/6(Th1- biased) and BALB/c (Th2-biased) mice.

20 **Methods and Results:** Following treatment with vancomycin till day 4, we observed reduction  
21 in abundance of phyla Firmicutes and Bacteroides and increase in Proteobacteria in the gut for  
22 both strains of mice. Results further revealed a significant increase in the phylum  
23 Verrucomicrobia, from day 5 onwards following treatment with vancomycin led to decreased  
24 inflammation and increased rate of glucose tolerance in the host.

25 **Conclusions:** Continued dosage of vancomycin was more beneficial in C57BL/6 than BALB/c  
26 mice

27 **Significance and Impact of the study:** The current study established that initial doses of  
28 vancomycin increased pathogenic bacteria but the continued doses of vancomycin provided  
29 significant health-related benefits to the host by decreasing pathogenic load and by increasing  
30 beneficial microbes of Verrucomicrobia phylum (*A. muciniphila*) more in C57BL/6 (Th-1) than  
31 BALB/c (Th-2) mice.

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33 **Keywords:** Antibiotics; Microbiome; Glucose metabolism; Insulin resistance; Vancomycin;  
34 Mouse; *Akkermansia muciniphila*; Gut permeability; Cecal microbiota transplantation (CMT)

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## 37 **Introduction**

38 More reports have started revealing that gut microbiota play an important role in  
39 maintaining health (Maldonado *et al.*, 2012; Jandhyala *et al.*, 2015; Andoh, 2016). Perturbation  
40 of gut microbiota can be used as an effective tool to understand its role in the host (Willing,  
41 Russell and Finlay, 2011). Abundance and diversity of gut microbiota change with different  
42 factors like age (Hill *et al.*, 2017), diet (Singh *et al.*, 2017), geography (Morton *et al.*, 2015;  
43 Falony *et al.*, 2016), stress (Bendtsen *et al.*, 2012), pathogen (Khan, 2014), and antibiotics  
44 (Lange *et al.*, 2016). Since treatment with antibiotics is still the most important and major  
45 avenue of dealing with many diseases, it is reported that the antibiotics could perturb the general  
46 taxonomy of the abundant and diverse gut microbes. Antibiotics are, therefore, being used as one  
47 of the most potent agents to study the role of gut microbiota (Jernberg *et al.*, 2010). The altered  
48 microbial profile may lead to different diseases including metabolic syndromes like diabetes,  
49 obesity, and Inflammatory Bowel Disease (Bosi *et al.*, 2006; Dunlop *et al.*, 2006). Among  
50 various antibiotics, vancomycin can cause drastic changes in the human gut microbiota by  
51 increasing pathogens and by decreasing the commensal healthy microbes (Isaac *et al.*, 2016).  
52 Vancomycin is majorly prescribed orally against the infection of two multi-drug resistant strains;  
53 i.e., *Clostridium difficile* and *Staphylococcus* (Bernard *et al.*, 2003; Pepin, 2008; Tang *et al.*,  
54 2015). Reports also revealed that vancomycin could reduce gut microbial diversity and adversely  
55 affect metabolism and immunity of host (Vrieze *et al.*, 2014; Isaac *et al.*, 2016; Reijnders *et al.*,  
56 2016). The kinetics and dynamics of treatment with vancomycin on gut microbiota, however, are  
57 yet to be established. Especially, if treatment with vancomycin could affect the gut microbiome  
58 of Th1 and Th2-biased individuals differently to alter host metabolism and innate immunity.  
59 There were some earlier reports that elucidated the intrinsic differences in immune and immune-  
60 metabolic responses between C57BL/6 (Th1-biased) and BALB/c (Th2-biased) mice (Watanabe  
61 *et al.*, 2004; Jovicic *et al.*, 2015). The current laboratory reported earlier the differences in the  
62 abundance and composition of the gut microbiota between the two strains (Th1- and Th2-biased)

63 of mice (Pradhan *et al.*, 2018). Gut microbiota composition and diversity significantly influenced  
64 the immune system, gut barrier integrity and SCFA production of the host (Cani and Delzenne,  
65 2009; Turner, 2009; Ulluwishewa *et al.*, 2011; Wu and Wu, 2012). In this current study, we  
66 reported the role of vancomycin on the gut microbiota of both Th1- and Th2-biased mice. Time-  
67 dependent increase in specific gut microbes like Proteobacteria and Verrucomicrobia might be  
68 associated with the alteration of immune regulation, gut barrier maintenance, glucose  
69 metabolism and SCFA production of the host. The alteration pattern of gut microbes through  
70 vancomycin treatment and its effect on the host could be varied significantly between BALB/c  
71 and C57BL/6 mice. The results from the present study revealed that the earlier part of the  
72 vancomycin treatment caused i) an increase in abundance of specific pathogens, and ii) a  
73 decrease in native microbes. In the later stage of the treatment, some beneficial microbes started  
74 appearing and pathogens started declining, which provided health benefits to the host.

## 75 **Material and methods**

76 **Animals Used in the study:** All mice of the same strain used in the present study were co-  
77 housed in polysulfone cage, and corncob was used as bedding material. Food and water were  
78 provided *ad libitum*. Animals were co-housed in a pathogen-free environment with a 12h light-  
79 dark cycle at temperature  $24 \pm 3^\circ$  with the humidity around 55%. The guidelines for animal  
80 usage were as per CPCSEA (Committee for the Purpose of Control and Supervision of  
81 Experiments on Animals, Govt. of India). All protocols were approved by the Institute Animal  
82 Ethics Committee constituted by CPCSEA (Reg. No.- 1643/GO/a/12/CPCSEA). 6-8 weeks old  
83 male C57BL/6 (Th1) and BALB/c (Th2) mice were used in the present study. Unless otherwise  
84 stated, we used at least 3 mice per treatment condition per time point.

85 **Antibiotic treatment:** Both Th1-(C57BL/6) and Th2- (BALB/c) biased mice were treated (n=3  
86 per group) with vancomycin (Cat#11465492) twice daily for 6 consecutive days. Vancomycin

87 was administered by oral gavaging at 50 mg per kg of body weight. The dosage was selected as  
88 per previous reports and FDA guidelines(Erikstrup *et al.*, 2015; Patel, Preuss and Bernice, 2019)

89 **Mice treatment and sample collection:** Mice were separated into two different groups: Control  
90 (untreated) and Treatment (groups that were treated with vancomycin). Each day of the  
91 experiment for 6 days, time-matched control and treated mice were euthanized by cervical  
92 dislocation. Colon tissue, serum and cecal materials were isolated from each mouse following  
93 the protocols described elsewhere. Tissue samples, not used immediately, were stored (-80°C)  
94 either by snap freezing for protein analysis or in RNAlater for RNA analysis until further used  
95 (Pradhan *et al.*, 2018; Ghosh *et al.*, 2019)

96 **Cecal Sample plating:** Cecal sample was collected from both strains of mice on day 4 following  
97 treatment with vancomycin. 50 mg of each sample was homogenized in 1ml of deionized MilliQ  
98 water and plated at a dilution of 10<sup>4</sup> fold on Salmonella-Shigella specific media and EMB (Eosin  
99 methylene blue agar plate) agar plate (Dekker and Frank, 2015).

100 **RNA extraction:** RNA was extracted from the colon tissue by using RNeasy mini kit (Cat#  
101 74104, Qiagen, Germany) following the manufacturer's protocol. 20-23 mg of tissue was  
102 processed using liquid nitrogen followed by homogenization in 700 µl of RLT buffer. An equal  
103 volume of 70% ethanol was added and mixed well. The solution was centrifuged at 8000g for 5  
104 minutes at room temperature. The clear solution containing lysate was passed through RNeasy  
105 mini column (Qiagen, Germany), which leads to the binding of RNA to the column. The column  
106 was washed using 700 µl RW1 buffer and next with 500 µl of RPE buffer. RNA was eluted using  
107 30 µl of nuclease-free water. RNA was quantified using NanoDrop 2000 (ThermoFisher  
108 Scientific, Columbus, OH, USA).

109 **cDNA preparation:** cDNA was synthesized by using Affinity Script One-Step RT-PCR Kit  
110 (Cat# 600559, Agilent, Santa Clara, CA, USA). RNA was mixed with random nonamer primers,

111 Taq polymerase, and NT buffer. The mixture was kept at 45 °C for 30 min for the synthesis of  
112 cDNA and temperature increased to 92 °C for deactivating the enzyme.

113 **Real-time PCR (qRT-PCR):** Real-time PCR was performed in 96-well plate, using 25 ng  
114 cDNA as template, 1 µM of each of forward (\_F) and reverse (\_R) primers for genes mentioned  
115 in Table 2, SYBR green master mix (Cat#A6002, Promega, Madison, WI, USA), and nuclease-  
116 free water. qRT-PCR was performed in Quantstudio 7 (Thermo Fisher Scientific, Columbus,  
117 OH, USA). All values were normalized with cycle threshold (Ct) value of GAPDH (internal  
118 control) and fold change of the desired gene was calculated with respect to the control C<sub>t</sub>-value  
119 as mentioned elsewhere (Pradhan *et al.*, 2016, 2018).

#### 120 **Cytokine Analysis at the protein level**

121 Colon tissues were collected from mice on day 0 (untreated control) and on days 3 and 6  
122 following treatment with vancomycin. After washing the colon tissues thoroughly, lysis buffer  
123 (Tris-hydrochloric acid, sodium chloride, and Triton X-100 in distilled water) containing 1X  
124 protease inhibitor cocktail (PIC) (Cat#ML051, Himedia, India) was used to churn the tissue [40].  
125 The supernatant was collected following centrifuging the churned mixture at 20,000g for 20  
126 minutes. ELISA (BD Biosciences, San Diego, CA, USA) was performed using the  
127 manufacturer's protocol for TNF $\alpha$  (Cat#560478) and IL10 (Cat#555252) expression [40].  
128 Protein concentration was normalized through Bradford assay (BD Biosciences, San Diego, CA,  
129 USA). The absorbance was taken using Multiskan Go (Thermo Fisher Scientific, Columbus, OH,  
130 USA).

131 **Serum collection:** Mice were anaesthetized and whole blood was collected by cardiac puncture.  
132 Blood was kept on ice for 30 mins and centrifuged at 1700g for 15 min at 4°C, and serum was  
133 collected for further analysis. If required, serum was stored at -80°C until further use.

134 **Genomic DNA extraction:** Cecal sample was collected from mice of both strains and gDNA  
135 was extracted using the phenol-chloroform method. 150-200 mg of cecal sample was used to

136 homogenize using 1ml of 1X PBS and centrifuged at 6700g for 10 minutes. The precipitate was  
137 lysed by homogenizing it in 1ml of lysis buffer (containing Tris-HCl 0.1M, EDTA 20 mM, NaCl  
138 100 mM, 4% SDS (at pH 8) and thereafter heating it at 80 °C for 45min. Lipid and protein were  
139 removed from the supernatant using an equal volume of phenol-chloroform, this process was  
140 repeated until the aqueous phase became colourless. DNA was precipitated overnight at -20 °C  
141 with 3 volumes of absolute chilled ethanol. Finally, it was washed with 500 µl of 70% chilled  
142 ethanol and briefly air-dried. The gDNA was dissolved in nuclease-free water and quantified  
143 using NanoDrop 2000.

#### 144 **16S-rRNA sequencing (V3-V4 Metagenomics):**

145 Using cecal DNA samples, V3-V4 regions of the 16S rRNA gene were amplified. For  
146 this amplification, V3F (Forward primer): 5'-CCTACGGGNBGCASCAG-3' and V4R (Reverse  
147 primer): 5'-GACTACNVGGGTATCTAATCC-3' primer pair was used. In Illumina Miseq  
148 platform, amplicons are sequenced using paired-end (250bpX2) with a sequencing depth of  
149  $500823.1 \pm 117098$  reads (mean  $\pm$  SD). Base composition, quality and GC content of FASTQ  
150 sequence were checked. More than 90% of the sequences had Phred quality scores above 30  
151 and GC content nearly 40-60%. Conserved regions from the paired-end reads were removed.  
152 Using the FLASH program, a consensus V3-V4 region sequence was constructed by removing  
153 unwanted sequences (Kim *et al.*, 2012; Mysara *et al.*, 2017). Pre-processed reads from all the  
154 samples were pooled and clustered into Operational Taxonomic Units (OTUs) by using de novo  
155 clustering method based on their sequence similarity using UCLUST program. QIIME was used  
156 for the OTU generation and taxonomic mapping (Caporaso *et al.*, 2010; Purcell *et al.*, 2017). A  
157 representative sequence was identified for each OTU and aligned against the Greengenes core set  
158 of sequences using the PyNASt program (DeSantis *et al.*, 2006b, 2006a; Frank *et al.*, 2007;  
159 Kastenberger *et al.*, 2012). Alignment of these representative sequences against reference  
160 chimeric data sets was done and the RDP classifier against the SILVA database was used for  
161 taxonomic classification to get rid of hybrid sequences.

162 **Cecal Microbiota Transplantation ( CMT):** Cecal sample was collected from sixth-day  
163 vancomycin treated mice and diluted with PBS (1gm per10ml) to make stock. 400  $\mu$ l of the stock  
164 of cecal material was orally gavaged to each of third-day vancomycin treated mice.

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

172 The same procedure was adopted for oral glucose tolerance test was done for third day CMT  
173 recipient mice following 24h of CMT procedure.

#### 174 **Sample preparation and NMR data acquisition for metabolomics study**

175 Serum was isolated from the blood of vancomycin treated and control mice as described  
176 before. Proteins in the serum were removed by passing it through a pre-rinsed (7 times washed)  
177 Amicon Ultra-2ml 3000 MWCO (Merck Millipore, USA) column. Centrifugation was done at  
178 4°C at 12,000g. Total of 700  $\mu$ L solution (containing serum sample, D<sub>2</sub>O, pH maintenance buffer  
179 and DSS) was taken in 5 mm Shigemi tubes. NMR for all samples were performed at 298K on a  
180 Bruker 9.4 T (400 MHz) AVANCE-III Nanobay liquid-state NMR spectrometer equipped with 5  
181 mm broadband (BBO) probe. The pre-saturation technique was used with a moderate relaxation  
182 delay of 5 seconds to ensure complete water saturation. Offset optimization was performed using  
183 a real-time 'gs' mode for each sample. Topspin 2.1 was used to record and process the acquired  
184 spectra.

#### 185 **Metabolomic Analysis of NMR data**



186           ChenomX (Canada) was used for the analysis of NMR data. The Bayesian approach is  
187 used to derive metabolite concentration in serum. The phase and baseline of the raw spectrum  
188 were corrected and concentrations of metabolites were obtained through a profiler using  
189 Metaboanalyst (Hapfelmeier *et al.*, 2005; DeSantis *et al.*, 2006a; Frank *et al.*, 2007; Xia and  
190 Wishart, 2011; Xia *et al.*, 2015; Zhou and Zhi, 2016). To normalize the data across the study,  
191 the samples were log-transformed and compared with the control sample. Relative fold-change  
192 values in metabolite expression analysis were performed for each treated samples with respect to  
193 the untreated time-matched control. More than 2-Fold change values (above or below reference  
194 value) with  $p \leq 0.05$  were considered for further analysis.

195 **Calculation of Cecal index:** The body weight of each individual mouse was measured and  
196 recorded. The whole cecal content was collected in a microfuge tube and weighed for each  
197 individual mouse. The cecal index was measured by taking the ratio of cecal content to the body  
198 weight of each mouse and used by normalizing the data with respect to the average body weight  
199 of mice used(Shi *et al.*, 2018).

200 **Gut permeability test by FITC dextran:** Gut permeability was determined by measuring the  
201 concentration of non-digestible dextran conjugated with fluorescein isothiocyanate in the serum.  
202 After oral administration, FITC dextran transits through the GI tract and crosses the intestinal  
203 epithelium. Mice used for this experiment were water-starved overnight. Next day morning,  
204 FITC-dextran (Cat#F7250, Sigma-Aldrich, Missouri, US) at a concentration of  $100\text{mg ml}^{-1}$  was  
205 dissolved in PBS and oral gavaged. After 4h, mice were anaesthetized by isoflurane inhalation  
206 and the blood was collected by cardiac puncture. Serum was collected from blood and  
207 concentration of FITC in serum was measured by spectrofluorometer (Varioskan,  
208 ThermofisherScientific) with an excitation wavelength of 485 nm (20 nm bandwidth) and  
209 emission of 528 nm (20 nm bandwidth)(Woting and Blaut, 2018).

210 **Endotoxin detection assay from serum:** Limulus Amebocyte Lysate (LAL) test was used for  
211 the detection of lipopolysaccharides located in the outer cell membrane of gram-negative  
212 bacteria. For this test, mice were sacrificed on days 0, 3 and 6 following treatment of mice with  
213 vancomycin and blood was collected by cardiac puncture in an endotoxin-free vial. Toxin sensor  
214 chromogenic LAL endotoxin assay kit from GeneScript (Cat#L00350, Piscataway, NJ, USA)  
215 was used for detecting endotoxin level in the serum of mice using the manufacturer's protocol  
216 (Holzheimer, 2014).

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

224 **Hormonal assay:** Leptin (Cat# ELM-Leptin), and Insulin (Cat# ELM-Insulin) hormone levels  
225 were assayed in serum samples. PYY (Cat# EIAM-PYY) was assayed in the colon tissue  
226 samples by using Raybiotech mouse hormonal assay kit (Norcross, Georgia, USA).

#### 227 **Statistical Analysis:**

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

#### 231 **Results**

##### 232 **Vancomycin treatment alters the abundance and diversity of gut microbiota**

233 Effective gut microbial perturbation by vancomycin treatment was previously reported (Isaac  
234 *et al.*, 2016) but the role of the immune profile of the host was not addressed. The mammalian  
235 hosts could be broadly discriminated based on the immune profile in terms of pro-inflammatory  
236 (Th1) and tolerogenic (Th2) responses. We, therefore, compared the differential effects, if any,  
237 of vancomycin on Th1- and Th2-immune biased mice strains (C57BL/6 and BALB/c). Changes  
238 in the gut (specifically small and large intestine) associated physical and morphological  
239 parameters are usually the first important signs to look for following antibiotic treatment  
240 (Jernberg *et al.*, 2010). We observed that the vancomycin treatment significantly increased the  
241 cecal index (an important parameter determined by cecum weight/body weight) in both strains of  
242 the mice. The increase in the cecal index also suggested the alteration of bacterial abundance in  
243 the cecum (Patel *et al.*, 2012; Shi *et al.*, 2018) (Table.1). We used 16S rRNA (metagenomic)  
244 based sequencing protocol to understand the kinetics of altered microbiota profile in the cecum  
245 following treatment with vancomycin. Metagenomic analysis of the cecal content revealed that  
246 the microbial composition was significantly altered in both BALB/c and C57BL/6 mice  
247 following treatment with vancomycin (Figure 1). The results of untreated mice, shown in Figure  
248 1A and Figure 1C, mainly revealed that in both BALB/c and C57BL/6 the gut microbiota overtly  
249 belong to the phyla, Firmicutes and Bacteroidetes. The abundance of the phyla, Firmicutes and  
250 Bacteroidetes, reduced while the abundance of Proteobacteria phylum increased significantly by  
251 the second day following treatment with vancomycin. The Proteobacteria level reached  
252 maximum by day five (93% of total abundance) in BALB/c mice and day four (81% of total  
253 abundance) in C57BL/6 following treatment with vancomycin (Figure 1B and Figure 1D). On  
254 the contrary, Firmicutes level decreased from 70-80% (untreated control group) to below 10%  
255 (fourth day following treatment with vancomycin) and Bacteroidetes level from 25-30 %  
256 (untreated group) to 1% (fourth day following treatment with vancomycin) in both BALB/c and  
257 C57BL/6 mice (Figure 1B and Figure 1D). After day 4 of treatment with vancomycin, BALB/c  
258 and C57BL/6 mice showed a significantly different gut microbiota profile with the appearance of  
259 phylum, Verrucomicrobia. A sudden increase in Verrucomicrobia phylum, from day five

260 onwards, in C57BL/6 and from day six onwards, in BALB/c mice following vancomycin  
261 treatment replaced the previously predominant Proteobacteria phylum. However, the abundance  
262 of Verrucomicrobia phylum was found to be significantly higher, in C57BL/6 mice (72% of total  
263 abundance), than in BALB/c mice (30% of total abundance) on the day six following treatment  
264 with vancomycin. This result was significant to understand the differential response exhibited in  
265 two different strains of mice (C57BL/6 and BALB/c) used in this study following treatment with  
266 vancomycin.

267 In addition, we further determined the Shannon diversity of the cecal microbiota.  
268 Shannon equitability index at the phylum level showed a decrease in diversity up to the fifth day  
269 in BALB/c (Figure 1E) and up to the fourth day in C57BL/6 mice (Figure 1F) following  
270 treatment with vancomycin. Microbial diversity was increased on day 6 for BALB/c and day 5  
271 for C57BL/6 following treatment with vancomycin.

272 Since each phylum contains various genera, we were interested to find out the changes in  
273 the abundance and diversity at the genus level following vancomycin treatment. At the genus  
274 level, the gut microbiota of untreated time-matched control mice majorly composed of Blautia,  
275 Intestinimonas genera of Firmicutes phylum and Bacteroides, Alistipes genera of Bacteroidetes  
276 phylum in both strains (Figure 2A and Figure 2C). Whereas, on day four, the gut microbiota of  
277 vancomycin treated mice showed mostly Escherichia-Shigella and Desulfovibrio genus from  
278 Proteobacteria phylum in both strains of mice(Figure 2B and Figure 2D). These results were  
279 further validated by plating day four cecal homogenate in specific media- EMB agar (*E.coli*) and  
280 Salmonella-Shigella agar plate (*Shigella sp.*). Plating data of cecal samples from day four  
281 following vancomycin treatment showed overgrown colonies compared to untreated mice on the  
282 specific media (Figures 2E and 2F). On the day six following vancomycin treatment, the genus  
283 level data showed a predominance of *Akkermansia* in both strains of mice. However,  
284 *Akkermansia muciniphila* level was higher in C57BL/6 mice than BALB/c mice. We performed  
285 16S based qPCR by using *Akkermansia muciniphila* species-specific primers to confirm

286 metagenomic data (Table 4). The qPCR results revealed that there were nearly 21 fold and 24833  
287 fold increase in *A. muciniphila* abundance in vancomycin treated BALB/c and C57BL/6 mice  
288 respectively on day six compared to their time-matched untreated control mice. While on day  
289 three following vancomycin treatment, due to very low abundance of *A. muciniphila*, the value  
290 of threshold cycle ( $C_t$ ), from qPCR data, could not be determined for either of BALB/c or  
291 C57BL/6 mice. The change in the abundance of *A. muciniphila* was found to be comparable in  
292 both metagenomic and qPCR analysis.

293 Above results indicated that both the mice strains showed an initial increase in Proteobacteria  
294 abundance following treatment with vancomycin in a time-dependent manner followed by an  
295 increase in abundance of Verrucomicrobia Phylum by day six. Also, the differential abundance  
296 of *A. Muciniphila* on day six showed a difference in the response of vancomycin perturbation of  
297 gut microbiota between two strains of mice.

### 298 **The inflammatory response in the colon changes during vancomycin mediated microbiota** 299 **perturbation in a time-dependent manner.**

300 Antibiotic induced gut microbiota perturbation is associated with different inflammatory  
301 disorders (Willing *et al.*, 2011; Miyoshi *et al.*, 2017). We checked the effect of vancomycin  
302 mediated microbial perturbation on the expression of various pro-(*tnfa*, *il6*, *il1a*, *il17*) and anti-  
303 inflammatory (*tgfb* and *il10*) genes in both mice strains. The mRNA level expression data from  
304 colonic tissue revealed the time-dependent increase of pro-inflammatory cytokines till day four  
305 of treatment in both mice strains (Figures 3A and 3B). A decrease in the expression of the pro-  
306 inflammatory cytokines was associated with the decrease of Proteobacteria abundance after day  
307 four of vancomycin treatment (Figures 3C and 3D). Next, we also observed a marked increase of  
308 *tlr4* expression, upstream regulator of the inflammatory response (Wahid *et al.*, 2015) on the  
309 third day and decreased by the sixth day of treatment in both mice strains (Figures 3E and 3F).  
310 However, we found a significant increase in the expression of *tlr2* on day five and day six

311 following vancomycin treatment in C57BL/6 mice compared to BALB/c mice. The increase of  
312 *tlr2* gene expression was correlated with the higher abundance of *A. muciniphila* during the day  
313 five and day six following vancomycin treatment in C57BL/6 mice. Validation of qRT-PCR  
314 results was done, at the protein level expression by ELISA (Figures 4A and 4C). ELISA results  
315 revealed that on the third day following vancomycin treatment, TNF $\alpha$  level was significantly  
316 more in both BALB/c and C57BL/6 mice with respect to the third day time-matched untreated  
317 groups of mice. Similarly, IL10 cytokine level was more in BALB/c and C57BL/6 on day six  
318 following treatment with vancomycin compared to the day sixth time-matched untreated mice  
319 (Figures 4A and 4C). Both qRT PCR and ELISA data showed nearly similar results for the  
320 expression of pro- and anti-inflammatory cytokines (Figures 4B and 4D). In summary, our data  
321 suggest that the pro-inflammatory response in colonic tissue is linked with increased  
322 Proteobacteria abundance during vancomycin mediated microbial disruption. The emergence of  
323 Verrucomicrobia phyla from the fifth day onwards may lead to a transition from pro-  
324 inflammatory to anti-inflammatory response irrespective of initial immune bias of the mice.  
325 However, on the sixth day following vancomycin treatment, the decrease of pro-inflammatory  
326 cytokine and an increase of anti-inflammatory cytokine expression was more significant in  
327 C57BL/6 mice compared to BALB/c mice. This result can be correlated with the difference in  
328 the abundance of Verrucomicrobia phylum between BALB/c and C57BL/6 mice on the sixth  
329 day following vancomycin treatment.

### 330 **Effect of vancomycin treatment on gut barrier integrity :**

331 Previous reports stated that gut microbiota is responsible to maintain the gut barrier  
332 integrity(Ulluwishewa *et al.*, 2011; Feng *et al.*, 2019).Although the effect of microbiota  
333 perturbation on gut barrier integrity of different immune biased mice ( Th1 and Th2) is not clear.  
334 The current results revealed that treatment, with vancomycin for three days, massively disrupts  
335 the gut barrier integrity as was evident from FITC-dextran based gut permeability assay. Serum  
336 FITC-dextran level was significantly higher in day 3 treated mice compared to the day 0 control

337 mice. But surprisingly the level decreased to normal (day 0 control) on the sixth day of treatment  
338 in both mice strains (Figure 5C). These results prompted us to evaluate the gene expression of  
339 different colonic tight junction proteins (occludin and claudin1) that maintain the barrier function  
340 of gut (Chelakkot, Ghim and Ryu, 2018). Results revealed that the expression of claudin1 gene  
341 decreased continuously from day one to day six following treatment with vancomycin in  
342 BALB/c and C57BL/6 mice (Figures 5A and 5B). However, the expression of occludin gene  
343 decreased continuously from day zero to day six in BALB/c mice and from day zero to day four  
344 in C57BL/6 mice. On day five and six, in C57BL/6 mice, we observed a slight increase in  
345 occludin gene expression compared to its day three following vancomycin treatment.

346 Till day three following vancomycin treatment, both FITC dextran data and expression of  
347 tight junction genes showed almost similar results, i.e. decrease in gut barrier integrity. While on  
348 day six following vancomycin treatment, FITC-dextran studies suggested restoration of the gut  
349 barrier for both BALB/c and C57BL/6 mice, but the expression of claudin1 and occludin genes  
350 comparatively remained repressed with respect to their time-matched control mice. Further  
351 studies are required to understand this apparent discrepancy which may be due to a different  
352 mechanism, independent of these two genes that may exist to re-establish the gut barrier  
353 function.

354 In agreement with increased gut permeability, we intended to see whether it also induce  
355 the translocation of microbial products into the systemic circulation, as a result of barrier  
356 disruption. In both strains, serum endotoxin level was highest on the third day following  
357 vancomycin treatment compared to control mice and it was reduced on the sixth day of treatment  
358 (Figure 5D). On day six, BALB/c mice had slightly higher endotoxin level in serum compared to  
359 C57BL/6 mice. From the above findings, it is clear that the disruption of gut barrier integrity is  
360 strongly associated with the Proteobacteria level, whereas restoration is associated with  
361 Verrucomicrobia abundance in both mice strains



362 **Differential level of Verrucomicrobia in gut regulates blood glucose level following**  
363 **vancomycin treatment.**

364 Antibiotic mediated gut microbiota perturbation can affect different host metabolic  
365 functions, one such measurement involves the regulation of blood glucose homeostasis (Vrieze  
366 *et al.*, 2014; Zarrinpar *et al.*, 2018; Khan *et al.*, 2019). Our results showed a high abundance of  
367 Verrucomicrobia phylum on the 6th day of vancomycin antibiotic treatment. Since previous  
368 studies reported that *Akkermansia* sp. from Verrucomicrobia phylum positively regulated  
369 glucose metabolism(Dao *et al.*, 2016; Plovier *et al.*, 2017) we intended to see how vancomycin  
370 induced time-dependent change in microbiota profile regulates blood glucose level. We  
371 performed an oral glucose tolerance test (OGTT) from 0 to 90 minutes in both strains of mice.  
372 From the glucose tolerance test, it was revealed that glucose metabolism was different in control  
373 and vancomycin treated mice (Figures 6A and 6B). Important to note that the results from  
374 OGTT studies for control animals for both BALB/c and C57BL/6 remain unchanged on days  
375 zero, three and six (data not shown). On the day third following vancomycin treatment, fasting  
376 blood glucose (0<sup>th</sup> min) levels in the Th2- and Th1-biased mice (BALB/c  $194.6 \pm 6.3$  mg dl<sup>-1</sup> and  
377 C57BL/6  $186 \pm 6$  mg dl<sup>-1</sup>) were significantly higher than their respective zero-day untreated  
378 (BALB/c  $115 \pm 3$  mg dl<sup>-1</sup> and C57BL/6  $126 \pm 4$  mg dl<sup>-1</sup>) mice. On day sixth following vancomycin  
379 treatment glucose levels dropped (BALB/c  $148.6 \pm 7$  mg dl<sup>-1</sup> and C57BL/6  $103 \pm 5$  mg dl<sup>-1</sup>). The  
380 reduction of blood glucose level on day six following vancomycin treatment was more  
381 prominent in C57BL/6 compared to BALB/c mice (Figures 6A and 6B).The metabolism rate of  
382 glucose in the blood of the sixth-day vancomycin treated mice was faster than the third day  
383 treated mice. This rate was higher in vancomycin treated C57BL/6 mice compared to BALB/c  
384 mice. Next, we hypothesized that the differential level of Verrucomicrobia of C57BL/6 and  
385 BALB/c mice has an effect on the blood glucose level. To prove the causal role of  
386 Verrucomicrobia, we transplanted the cecal microbiota from sixth-day vancomycin treated mice  
387 (high Verrucomicrobia) to third-day vancomycin treated mice. We observed a significant drop in



388 blood glucose level in the third-day vancomycin treated C57BL/6 mice after CMT. However, the  
389 blood glucose level remained unchanged even after CMT in the third-day vancomycin treated  
390 BALB/c mice (Figures 6A and 6B). These data together suggest that high Verrucomicrobia level  
391 on the sixth day treated C57BL/6 mice actually improves blood glucose level, which is not  
392 possible with a comparatively low level of Verrucomicrobia present in BALB/c mice following  
393 sixth-day vancomycin treatment.

#### 394 **Metabolites level in serum changes following vancomycin treatment**

395 Antibiotic treatment can drastically reduce the Short-chain fatty acids (SCFAs) level,  
396 which are important regulators of several host physiological functions (Zarrinpar *et al.*, 2018;  
397 Venegas *et al.*, 2019). We measured the amount of SCFAs in the host serum using NMR based  
398 metabolomics. The current results showed that the ratios of the abundance of butyrate/lysine  
399 (Figure 6C) and propionate /threonine (Figure 6D) (Table.3) in serum were reduced significantly  
400 on day six following treatment with vancomycin. This might indicate the significant decrease in  
401 the conversion of SCFAs such as butyrate from lysine and propionate from threonine of  
402 vancomycin treated mice. The results further suggested the accumulation of the substrate (lysine,  
403 threonine) in blood and less detection of SCFA in the vancomycin treated day six group of mice  
404 (VB and VC on D6). We further measured the abundance of acetate in the serum of both  
405 BALB/c and C57BL/6 mice by using an acetate detection kit (Figure 6E). In both BALB/c and  
406 C57BL/6 mice, the concentrations of acetate on the day six following vancomycin treatment was  
407 found to be significantly lower than their respective time match untreated group of mice. Hence,  
408 these results indicate that the serum SCFA level decreased with vancomycin treatment and high  
409 Verrucomicrobia abundance on the 6th day cannot restore the SCFA level in both Th1 and Th2  
410 biased mice.

#### 411 **Effect of vancomycin treatment on metabolic hormones**

412 Our previous results indicated that the rate of glucose metabolism and SCFA concentration in  
413 serum altered during vancomycin treatment. Circulating SCFAs are also related with different  
414 metabolic hormones(Larraufie *et al.*, 2018; Müller *et al.*, 2019) Considering that we further  
415 measured associated metabolic hormones such as insulin, Peptide tyrosine tyrosine (PYY) and  
416 leptin (regulates appetite) in mice during vancomycin treatment. Results revealed that insulin  
417 level decreased significantly on the day six compared to day three following treatment in  
418 C57BL/6 mice, but not in the BALB/c mice (Figure 7A). Hence, vancomycin treatment on day  
419 six showed a reduced amount of serum insulin concomitant with the blood glucose level in  
420 C57BL/6 mice. However, insulin levels on day three following vancomycin treatment were  
421 significantly higher compared to their respective day zero untreated group of mice for both  
422 strains. No significant changes were observed in serum leptin concentration during vancomycin  
423 treatment in both BALB/c and C57BL/6 mice with respect to their respective untreated controls  
424 (Figure 7B). Further results revealed that the concentration of PYY hormone in the gut  
425 decreased in both BALB/c and C57BL/6 mice from day zero to day six following vancomycin  
426 treatment (Figure 7C) Hence, our results suggest that vancomycin mediated gut microbiota  
427 perturbation may regulate blood glucose and insulin level differentially between C57BL/6 and  
428 BALB/c mice in a time-dependent manner.

429 .

## 430 **Discussion**

431 It was reported earlier that the treatment with vancomycin reduced the abundance and  
432 diversity of the gut microbiota (Vrieze *et al.*, 2014; Isaac *et al.*, 2016; Sun *et al.*, 2019). The  
433 current study corroborated with the fact that mice treated with vancomycin had decreased levels  
434 of Firmicutes and Bacteroidetes and elevated levels of Proteobacteria in the initial four days. It is  
435 apparent that the increase in the one phylum would repress the abundance of other phyla and as a  
436 sequel, the overall diversity of the gut microbiota would decrease (Mosca, Leclerc and Hugot,

437 2016). In the current study, we observed an increase in only Proteobacteria phylum caused a  
438 decrease in the diversity of gut microbiota. Escherichia and Shigella genera of Proteobacteria  
439 phylum belong to the Gram-negative group of bacteria which caused an increase in endotoxin  
440 level in the blood through their LPS (Steimle, Autenrieth and Frick, 2016). These bacteria  
441 activate the TLR4 receptor present in the gut epithelial cell, and this causes increases in the  
442 expression of pro-inflammatory cytokines (Akira and Hemmi, 2003; Rallabhandi *et al.*, 2008). In  
443 the current study, concerted effects of the increase in pathogenic Proteobacteria and a decrease in  
444 Firmicutes in the gut caused increased inflammation and endotoxin level of mice during initial  
445 days of vancomycin treatment. Firmicutes, specifically the Clostridium group present in the gut,  
446 produces short-chain fatty acids like acetate, butyrate, propionate from complex carbohydrate  
447 foods (Venegas *et al.*, 2019). Bacteria belong to Instentimonas (Firmicutes phylum), produce  
448 butyrate from lysine and Bacteroidetes produces propionate from threonine in the gut (Bui *et al.*,  
449 2015; Neis, Dejong and Rensen, 2015).The production of these SCFAs in the gut suppresses the  
450 LPS and pro-inflammatory cytokines like TNF- $\alpha$ , IL-6 level and enhances the release of the anti-  
451 inflammatory cytokine like IL10 (Vinolo *et al.*, 2011; Morrison and Preston, 2016). The current  
452 study revealed a decrease in Firmicutes and Bacteroidetes levels following treatment with  
453 Vancomycin till day four. The decrease, in the levels of Firmicutes and Bacteroidetes, may result  
454 in less yield of SCFAs and higher production of inflammatory cytokines in mice.

455 It is well established that over-expression of Inflammatory cytokine like TNF $\alpha$  is associated with  
456 the higher gut permeability by suppressing the expression of tight junction proteins like occludin  
457 and claudin 1 (Al-Sadi *et al.*, 2013; Rios-Arce *et al.*, 2017). Initial days of vancomycin  
458 treatment caused the lower expression of tight junction protein resulting in increased gut  
459 permeability. During obesity and diabetic condition, metabolic endotoxemia had been observed  
460 where endotoxin (LPS) level increased in the blood to cause inflammation and impaired glucose  
461 metabolism of the host (Hawkesworth *et al.*, 2013; Boutagy *et al.*, 2016). The current report  
462 revealed a higher concentration of fasting insulin and glucose in the serum of BALB/c and

463 C57BL/6 mice on day 3 following vancomycin treatment compared to the control mice. The  
464 increased glucose and serum insulin level hinted at insulin resistance. The changes, in glucose  
465 and insulin levels, were associated with the higher abundance of Proteobacteria and endotoxin  
466 level on the day three following vancomycin treatment in both strains of mice.

467 The changes, in gut microbiome profile till day 4, were different from post day four of treatment,  
468 with Vancomycin. The profile of the changes in gut microbiota was distinct and varied between  
469 C57Bl/6 and BALB/c mice. It was reported that an increased abundance of Verrucomicrobia  
470 caused a decrease in inflammation and enhanced glucose metabolism of the host(Fujio-Vejar *et*  
471 *al.*, 2017; Plovier *et al.*, 2017; FUJISAKA *et al.*, 2018). On the sixth day of vancomycin  
472 treatment, in the current study, a decrease in Proteobacteria and increase in Verrucomicrobia  
473 caused an anti-inflammatory effect and enhanced glucose metabolism in the gut. Specifically in  
474 C57BL/6 mice, with a significant decrease in Proteobacteria and increase in Verrucomicrobia  
475 phylum on day six following vancomycin treatment caused decreased tlr4 expression and  
476 increased tlr2 expression in the gut. However, on the sixth day following vancomycin treatment,  
477 replacement of Proteobacteria by Verrucomicrobia caused significant improvement in glucose  
478 metabolism of mice by bringing back the fasting glucose and insulin level in the blood to normal  
479 level. Following a successful transfer of the cecal sample from the sixth day of vancomycin  
480 treated C57BL/6 mice (*A. muciniphila* level is above 70%) to the third day of vancomycin  
481 treated mice caused a significant decrease in blood glucose level of third-day vancomycin treated  
482 mice. The glucose level decrease following cecal microbiota transplant was comparable to the  
483 control glucose level. Since sixth-day vancomycin treated mice had a significantly higher level  
484 of Akkermansia, the current report prospectively hinting at the effective causal role of *A.*  
485 *muciniphila* in controlling the blood glucose level. On the sixth day of vancomycin treatment, *A.*  
486 *muciniphila* level was significantly higher in C57BL/6 mice than BALB/c. This higher level of  
487 Akkermansia is a very good supportive evidence of our suggestion of the role of *A. muciniphila*  
488 in reversing the glucose level to normal. This higher level of Verrucomicrobia in C57BL/6 could

489 have caused a more prominent effect in decreasing glucose level and increasing insulin  
490 sensitivity in the blood of C57BL/6 than BALB/c mice.

491 Reports also suggested that higher abundance or production of SCFA usually leads to anti-  
492 inflammatory response (Vinolo *et al.*, 2011). The current study further confirmed the  
493 proposition by showing higher SCFA yield in C57BL/6 than BALB/c. This observation is also in  
494 corroboration with the Th1-bias of C57BL/6 over Th2-immune bias of BALB/c mice. Reports  
495 also revealed that SCFA could stimulate PYY hormone (explain briefly the function or  
496 importance of PYY) production by activating Gq-coupled receptor, FFAR2, of endocrine cells  
497 present in the gut (Cahill *et al.*, 2014; Larraufie *et al.*, 2018). The current results revealed that  
498 following treatment with vancomycin the level of SCFA decreased. The decrease in SCFA level  
499 is concomitant with the reduction in the production of serum PYY. The observations so far  
500 prompted us to conclude the following proposition.

501 Host genetics is one of the major factors that regulate the gut microbiota composition and  
502 ecosystem (Korach-Rechtman *et al.*, 2019). C57BL/6 and BALB/c are two genetically different  
503 inbred mouse lines, which are respectively Th1 and Th2 immune biased mouse strains and differ  
504 in their baseline microbiota composition (Watanabe *et al.*, 2004; Fransen *et al.*, 2015). Here we  
505 found that the gut microbial population responds differentially against the vancomycin challenge,  
506 which is associated with higher a) abundance of Verrucomicrobia and b) production of SCFAs  
507 in C57BL/6 compared to BALB/c mice. The changes in the gut microbiota through vancomycin  
508 perturbation can alter host metabolism like glucose tolerance significantly between two strains of  
509 mice. Overall, the time-dependent perturbation of gut microbiota by vancomycin was not  
510 random. It followed a particular pattern. It affects the host in two different ways; Initial doses  
511 caused increased in pathogenic bacteria in the gut which caused a most deleterious effect on the  
512 host while continued later doses of vancomycin caused in increased Verrucomicrobia phylum in  
513 the gut which showed some beneficial effects on the host.

514 **Author Contribution Statement**

515 PR performed all experiments and drafted the manuscript. PR and PA designed the  
516 experiments. UP designed critical parts of some experiments and also contributed to the  
517 manuscript preparation. PA conceptualized, supervised the studies and finalized the manuscript.

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524 **Conflict of Interest**

525 The authors declare that there is no conflict of interest.

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## 761 **Figure Legends**

762 Figure 1. Phylum level changes in the gut microbiota. Time dependent changes in the phyla of  
763 gut microbiota, in the control A. BALB/c and C. C57BL/6 and in vancomycin treated B.  
764 BALB/c and D. C57BL/6 mice are shown. In the figure, only major phyla are shown to avoid  
765 clutter. Data shown are average of 3 biological replicates. To avoid clutter, standard deviation  
766 (SD) calculated using 2-way ANOVA is not shown. However, SD was less than 10% on average.  
767 Kinetics of changes in phylum level Equitability index (E) (diversity) of the gut microbiota  
768 following treatment with vancomycin in E. BALB/c and F. C57/BL6 mice. Statistical  
769 significance of diversity in panels E. And F. was calculated by two-way ANOVA. (‘\*\*\*\*’  
770 corresponds to  $P \leq 0.001$ , ‘\*\*\*’ corresponds to  $P \leq 0.01$ , ‘\*’ corresponds to  $P \leq 0.05$  level of  
771 significance). Error bars are one standard deviation of the mean value and determined from the  
772 average values of biological replicates.



773 Figure 2. Metataxonomic studies of genus level variation of gut microbiota in vancomycin  
774 treated and its respective control group. Kinetics of changes in genera of gut microbiota, in the  
775 control A. BALB/c, and C. C57BL/6 and in vancomycin treated B. BALB/c, and D. C57BL/6  
776 mice are shown. Data shown are average of 3 biological replicates and standard deviation was  
777 less than 10% on average. Percentage abundance of different genera for various treatment  
778 conditions are shown on the 'Y'-axis and the days elapsed post treatment or for time matched  
779 control are shown on the 'X'-axis. Colony of culturable Proteobacteria by plating of cecal  
780 samples from both strains of mice on selective and differential media. Evidence of *E. Shigella*  
781 colonies growth on day 4 on Salmonella-Shigella specific media agar plate 1. control C57BL/6,  
782 2. vancomycin treated C57BL/6, 3. control BALB/c and 4. vancomycin treated BALB/c and F.  
783 Growth of *E. coli*. colonies on day 4 on EMB (Eosin methylene blue agar plate), 1. vancomycin  
784 treated C57BL/6 and 2. control C57BL/6.

785 Figure 3. Transcriptional gene expression profile. Kinetics of transcriptional (by qRT-PCR)  
786 expression of genes categorized as, pro-inflammatory in A. BALB/c, and B. C57BL/6, anti-  
787 inflammatory in C. BALB/c, and D. C57BL/6 as Toll like receptors *tlr4* and *tlr2* in E. BALB/c,  
788 and F. C57BL/6 mice. Statistical significance was calculated by two-way ANOVA. ('\*\*\*'  
789 corresponds to  $P \leq 0.001$ , '\*\*' corresponds to  $P \leq 0.01$ , '\*' corresponds to  $P \leq 0.05$  level of  
790 significance). Error bars are one standard deviation of the mean value and determined from the  
791 average values of three biological replicates.

792 Figure 4. Protein level gene expression and comparative analysis of qRT PCR and ELISA data  
793 for TNF $\alpha$  and IL10. Mean values (n=3) of protein level concentration (in pg mg<sup>-1</sup>) with standard  
794 deviation of TNF $\alpha$  (blue) and IL10 (red) expression on days 0, 3 and 6 for control and  
795 vancomycin treated A. BALB/c (CB and VB) and C. C57BL/6 (CC and VC) are shown.  
796 Statistical significance was calculated by two-way ANOVA ('\*\*\*' corresponds to  $P \leq 0.001$ ,  
797 '\*\*' corresponds to  $P \leq 0.01$ , '\*' corresponds to  $P \leq 0.05$  level of significance). Fold change  
798 values of expression of TNF $\alpha$  (blue) and IL10 (red) to compare the values obtained from qRT-

799 PCR and ELISA studies are shown for B. BALB/c, and D. C57BL/6. Error bars of the data are  
800 already shown in preceding figures.

801 Figure 5. Measurement of intestinal integrity of BALB/c and C57BL/6 mice following treatment  
802 with Vancomycin. Transcriptional expression levels of tight junction genes, claudin 1 and  
803 occludin, in gut tissue by qRT-PCR are shown in vancomycin treated and untreated (control)  
804 groups for A. BALB/c and B. C57BL/6 mice C. Gut permeability data by measuring FITC  
805 dextran concentration in serum. D. Endotoxin concentration in the serum for both strains of mice  
806 are shown.

807 where CB, VB3 and VB6 implies untreated (control), day 3 (D3) and day 6 (D6) post  
808 vancomycin treated BALB/c and CC, VC3 and VC6 denote the same for C57BL/6 mice. The  
809 colors corresponding to different days are shown above the panels A. and B. Comparisons  
810 among the groups were calculated by two-way ANOVA. In the figure for panels 'A.', 'B.', 'C.'.  
811 and 'D.', '\*\*\*' corresponds to  $P \leq 0.001$ , '\*\*' corresponds to  $P \leq 0.01$ , '\*' corresponds to  $P \leq$   
812 0.05 level of significance.

813 Figure 6. Glucose tolerance and abundance of select metabolites in serum. Kinetics of fasting  
814 blood sugar in A. BALB/c B. C57BL/6 mice following treatment with Vancomycin on days 0, 3  
815 and 6 and following treatment with CMT from day 6 vancomycin treated mice transferred to  
816 vancomycin treated day 3 group of mice. Ratio of abundance, from chemometric<sup>1</sup>H-NMR  
817 studies for major short chain fatty acids, of C. butyrate production over lysine and D. propionate  
818 production over threonine in untreated control BALB/c (CB) and C57BL/6 (CC) and  
819 Vancomycin treated BALB/c (VB) and C57BL/6 (VC) are compared for Day 0 and Day 6  
820 following treatment with vancomycin. In addition, E. acetate concentration in the serum by using  
821 acetate detection kit on day 6 in vancomycin treated groups of mice (VB6, VC6) along with the  
822 time matched control mice (CB6, CC6) of BALB/c and C57BL/6, respectively. In the figure,

823 ‘\*\*\*\*\*’ corresponds to  $P \leq 0.0001$ , ‘\*\*\*’ corresponds to  $P \leq 0.001$ , ‘\*\*’ corresponds to  $P \leq 0.01$ ,  
824 level of significance).

825 Figure 7. Changes in select hormones in serum. Abundance of A. Insulin ( $\text{ng ml}^{-1}$ ), B. Leptin ( $\text{ng}$   
826  $\text{ml}^{-1}$ ) and C. PYY ( $\text{pg mg}^{-1}$ ) in the serum of control BALB/c (CB) or C57BL/6 (CC) and  
827 vancomycin treated mice on third day (VB3, VC3) and sixth day (VB6, VC6) of BALB/c and  
828 C57BL/6 mice respectively. Comparisons among the groups were calculated with two-way  
829 ANOVA. In the figure, ‘\*\*\*’ corresponds to  $P \leq 0.001$ , ‘\*\*’ corresponds to  $P \leq 0.01$ , ‘\*’  
830 corresponds to  $P \leq 0.05$  level of significance). Error bars shown are one standard deviation from  
831 the mean value of four replicates ( $n=4$ ).

## 832 **Tables**

### 833 **Table 1.**

834 Measurement of Cecal index and cecal liquid content at different time points of BALB/c and C57BL/6  
835 mice.

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Mice	Conditions	Day	Cecal index( $\pm$ SD)	Cecal liquid content in $\mu\text{l}$ ( $\pm$ SD)
BALB/c	Control	0	0.01 ( $\pm$ 0.001)	6.1 ( $\pm$ 1.4)

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	Vancomycin treated	3	0.02 ( $\pm 0.005$ )	200 ( $\pm 26$ )
	Vancomycin treated	6	0.02 ( $\pm 0.002$ )	211 ( $\pm 20$ )
C57BL/6	Control	0	0.008 ( $\pm 0.002$ )	7.3 ( $\pm 1.9$ )
	Vancomycin treated	3	0.03 ( $\pm 0.003$ )	245 ( $\pm 40$ )
	Vancomycin treated	6	0.03 ( $\pm 0.002$ )	232 ( $\pm 34$ )

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**Table 2:** Sequences of forward (\_F) and reverse (\_R) primers for PCR studies to confirm

841 presence and expression level of various genes used in this study.

Genes specific for	Sequence of the primers used
<i>A. muciniphila</i> _F	5'-CAGCACGTGAAGGTGGGGAC-3'
<i>A. muciniphila</i> _R	5'- CCTTGCGGTTGGCTTCAGAT-3'
<i>il10</i> _F	5'-AGGCAGTGGAGCAGGTGAAGAGTG-3'
<i>il10</i> _R	5'-GCTCTCAAGTGTGGCCAGCCTTAG-3'
<i>tnf</i> _F	5'-CCACGTCGTAGCAAACCACCAAAG-3'
<i>tnf</i> _R	5'- TGCCCGGACTCCGCAAAGTCTAAG-3'
<i>cldn1</i> _F	5'-TGCCCCAGTGGAAGATTTACT-3'
<i>cldn1</i> _R	5'-CTTTGCGAAACGCAGGACAT-3'
<i>tlr4</i> _F	5'- CGCTGCCACCAGTTACAGAT-3'
<i>tlr4</i> _R	5'-AGGAACTACCTCTATGCAGGGAT-3'
<i>ocln</i> _F	5'- GTTGAACTGTGGATTGGCAG -3'
<i>ocln</i> _R	5'- AAGATAAGCGAACCTTGGCG -3'
<i>il6</i> _F	5'-AGACAAAGCCAGAGTCCTTCAGAG-3'
<i>il6</i> -R	5'-CCACAGTGAGGAATGTCCACAAAC-3'
<i>tlr2</i> -F	5'-GCCCCTAGATGAAGTCAGCTCACC-3'
<i>tlr2</i> -R	5'-CGGGCATCTACTTCAGTCGAGTGG-3'

*il17\_F* 5'-TCCAGAAGGCCCTCAGACTA-3'  
*il17\_R* 5'-ACACCCACCAGCATCTTCTCA-3'  
*tgfb\_F* 5'-CCCAGCATCTGCAAAGCT-3'  
*tgfb\_R* 5'-GTCAATGTACAGCTGCCGCA-3'  
*illa\_F* 5'-ATCAGTACCTCACGGCTGCT-3'  
*illa\_R* 5'-TGGGTATCTCAGGCATCTCC-3'

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

Days post treatment	Mouse	Treatment	Condition	Mean concentration [ $\mu$ M] ( $\pm$ SD)				
				Acetate	Butyrate	Lysine	propionate	Threonine
0	BALB/c	None (Control)	CB0	149( $\pm$ 9.5)	231.4( $\pm$ 11.4)	183.8( $\pm$ 7.8)	216.6( $\pm$ 2)	105.1( $\pm$ 6.9)
0	BALB/c	Vancomycin	VB0	136.2( $\pm$ 7.4)	242( $\pm$ 13.6)	187.1( $\pm$ 1.3)	221.5( $\pm$ 5.7)	102.5( $\pm$ 10)
6	BALB/c	None (Control)	CB6	157.5( $\pm$ 4.9)	223.2( $\pm$ 18.3)	179( $\pm$ 8.5)	223.2( $\pm$ 5.9)	115.7( $\pm$ 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)

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

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860 **Table 4.** 16S qPCR detection of *A. muciniphila* bacteria abundance in cecal sample of treated and control  
861 mice.

Mice	Conditions	Day	Ct Value ( $\pm$ SD) through qRT PCR	Remarks	OTU number ( $\pm$ SD) through NGS
BALB/c	Control	0	27.4 ( $\pm$ 0.6)	Low abundance	300 ( $\pm$ 86)
	Vancomycin treated	3	Could not be determined	Diminished	3 ( $\pm$ 1)
	Vancomycin treated	6	23 $\pm$ (0.7)	Increased by 21 fold wrt Day 0	12531 ( $\pm$ 2892)
C57BL/6	Control	0	29 $\pm$ (0.9)	Low abundance	10 ( $\pm$ 4)
	Vancomycin treated	3	Could not be determined	Diminished	5 ( $\pm$ 2)



Vancomycin treated	6	14.4± (0.5)	Increased by 24833 fold wrt Day 0	217482± (10926)
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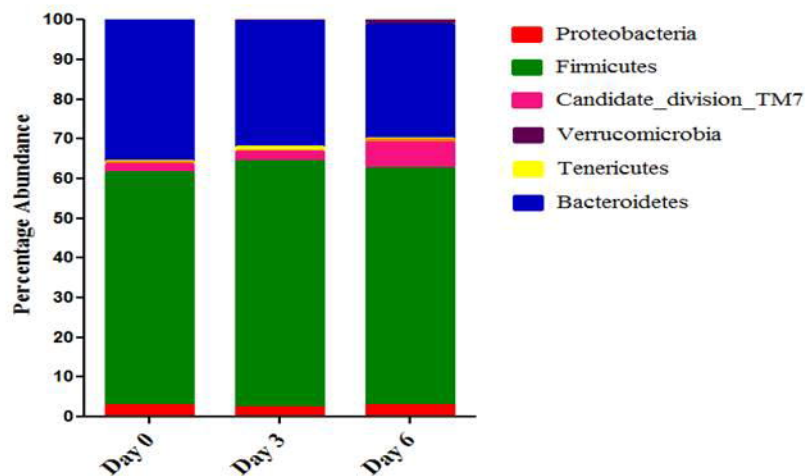
863 Ct value of cecal DNA using *A. muciniphila* specific primer in qPCR for untreated, Day 3 and Day 6  
864 following vancomycin treatment in BALB/c and C57BL/6 mice.

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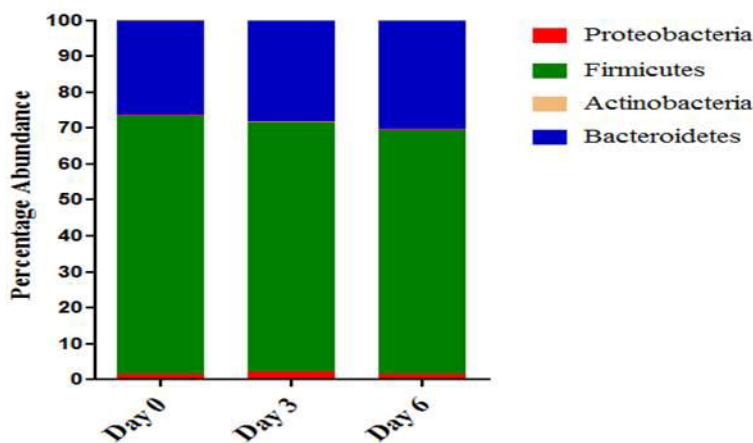
A.

BALB/c

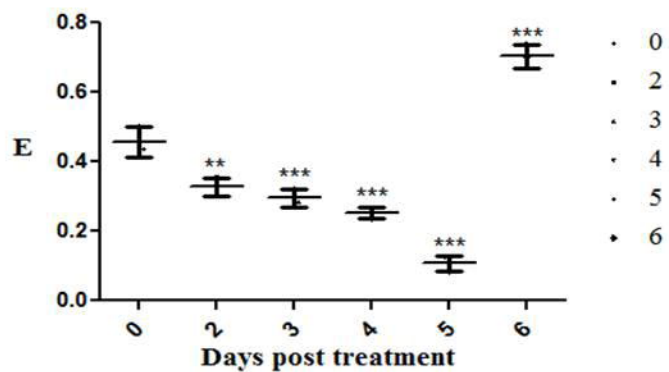


C.

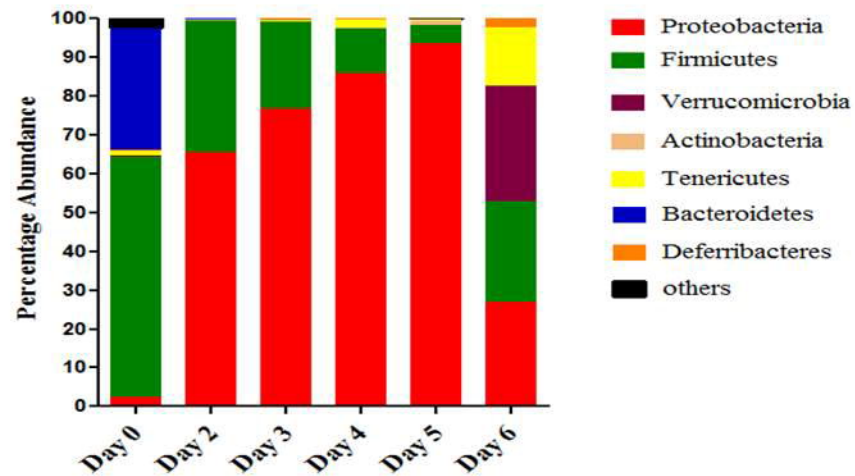
C57BL/6



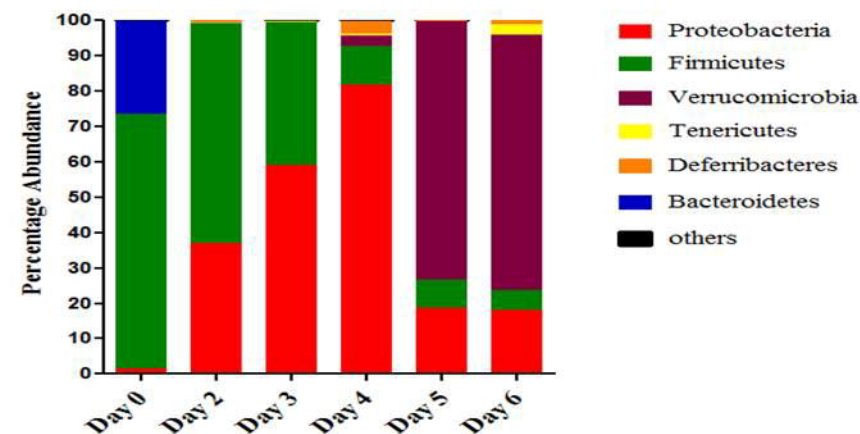
E.



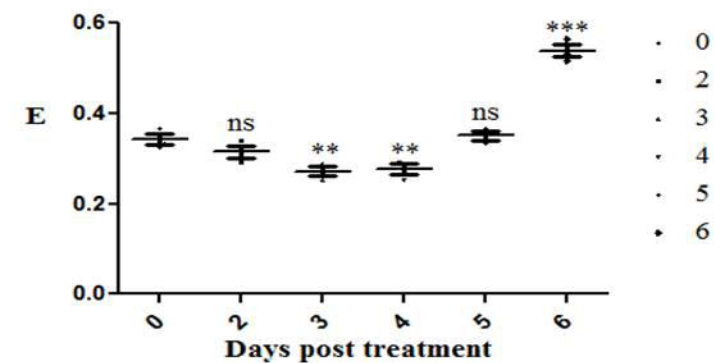
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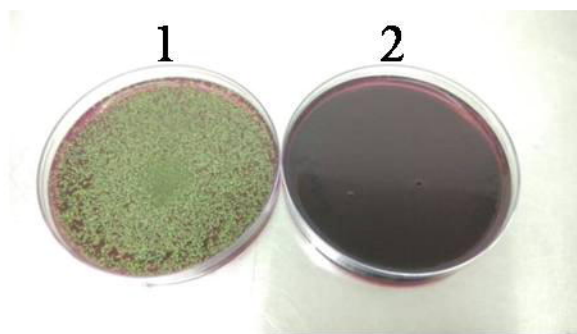
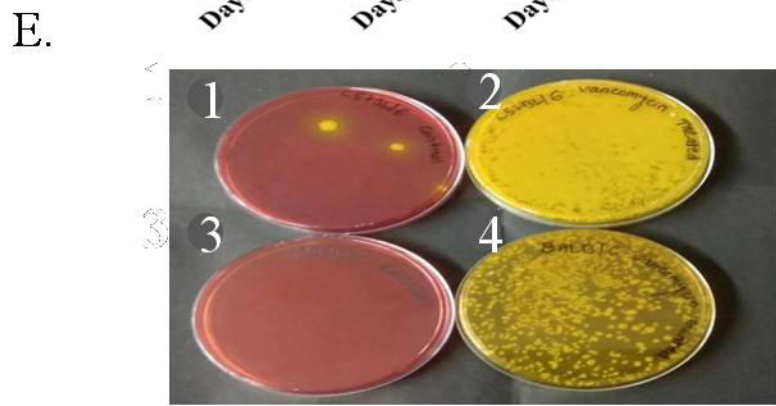
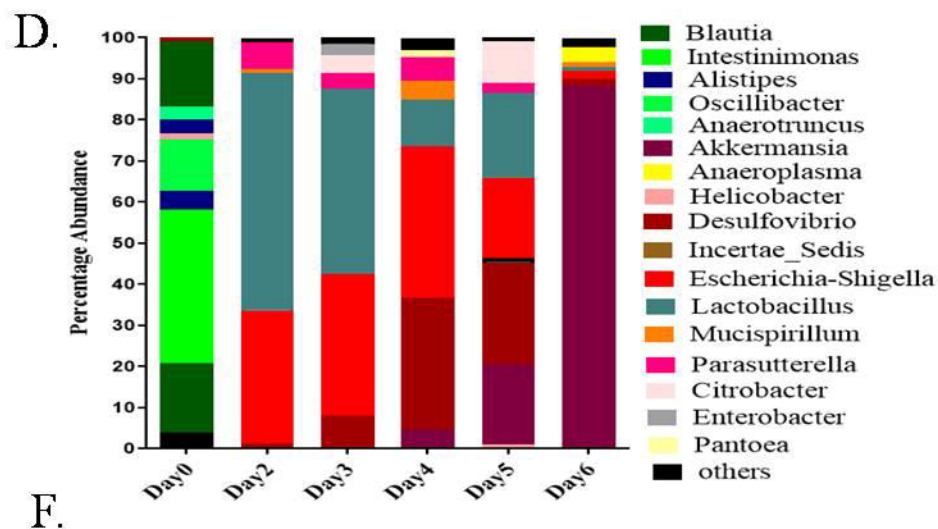
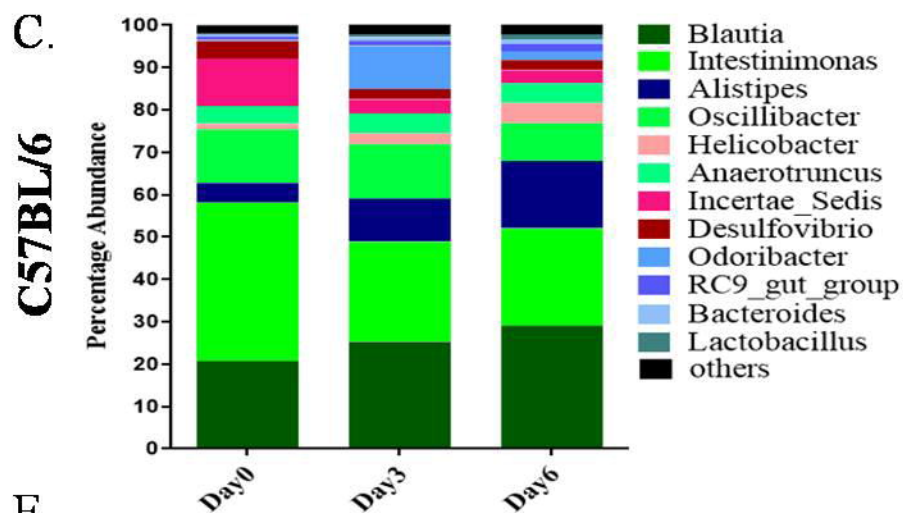
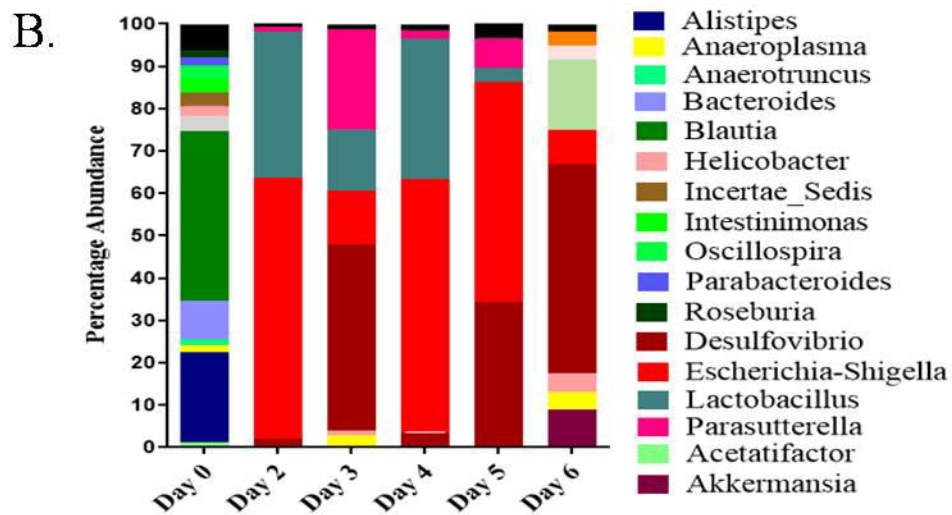
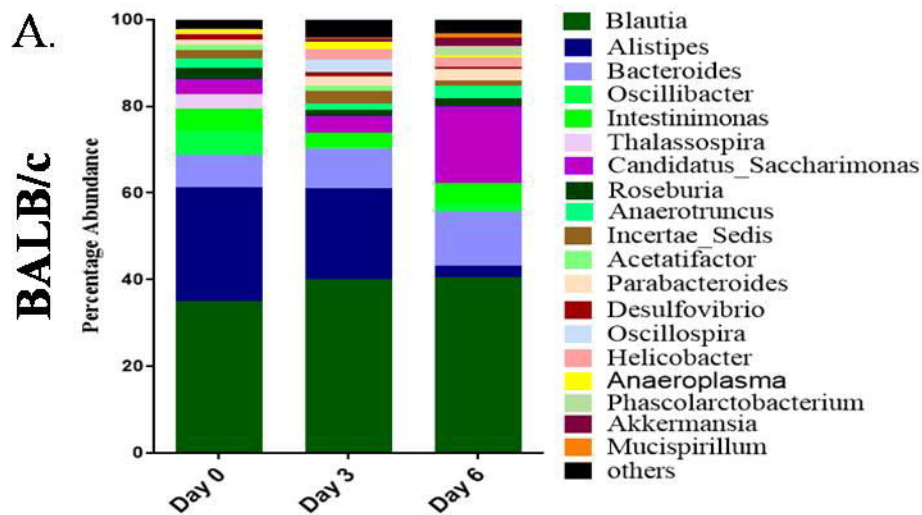


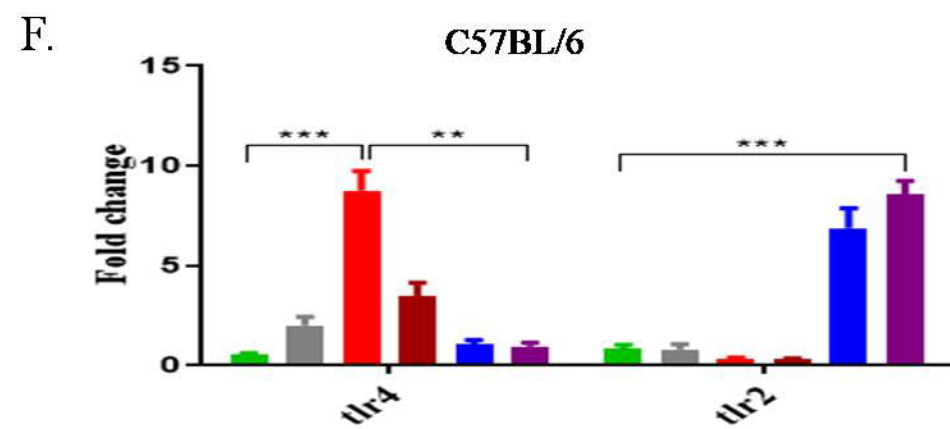
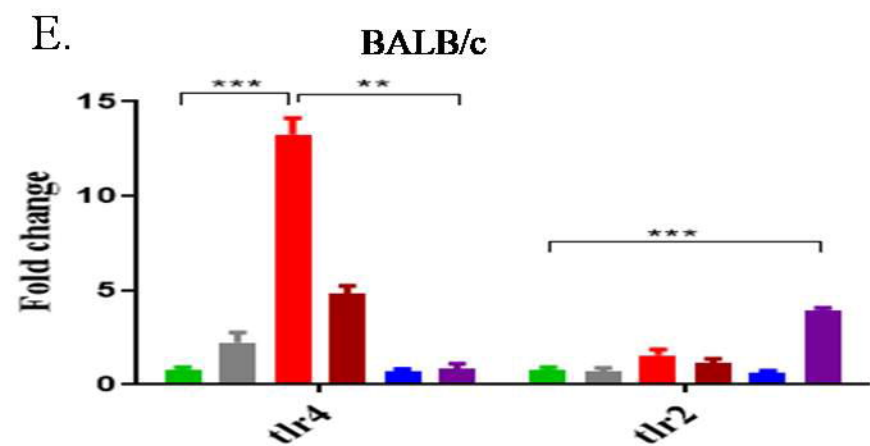
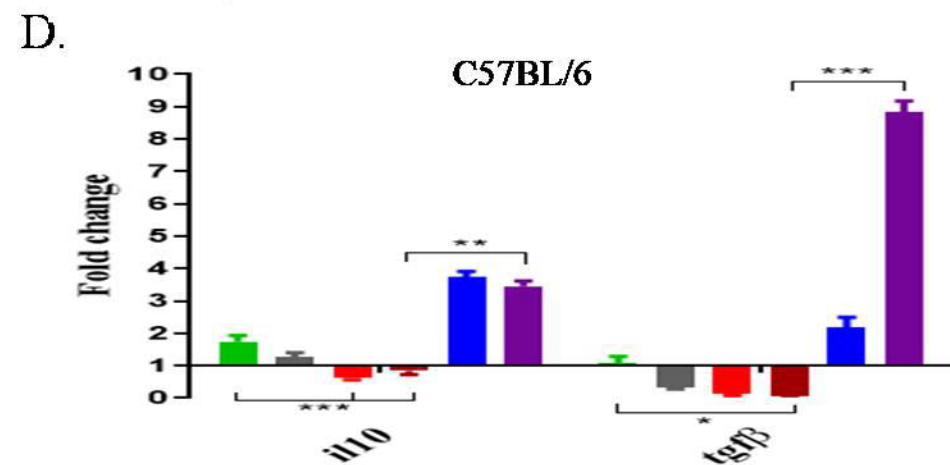
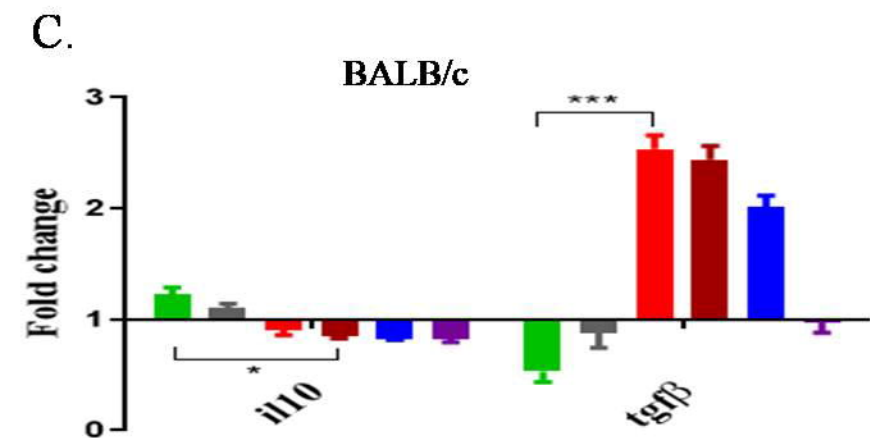
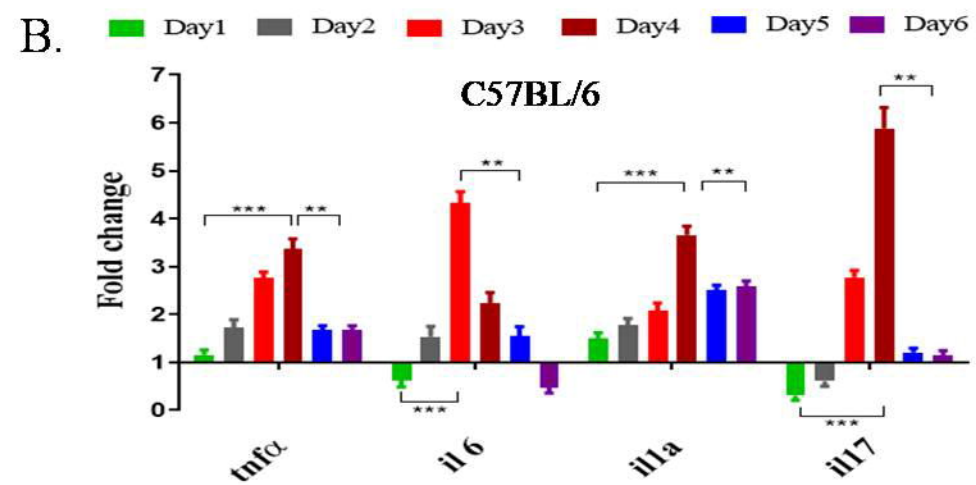
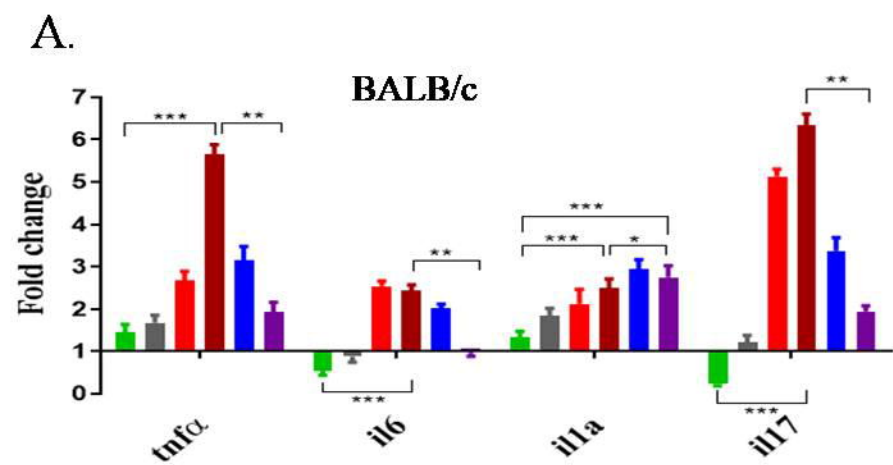
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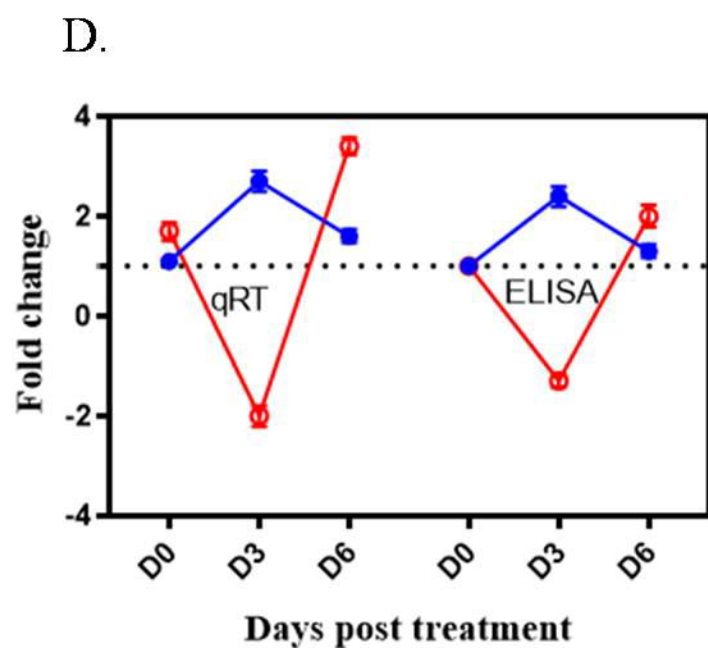
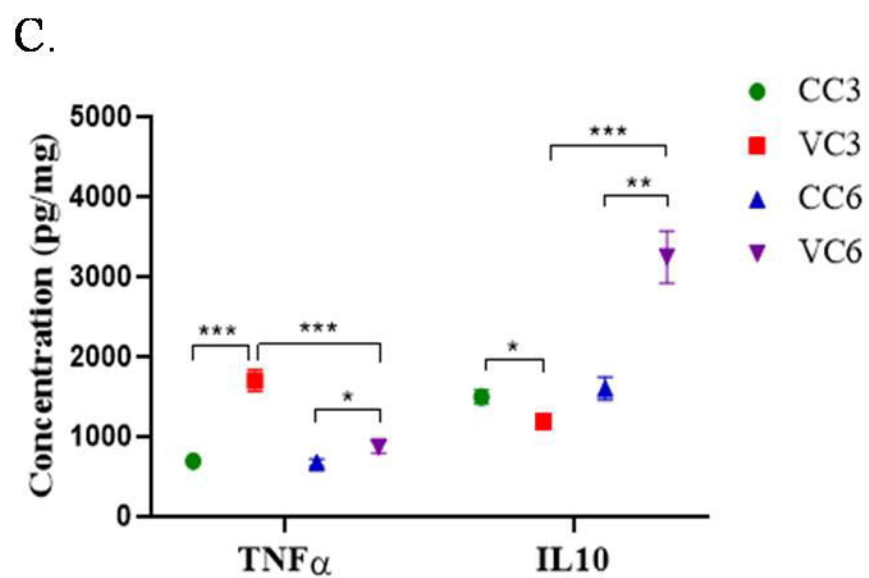
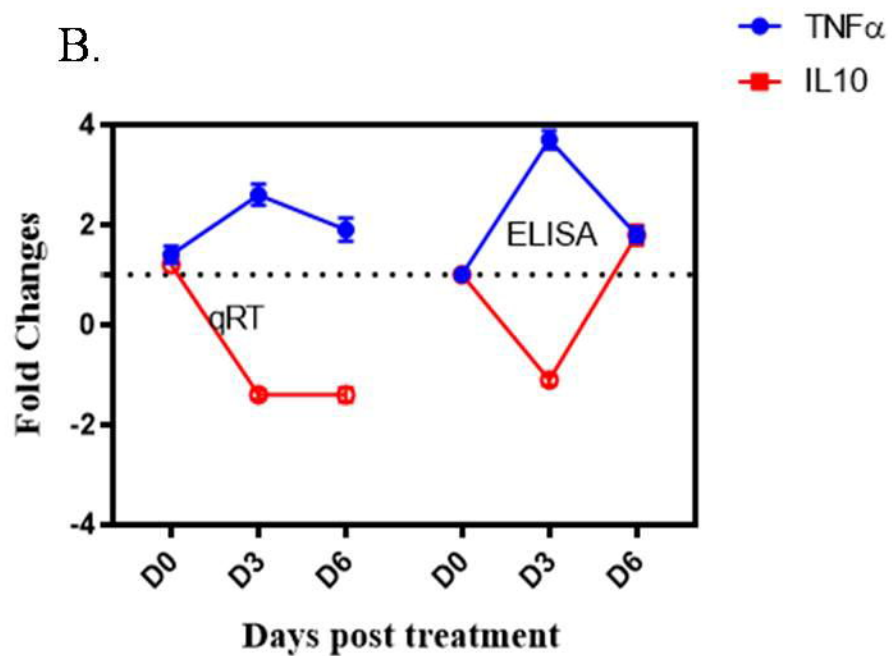
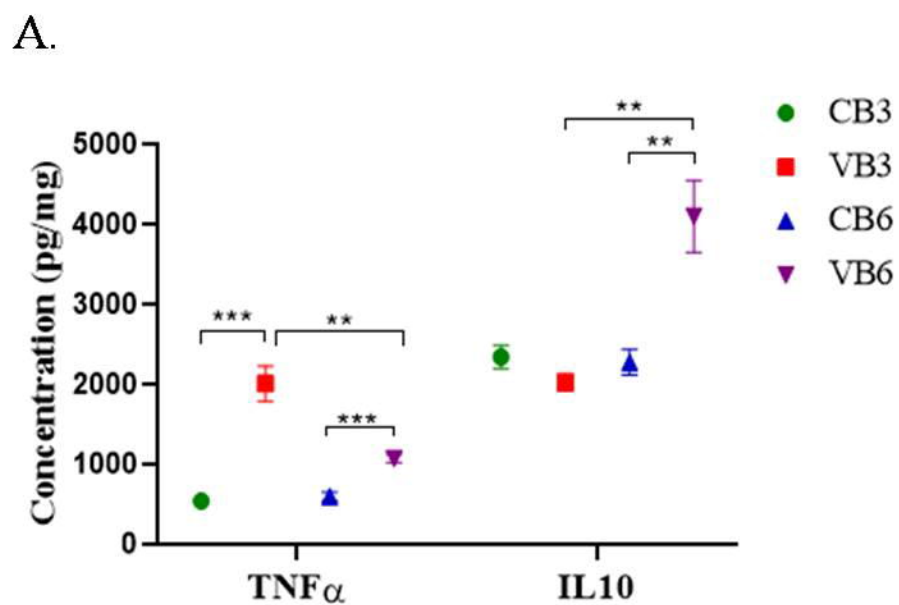


F.



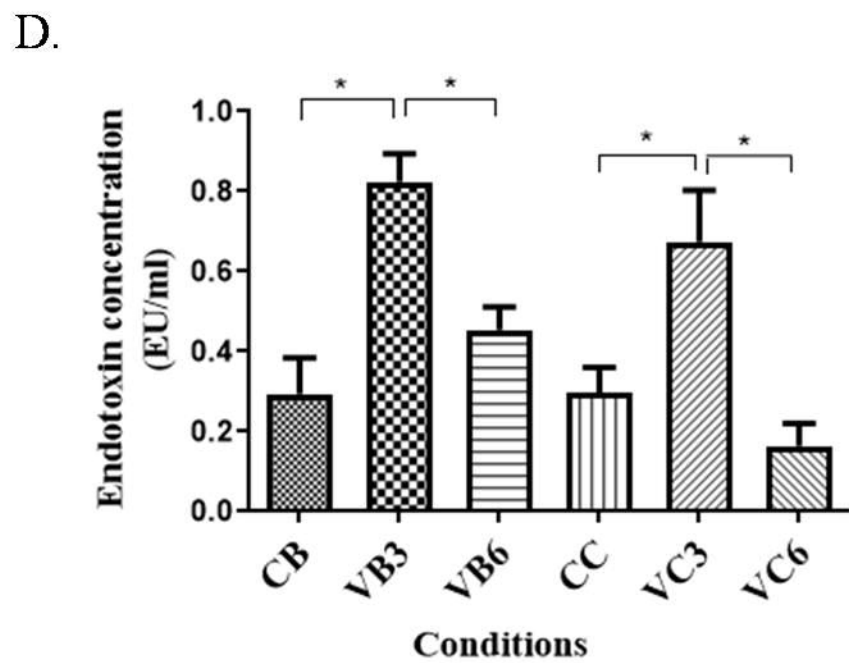
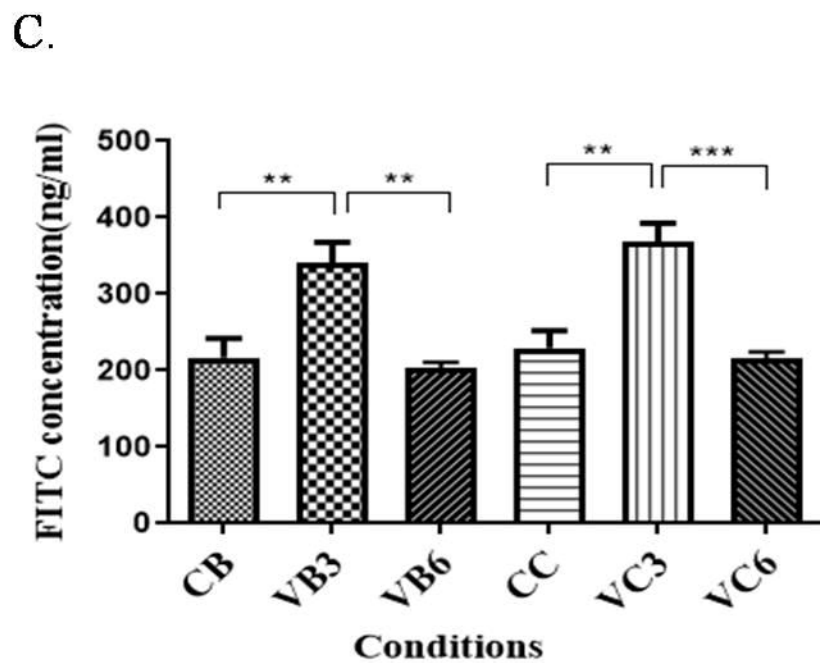
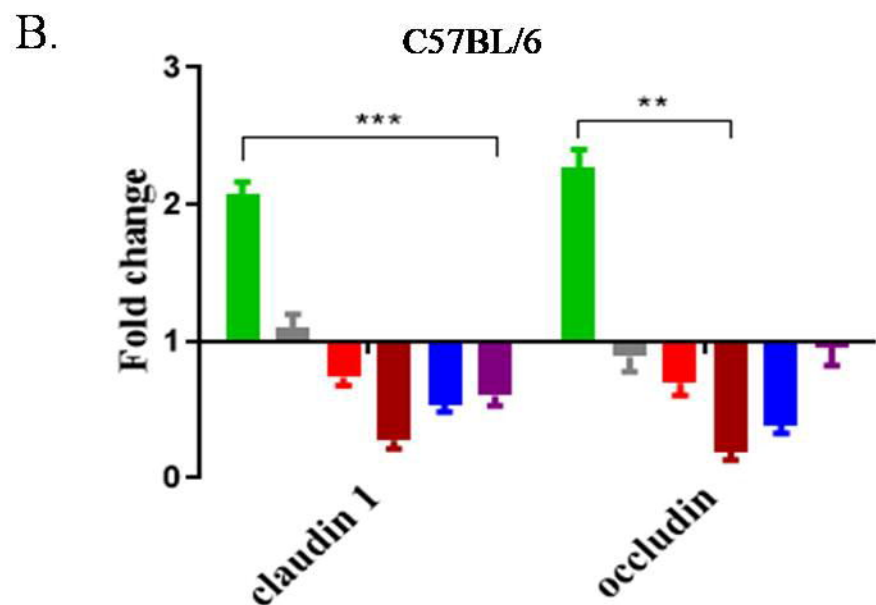
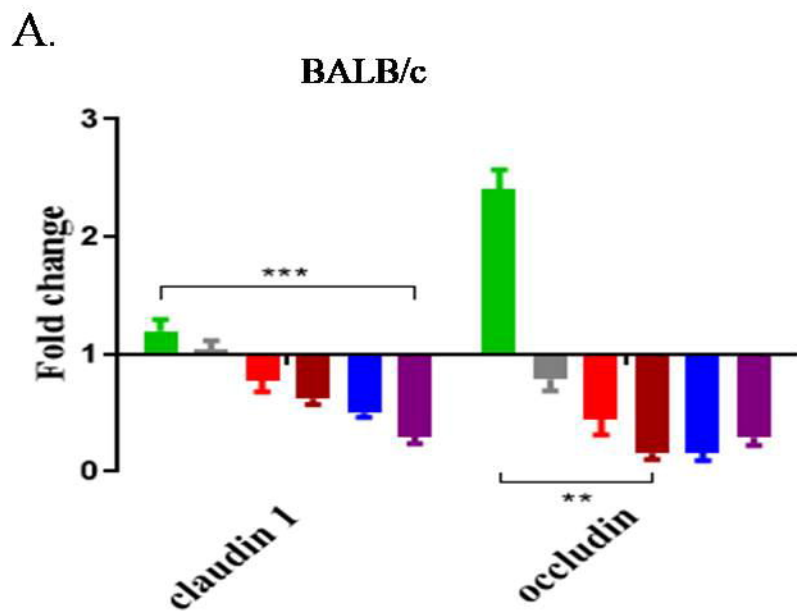




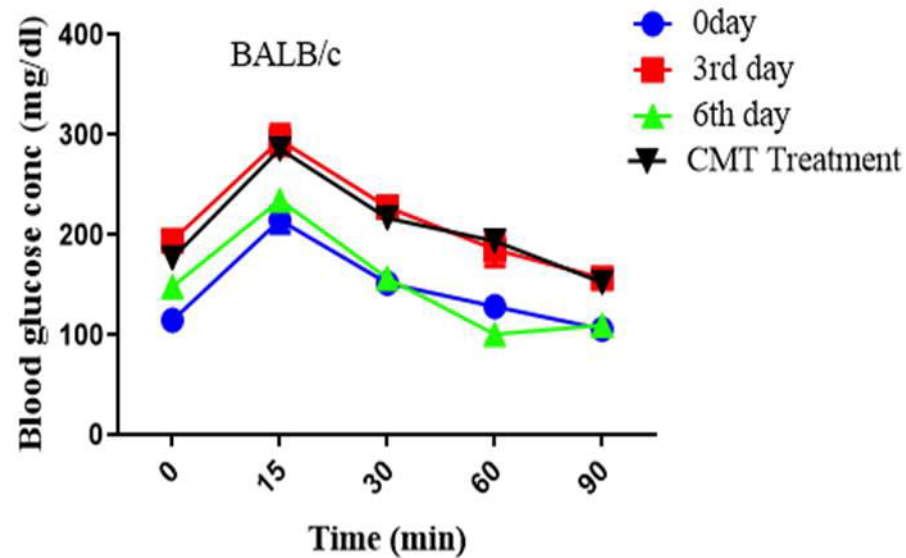




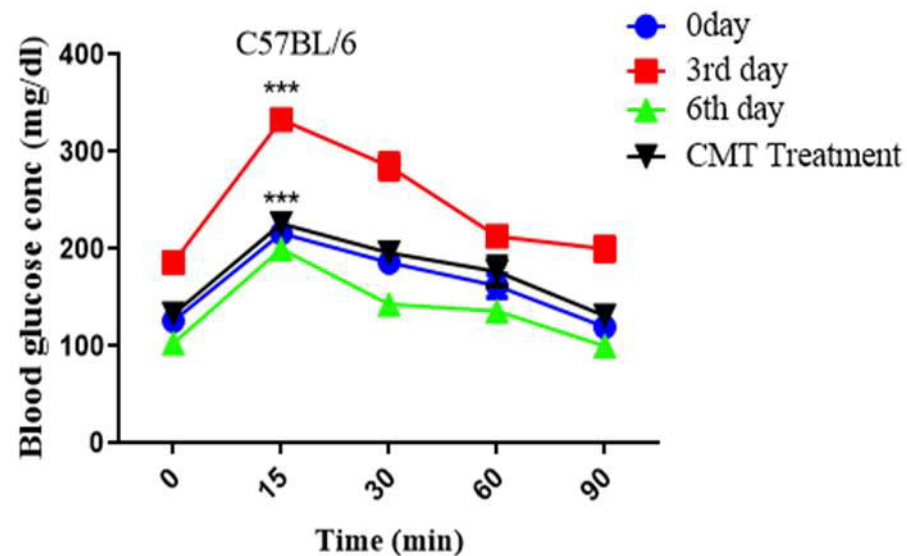
Legend for Days: Day1 (Green), Day2 (Grey), Day3 (Red), Day4 (Dark Red), Day5 (Blue), Day6 (Purple)



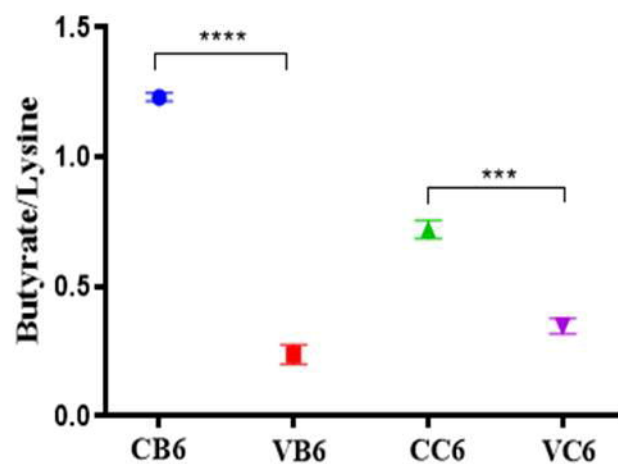
A.



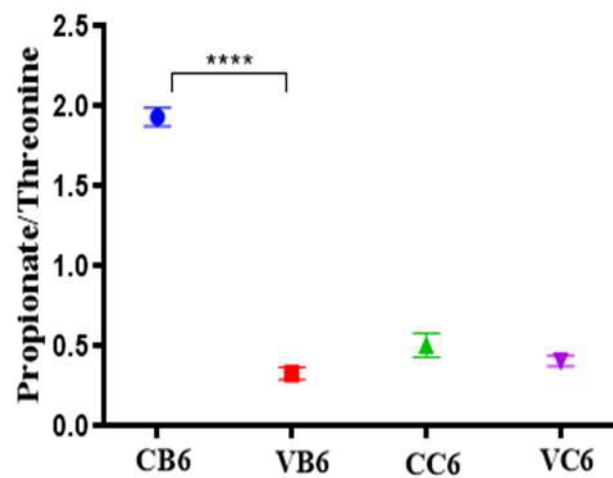
B.



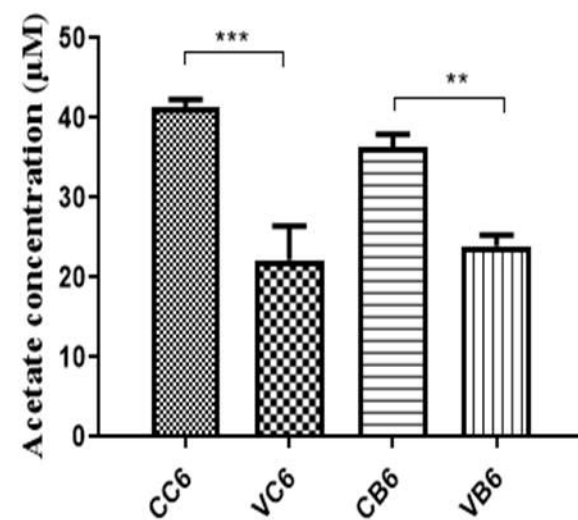
C.

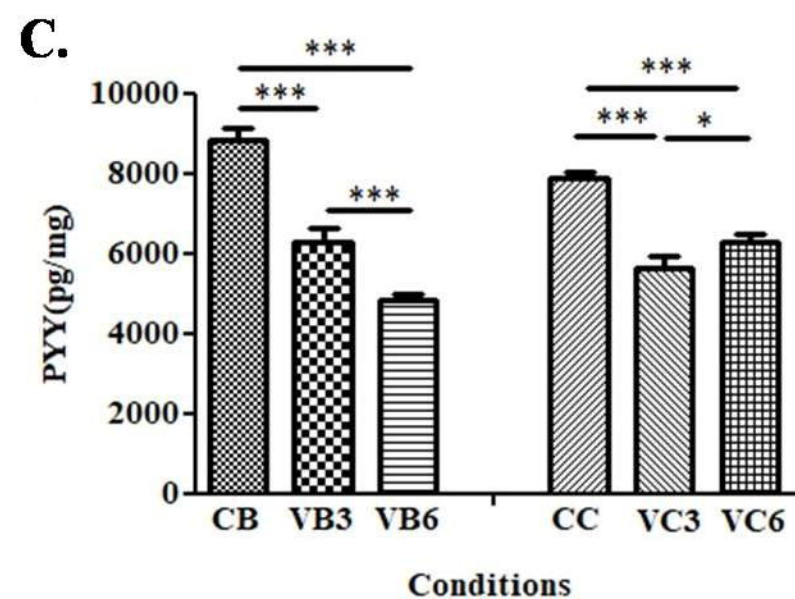
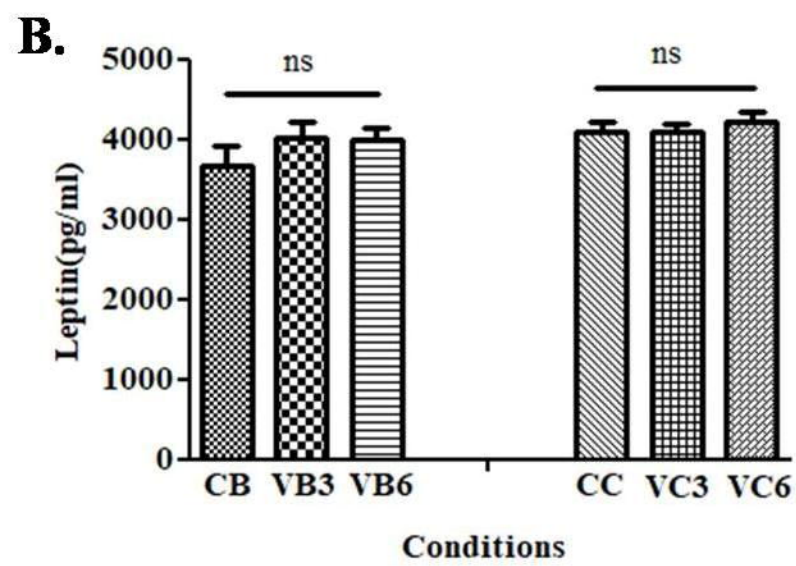
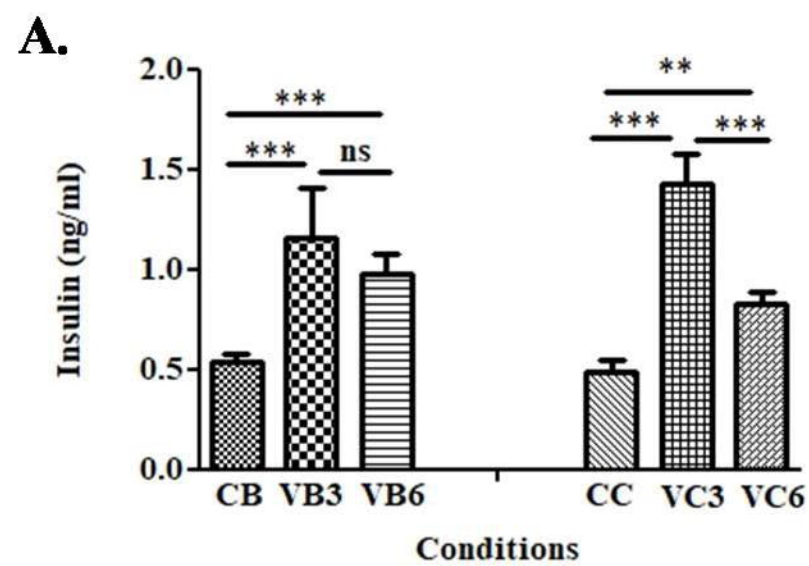


D.



E.







Proteobacteria Firmicutes  
Verrucomicrobia Bacteroidetes

