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

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Running title: Role of vancomycin on gut microbiota of Th1- and Th2- biased mice

Abstract

Use and abuse of antibiotics not only cause microbial resistance but also influence abundance and diversity of gut microbiota. Gut microbiota not only are the largest reservoir of beneficial microbes in mammals but also play major role in maintaining health and homeostasis. To understand the role of gut microbiota and mechanism by which they 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. In this study, we screened four antibiotics (amoxicillin, ciprofloxacin, neomycin, and vancomycin) and finally selected vancomycin for further studies to establish the effects on host immunity and metabolism in Th2-(BALB/c) and Th1- (C57BL/6) biased mice models. The screening study revealed that vancomycin was the most effective perturbing agent. We found that initial doses of vancomycin caused reduction in abundance of phylum Firmicutes and Bacteroides in contrast to significant increase in the phylum Proteobacteria in the gut. These changes in microbial profile could be correlated with observed increase in inflammation and permeability of gut and cecal index in oppose to a decrease in the rate of glucose metabolism, and change in SCFA metabolic profile of the host. Interestingly, at later stages (day 5 onwards) of vancomycin treatment we observed significant increase in the phylum Verrucomicrobia for the genus Akkermansia that led to restoration of gut environment by decreased inflammation and increased rate of glucose metabolism. The effects of Akkermansia was more pronounced in C57BL/6 than BALB/c.

Introduction:

Role of commensal microbiota in maintaining health is becoming more confluent with newer reports and insights (1)(2)(3). The mammalian gastrointestinal tract is inhabited by a hundred trillions of highly diverse and complex microbial cells (4) , and huge number of these microbes

have co-evolved with the host over thousands of years (5), in fact, these microbes have at least hundred times more genomic content than the human genome (6)(7) . The abundance and diversity of gut microbiota have a significant role in maintaining various host physiology – for example, harvesting energy (8), maintaining immune homeostasis (9), regulating metabolism(10), and giving protection against pathogen (11). While microbiota profile may be huge and diverse, reports suggested only a handful of major phyla of gut microbes that include Firmicutes, Bacteroidetes, and Proteobacteria that are accounting for at least 90– 95% of the gut microbiome. Among which the anaerobic genera which constitute the majority of gut microbe include Bacteroides, Eubacterium, Clostridium, Ruminococcus, and Faecalibacterium (12).

It is believed and 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 varies with different factors like age (13), diet(14), geography (15)(16), stress (17), pathogen (18), and antibiotics (19). Since, antibiotics is still a most important and major avenue of treating 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 (20). In fact, 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 host (21)(22) and thus, lead to different metabolic syndromes like diabetes, obesity, and IBD (23)(24). Intensity of the perturbation of commensal microbes by antibiotics depends on different factors like the mode of action of a particular antibiotic and the presence of various resistant group of microbes in gut against that antibiotic (20).

Among various antibiotics, vancomycin is majorly prescribed orally against the infection of two multi-drug resistant strains; i.e., *Clostridium difficile* and *Staphylococcus aureus* (25) (26)(27). 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 (28). However, the dose and time dependent effects of vancomycin on gut microbiota of mice 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, happen in gut microbiota following treatment of mice with vancomycin, are not as direct 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 treatment, some healthy microbes start appearing 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 (29). In the current study, we compared the effect of perturbation of gut microbiota between Th1 and Th2 biased mice by using four different antibiotics (amoxicillin, ciprofloxacin, neomycin, and vancomycin). The initial screening result revealed that out of four antibiotics, vancomycin has highest potential for perturbing gut microbiota. The microbiota present in the gastrointestinal tract is important for the development of a mature immune system of the host (30). 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 (31). In this study, after vancomycin

85 treatment, we observed and correlated the kinetic of immunological changes of host at both
86 transcriptional and protein level with metagenomic changes of gut.

87 Microbiota present in gut controls the metabolism of major nutrients like carbohydrate, amino
88 acid, fatty acid, and vitamins. Moreover, it was already reported that some microbes convert
89 complex carbohydrate food into short chain fatty acids (SCFAs) like acetate, propionate, and
90 butyrate, and these SCFAs play an important role in regulating inflammatory response and
91 metabolism of the host by binding to free fatty acid receptors (FFAR2/3) present in intestinal
92 epithelial cells, immune cells, adipocytes(32).

93 Intestinal epithelium acts as a barrier to the entry of different pathogens, inflammatory molecules
94 and toxins into the mucosal tissue (33). The commensal microbes present on intestinal epithelial
95 cell maintain the integrity of +-- it by controlling the expression of various tight junctions by
96 occludin, claudin etc (34). The defect in barrier due to perturbation of gut microbiota leads to
97 different metabolic diseases like IBD, obesity, diabetes 1 (23)(24). Here in our study, we
98 observed how the prevalence of different group of microbes differentially controls the expression
99 of tight junction protein and subsequent inflammation.

100 **Result:**

101 **Selection of antibiotics:**

102 Plating data of fecal samples of different antibiotic-treated mice (Fig.1A and 1B) showed
103 changes in colony count from the first day to the seventh day of treatment. Clinical dose of
104 vancomycin-treated group showed the most significant changes in colony count compared to
105 other antibiotic treated groups (neomycin, amoxicillin, and ciprofloxacin). So, we chose to use
106 vancomycin as the perturbing agent among other antibiotics for further study. A sudden increase

in certain bacterial colonies was observed on NB agar plate after second day or fourth dose of vancomycin treatment (Fig.1C and Fig. 1D). The bacterial colony count on plate from second day to fourth day was one log higher in BALB/c (Fig. 1A) and two log higher in C57BL/6 mice (Fig. 1B) of vancomycin treated group than other antibiotic treated and control groups of mice. After fifth day of treatment, bacterial population started decreasing on the plate.

Vancomycin treatment alters the abundance and diversity of gut microbiota

Metagenomic (16S rRNA) analysis of cecal sample from vancomycin treated mice showed a decrease in major phyla of bacteria like Firmicutes and Bacteroidetes and increase in proteobacteria level (Fig. 2B and Fig. 2D) compared to untreated mice. 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). 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 2B and 2D). Genus level data (Figs 3B and 3D) showed an increase in Escherichia and Shigella group of bacteria that belong to Proteobacteria phylum and decrease in Clostridia (Blautia, Instentimonas, Roseburia) group of bacteria from Firmicutes phylum in vancomycin treated mice following the fourth day of treatment with vancomycin with respect to time matched control mice, It is further observed that the phylum, Verrucomicrobia started increasing from day 5 following vancomycin treatment. 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.3D). Verrucomicrobia level also started increasing in BALB/c mouse (Fig.3B) on the sixth day following treatment with vancomycin but to a much lower level (20-25%). 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. We, therefore, tested for *A. muciniphila* on day 6 following treatment with vancomycin of BALB/c and C57Bl/6. Species specific primer (Table 1) of *Akkermansia muciniphila* was used to confirm its presence. 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 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.

We have further determined the diversity of the cecal microbiota. Shannon diversity index 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 the sixth day for BALB/c and fifth day for C57BL/6 following treatment with vancomycin.

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

Results revealed that expression of pro-inflammatory cytokine genes (tnfα, il17,il6,il1a) in the gut at transcriptional level, increased till day 4 for C57BL/6 mice (Fig. 4B) and day 5 for BALB/c mice (Fig. A) following treatment with vancomycin. Concentration of anti-inflammatory cytokines (TGFB, IL10) started increasing and pro-inflammatory cytokines started decreasing from day 5 in C57BL/6 mice (Fig. 4D) and from day 6 in BALB/c mice (fig. 4C) following treatment with vancomycin. Concomitant to the increase in pro-inflammatory cytokines we observed increase in expression oftlr4 (Fig. 4F and 4G) at transcriptional level till day 4 and tlr2 till day 6 in both BALB/C and C57BL/6 mice following treatment with vancomycin.

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. 5). ELISA results revealed that, on the third day of vancomycin treatment, TNFα level was significantly more in both BALB/C (Fig. 5A) and C57BL/6 (Fig. 5B) mice with respect to day 3 time matched untreated control group of mice. Similarly, IL10 cytokine level was more in BALB/c (Fig. 5C) and C57BL/6 (Fig.5D) on the six day following treatment with vancomycin compared to the day 6 time matched untreated control mice.

Gut permeability increased after vancomycin treatment:

In vancomycin treated mice, a large cecum with more fluid accumulation was observed than control mice (Fig. 6D). Gut microbiota abundance and diversity regulates the cecal index of host.. The cecal index was calculated and the ratio was significantly more in vancomycin treated C57BL/6 and BALB/c mice than their respective control groups (Fig. 6A).

Cecum is known to be a better representation than fecal sample for understanding intestinal microbiota profile (35) and cecum usually gets enlarged following treatment with antibiotic by accumulating fluid and solid matter (36). The changes in the bacterial composition due to antibiotic treatment in cecum 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 control mice (Fig.6B). For further confirmation of gut permeability, FITC conjugated dextran was gavaged on day three following treatment with vancomycin and in respective control mice. FITC dextran Concentration was detected significantly higher in the serum of third day mice 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.6C).

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 than control mice. Their expression of claudin gene decreased continuously from day zero to day six following treatment of vancomycin in BALB/c (from 1.2 fold upregulation on day zero to 3.4 fold down regulation on day six) and C57BL/6 (From two fold upregulation on day zero to 1.5 fold downregulation on day six) mice (Fig. 6E and Fig. 6F). Similarly occludin gene expression decreased in BALB/c mice (from 2.5 fold upregulation on day zero to 6.5 fold downregulation on day six) and C57BL/6 (from 2.3 fold upregulation on day zero to one fold downregulation on day six) following vancomycin treatment. The expression of claudin and occludin gene is lowest on day three and day four following vancomycin treatment.

The endotoxin level in serum correlates with increase in pathogenic gram-negative bacteria (proteobacteria) in gut and compromised permeability and inflammation of gut tissue The endotoxin level was measured in the serum of control and vancomycin treated mice. On the third day of vancomycin treatment, endotoxin level was 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.6G).

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. A glucose tolerance test is usually done following 12h fasting. From glucose tolerance test, it was found that glucose metabolism was different in control and vancomycin treated mice (Fig. 7A and 7B). on the third day following vancomycin treatment, fasting glucose level in the blood of both Th2 and Th1 biased mice (BALB/c 194.6± 6.3 mg/dl and C57BL/6 186±6 mg/dl) have significantly higher than their time matched control (BALB/c 115±3 mg/dl and C57BL/6 126±4 mg/dl) and the sixth day treated (BALB/c 148.6±7 mg/dl and C57BL/6 103±5 mg/dl) mice. 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 (VB6 235±5 mg/dl, VC6 200±9 mg/dl) mice. The metabolism rate of glucose in the blood of the sixth day 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.

In CMT group of mice on the third day following vancomycin treatment (after cecal microbiota transplantation from 6th day to 3rd day vancomycin treated group of mice), glucose level in serum decreases drastically in C57BL/6 mice and became equivalent to control groups of mice but in BALB/c mice the effect is insignificant.

Metabolite level in serum changes after vancomycin treatment

The production of short chain fatty acids (SCFA) like acetate, butyrate, and propionate from lysine and threonine was disturbed in the gut of vancomycin-treated mice. The concentration of acetate, butyrate, and glutamate (Fig. 7C, 7D, 7E) in the blood of vancomycin treated mice were very low but their respective substrate concentration (lysine, threonine) were higher than control, which showed accumulation of the substrate in blood and less production of SCFA from them in treated group of mice. Among vancomycin treated groups, BALB/c mice had lower SCFA production rate than C57BL/6 mice. Further validation of acetate production in the serum of both BALB/c and C57BL/6 mice by using acetate detection kit (EOAC-100,San Francisco,USA) also showed similar result i.e, lower concentration of acetate on vancomycin treated group (VB6 23.9±1.4, VC6 22.2±4) with respect to their time matched control group (CB6 35.3±1.6, CC6 41.3±1.02) of mice (Fig.7F).

Gut hormonal level during vancomycin treatment

We already reported rate of glucose metabolism that increased on day 6 following treatment with vancomycin. In addition to SCFAs it is also important to understand the role of related hormones such as Peptide tyrosine tyrosine (PYY), leptin and insulin following treatment with vancomycin in Th1- and Th2-biased mice (37). The results revealed that the concentration of PYY hormone in the serum decreased following vancomycin treatment in both BALB/c (from 8836±311pg/mg

on day zero to 4843.9±149 on day six) (Fig. 7F) and C57BL/6 mice (from 7883±155pg/mg on day zero to 6283.8±209 on day six) (Fig.8C). There was no significant change observed in serum leptin concentration of vancomycin treated (Fig.8B) BALB/c and C57BL/6 mice. Insulin concentration in serum was the highest on the third day of vancomycin treated BALB/c (1.16±0.25 ng/ml) and C57BL/6(1.43±0.15 ng/ml) but decreased significantly on sixth day of C57BL/6 mice (0.83±0.06 ng/ml) (Fig.8A). Local tissue level concentrations could not be determined to ascertain their immediate effects, if any, however we measured blood level of these hormones to ascertain their roles as described elsewhere (38).

Discussion

The extent of perturbation of gut microbiota varies differently with the use of different antibiotics. Metagenomic (16S rRNA) analysis of cecal sample of mice showed that vancomycin altered the gut microbiota most extensively by decreasing the phylum like Firmicutes and Bacteroidetes and increasing Proteobacteria level. By correlating the NGS data with plating data, it was observed that the group of bacteria that overpopulated on the plate after the second day and decreased after the fifth day of vancomycin treatment might be from Proteobacteria phylum. It was later proved by plating the cecal sample of third day mice on specific media, i.e. salmonella-shigella specific agar plate and EMB agar plate (Fig 3E and 3F). It was found that those overgrown colonies were mostly Shigella and E.coli – which was correlated with metagenomic data of cecal sample at genus level. On the fifth and sixth day of treatment, Verrucomicrobia phylum replaced the Proteobacteria phylum in the gut. The Verrucomicrobia phylum has Akkermansia genus, which is an obligate anaerobe and nonculturable microbe (39). So, on the fifth and sixth day of vancomycin treatment showed a decrease in colony count on the plate.

During vancomycin treatment, Proteobacteria phylum started increasing drastically up to the fourth day and it replaced all other phyla like Firmicutes, Bacteroidetes, Actinobacteria, Tenericutes, Verrucomicrobia (Fig.2D). 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 were 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. 2E and Fig. 2F).

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 (40). Instentimonas bacteria (Firmicutes phylum) produces butyrate from lysine (41) and Bacteroidetes produces propionate from threonine in the gut (41)(42). 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. (32)(43). vancomycin treatment causes the decrease in Firmicutes and Bacteroidetes level

283 significantly in mice gut, which results in less SCFA production and increases level of
284 inflammation (Fig 5 and 7)in vancomycin treated mice than control.

285 Overexpression of Inflammatory cytokine like TNFα is associated with higher gut permeability
286 by suppressing the expression of tight junction proteins like occludin and claudin 1 (44)(45).
287 Lower expression of tight junction protein in vancomycin treated mice caused the increase in gut
288 permeability resulting in higher cecal index and fluid accumulation of cecal sample (Fig. 6). On
289 the sixth day of vancomycin treatment, elevated Verrucomicrobia caused an anti-inflammatory
290 effect in the gut. Verrucomicrobia causes a decrease in inflammation and enhances glucose
291 metabolism of host (46)(47). During obesity and diabetic condition, metabolic endotoxemia had
292 been observed where endotoxin (LPS) level increased in the blood to cause inflammation and
293 impaired glucose metabolism in the host (40)(48). The increase in Proteobacteria phylum in gut
294 during vancomycin treatment elevated the endotoxin level of blood in mice. This resulted in
295 impaired glucose metabolism and insulin resistance (highest level of both insulin and glucose on
296 the day 3 following treatment with vancomycin till day 4 of vancomycin treatment. On the sixth
297 day following vancomycin treatment, in C57BL/6, replacement of Proteobaceria by
298 Verrucomicrobia caused significant improvement in glucose metabolism in mice; fasting glucose
299 (Fig.7A and 7B) and insulin level (Fig. 8A) in blood came to normal. This effect is more
300 prevalent in C57BL/6 than BALB/c mice. Following successful transfer of cecal sample from
301 sixth day of vancomycin treated C57BL/6 mice (*A. muciniphila* level is above 70%) to third day
302 of vancomycin treated mice,blood glucose level decreased significantly on the third day mice,
303 which showed the effective role of *Akkermansia muciniphila* in controlling the blood glucose
304 level. Higher fasting Insulin concentration in serum of the third day vancomycin treated BALB/c
305 and C57BL/6 than control and sixth day vancomycin treated C57BL/6 mice (Fig.8a) showed the

insulin resistance after 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.7A and 7B).

SCFA stimulates PYY hormone production by activating Gq coupled receptor FFA2 of endocrine cells present in the gut (49). The current results revealed that following treatment with vancomycin the level of SCFA decreased, which cause a reduction in serum PYY production (Fig.8C) that might have an effect of observed enhanced glucose metabolism or increased insulin sensitivity (38). 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 in a very distinct way - by depleting the healthy microbes and increasing the infectious microbes in initial days of treatment, which causes disruption in the equilibrium of various physiological processes (immunological and metabolic) of body; however, at the later stages of treatment, the body tries to restore the equilibrium in various physiological processes by increasing 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; they help to restore the equilibrium by alleviating the adverse effect of Proteobacteria.

Material and methods:

Animal Used in study: All mice used in the present study were 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), temperature 24 ± 3° and humidity 40-70% maintained. Guideline 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 were used for the present study.

Antibiotic treatment: Both Th1(C57BL/6) and Th2(BALB/c) biased mice were treated twice daily by oral gavaging with one of the antibiotics of Amoxicillin (Cat#190145) (at 10 mg per kg of body weight), or Ciprofloxacin (Cat#11416981) (at 10 mg per kg of body weight) [29], or vancomycin (Cat#11465492) (at 50 mg per kg of body weight) (50), or Neomycin (Cat#12787149) (at 50 mg per kg of body weight). All antibiotics, of molecular biology grade, were purchased from MP Biomedical, France.

Fecal Sample plating: Fresh fecal samples were collected each day from mice following treatment with antibiotics until day 7, and 50 mg of each fecal sample was dissolved in 1ml of sterile distilled water and plated at a dilution of 10^3- and 10^5-fold on Nutrient agar medium. Colonies were counted after 24h of incubation at 37°C.

Mice treatment and sample collection: Mice were grouped into two different groups: untreated (control) and treated. Treated group of mice were orally gavaged 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 protocol approved by the Institutional Animal Ethics Committee.

Duodenum, jejunum, ileum, colon, spleen, liver, blood and fecal samples were collected from each mice and stored in a respective way until further used.

RNA extraction: RNA was extracted from gut wall tissue by using RNeasy mini kit (Cat# 74104, Qiagen, India). 20-23mg of tissue were taken ground using liquid nitrogen and 700ul of RLT buffer was added and homogenized well. An equal volume of 70% ethanol was added and mixed well. The solution was centrifuged at 13,000rpm for 5min at room temp. The clear solution containing lysate was passed through RNeasy mini column (Qiagen, Germany), which leads to binding of RNA to the colunm. The column was washed using 700ul RW1 buffer and next with 500ul of RPE buffer. RNA was eluted using 30ul of nuclease-free water. RNA was quantified using NanoDrop 2000 (Thermo Fisher 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 9mer primer, Taq polymerase, and NT buffer, the mixture was kept at 45°C for 30min 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 1, SYBR green master mix (Cat#A6002, Promega, Madison, WI, USA), and nuclease free water. qRT-PCR performed in Applied bioscience. 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 control.

Cytokine Analysis at protein level

Colon tissues were collected from third and sixth day following vancomycin treatment and control mice. After washing colon tissue 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 (51). Then mixture was centrifuged and the supernatant was collected. ELISA (BD Biosciences, San Diego, CA, USA) was performed using manufacturer's protocol for TNFα (Cat#560478) and IL10 (Cat#555252) expression. The protocol was adapted from Kim et al. 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 and gDNA was extracted using phenol-chloroform method. 150-200mg of cecal sample was taken, it was homogenized using 1ml of 1X PBS and centrifuged. The precipitate was lysed by homogenizing it in 1ml of lysis buffer (Tris-HCl 0.1M, EDTA 20 mM, NaCl 100mM, 4% SDS) and thereafter heating it at 80°C for 45min. Lipid and protein removed from the supernatant using an equal volume of phenol-chloroform, this process repeated until the aqueous phase becomes colorless. DNA was precipitated overnight at -20°C with 3 volumes of 70% chilled ethanol. At last, it was washed with 500ul of 70% chilled ethanol and 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 30mins and centrifuged at 1700rpm for 15min at 4°C, and serum was collected and stored at -80°C till further used.

16srRNA sequencing (V3-V4 Metagenomics):

From cecal DNA samples, V3-V4 regions of 16s rRna gene was amplified. For this amplification, V3F: 5'-CCTACGGGNBGCASCAG-3' and V4R: 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. 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 (52)(53). Representative sequence was identified for each OTU and aligned against Greengenes core set of sequences using PyNAST program(54)(55)(56)(57). Alignment of these representative sequences against reference chimeric data sets was done and RDP classifier against SILVA OTUs database was used for taxonomic classification.

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

Glucose tolerance test (GTT): It was performed at the 0^{th}, 3^{rd} and 6^{th} day of vancomycin treatment in mice. Mice were starved for 6h. After 6h of starvation, Fasting blood glucose level was measured from tail vein using glucometer. After those mice were orally gavaged with glucose at a dose of 1g/kg, blood glucose level was measured in serum at 15, 30, 60 and 90mins post glucose gavage by using blood glucose monitoring system (ACCU-CHEK Active, Roche Diabetes Care GmbH,Mannheim,Germany).

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

Sample preparation and NMR data acquisition for metabolomics study

Blood of vancomycin treated and control mice was extracted by cardiac puncture method and kept for clotting. Then blood samples were centrifuged at 1700g for 15min and supernatant was collected as serum. 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 uL solution (containing serum sample, D_2O, pH maintenance buffer and DSS) was taken in 5mm 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, (58)(59)(60)(61) 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. Fold change having Cutoff value of above two log fold (both up and down regulation) were considered to be significance.

Calculation of Cecal index: Body weight of individual mice was measured and recorded. The whole cecal content was collected in an eppendrof and weighed for each individual mice. The

cecal index was measured by taking the ratio of cecal content to the body weight of respective mice (61).

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 collected the blood by cardiac puncture. Serum was collected from blood and concentration of FITC in serum was measured by spectrophoto fluorometry with an excitation wavelength of 485nm (20nm bandwidth) and emission of 528nm (20nm bandwidth) (62).

Endotoxin detection assay from serum: Mice were sacrificed at zero, 3rd and 6th day of vancomycin treatment 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.

Hormonal assay: PYY(Cat# EIAM-PYY), Leptin (Cat# ELM-Leptin), and Insulin(Cat# ELM-Insulin) hormone level were checked from serum samples of zero, third and sixth day of vancomycin treated and control mice by using Raybiotech mouse hormonal assay kit (Norcross, Georgia, USA). Mice were food and water starved for six hours, then fasting insulin and leptin level were checked in the serum of both BALB/c and C57BL/6 mice.

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626 **Figure Legends**

627 Fig1.

628 Colony count of culturable gut bacteria & phyla from metataxonomic studies. Kinetics of culturable gut
629 bacteria in fecal material following treatment with different antibiotics e.g. Vancomycin, Neomycin,
630 Amoxicillin, Ciprofloxacin and control mice in A. BALB/c, B. C57BL/6. (Values on the Y-axis are
631 given in log scale in cfu/ml/mice. Statistical significance was calculated by using two-way ANOVA, The
632 statistical analysis revealed that Vancomycin treated mice had significantly ($P \leq 0.001$) larger amount of

633 culturable bacteria emerged compared to control and other antibiotic treated mice. Kinetics of changes in
634 major phyla of gut bacteria determined from metataxonomic studies following treatment with
635 Vancomycin in C. BALB/c D. C57BL/6 mice.

636 Fig2.

637 Phylum level changes in the gut microbiota. Time dependent changes in the phyla of gut microbiota in the
638 control A. BALB/c, C. C57BL/6 and in vancomycin treated B. BALB/c, D. C57BL/6 mice are shown. In
639 the figure only major phyla are shown to avoid clutter. Kinetics of changes in phylum level Equitability
640 (E) (diversity) of the gut microbiota following treatment with vancomycin in E. BALB/c and F. C57/BL6
641 mice. Statistical significance of diversity in panels E. and F. are shown by comparing treatment conditions
642 with respect to untreated (control) are shown with *, where ** denotes $P \leq 0.01$ and *** signifies $P \leq$
643 0.001.

644 Fig3.

645 Metataxonomic studies of genus level variation of gut microbiota. in vancomycin treated and its
646 respective control group. Kinetics of changes in genera of gut microbiota in the control A. BALB/c, C.
647 C57BL/6 and in vancomycin treated B. BALB/c, D. C57BL/6 mice are shown. Percentage abundance of
648 different genera for various treatment conditions are shown on the 'Y'-axis and the days elapsed post
649 treatment or for time matched control are shown on the 'X'-axis. Colony of culturable Proteobacteria by
650 plating of cecal samples from both strains of mice on selective and differential media. Evidence of E.
651 Shigella colonies growth on Salmonella-Shigella specific media agar plate on day 4 (CC) control
652 C57BL/6, (VC) vancomycin treated C57BL/6, (CB) control BALB/c and (VB) vancomycin treated
653 BALB/c and F. Growth of *E. coli*. colonies on EMB (Eosin methylene blue agar plate) on day 4 (VC)
654 vancomycin treated C57BL/6 and (CC) control C57BL/6.

655

656 Fig4.

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

663 Fig5.

664 Protein level gene expression. Concentration (pg/mg of tissue) of cytokines expressed in control (CB or
665 CC for BALB/C or C57BL/6), Vancomycin treated mice on day 3 (VB3) and day 6 (VB6) in A. BALB/c
666 and B. C57BL/6 mice for TNFA C. BALB/c and D. C57BL/6 mice for IL10 are shown as Box-Whisker
667 plot. Statistical significance is expressed by '***' corresponding to $P \leq 0.001$, and '*' corresponding to P
668 ≤ 0.05). Error bars shown are one standard deviation from the average value of four replicates.

669 Fig6.

Measurement of intestinal integrity of BALB/c (Th2-biased) and C57BL/6 (Th1-biased) mice following treatment with Vancomycin. Values of A. Cecal index (total cecal mass/total body mass), B. Cecal liquid content and C. FITC dextran concentration in serum. D. Representative raw images of various sizes of Cecum from both BALB/c and C57BL/6 mice for vancomycin treated day 6, 1. C57BL/6, 2. BALB/c and time matched control 3. C57BL/6 and 4. BALB/c. Transcriptional expression of tight junction genes 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. 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).

Fig7.

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). Error bars shown are one standard deviation from the mean value of four replicates (n=4).

Fig8.

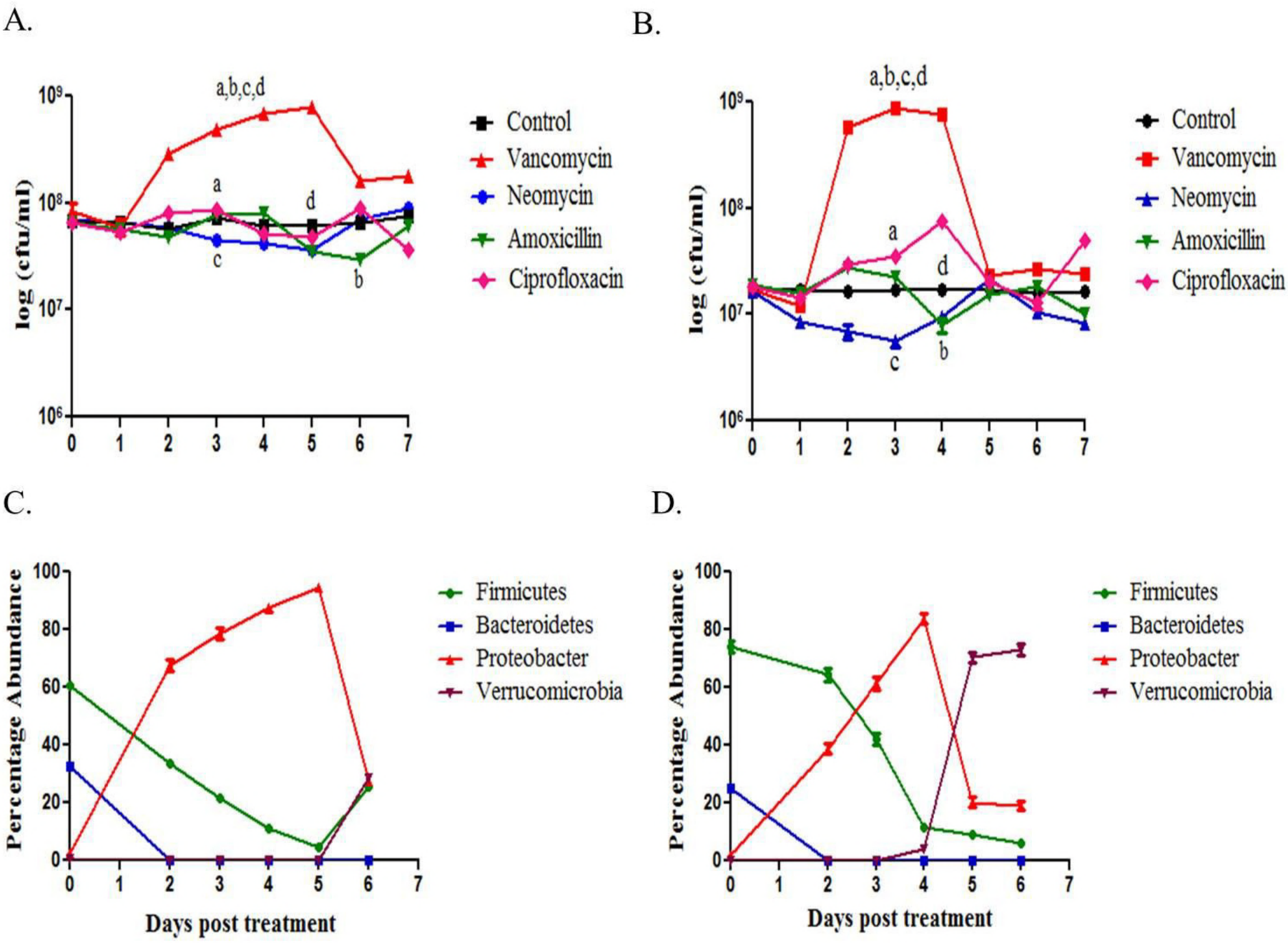
Changes in select hormones in serum. Abundance of A. Insulin (ng/ml), B. Leptin (ng/ml) and C. PYY (pg/mg) in the serum of control BALB/c (CB) or C57BL/6 (CC) and vancomycin treated mice on third day (VB3, VC3) and sixth day (VB6, VC6) of BALB/c and C57BL/6 mice respectively. Comparisons among the groups were calculated with two-way ANOVA. In the figure, '***' corresponds to $P \leq 0.001$,

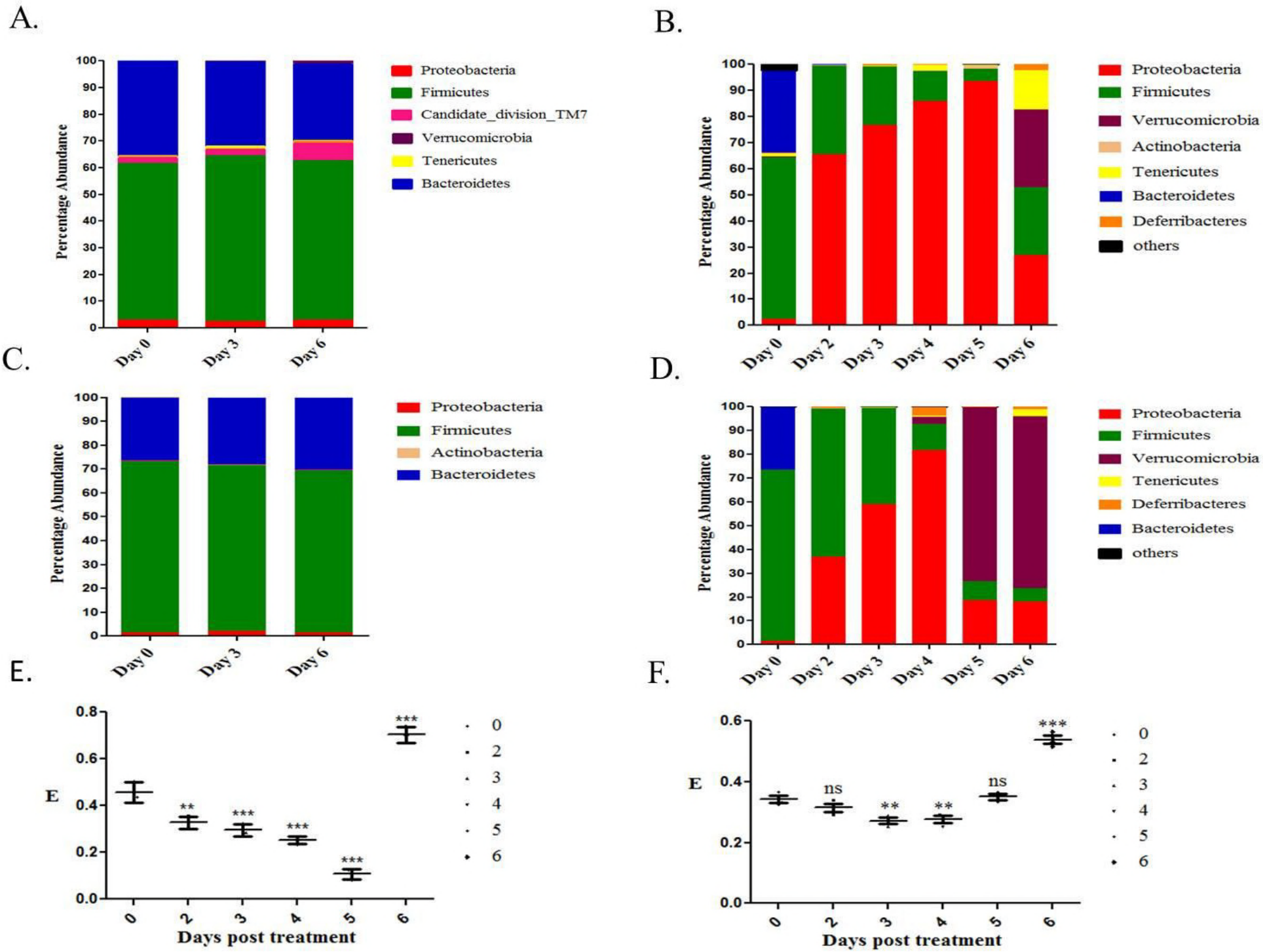
701 '**' corresponds to $P \leq 0.01$, '*' corresponds to $P \leq 0.05$ level of significance). Error bars shown are one
702 standard deviation from the mean value of four replicates (n=4).

Table 1: 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'


A.
B.
C.
D.
log (cfu/ml)
Control
Vancomycin
Neomycin
Amoxicillin
Ciprofloxacin
a,b,c,d
a
b
c
d
Percentage Abundance
Firmicutes
Bacteroidetes
Proteobacter
Verrucomicrobia
Days post treatment


A.
B.
C.
D.
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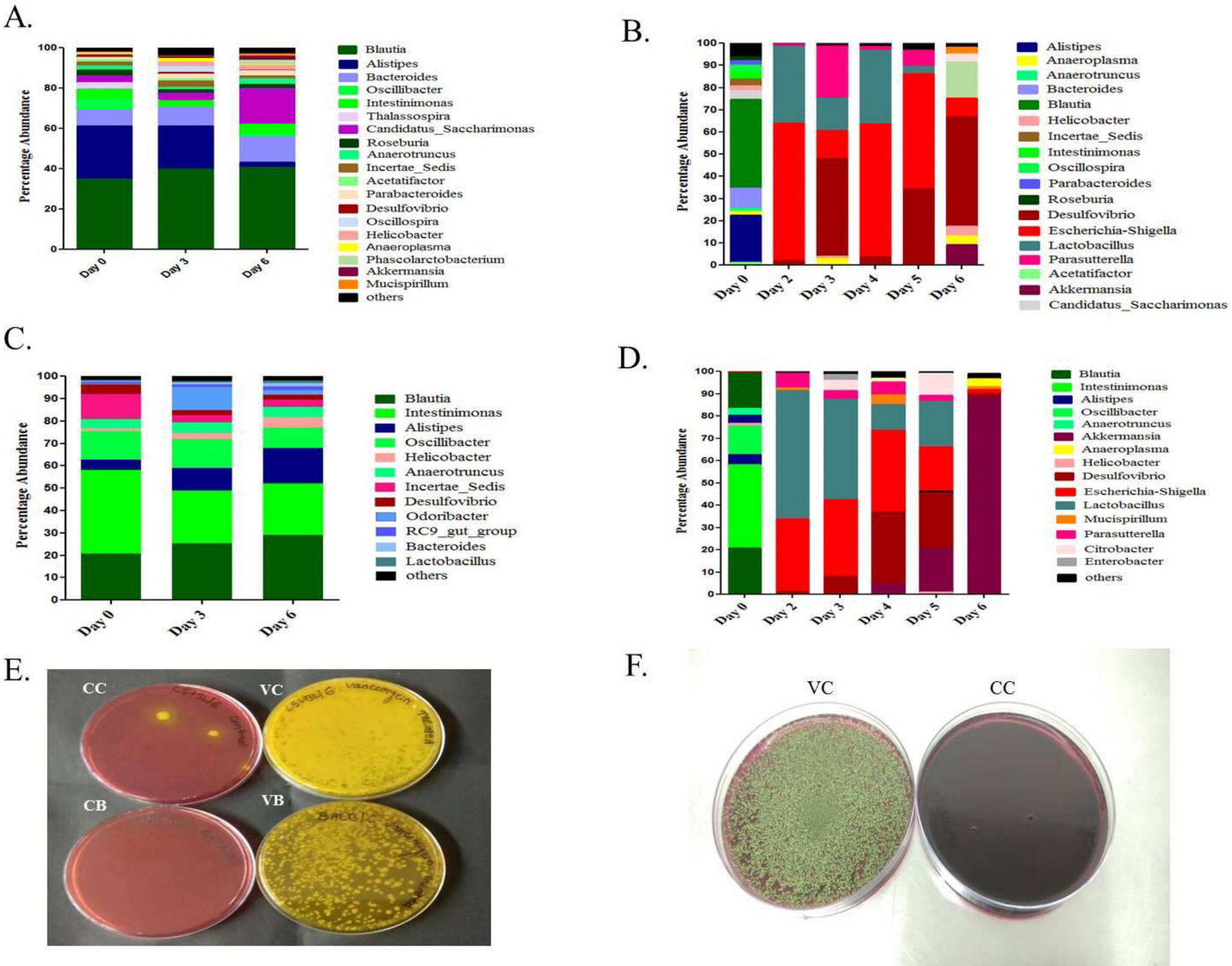
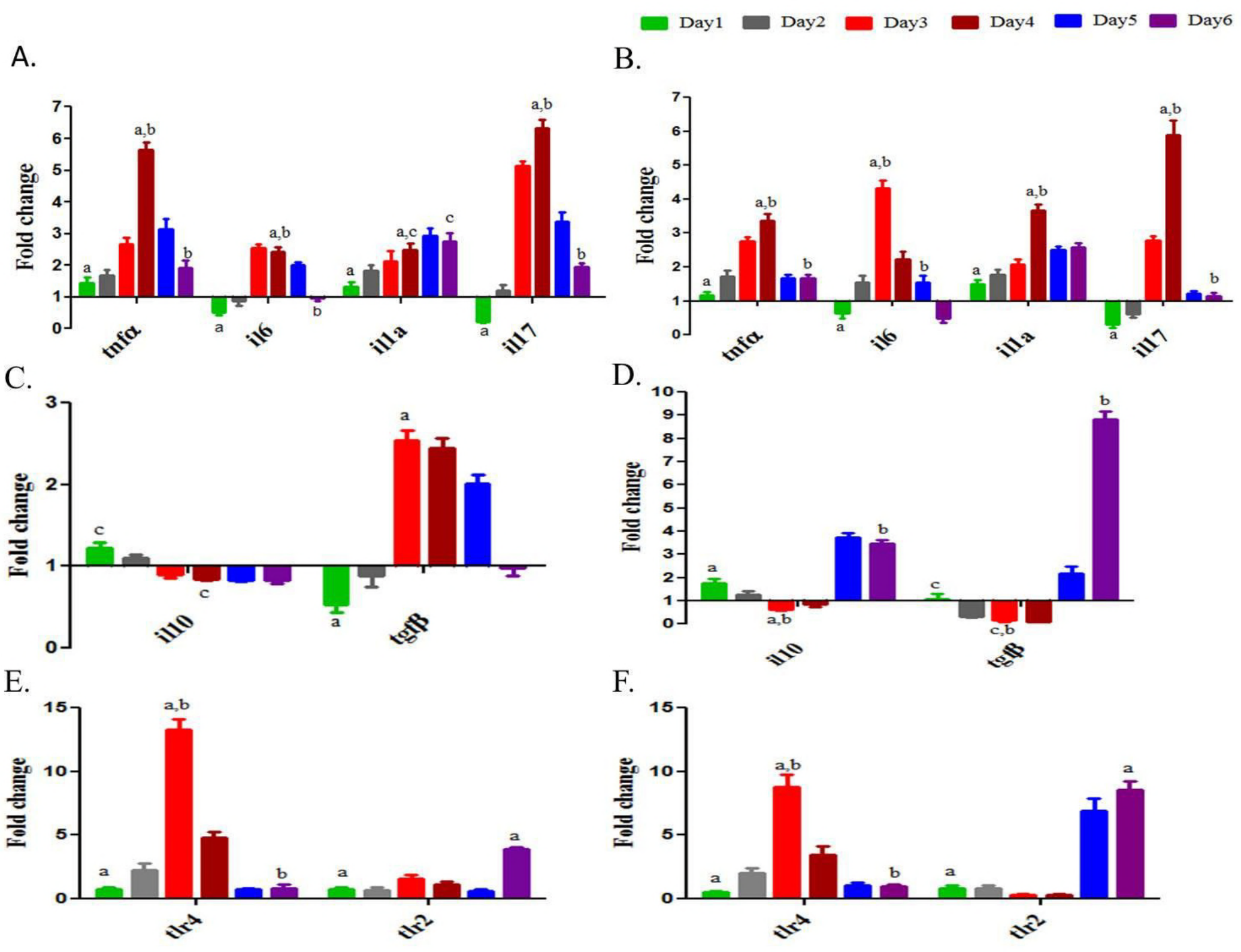
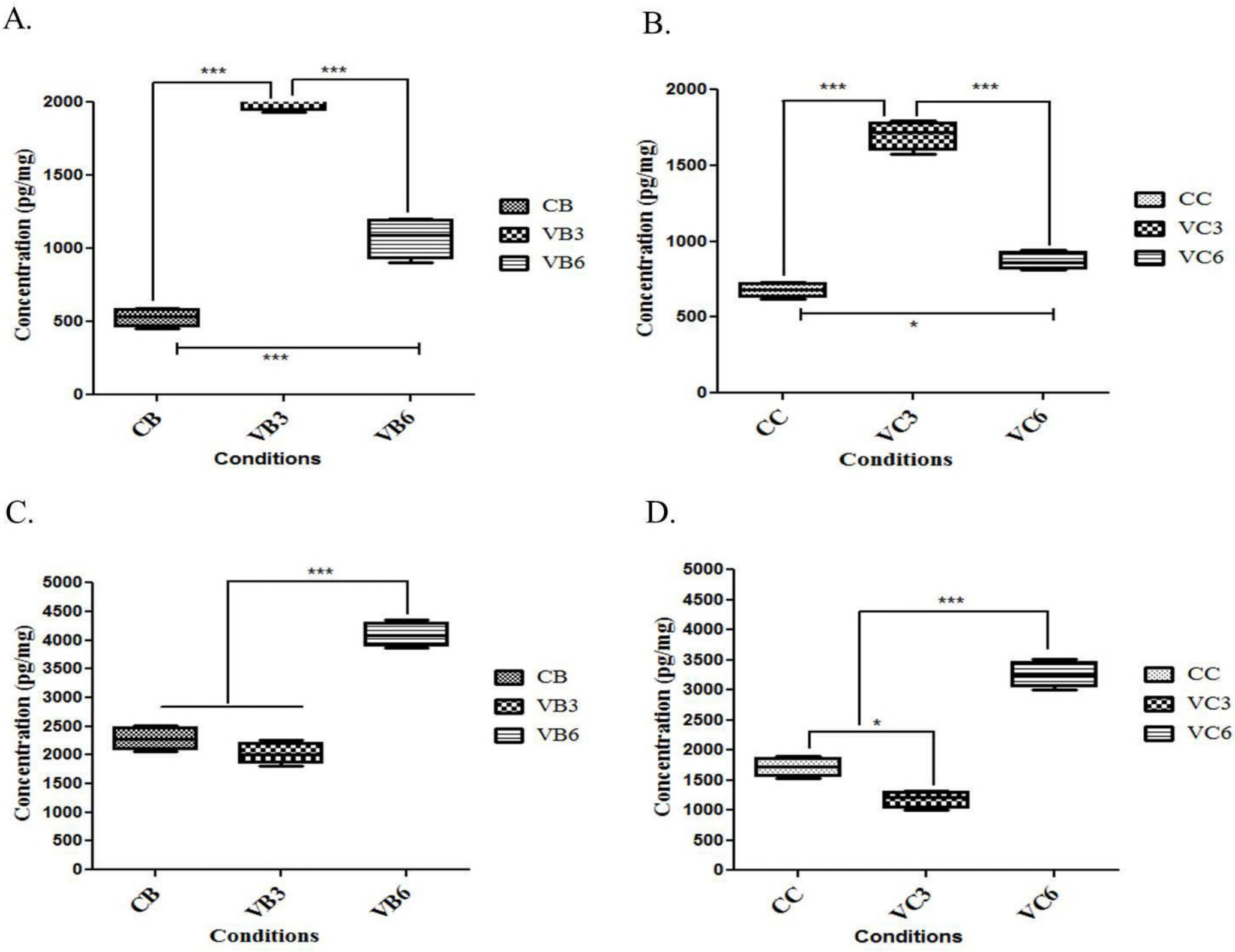
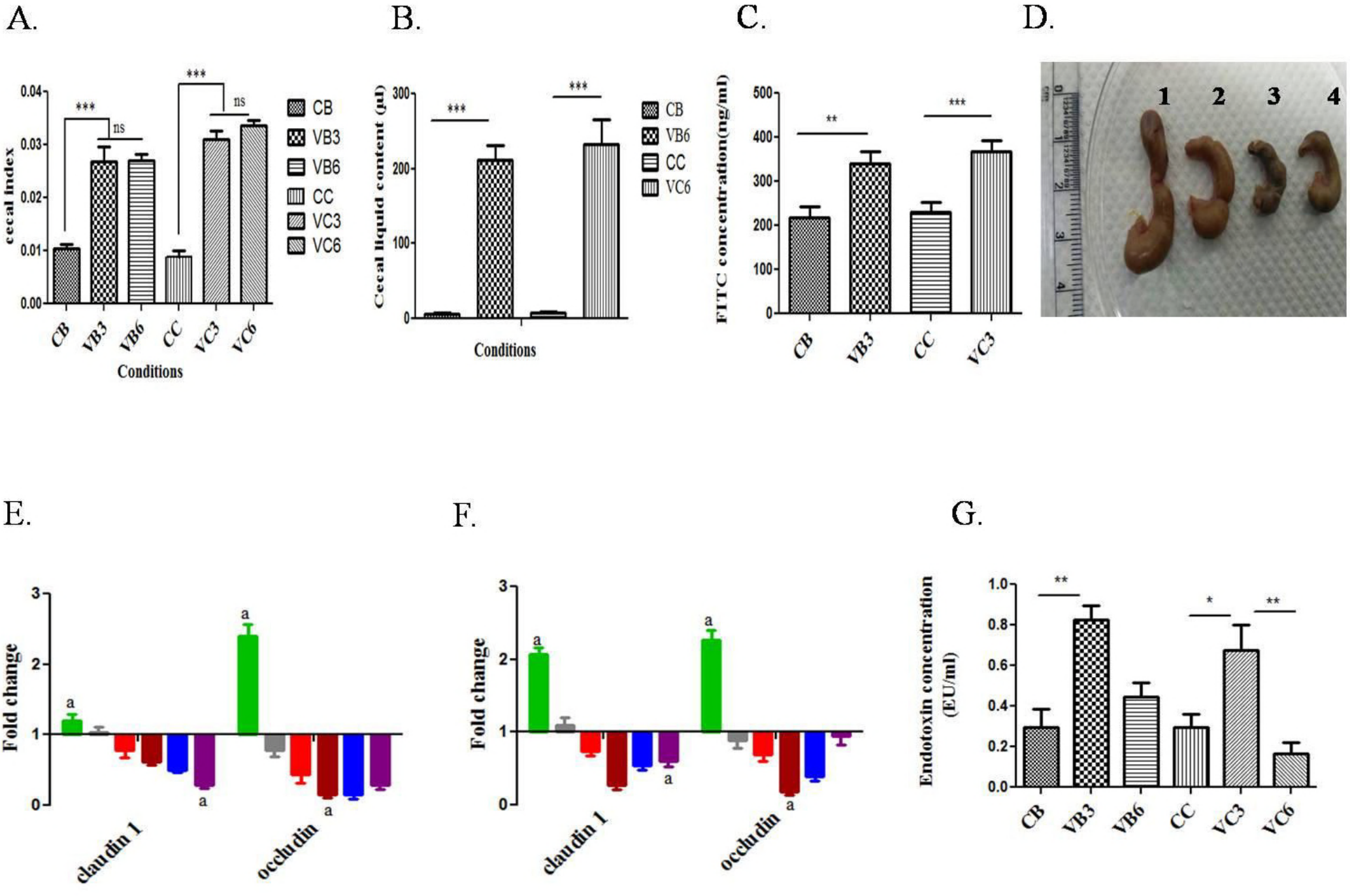
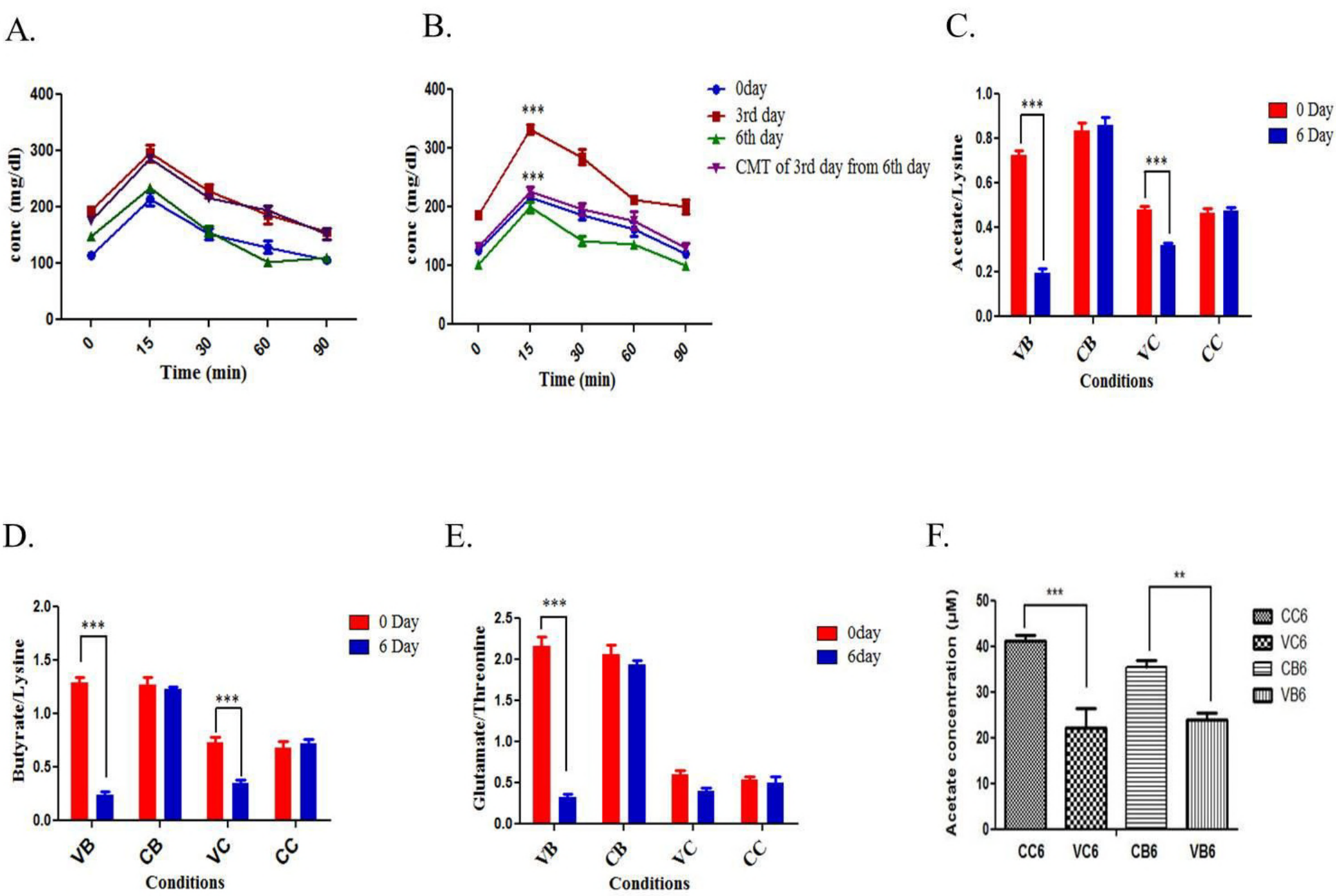
Percentage Abundance
Proteobacteria
Firmicutes
Candidate_division_TM7
Verrucomicrobia
Tenericutes
Bacteroidetes
Actinobacteria
Deferribacteres
others
Day 0
Day 2
Day 3
Day 4
Day 5
Day 6
E
Days post treatment
ns
**
***


A.
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.
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
Parasutterella
Acetatifactor
Akkermansia
Candidatus_Saccharimonas
C.
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.
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
Parasutterella
Citrobacter
Enterobacter
others
E.
CC
VC
CB
VB
F.
VC
CC

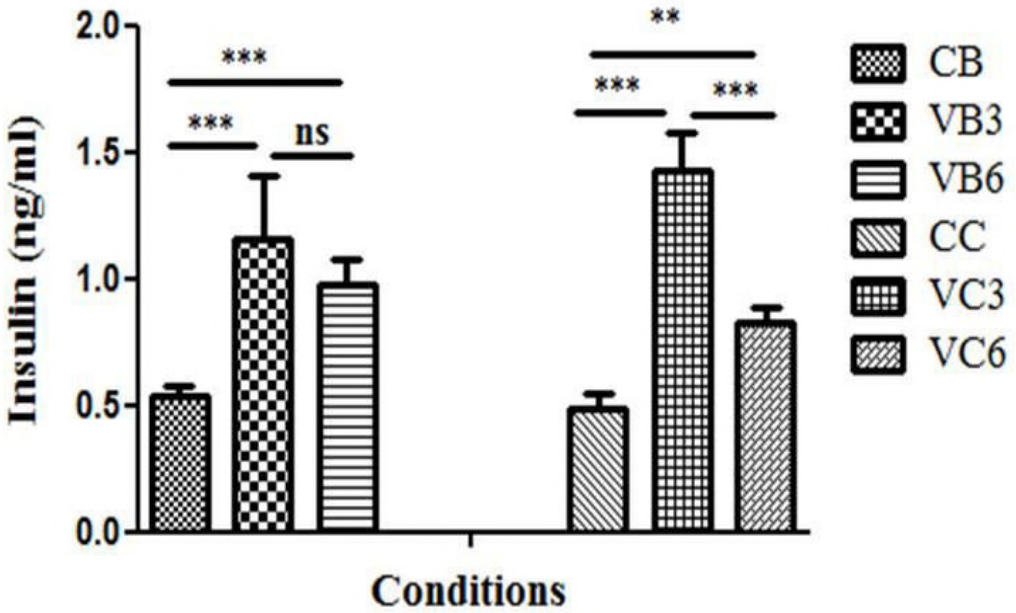

Day1
Day2
Day3
Day4
Day5
Day6
A.
B.
C.
D.
E.
F.
Fold change
tnfα
il6
il1a
il17
il10
tgfβ
tlr4
tlr2


A.
B.
C.
D.
Concentration (pg/mg)
Conditions
CB
VB3
VB6
CC
VC3
VC6
***
*
0
500
1000
1500
2000
2500
3000
3500
4000
4500
5000

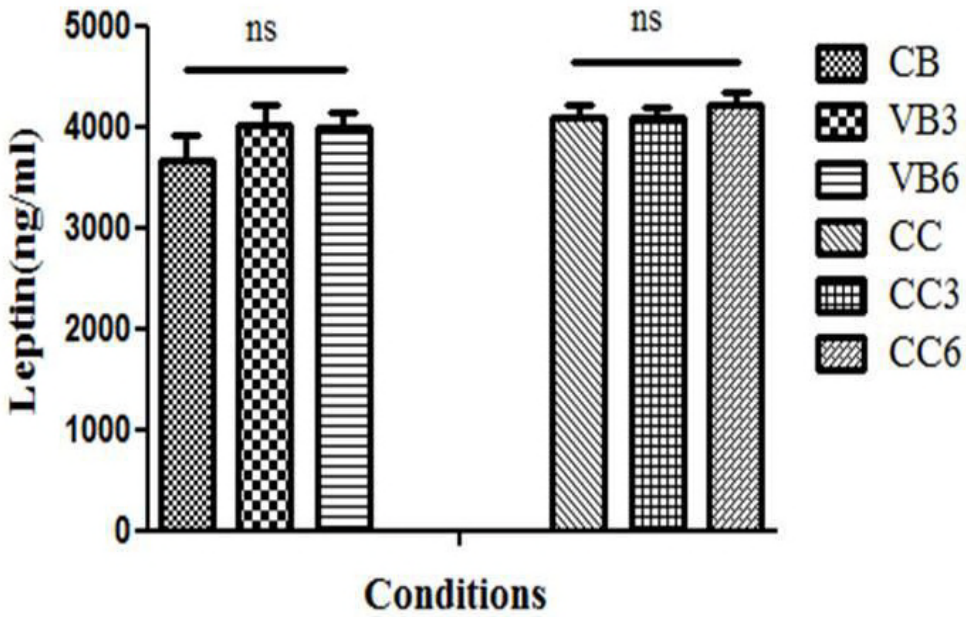

A.
B.
C.
D.
E.
F.
G.
cecal index
0.04
0.03
0.02
0.01
0.00
***
ns
***
ns
CB
VB3
VB6
CC
VC3
VC6
Conditions
Cecal liquid content (μl)
300
200
100
0
***
***
CB
VB6
CC
VC6
Conditions
FITC concentration(ng/ml)
500
400
300
200
100
0
**
***
CB
VB3
CC
VC3
1
2
3
4
Fold change
3
2
1
0
a
a
a
claudin 1
occludin
Fold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


A.
B.
C.
D.
E.
F.
conc (mg/dl)
Time (min)
0day
3rd day
6th day
CMT of 3rd day from 6th day
***
Acetate/Lysine
0 Day
6 Day
VB
CB
VC
CC
Conditions
Butyrate/Lysine
Glutamate/Threonine
0day
6day
Acetate concentration (μM)
**
CC6
VC6
CB6
VB6

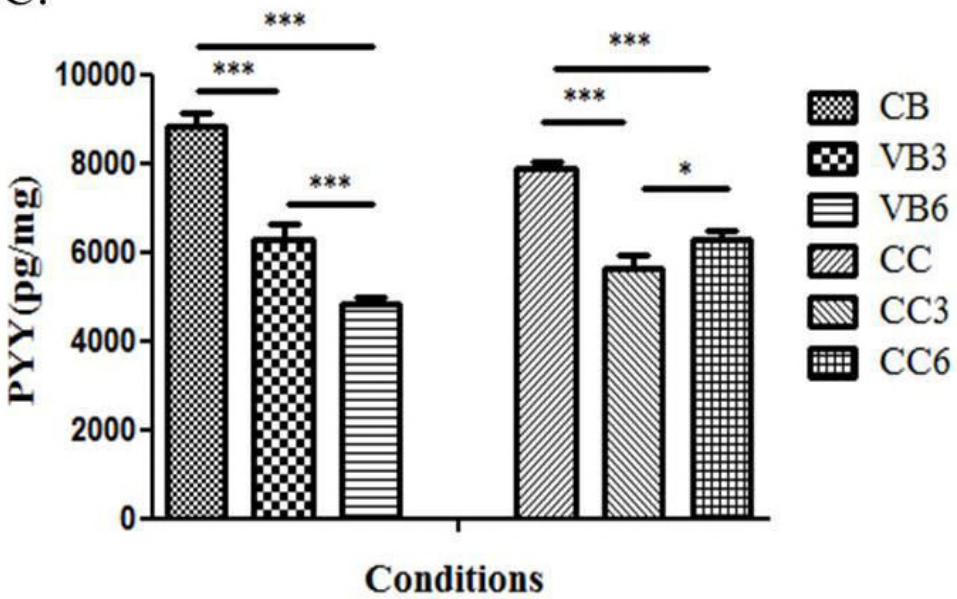
A.


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

B.


5000
4000
3000
2000
1000
0
Leptin(ng/ml)
ns
ns
Conditions
CB
VB3
VB6
CC
CC3
CC6

C.


10000
8000
6000
4000
2000
0
PYY(pg/mg)
***
***
***
***
***
*
Conditions
CB
VB3
VB6
CC
CC3
CC6